universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Community dynamics of bacteria, with special focus on denitrifying bacteria, in a wastewater treatment plant subjected to a new operational mode „controlled disturbances“

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

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A. Introduction

A.1. Significance of prokaryotes for the biogeochemical nitrogen cycle

Nitrogen is an element of great importance for living organisms as it is a component of key compounds like DNA, proteins and vitamins. Consequently, the nitrogen cycle (see Fig. A.1) is one of the most important global biogeochemical cycles. Prokaryotes are of particular importance for the biogeochemical nitrogen cycle, as they are key players in most conversions. Some conversions are even exclusively accomplished by prokaryotes (see Fig. A.1).

Dinitrogen (N_2) is the most abundant gas species in the atmosphere, making up 78 % of its volume, however since it is chemically inert it can be used as a nitrogen source by only a limited number of organisms. Those are some groups of symbiotic or free-living bacteria and archaea that are able to convert dinitrogen into ammonia (NH_3), a compound that can be used to build up biomass (Kneip et al. 2007, Zehr et al. 2000). Ammonia and ammonium (NH_4^+) are used as nitrogen sources by many prokaryotic and eukaryotic species. Moreover, ammonia is used as an energy source by ammonia-oxidizing bacteria and archaea (AOB, AOA, Bock and Wagner 2001, Könneke et al. 2005). Those are chemolithoautotrophic prokaryotes that use ammonia as an electron donor and oxidize it to nitrite (NO_2^-) with oxygen (O_2) as electron acceptor. Nitrite can be further oxidized to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB) also being chemolithoautotrophic bacteria. The sequential oxidation of ammonia to nitrate is referred to as nitrification. Nitrate can be assimilated and used for biomass production by a variety of organisms, but it is also used as an electron acceptor by a broad variety of prokaryotes and some eukaryotes which are able to reduce oxidized nitrogen species when oxygen is lacking. In this way, nitrate is reduced stepwise to dinitrogen in a process called denitrification (Zumft 1997). A second type of metabolism that produces dinitrogen from fixed nitrogen species is the ANAMMOX (anaerobic ammonium oxidation) metabolism. It comprises the oxidation of ammonium with nitrite under anaerobic conditions and is carried out by a group of *Planctomycetales* (Jetten et al. 1998, Strous et al. 1999). The activities of denitrifying prokaryotes and of ANAMMOX bacteria close the nitrogen cycle with the production of dinitrogen gas.

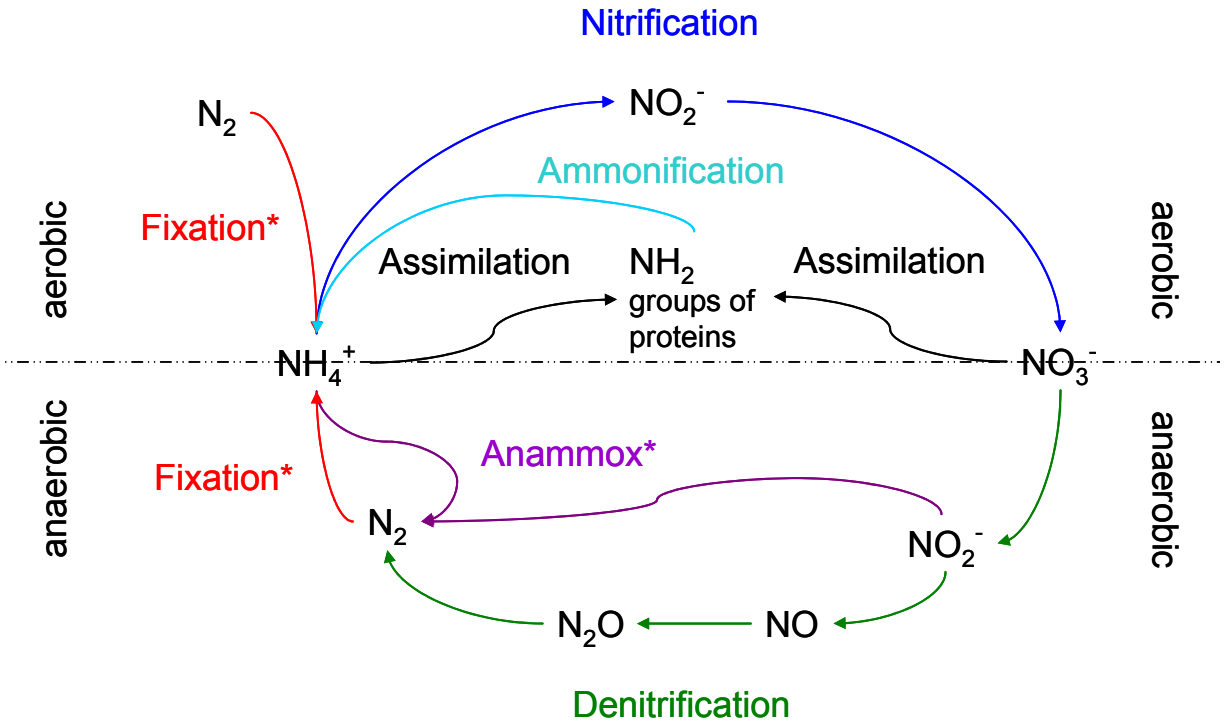

Figure A.1. The biogeochemical nitrogen cycle.
*** steps that are exclusively catalyzed by prokaryotes.**

A.2. Denitrifying prokaryotes

Denitrification is the stepwise dissimilatory reduction of nitrate to dinitrogen, involving four enzyme complexes (Fig. A.1 and A.2) It is a nearly exclusively facultative trait of otherwise aerobically growing prokaryotes which use oxidized nitrogen compounds as terminal electron acceptors in the absence of oxygen (Zumft 1997). Some strains perform aerobic denitrification, i.e. co-respiration of O_2 and nitrogen oxides (Patureau et al. 2000). Not all denitrifying prokaryotes carry out full denitrification, i.e. the production of dinitrogen as the end product, but instead produce nitric oxide (NO) or nitrous oxide (N_2O). Reduction of nitrate to nitrite is termed nitrate respiration and is not considered to be denitrification since no gas compound is produced (Zumft 1997).

Denitrifying prokaryotes can be found in a broad variety of environments including soil, sediment, aquatic environments and earthworm guts (Gamble et al. 1977, Miskin et al. 1998, Bothe et al. 2000, Drake and Horn 2007). The activity of denitrifying prokaryotes is of high ecological significance. Besides being the main biological process responsible for the conversion of fixed nitrogen to dinitrogen, denitrification causes the release of the greenhouse gases nitric oxide and nitrous oxide which are considered to enhance global warming (Lashof and Ahuja 1990) as well as destruction of the ozone layer (Waibel et al. 1999) into the atmosphere. Denitrification accounts for losses of nitrate from soils and therefore influences

plant growth and causes the nutrient depletion of fertilized soils. Denitrification is further responsible for the removal of nitrogen compounds from wastewater, a process that is important to prevent the eutrophication of surface waters.

Denitrifying prokaryotes comprise both bacteria and archaea and are taxonomically (Cabello et al. 2004, Heylen et al. 2006b) and physiologically (Zumft 1997) very diverse. The widespread occurrence of the denitrifying trait in many different lineages is thought to have - at least partly - resulted from horizontal gene transfers of the involved genes. Partial incongruences of denitrification gene (*nir*, *nor*, *nos* genes) and 16S rDNA phylogeny of bacteria (Casciotti and Ward 2001, Philippot 2002, Song and Ward 2003, Goregues et al. 2005, Heylen et al. 2006a, Heylen et al. 2007) and archaea (Cabello et al. 2004) indicate that horizontal gene transfer took place in different lineages. This finding has an important consequence for studies of denitrifying prokaryotes, since it hampers the identification of denitrifiers based on their *nir*, *nor* or *nos* gene sequences. These sequences can only be used for comparing the community structures of denitrifying prokaryotes between different samples using fingerprinting techniques like denaturant gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP). *Nir* genes are considered to be the best target genes for this kind of studies since nitrite reductase is the key enzyme of the denitrifying metabolism, being the enzyme that produces the first gaseous product (Zumft 1997, Philippot and Hallin 2005, see Fig. A.2). Two kinds of nitrite reductases are known, the cytochrome cd1-containing enzyme NirS and the Cu-containing enzyme NirK. They are different in structure but functionally equivalent and appear to occur mutually exclusive in any given denitrifying strain.

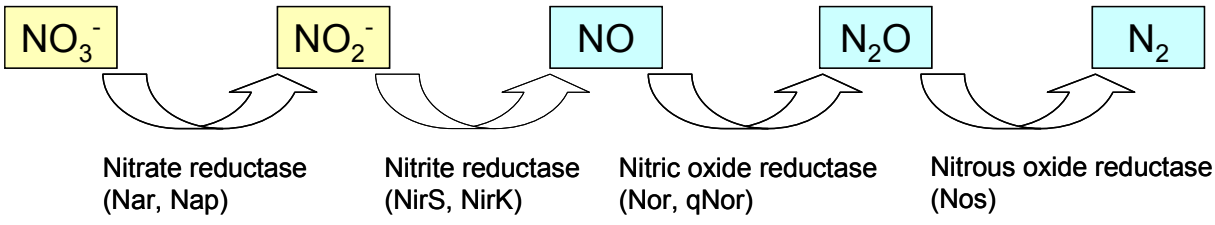

Figure A.2. Enzymes involved in denitrification.
Blue boxes indicated gaseous compounds, yellow boxes indicate solid compounds.

A.3.Wastewater treatment and microbial ecology

Human activities continuously produce wastewater of domestic and industrial origin containing carbon, nitrogen and phosphorus compounds, as well as toxic substances and pathogenic microorganisms. To prevent negative effects on natural ecosystems and human health, wastewater is collected and treated prior to discharge into the environment. Removal of nutrients and toxins from wastewater is achieved by the activity of microorganisms, therefore wastewater treatment is considered to be one of the most important biotechnological processes in the world. Until recently, microorganisms involved in wastewater treatment have been treated as a "black box" due to the fact that research in this field was usually done by engineers not trained in microbiology. During the last years, microbiologists became interested in wastewater treatment plants as model ecosystems (Wagner and Loy 2002, Wagner et al. 2002, Daims et al. 2006). The limited scale, the homogeneous content, the stable chemical and physical conditions, the continuous measurement of environmental parameters and the possibility of controlled manipulation provide an ideal setup to test ecological hypotheses. Going one step further, environmental engineering and microbial ecology may benefit from each other by combining knowledge of wastewater treatment plant function with observations of community dynamics and ecophysiology of microorganisms and ecological theory.

In wastewater treatment research continuous effort is made to improve wastewater treatment function and reliability. Unfavorable microbial population dynamics, treatment failures like malfunction of aeration, inappropriate wastewater composition or introduction of toxic substances can cause instabilities of treatment processes. Malfunctions of treatment processes that are frequently observed are for example breakdowns of nitrification and sludge bulking and foaming caused by extensive growth of filamentous bacteria. In this context microbial ecology may be able to give hints for optimized process design.

A.4. Implementation of new operational mode "controlled disturbances" on the wastewater treatment plant Weißtal

In an effort to optimize wastewater treatment plant processes on the basis of ecological theory, a new operational mode termed "controlled disturbances" was developed by Dr. Niels Holm (LimnoTec), Dr. Holger Daims and Prof. Michael Wagner. It was designed to increase the stability of the treatment processes by enhancing the diversity of microorganisms. As mentioned before wastewater treatment plant function is sometimes unstable due to naturally

occurring disturbances or unfavourable population dynamics. Enhancement of diversity may counteract this problem. According to the "intermediate disturbance hypothesis" the highest diversity in an ecosystem is expected to occur when disturbances of the system are intermediate in intensity and frequency (Connell 1978). The operational mode "controlled disturbances" follows this principle. It introduces varying wastewater loads and periodic shutdown of the aeration of nitrification tanks. The resulting feast/famine conditions and fluctuating oxygen availability are thought to enlarge the spectrum of available ecological niches for colonizing bacteria and thus to enhance microbial diversity. Ecosystem stability is thought to be linked to diversity due to the following reasons: I) Increasing diversity increases the probability of functional redundancy of microorganisms, i.e. the presence of different microorganisms exerting the same function (e.g. nitrification or denitrification) (McCann 2000, Briones and Raskin 2003). Functional redundancy is thought to enhance the stability of ecosystem processes because if one species goes extinct or is unable to perform the respective function under certain environmental conditions, others can sustain its function. II) Increasing diversity increases the probability that species are present that respond differently to variable environmental conditions and perturbations (McCann 2000). These two points make up the so called "insurance hypothesis" (Yachi and Loreau 1999). III) Increasing diversity may decrease interaction strengths between species and therefore dampen the destabilizing potential of strong interspecies interactions (McCann 2000, Kato et al. 2008).
The new operational mode was implemented on the full-scale wastewater treatment plant Weißtal. Parallel to the evaluation of the effects of "controlled disturbances" on wastewater treatment plant performance, samples were taken for analyzing its effects on the community composition of sludge bacteria. A part of these microbiological analyses was conducted in this study.

A.4.1. Wastewater treatment plant setup

The wastewater treatment plant Weißtal consists of two independent plants for carbon, nitrogen and phosphorus removal. One plant is a conventional activated sludge system with an upstream denitrification stage, the other plant consists of two sequencing batch reactors (SBRs) (Fig. A.2). The conventional plant comprises - in the sequence of wastewater treatment - a buffer tank, a denitrification tank, two nitrification tanks and a settling tank. Phosphorus removal is accomplished by a combination of enhanced biological phosphorus removal (EBPR) and chemical precipitation.

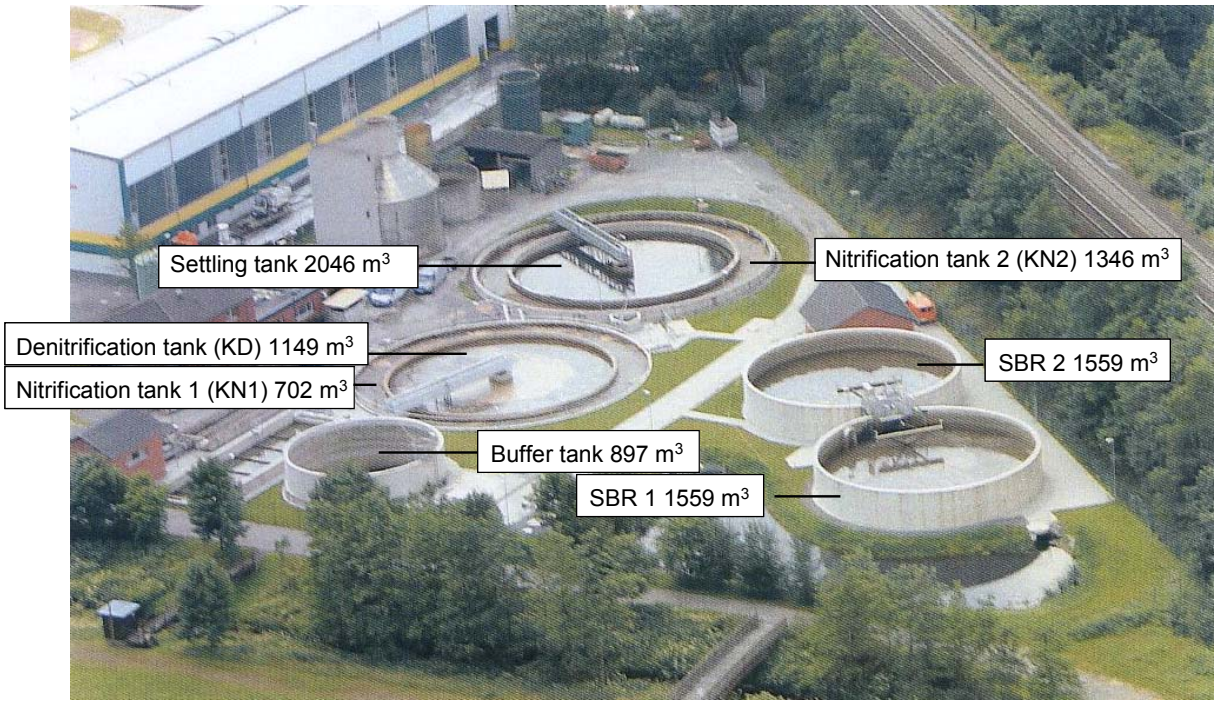

Figure A.2. Wastewater treatment plant Weißtal (LimnoTec).

A.4.2. Operational mode "controlled disturbances"

The new operational mode "controlled disturbances" was implemented in the conventional plant. Whereas during regular operation, wastewater was continuously discharged into the plant and both nitrification tanks were continuously aerated, "controlled disturbances" comprised cyclic variation of load and aeration. It was subdivided into three phases, each spanning one hour (see Fig A.3). A phase of regular load and reduced aeration of nitrification tanks was followed by a phase of reduced load (18 % of regular load), whereas the excess wastewater influent was stored in the buffer tank. During this phase aeration in nitrification tank 2 was shut off, which enabled denitrification. At the end of the reduced load phase ammonium and nitrate concentrations in the tanks were extremely low resulting in pronounced famine conditions for bacteria residing therein. In the following phase of elevated load the wastewater stored in the buffer tank was discharged into the tanks together with the normal influent resulting in elevated load making up 181 % of regular load. Aeration of nitrification tanks was stopped until 20 min after the beginning of this phase or until the ammonium concentration reached a critical value. This should result in further denitrification in the nitrification tanks, pronounced anaerobic conditions in the denitrification tank which have a positive effect on EBPR performance, as well as highly increased ammonium concentrations. When aeration was started again the microorganisms were facing feast

conditions with respect to ammonium and organic carbon. After the end of the elevated load phase, the cycle started again with the normal load phase.

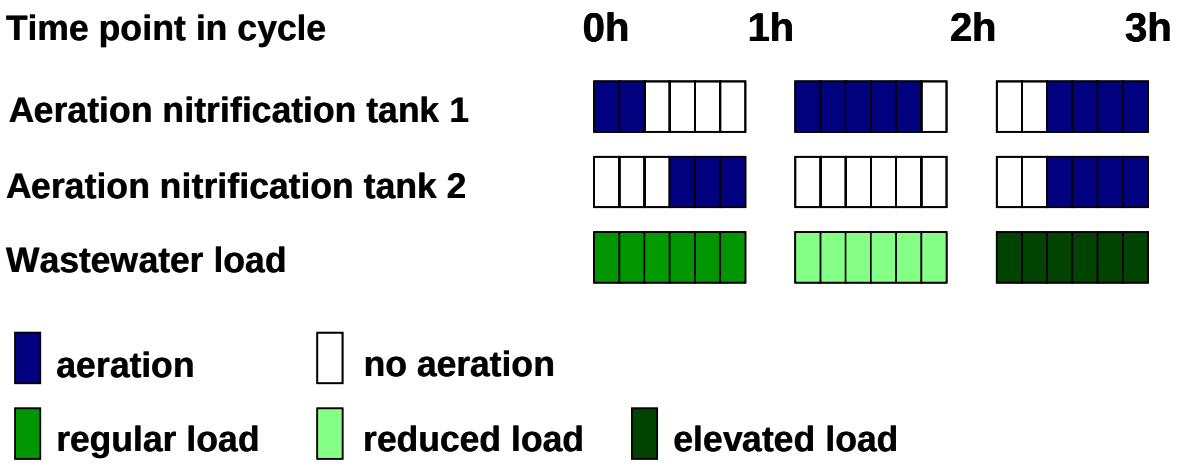

Figure A.3. Operational mode "controlled disturbances".

A.4.3. Positive effects of „controlled disturbances" on wwtp performance

Several improvements in terms of costs and efficiency of wastewater treatment were observed during the implementation of "controlled disturbances". The reduced aeration of nitrification tanks had positive effects on nitrogen and phosphorus removal. It resulted in lower energy requirements for nitrification but constantly high nitrification performance. Denitrification performance increased compared to regular operation, most likely because the introduction of anaerobic phases in the nitrification tanks enabled further nitrate removal. Enhanced biological phophorus removal (EBPR) performance increased, most probably because pronounced anaerobic conditions in the denitrification tank at the beginning of the high load phase had a beneficial effect on the metabolism of EBPR-performing microorganisms. The function of the settling tank was improved by a strong decrease of the sludge settling index.

A.5. Aims of this study

A.5.1. Characterization of community dynamics of denitrifying bacteria in a wastewater treatment plant subjected to the new operational mode "controlled disturbances"

As the denitrification performance of the wastewater treatment plant Weißtal increased when "controlled disturbances" was implemented, the first objective of this study was an examination of denitrifying community structure dynamics accompanying the change in denitrifying activity. For this purpose terminal restriction fragment length polymorphism (T-

RFLP) analyses of the functional genes *nirS* and *nirK* were performed for samples taken from the denitrification tank before and during the implementation of "controlled disturbances". These genes are, as discussed above, encoding the key enzymes of the denitrifying metabolism, cytochrome cd1- and copper-containing nitrite reductases, and are therefore considered to be the best target genes for studying denitrifiers. T-RFLP is a sensitive high-throughput technique for the production of community fingerprints (Kitts 2001, Marsh 2005). It has the disadvantage that the results cannot be directly linked to sequence information, but given the limited usability of *nir* genes as phylogenetic markers this was not of main interest.

A.5.2. Characterization of community dynamics of bacteria in a wastewater treatment plant subjected to the new operational mode "controlled disturbances"

The new operational mode "controlled disturbances" resulted in improved efficiency of all wastewater treatment processes, including nitrification, denitrification and EBPR. The second aim of this study was to investigate whether the observed change in ecosystem function was associated with a change in composition and/or diversity of the bacterial community. This was accomplished by performing denaturant gradient gel electrophoresis (DGGE) of sludge samples taken before and during implementation of the new operational mode, using PCR-amplified bacterial 16S genes. This is a classical approach for comparison of community fingerprints (Muyzer et al. 1993, Muyzer and Smalla 1998). Although there may be more sensitive fingerprinting techniques available than DGGE, namely T-RFLP (Moeseneder et al. 1999), DGGE offers the big advantage of the possibility of the direct phylogenetic characterisation of bacteria that were affected by the implementation of "controlled disturbances". Excision and sequencing of bands that appeared or disappeared during "controlled disturbances" enables a rough phylogenetic classification and the possibility to design probes for fluorescence in situ hybridization (FISH) to verify the results obtained by DGGE analysis by the use of a non PCR-biased technique. This results in a kind of modified full-cycle rRNA approach.

B. Materials and Methods

All chemicals used in this study were of *p.a.* quality, if not stated otherwise. All buffers, media and solutions were prepared using double distilled and filtered water ($H_2O_{bidist.}$) produced by a water purification facility (MQ Biocel, Millipore Corporation, Billerica, MA, USA). They were autoclaved in a watervapour-high pressure autoclave (Varioclav 135S, H+P, München Germany) for 20 min at 121°C and 1.013×10^5 Pa pressure and stored at room temperature (RT), unless stated otherwise. Substances that are unstable at high temperatures were sterile filtered and added after autoclaving and cooling. All centrifugation steps were conducted by using a table-top centrifuge (Mikro 22R, Hettich, Tuttlingen, Germany) at RT unless stated otherwise.

B.1. Technical equipment

Table B.1. Technical equipment.

Equipment	Source of supply
Capillary electrophoresis for sequencing and T-RFLP: 3130xl Genetic Analyzer	Applied Biosystems Lincoln, USA
Centrifuges: Mikro 20 Rotina 35 R Galaxy Mini Centrifuge	 Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany VWR international, West Chester, PA, USA
Concentrator 5301 (Vacufuge)	Eppendorf, Hamburg, Germany
Confocal Laser Scanning Microscope LSM 510 Meta	Zeiss, Jena, Germany
DCodeTM system for DGGE	Biorad, München, Germany
Econo model EP-1 gradient pump	Biorad, München, Germany
Gel Dokumentationsystem MediaSystem FlexiLine 4040	Biostep, Jahnsdorf, Germany
Gelcarriage: HoeferTM HE 33 - gel running tray (7x10cm) Sub-Cell GT UV-Transparent Gel Tray (15x15cm)	 Amersham Biosciences (SF) Corp., USA Biorad, Munich, Germany
Gelelectrophoresis: HoeferTM HE 33 Mini Horizontal submarine unit Sub-Cell GT	 Amersham Biosciences (SF) Corp., USA Biorad, Munich, Germany
Hybridisation oven UE-500	Memmert GmbH, Schwabach, Germany
Laminar flow hood Safe 2010 Modell 1.2	Holten, Jouan Nordic, Allerød, Dänemark
Magnetic stirrer: RCT basic Variomag® Maxi	 IKA® Werke GmbH, Schwabach, Deutschland Variomag®, Dayton Beach, FL, USA
Microwave MD6460	Microstar
Mini Protean 3-Cell	Biorad, Munich, Germany
PCR thermocyclers: Icycler Mastercycler gradient	 Biorad, Munich, Germany Eppendorf, Hamburg, Germany

pH-Meter WTW inoLab Level 1	Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Photometers: NanoDrop® ND-1000 Spectralphotometer SmartSpecTM 3000	 NanoDrop Technologies, Wilmington, USA Biorad, Munich, Germany
Platform Shaker Innova 2300	New Brunswick Co., Inc., Madison NJ, USA
Power device for gelelectrophoresis PowerPac Basic	Biorad, Munich, Germany
Scale: OHAUS Analytic Plus Sartorius	 Ohaus Corp., NY, USA Sarotius AG, Göttingen, Germany
Transilluminator UST-30M-8E (312 nm)	Biostep GmbH, Jahnsdorf, Germany
Ultraviolet Sterilizing PCR Workstation	Peqlab Biotechnology GmbH, Germany
Vortex Genie 2	Scientific Industries, New York, USA
Water purification facility: MQ Biocel	Millipore Corporation, Billerica, MA, USA
Waterbaths: DC10 GFL Typ 1004	Thermo Haake, Karlsruhe, Germany Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Watervapour high pressure autoclaves: Varioclav 135S H+P Varioclav 25T H+P	 H+P, Munich, Germany H+P Munich, Germany

B.2. Expendable items

Table B.2. Expendable items.

Expendable item	Source of supply
Cover slips 24×50 mm	Paul Marienfeld, Bad Mergentheim, Germany
Eppendorf Reaktionsgefäße (ERT), various sizes	Eppendorf AG, Hamburg, Germany
Erlenmeyer-Kolben DURAN®, various sizes	Schott Glas, Mainz, Germany
Glascapillares (50 µl in 5.1 cm)	Idaho Technology Inc., Salt Lake City, UT, USA
Microseal „A“ Film	MJ Research, Waltham, MA, USA
Microseal® “B” film	Biorad, München, Germany
Mikrotitterplatte MicrosealTM 96, V-Boden	MJ Research, Waltham, MA, USA
MultiScreen 96-well plates	Millipore Corporation, Billerica, MA, USA
Petri dishes 94/16	Greiner Bio-one GmbH, Frickenhausen, Germany
Sampling vessels, 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
slides, 10 Well	Paul Marienfeld, Bad Mergentheim, Germany
Tips, various volumes	Carl Roth GmbH & Co., Karlsruhe, Germany

B.3. Chemicals

Table B.3. Chemicals.

Chemical	Source of supply
6x DNA Loading Dye	Fermentas, St. Leon-Rot, Germany
Acetic acid(pure)	Carl Roth GmbH & Co., Karlsruhe, Germany
Acrylamide/Bisacrylamid (40%, 37.5:1)	Biorad, Munich, Germany
Agar	FLUKA Chemie AG, Buchs, Switzerland

Agarose: Agarose, elecrophoresis Grade NuSieve® 3:1 Agarose (low melting)	 Invitrogen Corp., Carlsbad, CA, USA Bio Science, Rockland, Inc., Rockland, ME, USA
Ammonium peroxy-di-sulfate	(APS) Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Boric acid	Fluka Chemie AG, Buchs, Switzerland
Bromphenol Blue	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Cetyltrimethylammoniumbromid (CTAB)	Carl Roth GmbH & Co., Karlsruhe, Germany
Cititfluor AF1	Agar Scientific Limited, Essex, England
Ethanol absolute	Merk KGaA, Darmstadt, Germany
Ethidium Bromide (EtBr)	FLUKA Chemie AG, Buchs, Switzerland
Ethylene-di-amine-tetra-acetic acid (EDTA), disodium salt	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Ficoll® 400	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Formamide (FA)	FLUKA Chemie AG, Buchs, Switzerland
Glycerol	Carl Roth GmbH & Co., Karlsruhe, Germany
Hi-Di Formamide	Applied Biosystems Lincoln, USA
Hydrochloric acid (HCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Hydrogen Peroxid (H_2O_2), 30%	Carl Roth GmbH & Co., Karlsruhe, Germany
Imidazol	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropanol (2-propanol)	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropyl-ß-D-thiogalactopyranoside	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Kanamycin	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Lysozyme	Fluka Chemie AG, Buchs, Switzerland
Magnesium chloride	Carl Roth GmbH & Co., Karlsruhe, Germany
N,N,N',N'-tetra-methyl-ethylene-di-amine (TEMED)	FLUKA Chemie AG, Buchs, Switzerland
Paraformaldehyde (PFA)	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Potassium acetate (KCl)	J. T. Baker, Deventer, Holland
Sephadex G50	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Sodium acetate (Na-acetate)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium Chloride (NaCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	FLUKA Chemie AG, Buchs, Switzerland
Sodium hydroxide (NaOH)	J.T. Baker, Deventer, Holland
Sodium-di-hydrogenphosphate (NAH_2PO_4)	J.T. Baker, Deventer, Holland
SYBR® Green I	Cambrex Bio Science, Rockland, Inc., Rockland, ME, USA
Tris	Carl Roth GmbH & Co., Karlsruhe, Germany
Tryptone	Oxoid LTD., Hampshire, England
Urea	USB, Corp., Cleveland, USA
X-Gal (5-brom-4-chlor-3-indolyl-ß-Dgalactopyranoside)	Carl Roth GmbH & Co., Karlsruhe, Germany
Xylencyanol	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Yeast extract	Oxoid LTD., Hampshire, England

B.4. Kits

Table B.4. Kits used.

Kit	Source of supply
UltraClean™ Soil DNA Kit	MOBio Lab. Inc., Salana Beach, CA, USA
QIAquick Gel Extraction Kit	QIAgen, Hilden, Germany
QIAquick PCR Purification Kit	QIAgen, Hilden, Germany
TOPO TA Cloning®	Invitrogen Corporation, Carlsbad, CA, USA

B.5. Software

Table B.4 Used software tools.

Software	URL	Reference
ARB software-package	http://www.arb-home.de/	(Ludwig et al. 2004)
Basic Local Alignment Search Tool	http://www.ncbi.nlm.nih.gov/BLAST/	(Altschul et al. 1990)
Primer	http://www.primer-e.com/	PRIMER-E Ltd, UK
probeBase	http://www.microbial-ecology.net/probebase/	(Loy et al. 2003)
probeCheck	http://www.microbial-ecology.net/probecheck/	(Loy et al. 2008)
Ribosomal Database Project	http://rdp.cme.msu.edu/	(Cole et al. 2003)
Peak Scanner	http://appliedbiosystems.com/peakscanner	Applied Biosystems, USA
REMA	http://www.macaulay.ac.uk/rema	(Szubert et al. 2007)
Finch TV	http://www.geospiza.com/finchtv/	Geospiza, USA

B.6. Sampling of wastewater treatment plant

Activated sludge was sampled by our cooperation partners (Limnotec). Samples were taken from the denitrification tank and the nitrification tank 2 before and during the implementation of the operational mode “controlled disturbances” was implemented. The sampling scheme is summarized in table B.6. Samples were stored in Greiner tubes at -20 °C. Samples for FISH analyses were fixed and stored according to section B.19.1.

Table B.6. Sampling of wastewater treatment plant Weißtal.

Samples taken before "controlled disturbances"			
Sampling date	sampled tanks		name of sample
	nitrification 2 (KN2)	denitrification (KD)	
9th March 2006	+		A
21st March 2006	+		B
22nd May 2006	+	+	D
Samples taken during "controlled disturbances"			
Sampling date	sampled tanks		name of sample
	nitrification 2 (KN2)	denitrification (KD)	
16th August 2006	+	+	E
18th September 2006	+	+	F

+ means that sample was taken

B.7. Buffers, media and solutions

B.7.1. General buffers

a) Phosphate buffered saline (PBS)

(i) PBS stock solution (Na_xPO_4)

NaH_2PO_4 200 mM 35.6 g/l

Na_2HPO_4 200 mM 27.6 g/l

pH of NaH_2PO_4 solution was adjusted to 7.2 -7.4.

(ii) 1 x PBS

NaCl 130 mM 7.6 g/l

PBS stock solution 10 mM 50 ml/l

H_2O_{bidist} ad 1000 ml

pH 7.2–7.4

(iii) 3 x PBS

NaCl 390 mM 22.8 g/l

PBS stock solution 30 mM 150 ml/l

H_2O_{bidist} ad 1000 ml

pH 7.2 –7.4

B.7.2. Buffers, solutions and standards for gel electrophoresis

a) TAE buffer

(i) 50 x TAE

Tris 2 M

Sodium acetate 500 mM

EDTA 50 mM

pH was adjusted to 8.0 with pure acetic acid.

(ii) 1 x TAE

50 x TAE 20 ml/l

$H_2O_{bidist.}$ ad 1000 ml

b) TBE buffer

(i) 10 x TBE

Tris 890 mM 162.0 g/l

Boric acid 890 mM 27.5 g/l

EDTA 20 mM 9.3 g/l

H_2O_{bidist} ad 1000 ml

pH 8.3 – 8.7

(ii) 1 x TBE

10 x TBE 100 ml/l

H_2O_{bidist} ad 1000 ml

c) Loading buffer

Ficoll 25% (w/v)

Bromphenol blue 0.5% (w/v)

Xylencyanol 0.5% (w/v)

EDTA 50 mM

d) Ethidium bromide solution

(i) Ethidium bromide stock solution

10 mg/ml Ethidium bromide (EtBR) in H_2O_{bidist}

(ii) Ethidium bromide staining solution

EtBr-stock solution diluted 1:10,000 in H_2O_{bidist}

e) SYBR® Green I solution

(i) SYBR® Green I stock solution

SYBR® Green I 10,000 x concentrate in DMSO

(ii) SYBR® Green I staining solution

SYBR® Green I stock solution diluted 1:10,000 in 1x TAE buffer

f) DNA ladder

GeneRuler™ 1kb DNA Ladder (Fermentas, St. Leon-Rot, Germany)

GeneRuler™ 100 bp DNA Ladder (Fermentas, St. Leon-Rot, Germany)

B.7.3. Culture media for *Escherichia coli* (*E. coli*) strains

a) Luria Bertani medium (LB medium)

Tryptone 10.0 g/l

Yeast extract 5.0 g/l

NaCl 5.0 g/l

H_2O_{bidist} ad 1000 ml

For solid media: 15 g/l agar

pH 7.0-7.5

Autoclaved LB medium was stored at 4°C.

b) SOC medium (component of TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA))

Tryptone 2 % w/v

Yeast extract 0.5 % w/v

NaCl 10 mM

KCl 2.5 mM

$MgCl_2$ 10 mM

$MgSO_4$ 10 mM

Glucose 20 mM

SOC medium was stored at -20°C.

B.7.4. Antibiotics

Kanamycin solution was added to autoclaved solid media at a temperature of ~50°C and to liquid media right before usage.

Kanamycin stock solution (Kan)
Kanamycin 100 mg/ml
Kan was dissolved in $H_2O_{bidist.}$
Kan was added to medium reaching a final concentration of 100 µg/µl.

B.7.5. Selection solutions

X-Gal stock solution
X-Gal (5-brom-4-chlor-3-indolyl-β-D-galactopyranoside) was dissolved in di-methylformamide (DMF) in a concentration of 40 mg/ml, filtrated sterile (0.22 µm pore size) and stored in the dark at -20°C.

B.7.6. Solutions for plasmid isolation

a) P1 buffer
Tris-HCl, pH 8.0 50 mM
EDTA 10 mM
RNAse A 100 µg/ml

b) NaOH/SDS solution
H_2O_{bidist} 8 ml
NaOH (2 M) 1 ml
10% SDS 1 ml

c) Potassium acetate/acetate solution
KCl (5 M) 6 ml
$H_2O_{bidist.}$ 2.85 ml
Acetic acid (pure) 1.15 ml

B.8. Cultivation and maintenance of recombinant *E. coli* strains

B.8.1. Culturing and cell harvesting

Solutions

LB medium

Kanamycin stock solution

Liquid cultures of recombinant *E. coli* cells for Plasmid Miniprep were established in 5 ml liquid LB medium in test-tubes. In a laminar flow 5 µl kanamycin stock solution was added to the LB medium. Cells were picked from plates using tooth sticks and were transferred into the medium. Cells were grown at 37 °C on an orbital shaker at 200 rpm overnight. Cells were harvested by two centrifugation steps (14000 rpm, 1 min) in a sterile ERT.

B.8.2. Maintenance

For short-term maintenance cells were cultivated on master plates: Cells were transferred to LB-Kan master plates with tooth sticks, grown overnight at 37 °C and stored at 4 °C.

For long-term maintenance cryostocks were prepared: 700 µl liquid overnight culture of cells produced as described above (Sec. B.8.1) was transferred into sterile 2 ml screw tubes under sterile conditions. 300 µl 50 % glycerol was added. The tube was incubated at 4 °C for 1 hour and stored at -80 °C.

B.9. Methods for isolation of DNA

B.9.1. Isolation of genomic DNA from activated sludge using the UltraClean™ Soil DNA Kit

Genomic DNA from 0.25 – 1g of activated sludge samples was isolated using the UltraClean™ Soil DNA Kit (MOBio Lab. Inc., Salana Beach, CA, USA) according to the manufacturers instructions.

B.9.2. Isolation of plasmid DNA from recombinant *E. coli* cells with Plasmid Miniprep

Plasmid DNA was isolated from *E. coli* cells using alkaline lysis, followed by organic precipitation of proteins and precipitation of DNA with isopropanol.

Solutions

Buffer P1

NaOH/SDS solution

Potassium acetate/acetate solution

Procedure

Overnight cultures of recombinant *E. coli* cells were established and harvested as described above (Sec. B.8.1). The pellet was resuspended in 100 µl P1 buffer and incubated for 5 min at RT for RNA digestion. Afterwards cells were lysed by addition of 200 µl NaOH/SDS solution, mixing by inversion of the tube and incubation on ice for 5 min. Tubes were inverted again during the incubation step. For precipitation of proteins, 150 µl potassium acetate/acetate solution was added, mixed by inversion of tube and incubated on ice for 5 min. The precipitated proteins were pelletized by centrifugation (14000 rpm, 1 min). The supernatant, which contained the DNA, was transferred to a sterile ERT. After addition of one volume of isopropanol and mixing DNA was precipitated at RT for 10 min. DNA was sedimented by centrifugation (14000 rpm, 1min) and the supernatant discarded. The pellet was washed in 500 µl ice-cold 70 % ethanol and centrifuged at 14000 rpm for 1 min. The supernatant was discarded and the DNA pellet dried by one further centrifugation step followed by removal of supernatant and drying on air. DNA was dissolved in 50 µl H_2O_{bidist} and stored at -20 °C until further analysis.

B.10. Quantitative und qualitative analysis of nucleic acids

B.10.1. Quantitative photometric analysis of nucleic acids

Quantitative photometric analysis of nucleic acids was performed using a NanoDrop® ND-1000 spectralphotometer (NanoDrop Technologies, Wilmington, USA). 1.5 µl of DNA solution was pipetted onto the end of the fiber optic cable. The DNA concentration was measured at $\lambda = 260$ nm.

B.10.2. Qualitative analysis of nucleic acids using agarose gel electrophoresis

For qualitative and semi-quantitative analysis of nucleic acids horizontal agarose gel electrophoresis was performed. This technique involves the separation of nucleic acid molecules in an agarose gel by size-dependent differences in migration speed when exposed to an electric field.

Reagents

1 - 3 % (w/v) agarose in 1 x TBE buffer
Loading buffer
DNA-ladder (1 kb, 100 bp ladder)
EtBr staining solution
SYBR® Green I staining solution

Procedure

Depending on the size of DNA fragments to be analyzed, 1 - 3 % agarose gels were produced. An appropriate amount of agarose was dissolved in 1x TBE buffer by heating in a microwave oven. The solution was subsequently cooled to ~50 °C and poured into a gel tray (Sub-Cell GT UV-Transparent Gel Tray (15x15cm), Biorad, Munich, Germany) equipped with a comb for the production of gel pockets. The gel was allowed to solidify for 30 – 45 min and then put into an electrophoresis apparatus (Sub-Cell GT, Biorad, Munich, Germany) filled with 1x TBE. DNA solutions were mixed with loading dye and applied to the pockets of the gel. For size determination of DNA fragments a DNA size standard was applied to the outer gel pockets. Gels were run at 80 – 120 V voltage for 40 – 90 min, depending on agarose concentration and size of DNA fragments. Gels were stained in EtBr or SYBR® Green I staining solution. SYBR® Green I staining solution was used if bands were excised for gel purification. DNA was visualized by placing the gel on a transilluminator (Biostep GmbH, Jahnsdorf, Germany) emitting UV light (λ = 312 nm) and documented by digital photography using a gel-documentation system (Biostep, Jahnsdorf, Germany).

B.11. In vitro amplification of DNA fragments via polymerase chain reaction (PCR)

Polymerase chain reaction was used to amplify genes of interest. This technique involves amplification of DNA by Taq DNA Polymerase starting at defined sequence positions after binding of oligonucleotides ("primers") to denaturized DNA.

B.11.1. Amplification of target gene fragments

B.11.1.1. Primers

Table B.7. Primers used for amplification of 16S rRNA genes.

Primer	Sequence (5' - 3')[a]	Binding position[b]	Annealing temperature [°C]	Specificity	Reference
907r	CCG TCA ATT CMT TTG AGT TT	907 - 926	65-55	Bacteria	Muyzer and Smalla 1998
518r	ATT ACC GCG GCT GCT GG	518 - 534	65-55	Bacteria	Muyzer et al. 1993
341f-GC[c]	CCT ACG GGA GGC AGC AG	341 - 357	65-55	Bacteria	Muyzer et al. 1993
616V	AGA GTT TGA TYM TGG CTC	8 - 27	52/56[d]	Bacteria	Juretschko et al. 1998
630R	CAK AAA GGA GGT GAT CC	1528 - 1542	52	Bacteria	Juretschko et al. 1998
1492r	GGY TAC CTT ACG ACT T	1492 - 1511	56	Bacteria	Lane 1991

[a] abbreviations according to IUPAC: M = A/C, K = G/T , Y = C/T

[b] according to *E.coli* 16S rRNA (Brosius *et al.* 1981)

[c] Primer with GC-clamp attached to 5' end: 5' - CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G - 3'

[d] 52 °C and 56 °C were used when combined with 630R and 1492r, respectively

Table B.8. Primers used for amplification of *nirS* and *nirK* genes.

Primer	Sequence (5' - 3')[a]	Binding position[b]	Annealing temperature [°C]	Specificity	Reference
cd3aF[c]	GTS AAC GTS AAG GAR ACS GG	916–935	57	*nirS*	Michotey et al. 2000
R3cd[d]	GAS TTC GGR TGS GTC TTG A	1322–1341	57	*nirS*	Throbäck et al. 2004
F1aCu[d]	ATC ATG GTS CTG CCG CG	568–584	57	*nirK*	Hallin and Lindgren 1999
R3Cuf[c]	GCC TCG ATC AGR TTG TGG TT	1021–1040	57	*nirK*	Hallin and Lindgren 1999

[a] abbreviations according to IUPAC:R = A/G, S = C/G

[b] Positions in the *nirS* gene of *Pseudomonas stutzeri* ZoBell ATCC 14405 (X56813) or in the *nirK* gene of *Alcaligenes faecalis* S-6 (D13155)

[c] for T-RFLP analysis primers were labelled with Fam at 5' end

[d] for T-RFLP analysis primers were labelled with Joe at 5' end

B.11.1.2. Reagents

$MgCl_2$ (25 mM) (Fermentas Inc., Hanover, MD, USA)

10 x Ex Taq polymerase-buffer (Fermentas Inc., Hanover, MD, USA)

Nucleotide-Mix (2 mM/dNTP) (Fermentas Inc., Hanover, MD, USA)

Bovine Serum Albumine (BSA; 20 mg/ml) (New England BioLabs Inc., Beverly, MA, USA)

Taq DNA-polymerase (5 units/µl) (Fermentas Inc., Hanover, MD, USA)

$H_2O_{bidist.}$

Promega PCR Mastermix (Promega Corporation, Madison, WI, USA)

B.11.1.3. Reaction Mixes

Table B.9. Reaction mix for amplification of 16S rRNA genes with primer pair 341f-GC - 518r.

	volume per reaction [µl]	stock concentration	end concentration
Template	1		
$MgCl_2$	4[a]	25 mM	2 mM
Taq buffer 10x	5	10 x	1 x
dNTP Mix	5	2 mM	0.2 mM
341f-GC	0.25	50 pmol / µl	0.25 pmol / µl
907r	0.25	50 pmol / µl	0.25 pmol / µl
BSA	0.25	10 mg / ml	0.05 mg / ml
Taq polymerase	0.2	5 units / µl	0.02 units / µl
$H_2O_{bidist.}$	34.05		
total volume	50 µl		

[a] $MgCl_2$ concentrations have been reduced for some experiments, in this case the amount of $H_2O_{bidist.}$ was increased to reach a final volume of 50 µl.

Table B.10. Reaction mix for amplification of 16S rRNA genes with primer pair 341fGC - 518r with Promega PCR Mastermix.

	volume per reaction [µl]	stock concentration	end concentration
Template	0.5		
Promega PCR mastermix	25	$MgCl_2$ - 3mM Taq buffer - 2x Taq polymerase – 0.05 units / µl dATP - 400 µm dGTP - 400 µm dCTP - 400 µm dTTP - 400 µm	$MgCl_2$ – 1.5 mM Taq buffer - 1x Taq polymerase - 0,025 units / µl dATP - 200 µm dGTP - 200 µm dCTP - 200 µm dTTP - 200 µm
341f-GC	0.25	50 pmol / µl	0.25 pmol / µl
907r	0.25	50 pmol / µl	0.25 pmol / µl
BSA	0.25	10 mg / ml	0.05 mg / ml
$H_2O_{bidist.}$	23.65		
total volume	50 µl		

Table B.11. Reaction mix for amplification of *nirS* genes.

	volume per reaction [µl]	stock concentration	end concentration
Template	1		
$MgCl_2$	3	25 mM	1.5 mM
Taq buffer	5	10 x	1 x
dNTP Mix	5	2 mM	0.2 mM
cd3aF	0.25	50 pmol / µl	0.25 pmol / µl
R3cd	0.25	50 pmol / µl	0.25 pmol / µl
BSA	0.25	10 mg / ml	0.05 mg / ml
Taq polymerase	0.2	5 units / µl	0.02 units / µl
$H_2O_{bidist.}$	35.05		
total volume	50 µl		

Table B.12. Reaction mix for amplification of *nirK* genes.

	volume per reaction [µl]	stock concentration	end concentration
Template	1		
$MgCl_2$	3	25 mM	1.5 mM
Taq buffer	5	10 x	1 x
dNTP Mix	5	2 mM	0.2 mM
cd3aF	0,5	50 pmol / µl	0.5 pmol / µl
R3cd	0.5	50 pmol / µl	0.5 pmol / µl
BSA	0.25	10 mg / ml	0.05 mg / ml
Taq polymerase	0.2	5 units / µl	0.02 units / µl
$H_2O_{bidist.}$	35.05		
total volume	50 µl		

Table B.13. Reaction mix for amplification of 16S rRNA genes with primer pair 341fGC - 907r.

	volume per reaction [µl]	stock concentration	end concentration
Template	1		
$MgCl_2$	3	25 mM	1.5 mM
Taq buffer	5	10 x	1 x
dNTP Mix	5	2 mM	0.2 mM
341f-GC	0.25	50 pmol / µl	0.25 pmol / µl
907r	0.25	50 pmol / µl	0.25 pmol / µl
BSA	0.25	10 mg / ml	0.05 mg / ml
Taq polymerase	0.2	5 units / µl	0.02 units / µl
$H_2O_{bidist.}$	35.05		
total volume	50 µl		

B.11.1.4. PCR Programs

A hot start at 95 °C was performed for all PCRs.

Table B.14. PCR-program for amplification of *nirS* and *nirK* genes.

PCR step	Annealing temperature °C	time (min)	
Denaturation	95	05:00	1 x
Denaturation	95	00:30	35 x
Annealing	57	00:30	
Elongation	72	01:00	
Final Elongation	72	10:00	1 x
Hold	20	inf.	1 x

Table B.15. PCR-program for amplification of 16S rRNA genes with primer pairs 616V - 1492r and 616V - 630R.

PCR step	Annealing temperature °C	time (min)	
Denaturation	95	05:00	1 x
Denaturation	95	00:40	35 x
Annealing	[a]	00:40	
Elongation	72	01:30	
Final Elongation	72	10:00	1 x
Hold	20	inf.	1 x

[a] Annealing temperatures according to Tab. B.7.

B.11.1.5. Procedure

The reaction mix (Sec. B.11.1.3) was prepared inside a PCR hood (Ultraviolet Sterilizing PCR Workstation, Peqlab Biotechnology GmbH, Germany) after decontamination with UV light for 10 – 15 min and cleaning with H_2O_2. Templates were added inside the PCR hood. Positive controls (PCR with template known to contain the gene of interest) and negative controls (PCR without addition of template) were always included. Templates for positive controls were added outside the PCR hood. Taq polymerase and reaction mix containing Taq polymerase were kept on ice as long as possible prior to the start of the PCR. PCRs were performed using either an Icycler (Biorad, Munich, Germany) or a Mastercycler gradient (Eppendorf, Hamburg, Germany) PCR cycler.

B.11.2. Gradient PCR

To determine the optimal annealing temperatures of primers, a gradient PCR was performed. A gradient PCR is a series of PCR reactions performed under equal conditions except for the variation of annealing temperature along a temperature gradient.

Table B.16. PCR-program for amplification of 16S rRNA genes with primer pair 341fGC – 518r.

PCR step	Annealing temperature °C	time (min)	
Denaturation	95	05:00	1 x
Denaturation	95	00:30	35 x
Annealing	[a]	00:30	
Elongation	72	00:30	
Final Elongation	72	10:00	1 x
Hold	20	inf.	1 x

[a] Gradient of annealing temperatures ranged from 57 – 64 °C.

B.11.3. Touchdown PCR

Touchdown PCR was performed to increase PCR specificity. In this approach a high annealing temperature is used in the first PCR cycle which is gradually lowered and than maintained for the remaining cycles.

Table. B.17. Touchdown PCR-program for amplification of 16S rRNA genes with primer pairs 341fGC-518r and 341fGC - 907r.

PCR step	Annealing temperature °C	time (min)	
Denaturation	95	05:00	1 x
Denaturation	95	00:30	20 x
Annealing	65 -55 [a]	00:30	
Elongation	72	00:30	
Denaturation	95	00:30	15 x
Annealing	55	00:30	
Elongation	72	00:30	
Final Elongation	72	10:00	1 x
Hold	20	inf.	1 x

[a] temperature was decreased every cycle by 0.5 °C.

B.11.4. Addition of dATP-overhangs to PCR products

Taq polymerase adds dATPs which are essential for cloning to the ends of amplified strands PCR products into TOPO TA cloning vectors. During storage dATP-overhangs are easily degraded, therefore it is required to add dATPs to PCR products that were stored longer than one day prior to cloning.

Reagents

TaKaRa Ex Taq buffer (10 x) (TaKaRa, Otsu, Japan)

dATP (25µM) (TaKaRa, Otsu, Japan)

TaKaRa Taq polymerase (5 units/µl) (TaKaRa, Otsu, Japan)

Procedure

The reaction mix was prepared according to table B.18 and incubated in a Thermocycler (Icycler Biorad, Munich, Germany) at 72 °C for 10 min.

Table B.18. Reaction mix for addition of dATP-overhangs to PCR products.

	volume per reaction [µl]	stock concentration	end concentration
PCR - product	13.9		
dATP	1.75	25 µM	2.5 µM
Taq buffer	1.75	10 x	1 x
TaKaRa Taq polymerase	0.1	5 units / µl	0.03 units / µl
total volume	17.5 µl		

B.12. Purification of PCR products

Prior to sequencing reactions and T-RFLP analysis PCR products have to be purified to remove oligonucleotides, salts and enzymes.

B.12.1. Purification of PCR products with QIAquick PCR Purification Kit (QIAGEN GmbH, Germany)

PCR products were purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions.

B.12.2. Gel purification of PCR products with QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany)

Agarose gels were prepared and the run was performed as described above (Sec. B.10.2). Gels were stained in SYBR® Green I staining solution as described above (Sec. B.10.2). DNA was visualized on a transilluminator (Biostep GmbH, Jahnsdorf, Germany) and bands of correct size were cut out using a scalpel. Cut out bands were purified using the QIAquick Gel Extraction Kit according to the manufacturer's instructions.

B.13. Cloning of gene amplicons using the TOPO TA cloning® kit

For sequence analysis of PCR products, which contain a mix of DNA sequences it is necessary to isolate sequence types before performing the sequencing reaction. For this purpose, PCR products are ligated into cloning vectors which are subsequently transformed into *E. coli* TOP10 cells. *E. coli* cells are added to this reaction in a far higher proportion than vectors to make sure that no cell takes up more than one vector. In this way PCR products are isolated and can be used for sequencing.

B.13.1. Ligation

The TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) uses self-ligating high copy plasmids into which Taq-polymerase amplified PCR products are ligated. TOPO TA vectors contain PolyT overhangs which bind PolyA overhangs of PCR products produced by Taq-polymerase. A vector bound Topoisomerase I ligates the PCR product into the vector.

Procedure

PCR products of *nirS* and *nirK* genes were produced from sample KDF according to B.11. For ligation either freshly produced PCR products were used or PCR products older than one day with newly attached PolyA overhangs (see Sec. B.11.6).

A low melting agarose gel was produced as follows: 0.4 g of agarose (NuSieve® 3:1 Agarose low melting) was dissolved in 0.35 ml 1x TAE buffer by heating in a microwave oven. The liquid was cooled to ~50 °C and then poured into a gel tray (HoeferTM HE 33 gel running tray (7x10cm)) equipped with a comb to produce gel pockets. The gel was allowed to polymerize for 30 – 45 min and then put into a cooled electrophoresis apparatus (HoeferTM HE 33 Mini Horizontal submarine unit) filled with 1x TAE. 10 – 20 µl of PCR products was mixed with loading dye and applied to the gel pockets. A sizemarker (GeneRuler™ 1kb DNA Ladder, Fermentas, St. Leon-Rot, Germany) was applied to the outer gel pockets. The gel was run at 100 mA for 90 min and afterwards stained in SYBR® Green I staining solution for 45 min. Stained DNA was visualized by placing the gel on a transilluminator (Biostep GmbH, Jahnsdorf, Germany) emitting UV light (λ = 312 nm). Bands of correct size were excised with a glass capillary and subsequently blown out of the capillary into an ERT by the use of a 200 µl pipette and a yellow tip. The agarose was melted by putting the tube into a waterbath at 70 °C. 100 µl of sterile $H_2O_{bidist.}$ was added and mixed by finger flipping the tube. The

solution was put into the waterbath again for 2 min. Afterwards 10 µl of the solution was used for the ligation reaction:

Reaction mixture for ligation (melted PCR product):

melted PCR product	10 µl
TOPO TA vector (pCR®II)	1.5 µl
(Invitrogen Corporation, Carlsbad, CA, USA)	
salt solution	1.5 µl

Alternatively, PCR product was used directly for ligation:

Reaction mixture for ligation (direct use of PCR product):

melted PCR product	4 µl
TOPO TA vector	1 µl
(Invitrogen Corporation, Carlsbad, CA, USA)	
salt solution	1 µl
(Invitrogen Corporation, Carlsbad, CA, USA)	

Ligation was performed for 15 - 20 min at RT. Afterwards the reaction mix was centrifuged and put on ice.

B.13.2. Transformation

Transformation of plasmids into chemically competent *E. coli* cells was done by heat shocking of cells. Transformed cells were identified by growth on kanamycin plates, as the vector contains a kanamycin resistance gene.

Preparations

SOC medium was thawed and kept at RT. Chemically competent *E. coli* cells were thawed on ice. Two LB-Kan plates were dried at 46 °C in an incubation oven. A waterbath was heated to 42 °C.

Procedure

The reaction mix was added to the competent cells and carefully stirred. The mixture was incubated on ice for 30 min. Afterwards the cells were heatshocked for 30 sec at 42 °C in a waterbath and immediately put on ice for 2 min. 250 µl of SOC medium at RT was added and

the cells were incubated at 37 °C for 60 – 90 min on a shaker (Platform Shaker Innova 2300, New Brunswick Co., Inc., Madison NJ, USA). Inside a laminar flow (Laminar flow hood Safe 2010 Modell 1.2, Holten, Jouan Nordic, Allerød, Dänemark) 40 µl of X-Gal solution was plated on pre-warmed LB-Kan-plates. 100 µl and 150 µl of cell suspension was plated on an X-Gal/LB-Kan plate. Plates were incubated at 37 °C overnight.

B.13.3. Identification of recombinant clones

Insert-positive cells were identified by blue/white screening. The LacZα gene harboured by TOPO TA vectors complements the LacZ gene fragment encoded by the *E coli* host to form α-Galactosidase which will subsequently cleave X-Gal to form a bluish dye. By ligating inserts into the vector the LacZα gene gets disrupted. Therefore insert-positive colonies are recognizable by white or light blue colour. Incubation of plates at 4 °C intensifies the blue colour. Inside a laminar flow (Laminar flow hood Safe 2010 Modell 1.2, Holten, Jouan Nordic, Allerød, Dänemark) white or light blue colonies were picked with a tooth stick and transferred to a LB-Kan master plate. Residual cells on the tooth stick were used for insert screening PCR.

B.13.4. Test of insert size via M13-screening PCR and agarose gel electrophoresis

Inserts of TOPO TA vectors were amplified by PCR using M13 primer binding sites located on the vector. Insert size was checked by agarose gel electrophoresis.

Procedure

A PCR reaction mix (Tab. B.19) was prepared inside a PCR hood (Ultraviolet Sterilizing PCR Workstation, Peqlab Biotechnology GmbH, Germany) after decontamination with UV light for 10 – 15 min and cleaning with H_2O_2. After plating on a masterplate (see Sec. B.8.2) residual cells on the tooth stick were suspended in PCR reaction mix. A PCR program according to table B.21 was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) PCR cycler. 5 µl of PCR product were used for agarose gel electrophoresis according to section B.10.2. Clones harbouring plasmids with inserts of correct size (~410 - 420 bp for *nirS*, 473 bp for *nirK*) were used for further analysis.

Table B.19. Reaction mix for amplification of plasmid inserts.

	volume per reaction [µl]	stock concentration	end concentration
$MgCl_2$	2	25 mM	1.5 mM
Taq buffer	2.5	10 x	1 x
dNTP Mix	2.5	2 mM	0.2 mM
M13f	0.125	50 pmol / µl	0.25 pmol / µl
M13r	0.125	50 pmol / µl	0.25 pmol / µl
Taq polymerase	0.1	5 units / µl	0.02 units / µl
$H_2O_{bidist.}$	17.65		
total volume	25 µl		

Table B.20. Primers used for amplification of plasmid inserts.

Primer	Sequence (5' - 3')	Annealing temperature [°C]	Reference
M13 reverse	GTA AAA CGA CGG CCA G	60	TOPO cloning kit (Invitrogen Corporation, Carlsbad, CA, USA)
M13 forward	CAG GAA ACA GCT ATG AC	60	

Table B.21. PCR-program for amplification of plasmid inserts.

PCR step	Annealing temperature °C	time (min)	
Denaturation	95	05:00	1 x
Denaturation	95	00:30	35 x
Annealing	60	00:30	
Elongation	72	01:30	
Final Elongation	72	10:00	1 x
Hold	20	inf.	1 x

B.14. Restriction fragment length polymorphism (RFLP)

RFLP analysis was performed with products of insert screening PCR to identify different sequence types by differences in locations and abundance of restriction endonuclease cutting sites. PCR products were cut with restriction endonuclease *Msp*I (Tab.B.22) and applied to an agarose gel to visualize restriction fragments.

RFLP reaction mix was prepared according to table B.23 and incubated at 37 °C for 3 hours. Restriction reaction was stopped by adding 5 µl of loading dye which contains EDTA to inhibit endonuclease activity. The whole reaction mix was applied to a 3 % agarose gel and run at 80 V voltage for 90 min (for details on agarose gel electrophoresis see B.10.2). 1 – 3 clones per banding pattern were further analyzed by isolating the plasmid from the cells

(B.9.2) and using it for sequencing reaction (B.15). Sequence analysis was perfomed according to sections B.16.1 and B.16.2.

Tab. B.22. Restriction enzyme used for RFLP.

Enzyme	restriction site[a]	Enzyme buffer	incubation temperature (°C)	incubation time	source of supply
MspI (HpaII)	C↓CGG	Tango	37	3 h	Fermentas Life Sciences Inc., Hanover, MD, USA

[a] arrow indicates site of restriction

Table B.23. Reaction mix for RFLP.

	volume per reaction [µl]	stock concentration	end concentration
PCR - product	5		
Enzyme (HaeIII)	0.2	10 u / µl	1 u / µl
Enzyme buffer Tango	1	10 x	1 x
$H_2O_{bidist.}$	3.8		
total volume	10 µl		

B.15. DNA sequencing

PCR products and plasmid inserts were sequenced following the principle of cycle-sequencing which is a combination of the di-deoxy mediated chain termination method (Sanger et al. 1977) and PCR (Saiki et al. 1988). PCR is performed with template specific primers in a reaction mix containing dNTPs as well as ddNTPs. ddNTPs lack the 3' OH group which is needed for further extension of the PCR product, therefore the PCR stops at this particular point. Because the four different ddNTPs are labelled with 4 different dyes, it is possible to infer the identity of the nucleotide at the 3' end from the colour of the PCR product. PCR products of different length and colour are electrophoretically separated and colours are detected in a capillary of the sequencing machine. The nucleotide sequence can subsequently be inferred from the sequence of detected colours.

DNA sequencing was performed using a 3130xl Genetic Analyzer (Applied Biosystems Lincoln, USA) following the instructions of the manufacturer. For sequencing of plasmid inserts vector-specific primers were used, for sequencing of PCR products purified from DGGE bands, the respective reverse primers were used (Tab. B.20, Tab. B.7).

B.16. Analyses of sequences

B.16.1. Proofreading of sequences using Finch TV

The software program Finch TV (http://www.geospiza.com/finchtv/) was used to visualize the chromatograms of sequences. Sequences determined automatically by the software of the sequencing machine were checked and corrected if necessary.

B.16.2. Quick analyses of sequences using BLAST

To check for primer specificity sequences of cloned PCR products of *nirS* and *nirK* primers were analyzed by comparing them against the NCBI database using the search algorithm BLAST (basic local alignment search tool, Altschul et al. 1990, www.ncbi.nlm.nih.gov/BLAST). BLAST was designed to find regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer evolutionary relationships between sequences.
BLAST search was further used for a rough phylogenetic characterization of 16S rDNA sequences derived from DGGE bands to find related sequences that could subsequently be used for alignment in ARB (see Sec. B.16.3.1)

B.16.3. Comparative sequence analyses using the ARB software package

16S rDNA sequences derived from DGGE bands were analyzed using the software package ARB (Ludwig et al. 2004). The ARB software package comprises different programs and tools for sequence database maintenance and analysis, as for example tools for import and export of sequences, sequence alignment, primary and secondary structure editing, filter calculation, phylogenetic analyses, and primer and probe design.

B.16.3.1. Alignment of sequences using the ARB software package

Sequences were aligned against related sequences determined by BLAST searches. Automated alignment was performed (if possible) by using the fast aligner function of the editor tool. Manual alignment was performed if the fast aligner function did not work.

B.16.3.2. Phylogenetic analyses using ARB

After alignment sequences were added to an up-to-date 16S rDNA tree by using the "quick add marked" function.

B.16.4. Design of 16S rRNA-targeted oligonucleotide probes using ARB and probe evaluation

FISH probes targeting organisms which were represented by excised DGGE bands, were designed using the Probe design and Probe match tools of the ARB software package. The Probe design tool uses sequences contained in a server (PT-server) to search for specific target regions of specified target sequences. The Probe match tool was used to verify the specificity of probes suggested by the Probe design tool by aligning the probes against the whole database. The specificity of probes was furthermore evaluated using the probeCheck tool (http://www.microbial-ecology.net/probecheck/, Loy et al. 2008) as well as the probe match tool of the ribosomal database project (http://rdp.cme.msu.edu/, Cole et al. 2003). The accessibility of the respective target sites due to secondary structures as determined for the rRNA molecule of *E .coli* (Fuchs et al. 1998) and consequently the brightness of FISH signal to be expected was also taken into account for probe evaluation. The goal was to find FISH probes that are highly specific for the respective DGGE band sequence and target no or few other sequences in the database. Furthermore, probes were supposed to have a length of 18 nucleotides and a GC content of approximately 50 %, if possible.

B.16.5. Selection of restriction endonucleases for T-RFLP

Restriction endonucleases for T-RFLP analyses were chosen by their ability to discriminate sequences in the *nirS* and *nirK* clone libraries according to their terminal restriction sites. Heterogeneity of terminal restriction fragments (T-RFs) produced by different enzymes was evaluated *in silico* by manual comparison of restriction patterns using the software program "Clone manager" (Scientific & Educational Software) as well as using the web based program "REMA" (http://www.macaulay.ac.uk/rema, Szubert et al. 2007). Three restriction enzymes expected to produce the highest heterogeneity were selected.

B.17. Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis is a technique that is frequently used for producing fingerprints of microbial communities which can be compared over temporal or spatial scales. It is based on the different melting behaviour of nucleic acid duplexes due to differences in GC and accordingly AT content. PCR products of equal length and different sequence are electrophoretically separated in a polyacrylamide gel along an increasing concentration of denaturating substances (urea and formamide). When a duplex reaches a position in the gel, where the denaturant concentration is high enough, it melts and gets stuck in the gel. To improve sticking of PCR products in the gel, primers with so called GC-clamps, i.e. a GC-rich sequence attached to the 5' end of the primer, are used. That way a banding pattern is produced on the gel that represents the sequence variation of the applied PCR products.

B.17.1. PCR

PCR was performed according to section B.11 using primer pairs 341fGC/907r and 341fGC/518r (Tab. B.7).

B.17.2. Preparation of acrylamide stock solutions

Stock solutions containing 0 % and 80 % denaturants for 6 % and 8 % acrylamide gels were prepared according to the following recipes.

0 % denaturant stock solution for 6 % and 8 % acrylamide gels:

AA/Bis Solution 40 % (37.5:1)	37.5 ml (6 % gels)/ 50 ml (8 % gels)
50 x TAE	5 ml
H_2O_{bidist}	ad 250 ml

80% denaturant stock solution for 6 % and 8 % acrylamide gels:

Urea	84 g
AA/Bis Solution 40 % (37.5:1)	37.5 ml (6% gels)/ 50 ml (8% gels)
50 x TAE	5 ml
Formamide	80 ml
$H_2O_{bidist.}$	ad 250 ml

Important: Urea was first added to and dissolved in 60-100 ml of H_2O_{bidist}, afterwards the remaining reagents were added.

The stock solutions were mixed and transferred to a dark bottle. They were stored in the dark at 4 °C.

B.17.3. Preparation of polyacrylamide gels

Solutions

Acrylamide stock solution with 0 % urea
Acrylamide stock solution with 80 % urea
Ammonium peroxydisulfate (APS)
N,N,N',N'-tetra-methyl-ethylene-di-amine (TEMED)

Procedure

Glass plates were washed with soap and subsequently cleaned with 70 % Ethanol. The gel apparatus (DCodeTM system for DGGE, Biorad, München, Germany) was assembled. 2 ml of acrylamide stock solutions containing 80 % denat. were mixed with 25 µl APS and 8 µl Temed and subsequently pipetted between the glass plates. Acrylamide solutions corresponding to the endpoints of the respective gradient were mixed according to table B.24. Gradient solutions were poured into the gradient mixer (Econo model EP-1 gradient pump, Biorad, München, Germany) and 30 µl APS and 8 µl Temed was added to the gradient solutions. The mixed solutions were poured between the glass plates. 5 ml of acrylamide stock solutions containing 0 % denat. was mixed with 20 µl APS and 8 µl Temed and pipetted onto the gradient gel. The gel was allowed to polymerize for 1 – 2 hours. Gels could be stored at 4 °C and wrapped in plastic foil for up to one week.
For separation of PCR products amplified with primer pairs 341fGC/907r or 341fGC/518r 6 % or 8 % gels were used, respectively.

Table B.24. Mixing ratio of acrylamid stock solutions for production of acrylamid solutions with different denaturant concentrations.

AA solution 0 % denat. [ml]	AA solution 80 % denat. [ml]	Final volume 11 ml: denat. end concentration [%]
0.0	11.0	80.00
0.5	10.5	76.36
1.0	10.0	72.73
1.5	9.5	69.09
2.0	9.0	65.45
2.5	8.5	61.82
3.0	8.0	58.18
3.5	7.5	54.55
4.0	7.0	50.91
4.5	6.5	47.27
5.0	6.0	43.64
5.5	5.5	40.00
6.0	5.0	36.36
6.5	4.5	32.73
7.0	4.0	29.09
7.5	3.5	25.45
8.0	3.0	21.82
8.5	2.5	18.18
9.0	2.0	14.55
9.5	1.5	10.91
10.0	1.0	7.27
10.5	0.5	3.64
11.0	0.0	0.00

B.17.4. Electrophoresis

The polymerized gel was put into the electrophoresis chamber (DCodeTM system for DGGE, Biorad, München, Germany) which was filled with pre-warmed (63°C) 1x TAE buffer and pre-run at 100V for 15 – 30 min. Afterwards the slots were rinsed with prewarmed 1x TAE buffer. Equal amounts of PCR product, estimated based on the intensity of bands on an agarose gel, of the different environmental samples were mixed with loading dye (6x DNA Loading Dye, Fermentas, St. Leon-Rot, Germany) and applied to the slots of the gel. Electrophoresis was performed at 100V and 60 °C for 16 hours. Gels were stained with SYBR® Green I (0.1 µl per ml 1x TAE) for 45 min. Evaluation of stained gels was done as described above (Sec. B.10.2).

B.17.5. Excision of DGGE bands for sequencing

Bands of interest were cut out of the stained gel with a scalpel and placed in a sterile 1.5 ml ERT. 50 µl of sterile $H_2O_{bidist.}$ was added to elute the DNA. The bands were incubated at 4 °C for at least 24 hours. 1 µl of eluted PCR product was used for PCR according to section B.17.1. PCR products were purified according to section B.12.1 and used for sequencing. Sequencing was done according to section B.15, sequence analysis was performed according to sections B.16.1 and B.16.3.

B.18. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a method frequently used to produce fingerprints of microbial communities. It is based on differences in the location of the terminal restriction endonuclease cutting site between sequences. Target genes are PCR-amplified using fluorescently labelled primers. The PCR products are subsequently digested by restriction endonucleases. Different sequences are expected to harbour terminal restriction sites, i.e. restriction sites next to the ends of the PCR product, at different positions, resulting in a mixture of labelled DNA fragments of different lengths. This mixture is separated by electrophoresis in a capillary, occurrence of fragments is photometrically detected and the length of the fragments is inferred by comparison with a size standard.

B.18.1. PCR

PCR was performed according to section B.11 using labeled primers according to table B.8. For environmental samples, products of two individual DNA extractions were pooled and used as template for PCR. For plasmid mixes, similar amounts of plasmids representing the OTUs defined in tables C.1 and C.2 were mixed and used as template for PCR. One clone per OTU was included in the analysis. Plasmid concentrations were determined by measurement with NanoDrop ND-1000 photometer before producing the template mixture. PCR products of environmental samples and plasmid mixes were purified via gel extraction according to section B.12.2. PCR products of plasmids were purified using a PCR purification kit according to section B.12.1. For PCR products of plasmid mixes, products of two PCRs were pooled before purification, for PCR products of environmental samples, products of four PCRs were pooled. Before application onto the gel, pooled PCR products were concentrated

to a volume of 20-30 µl using a Concentrator 5301 Vacufuge (Eppendorf, Hamburg, Germany).

B.18.2. Klenow fragment incubation

Purified PCR products were incubated with Klenow fragment exo$^-$ in a reaction mix supplemented with dNTPs prior to digestion to fill in bases in partially single stranded amplicons (Egert 05). Reaction mixes were prepared according to table B.25 in 0,2 ml ERTs. Klenow fragment reaction was performed in an Icycler (Biorad, Munich, Germany) according to table B.26.

Table B.25. Reaction mix for Klenow fragment exo$^-$ incubation of PCR products and restriction digest for T-RFLP

Restriction enzyme used for digestion	*Mnl*I		*Hae*III		*Mbo*II		
	volume	stock concentration	volume	stock concentration	volume	stock concentration	end concentration
Klenow fragment exo$^-$	0.2 µl	50 units / µl	0.2 µl	50 units / µl	0.2 µl	50 units / µl	~0.1 units / µl
Enzyme buffer (NEB buffer 2)	10 µl	10 x	10 µl	10 x	10 µl	10 x	1 x
dNTP Mix	5 µl	2mM	5 µl	2mM	5 µl	2mM	0.1mM
PCR product[a]	x µl		x µl		x µl		
H_2O_{bidist}	ad 98 µl		ad 99.5 µl		ad 99 µl		
Reagents added after Klenow fragment exo$^-$ incubation for restriction digest:							
Restriction enzyme	1 µl	5 units / µl	0.5 µl	10 units / µl	1 µl	5 units / µl	0.05 units / µl
BSA (NEB)	1 µl	100x					1 x
final volume	100 µl		100 µl		100 µl		

[a] volume depended on DNA concentration evaluated by agarose gel electrophoresis.

Table B.26. Thermocycler program for Klenow fragment exo$^-$ incubation of PCR products.

Incubation step	temperature [°C]	time [min]
incubation	37	60
inactivation of enzyme	75	20
hold	15	inf.

B.18.3. Restriction digest

For digestion of PCR products without Klenow fragment preincubation. reaction mixes were prepared according to table B.27 for PCR products of environmental samples and plasmid mixes and according to table B.28 for PCR products of plasmids. For PCR products digested after Klenow fragment preincubation restriction enzyme (and BSA for digests with *Mnl*I) was

added to the reaction mix (Tab. B.25) after inactivation of Klenow fragment and cooling of reaction mix to RT. Reaction mixes were kept on ice until digestion. Digestion was performed in an Icycler (Biorad, Munich, Germany) according to table B.29. Digested PCR products were concentrated to a volume of approximately 20 µl in Concentrator 5301 Vacufuge (Eppendorf, Hamburg, Germany).

Table B.27. Reaction mix for restriction digest of PCR products of environmental samples and plasmid mixes for T-RFLP.

	***Mnl*I**		***Hae*III**		***Mbo*II**		
		stock concentration		**stock concentration**		**stock concentration**	**end concentration**
Restriction enzyme	1 µl	5 units / µl	0.5 µl	10 units / µl	1 µl	5 units / µl	0.05 units / µl
Enzyme buffer (NEB buffer 2)	10 µl	10 x	10 µl	10 x	10 µl	10 x	1 x
PCR product[a]	x µl		x µl		x µl		
BSA (NEB)	1 µl	100x					1 x
H_2O_{bidist}	ad 100 µl		ad 100 µl		ad 100 µl		

[a] volume depended on DNA concentration evaluated by agarose gel electrophoresis.

Table B.28. Reaction mix for restriction digest of PCR products of plasmids for T-RFLP.

	***Mnl*I**		***Hae*III**		***Mbo*II**		
		stock concentration		**stock concentration**		**stock concentration**	**end concentration**
Restriction enzyme	1 µl	5 units / µl	0.5 µl	10 units / µl	1 µl	5 units / µl	0.05 units / µl
Enzyme buffer (NEB buffer 2)	2 µl	10 x	2 µl	10 x	2 µl	10 x	1 x
PCR product[a]	x µl		x µl		x µl		
BSA (NEB)	1 µl	100x					1 x
H_2O_{bidist}	ad 20 µl		ad 20 µl		ad 20 µl		

Table B.29. Thermocycler program for restriction digests for T-RFLP.

	temperature [°C]			**time [min]**		
Incubation step	***Mnl*I**	***Hae*III**	***Mbo*II**	***Mnl*I**	***Hae*III**	***Mbo*II**
restriction	37	37	37	240	240	60
inactivation of enzyme	80	80	65	20	20	20
hold	15	15	15	inf.	inf.	inf.

B.18.4. Purification of restriction digests

Concentrated restriction digests were purified using Sephadex. 75 g of Sephadex G50 (Sigma-Alderich Chemie GmbH, Steinhausen, Germany) was dissolved in 1 l H_2O_{bidist}. 200 µl of Sephadex solution was pipetted into a well of a MultiScreen 96–well plate (Millipore

Corporation, Billerica, MA, USA) and centrifuged for 1 min at 250 g. This procedure was repeated with 200 µl Sephadex solution. The concentrated restriction digest was pipetted into the well and centrifuged for 1 min at 420 g. The purified restriction digest was concentrated in a Concentrator 5301 Vacufuge (Eppendorf, Hamburg, Germany) until all liquid was gone. The dried DNA was resuspended in 10 µl Hi-Di Formamide(Applied Biosystems Lincoln, USA) and mixed with 0.5 µl Genescan 350 Rox sizemarker (Applied Biosystems Lincoln, USA). The DNA was denatured at 95 °C for 5 min and immediately placed on ice to avoid renaturation.

B.18.5. Electrophoresis

The denatured restriction digest was electrophoretically separated for 3000 s at 15 kV in a 3130xl Genetic Analyzer (Applied Biosystems Lincoln, USA) using 50 cm capillaries equipped with polymer POP7. Calibration was done using the Multi-Capillary DS-32 (Dye Set F) Matrix Standard Kit (Applied Biosystems Lincoln, USA).

B.18.6. Analysis of T-RFLP profiles

T-RFLP profiles were analyzed using the software program “Peak Scanner” (Applied Biosystems). A peak height of 25 relative fluorescence units (rfu) was determined as minimum peak height threshold. The analysis of the program was manually checked and adjusted if necessary with respect to peak area considered for analysis and peaks considered in the size standard profile. T-RFs with a predicted size of less than 30 bp were omitted from the profiles because such fragments may represent primers. Only profiles with a summed peak area greater than 20000 rfu were analyzed further. To account for differing amounts of DNA used for different digests and to minimize noise, peaks with an area smaller than 1 % of the summed peak area were omitted. PCR products of environmental samples were digested in triplicates, those of plasmid mixes in duplicates. Replicate profiles were aligned manually and the presence or absence of peaks was determined. Peaks present in two of three replicates (environmental samples) or two of two replicates (plasmid mixes) were considered for profile consensus. Consensus profiles of environmental samples were subjected to statistical analysis according to section B.20.

B.19. Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization is a cultivation independent approach that allows the detection of microorganisms in their natural habitat using fluorescently labeled oligonucleotide probes which bind the 16S or 23S rRNA. FISH involves cell fixation, immobilization of cells to a microscopie slide, hybridization of oligonucleotides to rRNA under stringent conditions and finally detection of fluorescently labelled cells using epifluorescence microscopy.

B.19.1. Cell fixation

For stabilization of bacterial cell morphology, environmental samples were fixed with PFA (for fixation of gram negative bacteria) or ethanol (fixation of gram positive bacteria).

B.19.1.1. Cell fixation with paraformaldehyde (PFA)

a) Production of a 4 % PFA solution

Reagents

3 x PBS

1 M NaOH

1 M HCl

Procedure

33 ml of $H_2O_{bidist.}$ was heated to 60-65 °C. 2 g PFA was added and subsequently 1 M NaOH was added drop by drop until PFA had dissolved and the solution cleared. 16.6 ml 3 x PBS was added and the solution cooled until it reached RT. The pH was adjusted to 7.2-7.4 by addition of 1 M HCl. The solution was filtered sterile (0.22 µm pore size), cooled to RT and stored at -20 °C.

b) Cell fixation with paraformaldehyde (PFA)

Three volumes of 4 % PFA solution was added to one volume of sludge sample and incubated at 4 °C for 30 min to 12 h (done by our cooperation partners at Limnotec). Afterwards samples were centrifuged at 11000 rpm for 15 min. After removal of supernatant, the pellet was washed in 1x PBS and centrifuged at 11000 rpm for 15 min. After removal of

supernatant, the pellet was resuspended in one volume 1x PBS and one volume of Ethanol abs. Samples were stored at -20 °C.

B.19.1.2. Cell fixation with ethanol (done by our cooperation partners)

Sludge samples were fixed by adding one volume of Ethanol abs. and stored at -20 °C.

B.19.2. *In situ* hybridization

B.19.2.1. 16S rRNA targeted oligonucleotide probes

B.19.2.1.1. 16S rRNA targeted oligonucleotide probes used

For selection of appropriate FISH probes the online database probeBase (http://www.microbial-ecology.net/probebase/, Loy et al. 2003) was used. If no probes were found, new probes were designed. (see Sec. B.16.4). The characteristics of oligonucleotide probes and fluorescence dyes used are listed in tables B.30 and B.31, respectively.

Table B.30. Oligonucleotides used for FISH analyses.

Probe	Sequence 5'-3'	Target molecule	Binding position[a]	Formamid [%]	Specificity	Reference	Competitor Sequence 5'-3'
Ac442	GCC CAC CTT CGT CCC TGA	16S rRNA	442 - 459	no signel	DGGE band A5_2 and some related *Acidobacteria*	this study	
Aqua444	CCA GAA GAT TTC TTC CCT	16S rRNA	445 - 461	no signal	bacteria related to DGGE band A2_1 and other *Burkholderiales*	this study	
Bact414	TAC AAG GCG TAG CCC CTT	16S rRNA	414 - 431	30	DGGE band C1_1	this study	
Bact448	AGA ATG AGC TTT TTT CGT	16S rRNA	448 - 465	35	some *Bacteroidetes* related to DGGE band A7_2	this study	
Bet42a	GCC TTC CCA CTT CGT TT	23S rRNA	1027 - 1043	35	*Betaproteobacteria*	Manz 1992	GCC TTC CCA CAT CGT TT
Cd847	TAG CTT CAT GAC ACA GAG	16S rRNA	847 - 864	no signal	DGGE band A3	this study	
EUB338	GCT GCC TCC CGT AGG AGT	16S rRNA	338 - 355	0-50	most *Bacteria*	Amann *et al.* 1990	
EUB338II	GCA GCC ACC CGT AGG TGT	16S rRNA	338 - 355	0-50	*Planctomycetales*	Daims *et al.* 1999	
EUB338III	GCT GCC ACC CGT AGG TGT	16S rRNA	338 - 355	0-50	*Verrucomicrobia*	Daims *et al.* 1999	
HGC69a	TAT AGT TAC CAC CGC CGT	23S rRNA	1901 - 1918	25	*Actinobacteria*	Roller *et al.* 1994	TAT AGT TAC GGC CGC CGT
Meth444	GCA GAC CGT TTC TTC CCT	16S rRNA	444 - 461	40	some *Burkholderiaceae* related to *Methylibium aquaticum* and DGGE band A10_2	this study	
NONEUB	ACT CCT ACG GGA GGC AGC	-	-	0 - 30	control (complementary to EUB338)	Wallner *et al.* 1993	
Par651	ACC TCT CTC GAA CTC CAG	16S rRNA	651 - 668	40	*Paracoccus* sp.	Neef *et al.* 1996	
RHC445	CCC ATG CGA TTT CTT CCC	16S rRNA	445 - 462	20	Activated sludge clones related to *Dechloromonas*	Hesselsoe unpublished	
RHC827	TTA CCC ACC CAA CAC CTA	16S rRNA	827 - 844	20	Activated sludge clones related to *Dechloromonas*	Hesselsoe unpublished	

[a] according to *E. coli* 16S rRNA or 23S rRNA (Brosius et al., 1981)

Table B.31. Characteristics of fluorescence dyes used.

Fluorescence dye	Max. of absorption [nm]	Max. of emission [nm]	ε [l/mol×cm][a]
Fluos	494	518	7.5×10^4
Cy3	554	570	1.3×10^5
Cy5	650	667	$\geq2\times10^5$

[a] molar extinction coefficient.

B.19.2.1.2. Evaluation of hybridization and washing conditions

To evaluate the appropriate stringency conditions for newly designed probes, formamide series were performed using sludge samples. Formamide concentrations between 5 and 50 percent were evaluated in 5 percent intervals. For each probe a sample was selected that showed presence of the DGGE band for which the respective probe had been designed. Probes of wider specificity were hybridized together with designed probes to confirm binding of the probes to target organisms. If fluorescently labelled structures were detected that could not be verified to be cells by this hierarchical probe approach, a control probe consisting of a sequence that is not likely to bind to the rRNA of any bacterium (NONEUB) labelled with the same fluorescent dye as the designed probe was hybridized to the sample to exclude the possibility of unspecific binding.

B.19.2.2. Cell immobilization

Two times 10 µl of sludge samples was pipetted onto a slide and dried at 46 °C successively until all liquid was gone.

B.19.2.3. Dehydration of the sample

For further dehydration of the samples an ethanol series was performed. The slides were put into 50 %, 80 % and 96 % ethanol successively, 3 min at a time.

B.19.2.4. Permeabilization of bacterial cell walls with lysozyme

Gram positive cell walls were digested with lysozyme before cells were hybridized to probe HGC69a. 15 µl of lysozyme solution (5 mg lysozyme in 1 ml TE puffer) was pipetted onto ethanol fixed dehydrated biomass. The slide was incubated in a moist chamber for 45 min at 37 °C. Afterwards the slide was washed in icecold MilliQ for 1 min and dehydrated in ethanol abs. for 1 min. Residual ethanol was removed with compressed air.

B.19.2.5. Hybridization and washing buffers

Hybridization and washing buffers were prepared according to tables B.32 and B.33, respectively. To obtain stringent hybridization conditions, defined proportions of formamide and NaCl were added to hybridization and washing buffers, respectively. Formamide increases stringency by destabilizing hydrogen bonds between nucleic acids, Na^+ ions decrease stringency due to a stabilizing effect on nucleic acid duplexes which is caused by masking of the negatively charged nucleic acid backbone. EDTA, which binds bivalent cations, was added to washing buffers containing ≥225mM NaCl to ensure that stabilization of nucleic acid duplexes could only be achieved by Na^+ ions.

Solutions

5 M NaCl

1 M Tris/HCl, pH 8.0

0.5 M EDTA, pH 8.0

10 % (w/v) SDS

Formamide (FA)

Tab. B.32. Hybridization buffer (46°C).

Formamide [%]	5M NaCl [µl]	1M Tris/HCl pH 8 [µl]	Formamide (µl)	$H_2O_{bidist.}$ [µl]	10 % SDS (w/v) [µl]
0	180	20	800	0	1
5	180	20	750	50	1
10	180	20	700	100	1
15	180	20	650	150	1
20	180	20	600	200	1
25	180	20	550	250	1
30	180	20	500	300	1
35	180	20	450	350	1
40	180	20	400	400	1
45	180	20	350	450	1
50	180	20	300	500	1

Tab. B.33. Washing buffer (48 °C).

% Formamide in hybridization buffer	NaCl (mM)	5M NaCl [µl]	1M Tris/HCl pH 8 [µl]	0,5M EDTA pH 8	H_2O_{bidist}
0	900	9000	1000	-	ad 50 ml
5	630	6300	1000	-	ad 50 ml
10	450	4500	1000	-	ad 50 ml
15	318	3180	1000	-	ad 50 ml
20	225	2150	1000	500	ad 50 ml
25	159	1490	1000	500	ad 50 ml
30	112	1020	1000	500	ad 50 ml
35	80	700	1000	500	ad 50 ml
40	56	460	1000	500	ad 50 ml
45	40	300	1000	500	ad 50 ml
50	28	180	1000	500	ad 50 ml

B.19.2.6. *In situ* hybridization

9 µl of hybridization buffer was applied to the dehydrated biomass. 1 µl of each probe was pipetted into the buffer and mixed by pipetting up and down. A hybridization chamber was constructed by putting a paper tissue into a 50 ml Greiner tube and wetting the tissue with the residual hybridization buffer. The slide was enclosed in the hybridization chamber and put into a hybridization oven at 46 °C for 180 min. Afterwards the slide was transferred into a tube with pre-warmed washing buffer (48 °C) and incubated in a waterbath (48 °C) for 10 min. Subsequently the slide was washed for a few seconds in ice-cold H_2O_{bidist} and dried with compressed air. Slides were stored at -20 °C in the dark until microscopic analysis.

B.19.2.6.1. Simultaneous hybridization of several probes requiring different stringencies

If probes requiring different stringencies were used on the same slide, probes requiring higher stringencies were hybridized first as described in section B.19.2.6, afterwards the whole procedure was repeated for probes requiring lower stringencies.

B.19.3. Confocal laser scanning microscopy

Confocal laser scanning collects the light emitted or reflected by a single plane of an examined sample, because light from outside the current focal plane is stopped by a pinhole in the beampath. Therefore only light from objects in focus can reach the detector. Objects are scanned with a laser and reconstructed by software.

B.19.3.1. Detection of fluorescently labelled cells

Samples were embedded in Citifluor before the application of the coverslip, to decrease bleaching effects during microscopic analysis. Samples were analyzed using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) equipped with an Ar-laser (430-514 nm; for excitation of the Fluos-fluorophore) and two He-Ne-lasers (543 nm and 633 nm; for excitation of Cy3 and Cy5, respectively), using a Plan-Neoflar objective with 63x magnification and a 10x ocular. Analysis and documentation was done using the provided software.

B.19.3.2. Evaluation of FA series

The highest stringency conditions yielding a bright FISH signal of a probe in an environmental sample were determined to be the optimal stringency conditions for the respective probe.

B.20. Statistical analysis of DGGE and T-RFLP data

To determine how the community structure of target organisms changed during the "controlled disturbances" experiment, community fingerprints obtained by DGGE and T-RFLP were statistically analyzed using the software program Primer. For DGGE fingerprints, presence and absence of DGGE bands in the different samples were determined and used for calculation of Bray-Curtis similarity. For T-RFLP analysis, Bray-Curtis similarities of consensus profiles were detemined. Based on Bray-Curtis similarities, Cluster analysis was performed.

C. Results

C.1. Investigation of community dynamics of denitrifying bacteria associated with the implementation of "controlled disturbances" by T-RFLP analysis of nitrite reductase (*nir*) genes

The effect of the implementation of the operational mode "controlled disturbances" on the population structure of denitrifying bacteria was assessed by T-RFLP fingerprinting of nitrite reductase genes (*nirS*, *nirK*). A T-RFLP approach was developed to achieve the highest possible amount of information and reliability of results.

C.1.1. Choice of primers for T-RFLP analysis

Primer sets F1aCu – R3Cu and cd3aF – R3cd have been chosen for amplification of *nirK* and *nirS*, respectively, because they managed to amplify *nir* genes from most environmental samples and pure cultures tested in recent studies (Throbäck et al. 2004, Heylen et al. 2006a).

C.1.2. Check of primer specificity - Cloning of *nir* genes

To confirm the specificity of the primers for the respective target genes, cloning of amplicons obtained from sample KDF was performed. This sample was chosen since it was expected to harbour the highest diversity of organisms and therefore of target and non-target genes, according to the "intermediate disturbance hypothesis" (see Sec. A.4). RFLP analysis with restriction enzyme *Hae*III was performed on PCR products derived from insert screening PCR. For the *nirK* clone library RFLP analysis was performed on 87 clones, from which 17 different RFLP patterns were obtained. Sequencing was performed for one to three representatives of each pattern. For the *nirS* clone library RFLP analysis was performed on 67 clones, from which 38 different RFLP patterns were obtained. Sequencing was performed for one to two representatives of each pattern. In total 44 sequences were obtained for the *nirS* clone library and 33 for the *nirK* clone library. The sequences were analyzed using the BLAST algorithm (blastn), the results are summarized in tables F.1 and F.2. All of the 33 sequences of the *nirK* clone library had highest sequence homologies to *nirK* sequences in the NCBI database. 43 of 44 sequences of the *nirS* clone library had highest sequence homologies to *nirS* sequences in the database. One sequence had a low homology to DNA gyrase subunit

B of *Rikenella microfusus*. These results confirm a high specificity of the primers for *nir* genes.
The length of sequences was 473 bp and ~410 - 420 bp for *nirK* and *nirS*, respectively.

C.1.3. Choice of restriction enzymes for T-RFLP

Restriction endonucleases for T-RFLP analyses were selected according to their ability to discriminate sequences in the *nirS* and *nirK* clone libraries according to their terminal restriction sites. Heterogeneity of terminal restriction fragments produced by different enzymes was evaluated *in silico* by manual comparison of restriction patterns using the software program "Clone manager" (Scientific & Educational Software) as well as using the web based program "REMA" (http://www.macaulay.ac.uk/rema, Szubert et al. 2007). Sequences that had a similarity of ≥ 97 % produced same-sized terminal restriction fragments (T-RFs) and thus could not be discriminated by this approach. They were therefore grouped into OTUs (operational taxonomic units). The production of a high diversity of T-RFs and the production of T-RFs that are unique for a respective OTU were criteria for restriction enzyme selection. T-RFs with an expected size of less than 30 bp were not considered for *in silico* evaluation of enzymes, since shorter T-RFs were not considered for T-RFLP analysis to avoid detection of primers. Parallel single digests with enzymes *Hae*III, *Mnl*I and *Mbo*II produced a high diversity of T-RFs as well as the highest number of unique T-RFs for either library (Tab. C.1 and C.2). 56 and 49 T-RFs were expected to be produced for the *nirS* and *nirK* clone libraries, respectively, in total for all profiles. Unique T-RFs were expected to be produced for 20 of 21 OTUs in case of the *nir*S clone library, and for 14 of 21 OTUs in case of the *nirK* clone library (Tab. C.1 and C.2). For *nirK* sequences T-RFLP analysis with labeled reverse primers and restriction endonuclease *Mnl*I was not possible because the primer contained a restriction site for the enzyme.

Table C.1. Terminal restriction fragment lengths of sequences of *nirS* clone library. Unique T-RFs are labelled in red.

Sequences/ OTUs	**S1_1**		**S1_8**		**S1_9,** S2_7		**S1_10,** S3_15, S3_8, S2_2, S3_9, S2_25		**S1_30,** S2_17, S2_19, S1_27, S2_27, S2_4, S3_5, S3_7, S4_12, S4_19, S4_9, S2_13, S2_18	
	f [b]	**r** [b]	**f**	**r**	**f**	**r**	**f**	**r**	**f**	r
***Hae*III**	79 bp	42 bp	137 bp	144 bp	226 bp	42 bp	19 bp [a]	144 bp	31 bp	139 bp
***Mnl*I**	68 bp	345 bp	120 bp	267 bp	34 bp	344 bp	85 bp	300 bp	57 bp	126 bp
***Mbo*II**	189 bp	224 bp	70 bp	81 bp	-	-	70 bp	81 bp	71 bp	221 bp
Sequences	**S1_31**		**S2_15**		**S2_16,** S4_31		**S3_4**		**S3_10**	
	f	**r**	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	20 bp [a]	151 bp	20 bp [a]	151 bp	20 bp [a]	151 bp	19 bp [a]	162 bp	19 bp [a]	151 bp
***Mnl*I**	99 bp	131 bp	99 bp	323 bp	86 bp	57 bp	154 bp	267 bp	154 bp	131 bp
***Mbo*II**	71 bp	351 bp	71 bp	201 bp	71 bp	81 bp	70 bp	351 bp	70 bp	351 bp
Sequences	**S3_16,** S3_21		**S3_18**		**S3_19**		**S3_20**		**S3_22,** S1_6	
	f	**r**	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	79 bp	42 bp	278 bp	132 bp	208 bp	42 bp	31 bp	42 bp	19 bp [a]	150 bp
***Mnl*I**	325 bp	85 bp	58 bp	119 bp	368 bp	17 bp [a]	57 bp	126 bp	98 bp	241 bp
***Mbo*II**	-	-	305 bp	66 bp	188 bp	33 bp	71 bp	339 bp	70 bp	200 bp
Sequences	**S3_23,** S3_3		**S3_27**		**S3_30**		**S4_8**		**S4_10**	
	f	**r**	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	138 bp	195 bp	52 bp	70 bp	193 bp	42 bp	79 bp	70 bp	-	-
***Mnl*I**	154 bp	267 bp	-	-	284 bp	126 bp	68 bp	126 bp	221 bp	96 bp
***Mbo*II**	70 bp	351 bp	71 bp	221 bp	71 bp	105 bp	63 bp	224 bp	70 bp	201 bp
Sequences	**S4_14**									
	f	**r**								
***Hae*III**	20 bp [a]	147 bp								
***Mnl*I**	155 bp	134 bp								
***Mbo*II**	71 bp	204 bp								

[a] T-RFs <30 bp were not considered for *in silico* evaluation of enzymes.

[b] f: T-RF including labelled forward primer, r: T-RF including labelled reverse primer.

Table C.2. Terminal restriction fragment lengths of sequences of *nirK* clone library. Unique T-RFs are labelled in red.

Sequences/ OTUs	**K2_32**		**K5_14**, K6_8 K6_12		**K6_13,** K6_1, K6_2, 6_4		**K6_14,** K6_19		**K6_15,** K6_7 K6_11, K6_16	
	f [b]	**r** [b]	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	317 bp	70 bp	23 bp [a]	219 bp	23 bp [a]	190 bp	235 bp	36 bp	23 bp [a]	137 bp
***Mnl*I**	461 bp	12 bp [a]	98 bp	12 bp [a]	98 bp	12 bp [a]	11 bp [a]	12 bp [a]	33 bp	12 bp [a]
***Mbo*II**	38 bp	435 bp	38 bp	125 bp	348 bp	125 bp	117 bp	216 bp	162 bp	285 bp
Sequences	**K6_18,** K6_3, K7_20, K6_25		**K6_23**		**K7_3**		**K7_4,** K7_13		**K7_6**	
	f	**r**	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	23 bp [a]	173 bp	23 bp [a]	70 bp	136 bp	82 bp	145 bp	156 bp	23 bp [a]	190 bp
***Mnl*I**	12 bp [a]	12 bp [a]	329 bp	12 bp [a]	101 bp	12 bp [a]	134 bp	12 bp [a]	98 bp	12 bp [a]
***Mbo*II**	188 bp	285 bp	153 bp	216 bp	117 bp	356 bp	-	-	348 bp	125 bp
Sequences	**K7_18**		**K7_29**		**K7_31**		**K8_2**		**K8_3**	
	f	**r**	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	176 bp	229 bp	23 bp [a]	190 bp	134 bp	229 bp	23 bp [a]	70 bp	23 bp [a]	58 bp
***Mnl*I**	461 bp	12 bp [a]	381 bp	12 bp [a]	174 bp	12 bp [a]	185 bp	12 bp [a]	33 bp	12 bp [a]
***Mbo*II**	56 bp	417 bp	162 bp	311 bp	59 bp	285 bp	-	-	-	-
Sequences	**K8_10**		**K8_14**		**K8_16**		**K8_18**		**K8_20**	
	f	**r**	**f**	**r**	**f**	**r**	**f**	**r**	**f**	**r**
***Hae*III**	23 bp [a]	70 bp	23 bp [a]	102 bp	136 bp	70 bp	23 bp [a]	156 bp	136 bp	136 bp
***Mnl*I**	33 bp	12 bp [a]	33 bp	12 bp [a]	101 bp	12 bp [a]	154 bp	12 bp [a]	185 bp	12 bp [a]
***Mbo*II**	182 bp	291 bp	-	-	-	-	38 bp	125 bp	182 bp	291 bp
Sequences	**K8_30**									
	f	**r**								
***Hae*III**	176 bp	262 bp								
***Mnl*I**	165 bp	12 bp [a]								
***Mbo*II**	-	-								

[a] T-RFs <30 bp were not considered for *in silico* evaluation of enzymes.

[b] f: T-RF including labelled forward primer, r: T-RF including labelled reverse primer.

C.1.4. Evaluation of the T-RFLP protocol by analysis of plasmid mixes

For evaluation of the usability of the T-RFLP protocol to detect the whole diversity of *nir* genes in environmental samples, PCR products prepared from plasmid mixes were analyzed and the obtained T-RFLP profiles were compared to the T-RFs predicted by *in silico* digestion. Plasmid mixes consisted of similar amounts of plasmids representing the OTUs defined in tables C.1 and C.2. PCR products of correct size were excised from agarose gels and purified. This purification procedure was chosen to exclude wrong sized unspecific amplicons that were sometimes observed and could result in the production of artefactual T-RFs. To reduce the effect of PCR bias, pooled products of two PCRs were used for T-RFLP

analysis. For normalizing differences in data sets produced from loading different amounts of DNA for electrophoresis and for eliminating background noise, peaks with an area of less than 1 % of the total peak area were not considered for further analysis. T-RFLP analysis was performed in duplicates. Duplicate profiles were manually aligned and consensus profiles that contained T-RFs with an area of more than 1 % of the total peak area in both replicates created. Abundance data was converted into presence/absence data for consensus profiles.

Observed T-RF lengths were in most cases shorter than predicted by *in silico* digests (Tab. C.3 – C.8). This phenomenon is frequently observed (Osborn et al. 2000, Kitts 2001) and is referred to as T-RF drift (Kaplan and Kitts 2003).

In most cases the applied detection threshold (1 % of the total peak area) discriminated expected from artefact peaks. However some expected peaks were below the threshold or completely undetectable and some unexpected peaks had equally high proportion of the total peak area as expected peaks. These observations will be reported in detail and efforts made to optimize the protocol are described.

In some T-RFLP profiles peaks were missing hat were predicted by *in silico* digestion (Tab. C.9). Some of these absent T-RFs had a predicted length of 30-35 bp and were thus due to underestimation of T-RF length caused by T-RF drift most likely below the threshold of 30 bp chosen for consideration of peaks. In five cases T-RFs with a length of more than 35 bp were absent or below the threshold of 1 % of the total peak area. In these cases PCR products of cloned sequences were digested separately to verify the predicted T-RF length. Furthermore, it was checked whether unique T-RFs corresponding to the respective sequences were present in the other plasmid mix T-RFLP profiles. For all sequences, T-RFs were produced as predicted when the PCR products were digested separately. In cases of sequences S4_14 and S1_9 whose T-RFs were missing from profile *nirS Mnl*I reverse, unique T-RFs corresponding to these sequences were present in other profiles. In case of sequence S4_14 a unique T-RF of 202 bp was present in profile *nirS Mbo*II reverse with an abundance of ~3 % of the total peak area. A unique T-RF (224 bp) of sequence S1_9 was present in profile *nirS Hae*III forward with an abundance of ~17 % of the total peak area. The T-RF of sequence S3_16 was missing from profile *nirS Mnl*I reverse after application of the 1 % threshold. In profile *nirS Mnl*I forward the abundance of the unique T-RF (323 bp) corresponding to the same sequence was slightly above the threshold. The T-RF of sequence S3_22 was missing from profile *nirS Mnl*I reverse. This sequence was not represented by a unique T-RF in one of the other profiles. The T-RF of sequence S4_10 was below the threshold in profile *nirS Mnl*I forward. When digested separately this sequence produced two peaks with a length of 222

and 226 bp, the latter one being slightly higher than the first. In profile *nirS Mnl*I forward a peak with a length of 226 bp was present with an abundance sightly above the threshold.

T-RFLP profiles of plasmid mixes contained peaks that did not correspond to predicted T-RFs of cloned sequences (labelled in grey in tables C.3 – C.8). Twelve artefact peaks were detected in the eleven profiles. Some of these artefactual restriction fragments had a length comparable to nonterminal restriction sites of cloned sequences contained in the mix which could be evidence for incomplete digestion of PCR products. Incomplete digestion might happen if the amount of applied restriction enzyme was too low or the incubation time was too short. This is most unlikely in the present case since enzyme was added in excess. It has been suggested that restriction fragments corresponding to nonterminal restriction sites, so-called "pseudo-T-RFs", can be the result of incomplete digestion of partially single stranded PCR products (Egert and Friedrich 2003, Egert and Friedrich 2005). Partially single stranded PCR products are thought to be produced when DNA polymerase stops amplifying before having reached the end of the strand. This might happen if it is hindered by secondary structures or simply falls off by accident. If the terminal restriction site resides within the single stranded amplicon region it cannot be cleaved as restriction endonucleases only cleave double strands. Consequently a labelled fragment will show up in the T-RFLP profile corresponding to that restriction site residing in the double stranded region of the amplicon which is closest to the labelled primer. To check for this possible origin of artefact peaks, restriction digests of PCR products of cloned sequences that harboured nonterminal restriction sites corresponding to artefact peaks were performed with shortened incubation time and thus under conditions expected to result in incomplete digestion. The results suggest that nine of twelve observed artefact peaks might have originated from incomplete digestion of some cloned sequences (Tab. C.10). The next question that had to be addressed was how to minimize this sort of bias. Egert and Friedrich suggested different ways of eliminating partially single stranded amplicons prior to T-RFLP analysis. The simplest approach is to minimize PCR cycle number, as a relationship between pseudo T-RF peak area and number of PCR cycles was suggested (Egert and Friedrich 2003). However, as the amount of PCR product obtained after 35 cycles of amplification was barely sufficient for T-RFLP analysis this approach was not practicable for the present study. Egert and Friedrich furthermore suggested two approaches for elimination of single stranded amplicon regions. The first approach involves digestion of single strands by incubation with mung bean nuclease (Egert and Friedrich 2003). This approach has the disadvantage of underestimation of real T-RF abundance. Furthermore the protocol includes a purification step that leads to a loss of DNA,

thus introducing new bias. The second approach involves incubation of PCR products with Klenow fragment exo$^-$ and dNTPs prior to restriction digest (Egert and Friedrich 2005). Klenow fragment exo$^-$ is the large subunit of DNA polymerase I of *E.coli* lacking exonuclease activity. It incorporates dNTPs into partially single stranded amplicons. In this way double strands could be restored and terminal restriction sites cleaved. Thus, artefact peaks could be removed and real T-RF abundance restored. This approach has been evaluated for clone mix T-RFLP analysis. Klenow fragment exo$^-$ preincubation led to a reduction of six artefact peaks below the threshold, five of which corresponded to nonterminal restriction sites and one of them was of unknown origin (Tab. C.10). Surprisingly, one new artefact peak was detected in the preincubated PCR products and three peaks of pseudo-T-RFs increased in area (data not shown). However, in total the approach resulted in a lower number of artefact peaks and was considered to be a useful addition to the T-RFLP protocol. All T-RFLP analyses of environmental samples were therefore performed including a Klenow fragment exo$^-$ preincubation step.

Table C.3. T-RFLP analysis of *nirK* plasmid mix with restriction enzyme *Mnl*I.

T-RFs including forward primer		
T-RF length in bp	**corresponding to OTU**	***in silico* T-RF length in bp**
96	K5_14, K6_13, K7_6	98
99	K7_3, K8_16	101
130	K7_4	134
153	K8_18	154
163	K8_30	165
167	K7_31	174
182	K8_2, K8_20	185
250 [a]		
325	K6_23	329
327 [a]		
375	K7_29	381
445 [a]		

[a] T-RF length does not correspond to OTU

Table C.4. T-RFLP analysis of *nirK* plasmid mix with restriction enzyme *Hae*III.

T-RFs including forward primer			T-RFs including reverse primer		
T-RF length in bp	**corresponding to OTU**	***in silico* T-RF length in bp**	**T-RF length in bp**	**corresponding to OTU**	***in silico* T-RF length in bp**
65 [a]			31 [b]	K6_14	36
131	K7_31	134	55	K8_3	58
135	K7_3, K8_16, K8_20	136	64	K2_32, K8_16	70
139-140	K7_4	145	67	K6_23, K8_2, K8_10	70
173	K8_30	176	78	K7_3	82
174	K7_18	176	99	K8_14	102
207 [a]			132-133	K8_20	136
234	K6_14	235		K6_15	137
244 [a]			154	K7_4, K8_18	156
279 [a]			171	K6_18	173
312	K2_32	317	189	K6_13, K7_6, K7_29	190
			220 [b]	K5_14	219
			230	K7_18, K7_31	229
			262	K8_30	262
			295 [a]		

[a] T-RF length does not correspond to OTU

[b] T-RF is missing from T-RFLP profile of sample KDF

Table C.5. T-RFLP analysis of *nirK* plasmid mix with restriction enzyme *Mbo*II.

T-RFs including forward primer			T-RFs including reverse primer		
T-RF length in bp	**corresponding to OTU**	***in silico* T-RF length in bp**	**T-RF length in bp**	**corresponding to OTU**	***in silico* T-RF length in bp**
31-32	K2_32, K5_14, K8_18	38	122	K5_14, K6_13, K7_6, K8_18	125
53	K7_18	56	216	K6_14, K6_23	216
55-56	K7_31	59	282	K6_15, K6_18	285
114	K6_14, K7_3	117	283	K7_31	285
151	K6_23	153	286	K8_10, K8_20	290
160	K6_15	162	311	K7_29	311
161	K7_29	162	354	K7_3	356
179-180	K8_10, K8_20	182	411	K7_18	417
187	K6_18	188	425	K2_32	435
348	K7_6, K6_13	348			

Table C.6. T-RFLP analysis of *nirS* plasmid mix with restriction enzyme *Mbo*II.

T-RFs including forward primer			T-RFs including reverse primer		
T-RF length in bp	**corresponding to OTU**	***in silico* T-RF length in bp**	**T-RF length in bp**	**corresponding to OTU**	***in silico* T-RF length in bp**
59	S4_8	63	61-62	S3_18	66
66	S1_8, S1_10, S3_4, S3_10, S3_22, S3_23, S4_10	70	77	S1_8, S1_10, S2_16	81
	S1_30, S1_31, S2_15, S2_16, S3_20, S3_27, S3_30,S4_14	71	102	S3_30	105
188	S1_1	189	200	S3_22	200
189	S3_19	188		S2_15, S4_10	201
300	S3_18	305	202 [b]	S4_14	204
			221	S3_27	221
			223	S1_30	221
			225	S1_1, S4_8	224
			339	S3_20	339
			349	S1_31, S3_4, S3_10, S3_23	351

[b] T-RF is missing from T-RFLP profile of sample KDF

Table C.7. T-RFLP analysis of *nirS* plasmid mix with restriction enzyme *Mnl*I.

T-RFs including forward primer			T-RFs including reverse primer		
T-RF length in bp	**corresponding to OTU**	***in silico* T-RF length in bp**	**T-RF length in bp**	**corresponding to OTU**	***in silico* T-RF length in bp**
52	S1_30, S3_20	57	54 [b]	S2_16	57
	S3_18	58	95 [b]	S4_10	96
64	S1_1, S4_8	68	116	S3_18	119
82	S1_10	85	124	S1_30, S3_20, S3_30, S4_8	126
	S2_16	86	128	S1_31, S3_10	131
96	S3_22	98	267	S1_8, S3_4, S3_23	267
	S1_31, S2_15	99	298	S1_10	300
119	S1_8	120	322 [b]	S2_15	323
154	S3_4, S3_10, S3_23	154	342 [a]		
	S4_14	155	344	S1_1	345
226 [a]			353 [a]		
283	S3_30	284			
323 [b]	S3_16	325			
366-367 [b]	S3_19	368			

[a] T-RF does not correspond to OTU

[b] T-RF is missing from T-RFLP profile of sample KDF

Table C.8. T-RFLP analysis of *nirS* plasmid mix with restriction enzyme *Hae*III.

T-RFs including forward primer			T-RFs including reverse primer		
T-RF length in bp	**corresponding to OTU**	***in silico* T-RF length in bp**	**T-RF length in bp**	**corresponding to OTU**	***in silico* T-RF length in bp**
48 [b]	S3_27	52	36	S1_1, S1_9, S3_16, S3_19, S3_20, S3_30	42
74	S1_1, S3_16, S4_8	79	66	S3_27, S4_8	70
116 [a]			129	S3_18	132
137	S1_8	137	138	S1_30	139
	S3_23	138	143	S1_8, S1_10	144
191	S3_30	193		S4_14	147
209	S3_19	208	150	S3_22	150
224	S1_9	226		S1_31, S2_15, S2_16, S3_10	151
274	S3_18	278	161 [b]	S3_4	162
			193	S3_23	195

[a] T-RF does not correspond to OTU

[b] T-RF is missing from T-RFLP profile of sample KDF

Table C.9. T-RFs missing from plasmid mix profiles.

T-RFLP profile	OTU	***in silico* length of T-RF (bp)**	**detected length of T-RF (bp)**
*nirS Mbo*II reverse	S3_19	33	≤30 [a]
*nirS Mnl*I forward	S4_10	221	222
*nirS Mnl*I forward	S1_9	34	≤30 [a]
*nirS Mnl*I reverse	S3_22	241	243
*nirS Mnl*I reverse	S4_14	134	131
*nirS Mnl*I reverse	S3_16	85	82
*nirS Mnl*I reverse	S1_9	344	340
*nirS Hae*III forward	S1_30	31	≤30 [a]
*nirS Hae*III forward	S3_20	31	≤30 [a]
*nirK Mnl*I forward	K6_15	33	≤30 [a]
*nirK Mnl*I forward	K8_3	33	≤30 [a]
*nirK Mnl*I forward	K8_10	33	≤30 [a]
*nirK Mnl*I forward	K8_14	33	≤30 [a]

[a] T-RFs with a size of ≤30 bp were not considered for further analysis

Table C.10. Effect of Klenow fragment exo$^-$ preincubation on detectability of artefact peaks.

T-RFLP profile	artefact peak (length in bp)	presence of artefact peak [a]		remarks
		without Klenow fragment exo$^-$ preincubation	with Klenow fragment exo$^-$ preincubation	
*nirS Mnl*I forward	226	+	+	corresponds to nonterminal restriction site of S4_10
*nirS Mnl*I reverse	342	+	+	no correspondence to nonterminal restriction sites of cloned sequences
*nirS Mnl*I reverse	353	+	-	corresponds to nonterminal restriction site of S1_30
*nirS Hae*III forward	116	+	-	corresponds to nonterminal restriction site of S1_30, S3_27
*nirK Mnl*I forward	250	+	+	corresponds to nonterminal restriction site of K7_4
*nirK Mnl*I forward	327	+	+	corresponds to nonterminal restriction site of K8_18
*nirK Mnl*I forward	445	+	+	no correspondence to nonterminal restriction sites of clones
*nirK Mnl*I forward	448	-	+	corresponds to nonterminal restriction site of K6_13, K7_6
*nirK Hae*III forward	65	+	-	no correspondence to nonterminal restriction sites of cloned sequences
*nirK Hae*III forward	207	+	+	corresponds to nonterminal restriction site of K7_4, K8_30
*nirK Hae*III forward	244	+	-	corresponds to nonterminal restriction site of K7_29
*nirK Hae*III forward	279	+	-	corresponds to nonterminal restriction site of K6_23
*nirK Hae*III reverse	295	+	-	corresponds to nonterminal restriction site of K8_30, K6_13
artefact peaks in total		**12**	**7**	

[a] + indicates presence, - indicates absence; peaks with an area of at least 1 % of total peak area were considered

C.1.5. T-RFLP analysis of denitrification tank samples

T-RFLP analysis of denitrification tank samples KDD, KDE and KDF was performed as described for the plasmid mixes (Sec. C.1.4) except that four PCR products were pooled for digestion and analyzed in triplicates, for enhanced accuracy of results. To reduce the effect of bias that occurs during DNA extraction, two replicate DNA extracts were pooled for PCR. T-RFs that were above the detection threshold in at least two replicate profiles were included in the consensus profile. A complete list of consensus profiles can be found in table F.3 (Appendix). Profiles contained 7 - 19 T-RFs, respectively. The majority of T-RFs that were detected in the plasmid mix profiles was present in the consensus profiles of sample KDF, from which the clone library had been established. Plasmid mix profiles contained in total 96 T-RFs. Ten of those (~10 %) were absent from the profile of KDF after application of the 1 % threshold (marked in Tab. C.3 – C.8). All of these T-RFs were present before application of the threshold.

Bray Curtis similarities between the samples were determined for the whole set of profiles, as well as for the *nirS* and *nirK* profiles exclusively and cluster analysis was performed. When the whole data set was analyzed, samples KDE and KDF showed a higher similarity to each other with respect to detected T-RFs than to sample KDD (Tab. C.11, Fig. C.1). Similarity analysis of the *nirS* and *nirK* datasets indicated that the clustering pattern of the joined dataset was exclusively due to the *nirS* data. The *nirS* profiles had pronouncedly higher similarities between KDE and KDF than between these two samples and KDD (Tab. C.13, Fig. C.3). For the *nirK* genes, T-RFLP profiles of samples KDD and KDE were most similar (Tab. C.12, Fig. C.2).

171 T-RFs were detected for all profiles, 84 for *nirK* and 87 for *nirS* analysis. Diversity of T-RFs decreased during the implementation of "controlled disturbances" (Tab. C.14), as more T-RFs disappeared than appeared (Tab. C.15). The shift was more pronounced for *nirS* than for *nirK* genes, as 19 T-RFs disappeared and 13 appeared and twelve disappeared and six appeared, respectively (Tab. C.15). In total, the presence of 50 T-RFs (29 %) was affected by the implementation of the new operational mode. 8 % of T-RFs showed a pattern of presence and absence that could not be explained by the implementation of "controlled disturbances" (i.e. they were only present in either KDE or KDD and KDF).

Table C.11. Bray-Curtis similarity matrix of *nirS* and *nirK* T-RFLP profiles.

	KDD	KDE
KDD		
KDE	84,5	
KDF	81,5	85,3

Table C.12. Bray-Curtis similarity matrix of *nirK* T-RFLP profiles.

	KDD	KDE
KDD		
KDE	87,8	
KDF	86,4	79,7

Table C.13. Bray-Curtis similarity matrix of *nirS* T-RFLP profiles.

	KDD	KDE
KDD		
KDE	81,2	
KDF	76,8	90,9

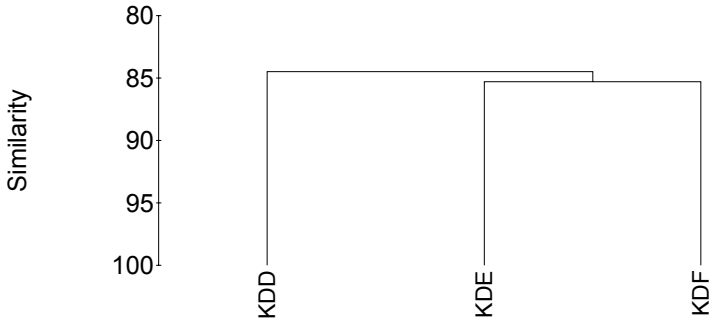

Figure C.1. Cluster analysis of T-RFLP analysis of *nirS* and *nirK* genes in denitrificaion tank samples.

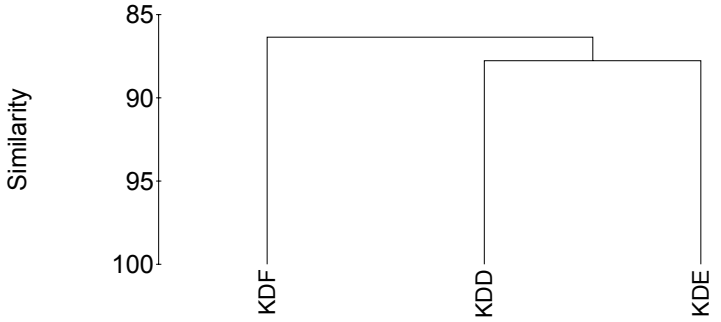

Figure C.2. Cluster analysis of T-RFLP analysis of *nirK* genes in denitrificaion tank samples.

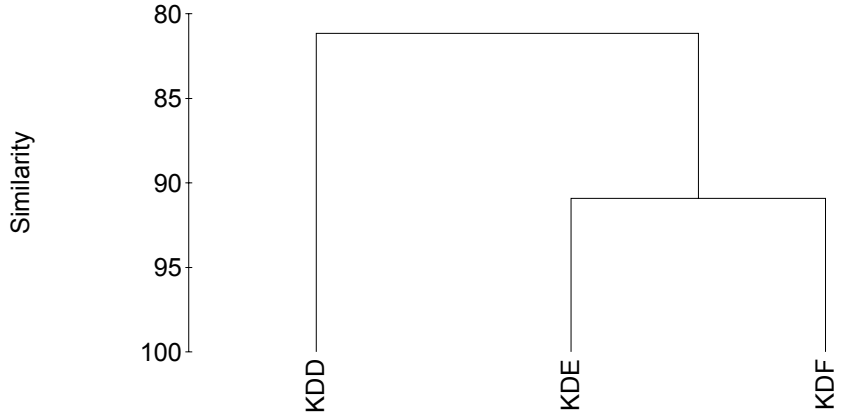

Figure C.3. Cluster analysis of T-RFLP analysis of *nirS* genes in denitrificaion tank samples.

Table C.14. Diversity of T-RFs in denitrification tank samples.

T-RFLP profile	number of T-RFs in sample		
	before „controlled disturbances“	during „controlled disturbances“	
	KDD	KDE	KDF
*nirS Mbo*II reverse	11	9	9
*nirS Mbo*II forward	8	7	7
*nirS Hae*III reverse	13	14	14
*nirS Hae*III forward	13	13	14
*nirS Mnl*I reverse	17	13	12
*nirS Mnl*I forward	10	10	10
total number of T-RFs *nirS*	**72**	**66**	**66**
*nirK Mbo*II reverse	10	10	10
*nirK Mbo*II forward	12	13	11
*nirK Hae*III reverse	14	14	11
*nirK Hae*III forward	17	14	16
*nirK Mnl*I forward	16	19	15
total number of T-RFs *nirK*	**69**	**70**	**63**
total number of T-RFs	**141**	**136**	**129**

Table C.15. T-RFs that appeared or disappeared during "controlled disturbances".

Profiles	number of T-RFs that disappeared during "controlled disturbances"			number of T-RFs that appeared during "controlled disturbances"		
	number of T-RFs only present in			number of T-RFs only present in		
	KDD	KDD and KDE	KDD or KDD and KDE	KDE and KDF	KDF	KDF or KDE and KDF
all *nirS*	15	4	**19**	8	5	**13**
all *nirK*	4	8	**12**	0	6	**6**
all	19	12	**31**	8	11	**19**

C.1.6. Identification of denitrifiers affected by “controlled disturbances” by T-RFLP

To infer the possible phylogenetic affiliation of T-RFs whose presence was affected by “controlled disturbances”, T-RF length was compared to sequences in the *nir* clone libraries. If sequences were found that produced a T-RF of the respective length for the respective T-RFLP profile, it was checked whether T-RFs corresponding to that sequence were present in the other profiles of the same samples. If this was the case, the sequence could have corresponded to the respective T-RF and was subjected to phylogenetic analysis using the BLAST algorithm. BLAST analysis was done using the nucleotide (blastn) as well as the protein (blastx) database of NCBI. The most closely related *nir* gene in the database derived from a bacterial isolate was determined (Tab. C.16).

Three T-RFs that appeared during "controlled disturbances" were determined to be possibly derived from bacteria harbouring cloned sequences (S3_22, S1_10, S3_23). blastn as well as blastx analysis of all three sequences revealed that most similar *nirS* genes derived from cultured bacteria belonged to different isolates affiliated to the genus *Dechloromonas* (Tab. C.16).

Two T-RFs that disappeared during "controlled disturbances" were possibly derived from bacteria harbouring cloned sequences (S4_8, K8_3). blastn and blastx analysis of sequence S4_8 revealed that most similar *nirS* genes of bacterial isolates contained in the database were *nirS* genes of *Paracoccus* sp. and *Stappia aggregata*, respectively. For sequence K8_3 most similar *nirK* genes of bacterial isolates belonged to *Mesorhizobium* spp.

As bacteria affiliated to the genera *Dechloromonas* and *Paracoccus* were previously detected in denitrifying wastewater treatment plant sludge or lab scale reactors treating wastewater (Juretschko et al. 2002, Loy et al. 2005, Heylen et al. 2006b, Osaka et al. 2006, Ahn et al. 2007), their presence in the denitrification tank of wastewater treatment plant Weißtal seemed plausible. FISH experiments with probes targeting members of these genera (see Tab. B.30) were performed using PFA-fixed sludge of denitrification tank samples taken before and during "controlled disturbances". No cells targeted by *Paracoccus* spp. specific probe Par651 were detected in any of the samples. Cells targeted by probe combination RHC445/RHC827 specific for some bacteria affiliated to *Dechloromonas* were detected in small numbers (10 - 15 colonies in 20 µl fixed sludge sample) in all samples (see Fig. C.4).

Table. C.16. BLAST analysis of *nir* sequences.

Sequence	next cultured relative (blastn)			next cultured relative (blastx)		
	organism	Accession nr.	identity (%)	organism	Accession nr.	Identity (%)
T-RF showed up during "controlled disturbances"						
KDD -, KDE +, KDF +						
S3_22	*Dechloromonas* sp. R-28400	AM230913	87	*Dechloromonas* sp. R-28400	AM230913	89
S1_10	*Dechloromonas* sp. R-28451	AM230919	86	*Dechloromonas aromatica* RCB	AM230919	90
KDD -, KDE -, KDF +						
S3_23	*Dechloromonas* sp. R-28400	AM230913	82	*Dechloromonas* sp. R-28400	AM230913	92
T-RF disappeared during "controlled disturbances"						
KDD +, KDE -, KDF -						
S4_8	*Paracoccus* sp. R-28241	AM230918	80	*Stappia aggregata* IAM 12614	ZP_01549666	84
KDD +, KDE +, KDF -						
K8_3	*Mesorhizobium* sp. 4FB11	AY078254	73	*Mesorhizobium* sp. BNC1	YP_665890	74

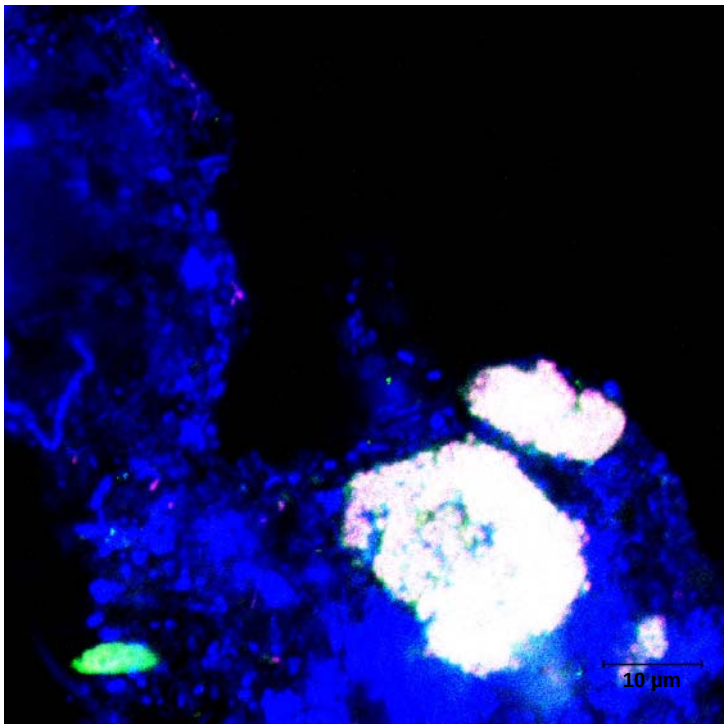

Fig C.4. Bacteria that hybridized to probes RHC445 (red), RHC827 (green) and EUB338 mix (blue).

C.2. Investigation of bacterial community dynamics by DGGE analysis of 16S rRNA genes and FISH analysis

To investigate whether the optimization of treatment processes in wastewater treatment plant Weißtal by the implementation of the operational mode "controlled disturbances" was associated with shifts in the composition of the total bacterial community, denaturant gradient gel electrophoresis (DGGE) of bacterial 16S genes was performed. For increased reliability of the detection of population shifts, two DGGE approaches involving the analysis of PCR products obtained from two different primer sets (341fGC compared with either 518r or 907r) were performed and the results compared. DGGE bands that possibly represented bacterial species that were affected by the implementation of "controlled disturbances", i.e. the bands appeared or disappeared in the samples taken during the experiment, were further analyzed. They were excised and the PCR products were eluted and sequenced. Highly specific FISH probes targeting these sequences were designed and applied to confirm an effect of the change of the operational mode on the respective bacteria.

C.2.1. DGGE analysis

C.2.1.1. Optimisation of PCR for DGGE

PCR conditions were optimized until PCR products were obtained that produced single distinct bands of correct size on an agarose gel.

C.2.1.1.1. Optimisation of PCR with primer set 341fGC – 518r

For optimization of PCR with primer set 341fGC – 518r, a gradient PCR with one sample was performed to determine the optimal annealing temperature which should result in the production of a single distinct band of ~180 bp on an agarose gel. Distinct bands of correct size could not be obtained (Fig. C.5). To overcome this, nested PCR approaches were performed. Primer sets 616V-630R and 616V-1492r targeting 16S of most bacteria were used for the first round of PCR. Products were used as templates for nested PCR with primer set 341fGC – 518r. To increase the stringency of PCR, a touchdown program was performed (see Sec. B.11.5). Both nested PCR approaches did not yield distinct bands of correct size (Fig. C.6, C.7). The $MgCl_2$ concentration was lowered to increase the specificity of PCR, but better results could not be obtained. The next approach that was evaluated was performing PCR with a premixed mastermix containing Taq polymerase, Taq buffer, $MgCl_2$ and dNTPs purchased from Promega. Using the Promega mastermix and a touchdown program according to section B.11.5 finally enabled the production of correctly sized amplicons without by-products (Fig. C.8).

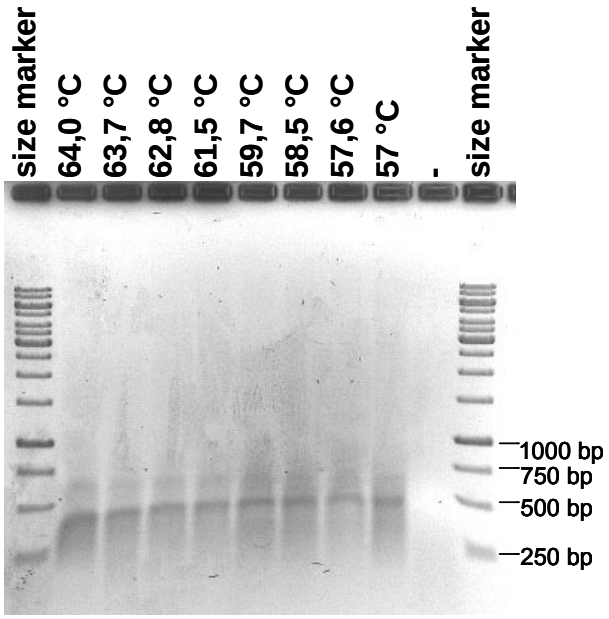

Figure C.5. Gradient PCR with primer set 341fGC – 518r for DGGE, - indicates negative control.

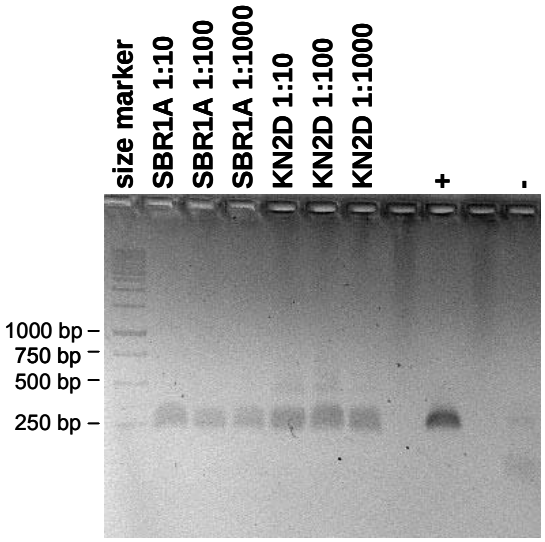

Figure C.6. Nested PCR from 616V – 630R products with primer set 341fGC – 518r, + indicates positive control, - indicates negative control.

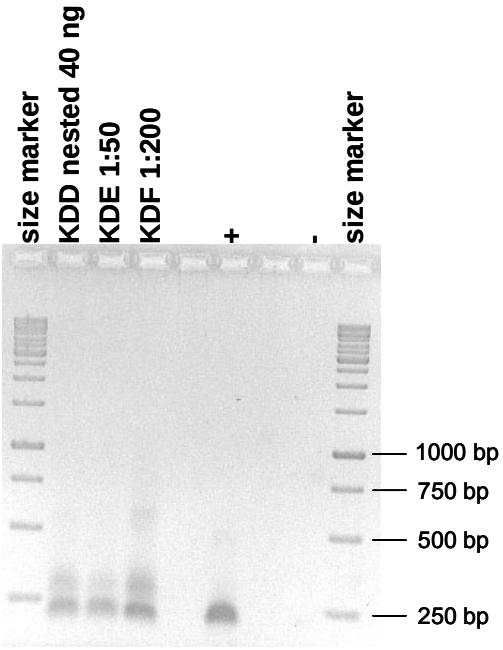

Figure C.7. Nested PCR from 616V – 1492r products with primer pair 341fGC – 518r, + indicates positive control, - indicates negative control.

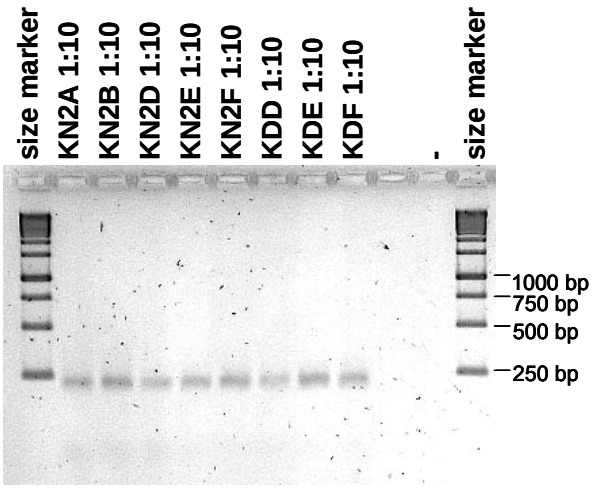

Figure C.8. PCR with primer pair 341fGC – 518r and Promega PCR mastermix, - indicates negative control.

C.2.1.1.2. Optimisation of PCR for DGGE with primer set 341fGC – 907r

For the amplification of 16S rRNA genes with primer set 341fGC – 907r a touchdown program according to section B.11.5 was performed. Different $MgCl_2$ concentrations were evaluated and the best results were achieved by the use of 1,5 mM $MgCl_2$ which led to the production of single distinct bands of the expected size (~570 bp, Fig. C.9).

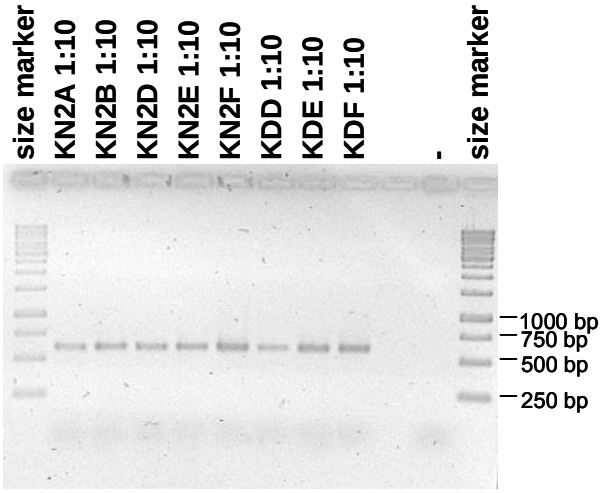

Figure C.9. PCR with primer pair 341fGC – 907r for DGGE, - indicates negative control.

C.2.1.2. Optimisation of DGGE conditions

At first, the appropriate amount of PCR product to be applied onto the gel was evaluated. The optimal amount of PCR product should produce clearly visible bands with a minimum intensity of background smear. For PCR products derived from PCR with primer set 341fGC – 518r, the optimal volume to be applied onto DGGE gels was determined to be 140 µl if band intensity on agarose gels was comparable to figure C.9, or more if band intensity was lower. For PCR products derived from PCR with primer set 341fGC – 907r, the optimal volume was determined to be 90 µl if band intensity on agarose gels was comparable to figure C.9, or more if band intensity was lower.

Next, the optimal gradient for DGGE separation was evaluated. At first, PCR products were separated on a wide gradient of 20 – 80 % denaturant. The optimal gradient was determined to be the narrowest gradient covering the whole range of denaturant concentrations where bands were visible. The optimal gradient should not be narrower than 33 %, since previous DGGE experiments in our lab conducted by Roland Hatzenpichler (Hatzenpichler 2006) and Christian Baranyi showed that quality of DGGE gels decreases for narrower gradients. The optimal gradients for PCR products derived from PCR with primer sets 341fGC – 518r and

341fGC – 907r were determined to be 35 – 70 % denat. and 35 – 75 % or 40 – 75 % denat., respectively.

C.2.1.3. DGGE analysis of wastewater treatment plant samples

Wastewater treatment plant samples were applied onto DGGE gels ordered by sampling date. Two gels loaded with 341fGC – 907r-PCR products and one gel loaded with 341fGC – 518r-PCR products were produced (Fig. C.10 – C.12).

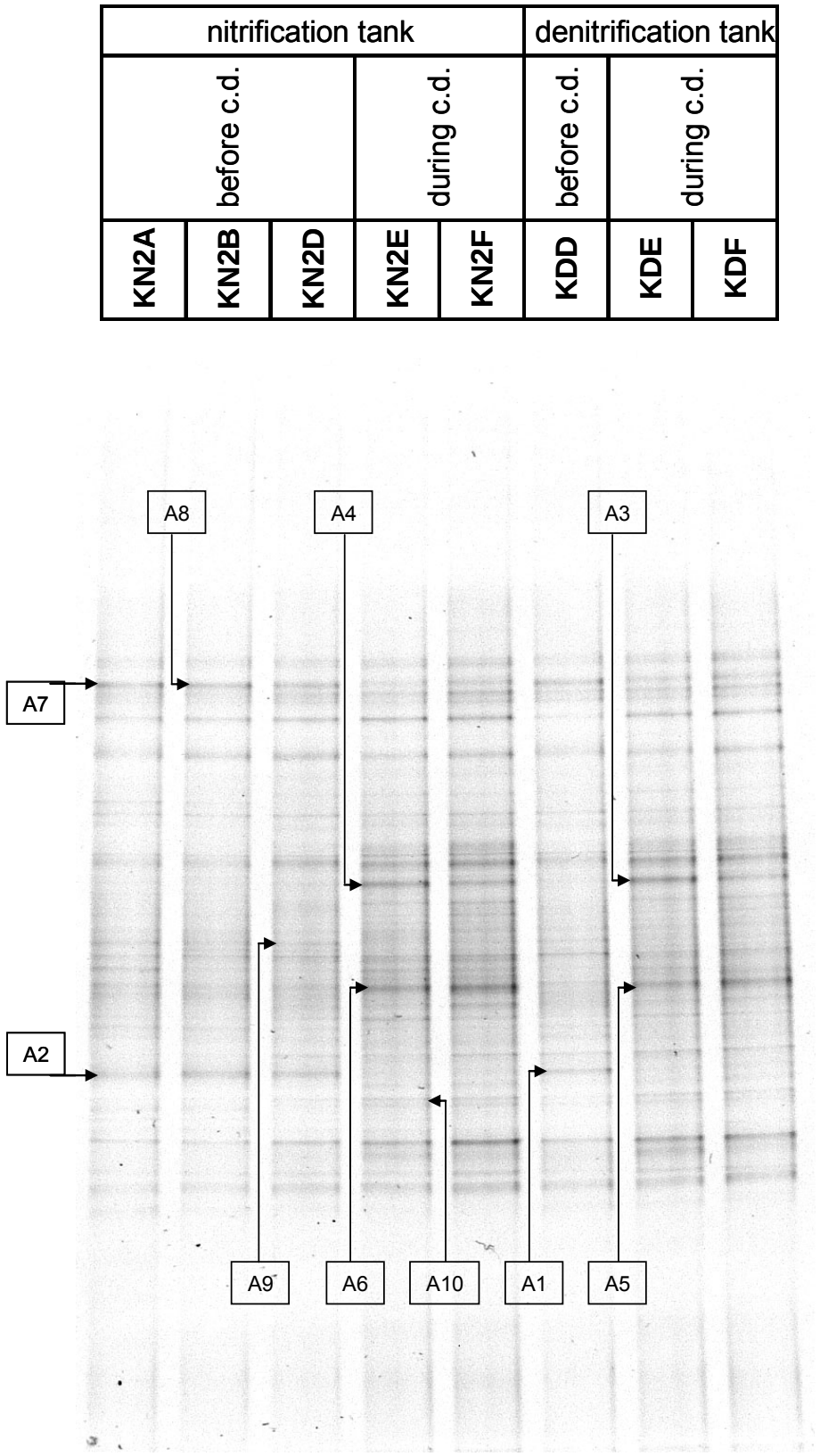

Figure C.10. DGGE gel (Gradient: 35 – 75 % denat.) with PCR products of primer pair 341fGC-907r. Excised bands are indicated by arrows, labels of bands are indicated in boxes. c.d. means "controlled disturbances".

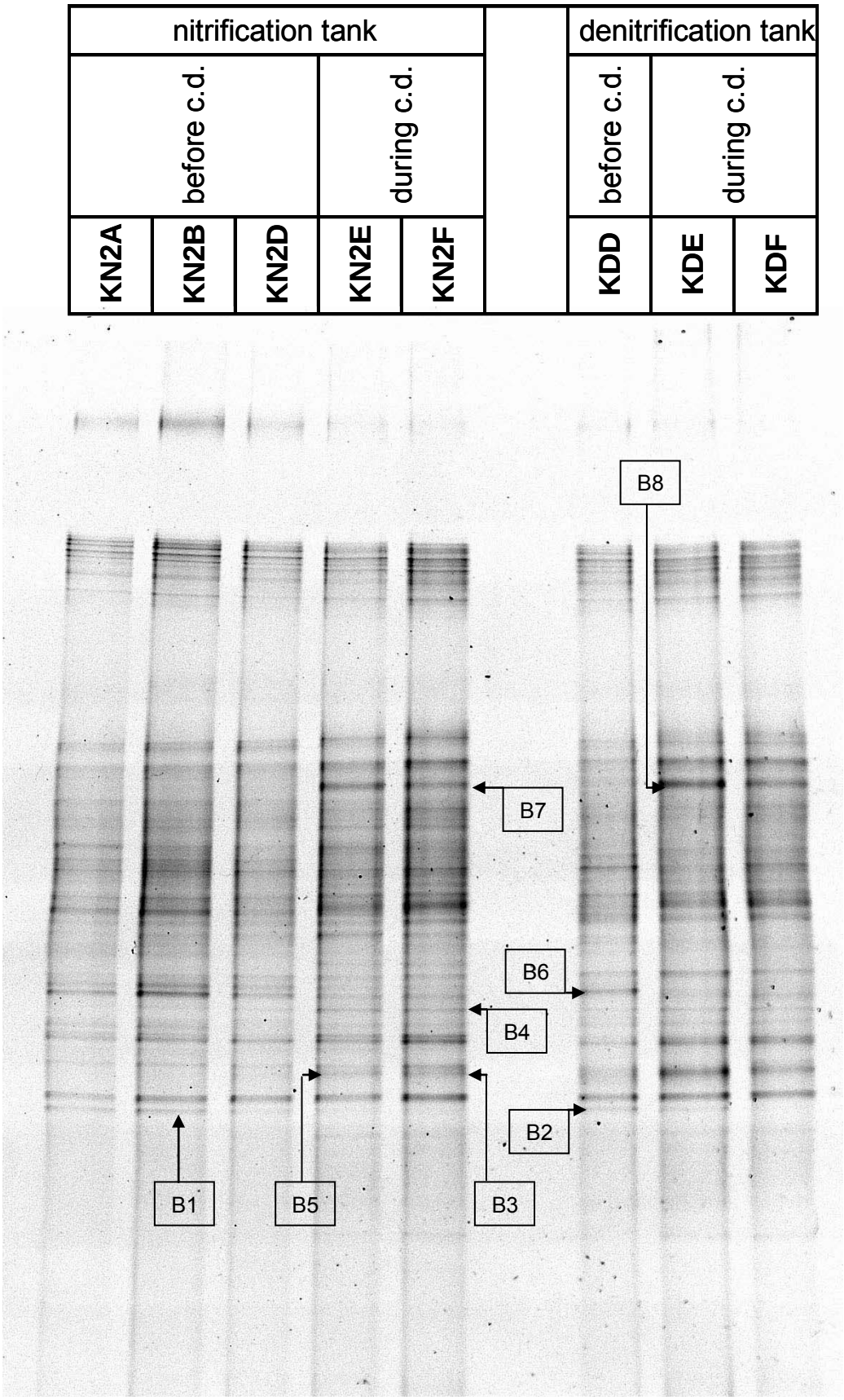

Figure C.11. DGGE gel (Gradient: 40 – 75 %) with PCR products of primer pair 341fGC-907r. Excised bands are indicated by arrows, labels of bands are indicated in boxes. c.d. means "controlled disturbances".

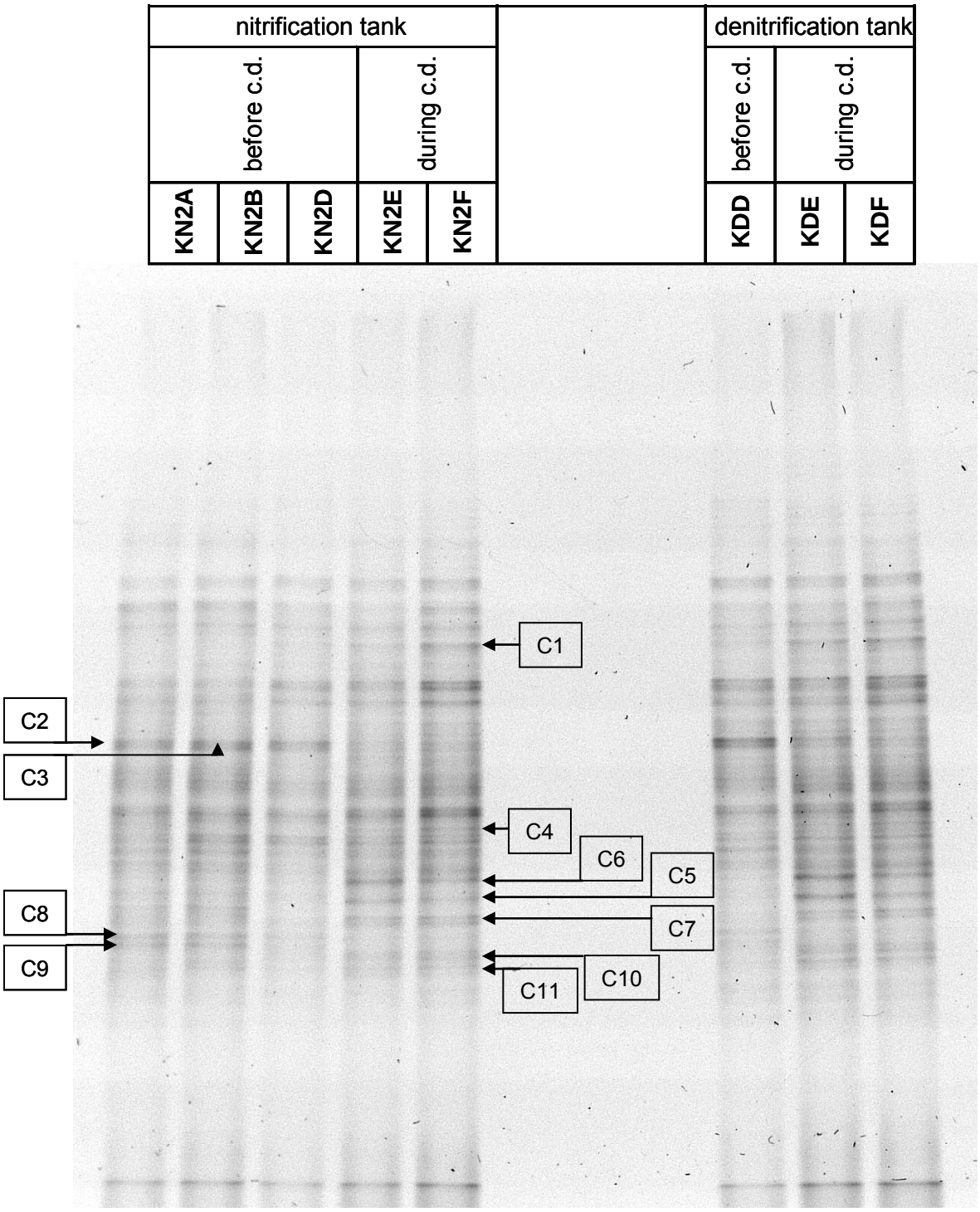

Figure C.12. DGGE gel (Gradient: 35 – 70 %) with PCR products of primer pair 341fGC-518r. Excised bands are indicated by arrows, labels of bands are indicated in boxes. c.d. means "controlled disturbances".

C.2.1.4. Statistical analysis of DGGE gels

Banding patterns obtained by DGGE analysis of the wastewater treatment plant samples were transformed into a presence-absence matrix from which a Bray-Curtis similarity matrix was calculated using the software program Primer (Tab. C.17, C.18). Based on this matrix, cluster analysis was performed (Fig. C.13, C.14). Statistical analyses were performed for the 341fGC-518r gel shown in figure C.12 as well as for the 341fGC-907r gel shown in figure C.10. The quality of the gel shown in figure C.11 was considered to be not good enough for statistical analysis.

Dendrograms obtained from either analyzed DGGE gel contained two clusters, which consisted of samples taken before and during the "controlled disturbances" experiment, respectively (Fig. C.13 and C.14). For either gel, diversity of bands was slightly but consistently higher for samples taken during "controlled disturbances" compared to samples taken before "controlled disturbances" (Tab. C.19 and C.20).

Table C.17. Bray-Curtis similarity matrix of DGGE gel with 341fGC-518r PCR products shown in Fig. C.12.

	KN2A	KN2B	KN2D	KN2E	KN2F	KDD	KDE
KN2A							
KN2B	100,0						
KN2D	93,0	93,0					
KN2E	69,4	69,4	75,0				
KN2F	69,4	69,4	75,0	100,0			
KDD	83,3	83,3	89,4	79,2	79,2		
KDE	66,7	66,7	72,0	96,4	96,4	83,6	
KDF	66,7	66,7	72,0	96,4	96,4	83,6	100,0

Table C.18. Bray-Curtis similarity matrix of DGGE gel with 341fGC-907r PCR products shown in Fig. C.10.

	KN2A	KN2B	KN2D	KN2E	KN2F	KDD	KDE
KN2A							
KN2B	100,0						
KN2D	92,3	92,3					
KN2E	77,2	77,2	83,6				
KN2F	77,2	77,2	83,6	100,0			
KDD	94,1	94,1	98,0	81,5	81,5		
KDE	78,6	78,6	85,2	98,3	98,3	83,0	
KDF	74,1	74,1	80,8	94,7	94,7	78,4	96,4

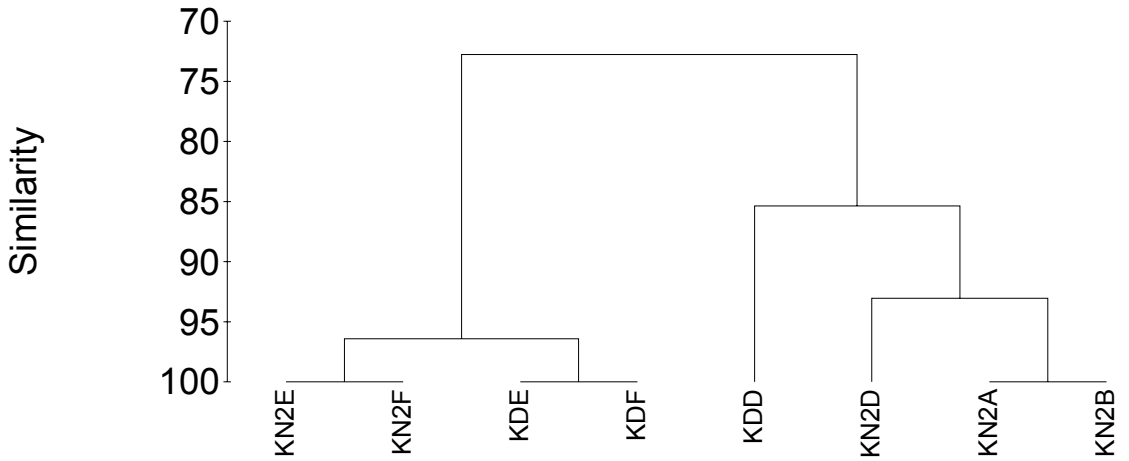

Figure C.13. Cluster analysis of banding pattern of DGGE gel with 341fGC-518r PCR products shown in Fig. C.12.

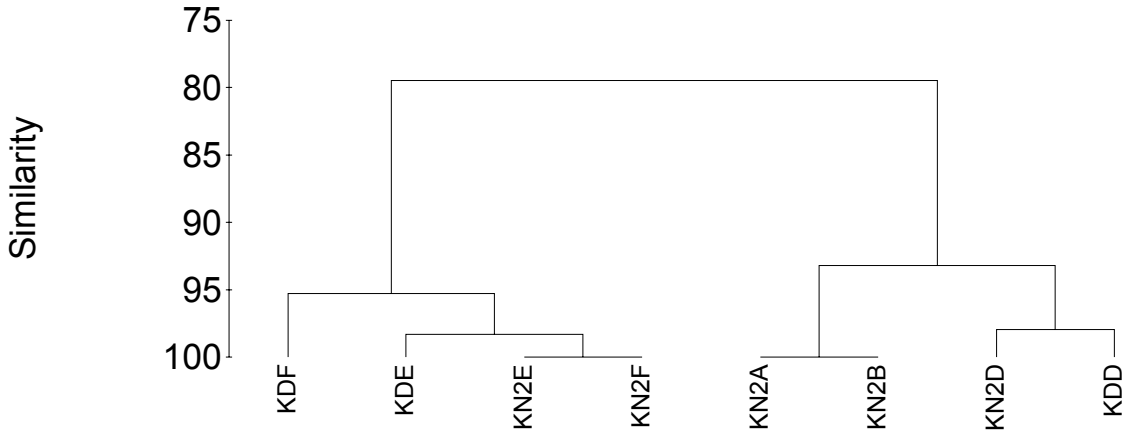

Figure C.14. Cluster analysis of banding pattern of DGGE gel with 341fGC-907r PCR products shown in Fig. C.10.

Table C.19. Diversity of bands in DGGE gel with 341fGC-518r PCR products shown in Fig. 12.

	Nitrification tank				
	before controlled disturbance			during controlled disturbance	
Sample	KN2A	KN2B	KN2D	KN2E	KN2F
Number of bands	22	22	21	27	27

	Denitrification tank		
Sample	KDD	KDE	KDF
Number of bands	26	29	29

Table C.20. Diversity of bands in DGGE gel with 341fGC-907r PCR products shown in Fig. 10.

	Nitrification tank				
	before controlled disturbance			during controlled disturbance	
Sample	KN2A	KN2B	KN2D	KN2E	KN2F
Number of bands	**27**	**27**	**25**	**30**	**30**

		Denitrification tank		
	Sample	KDD	KDE	KDF
	Number of bands	**24**	**29**	**27**

C.2.1.5. Excision of DGGE bands and sequencing of eluted PCR products

DGGE bands that were exclusively obtained with samples taken before or during the implementation of "controlled disturbances", or which showed a pronounced change in intensity associated with the implementation of the new operational mode, were selected for further analysis. Bands were excised, the PCR products eluted, purified and sequenced. Excised bands are labelled in figures C.10 – C.12.

C.2.1.6. Optimization of sequencing of PCR products eluted from excised DGGE bands

All bands excised from DGGE gels except bands A3 and A4 contained multiple sequences and it was therefore not possible to analyze the obtained sequence data. Reamplified PCR products of those bands were separated on a DGGE gel containing a narrower gradient of denaturants than the gel they were excised from (Fig. C.15 – C.17). Obtained bands were excised, eluted PCR products were reamplified, purified and sequenced. By doing so, sequences were obtained for 15 of 18 bands excised from DGGE gels produced from 907r-341fGC PCR products, and for 4 of 11 bands excised from the DGGE gel produced from 518r-341fGC PCR products (Tab. C.21). In 17 cases more than one prominent band showed up on the gel containing a narrower gradient (Fig. C.15 – C.17), in these cases all obtained bands were excised and sequenced.

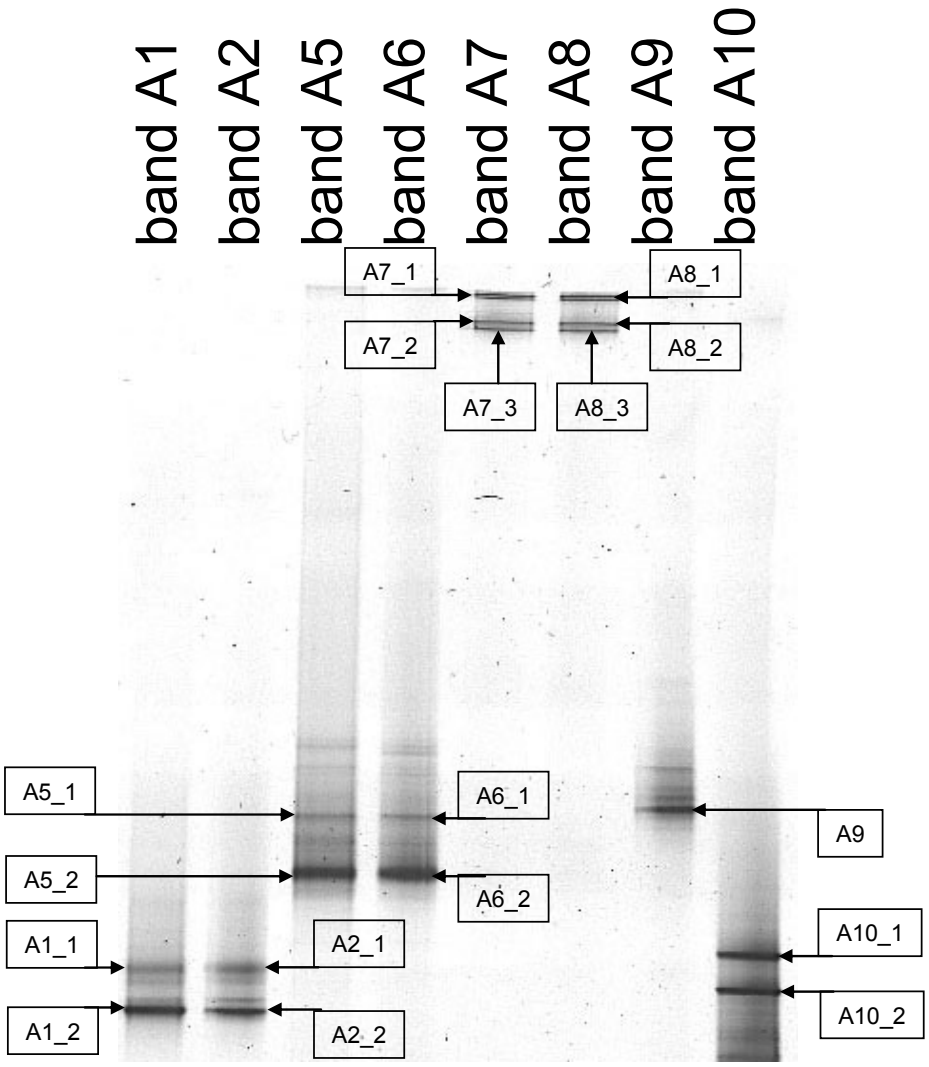

Figure C.15. DGGE gel (Gradient: 40 – 65 %, zoomed in) for further separation of bands excised from gel shown in Fig. C.10. Excised bands are indicated by arrows, labels of bands are indicated in boxes.

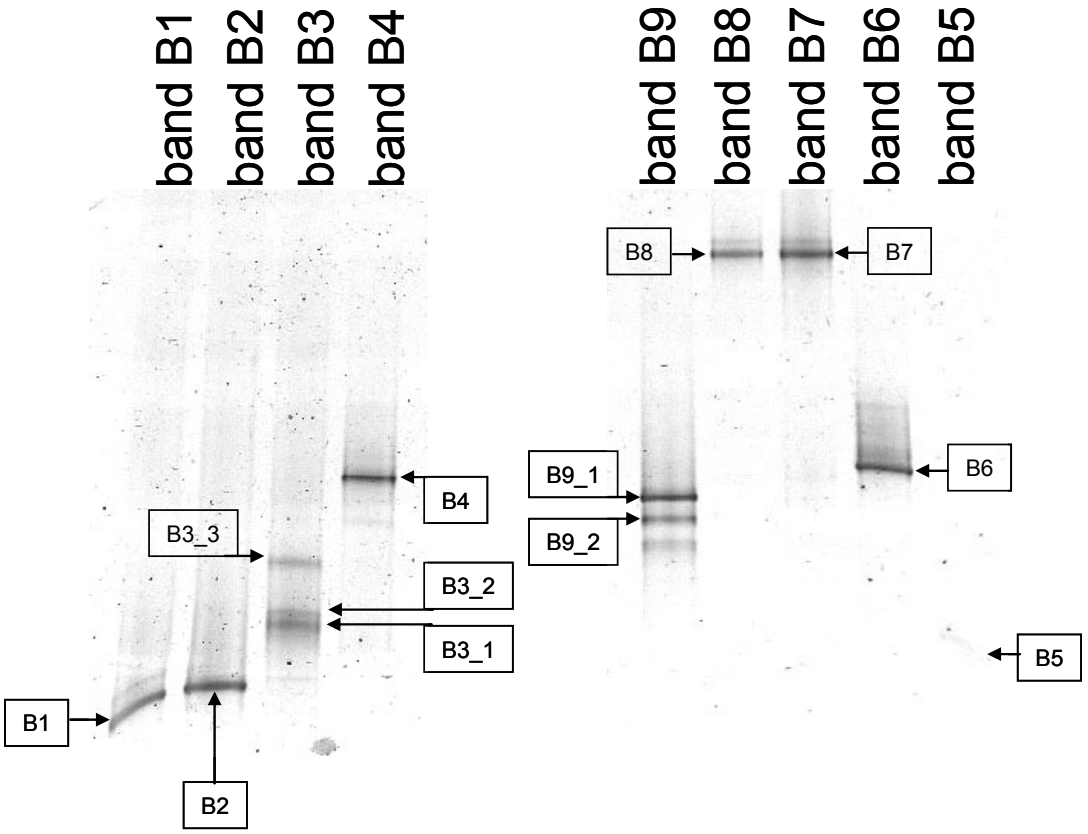

Figure C.16. DGGE gel (Gradient: 40 – 65 %, zoomed in) for further separation of bands excised from gel shown in Fig. C.11. Excised bands are indicated by arrows, labels of bands are indicated in boxes.

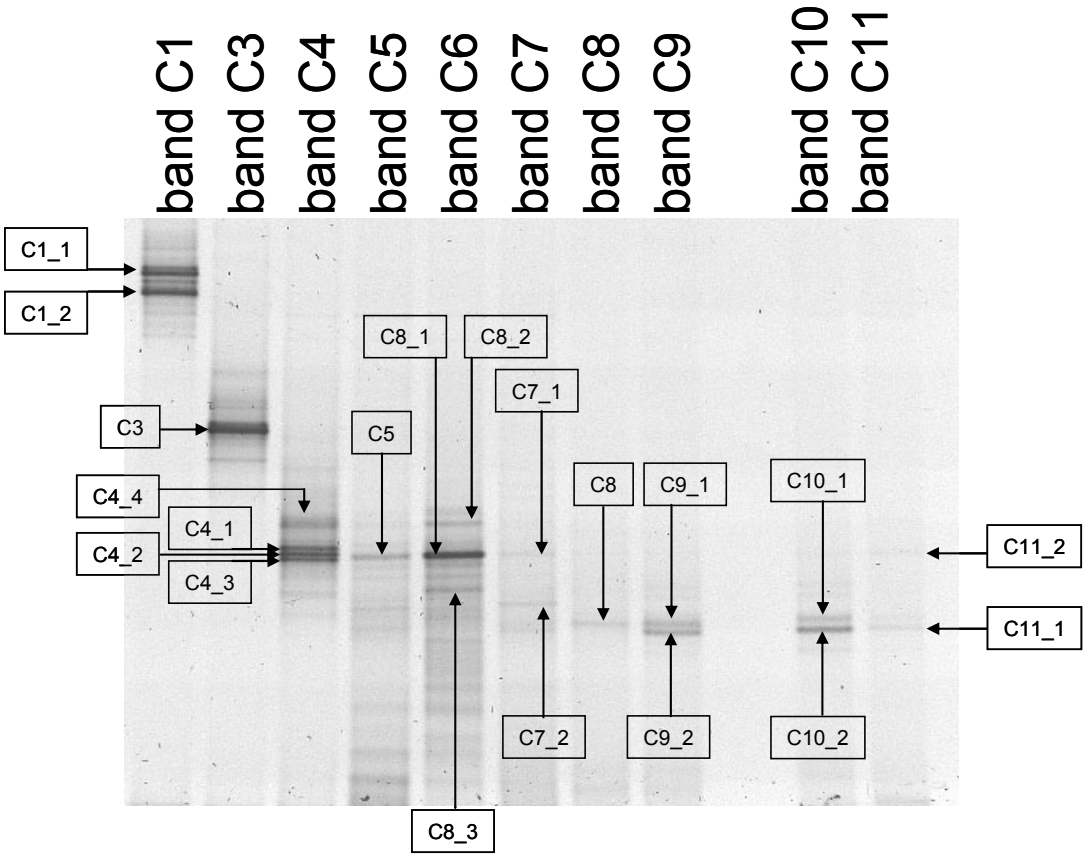

Figure C.17. DGGE gel (Gradient: 40 – 65 %, zoomed in) for further separation of bands excised from gel shown in Fig. C.12. Excised bands are indicated by arrows, labels of bands are indicated in boxes.

C.2.1.7. Analysis of sequences obtained from DGGE bands

The phylogenetic affiliations of sequences retrieved from DGGE bands was determined by BLAST and ARB analysis (Tab. C.21). Twelve sequence types affiliated to five phyla and two candidate phyla were obtained. One sequence type, represented by band A3, was isolated from all three gels.

As mentioned above, in some cases multiple prominent bands were obtained when eluted PCR products were separated on a gel with a narrower gradient. In 7 cases sequences were obtained for two of the multiple prominent bands, in three of these cases the two bands contained identical sequences (bands A7_2 and A7_3, A8_2 and A8_3, C6_1 and C6_2), in four cases sequences were different (identity of nucleotides ranged from 74,7 % for bands C1_1 and C1_2 to 74,8 % for bands A1_1 and A1_2, A2_1 and A2_2, A10_1 and A10_2).

Bands that migrated to the same position in a gel, like bands A1 and A2, always contained the same sequence. In the case of bands C9_1, C9_2 and C10_1, bands that migrated to different positions contained the same sequence.

Table C.21. Phylogeny of DGGE bands

Bands that appeared or increased in intensity during controlled disturbance						
Band [a]	**BLAST analysis (blastn)**			**ARB analysis**		**designed FISH probe**
	Next cultured relative	**Similarity**	**Accession number**	**Next cultured relative**	**Taxonomy**	
A3, A4, B7, B8, C6_1, C6_2	no cultured relatives			no cultured relatives	related to candidate phyla OD1 and TM7	Cd847
A5_2, A6_2	*Geothrix fermentans*	97%	GFU41563	*Geothrix fermentans*	*Acidobacteria*	Ac442
A10_2	*Methylibium aquaticum*	99%	DQ664244	*Methylibium aquaticum*	*β-Proteobacteria / Burkholderiales*	Meth444
A10_1	*Nitrospira moscoviensis*	97%	X82558	*Nitrospira moscoviensis*	*Nitrospirae / Nitrospira*	
B3_3	*Candidatus* Nitrospira defluvii	99%	DQ059545	*Candidatus* Nitrospira defluvii	*Nitrospirae / Nitrospira*	
C1_1	*Lewinella agarilytica*	87%	AM286229	*Haliscomenobacter hydrossis*	*Bacteroidetes / Saprospiraceae*	Bact414
C1_2	*Terrimonas lutea*	95%	AB192292	*Terrimonas lutea*	*Bacteroidetes*	
Bands that disappeared or decreased in intensity during controlled disturbance						
A1_1, A2_1	*Aquabacterium* sp. *Aqua2*	97%	AF089858	*Leptothrix* sp.	*β-Proteobacteria / Burkholderiales*	Aqua444
A1_2, A2_2, B6	*Propionicicella superfundia*	95%	DQ176646	*Aestuariimicrobium kwangyangensis*	*Actinobacteria / Propionibacteriaceae*	
A7_2, A7_3, A8_2, A8_3	*Flavobacterium ferrugineum* (*Terrimonas ferruginea*)	93%	AM230484	*Terrimonas lutea*	*Bacteroidetes*	Bact448
B1, B2	*Ferrimicrobium acidiphilum*	92%	AF251436	*Candidatus* Microthrix calida	*Actinobacteria / Acidimicrobiaceae*	
C10_1, 9_1, 9_2	no cultured relatives			no cultured relatives	candidate phylum TM7	

[a] labeling according to Figures C.10 - C.12, C.15 - C.17

C.2.2. FISH analysis

C.2.2.1. Design of FISH probes targeting rDNA sequences retrieved from DGGE bands

FISH probes were designed to target populations whose abundances were influenced by the implementation of "controlled disturbances". Those populations were represented by the following DGGE bands: A3, A5_2, A10_2, C1_1, A2_1 and A7_2. It was intended to design probes with the highest achievable specificity for the respective organisms containing a maximum number of central mismatches to non target organisms. Accessibility of target sites as determined for the 16S rRNA molecule of *E.coli* (Fuchs et al. 1998) was also considered to be an important issue for probe design. For the design of FISH probes that were supposed to target closely related populations that behaved differently under the "controlled disturbances" regime according to DGGE analysis, as was the case for the *Bacteroidetes* and *Burkholderiales* sequences, the most important criterion for probe design was the possibility to discriminate between these populations by FISH analysis.

No probes were designed for the *Nitrospira*, *Actinobacteria*, and TM7 affiliated sequences. FISH experiments with probes targeting *Nitrospira* Clusters I and II (Ntspa1431, Ntspa1151) into which the sequences from bands B3_3 and A10_1 fell, respectively, were already conducted in a previous study (Christiane Dorninger, unpublished results). Abundances of Ntspa1431- and Ntspa1151-targeted populations have been quantified and no shift induced by "controlled disturbances" has been detected. FISH experiments with probe HGC69a specific for *Actinobacteria* showed a very low abundance of actinobacterial cells (Fig. C.18) in nitrification and denitrification tank samples. Therefore a quantification of actinobacterial subpopulations was not considered to be a promising approach. In case of the TM7-related organism represented by bands C10_1 and C9_1 FISH analysis was not performed, because the respective sequences were present before as well as during the implementation of "controlled disturbances" and thus were not of interest for studying population dynamics induced by "controlled disturbances".

Characteristics of designed FISH probes are summarized in table B.30.

C.2.2.2. FISH experiments

Newly designed FISH probes were first hybridized to fixed sludge samples in the presence of 5 % formamide. If FISH signals could be detected, the highest formamide concentration that yielded bright signals of cells was determined. Probes were hybridized to nitrification or

denitrification tank samples according to the sample from which the respective DGGE bands had been obtained. Probes of broader specificities were always hybridized together with newly designed probes in order to confirm high probe specificity.

No cells hybridized to probes Aqua444, Cd847 and Ac442.

The optimal formamide concentration for hybridization of probe Meth444 was determined to be 40 %. Probe Bet42a specific for the ß-subdivision of *Proteobacteria* as well as the EUB338 probe mix targeting all bacterial cells hybridized to all cell clusters detected by probe Meth444 (Fig. C.19). Low numbers of cells (2 - 20 colonies per 20 µl of sample) that hybridized to probe Meth444 were detected in all nitrification tank samples. Hybridizations were done in duplicates. Higher colony numbers were detected in samples taken before (9-20) than in those taken during (2-15) "controlled disturbances".

The optimal formamide concentration for hybridization of probe Bact448 was determined to be 35 %. The EUB338 probe mix hybridized to all Bact448 detected cells (Fig. C.20). Very low numbers of cells that hybridized to probe Bact448 were detected in all nitrification tank samples (5-10 colonies per 20 µl of sample).

The optimal formamide concentration for hybridization of probe Bact414 was determined to be 30 %. Cells targeted by probe Bact414 were present in all nitrification tank samples and were in all cases detected by the EUB probe mix (Fig. C.21). 33 – 40 colonies were detected in 20 µl of samples taken before "controlled disturbances" (KN2A, KN2B, KN2D) and 6 - 10 colonies were detected in 20 µl of samples taken during "controlled disturbances".

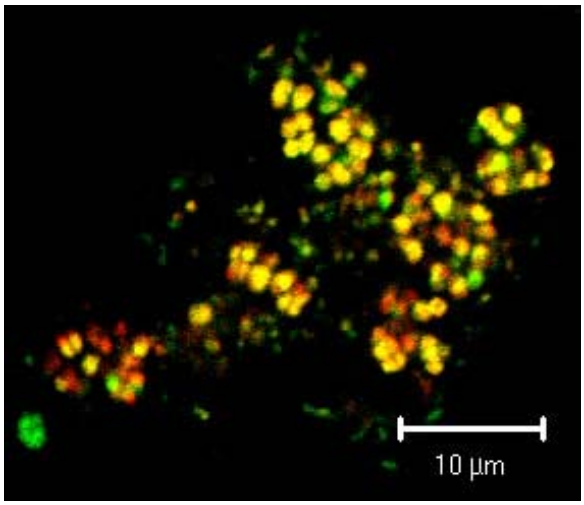

Fig. C.18. Actinobacterial cells detected by probe HGC69a (red) and EUBMix (green).

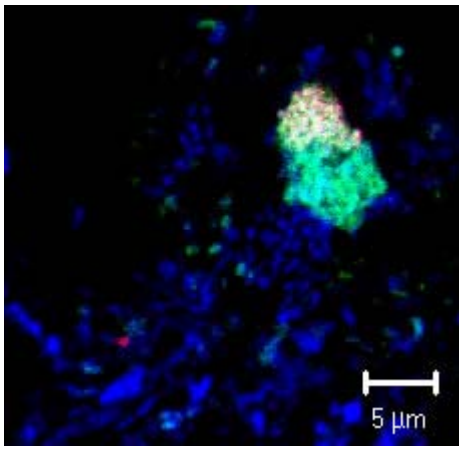

Fig. C.19. Bacteria detected by probes Meth444 (red), Bet42a (green) and EUBMix (blue).

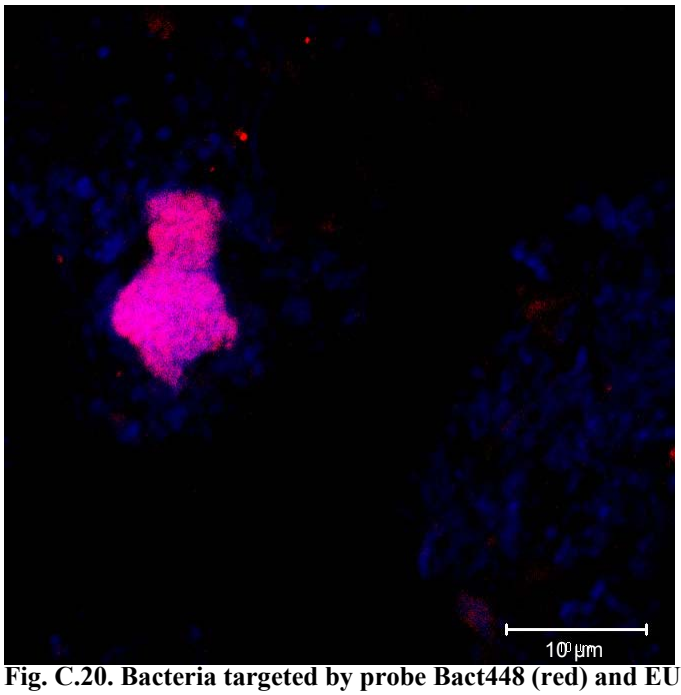

Fig. C.20. Bacteria targeted by probe Bact448 (red) and EUBMix (blue).

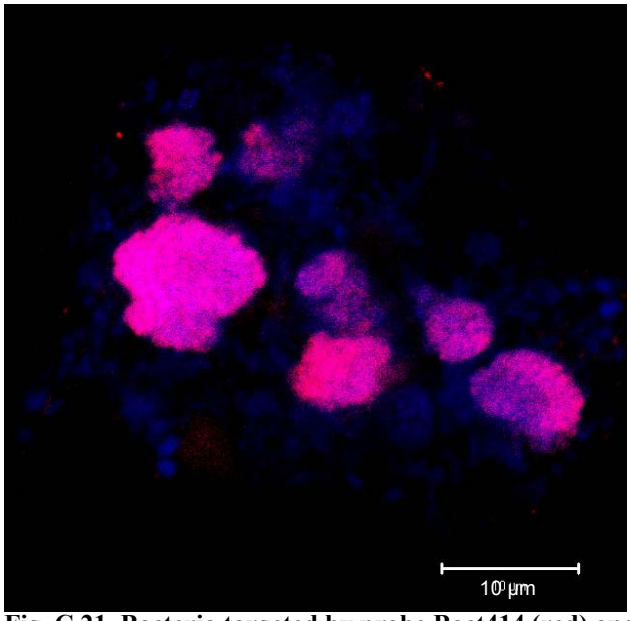

Fig. C.21. Bacteria targeted by probe Bact414 (red) and EUBMix (blue).

D. Discussion

D.1. Investigation of community dynamics of denitrifying bacteria associated with the implementation of "controlled disturbances" by T-RFLP analysis of nitrite reductase genes

D.1.1. T-RFLP: Design and optimization of the protocol

One aim of this study was to characterize the community dynamics of denitrifying bacteria associated with the implementation of "controlled disturbances". Since denitrifying bacteria are phylogenetically highly diverse (Zumft 1997, Heylen et al. 2006b), a diversity study based on 16S rDNA analysis alone is not possible. Functional genes involved in denitrification are the best target for this kind of analysis. Nitrite reductase genes were selected as target genes in this study since nitrite reductase is considered to be the key enzyme of denitrification (Zumft 1997). *Nir* gene diversity was investigated by T-RFLP fingerprints. For this purpose, a T-RFLP approach had to be developed that was suitable for reliably detecting the diversity of denitrifying bacteria in the denitrification tank samples.

D.1.1.1. Considerations about limitations of the method

It has been shown that T-RFLP analyses are more sensitive than other fingerprinting techniques (Moeseneder et al. 1999). Furthermore, T-RFLP is a potential high throughput technique, once a protocol has been established and carefully evaluated. For the establishment of a protocol it is necessary to consider and constrain all possible biases and limitations that are immanent to the method. Mechanisms that bias the depiction of the actual diversity of target organisms in a sample as T-RFLP data fall into two categories: mechanisms that lead to a loss of diversity information and those that feign a diversity that does not exist.

A loss of diversity information is expected to occur during different steps of the protocol. In the beginning DNA has to be isolated out of intact bacterial cells residing in the sample. Extraction efficiencies are expected to be different depending on cell wall composition (Frey et al. 2006) and might be to low for the detection of some species. During PCR a further reduction of detectable diversity occurs due to possibly limited primer coverage of target organisms and due to different amplification efficiencies of target sequences (von Wintzingerode et al. 1997). The conservation of restriction sites between disparately related

sequences further limits the resolution power of the approach. This problem can be overcome by the parallel use of different restriction enzymes (Schütte et al. 2008). However, this strategy may not facilitate the discrimination between highly related sequences (see Sec. D.1.1.2.1). During the electrophoresis step information may be lost due to the detection limit of the analyzing machine. Finally, information may be lost during T-RFLP profile analysis. It is necessary to set a threshold for consideration of peaks, as profiles that were produced from different amounts of DNA have to be normalized and small artefactual peaks need to be excluded from profiles (Osborn et al. 2000, Schütte et al. 2008). However, peaks derived from minor population members might have intensities below that threshold and will be removed.

On the other hand diversity may be overestimated by T-RFLP analysis due to different reasons. Artefactual amplicons, like chimeric amplicons or amplicons containing point mutations, can be produced during PCR (von Wintzingerode et al. 1997). These amplicons may contain terminal restriction sites at different positions than the non-artefactual amplicons in the mixture and will consequently produce T-RFs that do not correspond to organisms present in the sample. Incomplete digestion of PCR products can lead to the detection of labelled DNA fragments that do not represent T-RFs but PCR products cut at nonterminal restriction sites (Kitts 2001). Such fragments may be produced if digestion conditions are suboptimal, as the amount of restriction enzyme is too low and/or incubation time is too short, or if the PCR product is single stranded at the terminal restriction site (Egert and Friedrich 2003). Careful evaluation of the protocol is therefore necessary to limit the occurrence of artefactual T-RFs as far as possible. Another source of overestimation of diversity by T-RFLP analysis is the presence of multiple target gene copies in a bacterial genome (Crosby and Criddle 2003). If the sequences of these genes are different they may produce differently sized T-RFs and thus indicate the detection of multiple bacterial species. However, overestimation of diversity due to multiple gene copies per genome was not expected to be a big issue for *nir* gene T-RFLP analysis, since there is only one known case of a bacterium that possesses more than one *nir* gene, which is a *Thauera* species harbouring two different *nirS* genes (Etchebehere and Tiedje 2005).

D.1.1.2. Design of the T-RFLP protocol

D.1.1.2.1. Choice of primers and restriction enzymes

The first step for setting up a suitable T-RFLP protocol is the choice of PCR primers. Ideal primers should manage to target every target gene in the sample, and at the same time exclude non-target genes. The selected primers were shown to detect the broadest range of *nir* genes in a recent study (Throbäck et al. 2004). Moreover, the primers were shown to be highly specific for *nir* genes as determined by sequence analysis of PCR products (Sec. C.1.2), which is not the case for other primer sets regularly used for *nir* gene analysis (Prieme et al. 2002). The next important step was the choice of restriction enzymes that will make the diversity of amplified target sequences detectable via discrimination by different terminal restriction fragment lengths. It was shown that the choice of restriction enzymes can highly influence the composition of T-RFLP profiles and the conclusions drawn from them (Engebretson and Moyer 2003, Zhang et al. 2008). Consequently, careful evaluation of sequence data is required. All available restriction endonucleases were evaluated for their ability to discriminate cloned *nir* sequences by the position of their terminal restriction site and the three most suitable enzymes were selected. The resolution power was equally high for the *nirS* as for the *nirK* library. In both cases the 21 OTUs produced 48 T-RFs in total for all T-RFLP profiles, when plasmid mixes were analyzed. It is only possible to unambiguously determine the presence of a sequence in a sample if it produces a unique T-RF. If this is not the case its absence can be masked by the presence of other OTUs sharing the same terminal restriction sites. The ability of all restriction enzymes to discriminate between sequences by the production of unique T-RFs was therefore evaluated. Using the aforementioned enzymes, 19 of 21 OTUs and 14 of 21 OTUs were represented by a unique T-RF in at least one plasmid mix profile for the *nirS* and *nirK* clone library, respectively. This means that the presence of nearly all OTUs of the *nirS* library and of the majority of OTUs of the *nirK* library in a sample could be unambiguously determined using those three restriction enzymes provided that the sample contains the whole set of sequences represented in the library and no additional *nirS* sequences. However, as the clone library does not cover the whole diversity of target genes, which is indicated by the presence of T-RFs in sample KDF that do not correspond to any OTU, the discriminative power of the applied restriction enzymes is most probably lower in terms of production of unique T-RFs than estimated by analysis of cloned sequences.

During evaluation of the usability of restriction enzymes it became clear that a discrimination of sequences with more than 97 % sequence similarity is hardly possible. Therefore sequences with a similarity > 97 % were grouped into OTUs. Such OTUs may contain an unresolved microdiversity of sequences that represent different bacterial species.
In summary, *in silico* evaluation of restriction enzymes indicates that although a high resolution could be obtained, it is not possible to detect the whole diversity of *nir* genes by the compiled T-RFLP approach.

D.1.1.2.2. Methods for minimizing bias that occurs during DNA extraction, PCR, restriction digest and electrophoresis

To minimize the effect of random bias that occurs during DNA extraction, two replicate DNA extracts were pooled for PCR. To minimize the effect of PCR bias as differential amplification and formation of artefactual amplificates (von Wintzingerode et al. 1997), four replicate PCR products were pooled before they were used for the restriction digest. As the proportion of artefactual amplificates increases with PCR cycle number (von Wintzingerode et al. 1997), it was suggested to minimize PCR cycle number for T-RFLP analysis as far as possible (Egert and Friedrich 2003). In this study 35 PCR cycles have been performed which is a high cycle number compared to the literature. However as the amount of PCR product produced after 35 cycles from lowly diluted template DNA was barely sufficient for the following work steps a reduction of cycle number was not suitable. Moreover, a higher PCR cycle number may facilitate the detection of less abundant members of the bacterial population in the samples, when a fingerprinting technique such as T-RFLP is used.
To minimize the effects of biases that occur during restriction digest and electrophoresis, digests were performed and separated in triplicates and only peaks present in at least two replicate profiles were included in consensus profiles.

D.1.1.3. Evaluation and optimization of T-RFLP protocol by analysis of plasmid mixes

To evaluate the suitablility of the T-RFLP protocol for *nir* gene analysis of denitrification tank samples, a mimicked environmental sample was analyzed whose content of *nir* genes was known and consequently also the desired result of T-RFLP analysis. Plasmids harbouring the whole diversity of cloned sequences were mixed in comparable amounts and used as template for PCR and subsequently for restriction digest and electrophoresis in the same way as was planned for environmental samples. The detection of all predicted T-RFs and no

occurrence of other labelled DNA fragments in the T-RFLP profile would confirm the suitability of the protocol.

Observed T-RFs were in most cases shorter than predicted by *in silico* digests (Sec. C.1.4, Tab. C.3 – C.8). A difference between observed T-RF length and predicted T-RF length is frequently encountered (Osborn et al. 2000, Kitts 2001) and is referred to as T-RF drift (Kaplan and Kitts 2003). There are different explanations for this phenomenon. First, purin content of T-RFs was shown to be negatively correlated with T-RF drift (Kaplan and Kitts 2003). Second, sizing algorithms were shown to have an influence on calculated T-RF size (Osborn et al. 2000). Third, differences in the chemical structures of dye molecules used to label T-RFs and size standard DNA resulted in T-RF drift. Fragments labelled with fluorescein dye derivates like FAM and JOE that were used in this study to label T-RFs show a higher motility than fragments labelled with rhodamine dyes like TAMRA, which was the label of the size standard. Such a combination of dyes was shown to lead to an underestimation of T-RF size (Hahn et al. 2001, Pandey et al. 2007). However, as the aim of this study was a comparison of peak diversity between samples and T-RF drift was affecting T-RFLP analysis of all samples in the same way, no effort was undertaken to limit T-RF drift.

The protocol worked well for the detection of most of the diversity of *nir* sequences. Nevertheless, some bias has been observed. In some cases expected T-RFs were missing from profiles or were below the threshold (Sec. C.1.4, Tab. C.9). The majority of these T-RFs had a predicted length between 30 and 35 bp. Because of an underestimation of T-RF length due to T-RF drift they were most likely removed from the profiles, as it was decided to omit fragments with a length ≤ 30 bp. This is necessary and frequently done (Braker et al. 2000, Osborne et al. 2006, Zhang et al. 2008), because smaller fragments may represent primers. It has to be considered for restriction enzyme selection that enzymes producing T-RFs with a length between 30 and 35 bp may be undetectable, which leads to a loss of information. In five cases T-RFs with a predicted length of more than 35 bp were undetectable (Sec. C.1.4, Tab. C.9). For three of these sequences unique T-RFs were produced in one of the other plasmid mix profiles. In one case (sequence S3_16), the abundance of the unique T-RF was slightly above the threshold suggesting a low amplification efficiency of the respective sequence compared to other sequences in the mixture during PCR. For the other two sequences (S4_14, S1_9) abundances of unique T-RFs were higher, indicating that PCR products were present in sufficient quantities for T-RFLP detection. Therefore, a bias must have occurred after PCR. Bias might have occurred during the restriction digest, as probably the restriction site was due to structural features of the sequence difficult to access for the

enzyme. Although it was possible to cut PCR products of sequences S4_14 and S1_9 at the respective sites when they were digested separately, other sequences harbouring restriction sites that were easier to access were probably preferentially cut when sequences were digested in a mixture. In case of sequence S4_10 whose T-RF was missing from profile *nirS Mnl*I forward, two peaks with a length of 222 and 226 bp were produced when the sequence was digested separately, the latter one being slightly higher than the first. The 222 bp peak represented the T-RF, the 226 bp peak an artefactual restriction fragment that was present in profile *nirS Mnl*I forward with an abundance slightly above the threshold. The sequence contained two recognition sites on opposing strands that were situated close to each other. The cleavage sites were intersecting each other so that the cleavage of a cleaving site on one strand resulted in separation of recognition and cleavage site on the other strand, making cleavage impossible. For some reason cleavage of the restriction site at 226 bp happened slightly more often.

Some profiles contained peaks that could not be assigned to sequences contained in the template mixture (Sec. C.1.4, Tab. C.3 – C.8, C.10). In total, twelve of these so-called pseudo-T-RFs were found. In nine of these cases artefact peaks were probably the result of incomplete digestion of PCR products as their length was shown to correspond to restriction fragments obtained by incomplete digestion of PCR products of some cloned sequences (Sec. C.1.4, Tab. C.10). It was suggested that incomplete digestion might happen if PCR products are partially single-stranded (Egert and Friedrich 2003, Egert and Friedrich 2005). Incubation of PCR products with Klenow fragment exo$^-$ and dNTPs was performed prior to the restriction digest to fill up single stranded regions and make them cleavable for restriction enzymes. This approach led to a reduction of pseudo-T-RF peak area below the detection threshold in six cases, in five of which pseudo T-RFs corresponded to nonterminal restriction sites. One new pseudo-T-RF showed up that also most likely corresponded to a nonterminal restriction site. Four pseudo-T-RFs that were probably the result of incomplete digestion could not be removed by this approach, some of them were even present in larger amounts. Thus, restoring double strands by Klenow fragment exo$^-$ preincubation might help to remove artefact peaks in some cases, however it has no or even the contrary effect on pseudo-T-RF peak area in other cases. There is evidence that single stranded regions of PCR products may transiently form secondary structures during the restriction digest (Egert and Friedrich 2003). As conditions are the same in terms of temperature and buffering for restriction digests and Klenow fragment exo$^-$ preincubation, it can be speculated that formation of secondary structures may occur in both cases. Secondary structures will prevent the Klenow fragment

from incorporating nucleotides and restoring double strands. If the terminal restriction site resides in a single stranded region where secondary structures are formed, double strands will not be restored, thus the restriction site will not be cleaved and pseudo-T-RF amount stays the same. This scenario is of course only plausible if secondary structures are not formed in that way, that the restriction site resides in a double-stranded and thus cleavable region. An enlargement of pseudo-T-RF peak area by Klenow fragment exo$^-$ preincubation is imaginable if the terminal restriction site resides in a single stranded region of the PCR product that forms secondary structures hindering the enzyme, and the nonterminal restriction site responsible for pseudo-T-RF formation resides within a single stranded region without secondary structures. Double strands will be restored at the nonterminal restriction site but not at the terminal restriction site and consequently, after Klenow fragment incubation, more PCR product will be available that can be cut at the nonterminal restriction site, resulting in a higher amount of detectable pseudo-T-RF.

In summary it can be stated that Klenow fragment preincubation may introduce an additional source of bias. It is therefore not advisable to perform it without prior evaluation of its effect in the respective experimental setup. For the T-RFLP approach performed in this study, the detected bias was smaller with Klenow fragment preincubation than without it, so it was decided to include this preincubation step into the protocol.

D.1.1.4. Data analysis

Peak area as well as peak height have been used as measures of peak intensity in previous studies and there is no general agreement which approach is more suitable. However, as peak height is influenced by T-RF size and peak area is not (Kitts 2001), it was decided to use peak area.

For normalizing differences in data sets produced from loading different amounts of DNA for electrophoresis and eliminating background noise it is necessary to set a threshold for consideration of peaks (Kitts 2001). For this study, it was decided to omit peaks with an area of less than 1 % of the total peak area from the profiles. There are different, more sophisticated methods for threshold calculation available (Dunbar et al. 2001, Osborne et al. 2006), but it was decided to use this approach as it was used with success before (Rees et al. 2004) and worked well for the separation of true T-RFs from smaller artefact T-RFs when applied to plasmid mix profiles in this study. As discussed above some artefactual peaks were well above the threshold, but as their height was comparable to that of true T-RFs no kind of threshold would manage to sort them out. The threshold removed small

noise peaks in high numbers. Only in one case a peak representing a true T-RF was below the threshold. However, 10 % of T-RFs corresponding to cloned sequences were below the threshold in T-RFLP profiles of sample KDF, the sample from which the clone library has been established. Results of the plasmid mix analysis indicated that lowering the threshold would have led to the detection of further artefact peaks. It was therefore decided to leave the threshold at 1% of the total peak area, taking into account that a part of the diversity would be overlooked by this approach.

In recently published studies, binary data (presence/absence of T-RFs) as well as relative proportions of T-RFs were used for comparing the diversity of microorganisms between samples (Baniulyte et al. 2008, Magalhaes et al. 2008, Meier et al. 2008, Zhang et al. 2008). Some authors used the relative proportions of T-RFs to infer the original abundance of bacteria in environmental samples (Gentile et al. 2007b, Boyle-Yarwood et al. 2008). However, it has been shown that bias that occurs during PCR as well as during DNA extraction can lead to a shift of T-RF abundance compared to the real abundance of microorganisms represented by the respective T-RFs (Lueders and Friedrich 2003, Frey et al. 2006, Hartmann and Widmer 2008). Moreover, post-PCR analysis (digestion and electrophoresis) was shown to affect the abundance of T-RFs (Hartmann and Widmer 2008). There is evidence that such biases have affected the relative proportions of T-RFs in the present study. T-RFLP analysis of pooled plasmids showed differences in T-RF abundances. Even though the proportions of plasmids in the template mixture have not been totally equal but differed by up to twofold between OTUs, no T-RF abundance should have been below the threshold, as was the case for sequence S3_16. Moreover, two peaks were below the threshold due to bias that probably occurred during the restriction digest. This indicates that some causes of bias in T-RFLP analysis are still unexplored and it confirms the unsuitability of T-RF relative abundance to mirror the true abundance of an organism in an environmental sample. The results of Hartmann and Widmer suggest that although ratios of T-RFs might not reflect ratios of genes in the template mixture, relative changes of gene abundances could be determined by T-RFLP analysis (Hartmann and Widmer 2008). However, this might not be true in every case since the exact way in which PCR bias influences the abundance of PCR products of an unknown mixture of genes in an environmental sample is hard to predict. Another study has shown that relative T-RF abundances vary by about 11 % between profiles obtained from replicate DNA extracts, PCRs and electrophoretic separations (Osborn et al. 2000). After considering all this evidence it was decided that it is not possible to conclude with certainty that the variability of relative proportions of T-RFs can be used as a measure of

variability of the bacterial community between samples and consequently, presence/absence data were used for the comparison of diversity. This might lead to a loss of information but increases the reliability of the results.

D.1.2. T-RFLP analysis of *nir* genes in the denitrification tank before and during the implementation of "controlled disturbances"

D.1.2.1. Effect of "controlled disturbances" on the population structure of denitrifying bacteria

For assessing the effect of "controlled disturbances" on the population structure of denitrifying bacteria, cluster analysis was performed for presence/absence data of consensus profiles of all T-RFLP analyses. Samples taken during "controlled disturbances" (KDE and KDF) clustered together (Sec. C.1.5, Fig. C.1), but similarities between samples did not show pronounced differences (Sec. C.1.5, Tab. C.11). Cluster analysis was also separately performed on T-RFLP profiles of *nirS* and *nirK* genes. The results indicate that the clustering pattern of the whole dataset was due to a distinct clustering of samples KDE and KDF for *nirS* T-RFLP profiles (Sec. C.1.5, Fig. C.3, Tab. C.13). Thus, the population structure of *nirS* possessing bacteria experienced a shift when "controlled disturbances" were implemented. *nirK* profiles were most similar between samples KDD and KDE (Sec. C.1.5, Fig. C.2, Tab. C.12), which might indicate that the *nirK* population underwent a lagged population shift. This, however, cannot be concluded with certainty. Taken together, these data suggest that "controlled disturbances" induced a population shift of denitrifying bacteria with respect to the presence or absence of species, although it had different effects on *nirS* and *nirK* possessing bacteria. The presence of 29 % of T-RFs was affected by the implementation of the new operational mode, whereas only 8 % of T-RFs showed a pattern of presence and absence that could not be explained by the implementation of "controlled disturbances" (i.e. they were only present in either KDE or KDD and KDF). This finding supports the conclusion that a population shift in reaction to "controlled disturbances" was detected rather than some stochastic population dynamics.

It was hypothesized that the operational mode "controlled disturbances" might cause an increase in diversity of denitrifying bacteria, due to the creation of new ecological niches. Diversity of denitrifyig bacteria was investigated by T-RFLP analysis with respect to species richness. For *nirS* as well as for *nirK* numbers of T-RFs slightly decreased during the implementation of "controlled disturbances" (Sec. C.1.5, Tab. C.14). The new operational

mode introduced two kinds of modifications of regular operation, periodic changes of wastewater load and regular shutdown of aeration of the nitrification tanks. Both modifications have most likely affected the denitrifying community. The modified loading confronted the bacteria with fluctuating nitrate and organic carbon concentrations, whereas the shutdown of aeration of the nitrification tanks confronted the bacteria with fluctuating oxygen concentrations and most probably stimulated their denitrifying activity. Thus, new niches for denitrifiers were created in the denitrification tank as well as the nitrification tanks when "controlled disturbances" were implemented, most likely supporting the growth of microorganisms with different ecophysiological properties than those adapted to denitrify in the denitrification tank during regular operation. During phases of low load, denitrifiers with higher substrate affinities for nitrate and organic carbon compounds were presumably on the rise in the community, whereas phases of high load were favourable for denitrifiers with lower substrate affinities but higher growth rates. In ecological terms, low load phases selected for K-strategists and high load phases for r-strategists. The fluctuating oxygen levels in the nitrification tank may have promoted the growth of bacteria that are able to switch fast between using oxygen and oxidized nitrogen species as electron acceptors. Given the introduction of the described new ecological niches, it is surprising that an increase in species richness of denitrifying bacteria could not be detected by T-RFLP analysis of *nir* genes. Instead, numbers of T-RFs decreased. Since this decrease was not very pronounced it cannot be concluded with certainty that it mirrors a decrease in denitrifier species richness, given the limited resolution power and the detection limit of the T-RFLP approach. Nevertheless, the T-RFLP results indicate that an increase in denitrifier species richness, as was predicted by the "intermediate disturbance hypothesis" did not happen. If such an increase happened it could only have been due to an increase in the number of less abundant species which could not be detected by T-RFLP analysis or due to species whose *nir* genes did not produce T-RFs of different length than species that were present before the implementation of "controlled disturbances".

The implementation of "controlled disturbances" resulted in a higher denitrification performance of the wastewater treatment plant, meaning that the bacterial community denitrified at higher rates. Results of T-RFLP analysis suggest that this improved function was not linked to an increase in species richness of denitrifiers, but rather to a population shift. "Controlled disturbances" might therefore, instead of the predicted creation of more ecological niches have caused a more efficient utilization of nutrients by selecting bacterial species that were able to remove nitrate more efficiently, than those that were present before.

It has to be noted that changes in abundances of denitrifying populations and species evenness of denitrifiers could have affected the performance of the wastewater treatment plant. A population shift regarding the presence of species was detectable, so it is tempting to speculate that quantitative shifts did also occur. However, such changes could not be investigated by T-RFLP analysis as T-RF abundances do not reliably reflect the abundances of the corresponding bacteria in the samples.

D.1.2.2. Effect of "controlled disturbances" on the metabolic activity of denitrifiers

The implementation of "controlled disturbances" did most likely not only affect the population structure of denitrifiers but also the metabolic activity of the respective denitrifying populations. The presence of more denitrifying zones because of less aeration and the higher supply of organic carbon compounds during the phases of elevated load most likely stimulated the denitrifying activity of the bacteria and thus contributed to the observed increase in denitrification performance of the wastewater treatment plant. However, as DNA-based analyses were performed in this study, changes in the metabolic activity of the monitored denitrifying microorganisms could not be assessed. Whereas genes encoding proteins involved in denitrification are always present in an organism, the corresponding proteins may not be expressed at any time. Additionally, detected genes may not be functional. Thus, DNA-based analyses can only be used to infer a possible denitrifying potential of a community, but not its actual activity (Philippot and Hallin 2005). As denitrification is a facultative trait, this discrepancy between potential and actual activity is of particular importance. Bacteria that have the potential to denitrify might make use of this potential under different environmental conditions, as for example the expression of the denitrifying trait is in case of most bacteria dependent on the availability of oxygen as electron acceptor. However, the oxygen concentration at which bacteria perform denitrification varies between species. It is generally assumed that most bacteria denitrify only under anaerobic or microaerophilic conditions, but there are also known cases of bacteria that denitrify under aerobic conditions (Zumft 1997, Patureau et al. 2000). Given this ecophysiological versatility of denitrifying bacteria, it is hard to predict the activity of a community by the mere presence of certain denitrifying genes. The metabolic activity of denitrifiers could be studied by assessing the transcription of denitrifying genes by mRNA-based techniques (Nogales et al. 2002, Sharma et al. 2005). Even more reliable is the immunological detection of denitrification proteins of bacteria, as they are at the end of the regulatory cascade (Metz et al. 2003, Philippot and Hallin 2005). Other approaches involve

the detection of active denitrifiers based on organic substrates labelled with stable or radioactive isotopes that are built into the biomass under denitrifying conditions. In that way, denitrifiers were investigated in environmental samples by stable isotope probing and microautoradiography combined with FISH (Ginige et al. 2005, Osaka et al. 2006, Thomsen et al. 2007, Morgan-Sagastume et al. 2008).

D.1.2.3. Different effects of "controlled disturbances" on the population structures of *nirS*- and *nirK*-possessing bacteria

Cluster analysis indicated that the community of *nirS* possessing bacteria experienced a shift when "controlled disturbances" were implemented, whereas a pronounced shift of the *nirK* community could not be detected (Sec. C.1.5, Fig. C.2, C.3). These different responses are also reflected in numbers of T-RFs that appeared and disappeared during "controlled disturbances". For the *nirK* population 12 T-RFs disappeared and 6 appeared during "controlled disturbances", whereas 19 T-RFs disappeared and 13 appeared for the *nirS* population (Sec. C.1.5, Tab. C.15). Thus, the *nirK* community showed a higher stability regarding community structure while facing "controlled disturbances".

These different population dynamics may be due to a pronounced reduction of iron salt addition during the implementation of "controlled disturbances". Iron salts were used to precipitate phosphate during regular operation of the plant. As the EBPR performance increased dramatically during "controlled disturbances", iron salt addition was reduced. As iron is the prosthetic metal of *nirS*, the limited iron availability might have selected for bacteria with higher iron uptake efficiencies under low substrate concentrations, therefore affecting the structure of the *nirS* community. *nirK* on the other hand contains copper. Copper concentrations were equally high before as during the implementation of "controlled disturbances". Therefore, the observed population dynamic patterns might have – at least partly – been linked to the availability of the prosthetic metal of nitrite reductases.

Furthermore, it is tempting to speculate about ecophysiological differences between *nirS*- and *nirK*-possessing bacteria. The results obtained by T-RFLP analyses indicate a higher stability of the *nirK* population stucture when exposed to varying oxygen and nitrate concentrations, indicating that a higher proportion of the *nirK* community was able to adapt to the new environmental conditions compared to the *nirS* population which might have contained a higher number of specialists that were successful when either the regular operational mode or "controlled disturbances" were implemented. Indications of ecophysiological differences between *nirS* and *nirK* possessing bacteria can be found in different publications. In a

microbial mat, *nirS* genes were found only in deep anoxic layers, whereas *nirK* genes were detected throughout all layers of the mat, along pronounced gradients of environmental factors like oxygen concentration and pH (Desnues et al. 2007). This might indicate a higher ecophysiological versatility of *nirK* harbouring bacteria, too, although the population structure of the *nirK* community varied along the mat profile. By contrast, a higher variability of *nirK* than *nirS* genes was detected along oxygen and nitrate gradients in the suboxic zone of the Black Sea (Oakley et al. 2007). Abundances of *nirK* and *nirS* genes in a membrane aerated biofilm were shown to be differently influenced by oxygen and organic nutrient concentrations (Cole et al. 2004). A real time PCR assay suggested a higher abundance of *nirK* compared to *nirS* genes in wastewater treatment plants with higher nitrogen loads (Geets et al. 2007). In nitrate and uranium contaminated groundwater, a higher diversity of *nirK* genes was found in environments with higher nitrate levels, whereas a higher *nirS* diversity was detected in environments with relatively moderate nitrate levels (Yan et al. 2003). All these observations suggest different ecophysiological properties of *nirS* and *nirK* possessing bacteria, although yet a consistent trend cannot be seen.

Since the possession of *nirS* and *nirK* genes is widespread among distantly related and physiologically different bacteria (Zumft 1997, Heylen et al. 2006a) it might not be generally possible to assign ecophysiological traits to one of these groups. Physiological properties of denitrifying bacteria may vary greatly, therefore masking a possible ecophysiological effect of the type of nitrite reductase. One might speculate that only in highly selective habitats which limit the diversity of present denitrifiers, a single feature such as the type of nitrite reductase may have pronounced effects on community composition. Consequently, wastewater treatment plants that treat wastewater of less complex composition than the one investigated in this study might be a better setup for investigating niche differentiations between *nirS* and *nirK* possessing bacteria.

On the other hand, horizontal gene transfer might lead to the distribution of *nirS* and *nirK* genes encoding enzymes with similar properties within a habitat, at least among closely related bacteria, thus resulting in a niche differentiation specific for a community of bacteria in one specific place, at one specific time, a kind of dynamic niche differentiation. This theory is supported by the observation of habitat specific *nirK* and *nirS* clusters (Braker et al. 2000, Prieme et al. 2002, Sharma et al. 2005, Heylen et al. 2006a, Santoro et al. 2006). In summary, more research is needed before conclusions about possible niche differentiations between *nirS* and *nirK* possessing bacteria can be drawn.

D.2. Investigation of bacterial community dynamics before and during the implementation of "controlled disturbances" by DGGE analysis of 16S rRNA genes

Bacterial community dynamics associated with the implementation of "controlled disturbances" were monitored using 16S rRNA gene DGGE analysis. No data obtained by any fingerprinting approach available today is expected to mirror the true diversity of organisms in a sample as numerous sorts of biases are expected to act during different steps of the analysis, as was also discussed for T-RFLP (Sec. D.1.1.1). It was therefore decided to approach the complexity of the microbial community by two different DGGE experiments involving analysis of PCR products established by the use of different reverse primers. The use of two different reverse primers most likely resulted in different PCR bias effects. Additionally, due to different lengths and sequences of PCR products, separation efficiencies may have differed. To constrain PCR bias effects like stochastic variation of amplification efficiency of different sequences and occurrence of chimeric molecules, replicate PCR products were pooled for analysis.

Statistical analysis of DGGE gels obtained by both approaches showed consistent results. Cluster analysis of both gels suggested that the composition of the bacterial community was affected by "controlled disturbances", as two distinct clusters were formed which contained samples taken before or during "controlled disturbances" exclusively (Sec. C.2.1.4, Fig. C.13 and C.14). The combined information gives a strong hint that the composition of the bacterial community changed to a degree that exceeded the variation that occured during normal operation when "controlled disturbances" were implemented. Consequently, "controlled disturbances" selected for a differently structured bacterial community than the original operational mode. This community exerted the treatment processes more efficiently under conditions provided by "controlled disturbances" than the community present during regular operation was able to do under the respective environmental conditions.

Furthermore, the results of DGGE analysis suggest that the bacterial community structure changed when exposed to a different operational mode but stayed relatively constant when operating conditions were kept constant. Stability of bacterial community structure under constant operating conditions was observed in a full-scale wastewater treatment plant treating pharmaceutical wastewater (LaPara et al. 2002), in a full-scale pulp mill effluent treatment system (Smith et al. 2003), in a pilot-scale denitrifying fluidized bed reactor (Gentile et al. 2007a) and in a small-scale denitrifying reactor treating aquarium seawater (Labbe et al.

2003). By contrast, fluctuating community composition in spite of constant operating conditions was observed for bacterial communities in a lab-scale methanogenic bioreactor (Fernandez et al. 1999), a lab-scale anaerobic bioreactor (Zumstein et al. 2000) and for nitrifiers in a lab-scale sequencing batch reactor as well as a lab-scale membrane bioreactor (Wittebole et al. 2008). Community composition was relatively stable during constant operating conditions and shifted when environmental conditions were changed or disturbances introduced in the studies of LaPara *et al.* (2002) and Gentile *et al.* (2007a), resembling the results of this study. By contrast, in the study of Smith *et al.* (2003) variability of bacterial community composition was equally low during normal operation and disturbance. Thus, bacterial community dynamics in wastewater treatment facilities during constant operation and changing environmental conditions did not show consistent trends in recent studies, except from the fact that fluctuating bacterial community composition during constant operation was only observed in small-scale reactors, whereas all full-scale wastewater treatment facilities (including the one investiagted in this study) contained bacterial communities that showed high stability when conditions were kept constant. This could indicate a higher likelihood to encounter unstable bacterial communities in wastewater treatment facilities of smaller scales. An explanation for this trend is provided by the ecological theory of island biogeography which suggests that smaller wastewater treatment facilities harbour less diverse and more variable communities (MacArthur and Wilson 1967, Curtis et al. 2003). It might thus be problematic to extrapolate knowledge about microbial population dynamics gained from observing lab-scale reactors to full-scale wastewater treatment plants. This underlines the importance of studying full-scale wastewater treatment systems to understand the dynamics of microbial communities responsible for wastewater treatment. However, research on these issues is not yet exhaustive. Additionally, results of studies conducted so far might be difficult to compare, as they involved not only wastewater treatment facilities of different scales, but of totally different function (denitrification, nitrification, methanogenesis, treatment of paper mill effluents, treatment of pharmaceutical wastewater and treatment of artificial seawater). The pattern of microbial community dynamics may depend on the type of treatment process and the specific bacteria involved. Sampling was done in different intervals and methods used for characterization of community structure were different (DGGE, T-RFLP, RIS-LP, ARDRA, SSCP). Furthermore, data analysis was done in different ways and community stability and instability were differently defined. Thus, more studies involving comparable methods to determine bacterial community

structure and stability in wastewater treatment facilities of different scales would be needed to clarify the picture.

One idea behind the design of "controlled disturbances" was to increase the bacterial diversity in order to increase the resistance of the community against unfavourable conditions. Whether "controlled disturbances" selected for a more diverse community cannot be concluded with certainty. Numbers of detected DGGE bands were slightly higher for all samples taken during "controlled disturbances" than for those taken before (Sec. C.2.1.4, Tab. C.19 and C.20). This could be an indication of an increase of microbial diversity that was predicted by the "intermediate disturbance hypothesis". However, it is problematic to infer the diversity of bacteria from the diversity of bands on 16S DGGE gels due to methodological limitations. Those comprise besides preferential amplification during PCR, which has been discussed above, limitations inherent to electrophoresis. The necessity of visual detectability of bands constrains the analysis to genes that were amplified to higher extents. Additionally, limited separation of non identical sequences results in an underestimation of the diversity. Bands containing multiple sequences were observed before (Sekiguchi et al. 2001) and were also detected in this study (see Sec. C.2.1.7). Nucleotide similarities of non identical sequences obtained from the same excised band were in all cases about 75 %, indicating that these sequences represented bacteria which where not closely related. Consequently, a part of sequence heterogeneity was overlooked. Another important factor that biases diversity estimations based on 16S fingerprints is the presence of multiple rRNA operons in bacterial genomes. Up to 15 copies per genome have been found (Acinas et al. 2004). In most cases different copies contain nearly identical sequences, but in some cases copies were found that differed to a higher extent (Acinas et al. 2004). The opposite was also observed, as different species of bacteria were shown to possess completely identical 16S genes (Jaspers and Overmann 2004). For all of these reasons band diversity of 16S DGGE gels is not a reliable measure of species diversity in an environmental sample. As the number of detected bands was not considerably higher for samples taken during than for those taken before "controlled disturbances", it is not possible to exclude the possibility that the detected increase of bands due to "controlled disturbances" is caused by some methodological artefact.

As discussed for T-RFLP analysis of nitrite reductase genes (see Sec. D.1.2.2), effects of "controlled disturbances" on the bacterial community were only investigated regarding effects on community structure, not in terms of effects on metabolic activity of the community. Evidence for such effects were found in previous studies, where abundances of different groups of nitrifying and polyphosphate accumulating bacteria before and during the

implementation of "controlled disturbances" were determined (Christiane Dorninger, unpublished results; Karin Hace, unpublished results, Per H. Nielsen, unpublished results). Abundances of the dominant nitrifiers (AOB, NOB) and polyphosphate accumulating organisms (PAO) before and during "controlled disturbances" were comparably high. Nevertheless, their metabolic activities were influenced as can be inferred from the constant nitrification rate despite pronounced changes in oxygen and ammonia availabilities and a higher EBPR rate during "controlled disturbances".

D.3. Investigation of bacteria affected by "controlled disturbances" by FISH analysis

D.3.1. FISH analysis of bacteria represented by DGGE bands

D.3.1.1. Effect of "controlled disturbances" on bacterial populations targeted by newly designed FISH probes

This study was mainly focused on investigating the impact of "controlled disturbances" on the bacterial community structure by fingerprinting techniques. Nevertheless, identification of bacteria whose presence or abundance was affected by "controlled disturbances" was considered to be an interesting issue. Therefore, DGGE bands that appeared or disappeared in samples taken during "controlled disturbances" were excised and sequenced. FISH probes targeting the respective sequences were designed if possible and applied to the respective environmental samples.

Two sequences with a similarity of 95%, affiliated to the order *Burkholderiales* were obtained from bands A10_2 and A1_1. Band A10_2 disappeared and band A1_1 appeared during "controlled disturbances" (Sec. C.2.1.3, Fig. C.10). This indicated a niche differentiation of closely related bacteria. However, FISH analysis with probes designed to target the respective bacteria could not confirm this hypothesis. Probe Meth444 targeting bacteria represented by band A10_2 hybridized to low numbers of cells (2-20 colonies per 20 µl of sample) in all nitrification tank samples. By visual inspection an apparent trend towards higher colony numbers in samples taken before "controlled disturbances" was seen, but due to their low abundance cells could not be reliably quantified. Positive FISH signals for probe Aqua444 could not be obtained. Thus, it was not possible to confirm the population dynamics suggested by DGGE by FISH analysis.

Probes Bact414 and Bact448-targeted organisms affiliated to the phylum *Bacteroidetes* represented by DGGE bands C1_1 and A7_2, respectively. Band A7_2 appeared during "controlled disturbances" (Sec. C.2.1.3, Fig. C.10), however, the abundance of cells that hybridized to the corresponding probe Bact448 was not affected by the implementation of "controlled disturbances". Band C1_1 had a pronounced higher intensity in samples taken during controlled disturbances (Sec. C.2.1.3, Fig. C.12). In contrast, cells that hybridized to probe Bact414 decreased during the implementation of "controlled disturbances". Thus, in this case, a bacterial population that was affected by the implementation of "controlled disturbances" could be detected by FISH analysis, although it was most likely not the intended target population according to DGGE analysis (see Sec. D.3.1.2). As no close relatives of these bacteria were so far cultivated or ecophysiologically characterized it is not possible to speculate about why they were negatively affected by the implementation of "controlled disturbances" or about a possible positive effect of the inhibition of their growth on the function of the wastewater treatment plant. Closely related sequences with nucleotide similarities ≥ 96 % have been found in other wastewater treatment plants (EU177683, EU177682, EU177681 (Xia et al. 2008); DQ640703 (Kong et al. 2007); AB205940, AB205795, AB158696 (Osaka et al. 2006)) which indicates that these bacteria might play a role in wastewater treatment that could be a subject of further investigation.

No probes were designed to target the *Nitrospira* sequences obtained from bands A10_1 and B3_3, since the abundances of bacteria affiliated to *Nitrospira* sublineages I and II, in which the sequences fell, have been quantified before in nitrification tank samples using probes Ntspa1431 and Ntspa1151 (Maixner et al. 2006), respectively (Christiane Dorninger 2008, unpublished results). No difference in abundances could be detected between samples taken before and during "controlled disturbances". Representatives of *Nitrospira* sublineage I were the dominant *Nitrospira* population in all samples and representatives of sublineage II did only make up about one to five percent of the *Nitrospira* population. The observation that the change in operating conditions did not affect the structure of the *Nitrospira* population was unexpected, since evidence exists that the two sublineages are adapted to different environmental conditions. Representatives of sublineage II were found in higher proportions in wastewater treatment plants that were subjected to irregular change in load and aeration failures (Anneser 2004). Moreover, representatives of the two sublineages were shown to be adapted to different nitrite concentrations (Maixner et al. 2006). Therefore, a different effect of the implementation of "controlled disturbances" on the two *Nitrospira* populations was expected. Sequence analysis of DGGE bands suggests that representatives of both *Nitrospira*

sublineages that were not present before "controlled disturbances" or only to a minor extent were on the rise during "controlled disturbances". Consequently, *Nitrospira* sublineages I and II may contain some unknown microdiversity of differently adapted bacteria that would be a worthwhile subject of further studies.

No hybridized cells were detected for probes Aqua444, Cd847 and Ac442. This could indicate very low cell numbers below the FISH detection limit, malfunction of probes due to inaccessibility of target sites or low fluorescence signals of target cells due to a low ribosome content (Amann et al. 1995, Wagner et al. 2003, Hoshino et al. 2008).

D.3.1.2. Incongruence of results obtained by DGGE and FISH analysis

Three of the probes that were designed to target organisms represented by DGGE bands hybridized to cells residing in the samples. In none of these cases the targeted populations were affected by the implementation of "controlled disturbances" as predicted by DGGE analysis. In case of probes Meth444 and Bact448 no pronounced differences in numbers of targeted cells could be detected between samples taken before or during "controlled disturbances" whereas the corresponding DGGE bands were exclusively present before and during "controlled disturbances", respectively. In case of probe Bact414 higher cell numbers were detected in samples taken before "controlled disturbances" whereas the respective DGGE band had a much higher intensity in samples taken during "controlled disturbances". This inconsistency of results may be due to one of the following reasons. One possible cause could be a bias that occurred during PCR. Identical genes may have been amplified to different extent in different samples due to stochastic processes during PCR and thus may have shown different band intensities on DGGE gels. However, it is most unlikely that PCR bias has caused such pronounced differences in amplicon abundance as were encountered when band intensities or presence and absence of bands were compared between samples, especially, as multiple PCR products were pooled for DGGE analysis to counteract such problems. Additionally, it is even more unlikely that amplification of identical genes was biased towards higher amplification efficiency in all samples taken before "controlled disturbances" and towards lower amplification efficiencies in all samples taken during "controlled disturbances", or the other way round. It is thus most likely that the obviously encountered bias occurred during DGGE analysis. All bands from which sequences for FISH probe design have been obtained contained heterogeneous DNA that could only be sequenced after separation on a gel with a narrower gradient, where it produced multiple bands. This might be due to one of the following reasons. Firstly, although excision was done very

carefully, DNA from bands adjacent to or overlapping the target band may have been contained in the excised gel piece. Secondly, separation efficiency may have been insufficient on original DGGE gels, meaning that although PCR products with different sequences migrated to different positions when separated on a gel with a narrower gradient, they may well have migrated to the exact same position when separated on the original DGGE gel with a wider gradients. There are even known cases of PCR products that migrated to different positions on the same gel when applied alone but migrated to the same position when applied in a mixture (Sekiguchi et al. 2001). Furthermore, PCR products of prominent bands may be distributed all over the gel, contaminating all other bands (Nikolausz et al. 2005). For all of these reasons, sequence analysis alone is insufficient to infer the identity of dominant organisms, if sequences were obtained from bands that contained heterogenous DNA. Results of FISH analysis indicated that the organisms identified by sequence analysis were not the ones that were represented by the excised DGGE bands but rather represented comigrated PCR products. This may also be true for some of the organisms which could not be targeted by FISH probes, thus conclusions about any sequence data obtained by DGGE analysis have to be drawn very carefully.

D.3.2. FISH analysis of bacteria corresponding to T-RFs

Analysis of nitrite reductase genes by T-RFLP was performed to investigate the diversity of denitrifiers in wastewater treatment plant Weißtal when subjected to different operational modes. Analysis of the sequences of *nir* genes that appeared or disappeared during "controlled disturbances" was not the main intention of this study due to their limited usability as phylogenetic markers (Philippot 2002, Heylen et al. 2006a). Nevertheless, *nir* sequences may contain phylogenetic information to some extent. Consequently, phylogenetic analysis of OTUs whose occurrence might have been influenced by the implementation of "controlled disturbances" may not be sufficient for drawing reliable conclusions, but can provide hints for further studies. Cloned sequences possibly corresponding to T-RFs were therefore subjected to rough phylogenetic classification using BLAST analysis. The outcome of this analysis suggested an effect of "controlled disturbances" on bacteria affiliated to the genera *Paracoccus* and *Dechloromonas*. FISH probes targeting members of these genera did not confirm this evidence. However, the FISH probes applied to detect *Dechloromonas* spp. did not target all known organisms belonging to the genus. These FISH probes have been designed to target cloned sequences affiliated to the genus *Dechloromonas* (Hesselsoe et al. unpublished) and they have mismatches to a couple of *Dechloromonas* species, including

some next cultured relatives (as determined by BLAST analysis) of cloned sequences obtained in this study. Probe RHC445 has mismatches to *Dechloromonas* sp. R-28400 and *Dechloromonas aromatica* RCB, probe RHC827 targets *Dechloromonas* sp. R-28400 but has mismatches to *Dechloromonas aromatica* RCB. Whether the probes target *Dechloromonas* sp. R-28451 could not be determined because the 16S sequence of the organism deposited in the NCBI database did not include the respective target region. Consequently, the abundance of all *Dechloromonas* spp. and possible population shifts could not be determined with the applied FISH probes. Moreover, the *nirS* sequences of target organisms had similarities of 82 – 87 % and 89 – 92 % on nucleotide and amino acid level, respectively, to those of *Dechloromonas* isolates (Sec. C.1.6, Tab. C.16), indicating that they might belong to another, yet closely related genus, provided that *nirS* genes reflect the phylogenetic relationship of the respective organsims.

This approach for identification of denitrifiers affected by "controlled disturbances" was biased in two ways, firstly by the unreliability of the inferred *nir* phylogeny, secondly by the unreliability of T-RF assignment to cloned sequences. Nevertheless, the results obtained for three T-RFs that appeared during "controlled disturbances" were consistently suggesting that bacteria affiliated or related to the genus *Dechloromonas* were positively influenced by "controlled disturbances". It would therefore be worthwhile to continue the analysis with optimized FISH probes.

E. Summary and Conclusion

A new operational mode termed "controlled disturbances" was evaluated for wastewater treatment. Its characteristic features are periodic changes of wastewater load and periodic shutdown of aeration of nitrification tanks. The operational mode was designed to enhance microbial diversity by introducing fluctuating environmental conditions and thus providing more niches for bacteria. Enhanced microbial diversity should increase the functional redundancy of the bacterial community and thus increase its functional stability when subjected to unfavourable conditions. During its implementation, "controlled disturbances" enabled higher EBPR and denitrification rates as well as lower energy requirements for nitrification and also improved the sludge settling behaviour.

"Controlled disturbances" introduced anaerobic phases to nitrification tanks which stimulated the denitrifying activity of bacteria. To investigate whether the improved denitrification performance of the wastewater treatment plant was also linked to community dynamics of denitrifying bacteria, a T-RFLP approach was developed for monitoring of nitrite reductase gene (*nirS*, *nirK*) diversity. Results indicated that "controlled disturbances" induced a shift of the community composition of denitrifying bacteria but did not enhance denitrifier species richness. An effect of "controlled disturbances" on *nirS* diversity was detected whereas effects on *nirK* diversity were inconclusive. Those different trends were probably caused by a reduced iron salt addition during "controlled disturbances" and could additionally be due to a hitherto unexplored niche differentiation of *nirS* and *nirK* possessing bacteria.

The effect of "controlled disturbances" on the entire bacterial community structure was investigated by DGGE analysis of 16S rRNA genes. The obtained data suggested that "controlled disturbances" selected for a newly structured bacterial population that was able to exhibit wastewater treatment processes in a more efficient way under the implemented environmental conditions than the community present during normal operation was able to do under the respective environmental conditions. Whether this community was more diverse or just differently structured could not be concluded with certainty. FISH probes targeting sequences obtained from DGGE bands were designed to identify bacteria whose abundance was affected by "controlled disturbances". Three probes were successfully applied. None of these targeted populations showed a response to the implementation of "controlled disturbances" comparable to the DGGE bands they corresponded to. This finding suggests that it is problematic to draw conclusions about the occurrence of organisms in environmental

samples based on sequence data obtained from DGGE bands, especially if bands contain heterogeneous DNA.

F. Zusammenfassung

Der neue Betriebsmodus “controlled disturbances” wurde für die Abwasserbehandlung in einer Kläranage evaluiert. Seine charakteristischen Merkmale sind eine periodische Variation der den Klärbecken zugeleiteten Abwassermenge, sowie ein periodisches Aussetzen der Belüftung der Nitrifikationsbecken. Der Entwurf des Betriebsmodus zielte darauf ab, durch die Implementierung fluktuierender Umweltbedingungen die Anzahl an ökologischen Nischen für am Klärprozess beteiligte Mikroorganismen und folglich deren Diversität zu erhöhen. Eine Erhöhung der mikrobiellen Diversität sollte mit einer Erhöhung der funktionellen Redundanz der bakteriellen Lebensgemeinschaft einhergehen, wodurch deren funktionelle Stabilität unter ungünstigen Umweltbedingungen verbessert wird. Während seiner Implementierung ermöglichte der Betriebsmodus eine Erhöhung der EBPR- und Denitrifikationsraten, sowie einen niedrigeren Energieverbrauch für die Nitrifikation und eine Erniedrigung des Schlammindex.

“Controlled disturbances” stimulierte die denitrifiziernde Aktivität der Bakterien durch die Einführung anaerober Phasen in den Nitrifikationsbecken. Um zu untersuchen, ob die verbesserte Denitrifikationsleistung der Kläranlage auch mit Populationsdynamiken denitrifizierender Bakterien assoziiert war, wurde ein T-RFLP Experiment zur Untersuchung der Diversität der für die Nitrit-Reduktase kodierenden Genen (*nirS*, *nirK*) entwickelt. Die Resultate dieser Analyse deuten darauf hin, dass “controlled disturbances” eine Veränderung der Zusammensetzung der Population denitrifizierender Bakterien bewirkt, aber die Anzahl der vorhandenen Arten nicht erhöht hat. Des weiteren konnte ein Effekt auf die Diversität des *nirS* Gens beobachtet werde, während mögliche Auswirkungen auf die Diversität des *nirK* Gens uneindeutig waren. Diese unterschiedlichen Trends könnten durch die reduzierte Zugabe von Eisensalzen während “controlled disturbances” bedingt sein und könnten weiters von einer bis dato unerforschten Nischendifferenzierung *nirS* und *nirK* besitzender Bakterien herrühren.

Der Effekt von “controlled disturbances” auf die Struktur der gesamten bakteriellen Lebensgemeinschaft wurde mittels DGGE Analyse des 16S rRNS Gens untersucht. Die Ergebnisse dieser Experimente legen nahe, dass “controlled disturbances” zur Selektion einer neu strukturierten bakteriellen Lebensgemeinschaft geführt haben, welche die für die Abwasserreinigung nötigen Prozesse unter den neuen Umweltbedingungen effizienter ausgeführt hat, als jene Lebensgemeinschaft, die während der Implementierung des regulären Betriebsmodus anwesend war es unter den damals vorherrschenden Bedingungen tun konnte.

Ob diese neue Lebensgemeinschaft nur unterschiedlich strukturiert oder auch diverser war, konnte mit den eingesetzten Methoden nicht mit Sicherheit festgestellt werden.

Zur Identifizierung von Bakterien deren Abundanz von “controlled disturbances” beeinflusst wurde, wurden FISH Sonden entworfen, die aus DGGE Banden erhaltene Sequenzen detektieren. Drei dieser Sonden konnten erfolgreich eingesetzt werden. Bei keiner der detektierten Populationen zeigte die Abundanz in Reaktion auf die Implementierung von “controlled disturbances” einen Trend, welcher mit dem der korrespondierenden DGGE Banden vergleichbar gewesen wäre. Dieses Ergebnis legt nahe, dass Schlussfolgerungen über Abundanzveränderungen von Mikroorganismen basierend auf Sequenzen, die aus DGGE Banden gewonnen wurden, nicht ohne zusätzliche experimentelle Bestätigung getroffen werden können, im Speziellen dann wenn die betreffende Bande heterogene DNA beinhaltet hat.

G. Appendix

Table F.1. BLAST analysis of *nirK* clone library.

Sequence		First BLAST Hit (blastn)			
Name	**Length (bp)**	**Description**	**Accession number**	**Identity [a]**	**Putative gene [b]**
K2_32	473	Uncultured bacterium clone M71 nitrite reductase (nirK) gene, partial cds	AY121519	92%	***nirK***
K5_14	473	Uncultured bacterium clone KEP26 putative nitrite reductase (nirK) gene, partial cds	DQ182206	100%	***nirK***
K6_1	473	Uncultured bacterium clone DGGE band UK7D nitrite reductase (nirK) gene, partial cds	AY583392	79%	***nirK***
K6_2	473	Uncultured bacterium clone T1D1_0-7cm_043 NirK (nirK) gene, partial cds	DQ783105	78%	***nirK***
K6_3	473	Uncultured bacterium clone DGGE band HK3F nitrite reductase (nirK) gene, partial cds	AY583396	99%	***nirK***
K6_4	473	Uncultured bacterium clone DGGE band UK7D nitrite reductase (nirK) gene, partial cds	AY583392	79%	***nirK***
K6_7	473	Uncultured bacterium clone KRF3 putative nitrite reductase (nirK) gene, partial cds	DQ182213	99%	***nirK***
K6_8	473	Uncultured bacterium clone KEP26 putative nitrite reductase (nirK) gene, partial cds	DQ182206	100%	***nirK***
K6_11	473	Uncultured bacterium clone KRF3 putative nitrite reductase (nirK) gene, partial cds	DQ182213	99%	***nirK***
K6_12	473	Uncultured bacterium clone KEP26 putative nitrite reductase (nirK) gene, partial cds	DQ182206	99%	***nirK***
K6_13	473	Uncultured bacterium clone DGGE band UK7D nitrite reductase (nirK) gene, partial cds	AY583392	79%	***nirK***
K6_14	473	Uncultured bacterium clone KEP40 putative nitrite reductase (nirK) gene, partial cds	DQ182210	98%	***nirK***
K6_15	473	Uncultured bacterium clone KRF3 putative nitrite reductase (nirK) gene, partial cds	DQ182213	97%	***nirK***
K6_16	473	Uncultured bacterium clone KRF3 putative nitrite reductase (nirK) gene, partial cds	DQ182213	100%	***nirK***
K6_18	473	Uncultured bacterium clone DGGE band HK3F nitrite reductase (nirK) gene, partial cds	AY583396	99%	***nirK***
K6_19	473	Uncultured bacterium clone KEP40 putative nitrite reductase (nirK) gene, partial cds	DQ182210	98%	***nirK***
K6_23	473	Uncultured bacterium clone Ag08-55 putative nitrite reductase (nirK) gene, partial cds	DQ304283	90%	***nirK***
K6_25	473	Uncultured bacterium clone DGGE band HK3F nitrite reductase (nirK) gene, partial cds	AY583396	99%	***nirK***
K7_3	473	Uncultured bacterium clone Kasp5a putative nitrite reductase (nirK) gene, partial cds	DQ182161	89%	***nirK***
K7_4	473	Uncultured bacterium clone Kasp6a putative nitrite reductase (nirK) gene, partial cds	DQ182163	99%	***nirK***
K7_6	473	Uncultured bacterium clone T8R2_13-20cm_041 NirK (nirK) gene, partial cds	DQ784056	78%	***nirK***
K7_13	473	Uncultured bacterium clone Kasp6a putative nitrite reductase (nirK) gene, partial cds	DQ182163	99%	***nirK***
K7_18	473	Uncultured bacterium nirK gene for nitrite reductase, partial cds, clone: K-A9	AB162321	85%	***nirK***
K7_20	473	Uncultured bacterium clone DGGE band HK3F nitrite reductase (nirK) gene, partial cds	AY583396	99%	***nirK***
K7_29	473	Uncultured bacterium nirK gene for nitrite reductase, partial cds, clone: K-A1	AB162313	95%	***nirK***
K7_31	473	Uncultured bacterium clone Ag12-82 putative nitrite reductase (nirK) gene, partial cds	DQ304337	79%	***nirK***
K8_2	473	Uncultured bacterium clone DGGE band KK8H nitrite reductase (nirK) gene, partial cds	AY583400	93%	***nirK***
K8_3	473	Uncultured bacterium clone P7m_nirK-33 nitrite reductase (nirK) gene, partial cds	EF615340	79%	***nirK***
K8_10	473	Uncultured bacterium clone T7R3b_0-7cm_053 NirK (nirK)	DQ783672	88%	***nirK***

		gene, partial cds			
K8_14	473	Uncultured bacterium clone T8R1_13-20cm_115 NirK (nirK) gene, partial cds	DQ783915	94%	***nirK***
K8_16	473	Uncultured bacterium clone Kasp5a putative nitrite reductase (nirK) gene, partial cds	DQ182161	93%	***nirK***
K8_18	472	Uncultured bacterium clone KRF71 putative nitrite reductase (nirK) gene, partial cds	DQ182218	94%	***nirK***
K8_20	473	Uncultured bacterium clone Kasp1c putative nitrite reductase (nirK) gene, partial cds	DQ182157	90%	***nirK***
K8_30	473	Uncultured bacterium clone P7m_nirK-33 nitrite reductase (nirK) gene, partial cds	EF615340	78%	***nirK***

[a] query coverage was always ≥ 97 %.

[b] according to BLAST analysis

Table F.2. BLAST analysis of *nirS* clone library.

Sequence		**First BLAST Hit (blastn)**			
Name	**Length (bp)**	**Description**	**Accession number**	**Identity [a]**	**Putative gene [b]**
S1_1	413	Uncultured bacterium clone DGGE band US6F nitrite reductase (nirS) gene, partial cds	AY583441	92%	***nirS***
S1_6	422	Uncultured bacterium clone S14m_nirS-29 nitrite reductase (nirS) gene, partial cds	EF615511	91%	***nirS***
S1_8	420	Uncultured bacterium partial nirS gene for nitrite reductase, isolate LK70S20m	AM071463	93%	***nirS***
S1_9	409	Uncultured bacterium clone B39 dissimilatory nitrite reductase (nirS) gene, partial cds	EF558513	93%	***nirS***
S1_10	421	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S1_27	409	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S1_30	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S1_31	422	Uncultured bacterium clone Psedi_nirS-28 nitrite reductase (nirS) gene, partial cds	EF615459	98%	***nirS***
S2_1	428	Rikenella microfusus gyrB gene for DNA gyrase subunit B, partial cds, strain:JCM 2053	AB015034	75%	***gyrB***
S2_2	422	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S2_4	409	Uncultured bacterium partial nirS gene for nitrite reductase, isolate LK80S35m	AM071471	83%	***nirS***
S2_7	410	Uncultured bacterium clone B39 dissimilatory nitrite reductase (nirS) gene, partial cds	EF558513	93%	***nirS***
S2_13	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S2_15	422	Uncultured bacterium clone S14m_nirS-13 NirS (nirS) gene, partial cds	DQ337907	90%	***nirS***
S2_16	422	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S2_17	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S2_18	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S2_19	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S2_25	422	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S2_27	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S3_3	421	Uncultured bacterium clone B2m_nirS-01 NirS (nirS) gene, partial cds	DQ337813	88%	***nirS***
S3_4	421	Simplicispira psychrophila partial nirK gene for copper containing nitrite reductase type strain 5	AM269907	90%	***nirS***
S3_5	409	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene,	DQ337849	83%	***nirS***

		partial cds			
S3_7	411	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S3_8	421	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S3_9	421	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S3_10	421	Uncultured bacterium clone P1m_nirS-04 NirS (nirS) gene, partial cds	DQ337835	99%	***nirS***
S3_15	422	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***
S3_16	410	Uncultured bacterium clone DGGE band AS8A nitrite reductase (nirS) gene, partial cds	AY583422	85%	***nirS***
S3_18	410	Uncultured bacterium clone A3 dissimilatory nitrite reductase (nirS) gene, partial cds	EF558391	88%	***nirS***
S3_19	409	Uncultured bacterium nirS gene for cytochrome cd1 nitrite reductase, partial sequence, clone: NS12	AB378603	77%	***nirS***
S3_20	410	Uncultured bacterium clone A72 dissimilatory nitrite reductase (nirS) gene, partial cds	EF558435	82%	***nirS***
S3_21	410	Uncultured bacterium clone DGGE band AS8A nitrite reductase (nirS) gene, partial cds	AY583422	85%	***nirS***
S3_22	421	Uncultured bacterium clone S14m_nirS-29 nitrite reductase (nirS) gene, partial cds	EF615511	91%	***nirS***
S3_23	421	Uncultured bacterium clone B2m_nirS-01 NirS (nirS) gene, partial cds	DQ337813	89%	***nirS***
S3_27	410	Uncultured bacterium nirS gene for cytochrome cd1 nitrite reductase, partial cds, clone: NS76	AB378621	84%	***nirS***
S3_30	410	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone: IMR64SE5	AB296353	85%	***nirS***
S4_8	413	Uncultured bacterium clone SMP17 putative nitrite reductase (nirS) gene, partial cds	DQ182125	89%	***nirS***
S4_9	408	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S4_10	421	Uncultured bacterium clone P7m_nirS-05 NirS (nirS) gene, partial cds	DQ337841	83%	***nirS***
S4_12	409	Uncultured bacterium partial nirS gene for nitrite reductase, isolate LK80S35m	AM071471	83%	***nirS***
S4_14	425	Uncultured bacterium clone S12m_nirS-10 NirS (nirS) gene, partial cds	DQ337890	88%	***nirS***
S4_19	410	Uncultured bacterium clone Psedi_nirS-05 NirS (nirS) gene, partial cds	DQ337849	83%	***nirS***
S4_31	422	Uncultured bacterium nirS gene for nitrite reductase, partial cds, clone:S-Z10	AB162266	94%	***nirS***

[a] query coverage was always ≥ 97 %, except 2_1 where query coverage was 58 %.

[b] according to BLAST analysis.

Table F.3. Presence of T-RFs in T-RFLP profiles of denitrification tank samples.

T-RFLP profile	T-RF (length in bp)	sample		
		before controlled disturbances	during controlled disturbances	
		KDD	KDE	KDF
*nirS Mbo*II reverse	35	+	-	-
*nirS Mbo*II reverse	50	+	+	+
*nirS Mbo*II reverse	61	+	+	+
*nirS Mbo*II reverse	73	+	+	+
*nirS Mbo*II reverse	77	+	+	+
*nirS Mbo*II reverse	102	+	+	+
*nirS Mbo*II reverse	200	-	+	+
*nirS Mbo*II reverse	220	+	-	-
*nirS Mbo*II reverse	222	+	+	+
*nirS Mbo*II reverse	225	+	-	-

*nirS Mbo*II reverse	337	+	+	+
*nirS Mbo*II reverse	349	+	+	+
*nirS Mbo*II forward	50	-	+	+
*nirS Mbo*II forward	58	+	+	+
*nirS Mbo*II forward	66	+	+	+
*nirS Mbo*II forward	187	-	+	+
*nirS Mbo*II forward	221	+	+	+
*nirS Mbo*II forward	300	+	+	-
*nirS Mbo*II forward	328	+	-	-
*nirS Mbo*II forward	330	+	+	+
*nirS Mbo*II forward	342	+	-	-
*nirS Mbo*II forward	370	+	-	-
*nirS Mbo*II forward	374	-	-	+
*nirS Hae*III reverse	35	+	+	+
*nirS Hae*III reverse	51	+	-	-
*nirS Hae*III reverse	67	+	+	+
*nirS Hae*III reverse	74	+	+	+
*nirS Hae*III reverse	80	+	+	+
*nirS Hae*III reverse	129	+	+	+
*nirS Hae*III reverse	133	+	+	+
*nirS Hae*III reverse	138	+	+	+
*nirS Hae*III reverse	143	+	+	+
*nirS Hae*III reverse	145	+	+	+
*nirS Hae*III reverse	147	-	-	+
*nirS Hae*III reverse	150	+	+	+
*nirS Hae*III reverse	193	-	-	+
*nirS Hae*III reverse	214	+	+	+
*nirS Hae*III reverse	225	+	+	-
*nirS Hae*III reverse	234	-	+	-
*nirS Hae*III reverse	286	-	+	+
*nirS Hae*III forward	74	+	+	+
*nirS Hae*III forward	76	+	+	+
*nirS Hae*III forward	100	+	+	+
*nirS Hae*III forward	115	+	+	+
*nirS Hae*III forward	132	+	+	+
*nirS Hae*III forward	136	+	+	+
*nirS Hae*III forward	188	+	-	+
*nirS Hae*III forward	191	+	+	+
*nirS Hae*III forward	210	+	+	+
*nirS Hae*III forward	223	+	+	+
*nirS Hae*III forward	227	+	+	+
*nirS Hae*III forward	270	-	+	-
*nirS Hae*III forward	271	-	+	+
*nirS Hae*III forward	275	-	-	+
*nirS Hae*III forward	278	+	-	-
*nirS Hae*III forward	363	+	+	+
*nirS Mnl*I reverse	35	+	+	+
*nirS Mnl*I reverse	58	-	-	+
*nirS Mnl*I reverse	63	+	-	-
*nirS Mnl*I reverse	66	+	-	-
*nirS Mnl*I reverse	102	+	+	+
*nirS Mnl*I reverse	116	+	+	+
*nirS Mnl*I reverse	123	+	+	+
*nirS Mnl*I reverse	128	+	+	+
*nirS Mnl*I reverse	212	+	-	-
*nirS Mnl*I reverse	215	+	-	-

*nirS Mnl*I reverse	240	+	-	-
*nirS Mnl*I reverse	243	-	+	+
*nirS Mnl*I reverse	268	+	+	+
*nirS Mnl*I reverse	270	+	+	+
*nirS Mnl*I reverse	294	+	+	-
*nirS Mnl*I reverse	298	-	+	+
*nirS Mnl*I reverse	341	+	+	+
*nirS Mnl*I reverse	344	+	-	-
*nirS Mnl*I reverse	350	+	+	+
*nirS Mnl*I reverse	353	+	+	-
*nirS Mnl*I forward	53	+	+	+
*nirS Mnl*I forward	61	+	+	+
*nirS Mnl*I forward	63	+	+	+
*nirS Mnl*I forward	82	+	+	+
*nirS Mnl*I forward	96	-	+	+
*nirS Mnl*I forward	119	+	+	+
*nirS Mnl*I forward	154	+	+	+
*nirS Mnl*I forward	169	+	-	-
*nirS Mnl*I forward	283	+	+	+
*nirS Mnl*I forward	289	+	+	+
*nirS Mnl*I forward	364	+	+	+
*nirK Mbo*II reverse	52	-	-	+
*nirK Mbo*II reverse	122	+	+	+
*nirK Mbo*II reverse	216	+	+	+
*nirK Mbo*II reverse	234	+	+	+
*nirK Mbo*II reverse	283	+	+	+
*nirK Mbo*II reverse	286	+	+	+
*nirK Mbo*II reverse	311	+	+	+
*nirK Mbo*II reverse	318	+	+	-
*nirK Mbo*II reverse	353	+	+	+
*nirK Mbo*II reverse	409	+	+	+
*nirK Mbo*II reverse	424	+	+	+
*nirK Mbo*II forward	30	+	+	+
*nirK Mbo*II forward	32	+	+	+
*nirK Mbo*II forward	53	+	+	+
*nirK Mbo*II forward	55	+	+	+
*nirK Mbo*II forward	114	+	+	+
*nirK Mbo*II forward	152	+	+	-
*nirK Mbo*II forward	161	+	+	+
*nirK Mbo*II forward	179	+	+	+
*nirK Mbo*II forward	181	+	+	+
*nirK Mbo*II forward	186	+	+	+
*nirK Mbo*II forward	283	-	+	-
*nirK Mbo*II forward	285	+	+	+
*nirK Mbo*II forward	348	+	+	+
*nirK Hae*III reverse	55	+	+	-
*nirK Hae*III reverse	58	+	+	+
*nirK Hae*III reverse	61	+	+	+
*nirK Hae*III reverse	64	+	+	+
*nirK Hae*III reverse	66	+	+	+
*nirK Hae*III reverse	78	-	+	-
*nirK Hae*III reverse	99	+	+	+
*nirK Hae*III reverse	106	+	+	+
*nirK Hae*III reverse	133	+	+	+
*nirK Hae*III reverse	154	+	+	+
*nirK Hae*III reverse	171	+	+	+

*nirK Hae*III reverse	188	+	+	+
*nirK Hae*III reverse	229	+	+	+
*nirK Hae*III reverse	262	+	+	-
*nirK Hae*III reverse	439	+	-	-
*nirK Hae*III forward	35	+	+	+
*nirK Hae*III forward	42	-	-	+
*nirK Hae*III forward	56	+	+	-
*nirK Hae*III forward	59	+	+	+
*nirK Hae*III forward	108	-	+	-
*nirK Hae*III forward	112	+	-	+
*nirK Hae*III forward	120	+	+	+
*nirK Hae*III forward	131	+	+	+
*nirK Hae*III forward	139	+	+	+
*nirK Hae*III forward	173	+	+	+
*nirK Hae*III forward	175	+	+	+
*nirK Hae*III forward	188	-	-	+
*nirK Hae*III forward	202	+	+	-
*nirK Hae*III forward	209	+	+	+
*nirK Hae*III forward	234	+	+	+
*nirK Hae*III forward	241	+	-	-
*nirK Hae*III forward	244	+	-	-
*nirK Hae*III forward	245	-	-	+
*nirK Hae*III forward	279	+	+	+
*nirK Hae*III forward	294	+	-	+
*nirK Hae*III forward	312	+	+	+
*nirK Mnl*I forward	53	+	-	+
*nirK Mnl*I forward	95	+	+	+
*nirK Mnl*I forward	98	+	+	+
*nirK Mnl*I forward	103	+	+	+
*nirK Mnl*I forward	131	+	+	+
*nirK Mnl*I forward	141	+	+	+
*nirK Mnl*I forward	152	+	+	-
*nirK Mnl*I forward	153	+	+	+
*nirK Mnl*I forward	162	+	+	+
*nirK Mnl*I forward	167	+	+	+
*nirK Mnl*I forward	182	+	+	+
*nirK Mnl*I forward	234	-	+	-
*nirK Mnl*I forward	245	+	+	-
*nirK Mnl*I forward	248	-	+	-
*nirK Mnl*I forward	271	-	+	-
*nirK Mnl*I forward	325	-	+	-
*nirK Mnl*I forward	262	-	-	+
*nirK Mnl*I forward	375	+	+	+
*nirK Mnl*I forward	413	-	+	-
*nirK Mnl*I forward	414	-	-	+
*nirK Mnl*I forward	442	+	-	-
*nirK Mnl*I forward	444	-	+	-
*nirK Mnl*I forward	446	+	+	+
*nirK Mnl*I forward	447	+	-	+

+ indicates presence, - indicates absence

H. List of abbreviations

λ	Wavelength
µ	Mikro (10^{-6})
°C	Degree Celsius
%	Percent
A	Adenine
abs	Absolute
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
APS	Adenosine-5'-phosphosulfate
ARB	Software package for phylogenetic analyses
bidist.	Double distilled and filtered
BLAST	Basic local alignment search tool
bp	Base pair(s)
c	Centi (10^{-2})
C	Cytosine
c.d.	controlled disturbances
CLSM	Confocal laser scanning microscope (or microscopy)
Cy3	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy3.18-derivative N-hydroxysuccimidester
Cy5	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy5.18-derivative N-hydroxysuccimidester
DGGE	denaturing gradient gel electrophoresis
DNA	Desoxyribonucleic acid
dNTP	Desoxy-nucleotide-tri-phosphate
denat.	denaturant
E. coli	*Escherichia coli*
EDTA	Ethylene-di-amine-tetra-acetic acid
e.g.	Exempli gratia (lat., "example given")
ERT	Eppendorf reaction tube
et al.	Et alteri (lat., "and others")
EtBr	Ethidium bomide
f	Forward (used for labbeling of primers)
FA	Formamide
Fig.	Figure
FISH	Fluorescence in situ hybridisation
FLUOS	5,(6)-carboxfluorescein-N-hydroxysuccimidester
g	Gram(s)
G	Guanine
h	Hour(s)
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
k	Kilo (10^3)
Kan	Kanamycin
KBL	Kilobase-ladder (DNA length standard)
KCl	Potassium chloride
KDD	denitrification tank sample D

KDE	denitrification tank sample E
KDF	denitrification tank sample F
KN2A	nitrification tank sample A
KN2B	nitrification tank sample B
KN2D	nitrification tank sample D
KN2E	nitrification tank sample E
KN2F	nitrification tank sample F
l	Liter(s)
LB	Luria Bertani
m	Milli (10^{-3})
M	Molar
min	Minute(s)
mol	Mol
n	nano (10^{-9})
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NH3	Ammonia
NH_4^+	Ammonium
NirK, *nirK*	Cu-containing nitrite reductase and corresponding gene, respectively
NirS, *nirS*	Cytochrome cd1-containing nitrite reductase and corresponding gene, respectively
N2	Dinitrogen
N2O	Nitrous oxide
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-oxidizing bacteria
O_2	Molecular oxygen
o/n	Overnight
OTU	Operational taxonomic unit
p	pico (10^{-12})
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
r	Reverse (used for labelling of primers)
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	ribosomal RNA
rDNA	Ribosomal DNA
RT	Room temperature
SDS	Sodium dodecyl sulphate
SBR	Sequencing batch reactor
sec.	Second(s)
sp.	Species (singular)
spp.	Species (plural)
Tab.	Table
TAE	Tris-actetate-EDTA
Taq	Thermostable DNA-polymerase from Thermus aquaticus
TBE	Tris-boric acid-EDTA

TEMED	N,N,N',N'-tetra-methyl-ethylene-di-amine
Temp.	Temperature
T-RFLP	Terminal restriction fragment length polymorphism
T-RF	Terminal restriction fragment
U	Uracil
UV	Ultraviolet
V	Forward (used for labelling of primers)
Vol.	Volume(s)
w/v	Weight per volume
X-Gal	5-brom-4-chlor-3-indolyl-β-D-galactopyranoside

I. References

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K. Curriculum vitae

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