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Bacterial composition and dynamics of active communities in an anammox reactor treating wastewater at mainstream conditions

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PhD Thesis

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CERTIFICAMOS:

Que la bioingeniera y máster en ciencias con mención microbiología por la Universidad de Concepción (Chile) **PÍA KARINA OYARZÚA ALARCÓN** ha realizado bajo nuestra dirección el trabajo titulado "***Bacterial composition and dynamics of active communities in an anammox reactor treating wastewater at mainstream condition***s", que se presenta en esta memoria, y que constituye su tesis para optar al Grado de Doctor por la Universidad Autónoma de Barcelona.

Y para que se tenga conocimiento y conste a los efectos oportunos, presentamos en l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona la mencionada tesis y firmamos el presente certificado en Bellaterra y Montevideo, noviembre del 2021.

Dra. María Eugenia Suárez Ojeda

Dra. Claudia Etchebehere Arenas

A mi familia

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Contents

Summary

One of the critical points for the application of the anammox process at the mainstream of urban wastewater treatment plants is the need to work at rather low temperatures (10-15ºC) without reducing the nitrogen removal efficiency. With this in mind, this thesis has been developed within the framework of the HIPATIA project, aiming to demonstrate the operational viability of an anammox reactor treating real mainstream wastewater at low temperatures. Thus, the main objective of this work is to know the changes in the microbial community of an anammox reactor when working at mainstream conditions to understand the performance of the system during the operational changes the process has suffered.

Molecular techniques are widely used to assess the dynamics and the composition of microbial communities in anammox process. Nevertheless, biases on its application are not always considered. Moreover, we observed low resolution of the molecular techniques (amplicon sequencing and fluorescence *in situ* hybridization -FISH-) traditionally applied to follow the process in our research group. Therefore, chapter 4 consisted of evaluation, comparison, and standardization of different molecular approaches to study bacterial communities in a granular-sludge Upflow Anammox Sludge Blanket (UAnSB) reactor working at mainstream conditions and in a Sequencing Batch Reactor (SBR) working at controlled conditions with synthetic wastewater. FISH, 16S rRNA amplicon sequencing using different primers sets, metagenomics, and quantitative PCR (qPCR) were evaluated. All evaluated techniques were standardized generating, among others, an optimized FISH protocol to overcome sample autofluorescence and a proper choice of the primer sets for amplicon sequencing and qPCR. The importance of raw data reprocessing with updated databases and recommendations on the advantages of using one technique over another or a combination of them were outlined, depending on the research question to be addressed. From the first phylogenetic analysis, we propose a new "*Candidatus"* species of anammox, namely "*Candidatus*" Brocadia barcinensis, as the predominant species in the anammox enriched-culture developed in the SBR in our laboratories.

Then, in chapter 5, the structure of whole microbial and anammox communities and their dynamics along the application of a decreasing gradient of temperature from 20ºC to 10ºC in the granular-sludge UAnSB reactor was studied. Using, both, amplicon

sequencing and metagenomic approaches based on the 16S rRNA gene, the whole bacterial composition was established in three sludge bed sections located along the reactor height (S1-base, S3-middle part, S5-top). Both approaches showed the same predominant classes, namely: Ignavibacteria, Gammaproteobacteria, Anaerolinea, and Brocadiae along the different sludge bed sections of the reactor, which can be described as the "core microbiome". With the temperature decrease, the core microbiome was determined to be the same, but changes in the microbial community structure showed stratification along the reactor. Regarding anammox bacteria, a higher relative abundance was detected in the base of the reactor (S1) at both temperatures compared to S3 and S5. Moreover, at 10ºC the relative abundance was almost doubling of the one at 20ºC, despite the anammox specific activity, calculated as the specific Nitrogen Removal Rate (sNRR) was lower than that at 20ºC. Thus, more reliable information was needed to explain and confirm the hypothesis of reactor overcapacity by means of the specific study of its active community.

In this sense, the use of RNA-based techniques appears like an excellent alternative to detect active communities along operational changes. Thus, in chapter 6 the aim was to determine the dynamics of active bacterial and anammox communities along a granular-sludge UAnSB reactor treating real wastewater at mainstream conditions during the decrease of the operating temperature from 20ºC to 10ºC. Changes in stratification due to the temperature effect were recorded for Ignavibacteria and Latescibacteria at 20ºC, while at 10ºC the phylum Chloroflexi and the class Brocadiae were the ones contributing to the stratification due to both temperature and sludge-bed section effects. The community dynamics variation along the reactor would support the reactor overcapacity concept, described as a significant amount of biomass that is not contributing to the overall anammox activity, as an important factor for the good performance at low temperatures. The active anammox community showed that the genus differentiation along the reactor, changing between *Ca.* Brocadia to *Ca.* Kuenenia, being this last one could be the responsible for keeping the removal efficiency at low temperatures. In addition, it is striking that a greater diversity of anammox species was recorded at low temperatures and that the abundance of active *Ca*. Brocadia barcinensis increased at low temperatures. Therefore, the different physiological abilities of each anammox species might be influencing the reactor´s performance at each temperature and sludge bed section.

Finally, as there are still gaps in determining the role of the predominant bacterial groups, in chapter 7 we applied genome-centric metagenomics to obtain representative genomes to study the metabolic potential of microorganisms from the Brocadiales and Ignavibacteriales orders from the SBR and UAnSB anammox-reactors. Three metagenomics assembled genomes (MAGs) belonging to order Brocadiales and four MAGs belonging to the order Ignavibacteriales with high quality were obtained. From a phylogenetic analysis, one of the Brocadiales MAG bunched in the same clade of our recently proposed new species, *Ca*. Brocadia barcinensis, dominant in the enriched anammox culture and its metabolic potential was determined. Regarding the Ignavibacteriales, its metabolic potential was studied and the interactions with anammox bacteria were confirmed.

The high amount of bioinformatic data acquired through this thesis, would allow future investigations as the study of the metabolic potential of the metagenome along the UAnSB reactors. Futures lines of research could be achieved with a continuous assessment of the active communities at different operation conditions to understand changes in the dynamics of the anammox consortia, and finally, the use of new approaches as metatranscriptomics would be necessary to study the expression of the identified metabolic functions under certain operational conditions.

Resumen

Uno de los puntos críticos para la aplicación del proceso anammox en la corriente principal de las plantas de tratamiento de aguas residuales urbanas es la necesidad de trabajar a temperaturas bastante bajas (10-15ºC) sin reducir la eficiencia de eliminación de nitrógeno. Teniendo esto en cuenta, esta tesis se ha desarrollado en el marco del proyecto HIPATIA, con el objetivo de demostrar la viabilidad operativa de un reactor anammox que trata aguas residuales reales en condiciones de línea principal de aguas a bajas temperaturas. Así, el principal objetivo de este trabajo es conocer los cambios en la comunidad microbiana de un reactor anammox cuando se trabaja en condiciones de línea principal de aguas, para comprender el desempeño del sistema durante los cambios operacionales que enfrenta el proceso.

Las técnicas moleculares se utilizan ampliamente para evaluar la dinámica y la composición de las comunidades microbianas en el proceso anammox. Sin embargo, no siempre se tienen en cuenta los sesgos en su aplicación. Por otra parte, observamos una baja resolución de las técnicas moleculares que tradicionalmente se aplican para hacer el seguimiento microbiológico del proceso en nuestro grupo de investigación (secuenciación de amplicones e hibridación fluorescente *in situ* -FISH). Por lo tanto, el capítulo 4 consistió en la evaluación, comparación y estandarización de diferentes enfoques moleculares para estudiar comunidades bacterianas en un reactor anammox de lecho de lodos de flujo ascendente (UAnSB, por sus siglas en inglés) de biomasa granular que funciona en condiciones de corriente principal y en un reactor de carga secuencial (SBR, por sus siglas en inglés) que funciona en condiciones controladas con aguas residuales sintéticas. Se evaluaron FISH, secuenciación de amplicones del gen ARNr 16S utilizando diferentes conjuntos de cebadores, metagenómica y PCR cuantitativa (qPCR). Todas las técnicas evaluadas se estandarizaron, generando entre otros, un protocolo FISH optimizado para reducir la autofluorescencia de la muestra y la elección de los cebadores para secuenciación de amplicones y qPCR. Se destacó la importancia del reprocesamiento de datos brutos utilizando bases de datos actualizadas y se señalaron recomendaciones sobre las ventajas de utilizar una técnica sobre otra o una combinación de ellas, dependiendo de la pregunta de investigación a abordar. A partir del primer análisis filogenético, proponemos una nueva especie "*Candidatus*" anammox, denominada

"*Candidatus*" Brocadia barcinensis, como especie predominante en el cultivo enriquecido en anammox desarrollado en el SBR en nuestros laboratorios.

Luego, en el capítulo 5, se estudió la estructura de comunidades microbianas y anammox, y su dinámica a lo largo de la aplicación de un gradiente decreciente de temperatura desde 20ºC a 10ºC en el reactor de biomasa granular UAnSB. Utilizando tanto la secuenciación de amplicones como los enfoques metagenómicos basados en el gen del ARNr 16S, se estableció la composición bacteriana en tres secciones del lecho de lodo ubicadas a lo largo de la altura del reactor (base S1, parte media S3, parte superior S5). Ambos enfoques mostraron las mismas clases predominantes: Ignavibacteria, Gammaproteobacteria, Anaerolinea y Brocadiae a lo largo de las diferentes secciones del lecho de lodo del reactor, lo que se puede describir como el "microbioma central". Con la disminución de la temperatura, se determinó que este microbioma central se mantiene, pero los cambios en la estructura de la comunidad microbiana mostraron una estratificación a lo largo del reactor. En cuanto a las bacterias anammox, se detectó una mayor abundancia relativa en la base del reactor (S1) en ambas temperaturas, comparada con S3 y S5. A 10ºC la abundancia relativa fue casi el doble que a 20ºC, a pesar de que la actividad específica anammox, calculada como la tasa específica de eliminación de nitrógeno (sNRR) fue menor que a 20ºC. Por lo tanto, se necesitaba información más confiable para explicar y confirmar la hipótesis de sobrecapacidad del reactor, descrito como una cantidad significativa de biomasa "durmiente"que no contribuye a la actividad general de anammox, recurriendo entonces al estudio específico de la comunidad activa.

En este sentido, el uso de técnicas basadas en ARN aparece como una excelente alternativa para detectar comunidades activas a lo largo de cambios operacionales. Así, en el capítulo 6 el objetivo fue determinar la dinámica de las comunidades bacterianas y anammox activas a lo largo de un reactor UAnSB de biomasa granular que trata aguas residuales reales en condiciones de corriente principal durante la disminución de la temperatura de operación de 20ºC a 10ºC. Se registraron cambios en la estratificación por efecto de la temperatura en las clases Ignavibacteria y Latescibacteria a 20ºC, mientras que a 10ºC el filo Chloroflexi y la clase Brocadiae contribuyeron a la estratificación tanto por efecto de la temperatura como de la sección del lecho de lodos. Estos cambios en la dinámica de la comunidad a lo largo del reactor respaldarían el concepto de sobrecapacidad como un factor importante para el buen rendimiento del proceso a bajas temperaturas. La comunidad anammox activa mostró que la diferenciación a nivel de

género a lo largo del reactor, cambiando entre *Ca*. Brocadia y *Ca*. Kuenenia, siendo esta última la que podria ser responsable de mantener la eficiencia de eliminación de nitrógeno a bajas temperaturas. Además, llamó la atención que se registró una mayor diversidad de especies de anammox a bajas temperaturas y que la abundancia de la especie *Ca* Brocadia barcinensis activa aumentó a bajas temperaturas. Por lo tanto, las diferentes capacidades fisiológicas de cada especie anammox podrían estar influyendo en el desempeño del reactor a cada temperatura y en sección del lecho de lodos.

Por último, como todavía existen vacíos en la determinación del papel de los grupos bacterianos predominantes, en el capítulo 7 utilizamos la aproximación de metagenómica centrada en el genoma para obtener genomas representativos presentes en los reactores anammox SBR y UAnSB, para estudiar el potencial metabólico de los microorganismos de los órdenes Brocadiales e Ignavibacteriales. Se obtuvieron tres genomas ensamblados desde metagenomas (MAGs) de alta calidad pertenecientes al orden Brocadiales y cuatro MAGs pertenecientes al orden Ignavibacteriales. A partir del análisis filogenético se determinó que uno de los MAGs de Brocadiales se agrupó en el mismo clado de nuestra nueva especie, *Ca.* Brocadia barcinensis, dominante en el cultivo enriquecido de anammox (UAnSB), y se determinó su potencial metabólico. En cuanto a los Ignavibacteriales, se estudió su potencial metabólico y se confirmaron las interacciones con bacterias anammox.

La gran cantidad de datos bioinformáticos adquiridos a través de esta tesis permitirá futuras investigaciones como el estudio del potencial metabólico del metagenoma en los reactores UAnSB. También se podrían generar futuras líneas de investigación que contemplen una evaluación continua de las comunidades activas en diferentes condiciones de operación para comprender los cambios en la dinámica de los consorcios anammox. Finalmente, sería interesante utilizar nuevos enfoques moleculares como la metatranscriptómica para estudiar la expresión de las funciones metabólicas identificadas frente a determinadas condiciones de operación de los reactores.

Resum

Un dels punts crítics per l'aplicació del procés anammox en el corrent principal de les plantes de tractament d'aigües residuals urbanes és la necessitat de treballar a temperatures relativament baixes (10-15ºC) sense reduir l'eficiència d'eliminació de nitrogen. Tenint això en compte, aquesta tesi s'ha desenvolupat en el marc del projecte HIPATIA, amb l'objectiu de demostrar la viabilitat operativa d'un reactor anammox que tracta aigües residuals reals en condicions de línia principal a baixes temperatures. Així, el principal objectiu d'aquest treball es conèixer els canvis en la comunitat microbiana d'un reactor anammox quan es treballa en condicions de línia principal, per comprendre el comportament del sistema durant els canvis operacionals que enfronta el procés.

Les tècniques moleculars es fan servir àmpliament per avaluar la dinàmica i la composició de les comunitats microbianes en el procés anammox. No obstant això, no sempre es tenen en compte els biaixos en la seva aplicació. D'altra banda, observem una baixa resolució de les tècniques moleculars que tradicionalment s'apliquen per fer el seguiment microbiològic del procés al nostre grup de recerca (seqüenciació d'amplicons i hibridació fluorescent *in situ* -FISH). Per tant, el capítol 4 va consistir en l'avaluació, la comparació i l'estandarditzación de diferents enfocaments moleculars per estudiar comunitats bacterianes en un reactor anammox de llit de llots de flux ascendent (UAnSB, per les sigles en anglès) de biomassa granular que funciona en condicions de corrent principal i en un reactor de càrrega seqüencial (SBR, per les sigles en anglès) que funciona en condicions controlades amb aigües residuals sintètiques. Es van avaluar FISH, seqüenciació d'amplicons del gen ARNr 16S utilitzant diferents conjunts d'encebadors, metagenòmica i PCR quantitativa (qPCR). Totes les tècniques avaluades es van estandarditzar, generant entre d'altres, un protocol FISH optimitzat per reduir l'autofluorescència de la mostra i l'elecció dels encebadors per a seqüenciació d'amplicons i qPCR. Es va destacar la importància del reprocessament de dades brutes utilitzant bases de dades actualitzades i es van assenyalar recomanacions sobre els avantatges d'utilitzar una tècnica sobre una altra o una combinació d'aquestes, depenent de la pregunta de recerca a abordar. A partir de la primera anàlisi filogenètica, proposem una nova espècie "*Candidatus*" anammox, anomenada "*Candidatus*" Brocadia barcinensis, com a espècie predominant en el cultiu enriquit en anammox desenvolupat al SBR als nostres laboratoris.

Després, al capítol 5, es va estudiar l'estructura de comunitats microbianes i anammox, i la seva dinàmica al llarg de l'aplicació d'un gradient decreixent de temperatura des de 20ºC a 10ºC al reactor de biomassa granular UAnSB. Utilitzant tant la seqüenciació d'amplicons com els enfocaments metagenòmics basats en el gen de l'ARNr 16S, es va establir la composició bacteriana en tres seccions del llit de fang ubicades al llarg de l'alçada del reactor (base S1, part mitjana S3, part superior S5). Ambdós enfocaments van mostrar les mateixes classes predominants: Ignavibacteria, Gammaproteobacteria, Anaerolinea i Brocadiae al llarg de les diferents seccions del llit de fang del reactor, cosa que es pot descriure com el "microbioma central". Amb la disminució de la temperatura, es va determinar que aquest microbioma central es manté, però els canvis a l'estructura de la comunitat microbiana van mostrar una estratificació al llarg del reactor. Pel que fa als bacteris anammox, es va detectar una major abundància relativa a la base del reactor (S1) a les dues temperatures, comparades amb S3 i S5. A 10ºC l'abundància relativa va ser gairebé el doble que a 20ºC, malgrat que l'activitat específica anammox, calculada com que la taxa específica d'eliminació de nitrogen (sNRR) va ser menor que a 20ºC. Per tant, calia informació més fiable per explicar i confirmar la hipòtesi de sobrecapacitat del reactor, descrit com una quantitat significativa de biomassa que no contribueix a l'activitat general d'anammox, recorrent llavors a l'estudi específic de la comunitat activa.

En aquest sentit, l'ús de tècniques basades en ARN apareix com una alternativa excel·lent per detectar comunitats actives al llarg de canvis operacionals. Així, al capítol 6 l'objectiu va ser determinar la dinàmica de les comunitats bacterianes i anammox actives al llarg d'un reactor UAnSB de biomassa granular que tracta aigües residuals reals en condicions de corrent principal durant la disminució de la temperatura d'operació de 20ºC a 10ºC. Es van registrar canvis en l'estratificació per efecte de la temperatura a les classes Ignavibacteria i Latescibacteria a 20ºC, mentre que a 10ºC el tall Chloroflexi i la classe Brocadiae van contribuir a l'estratificació tant per efecte de la temperatura com de la secció del llit de llots. Aquests canvis en la dinàmica de la comunitat al llarg del reactor donarien suport al concepte de sobrecapacitat com un factor important per al bon rendiment del procés a baixes temperatures. La comunitat anammox activa va mostrar que la diferenciació a nivell de gènere al llarg del reactor, canviant entre *Ca.* Brocadia i *Ca*. Kuenenia, sent aquesta última la que podria ser responsable de mantenir l'eficiència d'eliminació de nitrogen a baixes temperatures. A més, va cridar l'atenció que es va

registrar una diversitat més gran d'espècies d'anammox a baixes temperatures i que l'abundància de l'espècie *Ca.* Brocadia barcinensis activa va augmentar a baixes temperatures. Per tant, les diferents capacitats fisiològiques de cada espècie anammox podrien estar influint en l'exercici del reactor a cada temperatura i en secció del llit de llots.

Per últim, com que encara existeixen buits en la determinació del paper dels grups bacterians predominants, al capítol 7 utilitzem l'aproximació de metagenòmica centrada en el genoma per obtenir genomes representatius presents als reactors anammox SBR i UAnSB, per estudiar el potencial metabòlic de els microorganismes dels ordres Brocadials i Ignavibacteriales. S'obtingueren tres genomes acoblats des de metagenomes (MAGs) d'alta qualitat pertanyents a l'ordre Brocadials i quatre MAGs pertanyents a l'ordre Ignavibacteriales. A partir de l'anàlisi filogenètica es va determinar que un dels MAGs de Brocadials es va agrupar al mateix clade de la nostra nova espècie, *Ca*. Brocadia barcinensis, dominant en el cultiu enriquit d'anammox (UAnSB), i se'n va determinar el seu potencial metabòlic. Pel que fa als Ignavibacterials, se'n va estudiar el potencial metabòlic i es van confirmar les interaccions amb els bacteris anammox.

La gran quantitat de dades bioinformàtiques adquirides a través d'aquesta tesi permetrà investigacions futures com l'estudi del potencial metabòlic del metagenoma dels reactors UAnSB. També es podrien generar futures línies de recerca que tinguin en compte una avaluació contínua de les comunitats actives en diferents condicions d'operació per comprendre els canvis en la dinàmica dels consorcis anammox. Finalment, seria interessant utilitzar nous enfocaments moleculars com la metatranscriptòmica per estudiar l'expressió de les funcions metabòliques identificades davant de determinades condicions d'operació dels reactors.

Thesis outline

This thesis was developed in the GENOCOV research group (Group of biological treatment and of liquid and gaseous effluents, nutrient removal, and odors and Volatile Organic Compounds) at the Department of Chemical, Biological and Environmental Engineering at Universitat Autònoma de Barcelona, and in collaboration with the LEM research group (Microbial Ecology Laboratory) at the Department of Microbial Biochemistry and Genomic, Biological Research Institute "Clemente Estable" of Uruguay.

The thesis manuscript is divided into eight chapters. The main content of each chapter is detailed below.

Chapter 1 present an overview of the state of the art in the field of biological nitrogen removal in urban wastewater treatment plants (WWTPs), focused on the microbiology of the anammox process, especially on microbial communities when working at mainstream conditions. Chapter 2 states the main objectives of the thesis. Chapter 3 describes the general materials and methods used during the experimental work of this thesis which were common to all the experiments presented in the following chapters of results; thus, the more specific materials and methods used in the specific chapters are described in the corresponding chapter. Chapters 4 to 7 contain the results obtained during the development of this thesis. Each chapter is presented in a self-contained manner (Fig. 1) to ease the reading. Therefore, the description of the reactor and samples in the materials and methods stated in Chapter 3, is then repeated in Chapters 5 and 6 as it is essential for subsequent publication and also to facilitate readability.

Chapters 4 and 5 respond to the first goal of this thesis. More specifically, Chapter 4 describes the standardization of different molecular approaches for the study of bacterial communities in anammox reactors treating real wastewater at mainstream conditions. Chapter 5 was focused on the use of standardized molecular approaches for determining the microbial and anammox community of an anammox reactor working at mainstream conditions during the decrease of operating temperature.

Chapter 6 respond to the second goal and comprises the study of the dynamics and changes in the stratification of the active microbial and anammox communities of the

anammox reactor working at mainstream conditions during the decrease of operating temperature.

Once the community structure was defined, Chapter 7 respond to the third goal and describes the metabolic potential of metagenome-assembled genomes belonging to two orders involved in the nitrogen removal metabolism of two anammox reactors.

Finally, Chapter 8 states the main conclusions and future work extracted from this thesis.

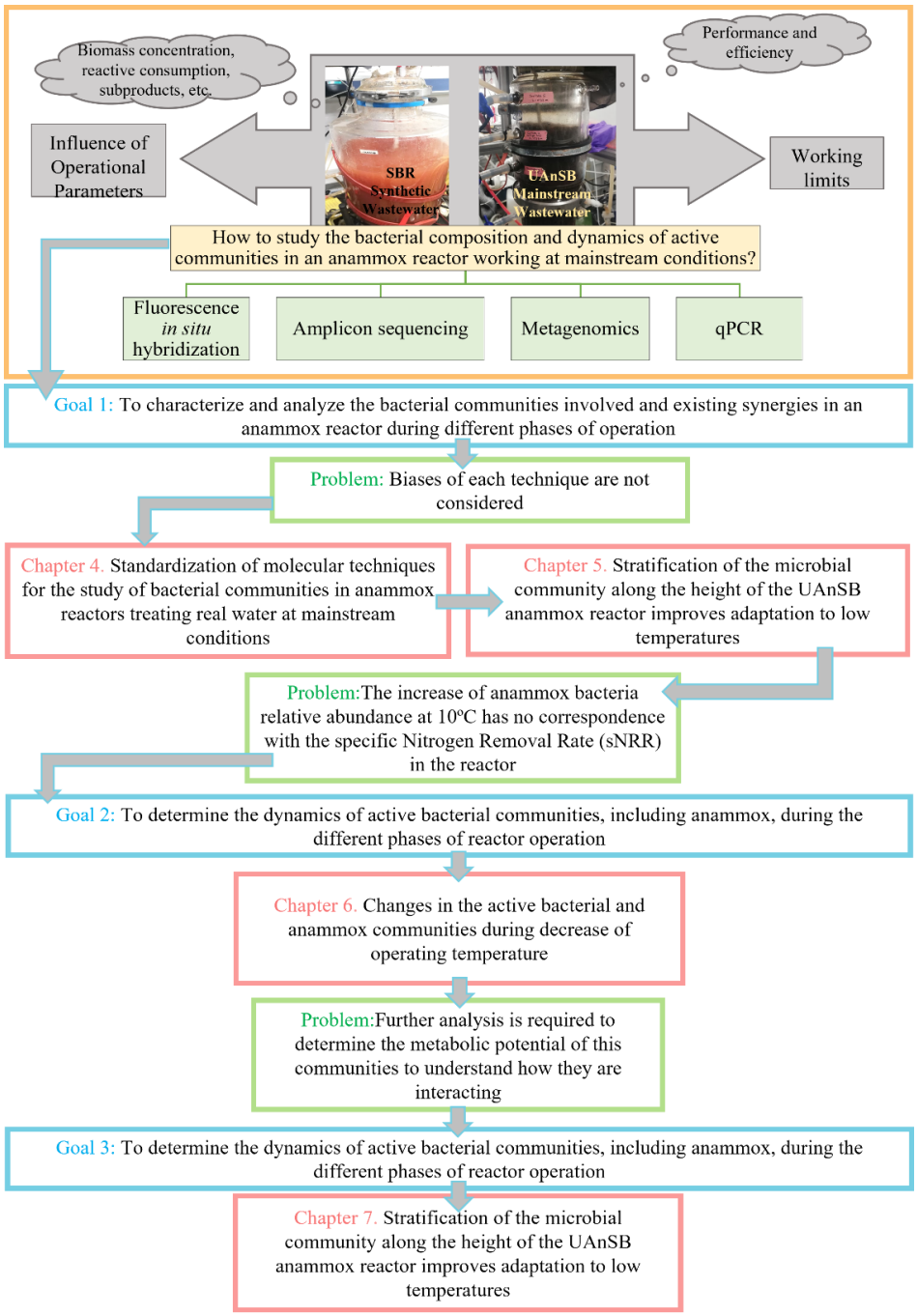

Figure 1.0. Graphical thesis outline.

Abbreviations and acronyms

aBNR	Autotrophic Biological Nitrogen Removal
ACC	Acetyl-Coa Carboxylase Complex
Acetyl-CoA	Acetyl- Coenzyme A
ACP	Acyl Carrier Protein
ANI	Average Nucleotide Identity
AOB	Ammonia-Oxidizing Bacteria
ASVs	Amplicon Sequence Variants
ATP	Adenosine Triphosphate
BNR	Biological Nitrogen Removal
bp	base pairs
Ca.	Candidatus
cDNA	Complementary DNA
CDS	Protein Coding Sequences
CHB	Chlorobi
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DNRA	Dissimilatory Nitrite Reduction To Ammonium
EfOM	Effluent Dissolved Organic Matter
EPS	Extracellular Polymeric Substances
Fab	Fatty Acid Biosynthesis
FISH	Fluorescent In-Situ Hybridization
GTDB	Genome Taxonomy Database
GTDB-Tk	Genome Tree Database
hao	Hydroxylamine Oxidoreductase
hdh	Hydrazine Dehydrogenase
HTS	High-Throughput Sequencing
hzo	Hydrazine Oxidoreductase
hzs	Hydrazine Synthase Enzyme
kb	kilobases
KEGG	Kyoto Encyclopedia Of Genes And Genomes
MA-EBR	Microbial Attached Expanded Bed Reactor
MAGs	Metagenome-Assembled Genomes
MBBR	Moving Bed Biofilm Reactor
MBR	Membrane Bioreactor
ML	Maximum Likehood
MWW	Municipal Wastewater
N	Nitrogen
N_2H_4	Hydrazine

NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
NGS	Next-Generation Sequencing
NH_2OH	Hydroxylamine
NH_4^+	Ammonia
nirK/nirS	Nitrite Reductase Enzyme
NLR	Nitrogen Loading Rate
NMDS	Non-Metric Multidimensional Scaling
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-Oxidizing Bacteria
NRE	Nitrogen Removal Efficiency
NRR	Nitrite Removal Rate
OCT	Optimal Cutting Technique
OTUs	Operational Taxonomic Units
PBS	Phosphate Saline Buffer
PCR	Polymerase Chain Reaction
PE	Pair-End
PN/A	Partial Nitritation Anammox
qPCR	Quantitative PCR
RGB	Red-Green-Blue
RNA	Ribonucleic Acid
ROIs	Region Of Interest
rRNA	Ribosomal Ribonucleic Acid
SAM	S-Adenosylmethionine
SBR	Sequential Batch Reactor
SM	Synthetic Medium
SMP	Soluble Microbial Products
SNAD	Simultaneous Partial Nitrification, Anammox And Denitrification
sNRR	Specific Nitrogen Removal Rate
ssDNA	Single-Strand DNA
SSU	Ribosomal Small Subnubit
tRNA	Transfer RNA
UAnSB	Up-Flow Anammox Sludge Bed
UASB	Up-Flow Anaerobic Sludge Blanket
UBF	Upflow Biofilter
WGS	Whole Genome Shotgun Sequencing
WLP	Wood–Ljungdahl Pathway
WRP	Water Reclamation Plant
WW	Wastewaters
WWTPs	Wastewater Treatment Plants

Chapter 1

Introduction

1.1. Nitrogen removal in wastewater treatment plants

Among all the elemental cycles, the Nitrogen cycle is, probably, the one where the human impact has been dramatic (Galloway, 2005). The rising demand for more food increases the use of fertilizers and land, but also, the need for more energy increases the combustion of fossil fuels. Both needs intensify the losses of reactive nitrogen to the environment (Erisman et al., 2013; Galloway et al., 2008). Much anthropogenic nitrogen is lost to the air, water, and land causing a cascade of environmental and human health problems. Most notably is the increase of nitrate levels in freshwater leading to a rise of nitrous oxide production to the atmosphere that can intensify global climate change (Duce et al., 2008).

An increasing population and industrialization will increase our water demand, placing even more pressure on water resources and therefore, in wastewater treatment. Most existing urban wastewater treatment plants (WWTPs) over the world only remove organic matter through conventional activated sludge systems. However, in the last decades, nutrients such as nitrogen and phosphorous have become an essential concern since they can be toxic to aquatic life (Karthik and Joseph, 2014; Reino, 2016).

The toxicity of the wastewater containing excessive nitrogen compounds in the form of ammonia (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and organic bound nitrogen would cause depleted dissolved oxygen levels and eutrophication in receiving water bodies, moreover, it will also affect the suitability of wastewater for reuse (Kumar and Lin, 2010). Thus, nitrogen removal from wastewater is of extreme importance to protect water resources. Nowadays, it is proven that nitrogen compounds present in wastewater can be only effectively removed by biological processes (Zhu et al., 2008).

Many countries have enforced stringent nitrogen discharge standards in recent years (Du et al., 2015). For example, the recognition of the relevance of nitrogen content in waters has been concretized in some increasingly strict European regulations. The current European legislation establishes a maximum total nitrogen concentration in the discharge of urban wastewaters of 10 mg N L^{-1} and a minimum percentage of reduction of 80 %, which requires applying technologies for nitrogen removal in WWTPs, including in some cases post-treatment of the produced effluents (Dapena, 2007).

However, many conventional WWTPs have not been designed from the outset for nitrogen removal, as a consequence, many of them do not meet current discharge limits.

(Jetten et al., 2002; Karthik and Joseph, 2014). Having this in mind, several nitrogen removal processes have been developed. These technologies remain challenging due to multiple variables to be controlled related to the functional microorganisms for nitrogen removal, among other critical process parameters (Zhu et al., 2008). In the following lines, a description of the biological processes for nitrogen removal is presented.

1.2. Biological nitrogen removal (BNR)

Based on the microbial nitrogen cycle and the metabolism of inorganic nitrogen compounds, many biological technologies and processes have been developed and implemented for nitrogen removal from wastewater (Zhu et al., 2008). Regarding the environmental regulations, biological nitrogen removal (BNR) is one of the crucial processes in WWTPs (Cui et al., 2019).

1.2.1. Nitrogen cycle

Nitrogen is present in the atmosphere in its stable, non-reactive form, as N_2 gas. Reactive forms of this element (ammonium, nitrite, and nitrate) are essential for plant growth, although their content is limited in soils. Most of the natural reactive nitrogen comes from lightning (2%) and biological fixation (98%) (van der Hoek et al., 2018).

Different microorganisms change the nitrogen oxidation state carrying different metabolic reactions. According to Stein and Klotz (2016), currently, the nitrogen cycle consists of five accepted nitrogen-transformation flows: ammonification, including nitrogen fixation, and assimilatory and dissimilatory reduction of nitrite; nitrification; denitrification, including canonical, nitrifier-dependent and methane-oxidation-dependent denitrification; anammox, as a form of coupled nitrification-denitrification; and nitrite–nitrate interconversion. The general processes of organic matter mineralization and assimilation by cellular life complete reactive nitrogen through the biosphere is summarized in Fig. 1.1.

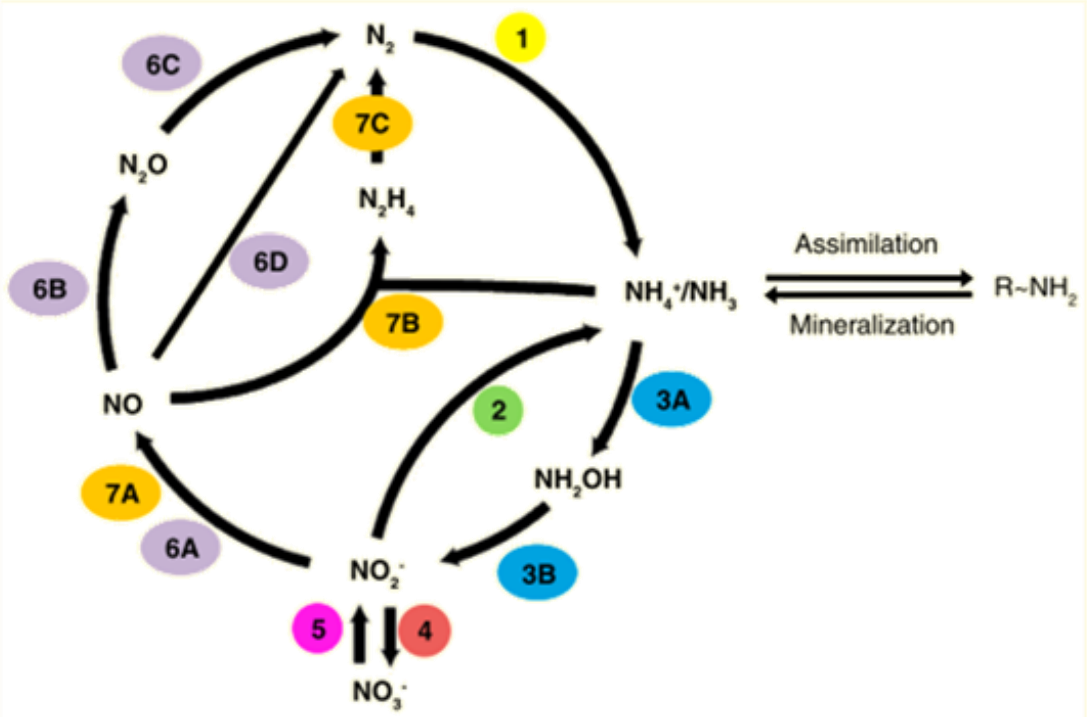

Figure 1.1.- Major processes of the nitrogen cycle. Reactions that comprise the seven major processes of the nitrogen cycle are represented by the numbered circles. Ammonification may be accomplished either by process 1, nitrogen fixation, or by process 2, dissimilatory nitrite reduction to ammonium (DNRA). Nitrification is composed of process 3, oxidation of ammonia to nitrite, and process 4, oxidation of nitrite to nitrate. Reduction of nitrate to nitrite (process 5), can be coupled to processes 2 (DNRA), 6 (Denitrification) or 7 (Anammox) in a population or in a community. Denitrification is shown as process 6. Anammox is shown as process 7. Adapted from Stein and Klotz (2016).

1.2.2. Conventional BNR: Nitrification-Denitrification

The conventional BNR process consists of two steps, based on autotrophic nitrification and heterotrophic denitrification (Zhu et al., 2008). On one side, nitrification consists of two sub-reactions catalyzed by physiologically distinct clades of microorganisms. In the first step, ammonia-oxidizing bacteria (AOB) oxidize ammonium (NH_4^+) to nitrite (NO_2^-) via hydroxylamine (NH_2OH). The ammonia oxidizing archaea (AOA), have also been discovered to harbor archaeal ammonia monooxygenase genes to encode corresponding enzyme for catalyzing ammonia oxidization (Ren et al., 2020). In the second step, nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate (NO_3^-).

On the other side, denitrification is an anoxic process where NO_3^- and NO_2^- are reduced to nitrogen gas using organic matter as electron donor (Ren et al., 2020; Zhu et al., 2008). The overall rate and extension of this process depend mainly on the biodegradability characteristics of the selected electron donor and on the final chemical oxygen demand (COD):N ratio in the bioreactor. For these reasons, adding readily biodegradable carbon sources, such as acetic acid and methanol can enhance the denitrification process. However, the addition of these carbon sources is expensive and increases the cost of treating the wastewater (Paredes et al., 2007).

Because different microorganisms carry out nitrification and denitrification under different conditions, the BNR process should be designed and operated in separated time sequences or spaces, needing more retention time, oxygen, and carbon source addition for a complete nitrogen removal. These limitations are the driving forces for developing new biological treatment processes for complete nitrogen removal (Jetten et al., 2002; Lee et al., 2001).

1.2.3. Autotrophic nitrogen removal

If the organic matter removal and nitrogen removal processes were independent treatments, all the organic matter present in the raw wastewater could be redirected to the anaerobic digestion process, and the subsequent energy recovery through biogas could be increased. (Reino, 2016). Therefore, autotrophic nitrogen removal, which can remove nitrogen without consuming organic matter in raw wastewater, is ideally considered a more sustainable way for wastewater treatment (Ma et al., 2011).

Autotrophic nitrogen removal can be achieved by autotrophic denitrification, photoautotrophic systems such as wetlands and ecological ditches (Wu et al., 2011), and anoxic ammonium-oxidizing (anammox) process. Among them, the anammox process is the most promising way of making wastewater treatment an energy-neutral or even energy-positive process (Kartal et al., 2010; Ma et al., 2011).

This process is carried out by an organism belonging to the order of the Planctomycetales (Strous et al., 1999). Although the Nitrosomonas species can also oxidize ammonium anaerobically, the term “anaerobic ammonium oxidation” and the abbreviation “anammox” are being used exclusively for nitrogen removal by these microorganisms from the phylum Planctomycete (Fux and Siegrist, 2004). Over the last

20 years, the autotrophic removal of nitrogen by anammox bacteria have emerged as a disruptive technology (J. Li et al., 2018).

1.2.4. Partial Nitrification/ Anammox

The discovery of anammox bacteria opens the possibility of performing autotrophic denitrification in urban WWTPs. The BNR via nitritation-autotrophic denitrification is a two-step process (Fig. 1.2). Firstly, half of the ammonium is oxidized to nitrite (partial nitritation) by aerobic ammonium oxidizing bacteria (AOB) under aerobic conditions. Secondly, the rest of the ammonium and the nitrite generated are directly converted to N_2 by the anammox bacteria without oxygen and organic matter consumption (Fux and Siegrist, 2004; Reino, 2016). When combined, nitritation and anammox are known as partial nitritation anammox (PN/A) systems (Van Hulle et al., 2010).

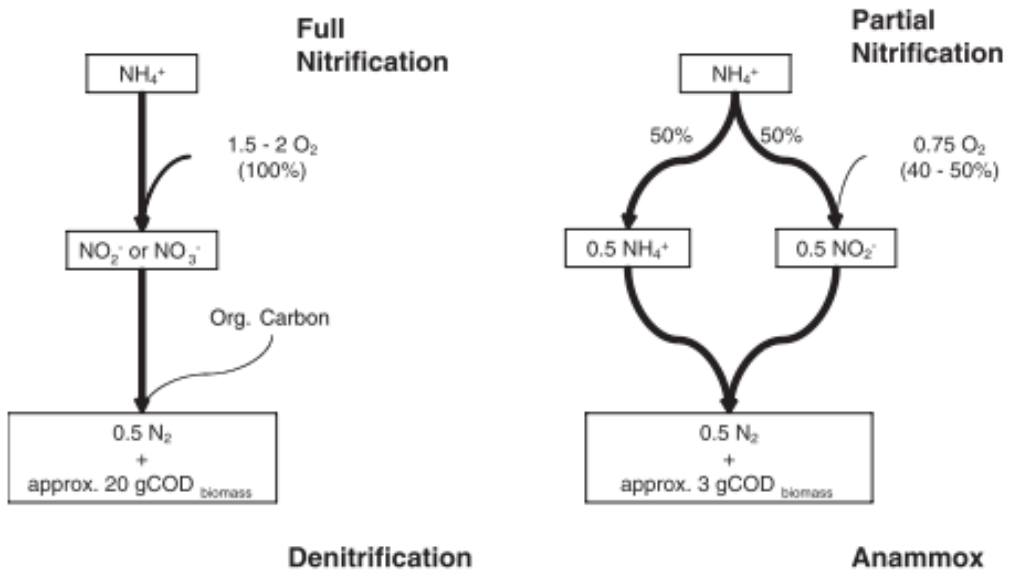

Figure 1.2.- Comparison of oxygen and organic carbon consumption of conventional nitrification/denitrification (left) and partial nitritation/anammox (right). Extracted from Fux and Siegrist (2004).

The application of anammox is contingent on the ability to shunt nitrification to nitrite effectively. Thereby, the balance between the different microbial groups involved is critical. Aside from growing and sustaining the slow-growing anammox bacteria, the balanced activity of aerobic AOB needs to be established in line with the suppression or

out-selection of NOB (Lackner et al., 2014). Several studies have proved that the PN/A process can be successfully applied for lab and full-scale designs for sidestream treatment in WWTPs due to the elimination of carbon demand for denitrification, that would result in having cost-effective benefits (Shourjeh et al., 2021).

Different PN/A configurations have been developed to achieve this synergy, including two-stage PN/A systems where nitritation and anammox occur in separate reactors or single-stage PN/A systems in which nitritation and anammox processes occur in a single reactor, being this process the most widespread approach for sidestream treatment (Bhattacharjee et al., 2017; Juan-Díaz et al., 2020).

Two-stage systems consist of two reactors in series, where partial nitritation is performed in a first aerobic reactor, followed by anaerobic ammonium oxidation in a second tank. With this configuration, the two biological processes can be controlled separately (Fux and Siegrist, 2004). Even though one-stage systems gained much interest due to the success reached in sidestream treatment and the lower investment costs, two-stage systems have been recognized as an excellent alternative to avoid the associated problems in terms of NOB proliferation by reduced nitrate production and of elimination of the exposure to oxygen of anammox bacteria. Thus, the anammox activity can be enhanced in the two-stage process, since the presence of oxygen was considered to inhibit the activity of anammox bacteria (Li et al., 2011). Based on the above, the two-stage process might have higher nitrogen removal rate than the one-stage process when dealing with wastewater at mainstream conditions (Juan-Díaz et al., 2020; Ma et al., 2011).

At present, these technologies for nitrogen removal have been worldwide implemented for treating industrial and sidestream wastewater at full scale (Lackner et al., 2014; Ni and Zhang, 2013). Moreover, Gonzalez-Martinez et al. (2018) have suggested that partial nitrification/anammox system is better in terms of efficiency than other autotrophic technologies since the rate of nitrogen removal is higher, the aeration required is lower, and nitrate concentrations in the effluent and nitrogen oxides emissions are lower. The application of the anammox process at mainstream conditions (low-strength ammonium concentrations and low temperatures at winter season) appears as a prerequisite for the implementation of a two-stage system for autotrophic BNR in urban WWTPs (Reino and Carrera, 2017).

1.3. Anammox process and microbiology

1.3.1. Mainstream anammox

Anammox process has been widely used for nitrogen removal from ammonium-rich wastewater for sidestream treatment of WWTPs and for industrial wastewater, including sludge liquor, landfill leachate, and pharmaceutical effluents (Hoekstra et al., 2019; Kartal et al., 2010; Lackner et al., 2014). However, its full-scale application is still relatively restricted to wastewaters from mesophilic anaerobic digesters, especially in the reject water line of WWTPs, due to the process limitations as temperature range and slow start-up. In fact, Lackner et al. 2014 reported that about 75% of the full-scale PN/A reactors were operated for sidestream treatment of municipal wastewater (MWW). Even so, more than 200 full-scale facilities mainly for the sidestream of municipal wastewater treatment and industrial wastewater treatment have been operating successfully in Europe and Asia (Yeshi Cao et al., 2017; Fernández et al., 2016; Lackner et al., 2014).

Recent research on autotrophic nitrogen removal has been focused on PN/A implementation in the mainstream line of urban WWTP, encouraged by the intention of achieving energy-neutral plants (Akaboci et al., 2018; Reino et al., 2018). Introduction of mainstream PN/A process enables the decoupling of carbon and nitrogen removal and maximizes energy recovery through carbon concentrating pretreatment process that channels more carbon to anaerobic digester (or an up-flow anaerobic sludge blanket (UASB) reactor) for biogas generation (Fig. 1.3) (Yeshi Cao et al., 2017).

In the last years, several studies have been focused on laboratory and pilot-scale research on the mainstream application of the anammox process to demonstrate its feasibility (Hoekstra et al., 2019; Laureni et al., 2016; Lotti et al., 2015; Reino, 2016). However, their potential for the direct treatment of MWW is still a challenge (X. Li et al., 2018). The main challenges of mainstream applications relate to low nitrogen concentrations, variable nitrogen loads, low temperatures, stringent effluent quality requirements, and long-term process stability (Laureni et al., 2016).

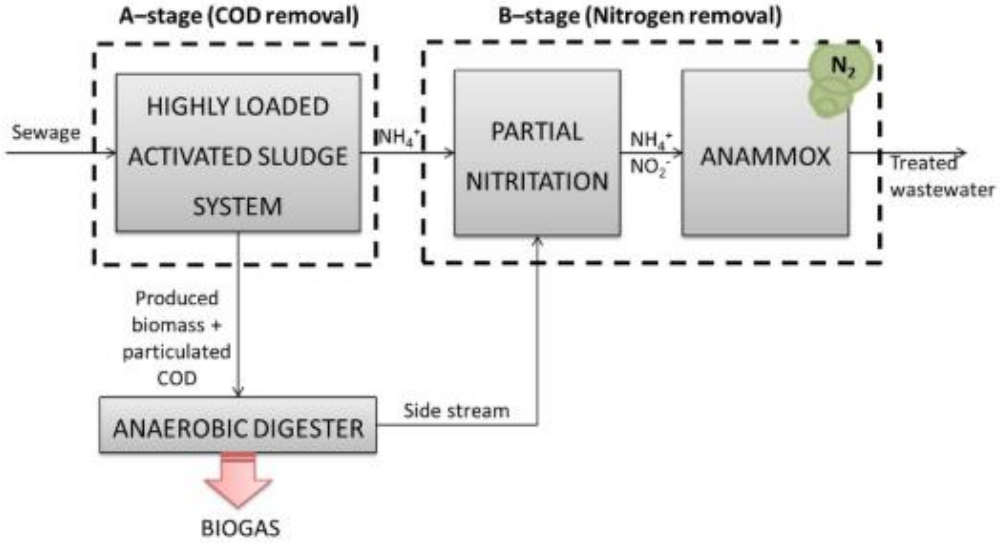

Fig 1.3.- Scheme of the implementation of autotrophic biological nitrogen removal process in the main water line of an urban wastewater treatment plant using a two-stage partial nitritation anammox system. Adapted from Reino et al., 2016.

The laboratory and pilot-scale results indicate that the main challenge of implementing the technology is the low water temperatures during winter, which can substantially affect the anammox growth rate and potentially interrupt the microbial community structure (Laureni et al., 2016; J. Li et al., 2018). At low temperatures (below 15ºC), it has been reported that the total conversion rates decrease as a consequence of the accumulation of substrates (ammonium and nitrite) due to the decrease of anammox activity, leading to a loss of stability in the system (Yeshi Cao et al., 2017; González-Martínez et al., 2018; Hoekstra et al., 2019). In a recent study, Juan-Díaz et al. (2020) demonstrate the effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater by roughly maintaining stable removal rates in a long-term operation process.

1.3.2. Anammox bacteria

The anammox process in which ammonium is oxidized under anaerobic conditions was discovered by serendipity in a denitrifying pilot plant (Güven et al., 2004; Mulder et al., 1995). Initially, it was unknown whether nitrite or nitrate was used as an electron donor. Later it was found that ammonium and nitrite are reacting and release nitrogen gas into the N-cycle (Mulder, 2003).

The process is mediated by obligately anaerobic chemolithoautotrophic bacteria (Schouten et al., 2004). Known species are divided over six genera, all belonging to the same order Brocadiales, that form a monophyletic clade, deeply branching inside the Planctomycetota phylum. So far, the anammox genus has received the *Candidatus* (*Ca*.) status. The term is used for microorganisms that have yet to be isolated and cultivated by classical microbiological methods (Konstantinidis and Rosselló-Móra, 2015; Pereira et al., 2017).The known species of anammox bacteria are listed in Table 1.1. All genera have been detected in different wastewater treatment systems, although only one genus (*Ca.* Scalindua) seems predominant in marine ecosystems ranging from arctic to tropical regions (Ali et al., 2013; Hu et al., 2010; Pereira et al., 2017).

Table 1.1.- Anammox bacteria genera and species most studied in literature.

Genus	**Species**	**Reference**
Ca. Kuenenia	*Ca.* K. stuttgartiensis	Strous et al. (2006)
Ca. Brocadia	*Ca.* B. anammoxidans	Strous et al. (1999)
	Ca. B. fulgida	Kartal et al. (2008)
	Ca. B. sinica	Oshiki et al., 2016
	Ca. B. caroliniensis	Park et al. (2017)
	Ca. B. sapporoensis	Narita et al. (2017)
	Ca. B. brasiliensis	Araujo et al. (2011)
	Ca. B. *barcinensis*	Oyarzúa et al. (2021)
Ca. Anammoxoglobus	*Ca.* A. propionicus	Kartal et al. (2007)
Ca. Jettenia	*Ca.* J. asiatica	Quan et al. (2008)
	Ca. J ecosi	Botchkova et al. (2018)
	Ca. J. caeni	Ali et al. (2015)
Ca. Scalindua	*Ca.* S. wagneri	Schmid et al. (2003)
	Ca. S. brodae	
	Ca. S. sorokinii	Kuypers et al. (2003)
Ca. Anammoximicrobium	*Ca.* Anammoximicrobium moscowii	Khramenkov et al. (2013)

1.3.3. Anammox metabolism

In anammox bacteria, ammonium and nitrite are processed in the anammoxosome (a central cellular compartment), where nitrite is reduced to nitric oxide (NO) and followed by hydrazine (N_2H_4) synthesis (Strous et al., 2006, 1999). The oxidation of hydrazine results in the formation of the end product (N_2) and electrons that are invested both in electron-transport phosphorylation and in the regeneration of the catabolic intermediates (N_2H_4, NO). Next to this, the electrons provide the reducing power for CO_2 fixation. In addition, some nitrite is oxidized to nitrate by a nitrate/nitrite oxidoreductase (*NarGHI*) to supply the cells with sufficient reducing equivalent for carbon dioxide fixation via the acetyl-coenzyme A pathway (Kartal et al., 2012). An analysis of mass balances showed that these organisms use carbon dioxide as their carbon source to produce biomass (Kartal et al., 2011).

Anammox bacteria can also consume organic compounds to sustain their metabolism, as formate, acetate, and propionate, which are completely oxidized to CO_2 (Kartal et al., 2007). The former two can employ already available acetyl-CoA pathway enzymes for CO_2 fixation.

To date, different pathways for nitrogen metabolism have been described (Fig. 1.4). The first was proposed by Strous et al. (2006) from the genome sequence of *Ca*. *K*. stuttgartiensis and it was experimentally supported by Kartal et al. (2011). These studies showed that nitrite is reduced to nitric oxide by a cytochrome cd1-type nitric oxide/nitrite oxidoreductase (nirS). Then a hydrazine synthase enzyme (*hzs*) produces hydrazine by coupling nitric oxide and ammonium, and finally, hydrazine is oxidized to produce dinitrogen gas by a hydrazine oxidoreductase (*hzo* or *hdh*) (Han et al., 2017; Harhangi et al., 2012).

An alternate pathway is the nitric oxide production through hydroxylamine (NH_2OH) oxidation by a hydroxylamine oxidoreductase (*hao*)-like protein, which could be activated under nitrite limitation. Similar protein-coding sequences have been previously found in *Ca*. Kuenenia, *Ca*. Scalindua, and *Ca*. Jettenia (Irisa et al., 2014; Park et al., 2017).

Another versatile trait of anammox bacteria metabolism includes denitrification using selected organic compounds, nitrate, and dissimilatory nitrate reduction to ammonia (DNRA) (Kartal et al., 2007). This pathway enables anammox bacteria to use organic

compounds and nitrate in the absence of ammonium, leading to three reactions: nitrate reduction to nitrite; dissimilatory reduction of nitrite to ammonium; and anammox reaction with ammonium and nitrite to form dinitrogen gas (Park et al., 2017).

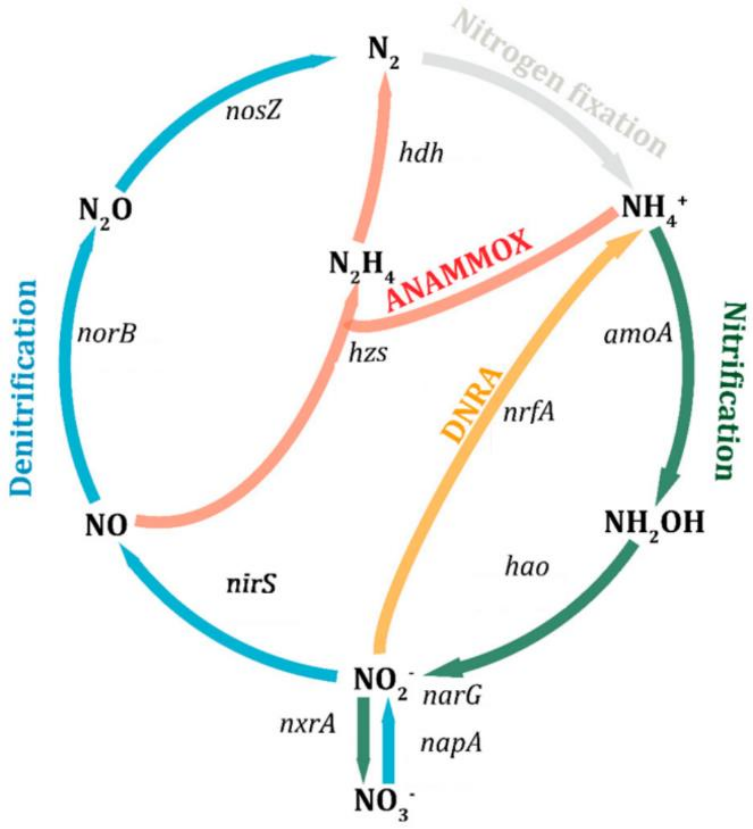

Figure 1.4. Nitrogen metabolic pathways and its related enzymes. amoA, bacterial ammonia monooxygenase subunit A; hzo, hydroxylamine oxidoreductase; narG, cytoplasmic nitrate reductase alpha subunit; napA, periplasmic nitrate reductase precursor; nrfA, ammonia-forming dissimilatory nitrite reductase; nxrA, nitrite oxidoreductase alpha subunit; nirS, cytochrome cd1 nitrite reductase; norB, nitric oxide reductase subunit B; nosZ, nitrous oxide reductase. Modified from Meng et al. (2019).

1.3.4. Growth physiology of anammox bacteria

Culturing anammox bacteria has been a challenge due to their long doubling times. Typical anammox doubling times in anammox reactors are 15-30 days at mesophilic temperatures (Fux and Siegrist, 2004; Strous et al., 1998). Nevertheless, lower doubling times have also been reported, with values as low as 3.3 days in a membrane

bioreactor at 30 ºC (Lotti et al., 2014). Therefore, culture techniques in which biomass is retained effectively at low substrate concentration, as is found in natural habitats, are required (Kartal et al., 2012). This proves that the versatility of anammox is far beyond the known metabolic pathways. Differences in physiological characteristics are important key factors to understand niche differentiation among anammox bacteria living in a wide variety of environments. In this sense, recent genomic studies of anammox bacteria are now providing an excellent opportunity to investigate the physiological characteristics of anammox bacteria (Narita et al., 2017; Oshiki et al., 2016).

Some of these characteristics have been determined from enriched cultures of five genera (excluding *Ca*. Anammoximicrobium) and for some species from *Ca.* Brocadia genus, including optimal growth temperature, optimal pH, biomass yield, substrate affinity, among others.

- **Temperature**

In the natural environment, anammox bacteria can be found in low (−5 to 4 ◦C) and high (60 to 80 ◦C) temperature ranges. This acclimation to a wide range of temperatures enables their applicability to various wastewater treatment systems. Nevertheless, the fluctuation in temperature in a bioreactor can change the physical response of anammox and affects nitrogen removal efficiency (Cho et al., 2019).

- **Substrate affinity**

The substrate affinity has a role in niche differentiation. For example, *Ca.* B. sinica would prefer environments rich in ammonia and nitrite because has low affinities for both substrates. Conversely, *Ca.* K. stuttgartiensis has higher affinity constants for NH_4+ and NO_2−, but the growth rate is lower than that of *Ca.* B. sinica. These physiological characteristics indicate that *Ca.* B. sinica and *Ca.* K. stuttgartiensis are r- and k-strategists respectively (Narita et al., 2017; Oshiki et al., 2016). The r-strategists microorganisms are defined by a fast growth rate at the high substrate availability and under low competition level with other microorganism, whereas K-strategists grow faster when they are faced with limited substrate concentrations and are exposed to the strong competitiveness (Shourjeh et al., 2021)**.**

- **pH**

The importance of the pH control relies on the fact that this would determine the dominant anammox bacteria in the anammox process. Some studies found that pH ranging from 6.5 to 8.3 can support growth and activity of anammox bacteria, while others suggested that a pH range of 7-8 is suitable to anammox and seems to be the ideal range for avoiding the inhibition of anammox by high free ammonia and free nitrous acid. For example, *Ca.* B. anammoxidans and *Ca.* A. propionicus were observed to be dominant in the enrichment from aerobic granules and leachate sludge, respectively, when the pH of the enrichment culture was controlled from 6.8–7.0. However, *Ca.* B. anammoxidans and *Ca.* K. stuttgartiensis were found predominantly in anammox systems with a pH of 7.8−8 (Cho et al., 2019; Oshiki et al., 2016).

- **Operational conditions regulating the anammox process**

Since anammox bacteria are phylogenetically, metabolically, and physiologically diverse, more enriched cultures are needed to improve our understanding of their ecology, physiology, and biochemistry. Environmental factors play an essential role in triggering high bacterial growth rates. Thus, high nitrogen removal performance of the anammox process can be enhanced by optimizing the operating conditions and limiting environmental stresses (Cho et al., 2019; Kartal et al., 2012).

Some studies on anammox technologies have reported that there would be one genus of anammox bacteria dominant under specific growth conditions (Kartal et al., 2008, 2007). For example, *Ca. K.* stuttgartiensis could adapt to effluent with salinity up to 3%, and *Ca.* B. fulgida or *Ca.* A. propionicus could be favored in their growth by the addition of organic acids such as acetate or propionate. Consequently, they suggested that in WWTPs, environmental and operational conditions may favor the enrichment of different anammox bacteria (Gonzalez-Martinez et al., 2018).

- **Granule formation**

The formation of granular sludge is considered an effective means to overcome the adverse effects caused by various environmental conditions and diverse inhibitors during the anammox process (Ali et al., 2013; Cho et al., 2019; Ni et al., 2010). In fact, anammox bacteria can secrete extracellular polymeric substances (EPS), which ease granules formation (Tang et al., 2017). Some authors suggested that the high performance and stability of anammox reactors largely depend on the quantity of granular sludge (Tang

et al., 2017, 2011). The mature active anammox granules are red in color due to the presence of heme c content. Heme c is an important constituent of enzymes hydroxylamine oxidoreductase and hydrazine oxidoreductase in anammox process (Fig. 1.5) (Manonmani and Joseph, 2018).

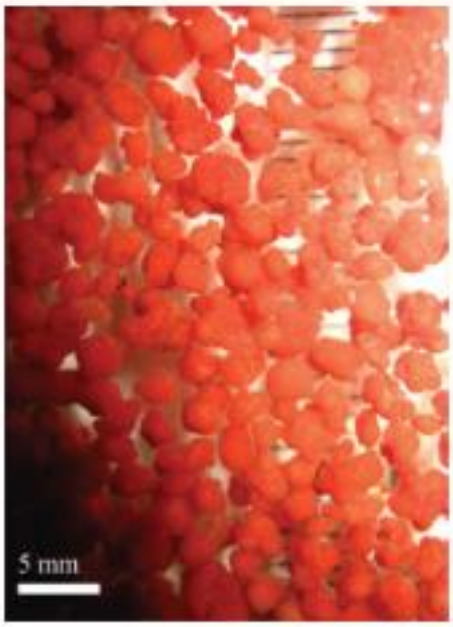

Figure 1.5. Anammox granules, adapted from Ali et al. (2013).

Besides anammox bacteria, several studies have been surveyed the microbial community in anammox reactors and have detected diverse 'non-anammox' microorganisms. These microbial communities usually include microorganisms performing nitrification and denitrification, as well as various heterotrophs. In this sense, it has been reported that anammox bacteria thrive by competition and by metabolic interactions with these organisms. Therefore, they may play important roles in such systems (Kartal et al., 2012; Lawson et al., 2017; Pereira et al., 2017).

The microorganisms belonging to the phylum Chloroflexi, Chlorobi, Proteobacteria, Acidobacteria, and Bacteroidetes comprise 30–70% of the anammox consortia. The members of the anammox consortia would share symbiotic relationships, which might be helpful for anammox metabolism, including growth factor supply or anammox metabolite scavenging. Although this phenomenon has been observed for a long time, the potential roles of these symbiotic bacteria in the consortia are still inconclusive (Lawson et al., 2017; Zhao et al., 2018). In addition, communities in anammox reactor have shown less diversity than granules and flocs found in other

wastewater treatment systems such as anaerobic reactors used to treat wastewater from industry and activated sludge systems. This suggests that a highly specialized community with dominant phylotypes is formed in anammox reactors because less diverse electron donors and acceptors are available in these systems compared to others (Pereira et al., 2017).

To date, significant changes to the design and operation of anammox-based systems have been done predominantly from an engineering perspective, vastly underestimating the importance of microbial communities as an integral component of these systems (Chu et al., 2015). Nevertheless, the performance of the wastewater treatment process is based on the exploitation of the concerted activity of the microorganisms involved. Thus, the knowledge of the microbial community structure, coexistence patterns, and the links to the changing environmental conditions is crucial for the development and optimization of biological systems by engineers, such as in the anammox process (Ferrera and Sánchez, 2016; Vlaeminck et al., 2012).

1.4. How to study the microorganisms involved in the anammox process?

Until now, microorganisms responsible for the anammox process could not be obtained in pure cultures, therefore, culture-independent methods must be used.

To date, different molecular biology techniques have been developed to study the microbial communities involved in the anammox process, including fluorescent *in-situ* hybridization (FISH), quantitative PCR, amplicon sequencing, and metagenomics (Ferrera and Sánchez, 2016; Schmid et al., 2005; Yang et al., 2020).

1.4.1. Fluorescent *in situ* hybridization (FISH)

This technique uses fluorescently-labeled oligonucleotide probes for the detection of 16S rRNA within prokaryotic cells. Microbial cells are first treated with appropriate chemical fixatives and then, hybridized under stringent conditions on a glass slide or in a solution with oligonucleotide probes (Fig. 1.6). Generally, these probes consist of short DNA sequences (usually 15-25 nucleotides in length), and they are designed to specifically hybridize to their complementary target sequence on the rRNA structures (16S and 23S subunits are typically used for Bacteria and Archaea) in the target cell. After

stringent washing, specifically stained cells are detected via microscope visualization (Nielsen et al., 2009; Wagner et al., 2003). A typical flowchart for FISH in activated sludge or biofilm is shown in Fig. 1.6, extracted from Nielsen et al. (2009), and includes sampling, possible pretreatment, fixation, possible enzyme treatment, hybridization, epifluorescence microscopy, and possible image analysis. The method makes possible to retrieve information on phylogenetic identities of the cells directly in a sample within a relatively short time. Since it also keeps the morphology of the cells, it could supply information on the spatial distribution and the number of identified organisms.

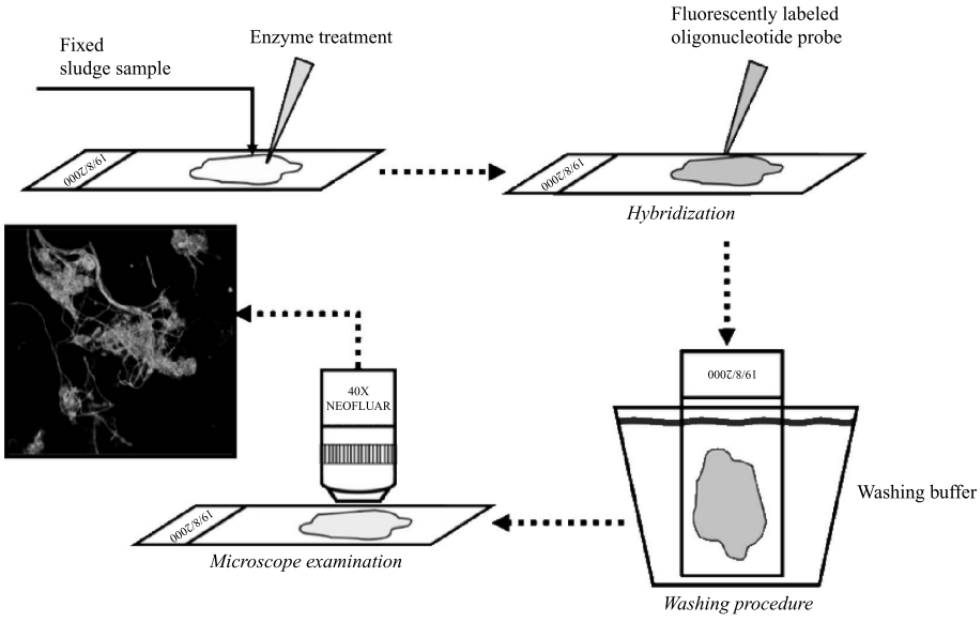

Figure 1.6. Schematic illustration of the FISH approach (Nielsen et al., 2009)

1.4.2. quantitative Polymerase Chain Reaction (qPCR)

The most substantial milestone in Polymerase Chain Reaction (PCR) utilization was introducing the concept of monitoring DNA amplification in real-time through the monitoring of fluorescence (Higuchi et al., 1992). In real-time PCR (also denoted as quantitative PCR or qPCR), fluorescence is measured after each cycle, and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time. In initial cycles, the fluorescence is too low to be distinguishable from the background. However, the point at which the fluorescence intensity increases above the detectable level corresponds proportionally to the initial number of template

DNA molecules in the sample. This point is called the threshold cycle (Ct). It allows the determination of the absolute quantity of target DNA in the sample according to a calibration curve constructed of serially diluted standard samples (usually decimal dilutions) with known DNA concentrations or DNA copy numbers. These values are then plotted against the amount of target in the standards to produce a calibration curve, and the amount of target in the unknown samples can be then interpolated from the graph (Fig. 1.7) (Kralik and Ricchi, 2017).

With the advent of PCR, also vanishingly small amounts of RNA, converted into cDNA by the reverse-transcription reaction, could be amplified. This amplification poses a challenge for accurately quantifying the initial amount of cDNA. Nevertheless, this technique has become the gold standard for measuring gene expression. Thus, accurate analysis of qPCR data is crucial for optimal results, and some well-defined methods are in use to calculate gene expression (Adamski et al., 2014).

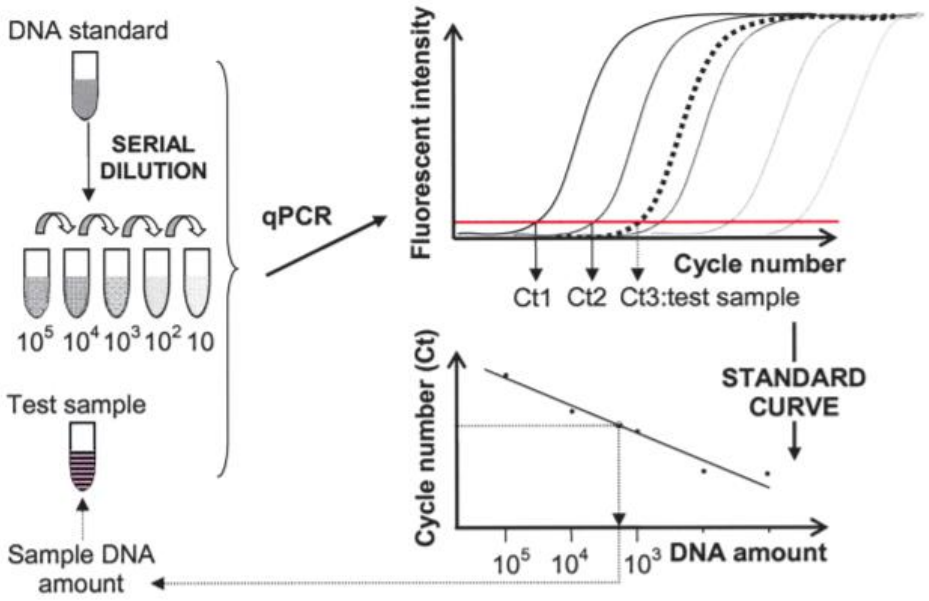

Figure 1.7.- schematic representation of the basis of real-time PCR using a quantitative standard. From (Keer, 2008)

1.4.3. High-throughput sequencing (HTS)

High-throughput sequencing technologies, also called next-generation sequencing (NGS) involve massively parallel sequencing of a number of templates of the same

sample in a single run and produce an enormous volume of data in an economic way (Ambardar et al., 2016). Recent progress in NGS and the rapid development of bioinformatics tools allow research groups of any size to generate large amounts of genomic sequences for organisms of interest (Young and Gillung, 2020). Different sequencing platform technologies have been commercialized; however, sample sequencing research is nowadays often performed on the Illumina platform (Liu et al., 2020).

The Illumina sequencing platform works as follows. The first step is the DNA library preparation, which involves random fragmentation of the template DNA and the ligation of oligonucleotide adaptors. Then, both forward and reverse primers, with complementarity to the adaptor, are attached to a flow cell by a flexible linker. The adaptor flanked DNA fragments are hybridized onto the forward and reverse primers attached to the flow cell (Fig. 1.8-a). A reverse strand of the DNA fragment is synthesized, the double-strand DNA molecule is denatured, and the original template is washed away (Fig. 1.8-b). The DNA amplification strategy involved is referred to as Bridge PCR, where the strand folds over, and its free end binds to the nearest oligo, the complementary DNA strand is synthesized, forming a double-stranded bridge, and then, the bridge is denatured, forming two single-strand DNA (ssDNA) (Fig. 1.8-c). The process is repeated over and over, resulting in a "cluster" of clonal amplicons. Amplicons produced from a single DNA fragment will cluster in a single physical location on the array (Fig. 1.8-d). Following cluster generation, the sequencing primer hybridizes to the universal sequence flanking the region of interest (Fig. 1.8-e). Sequencing then proceeds in cycles with a modified DNA polymerase and four nucleotides. Nucleotides are labeled with a chemically cleavable fluorescent reporter group at the 3′-OH end, thereby allowing only single base incorporation in each cycle. Each cycle extends a single base followed by the chemical cleavage of the fluorescent reporter that will identify the incorporated nucleotide. The activated fluorescence is detected by a camera and recorded on a computer (Yu Cao et al., 2017; Young and Gillung, 2020).

Only Illumina NGS platforms are capable of paired-end sequencing which allows users to sequence the DNA fragment from both ends resulting in high coverage, high numbers of reads, and more data as compared to single-end sequencing systems (Ambardar et al., 2016)

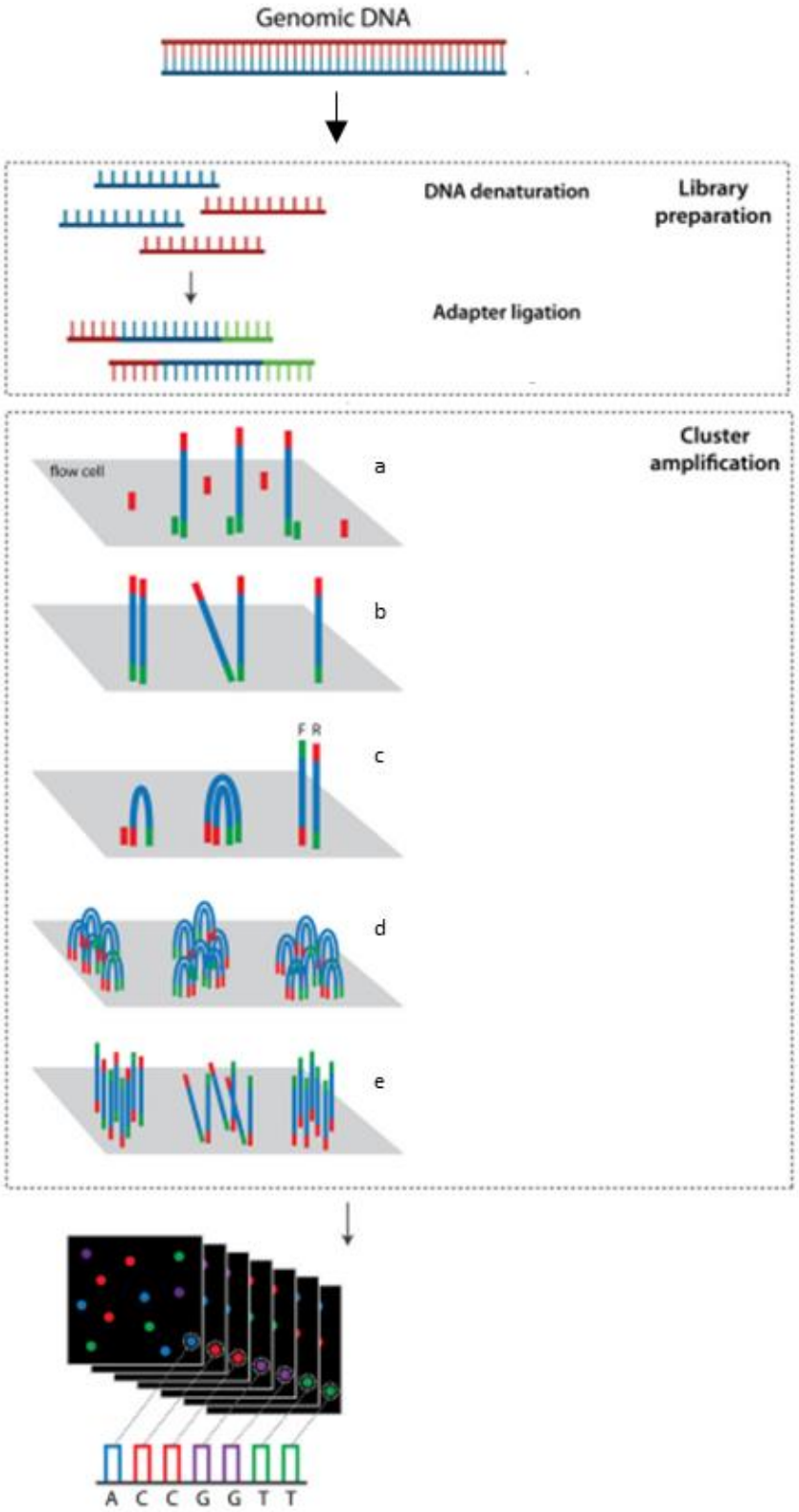

Figure 1.8.- Schematic representation of Illumina sequencing process, modified from Young and Gillung (2020)

1.4.3.1. HTS application on 16S rRNA sequencing

Most microbiome studies using NGS technologies have relied on a single gene, the 16S small subunit ribosomal RNA (rRNA), called 16S rRNA gene amplicon sequencing. There are nine different variable regions within the prokaryotes ubiquitous 16S rRNA gene (V1-V9), each one flanked by highly conserved stretches of DNA suitable for primer binding (Clooney et al., 2016; Poretsky et al., 2014). Sequencing of hypervariable sub-regions of this gene produces single short-reads of 100-500 nucleotides when the gene contains 1542 base pairs, missing variations out of sequencing area (Fig 1.9-A). Although some sub-regions provide a good approximation of 16S rRNA gene diversity, they allow genus identification in most cases but less so concerning species. On the other hand, a key limitation of using 16S rRNA gene amplicon sequencing is that related bacterial species may be indistinguishable due to not distinguish polymorphisms between closely related taxa sequences, which could be avoided by the complete sequencing of the gene (Bailén et al., 2020; Johnson et al., 2019).

Unfortunately, no standard approach exists for selecting the most appropriate primer pair suitable for all taxa and type of samples, and the decision is often made based on availability of the primers by the sequencing platforms, anecdotal evidence and/or advice from the published literature (Clooney et al., 2016).

PCR- amplified 16S rRNA gene sequences have typically been clustered based on similarity to generate operational taxonomic units (OTUs) and representative OTU sequences compared with reference databases to infer likely taxonomy. In general, OTUs are analyzed at the phyla or genera level and can be less precise at the species level (Ranjan et al., 2016). OTU- based approaches needed certain assumptions, e.g., the now historical assumption that sequences of> 95% identity represent the same genus. Instead, sequences of> 97% identity represent the same species and thus do not allow us to discriminate between closely related but different taxa (Johnson et al., 2019; Pérez-Cobas et al., 2020).

Several methodologies and software packages have been developed to improve the analysis of high-throughput sequencing data outputs due to the growing interest and importance of biodiversity analysis in microbial ecology. Therefore, recently, non- OTU-based methods have been developed to determine exact features named amplicon sequence variants (ASVs), which resolves the differences of as little as one nucleotide (Pérez-Cobas et al., 2020).

1.4.4. Whole Genome shotgun sequencing (WGS)

An alternative approach to the 16S rRNA amplicon sequencing method is whole genome shotgun sequencing (WGS) which uses sequencing of all the DNA fragments without primers bias (Fig 1.9-B). The metagenome approach is commonly used to describe microbial communities in different systems without the biases inherent to PCR amplification of a single gene, although it remains challenging to infer taxonomic origin from metagenomic reads (Poretsky et al., 2014). The major advantages of the WGS method are that the taxa can be more accurately defined at the species level (Ranjan et al., 2016).

Illumina sequencing technology, which is the most widely used sequencing method for metagenomics experiments today, generates read lengths in the range of 100-250 bp, with a typical sequencing run producing tens of millions of reads. Depending on the number of reads and the complexity of the microbial species in the sample, some genomes might be sequenced deeply, allowing the experimenter to try to assemble the original genome sequence, or parts of it, from the short reads (Breitwieser et al., 2017).

In a first step, genomic DNA is sheared into small random fragments. Depending on the technology, these are sequenced independently to a given length. Powerful computer algorithms are then utilized to piece the resulting sequence reads back together into longer continuous stretches of sequence (contigs), a process known as *de novo* assembly. Usually, longer fragments (several hundred base pairs) are sequenced from both ends (paired-end sequencing) to provide additional information on correct read placement in the assembly. After the initial assembly, contigs are typically joined to form long stretches of sequence (known as scaffolds). To achieve this, libraries from long DNA fragments spanning several kilobases (kb) of sequence in the genome are prepared and their endpoints sequenced (Ekblom and Wolf, 2014).

In general, there are two approaches for WGS metagenomics data analysis: read-based and assembly-based metagenomics. The former aims to classify single reads with regard to taxonomy and function. In the second, reads are first de novo assembled to contigs and hereafter clustered into so-called genome bins during a binning process. Thereby, it is possible to reconstruct genomes of highly abundant taxa from a metagenomic sample. For this purpose, the corresponding workflow includes an assembler that is well suited for the reconstruction of long contigs and a genome binner

to cluster such sequences from the same organism. For taxonomic profiling, the assembly is followed by taxonomic classification. If the metabolic potential is of special interest, a gene prediction, functional annotation, and metabolic reconstruction are done on assembled contigs. It is to be noted that binning, in terms of generally separating sequences into groups, is, of course, not limited to assembled contigs per se and can also be performed on unassembled reads (Jünemann et al., 2017)

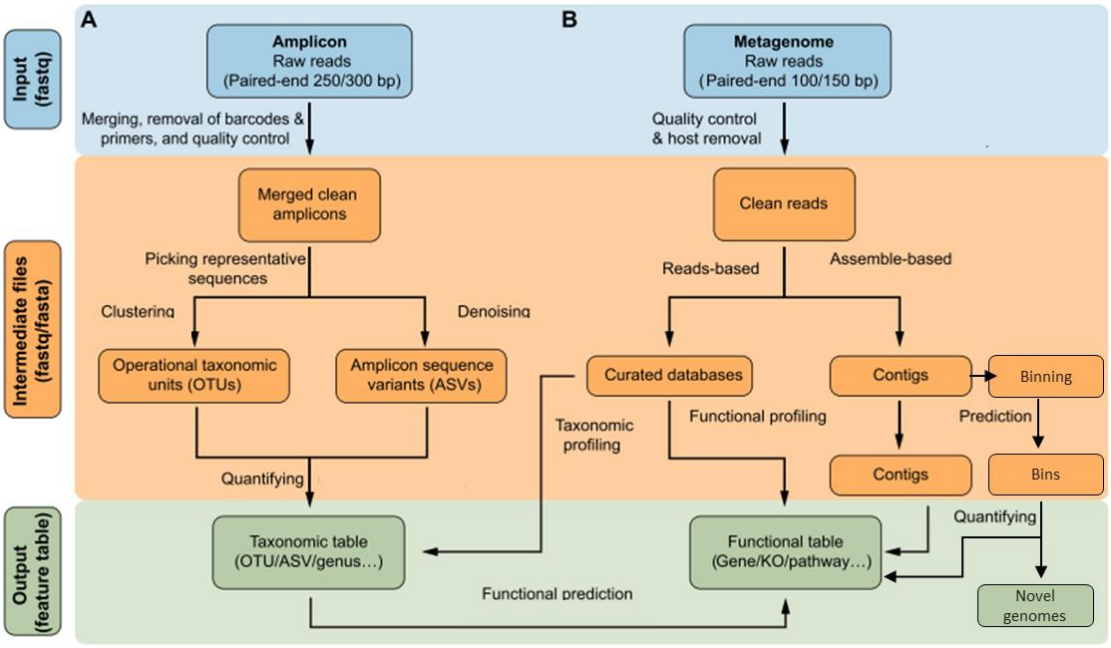

Figure 1.9.- Workflow of commonly used methods for amplicon (A) and metagenomic (B) sequencing. Adapted from Liu et al. (2020).

1.5. Motivations of this thesis

The project "Towards the implementation of the concept of biorefinery and energy self-sustainability in an urban wastewater treatment plant" (HIPATIA, for its acronym in Spanish) aims to redesign urban wastewater treatment by reducing the energy consumption to achieve a WWTP close to energy self-sufficiency or even a net energy producer, also reducing the carbon footprint of the WWTP through the design of mitigation strategies for N_2O emissions, and introducing the concept of biorefinery through the recovery of phosphorus (in the form of struvite) and hydrogen production.

To reach these goals, the proposed WWTP configuration uses anammox in the main water line to achieve an autotrophic nitrogen removal that allows maximizing the use of organic matter for energy production (biogas and hydrogen).

Thus, the project included regarding anammox process the development of the autotrophic nitrogen elimination in two stages, separating the partial nitritation from the anammox process to separately optimize each process and to provide stability in operation at low temperatures and the use of a small fraction of the wastewater to denitrify the nitrate produced by anammox (Fig. 1.10). All the above, conducted at a laboratory scale treating real wastewater from a WWTP.

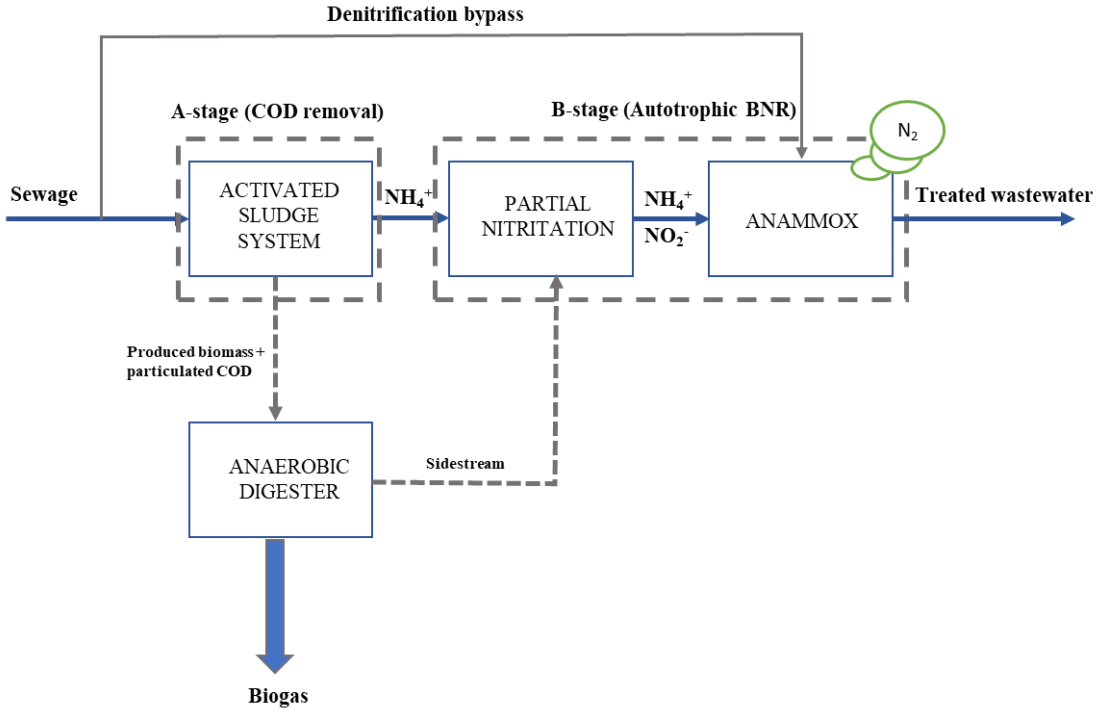

Figure 1.10. Scheme of the implementation of autotrophic biological nitrogen removal process in the main water line of an urban wastewater treatment plant for HIPATIA project. Modified from HIPATIA technical proposal.

In previous works of the research group, it has been demonstrated the feasibility to remove nitrogen using anammox process at low temperatures treating low-strength synthetic wastewater in a lab-scale Up-flow Anammox Sludge Blanket (UAnSB), achieving operate at 11ºC for more than six months (Reino et al., 2018; Reino and Carrera, 2017). Then, during the development of the HIPATIA project, Juan-Díaz et al. (2020), demonstrated the effective dampening of temperature effects in an anammox UAnSB lab-scale reactor treating real mainstream wastewater.

The previous results were obtained following the process from an engineering point of view. Although the microbial communities were studied, we observed a low resolution of the molecular/microbiological techniques applied to follow the process, leading to a high percentage of unknown Planctomycetes detected with amplicon sequencing, for example. In addition, macroscopical differences were observed between anammox granules from an enriched anammox culture (used as inoculum for several studies in the research group) and the granules of the reactor working at mainstream conditions using real wastewater (Fig. 1.11). According to this, and to the fact that most of the microbial communities' studies have been performed using synthetic wastewater, further research was needed to determine the whole microbial and the anammox community, its dynamics, and their potential interactions in the granular-sludge anammox reactor working at mainstream conditions and low temperatures.

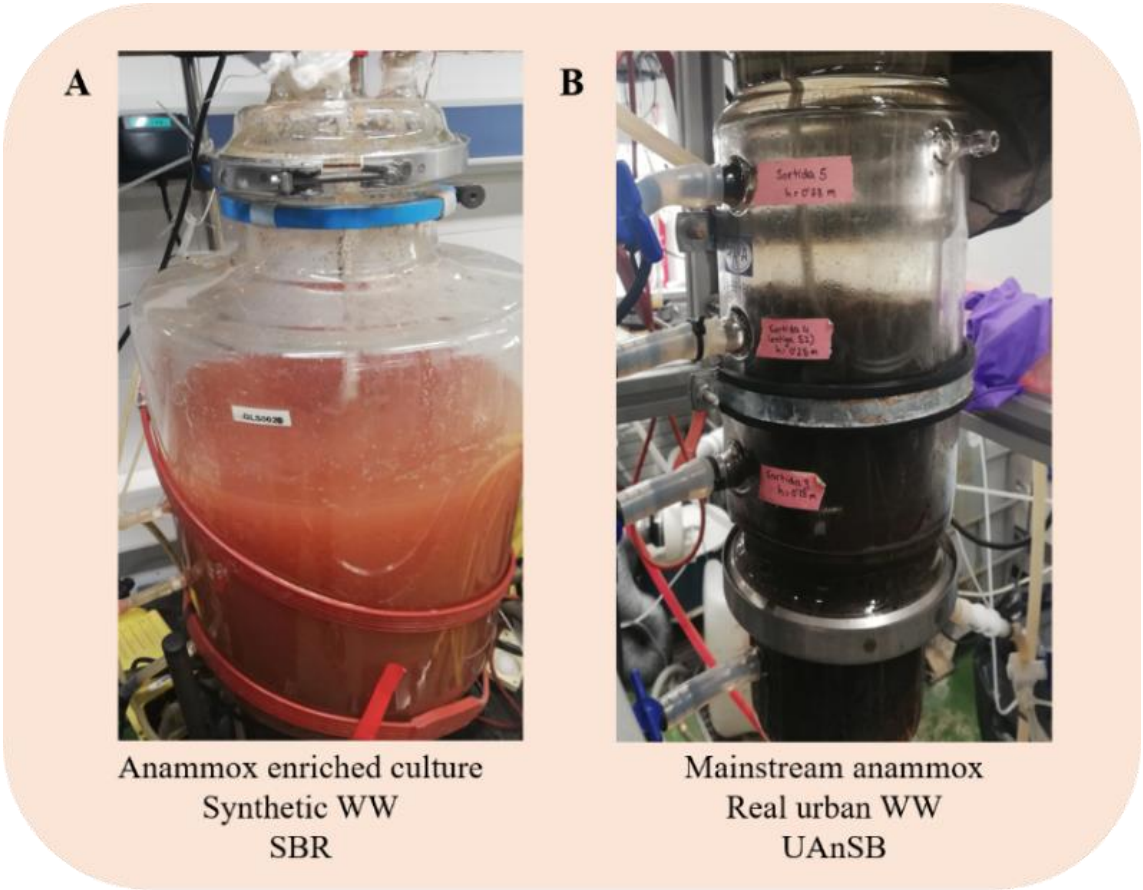

Figure 1.11.- Granular -sludge anammox reactors of this thesis. A) treating synthetic WW, B) treating mainstream line WW.

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Chapter 2

Objectives

This research has been developed within the framework of the HIPATIA project, which aims to redesign the current treatment of municipal wastewater and proposes the use of the anammox process in the main water line to achieve a stable autotrophic nitrogen removal at low temperatures. In this sense, a parallel investigation was studying how to reach the operational stability and proving an effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater.

Based on previous operational results, we hypothesized that the microbial communities of the anammox reactor change its composition and dynamics when operating at mainstream conditions with real wastewater.

Therefore, the main objective of this thesis is to know the changes in the microbial community of an anammox reactor when working at mainstream conditions (urban wastewater and low temperature) to understand the performance of the system during the application of the process.

More specifically, the goals of this thesis were:

- To characterize and analyze the bacterial communities involved and existing synergies in an anammox reactor during different phases of operation.
- To determine the dynamics of active bacterial communities, including anammox, during the different phases of reactor operation.
- To analyze and set up the possible metabolic roles of the bacterial communities involved in the elimination of nitrogen in anammox reactors.

Chapter 3

Materials and methods

3.1. Anammox reactors description

Samples were obtained from two different reactors. The first reactor corresponds to an anammox sludge sequential batch reactor (SBR) working at 35 ºC under stable conditions for more than six years (Isanta et al., 2015). This SBR was fed with a synthetic influent (180±30 mg $N\text{-}NH_4^+$ L^{-1}, 190±30 mg $N\text{-}NO_2^-$ L^{-1}). Samples from SBR reactor were labelled as "AM". The second reactor was a lab-scale up-flow anammox sludge bed (UAnSB) reactor inoculated with the biomass from the first reactor. This UAnSB was fed with real urban wastewater (26±8 mg $N\text{-}NH_4^+$ L^{-1}, 36±9 mg $N\text{-}NO_2^-$ L^{-1}, 2±2 mg $N\text{-}NO_3^-$ L^{-1}, 60 ± 14 mg COD L^{-1}, among other compounds), coming from an urban WWTP located in an industrial area of Catalonia, NE Spain (for more details, see the work of Juan-Díaz et al., 2020).

The design of the UAnSB reactor was previously described by Reino and Carrera (2017) and the operating conditions by Juan-Díaz et al. (2020). The temperature of this reactor was gradually lowered from 20°C (period I) to 10ºC (period III) using a cooling system around the bed. The reactor had five different sampling points at different heights from the bottom: 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) (Fig. 3.1). For the purposes of this thesis, 3 points along the bed were selected to take the samples, indicated in the Fig. 3.1 as "S1", "S3" and "S5", and labeled in the same way. The working temperature was added to the label when the temperature effect was studied.

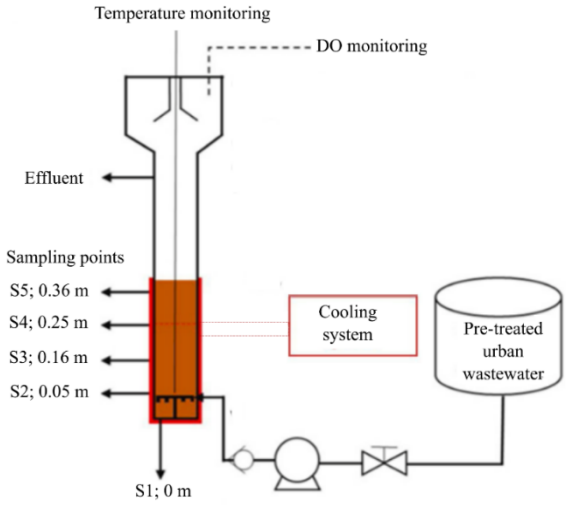

Figure 3.1.- Schematic diagram of the lab-scale UAnSB reactor. Adapted from Juan-Díaz et al. (2020).

The operational parameters of the UAnSB reactor such: Nitrogen Loading Rate (NLR), Nitrite Removal Rate (NRR), Nitrogen Removal efficiency (NRE), the anammox activity or specific NRR at the sample day or period were obtained from Juan-Díaz et al (2020) and Juan-Díaz (2021). According to them, NLRs and NRRs were calculated by using the effective UAnSB reactor volume of 13 L, excluding the gas-liquid-solid separator. NRR was calculated as the removal of ammonium and nitrite without considering the nitrate produced in the anammox reaction. Ammonium, nitrite and nitrate concentrations were measured along the different sampling points of the UAnSB reactor at each steady state conditions to evaluate nitrogen compounds concentrations distribution on the sludge bed. Specific NRRs corresponding to the different sludge bed sections delimited by the sampling points were calculated using the measured nitrogen compounds concentrations.

Samples taken from both reactors for microbial analyses were washed 3 times (5,000 g, 5 min) with 1 mL of 1X Phosphate Saline Buffer (PBS) and the pellet was stored in duplicate at -80ºC for nucleases extraction. A third part of each sample pellet was separately handled for the FISH technique.

3.2. Fluorescence in situ hybridization (FISH)

3.2.1. Sample preparation

Granular biomass samples were homogenized in a glass mortar or were maintained as they were, directly from reactors. Homogenized samples were fixed with a paraformaldehyde solution 4% (v/v) at 4ºC for 2.5 h, while latter samples were fixed for 24 h. Fixed samples were washed 3 times (5,000 g, 5 min) with 1X Phosphate Buffered Saline (PBS). Then, homogenized biomass was re-suspended in 1 mL of 1:1 PBS:ice cold ethanol 98% and stored at -20ºC. Granules samples were re-suspended in sucrose 30% in phosphate buffer 0.1 M and stored at 4ºC.

On the one hand, 5 µL of homogenized fixed biomass were spotted onto an 8-well microscope glass slide and dried 10 min at 46ºC. On the other hand, fixed granules were embedded in a block filled with Optimal Cutting Technique (OCT) compound (Tissue Tek®, Sakura 4583), froze at -20 ºC, and the blocks sectioned in 10 µm thick vertical slices with a cryostat Leica (CM3050S). Two consecutive slices were placed in the middle of adherent slides (Thermo Scientific® Menzel®, SuperFrost PlusTM), allowed to dry for 20 min on a 37ºC hot plate, and stored at -20ºC until its use.

Glass slides containing fixed samples (homogenized and granule slices) were subjected to a membrane permeabilization step including two enzymatic treatments. First, a digestion with 10 mg mL^{-1} of lysozyme at 37ºC for 1 h. Lysozyme was prepared with 1 mL of 0.5 M EDTA, 1 mL of 1 M Tris/HCl pH 8, 8 mL of Milli-Q-grade water and 100 mg of lysozyme (Sigma-Aldrich, ref.: L6876-5G). Then, a treatment with achromopeptidase 60 U mL^{-1} for 30 min at 37ºC. To prepare the achromopeptidase solution, 10 mL of achromopeptidase buffer (100 µL 5M NaCl, 500 µL 1M Tris/HCl pH 8 and 50 mL of Milli-Q-grade water, pH 8) were mixed with 20 µL achromopeptidase stock solution (the lyophilized powder as come from supplier (Sigma-Aldrich, ref.: A3547-100KU) was dissolved in Milli-Q-grade water to prepare a stock solution of 3KU mL^{-1}). Afterward, the slides were washed followed by a dehydration by immersion in increasing ethanol concentrations for 3 min each (50%, 80% and 96%) and finally allowed to dry (Nielsen et al., 2009; Suárez-Ojeda et al., 2011).

3.2.2. Fluorescence *in situ* hybridization using fluorochrome-labelled oligonucleotide probes

The hybridization protocol was adapted from Nielsen et al., 2009 and Suárez-Ojeda et al., 2011. A 2 mL microcentrifuge tube of hybridization buffer was prepared containing 360 µL of 5 M NaCl (sterilized by autoclave), 40 µL of 1 M Tris/HCl (sterilized by autoclave), 2 µL of 10% SDS, the corresponding amount of formamide required for each molecular probe, and Milli-Q-grade water up to 2 mL. Probes, sequences, specificity and optimal formamide concentrations used in the hybridization buffers are displayed in Table 3.1. The general bacteria probe was an equal mixture of probes EUB338I, EUB338II, EUB338III and UNIV1390 (EUBmix). The hybridization mix containing the probe working solution (50 ng μL^{-1}) and the hybridization buffer in a ratio of 1:10 was prepared at the moment to use. A volume of hybridization mix of 10 µl for each well (homogenized samples) and 160 µL for the granule slice sample was used to cover the sample. The remainder hybridization buffer was used to moist a cellulose tissue placed in a 50 mL Falcon tube, as a moisture chamber. The slice was placed horizontally into the tube, closed, and taken to the oven at 46ºC. Hybridization with probes requiring different stringency was realized by a successive hybridization procedure (Wagner et al., 1994). Hybridization rounds were 2 h for homogenized samples and 5 h for granule slice.

After hybridization, the slides were quickly transferred to a wash buffer tube by immersing the entire slide in the pre-warmed washing buffer at 48ºC for 15 min. Washing buffer composition is described in Table 3.2. After washing time, the slices were transferred to a 50 mL falcon tube containing cold Milli-Q-grade water. Afterwards, all droplets of water were removed from the slide by directly applying compressed air to the slide. To finish, a mounting medium (ProLong Diamond Antifade Mountant, Thermofisher) was applied over the sample and covered with a large coverslip for the subsequent microscope observation.

3.2.3. FISH visualization and quantification

Relative abundance of anammox bacteria were determined by FISH technique coupled with confocal laser scanning microscopy (CLSM). A Leica TCS-SP8 confocal laser scanning microscope using a Plan-Apochromatic 63X objective (NA 1.4-0.6, oil) was used to obtain at least 45 microscopic fields (randomly selected *xy* sections) of homogenized samples (15 images of each repetition) and at least 3 volumetric fields were taken of each granule slice sample (15 sections along *z* dimension, 3 channels, 45 frames in total).

Images were acquired using HyD detectors in combination with white-light laser, that allow time gating for added spectral selectivity (for example, removal of autofluorescence). On one hand, it was used a macro for the ImageJ® 1.51k software self-developed in the microscopy service of Universitat Autònoma de Barcelona. This macro considers the colocalization of the specific probe over the general probes and it was used to quantify hybridized biomass in random *xy* sections for homogenized samples and granules slices. On the other hand, two different software, LAS X from Leica and Imaris 9.3, were used to quantify the volumetric images (3D) of the granule slices samples. 2D maximum intensity projection of volumetric images were used for visualization purposes. Measurements of background intensity of samples without probes were used to stablish thresholds in each laser channel and NON EUB probe hybridization was used as negative control.

Table 3.1. 16S rRNA-targeted oligonucleotide probes sequences, target microorganisms, dye and formamide percent used, the reference was also included.

Probe	Sequence 5' to 3	Specificity	5' Dye	Formamide (%)	Reference
EUB338I	GCTGCCTCCCGTAGGAGT	Most bacteria	Alexa-488	0-50	Amann et al. (1990)
EUB338II	GCAGCCACCCGTAGGTGT	Planctomycetales	Alexa-488	0-50	Daims et al. (1999)
EUB338III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	Alexa-488	0-50	Daims et al. (1999)
UNIV1390	GACGGGCGGTGTGTACAA	All organisms	Alexa-488	0-50	Zheng et al., (1996)
NONEUB	ACTCCTACGGGAGGCAGC	Control probe complementary to EUB338	Alexa-488	Not determined	Wallner et al. (1993)
AMX368	CCTTTCGGGCATTGCGAA	All anammox bacteria	Alexa-647	15	Alm et al. (1996)
Bfu613	GGATGCCGTTCTTCCGTTAAGCGG	*Ca*. Brocadia fulgida	Alexa-594	35	Kartal et al. (2008)
Ban162	CGG TAG CCC CAA TTG CTT	*Ca.* Brocadia anammoxidans	Alexa-594	45	Schmid et al. (2001)

Table 3.2. Washing buffer composition

Component	Volume to prepare 50 mL			
	Formamide %			
	15	35	45	50
5M NaCl (autoclaved)	3.18 mL	700 µL	300 µL	180 µL
0.5M EDTA (autoclaved)	0	500 µL	500 µL	500 µL
1M Tris/HCl (autoclaved)	1 mL	1 mL	1 mL	1 mL
Milli-Q-grade water	up to 50 mL	up to 50 mL	up to 50 mL	up to 50 mL
10% SDS (not autoclaved)	50 µL	50 µL	50 µL	50 µL

3.3. DNA and RNA -based molecular approaches

3.3.1 DNA and RNA extraction

DNA and RNA were extracted separately from each sample. For total genomic DNA obtention, 2 mL of frozen samples (mainly granules) were thawed, centrifuged (5,000 x g, 5 min) and up to 250 mg of wet pellet were used for the Soil DNA Isolation Plus Kit (Norgen Biotek) according to the manufacturer's instructions. An earlier step of heat shock was included, consisting of put the tubes at 65ºC for 10 min followed by 5 min at 4ºC, to optimize the DNA extraction. Finally, to ensure a good DNA final concentration, the DNA elution step was made with 60 µl.

Total RNA was extracted from lysate biomass using the Pure Link RNA mini kit (Life Technologies). Frozen samples were carefully thawed by a gradual increase in temperature to -20ºC and then to 4ºC. Then, samples were centrifuged (5,000*g*, 5 min, 4ºC) and the supernatant was discarded. For better RNA yields, a step of lysis and homogenization was followed as recommended for purifying RNA from bacterial cells. For this, 100 µL of 10 mg mL^{-1} lysozyme solution was added for each 250 mg of sample, mixed and 0.5 ul of SDS10% was added for each 100 µL of lysozyme and mixed again and incubated for 5 min at room temperature. Then, the manufacturer protocol for the binding step was followed and during the washing step, the residual genomic DNA was removed using an On-column Pure Link DNase set (Life Technologies), to finally elute the RNA.

DNA and RNA quantities and integrities were checked with a ND-1000 Nanodrop spectrophotometer (Thermo Scientific) and stored at -80°C until further use. 10 µL of

total RNA was used to conduct reverse transcription to obtain the complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). A final quantification of DNA and cDNA was determined with Qubit fluorometric quantitative analyzer (Life Technologies, Carlsbad, CA, USA) for qPCR assays.

3.3.2 Bacterial 16S rRNA gene analysis by amplicon sequencing

Amplicon sequencing paired-end (PE) service of 16S rRNA genes on the Illumina MiSeq platform (2x 300 bp) was performed by RTL Genomics (Texas, USA) for detecting eubacteria and anammox bacteria using primers shown in Table 3.3. The Silva database Test Prime tool (Klindworth et al., 2013) was used to evaluate primer coverage with settings recommended by (Zhou et al., 2018), 2 maximum number mismatches and 1 site of 0-mismatch zone at 3' end .

3.3.2.1. Data processing and analysis of amplicon sequencing

FastQC v.0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess raw sequences quality control and adaptors contamination. Demultiplexed sequences were imported into QIIME2™ v.2019.10 (Bolyen et al., 2019). DADA2 denoise-paired plug-in (Callahan et al., 2016) was used to quality filtering, trimming of poor-quality bases, de-replication, chimera filtering, merging paired-end reads, and the identification of amplicon sequence variants (ASV) (Hall and Beiko, 2018). The resulting feature table was used for taxonomic assignment based on the SILVA SSU 138 rRNA database pre-clustered at 99% sequence identity with the QIIME2 q2-feature-classifier plugin. Each count table was then normalized to the lowest number of reads.

3.3.3. Quantitative real-time PCR (qPCR)

Two functional genes (hzsA and nirS) and one specific 16S rRNA method were used to quantify the proportion of anammox in both reactors. Moreover, the total bacterial population was quantify using 16S rRNA gene bacterial-target primers. Primers and annealing temperatures are shown in Table 3.3.

The calibration curves were constructed with positive controls. For these controls, plasmids pTOP_Blunt_V2 containing as insert specific anammox bacterial 16S rRNA and for the functional genes encoding hzsA and nirS were manufactured by Macrogen Inc (Seoul, Korea). Accession number of reference genes used for synthesis were: AB277765.1; JN703716.1 and CT573071.1. Genomic DNA previously extracted from a

strain of *Pseudomonas fluorescens* was used as standard for the 16S rRNA gene bacterial quantification. The standards were diluted to yield 10-fold dilutions in the range of 10^4-10^8 DNA copies μL^{-1} and subsequently used to generate q-PCR standard curves. The R^2 value for each standard curve exceeded 0.99. All PCR reactions were performed in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). Fluorescence was detected at the end of the extension step.

The qPCR protocol was as follow: 95°C for 5 min followed by 40 cycles at 95°C for 10 s, 20 s at the annealing temperature and 72ºC for 20 s. Each PCR mixture (20 µL) contained 2 µL of diluted DNA template, 10 µM of each primer, and 10 µL of 2X KAPA SYBR® FAST qPCR Master Mix Universal (Kapa Biosystems, USA).

The specificity of the amplified PCR product was assessed by performing a melting curve analysis; fluorescence readings were consecutively collected during the melting process from 60 to 95°C at a heating rate of 5°C s^{-1}. DNA concentrations were determined using the Qubit fluorometric quantitative analyzer (Life Technologies, Carlsbad, CA, USA) and gene copy numbers obtained were normalized by DNA concentrations in corresponding individual samples. All quantitative PCR reactions were carried out in triplicates and statistical analysis was performed with Infostat software. Log transformed gene copy numbers were subjected to ANOVA analysis. Tukey's test was used to determine which genes copies were statistically distinct. 5% of significance was used. The efficiencies of the quantitative PCR assays were over 80%, and the R^2 value always exceeded 0.98.

Table 3.3. Primers used in this study for amplicon sequencing and qPCR.

Application	Primer set	Sequence (5'-3')	Specificity	Annealing (ºC)	Reference
Amplicon sequencing	515F	GTGCCAGCMGCCGCGGTAA	Bacteria and Archaea	-	Caporaso et al. (2012)
	806R	GGACTACVSGGGTATCTAAT			
	909R	CCCCGYCAATTCMTTTRAGT	Bacteria and Archaea	-	Tuan et al. (2014)
	AMX368F	TTCGCAATGCCCGAAAGG	Anammox bacteria 16S rRNA gene	-	Gu et al. (2007)
	AMX820R	AAAACCCCTCTACTTAGTGCCC			
qPCR	519F	CAGCMGCCGCGGTAANWC	Eubacteria 16S rRNA gene	52	Stubner (2002)
	907R	CCGTCAATTCMTTTRAGTT			
	AMX809F	GCCGTAAACGATGGGCACT	Anammox bacteria 16S rRNA gene	56	Tsushima et al. (2007)
	AMX1066R	AACGTCTCACGACACGAGCTG			
	AnnirS379F	TCTATCGTTGCATCGCATTT	nitrite reductase gene	54	Li and Gu (2011)
	AnnirS821R	GGATGGGTCTTGATAAACA			
	AnhzsA1597F	WTYGGKTATCARTATGTAG	hydrazine synthase gene	51	Harhangi et al. (2012)
	AnhzsA1857R	AAABGGYGAATCATARTGGC			

3.3.4. Metagenomics

Shotgun sequencing was performed in Illumina Novaseq6000 sequencer (Macrogen, Seoul, South Korea) (2 × 100 bp, PE). To process and analyze the metagenomic data the following tools were used: FastQC (v0.11.9) for assessing sequence quality, Trimmomatic (v0.36) for removal of ambiguous reads, adapters, and low-quality sequences to obtain high quality reads (Bolger et al., 2014) with the settings SLIDINGWINDOW:4:25 and MINLEN:90. A schematic diagram of the metagenomics analysis is shown in Fig. 3.2.

3.3.4.1. Metagenome assembly and analysis

MEGAHIT (v1.2.3-beta) (Li et al., 2015) was used for metagenome assembly with minimum k-mer length 43, maximum k-mer 75, with steps of four. Contigs shorter than 1,000 bp were filtered. After this, on one side, metagenomes annotation and functional classification were predicted against the KEGG GENES database using MetaErg (Dong and Strous, 2019). On the other side, Ribosomal Small Subnubit (SSU) from 16S rRNA genes were reconstructed from metagenomic reads using Metaxa2 v.2.1 (Bengtsson-Palme et al., 2015) together with the SILVA SSU 138 rRNA database. Then, assembly of shotgun reads assigned to the 16S rRNA gene was performed using EMIRGE (Miller et al., 2011).

3.3.4.2. Genome assembly from metagenomes

After metagenome assembly with MEGAHIT, reads were mapped against the contigs with Bowtie2 (v2.3.4.1) for coverage calculations (to estimate the abundance per sample) using default parameters (Langmead and Salzberg, 2012). Then, MetaBAT2 (v2.12.1) (Kang et al., 2019)was used for contig binning based on differential coverage. CheckM (v1.0.13) (Parks et al., 2015) was used to assess the completeness and the contamination of bins. The automatic taxonomic classification of CheckM was then extracted to determine the nature of each bin considered for further refinement and validation. The bins classified as bacteria with an estimated completeness >80% and contamination <10% were reclassified using the Genome Tree DataBase (GTDB-Tk) (Parks et al., 2018). For interest genomes belonging to the order Brocadiae and Ignavibacteriales, an iteration of tools was done to obtain the metagenome-assembled genomes (MAGs): SPAdes (v3.1.1) (Bankevich et al., 2012) was used for reassembly, bins curation was performed with CheckM, and refinement of genomes with ESOM

(Ultsch and Mörchen, 2005), until the best conditions of completeness/contamination were obtained (over 90% of completeness and less than 5% contaminated).

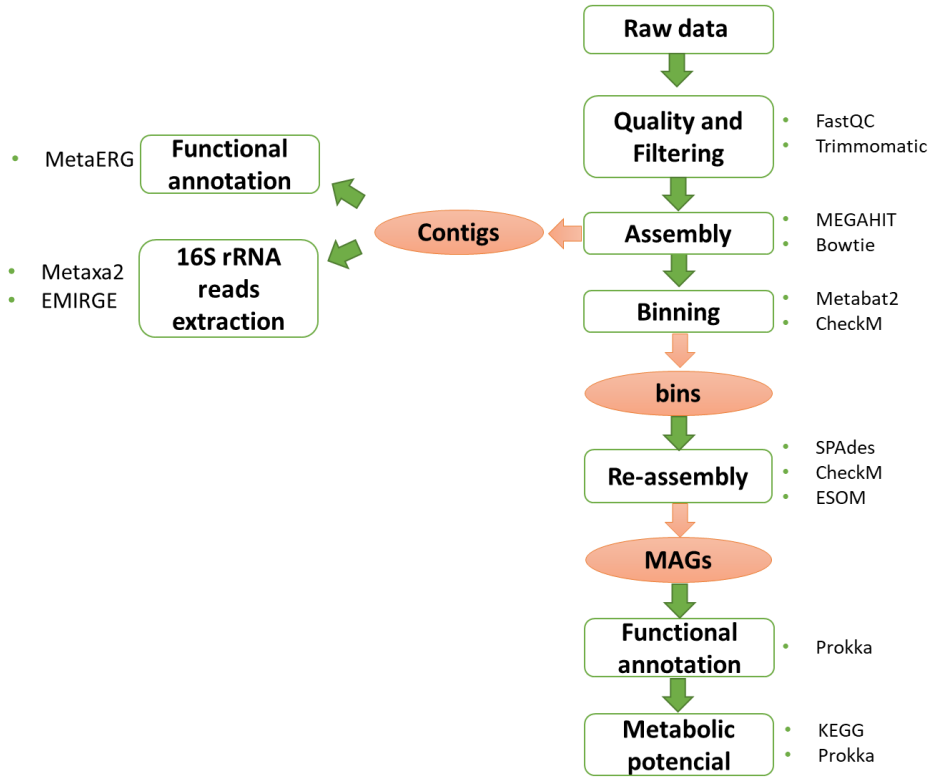

Figure 3.2.- Schematic diagram of the metagenomic analysis.

3.3.4.3. Metabolic potential analysis

Protein coding sequences (CDS) were determined with Prokka (1.14.5) (Seemann, 2014). Predicted amino acid sequences (amino acid sequences in FASTA format produced by PROKKA) were annotated with Prokka and KOALA (KEGG Orthology And Links Annotation) for K number assignment of KEGG Genes (Kanehisa et al., 2016). A deep analysis of the metabolic pathways was performed to obtain the metabolic potential of each MAG.

3.3.5. Phylogenetic analysis

3.3.5.1. 16S rRNA based phylogenetic trees

Phylogenetic trees were constructed to evaluate the phylogenetic diversity of anammox bacteria. 16S rRNA phylogenetic trees included: representative sequences of ASVs classified as Brocadiaceae family from amplicon sequencing for each primer set, full-length 16S rRNA gene sequences reconstructed from metagenome, sequences belonging to the *Ca.* Brocadiales order from different anammox studies (Luo et al., 2017;

Narita et al., 2017; Zhou et al., 2018) and related environmental sequences found in GenBank using BLASTN algorithm. All sequences were aligned using SINA v1.2.11 (Pruesse et al., 2012) and phylogenetic trees were performed using the maximum likelihood method in MEGA7 software (Kumar et al., 2016). *Pirellula staleyi* was used as an outgroup for rooting trees. Bootstrap values were obtained from data resampling of 500 replicates. The resultant phylogenetic trees were visualized in iTOL (Letunic and Bork, 2019).

3.3.5.2. Genome based phylogenetic trees

Genomes trees of the recovered MAGs and reference genomes of orders Brocadiales and Ignavibacteriales were constructed using the Genome Tree DataBase (GTDB-Tk) with a concatenated set of 120 bacterial-specific conserved marker genes (Parks et al., 2018). The obtained alignments were used to perform the trees using the JTT (Jones-Taylor-Thornton) model in MEGA7 software. *Acidobacterium capsulatum* ATCC51196 and *Streptococcus mutans* UA159 were used as outgroup. Bootstrap values were obtained from data resampling of 1000 replicates. The resultant phylogenomic trees were visualized in iTOL (Letunic and Bork, 2019).

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Chapter 4

Standardization of molecular techniques for the study of bacterial communities in anammox reactors treating real water at mainstream conditions

A modified version of this chapter has been sent for publication as:

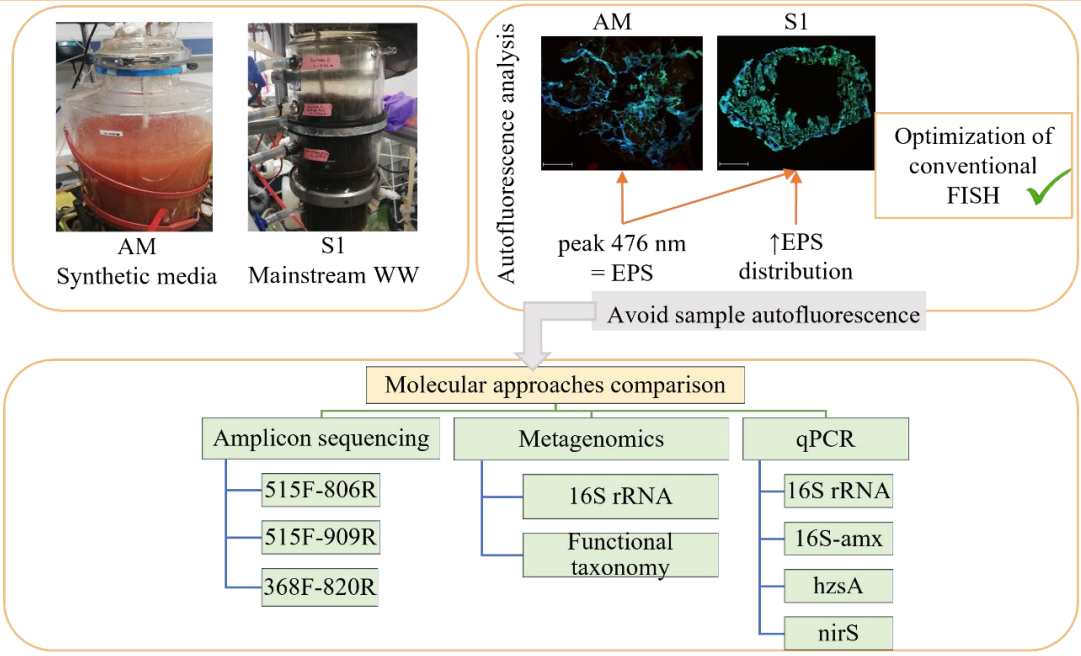
Oyarzúa, P., Suárez-Ojeda, M.E., Roldán, M. Anammox reactors working at mainstream conditions: The real behind-the scenes when using fluorescence in situ hybridization (FISH). Under revision.

Oyarzúa, P., Bovio-Winkler, P., Etchebehere, C., Suárez-Ojeda, M.E., 2021. Microbial communities in an anammox reactor treating municipal wastewater at mainstream conditions: Practical implications of different molecular approaches. J. Environ. Chem. Eng. 9, 106622. https://doi.org/10.1016/j.jece.2021.106622

Goal 1: To characterize and analyze the bacterial communities involved and existing synergies in an anammox reactor during different phases of operation

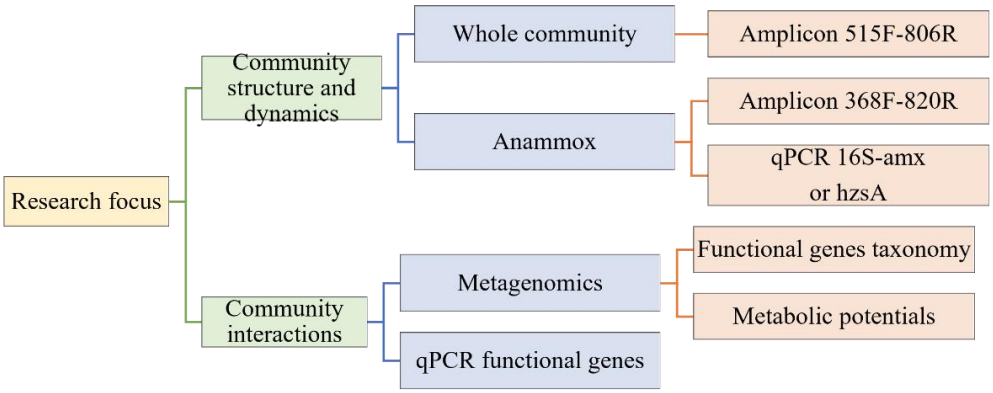
Problem: Biases of each technique are not considered

Chapter 4. Standardization of molecular techniques for the study of bacterial communities in anammox reactors treating real water at mainstream conditions



Main results:

- Modified and optimized FISH-CLSM protocol
- Importance of practical primer's choice
- 515F-909R had very low coverage of the Planctomycetota phylum
- Low abundance of anammox bacteria in S1, dominated by *Ca.* B fulgida.
- Recommendations depending on the research focus



Abstract

Molecular techniques are widely used to assess the dynamics and the composition of microbial communities in anammox process. Nevertheless, biases on its application are not always considered. These biases lead to inconsistency of the data retrieved with the different methods, which are not usually understood. This chapter aimed to evaluate, compare, and standardize different molecular approaches to study bacterial communities in a granular-sludge Upflow Anammox Sludge Blanket (UAnSB) reactor treating real wastewater from a two-step autotrophic biological nitrogen removal (aBNR) at mainstream conditions. For validation of the techniques, an anammox-enriched culture of a sequencing batch reactor (SBR) treating synthetic wastewater was used. Fluorescence in situ hybridization, 16S rRNA amplicon sequencing using different primers sets, metagenomics, and qPCR were evaluated.

Background fluorescence and sample autofluorescence are the main problems when handling FISH identification in anammox reactors. However, it has rarely been reported as a pitfall, and some works reported that individual case-by-case optimization is required based on sample type. Results detected a wide range of autofluorescence wavelengths in the biomass of UAnSB reactor, that was attributed to extracellular polymeric substances (EPS). The standard FISH protocol has shown to give unreliable results and therefore, different strategies were assessed to define an optimized protocol to study the UAnSB anammox samples. This consisted of sequential hybridizations of 5 h of the cryosectioned granules (previously fixed for 24 h and permeabilized by two enzymatic treatments) in addition to a volumetric image acquisition where the colocalization of the target population with the total bacteria was clearly seen. the anammox bacteria were quantified to be below 1% of the total bacteria, where almost half corresponds to *Candidatus* (*Ca.*) Brocadia anammoxidans and the other half to *Ca.* Brocadia fulgida. Despite the optimized protocol, it was decided to assess the anammox communities with other molecular techniques where EPS did not interfere, then, DNA-based method were standardized.

The main bias of PCR-based techniques (amplicon sequencing and qPCR) is related to the primer's choice. We reported that, despite its *in-silico* coverage, the universal primers 515F-909R had, in fact, very low coverage (1%) of the Planctomycetota phylum in the enriched anammox reactor, while the primers 515F-806R detected a

relative abundance of 79%. Regarding metagenomic, a higher taxonomic resolution at the species level was achieved with the functional genes approach, although the results could be limited by genomic database updates and completeness. From the phylogenetic analysis, the most abundant sequences retrieved with the different amplicon sequencing approaches, always cluster in the same branch with a sequence retrieved by the metagenomics, showing coherence among the approaches. The selected primers targeting anammox 16S rRNA and hydrazine synthase genes in qPCR showed consistency with the anammox absolute quantification in the reactor, making them useful for later comparisons with similar investigations.

Finally, all evaluated techniques were standardizer to study a mainstream anammox reactor. The obtained results were coherent, with a low abundance of anammox bacteria, mainly dominated by *Ca.* B fulgida. According to these, recommendations on the advantages of using one technique over another or a combination of them were outlined, depending on the research question to be addressed.

4.1. Introduction

Application of anaerobic ammonium oxidation bacteria (anammox) in wastewater treatment is now a proven technology and constitute a robust, reliable, and cost-effective treatment for wastewaters (WW) with high nitrogen concentrations (Kuenen, 2008; Lackner et al., 2014). Despite the known advantages, full-scale implementation of anammox based process for direct treatment of the mainstream of municipal WW (MWW) has still not been fully confirmed, but experimental evidence is increasing (Juan-Díaz et al., 2020; Laureni et al., 2016; Ni and Zhang, 2013; Pérez et al., 2015; Reino et al., 2018).

There is evidence that under the same operational conditions, the nitrogen removal efficiency of various anammox systems could differ owing to the difference in dominant anammox species (Cho et al., 2019). To date, six anammox genera has been discovered, including *Candidatus* (*Ca.*). Brocadia, *Ca.* Kuenenia, *Ca.* Scalindua, *Ca.* Anammoxoglobus, *Ca.* Jettenia and *Ca.* Anammoximicrobium, and all have been detected in many different WWTP (Hu et al., 2010). These lineages all have '*Candidatus*' status as they do not exist in pure culture and must be grown in laboratory enrichments (Lawson et al., 2017).

Given the metabolic differences among the anammox community, better identification of the existing anammox bacteria in the bioreactor is important to understand the process and improve its operation (Cho et al., 2019). Nevertheless, in granular-based anammox process, other microbial communities whose identity and relationship with the anammox bacteria are still under investigation (Gonzalez-Gil et al., 2015), and some metabolic networks between anammox and heterotrophic bacteria have been proposed (Lawson et al., 2017). Until now, mainstream anammox research have been done mainly from an engineering perspective, using the so-called "black box approach", usually underestimating the importance of microbial communities as an integral component of these systems (Chu et al., 2015).

Considering this lack and the slowly growth rate of anammox bacteria, culture-independent methods, specifically DNA/ RNA-based molecular techniques are the most widely used for studying this group of microorganisms, but also to determine the dynamics of the whole bacterial community diversity (Li and Gu, 2011; Schmid et al., 2001; Sun et al., 2014). These approaches include Fluorescence *in situ* hybridization

(FISH)-based techniques, which using fluorochrome-labelled oligonucleotide probes targeting 16S rRNA have been successfully applied for in situ detection of anammox bacteria activity (Kuenen, 2008); PCR-based methods, as amplicon sequencing and quantification by quantitative PCR (qPCR), which focused on 16S rRNA gene as a molecular marker are utilized for detection, identification, and/or quantification of bacterial communities, including anammox. But, since the 16S rRNA gene is not necessarily related to the physiology of anammox bacteria, the use of functional gene markers as hydrazine synthase (hzsA), nitrite reductase (nirS), hydrazine dehydrogenase (hzo), or hydroxylamine dehydrogenase (hao) may provide alternatives for assessing these communities in anammox reactors (Harhangi et al., 2012; Junier et al., 2010; Li and Gu, 2011; Zhou et al., 2018). The most recent developed molecular approach is metagenomic sequencing, which allows a functional and taxonomic analysis, besides a higher resolution of microbial and anammox diversity (Bhattacharjee et al., 2017; Lawson et al., 2017).

Despite the wide variety of molecular approaches, biases on each approach are not always considered. On one side, background fluorescence or sample autofluorescence in FISH, seems to be one of the most common challenges found in different studies (Table 4.1), however, to the best of our knowledge it is rarely reported as a pitfall. Noteworthy, some of these studies used FISH images for anammox quantification. In the cases where high sample autofluorescence was registered, biomass samples corresponded to biofilm and homogenized granules from enriched anammox cultures reactors treating synthetic influents. For example, a clear standard protocol for applying FISH on biological wastewater treatment samples was established by Nielsen et al. (2009), nevertheless, the use of a similar standard method by Pavlekovic et al. (2009) showed weak probe signals in biofilm samples, suggesting that individual optimization is required depending on the sample type (suspended or biofilm). So far, no evidence has been found for optimized FISH protocols for evaluating granular anammox communities in reactors treating real wastewater. On the other side, PCR-based methods have caused a rise in the integration of eco-physiological approaches into anammox research (Orschler et al., 2019). However, the reliability of the method predominantly depends on the specificity and efficiency of the PCR primers (Yang et al., 2020). Since the PCR primers were designed *in silico*, these characteristics depend on the biomass, the type of sample, and other interference factors for the PCR reaction to take place efficiently. Therefore, practical applications do not

always offer an acceptable performance (Zhou et al., 2018). The above has resulted in problems in the coherence and precision of the data when similar works are to be compared (Orschler et al., 2019). In the last years, the metagenome approach has been taken place since avoids PCR biases (Logares et al., 2014) and have resulted in a turning point in studying phylogenetic and functional diversity of wastewater treatment systems (Ferrera and Sánchez, 2016) and more insights into anammox evolution and metabolism have been obtained (Wang et al., 2019). But its limitations include that it remains a challenge to accurately infer taxonomic origin from metagenomic reads (Poretsky et al., 2014), a higher cost, and bioinformatics complexity (Liu et al., 2020).

Considering the pitfalls and biases of the available techniques to study anammox community, and how the results are affected by the type of sample, this work aimed to standardize different molecular approaches to determining the whole bacterial and the anammox communities' structure of a granular-sludge anammox reactor treating real wastewater at mainstream conditions. A comparison with an enriched anammox culture was used to validate the techniques and the scope and biases of each technique.

Table 4.1. Investigations using FISH technique to detect anammox populations in biological nitrogen removal (BNR) treatments.

Reactor	**Biomass sample**	**Influent**	**Sample autofluorescence Observed/Reported**	**Reference**
Fixed bed column	Biofilm	SM	+/-	Kindaichi et al. (2007)
Upflow fixed bed column	Biofilm	WWTP- SM	+/-	Tsushima et al. (2007)
SBR	Granule	WWTP-SM and acetate	+/+	Kartal et al. (2008)
MBR	Biofilm	WWTP- SM	+/-	Pavlekovic et al. (2009)
SBR	Suspended			
UASB	Sludge	WWTP- SM	+/-	Hu et al. (2010)
UBF	Biofilm			
CSTR	Suspended			
MA-EBR				
MBBR	Biofilm	WWTP- SM	+/+ Removed	Almstrand et al. (2014)
UASB	Granules	Swine manure treatment- SM	+/-	Ni and Yang (2014)
MBBR	Biofilm Suspended	Pre-treated mainstream WW and SM	+/-	Laureni et al. (2015)
MBR	Granules	WWTP- SM	+/-	Wang et al. (2016)
MBR	Granules	WWTP- SM	+/+ Studied	Hou et al. (2017)
SBR	Biofilm	SM	+/+ Studied	Böllmann et al. (2019)
SBR	Granules	WRP-SM	+/-	Lu et al. (2020)

SM: Synthetic medium, WWTP: Wastewater Treatment Plant; SBR: Sequencing Batch Reactor, MBR: Membrane BioReactor, UASB: Upflow Anaerobic Sludge Blanket reactor; UBF: Upflow biofilter; CSTR: Continuous Stirred Tank; MA-EBR: Microbial attached Expanded Bed Reactor; MBBR: Moving Bed Biofilm Reactor, WRP: Water reclamation plant.

4.2. Materials and methods

4.2.1. Anammox reactors description

Samples of anammox granular biomass were obtained from two different reactors. The first reactor was a sequencing batch reactor (SBR) working at 35 ºC under stable conditions and it was used as a positive control for standardization of techniques. The second reactor was a lab-scale Up-flow Anammox Sludge Blanked (UAnSB) working at 20 ºC. Details of both reactor feeding and performance were described in Section 3.1 of Chapter 3. Sample from SBR reactor was labelled as "AM" while the other coming from UAnSB reactor was labelled as "S1". S1 sample was taken of the bottom of this reactor during a stable operation period of 81 days.

4.2.2. FISH

Abundances of anammox bacteria were analyzed by FISH coupled to confocal laser scanning microscopy (CLSM). Two procedures were tested: the standard protocol using homogenized biomass and a modified protocol using granules slices. Modifications to the standard hybridization protocol were included as successive procedures when the probes required different stringency, from the higher to the lower, and each round were 2 h for homogenized samples and 5 h for granule slice. A mix of general probes (EUBmix) for all bacteria was 5´-Alexa-488-labeled. Specific probe for all anammox bacteria was 5´-Alexa-647-labeled, while specific probes for *Ca.* Brocadia fulgida y *Ca*. Brocadia anammoxidans were 5´-Alexa-594-labeled. For image visualization and quantification of homogenized samples, a macro for the ImageJ® 1.51k software jointly developed with the microscopy service of Universitat Autònoma de Barcelona. Instead, two different software were used to quantify the volumetric images (3D) of the granule slices samples. 2D maximum intensity projection of volumetric images were used for visualization purposes Hybridization protocol, probes and quantification protocol are fully described in Section 3.2 of Chapter 3.

4.2.2.1. Autofluorescence analysis

A spectral analysis (λ scan function) at 405 nm was made to each sample using a confocal microscope TCS SP8 (Leica) in a range between 425 and 785 nm, 5nm of spectral resolution and step size of 15 nm. Sample autofluorescence was calculated as an average of 10 region of interest (ROIs). Maximum emission wavelength was detected and

fluorochromes for each FISH probe were selected according to this, to avoid interference of samples autofluorescence with the real probe signal as Suárez-Ojeda et al. (2011) pointed out in their research.

4.2.2.2. Structural EPS extraction

To better understand sample autofluorescence, EPS was extracted from AM and S1 granular anammox biomass according to Lin et al. (2018). Granules (1 g) were heated for 30 min at 100 ºC in 300 mL of 0.1%(w/v) sodium dodecyl sulphate while stirred at 400 rpm. pH was maintained at 9 by adding 0.1 M NaOH. Then, samples where centrifugated (2000 g, 20 min) at 4 ºC. Supernatant was recovered and mixed with 100 mL of ethanol. Polymers in the precipitated were used for autofluorescence check using λ scan function.

4.2.3 DNA-based molecular approaches

Samples labelled as AM and S1 were taken in the same day that the FISH samples. Then, the genomic DNA was extracted, and the whole microbial composition was evaluated performing four molecular approaches. Two derived from 16S rRNA gene amplicon sequencing. This was performed using two sets of universal primers: 515F-806R and 515F-909R. Each primer set was considered an individual approach. Shot-gun sequencing metagenomic was also evaluated, including two different approaches. On the one hand, 16S rRNA sequences were extracted from the metagenomic data, classified and analyzed. On the other hand, metagenome contigs were annotated and functional taxonomy was assigned based on functional genes. The taxonomic assignment was performed using SILVA SSU 138 rRNA database. The applied protocol for each approach was previously described in Section 3.3 of Chapter 3. The raw data was deposited at NCBI under accessing number: PRJNA728853.

To specifically study the anammox community, a third primers set Amx368F-Amx820R which was designed to target anammox bacteria, was included for amplicon sequencing, as a fifth approach. Anammox sequences from each approach classified at the Brocadiae class were separately analyzed and relative abundances of anammox community at deeper taxonomic levels were determined.

The Bray-Curtis metric was used for calculation of beta diversity and a non-metric multidimensional scaling (NMDS) plot was constructed with metaMDS function (vegan

package) and ggplot function (ggplot2 package) using R software version 3.5.1 (R Core Team 2013) in R Studio environment Version 1.0.153. β-diversity refers to microbiota differences between samples or groups. It is used to understand whether differences in the microbiota compositions of two groups are significant. The Bray-Curtis dissimilarity is a statistical measure used to quantify the compositional dissimilarity between two samples or groups. Its value ranges from 0 to 1, where 0 means that the two samples or groups share all species, and 1 means that they do not share any (Qian et al., 2020).

4.2.4. Phylogenetic analysis

Three phylogenetic trees were constructed to determine the position of anammox bacteria retrieved by the different strategies, using the protocol described in Section 3.3.5 of Chapter 3 for 16S rRNA-based trees. As all primers set used for amplicon sequencing produced different length sizes PCR products, we decided to construct one tree for each primer set. This way, we avoid losing information by trimming sequences.

4.2.5. qPCR

Absolute quantification of whole bacterial and anammox population was obtained with qPCR technique. We use two different functional genes markers: hszA and nirS. Moreover, a specific 16S rRNA primer set targeting all the anammox 16S rRNA genes was also included in the analysis. The followed protocol, PCR conditions, primers and annealing temperatures are fully detailed in Section 3.3.3 of Chapter 3. Log transformed gene copy numbers were subjected to ANOVA analysis, after a Shapiro-Wilks test for normality. Tukey's test was used to determine which genes copies were statistically distinct. 5% of significance was used

4.3. Results and Discussion

4.3.1. FISH standardization to study anammox bacteria in a mainstream anammox reactor

4.3.1.1. Sample autofluorescence

Autofluorescence of two anammox biomass samples (AM and S1) was studied using the λ-scan function of a CLSM in a range of wavelengths from 425 nm to 770 nm. Using a 5% excitation of laser line 405, it was observed a fluorescence signal between 440 and 580 nm with a maximum emission wavelength at 476 nm for both samples (Fig.

4.1). If we translate this to the Red-Green-Blue (RGB) colour scheme of emission, we observed that autofluorescence was highly present in blue and green channels and decreases in the red channel, similar to the results observed by Böllmann et al. (2019) in anammox enriched biomass from two different reactors. Also, it was noted that autofluorescence was more intense in areas where EPS patches were observed, suggesting that the fluorescence emission does not come from single cells, but from the EPS matrix. For this reason, EPS was extracted from both samples and autofluorescence emission was recorded using the same protocol as for samples. The spectra showed the same maximum emission wavelength (Fig. 4.1), which confirms this EPS was responsible for the autofluorescence, although deeper research is needed to identify the exact composition of this EPS. This strong autofluorescence in anammox was first described by Kartal et al. (2008), reporting two emissions of 464 and 521 nm maximum while studying aggregates of *Ca.* Brocadia fulgida biofilms and defined it as a specie feature. Then, a similar emission peak of 464 nm was observed by Ruscalleda et al. (2014), while characterizing the effluent dissolved organic matter (EfOM) from an anammox reactor treating synthetic wastewater, and they identify it as humic acid-like substances, which belong to a soluble microbial product derived from the growth process. Our results, agreed with recent studies focused on two different enriched anammox reactors by Böllmann et al. (2019), which also noted that autofluorescent substances or structures were outside the cells, but strongly associated with them, and proposed that structural-linked EPS could be a likely explanation. In this sense, Lotti et al. (2019) went beyond and associated an increased intensity of fluorescence at λ 450 nm to the abundance of amyloid-like aggregates in anammox EPS.

Another important consideration about the autofluorescence was the variation on the EPS distribution among the granules of AM and S1 samples, presumably due to the absence or presence, respectively, of organic matter in the reactor's influent. Figure 4.2 showed a higher distribution of EPS all over the S1 samples and a denser EPS matrix compared to AM sample. These results are similar to those of Li et al. (2015) which described denser granules with a higher EPS production and distribution in anammox reactors after the addition of organic matter.

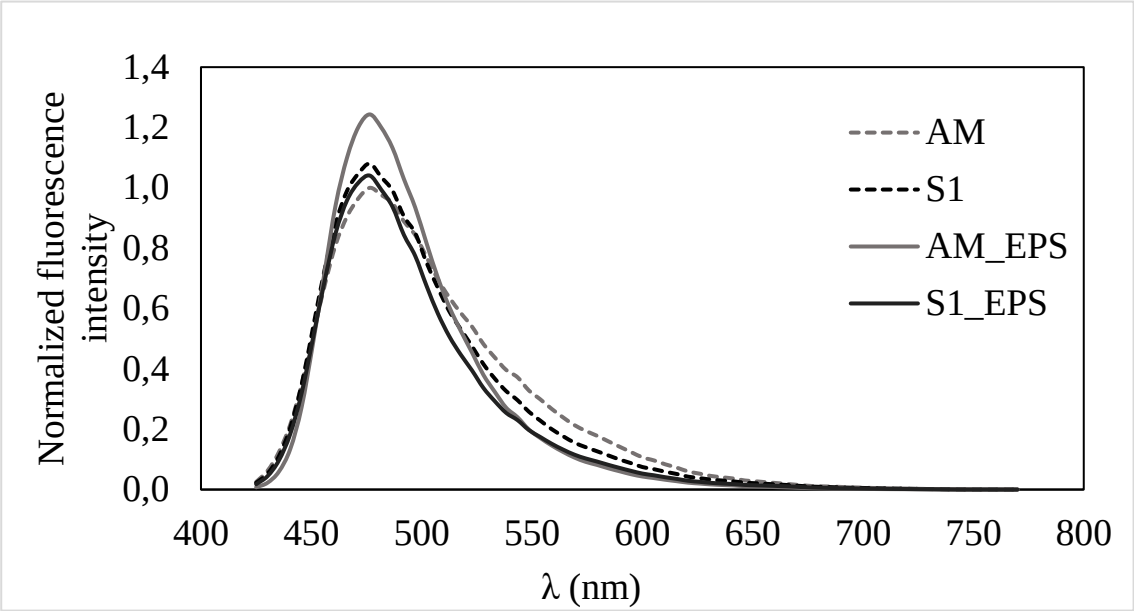


Figure 4.1.- Spectral analysis (λ-scan function) of a negative control sample of granule slices and structural EPS extracted of each sample. Data was normalized with maximum emission of AM sample.

Autofluorescence in anammox enriched lab-scale reactors have been reported before (Kartal et al., 2008; Schmid et al., 2005) and some investigations showed a relationship between anammox fluorescence and bacterial metabolism or reactor performances, determining, for example, a correlation between the production of humic acid-like fluorescent substances and nitrogen removal rate, however, a direct correlation with EPS fluorescence has not been detected and its fluctuation was associated with its fast production and degradation (Hou et al., 2017; Ruscalleda et al., 2014). FISH is a common tool to obtain qualitative and quantitative data of this community (Schmid et al., 2005), but autofluorescence has not been generally reported to cause interferences. Although recently, Böllmann et al. (2019) reported the misinterpretations and misidentification on FISH procedures caused by *Ca.* Brocadia fulgida autofluorescence. Until now, there are no reports on how to improve anammox detection despite the high autofluorescence recorded in complex samples, like the ones coming from real wastewater treating reactors.

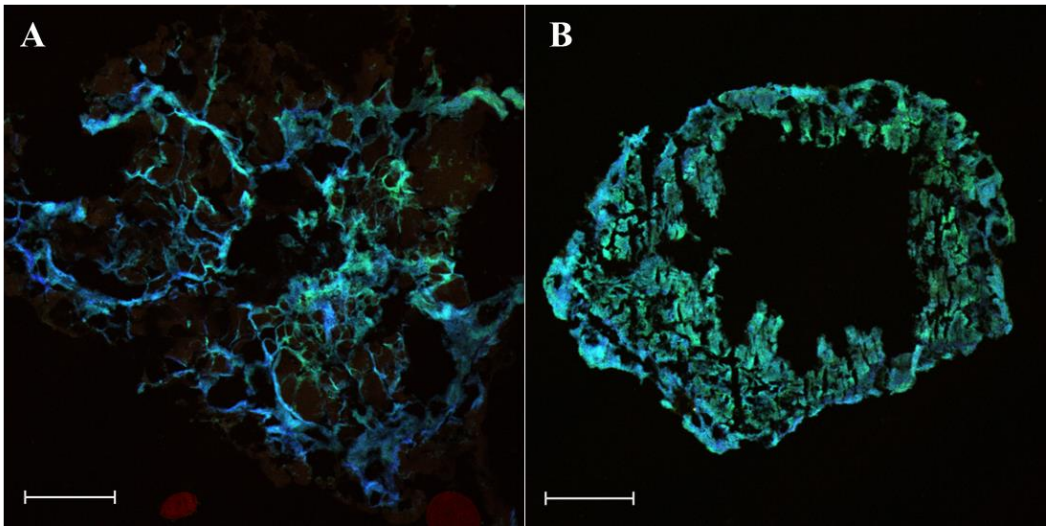


Figure 4.2.- Autofluorescence of AM (A) and S1 samples (B). Images were taken at 10x amplification, with a 5% laser power for lasers 405 (blue) and 488 (green), while laser 561 (red) was set to 50%. Overlapping of lasers signals (light blue and green) shows EPS autofluorescence. Scale bar 200 µm.

4.3.1.2. Conventional FISH with homogenized samples

As the previous analysis showed that the autofluorescent signal decreases from 580 nm onward, the fluorochromes for FISH probes to detect specific populations were selected to have emission in a wavelength higher than that value. Furthermore, considering that autofluorescence could support an erroneous quantification of the target groups, the acquisition of images should avoid areas with a high amount of EPS. Conventional FISH with homogenized samples AM and S1 was performed and selected images of a *xy* section in a single *z* position are shown in Fig. 4.3. Areas with high density of the EPS matrix were avoided as much as possible for image acquisition, but this was not possible in the S1 sample. Thus, when the samples were homogenized, EPS were fully distributed among the sample, and quantification of S1 anammox populations was not possible to be obtained, as it will overestimate the results. Fig. 4.3-H shows that it cannot be distinguished between the autofluorescence of EPS matrix from the fluorochrome signals of hybridized bacterial cells.

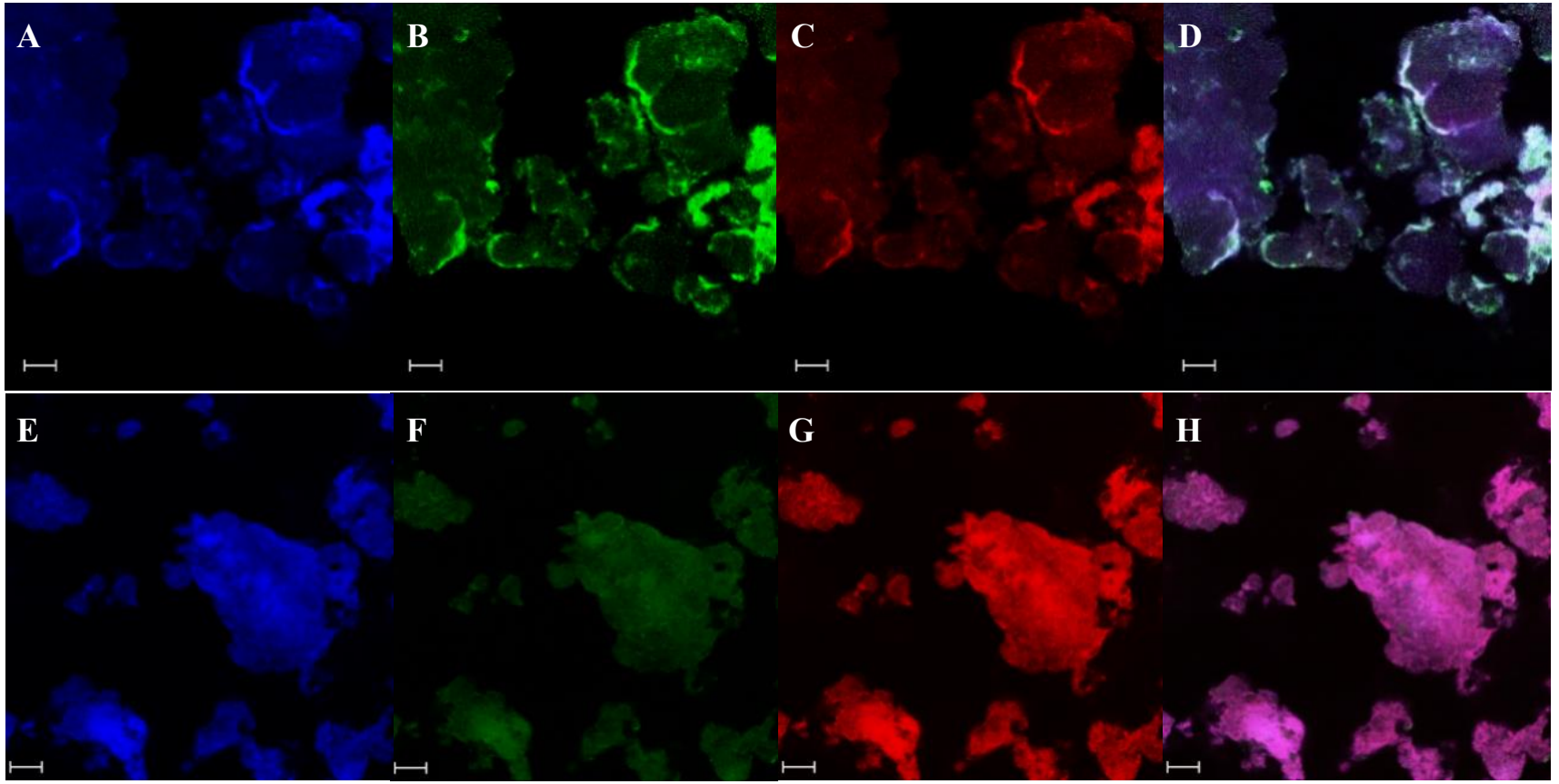


Figure 4.3.- Conventional FISH-CLSM images obtained from homogenized samples AM (A-D) and S1 (E-H). EUBmix in blue (A, E), all anammox in green (B, F), *Ca.* Brocadia anammoxidans in red (C, G) and merged image (D, H). Scale bar 20 µm

Images of homogenized AM sample were quantified considering the colocalization of probes. A 32 ± 12% of anammox population was estimated, where a 45±15% correspond to *Ca.* Brocadia anammoxidans and a 17±14% correspond to *Ca.* Brocadia fulgida.

The standard FISH protocol has shown to failing to distinguish between the EPS matrix's autofluorescence and fluorochrome signals from hybridized bacterial cells on homogenized samples coming from the UAnSB reactor. Nevertheless, the correctness of hybridization of the anammox probe was corroborated by taking 3D images of the AM sample (Fig. 4.4). These also allowed us to clearly observe the "donut-like" shape type structure characteristic of anammox bacteria (Nielsen et al., 2009). Thus, it was confirmed that FISH methodology was correctly used, but it was not reliable on homogenized samples.

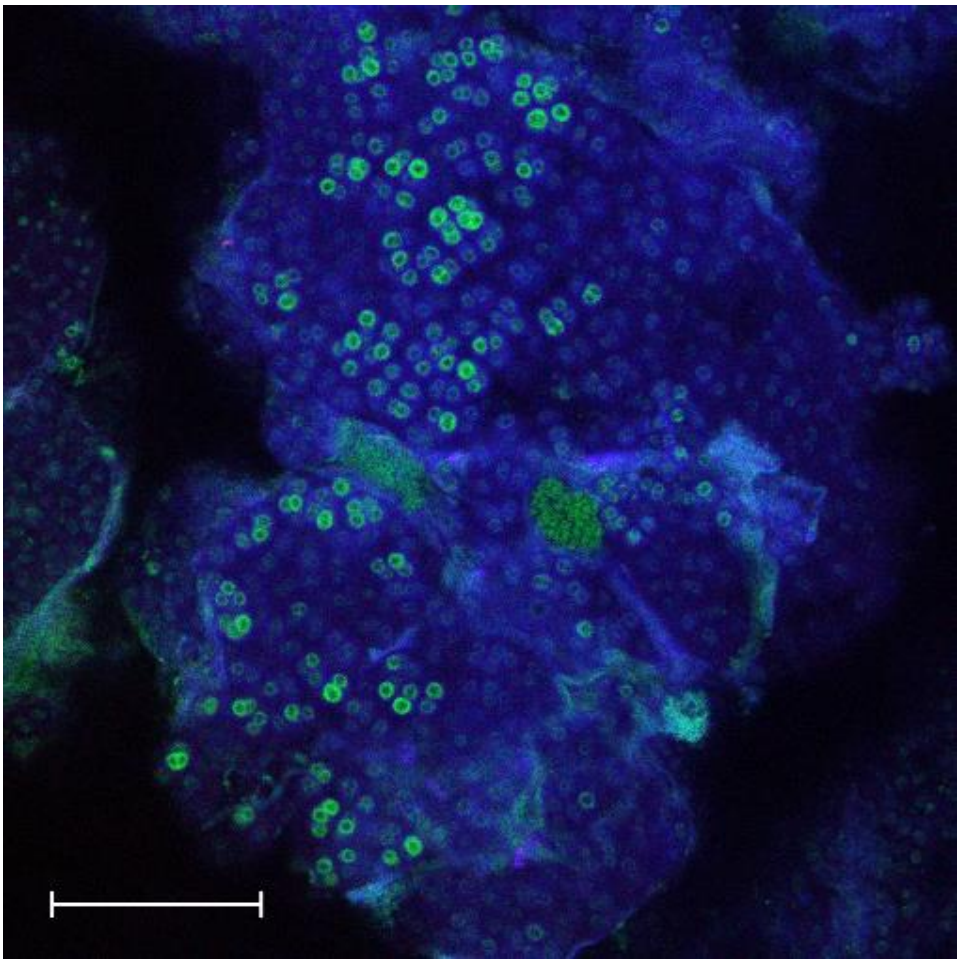


Figure 4.4.- 2D projection of AM sample using FISH-CLSM. All bacteria are depicted in blue and all anammox in green. Scale bar 20 µm.

4.3.1.3. Optimization of conventional FISH for anammox detection in samples coming from reactors performing autotrophic nitrogen removal at mainstream conditions with real wastewaters

Visualization and quantification of anammox bacteria using the FISH technique have been widely used and modified to avoid its limitations (Behnam et al., 2012; Kuypers et al., 2003; Li and Gu, 2011; Pavlekovic et al., 2009), including sample quantity, matrix interferences, among others. Here, we present a fully optimised protocol that includes i) a 24h fixation of sludge granules followed by ii) a cryosection of granules placed in adherent flat slides (this is important to decrease sample fluorescence), then, iii) two enzymatic pre-treatment were required to improve probes' accessibility to cells, iv) 5 h of successive hybridization with probes of different stringency, from the highest to the lowest, and finally, v) the image acquisition in several *z* positions and 2D projection of granule slice were implemented for volumetric quantification and visualization.

Hybridized granules slices were analysed using volumetric acquisition images, considering the distribution of EPS in the samples. Figure 4.5 is shown the 2D projected images. In the upper images, corresponding to the AM sample (A and B) can be distinguished the EPS fluorescence from the hybridization probe signal, so it was possible to capture images with low amounts of EPS. Instead, in the lower images of the S1 sample (C and D), the EPS still cannot be avoided, and the volumetric image acquisition allowed us to observe that anammox bacteria were surrounded by a dense layer of exopolymers, but despite this, it was, indeed, possible to detect the specific probes and anammox colocalization signals (Fig. 4.6).

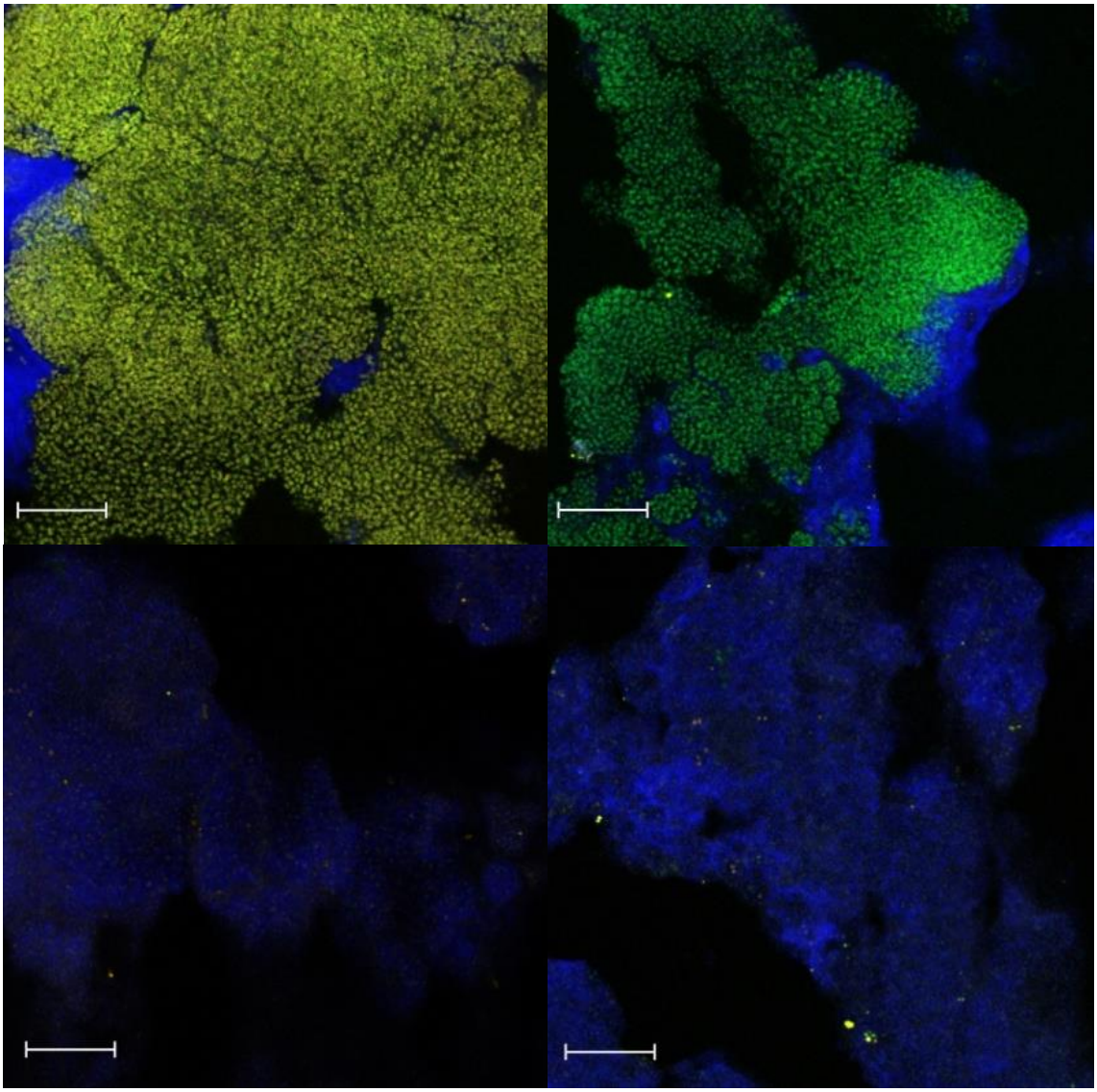


Figure 4.5.- FISH-CLSM merged and projected images obtained from successive hybridization of granule-sludge slices from AM (A and B) and S1 (C and D) samples. EUBmix (blue), all anammox (green), *Ca*. Brocadia anammoxidans (yellow) on A and C, and *Ca*. Brocadia fulgida (yellow) on B and D. Scale bar 20 µm.

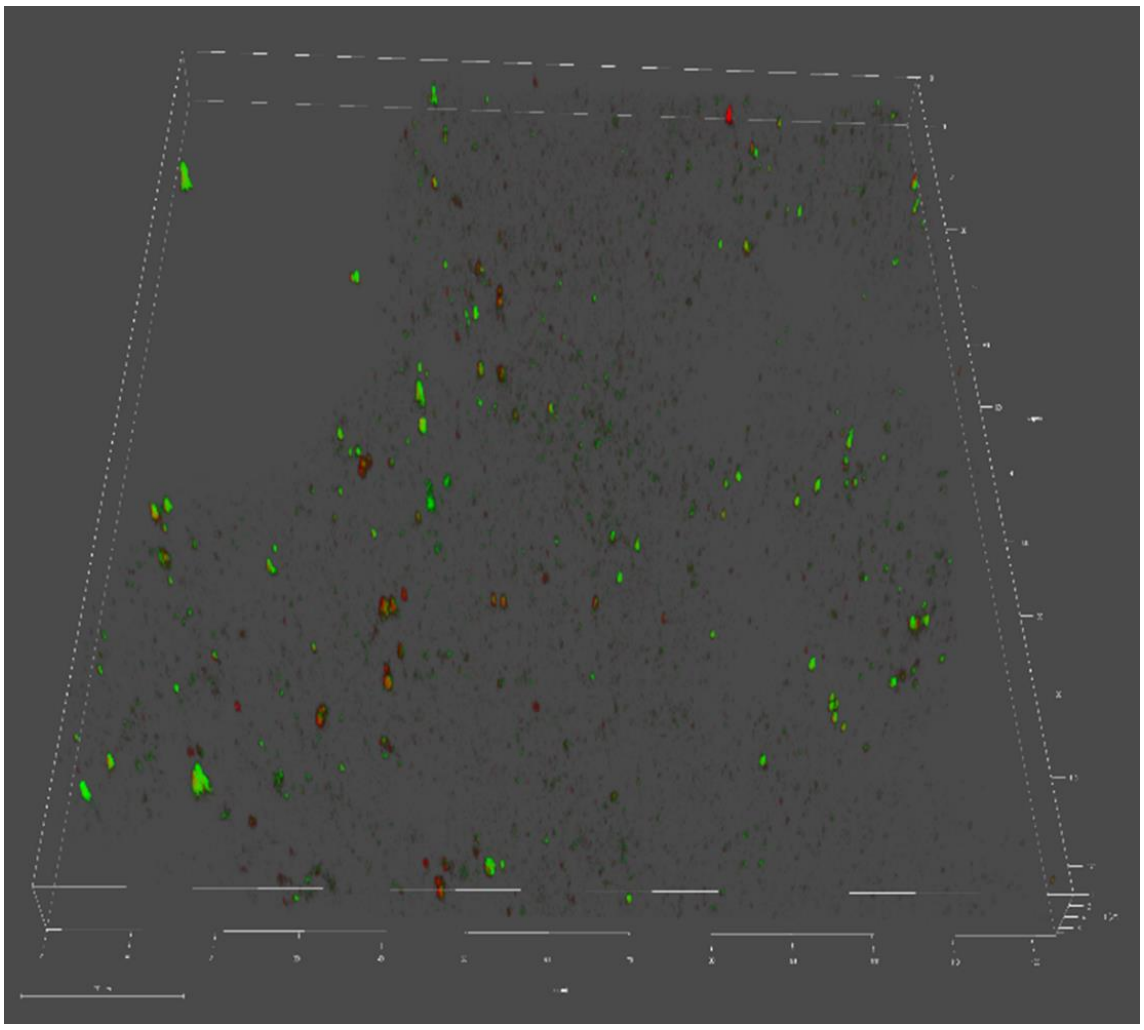


Figure 4.6.- 3D CLSM of S1 granule-slice FISH for volumetric quantification of probe colocalization using Leica software. Anammox bacteria in green, *Ca.* Brocadia fulgida in red. Scale bar 20 µm

After establishing an optimised methodology, total anammox bacteria in the samples and the percentage of the species of *Ca.* Brocadia fulgida and *Ca.* Brocadia anammoxidans in the anammox community were estimated with two different software (Fig. 4.7). There were no significant differences between these quantification methods. High error bars could indicate the heterogeneity of distribution of the target bacteria among the granules. According to this, the population of total anammox bacteria could be estimated over 50% for AM sample, where more than 70% correspond to *Ca.* Brocadia anammoxidans. Instead, in the S1 sample the total anammox bacteria count was lower than 1%, where almost 50% correspond to *Ca.* Brocadia anammoxidans and the other 50% correspond to *Ca.* Brocadia fulgida. The presence of *Ca.* Brocadia fulgida in the S1

reactor could explain the major distribution of fluorescent aggregates because it was described as a bacterium capable of forming an autofluorescent EPS (Kartal et al., 2008).

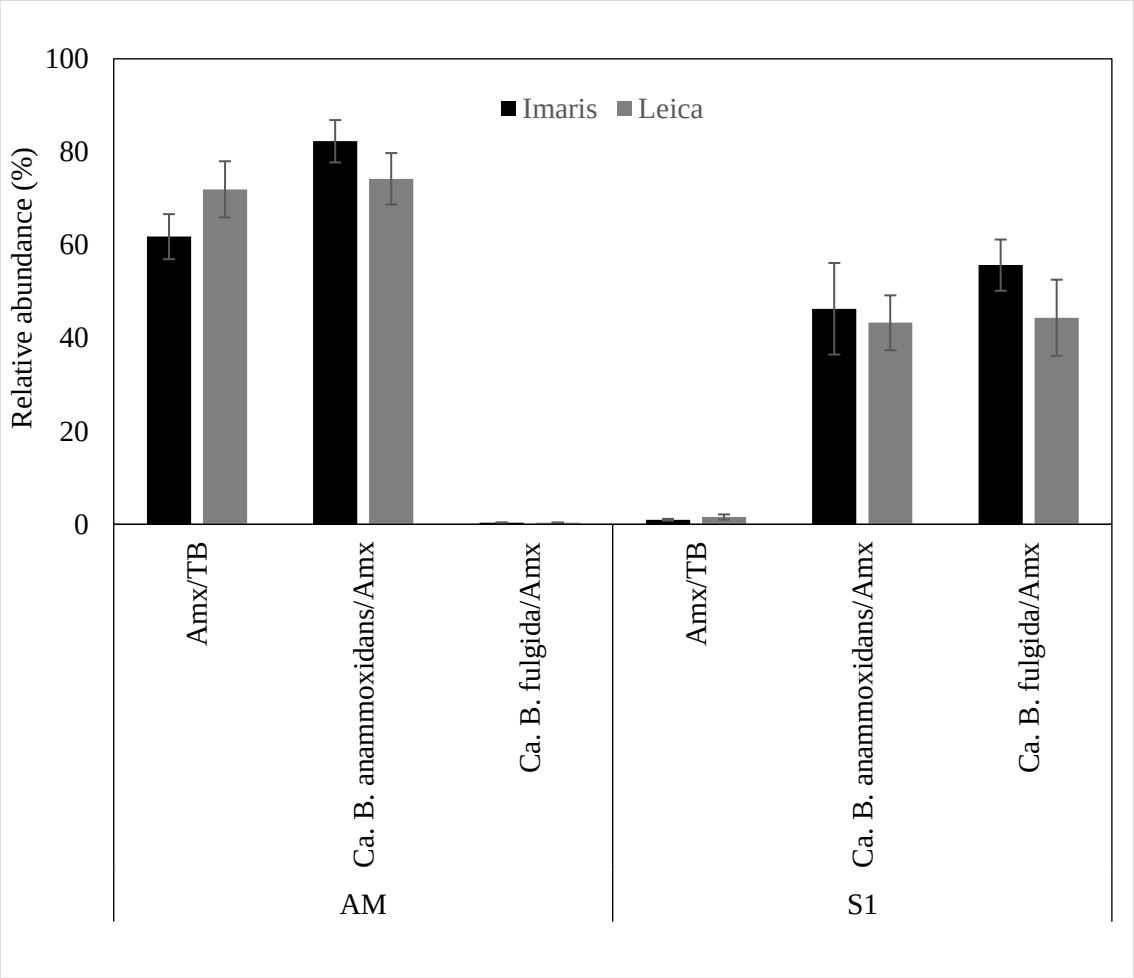


Figure 4.7.- Volumetric quantification of FISH-CLSM images using two different software. Black columns: Imaris, grey columns: Leica. Amx: total anammox bacteria, TB: Total bacteria. The error bars stand for the standard error. No significative difference between both software $p<0.05$.

Even though the low amount of anammox in the S1 reactor is striking (below 1%), the nitrite to ammonium consumption and the produced nitrate to consumed ammonium ratios (1.4 ± 0.1 and 0.20 ± 0.03, respectively), determined by Juan-Díaz et al. (2020), were close to the previously reported stoichiometric ratios for anammox cultures (Lotti et al., 2014; Strous et al., 1998; Yang et al., 2009), thus, the anammox process is taking place in the reactor. Although slightly, more nitrite has been consumed and less nitrate has been produced, indicating that heterotrophic denitrifiers have been using organic matter to reduce nitrite (de Almeida Fernandes et al., 2018) and that part of the nitrate produced by anammox has also been consumed by denitrifiers (Juan-Díaz et al., 2020).

In this sense, (Ni and Yang, 2014) suggest that organic matters enhanced the nitrogen removal by benefiting the denitrifiers, which could be the more active community in the reactor.

Until now, several studies have been using different FISH protocols for detecting, quantifying, or analysing the spatial distribution of the anammox community in wastewater samples, but usually, these samples have been passed through an enrichment process with a synthetic medium (Almstrand et al., 2013; Mardanov et al., 2019; Pavlekovic et al., 2009; Schmid et al., 2005). As we have observed, sludge-granules from a process treating real wastewater shown a higher amount of EPS, i.e, higher autofluorescence, than samples coming from reactors treating synthetic wastewaters, so, quantification of FISH images on this kind of samples is a complex issue. The modified protocol we propose, and the improvement of the image acquisition technique allow for a reliable estimation of the anammox population. We also observed that the FISH technique is useful to know the presence and distribution of the desired population among the granule, the same pointed out by Mardanov et al. (2019) where FISH was used to visualize various groups of microbial satellites of anammox bacteria of an anammox bioreactor, to understand and clarify the structure of the community.

In this sense, an exhaustive study of the autofluorescence properties of these complex samples combined with the continuous development of photon microscopy technologies, such as confocal white light microscopes that offer three-dimensional excitation-emission spectral maps (Borlinghaus, 2012), will allow a better optimization in the protocols of acquisition and quantification imaging in complex samples with variable autofluorescence due to different components.

4.3.2.- Comparison of different molecular approaches to study bacterial communities in a mainstream anammox reactor

Different molecular approaches to determining the whole bacterial and the anammox communities' structure in a granular-sludge anammox reactor treating real wastewater at mainstream conditions were evaluated. To a better understanding of the used strategies in every step of this work, we define six DNA-based approaches, coming from three techniques: amplicon sequencing, metagenomics, and qPCR. The amplicon sequencing technique was performed using three primers sets, where each primer was

considered an individual approach: a) universal primers 515F-806R, b) universal primers 515F-909R and c) specific anammox primers Amx368F-Amx820R. The shot-gun sequencing metagenomic was the second technique evaluated, including two different approaches: d) 16S rRNA sequences extracted from the metagenomic data, and e) functional taxonomy assigned based on functional genes. These five approaches were sequencing-related and relative abundances were obtained. The final technique performed was qPCR, where primers targeting 16S rRNA gene for anammox populations, and two primer set targeting functional genes were used. This last technique allowed an absolute quantification.

4.3.2.1 Microbial community composition

Four different molecular DNA-based approaches (a, b, d and e) were used to determine the microbial composition of the samples taken from two anammox reactors: reactor S1 (anammox reactor treating urban wastewater at mainstream conditions) and reactor AM (enriched anammox culture treating synthetic influent as a positive control).

The first evaluated technique was amplicon sequencing. This was performed using two sets of universal primers: 515F-806R and 515F-909R (approaches a and b). These primers have 94% and 96% of coverage for the Planctomycetota phylum, respectively, when checked with SILVA test prime SSU138 RefNR database (Table 4.2). A coverage of 94% and 96% was obtained for the universal primers 515F-806R and 515F-909R, respectively. Shot-gun sequencing metagenomic was the second technique evaluated, including two different approaches (approaches d and e).

Relative abundances of the four datasets were compared at the phylum and class level. At the phylum level, Planctomycetota, Bacteroidota, Chloroflexi, and Proteobacteria were the main phyla detected in all the datasets for both samples, although the relative abundances vary among techniques and, of course, reactor samples. A very low coverage of the phylum Planctomycetota was observed using the universal primers 515F-909R. In the anammox enriched culture treating synthetic influent (AM), the results showed that 16S rRNA amplicon with universal primers 515F-909R detected 1% of phylum Planctomycetota while the universal primers 515F-806R presented a relative abundance of 79% (Fig. 4.8)

Table 4.2. *In silico* primer coverage prediction for the primers used in this study restricted to de phylum Planctomycetes in SILVA Test Prime analysis (SSU Ref 138 NR).

			Target gene	Bacterial 16S rRNA			Anammox 16S rRNA	
			Primers set	515F-806R	515F-909R	519F-907R	Amx368F-Amx820R	Amx809F-Amx1066R
			References	(Caporaso et al., 2012)	Tuan et al. (2014)	Stubner (2002)	Gu et al. (2007)	Kindaichi et al. (2007)
		Planctomycetota		94	96	96	0.9	43
		Brocadiae		90	90	90	52	92
Brocadiales				91	90	91	54	92
Brocadiales	Brocadiaceae			85	85	85	93	92
Brocadiales	Brocadiaceae	Ca. Anammoxoglobus		67	67	67	67	100
Brocadiales	Brocadiaceae	*Ca.* Brocadia		91	91	91	97	97
Brocadiales	Brocadiaceae	*Ca.* Jettenia		94	94	94	88	88
Brocadiales	Brocadiaceae	*Ca.* Kuenenia		87	87	87	93	80
Brocadiales	Scalinduaceae			98	94	96	13	98
Brocadiales	Scalinduaceae	*Ca.* Scalindua		98	94	96	13	98

Concerning amplicon sequencing, there are fundamental biases related to the PCR amplification and the primer set used that impact the quantitative information that were obtained from these approaches, as also shown (Ferrera and Sánchez, 2016). We have selected two universal primers set targeting the same hypervariable region of the 16S rRNA gene (V4-V5), that showed similar coverage efficiencies and specificity for anammox bacterial detection on in silico evaluation with SILVA test prime, as recommended by various investigations (Liu et al., 2020; Yang et al., 2020; Zhou et al., 2018). Nevertheless, our results showed that primer set 515F-909R did not cover most of the organisms from the phylum Planctomycetota present in our reactors, underrepresenting the anammox community despite the estimated in silico coverage (96%). In this regard, the differences between in silico and in vitro results have been previously reported (Henriques et al., 2012; Morales and Holben, 2009). This may be due to *in silico* predictions do not consider chemical reactions and limitations that can occur in the PCR tube and therefore cannot truly approximate to the practical applications (Henriques et al., 2012).

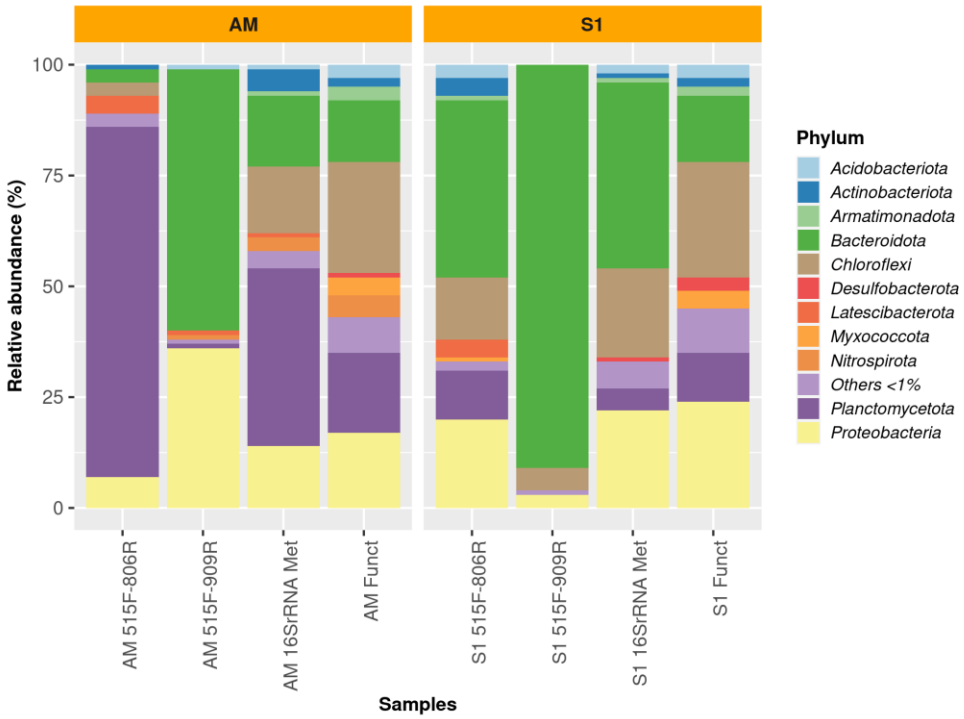


Figure 4.8. Relative abundance of identified phyla in anammox reactors using four different molecular approaches. Phyla whose relative abundance among all the techniques was less than 1% were grouped as “Others”.

In contrast, the primer set 515F-806R gave us a better representation of the anammox community but could lead to an underestimation of the other phyla on the enriched culture. This, due to the amplification bias among taxa with low estimate abundance in amplicons, as was demonstrated by Campanaro et al. (2018).

Differences in the community structure showed with universal primers in amplicon sequencing could be explained for the amplicon size, which is 394 bp and 291 bp for 515F-909R and 515F-806R, respectively. Huber et al. (2009) demonstrated that smaller amplicon libraries contained a higher diversity and the ability to detect lower abundant groups in the microbial community.

Also, differences between amplicon sequencing and metagenomics results were noted. Specifically, 16S rRNA extracted reads from metagenome showed 40%, whereas with functional taxonomy from metagenome 18% was estimated. For the S1 sample the results showed higher differences between the approach used, as primers 515F-909R did not detect phylum Planctomycetota and showed that Bacteroidota was the main phylum (91%) (Fig. 4.8). For the other 2 datasets based on 16S rRNA gene, no major differences were observed between the relative abundances of the different phyla core mentioned above, except for Planctomycetota, where 5% was estimated with metagenomic 16S rRNA versus 11% with 16S rRNA amplicon sequencing using the 515F-806R set of primers. Yet, Bacteroidota, Proteobacteria and, Chloroflexi dominated the microbiome core. Differences between amplicon sequencing and metagenomic and even between the two metagenomics approaches were obtained. This could be explained because a common bias for all the techniques studied is the databases limitations, although there are several high quality, comprehensive and curated 16S rRNA gene databases compared to genomic databases (Poretsky et al., 2014).

The main differences in terms of relative abundance were obtained with functional taxonomy when all approaches were compared. Metagenomics approaches, on the contrary, avoids PCR biases (Logares et al., 2014) and have resulted in a turning point in studying phylogenetic and functional diversity of wastewater treatment systems (Ferrera and Sánchez, 2016) since offers the potential to investigate 16S rRNA gene fragments, reconstruction of full-length 16S rRNA gene sequences and functional genes annotation without amplification and primers biases, reaching a much higher resolution of taxonomic annotation (Liu et al., 2020; Poretsky et al., 2014; Speth et al., 2016). But, on the other

hand, its limitations include a higher cost and bionformatics complexity than PCR (Liu et al., 2020).

The analysis at the class level (Fig. 4.9-A) revealed that Anaerolineae, Brocadiae, Gammaproteobacteria and Ignavibacteria were the most abundant classes in both reactors, corresponding to the phyla Chloroflexi, Planctomycetota, Proteobacteria and Bacteroidota, respectively. The S1 reactor, was dominated by heterotrophs classes, not anammox. For this reactor (S1) Brocadiae was not detected with the universal primers 515F-909R. In both reactors the same differences were detected between the determinations of the approaches at the class level as at the phylum level. According to both metagenomics approaches, 10% of the relative abundance of Planctomycetota phylum detected in AM sample correspond to the non-anammox class Phycisphaerae while showed 1% with amplicon techniques.

The relative abundance of the class Brocadiae, which includes all anammox bacteria, varies between reactors and the approaches used. A low abundance of this class was observed in the mainstream anammox reactor (S1). Important differences in the relative abundance at this class were observed among techniques in the AM reactor, and nevertheless, in the S1 reactor were in a similar range (3-11%).

To determine how the samples analyzed with different approaches were grouped, an NMDS analysis was performed. The samples from amplicon sequencing using the primers 515F-909 were excluded of the analysis as they do not detect the class Brocadiae in the S1 reactor. The results obtained using the relative abundance of the different classes showed that the communities were grouped by reactor (S1 and AM) (Fig. 4.9-B). Thus, major difference between techniques were observed in AM reactor, where the class Anaerolineae was underrepresented in the amplicon sequencing compared to the metagenome in the AM reactor, but, on the contrary, Brocadiae class was overrepresented comparing with the metagenomic approach (Table 4.3). These differences would be explained because 16S rRNA sequencing could detect only a part of the microbial community, while shotgun sequencing (metagenomics) has more power to identify less abundant taxa (Durazzi et al., 2021). In our study, the concordance between the functional and amplicon taxonomic assignment in the S1 reactor could indicate that this last it is a good method to visualize the functional abilities of our reactor. In agree with (Ferrera and Sánchez, 2016), more effort is needed to better understand the relationship between structure and function in these microbially mediated processes.

In terms of the composition of the bacterial community, these results revealed four predominant phyla in both reactors, beyond the differences in relative abundances, which have been recently described by different authors as a core microbiome. This core formed mainly by Proteobacteria, Bacteroidota, Chlorofexi, and Planctomycetota phyla, have been found in the microbial communities of anammox bioreactors, being similar in several types of reactors, despite the differences in the operation of the reactor and the composition of the influent wastewater (Bhattacharjee et al., 2017; Lawson et al., 2017; Mardanov et al., 2019; Speth et al., 2016; Zhao et al., 2018).

The S1 reactor, instead, was dominated by the heterotroph's classes Ignavibacteria, Anaerolineae and Gammaproteobacteria, not anammox. Brocadiae class was found in very low abundance and was not detected with the universal primers 515F-909R. Function of classes Ignavibacteria and Anaerolinea (Bacteroidota and Chloroflexi phyla, respectively) are not yet completely understood, but based on transcriptional insights on interactions with anammox, they seem to be polymer-degrading, fermentative anaerobes, and participates in the nitrogen removal supporting complete denitrification (Gonzalez-Gil et al., 2015; Lawson et al., 2017; Zhao et al., 2018). Also, they could supply short-chain volatile fatty acids and alcohols to support other communities as Proteobacteria, due to the ability to hydrolysate extracellular compounds of anammox bacteria (Bhattacharjee et al., 2017; Juan-Díaz et al., 2020). A similar community dynamic was showed by Yang et al. (2018), where heterotrophs constituted the majority of microorganisms in a mainstream Partial nitritation/Anammox (PN/A) reactor and a low amount of anammox bacteria was detected. This latter has been also reported in similar works, as in Laureni et al. (2016) using pre-treated MWW effluent on biofilms PN/A reactors; Luo et al. (2017) in anammox granules treating effluent from a primary clarifier from a full-scale WWTP and Miao et al. (2020) in granules from a simultaneous partial nitrification, anammox and denitrification (SNAD) reactor treating municipal wastewater. Likewise, our findings support and detail from a bioinformatics point of view the operational results of Juan-Díaz et al. (2020), where it was demonstrated that there was heterotrophic denitrification activity in the reactor due to the presence of COD in the influent, but that anammox activity dominated the overall nitrogen conversion despite its abundance.

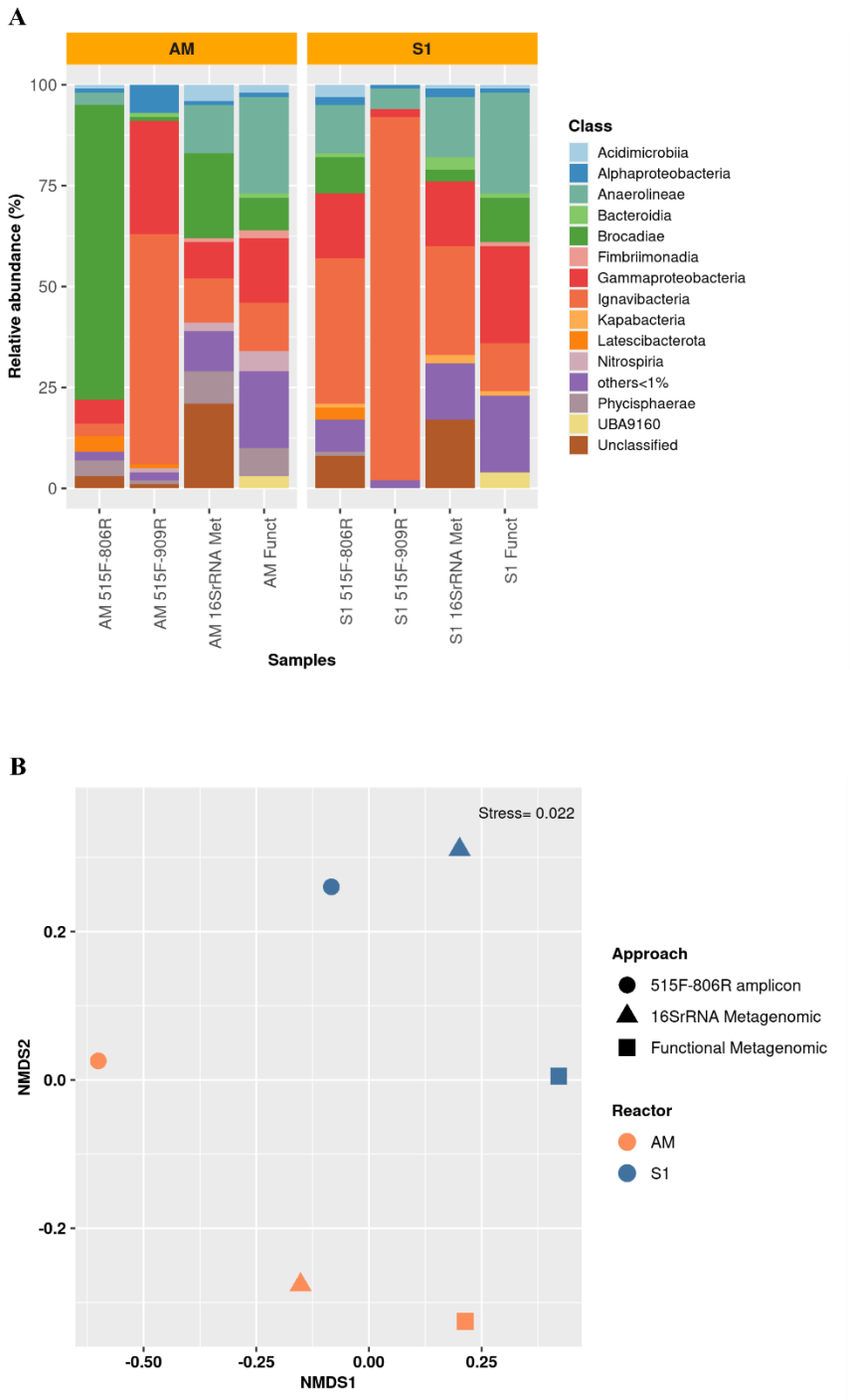


Figure 4.9.- A) Relative abundance of identified classes of the microbial community in anammox reactors using four different molecular approaches. Classes whose relative abundance among all the techniques were less than 1% were grouped as "Others". B) NMDS ordination using the class level relative abundance data of the whole microbial community. Communities similarities are based on the Bray–Curtis index which includes the relative abundances on each approach

4.3.2.2. Anammox bacteria community analysis

To specifically study the anammox community, a third set of primers was included (approach c). The primers Amx368F-Amx820R were designed to target anammox bacteria and have 93% of coverage for the Brocadiaceae family when checked with SILVA test prime SSU138 RefNR database (Table 4.2). Then, five approaches were used: amplicon sequencing using three primers sets (approaches a, b and c) and two taxonomic classification methods derived from metagenomics data (approaches d and e). Anammox sequences from each approach classified at the Brocadiae class were separately analyzed and relative abundances of anammox community at deeper taxonomic levels were determined.

Leaving aside amplicon sequencing data obtained with primers 515F-909R which did not detect Planctomycetota in S1 sample, 100% of Brocadiales order was detected. At family level, all approaches detected mainly Brocadiaceae in the two samples. 1% of Scalinduaceae was determined in AM sample with functional taxonomy (Table 4.3). Approximately 10% of the sequences from both reactors' samples were not classified at the family level with the16S rRNA metagenomic approach.

Regarding the approach, in the whole community overview, it was observed that the 515F-909R primers did not cover the anammox community developed in our reactors, but all other methods studied were consistent in the composition of the anammox community down to the family taxonomic level (phylum, class, order, and family).

The classification at the genus level reveals that the anammox community in both reactors´ samples correspond mainly to genus *Ca.* Brocadia (Fig. 4.10-A). All the approaches showed similar detection abilities in both reactors. The communities obtained by amplicon sequencing with the three primers sets showed no other genus than *Ca.* Brocadia in the AM sample, while a low abundance of *Ca.* Kuenenia was detected in the S1 reactor. The primers 515F-909R did not detected anammox at any taxonomic level. The 16S rRNA metagenomic approach detected mainly *Ca. Brocadia* and small abundances of *Ca.* Jettenia and/or *Ca.* Kuenenia in both reactors, nevertheless, showed a low resolution at the genus level. A high proportion of the sequences were classified at previous taxonomic levels as order and family (41% and 40%, for AM and S1 reactors, respectively). *Ca.* Brocadia was also the genus of anammox with the highest abundance detected by functional taxonomy in both reactors. The NMDS analysis (Fig. 4.10-B)

showed that S1 samples grouped together while for AM samples the communities retrieved by the two-metagenomics approach were closer positioned and the communities obtained using the 16S rRNA approaches were identical for the two set of primers.

About the anammox bacteria community on S1 reactor, the dominant genus was *Ca.* Brocadia. Hugh contrast are reported on mainstream studios about the predominance of certain genus. For example, Yang et al. (2018), observed that despite the presence of *Ca.* Brocadia, *Ca.* Kuenenia was de dominant anammox bacteria on mainstream conditions in a PN/A reactor, and de Almeida Fernandes et al. (2018) showed that *Ca.* Anammoximicrobium was the dominant anammox population in a reactor treating a real anaerobic MWW effluent. Contrariwise, the predominance of *Ca.* Brocadia has been reported in many mainstream studies (Cao et al., 2017; Laureni et al., 2016; Liu et al., 2018; Lotti et al., 2015; Miao et al., 2020; Nejidat et al., 2018). Some studies on anammox WW treatment technologies have reported that there would be a dominant genus of anammox bacteria under specific growth conditions (Kartal et al., 2008), so this will depend on the capacity to adapt to the effluent (Gonzalez-Martinez et al., 2018).

Functional taxonomy from metagenomes allowed obtaining a classification at the species level (Fig. 4.11). The predominant species of anammox bacteria in the reference reactor, AM, were *Ca.* Brocadia sinica and *Ca.* Brocadia fulgida (45% each). In the S1 reactor, *Ca.* Brocadia fulgida (66%) and *Ca.* Brocadia caroliniensis (18%) had higher relative abundance.

Down genus level, all techniques cover the same/similar anammox bacteria, but the 16S rRNA sequences extracted from the metagenome approach lost resolution in the taxonomic assignment and on the contrary, functional taxonomy of the metagenome had a better resolution on assignment at the specie level. Nevertheless, considering that functional taxonomy from metagenomes is a new approach, the obtained results depend on database updates and completeness, and genome annotations pipelines are nowadays on constant challenge (Dong and Strous, 2019).

Table 4.3 Comparative table of the relative abundance (in percentage) obtained for the anammox microorganisms with the different approaches applied in this work. The relative abundance of the higher taxonomic levels of phylum and class was determined as the percentage of ASVs of each level within the total of ASVs. From the taxonomic level of order onwards, the relative abundance was defined as the ASVs of the level within the Brocadiae class.

		AM					S1				
Taxonomic Classification		16S rRNA Amx368F-Amx820R	16S rRNA 515F-909R	16S rRNA 515F-806R	16S rRNA metagenomic	Functional genes	16S rRNA Amx368F-Amx820R	16S rRNA 515F-909R	16S rRNA 515F-806R	16S rRNA metagenomic	Functional genes
Phylum	Plancomycetota	100	1	79	40	18	100	0	11	5	11
Class	Brocadiae	100	1	75	27	8	100	0	9	3	11
Order	Brocadiales	100	100	100	100	100	100	0	100	100	100
Family	Brocadiaceae	100	100	100	90	99	100	0	100	89	99
Genus	*Ca.* Brocadia	100	100	99,8	55	95,4	99	0	99	56	98
	Ca. Jettenia	0	0	0	3	2	0	0	0	0	0,4
	Ca. Kuenenia	0	0	0,2	1	0,5	1	0	1	4	0,9

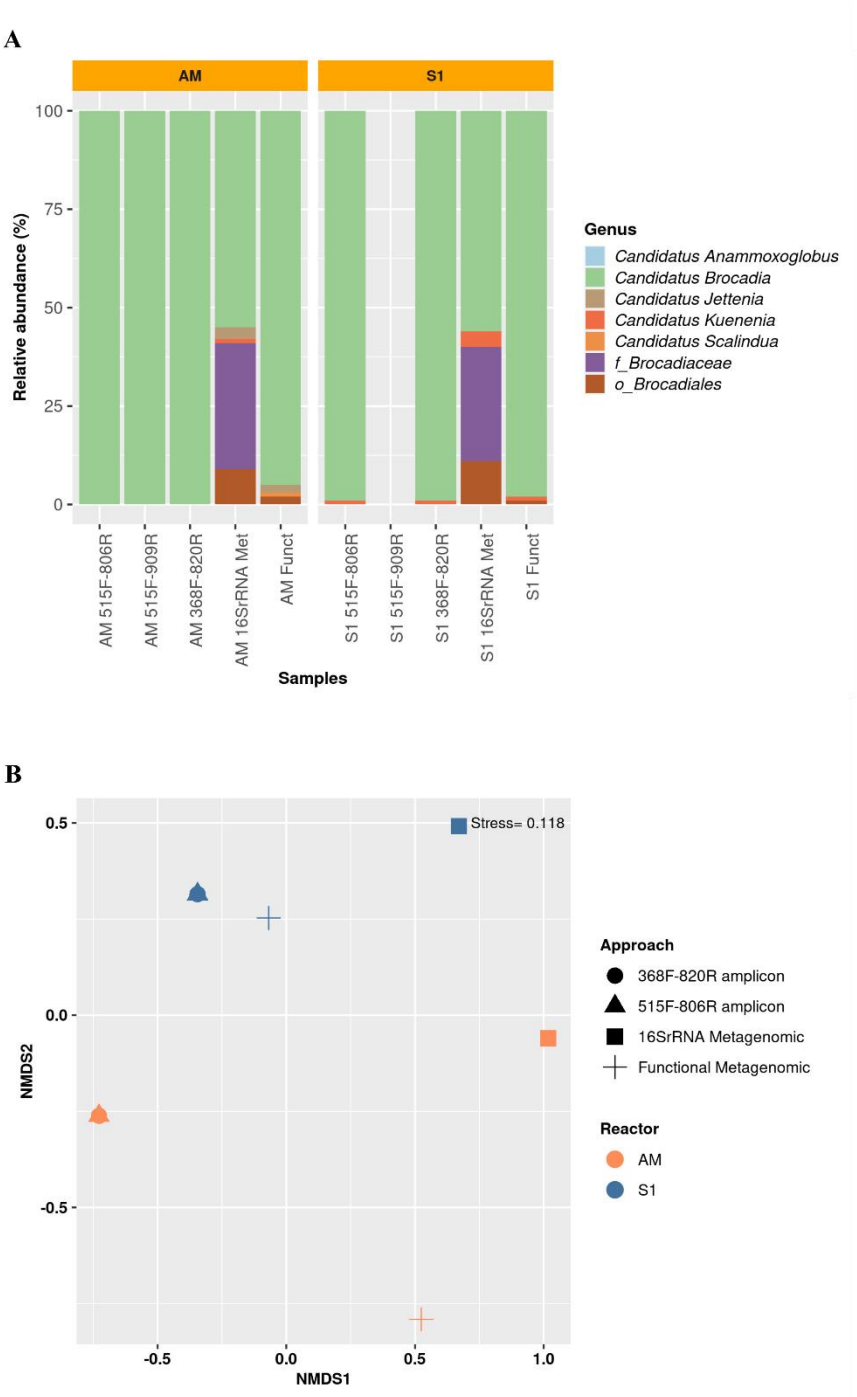


Figure 4.10.- A) Relative abundance of identified anammox bacteria at the genus level using five different molecular approaches. When classification at the genus level was not obtained, the previous taxonomic level was indicated as f (family) and o (order). B) NMDS ordination using the genus level relative abundance data of detected anammox bacteria. Communities' similarities are based on the Bray–Curtis index which includes the relative abundances on each approach.

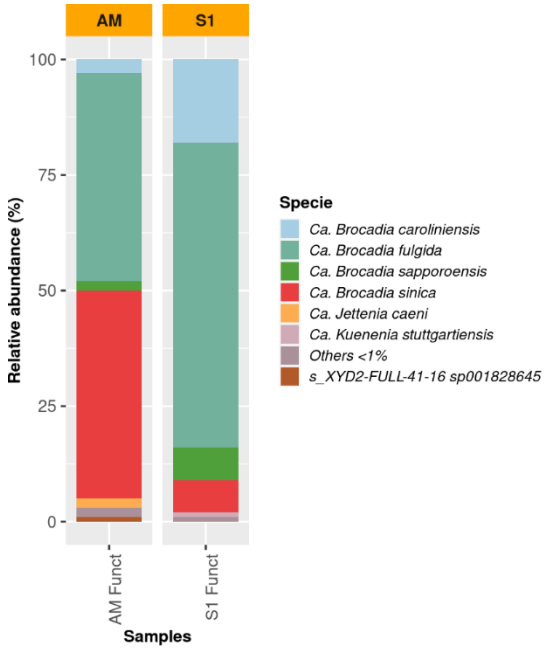


Figure 4.11.- Relative abundance of identified anammox bacteria at the specie level using functional genes approach from metagenomes. Contigs with abundance below 1% were grouped as "Others". Results presented as "s" (specie) are not associated to a known *Candidatus* specie.

4.3.2.3. Phylogeny of anammox microorganisms predominant in the samples

A phylogenetic analysis was performed to elucidate the relationship between the anammox sequences obtained by the different 16S rRNA-based approaches and the complete 16S rRNA genes retrieved from the metagenome without any primer's bias. For the amplicon sequence analysis, the sequences were grouped in ASVs (sequences with 100% similarity). As different regions were target by the different primers sets, three phylogenetic trees were constructed, one for each primers sets (Fig. 4.12). Comparing the three phylogenetic trees it can be observed that for the specific set of primers (AMX368F-AMX820R) and for the universal primers (515F-806R) most of the ASV sequences cluster in the same branch with sequences retrieved from the metagenomic approach. Some ASVs sequences retrieved with the specific primers did not cluster with any of the sequences retrieved from the metagenomic approach. Due to the low coverage of the set of primers (515F-909R) only one ASV was retrieved which cluster with a sequence retrieved by the metagenomics approach.

Some ASVs sequences retrieved with the specific primers did not cluster with any of the sequences retrieved from the metagenomic approach. Due to the low coverage of

the set of primers (515F-909R) only one ASV was retrieved which cluster with a sequence from the metagenomic approach.

Most of the ASV sequences were positioned in two clusters (A and B) within the genus *Ca.* Brocadia. On one side, Cluster A grouped the most abundant ASVs of the S1 sample with *Ca.* Brocadia fulgida reference sequences (around 70% of relative abundance of ASVs retrieved with specific primers and with universal primers 515F-806R) and with two sequences retrieved from the metagenome (Full length AM metagenome 1 and Full length S1 metagenome 1).

The dominant anammox in the S1 reactor was *Ca.* Brocadia fulgida. Anammox communities dominated by this specie have been reported in a variety of studies on mainstream effluent treated by anammox and PN/A processes, though, it was mainly detected by fluorescence in situ hybridization (FISH) (Hoekstra et al., 2018; Laureni et al., 2016; Lotti et al., 2015; Reino et al., 2018) and others by denaturing gradient gel electrophoresis (DGGE) (Nejidat et al., 2018) and metagenomics (Lawson et al., 2017). So, to the authors' knowledge, there is a lack of DNA sequencing data available about the anammox "*Candidatus*" species present in mainstream anammox which allows a comparison of our results.

The next ASVs in decreasing abundance order (12% in Fig. 4.12-A and 17% in Fig. 4.12-B) were bunched in the same clade as the environmental clone Pla PO55-3 sequence (GQ356105), and close to and the sequence from the recently described "*Candidatus"* Brocadia pituitae (Okubo et al., 2021). On the other side, Cluster B involved the dominant ASVs of the AM sample (100%) which were also present in the S1 sample (relative abundance of 8% in Fig. 4.12-A and 4.12-B). These ASVs and two sequences retrieved from the metagenome (Full length AM metagenome 2 and 3) were bunched in a clade, close but in a different branch to the environmental clone ZMP-3 sequence (GQ175287) and distant to the reference's sequences of *Ca.* Brocadia sinica, *Ca.* Brocadia anammoxidans and *Ca.* Brocadia brasiliensis. Clusters C, D, E and F grouped sequences from other anammox genera: *Ca.* Kuenenia, *Ca.* Anammoxoglobus, *Ca.* Jettenia and *Ca.* Scalindua respectively. From these clusters, only in cluster C, sequences from ASVs (1% of relative abundance) and from the genome were positioned. As expected by the low coverage of this primers set, the sequences obtained with the primers 515F-909R detected only one ASV positioned in cluster B for the AM sample. The most abundant ASVs retrieved with the different amplicon sequencing approaches,

always cluster in the same branch with a sequence retrieved by the metagenomics approach, showing coherence between all approaches in the phylogenetic analysis.

From this phylogenetic analysis, we were struck by the fact that the sequences from the entire anammox population of our reference reactor (AM) and, also detected in the S1 reactor (8% of relative abundance), were positioned into a clade that is not directly associated with any previously described species. According to Whitman, (2016), sequence of genomic DNA may also serve as the type of material that unambiguously identifies the species. Herein, we propose a new anaerobic ammonium oxidation (anammox) bacterium, namely "*Candidatus*" Brocadia barcinensis. Representative sequences were submitted to GeneBank with accession numbers: MZ242076 and MZ242077.

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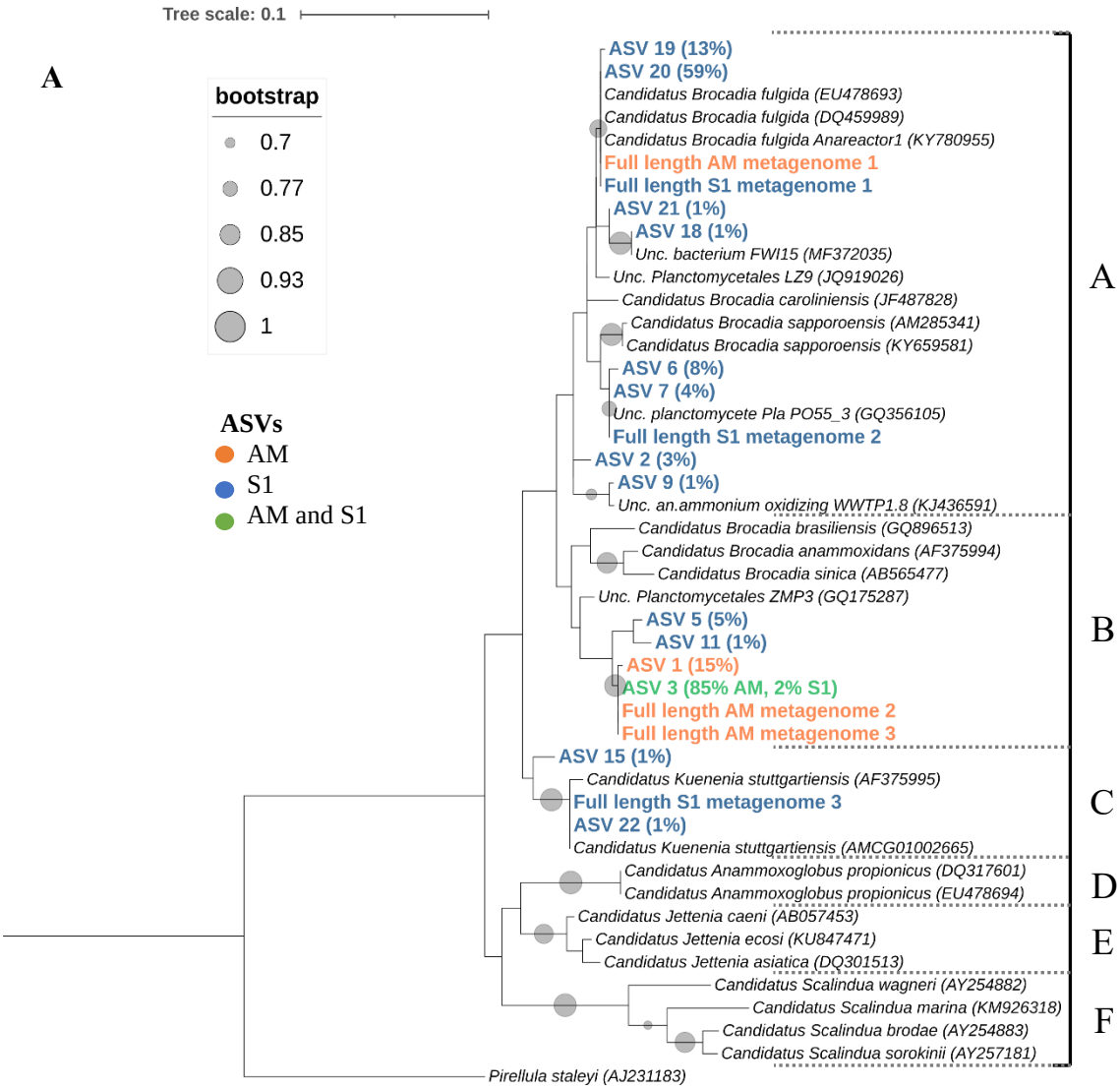

Figure 4.12. Phylogenetic Maximum Likelihood trees based on 16S rRNA gene sequences from amplicon sequencing with primers set Amx368F-Amx820R (A) and 515F-909R (B). ASVs of this study and full-length sequences of 16S rRNA gene reconstructed from metagenomes are shown in colors for each reactor. The relative abundance of each ASV is shown in parentheses. Accession numbers of the reference sequences and environmental clones of anammox bacteria are shown in parentheses. Bootstrap values (greater than or equal to70%) based on 500 replications are shown at branch nodes. *Pirellula staleyi* was used as an outgroup for rooting trees. Bar indicates 0.01 substitutions per site.

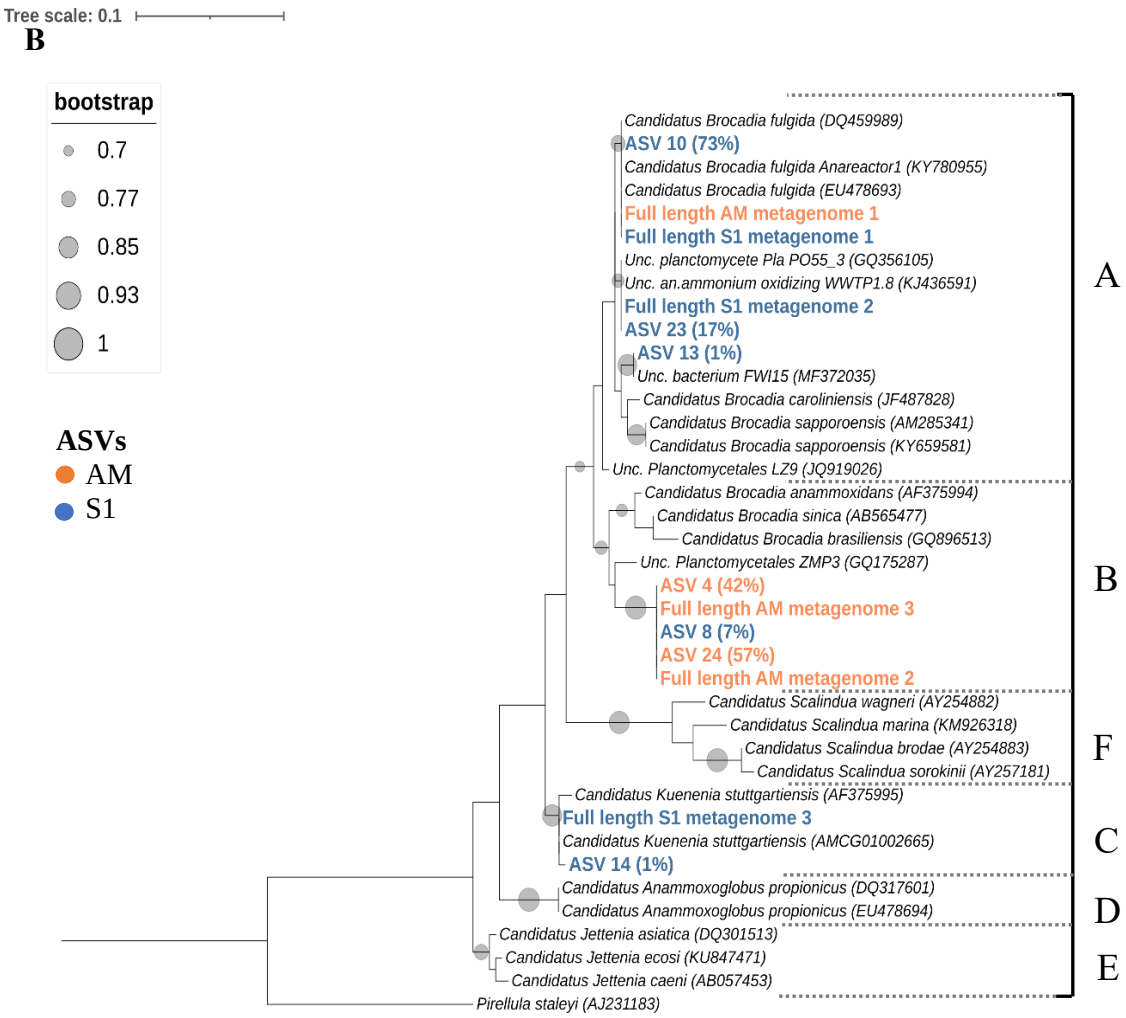

Figure 4.12. Cont.

C

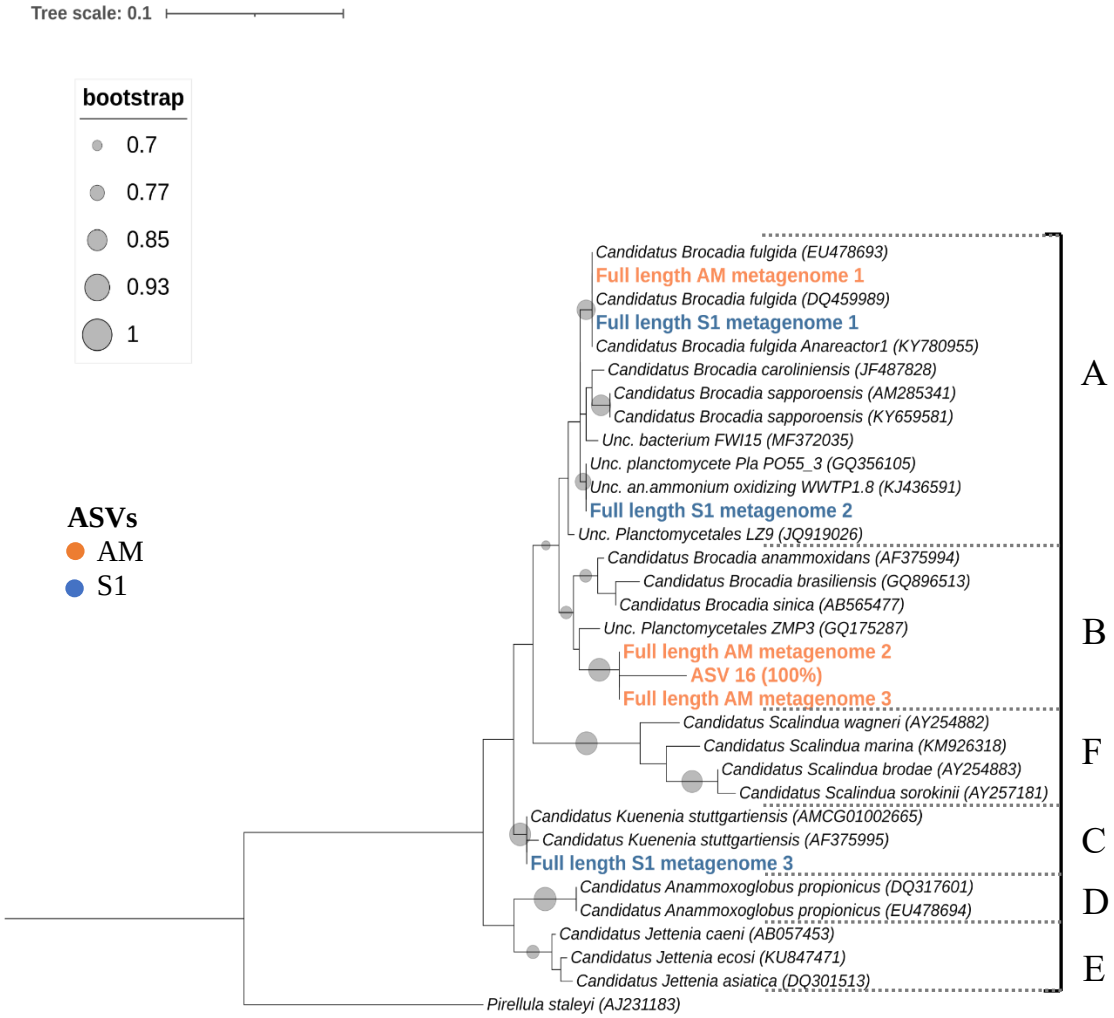

Figure 4.12. Cont.

4.3.2.4. 16S rRNA and functional genes quantification by qPCR

To specifically quantify our target population, the main method used is quantitative PCR (qPCR) (Li &Gu, 2011). The 16S rRNA gene is generally the most used biomarker to design primers for detecting anammox bacteria (Z. bin Wang et al., 2016; Yang et al., 2020). But earlier studies on this field, referred that targeting the functional genes of anammox bacteria provides a more reliable method of detection, especially in environments with low anammox abundance (Kartal et al., 2011). In this case, functional genes specific for the metabolic pathway can be used as markers. We use two different functional genes markers: hszA and nirS. Moreover, a specific 16S rRNA primer set targeting all the anammox 16S rRNA genes was also included in the analysis and first checked on a good performance *in silico* (see primer coverage in Table 4.2) and in practical test according to different authors (Li and Gu, 2011; Yang et al., 2020; Zhou et al., 2018). In order to compare the values from the different reactors a boxplot was constructed, and statistical analysis was performed (Fig. 4.13). The results showed that 16S rRNA gene copies were significant different for both reactors with higher values for the S1 reactor. In the enriched anammox reactor treating synthetic influent, values of 1.04 x 10^6- 1.17 x 10^6 gene copies ng^{-1} DNA of the 16S rRNA-amx gen were obtained. In the anammox reactor treating urban wastewater at mainstream conditions, values ranging from 2.32 x10^5 to 2.58 x 10^5 gene copies ng^{-1} DNA of the 16S rRNA-amx gen were obtained. There were no statistically significant differences between the anammox marker genes (16S rRNA-amx and hzsA) in both reactors. Comparing both reactors, a lower abundance of 16S rRNA-amx was obtained in S1. Consistency in the anammox community quantification of either 16S-amx gene and hzsA gene on both reactors showed that these primers are a good choice for estimating our target and serve as a guide to further works that use anammox biomass for treating mainstream MWW. Regarding nirS gene, a low quantification was obtained in both reactors compared to the other studied genes. Also, a statistically different quantification between both reactors was obtained, being lower in the AM reactor. Han et al. (2017) showed that for a sample of WWTP, nirS primer set presented 100% efficiency, though, it was able to detect a large portion of denitrifiers. Other works evaluating this gene, showed that it was absent in some genomes of *Ca.* Brocadia (Oshiki et al., 2015), therefore, is not available for the detection and quantitative analysis of this genus (Yang et al., 2020), which is the main genus detected in our reactors.

Beyond the target gen, it is important to understand that the primers influence the PCR based methods for relative quantification (as we have shown on amplicon sequence analysis) and for absolute quantification (Orschler et al., 2019; Yang et al., 2020). Besides, the differences between *in silico* and practical application have been proved, which is why we pointed out that standardization is required in this field, before conducting more in-depth analyzes, such as changes in communities over time.

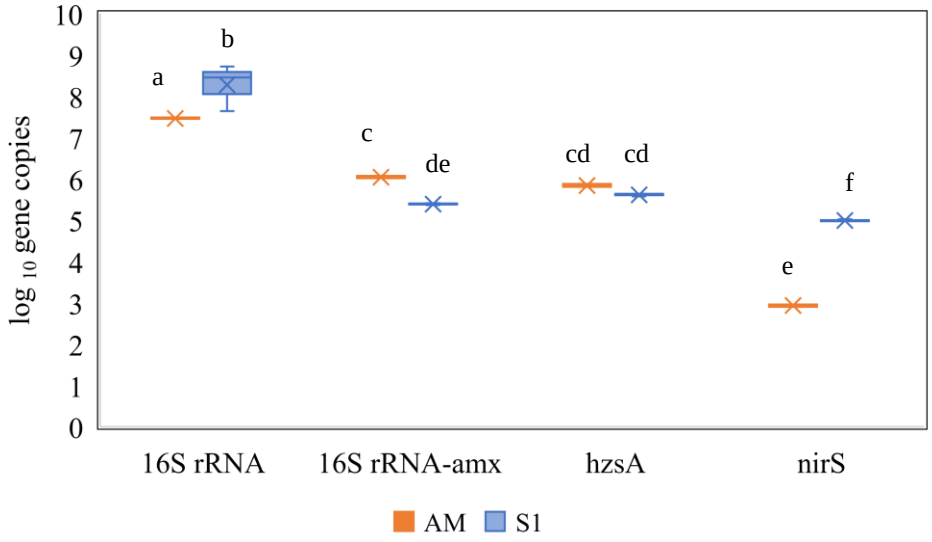

Figure 4.13.- Boxplot of quantification of bacterial genes in anammox reactors by qPCR. 16S rRNA gene for total bacteria, 16S rRNA-amx for anammox bacteria, and functional genes hzsA and nirS were quantified. A logarithmic scale was chosen for the y-axis. The X on each box represents the mean value from triplicate qPCR reactions. The error bars stand for the standard deviations. Means with one letter in common are not significantly different ($p>0.05$).

In addition to the primer bias, it is difficult to compare our results with similar studies. This, because in general, the results are reported as the relative abundance of certain microbial groups (i. e., ammonium-oxidizing bacteria-AOB, nitrite-oxidizing bacteria NOB, others) normalized to the abundance of total bacteria. So, it should not be compared with other works, unless the same primers are used (Orschler et al., 2019). Furthermore, when the results have been reported as absolute quantification, since there is no consensus on the normalization of the data, they have been expressed as a function of the sample volume (Orschler et al., 2019; Z. bin Wang et al., 2016), ng of DNA (Li et al., 2009; this study), g of dry weight (Han et al., 2017), among others.

4.3.3. Defining scopes of different molecular approaches for the study of anammox reactor treating mainstream urban WW

Even when the implementation of and feasibility of the mainstream anammox process has been studied in the last decade (Cao et al., 2017; Miao et al., 2020), few studies are focused on whole the community structure, but on particular bacterial groups as anammox, AOB and NOB, (Laureni et al., 2016; Miao et al., 2020; Nejidat et al., 2018). Also, when the whole community was studied, despite the different variables among the anammox process (the type of biomass, operation parameters, type or reactor, etc), there was a lack of consensus on how to carry out this research, recently reported by Orschler et al. (2019). Furthermore, once the investigations were developed, as many of them used different techniques (FISH, DGGE, amplicon sequencing, metagenomics, among others), comparison of results is difficult. For example, relative abundances on microbial diversity obtained by Yang et al. (2018) in a PN/A reactor, are not comparable with this study's results, because amplicon sequencing was made with other primers set. No study is available yet that specifically looked at the extent of molecular approaches selection and its influence on determining the microbial communities in granular anammox systems at the mainstream condition, and these results serve as a basis, regarding that the influence of the molecular approach varied between the samples with different influent feeding. This agrees with Liu et al. (2020), on relying on the primers used in similar published studies, allowing an optimization of the methods, and facilitating the comparison of results between studies.

As we have seen throughout this study, we have a wide variety of approaches for the detection and quantification of anammox communities, however, each one has biases that will influence the reported results. Also, as Orschler et al. (2019) pointed out, before choosing to use one technique over another, we must define what the focus of the study is (Fig. 4.14). Regarding all the approaches evaluated in a granular anammox reactor at mainstream conditions, these two objectives should be considered for method selection: 1) focus on community structure and dynamics and 2) focus on community interactions. If the study is centered on whole community structure, amplicon sequencing with primers set 515F-806R showed a good performance and have been widely used in related published research (Bhattacharjee et al., 2017; Laureni et al., 2016; Zhao et al., 2018). If instead it is focused on the anammox community, FISH would be used for a quick screening, and also, a combination of amplicon sequencing with specific primers and

qPCR, targeting both the 16S rRNA gene, would be reliable. Both approaches allowed a comparison to determine the community dynamics. Metagenomics approaches have also solved this goal but are more expensive to use for that purpose. On the other side, if the objective is focused on understanding the relationship between the communities, like how the presence or absence of determined genes would collaborate to define interactions between the communities, or how physiology information could contribute to comprehend the existent synergies; metagenomics (using gene annotation tools) and qPCR targeting functional genes, have shown the best performance.

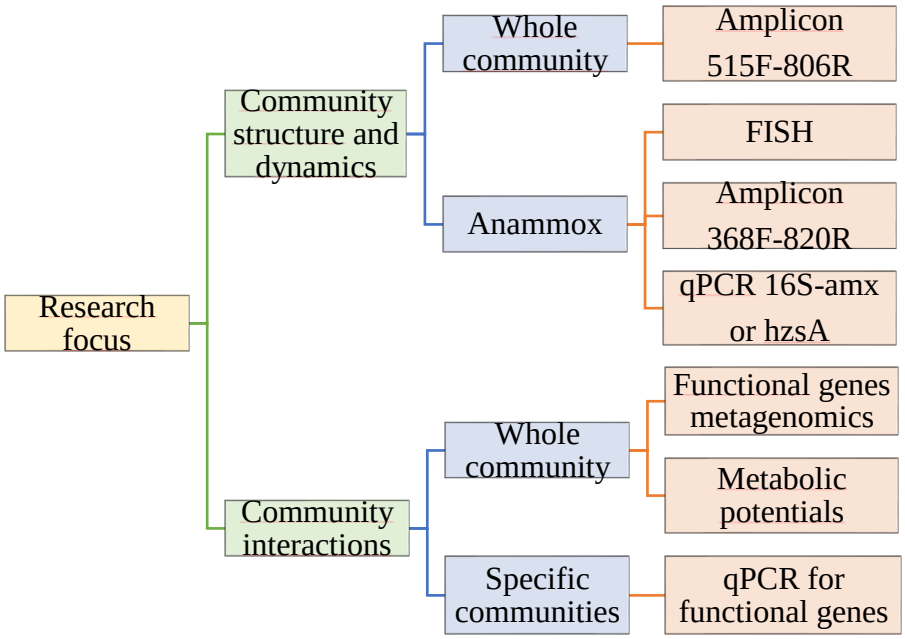

Figure 4.14: Schematic representation of molecular approaches to use according to the research focus.

4.4. Conclusions

A modified FISH-CLSM protocol was established for identification and quantification of anammox communities in granular-sludge coming from an UAnSB reactor performing autotrophic nitrogen removal at mainstream conditions with real wastewaters. Using this methodology, the high autofluorescence of the sample was avoided or could be distinguished and filtered from probes' signals. Colocalization of the target population was clearly observed, even when anammox bacteria were below 1% of the total bacteria.

As a high autofluorescence was detected in this type of samples, it was decided to assess the anammox communities with different DNA-based molecular approaches,

where EPS did not interfere. Furthermore, considering the low amount of anammox bacteria detected, these techniques were standardized to also study the whole bacterial community. Considering biases and scopes at the time of interpreting the obtained results, recommendations on the advantages of using one technique over another or a combination of them were outlined, depending on the research question.

This work serves as a basis for studying the microbial ecology and relationships in anammox reactors working at mainstream conditions. Thus, based on our previous analysis we are able to recommend amplicon sequencing with primers set 515F-806R if the research wants to determine the whole community and Amx-368F-820R for anammox microorganisms. However, if the focus is on the anammox community relationships, a combination of amplicon sequencing with specific primers and qPCR, targeting 16S rRNA or hzsA genes, would be more reliable. Both approaches allowed a comparison to determine the community dynamics. Metagenomics approaches have also solved this goal but are more expensive to use for that purpose, but, with the drastic decrease in sequencing costs this approach will be used in a near future.

In an anammox process treating real wastewater, this optimized protocol for FISH will be useful for a quick monitorization of the anammox and bacterial dynamics, while the standardization of the DNA-based molecular approaches will facilitate the processing of samples and analysis of results in a reliable and repeatable way to finally assess how operational changes can affect this population.

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Chapter 5

Stratification of the microbial community along the height of the UAnSB anammox reactor improves adaptation to low temperatures

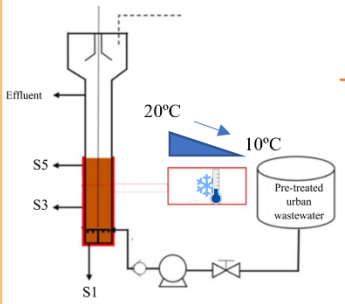
Goal 1: To characterize and analyze the bacterial communities involved and existing synergies in an anammox reactor during different phases of operation

Problem: Lack of the use of standardized molecular approaches

Chapter 5. Stratification of the microbial community along the height of the UAnSB anammox reactor improves adaptation to low temperatures

Predominant microorganisms?

Stratification?



Reactor overcapacity?

Dynamics through the sludge-bed (S1-S3-S5)

20ºC

Microbial communities

Amplicon sequencing 515F-806R

16S rRNA-Met

Anammox

Amplicon sequencing Amx368F-Amx820R

qPCR functional genes

Temperature effect into dynamics

20ºC to 10ºC

Microbial communities

Amplicon sequencing 515F-806R

Anammox

Amplicon sequencing Amx368F-Amx820R

qPCR functional genes

Ratio 16S rRNA-amx: 16S rRNA

Main results:

Whole community along UAnSB reactor

Same core Microbiome

Ignavibacteria, Brocadiae, Gammaproteobacteria, Anaerolinea

Different relative abundances

Stratification

Low temperature = change in stratification

Support overcapacity

Anammox community

Higher abundance in S1

Main genus: *Ca.* Brocadia

Ca. Kuenenia increased in S3 at 10ºC

High abundance of anammox at 10ºC

No correspondence with anammox activity from sNRR

Abstract

The implementation of the anammox process for the mainstream treatment of urban wastewater has been extensively studied in the last years to demonstrate its feasibility. One of the critical points is the need to work at low temperatures without reducing the nitrogen removal efficiency. The structure of the microbial community is known to be important in maintaining stable nitrogen removal, but not is enough known about how temperature changes this structure. In a previous work (Juan-Díaz et al., 2020), it was demonstrated that a granular-sludge Upflow Anammox Sludge Blanket (UAnSB) reactor treating real wastewater at mainstream conditions adapts to low temperature due to an overcapacity along the sludge bed, described as a significant amount of biomass that was not contributing to the overall anammox activity. To understand this effect, the microbial communities from different highs of the reactor were studied along a decreasing gradient of temperatures from 20ºC to 10ºC.

Using amplicon sequencing and metagenomic approaches based on 16S rRNA gene the whole bacterial composition was established in tree sludge bed sections located along the reactor height (S1-base, S3-middle part, S5-top). Both approaches showed the same predominant classes, namely: Ignavibacteria, Gammaproteobacteria, Anaerolinea and Brocadiae along the different sludge bed sections of the reactor, whose can be described as the "core microbiome". Only with amplicon sequencing, other abundant class was detected: Latescibacterota. Despite the differences in relative abundances, both techniques exhibit changes in the microbial community structure showing a stratification due to the heterogeneous distribution of the anammox specific activity in the sludge bed. With the temperature decrease, the core microbiome was determined to be the same.

Regarding specifically to the anammox bacteria, the stratification was studied using amplicon sequencing and gene quantification by qPCR of the 16S rRNA gene (targeted both bacteria and anammox) and two functional genes encoding hydrazine synthase (hzsA) and nitrite reductase (nirS). The higher abundance of anammox bacteria was detected in the base of the reactor (S1) at both temperatures, where the nitrogen removal was mainly performed according to the anammox specific activity. *Ca.* Brocadia was the main genus in the reactor during the whole period of operation, but a shift for *Ca.* Kuenenia was observed in the central sludge bed section (S3) when working at 10ºC, probably due to an accumulation of nitrite in that section. Despite that, the relative

abundance at 10ºC was almost doubling in each sludge bed section, the anammox activity was lower than at 20ºC. Thus, more reliable information to confirm a reactor overcapacity would be obtained from active community studies instead of using amplicon sequencing and metagenomic approaches based on 16S rRNA gene.

Exploring in detail the microorganisms contributing to the stratification, Gammaproteobacteria was the unique class that exhibited a statistical significative change in the reactor community structure when decreasing the temperature of operation. Comparing the community structure by sludge bed section, Latescibacteria and Anaerolinea were the major classes contributing at the upper sludge bed section (S5), while in the base (S1) the Brocadiae class was predominant. This shift in the community structure could be explained by a multiple cross-feeding during the operation caused by the temperature decrease, however the mechanism that regulates these interactions remains unclear. From these results, the community stratification along the reactor would confirm a latent overcapacity of the reactor depending on the studied sludge section and temperature of operation.

5.1. Introduction

Since anaerobic ammonium oxidation (anammox) was discovered in the 90's decade as a new metabolic pathway in the removal of inorganic N species, the anammox-based process has been successfully implemented for the sidestream or reject water treatment of urban wastewater treatment plants (WWTP) (Lackner et al., 2014; Siegrist et al., 2008). In the last years, extensive efforts to demonstrate the feasibility for direct treatment of municipal wastewater (MWW) (known as "mainstream anammox") has have been made, particularly due to its potential of transforming current WWTPs from energy-consuming to energy-neutral or even positive facilities (Juan-Díaz et al., 2020; Laureni et al., 2016; Liu et al., 2017; Lotti et al., 2015a; Reino et al., 2018). Nevertheless, its application stays a challenge (N. Li et al., 2018).

One of the bottlenecks of the mainstream implementation of the anammox process is the need to deal with low wastewater temperatures (as those occurring in the winter season) compared to that of the sidestream processes (>30 °C) (Reino et al., 2018). In this sense, only few studies have evaluated the performance of Partial Nitritation/ Anammox (PN/A) processes treating real MWW at low temperatures (<15ºC). Among them, a simulation of decreasing temperatures in urban WWTPs in moderate climates applying a gradient from 20 to 10ºC was proposed by Gilbert et al.(2014) and Laureni et al. (2016), where they assessed the long-term stability of PN/A processes working at low temperatures (15 ºC) on pre-treated MWW showing stable nitrogen removal efficiencies (NRE) but, a sudden temperature drop to 11ºC resulted in significant suppression of anammox activity. Different anammox technologies have been reported to operate at different temperatures, but the main problem occurred when the temperature dropped below 15°C was the accumulation of nitrite and a subsequent loss of stability of the system (Dosta et al., 2008; Gonzalez-Martinez et al., 2018). Therefore, the NRE was drastically reduced (González-Martínez et al., 2018).

The anammox process is carried out by anammox bacteria, described as a monophyletic order (*Candidatus-Ca.*- Brocadiales) in the Planctomycetes phylum that has the metabolic ability to catalyze the one-to-one conversion of ammonium and nitrite to form dinitrogen gas under anoxic conditions (Li et al., 2009; Strous et al., 1998; Sun et al., 2014). These microorganisms can self-aggregate and form granular structures that give them good sedimentation properties and, therefore, improving biomass retention that ensures the success of the process (X. Li et al., 2018; Zhao et al., 2018a). Moreover,

granular biomass also provides a favorable anoxic microenvironment that promotes the enrichment of anammox bacteria (Miao et al., 2020; Winkler et al., 2012).

Besides anammox bacteria, other microbial communities are present in anammox granules. It has been reported that anammox bacteria thrive by competition as well as by metabolic interactions with other organisms associated with the nitrogen cycle (Kartal et al., 2012). Also, the presence of organic matter in the influent promotes the proliferation of heterotrophic bacteria, which, depending on the concentration, would compete for nitrite with the anammox bacteria. These groups have been found in dynamic equilibrium to achieve simultaneous nitrogen and COD removal in some systems (Chen et al., 2009; Liu et al., 2017).

Hence, the complex microbial community structure and taxa interactions are crucial for the stable removal of nitrogen from wastewater (Wang et al., 2019). Besides, environmental factors such as temperature, light, pH, influent characteristics, among others, would influence microbial community structures and diversity of these bio-systems. So that, changes in these factors should lead to variations in microbial species and abundances (González-Martínez et al., 2015; Liu et al., 2018; Miao et al., 2020; Wang et al., 2019). Considering this, a comprehensive understanding of the microbial community composition of the anammox-based processes under different operational conditions is one of the premises for optimizing the PN/A process (Vlaeminck et al., 2012).

Until now, little is known about the microbial community composition and dynamics in anammox reactors under mainstream conditions. Usually, studies are focused on particular bacterial groups as anammox, ammonium oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) (Laureni et al., 2016; Miao et al., 2020; Nejidat et al., 2018). But, very few studies have dealt with whole microbial community diversity and process performance (de Almeida Fernandes et al., 2018; Laureni et al., 2016) when temperature variations occurs. In this sense, Juan-Díaz et al. (2020) recently demonstrated the effective dampening of temperature effects in a granular-sludge Upflow Anammox Sludge Blanket (UAnSB) reactor treating real mainstream wastewater by roughly maintaining stable removal rates during 350 days of operation, despite applying a temperature gradient from 20 to 10ºC. The microbial community on its work was studied; nevertheless, a low resolution of the taxonomic assignment was achieved, showing around 30% down to genus level and a high proportion of unclassified bacteria, both

probably due to the reprocessing of the raw data. However, results in chapter 4 of this thesis point out the standardization of different molecular approaches to study the microbiome of a mainstream anammox reactor, including PCR-based methods (amplicon sequencing and qPCR) and metagenomics. Moreover, the taxonomic assignment was performed using the recently released SILVA SSU 138 rRNA database. Thus, based on the practical choice of the best primers to perform amplicon sequencing and qPCR, it was possible to determine the whole community structure and the anammox bacteria diversity at deep taxonomic levels. The metagenomic approach also solved this goal. In addition, the results allowed us to identify an uncharacterized Planctomycetes in high proportion leading us to propose a new *Candidatus* specie: Brocadia barcinensis.

For that reason, in this work, the microbiome structure along the height of an UAnSB reactor working at mainstream conditions was assessed in samples taken during the operation at decreasing temperatures (from 20ºC to 10ºC). The research questions to be addressed were: 1) What are the predominant microorganisms in the anammox reactor working at mainstream conditions? 2) Is there a stratification (differential vertical distribution) of the communities throughout the reactor height? 3) Is there a change of these communities in abundance when the operating temperature is lowered from 20 to 10ºC?

5.2. Materials and methods

5.2.1. Description of the samples from the UAnSB reactor

Samples of anammox granular biomass were obtained from three sample points along the sludge bed of an UAnSB reactor during it operation at mainstream conditions. This UAnSB was fed with real urban wastewater (26±8 mg N-NH_4^+ L^{-1}, 36±9 mg N-NO_2^- L^{-1}, 2±2 mg N-NO_3^- L^{-1}, 60 ± 14 mg COD L^{-1}, among other compounds), coming from an urban WWTP located in an industrial area of Catalonia, NE Spain (for more details, see the work of Juan-Díaz et al., 2020). The samples were taken at different operational days representative from steady state of operational period I and III (Juan-Díaz et al., 2020), while temperature of the reactor was gradually lowered from 20ºC (period I) to 10ºC (period III). The reactor had five different sampling points at heights of 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) (Fig. 5.1). For the purposes of this thesis, 3 points along the bed were selected to take the samples, indicated in the Fig. 5.1 as “S1”, “S3” and “S5”, and labeled in the same way, followed by the reactor temperature

at the time of sampling. The operational parameters of the UAnSB reactor such: Nitrogen Loading Rate (NLR), Nitrogen Removal Rate (NRR), Nitrogen Removal efficiency (NRE), the anammox activity or specific NRR (sNRR) at the sample day or period were obtained from Juan-Díaz et al. (2020) and Juan-Díaz (2021). The anammox activity determined by them as Specific NRRs, was calculated for the different sludge bed sections delimited by the sampling points using measured nitrogen compounds concentrations at each point (more details in Juan-Díaz et al., 2020).

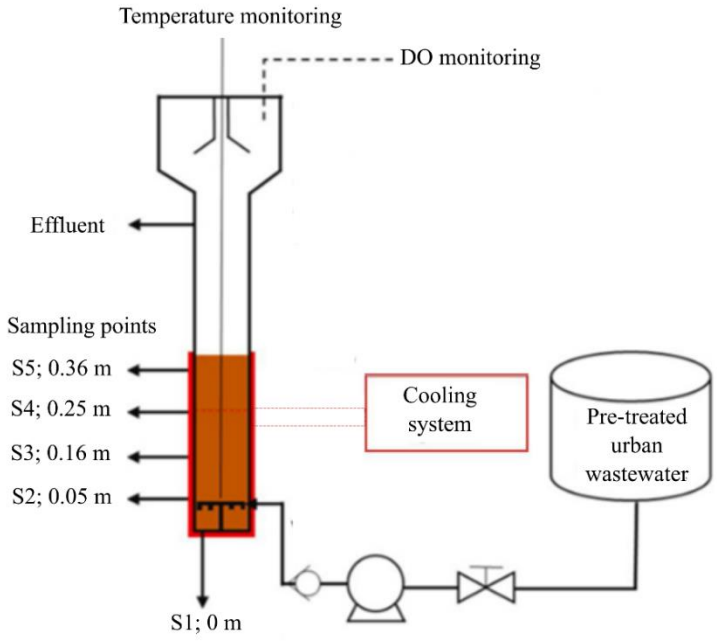


Figure 5.1. Schematic diagram of the lab-scale UAnSB reactor. Adapted from Juan-Díaz et al. (2020).

Table 5.1. Samples and operational parameters of the anammox reactor when sampling to study the microbial communities.

	Operational data for the specified sampling day					Operational Parameters at each steady state by period				
Sample ID	Day[a]	Temp. (ºC)	NLR[b] (g L^{-1} d^{-1})	NRR[b] (g L^{-1} d^{-1})	NRE[b] (%)	Operation period[a] (days)	NLR (g L^{-1} d^{-1})	NRR (g L^{-1} d^{-1})	NRE (%)	sNRR[c] (g N g^{-1} VS h^{-1})
S1-20										100
S3-20	17	20	0.11	0.10	90	I (0-81)	0.10 ± 0.02	0.10 ± 0.02	87 ± 3	2
S5-20										0
S1-18	105	18	0.10	0.01	90	II (81-155)	0.09 ± 0.01	0.09 ± 0.01	86 ± 5	N.A
S1-16	130	16	0.090	0.09	91	III (155-260)	0.11 ± 0.01	0.09 ± 0.01	71 ± 6	N.A
S1-10										62
S3-10	203	10	0.10	0.06	49	III (155-260)	0.11 ± 0.01	0.09 ± 0.01	71 ± 6	8
S5-10										0

[a] Operation period and parameters such as: Nitrogen Loading Rate (NLR), Nitrogen Removal Rate (NRR), and Nitrogen Removal Efficiency (NRE) as described by Juan-Díaz et al. (2020). [b] Operational parameters for the sampling day were obtained from Juan-Díaz (2021). [c] Specific NRR (sNRR) estimated from graphical data in Juan-Díaz et al. (2020). N.A.: Not Available data.

5.2.2. Microbial community analysis

In order to solve the low taxonomic resolution in related microbiome studies performed in mainstream anammox reactors, the identification and characterization of the microbial community along the height of the UAnSB reactor was performed using four different approaches shown in Table 5.2. For more details see sections 3.3.2 to 3.3.4 of Chapter 3.

Table 5.2. DNA-based approaches used in this chapter to determine the whole bacterial and the anammox communities' structure in a granular-sludge anammox reactor treating real wastewater at mainstream conditions.

Molecular technique	Details	Approach
Amplicon sequencing	Set of primers used: 515F-806R	A
	Set of primers used: Amx368F-Amx820R	B
Shot-gun sequencing metagenomic	16S rRNA sequences from metagenomic data	C
qPCR	Functional genes (hydrazine synthase gen and nitrite reductase gen) + Bacteria 16S rRNA gen + Anammox bacteria 16S rRNA gen	D

Data reprocess was performed using QIIME2™ v.2019.10 (Bolyen et al., 2019), and the taxonomic assignment was performed using the recently released SILVA SSU 138 rRNA database.

To determine if there is a stratification of the microbial communities in the reactor along with the reactor height, samples from stable operation at 20ºC using all the approaches were analyzed. Then, approaches that provided more facilities in terms of analysis, besides reliable results were used to study how the decrease in temperature affects the system and the dynamics of the communities. On one side, DNA from biomass samples collected from the bottom sludge bed section (S1) was taken at different temperatures, and a qPCR analysis was performed to determine the temperature effect on the anammox abundance. On the other side, when the lowest temperature was reached,

DNA from the three sample points (S1, S3, and S5) of the reactor working at 10ºC was studied using three approaches as shown in Table 5.3, to understand changes in microbiome structure and anammox community.

Table 5.3. Molecular approaches used for each DNA sample. A-D approaches details are shown in Table 5.2

	Molecular Approaches used for each sample			
Sample ID	A	B	C	D
S1-20	x	x	x	x
S3-20	x	x	x	x
S5-20	x	x	x	x
S1-18				x
S1-16				x
S1-10	x	x		x
S3-10	x	x		x
S5-10	x	x		x

5.2.3 Statistical analysis

The Statistical Analysis of Metagenomic Profiles (STAMP) program (Parks et al., 2014) was used to identify differentially abundant communities based on relative abundances from amplicon sequencing. Two groups comparison was performed with Whites non parametric t-test ($p<0.05$) and two samples comparison with the Fisher's exact test ($p<0.05$).

All quantitative PCR reactions were carried out in triplicates and statistical analysis was performed with Infostat software v.2017. Log transformed gene copy numbers were calculated. Samples grouped by gene were subjected to the normality test of Shapiro-Wilks. When normality was confirmed, data was subjected to ANOVA analysis and Tukey's test for media comparison was used. When no normality was observed, a Kruskal Wallis test was performed. In both cases, 5% of significance was used.

5.3. Results and Discussion

5.3.1. Microbial community in an anammox reactor at mainstream conditions working at 20ºC

Whole community of an anammox reactor treating real wastewater at mainstream conditions was studied using two approaches based on 16S rRNA gen (approaches A and C) at class level (Fig. 5.2). Both approaches showed the same predominant classes Ignavibacteria, Gammaproteobacteria, Anaerolinea and Brocadiae along the different sludge bed sections of the reactor. These four classes belong to the phyla that make up the so-called "core microbiome": Bacteroidota, Proteobacteria, Chloroflexi and Planctomycetota, whose presence in anammox reactors has been previously reported and discussed in Chapter 4 (Bhattacharjee et al., 2017; González-Martínez et al., 2015; Lawson et al., 2017; Mardanov et al., 2019; Speth et al., 2016; Zhao et al., 2018b). Nevertheless, using amplicon sequencing other abundant class was detected, Latescibacterota, which was not observed in the 16S rRNA gene extracted from metagenomes. This class, belonging to the Latescibacterota phylum, previously named *Candidate* division WS3, have been found in a wide range of habitats, including wastewater treatment bioreactors (de Almeida Fernandes et al., 2018; Pereira et al., 2014; Youssef et al., 2015). Little is known about this phylum, but Youssef et al. (2015) made a metabolic reconstruction in silico, suggesting its capability to degrade multiple polysaccharides, glycoproteins and extracellular polymeric substances.

Regarding amplicon sequencing, differences in abundance were observed between the three studied sludge bed sections of the reactor. Ignavibacteria was the major class present in the reactor, being its relative abundance higher in the top of the reactor (S5-20 sample), while the lower abundance was observed in the centre (S3-20 sample). This class have been reported to be present in substantial numbers in full-scale anammox and in lab-scale anammox bioreactors (González-Gil et al., 2015; Lawson et al., 2017). Considering that at 20ºC the NRR was 50 times higher at the base of the reactor (S1-20 sample) than in the higher bed sections (S3-20 and S5-20; Juan-Díaz et al., 2020), the high abundance of Ignavibacteria could be explained because this class has been related to the consumption of organic compounds known as soluble microbial products (SMP) released by nitrifiers and anammox bacteria (Yang et al., 2018). Also, this class harbor genes capable of reducing the nitrous oxide to nitrogen gas, thus facilitating a nitrite loop

with anammox bacteria or supporting complete denitrification (Akaboci et al., 2018; Lawson et al., 2017a).

By contrast, the class Gammaproteobacteria was more abundant in the centre (21%) of the reactor and showed lower abundance at the highest point (15%). The class Latescibacterota showed was more abundant in the upper sludge bed sections of the reactor, being greater in the centre (14%). The class Anaerolinea abundance was decreasing along the reactor from de bottom to the top from 13% to 7%, and the class Brocadiae abundance was similar in the three sludge bed sections (9% in S1-20 and S5-20 samples), and the lower abundance was observed in S3-20 sample (7%).

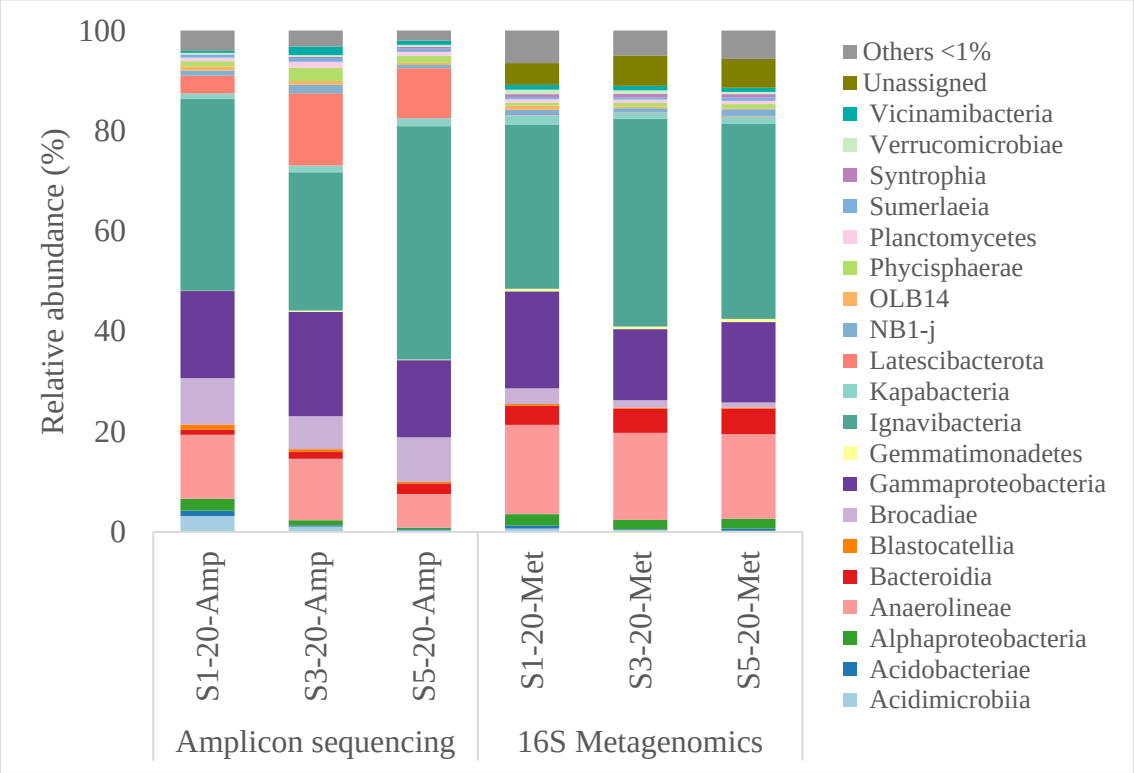


Figure 5.2. Relative abundance of identified classes in three sludge bed sections of an anammox reactor using two different molecular approaches. Classes whose relative abundance among the techniques was less than 1% were grouped as "Others".

On the contrary, the metagenomic approach (approach b) showed that the S3-20 sample exhibit the highest abundance of Ignavibacteria and the lowest abundance of Gammaproteobacteria in comparison with the other sludge bed sections (S1-20 and S5-20). Also, higher abundance of the class Bacteroidea was observed in the metagenomic approach than that obtained in amplicon sequencing. Differences in abundances of both techniques have been previously reported in Chapter 4, but these differences were lower

in the sample S1-20 of the mainstream anammox reactor when was studied with both approaches. Although this analysis was not conclusive regarding the stratification, more information about it could be obtained with amplicon sequencing, due to the observed differences in the taxonomic classifications. Relating the operational parameters obtained previously by Juan-Díaz et al. (2020) with the reprocessed microbial analysis in this work, it can be noted that at 20ºC, changes in the microbial stratification were clear at S3-20 sludge section, as a product of the heterogeneous distribution of the anammox activity in the sludge bed, where nitrogen was mainly removed in the bottom of the reactor (S1-20 sludge section). Thus, in the upper sections, the biomass did not contribute to the general specific activity of anammox (sNRR), but the community was restructured, increasing the abundance of Latescibacteria and Gammaproteobacteria, while Ignavibacteria, Anaerolinea and Brocadiae decreased. These shift in the community structure could be explained by a potential multiple cross-feeding during the operation, as described by Zhao et al. (2018b).

5.3.2. Comparison of bacterial communities through the bed high with the decrease in temperature of operation from 20ºC to 10ºC

One of the goals pursued by the implementation of the anammox process to mainstream wastewater is to maintain a good NRE performance while the temperature of the system decreases because of the seasonal temperatures variations. To understand changes in the dynamics of the bacterial community, amplicon sequencing of the three sludge bed sections of the reactor at 20ºC and at 10ºC were compared in this section at class level (Fig. 5.3).

Few studies have explored the entire bacterial community in anammox reactors working under mainstream conditions as in this study. However, a common factor among these studies is the high abundance of heterotrophic bacteria from phyla Chloroflexi, Proteobacteria and Bacteroidota (Akaboci et al., 2018; Laureni et al., 2016; Luo et al., 2017; Miao et al., 2020) as we report below. Akaboci et al. (2018) suggest that the anammox process was not the exclusive pathway responsible for nitrogen removal, but endogenous denitrification might also contribute. Regarding how the temperature affects the microbial structure, Akaboci et al. (2018) reported that a temperature drop (15ºC) only affected bacterial abundance, but not the main bacteria involved in the process. On the one hand and despite the decrease of the temperature, the core microbiome in the reactor

at 10ºC was the same as at 20ºC: Ignavibacteria, Gammaproteobacteria, Anaerolinea, Latescibacterota and Brocadiae.

Ignavibacteria was, as such as at 20°C, the main class present in the reactor. The relative abundances were similar at 10ºC than at 20ºC (between 33% and 43%) but their dynamic was different. At 10ºC, the abundance increased towards the upper sludge bed sections of the reactor. On the contrary, Gammaproteobacteria abundance decreased from S1-10 to S5-10. In comparison with the dynamic of this class at 20ºC, the major difference was observed in the S3 sludge bed section, being lower at 10ºC.

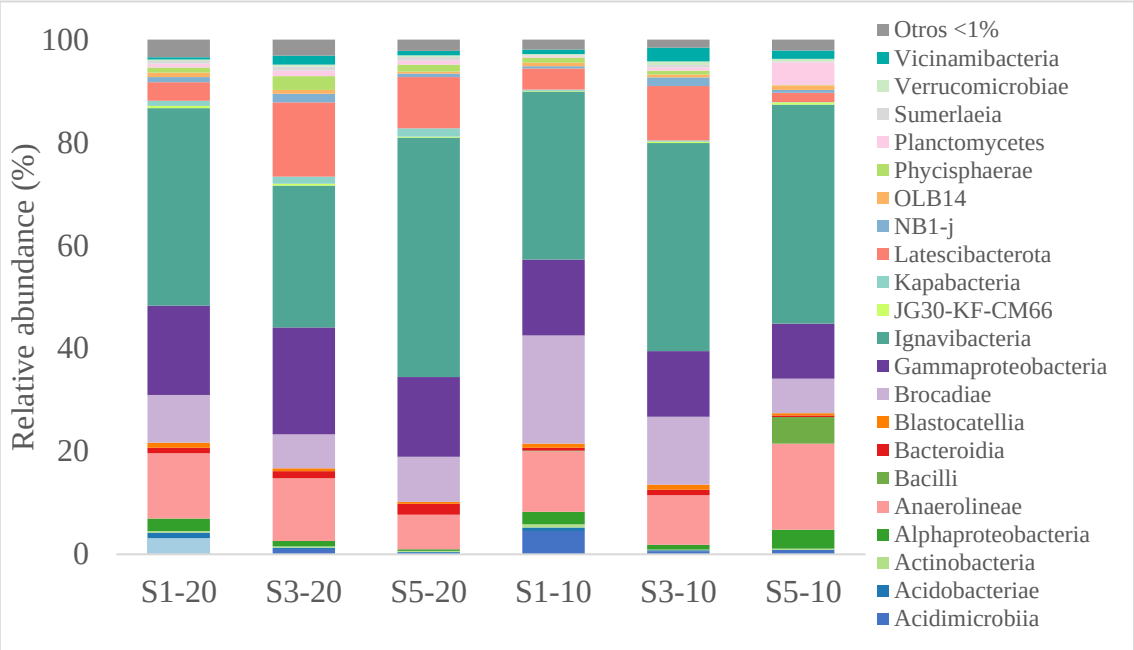


Figure 5.3. Relative abundance of identified classes in three sludge bed sections of an anammox reactor at 20ºC and 10ºC using amplicon sequencing of 16S rRNA gen. Classes whose relative abundance among the heights was less than 1% were grouped as "Others".

On the other hand, the abundance of Anaerolinea was lower in the middle part (S3) of the reactor and higher in the upper sludge bed section (S5), reaching 17% for sample S5-10, while at the bottom (sample S1-10) it was similar to the abundance at 20ºC (sample S1-20). Ending with the predominant classes, Brocadiae was the class with major differences between the two evaluated temperatures. The abundance of this class was the double (21%) at 10ºC than at 20ºC in the lower part of the reactor (S1-10). Then, in the upper sludge bed sections, it was decreasing from 13% in S3-10 to 7% in S5-10, while at 20ºC the abundances were 7% and 9% for S3-20 and S5-20, respectively. Regarding the class Latescibacterota, it was more abundant in the S3-10 sample. Only 2% was present in the top sludge bed section, being lower than in the same sample point at 20ºC.

Hence, the dynamics of the different classes through the reactor at 10ºC evidenced a possible stratification, where Ignavibacteria and Anaerolinea abundances increase towards the top sludge bed section, while instead, Gammaproteobacteria and Brocadiae decreased. To determine significant changes in the communities through the reactor, a statistical differential abundance analysis was performed between the heights (Fig. 5.4). Thus, at 20ºC, the class Latescibacteriota increased from S1-20 to S3-20 and class Ignavibacteria increased from S3-20 to S5-20. Instead, at 10 ºC, there were no significant differential abundances of classes in the bottom sludge-bed sections (S1-10 and S3-10), while classes Latescibacteriota and Bacili increased from S3-10 to S5-10.

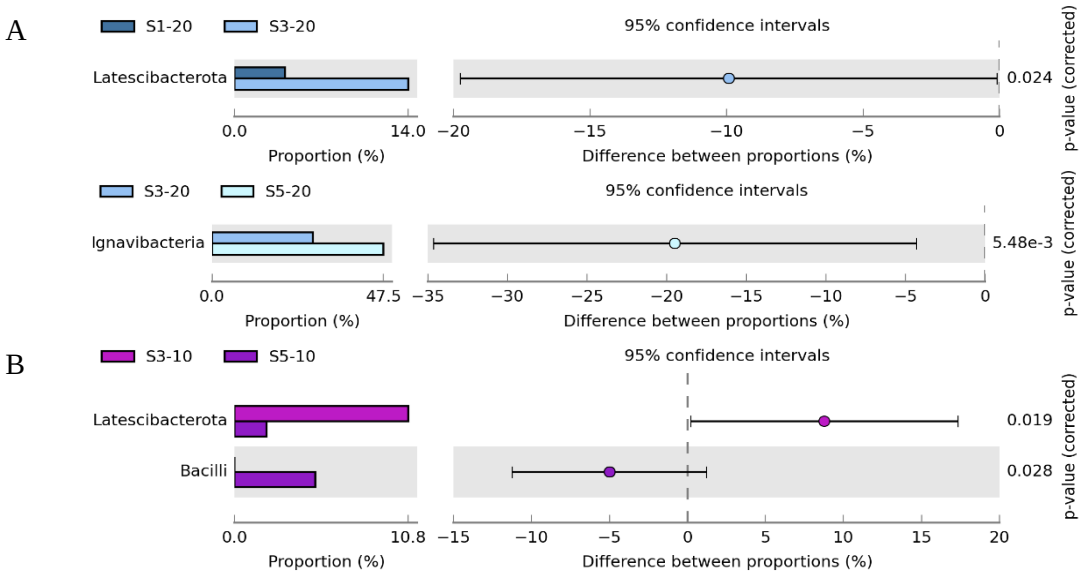


Figure 5.4.- Extended error plot for difference analysis at class level. A) Fisher's exact test for comparison of sludge bed sections at 20ºC ($p<0.05$); B) Fisher's exact test for comparison of sludge bed sections at 10ºC ($p<0.05$). S1-10 to S3-10 comparison did not showed significant differences.

The same statistical analysis was performed comparing each sludge bed section at both temperatures (20ºC and at 10ºC) to determine which specific members were contributing to the community stratification in the reactor (Fig. 5.5). In the comparison of the changes in the structure of the community with the decrease in temperature, the classes of Gammaproteobacteria, Kapabacteria, and Sumerlaeia showed differences. Of these classes, only Gammaproteobacteria belong to the core microbiome, being more abundant at 20ºC than at 10ºC.

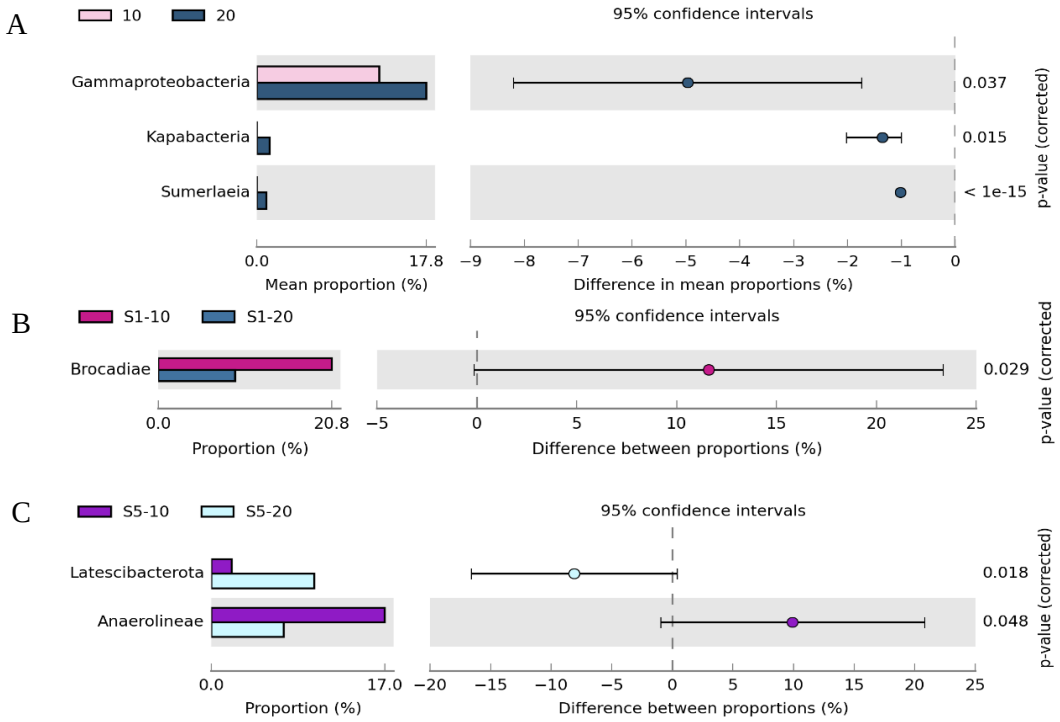


Figure 5.5.- Extended error plot for difference analysis at class level. A) White's non parametric t-test for two groups comparison (10ºC and 20ºC) ($p<0.05$); B) Fisher's exact test for comparison of S1 sludge bed section at 20ºC and 10ºC ($p<0.05$); C) Fisher's exact test for comparison of S5 sludge bed section at 20ºC and 10ºC ($p<0.05$). S3 samples did not showed statistical differences.

From a global point of view, the comparison of the communities at 20ºC and 10ºC showed that Gammaproteobacteria was the unique class that exhibited a meaningful change in their abundance (Fig. 5.5-A). Thus, going deeper in its taxonomy, it was observed that in this class, there were two main families whose abundances changes with the temperature (Table 5.4). On one side, Rhodocyclaceae decrease at 10ºC and, on the contrary, Burkholderiaceae increased. Our findings coincide with the hypothesis suggested by Speth et al. (2016) about the existence of variable macro- and micro-environments that allows the coexistence of such large diversity of heterotrophic bacteria in the reactors. In this sense, the high abundance of the families Rhodocyclaceae and

Burkholderiaceae have been reported by several authors in different anammox reactors (PN-A or anammox reactors treating synthetic or mainstream feeding) (Feng et al., 2019; Mardanov et al., 2019; Pereira et al., 2017; Val del Rio et al., 2018; Yang et al., 2018). Also, some of these authors suggest that these families are known to be complete or partial denitrifiers and denitrification intermediate reducers (Pereira et al., 2017; Val del Rio et al., 2018). According to Grießmeier et al. (2017), members of Rhodocyclales order have and express all genes involved in the denitrification process and seem to play a relevant role in denitrification at high nitrate concentration (1.18 mmol L^{-1}) in denitrification systems. This behavior is consistent with the results of Juan-Díaz et al. (2020), where in the first period (20ºC) the concentration of nitrates was higher (average 0.36 – 0.64 mmol N L^{-1}) and in the following periods it decreased, as well as the abundance of Rhodocyclales in this work. The results obtained by Juan-Díaz et al. (2020) showed that the heterotrophic activity achieved in batch reactors with nitrate was almost twice that achieved with nitrite. Therefore, we could hypothesize that this group (Rhodocyclales) has a role on reduce the nitrate produced by anammox bacteria without competing for nitrite, while the reactor is working at 20ºC.

Table 5.4.- Relative abundance (%) at family level of class Gammaproteobacteria found in anammox reactor working under mainstream conditions.

Family	S1-20	S3-20	S5-20	S1-10	S3-10	S5-10
Rhodocyclaceae	10	11	9	8	3	1
Burkholderiaceae	3	3	2	3	6	7
Xanthomonadaceae	0	1	1	0	2	1
Comamonadaceae	1	1	1	1	0	0
Nitrosomonadaceae	1	1	1	1	0	0
Rhodanobacteraceae	1	1	1	0	0	0
Class Gammaproteobacteria	17	20	15	14	12	10

Following the comparison of the community changes by sludge bed section with temperature decrease, the class Brocadiae from S1 samples was the unique taxonomic group statistically different at the two studied temperatures, being more abundant at 10ºC (Fig. 5.5-B). In this sense, Lotti et al. (2014)have reported that when anammox bacteria have adapted to a temperature of 15ºC, their biomass can easily increase and result in a higher NRR than non-adapted anammox. This was also observed by Juan-Díaz et al. (2020), where the specific NRR associated with the S1-S2 sludge bed section was 50

times higher than in the upper sections, therefore a significant amount of biomass was not contributing to the overall anammox activity at 20ºC, which they named as reactor overcapacity. However, in our study, the increase in abundance at 10ºC does not correlate with the sNRR reported by Juan-Díaz et al. (2020), who detected a decrease in biomass specific activity (sNRR) at 10ºC. Considering this gap, further studies should be performed on metabolically active bacteria to obtain more reliable information that allows to confirm the reactor overcapacity and the stratification along the reactor.

In the case of S3 samples, there were not differences in any taxonomic group. In S5 samples, the differences observed between the two temperatures was in the classes Anaerolinea and Latescibacterota, the former with higher abundance at 10ºC than at 20ºC, while the last was more abundant at 20ºC than at 10ºC (Fig 5.5-C). These two classes belong to the Latescibacterota and Chloroflexi phylum, respectively. Members of these phyla, as well as Ignavibacteria class, have been frequently associated to the consumption of SMP and polymers (Akaboci et al., 2018; de Almeida Fernandes et al., 2018; Kindaichi et al., 2012; Lawson et al., 2017b; Youssef et al., 2015). The increase in the abundance of Anaerolinea at 10ºC would be explained on the one hand because they might play an important role in the structure of the granules due to their filamentous morphology and/or adhesive properties (Bovio-Winkler et al., 2021) and could then be reinforcing the granular structure (Li et al., 2009) that, according to Juan-Díaz et al., (2020), was stable at low temperature. On the other hand, the main carbon source used by the Anaerolinea group would come from cell lysis and decay (de Almeida Fernandes et al., 2018), which could be higher in the upper section of the reactor (S5) considering that the activity of anammox is higher in S3 section at 10ºC. According to Bhattacharjee et al. (2017), the biomass degradation into smaller organic molecules may support denitrification activity of heterotrophic bacteria abundant in the system while recycling dead biomass and reducing sludge yields. In this regard, different investigations have been working to reveal the interactions throughout the community and especially in the core microbiome, however, until now, more research is required to understand the mechanisms that modify these interactions (Akaboci et al., 2018; Keren et al., 2019; Lawson et al., 2017b). This is why a deeper analysis taking into account the expressed genes would be of paramount importance.

5.3.3.- Stratification analysis in the anammox community during reactor operation at different temperatures

To study specifically the anammox community along the reactor, two other approaches were used for samples taken at 20ºC. On one side, amplicon sequencing using the specific primers for anammox Amx368F-Amx820R (approach B) was performed and the classification at genus level was analyzed. On the other side, anammox sequences retrieved from amplicon sequencing with the universal primers 515F-806R that classified as Brocadiae class were analyzed and abundances at genus level were recalculated (approach A). Figure 5.6 shown that there were no differences at genus level between the approaches used nor at the genus classification. *Ca.* Brocadia was the predominant genus, while abundances below 2% of *Ca.* Kuenenia were present at each height of the reactor working at 20ºC. Predominance of *Ca*. Brocadia in mainstream anammox process has been reported (Cao et al., 2017; Laureni et al., 2016; Liu et al., 2018; Lotti et al., 2015a; Miao et al., 2020; Nejidat et al., 2018), and as it was previously discussed in chapter 4 (Section 4.3.2.2), the dominant anammox genus depends on its ability to adapt to the influent conditions (González-Martínez et al., 2018). As can be seen, both molecular approaches (amplicon sequencing and 16S rRNA extracted from metagenomes) could not explained the stratification but allowed to understand the microbiome composition variations between the three sludge-bed sections.

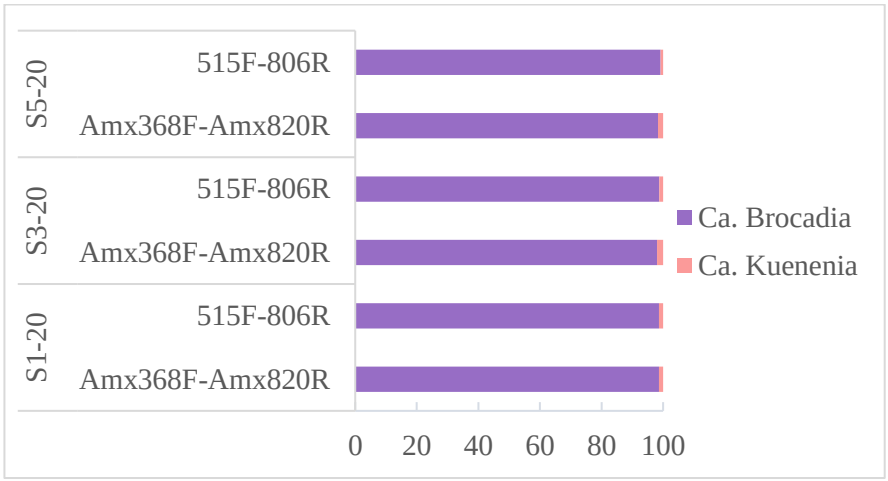


Figure 5.6.- Relative abundance of identified anammox bacteria at the genus level in three sludge bed sections of a mainstream anammox reactor working at 20ºC, using two different molecular approaches.

Hence, to better understand the dynamics of the anammox community along the reactor, a qPCR quantification of the 16SrRNA gen, the 16S rRNA gen from anammox, and the functional genes hszA and nirS was performed (approach D). Log transformed copy genes number was calculated for each reactor height and compared by gen to assess statistical differences among genes quantification. Figure 5.7 showed statistically significant differences in the abundance of the 16S rRNA gene between the bottom and the middle part of the reactor (S1-20 and S3-20 samples), but not with S5-20 sample. Regarding the abundance of anammox bacteria with 16S rRNA-amx and hzsA, significant differences were found between each sludge bed section. Abundance of nirS was statistically lower in the S3-20 sample.

Beyond the statistical dissimilarities, this approach showed a higher abundance of anammox bacteria independently of the three specific genes quantified (16S rRNA-amx hzsA and nirS) compared to approaches A and B (Fig. 5.7) for both, the S1-20 and S5-20 sludge bed sections. Therefore, the analysis of this reactor with specific genes quantified (16S rRNA-amx hzsA and nirS) show a clear stratification of the anammox community, not observable with the amplicon sequencing approaches (A and B, in Fig. 5.4 and 5.6). Today, qPCR gene quantification is considered a rapid, simple, and sensitive method to reveal the diversity and abundance of anammox bacteria in various environmental samples. Although a wide variety of primers have been designed, their results *in silico* may differ from those obtained in the actual application (Yang et al., 2020). Also, because of the high divergence among different genera of anammox bacteria and the others, the use of functional gene primers, besides the primers targeting the 16S rRNA gene, could give us more reliable information on detecting specific anammox bacteria (Li and Gu, 2011; Yang et al., 2020).

In this work, three different genes (two of them being functional genes) were used to figure out the structure of the anammox population. Besides the differences in abundances expressed as log copy genes, the dynamics observed in the three sections of the sludge bed studied were similar between the 16S rRNA-amx and hzsA genes but differ in the evaluation of the nirS gene. This behavior was previously detected in chapter 4, where nirS gene showed a lower quantification in comparison to the other genes evaluated, probably due to the absence of this gene in some genomes of *Ca*. Brocadia (Oshiki et al., 2016), which is the main genus detected in our mainstream anammox reactor.

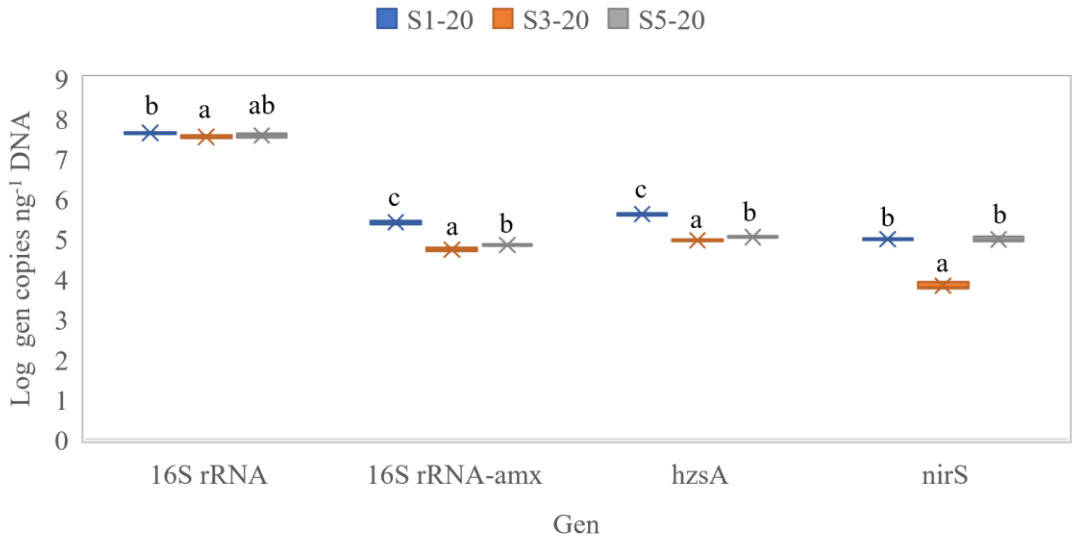


Figure 5.7.- Boxplot of absolute quantification of bacterial genes in three sludge bed sections of an anammox reactor working at mainstream conditions at 20ºC by qPCR. 16S rRNA gene for total bacteria, 16S rRNA-amx for anammox bacteria, and functional genes hzsA and nirS were quantified. A logarithmic scale was chosen for the y-axis. The X on each box represents the mean value from triplicate qPCR reactions. The error bars stand for the standard deviations. Means with one letter in common are not significantly different (p>0.05).

5.3.4. Changes in anammox community structure at 10ºC

To understand the influence of the temperature on the anammox community structure, the same approaches (A and B) performed at 20ºC were applied to the samples at 10ºC and analyzed at genus level (see section 5.3.3). Figure 5.8 showed that in the three sample points of the reactor the predominant genus was *Ca.* Brocadia. However, unlike of what was observed at 20ºC, in the upper sludge bed sections (S3-10 and S5-10) there was an increase of *Ca.* Kuenenia abundance. The highest percentage was observed in S3-10 sample, reaching between 9% and 18%, depending on the primer used. Yang et al. (2018) reported similar observations, experiencing a change in the anammox population from *Ca.* Brocadia to *Ca.* Kuenenia in a PN/A system when feeding change from sidestream to mainstream at room temperature. This genus (*Ca.* Kuenenia) has shown a greater affinity for nitrite than *Ca.* Brocadia (Park et al., 2015; van der Star et al., 2007) and its increase in relative abundance when working at 10ºC could be related to the results pointed out by Juan-Díaz et al. (2020), where a decrease in the sNRR in the bottom sludge section (S1) compared to the one at 20ºC would results in higher substrate availability for the S3 sludge bed section, that would lead to the observed increase in *Ca.* Kuenenia.

However, as explained in the previous section the approaches A and B fail to describe certainty if microbial stratification occurs in the bed sections.

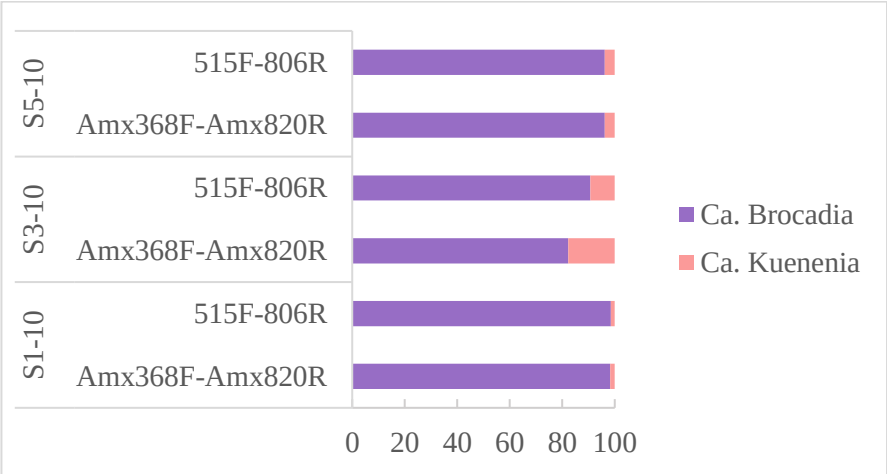


Figure 5.8.- Relative abundance of identified anammox bacteria at the genus level in three sludge bed sections of a mainstream anammox reactor working at 10ºC, using two different molecular approaches.

To understand the effect of the gradual decrease of the temperature affect the anammox community, the approach *d* was directly performed. For this, the 16S rRNA, 16S-amx, hzsA, and nirS genes were quantified from samples taken from the lower sludge bed section (S1) at different temperatures in the stable operating period at 20ºC (S1-20), 18ºC (S1-18), 16ºC (S1-16) and 10ºC (S1-10). Figure 5.9 showed that there were not significant differences in the 16S rRNA gen quantification at the different temperatures. The other quantified genes, instead, showed statistical differences. In the cases of the 16S rRNA specific for anammox population (16S rRNA-amx) and the functional gene hzsA, the copy genes quantification increased as the temperature decreased until 16ºC. Then, towards 10ºC, the abundance of these genes decreased, but it was still higher than that recorded at 20ºC. The adaptation of anammox bacteria to low temperatures has been reported previously (Lotti et al., 2015b), but normally it is reported as a stably maintained population (Laureni et al., 2016; Ma et al., 2013). Instead, Liu et al. (2018) showed a slight decrease in anammox abundance at low temperatures (close to 10ºC), nevertheless, they main objective was to compare diversity shifts with the influent change from synthetic to mainstream. Here, we confirm that the effective dampening of temperature reached by Juan-Díaz et al. (2020) was due to the progressive acclimatization of the anammox bacteria to low temperatures, as can be extracted from Fig. 5.9. This anammox increase at low temperatures could explain that despite the overall sNRR decrease, a good

nitrogen removal performance was reached (as can be seen in Table 5.1). With respect to the nirS gene, there was clearly a significant decrease as a function of temperature. Although the primer set used to quantify the nirS gen was designed to detect anammox bacteria in the environment (Li et al., 2011; Li and Gu, 2011), it have been reported that also detect as well a large portion of denitrifiers (Han et al., 2017; Yang et al., 2020). According to (Oshiki et al., 2016) this gene was absent in the genomes of *Ca.* Brocadia. Considering that the predominant genus in our reactor was *Ca.* Brocadia, the reported abundance of nirS gene might be a representation of denitrifiers, which decrease its relative abundance along with the decrease of temperature.

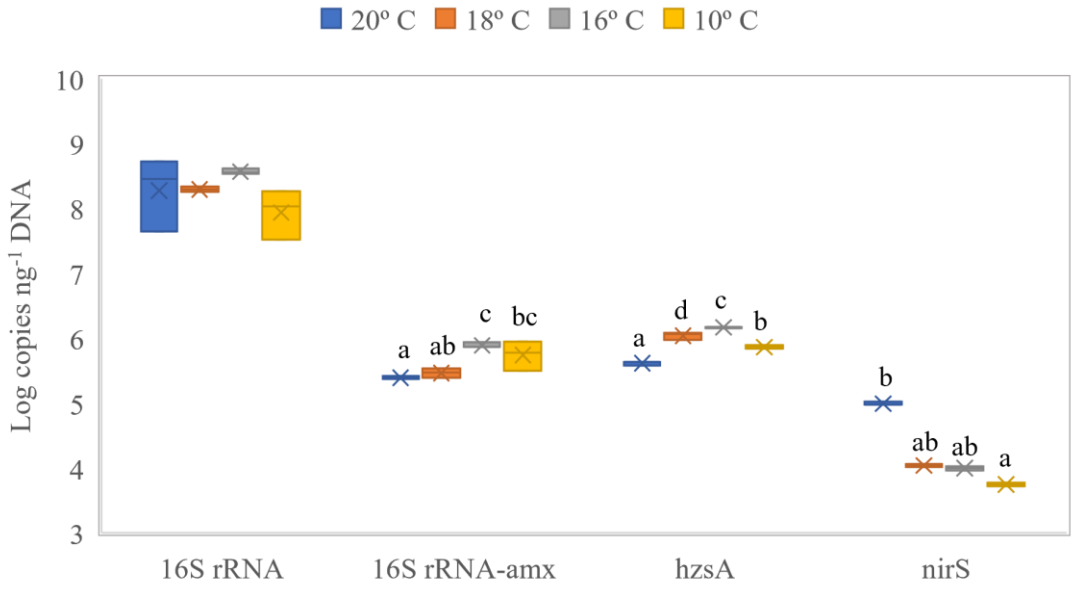


Figure 5.9.- Boxplot of absolute quantification of bacterial genes in the S1 sludge bed section of an anammox reactor working at mainstream conditions during temperature decrease by qPCR. 16S rRNA gene for total bacteria, 16S rRNA-amx for anammox bacteria, and functional genes hzsA and nirS were quantified. A logarithmic scale was chosen for the y-axis. The X on each box represents the mean value from triplicate qPCR reactions. The error bars stand for the standard deviations. Means with one letter in common are not significantly different ($p>0.05$).

The comparison performed in the previous section about bacterial communities through the bed height at the two temperatures of operation (20ºC to 10ºC), did not allow to determine if there is a stratification of the anammox community. In this sense, the ratio between the copy number of the 16S rRNA-amx gene (specific for anammox) to the 16S rRNA gene (for whole bacteria) was used to look for significant changes in the structure of the microbial population throughout the reactor, as suggested by Powell et al. (2006) (Fig. 5.10). A higher ratio of 16S-amx was obtained in the bottom sludge bed section (S1-20), while there were no differences on these ratios between the upper sludge bed sections of the reactor at this temperature (20ºC). The same dynamics was observed at 10ºC. Also, in all sludge bed sections at 10ºC, the ratio was higher than at 20ºC.

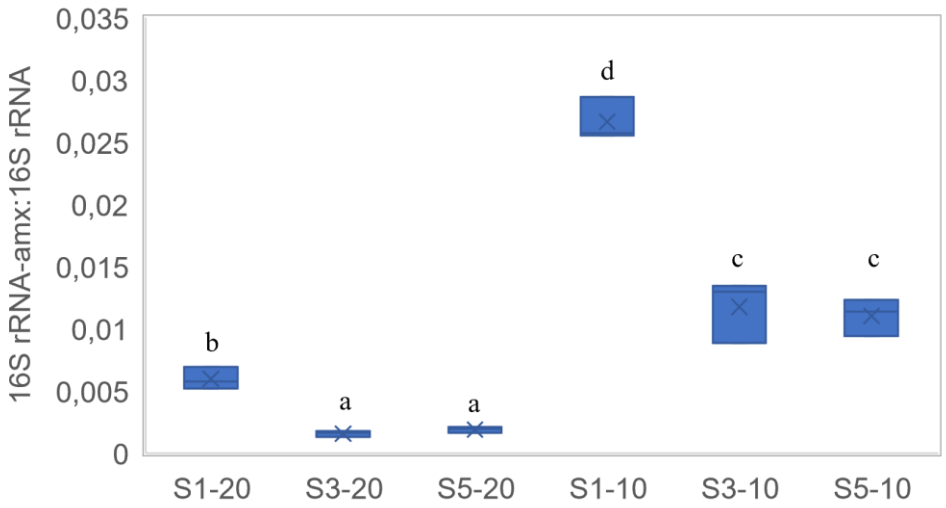


Figure 5.10.- Boxplot of the ratio between the gene copy numbers determined by qPCR of the 16S rRNA-amx over the 16S rRNA of three sludge bed sections of an anammox reactor working at mainstream conditions at 20ºC and 10ºC. 16S rRNA-amx gene for anammox bacteria and 16S rRNA gene for total bacteria were quantified and its ratio was calculated. The X on each box represents the mean value from triplicate qPCR reactions. The error bars stand for the standard deviations. Means with one letter in common are not significantly different ($p>0.05$).

Finally, if we compare the abundances of the anammox community obtained by the approaches a and d, based on the 16S rRNA gen (Fig. 5.11), we can deduce that there is a stratification of the anammox community along the reactor height at both studied temperatures, where the major abundance was detected in the bottom sludge bed section (feeding spot), followed by an abundance reduction in the middle part of the reactor.

Nevertheless, there is no significant differences between the upper sludge bed sections (S3 and S5) in the reactor. The stratification and the dynamics showed by the anammox community of this work agree with the conclusions of Juan-Díaz et al. (2020), where they determine that the nitrogen in the wastewater was mainly consumed in the base of the reactor. This, and the higher abundances exhibited in the upper sections at low temperatures confirm the reactor`s overcapacity. Nevertheless, due to the increased abundance of *Ca*. Kuenenia observed in S3-10, we suggest that shift in the anammox structure could play a role in the overcapacity (not only the substrate distribution), maintaining the nitrogen removal performance at low temperatures. Further investigation based on metabolically active anammox bacteria, is needed to probe this hypothesis.

Although the DNA-based methods used in this work provided us with important information on the diversity and dynamics of bacterial communities throughout the anammox reactor, more RNA-based research is needed to discover patterns of gene expression and the dynamics of functionally active communities (Ferrera and Sánchez, 2016).

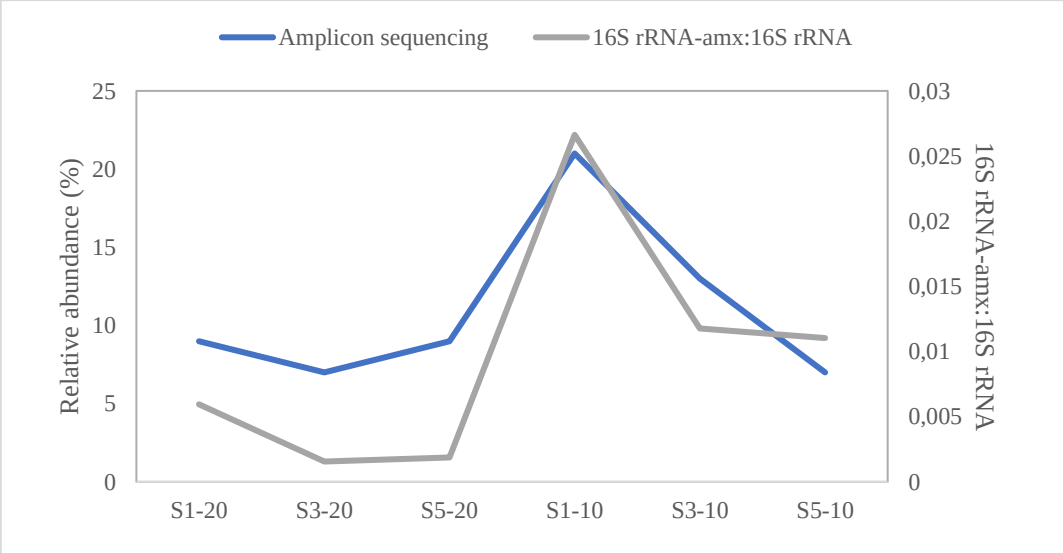


Figure 5.11.- Comparison of anammox community abundance in three sludge bed sections of a mainstream anammox reactor working at 20ºC and 10ºC determined by two molecular approaches.

5.4. Conclusions

Results obtained in this chapter demonstrated the influence of temperature dampening on the microbial community structure. The stratification of the community change, although the core microbiome was the same. This is the first report on how the microbial community can vary along the height of an UAnSB anammox reactor working at mainstream conditions with the gradual decrease in temperature from 20ºC to 10ºC. The major differences in the whole community were obtained in the top bed sections at different temperatures when comparing all the samples. These differences were given mainly by heterotrophic bacteria, while anammox bacteria only contributed to the whole community stratification in the base of the reactor. Despite the knowledge on the changes in the community, there is no clear information on the eco-physiological mechanisms that regulates the interactions between the whole members of the community.

Stratification of the anammox community throughout the reactor height at 20°C showed higher abundance of this bacteria at the bottom of the reactor (S1) than in the upper sections (S3, S5). The same trend was found at low temperatures, but with even double abundances This and the whole community stratification would confirm the overcapacity of the anammox reactor which allowed its good performance in NRE at low temperature. Nevertheless, the abundance increase of anammox bacteria at 10ºC has no correspondence with the sNRR in the reactor, therefore, a better and reliable confirmation would be obtained by studying the metabolically active communities from RNA-based methods.

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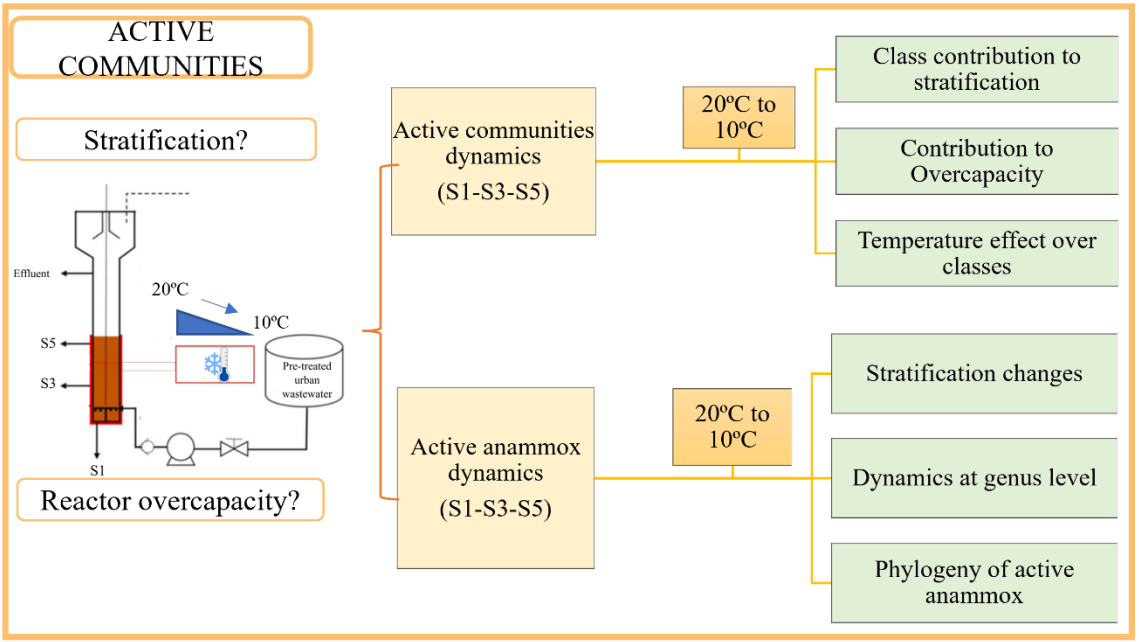
Chapter 6

Changes in the active bacterial and anammox communities during decrease of operating temperature

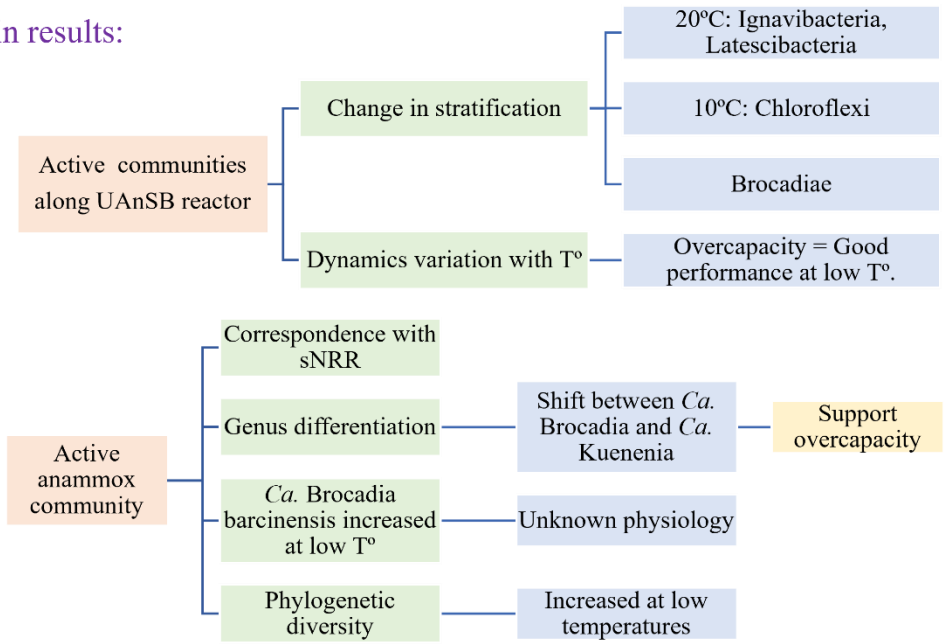
Goal 2: To determine the dynamics of active bacterial communities, including anammox, during the different phases of reactor operation

Problem: The increase of anammox bacteria relative abundance at 10ºC has no correspondence with the specific Nitrogen Removal Rate (sNRR)

Chapter 6. Changes in the active bacterial and anammox communities during decrease of operating temperature



Main results:



Abstract

In chapter 5 it was demonstrated that the microbial community presented stratification in the granular-sludge Upflow Anammox Sludge Blanket (UAnSB) reactor treating real wastewater at mainstream conditions, and that this stratification explain the performance during decrease of the operating temperature from 20ºC to 10ºC. But as these studies were made with DNA, it was not possible to known if all the detected microorganisms were active or not. Then, this chapter aimed to determine the active microbial and anammox communities along the same UAnSB reactor at the same conditions. For that, RNA was extracted from samples taken at different height of the reactor during operation at 20ºC and 10ºC and the active communities were analyzed by complementary DNA (cDNA) based 16S rRNA gene amplicon sequencing using both, universal primers and specific primers for anammox. Moreover, the proportion of active anammox communities in the different samples were assessed by specific qPCR.

The results showed that changes in stratification were explained by the temperature effect in the classes Ignavibacteria and Latescibacteria at 20ºC, while at 10ºC by two classes belonging to the phylum Chloroflexi. Class Brocadiae was contributing to the stratification due to both temperature and sludge-bed section effects. The community dynamics variation along the reactor could be supporting the reactor overcapacity as an important factor for maintaining the good performance at low temperature. Active anammox community analysis agrees with the anammox activity calculated as specific Nitrogen Removal Rate (sNRR) by Juan-Díaz et al (2020) for the same reactor. However, besides the substrate availability shift at 10ºC determined by them, a deeper study of the active anammox community showed that the genus differentiation along the reactor, changing between *Ca.* Brocadia and *Ca.* Kuenenia could be responsible for the maintained removal efficiency at low temperatures. Also, draws the attention that relative abundance of active *Ca.* B. barcinensis increased at low temperatures, and that the phylogenetic analysis showed higher diversity of anammox species at low temperatures. Therefore, the different physiological abilities of each anammox species might be influencing the reactor´s performance. Thus, amplicon sequencing of 16S rRNA approach works better to correlate operational parameters with a metabolically active community profile. Further metagenomics analysis would be useful to determine the communities' potential metabolic interactions.

6.1. Introduction

Universal 16S rRNA gene-based amplicon sequencing is widely used to assess microbial communities due to its low cost, time efficiency, and ability to produce a nearly quantitative profile of community membership. The disadvantage is that it provides limited information on non-dominant guilds because of their low contribution to the total sequence pool (Bowman, 2018; Diwan et al., 2018). Despite known biases, amplicon sequencing has been used successfully for quantitative assessment of the ubiquitous taxa in the bacterial community (Ibarbalz et al., 2014).

All the above takes relevant on microbial ecology research of unculturable bacteria that play an essential role in the biochemical cycling of nitrogen (Harhangi et al., 2012), such as anammox bacteria. Until now, research on this field has not resulted in any pure cultures in a laboratory setting due to their extremely slow growth rates, relatively low biomass yields, and inactivation in the presence of oxygen and high concentrations of nitrite (Kartal et al., 2012). Thus, requiring culture-independent methods for its study, as PCR-based methods (Yang et al., 2020).

In chapter 4 we evaluate a wide variety of approaches DNA-based including Fluorescence *in situ* hybridization (FISH), amplicon sequencing, metagenomics and quantitative PCR (qPCR), to detect and quantify anammox communities in an anammox reactor working at mainstream conditions. Each method has its biases that will influence the reported results. Standardization of these approaches to study a granular-sludge anammox reactor was outlined, concluding that amplicon sequencing with the universal primers 515F-806R and qPCR targeting 16SrRNA specific for anammox, both were a good choice for practical determinations such as relative abundance of bacteria and phylogeny. However, in chapter 5, it was determined that the increase in abundance of anammox bacteria at 10ºC using universal primers 515F-806R and qPCR targeting 16SrRNA specific for anammox, but it has no correspondence with the anammox activity calculated from operation parameters as specific Nitrogen Removal Rate (sNRR) in a mainstream anammox reactor (Juan-Díaz et al., 2020) .

Considering that DNA-based analysis cannot discriminate between active and inactive microorganisms, RNA-based studies have become popular in microbial ecology investigations as an alternative methodological approach to generate information of active members in the communities of different environments (Kim et al., 2013; Salgar-

Chaparro and Machuca, 2019). Nevertheless, the suitability of amplicon sequencing of 16S rRNA gene for identifying the active microbial populations in anammox reactors working under mainstream conditions systems has rarely, if ever, been explicitly addressed.

Therefore, this study aims to provide us with more reliable information to understand the relationship between microbial ecology of active communities and operating conditions. In this sense, the whole microbial and specific anammox active communities during the decrease of operating temperature of an anammox reactor working under mainstream conditions is addressed.

6.2. Materials and methods

6.2.1. Anammox reactor: description and samples taken

Samples of anammox granular biomass were obtained from three sample points along the sludge bed of an UAnSB reactor during it operation at mainstream conditions. This UAnSB was fed with real urban wastewater (26±8 mg N-NH4+ L-1, 36±9 mg N-NO2- L-1, 2±2 mg N-NO3- L-1, 60 ± 14 mg COD L^{-1}, among other compounds), coming from an urban WWTP located in an industrial area of Catalonia, NE Spain (for more details, see the work of Juan-Díaz et al., 2020). The samples were taken at different operational days representative from steady state of operational period I and III (Juan-Díaz et al., 2020), while temperature of the reactor was gradually lowered from 20º C (period I) to 10º C (period III). The reactor had five different sampling points at heights of 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) (Fig. 6.1). For the purposes of this thesis, 3 points along the bed were selected to take the samples, indicated in the Fig. 6.1 as “S1”, “S3” and “S5”, and labeled in the same way, followed by the reactor temperature at the time of sampling. The operational parameters of the UAnSB reactor such: Nitrogen Loading Rate (NLR), Nitrogen Removal Rate (NRR), Nitrogen Removal efficiency (NRE), the anammox activity or specific NRR (sNRR) at the sample day or period were obtained from Juan-Díaz et al (2020) and Juan-Díaz (2021). The anammox activity determined by them as specific NRRs, was calculated for the different sludge bed sections delimited by the sampling points using measured nitrogen compounds concentrations at each point (more details in Juan-Díaz et al., 2020). Table 6.1 shown the

samples taken, associated to the operational parameters such: NRL, NRR, NRE, and the anammox activity or sNRR.

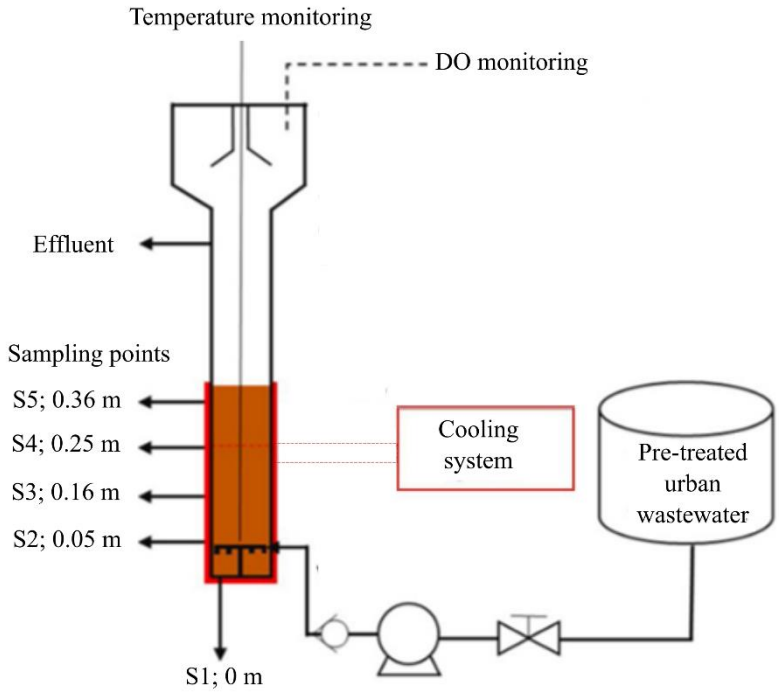


Figure 6.1.- Schematic diagram of the lab-scale UAnSB reactor. Adapted from Juan-Díaz et al. (2020).

Table 6.1.- Samples and operational parameters.

	Operational data for the specified sampling day					Operational Parameters at each steady state by period				
Sample ID	Day[a]	Temp. (ºC)	NLR[b] (g L^{-1} d^{-1})	NRR[b] (g L^{-1} d^{-1})	NRE[b] (%)	Operation period[a] (days)	NLR (g L^{-1} d^{-1})	NRR (g L^{-1} d^{-1})	NRE (%)	sNRR[c] (g N g^{-1} VS h^{-1})
S1-20										100
S3-20	17	20	0.11	0.10	90	I (0-81)	0.10 ± 0.02	0.10 ± 0.02	87 ± 3	2
S5-20										0
S1-10										62
S3-10	203	10	0.10	0.06	49	III (155-260)	0.11 ± 0.01	0.09 ± 0.01	71 ± 6	8
S5-10										0

[a] Operation period and parameters such as: Nitrogen Loading Rate (NLR), Nitrogen Removal Rate (NRR), and Nitrogen Removal Efficiency (NRE) as described by Juan-Díaz et al. (2020). [b] Operational parameters for the sampling day were obtained from Juan-Díaz (2021). [c] Specific NRR (sNRR) estimated from graphical data in Juan-Díaz et al. (2020).

6.2.2. RNA extraction and reverse transcription

Total RNA was extracted using the Pure Link RNA mini kit (Life Technologies) following the protocol described in section 3.3.1. Reverse transcription to obtain the complementary DNA (cDNA) was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific), and the quantification of the cDNA was determined with Qubit fluorometric quantitative analyzer (Life Technologies, Carlsbad, CA, USA) to normalize the qPCR assays.

6.2.3. Active microbial communities

Identification and determination of the active microbial community along the heights of the anammox reactor at different working temperatures was performed with amplicon sequencing of cDNA using 16S rRNA universal primers 515F-806R. Samples were sequenced by RTL genomics and the reprocess of the raw data was performed using QIIME2™ v.2019.10 (Bolyen et al., 2019) and DADA2 denoise-paired plug-in (Callahan et al., 2016). The obtained amplicon sequence variants (ASV) were taxonomically assigned with SILVA SSU 138 rRNA database, as described in section 3.3.2 of Chapter 3. Additionally, quantification of the anammox active microorganisms was performed using qPCR with specific primers targeting 16S rRNA of anammox. Both approaches were applied to samples taken at 20ºC and at 10ºC.

6.2.4. Statistical analysis

The Statistical Analysis of Metagenomic Profiles (STAMP) program (Parks, 2014) was used to identify differentially abundant communities. Two groups comparison was performed with Whites nonparametric t-test ($p<0.05$) and two samples comparison with the Fisher's exact test ($p<0.05$).

For active bacteria determined by qPCR, statistical analysis was performed with Infostat software. Copy number ratios of 16S rRNA-amx gene to the 16S rRNA gene were calculated. Samples were subjected to the normality test of Shapiro-Wilks. When normality was confirmed, data was subjected to ANOVA analysis and Tukey's test for media comparison was used. Significance 5% was used.

6.3. Results and Discussion

6.3.1. Active bacterial community through the sludge bed with decrease of reactor operation temperature

According to the 16S rRNA sequence analysis performed in cDNA samples (Fig. 6.2), the most active classes at both temperatures were Brocadiae, Gammaproteobacteria, Ignavibacteria, and Anaerolineae, although its relative abundances varied between sludge bed-sections. Coincidentally, these active classes were the same that has been described as predominant in anammox reactors from DNA-based approaches from other studies, despite the operational conditions, feeding (synthetic or real wastewater and type of reactor (anammox or partial nitritation- anammox) (Bhattacharjee et al., 2017; Lawson et al., 2017; Mardanov et al., 2019; Zhao et al., 2018).

Brocadiae was the most abundant of the active classes detected in the reactor's bottom (27%, sample S1-20), on the contrary to the observed with DNA sequencing at 20ºC (9%). This class was decreasing its abundance in the upper sections to 12% and 1% in S3-20 and S5-20, respectively. A similar behavior was observed in Gammaproteobacteria and Ignavibacteria. Conversely, the abundance of active Anaerolinea increased from the bottom to the upper sludge-bed section from 13% (sample S1-20) to 18% in S5-20.

The predominant active classes at 10ºC in the reactor lower section (S1-10) were Anaerolinea, Gammaproteobacteria, Ignavibacteria, and JG30-KF-CM66. Brocadiae was present only in 3%; however, its abundance increases in the S3-10 sludge bed section to 16%, to decrease again in the S5-10 sludge bed section to 4%. The classes belonging to the phylum Chloroflexi (Anaerolinea and JG30-KF-CM66) and class Ignavibacteria fall in abundance to the upper sections, while Gammaproteobacteria abundance was between 19% and 22%, being lower in the middle section (S3-10).

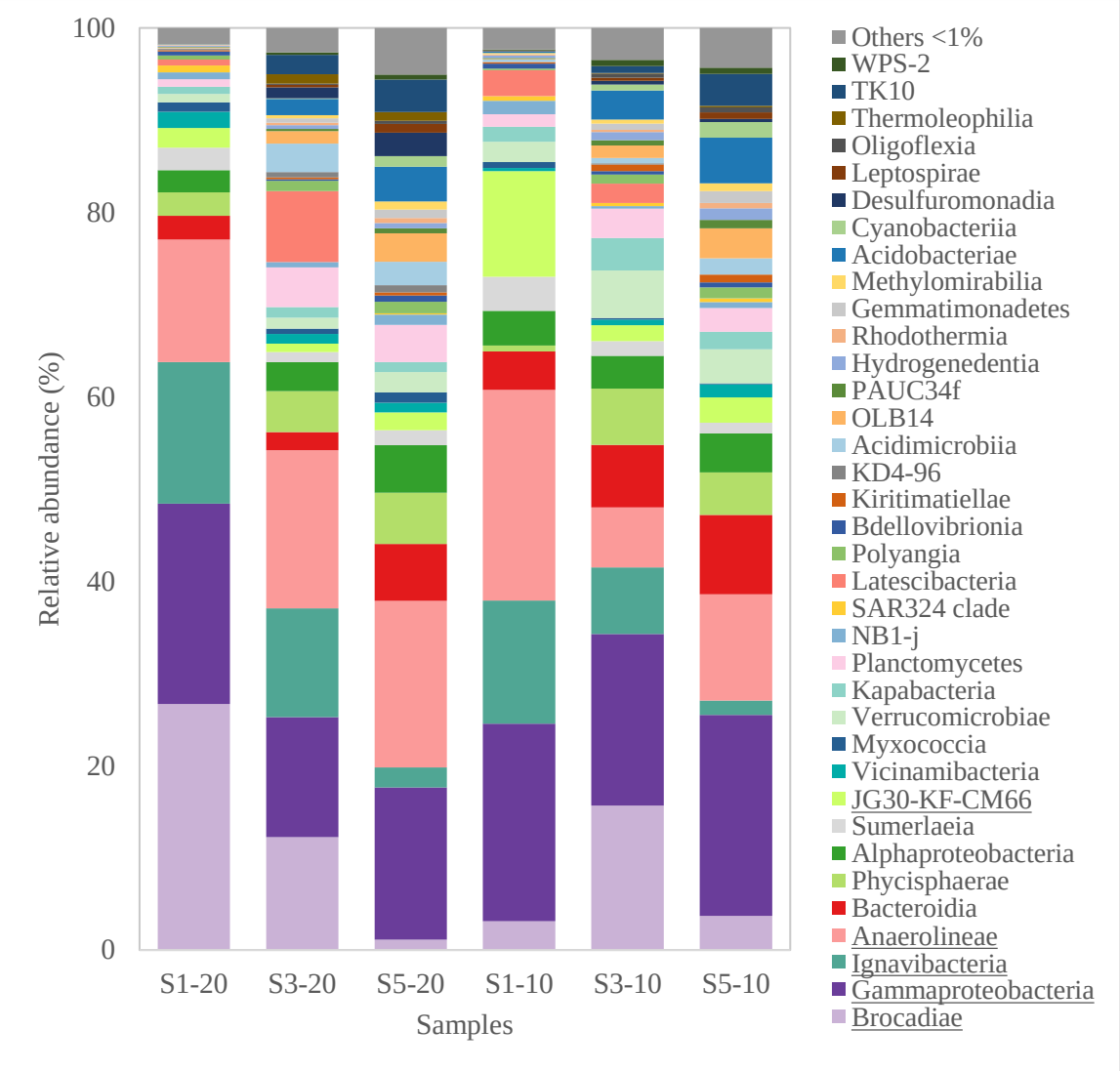


Figure 6.2.- Relative abundance of identified classes in three sludge bed sections of an anammox reactor at 20ºC and 10ºC obtained from RNA-based amplicon sequencing. Predominant classes were underlined in legend. Classes whose relative abundance among the techniques was less than 1% were grouped as "Others".

Therefore, the overview of the whole microbial community obtained from RNA-based amplicon sequencing performed in this work showed clear differences with the observed using DNA-based methods (Fig. 6.3). Analyzing our reactor, the relative abundance classes Brocadiae, JG30-KF-CM66 and Gamaproteobacteria was underestimated with DNA sequencing, while class Ignavibacteria was overestimated, in contrast with the microbial profile of active communities determined with cDNA sequencing. Wang et al. (2016) and Wu et al. (2016) used both, DNA and cDNA-based methods for studying anammox population, but not the entire community as in this study.

To the best of our knowledge, Salgar-Chaparro and Machuca (2019) performed this method comparison for assessing the profile of corrosive microbial communities in an oil production facility, and they detected that DNA based results lead to the underestimation of active members in the community, as we also observed.

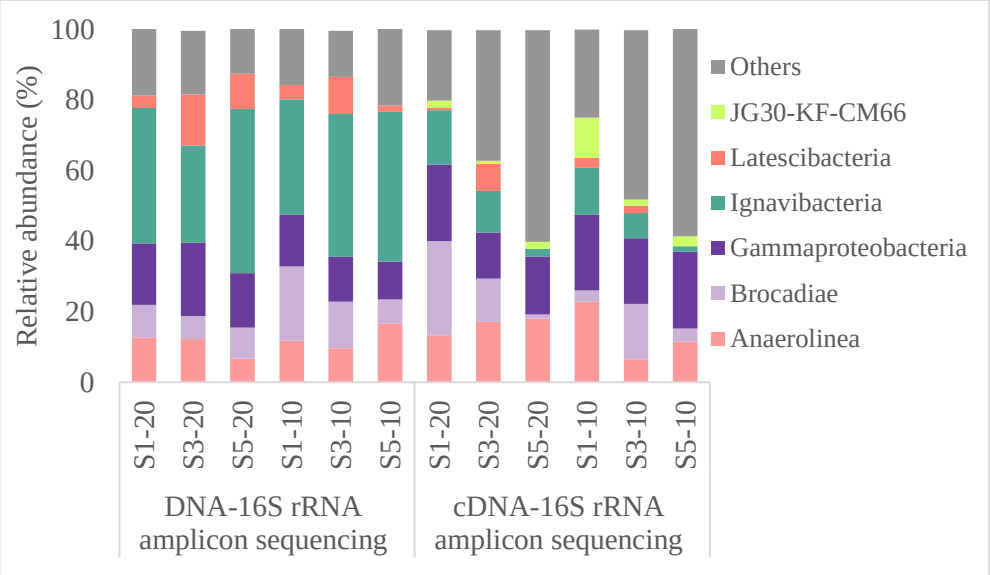


Figure 6.3. Comparison of relative abundances of predominant classes determined by the analysis of DNA and cDNA amplicon sequencing in an UAnSB anammox reactor working at mainstream conditions. Both approaches were performed with primers 515F-806R.

6.3.1.1. Stratification of the active community along sludge bed sections

To determine significant changes in the active communities through the reactor, a statistical differential abundance analysis was performed between the heights (Fig. 6.4). This stratification was studied comparing the relative abundance of active bacteria classes presented in Fig. 6.2 and obtaining the ones with significant statistical contribution to the microbial dynamics at each temperature for each sludge-bed section.

The class Brocadiae showed significant changes in the abundance of active bacteria between sludge sections at both temperatures (Fig. 6.4). Differences with the detected in the DNA-based study were noted, confirming the importance of performing this analysis using RNA-based methods. These contrast in the relative abundances reinforce that it may be important to distinguish between viable and non-viable cells in some studies, which is an advantage of using the cDNA sequencing to correlate with the reactor´s performance data. These results agree with the anammox activity calculated as

specific NRR, based on nitrogen compounds concentrations in the different sludge bed sections by Juan-Díaz et al. (2020). The determined active anammox community could be supporting the reactor overcapacity suggested previously, although further analysis of the temperature effect in the active anammox community profile at deeper taxonomic levels, could be key in understanding the causes of variations in the overall performance in the mainstream process.

On the one hand, Fig. 6.4-A showed that at 20ºC, from S1 to S3 section, the active community of Brocadiaceae decrease while the Latescibacterota increased. From S3 to S5 section, there was a decrease in the classes Brocadiaceae, Latescibacteriota, and Ignavibacteria. Regarding the activity of Latescibacteriota through the reactor, differences were detected comparing these results with those obtained with DNA amplicon sequence (Fig. 6.3). In chapter 5, from DNA amplicon sequencingresults we have reported that its dynamic was important from S1 to S3 section at 20ºC and from S3 to the S5 section at 10ºC. Nevertheless, the differential analysis of active community showed that class Latescibacteria contributed to the changes through the entire reactor only at 20ºC. On the other hand, Fig. 6.4-B showed that at low temperature (10ºC), from the reactor base (S1) to the middle section (S3), there was a decrease of Anaerolinea and JG30-KF-CM66, while Brocadiae increases significantly. Then, between the upper sections of the reactor (S3 and S5) at 10ºC, the main change was performed by Brocadiae, in which abundance decreases. Therefore, from a general perspective, the changes in the active community observed at 20°C were related to the classes Latescibacteriota and Ignavibacteria in the upper sections of the reactor. As we described earlier in Chapter 5, these classes have often been associated with the consumption of soluble molecular products (SMPs), polymers, or cell biomass degraders (Yang et al., 2018; Youssef et al., 2015). Therefore, they become more active in the sections where the anammox was less abundant, making sense considering that they might be consuming SMP produced by anammox. In the case of Ignavibacteria, they could also be supporting complete denitrification. (Akaboci et al., 2018; Lawson et al., 2017; Yang et al., 2018).

In contrast, the changes in the active community profile at 10°C were mainly due to the decrease in the abundance of two classes belonging to the phylum Chloroflexi (Anaerolinea and JG30-KF-CM66) in the middle section of the reactor. These classes were less active in the section where anammox were most abundant. These marked differences in terms of abundance were not observed in the DNA-based community

analysis (Fig. 6.3). The functional interaction of these communities remains unclear, but it has been described the role of Chloroflexi in reinforcing the granular structure (Bovio-Winkler et al., 2021; Li et al., 2009). Also, a recent study of the metabolic potential of MAG obtained from this anammox reactor showed that this phylum could be collaborating with the reactor removal performance due to the ability to perform the dissimilatory reduction of nitrate to ammonia (DNRA). Genes encoding both dissimilatory nitrate and nitrite reductases were found (Bovio-Winkler, 2021).

The determination of active communities' stratification along sludge bed sections increases the understanding of how other active communities in addition to anammox could improve the system performance through the possible interactions between them.

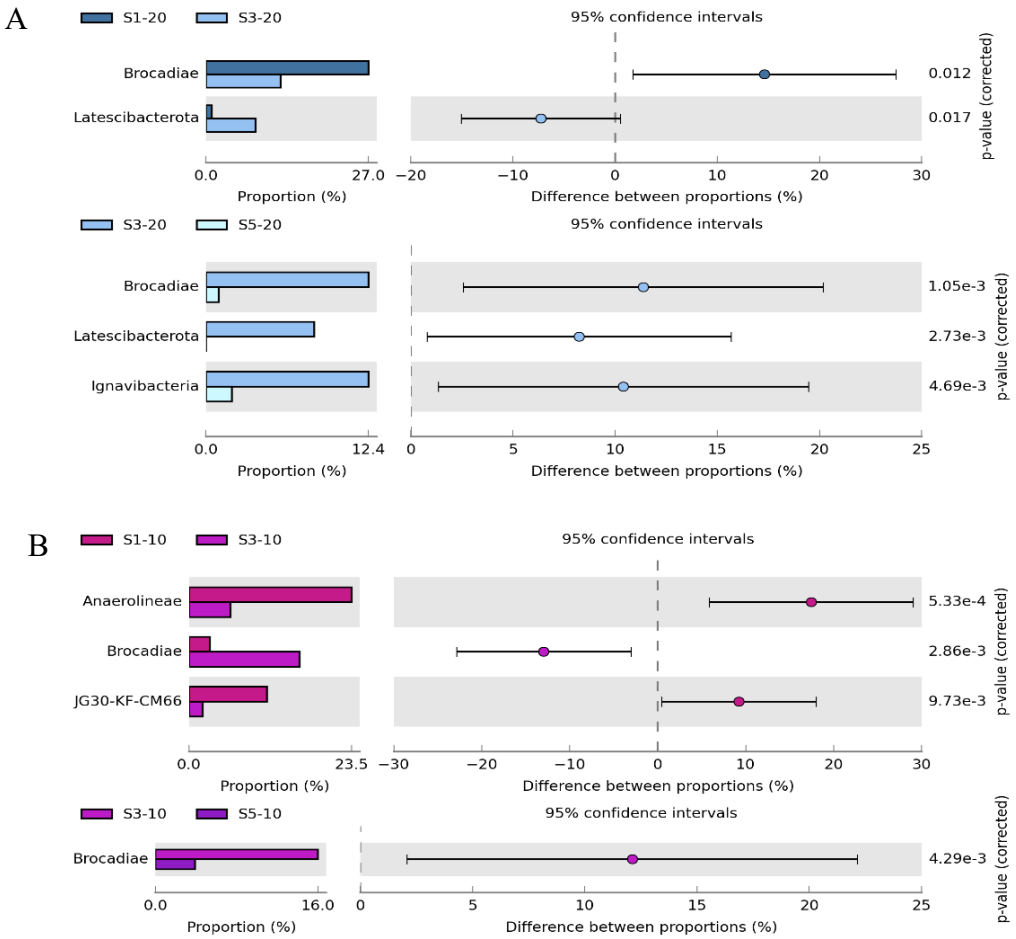


Figure 6.4.- Extended error plot for difference analysis of relative abundance of active microorganisms by sludge bed section at two different working temperatures. The analysis was performed with the sequences classified at the class level. Fisher's exact test for comparison of S1 with S3 and S3 with S5 sludge bed sections, A) at 20ºC and B) at 10ºC. ($p<0.05$).

6.3.1.2 Changes in the active community on the sludge bed sections by temperature effect

The microbial community structure changes with the temperature decrease were evaluated. A first comparison of the temperature effect between all the reactor's communities at 10ºC and 20ºC was performed, and the were no significant differences. Then, the microbial structure at the two evaluated temperatures for each sludge bed section was analyzed to understand which classes contributed to the reactor's performance when the temperature decreases (Fig. 6.5). In the case of the S1 sludge bed section, two classes impact the active microbial community. At low temperatures, class Brocadiae significantly decreases while JG30-KF-CM66 increases. In the S3 section, Anaerolineae was the only class affected by the temperature decrease, being less abundant at 10ºC. There were no differences in the community structure of the S5 section, despite the temperature difference.

The contribution of the anammox bacteria to the community structure dynamics in the sludge sections (stratification) at both temperatures was demonstrated. Nevertheless, the temperature effect over the class Brocadiae was observed only in the bottom section, which slightly differs from the proposed by Juan-Díaz et al. (2020). According to their analysis, the specific NRR of the S1 section at 20ºC was 50 times higher than those determined for upper sludge bed sections but decreased significatively at 10ºC. This resulted in a substrate availability increase in the upper layers, which led to the activation of starved anammox biomass of the upper sections. Our results also showed higher activity in the S1 section at 20ºC than at 10ºC. However, they did not detect a significant change in the abundance of active anammox bacteria in the middle and upper layers of the reactor caused by the temperature decrease. Thus, besides the substrate availability shift, other factors could allow a good reactor performance at low temperatures requiring a detailed analysis of the anammox community structure.

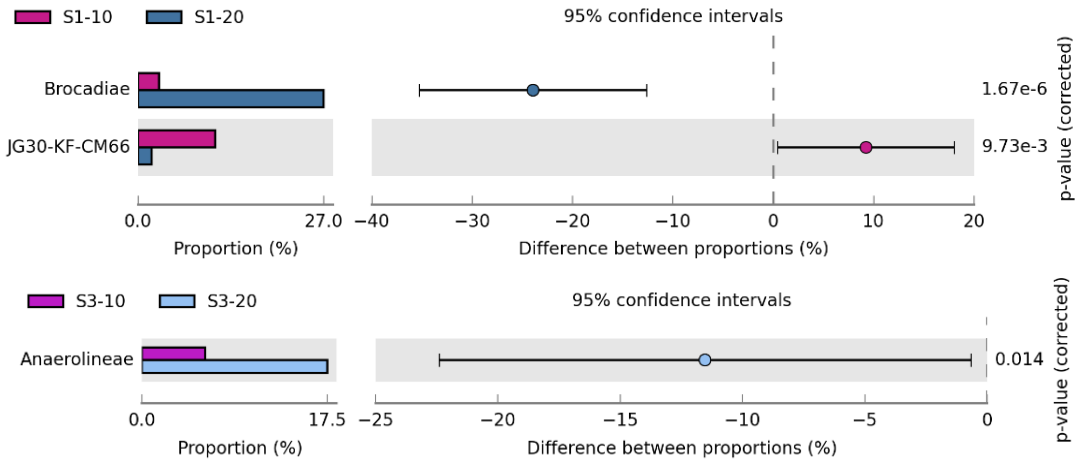


Figure. 6.5.- Extended error plot for difference analysis of active communities by temperature at the class level of each sludge bed section. Fisher's exact test for comparison of S1-10 with S1-20 and S3-10 with S3-20. Samples without significant differences were not showed. ($p<0.05$)

6.3.2. Stratification changes in anammox community through the sludge bed with decreasing operation temperatures from 20ºC to 10ºC

Relative quantification of the active bacteria was performed by calculating the copy number ratios of the 16S rRNA-amx gene to the 16S rRNA gene to determine stratification changes in the anammox community structure. Figure 6.6 confirmed that at 20ºC, anammox community abundance had a significant decrease from the bottom towards the higher layers of the reactor. Instead, when the temperature drops to 10ºC, the stratification of the reactor changes. The active anammox bacteria in the S1-10 section decreases, statistically similar to the lower abundance at 20ºC (S5-20). Then, the higher quantity was observed in section S3-10 without significant differences with the same section at 20ºC (S3-20) and similar to the observed at S1-20 section. Finally, this abundance decreases in the S5-10 section but still is similar to the showed in S3-20. This increase of active anammox population in the upper sections should support the reactor's nitrogen removal performance at low temperatures, confirming the proposed reactor's overcapacity proposed by Juan-Díaz et al. (2020). However, due we detected the same active population in the middle of the reactor at both temperatures, despite the higher substrate availability at 10ºC, this analysis led to infer that the heterogeneous substrate concentrations gradients along the reactor are not the critical factor causing the overcapacity.

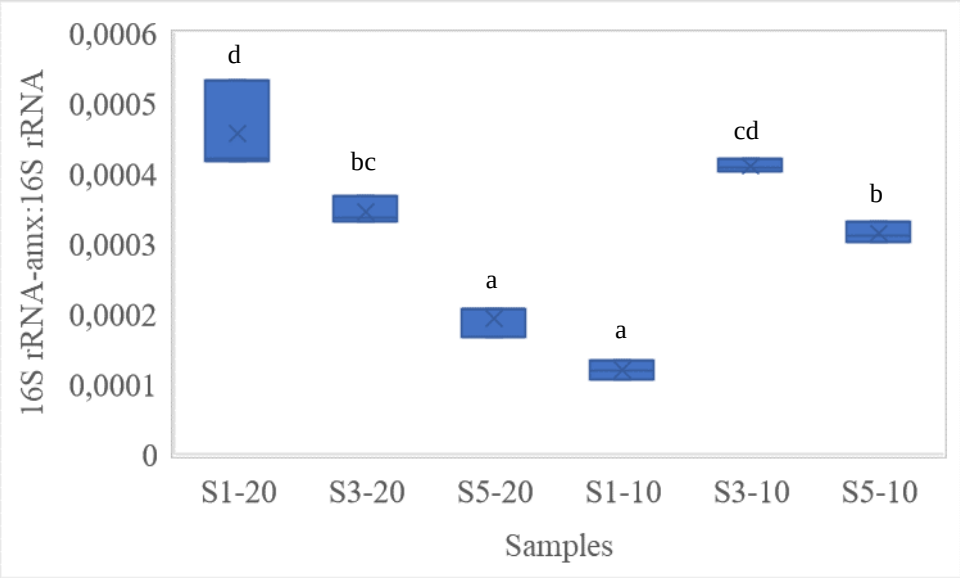


Figure 6.6. Boxplot of ratio 16S rRNA-amx:16S rRNA copy numbers determined by qPCR using RNA from three sludge bed sections of an anammox reactor working at mainstream conditions at 20ºC and 10ºC. The X on each box represents the mean value from triplicate qPCR reactions. The error bars stand for the standard deviations. Means with one letter in common are not significantly different ($p>0.05$).

6.3.2.1. Dynamics of the active anammox community at genus level

Changes in the active anammox bacteria was observed in the community structure regarding the temperature and throughout the sludge bed sections. The ASVs classified into the class Brocadiae retrieved from cDNA 16S rRNA amplicon sequencing, were analyzed at the genus level to understand changes in this community. Figure 6.7 showed that at 20ºC, sections S1 and S3 were dominated by *Ca.* Brocadia genus, while S5-sludge bed section was entirely dominated by *Ca.* Kuenenia. A possible explanation for the enrichment of the upper section of the anammox-mainstream reactor with *Ca.* Kuenenia at 20ºC could be its ability to adapt to low-strength wastewater due it is a K-strategist microorganism. This means that they grow faster when faced with limited substrate concentrations and are exposed to a strong competitiveness. Therefore, under limited substrate conditions (as in S5-20 section), it would outcompete with *Ca.* Brocadia (strategist r, a fast growth rate at the high substrate availability and under low competition level with other microorganisms (Shourjeh et al., 2021; Sun et al., 2018).

When temperature decreases to 10ºC, the dominance of *Ca.* Brocadia was seen in all sections, although S3-10 showed a high abundance (32%) of *Ca.* Kuenenia. A

significant shift in the anammox community was noted between both temperatures in the upper section, from 100% *Ca.* Kuenenia in S5-20 to 18% in S5-10. Thus, in agreement with (Narita et al., 2017), the differences in physiological characteristics among anammox bacteria are one of the essential key factors to understand niche differentiation, and our result could support the role of the genus differentiation in the reactor overcapacity and therefore in the good removal efficiency at low temperatures.

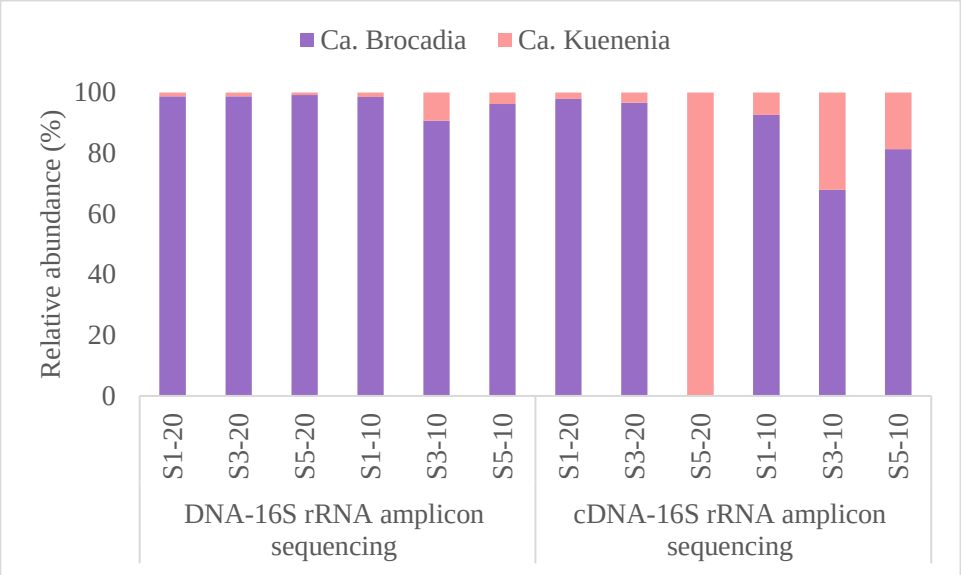


Figure 6.7.- Relative abundance of identified anammox bacteria at the genus level in three sludge bed sections of a mainstream anammox reactor working at 20ºC and 10ºC. The relative abundance was determined as the percentage of ASVs within the upper taxonomy level (Brocadiae class).

6.3.2.2. Phylogeny of active anammox communities

A phylogenetic analysis was performed to elucidate the anammox diversity at the species level. The tree was constructed with the ASVs retrieved from amplicon 16S rRNA sequencing of cDNA (Fig. 6.8). Relative abundances on each sample were included in determining specific changes in the active community with the temperature decrease. Five ASVs (from ASV1_c to ASV5_c) were present at both temperatures, at different proportions in the different sludge-bed sections.

Thus, for example, ASV3_c that clustered with *Ca.* Brocadia fulgida, was present in S1-20 in low abundance (11%), in S3-20 reached 68%, 52% in S1-10 and 11% in S3-10. From the tree, it can be observed that a higher diversity of the anammox community was obtained at lower temperatures. There were more ASVs clustered with different

species, mainly in the S5-10 section. According to the phylogenetic tree, the assignment of each ASV to a species taxonomic level and the relative abundances of each one is shown in Table 6.2.

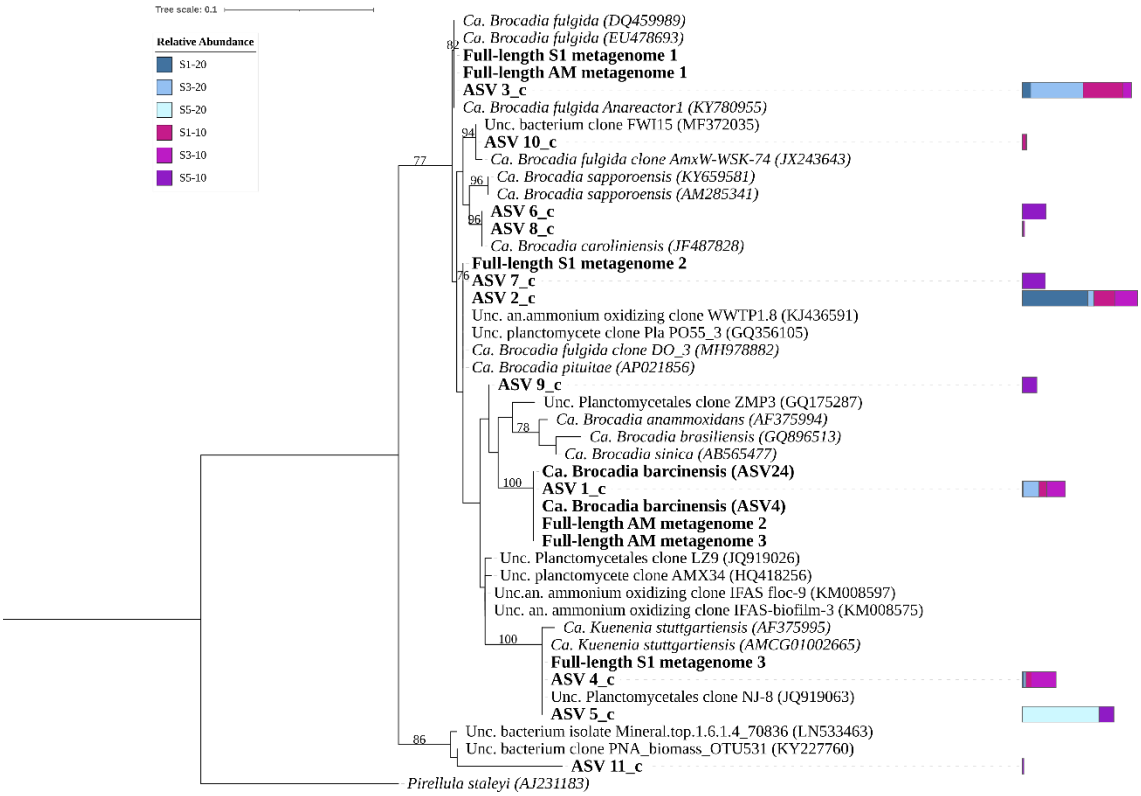


Figure 6.8.- Phylogenetic tree based on 16S rRNA gene sequences from cDNA amplicon sequencing. ASVs of this study and some ASVs retrieved from amplicon sequencing of DNA and full-length sequences of 16S rRNA gene reconstructed from metagenomes are shown in bold. The relative abundance of each ASV is shown as multiple bar dataset, in different color for each sample. Accession numbers of the reference sequences and environmental clones of anammox bacteria are shown in parentheses. Maximum Likehood method was used to construct the tree. Bootstrap values (greater than or equal to70%) based on 500 replications are shown at branch nodes. 16S rRNA gene sequence from *Pirellula staleyi* was used as an outgroup for rooting the tree. Bar indicates 0.01 substitutions per site.

Table 6.2.- Taxonomic classification of ASVs retrieved from cDNA amplicon sequencing according to SILVA 138 nR database at genus level and the phylogenetic tree. The relative abundance in percentage was defined as the ASVs of the level within the Brocadiae class.

ASV ID	Genus	Phylogenetic tree assignment	Relative abundance (%)					
			S1-20	S3-20	S5-20	S1-10	S3-10	S5-10
ASV1_c	*Ca.* Brocadia	*Ca.* Brocadia barcinensis	1	21	0	10	24	0
ASV2_c	*Ca.* Brocadia	*Ca.* Brocadia pituitae	85	8	0	27	30	0
ASV3_c	*Ca.* Brocadia	*Ca.* Brocadia fulgida	11	68	0	52	11	0
ASV4_c	*Ca.* Kuenenia	*Ca.* Kuenenia stuttgartiensis	2	3	0	7	32	0
ASV5_c	*Ca.* Kuenenia	*Ca.* Kuenenia stuttgartiensis	0	0	100	0	0	19
ASV6_c	*Ca.* Brocadia	*Ca.* Brocadia caroliniensis	0	0	0	0	0	31
ASV7_c	*Ca.* Brocadia	*Ca.* Brocadia pituitae	0	0	0	0	0	30
ASV8_c	*Ca.* Brocadia	*Ca.* Brocadia caroliniensis	1	0	0	0	2	0
ASV9_c	*Ca.* Brocadia	*Ca.* Brocadia (unclassified)	0	0	0	0	0	19
ASV10_c	*Ca.* Brocadia	Unc. bacterium clone FWI15	0	0	0	5	1	0
ASV11_c	*Ca.* Brocadia	Unc. bacterium clone PNA biomass OTU531	0	0	0	0	0	2

[a] *Ca.* Brocadia barcinesis correspond to a new *Candidatus* species derived from Chapter 4.

From the phylogenetic analysis, there were determined important changes in the anammox population at the species level. The S1-20 section, which presents the higher specific NRR according to Juan-Díaz et al. (2020) determinations, was dominated by *Ca.* Brocadia pituitae. Okubo et al. (2021), recently assembled a complete genome of this species describing an essential characteristic of its metabolism, the lack of the nitrite reductase genes responsible for reducing nitrite to nitric oxide, a key intermediate in anammox bacteria.

The most abundant anammox species in the S3-20 section was *Ca.* Brocadia fulgida. This species has been reported to be dominant on different de-ammonification systems (Laureni et al., 2015; Liu et al., 2018; Winkler et al., 2012). Interestingly, Winkler et al. (2012), reported an accumulation of this species in granular sludge once nitrite was omitted from the feed, similar to the low availability of this compound in the S3-20 section, as reported by Juan-Díaz et al. (2020).The high abundance of *Ca.* Kuenenia in S5-20 was previously discussed.

When the temperature change to 10ºC, the active population of S1-10 section was dominated by *Ca.* B. fulgida in 58%. Therefore, nitrite concentration along the reactor at this temperature does not explain the shift in the dominant species.

The sludge-section S3-10 showed similar abundance of three species: *Ca.*B. barcinensis, *Ca.* B. pituitae and *Ca.* K. stuttgartiensis. The increased abundance of *Ca.* B. barcinensis draws attention because as was identified as a new Candidatus species (Chapter 4), their physiology has not been studied yet, but according to this result, has a good adaptation to low temperature and could be outcompeting with other anammox species under adverse conditions.

The greatest difference in the diversity of active anammox was observed in section S5 when the temperature changes from 20ºC to 10ºC, being dominated by four species. These species were hardly present in the other samples, and even when they were, they corresponded to different clones: *Ca.* K. sttutgartiensis, *Ca.* B. caroliniensis, *Ca.* B. pituitae and a *Ca.* Brocadia, which does not match any sample environmental. According to a genome assembly of *Ca.* B. caroliniensis, a pathway for dissimilatory nitrate reduction to ammonium (DRNA) was found, which enables to utilize organic compounds and nitrate in the absence of ammonium (Park et al., 2017). Thus, using this pathway, are likely to prevail over other anammox bacteria when such volatile fatty acids are present

(Li et al., 2018), and therefore could enhance their ability to survive under limiting conditions.

Hence, we could be hypothesized that changes in the community stratification depend on several factors, given by the interactions with other communities and the physiological characteristics, which led the adaptability of the anammox species to the environmental changes. Also, the higher diversity of anammox bacteria observed at low temperatures could be causing the NRR decrease from 0.10 ± 0.02 g $L^{-1}d^{-1}$ to 0.09 ± 0.01 g $L^{-1}d^{-1}$ considering the operation period, or 0.06 g $L^{-1}d^{-1}$ considering the operation day data (Juan-Díaz et al., 2020), as suggested by Reino et al. (2018).

From a process perspective, our findings allowed to understand the microbial behavior along the different sections of an anammox reactor, which have been demonstrated to be an essential focus to develop rapid responses to process changes or upsets, as suggested by Park et al. (2010). With this method RNA-based, it would be interesting to be able to correlate the operational data with the microbial profile in the mainstream operation, given the differences observed in the NRR and NRE data recovered from the operation day or the operation period at work by Juan-Diaz (2020). However, more periodic sample acquisition would be required.

6.4. Conclusions

Important differences on the active microorganisms were detected in comparison with DNA-based methods. Changes in the community structure through the sludge bed were demonstrated, and it was proven that different populations are contributing to these changes, depending on the temperature and also depending on the sludge-bed section. The observed bacterial dynamics clearly support the denominated reactor's overcapacity.

According to our results, the heterogeneous nutrient distribution in the reactor was not the only factor involved in the reactor overcapacity and removal performance. The anammox stratification at the genus and species level, given by the shift from *Ca.* Brocadia to *Ca*. Kuenenia in the middle section (S3-10) at low temperature, and a higher diversity of anammox bacteria in S5-10, could be supporting this overcapacity too. Physiological differences among anammox community which led a variable interaction

with other communities and adaptability to the environmental changes may explain these changes.

The implementation RNA-based studies would enable a better prediction of microbial and/or anammox activity under operational parameter changes. More periodic samples would be necessary to link the operational data with the microbial dynamics. Further analysis would include the determination of the metabolic potential of this communities to understand how they are interacting.

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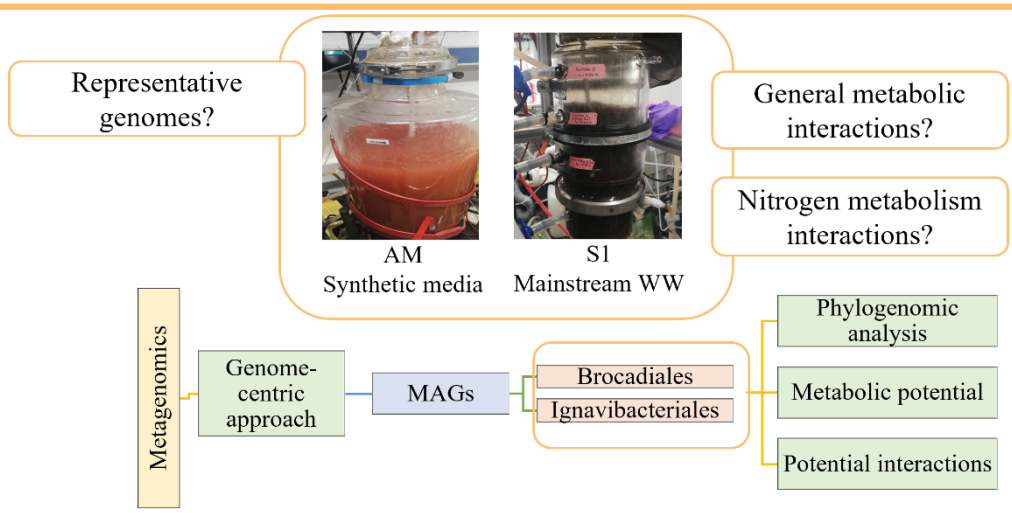
Chapter 7

Metabolic potential of metagenome-assembled genomes (MAGs) retrieved from two anammox reactors working at different conditions

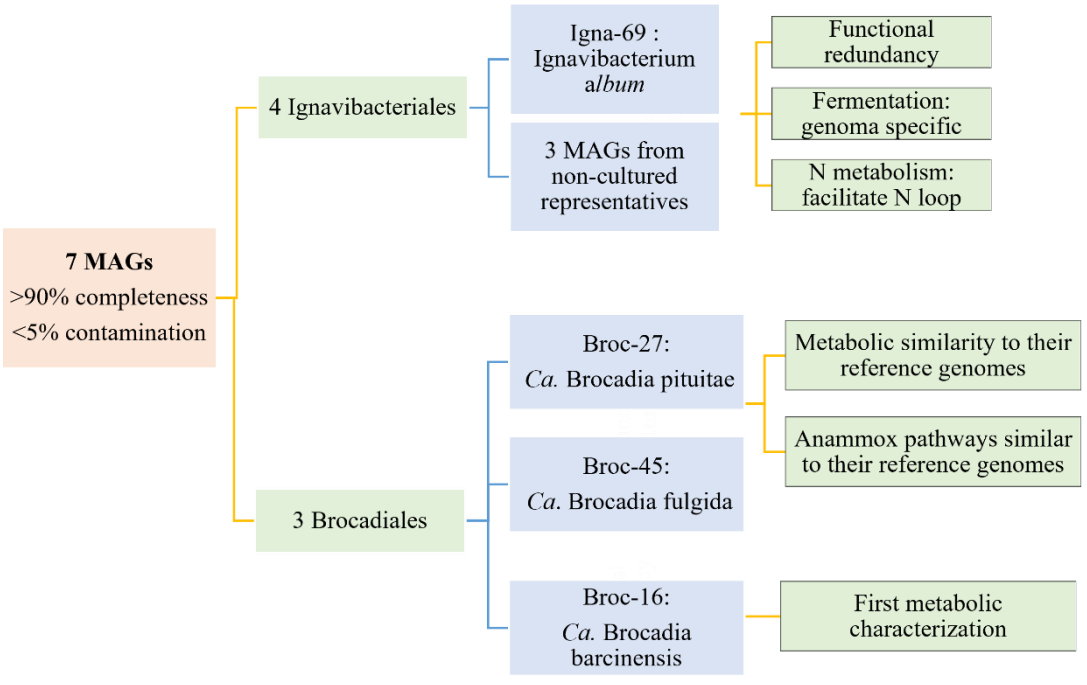
Goal 3: To analyze and set up the possible metabolic roles of the bacterial communities involved in the elimination of nitrogen in anammox reactors.

Problem: Further analysis is required to determine the metabolic potential of this communities to understand how they are interacting

Chapter 7. Metabolic potential of metagenome-assembled genomes (MAGs) retrieved from two anammox reactors working at different conditions



Main results:



Abstract

The genome-centric approach has been applied to obtain genomes from highly enriched anammox bioreactors. While most of these studies have focused on understanding the ecophysiology of anammox bacteria and their interactions with autotrophic nitrifying bacteria, recent studies have been focused also on the interactions with heterotrophic bacteria present in the anammox consortia, such as the phyla Chlorobi and Chloroflexi. However, there are still many gaps in determining the role of these groups among different anammox reactors.

Based on the possibility to reconstruct genomes (metagenome-assembled genomes, MAGs) of highly abundant taxa from a metagenomic sample, this work aimed to apply genome-centric metagenomics to obtain representative genomes to study the metabolic potential and interactions of microorganisms from the Brocadiales and Ignavibacteriales orders in two granular sludge anammox reactors, which were the most abundant groups detected through this thesis. One of the reactors was operated with synthetic influent for over six years. The other one was operated at mainstream conditions treating real wastewater from a partial nitritration system.

Three MAGs belonging to order Brocadiales (Broc-16, Broc-27, and Broc-45) and four MAGs belonging to the order Ignavibacteriales (Igna-33, Igna-21, Igna-39, and Igna-69) were obtained with over 90% completeness and less than 5% contamination. The phylogenomic analysis was performed based on concatenated alignments of 120 single-copy marker genes and compared with near-completed reference genomes. According to this, Broc-27 bunched with *Candidatus* (*Ca*.) Brocadia pituitae and Broc-45 with *Ca*. Brocadia fulgida. Broc-16 was not directly associated with any previously described species. Thus, a phylogenetic tree based on the 16S rRNA gene was constructed. Broc-16 bunched in the same clade of our recently proposed new species, *Ca*. Brocadia barcinensis, dominant in the anammox reactor feed with synthetic influent. From the potential metabolic analysis, the functional genes hydrazine synthase (hzs) and hydrazine dehydrogenase (hdh) were found, however, this MAG lacks nitrite reductase genes (nirK and nirS). Nevertheless, the hydroxylamine oxidoreductase gene (hzo) was found, which could be used as nitrite reductase. In addition, marker genes for dissimilatory nitrate reduction to ammonia (DNRA) were also found.

Regarding the Ignavibacteriales, only one MAG (Igna-69) clustered with a described species Ignavibacterium album JCM16511 genome while the others clustered with genomes from non-cultured representatives. Our results have shown functional redundancy in the potential metabolism of central carbohydrates, carbon fixation, and fatty acid metabolism of this group in anammox reactors. Instead, fermentation products and nitrogen metabolism seem to be a genome-specific features. Its role as protein degraders, fermentative lifestyle, and auxotrophic and cross-feeding interactions with anammox bacteria was confirmed. They could also facilitate a nitrite loop with anammox bacteria or support complete denitrification, enhancing nitrogen removal performance.

This metagenomic study allowed us to expand the knowledge about diversity, metabolic potential, and to understand interactions between the studied groups and is a fresh start for further investigations in search of strategies for improving anammox reactors' performance at mainstream conditions.

7.1. Introduction

Metagenomics is commonly used nowadays to describe microbial communities in different systems without the biases inherent to PCR amplification of a single gene (Poretsky et al., 2014). Whole-genome shotgun sequencing (WGS) metagenomics aims to sequence all genomes existing in an environmental sample to analyze the biodiversity and the functional capabilities of the microbial community studied (Pérez-Cobas et al., 2020). This technique also allows the assembly of full genomes from metagenome data (genome-centric approach) to gain insights into the genomic diversity of microbial ecosystems and obtain draft genomes of uncultured organisms (Pérez-Cobas et al., 2020).

In anammox communities, the complex microbial structures, life strategies, and interactions between taxa are crucial for stable removal of nitrogen from wastewater. Therefore, a better understanding of this community will provide new insights into this process and will support its further optimization (Wang et al., 2019) a crucial step for anammox implemetation at mainstream conditions.

The genome-centric approach has been applied to obtain genomes from highly enriched anammox bacterial bioreactors using high throughput sequencing technologies (Park et al., 2017). Unfortunately, few complete anammox bacterial genomes are currently available from biological systems studies (Okubo et al., 2021; Strous et al., 2006). However, near-complete draft genomes are commonly obtained (Mardanov et al., 2019; Oshiki et al., 2016; Speth et al., 2016). Furthermore, better insights into anammox evolution and its versatile metabolism have been obtained using it (Wang et al., 2019).

The first genome of an anammox bacterium came from an enrichment culture of *Candidatus (Ca.)* Kuenenia stuttgartiensis in 2006 (Strous et al., 2006). In silico analysis of this genome assembly led to the postulation of a minimal set of three redox reactions essential for anammox catabolism (Kartal et al., 2011; Strous et al., 2006). According to this (Fig. 7.1), the first reaction is the reduction of nitrite to nitric oxide by a cd1 nitrite reductase (nirS) or by a copper-containing nitrite reductase (nirK) (Hu et al., 2012; Okubo et al., 2021), which is followed by the conversion of equimolar amounts of ammonium and nitric oxide into hydrazine catalyzed by hydrazine synthase (hzs), and finally, the oxidation of hydrazine into dinitrogen gas by a hydrazine oxidoreductase (hzo) or hydrazine dehydrogenase (hdh) (de Almeida et al., 2011; Van de Vossenberg et al., 2013).

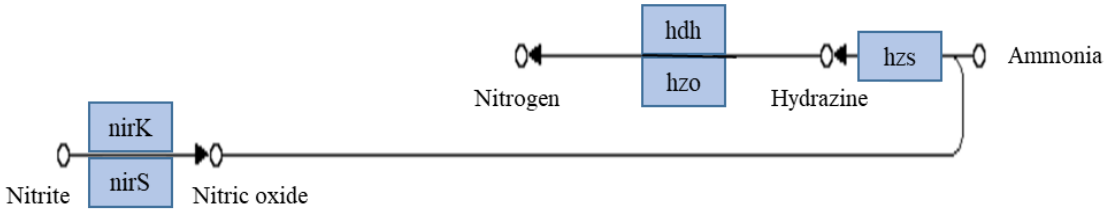


Figure 7.1. Reference metabolic anammox pathway. Modified from KEGG pathway maps (Kanehisa et al., 2016).

Okubo et al. (2021) pointed out that anammox bacteria do not necessarily possess the genes encoding the enzymes that catalyze the first reaction. Interestingly, the nirK gene has been detected in the draft genome of *Ca*. Brocadia caroliniensis (Park et al., 2017), but neither nirK nor nirS genes have been detected in complete *Ca*. Brocadia pituitae and nearly complete *Ca*. Brocadia sinica genomes. Instead, they detected a core genome structure involving hydroxylamine oxidoreductase (hao)-like proteins, which are thought to be the most likely candidate enzymes for the elusive nitrite reductases producing nitrous oxide in anammox bacteria (Kartal and Keltjens, 2016; Okubo et al., 2021)

Most studies have focused on understanding the ecophysiology of anammox bacteria (Ali et al., 2015; Kartal et al., 2013; Strous et al., 2006) and their interactions with autotrophic nitrifying bacteria. However, nowadays, it is clear that other microbial communities flank anammox bacteria. Thus, Lawson et al. (2017) studied the activity of heterotrophic bacteria in anammox bioreactors, finding that Chlorobi-affiliated bacteria may be highly active protein degraders while recycling nitrate to nitrite. Furthermore, other heterotrophs as Chloroflexi may also contribute to the scavenging of detritus and peptides produced by anammox bacteria. Later, Zhao et al. (2018), pointed out that the members of the anammox consortia (including microorganisms belonging to the phylum Chloroflexi, Proteobacteria, and the orders Ignavibacteriales and Brocadiales might share symbiotic relationships, as such growth factor supply or anammox metabolite scavenging.

In chapter 6, we described changes in the structure of the active community of an anammox reactor working at mainstream conditions during the decrease of its operating temperature. The significant contribution to community stratification caused by temperature effect was performed by the classes Ignavibacteria and Latescibacteria at 20ºC, while at 10ºC was performed by two classes: Anaerolinea and JG30-KF-CM66.

belonging to the phylum Chloroflexi. In addition, the class Brocadiae contributed to the stratification because of temperature and also because the bed sections along the reactor height. It draws attention that despite this phenomenon of symbiotic interactions among the anammox consortia has been observed for a long time, the potential roles of these bacteria in the consortia are still elusive (Zhao et al., 2018). Besides, in the same Chapter 6, it was detected increased activity of *Ca*. Brocadia barcinensis (new anammox *Candidatus* species we propose, see Chapter 4) in the middle section of the reactor at a lower temperature. Thus, its unknown metabolism could be revealed through metagenomics tools as a first step to further understand the niche differentiation ability of this new specie.

Considering that it is possible to reconstruct genomes (metagenome-assembled genomes, MAGs) of highly abundant taxa from a metagenomic sample (Jünemann et al., 2017), this molecular approach appears like an excellent method to obtain a metabolic reconstruction of MAGs belonging to the more abundant classes detected through this thesis.

In this sense, Bovio-Winkler (2021) performed a metagenomic analysis of members of Chloroflexi phylum from different wastewater treatment systems, including activated sludge, methanogenic, and anammox reactors. They obtained a total of 17 MAGs, being 9 of them from the anammox reactors (same sampled through this thesis). Comparing the metabolic potential of these 17 MAGs, they found a functional redundancy of the Chloroflexi members among the systems, including: 1) anaerobic respiration with a versatile metabolism related to the hydrolysis and fermentation of complex and simple organic compounds, 2) a crucial structural role in flocs and granules in all reactors, and 3) an important role in nitrogen cycle in anammox reactors, performing dissimilatory nitrate reduction to ammonium (DNRA) and/or partial denitrification, thus enhancing overall nitrogen removal performance in these bioreactors.

Based on the above exposed ideas. this work aimed to apply genome-centric metagenomics to obtain representative genomes of two granular sludge anammox reactors to study the metabolic potential of microorganisms from the Brocadiales and Ignavibacteriales orders. The first studied reactor was operated with synthetic influent for over six years (Isanta et al., 2015; Reino et al., 2018). The second reactor was operated at mainstream conditions treating real wastewater from a partial nitritation process feed with municipal wastewater (MWW) (Juan-Díaz et al., 2020). This second reactor was

inoculated with the biomass from the first reactor. We hypothesized that a near-complete draft genome of our suggested new anammox *Candidatu*s species (*Ca*. Brocadia barcinensis) could be obtained revealing its metabolic potential. In addition, recovering genomes of Ignavibacteriales would reveal interesting interactions between them especially regarding nitrogen metabolism.

7.2. Material and Methods

7.2.1. Description of the anammox reactors

Samples of anammox granular biomass were obtained from two different reactors. The first reactor was a sequencing batch reactor (SBR) operating under stable conditions with an enriched anammox culture for more than 6 years. This reactor was fed with synthetic wastewater at 35ºC. Samples taken from SBR reactor were labelled as "AM". The second reactor was a lab-scale Up-flow Anammox Sludge Blanked (UAnSB) operated at mainstream conditions treating real wastewater from a partial nitritation process feed with municipal WW. This second reactor was inoculated with the biomass from the first reactor. Samples from UAnSB reactor were taken from three sample points at different heights along the bed height during a stable operation period of 81 days working at 20 ºC (Juan-Díaz et al., 2020). Samples of the bottom, middle and top of the reactor were labelled as S1, S3, and S5, respectively. Details about feeding and performance are described in Section 3.1 of Chapter 3 for both reactors. For the purpose of referring to each reactor in this chapter, they will be indicated according to the influent used, that is, synthetic (AM sample, SBR reactor) or mainstream influent (S1-S3-S5 samples, UAnSB reactor). Samples from the biomass (2 mL) were taken and stored at -20ºC until DNA extraction.

7.2.2. DNA extraction and metagenomic sequencing

Biomass samples were thaw, and the biomass were separated by centrifugation (5,000 x g, 5 min) and up to 250 mg of wet pellet were used for the Soil DNA Isolation Plus Kit (Norgen Biotek) according to the manufacturer's instructions. DNA quantity and integrity was checked with a ND-1000 Nanodrop spectrophotometer (Thermo Scientific) and stored at -80 °C until further use. The four metagenomes (AM, S1, S3 and S5) were shotgun sequenced by Illumina Novaseq6000 sequencer (Macrogen, Seoul, South Korea)

using Library Kit TruSeq Nano DNA Kit (350bp). The yield was approximately 50 Gb (~5Gb/sample) of raw short-read sequences per library (2 × 100 bp, paired-end).

7.2.3. Genome assembly, binning, and genome annotation

Raw data was processed using the following tools: FastQC (v0.11.9) and Trimmomatic (V0.36) for assessing quality and filtering low quality reads; MEGAHIT (v1.2.3-beta) for metagenome assembly. Then, reads for each metagenome were mapped to the co-assembly of short reads using Bowtie2 (v2.3.4.1). MetaBAT 2 (v2.12.1) was used for contig binning. The completeness levels and contamination of the bins were assessed using CheckM (v1.0.13). Bins classified into the interest taxonomic groups (Brocadiales and Ignavibacteriales order) were curated using different tools to obtain the best conditions of completeness/contamination. More details of the bin curations were described in section 3.3.4 of chapter 3.

7.2.4.- Phylogenomic analysis

We performed taxonomic annotation on recovered genomes sequences using Genome Taxonomy Database GTDB-Tk (version v1.3.0 and GTDB-Tk reference data version r95) (Parks et al., 2018).

The phylogenomic analysis was performed based on concatenated alignments of 120 single-copy marker genes using the genome taxonomy database (GTDB) and compared with 24 and 22 near-completed reference genomes for Brocadiales and Ignavibacteriales order, respectively.

Phylogenomic and phylogenetic trees were performed using MEGA7 software (Kumar et al., 2016) and the resultant phylogenetic trees were visualized in iTOL (Letunic and Bork, 2019).

7.2.5. Metabolic potential of MAGs

Protein coding sequences (CDS) were determined with Prokka (1.14.5) for each MAG of interest (Seemann, 2014). These were annotated with Prokka and KOALA (KEGG Orthology And Links Annotation) for K number assignment of KEGG Genes (Kanehisa et al., 2016), and the potential metabolic pathways were analyzed.

7.3. Results and discussion

7.3.1. General genomic features of metagenomic assembled genomes

Differential coverage binning (Mads Albertsen et al., 2013) was used to obtain metagenomic assembled genomes (MAGs) from the four samples. As a result, a total of 28 assembled genomes were retrieved with >90% completeness and <5% contamination, thus meting the recently proposed criteria by Bowers et al. (2017) for the high-quality metagenome-assembled genomes (> 90% completeness with < 5% contamination and the presence of rRNA genes). According to the taxonomic classification with the genome taxonomy database (GTDB), selected MAGs of interest belonging to the orders Brocadiales and Ignavibacteriales were used for further analysis and statistic overview of them is shown in Table 7.1. Thus, nine of them, which belong to the phylum Chloroflexi were studied in a parallel work by Bovio-Winkler (2021).

Three MAGs which belong to the order Brocadiales were obtained, with coverages ranging 36X to 211X, and four MAGs belonging to the order Ignavibacteriales were obtained, with coverages ranging 18X to 1,223X. These coverages reflect the abundances of the underlying organisms across samples (Alneberg et al., 2014). The formers were labeled as “Broc” followed by the bin number: Broc-27, Broc-16, and Broc-45, and those of the second group were labeled as “Igna” followed by the bin number: Igna-69, Igna-21, Igna-39, and Igna-33.

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Table 7.1. Statistics overview of MAGs of interest. MAGs belonging to order Ignavibacteriales are shown in green, and those belonging to order Brocadiales are shown in orange. CDS: Protein coding sequences; rRNA: ribosomal RNA; tRNA: transfer RNA

MAG	Genome size (bp)	CDS	rRNA (16S/23S/5S)[a]	tRNA[a]	Completeness[b]	Contamination[b]	Contigs	N50 (bp)	Coverage	%GC	Taxonomic annotation[c]
Igna-69	3,536,683	3015	2	40	99.44	0	41	127222	18.7	33.9	Ignavibacteriales
Broc-27	3,944,836	3457	1	37	98.9	2.75	203	48630	36.4	43.4	Brocadiales
Igna-21	3,929,532	3381	1	39	98.32	0	183	73890	122.0	34.8	Ignavibacteriales
Broc-16	3,697,033	3253	1	42	96.7	1.65	141	56838	136.1	44.1	Brocadiales
Igna-39	3,464,169	2884	2	42	96.64	0	58	223121	210.9	33.3	Ignavibacteriales
Igna-33	2,442,297	2101	2	41	95.08	0	38	211136	1223.8	37.6	Ignavibacteriales
Broc-45	3,124,986	2806	2	44	93.41	2.75	156	28581	211.4	45.1	Brocadiales

[a] Predicted using Prokka. The rRNA count refers to the occurrence of complete rRNA operons (5S, 16S, 23S).

[b] Genome quality estimates from CheckM.

[c] Taxonomic assignments from GTDB-Tk at order level.

7.3.2. Phylogenomics of Brocadiales and Ignavibacteriales MAGs

7.3.2.1. Phylogenomic analysis of Brocadiales MAGs

A complete phylogenomic tree based on concatenated alignments of 120 single-copy marker genes was constructed using 24 near-complete reference genomes retrieved from GTDB (May 2021) and the three Brocadiales genomes obtained in this work (Fig. 7.2).

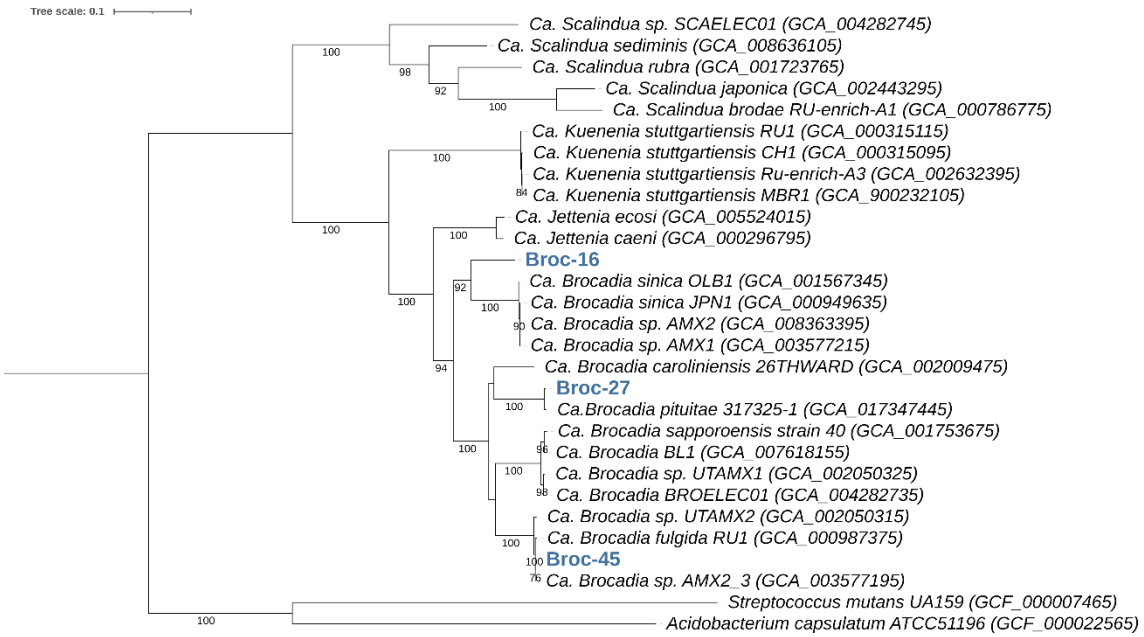

Figure 7.2. Phylogenomic analysis of obtained Brocadiales MAGs. The tree was reconstructed using the Maximum Likehood method and the JTT model. Bootstrap values greater than or equal to 70% are shown at each node. NCBI accession numbers of reference genomes are shown in parenthesis. MAGs of this works are shown in blue. Sequences from *Acidobacterium capsulatum* ATCC 51196 and *Strepococcus mutans* UA159 were used as outgroup. Bar, 0.01 substitutions per site.

According to this, Broc-27 bunched with *Candidatus* (*Ca*.) Brocadia pituitae and Broc-45 with *Ca*. Brocadia fulgida. Instead, Broc-16 was positioned close to *Ca*. Brocadia sinica clade, but it was not directly associated with any previously described species. Thus, the 16S rRNA gene of this MAG was found and used to construct a phylogenetic tree to elucidate if we have assembled a genome of a new *Candidatus* anammox species.

The phylogenetic tree based on the 16S rRNA gene (Fig. 7.3) showed that Broc-16 bunched in the same clade that ASVs retrieved from amplicon sequencing presented in Chapter 4. Furthermore, these ASVs showed 100% abundance in the anammox

enriched culture reactor (AM) and 15% in the mainstream anammox reactor. More interestingly, according to the 16S rRNA gene analysis we have proposed that correspond to a new anammox *Candidatus* named *Ca.* Brocadia barcinensis (see Chapter 4, section 4.2.3). Also, using the defined average nucleotide identity (ANI) species cutoff of 95% (Konstantinidis and Tiedje, 2005), only Broc-16 genome represent a novel species with no cultured representatives or Candidatus described genome (Table 7.2). Thus, this is the first draft genome of this new anammox *Candidatus* species.

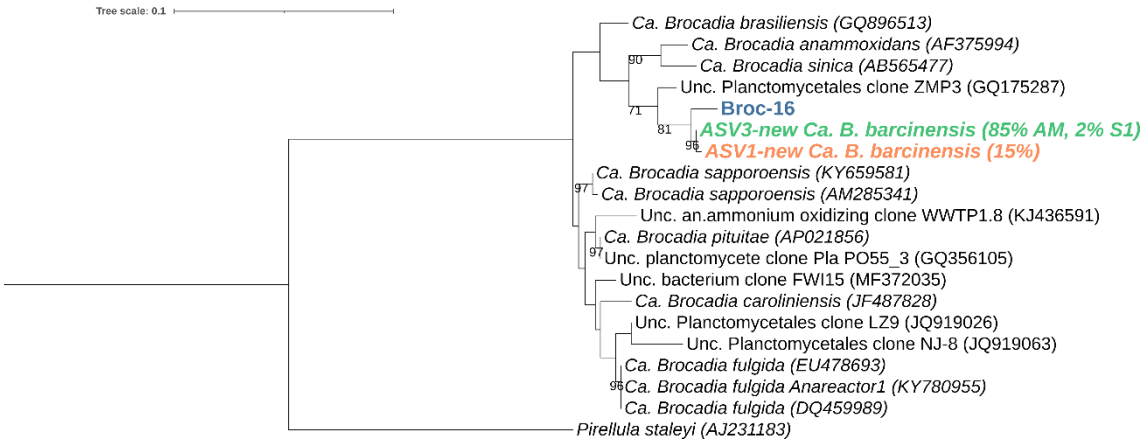

Figure 7.3. Phylogenetic Maximum Likelihood (ML) tree based on 16S rRNA gene sequences from *Candidatus* Brocadia genus. The tree was reconstructed using ASVs retrieved from a previous 16SrRNA gene amplicon sequencing of the samples. Relative abundances of ASVs and accession numbers of the reference sequences are shown in parentheses. Bootstrap values (greater than or equal to 70%) are shown at branch nodes. 16S rRNA gene sequence from *Pirellula staleyi* ATCC 35122 was used as outgroup. Bar indicates 0.01 substitutions per site.

Table 7.2. ANI values between Brocadiales MAGs and between reference genomes from the order Brocadiales. The highest ANI values obtained between genomes are shown. The reference genomes were annotated for subsequent metabolic comparisons with the MAGs.

MAGs	Most related genomes	ANI (%)
Broc-16	*Ca.* Brocadia sinica GCA_001567345	82.5
	Ca. Brocadia sp. AMX1 GCA_003577215	82.4
Broc-27	*Ca.* Brocadia pituitae GCA_017347445	98.4
	Ca. Brocadia caroliniensis GCA_002009475	83.8
Broc-45	*Ca.* Brocadia sp. AMX2_3 GCA_003577195	99.7
	Ca. Brocadia fulgida RU1 GCA_000987375	99.5

7.3.2.2. Phylogenomic analysis of Ignavibacteriales MAGs

Using the same previous strategy as for Brocadiales (concatenated alignments of 120 single-copy marker genes), a phylogenomic tree was constructed using 22 near-complete reference genomes retrieved from GTDB (May 2021) and the four Ignavibacteriales genomes assembled in this work (Fig. 7.4). According to this, only one of the retrieved MAGs (Igna-69) was positioned in a clade close to the genome from a described species (*Ignavibacterium album*), the other three MAGs were positioned with genomes from non-cultured representatives. Nevertheless, as can be observed also with the ANI values (Table 7.3), Igna-33 and Igna-39 coincided with genomes assembled from metagenomes retrieved from anammox cultures (GCA_013285425 and GCA_002050165), while Igna-21 and Igna-69 genomes represent novel species with no cultured representatives These could be explained because, the vast majority of the Ignavibacteriales genomes have been analyzed as a part of a metagenomic community study (Lawson et al., 2017; Wang et al., 2019; Zhao et al., 2018) , which brig to the fore the lack of knowledge on the Ignavibacteriales community in these types of ecosystems. On the other hand, the classification of the phylum Chlorobi as a novel taxonomic group is not yet clear as it was performed based on phylogenetic analysis of the 16S rRNA gene sequence, but the phylum shares a common root with the phylum Bacteroidetes. Thus, the observation of close phylogenetic relationships between proteins encoded in the genomes (using concatenated marker gene phylogenetic studies) could provide a promising route to building a more detailed evolutionary model linking the Bacteroidetes and the Chlorobi (Hiras et al., 2016; Iino et al., 2010). Therefore, more studies based on the metabolic potential analysis of genomes are needed to clarify these taxonomic gaps. It is remarkable that nowadays, there are still differences on classification of the order Ignavibacteriales into the phylum Chlorobi or Bacteroidota, depending on the database.

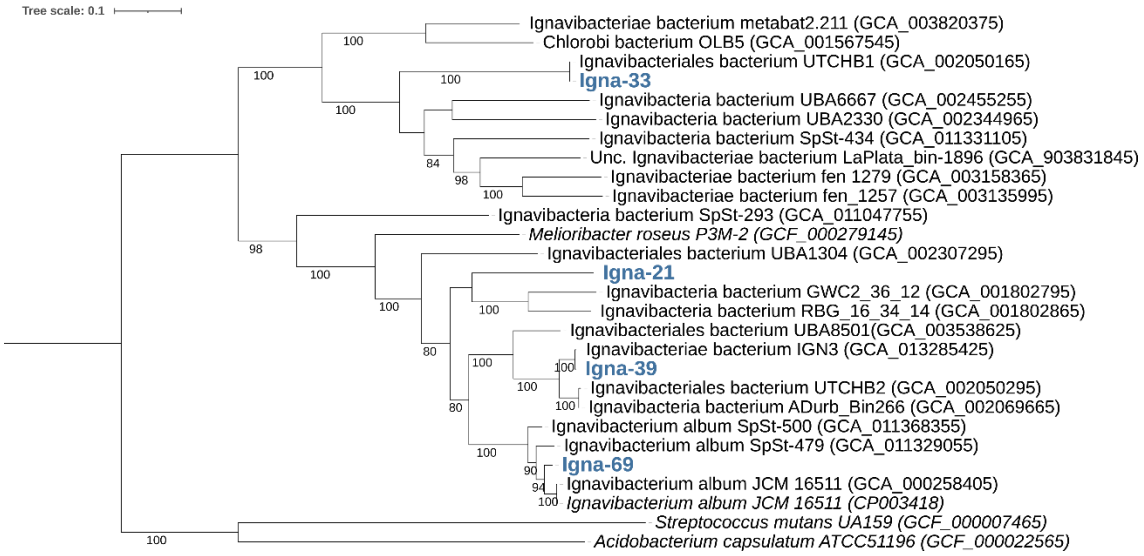

Figure 7.4. Phylogenomic analysis of obtained Ignavibacteriales MAGs. The tree was reconstructed using the Maximum Likehood method and the JTT model. Bootstrap values greater than or equal to 70% are shown at each node. NCBI accession numbers of reference genomes are shown in parenthesis. MAGs of this works are shown in blue. Sequences from *Acidobacterium capsulatum* ATCC 51196 and *Strepococcus mutans* UA159 were used as outgroup. Bar indicates 0.01 substitutions per site.

Table 7.3. ANI values between Ignavibacteriales MAGs and between reference genomes of the order Ignavibacteriales. The highest ANI values obtained between genomes are shown. The reference genomes were annotated for subsequent metabolic comparisons with the MAGs.

MAGs	Most related genomes	ANI (%)
Igna-39	Ignavibacteriae bacterium IGN3 GCA_013285425	99.0
	Ignavibacteria bacterium Adurb Bin266 GCA_002069665.1	82.9
Igna-33	Ignavibacteriales bacterium UTCHB1 GCA_002050165	99.9
	Igna-69	76.8
Igna-21	Ignavibacteriales bacterium UTCHB2 GCA_002050295	78.5
	Ignavibacteriae bacterium IGN3 GCA_013285425	76.6
Igna-69	Ignavibacterium album JCM16511 CP003418	89.1
	Ignavibacterium album JCM16511 GCA_000258405	89.1

7.3.3. General metabolic pathways and genes according to the MAGs

To infer the Brocadiales MAGs metabolic potential in our work, we annotated genes within each of the MAGs using different tools. We combined the annotation information-based to obtain more reliable identification of the metabolic pathways, as suggested by Bengtsson-Palme (2018). In addition, reference genomes of *Ca*. Brocadia pituitae, *Ca*. Brocadia fulgida, and *Ca*. Brocadia sinica, which bunched or were closely related with our assembled genomes, were included to overcome the lack of annotation of some genes that could be important for the metabolic pathways due to the completeness reached of the MAGs between 93% and 99%. The metabolic potential of the Ignavibacteriales MAGs of the present study was also analyzed. Nevertheless, these were compared between them considering the lack of reference species metabolically characterized of this group. All these results are presented in Fig. 7.4. In the following lines, each metabolic potential will be analyzed.

- **Respiration**

All MAGs showed a strict anaerobic metabolism due to the incompleteness of at least one of the following pathways: tri-carboxylic acid (TCA) cycle, NADH:quinone oxidoreductase, cytochrome c oxidase, and F-type ATPase (incomplete respiration machinery). Figure 7.5 showed that all MAGs have a complete set of genes for the ATPase system. Also, the Brocadiales MAGs presented one missing block in the bc1 respiratory unit and the absence of cytochrome c oxidase. In contrast, Kartal et al. (2012) described the presence of a quinol-cytochrome c oxidoreductase (bc1, complex III) and an ATPase complex, suggesting a chemiosmotic mechanism by which the energy derived from the anammox reaction is conserved as ATP. Instead, in the case of Ignavibacteriales MAGS, they showed a complete module of cytochrome c oxidase and the absence of bc1 complex. In addition, different genes for oxidative stress protection were found, such as catalase, superoxide dismutase, and peroxidases. This last was present in all MAGs and reference genomes and the unique stress-protective enzyme found in Broc-16.

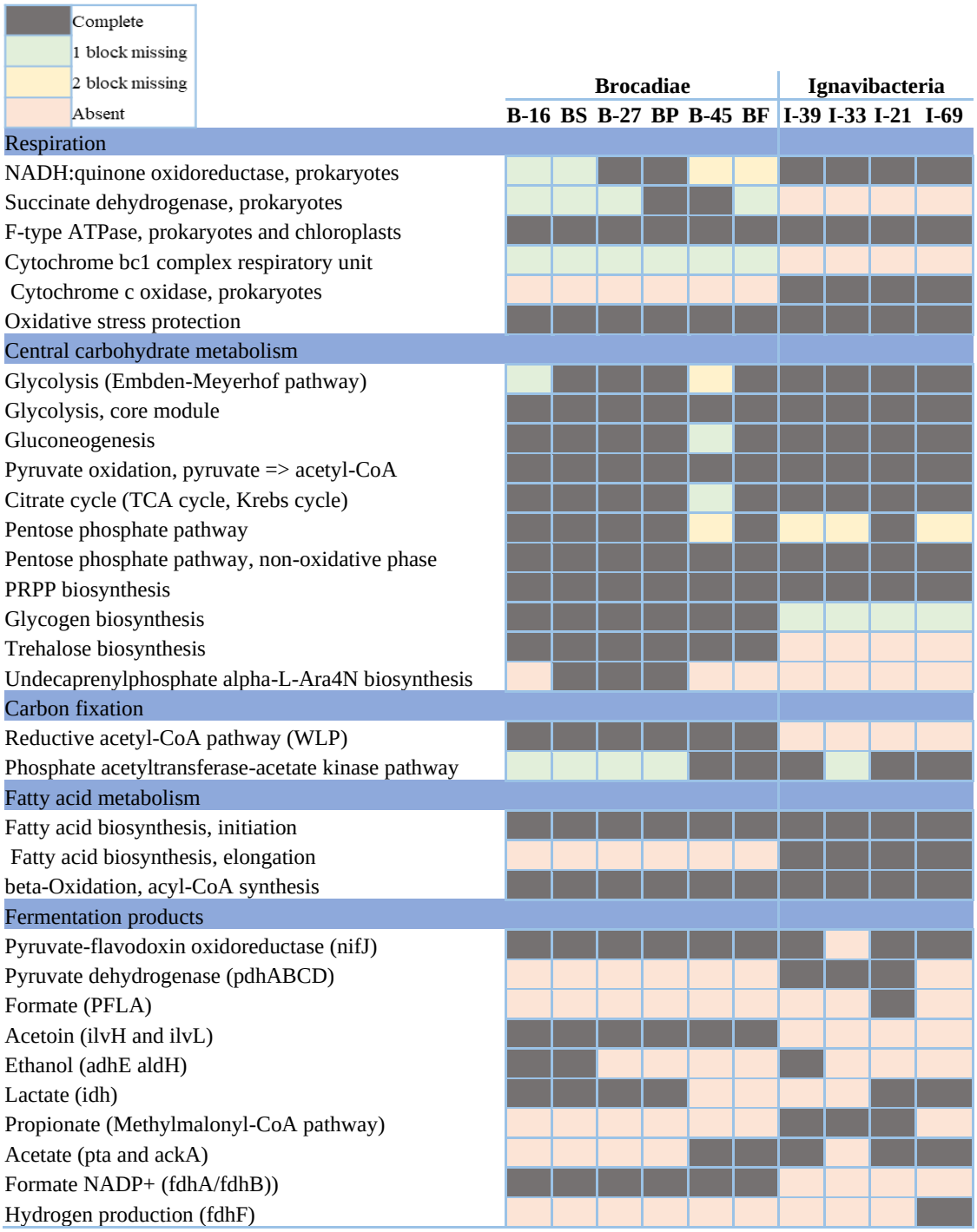

Figure 7.5. Heatmap showing the completeness and the incompleteness of each metabolic pathway for the recovered MAGs and reference genomes. B-: Broc-; I-: Igna-; BS: *Ca.* Brocadia sinica; BP: *Ca.* Brocadia pituitae; BF: *Ca.* Brocadia fulgida. Pathways with more than 2 blocks missing were considered as absent.

- **Central carbon metabolism**

In the central carbohydrate metabolism of Brocadiales (Fig.7.5), it was observed some missing blocks of the Embden-Meyerhof pathways of glycolysis in the MAGs Broc-16 and Broc-45. The incompleteness of the MAGs could explain this difference with the other genomes because all the downstream key enzymes were present. The absence of the module for undecaprenyl phosphate alpha-L-Ara4N biosynthesis (involved in amino and nucleotide sugar metabolism) was observed in MAGs Broc-16, Broc-45, and *Ca*. Brocadia fulgida. This module has been found in Brocadia, even though this is a γ-proteobacteria-specific rare module (Okubo et al., 2021).

Since there was no noticeable difference in the pathways for central carbohydrate, carbon fixation, and fatty acid metabolism, it could be inferred that there is no difference in the basic anabolic potential of the recovered Brocadiales MAGs and their closer phylogenetically anammox bacteria. The same was observed between Ignavibacteriales MAGs, also suggesting a functional redundancy in anammox reactors. Therefore, the analysis of the metabolic potential was focused on determining the possible interaction of both groups due to the differential comparison of complete or near-complete pathways.

According to this, Ignavibacteriales MAGs showed one block missing in the glycogen biosynthesis module and the absence of trehalose biosynthesis in all Ignavibacteriales MAGs, which instead were complete in Brocadiales MAGs. Trehalose is a gel-forming exopolysaccharide that helps maintain protein integrity (Barr et al., 2016), which is why its biosynthesis has been related to overcoming high-stress conditions in bacteria, as heat, osmotic stress, starvation, among others (Barr et al., 2016; Ruhal et al., 2013). Moreover, it has been reported that *Ca*. Brocadia sinica utilized glycogen as an energy and carbon source for trehalose biosynthesis under starvation conditions (Okabe et al., 2021).

- **Carbon fixation**

CO_2 fixation is a metabolic pathway, where CO_2 is assimilated to acetyl-coenzyme (Acetyl-CoA) through the reductive Acetyl-CoA pathway (Strous et al., 2006), also known as Wood–Ljungdahl pathway (WLP) (Ali et al., 2020). To complete this pathway, acetyl-CoA is converted to acetate by the phosphate acetyltransferase-acetate kinase pathway, which is a critical energy conservation step in the WLP (Smith et al., 2019). The complete WLP was observed (Fig. 7.5) in two Brocadiales MAGs (Broc-45

and *Ca*. Brocadia fulgida). In contrast, it was near-complete in the others, suggesting that this pathway is used primarily for carbon fixation rather than energy generation in this community, as described by Smith et al. (2019). The presence of genes for carbon fixation using the WLP has been described before as a core metabolic pathway in anammox bacteria (Kartal et al., 2013; Strous et al., 2006). In the Ignavibacteriales MAGs, the reductive acetyl-CoA pathway was absent, while three MAGs (Igna-39, Igna-21, and Igna-69) showed the complete phosphate acetyltransferase-acetate kinase (pta-ak) pathway. The presence of this pathway was also found in a Chlorobi genome (usually referred to as CHB as the abbreviation of Chlorobi) by Lawson et al. (2017), which suggested that the reactions encoded by this microorganism are used for acetate consumption rather than production, which can be explained due to the reversible function of this pathway, meaning that they could also synthesize acetyl-CoA from acetate or to generate ATP from excess acetyl-CoA (Ingram-Smith et al., 2006). Based on the results, the presence of the key enzymes for CO_2 fixation via WLP in Brocadiales and via pta-ak pathways would reveal a mixotrophic growth, considering the lack or low concentration of organic carbon in the influent of the anammox reactor in this study (0 mg COD L^{-1} and 60 ± 14 mg COD L^{-1} in the synthetic and mainstream influents, respectively). This assumption agrees with the results obtained by Bovio-Winkler (2021) in Chloroflexi genomes, where genes encoding for the key enzymes required for CO_2 fixation via WLP were found.

- **Fatty acid metabolism**

Fatty acid synthesis in bacteria and plants usually occurs via type II fatty acid synthesis (FASII) to generate the fatty acid components of phospholipids. The first step reaction is performed by the acetyl-CoA carboxylase complex (ACC). A crucial enzyme in this pathway is the acyl carrier protein (ACP), which transports lipid intermediates between different enzymes in the FASII pathway. The resulting malonyl-CoA is used to prime the elongation module where four enzymatic reactions catalyze the growth of the lipid chain by two carbons per pathway cycle, with consecutive reduction, dehydration, reduction, and condensation reactions by the Fatty acid biosynthesis (Fab) enzymes (Parsons and Rock, 2011; Rattray et al., 2009). Figure 7.5 showed that Brocadiales MAGs had a complete fatty acid biosynthesis initiation cycle, while elongation was absent. However, it was found the beta-oxidation pathway (synthesis or degradation of acyl-CoA). This could imply that anammox bacteria could produce a short-chain fatty acid,

and long chains could be obtained by acyl-CoA ligase or being provided by other microorganisms, considering that some bacterium can incorporate extracellular fatty acids (Parsons and Rock, 2011).

In addition, they could be able to use different metabolic pathways. This last could be in line with the metabolic versatility of anammox bacteria. For example, ladderane lipids are the most abundant membrane lipids of anammox bacteria, containing unique linearly concatenated cyclobutane (3 or 5 rings) (Sinninghe Damsté et al., 2002). The biosynthesis of these lipids has been deeply studied. The first approaches were performed by Strous et al. (2006), suggesting that this is synthesized by combining known fatty acid biosynthesis genes of the FASII pathways and S-adenosylmethionine (SAM) radical enzyme genes. Nevertheless, the latest studies have shown that ladderane lipids are not biosynthesized via the known pathways of fatty acid biosynthesis and that the ladderane biosynthetic pathway remains unknown (Javidpour et al., 2016; Rattray et al., 2009).

Ignavibacteriales MAGs showed both complete cycles (initiation and elongation) and beta-oxidation, suggesting that it might be an alternative pathway to obtain energy for these microorganisms or to supply organic compounds for the metabolism of anammox consortia. Although, few studies have been analyzed the metabolic potential of Ignavibacteriales MAGs in the anammox core microbiome, fatty acid metabolism has not been mentioned (Huo et al., 2020; Lawson et al., 2017; Zhao et al., 2018).

- **Fermentation**

The potential ability to obtain energy from fermentation by-products from pyruvate and acetyl-CoA was studied, including acetate, ethanol, lactate, acetoin, formate, and/or propionate (via methylmalonyl-CoA). Figure 7.5 showed that genes encoding pyruvate oxidation were annotated in all recovered MAGs, such as some of the enzymes nifJ, pdhABCD, and PFLA.

The potential for the synthesis of formate and acetoin was observed only in Brocadiales MAGs. It draws the attention that only Brocadiales encoded the acetoin production due to its reported physiological implications, including avoiding acidification, taking part in the regulation of NAD / NADH ratio, and storing carbon (Xiao and Xu, 2007). Therefore, it can play a role in protecting anammox bacteria against sudden changes in pH in the environment. The ability of individual species of bacteria belonging to the phyla Chloroflexi and Gammaproteobacteria to produce acetoin was

previously determined by (Yasuda et al., 2021) in an activated sludge microbial community treating leachate of a completed landfill.

However, other fermentation products observed varied between the recovered MAGs. As an example, *Ca.* Brocadia barcinensis (Broc-16) and *Ca*. Brocadia sinica showed the potential to produce more fermentation products than the rest of Brocadiales MAGs, including acetoin, lactate, ethanol, and formate. In this sense, it has been shown that anammox bacteria can perform cometabolism since they convert short-chain fatty acids (as propionate, acetate, and formate) to CO_2 as an additional energy source while nitrite is reduced to N_2 via ammonium. In this way, anammox bacteria can produce their own ammonium (and nitrite) to perform their "standard" catabolism (Güven et al., 2005; Kartal et al., 2008, 2007; van der Star, 2008).

Various recovered MAGs contained genes indicating the capability for fermentative production of ethanol via alcohol dehydrogenase (Broc-16, Broc 27 and Igna-39), lactate (Broc-16, Broc27, Igna-21, and Igna-69), propionate via methylmalonyl-CoA (Igna-39, Igna21, and Igna-33) and acetate (Broc-45, Igna-39, Igna-21, and Igna-69), although this last can also be produced by acetogenic CO_2 reduction through the Wood−Ljungdahl pathway using a range of inorganic and organic substrates (Dong et al., 2019). Also, only Igna-69 holds the gene (fdhF) that encodes for formate dehydrogenase H that facilitates the decomposition of formic acid to H_2 and CO_2 under anaerobic conditions in the absence of exogenous electron acceptors (Ali et al., 2020). Considering that this MAG was phylogenetically close to the Ignavibacterium album, the results agree with Liu et al. (2012), were pathways for the fermentative production of propionate, ethanol, and formate (via pyruvate formate lyase) were apparently incomplete or missing altogether.

- **Potential metabolic interactions between Brocadiales and Ignavibacteriales determined by auxotrophia and cross-feeding**

An important process that governs the growth and composition of microbial ecosystems is the exchange of essential metabolites (primary and secondary), known as metabolic cross-feeding, where the metabolites utilized by one type of microorganism are produced from other members of the community (Lawson et al., 2017; Mee et al., 2014; Zhao et al., 2018). According to this, the potential metabolic interaction between anammox bacteria and Ignavibacteriales was determined based on the ability to

synthesize metabolites, transport them, or degrade them (Fig. 7.6). On one side, it was observed that Brocadiales MAGs contain complete or near-complete pathways for the biosynthesis of a large number of amino acids. In contrast, there were absent in the Ignavibacteriales MAGs, except for some amino acid degradation pathways. These differences in the synthesize of amino acids and vitamins in microbes might drive the interspecies interactions (Ji et al., 2021). Lawson et al. (2017) observed a lack of hydrophobic amino acid synthesis in heterotrophic bacteria MAGs from Chlorobi and Chloroflexi in an anammox reactor. Nevertheless, we observed that only Ignavibacteriales MAGs contain genes encoding amino acid and peptide transporters, implying that Ignavibacteriales use amino acids as a carbon and energy source, in addition to direct assimilation into protein biosynthesis. These results agree with the proposed by Lawson et al. (2017). Also, more genes encoding extracellular peptidases families were found in these MAGS, similar to the metabolic cross-feeding proposed by Zhao et al. (2018), who established that Chlorobi microorganisms can degrade the extracellular proteins and peptides secreted by the anammox bacteria and use the resultant amino acids that they cannot biosynthesize. Regarding the metabolism of cofactors and vitamins, it was observed that anammox bacteria mainly synthesize these. Still, a major diversity and abundance of transporters were found in the Ignavibacteriales MAGs, thus showing an auxotrophic metabolism in agreement with Lawson et al. (2017).

Another important source of metabolite exchange is the breakdown of extracellular polymeric substances (EPS) produced by anammox and other bacteria, which has been suggested to be a primary source of organic carbon for heterotrophic bacterial growth in anammox bioreactors. (Ali et al., 2020; Lawson et al., 2017). Although EPS of anammox-enriched cultures have been studied at the chemical composition and structural levels, the key genetic pathway mechanism of the aggregation behavior of anammox microorganisms still is unclear. Therefore, different genes involved in the formation of EPS were searched, including genes for alginate metabolism and exopolysaccharide biosynthesis (M. Albertsen et al., 2013), biofilm formation and aggregation (Jia et al., 2021), and sticky macromolecular EPS (Zhao et al., 2018). The results showed that a major number of genes encoding alginate precursors and biofilm formation and aggregation genes were found in Brocadiales MAGs. The presence of genes involved in biofilm formation and aggregation agrees with the recently proposed pathway for anammox EPS by Jia et al. (2021), which is regulated by quinolone and

transport to the extracellular environment through a type II secretion system. The strong ability of c- di- GMP (bis- (3′- 5′)- cyclic dimeric guanosine monophosphate) synthesis enabled a stable structure of aggregation (Detailed annotated enzymes can be found in Annex 1, Table A.I.1). This pathway was not completely found (missing or absent) in the Ignavibacteriales MAGs.

Ca. Brocadia barcinensis (Broc-16) showed more genes encoding different glycosyltransferase enzymes for the biosynthesis of EPS. This is interesting regarding those different classes of EPS that can be distinguished based on their biosynthesis mechanisms and precursors (Sutherland, 1993). Thus, this genome could have the potential to synthesize a bigger range of EPS classes.

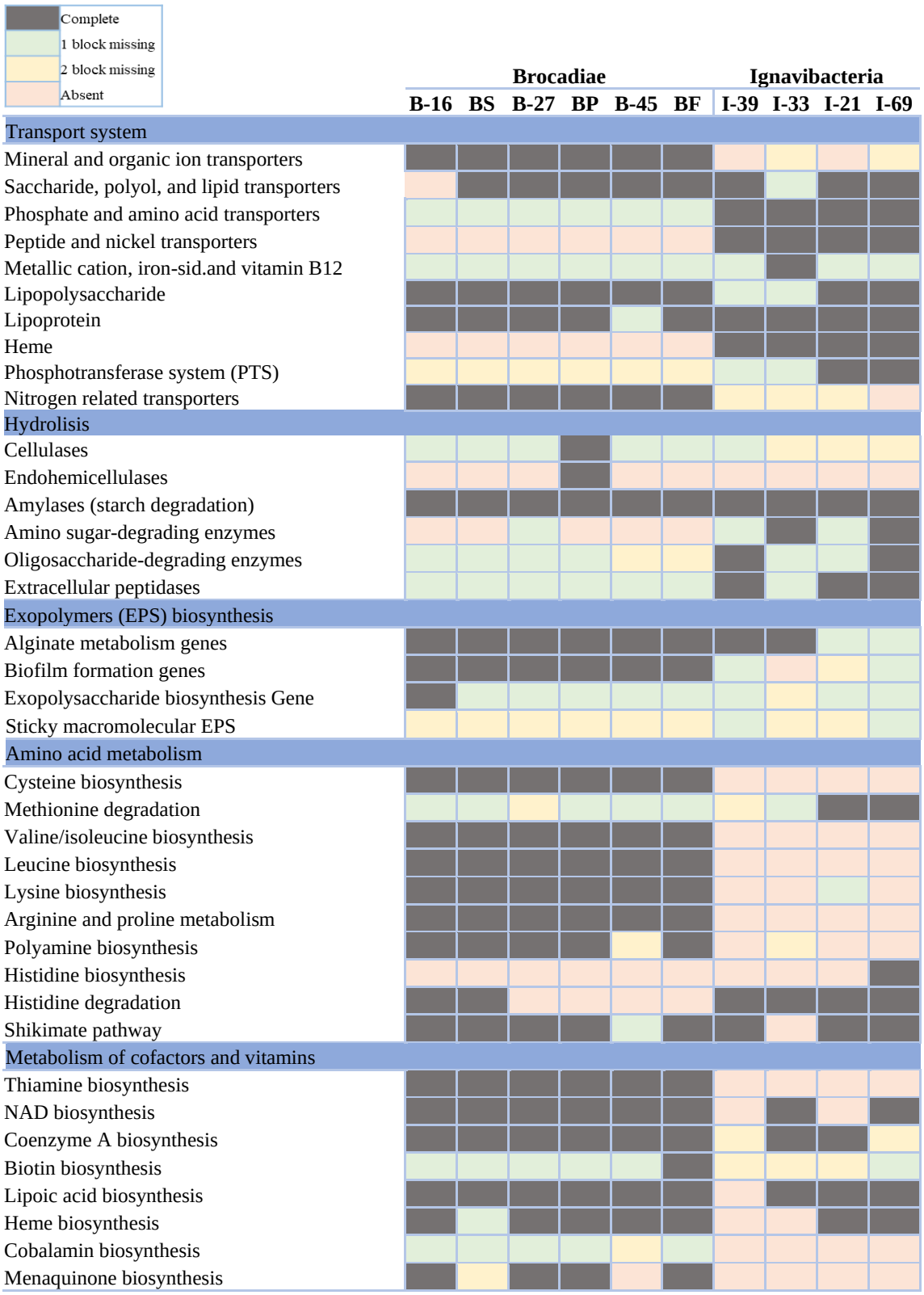

Figure 7.6. Heatmap showing the completeness and the incompleteness of each metabolic pathway for the recovered MAGS and reference genomes. B-: Broc-; I-: Igna-; BS: *Ca.* Brocadia sinica; BP: *Ca.* Brocadia pituitae; BF: *Ca.* Brocadia fulgida. Pathways with more than 2 blocks missing were considered as absent.

Different genes for EPS formation were observed in Ignavibacteriales; however, the pathways were less complete than in Brocadiales MAGs. For example, only two Ignavibacteriales MAGs (Igna-21 and Igna-69) encoded the enzyme (ldh) for the biosynthesis of lactate, which is secreted into the extracellular media to maintain acidity, a prerequisite for exopolysaccharide production (Silva et al., 2017; Zhao et al., 2018), instead it was found in Broc-16, Broc-21 and in the reference genomes of *Ca*. Brocadia sinica and *Ca*. Brocadia pituitae. Therefore, Ignavibacteriales could support cell aggregation and the subsequent formation of cores or carriers, but not the whole process. The same trend was observed by Bovio-Winkler (2021) in Anaerolineales MAGs (phylum Chloroflexi) retrieved from three different wastewater treatment bioreactors. Thus, it could be in line with the heterotrophic bacteria functions within the anammox consortia.

In addition, Lawson et al. (2017), suggested that hydrolysis of EPS into soluble compounds and/or the secretion of soluble microbial products by anammox bacteria during cell growth is believed to support the heterotrophic growth in anammox bioreactors that receive no external organic carbon substrates as in the case of the AM sample. This could be confirmed with the highest number of hydrolytic enzymes, including amylases, amino sugar-degrading, oligosaccharide-degrading, and extracellular peptidases found in the Ignavibacteriales MAGs.

- **Nitrogen metabolism**

Different interactions of anammox bacteria with other members of the anammox consortia have been studied in the last years to understand the physiological capabilities of each member (Ji et al., 2021; Lawson et al., 2017; Speth et al., 2016; Zhao et al., 2018). Until now, our results have shown functional redundancy in the potential metabolism of central carbohydrates, carbon fixation, and fatty acid metabolism of Ignavibacteriales in our anammox reactors, as well as studies (Lawson et al., 2017; Zhao et al., 2018) on heterotrophic bacteria from phyla Chloroflexi and Chlorobi in different anammox reactors, despite the wastewater feeding or reactor configuration(Lawson et al., 2017; Zhao et al., 2018).

As the importance of applying the anammox process is focused on the nitrogen removal performance, a recent metagenomic study revealed that most of the heterotrophic organisms in anammox granules encode the ability to respire nitrate via partial

denitrification. Thus, these denitrifiers might mitigate excess nitrate accumulation in the system, completing a nitrite loop with anammox and nitrite-oxidizing bacteria (NOB) by reducing nitrate back to nitrite (Lawson et al., 2017; Speth et al., 2016).

Figure 7.7 showed that two Ignavibacteriales MAGs (Igna-21 and Igna-29) have the potential to perform DNRA. However, all studied MAGs showed at least one gene involved in the conversion of nitrate to nitrite. Also, all Ignavibacteriales MAGs contained genes capable of reducing the nitrous oxide to nitrogen gas via nitrous oxide reductase (nosZ). Still, only one Ignavibacterial genome (Igna-69) encoded to a hydroxylamine oxide reductase enzyme. These functions could facilitate a nitrite loop with anammox bacteria or support complete denitrification, thus enhancing overall nitrogen removal performance in the bioreactor as also observed by Lawson et al. (2017) in Chlorobi genomes. Therefore, the contribution of this group within the nitrogen removal is undeniable, but it seems that the nitrogen-related genes encoded are a genome-specific feature.

In the case of anammox bacteria, due to its versatile metabolism, genes encoding enzymes of all pathways involved in the nitrogen metabolism were searched, and recovered MAGs were compared with the closest reference genomes from the phylogenomic analysis. The recovered MAGs Broc-27 and Broc-45 encoded the same genes as its related anammox species, *Ca*. Brocadia pituitae and *Ca*. Brocadia fulgida, respectively. However, Broc-16 showed differences with *Ca*. Brocadia sinica, which makes sense because phylogenomically, they belong to different clades, and we proposed it as the new *Candidatus* species: *Ca*. Brocadia barcinensis.

In general terms, all Brocadiales MAGs encoded the complete pathway to perform dissimilatory nitrate reduction to ammonia. This was described by Kartal et al. (2007) as another versatile trait of anammox bacteria metabolism. According to Keren et al. (2019), despite having the genes for this pathway, they are expected to use the anammox pathway for energy generation while using DNRA for detoxification by recycling excess potentially toxic nitrite back to ammonium, which could then participate in the anammox reactions.

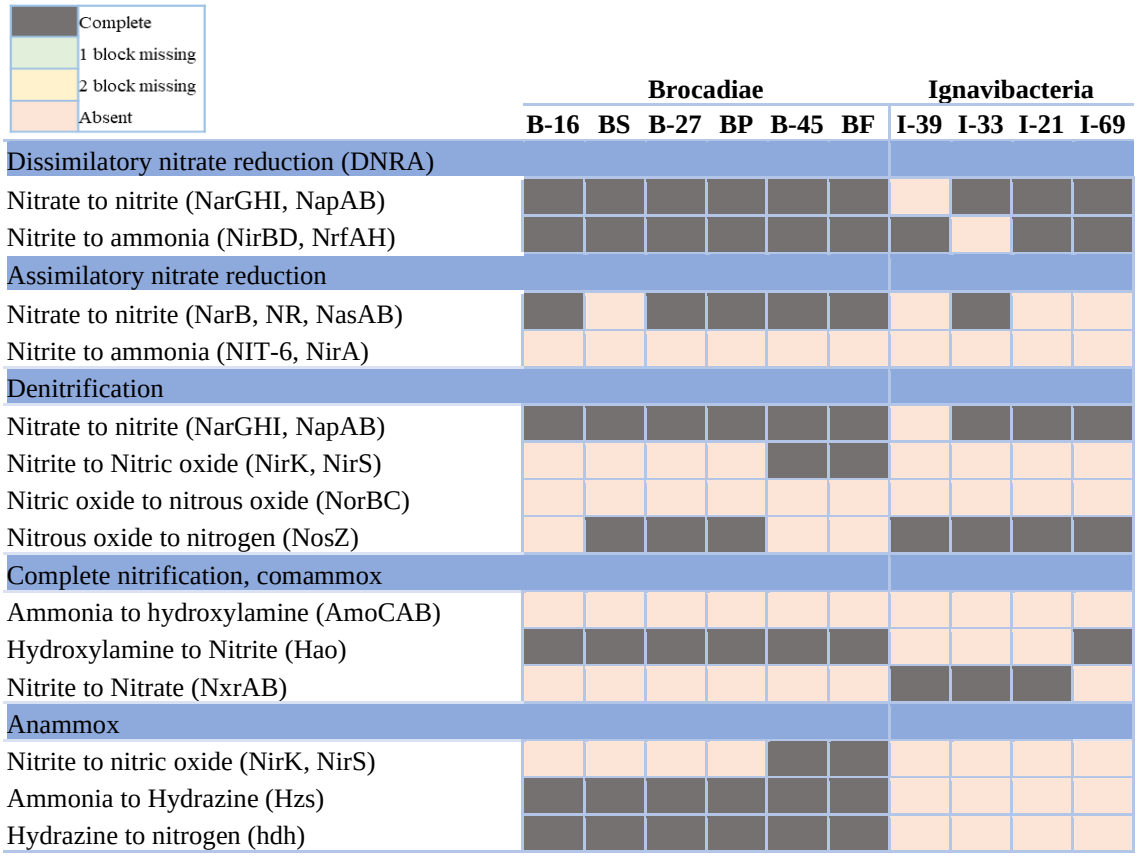

Figure 7.7. Heatmap showing the completeness and the incompleteness of nitrogen metabolism pathways for the recovered MAGS and reference genomes. B-: Broc-; I-: Igna-; BS: *Ca.* Brocadia sinica; BP: *Ca.* Brocadia pituitae; BF: *Ca.* Brocadia fulgida. Pathways with more than 2 blocks missing were considered as absent.

- **Potential nitrogen metabolism of *Ca.* Brocadia barcinensis**

The metabolic potential in the anammox metabolism of *Candidatus* Brocadia barcinenis (Broc-16) is shown in Figure 7.8. This genome lacks nitrite reductase genes (nirK and nirS), which is one of the pathways for nitric oxide production, a key intermediate in the process. Nevertheless, the hydroxylamine oxidoreductase gene was found, thus, could be using another pathway for NO production, through hydroxylamine (NH_2OH) oxidation by a hydroxylamine oxidoreductase hao-like protein (1) (Han et al., 2017; Harhangi et al., 2012; Park et al., 2017), or using the same pathway of *Ca*. Brocadia sinica, where nitrite is reduced to hydroxylamine and not to NO, and then utilized NH_2OH and NH_4^+ for hydrazine synthesis. Although the enzyme responsible for this reaction is still unidentified, according to Oshiki et al. (2016) the most likely candidate enzyme is a hao-like protein (2).

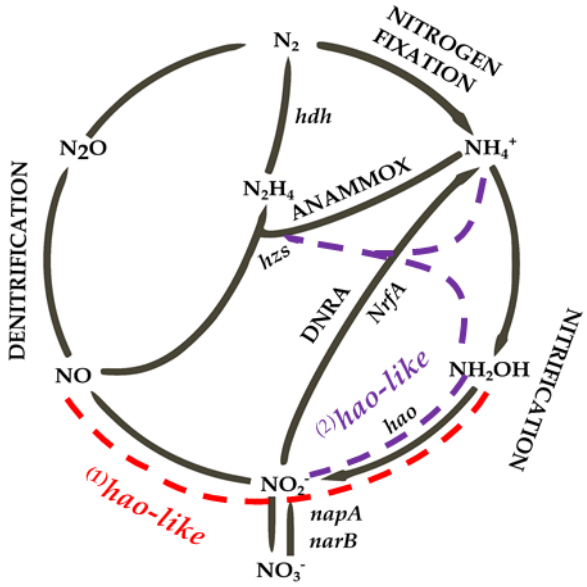

Figure 7.8. Metabolic potential of new *Candidatus* Brocadia barcinensis.

7.4. Conclusions

The metabolic potential of seven metagenome-assembled genomes (MAGs) from two anammox reactors was studied, which belong to the order Brocadiales or Ignavibacteriales.

Regarding the Ignavibacteriales MAGs, our results have shown functional redundancy in the potential metabolism of central carbohydrates, carbon fixation, and fatty acid metabolism in anammox reactors. Instead, fermentation products and nitrogen metabolism seem to be a genome-specific features. Its role as protein degraders, fermentative lifestyle, and auxotrophic and cross-feeding interactions with anammox bacteria was confirmed. They could also facilitate a nitrite loop with anammox bacteria or support complete denitrification, enhancing nitrogen removal performance.

Within the Brocadiales, we recovered a near-complete genome that was not directly associated with any previously described species but whose 16S rRNA gene allowed us to determine that bunched in the same clade of our recently proposed new species, *Ca*. Brocadia barcinensis, dominant in the enriched anammox reactor and also

present in the mainstream anammox reactor. Therefore, this is the first metabolic characterization of this new proposed species.

While MAGs are required to expand the diversity, potential function, and potential interactions between the studied orders, further studies could improve the knowledge on the bacterial communities, which could help develop strategies for strengthening anammox reactor performance at mainstream conditions.

On one side, we could study the differences in the metabolic potential of the metagenome along the sludge bed sections at different operational conditions. This would let to know how changes in working conditions could change the structure of the anammox consortia and the potential metabolism. On the other side, if these differences are important, experiments as metatranscriptomics are necessary to confirm the expression of the identified metabolic functions under certain operational conditions.

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Chapter 8

General conclusions and future work

This thesis was performed in the framework of the HIPATIA project, which aims to redesign the current treatment of municipal wastewater and proposes the use of the anammox process in the main water line to achieve a stable autotrophic nitrogen removal at low temperatures. The main objective was to understand how the communities adapt to the operational conditions in mainstream conditions and at lower temperature. It was previously postulated that the UAnSB operated at mainstream condition presented a stratification of the biomass along the high of the reactor and due to this stratification, an overcapacity was produced which was used when the temperature decrease. By this way, the adaptation of the anammox to mainstream conditions was achieved. Then, this thesis was focus in trying to understand this stratification and overcapacity from the microbiological point of view. A deep analysis of the structural changes in the microbiome structure of an anammox reactor treating real wastewater from a two-step autotrophic biological nitrogen removal at mainstream conditions was performed using different molecular tools. The analysis performed confirm the biomass stratification hypothesis and showed how the microbial communities adapt to the new condition by selecting different microorganisms.

In the following paragraphs we will describe the main conclusions obtained for each proposed goal.

- Goal 1: To characterize and analyze the bacterial communities involved and existing synergies in an anammox reactor during different phases of operation

In the first place, the standardization of different molecular approaches used to characterize the composition of the whole community and the anammox community serves as a basis for studying the microbial ecology and dynamics of anammox reactors working at mainstream conditions. Furthermore, the advantages of using one approach over another were outlined, depending on the research question to be addressed. The use of a combination of molecular approaches, allowed to obtain more reliable information about the community structure and anammox diversity at deep taxonomic levels. In this sense, we identified a new Candidatus anammox species, named *Ca.* Brocadia barcinensis, which was the most abundant bacteria found in the enriched-anammox reactor treating synthetic wastewater.

Secondly, molecular DNA-based methods showed to be a good approach to study the microbiome composition and to detect variations on its structure along the height of an anammox reactor treating real wastewater at mainstream conditions, including different phases of operation at decreasing temperature. The same core microbiome was detected, despite the changes in the operating temperature. The bacterial dynamics along the reactor would confirm its overcapacity, which allowed to demonstrate a good NRE performance at low temperature. However, the high relative abundance of anammox bacteria at low temperature did not correlate with anammox specific activity (NRR) calculated from operational assessment).

- Goal 2: To determine the dynamics of active bacterial communities, including anammox, during the different phases of reactor operation.

In this sense, and as third step in this research, the use of RNA-based methods showed important differences on the active microorganisms in comparison with DNA-based methods. This research is the first report on the use of amplicon sequencing of 16S rRNA transcripts for identifying the active microbial communities in anammox reactors working under mainstream conditions systems. Different populations were found contributing to changes in the community structure, depending on the temperature and also depending on the sludge-bed section. The anammox stratification at the genus and specie level observed at low temperatures in the top sections bed of the reactor and the observed bacterial dynamics of the active communities clearly support the denominated reactor's overcapacity.

- Goal 3: To analyze and set up the possible metabolic roles of the bacterial communities involved in the elimination of nitrogen in anammox reactors.

Finally, we reported the metabolic potential of seven metagenome-assembled genomes (MAGs) from the two anammox reactors studied, which belong to the order Brocadiales or Ignavibacteriales. These results allowed to infer the potential interactions between both communities. Also, we recovered a near-complete genome that phylogenetically bunched in the same clade of our recently proposed new species, *Ca.* Brocadia barcinensis. Therefore, this is the first metabolic characterization of this new proposed species.

Our results give an explanation on how the anammox microbial communities could adapt to mainstream conditions and demonstrate that the technology is adaptable to real systems. New knowledge about not-yet described microorganisms and their interaction in the communities were obtained. This knowledge will be used in the future to understand the behavior of the microbial communities in this kind of systems applied to real conditions.

- Future work

Regarding the high amount of data acquired with the molecular approaches used in this thesis, future work could be focused first on the study of the metabolic potential the metagenome along the UAnSB reactors. Secondly, a continuous assessment of the active communities at different operation conditions would let to know how these changes could affect the structure of the anammox consortia. Finally, if these differences are important, experiments as metatranscriptomics are necessary to confirm the expression of the identified metabolic functions under certain operational conditions.

Annex I

A.I.1. Heatmap showing the completeness and the incompleteness of each metabolic pathway and annotated enzymes for the recovered MAGS and reference genomes. B-: Broc-; I-: Igna-; BS: Ca. Brocadia sinica; BP: Ca. Brocadia pituitae; BF: Ca. Brocadia fulgida. Pathways with more than 2 blocks missing were considered as absent.

Complete	3
1 Block missing	2
2 Block missing	1
Absent	0

Potential metabolic pathways	Broc -16	BS	Broc -27	BP	Broc -45	BF	Igna-39	Igna-33	Igna-21	Igna-69
Respiration										
NADH:quinone oxidoreductase, prokaryotes	2	2	3	3	1	1	3	3	3	3
Succinate dehydrogenase, prokaryotes	2	2	2	3	3	2	0	0	0	0
F-type ATPase, prokaryotes and chloroplasts	3	3	3	3	3	3	3	3	3	3
Cytochrome c oxidase, prokaryotes	0	0	0	0	0	0	3	3	3	3
V-type ATPase, prokaryotes	0	2	0	0	0	0	0	0	0	0
Cytochrome c oxidase, cbb3-type	0	0	0	0	0	0	0	0	2	2
Cytochrome bc1 complex respiratory unit	2	2	2	2	2	2	0	0	0	0
Superoxide dismutase	0	3	3	3	0	0	3	3	3	3
Catalase peroxidase	0	0	0	0	0	0	0	0	3	3
Catalase	0	3	3	3	3	3	3	0	0	0
Glutathione peroxidase	0	0	0	0	0	0	3	0	3	3
Peroxidases	3	3	3	3	3	3	3	3	0	0
TransporterS										
Prokaryotic- type ABC transporters										
Mineral and organic ion transporters										
Molybdate	3	3	3	3	3	3	0	0	0	0

Iron	3	3	3	3	3	3	0	3	0	0
NitT/TauT family transporter	3	3	3	3	3	3	0	0	0	3
Saccharide, polyol, and lipid transporters										
Trehalosa/maltosa	3	3	3	3	3	3	3	0	3	3
Phospholipid	3	3	3	3	3	3	3	3	3	3
gamma-Hexachlorocyclohexane transporter	3	3	3	3	3	3	3	3	3	3
Mce system transporter	3	3	3	3	3	3	3	3	3	3
Phosphate and amino acid transporters										
Phosphate	3	3	3	3	3	3	0	3	3	3
phosphonate	0	0	0	0	0	0	3	0	0	3
Urea	3	3	3	3	3	3	3	3	3	0
Branched-chain amino acid	0	0	0	0	2	2	3	3	3	3
Peptide and nickel transporters										
Peptide and nickel transporters	0	0	0	0	0	0	3	3	3	3
Metallic cation, iron-siderophore and vitamin B12 transporters										
Nickel	3	3	3	3	3	3	0	0	0	3
Cobalt	3	3	3	3	3	3	0	0	0	0
Biotin	0	0	0	0	0	0	0	0	3	0
zinc	0	0	0	0	0	0	3	3	3	3
Fe	0	0	0	0	0	0	0	3	0	0
Vitamin B12 transporter	3	3	3	3	3	3	3	3	3	3
Mn	0	0	0	0	0	0	0	3	0	0
ABC-2 type and other transporters										
Lipopolysaccharide transport	3	3	3	3	3	3	0	0	3	3
Lipopolysaccharide export	3	3	3	3	3	3	3	3	3	3
Lipoprotein releasing	3	3	3	3	2	3	3	3	3	3
Putative ABC transporter	3	3	3	3	3	3	3	3	3	3
heme	0	0	0	0	0	0	3	3	3	3
ABC-2 type transporter	3	3	3	3	3	3	3	3	3	3

Copper-processing transporter	0	0	0	0	0	0	3	3	3	3
Phosphotransferase system (PTS)										
Nitrogen regulatory II component	3	3	3	3	3	3	0	0	3	3
Phosphoenolpyruvate-protein phosphotransferase	3	3	3	3	3	3	3	3	3	3
Phosphocarrier protein HPr	0	0	0	0	0	0	3	3	3	3
Multiphosphoryl transfer protein	0	0	0	0	0	0	3	3	3	3
Nitrogen related transporters										
Formate transporter (focA)	3	3	3	3	3	3	0	0	0	0
Nitrite/nitrate transporter (NarK)	3	3	3	2	3	2	3	3	3	0
Ammonia channel (amtB)	3	3	3	3	3	3	0	0	0	0
Nitrite transporter (NirC)	3	3	3	3	3	3	0	0	0	0
Putative nitrate transporter (narT)	3	3	3	3	3	3	0	0	0	0
Drug transporters										
Fosmidomycin resistance	0	0	3	3	3	3	0	3	0	0
Antibiotic resistance protein	3	3	3	3	3	3	0	3	3	3
Multidrug resistance protein	3	3	3	3	3	3	0	3	0	0
Others										
Long-chain fatty acid transporter	3	3	3	3	3	3	3	0	3	3
Polysaccharide biosynthesis	3	3	3	3	3	0	3	0	3	3
Biopolymer transport protein	3	3	3	3	3	3	3	3	3	3
polysaccharide biosynthesis transport	3	3	3	3	3	3	3	3	3	3
Nitrogen metabolism										
Dissimilatory nitrate reduction										
Nitrate to nitrite (NarGHI, NapAB)	3	3	3	3	3	3	3	3	3	3
Nitrite to amonia (NirBD, NrfAH)	3	3	3	3	3	3	3	0	3	3
Assimilatory nitrate reduction										
Nitrate to nitrite (NarB, NR, NasAB)	3	0	3	3	3	3	0	3	0	0
Nitrite to ammonia (NIT-6, NirA)	0	0	0	0	0	0	0	0	0	0
Denitrification										

Nitrate to nitrite (NarGHI, NapAB)		3	3	3	3	3	3	3	3	3	3
Nitrite to Nitric oxide (NirK, NirS)		0	0	0	0	3	3	0	0	0	0
Nitric oxide to nitrous oxide (NorBC)		0	0	0	0	0	0	0	0	0	0
Nitrous oxide to nitrogen (NosZ)		0	3	3	3	0	0	3	3	3	3
Complete nitrification, comammox											
Ammonia to hydroxylamine (AmoCAB)		0	0	0	0	0	0	0	0	0	0
Hydroxylamine to Nitrite (Hao)		3	3	3	3	3	3	0	0	0	3
Nitrite to Nitrate (NxrAB)		0	0	0	0	0	0	3	3	3	0
Anammox											
Nitrite to nitric oxide (NirK, NirS)		0	0	0	0	3	3	0	0	0	0
Ammonia to Hydrazine (Hzs)		3	3	3	3	3	3	0	0	0	0
Hydrazine to nitrogen (hdh)		3	3	3	3	3	3	0	0	0	0
Cellulases											
GH5	cellulase	3	3	3	3	3	3	3	0	3	3
GH9	endoglucanase	3	3	3	3	3	3	3	3	0	0
GH10	endo-xylanase	0	0	0	3	0	0	0	0	0	0
GH28	polygalacturonase	0	0	0	0	0	0	0	0	0	0
GH74	endoglucanase	0	0	0	0	0	0	0	0	0	0
Endohemicellulases											
GH8	endo-xylanase	0	0	0	3	0	0	0	0	0	0
Amylases (starch degradation)											
GH13	alpha-amylase	3	3	3	3	3	3	3	0	3	3
GH57	alpha-amylase	0	0	0	0	0	0	0	0	0	0
Debranching enzymes											
GH51	alpha-L-arabinofuranosidase	0	0	0	0	0	0	0	0	0	0
GH78	alpha-L-rhamnosidase	0	0	0	0	0	0	0	0	0	0
Amino sugar-degrading enzymes											
GH20	beta-hexosaminidase	0	0	0	0	0	0	3	3	0	0
GH23	lysozyme	0	0	0	0	0	0	0	0	0	3

GH24	lysozyme	0	0	0	0	0	0	0	0	0	0
GH25	lysozyme	0	0	0	0	0	0	0	0	0	0
GH46	chitosanase	0	0	0	0	0	0	0	0	0	0
GH73	peptidoglycan hydrolase	0	0	0	0	0	0	0	0	0	0
GH103	peptidoglycan lytic transglycosylase	0	0	3	0	0	0	0	3	3	3
GH109	alpha-N-acetylgalactosaminidase	0	0	0	0	0	0	0	0	0	0
GH114	endo-alpha-1,4-polygalactosaminidase	0	0	0	0	0	0	0	0	0	0
Oligosaccharide-degrading enzymes											
GH1	beta-glucosidase	0	0	0	0	0	0	0	3	0	3
GH1	beta-galactosidase	0	0	0	0	0	0	3	3	0	3
GH3	beta-glucosidase	0	0	0	0	0	0	0	0	0	0
GH4	alpha-glucosidase	0	0	0	0	0	0	3	0	3	3
GH29	alpha-L-fucosidase	0	0	0	0	0	0	0	0	0	0
GH31	alpha-glucosidase	0	0	0	0	0	0	0	0	0	0
GH32	invertase	0	0	0	0	0	0	0	0	0	0
GH33	sialidase	0	0	0	0	0	0	0	0	0	0
GH35	beta-galactosidase	0	0	0	0	0	0	0	0	0	0
GH36	alpha-galactosidase	0	0	0	0	0	0	0	0	0	0
GH37	alpha,alpha-trehalase	3	3	3	3	3	3	0	0	0	0
GH38	alpha-mannosidase	0	0	0	0	0	0	3	0	0	0
GH42	beta-galactosidase	0	0	0	0	0	0	0	0	3	0
GH43	beta-xylosidase	3	3	3	3	0	0	0	0	0	0
GH63	alpha-glucosidase	0	0	0	0	0	0	0	0	0	0
GH65	alpha,alpha-trehalase	0	0	0	0	0	0	0	0	0	0
GH76	alpha-1,6-mannanase	0	0	0	0	0	0	0	0	0	0
GH94	cellobiose phosphorylase	0	0	0	0	0	0	0	0	0	3
GH100	invertase	0	0	0	0	0	0	0	0	0	0
GH120	beta-xylosidase	0	0	0	0	0	0	0	0	0	0
Extracellular peptidases											

Family M14C LysM	0	0	0	0	0	0	3	3	3	3
alfa/beta hydrolase, family SS3	0	0	0	0	0	0	0	0	0	0
sedolisins, samily s53	0	0	0	0	0	0	0	0	0	0
peptidase family M48C	3	3	3	3	3	3	3	0	3	3
Potential fermentation by-products from acetyl-CoA										
Pyruvate oxidation, pyruvate => acetyl-CoA										
1										
pyruvate dehydrogenase E1 component alpha subunit PDHA, pdhA K00161 O K00163	0	0	0	0	0	0	0	0	0	0
pyruvate dehydrogenase E1 component beta subunit PDHB, pdhB K00162 O K00163	0	0	0	0	0	0	3	3	3	3
pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) pdhC K00627	0	0	0	0	0	0	3	3	3	3
dihydrolipoamide dehydrogenase DLD, lpd, pdhD K00382	3	0	3	3	3	3	3	3	3	3
Dihydrolipoamide dehydrogenase-binding protein of pyruvate dehydrogenase complex DHX K13997	0	0	0	0	0	0	0	0	0	0
2										
pyruvate ferredoxin oxidoreductase alpha subunit [EC:1.2.7.1] pforA K00169	3	3	0	0	0	0	3	0	3	3
pyruvate ferredoxin oxidoreductase beta subunit [EC:1.2.7.1] pforB K00170	3	3	0	0	0	0	0	0	0	0
pyruvate ferredoxin oxidoreductase delta subunit [EC:1.2.7.1] pforD K00171	3	3	0	0	0	0	0	0	0	0
pyruvate ferredoxin oxidoreductase gamma subunit [EC:1.2.7.1] pforG K00172 O K00189	0	0	0	0	0	0	0	0	0	0
3										
Pyruvate formate-lyase (PFLA) K00656 EC:2.3.1.54 conversion of pyruvate to acetyl-CoA and formate	0	0	0	0	0	0	0	0	3	0
pflA, pflC, pflE; pyruvate formate lyase activating enzyme EC:1.97.1.4 K04069	3	3	3	3	3	3	3	0	3	3
4										
pyruvate-flavodoxin oxidoreductase EC 1.2.7.1 K03737 por, nifJ	3	3	3	3	3	3	3	0	3	3
Others										
formate dehydrogense fdhA /B	3	3	3	3	3	3	0	0	0	0

formate dehydrogenase H (fdhF) K00123 formic acid to H2 and CO2 1.17.1.9	0	0	0	0	0	0	0	0	0	3
Aldehyde dehydrogenase eutE EC:1.2.1.10 K04021 aldH k00128 1,2,1,3	3	3	3	3	3	3	3	3	3	3
Alcohol dehydrogenase adh EC:1.1.1.1 K00001	3	3	0	0	0	0	3	0	0	0
Aldehyde/alcohol dehydrogenase adhE EC:1.2.1.10 1.1.1.1 K04072	0	0	0	0	0	0	0	0	3	0
L-lactate dehydrogenase ldh K00016 EC:1.1.1.27	0	0	0	0	0	0	0	0	3	3
D-lactate dehydrogenase ldhd k00102 ec 1.1.2.4	3	3	3	3	0	0	0	0	0	0
Acetolactate synthase, small sub-unit ilvH 2.2.1.6 K01653	3	3	3	3	3	3	0	0	0	0
Acetolactate synthase, large sub-unit ilvI 2.2.1.6 K01652	3	3	3	3	3	3	0	0	0	0
phosphotransacetylase pta EC:2.3.1.8 K00625, K13788 o K15024	0	0	0	0	3	3	3	3	3	3
acetate kinase ackA EC:2.7.2.1 K00925	3	3	3	3	3	3	3	0	3	3
Methylmalonyl-CoA pathway	0	0	0	0	0	0	0	0	0	0
Propionyl-CoA carboxylase alpha subunit pccA 6.4.1.3 K01965	0	0	0	0	0	0	0	0	0	0
Propionyl-CoA carboxylase beta subunit pccB 6.4.1.3 K01966	0	0	0	0	0	0	3	3	3	0
Methylmalonyl-CoA/ethylmalonyl-CoA epimerase epi 5.1.99.1 K05606	0	3	3	0	0	0	3	3	3	0
Methylmalonyl-CoA mutase mcm 5.4.99.2	0	3	0	0	0	0	3	3	3	0
aldehyde:ferredoxin oxidoreductase aor EC:1.2.7.5 K03738	0	0	0	0	0	0	0	0	0	0
EPS										
Alginate metabolism Exopolysaccharide										
Phosphomannomutase (AlgC)	3	3	3	3	3	3	3	3	3	3
Mannose-6-P isomerase (AlgA)	3	3	3	3	3	3	3	3	0	0
Exopolysaccharide biosynthesis Gene										
Glycogen(starch) synthase (glgA)	3	3	3	3	3	3	3	3	3	3
Glycosyltransferase (epsA)	3	3	3	3	3	3	3	0	3	3
Glycosyltransferase (epsC)	0	3	3	3	3	3	3	3	3	0
Glycosyltransferase (epsD)	3	3	3	3	3	3	0	3	0	3
Glycosyltransferase (epsF)	3	3	3	3	3	3	0	0	0	0
Glycosyltransferase (epsE)	3	0	0	0	0	0	0	0	0	0
Glycosyltransferase (epsJ)	3	0	0	0	0	0	3	0	3	0

Glycosyltransferase (epsP)	3	0	0	0	0	0	3	0	3	3
Glycosyltransferase (epsB)	3	0	0	0	0	0	3	3	3	3
Biofilm formation										
Diguanylate cyclase (PleD/wspR)	0	3	3	3	3	3	0	0	0	0
CRP/FNR family transcriptional regulator (crp)	3	3	3	3	3	3	3	0	3	3
RNA polymerase sigma-54 factor (rpoN)	3	3	3	3	3	3	3	3	3	3
Carbon storage regulator (csra)	3	3	3	3	3	3	3	0	0	3
Sticky macromolecular eps										
UDP-Gal Synthesis										
Glucokinase (glk)	3	3	3	3	3	3	3	3	3	3
UDP-glucose-4-epimerase (GalE)	3	3	3	3	3	3	3	3	3	3
UDP-GlcNAcA Synthesis										
Glucokinase (glk)	3	3	3	3	3	3	3	3	3	3
Glucose-6-phosphate isomerase (gpi)	3	3	3	3	0	3	3	3	3	3
UDP-N-acetyl-D-glucosamine dehidrogenase (wbpA)	3	3	3	3	3	3	3	3	3	3
GDP-Rha										
Glucose-6-phosphate isomerase (gpi)	3	3	3	3	0	3	3	3	3	3
Mannose-6-P isomerase (ManA)	0	0	0	0	0	0	3	3	0	3
Assembly										
Polyisoprenyl-phosphate glycosyltransferase	0	0	0	3	0	0	0	0	0	0
Protein-tyrosine phosphatase	3	3	3	3	3	3	3	0	3	3
Phosphoglycolate phosphatase (gph)	3	3	3	3	3	3	3	3	3	3
Central carbohydrate metabolism										
Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate	2	3	3	3	1	3	3	3	3	3
Glycolysis, core module involving three-carbon compounds	3	3	3	3	3	3	3	3	3	3
Gluconeogenesis, oxaloacetate => fructose-6P	2	3	3	3	2	3	3	3	3	3
Pyruvate oxidation, pyruvate => acetyl-CoA	3	3	3	3	3	3	3	3	3	3

Citrate cycle (TCA cycle, Krebs cycle)	3	3	3	3	2	3	3	3	3	3
Citrate cycle, first carbon oxidation, oxaloacetate => 2-oxoglutarate	3	3	3	3	2	3	3	3	3	3
Citrate cycle, second carbon oxidation, 2-oxoglutarate => oxaloacetate	3	3	3	3	3	3	3	3	3	3
Pentose phosphate pathway (Pentose phosphate cycle)	3	3	3	3	1	3	1	1	3	1
Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P	3	3	3	3	2	3	0	0	3	0
Pentose phosphate pathway, non-oxidative phase, fructose 6P => ribose 5P	3	3	3	3	3	3	3	3	3	3
Pentose phosphate pathway, archaea, fructose 6P => ribose 5P	0	0	0	0	0	0	2	2	2	2
PRPP biosynthesis, ribose 5P => PRPP	3	3	3	3	3	3	3	3	3	3
Other carbohydrate metabolism										
Glycogen biosynthesis, glucose-1P => glycogen/starch	3	3	3	3	3	3	2	2	2	2
Trehalose biosynthesis, D-glucose 1P => trehalose	3	3	3	3	3	3	0	0	0	0
Nucleotide sugar biosynthesis, galactose => UDP-galactose	2	2	2	2	2	2	2	0	0	0
UDP-N-acetyl-D-glucosamine biosynthesis, prokaryotes, glucose => UDP-GlcNAc	2	2	2	2	1	2	2	2	2	2
Propanoyl-CoA metabolism, propanoyl-CoA => succinyl-CoA	0	0	0	0	0	0	2	2	2	2
Undecaprenylphosphate alpha-L-Ara4N biosynthesis, UDP-GlcA => undecaprenyl phosphate alpha-L-Ara4N	0	3	3	3	0	0	0	0	0	0
Carbon fixation										
Reductive acetyl-CoA pathway (Wood-Ljungdahl pathway)	3	3	3	3	3	3	0	0	0	0
Phosphate acetyltransferase-acetate kinase pathway, acetyl-CoA => acetate	2	2	2	2	3	3	3	2	3	3
Fatty acid metabolism										
Fatty acid biosynthesis, initiation	3	3	3	3	3	3	3	3	3	3
Fatty acid biosynthesis, elongation	0	0	0	0	0	0	3	3	3	3
beta-Oxidation, acyl-CoA synthesis	3	3	3	3	3	3	3	3	3	3
Lipid metabolism										
Phosphatidylcholine (PC) biosynthesis, PE => PC	3	3	3	3	3	3	0	0	0	0
Phosphatidylethanolamine (PE) biosynthesis, PA => PS => PE	3	3	3	3	3	3	3	3	3	3
Amino acid metabolism										
Serine biosynthesis, glycerate-3P => serine	2	2	2	2	2	2	2	2	2	2
Threonine biosynthesis, aspartate => homoserine => threonine	2	2	2	2	2	2	0	0	0	0

Cysteine biosynthesis, serine => cysteine	3	3	3	3	3	3	0	0	0	0
Methionine degradation	2	2	1	2	2	2	1	2	3	3
Valine/isoleucine biosynthesis, pyruvate => valine / 2-oxobutanoate => isoleucine	3	3	3	3	3	3	0	0	0	0
Leucine biosynthesis, 2-oxoisovalerate => 2-oxoisocaproate	3	3	3	3	3	3	0	0	0	0
Lysine biosynthesis, DAP aminotransferase pathway, aspartate => lysine	3	3	3	3	3	3	0	0	2	0
Arginine and proline metabolism	3	3	3	3	3	3	0	0	0	0
Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine	3	3	3	3	1	3	0	1	0	0
Polyamine biosynthesis, arginine => ornithine => putrescine	0	0	0	0	0	0	2	2	2	2
Histidine biosynthesis, PRPP => histidine	0	0	0	0	0	0	0	0	0	3
Histidine degradation, histidine => N-formiminoglutamate => glutamate	3	3	0	0	0	0	3	3	3	3
Shikimate pathway, phosphoenolpyruvate + erythrose-4P => chorismate	3	3	3	3	2	3	3	0	3	3
Tryptophan biosynthesis, chorismate => tryptophan	2	2	2	2	2	2	0	0	0	2
Phenylalanine biosynthesis, chorismate => phenylpyruvate => phenylalanine	2	2	2	2	2	2	2	0	0	2
Tyrosine biosynthesis, chorismate => HPP => tyrosine	2	2	2	2	2	2	0	0	0	2
Lipopolysaccharide metabolism										
KDO2-lipid A biosynthesis, Raetz pathway, non-LpxL-LpxM type	1	1	0	1	1	2	2	2	2	2
CMP-KDO biosynthesis	3	3	3	3	3	3	3	3	3	3
ADP-L-glycero-D-manno-heptose biosynthesis	2	2	2	2	2	2	3	3	3	3
Metabolism of cofactors and vitamins										
Thiamine biosynthesis, archaea, AIR (+ NAD+) => TMP/TPP	3	3	3	3	3	3	0	0	0	0
Riboflavin biosynthesis, plants and bacteria, GTP => riboflavin/FMN/FAD	3	3	3	3	3	3	3	3	3	3
NAD biosynthesis, aspartate => quinolinate => NAD	3	3	3	3	3	3	0	3	0	3
Coenzyme A biosynthesis, pantothenate => CoA	3	3	3	3	3	3	1	3	3	1
Biotin biosynthesis, pimeloyl-ACP/CoA => biotin	2	2	2	2	2	3	1	1	1	2
Lipoic acid biosynthesis, plants and bacteria, octanoyl-ACP => dihydrolipoyl-E2/H	3	3	3	3	3	3	0	3	3	3
Heme biosynthesis, archaea, siroheme => heme	3	2	3	3	3	3	0	0	1	0
Heme biosynthesis, plants and bacteria, glutamate => heme	0	0	0	0	0	0	0	0	3	3
Cobalamin biosynthesis, cobyrinate a,c-diamide => cobalamin	2	2	2	2	1	2	0	0	0	0
Menaquinone biosynthesis, futalosine pathway	3	1	3	3	0	3	0	0	0	0

Terpenoid backbone biosynthesis										
C5 isoprenoid biosynthesis, non-mevalonate pathway	1	1	1	1	1	1	3	2	3	3
C10-C20 isoprenoid biosynthesis, bacteria	2	2	0	0	0	0	3	2	3	3
Polyketide sugar unit biosynthesis										
dTDP-L-rhamnose biosynthesis	3	3	3	3	3	3	3	3	3	3