SULFIDE AND METHANE PRODUCTION IN ANAEROBIC SEWER PIPES: FROM MICROBIAL COMMUNITY CHARACTERIZATION TO EFFECTIVE MITIGATION STRATEGIES

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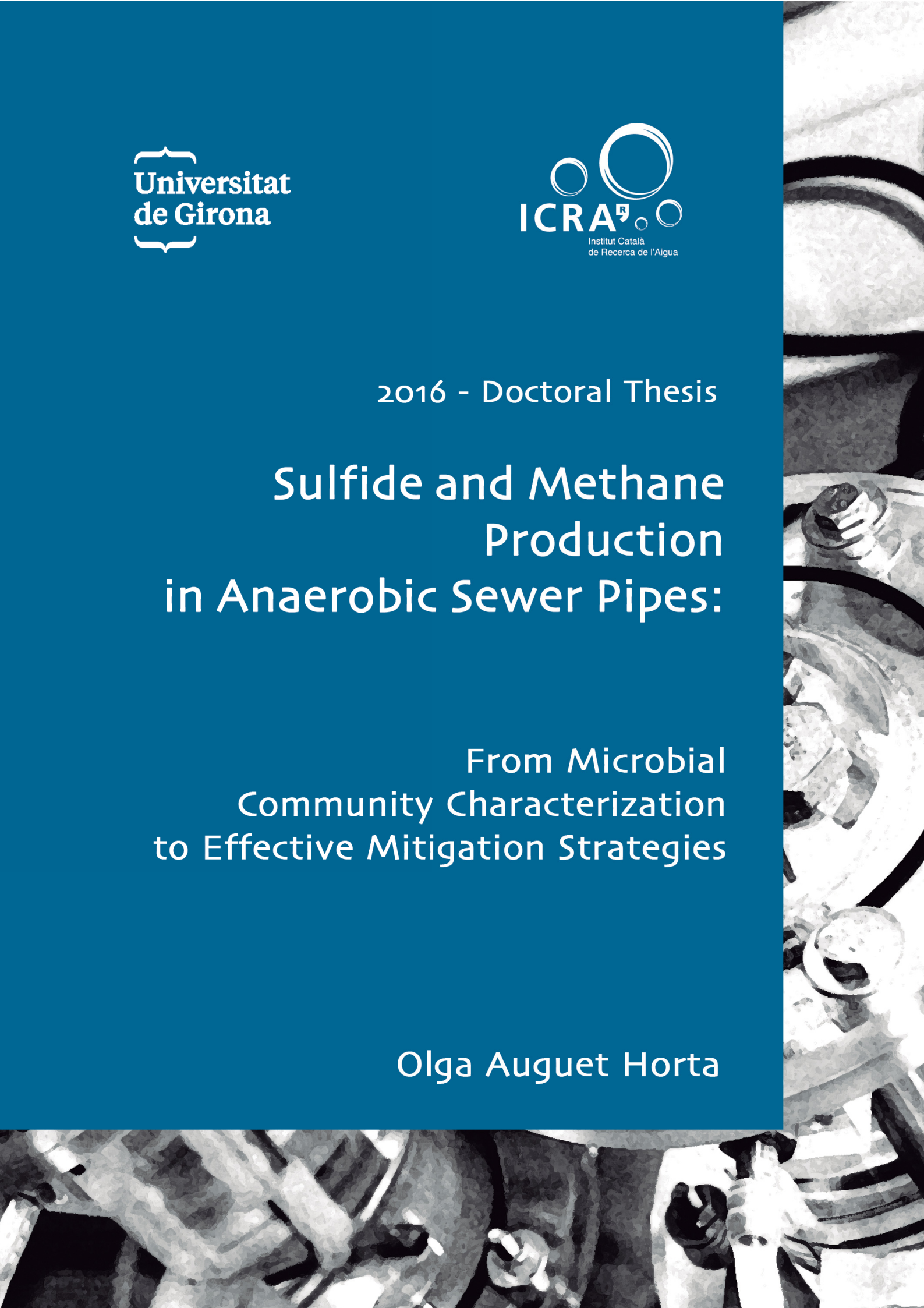

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Sulfide and methane production in anaerobic sewer pipes: from microbial community characterization to effective mitigation strategies

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Doctoral Programme in Water Science and Technology

Supervised by: Dr. Oriol Gutierrez Garcia-Moreno, Dr. Carles Borrego Moré and Dr. Maite Pijuan Vilalta

Tutor: Dr. Carles Borrego Moré

Thesis submitted in fulfillment of the requirements for the degree of Doctor from the University of Girona

El Dr. Oriol Gutierrez Garcia-Moreno i la Dra. Maite Pijuan Vilalta, investigadors de l'Institut Català de Recerca de l'Aigua (ICRA) i el Dr. Carles Borrego Moré, professor del departament de Microbiologia de la Universitat de Girona i investigador de l'ICRA.

Declarem

Que la llicenciada en Biologia **Olga Auguet Horta** ha realitzat, sota la direcció del Dr. Oriol Gutierrez Garcia-Moreno, el Dr. Carles Borrego Moré i la Dra. Maite Pijuan Vilalta el treball titulat "**Sulfide and methane production in anaerobic sewer pipes: from microbial community characterization to effective mitigation strategies**", que es presenta en aquesta memòria la qual constitueix la seva tesi per optar al Grau de Doctora per la Universitat de Girona.

I perquè en prengueu coneixement i tingui els efectes que corresponguin, presentem davant la Facultat de Ciències de la Universitat de Girona l'esmentada tesi, signant aquesta document.

Girona, 30 de Maig de 2016

Dr. Oriol Gutierrez Garcia-Moreno

Dr. Carles Borrego Moré

Dra. Maite Pijuan Vilalta

Agraïments

Arribar fins al final d'aquesta etapa ha estat possible perquè he tingut gent molt important al meu costat que m'ha ajudat i m'han donat forces per continuar.

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List of publications derived from the thesis

This doctoral thesis is a compendium of research articles. The publications presented as chapters of this PhD thesis are listed below:

Auguet, O., Pijuan, M., Batista, J., Borrego, C.M., Gutierrez, O., 2015. Changes in Microbial Biofilm Communities during Colonization of Sewer Systems. *Appl. Environ. Microbiol*. 81, 7271–7280. Impact factor 2014: 3.668 (5-year 4.359). 1st Quartile in Applied Microbiology and Biotechnology.

Auguet, O., Pijuan, M., Guasch-Balcells, H., Borrego, C.M., Gutierrez, O., 2015. Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions. *Water Res*. 68, 522–532. Impact factor 2014: 5.528 (5-year 6.279). 1st Quartile in Water Science and Technology.

Auguet, O., Pijuan, M., Borrego, C.M., Gutierrez, O. Control of sulfide and methane production in anaerobic sewer systems by means of Downstream Nitrite Dosage. *Sci. Total Environ.* 550, 1116-1125. Impact factor 2014: 4.099 (5-year 4.414). 1st Quartile in Water Science and Technology. *

** This paper was proposed as a special recommended read by the edithor of the Journal.*

List of acronyms

2D: two-dimensional
ARG: antibiotic resistance genes
BLAST: Basic Local Alignment Search Tool
BSA: bovine serum albumin
BT: batch test
COD: chemical oxygen demand
DGGE: denaturing gradient gel electrophoresis
DNA: deoxyribonucleic acid
DND: downstream nitrate dosage
DNO_2D: downstream nitrite dosage
dsrA: dissimilatory sulfate reductase subunit A
hNRB: heterotrophic, nitrate reducing bacteria
HRT: hydraulic retention time
IC: ion chromatography
MA: methanogenic archaea
NF: normal functioning
NF-BT: normal functioning-batch tests
NMDS: Nonmetric multidimensional scaling
NRB: nitrate reducing bacteria
NR-SOB: nitrate-reducing, sulfide-oxidizing bacteria
OTU: operational taxonomic unit
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
qPCR: quantitative polymerase chain reaction
RNA: ribonucleic acid
SAOB: solution antioxidant buffer
SCFA: short-chain fatty acids
sCOD: soluble chemical oxygen demand
SCORe-CT: sewer corrosion and odour research–chemical testing
soNRB: sulfide-oxidizing nitrate-reducing bacteria
SRB: sulfate reducing bacteria
SSU: small subunit
TAE: Tris acetate ethylenediaminetetraacetic acid
TSS: total suspended solids
VFA: volatile fatty acid
VSS: volatile suspended solids
WWTP: wastewater treatment plant

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Summary

Sewer systems consist of an underground network of pipelines, pumping stations, manholes, and channels that convey wastewaters from their source to the discharge point, usually a wastewater treatment plant (WWTP). However, sewers are not conduits that passively transport wastewater but artificial ecosystems characterized by complex microbial networks that transform wastewater along its transport. Besides, microorganisms in wastewater readily colonize inner surfaces of sewer pipelines, forming biofilms that have major effects on the quality of flowing water. In these biofilms, sulfate-reducing bacteria (SRB) and methanogenic archaea (MA) causes the build-up of sulfide (H_2S) and methane (CH_4), respectively. Both compounds may have undesired consequences for both the environment and the human health. The release of H_2S to the sewer atmosphere causes malodour, corrosion and health problems. Moreover, CH_4 is a potent greenhouse gas with higher global warming potential than carbon dioxide and it poses an additional risk in confined spaces due to its low explosion limit. For these reasons a good control of H_2S and CH_4 emissions from sewer systems is essential for the optimal management of these facilities.

Although different studies have been done to identify the main microbial players involved in the production of H_2S and CH_4 in sewers, very little information is available on the colonization dynamics and activity of SRB and MA in these systems. To fill this gap, the first part of this thesis was focused on the study of microbial biofilm colonisation (mainly SRB and MA) and H_2S and CH_4 production capabilities in pressure pipes during the early stages of system operation compared with mature biofilms. The results showed that SRB were present and active from the second week of system operation but methanogenic community was progressively adapted to sewer conditions along time. These changes greatly affected CH_4 emission, which increased after one year of system functioning.

Another important aspect of sewers is the development and optimization of different mitigation strategies to reduce H_2S and CH_4 production in sewers. Concretely, two comparative studies were done to test the addition of nitrate (NO_3^-, Chapter III) and nitrite (NO_2^-, Chapter IV) at different locations. The addition of nitrate and nitrite at downstream sections of the system were tested for the first time in this thesis to determine its effects on H_2S and CH_4 emissions and to compare their effectiveness. Downstream Nitrate Dosage (DND) and Downstream Nitrite Dosage (DNO_2D) caused a complete abatement of H_2S emissions and a reduction of approximately 50% (DND) and 80% (DNO_2D) in CH_4 emissions during the addition period.

Although DNO_2D seems to be the best option, a high H_2S overproduction was detected after ceasing nitrite addition in comparison to nitrate. This response was probably a

consequence of the large accumulation of sulfur compounds accumulation (*e.g.* elemental sulfur) in the biofilm during the dosage period due to the biological oxidation of sulfide by nitrogen oxides (nitrate/nitrite). Moreover, the addition of these two compounds triggered changes on the relative abundance of *Betaproteobacteria* and *Gammaproteobacteria,* members of which could be involved in denitrification, H_2S oxidation and CH_4 oxidation processes. Furthermore, archaeal communities also undergone important changes in their composition during nitrite addition, favouring hydrogenotrophic over acetoclastic methanogens.

Overall, the results of this work can be useful for sewer managers to determine the suitable alternative to control H_2S and CH_4 emissions in these systems. Also, this thesis provides fundamental knowledge on the microbial communities present in sewer biofilms and their role in biotransformation processes that can be highly relevant in terms of environmental and public health issues.

Resum

Els col·lectors d'aigües residuals són xarxes subterrànies de tubs, pous i canals que s'utilitzen pel transport d'aigües residuals des del seu origen fins el punt de descàrrega, normalment una estació depuradora d'aigua residual (EDAR). Ara bé, el clavegueram no és només una xarxa de conductes per el transport passiu d'aigües residuals sinó que pot considerar-se un ecosistema complex on les diferents comunitats microbianes transformen activament l'aigua residual durant el seu transport. A més, aquests microorganismes colonitzen fàcilment les superfícies interiors de les canonades del clavegueram, formant biopel·lícules que tenen efectes importants sobre la qualitat de l'aigua. En aquestes biopel·lícules, els bacteris sulfato reductors (SRB) i els arqueus metanògens (MA) produeixen sulfhídric (H_2S) i metà (CH_4), respectivament, els quals s'acumulen en l'atmosfera dels col·lectors, representant un risc tant per el medi ambient com per la salut humana. L'alliberació del H_2S a l'atmosfera del clavegueram provoca males olors, corrosió i toxicitat per els organismes superiors. A més, el CH_4 és un potent gas d'efecte hivernacle amb un major efecte que el diòxid de carboni en l'escalfament global. El metà també constitueix un risc addicional en espais tancats degut al seu baix límit d'explosió. Per totes aquestes raons, un bon control de l'acumulació de H_2S i CH_4 en els sistemes de clavegueram és essencial per la bona gestió d'aquestes infraestructures.

Encara que s'han realitzat diferents estudis per identificat els principals microorganismes implicats en la producció de H_2S i CH_4 a les xarxes de clavegueram, hi ha molt poca informació sobre la dinàmica de colonització i activitat de SRB i MA en aquests sistemes. El treball experimental dut a terme en aquesta tesi pretén, precisament, omplir aquest buit. En primer lloc es va realitzar un estudi comparatiu de la colonització microbiana de les superfícies de col·lectors experimentals de laboratori per investigar amb detall la formació de biopel·lícules i la seva capacitat per produir H_2S i CH_4 durant les primeres etapes d'operació del sistema i en biopel·lícules madures. Els resultats van mostrar que els SRB ja estaven actius des de la segona setmana de funcionament del sistema però que les comunitats metanogèniques de les biopel·lícules s'anaven adaptant progressivament a les condicions del clavegueram. Aquesta successió va afectar considerablement les emissions de CH_4 les quals van incrementar després d'un any de funcionament del sistema.

Un altre aspecte rellevant del clavegueram és el desenvolupament i optimització de diferents estratègies encaminades a la reducció de les emissions de H_2S i CH_4 en aquests sistemes. En concret, es van realitzar dos estudis comparatius on es va provar l'aplicació de nitrat (NO_3^-, Capítol III) i nitrit (NO_2^-, Capítol IV) a diferents punts de dosificació. L'addició de nitrat i nitrit en les seccions terminals del sistema es va provar per primera vegada en aquesta tesi amb l'objectiu d'avaluar el seu efecte sobre les emissions de H_2S i CH_4, tot

comparant-ne la seva efectivitat. L'addició de nitrat (DND) i nitrit (DNO_2D) en seccions terminals van causar una reducció completa de les emissions de H_2S i una reducció d'aproximadament el 50% (DND) i el 80% (DNO_2D) en les emissions de CH_4 durant el període de dosificació.

Tot i que la DNO_2D sembla la millor opció, aquest tractament va donar a lloc a una sobreproducció de H_2S al finalitzar el període d'addició. Aquesta resposta va ser deguda probablement a la gran acumulació de compostos de sofre (per exemple, sofre elemental) en les biopel·lícules durant el període de dosificació a causa de l'oxidació biològica del sulfhídric a partir de les espècies oxidades de nitrogen (nitrat/nitrit). Per altra banda, l'addició dels dos compostos va provocar canvis en l'abundància relativa de *Betaproteobacteria* i *Gammaproteobacteria,* els membres dels quals podrien estar implicats en processos de desnitrificació, oxidació de H_2S i oxidació de CH_4. A més, les comunitats d'arqueus també van registrar canvis importants en la seva composició durant l'adició de nitrit, afavorint les espècies hidrogenotròfiques respecte els acetoclàstiques.

Els resultats d'aquest treball poden ser de gran utilitat per als gestors de les xarxes de clavegueram ja que permeten valorar amb dades reals quina és la millor estratègia per al control adequat de les emissions de H_2S i CH_4 en aquests sistemes. A més, aquesta tesi doctoral representa una contribució significativa pel coneixement de les comunitats microbianes que formen les biopel·lícules en els col·lectors del clavegueram i la seva participació en processos de biotransformació que són de gran rellevància des del punt de vista ambiental i de salut pública.

Resumen

Los colectores de aguas residuales son redes subterráneas de tubos, pozos y canales que se utilizan para el transporte de aguas residuales desde su origen hasta el punto de descarga, normalmente una estación depuradora de aguas residuales (EDAR). Sin embargo, el alcantarillado no es solo una red de conductos donde esta agua se transporta pasivamente sino que puede considerarse un ecosistema complejo donde las comunidades microbianas la transforman activamente durante el transporte. Además, estos microorganismos colonizan fácilmente las superficies interiores de tuberías de alcantarillado, formando biopelículas cuya actividad tiene efectos importantes sobre la calidad del agua. En estas biopelículas, las bacterias sulfato reductoras (SRB) y las archaea metanógenas (MA) producen sulfhídrico (H_2S) y metano (CH_4), respectivamente, que se acumulan en la atmósfera del alcantarillado representando un riesgo tanto para el medio ambiente como para la salud humana. La liberación de H_2S a la atmósfera del alcantarillado provoca malos olores, corrosión y toxicidad para los organismos superiores. Además, el CH_4 es un gas de efecto invernadero con mayor efecto que el dióxido de carbono en el calentamiento global. El metano también constituye un riesgo adicional en espacios cerrados debido a su bajo límite de explosión. Por todas estas razones, un buen control de la acumulación de H_2S y CH_4 en los sistemas de alcantarillado es esencial para la buena gestión de estas infraestructuras.

Aunque se han realizado diferentes estudios para identificar los principales microorganismos implicados en la producción de H_2S y CH_4 en las redes de alcantarillado, existe muy poca información sobre la dinámica de colonización y la actividad de SRB y MA en estos sistemas. El trabajo experimental llevado a cabo en esta tesis pretende pues llenar este vacío. En primer lugar se realizó un estudio comparativo de la colonización microbiana de las superficies de colectores experimentales de laboratorio para investigar con detalle la formación de biopelículas y su capacidad para producir H_2S and CH_4 tanto durante las primeras etapas de colonización como en biopelículas maduras. Los resultados confirmaron que las SRB eran activas ya desde la segunda semana de funcionamiento del sistema aunque las comunidades metanogénicas que formaban las biopelículas mostraron una adaptación progresiva a las condiciones del alcantarillado. Esta sucesión afectó considerablemente las emisiones de CH_4, las cuales incrementaron después de un año de funcionamiento del sistema.

Otro aspecto relevante del sistema de alcantarillado es el desarrollo y optimización de diferentes estrategias encaminadas a la reducción de las emisiones de H_2S and CH_4 en estos sistemas. En concreto se realizaron dos estudios comparativos donde se ensayó la aplicación de nitrato (NO_3^-, Capítulo III) y nitrito (NO_2^-, Capítulo IV) a diferentes

concentraciones y en diferentes puntos de dosificación. La adición de nitrato o nitrito en las secciones terminales del sistema fue ensayada por primera vez en esta tesis con el objetivo de evaluar su efecto en las emisiones de H_2S and CH_4 y comparar su efectividad. La adición de nitrato (DND) y nitrito (DNO_2D) en secciones terminales causaron una reducción completa de las emisiones de H_2S y una reducción del 50% (DND) y 80% (DNO_2D) en las emisiones de CH_4 durante el periodo de adición, aproximadamente.

Si bien a partir de los resultados la DNO_2D parece la mejor opción, este tratamiento resultó en una sobreproducción de H_2S al finalizar el periodo de adición. Esta respuesta fue debida probablemente a la gran acumulación de compuestos de azufre (por ejemplo, azufre elemental) en las biopelículas durante el período de dosificación a causa de la oxidación biológica del sulfhídrico a partir de las especies oxidadas de nitrógeno (nitrato/nitrito). Por otra parte, la adición de ambos compuestos provocó cambios en la abundancia relativa de *Betaproteobacteria* y *Gammaproteobacteria*, cuyos miembros podrían estar implicados en procesos de desnitrificación, oxidación de H_2S y oxidación de CH_4. Además, la composición de las comunidades de archaea también registró cambios importantes durante la adición de nitrito, favoreciendo a las metanógenas hidrogenotróficas respecto las acetoclásticas.

Los resultados de este trabajo pueden ser de utilidad para los gestores de las redes de alcantarillado ya que permiten valorar, con datos reales, cuál es la mejor estrategia para el control adecuado de las emisiones de H_2S y CH_4 por estos sistemas. Esta tesis doctoral representa, además, una contribución significativa en el conocimiento de las comunidades microbianas que forman biopelículas en los colectores del alcantarillado y su participación en procesos de biotransformación que son de gran relevancia desde el punto de vista ambiental y de salud pública.

Block I

INTRODUCTION AND OBJECTIVES

Chapter 1
General introduction

1.1. Sewer systems

1.1.1. Development of sewer systems

The generation of wastewater as a result of human activity and its collateral effects generated the need for its management long time ago. The development of sewers and drainage systems became necessary when urban settlements were established during the urban revolution (≈ 7,000 before Christ) in order to eliminate the wastewater from houses or surface runoff. The construction of underground wastewater collection systems was not common before the Second Industrial Revolution (≈ 1,830 in Europe and America). Underground systems used then to collect wastewater were developed to solve the enormous problem of the unpleasant smell from the open sewers, cesspools, and privies and to save space in the streets (Hvitved-Jacobsen et al., 2013).

Moreover, sewers systems were also considered as a hygienic and sanitary installation for the prevention of pandemics and infections. This concept started to be developed from the late 18th century; one example well related with wastewater contamination of drinking water sources was the cholera pandemics (1848 and 1854). At that time, the English physician John Snow detected that outbreaks of cholera occurred in a specific area which used the same water supply. He concluded that the infected humans excreted a *materia morbus* that was transported into the drinking water system. Although he identified that the polluted water (contaminated with human excreta) was the vector of the cholera disease, it was not until the isolation of *Vibrio cholera* by Robert Koch when the cause of the disease was finally established (Hvitved-Jacobsen et al., 2013). Therefore, the development of sewer systems was useful for the separation of wastewater and drinking water.

Another important aspect is that the wastewater collected in cities was directly discharged into the environment without being treated, causing alterations such as bacterial contamination, toxicity, eutrophication and disturbances in animal species. Wastewater treatment was not developed until after World War II (Hvitved-Jacobsen et al., 2013). Nowadays, sewer networks are systems used to transport wastewater from cities (the source of generation) to the wastewater treatment plants (WWTPs) where it is treated before its release into the environment.

The human population growth and the environment contamination highlight the need to develop more efficient sewage systems, improving wastewater treatment technologies. This has become the most important purpose of the wastewater industry until today.

1.1.2. Different types of sewer pipes

Sewer pipes can be classified into two categories depending on the topography of the zone: gravity and pressure sewers (Fig. 1.1). Wastewater generated in the different sources convey by gravity to a central location were the wastewater is accumulated using gravity sewers. The slope of these pipes allow the flow of the wastewater naturally through the system generating aerobic conditions most of the times due to the presence of gas phase (sewer atmosphere) that facilitate the re-aeration of wastewater. However, when the transport of the wastewater by gravity is not possible because of *e.g.* the presence of a hill or the different level location of the WWTP, then the sewage has to be pumped in pump stations (where wastewater is accumulated) through a pressure pipe to another gravity sewer. The main characteristic of pressure pipes is that they are always full of wastewater allowing anaerobic conditions. In both types of pipes, the environmental conditions and the wastewater characteristics favour the formation of biofilm on the wall surfaces.

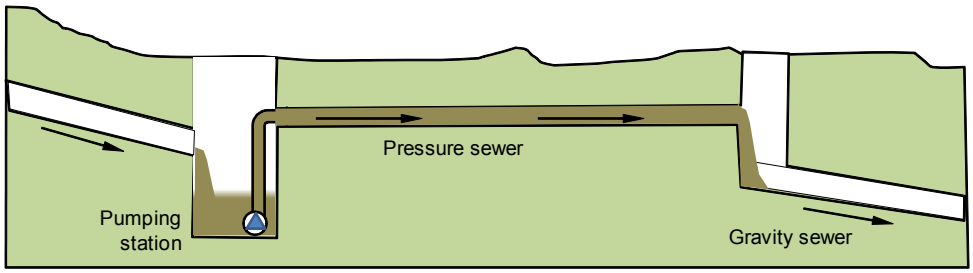

Figure 1.1. Scheme of the wastewater transport through gravity and pressure sewers using pumping stations (Gutierrez et al., 2016).

1.2. Microbial processes in sewer systems: sulfide and methane production.

Sewer systems should not only be considered as transport systems due to the active biomass present in the wastewater, in sewer deposits (sediments), in the biofilm attached to the wall of the pipe and walls exposed to the sewer atmosphere (Hvitved-Jacobsen et al., 2013). Microorganisms play a central role in the biotransformation of wastewater components during its transport along sewers before it is discharged to WWTP. Several microbiological transformations occur in sewer systems, basically in the biofilm developed in sewer walls. Transported wastewater contains the required organic matter and inorganic compounds that can be used by these microorganisms to grow and to survive.

Biofilms are structures formed by microbial communities that grow attached on surfaces. Different factors, such as large surface area, low flow velocity near pipe walls, and nutrient availability, favor microbial colonization of sewer surfaces and biofilm growth. Formation of fully functional biofilms occurs in six different steps: surface conditioning, adhesion of

microbial "colonizers," initial growth, and glycocalyx formation, followed by secondary colonization and growth (Dreeszen, 2003). Different reasons have been proposed to explain the advantages that confer the formation of biofilms. First, biofilm matrix protects cells, increasing survival and gaining resistance. Second, biofilm structures allow cells to remain in a favourable place. Finally, biofilm formation allows microbial communities to live in association and interact thus favouring syntrophic relationships (Madigan et al., 2012). For that reason, a complex interaction of different metabolisms occurs within biofilms.

Respiration and fermentation provide energy source to microorganisms that growth under anaerobic conditions. During anaerobic respiration, electron acceptors are needed to transfer electrons. On the other hand, different series of oxidative and reductive processes occur during fermentation, generating by-products with low molecular weight such as volatile fatty acids (VFA), carbon dioxide (CO_2) or hydrogen (H_2) which can then be used by other microorganisms, allowing the interaction of different microbial communities (*e.g.* sulfate reducing bacteria (SRB) and methanogenic archaea (MA)). Unfortunately, problematic compounds such as sulfide (H_2S) and methane (CH_4) are produced during these transformation processes. A summary of the main reactions that can occur in sewer systems is shown in Figure 1.2.

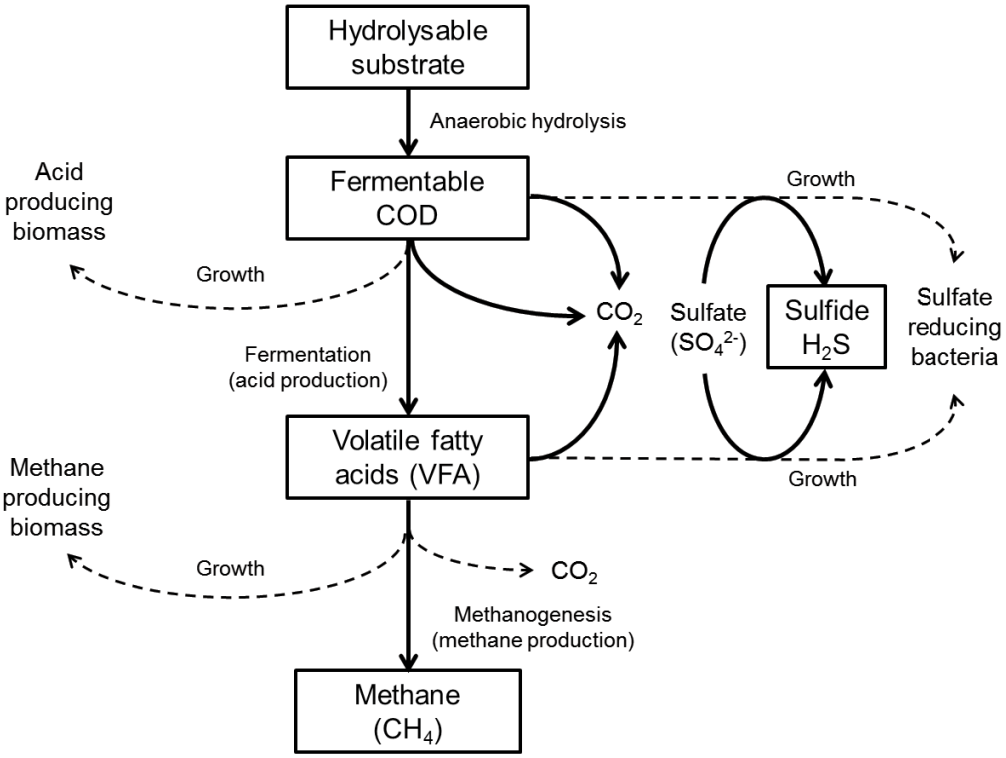

Figure 1.2. Scheme of the theoretical anaerobic transformations in wastewater and biofilm sewer systems (adapted from Hvitved-Jacobsen et al., 2013).

1.2.1. Sulfide production in sewer systems

Sulfate reducing bacteria are strict anaerobes thriving in anaerobic habitats and using sulfate (SO_4^{2-}) as electron acceptor for anaerobic respiration of organic matter that produce sulfide as end-product. Sulfate reducing bacteria are detected in different habitats such as oil fields (Nilsen et al., 1996), deep sub-surface (Kovacik et al., 2006), fresh water sediments (Sass et al., 1998), rhizosphere of plants (Bahr et al., 2005; Hines et al., 1999) and also in sewer systems (Gutierrez et al., 2008; Mohanakrishnan et al., 2011, 2009a, 2009b; Sun et al., 2014). Sulfide could also be produced during the degradation of organic sulfur compounds although this reaction is not considered as the main source of this compound in sewers (Hvitved-Jacobsen et al., 2013).

The general sequential pattern of microbial degradation of complex organic matter and the use of end products by SRB is summarized in Figure 1.3.

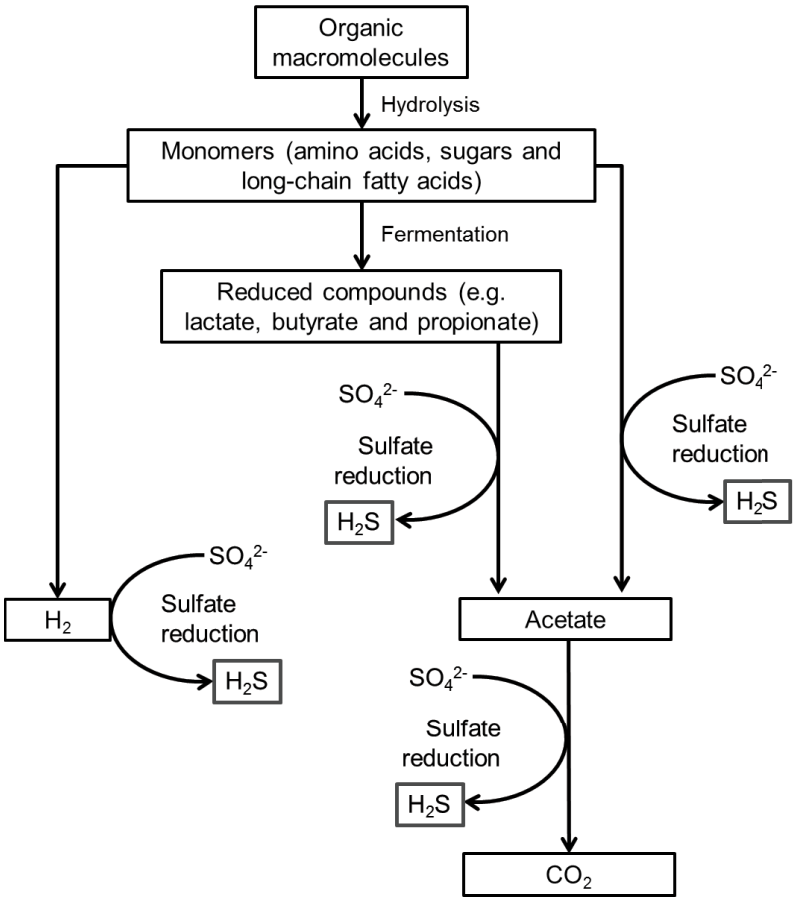

Figure 1.3. Organic matter degradation in anoxic environments carried out by sulfate reducing bacteria (adapted from Muyzer and Stams, 2008).

Organic macromolecules (*e.g.* proteins, polysaccharides and lipids) are degraded by hydrolytic bacteria producing monomers (*e.g.* amino acids, sugars and long-chain fatty

acids). Subsequently, these monomers are further degraded by fermentative bacteria into different end products (*e.g.* acetate, propionate, butyrate, lactate and H_2). Monomers, reduced compounds and H_2 resulting from fermentation can then be used by SRB to grow, producing H_2S that accumulates in the surroundings. If sulfate is used as electron acceptor and organic matter (*e.g.* acetate) as electron donor, SRB are considered strict heterotrophs (organotrophic microorganisms). If the reaction is carried out by lithotrophic microorganisms, sulfate is used as electron acceptor and hydrogen as electron donor. The main reactions are listed in Table 1.

Table 1.1. The main dissimilatory sulfate reduction reactions (Gutierrez et al., 2016).

Sulfate-reducing reactions	**ΔGo' (kJ/reaction)**
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	−151.9
$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−47.6
$Propionate^- + 0.75\ SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$	−37.7
$Butyrate^- + 0.5\ SO_4^{2-} \rightarrow 2\ Acetate^- + 0.5\ HS^- + 0.5\ H^+$	−27.8
$Lactate^- + 0.5\ SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.5\ HS^-$	−80.2

Sulfide is mainly produced in pressure sewers because of the anaerobic conditions present in this environment. In gravity sewers, sulfide production can also occur in deeper layers of sewer biofilms during slow water flow. However, during wastewater transport, H_2S is normally oxidized due to the presence of an air-phase and re-aeration processes. Under these conditions, different factors can determine sulfide production:

Sulfate concentration: Sulfate concentration is an important variable to take into account: the normal concentration detected in municipal wastewater is between 10 and 25 mgS/L (Gutierrez et al., 2010; Jiang et al., 2013, 2011a, 2010, 2009; Mohanakrishnan et al., 2009a, 2008). Moreover, thiosulfate ($S_2O_3^{2-}$) and sulfite can also be reduced by SRB. Indeed, bacteria prefer $S_2O_3^{2-}$ than SO_4^{2-} during H_2S production, increasing the rate production around two times (Nielsen, 1991). However, the concentrations of both compounds are usually negligible in wastewater transported in sewers (Auguet et al., 2015b; Gutierrez et al., 2008; Mohanakrishnan et al., 2009a). Moreover, when SO_4^{2-} is depleted then SRB can ferment organic compounds, producing H_2, acetate and CO_2 as end products which can be used by hydrogen- and acetate-scavenging methanogens to convert organic compounds to CH_4 (Plugge et al., 2011).

Biodegradable organic matter: biodegradable organic matter is used as substrate for growth and also as electron donor for SRB. In wastewater, organic compounds achieve high concentrations leading to a high potential for H_2S formation. Some organic compounds used during sulfate reduction are acetate, propionate, lactate and butyrate.

Temperature: temperature can also affect microbial metabolism and, particularly in this case, H_2S production. However, the development of different species that are adapted to different temperatures may allow the production of H_2S under a wide range of temperature conditions (Hao et al., 2014; Mackenzie, 2005).

pH: growth of SRB can be influenced by changes in pH. SRB can grow in a pH range from 3 to 9.8 (Barton and Tomei, 1995; Mackenzie, 2005). However, sudden changes in pH can reduce H_2S production by the adapted SRB at sewer conditions (Gutierrez et al., 2014, 2009). Moreover, pH also affects the sulfur speciation. The pKa of the equilibrium between H_2S and HS^- is 7.05, indicating that 50% of each form can be found at pH 7.05.

Area/volume ratio of the pipe: sulfide is mainly produced by SRB present in biofilms grown on pressure sewer pipes. For this reason, high A/V ratios (where A is the area surface of the pipe and V is the volume of the bulk water) result in high H_2S concentrations in the water phase.

Flow velocity and hydraulic retention time: the flow velocity also impacts the thickness of the biofilm thus affecting H_2S production rates. This factor also changes the anaerobic residence time or hydraulic retention time (HRT). The level of H_2S produced depends on the daily variation of the wastewater pumping: with higher HRT, more H_2S can be produced.

Impacts of H_2S production in sewer systems are diverse, including odour, corrosion and health problems. Release of H_2S produced during wastewater transport into the sewer atmosphere in gravity sewers (Fig. 1.4) causes concrete corrosion, malodour and toxicity. Biogenic H_2S corrosion is a microbial process in which H_2S gas is biochemically oxidized to sulfuric acid that induces pipe corrosion (Jiang et al., 2015). This phenomenon has a great economic impact because sewer structures need replacement after few years of operation. The H_2S produced can also cause structural damages in WWTP inflows. Sulfide in the gas phase also poses a risk for human health causing eye irritation, headache, memory and breathing problems and eventually death (Hvitved-Jacobsen et al., 2013).

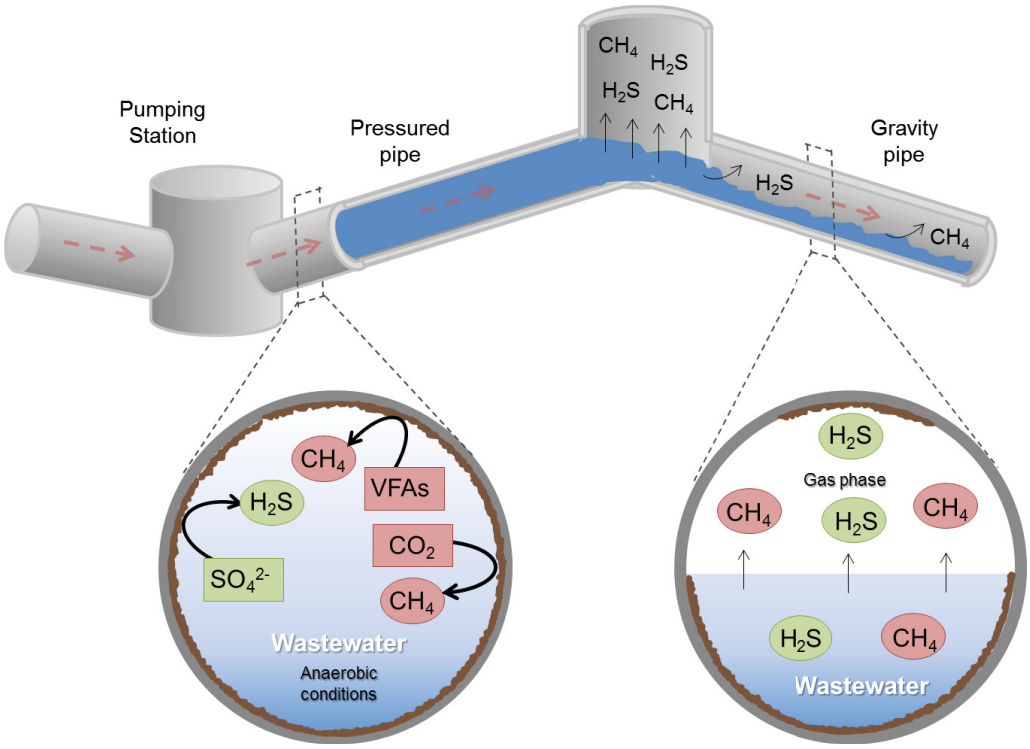

Figure 1.4. Scheme of a sewer system consisting of pressure and gravity pipes with the main reactions and processes that occur.

1.2.2. Methane production in sewer systems

Methanogenesis is a type of anaerobic respiration which is carried out by MA. The production of CH_4 was detected in different natural and anthropogenic environments such as freshwater sediments, swamps, paddy fields, landfills, the intestinal tracts of humans, ruminants and termites (Thauer et al., 2008). Moreover, CH_4 formation in sewer systems was firstly identified by Guisasola and collaborators a few years ago (Guisasola et al., 2008).

The different pathways for the degradation of organic matter and the use of resultant by-products by MA are summarized in Figure 1.5.

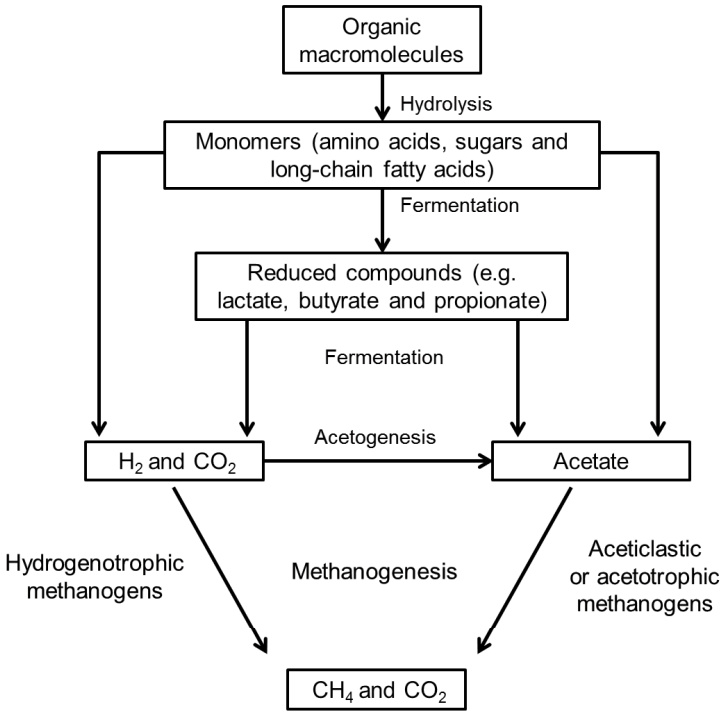

Figure 1.5. Organic matter degradation in anoxic environments and the utilization of the end-products by methanogenic archaea (adapted from Muyzer and Stams, 2008).

Acetate, hydrogen and carbon dioxide are produced during the degradation and fermentation of monomers. Moreover, acetate can be produced using H_2 and CO_2 during acetogenesis. Hydrogenotrophic methanogens produce CH_4 from H_2 and CO_2. In turn, acetoclastic (also known as acetotrophic) methanogens use acetate (which is simultaneously reduced and oxidized) to produce CH_4 and CO_2. Table 1.2 compiles the main CH_4 production reactions known to date.

Table 1.2. Main methane production reactions (Gutierrez et al., 2016)

Methane production reactions	**ΔGo' (kJ/reaction)**
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	−135.6
$CO_2 + H_2 \rightarrow CH_4 + 2\ H_2O$	−130.7
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31.0

In recent studies, different factors that affect CH_4 production have been identified (Foley et al., 2009; Guisasola et al., 2008; Y. Liu et al., 2015), namely:

Biodegradable organic matter: organic compounds used to produce directly or indirectly CH_4 are usually measured at high concentrations in wastewater. Moreover, a correlation between high concentrations of fermentable chemical oxygen demand (COD) and high CH_4 concentrations was identified (Sudarjanto et al., 2011).

Temperature: temperature also plays an important role on methane production. Higher CH_4 production was observed in summer as compared to winter in the study of Liu and collaborators (Liu et al., 2014).

pH: pH also affects CH_4 production. Methanogens can growth at a wide pH range (3–9 (Williams and Crawford, 1985; Worakit et al., 1986)). However, microorganisms adapted to wastewater conditions have an optimal pH for growth (normally, between 7-8). For that reason, pH disequilibria might suppress methanogenic growth and activity in sewer systems (Gutierrez et al., 2014, 2009).

Area/volume ratio: methane concentration in sewer systems is also affected by the A/V ratio: higher A/V ratios enhance biofilm growth per volume of wastewater, thus stimulating higher CH_4 production rates (Foley et al., 2009; Guisasola et al., 2009).

Flow velocity and hydraulic retention time: dissolved CH_4 production can also be affected by HRT of wastewater in sewers (Guisasola et al., 2009). Obviously, high sewer length increase the HRT, allowing more CH_4 production (Foley et al., 2009). Moreover, the diurnal pumping pattern (which depends on the wastewater volume produced on a daily basis) affects HRT and also CH_4 production (Liu et al., 2014).

Methane production in sewer systems can also cause severe problems in the environment. Methane is a potent greenhouse gas with higher global warming potential than CO_2 (21–23 times) (IPCC, 2013). This compound can be released into the atmosphere through sewer valves and also during wastewater discharges in WWTPs. Methane is also a safety problem when it is released to the sewer air fraction due to its low explosion limit (Spencer et al.,

2006) (Fig. 1.4). Finally, the consumption of organic compounds in wastewater during CH_4 formation could has several detrimental effects on biological nutrient removal in WWTPs (Guisasola et al., 2008).

1.3. Microbial communities in sewer systems

The simultaneous production of H_2S and CH_4 in sewer systems suggested the coexistence of SRB and MA in sewer biofilms (Guisasola et al., 2008). Although the competition between SRB and MA had been established in some environmental systems due to the limitation of substrates (Lovley and Klug, 1983; Lovley et al., 1982; Omil et al., 1998), the abundance of low molecular weight compounds in wastewater and the stratification of biofilm microbial communities allows the coexistence of both metabolisms/microorganisms in sewer biofilms.

The hypothesis of the biofilm stratification presented by Guisasola and colleagues (Guisasola et al., 2008) states that SRB are predominant in upper layers of the biofilm (close to the bulk wastewater) according to H_2S profiles measured within the biofilm matrix in the study of Mohanakrishnan et al. (Mohanakrishnan et al., 2009b). These vertical profiles indicates a partial penetration of SO_4^{2-} into the biofilm, allowing the growth of MA in the deeper layers of the biofilm near the sewer pipe (Fig. 1.6). This hypothesis has been recently verified by Sun and colleagues (Sun et al., 2014) after demonstrating that the relative abundance of SRB at the biofilm surface was 20%, decreasing to 3% at deeper layers. In turn, MA accounted for only 3% at the biofilm surface and increased up to 75% at deeper layers.

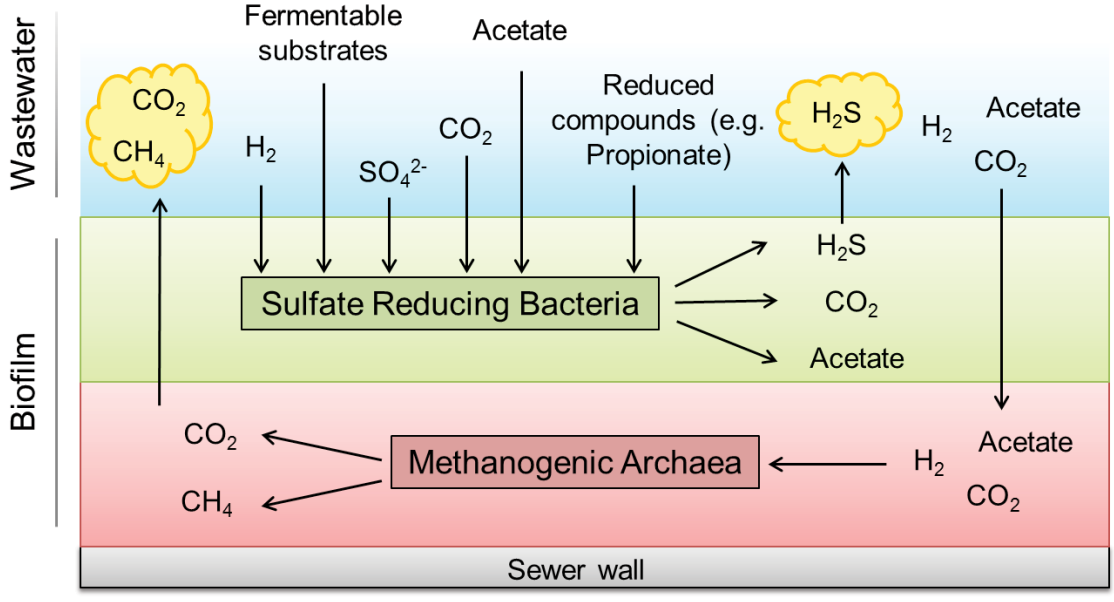

Figure 1.6. Theoretical stratification of the different microbial groups in biofilm sewers.

Moreover, when an electron acceptor is externally added (*e.g.* nitrate (NO_3^-)), heterotrophic nitrate reducing bacteria (hNRB) and sulfide-oxidizing nitrate-reducing bacteria (soNRB) grow on the biofilm surface thus preventing nitrate toxicity on methanogens. Under these conditions, MA may persist in deeper zones because NO_3^- and SO_4^{2-} cannot completely diffuse into the biofilm matrix.

Identification of microbial communities inhabiting sewer biofilms has been carried out using different molecular methods to properly identify the main microbial players involved in wastewater biochemical transformations (mainly H_2S and CH_4 production).

Mohanakrishnan and co-workers (Mohanakrishnan et al., 2009b) combined denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH). These techniques have complementary purposes: whereas DGGE is mainly used to resolve the composition of microbial communities, FISH is intended to both quantify and locate target microbial groups by using specific rRNA probes. In the study by Mohanakrishnan and co-workers DGGE identified few species, namely: uncultured *Clostridiales*, *Acetobacterium paludosum*, *Stenotrophomonas maltophila* and uncultured *Gammaproteobacteria*. The low richness detected was probably related with the low resolution of DGGE rather than to the intrinsic low diversity of the microbial community under study (Kisand and Wikner, 2003). On the other hand, different SRB (*Desulfovibrionaceae*, *Desulfobacteraceae* and *Syntrophobacteraceae*) and MA (*Methanosaetaceae, Methanomicrobiales, Methanosarcinaceae, Methanococcales, Methanocaldococcaceae*) were detected in sewer biofilm samples using FISH. These results provided extra evidence that the recovered microbial diversity was highly dependent on the methodological approach used and that a large fraction of the true diversity in these complex systems remains hidden.

Application of high-throughput sequencing techniques (*e.g.* 454 pyrosequencing and Illumina) has allowed a better estimation of the real richness and diversity of microbial communities due to a huge increase in sequencing effort that exceed old techniques (DGGE, cloning) by several orders of magnitude. A recent study using 454 pyrotag libraries allowed the identification of a broad diversity of SRB and MA in sewer biofilms (Sun et al., 2014). SRB detected were mainly affiliated to genera *Desulfobulbus* (33%), *Desulfomicrobium* (19%), *Desulfovibrio* (24%), *Desulfatiferula* (7%) and *Desulforegula* (16%) whereas 90% of MA affiliated to genus *Methanosaeta.* The remaining 10% of Archaea affiliated to genera *Methanospirillum, Methanomethylovorans, Methanobrevibacter, Methanobacterium* and *Candidatus* Methanomethylophilus.

1.4. Control of sulfide and methane emissions in sewers

Different strategies are being applied to reduce H_2S and CH_4 production in sewer systems. For example, the usage of new technologies that involve the construction of brand new sewers with materials that prevent corrosion. Another option is the application of antimicrobial products in new pipe walls to inhibit microbial growth and prevent microbial activity (Rivera-Garza et al., 2000; Yamanaka et al., 2002).

However, the need to reduce H_2S and CH_4 production in old sewer networks has led to the development of different mitigation strategies using chemical compounds (Ganigue et al., 2011; Zhang et al., 2008). Addition of iron salts (Firer et al., 2008; Zhang et al., 2010, 2009, 2008), the use of magnesium or sodium hydroxides (Gutierrez et al., 2014, 2009; Zhang et al., 2008), the injection of air/pure oxygen (Gutierrez et al., 2008; Zhang et al., 2008), or the addition of nitrogen oxides (Gutierrez et al., 2010; Jiang et al., 2013, 2011a, 2011b, 2010; Mohanakrishnan et al., 2011, 2009a, 2008) are among the most used mitigation strategies in full-scale sewers.

1.4.1. Addition of chemicals

Iron salts

The addition of iron salts (*e.g.* ferrous chloride, ferric chloride) is commonly used in Australia and Europe (Ganigue et al., 2011). Ferrous iron (Fe^{2+}) forms highly insoluble metallic sulfide (FeS) allowing the removal of H_2S by precipitation of this generated compound (WERF, 2007). On the other hand, when ferric iron (Fe^{3+}) is added, H_2S is oxidized to elemental sulfur (S^0) while Fe^{3+} is reduced to Fe^{2+} (D.A. and J.A., 1983). Laboratory studies have demonstrated that iron salts affect biofilm activities. The activity of SRB was reduced by ≈50% when ferric chloride ($FeCl_3$) was added. Besides, addition of ferrous chloride ($FeCl_2$) also caused a reduction of the chemical demand that almost achieved a complete control of H_2S production (Zhang et al., 2010, 2009). Regarding CH_4, its concentration in the effluent wastewater was reduced by 43% when $FeCl_3$ was added (Zhang et al., 2009).

Increase in pH

The increase in pH reduces the transfer/release of H_2S from the liquid to the gas phase and it also affects SRB and MA activity. Laboratory studies showed a 70–90% reduction in H_2S production and a reduction of 95–100% in CH_4 production when pH increased from 9 to 12.5 (Gutierrez et al., 2014). In another study, SRB activity was reduced by 30–50% when pH was raised to 8.6–9, which also caused a suppression of CH_4 production (Gutierrez et al., 2009). The recovery of SRB and MA was not immediate after pH control stopped,

indicating a long-term effect of pH on the activity and viability of SRB and MA populations.

Oxygen

The addition of air or oxygen has been widely used to prevent anaerobic conditions and to oxidize H_2S (Ganigue et al., 2011; Hvitved-Jacobsen et al., 2013). Sulfide oxidation can be chemical or biological because it can occur in the bulk liquid-water phase and also in the biofilm (Gutierrez et al., 2008). However, oxygen does not affect SRB activity since these microbes are embedded in a dense polysaccharide matrix that impedes oxygen diffusion within the biofilm. For that reason, H_2S production was immediately reestablished after oxygen depletion. On the other hand, CH_4 emissions were reduced by 47% in a laboratory-scale sewer system after aeration (Ganigué and Yuan, 2014). In this latter case, the reestablishment of CH_4 production after some days of oxygen injection suggested that oxygen did not reached deeper biofilm layers thus not affecting MA.

Nitrate

The addition of NO_3^- is commonly used in Australia and Europe to reduce H_2S and CH_4 emission (Ganigue et al., 2011). Nitrate prevents anaerobic conditions in sewer systems and also increases redox potential and suppresses anaerobic processes (*e.g.* SO_4^{2-} reduction). Effects of NO_3^- on H_2S production can be related to: *i)* the competition for electron donors between SRB and NRB; *ii)* the increase of nitrate reduction intermediates which can affect H_2S production; *iii)* the increase in pH caused by the activity of NRB which reduce the release of H_2S to the sewer atmosphere; and *iv)* the H_2S oxidation by soNRB (He et al., 2010; Mohanakrishnan et al., 2011; Zhang et al., 2008).

Different studies reported that H_2S could be oxidized during nitrate addition (Gutierrez et al., 2010; Jiang et al., 2009; Mohanakrishnan et al., 2011, 2009a) while no nitrous oxide (N_2O) production was detected (Jiang et al., 2013). Although H_2S levels at the outlet of the system were reduced by 66%, nitrate did not have a long-term inhibitory effect on H_2S production and this chemical only controlled H_2S emission during its continued presence in the bulk wastewater (Mohanakrishnan et al., 2009a). In a more recent study, Mohanakrishnan and colleagues (Mohanakrishnan et al., 2011) observed that H_2S production and *dsrB* gene transcription —*dsrA* and *dsrB* genes encode A and B subunits of dissimilatory sulfite reductase that is used by SRB to reduce sulfite (SO_3^{2-}) to H_2S and both genes are used as functional markers for SRB (Klein et al., 2001; Wagner et al., 1998)— was reduced after few hours of NO_3^- dosing, indicating an immediate effect of nitrate on sulfate reduction. Although SO_4^{2-} consumption and *dsrB* transcription were reduced to negligible levels, some SRB persisted in the biofilm allowing the production of H_2S at low rates. These SRB communities changed during nitrate addition, being members of genera *Desulfomicrobium* and *Desulfovibrio* those that persisted in the biofilm. Moreover,

different NRB grew during the addition of nitrate (*e.g. Thauera*, *Deferribacter*, *Citrobacter* and *Sulfurospirillum*).

Interestingly, Jiang and co-workers determined that S^0 is an important intermediate product during H_2S oxidation and could be accumulated in the biofilm. Elemental sulfur can also be oxidized to SO_4^{2-} in the presence of nitrate and reduced to H_2S when nitrate is depleted (Jiang et al., 2009).

Furthermore, nitrate addition also reduces CH_4 production in pressure sewers although it has not a long-term inhibitory effect (Jiang et al., 2013; Mohanakrishnan et al., 2009a). Methanogens can coexist with SRB and NRB because MA could persist in deeper layers of the biofilm due to the low penetration of SO_4^{2-} and NO_3^- compared with the full penetration of soluble organic substrates (Jiang et al., 2013).

Nitrite

Nitrite (NO_2^-) is a nitrogen oxide which could be easily added to the inlet of pressure sewers due to its high solubility in water (0.82 g/mL at 20ºC) and its cost (Mohanakrishnan et al., 2008). The main difference compared with nitrate is that nitrite blocks sulfate reduction by inhibiting the reduction of SO_3^{2-} to H_2S by the dissimilatory sulfite reductase (Hubert et al., 2005). Nevertheless, some SRB can overcome this inhibition by reducing nitrite *via* nitrite reductase as detoxification system (Greene et al., 2003; Mohanakrishnan et al., 2008). Nitrite could also prevent CH_4 production in sewers because some methanogens are sensitive to NO_2^- (Kaster and Voordouw, 2006) and the duration of the inhibition depend on the concentration of NO_2^- and the methanogenic species present (Klüber et al., 1998).

Mohanakrishnan and co-workers (Mohanakrishnan et al., 2008) observed a long-term inhibitory effect of NO_2^- on H_2S and CH_4 production. Reduction of nitrite was accompanied by sulfide oxidation thus suggesting the activity of autotrophic, sulfide-oxidizing nitrite-reducing bacteria. Moreover, the observation of a remarkable accumulation of intermediate sulfur compounds in biofilms also supported the presence of sulfide-oxidizing nitrite-reducing bacteria although these compounds were quickly depleted after stopping NO_2^- addition. Additionally, Jiang and colleagues (Jiang et al., 2010) showed that the inhibition of SRB was affected both by the NO_2^- concentrations used and by the exposure time. They also suggested that MA were more susceptible to NO_2^- than SRB. Further studies showed that nitrite dosing with acid or hydrogen peroxide caused the formation of free nitrous acid which may have a biocidal effect on SRB and MA populations (Jiang and Yuan, 2013; Jiang et al., 2011a, 2011b).

In brief, the effective sulfide and methane emission reduction and the slow recovery of SRB and MA indicated advantages of nitrite dosing compared with other used oxidant

compounds (*e.g.* oxygen or nitrate).

1.4.2. Optimizing dosing location and dosing rate

While the added chemical is an important factor to take into account, both the location where this compound is dosed and the dosage rate may also determine its effectiveness and the associated cost (Ganigue et al., 2011). For instance, nitrate dosage or oxygen injection at upstream sewer sections stimulated the activity of SRB at downstream sewer sections (Gutierrez et al., 2008; Mohanakrishnan et al., 2009a). This might be explained by the depletion/consumption of the added chemicals during wastewater transport along upstream sewer sections, increasing SO_4^{2-} concentration in wastewater and thus fuelling sulfate-respiration (*i.e.* H_2S production) by SRB at downstream parts of the sewer. Gutierrez and colleagues compared different dosage locations and dosage rates using nitrate (Gutierrez et al., 2010). Their results showed the benefits of adding NO_3^- at a point close to the end of the pipe rather than at the beginning of the pressure sewer. This strategy reduced the associated costs and improved the control of H_2S emissions (Gutierrez et al., 2010). Moreover, the dosage rate is crucial for the optimization of the mitigation strategy because the sewer dynamics may vary in a daily basis (different HRTs). Thus, chemical dosing based on the on-line measurement of H_2S and CH_4 production may allow a better cost-effective strategy (Ganigue et al., 2011). For example, the effectiveness of an on-line method used to reduce sulfide emissions based on ensuring the required distribution of the dosed chemical along the different pipe sections was recently demonstrated (Liu et al., 2016).

1.4.3. Other mitigation approaches

Other mitigation strategies have also been tested to control H_2S and CH_4 emissions although they have not gained attention probably due to the difficult implementation and their controversial results. For instance, different compounds (ozone, hydrogen peroxide, chlorine compounds or permanganate) have been used to oxidize odourous compounds. However, most of these compounds are toxic (difficult handling and transport), expensive and, besides, some of them can also stimulate the release of unwanted salts to the wastewater (Boon, 1995; Charron et al., 2004; Firer et al., 2008; Tomar and Abdullah, 1994). An alternative strategy relies on activated carbon adsorption or biofiltration reactors to remove malodourous compounds. Although these procedures may effectively prevent odour problems do not avoid corrosion and they requires regular maintenance (Firer et al., 2008; Hvitved-Jacobsen et al., 2013). Ventilation of headspace sewers is also an alternative option to control H_2S and CH_4 accumulation but it requires an important investment to construct new infrastructures (Boon, 1995; Boon et al., 1998; Firer et al., 2008; Hvitved-Jacobsen et al., 2013; Olson et al., 1997). Another option is the production of oxygen using

electrodes (electrochemical method) in order to remove sulfide from wastewater (Pikaar et al., 2011). Moreover, new emerging technologies such are photocatalytic systems, non-thermal plasmas and hollow fiber membrane bioreactors are being tested to be used for H2S and non-H2S odour volatile compounds (Chen and Xie, 2013; Lebrero et al., 2014; Wei et al., 2013). Mechanical and hydraulic removal of biofilms has also been proposed as a low cost alternative although biofilm re-growth poses a serious drawback in the long-term.

Chapter 2
Objectives

The research on sewer systems is gaining attention during the last years due to the environmental problems and the economic losses related to their management. The main problem faced by researchers is the design and implementation of new mitigation strategies to reduce sulfide and methane emissions and to resolve the associated problems in sewers facilities (*e.g.* concrete corrosion). However, optimization of dosing conditions (*i.e.* the type of chemicals used, the dosing location and the dosage rate) may overcome some of the problems and provide a better knowledge on the advantages and disadvantages of the different strategies. Moreover, a better understanding of what is happening during wastewater transport and the proper identification of the main microbial players involved in the different biotransformation routes (*e.g.* fermentation, sulfate reduction, methane production, nitrate reduction, sulfide oxidation, among others) may allow a better management of wastewater in sewer systems.

The main objectives of this thesis were to study the microbial diversity in biofilms associated to wastewater sewer systems and to test different mitigation strategies to reduce sulfide and methane emissions.

To accomplish these main goals, different secondary objectives were defined:

- To determine changes in microbial biofilm communities during colonization of a laboratory sewer pilot plant and full-scale sewer systems, particularly:
    - To compare sulfide and methane production/emission between young and mature biofilms in laboratory sewer systems.
    - To investigate changes in the composition of microbial communities during biofilm development.
    - To compare sulfide/methane emissions and microbial biofilm communities between mature biofilms in laboratory and full-scale sewer systems.
- To test the effects of Downstream Nitrate Dosage (DND) on anaerobic sewer biofilms with regards to sulfide and methane production in a laboratory sewer pilot plant, specifically:
    - To compare sulfide and methane production before, during and after nitrate addition.
    - To determine the possible negative effects of nitrate addition.
    - To investigate changes of bacterial and archaeal biofilm communities exposed to nitrate.

- To test the effectiveness of Downstream Nitrite Dosage (DNO_2D) in pressure sewer systems to reduce sulfide and methane production in a laboratory pilot plant, focusing on:
    - To compare sulfide and methane production before, during and after nitrite addition.
    - To determine potential collateral effects of nitrite addition.
    - To assess the effects of nitrite addition on the composition of the active and the bulk fraction of biofilm microbial communities.
    - To compare the effects of DNO_2D and DND strategies regarding their ability to control sulfide and methane emissions.

According to these objectives, the research work of this thesis has been structured in three chapters based on the published articles (see List of publications derived from the thesis, page iii):

Block II: Biofilm development and mitigation strategies

Chapter 4: *Changes in microbial biofilm communities during colonization of sewer systems*

Chapter 5: *Implications of downstream nitrate dosage in anaerobic sewers to control sulfide and methane emissions*

Chapter 6: *Control of sulfide and methane production in anaerobic sewer systems by means of downstream nitrite dosage*

Block II

BIOFILM DEVELOPMENT AND MITIGATION STRATEGIES

Chapter 3

Changes in microbial biofilm communities during colonization of sewer systems

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Changes in Microbial Biofilm Communities during Colonization of Sewer Systems

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The coexistence of sulfate-reducing bacteria (SRB) and methanogenic archaea (MA) in anaerobic biofilms developed in sewer inner pipe surfaces favors the accumulation of sulfide (H_2S) and methane (CH_4) as metabolic end products, causing severe impacts on sewerage systems. In this study, we investigated the time course of H_2S and CH_4 production and emission rates during different stages of biofilm development in relation to changes in the composition of microbial biofilm communities. The study was carried out in a laboratory sewer pilot plant that mimics a full-scale anaerobic rising sewer using a combination of process data and molecular techniques (e.g., quantitative PCR [qPCR], denaturing gradient gel electrophoresis [DGGE], and 16S rRNA gene pyrotag sequencing). After 2 weeks of biofilm growth, H_2S emission was notably high (290.7 ± 72.3 mg S-H_2S liter^{-1} day^{-1}), whereas emissions of CH_4 remained low (17.9 ± 15.9 mg COD-CH_4 liter^{-1} day^{-1}). This contrasting trend coincided with a stable SRB community and an archaeal community composed solely of methanogens derived from the human gut (i.e., *Methanobrevibacter* and *Methanosphaera*). In turn, CH_4 emissions increased after 1 year of biofilm growth (327.6 ± 16.6 mg COD-CH_4 liter^{-1} day^{-1}), coinciding with the replacement of methanogenic colonizers by species more adapted to sewer conditions (i.e., *Methanosaeta* spp.). Our study provides data that confirm the capacity of our laboratory experimental system to mimic the functioning of full-scale sewers both microbiologically and operationally in terms of sulfide and methane production, gaining insight into the complex dynamics of key microbial groups during biofilm development.

Wastewater collection systems, or sewers, consist of an underground network of physical structures-installations composed of pipelines, pumping stations, manholes, and channels that convey wastewaters from their source to the discharge point, usually a wastewater treatment plant (WWTP). Sewer systems thus prevent the direct contact of urban populations to fecal material and potential microbial pathogens, greatly reducing the spread of infectious diseases. Sewers have traditionally been considered only hydraulic transport systems for sewage, although they are in fact "reactors" where complex physicochemical and microbial processes take place. Wastewater microorganisms are diverse and abundant, and they are exposed to a wide range of both inorganic and organic substrates as well as changing conditions along their transport through sewers (1). In this regard, wastewater transport through the pipes facilitates the formation of microbial biofilms that grow attached to the inner surface of sewer pipes (2). Different factors, such as large surface area, low flow velocity near pipe walls, and nutrient availability, may favor microbial colonization of pipe surfaces and biofilm growth. Formation of fully functional biofilms occurs in different steps, from surface conditioning, adhesion of microbial "colonizers," initial growth, and glycocalyx formation, followed by secondary colonization and growth (3).

Anaerobic conditions in sewer pipes favor the accumulation of both sulfide (H_2S) and methane (CH_4) as end products of different microbial metabolisms, i.e., anaerobic respiration of organic matter by sulfate-reducing bacteria (SRB) and methanogenic archaea (MA), respectively. Both compounds have detrimental effects on the sewer system, with different consequences for both the installation and its surroundings (2). Accumulation of H_2S in the sewer atmosphere causes malodor in the whole system, health hazards due to the well-known toxicity of H_2S, and corrosion of both the inner surface of pipes and the inlet zones of WWTPs (4, 5). H_2S accumulation also impacts the structural integrity of the sewerage by microbial-mediated corrosion processes, which severely affect the performance and cost of downstream processes at the WWTPs (2, 6). Remediation or replacement of corroded pipes requires a high economic investment for large systems, ranging from several hundreds to several thousands of Euros per meter depending on pipe diameter and location depth (7). On the other hand, buildup of CH_4 in sewers results from the activity of MA that colonize inner pipe surfaces and develop within the biofilm matrix under strict anaerobic conditions (8–10). In addition to being explosive at low concentrations, CH_4 is a major greenhouse gas with a life span of ~12 years and a global warming potential roughly 21 to 23 times higher than that of carbon dioxide (11). Recent reports suggest that CH_4 emissions from sewers contribute significantly to the total greenhouse gas footprint of wastewater systems (12, 13). Accordingly, different mitigation strategies have been used to reduce H_2S and CH_4 production in sewers (14–24).

Although competition between SRB and MA has been reported in some environments such as freshwaters (25), sediments (25), and WWTPs (26), CH_4 production in sewers containing high sulfate concentrations was first detected by Guisasola and coworkers

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(8). Assuming that SRB and MA may compete for the same substrates (e.g., complex organic matter, acetate, and hydrogen), their cooccurrence in sewer systems is probably the rule rather than the exception, especially considering the large amount of organic matter in wastewater and the prevalence of anaerobic conditions in many sections of sewer networks. In biofilms, this coexistence may be explained by processes of mass transfer of required substrates (e.g., sulfate and organic matter) into the biofilm matrix, which results in a physicochemical stratification along its thickness. Very recently, Sun and coworkers (10) investigated the stratification pattern of SRB and MA in sewer biofilms thicker than 800 μm, locating the former closer to the biofilm surface and locating the latter in greater abundance at deeper, highly anaerobic layers.

Despite these findings, little information is available on the colonization dynamics and activity of SRB and MA relating to biofilm development in sewer systems. Particularly, processes behind early biofilm colonization by SRB and MA in sewer pipes are still not fully understood. In this regard, a better understanding of how these processes take place and how they affect H_2S and CH_4 production rates during biofilm development is necessary to design effective biofilm control strategies for the commissioning of sewers. This information could be crucial to the development and application of optimal control methods to reduce odor, corrosion, and global warming issues generated by sewer biofilms.

The aim of this study was to investigate the initial stages of microbial biofilm development in sewer systems, with a special focus on the interactions between SRB and MA. Biological activities and phylogenetic community structure during the colonization phase were investigated by using a combination of molecular techniques (denaturing gradient gel electrophoresis [DGGE], quantitative PCR [qPCR], and massive parallel sequencing of 16S rRNA genes from target groups) and process data (H_2S and CH_4 production). The work was carried out by using a laboratory sewer pilot plant fed with wastewater that reproduced a full-scale anaerobic pressured sewer. The microbial community composition was compared with that of a biofilm from a full-scale sewer to validate the data obtained from our laboratory experiments.

MATERIALS AND METHODS

Anaerobic sewer biofilm reactor system. The study was carried out in a specially designed pilot system validated previously, the SCORe-CT method (Sewer Corrosion and Odour Research—Chemical Testing) (27), that mimics the H_2S and CH_4 production capacity of full-scale rising main sewers by reproducing its main characteristics, including (i) hydraulic features, such as hydraulic retention times (HRTs), turbulence, and area-to-volume ratios, and (ii) wastewater characteristics associated with real sewage. The laboratory system consisted of 3 airtight reactors (reactor 1 [R1], R2, and R3), each of them mimicking a section of an anaerobic sewer pipe (see Fig. S1 in the supplemental material). Each reactor had a volume of 0.75 liters, an 80-mm diameter, and a height of 149 mm. The system was fed with fresh sewage (domestic fresh sewage collected in the upstream sections of the sewer network in the municipality of Girona, Spain, close to its source in households) by a peristaltic pump (Masterflex model 7518-10). Sewage was collected on a weekly basis and kept at 4°C to minimize variation in its composition. Wastewater contained 26.5 ± 2.6 mg $S\text{-}SO_4^{2-}$ $liter^{-1}$ and 0.1 ± 0.1 mg $COD\text{-}CH_4$ $liter^{-1}$. Volatile fatty acid (VFA) and soluble and total chemical oxygen demand (COD) concentrations were 42.3 ± 8.3 mg COD-VFA $liter^{-1}$, 325.8 ± 40.8 mg $liter^{-1}$, and 672 ± 93.2 mg $liter^{-1}$, respectively. Sewage was heated to 20°C before entering the reactors. Magnetic stirrers (Mr Hei-MixS; Heidolph) were used to ensure homogeneous conditions and to produce a shear within the reactors. Wastewater was pumped 15 times a day in uneven periods (between 1 and 3 h). During these intervals, wastewater was transferred from the storage tank to R1 and then from R1 to R2 and finally from R2 to R3 in order to simulate the HRT pattern observed in a full-scale rising main used as a reference sewer pipe, the Radin collector (lat 42.101843, long 3.131631 [L'Escala municipality, Spain]). The Radin anaerobic pipe is 2,930 m long and has a 0.5-m diameter with an HRT of between 3 and 7 h.

Plastic carriers (Anox Kaldnes, Norway) with a 1-cm diameter were clustered on three stainless steel rods inside each reactor to increase biofilm growth surface area and to provide easily extractable biofilm samples. Taking into consideration the reactor wall and carriers, the total biofilm growth area in each reactor was 0.05 m^2 (area/volume ratio of 65 m^2 m^{-3}). The system was operated continuously for 48 weeks. The colonization period was monitored during the first 12 weeks after start-up of the system. In addition, characterization of mature biofilms was undertaken during the 12th month after start-up. The microbial community composition of mature biofilms was compared to the composition of the biofilm extracted from the upstream reference section of the Radin sewer pipe. A biofilm sample from the full-scale sewer pipe was obtained from a sewer air scour valve that was constantly in contact with the flowing wastewater. The valve was disassembled, and the biofilm grown on its surface was scraped by using a sterile spatula and collected into a sterile Falcon tube containing 5 ml of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), in which the collected biomass was resuspended. The sample tube was maintained at 4°C in a portable icebox until arrival at the laboratory (1 h after collection), where it was immediately frozen at −20°C until DNA extraction.

H_2S generation, CH_4 production, and VFA production/consumption in the laboratory system were monitored as the wastewater was transported through the system. Liquid-phase sampling from R3 and offline chemical analyses were done weekly during normal-functioning (NF) tests for the determination of sulfur species (sulfate, sulfide, sulfite, and thiosulfate), CH_4, COD, and VFAs. Sampling hours covered the entire HRT range (3 h to 7 h). Also, 10 batch tests (BTs) were performed to monitor H_2S and CH_4 production by biofilms. Batch tests were carried out once every 1 to 2 weeks. During BTs, the continuous operation of the reactors was stopped. The feed pump was activated for 10 min to ensure that each reactor was filled with fresh sewage. After this, the feed was stopped, and liquid samples were withdrawn every hour for a 3-h period by using a 10-ml syringe connected through a sampling port fitted with a valve and Tygon tubing. Samples were analyzed for sulfur species, CH_4, VFAs, and COD, as described below. Using linear regression, H_2S and CH_4 production rates were calculated from the sampling-point data. A special 6-h batch test was run in order to investigate changes in methane production depending on the presence of sulfate in R1 and R3. Samples were analyzed every hour over a 3-h period for sulfur species and every hour for a 6-h period for methane in order to determine changes in methane production when sulfate was totally reduced to sulfide.

Daily H_2S and CH_4 emissions (calculated from NF test data) were also determined after 1 year of biofilm development to detect changes in activity between early and mature stages of biofilm development in the system.

Chemical analysis. Dissolved sulfide in R1 and R3 was measured continuously by using an s::can spectro::lyser UV-visible (UV-Vis) spectrometer probe (Messtechnik GmbH, Austria) (28). For the analysis of dissolved sulfur species, 1.5 ml of wastewater was filtered through disposable Millipore filter units (0.22-μm pore size) and added to 0.5 ml preserving solution antioxidant buffer (SAOB) (29). Samples were analyzed within 24 h in an ion chromatograph (IC) with a UV and conductivity detector (ICS-5000; Dionex). VFAs were measured by gas chromatography (Thermo Fisher Scientific) (coupled with a flame ionization detector). For CH_4 samples, 5 ml of sewage was filtered through disposable Millipore filter units (0.45-μm pore size) and injected into vacuumed glass tubes with the help of a hypodermic needle attached to a plastic syringe. After reaching liquid-gas equilibrium inside the tubes, the samples were ana-

lyzed by gas chromatography (Thermo Fisher Scientific) (coupled with an flame ionization detector). COD analyses were performed by using a standard photometric test kit with a commercially available reagent (LCK 114; Hach Lang). Absorbance readings were obtained by using an LCK 314 cuvette test with a DR2800 Hach Lang spectrometer. During start-up, Anox Kaldnes plastic carriers were regularly withdrawn to quantify changes in biomass content as a result of microbial biofilm formation. The biomass attached to each carrier was suspended in MilliQ water by vortexing (Genius-3; IKA) until complete detachment occurred (≈2 min). Concentrations of total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed by using standard methods (30). Biomass content was referred to the carrier surface by using values for volatile suspended solids.

DNA extraction. DNA was extracted from biofilm biomass collected in R1 and from sewage at different-week intervals during the study period. The biomass attached to each carrier was suspended in 5 ml 1× PBS by vortexing (Genius-3; IKA). Suspended biomass from carriers and samples of wastewater (45 ml) were centrifuged at 11,000 rpm for 5 min at 25°C in an Eppendorf 5804R centrifuge equipped with an F-34-6-38 rotor (Eppendorf, Hamburg, Germany). DNA was then extracted from pelleted biomass by using the FastDNA Spin kit for soil (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's instructions. Genomic DNA concentrations of biofilm samples were measured by using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR amplification and 16S rRNA gene fingerprinting. The microbial composition of biofilms formed on carrier surfaces was studied by combining specific amplification of 16S rRNA gene fragments and fingerprinting by denaturing gradient gel electrophoresis (DGGE) (31). Bacterial and archaeal 16S rRNA gene fragments were amplified from DNA extracts by using primer pairs 357F-GC/907R (32) and 109(T)F/515R-GC (33), respectively. PCR amplification mixtures (final volume of 50 μl) contained 10 μl of $MgCl_2$ buffer (15 mM), 1 μl of deoxynucleoside triphosphates (dNTPs) (10 mM), 2 μl bovine serum albumin (BSA), 1 μl of each primer (10 μM), 0.25 μl of *Taq* polymerase, and 2 μl of the DNA sample. DNA extracts were diluted with sterile MilliQ water to a final concentration of 10 to 50 ng μl^{-1} to avoid inhibition of amplification reactions. Sequences of the different primer pairs used during the study and PCR conditions are summarized in Tables S1 and S2 in the supplemental material, respectively.

DGGE analyses were performed with an Ingeny phorU-2 DGGE system (Ingeny International BV, Netherlands). Samples were loaded onto 6% polyacrylamide gels and run with 1× Tris-acetate-EDTA (TAE) buffer using 30 to 70% (bacterial 16S rRNA) and 30 to 50% (archaeal 16S rRNA) linear denaturing gradients of urea-formamide (100% denaturant agent contained 7 M urea and 40% deionized formamide). A molecular ladder composed by a mixture of known small-subunit (SSU) rRNA gene fragments was loaded into all gels to allow intergel comparisons of band migration. Electrophoreses were performed overnight at 60°C at a constant voltage of 120 V. After electrophoresis, gels were stained for 30 min with 1× SYBR gold nucleic acid stain (Molecular Probes Inc.) in 1× TAE buffer, rinsed, and visualized under UV radiation. DGGE fingerprints were analyzed by using GelCompar II (Applied Maths, Belgium). For sample comparison, a presence-absence matrix was used to calculate similarities between patterns, and statistical analysis based on hierarchical cluster analysis was performed with the Dice distance and the unweighted pair group method using average linkages (UPGMA) grouping algorithm.

DNA from excised bands of wastewater samples was eluted as previously described (34). DNA was then amplified by using the same primer pairs (without a GC clamp) and PCR conditions as those described above but sizing down the number of PCR cycles up to 20. PCR products were directly sent to Genoscreen (Lille, France) for sequencing on both strands. Sequences were checked for chimeras by using Uchime (35), aligned by using BioEdit (36), manually curated, and then compared for the closest relatives in the NCBI sequence database (http://www.ncbi.nlm.nih.gov/blast/) using the Basic Local Alignment Search Tool (BLAST) (37).

Real-time quantitative PCR. Real-time quantitative PCR (qPCR) assays were used to quantify gene copies of bacterial and archaeal 16S rRNA and *dsrA* functional genes. All qPCRs were run with a Stratagene MX3005P instrument (Agilent Technologies). For all tests, qPCR standards contained a known number of target 16S rRNA genes. qPCR mixtures for bacterial genes contained 15 μl Brilliant III Ultra Fast SYBR green qPCR master mix (Agilent Technologies), 400 nM (each) forward (1048F) and reverse (1194R) primers (38), and 1 μl of template and were adjusted to a final volume of 30 μl with molecular biology-grade sterile water. DNA sample stocks were diluted with water to a final concentration of 10 to 20 ng μl^{-1}. qPCR for archaeal 16S rRNA genes was carried out under the same conditions as those for bacteria but using forward primer 806F (39) and reverse primer 915R (40) and reducing the number of cycles to 35. Quantification of SRB was based on the dissimilatory sulfate reductase subunit A gene (*dsrA*) according to methods described previously by Ben-Dov et al. (41). Primer sequences, reaction temperatures, R^2 values, and amplification efficiencies for each qPCR are compiled in Tables S1 and S3 in the supplemental material. All qPCR analyses were carried out according to MIQE rules for quantitative PCR analyses (42), and all essential information is included in this section.

Pyrosequencing and phylogenetic analyses of microbial diversity. DNA extracts from biofilms at early stages (weeks 1, 5, and 13), mature biofilms (1 year old), and full-scale sewers were analyzed by tag-encoded FLX-Titanium amplicon pyrosequencing at the Research and Testing Laboratory (RTL; Lubbock, TX, USA). Briefly, genomic DNA from biofilm samples was used as a template in PCRs using universal bacterial (28F/519R) (33) and archaeal (341F/958R) (43, 44) primer combinations complemented with 454 adapters and sample-specific barcodes. Raw sequence data sets were preprocessed at RTL facilities to reduce noise and sequencing artifacts, as previously described (45). Demultiplexing according to sample barcodes, sequence quality assessments, chimera detection, and downstream phylogenetic analyses were conducted with mothur (46). Bacterial and archaeal curated sequence data sets were then aligned in mothur by using the bacterial and archaeal SILVA reference alignments, respectively, available at the mothur website (http://www.mothur.org/). Taxonomic classification of bacterial sequences was carried out by using the RDP taxonomy reference database with a cutoff value of 80% for valid assignments. Classification of archaeal sequences was carried out by using the SILVA reference database and taxonomy files using the same cutoff as that used for bacteria (80%). Operational taxonomic units (OTUs) (97% cutoff) and representative sequences of each OTU were delineated and taxonomically assigned by using mothur. For community analysis, the number of sequences in each sample was normalized by using a randomly selected subset of 1,500 sequences (for bacteria) and 6,000 sequences (for archaea) from each sample to standardize the sequencing effort across samples and minimize any bias due to a different number of total sequences. These normalized sequence data sets were then used in mothur to calculate α-diversity indicators of richness (Chao1) and diversity (Shannon) and to calculate community similarity among sites (β-diversity) based on the weighted UniFrac distance (47). Nonmetric multidimensional scaling (nMDS) analysis was performed on the UniFrac similarity matrices to visualize patterns of community composition. The relative abundance of the most populated OTUs (OTUs with relative abundances of ≥4% of total sequences in at least one sample) across samples was visualized as bubble plots by using bubble.pl (http://www.cmde.science.ubc.ca/hallam/bubble.php).

After taxonomic classification of bacteria, sequences affiliated with the class *Deltaproteobacteria* were selected and further grouped into 149 OTUs (97% cutoff). Representative sequences of each deltaproteobacterial OTU were delineated and assigned by using mothur and then compared for the closest cultured relative by using BLAST. Phylogenetic trees were constructed in MEGA 5 (48) by using representative sequences of abundant OTUs, defined as those having a relative abundance of ≥4% of

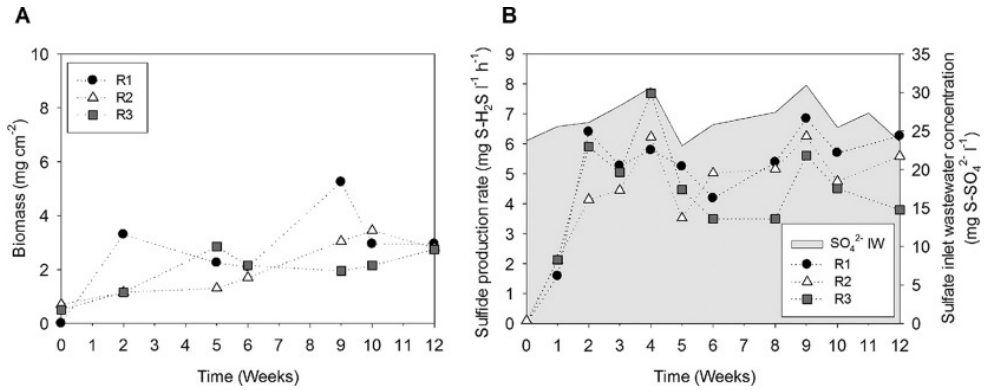

FIG 1 (A) Temporal changes of microbial biomass in R1, R2, and R3. (B) Sulfide production rates determined in the batch tests on R1, R2, and R3 and sulfate concentrations in inlet wastewater (IW).

total deltaproteobacterial and archaeal sequences in at least one sample and the closest cultured representative sequences.

Statistical analyses. Statistical analyses were carried out by using SPSS software (version 15.0; SPSS, Chicago, IL, USA). Normality of data was assessed by the Kolmogorov-Smirnov test for values obtained for batch testing and inlet wastewater (sulfate and sulfur balance). The correlation between the sulfate concentration in wastewater and sulfate reduction rates was assessed by the Pearson test.

Accession numbers. Bacterial and archaeal 16S rRNA gene sequences obtained by DGGE fingerprinting were deposited in GenBank under accession numbers KR080151 to KR080166. Pyrosequencing data from this study have been deposited in the NCBI database via the BioSample submission portal (http://www.ncbi.nlm.nih.gov/biosample/) under accession number PRJNA279227.

RESULTS

Differences in sulfide and methane production/emission between young and mature biofilms in laboratory and full-scale sewer systems. Changes in microbial biomass were continuously monitored for 12 weeks after the beginning of the experiment to assess biofilm formation within bioreactors (Fig. 1A). Initial biofilm growth was detected after stabilization of the biomass content in the range between 2.1 and 3.5 mg VSS cm^{-2}.

The daily profile of H_2S measured by using the s::can spectro::lyser UV-Vis spectrometer probe showed a gradual increase of H_2S production during the first 12 weeks of biofilm development in R1 and R3 (see Fig. S2 in the supplemental material). The higher H_2S production rate determined for R1 than for R3 was probably related to the low sulfate concentration in the wastewater arriving at R3. H_2S and CH_4 production rates were calculated for the same time period to assess the activity of recently formed biofilms. Figure 1B shows the H_2S production capacity within reactors in batch test experiments. H_2S production increased immediately after the start-up of the system. After the second week of operation, the capacity of the biofilm to produce H_2S stabilized at rates of between 3.5 and 7.7 mg S-H_2S liter^{-1} h^{-1}. Sulfate reduction rates were between 3.2 and 7.7 mg S-SO_4^{2-} liter^{-1} h^{-1}, which were positively related to H_2S production rates in each reactor (see Fig. S3 in the supplemental material). Differences in H_2S production showed a good correlation with the sulfate concentration in inlet wastewater (Pearson correlation index $[R] = 0.881$; $P = 0.02$). Interestingly, from week 8 to week 12, H_2S production in R1 was higher than that in R2 and R3. Regarding CH_4 production, low rates were detected in all reactors during these early stages of development (0.08 ± 0.11, 0.12 ± 0.16, and 0.16 ± 0.16 mg COD-CH_4 liter^{-1} h^{-1} in R1, R2, and R3, respectively).

Sulfide emission was measured weekly for 24 h to evaluate the impact of SRB activity in the system, as an accurate representation of full-scale sewer conditions. After the second week of operation, the H_2S emission rate ranged between 195.7 and 388.8 mg S-H_2S liter^{-1} day^{-1} (see Fig. S4A in the supplemental material), representing 78.6% ± 14.0% of the inlet sulfate. Therefore, some SO_4^{2-} was still present in the effluent wastewater (75.3 ± 33.0 mg S-SO_4^{2-} liter^{-1} day^{-1}) because not all sulfate in the influent wastewater was reduced within the system. On the other hand, CH_4 emissions were very low (between 0 and 8.7 mg COD-CH_4 liter^{-1} day^{-1}) for the first 6 weeks (see Fig. S4B in the supplemental material) but increased to values as high as 44.5 mg COD-CH_4 liter^{-1} day^{-1} from week 8 to week 12.

A 6-h batch test experiment was carried out during week 14 (see Fig. S5 in the supplemental material) to assess if CH_4 production was limited by the presence of sulfate. For the first 4 h, the CH_4 production rate in R3 was twice that of R1 (0.37 and 0.88 mg COD-CH_4 liter^{-1} h^{-1} for R1 and R3, respectively). Remarkably, CH_4 production increased after 4 h of testing (1.06 and 2.07 mg COD-CH_4 liter^{-1} h^{-1} for R1 and R3, respectively), coinciding with the reduction of all sulfate available.

A high level of variability of VFA production rates was observed due to the simultaneous production and consumption of these compounds during batch test experiments (see Fig. S6A in the supplemental material). Nevertheless, VFA production rates were remarkably low for the first 2 weeks of biofilm development. Furthermore, the concentration of VFA exiting the system was higher than those measured in inlet wastewater (see Fig. S6B in the supplemental material).

Comparison of H_2S and CH_4 emissions measured after 1 year of biofilm development with those calculated during the first 3 months of operation in the laboratory suggested similar activities of SRB but clear differences in methanogenesis. After 1 year of growth, emissions of H_2S by laboratory biofilms were slightly different (204.7 ± 14.6 mg S-H_2S liter^{-1} day^{-1}) from those measured at the initial stage (316.5 ± 61.0 mg S-H_2S liter^{-1} day^{-1}). This discrepancy may have been caused by differences in sulfate concentrations in the inlet wastewater between the two periods (26.7 ±

2.5 mg S liter^{-1} and 16.0 ± 1.0 mg S liter^{-1} during the first weeks and after 1 year, respectively). Regardless of these differences in absolute values, mature biofilms performed better when these concentrations were compared in relative terms (~80% and 100% of SO_4^{2-} reduced to H_2S during the initial weeks and after 1 year of operation, respectively). In turn, CH_4 emissions largely increased after 1 year of biofilm growth (from 17.9 ± 15.9 mg COD-CH_4 liter^{-1} day^{-1} to 327.6 ± 16.6 mg COD-CH_4 liter^{-1} day^{-1}).

To determine if the high levels of production of H_2S and CH_4 in mature biofilms under laboratory conditions were similar to the emissions of these compounds under natural conditions (e.g., full-scale sewers), we calculated the daily production of both compounds in both systems. Whereas full-scale sewers discharged 4.56 g S-H_2S day^{-1} m^{-2}, laboratory systems produced 1.58 g S-H_2S day^{-1} m^{-2}. Similar values were obtained for CH_4 production; whereas the full-scale sewer produced 4.24 g COD-CH_4 day^{-1} m^{-2}, laboratory systems emitted 1.65 g COD-CH_4 day^{-1} m^{-2}.

Changes in the composition of microbial communities during biofilm development. DGGE fingerprints showed compositional differences between the bacterial community in the inlet wastewater and that of biofilms grown in R1 over the study period (Fig. 2A). Even though several bands were consistently detected at different time intervals, the variation in the banding pattern suggested changes in the composition of bacterial communities during biofilm development. Hierarchical clustering of samples according to the Dice similarity index clearly segregated wastewater samples from laboratory biofilms. Moreover, biofilm samples clustered according to date of collection (e.g., developmental stage). Less variation between wastewater and biofilm samples was observed for archaeal communities, although a similar clustering of biofilm samples according to date was distinguished (Fig. 2B). A total of 16 of the 23 excised bands (9 and 7 bands from the bacterial and archaeal wastewater communities, respectively) (see Fig. S7 in the supplemental material) yielded good-quality sequences. Differences in the bacterial closest relatives identified and band patterns showed high variability of wastewater bacterial communities. On the other hand, the closest relatives of the identified archaea were less diverse, belonging to *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (see Table S4 in the supplemental material).

Variations in bacterial and archaeal abundance in R1 biofilms during the study period were assessed by qPCR devoted to monitoring biofilm development. Although bacterial 16S rRNA gene copy numbers were always higher than archaeal 16S rRNA copy numbers, both genes showed similar trends in increases of copy numbers for the first 2 weeks of growth, followed by a steady state, which suggested a balanced composition of biofilm communities for the rest of the study period (see Fig. S8 in the supplemental material). Remarkably, *dsrA* gene abundance showed a time course similar to that of bacterial 16S rRNA genes (see Fig. S8 in the supplemental material), suggesting similar growth dynamics of SRB for the first 2 weeks of experiment.

The compositions of microbial communities from R1 and full-scale sewer biofilms were assessed by massively parallel sequencing to determine whether or not H_2S and CH_4 production rates measured over time were related to compositional changes of bacterial and archaeal biofilm communities. Bacterial and archaeal 16S rRNA gene libraries were constructed by using pyrotags from different samples collected during the study period (week 1, week 5, week 13, 1-year, and full-scale sewer samples). The relative contributions of bacterial phyla changed during biofilm development (Fig. 3A). Furthermore, the composition of the bacterial community in 1-year-old biofilms was clearly different from that of the full-scale sewer system (Fig. 3A). Sequences affiliated with the bacterial classes *Bacilli*, *Fusobacteria*, and *Gammaproteobacteria* progressively decreased during biofilm maturation. It is noteworthy that no sequences affiliated with these classes were identified in the bacterial community from the full-scale sewer biofilm. In turn, sequences affiliated with the class *Betaproteobacteria* were prevalent in the full-scale sewer biofilm and in R1 samples collected at

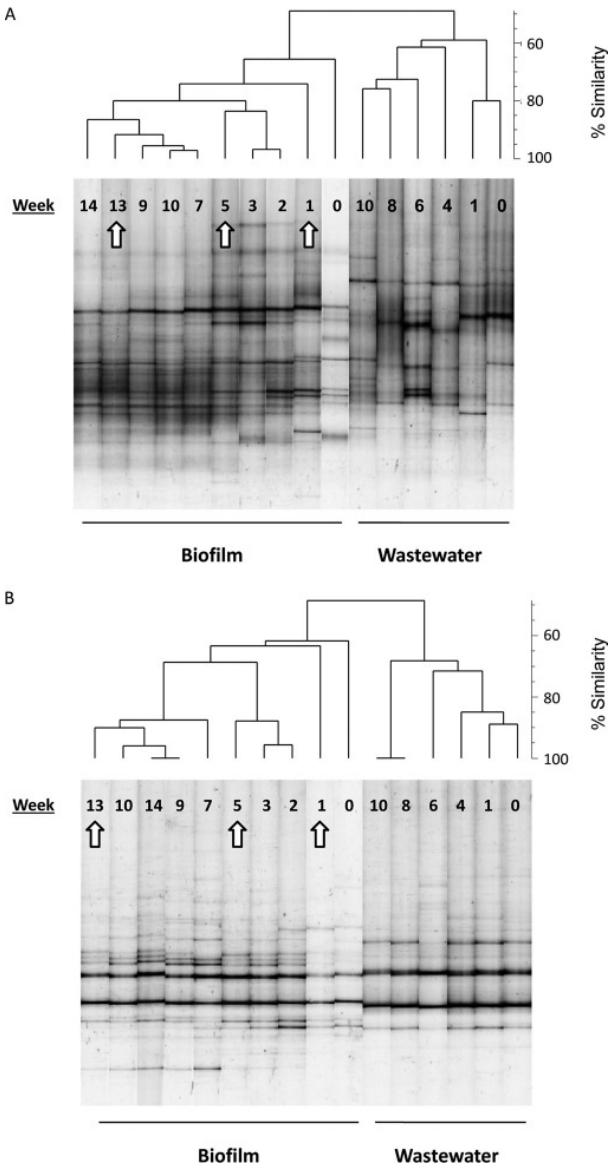

FIG 2 Negative images of DGGE gels of 16S rRNA gene fingerprints for *Bacteria* (A) and *Archaea* (B) from wastewater and biofilms grown in R1. Hierarchical clustering of samples based on Dice similarity indexes of the banding patterns are also shown. White arrows indicate biofilm samples used for further pyrosequencing analyses (weeks 1, 5, and 13).

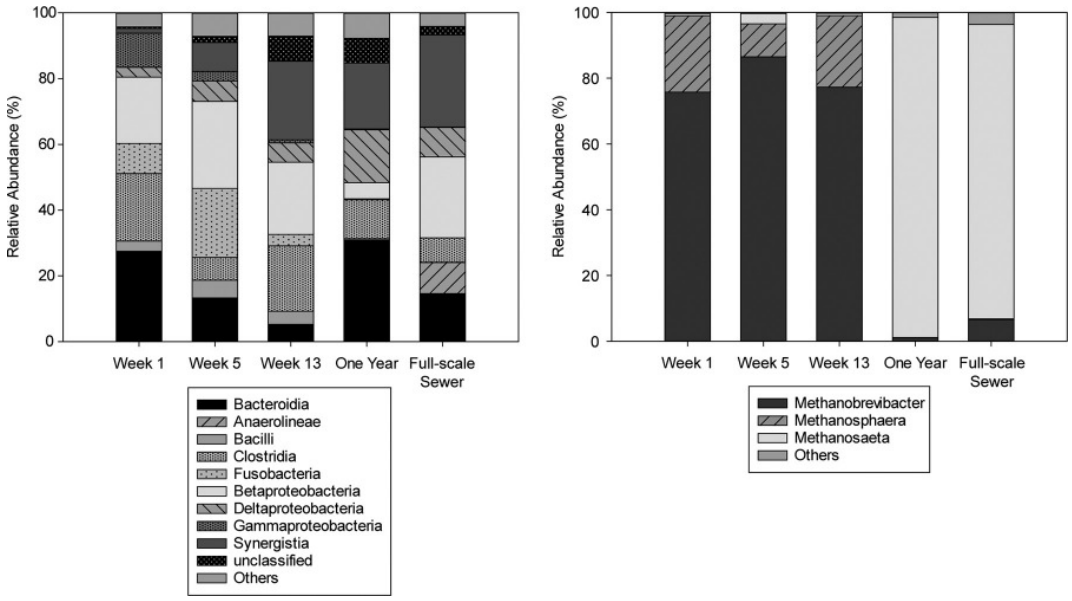

FIG 3 Relative abundances of sequences (percent) affiliated with main bacterial classes (A) and main archaeal genera (B) in week 1, week 5, week 13, 1-year, and full-scale sewer biofilm samples.

the first stages of biofilm development (20 to 26% of total sequences), but they showed less representativeness after 1 year of operation (4.9% of total sequences). On the other hand, the prevalence of sequences affiliated with the classes *Synergistia* and *Deltaproteobacteria* increased during biofilm colonization, reaching similar relative abundances as those found in the full-scale sewer biofilm. Concerning archaeal communities, no archaea other than methanogens were identified in pyrotag libraries from biofilm samples. Specifically, archaeal sequences were affiliated with three main genera, *Methanosphaera*, *Methanobrevibacter*, and *Methanosaeta*. Whereas sequences affiliated with *Methanosphaera* (relative abundances ranging from 10 to 23%) and *Methanobrevibacter* (76 to 86%) were prevalent during the first weeks of biofilm development (Fig. 3B), the archaeal community in 1-year-old biofilms was dominated mainly by sequences affiliated with the genus *Methanosaeta*, which were also prevalent in the biofilm collected from the full-scale sewer (Fig. 3B).

Grouping of sequences into OTUs (97% cutoff) resulted in 1,283 and 137 OTUs for *Bacteria* and *Archaea*, respectively (see Table S5 in the supplemental material). OTU delineation allowed us to identify potentially those OTUs (i.e., species) that may make a relevant contribution to the development and activity of sulfidogenic and methanogenic biofilms. Because of the high diversity of the sample and nutrient availability in the system, OTUs were considered relevant in terms of abundance if their relative abundance was ≥4% in at least one sample. Whereas the relative abundance of some OTUs increased only at the end of the incubation period (OTU-B1, OTU-B6, and OTU-B7), that of others clearly decreased during this time (OTU-B3, OTU-B8, OTU-B10, OTU-B12, and OTU-B20) (Fig. 4A). One of the most prevalent OTUs in early stages of biofilm development (OTU-B3; >10% of total sequences) showed 100% sequence identity to *Macellibacteroides fermentans*, a fermentative member of the *Porphyromonadaceae* (*Bacteroidetes*) (49). Other common OTUs identified during this period (e.g., OTU-B8 and OTU-B20) were rare in mature and full-scale sewer biofilms. In turn, most prevalent OTUs in full-scale sewer biofilms were rare in the laboratory system, with the exception of OTU-B1 (83% sequence identity to *Rikenella microfusus* strain Q-1, an obligate anaerobic fermentative microorganism) (50). The bacterial community in the biofilm collected from the full-scale sewer was composed mainly of microorganisms affiliated with the class *Betaproteobacteria* (OTU-B2, OTU-B14, and OTU-B18) and the phyla *Synergistetes* (OTU-B4, OTU-B5, and OTU-B13) and *Chloroflexi* (OTU-B9) (Fig. 4A). Only OTU-B6 was affiliated with the class *Deltaproteobacteria*, having 99% sequence identity to *Desulfobacter postgatei* strain 2ac9.

In order to study the phylogenetic structure of the SRB community during biofilm development in more detail, sequences affiliated with the class *Deltaproteobacteria*, which includes most of the sulfate reducers known to date, were retrieved and grouped into OTUs that were then used to construct a phylogenetic tree (see Fig. S9A in the supplemental material). Whereas abundant OTUs in the first weeks of incubation (OTU-D3 and OTU-D4) were phylogenetically related to *Desulfobulbus propionicus* strain DSM2032 (see Fig. S10 in the supplemental material), the composition of the SRB community changed as the biofilm developed. After 1 year of operation, the community was dominated mainly by OTU-D1 (36% of total deltaproteobacterial sequences), which showed 99% sequence identity to *Desulfobacter postgatei* strain 2ac9 (see Fig. S9A and Table S6 in the supplemental material). Although this OTU was also present in biofilms collected from a full-scale sewer, the deltaproteobacterial community under natural conditions was more diverse than that grown under laboratory conditions.

In turn, abundant archaeal OTUs (>4% of total sequences) were all affiliated with methanogenic lineages. Particularly, OTU-A1, which showed 99% sequence similarity to *Methanosaeta concilii*, was detected only in mature biofilms and in biofilms from the

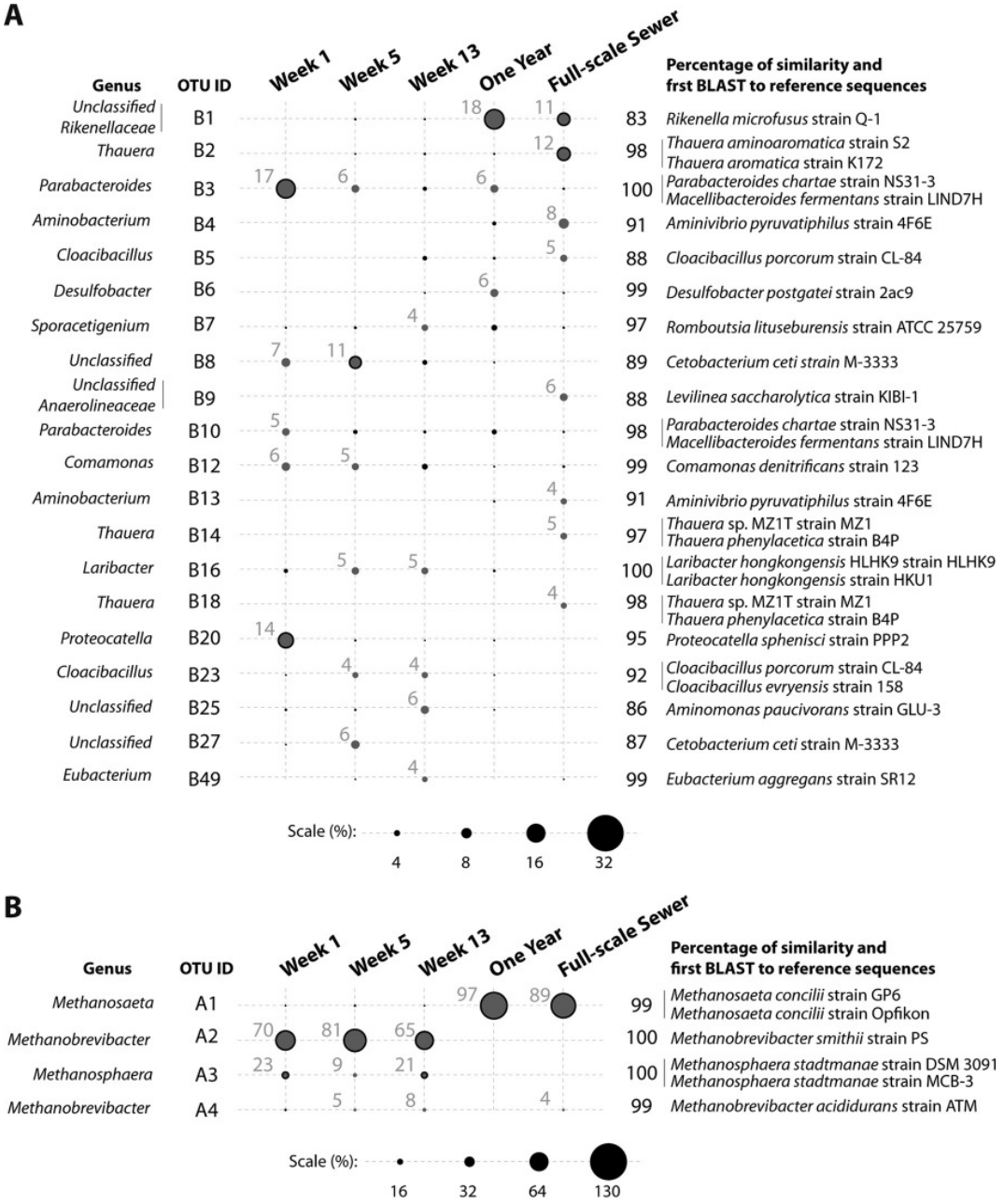

FIG 4 Bubble plots of bacterial (A) and archaeal (B) OTUs showing their relative abundances across samples, their taxonomy affiliation (at the genus level), and the percent identity to the first BLAST hit against reference sequence databases. Data are proportional to the radius and plotted on a logarithmic scale, as indicated below the graph. The relative abundance (percent) of each OTU at different sampling points is indicated next to the corresponding bubble (gray figures).

full-scale sewer (Fig. 4B; see also Fig. S9B in the supplemental material). In turn, OTU-A2 and OTU-A3 were detected mainly during the first weeks of biofilm growth. Both OTUs had 100% sequence identity to *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, respectively. Finally, OTU-A4 (showing 99% sequence similarity to *Methanobrevibacter acididurans*) was detected at low relative abundances in all pyrotag libraries analyzed.

Richness and diversity metrics calculated for the bacterial biofilm communities increased during the experimental period (see Table S7 in the supplemental material). However, the bacterial community in the biofilm from the full-scale sewer was less rich and diverse than that from biofilms under laboratory conditions. In turn, the richness of the archaeal community showed an opposite trend, clearly decreasing during the 13 weeks of incubation,

but remained at a similar level in mature biofilms (see Table S7 in the supplemental material). Despite these changes in richness, archaeal diversity remained fairly constant from the start-up to the end of the monitoring period and decreased in mature biofilms. Moreover, both the richness and diversity of archaeal biofilm communities in the full-scale in-sewer biofilm were higher than the levels estimated for biofilms after 1 year of operation under laboratory conditions.

To easily compare bacterial and archaeal biofilm communities, samples were distributed in a nMDS two-dimensional (2D) ordination space according to their similarity based on the weighted UniFrac distance (see Fig. S11 in the supplemental material). The ordination segregated biofilm samples collected at early stages of development (weeks 1, 5, and 13) from those collected at mature stages from the laboratory-scale sewer and from the biofilm samples from the full-scale sewer. It is noteworthy that bacterial and archaeal communities in mature biofilms (i.e., 1 year of incubation) were similar to those occurring in biofilms from full-scale sewers.

DISCUSSION

Sulfide and methane production rates during biofilm formation. In this study, we investigated the association between H_2S and CH_4 production and the corresponding biofilm development stage in a laboratory-scale anaerobic sewer pilot plant. H_2S production rates suggested a fully adapted and functional SRB community after 2 weeks of biofilm colonization. The low level of production of H_2S for the first 2 weeks may have been a consequence of the low abundance of SRB in young biofilms after the experimental setup (Fig. 4; see also Fig. S8 in the supplemental material). In turn, the higher level of H_2S production in R1 than in R2 and R3 may have resulted from the system design, considering that the bioreactors were connected in series and that wastewater that entered R2 and R3 contained only trace amounts of sulfate because of its consumption in R1.

Methane production rates measured in batch tests were minimal for the first 12 weeks, probably because reactors were filled with fresh wastewater (containing high concentrations of sulfate) just before the start-up of each batch test. The differences in CH_4 production and emission rates might be a consequence of biofilm adaptation under each reactor condition, which varied mainly in terms of the sulfate concentration and HRT. During normal functioning, the small quantity of sulfate in the R1 effluent could have promoted active methanogenesis in R2 and R3, whereas conditions in R1 (high sulfate and organic matter concentrations), in turn, favored SRB over MA (25, 51). Results from 6-h batch test experiments confirmed a stimulation of CH_4 production after 3 to 4 h of wastewater retention in the system (when sulfate was depleted), especially in R3, where the sulfate concentration was already low (see Fig. S4 in the supplemental material). These results point to a spatial segregation of microbial communities responsible for H_2S and CH_4 production along the length of the anaerobic sewer, although no direct evidences of this differential distribution were obtained. Further work is then needed to validate if both the composition and activity of SRB and MA communities in sewer biofilms vary along the length of full-scale sewer systems.

Sulfide and methane emissions by mature biofilms. Comparison of H_2S emissions from young biofilms and those from mature biofilms showed a decrease as a consequence of the smaller amount of sulfate available in the influent wastewater. Notwithstanding this, the relative amount of sulfate reduced to H_2S increased in mature biofilms (from ≈80% to 100%). Concerning CH_4 emission, several factors could account for its increase in mature biofilms (from 17.9 ± 15.9 to 327.6 ± 16.6 mg COD-CH_4 liter^{-1} day^{-1}), namely (i) the low sulfate concentration in the inlet wastewater after 1 year of the experiment favoring a higher methanogenic activity, (ii) the high rate of consumption of sulfate by SRB in mature biofilms stimulating CH_4 production, or (iii) a change in the composition of the methanogenic community over time toward species more adapted to local conditions, resulting in a higher level of production of CH_4.

Compositional changes of microbial communities. DGGE fingerprints showed differences in the overall compositions of bacterial and archaeal communities between inlet wastewater and biofilm samples. Despite the inherent limitations of the PCR-DGGE approach (52), similarity analysis of both bacterial and archaeal communities based on DGGE band patterns grouped samples according to sampling date (i.e., stage of biofilm development), showing that the structure of microbial biofilm communities progressively adapted to local conditions in the system. The fact that both bacterial and archaeal communities showed similar clustering patterns suggests potential interactions (e.g., synergy or competition) that deserve further investigation.

During the first weeks of biofilm development, the most abundant OTUs belonging to the class *Deltaproteobacteria* (OTU-D3 and OTU-D4) were closely related to *Desulfobulbus propionicus*. Interestingly, this species was recently identified by Sun and coworkers as the main SRB in the outer layers of sewer biofilms (10). *D. propionicus* reduces sulfate via the incomplete oxidation of organic acids such as lactate, propionate, butyrate, and ethanol to acetate (53), all of which were available in the inlet wastewater. In turn, the SRB community in mature biofilms was composed mainly of a deltaproteobacterium closely related to *Desulfobacter postgatei* (OTU-D1), whereas sequences affiliated with SRB colonizers (i.e., OTU-D3 and OTU-D4) were rare after 1 year of incubation (see Fig. S10 in the supplemental material).

Hydrogenotrophic methanogens (belonging to the order *Methanomicrobiales* or *Methanobacteriales*) may use H_2 generated in fermentative metabolisms or act as hydrogen scavengers in syntrophic growth with acetate-oxidizing microorganisms (54–57). Also, acetate produced during fermentation of organic substrates by anaerobic heterotrophs within the biofilm matrix would be used by acetoclastic methanogens (*Methanosarcinaceae* and *Methanosaetaceae*) (58). The identification of sequences belonging to both groups of methanogens (hydrogenotrophic and acetoclastic) in our experimental system during the study period lends support to a progressive change of methanogenic pathways over time in relation to both local environmental conditions and the composition of the archaeal community at each stage of biofilm development.

Methanobrevibacter smithii and *Methanosphaera stadtmanae* (*Methanobacteriales*) are considered to be the prevalent methanogens in the human gut (59). In our study, sequences belonging to both species were identified in DGGE fingerprints from inlet wastewater samples and in pyrotag libraries from the first weeks of biofilm development, suggesting that archaeal colonizers at early stages of biofilm development derive from human fecal material in wastewater. These human-derived methanogens were probably outcompeted later on by acetoclastic methanogens (e.g., *Methanosaeta concilii*), which would probably be more adapted to envi-

ronmental conditions in the pilot plant. The time needed by these better-adapted methanogens to be established in the biofilm matrix is consistent with the low level of CH_4 production during the initial phases of biofilm development. During this first stage, methanogenesis was also probably inhibited by sulfate reducers, which decrease the H_2 potential pressure below levels required by methanogens when sulfate is not limiting (60). Despite the well-known competitive interaction between SRB and MA, several studies have demonstrated that both groups coexist under certain conditions (60, 61). Particularly, Struchtemeyer and coworkers reported that low levels of sulfate may favor acetate consumption by MA rather than by SRB (62). In this regard, and although it is always risky to infer functional properties from phylogeny (63), sequences affiliated with both *Deltaproteobacteria* and MA identified in mature biofilms were closely related to species that are able to use acetate (i.e., *D. postgatei* and *M. concilii*, respectively). Accordingly, the increase in CH_4 production measured after 1 year of incubation might be explained by the establishment of acetoclastic methanogens in the biofilm, favored by a greater availability of acetate in wastewater. Besides, the increase in CH_4 production also could have been favored by the stratification of both groups within the biofilm matrix, as recently reported (10), although in our case, no measurements aimed at resolving the spatial organization of SRB and MA in the studied biofilms were carried out.

Altogether, this study provides data that confirm the capacity of our laboratory experimental system to mimic the functioning of full-scale sewers both microbiologically and operationally in terms of H_2S and CH_4 production and the composition of microbial communities during biofilm growth. Whereas H_2S emission was notably high during early stages of biofilm development, CH_4 emissions increased after biofilm maturation, coinciding with an establishment of a methanogenic community better adapted to sewer conditions; for this reason, it is important to take into account that the management of sewer systems is very important from the first stages of sewer functioning. Although further research is needed to better resolve the dynamics of the bacterial communities in biofilms and to identify the key bacterial players involved in both nutrient transformations and potential syntrophic interactions that occur in these complex ecosystems, our results should be valuable when designing optimal strategies to mitigate H_2S and CH_4 emissions from sewer systems.

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REFERENCES

1. **Tchobanoglous G, Burton FL, Stensel HD (ed).** 2003. Metcalf & Eddy wastewater engineering, treatment and reuse, 4th ed. McGraw-Hill, New Delhi, India.
2. **Hvitved-Jacobsen T.** 2002. Sewer processes—microbial and chemical process engineering of sewer networks, 1st ed. CRC Press, Boca Raton, FL.
3. **Dreeszen PH.** 2003. The key to understanding and controlling bacterial growth in automated drinking water systems, 2nd ed. Edstrom Industries, Waterford, WI.
4. **Boon AG.** 1995. Septicity in sewers: causes, consequences and containment. Water Sci Technol **31**(7)**:**237–253. http://dx.doi.org/10.1016/0273-1223(95)00341-J.
5. **Boon AG, Vincent AJ, Boon KG.** 1998. Avoiding the problems of septic sewage. Water Sci Technol **37**(1)**:**223–231. http://dx.doi.org/10.1016/S0273-1223(97)00773-7.
6. **Thistlethwayte DKB.** 1972. The control of sulfides in sewerage systems. Butterworth, Sydney, Australia.
7. **Ganigue R, Gutierrez O, Rootsey R, Yuan Z.** 2011. Chemical dosing for sulfide control in Australia: an industry survey. Water Res **45:**6564–6574. http://dx.doi.org/10.1016/j.watres.2011.09.054.
8. **Guisasola A, de Haas D, Keller J, Yuan Z.** 2008. Methane formation in sewer systems. Water Res **42:**1421–1430. http://dx.doi.org/10.1016/j.watres.2007.10.014.
9. **Foley J, Yuan Z, Lant P.** 2009. Dissolved methane in rising main sewer systems: field measurements and simple model development for estimating greenhouse gas emissions. Water Sci Technol **60**(11)**:**2963–2971. http://dx.doi.org/10.2166/wst.2009.718.
10. **Sun J, Hu S, Sharma KR, NiB-J, Yuan Z.** 2014. Stratified microbial structure and activity in sulfide- and methane-producing anaerobic sewer biofilms. Appl Environ Microbiol **80:**7042–7052. http://dx.doi.org/10.1128/AEM.02146-14.
11. **Intergovernmental Panel on Climate Change.** 2013. Working group I contribution to the assessment report climate change 2013: the physical science basis. Final draft underlying scientific-technical assessment. Cambridge University Press, Cambridge, United Kingdom.
12. **Foley J, Yuan Z, Keller J, Senante E, Chandran K, Willis J, Shah A, van Loosdrecht M, van Voorthuizen E.** 2011. N_2O and CH_4 emission from wastewater collection and treatment systems: state of the science report. IWA Publishing, London, United Kingdom.
13. **Rodriguez-Caballero A, Aymerich I, Poch M, Pijuan M.** 2014. Evaluation of process conditions triggering emissions of green-house gases from a biological wastewater treatment system. Sci Total Environ **493:**384–391. http://dx.doi.org/10.1016/j.scitotenv.2014.06.015.
14. **Auguet O, Pijuan M, Guasch-Balcells H, Borrego CM, Gutierrez O.** 2015. Implications of downstream nitrate dosage in anaerobic sewers to control sulfide and methane emissions. Water Res **68:**522–532. http://dx.doi.org/10.1016/j.watres.2014.09.034.
15. **Jiang G, Sharma KR, Guisasola A, Keller J, Yuan Z.** 2009. Sulfur transformation in rising main sewers receiving nitrate dosage. Water Res **43:**4430–4440. http://dx.doi.org/10.1016/j.watres.2009.07.001.
16. **Mohanakrishnan J, Gutierrez O, Sharma KR, Guisasola A, Werner U, Meyer RL, Keller J, Yuan Z.** 2009. Impact of nitrate addition on biofilm properties and activities in rising main sewers. Water Res **43:**4225–4237. http://dx.doi.org/10.1016/j.watres.2009.06.021.
17. **Mohanakrishnan J, Gutierrez O, Meyer RL, Yuan Z.** 2008. Nitrite effectively inhibits sulfide and methane production in a laboratory scale sewer reactor. Water Res **42:**3961–3971. http://dx.doi.org/10.1016/j.watres.2008.07.001.
18. **Jiang G, Gutierrez O, Sharma KR, Yuan Z.** 2010. Effects of nitrite concentration and exposure time on sulfide and methane production in sewer systems. Water Res **44:**4241–4251. http://dx.doi.org/10.1016/j.watres.2010.05.030.
19. **Jiang G, Gutierrez O, Yuan Z.** 2011. The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res **45:**3735–3743. http://dx.doi.org/10.1016/j.watres.2011.04.026.
20. **Firer D, Friedler E, Lahav O.** 2008. Control of sulfide in sewer systems by dosage of iron salts: comparison between theoretical and experimental results, and practical implications. Sci Total Environ **392:**145–156. http://dx.doi.org/10.1016/j.scitotenv.2007.11.008.
21. **Gutierrez O, Mohanakrishnan J, Sharma KR, Meyer RL, Keller J, Yuan Z.** 2008. Evaluation of oxygen injection as a means of controlling sulfide production in a sewer system. Water Res **42:**4549–4561. http://dx.doi.org/10.1016/j.watres.2008.07.042.
22. **Gutierrez O, Park D, Sharma KR, Yuan Z.** 2010. Iron salts dosage for sulfide control in sewers induces chemical phosphorus removal during wastewater treatment. Water Res **44:**3467–3475. http://dx.doi.org/10.1016/j.watres.2010.03.023.
23. **Gutierrez O, Park D, Sharma KR, Yuan Z.** 2009. Effects of long-term pH elevation on the sulfate-reducing and methanogenic activities of anaero-

bic sewer biofilms. Water Res **43:**2549–2557. http://dx.doi.org/10.1016/j.watres.2009.03.008.
24. **Gutierrez O, Sudarjanto G, Ren G, Ganigué R, Jiang G, Yuan Z.** 2014. Assessment of pH shock as a method for controlling sulfide and methane formation in pressure main sewer systems. Water Res **48:**569–578. http://dx.doi.org/10.1016/j.watres.2013.10.021.
25. **Lovley DR, Klug MJ.** 1983. Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. Appl Environ Microbiol **45:**187–192.
26. **Omil F, Lens P, Visser A, Hulshoff Pol LW, Lettinga G.** 1998. Long-term competition between sulfate reducing and methanogenic bacteria in UASB reactors treating volatile fatty acids. Biotechnol Bioeng **57:**676–685.
27. **Gutierrez O, Sudarjanto G, Sharma KR, Keller J, Yuan Z.** 2011. SCORe-CT: a new method for testing effectiveness of sulfide-control chemicals used in sewer systems. Water Sci Technol **64**(12):2381–2388. http://dx.doi.org/10.2166/wst.2011.809.
28. **Gutierrez O, Sutherland-Stacey L, Yuan Z.** 2010. Simultaneous online measurement of sulfide and nitrate in sewers for nitrate dosage optimisation. Water Sci Technol **61**(3):651–658. http://dx.doi.org/10.2166/wst.2010.901.
29. **Keller-Lehmann B, Corrie S, Ravn R, Yuan Z, Keller J.** 2006. Preservation and simultaneous analysis of relevant soluble sulfur species in sewage samples. Proceedings of the Second International IWA Conference on Sewer Operation and Maintenance. BOKU-SIG, Vienna, Austria.
30. **APHA.** 1998. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Washington, DC.
31. **Muyzer G, de Waal EC, Uitterlinden AG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol **59:**695–700.
32. **Muyzer G, Ramsing NB.** 1995. Molecular methods to study the organization of microbial communities. Water Sci Technol **32**(8)**:**1–9. http://dx.doi.org/10.1016/0273-1223(96)00001-7.
33. **Lane DJ.** 1991. 16S/23S rRNA sequencing, p 115–175. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY.
34. **Llirós M, Casamayor EO, Borrego C.** 2008. High archaeal richness in the water column of a freshwater sulfurous karstic lake along an interannual study. FEMS Microbiol Ecol **66:**331–342. http://dx.doi.org/10.1111/j.1574-6941.2008.00583.x.
35. **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R.** 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics **27:**2194–2200. http://dx.doi.org/10.1093/bioinformatics/btr381.
36. **Hall T.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser (Oxf) **41:**95–98.
37. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J Mol Biol **215:**403–410. http://dx.doi.org/10.1016/S0022-2836(05)80360-2.
38. **Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S.** 2003. Quantitative real-time PCR using TaqMan and SYBR green for *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ* gene and total bacteria. FEMS Immunol Med Microbiol **39:**81–86. http://dx.doi.org/10.1016/S0928-8244(03)00224-4.
39. **Takai K, Horikoshi K.** 2000. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl Environ Microbiol **66:**5066–5072. http://dx.doi.org/10.1128/AEM.66.11.5066-5072.2000.
40. **Stahl D, Amman R.** 1991. Development and application of nucleic acid probes, p 205–248. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY.
41. **Ben-Dov E, Brenner A, Kushmaro A.** 2007. Quantification of sulfate-reducing bacteria in industrial wastewater, by real-time polymerase chain reaction (PCR) using *dsrA* and *apsA* genes. Microb Ecol **54:**439–451. http://dx.doi.org/10.1007/s00248-007-9233-2.
42. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem **55:**611–622. http://dx.doi.org/10.1373/clinchem.2008.112797.
43. **Øvreås L, Forney L, Daae FL, Torsvik V.** 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol **63:**3367–3373.
44. **DeLong EF.** 1992. Archaea in coastal marine environments. Proc Natl Acad Sci U S A **89:**5685–5689. http://dx.doi.org/10.1073/pnas.89.12.5685.
45. **Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeehan T, Hagevoort RG, Edrington TS.** 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol **8:**125–132. http://dx.doi.org/10.1186/1471-2180-8-125.
46. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.** 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **75:**7537–7541. http://dx.doi.org/10.1128/AEM.01541-09.
47. **Lozupone C, Knight R.** 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol **71:**8228–8235. http://dx.doi.org/10.1128/AEM.71.12.8228-8235.2005.
48. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
49. **Jabari L, Gannoun H, Cayol J-L, Hedi A, Sakamoto M, Falsen E, Ohkuma M, Hamdi M, Fauque G, Ollivier B, Fardeau M-L.** 2012. *Macellibacteroides fermentans* gen. nov., sp. nov., a member of the family *Porphyromonadaceae* isolated from an upflow anaerobic filter treating abattoir wastewaters. Int J Syst Evol Microbiol **62:**2522–2527. http://dx.doi.org/10.1099/ijs.0.032508-0.
50. **Kaneuchi C, Mitsuoka T.** 1978. *Bacteroides microfusus*, a new species from the intestines of calves, chickens, and Japanese quails. Int J Syst Bacteriol **28:**478–481. http://dx.doi.org/10.1099/00207713-28-4-478.
51. **Oremland RS, Polcin S.** 1982. Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. Appl Environ Microbiol **44:**1270–1276.
52. **Kisand V, Wikner J.** 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J Microbiol Methods **54:**183–191. http://dx.doi.org/10.1016/S0167-7012(03)00038-1.
53. **Holmes DE, Bond DR, Lovley DR.** 2004. Electron transfer by *Desulfobulbus propionicus* to Fe(III) and graphite electrodes. Appl Environ Microbiol **70:**1234–1237. http://dx.doi.org/10.1128/AEM.70.2.1234-1237.2004.
54. **Hattori S, Kamagata Y, Hanada S, Shoun H.** 2000. *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int J Syst Evol Microbiol **50:**1601–1609. http://dx.doi.org/10.1099/00207713-50-4-1601.
55. **Petersen SP, Ahring BK.** 1991. Acetate oxidation in a thermophilic anaerobic sewage-sludge digestor: the importance of non-aceticlastic methanogenesis from acetate. FEMS Microbiol Lett **86:**149–152. http://dx.doi.org/10.1111/j.1574-6968.1991.tb04804.x.
56. **Schnürer A, Svensson BH, Schink B.** 1997. Enzyme activities in and energetics of acetate metabolism by the mesophilic syntrophically acetate-oxidizing anaerobe. FEMS Microbiol Lett **154:**331–336. http://dx.doi.org/10.1016/S0378-1097(97)00350-9.
57. **Karakashev D, Batstone DJ, Trably E, Angelidaki I.** 2006. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of *Methanosaetaceae*. Appl Environ Microbiol **72:**5138–5141. http://dx.doi.org/10.1128/AEM.00489-06.
58. **Ferry JG.** 1992. Methane from acetate. J Bacteriol **174:**5489–5495.
59. **Dridi B, Henry M, El Khéchine A, Raoult D, Drancourt M.** 2009. High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. PLoS One **4:**e7063. http://dx.doi.org/10.1371/journal.pone.0007063.
60. **Lovley DR, Dwyer DF, Klug MJ.** 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl Environ Microbiol **43:**1373–1379.
61. **Kristjansson JK, Schönheit P, Thauer RK.** 1982. Different K_s values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Arch Microbiol **131:**278–282. http://dx.doi.org/10.1007/BF00405893.
62. **Struchtemeyer CG, Elshahed MS, Duncan KE, McInerney MJ.** 2005. Evidence for aceticlastic methanogenesis in the presence of sulfate in a gas condensate-contaminated aquifer. Appl Environ Microbiol **71:**5348–5353. http://dx.doi.org/10.1128/AEM.71.9.5348-5353.2005.
63. **Gray ND, Head IM.** 2001. Linking genetic identity and function in communities of uncultured bacteria. Environ Microbiol **3:**481–492. http://dx.doi.org/10.1046/j.1462-2920.2001.00214.x.

Chapter 4

Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions

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Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions

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ABSTRACT

Nitrate (NO_3^-) is commonly dosed in sewer systems to reduce sulfide (H_2S) and methane (CH_4) produced in anaerobic rising main pipes. However, anoxic conditions along the whole rising pipes are difficult and costly to maintain since nitrate is added at the upstream sections of the sewer. In this study we tested the effects of the Downstream Nitrate Dosage strategy (DND) in anaerobic pipes in a specially designed laboratory-scale systems that mimics a real rising main. Effectiveness of the strategy was assessed on H_2S and CH_4 abatement on the effluent of the lab sewer system. A combination of process (Normal Functioning monitoring and batch tests) and molecular (by 454-pyrosequencing) methods were used to investigate the impacts and microbial activities related to the nitrate addition. Results showed a complete abatement of H_2S generated, with a fraction transformed to elemental sulfur (S^0). Methane discharged was reduced to 50% while nitrate was added, due to the CH_4 oxidation in the anoxic conditions established at the end of the pipe. Both sulfidogenic and methanogenic activities resumed upon cessation of NO_3^- dosage. An increase of microorganisms of the genera *Simplicispira*, *Comamonas*, *Azonexus* and *Thauera* was detected during nitrate addition. Regarding anoxic methane oxidation, only one Operational Taxonomic Unit (OTU) was identified, which is likely related with this metabolism. Obtained results are relevant for the optimal management of nitrate dosage strategies in sewer systems.

1. Introduction

Sewer networks are important infrastructures aimed to collect and transport wastewater to wastewater treatment plants (WWTP). Wastewater is transported either through aerobic (gravity) or anaerobic (rising-pressured) sewer pipes where biofilms are usually developed. The activity of microorganisms that are part of these biofilms produces changes in wastewater characteristics during its transport. These

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Abbreviations

CH_4	Methane
COD	Chemical oxygen demand
DND	Downstream Nitrate Dosage
H_2S	Sulfide
hNRB	Heterotrophic, nitrate reducing bacteria
HRT	Hydraulic retention time
IC	Ion chromatography
MA	Methanogenic archaea
MOR	Methane oxidation rate
N_2O	Nitrous oxide
NF	Normal functioning
NO_2^-	Nitrite
NO_3^-	Nitrate
NRR	Nitrate reduction rate
OTU	Operational taxonomic unit
S^0	Elemental sulfur
SBR	Sulfate reducing bacteria
SCR	Sulfide consumption rate
sCOD	Soluble chemical oxygen demand
SO_4^{2-}	Sulfate
soNRB	Sulfide-oxidizing nitrate-reducing bacteria
SPR	Sulfate production rate
TSS	Total suspended solids
VFAs	Volatile Fatty Acids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plants

changes may affect on the subsequent wastewater treatment, the integrity of sewers and also produce health and environmental risks (Hvitved-Jacobsen, 2002).

A widely reported problem in anaerobic sewers is the production of sulfide (H_2S) as a result of oxidation of organic matter by sulfate reducing bacteria (SRB) (Boon, 1995; Hvitved-Jacobsen, 2002). When H_2S is released to the sewer atmosphere it causes malodor, corrosion and health problems. However, H_2S is not the only problematic compound produced under these conditions. Recently, the generation of methane (CH_4) by methanogenic archaea was also detected in sewers systems (Foley et al., 2009; Guisasola et al., 2008). Management of CH_4 is very important as it is a potent greenhouse gas with higher global warming potential than carbon dioxide (IPCC, 2013) and it is also a safety problem in confined spaces due to its low explosion limit (Spencer et al., 2006). Another problem caused as a consequence of methane production is the consumption of part of the chemical oxygen demand (COD) in wastewater that is needed for biological nutrient removal in WWTP.

Different mitigation strategies have been used to reduce H_2S and CH_4 production in sewers. Those include the addition to the sewer-liquid-phase of nitrate (Jiang et al., 2009; Mohanakrishnan et al., 2009a,b; Zhang et al., 2008), nitrite (Jiang et al., 2011a, 2010; Mohanakrishnan et al., 2008), free nitrous acid (Jiang et al., 2011b), iron salts (Firer et al., 2008; Gutierrez et al., 2010a), oxygen (Boon, 1995; Gutierrez et al., 2008), magnesium hydroxide (Gutierrez et al., 2009) or sodium hydroxide (Gutierrez et al., 2014). Although primarily designed to control sulfide, these chemical-dosing practices may also induce inhibitory effects on methanogens in sewers.

Nitrate (NO^{3-}), for instance, is an effective and widely used chemical especially in Europe (Ganigue et al., 2011; Zhang et al., 2008). Two main mechanisms have been suggested to control sulfide production by nitrate addition in sewers: anoxic sulfide oxidation and competitive exclusion of SRB. The first involves the growth of a chemolithotrophic sulfide-oxidizing nitrate-reducing community, able to oxidize sulfide to elemental sulfur as a major intermediate coupled to nitrate reduction. The latter triggers the development of a heterotrophic, nitrate reducing bacteria (hNRB) community, competing with SRB for organic electron donors. Jiang et al. (2013) proposed a conceptual biofilm model with competitive and synergistic interactions among hNRB, sulfide-oxidizing nitrate-reducing bacteria (soNRB), SRB and methanogenic archaea (MA) occurring in upstream sections of a sewer pipe. They suggested that microbial stratification within the biofilm plays a major role and that methane control is related to penetration of nitrate into the biofilm. Methanogenesis would persist in the deeper parts of the biofilm where soluble chemical oxygen demand (sCOD) would penetrate but not nitrate and/or sulfate (SO_4^{2-}).

Nitrate is normally dosed at wet wells or pumping stations. However the main limitation of this approach is that anoxic conditions must be continuously kept through the whole pipe, otherwise H_2S build-up resumes immediately after the depletion of the dosed nitrate (Mohanakrishnan et al., 2009a,b). This implies very high costs in chemicals, typically between 48.7 and 159.3 $/ML according to Ganigue et al. (2011), since the presence of nitrate has to be ensured along all the sewer pipe. The need for more cost-effective methods for H_2S and CH_4 mitigation has led to the development of new nitrate dosing strategies based on improved dosage rates and dosing locations. For instance, Gutierrez et al. (2010b) tested seven different nitrate dosing strategies in a laboratory-scale sewer system, providing strong support to H_2S-control optimization. The results showed the benefits of adding nitrate at a point close to the end of the pipe, named Downstream Nitrate Dosage (DND). With this strategy, H_2S production was still occurring in the first sections of the pipe not exposed to nitrate but was immediately consumed as soon as passing through the anoxic sections. Nitrate consumption was reduced by 42% while still ensuring complete abatement of H_2S. However the effects of the DND on methane production from sewers have not been established yet. The simultaneous presence of H_2S, CH_4 (generated in upstream pipe sections) plus NO_3^- (added in downstream sections) would lead to different conditions, not reported to date, that could be important to validate the overall effectiveness of the DND strategy.

The aim of this study is to determine the effects of Downstream Nitrate Dosage on anaerobic sewer biofilms with regards to sulfide oxidation and, for the first time, on methane production. The work was carried out in a laboratory main sewer system previously validated to mimic the main features of sewer rising mains (Gutierrez et al., 2011). The DND testing involved three phases, namely: the baseline phase, the dosage phase during which NO_3^- was added to the downstream section of the pipe, and a recovery phase, during which the performance of the pipe was monitored subsequent to the

termination of the dosage. The work provides further information on the optimal dosage of nitrate for H_2S and CH_4 control, both at process and microbiological levels. The production of nitrogen-related greenhouse gas, nitrous oxide (N_2O), was also monitored to evaluate a potential greenhouse gas effect of the DND strategy. Besides, the changes in biofilm bacterial and archaeal communities exposed to nitrate were investigated by 454-pyrosequencing over the study period.

2. Materials and methods

2.1. Laboratory sewer system

A laboratory reactor system simulating a real rising main in terms of operation and performance was used. The lab-scale system was based on the SCORE-ct system (Gutierrez et al., 2011) previously demonstrated to successfully mimic the main features of anaerobic sewer rising mains described in Hvitved-Jacobsen (2002). Those consist of: (i) hydraulic features: hydraulic retention times (HRT), turbulence and area-to-volume ratio (A/V), and (ii) wastewater characteristics: sulfate concentration, biodegradable organic matter concentration, pH and temperature. The system consisted of three completed-sealed PersPex™ reactors (R1, R2 and R3) connected in series to simulate three different sections of a real rising main sewer pipe (Fig. 1). Each reactor had a volume of 0.75 L and an inner diameter of 80 mm. Typical sewer turbulent conditions were mimicked using magnetic stirrers (Heidolph MR Hei Mix S). To provide easily extractable biofilm samples, 51 Plastic carriers (Anox Kaldnes, Norway) of 1 cm^2 of surface were also clustered on three stainless-steel rods inside each reactor. Total biofilm growth area was 0.05 m^2 (including carriers and wall area) with a final area to volume ratio (A/V) of 65 m^2/m^3.

The system was fed with domestic wastewater collected weekly in the upstream section of a sewer network in the municipality of Girona. The sewage was stored at 4 °C to minimize biological transformations, and heated to 20 °C before it was transferred into R1 (see Fig. 1). The system was intermittently fed through a peristaltic pump (Masterflex model 7520-47) following a typical pumping pattern observed in a real sewer pipe used as a reference (Radin sewer pipe, municipality of l'Escala, North East Catalonia, Spain). The system was daily exposed to 12 sewage pump cycles and the diurnal variation of the sewage HRT was maintained within a minimum and maximum of 4–9 h respectively.

Influent wastewater was characterized in a weekly basis. The sewage typically contained sulfate at concentration of 16.4 ± 1.6 mg S-SO_4^{2-}/L, 529.0 ± 40.0 mg COD/L of total COD and 277.7 ± 9.8 mg COD/L of soluble COD. Negligible amounts (<1 mg/L) of sulfide, sulfite, thiosulfate and methane were present. The system was operated at 23.3 ± 1.7 °C.

2.2. Normal functioning and microbial activity monitoring

The testing lasted approximately 4 months and was divided in three different phases:

- Base line monitoring (Phase 1). The pilot plant had been in operation for one year before this study was conducted. Anaerobic biofilms were present on the walls and carriers of the reactors. A baseline monitoring was performed for 1 month before starting the nitrate addition to confirm the pseudo-state conditions in terms of sulfide and methane production.
- Nitrate addition (Phase 2). Nitrate was added into R3 for a period of 49 days. A solution of nitrate (3.9 g N-NO_3^-/L) was added in an on/off mode controlled by the concentration of nitrate present in R3. When the nitrate concentration was lower than the set-point (5 mg N-NO_3^-/L), the nitrate pump was activated for a 2 min period resulting in an increase of the nitrate concentration (47 ± 13 mg N-NO_3^-/L). With this strategy, the presence of nitrate was always ensured in R3.

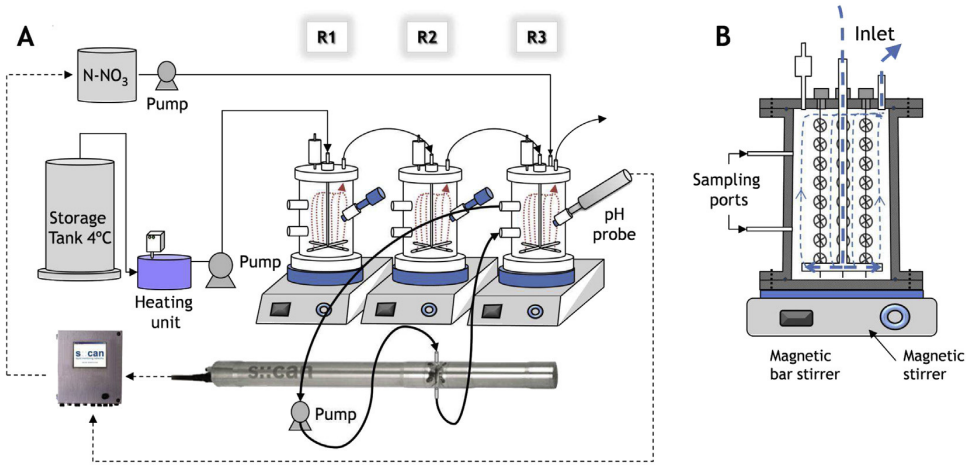

Fig. 1 – (A) Schematic representation of the laboratory scale sewer system used in this study. (B) Schematic view of one of the reactors of the laboratory scale sewer system.

Recovery period (Phase 3). The monitoring of the system was continued for a period of 36 days after the termination of the nitrate dosage.

The system was operated in two different regimes: normal functioning (NF) and batch test (BT).

2.2.1. *Normal functioning (NF)*

During NF mode changes in the wastewater characteristics were monitored as the wastewater was transported through the system. H_2S and NO_3^- concentrations were continuously measured in reactor R3 by means of a s::can spectrophotometer (model spectro::lyser). Offline samples were also taken in a weekly basis for the determination of sulfur species (sulfate, sulfide, sulfite and thiosulfate), CH_4 and N_2O concentrations exiting the system. Two carriers were extracted from R3 on the last day of Phase 1 (Day-1) and on the 36th day of Phase 2 to identify the microbial community composition and to monitor changes induced by the exposure to nitrate. Carriers were also collected from R1 and R3 on the last day of Phase 1 (Day-1); on the 17th and 49th days of Phase 2; and on the 36th day of Phase 3 for the analysis of the elemental sulfur (S^0) content in the biofilm and for biomass quantification.

2.2.2. *Batch tests (BT)*

Five batch tests were carried out on days 1, 2, 14, 22, and 35 during nitrate addition to investigate in detail the microbial biofilm activities in reactor R3. During these tests, the continuous operation of the system was temporarily interrupted. Batch tests in R3 were carried out with sewage coming from R2, which had been retained into the system for 7 h (3 and 4 h in R1 and R2, respectively), thus mimicking the conditions of an HRT of 9 h. In all tests, the initial wastewater composition entering R3 had an average of 5.8 ± 1.3 mg S-H_2S/L, 7.5 ± 1.5 mg S-SO_4^{2-}/L and 35.0 ± 10.4 mg COD-CH_4/L. At the beginning of each batch test, 5.8 mL of the concentrated NO_3^- solution were added to R3, obtaining an initial concentration of 30 mg N-NO_3^-/L. Each batch test lasted for 4 h where liquid samples were taken at 0, 0.25, 0.5, 1, 2 and 4 h for the analysis of sulfur species (sulfide, sulfite, thiosulfate and sulfate), nitrate and methane. Rates for the production and consumption of the various compounds analyzed were calculated from the slopes of the data points using linear regression (SPR: sulfate production rate, MOR: methane oxidation rate, SCR: sulfide consumption rate, NRR: nitrate reduction rate). A summary of all tests performed in phase is presented in Table 1.

Table 1 – Tests carried out in each phase of the experimental period.

	Length (days)	R3 NO_3^- exposure range	Batch tests	NF monitoring
Phase 1. Base line monitoring	30	0	0	5
Phase 2. Nitrate addition	49	5–60	5	3
Phase 3. Recovery period	36	0	3	3

2.3. *Statistical analysis*

Statistical analyses were carried out using SPSS 15.0v software. Normal distribution analysis was conducted by the Shapiro–Wilk test for quantitative variables of the normal functioning and inlet wastewater (sulfur balance, sulfide, sulfate, methane and nitrate concentrations). Variance uniformity was evaluated with Levene test and *T* test for independent samples was used to compare differences in compounds concentrations between phases and inlet wastewater.

2.4. *Chemical analysis*

Dissolved sulfide and nitrate was simultaneously measured in continuous by means of an UV–VIS spectrometer probe s::can spectro::lyser (Messtechnik, GmbH, Austria) (Gutierrez et al., 2010b). Dissolved sulfur species (sulfate, sulfide, sulfite and thiosulfate) were measured offline via ion chromatography (IC) with a UV and conductivity detector (Dionex ICS-5000). These samples were filtered through 0.22 μm and added into an air-tight vial with 0.5 mL antioxidant buffer (SAOB) preserving solution (Keller-Lehmann et al., 2006). Nitrate was also measured by IC. Samples for CH_4 analysis were filtered at 0.45 μm and injected into glass vacuumed tubes, according to the methodology proposed in Guisasola et al. (2008). CH_4 concentrations were analyzed by gas chromatography (Thermofisher Scientific, coupled with FID detector). N_2O analyses were carried out with an N_2O-R microsensor (Unisense, Aarhus, Denmark). Elemental sulfur (S^0) present in the biofilm from R1 (not exposed to nitrate) and R3 (exposed to nitrate) was analyzed according to Goehring et al. (1949) and (Jiang et al., 2009). For the analysis of total and suspended solids content, biomass attached to each carrier was suspended in MilliQ water by vortexing (IKA, genius 3) until complete detachment (≈2 min). Total and volatile suspended solids content (TSS, VSS) were then analyzed as per standard methods 2510D (APHA, 1998). Biomass content was referred to carrier surface.

2.5. *Microbial analysis*

DNA was extracted from collected biofilm samples using RNA PowerSoil™ Total RNA Isolation Kit and DNA Elution Accessory kit according to manufacturer instructions (MOBIO Laboratories, Inc, Carlsbad, CA). The extracted DNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen Molecular probes Inc., Oslo, Norway) and were subsequently analyzed through tag-encoded FLX-Titanium amplicon pyrosequencing at the Research and Testing Laboratory (Lubbock, TX, USA). Briefly, genomic DNA from the biofilm communities was used as a template in PCR reactions using primers 28F/519R (Bacteria) and 341F/958R (Archaea) targeting the V1-3 region of the 16S rRNA gene complemented with 454-adapters and sample-specific barcodes. Raw sequence dataset was pre-processed at RTL facilities to reduce noise and sequencing artifacts as previously described (Dowd et al., 2008). Demultiplexing according to sample barcodes, sequence quality assessments, chimera detection and downstream phylogenetic analyses were conducted in MOTHUR

(Schloss et al., 2009). High quality sequences were aligned against the bacterial and archaeal SILVA reference alignments available at the Mothur website (http://www.mothur.org) and then checked for chimeras using Uchime (Edgar et al., 2011) in Mothur. Taxonomical assignments for bacterial sequences were performed using the RDP classifier (Wang et al., 2007) using the last RDP database (trainset9_032012). For archaeal sequences, taxonomical assignments were done using the last SILVA reference database and taxonomy files available at Mothur website. In both cases, the cutoff value for valid assignments was 80%. Mothur was also used to delineate Operational Taxonomic Units (OTUs) at 97% cutoff and to identify representative sequences for each OTU (OTUreps). For community analysis, the number of sequences in each sample was normalized using a randomly selected subset of 4000 sequences from each sample to standardize the sequencing effort across samples and minimize any bias due to a different number of total sequences. Mothur was used to calculate α-diversity indicators of richness (Chao1) and diversity (Shannon–Wiener index) for each community at different sampling intervals. Abundance data are visualized as a bubble plots using bubble.pl (http://www.cmde.science.ubc.ca/hallam/bubble.php). Pyrosequencing data from this study was deposited in the NCBI under accession number SRP043373.

3. Results and discussion

3.1. Sulfide and methane accumulation before, during and after Downstream Nitrate Dosage

Changes in sulfide concentrations were continuously monitored during the normal functioning of the system across the experimental period. Fig. 2 shows a typical 24 h profile obtained in R3 before nitrate addition (A), during nitrate addition (B) and during the recovery period (C) using s::can spectrophotometer. Before nitrate addition, all sulfate (SO_4^{2-}) in the inlet wastewater was converted to H_2S during its transport through the system. The H_2S concentration in R3 had an average value of 17.7 ± 1.0 mg S-H_2S/L, which was slightly reduced during each pumping even, when the wastewater present in R2 (which still had some SO_4^{2-} left) entered into R3 (where this remaining SO_4^{2-} was completely reduced to H_2S).

NO_3^- addition in R3 (Fig. 2B) caused a substantial decrease of H_2S in this reactor due to the suppression of SO_4^{2-} reducing activity by NO_3^-. Only a small increase of H_2S was detected after each pumping event due to the arrival of wastewater from R2 containing H_2S. This H_2S was immediately oxidized in R3 by bacteria that was able to use NO_3^- as electron acceptor.

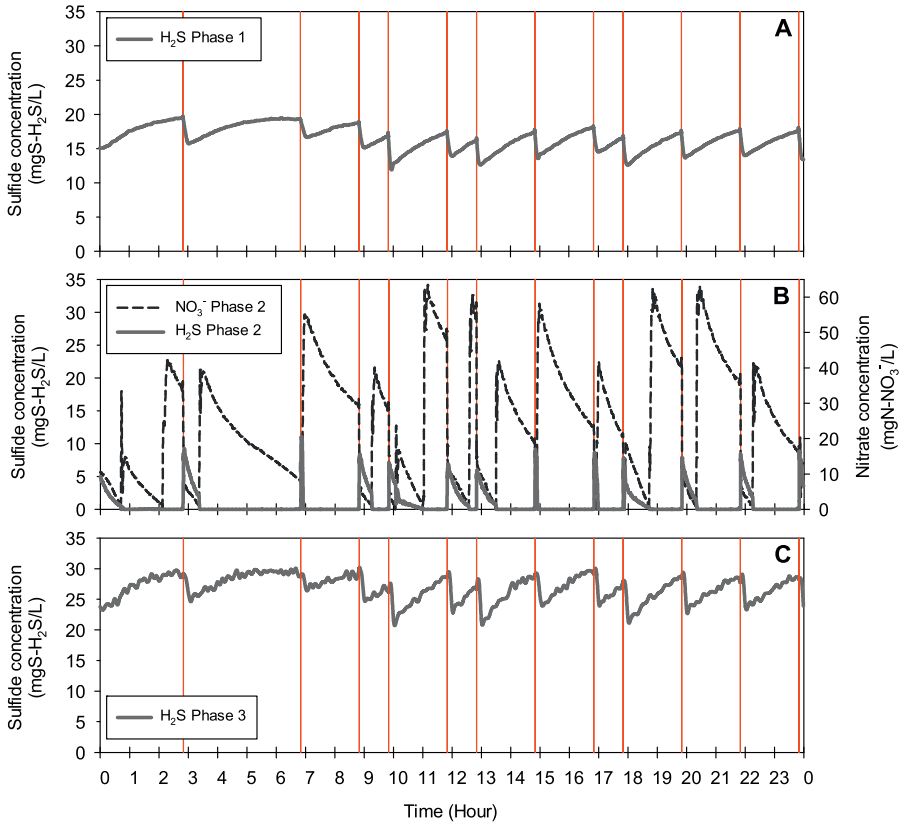

Fig. 2 – Typical daily profiles in R3 of (A) sulfide during baseline monitoring; (B) sulfide and nitrate during the nitrate addition period; and (C) sulfide during the recovery period. Vertical lines represent the pumping events, when wastewater present in R2 was moved to R3.

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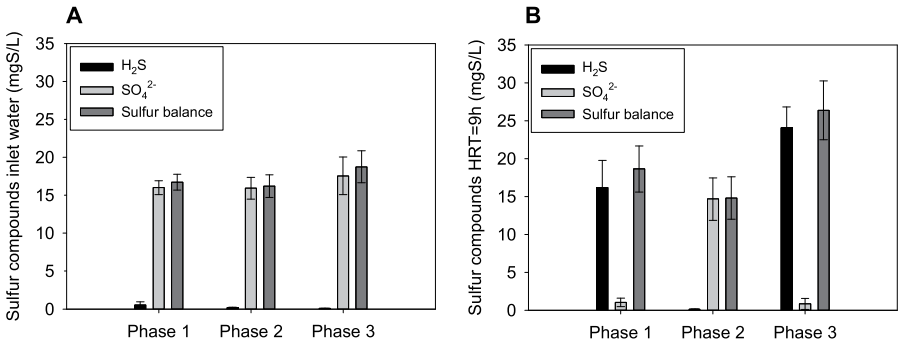

Fig. 3 – Sulfide (H_2S), Sulfate (SO_4^{2-}) and Sulfur balance (sum of sulfur species) in each phase in (A) inlet wastewater and (B) outlet wastewater. Error bars indicate standard deviation of the mean.

This observation is in agreement with other studies where a similar observation has been reported (Gutierrez et al., 2010b; Jiang et al., 2013, 2009; Mohanakrishnan et al., 2009a,b). Interestingly, even after H_2S depletion, NO_3^- continued to be reduced at a similar rate.

The recovery of H_2S production was also monitored after NO_3^- addition stopped. Fig. 2C shows the H_2S concentrations in R3 on the 5th day into the recovery period, which had an average value of 28.9 ± 0.8 mg S-H_2S/L that was higher than in Phase 1. These results are in agreement with offline results.

During Phase 1, all the SO_4^{2-} of the influent wastewater (Fig. 3A) was transformed to H_2S during its transport through the pilot sewer system (Fig. 3B). In contrast, when NO_3^- was added in R3, H_2S produced along the transport of wastewater through the system was oxidized to SO_4^{2-}. Although sulfur species in the inlet wastewater did not significantly change across the study, H_2S present in the effluent wastewater during the recovery period was higher than the one present in the influent. This significantly difference ($p < 0.05$; $p = 0.04$) could be explained by the production of elemental sulfur (S^0) during NO_3^- addition which precipitated into the biofilm and was transformed into H_2S by SRB when anaerobic conditions were reestablished.

To validate this hypothesis, S^0 was quantified in the biofilm present in R3 and compared to the one present in R1, which had not been exposed to NO_3^- (Fig. 4). Biofilm extracted from R1 showed stable S^0 concentrations during all the monitoring period (7.5 ± 1.0 mg S-S^0/g biomass). In contrast, S^0 concentration in R3 varied and was clearly related with the presence or absence of NO_3^-. Before NO_3^- addition, S^0 concentration in R3 (~8.9 mg S-S^0/g of biomass) was very similar with S^0 concentration in R1. However, towards the end of the NO_3^- exposure period (Phase 2) S^0 concentration doubled the amount initially present (16.23 ± 0.04 mg S-S^0/g biomass). Finally, after 36 days of stopping NO_3^- addition, S^0 concentration in the biofilm of R3 decreased to levels found before being exposed to NO_3^-. These results confirmed our initial hypothesis and are also in agreement with the results of Jiang et al. (2009).

CH_4 concentration in the influent and effluent wastewater was also periodically monitored (Fig. 5). CH_4 in the inlet wastewater was very low during all periods (an average of 0.3 ± 0.2 mg COD-CH_4/L). Production of CH_4 occurred during the transport of wastewater through the system. Under baseline conditions (Phase 1), and with an HRT of 9 h, CH_4 concentration in the effluent was 39.6 ± 5.2 mg COD-CH_4/L. Interestingly, when NO_3^- was added, a reduction of 46.2% was observed, reaching a concentration of 21.3 ± 0.3 mg COD-CH_4/L. Over Phase 3, CH_4 concentration in the outlet of the system incremented again, reaching a concentration of 49.4 ± 14.1 mg COD-CH_4/L, which was significantly equal ($p > 0.05$; $p = 0.32$) to the one found before NO_3^- addition (Fig. 5).

The application of the DND strategy was not effective to completely suppress the dissolved methane discharged from the system. The nitrate exposure reduced the methanogenic activity in R3, but the CH_4 generated in R1 and R2 upstream was still sufficient to detect a certain amount discharged. However, the magnitude of the CH_4 reduction (≈50%) obtained in only a third of the system indicated that more-than-just CH_4 suppression occurred in R3 (see following sections). Once in Phase 3, the recovery of R3 methanogenic activity occurred in a short period of time (7 days) indicating that MA was not severely affected by the nitrate dosage.

In a longer study (over 200 consecutive days of nitrate exposure), Jiang et al. (2013) reported a complete suppression of methane production only after increasing the nitrate dose from 30 to 130 mg N-NO_3^-/L in their system. In their study, CH_4 consumption was never observed.

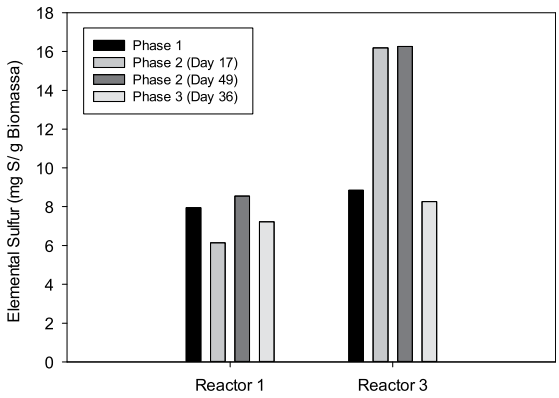

Fig. 4 – Elemental sulfur content in reactor 1 and reactor 3 during the monitoring period.

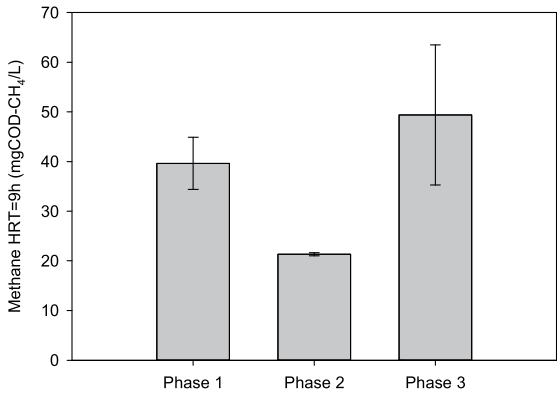

Fig. 5 – Methane concentration in the effluent wastewater over the monitoring period measured with an HRT of 9 h during normal functioning tests. Error bars indicate standard deviation of the mean.

It has to be noted that nitrate application periods between 30 and 60 days are typical in the Mediterranean area (Consorci Costa Brava, Local Water Utility personal communication) due to the increase of odor problems in warmer holyday seasons. Therefore, the limitations of DND for methane control are relevant to wastewater managers that would need to take into account the effects of NO_3^- seasonal dosage.

3.2. *Biotransformation of sulfur compounds and methane under anoxic conditions*

Three representative batch tests conducted in R3 over the NO_3^- exposure period are presented in Fig. 6. H_2S in the wastewater was oxidized to SO_4^{2-} from the first day of NO_3^- addition, suggesting the presence in the biofilm of an H_2S oxidizing microbial community despite that this biofilm had never been previously exposed to NO_3^-. SO_4^{2-} production continued after H_2S depletion, most likely as a result of the oxidation of the elemental sulfur present in the biofilm.

CH_4 oxidation was not observed until the second day of NO_3^- exposure (Fig. S1). This might be due to the fact that microorganisms able to oxidize CH_4 probably needed a certain time to growth and to express the enzymes needed to oxidize CH_4. CH_4 oxidation increased over the period of NO_3^- exposure, removing around 45.7 mg COD-CH_4/L·h of the CH_4 arriving to R3 after 35 days of exposure to NO_3^- (Table 2). Oxidation of CH_4 has been reported in some environments where nitrate or nitrite are available and can be conducted by either methane oxidizing bacteria (Ettwig et al., 2010, 2009, 2008; Zhu et al., 2012) or methane oxidizing archaea (Haroon et al., 2013). This is the first time that CH_4 oxidation is reported in a sewer system, suggesting that nitrate addition is not only a good strategy to control H_2S and CH_4 production but also to reduce any CH_4 formed in previous sections of the sewer network not exposed to NO_3^-.

Table 2 – Oxidation, reduction and production rates of H_2S, CH_4, NO_3^-, SO^{2-} during the batch tests conducted in R3 during the period of nitrate addition.

Time (days)	H_2S (mg S–H_2S/L·h)	SO_4^{2-} (mg S-SO_4^{2-}/L·h)	CH_4 (mg COD-CH_4/L·h)	NO_3^- (mg N-NO_3^-/L·h)
1	−6.4	2.45	0	−7.2
2	−9.2	2.9	−8.3	−14.8
14	−8.2	6.86	−15.6	−26.9
22	−17.9	9.31	−9.0	−15.4
35	−24.7	7.57	−45.7	−31.5

NO_3^- was reduced from the beginning of Phase 2, suggesting that sewer biofilms were able to use NO_3^- as electron acceptor without any lag phase. Besides, the NO_3^- reduction rate rapidly increased in the system, causing a complete NO_3^- consumption in the first hour of the batch tests from day 14th onwards. This NO_3^- depletion was followed by a short-term recovery of H_2S formation. In fact, H_2S formation started as soon as NO_3^- was depleted in R3 (Fig. 6B–C). In clear contrast, CH_4 production did not occurred, suggesting that the methanogenic community was impaired after NO_3^- exposure. These results are in agreement with Jiang et al. (2013) that also observed a suppression of CH_4 production during the first 4 weeks of upstream nitrate addition in a sewer pilot plant. However, after that time, CH_4 production restarted despite the presence of nitrate. They suggested that the long-term exposure to nitrate (around 200 days in their study) enhanced the

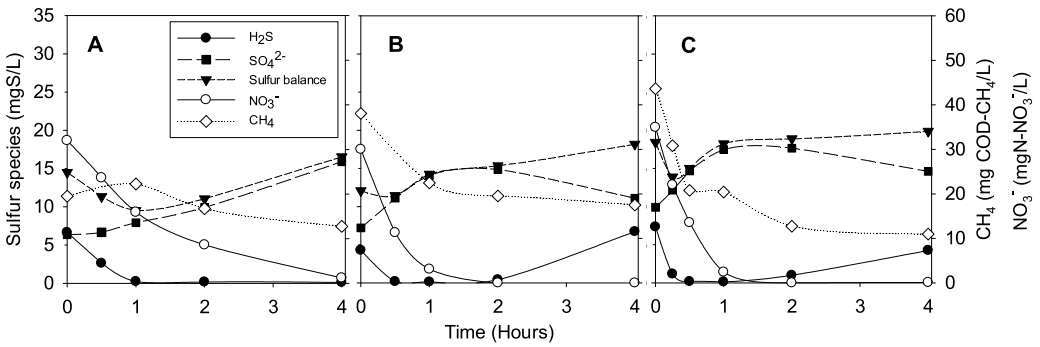

Fig. 6 – Profiles of sulfide, sulfate, sulfur balance, nitrate and methane concentrations measured during batch tests carried out throughout Phase 2. Day 1 (A), Day 14 (B), and Day 35 (C).

growth of microbial consortia located in the upper part of the biofilm responsible for nitrate reduction. That caused a reduction on the nitrate penetration into the biofilm, allowing the reestablishment of methanogenic archaea in the deeper layers of this biofilm. This observation might suggests that nitrate based control strategy would only be effective for CH_4 control under short exposure times (1–2 months). However, it has to be noted that nitrate application periods between 30 and 60 days are typical in the Mediterranean area (Consorci Costa Brava, Local Water Utility personal communication with Consorci Costa Brava, Local Water Utility) due to the increase of odor problems in warmer holyday seasons.

Table 2 compiles the production and reduction rates of the different compounds analyzed in the batch tests. H_2S, CH_4 oxidation, SO_4^{2-} production and NO_3^- reduction rates increased gradually during Phase 2 indicating that biofilm had been adapted to anoxic conditions.

Finally, N_2O was only detected until day 14 and at very low concentrations (0.13 ± 0.06 mgN-N_2O/L). This indicates that the production of this strong greenhouse gas is not a concern when nitrate is being added in downstream sections of anaerobic sewer pipes.

3.3. *Changes in biofilm microbial communities related with DND*

The effect of DND in the microbial community in R3 was assessed by pyrosequencing analysis. Main changes on bacterial biofilm communities affected members of the class *Betaproteobacteria* (Fig. 7A), with different genus that showed a substantial increase in their relative abundance (*Simplicispira*, *Comamonas*, *Azonexus* and *Thauera*) (Fig. 7B). These genera were possibly responsible for changes in nitrate, sulfide and methane transformations. Interestingly, the relative abundance of class *Deltaproteobacteria*, which includes most of the sulfate reducing bacteria known so far, decreased during nitrate addition in agreement with the low sulfate-reduction activity measured during this phase.

The most abundant OTUs (1, 7, 10, 11, 30, 32 and 35) with a total relative abundance of 50.6% in Phase 2 were related to microorganisms involved in heterotrophic nitrate reduction

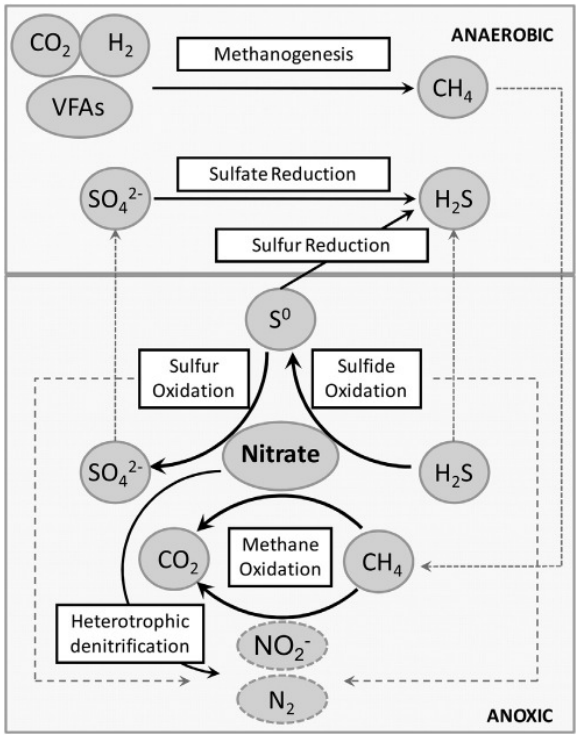

Fig. 8 – Schematic model of sulfur species and methane transformations due to nitrate addition modified from model of Jiang et al. (2009).

(Table S1) suggesting that nitrate addition stimulated anaerobic heterotrophs capable of nitrate reduction. Within these groups, OTU-11 and OTU-35 (with a total relative abundance of 4.7% when nitrate was present) affiliated to *Thauera*. Since members of this genus have been suggested to oxidize sulfide under anoxic conditions (Cytryn et al., 2005) it is plausible that they were involved in the consumption of sulfide and nitrate observed in the batch tests conducted during nitrate addition.

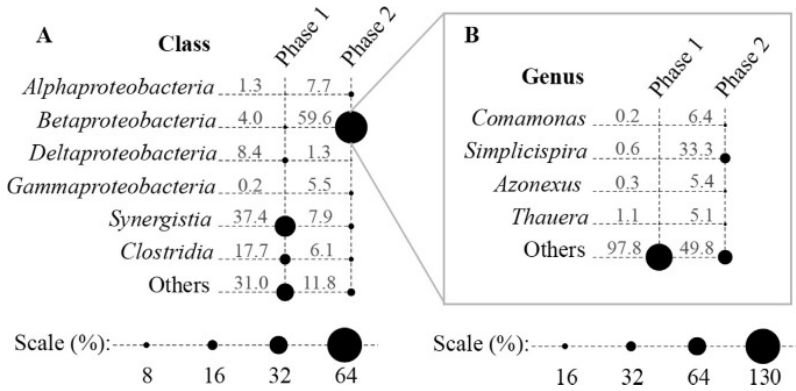

Fig. 7 – Relative abundance of sequences (%) affiliated to (A) main bacterial classes and (B) main betaproteobacterial genera in Phase 1 and Phase 2. Data values are proportional to radius and plotted in a logarithmic scale as indicated below the graph.

OTU-16 (with a relative abundance of 3.1%) had a 100% sequence identity to *Dechloromonas agitata* strain CKB, a facultative anaerobe capable of sulfide oxidation accompanied by the formation of yellow-white precipitates (apparently elemental sulfur) (Bruce et al., 1999). The increase of its relative abundance (from 0.5% to 3.1%) during nitrate exposure could also be explained by its ability to oxidize sulfide into S^0 while nitrate is co-reduced by perchlorate reductase (Chaudhuri et al. (2002). Besides, the appearance of OTU-28 only after nitrate exposure (with a relative abundance of 1.8%) can be related to the oxidation of methane measured after the nitrate treatment. OTU-28 showed a 92% similarity both to *Methylomicrobium album* BG8 and to *Methylobacter whittenburyi* 1521, two bacterial species involved in methane oxidation. Whereas the former has the ability to reduce nitrate to nitrite (Bowman et al., 1993) and it also contains genes encoding enzymes involved in methane oxidation (Kits and Kalyuzhnaya, 2013), the latter can use methane as carbon source wherever an electron acceptor is provided (Bowman et al., 1993). First hit of OTU-28 against BLAST database (Table S2), showed a 98% similarity to a phylotype from a DEAMOX reactor where sulfide oxidation to ^0S occurred with nitrate as electron acceptor (Kalyuzhnyi et al., 2010). The involvement of OTU-28 in both methane and sulfide oxidation cannot be then formally ruled out.

On the other hand, variations in the relative abundance of main archaeal phyla were less pronounced, suggesting that the archaeal community was not affected under the conditions imposed by the NO_3^- addition. In this regard, only minor changes occurred between the relative abundances of genera *Methanobacterium* (from 6.6% in Phase 1–1.1% in Phase 2) and *Methanosaeta* (from 93.1% in Phase 1–98.8% in Phase 2).

The richness and diversity of bacterial and archaeal communities were higher in Phase 1 than in Phase 2 as indicated by the correspondent estimators (Chao1 and Shannon indexes, Table 3). The obtained values not only indicate that nitrate addition severely impacted microbial communities in the reactors, greatly reducing both their richness and diversity, but also evidence that archaeal communities were less rich and diverse than bacterial ones as previously indicated for other systems (Aller and Kemp, 2008). In fact, the archaeal community was mainly composed of methanogenic classes whereas the bacterial community was composed of a large number of sequences spread over different bacterial classes.

3.4. *Microbial interactions and chemical transformations*

The complexity of sewer systems and the high nutrient load of wastewater favor the development of highly diverse microbial biofilms that grow using and transforming a wide range of organic and inorganic compounds. Our results allow us to represent the potential processes responsible for microbial transformations of methane and sulfur species in the studied system (Fig. 8).

Table 3 – Diversity and richness estimators of bacterial and archaeal biofilm communities in Phase 1 and Phase 2 samples.

Domain	Index	Phase 1	Phase 2
Bacteria	*Chao1*	727	403
	Shannon	4.6	3.6
Archaea	*Chao1*	60	25
	Shannon	0.4	0.1

Under anaerobic conditions fermentation products in wastewater can be readily metabolized by a wide range of microorganisms, either facultative or strict anaerobes. Among the latter, methanogenic archaea (mainly within genus *Methanosaeta*) uses Volatile Fatty Acids or CO_2 to obtain energy, producing CH_4. Sulfate-reducing bacteria (most of them belonging to the class *Deltaproteobacteria*) anaerobically oxidize organic matter using sulfate as electron acceptor and producing H_2S. Although inhibition of methane production by sulfate reducing bacteria was detected in other studies (Dar et al., 2008; Lovley et al., 1982; Lovleyt and Klug, 1983; Omil et al., 1998; Oremland and Polcin, 1982), the high abundance of organic and inorganic compounds possibly allow the coexistence of SRB and MA in these systems (Guisasola et al., 2008; Mohanakrishnan et al., 2009a,b).

Addition of nitrate to the system re-establishes the anoxic conditions in the sewers, severely impacting microbial biofilm communities, which changes their composition to cope with the new conditions. According to our results, the most important change was the stimulation of anaerobic bacterial respiration using nitrate as electron acceptor in detriment of sulfate-reduction. Also, NO_3^- can also be used by soNRB that oxidize H_2S to S^0 and then, the produced S^0 can be readily oxidized to SO_4^{2-}. However, S^0 was reduced to H_2S by sulfate-reducers when anaerobic conditions resumed. All groups of heterotrophic and autotrophic nitrate reducing bacteria are present in the initial biofilm (before nitrate addition) but at low relative abundances. Some of these microorganisms putatively involved in nitrate and sulfur transformations (within genus *Thauera*) have also been detected in sulfidogenic wastewater biofilms (Mohanakrishnan et al., 2011). Finally, the CH_4 accumulated during anaerobic conditions can be oxidized using nitrate or nitrite as electron acceptor by methanotrops (either bacteria or archaea), which are not present before the addition of nitrate. Some microorganisms associated with this metabolism were described in other studies. Metagenomic sequencing of bacterium *Candidatus* '*Methylomirabilis oxyfera*' pointed to a possible anaerobic oxidation of methane with the reduction of nitrite (Ettwig et al., 2010) after its enrichment (Ettwig et al., 2009, 2008). Morover, the enrichment of *Candidatus* '*Methanoperedens nitroreducens*' was capable of anaerobic oxidation of methane using nitrate as electron acceptor (Haroon et al., 2013). Difficulties associated with the isolation and characterization of microorganisms inhabiting complex systems such as those studied in this work lessen the progress towards the precise identification of the main microbial players involved in the different biotransformation processes occurring in these systems.

4. Conclusions

The results of this study reveal the main effects of Downstream Nitrate Dosage on sulfide and methane production:

- The production of sulfide was completely reduced when nitrate was added at a point close to the end of the pipe because sulfide oxidation occurred immediately. During this process, elemental sulfur was accumulated in the biofilm which can be also oxidized to sulfate.
- The immediate response to nitrate addition could be explained by the presence of heterotrophic and autotrophic nitrate reducing bacteria in the biofilm before the use of nitrate.
- When anaerobic conditions restarted after the nitrate addition period, sulfide concentration in the effluent wastewater was higher than sulfate present in the influent as a consequence of elemental sulfur reduction.
- Part of the methane produced during the transport of wastewater along the system was consumed at the last zone of the pipe in consequence of nitrate addition, possibly by methane oxidizing microorganisms that use nitrate or nitrite as electron acceptors. These potential methane oxidizers were not detected in the biofilm before nitrate addition.
- Nitrous oxide production was detected at negligible levels.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.09.034.

REFERENCES

Aller, J.Y., Kemp, P.F., 2008. Are Archaea inherently less diverse than Bacteria in the same environments? FEMS Microbiol. Ecol. 65 (1), 74–87.

APHA, 1998. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Administration, Washington, DC.

Boon, A., 1995. Septicity in sewers: causes, consequences and containment. Water Sci. Technol. 31 (7), 237–253.

Bowman, J.P., Sly, L.I., Nichols, P.D., Hayward, A.C., 1993. Revised taxonomy of the methanotrophs: description of Methylobacter gen. nov., emendation of Methylococcus, validation of Methylosinus and Methylocystis species, and a proposal that the family Methylococcaceae includes only the group I Methanotrophs. Int. J. Syst. Bacteriol. 43 (4), 735–753.

Bruce, R.A., Achenbach, L.A., Coates, J.D., 1999. Reduction of (per) chlorate by a novel organism isolated from paper mill waste. Environ. Microbiol. 1 (4), 319–329.

Chaudhuri, S.K., Connor, S.M.O., Ruth, L., Achenbach, L.A., Coates, J.D., Gustavson, R.L., 2002. Environmental factors that control microbial perchlorate reduction environmental factors that control microbial perchlorate reduction. Appl. Environ. Microbiol. 68 (9), 4425–4430.

Cytryn, E., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., Minz, D., 2005. Identification of bacteria potentially responsible for oxic and anoxic sulfide oxidation in biofilters of a recirculating mariculture system. Appl. Environ. Microbiol. 71 (10), 6134–6141.

Dar, S.A., Kleerebezem, R., Stams, A.J.M., Kuenen, J.G., Muyzer, G., 2008. Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. Appl. Microbiol. Biotechnol. 78 (6), 1045–1055.

Dowd, S.E., Callaway, T.R., Wolcott, R.D., Sun, Y., McKeehan, T., Hagevoort, R.G., Edrington, T.S., 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol 8, 125.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H.J.C.T., van Alen, T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., Op den Camp, H.J.M., Janssen-Megens, E.M., Francoijs, K.-J., Stunnenberg, H., Weissenbach, J., Jetten, M.S.M., Strous, M., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464 (7288), 543–548.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27 (16), 2194–2200.

Ettwig, K.F., Shima, S., van de Pas-Schoonen, K.T., Kahnt, J., Medema, M.H., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2008. Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. Environ. Microbiol. 10 (11), 3164–3173.

Ettwig, K.F., van Alen, T., van de Pas-Schoonen, K.T., Jetten, M.S.M., Strous, M., 2009. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. Appl. Environ. Microbiol. 75 (11), 3656–3662.

Firer, D., Friedler, E., Lahav, O., 2008. Control of sulfide in sewer systems by dosage of iron salts: comparison between theoretical and experimental results, and practical implications. Sci. Total Environ. 392 (1), 145–156.

Foley, J., Yuan, Z., Lant, P., 2009. Dissolved methane in rising main sewer systems: field measurements and simple model development for estimating greenhouse gas emissions. Water Sci. Technol. 60 (11), 2963–2971.

Ganigue, R., Gutierrez, O., Rootsey, R., Yuan, Z., 2011. Chemical dosing for sulfide control in Australia: an industry survey. Water Res. 45 (19), 6564–6574.

Goehring, M., Feldmann, U., Helbing, W., 1949. Quantitative bestimmung der polythionate (trithionat, tetrathionat, pentathionat und hexathionat) nebeneinander. Fresenius' Z. Anal. Chem. 129 (4–5), 346–352.

Guisasola, A., de Haas, D., Keller, J., Yuan, Z., 2008. Methane formation in sewer systems. Water Res. 42 (6–7), 1421–1430.

Gutierrez, O., Mohanakrishnan, J., Sharma, K.R., Meyer, R.L., Keller, J., Yuan, Z., 2008. Evaluation of oxygen injection as a means of controlling sulfide production in a sewer system. Water Res. 42 (17), 4549–4561.

Gutierrez, O., Park, D., Sharma, K.R., Yuan, Z., 2009. Effects of long-term pH elevation on the sulfate-reducing and methanogenic activities of anaerobic sewer biofilms. Water Res. 43 (9), 2549–2557.

Gutierrez, O., Park, D., Sharma, K.R., Yuan, Z., 2010a. Iron salts dosage for sulfide control in sewers induces chemical phosphorus removal during wastewater treatment. Water Res. 44 (11), 3467–3475.

Gutierrez, O., Sudarjanto, G., Ren, G., Ganigué, R., Jiang, G., Yuan, Z., 2014. Assessment of pH shock as a method for controlling sulfide and methane formation in pressure main sewer systems. Water Res. 48 (1), 569–578.
Gutierrez, O., Sudarjanto, G., Sharma, K.R., Keller, J., Yuan, Z., 2011. SCORe-CT: a new method for testing effectiveness of sulfide-control chemicals used in sewer systems. Water Sci. Technol. 64 (12), 2381–2388.
Gutierrez, O., Sutherland-Stacey, L., Yuan, Z., 2010b. Simultaneous online measurement of sulfide and nitrate in sewers for nitrate dosage optimisation. Water Sci. Technol. 61 (3), 651–658.
Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z., Tyson, G.W., 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500 (7464), 567–570.
Hvitved-Jacobsen, T., 2002. Sewer Processes – Microbial and Chemical Process Engineering of Sewer Networks. CRC Press, Boca Raton, FL.
IPCC, 2013. Working Group I Contribution to the Assessment Report Climate Change 2013: the Physical Science Basis: Final Draft Underlying Scientific-Technical Assessment. Cambridge University, Cambridge, U.K. and New York.
Jiang, G., Gutierrez, O., Sharma, K.R., Keller, J., Yuan, Z., 2011a. Optimization of intermittent, simultaneous dosage of nitrite and hydrochloric acid to control sulfide and methane productions in sewers. Water Res. 45 (18), 6163–6172.
Jiang, G., Gutierrez, O., Sharma, K.R., Yuan, Z., 2010. Effects of nitrite concentration and exposure time on sulfide and methane production in sewer systems. Water Res. 44 (14), 4241–4251.
Jiang, G., Gutierrez, O., Yuan, Z., 2011b. The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res. 45 (12), 3735–3743.
Jiang, G., Sharma, K.R., Guisasola, A., Keller, J., Yuan, Z., 2009. Sulfur transformation in rising main sewers receiving nitrate dosage. Water Res. 43 (17), 4430–4440.
Jiang, G., Sharma, K.R., Yuan, Z., 2013. Effects of nitrate dosing on methanogenic activity in a sulfide-producing sewer biofilm reactor. Water Res. 47 (5), 1783–1792.
Kalyuzhnyi, S.V., Shestakova, N.M., Tourova, T.P., Poltaraus, A.B., Gladchenko, M.A., Trukhina, A.I., Nazina, T.N., 2010. Phylogenetic analysis of a microbial community involved in anaerobic oxidation of ammonium nitrogen. Microbiology 79 (2), 237–246.
Keller-Lehmann, B., Corrie, S., Ravn, R., 2006. Preservation and simultaneous analysis of relevant soluble sulfur species in sewage samples. In: Conference Proceedings Sewer Operation & Maintenance (SOM), Vienna, Austria, October 26-28, 2006.
Kits, K., Kalyuzhnaya, M., 2013. Genome sequence of the obligate gammaproteobacterial methanotroph Methylomicrobium album strain BG8. Genome Announc. 1 (2), 11–12.
Lovley, D.R., Dwyer, D.F., Klug, M.J., 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl. Environ. Microbiol. 43 (6), 1373–1379.
Lovleyt, D.R., Klug, M.J., 1983. Sulfate reducers can Outcompete methanogens sulfate concentrations at freshwater. Appl. Environ. Microbiol. 45 (1), 187–192.
Mohanakrishnan, J., Gutierrez, O., Meyer, R.L., Yuan, Z., 2008. Nitrite effectively inhibits sulfide and methane production in a laboratory scale sewer reactor. Water Res. 42 (14), 3961–3971.
Mohanakrishnan, J., Gutierrez, O., Sharma, K.R., Guisasola, A., Werner, U., Meyer, R.L., Keller, J., Yuan, Z., 2009a. Impact of nitrate addition on biofilm properties and activities in rising main sewers. Water Res. 43 (17), 4225–4237.
Mohanakrishnan, J., Kofoed, M.V.W., Barr, J., Yuan, Z., Schramm, A., Meyer, R.L., 2011. Dynamic microbial response of sulfidogenic wastewater biofilm to nitrate. Appl. Microbiol. Biotechnol. 91 (6), 1647–1657.
Mohanakrishnan, J., Sharma, K.R., Meyer, R.L., Hamilton, G., Keller, J., Yuan, Z., 2009b. Variation in biofilm structure and activity along the length of a rising main sewer. Water Environ. Res. 81 (8), 800–808.
Omil, F., Lens, P., Visser, A., Hulshoff Pol, L.W., Lettinga, G., 1998. Long-term competition between sulfate reducing and methanogenic bacteria in UASB reactors treating volatile fatty acids. Biotechnol. Bioeng. 57 (6), 676–685.
Oremland, R.S., Polcin, S., 1982. Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. Appl. Environ. Microbiol. 44 (6), 1270–1276.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75 (23), 7537–7541.
Spencer, A.U., Noland, S.S., Gottlieb, L.J., 2006. Bathtub fire: an extraordinary burn injury. J. Burn Care Res. 27 (1), 97–98.
Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol 73 (16), 5261–5267.
Zhang, L., De Schryver, P., De Gusseme, B., De Muynck, W., Boon, N., Verstraete, W., 2008. Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: a review. Water Res. 42 (1–2), 1–12.
Zhu, B., van Dijk, G., Fritz, C., Smolders, A.J.P., Pol, A., Jetten, M.S.M., Ettwig, K.F., 2012. Anaerobic oxidization of methane in a minerotrophic peatland: enrichment of nitrite-dependent methane-oxidizing bacteria. Appl. Environ. Microbiol. 78 (24), 8657–8665.

Chapter 5

Control of sulfide and methane production in anaerobic sewer systems by means of Downstream Nitrite Dosage

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Control of sulfide and methane production in anaerobic sewer systems by means of Downstream Nitrite Dosage

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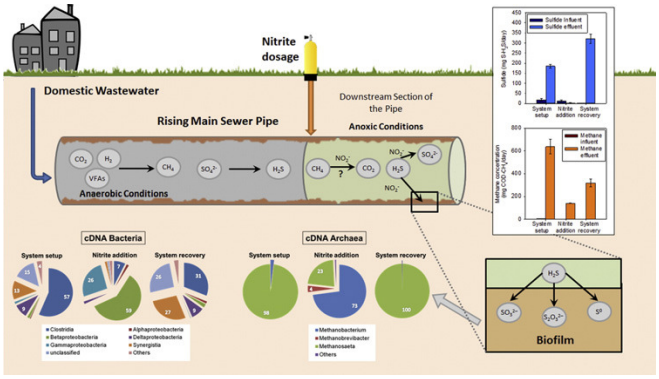
HIGHLIGHTS

- Sulfide emissions were completely reduced during Downstream Nitrite Dosage.
- Sulfide emissions were higher after the cessation of nitrite dosage.
- Methane emissions were reduced by ≈80% during Downstream Nitrite Dosage.
- Changes in the active microbial communities were detected during nitrite addition.

GRAPHICAL ABSTRACT



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ABSTRACT

Bioproduction of hydrogen sulfide (H_2S) and methane (CH_4) under anaerobic conditions in sewer pipes causes detrimental effects on both sewer facilities and surrounding environment. Among the strategies used to mitigate the production of both compounds, the addition of nitrite (NO_2^-) has shown a greater long-term inhibitory effect compared with other oxidants such as nitrate or oxygen. The aim of this study was to determine the effectiveness of a new method, the Downstream Nitrite Dosage strategy (DNO_2D), to control H_2S and CH_4 emissions in sewers. Treatment effectiveness was assessed on H_2S and CH_4 abatement on the effluent of a laboratory sewer pilot plant that mimics a full-scale anaerobic rising sewer. The experiment was divided in three different periods: system setup (period 1), nitrite addition (period 2) and system recovery (period 3). Different process and molecular methods were combined to investigate the impact of NO_2^- addition on H_2S and CH_4 production. Results showed that H_2S load was reduced completely during nitrite addition when compared to period 1 due to H_2S oxidation but increased immediately after nitrite addition stopped. The H_2S overproduction during recovery period was associated with the bacterial reduction of different sulfur species (elemental sulfur/thiosulfate/sulfite) accumulated within the sewer biofilm matrix. Oxidation of CH_4 was also detected during period 2 but, contrary to sulfide production, re-establishment of methanogenesis was not immediate after stopping nitrite dosing. The analysis of

Abbreviations: BT, batch test; CH_4, methane; DND, downstream nitrate dosage; DNO_2D, Downstream Nitrite Dosage; H_2S, sulfide; HRT, hydraulic retention time; MA, methanogenic archaea; N_2O, nitrous oxide; NF, normal functioning; NF-BT, normal functioning-batch tests; NMDS, nonmetric multidimensional scaling; NO_2^-, nitrite; NO_3^-, nitrate; NR-SOB, nitrate-reducing, sulfide-oxidizing bacteria; OTU, operational taxonomic unit; S°, elemental sulfur; SO_3^{2-}, sulfite; SO_4^{2-}, sulfate; SRB, sulfate reducing bacteria; WWTP, wastewater treatment plant.

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bulk and active microbial communities along experimental treatment showed compositional changes that agreed with the observed dynamics of chemical processes. Results of this study show that DNO_2D strategy could significantly reduce H_2S and CH_4 emissions from sewers during the addition period but also suggest that microbial agents involved in such processes show a high resilience towards chemical stressors, thus favoring the re-establishment of H_2S and CH_4 production after stopping nitrite addition.

1. Introduction

Microbial biofilms developed on the inner surface of sewer pipes produce changes of wastewater characteristics during its transport from urban settlements to wastewater treatment plants (WWTP). The microbial colonization of inner pipe surfaces and biofilm growth depends on different factors such as surface area, low flow velocity near pipe walls and nutrient resources. Availability of organic matter and sulfate (SO_4^{2-}) in wastewater favor the growth and activity of sulfate reducing bacteria (SRB) (Gutierrez et al., 2016; Sun et al., 2014). Despite their significant contribution in sulfide production, recent results revealed that SRB are ≈9–16% of total bacteria in sewer biofilms, which are mainly dominated by fermentative microorganisms (Auguet et al., 2015a,b). Furthermore, availability of acetate allows the development of acetoclastic methanogens in deeper layers of the biofilm matrix (Sun et al., 2014). The activity of both microbial groups causes a build-up of sulfide (H_2S) and methane (CH_4) that has severe consequences to both the flowing wastewater and the sewer environment. The accumulation of H_2S is a widely reported problem in sewer systems causing corrosion, malodour and health problems (Hvitved-Jacobsen, 2002; Pikaar et al., 2014). In turn, production of CH_4 by methanogenic archaea (MA) is also troublesome due to both its low explosive limit and its global warming potential, which is 21–23 times higher than that for carbon dioxide (Foley et al., 2011; IPCC, 2013; Liu et al., 2015; Spencer et al., 2006).

Several strategies have been developed to reduce H_2S and CH_4 production from sewers and to minimize their consequences (Ganigue et al., 2011; Zhang et al., 2008). Addition of iron salts to precipitate sulfide (Firer et al., 2008; Gutierrez et al., 2010a; Zhang et al., 2008), the use of magnesium or sodium hydroxides to modify the pH of wastewater and thus impairing microbial activity (Gutierrez et al., 2009, 2014; Zhang et al., 2008), or the injection of air/pure oxygen as oxidant agent to prevent anaerobic conditions (Gutierrez et al., 2008; Zhang et al., 2008) are among the most used mitigation strategies in full-scale sewers. Also, the use of oxidants in the form of nitrogen oxides is a widely applied strategy to induce anoxic conditions in sewers (Ganigue et al., 2011). For instance, the dosage of nitrate (NO_3^-) consists in the addition of an electron acceptor to prevent anaerobic activity. Nitrate stimulates biological sulfide oxidation by nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) thriving in biofilms. Several studies have reported that NO_3^- addition induced significant changes in the composition of biofilm bacterial communities but the inhibitory effects on SRB and MA in sewer biofilms are temporary (Auguet et al., 2015b; Jiang et al., 2013; Mohanakrishnan et al., 2009, 2011).

Whereas the added chemical is an important parameter to take into account, the dosage location is crucial to obtain an optimal mitigation effectiveness and reduce associated costs (Ganigue et al., 2011). For instance, NO_3^- is normally dosed at the pump stations or wet wells in upstream sections of sewer pipes, thus stimulating the activity of SRB in downstream biofilms as a collateral effect (Mohanakrishnan et al., 2009). This rebound phenomenon compels to increase the dosing rate (i.e. the total cost) to ensure the presence of NO_3^- along the sewer (Mohanakrishnan et al., 2009). In contrast, some studies showed the benefits of dosing NO_3^- at downstream sections of sewer pipes (known as DND) close to the discharging point (Auguet et al., 2015b; Gutierrez et al., 2010b). With this strategy, H_2S production is still occurring in the first sections of the pipe not exposed to NO_3^- but is immediately consumed as soon as it passes through anoxic sections. With DND, NO_3^- consumption is reduced by 42% while still ensuring complete abatement of H_2S and 50% reduction in CH_4 emissions. The need for more cost-effective methods for H_2S and CH_4 mitigation has led to the development of new dosing strategies based on improved dosage rates and dosing locations.

Nitrite (NO_2^-) is a nitrogen oxide that is gaining attention as a suitable product for H_2S and CH_4 control in sewers (Jiang et al., 2010; Mohanakrishnan et al., 2008). Similarly, to NO_3^-, NO_2^- has been showed to reduce H_2S and CH_4. However, the stronger long-term inhibitory effects of NO_2^- on both SRB and MA are advantageous compared with other oxidants such as NO_3^- or oxygen. Several studies have provided evidences that NO_2^- has a lethal effect on both SRB and MA although the latter appear to be more susceptible than the former (Jiang et al., 2010; Mohanakrishnan et al., 2008). To date, the effectiveness of addition of NO_2^- in downstream sections of a pipe to control H_2S/CH_4 and its impacts on the composition of microbial biofilms communities are not well established. The simultaneous presence of H_2S and CH_4 (generated in upstream pipe sections) plus NO_2^- (added in downstream sections) would lead to different conditions, still not reported, that could be important to validate the overall effectiveness of this strategy.

The aim of this study is to test the effectiveness of Downstream Nitrite Dosage (DNO_2D) in pressured sewer systems to reduce the production of H_2S and CH_4 and to assess its effects on the composition of biofilm microbial communities. The work was carried out in a laboratory system previously demonstrated to mimic the main features of a sewer rising main. The work has been carried out in three different phases: system setup, NO_2^- addition and system recovery. Normal functioning monitoring and batch tests were carried out during the three experimental periods to determine the changes produced by NO_2^- in the last section of the pipe. Biofilm samples were collected to assess changes in the composition of the active and bulk fractions of microbial communities through pyrotag sequencing of both 16S rRNA and its genes, respectively. This work provides valuable insights of DNO_2D effects on both H_2S and CH_4 production and allows a useful comparison with downstream NO_3^- dosage (DND) (Auguet et al., 2015b) that would help to identify the main advantages and limitations of each strategy.

2. Materials and methods

2.1. Laboratory sewer system and functioning settings

A laboratory system which simulates a real rising main sewer pipe was used to test the effects of DNO_2D strategy (Gutierrez et al., 2011; Sudarjanto et al., 2013). The laboratory system was composed of three PersPex™ reactors (R1, R2 and R3) serially connected to mimic different sections of real anaerobic sewer pipe (Fig. 1). Each reactor had a volume of 0.75 L and an internal diameter of 80 mm. Plastic carriers (Anox Kaldnes, Norway) of 1 cm^2 of surface were placed on rods inside each reactor and were used to provide easily extractable biofilm samples. The total area covered by biofilm was 0.05 m^2 taking into account reactor wall and carrier surfaces; the final area to volume ratio (A/V) was 65 m^2/m^3. Magnetic stirrers (Heidolph Mr Hei-MixS) were used to mimic sewer turbulent conditions in the reactors.

Domestic wastewater collected weekly in the upstream section of a sewer network in Girona (Spain) was used as intermittent fed by means of peristaltic pump (Masterflex model 7520–47). A pattern of 12

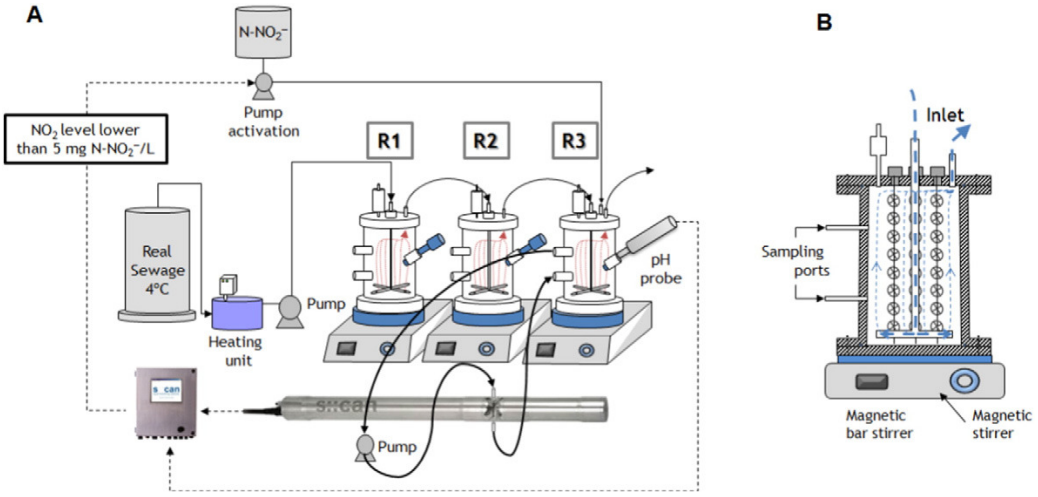

Fig. 1. Scheme of laboratory scale sewer system used in the nitrite dosing study (A) and sectional view of one reactor of the system (B).

uneven pump events was applied to reproduce the hydraulic retention time (HRT) of the real sewer used as reference (Radin sewer pipe, l'Escala, Spain). Depending on the variation of pump events, the minimum and maximum hydraulic retention times of wastewater in the system were between 4 and 9 h, respectively. The sewage was stored at 4 °C (to minimize undesired biotransformations) and heated to 20 °C before being pumped into R1 (Fig. 1A).

Sulfate concentration of the influent wastewater used during the experimental period was 18.6 ± 0.4 mg S–SO_4^{2-}/L. Negligible amounts of sulfide (1.1 ± 0.2 mg S–H_2S/L), sulfite (0 mg S–SO_3^{2-}/L), thiosulfate (0.3 ± 0.1 mg S–$S_2O_3^{2-}$/L) and methane (0.4 ± 0.2 mg COD–CH_4/L) were detected in source wastewater.

The study was conducted during approximately 6 months and was operated in three different periods: system setup, nitrite addition and system recovery.

- *Period 1, system setup*: anaerobic biofilms were developed on the walls and carriers of the laboratory sewer system prior to NO_2^- addition. The system was operated for >1 year to reach pseudo steady-state conditions. Intense the monitoring for this study was carried out for 34 days before the dosage of NO_2^- (days-34 to 0).
- *Period 2, nitrite addition*: nitrite solution (3.8 g N–NO_2^-/L) was automatically injected to R3 to achieve a set point of 20 mg N–NO_2^-/L. Nitrite and sulfide concentrations were continuously monitored by means of s::can spectrophotometer (model spectro::lyser) which was also programed to trigger the addition of NO_2^- to ensure anoxic conditions during the whole period 2 (Fig. 1A). Period 2 lasted 76 days (days 0 to 76).
- *Period 3, system recovery*: the system was monitored after terminating NO_2^- addition. Period 3 lasted 78 days (days 77 to 155).

2.2. Laboratory sewer system monitoring: normal functioning and biotransformations produced by the biofilm

The system was operated in two different regimes: normal functioning (NF) and batch tests (BT). Differences in wastewater characteristics exiting the system were analyzed during NF experiments while it was transported along the lab sewer system. Sulfide and nitrite concentrations were continuously measured in reactor R3 using a s::can spectrophotometer (model spectro::lyser). Wastewater samples were taken in R3 before exiting the system during different HRT to determine the concentrations of total dissolved sulfur species (calculated as the sum of measured sulfide (H_2S), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-})) and CH_4. Carriers from the initial (R1) and final (R3) sections of the lab sewer system were extracted to analyze the accumulation of elemental sulfur/thiosulfate/sulfite (S°, $S_2O_3^{2-}$ and SO_3^{2-}, respectively) in the biofilm and to quantify biofilm biomass during the different experimental periods (day-14 of period 1; day 22 and 57 days of period 2; and day 8 of period 3).

To complement the NF, additional normal functioning-batch tests (NF-BT) were carried out during the transport of the same wastewater volume along the system (R1, R2 and R3) in order to detect the different transformations of sulfur compounds and CH_4 production in each section of the lab sewer system as in a real pipe in each period during an HRT of 9 h. Fresh wastewater was pumped to R1 and offline samples were taken during its retention inside the reactor (0 h, 1.5 h and 3 h). Then, this wastewater was pumped to R2 where offline samples were also taken (3 h, 5 h and 7 h). Finally, wastewater from R2 (which was retained during 7 h in the system) was pumped to R3 to perform the BT as explained below. This monitoring was carried out during day-14 of period 1, day 71 of period 2 and day 14 of period 3.

Batch tests were done in order to detect differences of microbial biofilm activities in R3 among the three experimental periods (period 1, n = 3; period 2, n = 5; and period 3, n = 4). These tests were carried out in R3 using wastewater retained during 7 h in the system (3 h in R1 and 4 h in R3) to simulate the wastewater which entered in the final section of a sewer pipe; the initial wastewater composition entering R3 had an average of 22.9 ± 1.1 mg S–H_2S/L, 0.9 ± 0.3 mg S–SO_4^{2-}/L and 99.6 ± 9.9 mg COD–CH_4/L. The continuous pumping pattern of the system was temporarily interrupted and NO_2^- was added in R3 at the beginning of each batch test to reach initial bulk concentration of ≈30 mg N–NO_2^-/L. The duration of batch tests was 4 h. Wastewater samples were taken at 0, 0.25, 0.5, 0.75, 1, 2 and 4 h in all batch tests for the analysis of total dissolved sulfur (H_2S, SO_3^{2-}, $S_2O_3^{2-}$ and SO_4^{2-} concentrations) CH_4 and NO_2^- (the last one only during period 2). Rates for the production and consumption of the various compounds analyzed were calculated from the slopes of the data points using linear regression.

2.3. Chemical analysis

Dissolved H_2S and NO_2^- were simultaneously measured in continuous by means of an UV–VIS spectrometer probe s::can spectro::lyser (Messtechnik, GmbH, Austria). Total dissolved sulfur species were measured offline via ion chromatography (IC) with a UV and conductivity detector (Dionex ICS-5000). These samples were filtered through 0.22 μm and added into an air-tight vial with 0.5 mL antioxidant buffer

(SAOB) preserving solution (Keller-Lehmann et al., 2006). Nitrite samples were also filtered at 0.22 μm and measured by IC. Samples for CH_4 analysis were filtered at 0.45 μm and injected into glass vacuumed tubes (Guisasola et al., 2008). Methane concentrations were analyzed by gas chromatography (Thermofisher Scientific, coupled with FID detector). Nitrous oxide (N_2O) analyses were carried out with an N_2O-R microsensor (Unisense, Aarhus, Denmark). Biomass attached to each carrier was suspended in MilliQ water by vortexing (IKA, genius 3) until complete detachment (≈2 min) for the analysis of total and suspended solids content. Total and volatile suspended solids content (TSS, VSS) were then analyzed as per standard methods 2510D (APHA, 1998) to calculate biomass content (Table S1). Elemental sulfur, thiosulfate and sulfite present in the biofilm from R1 (not exposed to NO_2^-) and R3 (exposed to NO_2^-) was measured using the conversion of S° and SO_3^{2-} to $S_2O_3^{2-}$ at high pH (Goehring et al., 1949; Jiang et al., 2009).

2.4. DNA samples and microbial community study

Three carriers were extracted from R3 on the last day of period 1 (day-1), on the 35th day of period 2 and on the 10th day of period 3 to analyze the composition of microbial communities and potential changes induced by exposure to NO_2^-. Biofilm attached to the carriers was resuspended in 3 mL of LifeGuard™ Soil Preservation Solution (MO BIO Laboratories, Inc., Carlsbad, CA) and immediately frozen at −20 °C until DNA and RNA extraction. Nucleic acids were extracted from collected biofilm samples using RNA PowerSoil™ Total RNA Isolation Kit and DNA Elution Accessory kit according to manufacturer instructions (MO BIO Laboratories, Inc., Carlsbad, CA). TURBO DNA-free™ Kit (Ambion, Austin, TX, USA) was used to remove any remaining contaminating DNA in RNA extracts. Reverse transcription of RNA to cDNA was done using random hexamer primers and Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The extracted DNA and treated RNA and cDNA were quantified using a Qubit® 2.0 Fluorometer (Invitrogen Molecular probes Inc., Oslo, Norway) (Table S2).

Amplicon pyrosequencing of DNA and cDNA biofilm extracts was done using 454 GS-FLX Plus system by an external company (Macrogen Inc., Seoul, South Korea). Gene fragments of bacterial and archaeal 16S rRNA were amplified using universal bacterial (27F-GAGTTTGA TCMTGGCTCAG and 518R-WTTACCGCGGCTGCTGG (Lee et al., 2010)) and archaeal (340F-CCCTAYGGGGYGCASCAG (Gantner et al., 2011) and 958R-YCCGGCGTTGAMTCCAATT (DeLong, 1992)) primer sets, respectively. Different barcodes were used to allow demultiplexing of sequences on a per sample basis.

Demultiplexing according to sample barcodes, sequence quality assessments, chimera detection, and downstream phylogenetic analyses were conducted in Mothur (Schloss et al., 2009). Bacterial and archaeal curated sequence datasets were then aligned in Mothur using the bacterial and archaeal SILVA reference alignments, respectively, available online (http://www.mothur.org/). Taxonomic assignment of bacterial sequences was carried out using the RDP taxonomy reference database and the SILVA taxonomy file (silva.bacteria.rdp.tax) with a cutoff value of 80% for valid assignments. Classification of archaeal sequences was carried out by using the SILVA reference database and taxonomy files using the same cutoff as that used for bacteria. Operational taxonomic units (OTUs) were delineated at 97% cutoff and then representative sequences of each OTU were taxonomically assigned against the same reference taxonomies used previously. Relative abundances of most populated OTUs (OTUs with relative abundances of >4% of total sequences in at least one sample) across samples were visualized as bubble plots using a perl script (Zaikova et al., 2010; http://www.cmde.science.ubc.ca/hallam/bubble.php). For community analysis, the number of sequences in each sample was normalized using a randomly selected subset of 2400 sequences (for bacteria) and 6200 sequences (for archaea) from each sample to standardize the sequencing effort across samples and minimize bias. These normalized sequence datasets were then used in Mothur to calculate community similarity among sites (β-diversity) based on the weighted UniFrac distance (Lozupone and Knight, 2005). Nonmetric multidimensional scaling (NMDS) analysis was performed on the UniFrac similarity matrices to visualize patterns of community composition. Pyrosequencing data from this study was deposited in the NCBI under accession number PRJNA304277.

3. Results and discussion

3.1. Comparison of sulfur compounds and methane concentrations exiting the sewer system before, during and after Downstream Nitrite Dosing

Changes in daily sulfur compounds exiting the system were checked during the normal functioning of the laboratory sewer system in the course of the experimental period (Fig. 2A and Fig. S1). During period 1, 95.1% of SO_4^{2-} present in the influent wastewater was reduced to H_2S along the transport of wastewater through the system. In period 2, conversely, H_2S concentrations were nearly 0 as long as NO_2^- was added in R3. This shows that DNO_2D was able to completely control H_2S at the outlet of the system. The reduction of H_2S emission in lab-scale sewer pipes was also detected in other studies although the concentration of nitrite was higher (Jiang et al., 2010; Mohanakrishnan et al., 2008). During NO_2^- addition, it was also observed that only 41.0% of total dissolved sulfur from the influent wastewater was released at the discharge point of the system (mainly as SO_4^{2-}). Finally, in period 3, sulfide production resumed immediately once NO_2^- addition was stopped. During the first week of period 3, the amount of total dissolved sulfur discharged from the system increased dramatically, 120.4% higher than the concentration in the influent wastewater. During these days, the distribution of sulfur species was 82.5% as H_2S, 6.9% as SO_4^{2-}, 9.3% as $S_2O_3^{2-}$ and 1.3% SO_3^{2-}. From day 85-period 3 onwards, the concentrations of total dissolved sulfur at the effluent of the system decreased and stabilized in lower levels but remained consistently higher than the total dissolved sulfur in the inlet. The results clearly showed that the cessation of NO_2^- dosage produced a significant temporary-initial increase of H_2S discharged from the system followed by a longer-lower release of H_2S. Typical 24 h online profiles of H_2S and NO_2^- concentrations corresponding to all 3 periods can be found in supplementary information (Fig. S2).

Sulfide overproduction and the increase of total dissolved sulfur (mainly SO_4^{2-} and $S_2O_3^{2-}$) during recovery period was associated with $S°/S_2O_3^{2-}/SO_3^{2-}$ accumulation in the biofilm (Fig. 3). Elemental sulfur/thiosulfate/sulfite concentration in Reactor 1 biofilm (not exposed to NO_2^-) was stable during the experiment (9.8 ± 1.1 mg S/g Biomass). On the other hand, $S°/S_2O_3^{2-}/SO_3^{2-}$ accumulation in Reactor 3 was changing depending on the presence/absence of NO_2^-. Before the dosing of NO_2^- (day-14) the concentration was 7.8 mg S/g Biomass and it increased during NO_2^- addition period (from 245.2 to 424.7 mg S/g Biomass). This accumulation could be related with the loss of 59.0% of total dissolved sulfur detected in NF tests (Fig. 2A and Fig. S1). However, during recovery period, this amount of $S°/S_2O_3^{2-}/SO_3^{2-}$ in the biofilm decreased to similar levels as in period 1. As a result of the possible $S°/S_2O_3^{2-}/SO_3^{2-}$ release from the biofilm and its partial reduction to SO_4^{2-} and/or H_2S, the amount of total dissolved sulfur exiting the system during recovery period (mainly composed by H_2S, SO_4^{2-} and $S_2O_3^{2-}$) increased to higher concentrations than total dissolved sulfur amounts quantified in the inlet wastewater (Fig. 2A and Fig. S1). The accumulation of intermediate sulfur compounds (basically S°) during NO_2^- addition was also lightly detected by Mohanakrishnan and co-authors (Mohanakrishnan et al., 2008), although an increase of total dissolved sulfur during recovery period was not detected in their study probably as a consequence of different system operation and dosage pattern.

Changes in CH_4 emissions were also monitored throughout the experimental period in order to detect differences on CH_4 production

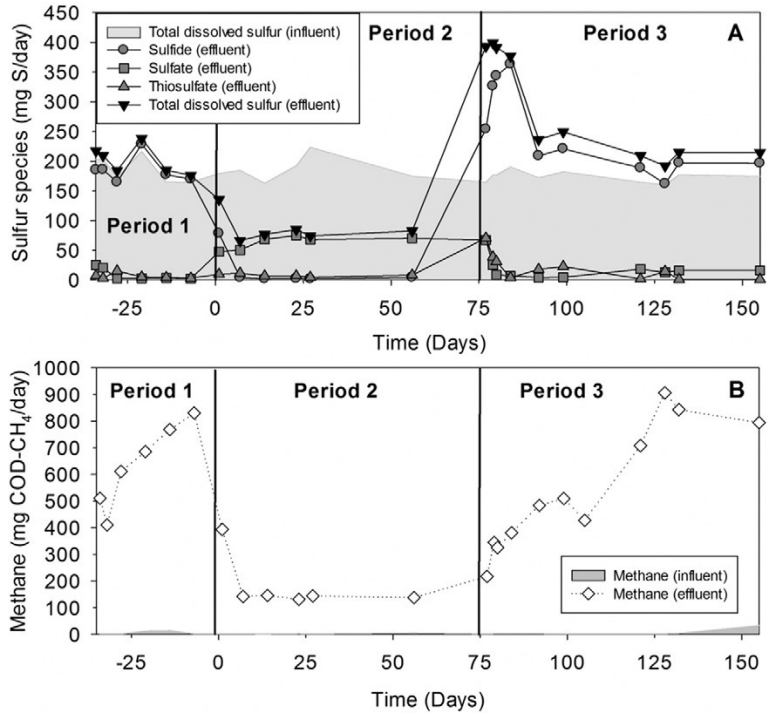

Fig. 2. Daily sulfur species loads (A) and methane emissions (B) during the different periods of the study measured during a 24 hour-period approach.

during NO_2^- addition period (Fig. 2B). Although the amount of CH_4 in inlet wastewater was close to 0 during the experimental period (3.8 ± 1.5 mg COD–CH_4/day), CH_4 levels discharged from the system during period 1 were 636.1 ± 64.6 mg COD–CH_4/day because of the production of CH_4 by methanogenic archaea. When NO_2^- was added at the downstream part of the system CH_4 discharged was reduced to 140.0 ± 2.7 mg COD–CH_4/day (≈78% of reduction) (Fig. S3) meaning that DNO_2D was able to largely control its release. Finally, in period 3, CH_4 exiting the system increased gradually for 50 days until reach similar levels (848.0 ± 32.2 mg COD–CH_4/day) as in period 1 (830.6 mg COD–CH_4/day). These results indicated the inhibitory effect of NO_2^- in methanogenic community and also suggested slower recovery of this microbial group compared with SRB, which started their activity from the first day of system recovery period as was reported in other studies (Jiang et al., 2010).

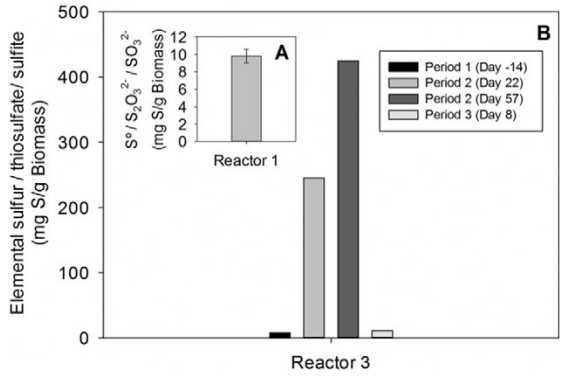

Fig. 3. Elemental sulfur (S°)/thiosulfate ($S_2O_3^{2-}$)/sulfite (SO_3^{2-}) concentration in biofilm from reactor 1 (A) and reactor 3 (B) during the monitoring period. Error bars indicate standard error of the mean.

Finally, negligible daily N_2O loads were measured in the effluent wastewater during the normal functioning monitoring (0.61 ± 0.14 mg N–N_2O/day), indicating that DNO_2D strategy did not promote the accumulation or emission of this strong greenhouse gas in sewer systems.

3.2. Transformation of sulfur compounds and methane production/consumption during wastewater transport in sewer systems

Transformation of sulfur compounds were monitored during transport of wastewater across the system. During period 1 (Fig. 4A), SO_4^{2-} present in the inlet wastewater was reduced to H_2S in Reactor 1 during an HRT of 3 h. This wastewater was pumped to Reactor 2 and then to Reactor 3 (after 3 h of retention) where remaining SO_4^{2-} was completely reduced to H_2S within a 4-hour period. These transformations in R1 and R2 were also detected during period 2, but H_2S was mainly oxidized to SO_4^{2-} in R3 when NO_2^- was added (Fig. 4B). However, the NO_2^- depletion promoted the reduction of $S°/S_2O_3^{2-}/SO_3^{2-}$ causing the increase of H_2S production. In period 3, the trend of H_2S production in R1 and R2 was similar as in period 1 and also was reestablished in R3 although it was slightly higher because of $S°/S_2O_3^{2-}/SO_3^{2-}$ accumulation (Fig. 4C).

During period 1, CH_4 production was detected in each reactor but mainly in R1 and R2 (Fig. 4D). When NO_2^- was added at the downstream part of the system, CH_4 was consumed as long as NO_2^- was present (Fig. 4E). In contrast to sulfur species, CH_4 production in R3 was not reestablished during the recovery period after more than 14 days of NO_2^- addition stopping (Fig. 4F).

3.3. Biotransformation of sulfur compounds and methane before, during and after nitrite addition at downstream section of the sewer system

Biotransformation of SO_4^{2-}, H_2S, CH_4 and NO_2^- were evaluated in R3 using the results of batch tests. Calculations of H_2S–CH_4 production rates and SO_4^{2-}–NO_2^- consumption rates were used to determine

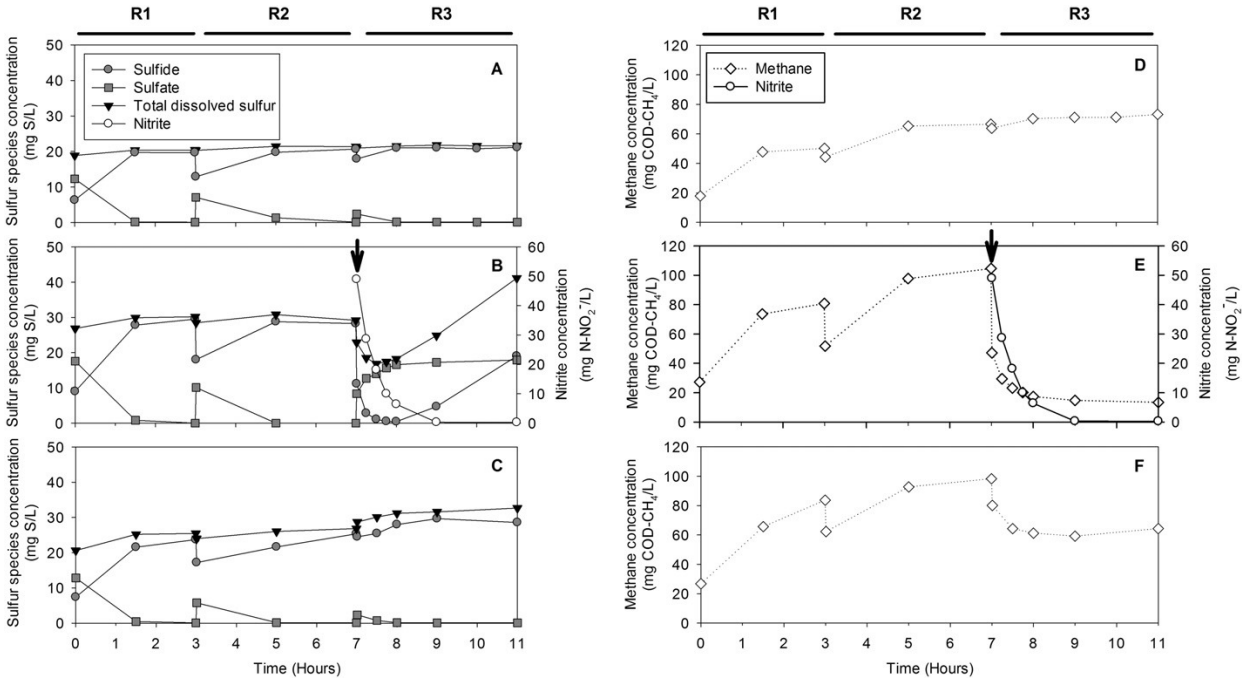

Fig. 4. Monitoring of sulfur transformations and methane production/consumption during the transport of wastewater along the different sections of the sewer system (R1, R2 and R3) in day-14 of period 1 (A, D), day 71 of period 2 (B, E) and day 14 of period 3 (C, F). Bold arrow indicates nitrite addition point.

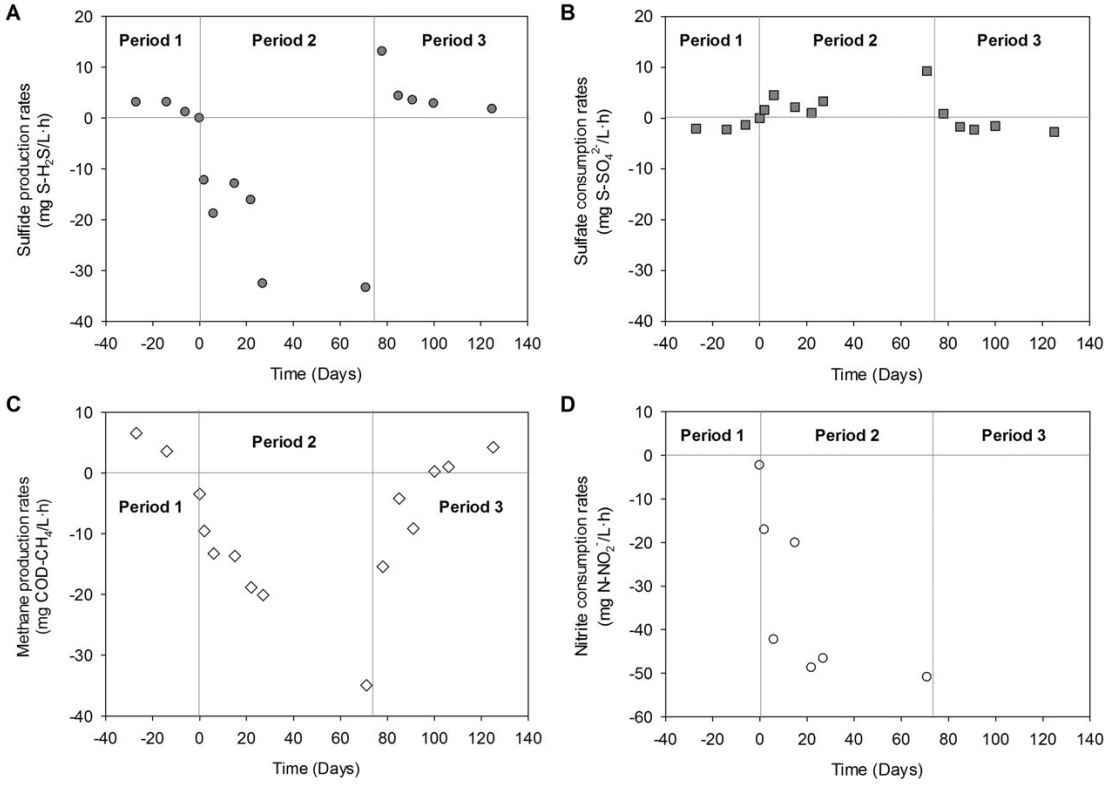

Fig. 5. Sulfide production rates (A), sulfate consumption rates (B), methane production rates (C) and nitrite consumption rates (D) determined using batch tests during the different periods of the study. Positive and negative values indicated production and consumption rates, respectively.

microbial biofilm activities over the experimental period (Fig. 5). During period 1, H_2S was produced in R3 at an average rate of 2.4 ± 0.6 mg S–H_2S/L·h and SO_4^{2-} reduction occurred at 1.9 ± 0.3 mg S–SO_4^{2-}/L·h. During this period, CH_4 was produced at an average rate of 5 ± 1.5 mg COD–CH_4/L·h.

In period 2, the consumption of NO_2^- increased drastically during the first 15–20 days of addition and then it stabilized for the rest of the period at levels around 45–50 mg N–NO_2^-/L·h. Sulfide production rates showed a very similar pattern with a sudden drop (down to −30 mg S–H_2S/L·h) followed by a plateau, indicating H_2S consumption was occurring. Methane production was likewise reversed due to NO_2^- addition. Consumption of CH_4 increased constantly during period 2 up to a maximum level of 34.9 mg COD–CH_4/L·h on day 71. Production of SO_4^{2-} at an average rate of 3.1 ± 1.2 mg S–SO_4^{2-}/L·h was also detected.

Finally, during the recovery period, H_2S production rate recovered immediately. It was higher during the first day (13.1 mg S–H_2S/L·h) compared to the next days (3.1 ± 0.5 mg S–H_2S/L·h) probably as a consequence of the reduction of S°/$S_2O_3^{2-}$/SO_3^{2-} accumulated in the biofilm. Sulfate reduction rate decreased to levels found in system setup (2.1 ± 0.2 mg S–SO_4^{2-}/L·h). Conversely, CH_4 production capacity of R3 remained negative for 23 days, a period when CH_4 was still being consumed despite the absence of NO_2^-. After day 23 of period 3, CH_4 production became positive again and kept increasing until 4.2 mg COD–CH_4/L·h in day 48 of period 3, reaching similar values than in period 1 (5 ± 1.5 mg COD–CH_4/L·h).

Results of the nitrite BTs' (Fig. S4) showed that H_2S was totally oxidized before NO_2^- depletion, which was then probably used both in heterotrophic denitrification and in CH_4 oxidation processes. When NO_2^- was completely consumed, then CH_4 oxidation stopped and H_2S was produced again but without an apparent SO_4^{2-} reduction. As was reported before, the sulfur stores were used preferentially as an electron acceptor when there was excess of SO_4^{2-} in the bulk (Mohanakrishnan et al., 2008). Therefore, the increase in total dissolved sulfur concentration is again an evidence of a possible reduction of S°/$S_2O_3^{2-}$/SO_3^{2-} accumulated in the biofilm during H_2S oxidation by means of NO_2^-. This increase of total dissolved sulfur was also detected in BTs performed during first days of period 3 (Fig. S5).

3.4. Impact of nitrite addition on microbial communities

The effect of NO_2^- addition on the composition and activity of biofilm microbial communities was assessed by 16S rRNA gene pyrotag sequencing of DNA (i.e. the bulk community) and cDNA (i.e. the active fraction of community members) extracts. These analyses allowed us to determine: *i*) the identity of active microbial groups involved in H_2S and CH_4 production, and *ii*) the impact of NO_2^- addition on the composition and activity of the biofilm communities. Results of pyrotag libraries showed a completely adaptation of bacterial and archaeal communities during the three different experimental periods.

Analyses of active communities (cDNA fraction) revealed that during system setup the majority of the bacterial sequences affiliated to class *Clostridia* (57%) although only 14% of sequences were assigned to this class in the DNA fraction (Fig. 6A). This result indicated that anaerobic *Clostridia* might have an important contribution in sewage water transformations. Interestingly, the relative abundance of sequences affiliated to *Clostridia* plummeted from 57% to 7% during NO_2^- period, suggesting that the oxidative damage caused by NO_2^- severely affected these strict anaerobes. Clostridial sequences regained their abundance once NO_2^- addition stopped (period 3). The main OTU affiliated with the class *Clostridia* (OTU-B1) showed 97% sequence similarity to obligate anaerobic bacterium *Romboutsia ilealis*, which ferments complex polysaccharides and produce acetate (Gerritsen et al., 2014) (Fig. S6A). Sequences affiliated to class *Synergistia* also showed a decrease in their relative abundance during NO_2^- addition (mainly in the cDNA fraction) but their prevalence increased during period 3 (Fig. 6A). Only one of the most abundant OTUs affiliated to this class

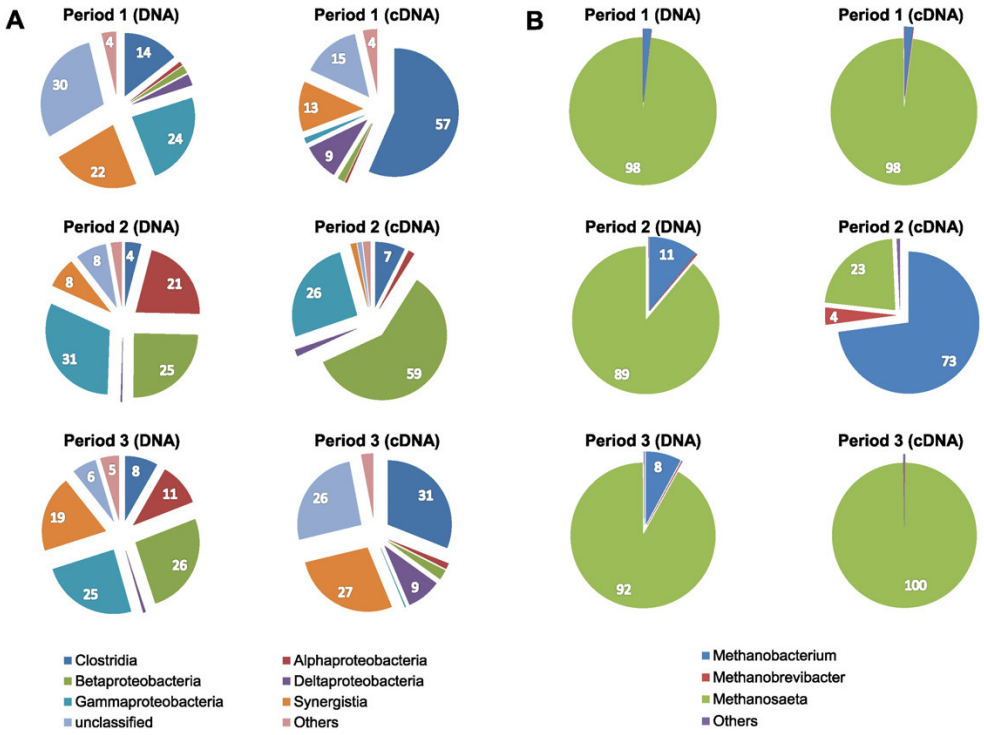

Fig. 6. Relative abundance of sequences (%) affiliated to main bacterial classes (A) and main archaeal genus (B) during the experimental period in the different acid nucleic fractions (DNA and cDNA).

(OTU-B5) had >97% of similarity to a cultured representative. Particularly, OTU-B5 was prevalent during period 3 (Fig. S6A) and showed a 100% similarity to *Aminivibrio pyruvatiphilus*, a strict fermenter that produce acetate, propionate, H_2 and CO_2 from organic matter (Honda et al., 2013). Sequences affiliated to class *Deltaproteobacteria* showed an important decrease during NO_2^- addition (Fig. 6A), agreeing with the substantial reduction of H_2S emission/production in this period (Fig. 1A). Despite this reduction, the sulfate-reducing community increased its relative abundance during the recovery period to same levels that those observed during period 1 (Fig. 6A). This observation agreed with the resumption of H_2S production during period 3.

Nitrite addition also stimulated the proliferation of members of classes *Betaproteobacteria* and *Gammaproteobacteria*. The total relative abundance of sequences affiliated to class *Betaproteobacteria* increased from 2% to 59% in the active microbial community (cDNA) in period 2 (NO_2^- addition) although their increase in the DNA fraction was less pronounced (from 2% to 25%) (Fig. 6A). During period 3, the population returned to the initial values pointing to a possible role of this class in H_2S oxidation, CH_4 oxidation and/or NO_2^- reduction. The main genera within this class identified during NO_2^- dosage were *Thauera*, *Comamonas* and *Azonexus* (29%, 13% and 9% of total relative abundances in the cDNA fraction during period 2, respectively). OTU sequences affiliated to genus *Thauera* (OTU-B2, Fig. S6A) matched with sequences of microorganisms capable of using nitrate, nitrite and oxygen as electron acceptors in anaerobic respiration of organic matter and it is then possible that they were involved in NO_2^- consumption (Mechichi et al., 2002). Moreover, Cytryn and collaborators suggested that some *Thauera*-related phylotypes were capable of anoxic, nitrate-dependent sulfide oxidation (Cytryn et al., 2005). Although we did not use NO_3^-, intermediate products during denitrification process (e.g. NO_2^-) could be used to oxidize H_2S as reported by several authors (Doğan et al., 2012; Mahmood et al., 2007). Sequences affiliated to genus *Comamonas* and *Azonexus* (OTU-B4 and OTU-B13, respectively, Fig. S6A) were also related with species capable of NO_3^- reduction (Chou et al., 2008; Gumaelius et al., 2001). The representative sequence of OTU-B13 had also a 100% similarity with sequences retrieved from planktonic methane-oxidizing bacteria (Kojima et al., 2014) and could indicate that this metabolism also occurred during period 2. The increase in the relative abundance of sequences affiliated to class *Gammaproteobacteria* (from 1% to 26% during period 2) is also worth to mention since most sequences within this class were related with species able to reduce NO_3^- (OTU-B3 and OTU-B14, Fig. S6A) (Tao et al., 2014; Xiao et al., 2009).

Regarding archaeal communities, 98% of the sequences recovered in period 1 affiliated to genus *Methanosaeta* (Fig. 6B). Particularly, OTU-A1 and OTU-A2 were related with *Methanosaeta concilii* (Fig. S6B), which use acetate as sole energy source (Patel and Sprott, 1990). In turn, the relative abundance of sequences affiliated to genus *Methanobacterium* increased from 2% to 73% during period 2 in the cDNA fraction, suggesting a drastic change in the composition and activity of the archaeal community as a result of NO_2^- addition (Fig. 6B). Most abundant OTUs (OTU-A3, OTU-A4 and OTU-A5, Fig. S6B) affiliated to *Methanobacterium* and showed a 99–100% similarity to species that use H_2 and CO_2 to produce CH_4 (Borrel et al., 2012; Bryant and Boone, 1988; Cadillo-Quiroz et al., 2014; Maus et al., 2014; Zellner et al., 1988). These results indicated that hydrogenotrophic methanogens were prevalent during period 2 in detriment of acetoclastic counterparts, which were apparently more affected by NO_2^- addition (e.g. *Methanosaeta*). Recovery of the system after cessation of NO_2^- addition caused a re-establishment of the prevalence of *Methanosaeta*-related methanogens in the biofilm community (Fig. 6B).

A two-dimensional NMDS ordination based on the weighted UniFrac distance was computed to easily compare bulk and active bacterial and archaeal biofilm communities in each period (Fig. S7). Bacterial and archaeal bulk communities (i.e. DNA fraction) from period 1 clearly segregated from bulk communities from period 2 and 3. On the other hand, active communities (i.e. cDNA fraction) from period 1 and 3 clustered together suggesting similar activity patterns before and after NO_2^- addition. In turn, cDNA samples from period 2 clearly segregated from the rest indicating a severe effect of NO_2^- treatment on the activity of both bacterial (large decrease in *Clostridia*) and archaeal (*Methanobacterium* outcompeted *Methanosarcina* in this period) communities. These results substantiate the importance to analyze both DNA and RNA fractions to accurately describe changes of microbial communities and their active contribution in wastewater biochemical transformations.

In conclusion, the most abundant bacterial sequences were affiliated to classes *Clostridia* and *Synergistia*, which are mainly involved in fermentative processes. It is then possible that the abatement of bacterial fermenters during period 2 caused a reduction in the amount of acetate available to fuel acetoclastic methanogenesis (e.g. *Methanosaeta*) that, in turn, favored hydrogenotrophic representatives (e.g. *Methanobacterium*) during NO_2^- treatment. Besides, the growth of denitrifying communities may cause either a competition with sulfate-reducing bacteria and methanogens for organic substrates (Achtnich et al., 1995) or its inhibition by reduced nitrogen forms (Clarens et al., 1998). The re-establishment of both CH_4 and H_2S production during period 3 might be regarded as a consequence of the resumption of fermentative metabolisms, the reduction of denitrifying activity, and the cessation of NO_2^- toxicity imposed during the dosage period. Altogether, our results suggest a notable resilience of biofilm microbial communities under disturbance episodes as previously stated (Shade et al., 2012). Moreover, the attachment of microbes to abiotic or biotic surfaces and the development of biofilms is a well-known survival strategy under the presence of both chemical and environmental stressors (Costerton et al., 1978; Donlan, 2002; Gilbert et al., 2002).

3.5. Differences between nitrate and nitrite addition downstream sections of sewer pipes

The effectiveness of DND strategy was previously tested in a lab-scale sewer system. (Auguet et al., 2015b). Although NO_2^- addition was also tested in other studies, the effects of its addition at the last sections of sewer pipes were not reported until today. Both NO_3^- and NO_2^- addition at the downstream sections of sewer pipes allows the

Table 1
Differences between downstream nitrite and nitrate dosage strategies.

	Nitrite DNO_2D	Nitrate DND
Sulfide control effectiveness during application	98.5%	99.2%*
Decrease of total dissolved sulfur during application	59%	8.6%*
Accumulation of elemental sulfur/thiosulfate/sulfite in the biofilm	245.2–424.7 mg/g Biomass	16 mg/g Biomass
Significant changes in total dissolved sulfur during batch tests	Yes	No
Increase of total dissolved sulfur exiting the sewer system after cessation of dosage	120.4%	40.6%*
Methane control effectiveness during application	78.0%	46.2%*
Progressive increase of production/consumption rates (sulfide, sulfate, methane and nitrite/nitrate)	Yes	Yes
Nitrous oxide production	Negligible	Negligible
Changes in bacterial communities in the three different periods	Yes	Yes
Changes in archaeal communities in the three different periods	Yes	No
Nitrite/nitrate daily utilization	412.8 ± 12.9 mg N–NO_2^-/day	385.7 ± 24.8 mg N–NO_3^-/day

* Calculated with an HRT of 9 h.

control of H_2S and CH_4 emissions during the presence of these compounds. However, some differences have been detected in the study of NO_2^- addition compared with the study carried out with NO_3^-. All these results are summarized in Table 1.

DNO_2D and DND strategies are useful to control H_2S release at the downstream sections of sewer pipes because H_2S loads in effluent wastewater were reduced by 98.5% and 99.2% respectively. Furthermore, total dissolved sulfur load exiting the system was similar to those in inlet wastewater during NO_3^- addition (8.6% reduction). However, ≈60% of total dissolved sulfur was missing after NO_2^- addition, likely because of H_2S was transformed to S°, $S_2O_3^{2-}$ or SO_3^{2-} and accumulated in the biofilm. This was confirmed by the quantification of these elements in the biofilm, especially abundant during NO_2^- dosage (245.2–424.7 mg S/g Biomass) compared with their amount during NO_3^- addition (16 mg S/g biomass). Moreover, a 40.6% more of total dissolved sulfur was released from the system after NO_3^- addition stopped; however, 120.4% more of total dissolved sulfur was measured when NO_2^- was the added compound, confirming our hypothesis of S°/$S_2O_3^{2-}$/SO_3^{2-} accumulation in the biofilm. Regarding CH_4 emission, it was reduced a 46.2% with DND; nevertheless, a reduction of 78.0% was detected with DNO_2D. In both cases, CH_4 emissions were re-established during the recovery period, although methanogenic community treated with NO_2^- required more time to reach similar CH_4 production capabilities as in period 1. H_2S–CH_4 production rates and SO_4^{2-}, NO_3^-/NO_2^- consumption rates gradually increased in both studies, showing a progressive adaptation of the biofilm at these new conditions. Interestingly, neither of the two mitigation strategies promotes N_2O emissions, a possible simultaneous negative effect because it is also a potent greenhouse gas.

Regarding bacterial biofilm communities, an increase of relative abundances of classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were detected in the DNA fraction of both studies. The analysis of cDNA fractions in the present study allowed us to identify the main bacterial and archaeal groups affected by NO_2^- addition and their potential contribution to the system metabolism during the treatment. In this regard, a drastic increase in the relative abundances of class *Betaproteobacteria* and *Gammaproteobacteria* and a drop in the relative contribution of classes *Deltaproteobacteria*, *Synergistia* and *Clostridia* during NO_2^-/NO_3^- addition period were detected. Regarding *Archaea*, NO_2^- treatment caused severe consequences on the methanogenic community, with a raise in the relative abundance of *Methanobacterium* in detriment of *Methanosaeta*, thus suggesting a substitution of acetoclastic by hydrogenotrophic methanogens during NO_2^- treatment. Notwithstanding these effects, both biofilms communities recovered from the oxidative stress imposed by NO_2^-/NO_3^- addition in both community composition and activity (H_2S and CH_4 production) pointing to a high resilience and resistance to environmental disturbances.

These results confirm the different effects of NO_2^- and NO_3^- addition in sewer systems although both strategies could be useful for H_2S and CH_4 control during its addition and similar concentrations of the compounds are required to obtain these results (≈ 400 mg N/day). While NO_2^- produce a higher accumulation of S°/$S_2O_3^{2-}$/SO_3^{2-} potentially releasable upon dosage cessation, it's also true that has a higher control on CH_4 production and emission. At a practical level, higher toxicity and handling risks associated to nitrite compared to nitrate must be also considered by sewer practitioners. Besides, the different costs of NO_2 and NO_3, varying from country to country, would also be a key factor when choosing the most adequate control strategy. This information is valuable for sewer managers to fully understand benefits and limitations of the available control strategies based on addition of nitrogen oxides.

4. Conclusions

The effectiveness and effects of Downstream Nitrite Dosage were evaluated in a laboratory anaerobic sewer system. The main conclusions formulated are:

- Sulfide emission was completely reduced during the addition of nitrite in downstream sections of the system.
- Most of the oxidized sulfide was accumulated in the biofilm as elemental sulfur/thiosulfate/sulfite during the nitrite addition period.
- Sulfide emissions were higher during the recovery period compared with emissions during baseline period as a consequence of elemental/thiosulfate consumption.
- Methane emissions were reduced during nitrite addition period probably as a consequence of methane oxidation.
- Nitrous oxide emissions at the last part of the sewer system were detected at negligible levels.
- The decrease of sequences affiliated with classes *Clostridia*, *Deltaproteobacteria* and *Synergistia* during nitrite addition revealed an important effect of this compound on this part of the community.
- The increase of sequences affiliated with classes *Betaproteobacteria* and *Gammaproteobacteria* during nitrite addition showed an important role of these groups in nitrite, methane and sulfur species transformations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.01.130.

References

Achtnich, C., Bak, F., Conrad, R., 1995. Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers, and methanogens in anoxic paddy soil. Biol. Fertil. Soils 19 (1), 65–72.
APHA, 1998. Standard Methods for the Examination of Water and Wastewater. 21st ed. American Public Health Administration, Washington, DC.
Auguet, O., Pijuan, M., Batista, J., Borrego, C.M., Gutierrez, O., 2015a. Changes in microbial biofilm communities during colonization of sewer systems. Appl. Environ. Microbiol. 81 (20), 7271–7280.
Auguet, O., Pijuan, M., Guasch-Balcells, H., Borrego, C.M., Gutierrez, O., 2015b. Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions. Water Res. 68, 522–532.
Borrel, G., Joblin, K., Guedon, A., Colombet, J., Tardy, V., Lehours, A.C., Fonty, G., 2012. *Methanobacterium lacus* sp. nov., isolated from the profundal sediment of a freshwater meromictic lake. Int. J. Syst. Evol. Microbiol. 62 (7), 1625–1629.
Bryant, M.P., Boone, D.R., 1988. Isolation and characterization of *Methanobacterium formicicum* MF. Int. J. Syst. Bacteriol. 38 (4), 453.
Cadillo-Quiroz, H., Brauer, S.L., Goodson, N., Yavitt, J.B., Zinder, S.H., 2014. *Methanobacterium paludis* sp. nov. and a novel strain of *Methanobacterium lacus* isolated from northern peatlands. Int. J. Syst. Evol. Microbiol. 64 (Pt 5), 1473–1480.
Chou, J.H., Jiang, S.R., Cho, J.C., Song, J., Lin, M.C., Chen, W.M., 2008. *Azonexus hydrophilus* sp. nov., a nifH gene-harbouring bacterium isolated from freshwater. Int. J. Syst. Evol. Microbiol. 58 (4), 946–951.
Clarens, M., Bernet, N., Delgenès, J.P., Moletta, R., 1998. Effects of nitrogen oxides and denitrification by *Pseudomonas stutzeri* on acetotrophic methanogenesis by *Methanosarcina mazei*. FEMS Microbiol. Ecol. 25 (3), 271–276.
Costerton, J.W., Geesey, G.G., Cheng, K.J., 1978. How bacteria stick. Sci. Am. 238, 86–95.
Cytryn, E., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., Minz, D., 2005. Identification of bacteria potentially responsible for oxic and anoxic sulfide oxidation in biofilters of a recirculating mariculture system. Appl. Environ. Microbiol. 71 (10), 6134–6141.
DeLong, E.F., 1992. Archaea in coastal marine environments. Proc. Natl. Acad. Sci. U. S. A. 89 (12), 5685–5689.
Doğan, E.C., Türker, M., Dağaşan, L., Arslan, A., 2012. Simultaneous sulfide and nitrite removal from industrial wastewaters under denitrifying conditions. Biotechnol. Bioprocess Eng. 17 (3), 661–668.
Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8 (9), 881–890.

Firer, D., Friedler, E., Lahav, O., 2008. Control of sulfide in sewer systems by dosage of iron salts: comparison between theoretical and experimental results, and practical implications. Sci. Total Environ. 392 (1), 145–156.

Foley, J., Yuan, Z., Keller, J., Senante, E., Chandran, K., Willis, J., Shah, A., van Loosdrecht, M., van Voorthuizen, E., 2011. N_2O and CH_4 emission from wastewater collection and treatment systems: state of the science report. Technical Report. Global Water Research Coalition, London, UK.

Ganigue, R., Gutierrez, O., Rootsey, R., Yuan, Z., 2011. Chemical dosing for sulfide control in Australia: an industry survey. Water Res. 45 (19), 6564–6574.

Gantner, S., Andersson, A.F., Alonso-Sáez, L., Bertilsson, S., 2011. Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. J. Microbiol. Methods 84 (1), 12–18.

Gerritsen, J., Fuentes, S., Grievink, W., van Niftrik, L., Tindall, B.J., Timmerman, H.M., Rijkers, G.T., Smidt, H., 2014. Characterization of *Romboutsia ilealis* gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus *Clostridium* into the genera *Romboutsia gen.* nov., *Intestinib*. Int. J. Syst. Evol. Microbiol. 64 (Pt 5), 1600–1616.

Gilbert, P., Maira-Litran, T., McBain, A.J., Rickard, A.H., Whyte, F.W., 2002. The physiology and collective recalcitrance of microbial biofilm communities. Adv. Microb. Physiol. 46, 203–256.

Goehring, M., Feldmann, U., Helbing, W., 1949. Quantitative Bestimmung der Polythionate (Trithionat, Tetrathionat, Pentathionat und Hexathionat) nebeneinander. Fresenius' Z. Anal. Chem. 129 (4–5), 346–352.

Guisasola, A., de Haas, D., Keller, J., Yuan, Z., 2008. Methane formation in sewer systems. Water Res. 42 (6–7), 1421–1430.

Gumaelius, L., Magnusson, G., Pettersson, B., Dalhammar, G., 2001. *Comamonas denitrificans* sp. nov., an efficient denitrifying bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 51 (3), 999–1006.

Gutierrez, O., Mohanakrishnan, J., Sharma, K.R., Meyer, R.L., Keller, J., Yuan, Z., 2008. Evaluation of oxygen injection as a means of controlling sulfide production in a sewer system. Water Res. 42 (17), 4549–4561.

Gutierrez, O., Park, D., Sharma, K.R., Yuan, Z., 2009. Effects of long-term pH elevation on the sulfate-reducing and methanogenic activities of anaerobic sewer biofilms. Water Res. 43 (9), 2549–2557.

Gutierrez, O., Park, D., Sharma, K.R., Yuan, Z., 2010a. Iron salts dosage for sulfide control in sewers induces chemical phosphorus removal during wastewater treatment. Water Res. 44 (11), 3467–3475.

Gutierrez, O., Sutherland-Stacey, L., Yuan, Z., 2010b. Simultaneous online measurement of sulfide and nitrate in sewers for nitrate dosage optimisation. Water Sci. Technol. 61 (3), 651–658.

Gutierrez, O., Sudarjanto, G., Sharma, K.R., Keller, J., Yuan, Z., 2011. SCORe-CT: a new method for testing effectiveness of sulfide-control chemicals used in sewer systems. Water Sci. Technol. 64 (12), 2381–2388.

Gutierrez, O., Sudarjanto, G., Ren, G., Ganigué, R., Jiang, G., Yuan, Z., 2014. Assessment of pH shock as a method for controlling sulfide and methane formation in pressure main sewer systems. Water Res. 48, 569–578.

Gutierrez, O., Jiang, G., Keshab, S., Yuan, Z., 2016. Biofilm development in sewer networks. In: Romani, A.M., Guasch, H., Balaguer, M.D. (Eds.), Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment. Caister Academic Press, Norfolk, UK, pp. 145–164.

Honda, T., Fujita, T., Tonouchi, a., 2013. *Aminivibrio pyruvatiphilus* gen. nov., sp. nov., an anaerobic, amino-acid-degrading bacterium from soil of a Japanese rice field. Int. J. Syst. Evol. Microbiol. 63 (Pt 10), 3679–3686.

Hvitved-Jacobsen, T., 2002. Sewer Processes — Microbial and Chemical Process Engineering of Sewer Networks. first ed. CRC Press. CRC Press, Boca Raton, FL, Boca Raton.

IPCC, 2013. Working Group I Contribution to the Assessment Report Climate Change 2013: The Physical Science Basis: Final Draft Underlying Scientific-Technical Assessment. Cambridge University, Cambridge, U.K. and New York.

Jiang, G., Sharma, K.R., Guisasola, A., Keller, J., Yuan, Z., 2009. Sulfur transformation in rising main sewers receiving nitrate dosage. Water Res. 43 (17), 4430–4440.

Jiang, G., Gutierrez, O., Sharma, K.R., Yuan, Z., 2010. Effects of nitrite concentration and exposure time on sulfide and methane production in sewer systems. Water Res. 44 (14), 4241–4251.

Jiang, G., Sharma, K.R., Yuan, Z., 2013. Effects of nitrate dosing on methanogenic activity in a sulfide-producing sewer biofilm reactor. Water Res. 47 (5), 1783–1792.

Keller-Lehmann, B., Corrie, S., Ravn, R., 2006. Preservation and simultaneous analysis of relevant soluble sulfur species in sewage samples. Second International IWA Conference on Sewer Operation and Maintenance.

Kojima, H., Tokizawa, R., Kogure, K., Kobayashi, Y., Itoh, M., Shiah, F., Okuda, N., Fukui, M., 2014. Community structure of planktonic methane-oxidizing bacteria in a subtropical reservoir characterized by dominance of phylotype closely related to nitrite reducer. Sci. Rep. 4, 1–7.

Lee, T.K., Van Doan, T., Yoo, K., Choi, S., Kim, C., Park, J., 2010. Discovery of commonly existing anode biofilm microbes in two different wastewater treatment MFCs using FLX Titanium pyrosequencing. Appl. Microbiol. Biotechnol. 87 (6), 2335–2343.

Liu, Y., Ni, B.-J., Sharma, K.R., Yuan, Z., 2015. Methane emission from sewers. Sci. Total Environ. 524–525, 40–51.

Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71 (12), 8228–8235.

Mahmood, Q., Zheng, P., Cai, J., Wu, D., Hu, B., Li, J., 2007. Anoxic sulfide biooxidation using nitrite as electron acceptor. J. Hazard. Mater. 147 (1–2), 249–256.

Maus, I., Stantscheff, R., Wibberg, D., Stolze, Y., Winkler, A., Pühler, A., König, H., Schlüter, A., 2014. Complete genome sequence of the methanogenic neotype strain *Methanobacterium formicicum* MF T. J. Biotechnol. 192, 40–41.

Mechichi, T., Stackebrandt, E., Gad'on, N., Fuchs, G., 2002. Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. Arch. Microbiology 178 (1), 26–35.

Mohanakrishnan, J., Gutierrez, O., Meyer, R.L., Yuan, Z., 2008. Nitrite effectively inhibits sulfide and methane production in a laboratory scale sewer reactor. Water Res. 42 (14), 3961–3971.

Mohanakrishnan, J., Gutierrez, O., Sharma, K.R., Guisasola, A., Werner, U., Meyer, R.L., Keller, J., Yuan, Z., 2009. Impact of nitrate addition on biofilm properties and activities in rising main sewers. Water Res. 43 (17), 4225–4237.

Mohanakrishnan, J., Kofoed, M.V.W., Barr, J., Yuan, Z., Schramm, A., Meyer, R.L., 2011. Dynamic microbial response of sulfidogenic wastewater biofilm to nitrate. Appl. Microbiol. Biotechnol. 91 (6), 1647–1657.

Patel, G.B., Sprott, G.D., 1990. Methanosaeta concilii gen. nov., sp. nov. ("*Methanothrix concilii*") and *Methanosaeta thermoacetophila* nom. rev., comb. nov. Int. J. Syst. Bacteriol. 40 (1), 79–82.

Pikaar, I., Sharma, K.R., Hu, S., Gernjak, W., Keller, J., Yuan, Z., 2014. Reducing sewer corrosion through integrated urban water management. Science 345 (6198), 812–814.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R. a, Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75 (23), 7537–7541.

Shade, A., Peter, H., Allison, S.D., Baho, D.L., Berga, M., Bürgmann, H., Huber, D.H., Langenheder, S., Lennon, J.T., Martiny, J.B.H., Matulich, K.L., Schmidt, T.M., Handelsman, J., 2012. Fundamentals of microbial community resistance and resilience. Front. Microbiol. 3, 1–19.

Spencer, A.U., Noland, S.S., Gottlieb, L.J., 2006. Bathtub fire: an extraordinary burn injury. J. Burn Care Res. 27 (1), 97–98.

Sudarjanto, G., Gutierrez, O., Ren, G., Yuan, Z., 2013. Laboratory assessment of bioproducts for sulphide and methane control in sewer systems. Sci. Total Environ. 443, 429–437.

Sun, J., Hu, S., Sharma, K.R., Ni, B.-J., Yuan, Z., 2014. Stratified Microbial structure and activity in sulfide- and methane-producing anaerobic sewer biofilms. Appl. Environ. Microbiol. 80 (22), 7042–7052.

Tao, Y., Zhou, Y., He, X., Hu, X., Li, D., 2014. *Pseudomonas chengduensis* sp. nov., isolated from landfill leachate. Int. J. Syst. Evol. Microbiol. 64 (Pt 1), 95–100.

Xiao, Y.-P., Hui, W., Wang, Q., Roh, S.W., Shi, X.-Q., Shi, J.-H., Quan, Z.-X., 2009. *Pseudomonas caeni* sp. nov., a denitrifying bacterium isolated from the sludge of an anaerobic ammonium-oxidizing bioreactor. Int. J. Syst. Evol. Microbiol. 59 (Pt 10), 2594–2598.

Zaikova, E., Walsh, D.a., Stilwell, C.P., Mohn, W.W., Tortell, P.D., Hallam, S.J., 2010. Microbial community dynamics in a seasonally anoxic fjord: Saanich Inlet, British Columbia. Environ. Microbiol. 12 (1), 172–191.

Zellner, G., Bleicher, K., Braun, E., Kneifel, H., Tindall, B.J., de Macario, E.C., Winter, J., 1988. Characterization of a new mesophilic, secondary alcohol-utilizing methanogen, *Methanobacterium palustre* spec. nov. from a peat bog. Arch. Microbiol. 151 (1), 1–9.

Zhang, L., De Schryver, P., De Gusseme, B., De Muynck, W., Boon, N., Verstraete, W., 2008. Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: a review. Water Res. 42 (1–2), 1–12.

Block III

SUMMARY OF RESULTS, GENERAL DISCUSSION, FUTURE PERSPECTIVES AND CONCLUSIONS

Chapter 6

Summary of results

This PhD thesis has been prepared as a compendium of publications. The different studies contain their own results and discussion and are distributed in the chapters listed below:

Biofilm development:

Changes in Microbial Biofilm Communities during Colonization of Sewer Systems (***Chapter 3***)

Mitigation strategies:

Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions (***Chapter 4***)

Control of sulfide and methane production in anaerobic sewer systems by means of Downstream Nitrite Dosage (***Chapter 5***)

Each study contributed to obtain a better understanding on both the identity and dynamics of microbial communities during biofilm development in sewer systems and the effects of different mitigation strategies (*e.g.* downstream nitrate and nitrite dosage) on these microbial communities and associated biotransformation routes. This chapter summarizes and discuss the main results of the experimental work under a more general perspective.

6.1. Changes in microbial biofilm communities during colonization of sewer systems

The first objective of this thesis was to investigate the initial stages of microbial biofilm development in sewer systems, with a special focus on the interactions between SRB and MA and associated activities (*i.e.* H_2S and CH_4 production, respectively) during biofilm development. In this study, mature biofilms developed in both a laboratory pilot plant that mimics the functioning of a real sewer and a full-scale sewer system were compared to validate the data obtained from our laboratory experiments.

Results showed that after the second week of lab-sewer system operation, the capacity of the biofilm to produce H_2S was stabilized (3.5–7.7 mg S–H_2S/L·h) and SO_4^{2-} reduction rates were positively related to sulfide production rates in each reactor (3.2 –7.7 mg S – SO_4^{2-}/L·h) indicating that reduced sulfate was readily transformed to H_2S. Furthermore, H_2S emissions ranged between 195.7 and 388.8 mg S – SO_4^{2-}/L·day after the second week of system functioning. These production rates suggested the presence of a fully adapted SRB community after 2 weeks of biofilm development. Moreover, H_2S production rates in R1

(upstream section of the sewer) were higher than in R2 (middle section of the sewer) and R3 (downstream section of the sewer) from week 8 to week 12 suggesting that biofilm at upstream sections of the sewer was more adapted to reduce sulfate.

On the other hand, CH_4 production rates were low in all reactors during early stages of biofilm development (0.08±0.11, 0.12±0.16, and 0.16±0.16 mg COD–CH_4/L·h, respectively). Methane emission from the system was also low during the first 6 weeks of system operation (0-8.7 mg COD–CH_4/L·day) and increased to high levels (44.5 COD–CH_4/L·day) from week 8 to week 12.

After one year of biofilm development, results showed similar activities for SRB but clear differences in methanogenesis: a ≈80% and ≈100% of SO_4^{2-} was reduced to H_2S during the first weeks and after 1 year of system functioning, respectively. However, CH_4 emissions increased significantly after 1 year of system operation (from 17.9 ± 15.9 mg COD – CH_4/L·day to 327.6 ± 16.6 mg COD – CH_4/L·day) possibly as a consequence of different SO_4^{2-} concentrations in inlet wastewater, high consumption capability of SRB in mature biofilms or changes in the composition of methanogenic communities at different development stages. Comparison of these values with H_2S and CH_4 emissions measured in a full-scale real sewer showed similar loads (1.58 g S – H_2S/L·m^2 and 4.56 g S – H_2S/L·m^2 of H_2S emissions and 1.65 g COD – CH_4/L·m^2 and 4.24 g COD – CH_4/L·m^2 of CH_4 emissions in the laboratory and full-scale sewer systems, respectively) validating the results obtained under laboratory conditions.

Molecular analysis using gene fingerprinting (*i.e.* DGGE of bacterial and archaeal 16S rRNA gene fragments) revealed differences between the composition of bacterial communities in the inlet wastewater and the biofilms grown at upstream section of the sewer (R1). Moreover, changes on the bacterial communities were also detected during biofilm development, indicating a gradual adaptation of bacterial biofilm communities to local conditions. Less variation in diversity was detected between archaeal communities in the inlet wastewater and biofilm samples. Moreover, quantification of marker genes for SRB and archaea showed similar trends during the first 2 weeks of operation followed by a steady state. The fact that both bacterial and archaeal communities showed similar clustering patterns suggests potential interactions (*e.g.*, synergy or competition) between communities.

The phylogenetic composition of biofilm microbial communities was also assessed by high-throughput sequencing (*i.e.* 454-pyrosequencing) to resolve the identity of bacterial and archaeal communities involved in H_2S and CH_4 production. Sequences affiliated to bacterial classes *Bacilli*, *Fusobacteria*, and *Gammaproteobacteria* decreased during biofilm maturation but they were not detected in the full-scale sewer. In turn, sequences affiliated to class *Betaproteobacteria* were detected in the full-scale sewer biofilm and

in biofilm samples collected from R1 (this reactor mimics the upstream section of the sewer) during early stages of biofilm development. Finally, the relative abundance of sequences affiliated to classes *Synergistia* and *Deltaproteobacteria* increased during biofilm development to same values detected in the full-scale sewer system. Members affiliated to class *Deltaproteobacteria* (which includes most SO_4^{2-} reducers) were detected during the first week of biofilm development (3.0–5.8% of relative abundance) showing an increase in their relative contribution in mature biofilms (16.1%). This observation agreed with both the increment in H_2S production after 2 weeks of system operation and the increase of SO_4^{2-} reduction activity in mature biofilms (from 80% to 100% in young and mature biofilm, respectively). Overall, most of the microorganisms identified in sewer biofilms were closely similar (in terms of 16S rRNA gene sequence identity) to both well-known fermenters and sulfate reducers (the latter in a lesser extent).

Less diversity was detected for the archaeal community, which were solely composed of methanogenic lineages. During the first weeks of biofilm development the archaeal community was dominated by sequences affiliated to genera *Methanosphaera* (10–23%) and *Methanobrevibacter* (76–86%). *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (belonging to order *Methanobacteriales*) were identified during early stages of biofilm development and also in the inlet wastewater. However, the archaeal community was completely different in mature and full-scale sewer biofilms, being members of the genus *Methanosaeta* the most abundant archaeal representatives. The differences in these two archaeal communities could also favour the different CH_4 emissions during the different stages of biofilm development.

6.2. Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions

The second goal of this thesis was to determine the effects of downstream nitrate dosage both on the H_2S and CH_4 productions/emissions from anaerobic sewers and on the microbial biofilm communities. This work was carried out in a laboratory pilot plant that mimics the main characteristics of a full-scale sewer pipe. The experiment was divided in three phases: baseline monitoring (phase 1), nitrate addition (phase 2) and recovery period (phase 3). Changes of microbial communities in the biofilm were also monitored by 454-pyrosequencing during the study period.

Results showed that H_2S produced in R1 and R2 (upstream and middle sections of the sewer pipes) was completely oxidized in R3 (downstream section of the sewer pipe) when NO_3^- was added. However, total dissolved sulfur in the inlet wastewater was higher compared with the concentrations measured in the effluent wastewater. Interestingly, elemental sulfur accumulation was detected in the biofilm. Sulfide production was re-established

immediately after ceasing nitrate addition. Besides, concentrations of H_2S clearly exceeded SO_4^{2-} concentrations in inlet wastewater suggesting that the S^0 accumulated in the sewer biofilm matrix was reduced to H_2S by SRB. Regarding CH_4, a 46.2% reduction in its emission was measured when nitrate was added although it readily increased when nitrate addition stopped.

Different biotransformation routes occurring in the reactor were monitored during the study by means of batch tests (BTs) analyses. Sulfide oxidation, methane oxidation and nitrate reduction were detected during phase 2. The reduction rates of these reactions increased gradually during the nitrate addition phase. Sulfide formation started immediately as soon as NO_3^- was depleted in R3. In contrast, CH_4 production was not re-established immediately. Moreover, N_2O was also measured and it was detected at negligible levels.

Nitrate addition mainly caused an increase in the relative abundance of *Betaproteobacteria*, especially of members of genera *Simplicispira, Comamonas, Azonexus* and *Thauera*. Classes *Alphaproteobacteria* and *Gammaproteobacteria* showed a minor increase in their relative abundances. Furthermore, a reduction of sequences affiliated to classes *Deltaproteobacteria*, *Synergistia* and *Clostridia were observed* during phase 2 (nitrate addition period). Less pronounced changes were observed for archaeal biofilm communities, being *Methanosaeta* the most abundant genus during both phases (93.1% and 98.8% in phase 1 and phase 2, respectively).

6.3. Control of sulfide and methane production in anaerobic sewer systems by means of Downstream Nitrite Dosage

The last objective of this thesis was to test the effectiveness of Downstream Nitrite Dosage (DNO_2D) in anaerobic sewer systems to reduce H_2S and CH_4 emissions and to identify potential changes on the active members of the biofilm microbial community. The work was also carried out in the laboratory sewer system cited above. The study was divided in three periods: system setup (period 1), nitrite addition (period 2) and system recovery (period 3).

During the transport of wastewater through the upstream and middle sections of the sewer (R1 and R2) a 95.1% of the SO_4^{2-} was reduced to H_2S. Remarkably, H_2S emission was completely supressed during NO_2^- addition at the downstream section of the system (R3). During this period only a 41% of the total dissolved sulfur from the influent wastewater was released at the end point. A large increase in the concentration of S^0/ $S_2O_3^{2-}$/ SO_3^{2-} was detected in biofilms (from 7.8 to 424.7 mg S/g biomass). Sulfide production was immediately re-established during period 3 and during the first week, the total dissolved

sulfur concentration exiting the system was 120.4% higher than the concentration detected in influent wastewater. Methane emission was reduced by ≈80% during NO_2^- addition and increased gradually for the first 50 days of period 3 until reach similar levels as in period 1. Finally, N_2O emissions were detected at negligible levels.

Results from batch tests indicated that H_2S and CH_4 were produced at rates of 2.4±0.6 mg $S\text{-}H_2S/L\cdot h$ and 5±1.5 mg $COD{-}CH_4/L\cdot h$, respectively. During period 2, nitrite consumption increased during the first days of NO_2^- addition and was stabilized from the 20th day at a rate of $45-50$ mg $N-NO_2^-/L\cdot h$. On the other hand, H_2S and CH_4 were consumed during the presence of NO_2^- (30 mg $S-H_2S/L\cdot h$ and 35 mg $COD-CH_4/L\cdot day$). Finally, H_2S started immediately after nitrite addition stopped although it was higher during the first day compared with the other days (13.1 and 3.1±0.5 mg $S\text{-}H_2S/L\cdot h$, respectively). In the case of CH_4, there was consumption during the first 23 days even though the absence of NO_2^- and after day 48 was re-established as similar levels as in period 1.

Molecular analyses showed that the majority of the active bacterial community (in the cDNA fraction) was composed by microorganisms affiliated to class *Clostridia* (57% of relative abundance) and during nitrite addition its relative abundance decreased to 7%. Interestingly, this bacterial group recovered its abundance during period 3 (recovery after nitrite dosage). A similar decrease and recovery was observed for members of classes *Synergistia* and *Deltaproteobacteria*. It is important to remark that sequences affiliated to classes *Betaproteobacteria* (specifically the genera *Thauera, Comamonas* and *Azonexus*) and *Gammaproteobacteria* underwent an increase in their relative abundance during NO_2^- addition. Regarding archaeal communities, most sequences affiliated to genus *Methanosaeta* (98% of relative abundance) during period 1 (system setup). However, addition of NO_2^- drastically changed the archaeal community, being members of genus *Methanobacterium* the most abundant during nitrite treatment. During the recovery period the composition of the archaeal community was similar to that observed in period 1.

Chapter 7

General discussion

7.1. Biofilms in sewer systems

Several studies investigated the composition of microbial communities in mature sewer biofilms (Mohanakrishnan et al., 2009b) and also during the utilization of different mitigation strategies to prevent H_2S and CH_4 emission (Gutierrez et al., 2008; Mohanakrishnan et al., 2011, 2009a). However, since most of these studies used molecular techniques characterized by a low resolution (*e.g.* DGGE and FISH) the precise characterization of the microbial diversity was severely limited. The combination of two independent molecular methods (FISH and pyrosequencing) allowed the confirmation of SRB and MA community stratification in sewer biofilms (Sun et al., 2014). In general, the results of these studies showed the presence of SRB and MA that are involved in H_2S and CH_4 production, respectively.

Little information is available, however, about the composition of microbial biofilm communities at early stages of colonization and its impact on H_2S and CH_4 production in sewer pipes.

7.1.1. Development of biofilms in sewer systems

The results of **Chapter 3** (*Changes in Microbial Biofilm Communities during Colonization of Sewer Systems*) showed differences on sulfide and methane emissions and also changes on the microbial community composition during the different stages of biofilm development.

Reasons behind the changes on sulfide and methane production during biofilm development

The ability to reduce SO_4^{2-} to H_2S (measured as H_2S production rate) was acquired after two weeks of functioning of the laboratory sewer system. This implied that SRB colonized the inner pipe walls with ease. Results from qPCR and pyrosequencing analyses confirmed the presence of SRB during initial stages of biofilm growth. Moreover, the H_2S production capacity was different depending on the development stage of the biofilm: during the first weeks the total SO_4^{2-} in the influent wastewater was not completely reduced to H_2S (80%) but it changed after one year of system operation, when a total reduction was measured. This change was possibly related to the full adaptation of the SRB community to local conditions. According to this reasoning, the relative abundance of *Deltaproteobacteria* progressively increased from 3–6% in young biofilms to 16% in mature biofilms whereas the remaining fraction of the bacterial community was composed basically by fermenters, which were probably involved in the production of simple fermentation end products (*e.g.* VFA, CO_2, H_2, lactate, butyrate, propionate, among others).

Different *Deltaproteobacteria* were identified in each studied period, suggesting an ecological succession within the SRB community, from a community dominated by *Desulfobulbus propionicus* during early stages to a mature community dominated by *Desulfobacter postgatei* after one year of biofilm development. However, *Desulfobulbus* was the most abundant SRB in full-scale sewers and also in laboratory biofilms in the Sun and co-workers study (10 months of system operation) (Sun et al., 2014). This result indicates that the population change does not only depend on the development stage but it is also affected by system conditions. Nevertheless, sulfate was almost completely reduced in all cases demonstrating that different SRB community composition behaves similarly in terms of H_2S production.

On the other hand, CH_4 emissions were considerably higher after one year of biofilm development compared to early stages. To explain these difference we may draw three different hypotheses, namely: *(i)* the low sulfate concentration in inlet wastewater favours high methanogenic activity; *(ii)* the high relative abundance of SRB in mature biofilms could explain the high rate of sulfate consumption in these biofilms thus stimulating CH_4 production, or *(iii)* the change in the composition of the methanogenic community over time favours species more adapted to local conditions and CH_4 production. Moreover, a fourth hypothesis can be stated being the competition for key substrates the factor that causes changes of microbial communities and consequently, affects CH_4 production.

Different results of CH_4 production were obtained during the performance of batch tests (BT) and normal functioning (NF) experiments, showed that the impact of H_2S consumption or other inhibitors during wastewater transport. The BT indicated a poor development of methanogenic community during the first 12 weeks of biofilm development because CH_4 production rates were really low in each section of the sewer (0-0.48 mg $COD-CH_4$/L·h). However, results of NF showed an increase of CH_4 emissions from week 8 to week 12 (from 8.7 to 44.5 mg $COD-CH_4$/L·day). This discrepancy was related to the different experimental design between BT and NF. The low CH_4 production rates measured in BTs are probably due to the use of fresh, sulfate-rich wastewater in each reactor, thus hindering CH_4 production in all sections. However, CH_4 concentrations were analysed only in the last section of the system before wastewater was pumped outside the system to know the total emission during NF experiments. In this case wastewater was transported through the reactors thus allowing the consumption of SO_4^{2-} in the upstream sections (R1 and R2) and favouring CH_4 production in downstream sections (R3). This length-pipe spatial adaptation was also confirmed in a special BT (which lasted 6 hours) that showed an increase of CH_4 production when SO_4^{2-} was consumed, especially in R3 (the last section of the sewer which was adapted to receive wastewater without SO_4^{2-}). These results show that the high rate of sulfate consumption by SRB in mature biofilms could stimulate CH_4 production indicating a potential competition between these two communities for the

same substrates or energy sources as was reported in other studies (Lovley and Klug, 1983; Omil et al., 1998), basically during early stages of biofilm development.

Secondly, changes of the methanogenic community composition can also lead to an increase of CH_4 emissions in sewer systems. During the early stages of biofilm development the most abundant species identified were *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, two species that are considered to be the prevalent in the human gut (Dridi et al., 2009). In turn, members of the genus *Methanosaeta* were the most abundant archaeal microorganisms in mature and full-scale sewer biofilms; particularly, the majority of the sequences affiliated to *Methanosaeta concilii* (97% and 89% in mature and full-scale sewer biofilms, respectively). This change in the archaeal community might explain the differences measured in CH_4 emissions at different stages of biofilm development.

Human gut microbiota as source of methanogenic colonizers of sewer biofilms

In the human gut, complex carbohydrates from dietary are degraded by microbiota to smaller oligomers or monomers, which are subsequently fermented mainly to short-chain fatty acids (SCFA, such as acetate, propionate, and butyrate), H_2 and CO_2 (Nakamura et al., 2010). The accumulation of H_2 produced during fermentation can inhibit the transformation of some fermentable products (*e.g.* propionate, acetate…) (Fukuzaki et al., 1990). For that reason, the syntrophic interaction between H_2 producers and consumers (*i.e.* fermentative bacteria and hydrogenotrophic microorganisms, respectively) is crucial to sustain the process. Particularly, the three most important types of H_2-scavengers in the human colon are methanogens, SRB and acetogenic bacteria (Christl et al., 1992). It is important to remark that these H_2 consumers are typically present at much lower densities than fermentative bacteria, but its presence is crucial to maintain an optimal H_2 pressure to regulate fermentation (Nakamura et al., 2010).

Methanogenic archaea in the human gut are mainly hydrogenotrophs. The most abundant methanogen detected is *Methanobrevibacter smithii*, and, occasionally, *Methanosphaera stadtmanae* (Dridi et al., 2009). The prevalence of *M. smithii* can be explained by its metabolic versatility since it can use H_2, CO_2, and formate to produce CH_4. This archaeon can also assimilate other end products from fermentation (non-methanogenic removal of methanol and ethanol) (Samuel et al., 2007). In turn, *M. stadtmanae* has more restricted energy metabolism because it is limited to use H_2 to reduce methanol to CH_4 (Fricke et al., 2006; Miller and Wolin, 1985). The predominant SRB in the human gut were species belonging to genera *Desulfovibrio* and *Desulfobulbus,* accounting approximately 66% and 16% of all colonic SRB, respectively (Gibson et al., 1993a). In another study of Gibson and co-workers, they identified *Desulfovibrio* spp. (lactate and H_2 utilizing bacteria: 64–81%), *Desulfobacter* spp. (acetate utilizing bacteria: 9–16%), *Desulfobulbus* spp. (propionate and H_2 utilizing bacteria: 5–8%), lactate utilizing *Desulfomonas* spp. (lactate utilizing bacteria:

3–10%) and acetate and butyrate utilizing *Desulfotomaculum* spp. (acetate and butyrate utilizing bacteria: 2%) in human feces (Gibson et al., 1993b)

All these results dealing with the composition of microbial communities in the human gut show striking similarities to those found during early stages of biofilm development (Fig. 7.1).

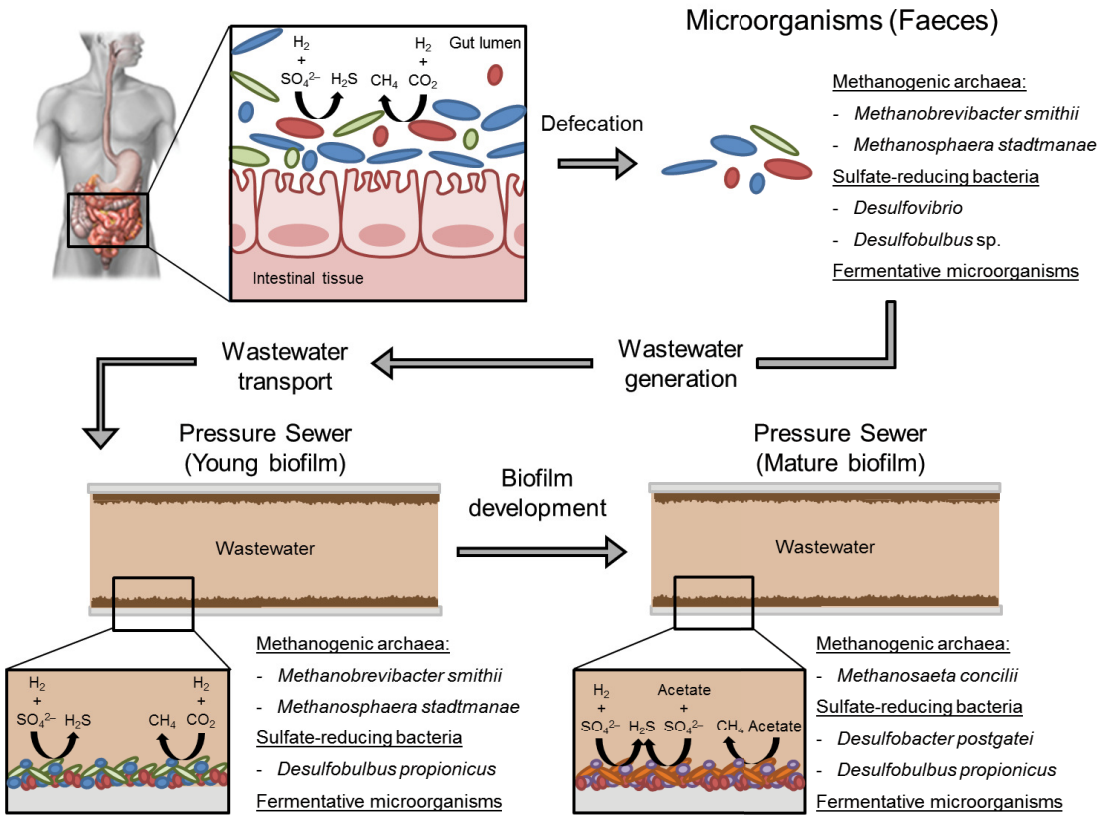


Figure 7.1. Scheme of microbial community evolution from the source (human gut) to the sewer system.

The main methanogenic species found in young sewer biofilms were the same found in the human gut: *M. smithii* and *M. stadtmanae*. These species were probably responsible of pipe wall colonization and biofilm formation during the first weeks of system operation. During the further growth of the biofilm both species (hydrogenotrophic methanogens) could be outcompeted by hydrogenotrophic SRB also found in human faeces (*e.g. Desulfobulbus*) thus affecting the overall CH_4 production (which remained low during the first 12 weeks of system operation). SRB have a more favourable kinetics for H_2 than methanogens (Kristjansson et al., 1982; Lovley and Klug, 1983; Lovley et al., 1982) thus favouring in the competition for H_2. Moreover, the short retention time in the colon does not allow the development of slow-growing acetoclastic methanogens (Bryant, 1979; McInerney et al., 1979) similarly to processes occurring in young biofilms. However, the HRT found in

many sewer pipes (*e.g.* 9 hours) may favour slow-growing acetoclastic methanogens (Fig. 7.1) especially considering that acetate is not limiting. These conditions may favour the growth of acetoclastic *Methanosaeta* spp*.,* which became the most abundant methanogenic species in mature sewer biofilms (Auguet et al., 2015a; Sun et al., 2014) and thus being the main responsible of CH_4 emissions.

Results of **Chapter 3** might be of special interest to develop optimal methods to control and reduce malodour, corrosion, and emissions from sewer systems. The most important factor to take into account is that SRB in sewers are able to convert ≥80% of SO_4^{2-} to H_2S after just two weeks of sewer functioning. On the other hand, the increase in CH_4 production started after the 8th week of system operation, reaching maximal production after one year of system operation. These data would be of interest for sewer managers and the water industry to identify, more precisely, at which time-point sewer biofilms begin to produce H_2S and CH_4. According to the results, the control of H_2S and CH_4 production should be performed from the very beginning of system operation despite of the delayed kinetics of CH_4 emissions. Moreover, these findings are also relevant for the scientific community interested in the microbial ecology of sewer systems. A proper characterization of microbial communities in sewer biofilms may appear as an indicator of the capacity of the system to produce H_2S and CH_4.

7.1.2. Changes in the biofilm during nitrate and nitrite addition in sewer systems

The results from **Chapters 4** (*Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions*) and **Chapter 5** (*Control of sulfide and methane production in anaerobic sewer systems by means of Downstream Nitrite Dosage*) provide a comprehensive view of how the chemical treatments applied to mitigate H_2S and CH_4 emissions affect the microbial communities that constitute sewer biofilms.

Before nitrate and nitrite addition the bacterial community was mainly composed of microorganisms affiliated with classes *Synergistia*, *Clostridia* and *Deltaproteobacteria*. *Synergistia* and *Clostridia* probably rely on fermentation processes to obtain energy providing substrates for SRB and MA (*e.g.* H_2, CO_2, acetate, formate, lactate, propionate and butyrate). Whereas members of the class *Deltaproteobacteria* are responsible for H_2S production, the most abundant methanogenic genus before NO_3^-/ NO_2^- addition was *Methanosaeta*, using acetate from bacterial fermentation to generate CH_4.

Addition of nitrate or nitrite to the system caused changes on the composition of microbial biofilm communities. According to our results, the most important change was the decrease in the relative abundance of fermenters and the increase in the relative contribution of NO_3^-/ NO_2^- reducing bacteria (mainly grouped within classes *Betaproteobacteria* (genera

Comamonas, *Azonexus*, *Thauera* and *Simplicispira*) and *Gammaproteobacteria*). It is important to remark that both NO_3^- and NO_2^- were readily reduced from the first days of addition, indicating the presence of nitrate/nitrite reducing bacteria before dosing period. These changes may cause a competition for organic compounds between nitrate/nitrite reducing bacteria and SRB and MA (Achtnich et al., 1995) thus affecting H_2S and CH_4 production. Interestingly, NO_3^- addition did not have a direct effect on the biofilm archaeal community because only the DNA fraction (bulk community) was analysed. However, the results obtained during the addition of NO_2^- showed that nitrite (analysis of cDNA fraction which represents the active microbial community) favoured the activity of hydrogenotrophic methanogens (*i.e. Methanobacterium formicicum*), which were then outcompeted by other H_2-scavengers thus causing a drop in CH_4 production.

The detection of H_2S oxidation from the first day of chemical addition may suggest the presence of denitrifying sulfide-oxidizing bacteria in sewer biofilms. The oxidation of H_2S to S^0 measured during the addition of both NO_3^- and NO_2^- could be attributed to bacteria affiliated to genus *Thauera* (Cytryn et al., 2005; C. Liu et al., 2015). However, this assignment is risky according to the huge microbial diversity (including a large number of uncultured species with unknown capabilities) in sewers and the vast array of different metabolisms occurring in the system.

Another interesting aspect of this thesis is the lack of any direct evidence of microorganisms involved in either aerobic or anaerobic CH_4 oxidation although oxidation of CH_4 was measured during NO_3^-/NO_2^- addition. This oxidation capability was low during the first days of NO_3^-/NO_2^- dosing, indicating that the community able to do this reaction need more time to be adapted compared with sulfide oxidizing microorganisms. In this regard, a sequence having a 92% similarity to *Methylomicrobium album* (an aerobic CH_4 oxidizer) was identified during NO_3^- addition but no other results point to the presence of CH_4 oxidizers in the system. However, it is possible that the microorganisms involved in this methane oxidation that occurred in these experiments have not been identified and characterized yet.

Finally, the reestablishment of anaerobic conditions (samples only taken during NO_2^- experiment) favours the resumption of fermentation metabolisms and the production of H_2S and CH_4. Under these new conditions, the S^0 accumulated in the biofilm during the oxidative treatment boosted the production of H_2S by SRB (probably *Desulfobulbus propionicus* (Lovley and Phillips, 1994)) thus increasing its emission from the system.

Figure 7.2 summarizes these results including hypothesis on the potential metabolisms and microorganisms involved in the biotransformations occurring in sewer biofilms under the different conditions tested.

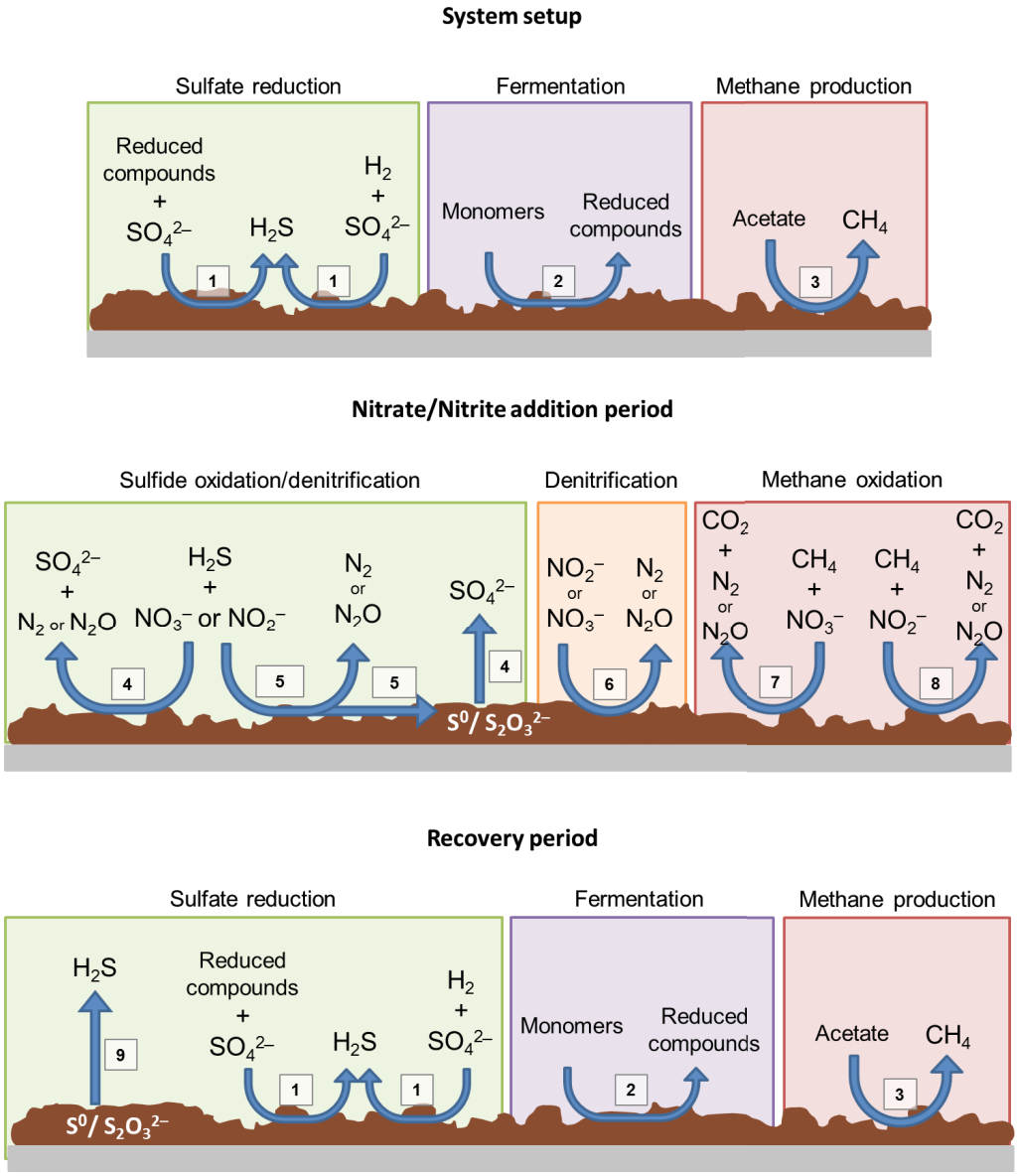


Figure 7.2. Hypothetical scheme of the different processes carried out by biofilm microbial communities in sewer systems during nitrate/nitrite addition. 1: Sulfate reduction (*Deltaproteobacteria*). 2: Fermentation (*Synergistia* and *Clostridia*). 3: Methane production (*Methanosaeta).* 4: Biological oxidation of sulfide, elemental sulfur or thiosulfate to sulfate with nitrate or nitrite. 5: Biological oxidation of sulfide to elemental sulfur with nitrate or nitrite (similar microorganisms like *Thauera*). 6: Denitrification (*Comamonas*, *Azonexus*, *Thauera* and *Simplicispira)*. 7: Methane oxidation with nitrate. 8: Methane oxidation with nitrite. 9: Sulfur reduction (*Deltaproteobacteria*).

The molecular results obtained in this thesis (**Chapter 4** and **Chapter 5**) can be useful for researches to know the most important microbial communities developed during the different conditions (anaerobic/aerobic) and to understand the potential processes that can occur during nitrate and nitrite addition. However, more field tests have to be done to confirm these findings because the system operation could be different (e.g. differences on the gradient of nitrite/nitrate concentrations along the end of the sewer pipe).

7.2. Mitigation strategies used to reduce sulfide and methane emissions

Different mitigation strategies have traditionally been used to reduce H_2S and CH_4 emissions from sewer systems. However, the need for more cost effective methods has led the development of new dosing strategies based not only in the chemical used but also on the optimization of dosage rates and dosing locations (Ganigue et al., 2011). For example, the addition of chemicals at a point close to the end of the pressure sewer in laboratory preliminary studies showed the benefits in terms of better control of H_2S emissions and also on the reduction of the amounts needed during the addition period (Gutierrez et al., 2010). However, more investigation and optimization are still required before application in the field.

For all these reasons and because the use of nitrogen oxides (NO_3^- and NO_2^-) revealed that these compounds allow a good control on H_2S and CH_4 emissions (Jiang et al., 2010; Mohanakrishnan et al., 2008), the effectiveness of its addition was tested at downstream sections of the sewer pipes and results of these experimental activities are included in **Chapter 4** (Downstream Nitrate Dosage) and **Chapter 5** (Downstream Nitrite Dosage).

7.2.1. Comparison between downstream nitrate and nitrite dosage strategies.

During nitrate and nitrite addition, H_2S concentrations exiting the system were completely reduced indicating that DND and DNO_2D strategies were able to completely reduce the emission of this problematic compound from the sewer. However, the accumulation of potential S^0 in the biofilm during NO_3^-/NO_2^- (or also $S_2O_3^{2-}$ and SO_3^{2-}) which was reported before (Jiang et al., 2009) produced an increase of H_2S production when chemical addition ceased, indicating that this strategy has some side effects that need to be considered. Interestingly, the accumulation of S^0 was higher during NO_2^- addition than during NO_3^- dosage thus causing an overproduction of H_2S during the first days of recovery period. In a previous study (Jiang et al., 2009), the formation of these intermediate sulfur compounds were also observed during nitrate addition in sewers. For nitrite treatment, the accumulation of S^0 was suggested by Mohanakrishnan and co-workers (Mohanakrishnan et al., 2008) although this is the first time that its presence at high levels has been experimentally demonstrated.

Methane emission was reduced during the addition of both NO_3^- and NO_2^- although it was not completely supressed (a 50% and 20% of CH_4 remained in the effluent wastewater during NO_3^- and NO_2^-, respectively). However, we reported for the first time that CH_4 oxidation occurred in sewer systems during both oxidative treatments although it is a well-known process in other environments (Ettwig et al., 2010, 2009, 2008; Haroon et al.,

2013; Zhu et al., 2012). Once nitrate addition ceased (period 3), CH_4 production resumed immediately. In turn, the recovery of methanogenic activity was more progressive after the cessation of nitrite addition, suggesting that this chemical inhibited methanogenesis to a certain extent probably because of its well-known toxicity as previously reported (Jiang et al., 2010).

In summary, our results demonstrate the different effects of DND and DNO_2D strategies in sewer systems. Although both mitigation methods could be useful to reduce H_2S and CH_4 emissions, NO_2^- produce higher accumulation of sulfur intermediates (*e.g.* S^0 or $S_2O_3^{2-}$) compared to NO_3^-, resulting in an increase of H_2S production upon dosage cessation. For that reason, the water industry and sewer managers should also consider this issue since nitrite dosage induced a partial delay on sulfide emission and only allow the control of sulfide during the addition period. On the other hand, the most important advantage of NO_2^- addition compared with NO_3^- addition is that allows a better control in terms of CH_4 production and emission.

The addition of NO_3^-/NO_2^- at downstream sections was able to achieve completely control of H_2S emissions but not for CH_4. If nitrate/nitrite is added at the upstream sections of the sewer system, they can be consumed along transport favouring the production of H_2S and CH_4 at downstream sewer sections (Jiang et al., 2010; Mohanakrishnan et al., 2009a). For that reason, the application of chemicals at downstream sections could reduce overall costs since fewer amounts of product are needed to control of H_2S and CH_4 emissions. Regarding costs, it is difficult to make a valid comparison between the price of NO_3^- and NO_2^- worldwide since they largely vary between countries. Thus, a careful evaluation of costs and benefits should be done at each occasion. Moreover, the need of large amounts of NO_3^-/NO_2^- also has a direct influence on the organic matter consumption thus affecting the subsequent wastewater treatment efficiency.

Finally, potential hazards associated with nitrite usage have to be considered by sewer managers when planning and designing which strategy to use. Moreover, the complete abatement of NO_3^- and NO_2^- has to be ensured in the sewer to avoid subsequent problems (*e.g.* increase of NO_3^-/ NO_2^-). Therefore, a careful dosage optimization is mandatory to avoid side effects and should be evaluated for each sewer network under study. However, if some nitrate or nitrite reaches the WWTP it could be removed during denitrification processes for nitrogen removal.

These findings can be useful for sewer managers to determine the suitable alternative to control H_2S and CH_4 emissions in these systems. Moreover, field tests are also needed to validate the results obtained in lab-scale sewer systems and to properly determine the effects of NO_3^- and NO_2^- not only in sewer pipes but also in the receiving WWTP. The results could then be valuable to fully understand the benefits and

limitations of the available control strategies based on addition of nitrogen oxides and to find the optimal solution before its application to full-scale sewer systems.

Chapter 8

Future perspectives

This PhD thesis compiles for the first time valuable information about the early stages of development of sewer biofilms and the growth dynamics of microorganisms involved in H_2S and CH_4 production, SRB and MA, respectively. These results demonstrate that H_2S emissions start after only two weeks of sewer operation but CH_4 emissions proceed after a delay of approximately one year of system functioning. Besides, the two mitigation strategies tested in the laboratory sewer system (DND and DNO_2D) provide a good control on H_2S and CH_4 emissions.

In this context, it could be of great interest to carry out several field-studies using full-scale sewer systems aimed to validate laboratory results, namely: *i)* to determine real H_2S and CH_4 production rates during the first stages of system operation; and *ii)* to assess the effectiveness of both DND and DNO_2D strategies and its effects *in situ*. Further research on these two aspects is therefore needed.

Some gaps still exist regarding the functioning of the laboratory pilot plant. Although we studied the biofilm development at upstream sewer sections (*i.e.* reactor 1) to monitor changes in the composition and activity of microbial communities during colonization, a more detailed description is needed to describe these aspects at different sewer sections. For instance, it would be of interest to determine if the composition of archaeal communities varies along sewer sections and if these potential variations affect CH_4 production.

Moreover, the monitoring of the expression of functional genes involved in SO_4^{2-} reduction (*e.g. dsrA*) or methanogenesis (*e.g. mcrA*) as well as the phylogenetic markers of both groups (bacteria *vs.* archaea) might be useful to understand the dynamics and kinetics of both processes along space (sewer sections) and time (different stages of biofilm development). Additionally, this quantification could be helpful to determine the effects of NO_3^- and NO_2^- treatment on the activity of these communities in situ not only during the treatment (addition period) but also, and especially, after treatment stops (recovery period).

Unfortunately, we were unable to identify any microorganism involved in the anaerobic oxidation of CH_4. For that reason, the quantification of a functional marker for this process (*e.g. pmoA*, which encodes the alpha subunit of the particulate methane monooxygenase, pMMO) would be of great interest to confirm or discard the occurrence of anaerobic CH_4 oxidizers in the sewers and to determine under which conditions they prevail.

A final aspect refers to the potential role of sewer biofilms as reservoirs of antibiotic resistance. The main causes of the emergence and spread of antibiotic resistance among bacterial pathogens are due to the extensive use and misuse of antibiotics that exert a selective pressure favoring the selection of drug-resistant strains and compromising the treatment effectiveness. As a consequence, the human microbiota is usually altered and

enriched in antibiotic-resistant bacteria (ARB). Antibiotics and ARB are excreted and then transported from individual toilets to sewage treatment plants via the sewer system, which collects wastewater from domestic, hospital and industrial sources. Although many studies have addressed the study of antibiotic resistance in environmental settings (*e.g.* water or soil), less information is available on the diversity, abundance and spread of antibiotic resistance genes (ARG) in sewage systems, even considering that sewers directly collect raw sewage from households and hospital settings. For that reason it could be interesting to investigate how antibiotics and ARB in raw sewage favor the generation and spread of resistance within the sewer systems and how this "sewer resistome" varies under different conditions.

Chapter 9

General conclusions

The conclusions drawn from the present work are:

Changes in Microbial Biofilm Communities during Colonization of Sewer Systems

1. Sulfate reducing bacteria readily colonized sewers and formed active populations in biofilms that produce H_2S after two weeks of system functioning. The production of H_2S was maintained after one year of system operation.

2. The emission of methane increased after 8 weeks of system operation, most likely because sulfate was depleted in upstream sections of the sewer.

3. Methane production was inhibited during early stages of biofilm development as a consequence of competition for H_2 between hydrogenotrophic methanogenic archaea (i.e. *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*) and sulfate reducing bacteria.

4. Methane emissions were significantly higher after one year of system operation compared to the initial weeks of operation, indicating a change in the capacity of biofilm to produce methane. This change in activity was related to a progressive displacement of hydrogenotrophic methanogens derived from the human gut by acetoclastic representatives (*i.e. Methanosaeta concilii*), which appear to be better adapted to local sewer conditions.

5. Emissions of methane and sulfide in full-scale sewer were similar to those measured in the laboratory-scale anaerobic sewer system. Composition of microbial communities also showed similarities thus confirming the capacity of our laboratory experimental system to mimic the functioning of full-scale sewers in terms of microbial diversity and activity.

Downstream nitrate and nitrite dosage

6. Sulfide emission was completely reduced during downstream nitrate and nitrite dosage due to biological sulfide oxidation in the biofilm. As a consequence, intermediate sulfur compounds (*e.g.* elemental sulfur) accumulated during this period being higher during nitrite addition.

7. Methane emissions were reduced approximately by 50% and 80% during nitrate and nitrite addition, respectively. Moreover, methane produced at upstream sewer sections was reduced at downstream sections as a consequence of nitrate/nitrite dosage.

8. Sulfide emission was higher after ceasing nitrate/nitrite additions (*i.e.* recovery period) probably as a consequence of the reduction of intermediate sulfur compounds accumulated in the biofilm. The latter occurrence was larger when using nitrite, thus causing a higher emission of sulfide after stopping nitrite addition.

9. The immediate consumption of nitrate and nitrite after their addition suggested the presence of potential heterotrophic and autotrophic nitrate/nitrite reducing bacteria in the biofilm.

10. The bacterial community was mainly composed of microorganisms affiliated with classes *Synergistia*, *Clostridia* and *Deltaproteobacteria* before nitrate/nitrite addition. The addition of oxidized nitrogen species caused a drastic change in the bacterial community composition, favoring an increase in the relative abundance of members of classes *Betaproteobacteria* and *Gammaproteobacteria*.

11. The most abundant archaeal species before nitrate/nitrite addition was *Methanosaeta concilii*, an acetoclastic methanogen. Addition of nitrite favored the growth of hydrogenotrophic methanogens (*e.g. Methanobacterium formicicum*).

12. Microbial communities recovered from the oxidative stress imposed by nitrate/nitrite addition in both community composition and activity (H_2S and CH_4 production) pointing to a high resilience and resistance to environmental disturbances. Nevertheless, the recovery of methanogenic activity was slower than sulfate reduction.

Chapter 10

References

Achtnich, C., Bak, F., Conrad, R., 1995. Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers, and methanogens in anoxic paddy soil. Biol. Fertil. Soils 19(1), 65–72.

Auguet, O., Pijuan, M., Batista, J., Borrego, C.M., Gutierrez, O., 2015a. Changes in Microbial Biofilm Communities during Colonization of Sewer Systems. Appl. Environ. Microbiol. 81(20), 7271–7280.

Auguet, O., Pijuan, M., Guasch-Balcells, H., Borrego, C.M., Gutierrez, O., 2015b. Implications of Downstream Nitrate Dosage in anaerobic sewers to control sulfide and methane emissions. Water Res. 68, 522–532.

Bahr, M., Crump, B.C., Klepac-Ceraj, V., Teske, A., Sogin, M.L., Hobbie, J.E., 2005. Molecular characterization of sulfate-reducing bacteria in a New England salt marsh. Environ. Microbiol. 7(8), 1175–1185.

Barton, L.L., Tomei, F.A., 1995. Characteristics and Activities of Sulfate-Reducing Bacteria., in: Sulfate-Reducing Bacteria. pp. 1–32.

Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M., Coleman, M.A., Wood, A.P., Kelly, D.P., 2006. The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium Thiobacillus denitrificans. J. Bacteriol. 188(4), 1473–1488.

Boon, A., 1995. Septicity in sewers: Causes, consequences and containment. Water Sci. Technol. 31(7), 237–253.

Boon, A.G., Vincent, A.J., Boon, K.G., 1998. Avoiding the problems of septic sewage. Water Sci. Technol. 37(1), 223-231.

Brandis-Heep, A., Gebhardt, N.A., Thauer, R.K., Widdel, F., Pfennig, N., 1983. Anaerobic acetate oxidation to CO2 by Desulfobacter postgatei. Arch. Microbiol. 136(3), 222–229.

Bryant, M.P., 1979. Microbial methane production – Theoretical aspects. J. Anim. Sci. 48(1), 193–201.

Bryant, M.P., Boone, D.R., 1988. Isolation and Characterization of Methanobacterium formicicum MF. Int. J. Syst. Bacteriol. *37*(2), 171-171.

Charron, I., Féliers, C., Couvert, a, Laplanche, a, Patria, L., Requieme, B., 2004. Use of hydrogen peroxide in scrubbing towers for odor removal in wastewater treatment plants. Water Sci. Technol. 50(4), 267–74.

Chen, J., Xie, Z., 2013. Removal of H2S in a novel dielectric barrier discharge reactor with photocatalytic electrode and activated carbon fiber. J. Hazard. Mater. 261, 38–43.

Christl, S.U., Murgatroyd, P.R., Gibson, G.R., Cummings, J.H., 1992. Production, metabolism, and excretion of hydrogen in the large intestine. Gastroenterology 102(4), 1269–1277.

Cui, M., Ma, A., Qi, H., Zhuang, X., Zhuang, G., 2015. Anaerobic oxidation of methane: An "active" microbial process. Microbiologyopen 4(1), 1–11.

Cytryn, E., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., Minz, D., 2005. Identification of Bacteria Potentially Responsible for Oxic and Anoxic Sulfide Oxidation in Biofilters of a Recirculating Mariculture System. Appl. Environ. Microbiol. 71(10), 6134–6141.

D.A., D., J.A., F., 1983. The chemistry of reduced sulfur species and their removal from groundwater supplies. J. / Am. Water Work. Assoc. 75(6), 298–309.

Dannenberg, S., Kroder, M., Dilling, W., Cypionka, H., 1992. Oxidation of H2, organic compounds and inorganic sulfur compounds coupled to reduction of O2 or nitrate by sulfate-reducing bacteria. Arch. Microbiol. 158(2), 93–99.

Dilling, W., Cypionka, H., 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiol. Lett. 71(1-2), 123–128.

Dreeszen, P.H., 2003. The key to understanding and controlling bacterial growth in Automated Drinking Water Systems, 2nd ed. Edstrom Industries Incorporated, Wisconsin.

Dridi, B., Henry, M., El Khéchine, A., Raoult, D., Drancourt, M., 2009. High prevalence of Methanobrevibacter smithii and Methanosphaera stadtmanae detected in the human gut using an improved DNA detection protocol. PLoS One 4(9), e7063.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H.J.C.T., van Alen, T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., Op den Camp, H.J.M., Janssen-Megens, E.M., Francoijs, K.-J., Stunnenberg, H., Weissenbach, J., Jetten, M.S.M., Strous, M., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464(7288), 543–548.

Ettwig, K.F., Shima, S., van de Pas-Schoonen, K.T., Kahnt, J., Medema, M.H., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2008. Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. Environ. Microbiol. 10(11), 3164–3173.

Ettwig, K.F., van Alen, T., van de Pas-Schoonen, K.T., Jetten, M.S.M., Strous, M., 2009. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. Appl. Environ. Microbiol. 75(11), 3656–3662.

Firer, D., Friedler, E., Lahav, O., 2008. Control of sulfide in sewer systems by dosage of iron salts: comparison between theoretical and experimental results, and practical implications. Sci. Total Environ. 392(1), 145–156.

Foley, J., Yuan, Z., Lant, P., 2009. Dissolved methane in rising main sewer systems: field measurements and simple model development for estimating greenhouse gas emissions. Water Sci. Technol. 60(11), 2963–2971.

Fricke, W.F., Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., Gottschalk, G., Thauer, R.K., 2006. The genome sequence of Methanosphaera stadtmanae reveals why this human intestinal archaeon is restricted to methanol and H2 for methane formation and ATP synthesis. J. Bacteriol. 188(2), 642–658.

Fukuzaki, S., Nishio, N., Shobayashi, M., Nagai, S., 1990. Inhibition of the fermentation of propionate to methane by hydrogen, acetate, and propionate. Appl. Environ. Microbiol. 56(3), 719–723.

Fuseler, K., Cypionka, H., 1995. Elemental sulfur as an intermediate of sulfide oxidation with oxygen by Desulfobulbus propionicus. Arch. Microbiol. 164(2), 104–109.

Ganigue, R., Gutierrez, O., Rootsey, R., Yuan, Z., 2011. Chemical dosing for sulfide control in Australia: An industry survey. Water Res. 45(19), 6564–6574.

Ganigué, R., Yuan, Z., 2014. Impact of oxygen injection on CH4 and N2O emissions from rising main sewers. J. Environ. Manage. 144, 279–285.

Gibson, G.R., Macfarlane, G.T., Cummings, J.H., 1993a. Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34(4), 437–439.

Gibson, G.R., Macfarlane, S., Macfarlane, G.T., 1993b. Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. FEMS Microbiol. Ecol. 12(2), 117–125.

Greene, E. a, Hubert, C., Nemati, M., Jenneman, G.E., Voordouw, G., 2003. Nitrite reductase activity of sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. Environ. Microbiol. 5(7), 607–617.

Guisasola, A., de Haas, D., Keller, J., Yuan, Z., 2008. Methane formation in sewer systems. Water Res. 42 (6-7), 1421–1430.

Guisasola, A., Sharma, K.R., Keller, J., Yuan, Z., 2009. Development of a model for assessing methane formation in rising main sewers. Water Res. 43(11), 2874–2884.

Gutierrez, O., Jiang, G., Keshab, S., Yuan, Z., 2016. Biofilm Development in Sewer Networks, in: Romani, A.M., Guasch, H., Balaguer, M.D. (Eds.), Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment. Caister Academic Press, Norfolk, UK, pp. 145–164.

Gutierrez, O., Mohanakrishnan, J., Sharma, K.R., Meyer, R.L., Keller, J., Yuan, Z., 2008. Evaluation of oxygen injection as a means of controlling sulfide production in a sewer system. Water Res. 42(17), 4549–4561.

Gutierrez, O., Park, D., Sharma, K.R., Yuan, Z., 2009. Effects of long-term pH elevation on the sulfate-reducing and methanogenic activities of anaerobic sewer biofilms. Water Res. 43(9), 2549–2557.

Gutierrez, O., Sudarjanto, G., Ren, G., Ganigué, R., Jiang, G., Yuan, Z., 2014. Assessment of pH shock as a method for controlling sulfide and methane formation in pressure main sewer systems. Water Res. 48, 569–578.

Gutierrez, O., Sutherland-Stacey, L., Yuan, Z., 2010. Simultaneous online measurement of sulfide and nitrate in sewers for nitrate dosage optimisation. Water Sci. Technol. 61(3), 651–658.

Hao, T., Xiang, P., Mackey, H.R., Chi, K., Lu, H., Chui, H., van Loosdrecht, M.C.M., Chen, G.-H., 2014. A review of biological sulfate conversions in wastewater treatment. Water Res. 65, 1–21.

Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z., Tyson, G.W., 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature 500(7464), 567–570.

Hines, M., Evans, R., Sharak Genthner BR, Willis, S., Friedman, S., Rooney-Varga, J., Devereux, R., 1999. Molecular phylogenetic and biogeochemical studies of sulfate-reducing bacteria in the rhizosphere of spartina alterniflora. Appl. Environ. Microbiol. 65(5), 2209–2216.

Hubert, C., Nemati, M., Jenneman, G., Voordouw, G., 2005. Corrosion risk associated with microbial souring control using nitrate or nitrite. Appl. Microbiol. Biotechnol. 68(2), 272–282.

Hvitved-Jacobsen, T., Vollertsen, J., Nielsen, A.H., 2013. Sewer Processes: Microbial and Chemical Process Engineering of Sewer Networks, 2ond ed. CRC Press, Boca Raton, FL.

IPCC, 2013. Working group I contribuition to the assessment report climate change 2013: The physical science basis: Final Draft Underlying Scientific-Technical Assessment. Cambridge University. Cambridge, U.K. and New York.

Jiang, G., Gutierrez, O., Sharma, K.R., Keller, J., Yuan, Z., 2011a. Optimization of intermittent, simultaneous dosage of nitrite and hydrochloric acid to control sulfide and methane productions in sewers. Water Res. 45(18), 6163–6172.

Jiang, G., Gutierrez, O., Sharma, K.R., Yuan, Z., 2010. Effects of nitrite concentration and exposure time on sulfide and methane production in sewer systems. Water Res. 44(14), 4241–4251.

Jiang, G., Gutierrez, O., Yuan, Z., 2011b. The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res. 45(12), 3735–3743.

Jiang, G., Sharma, K.R., Guisasola, A., Keller, J., Yuan, Z., 2009. Sulfur transformation in rising main sewers receiving nitrate dosage. Water Res. 43(17), 4430–4440.

Jiang, G., Sharma, K.R., Yuan, Z., 2013. Effects of nitrate dosing on methanogenic activity in a sulfide-producing sewer biofilm reactor. Water Res. 47(5), 1783–1792.

Jiang, G., Sun, J., Sharma, K.R., Yuan, Z., 2015. Corrosion and odor management in sewer systems. Curr. Opin. Biotechnol. 33, 192–197.

Jiang, G., Yuan, Z., 2013. Synergistic inactivation of anaerobic wastewater biofilm by free nitrous acid and hydrogen peroxide. J. Hazard. Mater. 250-251, 91–98.

Kaster, K.M., Voordouw, G., 2006. Effect of nitrite on a thermophilic, methanogenic consortium from an oil storage tank. Appl. Microbiol. Biotechnol. 72(6), 1308–1315.

Kisand, V., Wikner, J., 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J. Microbiol. Methods 54(2), 183–191.

Klein, M., Friedrich, M., Roger, A.J., Hugenholtz, P., Fishbain, S., Abicht, H., Blackall, L.L., Stahl, D.A., Wagner, M., 2001. Multiple Lateral Transfers of Dissimilatory Sulfite Reductase Genes between Major Lineages of Sulfate-Reducing Prokaryotes 183(20), 6028–6035.

Klüber, H.D., Conrad, R., Detlef Kluber H.; Conrad, R., 1998. Inhibitory effects of nitrate , nitrite, NO and N2O on methanogenesis by Methanosarcina barkeri and Methanobacterium bryantii. FEMS Microbiol. Ecol. 25(4), 331–339.

Kovacik, W.P., Takai, K., Mormile, M.R., McKinley, J.P., Brockman, F.J., Fredrickson, J.K., Holben, W.E., 2006. Molecular analysis of deep subsurface Cretaceous rock indicates abundant Fe(III)- and S^0-reducing bacteria in a sulfate-rich environment. Environ. Microbiol. 8(1), 141–155.

Kristjansson, J.K., Schönheit, P., Thauer, R.K., 1982. Different Ks values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: An explanation for the apparent inhibition of methanogenesis by sulfate. Arch. Microbiol. 131(3), 278–282.

Lebrero, R., Gondim, A.C., Pérez, R., García-Encina, P.A., Muñoz, R., 2014. Comparative assessment of a biofilter, a biotrickling filter and a hollow fiber membrane bioreactor for odor treatment in wastewater treatment plants. Water Res. 49, 339–350.

Liu, C., Zhao, D., Yan, L., Wang, A., Gu, Y., Lee, D.J., 2015. Elemental sulfur formation and nitrogen removal from wastewaters by autotrophic denitrifiers and anammox bacteria.

Bioresour. Technol. 191, 332–336.

Liu, Y., Ganigué, R., Sharma, K., Yuan, Z., 2016. Event-driven model predictive control of sewage pumping stations for sulfide mitigation in sewer networks. Water Res. 98, 376–383.

Liu, Y., Ni, B.-J., Sharma, K.R., Yuan, Z., 2015. Methane emission from sewers. Sci. Total Environ. 524-525, 40–51.

Liu, Y., Sharma, K.R., Fluggen, M., O'Halloran, K., Murthy, S., Yuan, Z., 2014. Online dissolved methane and total dissolved sulfide measurement in sewers. Water Res. 68, 109–118.

Lovley, D.R., Dwyer, D.F., Klug, M.J., 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl. Environ. Microbiol. 43(6), 1373–1379.

Lovley, D.R., Klug, M.J., 1983. Sulfate Reducers Can Outcompete Methanogens at Freshwater Sulfate Concentrations. Appl. Environ. Microbiol. 45(1), 187–192.

Lovley, D.R., Phillips, E.J.P., 1994. Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. Appl. Environ. Microbiol. 60(7), 2394–2399.

Mackenzie, F. T. (Ed.). (2005). Sediments, diagenesis, and sedimentary rocks: Treatise on Geochemistry (Vol. 7). Elsevier.

Madigan, M.T., Martinko, J.M., Stahl, D.A., Clark, D.P., 2012. Brock biology of microorganisms, 13th ed. Pearson Benjamin-Cummings, San Francisco.

Magingo, F.S.S., Stumm, C.K., 1991. Nitrogen fixation by Methanobacterium formicicum. FEMS Microbiol. Lett. 81(3), 273–277.

McInerney, M.J., Bryant, M.P., Pfennig, N., 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. 122(2), 129–135.

Miller, T.L., Wolin, M.J., 1985. Methanosphaera stadtmaniae gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. Arch. Microbiol. 141(2), 116–122.

Mohanakrishnan, J., Gutierrez, O., Meyer, R.L., Yuan, Z., 2008. Nitrite effectively inhibits sulfide and methane production in a laboratory scale sewer reactor. Water Res. 42(14), 3961–3971.

Mohanakrishnan, J., Gutierrez, O., Sharma, K.R., Guisasola, A., Werner, U., Meyer, R.L., Keller, J., Yuan, Z., 2009a. Impact of nitrate addition on biofilm properties and activities in rising main sewers. Water Res. 43(13), 4225–4237.

Mohanakrishnan, J., Kofoed, M.V.W., Barr, J., Yuan, Z., Schramm, A., Meyer, R.L., 2011. Dynamic microbial response of sulfidogenic wastewater biofilm to nitrate. Appl. Microbiol. Biotechnol. 91(6), 1647–1657.

Mohanakrishnan, J., Sharma, K.R., Meyer, R.L., Hamilton, G., Keller, J., Yuan, Z., 2009b. Variation in biofilm structure and activity along the length of a rising main sewer. Water Environ. Res. 81(8), 800–8.

Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6(6), 441–454.

Nakamura, N., Lin, H.C., McSweeney, C.S., Mackie, R.I., Gaskins, H.R., 2010. Mechanisms of

Microbial Hydrogen Disposal in the Human Colon and Implications for Health and Disease. Annu. Rev. Food Sci. Technol. 1, 363–395.

Nielsen, P.H., 1991. Sulfur sources for hydrogen sulfide production in biofilms from sewer systems. Proc. 15th Bienn. Conf. Int. Assoc. Water Pollut. Res. Control 23(7-9), 1265–1274.

Nilsen, R.K., Beeder, J., Thorstenson, T., Torsvik, T., 1996. Distribution of thermophilic marine sulfate reducers in north sea oil field waters and oil reservoirs. Appl. Environ. Microbiol. 62(5), 1793–1798.

Olson, D., Rajagopalan, S., Corsi, R.L., 1997. Ventilation of industrial process drains: Mechanisms and effects on VOC emissions. J. Environ. Eng. 123(9), 939–947.

Omil, F., Lens, P., Visser, A., Hulshoff Pol, L.W., Lettinga, G., 1998. Long-term competition between sulfate reducing and methanogenic bacteria in UASB reactors treating volatile fatty acids. Biotechnol. Bioeng. 57(6), 676–685.

Pikaar, I., Rozendal, R.A., Yuan, Z., Keller, J., Rabaey, K., 2011. Electrochemical sulfide removal from synthetic and real domestic wastewater at high current densities. Water Res. 45, 2281–2289.

Plugge, C.M., Zhang, W., Scholten, J.C.M., Stams, A.J.M., 2011. Metabolic Flexibility of Sulfate-Reducing Bacteria. Front. Microbiol. 2, 1–8.

Raghoebarsing, A. a, Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., Schouten, S., Damsté, J.S.S., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2006. A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440(7086), 918–921.

Rivera-Garza, M., Olguín, M.T., García-Sosa, I., Alcántara, D., Rodríguez-Fuentes, G., 2000. Silver supported on natural Mexican zeolite as an antibacterial material. Microporous Mesoporous Mater. 39, 431–444.

Samuel, B.S., Hansen, E.E., Manchester, J.K., Coutinho, P.M., Henrissat, B., Fulton, R., Latreille, P., Kim, K., Wilson, R.K., Gordon, J.I., 2007. Genomic and metabolic adaptations of Methanobrevibacter smithii to the human gut. Proc. Natl. Acad. Sci. U. S. A. 104(25), 10643– 10648.

Sass, H., Wieringa, E., Cypionka, H., Babenzien, H.D., Overmann, J., 1998. High genetic and physiological diversity of sulfate-reducing bacteria isolated from an oligotrophic lake sediment. Arch. Microbiol. 170(4), 243–251.

Schedel, M., Trüper, H.G., 1980. Anaerobic oxidation of thiosulfate and elemental sulfur in Thiobacillus denitrificans. Arch. Microbiol. 124(2-3), 205–210.

Spencer, A.U., Noland, S.S., Gottlieb, L.J., 2006. Bathtub fire: An extraordinary burn injury. J. Burn Care Res. 27(1), 97–98.

Sudarjanto, G., Sharma, K.R., Gutierrez, O., Yuan, Z., 2011. A laboratory assessment of the impact of brewery wastewater discharge on sulfide and methane production in a sewer. Water Sci. Technol. 64(8), 1614–1619.

Sun, J., Hu, S., Sharma, K.R., Ni, B.-J., Yuan, Z., 2014. Stratified Microbial Structure and Activity in Sulfide- and Methane- Producing Anaerobic Sewer Biofilms. Appl. Environ. Microbiol. 80(22), 7042–7052.

Takai, K., Suzuki, M., Nakagawa, S., Miyazaki, M., Suzuki, Y., Inagaki, F., Horikoshi, K., 2006. Sulfurimonas paralvinellae sp. nov., a novel mesophilic, hydrogen- and sulfur-oxidizing chemolithoautotroph within the Epsilonproteo-bacteria isolated from a deep-sea hydrothermal vent polychaete nest, reclassification of Thiomicrospira denitrificans as S. Int. J. Syst. Evol. Microbiol. 56(8), 1725–1733.

Tasaki, M., Kamagata, Y., Nakamura, K., Okamura, K., Minami, K., 1993. Acetogenesis from pyruvate by Desulfotomaculum thermobenzoicum and differences in pyruvate metabolism among three sulfate-reducing bacteria in the absence of sulfate. FEMS Microbiol. Lett. 106(3), 259–263.

Thauer, R.K., Kaster, A.-K., Seedorf, H., Buckel, W., Hedderich, R., 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6(8), 579–591.

Tomar, M., Abdullah, T.H.., 1994. Evaluation of chemicals to control the generation of malodorous hydrogen sulfide in waste water. Water Res. 28(12), 2545–2552.

Wagner, M., Roger, a J., Flax, J.L., Brusseau, G. a, Stahl, D. a, 1998. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J. Bacteriol. 180(11), 2975–2982.

Wei, Z.S., Li, H.Q., He, J.C., Ye, Q.H., Huang, Q.R., Luo, Y.W., 2013. Removal of dimethyl sulfide by the combination of non-thermal plasma and biological process. Bioresour. Technol. 146, 451–456.

WERF, 2007. Minimization of Odors and Corrosion in Collection Systems, Phase I. Water Environment Research Foundation WERF, IWA, Alexandria, VA 22314-1177.

Widdel, F., Pfennig, N., 1982. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids II. Incomplete oxidation of propionate by Desulfobulbus propionicus gen. nov., sp. nov. Arch. Microbiol. 131(4), 360–365.Williams, R.T., Crawford, R.L., 1985. Methanogenic bacteria, including an Acid-tolerant strain, from peatlands. Appl. Environ. Microbiol. 50(6), 1542–1544.

Worakit, S., Boone, D.R., Mah, R. a., Abdel-Samie, M.-E., El-Halwagi, M.M., 1986. Methanobacterium alcaliphilum sp. nov., an H2-Utilizing Methanogen That Grows at High pH Values. Int. J. Syst. Bacteriol. 36(3), 380–382.

Yamanaka, T., Aso, I., Togashi, S., Tanigawa, M., Shoji, K., Watanabe, T., Watanabe, N., Maki, K., Suzuki, H., 2002. Corrosion by bacteria of concrete in sewerage systems and inhibitory effects of formates on their growth. Water Res. 36, 2636–2642.

Zhang, L., De Schryver, P., De Gusseme, B., De Muynck, W., Boon, N., Verstraete, W., 2008. Chemical and biological technologies for hydrogen sulfide emission control in sewer systems: a review. Water Res. 42(1), 1–12.

Zhang, L., Keller, J., Yuan, Z., 2010. Ferrous salt demand for sulfide control in rising main sewers: Tests on a laboratory-scale sewer system. J. Environ. Eng. 136(10), 1180–1187.

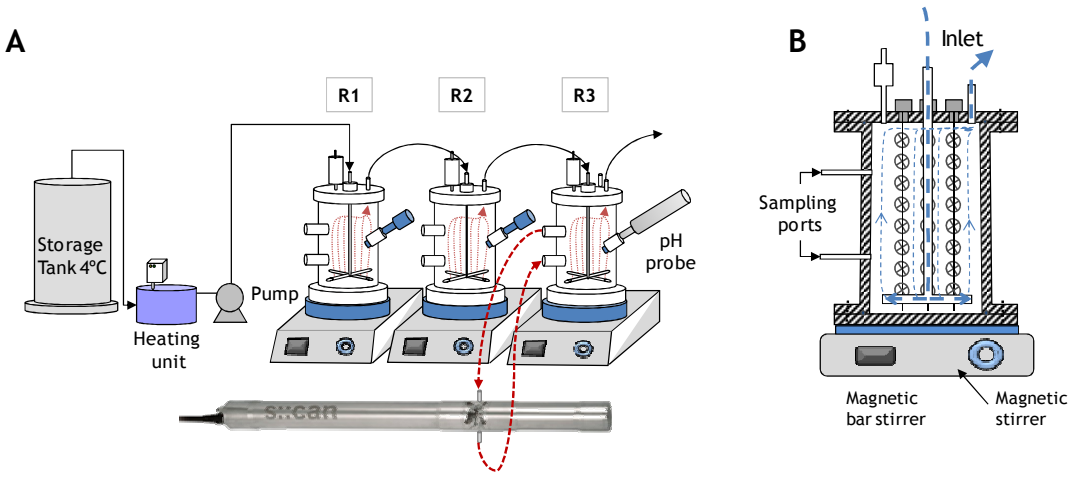
Zhang, L., Keller, J., Yuan, Z., 2009. Inhibition of sulfate-reducing and methanogenic activities of anaerobic sewer biofilms by ferric iron dosing. Water Res. 43(17), 4123–4132.

Zhu, B., van Dijk, G., Fritz, C., Smolders, A.J.P., Pol, A., Jetten, M.S.M., Ettwig, K.F., 2012. Anaerobic oxidization of methane in a minerotrophic peatland: enrichment of nitrite-dependent methane-oxidizing bacteria. Appl. Environ. Microbiol. 78(24), 8657–8665.

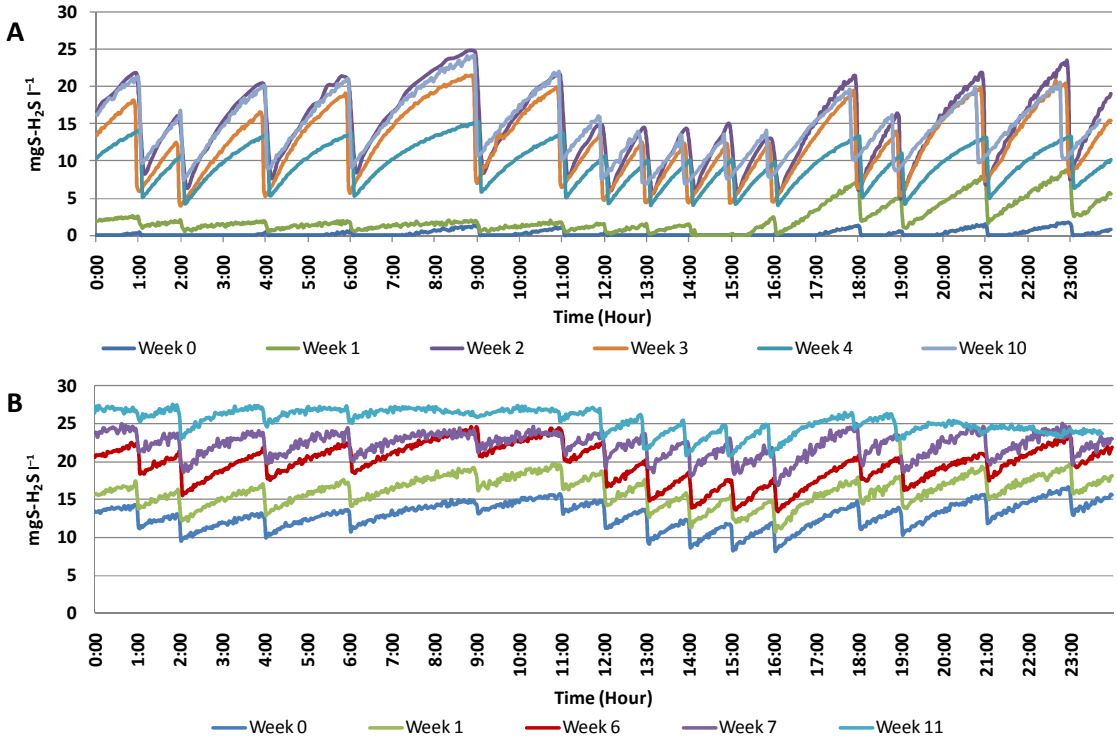
ANNEX

Supplementary material

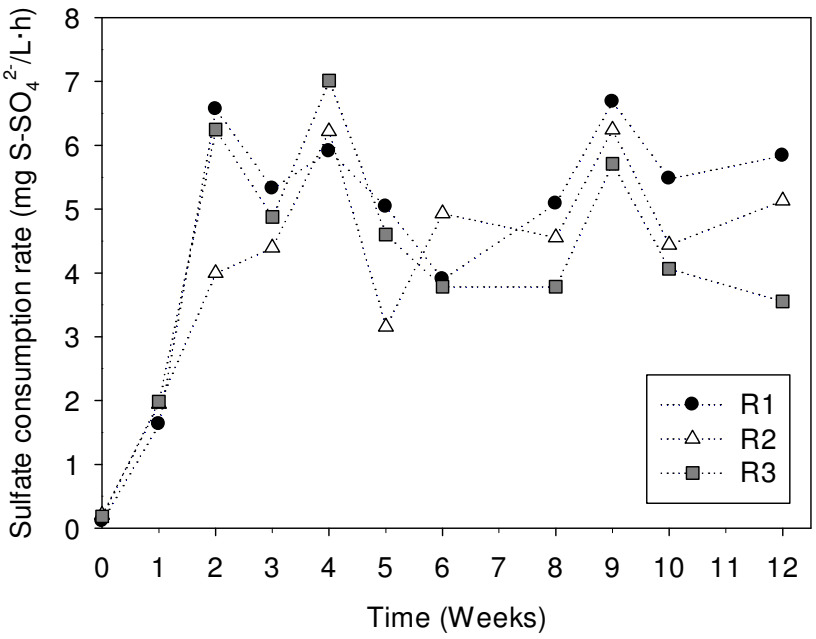
Chapter 3



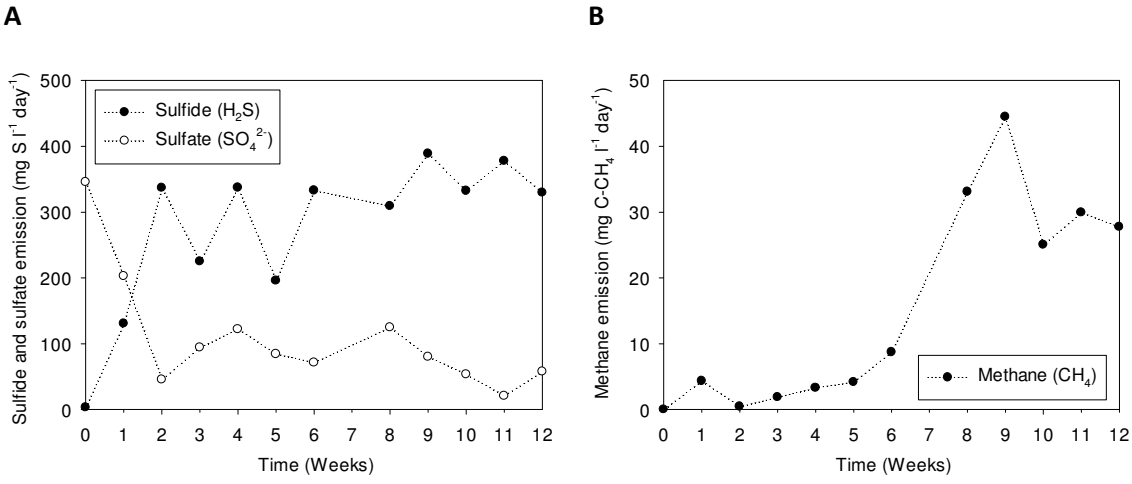
Supplementary Figure S1. (A) Schematic representation of the laboratory scale sewer system used in this study. (B) Sectional view of the reactors of the laboratory scale sewer system.



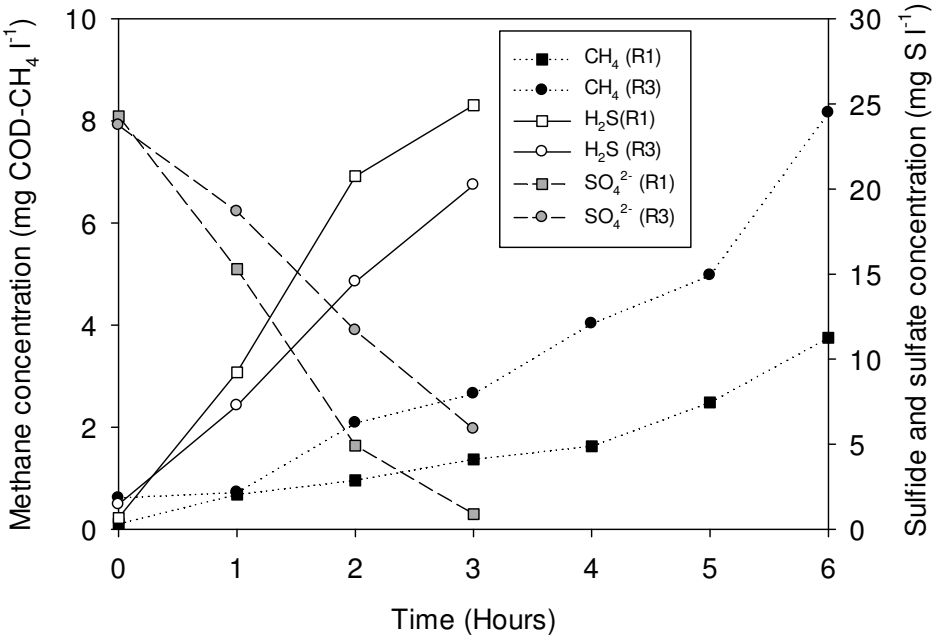
Supplementary Figure S2. Daily profiles of sulfide concentration in (A) R1 and (B) R3 during the first weeks of biofilm growth.



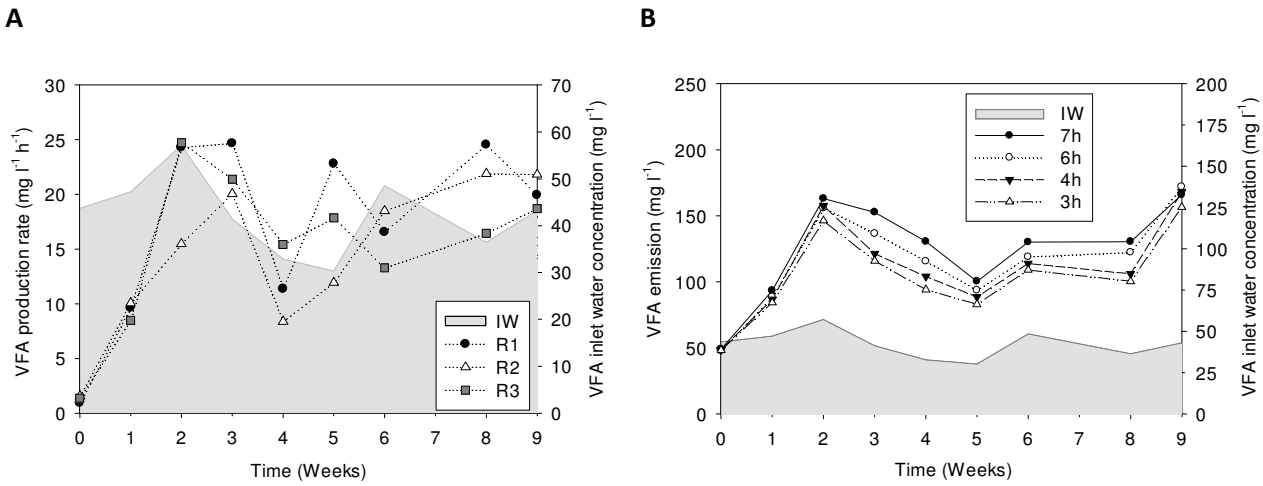
Supplementary Figure S3. Sulfate reduction rates determined in batch tests carried out in reactors R1, R2 and R3



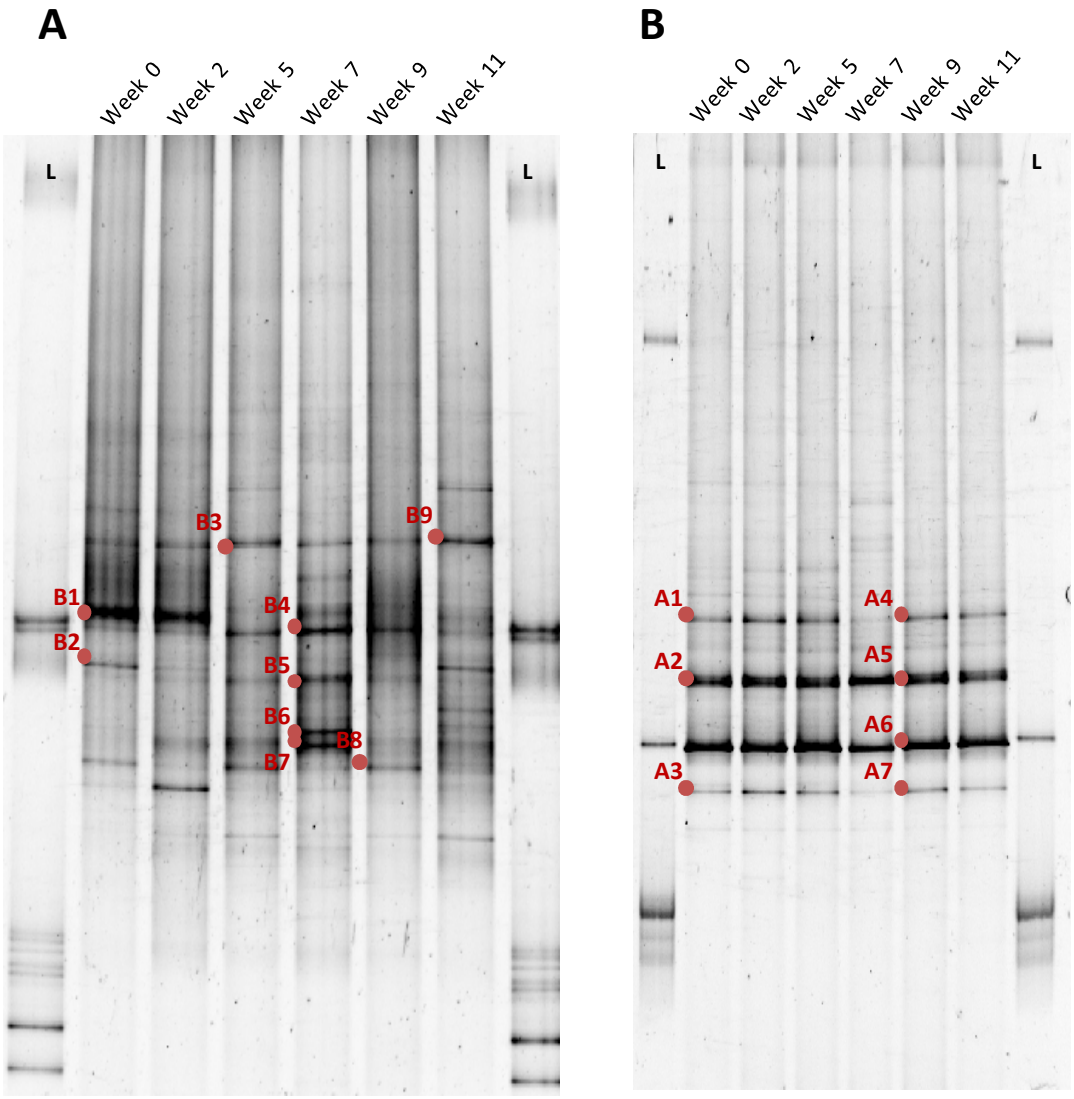
Supplementary Figure S4. Concentrations of (A) sulfide and sulfate and (B) methane in the system effluent during the study period (12 weeks).



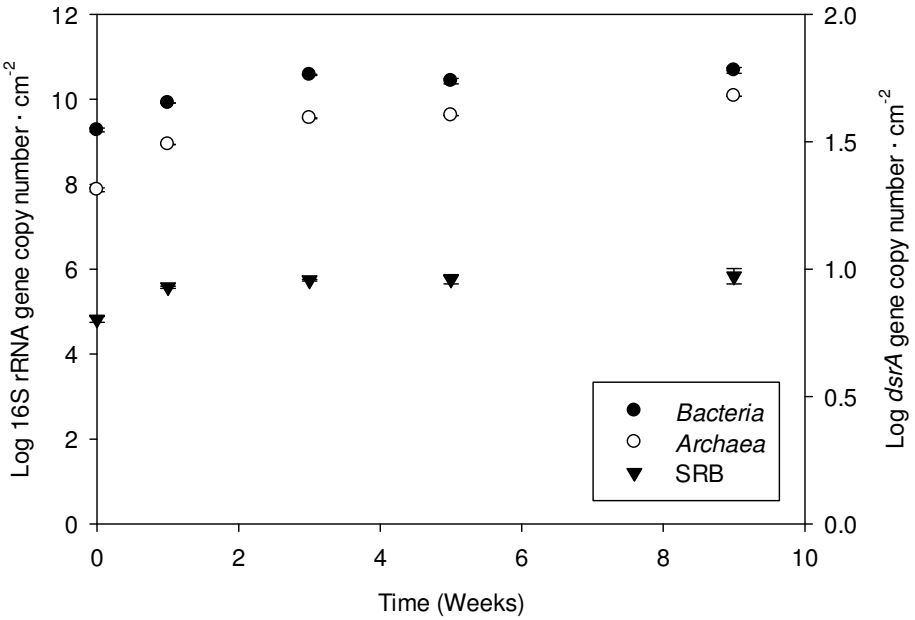
Supplementary Figure S5. Sulfide, sulfate and methane concentrations measured in R1 and R3 during 6-hour batch test experiment carried out on week 14.



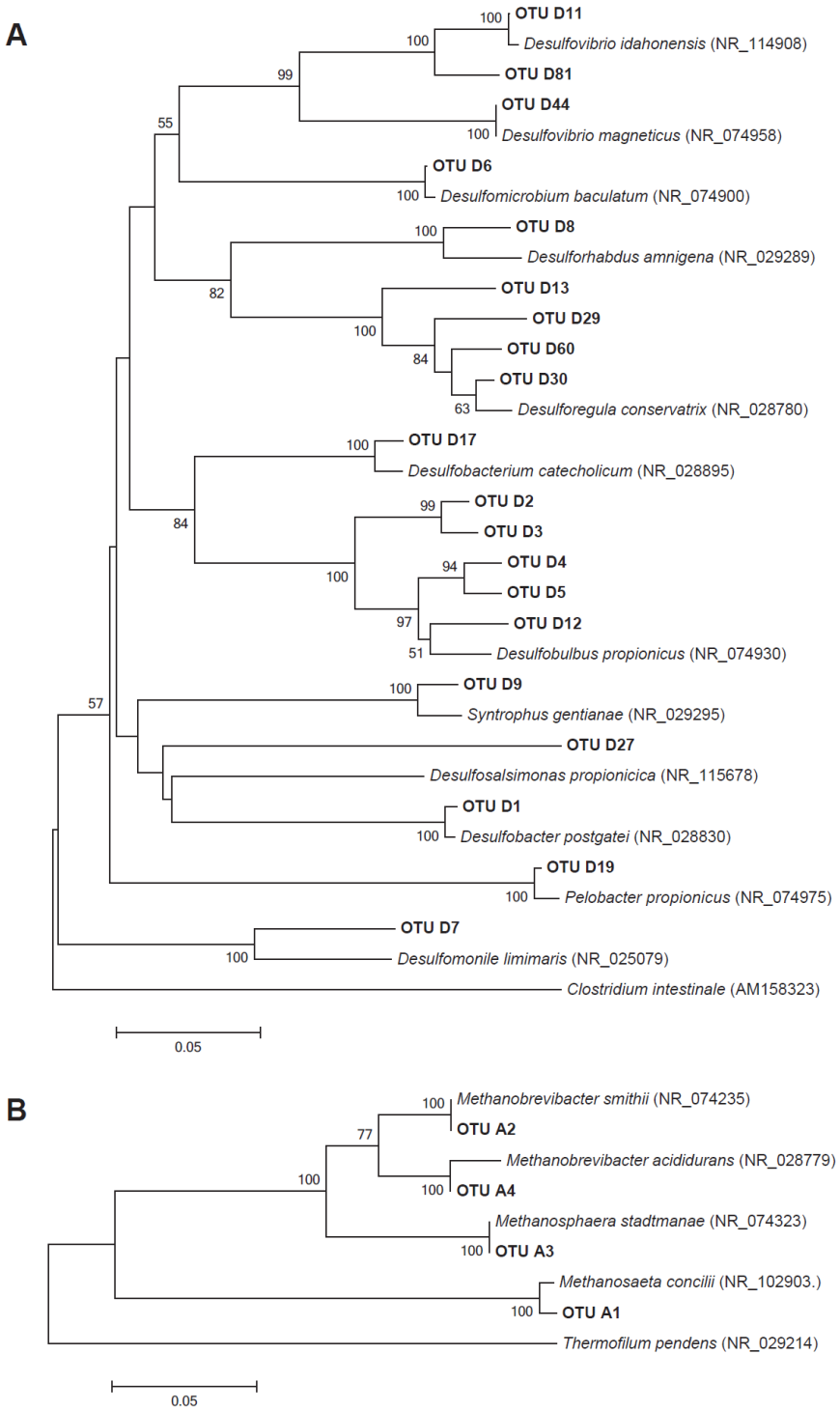
Supplementary Figure S6. (A) Volatile Fatty Acids (VFA) production rates determined in the batch tests on reactors R1, R2 and R3 and in inlet wastewater (IW). (B) VFA concentration in the system effluent and in inlet wastewater at different HRT.



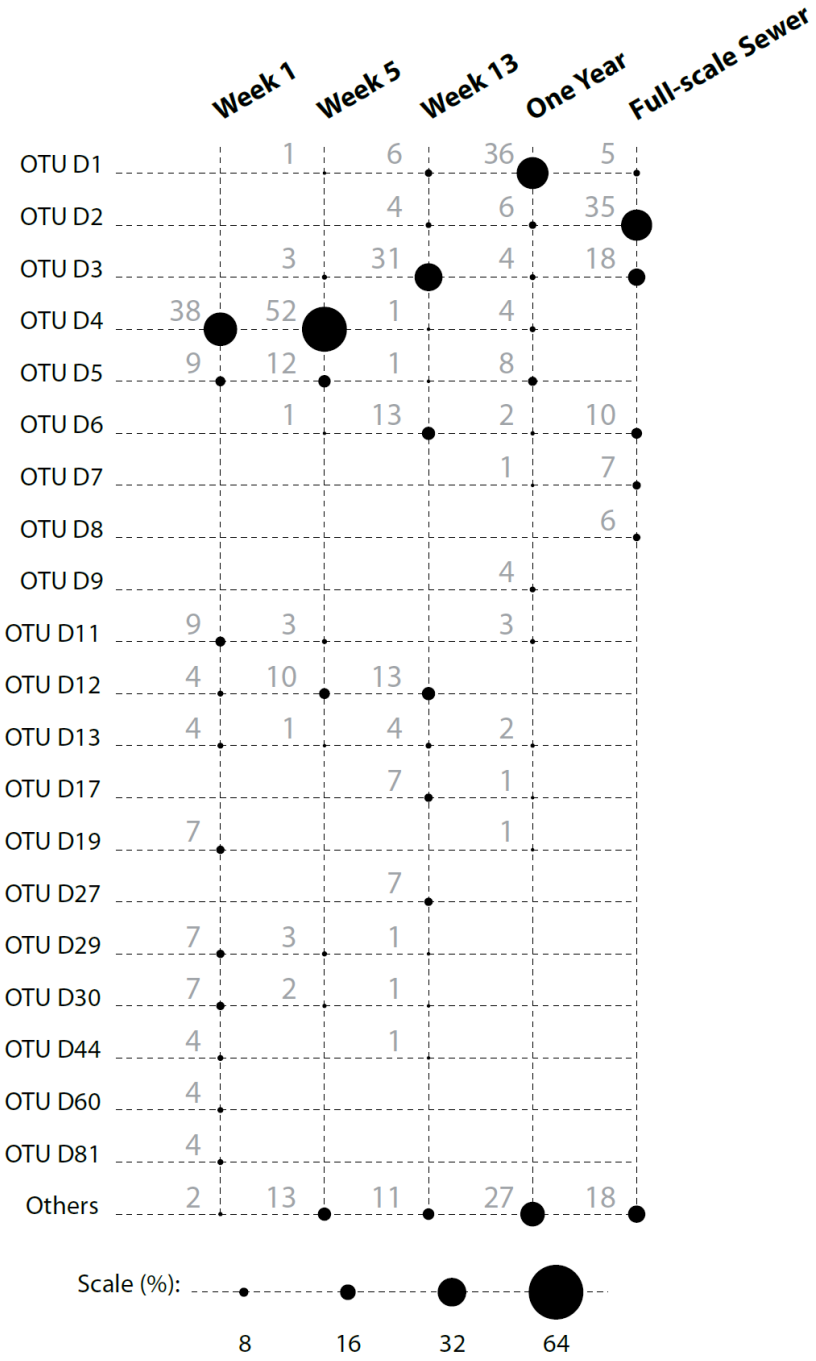
Supplementary Figure S7. Negative image of DGGE gels of (A) bacterial and (B) archaeal 16S rRNA gene fingerprinting of wastewater samples at different time intervals (weeks) during the study period. Representative bands of each lane were sequenced and used for phylogenetic analysis. Percentage identities of each sequence to their first BLAST hit against a reference sequence database are compiled in Table S4.



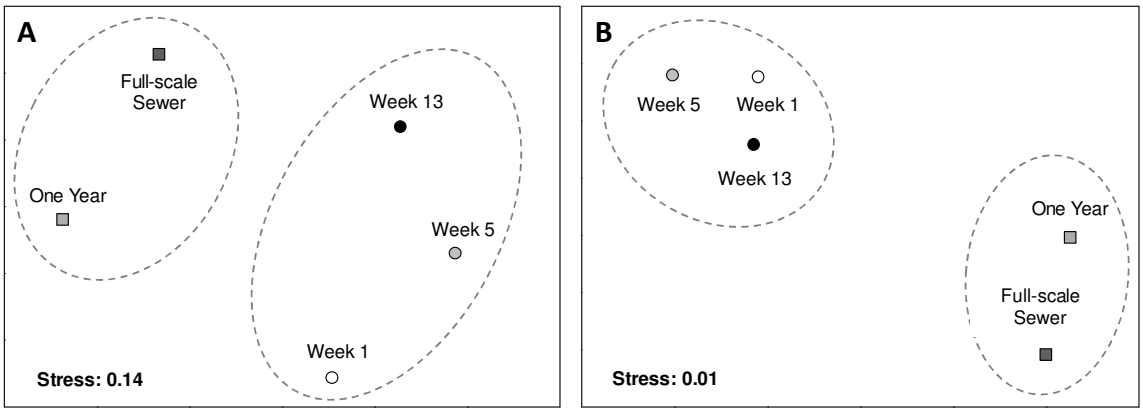
Supplementary Figure S8. Time-course of 16S rRNA gene copy numbers of target groups (*Bacteria*, *Archaea* and SRB) and *dsrA* gene copy numbers in R1 during the first 10 weeks of biofilm development. Symbols represent the mean of duplicate quantifications and bars represent the standard error of the mean.



Supplementary Figure S9. Neighbor-Joining phylogenetic trees of (A) bacterial and (B) archaeal 16S rRNA gene fragments of representative sequences of most abundant OTUs identified in biofilm samples. NJ trees were constructed using MEGA software (1). Bootstrap values greater than 50% (1,000 replicates) are indicated at the nodes.



Supplementary Figure S10. Bubble plot showing the relative abundance of OTUs affiliated to *Deltaproteobacteria* across samples. Data values are proportional to radius and plotted in a logarithmic scale as indicated below the graph. The relative abundance was calculated as % of deltaproteobacterial sequences as indicated (grey figures next to the corresponding bubble).



Supplementary Figure S11. Non-metric multidimensional scaling (NMDS) of (A) bacterial and (B) archaeal biofilm communities in different samples based on the weighted UniFrac distances.

Supplementary Table S1. Information of primers used in this study.

Technique	Target	Primer name	Sequence (5'-3')	Ref.
PCR	Universal *Bacteria*	357F*	CCTACGGGAGGCAGCAG	(2)
		907R	CCGTCAATTCMTTTGAGTTT	
PCR	Universal *Archaea*	109F	ACTGCTCAGTAACACGT	(3)
		515R*	ATCGTATTACCGCGGCTGCTGGCA	
qPCR	Universal *Bacteria*	1048F	GTGSTGCAYGGYTGTCGTCA	(4)
		1194R	ACGTCRTCCMCACCTTCCTC	
qPCR	Universal *Archaea*	806F	ATTAGATACCCSBGTAGTCC	(5)
		915R	GTGCTCCCCCGCCAATTCCT	(6)
qPCR	*dsrA* gene	DSR1F	ACBCAYTGGAARCACG	(7)
		RH3-dsr-R	gGTGGAGCCGTGCATGTT	

* A GC-rich clam was added to the 5' end of the primers used in amplification reactions for DGGE fingerprinting analyses (8).

Supplementary Table S2. PCR conditions used for bacterial and archaeal 16S rRNA gene amplification.

***Bacteria*: PCR Conditions**				
		Temperature	Time	Cycles
Initial denaturation		94	4'	1
First step	Denaturation	94	30''	10
	Annealing	61	45''	
	Elongation	72	60''	
Second step	Denaturation	94	30''	25
	Annealing	56	45''	
	Elongation	72	60''	
Final elongation		72	15'	1
***Archaea*: PCR Conditions**				
		Temperature	Time	Cycles
Initial denaturation		94	5'	1
Denaturation		94	30''	35
Annealing		56	40''	
Elongation		72	60''	
Final elongation		72	5	1

Supplementary Table S3. Conditions used for bacterial and archaeal 16S rRNA and *dsrA* genes quantification by qPCR.

Target Primers	Primers	Cycles	qPCR program		R2	Efficiency (%)
Universal *Bacteria* 16S rRNA	1048F / 1194R	35	3'	95ºC	0.99	96.3
			15''	95ºC		
			60'	60ºC		
Universal *Archaea* 16S rRNA	806F / 915R	40	3'	95ºC	0.97	95.3
			20''	95ºC		
			60'	60ºC		
dsrA gene	DSR1F / RH3-dsr-R	40	3'	95ºC	0.99	88.0
			15''	95ºC		
			60'	60ºC		

Supplementary Table S4. First BLAST hit against the reference database of 16S rRNA bacterial and archaeal gene sequences and % identity for bands excised from DGGE fingerprints (see Fig. S6 for band coding).

Bacteria			
OTU ID	**Cultured representative**	**Accession number**	**% identity**
B1	*Acinetobacter johnsonii* strain ATCC 17909	NR_117624	100
B2	*Comamonas denitrificans* strain 123	NR_025080	99
B3	*Faecalibacterium prausnitzii* strain ATCC 27768	NR_028961	97
B4	*Gemmiger formicilis* strain X2-56	NR_104846	97
B5	*Romboutsia ilealis* strain CRIB	NR_125597	98
B6	*Clostridium sticklandii* strain DSM 519	NR_102880	99
B7	*Proteocatella sphenisci* strain PPP2	NR_041885	99
B8	*Trichococcus pasteurii* strain KoTa2	NR_036793	97
B9	*Faecalibacterium prausnitzii* strain ATCC 27768	NR_028961	98
Archaea			
OTU ID	**First Blast match**	**Accession number**	**% identity**
A1	*Methanobrevibacter smithii* strain PS	NR_074235	100
A2	*Methanosphaera stadtmanae* strain DSM 3091	NR_074323	100
A3	*Methanobrevibacter smithii* strain PS	NR_074235	99
A4	*Methanobrevibacter smithii* strain PS	NR_074235	100
A5	*Methanosphaera stadtmanae* strain DSM 3091	NR_074323	100
A6	*Methanobrevibacter smithii* strain PS	NR_074235	100
A7	*Methanobrevibacter smithii* strain PS	NR_074235	99

Supplementary Table S5. Number of bacterial and archaeal OTUs identified in biofilm samples and number of sequences affiliated into each OTU across samples.

Bacteria					
Group	**Week 1**	**Week 5**	**Week 13**	**One Year**	**Full-scale Sewer**
Number of OTUs	164	300	282	718	357
			Number of sequences		
OTU B1	0	21	12	1460	1017
OTU B2	0	10	21	41	1061
OTU B3	257	167	53	477	75
OTU B4	0	0	0	182	701
OTU B5	0	1	62	101	450
OTU B6	0	1	8	484	40
OTU B7	8	35	102	317	62
OTU B8	100	314	71	21	0
OTU B9	0	0	0	1	499
OTU B10	81	72	30	254	62
OTU B12	94	145	88	22	88
OTU B13	0	0	0	50	376
OTU B14	0	0	0	0	408
OTU B16	36	154	111	83	2
OTU B18	0	0	0	1	357
OTU B20	207	18	3	27	0
OTU B23	3	117	99	6	0
OTU B25	13	20	140	13	1
OTU B27	1	182	0	0	0
OTU B49	0	3	84	3	11
Other	714	1645	1451	4719	3752
Total	1514	2905	2335	8262	8962
Archaea					
Group	**Week 1**	**Week 5**	**Week 13**	**One Year**	**Full-scale Sewer**
Number of OTUs	44	39	27	30	71
			Number of sequences		
OTU A1	40	358	53	24129	18185
OTU A2	4439	9245	4838	0	30
OTU A3	1425	1001	1574	3	15
OTU A4	180	597	558	0	810
Other	249	266	386	726	1303
Total	6333	11467	7409	24858	20343

Supplementary Table S6. First BLAST hit and % sequence identity for the OTUs affiliated to *Deltaproteobacteria* in sewer biofilm samples.

Deltaproteobacteria			
OTU ID	**Cultured representative**	**Accession number**	**% identity**
D1	*Desulfobacter postgatei* strain 2ac9	NR_028830	99
D2	*Desulfobulbus propionicus* strain DSM 2032	NR_074930	93
D3	*Desulfobulbus propionicus* strain DSM 2032	NR_074930	94
D4	*Desulfobulbus propionicus* strain DSM 2032	NR_074930	96
D5	*Desulfobulbus propionicus* strain DSM 2032	NR_074930	97
D6	*Desulfomicrobium baculatum* strain DSM 4028	NR_074900	99
D7	*Desulfomonile limimaris* strain DCB-M	NR_025079	93
D8	*Desulforhabdus amnigena* strain ASRB1	NR_029289	95
D9	*Syntrophus gentianae* strain HQgoe1	NR_029295	97
D11	*Desulfovibrio idahonensis* strain CY1	NR_114908	99
D12	*Desulfobulbus propionicus* strain DSM 2032	NR_074930	97
D13	*Desulforegula conservatrix* strain Mb1Pa	NR_028780	94
D17	*Desulfobacterium catecholicum* strain NZva20	NR_028895	98
D19	*Pelobacter propionicus* strain DSM 2379	NR_074975	99
D27	*Desulfosalsimonas propionicica* strain PropA	NR_115678	84
D29	*Desulforegula conservatrix* strain Mb1Pa	NR_028780	97
D30	*Desulforegula conservatrix* strain Mb1Pa	NR_028780	99
D44	*Desulfovibrio magneticus* strain RS-1	NR_074958	100
D60	*Desulforegula conservatrix* strain Mb1Pa	NR_028780	97
D81	*Desulfovibrio idahonensis* strain CY1	NR_114908	93

Supplementary Table S7. Richness and diversity estimators of bacterial and archaeal biofilm communities at different time intervals along the experimental period. Chao1 and Shannon indices for biofilm communities in the real sewer are shown to allow comparison.

Domain	**Index**	**Week 1**	**Week 5**	**Week 13**	**One Year**	**Full-scale Sewer**
Bacteria	Chao1	202	365	420	631	313
	Shannon	3.7	4.2	4.4	4.3	3.7
Archaea	Chao1	72	45	31	28	78
	Shannon	1.0	0.8	1.1	0.2	0.5

References in supplementary tables and figures

1. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S**. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**:2731–2739.

2. **Muyzer G, Ramsing NB**. 1995. Molecular methods to study the organization of microbial communities. Water Sci Technol **32**:1–9.

3. **Lane DJ**. 1991. 16S/23S rRNA sequencing, p. 115–175. *In* Stackebrandt, E., Goodfellow, M (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

4. **Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, Arai H, Tanimoto I, Nishimura F, Takashiba S**. 2003. Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans , Porphyromonas gingivalis , Prevotella intermedia , tetQ gene and total bacteria. FEMS Immunol. Med. Microbiol. **39**:81–86.

5. **Takai K, Horikoshi K**. 2000. Rapid Detection and Quantification of Members of the Archaeal Community by Quantitative PCR Using Fluorogenic Probes Rapid Detection and Quantification of Members of the Archaeal Community by Quantitative PCR Using Fluorogenic Probes. Appl. Environ. Microbiol. **66**:5066–5072.

6. **Stahl D, Amman**. 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

7. **Ben-Dov E, Brenner A, Kushmaro A**. 2007. Quantification of sulfate-reducing bacteria in industrial wastewater, by real-time polymerase chain reaction (PCR) using dsrA and apsA genes. Microb. Ecol. **54**:439–451.

8. **Muyzer G, de Waal EC, Uitterlinden AG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol **59**:695–700.

Supplementary material

Chapter 4

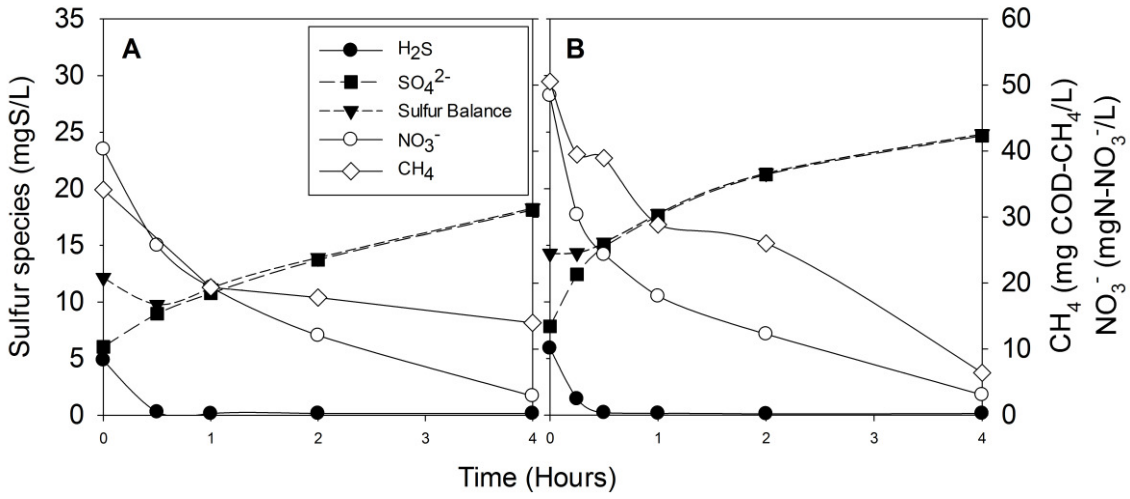


Figure S1. Profiles of sulfide, sulfate, sulfur balance, nitrate and methane concentrations measured during Batch tests carried out throughout Phase 2 (Days 2 and 22).

Table S1. Number for the assigned bacterial OTUs (OUT-ID), relative abundances in each sample (Phase 1 and Phase 2 (Day 37) %), the closest cultured representative hit after BLAST search (Blast match excluding uncultured/environmental sample sequences), percentage of similarity (% Sim.) and accession number (Acc). Taxonomy affiliation (Class level); OTU 1, 7, 10, 11, 16, 30, 32, 35: *Betaproteobacteria* ; OTU 2: *Synergistia* ; OTU 6: *Clostridia* ; OTU 23: *Alphaproteobacteria* ; OTU 28, 29: *Gammaproteobacteria*.

OTU-ID	Phase 1 (%)	Phase 2 (%)	Cultured representative	Sim. (%)	Acc.	Main features
1	0.7	31.4	*Simplicispira limi* strain EMB325 *Simplicispira metamorpha* strain DSM 1837	100 100	NR_043773 NR_044941	Facultative anaerobes, chemoorganoheterotrophs and denitrifiers (Grabovich et al., 2006)
2	8.2	2.3	*Aminobacterium colombiense* strain DSM 12261	88	NR_074624	Relative abundance was reduced when nitrate was added
6	3.7	1.8	*Clostridium bartlettii* DSM 16795	94	NR_027573	Relative abundance was reduced when nitrate was added
7	0.2	6.4	*Comamonas denitrificans* strain 123	99	NR_025080	Facultative anaerobe and denitrifier (Gumaelius et al., 2001)
10	0.2	4.7	*Azonexus hydrophilus* DSM 23864 strain d8-1	96	NR_044125	Grows under anaerobic conditions (not as well as in aerobic conditions). Positive for nitrate reduction and malate assimilation. This strain also harboured nifH gene but nitrogen fixation activity was not detected (Chou et al., 2008)
11	1.1	3.2	*Thauera aminoaromatica* strain S2 *Thauera aromatica* strain DSM 6984	99 99	NR_027211 NR_026153	*T. aminoaromatica* strain S2: grows with aminoaromatic compounds and nitrate and nitrite are used as electron acceptors under anoxic conditions. (Mechichi et al., 2002) *T. aromatica strain* DSM 6984: grows aerobically or anaerobically with nitrate and many aromatic compounds are used as a carbon source under denitrifying conditions, producing mainly N_2O. (Anders et al., 1995)
16	0.5	3.1	*Dechloromonas agitata* strain CKB	100	NR_024884	Facultative anaerobe that can oxidixe sulfide. Cannot growth by nitrate reduction according to Bardiya and Bae (2011) but nitrate coreduction by perchlorate reductase was also suggested (Chaudhuri et al., 2002)
23	0.1	2.5	*Gemmobacter caeni* strain DCA-1	98	NR_108321	Previously described as *Catellibacterium caeni,* this specie was defined as strictly aerobic bacterium. (Zheng et al., 2011)
28	0.0	1.8	*Pseudohaliea rubra* strain CM41_15a *Methylomicrobium album* BG8 *Methylobacter whittenburyi* strain 1521	93 92 92	NR_044426 NR_029244 NR_029242	*M. album*: reduces nitrate to nitrite (Bowman et al., 1993) and also contains genes that encoding enzymes involved in methane oxidation (*pmoCAB*) and in denitrification (*nirS* and *norCB*) (Kits and Kalyuzhnaya, 2013) *M. whittenburyi*: utilizes methane and methanol as a carbon sources (Bowman et al., 1993)
29	0.0	1.8	*Burkholderia thailandensis* strain E264	91	NR_074312	
30	0.0	1.8	*Simplicispira psychrophila* strain CA 1	97	NR_028712	*Simplicispira*: Genus capable of denitrification
32	0.1	1.6	*Alicycliphilus denitrificans* strain BC *Alicycliphilus denitrificans* K601	99 99	NR_074585 NR_025510	This specie grows under aerobic or anoxic conditions and electron acceptors are nitrate, nitrite and oxigen (Mechichi, 2003)
35	0.0	1.5	*Thauera* sp. MZ1T strain MZ1 *Thauera phenylacetica* strain B4P	98 98	NR_074711 NR_027224	*T. phenylacetica*: Nitrate, nitrite and oxygen are used as electron acceptor; nitrogen is not fixed (Mechichi et al., 2002)
Other OTUs	85.4	36.2	-	-	-	-

Table S2. Assigned bacterial OTUs (OTU ID), relative abundance in each sample (Phase 1 and Phase 2 (Day 37) %), first BLAST hit (blast match including uncultured/environmental sample sequences), percentage of similarity (%) and accession number (Acc). Taxonomy affiliation (Class level); OTU-1, -7, -10, -11, -16, -30, -32, -35: *Betaproteobacteria*; OTU-2: *Synergistia*; OTU-6: Clostridia; OTU 23: *Alphaproteobacteria*; OTU-28, -29: *Gammaproteobacteria*.

OTU-ID	Before (%)	Day 37 (%)	First Blast Hit	Sim. (%)	Acc.
1	0.7	31.4	Uncultured bacterium clone D52	100	KF756904
2	8.2	2.3	Uncultured *Aminanaerobia* bacterium clone QEDN9DC12	100	CU925673
6	3.7	1.8	Uncultured bacterium clone PCS439_32	100	JX851723
7	0.2	6.4	Uncultured *Comamona*s sp. clone Inoculum-OTU4	100	KF956396
10	0.2	4.7	Uncultured *Azonexus* sp. clone SD2	100	JN860153
11	1.1	3.2	Uncultured bacteria clone 30e	100	HE650068
16	0.5	3.1	Uncultured *Dechloromonas* sp. clone NS-OTU33	100	KF956462
23	0.1	2.5	Uncultured bacterium clone D78	100	KC683213
28	0.0	1.8	Uncultured *Desulfobacteraceae* bacterium clone S24 Uncultured gamma proteobacterium clone MB-51	99 98	KC769119 DQ507152
29	0.0	1.8	Uncultured bacterium clone HKT_30B9	100	JX170178
30	0.0	1.8	Uncultured bacterium clone EMIRGE_OTU_s3t2d_1519	100	JX222725
32	0.1	1.6	Uncultured bacterium clone: SludgeB_bottom_93	100	AB516154
35	0.0	1.5	Uncultured bacterium clone W4-74	100	KF802937
Other OTUs	85.2	36.1	-	-	-

Table S3. Number of sequences and relative abundance (%) of sequences affiliated to different bacterial and archaeal classes in Phase 1 and Phase 2.

	Phase 1		Phase 2	
Bacterial classes	**Number of Sequences**	**Relative Abundance (%)**	**Number of Sequences**	**Relative Abundance (%)**
Acidobacteria_Gp7	17	0.3	6	0.1
Holophagae	3	0.0	3	0.1
Actinobacteria	59	0.9	2	0.0
Armatimonadetes_gp2	5	0.1	0	0.0
Bacteroidia	272	4.1	35	0.9
Sphingobacteria	27	0.4	20	0.5
Flavobacteria	5	0.1	10	0.2
Chlorobia	4	0.1	2	0.0
Anaerolineae	36	0.5	10	0.2
Fusobacteria	6	0.1	4	0.1
Lentisphaeria	1	0.0	0	0.0
Alphaproteobacteria	85	1.3	309	7.7
Betaproteobacteria	264	4.0	2405	59.6
Deltaproteobacteria	556	8.4	53	1.3
Epsilonproteobacteria	16	0.2	53	1.3
Gammaproteobacteria	11	0.2	222	5.5
Spirochaetes	0	0.0	2	0.0
Synergistia	2473	37.4	320	7.9
Subdivision3	1	0.0	0	0.0
Verrucomicrobiae	1	0.0	0	0.0
BRC1_genera_incertae_sedis	7	0.1	4	0.1
Bacilli	2	0.0	61	1.5
Clostridia	1171	17.7	247	6.1
Erysipelotrichia	104	1.6	24	0.6
Negativicutes	103	1.6	3	0.1
Unclassified	1383	20.9	239.0	5.9
Total	6612	100	4034	100

	Phase 1		Phase 2	
Archaeal classes	**Number of Sequences**	**Relative Abundance (%)**	**Number of Sequences**	**Relative Abundance (%)**
Methanobacteria	847	6.7	293	1.2
Methanomicrobia	11795	93.1	24999	98.8
Thermoplasmata	21	0.2	0	0.0
Total	12663	100	25292	100.0

References

Anders, H., Kaetzke, A., Kampfer, P., Ludwig, W., Fuchs, G., 1995. Taxonomic Position of Aromatic-Degrading Denitrifying Pseudomonad Strains K 172 and KB 740 and Their Description as New Members of the Genera Thauera , as Thauera aromatica sp . nov ., and Azoarcus , as Azoarcus evansii sp . nov ., Respectively , Members. Int. J. Syst. Bacteriol. 45 (2), 327–333.

Bardiya, N., Bae, J.H., 2011. Dissimilatory perchlorate reduction: a review. Microbiol. Res. 166 (4), 237–254.

Chou, J.H., Jiang, S.R., Cho, J.C., Song, J., Lin, M.C., Chen, W.M., 2008. Azonexus hydrophilus sp. nov., a nifH gene-harbouring bacterium isolated from freshwater. Int. J. Syst. Evol. Microbiol. 58 (Pt 4), 946–951.

Grabovich, M., Gavrish, E., Kuever, J., Lysenko, A.M., Podkopaeva, D., Dubinina, G., 2006. Proposal of Giesbergeria voronezhensis gen. nov., sp. nov. and G. kuznetsovii sp. nov. and reclassification of [Aquaspirillum] anulus, [A.] sinuosum and [A.] giesbergeri as Giesbergeria anulus comb. nov., G. sinuosa comb. nov. and G. giesbergeri comb. nov. Int. J. Syst. Evol. Microbiol. 56 (Pt 3), 569–576.

Gumaelius, L., Magnusson, G., Pettersson, B., Dalhammar, G., 2001. Comamonas denitrificans sp. nov., an efficient denitrifying bacterium isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 51 (3), 999–1006.

Mechichi, T., 2003. Alicycliphilus denitrificans gen. nov., sp. nov., a cyclohexanol-degrading, nitrate-reducing beta-proteobacterium. Int. J. Syst. Evol. Microbiol. 53 (1), 147–152.

Mechichi, T., Stackebrandt, E., Gad'on, N., Fuchs, G., 2002. Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of Thauera phenylacetica sp. nov., Thauera aminoaromaticasp. nov., and Azoarcus buckelii sp. nov. Arch. Microbiol. 178 (1), 26–35.

Zheng, J.W., Chen, Y.G., Zhang, J., Ni, Y.Y., Li, W.J., He, J., Li, S.P., 2011. Description of Catellibacterium caeni sp. nov., reclassification of Rhodobacter changlensis Anil Kumar et al. 2007 as Catellibacterium changlense comb. nov. and emended description of the genus Catellibacterium. Int. J. Syst. Evol. Microbiol. 61 (Pt 8), 1921–1926.

Supplementary material

Chapter 5

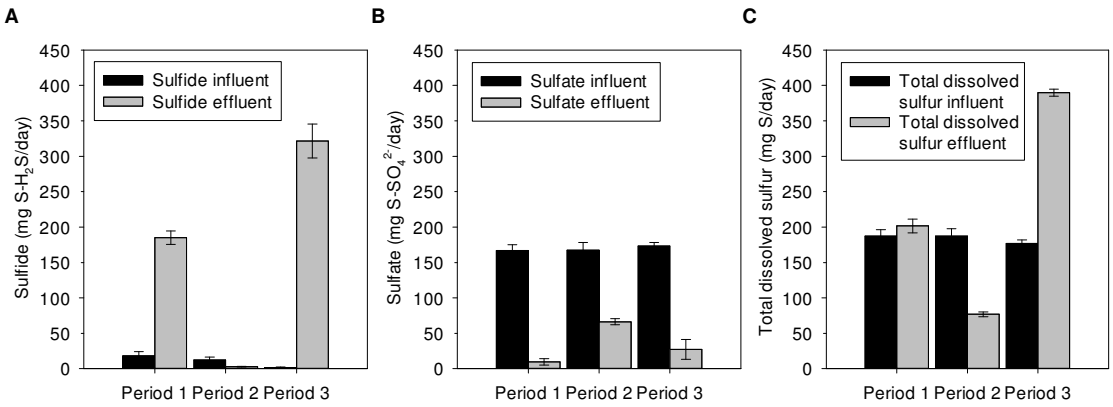


Figure S1. Daily sulfide (A), sulfate (B) and total dissolved sulfur (C) load in the influent and the effluent wastewater during each study period measured during a 24 hour-period approach. Error bars indicate standard error of the mean.

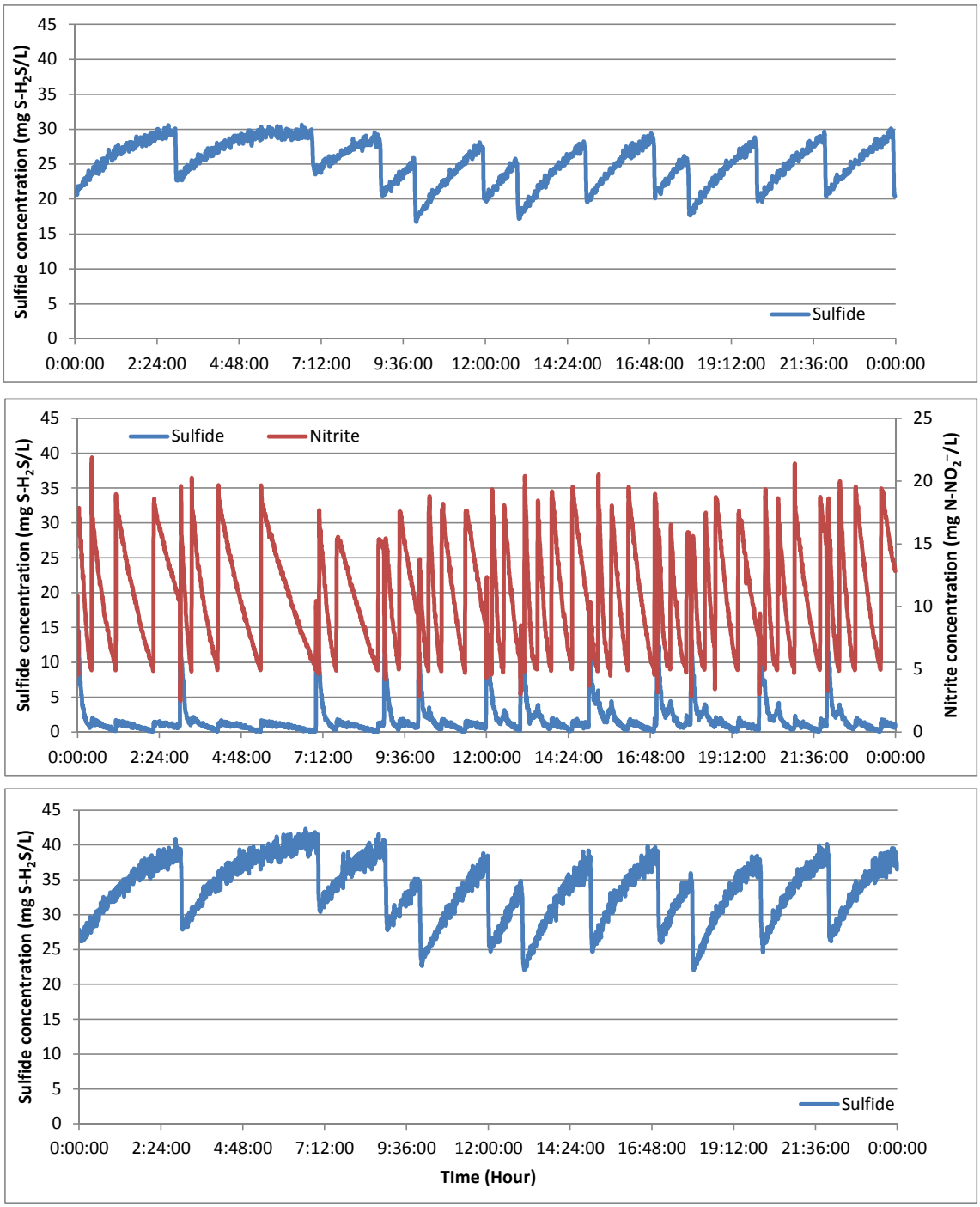


Figure S2. Daily profiles of sulfide production during period 1 (A), sulfide and nitrite consumption during period 2 (B), and sulfide production during the recovery period (C).

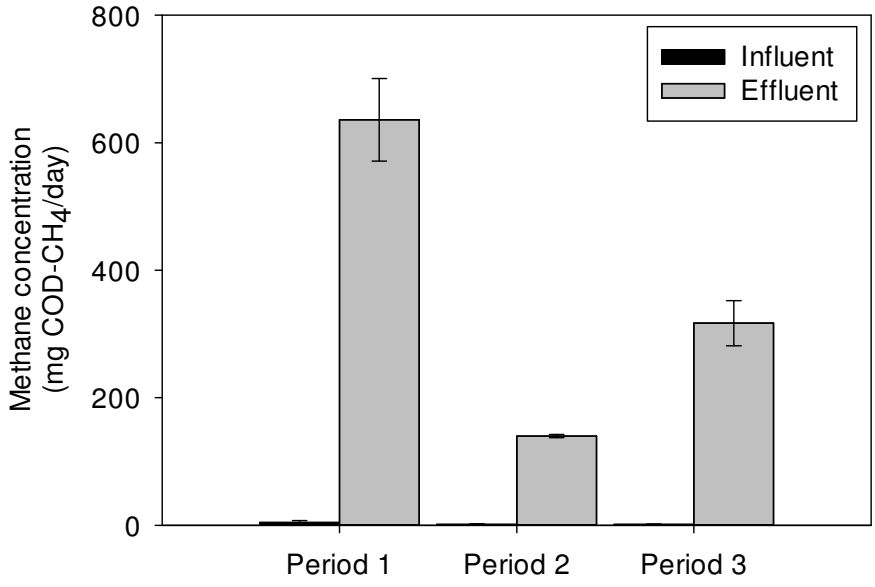


Figure S3. Daily methane emission over the monitoring of the system measured during 24 hour-periods approach. Error bars indicate standard error of the mean.

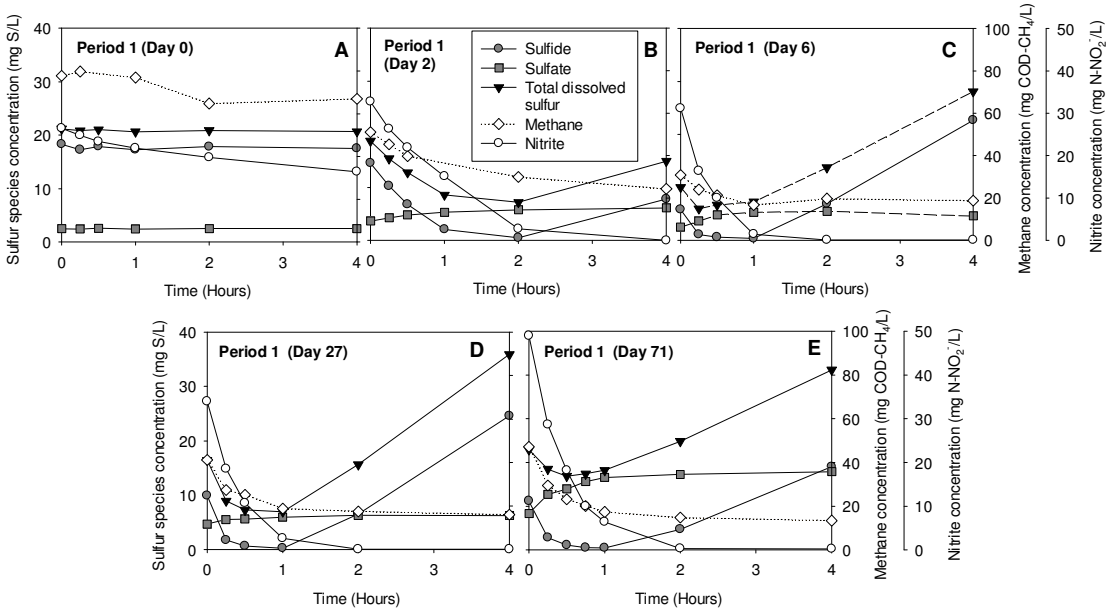


Figure S4. Profiles of sulfide, sulfate, sulfur balance, methane and nitrite concentrations measured in batch tests during day 0 (A), day 2 (B), day 6 (C), day 27 (D) and day 71 (E) of period 2.

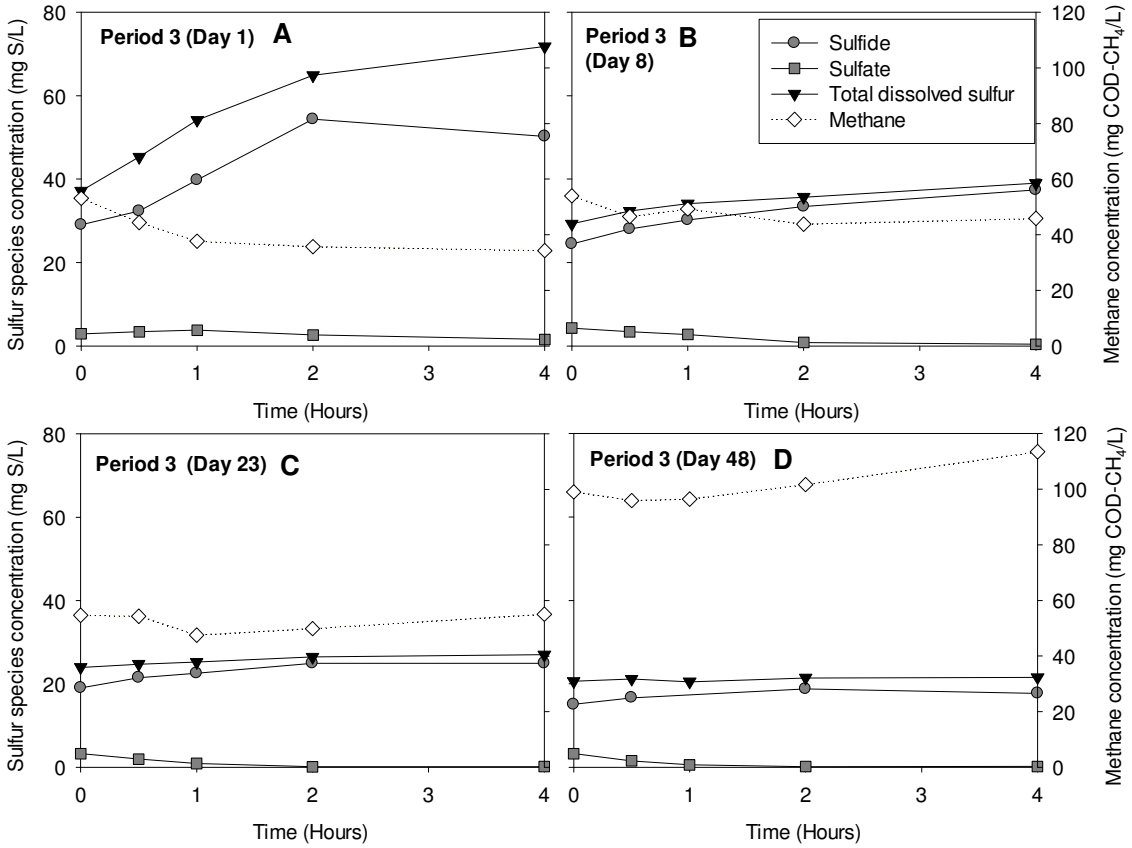


Figure S5. Profiles of sulfide, sulfate, total dissolved sulfur and methane concentrations measured in batch tests during day 1 (A), day 8 (B), day 23 (C) and day 48 (D) of period 3.

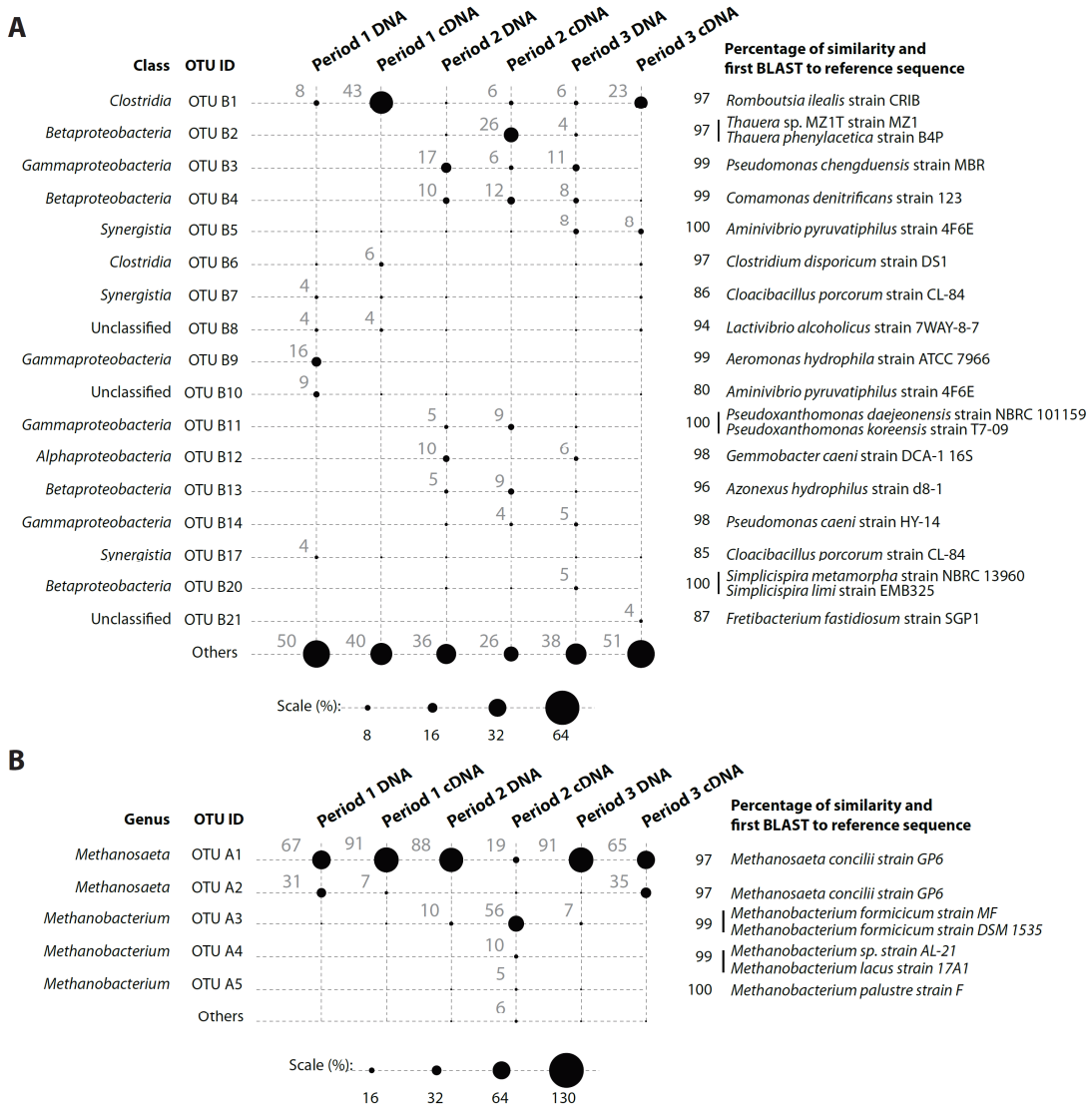


Figure S6. Relative abundance plots of bacterial (A) and archaeal (B) sequences grouped into OTUs during different periods and nucleic acid samples, the taxonomy groups and the percentage identity to the first BLAST hit against reference database. Bubble plots are proportional to the radius in a logarithmic scale (showed below the graph). Percentage values higher than 4% are indicated next to the corresponding bubble.

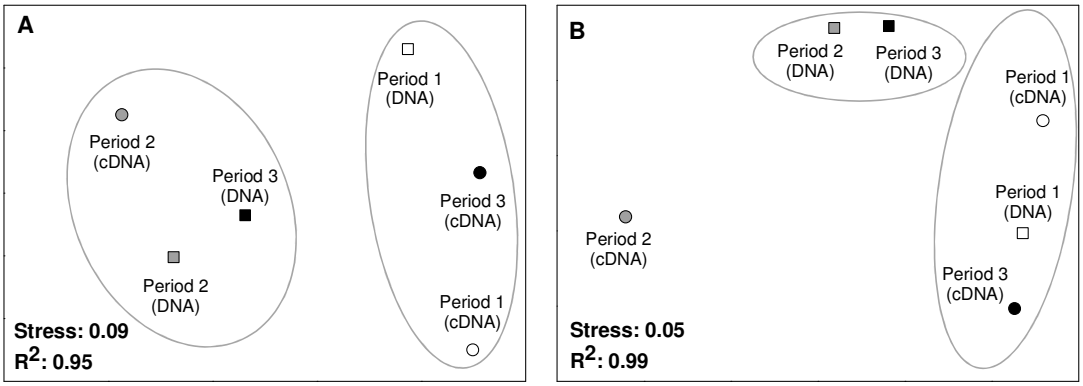


Figure S7. Non-metric multidimensional scaling (NMDS) based on weighted UniFrac distances of bacterial (A) and archaeal (B) biofilm communities

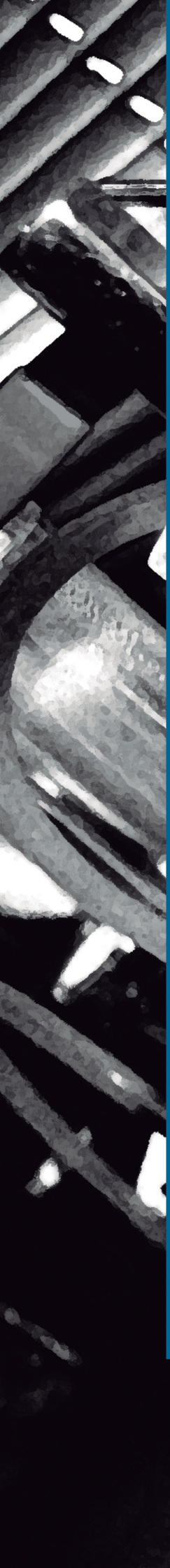
Table S1. Total and volatile suspended solids used to calculate biomass content and elemental sulfur/thiosulfate/sulfide concentrations in the biofilm.

	TSS (mg/cm^2)		VSS (mg/cm^2)	
	R1	**R3**	**R1**	**R3**
Before	8.7	8.5	7.5	7.7
During nitrite addition	9.2±0.8	12.2±1.9	8.0±0.6	7.6±0.9
Recovery	12.8	8.1	11.1	5.8

Table S2. DNA quantification and RNA quantification during different treatment steeps using QUBIT assays.

Sample	**After DNA/RNA extraction (ng/µl)**		**After DNase treatment (ng/µl)**	**cDNA synthesis (ng/µl)**
	DNA[1]	RNA[2]	RNA[2]	cDNA[3]
Before	52.0	70.8	30.8	4.63
During	298.0	162.0	72	8.81
Recovery	33.0	48.0	21.4	2.85

[1] Qubit® dsDNA BR Assay Kit / [2] Qubit® RNA HS Assay Kit / [3] Qubit® ssDNA Assay Kit



Sewer systems transport wastewater from source to treatment plants. During this transport, wastewater microorganisms colonize pipe walls and develop biofilms. The activity of microbial communities in sewer biofilms produces changes in wastewater characteristics resulting in different problems. One of them is the production of sulfide (H_2S) by sulfate-reducing bacteria (SRB). Sulfide causes corrosion in sewer pipes, malodour and health problems. Another worrying problem is the production of methane (CH_4) by methanogenic archaea (MA). Methane is a strong greenhouse gas with higher global warming potential than carbon dioxide and it poses an additional risk in confined spaces due to its low explosion limit. For these reasons a good control of H_2S and CH_4 emissions from sewer systems is essential for the optimal management of these facilities.

The results of this PhD Thesis are useful for sewer managers to determine the suitable strategy to control H_2S and CH_4 emissions in these systems. Also, this thesis provides fundamental knowledge on the microbial communities present in sewer biofilms and their role in biotransformation processes that can be highly relevant in terms of environmental and public health issues.