Anaerobic degradation of anionic surfactants by denitrifying bacteria

Ana Paulo

Thesis committee

Promotor
Prof. Dr A.J.M. Stams
Personal chair at the Laboratory of Microbiology
Wageningen University

Co-promotor
Prof. Dr P. A. García-Encina
Professor at the Department of Chemical Engineering and Environmental Technology
University of Valladolid, Spain

Dr C.M. Plugge
Assistant Professor at the Laboratory of Microbiology
Wageningen University

Other members
Prof. Dr B. Philipp, University of Münster, Germany
Dr F.P. van der Zee, Veolia Water Technologies / Biothane Systems International B.V., Delft, The Netherlands
Prof. Dr H. H. M. Rijnaarts, Wageningen University
Prof. Dr M. M. Alves, University of Minho, Portugal

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Anaerobic degradation of anionic surfactants by denitrifying bacteria

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Ana Paulo
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SUMMARY

Surfactants are produced and used in the formulation of many different commercial products. After use, these compounds end up in wastewater treatment plants (WWTPs) or in the environment. Although many surfactants can be degraded in aerobic conditions, anaerobic conditions are also common in Nature and in WWTPs. For achieving nutrients removal from wastewater, biological removal of nitrogen and phosphorus can be performed in a WWTP using the anaerobic-anoxic-aerobic (A^2/O) concept. Using the A^2/O process sequence, surfactants can be degraded anaerobically before reaching the aerobic compartment. In the anoxic compartment, facultative anaerobic bacteria can degrade surfactants by using nitrate/nitrite as electron acceptor. However, not much is known about surfactant-degrading denitrifying bacteria. In this thesis, *Pseudomonas stutzeri* strain SN1 and *Pseudomonas nitroreducens* strain SN2 were isolated from activated sludge of a WWTP with the A^2/O process, using the anionic surfactant sodium dodecyl sulfate (SDS) as sole carbon and energy source. Both strains were able to completely degrade SDS coupled to nitrate reduction to dinitrogen gas (Chapter II).

In the A^2/O process, the diversity of bacterial communities involved in the degradation of surfactants may differ between anoxic and oxic compartments, where two different electron acceptors are involved. Surfactants can directly affect the biological activity of microorganisms present in WWTPs and disturb the treatment efficiency. In this way, increased concentrations of surfactants may give rise to a different bacterial diversity selection in anaerobic, anoxic and oxic conditions. The degradation of the anionic surfactant sodium lauryl ether sulfate (SLES) in aerobic conditions is known, but not in denitrifying conditions. In this thesis, the bacterial diversity of enrichments cultures able to degrade different concentrations of SLES in anoxic and aerobic conditions was determined. *Aeromonas hydrophila* strain S7, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 were isolated from anoxic enrichments. *Comamonas testosteroni* strain S13 and *Acinetobacter* sp. S15 were isolated from aerobic enrichments (Chapter III). SLES initial degradation steps by pure bacterial cultures were previously investigated, but much is still unknown about how the cleavage of ether bonds from chemical compounds is catalyzed by bacterial enzymes. *Aeromonas hydrophila* strain S7, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 are able to use SLES in anoxic conditions coupled to nitrate reduction (Chapter III). SLES degradation in anoxic conditions was compared between the three strains. *P. nitroreducens* strain S11 was found to be the best SLES degrader in anoxic conditions and also to be an excellent aerobic SLES degrader (Chapter IV). Sulfatases and ether cleaving enzymes were probably used by *P. nitroreducens* strain S11 in both conditions, although differences between SLES

degradation in aerobic and anoxic conditions indicated that ether cleavage and following SLES complete degradation is faster under aerobic conditions.

Although surfactants can be toxic to microorganisms, surfactant-degrading bacteria are known to be resistant to high surfactants concentration, in aerobic conditions. This was not previously investigated using surfactant-degrading denitrifying bacteria. Surfactant-resistant bacteria, with the ability to couple surfactant degradation to nitrate reduction, can be very useful for degrading the surfactants arriving to the anoxic compartments of a WWTP at high concentration. In this thesis, high concentrations of SDS and SLES were used to investigate the effect of these on SDS/SLES-degrading bacteria (*P. stutzeri* strain SN1, *P. nitroreducens* strain SN2, *P. stutzeri* strain S8 and *P. nitroreducens* strain S11), under anoxic conditions (Chapter V). *P. stutzeri* strain SN1 was inhibited by increasing SDS and SLES concentrations, after degrading a certain amount of the surfactants. Overall, *P. nitroreducens* strains showed to be more resistant to high surfactant concentrations compared to *P. stutzeri* strains. Nevertheless, high concentrations of SDS and SLES did not inhibit growth and nitrate reduction ability of any of the tested *Pseudomonas* sp..

Protein domains represent the evolutionary conserved autonomously folding functional building blocks of the proteins. Prediction of protein domains from genomes can be used for species classification and validation of known physiological abilities. *P. nitroreducens* are facultative anaerobic bacteria from the *P. aeruginosa* group, which can degrade complex compounds. *P. nitroreducens* DSM 14399^T shares with *P. nitroreducens* strain SN2 the ability for SDS degradation in anoxic conditions. For increasing the insight into *P. nitroreducens* DSM 14399^T phylogenetic classification and physiological properties (e.g. SDS degradation) its genome was sequenced, annotated and compared to other *Pseudomonas* spp. genomes. This was performed by comparing functional profiles, based on protein domains presence or absence, with physiological data (Chapter VI). Functional profile comparison confirmed *P. nitroreducens* classification. Protein domain analysis and genes annotation validated SDS degradation by *P. nitroreducens* DSM 14399^T. This study showed that protein domains prediction and functional profiles comparison can be used for studying and comparing different *Pseudomonas* species at the physiological level.

CHAPTER I

GENERAL INTRODUCTION

Surfactants can be found in industrial and domestic wastewater and may negatively affect biological treatment processes. Wastewater treatment plants (WWTPs) have often an anaerobic-anoxic-oxic (A^2/O) regime, used for organic matter and nutrients removal from the wastewater. Therefore, it is important to get insight into the biodegradation and toxicity of surfactants in the anaerobic and anoxic compartments. This thesis describes research on the degradation of anionic surfactants in the anoxic compartment of WWTPs.

1. SURFACTANTS

Surfactants constitute a diverse group of chemicals designed mainly for cleaning and solubilization purposes. These compounds can be used in personal care products and pesticide formulations, among other applications (Lara-Martin et al. 2008). The name *surfactant* results from the shortening of the term *surface active agent* due to the ability of these compounds to change surface properties (Holmberg et al. 2002). A surfactant is characterized by its tendency to adsorb to surfaces and interfaces (Holmberg et al. 2002). Soaps are the first known and oldest surfactants used for cleaning purposes. These are composed of sodium or potassium salts of fatty acids produced from the hydrolysis of fats in a chemical process called saponification (Stalmans et al. 2007) (Figure 1).

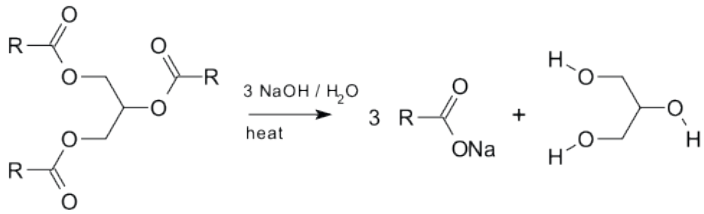

Figure 1 Saponification reaction where fats are hydrolyzed to produce soap (sodium salt of fatty acid) and glycerol.

The first synthetic soap was made in Germany in 1916, due to shortage of natural materials. After this moment, synthetic soaps commonly referred to as detergents, have undergone many improvements. Detergents and soaps are used for removing dirt particles, and suspend them to be rinsed away with water (Stalmans et al. 2007). Commercial detergents are composed of 10 to 30 % of surfactants (often referred to as the *active* components), larger proportions of builders used for softening the water by removing cations from it (e.g. sodium tripolyphosphate and/or other suitable chelates) and a number of other ingredients in smaller amounts (Swisher 1987).
Surfactants started to be produced after the chemical revolution that occurred in the detergent industry, around 1950, when more economical and more efficient chemical

compounds were needed (Swisher 1987). Surfactants have both hydrophilic and hydrophobic properties, consisting of a polar head (either charged or uncharged), which is well soluble in water, and a nonpolar hydrocarbon tail that is less water soluble (Ying 2006). When present in water at low concentrations, surfactant molecules exist as monomers, but at higher concentrations the presence of both hydrophobic and hydrophilic groups enables these compounds to form micelles in solution (Haigh 1996). The concentration above which surfactant molecules form micellar aggregates is defined as the critical micelle concentration (CMC) (Ruckenstein and Nagarajan 1975). This fundamental characteristic is the basis of surfactants detergency and solubilization properties. The clusters (micelles) are formed with the hydrophobic groups located in the center of the cluster and the hydrophilic head groups towards the water. When used at concentrations higher than the CMC level, surfactants are able to better solubilize hydrophobic compounds that would be poorly dissolved in water alone (Figure 2) (Haigh 1996).

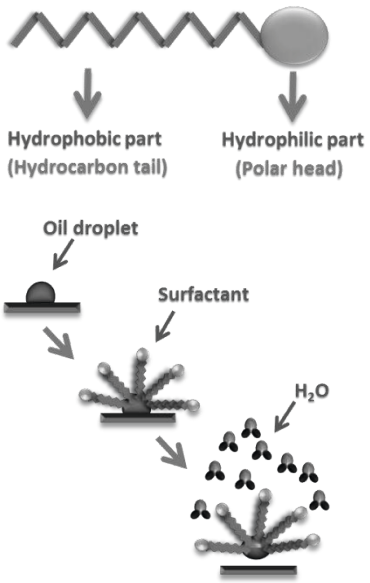

Figure 2 Surfactant general molecular structure and solubilization mechanism of oil droplets using surfactants.

Surfactants are classified according to their charge as anionic, non-ionic, cationic and amphoteric (Figure 3) (Swisher 1987; Van Hamme et al. 2006).

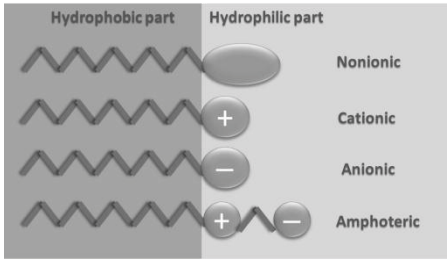

Figure 3 Main classes of surfactants regarding the different hydrophilic molecular structure (polar head) of the molecules.

Non-ionic, anionic and cationic surfactants are the most commonly used classes (Ying 2006). Examples of surfactants from these three classes are presented in Table 1.

Table 1
Main surfactant sub-classes within anionic, non-ionic and cationic surfactants classes (Ying 2006)

Class	Sub-classes
Anionic	Linear alkyl benzene sulfonates (LAS) Alkyl ether sulfates (AES) Alkyl sulfates (AS) Secondary alkane sulfonates (SAS)
Nonionic	Alkylphenol ethoxylates (APE) Alkyl ethoxylates (AE)
Cationic	Quaternary ammonium compounds (QAC)

Worldwide, 60% of surfactants production was estimated to be of anionic surfactants (Holmberg et al. 2002). According to the *Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques* (CESIO), in 2012 anionic surfactants accounted for 41 % and together with non-ionic surfactants these accounted for almost 90% of the total production of surfactants in Western Europe (Figure 4). Surfactants production is increasing every year in Western Europe. The major reason for anionic surfactants production is the easy and cheap manufacture of anionic surfactants. Anionic surfactants present a polar head group (carboxylate, sulfate, sulfonate or phosphate) and a hydrophobic chain (alkyl or alkyl aryl chain in the C12-C18 range) (Holmberg et al. 2002). Percentages of main anionic surfactants produced in Western Europe are presented in Figure 5.

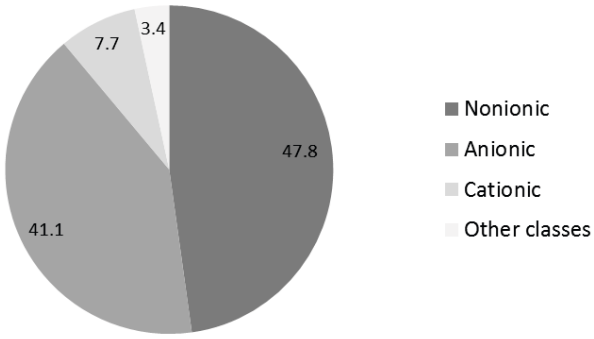

Figure 4 Percentages of classes of surfactants produced in 2012 in Western Europe (CESIO 2012).

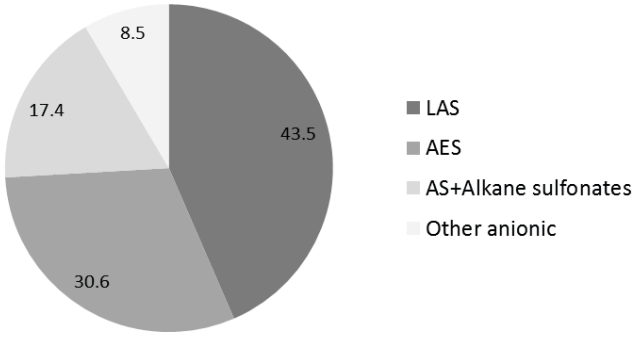

Figure 5 Percentages of sub-classes of anionic surfactants produced in Western Europe (REACH 2003). Legend: LAS – linear alkyl benzene sulfonates; AES – alkyl ether sulfates; AS – alkyl sulfates.

In many studies, linear primary alkyl sulfates (LPAS) are represented by sodium dodecyl sulfate (SDS) (Figure 6) as model compound (Abboud et al. 2007). SDS is present in many cosmetics and personal hygiene products (Sirisattha et al. 2004). Linear alkyl ether sulfate (AES) surfactants, used in products formulation, can vary in chemical structure, according to the number of ethoxy groups (Figure 7). Sodium lauryl ether sulfate (SLES) or sodium laureth sulfate (SLS) is a commercial mixture of AES homologs. Its homologs composition can vary according to the manufacturer, in the length of the alkyl chains and number of ethoxy groups.

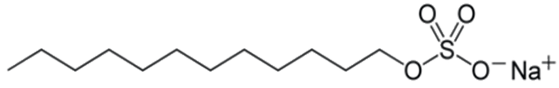

Figure 6 Sodium dodecyl sulfate molecular structure.

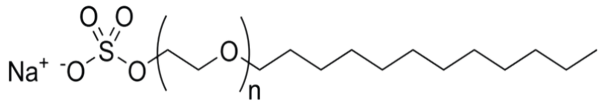

Figure 7 Linear AES surfactant molecular structure; n (number of ethoxy groups) = 0-8.

2. TOXICITY OF SURFACTANTS

Surfactants are produced worldwide and after usage they end up in WWTP and surface waters. The toxic effect of surfactants has been extensively studied using diverse aquatic organisms. The toxic effect depends on the type of surfactant and on the type of organism tested (Glover et al. 1999). However, by testing the toxicity of seven surfactants on freshwater organisms, such as fish, shrimp and fly larva, cationic surfactants were found more toxic than anionic surfactants (Singh et al. 2002). Non-ionic surfactants were less toxic to these organisms than the two previous classes of surfactants. Within the class of anionic surfactants, the AS subclass is expected to be more toxic to the aquatic environment compared to the AES or LAS subclasses, being AES the less toxic of these three subclasses (REACH 2003). For all AS homologues, invertebrates were found to be the most sensitive taxonomic group, and *Brachionus calyciflorus,* a planktonic rotifer, the most sensitive species to C12 AS (HERA 2002). AS surfactants with alkyl chains longer than 12 carbons seem to be more toxic to aquatic flora and fauna when compared to shorter alkyl chains (Konnecker et al. 2011). SDS can affect negatively bivalves (Ostroumov 2003; Ostroumov and Widdows 2006), aquatic plants (Forni et al. 2012), algae (Ostroumov and Widdows 2006) and fish (Rosety et al. 2001). SDS was more toxic to planarian (flatworm) compared to LAS and cetrimonium bromide (CTAB) (Li 2008).

AES subclass seems to affect more invertebrates and fish (HERA 2004). During an ecological assessment, a 10 mg L^{-1} concentration of a C12-15 triethoxy sulfate affected different types of invertebrates, but did not affect macrophytes (aquatic plants),and periphyton (mixture of algae, cyanobacteria, heterotrophic microbes and detritus). At lower concentrations than 10 mg L^{-1}, this AES surfactant affected fish growth and reproduction (Lizotte et al. 2002). Acute toxicity (48-h LC50) of 18 AES surfactants tested using *Ceriodaphnia dubia* (water flea) showed that, in general, acute toxicity increased

with alkyl carbon chain and decreased with increased numbers of ethoxy units (Dyer et al. 2000). C12-14 diethoxy sulfate inhibited microalgae from pristine sea water at a concentration ranging between 5 mg L^{-1} and 38 mg L^{-1} (Sibila et al. 2008).

Surfactants can directly affect the biological activity of microorganisms by binding to enzymes, proteins, phospholipids or by changing the hydrophobicity of the bacterial cell (Cserhati et al. 2002). The toxic effect of surfactants on bacteria depends on their chemical structure and their concentration (Jin et al. 2007; Li and Chen 2009). Cationic surfactants have been reported to be the most toxic surfactants to bacteria, even at low concentrations. Anionic surfactants are known to be more effective to Gram-positive than to Gram-negative bacteria. Non-ionic surfactants are often considered non-toxic to bacteria (Louvado et al. 2012).

The effect of surfactants in wastewater treatment plants (WWTPs) has also been studied. In WWTPs the floc size of activated sludge decreased after contact with LAS, AS and AES surfactants (Liwarska-Bizukojc and Bizukojc 2006). The release of untreated effluents containing surfactants such as AS or AES to the environment can lead to ecotoxicological problems. Surfactants can severely disturb the efficiency of treatment in WWPTs. Fast degradation of synthetic surfactants is, obviously, very important to avoid its negative impact.

3. BIODEGRADATION TESTS OF CHEMICALS

When new substances are produced these should not constitute any danger to the environment. To make sure that chemicals and products are environmentally safe, there are numerous administrative instruments such as European directives, and national laws, for regulating directly the ecological impact of the chemicals and products, indicating also which tests have to be done (Pagga 1997). The information associated to the ecological behavior of new chemicals or products needs to be provided by the manufacturer (Pagga 1997). The extent of biodegradation of chemicals and products is one of the most important aspects of their environmental behavior, since a biodegradable substance will cause less ecological problems than a persistent one (Merrettig-Bruns and Jelen 2009). As the natural environment was considered to be predominately aerobic, the development of a number of international recognized standard methods regarding aerobic biodegradability of substances were developed (Merrettig-Bruns and Jelen 2009). However, the environmental fate of chemicals and products in anaerobic conditions is also important to consider. Environments where molecular oxygen is present at very low concentrations or is even absent are common in Nature. Strict anaerobic and/or anoxic environments are found in organic rich sediments, landfills and in sub-surface soil layers

(Nuck and Federle 1996). Anaerobic conditions are common in wastewater treatment such as anaerobic digestion of sewage sludge stabilization, denitrification and activated sludge systems with simultaneous aerobic/anaerobic conditions and septic tanks (Merrettig-Bruns and Jelen 2009). In the absence of oxygen, bacteria may use nitrate, sulfate or carbon dioxide as electron acceptors for growth. Since chemical compounds can be transported from aerobic to anaerobic zones in the environment (Merrettig-Bruns and Jelen 2009) these can be degraded by aerobic but also by anaerobic communities (Birch et al. 1989).

The international Organization for Economic Co-operation and Development (OECD) has defined several guidelines for testing chemical compounds biodegradation in aerobic and anaerobic conditions. Examples of these tests are OECD tests no. 301 and no. 311, indicated for aerobic and strict anaerobic biodegradation, respectively. The OECD test no. 301 test is used to measure ready biodegradability in an aerobic aqueous medium, describing 6 different methods: the DOC Die-Away, the CO_2 Evolution (Modified Sturm Test), the MITI (I) (Ministry of International Trade and Industry, Japan), the Closed Bottle, the Modified OECD Screening and the Manometric Respirometry. Parameters such as dissolved organic carbon (DOC), carbon dioxide production and oxygen uptake are generally determined (OECD 1992). The OECD test no. 311 can be used for measuring the anaerobic biodegradability of organic compounds in digested sludge. Production of carbon dioxide and methane is measured (OECD 2006). Other examples of tests related to the biodegradability in aerobic and anaerobic conditions defined by the OECD are related to transformation in soil (test no. 307) (OECD 2002a) and transformation in aquatic sediments systems (test no. 308) (OECD 2002b). LAS, being the most important anionic surfactants, have been subject of several biodegradability tests. Different OECD tests have been used for measuring its primary (>99%) and ultimate biodegradation (80% to >95%) in aerobic conditions (HERA 2013).

4. BIODEGRADATION OF SURFACTANTS

After use, surfactants can be found together with the distinct mixture of pollutants that arrive to WWTPs or are discharged directly into the aquatic environment (Ying 2006; Gonzalez et al. 2007). Influent wastewater concentrations of the most important non-ionic surfactants (AE and APE) can go up to 4 mg L^{-1} (Melcer et al. 2006; HERA 2009), while the average surfactant concentration for several individual classes of anionic surfactants (LAS, AES and AS) in influent wastewater can vary between 0.4 - 12 mg L^{-1} (HERA 2002, 2004, 2013). Surfactants can be applied in pollution control (Zhang et al. 1999) and are used for cleaning up contaminated soils and aquifers, by enhancing groundwater pump-and-treat

(Roy et al. 1995) and as dispersants for fuel oil spillages (Scott and Jones 2000). Conventional foams made from surfactants, micro-gas suspensions and micelles have been used in wastewater treatment. However, due to these surfactant-based technologies, residual surfactants in concentrations above its CMC will remain in the surface water, produced water or in the subsurface environment and need to be removed (Zhang et al. 1999). The removal of surfactants from wastewater can be performed using microbial processes, which present several advantages when compared to physicochemical processes, such as adsorption on activated carbon and coagulation/precipitation, mainly because of easy application and of relatively low costs (Cserhati et al. 2002). The primary biodegradation of surfactants occurs when the surfactant molecular structure is changed by the action of microorganisms, resulting in the loss of its surface-active properties (REACH 2003). The complete or ultimate degradation of a surfactant is reached when the surfactant is mineralized by microorganisms. In presence of oxygen, surfactants can be mineralized coupled to microbial growth (REACH 2003). The first step in the biodegradation pathway of surfactants is, in many cases, the most crucial one. Once the parent surfactant is degraded, the metabolites formed (often alcohols and carboxylic acids) can be easily degraded by enzymes common in the cells (REACH 2003). Aerobic treatment processes seem to provide conditions for rapid primary and complete biodegradation of many surfactants (Scott and Jones 2000; Ying 2006). Less is known about anaerobic degradation of surfactants. While anionic, non-ionic and cationic are generally well aerobically degraded, their anaerobic degradation varies according to the different subclasses and different molecular structures or is not feasible. LAS are completely degraded under aerobic conditions, but considered persistent under anaerobic conditions such as the ones found in anaerobic sewage-sludge digesters and sewage-contaminated groundwater (Krueger et al. 1998; Scott and Jones 2000; Garcia et al. 2005). The most easily degraded subclasses of anionic surfactants in aerobic conditions are AS, AES, LAS and SAS, of which AS and AES can also be degraded anaerobically (Ying 2006). LAS and SAS seem to be persistent in anaerobic conditions (Scott and Jones 2000). Linear primary AS (LPAS) have been reported to undergo biodegradation in anaerobic digestion processes (Salanitro and Diaz 1995; Nuck and Federle 1996; Feitkenhauer and Meyer 2002a). However, severe inhibition of methanogenesis can occur when concentrations of LPAS are high, unless lower surfactant/biomass ratios are used (Feitkenhauer and Meyer 2002a, b). Aerobically, linear primary AES (LPAES) are more easily degraded than LPAS (Swisher 1987; Scott and Jones 2000). The non-ionic surfactants AE were found to be more easily degraded than APE, both in aerobic and anaerobic conditions. The cationic surfactants QAC are known to be degradable under aerobic conditions, but in general these are not degradable under anaerobic conditions.

They are even inhibitory to the microorganisms in the anaerobic process (Ying 2006; Merrettig-Bruns and Jelen 2009).

5. DENITRIFICATION AND SURFACTANTS

Denitrification is a microbiological process that constitutes one of the main branches of the global nitrogen cycle. During denitrification, nitrate (or nitrite) is reduced to dinitrogen, through a sequence of steps, mainly performed by heterotrophic bacteria. The use of nitrate (or nitrite) as electron acceptor coupled to substrate degradation by microorganisms is thought to have evolved before the use of molecular oxygen (Gerardi 2002). Denitrification is the process where nitrate and nitrite act as terminal electron acceptors and the gaseous nitrogen species (NO, N_2O and N_2) are the major products. In a complete denitrification process, nitrate (NO_3^-) is reduced to nitrite (NO_2^-), which is reduced to nitric oxide (NO), later reduced to nitrous oxide (N_2O) and finally resulting in the production of dinitrogen (N_2), according to the following scheme (Knowles 1982; Gerardi 2002):

$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

In each reduction step of the process, different enzymes and cofactors are involved, including: nitrate reductases (respiratory nitrate reductase *nar* and periplasmic nitrate reductase *nap*), nitrite reductase (*nir*), nitric-oxide reductase (*nor*) and nitrous oxide reductase (*nos*) (Lalucat et al. 2006). The study of denitrification is relevant for several reasons: it reduces the amounts of fertilizer nitrogen in agricultural soils, it can be applied in the removal of nitrogen from high-nitrogen content wastes, it balances the global nitrogen cycle and an incomplete denitrification results in the release to the atmosphere of N_2O, a strong greenhouse gas, which contributes to the depletion of ozone layer (Knowles 1982). The detailed knowledge of denitrification is essential not only because of its role in the nitrogen cycle but also because of the cellular bioenergetics of nitrate reducing microorganisms (Zumft 1997). Denitrifiers are from different phylogenetic groups, such as fungi, protozoa and bacteria (Zumft 1997). However, most denitrifying microorganisms consist of facultative anaerobic bacteria. Denitrifying bacteria can use oxygen or nitrate as electron acceptors. Since oxygen provides more energy for bacterial activity, denitrifying bacteria generally use oxygen over nitrate. The largest number of denitrifying bacteria belongs to the genera *Alcaligenes*, *Bacillus* and *Pseudomonas* (Gerardi 2002). Other examples of genera that harbor denitrifying species are *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Chromobacterium*,

Corynebacterium, *Flavobacterium*, *Hyphomicrobium*, *Halobacterium*, *Neisseria*, *Propionibacterium*, *Rhizobium*, *Rhodopseudomonas*, *Spirillum*, *Moraxella*, *Paracoccus* and *Vibrio* (Metcalf and Eddy 2003). Some genera of denitrifying bacteria are microaerophillic, tolerating only low levels of oxygen while some species of *Corynebacterium* and *Pseudomonas* do not denitrify completely and produce only N_2O (Gerardi 2002).

6. ANAEROBIC-ANOXIC-OXIC (A^2/O) PROCESS

Eutrophication is a phenomenon that occurs when nutrients accumulate in surface waters due to human activities, being nitrogen and phosphorus the main causes of this problem. Surface waters affected by eutrophication present low dissolved oxygen, murky water and the absence of desirable flora and fauna. This excess of nutrients can induce algae blooms and growth of microorganisms that can cause human health problems (EPA 2007). Once accumulation of nutrients became a relevant public health and environmental issue, lower WWTP effluent limits for nitrogen and phosphorous were defined (EPA 2007). This required an improvement of the wastewater treatment efficiency, by increasing the removal of nitrogen and phosphorus from the influent wastewater. Nitrogen and phosphorus can be removed from wastewater by using biological nutrient removal (BNR) (Metcalf and Eddy 2003). In a WWTP the BNR and elimination of organic matter can be performed using an anaerobic-anoxic-aerobic (A^2/O) biological process (Figure 8).

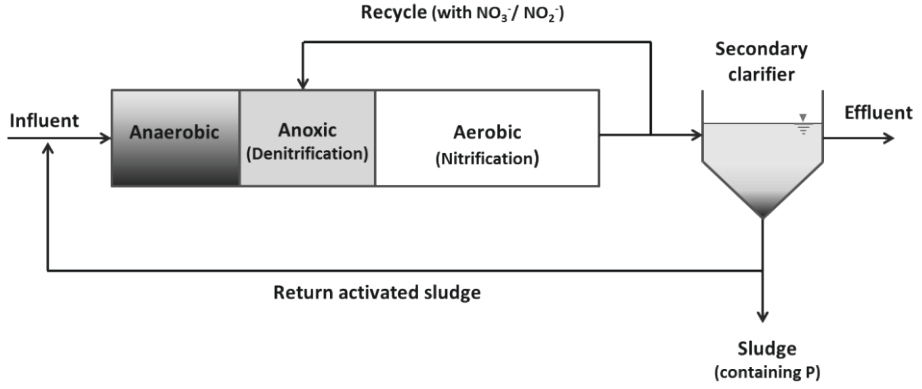

Figure 8 A^2/O biological process description (adapted from Metcalf and Eddy (2003)).

In this biological process, anaerobic treatment occurs first, followed by anoxic and aerobic processes. In this way, the majority of the organic matter is degraded in the first two steps (anaerobic and anoxic), without extra additions. Nitrate, resulting from nitrification, is

recycled from the aerobic reactor effluent to the anoxic reactor, where denitrifying bacteria reduce nitrate to dinitrogen gas (Metcalf and Eddy 2003). The A^2/O process is also used for phosphorus removal. During biological phosphorus removal, phosphorus is incorporated in the cell biomass by phosphorus accumulating organisms (PAOs) present in the anaerobic digestion process. While in anaerobic conditions, PAOs assimilate fermentation products and release orthophosphate ($O\text{-}PO_4$). Orthophosphate is removed from solution and incorporated back into bacterial cells in the anoxic and aerobic zones of the A^2/O process (Metcalf and Eddy 2003).

Conventional wastewater treatment processes under optimized conditions are able to eliminate more than 90% of the surfactants, although this efficiency may vary according to the operating characteristics of the WWTP (Matthijs et al. 1999; Gonzalez et al. 2007). Although there are advantages in the use of an activated sludge process for wastewater treatment, its use has been proved to be more costly in terms of operation when compared to anaerobic and other processes (Gerardi 2002). One of the biggest costs in activated sludge processes is aeration (Metcalf and Eddy 2003). Surfactants affect the gas transfer from the air to the liquid phase and in this way, the energy efficiency of the aeration (Swisher 1987). Surfactants also affect the efficiency of activated sludge plants by increasing foaming in the aeration basin and changing the settling properties of sludge flocs (Wagener and Schink 1987). Depending on the concentration and type of surfactants, some might be degraded in the anaerobic reactor while many can be fully degraded in the anoxic reactor in an A^2/O process. In this way, effects of extra foaming and changes in gas transfer would not be so relevant for the aerobic system operation.

7. SURFACTANTS-DEGRADING BACTERIA

For a good prediction of surfactant biodegradation in Nature or in biological treatment processes the study of its degradation by mixed bacterial cultures is required. In addition, pure cultures are necessary for having a better insight in the individual steps of biodegradation (Swisher 1987). Surfactant-degrading bacteria are mainly Gram-negative bacteria. Besides *Bacillus*, Gram-positive bacteria able to use and grow with surfactants are not often found (Swisher 1987). *Pseudomonas* and *Acinetobacter* were identified as being able to grow with tetrapropylene derived alkyl benzene sulfonate (TBS) and these were also the predominant bacterial genera with ability to use different nonionic and anionic surfactants as carbon source (Swisher 1987). LAS can be primarily degraded by pure bacterial cultures, but only mixed cultures are able to mineralize LAS (van Ginkel 1996). Bacteria involved in LAS biodegradation can be found in different genera, such as: *Bacillus*, *Vibrio*, *Parvibaculum, Comamonas* and *Pseudomonas* (van Ginkel 1996; Schleheck

et al. 2004; Schleheck et al. 2010; Asok and Jisha 2012). Linear AS can be degraded by *Pseudomonas* strains (Swisher 1987). Since SDS is often used as model compound for AS degradation (Abboud et al. 2007) and is present in many cosmetics and personal hygiene products (Sirisattha et al. 2004), many studies focused on bacterial degradation of SDS. *Pseudomonas* are by far more frequently associated with SDS degradation (Figure 9a). Within the *Pseudomonas* genus, *Pseudomonas aeruginosa* is often described for its ability to use SDS (Figure 9b).

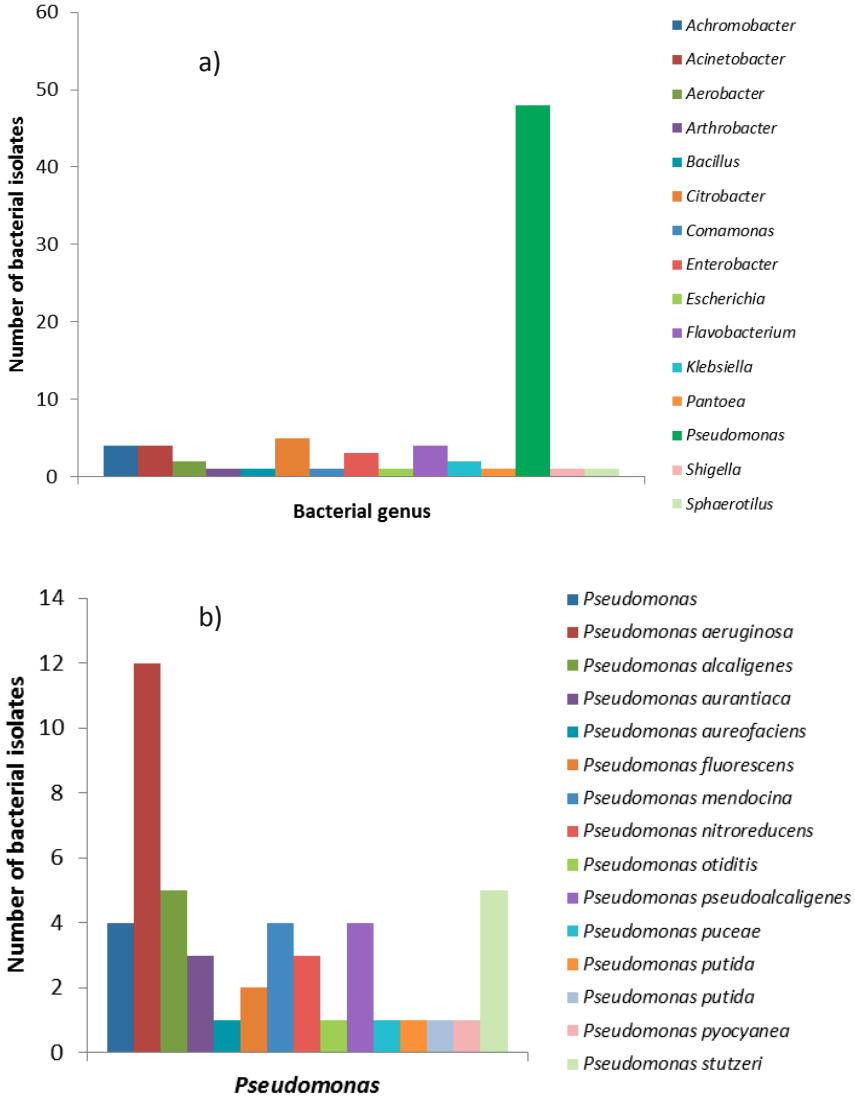

Figure 9 a) Number of isolates from different bacterial genera with ability for SDS degradation; **b)** Number of isolates from different *Pseudomonas* species with ability to degrade SDS. Literature references: Hsu (1963); Swisher (1987); Marchesi et al. (1994); Klebensberger et al. (2006); Abboud et al. (2007); Margesin and Shinner (1998); Singh et al. (1998); Shukor et al. (2009); Yeldho et al. (2011); Chaturvedi and Kumar (2011); Ambily and Jisha (2012).

SDS degradation by aerobic bacteria is well described (Swisher 1987; Scott and Jones 2000). SDS degradation steps by *Pseudomonas* C12B were studied by Thomas and White

(1989). In the pathway, SDS is first cleaved by an alkylsulfatase into sulfate and 1-dodecanol and further converted to dodecanal and lauric acid, which is degraded by β-oxidation and partly used for lipid biosynthesis (Figure 10).

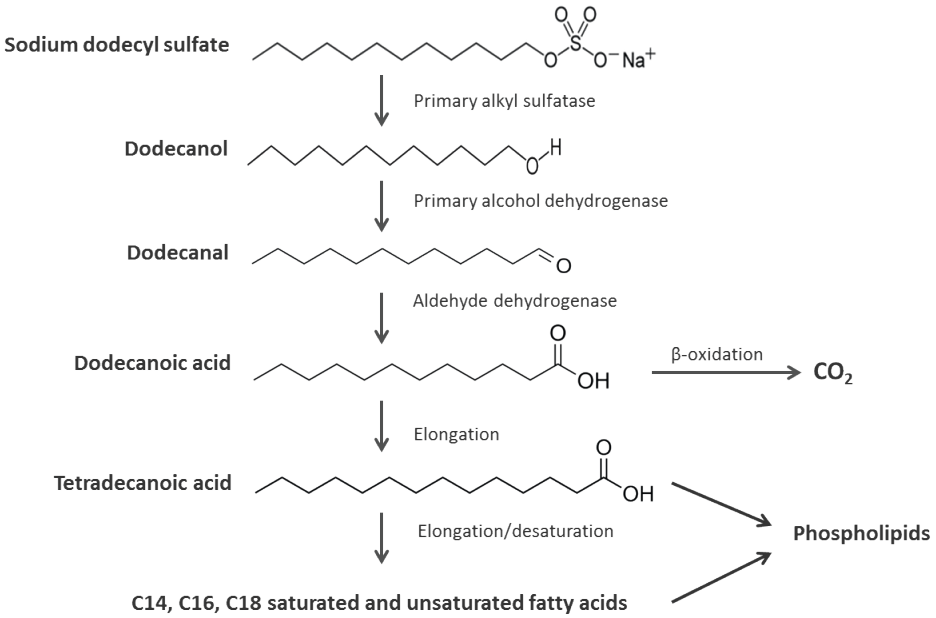

Figure 10 Metabolic pathway for degradation of SDS by *Pseudomonas* C12B (adapted from Thomas and White (1989)).

AES degraders are less studied than AS-degraders. So far, AES degraders were found among different bacterial genera such as: *Acinetobater*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Serratia*, *Citrobacter* and *Pseudomonas* (Hales et al. 1986; Swisher 1987; Dhouib et al. 2003; Khleifat 2006). Initial degradation steps of sodium dodecyl triethoxy sulfate (SDTES) have been studied for *Pseudomonas* DES1, but SDTES is not completely mineralized by this bacterium (Hales et al. 1982; Hales et al. 1986). SDTES primary degradation can be catalyzed by different enzymes, such as ether cleaving enzymes and ester cleaving enzymes. Ether cleavage was found to be the main mechanism for SDTES cleavage in several *Pseudomonas* isolates, but sulfatase action on the parent surfactant could also occur simultaneously with ether cleavage (Hales et al. 1986). Previously it was thought that ether cleaving enzymes could be hydrolytic. For that reason these were referred as *etherases*. However, there are not clear observations on ether bonds hydrolysis (White et al. 1996). Different mechanisms have been associated to ether bond cleavage, such as:

oxygenation, dehydrogenation, hydroxyl group transfers, hydrolysis, reduction, nucleophilic substitution and dismutation. But most of these mechanisms do not present good evidence of their involvement in the metabolic pathways (White et al. 1996). Ether cleaving enzymes from different *Pseudomonas* sp. were shown to be selective and attack preferentially one of the three ether bonds of SDTES or not to be selective and attack the three ether bonds in somewhat similar proportions (Hales et al. 1986). For *Pseudomonas* DES1, ether cleaving enzymes were not selective and different intermediate compounds were formed (Figure 11).

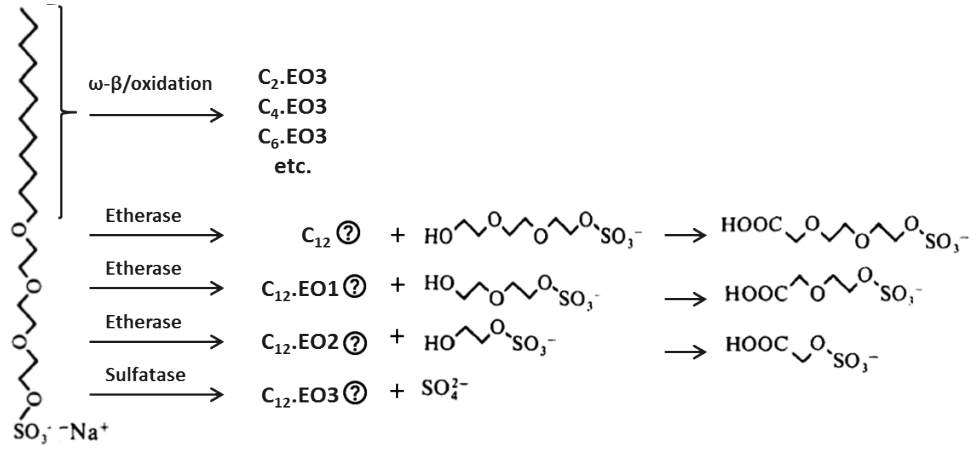

Figure 11 Possible routes for SDTES degradation by *Pseudomonas* DES1 (adapted from Hales et al. (1986)). Symbol ? indicates that the terminal group of the intermediate compound formed may be an alcohol, aldehyde or carboxylic acid. EO1, EO2 and EO3 refer to 1, 2 or 3 number of ethoxy units, respectively, present in the intermediate compound formed.

The cleavage of the first ether bond connected to the alkyl chain, released a compound that could be an alcohol, aldehyde or carboxylic acid with the alkyl chain and also triethylene glycol monosulfate, which can be further converted to acetic acid 2-(diethoxy sulfate). An attack on the other ether bonds gave origin to different intermediate compounds formation and accumulation (Hales et al. 1982; Hales et al. 1986). Glycol sulfates were formed by *Pseudomonas* strains while degrading SDTES (Hales et al. 1986). However, a mixed bacterial culture could degrade all the intermediate compounds formed after SDTES degradation, being the first ether bond linked to the alkyl chain preferred for initial SDTES cleavage (Griffiths et al. 1986). Sulfatase action on the ester bond also occurred, in different proportions, during SDTES degradation by several *Pseudomonas*

isolates (Hales et al. 1982; Hales et al. 1986). Sulfatase activity released a compound that contained three ethoxy units, which could be an alcohol, aldehyde or carboxylic acid, and released sulfate directly from SDTES. ω-/β-oxidation, which occurs when bacteria directly act on the ω-terminal of the alkyl chain of the parent surfactant, was also observed with one *Pseudomonas* strain. However, it is not expected to be a very important mechanism in AES degradation (Hales et al. 1986).

Pseudomonas sp. were also isolated and studied with other classes of surfactants. The description of degradation steps of the non-ionic surfactants alcohol ethoxylates was demonstrated with *Pseudomonas* sp. SC25A (Tidswell et al. 1996). Different *Pseudomonas* spp. able to grow on nonylphenol ethoxylate were isolated and degradation steps were further studied (van Ginkel 2007). Related to cationic surfactants, a *Pseudomonas putida* strain could mineralize the cationic surfactant esterquat, while a QAC served as sole carbon and energy source to a *Pseudomonas* sp. isolated from activated sludge (van Ginkel 2007).

8. *PSEUDOMONAS*

The *Pseudomonas* genus is composed of ubiquitous bacteria in Nature, occupying numerous and diverse ecological niches. Phylogenetically grouped in the class of the *Gammaproteobacteria*, the taxonomy of this genus is complex, since species are metabolically versatile and heterogeneous in terms of substrates degradation. *Pseudomonas* are of great importance for the carbon and nitrogen cycles and while some species can be beneficial to plants and as bioremediation or biocontrol agents, other species are known as important animal or plant pathogens (Mulet et al. 2009). *Pseudomonas* was named by professor Migula from the Karlsruhe Institute in Germany in the end of the nineteenth century (Kersters et al. 1996). The remarkable metabolic versatility of *Pseudomonas* species was described in early studies performed by several bacteriologists such as Beijerinck and Winogradsky during the first half of the 20th century (Peix et al. 2009). The general characteristics of the *Pseudomonas* genus are: straight or slightly curved, rod-shaped, Gram-negative cells; many species accumulate polyhydroxybutyrate as carbon reserve material; rarely non-motile; aerobic, having a strictly respiratory type of metabolism with oxygen as terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor; some species are facultative chemolithotrophic, able to use H_2, H_2S or CO as energy sources (Kersters et al. 1996). *Pseudomonas* species were divided into seven different groups based on 16S rRNA sequence comparison: *Pseudomonas aeruginosa* group, *Pseudomonas chlororaphis* group, *Pseudomonas fluorescens* group, *Pseudomonas pertucinogena* group, *Pseudomonas*

putida group, *Pseudomonas stutzeri* group and the *Pseudomonas syringae* group (Anzai et al. 2000).

P. stutzeri and *P. nitroreducens* are known denitrifiers, which can be present in activated sludge from WWTPs. *P. stutzeri* are non-fluorescent bacteria distinguished from other *Pseudomonas* spp. mainly by a strong denitrifying ability, the use of maltose, starch and ethylene glycol as carbon sources, by negative reaction in arginine dihydrolase and glycogen hydrolysis tests and by an unusual colony morphology (Palleroni et al. 1970; Lalucat et al. 2006). Besides, *P. stutzeri* are able to metabolize a wide range of pollutants, such as aromatic compounds, aliphatic hydrocarbons, biocides, and, in the case of some strains, are also able to fix nitrogen (Cladera et al. 2004; Lalucat et al. 2006). *P. stutzeri* are ecologically relevant. They are found in the natural environment and in the human body (Cladera et al. 2004). *P. stutzeri* was first discovered and described by Burri and Stutzer in 1895 (Lalucat et al. 2006). The definitive designation as *P. stutzeri* was proposed in 1896 by Lehmann and Neumann (Lalucat et al. 2006) and van Niel and Allen (in 1952) defined more precisely the phenotypic features. *P. stutzeri* strains present phenotypic heterogeneity (Rossello et al. 1991). At least nine genomovars, which are closely related, were classified within *P. stutzeri* species (Cladera et al. 2004; Lalucat et al. 2006). *P. nitroreducens* belongs to the *P. aeruginosa* group and are characterized as non-pathogenic, non-fluorescent bacteria are not able to liquefy gelatine, neither to use mannitol and N-Acetyl-glucosamine as carbon sources, opposite to *P. aeruginosa* (Iizuka and Komagata 1964; Clark et al. 2006; Lang et al. 2007). *P. nitroreducens* cannot use maltose but can use L-arginine as carbon source, opposite to *P. stutzeri* strains (Iizuka and Komagata 1964; Clark et al. 2006; Lang et al. 2007). *P. nitroreducens* strains are able to degrade several xenobiotic compounds, such as azelaic acid (Janota-Bassalik and Wright 1964), resin acids (Mohn et al. 1999), biphenyl compounds (Kohler et al. 1988), isoeugenol and eugenol (Unno et al. 2007), phenolic compounds (Zhang et al. 2010), alkylphenol ethoxylates (Chen et al. 2006) and to produce polyhydroxyalkanoates (PHA) (Yao et al. 1999) and rhamnolipids (Onwosi and Odibo 2013). *P. nitroreducens* was first discovered and described by Iizuka and Komagata (1964). When first isolated, *P. nitroreducens* was included in the *P. fluorescens* group since it produced diffusible greenish yellow pigments (Iizuka and Komagata 1964). Recently, in a study by Lang et al. (2007) fluorescence was absent in the *P. nitroreducens* type strain. In the study of Anzai et al. (1997) *P. nitroreducens* was included in the *P. aeruginosa* group (Anzai et al. (2000). The fatty acid pattern of *P. nitroreducens* type strain is also similar to that of the *P. aeruginosa* group (Lang et al. 2007). Although initially considered a different species, it was shown that *P. multiresinivorans* can be considered as a later heterotypic synonym of *P. nitroreducens* (Lang et al. 2007).

`Pseudomonas denitrificans´ was named by Bergey and his co-workers in 1923. The specific epithet was selected after Christensen, in 1903, who initially gave the name *Bacillus denitrificans fluorescens* (not validly published) to two organisms. Both microorganisms were able to denitrify, to liquefy gelatin and to produce soluble fluorescent pigment(s) (Doudorof et al. 1974; Anzai et al. 2000). Because 18 strains classified as *`P. denitrificans´* did not match the description proposed by Christensen, and were also different in terms of substrates degradation, DNA and rRNA, these appeared to belong to several species and at least two different bacteria genera (Doudorof et al. 1974). Thus, *`P. denitrificans´* is not a valid bacterial name (http://www.bacterio.net/). *`P. denitrificans´* ATCC 19244 was proposed as neotype of the species by Lysenko (1961).

9. *PSEUDOMONAS* GENOME ANALYSIS

In the last decades, microbiological research has changed considerably with the sequencing of complete genomes. The revolution in computer technology made it possible to deal with the enormous amount of genomic data, increasing genome analysis all over the world. This enabled new discoveries and tools not possible to use in wet-lab experiments (Bansal 2005). Bioinformatics analysis have been developed to identify and analyze various components of cells such as gene and protein function, interaction and metabolic and regulatory pathways, facilitating and making faster the analysis of the genome data (Bansal 2005).

Pseudomonas aeruginosa is used as a reference of the *Pseudomonas* genus, due to the relative phenotypic homogeneity of this species. It was suggested that *P. aeruginosa* is the most abundant bacterial species on the planet: in the environment and as a peaceful inhabitant or as an opportunistic human pathogen (Palleroni 2010). *P. aeruginosa* PAO1, a chloramphenicol-resistant mutant of the original PAO strain, is a common reference strain for *Pseudomonas* genetics and functional analysis of the physiology (Klockgether et al. 2010). The *P. aeruginosa* PAO1 genome was the 25th bacterial genome sequence to be completed (Stover et al. 2000). Since then, genomes of other strains from the *P. aeruginosa* genus were sequenced, such as the ones from: *P. aeruginosa* LESB58 (Winstanley et al. 2009), *P. aeruginosa* PA7 (Roy et al. 2010) and *P. aeruginosa* DK2 (Rau et al. 2012). Also genomes from strains of several other *Pseudomonas* species were sequenced, such as: *P. syringae* (Buell et al. 2003), *P. putida* (Nelson et al. 2002), *P. fluorescens* (Paulsen et al. 2005), *P. entomophila* (Vodovar et al. 2006), *P. stutzeri* (Yan et al. 2008) and *`P. denitrificans´* (Ainala et al. 2013). Only recently, a draft genome of *P. nitroreducens* strain TX1, which degrades non-ionic surfactants and estrogen-like alkylphenols, has been published (Huang et al. 2014). The size of *P. aeruginosa* PAO1

genome and the number of regulatory genes involved in catabolism, transport and efflux of organic compounds and in chemotaxis explained the ability of this species to thrive in different environments and resistance to antimicrobial substances (Stover et al. 2000). The genome sequencing of the plant pathogen *P. syringae* pathovar *tomato* DC3000 allowed identifying virulence genes, transporters for the acquisition of nutrients, mainly sugars, and genes related to the attachment to plant surfaces (Buell et al. 2003). *P. putida* KT2440 is a non-pathogenic saprophytic soil bacterium that shares 85% of predicted coding regions with *P. aeruginosa*. The genome sequence of this strain revealed diverse transport and metabolic systems, which did not present key virulence factors (Nelson et al. 2002). *P. fluorescens* Pf-5 is a plant commensal bacterium that suppresses soil borne plant pathogens. The complete genome sequence of this strain allowed identifying its broad catabolic and transport capabilities for using plant-derived compounds, diversity in iron siderophores, detoxification systems and genes that might contribute to its biocontrol abilities (Buell et al. 2003). *P. entomophila* kills insects after being digested by these, which makes it an entomopathogenic bacterium. Its genome sequence revealed that many of its catabolic genes are shared with *P. putida* KT2440. *P. entomophila* relies on potential insecticidal toxins and other virulence factors to infect and kill insects (Vodovar et al. 2006). *P. stutzeri* A1501 is a nitrogen-fixing root associated bacterium. Its genome contains genes involved in nitrogen fixation, denitrification, degradation of different carbon sources including aromatic compounds and in functions that are presumed to help in root colonization (Yan et al. 2008). `*P. denitrificans*´ ATCC 13867 genome was sequenced due to the ability of this strain to produce high amounts of vitamin B12 in aerobic conditions. Genes associated to this ability have been identified in its genome (Ainala et al. 2013). *P. nitroreducens* TX1 is able to degrade octyphenol polyethoxylates ($OPEO_n$) and nonylphenol polythoxylates ($NPEO_n$) which are nonionic surfactants. Several genes associated to ethoxylate chain degradation were identified in *P. nitroreducens* TX1 draft genome (Huang et al. 2014).

Phylogenetic relationships between microorganisms have been traditionally defined by phenotypic characters. Nowadays, using comparative genomics, evolutionary distances may be inferred more meticulously, as they are independent from the variability in expression profiles or from morphological characteristics. Whole genomic DNA content can be used for establishing more proper evolutionary relationships (Coenye et al. 2005). The 16S rRNA gene sequence has been used as the gold standard for the determination of phylogenetic relationships due to the fact that it is universally conserved in all organisms and is hardly affected by horizontal gene transfer (Bodilis et al. 2012). However, there are evidences in nature and in experiments using *E. coli* that 16S rRNA gene is occasionally amenable to horizontal gene transfer (Kitahara and Miyazaki 2013). The poor resolution of

the 16S rRNA gene was observed at the intra-genus level for some genera, such as *Pseudomonas* (Bodilis et al. 2012). In the past, the assignment of bacterial isolates to the *Pseudomonas* genus was mainly determined by using phenotypic characteristics and biochemical capabilities. Later on, DNA-DNA hybridization, analysis of rRNA gene and of housekeeping genes sequences aided taxonomic definition and species reorganization within the *Pseudomonas* genus. Nowadays whole-genome sequences provide information that allows a further improvement in the taxonomy of this genus (Silby et al. 2011).
Protein domains can be defined as independent and evolutionary units that can form a single-domain protein or be part of one or more different multidomain proteins. If these units can evolve, function and exist independently (Vogel et al. 2004) these can also be used for establishing comprehensive phylogenetic relationships, as an alternative to 16S rRNA comparisons.

10. OUTLINE OF THE THESIS

Xenobiotic compounds, such as surfactants, can be degraded by aerobic bacteria. However, these compounds can end up in environments where oxygen is absent. In a WWTP with the A^2/O concept, surfactants can be degraded anaerobically before reaching the aerobic compartment. In the absence of oxygen, facultative anaerobic bacteria can degrade surfactants by using nitrate/nitrite as electron acceptor. The main aim of the research described in this thesis was to get more insight into the ecology and physiology of anaerobic bacteria able to degrade anionic surfactants coupled to nitrate reduction. Research described here is presented in five experimental chapters.
Enrichment, isolation and characterization of two *Pseudomonas* strains with ability to couple SDS degradation to nitrate reduction are described in Chapter II. The diversity of bacteria enriched from activated sludge with ability to degrade and/or to tolerate SLES at aerobic and anoxic conditions is presented in Chapter III. Three different bacterial strains able to use SLES as sole carbon and energy source coupled to nitrate reduction were investigated (Chapter IV). In chapter V SDS-degrading and SLES-degrading bacteria were tested for their ability to grow with high SDS or SLES concentrations, coupled to nitrate reduction. *P. nitroreducens* strains were isolated that can use both SDS and SLES with nitrate. This ability is shared by *P. nitroreducens* DSM 14399^T. The genome of *P. nitroreducens* DSM 14399^T was sequenced, annotated and compared to the genome of several *Pseudomonas* spp.. (Chapter VI). The main findings of this thesis are comprehensible discussed and further research gaps are defined in Chapter VII.

CHAPTER II

ANAEROBIC DEGRADATION OF SODIUM DODECYL SULFATE (SDS) BY DENITRIFYING BACTERIA

A. M. S. Paulo, C. M. Plugge, P. A. García Encina and A. J. M. Stams

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ABSTRACT

Two denitrifying bacteria were isolated using sodium dodecyl sulfate (SDS) as substrate. Strains SN1 and SN2 were isolated from an activated sludge reactor of a wastewater treatment plant (WWTP) with anaerobic-anoxic-oxic (A^2/O) steps. Based on 16S rRNA gene analysis strain SN1 is 99% similar to *Pseudomonas stutzeri* (CCUG 11256^T), while strain SN2 is 99% similar to *Pseudomonas nitroreducens* (DSM 14399^T). The two novel strains are able to grow with a variety of organic compounds, including intermediates of SDS degradation. Alkylsulfatase activity was induced by SDS in strain SN1 and was enhanced by SDS in strain SN2. The type strain *P. stutzeri* (CCUG 11256^T) is not able to grow with SDS. However, *P. nitroreducens* (DSM 14399^T) is able to degrade and grow with SDS and nitrate or oxygen as electron acceptors, adding new information to the physiological abilities of this type strain.

KEYWORDS

Anionic surfactants, denitrification, detergents, nitrate, *Pseudomonas*, sodium dodecyl sulfate.

1. INTRODUCTION

Surfactants are surface-active agents used in the formulation of detergents, personal care products, paints, polymers and other products. Surfactant molecules typically consist of a polar head group (charged or not charged) and a nonpolar hydrocarbon tail. There are three main classes of surfactants: anionic, nonionic and cationic. The hydrophilic and hydrophobic properties make these compounds useful for cleaning/solubilization purposes (Ying 2006). Surfactants are widely used for domestic and industrial applications and after use they are discharged to wastewater treatment plants (WWTP's) (Gonzalez et al. 2007).

Aerobic degradation of surfactants has been described in several studies (Swisher 1987; Scott and Jones 2000). Less is known about anaerobic degradation of surfactants but many surfactants, especially linear alkyl benzene sulfonates, secondary alkane sulfonates and cationic surfactants are known to be persistent under anaerobic conditions (Ying 2006).

Sodium dodecyl sulfate (SDS) is present in many cosmetics and personal hygiene products (Sirisattha et al. 2004) and is often used as model compound for aliphatic alkyl sulfates degradation (Abboud et al. 2007). SDS degradation in the presence of oxygen is known

(Swisher 1987; Scott and Jones 2000). Initial studies were focused on aspects associated to the alkyl sulfate degradation in polluted rivers sites, such as SDS-degraders occurrence and distribution, alkylsulfatase activity and attachment of alkyl sulfate-degraders to river sediments (Anderson et al. 1988; White et al. 1989; Marchesi et al. 1994). However, SDS degradation by denitrifying bacteria is only scarcely documented. Thus far, SDS degradation coupled to nitrate reduction was only described for unidentified bacteria isolated from river sediments (Dodgson et al. 1984). In that study, alkylsulfatase activity of an isolate able to degrade SDS and other alkyl sulfates, in anoxic and oxic conditions, was investigated. The isolate degraded SDS coupled to nitrate reduction to N_2, not accumulating N_2O.

Nowadays, most of the developed countries have wastewater collection connected to municipal WWTP's. In a WWTP elimination of organic matter and nitrogen removal together with phosphorus accumulation can be performed using an A^2/O biological process. In this biological process, anaerobic treatment occurs first, followed by the anoxic and the oxic processes. In this way, most of the organic matter is degraded in the first two steps (anaerobic and anoxic), with no need for extra addition of carbon source to the anoxic reactor. Nitrate, resulting from nitrification, is recirculated from the oxic reactor to the anoxic reactor, where denitrifying bacteria reduce nitrate to dinitrogen gas (Metcalf and Eddy 2003).

A degradation pathway for SDS has been proposed for *Pseudomonas* C12B (Payne and Feisal 1963; Thomas and White 1989). In this pathway, the initial cleavage occurs via hydrolysis of the ester that bonds the alkyl chain to the sulfate. Since degradation of SDS does not require oxygenases, it is expected that in an A^2/O process such surfactants will be degraded in the anaerobic or anoxic compartment before reaching the oxic step. The aim of the present study was to get insight into occurrence and activity of microorganisms capable of SDS degradation coupled to denitrification in a WWTP with the A^2/O concept.

2. MATERIALS AND METHODS

2.1 Enrichment, isolation and identification

Activated sludge from a wastewater treatment plant (Valladolid, Spain) was used as inoculum. The initial enrichment was done in batch culture. The batches were prepared in 120-ml serum bottles and contained 50 ml of medium and a gas phase of helium (1.4 atm). Bottles were sealed with butyl-rubber stoppers and crimp seals. The standard mineral salts medium contained (per liter distilled water): 1 g KH_2PO_4, 3.48 g

$Na_2HPO_4.2H_2O$, 1 g $(NH_4)_2SO_4$, 0.033 g $MgCl_2.6H_2O$, 0.0090 g $CaCl_2.2H_2O$, 0.01 g $Fe(NH_4)$ citrate. Vitamins and trace elements were added in the final concentrations as described by Holliger et al. (1993). SDS (0.35 mM) was used as sole carbon and energy source and added from a filter sterilized anoxic stock solution. KNO_3 (4.3 mM) was added as electron acceptor from a sterilized stock solution. Initial inoculum concentration in the bottles was about 215 mg VSS L^{-1}. Batch enrichments were incubated at 30 °C and the pH was 7.3 ± 0.1. After several transfers of 5 % (v/v) of the enriched culture to fresh medium, dilutions of the last enriched culture were streaked on agar plates to isolate the microorganisms. Agar plates contained standard mineral salts medium, 0.4 mM of SDS and 20 g L^{-1} of agar. The plates were incubated at 30 °C, under air. Colonies with different morphology were selected and streaked on new plates until single colonies were obtained. The ability of each of the pure cultures to degrade SDS in anoxic conditions was tested by transferring the single colonies back to anoxic conditions in batch liquid cultures.

Purity and morphology of the isolated microorganisms was routinely observed by phase contrast microscopy. Gram staining was performed using standard techniques. For phylogenetic analysis DNA was extracted with the FastDNA® Spin kit for soil (MP Biomedicals, USA) according to the manufacturer protocol. The bacterial 16S rRNA genes were amplified by PCR (Polymerase Chain Reaction) using the universal bacterial primers 7f and 1510r (Lane 1991) and the GoTaq DNA Polymerase Kit (Promega, USA). Each PCR mixture contained: 1 µL of DNA template, 0.25 µL of Taq DNA polymerase, 1 µL dNTPs, 1 µL primer 7f, 1 µL primer 1510r, 10 µL PCR buffer and 35.75 µL PCR water. The settings for PCR were: initial denaturation for 2 min at 95 °C, followed by 25 cycles of 30 s denaturation at 95 °C, 40 s annealing at 52 °C and 1.5 min elongation at 72 °C. Post-elongation was 5 min at 72 °C. PCR products were purified with DNA Clean & Concentrator™-5 kit (Zymo Research, USA). Sequencing was performed by Baseclear (The Netherlands). The sequences were checked with the alignment programs of BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Closely related 16S rRNA gene sequences were identified using BLAST for homology searches (Altschul et al. 1990). The 16S rRNA gene sequences of strains SN1 and SN2 have been deposited in the GenBank database under accession numbers JF461537 and JF461538, respectively. Both strains SN1 and SN2 have been deposited in the open collection of DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) under the DSM numbers 26095 and 26096, respectively.

2.2 Growth with SDS

Anoxic biodegradation of SDS with nitrate as electron acceptor was studied with the enriched culture and the obtained isolates. For this, 1.1 L glass bottles were used containing 750 ml of standard mineral medium with SDS (0.35 mM) and KNO_3 (4.3 mM). The incubations were performed in triplicate while appropriate duplicate controls were included, lasting around 50 hours. All experiments were conducted at 30 °C. In time, liquid and gas samples were taken. Biomass increase was monitored using optical density (OD) measurements at 600 nm. NO_3^-, NO_2^- and N_2 were also analyzed. SDS degradation was indirectly monitored by quantifying the dissolved organic carbon (DOC) in the medium. At the beginning of the growth experiments 2 % (v/v) of inoculum of enriched culture, strain SN1 or strain SN2 was added. In all growth experiments the initial concentration of inoculum was similar. Carbon mass balances were calculated at the beginning of incubation and at the end of the exponential growth phase. Total organic carbon (TOC), inorganic carbon (IC), dissolved organic carbon (DOC) and CO_2 were measured for this purpose. The carbon present as biomass was determined by subtracting DOC from TOC values. The amount of carbon present as biomass at the beginning of the tests was disregarded. Total inorganic carbon in the bottles was calculated as the sum of CO_2 (g) + CO_2 (aq) + HCO_3^- (aq). The Henry's law was used to calculate the expected dissolved CO_2 in the medium. Growth of isolated strains was studied with concentrations of SDS up to 40 g L^{-1} (139 mM) with NO_3^- (20 mM). Growth was determined by measuring increase in OD at 600 nm, NO_3^- reduction and NO_2^- and SO_4^{2-} accumulation, after a period of incubation of 5 days.

2.3 Optimal temperature and pH

Temperature and pH ranges were tested in 120-ml bottles with 40 ml standard medium, acetate (10 mM) and NO_3^- (10 mM). Isolates were incubated at a pH of 7.3 ± 0.1 from 4 °C to 55 °C and at a pH range between 4 and 10.5, at 30 °C, during two weeks. The optimal temperature and pH were determined by measuring the OD at 600 nm and nitrate depletion over the exponential growth phase, in duplicate.

2.4 Other electron donors

Different organic carbon sources such as sugars, alcohols, amino acids, volatile fatty acids and intermediates of the citric acid cycle were tested as growth substrates. Substrates were added to the medium from sterilized anoxic stock solutions to a final concentration

of 10 mM. Peptone and yeast extract (final concentration 1 g L^{-1}) were also tested as growth substrates. Possible intermediates of SDS degradation (dodecanol, dodecanal and lauric acid) were directly added to the bottles before sterilization and tested at a final concentration of 0.2 mM.

Other anionic surfactants were also tested, including sodium dodecyl sulfonate and linear alkyl benzene sulfonate. Triton X-100, a nonionic surfactant, was tested as well. All the surfactants were applied in two concentrations (25 and 50 mg L^{-1}) and added to the bottles from filter sterilized stock solutions. Alkylamine degradation, such as hexylamine (0.25 and 0.5 mM) and dodecylamine (0.15 and 0.3 mM), was also evaluated. Hexylamine was added from a filter sterilized anoxic stock solution, while dodecylamine was added directly to the bottles before sterilization. Alkane degradation was tested by adding decane (0.6 mM) and dodecane (0.6 mM) from anoxic sterilized stock solutions. The use of electron donors was studied with NO_3^- (10 mM) or with oxygen (air gas phase), at 30 °C, using biological duplicates. Growth was determined by measuring increase in OD at 600 nm and/or the NO_3^- or O_2 decrease. API strip tests 20NE (bioMérieux SA, France) were used to compare the isolated strains with related type strains based on the results for specific reactions and substrates use. The related type strains *P. stutzeri* (CCUG 11256^T) and *P. nitroreducens* (DSM 14399^T) were both tested for the use of SDS as carbon source in the presence of oxygen or nitrate. Acetate was also used as substrate for comparison. Growth was measured by OD measurements and nitrate was analyzed. All tests were performed in triplicate. *P. stutzeri* strain ZN6, isolated by its ability to degrade long chain alkyl amines with nitrate (Nguyen et al. 2008), was also tested for SDS degradation.

2.5 Other electron acceptors

Different electron acceptors were tested using the standard medium and sodium acetate (10 mM) as electron donor and carbon source. NO_3^-, NO_2^-, SO_4^{2-}, ClO_3^-, ClO_4^-, $S_2O_3^-$, FeOOH, Fe-citrate, MnO_2 and fumarate were added from anoxic stock solutions to a final concentration of 10 mM. Pure O_2 was autoclaved in serum bottles and later added in sterile conditions by syringe to serum bottles with medium. Incubations were performed at 30 °C. Amorphous Fe(III) oxyhydroxide (FeOOH) was formed by neutralizing a 0.4 M solution of $FeCl_3$ to a pH of 7 with NaOH (Lovley and Phillips 1986). Amorphous manganese (IV) oxide (MnO_2) was made by mixing equal amounts of 0.4 M $KMnO_4$ and 0.4 M $MnCl_2$ and adding 1 M NaOH to obtain a pH 10 (Burdige and Nealson 1985). After preparation, the FeOOH and MnO_2 suspensions were washed four times with deionized water and separated by centrifugation (10 min at 10000 *g*). Growth with electron

acceptors was determined by measuring increase in OD at 600 nm and the acetate decrease in duplicate.

2.6 Alkylsulfatase activity

Alkylsulfatase activity was calculated after measuring the amount of inorganic SO_4^{2-} released during the reaction of cell free extract (CFE) with acetate or SDS, in buffer solution. For CFE preparation, strains SN1 and SN2 were grown aerobically in the standard medium. SDS (0.7 mM) and acetate (10 mM) were used as substrates. Whole cells were harvested in the mid exponential phase from 400-ml cultures by centrifugation at 18600 *g* for 20 min at 4 °C. The cell pellet was washed twice with 0.1 M Tris-HCl buffer, pH 7.5. Cells were resuspended in the same buffer (2 ml) and stored at -20 °C (Dodgson et al. 1984). To prepare cell extracts, 2 ml cell suspension was sonicated for 20 seconds at 20 W with a Sonifier B12 (Branson Ultrasonics, USA) with intervals of 20 seconds while cooling on ice, for 4 times. Cell-debris was removed by centrifugation at 15000 *g* for 10 min at 4 °C. CFEs were used the same day. Sulfatase activity was tested at 30 °C, using 5 ml of 0.1 M Tris-HCl buffer (pH 7.5), 200 µL of CFE and 130 µL of SDS (0.7 mM, final concentration). Tests with CFE and SDS were performed in triplicate. Controls without CFE and without SDS were performed in duplicate. Previously cleaned and autoclaved 10-ml serum bottles, sealed with butyl-rubber stoppers and crimp seals, were used for the tests. Samples were taken discontinuously and immediately frozen and stored for later analysis. Samples were diluted with NaOH (20 mM) and analyzed for SO_4^{2-} release.

2.7 Analytical methods

OD at 600 nm was determined using a Hitachi U2000 UV/visible spectrophotometer (Hitachi, Japan). NO_3^-, NO_2^- and SO_4^{2-} were analyzed by suppressor mediated ion chromatography using a conductivity detector and an IonPac AS9-SC 4 x 50 mm column (Dionex, USA). The mobile phase (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$) was used at a flow rate of 1 ml min^{-1}. Mannitol was used for stabilization of the samples and sodium fluoride was used as the internal standard. The analysis was conducted at a temperature of 35 °C. A TOC-5050A analyzer (Shimadzu, Japan) with automatic sampler ASI-5000A (Shimadzu, Japan) was used for TOC, IC and DOC measurements of the liquid samples, according to the manufacturer instructions. N_2 and CO_2 concentrations in the headspace were measured using a GC CP-2800 (Varian, USA) equipped with a Mol Sieve 5A (15 m x 0.53 mm x 15 µm), a CP-PoraBand Q (25 m x 0.53 mm x 10 µm) capillary column and a thermal conductivity detector. The temperatures of injector, detector and oven were maintained

at 150, 175 and 40 °C, respectively. Helium was used as the carrier gas (13.7 mL min^{-1}). Acetate was analyzed by HPLC using a Varian Metacarb 67H 300 x 6.5 mm column and a SpectraSYSTEM RI-150 detector (Thermo Electron Corporation, USA). The mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.8 ml min^{-1}. The analysis was conducted at a temperature of 30 °C. Crotonate was used as internal standard. N_2O was measured using a GC-2014 (Shimadzu, Japan) equipped with a CP Poraplot Q column (25 m x 0.53 mm x 20 µm) and a thermal conductivity detector. The temperatures of injector, detector and oven were maintained at 60, 130 and 40 °C, respectively. Samples for DOC and anions measurements were centrifuged and filtrated using a membrane filter (0.22 µm) before analysis. Protein content of CFEs was estimated according to (Bradford 1976) with bovine serum albumin as the standard.

2.8 Other strains

Pseudomonas stutzeri (CCUG 11256^T) was obtained from the Culture Collection of the University of Göteborg (Sweden). *Pseudomonas nitroreducens* (DSM 14399^T) was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *P. stutzeri* strain ZN6 was isolated at our laboratory (Nguyen et al. 2008).

2.9 Chemicals

Decane (purity ≥ 94 %), dodecane (purity ≥ 99 %) and hexylamine (purity ≥ 99 %) were purchased from Merck (Germany). Dodecylamine (purity ≥ 98 %) was purchased from Acros Organics (Belgium). Sodium dodecyl sulfate (purity ≥ 99 %) and other chemicals used in this study were purchased from Sigma-Aldrich (Germany).

2.10 Statistical analysis

All average values were obtained using biological triplicates. The variation of the mean of the triplicate values was calculated using the standard deviation (SD), which is presented as average ± SD in the text (sections 2.1, 2.3 and 3.4) and tables. In the figures, SD is presented as error bars associated to the average values.

3. RESULTS

3.1 Enrichment, isolation and identification

An enriched culture capable of degrading SDS coupled to complete nitrate reduction to N_2 was obtained from activated sludge. Two pure cultures designated strains SN1 and SN2 were obtained. SN1 colonies were circular and dense, while SN2 colonies were less dense and expanding through ramifications. When transferred back to anoxic conditions, both strains were able to use SDS as sole carbon and energy source with nitrate as electron acceptor. Strains SN1 and SN2 are Gram negative, motile and rod-shaped. Both strains are 0.5-1µm wide and 1.5-3 µm large. The 16S rRNA gene sequence (1401 bp) of strain SN1 shows 99 % homology with several *P. stutzeri* strains, including the type strain CCUG 11256^T (Stanier et al. 1966) and the 16S rRNA gene sequence of SN2 (1412 bp) is 99 % similar to *P. nitroreducens* (DSM 14399^T) (Lang et al. 2007). According to these results, strain SN1 was identified as a *P. stutzeri* strain and strain SN2 as a *P. nitroreducens* strain.

3.2 Growth with SDS

SDS degradation and growth of strains SN1 and SN2 with nitrate is shown in Figure 1. A similar growth pattern was obtained for the enrichment (data not shown). Growth was complete within 16 hours and similar final OD values were obtained. The doubling time was approximately 2 hours for both strains. Table 1 presents the results of the carbon mass balances for strains SN1 and SN2, between the beginning of the incubation and the end of exponential growth. Results show mineralization of SDS (0.35 mM) to biomass and CO_2. Approximately half of the carbon in SDS was converted to CO_2 and the remainder was incorporated in microbial biomass. The values calculated for the yields are in agreement with the results obtained for other pure and mixed cultures in anoxic conditions (Copp and Dold 1998). Results for growth with higher concentrations of SDS showed that strains SN1 and SN2 are able to reduce nitrate with 40 g L^{-1} of SDS as initial concentration. While strain SN1 reduced part of the nitrate and accumulated nitrite, strain SN2 reduced all nitrate and nitrite to nitrogen gases. Sulfate formation increased with increasing SDS concentration in the medium up to 10 g L^{-1} for strain SN2. For strain SN1, sulfate formation reached similar values for all tested SDS concentrations, identical to the lower tested concentration (1 g L^{-1}), indicating inhibition of alkylsulfatase activity with increased SDS concentrations (data not shown).

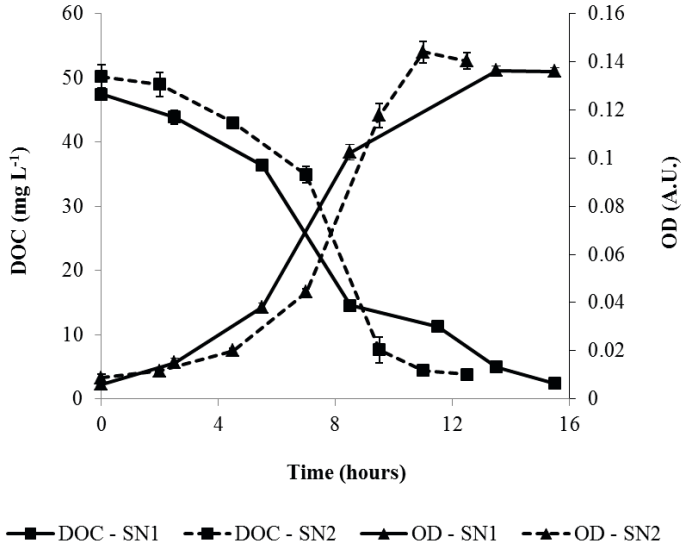

Figure 1 Growth and SDS degradation by strains SN1 and SN2. 2 % (v/v) of inoculum of strain SN1 or strain SN2 was inoculated with SDS (0.35 mM) and KNO_3 (4.3 mM). Incubations were performed in triplicate and conducted at 30 °C. Liquid samples were taken in time. Biomass increase was monitored using optical density (OD) measurements at 600 nm. SDS degradation was indirectly monitored by quantifying the dissolved organic carbon (DOC) in the medium. SD values are represented as error bars.

Table 1
Carbon mass balances for SDS degradation by strains SN1 and SN2 at the end of exponential growth. Average values ± SD.

	$DOC_{initial}$ [a]	DOC_{final} [b]	IC_{final} [c]	$C_{biomass}$ [d]	$DOC_{removal}$ [e]	Yield [f]
Strain	mg C L^{-1}	mg C L^{-1}	mg C L^{-1}	mg C L^{-1}	%	mg C mg C^{-1}
SN1	47.4 ± 0.1	2.4 ± 0.3	22.7 ± 0.2	23.4 ± 1.1	94.9 ± 0.7	0.52 ± 0.02
SN2	50.2 ± 1.9	3.8 ± 0.0	23.6 ± 0.7	23.4 ± 0.4	92.4 ± 0.3	0.50 ± 0.01

[a] Initial dissolved organic carbon added as carbon source (SDS).
[b] Dissolved organic carbon at the end of the exponential growth.
[c] Total inorganic carbon (CO_2 (g) + CO_2 (aq) + HCO_3^- (aq)) at the end of the exponential growth.
[d] Difference between TOC and DOC.
[e] Percentage of carbon removed from the medium in the dissolved form.
[f] Ratio of $C_{biomass}$ to the substrate consumed ($DOC_{initial} - DOC_{final}$), in terms of carbon.

3.3 Denitrification

Nitrate was reduced to N_2 in all experiments (Figures 2a and 2b). Slow formation of N_2 during growth was observed, followed by a fast production at the end of the exponential growth. During nitrate reduction by both strains some nitrite accumulation was observed. The nitrogen balance was calculated at the end of exponential phase and also at the end of the incubation (Table 2). The difference between the initial nitrate and the final nitrate and nitrite concentration was similar to the amount of nitrogen in N_2 that had been produced at the end of the stationary phase for strain SN1. Less recovery in terms of N_2 was found for strain SN2. Hence, SN1 and SN2 were able to degrade SDS coupled to denitrification.

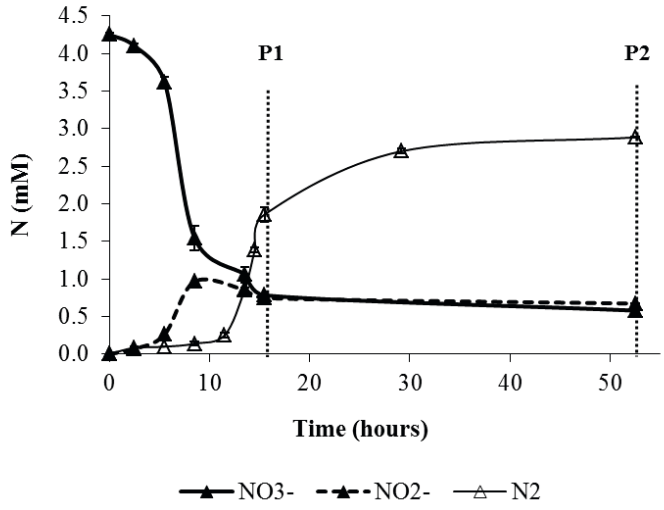

Figure 2a Nitrogen concentration in the liquid (NO_3^- and NO_2^-) and N-N_2 production for strain SN1. During growth and until the end of the incubation, nitrate, nitrite and nitrogen gas were analyzed. P1 is the sampling point at the end of the exponential phase; P2 is the last sampling point of the incubation. SD values are represented as error bars.

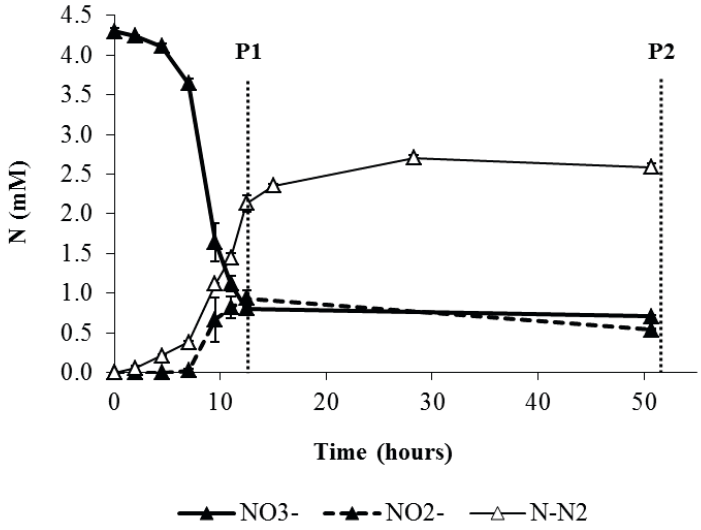

Figure 2b Nitrogen concentration in the liquid (NO_3^- and NO_2^-) and N-N_2 production for strain SN2. During growth and until the end of the incubation, nitrate, nitrite and nitrogen gas were analyzed. P1 is the sampling point at the end of the exponential phase; P2 is the last sampling point of the incubation. SD values are represented as error bars.

Table 2

Nitrogen balances for SDS degradation by strains SN1 and SN2 at the end of the exponential growth (P1) and at the end of the incubation (P2). Average values ± SD. P1 and P2 are different moments of sampling as indicated in figures 2a and 2b.

	$NO_{3\ initial}^{-}$ [a]	$NO_{3\ final}^{-}$ [b]	$NO_{2\ final}^{-}$ [c]	$N\text{-}N_{2prod}$ [d]	$N_{recovery}$ [e]
Strain	mmol L^{-1}	mmol L^{-1}	mmol L^{-1}	mmol L^{-1}	%
SN1 (P1)	4.3 ± 0.02	0.78 ± 0.02	0.74 ± 0.05	1.86 ± 0.19	72.8 ± 12.7
SN1 (P2)	4.3 ± 0.02	0.58 ± 0.09	0.67 ± 0.05	2.88 ± 0.00	96.6 ± 0.4
SN2 (P1)	4.3 ± 0.04	0.80 ± 0.03	0.93 ± 0.10	2.13 ± 0.22	78.2 ± 7.0
SN2 (P2)	4.3 ± 0.04	0.71 ± 0.02	0.54 ± 0.01	2.59 ± 0.09	83.1 ± 3.0

[a] Initial concentration of NO_3^- in the medium (no NO_2^- was present at the beginning of the tests).
[b] Final concentration of NO_3^- in the medium.
[c] Final concentration NO_2^- in the medium.
[d] Nitrogen produced in terms of mmol N L^{-1}.
[e] Percentage of removed nitrogen which was reduced to N_2.

3.4 Optimal temperature and pH

Strains SN1 and SN2 presented an optimal growth between 30 °C and 37 °C. Strain SN1 is able to grow from 15 °C to 45 °C, while strain SN2 can grow from 15 °C to 37 °C. The optimal pH for strain SN1 is 8 ± 0.5, being able to grow in a pH range from 6.5 to 9.8. When tested at pH 9.8, strain SN1 showed a slight increase in OD in time. Also nitrate reduction to nitrite was observed at this pH. At pH above 10 no OD increase or nitrate reduction were found. For strain SN2 fastest growth was obtained at pH 7.0 ± 0.5, being able to grow at a pH range from 6.0 to 9.0. OD values of strains SN1 and SN2 at the end of exponential growth, at different temperatures and pH are presented in Table 3.

Table 3
OD values of strains SN1 and SN2 at the end of exponential growth, at different temperatures and pH. Average values ± SD.

	Temperature (°C)				
Strain	15	20	30	37	45
SN1	0.06 ± 0.00	0.10 ± 0.01	0.20 ± 0.00	0.23 ± 0.01	0.10 ± 0.00
SN2	n.a.	0.03 ± 0.00	0.11 ± 0.01	0.12 ± 0.01	n.s.

	pH					
	6.5	7.0	7.5	8.0	8.5	9.0
SN1	0.09 ± 0.00	0.19 ± 0.00	0.28 ± 0.00	0.30 ± 0.01	0.31 ± 0.00	0.24 ± 0.03
SN2	0.11 ± 0.01	0.12 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	n.a.	n.a.

n.a. – not available due to slow growth.
n.s. – not significant (no OD increase occurred during the incubation period).

3.5 Other electron donors and electron acceptors

Strains SN1 and SN2 were able to degrade diverse types of carbon sources in both aerobic and anoxic conditions. Table 4 includes the differences in substrates utilization by strains SN1 and SN2 and comparison with the results obtained with API strip tests 20NE and from literature for *P. stutzeri* CCUG 11256^T and *P. nitroreducens* DSM 14399^T. Peptone, acetate and succinate were used by isolated strains and type strains. API strip tests 20NE results showed that all strains were able to grow on glucose, gluconate, capric acid, malic acid and citrate. However, none of the strains presented growth on arabinose, mannose and N-

acetyl-glucosamine. Using the reactions tested with the API strip tests 20NE none of the strains could produce indole from L-tryptophane, ferment glucose, use urea and hydrolyze esculin, gelatin or 4-nitrophenyl-βD-galactopyranoside. 1-dodecanol and lauric acid were used by SN1 and SN2 strains in both aerobic and anoxic conditions. Dodecanal was used by both isolated strains only in aerobic conditions. Decane, sodium dodecyl sulfonate, linear alkyl benzene sulfonate and Triton X-100 were not used as carbon sources by strains SN1 and SN2. Dodecylamine was not used as carbon source and this compound was found to be toxic to both isolates in the presence of acetate as extra carbon source. Dodecane was only used by strain SN2 in aerobic conditions. Reduction of nitrate and decrease of O_2 was observed with hexylamine for both strains. However, these results were not reproducible after several transfers of the strains to fresh medium with acetate. Strains SN1 and SN2 were able to use O_2, NO_3^-, and NO_2^- as electron acceptors, but both strains could not grow in the presence of SO_4^{2-}, ClO_3^-, ClO_4^-, $S_2O_3^-$, FeOOH, Fe-citrate, MnO_2 and fumarate. SDS degradation in aerobic and anoxic conditions was observed with the type strain *P. nitroreducens* DSM 14399^T, but not with the type strain *P. stutzeri* CCUG 11256^T. Both strains denitrified using acetate as sole carbon and energy source, although *P. nitroreducens* DSM 14399^T grew slower than *P. stutzeri* CCUG 11256^T with this carbon source. The type strain *P. nitroreducens* DSM 14399^T showed faster growth with SDS and nitrate when compared to the growth with acetate and nitrate. *P. stutzeri* strain ZN6 was not able to degrade SDS.

Table 4
Substrates tested for growth of SN1 and SN2 strains under aerobic and nitrate reducing conditions and comparison with *P. stutzeri* CCUG 11256^T and *P. nitroreducens* DSM 14399^T.

Substrates	SN1 strain	*P. stutzeri* CCUG 11256^T	SN2 strain	*P. nitroreducens* DSM 14399^T
Mannitol[a]	-	+[b]	-	-[c]
Maltose[a]	+	+[b]	-	-[c]
Adipic Acid[a]	-	-[b]	-	+[c]
Phenylacetic Acid[a]	+	-[b]	-	+[c]
Fructose	+	+[b]	+	+[c]
Xylose	-	n.a.	-	-[c]
Ethanol	+	+[b]	+	+[c]
Methanol	-	-[b]	-	n.a.
Propanol	+	-[b]	+	+[c]
Yeast extract	+	+[b]	+	n.a.
L-Glutamate	+	+[b]	+	+[c]
Alanine	+	+[b]	+	+[c]
Butyrate	+	+[b]	+	+[c]
Propionate	+	-[b]	+	-[c]
Fumarate	+	+[b]	+	+[c]
Pyruvate	+	+[b]	+	+[c]
DL-Lactate	+	+[b]	+	+[c]
2-Oxoglutarate	+	+[b]	+	+[c]
Arginine	-	-[b]	+	+[c]

[a] Substrates tested with API strip tests 20NE (performed in aerobic conditions).
[b] Data from Stanier et al. (1966).
[c] Data from Iizuka and Kimagata (Iizuka and Komagata 1964) and from Lang et al. (2007) (data adapted from the published version).
n.a.– not available.

3.6 Alkylsulfatase activity

Alkylsulfatase activity of the isolated strains, after growth with SDS or acetate is presented in Table 5. Results of the activity measurements show that the CFE of strain SN2 presented higher activity when compared to the CFE of strain SN1, when both strains were grown in SDS. CFE of strain SN1 did not show alkylsulfatase activity when acetate was used as

growth substrate. CFE of strain SN2 still showed activity towards SDS cleavage after growth with acetate, even though this activity was twofold lower when compared to the activity of the CFE of the strain after growth with SDS.

Table 5
Specific activity of alkylsulfatase in strains SN1 and SN2 after growth with SDS and acetate. Average values ± SD.

Strain + growth substrate	SN1+SDS	SN1+Acet	SN2+SDS	SN2+Acet
Activity (nmol SO_4^{2-} mg^{-1} protein min^{-1})	2.00 ± 0.07	0	3.40 ± 0.30	1.68 ± 0.17

4. DISCUSSION

Two denitrifying SDS-degrading *Pseudomonas* sp. were isolated from activated sludge, collected at a WWTP with the A^2/O concept. Dodgson et al. (1984) enriched and isolated bacteria that grow with SDS and nitrate, but their physiological and phylogenetic properties were not studied further. Results of SDS biodegradation studies with strain SN1 were similar to those found by Dodgson et al. (1984) where stoichiometric amounts of N_2 were formed from nitrate. Although initially only NO_3^-, NO_2^- and N_2 were measured in our experiments, additional experiments showed the presence of N_2O in cultures of strain SN2 in the stationary phase. N_2O accumulation can explain the deficit in the nitrogen balances for this strain. N_2O was not detected during growth of strain SN1 with SDS in anoxic conditions.

Although results did not show a broad utilization of surfactants by both isolates, indications for degradation of other pollutants were found. Similarly to *P. stutzeri* strain ZN6, dodecylamine was toxic to our two strains. Strain ZN6 grew well up to 0.5 mM hexylamine, but was inhibited by the presence of 0.1 mM dodecylamine (Nguyen et al. 2008).

Thus far, besides *P. nitroreducens* type strain DSM 14399^T and strain SN2 only a few other *P. nitroreducens* strains have been tested for the use of SDS (Owen et al. 1997; Asok and Jisha 2012). Therefore it is not clear yet if SDS degradation is a key characteristic of *P. nitroreducens*. However, our findings indicate that the ability to degrade SDS is certainly not a general property of *P. stutzeri*. It remains to be determined if *P. nitroreducens* DSM 14399^T can also grow with alkanes, but this seems likely as this strain was isolated from a

petroleum and natural gas field in Japan and was enriched with kerosene and crude oil as sources of carbon and energy (Iizuka and Komagata 1964).

Several *Pseudomonas* strains with the ability to degrade SDS aerobically have been described (Payne and Feisal 1963). *Pseudomonas* C12B which was later identified as a *P. aeruginosa* strain (Lee et al. 1995) is able to grow aerobically with SDS. It is known that the initial cleavage of alkyl sulfates is catalyzed by sulfatases. These enzymes belong to the group III sulfatases, according to a classification based on its mode of action (Gadler and Faber 2007). These sulfatases activate a water molecule to form a hydroxyl ion which performs a nucleophilic attack on the substrate. By this attack, inorganic sulfate and the correspondent alcohol are formed (Gadler and Faber 2007). Alkylsulfatases produced by *Pseudomonas* C12B have been studied (Cloves et al. 1980; Shaw et al. 1980; Dodgson et al. 1984). This bacterium produces two alkylsulfatases designated P1 and P2, which hydrolyze linear primary alkyl sulfates, cleaving SDS into inorganic sulfate and 1-dodecanol. P1 is a constitutive enzyme, while synthesis of P2 is induced by primary alkyl sulfates, although induction does not seem to be limited to these compounds (Cloves et al. 1980). In our study, the alkylsulfatase activity is different in both isolated strains. Alkylsulfatase activity is inducible in strain SN1, while in strain SN2 the activity is constitutively present, but enhanced during growth with SDS. These results indicate different regulatory mechanisms in the isolated strains. The different alkylsulfatase activity of the two *Pseudomonas* strains could also explain the distinct behavior observed with increased concentrations of SDS, since strain SN2 was able to hydrolyze SDS at concentrations higher than 1 g L^{-1}, while strain SN1 could not.

In *Pseudomonas* C12B 1-dodecanol is further converted to dodecanal and lauric acid. Lauric acid is degraded by β-oxidation and partly used for lipid biosynthesis (Thomas and White 1989). The ability of both SN1 and SN2 strains to degrade 1-dodecanol, dodecanal and lauric acid as carbon and energy source in the presence of oxygen, validates the hypothesis that SDS is degraded using a similar pathway as described for *Pseudomonas* C12B. Nevertheless, dodecanal was not used for growth coupled to nitrate reduction by any of the two isolated strains. During degradation of n-alkanes by *P. chloritidismutans,* different alcohol and aldehyde dehydrogenases were expressed depending on the use of O_2 or ClO_3^- as electron acceptors (Mehboob 2010). The type of electron acceptor may also affect the type of alcohol and aldehyde dehydrogenases required for the degradation of SDS in strains SN1 and SN2. Dodecanal is a hydrophobic compound and its addition at higher concentrations than usually present during SDS degradation could cause a negative effect on the enzymatic mechanisms required for dodecanal oxidation coupled to nitrate reduction. This may be an explanation why dodecanal was degraded only with O_2.

Although agriculture represents the largest anthropogenic source of N_2O (Reay et al. 2012) its emission by biological processes in WWTP's is also subject of mitigation policies, since N_2O is an important greenhouse gas (Rodriguez-Garcia et al. 2012). Since *P. stutzeri* and *P. nitroreducens* are known denitrifiers and both species were co-enriched with SDS and nitrate, the relative contribution of these two *Pseudomonas* to complete denitrification might be relevant.

As anionic surfactants directly affect the biological activity of microorganisms due to binding to enzymes, proteins, phospholipids or by changing the hydrophobicity of the bacterial cell during its biodegradation (Cserhati et al. 2002), occurrence of bacteria with the ability to degrade detergents, such as strains SN1 and SN2, will be of importance to maintain a high activity of sludge and will influence the general microbial community composition of the sludge of WWTP´s. Further microbial ecological research is needed to study the population dynamics of denitrifying surfactant-degrading *Pseudomonas* species in WWTP´s with and without the A^2/O concept.

5. CONCLUSIONS

Pseudomonas sp. are described for the first time for its ability to use SDS coupled to nitrate reduction. *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2 were obtained from an A^2/O biological process. The two strains are physiologically different in terms of optimal pH for growth, utilization of carbon sources and accumulation of N_2O. Different regulation mechanisms may explain the differences in alkylsulfatase activity between the two strains. With this work, more insight of surfactant-degrading denitrifiers from an A^2/O process is obtained.

ACKNOWLEDGMENTS

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CHAPTER III

BACTERIAL DIVERSITY IN ANOXIC AND AEROBIC ENRICHMENTS DEGRADING SODIUM LAURYL ETHER SULFATE (SLES)

A. M. S. Paulo, R. Aydin, M. R. Dimitrov, P. A. García-Encina, A. J. M. Stams and C. M. Plugge

ABSTRACT

Sodium lauryl ether sulfate (SLES) was used as sole carbon and energy source to enrich bacteria from activated sludge at anoxic (denitrifying) and oxic conditions. Actively growing enrichments were obtained at concentrations of SLES from 50 to 1000 mg L^{-1}. The bacterial diversity of the enrichments was evaluated using Denaturant Gradient Gel Electrophoresis (DGGE). For identification of the bacterial communities present in the 50, 250 and 1000 mg SLES L^{-1} enrichments, pyrosequencing was performed. In addition, bacteria were isolated and identified from enrichments with 50, 250 and 1000 mg SLES L^{-1}. DGGE results showed a decrease in bacterial diversity with increasing SLES concentration. Pyrosequencing confirmed the change in bacterial diversity with concentration of SLES. *Pseudomonas*, *Comamonas* and *Alicycliphilus* were identified by pyrosequencing in the anoxic enrichments, while *Pseudomonas*, *Comamonas* and *Acinetobacter* were identified in the oxic enrichments. *Comamonas* were identified in the anoxic and oxic enrichments with lower SLES concentration, while *Acinetobacter* were identified in the oxic enrichments with lower SLES concentration. Mostly *Pseudomonas* were identified in the 1000 mg SLES L^{-1} enrichments. *Aeromonas hydrophila* strain S7, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 were isolated from the anoxic enrichments, while *Comamonas testosteroni* strain S13, *Acinetobacter* sp. strain S15, *Pseudomonas nitroreducens* strain S20 and *Aeromonas hydrophila* strain S21 were isolated from oxic enrichments. *Pseudomonas stutzeri* strain S8, *Pseudomonas nitroreducens* strain S11 and *Acinetobacter* sp. strain S15 grew within one day using SLES as sole carbon and energy source. *Aeromonas hydrophila* strain S7 had a longer lag phase using SLES compared to the *Pseudomonas* and *Acinetobacter* strains, while *Comamonas testosteroni* strain S13 could not grow using SLES as sole carbon and energy source.

KEYWORDS

Acinetobacter, *Aeromonas*, anionic surfactants, *Comamonas*, denitrification, detergents, nitrate, *Pseudomonas*, sodium lauryl ether sulfate.

1. INTRODUCTION

Anionic surfactants are largely used in pharmaceutical, agricultural and industrial applications. Alkyl ether sulfates (AES) are a subclass of anionic surfactants. Linear primary AES can be rapidly primary and ultimately biodegraded by mixed cultures in aerobic conditions (Griffiths et al. 1986; Swisher 1987). Sodium lauryl ether sulfate (SLES) is a mixture of linear primary AES commonly used in the formulation of several commercial products (Khleifat 2006). *Acinetobacter calcoacetiacus*, *Klebsiella oxytoca* and *Serratia odorifera* consortia (Khleifat 2006) and *Citrobacter braakii* (Dhouib et al. 2003) are able to degrade SLES in aerobic conditions. However, not much is known about the biodegradation of AES under anaerobic conditions. In a wastewater treatment plant (WWTP) the elimination of organic matter and nitrogen removal, together with phosphorus accumulation, can be performed using an anaerobic-anoxic-oxic (A^2/O) biological process (Metcalf and Eddy 2003). Both aerobic and facultative anaerobic bacteria are known to be versatile in terms of carbon sources degradation in an activated sludge process (Gerardi 2002). However, diversity of bacterial communities involved in the degradation of surfactants may differ between anoxic and oxic compartments of a WWTP with the A^2/O process, since different electron acceptors are involved. In addition, the presence of surfactants can directly affect the biological activity of microorganisms by binding to enzymes, proteins, phospholipids or by changing the hydrophobicity of the bacterial cell during its biodegradation (Cserhati et al. 2002). The toxicity of a certain surfactant depends also on the type and the physiological state of the microorganism (Glover et al. 1999; Li and Chen 2009). Surfactants have been found to significantly deteriorate biological wastewater treatment processes at high concentrations, typical for some industrial wastewaters (Liwarska-Bizukojc and Bizukojc 2006). Floc size of activated sludge decreased after contact with AES surfactants (Liwarska-Bizukojc and Bizukojc 2006). Every surfactant has a critical micelle concentration (CMC) at which the surfactant molecules start to form micelles. When applied at concentrations above the CMC many surfactants can become toxic to microorganisms in soil and water systems (Willumsen et al. 1998). In this way, increased concentrations of surfactants may give rise to a different bacterial diversity selection in anaerobic, anoxic and oxic conditions. The main objectives of this study were to identify bacteria involved in anoxic SLES degradation and compare the bacterial diversity of SLES-enriched cultures under anoxic and oxic conditions. SLES concentrations below and above the CMC (300 mg L^{-1}) were tested in both conditions. In this way, we aimed to get a better understanding of how microbial communities develop starting with the same inoculum.

2. MATERIALS AND METHODS

2.1 Enrichments

Activated sludge from a WWTP with an A^2/O biological process (Valladolid, Spain) was used as inoculum. Enrichments were performed in batch culture. The batches were prepared in 120-mL serum bottles containing 40 mL of medium. Anoxic bottles were flushed with dinitrogen gas (1.5 atm). Oxic bottles were prepared in aerobic conditions keeping air as gas phase. Bottles were sealed with butyl-rubber stoppers and crimp seals. The standard mineral salts medium contained (per liter demineralized water): 1 g KH_2PO_4, 3.48 g $Na_2HPO_4.2H_2O$, 1 g $(NH_4)_2SO_4$, 0.033 g $MgCl_2.6H_2O$, 0.0090 g $CaCl_2.2H_2O$, 0.01 g $Fe(NH_4)$ citrate. Vitamins and trace elements were added in the final concentrations as described by Holliger et al. (1993). SLES was used as sole carbon and energy source and added from a filter sterilized anoxic stock solution. Final concentrations of 50-100-250-500-1000 mg SLES L^{-1} were added to the serum bottles. For anoxic enrichments KNO_3 (10 mM) was added as electron acceptor from a sterilized stock solution. Batches were incubated statically at 30 °C and the pH was 7.3 ± 0.1. After growth, 10 % (v/v) of culture was transferred to fresh medium, always to the same SLES concentration.

2.2 Isolation, SLES degradation and growth inhibition tests

After several transfers to fresh medium, dilutions of the enriched cultures with 50, 250 and 1000 mg SLES L^{-1} were streaked on agar plates to isolate the microorganisms. Agar plates contained tryptic soy broth and 20 g L^{-1} of agar noble (BD Difco, USA). The plates were incubated at 30 °C, under air. Colonies with different morphology were selected and streaked on new plates until single colonies were obtained. The ability of each isolated strain to degrade SLES was tested by transferring the single colonies back to anoxic and or oxic conditions in batch liquid cultures, using 100 or 200 mg SLES L^{-1} as final concentration in the medium. Bacterial growth was monitored during 2 weeks of incubation. All tests were performed in duplicate. Effect on growth of increased concentrations of SLES (0 – 50 – 250 – 1000 mg SLES L^{-1}) of strains S7, S13 and S15 was studied during two weeks of incubation. Samples were taken after inoculation and after 1, 7 and 14 days of incubation. Strain S7 and strain S15 were tested using increasing concentrations of SLES as sole energy and carbon source. Strain S7 was tested with nitrate as electron acceptor and strain S15 at oxic conditions. Strain S13 was tested using acetate (10 mM) together with increased SLES concentrations at oxic conditions. The possible effect of SLES on bacterial growth was monitored using OD, nitrate, nitrite or oxygen and acetate measurements. Sulfate

accumulation in the medium was also measured. *Comamonas denitrificans* DSM 17887^{T} was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). This strain was tested for growth with SLES as sole carbon and energy source and nitrate as electron acceptor. To test if increased concentrations of SLES inhibited growth of this strain, acetate (10mM) was added together with 0 and 1000 mg SLES L^{-1}.

2.3 DNA extraction and isolates identification

Cells of each isolate were picked from single colonies, diluted in 10 µL of sterilized DNA free distilled water and lysed for 10 min at 95 °C. Lysates were stored at -20 °C. The bacterial 16S rRNA genes of lysates were amplified by Polymerase Chain Reaction (PCR) using primers 27F and 1492R (Lane 1991) and the GoTaq DNA Polymerase Kit (Promega, USA). Each PCR mixture contained: 1 µL of DNA template (lysate), 0.25 µL of Taq DNA polymerase (1.25 U), 1 µL dNTPs (0.2 µM), 1 µL of each primer (0.2 µM), 10 µL PCR buffer and 35.75 µL PCR water. The cycle conditions of the PCR were: 2 minutes of pre-denaturation at 95 °C, 25 cycles consisting of denaturation at 95 °C for 30 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 1 min, with a final 5 min elongation at 72 °C. PCR products were checked on agarose gel. PCR products were purified with DNA Clean & ConcentratorTM-5 kit (Zymo Research, USA), according to the manufacturer´s instructions, and sent for sequencing. Sequencing of the 16S rRNA was performed by BaseClear (The Netherlands). Sequences were aligned using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Closely related 16S rRNA gene sequences were identified using BLAST for homology searches (Altschul et al. 1990).

2.4 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE was used to compare the bacterial community structure of the enriched anoxic and oxic enrichments. Approximately 40-mL aliquots of well-homogenized enriched cultures were concentrated by centrifugation (10000 g, 10 min) and immediately stored at -20 °C. Genomic DNA from enriched cultures was extracted with the FastDNA® Spin kit for soil (MP Biomedicals, USA) according to the manufacturer's protocol. DNA was stored at -20 °C. PCR of partial bacterial 16S rRNA genes was performed using the primers 968-F-GC and 1401-R (Nubel et al. 1996). PCR conditions were the same as mentioned in section 2.3, except that 35 cycles were applied, an annealing temperature of 56 °C was used and the forward primer had a GC-clamp attached to its 5' end. PCR products were checked on agarose gel. PCR products were analyzed DGGE using a Dcode Universal Mutation

Detection System (Bio-Rad, Hercules, CA, USA) (Muyzer et al. 1993). DGGE was performed on polyacrylamide gels with a denaturing gradient from 30% to 60% (100% denaturing acrylamide was defined as 7 M urea and 40% (v/v) formamide). Aliquots of PCR products were loaded on the gel and electrophoresis was carried out with 0.5X Tris-acetate-EDTA buffer at 60 °C and at 85 V for 16 h. After the completion of the electrophoresis, gels were silver-stained (Sanguinetti et al. 1994) and scanned. DGGE analysis was performed using Bionumerics software version 4.61 (Applied Maths, Belgium) where a dendrogram was created using Unweighted Pair Group Analysis (UPGMA) and Pearson coefficient. The diversity of the bacterial community of each sample analyzed using DGGE was evaluated in terms of number of operational taxonomical units presents (OTUs).

2.5 454-Pyrosequencing

Bacterial diversity of selected samples (50-250-1000 mg SLES L^{-1} enriched cultures in anoxic and oxic conditions) was investigated by using 454-pyrosequencing. Barcoded amplicons covered the V1-V2 region of the bacterial 16S rRNA gene and were generated using the 27F-DegS primer and an equimolar mix of two reverse primers 338R I and II (Table 1). Forward primer was appended with the titanium sequencing adaptor A and a barcode sequence of eight nucleotides "NNNNNNNN" at its 5' end (Table 1). Barcode sequences were unique for each sample (Hamady and Knight 2009). The PCR mix (100 µl final volume) contained 20 µl of 5× HF buffer (Finnzymes, Finland), 2µl PCR Grade Nucleotide Mix (Roche Diagnostics, Germany), 1µl of Phusion hot start II High-Fidelity DNA polymerase (2U/µl) (Finnzymes, Finland), 500 nM of the reverse primer mix and forward primer (Biolegio, The Netherlands), 2 µl (i.e. 40 ng) of template and 65 µl nuclease free water. PCR was performed using the following conditions: 98°C for 30 s to activate the polymerase, 30 cycles consisting of denaturation at 98 °C for 10 s, annealing at 56 °C for 20 s, elongation at 72 °C for 20 s, and final extension at 72 °C for 10 min. PCR products (5 µl) with approximately 450 bp in length were analyzed on 1 % (w/v) agarose gel by electrophoresis, containing 1× SYBR® Safe (Invitrogen, USA). PCR products were purified from gel using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. DNA concentration of gel-purified amplicons was measured by Qubit 2.0 Fluorometer (Life Technologies, Germany). Purified PCR products were mixed in equimolar amounts and gel purified (Millipore, USA). The pooled amplicons were analyzed using an FLX genome sequencer in combination with titanium chemistry (GATC-Biotech, Germany).

Table 1
Oligonucleotides primers and sequence adapters used in 454-pyrosequencing

Primer[a]	Oligonucleotide sequence (5'-3')[b]	Reference
Adaptor A	CCATCTCATCCCTGCGTGTCTCCGACTCAG	Provided by GATC-Biotech
Adaptor B	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG	Provided by GATC-Biotech
27F-DegS	GTTYGATYMTGGCTCAG	(van den Bogert et al. 2011)
338R-I	GCWGCCTCCCGTAGGAGT	(Daims et al. 1999)
338R-II	GCWGCCACCCGTAGGTGT	(Daims et al. 1999)

[a] Primer names may not correspond to original publication; [b] M = A or C; R = A or G; W = A or T; Y = C or T

2.6 Analysis of the pyrosequencing data

Sequencing information was processed and sorted using default parameters in the QIIME pipeline version 1.6.0 (Caporaso et al. 2010). USEARCH version 5.2.236 (Edgar 2010) was used to cluster high-quality sequences into operational taxonomical units (OTUs) at 97% sequence identity. UCHIME (Edgar et al. 2011) was used for chimera removal. Taxonomy assignment of the rRNA sequences was done using the QIIME pipeline. RDP classifier (Wang et al. 2007) was used for the taxonomical assignment of the 16S rRNA sequences. Greengenes 12_10 release was used as reference database for taxonomy assignments. In order to cross-check the taxonomical classification obtained from QIIME pipeline for some of the most abundant OTUs, each selected representative OTU was matched with the GenBank nucleotide database using BLASTN (http:// ncbi.nlm.nih.gov/blast). Only results with at least 98 % maximum similarity were used. 16S rRNA sequences of the isolated strains S8, S11, S13 and S15 were compared to the more abundant OTU sequences of the same genus or species resulting from pyrosequencing results analysis. BLAST alignment tool was used to compare sequences (Altschul et al. 1990).

2.7 Analytical methods

OD at 600 nm was determined using a Hitachi U2000 UV/visible spectrophotometer (Hitachi, Japan). NO_3^-, NO_2^- and SO_4^{2-} were analyzed by suppressor mediated ion chromatography using a conductivity detector and an IonPac AS9-SC 4 x 50 mm column (Dionex, USA). The mobile phase (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$) was used at a flow rate of 1 mL min^{-1}. Mannitol was used for stabilization of the samples and sodium fluoride was used as the internal standard. The analysis was conducted at 35 °C. Acetate was analyzed by HPLC using a Varian Metacarb 67H 300 x 6.5 mm column and a SpectraSYSTEM RI-150 detector (Thermo Electron Corporation, USA). The mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.8 mL min^{-1}. The analysis was conducted at 30 °C.

Crotonate was used as internal standard. Oxygen was monitored using gas chromatography with a GC-14B (Shimadzu, Japan) equipped with a packed column Molsieve 13X 60/80 mesh 2 m x 2.4 mm (Varian, The Netherlands) and a thermal conductivity detector. The oven, injector and detector temperature were 100 °C, 90 °C and 150 °C, respectively. Argon was used as carrier gas with a flow rate of 30 mL min^{-1}.

2.8 Chemicals

Commercial SLES (M_w = 385 g mol^{-1}; 2 degrees of ethoxylation; 70 % active) with the name Marlinat 242/70 was purchased from Sasol (Germany). CMC of SLES is 0.8 mM (300 mg L^{-1}) (Aoudia et al. 2009). Other chemicals where purchased at Sigma-Aldrich (Germany).

3. RESULTS

After obtaining active enrichment cultures, DNA was extracted from each enrichment culture for DGGE and pyrosequencing analysis and bacteria were isolated from anoxic and aerobic enrichments. DGGE and pyrosequencing analysis were performed not only to study bacterial diversity differences between enrichments, but also to better understand the relevance of the isolation results. Overall results indicated possible sensibility of certain isolates to increased SLES concentration, being these isolates used in inhibition tests.

3.1 Isolated strains and SLES degradation

Five enrichment cultures were obtained for each anoxic and oxic condition, using SLES applied at different concentrations as sole carbon and energy source. Although in the aerobic enrichments growth was faster compared to the anoxic enrichments, the time difference at which exponential growth started was not of more than 1 day. The use of mineral medium with SLES as sole substrate for isolation on agar plates was tried without success, since no bacteria were able to grow on the plates. Tryptic soy broth was used instead. Three pure cultures were isolated from anoxic enrichments and designated strains S7, S8 and S11. Four strains designated S13, S15, S20 and S21 were isolated from oxic enrichments. All isolated strains were identified according to their closest related strain, with a maximum of 99% similarity based on 16S rRNA gene sequence. The 16S rRNA gene sequences of strains S7, S8, S11, S13 and S15 have been deposited in the GenBank database under accession numbers KJ152584, KJ152585, KJ152586, KJ152587 and KJ152588, respectively. Strains S7, S8, S11 and S13 have been deposited in DSMZ with

the accession numbers DSM 28624, DSM 28647, DSM 28648 and DSM 28596, respectively. Table 2 shows the identification for each isolated strain, the results for growth with SLES as sole carbon and energy source, in the presence of nitrate or oxygen and indicates from which enriched cultures these strains were isolated from.

Table 2
Identification of isolates from each condition and growth observations after transfer back to liquid medium with SLES under anoxic or oxic conditions

Condition	Strain code	Identification	Growth[1]	Enrichment (mg SLES L^{-1})[2]
Anoxic	S7	*Aeromonas hydrophila* (99%)	+/–	50, 250
	S8	*Pseudomonas stutzeri* (99%)	+	1000
	S11	*Pseudomonas nitroreducens* (99%)	+	250, 1000
Oxic	S13	*Comamonas testosteroni* (99%)	–	50
	S15	*Acinetobacter* sp. (99%)	+	50
	S20	*Pseudomonas nitroreducens* (99 %)	+	1000
	S21	*Aeromonas hydrophila* (99%)	+/–	1000

[1]Growth observations were performed using SLES as substrate, compared to controls without substrate and related only to the initial enrichment condition; – No growth; +/– Weak but visible growth; + Growth;
[2]Presence in original enrichment; Note: strains isolated in anoxic or aerobic conditions were tested in anoxic or aerobic conditions for SLES degradation, respectively.

Strains S7 and S21 were both identified as *Aeromonas hydrophila* strains and are both able to grow weakly with SLES and nitrate or oxygen as electron acceptor. Strain S8 was identified as a *Pseudomonas stutzeri* strain; it is also able to degrade SLES in both anoxic and oxic conditions. Strains S11 and S20 were both identified as *Pseudomonas nitroreducens* strains; they are able to grow with SLES in anoxic and oxic conditions. Strain S13 was identified as a *Comamonas testosteroni* strain. That strain is not able to use SLES as sole carbon and energy source. Strain S15 was identified as an *Acinetobacter* sp. strain; it is able to use SLES in oxic conditions. 16S rRNA sequences of strains S7 and S21 were 99 % similar to each other. Hence, one of the strains (S7) was selected for further studies. The 16S rRNA sequences of strains S20 and S11 were also 99 % similar to each other. Strain

S11 was selected for further studies. For all tested *Pseudomonas* strains the lag phase was larger under anoxic conditions when compared to oxic conditions.

3.2 DGGE analysis

Five enriched cultures were obtained from activated sludge, for both anoxic and oxic conditions. Figure 1 (Anoxic and Oxic) shows the bacterial diversity profiles of the enrichments and the obtained isolates for anoxic and oxic conditions.

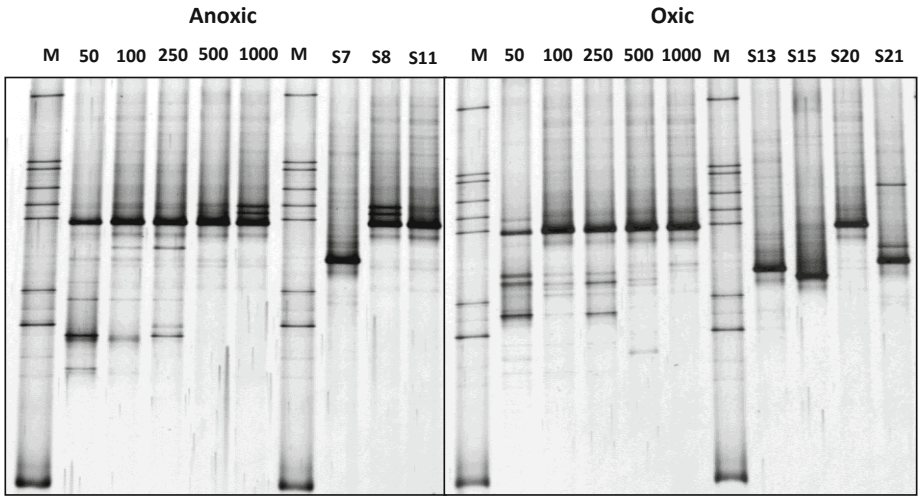

Figure 1 DGGE analysis of bacterial 16S rRNA amplicons from enriched cultures under anoxic and oxic conditions and from obtained isolates. Numbers from 50 to 1000 refer to the different SLES concentrations in mg SLES L^{-1};
M – Marker;
S7 – *Aeromonas hydrophila* strain S7;
S8 – *Pseudomonas stutzeri* strain S8;
S11 – *Pseudomonas nitroreducens* strain S11;
S13 – *Comamonas testosteroni* strain S13;
S15 – *Acinetobacter* sp. strain S15;
S20 –*Pseudomonas nitroreducens* strain S20;
S21 –*Aeromonas hydrophila* strain S21.

The number of bands in the oxic and anoxic enrichments decreased with an increase in SLES concentration. The bacterial community diversity is similar among 1000 mg SLES L^{-1} enrichments. An intense band is present at the same migration position in all the enriched cultures. This intense band points to the presence of *Pseudomonas* in all enrichments, as

can be inferred by comparison with the migration of bands of *Pseudomonas stutzeri* (S8) and *Pseudomonas nitroreducens* (S11 and S20) in the DGGE gels. When comparing the diversity of the enriched cultures using Bionumerics and the Pearson coefficient a similarity of 99.5 % was obtained between 1000 mg SLES L^{-1} anoxic enrichment and *Pseudomonas stutzeri* strain S8 (see Figure S1 from Supplementary Materials section). In the DGGE gels only vague bands at the position corresponding to *Aeromonas hydrophila* strain S7 are visible in most lanes. In Figure 1-Oxic, for enrichments with 50 and 250 mg SLES L^{-1} the presence of very close intense bands at the same migration position as for *Comamonas testosteroni* strain S13 and *Acinetobacter* sp. strain S15 are present.

3.3 454-Pyrosequencing analysis

Taxonomical classification by the QIIME pipeline of the OTUs present in the enrichments was performed. *Proteobacteria* was the only phylum detected in the anoxic enrichments, and was also dominant in the oxic enrichments. In both conditions *Pseudomonadales* and *Burkholderiales* were the dominant orders. With increase in SLES concentration the relative abundance of the *Pseudomonadales* order increased whereas *Burkholderiales* order decreased, for both oxic and anoxic conditions. The bacterial family diversity decreased with increase in SLES concentration in the enrichments. In the anoxic enrichments the *Pseudomonadales* order was mainly composed by bacteria from the *Pseudomodaceae* family, while in oxic conditions the *Pseudomonadales* order was composed by members of the *Pseudomonadaceae* and *Moraxelaceae* families. The *Burkholderiales* order was mainly composed of bacteria from the *Comamonadaceae* family. A summary of the results for relative abundance of the taxonomically classified OTUs up to the family level can be found in Table S1 (from Supplementary Materials section).

Figure 2 shows the relative abundance of identified bacteria classified in terms of order but also family, and in most of the cases, also in terms of genus, for anoxic and oxic enrichments, using the QIIME pipeline. A few bacterial species were also possible to identify. This taxonomical classification was obtained considering the complete classification of each OTU. Not all OTUs reached the same level of classification due to differences in sequence quality. The decrease in bacterial diversity with increase in SLES concentration is clearly visible, for both conditions. *Comamonas* species were detected with high relative abundance in the 50 mg SLES L^{-1} anoxic and oxic enrichments. Their relative abundance was lower in the 250 mg SLES L^{-1} enrichments, while they were absent in the 1000 mg SLES L^{-1} enrichments. *Alicycliphilus* was detected with high relative abundance only in the 50 mg SLES L^{-1} anoxic enrichment. *Acinetobacter* was detected in

the oxic enrichments with 50 mg SLES L^{-1} and 250 mg SLES L^{-1} but absent in 1000 mg SLES L^{-1} enrichment. Bacteria from the *Pseudomonadaceae* family and the *Pseudomonas* genus were identified in all the enrichments. Bacteria from the *Pseudomonas* genus increased their relative abundance from the 50 to the 1000 SLES L^{-1} enrichment. Only *Pseudomonas nitroreducens* and *Pseudomonas alcaligenes* were identified up to the species level, using the QIIME pipeline. *Pseudomonas nitroreducens* was identified in the 250 and 1000 mg SLES L^{-1} oxic enrichments, showing a dominance of these bacteria in the enriched cultures.

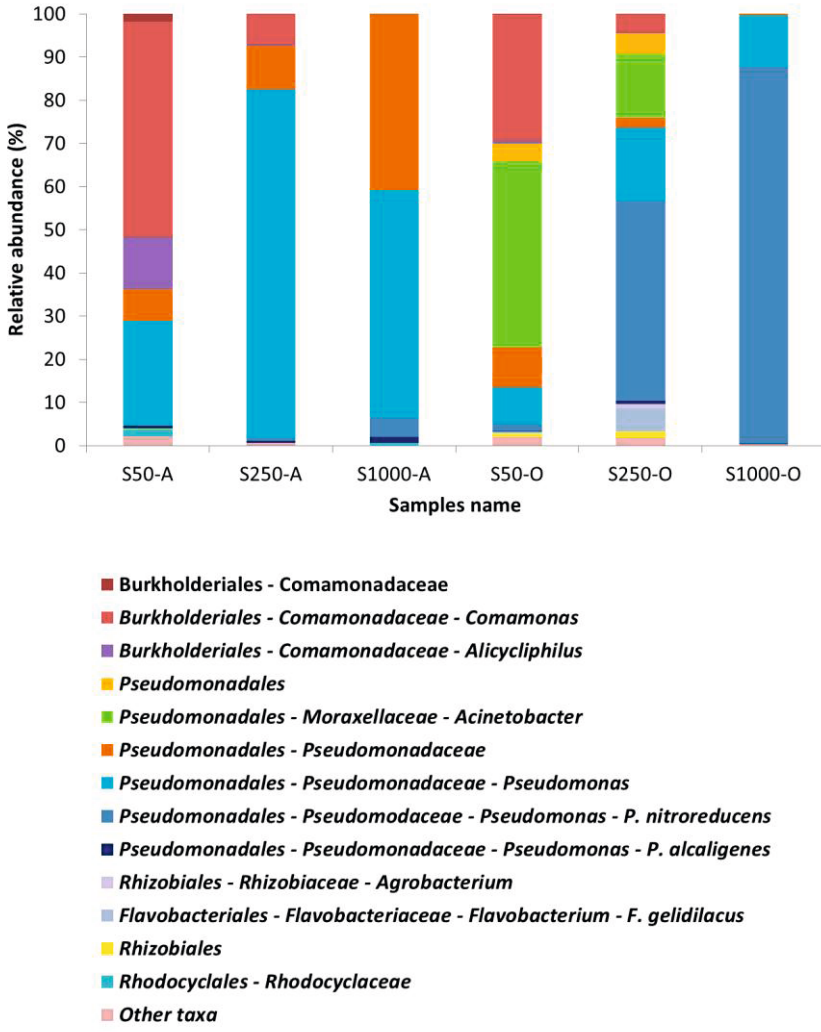

Figure 2 Relative abundance of taxa present in the 50, 250 and 1000 mg SLES L^{-1} enrichments for both conditions (A-Anoxic; O-Oxic). Taxonomy up to the species level was also possible to identify for some bacteria. Taxa with relative abundance ≤1% and with classification above the order level were included in *Other taxa*.

Table 3 presents a comparison between QIIME (Figure 2) and BLASTN taxonomical classification for dominant OTUs where BLASTN classification was used for indication of the genus but also of the bacterial species. Thus, *Pseudomonas stutzeri* and *Comamonas denitrificans* where identified by BLASTN in anoxic enrichments, while *Comamonas testosteroni* were identified in aerobic enrichments. Taxonomical classification of OTU sequences using BLASTN confirmed QIIME classification for *Pseudomonas nitroreducens* (100%), *Pseudomonas alcaligenes* (99%), Acinetobacter (99%), *Alycicliphilus* (99%) and Flavobacterium (99%).

Table 3
Comparison between taxonomical classification given by QIIME and BLASTN, for selected OTUs

QIIME[1]	BLASTN	Enrichment[2]
Pseudomonadaceae	*P. stutzeri* (98%)	1000-A
Pseudomonas	*P. stutzeri* (98%)	1000-A
Comamonas	*C. denitrificans* (99%)	50-A
Comamonas	*C. testosteroni* (99%)	50-O
Comamonas	*C. testosteroni* (99%)	250-O

[1] Complete taxonomical classification given by QIIME for selected OTUs as indicated in Figure 2;
[2] Enrichments where the OTUs were identified (Figure 2); 50-A – 50 mg SLES L^{-1} anoxic enrichment; 1000-A – 1000 mg SLES L^{-1} anoxic enrichment; 50-O – 50 mg SLES L^{-1} oxic enrichment; 250-O – 250 mg SLES L^{-1} oxic enrichment.

3.4 SLES growth inhibition tests

Aeromonas hydrophila strain S7, *Comamonas testosteroni* strain S13 and *Acinetobacter* sp. strain S15 were specifically tested for SLES growth inhibition since none of these isolated strains was identified by pyrosequencing in the 1000 mg L^{-1} enrichments (Table 4).
Comamonas denitrificans DSM 17887^T, as a close relative of dominant OTUs identified by BLASTN (Table 3), was also tested for SLES degradation and for SLES growth inhibition. *Comamonas denitrificans* DSM 17887^T could not use SLES as sole carbon and energy source. A greater lag phase occurred in the presence of acetate and 1000 mg SLES L^{-1} compared to the presence of acetate alone.

Table 4
SLES inhibition tests for strain S7, strain S13 and strain S15*

Strains	Co-substrate	mg SLES L^{-1}			
		0	**50**	**250**	**1000**
Strain S7	---	**NO_3^- (mM)**			
		0.6±0.2	1.2±0.0	5.1±0.1	7.1±0.5
		NO_2^- (mM)			
		0.4±0.1	1.0±0.1	4.7±0.4	5.6±0.6
		OD			
		0.02±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Strain S13	**Acetate**	**Acetate (mM)**			
		7.0±1.4	10.4±0.4	9.6±0.5	9.3±0.6
		OD			
		0.12±0.02	0.04±0.01	0.04±0.00	0.03±0.00
Strain S15	---	**O_2 (mmol L^{-1})**			
		0.0±0.0	0.4±0.0	5.5±0.3	12.7±0.2
		OD			
		0.01±0.00	0.05±0.00	0.09±0.00	0.07±0.00

* Shown are average values and standard deviation of duplicate cultures for NO_3^- reduced and NO_2^- accumulated by strain S7; acetate degraded by strain S13 and; O_2 used by strain S15, after two weeks of incubation. OD values were measured after one day of incubation.

Aeromonas hydrophila strain S7 reduced nitrate to nitrite, which accumulated in the medium, with all tested SLES concentrations. Nitrate reduction to nitrite increased with increasing SLES concentration. Growth of strain S7 was still very low at all tested SLES concentrations, after 1 day of incubation. Growth of *Comamonas testosteroni* strain S13 was slowest with 1000 mg SLES L^{-1} throughout the incubation (Figure 3a). Acetate degradation by strain S13 was faster in the presence of 0 and 50 mg SLES L^{-1}, after one day

of incubation (Figure 3b), although less acetate was degraded with 0 mg SLES L^{-1} at the end of the test. *Acinetobacter* sp. strain S15 presented the highest OD value with 250 mg SLES L^{-1} compared to other tested concentrations, after 1 day of incubation. For all three strains, sulfate accumulated in the medium was higher for the higher initial SLES concentration, at the end of the tests.

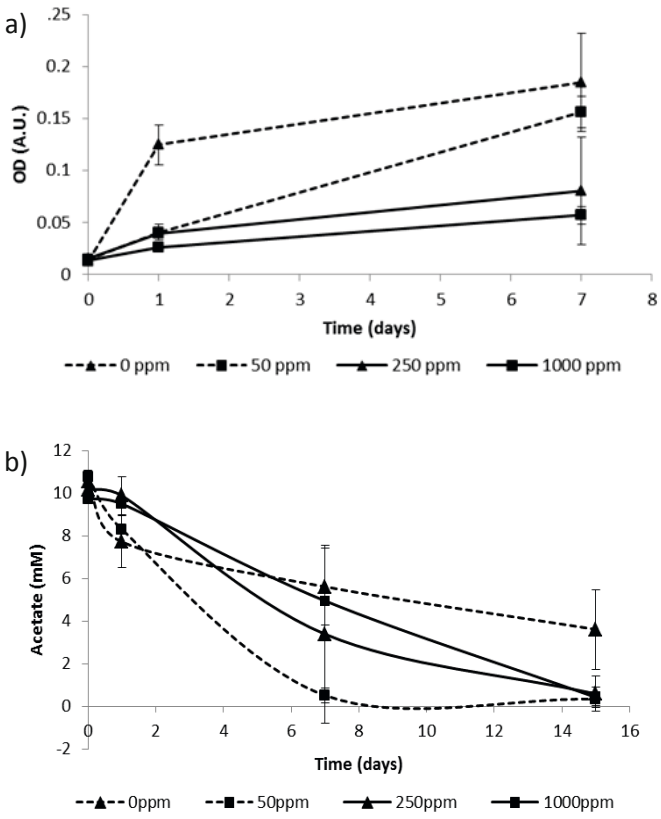

Figure 3 Inhibition of SLES on a) OD increase of *Comamonas testosteroni* strain S13 and on b) acetate degradation of *Comamonas testosteroni* strain S13.

4. DISCUSSION

Several bacterial enrichments were obtained that grow with SLES at anoxic and oxic conditions. *Aeromonas hydrophila* strains S7 and S21, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strains S11 and S20 were isolated from the anoxic enrichments. These strains can use SLES as carbon and energy source with nitrate and oxygen as electron acceptors. From the strains isolated from oxic cultures, *Acinetobacter* strain S15 is able to use SLES while *Comamonas testosteroni* strain S13 is not. *Pseudomonas*, *Comamonas* and *Acinetobacter* genera were identified in the enrichments by pyrosequencing analysis. Pyrosequencing analysis showed that all isolates, except *Aeromonas hydrophila*, were present in the enrichments where these bacteria were isolated from. The identification of *Pseudomonas stutzeri*, *Comamonas testosteroni* and *Comamonas denitrificans* using BLASTN was also obtained. Interestingly, in our studies *Aeromonas hydrophila* was isolated from several enrichments using agar plates which contained tryptic soy broth, but these bacteria were not detected in DGGE profiles or by pyrosequencing analysis. By contrast, *Alycicliphilus*, *Comamonas denitrificans* and *Flavobacterium* were identified by sequencing analysis, but not isolated using our isolation strategy.

Both DGGE and pyrosequencing results showed that diversity of the anoxic and oxic enrichments was lower with increased SLES concentration. Surfactants can cause inhibitory effects on bacteria when present between 100 and 1000 mg L^{-1} (Swisher, 1987). Such compounds negatively affect bacteria by increasing membrane permeability and disturbing the membrane function (Glover et al. 1999). At high concentration, surfactants (near or above the CMC) may form mixed micelles with the cell membrane lipids and solubilize them, leading to the necrosis and cell lysis (Li and Chen 2009). Bacterial diversity in anoxic and oxic enrichments decreased at 250 mg SLES L^{-1}, a concentration very close to the CMC of SLES (300 mg L^{-1}). The lower bacterial diversity in the 250 mg SLES L^{-1} anoxic enrichment compared to the homologous oxic enrichment indicates that more aerobic than facultative anaerobic bacteria are able to tolerate and degrade 250 mg SLES L^{-1} and/or SLES degradation products. Molecular analysis results also showed a clear difference in surfactant resistance between *Pseudomonas* and the other identified genera. *Comamonas, Alicycliphilus*, *Acinetobacter* and *Flavobacterium* seem to be more surfactant-sensitive when compared to *Pseudomonas*. However, with 50 mg SLES L^{-1} in oxic conditions, *Acinetobacter* bacteria were identified in higher relative abundance compared to *Pseudomonas*. In a previous study, *Acinetobacter* was found to better degrade a low concentration of linear alkyl benzene sulfonate (LAS) (50 mg L^{-1}) than *Pseudomonas.* However, *Pseudomonas* were found to be more resistant to the

bacteriostatic action of this surfactant (Swisher 1987). Several *Pseudomonas* sp. have been enriched and isolated using surfactants as sole carbon and energy source, even at high concentration (Swisher 1987; Jovčić et al. 2009). *Pseudomonas stutzeri* (Swisher 1987; Chaturvedi and Kumar 2011; Paulo et al. 2013) and *Pseudomonas nitroreducens* (Owen et al. 1997; Asok and Jisha 2012; Paulo et al. 2013) are known for their ability to degrade anionic surfactants. 1000 mg SLES L^{-1} was not toxic to any of our tested strains. 1000 mg SLES L^{-1} did not affect nitrate reduction by *Aeromonas hydrophila* strain S7 but delayed growth of *Acinetobacter* sp. strain S15. *Comamonas testosteroni* strain S13 growth on acetate was partially inhibited in the presence of 1000 mg SLES L^{-1}, while *Comamonas denitrificans* DSM 17887^T growth on acetate was delayed in the presence of acetate and 1000 mg SLES L^{-1}.

Acinetobacter (Swisher 1987; Khleifat 2006; Abboud et al. 2007), *Comamonas* (Taranova et al. 2004; Weiss et al. 2012), *Aeromonas* (Jimenez et al. 1991; Sacco et al. 2006) and also *Flavobacterium* (Swisher 1987; Okpokwasili and Olisa 1991; Amund et al. 1997; Sacco et al. 2006) are known to be involved in the degradation of surfactants. Yet, surfactants degradation by *Alicycliphilus* is not known.

Being *Acinetobacter* strict aerobic bacteria it explains why this genus was isolated and identified only in the aerobic enrichments. DGGE and pyrosequencing analysis together with BLASTN classification indicated that *Pseudomonas stutzeri* were most abundant in the 1000 mg SLES L^{-1} anoxic enrichment. On the other hand, pyrosequencing results show that *Pseudomonas nitroreducens* dominated the 1000 mg SLES L^{-1} oxic enrichment, although being also facultative anaerobic bacteria. The identification of two species of *Comamonas* was probably associated to different physiological abilities. *Comamonas denitrificans* strains are known to be able to denitrify, while the *Comamonas testosteroni* strains are not all able to reduce nitrate and not able to reduce nitrite to dinitrogen (Gumaelius et al. 2001). Physiological differences associated to electron acceptor utilization could explain the presence of different bacterial species in anoxic and oxic enrichments.

Pyrosequencing results confirmed in many aspects isolation and DGGE results. However, the taxonomical classification of short 16S rRNA sequences up to the species level has to be interpreted with caution. The combination of cloning and sequencing of full rRNA genes and the isolation and characterization of strains allowed us to confirm and add information about the bacterial composition of each enrichment culture. In this study insight was obtained into the bacteria involved in SLES degradation at oxic and anoxic conditions and into differences in surfactant resistance of bacteria.

5. CONCLUSIONS

SLES enrichments and bacterial isolates were obtained under oxic and anoxic conditions. Bacterial diversity of enrichments decreased with increased SLES concentration, in anoxic and oxic conditions. *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 were able to grow well by coupling SLES degradation to nitrate reduction. *Acinetobacter* sp. strain S15 was able to grow well using SLES in oxic conditions. SLES is not toxic to the isolated bacteria even at high concentrations. *Pseudomonas* resisted better than the other bacteria to increased SLES concentrations. Bacterial diversity differences associated to electron acceptor preference were found mostly between the 50 mg SLES L^{-1} oxic and anoxic enrichments.

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SUPPLEMENTARY MATERIALS

Table S1

Relative abundance of OTUs present in the enrichments with 50, 250 and 1000 mg SLES L^{-1} for both conditions (anoxic and oxic) in terms of phylum, class, order and family, after using the QIIME pipeline; obtained sequences per sample are indicated

Phylum	**Anoxic**			**Oxic**		
	50	**250**	**1000**	**50**	**250**	**1000**
Proteobacteria (%)	100.0	100.0	100.0	99.5	94.5	100.0
Bacteroidetes (%)	0.0	0.0	0.0	0.5	5.5	0.0
Class						
Flavobacteria (%)	0.0	0.0	0.0	0.5	5.5	0.0
Alphaproteobacteria (%)	0.3	0.1	0.0	1.8	3.5	0.0
Betaproteobacteria (%)	65.7	7.6	0.6	30.7	5.1	0.0
Gammaproteobacteria (%)	33.1	92.1	99.4	67.1	85.8	99.9
Other (%)	0.9	0.2	0.0	0.0	0.0	0.0
Class-Order-Family						
Flavobacteria - Flavobacteriales-Flavobacteriaceae (%)	0	0	0	0.5	5.4	0
Alphaproteobacteria-Rhizobiales-Rhizobiaceae (%)	0.1	0.0	0.0	0.8	2.1	0.0
Betaproteobacteria-Burkholderiales-Comamonadaceae (%)	64.0	7.4	0.0	30.1	4.6	0.0
Betaproteobacteria-Rhodocyclales-Rhodocyclaceae (%)	1.7	0.0	0.6	0.0	0.0	0.0
Gammaproteobacteria-Pseudomonadales-Moraxellaceae (%)	0.0	0.0	0.0	43.2	14.9	0.0
Gammaproteobacteria-Pseudomonadales-Pseudomonadaceae (%)	32.2	91.9	99.4	19.5	66.2	99.7
Less abundant (< 1%)[1]	1.9	0.6	0.0	1.0	0.7	0.3
Others (%)[2]	0.2	0.1	0.0	5.0	6.2	0.0
Sequences per sample	18254	19524	27899	19866	12313	27759

[1] Taxa with less than 1% of relative abundance were included in *Less abundant*;

[2] Taxa with classification higher than order were included in *Others*.

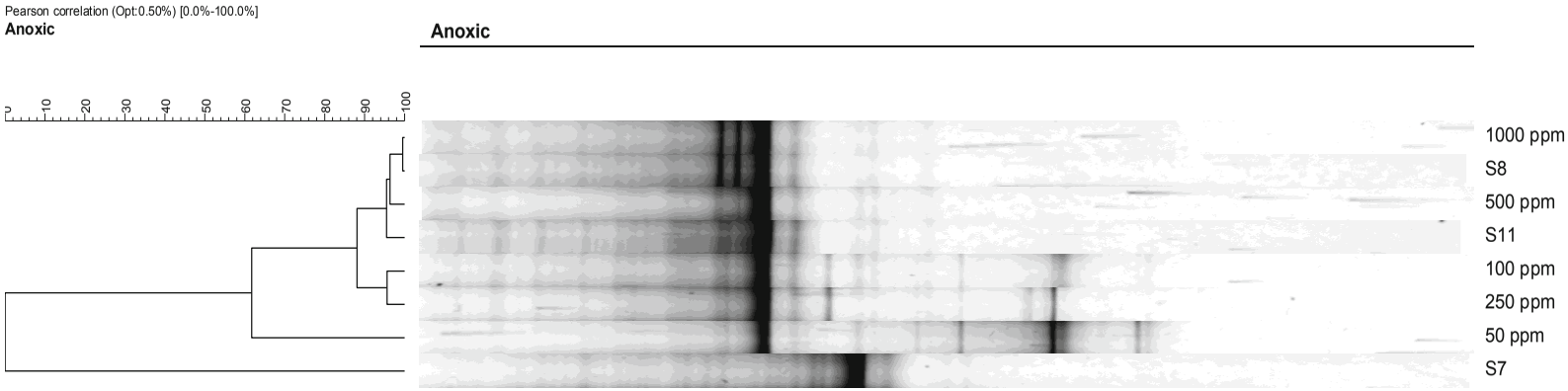

Figure S1. Dendogram of enrichment samples and isolates obtained in anoxic conditions.
Legend: 50, 100, 250, 500 and 1000 ppm – enrichment cultures with different SLES concentration; S7, S8 and S11 – Isolated strains.

CHAPTER IV

DEGRADATION OF SODIUM LAURYL ETHER SULFATE (SLES) BY FACULTATIVE ANAEROBIC BACTERIA

A. M. S. Paulo, P. A. García-Encina, C. M. Plugge and A. J. M. Stams

ABSTRACT

Alkyl ether sulfates (AES) biodegradation in aerobic conditions is a well-studied subject. However, anaerobic degradation of AES using nitrate as alternative electron acceptor is not so well known. *Aeromonas hydrophila* strain S7, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 were previously isolated from anoxic enrichments using sodium lauryl ether sulfate (SLES) as sole carbon and energy source and nitrate as electron acceptor. Strain S11 degraded SLES coupled to nitrate reduction to nitrogen gas, while strains S7 and S8 formed mainly nitrite. During growth with SLES, both strains S8 and S11 had a doubling time of 5 hours. Strain S7 grew slower with SLES presenting a doubling time of 32 hours. Both SLES primary and complete degradation were measured at the beginning of stationary growth. SLES primary degradation was higher for strain S11 (41.6±0.6 %) compared to strains S8 (29.7±2.2 %) and S7 (33.1±1.7 %). SLES complete degradation was also higher for strain S11 (29.6±1.8 %) compared to strains S8 (24.8±0.9 %) and S7 (19.4±1.2 %). Differences between SLES primary and complete degradation indicate that strains S7 and S11 produced intermediate compounds from SLES, which were not further degraded. When comparing anoxic versus aerobic degradation of SLES using strain S11, for a period of two weeks, aerobic SLES primary degradation reached 100 %, while anoxic primary degradation reached around 66 %. SLES complete degradation by strain S11 in aerobic conditions (≈ 78 %) was also higher than the anoxic complete degradation (≈ 40 %). Sulfatase activity was lower in aerobically grown cells than in cells grown anaerobically with nitrate. We show here for the first time that facultative anaerobic bacteria are able to grow by degrading SLES coupled to nitrate reduction. *Pseudomonas nitroreducens* strain S11 is an excellent aerobic and denitrifying SLES degrader.

KEYWORDS

Aeromonas hydrophila, anionic surfactants, denitrification, detergents, nitrate, *Pseudomonas nitroreducens*, *Pseudomonas stutzeri*, sodium lauryl ether sulfate.

1. INTRODUCTION

Alkyl ether sulfates (AES) are a subclass of anionic surfactants which present as general molecular formula $C_nH_{2n+1}O(C_2H_4O)_mSO_3X$, where n=10-18 and m = 0-8 and X = sodium, ammonium or triethanolamine (HERA 2003). The molecular structure of sodium dodecyl triethoxy sulfate (SDTES) is presented in Figure 1. The ether bond gives these xenobiotic surfactants a high degree of resistance to complete biological degradation, as microbial ether bond cleavage demands a considerable investment of energy (White et al. 1996). However, linear primary AES can be rapidly biodegraded aerobically (Swisher 1987). The initial aerobic degradation steps of sodium dodecyl triethoxy sulfate (SDTES) by some *Pseudomonas* strains were studied. These bacteria accumulated intermediate compounds in the medium, which could not be further degraded (Hales et al. 1982; 1986). Degradation of SDTES by these *Pseudomonas* strains takes place by ether bond cleavage although sulfatase action on the ester bond of the parent surfactant also occurs in some strains. Complete mineralization of SDTES by a mixed culture was found by Griffiths et al. (1986). Sodium lauryl ether sulfate (SLES) is commonly used in the formulation of several commercial products (Khleifat 2006). *Acinetobacter calcoacetiacus*, *Klebsiella oxytoca* and *Serratia odorifera* (Khleifat 2006) degraded SLES in bacterial consortia, while *Citrobacter braakii* degraded SLES alone (Dhouib et al. 2003), in aerobic conditions. However, not much is known about anaerobic biodegradation of AES or SLES. Early studies indicated that primary AES degradation could be merely hydrolytic involving ether cleaving enzymes designated as *etherases* (Hales et al. 1982; 1986). Nevertheless, further research lead to the idea that multi-component enzyme systems could be involved in ether cleavage by microorganisms, being some of these mechanisms dependent on the presence of oxygen (White et al. 1996). Because not much is known about alkyl ether sulfates degradation in the absence of oxygen, the main objective was to study bacteria involved in

SLES degradation under anoxic conditions, using nitrate as electron acceptor, and to compare aerobic and anoxic SLES degradation with selected SLES-degrading denitrifying bacteria.

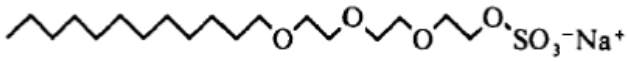

Figure 1 Molecular structure of SDTES (m=3; n=12; X = sodium).

2. MATERIALS AND METHODS

2.1 Strains

Strains used in this study were enriched and isolated with SLES as substrate and nitrate as electron acceptor (chapter III of this thesis). These include: *Aeromonas hydrophila* strain S7, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11. *Aeromonas hydrophila* DSM 30187^T and *Pseudomonas nitroreducens* DSM 14399^T were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *Pseudomonas stutzeri* CCUG 11256^T was obtained from the Culture Collection of the University of Göteborg (Sweden).

2.2 Growth and degradation tests

Growth of strains S7, S8 and S11 during SLES degradation coupled to nitrate reduction was compared. For growth curves, batches were prepared in 250-mL serum bottles and contained 80 mL of medium. Bottles were sealed with butyl-rubber stoppers and crimp seals and flushed with dinitrogen gas (1.5 atm). The standard sulfate-free mineral salts medium contained (per liter distilled water): 1 g KH_2PO_4, 3.48 g $Na_2HPO_4.2H_2O$, 0.81 g NH_4Cl, 0.033 g $MgCl_2.6H_2O$, 0.0090 g $CaCl_2.2H_2O$, 0.01 g $Fe(NH_4)$ citrate. Vitamins and trace elements were added in the final concentrations as described by Holliger et al. (1993). SLES was used as sole carbon and energy source and added from a filter sterilized anoxic stock solution, with a final concentration of about 500 mg SLES L^{-1} (1.3 mM). KNO_3 (around 30 mM) was added as electron acceptor from a sterilized stock solution. Batches were incubated at 30°C and the pH was 7.3 ± 0.1. The incubations were performed in triplicate while appropriate duplicate controls were included. In all tests, 2 % of an active bacterial

culture grown with SLES and nitrate was added as inoculum. Cultures were monitored until stationary growth phase was reached. Liquid samples were taken in time for OD, anions, anionic surfactants measurements and dissolved organic carbon (DOC) analysis.
SLES degradation tests using strain S11 in anoxic and oxic conditions were performed in triplicate. Batches were prepared using 1000-ml serum bottles containing 400 ml of medium. Batches were prepared according to the procedure above described, though oxic bottles had air in the headspace. Incubation was conducted at 30°C during 2 weeks. Liquid samples were taken in the beginning, after one day of incubation and at the end of the incubation period for anions, anionic surfactants measurements and DOC analysis. Batches were gently stirred (60 rpm) during the incubation period, avoiding foam production. Tests for comparison between the consortium of the three isolated strains and of the strains alone were performed in duplicate in 250-ml serum bottles, using 80 ml medium. Incubation was conducted at 30°C, during 3 weeks. Samples were taken at the beginning and at the end of the incubation for anions and DOC analysis. Biomass increase was monitored using optical density (OD) measurements at 600 nm. NO_3^-, NO_2^- and SO_4^{2} were also analyzed. N_2O was measured at the beginning and end of growth tests.
SLES primary degradation was monitored by quantifying the amount of anionic surfactant present in the medium at the beginning and at the end of the incubation. To test if SLES was completely or partially degraded, the DOC in the medium was determined at the end and at the beginning of the incubation. Anoxic SLES degradation coupled to nitrate reduction by the type strains was tested in 120-ml serum bottles containing 40 ml of medium. Batches were prepared as referred above, with an initial SLES concentration of 250 mg L^{-1} (0.65 mM) and 10 mM of nitrate. Incubation was performed during two weeks, at 30°C. Samples were taken at the beginning and at the end of the incubation period, for anion analysis.

2.3 Analytical methods

OD at 600 nm was determined using a Hitachi U2000 UV/visible spectrophotometer (Hitachi, Japan). Samples for DOC, anionic surfactants and anions measurements were centrifuged and filtrated using a membrane filter (0.22 µm) before analysis. Samples for DOC and anionic surfactant measurements were acidified by adding 0.5 ml of H_2SO_4 (1 M). NO_3^-, NO_2^- and SO_4^{2-} were analyzed by suppressor mediated ion chromatography using a conductivity detector and an IonPac AS9-SC 4 x 50 mm column (Dionex, USA). The mobile phase (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$) was used at a flow rate of 1 mL min^{-1}. Mannitol was used for stabilization of the samples and sodium fluoride was used as the internal standard. The analysis was conducted at 35 °C. A TOC analyzer (TOC-VCSH, Shimadzu) was

used for DOC measurements of the liquid samples. N_2O was measured using a GC (GC-2014, Shimadzu) equipped with a CP Poraplot Q column (25 m x 0.53 mm x 20 µm) and a thermal conductivity detector. SLES concentration measurements were performed using cell test kits for anionic surfactants measurement according to the manufacturer instructions (Merck, Germany). A Spectroquant Multy photometer (Merck, Germany) was used. Since surfactant concentration units were given in terms of MBAS (Methylene Blue Active Substances) (mg L^{-1}), a correspondence between concentration of SLES and MBAS in mg L^{-1} was obtained measuring the absorbance of solutions with a known concentration of SLES.

2.4 Chemicals

Commercial SLES (M_w = 385 g mol^{-1}; 2 degrees of ethoxylation; 70 % active) with the name Marlinat 242/70 was purchased from Sasol (Germany). Other chemicals where purchased at Sigma-Aldrich (Germany).

3. RESULTS

3.1 Growth, nitrate reduction and SLES degradation by strains S7, S8 and S11

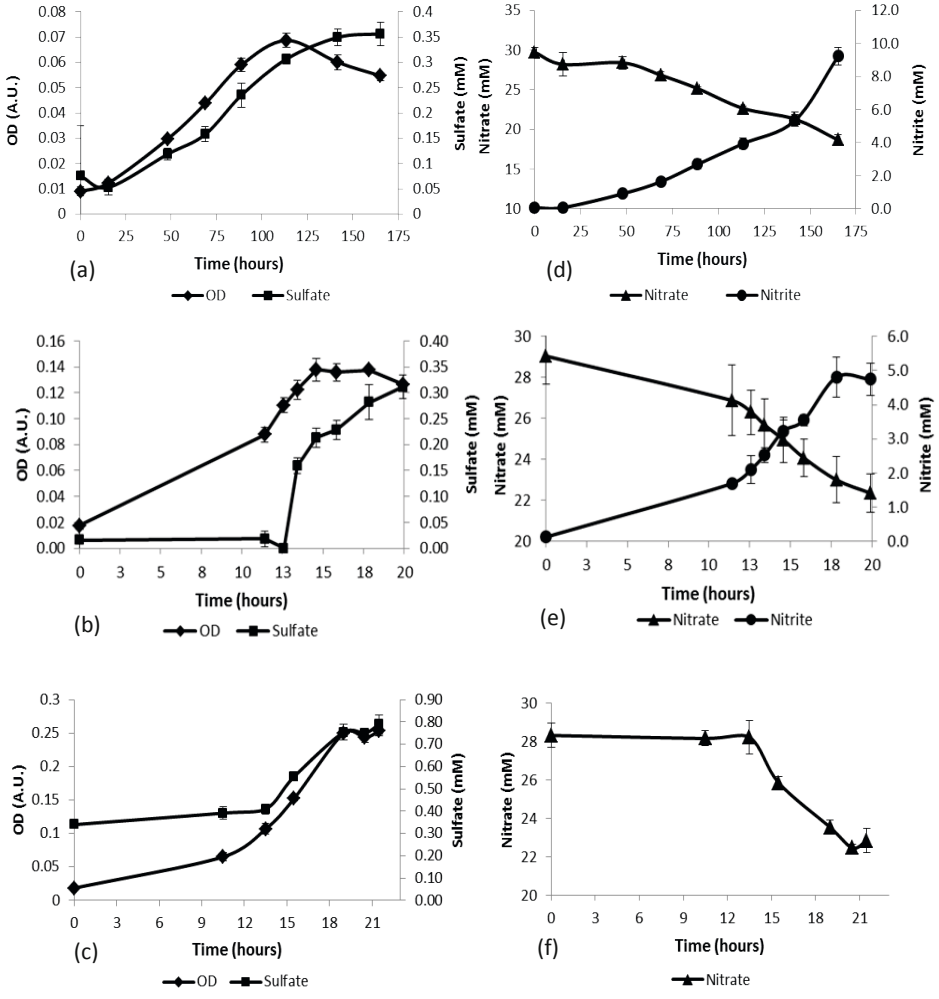

Figure 2 OD_{600} increase and sulfate accumulation during growth of strains S7 (a), S8 (b) and S11 (c); Nitrate reduction and nitrite accumulation during growth of strains S7 (d), S8 (e) and S11 (f); No accumulation of nitrite was detected during growth of strain S11. Symbols: Black lozenge - OD; Black square – Sulfate; Black triangle – nitrate; black circle – nitrite.

Table 1
Nitrate reduced, nitrite and sulfate accumulated in the medium and maximum OD for the 3 isolates

Strain	$NO_3^-{}_{red}$ (mM)	NO_2^- (mM)	SO_4^{2-} (mM)	OD_{max} (A.U.)
Aeromonas hydrophila S7	11.1±0.6	9.1±0.5	0.36±0.02	0.07±0.00
Pseudomonas stutzeri S8	6.7±0.5	4.6±0.5	0.31±0.02	0.14±0.01
Pseudomonas nitroreducens S11	5.5±1.1	0.0±0.0	0.45±0.05	0.25±0.01

Notes: $NO_3^-{}_{red}$ – Amount of nitrate reduced; NO_2^- – Nitrite accumulated in the medium; SO_4^{2-} – Sulfate accumulated in the medium.

Table 2
SLES primary and complete degradation for all 3 isolates

Strain	$SLES_{deg}$ (%)	$SLES_{deg}$ (mmol L^{-1})*	DOC_{deg} (%)
Aeromonas hydrophila strain S7	33.1±1.7	0.42±0.02	19.4±1.2
Pseudomonas stutzeri strain S8	29.7±2.2	0.33±0.03	24.8±0.9
Pseudomonas nitroreducens strain S11	41.6±0.6	0.61±0.04	29.6±1.8

Legend: $SLES_{deg}$– Percentage of SLES primary degradation; DOC_{deg} – Percentage of SLES completely degraded; * Estimated taking into account $SLES_{deg}$ and that the average molecular weight of SLES is 385 g mol^{-1}.

From Figure 2, Table 1 and 2 it can be seen that *Aeromonas hydrophila* strain S7 reduced almost all nitrate to nitrite. From the 3 isolates, strain S7 grew slower and reached lower optical densities. Strain S8 also accumulated nitrite in the medium. Strain S11 reached higher optical densities when compared to strains S7 and S8. Strain S11 did not accumulate nitrite in the medium, and reduced nitrate to nitrogen gases. In all tests, sulfate accumulation in the medium followed bacterial growth in time. Sulfate accumulation in the medium stabilized with stationary growth for both strains S8 and S11. SLES primary and complete degradation were similar for strain S8. Strains S7 and S11 presented SLES primary degradation values that were higher than SLES complete degradation values. Both strains S7 and S8 coupled SLES degradation to equimolar sulfate production, where sulfate accumulation in the medium by strain S11 was lower compared to SLES degradation (mM). N_2O formation by strain S11 accumulated in the headspace.

3.2 Anaerobic and aerobic SLES degradation by strain S11

Table 3 shows the results obtained for strain S11, which was selected for further studies, growing with SLES in anoxic (with nitrate) and aerobic conditions.

Table 3
SLES primary degradation, SLES complete degradation and sulfate accumulated by strain S11 in anoxic (nitrate reducing) and aerobic conditions, after 1 day and 14 days of incubation

	Time (days)	$SLES_{deg}$ (%)	$SLES_{deg}$ (mmol L^{-1})*	SO_4^{2-} (mM)	DOC_{deg} (%)
Anoxic, nitrate reducing	1	38.7±4.8	0.42±0.08	0.49±0.01	28.6±0.6
	14	65.6±2.8	0.71±0.09	0.82±0.04	40.7±5.2
Aerobic	1	72.4±2.3	0.80±0.02	0.62±0.03	56.4±2.5
	14	99.6±0.1	1.10±0.02	0.70±0.02	78.1±0.8

Legend: $SLES_{deg}$– Percentage of SLES primary degradation; SO_4^{2-} – Sulfate accumulated in the medium; DOC_{deg} – Percentage of SLES completely degraded; * Estimated taking into account $SLES_{deg}$ and that the average molecular weight of SLES is 385 g mol^{-1}.

After a period of two weeks, SLES concentration in the medium was close to zero in aerobic conditions. Anoxic primary degradation of SLES was not completed in the same period. According to results obtained after one day of incubation, high percentage of SLES primary and complete degradation were obtained in both conditions, but degradation was faster and more extended in the aerobic incubation. While sulfate accumulation in the medium did not increase significantly between one day and the end of the incubation in oxic conditions, it increased significantly in anoxic conditions.

3.3 Consortium of strains versus strain S11 alone

A comparison between the consortium of strains S7, S8 and S11 and the strains alone was performed in order to evaluate differences in nitrate reduction, sulfate accumulation in the medium and SLES complete degradation. Results showed that nitrite accumulation occurred in the consortium although to a lesser extent when compared to the individual nitrite accumulation of strain S7 or strain S8. Sulfate accumulation in the medium by the consortium was not significantly higher from the sulfate accumulation of any of the

individual strains. However, SLES complete degradation was higher for the consortium (85.9 ± 2.8 %) compared to the complete degradation by strain S11 alone (49.0 ± 1.6 %).

3.4 SLES degradation by type strains

Aeromonas hydrophila DSM 30187^T and *Pseudomonas stutzeri* CCUG 11256^T did not reduce nitrate in the presence of SLES and no visible growth was noted in comparison to the controls without SLES. *Pseudomonas nitroreducens* DSM 14399^T was able to use SLES, reducing nitrate to nitrite and dinitrogen gases and visible growth was observed in comparison to the controls without SLES.

4. DISCUSSION

Aeromonas hydrophila strain S7, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 were isolated from activated sludge using SLES as sole carbon and energy source and nitrate as electron acceptor (Chapter III). In this previous work, bacteria related to *Pseudomonas nitroreducens* were identified in anoxic and oxic enrichments with high relative abundance. This shows that *Pseudomonas nitroreducens* is important in SLES degradation. Indeed, *Pseudomonas nitroreducens* strain S11 presented the best performance among the tested strains in SLES primary and complete degradation, being able to reduce nitrate to dinitrogen gases. SLES degradation coupled to nitrate reduction might even be a general property of *Pseudomonas nitroreducens*, but it was not observed in *Aeromonas hydrophila* and *Pseudomonas stutzeri* type strains. Sulfate accumulation in the medium has been used previously as an indication of the sulfatase activity associated to primary and possibly complete degradation of SLES. With pure cultures sulfate was only released from initial SDTES cleavage, while mixed bacteria released sulfate mostly after complete degradation (Griffiths et al. 1986; Hales et al. 1986). Sulfate accumulated in the medium by strain S11 from the start of the different incubations, indicating the use of sulfatases in SLES degradation for both anoxic and aerobic conditions. Strain S11 showed lower sulfate accumulation compared to the estimated SLES primary degradation values, until its stationary growth, in anoxic conditions and also in aerobic conditions when incubated for longer. However, sulfate accumulation and estimated SLES primary degradation values were similar in anoxic conditions after a longer period of incubation. Sulfate required for bacterial growth, in all tests, was neglected in sulfate accumulation calculation, using SLES as carbon source. Estimation of sulfur required for bacterial growth was less than 0.05 mmol S L^{-1} (based on estimation of biomass production from carbon consumed). Therefore, when the amount of sulfate accumulated was less than the

estimated SLES degradation, this indicated the occurrence of ether cleavage besides ester cleavage performed by sulfatases. Hence, while ether and ester cleavage contributed for aerobic SLES degradation, ether cleavage was not so clear in anoxic conditions, using strain S11. Although sulfatases can cleave AES, additional ether cleavage might be necessary for bacteria to reach the alkyl chain of the surfactant (Hales et al. 1982; Hales et al. 1986). A higher accumulation of intermediate compounds after sulfatase action on SLES could be expected if ether cleaving enzymes are not available or active. The presence of alkyl sulfates together with SDTES enhanced sulfatase activity of several *Pseudomonas* strains on the initial SDTES cleavage (Hales et al. 1982; Hales et al. 1986). Comparably, sulfatase activity on SLES might have been enhanced by strain S11 due to SLES formulation. Aerobic SLES primary and complete degradation by strain S11 was better than anoxic degradation, removing all SLES from the medium in the same incubation period. However, strain S11 could still reach significant SLES degradation. Not much is known about ether cleaving enzymes and hydrolytic ether cleavage has never been clearly observed (White et al. 1996). However, our results indicate that oxygen is probably required by strain S11 for further ether bond cleavage. DOC measurements indicated a lower complete degradation compared to the primary degradation of SLES by strain S11, in both anoxic and aerobic. Therefore, DOC values measured in anoxic conditions result from the sum of not cleaved SLES and formed intermediate compounds after SLES cleavage, while only intermediate compounds (≈ 20 %) were accumulated in the medium in aerobic conditions. During a similar incubation period, four *Pseudomonas* strains were able to remove 99 % of SDTES from the medium in aerobic conditions, accumulating intermediate compounds in the medium (Hales et al. 1986). Extraction and measurement of accumulated intermediate compounds by strain S11 in both conditions was tried. Possible interference of SLES and/or biological material with the extraction procedure could explain our unsuccessful attempts. Although less effective in SLES degradation, strains S7 and S8 could both perform partial SLES cleavage coupled to nitrate reduction. Results on sulfate accumulation, Overall, SLES degradation values indicate that mostly sulfatases were used for SLES cleavage by both strains S7 and S8. While strain S7 also accumulated nitrite with acetate (data not shown) strain S8 is a complete denitrifier. Strain S7 was able to increase its nitrate reduction and nitrite accumulation with increased SLES concentration added to the medium (Chapter III). A low amount of easily degradable compounds present in 500 mg L^{-1} of SLES probably limited strains S7 and S8 growth. However, SLES complete degradation in anoxic conditions was improved by the presence of all three isolated strains. Although enzymes required for anoxic SLES degradation might be different from the ones of aerobic degradation, SLES and its intermediate compounds

are not expected to accumulate without biodegradation in the anoxic compartments of a WWTP.

5. CONCLUSIONS

We show here for the first time that facultative anaerobic bacteria are able to grow by degrading SLES coupled to nitrate reduction. Sulfatases were used by the 3 isolated strains for SLES degradation in anoxic conditions. *Pseudomonas nitroreducens* strain S11 was the best SLES-degrading bacteria in anoxic conditions. Aerobic SLES primary and complete degradation was better than anoxic degradation by strain S11. Although oxygen might be required for higher SLES degradation by strain S11 alone, a consortium of facultative anaerobic bacteria present in activated sludge is expected to completely degrade SLES.

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CHAPTER V

BEHAVIOR OF SURFACTANT-DEGRADING BACTERIA AT INCREASING ANIONIC SURFACTANT CONCENTRATIONS IN DENITRIFYING CONDITIONS

A. M. S. Paulo, H. B. Vreeling, C. M. Plugge, A. J. M. Stams and P. A. García-Encina

ABSTRACT

Sodium dodecyl sulfate (SDS) and sodium lauryl ether sulfate (SLES) are anionic surfactants, present in the composition of many commercial compounds. In contact with bacteria, these surfactants can damage bacterial cell membranes, affecting bacterial growth and activity. In this study, surfactant-degrading denitrifying bacteria were tested for their ability to reduce nitrate coupled to the degradation of high SDS and SLES concentration. Growth of *Pseudomonas stutzeri* strain SN1, *Pseudomonas nitroreducens* strain SN2, *Pseudomonas stutzeri* strain S8 and *Pseudomonas nitroreducens* strain S11 was studied with 1, 5, 10 and 20 g L^{-1} of SDS or SLES. Both *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2 were affected by SDS increased concentration. However, the growth of *P. stutzeri* strain SN1 was more affected than that of *P. nitroreducens* strain SN2. While *P. nitroreducens* strain SN2 reduced all nitrate to dinitrogen gas, *P. stutzeri* strain SN1 could not completely reduce nitrate to dinitrogen gas, accumulating nitrite in the medium, in all tests. Sulfatase activity of *P. stutzeri* strain SN1 was inhibited in all tests, after reaching a maximum concentration of sulfate in the medium close to 3.2 mM. With increasing SLES concentrations, *P. stutzeri* strain S8 growth was negatively affected while growth of *P. nitroreducens* strain S11 improved. *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 were able to completely reduce nitrate to dinitrogen gas. 1 and 10 g L^{-1} concentrations of SDS and SLES were used to perform comparative analysis of all 4 strains. *P. stutzeri* strain SN1 was similarly affected in nitrate reduction and sulfate accumulation with both SLES and SDS. *P. nitroreducens* SN2, *P. stutzeri* S8 and *P. nitroreducens* S11 behaved identically in terms of denitrification with SDS and SLES. *P. nitroreducens* strains (SN2 and S11) were more resistant to the surfactants when compared to the *P. stutzeri* strains (SN1 and S8). Nevertheless, high concentrations SDS and SLES did not inhibit growth and nitrate reduction ability of any of the tested *Pseudomonas* sp. This study helps to increase our knowledge related to highly surfactant-resistant denitrifying bacteria and how different strains are affected in growth, nitrate reduction and sulfatase activity.

KEYWORDS

Nitrate reduction, *Pseudomonas nitroreducens*, *Pseudomonas stutzeri*, sodium dodecyl sulfate, sodium lauryl ether sulfate, toxicity.

1. INTRODUCTION

Most commonly used anionic surfactants are: linear alkyl benzene sulfonates (LAS), alkyl ether sulfates (AES) and alkyl sulfates (AS) (Liwarska-Bizukojc and Bizukojc 2006). AS and AES degradation by aerobic bacteria is well described (Swisher 1987; Scott and Jones 2000). However, anionic surfactants can directly affect the biological activity of microorganisms (Cserhati et al. 2002). Sodium dodecyl sulfate (SDS) and sodium lauryl ether sulfate (SLES) are AS and AES surfactants, respectively. SDS and SLES are sulfated anionic surfactants used in the formulation of several commercial products (Sirisattha et al. 2004; EPA 2009). At low concentrations, SDS was found to be toxic to phosphate accumulating bacteria present in the activated sludge of a WWTP (Hrenovic and Ivankovic 2007). Several bacteria are able to degrade 1 g SDS L^{-1} in less than one day (Klebensberger et al. 2006; Chaturvedi and Kumar 2011), but at concentrations higher than its CMC (8.2 mM = 2.4 g L^{-1}) SDS can become inhibitory. *Pseudomonas* sp. LBBMA 101B strain was completely inhibited at a SDS concentration 4 times its CMC (Lima et al. 2011). *Klebsiella oxytoca*, able to reach its optimal growth between 1 and 2 g SDS L^{-1}, was completely inhibited with 10 g SDS L^{-1} (Shukor et al. 2009). *Acinetobacter calcoaceticus*, able to use SDS as carbon source in consortium with *Pantoea agglomerans*, was inhibited in the presence of 8 g SDS L^{-1} (Abboud et al. 2007). Studies testing high concentrations of SLES are scarcer than those with SDS. SLES was found to be non-toxic at 7 g SLES L^{-1} for bacterial consortia able to completely degrade 3 g SLES L^{-1} (Khleifat 2006). So far, *Pseudomonas* are the most frequently isolated bacteria when anionic surfactants were used as sole carbon and energy source (Swisher 1987; Chaturvedi and Kumar 2011). Some *Pseudomonas* strains were able to grow at concentrations of 10 g SDS L^{-1} (Jovčić et al. 2009) and up to 20 g SDS L^{-1} (Payne and Feisal 1963). Several *Pseudomonas stutzeri* strains can degrade SDS (Swisher 1987; Chaturvedi and Kumar 2011; Paulo et al. 2013) and can tolerate LAS up to 1 g L^{-1} (Eniola and Olayemi 2008). *Pseudomonas nitroreducens* strains are able to degrade LAS (Asok and Jisha 2012), SDS (Paulo et al. 2013) and AES (Owen et al. 1997). The average surfactant concentration for several individual classes of anionic surfactants (LAS, AES and AS) in influent wastewater can vary between 0.4 - 12 mg L^{-1} (HERA 2002, 2004, 2013). However, surfactant-based technologies use high concentrations of surfactants to enhance the cleanup of contaminated soils and aquifers, and for improving wastewater treatment. After use, these surfactants can be left in the produced water or in the subsurface environment, in concentrations closer to their CMC (Zhang et al. 1999). Surfactants degradation coupled to nitrate reduction is not well-studied and the way surfactant-degrading denitrifying bacteria can be affected by the presence of surfactants it is not known. Surfactant-resistant bacteria, with the ability to couple surfactant

degradation to nitrate reduction, can be very useful not only to degrade the surfactants arriving to a WWTP at high concentration, but also in sub-surface environments after the cleanup, both in anoxic and oxic conditions. In this study we tested sub- and supra-critical micelle concentrations of SDS (CMC of 8.2 mM = 2.4 g L^{-1}) and supra-critical micelle concentrations of SLES (CMC of 0.8 mM = 0.3 g L^{-1}) in order to investigate the effect of these on SDS/SLES-degrading bacteria, under anoxic conditions. This study allowed comparing the effect of increasing concentration of two anionic surfactants on different surfactant-degrading *Pseudomonas stutzeri* and *Pseudomonas nitroreducens* strains.

2. MATERIALS AND METHODS

2.1. Strains

Strains used in this study were previously isolated from anoxic enrichments using SDS or SLES as sole carbon and energy source. *Pseudomonas stutzeri* strain SN1 (DSM 26095) and *Pseudomonas nitroreducens* strain SN2 (DSM 26096) were enriched with SDS (Chapter II) while *Pseudomonas stutzeri* strain S8 (DSM 28647) and *Pseudomonas nitroreducens* strain S11 (DSM 28648) were enriched with SLES (Chapter III). Enrichments were performed in anoxic conditions, using nitrate as electron acceptor. The 16S rRNA gene sequences of *P. stutzeri* strains SN1 and S8 were compared to each other using the BLASTN alignment tools (http:// ncbi.nlm.nih.gov/blast). The same comparison was performed for *P. nitroreducens* SN2 and S11 strains.

2.2. Growth with SDS and SLES

Different types of tests were performed to study the effect of SDS and SLES on *Pseudomonas* strains. Preliminary tests were performed with different SDS and SLES concentrations, ranging from 1 to 40 g L^{-1} (3.5 to 138.7 mM of SDS; and 2.6 to 103.7 mM of SLES), for 5 days. Liquid and gas analyses were carried out at the beginning and at the end of the tests. Growth tests were performed in triplicate with 1, 5, 10 and 20 g L^{-1} of SDS and SLES (3.5, 17.3, 34.7, 69.4 mM of SDS; 2.6, 13.0, 26.0 and 51.9 mM of SLES), for 2 weeks. A complementary test was performed using only 1 and 10 g L^{-1} of SDS and SLES, for 2 weeks, for comparing all four strains at the same time. Batches for preliminary and complementary tests were prepared in 120-ml serum bottles and contained 50 ml of medium. For the growth experiments, 250-ml serum bottles were used, containing 100 ml of medium. Bottles were sealed with butyl-rubber stoppers and crimp seals and flushed

with nitrogen gas phase (1.5 atm). A sulfate-free standard mineral salts medium was used containing (per liter distilled water): 1 g KH_2PO_4, 3.48 g $Na_2HPO_4.2H_2O$, 0.81 g NH_4Cl, 0.033 g $MgCl_2.6H_2O$, 0.0090 g $CaCl_2.2H_2O$, 0.01 g $Fe(NH_4)$ citrate. Vitamins and trace elements were added to a final concentration as described by Holliger et al. (1993). Stock solutions of surfactant were prepared in the phosphate buffer with the same concentration as used in the standard mineral medium and added to the batches from a filter sterilized anoxic stock solution. KNO_3 (20 mM for preliminary and growth tests and 25 mM for complementary tests) was added as electron acceptor from a sterilized stock solution. Batch enrichments were incubated at 30 °C and the pH was 7.3 ± 0.1. In all experiments, 2 % of the pure cultures was added as inoculum. All incubations were performed at 30 °C. Biomass increase was monitored during the preliminary, growth experiments and at the beginning and after 30 hours in complementary tests. For preliminary and growth tests, *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2 were tested using SDS and *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 were tested using SLES. SDS degradation by SLES-degraders *P. stutzeri* S8 and *P. nitroreducens* S11 was tested. Also SLES degradation by SDS-degraders *P. stutzeri* SN1 and *P. nitroreducens* SN2 was tested. For the complementary test, two concentrations (1 and 10 g L^{-1}) of both surfactants were used as substrate for all four strains.

2.3. Analytical methods

Biomass increase was monitored using optical density (OD) measurements at 600 nm, using a Hitachi U2000 UV/visible spectrophotometer (Hitachi, Japan). NO_3^-, NO_2^- and SO_4^{2-} were analyzed by suppressor mediated ion chromatography using a conductivity detector and an IonPac AS9-SC 4 x 50 mm column (Dionex, USA). The mobile phase (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$) was used at a flow rate of 1 ml min^{-1}. Mannitol was used for stabilization of the samples and sodium fluoride was used as the internal standard. The analysis was conducted at a temperature of 35 °C. Samples used for NO_3^-, NO_2^- and SO_4^{2-} analysis, were diluted with alkaline demineralized water in order to avoid bacterial and enzymatic activity during the analysis. N_2O concentration in the headspace was measured using a GC-2014 (Shimadzu, Japan) equipped with a CP Poraplot Q column (25 m x 0.53 mm x 20 µm) and a thermal conductivity detector. The temperatures of injector, detector and oven were maintained at 60, 130 and 40 °C, respectively.

2.4. Chemicals

Commercial sodium lauryl ether sulfate (SLES) (M_w = 385 g mol^{-1}; 2 degrees of ethoxylation; 70 % active) with the name Marlinat 242/70 was purchased from Sasol (Germany). According to the manufacturer, SLES is a mixture of alkyl ether sulfate compounds, with an average number of two ethoxy units. Hence, alkyl sulfates (no ethoxy units) might be present up to 20 % in the commercial mixture. Sodium dodecyl sulfate (SDS) (99% purity) and other chemicals where purchased at Sigma-Aldrich (Germany).

3. RESULTS

3.1. Preliminary tests

P. stutzeri strain SN1 and *P. nitroreducens* strain SN2 were tested using SDS as substrate, while *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 were tested using SLES as substrate. Table 1 shows the overall denitrification and sulfate accumulation behavior with all tested SDS or SLES concentrations.

Table 1
Overall results in terms of nitrate reduction, nitrite and sulfate accumulation and N_2O formation for the four strains with all tested SDS or SLES concentrations (range between 1 and 40 g SDS/SLES L^{-1})

Strains	Surfactant	$NO_{3\ red}^{-}$ [1]	$NO_{2\ accum}^{-}$ [2]	N_2O [3]	$SO_{4\ accum}^{2-}$ (mM)
P. stutzeri strain SN1	SDS	+	+/-	-	≈ 2
P. nitroreducens strain SN2	SDS	+	-	+	≈ 8
P. stutzeri strain S8	SLES	+	+/-	-	≈ 4
P. nitroreducens strain S11	SLES	+	-	+	≈ 2

[1] Nitrate reduction with all tested SDS/SLES concentrations;
[2] Nitrite accumulation with tested SDS/SLES concentrations; +/- indicates that nitrite accumulation occurred with concentrations above 10 g L^{-1};
[3] Nitrous oxide detection in the headspace with all tested SDS/SLES concentrations;
[4] Maximum sulfate concentration accumulated with all tested SDS/SLES concentrations.

All tested strains were able to grow and reduce nitrate up to 40 g SDS/SLES L^{-1}. The *P. nitroreducens* strains SN2 and S11 could reduce all nitrate to dinitrogen gases, with all tested SDS/SLES concentrations, while *P. stutzeri* strains accumulated some nitrite with higher surfactant concentrations. *P. stutzeri* strain SN1 accumulated around 2 mM of sulfate, independently of the initial SDS concentration, while the other strains accumulated variable sulfate concentrations in the medium.

In terms of 16S rRNA gene comparison, *P. stutzeri* strains SN1 and S8 were 99% similar, and *P. nitroreducens* strains SN2 and S11 were also 99% similar.
All strains were able to use SDS or SLES as sole carbon and energy source coupled to nitrate reduction.

3.2. Growth tests

Results from the growth tests experiments using 1, 5, 10 and 20 g L^{-1} of SDS/SLES, for *P. stutzeri* strain SN1, *P. nitroreducens* strain SN2, *P. stutzeri* strain S8 and *P. nitroreducens* strain S11, are presented in Table 2.

Table 2

Nitrate reduced, nitrite and sulfate accumulated in the medium, maximum OD at the end of the experiment, and estimated SDS/SLES complete degradation by the four strains with 1, 5, 10 and 20 g L^{-1} of SDS/SLES

				SDS	
Strains		**1 g L^{-1} (3.5 mM**	**5 g L^{-1} 17.3 mM**	**10 g L^{-1} 34.7 mM**	**20 g L^{-1} 69.4 mM)**
	$NO_{3\ red}^-$ (mM)	12.0±0.4	11.1±0.3	11.8±2.5	11.8±0.2
	NO_2^- (mM)	4.8±0.3	4.1±0.6	4.6±0.3	4.8±0.5
SN1	SO_4^{2-} (mM)	2.2±0.1	2.3±0.1	1.9±0.0	2.3±0.1
	OD (A.U.)[1]	0.193±0.003	0.138±0.006	0.130±0.003	0.113±0.004
	SDS_{deg} (mg L^{-1})	261.0±7.7	247.0±6.5	255.5±75.6	255.4±4.0
	$NO_{3\ red}^-$ (mM)	18.7±0.2	18.6±0.3	18.5±0.5	18.5±0.4
	NO_2^- (mM)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
SN2	SO_4^{2-} (mM)	2.8±0.1	11.5±0.1	21.5±4.6	11.8±1.1
	OD (A.U.)[2]	0.711±0.141	0.631±0.025	0.514±0.037	0.285±0.083
	SDS_{deg} (mg L^{-1})	576.0±7.6	571.9±8.1	569.8±14.3	569.9±12.7
				SLES	
Strains		**1 g L^{-1} (2.6 mM**	**5 g L^{-1} 13.0 mM**	**10 g L^{-1} 26.0 mM**	**20 g L^{-1} 51.9 mM)**
	$NO_{3\ red}^-$ (mM)	16.7±2.5	18.0±0.4	18.7±0.4	18.9±0.3
	NO_2^- (mM)	10.9±0.2	0.0±0.0	0.0±0.0	0.0±0.0
S8	SO_4^{2-} (mM)	1.2±0.5	4.5±0.1	7.2±0.1	3.9±0.3
	OD (A.U.)[3]	0.072±0.004	0.203±0.012	0.139±0.007	0.072±0.005
	$SLES_{deg}$ (mg L^{-1})	314.2±72.0	572.7±2.7	583.5±12.1	589.8±10.1
	$NO_{3\ red}^-$ (mM)	19.1±0.1	19.3±0.6	18.6±0.3	18.8±0.9
	NO_2^- (mM)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S11	SO_4^{2-} (mM)	1.3±0.1	3.6±0.3	6.5±0.1	3.0±0.2
	OD (A.U.)[4]	0.354±0.024	0.468±0.046	0.568±0.65	0.580±0.083
	$SLES_{deg}$ (mg L^{-1})	553.1±3.7	559.6±16.0	539.6±8.4	544.2±25.5

Notes: $NO_{3\ red}^-$ – Amount of nitrate reduced; NO_2^- – Nitrite accumulated in the medium; SO_4^{2-} – Sulfate accumulated in the medium, which also indicates primary SDS/SLES degradation; [1] Maximum OD after 25 hours of experiment; [2] Maximum OD after 25 hours of experiment for 1 and 5 g SDS L^{-1} and after 14 hours for 10 and 20 g SDS L^{-1}; [3] Maximum OD after 22 hours; [4] Maximum OD after 40 hours of experiment; Complete $SLES_{deg}$ was estimated taking into account previous studies for strains growing with SDS (Chapter II) and growing with SLES (Chapter IV), using values of nitrate reduction to nitrite and to dinitrogen for estimation; Initial NO_3^- concentration was around 19 mM for all tests.

P. stutzeri strain SN1 reached its maximum OD value with 1 g SDS L^{-1}, decreasing with increase in SDS concentration (see also Figure S1 from Supplementary Materials). Nitrate was partially reduced to nitrite, which accumulated in the medium. Nitrate reduction and nitrite accumulation profile was similar for all tested concentrations, being faster with 1 and 5 g SDS L^{-1} and slower with 20 g SDS L^{-1} (see Figure S2 from Supplementary Materials). Sulfate accumulation remained stable in the medium after reaching a concentration

around 2 mM, for all tested SDS concentrations. Similar complete SDS degradation was estimated for all tested concentrations and according to this estimation, sulfate accumulation due to complete degradation should be 0.9 mM. Higher sulfate accumulation in the medium (≈ 2 mM) at the end of the test indicated faster SDS primary degradation compared to SDS complete degradation.

P. nitroreducens strain SN2 reached its highest OD with 1 g SDS L^{-1}, decreasing with increase in SDS concentration (see also Figure S3 from Supplementary Materials). Strain SN2 reduced nitrate completely (after 8 to 10 hours of incubation) and nitrite accumulated only temporarily with all tested SDS concentrations (see Figure S4 from Supplementary Materials). Sulfate accumulation increased with initial SDS concentration up to 10 g SDS L^{-1} (maximum sulfate release). SDS complete degradation was estimated for all tested concentrations, being higher for *P. nitroreducens* strain SN2 than for *P. stutzeri* strain SN1.

P. stutzeri strain S8 reached its maximum OD with 5 g SLES L^{-1}. Very similar and low maximum OD was obtained in the tests with 1 and 20 g SLES L^{-1} (see also Figure S5 from Supplementary Materials). Nitrate was completely reduced in the tests with the 5, 10 and 20 g SLES L^{-1}. Nitrite accumulation was temporary and longer for lower SLES concentration, except in the test with 1 g SLES L^{-1} (see Figure S6 from Supplementary Materials). Sulfate accumulation increased with initial SDS concentration up to 10 g SLES L^{-1} (maximum sulfate release). Except for the test with 1 g SLES L^{-1}, estimated complete SLES degradation was similar among tested SLES concentrations.

OD increased with increased SLES concentration for *P. nitroreducens* strain S11, which reached its maximum OD with 20 g SLES L^{-1} (see also Figure S7 from Supplementary Materials). Nitrate was completely reduced in all experiments with *P. nitroreducens* strain S11. Nitrate reduction was faster in the 20 g SLES L^{-1} test compared to the 1 g SLES L^{-1} test. Nitrite was completely reduced in the 1 g SLES L^{-1} test only after 330 hours of experiment (almost two weeks) (see Figure S8 from Supplementary Materials). Sulfate accumulation increased with initial SLES concentration up to 10 g SLES L^{-1} (maximum sulfate release). Estimated SLES complete degradation values obtained by for *P. nitroreducens* strain S11 were similar for all tested concentrations, being also similar to the SLES degradation values obtained with *P. stutzeri* strain S8.

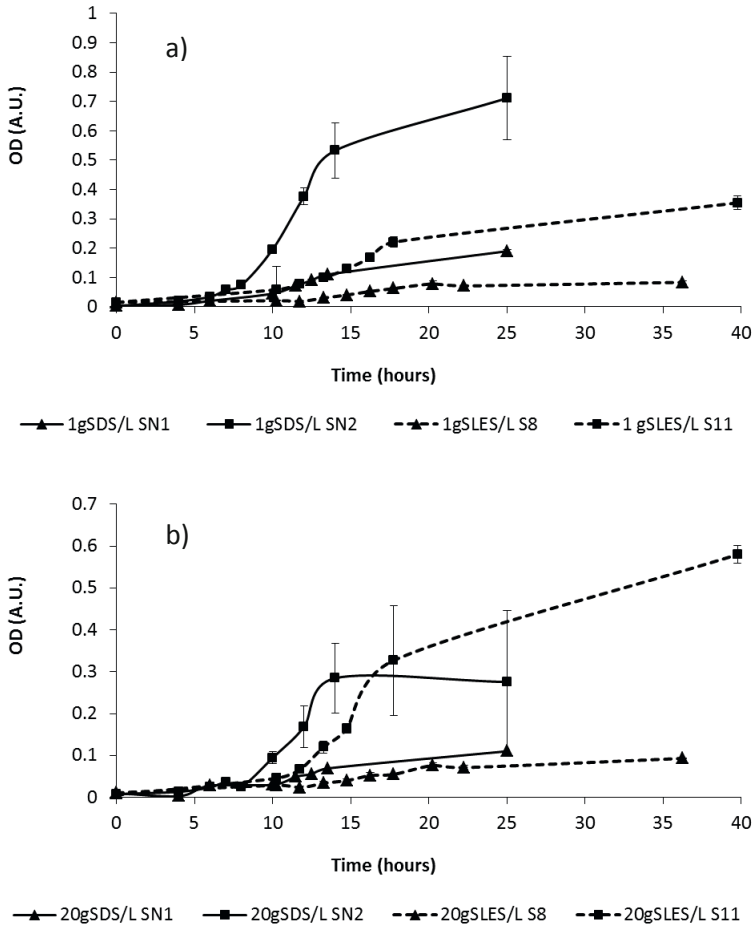

Figure 1 Time course of OD increase for *P. stutzeri* strain SN1, *P. nitroreducens* strain SN2, and *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 with a) 1 g SDS/SLES L^{-1} and b) 20 g SDS/SLES L^{-1}.

P. stutzeri strain SN1 reached a much lower OD compared to *P. nitroreducens* strain SN2 when growing with any tested SDS concentrations, as illustrated in Figures 1a and 1b with 1 and 20 g L^{-1} of SDS/SLES, respectively. For both SN1 and SN2 strains, maximum OD decreased with increase in SDS concentration. On the other hand, *P. nitroreducens* strain S11 increased its maximum OD with increase in SLES, while *P. stutzeri* strain S8 increased the maximum OD with a SLES concentration increase up to 5 g SLES L^{-1}. *P. nitroreducens* strain S11 always reached a much higher OD with SLES compared to *P. stutzeri* strain S8 (Figures 1a and 1b).

3.3. SDS and SLES comparative degradation tests with the four *Pseudomonas* strains

P. stutzeri strains SN1 and S8 and *P. nitroreducens* strains SN2 and S11 behavior was compared using both SDS and SLES as substrates, in anoxic conditions, in simultaneous tests. Table 3 shows the results for nitrate reduction, nitrite and sulfate accumulation, and OD obtained by each strain with 1 and 10 g L^{-1} of SDS and SLES.

Table 3
Nitrate reduced, nitrite and sulfate accumulated and OD in the medium for the four strains, with 1 and 10 g L^{-1} of SDS and SLES, after two weeks of incubation

Strains		SDS 1 g L^{-1} (3.5 mM	SDS 10 g L^{-1} 34.7 mM	SLES 1 g L^{-1} 2.6 mM	SLES 10 g L^{-1} 26.0 mM)
SN1	$NO_{3\ red}^-$ (mM)	12.5±0.3	11.0±0.5	13.5±0.0	18.4±0.8
	NO_2^- (mM)	2.4±0.1	1.2±0.2	4.6±0.4	6.3±0.1
	SO_4^{2-} (mM)	3.0±0.1	2.5±0.2	1.5±0.0	2.1±1.0
	OD (A.U.)	0.179±0.003	0.133±0.005	0.145±0.001	0.277±0.012
SN2	$NO_{3\ red}^-$ (mM)	21.2±0.3	22.5±0.3	17.7±0.3	22.0±0.0
	NO_2^- (mM)	0.0±0.0	0.0±0.0	3.3±0.6	0.0±0.0
	SO_4^{2-} (mM)	3.0±0.0	16.0±0.4	1.1±0.1	8.0±0.4
	OD (A.U.)	0.513±0.011	0.800±0.052	0.091±0.004	0.401±0.066
S8	$NO_{3\ red}^-$ (mM)	21.0±3.0	22.7±1.4	15.8±0.9	21.6±0.9
	NO_2^- (mM)	1.1±0.2	0.0±0.0	3.5±0.2	0.4±0.6
	SO_4^{2-} (mM)	3.5±1.9	5.8±0.1	1.2±0.0	8.8±0.6
	OD (A.U.)	0.045±0.023	0.128±0.045	0.079±0.004	0.087±0.010
S11	$NO_{3\ red}^-$ (mM)	24.5±2.2	22.3±0.6	22.6±0.6	21.7±0.7
	NO_2^- (mM)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	SO_4^{2-} (mM)	1.8±0.2	8.9±1.1	1.2±0.1	6.1±0.1
	OD (A.U.)	0.470±0.059	0.512±0.025	0.379±0.056	0.949±0.285

Notes: $NO_{3\ red}^-$ – Amount of nitrate reduced; NO_2^- – Nitrite accumulated in the medium; SO_4^{2-} – Sulfate accumulated in the medium; OD was measured after 30 hours of incubation; initial NO_3^- concentration was similar for all tests (between 21-25 mM).

P. stutzeri strain SN1 presented a similar behavior in nitrate reduction and nitrite and sulfate accumulation with SDS and SLES. The main difference was observed with 10 g SLES L^{-1}, where *P. stutzeri* strain SN1 reached a higher OD after 30 hours of incubation. *P. nitroreducens* strain SN2, *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 reduced most of the nitrate added to the medium, accumulating nitrite at the beginning of the

incubation. With 1 and 10 g SDS L^{-1} the highest OD value was obtained by *P. nitroreducens* strain SN2, while with 1 and 10 g SLES L^{-1} the highest OD value was obtained by *P. nitroreducens* strain S11. *P. stutzeri* strain S8 grew less in all tests compared to the remaining strains. With 1 g SLES L^{-1} the amount of sulfate accumulated in the medium was similar between all strains, not being higher than 1.5 mM.

Comparing *P. stutzeri* strain SN1 and *P. stutzeri* strain S8, these showed a different behavior when growing with 10 g SDS/SLES L^{-1} (see Figures S9a and S9b from Supplementary Materials). *P. stutzeri* strain SN1 reached lower nitrate and nitrite reduction with both surfactants, when compared to *P. stutzeri* strain S8. Sulfate release was higher for *P. stutzeri* strain S8 with 10 g SDS/SLES L^{-1}.

Comparing *P. nitroreducens* strain SN2 and *P. nitroreducens* strain S11, these presented different behavior mostly in sulfate accumulation in the medium when growing with 10 g SDS/SLES L^{-1}, since nitrate reduction and nitrate accumulation were similar (see Figures S10a and S10b from Supplementary Materials).

4. DISCUSSION

P. stutzeri strains SN1 and S8 and *P. nitroreducens* strains SN2 and S11 were able to grow with very high SDS or SLES concentrations. Increased SDS concentrations had visible effects on the maximum OD of both *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2, being *P. stutzeri* strain SN1 the most affected. However, when growing with 0.1 g SDS L^{-1}, *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2 reached a comparable maximum OD and presented similar nitrate and nitrite reduction (Paulo et al. 2013). Surfactants are amphiphilic molecules and their first interaction locus with any kind of cell is its membrane. Increase of the membrane permeability, associated to the loss of membrane function, is one of the several ways of action of surfactants that can contribute to bacterial death (Glover et al. 1999). A high concentration of surfactant (near of above the CMC) forms mixed micelles with the cell membrane lipids and solubilize them, leading to the necrosis and cell lysis (Li and Chen 2009). This can explain the effect of high surfactant concentrations on *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2. Yet, growth of *P. stutzeri* strain SN1 with SDS was more affected compared to *P. nitroreducens* strain SN2 even below the CMC value of SDS (2.4 g L^{-1}). Surfactants below its CMC value will be present in solution as monomers, not forming micelles. However, these monomers can still be incorporated in the cell membrane and interfere with its phospholipid bilayer, disturbing enzymatic processes or even penetrating into the cell (Li and Chen 2009). For the whole tested SDS concentrations range, *P. nitroreducens* strain SN2 presented no differences in nitrate and nitrite reduction to dinitrogen gases, while *P. stutzeri* strain SN1

accumulated nitrite in the medium in similar amounts. *P. stutzeri* strain SN1, a complete denitrifier, was unable to continue nitrate and nitrite reduction to dinitrogen gases with any of the tested concentrations, although there was still substrate and electron acceptor available. Associated to this is the similar sulfate accumulation obtained by strain SN1, with any SDS concentration. The formation of sulfate during SDS degradation can be directly associated to SDS primary degradation, since from 1 mole of SDS degraded, 1 mole of sulfate should be formed. Also, sulfate accumulation is only possible when sulfatases present in the bacteria are active, since these are responsible for SDS hydrolysis (Thomas and White 1989). Thus, sulfate accumulation in the medium was used as an indirect measurement not only of SDS degradation but also of sulfatase activity. The hydrolytic activity of sulfatases was completely inhibited in *P. stutzeri* strain SN1, reaching the same level of SDS degradation and nitrate reduction, despite the initial SDS concentration. Once sulfatase activity is inhibited, SDS cannot be further hydrolyzed to be converted into 1-dodecanol and sulfate, being 1-dodecanol formation required for bacterial growth (Thomas and White 1989). Surfactants can also interact with proteins produced by microorganisms, by altering their conformation and changing their physiological functions, modifying enzymes activity, specificity and stability (Van Hamme et al. 2006). However, according to estimations of SDS complete degradation for *P. stutzeri* strain SN1, this was lower compared to SDS primary degradation, based on sulfate accumulation in the medium. Apparently, sulfatase activity was faster than 1-dodecanol degradation by the bacteria. In fact, SDS alone did not inhibit sulfatase activity of strain SN1 growth. 1-dodecanol accumulation, due to a slow metabolism of *P. stutzeri* strain SN1, and/or its interaction with the high concentration of SDS present in the medium could have inhibited bacterial growth and sulfatase activity. 1-dodecanol can inhibit Gram-positive bacteria (Kubo et al. 1993) and Gram-negative bacteria (Kabelitz et al. 2003) due to its hydrophobicity. Long-chain alkylsulfatases are suspected to be located at the cell periphery or in the periplasmic space (Thomas et al. 1988). Hence, bacterial growth and sulfatase activity of *P. stutzeri* strain SN1 might have been inhibited by increased cell membrane hydrophobicity and effects on its fluidity. Sulfate accumulation in the medium was also used as an indication of sulfatase activity associated to the degradation of AS and AES present in the SLES commercial mixture. Depending on the degree of tolerance of sulfatases to SDS or SLES concentration, the release of sulfate can increase continuously until the exhaustion of the surfactants, even after stationary bacterial growth. This increase varied between strains and type of surfactants. Sulfate accumulation by *P. nitroreducens* strains SN2 and S11 and by *P. stutzeri* strain S8 was not completely inhibited with any tested concentration of SDS or SLES until the end of incubation, indicating that sulfatases produced by these three strains are surfactant-resistant, differently from *P.*

stutzeri strain SN1. Several proteins have been studied for their resistance to SDS (Manning and Colon 2004) and there are enzymes resistant to the presence of surfactants (Dong et al. 1997). With SLES, *P. stutzeri* strain S8 was affected by an increase in SLES concentration above 5 g L^{-1}, while *P. nitroreducens* strain S11 increased its maximum growth with higher SLES concentrations. This shows that *P. nitroreducens* strain S11 is more SLES-resistant than *P. stutzeri* strain S8. *P. nitroreducens* strain S11 was also able to use more surfactants present in SLES than *P. stutzeri* strain S8 when comparing growth, nitrate reduction and estimated SLES complete degradation with 1 g SLES L^{-1}. Besides being less resistant, *P. stutzeri* strain S8 is probably only able to reach a part of the compounds present in SLES, which is a mixture of different alkyl ether sulfate surfactants together with a small percentage of alkyl sulfates. An increased amount of SLES provides more easily degradable surfactants, which can be taken up first depending also on the enzymes produced by each bacterial strain.

Complementary tests with 1 and 10 g L^{-1} of SDS and SLES showed that all four strains were able to grow with SDS and SLES as sole carbon and energy source coupled to nitrate reduction. *P. stutzeri* strain SN1 behavior was different from those of the other three strains, showing a similar trend in anions concentrations in the medium with any SDS or SLES concentration. *P. nitroreducens* strain SN2, *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 did not show significant differences related to nitrate reduction between SDS and SLES tests. *P. stutzeri* strains are known to be genetically and phenotypically diverse inside the species (Lalucat et al. 2006), what can explain the differences in physiology between strains SN1 and S8. Comparing OD values of *P. stutzeri* strain SN1 and *P. nitroreducens* strain S11, when growing with SDS and SLES, indicates that for the same concentration (g L^{-1}) SLES could be better tolerated by these two bacteria when compared to SDS. This occurred even though *P. stutzeri* strain SN1 was enriched and isolated using SDS as initial substrate. When testing toxicity of different surfactants on *Shewanella*, SLES was found to be less toxic to these bacteria compared to SDS (Bailey et al. 2012). Hisano and Oya (2010) showed that binary mixtures of surfactants could be less toxic to aquatic organisms than the surfactants alone. However, *P. nitroreducens* strain SN2, enriched and isolated with SDS, showed to be better adapted to SDS than to SLES.

For each tested surfactant, *P. stutzeri* strains SN1 and S8 have shown to be less surfactant resistant when compared to *P. nitroreducens* strains SN2 and S11. Growth with acetate of *P. stutzeri* CCUG 11256^{T}, a strain not able to use SDS as sole carbon and energy source, was also negatively affected by increased SDS concentrations (data not shown). *P. stutzeri* strains have shown to be more susceptible than *Pseudomonas aeruginosa* to antibiotics (Russell and Mills 1974). As *P. nitroreducens* is part of the *P. aeruginosa* group, *P. nitroreducens* might share this resistance. This study shows how different facultative

anaerobic *Pseudomonas* strains, highly resistant to surfactants, grow and reduce nitrate with high surfactant concentrations.

5. CONCLUSIONS

All four tested strains are resistant to high SDS or SLES concentrations, being able to use both surfactants as sole carbon and energy source coupled to nitrate reduction. *P. stutzeri* strains (SN1 and S8) were more affected in terms of growth by high surfactant concentrations than *P. nitroreducens* strains. *P. nitroreducens* strains (SN2 and S11), proved to be highly surfactant-resistant, coupling surfactant degradation to fast nitrate reduction. Enzymes associated to surfactant cleavage, such as sulfatases, can be inhibited by compounds present in a highly concentrated surfactant medium.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

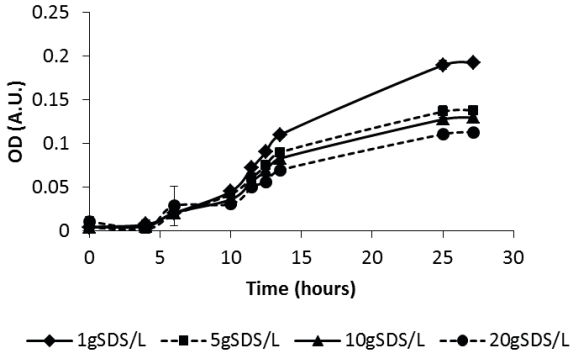

Figure S1 OD increase of *P. stutzeri* strain SN1 with 1, 5, 10 and 20 g SDS L^{-1}.

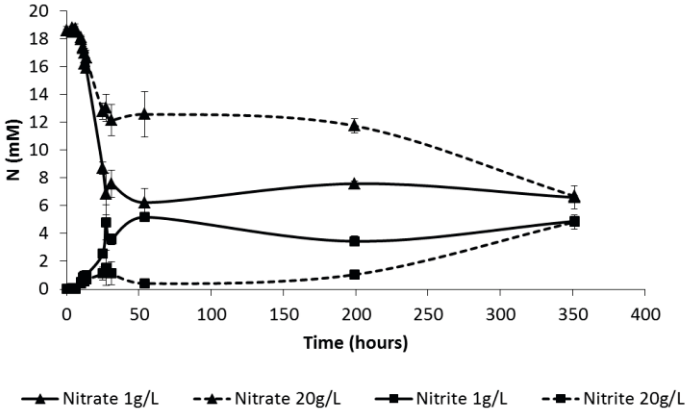

Figure S2 Nitrate reduction and nitrite accumulation of *P. stutzeri* strain SN1 with 1 and 20 g SDS L^{-1}).

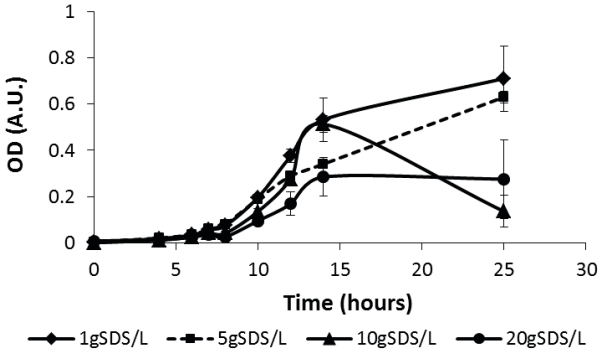

Figure S3 OD increase of *P. nitroreducens* strain SN2 with 1, 5, 10 and 20 g SDS L^{-1}.

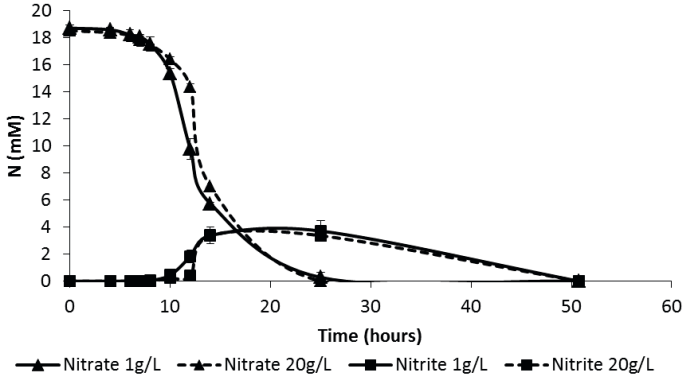

Figure S4 Nitrate reduction and nitrite accumulation of *P. nitroreducens* strain SN2 with 1 and 20 g SDS L^{-1}.

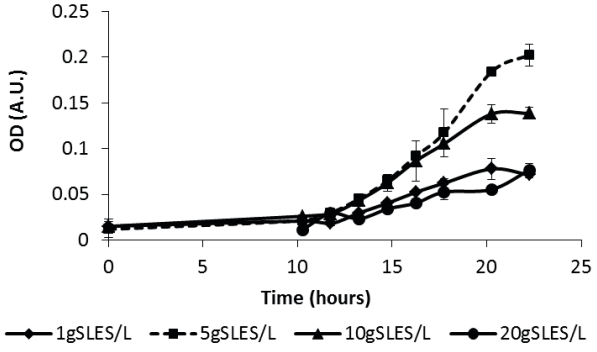

Figure S5 OD increase of *P. stutzeri* strain S8 with 1, 5, 10 and 20 g SLES L^{-1}, until reaching stationary growth.

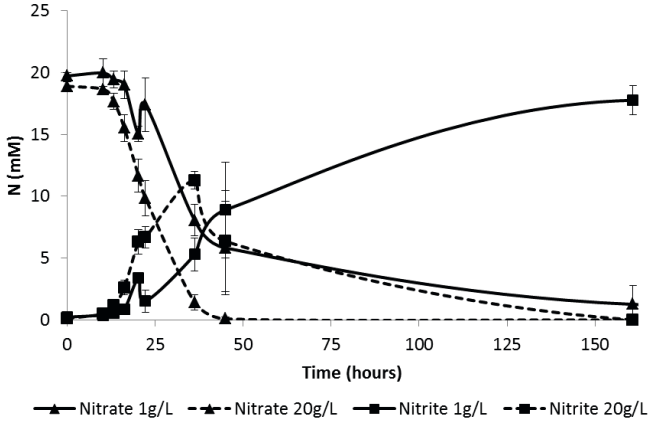

Figure S6 Nitrate reduction and nitrite accumulation of *P. stutzeri* strain S8 with 1 and 20 g SLES L^{-1}, until 160 hours of incubation of a total of almost 350 hours of incubation.

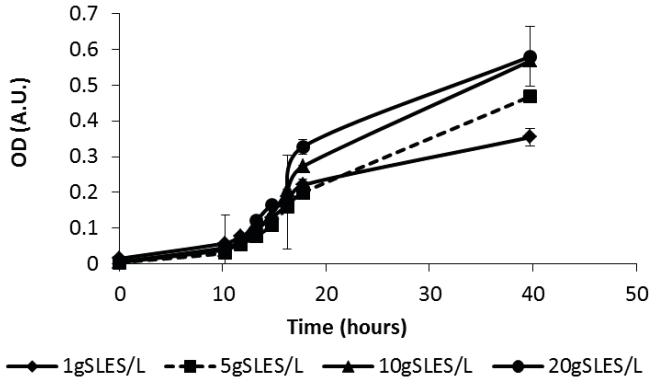

Figure S7 OD increase of *P. nitroreducens* strain S11 with 1, 5, 10 and 20 g SLES L^{-1}, until reaching stationary growth.

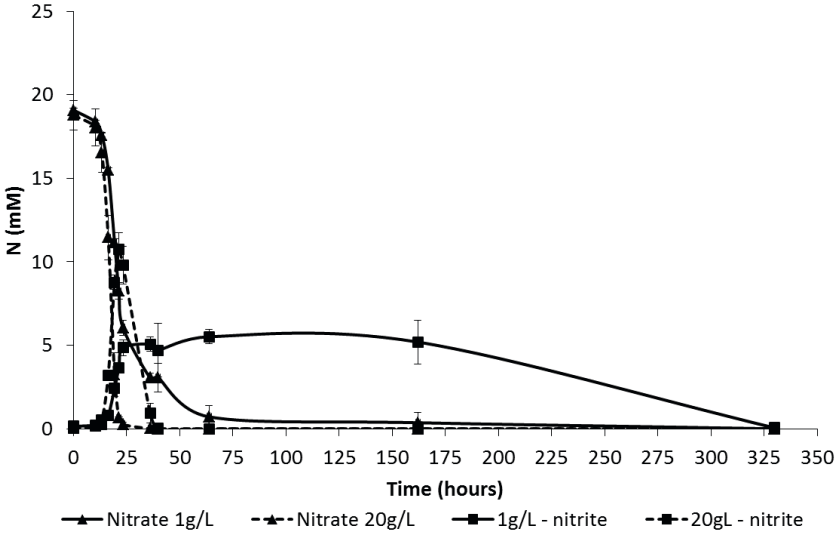

Figure S8 Nitrate reduction and nitrite accumulation of *P. nitroreducens* strain S11 with 1 and 20 g SLES L^{-1}, during around 325 hours of incubation.

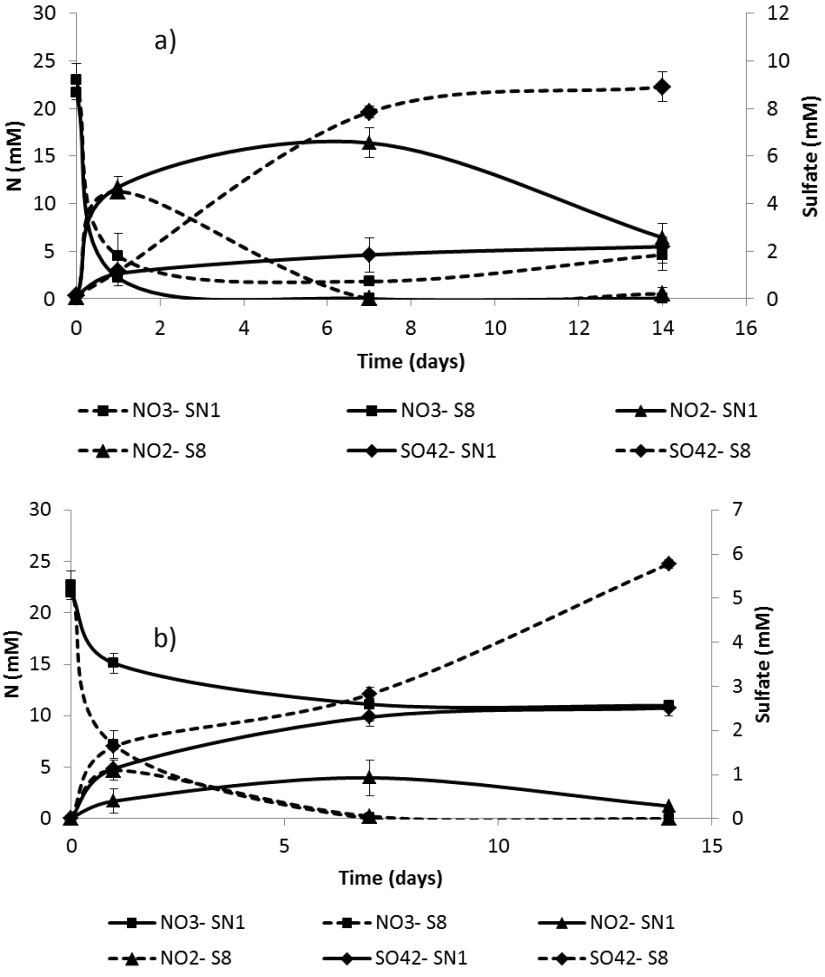

Figure S9 Comparison between *P. stutzeri* strain SN1 and *P. stutzeri* strain S8 for nitrate and nitrite reduction and sulfate accumulation, with a) 10 g SDS L^{-1} and b) 10 g SLES L^{-1}.

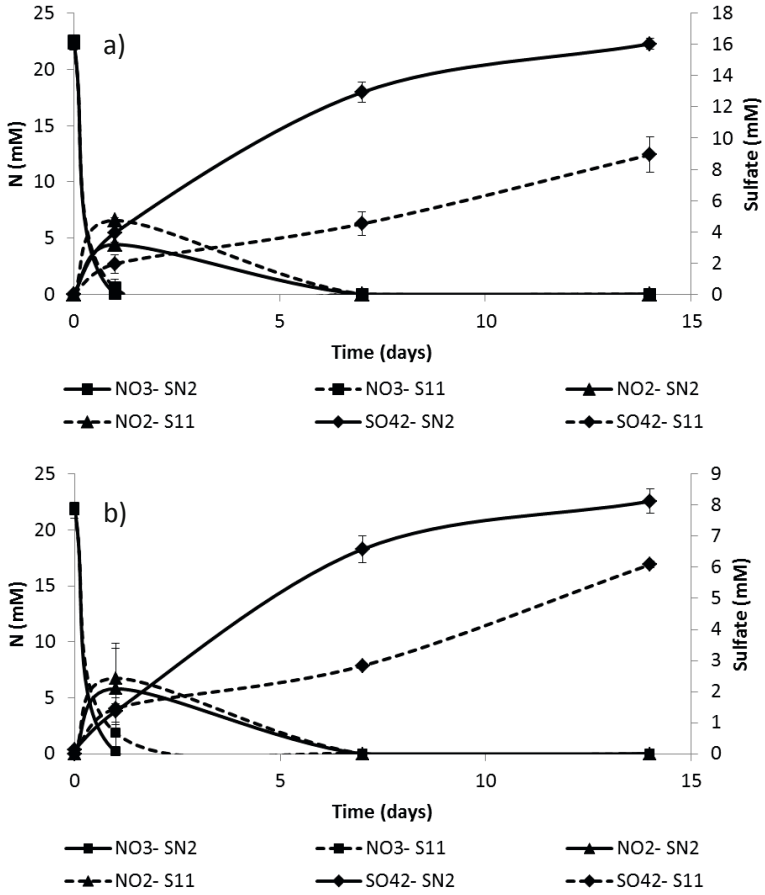

Figure S10 Comparison between *P. nitroreducens* strain SN2 and *P. nitroreducens* strain S11 for nitrate and nitrite reduction and sulfate accumulation, with a) 10 g SDS L^{-1} and b) 10 g SLES L^{-1}.

CHAPTER VI

DOMAINOME ANALYSIS OF *PSEUDOMONAS NITROREDUCENS* DSM 14399^{T}

J. J. Koehorst[◊], A. M. S. Paulo[◊], B. Nijsse, P. A. García-Encina, C. M. Plugge, A. J. M. Stams, P. J. Schaap

[◊]These authors contributed equally to this work.

ABSTRACT

The genus *Pseudomonas* includes a number of species with a vast metabolic versatility, interesting for biotechnological and environmental purposes. *Pseudomonas nitroreducens* strains have been isolated for their ability to degrade different types of pollutants, including anionic surfactants, sharing this ability with *P. nitroreducens* DSM 14399^T. In this study, the genome of *P. nitroreducens* DSM 14399^T was sequenced and compared to complete *Pseudomonas* genomes from the NCBI genome repository. Functional profiles, based on the presence or absence of protein domains, were used for establishing functional relationships between *Pseudomonas* species. This comparison validated *P. nitroreducens* DSM 14399^T and other *Pseudomonas* to the original taxonomical classifications. Functional profile comparison revealed that *P. nitroreducens* DSM 14399^T is closely related to `*P. denitrificans´* ATCC 13867, a bacterial strain initially identified as a `*P. denitrificans´* species, an invalid bacterial name. Biotechnological relevant genes coding for enzymes involved in surfactants and aromatic compounds degradation and related to polyhydroxyalkanoates (PHA) and rhamnolipid production were identified in *P. nitroreducens* DSM 14399^T. *SdsA1* hydrolase involved in SDS hydrolysis in several *P. aeruginosa* strains is also present in *P. nitroreducens* DSM 14399^T and *P. denitrificans´* ATCC 13867. The presence or absence of domains belonging to key enzymes required for substrates degradation, could be correlated with physiological data available for *P. nitroreducens* DSM 14399^T and for several other *Pseudomonas* strains. In this study we show how protein domains prediction and functional profiling can be used for studying and comparing different *Pseudomonas* species at the physiological level.

KEYWORDS

16S rRNA, domainome, genome, physiology, protein domains, *Pseudomonas nitroreducens*.

1. INTRODUCTION

Genome-enabled functional prediction has significantly changed the landscape of biological research. The revolution in computational biology made it possible to deal with the enormous amount of genomic data, enabling new discoveries beyond the realm of laboratory experiments. So far, the 16S rRNA gene has been the gold standard for the determination of evolutionary relationships and microbial diversity. 16S rRNA is universally conserved in all organisms and is considered to be only weakly affected by

horizontal gene transfer (Bodilis et al. 2012). However, there are evidences in nature and in experiments using *E. coli* that 16S rRNA gene is occasionally amenable to horizontal gene transfer (Kitahara and Miyazaki 2013). The availability of a complete genome sequence enables an in-depth investigation of what is missing and what is common at the genetic level between species, within species and even within strains of the same species. Whole genomic DNA content can be used to establish evolutionary relationships (Coenye et al. 2005). Microbial physiology depends on the microbial genotype, since it results from gene presence and activation subject to metabolic and genetic regulation in response to the environment (Liu et al. 2010). Protein domains represent the evolutionary conserved autonomously folding functional building blocks of the proteins (Bornberg-Bauer et al. 2005). The domain content of a species is highly dynamic; new building blocks are recruited by horizontal transfer while others are lost under selective pressures. Domains are also frequently duplicated and reused to build novel functions. The evolutionary dynamics of domain gain and loss, domain duplication and reuse impacts the proteome and therefore the breath of the metabolic capabilities and capacities of a specific species. With the increasing availability of physiological data in conjunction with whole genome data, classification of a species can also be performed by correlating protein domain presence with its *in vivo* physiological abilities. Such a comprehensive approach would be able to better capture the metabolic similarities and differences between strains of the same species and aids to predict common physiological capabilities of species belonging to the same genus.

Within Gram negative γ-Proteobacteria, the genus *Pseudomonas* is well studied as *Pseudomonas* species show a noticeable metabolic versatility and it includes species with serious pathogenic propensities (Palleroni 1993). *P. nitroreducens* strains are facultative anaerobic, non-pathogenic, non-fluorescent bacteria. They are able to use phenylacetate as carbon source, but are not able to liquefy gelatine, neither to use mannitol and N-acetyl-glucosamine as carbon sources, opposite to *P. aeruginosa* (Iizuka and Komagata 1964; Clark et al. 2006; Lang et al. 2007). *P. nitroreducens* is not able to use maltose but can use L-arginine as carbon source, opposite to *P. stutzeri* strains (Iizuka and Komagata 1964; Clark et al. 2006; Lang et al. 2007). *P. nitroreducens* were first discovered and described by Iizuka and Komagata (1964). The *P. nitroreducens* type strain (DSM 14399^T; IAM 1439^T; ATCC 33634^T) was isolated from an area used in petroleum and natural gas prospection, production and refinery. Initially, *P. nitroreducens* was assigned to the *P. fluorescens* group (Iizuka and Komagata 1964). Later on, Anzai et al. (1997) and Anzai et al. (2000) studied the phylogenetic affiliation of several *Pseudomonas* species by comparison of the 16S rRNA. According to these studies, *P. nitroreducens* was assigned to the *P. aeruginosa* group, and this classification is still valid today. *P. nitroreducens* is very

versatile in terms of substrate degradation. Many studies show the ability of *P. nitroreducens* to degrade compounds such as azelaic acid (Janota-Bassalik and Wright 1964), biphenyl compounds (Kohler et al. 1988), resin acids (Mohn et al. 1999), octylphenol polyethoxylates (Chen et al. 2006), isoeugenol and eugenol (Unno et al. 2007) and phenolic allelochemicals (Zhang et al. 2010). Other studies have shown that *P. nitroreducens* strains can produce polyhydroxyalkanoates (PHA) (Yao et al. 1999) and rhamnolipid (Onwosi and Odibo 2013). *P. nitroreducens* strain SN2, highly similar to *P. nitroreducens* DSM 14399^T regarding 16S rRNA gene comparison, was isolated for its ability to degrade SDS coupled to nitrate reduction, being this ability also observed in *P. nitroreducens* DSM 14399^T (Paulo et al. 2013). This indicates that the metabolic capabilities of *P. nitroreducens* are very broad and are relevant for bioremediation purposes.

The aims of this study were: to confirm phylogenetic classification of *P. nitroreducens* DSM 14399^T using functional profiling and other genomic analyses; to search for protein domains and genes encoding physiological capabilities of *P. nitroreducens* DSM 14399^T involved in surfactant and other compounds degradation; to find specificities only present in *P. nitroreducens* DSM 14399^T based on presence or absence of protein domains; and to start establishing a bottom up relationship between functional capability and capacity profiles and physiological abilities within the *Pseudomonas* genus. For this purpose, *P. nitroreducens* DSM 14399^T genome was sequenced, annotated and compared to other *Pseudomonas* spp. genomes using five different approaches: 16S RNA phylogenetic profiling, whole genome DNA comparison, standard protein comparison, functional profile comparison based on protein domain presence or absence and its comparison with physiological data.

2. MATERIALS AND METHODS

2.1 Strain, growth conditions and DNA isolation

Pseudomonas nitroreducens (DSM 14399^T) was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Growth of bacteria was performed at 30 °C on tryptic soy broth and agar (2%) plates, under aerobic conditions. DNA isolation was performed using the Zymo Research Fungal/Bacterial DNA MiniPrep™ kit. DNA quantity was measured after running 1 µl of genomic DNA with loading buffer on a 1 % (w/v) agarose gel using Quant-iT™ dsDNA Broad-Range Assay Kit (BR kit) (Invritogen, USA). Measurements were performed according to the manufacturer instructions.

2.2 Tests with surfactants

Nitrate reduction of *P. nitroreducens* DSM 14399^T with sodium dodecyl sulfate (SDS) and sodium lauryl ether sulfate (SLES) was tested. Nitrate reduction and sulfate accumulation of *P. nitroreducens* DSM 14399^T with high SDS concentrations was measured. 120-ml serum bottles were used for SDS/SLES degradation tests, containing 50 ml of medium. 250-ml serum bottles were used for high SDS concentration tests, containing 100 ml of medium. Dinitrogen gas was used a gas phase (1.5 atm). Bottles were sealed with butyl-rubber stoppers and crimp seals. The standard mineral salts medium used for SDS and SLES degradation tests contained (per liter distilled water): 1 g KH_2PO_4, 3.48 g $Na_2HPO_4.2H_2O$, 1 g $(NH_4)_2SO_4$, 0.033 g $MgCl_2.6H_2O$, 0.0090 g $CaCl_2.2H_2O$, 0.01 g $Fe(NH_4)$ citrate. A sulfate-free mineral salts medium used for high SDS concentrations tests contained (per liter distilled water): 1 g KH_2PO_4, 3.48 g $Na_2HPO_4.2H_2O$, 0.81 g NH_4Cl, 0.033 g $MgCl_2.6H_2O$, 0.0090 g $CaCl_2.2H_2O$, 0.01 g $Fe(NH_4)$ citrate. Vitamins and trace elements were added in the final concentrations as described by Holliger et al. (1993). SDS (0.35 mM) and SLES (0.26 mM), used for growth tests, and SDS (3.5 to 140 mM), used for high SDS concentration tests, were added as sole carbon and energy sources from a filter sterilized anoxic stock solution. KNO_3 (5 mM with 0.35 mM of SDS; 10 mM with 0.26 mM SLES; 20 mM with high SDS concentrations) was added as electron acceptor from a sterilized stock solution. Batch flasks were incubated, during a week, at 30 °C and the pH was 7.3 ± 0.1. Control batch tests without substrate were also prepared and analyzed. NO_3^-, NO_2^- and SO_4^{2-} were quantified by suppressor mediated ion chromatography using a conductivity detector and an IonPac AS9-SC 4 x 50 mm column (Dionex, USA). The mobile phase (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$) was used at a flow rate of 1 ml min^{-1}. Mannitol was used for stabilization of the samples and sodium fluoride was used as the internal standard. The analysis was conducted at a temperature of 35 °C. Samples used for NO_3^- and NO_2^- analysis, were diluted with alkaline demineralized water in order to avoid bacterial and enzymatic activity during the analysis.

2.3 Genome sequencing, assembly and annotation

The *P. nitroreducens* DSM 14399^T genomic library was created according to the LT TruSeq DNA sample preparation protocol (Illumina, USA). Size selection was according to standard protocol (400 – 420 bp, including 2 x 60 bp adapters). The quality of the finished library was verified using Agilent DNA 1000 Kit (Agilent, USA). The library was paired-end sequenced using the Illumina HiSeq2000 (Illumina Inc., USA) (Baseclear, The Netherlands). FASTQ sequence reads were generated using the Illumina Casava pipeline version 1.8.2.

Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing adapters and/or PhiX control signal were removed using an in-house filtering protocol, followed by a quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0 yielding 2,538 Mb with an average Phred score of 33.73. Reads were assembled using the Ray assembler (Boisvert et al. 2010). A Kmer size of 51 was selected to perform the assembly yielding a N50 of 156,007. After the assembly scaffolding was performed with SSPACE (Boetzer et al. 2011). Assembled contigs were annotated as follows: tRNA genes were predicted with tRNAscan (Lowe and Eddy 1997), and RNA genes with RNAmmer (Lagesen et al. 2007). Protein encoding genes were predicted using Prodigal (Hyatt et al. 2010). For domains prediction protein coding sequences were further annotated using InterProScan 5-RC7 (Jones et al. 2014). For EC retrieval a protein domain comparison was performed against the Swiss-Prot database (Boeckmann et al. 2003).

2.4 Phylogenetic positioning of *P. nitroreducens*

2.4.1 ***Functional profile comparison***

A universal workflow was used for functional profiling using the *P. nitroreducens* genome and 47 publically available *Pseudomonas* strains obtained from the NCBI repository as input (Figure 1). For all genomes, protein encoding regions were predicted with prodigal v2.5 (Hyatt et al. 2010), followed by a complete domainome analysis on the life science grid (SURF-SARA Amsterdam: https://surfsara.nl/) using InterProScan 5-RC7 (Jones et al. 2014). For each genome the presence or absence of each domain was scored resulting in a presence or absence matrix, building a functional profile. A distance tree based on the comparison between functional profiles was performed using an unweighted pair-group average method.

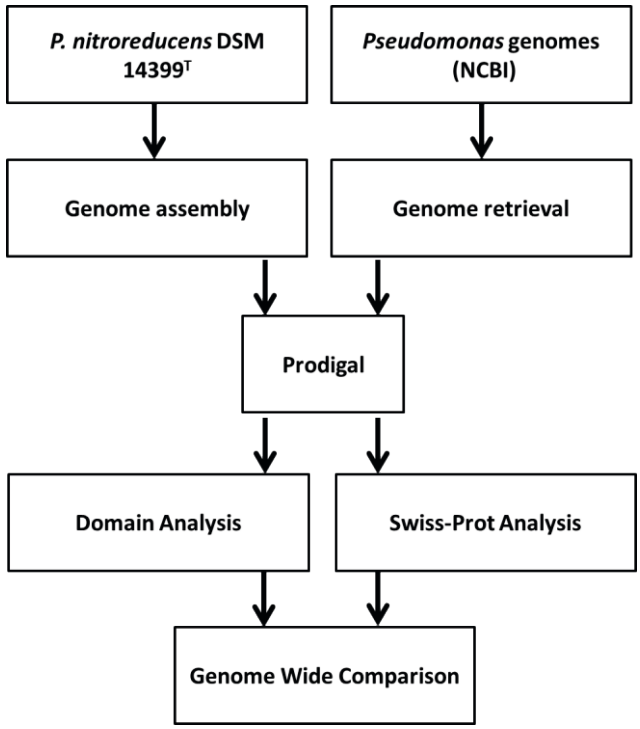

Figure 1 Overview of the universal workflow.

2.4.2 ***16S rRNA and whole genome comparison at nucleotide level***

All 16S rRNA genes were extracted from *P. nitroreducens* DSM 14399^T and from 44 *Pseudomonas* complete genomes obtained from the NCBI repository (http://www.ncbi.nlm.nih.gov/) using a ribosomal RNA prediction application (RNAmmer V1.2 (Lagesen et al. 2007)). A multiple alignment of the 16S rRNA genes obtained from the RNA prediction was performed using Clustal W 2.0 (Larkin et al. 2007). A Neighbour Joining tree of the multiple sequence alignment was performed using Clustal W 2.0 and the species tree was created using STRAW (Species TRee Analysis Web server) (Shaw et al. 2013), using the NJ_{st} method (Liu and Yu 2011). Furthermore, whole genome comparison was performed between 6 strains from the NCBI repository and the newly sequenced *P. nitroreducens* DSM 14399^T, using Pairwise Jspecies Anib analysis (Richter and Rossello-Mora 2009).

2.4.3 ***Standard protein comparison***

Single copy proteins containing a domain that occurred only once in a protein per genome in all *Pseudomonas* genomes were used for phylogenetic comparison as described by Aylward et al. (2013). Concatenated proteins were aligned using Clustal Omega (Sievers et al. 2011) and a phylogenetic tree was drawn using iTol (Letunic and Bork 2007, 2011).

2.5 Phenotypic and genotypic comparisons

Physiological data regarding general properties, substrate degradation, acid production and hydrolysis activity of *P. nitroreducens* DSM 14399^T were collected from literature (see Table S1 and Table S2 from Supplementary Materials). Physiological data obtained by Doudorof et al., (1974) for *`P. denitrificans´* ATCC 13867 were used for comparing substrate degradation between *P. nitroreducens* DSM 14399$^T$ and *`P. denitrificans´* ATCC 13867. Gene prediction for *P. nitroreducens* DSM 14399^T and *`P. denitrificans´* ATCC 13867 was performed using the Swiss-Prot database (Boeckmann et al. 2003). Only genes with a percentage of similarity ≥ 40% were considered. InterProScan 5-RC7 (Jones et al. 2014) was used for protein domains prediction. Genes and protein domains were compared between strains regarding: SDS hydrolysis, alkane sulfonates degradation, nitrate reduction, PHA production and vitamin B12 aerobic biosynthesis. *SdsA1* hydrolase from *P. aeruginosa* is involved in SDS degradation (Hagelueken et al. 2006; Jovčić et al. 2009). *SdsA1* hydrolase gene sequence from *P. aeruginosa* (PAO1, LESB58 and M18 strains) was compared to the NCBI database. *SdsA1* hydrolase gene sequence was predicted from *P. nitroreducens* DSM 14399^T genome and compared to the NCBI database. Prediction of genes encoding for enzymes presence or absence was based on prediction of protein domains from the complete domainome of selected *Pseudomonas*. A list of substrate degradation profiles was extracted from literature from different *Pseudomonas* species with complete sequenced genomes, including: *P. nitroreducens* DSM 14399^T, *`P. denitrificans´* ATCC 13867, *P. aeruginosa* PAO1, *P. syringae* pv. *phaseolicola*, *P. putida* KT2440, *P. fluorescens* SBW25 and *P. stutzeri* ATCC 17588. Substrate degradation pathways were searched using MetaCyc (http://www.metacyc.org/). Substrates were selected and enzymes required for their degradation pathways were checked if present in the genomes and information about degradation was verified and compared.

3. RESULTS

3.1 Tests with surfactants

P. nitroreducens DSM 14399^T was able to grow using low concentration of SDS and SLES as sole carbon and energy sources coupled to nitrate reduction to nitrite and to nitrogen gases. *P. nitroreducens* DSM 14399^T reduced all nitrate to nitrogen gases, without nitrite accumulation, while growing with SDS concentrations up to 139 mM (
Table **1**). Bacterial growth was observed in batch tests with SDS and SLES, opposite to batch control tests without substrate.

Table 1
Nitrate reduced, nitrite accumulated and sulfate accumulated by *P. nitroreducens* DSM 14399T when growing with SDS and SLES

	SDS (0.35 mM)	SDS (139 mM)	SLES (0.26 mM)
NO_3^- red	4.8±0.3	21.4±1.2	2.3±0.3
NO_2^- accum	3.1±1.3	0.0±0.0	1.0±0.3
SO_4^{2-} accum	*	3.5±0.2	*

* Not measured

3.2 General properties of the *P. nitroreducens* DSM 14399^T genome

Genome sequencing of *P. nitroreducens* DSM 14399^T resulted in a draft genome of 77 contigs with a total length of 6,141,600 bp, harboring 5,616 protein-encoding genes (Table 2).

Table 2
General properties of the *P. nitroreducens* DSM 14399^T genome

Attribute	**Value**
Length (bp)	6,141,600
Contigs	77
N50	156,007
G+C content (bp)	3,990,296
Nr. of predicted proteins	5,616
Nr. of predicted RNA genes	60
Total number of domains detected	27,047

3.3 The breath of a species: complete domainome profiling

Annotation of the *Pseudomonas* proteomes was performed using a universal workflow (see *Methods* section 2.4.1 for details). On average, 5,430 (±633) proteins, 24,864 (±2529) total domains of which 4,663 (±190) single domains (only occurring once), were predicted *per Pseudomonas* genome. Comparison of functional profiles of 48 *Pseudomonas* genomes suggests that the *P. nitroreducens* DSM 14399^{T} is closely related to `*P. denitrificans´* DSM 13867 (Figure 2). These two strains form a clearly separated subgroup in the *P. aeruginosa* group.

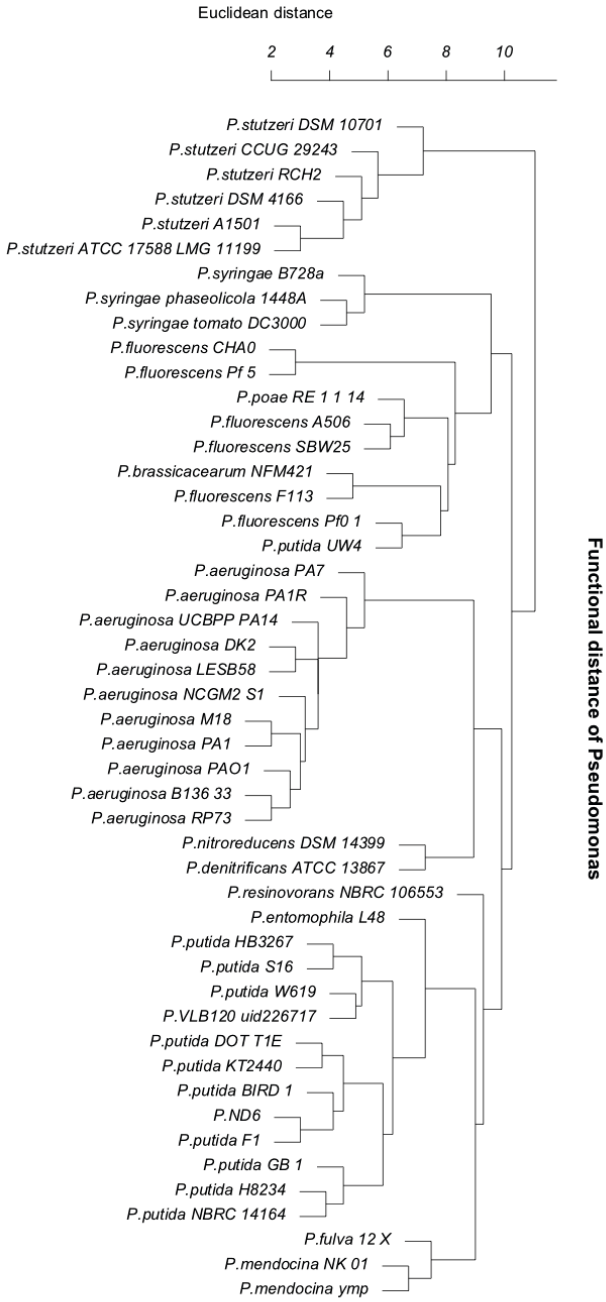

Figure 2 Distance tree resulting from the comparison between functional profiles.

An overview of total number of genes, total domains and single domains present in *P. nitroreducens* DSM 14399^T and related strains is presented in Table 3. It was revealed that the domainome core of *Pseudomonas* consists of 3,057 domains. 32 Domains were identified to be specific for *P. nitroreducens* DSM 14399^T such as chorismate synthase (see Table S4 in Supplementary Materials). Chorismate is used in the biosynthesis of aromatic compounds such as aromatic amino acids (e.g. tyrosine, phenylalanine and tryptophan) (Kitzing et al. 2004). In total, 4,268 single domains were shared by *P. nitroreducens* and `*P. denitrificans´*. Only 2 domains revealed to be specific for both strains in comparison to the *Pseudomonas* group: D-cysteine desulfhydrase (IPR005966) and Glutamyl-tRNA (Gln) amidotransferase subunit D (IPR011878). D-cysteine desulfhydrase is used for catalyzing the reaction of D-cysteine into pyruvate, H_2S and NH_3, being its physiological role unknown. However, it is hypothesized that this enzyme can be used for detoxification purposes in *E. coli* (Soutourina et al. 2001). Glutamyl-tRNA(Gln) amidotransferases are necessary for the correct formation of L-glutaminyl-tRNA^{gln} in Gram-positive bacteria (Curnow et al. 1997).

Table 3
Total number of genes, domains and single domains predicted for *P. nitroreducens* DSM 14399^T and related *Pseudomonas* genomes

Strains	Genes	NCBI	Total domains	Single domains
P. nitroreducens DSM 14399	5,616		27,047	4,689
P. denitrificans ATCC 13867	5,061	5,108	23,799	4,505
P. aeruginosa PAO1	5,681	5,616	26,675	4,851
P. resinovorans NBRC 106553	5,770	5,930	27,305	4,578
Total of *Pseudomonas*	255,727		1,338,246	7,655

Legend: Genes – identified using prodigal via the universal workflow; NCBI – the amount of genes predicted in the original strains as published in the NCBI database; Total domains – the total amount of domains found in the proteome of selected strains, considering repeated domains; Single domains – the total amount of non-repeated domains found in the proteome of the selected strains.

3.4 Phylogenetic positioning of *P. nitroreducens* DSM 14399^T via 16S rRNA genes, whole genome and standard protein comparison

The 16S rRNA phylogenetic tree (see Figure S1 from Supplementary Materials) shows that *P. nitroreducens* DSM 14399^T is closely related to `*P. denitrificans´* ATCC 13867. *P. nitroreducens* DSM 14399^T is also closely related to *P. resinovorans* NBRC 106553. The *P. nitroreducens* DSM 14399^T genome sequence was compared with other *Pseudomonas* genomes at DNA level (Figure 3). Results obtained from the whole genome comparison based on DNA similarity show that *P. nitroreducens* DSM 14399^T was found to be the closest related to `*P. denitrificans´* ATCC 13867. Both strains were closely related to *P.*

aeruginosa PAO1. *P. fulva* 12X clustered together with the two *P. mendocina* strains, using whole genome comparison.

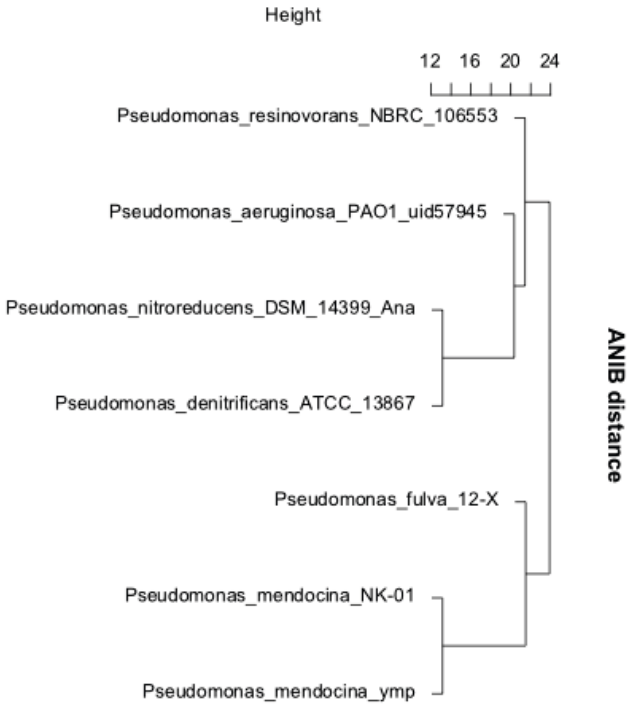

Figure 3 Whole genome sequence comparison at DNA level between pair of genomes was determined using Pairwise Jspecies Anib analyses.

3.5 Standard protein comparison

According to the alignment profiles shown in Figure 4, *P. nitroreducens* DSM 14399^T is closely related to `*P. denitrificans*´ ATCC 13867 and to *P. aeruginosa* strains.

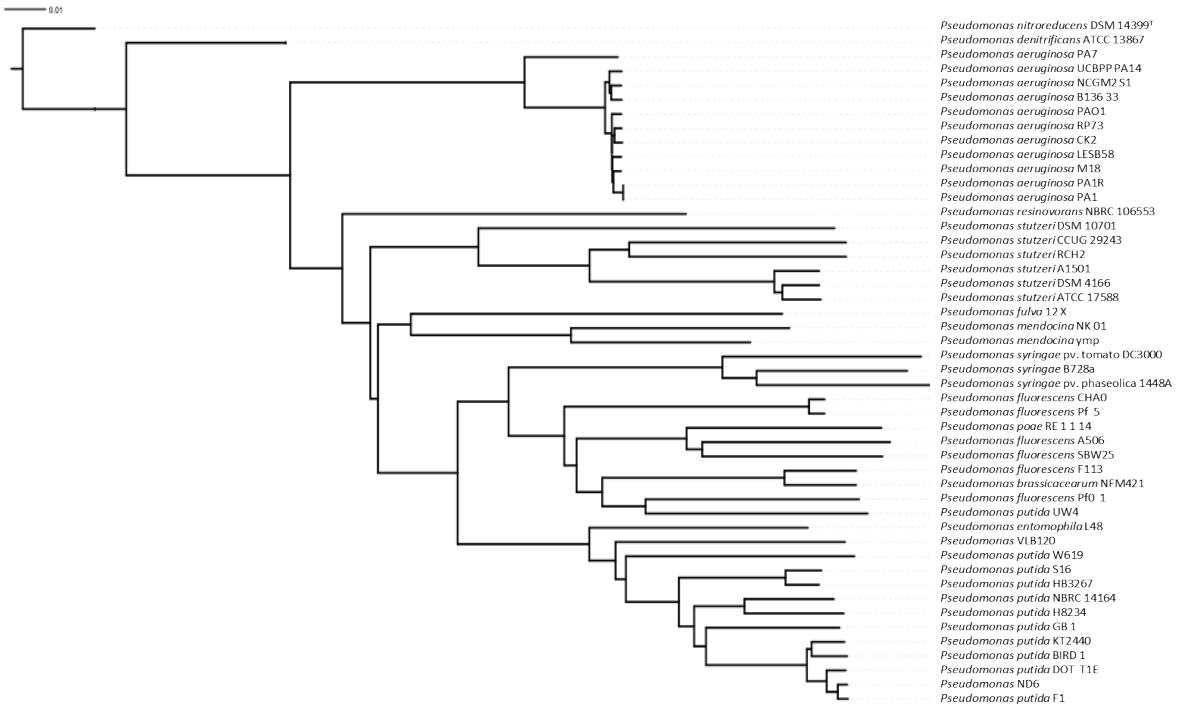

Figure 4 Multiple sequence alignment comparison of single copy genes from *Pseudomonas.*

3.6 Genes and protein domains associated to compounds degradation and production

3.6.1 ***Surfactants degradation***

The initial cleavage of alkyl sulfates such as SDS is catalyzed by sulfatases. These sulfatases activate a water molecule to form a hydroxyl ion which performs a nucleophilic attack on the substrate. By this attack, sulfate and the correspondent alcohol are formed (Gadler and Faber 2007). In *Pseudomonas* C12B, 1-dodecanol is further converted to dodecanal by alcohol dehydrogenases. Dodecanal is further converted to lauric acid by the action of aldehyde dehydrogenases. Lauric acid is degraded by β-oxidation and partly used for lipid biosynthesis (Thomas and White 1989). Several protein domains and genes associated with SDS degradation were identified in the genome of *P. nitroreducens* DSM 14399^T (Table 4). *SdsA1* hydrolase gene sequence present in *P. aeruginosa* PAO1, LESB58 and M18 strains (99% similar among each other) besides being 99% similar to genes present in other *P. aeruginosa* strains is also similar to genes present in 'P. denitrificans' ATCC 13867 (76%), *P. nitroreducens* (75%) and *Pseudomonas knackmussii* B13 (75%). The *SdsA1* hydrolase gene sequence also predicted for *P. nitroreducens* DSM 14399^T genome is highly similar to the *sdsA1* hydrolase present in another *P. nitroreducens* (100 %), in '*P. denitrificans*' ATCC 13867 strain (86%), in *P. knackmussii* B13 strain (83%) and in *P. aeruginosa* strains (76%). *P. knackmussii* B13 strain is also highly similar to *P.* nitroreducens DSM 14399^T in terms of 16S rRNA (Stolz et al. 2007). No other *Pseudomonas* species were retrieved in this comparison. Ester hydrolases, such as *tesA* and *estA,* are present in *P. aeruginosa* and known to be active towards tween nonionic surfactants and *p*-nitrophenyl esters of short acyl chain length (Asler et al. 2010). Protein domains related to esterases (IPR000801, IPR013831) and *tesA* and *estA* genes were identified in the *P. nitroreducens* DSM 14399^T genome. Tween 40 and Tween 80 were used as carbon sources by *P. nitroreducens* DSM 14399^T (Table S2 in Supplementary Materials), supporting these findings. Protein domain associated to a putative esterase (IPR000801) is present in all selected *Pseudomonas* genomes.

Table 4
List of genes and protein domains associated to SDS degradation predicted in *P. nitroreducens* DSM 14399^T genome

Gene name*	Product	Protein domain
sdsB	SDS degradation transcriptional activation protein	---
BDS1	Alkyl/aryl-sulfatase BDS1	---
---	Sulfatases, sulfatase conserved site, membrane sulfatases, choline sulfatase	IPR000917, IPR024607, IPR012160, IPR025863
adh, *alkJ, adhA, adhC2*	Alcohol dehydrogenases	IPR013154, IPR013149, IPR018211, IPR011047, IPR001670, IPR002328, IPR014183, IPR014182, IPR027295, IPR014353
aldA, ALDH7B4, *aldH*	Aldehyde dehydrogenase	IPR015590, IPR016160, IPR016163, IPR016162, IPR012394
fadD	Long-chain-fatty-acid--CoA ligase	---
fadB	Fatty acid oxidation complex enzymes	IPR012799

* Genes predicted using Swiss-Prot.

Alkyl ether sulfates, such as SLES, are known to be mainly cleaved by ether cleaving enzymes and sulfatases, depending on the microorganism and conditions (Hales et al. 1986). However, enzymes required for cleaving ether bonds are not well-known (White et al. 1996). Carboxymethyloxysuccinate lyase (EC 4.2.99.12) and 4-methoxybenzoate monooxygenase (EC 1.14.99.15) are the few known purified enzymes associated to ether cleavage (White et al. 1996) and were not predicted in the genome of *P. nitroreducens* DSM 14399^T. Although the hydrolytic cleavage of ether bonds has not been clearly observed, the enzyme isochorismate pyruvate-hydrolase (EC 3.3.2.1) has been indicated as responsible for the hydrolytic ether cleavage of isochorismic acid to 2,3-dihydro-2,3-dihydroxybenzoic acid (White et al. 1996). Using protein domains analysis, this enzyme was predicted in all *P. aeruginosa* strains selected in our study, but not in any other *Pseudomonas* spp.. Also isochorismate synthase (5.4.4.2), which catalyzes the reaction of chorismate to isochorismate, was found to only be present in *P. aeruginosa* strains.
Alkane sulfonates are a subclass of anionic surfactants (Koennecker et al. 2011) and have been shown to be used by *P. aeruginosa* as sulfate and carbon sources (Kertesz 1996). Alkane sulfonates degradation by *P. nitroreducens* strains is not described. Genes coding for this physiological ability described in *P. aeruginosa* strains were identified in *P. nitroreducens* DSM 14399^T genome (Table 5). Protein domains associated to sulfate binding proteins (IPR005669, IPR017875, IPR010067) and aliphatic sulfonates import ATP-binding proteins (IPR017875, IPR010067) were found to be present in all analyzed

Pseudomonas genomes. Taurine transporter protein domain (IPR010068) is present in all analyzed *Pseudomonas* genomes except in *P. stutzeri* genomes used in this study. Taurine catabolism dioxygenase protein domain (IPR003819) is present also in the genome of *P. aeruginosa* and *P. stutzeri* strains.

Table 5
List of genes and protein domains associated to alkane sulfonates degradation predicted in *P. nitroreducens* DSM 14399^T genome (Kertesz 1996)

Gene name*	Product	Protein domains
sbp	Sulfate-binding protein	IPR005669
ssuD, *msuD*	Alkanesulfonate monooxygenases	IPR019911
ssuB, *ssuB2*	Aliphatic sulfonates import ATP-binding proteins	IPR017875, IPR010067
tauA	Taurine-binding periplasmic protein	IPR010068, IPR015859, IPR003819
tauB	Taurine import ATP-binding protein TauB	
tauC	Taurine transport system permease protein TauC	
tauD	Alpha-ketoglutarate-dependent taurine dioxygenase	

* Genes predicted using Swiss-Prot.

3.6.2 ***Degradation of other compounds***

Different *P. nitroreducens* strains are able to degrade xenobiotics that contain aromatic rings in their molecular formula, such as nonionic surfactants (Chen et al. 2006), eugenol and isogenol (Unno et al. 2007) and phenolic compounds (Zhang et al. 2010). Several protein domains and genes related to aromatic compounds degradation were identified in *P. nitroreducens* DSM 14399^T genome (Table 6). Many of the protein domains associated to aromatic compounds degradation are common to the analyzed *Pseudomonas* genomes. Besides protein domains identified in Table 6, several protein domains related to aromatic rings hydroxylation were predicted in *P. nitroreducens* DSM 14399^T genome (IPR000391, IPR017641, IPR003042, IPR015881, IPR015879) and degradation (IPR005017), being most domains present also in many analyzed *Pseudomonas* genomes. Protein domains associated to phenolic compounds degradation were also predicted in *P. nitroreducens* DSM 14399^T genome (IPR003730, IPR001221). This agrees with observations regarding degradation of aromatic compounds such as *o*-cresol, benzylamine, protocatechuate and benzoate by *P. nitroreducens* DSM 14399^T (see Table S2 in Supplementary Materials).
P. nitroreducens DSM 14399^T is expected to be able to degrade alkanes, such as heptane (see Table S2 from Supplementary Materials). The gene encoding enzyme alkane 1-monooxygenase 2 (*alkB2*), was predicted in *P. nitroreducens* DSM 14399^T genome.

Table 6

List of genes and protein domains associated to aromatic compounds degradation predicted in *P. nitroreducens* DSM 14399^T genome

Gene name*	Product	Protein domain
catA	Catechol 1,2-dioxygenase	IPR012801
catB	Muconate cycloisomerase 1	IPR013370
catC	Muconolactone Delta-isomerase	IPR003464, IPR026029
catD	3-oxoadipate enol-lactonase 2	IPR026968
catI	3-oxoadipate CoA-transferase subunit A	---
catJ	3-oxoadipate CoA-transferase subunit B	---
xylX	Toluate 1,2-dioxygenase subunit alpha	---
xylY	Toluate 1,2-dioxygenase subunit beta	---
xylZ	Toluate 1,2-dioxygenase electron transfer component	---
xylL	1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase	---
xylS	*XylDLEGF* operon transcriptional activator	---
pcaB	3-carboxy-cis,cis-muconate cycloisomerase	IPR012789
pcaC	4-carboxymuconolactone decarboxylase	IPR012788, IPR003779
pcaF	Beta-ketoadipyl-CoA thiolase	IPR012793
pcaG	Protocatechuate 3,4-dioxygenase alpha chain	IPR012786
pcaH	Protocatechuate 3,4-dioxygenase beta chain	IPR024756, IPR012785
pcaK	4-hydroxybenzoate transporter *PcaK*	IPR012733
pcaR	Pca regulon regulatory protein	---

* Genes predicted using Swiss-Prot.

3.6.3 ***Rhamnolipids and PHA biosynthesis***

Rhamnolipids are glycolipid biosurfactants produced by *P. aeruginosa* (Soberon-Chavez et al. 2005). Rhamnolipids can be produced by *P. aeruginosa* using a first reaction catalyzed by rhamnosyltransferase 1 (Rt1) and a second reaction catalyzed by rhamnosyltransferase 2 (Rt2). Rt1 is composed of two polypeptides, RhlA and RhlB (Soberon-Chavez et al. 2005). Rhamnolipid production by *P. nitroreducens* has also been studied (Onwosi and Odibo 2013). Rhamnosyltransferase protein domain (IPR006446) was only predicted in some *P. aeruginosa* and *P. putida* genomes. Rhamnolipids biosynthesis related gene 3-oxoacyl-[acyl-carrier-protein] reductase (*rhlG*) was predicted in *P. nitroreducens* DSM 14399^T genome, using Swiss-Prot. Esterase *estA*, also identified in *P. nitroreducens* DSM 14399^T genome was described as being required for rhamnolipid biosynthesis (Wilhelm et al. 2007).

PHAs are biopolymers synthesized by many bacteria as carbon and energy storage compounds. Possible applications focus on the production of PHAs as biodegradable plastics (Yao et al. 1999). PHA biosynthesis was observed in a *P. nitroreducens* strain (Yao et al. 1999). Protein domains associated with poly-β-hydroxybutyrate polymerase (IPR010941), poly(R)-hydroxyalkanoic acid synthase (IPR011287, IPR013433) and to poly(3-hydroxyalkanoate) depolymerase (IPR011942) were predicted in P. nitroreducens DSM 14399^T genome. Protein domain related to poly(3-hydroxyalkanoate) depolymerase (IPR011942) was also predicted in P*. aeruginosa* genomes, but not in other analyzed *Pseudomonas* genomes. Genes coding for this physiological ability (*phA*, *phB*, *phC* and *phG*) were predicted using Swiss-Prot in *P. nitroreducens* DSM 14399^T genome.

3.6.4 ***Amino acids and vitamin B12 production***

Many bacteria can synthesize various aromatic compounds, such as aromatic amino acids (Gosset 2009). Genes encoding for enzymes associated to aromatic amino acids production in *E. coli* (Bongaerts et al. 2001) were predicted in *P. nitroreducens* DSM 14399^T genome using the Swiss-Prot database, such as: *aroA*, *aroC*, *aroE*, *aroF*, *aroK*, *aspC*, *trpA*, *trpB*, *trpC*, *trpD*, *trpE* and *pheA*. Aerobic biosynthesis of vitamin B12 is well studied for `*P. denitrificans´* strains (Li et al. 2008). Several genes encoding for aerobic vitamin B12 production (Scott and Roessner 2002) were predicted in *P. nitroreducens* DSM 14399^T genome using the Swiss-Prot database, such as: *hemB, hemC, hemD, cobA, cobI, cobJ, cobM, cobL, cobH, cobNST, cobO, cobQ, cobC, cobD,* and *cobP*.

3.7 Denitrification genes

P. nitroreducens DSM 14399^T is a denitrifier (Iizuka and Komagata 1964; Lang et al. 2007). Several protein domains and genes involved in denitrification were predicted in *P. nitroreducens* DSM 14399^T genome (Table 7). *P. nitroreducens* DSM 14399^T seems to present periplasmic nitrate reductases (*nap*), cytoplasmic assimilatory nitrate reductases (*nas*), respiratory nitrate reductase genes (*nar*), nitrite reductase genes (*nir*), nitric oxide reductase genes (*nor*) and nitrous oxide reductase genes (*nos*). Some protein domains were found to be common to all analyzed *Pseudomonas* genomes, indicating that most of these *Pseudomonas* are capable of nitrate reduction.

Table 7
Genes and protein domains involved in denitrification identified in the genome of *P. nitroreducens* DSM 14399^T

Gene name*	*Present in P. nitroreducens* DSM 14399^T*	Protein domains
nas	*nasA; nasD*	---
nar	*narK; narV; narJ; narH; narG; narL; narB*	IPR020945, IPR003765, IPR006468, IPR028189, IPR006547, IPR003816, IPR004737
nap	*napA; napB; napC; napF*	IPR005623, IPR004448, IPR005591, IPR010649, IPR011885, IPR027406, IPR010051
nir	*nirK; nirQ*	IPR012744, IPR012748, IPR017121, IPR001287, IPR005117, IPR006067, IPR006066
nor	*norD; norB; norR2; norC*	IPR008254
nos	*nosZ; nosD; nosR; nosY; nosF*	IPR008719, IPR011399, IPR026464, IPR011045, IPR023644

* Predicted using Swiss-Prot.

3.8 Comparison between *P. nitroreducens* DSM 14399^T and `*P. denitrificans´* ATCC 13867

Substrate degradation profiles of *P. nitroreducens* DSM 14399^T and `*P. denitrificans´* ATCC 13867 were compared (see
Table **S5** in Supplementary Materials). The main difference found between both strains is the incapability of `*P. denitrificans´* ATCC 13867 to degrade some alcohols and amine-related compounds when compared to *P. nitroreducens* DSM 14399^T, presenting overall a very similar substrate degradation profile. Besides, both strains share the same genes and protein domains regarding alkyl sulfates degradation, alkane sulfonates degradation, PHA biosynthesis, aromatic amino acids and vitamin B12 production and denitrification (excluding *nap* genes in `*P. denitrificans´* ATCC 13867).

3.9 Genotype *versus* phenotype

The analysis of protein domains allowed the prediction of enzymes presence or absence, which was compared for several *Pseudomonas* strains: *P. nitroreducens* DSM 14399^T, *P. denitrificans* DSM 13867, *P. aeruginosa* PAO1, *P. fluorescens* SBW25, *P. putida* KT2440, *P. stutzeri* ATCC 17588 and *P. syringae* pv. *phaseolicola.* All selected *Pseudomonas*

present known physiological abilities and a sequenced genome. Results are presented in Table S6 (Supplementary Materials), in which enzymes predicted in the different genomes and substrate degradation profile were compared. Several substrate degradation profiles agreed with associated enzymes presence or absence in these *Pseudomonas* strains, such as the degradation of N-acetyl-glucosamine, D-gluconate, L-arginine, L-alanine, pyruvate, L-proline and L-aspartate. For other cases, the substrate degradation profile confirmed almost all enzymes profiles, such as for degradation of mannitol, L-isoleucine, saccharic acid, inositol, L-histidine, L-isoleucine and α-keto-glutaric acid. Although for ribose degradation only the second enzyme was found, the substrate degradation profile confirmed the enzymes presence or absence. Some enzymes expected to be present in all selected *Pseudomonas* genomes were not found after annotation, such as the ones related to glucose, L-glutamate, D-fructose and L-asparagine degradation.

4. DISCUSSION

P. nitroreducens strains can degrade different types of pollutants. When studying *P. nitroreducens* strains able to degrade anionic surfactants coupled to denitrification it was observed that the ability of *P. nitroreducens* SN2 strain for SDS degradation (Paulo et al. 2013) is shared with *P. nitroreducens* DSM 14399^T. The genome of *P. nitroreducens* DSM 14399^T was found to be closely related to `*P. denitrificans´* ATCC 13867. Functional profiles comparison and other genomic analyses (whole genome comparison, 16S rRNA genes comparison and standard protein comparison) indicated that both genomes can be included in the *P. aeruginosa* group, validating *P. nitroreducens* and other *Pseudomonas* strains classification according to Anzai et al., (2000). *P. nitroreducens* DSM 14399^T and `*P. denitrificans´* ATCC 13867 share many physiological and genomic features. Doudorof et al. (1974) found that `*P. denitrificans´* ATCC 13867 was a different species from `*P. denitrificans´* ATCC 19244, the neotype of this species. According to the 16S rRNA sequence (sequence comparisons were made in 2007 based on a stretch of 480 nucleotides) and biochemical tests, DSMZ concluded that `*P. denitrificans´* ATCC 13867 is closely related to *P. nitroreducens* species, being designated in their collection as *P. nitroreducens* DSM 1650 (http://www.dsmz.de/catalogues/catalogue-microorganisms.html). Complementing these findings with the results obtained in our study, it can be concluded that `*P. denitrificans´* ATCC 13867 is indeed a *P. nitroreducens* strain.

Functional profiles and 16S rRNA genes comparison disagree on the classification of *P. resinovorans* NBRC 106553 and of *P. putida* DOT T1E. Several *Pseudomonas* species have not one but several different 16S rRNA genes, what is sometimes overlooked and can

introduce differences in phylogeny (Mulet et al. 2009). Still, 16S rRNA genes comparison is only focused on a single type of gene and does not capture the breath of a species, as functional profiling can reveal a different classification. According to all genomic analyses, *P. putida* UW4 is placed within the *P. fluorescens* group, agreeing with the conclusions by Duan et al. (2013). Also in none of the genomic analyses *P. fulva* 12X was positioned close to the *P. putida* group, according to Anzai et al. (2000). *P. mendocina* strains did not cluster with strains from the *P. aeruginosa* group, when using 16S rRNA genes comparison or functional profiling. These findings confirm the strength of classification by capability.

Protein domain analysis and genes annotation using Swiss-Prot validated known physiological capabilities of *P. nitroreducens* DSM 14399^T, related to SDS degradation, denitrification and several compounds degradation. Biosynthesis of PHA and rhamnolipids and degradation of alkane sulfonates are also possibly performed by *P. nitroreducens* DSM 14399^T. The use of *SdsA1* hydrolase in SDS degradation was identified in *P. aeruginosa* and *P. nitroreducens* strains, but not identified among other *Pseudomonas* spp..

Physiological characteristics of bacteria have been very useful is the past and still today for distinguishing bacteria and help classifying them. Substrate degradation profiles are used in standard tests such as API 20 NE strip tests (bioMérieux SA, France) for bacterial classification. Agreement between substrate degradation and enzyme profiles for degradation of some substrates present in API 20 NE strip tests was obtained (D-mannitol, L-arginine, N-acetyl-glucosamine), confirming known differences between *P. nitroreducens* DSM 14399^T, *P. aeruginosa* and *P. stutzeri* strains (Clark et al. 2006; Lalucat et al. 2006; Lang et al. 2007).

When looking for specific domains in *P. nitroreducens* DSM 14399^T, 32 domains were found to be specific for *P. nitroreducens* DSM 14399^T. However, it does not indicate the absence of identical metabolic functions in other *Pseudomonas* strains. Thus, at this stage, we could not yet find or validate the presence of metabolic pathways in *P. nitroreducens* DSM 14399^T not present in other *Pseudomonas* spp. by analyzing the presence or absence of protein domains.

5. CONCLUSIONS

P. nitroreducens DSM 14399^T is closely related to `*P. denitrificans*´ ATCC 13867 which needs to be properly reclassified as a `*P. nitroreducens*´ strain. Functional profile comparisons and other genomic analyses validated *P. nitroreducens* DSM 14399^T and most selected *Pseudomonas* species classification. Physiological abilities associated to surfactants degradation were supported by identification of related protein domains and genes in *P. nitroreducens* DSM 14399^T genome. The presence of *SdsA1* hydrolase gene in

P. aeruginosa and *P. nitroreducens* strains indicates that its presence is specific to both two *Pseudomonas* spp. Functional profiling can be used for re-classifying or classifying *Pseudomonas* strains while simultaneously giving information related to the physiological abilities. The results show a strong correlation between domain presence, phylogeny and prediction of the metabolic phenotype.

SUPPLEMENTARY MATERIALS

Table S1
General features of *P. nitroreducens* DSM 14399[T]

Property	Term	References
Gram strain	Negative	1,2
Cell shape	Rods	1,2
Cell size (L × W μm)	1.0×3.0	3
Motility	Motile	1,2
Sporulation	Nonsporulating	1
Optimum temperature	25 °C to 30 °C	1
Growth at 4 °C	Positive[3] ; negative [4]	3,4
Growth at 41 °C	Negative	3,4
Salinity	Growth up to 5 % of NaCl, does not grow at 7 % of NaCl	1,3,4
Oxygen requirement	Facultative anaerobic	1,2
Carbon source	Organic	1
Energy source	Organic	1
Habitat	Soil, water	1
Biotic relationship	Free living	1
Pathogenicity	Nonpathogenic	5
Biosafety level	1	5
Isolation	Oil brine	1
Geographic location	Japan	1
Sample collection time	1957	1

[1] Iizuka and Komagata (1964); [2] this study; [3] Prakash et al. (2007); [4] Lang et al. (2007); [5] DSMZ culture collection https://www.dsmz.de/.

Table S2

Physiological characteristics of *P. nitroreducens* DSM 14399^T

Utilization of substrates					
Growth			**No growth**		
Glucose	Heptane	γ-Aminobutyric acid	D-Saccharate	D-Mannose	Starch
Fructose	Triton X-100	α-Ketoglutaric acid	Methylamine	Urea	Glycogen
Ethanol	α – Naphthol (w)	Urocanic acid	Citraconate	α-Cyclodextrin	Xylitol
1-Butanol	L-glutamic acid (w)	DL-Lactic acid	D-Tartrate	Dextrin	β-Gentiobiose
Propanol	Hydroxy-L-proline	Phenylethylamine	Nicotinate	α-D-Lactose lactulose	D-Turanose
Propanediol	N-Acetylgalactosamine	Propionic acid	Salicylate	Methyl β-D-glucoside	D-Lyxose
Glycerol	Putrescine	Sebacic acid	2-Hydroxybenzoate	Psicose	D-Tagatose
D-glucoronate	*trans*-Aconitate	2-Aminoethanol	Citronellol	D-Galactonic acid lactone	D-Fucose
Gentisate	Mesaconate	Bromosuccinic acid	D-galactose	D-Galacturonic acid	L-Fucose
Succionate	cis-Aconitate	Allantoin	Toluene	D-Glucosaminic acid	Uridine
meso-Tartrate	Fumarate	Phenylacetate	Pyridine	D-Glucuronic acid	Thymidine
Itaconate	Glutarate	Adipic acid	Propionate	Glucuronamide	2,3-Butanediol
Adipate	DL-3-hydroxybutyrate	Malic acid	Pyrocatechol	Dulcitol	Glycerol
Azelate	DL-Lactate	D-gluconate	Aniline	Inositol	DL-α-Glycerol phosphate
Butyrate	L-Malate	Phenylacetic acid	*para*-Nitrophenol	Sorbitol	Glucose 1-phosphate
n-Valerate	2-Oxoglutarate	Tween 40	Acetic acid	Methyl α-D-mannoside	Glucose 6-phosphate
Pimelate	Sodium dodecyl sulfate*	L-alanylglycine	Maltitol	Methyl α-D-glucoside	L-Phenylalanine
Suberate	Sodium laureth sulfate*	Tween 80	Tryptophan	*N*-Acetylglucosamine	D-Serine
Pyruvate	Alanine	L-asparagine	L-arabinose		

Table S3 *(continued)*

Physiological characteristics of *P. nitroreducens* DSM 14399^T

Utilization of substrates					
Growth			**No growth**		
Citrate	Butylamine	4 –Hydroxybenzoate	Malonate	L-Arabitol	Inosine
D-gluconate	Histamine	Methylpyruvic acid	Erythritol	5-Ketogluconate	N-Acetylglucosamine
4-aminobutyrate	L-Leucine	L-aspartic acid	D-Arabinose	D-Arabitol	Mannitol
L-Valine	L-Arginine	Methylsuccinic acid	D-Ribose	Amygdalin	Melezitose
L-Serine	L-threonine	Acetic acid	D-Xylose	Arbutin	D-Raffinose
O-cresol	L-histidine	cis-Aconitic acid	L-Xylose	Salicin	
Geraniol	Cystine (w)	Formic acid	Adonitol	Cellobiose	
Benzylamine	L-ornithine	β-Hydroxybutyric acid	Methyl β-xyloside	Lactose	
Benzoate	Sarcosine	DL-Carnitine	D-Mannose	Melibiose	
3,4-Hydroxybenzoate	L-Citrulline	Itaconic acid	Sorbose	Sucrose	
Liquid paraffin	Betaine	D-Alanine	Rhamnose	Trehalose	
Caprate		Protocatechuate	Maltose	Inulin	
		L- Proline			

Table S2 (*continued*)

Acid production			**Presence of reactions and/or enzymes**	
Positive	**Negative**		**Positive**	**Negative**
Fructose	Arabinose	Sorbitol	DNase	Ornithine decarboxylase
Glucose	Maltose	Pyridine	Catalase	Indole production
L-alanine	Xylose	Heptane	Oxidase	β-Galactosidase (Para-NitroPhenyl - βD-Galactopyranosidase)
β-alaline	Ribose	Cystine	Aerobic NO_3^- reduction	
L-aspartate	Galactose	L-histidine	Anaerobic NO_3^- reduction	Lecithinase
Phenylacetate	Mannitol	Glycerol	Arginine dihydrolase	Aerobic NO_2^- reduction
	Sucrose	Toluene	Denitrification	Urease
	Adonitol	Dulcitol	Hydrolysis of bis-*p*NP phosphate	Fluorescence on King's B medium
	Sorbose	Salicin	Hydrolysis of *p*NP phosphorylcholine	Hydrolysis of *p*NP β-D-galactopyranoside
	Triton X-100	Raffinose	Hydrolysis of L-alanine *p*NA	Hydrolysis of *p*NP β -D-glucuronide
	α-Naphthol	Cellobiose	Hydrolysis of L-glutamate-3-carboxy-*p*NA	Hydrolysis of *p*NP α-D-glucopyranoside
	Inositol	Methyl α-D-glucoside	Hydrolysis of L-proline *p*NA	Hydrolysis of *p*NP β-D-glucopyranoside
	Rhamnose	Erythritol	Hydrolysis of Hypoxanthine	Hydrolysis of *p*NP β -D-xylopyranoside
	Trehalose	Melibiose	Tween 80 hydrolysis	Hydrolysis of *p*NP, paranitrophenyl
	Propionate	D-arabitol		Hydrolysis of *p*NA, para-nitroanilide
	D-mannose	Lactose		Hydrolysis of Aesculin
	Acetate			

Legend - (w) weak growth;
References: *this study; Iizuka and Komagata (1964); Lang et al. (2007); Prakash et al. (2007); Stolz et al. (2007); Gupta et al. (2008); Wang et al. (2012).

Table S4

List of protein domains unique in *P. nitroreducens* DSM 14399^{T}

IPR000417	Hydroxyethylthiazole kinase
IPR000673	Signal transduction response regulator, chemotaxis, protein-glutamate methylesterase
IPR000700	PAS-associated, C-terminal
IPR001111	Transforming growth factor-beta, N-terminal
IPR001315	CARD domain
IPR001505	Copper centre Cu(A)
IPR001827	Homeobox protein, antennapedia type, conserved site
IPR002782	Mut7-C RNAse domain
IPR004134	Peptidase C1B, bleomycin hydrolase
IPR004394	Protein Iojap/ribosomal silencing factor RsfS
IPR004421	Carbamoyltransferase, HypF-type
IPR004691	Malonate/sodium symporter MadM subunit
IPR004948	Nucleoside-triphosphatase, THEP1 type
IPR006901	tRNA methyltransferase TrmK
IPR007969	Domain of unknown function DUF732
IPR010383	Glycosyltransferase 36
IPR010895	CHRD
IPR011342	**Shikimate dehydrogenase**
IPR011856	tRNA endonuclease-like domain
IPR013519	Integrin alpha beta-propellor
IPR013525	ABC-2 type transporter
IPR018198	ATP phosphoribosyltransferase, conserved site
IPR018658	Protein of unknown function DUF2089
IPR019639	Protein of unknown function DUF2505
IPR019814	Translation initiation factor 3, N-terminal
IPR020541	**Chorismate synthase, conserved site**
IPR021778	Protein of unknown function DUF3343
IPR023435	D-tagatose-bisphosphate aldolase, class II, non-catalytic subunit KbaZ
IPR023998	Siderophore ferric iron reductase, AHA1954 family
IPR025499	Pectin degradation protein KdgF
IPR025692	Mechanosensitive ion channel inner membrane domain 1
IPR027396	DsrEFH-like

Table S5

Substrate degradation differences between *P. nitroreducens* DSM 14399^T and *`P. denitrificans´* ATCC 13867

Compound	***`P. denitrificans´*** **ATCC 13867**	***P. nitroreducens*** **DSM 14399^T**
Acetate	**+**	**-/+***
D-arabinose	**+**	**-**
Propionate	**+**	**-**
meso-tartrate	**-**	**+**
Phenylacetate	**-**	**+**
Valine	**-**	**+**
Citrulline	**-**	**+**
Histamine	**-**	**+**
Butylamine	**-**	**+**
Suberate	**-**	**+**
Ethanol	**-**	**+**
Propanol	**-**	**+**
Butanol	**-**	**+**

* Degradation was found positive or negative according to different authors.

Table S6

Comparison between enzymes absence/presence profile and substrate degradation in several *Pseudomonas* spp.

Substrate[1]	**Enzyme**[2]	***P. nitroreducens* DSM 14399^T**	***P. denitrificans* ATCC 13867**	***P. aeruginosa* PAO1**	***P. fluorescens* SBW25**	***P. putida* KT2440**	***P. stutzeri* ATCC 17588**	***P. syringae phaseolicola* 1448A**
Mannitol	mannitol-1-phosphate 5-dehydrogenase (1.1.1.17)	0	0	1	1	0	1	1
Degraded?		**NO**	NO	YES	YES		YES	NO
N-acetyl-glucosamine	N-acetylglucosamine-6-phosphate deacetylase (3.5.1.25; 1st)	0	0	1	1	0	0	0
	glucosamine-6-phosphate deaminase (3.5.99.6; 2nd)	1	1	1	1	1	1	1
Degraded?		NO		YES	YES		NO	NO
D-gluconate	D-gluconate kinase, thermosensitive (2.7.1.12)	1	1	1	1	1	1	1
Degraded?		YES	YES	YES	YES		YES	YES
D-malate	D-malate dehydrogenase (decarboxylating) (1.1.1.83)	1	0	0	1	0	0	1
Degraded?		YES	YES	YES	YES		YES	

Table S6 (*continued*)

Substrate[1]	**Enzyme**[2]	***P. nitroreducens* DSM 14399^T**	***P. denitrificans* ATCC 13867**	***P. aeruginosa* PAO1**	***P. fluorescens* SBW25**	***P. putida* KT2440**	***P. stutzeri* ATCC 17588**	***P. syringae phaseolicola* 1448A**
L-arginine (II)	arginine succinyltransferase (2.3.1.109; 1st)	1	1	1	1	1	0	1
	succinylarginine dihydrolase (3.5.3.23; 2nd)	1	1	1	1	1	0	1
	aldehyde dehydrogenase (1.2.1.71; 4th)	1	1	1	1	1	0	1
	succinylglutamate desuccinylase(3.5.1.96; 5th)	1	1	1	1	1	1	1
Degraded?		YES		YES	YES		NO	
Ribose	Ribokinase (2.7.1.15; 2nd)	0	0	1	1	1	0	1
Degraded?		NO	NO	YES	YES			
L-alanine (I)	alanine racemase 2, PLP-binding (5.1.1.1; 1st)	1	1	1	1	1	1	1
Degraded?		YES	YES	YES	YES		YES	YES
D-serine	D-serine ammonia-lyase (4.3.1.18)	0	0	1	0	0	0	0
Degraded?		NO		NO			NO	NO

Table S6 *(continued)*

Substrate[1]	Enzyme[2]	*P. nitroreducens* DSM 14399^T	*P. denitrificans* ATCC 13867	*P. aeruginosa* PAO1	*P. fluorescens* SBW25	*P. putida* KT2440	*P. stutzeri* ATCC 17588	*P. syringae phaseolicola* 1448A
Saccharic acid (II)	D-glucarate dehydratase (4.2.1.40; 1st)	0	0	0	1	1	0	0
	5-dehydro-4-deoxy-D-glucarate dehydratase (4.2.1.41; 2nd)	0	0	0	1	1	0	1
Degraded?		NO	NO	NO	YES			YES
Inositol (I)	inositol 2-dehydrogenase (1.1.1.18; 1st)	0	0	0	1	0	0	1
Degraded?		NO	NO	NO	YES		NO	NO
Phenylalanine	phenylalanine 4-monooxygenase	1	1	1	1	1	1	1
	4a-hydroxytetrahydrobiopterin dehydratase	1	1	1	1	1	1	1
Degraded?		NO	NO	YES	YES	YES	NO	NO
L-isoleucine (I)	Enoyl-CoA hydratase (4.2.1.17; 4th)	1	1	1	1	1	1	1
L-isoleucine (II)	Alcohol dehydrogenase IV (1.1.1.1; 3rd)	1	1	1	1	1	1	1
L-isoleucine (I,II)	branched-chain-amino-acid aminotransferase (2.6.1.42; 1st)	1	1	1	1	1	1	1
Degraded?			YES	YES	YES	YES	NO	

Table S6 *(continued)*

Substrate[1]	Enzyme[2]	*P. nitroreducens* DSM 14399^T	*P. denitrificans* ATCC 13867	*P. aeruginosa* PAO1	*P. fluorescens* SBW25	*P. putida* KT2440	*P. stutzeri* ATCC 17588	*P. syringae phaseolicola* 1448A
L-histidine (I, II, III)	Histidase (4.3.1.3; 1st)	1	1	1	1	1	0	1
	Urocanase (4.2.1.49; 2nd)	1	1	1	1	1	0	1
	Imidazolonepropionase (3.5.2.7; 3rd)	1	1	1	1	1	0	1
L-histidine I	Formimidoylglutamase (3.5.3.8; 4th)	0	0	1	0	0	0	0
	formimidoyltetrahydrofolate cyclodeaminase / glutamate formimidoyltransferase (4.3.1.4; 5th)	0	0	0	0	0	1	0
	methylenetetrahydrofolate dehydrogenase [multifunctional] (3.5.4.9; 6th)	1	1	1	1	1	1	1
Degraded?		YES	YES	YES	YES	YES		NO
L-aspartate (I,II)	aspartate aminotransferase (2.6.1.1; II – 1st)	1	1	1	1	1	1	1
L-aspartate (II)	cytosolic malate dehydrogenase (1.1.1.37; 2nd)	0	0	0	0	1	0	0
Degraded?		YES	YES	YES	YES	YES		YES

Table S6 *(continued)*

Substrate[1]	Enzyme[2]	*P. nitroreducens* DSM 14399^T	*P. denitrificans* ATCC 13867	*P. aeruginosa* PAO1	*P. fluorescens* SBW25	*P. putida* KT2440	*P. stutzeri* ATCC 17588	*P. syringae phaseolicola* 1448A
L-valine (I, II)	branched-chain-amino-acid aminotransferase (2.6.1.42; 1st)	1	1	1	1	1	1	1
L-valine (I)	enoyl-CoA hydratase (4.2.1.17; 4th)	1	1	1	1	1	1	1
	3-hydroxyisobutyrate dehydrogenase (1.1.1.31; 6th)	1	1	1	1	1	1	0
	(S)-3-amino-2-methylpropionate transaminase (2.6.1.22; 7th)	1	1	1	1	1	1	1
	methylmalonate-semialdehyde dehydrogenase (1.2.1.27; 7th)	1	1	1	1	1	1	1
L-valine (II)	alcohol dehydrogenase IV (1.1.1.1; 3rd)	1	1	1	1	1	1	1
Degraded?		YES	NO	NO	YES	YES		
L-proline	proline dehydrogenase(1.5.99.8; 1st)	1	1	1	1	1	1	1
Degraded?		YES	YES	YES	YES	YES	YES	YES

Table S6 *(continued)*

Substrate[1]	Enzyme[2]	*P. nitroreducens* DSM 14399[T]	*P. denitrificans* ATCC 13867	*P. aeruginosa* PAO1	*P. fluorescens* SBW25	*P. putida* KT2440	*P. stutzeri* ATCC 17588	*P. syringae phaseolicola* 1448A
Pyruvate	pyruvate dehydrogenase (1.2.4.1; 1st)	1	1	1	1	1	1	1
	pyruvate dehydrogenase E2 component (2.3.1.12; 2nd)	1	1	1	1	1	1	1
	dihydrolipoyl dehydrogenase component (1.8.1.4; 3rd)	1	1	1	1	1	1	1
Degraded?		YES	YES	YES	YES		YES	
α-keto- glutaric acid	2-oxoglutarate decarboxylase, thiamine-requiring (1.2.4.2; 1st)	1	1	1	1	1	1	1
	2-oxoglutarate dehydrogenase complex, (E2) inner core (2.3.1.61; 2nd)	1	1	1	1	1	1	1
	dihydrolipoyl dehydrogenase component (1.8.1.4; 3rd)	1	1	1	1	1	1	1
Degraded?		YES	YES	NO	YES	YES		NO

Table S6 *(continued)*

Substrate[1]	Enzyme[2]	*P. nitroreducens* DSM 14399^T	*P. denitrificans* ATCC 13867	*P. aeruginosa* PAO1	*P. fluorescens* SBW25	*P. putida* KT2440	*P. stutzeri* ATCC 17588	*P. syringae phaseolicola* 1448A
L-leucine (I, III)	Branched-chain-amino-acid aminotransferase (2.6.1.42; 1st)	1	1	1	1	1	1	1
L-leucine (I)	Mitochondrial hydroxymethylglutaryl-CoA lyase (4.1.3.4; 6th)	1	1	1	0	1	1	1
L-leucine (III)	alcohol dehydrogenase IV (1.1.1.1; 3rd)	1	1	1	1	1	1	1
Degraded?		YES	YES	NO		YES	YES	NO
Glycogen (I,II,III)	glycogen phosphorylase (2.4.1.1; 1st)	1	0	1	1	1	1	1
	glycogen debranching enzyme (2.4.1.25; 5th (I), 2nd (II) and 2nd (III))	1	0	1	1	1	1	1
Glycogen (I)	Glucokinase (2.7.1.2; 6th)	1	1	1	1	1	1	1
Degraded?		NO		NO			YES	

Legend: [1] between brackets it can be mentioned the pathway number according to MetaCyc ((http://www.biocyc.org/); [2] between brackets it is mentioned the EC number and it can be mentioned the enzyme position in the pathway; *P. nitroreducens* DSM 14399^T (substrate degradation profile table 2 in Supplementary Materials); `*P. denitrificans*´ DSM 13867 (Doudorof et al. 1974); *P. aeruginosa* PAO1 (Johnson et al. 2008); *P. fluorescens* SBW25 (Zhang and Rainey 2008); *P. putida* KT2440 (Nogales et al. 2008); *P. stutzeri* ATCC 17588 (Clark et al. 2006); *P. syringae* pv. *phaseolicola* 1448A (Guven et al. 2004); Degradation result in grey color - substrate degradation profile does not agree with enzymes profile.

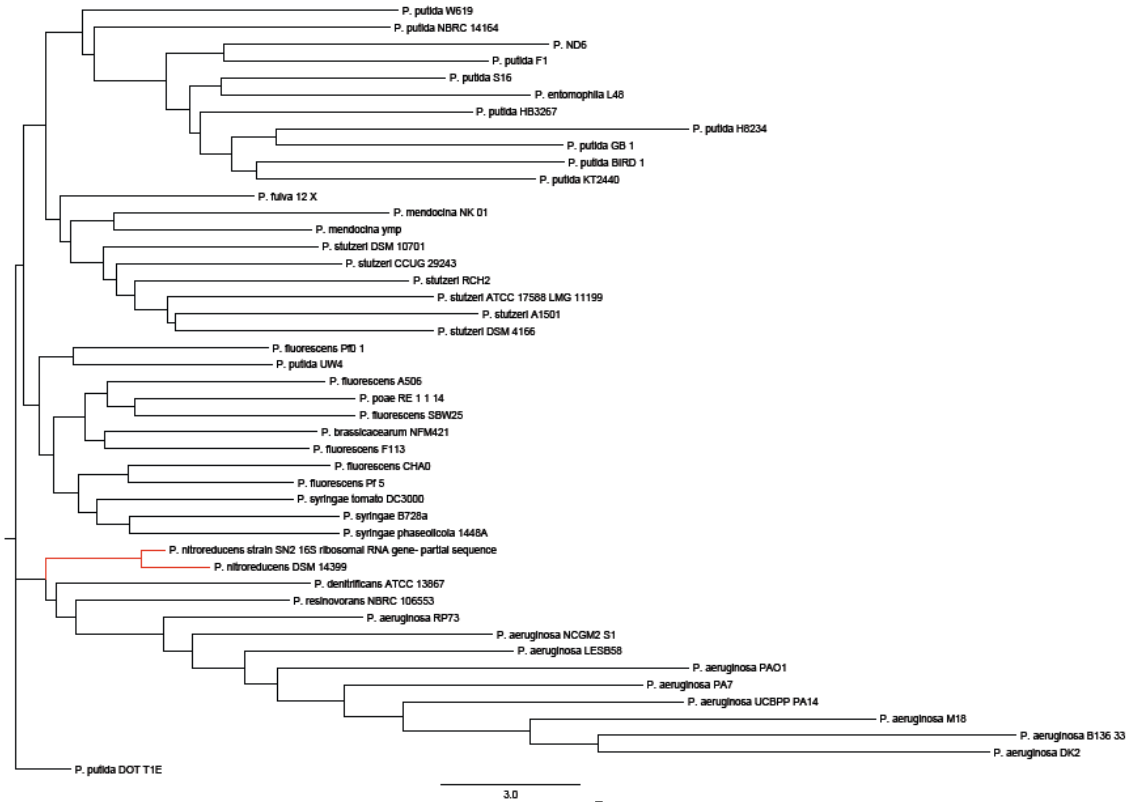

Figure S1 16S rRNA genes comparison between *P. nitroreducens* DSM 14399^{T} and completely sequenced *Pseudomonas* genomes.

CHAPTER VII

GENERAL DISCUSSION

Surfactants can be degraded in different compartments of wastewater treatment plants (WWTPs) in the presence or in the absence of oxygen. Therefore, it is of great importance to understand surfactants degradation in aerobic and anaerobic environments.

Surfactants degradation in aerobic environments has been extensively studied (Swisher 1987; Scott and Jones 2000; Ying 2006), but not in anaerobic environments. Sodium dodecyl sulfate (SDS) primary and complete aerobic degradation by *Pseudomonas* species is well-known (Thomas and White 1989). Sulfatases associated to SDS degradation in *Pseudomonas* have been further studied (Cloves et al. 1980; Shaw et al. 1980; Hagelueken et al. 2006; Jovčić et al. 2009). Little is known about bacteria able to degrade SDS using nitrate as electron acceptor. In this thesis, *Pseudomonas stutzeri* strain SN1 and *Pseudomonas nitroreducens* strain SN2 were isolated with ability to completely degrade SDS under anoxic conditions, using nitrate as electron acceptor (Chapter II). The results indicated that SDS degradation pathway present in *P. stutzeri* SN1 and *P. nitroreducens* SN2 strains growing anaerobically is the same as SDS aerobic metabolic pathway as proposed for *Pseudomonas* C12B (Thomas and White 1989). Besides several phenotypic differences between *P. stutzeri* strain SN1 and *P. nitroreducens* strain SN2, differences were also found at the sulfatase activity level. A lower and induced sulfatase activity by SDS in *P. stutzeri* SN1 strain and a higher and enhanced sulfatase activity by SDS in *P. nitroreducens* SN2 strain were observed.

Compared to SDS, sodium lauryl ether sulfate (SLES) degradation has been little studied. SLES degradation under aerobic conditions was studied using pure bacterial cultures (Dhouib et al. 2003) and using mixed cultures (Khleifat 2006). Sodium dodecyl triethoxy sulfate (SDTES) degradation was studied in aerobic conditions (Hales et al. 1982; Griffiths et al. 1986). SLES degradation by denitrifying bacteria was not studied before. In order to fill these gaps and to obtain extra information related to surfactant-resistant bacteria, the bacterial community diversity of enrichment cultures obtained with different SLES concentrations and redox condition (anoxic or aerobic) was analyzed (Chapter III). With increased SLES concentration, bacterial diversity decreased, *Pseudomonas* being enriched in both anoxic and aerobic conditions. Besides the ability for SLES degradation, the enrichment of the types of bacteria was also based on their adaptation to the bacteriostatic action of the surfactant. *Aeromonas hydrophila* strain S7, *P. stutzeri* strain S8 and *P. nitroreducens* strain S11 were isolated from anoxic enrichments with SLES and nitrate. *Acinetobacter* strain S15 was able to grow aerobically with SLES.

P. stutzeri and *P. nitroreducens* are known denitrifiers (Lalucat et al. 2006; Lang et al. 2007). *P. aeruginosa*, *P. fluorescens* biotype II, *P. alcaligenes* and *P. mendocina* are also able to denitrify (Gamble et al. 1977) and they were detected in activated sludge (Srinandan et al. 2011; Waheed et al. 2013). *P. aeruginosa* strains are able to degrade

surfactants (Swisher 1987), but they likely perform a slower nitrate reduction and/or have a larger sensitivity to nitrite compared to *P. stutzeri* and to *P. nitroreducens* (Carlson and Ingraham 1983). *P. stutzeri* and *P. nitroreducens* strains were enriched and isolated probably due to the combined ability to degrade anionic surfactants while efficiently reducing nitrate to dinitrogen gas.

Bacterial mechanisms used for cleaving alkyl ether sulfates are not as well-known as for alkyl sulfates. SDTES degradation was studied using pure and mixed bacterial cultures revealing that pure bacterial cultures were not able to mineralize SDTES as opposed to mixed bacterial cultures (Griffiths et al. 1986; Hales et al. 1986). SDTES initial steps of degradation by *Pseudomonas* under aerobic conditions indicated that ether cleaving enzymes were preferred compared to other cleaving mechanisms, such as sulfatases (Griffiths et al. 1986; Hales et al. 1986). Different mechanisms have been proposed for ether cleavage, but only for the involvement of oxygenation, hydroxyl group transfer, methyl transfer and carbon-oxygen lyase evidence has been obtained (White et al. 1996). In this study, *P. nitroreducens* strain S11 was the best SLES degrader under anoxic conditions compared to *A. hydrophila* strain S7 and to *P. stutzeri* strain S8 (Chapter IV). Aerobic SLES primary degradation was faster compared to anoxic SLES primary degradation by *P. nitroreducens* strain S11. *P. nitroreducens* strain S11 was also able to reach better SLES complete degradation in aerobic conditions compared to anoxic conditions. Results of this study indicate that ether cleavage in *P. nitroreducens* strain S11 occurs in both anoxic and aerobic conditions, but being at a higher rate in aerobic conditions. The differences found between the anoxic and aerobic SLES degradation indicate that at least some enzymes required for ether cleavage might be oxygen-dependent. Sulfatases can be used to hydrolyze both alkyl sulfates and alkyl ether sulfates, although ether cleavage is considered the preferred mechanism for alkyl ether sulfates degradation (Hales et al. 1982; Hales et al. 1986). Our results indicated that once sulfatases are produced by bacteria, they can continuously (slower or faster according to bacterial growth) perform SDS and SLES primary degradation, independently from complete degradation.

Resistance of surfactant-degrading bacteria to SDS and SLES has been studied in aerobic conditions (Khleifat 2006; Shukor et al. 2009; Lima et al. 2011). However, a comparison between tolerance of different surfactant-degrading *Pseudomonas* spp. to high surfactant concentrations under anoxic conditions has never been done. *P. stutzeri* strains (SN1 and S8) were compared to *P. nitroreducens* strains (SN2 and S11) for the ability to grow with high surfactant concentrations as carbon source and nitrate as electron acceptor (Chapter V). *P. stutzeri* S8, *P. nitroreducens* SN2 and *P. nitroreducens* S11 were able to reduce all nitrate to nitrogen gases, while accumulating sulfate in the medium until the end of the

experiments, in most tests. Maximum nitrate reduction and sulfate accumulation of *P. stutzeri* SN1 were similar in all tests, and lower compared to other strains, indicating inhibition of bacterial and sulfatase activity. In this study, *P. nitroreducens* strains were able to reach higher OD values compared to *P. stutzeri* strains while degrading the same amount of SDS and SLES. *P. stutzeri* strains are known to be genetically and phenotypically diverse (Lalucat et al. 2006), what can explain the differences in physiology between strains SN1 and S8. *P. nitroreducens* isolates SN2 and S11 and the type strain DSM 14399^T are able to degrade SDS and SLES coupled to nitrate reduction, sharing high surfactant concentration tolerance. Differences between *P. stutzeri* and *P. nitroreducens* strains during growth with high concentration of surfactants might be related to a different cell membrane resistance (Glover et al. 1999) or to an effect of surfactants on produced enzymes (Van Hamme et al. 2006). *P. stutzeri* were shown to be more sensitive to antimicrobial agents (Russell and Mills 1974; Tattawasart et al. 1999) and to be more susceptible to mechanical disintegration, when compared to other *Pseudomonas* (Wilkinson 1970). But overall, the *Pseudomonas* genus can often regulate the expression of their resistance to antibiotics, detergents and solvents (Moore et al. 2006), what can explain the results obtained in this thesis.

Most surfactants degradation can be accomplished in WWTPs (Matthijs et al. 1999; Gonzalez et al. 2007). In WWTPs using the A^2/O biological process, surfactants can be degraded in the anoxic compartment before reaching the aerobic one. Results show that denitrifying bacteria such as *P. stutzeri* and *P. nitroreducens* have a high tolerance to surfactants and achieve a fast degradation of SDS and SLES. This avoids possible inhibitory effects of anionic surfactants on other microbes relevant to the biological processes.

The *P. nitroreducens* type strain was isolated from hydrocarbon contaminated soil (Iizuka and Komagata 1964). Despite the different studies where *P. nitroreducens* strains have been identified for their ability to degrade complex compounds (Janota-Bassalik and Wright 1964; Chen et al. 2006; Zhang et al. 2010) this *Pseudomonas* species is less studied than other *Pseudomonas* species. How similar this species is to other *Pseudomonas* species was only known in terms of 16S rRNA gene comparison (Anzai et al. 2000). The 16S rRNA gene has been used as a classical method for studying the phylogenetic relationships between microorganisms (Bodilis et al. 2012). The use of this single gene to compare strains and to position them in terms of phylogeny might not be sufficient (Mulet et al. 2009), as also shown in chapter VI. With the increasing availability of physiological and genomic data, classification of a species can also be performed by correlating protein domain presence and absence with *in vivo* physiological abilities (Chapter VI). Together with functional profile comparison based on protein domain analysis, different comparative analyses (whole genomic DNA, 16S rRNA genes, standard proteins)

corroborated that *P. nitroreducens* is closely related to *P. aeruginosa* strains. The same analyzes showed that *P. nitroreducens* DSM 14399^T is very close related to `*P. denitrificans´* ATCC 13867, which could be properly re-classified as *P. nitroreducens* strain.
Many physiological features of *P. nitroreducens* DSM 14399^T have been described (Iizuka and Komagata 1964; Lang et al. 2007). However, none of these abilities has been compared to genomic data. Protein domain analysis was used to capture metabolic similarities and differences between *Pseudomonas* spp. by correlating protein domains prediction to known physiological data (Chapter VI). The degradation of several substrates by *P. nitroreducens* DSM 14399^T was corroborated by comparing presence or absence of encoding enzymes, predicted by protein domains analysis. This study allowed testing the use of protein domains analysis for establishing taxonomical relationships between *Pseudomonas* strains by using functional profiles, confirming also several physiological abilities of different *Pseudomonas* strains.
While SDS degradation by *P. aeruginosa* strains was described (Swisher 1987; Thomas and White 1989; Jovčić et al. 2009) degradation of anionic surfactants by *P. nitroreducens* DSM 14399^T was not known. Results described here revealed the ability of isolated *P. nitroreducens* strains and of *P. nitroreducens* DSM 14399^T to degrade and grow using SDS and SLES coupled to nitrate reduction. If this ability is a common property among *P. nitroreducens* strains is currently not known. However, genome analysis indicated that the *sdsA1* hydrolase related to SDS degradation and found in several *P. aeruginosa* strains (Hagelueken et al. 2006; Jovčić et al. 2009) is also present in *P. nitroreducens* DSM 14399^T and in `*P. denitrificans´* ATCC 13867 (Chapter VI).

FUTURE RESEARCH PERSPECTIVES

SDS and SLES degradation coupled to denitrification at high surfactant concentration was achieved by *P. stutzeri* strain SN1, *P. nitroreducens* strain SN2, *P. stutzeri* strain S8 and *P. nitroreducens* strain S11. However, differences that were found between *P. stutzeri* and *P. nitroreducens* isolates could be further investigated by testing surfactant concentration range required for optimal growth. Because enzymes required for surfactant cleavage may be more sensitive to the surfactant than whole cells, sulfate accumulation by cell-free extracts of all four strains could also be compared for different surfactant concentrations. These tests would give a better insight into the inhibition effects observed with *P. stutzeri* SN1. Further tests are required to better characterize the four strains physiologically and biochemically and to understand how different or similar the two pairs of *Pseudomonas* spp. are.

Comparison between SLES degradation under aerobic and anoxic conditions requires in-depth research. Monitoring SLES degradation using also sulfur isotope measurements might be important for understanding how much sulfate is liberated from SLES cleavage or from SLES mineralization in aerobic and anoxic conditions. These tests could be performed using pure bacterial cultures (e.g. *P. nitroreducens* S11) and mixed bacterial cultures (activated sludge). Also, detection of intermediate compounds formed during SLES degradation in both anoxic and aerobic conditions is needed. *Pseudomonas* were enriched as the dominant SLES-degraders in both anoxic and aerobic conditions. However, aerobic SLES degradation between *Acinetobacter* strain S15 and *P. nitroreducens* S11 needs to be compared.

To make a link with practice, the results obtained with enrichments and pure cultures need to be compared with information that can be obtained in a continuous reactor with activated sludge operated with oxygen and nitrate and different concentrations of SDS and SLES. Testing different concentrations would allow understanding if bacterial enrichment and selection would be similar or different to our results obtained with batch tests.

Surfactants degradation in a WWTP using the A^2/O process can occur during anaerobic digestion, anoxic and aerobic processes. Degradation of SDS and SLES could be tested, together with real wastewater, using a lab scale anaerobic-anoxic-aerobic reactors sequence. This would allow identifying the surfactant tolerant microorganisms present in these three conditions with ability for surfactants degradation. It would also allow understanding in which reactor surfactants are mainly degraded and which surfactant concentration could disturb the different biological processes.

Another relevant study would be to test SDS and SLES degradation using anaerobic sludge without adding a sulfate source and without adding any other electron acceptor added to the medium. In this way, sulfate released from surfactants due to hydrolysis could be used as electron acceptor by sulfate-reducing bacteria. In this case, surfactant degradation could be coupled to sulfate reduction and methanogenesis. Surfactants can inhibit methanogens and subsequently the methane production in an anaerobic digestion process. Different concentrations of surfactant could be tested to determine the maximum concentration at which methanogens are active.

The degradation of surfactants in interaction with other compounds also found in domestic wastewaters (e.g. lipids, cationic surfactants, real domestic wastewater) is important to investigate as well, by comparing batch with continuous reactor experiments.

Surfactants can be used for enhancing bioremediation efficiency in soils contaminated with hydrophobic compounds (Zhang and Zhu 2014). Tests could be performed using

SDS/SLES degrading denitrifying bacteria to evaluate SDS or SLES degradation alone and together with different hydrophobic compounds in anaerobic conditions, using nitrate as electron acceptor.

P. nitroreducens is closely related to *P. aeruginosa*, possibly sharing the ability for SDS degradation between the two species. Different *P. nitroreducens* and *P. aeruginosa* strains could be tested using SDS as sole carbon and energy source coupled to nitrate reduction. This would give a better insight into differences found between these two *Pseudomonas* species related to the degradation of surfactants in anoxic conditions. In that case it will become clear if SDS degradation is a widespread property in these two *Pseudomonas* species.

Sequencing and analysis of *P. nitroreducens* DSM 14399^{T} genome confirmed not only its taxonomical classification but also its ability for degradation of SDS and other substrates. Continuing protein domain analysis of this genome would give a better insight of special features of *P. nitroreducens* compared to other *Pseudomonas* species, namely *P. aeruginosa* and other species close related to *P. nitroreducens*. Being *P. aeruginosa* closely related to *P. nitroreducens*, it could be relevant to identify differences and similarities between these two species. For this, genome data analysis could be complemented using laboratory experiments, related to, for example, different xenobiotics degradation.

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SAMENVATTING

Oppervlakte-actieve stoffen worden geproduceerd en gebruikt voor de synthese van verschillende commerciële producten. Na gebruik komen deze stoffen vaak in rioolwaterzuiveringsinstallaties (RWZI) of in het milieu terecht. Hoewel veel oppervlakte-actieve stoffen kunnen worden afgebroken met zuurstof (aërobe omstandigheden), komen anaërobe omstandigheden veelvuldig in de natuur en in RWZI voor. De verwijdering van organische stof alsook van stikstof en fosfor wordt in RWZI vaak bewerkstelligd middels een anaëroob-anoxisch-aëroob (A^2/O) proces. Met behulp van het A^2/O proces kunnen oppervlakte-actieve stoffen al in het anaërobe dan wel anoxische compartiment afgebroken worden. In het anoxische compartiment kunnen facultatief anaërobe bacteriën oppervlakte-actieve stoffen afbreken met behulp van nitraat/nitriet als elektron acceptor. Er is echter niet veel bekend over denitrificerende bacteriën die oppervlakte-actieve stoffen kunnen afbreken.

In dit proefschrift wordt de isolatie van *Pseudomonas stutzeri* stam SN1 en *Pseudomonas nitroreducens* stam SN2 uit actief slib van een RWZI met een A^2/O proces beschreven. Deze twee denitrificerende bacteriën kunnen op dodecyl sulfaat (SDS) als enige koolstof- en energiebron groeien (hoofdstuk II).

In de verschillende compartimenten van het A^2/O proces, waar verschillende elektron acceptoren worden gebruikt, kan de microbiële diversiteit van micro-organismen die oppervlakte-actieve stoffen afbreken verschillen. Tevens kan de aanwezigheid van oppervlakte-actieve stoffen van invloed zijn op de activiteit van micro-organismen en op die manier de efficiëntie van het zuiverigsproces beinvloeden. Hierdoor kunnen verhoogde concentraties van oppervlakte-actieve stoffen aanleiding geven tot verschilllen in de bacteriële diversiteit in de compartimenten van het A^2/O proces. De afbraak van laurylsulfaat (SLES) onder aërobe omstandigheden is bekend. Dit is niet het geval voor denitrificerende condities. De bacteriële diversiteit in ophopingsculturen die SLES met zuurstof en nitraat afbreken is onderzocht in dit proefschrift. *Aeromonas hydrophila* stam S7, *Pseudomonas stutzeri* stam S8 en *Pseudomonas nitroreducens* stam S11 werden met nitraat geïsoleerd. *Comamonas testosteroni* stam S13 en *Acinetobacter* stam S15 werden met zuurstof geïsoleerd (hoofdstuk III). De eerste biochemische stappen van de afbraak van SLES zijn eerder al onderzocht, maar veel is nog onbekend over de afbraak van ether structuren in chemische verbindingen en welke bacteriële enzymen daarbij betrokken zijn. *Aeromonas hydrophila* stam S7, *Pseudomonas stutzeri* stam S8 en *Pseudomonas nitroreducens* stam S11 zijn in staat SLES met nitraat af te breken (hoofdstuk III). De anaërobe afbraak van SLES door de drie stammen is vergeleken. *P. nitroreducens* stam S11 bleek het beste in staat SLES met nitraat af te breken en is ook een uitstekende aërobe

SLES afbreker (Hoofdstuk IV). Waarschijnlijk zijn dezelfde sulfatases en ether-splitsende enzymen betrokken bij de afbraak van SLES met nitraat en met zuurstof, hoewel de resultaten laten zien dat de afbraak met zuurstof sneller verloopt.

Hoewel oppervlakte-actieve stoffen toxisch kunnen zijn voor micro-organismen zijn er oppervlakte-actieve stof-afbrekende bacteriën bekend die bestand zijn tegen zeer hoge concentraties. Het betreft veelal aërobe bacteriën; de afbraak door denitrificerende bacteriën is echter nauwelijks onderzocht. Bacteriën die hoge concentraties oppervlakte-actieve stoffen kunnen afbreken met nitraat kunnen erg bruikbaar zijn in RWZI. Het effect van hoge concentraties SDS en SLES op SDS/SLES-afbrekende bacteriën (*P. stutzeri* stam SN1, *P. nitroreducens* stam SN2, *P. stutzeri* stam S8 en *P. nitroreducens* stam S11), is onderzocht (hoofdstuk V). *P. stutzeri* stam SN1 werd in groei geremd bij toenemende SDS en SLES concentraties. In het algemeen bleken de *P. nitroreducens* stammen beter bestand te zijn tegen hoge concentraties oppervlakte-actieve stoffen dan *P. stutzeri* stammen. Echter, hoge concentraties van SDS en SLES remden de groei en nitraatreductie van de geteste *Pseudomonas* stammen niet volledig.

Eiwitdomeinen vertegenwoordigen de evolutionair geconserveerde, onafhankelijk vouwende, functionele bouwstenen van de eiwitten. Vaststelling van eiwitdomeinen op basis van genoominformatie kan worden gebruikt om bacteriesoorten in te delen en te valideren op basis van bekende fysiologische eigenschappen. Deze methode is gebruikt voor *P. nitroreducens,* facultatief anaërobe bacteriën uit de *P. aeruginosa* groep, die complexe verbindingen kunnen afbreken. *P. nitroreducens* DSM 14399T en *P. nitroreducens* stam SN2 kunnen beide SDS onder zuurstofloze condities met nitraat afbreken. Om meer inzicht te krijgen in de fylogenetische classificatie en fysiologische eigenschappen (waaronder SDS afbraak) van *P. nitroreducens* DSM 14399T is het genoom sequenced en vergeleken met de genomen van andere *Pseudomonas* soorten. Functionele profielen op basis van de aan- en afwezigheid van eiwit domeinen zijn vergeleken met fysiologische eigenschappen (hoofdstuk VI). Met deze benadering werd de *P. nitroreducens* classificatie bevestigd. Eiwitdomein analyse en de annotatie van genen werden in verband gebracht met het vermogen om SDS af te breken. Dit onderzoek toonde aan dat eiwitdomeinen en functionele profielen gebruikt kunnen worden voor het bestuderen en vergelijken van verschillende *Pseudomonas* soorten.

ABOUT THE AUTHOR

Ana Paulo was born on February 1st 1980, in Coimbra (Portugal). She studied Environmental Engineering at the University of Aveiro (Portugal) between 1998 and 2004. At the beginning of 2005, she volunteered to work in the PhD project of Luís Fonseca for 3 months, while getting laboratory skills related to the study of the pine wood nematode Bursaphelencus Xylophilus, in the Department of Zoology of the University of Coimbra (Portugal), under the supervision of Isabel Abrantes. Still during 2005 and 2006, she started working at the University of Minho (Portugal), on the optimization of the anaerobic degradation of textile effluents, in collaboration with CITEVE (Textile and Clothing Industry Technologic Center of Portugal). This project was supervised by Madalena Alves and Frank van der Zee. From the end of 2006 until the end of 2008, Ana Paulo worked in a project related to the monitoring of biological wastewater treatment systems, employing spectrometric techniques based on the use of UV-visible and near-infrared immersible probes in-situ, at the University of Minho, under the supervision of Eugénio Ferreira and Ana Dias. Also during 2006 and 2008, she got her Master degree on Environmental Management, at the University of Minho. Between the end of 2008 and beginning of 2013, she worked in a PhD project related to the study of anaerobic degradation of surfactants. This project, financed by the NOVEDAR_Consolider network, resulted from the collaboration between the University of Valladolid (Spain), under the supervision of Pedro García Encina, and the University of Wageningen (The Neterlands), under the supervision of Fons Stams and Caroline Plugge. In July 2014 she started her collaboration in the project OIL LESS, related to the anaerobic degradation of oils, at the University of Minho (Portugal) under the supervision of Ana Júlia Cavaleiro.

LIST OF PUBLICATIONS

Sarraguça, M.C., **Paulo, A.,** Alves, M.M., Dias, A.M.A., Lopes, J.A., Ferreira, E.C., 2009. Quantitative monitoring of an activated sludge reactor using on-line UV-visible and near-infrared spectroscopy. Analytical and Bioanalytical Chemistry vol. 395 (4), pag. 1159-1166.

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Paulo, A. M. S., García-Encina, P. A., Plugge, C. M. and Stams, A. J. M.. Degradation of sodium lauryl ether sulfate (SLES) by facultative anaerobic bacteria. *To be submitted*.

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Koehorst, J. J., **Paulo, A. M. S.**, Nijsse, B., García-Encina, P. A., Plugge, C. M., Stams, A. J. M., Schaap, P. J..Domainome analysis of *Pseudomonas nitroreducens* DSM 14399^{T}. *To be submitted*.

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