THÈSE / UNIVERSITÉ DE RENNES 1

sous le sceau de l'Université Bretagne Loire pour le grade de

DOCTEUR DE L'UNIVERSITÉ DE RENNES 1

Mention : Chimie

Ecole doctorale SDLM
Ecole Doctorale Sciences De La Matière

Romain CONNAN

Préparée à l'unité de recherche OPAALE
Optimisation des procédés en Agriculture, Agroalimentaire et Environnement - Irstea

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Feasibility of anammox for the treatment of sewage sludge digester supernatant: From inoculum enrichment and cultivation to process configurations and emissions

Thèse soutenue à Rennes
le 21 décembre 2016

Devant le jury composé de :

Siegfried VLAEMINCK
Professeur, Université d'ANVERS / *Rapporteur*
Mathieu SPERANDIO
Professeur, INSA / *Rapporteur*
Francesc PRENAFETA
Chercheur, IRTA / *Examinateur*
Gilbert BRIDOUX
Ingénieur de recherche, SAUR / *Examinateur*
Nicolas BERNET
Directeur de recherche, INRA / *Examinateur*
Ahlem FILALI
Ingénieur de recherche, Irstea / *Examinatrice*
Albert MAGRI
Chercheur, Irstea / *Encadrant*
Fabrice BELINE
Directeur de recherche, Irstea / *Directeur de thèse*

Remerciements

Je tiens dans un premier temps à remercier les membres de mon jury de thèse. Nicolas Bernet en tant que président, Siegfried VLAEMINCK et Mathieu SPERANDIO en tant que rapporteurs, Francesc PRENAFETA ainsi que Ahlem FILALI en tant qu'examinateurs. Je tenais à vous remercier d'avoir accepté de vous impliquer de ce processus d'examination et pour votre bienveillance à mon égard lors de cette journée toute particulière.

À mon directeur de thèse, Fabrice BELINE. De près ou de loin tu as toujours su garder un œil sur mon travail, le guider et l'orienter lorsque cela était nécessaire et pour cela je t'adresse un grand merci, ce fut un très un vrai plaisir de travailler avec toi toujours dans la bonne humeur. Je ne pourrais plus faire une présentation sans me demander, " Est-ce que Fabrice penserait que j'en mets trop ? "

À mon co-directeur de thèse, Albert MAGRÍ. Je pense qu'à ce jour je ne t'ai toujours pas assez remercié pour toute l'aide, le soutien et la richesse de ce que tu m'as apporté. Ces trois années où j'ai eu la chance de travailler avec toi ont été pour moi extrêmement formatrices et m'ont énormément apportées tant sur le plan personnel que scientifique. Cette période française s'est déroulée dans des conditions assez particulières pour toi et malgré tout tu as su demeuré toujours plein d'entrain et de bonne humeur et pour ton accompagnement sans faille je te remercie du fond du cœur.

À mon co-co- directeur de thèse, Patrick DABERT. Car toi aussi, pour un temps, ton bureau c'est transformé en bureau des pleurs, « je ne sais pas ce qu'elles ont aujourd'hui… », « je suis sûr que c'est le polymère qui les tuent… », « tu peux être mon référent ce week-end ? ». Tu as suivi ce projet de A à Z et grâce à ton aide j'ai pu m'introduire avec aise dans ces beaux domaines que sont l'écologie microbienne et la bioinformatique. Pour ta sympathie et tout ton soutien, un grand merci à toi.

À Ahlem FILALI, Mathieu SPERANDIO et Evelyne VOLCKE. Je tenais a vous remercier d'avoir fait le déplacement jusqu'à Rennes à l'occasion d'un point à mi-parcours. Je m'en rappelle comme d'une journée positive, aux riches discussions, qui a su remettre le projet et ses objectifs en perspective. Pour votre implication et vos encouragements, un merci particulier à vous trois.

À Gilbert BRIDOUX et à la SAUR. Ca a été un réel plaisir de travailler en lien avec vous. Tu nous as accompagné tout au long de ce projet et ouvert les portes des stations SAURiennes bretonnes. Pour ton implication et ta bonne humeur sans faille, un grand merci à toi.

À Olivier CHAPLEUR. Je tenais à t'adresser un grand merci pour ton aide, très précieuse, qui m'a littéralement tiré d'affaire pour le traitement de ces "satanés" séquences.

A l'incroyable équipe d'IRSTEA Rennes. Pour l'anecdote j'étais encore à l'institut d'ingénierie de la faculté de Paris EST - Marne la Vallée que l'on m'avait déjà vanté les mérites de ce centre de recherche, tant sur la bonne humeur qui y règne que sur les compétences de ceux qui y travaillent. Le fait est je dois reconnaitre que tout était fondé. Il doit exister peu d'endroit plus propice à un doctorant pour réaliser sa thèse, à tous ceux que je ne citerais pas ci-après, merci à vous.

A Fabrice GUIZIOU, mon papa IRSTEA. Merci pour tous les coups de pied au cul, les rêveries de voyage et les points sémantiques, toujours le mot juste. Nous n'avons pas eu l'occasion de travailler communément sur un projet (à mon grand regret, j'aurais vraiment aimé faire une campagne de terrain avec toi) et pourtant tu m'as énormément apporté dans la découverte de « l'ingénierie de la bricole », où comment assurer la transition du banc de l'école aux mains dans le cambouis ! Un très très grand merci à toi.

À Christine Ziebal, ma maman IRSTEA, #Staline. Car, sans aucun doute, la vie au centre ne serait absolument pas la même sans toi ! Un énorme merci pour toute la bonne humeur et le soutien que tu m'as apporté pendant ces trois années. C'est pas rose tous les jours mais on s'en sort toujours.

À ma co-bureau, Julie BUFFET #NousSommesBrillants. La liste est longue mais pour ces trois super années, ton aide au quotidien, les trente commandes que j'ai du te demander de passer, les visios-conf en anglais dans le bureau, les 1000 et une petites pièces que je ne trouvais pas, les sorties prélèvement super funcky et toute ta bonne humeur, un énorme merci à toi. :)

À Sophie LEROUX. Tu m'as accompagné tout au long de cette thèse, que ce soit pour me former, accompagner un stagiaire où me dépanner tu as eu une place très importante dans ce travail. Je te remercie énormément pour toute ton aide.

À Sylvie PICARD, Patricia SAINT-CAST et Milène DAUMOIN. Un grand merci à vous trois pour votre aide au laboratoire. Merci pour le temps que vous avez offert pour me former et m'accompagner. Un merci tout particulier à toi Sylvie pour ton aide avec la Chromato maudite.

Un grand merci à Pascal PEU, Jean Claude BENOIST, Céline DRUILHE, Anne Marie POURCHER et Amaury DE GARDIA pour leur aide dans ce projet.

À tous les collègues en appui, Régine LOUBAT, Julie DEULEURME, Isabelle MESLE, Philippe AUGUSTE, Brigitte MARCHIX, Fabrice EGIDO. Vous êtes toujours prêt à nous donner un coup de main et pour cela, un grand merci à vous tous.

À mes colloques et collègues, car chez nous l'un ne va jamais sans l'autre, Henry, Christelle, Marina, Caroline, Amine (cela fait très auberge espagnole dit comme ça) vous avez eu l'occasion de la vivre de l'intérieur cette thèse. Cette époque Vignoc-quoise restera comme la période la plus agréable de cette aventure Rennaise, un grand merci à vous tous pour ces beaux moments.

And the last, but not the least, à la super équipe de doctorants, post-doc et CDD et tous ceux qui passent trop vite, Axelle, Jerem, Henry, Tian, Stéph, Simon, Anne-Laure, Cyril, Nicolas, Geoffrey, Hassan, Amandine, Benjamin, Sam, Carlos et Carlos, Antoine, Ruzica et Younes.

Table des matières

Liste des Figures

Liste des Tableaux

Lexique

AOB : Ammonium-Oxidizing Bacteria

BOD_5 : Biological Oxygen Demand after 5 days

COD : Chemical Oxygen Demand

C_{org} : Carbone Organique

CR : Citation Rate

CSTR : Continuously Stirred Tank Reactor

DGGE : Denaturing Gradient Gel Electrophoresis

EGSB : Expanded Granular Sludge Bed

GAO : Glycogen-Accumulating Organisms

MBBR : Moving Bed Biofilm Reactor

NGS : Next Generation Sequencing

NDN : Nitrification – Dénitrification

NI : Number of Item

NOB : Nitrite-Oxidizing Bacteria

NP : Nitritation Partielle

OTU : Operational Taxon Unit

OD : Oxygène Dissous

PAO : Polyphosphate-Accumulating Organisms

RBC : Rotating Biological Contactor

S&T : Science and Technology

SBR : Sequencing Batch Reactor

TS : Total Solid

TSS : Total Suspended Solid

UASB : Up-Flow Anaerobic Sludge Blanket

VS : Volatile Solids

VSS : Volatile Suspended Solids

WWTP : Wastewater Treatment Plant

Communications liées à la thèse :

Publications scientifiques

R. Connan, P. Dabert, H. Khalil, G. Bridoux, F. Béline, A. Magrí, Batch enrichment of anammox bacteria and study of underlying microbial community dynamics, Chemical Engineering Journal 297 (2016), Pages 217-228.

A. Magrí, F. Giovannini, **R. Connan**, G. Bridoux, F. Béline, Nutrient management from biogas digester effluents: a bibliometric-based analysis considering publications and patents, International Journal of Environmental Science and Technology (submitted, JEST-D-16-01427).

R. Connan, P. Dabert, C. Bureau, O. Chapleur, G. Bridoux, M.B. Vanotti, F. Béline, A. Magrí, Characterization of a combined batch-continuous procedure for the culture of anammox biomass (Submitted for review).

R. Connan, M. Moya-Espinosa, G. Bridoux, P. Dabert, F. Béline, A. Magrí, Application of autotrophic nitrogen removal in one- and two-stage systems using the sequencing batch reactor technology (in preparation).

Communication affichées

R. Connan, P. Dabert, G. Bridoux, F. Béline, A. Magrí, Anammox bacteria enrichment and study of microbial community dynamics by q-PCR and pyrosequencing, International Water Association (IWA), 14th World Congress on Anaerobic Digestion (AD14), 15-18 November 2015, Viña del Mar, Chile, 4 pages (poster presentation).

A. Magrí, **R. Connan**, Procés anammox per al tractament de sobrenedants de digestors anaerobis, Government of Catalonia, International Symposium: "Reptes i Oportunitats en Fertilització i Gestió de les Dejeccions Ramaderes", 17-18 December 2014, Vic (Barcelona), Spain, Pages 89-92 (invited oral presentation, in Catalan).

Participation à des formations spécialisées

Advanced Course on Environmental Biotechnology, TU-Delft, 9-19 June 2015

Avant-propos :

Depuis des décennies l'urbanisation croissante n'a de cesse de concentrer des densités de populations de plus en plus importantes. Cette dynamique couplée au mode de vie moderne nécessite la mise en place d'un torrent de biens et de denrées produits sur de larges territoires et déferlant dans de fines mégapoles. Quelques soit leur nature et le nom qu'on leur prête, les sous-produits de cette consommation doivent trouver des cheminements hors de ces zones urbaines. Et quand bien même ces flux sont redirigés vers l'extérieur, une pure et simple dissémination dans la nature est trop bouleversante pour l'écosystème. L'eau, en tant que ressource essentielle à tout être, se doit d'être protégée contre les contaminations. Ce n'est qu'au début du siècle dernier que les premiers traitements ont vu le jour, s'attaquant à réduire la charge organique des effluents. Le traitement des eaux usées était né. Depuis lors, pour faire face à une charge polluante ne cessant de grandir, chercheurs et industriels n'ont eût de cesse de perfectionner leurs procédés qu'ils soient physico-chimiques, ou comme dans ces travaux biologiques. Ceci en domptant sans relâche de nouveaux microorganismes dont les aptitudes semblent sans limites. La nature dans son infinie prescience nous offre chaque jour les armes pour lutter un peu mieux contre nous même.

Introduction générale

L'eau est sans aucun doute la ressource la plus précieuse pour une communauté ou bien même pour un état. Or, de la qualité de l'eau dont nous disposons découle l'usage qui peut en être fait (Directive 98/83/CE, 1998). Le professeur Albert Calmette écrivait déjà en 1904 : « L'un des plus graves problèmes dont la solution s'impose aux villes et aux grandes industries est celui de l'épuration des eaux résiduaires ». Indéniablement, les sources de polluants au sein d'un bassin versant sont nombreuses. Elles vont être constituées principalement des rejets urbains au sens large, des rejets des exploitations agricoles et des retombées atmosphériques. La pollution diffuse due aux nitrates émanant des effluents agricoles et d'élevage a donné lieu à une directive européenne 91/676/CEE afin de limiter leur impact sur les cours d'eau et les littoraux. La volonté de l'Europe d'imposer à ses états membres une politique globale et cohérente en matière de gestion de l'eau à été encore renforcée par la directive cadre sur l'eau (DCE) 2000/60/CE. Dans le cadre de mes travaux, ce sont les pollutions azotées qui sont au centre du sujet. Majoritairement, les flux d'azote dans les eaux de surface vont provenir des rejets urbains en eaux usées, des sites d'enfouissement ainsi que des amendements agricoles excédentaires. Nutriment ou polluant, l'élément « azote – N » revêt deux visages. Son pouvoir fertilisant en fait un atout pour amender les sols agricoles, tandis que sa présence à trop fortes concentrations dans les eaux souterraines et les rivières viendra perturber le bon état écologique des milieux. A travers les phénomènes d'eutrophisation et de « marée verte », l'azote, au même titre que d'autres nutriments, est au cœur de cette problématique de gestion. Développer une filière d'élimination de l'azote s'impose donc pour éliminer le trop plein généré faute de pouvoir le faire absorber par les milieux naturels.

Au sens large, une autre facette du « déchet » est l'enjeu de sa valorisation. « Peut-on donner de la valeur à quelque chose qui n'en a pas et/ou pose problème ?» et faire ainsi d'une pierre deux coups en recouvrant un produit, comme de l'énergie ou un fertilisant, tout en réduisant le pouvoir polluant d'un déchet. C'est donc dans ce contexte qu'un procédé se développe de façon importante à travers le monde et notamment en Europe et en France ; la digestion anaérobie, plus communément appelée méthanisation. La méthanisation repose sur un enchainement de voies métaboliques microbiennes, et à ce titre, est un bioprocédé. Au cours de ce dernier, la matière organique (MO) présente dans la matrice va être transformée en biogaz. Les premières étapes du processus global consistent en une hydrolyse de la MO complexe et une étape d'acidogénèse via la formation d'acides gras volatiles (AGV). Dans un second temps les AGV vont être transformés soit en acétate soit en dihydrogène. L'étape finale entraine la consommation de l'acétate et du dihydrogène

par les guildes de microorganismes méthanogènes acétotrophes et hydrogénotrophes respectivement afin de produire un mélange de bio-méthane et de dioxyde de carbone. Après purification, le bio-méthane pourra être utilisé comme gaz de ville, biofuel ou pour la production électrique.

En parallèle de la production de biogaz, la méthanisation conduit à un produit communément appelé un digestat, composé des fractions organiques et minérales non dégradées au cours de la digestion. Le procédé de méthanisation peut être mis en place pour traiter divers types d'intrants, à la fois des effluents industriels, d'élevages ou bien de stations d'épuration. Cette diversité d'intrants va imposer une forte variabilité quant aux caractéristiques du digestat obtenu. Cette variabilité pourra constituer dans une certaine mesure une limitation forte et orientera conjointement avec les contraintes locales du territoire sur le choix des procédés les plus adaptés pour une gestion rationnelle des digestats. Cependant, la forme azotée majoritaire dans l'effluent est invariablement l'ammonium. Historiquement, les effluents riches en ammonium sont principalement traités par un procédé biologique de nitrification-dénitrification, bien que d'autres procédés aient été développés plus récemment (A. Magrí *et al.*, 2013). Une représentation non exhaustive des réactions biologiques du cycle biogéochimique de l'azote est présentée au travers de la Figure 1.

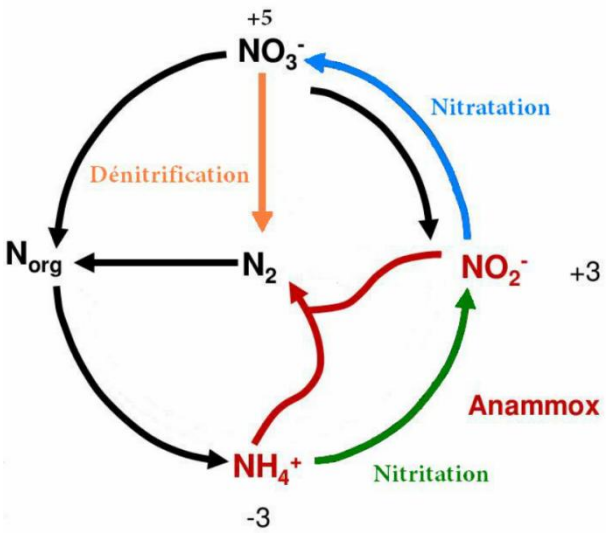

Figure 1 : Principales voies métaboliques impliquées dans le traitement biologique de l'azote.

La finalité des traitements biologiques de l'azote est sa transformation en diazote ($N_{2(g)}$) du fait de son caractère inerte et de son abondance dans l'atmosphère (i.e. 78 %). Pour atteindre cet objectif, les étapes de nitrification (i.e. nitritation et nitratation) assurées par des microorganismes autotrophes consistent en une oxydation successive de l'ammonium (NH_4^+) en nitrite (NO_2^-) puis en nitrate (NO_3^-). Ces étapes d'oxydation se traduisent en pratique par des bassins biologiques de

traitement nécessitant une aération suffisante pour apporter de l'oxygène dissous (OD, O_2) aux microorganismes (M. Henze, 1992). La troisième et dernière étape du traitement est la dénitrification, réalisée par des microorganismes hétérotrophes, elle consomme le nitrate préalablement formé (i.e. une voie de dénitrification à partir du nitrite est aussi possible) conjointement avec la matière organique présente dans l'effluent, aboutissant à la production de diazote (N_2) qui s'échappe du système (M. Henze, 2012).

Cependant une autre voie métabolique permettant le traitement de l'ammonium a été découverte à la fin des années 1980 (A. Mulder *et al.*, 1995) intitulée anammox pour « ANaerobic AMMonium OXydation », elle permet de réaliser une oxydation de l'ammonium en absence d'oxygène en utilisant le nitrite comme accepteur final d'électrons avec un ratio molaire approximativement de 1.00:1.32 (NH_4^+:NO_2^-) (M. Strous *et al.*, 1998) le tout mis en œuvre par une bactérie autotrophe (i.e. chemolithoautotrophe). Bien que plus complexe à réaliser, cette réaction permet le développement de procédés de traitement de l'azote qui présentent des avantages notoires en comparaison avec la nitrification/dénitrification classique. En effet, les procédés anammox ne nécessitent qu'une nitritation préalable partielle de l'effluent limitant ainsi les besoins en oxygène apporté² au système qui peuvent représenter jusqu'à 60% de l'énergie consommée en station d'épuration (H. Siegrist *et al.*, 2008)). De plus, contrairement aux bactéries dénitrifiantes majoritairement hétérotrophes, les bactéries anammox ne nécessitent pas de source de carbone organique (C_{org}) pour leur croissance, permettant ainsi de traiter à moindre coût des effluents possédant un ratio C_{org}/N faibles comme les effluents de digestion anaérobie (A. Magrí *et al.,* 2013). Il apparait aussi que les systèmes anammox sont capables de développer de plus fortes capacités épuratoires par unité de volume, réduisant l'emprise au sol nécessaire (S. Lackner *et al.*, 2015). Un autre avantage réside dans la faible quantité de boues produites, limitant le coûteux besoin de maintenance des systèmes et l'extraction des surplus de matières biologiques produites. Un bilan chiffré des besoins pour les différentes voies métaboliques d'élimination biologique de l'azote est présenté dans le Tableau 1.

Tableau 1 : Comparaison de données métaboliques entre différentes voies biologiques impliquées dans l'élimination de l'azote (E.I.P. Volcke, 2006).

Procédé	Consommation d'oxygène ($kg\ O_2/kg\ N$)	Consommation de matière organique (kg DCO/kg N)	Emissions directs de dioxyde carbone ($kg\ CO_2/kg\ N$)	Production de boues (kg poids sec / kg N)
Nitrification - dénitrification (nitrates)	4.57	2.86	5.76	1-1.2
Nitritation - dénitrification	3.43	1.71	4.72	0.8-0.9
Nitrification partielle - anammox (traitement autotrophe de l'azote)	1.71	0	3.14	<0.1

L'intérêt porté au procédé anammox vient donc du fait des avantages combinés qu'il présente par rapport au procédé classique de nitrification-dénitrification et aux aspects de réduction des coûts opérationnels de telles installations. La technologie anammox est d'autant plus intéressante lorsque la quantité de carbone organique dans l'effluent est faible et donc particulièrement dans le cas des effluents de digestion anaérobie. Plus précisément, le choix technologique le moins couteux pour le traitement de l'azote dans un effluent va être directement dépendant de sa concentration en ammonium. Basée sur l'énergie nécessaire pour traiter un effluent à une concentration donnée en ammonium, il apparait que la nitrification partielle-anammox reste la solution nécessitant le moindre apport, tant que la concentration en ammonium n'excède pas les 2 g $N\text{-}NH_4^+/L$ (A. Magrí *et al.*, 2013). Au-delà de cette concentration d'autres procédés comme le *stripping*-absorption ou l'osmose inverse deviendront plus compétitif en terme d'énergie nécessaire.

Toutefois, au-delà des intérêts exposés ci-dessus, sa mise en œuvre reste complexe. En effet, le taux de croissance de la bactérie anammox est particulièrement faible (i.e. entre 5 et 20 jours, en fonction des conditions et de l'espèce considérée). De ce fait, l'initialisation d'un système anammox reste encore aujourd'hui une tâche longue et demandant une attention toute particulière, appelant à une meilleure compréhension des facteurs impactant cette phase, même si la possibilité d'utiliser une fraction de biomasse pré-enrichie, lorsque cela est possible, est venue raccourcir considérablement cette étape (W.R.L. van der Star, 2007). De plus, la bactérie étant relativement sensible à de nombreux composés, il faudra minutieusement s'assurer que les constituants de l'effluent ne représentent pas, une fois leurs effets sommés, une inhibition excédant la tolérance de l'espèce en présence (R.C. Jin *et al.*, 2012).

Dans ce contexte, ce travail de thèse s'intéresse au développement et à la conduite du procédé anammox appliqué aux digestats liquides municipaux ainsi qu'aux enjeux environnementaux associés. Dans un premier temps, une méthodologique expérimentale a été mise en œuvre pour l'obtention sûre et rapide d'une biomasse anammox pré-enrichie. Tout en permettant l'obtention d'une biomasse anammox fortement active, pouvant être employée au démarrage d'unités de traitement ou pour l'étude en laboratoire, ce travail a permis d'étudier les aspects d'écologie microbienne des processus mis en jeu. Dans un second temps, une attention particulière a été portée sur les aspects d'ingénierie et d'application, sur les inhibitions rencontrées lors du traitement d'effluents réels ainsi que sur les émissions gazeuses du procédé.

Plan de la thèse

Faisant suite à cette introduction, le premier chapitre présente une revue de l'état actuel des connaissances en deux parties. Dans un premier temps, les considérations microbiologiques et physiologiques des genres bactériens présentant un métabolisme d'oxydation anaérobie de l'ammonium (anammox) sont abordées. Cette première partie vise donc à recenser les points d'intérêt biologique qui auront des implications sur la mise en application du procédé. La seconde partie s'intéresse davantage à la sensibilité de la bactérie à différents composés communément rencontrés dans les effluents et au rôle primordial joué par la structuration de la biomasse. Du fait du besoin d'une nitritation partielle (NP) en tête de procédé, les faisabilités de couplage entre la NP et anammox sont finalement détaillées ainsi que les solutions techniques associées (i.e. une et deux étapes) et les émissions gazeuses des différents types de procédés.

Le second chapitre revêt la forme d'une publication bibliométrique. Ces travaux s'intéressent à l'intérêt porté, à la fois par la recherche académique et par l'industrie, sur la gestion des effluents, et plus précisément des nutriments, issus de la digestion anaérobie. Cette étude bibliométrique s'appuie sur le volume de production annuel de contenu, soit sous forme de publication scientifique soit de brevet, afin d'étudier les tendances portées par ces deux domaines sur la période allant de 1995 à 2014. Une attention particulière est apposée sur la place occupée par le procédé anammox au sein de cette gestion.

Le troisième chapitre s'attache à décrire l'étape d'enrichissement initial des bactéries anammox à partir d'échantillons biologiques d'origines différentes. Présenté sous la forme d'une publication, il décrit en détail une méthodologie en batch et les points clés pour réussir dans cette opération. Ce chapitre constitue donc la première des trois étapes vers l'établissement d'un système anammox opérationnel. Comme un point de départ, on y trouve décrit des aspects de biologie moléculaire, d'écologie microbienne et d'ingénierie des procédés au service de la sélection de l'inoculum et des conditions opératoires favorables à leur enrichissement en batch.

Le quatrième chapitre s'inscrit dans la suite chronologique, et vise cette fois à poursuivre l'enrichissement par la mise en œuvre d'un réacteur continu avec un support inoculé à partir d'une biomasse anammox partiellement enrichie durant la période de batch précédemment décrite. Ce chapitre revêt lui aussi la forme d'une publication, qui présente les conditions opératoires favorables à la mise en place d'une culture de microorganismes anammox ainsi que les modifications subies par la communauté microbienne au cours de cette période de fonctionnement étendue.

Le cinquième chapitre présente des essais de mise en œuvre d'un procédé anammox de traitement de la fraction liquide d'un effluent de digestion anaérobie (i.e. boues de station de traitement d'eaux usées). Sous la forme d'une publication, il fait ainsi état d'une comparaison des performances obtenues entre deux procédés de nitrification partielle-anammox fonctionnant en une et deux étapes en utilisant des réacteurs séquentiels. La biomasse anammox enrichie dans le réacteur en colonne a été utilisée pour l'inoculation. Trois points sont particulièrement évalués pendant cette étude : les performances et conditions opérationnelles associées à chacune des configurations, les émissions de protoxyde d'azote ainsi que les effets de certains nutriments et constituants du digestat sur la stabilité du procédé.

Finalement, le sixième et dernier chapitre de conclusions et de perspectives vient clore ce travail et présente à la fois un regard critique sur ce qui a été entrepris et sur des pistes de réflexions pouvant être développées à l'avenir. Cet espace est aussi l'occasion d'introduire certains aspects et observations qui n'ont pas trouvé leur place dans les publications et qui restent néanmoins susceptibles d'alimenter une discussion nourrie.

CHAPITRE 1 : Etat de l’art

CHAPTER 1: State of the art

L'état de l'art présenté ci-après est séparé en deux parties. Une première intitulée « La bactérie et le processus d'oxydation anaérobie de l'ammonium (anammox) » s'attache à retracer l'histoire de la découverte de la bactérie jusqu'à aujourd'hui en explicitant les aspects physiologiques et microbiens afin de permettre une compréhension globale et d'aborder ensuite la partie dédiée au génie des procédés. Cette seconde partie détaille comment le procédé anammox peut être combiné avec l'étape de nitritation partielle de l'effluent ainsi que d'autres considérations relatives aux inhibitions et aux émissions gazeuses.

Partie 1.1 La bactérie et le processus anammox

1.1.1. Historique

L'oxydation de l'ammonium en conditions anaérobies a tout d'abord été mis en avant par le Professeur E. Broda (1977) comme étant une réaction thermodynamiquement favorable (i.e. exergonique) et à ce titre pouvant exister dans la nature, la qualifiant à l'époque de variante de la dénitrification. Cependant il fallut attendre la fin des années 80 pour qu'une bactérie capable de catalyser biologiquement cette réaction soit clairement mise en évidence. Elle a été involontairement enrichie dans un réacteur de dénitrification (A. Mulder *et al.*, 1995) et, faisant dévier les bilans d'azote, a attiré l'attention sur elle. Depuis ce moment, anammox a apporté un regard nouveau sur notre compréhension du cycle naturel de l'azote tout en apportant de grands espoirs pour le traitement des flux anthropiques riches en azote.

En effet une découverte majeure qui s'en est suivie est le rôle joué par anammox dans le recyclage de l'azote dans les sédiments océaniques. De récentes études attribuent à anammox près de 50% des émissions de diazote (N_2) (T. Dalsgaard *et al.*, 2005) en milieu océanique, venant redéfinir notre compréhension du cycle biogéochimique de l'azote. En parallèle les premières études sur l'enrichissement de systèmes de traitement en bactéries anammox sont apparues dans les années 90 (A.A. van de Graaf *et al.*, 1996 ; M. Strous *et al.,* 1998), venant poser les bases de l'enrichissement de ces microorganismes. Toutefois, tous les essais pour réaliser une culture pure de ces organismes sont restés infructueux (M. Strous *et al.*, 1999). Des méthodes d'enrichissement ont été proposées, comme M. Strous *et al.* (2002), modernisant l'approche de Winogradsky/Beijerink pour la culture mixte de microorganismes. L'intérêt unanime suscité par la découverte de cette bactérie a conduit assez rapidement à la découverte de différents genres anammox, aux niches écologiques variées. Les genres Candidatus *Brocadia* (M. Strous *et al.*, 1999) et Ca. *Kuenenia* (M. Schmid *et al.*, 2000) furent rapidement identifiés dans les systèmes de traitement d'eaux usées, tandis que Ca. *Scalindua* (M.M.M. Kuypers *et al.*, 2003) fut identifié dans des sédiments océaniques. Par la suite, d'autres genres bactériens furent encore découverts : Ca. *Anammoxoglobus* (B. Kartal *et al.*, 2007), Ca.

Jettenia (M.S.M. Jetten *et al.*, 2010) et plus récemment Ca. *Anammoximicrobium (*S.V. Khramenkov *et al.*, 2013).

Il faudra ensuite attendre 2002 pour que le premier réacteur anammox à échelle industrielle prenne place à la station d'épuration de Sluisjesdijk, à Rotterdam aux Pays-Bas, (W.R.L. van der Star *et al.*, 2007). Il aura fallu un peu plus de 2 ans afin d'atteindre un régime stationnaire, traitant 8 à 10 kg N/m^3/jour, soit près de 2 fois la capacité attendue initialement. Une seconde étape majeure a été franchie cette année-là, à travers le premier séquençage du génome de Ca. *Kuenenia stuttgartiensis* par M. Strous et ses collègues (2006). La séquence ainsi obtenue du génome représente une mine d'informations quant aux mécanismes biochimiques mis en œuvre par la bactérie, permettant de corroborer les observations réalisées in situ aux voies métaboliques connues à ce jour.

Un frein majeur à l'étude, comme à l'exploitation, de ces microorganismes se situe au niveau de leur temps de doublement. Ce paramètre clé, va être déterminant tant pour le temps nécessaire aux études réalisées, qu'aux contraintes sur l'application pratique du procédé. Au moment de la découverte de ce groupe bactérien, il fut rapporté un faible taux de croissance de 0.065 j^{-1} à 32-33°C (M. Strous *et al.,* 1998) selon les conditions optimales de l'époque, soit près d'une division tous les 11 jours en moyenne. Plus récemment, le développement de méthodes de biologie moléculaire permettant un suivi plus précis, ainsi qu'une meilleure compréhension de sa physiologie, ont permis la mesure des taux de croissance nettement supérieurs de l'ordre de 0.2 à 0.3 j^{-1} (I. Tsushima *et al.*, 2007 ; T. Lotti *et al.*, 2014b). En comparaison, les bactéries nitrifiantes (i.e. Ammonium-Oxidizing Bacteria (AOB) et Nitrite-Oxidizing Bacteria (NOB)) se développent à des taux de croissances variant entre 1.0 et 2.1 j^{-1} selon les conditions du milieu (C. Hellinga *et al.*, 1999). Les bactéries hétérotrophes (dont les dénitrifiantes) ont des taux de croissance encore supérieurs de l'ordre de 3 à 6 j^{-1} à 20°C (M. Henze *et al.*, 2000) soit un temps de doublement allant de 4 à 8 heures.

Fort de cette expérience, en plus de disposer maintenant de boues anammox déjà enrichies, raccourcissant grandement le démarrage des installations, l'établissement de nouvelles installations anammox s'est vu grandement facilité. La dynamique de recherche et de mise en application du procédé est représentée dans la Figure 2 sous la forme du nombre cumulé de publications et d'installations à tailles industrielles. En effet, après une vingtaine d'années, ce sont plus de 100 installations utilisant le procédé anammox qui ont été répertoriées à travers le monde (S. Lackner *et al.*, 2014).

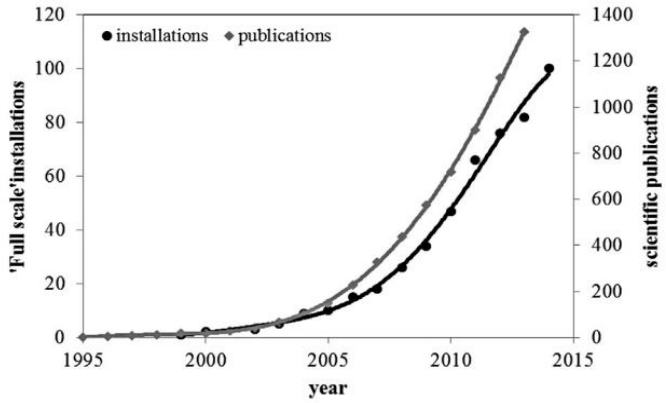

Figure 2 : Nombre cumulé de publications et d'installations utilisant le procédé anammox dans le monde (S. Lackner et al., 2014).

Compte tenu de l'intérêt du procédé en termes de coût et de performance, la recherche comme l'industrie se sont attachées à perfectionner techniquement le procédé. De nombreux types de réacteurs ont été développés, ceci venant s'ajouter à la possibilité de séparer ou de combiner les deux étapes nécessaires au procédé (i.e. nitrification partielle et anammox), ouvrant ainsi un champ de possibilités conséquent en termes de solutions techniques. Malgré le panel de faisabilité technique, on peut voir une tendance claire se profiler dans la réalisation des installations. Le procédé majoritaire est le réacteur séquentiel (i.e. SBR) avec la moitié des installations répertoriées suivi des systèmes granulaires (20%) et MBBRs (~10%). Les systèmes en une étape (i.e. cooccurrence de la nitrification partielle (NP)-anammox) représentent la majorité avec près de 90% du nombre total des systèmes en place. De plus, l'application du procédé, en nombre, est plus souvent faite pour le traitement d'effluents secondaires municipaux (75%), tandis que les installations industrielles, bien que minoritaires, traitent en moyenne des charge azotées nettement supérieures (S. Lackner *et al.*, 2014).

1.1.2. Caractéristiques cellulaires et physiologiques

La conduite de bioprocédés nécessite de connaître un certain nombre de paramètres spécifiques aux processus et bactéries mis en œuvre. Après une vingtaine d'années de recherche sur le procédé anammox, suffisamment d'informations clés sont disponibles afin de le mettre en application. Cependant les contraintes opérationnelles ainsi que l'impossibilité de réaliser des cultures pures rendent difficile une caractérisation fine de sa physiologie.

Composition et structure de la biomasse

Toute structure biologique est assemblée à partir de briques élémentaires au cours de réactions biochimiques qualifiées d'anaboliques. L'analyse élémentaire d'une matrice biologique permet d'identifier la part respective des différents éléments la constituant. Récemment, T. Lotti et ses collègues (2014b) ont réussi à enrichir une biomasse anammox en cellules libres à un degré de pureté encore jamais atteint de 98 ±1%. Ceci a permis d'accroître la précision sur la teneur respective en éléments de la biomasse anammox et d'en énoncer la composition suivante : $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.01}$.

A ce jour, les seuls microorganismes identifiés capables de catalyser biologiquement la réaction anammox appartiennent au phylum des *Planctomycetes*. Cette famille microbienne est singulière et réputée car elle est la seule, dans l'état actuel des connaissances, à posséder des compartiments intracellulaires proches des organites des métazoaires (i.e. la mitochondrie et le chloroplaste). Les bactéries anammox n'échappent pas à cette règle et possèdent une structure interne propre, un sous compartiment lié à la membrane cellulaire, appelée anammoxosome qui représente 30 à 60% (M.S.M Jetten *et al.*, 2001) du volume cellulaire (Figure 3). Bien que le rôle joué par ce compartiment ne soit pas encore complètement déchiffré, il a été montré qu'il contient près de 10 à 15% du contenu protéique de la cellule et qu'il est particulièrement riche en enzymes impliquées dans le métabolisme. Ceci amène a représenter l'anammoxosome comme le siège du catabolisme anammox (M. Strous *et al.*, 2008).

La membrane de l'anammoxosome contient des lipides singuliers, retrouvés uniquement chez cet organite : les ladderanes, formant comme une barrière moléculaire, qui permettraient l'imperméabilité de cette zone à l'hydrazine (N_2H_4) (cf. 1.1.5. Le métabolisme anammox), molécule connue pour sa toxicité. Jusqu'à présent, toutes les observations microscopiques d'organismes anammox ont conclu à des cellules de type coccus, d'une taille d'environ un demi-micromètre.

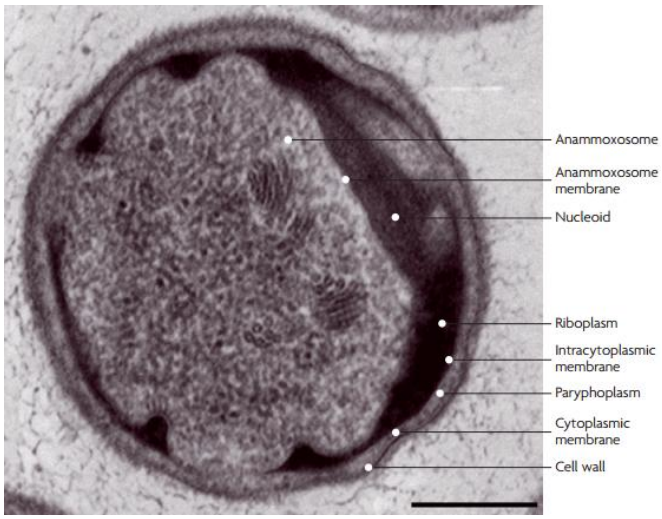

Figure 3 : Représentation de la structure cellulaire de Ca. Kuenenia stuttgartiensis au microscope électronique à transmission (J.G. Kuenen, 2008). La barre en échelle représente 200nm.

Stœchiométrie de la réaction

Une première estimation de l'équation bilan d'anammox a été proposée par M. Strous et ses collègues (1998) (éq. 1) dans un réacteur SBR peu de temps après la découverte de la bactérie.

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

Cette stœchiométrie a été très largement observée et acceptée dans de nombreuses études. Bien que non immuable, pour un organisme donné, la stœchiométrie apparente est la superposition de réactions cataboliques et anaboliques. En fonction de l'état du microenvironnement entourant la cellule, les conditions plus ou moins favorables peuvent entrainer des dépenses énergétiques augmentant le nombre de réactions cataboliques nécessaires par réaction anabolique. Ceci viendra ainsi modifier les coefficients apparents de la réaction. Toutefois, elle a été remise profondément en question par T. Lotti *et al.* en 2014(b) (éq. 2) rapportant des coefficients nettement différents dans un système de culture de cellules suspendues très enrichie en bactéries anammox.

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ + \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O \quad (2)$$

Il semblerait qu'aucune de ces deux stœchiométries ne soit plus légitime que l'autre, elles dépendent uniquement des conditions dans lesquelles on se place. Dans l'exemple de M. Strous *et al.* (1998), ainsi que dans la plupart des études, les réacteurs mis en œuvre possèdent une biomasse hétérogène, comportant un pourcentage non négligeable de microorganismes satellites (i.e. bactéries hétérotrophes dénitrifiantes, etc.) susceptibles de venir influencer les coefficients

stœchiométriques observés. De la même façon, ces communautés hétérogènes mènent à une erreur initiale sur la composition de la biomasse anammox. Donc même si la stœchiométrie initialement proposée par M. Strous *et al.* (1998) est la plus largement observée *in situ*, une fois le procédé en application, celle proposée par T. Lotti *et al.* (2014) serait davantage proche de la stœchiométrie réelle d'anammox débarrassée du bruit parasite des microorganismes satellites.

1.1.3. Phylogénie

Jusqu'à présent aucune bactérie possédant un métabolisme anammox n'a pu être cultivée sous forme d'une culture pure. De par le fait, tous les genres bactériens identifiés portent encore la mention Candidatus. Le premier genre bactérien identifié a été nommé *Brocadia*, et le nom complet donné à l'organisme fut Ca. *Brocadia anammoxidans* (M. Strous *et al.*, 1999). Ensuite, des groupes bactériens aux différences significatives d'un point de vue phylogénétique furent identifiés. Ce fut le cas pour Ca. *Kuenenia* (M. Schmid *et al.*, 2000) puis Ca. *Scalindula* (M.M.M. Kuypers *et al.*, 2003). Ca. *Jettenia* (M.S.M. Jetten *et al.*, 2010) et Ca. *Anammoxoglobus* (B. Kartal *et al.*, 2007) portant à cinq le nombre de genres bactériens clairement identifiés à ce jour. Les différences relatives mesurées entre les différents genres sont représentées sous la forme d'un arbre phylogénétique sur la Figure 4. Il est important de noter que ces 5 genres bactériens forment un groupe monophylétique au sein de la famille des *Brocadiaceae.*

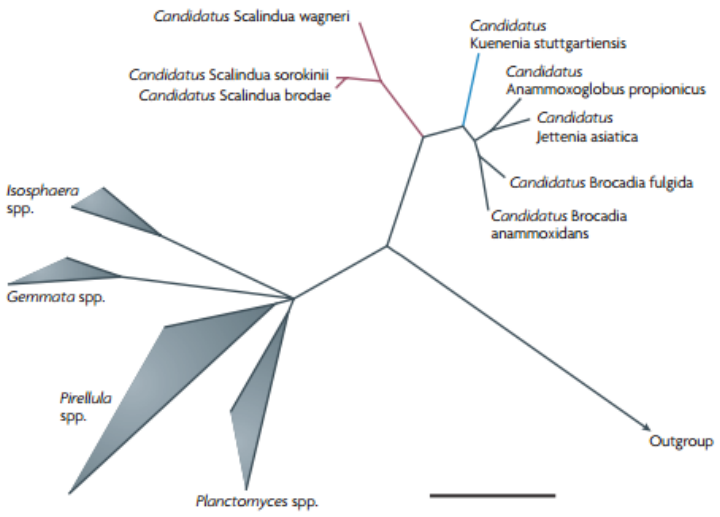

Figure 4 : Arbre phylogénétique représentant la diversité des genres bactériens possédant un métabolisme anammox (la légende représente 10% de divergence au niveau des séquences) (J.G. Kuenen, 2008).

Plus récemment, un sixième genre bactérien possédant un métabolisme anammox, Ca. *Anammoximicrobium*, a été présenté par S.V. Khramenkov *et al.* (2013). L'association taxonomique qui est proposée dans ces travaux viendrait remettre en question l'actuelle organisation des microorganismes anammox en un groupe monophylétique. En effet la souche identifiée serait davantage éloignée des genres connus, tout en restant dans l'ordre des *Planctomycetales*, son affiliation le placerait à proximité de *Pirellula staleyi*.

La principale distinction qui peut être établie entre ces différents genres est la niche écologique qu'ils occupent, c'est à dire l'environnement au sens physico-chimique du terme, qui leur convient le mieux. Cependant même si de grandes tendances apparaissent rapidement, il reste impossible de généraliser. On retrouvera principalement les genres *Brocadia* et *Kuenenia* dans les systèmes anthropisés, aux températures mésophiles. Il est actuellement admis que la grande distinction entre les deux genres réside dans le fait que les *Brocadia* possèdent un taux de croissance (μ_{max}) supérieur, et sont donc qualifiés de «r-strategist» (W.R.L. van der Star *et al.* 2008 ; E. Isanta *et al.*, 2015), tandis que les *Kuenenia* possèderaient une meilleure affinité pour le substrat (nitrites) (cf. Tableau 2), et sont donc qualifiés de «K-strategist». Cependant au sein même d'un genre il existe de fortes différences. Par exemple, la matière organique est décrite comme inhibitrice pour la plupart des espèces anammox, cependant deux espèces, *Brocadia fulgida* et *Anammoxoglobus propionicus* sont connues pour tirer un avantage sélectif à travers l'oxydation (dans un but uniquement catabolique) de l'acétate et du propionate respectivement (D. Güven *et al.*, 2005). A l'inverse il semblerait que les genres *Scalindula* et *Anammoximicrobium* (M.M. Kuypers *et al.*, 2005 ; S.V. Khramenkov *et al.*, 2013 ; R. Connan *et al.*, 2016) soient davantage retrouvés dans les milieux naturels. Ils seraient plus compétitifs à basse température, et dans le cas de *Scalindula* à plus forte salinité.

1.1.4. Paramètres physiologiques

La multiplicité des genres bactériens réalisant la réaction anammox mène à des fluctuations des paramètres physiologiques pouvant présenter des avantages ou des inconvénients lorsqu'il s'agit de les mettre en œuvre dans un procédé de traitement. En effet, des paramètres simples comme la température, le pH, ou la force ionique vont venir influencer très fortement les performances de tel ou tel genre ou espèce. L'influence de ces paramètres est présentée ici dans le Tableau 2.

Il est possible de trouver dans la littérature de larges gammes de pH et de températures données comme viables. Cependant il est important de souligner que ces valeurs sont le plus souvent issues de tests d'activité réalisés en batch sur de courtes périodes (quelques heures) et représentant davantage une mesure de l'inhibition instantanée dans des conditions données. Il se peut tout à fait

que les extrémums des gammes ainsi testées ne soient pas viables sur de plus longues périodes ou en routine. A l'inverse, une augmentation progressive de la concentration en inhibiteur peut permettre une adaptation des microorganismes et/ou des communautés microbiennes à des concentrations finales supérieures à celles mesurées en inhibition instantanée. De plus, une certaine hétérogénéité transparait dans les valeurs d'affinité aux substrats (K_S pour NH_4^+ et NO_2^-) obtenues. Ceci peut être intrinsèque au genre et à l'espèce considérée, mais peut aussi être fortement affecté par l'état de la biomasse. En effet, en fonction de la taille des agrégats, une limitation peut apparaitre à travers la diffusion du substrat vers les couches intérieures d'un biofilm ou d'un granule, entrainant une surestimation inévitable de l'affinité mesurée.

Tableau 2 : Principaux paramètres physiologiques recensés pour les différents genres et espèce présentant un métabolisme anammox dans la litérature. (n.c. correspond à "non caractérisé").

	Temps de doublement (j) (et taux de croissance (j^{-1}))	Température considérée pour le temps de doublement (°C)	Constante d'affinité au substrat - K_S (µM)		Gamme pH	Gamme de températures (°C)	Références
			NH_4^+	NO_2^-			
Ca. *Kuenenia stuttgartiensis*	5.8 - 7.5 (0.12 – 0.092)	38 30	n.c.	0.2-3	6.5-9	25-37	W.R.L. Van der Star *et al.* (2008) A. Dapena-Mora *et al.* (2007)
Ca. *Brocadia sinica*	7 (0.099)	37	28 ± 4	34 ± 21	6.5-8.5	25-45	M. Oshiki *et al.* (2011)
Ca. *Brocadia anammoxidans*	10.6 (0.09)	20 - 43	5	. < 5	n.c.	n.c.	M. Strous *et al.* (1998)
Ca. *Brocadia sp.*	3.3 (0.21)	30	n.c.	8.2	6.8-7.5	30	T. Lotti *et al.* (2014)
Ca. *Brocadia* sp.	5.5 – 8.3 (0.12 - 0.084)	30	640 ± 130	350 ±90	7.2	30 ± 1	D. Puyol *et al.* (2013)
Ca. *Anammoximicrobium*	32 (0.022)	20	29.3	27.1	7.8-8.3	20	S.V. Khramenkov *et al.* (2013)
Ca. *Scalindua profunda*	n.c.	20 - 22	n.c.	n.c.	7.4	15-45	J. Van de Vossenberg *et al.* (2008)
Ca. *Jettenia caeni*	14.2 (0.049)	37	17.1 ± 4.3	35.6 ± 0.92	6.5-8.5	20 – 42.5	M. Ali *et al.* (2015)

1.1.5. Le métabolisme anammox

Les observations préliminaires d'A.A. van de Graaf *et al.* (1996) puis celles de M. Strous *et al.* (1998) durant leurs travaux, couplés au séquençage du génome de Ca. *Kuenenia stuttgartiensis* en 2006 (M. Strous *et al.*, 2006) et aux valeurs thermodynamiques, ont permis la reconstruction des voies métaboliques les plus probables du métabolisme anammox présentées dans la Figure 5.

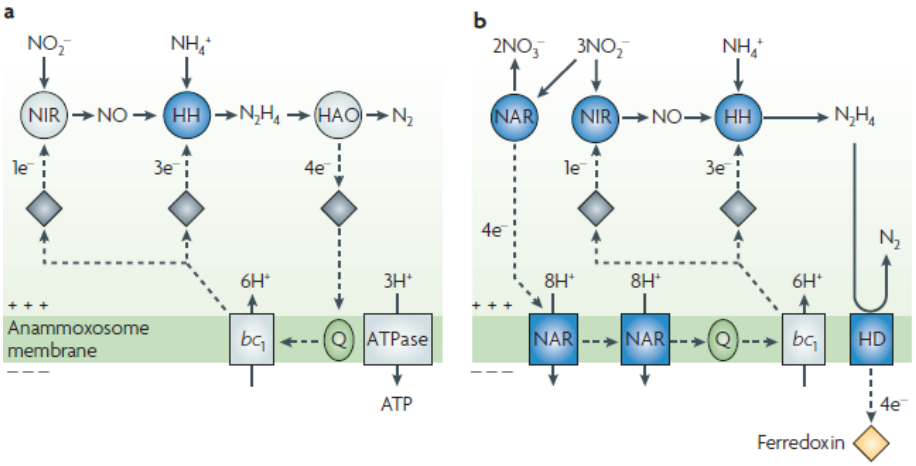

Figure 5 : Hypothétique catabolisme (a) et anabolisme (b) de la réaction anammox (M. Strous et al., 2008). HAO : hydrazine oxydase, HH hydrazine hydrolase, HD : hydrazine dehydrogenase, NIR : nitrite réductase, NAR : nitrate réductase, bc1 : complexe cytochrome bc1.

La Figure 5a représente la partie catabolique de la réaction dite « anammox », qui vise à l'établissement d'une force proton motrice (FPM) via l'oxydation de l'ammonium en utilisant le nitrite comme accepteur d'électrons. Le nitrite est initialement réduit en oxyde nitreux (NO) par une nitrite réductase (NirS). Cette réaction préalable permet à une seconde enzyme, l'hydrazine hydrolase, de conjointement combiner ammonium et oxyde nitreux afin de former de l'hydrazine. Ces deux premières étapes nécessitent l'incorporation de 4 électrons de faible énergie. Par la suite l'hydrazine est oxydée en diazote, libérant 4 électrons de haute énergie pouvant circuler le long des quinones de la chaine de transmission d'électrons, ainsi que 4 protons. La coenzyme Q-cytochrome c réductase (aussi appelée complexe bc1) transfert les protons vers la lumière de l'anammoxosome, générant ainsi la FPM qui permettra la régénération de ADP en ATP en énergisant une ATP-ase pompe à protons. Les électrons sont alors recyclés à partir de la quinone-cytochrome bc1 afin de réaliser la formation *de novo* de l'hydrazine.

La Figure 5b illustre la partie anabolique du métabolisme anammox. La FPM liée au transport inverse d'électrons combinée au catabolisme central avec un ion nitrate (NO_3^-) réductase permet de générer de la ferrédoxine utilisée pour la fixation du dioxyde de carbone *via* la voie de l'acétyl-CoA. L'hydrazine est capable de donner des électrons de haute énergie à la ferredoxinne, mais ces électrons ne sont pas recyclés. L'oxydation du nitrite en nitrate *via* une nitrate réductase (NAR) compense cette perte,

mais procure des électrons de basse énergie qui devront être énergisés par la FPM avant d'être réinjectés dans le cycle catabolique classique. Cette élévation du niveau d'énergie est opérée par une nitrate réductase s'appuyant sur l'énergie provenant de la FPM.

Comme il en a été fait mention plus amont, le séquençage du génome de Ca. *Kuenenia stuttgartiensis* s'est révélé une mine d'information sur le métabolisme des bactéries anammox et sur leur, jusqu'alors, insoupçonné versatilité. De par un intérêt évolutif certain, les bactéries sont connues pour être capables de merveilles d'adaptation quand il s'agit d'exploiter au mieux les ressources de leur milieu. Il semble dès lors assez attendu de retrouver une diversité métabolique chez les bactéries anammox comme tout autre organisme. Cependant, ce sont plus de 200 gènes, liés aussi bien au métabolisme anammox qu'à la respiration cellulaire, qui ont été retrouvés dans son génome (M. Strous *et al.*, 2006).

Une explication à cette résilience et cette capacité d'adaptation trophique réside dans la diversité des accepteurs finaux d'électrons que leur chaine respiratoire est capable d'admettre. Il a été montré que Ca. *Kuenenia stuttgartiensis* est capable notamment d'utiliser les oxydes de fer et de manganèse comme accepteur final d'électrons avec le formate comme donneur. De même le fer peut être oxydé en utilisant le nitrate comme accepteur final d'électrons (M. Strous *et al.*, 2006). Peu d'informations sont disponibles à ce sujet, mais ces spécificités, si elles s'avèrent réellement genre dépendant, pourraient constituer un atout afin de sélectionner le genre anammox enrichi dans un système.

Ces dernières années, le séquençage de génome s'est grandement démocratisé. D'autres études sont venues apporter davantage d'informations sur d'autres génomes de bactéries anammox, permettant d'identifier les différences en termes de gènes, d'un génome à l'autre. Cela a été le cas pour Ca. *Brocadia fulgida* (F. Gori *et al.*, 2011), Ca. *Scalindua profunda* (J. van de Vossenberg *et al.*, 2008) et Ca. *Jettenia asatica*, anciennement désignée par KSU-1 (D. Hira *et al.*, 2012). Le croisement de ces études montre tout d'abord une différence sur la première réaction du cycle catabolique. En effet la réduction du nitrite en oxyde nitreux est catalysée par une enzyme de type NIR. Chez les bactéries dénitrifiantes, deux métalloprotéines jouant un rôle de nitrite réductase sont bien connues, le gène *nirS* codant pour le cytochrome cd1NIR (possédant deux noyaux hemes) et le gène *nirK* codant pour CuNIR (possédant deux atomes de cuivre) (P. Tavares *et al.*, 2006). Les travaux de séquençage ont révélé que Ca. *Kuenenia stuttgartiensis* utilise le gène *nirS* et donc cd1NIR pour catalysé cette première étape, tout comme Ca. *Scalindua profunda* et Ca. *Brocadia fulgida*. Cependant il a été montré que Ca. *Jettenia asiatica* utilise quant à elle une CuNIR et qu'elle possède un gène de type *nirK* à la place de *nirS*.

De manière générale, un recoupement des gènes retrouvés chez Ca. *S. profunda* et chez Ca. *K. stuttgartiensis* montre que sur 4756 gènes identifiés chez cette première, 2016 ne trouvent pas leur équivalent chez la seconde, soit près de la moitié. Des différences ont été clairement identifiées pour les gènes *narK* codant pour le transport du nitrite, plus variés chez Ca. *K. stuttgartiensis*, possédant une forte

affinité pour le nitrite. De même, de nombreux gènes ont été retrouvés chez Ca. *S. profunda* relatifs au transport et l'utilisation de peptides organiques (J. van de Vossenberg *et al.*, 2013).

Partie 1.2. De la bactérie au procédé

1.2.1. Structure de la biomasse

Récemment, un intérêt accru est porté vers l'utilisation de biomasses granulées dans les systèmes de traitement. De telles structures sont retrouvées en conditions aérobie, en anaérobie, ou même dans des conditions intermédiaires. Ce type de structure est utilisé par une large gamme d'organismes comme les « Polyphosphate Accumulating Organisms » (PAO) pour la bioconcentration du phosphore (M.K.H. Winkler *et al.*, 2011), pour la production de bio-hydrogène (K.-S. Lee *et al.,* 2004), ou même dans des systèmes de co-traitement, ici pour le nitrate, le sulfate et le lactate (C. Chen *et al.*, 2008). De par la forte rétention dans les systèmes (temps de séjour solide élevé), ce type de structure constitue un avantage majeur lors de l'implication d'organismes à faible taux de croissance. D'un point de vu évolutif, les biofilms comme les granules peuvent être interprétés comme des stratégies présentant un intérêt à fixer une communauté dans un microenvironnement aux conditions favorables, là ou des cellules libres seraient systématiquement dispersées. Les organismes développant un métabolisme anammox n'échappent pas à cette règle. Ainsi on peut retrouver 4 grands types de structuration de la biomasse, présentés ici dans un ordre croissant d'organisation.

Sous forme de cellules libres

Récemment, une méthode a été proposée par T. Lotti *et al.* (2014b) visant à la culture de microorganismes anammox en cellules libres (i.e. cellules indépendantes physiquement). Cet état de la biomasse ne représente pas forcément un avantage pour les systèmes de traitement, en effet l'état dispersé implique une moindre retenue dans un bioréacteur et une exposition maximale aux solutés contenus dans l'effluent. Il revêt néanmoins un attrait particulier pour l'étude des caractéristiques physiologiques des microorganismes ainsi isolés et cultivés.

Sous forme de boues activées

Une boue activée (Figure 6) est une structure composée par un ensemble de microorganismes formant un agrégat le plus souvent autour d'un support organique ou minéral que l'on nomme un floc. Cette structure, appelée floculée, résulte donc de la synthèse d'exopolysaccharides combinés à la prolifération contrôlée de bactéries filamenteuses donnant une cohésion à l'ensemble.

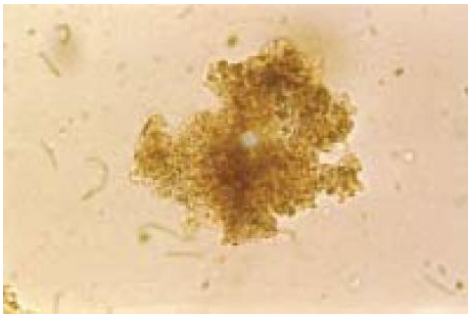

Figure 6 : Floc bactérien dans une boue activée (FNDAE n°33 - Dysfonctionnement biologiques des stations d'épuration, 2005).

Cet état de la biomasse est communément rencontré dans les stations d'épuration biologique, il permet un découplement entre le temps de séjour hydraulique et le temps de séjour du solide, permettant d'accumuler de la biomasse dans le système. Il est employé dans le procédé de nitrification / dénitrification pour l'abattement de la matière organique, ainsi que de l'ammonium.

Sous forme de biofilm

Un biofilm bactérien est une structure communément observée à la surface des matériaux. Similairement aux flocs, il résulte de l'agglutinement de bactéries au sein d'une matrice extracellulaire constituée d'exopolysaccharides (Figure 8). Cependant, à la différence d'une boue activée, le biofilm va venir se déposer sur une surface potentiellement étendue. Son épaisseur et son âge peuvent être assez variable et vont principalement dépendre du stress mécanique imposé par le milieu. Ce dernier sera aussi susceptible d'impacter la sélection de certaines populations microbiennes (A. Rochex *et.al.*, 2008). De même, la diffusivité des nutriments vers la base du biofilm va aussi constituer une limitation forte quant à son épaississement (C. Picioreanu *et al.*, 1998). C'est dans cette stratification cellulaire, cette rupture entre milieu extérieur et le microenvironnement ainsi créé au cœur du biofilm, que réside un intérêt fort de cet état de la biomasse.

En effet, si la diffusivité des nutriments est ralentie, cela signifie que la concentration réelle du milieu n'est pas la concentration ressentie par les organismes au cœur du biofilm. Cette nuance peut jouer un rôle capital lors de l'implémentation de systèmes de traitement, permettant ainsi d'exposer le biofilm à des concentrations qui seraient inhibitrices pour des cellules libres.

Sous forme de granules

Une particularité unanimement rencontrée et exploitée chez les organismes anammox est leur capacité à former des agrégats compacts sous forme de granules lorsque la biomasse atteint un degré d'enrichissement suffisant. Récemment, des analyses de transcriptomique sont venues montrer que

certains gènes impliqués dans le métabolisme et le transport des nutriments sont surexprimés lorsque le système granulaire est en place (i.e. tardivement dans le processus d'enrichissement) ceci venant conforter l'hypothèse initialement émise comme quoi les granules ainsi formés voient leur taille augmenter à cause d'une multiplication interne.

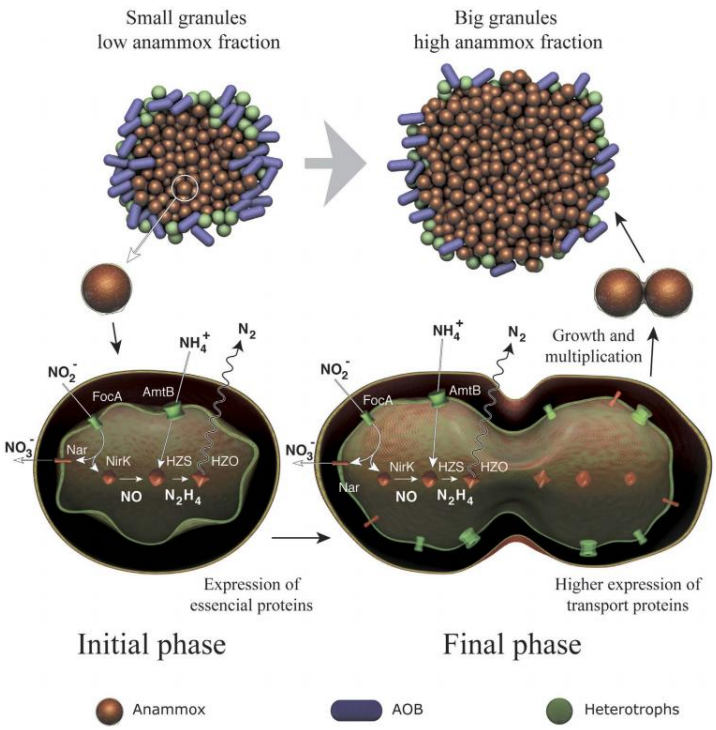

Figure 7 : Schéma de la structure d'un granule et de son mécanisme d'élargissement (S. Bagchi et. al., 2015)

Comme représenté sur la Figure 7, la périphérie du granule est principalement occupée par d'autres guildes[1] de microorganismes, dont deux grands groupes qui se distinguent particulièrement. En effet, la présence d'oxygène dans l'effluent, à l'état de traces (système anammox dédié) ou volontairement insufflé, (NP et anammox en une seule étape) va conduire au développement de bactéries nitrifiantes (i.e. AOB et NOB). Le développement des NOB sera volontairement inhibé (cf. partie 1.2.3.) afin d'éviter la formation de nitrates. De plus, la formation de nitrates par les bactéries anammox, due à leur anabolisme, va permettre la subsistance d'un second groupe, les bactéries dénitrifiantes hétérotrophes, s'appuyant sur la présence résiduelle de matières organiques exogènes, du recyclage cellulaire, ainsi que des sulfates présents en proportions variables.

Plusieurs paramètres intrinsèques du milieu sont susceptibles de venir favoriser ou inhiber la granulation. Comme dans le cas du biofilm, le stress mécanique provenant de la turbulence du milieu va être un point essentiel pour initier la réponse biologique adaptée qui est la granulation. Cependant un stress mécanique trop important pourra entrainer la rupture des granules et la déstabilisation du procédé

[1] Guilde microbienne se compose d'espèces étroitement apparentées exploitant en même temps, de la même manière et au même endroit une ressource commune.

(B. Arrojo *et al.*, 2006 ; C.J. Tang *et al.*, 2009). Il a été montré que lors de la granulation, la structure biologique est susceptible d'incorporer une part de précipités salins et repose sur leur présence (A. Dapena-Mora *et al.*, 2010). A l'inverse T. Lotti *et al.* (2014b) ont montré que de faibles teneurs en cations bivalents (i.e. calcium, magnésium) permet la culture en cellules libres et tend à inhiber la formation des granules.

Diffusion dans les structures

Comme il en a été fait mention dans les sections précédentes, des structures compactes comme un biofilm ou un granule constituent un microenvironnement distinct du fluide qui l'entoure (Figure 8). Ceci vient du fait que les éléments du milieu extérieur doivent être transportés jusqu'à l'intérieur de la structure et que cette dernière va imposer une résistance au transfert, introduisant des aspects cinétiques. Pour certains métabolites comme le nitrite ou l'ammonium, une consommation concomitante est un facteur qui tend à réduire davantage la progression de ces composés au cœur des biomasses structurées, limitant d'autant plus leur caractère potentiellement inhibiteur.

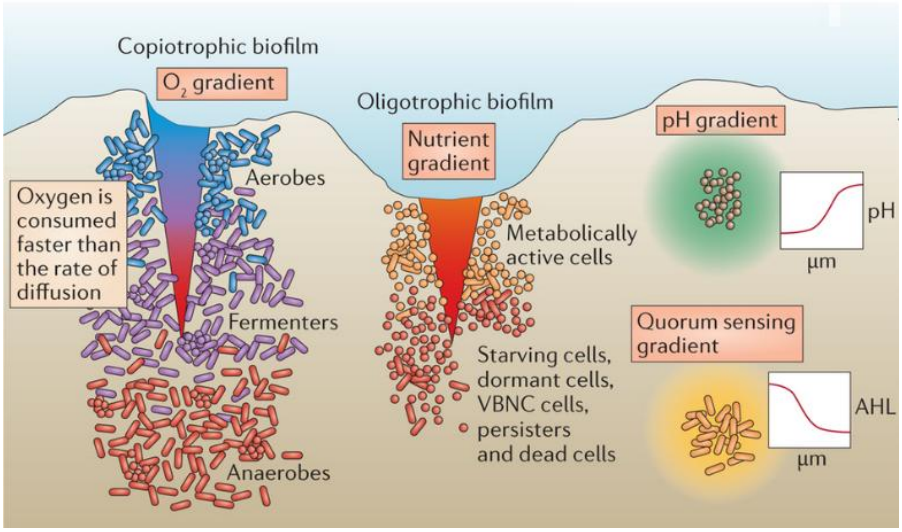

Figure 8 : Resprésentation schématique de la diffusion d'éléments dans une structure de type biofilm (H.-C. Flemming et al., 2016).

Comme le montre la Figure 8, de nombreux paramètres essentiels au bon fonctionnement d'un procédé microbien vont être affectés par ce transfert, comme la concentration en oxygène dissous, les concentrations en nutriments (ammonium, nitrites, etc.), ainsi que le pH. Comme il en est fait mention dans la section précédente, l'organisation stratifiée du granule anammox n'est que la résultante de cette diffusion limitée et du potentiel redox décroissant de l'extérieur vers l'intérieur. La diffusion dans la matrice biologique se fait principalement *via* des mécanismes lents de diffusion, plus rapides dit d'advection, et nécessite le franchissement de différentes interfaces de transfert, dont les épaisseurs respectives vont être principalement impactées par la viscosité du milieu, la rugosité du biofilm et le régime hydraulique (i.e., la turbulence du milieu) (C. Picioreanu *et al.*, 1998). En effet dans le cas d'un granule anammox, au fur et à

mesure du processus de diffusion, l'oxygène apporté par le milieu extérieur est rapidement consommé par les AOB, puis le nitrite nouvellement formé est consommé par les bactéries anammox sous-adjacentes, et enfin il a même été rapporté des traces de bactéries méthanogènes habitant le cœur du granule dépeuplé par la déplétion progressive de l'apport en nutriments extérieurs (Z. Hu *et al.*, 2012).

1.2.2. Inhibitions

La physiologie bactérienne repose sur une série de processus visant à assurer la survie de l'organisme unicellulaire dans son microenvironnement. Comme explicité précédemment, ces processus peuvent être découpés en plusieurs groupes ; ceux à la base d'une production énergétique, appelés catabolisme et ceux appelés anabolisme, à la base du renouvellement des constituants de l'organisme.

Cependant cette vision binaire de l'activité microbienne reste fortement réductrice, pour plus de justesse il faudrait prendre en compte d'autres processus impliqués dans les activités parallèles situationnelles. Il a été fait mention de différents états possibles de la biomasse anammox, ces états sont la résultante de l'expression de certains gènes aboutissant à la synthèse de molécules permettant une agrégation en granules ou en biofilms à l'aide de composés extracellulaires exportés par la bactérie elle-même dans son milieu. Cette matrice extracellulaire est un investissement réalisé par la bactérie, sur le plan énergétique, lui assurant un maintien géographique dans un microenvironnement lui étant davantage favorable. A l'instar de ces mécanismes, les stratagèmes mis en place par une bactérie afin de maintenir son homéostasie face à certains composés exogènes, que l'on appelle communément des inhibiteurs, vont déclencher l'activation ou la répression de gènes. Plusieurs stratégies sont retrouvées dans le monde microbien, telles que l'excrétion, le rejet dans le milieu extérieur *via* des transporteurs actifs ou la synthèse de chélateurs afin de rompre la réactivité d'une molécule pénétrant son milieu intérieur. Le point important est que plus un organisme doit mettre en place des voies d'expressions, moins il aura d'énergie chimique à investir dans sa croissance. En d'autres termes, le nombre de réactions cataboliques par réaction anabolique devra augmenter. Ceci peut être comparé à de l'inefficacité métabolique à travers un effort supplémentaire pour croitre. Cependant la mise en place de stratégies d'adaptation à un milieu peu conventionnel, aux constantes physico-chimiques peu communes et défavorables constitue un levier à la colonisation de niches écologiques vacantes.

Inhibition par les substrats

Afin de conduire un procédé dans des conditions optimales, et donc mettre en place l'activité microbienne la plus importante possible, il sera donc préférable d'utiliser le ou les microorganismes les plus adaptés à l'effluent à traité. Il est donc nécessaire de quantifier l'impact des différents composés sur le microorganisme. Pour anammox, les premiers composants qui nous intéressent sont l'ammonium et le nitrite. A la fois substrats et inhibiteurs, il faut veiller à ne pas dépasser certaines teneurs afin de ne pas

risquer une inhibition, qui si elle se prolonge, peut entrainer une mort cellulaire. Un aperçu des valeurs d'inhibition rencontrées dans la littérature est présenté dans le Tableau 3.

Tableau 3 : Impact de différentes concentrations en nitrite sur l'activité microbienne anammox.

Conditions	Biomasse (genre anammox)	Concentration en nitrites (mg $N\text{-}NO_2^-$/L)	Inhibition	Référence
EGSB flux continu	Granular (Ca. *Kuenenia stuttgartiensis*)	233.7	24%	T.T. Chen *et al.* (2011)
SBR	n.c.	4.8	~30 %	B. Wett *et al.* (2007)
Upflow flux continu	Granular (Ca. *Brocadia*)	115.7	31%	C.J. Tang *et al.* (2010)
Batch	Granular (Ca. *Brocadia*)	121.7	50%	T. Lotti *et al.* (2012)
Batch	Biofilm (Ca. *B. sinica)*	68.2	50%	M. Oshiki *et al.* (2011)
Batch	Granules (Ca. *Brocadia*)	400.0	50%	T. Lotti *et al.* (2012)
SBR	Biofilm	73.0	0%	I. Fernández *et al.* (2012)
SBR	Granule[a]	> 30.4	100%	M. Strous *et al.* (1999)
RBC	Biofilm (*B. anammoxidans*)	> 56.3	100%	K. Egli *et al.* (2001)

(a) : la biomasse granulaire a été préalablement fractionnée (mécaniquement) afin de réduire la résistance au transfert.
EGSB : Expanded Granular Sludge Bed ; RDC : Rotating Bed Contactor.
n.c. : non caractérisé

Les bactéries anammox utilisent des nitrites comme accepteur final d'électrons au cours de leurs voies métaboliques. Toutefois, demeurer sous une valeur seuil de concentration en nitrites semble être important pour éviter tout blocage du métabolisme. De nombreuses études ont été publiées concernant cet aspect clé du métabolisme anammox. Malgré ce sujet bien décrit, aucun seuil consensus n'a encore été défini, les résultats montrent une plage très variable de valeurs pour à la fois l'IC_{50} (i.e. concentration provoquant une baisse d'activité de 50%) et l'inhibition complète allant de 68.2 à 400 mg $N\text{-}NO_2^-$/L et 30.4 à 56.3 mg $N\text{-}NO_2^-$/L, respectivement (Table 3). Il est mis en avant que la structure de la biomasse (i.e. épaisseur du biofilm, diamètre moyen des granules), que le genre ou l'espèce anammox considéré, ainsi que la situation globale d'inhibition, la charge en azote et le temps d'exposition sont autant de paramètres qui viendront influencer très fortement la sensibilité au nitrite, comme à tout autre composé inhibiteur (T. Lotti *et al.*, 2012 ; I. Fernández *et al.*, 2012). Il apparaît donc que le paramètre concentration, pris indépendant, reste peu informatif et qu'un schéma plus global sera nécessaire pour évaluer l'impact sur un système donné.

Pendant longtemps, la question sur l'acteur de l'inhibition entre le nitrite et l'acide nitreux est restée sans réponse. Récemment les travaux publiés semblent désigner de plus en plus le nitrite comme étant l'agent de cette inhibition (T. Lotti *et al.*, 2012, D. Puyol *et al.*, 2014). Il fait sens de préciser qu'un temps d'exposition croissant va exacerber l'impact de l'inhibition (I. Fernández *et al.*, 2012). Cependant des résultats contraires apparaissent sur le rôle protecteur (T. Lotti *et al.*, 2012) ou aggravant (J.M. Carvajal-Arroyo *et al.*, 2014) de la déplétion concomitante en ammonium.

Depuis le début des études réalisées sur la physiologie anammox, l'inhibition due à l'ammonium a toujours été rapportée comme secondaire face au nitrite. Si M. Strous *et al.* (1999) ne rapporte aucune inhibition due à l'ammonium ou au nitrate jusqu'à 1 g N/L, d'autres études se sont ensuite intéressées à l'impact que peut avoir l'ammoniac (NH_3). Cependant cela implique un second paramètre, le pH, qui joue un rôle déterminant dans l'équilibre acido-basique entre NH_4^+ et NH_3. Etant donné que le pH impacte fortement l'activité microbienne, cela vient compliquer l'analyse. Récemment une étude s'est attachée à démontrer à l'aide d'un large criblage que le pH était le seul responsable de cette inhibition dans la gamme de concentrations classiquement rencontrée (D. Puyol *et al.*, 2014).

Effets de la matière organique

Que ce soit dans les eaux usées urbaines, les effluents de digestion anaérobie ou les effluents d'élevage, une part variable de matières organiques va être systématiquement retrouvée. Ce pool est très hétérogène dans sa constitution et est souvent quantifié sans distinction sous forme d'équivalent demande chimique en oxygène (DCO). Le Tableau 4 présente des valeurs d'inhibition rencontrées lors du traitement d'effluents aux contenus en matières organiques différents.

Si les composés organiques montrent potentiellement un effet inhibiteur sur les microorganismes anammox, plusieurs points rendent difficile la caractérisation de leurs effets. Le premier réside dans le fait que les matières organiques représentent un ensemble presque infini de molécules diverses, qui auront chacune un effet propre sur un microorganisme. Deuxièmement, si certains composés comme le glucose ou le méthanol montrent une inhibition claire, d'autres molécules comme le propionate, l'acétate ou le formate peuvent jouer le rôle de substrat pour certaines bactéries anammox, voire même leur donner un avantage sélectif, comme il en a été fait état précédemment. Enfin, les biomasses utilisées pour ces tests de sensibilité sont le plus souvent enrichies avec des substrats synthétiques, de ce fait l'exposition à la matière organique devient un élément ponctuel perturbateur. Cette exposition pourrait assez mal refléter l'effet réel sur une biomasse anammox lors d'une exposition continue et en parallèle de phénomènes d'adaptations biologiques.

Bien que certaines études récentes font état de systèmes de traitement stables à de fortes charges organiques, il reste établi qu'une charge entrante en DCO importante est source de perturbations pour le

système, notamment à travers un développement important d'une biomasse hétérotrophe (B. Molinuevo *et al.*, 2009), pouvant dans certains cas devenir le processus principal d'élimination de l'azote du système, excluant progressivement les populations anammox (M. Ruscalleda *et al.*, 2008).

Tableau 4 : Impact de la matière organique sur l'activité anammox.

Réacteur	Matière organique	Teneur	Effets	Références
Réacteur anaérobie	Glucose	0.5-3 mmol/L	0%	D. Güven *et al.* (2005)
	Formate, acétate and alanine	0.5-3 mmol/L	0%	D. Güven *et al.* (2005)
	Propionate	<3 mmol/L	0%	D. Güven *et al.* (2005)
UASB en continu	DCO	>300 mg/L	-100%	N. Chamchoi *et al.* (2008)
UASB en continu	Sucrose (exprimé en DCO)	700 mg/L	-98%	C.J. Tang *et al.* (2010)
UASB en semi-continu	Lisiers (après post-digestion)	>237 mg DCO/L (112 mg DCO/L/d)	-100%	B. Molinuevo *et al.* (2009)
	Lisiers (après une oxydation partielle)	>290 mg DCO/L (136 mg DCO/L/j)	-100%	B. Molinuevo *et al.* (2009)
CSTR	Méthanol	24.0 mg DCO/L	-100%	D Güven *et al.* (2004)

CSTR : Continuously Stirred Tank Reactor ; UASB : Upflow Anaerobic Sludge Bed ; DCO : Demande chimique en oxygène.

Effet du pH

Pour fonctionner de manière nominale une cellule repose, entre autre, sur des fonctions métaboliques assurées par des d'enzymes. Hors, une enzyme possède une plage de pH sur laquelle elle peut fonctionner de façon plus au moins optimale, poussant la cellule à établir un gradient de pH avec le milieu extérieur, même si localement, le pH cytoplasmique connait des fluctuations. De la même façon, le métabolisme anammox repose sur l'établissement d'une force proton motrice qui pourra être perturbée si le pH extérieur s'éloigne trop de la valeur optimale. La Figure 9 présente la variation d'activité spécifique en fonction de la variation en pH du milieu. A travers cette représentation graphique, il est possible d'appréhender le caractère capital du contrôle du pH pour la mise en place d'un procédé anammox stable et performant. Une faible variation de celui-ci par rapport à l'optimum peut entrainer une diminution drastique de l'activité spécifique, pouvant aller jusqu'à l'inhibition totale pour une variation de moins d'une unité pH. Si l'optimum pour des cellules libres se situe aux alentours de 7.5, celui d'une biomasse granulaire semble se situer à 7.3. Ceci illustre le fait que très souvent la valeur optimum va plus ou moins varier en

fonction des paramètres du système étudié. Il est possible de voir que l'état de la biomasse fait varier l'optimum de pH ainsi que la tolérance de la bactérie (i.e. chute plus importante de l'activité pour une variation de pH donnée). Cela vient surement du fait que l'alcalinisation du milieu dû à la réaction anammox permet à la bactérie de tolérer un pH du milieu plus acide en se créant un microenvironnement plus favorable lorsqu'elle est granulée ou en biofilm (i.e. gradient de pH entre l'effluent et le granule ou le biofilm). Alors qu'une cellule libre subit plus directement les conditions imposées par l'effluent.

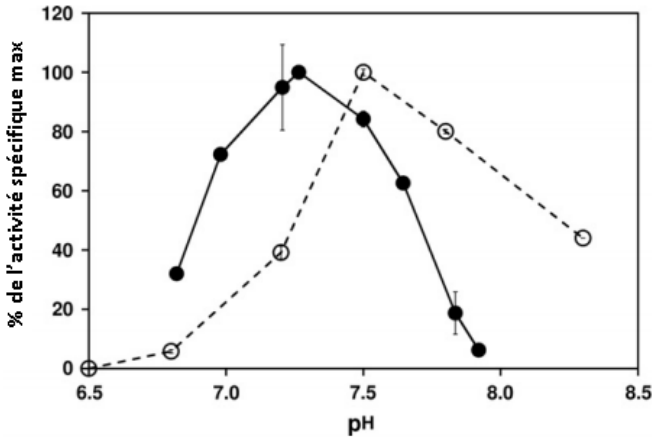

Figure 9 : Impact du pH sur l'activité anammox (plein : biomasse granulaire, vide : biomasse libre) (J.M. Carvajal-Arroyo et al., 2013)

Impact des composés ioniques.

De nombreux autres composés communs sont capables d'entrainer une inhibition de l'activité microbienne anammox. C'est le cas notamment du phosphore, sa présence en tant qu'oligoélément est capitale tant pour la synthèse de l'ADN ou de l'ATP, tandis qu'une trop forte concentration mènera à l'inhibition. Cependant une large gamme de seuils inhibiteurs est rapportée dans la littérature : 31-620 mg $P\text{-}PO_4^{3-}$/L (C. Trigo *et al.*, 2006 ; A. Magrí *et al.*, 2013), et il semblerait qu'une biomasse structurée (i.e. biofilm ou granule) soit moins sensible à l'impact du phosphore (J.M. Carvajal-Arroyo *et al.*, 2013). Un autre composant communément retrouvé quel que soit le type d'effluent est le sulfate (SO_4^{2-}). La toxicité du sulfate est assez peu impactant sur les gammes de concentrations rencontrées. Cependant, en conditions anaérobies strictes, après une déplétion totale en oxygène, nitrites et nitrates, leur réduction en sulfures (S^{2-}) par des bactéries sulfato-réductrices expose les bactéries anammox à un composé bien plus toxique (R.C. Jin *et al.*, 2013).

Ubiquitaire dans les eaux usées, et fortement présent dans les percolâts de sites d'enfouissement (F. Fu and Q. Wang, 2011), les métaux lourds et métalloïdes sont aussi susceptibles de devenir des sources d'inhibition potentielles pour anammox, même si cela reste un domaine très peu documenté (R.C. Jin *et al.*, 2012). Si certains d'entre eux comme le fer, le cuivre, le zinc, etc. sont des oligoéléments, utilisés

notamment pour la synthèse de métalloprotéines, de trop fortes concentrations viendront perturber l'homéostasie cellulaire. T. Lotti *et al.* (2012) se sont intéressés à la toxicité du zinc et du cuivre sur une biomasse anammox. Il apparait que l'effet inhibiteur est de type dose-réponse avec une sensibilité plus forte pour le cuivre. De même il a été montré que l'impact de l'inhibition est directement lié au temps d'exposition préalable.

Composés divers

Le procédé anammox a été largement mis en avant pour le traitement des effluents de digestion anaérobie (i.e. la fraction liquide du digestat), cependant une étape préalable de séparation de phase est nécessaire. L'utilisation de floculant est souvent nécessaire afin de diminuer le plus possible le taux de matières en suspension dans la fraction liquide. Cependant les composés utilisés comme surfactant (i.e. tensioactifs) sont susceptibles de perturber l'homéostasie bactérienne voir de désagréger les structure bactérienne (i.e. biofilms et granules). Il a été montré que les surfactants cationiques (A. Dapena-Mora *et al.*, 2007) comme les surfactants anioniques (S. Qiao *et al.*, 2016) sont susceptibles de causer des baisses d'activité irréversibles sur les biomasses anammox. Cependant le peu d'études publiées sur le sujet, combiné au fait que de très nombreux surfactants existent sur le marché, ne permet pas à ce stade d'établir de réelle comparaison ni de recommandations sur le choix d'un produit.

1.2.3. Aspects de génie des procédés

La variabilité métabolique des différents genres anammox, la présence simultanée de plusieurs guildes bactériennes, et l'ingéniosité déployée dans le design de procédés toujours plus performants ont abouti à un grand nombre de solutions techniques proposées pour mettre en œuvre un système anammox.

De manière générale, quand l'effluent à traiter provienne d'une unité de digestion anaérobie, d'un percolât de site d'enfouissement ou d'une eau vanne urbaine, la charge entrante en azote se fera majoritairement sous forme d'ammonium. La concentration de ces effluents en ammonium varie principalement selon leur source (municipal, industrielle ou agricole) entre 0.4-4.0 g $N\text{-}NH_4^+$/L (I. Jubany *et al.*, 2009 ; A. Magrí *et al.*, 2013). Comme mentionné préalablement, une étape de nitritation partielle devra impérativement être réalisée du fait de la stœchiométrie réactionnelle anammox. Il faut amener l'effluent dans un état où le ratio molaire NO_2^-:NH_4^+ sera satisfaisant (e.g. 1.32:1). Un intérêt fort est actuellement porté aussi sur la mise en place de systèmes de traitement stables pour les effluents appelés « mainstream » et correspondant au traitement des eaux vannes en entrée de station d'épuration urbaine (T. Lotti *et al.*, 2014a ; H. De Clippeleir *et al.*, 2015).

Couplage avec la nitritation partielle

De manière générale, que l'effluent à traiter provienne d'une unité de digestion anaérobie, d'un percolât de site d'enfouissement ou d'une eau vanne urbaine, la charge entrante en azote se fera majoritairement sous forme d'ammonium. La concentration de ces effluents en ammonium varie principalement selon leur source 0.4-4.0 g N-NH_4^+ / L (I. Jubany *et.al.*, 2009 ; A. Magrí *et.al.*, 2013). Comme mentionné préalablement, une étape de nitrification partielle devra impérativement être réalisée du fait de la stœchiométrie réactionnelle anammox. Il faut amener l'effluent dans un état où le ratio NO_2^-/NH_4^+ sera satisfaisant (i.e. 1.32 :1).Il est nécessaire de préciser que classiquement la nitrification regroupe deux réactions successives :

La première, qualifiée de nitritation, consiste à l'oxydation de l'ammonium en nitrite (éq. 3), réalisée par les ammonium-oxidizing bacteria (AOB) et plus minoritairement par les ammonium-oxidizing archaea (AOA).

$$NH_4^+ + 1.5\ O_2 \ \rightarrow \ NO_2^- + H_2O + 2H^+ \qquad (3)$$

La seconde, appelée nitratation, consiste en l'oxydation du nitrite en nitrate (éq. 4), réalisée par les nitrite-oxidizing bacteria (i.e. NOB). A l'inverse de la nitritation, aucune espèce d'archée capable de réaliser cette réaction n'a encore été découverte.

$$NO_2^- + 0.5\ O_2 \ \rightarrow \ NO_3^- \qquad (4)$$

Si l'on considère une nitritation partielle avec une conversion de 57% de l'ammonium en nitrite (effluent avec un ratio molaire approprié pour la réaction anammox ; i.e. 1.32:1), la réaction correspondante peut être écrite comme présentée ci-dessous (éq. 5) (Magrí *et al.*, 2012):

$$NH_4^+ + 0.86\ O_2 \ \rightarrow \ 0.57\ NO_2^- + 0.43\ NH_4^+ + \ 0.58\ H_2O + 1.14\ H^+ \ (5)$$

Finalement, si on considere une nitrification complète, en incorporant le carbone inorganique et la synthèse cellulaire ($C_5H_7O_2N$), la réaction de nitrification peut être écrite comme suivant : (éq. 6) (US EPA, 1993):

$$NH_4^+ + 1.89\ O_2 + \ 1.98\ HCO_3^- \ \rightarrow \ 0.0161\ C_3H_7O_2N + 0.984\ NO_3^- + \ 1.032\ H_2O + 1.899\ H_2CO_3 \ (6)$$

Le nitrate accumulé pendant la phase préliminaire de nitrification partielle ne pourra pas être éliminé par l'étape anammox, et de par le fait, viendra diminuer le rendement épuratoire du procédé. Ainsi, plusieurs stratégies ont été proposées pour sélectionner spécifiquement les AOB en réprimant le développement des NOB. En effet il a été montré qu'en s'appuyant sur le temps de séjour couplé à la température dans un système chemostat (système continu où le temps de séjour hydraulique et solide sont similaires) pour promouvoir spécifiquement les AOB (A. Magrí *et al.*, 2007). Comme explicité dans la Figure

10, il a été montré que les AOB possèdent un taux de croissance plus fort que les NOB à des températures avoisinant les 35°C et cela peut être utilisé pour faire une culture sélective des AOB dans le système (C. Hellinga *et al.*, 1998).

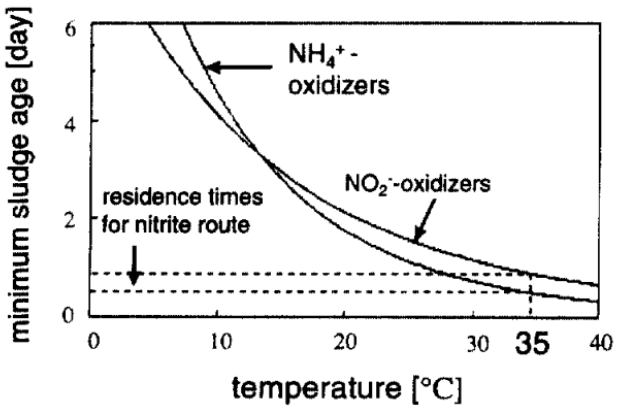

Figure 10 : Temps de séjour minimum requis pour les AOB et NOB en fonction de la température du système (C. Hellinga et al., 1998).

De plus, en ajustant la concentration en oxygène dissous du système à de faibles concentrations, il est possible de favoriser les AOB qui possèdent une plus grande affinité pour l'oxygène (i.e. K_{O2}) (I. Jubany *et al.*, 2009). Il est aussi possible de s'appuyer sur le fait que les NOB sont plus sensibles à de fortes concentrations en ammoniac et en acide nitreux (A.C. Anthonisen *et al.*, 1976). En effet pour se maintenir dans un système chemostat il faut que le taux de doublement d'un organisme soit inférieur au temps de séjour du solide (SRT). En favorisant des paramètres qui avantagent les AOB au détriment des NOB, il est possible de faire en sorte que le SRT soit inférieur au temps de doublement théorique des NOB et qu'ils soient ainsi incapables de se développer dans le système.

Concernant les effluents de digestion anaérobie traitant des boues activées en station d'épuration municipales, une concentration en azote d'environ 1.0-1.5 g $N\text{-}NH_4^+$/L et un ratio molaire bicarbonate/ammonium de 1-1.2 sont communément observés. Dans ce cas, une nitritation complète est limitée en premier lieu par le pH (i.e. manque de bicarbonate) plutôt que par l'ammonium et un taux de nitritation final de l'ammonium autour de 50-60% peut être alors attendu (U. van Dongen *et al.*, 2001). Cependant, la quantité d'alcalinité contenue dans l'effluent à traiter va jouer un rôle capital et si la valeur est trop importante, ceci entrainera un ratio trop fort, alors qu'une valeur trop faible d'alcalinité ne permettra pas une nitritation suffisante. De plus, l'aération mise en place dans le réacteur va entrainer un stripping partiel du carbone inorganique dissous, tandis que les bactéries nitrifiantes réaliseront un prélèvement pour la croissance de leur biomasse. Pour que la nitritation soit partielle et qu'elle respecte un ratio molaire proche de 1.32:1 (NH_4^+:NO_2^-), elle doit être arrêtée soit spontanément lorsque le pH chute suite à l'abaissement du pouvoir tampon de l'effluent sous une valeur physiologique seuil (environ 5.5-6),

ou suite à une vidange préprogrammée du réacteur. En effet, dans le cas d'un excès en alcalinité, certains travaux ont montré qu'il est possible de piloter empiriquement le procédé en utilisant le pH comme indicateur de fin de réaction pour un effluent donné (L. Zhang *et al.*, 2011 ; A. Magrí *et al.*, 2012). Alternativement, une source externe de carbone inorganique peut être utilisée pour réguler le degré de nitritation partielle souhaité.

D'un point de vu technique, la nitritation partielle peut être réalisée dans différents types de systèmes allant de simples CSTR (i.e. Completly Stirred Tank Reactor) sans rétention du solide type SHARON (i.e. Single reactor system for High Ammonia Removal Over Nitrite) à des systèmes membranaires (MBBRs) plus complexes ou des réacteurs séquentiels de types SBR (i.e. Sequencing Batch Reactor) qui présentent l'avantage de découpler temps de séjour hydraulique (HRT) et le SRT en retenant la biomasse dans le système.

Procédé anammox en deux étapes

Un procédé optant pour les deux étapes va réaliser dans des compartiments séparés la nitritation partielle puis anammox. Ce fut en effet le cas pour le premier réacteur anammox grande échelle mis en service à Rotterdam (W.R.L. van der Star *et al.*, 2007).

L'avantage intrinsèque de séparer les deux étapes du procédé est qu'il apparait possible de mieux maitriser les conditions physico-chimiques du milieu afin de placer chaque guilde microbienne dans les conditions qui lui sont le plus favorable, augmentant ainsi leurs performances respectives. De plus, comme explicité dans le sous chapitre précédent, le nombre de substances potentiellement inhibitrices et limitantes pour les bactéries anammox est important, le fait d'intercaler au préalable un réacteur de prétraitement ouvre un certain nombre de possibilités. En effet, un des composants régulièrement retrouvés et aux effets néfastes sur le procédé anammox est la matière organique. Lors de la nitritation partielle, des phases d'aérations nécessaires à l'oxydation de l'ammonium par les AOB, vont permettre l'établissement de guildes de bactéries et d'archées hétérotrophes (M. Ruscalleda *et al.*, 2008). L'abattement partiel de la matière organique qui va en résulter présente un intérêt évident compte tenu de l'effet délétère de certains composés organiques en termes d'inhibition et de limitation sur les bactéries anammox. Alternativement, un procédé intitulé anammox – partial dénitrification (i.e. anammox-PD) propose notamment d'intercaler une étape de dénitrification préalable (R. Du *et al.*, 2016) afin de réduire la charge entrante en DCO, nuisible pour anammox, en éliminant conjointement une part de l'azote entrant (nitrate produit). Compte tenu de la forte perturbation induite par la matière organique, ce type de procédé pourrait admettre de façon stable des effluents contenant des valeurs de DCO importantes comme les lisiers. Cela permet de plus un abattement concomitant de la matière organique soumis à des obligations réglementaires dans les rejets (M. Kumar *et al.*, 2010 ; E.A. Giustinianovich *et al.*, 2016).

Tout comme pour la nitritation, plusieurs designs de réacteurs anammox ont vu le jour. Cependant la problématique de la rétention de la biomasse est centrale du fait du faible taux de croissance de la bactérie. L'accent est donc mis sur des systèmes avec une biomasse fortement structurée. Initialement proposé par M. Strous *et al.* (1998), le réacteur séquentiel permet, avec une attention particulière sur la phase de sédimentation, d'éviter de vidanger la biomasse enrichie en bactéries anammox. De même les systèmes membranaires de type MBBRs permettent une rétention de la biomasse mais nécessitent un entretien supplémentaire à cause des colmatages. D'autres systèmes développés plus récemment comme l'UASB (Up-flow Anaerobic sludge Blanket) ou l'EGSB (Expended Granular Sludge Bed) permettent une concentration très importante de la biomasse amenant ainsi des taux de traitement par unité de volume plus importants. Le système le plus performant recensé à ce jour est un réacteur granulaire de type UASB travaillant à 42.0-57.7 g VSS/L et développant une activité de 74.3 - 76.7 kg N/m^3/j (C.-J. Tang *et al.*, 2011). Comme explicité par S. Lackner *et al.* (2015), les installations en deux étapes représentent moins de 20% des installations à grande échelle mises en place. Bien qu'initialement commercialisé en deux étapes, le procédé a été réorienté vers des technologies impliquant une étape en simultané pour des raisons techniques et économiques.

Procédé anammox en une étape

Avec plus de 80% des installations à échelle réelle répertoriées actuellement, la réalisation en une étape du procédé est celle majoritairement mise en place (S. Lackner *et al.*, 2015). En effet, le fait de combiner les deux réacteurs en un seul réduit considérablement l'emprise au sol, le nombre de sondes en ligne nécessaires et vient ainsi réduire le coût opérationnel des installations (S. Lackner *et al.*, 2015). De plus, la conduite des deux étapes simultanément permet d'éviter certaines complications liées à l'obtention d'un ratio NO_2^-:NH_4^+ compatible avec la stœchiométrie anammox comme explicitée précédemment (L.W. Jaroszynski *et al.*, 2011) Cependant réussir à faire cohabiter les deux guildes microbiennes nécessaires à la conduite du procédé (i.e. AOB et anammox) nécessite des précautions particulières. En effet alors que l'activité des AOB va nécessiter un apport en oxygène dissous, d'autant plus important que la charge en azote sera élevée, les bactéries anammox seront totalement inhibées pour des concentrations en oxygène dissous très faibles (A. Dapena-Mora *et al.*, 2007 ; M. Strous *et al.*, 1999). De par le fait, la totalité des systèmes étudiés ou commercialisés s'appuient sur des biomasses structurées sous forme de biofilms ou de granules limitant ainsi l'exposition des bactéries anammox à de trop fortes concentrations en oxygène dissous (cf. 1.2.2.). La stœchiométrie de la réaction combinée NP-anammox est explicititée dans l'éq. 7 (A.O. Sliekers *et al.*, 2003).

$$NH_4^+ + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O \quad (7)$$

Du fait de la présence des bactéries anammox, les concentrations en ammonium et en nitrites devront, elles aussi, être conservées relativement faibles. Ceci complique en partie la limitation de

croissance imposée aux NOB basée entre autre sur les concentrations élevées en ammoniac et en acide nitreux. Cette complication sera encore accentuée lorsque la température de l'effluent à traiter est faible. Un intérêt fort est actuellement porté sur la mise en place de systèmes de traitement stables pour les effluents appelés « mainstream » et correspondant au traitement des eaux vannes en entrée de station d'épuration urbaine (T. Lotti *et.al.*, 2014a ; H. De Clippeleir *et.al.*, 2015).

Emissions gazeuses

Etant donné que l'on parle ici de procédés biologiques pour le traitement de formes azotées, il est nécessaire de prendre en compte les émissions potentielles de certains composés gazeux polluants, comme l'ammoniac, le monoxyde d'azote (NO) et le protoxyde d'azote (N_2O). Les émissions d'ammoniac des systèmes anammox sont l'objet d'assez peu d'intérêt. Son transfert vers l'atmosphère dépend principalement de la température et du pH. Hors le pK_a du couple NH_4^+/NH_3 est d'environ 9.25, ce qui est relativement éloigné des pH optimum des systèmes anammox (i.e. 7-8) limitant ainsi la part d'ammoniac dans ces systèmes. Par contre, certaines guildes microbiennes, comme les nitrifiantes et les dénitrifiantes, sont susceptibles d'émettre des quantités importantes de NO et de N_2O en fonction des conditions du milieu. Par ailleurs, des réactions chimiques parasites peuvent aussi être à l'origine d'émissions significatives de protoxyde d'azote. (Kampschreur *et al.*, 2011).

Les émissions de NO des systèmes anammox sont l'objet de peu d'intérêt au sein de la communauté scientifique, probablement du fait que les flux issus du traitement des effluents sont très faibles par rapport aux flux azotés globaux et du fait d'un pouvoir de réchauffement global (PRG) non significatif. En effet, bien que le NO soit proposé comme étant un intermédiaire métabolique d'anammox (J.G. Kuenen *et al.*, 2006), les émissions enregistrées restent relativement faibles avec 0.2% N-NO/N-entrant (anammox deux étapes ; M.J. Kampschreur *et al.*, 2008) et 0.005% N-NO/N-entrant (anammox une étape ; M.J. Kampschreur *et al.*, 2009).

Il a été montré dans des systèmes de nitrification-dénitrification (NDN) classiques que les émissions de N_2O sont en partie dues aux bactéries nitrifiantes et dénitrifiantes. En effet bien que le N_2O ne soit pas un intermédiaire réactionnel de la voie catabolique attendue des bactéries nitrifiantes, il apparait qu'elles contribuent significativement aux émissions de ce dernier (J.E. Burgess *et al.*, 2002 ; S. Tsuneda *et al.*, 2005). A l'inverse, le N_2O fait partie des intermédiaires réactionnels de la dénitrification, et est strippé lorsque du gaz est insufflé dans un réacteur. Le travail réalisé par G. Tallec *et al.* (2006) évalue entre 58% et 83% la part relative des émissions dues à l'activité des nitrifiantes et entre 17% et 42% celle due à la dénitrification hétérotrophe. Cependant ces chiffres vont être directement dépendants du système en question et permettent juste de dresser une liste des organismes potentiellement émetteurs.

Le N_2O revêt un intérêt fort de la part de la communauté scientifique du fait de son fort PRG, 298 fois plus élevé que celui du CO_2. Même si le N_2O ne représente qu'environ 0.03% des flux totaux de gaz à effet de serre, une fois son PRG pris en compte, son impact représente jusqu'à 10% de l'impact de réchauffement total (B.C. Bates *et al.*, 2008). Une étude récente de M. Ali *et al.* (2016) dresse un bilan des émissions de N_2O observées dans les systèmes anammox en une et deux étapes. Il apparait que les émissions de N_2O sont plus élevées dans les réacteurs de nitrification partielle avec entre 0.8% et 6.6% (moyenne : 2.8%) de N-N_2O/N-entrant soit entre 3% et 15.5% (moyenne : 5.2%) de N-N_2O/N-abattu. Les émissions de la partie anammox du système sont évaluées entre 0.1 et 0.6% (moyenne 0.27%) de N-N_2O/N-entrant soit entre 0.1% et 0.68% (moyenne : 0.31%) de N-N_2O/N-abattu. Quant aux émissions de N_2O des systèmes en une étape, elles représentent entre 0.1% et 6% (moyenne : 1.4%) de N-N_2O/N-entrant soit entre 0.1% et 19.1% (moyenne : 3.1%) de N-N_2O/N-abattu. Comparativement, en moyenne les émissions de N_2O semblent être plus importantes dans les systèmes en deux étapes avec 5.2% + 0.31% de N-N_2O/N-abattu contre seulement 3.1% de N-N_2O/N-abattu pour les systèmes en une étape.

Une façon d'expliquer cette différence réside dans l'étude des paramètres affectant les émissions biologiques de N_2O. Dans ce sens, la concentration en nitrite du milieu va être un paramètre crucial vis-à-vis des émissions. De fortes concentrations en nitrite vont favoriser de fortes émissions de N_2O vis-à-vis des bactéries nitrifiantes comme dénitrifiantes (M.J. Kampschreur *et al.,* 2008 ; A. Alinsafi *et al.*, 2008 ; S. Okabe *et al.*, 2011). De plus, la concentration en oxygène va elle aussi impacter les émissions car les bactéries nitrifiantes activeront leurs voies de dénitrification lorsque la concentration en oxygène dissous sera suffisamment faible, induisant de fortes émissions de N_2O (G. Tallec *et al.*, 2006) tandis que de trop fortes teneurs en oxygène dissous favoriseront les émissions de la part des bactéries dénitrifiantes (M.J. Kampschreur *et al.*, 2008 ; M. Pijuan *et al.*, 2013). De plus, la N_2O réductase, enzyme associée à la chaine métabolique des bactéries hétérotrophes dénitrifiantes est inhibée par l'acide nitreux (HNO_2) (Y. Zhou *et al.*, 2008), qui tend à se former à des pH acides (pK_a HNO_2/NO_2^- : 3.398). De ce fait, des pH acides vont bloquer la chaine métabolique et tendre à accumuler du N_2O, favorisant son émission par stripping (S. Okabe *et al.*, 2011). De plus faibles émissions de N_2O sont quand même mesurées dans les systèmes anammox (i.e., étape anammox seule). Récemment une étude réalisée à l'aide de microélectrodes a mis en évidence une spatialisation des émissions de N_2O au niveau des granules anammox. En effet, la partie la plus interne du granule serait responsable des émissions de N_2O. Cette partie dépourvue d'AOB (i.e., faible proportion sur la partie externe) présente une abondance significative de bactéries hétérotrophes qui ont été identifiées comme responsables de ces émissions (S. Okabe *et al.*, 2011).

Ainsi, la raison principale aux fortes émissions de N_2O lors de l'étape de nitritation partielle des procédés en deux étapes semble résider dans le fait que l'air est insufflé dans un liquide contenant une très forte concentration en nitrites (i.e., plusieurs centaines de mg N-NO_2^-/L dissous) à des pH acides (e.g.,

5.5-7). Ces conditions entrainent, malgré des concentrations en oxygène dissous plus importantes que dans un réacteur NP-anammox une étape, des émissions de N_2O presque deux fois supérieures en moyenne.

Conclusion et ouverture

Les recherches sur le thème du métabolisme anammox et de son couplage avec la nitrification partielle reste une thématique jeune, de mieux en mieux documentée, mais dans laquelle persiste encore des points à éclaircir.

L'évolution nette du taux de croissance maximum observé depuis la découverte de la bactérie démontre que les connaissances accumulées sur la physiologie de la bactérie et l'amélioration des systèmes de culture permettent de raccourcir le temps nécessaire au démarrage d'un système anammox pleinement opérationnel. Dans ce sens la majeure part des travaux présentés ci-après s'emploie à définir précisément une méthodologie claire et adaptée pour faciliter et raccourcir le temps nécessaire à l'obtention d'un système anammox afin de rendre cette technologie plus largement accessible et à moindre coût. Tout au long des étapes d'enrichissement, une attention particulière est apportée sur les techniques de biologie moléculaire permettant de mieux caractériser ces phases. Ces outils se révèlent être une mine d'information pour la compréhension de l'écologie microbienne, encore peu détaillée, des systèmes anammox et des microorganismes qui les occupent.

Dans un second temps, un effort est porté sur la mise en place opérationnelle du procédé, avec la comparaison des systèmes de NP-anammox en une et en deux étapes. Il apparait qu'afin de limiter les inhibitions et ainsi améliorer les performances de ces systèmes, un grand nombre d'études portent sur l'impact négatif de certains composants sur les bactéries anammox. Dans ce sens, à travers ces travaux, un regard particulier est apporté sur l'effet d'un composant courant des effluents de digestion anaérobie, le phosphore, dont la littérature fait assez peu état. Un cas pratique particulier est présenté pour une station d'épuration faisant état d'effluents particulièrement chargés en phosphore et sur l'effet potentiellement néfaste dans le cas de l'établissement d'un système de traitement anammox. De plus, à travers un suivi en continu des émissions gazeuses, ces travaux documentent davantage les données actuellement disponibles concernant les émissions de N_2O relatives aux configurations en une et en deux étapes de l'ANR, notamment dans le cas de conditions de traitement perturbées.

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CHAPITRE 2 : Analyse bibliométrique appliquée à thématique de la gestion des nutriments issus des effluents de digestion anaérobie.

CHAPTER 2: Bibliometric analysis applied to the field of nutrients management from biogas digester supernatants

Foreword:

This chapter is dedicated to the evaluation of the interest supported by both research and industry to nutrients management within anaerobic digester supernatants. Analysis of documentary production through scientific publications and industrial patents illustrate the growing interest toward this specific field during the timelap considered (i.e., 1995-2014); however important disparities have been assessed worldwide. This work provides an overview at national, institute and individual levels to locate the documentary production hot-spots. Words analysis of document titles and key-words gave better understandings on interest trends and thematic evolutions all over the considered period. As this study is not completely focused on the anammox process it gives an informative point of view on the place it occupied within other thematic in the field of nutrients management.

Nutrient management from biogas digester effluents: a bibliometric-based analysis considering publications and patents

Albert Magrí [a,*], Floriane Giovannini [b], Romain Connan [a], Gilbert Bridoux [c], Fabrice Béline [a]

[a] Irstea, UR OPAALE, 17 Avenue de Cucillé-CS 64427, F-35044 Rennes, France.

[b] Irstea, DP2VIST, 1 Rue Pierre Gille de Gennes-CS 100300, F-92761 Antony, France.

[c] SAUR, Recherche et développement, Atlantis, 1 Avenue Eugène Freyssinet, F-78280 Guyancourt, France.

Abstract

Interest in organic waste(water) processing by anaerobic digestion to produce biogas as renewable energy source has increased significantly. The characteristics of the digested effluents are heterogeneous depending on the treated feedstock, and therefore, different handling alternatives are applicable for such by-products. This study investigates the development of science and technology in the specific field of nutrient (nitrogen and phosphorus) management from biogas digester effluents during the last 2 decades (from 1995 to 2014) using a bibliometric-based approach. Information concerning relevant publications (as representatives of the scientific research outputs) and patents (as representatives of the technological development outputs) was retrieved from specialized databases and systematically analyzed. The number of publications doubled the number of patents (1211 vs. 597 items, respectively). The production followed a rising pattern (in both cases, partial productivity in the last 5 years was >45%). The USA, China and Japan were the 3 most prolific countries when considering the joint production of publications and patents. However, while concerning the USA the number of publications was higher than the number of patents, the opposite was true for China and Japan. Those institutions more active in publishing (also the most cited items) were mainly European, whereas, regarding patenting were Asian (albeit the USA patents were more cited). The relative interest for some particular nutrient management alternatives and its evolution could be identified. A decreased consumption of resources, the implementation of integral solutions and circular economy approaches will be some of the key issues in future studies and developments fostering sustainability.

Keywords

Digestate; Reject water; Nutrients; Fertilization; Anaerobic digestion post-treatment; Environmental science and technology

1. Introduction

Anaerobic digestion (AD) is a biochemical conversion process, mediated by a microbial consortium, widely applied to valorize waste and biomass sources into a renewable energy vector such as biogas (60-70% v/v of methane, CH_4). It has been known for several centuries that combustible gas is generated when organic material is naturally decomposed in marshes and wetlands, or it is allowed to rot in piles. Application of AD as an environmental biotechnology was developed during the 20th century (T. Abbasi *et al.*, 2012) and its use has increased significantly during the last years (P. Buffiere *et al.*, 2008; L.-H. Wang *et al.*, 2013). Co-digestion approaches based on the mixture of different organic feedstock (e.g., urban, industrial or agricultural sources) are often considered to increase the biogas yield (J. Mata-Alvarez *et al.*, 2014). There is also a growing interest in upgrading the present energy-consuming wastewater treatment plant configurations -based on the aerobic biodegradation of the organic carbon (C)- shifting to anaerobic technologies that may enable net power production (Y.D. Scherson and C.S. Criddle, 2014). Positive energy balance, robustness of the process, reduction of sludge handling requirements, or the associated mitigation of greenhouse gas (GHG) emissions are some of the reasons that justify the high interest for the AD process (L. Appels *et al.*, 2011). Conventional means for the valorization of the biogas consist in burning it in situ, i.e. in a boiler, producing heat, or in a combined heat and power plant, producing electricity and "residual" heat. However, more efficient options are feasible for the integration of the biogas in the energy market based on its upgrading and use as vehicle fuel, or injection into the natural gas grid (J.B. Holm-Nielsen *et al.*, 2009; P. Weiland, 2010).

Digested effluents are heterogeneous in nature depending on the characteristics of the processed sources (M.P. Bernal *et al.*, 2011; W. Fuchs and B. Drosg, 2013). AD does not affect the total nitrogen (N) and phosphorus (P) content of the material being processed although it favors its mineralization. Utilization of the digested effluents in agriculture as organic fertilizer according to the current legislation -e.g., the nitrate directive (The Council of the European Communities, 1991)- and crop needs is advisable but such management strategy may be limited by factors like transport requirements, water content, or presence of heavy metals and pathogen microorganisms. Otherwise, further treatment of this by-product may be needed to enhance transportability of valuable components, as well as to protect the human health and quality of the receiving agricultural ecosystems and water bodies. An inappropriate management of the digested effluents (linked to the storage, soil application or post-treatment) will also cause negative impacts on air quality due to increased ammonia (NH_3) and GHG -i.e., CH_4 and nitrous oxide (N_2O)- emissions.

A solid-liquid separation of the digested effluents provides two different material fractions that can be handled independently (M. Hjorth *et al.*, 2010). The solid fraction can be transported longer distances because of the reduction in the water content, or undergo further processing (e.g., composting, drying,

pyrolysis, etc.) to produce added value products. On the other hand, the liquid fraction can be used for the fertilization of adjacent croplands, or post-treated according to its typical low C/N ratio following nutrient removal or recovery strategies (S. Malamis *et al.*, 2014; C.M. Mehta *et al.*, 2015; J.P. Sheets *et al.*, 2015). N-removal consists in converting ammonium (NH_4^+) into dinitrogen gas (N_2), an innocuous gas which is released into the atmosphere. Basically, this group comprises biological treatments including both, conventional and advanced strategies such as nitrification-denitrification and partial nitrification-anaerobic ammonium oxidation (anammox), respectively. Recovery is based on the production of separate streams of nutrients that are eventually recyclable, for instance, as agricultural fertilizer. Processes such as biological nutrient accumulation by prokaryotic organisms and algae, NH_3 stripping-absorption, struvite (magnesium ammonium phosphate, $MgNH_4PO_4 \cdot 6H_2O$) or calcium phosphate precipitation, and concentration by vacuum evaporation, filtration or reverse osmosis, among others, belong to this group. This latter approach makes it possible to close the nutrient cycle, which is always interesting from the sustainability perspective. Yet, factors such as influent strength, process efficiency, energy and reagent consumption, environmental impacts, and local market prices must be considered in order to appraise final interest of these technologies (T. Rehl and J. Müller, 2011; A. Magrí *et al.*, 2013; G. Rodriguez-Garcia *et al.*, 2014; D.J. Batstone *et al.*, 2015; N. Paccanelli *et al.*, 2015).

Journal article publishing is the most important form of dissemination of the research outputs in many disciplines of science and engineering. On the other hand, patents represent an invention in a particular field of technology, and have important influence on technological innovation and development, allowing the inventor to protect and profit from the invention and society to gain from dissemination of the knowledge about the invention (H.F. Moed *et al.*, 2005). As information platforms, both publications and patents have analogous features which are ideal “proxy measures” to represent the contents of science and technology (S&T), respectively (A. Verbeek *et al.*, 2002; M.-H. Huang *et al.*, 2014).

Bibliometric studies provide an accurate and presumably objective method to measure the contribution of a publication to the advance of knowledge and are already widely used in the analysis of scientific production and research trends, also in the field of the environmental science and engineering (W. Huang *et al.*, 2012; L. Yang *et al.*, 2013; L.-H. Wang *et al.*, 2013; Z. Ye *et al.*, 2014; B. Zhang *et al.*, 2014; Z. Zhang and S. Liu, 2014; F. Qian *et al.*, 2015). Similarly, patent data can be analyzed in a variety of ways to fulfill different purposes such as determining novelty, analyzing trends, forecasting technological developments in a particular domain, strategic technology planning, extracting the information for identifying the infringements, determining quality analysis for research and development tasks, identifying the promising patents, technological road mapping, identification of technological vacuums and hotspots, and identifying technological competitors (N. Johnstone *et al.*, 2010; A. Abbas *et al.*, 2014). In this study, we aim to assess the development of S&T in the particular field of nutrient (N and P) management from biogas digester effluents during the last two decades (i.e., from 1995 to 2014) using a bibliometric-based

approach. Journal publications (as representatives of the scientific research outputs) and patents (as representatives of the inventions and technological development outputs) are analyzed separately, and a final comparison of the main findings regarding both information platforms is also presented.

2. Materials and methods

Search strategy

An in-depth search of both, publications and patents, was carried out according to the strategy shown in Figure 11. This can be summarized in the following multi-term topic search (TS, TS = TS1 AND TS2):

(i) TS_1. Topic search targeting the identification of the by-product in which the study is focused

TS_1 = (("*digestate*" *OR* "reject* *water*") *OR* (("digestion" *NEAR*/3 ("anaerobic" *OR* "biogas" *OR* "*methane")) *OR* "digested" *OR* "*digester*" *OR* "*digestor*") *OR* ((("treat*" *OR* "*reactor*") *NEAR*/3 "anaerobic*") *AND* ("biogas" *OR* "*methane"))) *AND* (("digestion" *OR* "digested" *OR* "*digester*" *OR* "*digestor*" *OR* "treat*" *OR* "*reactor*") *NEAR*/3 ("*waste*" *OR* "*water*" *OR* "sludge*" *OR* "manure*" *OR* "effluent*" *OR* "*slurr*" *OR* "residue*" *OR* "biosolid*" *OR* "leachate*" *OR* "supernatant*" *OR* "liquor*" *OR* "*stream*" *OR* "centrate*")),

(ii) TS_2. Topic search targeting the identification of those nutrients to be managed and fertilization issues

TS_2 = ("nitrogen*" *OR* "ammoni*" *OR* "phosph*" *OR* "nutrient*" *OR* "ferti*" *OR* "soil*" *OR* "land").

Where, TS1 gathers different designations that can be used for referring to the by-product in which the study is focused; i.e., direct name (e.g., digestate, reject water, etc.), or description resulting from the linkage between words referring to the production process / place and name of the raw / processed material (e.g., anaerobically digested manure, biogas digester effluent, etc.), whereas TS2 refers to the nutrients (e.g., nitrogen, phosphorus, etc.) to be managed, and also fertilization issues. Use of Boolean (AND, OR) and proximity (NEAR) operators, truncation (*), and string search (“ ”) was considered when appropriate. Applied timespan accounted for the last twenty years; i.e., from 1995 to 2014 (both included).

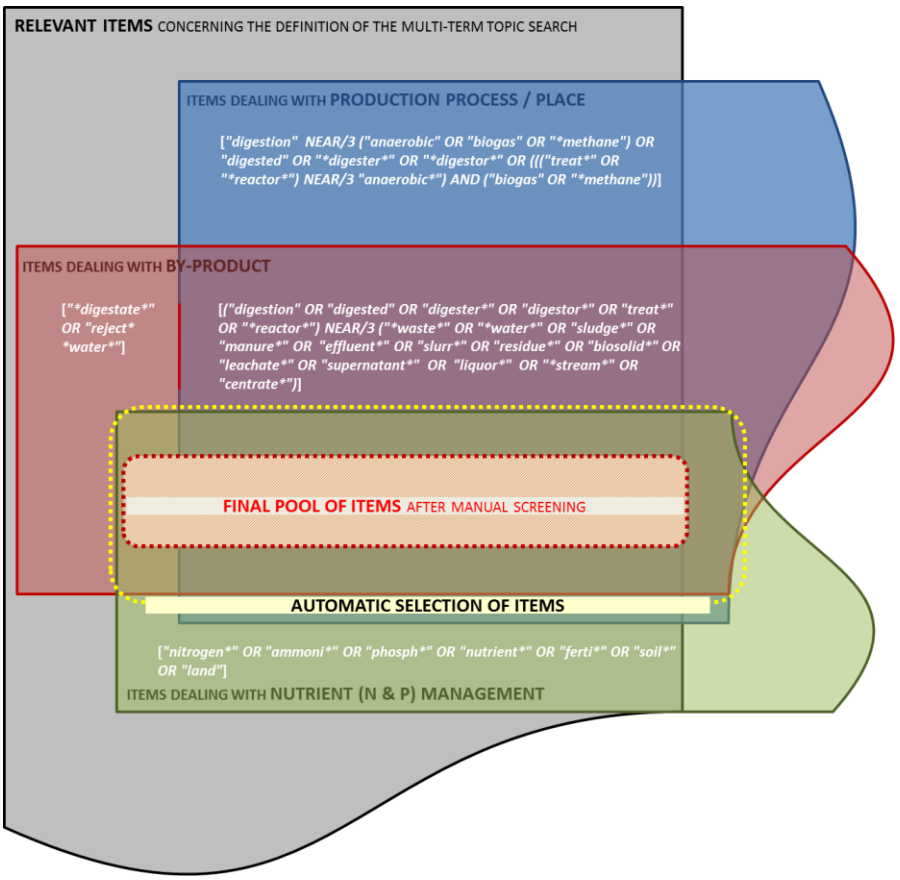

Figure 11: Approach followed for the definition of the multi-term topic search. Both, publications and patents were searched using the same approach.

Publications dataset

The publications analyzed in this research were gathered online using Science Citation Index Expanded (SCIE) accessed via Web of Science (WOS) Core Collection (Thomson Reuters, United States of America -USA-). This is the most widely accepted and frequently used database for the analysis of publications appeared in scientific journals. Particularly, this database indexed 8659 journals across 176 subject categories in 2014. Search was performed within the fields: title, abstract, author-provided keywords, and KeyWords Plus (i.e., additional words and phrases harvested from the titles of the cited publications). 3709 publications were retrieved after automatic search during June 2015 and main indexed contents were exported in an Excel (Microsoft, USA) file. Such list was then refined manually and only those publications that fitted the main objective of this work were selected; i.e., 1285 publications (34.6% of the initial) were considered after manual screening (consisting on the reading of the title and abstract, as needed, to confirm the selection of each item individually). Subsequently, only articles (and proceedings papers which were also published as articles) were selected, discarding reviews and other sort of documents. Thus, a total of 1211 items (32.7% of the initial) were finally considered. Final dataset was processed using the software Matheo Analyzer (Matheo Software, France). Research published by authors from England, Scotland, Northern Ireland and Wales was grouped under the United Kingdom (UK). For the source title analysis, all involved items were broken down in single words for further processing.

Conjunctions, prepositions, and other minor words (e.g., “and”, “with”, “of”, “by”, “in”, “on”, “from”, etc.) were then discarded, as they were meaningless for the analysis. Author-provided keywords were considered as independent items formed by one or more words. Use of abbreviations, acronyms, formulas, and misspelled terms, as well as the grammatical number, was reviewed when needed.

Patents dataset

The patent families (group of patents filed in different countries to protect the same invention) analyzed in this research were gathered online using PatBase (Minesoft Ltd. and RWS Group in partnership, UK). This database covered over 54 million patent families from 102 issuing authorities in 2015. Timespan was defined according to the priority date (first application filing date worldwide). In this case, search was performed within the fields: title, abstract, and claims. The main contents of those items written in other languages than English were automatically translated. 1440 patent families (5263 patents) were retrieved during July 2015 (and main indexed contents were exported in an Excel file) which resulted in a total of 597 items (41.5% of the initial) after manual screening (same procedure as for publications). Final dataset was partially processed using the software Matheo Patent (Matheo Software). Contents were processed following the same criteria than for the publications dataset.

Statistical analyses

For both datasets (i.e., publications and patents), the data within a given classification were analyzed in relative terms according to their rank and percentage value. The documentary production growth was assessed through regression analysis (J. Li *et al.*, 2009, L.-L. Li *et al.* 2009; L. Yang *et al.*, 2013) using in this case a power model such as $P(Y) = P_0 \cdot Y^k + \varepsilon$, where $P(Y)$ is the cumulative number of publications since 1995, Y is the number of years elapsed, and P_0, k, and ε are the characteristic parameters of the model (i.e., positive real values). For each dataset, 2 different curves were fitted, according to 10-year time intervals, using the Solver tool in Excel (allowing determination of different values for the power k in the time periods 1995-2004 (k_1) and 2005-2014 (k_2)).

3. Results and discussion

Results of this study are presented in two parts. The first part includes a bibliometric analysis of the two aforementioned datasets (i.e., publications and patents). As previously detailed, such datasets were defined by applying a multi-term search strategy in specialized databases and a subsequent manual screening (only those items which were identified as relevant for the study were considered). Target was to gather explicit knowledge concerning nutrient management from biogas digester effluents rather than all existing know-how in relation to generic nutrient management alternatives. Subsequently, the second part of this study focuses on the comparison of the main findings concerning both documentary sources.

Bibliometric analysis of publications and patents

Documentary production

Publications. In the initial years covered by this study (from 1995 to 2002), the annual number of research outputs did not change significantly, with a number of items ranging from 13 to 31 (Figure 12a). However, the publication rate increased fast in the following years; from 35 items in 2003 to 166 items in 2014 (which is about 13 times the number of publications appeared in 1995). In relative terms, documentary production accounted for 7.8%, 12.7%, 26.5%, and 53.0% in the 5-year periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively. This trend in research production concerning nutrient management from biogas digester effluents is quite similar to the evolution pointed out by Wang et al. (2013) when addressing bibliometric analysis of AD research. In such case, they identified as possible reasons for the increasing interest in AD the emergence of a fossil energy crisis, the rise of the energy prices, and the general improvement of the social consciousness, among others. The publication performance was also assessed through regression analysis using a power model (Figure 12b). Values of the power k obtained by fitting two different curves to the data according to 10-year intervals ($R^2 \geq 0.995$) indicate a faster publication rate during the later years ($k_2 > k_1$); i.e., $k_1 = 1.38$ (from 1995 to 2004) and $k_2 = 1.52$ (from 2005 to 2014). The same conclusion was also inferred using an exponential model, instead of a power model, but with a lower goodness of fit (data not shown). Otherwise, the average number of authors per publication rose slightly from 3.1 in 1995 to 4.0 in 2014, and the average number of references cited per publication rose from 25 to 38 in the same period.

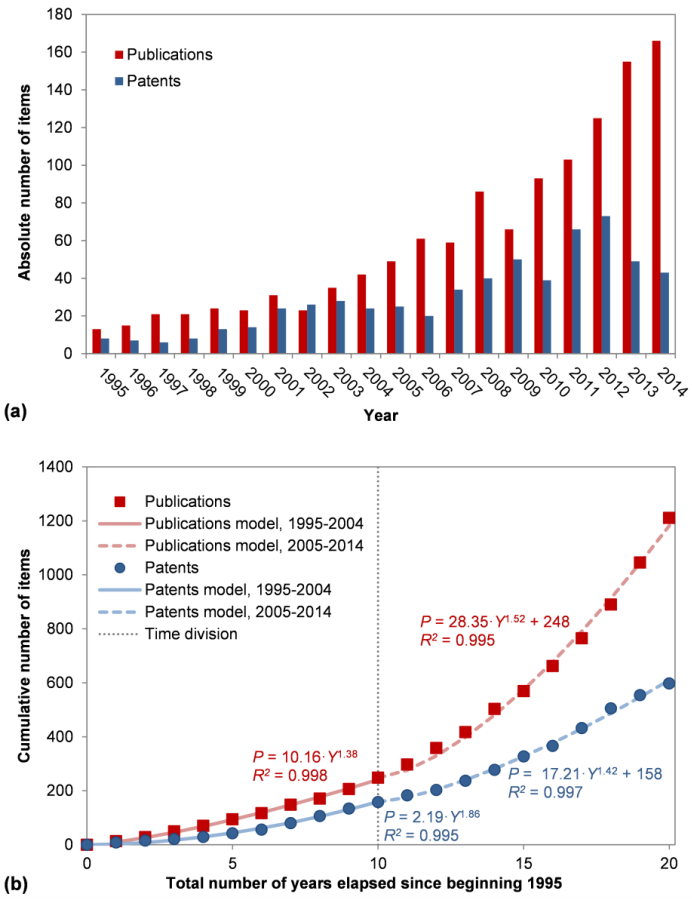

Figure 12: Number of items dealing with nutrient (N and P) management from biogas digester effluents (total timespan: 1995-2014). (a) Absolute number. (b) Cumulative number. The cumulative number of items was assessed using a power model according to 10-year time intervals.

Patents. The total number of patents (i.e., family patents) analyzed in this study was about half of the total number of publications (597 vs. 1211 items, respectively). Roughly, the number of items increased throughout the years (Figure 12a). In spite of this fact, two different plateaus were identified in the overall trend, particularly in the intervals 1995-1998 and 2003-2007. The year 2002 was the only one in which the number of patents was higher than the number of publications. Concerning 2014, the low number of patents in relation to previous years could be explained because of the 18 month delay required between the application filing and public disclosure (i.e., the search timespan was defined according to the priority date). In relative terms, productivity accounted for 7.1%, 19.4%, 28.3%, and 45.2% in the 5-year periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively. Finally, the highest value of the power *k* (belonging to the fitting model) was estimated for the interval 1995-2004 ($R^2 \geq 0.995$); i.e., $k_1 = 1.86$ (Figure 12b).

Documentary classification

Publications. According to SCIE, concerned articles were published in 53 different subject categories involving 246 journals. Table 5 summarizes the 10 subject categories with the highest number of published articles throughout the timespan 1995-2014. The most prevalent category was 'Environmental Sciences' with 604 items (49.9%), followed by 'Engineering, Environmental' and 'Water Resources' with 418 (34.5%) and 309 (25.5%) items, respectively. Relative values in 5-year partial periods (allowing the smoothing of yearly fluctuations) were also presented. The categories 'Energy & Fuels' (66.3%) and 'Engineering, Chemical' (64.9%) were those with the highest number of publications during the last 5-year period in relation to the total number of publications during the timespan 1995-2014 (i.e., these are the categories with the highest relative occurrences in the time period 2010-2014 in contrast to the overall timespan under study).

Tableau 5 : Top 10 most productive WOS SCIE subject categories (publications) and WIPO IPC groups (patents) based on the number of items dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014; results referred to total timespan and 5-year partial periods).

WOS SCIE Subject Categories (publications) [a]	**NI**	**R (%)** [c] **1995-2014**	**R (%) 1995-1999**	**R (%) 2000-2004**	**R (%) 2005-2009**	**R (%) 2010-2014**
Environmental Sciences	604	1 (49.9)	1 (52.1)	1 (64.9)	1 (52.6)	1 (44.5)
Engineering, Environmental	418	2 (34.5)	2 (34.0)	2 (46.7)	2 (39.2)	2 (29.3)
Water Resources	309	3 (25.5)	2 (34.0)	3 (44.8)	3 (24.6)	4 (20.1)
Biotechnology & Applied Microbiology	254	4 (21.0)	4 (14.9)	4 (13.0)	4 (21.2)	3 (23.7)
Energy & Fuels	187	5 (15.4)	6 (12.8)	8 (5.2)	6 (13.4)	5 (19.3)
Agricultural Engineering	183	6 (15.1)	6 (12.8)	7 (7.1)	5 (14.9)	6 (17.4)
Engineering, Chemical	94	7 (7.8)	13 (2.1)	16 (1.3)	7 (9.0)	7 (9.5)
Soil Science	88	8 (7.3)	5 (13.8)	5 (10.4)	9 (4.7)	8 (6.8)
Agriculture, Multidisciplinary	65	9 (5.4)	9 (4.3)	6 (7.8)	8 (5.0)	9 (5.1)
Agronomy	55	10 (4.5)	8 (7.4)	10 (3.2)	11 (4.0)	10 (4.7)
WIPO IPC Groups (patents) [b]	**NI**	**R (%) 1995-2014**	**R (%) 1995-1999**	**R (%) 2000-2004**	**R (%) 2005-2009**	**R (%) 2010-2014**
C02F 3/00	288	1 (48.2)	1 (54.8)	2 (60.3)	1 (51.5)	1 (40.0)
C02F 11/00	254	2 (42.5)	2 (31.0)	1 (61.2)	2 (43.8)	2 (35.6)
C02F 1/00	168	3 (28.1)	4 (23.8)	3 (37.9)	3 (31.4)	4 (22.2)
C02F 9/00	130	4 (21.8)	7 (11.9)	8 (11.2)	4 (20.7)	3 (28.5)
B09B 3/00	83	5 (13.9)	3 (26.2)	4 (23.3)	6 (14.2)	9 (7.8)
C05F 17/00	74	6 (12.4)	4 (23.8)	5 (16.4)	8 (10.7)	6 (10.0)
C12M 1/00	71	7 (11.9)	8 (7.1)	13 (4.3)	5 (16.0)	5 (13.3)
C05F 3/00	57	8 (9.5)	6 (16.7)	6 (13.8)	10 (8.3)	10 (7.4)
C05F 7/00	52	9 (8.7)	8 (7.1)	10 (9.5)	9 (9.5)	8 (8.1)
C12P 5/00	50	10 (8.4)	11 (4.8)	24 (2.6)	7 (12.4)	7 (8.9)

[a] Abbreviations: WOS, Web of Science; SCIE, Science Citation Index Expanded; WIPO, World Intellectual Property Organization; IPC, International Patent Classification; NI, number of items; R, ranking.

[b] Symbols X00Y 0/00: X, *Section*; X00, *Class*; X00Y, *Subclass*; X00Y 0/00, *Group*. *Section*: C, Chemistry - Metallurgy; B, Performing operations - Transporting. *Class*: C02, Treatment of water, wastewater, sewage, or sludge; B09, Disposal of solid waste - Reclamation of contaminated soil; C05, Fertilizers - Manufacture thereof; C12, Biochemistry - Beer - Spirits - Wine - Vinegar - Microbiology - Enzymology - Mutation or genetic engineering. *Subclass*: C02F, Treatment of water, wastewater, sewage, or sludge; B09B, Disposal of solid waste; C05F, Organic fertilizers not covered by subclasses C05B, C05C, e.g. fertilizers from waste or refuse; C12M, Apparatus for enzymology or microbiology; C12P, Fermentation or enzyme-using processes to synthesise a desired chemical compound or composition or to separate optical isomers from a racemic mixture. *Group*: C02F 3/00, Biological treatment of water, waste water, or sewage; C02F 11/00, Treatment of sludge - Devices therefor; C02F 1/00, Treatment of water, waste water, or sewage; C02F 9/00, Multistep treatment of water, waste water or sewage; B09B 3/00, Destroying solid waste or transforming solid waste into something useful or harmless; C05F 17/00, Preparation of fertilisers characterised by the composting step; C12M 1/00, Apparatus for enzymology or microbiology; C05F 3/00, Fertilisers from human or animal excrements, e.g. manure; C05F 7/00, Fertilisers from waste water, sewage sludge, sea slime, ooze or similar masses; C12P 5/00, Preparation of hydrocarbons.

[c] Total number of items: 1211 publications (94, 154, 321, and 642 publications for the periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively) and 597 patent families (42, 116, 169, and 270 patent families for the periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively).

Table 6 summarizes the 10 journals with the highest number of published articles from 1995 to 2014. These journals included 543 articles in total, accounting for 44.8% of all the publications dealing with nutrient management from biogas digester effluents. Water Science and Technology published the highest number of articles with 183 items (15.1%), followed by Bioresource Technology and Water Research with 119 (9.8%) and 53 (4.4%) items, respectively. Relative values in 5-year partial periods were also presented. The journals Biomass & Bioenergy (80.0%) and Waste Management (71.4%) were those with the highest number of publications during the last 5-year period in relation to the total number of publications during the overall timespan (i.e., these are the journals with the highest timespan-related relative occurrences in the partial period 2010-2014).

Tableau 6: Top 10 most productive WOS SCIE indexed journals based on the number of publications dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014; results referred to total timespan and 5-year partial periods).

WOS SCIE Journals [a]	**IF (2014)**	**NI**	**R (%)** [b] **1995-2014**	**R (%) 1995-1999**	**R (%) 2000-2004**	**R (%) 2005-2009**	**R (%) 2010-2014**
Water Science and Technology	1.106	183	1 (15.1)	1 (19.1)	1 (31.8)	1 (17.1)	2 (9.5)
Bioresource Technology	4.494	119	2 (9.8)	2 (8.5)	4 (4.5)	2 (11.8)	1 (10.3)
Water Research	5.528	53	3 (4.4)	2 (8.5)	2 (5.2)	4 (3.1)	3 (4.2)
Environmental Technology	1.560	49	4 (4.0)	5 (4.3)	2 (5.2)	3 (4.0)	4 (3.7)
Waste Management	3.220	28	5 (2.3)	41 (0.0)	24 (0.6)	8 (2.2)	5 (3.1)
Journal of Environmental Quality	2.652	26	6 (2.1)	4 (6.4)	6 (3.2)	7 (2.8)	20 (0.9)
Water Environment Research	0.865	25	7 (2.1)	6 (3.2)	24 (0.6)	4 (3.1)	7 (1.7)
Biomass & Bioenergy	3.394	25	7 (2.1)	6 (3.2)	24 (0.6)	47 (0.3)	5 (3.1)
Agriculture, Ecosystems & Environment	3.402	18	9 (1.5)	15 (1.1)	14 (1.3)	10 (1.2)	7 (1.7)
Water, Air, & Soil Pollution	1.554	17	10 (1.4)	15 (1.1)	11 (1.9)	16 (0.9)	9 (1.6)

[a] Abbreviations: WOS, Web of Science; SCIE, Science Citation Index Expanded; IF, Impact Factor; NI, number of items; R, Ranking.

[b] Total number of items: 1211 publications (94, 154, 321, and 642 publications for the periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively).

Patents. Patents are classified, according to the different areas of technology to which they pertain, using a hierarchical system of language independent symbols provided by the International Patent Classification (IPC) (WIPO, 2016). Thus, the 10 IPC groups with the highest number of items throughout the considered timespan are listed in Table 1 (total number of groups involved: 178). The top 4 positions were occupied by groups belonging to the subclass C02F 'Treatment of water, wastewater, sewage, or sludge'. Particularly, the most frequent group deals with the biological treatment of liquid streams (C02F 3/00; 288 items, 48.2%), and was followed by a group dealing with the treatment of sludge (C02F 11/00; 254 items, 42.5%). The subclass C05F 'Organic fertilizers […], e.g. fertilizers from waste or refuse' also appeared in multiple occasions (i.e., 3 times) in Table 5. In relative terms, the groups C02F 9/00 'Multistep treatment of water, waste water or sewage' (59.2%) and C12M 1/00 'Apparatus for enzymology or microbiology' (50.7%) were those with the highest number of patents during the last 5-year period in relation to the total number of patents during the timespan 1995-2014.

Geographical distribution

Publications. The contribution of different countries in a publication was estimated by the affiliation of at least 1 author, which implied the participation of 71 countries. According to this criterion, 989 publications (81.7%) were single-country publications and 222 publications (18.3%) resulted from international collaboration. Such kind of collaboration is expected to increase the citation impact of publications, although not to the same extent for all countries and all partners involved (W. Glänzel, 2001). The 10 most productive countries (2 from North America, 6 from Europe and 2 from Asia) were summarized in Table 7 (includes 60% of the contributions). The USA was the most productive country with 204 items (16.8%), followed by Spain (123 items, 10.2%) and China (118 items, 9.7%). France was the only country belonging to the G7 (group which includes the 7 leading industrial countries) not listed in this table, and occupied the 20th position of the ranking with 23 items (1.9%). Single-country publications were most produced in the USA (15.6%) whereas international collaborative publications were most produced in the USA and China (in equal percentage, 22.5%). Netherlands was the country with the widest gap when comparing rankings concerning single-country and international collaborative publications (12th vs. 5th position). The number of publications was also split in 5-year partial periods in Figure 13a. Italy (76.9%), China (74.6%) and Japan (60.3%) were those countries with the highest number of publications during the last 5-year period in relation to the total number of publications during the overall timespan.

Tableau 7: Top 10 most productive countries based on the number of items dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014).

Country (publications)	**NI**	**NI R (%)** [c]	**SI R (%)**	**CI R (%)**
USA	204	1 (16.8)	1 (15.6)	1 (22.5)
Spain	123	2 (10.2)	2 (9.2)	3 (14.4)
China	118	3 (9.7)	3 (6.9)	1 (22.5)
Japan	78	4 (6.4)	6 (5.0)	4 (13.1)
Canada	74	5 (6.1)	4 (5.4)	6 (9.5)
UK	73	6 (6.0)	4 (5.4)	8 (9.0)
Italy	65	7 (5.4)	7 (4.6)	8 (9.0)
Germany	57	8 (4.7)	8 (3.9)	10 (8.1)
Netherlands	54	9 (4.5)	12 (3.0)	5 (10.8)
Denmark	50	10 (4.1)	9 (3.8)	14 (5.4)
Country (patents) [a]	**NI**	**NI R (%)**	**-**	**-**
China	165	1 (27.3)	-	-
USA	103	2 (17.0)	-	-
Japan	102	3 (16.9)	-	-
South Korea	74	4 (12.2)	-	-
Germany	27	5 (4.5)	-	-
WIPO [b]	15	6 (2.5)	-	-
Spain	13	7 (2.1)	-	-
Russia	12	8 (2.0)	-	-
Brazil	11	9 (1.8)	-	-
France	9	10 (1.5)	-	-
Italy	9	10 (1.5)	-	-
UK	9	10 (1.5)	-	-

[a] List by earliest priority country.

[b] Abbreviations: WIPO, World Intellectual Property Organization; NI, number of items; NI R, number of items ranking; SI R, single-country items ranking; CI R, international collaborative items ranking.

[c] Total number of items: 1211 publications (989 and 222 publications for single-country and international collaborative items, respectively) and 597 patent families.

Patents. Patent families were analyzed according to the earliest priority country (first application filing country worldwide). Depending upon the office at which a patent application is filed, that application could either be an application for a patent in a given country, or may be an application for a patent in a range of countries. In a large percentage, patents analyzed here were initially filed at the national level (96.5%) although a minority number of applications were filed at the international level; i.e., European (1%) or worldwide (2.5%, named as WIPO in Table 7). The 10 most active countries (3 from Asia, 2 from America and 6 from Europe) are summarized in Table 7 (includes approximately 90% of the contributions) with China (165 items, 27.3%), USA (103 items, 17.0%), and Japan (102 items, 16.9%) at the top of the list. The Asian countries were especially productive with an absolute number of items higher than in the case of the publications. In contrast, Canada (8 items, 1.3%), Denmark (4 items, 0.7%) and Netherlands (2 items, 0.3%) were not listed in Table 7. Relative values in 5-year partial periods were also presented in Figure 13b. China (74.5%), Russia (66.7%) and Italy (55.6%) were those countries with the highest number of patents during the last 5-year period in relation to the total number of patents in the global period.

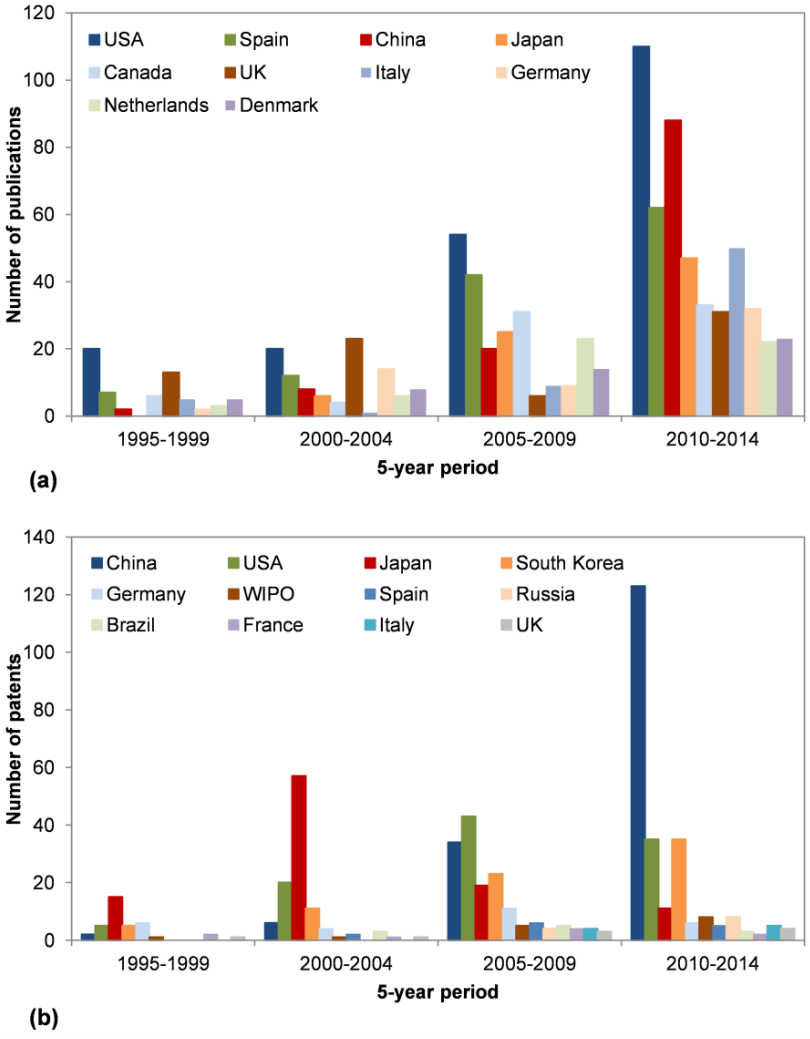

Figure 13: Geographical distribution of items in 5-year partial periods (total timespan: 1995-2014). (a) Publications. (b) Patents.

Institutional activity

Publications. The contribution of different institutions in a publication was estimated by the affiliation of at least 1 author. The 10 most productive institutions are listed in Table 8. The University of Ghent, Delft University of Technology, and Wageningen University were the 3 most productive institutions with 27 (2.2%), 22 (1.8%), and 22 (1.8%) items, respectively. A total of 569 items (47.0%) of the analyzed publications were single-institute publications whereas 642 items (53.0%) resulted from inter-institutional collaboration. Thus, the frequency of collaboration between institutes from the same country was notably higher that the frequency of collaboration between countries. It is worth to remark that no institutes from China, Canada, UK, Italy, Germany, or Denmark were found within the 10 most productive institutes although the aforementioned countries were ranked in the 3rd, 5th, 6th, 7th, 8th, and 10th position, respectively, in the total number of publications. On the other hand, a Belgian institute was identified as the most productive although Belgium was not identified within the top 10 most prolific countries. Three institutes from Spain and 2 institutes from Netherlands appeared in the ranking of the top 10 institutions.

Patents. Patent assignees (i.e., persons or corporate bodies to whom rights under a patent are legally transferred) were analyzed to provide the top 10 most productive institutions / organizations (Table 8). Except 1 case (which is a Russian academy), all the other assignees corresponded to Asian institutions such as companies (5), national institutes of S&T (2) and universities (2). Japan was the most represented country with 5 entries, including the 3 most productive institutions; i.e., Ebara (25 items, 4.2%), National Institute of Advanced Industrial Science & Technology (9 items, 1.5%) and Kubota (8 items, 1.3%). The most part of the analyzed patents were assigned to a single institution (496 items, 83.1%).

Tableau 8: Top 10 most productive institutions / organizations based on the total number of items dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014).

Institution (publications)	**NI** [a]	**NI R (%)** [b]	**SI R (%)**	**CI R (%)**
Ghent University, Belgium	27	1 (2.2)	5 (1.6)	1 (2.8)
Delft University of Technology, Netherlands	22	2 (1.8)	25 (0.7)	1 (2.8)
Wageningen University, Netherlands	22	2 (1.8)	2 (1.9)	7 (1.7)
United States Department of Agriculture	21	4 (1.7)	8 (1.2)	6 (2.2)
Swedish University of Agricultural Sciences	20	5 (1.7)	15 (0.9)	3 (2.3)
University of Santiago de Compostela, Spain	20	5 (1.7)	2 (1.9)	16 (1.4)
Consejo Superior de Investigaciones Científicas, Spain	18	7 (1.5)	35 (0.5)	3 (2.3)
University of Barcelona, Spain	18	7 (1.5)	1 (2.3)	49 (0.8)
Kumamoto University, Japan	17	9 (1.4)	58 (0.4)	3 (2.3)
Swiss Federal Institute of Aquatic Science and Technology	17	9 (1.4)	10 (1.1)	7 (1.7)
Institution / Organization (patents)	**NI**	**NI R (%)**	**SI R (%)**	**CI R (%)**
Ebara, Japan	25	1 (4.2)	1 (4.8)	24 (1.0)
National Institute of Advanced Industrial Science & Technology, Japan	9	2 (1.5)	2 (1.6)	24 (1.0)
Kubota, Japan	8	3 (1.3)	2 (1.6)	220 (0.0)
Mitsubishi, Japan	7	4 (1.2)	9 (0.8)	1 (3.0)
Russian Academy of Agricultural Sciences	7	4 (1.2)	4 (1.4)	220 (0.0)
BooKang Tech, South Korea	6	6 (1.0)	5 (1.2)	220 (0.0)
Korea Institute of Science & Technology, South Korea	6	6 (1.0)	7 (1.0)	24 (1.0)
Zhejian […] University, China	6	6 (1.0)	5 (1.2)	220 (0.0)
Fuji Electric, Japan	5	9 (0.8)	7 (1.0)	220 (0.0)
Tsinghua University, China	5	9 (0.8)	17 (0.6)	4 (2.0)

[a] Abbreviations: NI, number of items; NI R, number of items ranking; SI R, single-institution items ranking; CI R, collaborative items ranking.

[b] Total number of items: 1211 publications (569 and 642 publications for single-institution and collaborative items, respectively) and 597 patent families (496 and 101 patent families for single-institution and collaborative items, respectively).

Individual activity

Publications. The 10 most productive individuals are listed in Table 9. These authors represent 4 of the 10 most productive countries (Netherlands, Japan, Spain and Canada) (Table 7) and 6 of the 10 most productive institutions (Table 8). Particularly, affiliations mainly corresponded to European institutions (9); i.e., 5 authors developed their activity in Spain (3 in University of Barcelona and 2 in University of Santiago de Compostela) and 2 authors developed their activity in Belgium (Ghent University). Professor Mark van Loosdrecht from Delft University (Netherlands) was the most prolific individual with 20 publications (1.7%).

Patents. Patent inventors (i.e., persons or corporate bodies) were analyzed to assess the 10 most productive individuals. The resulting inventors are listed in Table 9 (only 2 individuals of a total of 20 individuals who are classified in the 9th position -invention of 4 patents- were listed in this table due to an issue of space; i.e., 28 individuals are involved in the following detailed analysis). Five of the 10 most productive countries were represented (Japan, Russia, USA, China and South Korea). Individuals were mainly involved with Asian institutions /organizations (10 of a total of 13 institutions, 76.9%); i.e., 15 individuals were related to Chinese institutions, 8 individuals were related to Japanese institutions and 1 individual was related to a South Korean institution (24 of a total of 28 individuals, 85.7%). Takao Hagino from Ebara (Japan) was the individual with the higher number of patented inventions (13 items, 2.2%).

Tableau 9 : Top 10 most productive authors / inventors based on the total number of items dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014).

Author (publications)	**Institution**	**NI** [b]	**R (%)** [c]
M.C.M. van Loosdrecht	Delft University of Technology, Netherlands	20	1 (1.7)
	Kumamoto University, Japan	17	2 (1.4)
K. Furukawa			
J. Mata-Alvarez	University of Barcelona, Spain	16	3 (1.3)
W. Verstraete	Ghent University, Belgium	16	3 (1.3)
D.S. Mavinic	University of British Columbia, Canada	15	5 (1.2)
J. Dosta	University of Barcelona, Spain	14	6 (1.2)
R. Mendez	University of Santiago de Compostela, Spain	13	7 (1.1)
H. Siegrist	Swiss Federal Institute of Aquatic Science & Technology	13	7 (1.1)
A. Gali	University of Barcelona, Spain	12	9 (1.0)
A. Mosquera-Corral	University of Santiago de Compostela, Spain	10	10 (0.8)
S.E. Vlaeminck	Ghent University, Belgium	10	10 (0.8)
Author / Inventor (patents)	**Institution / Organization**	**NI**	**R (%)**
T. Hagino	Ebara, Japan	13	1 (2.2)
K. Shimamura	Ebara, Japan	11	2 (1.8)
S. Sawayama	National Institute of Advanced Industrial Science & Technology, Japan	9	3 (1.5)
T. Tanaka	Ebara, Japan	7	4 (1.2)
E.N. Kamajdanov	Russian Academy of Agricultural Sciences	6	5 (1.0)
D.A. Kovalev	Russian Academy of Agricultural Sciences	6	5 (1.0)
J.C. Burnham	Vitag / Unified Environmental Services, USA	5	7 (0.8)
G.L. Dahms	Vitag / Unified Environmental Services, USA	5	7 (0.8)
Y. Jiang [a]	Beijing Drainage Group, China	4	9 (0.7)
Z. Li	Hebei University of Science and Technology, China	4	9 (0.7)
[…]	[…]	4	9 (0.7)

[a] Only 2 individuals of a total of 20 individuals with the same ranking (4 items) are listed.

[b] Abbreviations: NI, number of items; R, ranking.

[c] Total number of items: 1211 publications and 597 patent families.

Citations

Publications. The 10 most cited items are not older than 1997 (Table 10). After publication, such publications have been cited from 34.5 to 9.7 times per year, in average (total number of citations per item until end of 2014: 320-135). Six of these publications appeared in the journal Water Research, 4 included Delft University (Netherlands) as authors' affiliation institution and 3 were co-authored by the most prolific individual listed in Table 9 (Prof. van Loosdrecht). Although different topics were studied in these publications, N-removal using the anammox process was the most frequent (4 publications) gathering 1013 citations in total.

Patents. As in the case of publications, the older item that appears in the list of the 10 most cited patents (Table 10) is from 1997 (priority date). The frequency of citation considering the number of citing patent documents (i.e., not patent families), and the priority date, ranged from 15.2 to 4.8 times per year, in average (total number of citations per item until end of 2014: 219-67). With 8 items, the USA was the most usual earliest priority country within such list. It was surprising that Table 10 did not include any patent initially filed in Asia despite the aforementioned high productivity in terms of number of items in such continent; i.e., the most cited Asian patent was identified in the 23rd position (Japan, 44 citations). The idiomatic barrier could be an explanation for this fact. Since the patents filed in the Asian countries are not always translated into English, their citation within patents filed in other countries (with a different language) than the original filing country is more difficult. In addition, the high citation scores of the USA patents may be justified because of the characteristic referencing patterns in the USA patenting system, where a high number of patent references per patent are provided, mainly concerning earlier USA patents (Narin and Olivastro, 1998). Most of the top 10 cited patents referred to the use of the digested effluents in agricultural fertilization or in the production of fertilizers (even though in some cases an additional post-treatment was applied).

Tableau 10: Top 10 most cited items dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014).

Authors (publications)	**Title**	**Journal** [a]	**Publication year**	**Total citations** [b]	**Annual citations**	**Country**	**Institutions**
U. van Dongen, M.S.M. Jetten, M.C.M. van Loosdrecht	The SHARON®-Anammox® process for treatment of ammonium rich wastewater	*WST*	2001	320	22.9	NLD	Delft Univ Technol
W.R.L. van der Star, W.R. Abma, D. Blommers, J.W. Mulder, T. Tokutomi, M. Strous, C. Picioreanu, M.C.M. van Loosdrecht	Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam	*WR*	2007	272	34.0	NLD JPN	Delft Univ Technol, Paques BV, Waterschap Hollandse Delta, Kurita Water Ind Ltd, Radboud Univ Nijmegen
M. Strous, E. Van Gerven, P. Zheng, J.G. Kuenen, M.S.M. Jetten	Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (anammox) process in different reactor configurations	*WR*	1997	220	12.2	NLD	Delft Univ Technol
J.B. Holm-Nielsen, T. Al Seadi, P. Oleskowicz-Popiel	The future of anaerobic digestion and biogas utilization	*BT*	2009	207	34.5	DNK	Aalborg Univ, Univ So Denmark, Tech Univ

							Denmark
C. Fux, M. Boehler, P. Huber, I. Brunner, H. Siegrist	Biological treatment of ammonium-rich wastewater by partial nitritation and subsequent anaerobic ammonium oxidation (anammox) in a pilot plant	*JB*	2002	201	15.5	CHE	Swiss Fed Inst Aquatic Sci & Technol
B. Amon, V. Kryvoruchko, T. Amon, S. Zechmeister-Boltenstern	Methane, nitrous oxide and ammonia emissions during storage and after application of dairy cattle slurry and influence of slurry treatment	*AEE*	2006	170	18.9	AUT	Univ Bodenkultur Wien, Fed Res & Training Ctr Forests Nat Hazards Landscape
K.N. Ohlinger, T.M. Young, E.D. Schroeder	Predicting struvite formation in digestion	*WR*	1998	149	9.9	USA	Univ California, Sacramento Reg Cty Sanitat Dist
R.W. Holloway, A.E. Childress, K.E. Dennett, T.Y. Cath	Forward osmosis for concentration of anaerobic digester centrate	*WR*	2007	147	18.4	USA	Colorado Sch Mines, Univ Nevada, Tahoe Truckee Sanitat Agcy
E.V. Munch, K. Barr	Controlled struvite crystallisation for removing phosphorus from anaerobic digester sidestreams	*WR*	2001	136	9.7	AUS	Brisbane Water
M.J. Kampschreur, W.R.L. van der Star, H.A. Wielders, J.W. Mulder, M.S.M. Jetten, M.C.M. van Loosdrecht	Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment	*WR*	2008	135	19.3	NLD	Delft Univ Technol, Waterboard Hollandse Delta, Radboud Univ Nijmegen

Authors / Inventors (patents)	**Title**	**Priority year**	**Total citations** [c]	**Annual citations**	**Priority country**	**Institutions / Organizations**
L.P. Sower	Methods for producing fertilizers and feed supplements from agricultural and industrial wastes	1998	219	12.9	USA	Crystal Peak
J.D. Branson	System and method for extracting energy from agricultural waste	2001	213	15.2	USA	Best Biofuels
S.W. Dvorak	Method and apparatus for solids processing	1999	194	12.1	USA	Dvo
T. Bonde, L.J. Pedersen	Concept for slurry separation and biogas production	2000	139	9.3	DNK	Green Farm Energy
K. Stamper, R. Skinner	Process for producing energy, feed material and fertilizer products from manure	2001	99	7.1	USA	-
Y. Bouchalat	Optimized method for the treatment and energetic upgrading of urban and industrial sludge purifying plants	1997	86	4.8	FRA	-
R.A. Elefritz Jr., D.J. Barnes	Process to enhance phosphorus removal for activated sludge wastewater treatment system	2005	73	7.3	USA	Evoqua Water Technologies
P.J. Hirl	Process for producing ethanol and for energy recovery	2005	70	7.0	USA	Stanley Consultants
T. McKeeman, G. Karr, P. Vadlani	Method and apparatus for a multi-system bioenergy facility	2006	70	7.8	USA	-
R.H. Zhang, J.H. Turnbull	Treatment of swine wastewater by biological and membrane separation technologies	2003	67	5.6	USA	University of California

[a] Abbreviations. Journal: *WST, Water Science and Technology*; *WR, Water Research*; *BT, Bioresource Technology*; *JB, Journal of Biotechnology*; *AEE, Agriculture, Ecosystems & Environment*. Country: NLD, Netherlands; JPN, Japan; DNK, Denmark; CHE, Switzerland; AUT, Austria; USA, United States of America; AUS, Australia; FRA, France.

[b] Total citations until end of 2014.

[c] Citations are referred to patent documents, and not to patent families.

Words in title

Publications. Titles are supposed to include the information that authors would most like to express to the readers (J. Li et al., 2009). In this study, a total list of 1931 single words was retrieved from titles which were used 12975 times (only one use per title is considered). Particularly, 236 words (12.2%) were used 10 or more times and accumulated 71.9% of the total number of uses while 960 words (49.7%) were used only once accumulating 7.4% of the total number of uses. Hence, a minority group of words was widely used whereas most words were not employed frequently. The 30 most used words -including substantives (25 items), adjectives (2 items), verbal forms (2 items), and adverbs (1 item)- are summarized in Table 11 (such words are also ranked considering 5-year partial periods). According to the progressive increase in the number of published articles, use of these words in absolute terms basically increased throughout the period of study. Owing to the heterogeneous pool of publications identified by the search strategy applied (Figure 11), multiple interpretations for these words (Table 11) is usually feasible. Yet, some global ideas emerging from this analysis are: (i) “sludge” was probably the raw material most frequently processed through “anaerobic” “digestion” for “biogas” “production”, surpassing other organic sources such as “manure”; (ii) particular interest for “nitrogen” was higher than for “phosphorus”; (iii) preference for fertilizing-based management approaches was only identified through the word “soil” (meaning of the words “plant” and “application” is ambiguous); and (iv) “removal”-based “treatment” strategies were more frequently considered than those aiming to “nutrient” “recovery”. Considering the evolution of the relative number of uses of these words, some additional trends were also identified: (i) “sludge” and “manure”, two of the raw materials most processed, behaved oppositely; thus, while references to the first progressive decreased with time references to the second increased when comparing initial and final periods (1995-1999 vs. 2010-2014); (ii) interest for “nitrogen” became more focused in some species such as “ammonium” and interest for “phosphorus” progressively decreased; and (iii) consideration of “recovery”-based strategies progressively increased although still not attaining the level of the “removal”-based strategies in the last 5-year period (2010-2014).

Tableau 11: Top 30 most frequently used words in the title of publications dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014; results referred to total timespan and 5-year partial periods).

Title words (publications)	NI	R (%) 1995-2014	R (%) 1995-1999	R (%) 2000-2004	R (%) 2005-2009	R (%) 2010-2014
anaerobic	403	1 (33.3)	2 (23.4)	1 (27.3)	1 (34.9)	1 (35.0)
sludge	264	2 (21.8)	1 (41.5)	2 (25.3)	3 (22.4)	4 (17.6)
treatment	250	3 (20.6)	4 (18.1)	3 (23.4)	2 (26.2)	3 (17.9)
wastewater	209	4 (17.3)	15 (8.5)	6 (17.5)	6 (17.4)	2 (18.4)
nitrogen	197	5 (16.3)	4 (18.1)	4 (22.1)	5 (19.9)	9 (12.8)
removal	189	6 (15.6)	8 (13.8)	5 (20.1)	4 (21.2)	10 (12.0)
digested	174	7 (14.4)	11 (11.7)	11 (11.0)	10 (13.7)	5 (15. 9)
digestion	164	8 (13.5)	9 (12.8)	13 (10.4)	9 (14.3)	6 (14.0)
manure	158	9 (13.0)	17 (7.4)	16 (9.7)	7 (14.9)	7 (13.7)
waste	140	10 (11.6)	7 (16.0)	10 (13.6)	12 (11.5)	11 (10.4)
reactor	138	11 (11.4)	35 (4.3)	9 (14.9)	8 (14.6)	13 (10.3)
soil	133	12 (11.0)	6 (17.0)	8 (16.2)	25 (7.8)	11 (10.4)
process	125	13 (10.3)	27 (5.3)	16 (9.7)	11 (12.5)	14 (10.1)
production	121	14 (10.0)	27 (5.3)	45 (3.9)	22 (8.4)	8 (12.9)
sewage	118	15 (9.7)	3 (21.3)	6 (17.5)	19 (9.0)	32 (6.5)
effect	117	16 (9.7)	9 (12.8)	22 (7.8)	20 (8.7)	14 (10.1)
ammonium	105	17 (8.7)	44 (3.2)	27 (5.8)	16 (9.3)	19 (9.3)
nutrient	105	17 (8.7)	17 (7.4)	23 (7.1)	34 (6.8)	18 (9.7)
plant	100	19 (8.3)	15 (8.5)	11 (11.0)	15 (9.7)	29 (7.0)
using	100	19 (8.3)	122 (1.1)	27 (5.8)	22 (8.4)	17 (9.8)
anaerobically	99	21 (8.2)	22 (6.4)	23 (7.1)	22 (8.4)	20 (8.6)
digester	99	21 (8.2)	64 (2.1)	25 (6.5)	13 (10.3)	21 (8.4)
biogas	96	23 (7.9)	64 (2.1)	58 (3.2)	25 (7.8)	16 (10.0)
system	95	24 (7.8)	22 (6.4)	20 (9.1)	14 (10.0)	31 (6.8)
application	93	25 (7.7)	13 (10.6)	13 (10.4)	34 (6.8)	29 (7.0)
organic	93	25 (7.7)	35 (4.3)	27 (5.8)	16 (9.3)	25 (7.8)
water	93	25 (7.7)	22 (6.4)	13 (10.4)	38 (6.2)	24 (7.9)
ammonia	87	28 (7.2)	27 (5.3)	20 (9.1)	16 (9.3)	35 (6.1)
phosphorus	86	29 (7.1)	11 (11.7)	16 (9.7)	20 (8.7)	39 (5.3)
recovery	82	30 (6.8)	44 (3.2)	27 (5.8)	42 (5.6)	23 (8.1)

[a] Abbreviations: NI, number of items; R, ranking.

[b] Total number of items: 1211 publications (94, 154, 321, and 642 publications for the periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively).

Patents. A total list of 804 single words was retrieved from titles which were used 4288 times (only one use per title is considered). Thus, although the publication-to-patent ratio concerning the number of items analyzed in this study was 2.0 (i.e., double number of publications than patents), the corresponding ratios concerning the number of words and number of uses in the title increased to 2.4 and 3.0, respectively. Similarly to the case of the publications, only 77 words (9.6%) were used 10 or more times and accumulated 67.8% of the total number of uses while 450 words (56.0%) were used only once accumulating 10.5% of the total number of uses. The 30 most used words -including substantives (20 items), adjectives (5 items), and verbal forms (5 items)- are summarized in Table 12 (such words are also ranked considering 5-year partial periods). Some rough ideas that may derive from this table are: (i) application of “anaerobic” “digestion” for “biogas” “production” mainly concerned “treatment” of generic “liquid” / “solid” “organic” “waste”, “sludge”, and in some cases “waste” “water” (also referred as “wastewater” and “sewage”); (ii) titles dealing with “apparatus” and “devices” and the operational “method” thereof were frequent; (iii) particular interest for “nitrogen” was similar (i.e., slightly higher) than for “phosphorus”, and no specific N-compounds (e.g., ammonium, ammonia, etc.) could be found in the list; (iv) preference for “recovery”-based approaches, including production of “fertilizer”, was identified.

Tableau 12: Top 30 most frequently used words in the title of patent families dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014; results referred to total timespan and 5-year partial periods).

Title words (patents)	NI	R (%) 1995-2014	R (%) 1995-1999	R (%) 2000-2004	R (%) 2005-2009	R (%) 2010-2014
method	319	1 (53.4)	3 (35.7)	1 (63.8)	1 (44.4)	1 (57.4)
waste	194	2 (32.5)	2 (38.1)	3 (36.2)	2 (36.1)	4 (27.8)
treatment	193	3 (32.3)	1 (47.6)	4 (33.6)	3 (28.4)	2 (31.9)
organic	147	4 (24.6)	4 (26.2)	2 (41.4)	4 (19.5)	5 (20.4)
system	134	5 (22.4)	23 (4.8)	9 (14.7)	6 (18.3)	3 (31.1)
sludge	99	6 (16.6)	5 (19)	7 (17.2)	5 (18.9)	8 (14.4)
anaerobic	94	7 (15.7)	23 (4.8)	11 (11.2)	6 (18.3)	7 (17.8)
wastewater	93	8 (15.6)	23 (4.8)	7 (17.2)	12 (10.7)	6 (19.6)
process	83	9 (13.9)	7 (14.3)	9 (14.7)	6 (18.3)	11 (10.7)
apparatus	78	10 (13.1)	23 (4.8)	6 (21.6)	9 (17.2)	16 (8.1)
treating	78	10 (13.1)	15 (7.1)	5 (27.6)	17 (7.1)	9 (11.5)
water	69	12 (11.6)	5 (19)	19 (5.2)	9 (17.2)	14 (9.6)
fertilizer	62	13 (10.4)	8 (11.9)	19 (5.2)	11 (13)	11 (10.7)
biogas	48	14 (8)	44 (2.4)	23 (4.3)	15 (9.5)	14 (9.6)
nitrogen	48	14 (8)	not used	11 (11.2)	26 (4.7)	13 (10)
device	45	16 (7.5)	9 (9.5)	23 (4.3)	36 (3.6)	10 (11.1)
digestion	45	16 (7.5)	not used	17 (6)	12 (10.7)	17 (7.4)
liquid	42	18 (7)	23 (4.8)	13 (7.8)	14 (10.1)	25 (5.2)
production	40	19 (6.7)	9 (9.5)	15 (6.9)	15 (9.5)	27 (4.4)
sewage	38	20 (6.4)	9 (9.5)	15 (6.9)	26 (4.7)	18 (6.7)
using	31	21 (5.2)	15 (7.1)	23 (4.3)	26 (4.7)	21 (5.6)
phosphorus	29	22 (4.9)	44 (2.4)	36 (2.6)	26 (4.7)	19 (6.3)
producing	29	22 (4.9)	44 (2.4)	31 (3.4)	17 (7.1)	27 (4.4)
biological	28	24 (4.7)	9 (9.5)	36 (2.6)	36 (3.6)	21 (5.6)
digested	28	24 (4.7)	15 (7.1)	13 (7.8)	17 (7.1)	84 (1.5)
recovery	28	24 (4.7)	44 (2.4)	23 (4.3)	22 (6.5)	32 (4.1)
processing	27	27 (4.5)	23 (4.8)	not used	17 (7.1)	26 (4.8)
energy	26	28 (4.4)	44 (2.4)	31 (3.4)	36 (3.6)	21 (5.6)
plant	26	28 (4.4)	23 (4.8)	78 (0.9)	17 (7.1)	32 (4.1)
solid	25	30 (4.2)	44 (2.4)	48 (1.7)	31 (4.1)	21 (5.6)

[a] Abbreviations: NI, number of items; R, ranking.

[b] Total number of items: 597 patent families (42, 116, 169, and 270 patent families for the periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively).

Author-provided keywords

Publications. Author-provided keywords analysis is supposed to offer information about research trends that concern researchers (J. Li *et al.*, 2009). The examination of these keywords revealed a total list of 2330 items which were used 5672 times (163 publications did not include author-provided keywords information). It is interesting to remark that 1690 (72.5%) of these keywords were used only once, 284 (12.2%) were used twice, and only 82 (3.5%) were used ten or more times (37.7% of the uses). Thus, the higher complexity of the items analyzed in this section (formed by one or more words) favored a higher dispersion. According to other bibliometric studies (D. Uguloni *et al.*, 2001; K.-Y. Chuang *et al.*, 2007; J. Li *et al.*, 2009, L.-L. Li *et al.* 2009), the large number of once-cited keywords may indicate a lack of continuity in research and a wide disparity in the research aims. In addition, these keywords might not be standard or widely accepted by researchers (i.e., they are assigned by the author rather than following a systematic indexing method). Indeed, this lack of standardization hinders the analysis since use of language may be imprecise (individual authors may use the same term to mean different things or may define similar concepts with either synonymous or different terminology). The 30 most frequently used keywords are

listed in Table 13. In order to assist in the interpretation of such an heterogeneous list, the referred keywords may be grouped into the following main thematic packages: by-product production process ("anaerobic digestion", "biogas", "methane" and "wastewater treatment"), processed materials and produced by-products ("sewage sludge", "reject water", "biosolids", "digestate" and "wastewater"), N and P species ("struvite", "nitrogen", "phosphorus", "ammonia", "nitrous oxide" and "nitrite"), nutrient managing approaches ("nitrogen removal", "nutrient removal", "fertilizer" and "phosphorus recovery"), biological treatments ("anammox", "nitrification", "denitrification", "partial nitrification", "microalgae", "nitritation" and "compost") and environmental impact ("heavy metals" and "greenhouse gas").

Tableau 13: Top 30 most frequently author-provided keywords in publications dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014; results referred to total timespan and 5-year partial periods).

Author-provided keywords (publications)	NI	R (%) 1995-2014	R (%) 1995-1999	R (%) 2000-2004	R (%) 2005-2009	R (%) 2010-2014
anaerobic digestion	223	1 (18.4)	1 (17.0)	2 (12.3)	1 (17.1)	1 (20.4)
biogas	85	2 (7.0)	14 (3.2)	14 (4.6)	5 (5.9)	2 (8.7)
anammox	79	3 (6.5)	not used	17 (3.9)	2 (10.3)	3 (6.2)
struvite	73	4 (6.0)	6 (5.3)	5 (9.1)	3 (7.2)	4 (4.8)
nitrification	71	5 (5.9)	2 (11.7)	1 (13.0)	5 (5.9)	11 (3.3)
sewage sludge	66	6 (5.5)	3 (8.5)	3 (11.1)	7 (4.7)	7 (4.1)
nitrogen removal	57	7 (4.7)	6 (5.3)	7 (7.8)	4 (6.2)	15 (3.0)
denitrification	56	8 (4.6)	6 (5.3)	4 (9.7)	7 (4.7)	11 (3.3)
nitrogen	54	9 (4.5)	4 (6.4)	11 (5.2)	7 (4.7)	8 (3.9)
sequencing batch reactor	45	10 (3.7)	10 (4.3)	8 (7.1)	7 (4.7)	21 (2.3)
reject water	44	11 (3.6)	57 (1.1)	11 (5.2)	11 (3.7)	10 (3.4)
phosphorus	43	12 (3.55)	24 (2.1)	8 (7.1)	15 (2.8)	11 (3.3)
nutrient removal	42	13 (3.5)	10 (4.3)	10 (5.8)	32 (1.9)	9 (3.6)
biosolids	37	14 (3.1)	14 (3.2)	6 (8.4)	25 (2.2)	24 (2.2)
methane	35	15 (2.9)	24 (2.1)	19 (3.3)	15 (2.8)	18 (2.8)
ammonia	34	16 (2.8)	57 (1.1)	14 (4.6)	19 (2.5)	18 (2.8)
digestate	33	17 (2.7)	not used	not used	94 (0.6)	4 (4.8)
partial nitrification	32	18 (2.6)	not used	99 (0.7)	12 (3.1)	11 (3.3)
wastewater	31	19 (2.6)	57 (1.1)	17 (3.9)	15 (2.8)	21 (2.3)
nitrous oxide	30	20 (2.5)	57 (1.1)	30 (2.0)	25 (2.2)	15 (3.0)
microalgae	29	21 (2.4)	not used	not used	198 (0.3)	6 (4.4)
nitritation	29	21 (2.4)	not used	30 (2.0)	15 (2.8)	20 (2.7)
wastewater treatment	28	23 (2.3)	14 (3.2)	22 (2.6)	12 (3.1)	27 (1.7)
heavy metals	27	24 (2.2)	24 (2.1)	30 (2.0)	25 (2.2)	21 (2.3)
compost	25	25 (2.1)	10 (4.3)	30 (2.0)	25 (2.2)	27 (1.7)
nutrients	23	26 (1.9)	not used	99 (0.7)	58 (0.9)	15 (3.0)
fertilizer	22	27 (1.8)	not used	11 (5.2)	42 (1.3)	36 (1.6)
greenhouse gas	22	27 (1.8)	not used	50 (1.3)	58 (0.9)	27 (1.7)
nitrite	22	27 (1.8)	24 (2.1)	19 (3.3)	19 (2.5)	47 (1.1)
phosphorus recovery	22	27 (1.8)	not used	30 (2.0)	19 (2.5)	27 (1.7)

[a] Abbreviations: NI, number of items; R, ranking.

[b] Total number of items: 1211 publications (94, 154, 321, and 642 publications for the periods 1995-1999, 2000-2004, 2005-2009, and 2010-2014, respectively).

Those keywords provided by the authors concerning fertilization and treatment alternatives for the digested effluents were further processed, and subsequently represented as shown in Figure 14, in order to identify the main relative trends. Interest in those biotechnologies based on the anammox and algae cultivation has certainly grown during the last years. On the other hand, relative number of publications in other better known technologies such as precipitation (i.e., struvite and calcium phosphate crystallisation) seems to decline progressively, probably because of the extensive research already conducted (J. Greaves *et al.*, 1999; K.S. Le Corre *et al.*, 2009; Y.H. Liu *et al.*, 2013). Hence, over the past decade many technologies

concerning applicability of the partial nitrification-anammox concept have been developed and studied, and several of them are currently being implemented at full-scale (mainly linked to the sidestream treatment of reject water produced by dewatering anaerobically digested sewage sludge). Recently, mainstream wastewater treatment using energy efficient processes such as the anammox is gaining attention and has been identified as a priority for innovation and development by the water industry (S. Lackner *et al.*, 2014; J.D. Vela *et al.*, 2015). Concerning microalgae cultivation, multiple applications have been demonstrated as feasible, allowing combined biofuel production, carbon dioxide mitigation, and nutrient recovery from wastewater streams. The conversion of algae into biogas, and the subsequent utilization of the digestate for algae cultivation, makes it possible an attractive closed loop approach for bioenergy generation (T. Cai *et al.*, 2013; S.K. Prajapati *et al.*, 2014). In a similar way, research interest in the combination of organic farming and bioenergy production (i.e., focused on agricultural systems rather than bioenergy technology and engineering) has recently been emphasized by means of scientometric methods (T. Siegmeier and D. Möller, 2013).

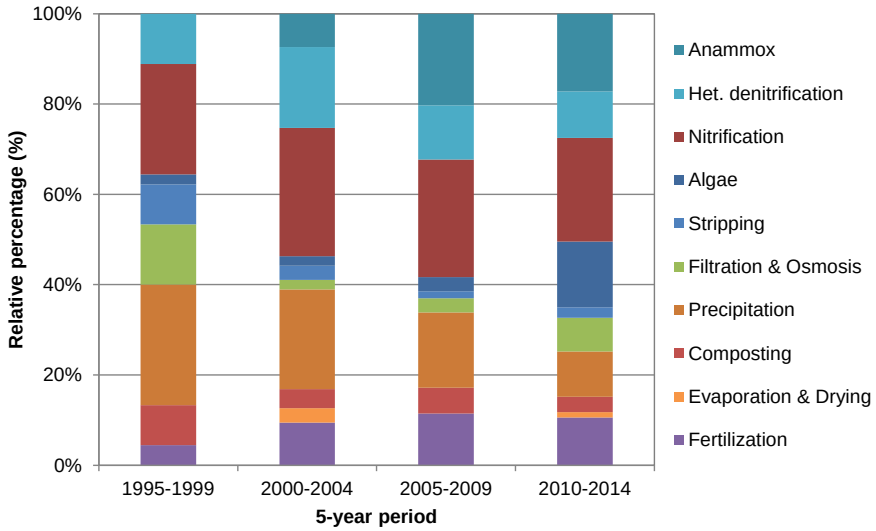

Figure 14: Relative trends concerning interest in particular treatment alternatives for the biogas digester effluents according to the analysis of the author-provided keywords in publications.

Patents. Inventors do not provide a list of keywords when filing a patent, so their analysis is not possible here. Otherwise, after automatically analyzing the abstract of the patents, the top 30 retrieved keywords matched 60% of the words included in Table 12 (obtained when analyzing the titles).

Comparison between publications and patents

Productivity assessment

The number of publications (1211 items) identified in this study was double than the number of patent families (597 items). The documentary production did not growth at a steady rate from 1995 to 2014, but according to a rising pattern (Figure 12). In this regard, more than 45% of the items for both documentary sources were produced in the last 5-year period (2010-2014). Only in 2002 the number of patents exceeded the number of publications. The USA, China and Japan were the 3 most prolific countries when considering the joint production of publications and patents. However, while regarding the USA the number of publications was higher than the number of patents, the opposite was true for China and Japan. Spain was the first European country in terms of productivity (holding the 4th position of the ranking). The most productive institutions concerning scientific publications (also the most cited items) were predominantly from European countries, whereas the most productive organizations concerning patents were from Asian countries (even though the USA patents were the most cited).

Lexical assessment

As aforementioned, the publication-to-patent ratio in terms of number of items was 2.0 while the corresponding ratios concerning number of words and number of word uses in the title increased to 2.4 and 3.0, respectively. This means that, in relative terms, titles in publications included more different words (1.6 vs. 1.3 words/item) which were also more used (10.8 vs. 7.2 uses/item). Hence, titles in publications were globally less uniform and more descriptive (longer) than in patents. Indeed, poorly informative original titles are a well-known attribute of patents, since a too precise description can be excessively informative for competitors and is also likely to narrow the scope of the invention (H.F. Moed et al., 2005). Regarding the most used words in titles, the relative usage in publications and patents is shown in Figure 15 (all words from Tables 11 and 12 are included). Those words with a higher difference in the relative percentage of use between both documentary sources usually showed higher frequencies for patents.

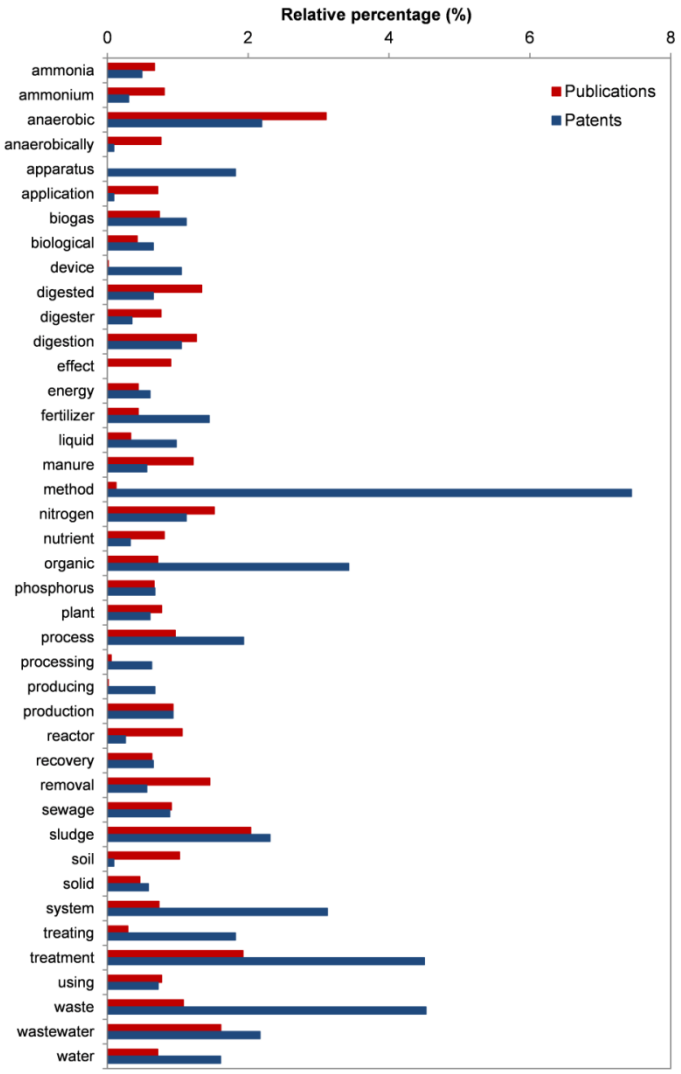

Figure 15: Relative number of uses concerning the most frequently used words in the titles of publications and patents.

Citation assessment

As a comparative example, a citation impact assessment for publications and patents is shown in Table 14. For both datasets, the analysis was performed considering all items, and also considering partial groups of items according to country and topic criteria. Those countries that were individually taken into account were the top 4 countries regarding joint production of publications and patents (i.e., according to Table 7 these countries are USA, China, Japan and Spain). On the other hand, those particular topics considered in the assessment were: anammox-based autotrophic N-removal, nutrient precipitation (involving struvite and calcium phosphate crystallisation), and soil application (including experimental developments concerning digestate characterization, soil amendment, agricultural fertilization, and production of fertilizers, among others; otherwise, those developments dealing with theoretical environmental assessment, digestate post-treatment and refinery approaches were discarded). The citation

rate refers to the number of cites per item and year (citations were counted for 3-year time periods beginning at the publication year). For publications, the global citation rate averaged 1.74. Similar partial citation rates were obtained for the 4 countries under study (range: 1.60-2.02). The topic 'anammox' showed the higher citation rates (average: 3.61), although for the countries under study such rates were lower than the average (range: 1.87-3.38). This is probably because of the high scores of those publications coming from the country where the process was initially developed (e.g., Netherlands). Concerning patents, in general terms, a significant higher citation rate (1.16) was obtained for those patents initially filed in USA in comparison with the rates obtained for the patents filed in other countries (range: 0.21-0.48; global average: 0.46). 'Anammox' was again the topic with the highest citation impact (USA was the exception).

Tableau 14: Citation impact assessment concerning publications and patents dealing with nutrient (N and P) management from biogas digester effluents (timespan: 1995-2014). For both datasets, the analysis was performed considering all items, and also considering partial groups of items according to country and topic criteria. Citations were counted for 3-year time periods beginning at the publication year. a, b

Publications								
Country ▼ | Topic ►	**All topics**		**Particular topics**					
			Anammox		**Precipitation**		**Soil application**	
	NI [c]	**CR**	**NI**	**CR**	**NI**	**CR**	**NI**	**CR**
All countries	1211	1.74	94	3.61	115	1.48	291	1.29
USA	204	1.81	5	1.87	17	1.82	44	1.17
China	118	1.60	13	2.28	12	1.50	18	0.54
Japan	78	1.75	15	3.38	11	1.45	23	1.13
Spain	123	2.02	11	3.27	11	1.52	29	1.87
Patents								
Country ▼ | Topic ►	**All topics**		**Particular topics**					
			Anammox		**Precipitation**		**Soil application**	
	NI	**CR** [d]	**NI**	**CR**	**NI**	**CR**	**NI**	**CR**
All countries	597	0.46	32	0.70	73	0.41	184	0.52
USA	103	1.16	3	0.67	15	0.96	31	1.53
China	165	0.48	20	0.73	6	0.17	68	0.46
Japan	102	0.23	4	0.25	31	0.23	16	0.21
Spain	13	0.21	1	1.00	2	0.33	3	0.00

[a] Individual countries taken into account were the top 4 countries regarding joint production of publications and patents. Particular topics taken into account were: anammox-based autotrophic N-removal, nutrient precipitation (involving struvite and calcium phosphate crystallisation), and soil application (including experimental developments concerning digestate characterization, soil amendment, agricultural fertilization, and production of fertilizers, among others).

[b] For those items published after 2013, citation counts were proportionally corrected according to the information retrieval date (mid-2016).

[c] Abbreviations: NI, number of items; CR, citation rate (average number of citations per item and year -during the next 3 years after publication-).

[d] Citations are referred to patent documents, and not to patent families.

Although it is out of the scope of this paper due to the relative small size of the assessed datasets, it is also worth pointing out that cross-citation analysis considering bi-directional links between publications and patents (i.e., publications citing patents and patents citing publications) has been proposed to identify eventual connections between S&T (F. Narin and D. Olivastro, 1998; A. Verbeek *et al.*, 2002; W. Glänzel and P. Zhou, 2011).

Overall, the use in this study of bibliometric tools resulted in an alternative approach for the review of the S&T productivities during the recent years in the field of nutrient management from anaerobic digester effluents. The growing interest in such environmental topic (frequently linked to waste-to-energy applications based on the production of biogas as renewable energy) was quantitatively evidenced. The

heterogeneity in terminology (different ways under which different authors and inventors refers to the same concept, frequently including use of multiple words), and the broad spectrum of the subject area (involving several management and treatment alternatives -which in turn are not of exclusive application in the field of the anaerobic digester effluents-), limited the definition of a simple search strategy for the identification of the S&T outputs in specialized databases, and made necessary a manual screening (which is always time-demanding and requires of the criterion of an specialist in the topic) of the datasets automatically retrieved before subsequent assessment. The in-depth analysis of publications and patents was useful for the identification of the most productive countries, institutions and individuals (confirming significant differences between both documentary sources). The relative interest for some particular nutrient management alternatives and its evolution could be identified (more explicit in the case of the publications). Future studies and developments are called to face the sustainability challenge by reducing resource consumption, considering integral approaches, and promoting circular economies, among other aspects.

4. Conclusion

Development of environmental S&T in the field of nutrient management from biogas digester effluents during the last twenty years (i.e., from 1995 to 2014) was investigated using a bibliometric-based approach. Information concerning relevant publications (as scientific research outputs) and patents (as technological development outputs) was retrieved from specialized databases (SCIE and PatBase, respectively) and systematically analyzed.

- The number of publications was double than the number of patents (1211 vs. 597 items, respectively). The production followed a rising pattern; i.e., more than 45% of the items were produced in the last 5-year period for both documentary sources.
- The USA, China and Japan were the 3 most prolific countries when considering the joint production of publications and patents. However, while concerning the USA the number of publications was higher than the number of patents, the opposite was true for China and Japan. The most prolific European country was Spain (in 4th position).
- The most prolific institutions concerning publishing (also the most cited items) were predominantly European, whereas the most prolific organizations concerning patenting were Asian (even though the USA patents were more cited).
- The relative interest for some particular nutrient management alternatives and its evolution could be identified (more explicit in the case of the publications). A decreased consumption of resources, the implementation of integral solutions and circular economy approaches will be key issues in future studies and developments fostering sustainability.

5. Highlights concerning the anammox process

The beginning of the timelap considered in this study, 1995, coincides with the first report made by A. Mulder *et al.* in 1995 concerning the anammox metabolism. As illustrated previously in Figure 14 the relative importance of anammox kept increasing rapidly eversince to reach approximatively 20% of academic documentary production after 10 years within the topic of biogas digester supernatant nutrients management. This trend maintained until today proving that interest is part of a momentum widely shared within the scientific community. Despite the relative youth of the anammox thematic and its inegal documentary production, ”anammox” remains the third keyword the most employed on the overall period. More impressive, 3 over the 4 most cited publications on all the timespan considered are directly related to the anammox process and its implementation. If the academic world clearly pays great attention to anammox, industrial aspects are also of importance. In fact, except for the US, anammox related patents possess the highest citation rates exceeding phosphate precipitation and soil application.

Disclosure statement

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by Rennes Métropole under the allocation d’installation scientifique number 14C0509.

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CHAPITRE 3 : Enrichissement de bactéries anammox en batch à partir de différents inoculum

CHAPTER 3: Anammox bacteria batch enrichment considering different inoculum sources

This chapter is dedicated to the study of anammox enrichment from various kind of inoculums in batch mode. Operational parameters are of importance as they dictate enrichment feasibility, time required for its achievement and the specific species selected. This is why it is of importance to improve understandings on the early anammox enrichment phase to better direct and optimize the procedure.

This enrichment procedure is particularly focused on effects of nitrite concentration on both anammox populations and global microbial community shifts. Use of molecular tools gave the opportunity to better describe and understand this changing during early enrichment phase. This chapter is presented as a scientific publication according to the following reference:

R. Connan, P. Dabert, H. Khalil, G. Bridoux, F. Béline, A. Magrí, Batch enrichment of anammox bacteria and study of underlying microbial community dynamics, Chemical Engineering Journal 297 (2016), Pages 217-228.

Batch enrichment of anammox bacteria and study of the underlying microbial community dynamics

Romain Connan [a], Patrick Dabert [a], Hassan Khalil [a], Gilbert Bridoux [b], Fabrice Béline [a], Albert Magrí [a,*]

[a] Irstea, UR OPAALE, 17 Avenue de Cucillé-CS 64427, F-35044 Rennes, France

[b] SAUR, Atlantis, 1, Avenue Eugène Freyssinet, F-78280, Guyancourt, France

ABSTRACT

The anaerobic ammonium oxidation (anammox) consists on the biological conversion of ammonium (NH_4^+) into dinitrogen gas under absence of oxygen. Nitrite (NO_2^-) is a substrate of the anammox reaction, but also an inhibitor at high concentrations. This study investigates the effect of nitrite on the microbial community during the batch enrichment of anammox sludge. Six inoculums collected from different environments were enriched after a conditioning pretreatment and under controlled conditions during 4 months. Concerning the mineral medium used, two different nitrite supply strategies were applied; i.e., (i) initially low concentration at 25 mg NO_2^--N/L and progressive increase to 150 mg NO_2^--N/L, and (ii) constant high concentration at 150 mg NO_2^--N/L. All tested inoculums developed anammox activity but only when the enrichment was started at low nitrite concentration. In such case, the specific ammonium conversion rates finally obtained ranged from 21 ±1 to 118 ±1 mg NH_4^+-N/g VS/d (VS, volatile solids). Abundance of the functional gene encoding for the enzyme hydrazine oxidoreductase (*hzo*) was assessed using the real-time quantitative polymerase chain reaction (q-PCR) showing positive correlation with the anammox activity finally reported. In addition, high-throughput DNA sequencing helped to elucidate the underlying microbial community dynamics. The raw inoculum source, the conditioning pretreatment, and the cultivation conditions applied were jointly determinants of the final microbial community structure of the enrichments despite a clear convergence at the end of the experimental period. On the other hand, the cultivation conditions alone determined the selection of anammox species belonging to the genus *Candidatus Brocadia*.

KEYWORDS

Anaerobic ammonium oxidation; Biomass enrichment; Nitrite; Real-time quantitative polymerase chain reaction; Next-generation sequencing.

1. INTRODUCTION

The anaerobic ammonium oxidation (anammox) consists on the biological conversion of ammonium (NH_4^+) into dinitrogen gas (N_2) under absence of oxygen. This is a chemolithoautotrophic microbial process where nitrite (NO_2^-) acts as the electron acceptor. The anammox reaction also involves the production of a minor fraction of nitrate (NO_3^-). According to M. Strous *et al.* (1998), the corresponding molar ratios for NH_4^+ consumption, NO_2^- consumption, N_2 production, and NO_3^- production are 1.00/1.32/1.02/0.26, respectively. Metagenomic studies also suggest that nitric oxide (NO) and hydrazine (N_2H_4) are intermediates in the anammox reaction (M. Strous *et al.*, 2006). The anammox process was discovered in the early 1990's in a denitrifying fluidized bed reactor (A. Mulder *et al.*, 2005) and the interest in this bioprocess has ever since been rising in fundamental and applied research fields, such as marine ecology and environmental biotechnology (K.R. Arrigo, 2005; B. Kartal *et al.*, 2010).

So far, six "Candidatus" anammox bacterial genera have been enriched (M.S.M. Jetten *et al.*, 2009; S.V. Khramenkov *et al.*, 2013) from wastewater treatment facilities and freshwater environments (*Brocadia*, *Kuenenia*, *Jettenia*, *Anammoxoglobus* and *Anammoximicrobium*), as well as from marine environments (*Scalindua*). In physiological terms, they feature a specific cytoplasmatic membrane-bound organelle known as anammoxosome, which is the locus of the anammox catabolism. They are also characterized by a low growth rate, with doubling times of 2.1-11 days (at ~30ºC) equivalent to a maximum specific growth rate of 0.065-0.334 d^{-1} (M. Strous *et al.*,1998; T. Lotti *et al.*, 2015). Because of this slow biomass development, and the specialized metabolism, anammox bacteria may be difficult to culture. Yet, phylotypes related to the anammox genera have increasingly been observed by molecular means in diverse environments, such as activated sludge from wastewater treatment plants (WWTP) (H. Bae *et al.*, 2010b; M. Waki *et al.*, 2010), marine sediments (B. Thamdrup *et al.*, 2002; M. Li *et al.*, 2010), freshwater environments (I. Yoshinaga *et al.*, 2011), and terrestrial ecosystems (A. Long *et al.*, 2013).

Anammox bacteria have not been isolated in pure culture yet, thus pointing to the fact that they may coexist with other microbial species, even in bioreactors fed exclusively with mineral substrates (T. Lotti *et al.*, 2015; H. Bae *et al.*, 2010a; S. Qiao *et al.*, 2009). The most frequent enrichment strategies have been based on different types of continuously operated bioreactors (M. Ibrahim *et al.*, 2016); e.g., sequencing batch reactor (SBR), rotating biological contactor, up-flow biofilm reactor, or membrane bioreactor (M. Strous *et al.*, 1998; K. Egli *et al.*, 2003; T. Fujii *et al.*, 2002; T. Wang *et al.*, 2009). Alternatively, enrichments have also been developed in batch cultures (H. Bae *et al.*, 2010b; S.K. Toh *et al.*, 2002; A. Sànchez-Melsió *et al.*, 2009; W. Sun *et al.*, 2011). Many studies have shown that successful cultivation of anammox bacteria from conventional sludge takes long time; i.e., generally from 4 months to 1 year (Y. Tao *et al.*, 2013a). Such time will be influenced by factors like (*i*) the ecological characteristics of the seeding sludge including initial concentration and relative abundance of anammox bacteria (Y. Tao *et*

al., 2013), (*ii*) effective biomass retention inside the reactor (C. Fux *et al.*, 2004), and (*iii*) the environmental conditions applied: temperature, pH, and concentration of NH_4^+, NO_2^-, dissolved oxygen (DO), organic carbon, sulphide and other inhibitors like metals and antibiotics (R.-C. Jin *et al.,* 2012; Y. Tao and D.-W. Gao, 2013; J.M. Carvajal-Arroyo *et al.*, 2013).

Monitoring the anammox activity usually involves the chemical analysis of relevant nitrogen (N) compounds (i.e., NH_4^+, NO_2^-, and NO_3^-) in the liquid phase. However, this strategy might be unsuccessful during the initial stages of the enrichment process, when the number of anammox cells is too low and their activity can still not be detected macroscopically. The use of culture-independent molecular methods has been proposed as a suitable method in such cases and various protocols for the DNA amplification by polymerase chain reaction (PCR) of target genes have been described in the literature, as summarized by M. Li *et al.* (2010). Anammox specific primers have been developed for amplifying ribosomal genes (16S rRNA) or functional genes such as that encoding for the enzyme hydrazine oxidoreductase (*hzo*) that dehydrogenates hydrazine to N_2. These primers have been used to assess the abundance of anammox bacteria genes within environmental samples by real-time quantitative polymerase chain reaction (q-PCR), and to analyse the microbial community diversity by molecular typing and sequencing methods (H. Bae *et al.*, 2010b; I. Tsushima *et al.*, 2008; C. Hao *et al.*, 2009; B.-L. Hu *et al.*, 2010; H. Park *et al.*, 2010). Emerging next-generation sequencing (NGS) have also been applied for providing an in-depth characterization of the microbial biodiversity in anammox systems (Z. Hu *et al.*, 2012; M.C.M.S. Costa *et al.*, 2014; A. Gonzalez-Martinez *et al.*, 2015; E. Isanta *et al.*, 2015). Yet, not so much information is available in the literature concerning the microbial community structure and dynamics during enrichment of the anammox biomass as determined by quantitative and qualitative culture-independent molecular methods.

Autotrophic nitrogen removal (ANR) applications based on anammox are promising for N-removal from municipal side-/mainstreams, industrial and agricultural wastewaters (S. Lackner *et al.*, 2015; G. Xu *et al.*, 2015; S.W.H. Van Hulle *et al.*, 2010; A. Magrí *et al.*, 2013), particularly after anaerobic digestion once biodegradable organic carbon is depleted. However, anammox enriched sludge is not always available, and biomass enrichment can become the critical point for the start-up of the process. An appropriate selection of the environmental conditions applied is decisive for a successful enrichment. In this regard, NO_2^- is a substrate of the anammox reaction but may also become an inhibitor at high concentrations. Such inhibition has been reported as highly case-specific; i.e., concentrations as low as 5 and 30 mg NO_2^--N/L were found as inhibitory in some studies (B. Wett *et al.*, 2007; E. Bettazzi *et al.*, 2010) whereas much higher inhibitory boundaries of 210-274 mg NO_2^--N/L were determined in other cases (A. Dapena-Mora *et al.*, 2007; Y. Kimura *et al.*, 2010; A. Magrí *et al.*, 2012a). Concerning this variability, Y. Kimura *et al.* (2010) suggested that differences in NO_2^- concentration tolerance may be caused by the cultivation conditions used. The aim of this study is to investigate the presence of anammox populations in different inoculum sources and to assess the feasibility of enrichment in batch under two different strategies concerning NO_2^-

supply. Thus, final concentrations of 150 mg NO_2^--N/L were targeted in the mineral medium used as feeding solution but testing two different supply strategies (i.e., initially low vs. high concentration) in order to evaluate the effect of the NO_2^- concentration when starting anammox batch enrichments. Use of molecular techniques will help to detect anammox bacteria and to establish correlations between macroscopically observed process parameters and the underlying microbial community dynamics. Microbial monitoring will be conducted using q-PCR and 16S rRNA gene targeted NGS.

2. MATERIALS AND METHODS

2.1. Inoculum sources

Six different biomass sources collected in conventional N-removal facilities were considered as inoculum (I) for batch enrichment; i.e., (I1) activated sludge collected in a municipal WWTP that combine the use of a Modified Ludzack-Ettinger (MLE) bioreactor unit and a membrane filtration loop (Betton, Brittany, France), (I2) mixture of activated and settled sludge collected in a pig slurry treatment plant with intermittent aeration and gravity settling (Meslin, Brittany, France), (I3) activated sludge collected in a pig slurry treatment plant with MLE configuration (Calldetenes, Catalonia, Spain), (I4) settled sludge -sediments- collected in a receiving lagoon treating municipal wastewater (Amanlis, Brittany, France), (I5) settled sludge -sediments- collected in polishing lagoons treating municipal wastewater (Amanlis, Brittany, France), and (I6) settled sludge collected in an intermittently aerated lagoon treating pig slurry (Almacelles, Catalonia, Spain). The volatile solids (VS) content of the samples was 0.44%, 1.82%, 0.62%, 0.82%, 0.48%, and 1.92% of the wet weight, respectively; whereas, the corresponding VS/TS ratio (TS, total solids) was 0.52, 0.64, 0.54, 0.09, 0.09, and 0.59, respectively. In order to favor biodegradation of available organic carbon before incubation for anammox biomass enrichment, a conditioning pretreatment based on promoting denitrification was carried out at room temperature during the first days after sampling by adding a NO_3^- source such as KNO_3 in pulses of 722 mg/L (100 mg N/L) and controlling the pH within the range 7.0-8.0 (HCl 2M). Batch enrichment was started once denitrification declined (after 2-4 weeks).

2.2. Mineral medium

The synthetic nutritive solution was prepared using tap water according to a modification of the mineral medium described by Magrí *et al.* (2012a); i.e., NH_4Cl (variable: 95-573 mg/L), $NaNO_2$ (variable: 123-739 mg/L), KNO_3 (361 mg/L), $KHCO_3$ (1000 mg/L), $FeSO_4 \cdot 7H_2O$ (9 mg/L), EDTA (5 mg/L), $MgSO_4 \cdot 7H_2O$ (240 mg/L), $CaCl_2 \cdot 2H_2O$ (143 mg/L), and trace element solution 0.3 mL/L. The trace element solution contained $ZnSO_4 \cdot 7H_2O$ (1247 mg/L), $MnSO_4 \cdot H_2O$ (1119 mg/L), $CuSO_4 \cdot 5H_2O$ (44 mg/L), $Al_2(SO_4)_3 \cdot 14H_2O$ (201.5 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (129 mg/L), $CoCl_2 \cdot 6H_2O$ (30 mg/L), KCl (100 mg/L), and EDTA (975 mg/L). Once dissolved the mineral salts, the DO was purged by bubbling with N_2 (< 0.2 mg/L) and the pH was adjusted to 7.0 (HCl 2M). The chemical KNO_3 was added aiming to strengthen the anoxic conditions and to prevent

potential sulphate reduction to sulphide (which could inhibit the anammox reaction) in case of total NO_2^- consumption during the enrichment. Unfortunately, no phosphorus source was added to the nutritive solution throughout the enrichment due to a mistake, discovered later, in the labeling of the corresponding chemical (K_2O (27 mg/L) was supplied instead of KH_2PO_4). Thus, it seems that the phosphorus released by the decaying biomass was enough to avoid limitation in the availability of this nutrient.

2.3. Enrichment procedure

Twelve glass bottles (total volume: 575 mL; working volume: 500 mL) containing inoculum and mineral medium were flushed with N_2, sealed with a rubber stopper plus an aluminum cap, and placed in an incubator shaker (KS4000i control, IKA, Germany) at 150 rpm, 35ºC, and in dark conditions. Initial VS content within the bottles was adjusted to 3 g/L. Biomass settling was allowed once per week (for 1h). Each bottle was then opened and the supernatant was manually withdrawn to avoid accumulation of inhibitory compounds while keeping the settled biomass inside of the bottles. Subsequently, the bottles were refilled with new nutritive mineral medium, closed, and flushed with N_2. Two different strategies concerning NO_2^- supply were applied (i.e., initially low vs. high concentration). Thus, mineral medium was prepared with low amount (25 mg NO_2^--N/L) or high amount (150 mg NO_2^--N/L) of NO_2^- whereas NH_4^+ was added at a constant rate of 25 mg NH_4^+-N/L. In those bottles running at low NO_2^- concentration, once anammox activity was detected, a second weekly addition of NO_2^- and NH_4^+ (NO_2^--N/NH_4^+-N = 1.2) was carried out. Following this procedure, NO_2^- content in the mineral medium was progressively increased from 25 to 150 mg N/L at increments of 12.5 mg N/L. For the six bottles running at high NO_2^- concentration, the NO_2^- content was kept constant at 150 mg N/L with only one feeding event per week throughout the experimental period. Concerning NH_4^+ content, it was proportionally increased at a ratio of 1.2 g NO_2^--N per gram of NH_4^+-N for those first six bottles initially running at low NO_2^- concentration but it was kept constant at 25 mg NH_4^+-N/L for the others. The pH within the bottles was controlled in the range from 7.0 to 8.0 using HCl 2M. N_2 flushing was used to displace air in the bottles headspace every time they were opened. The liquid volume exchanged when renewing the mineral medium was variable depending on the settling capability of the biomass, but usually between 60-80%. This is a higher value than those generally considered in anammox SBRs (H. López *et al.*, 2008; A. Magrí *et al.*, 2012b; Z. Hu *et al.*,2013). Enrichment lasted 4 months. Liquid samples were taken before and after each new feeding event and filtered using 0.45 µm polypropylene membrane filters prior to storage in the refrigerator. Biological samples were taken once per month, centrifuged at 10,000g for 4 min and supernatants were discarded. Pellets were stored at -20°C.

2.4. Final anammox activity test

Final anammox activity was assessed using batch tests at the end of the enrichment period. According to the aforementioned conditions, after renewing the mineral medium, liquid samples were collected using syringes at regular time intervals for N-compounds analysis. VS contents were also measured. The batch experiments were done in duplicate. Linear regression analyses were used to describe N-conversion.

2.5. Chemical analyses

NH_4^+, NO_2^- and NO_3^- were measured by ion chromatography (850 Professional IC, Metrohm, Switzerland). TS were measured after sample drying to constant weight at 105ºC and VS were measured after further ignition in a muffle furnace at 550ºC. The pH and DO were measured using portable meters pH 197i and Oxi 197 (WTW, Germany), respectively.

2.6. Molecular analyses

2.6.1. DNA extraction

Total DNA was extracted from approximately 0.25 g of pellet with the PowerSoilTM DNA Isolation Kit (MoBio Laboratories Inc., USA), according to the instructions of the manufacturer. The concentration and purity of the extracted DNA were checked spectrophotometrically (ND-1000, NanoDrop Technologies, USA) and in TBE 1X - 0.7% agarose gel. The extracted DNA was stored at -20ºC until further analysis.

2.6.2. Real-time q-PCR

All real-time PCR amplifications were performed using the iQ™ SYBR® Green supermix (2x) (Bio-Rad Laboratories, USA) and the CFX96 real-time system equipped with the CFX Manager™ software v3.1 (Bio-Rad Laboratories), according to the instructions of the manufacturer. Quantification of total bacteria used universal eubacterial forward 1055F (5'-ATGGCTGTCGTCAGCT-3') and reverse 1392R (5'-ACGGGCGGTGTGTAC-3') primers to amplify the hypervariable V3-V5 region from the 16S rRNA gene, as previously reported by M.J. Ferris *et al.* (1996). Concerning anammox bacteria, the gene encoding specific enzyme HZO (*hzo* gene) was used as functional biomarker. The primer set used to selectively amplify anammox bacterial fragment was hzocl1F1 (5'-TGYAAGACYTGYCAYTGG-3') and hzocl1R2 (5'-ACTCCAGATRTGCTGACC-3') as described by M.C. Schmid *et al.* (2008). The PCR reactions were run in a 25 μL volume containing 12.5 μL of iQ™ SYBR® Green supermix (2x), 1.5 or 0.625 μL of each primer (eubacteria-16S rRNA or anammox-*hzo*, respectively; 10 μM), 2 μL of diluted DNA template, and 7.5 or 9.25 μL of sterile water (eubacteria or anammox, respectively). To check against potential inhibition of the PCR amplification, reactions were done on DNA templates diluted 10- and 100-folds and each reaction was carried out in triplicates. The amplification program applied for eubacteria was 95ºC for 10 min, followed by 45 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 50 s, and extension at 72ºC for 30 s. In case

of anammox bacteria, the program applied was 95ºC for 2 min, followed by 40 cycles of denaturation at 95ºC for 30 s, annealing at 51ºC for 60 s, and extension at 72ºC for 60 s. The fluorescence intensity of the amplified DNA was measured after each extension step and a melting curve analysis was performed after completion of PCR. The microbial quantification was based on a mean slope value derived from standard curves obtained by q-PCR amplification of 10-fold successive dilutions of DNA fragments of the targeted genes (Y. Zeng *et al.*, 2012), and which were purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) according to the instructions of the manufacturer. The absolute copy number of DNA fragments per µL of standard solution was calculated from the concentration measured spectrophotometrically (ND-1000) and the molecular mass of the DNA fragment. The standard curves covered a range from 10^2 to 10^{10} gene copies per µL of reaction. These series of standard dilutions were amplified along with the unknown samples during each q-PCR run, which allowed building the standard curve (R^2 > 0.98) corresponding to each run. Results were expressed as gene copy number per mL of mixed liquor within the enrichment bottle.

2.6.3. High-throughput DNA sequencing

16S rRNA genes high-throughput DNA sequencing was performed at the NGS facility of the BIOMIC Team of Irstea (Antony, France) using Ion Torrent™ (Life Technologies, USA) technology and methods, according to the procedures described by S. Poirier *et al.* (2016). Briefly, the bacterial and archaeal hypervariable region V4-V5 of the 16S rRNA genes was amplified using the "universal" fusion-primers 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 928R (5'-CCCCGYCAATTCMTTTRAGT-3') (Y. Wang and P.-Y. Qian, 2009) modified to allow the tagging and sequencing of the amplified products. Amplification was performed in a 50 µL reaction mixture containing 5 µL of *Pfx* buffer (10X), 1.5 µL of dNTP mix (10 mM each), 1 µL of $MgSO_4$ (50 mM), 1.5 µL of each primer (10 µM), 0.4 µL of Platinum® *Pfx* DNA polymerase, 38.1 µL of water and 1 µL of extracted DNA (10 to 200 pg) (*Pfx* SuperMix protocol from Life Technologies). The mixture was held at 94°C for 5 min, followed by 30 cycles at 94°C for 15 s, 50°C for 30 s and 68°C for 1 min, and a final extension step at 68°C for 5 min. PCR products were cleaned using the Agencourt® AMPure® XP magnetic beads purification system (Beckman Coulter, USA) and quantified with a capillary electrophoresis bioanalyzer (2100 Electrophoresis Bioanalyzer, Agilent Technologies, USA). Purified libraries were diluted (in a first step at 500 pM and later at 100 pM). Equal volumes of amplicons were combined in equimolar concentrations (100 pM) for sequencing using the Ion OneTouch™ 2 Instrument with the Ion PGM™ Template OT2 400 Kit and using a Ion Personal Genome Machine (PGM™) System with the Ion 316™ Chip Kit v2 and the Ion PGM™ Sequencing 400 Kit, according to the instructions of the manufacturer. Low quality and polyclonal sequence reads were filtered out by the PGM™ System software, and resulting data was exported as a FastQ file. 16S rRNA genes high-throughput DNA sequencing was performed directly on total DNA extracts to analyze global microbial community structure, and when anammox bacteria were not detected on PCR products obtained by specific amplification of the

Planctomycetes phylum using the primers Pla46F and 1392R, as described in H. Bae *et al.* (2010b). Sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME v1.8.0) pipeline (J.G. Caporaso *et al.*, 2010). Sequences shorter than 200 bp, containing chimeras, and found as singletons were removed. Operational taxonomic units (OTUs) were subsequently defined using UPARSE implemented in USEARCH (v8.0.1623) (R.C. Edgar, 2013) at a 97% similarity level. MOTHUR (v1.25.0) (P.D. Schloss *et al.*, 2013) and SILVA (v119) (C. Quast *et al.*, 2013) were used as the classifier tool and database for taxonomic association (with a minimum similarity threshold of 80%), respectively.

2.6.4. Statistical analyses

Statistical analysis to evaluate microbial community structure evolution was carried out through the non-metric multidimensional scaling (NMDS) method using the open-source software R (v3.2.3) (W.N. Venables *et al.*, 2015) including functions from the vegan package (v2.3-2) (J. Oksanen *et al.*, 2015). Shannon-Weaver, Simpson, and Inverse Simpson diversity indices, as well as species richness and Pielou's evenness were calculated according to the procedures described in J. Oksanen *et al.* (2015).

3. RESULTS AND DISCUSSION

3.1. Performance of the anammox enrichment

All tested inoculums developed positive anammox activity throughout the 4-month experimental period when the enrichment was started at low NO_2^- concentration (Figure 16A). However, biomass still maintained overall appearance of activated sludge and brownish color at the end of the enrichment. Three main enrichment phases were observed: (P1) endogenous heterotrophic denitrification was the dominant process and NH_4^+ may even slightly increase during incubation due to the hydrolysis of the remaining organic matter, (P2) occurrence of NH_4^+ consumption and subsequent speed up at increasing NO_2^- concentration, and (P3) consolidation of NH_4^+ consumption at high NO_2^- concentration with evidence of NO_3^- production (while the NO_2^--N/NH_4^+-N reaction ratio became closer to the expected value of 1.32 (M. Strous *et al.*, 1998)). Time for detecting NH_4^+ consumption under anaerobic conditions (beginning of P2) was variable (Table 15), ranging from 0 days in bottle seeded with I3 to 92 days in bottle seeded with I2 (time for anammox activity appearance was longer in those bottles seeded with the inoculums containing higher VS). According to this fact, and the progressive increase in the NO_2^- supplied, concentrations of 150 mg NO_2^--N/L at the beginning of a new batch were applied at the end of the experimental period in all cases except in bottle with I2, where the maximum concentration used was 50 mg NO_2^--N/L. On the other hand, no anammox activity was developed by any of the inoculums when enrichment was started at high NO_2^- concentration (Figure 16B), which evidences the importance of the feeding strategy adopted when targeting anammox bacteria enrichment and although NO_2^- levels of 150 mg N/L were not previously

evaluated as inhibitory elsewhere (A. Dapena-Mora *et al.*, 2007; Y. Kimura *et al.*, 2010; A. Magrí *et al.*, 2012a).

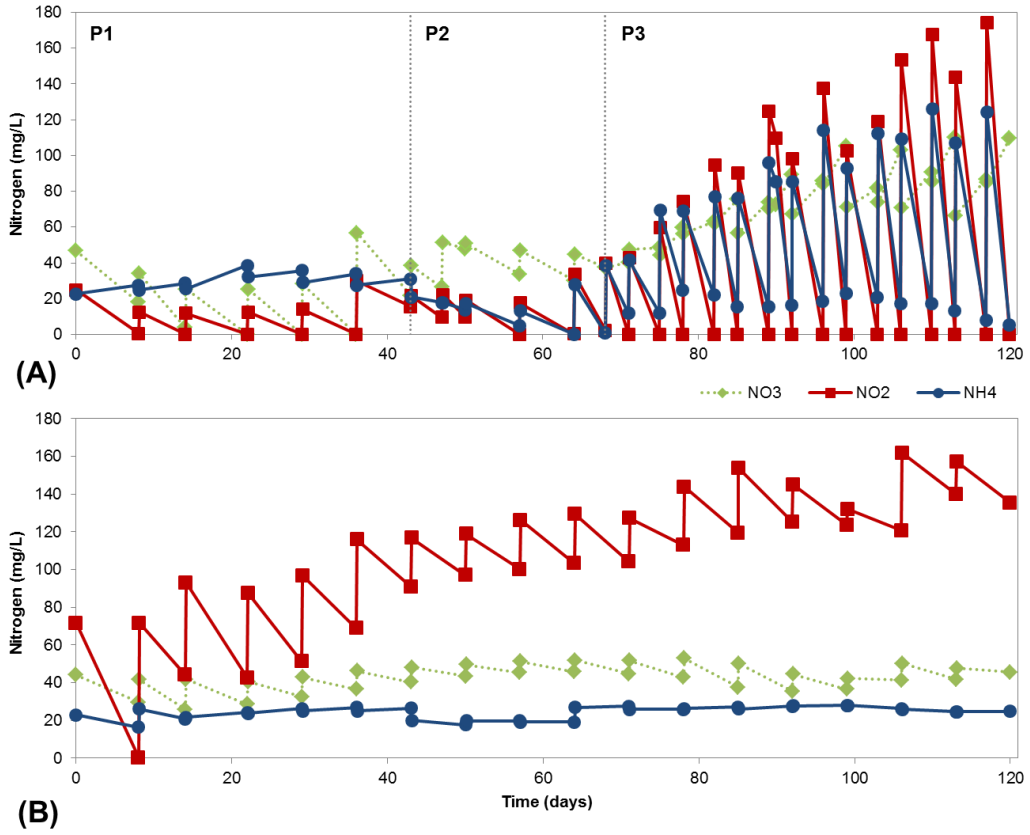


Figure 16: Example of evolution of the N-forms throughout the 4-month experimental period in the bottle seeded with I1 when process was started at (A) low nitrite concentration and (B) high nitrite concentration.

Tableau 15: Results of the anammox activity test at the end of the 4-month enrichment period when process was started at low nitrite concentration and comparison with the day of activity appearance and initial hzo gene copy number.

Inoculum (source)	Activity appearance (days)	ACR [a] (mg NH_4^+-N/L/d)	sACR (mg NH_4^+-N/g VS/d)	TNCR (mg N/L/d)	NO_2^--N/NH_4^+-N (-)	Initial *hzo* gene copy number (copies/mL) [b]
I1 (WWTP)	43	222 ±2	118 ±1	560 ±11	1.53 ±0.03	$6.3 \pm 1.4 \cdot 10^4$
I2 (PSTP)	92	60 ±9	42 ±6	160 ±25	1.67 ±0.01	$1.2 \pm 0.3 \cdot 10^5$
I3 (PSTP)	0	164 ±6	62 ±2	373 ±17	1.28± 0.02	$5.2 \pm 0.8 \cdot 10^4$
I4 (LWWTP)	57	78 ±1	26 ±0	208 ±8	1.64 ±0.08	$3.3 \pm 0.3 \cdot 10^{4*}$
I5 (LWWTP)	43	84 ±4	21 ±1	238 ±14	1.83 ±0.02	$5.4 \pm 0.9 \cdot 10^{4*}$
I6 (LPSTP)	77	162 ±12	70 ±5	370 ±37	1.28 ±0.05	$1.1 \pm 0.2 \cdot 10^5$

[a] ACR: ammonium conversion rate. sACR: specific ACR. TNCR: total nitrogen conversion rate. WWTP: wastewater treatment plant. PSTP: pig slurry treatment plant. LWWTP: lagoon WWTP. LPSTP: lagoon PSTP. [b] Quantification of those samples labeled with * was carried out at the detection limit.

Results of the final activity test did not evidence link between time of anammox activity appearance and measured NH_4^+ conversion rate (Table 15). Good linearity in the evolution of the N-forms was observed during the activity test, which was indicative of no substrate inhibition ($R^2 > 0.98$). Thus, specific activity was assessed within the range from 21 to 118 mg NH_4^+-N/g VS/d which is equivalent to total N-conversion rates of 59-297 mg N/g VS/d. These values are within the range of specific N-removal activities of 60-1600 mg N/g VSS/d (n=14; VSS, volatile suspended solids) obtained for a variety of anammox sludge of diverse origins as reported by S.W.H. Van Hulle *et al.* (2010).

The procedure followed in this study for the enrichment of anammox biomass consisted on the use of bottles as bioreactors (where substrates were supplied in pulses to the biomass). Eventual singularities of this method with respect to the use of the SBR technology are: long cycle (7 d), long hydraulic residence time (> 7 d), high volume exchange ratio (60-80%), and exposure of the biomass to a wide range of substrate concentrations (0-150 mg NO_2^--N/L). These factors could influence on the evolution of the microbial community since they may have implications on the biomass retention within the reactor, the existence of famine periods during the enrichment, and the tolerance of high substrate concentrations by the biomass.

3.2. Monitoring the enrichment of anammox bacteria by real-time q-PCR

The evolution of total and anammox bacteria was monitored by real-time q-PCR throughout the 4 months that lasted the experimental period. For a given batch culture, the bacterial 16S rRNA gene was the most abundant and the least fluctuant (Figure 17). Average values in the enrichments ranged from 2.5 $\pm 0.2 \cdot 10^6$ to 1.0 $\pm 0.2 \cdot 10^9$ copies/mL depending on the inoculum source and the NO_2^- supply strategy applied. Concerning the anammox *hzo* gene, it was quantified from the beginning of the experiment in all inoculums (Table 15), and a significant increase throughout the enrichment was evidenced in those cases where NO_2^- was initially supplied at low concentration (Figure 17).

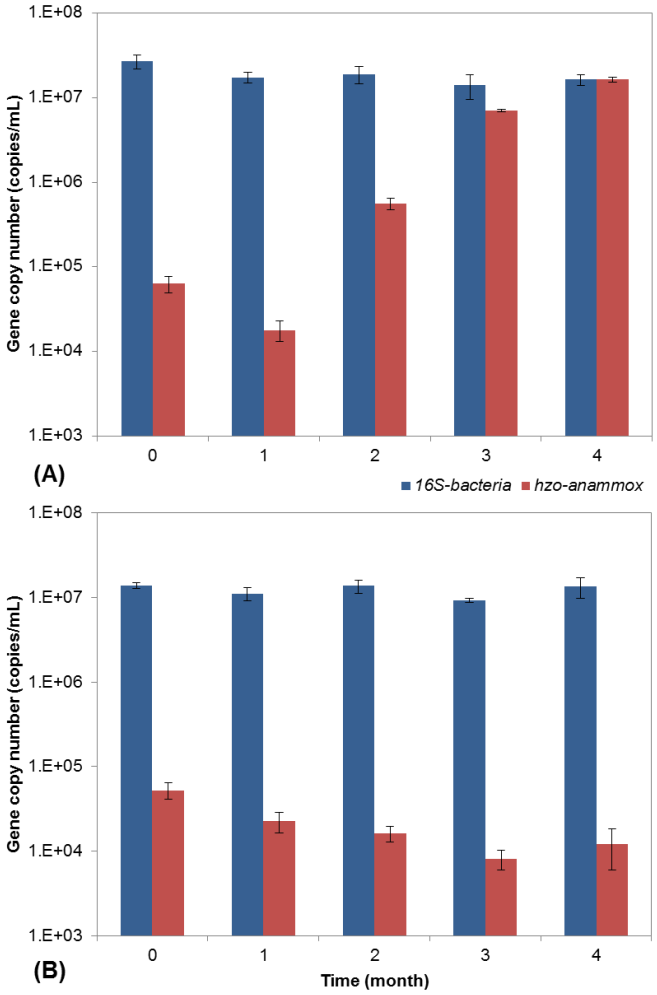


Figure 17: Average copy number of bacterial 16S rRNA and anammox hzo genes throughout the 4-month experimental period in the bottle seeded with I1 when process was started at (A) low nitrite concentration and (B) high nitrite concentration. Error bars represent ± standard deviation.

Conversely, that was not the case when NO_2^- was initially supplied at high concentration. Thus, at day 0, the *hzo* gene copy number in the enrichments started at low NO_2^- concentration ranged from 3.3 $\pm 0.3 \cdot 10^4$ to 1.2 $\pm 0.3 \cdot 10^5$ copies/mL, and after 4 months reached values from 1.1 $\pm 0.1 \cdot 10^6$ to 1.5 $\pm 0.1 \cdot 10^7$ copies/mL. At that time, it was also evidenced a positive correlation between the *hzo* gene copy number and the anammox activity (Figure 18), similarly as previously reported by I. Tsushima *et.al.* (2008) using primers targeting the 16S rRNA gene of the anammox bacteria, and although there was no evident link between the *hzo* gene copy number in the inoculum at day 0 and the time of anammox activity appearance (Table 15).

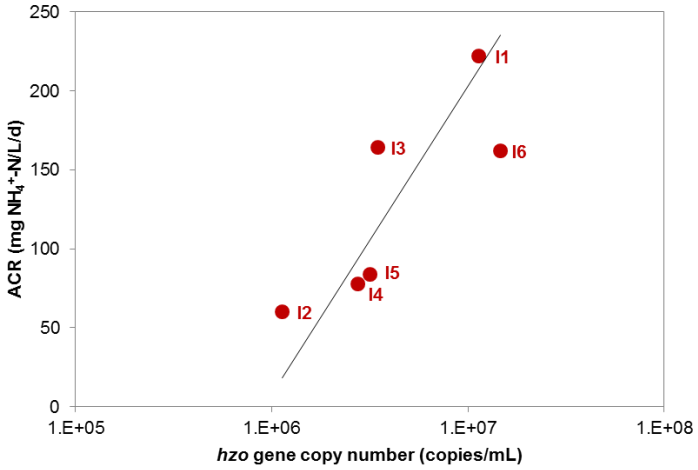


Figure 18: Correlation between the ammonium conversion rate (ACR) and the hzo gene copy number at the end of the enrichment period (4 months) in the 6 batch cultures positive in anammox activity.

Lack of linkage between the initial *hzo* gene copy number and the time of anammox activity appearance suggests that other factors besides the initial anammox biomass concentration (measured as hzo gene copy number) determined the time when such activity became evident macroscopically. Among these factors, we might include competition for substrate, coexistence of the anammox bacteria with other microbial populations, and change of the growing anammox species (see section 3.3). In this regard, mineralization of organic-N forms and denitrification could have masked an earlier detection of anammox activity. Furthermore, Y. Tao *et.al.* (2013) pointed out dependence of the lag phase length not only on the initial biomass concentration but also on other ecological characteristics of the inoculum used and concluded that an evenly distributed community benefits the start-up of the anammox process with shorter times and higher activities. Yet, differences exist between these findings and our study; i.e., we dealt with inoculums with much lower initial relative abundances of anammox bacteria and also we observed a change of the growing anammox species during the enrichment process (see section 3.3). On the other hand, final *hzo* gene copy numbers were below 2.0 ±0.2.10^5 copies/mL in all cultures started at high NO_2^- concentration.

3.3. Microbial community structure and dynamics during the enrichment

The 16S rRNA high-throughput DNA sequencing was performed for all inoculums (I1-I6) at initial time (t0 = 0 months) and final time (t4 = 4 months) when the enrichment was started at low NO_2^- (LN) and high NO_2^- (HN) concentrations, and for I3 at all times (t0-t4) and NO_2^- supply conditions (LN, HN). Reads obtained yielded between 1933 and 22475 high-quality sequences per sample that made up to 633 OTUs. A systematic random depletion was applied on the different datasets to equalize the number of sequences per sample; i.e., dataset for all inoculums including samples at initial and final time (18 samples) was

adjusted to 11058 sequences per sample whereas dataset for I3 including samples at all times (9 samples) was adjusted to 1933 sequences per sample. The representativeness and identification of these OTUs was analyzed to better understand the process of anammox enrichment.

3.3.1. Evolution of the microbial communities

The diversity indices reported in Table 16; i.e., Shannon-Weaver, Simpson, and Inverse Simpson, globally show a systematic decrease of the microbial diversity throughout the enrichment (average index reductions for the cultures started at low NO_2^- concentration of 25.7 ±4.5%, 8.8 ±3.4%, and 67.9 ±14.3%, respectively). In addition, both the species richness and Pielou's evenness decreased in this period (average reductions for the cultures started at low NO_2^- concentration of 30.2 ±7.6% and 20.5 ±4.7%, respectively). These data may imply concomitant disappearance of some species and larger segregation between the low and highly represented taxons (J. Oksanen, 2016; A.E. Magurran, 2004). The impact seems to be more important when the enrichment was started at low NO_2^- concentration (Table 16). No relation was found between the initial microbial diversity and the effectiveness of the anammox enrichment in terms of both time required and final activity achieved.

Statistical analysis of the microbial community structure evolution was also performed through the NMDS method. For a given inoculum, the evolution of the microbial community was different depending on the NO_2^- supply strategy applied during the enrichment. Relative differences in the composition of both microbiomes (enrichments started at low vs. high NO_2^- concentration) over time were evidenced as it is shown for the bottle seeded with I3 in Figure 19. Thus, although both microbiomes evolved similarly with a diminution in the diversity throughout the enrichment, divergence in the microbial community structure progressively increased and was maximal after 4 months of enrichment. Such divergence included the enrichment of anammox bacteria only under conditions of initially low NO_2^- concentration. In addition, the NMDS analysis also revealed that, despite the significant relative differences in the composition of the microbial community concerning the inoculums used in this study, the enrichment conditions applied forced the convergence of such communities (Figure 20). The most similar microbial community structures were observed for those inoculums coming from systems treating the same kind of wastewater; i.e., municipal (I1, I4, and I5) vs. pig slurry (I2, I3, and I6), at both initial and final time. The clear trend to converge independently of the inoculum source and the feeding strategy applied indicates the high selective pressure exerted by the experimental conditions here implemented.

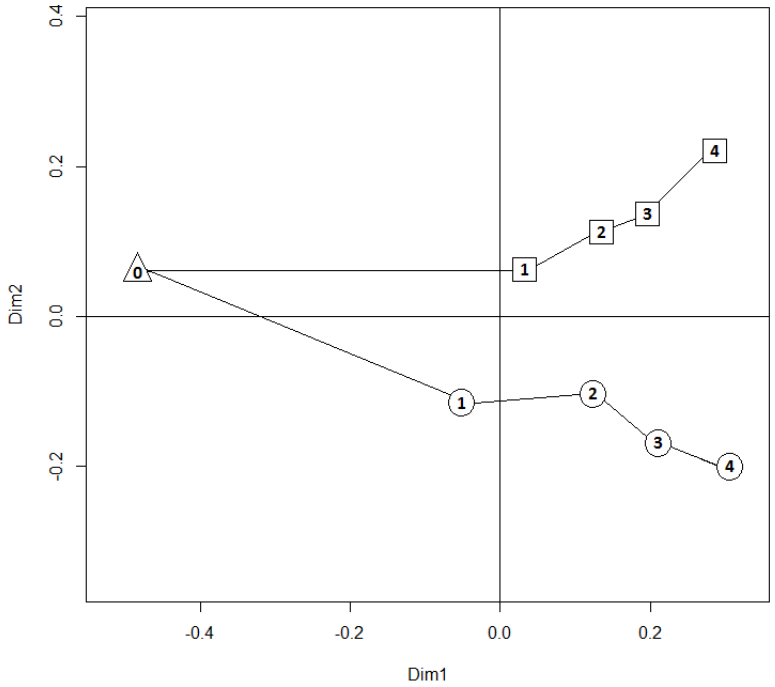


Figure 19: Non-metric multidimensional scaling (NMDS) plot showing the evolution of the microbial community structure in both bottles seeded with I3 throughout the 4-month experimental period. Circles correspond to the bottle started at low nitrite concentration whereas squares correspond to the bottle started at high nitrite concentration. Numbers inside circles and squares indicate the experimental time elapsed (in months).

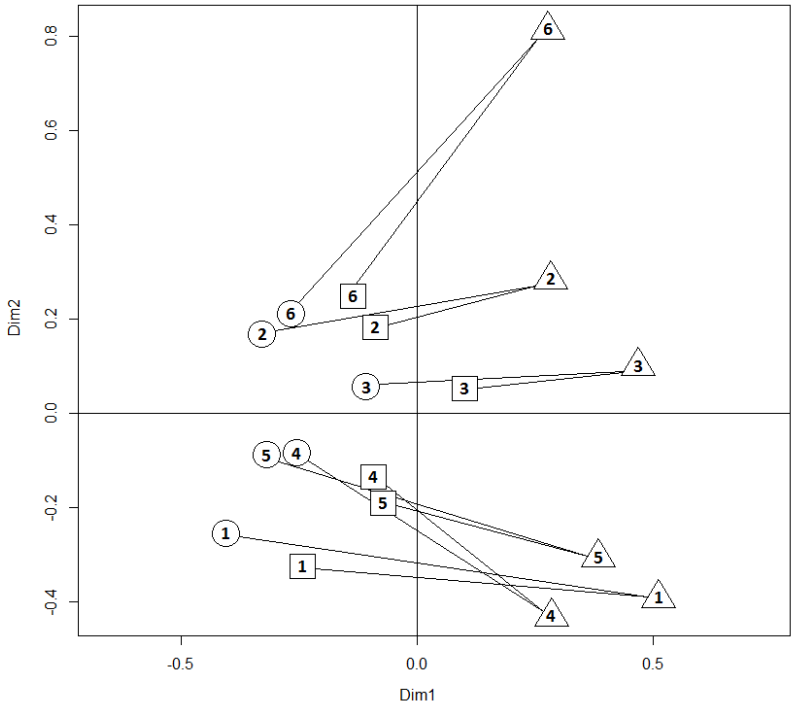


Figure 20: Non-metric multidimensional scaling (NMDS) plot showing the evolution of the microbial community structure for all the inoculums tested (1-6) throughout the 4-month experimental period. Triangles correspond to initial time, circles correspond to final time for cultures started at low nitrite concentration and squares correspond to final time for cultures started at high nitrite concentration.

3.3.2. Description of the microbial community structures

As aforementioned, the microbial community structure in the batch cultures evolved significantly throughout the enrichment. However, main phyla detected in inoculums such as *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Chloroflexi*, *Chlorobi*, *Acidobacteria*, and *Planctomycetes* were also present at the end of the enrichment period (Figure 21A-21B; Tables 17 and 18). The phylum *Proteobacteria*, which is commonly found in wastewater treatment bioreactors, anaerobic digesters, and soils (M. Hu *et al.*, 2012; D. Rivière *et al.*, 2009; N. Fierer *et al.*, 2007) was one of the most predominant at both initial time (relative abundances of 10.3-46.7%) and final time (relative abundances of 23.9-55.5%). For those enrichments started at low NO2- concentration, the evolution of this phylum in terms of abundance was dependent on the inoculum source. Thus, the proportion of sequences belonging to this phylum followed an increase of 93.1 ±60.4% (in average) for those inoculums coming from pig slurry treatment plants (I2, I3 and I6) whereas followed a decrease of 19.6 ±16.0% (in average) for those inoculums coming from municipal WWTPs (I1, I4, and I5). On the other hand, for those enrichments started at high NO_2^- concentration, the abundance of sequences belonging to this phylum increased at an average rate of 55.9 ±37.9% regardless the inoculum source. For I3 (Figure 21B), the increased proportion of Proteobateria is essentially attributable to the evolution of genera belonging to the class Betaproteobacteria and family Rhodocyclaceae (OTUs 2 and 11) that contain several denitrifying species; e.g. *Azoarcus*. *Bacteroidetes* was another of the most represented phyla at initial time with proportions ranging from 6.1% to 30.9% in I2 and I6, respectively. However, despite this initial high abundance, a systematic reduction was observed regardless the inoculum source and feeding strategy applied, with final relative abundances lower than 2.7%. This is not surprising since the phylum *Bacteroidetes* contains both aerobic and anaerobic bacteria such as *Sphingobacteriales* and *Bacteroides*, respectively. In the case of I3 (Figure 21B), the reduced proportion of *Bacteroidetes* is clearly linked to the disappearance of OTU 4 identified as a *Sphingobacteriales Saprospiraceae*. Conditions applied during the anoxic enrichment including the progressive abatement of residual organic compounds may be the cause of the depletion of this widespread phylum (M. Hu *et al.*, 2012; D. Rivière *et al.*, 2009; N. Fierer *et al.*, 2007). Concerning the phylum *Firmicutes*, relative abundance of sequences belonging to this phylum (0.7-18.6%) was higher for those inoculums coming from pig slurry treatment facilities than for those others coming from municipal WWTPs; i.e., 12.7 ±6.2% vs. 1.0 ±0.2% in average, respectively. The corresponding quantitative evolution was slightly different depending on the NO_2^- supply strategy. The coexistence of bacteria belonging to the phylum *Chloroflexi* is usually reported in anammox reactors being suggested that they can use decaying anammox bacterial cell materials (T. Kindaichi *et.al.*, 2012). Here, the evolution of this phylum (initial relative abundances of 2.1-12.9%) was highly variable ranging from 67.7% decrease to 302.4% increase for I2 enriched at initially low NO_2^- concentration and I6 enriched at high NO_2^- concentration, respectively. Sequences corresponding to the phylum *Chlorobi* (initial relative

Tableau 16: Diversity indices for the six tested inoculums (I1-I6) at initial time (t0 = 0 months) and final time (t4 = 4 months) when the enrichment was started at low nitrite (LN) and high nitrite (HN) concentrations.

	I1			I2			I3			I4			I5			I6		
Indices	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN
Shannon-Weaver	4.02	2.99	3.15	4.22	2.92	3.44	4.24	3.52	4.03	3.91	2.83	3.21	4.40	3.10	3.44	4.07	3.10	3.43
Simpson	0.96	0.89	0.87	0.97	0.88	0.94	0.96	0.93	0.97	0.95	0.83	0.92	0.98	0.85	0.93	0.97	0.90	0.93
Inverse Simpson	28.0	8.77	7.55	36.3	8.19	15.7	23.2	14.3	30.2	20.6	5.88	12.1	40.8	6.87	14.5	31.4	9.88	14.2
Species richness	245	165	187	255	163	209	358	235	287	262	177	199	303	206	221	239	207	226
Pielou's evenness	0.73	0.59	0.60	0.76	0.57	0.64	0.72	0.64	0.71	0.70	0.55	0.61	0.77	0.58	0.64	0.74	0.58	0.63

Tableau 17: Relative abundances (% of total sequences) at the phylum level in the six tested inoculums (I1-I6) at initial time (t0 = 0 months) and final time (t4 = 4 months) when the enrichment was started at low nitrite (LN) and high nitrite (HN) concentrations. "Others" includes all groups with relative abundance <1%.

	I1			I2			I3			I4			I5			I6		
	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN	t0	t4-LN	t4-HN
Euryarchaeota	0.09	0.04	0.07	0.18	0.20	0.25	0.59	0.15	0.43	0.14	0.05	0.13	0.42	0.30	0.38	1.90	0.58	0.95
Acidobacteria	3.74	0.43	2.18	1.43	1.10	1.26	0.62	1.95	2.04	1.08	1.49	1.63	1.74	2.32	0.94	0.08	2.00	0.77
Actinobacteria	5.29	1.01	4.00	5.47	0.32	3.04	0.86	1.65	2.60	0.30	0.55	0.75	0.49	1.12	1.29	0.04	0.82	1.22
Armatimonadetes	6.33	24.43	15.59	4.72	26.41	9.98	3.18	20.35	4.79	2.61	38.50	14.48	2.66	35.48	9.61	5.37	21.45	15.32
Bacteroidetes	13.07	0.95	0.48	6.13	0.99	0.51	29.75	2.13	2.58	7.12	0.44	0.53	4.98	1.38	1.36	30.87	2.42	2.71
Candidate_division_JS1	*	*	0.01	0.70	0.10	1.02	0.36	0.09	0.29	*	*	*	*	*	*	2.44	1.50	2.42
Chlorobi	2.93	14.12	3.46	3.50	4.41	8.73	0.19	4.75	2.55	9.26	5.36	4.32	4.73	5.29	4.92	0.09	2.85	1.82
Chloroflexi	12.90	11.15	7.31	12.78	4.12	9.36	6.62	11.39	12.77	12.59	6.80	7.46	9.37	10.10	10.03	2.07	4.80	8.34
Deinococcus-Thermus	*	0.03	2.31	8.75	0.88	4.83	1.37	0.64	2.64	*	0.54	7.55	*	0.05	0.68	0.86	1.99	2.77
Firmicutes	1.22	1.57	0.80	13.21	13.47	13.39	6.27	10.73	10.00	0.97	0.88	0.75	0.69	0.30	0.29	18.56	25.95	26.56
Gemmatimonadetes	1.24	0.96	3.11	1.44	0.58	2.50	1.90	0.91	5.77	0.44	0.58	1.63	0.59	2.18	5.27	*	0.38	0.51
Nitrospirae	*	*	*	0.00	*	*	0.00	*	*	0.54	0.09	0.25	1.07	0.70	0.38	*	*	*
Planctomycetes	4.54	7.68	3.74	4.19	2.95	1.73	2.62	5.97	4.26	1.47	1.74	0.62	2.57	3.39	0.71	0.45	1.44	3.08
Proteobacteria	32.47	31.68	49.15	22.04	39.40	36.43	25.07	31.94	37.40	45.24	38.28	53.77	46.71	27.61	55.55	10.30	28.14	23.86
SHA-109	*	0.27	*	*	0.01	*	0.01	*	0.01	*	0.37	0.08	*	0.34	0.06	*	*	1.49
Spirochaetae	*	*	*	*	*	*	0.00	*	*	*	*	*	*	*	0.01	2.95	0.01	*
Tenericutes	*	*	*	*	*	*	0.04	*	0.01	*	*	*	*	*	*	1.36	0.01	*
Others	16.18	5.69	7.78	15.45	5.05	6.96	20.56	7.33	11.88	18.27	4.32	6.04	23.97	9.45	8.53	22.65	5.69	8.16

* Relative abundance <0.01%.

Tableau 18: Identification of the main operational taxonomic units (OTUs) presented in Figure 21 and source of their closest relative sequences.

OTU	Identification (RDP-II)	Closest relative sequence (BLAST)	Accession number	Similarity (%)	Source of the closest relative sequence
1	*Bacteria; Armatimonadetes; Armatimonadetes_grp5*	clone AMX001BXFT7	LC094877	99	Sludge from an anammox UASB reactor
2	*Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Azoarcus*	Azoarcus sp. PA01 16S rRNA	KT784536	99	Unknown
3	*Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae*	clone AMX001BA4AW	LC094801	99	Sludge from an anammox UASB reactor
4	*Bacteria; Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Saprospiraceae*	clone E7-201bp	KJ993903	99	Earthworm gut
5	*Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae_1; Clostridium_sensu_stricto_1*	DGGE gel band RB2-79	KT835589	99	SBRs performing N-removal via nitrite treating swine wastewater
10	*Bacteria; Gemmatimonadetes; Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae; Gemmatimonas*	clone SEAB1AA061	KC432372	99	Wetland
11	*Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae*	clone L1A.6H12	AY989011	97	Soil
12	*Bacteria; Chlorobi; Ignavibacteria; Ignavibacteriales*	clone AMX001CIOAL	LC094916	99	Sludge from an anammox UASB reactor
18	*Bacteria; Proteobacteria; Gammaproteobacteria ;* unclassified	clone MISEQ01_89	KP356069	99	Biofilm in a bioelectrochemical system
19	*Bacteria; Bacteroidetes;* unclassified	cloneAMX001B2NAV	LC094861	99	Sludge from an anammox UASB reactor
21	*Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae*	clone HAD103	HG380607	99	Simultaneous autotrophic and heterotrophic denitrification process
36	*Bacteria; Planctomycetes; Planctomycetacia; Brocadiales; Brocadiaceae; Candidatus_Brocadia*	Candidatus Brocadia sinica clone MBR_day_30	KT023580	99	Anammox biomass in a membrane bioreactor
63	*Bacteria; Bacteroidetes;* unclassified	clone B252	KJ730164	99	Biogas digester sediment
74	*Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae*	clone: AMX001C54CS	LC094933	99	Sludge from an anammox UASB reactor
128	*Bacteria; Acidobacteria;* unclassified	DGGE band ANAMMOX11	AM900571	99	Anammox batch culture

abundances of 0.1-9.3%) represented from 1.8% to 14.1% of total sequences at final time for I6 and I1, respectively. Concerning the phylum *Acidobacteria*, final relative abundance was lower than 4.0% in all cases. *Planctomycetes*, which contains all known anammox genera, did not become dominant in any of the enrichments that developed anammox activity (final relative abundances ranged from 0.6% to 7.7% for I4 and I1, respectively), which is in accordance with other microbial characterizations performed in anammox dedicated reactors (M.C.M.S. Costa *et al.*, 2014; A. Gonzalez-Martinez *et al.*, 2015). Within this group, the final relative abundance of sequences belonging to anammox species was between 31.7% and 75.3% for I2 and I1, respectively. For I3 (Figure 21B), a significant enrichment of OTU 1 belonging to phylum *Armatimonadetes* (formally called the candidate phylum OP10) was observed (similarly for other inoculums). Species belonging to the phylum *Armatimonadetes* have been detected in different natural environments such as the Obsidian Pool in Yellowstone National Park and freshwater lakes and rivers (H. Tamaki *et al.*, 2011). However, the reason of their enrichment in this study is not clear. OTU 36 was identified as Candidatus_Brocadia. In addition,

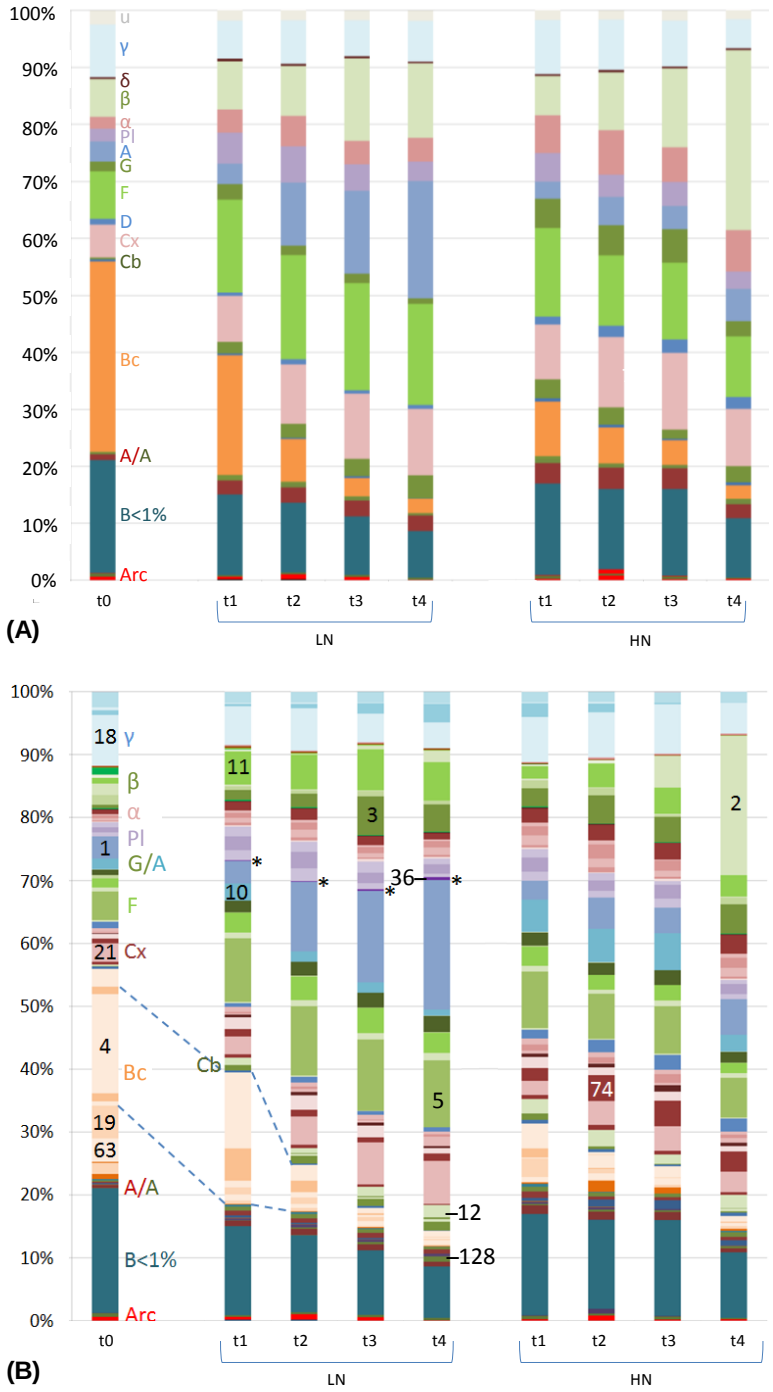


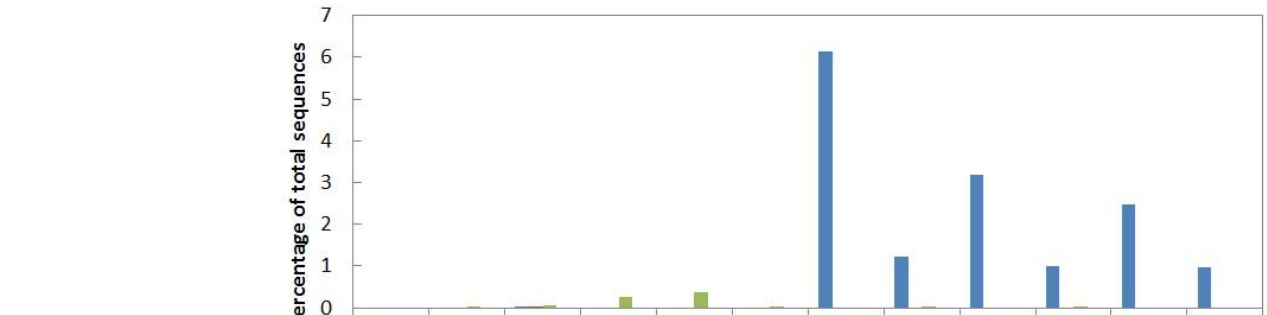
Figure 21: Evolution of the microbial community structure level in both bottles seeded with I3 (LN: process started at low nitrite concentration, HN: process started at high nitrite concentration) throughout the 4-month experimental period (t0-t4). Analysis was performed (A) at the phylum level and also (B) at the genus level. Abbreviations: Arc, Euryarchaeota; B<1%, sum of all operational taxonomic units (OTUs) with relative abundance <1%; A/A, Actinobacteria/Acidobacteria; Bc, Bacteroiedetes; Cb, Chlorobi; Cx, Chloroflexi; D, Deinococcus-Thermus; F, Firmicutes; G, Gemmatimonadetes; A, Armatimonadetes; Pl, Planctomycetes; Proteobacteria (α, Alphaproteobacteria; β, Betaproteobacteria; δ,

*Deltaproteobacteria; γ, Gammaproteobacteria); u, unknown. * is for Candidatus_Brocadia. Main OTUs are numbered and identified in Table 18.*

6 OTUs listed in Table 18 (from a total of 15) were identified as identical to sequences retrieved from anammox systems and 2 other OTUs were related to sequences retrieved from N-removal systems. This fact suggests that the conditions applied to perform the enrichment also favored selection of other specific microbial groups that could either behave as partners or competitors of the anammox bacteria.

3.3.3. Anammox genera

Concerning the presence of anammox genera in the inoculums at initial time, Ca. *Brocadia*, Ca. *Kuenenia*, Ca. *Anammoximicrobium* and Ca. *Jettenia* were all detected (Figure 22), although with an important disparity in terms of distribution and relative abundance.



	I1-t0	I2-t0	I3-t0	I4-t0	I5-t0	I6-t0	I1-t4	I2-t4	I3-t4	I4-t4	I5-t4	I6-t4
Candidatus_Brocadia	*	**	0.011	**	*	**	6.1	1.2	3.2	1.0	2.5	0.96
Candidatus_Kuenenia	*	*	0.011	*	*	*	*	**	*	*	*	*
Candidatus_Anammoximicrobium	*	0.008	0.056	0.27	0.37	0.009	*	0.009	**	0.009	**	*
Candidatus_Jettenia	*	*	**	*	*	*	*	*	**	*	*	**

*Figure 22: Percentage of sequences related to anammox genera in the enriched inoculums (I1-I6) at initial time (t0 = 0 months) and final time (t4 = 4 months). Only cultures started at low nitrite concentration are included. *, not detected when high-throughput sequencing was performed on DNA extracts using "universal" 16S rRNA primers and neither when sequencing was performed using PCR products obtained using Planctomycetes primers. **, only detected when sequencing was performed using PCR products obtained using Planctomycetes primers, so relative abundance cannot be quantified (<0.009%).*

Thus, for instance, these four genera were detected together in I3 but not in the other inoculums. Otherwise, any of these genera were detected in I1. Ca. *Jettenia* was only detected when DNA samples were first amplified by means of selective PCR using Planctomycetes

primers, which allowed lowering the detection threshold but preclude their relative quantification (Figure 22). Before enrichment, higher relative abundances were measured for Ca. *Anammoximicrobium* in I4 and I5 (lagoon WWTP), with 0.27% and 0.37%, respectively, of the total sequences. In fact, this anammox genus (which was recently described) may grow optimally at temperatures around 20°C (S.V. Khramenkov *et al.*, 2013). On the other hand, the genus Ca. *Brocadia* (maximum initial relative abundance of 0.011% for I3) became dominant at the end of the enrichment period regardless of the inoculum considered, with relative abundances ranging from 1.0 to 6.1% of the total sequences. Thus, similarly to M.C.M.S. Costa *et.al.* (2014), the obtained results indicated that while the type of inoculum and the culture conditions are both key determinants of the global microbial composition of the enriched biomass, the operational conditions alone determined the selection of the anammox species. The niche differentiation between Ca. *Brocadia* and other anammox genera often dominant in enrichments performed at lab-scale such as Ca. *Kuenenia* is still not clear. According to several studies (W.R.L. van der Star *et al.*, 2008; M. Oshiki *et al.*, 2011; D. Puyol *et al.*, 2013; E. Isanta *et al.*, 2015), the genus Ca. *Brocadia* would presumably be an r-strategist (i.e., relatively high growth rate and low substrate affinity), while the genus Ca. *Kuenenia* could be a K-strategist (i.e., relatively low growth rate and high substrate affinity). In this study, the feeding of the enrichments was based on the intermittent supply of substrates which would favour the proliferation of an r-strategist population.

Overall, our findings showed that anammox bacteria were ubiquitous in all the inoculums collected from different waste/water treatment environments which made feasible their eventual enrichment in batch under controlled conditions. However, such enrichment was only achieved when the process was started at low NO_2^- concentration (≤ 25 mg NO_2^--N/L) evidencing the importance of carefully controlling the conditions applied (i.e., substrate concentration). The procedure used prompted the enrichment of the anammox genus Ca. *Brocadia* regardless of the inoculum source. A higher initial anammox biomass concentration (measured as *hzo* gene copy number) did not necessarily imply a faster process start-up. Thus, other physicochemical and ecological characteristics of the inoculum affected the evolution of the enrichment since they determined issues such as the competition for substrate, coexisting microbial groups, dominant anammox species, and associated relative abundances at the beginning of the process.

5. CONCLUSIONS

The effect of NO_2^- supply during the batch enrichment (4 months) of anammox sludge was investigated using six different biomass sources. Concerning the mineral medium used as feeding solution, two different NO_2^- supply strategies were applied; i.e., (i) initially low concentration at 25 mg NO_2^--N/L and progressive increase to 150 mg NO_2^--N/L, and (ii) constant high concentration at 150 mg NO_2^--N/L.

- All tested inoculums developed anammox activity only when the enrichment was started at low NO_2^- concentration. In such case, the final specific NH_4^+ conversion rate was measured within the range from 21 to 118 mg NH_4^+-N/g VS/d.
- Abundance of the hzo functional gene showed positive correlation with the anammox activity finally reported.
- The biomass source, a conditioning pretreatment, and the cultivation conditions applied were determinant factors of the final microbial composition of the enrichments despite a clear convergence at the end of the experimental period. However, the cultivation conditions alone determined the selection of anammox species belonging to the genus Ca. *Brocadia*.

ACKNOWLEDGEMENTS

Authors thank Rennes Métropole for funding this research through the assignment of the allocation d'installation scientifique ref. 14C0509. Sophie Le Roux and Chrystelle Bureau are acknowledged for their technical assistance during microbial analyses whereas Olivier Chapleur is acknowledged for his help in processing DNA sequencing data.

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CHAPITRE 4 : Utilisation d'un système continu pour la culture de bactéries anammox.

CHAPTER 4: Establishment of continuous cultivation of anammox bacteria.

This chapter is dedicated to the cultivation of anammox microorganisms on a carrier material in a continuous packed-bed up-flow reactor. Due to batch enrichment limitations continuous reactors represent a viable solution to push further the anammox enrichment. Otherwise those systems are efficient tools in order to cultivate and produce excess sludge to be used for further purposes.

This cultivation procedure is particularly focused on the study of anammox population evolutions and global microbial community shifts. This was made possible through use of molecular tools helping better describe and understand this changing during the extended batch enrichment and continuous cultivation. This chapter is presented as a scientific publication under reviewing.

Characterization of a combined batch-continuous procedure for the culture of anammox biomass

Romain Connan [a], Patrick Dabert [a], Sophie Le Roux [a], Olivier Chapleur [b], Gilbert Bridoux [c], Matias B. Vanotti [d], Fabrice Béline [a], Albert Magrí [a,*]

[a] Irstea, UR OPAALE, 17 Avenue de Cucillé-CS 64427, F-35044 Rennes, France

[b] Irstea, UR HBAN, 1 rue Pierre-Gilles de Gennes-CS 10030, F-92761 Antony Cedex, France

[c] SAUR, Recherche et développement, 2 rue de la Bresle, F-78201 Maurepas, France

[d] USDA-ARS Coastal Plains Soil, Water and Plant Research Center, 2611 W. Lucas St., Florence, SC 29501, USA

E-mail addresses: *romain.connan@irstea.fr* (R. Connan); *patrick.dabert@irstea.fr* (P. Dabert); *sophie.leroux@irstea.fr (*S. Le Roux*); olivier.chapleur@irstea.fr (*O. Chapleur*); gbridoux@saur.fr* (G. Bridoux); *matias.vanotti@ars.usda.gov* (M.B. Vanotti)*; fabrice.beline@irstea.fr* (F. Béline); *albert.magri@gmail.com* (A. Magrí)

[*] Corresponding author: Albert Magrí, PhD. Institut national de Recherche en Sciences et Technologies pour l'Environnement et l'Agriculture (Irstea), 17 Avenue de Cucillé-CS 64427, F-35044 Rennes, France. Tel.: +33 223482121; Fax: +33 223482115. E-mail: *albert.magri@gmail.com*.

[1]

[1] **Abbreviations**

anammox, anaerobic ammonium oxidation; DO, dissolved oxygen; HRT, hydraulic residence time; N, nitrogen; NCE, nitrogen conversion efficiency; NCR, nitrogen conversion rate; NLR, nitrogen loading rate; NMDS, non-metric multidimensional scaling; OTU, operational taxonomic unit; PCR, polymerase chain reaction; SBR, sequencing batch reactor; VS, volatile solids; WWTP, wastewater treatment plant.

ABSTRACT

Interest in autotrophic nitrogen (N) removal through anaerobic ammonium oxidation (anammox) is increasing in the field of wastewater treatment as a more economic and sustainable alternative than conventional nitrification-denitrification. However, anammox biomass is difficult to enrich, and this can hinder the start-up of new applications. We carried out experimental work to characterize a combined batch-continuous procedure for the enrichment and culture of anammox biomass. In the first stage (time span: 120 d), the enrichment was started in batch mode using suspended activated sludge as inoculum. Anammox activity was clearly developed since the specific ammonium (NH_4^+) conversion rate increased from 0 to 118 ±1 mg NH_4^+-N/g VS/d (VS, volatile solids) -i.e., 560 ±11 mg N/L/d in terms of N-conversion rate (NCR)-. Subsequently, the sludge was transferred into a continuous upflow reactor packed with a polyester non-woven material to promote the attached growth of the biomass (initial biomass dilution rate of about 1/8). Such bioreactor was operated without interruption during 400 d. Under appropriate feeding, anammox activity increased fast, and a sustained NCR of 1166 ±118 mg N/L/d was reached according to the N-loading rate applied. Evolution of the microbial community structure was characterized using high-throughput DNA sequencing. The overall procedure selected for a community enriched in the anammox Candidatus *Brocadia sinica* species (70% of the total DNA sequences). Other enriched microbial groups belong to the *Rhodocyclaceae* family (class *β-Proteobacteria*), the *Anaerolineae* (*Chloroflexi* phylum) and the *Ignavibacteriaceae* (*Chlorobi* phylum).

Keywords

Anaerobic ammonium oxidation; Biomass culture systems; Batch enrichment;
Continuous upflow reactor; Biofilm; Microbial community structure.

1. Introduction

The anaerobic ammonium oxidation (anammox) is an interesting bioprocess for the removal of nitrogen (N) from wastewaters since it allows more economic and sustainable treatment than conventional approaches based on heterotrophic denitrification (B. Ma *et al.*, 2016; H. Siegrist *et al.*, 2008). In engineered systems, its coupling with partial nitritation results

in a complete autotrophic deammonification, which reduces by 60% the oxygen requirement, 100% the organic carbon requirement, and 90% the biosolids production with respect to classical nitrification-denitrification. In addition, it offers the chance of working with more compact reactors at higher loading rates (A. Magrí *et al.*, 2013; S.W.H. Van Hulle *et al.*, 2010).

The anammox process is mediated by chemolithoautotrophic bacteria that oxidize ammonium (NH_4^+) into dinitrogen gas (N_2) using nitrite (NO_2^-) as the electron acceptor (M. Strous *et al.*, 1998). Nitric oxide (NO) and hydrazine (N_2H_4) are two known intermediates of such reaction (B. Kartal *et al.*, 2011). This bioconversion takes place under absence of oxygen and presence of inorganic carbon. A small amount of nitrate (NO_3^-) is also produced due to the oxidation of nitrite linked to inorganic carbon fixation in anabolism (N. M. de Almeida *et al.*, 2011). According to the full stoichiometry proposed by M. Strous *et al.* (1998) -Eq. (8)-, ammonium and nitrite are converted into dinitrogen gas and nitrate under the molar ratios 1.00/1.32/1.02/0.26 for NH_4^+ consumption, NO_2^- consumption, N_2 production, and NO_3^- production, respectively -values obtained through mass balance in a sequencing batch reactor (SBR) running under stable conditions-.

$$1.00NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (8)$$

To date, six “Candidatus” anammox bacterial genera have been enriched from samples collected in wastewater treatment facilities and natural environments such as freshwater and marine zones; i.e., Ca. Brocadia (M. Strous *et al*., 1999), Ca. Kuenenia (M.C. Schmid *et al*., 2000), Ca. Scalindua (M.M.M. Kuypers *et al*., 2003), Ca. Anammoxoglobus (B. Kartal et al., 2007), Ca. Jettenia (Quan *et a*l., 2008), and Ca. *Anammoximicrobium* (Khramenkov *et al*., 2013). All these genera belong to the same phylum *Planctomycetes*. In spite of this fact, while the first five aforementioned genera form a deeply branched monophyletic group (family *Brocadiaceae*), the sixth is closely related to the genus *Pirellula* (family *Planctomycetaceae*). In physiological terms, the anammox bacteria feature a specific cytoplasmatic membrane-bound organelle known as anammoxosome, which is the locus of the anammox catabolism (L. van Niftrik and M. Jetten, 2012). They are also characterized by a low growth rate, with doubling times (at ~30ºC) of 2.1-11 days (T. Lotti *et al.*, 2015; M. Strous *et al.*, 1998). Owing to this slow biomass development and the specialized metabolism, the anammox bacteria may be difficult to culture.

The anammox bacteria have not been isolated in pure culture yet. Otherwise, such microorganisms have been enriched from various environments up to a culture purity degree of about 80-95% (L. van Niftrik and M. Jetten, 2012) -maximum value found in the literature is 98 ±1% in a suspended cell anammox culture (T. Lotti *et al*., 2014). Frequently, those strategies used to enrich anammox biomass consist on the utilization of different types of continuously operated reactors such as the SBR, rotating biological contactor, membrane bioreactor, upflow anaerobic sludge blanket reactor, or upflow fixed bed biofilm reactor, among others (K. Egli *et al.*, 2003; M. Strous *et al.*, 1998; I. Tsushima *et al.*, 2007; Y. Wang *et al.*, 2009; L. Xiong *et al.*, 2013). Alternatively, anammox enrichments have also been performed in batch mode (H. Bae *et al.*, 2010; R. Connan *et al.*, 2016; A. Sànchez-Melsió *et al.*, 2009). In all cases, appropriate selection of the environmental conditions such as temperature, pH, and levels of ammonium, nitrite, organic carbon, dissolved oxygen (DO), and other nutrients and inhibitors, is critical for a successful enrichment and mass culture (J.M. Carvajal-Arroyo *et al*., 2013). The enrichment of anaerobic ammonium-oxidizing biomass from conventional sludge is time-consuming and may take from several months to years depending on the seeding source, reactor setup, and operational conditions applied (M. Ibrahim *et al.*, 2016). Thus, biomass enrichment usually is the critical step for the start-up of new anammox applications (especially when pre-enriched sludge is not available).

The objective of this study is to characterize a culture of anammox biomass obtained from activated sludge using a combination of batch and continuous procedures. The enrichment was started under suspended biomass batch mode and subsequently continued using a continuous upflow reactor packed with a polyester non-woven material to promote the attached growth of the biomass. The evolution of the microbial community structure was characterized throughout the process by means of 16S rRNA gene high-throughput sequencing.

2. Material and methods

2.1. Biomass sources: collection and pretreatment

Activated sludge collected in a municipal wastewater treatment plant (WWPT) that combine the use of a Modified Ludzack-Ettinger bioreactor unit and a membrane filtration loop to perform N-removal was used as inoculum for the enrichment of anammox biomass. Such treatment facility is located in Betton (France). Before starting with the anammox

enrichment procedure, denitrification was favored during the first days after sampling in order to promote biodegradation of residual organic matter. Such pretreatment was carried out at room temperature by adding a nitrate source (KNO_3) in pulses equivalent to 100 mg N/L and controlling the pH within the range 7.0-8.0 (HCl). The anammox batch enrichment was started after 4 weeks, once denitrification activity decreased. For microbial characterization purposes, an alternative anammox biomass sample was obtained from a lab-scale 10 L jacketed upflow fixed bed biofilm reactor running at the USDA-ARS laboratory in Florence, South Carolina, USA (Vanotti et al., 2011, A. Magrí *et al*., 2012). At the time of sludge collection, the reactor was fed with mineral medium containing 153 mg NH_4^+-N/L and 153 mg NO_2^--N/L and operated with a hydraulic residence time (HRT) of 4 h, N-loading rate (NLR) of about 1800 mg N/L/d, and water temperature of 30ºC.

2.2. Batch enrichment in a vial

The batch enrichment was performed using a glass vial which contained the inoculum and mineral medium (working volume of 0.5 L). Such vial was sealed with a rubber stopper plus an aluminum cap and placed into an incubator shaker (KS4000i control, IKA, Germany) at 150 rpm, 35ºC, and in dark conditions. Initial solids content inside the vial was adjusted to 1.50 g VS (VS, volatile solids). Biomass settling was allowed weekly to withdrawn the supernatant and, subsequently, to refill the vial with new mineral medium (avoiding the accumulation of inhibitory compounds). The mineral medium was initially prepared with low amount of nitrite and ammonium (25 mg NO_2^--N/L + 25 mg NH_4^+-N/L). Once the anammox activity was detected, a second weekly addition of nitrogenous substrates was performed targeting a progressive increase in the concentration from 25 to 150 mg NO_2^--N/L (ammonium was added at a ratio of 1.2 g NO_2^--N/g NH_4^+-N). The pH within the vial was controlled in the range from 7.0 to 8.0 (HCl 2M). N_2 flushing was used to displace air in the vial headspace every time it was opened. Liquid samples were taken before and after each feeding event and filtered using 0.45 µm polypropylene membrane filters. A biological sample was taken once per month, centrifuged at 10000*g* for 4 min, and the pellet was stored at -20°C (after discarding the supernatant). This enrichment step lasted 120 days (4 months), and final anammox activity was evaluated in a batch test (in duplicates), as described elsewhere (R. Connan *et al*., 2016).

2.3. Continuous upflow reactor

The biomass enriched following the aforementioned method was seeded in a continuous upflow column reactor (0.94 g VS). This was a jacketed cylindrical reactor made of glass (Trallero & Schlee, Spain) with inner diameter of 9 cm and column height (to the effluent port) of 66 cm. Similarly to other works (K. Furukawa *et al.*, 2003; A. Magrí *et al*., 2012), a support made of polyester non-woven material coated with pyridinium-type polymer (Japan Vilene Co., Japan) was placed inside the column reactor to enhance the retention of the biomass (8 strips each one 4.5 cm wide and 57 cm long). Total liquid volume was 4.5 L whereas volume of the reaction zone (excluding the upper 9 cm zone without support) was 3.9 L. Process temperature was controlled at 35ºC using a water heating circulator (model EH-13, Julabo, Germany). Mineral medium was continuously pumped inside the reactor by the bottom-end through a low-flow peristaltic pump (model PD5001, Heidolph, Germany) equipped with a multichannel head (model C4, Heidolph), and treated liquid was discharged near the top of the reactor after passing through the matrix of immobilized biomass. Targeted NLRs ranged from 111 to 1551 mg N/L/d according to the nominal volumetric flow rate (3-12 mL/min) and influent concentration (50-175 mg NO_2^--N/L at a ratio of 1 g NO_2^--N/g NH_4^+-N). Corresponding HRTs (taking into account the reaction volume) were 21.7-5.4 h. The mineral medium was stored at room temperature in a polyethylene tank sealed at the top to prevent air from entering (a Tedlar bag filled with N_2 was connected with tubing to the top of the tank). Such influent tank was refilled weekly with fresh mineral medium. Tygon tubing was used for the setup. The reactor was placed in a dark chamber to avoid light. Liquid samples were regularly taken from the influent and the effluent lines for chemical analysis. Volumetric flow rate was measured by collecting the liquid in a graduated cylinder during a known period of time. When required, gas samples were taken from the headspace of the reactor for analysis. Off-gas flow rate in the effluent line was measured using the water displacement method. Finally, biological samples from the reactor (considering bottom and top ends of the support material) were taken during the experimental period and processed as aforementioned in section 2.2. The upflow reactor was uninterruptedly operated during 400 days (13.5 months).

2.4. Mineral medium

The synthetic nutritive solution used throughout the experiments was prepared with tap water according to a modification of the mineral medium described by Magrí et al. (2012); i.e., NH_4Cl (variable: 95-669 mg/L), $NaNO_2$ (variable: 123-862 mg/L), KNO_3 (variable: 0-361

mg/L), $KHCO_3$ (variable: 0-1000 mg/L), $NaHCO_3$ (variable: 0-1000 mg/L), KH_2PO_4 (variable: 0-27 mg/L), FeSO4·$7H_2O$ (9 mg/L), EDTA (5 mg/L), $MgSO_4·7H_2O$ (240 mg/L), $CaCl_2·2H_2O$ (143 mg/L), and trace element solution 0.3 mL/L. The trace element solution contained $ZnSO_4·7H_2O$ (1247 mg/L), $MnSO_4·H_2O$ (1119 mg/L), $CuSO_4·5H_2O$ (44 mg/L), $Al_2(SO_4)_3·14H_2O$ (201.5 mg/L), $Na_2MoO_4·2H_2O$ (129 mg/L), $CoCl_2·6H_2O$ (30 mg/L), KCl (100 mg/L), and EDTA (975 mg/L). KNO_3 was added to the mineral medium throughout the batch enrichment period in order to help maintaining anoxic conditions in case of total nitrite consumption (preventing sulfate reduction). Unfortunately, because of a wrong labeling by the chemical supplier (K_2O was supplied instead of KH_2PO_4) any phosphorus source was added to the mineral medium during the batch enrichment and also during the first 90 days of operation of the upflow reactor. In this regard, during batch culture, phosphorus released by the decaying biomass seemed to be sufficient to avoid limitation in the availability of this nutrient. Conversely, this was not the case when operating the upflow reactor as it will be discussed later in section 3.2.1. Once discovered after analysis, the aforementioned chemical was replaced by a real source of KH_2PO_4. Finally, the liquid flow regime in the upflow reactor was assessed by punctually replacing the bicarbonate source in the mineral medium ($KHCO_3$ and $NaHCO_3$ were interchanged) and subsequently monitoring the evolution of the concentration of the potassium (K^+) and sodium (Na^+) ions in the effluent line. Once dissolved all the mineral salts, the DO was purged by bubbling with N_2 (< 0.2 mg O_2/L) and the pH was adjusted to 6.8-7.0 (HCl).

2.5. Chemical analyses

NH4+, NO2-, NO3-, K+, Na+, and phosphate (PO4) were measured by ion chromatography (850 Professional IC, Metrohm, Switzerland). VS were measured after sample drying to constant weight at 105ºC and further ignition in a muffle furnace at 550ºC. The pH and DO were measured using portable meters pH 197i and Oxi 197 (WTW, Germany), respectively. Nitrous oxide (N_2O) in gaseous samples was measured by gas chromatography (6890N, Agilent Technologies, USA).

2.6. N-transformation calculations

Both, the N-conversion rate (NCR) and the N-conversion efficiency (NCE) were defined according to the removal of ammonium and nitrite from the liquid. During batch enrichment, the NCR was calculated from the corresponding time-dependent slopes for the evolution of

the concentrations (obtained through linear regression analysis). Specific activity (specific-NCR) was determined taking into account the VS content in the vial. Under continuous operation of the upflow reactor, the NCR was calculated from the difference in concentrations between the influent and the effluent, divided by the measured HRT. Similarly, the NCE was calculated from the difference in concentrations between the influent and the effluent, divided by the total concentration in the influent. On the other hand, reaction molar ratios were calculated according to the difference in concentrations of ammonium, nitrite, and nitrate between influent and effluent and expressed per unit of ammonium removal. The dinitrogen gas reaction ratio was calculated through mass balance (1 mol N_2 = 2 atoms N) considering the other three measured N-species.

2.7. Microbial community analyses

2.7.1. DNA extraction

Total DNA was extracted from approximately 0.25 g of pellet with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., USA), according to the manufacturer's instructions. The concentration and purity of the extracted DNA were checked spectrophotometrically (ND-1000, NanoDrop Technologies, USA) and in TAE 1X - 0.7% agarose gel. The extracted DNA was stored at -20ºC until further analysis.

2.7.2. High-throughput DNA sequencing

16S rRNA genes high-throughput DNA sequencing was performed by the BIOMIC team of Irstea (Antony, France) using an Ion PGM™ (Life Technologies, USA) platform, as described by Poirier et al. (2016a, 2016b). Briefly, the bacterial and archaeal hypervariable region V4-V5 of the 16S rRNA genes was amplified using the primer 515F (5'-GTGYCAGCMGCCGCGGTA-3') and a modified version of the primer 928R (T. Wang and Z.-X. Qian, 2009) that was named 928Ramx (5'-CCCCGYCAATTCHTTTRAGT-3'). The primer 928Ramx includes the nucleotide H (T, A or C) instead of the nucleotide M (A or C) in position 13, which does not change its universality but increases its similarity with anammox sequences. Amplification was performed in a 50 µL reaction mixture using from 10 to 200 pg of extracted DNA and the *Pfx* SuperMix protocol from Life Technologies, as described in S. Poirier *et al.* (2016b). Further processing of the polymerase chain reaction (PCR) products -purification, quantification, emulsion PCR, and sequencing on an Ion 316™ chip v2 using the Ion PGM™ System (Life Technologies)- was carried out according to the manufacturer's instructions, as described in Connan et al. (2016).

After sequencing, the PGM™ software filtered out low quality and polyclonal sequence reads. Filtered sequences were analyzed using the QIIME pipeline (v1.8.0) (J.G. Caporaso *et al.*, 2010). Sequences shorter than 200 bp or longer than 250 bp, chimeras, and singletons were removed from the dataset. Operational taxonomic units (OTUs) were subsequently defined using UPARSE implemented in USEARCH (v8.0.1623) (R.C. Edgar, 2013) at a 97% similarity level. MOTHUR (v1.25.0) (Schloss et al., 2009) and SILVA (v119) (C. Quast *et al.,* 2013) were used as the classifier tool and database for taxonomic association (with a minimum similarity threshold of 80%), respectively.

2.7.3. Statistical and phylogenetic analyses

Statistical analysis to evaluate the evolution of the microbial community structure was carried out through the non-metric multidimensional scaling (NMDS) method using the open-source software R (v3.2.3) (W.N. Venables *et al.*, 2016) including functions from the vegan package (v2.3-2) (Oksanen et al., 2016). Shannon-Weaver and Simpson diversity indices, as well as species richness and Equitability were calculated according to the procedures described in J. Oksanen *et al.* (2016).

3. Results and discussion

3.1. Batch stage

The batch enrichment developed significant anammox activity after 4 months (Figure 23), which was measured, in terms of NCR, as 560 ±11 mg N/L/d (ammonium conversion rate of 222 ±2 mg NH_4^+-N/L/d at a NO_2^-/NH_4^+ reaction molar ratio of 1.53 ±0.03) (Figure 24). If referred to the VS content, this is equivalent to a specific-NCR of 297 ±6 mg N/g VS/d. At the end of the enrichment, the biomass still maintained the aspect of activated sludge and brownish color, but tiny red granules could be identified already in the liquid bulk. Three main phases were observed throughout the enrichment period (Figure 23): (*Phase I*) endogenous heterotrophic denitrification was the dominant process and ammonium may even slightly increase during incubation due to the hydrolysis of the remaining organic matter, (*Phase II*) appearance of ammonium consumption (detected after 43 days) and subsequent speed up at increasing nitrite concentration, and (*Phase III*) consolidation of ammonium consumption at high nitrite concentration (attaining levels of 150 mg NO_2^--N/L) under NO_2^-/NH_4^+ reaction molar ratios approaching the value of 1.32 given in Eq. (1), and with evidence of nitrate production. As previously published (R. Connan *et al.*, 2016; S. Uyanik *et al.*, 2011), the nitrite supply

strategy applied when targeting anammox biomass enrichment is of upmost importance. In this study, nitrite levels of 150 mg NO_2^--N/L were feasible without inhibition (this was corroborated by the linear evolution of the N-forms observed during the final activity test, which was launched at nitrite concentrations slightly higher than 150 mg NO_2^--N/L; Figure 24) although the enrichment was started using low nitrite concentrations.

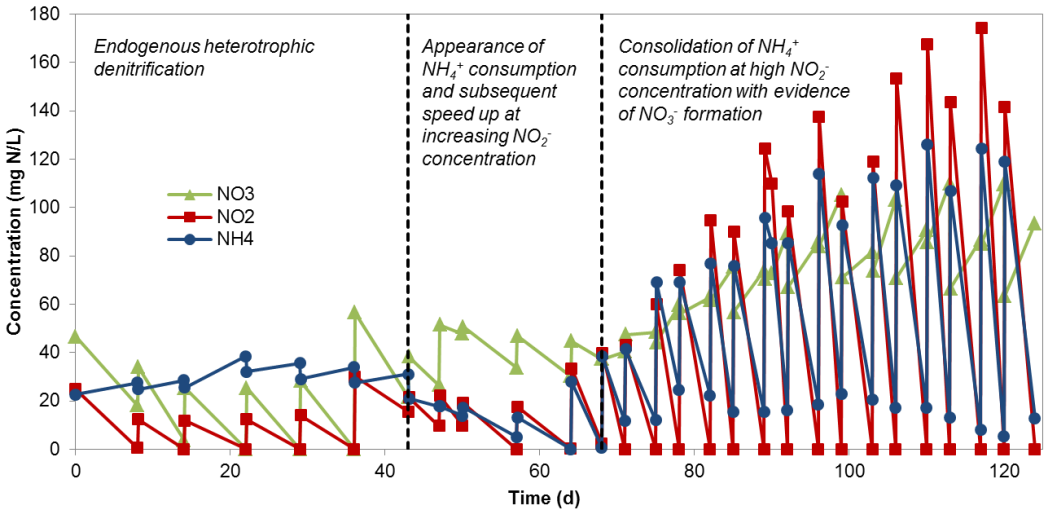

Figure 23: Evolution of the N-species during the batch enrichment of anammox biomass (4 months).

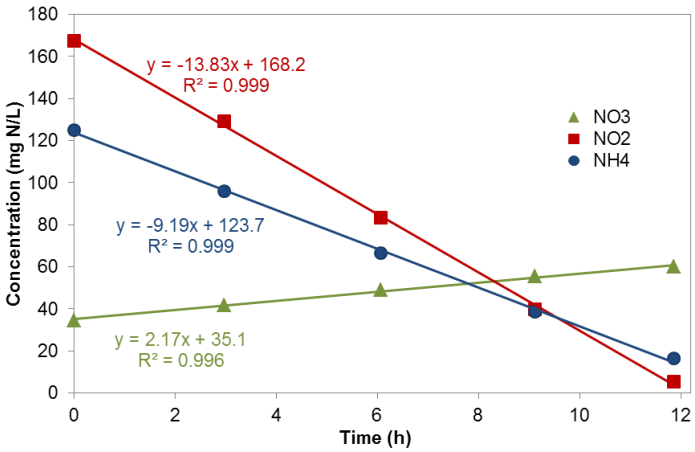

Figure 24: Batch test carried out to assess the anammox activity after 4 months of enrichment.

3.2. Continuous stage

3.2.1. N-removal performance

The suspended biomass with positive anammox activity was transferred into the upflow reactor. According to the corresponding reaction volumes, such handling implied an initial biomass dilution at a rate of about 1/8. The upflow reactor was subsequently operated without interruption for a period of 400 days (Figure 25, Figure 26, and Table 19). Two main phases can be identified throughout this long experimental period according to the availability of orthophosphate phosphorus in the mineral medium used for feeding the reactor (section 2.4).

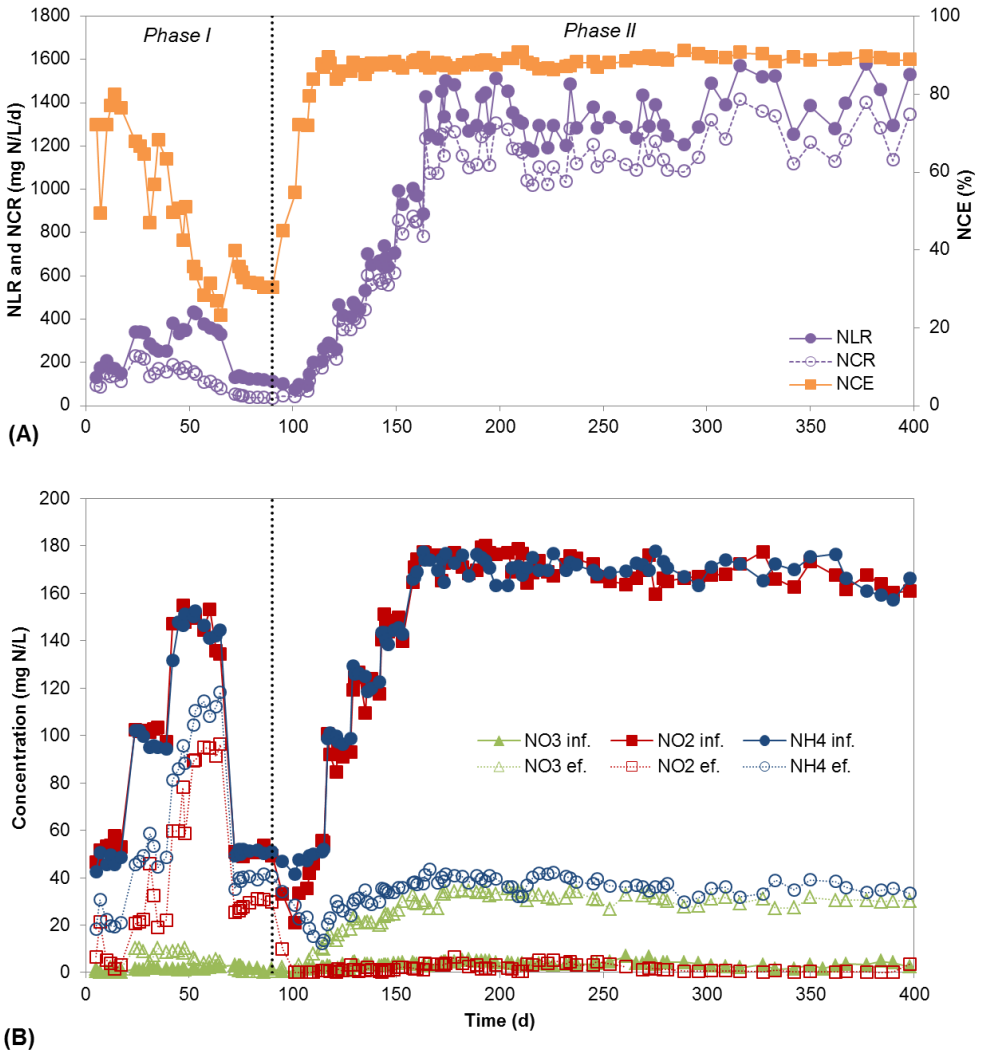

Figure 25: Time course of the N-removal performance in the continuous upflow reactor. (A) N-loading rate (NLR), N-conversion rate (NCR), and N-conversion efficiency (NCE). (B) Influent (inf.) and effluent (ef.) ammonium, nitrite, and nitrate concentrations.

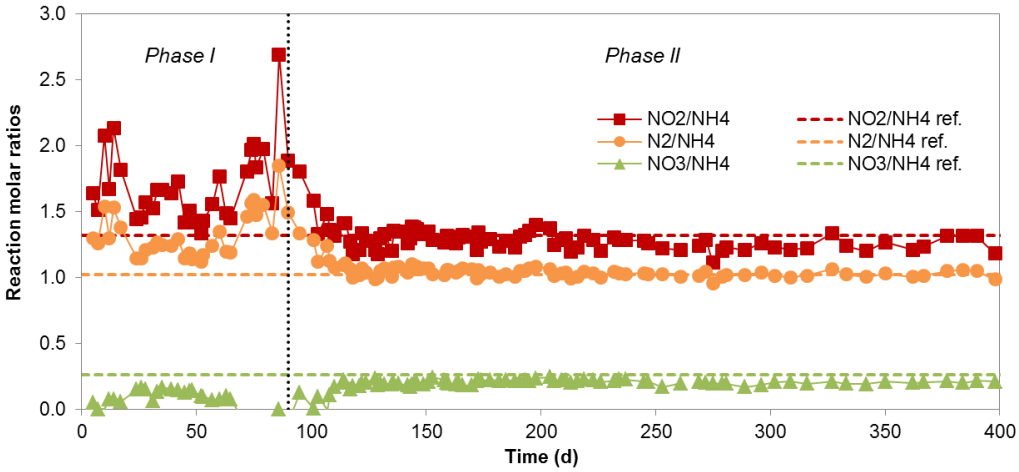

Figure 26: Time course of the nitrite-to-ammonium, dinitrogen gas-to-ammonium, and nitrate-to-ammonium reaction molar ratios in the continuous upflow reactor. Horizontal dashed lines indicate those reference (ref.) values provided in Eq. (1) (1.32, 1.02, and 0.26, respectively).

Phase I (90 days). The NLR was progressively increased (Figure 25A) targeting from 111 to 332 mg N/L/d according to the nominal volumetric flow rate (3 mL/min) and influent concentration (50-150 mg NO_2^--N/L at a ratio of 1 g NO_2^--N/g NH_4^+-N). Taking into account the activity of the biomass measured at the end of the batch enrichment and the dilution rate applied, NCRs not lower than 70 mg N/L/d were expected. Satisfactory N-removal performance was observed during the first 10 days of operation with low nitrite (limiting substrate) concentration in the effluent (Figure 25B). However, nitrite and ammonium started to accumulate in the reactor in the following days with the consequent decrease in the NCE. After 70 days of operation the nominal influent concentration was returned to 50 mg NO_2^--N/L but it did not imply the recovery of the system (NCE < 40%). As depicted in Figure 26, the reaction ratios during this phase did not match well those values given in Eq. (1), with higher values than expected for the NO_2^-/NH_4^+ and N_2/NH_4^+ reaction molar ratios but with lower values than expected for the NO_3^-/NH_4^+ reaction molar ratio (which may be explained due to the coexistence of anammox and heterotrophic denitrification processes). Finally, the problem with the phosphorus source was identified and the corresponding chemical used to prepare the mineral medium was replaced.

Phase II (310 days). In this second phase the NLR was progressively increased again (Figure 25A). Particularly, the targeted loads ranged initially from 111 to 1034 mg N/L/d according to the nominal volumetric flow rate (3-8 mL/min) and influent concentration (50-175

mg NO_2^--N/L at a ratio of 1 g NO_2^--N/g NH_4^+-N). Fast recovery of the N-removal (ammonium and nitrite) performance was observed, resulting in increased NCRs (maximum value of 870 mg N/L/d) and NCEs (maximum value of 89%). Nitrite was completely consumed (1 ±2 mg N/L in the effluent), nitrate was again detected in the outlet (1-33 mg N/L) (Figure 25B), and the reaction molar ratios evolved towards those given in Eq. (1) (Figure 26). After 73 days of operation (total time: 163 days) the targeted NLR was further increased up to 1551 mg N/L/d according to a nominal volumetric flow rate of 12 mL/min (maximum value planned for this study). Once arrived at this point, it was assumed that the anammox enrichment had been achieved successfully. The biomass growing at the bottom of the reactor and attached to the support material developed red color. During the following 237 days the reactor run at an average NCR of 1183 ±100 mg N/L/d and NCE of 88 ±1% (Table 19) in order to continue the culture of the anammox biomass. During this time, the gas produced within the reactor was repeatedly analyzed aiming to detect N_2O. In this regard, N_2O off-gas emissions never accounted for more than 0.2% of the applied N-load, which is quite similar to the values reported by other authors such as Okabe et al. (2011) (0.1% N-load in average) using similar bioreactor technology.

Table 19: Summary of the operating conditions applied and performance for the upflow reactor.

Parameter [a]	Phase I (90 d)	Phase II (310 d)	
	-	73 d	237 d
HRT (h)	18.1 (2.6) [b]	22.4-7.2	6.1 (0.5)
NLR (mg N/L/d)	110-431	73-1002	1353 (109)
NCR (mg N/L/d)	33-227	39-870	1183 (100)
NCE (%)	23-80	45-89	88 (1)
NH_4^+ inf. (mg N/L)	43-152	42-178	170 (5)
NO_2^- inf. (mg N/L)	47-155	21-178	170 (6)
NO_3^- inf. (mg N/L)	1 (1)	3 (1)	4 (1)
NH_4^+ ef. (mg N/L)	18-118	12-38	37 (3)
NO_2^- ef. (mg N/L)	2-96	0-10	2 (2)
NO_3^- ef. (mg N/L)	0-10	1-33	32 (2)
NO_2^-/NH_4^+ reaction molar ratio	1.70 (0.28)	1.34 (0.12)	1.27 (0.06)
N_2/NH_4^+ reaction molar ratio	1.33 (0.17)	1.08 (0.08)	1.03 (0.02)
NO_3^-/NH_4^+ reaction molar ratio	0.05 (0.10)	0.18 (0.05)	0.21 (0.02)

[a] Abbreviations: HRT, hydraulic residence time; NLR, N-loading rate; NCR, N-conversion rate; NCE, N-conversion efficiency; inf., influent; ef., effluent.

[b] Parameter values are provided according to the minimum-maximum range or in averages (standard deviation in parentheses).

3.2.2. Liquid flow regime assessment

Despite the satisfactory N-removal performance achieved, the existence of the support material, biomass growing, and rising gas bubbles within the reactor did result in a liquid flow regime far from the mixing patterns assumed in an ideal plug-flow reactor without axial mixing (where all the atoms of material leaving the reactor have been inside it for exactly the same amount of time (H.S. Fogler, 2006); i.e., the HRT). This was evidenced in a short-term experiment consisting on the introduction of a step perturbation into the system (Figure 27); i.e., increase in the inlet concentration of K^+ (which does not take part in the anammox reaction). The reacting fluid did not flow through the reactor uniformly, as shown by the temporal evolution of the concentration of K^+ in the outlet. At a time smaller than the HRT the K^+ concentration in the effluent started to increase (i.e., early exit of liquid) but at a time equal to the HRT the K^+ concentration in the effluent had not reached the inlet levels yet. This fact implies that, there were sections in the packed bed that offered little resistance to the flow, and as a result a major portion of the fluid channeled through this pathway. The molecules following this pathway did not spend as much time in the reactor as those flowing through the regions of high resistance to flow. Shortening HRT for a part of the nitrogen load may end up in release of non-treated nitrogen in the outlet and decrease of the system efficiency preventing further increase of NLR. This phenomenon was not observed in this work as the NLR was kept relatively low compared to the system maximum capacity; implications are that the TNCR measured in this work may be underestimated compared to reality.

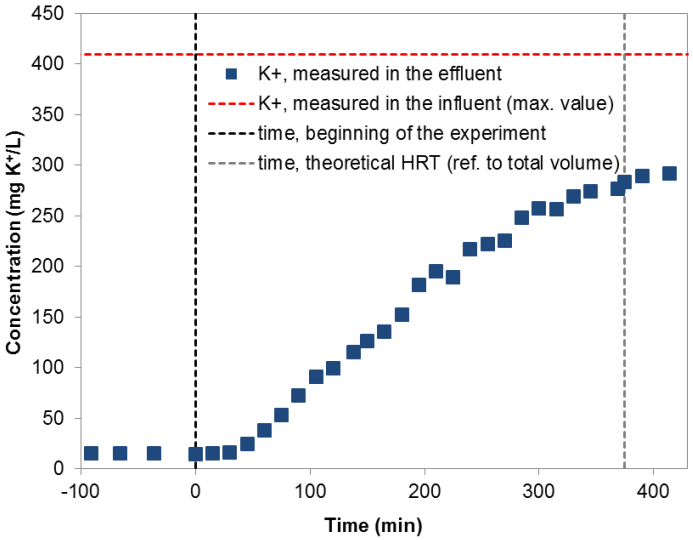

Figure 27: Assessment of the liquid flow regime in the upflow reactor by introducing a step perturbation into the feed stream entering the reactor (replacement of the bicarbonate source from NaHCO3 to KHCO3 in the mineral medium) and subsequent monitoring of the concentration of K+ in the outlet (day 266; volumetric flow rate of 12 mL/min). HRT, hydraulic residence time (here referred to total liquid volume).

Uneven repartition of biomass along the carrier was observed as a more covering and thick biofilm was present in the first 10 cm of the carrier material (i.e., bottom part of carrier support). This resulted in partial obstruction (i.e., uneven vertical flow resistance) of both liquid and gas flows. In operational conditions, the relative contribution of liquid preferential flow and N_2 gas production to axial mixing is delicate to assess. If liquid preferential flow is the main source of axial mixing, change in the carrier characteristics can be considered to improve the process. Smaller carrier wide or larger column diameter would avoid total obstruction at the bottom level of the column and subsequent variability in vertical flow resistance. In the same way it could trigger a more homogeneous repartition of the biomass along the full carrier height. If axial mixing is mainly due to gas production it seems not feasible to provide a plug-flow in this kind of tubular reactor as the N_2 gas production by anammox bacteria cannot be avoided. Compared to CSTR or SBRs, up-flow systems are known to provide greater polishing capacities as they allow concentrations of targeted compounds to decrease all along the flow as long as there is contact with the biomass. Anammox is an autotrophic process and, consequently, produces few amount of biomass. However, and according to these data, under long-term culture periods in upflow fixed bed biofilm reactors it may still be necessary to

periodically extract sludge from inside of the reactor in order to minimize the risk of clogging and enhance the liquid flow.

3.3. Microbial community characterization

Changes in the microbial community structure during the enrichment in batch (B) mode and subsequently in the continuous (C) upflow reactor, were monitored using 16S rRNA high-throughput DNA sequencing. An external (E) sample coming from the lab-scale upflow fixed bed biofilm anammox reactor running at the USDA-ARS (M.B. Vanotti et al., 2011, A. Magrí *et al*., 2012) was also analyzed using the same methodology. After quality filtering, the number of sequences per sample ranged from 2648 (sample B3) to 20555 (sample B2) with an average for all the libraries of 11670 ±5593 sequences per sample (13 samples). Coverage of all libraries, calculated after a systematic random depletion to equalize the number of sequences per sample to the one of the smallest library, was higher than 95 ±3%, which is high enough to validate the subsequent analysis.

3.3.1. Global evolution of the microbial community

Microbial diversity throughout the entire experimental period (i.e., 19 months) is assessed in Table 20 using the indices Shannon-Weaver, Simpson and number of OTUs. Such diversity indices exhibited a systematic decrease during both the batch stage (i.e., indices reductions between B0 and B4 of 23.3%, 5.1%, and 32.2%, respectively) and the continuous stage (i.e., indices reductions between C0 and C3T/C3B of 29.8-40.4%, 11.1-22.2%, and 34.5-68.3%, respectively). For the batch stage, the decrease occurred clearly after 2 months of enrichment with a drop of about 50% of the number of OTUs. This time corresponds to the transition of the ecosystem from a denitrifying to an anammox activity (Figure 23). Concerning the upflow reactor, the decrease was more variable (from 50% to 66% reduction in the number of OTUs) and seems to depend upon the sampling location in the column. According to the aforementioned values, higher indices reductions were found at the bottom side (where ammonium and nitrite concentrations were higher). Equitability also underwent a systematic decrease during both stages; i.e., 22.2% and 28.6%, respectively. These data may imply the concomitant disappearance of some species and a larger segregation between the low and highly represented taxons (A.E. Magurran, 2004; J. Oksanen, 2016).

Relative evolution of the microbial community structure throughout the experimental period was also assessed using the NMDS method (Figure 28). A progressive evolution of the

microbial communities was observed for both batch and continuous stages. Regarding the batch stage, the dynamic conditions aplied during the first 4 months induced a strong evolution of the community, especially between B1 and B3 which corresponds to the transition of the ecosystem from a denitrifying to an anammox activity (Figure 23). After transferring the biomass to the continuous upflow reactor, the microbial community continuated evolving. Initial lack of phosphorus in the feed stream resulted in a smaller relative change of the microbial community structure during the first 3.5 months (C0 vs. C1) than in the following 3 months (C1 vs. C2) after restoring the phosphorus supply. Concerning the next 7 months of culture (C2 vs. C3), the microbial community structure still evolved, but at a lower rate. Fast stabilization of the microbial diversity in anammox reactors was reported by previous studies, even after perturbation episodes in the feed stream (A.D. Pereira *et.al.*, 2014). The external sample coming from the USDA-ARS anammox reactor (E) was also included in the NMDS analysis. Its microbial community remains appart from the enrichment samples. The position of the different samples in the NMDS graph suggests that their vertical distribution is at least partly correlated with microbial diversity.

Tableau 20: Diversity indices for the microbial community during the batch (B0-B4) and continuous (C0-C3X) stage. Sampling position regarding the carrier within the upflow reactor is identified with the letter "B" for bottom or "T" for top. The external sample coming from USDA-ARS (E) is also included.

	Batch stage					Continuous stage							
Total time elapsed (months)	0	1	2	3	4	5	9	12		19			USDA-ARS
Sample	B0	B1	B2	B3	B4	C0	C1T	C1B	C2T	C2B	C3T	C3B	E
Shannon-Weaver	7.8	7.6	7.7	5.7	6.0	5.7	4.9	4.6	3.6	4.8	4.0	3.4	5.8
Simpson	0.99	0.99	0.99	0.95	0.94	0.90	0.90	0.90	0.70	0.90	0.80	0.70	0.96
Number of OTUs	552	515	576	271	374	319	192	154	147	207	209	101	231
Equitability	0.9	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.5	0.6	0.5	0.5	0.7

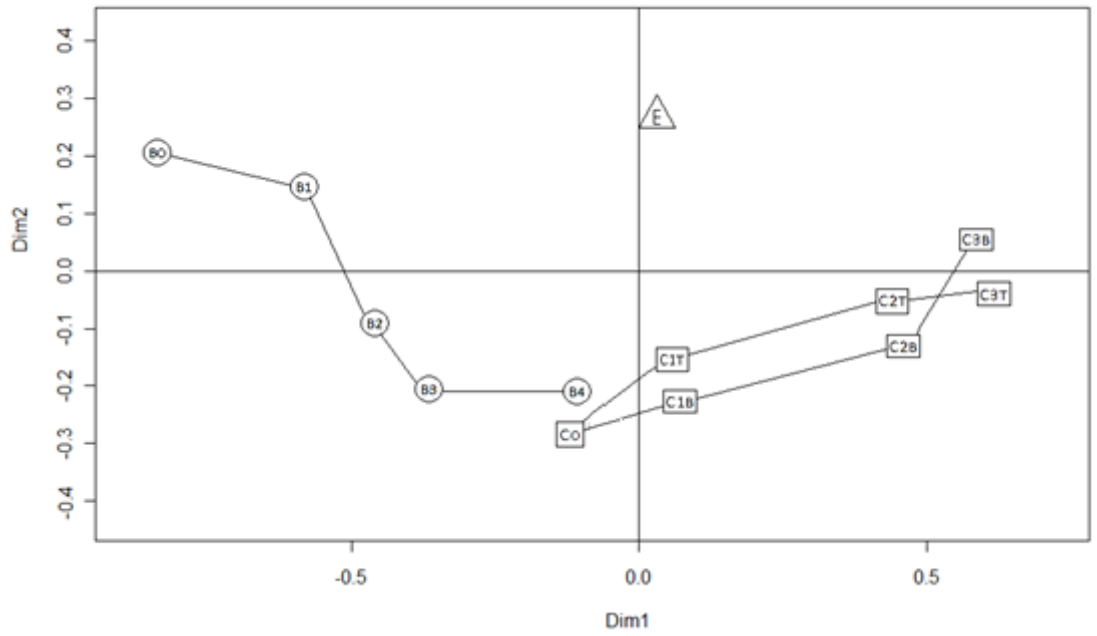

Figure 28: Non-metric multidimensional scaling (NMDS) plot showing the evolution of the microbial community structure during batch enrichment (circles; B0-B4) and subsequently in the continuous upflow reactor (squares; C0-C3X). Sampling time is quantified in Table 20. Sampling position regarding the carrier is identified with the letter "B" for bottom or "T" for top. For comparison, the external sample coming from USDA-ARS is also included (triangle; E).

3.3.2. Description of the microbial community

Because of the large set of data, only OTUs containing more than 1% of the total sequences are presented. The proportion of OTUs excluded from the analysis by choosing this threshold progressively decreased during the experimental period; i.e., from 28.8% at initial time (B0) to 11.1% at the end of the batch enrichment (B4), and to 3.2% at the end of the continuous culture (C3). None of the currently known anammox genus belonged to the excluded OTUs.

Dominant microbial groups (OTUs with > 1% sequences) exhibited progressive evolution during both batch and continuous stages (Figure 29). Such operational modes could affect differently the evolution of the microbial community.

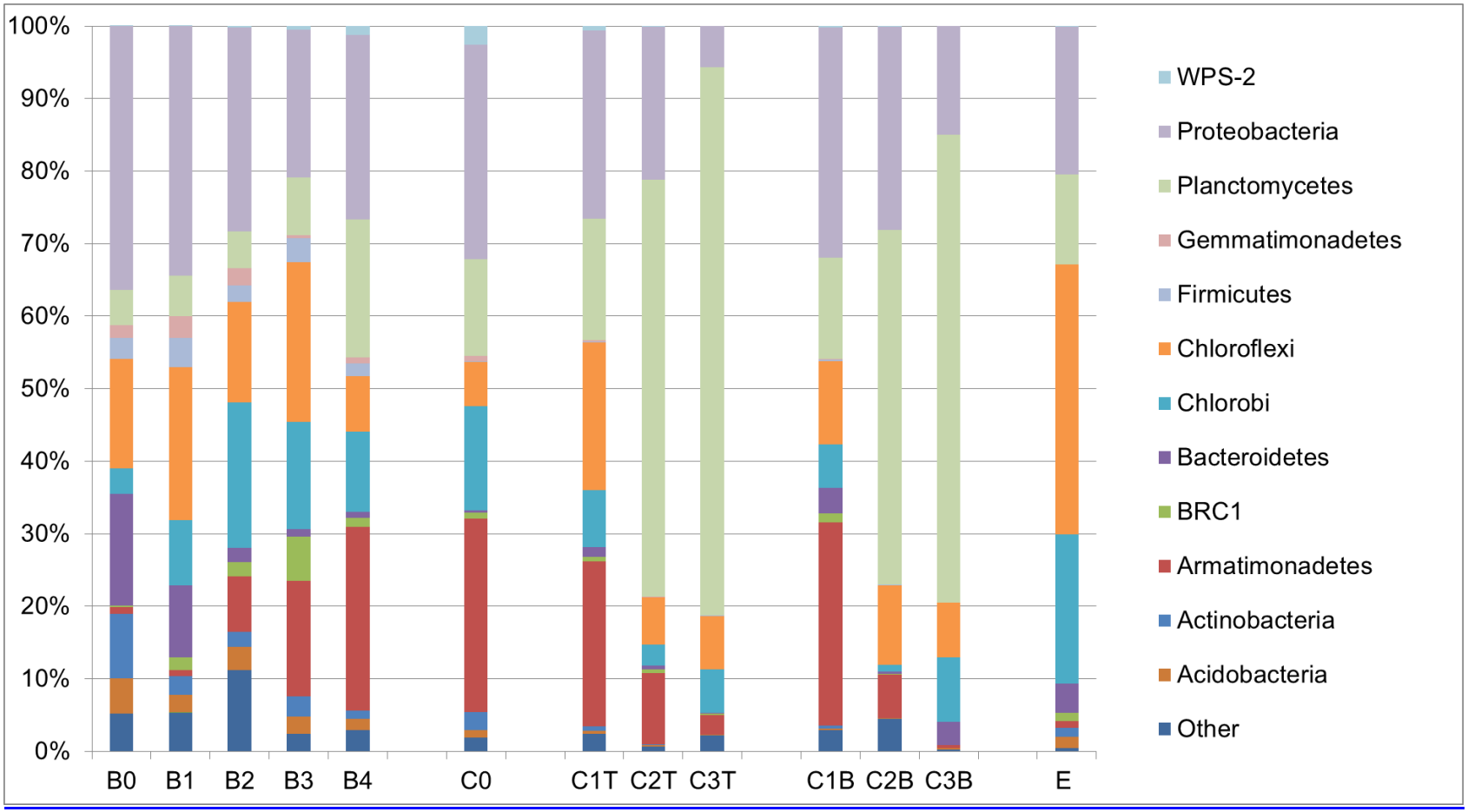

Figure 29: Changes in the microbial community structure at the phylum level during the enrichment in batch mode (B0-B4) and subsequently in the continuous upflow reactor (C0-C3X). Sampling time is quantified in Table 20. Sampling position regarding the carrier is identified with the letter "B" for bottom or "T" for top. For comparison, the external sample coming from USDA-ARS is also included.

The phylum *Proteobacteria*, which is commonly retrieved in wastewater treatment ecosystems (J.-C. Bertrand *et al.*, 2011; M. Hu *et al.*, 2012), was dominant in the activated sludge used as inoculum, but underwent a progressive decrease of relative abundance from 36.3% (B0) to about 10.4% (C3). Initially present as α-, β-, and γ-*Proteobacteria* at 2%, 13%, and 10% of the total sequences, respectively, the experimental conditions applied promoted the selection of genera from the *Rhodocyclaceae* family (class □-*Proteobacteria*). Thus, at the end of the experimental period, relative abundances for this family ranged from 1.3% (C3T) to 8.0% (C3B), while α- and γ-*Proteobacteria* classes represented less than 1% of the community. The most frequent *Rhodocyclaceae* genus was related to the uncultured bacterium clone Dok59 (FJ710778) which has been retrieved from a long-term operated anammox biofilm reactor and is closely related to the denitrifying bacteria *Denitratisoma oestradiolicum* clone 20b_15 (KF810114, 99% similarity). Such genus was also identified as the dominant proteobacterial genus in the external sample coming from the USDA-ARS anammox reactor (with relative abundance of about 10%).

The phyla *Bacteroidetes* (15.5%), *Chloroflexi* (15.1%), *Actinobacteria* (8.9%), *Acidobacteria* (4.9%), and *Planctomycetes* (4.9%) were also abundant in the seeding sludge. Regarding the phylum *Bacteroidetes*, a progressive reduction of its relative abundance was observed (up to about 1.6% of the total sequences at the end of the experimental period). It appears that the provided conditions were not favorable to the *Bacteroidetes*, mainly found in the seeding sludge as bacteria related to the family *Saprospiraceae* (which are known as aerobic and denitrifying chemoorganotrophs). The phylum *Chloroflexi*, which is composed of filamentous bacteria frequently present in activated sludge, was almost exclusively represented by genera belonging to the class *Anaerolineae*. The relative abundance of such class decreased from 12.6% to 6.8% at the end of the batch enrichment (B4). Similar abundance was found at the end of the continuous culture (C3). *Chloroflexi* is usually present in anammox reactors and it has been suggested that they can grow using materials derived from decaying anammox bacterial cells (T . Kindaichi *et al*., 2012). In the external sample coming from the USDA-ARS anammox reactor such phylum reached a relative abundance as high as 37.2%.

The phyla *Actinobacteria* and *Acidobacteria* rapidly diminished during the batch enrichment reaching a final relative abundance below 1.6% (B4). Such trend continued during the continuous culture and final percentages below 0.2% (C3) were obtained in all samples. Same behavior was observed for the *Firmicutes* and *Gemmatimonadetes* phyla that were poorly represented in the initial sludge (2.9% and 1.7% in B0 respectively).

On the opposite, batch enrichment promoted an increase in the relative abundance of *Plantomycetes* from 4.9% (B0) to 19.0% (B4) that further pursued in the continuous stage from 13.4%

(C0) to about 70.0% (C3). Interestingly, this phylum was initially dominated by the *Pirellulaceae* family in the seeding sludge, and anammox-like microorganisms were undetectable during the first 3 months of enrichment. However, with time, the *Pirellulaceae* abundance diminished in favor of the anammox family of *Brocadiaceae* whose relative abundance increased constantly reaching up to 15% of the total sequences at B4 and 66.0% at C3. The enriched species was Candidatus *Brocadia sinica* which was the only anammox microorganism detected during the whole enrichment except for one sample. The environmental and process conditions applied strongly selected for this species that is certainly responsible for the observed anammox metabolism. The other anammox species, identified as Candidatus *Brocadia fulgida*, was observed only in the C3 Bottom sample and represented only 0.5% of the total anammox sequences at this time.

Interestingly, the USDA-ARS sample had a much lower relative abundance of anammox related sequences of only 13% of the total microbial sequences. In this process, the dominant species was Candidatus *Brocadia fulgida* that represented 97% of the anammox sequences and, to a lesser extent, Candidatus *Brocadia caroliniensis* and Candidatus *Brocadia sinica* (2% and 1% of the total anammox sequences respectively).

Concerning other phyla less represented in the inoculum seeding sludge, the batch stage promoted the selection of *Chlorobi*, with relative abundances rising from 3.5% to 20.0% in 2 months, but it was followed by a decrease and stagnation at the end of the first stage and during the continuous culture (7.5%). Those genera enriched during the batch stage mainly belonged to the photoautotrophic classes C20 and OPB56 that disappeared during the continuous stage. Within the upflow reactor, *Chlorobi* was mostly represented by the non-phototrophic chemoheterotrophic order *Ignavibacteriaceae* (7.1 % of the total sequences, 100% similarity with uncultured clone KU000307). It is suggested that bacteria belonging to such order uses organic matter coming from other cells forming the biofilm. This is also supported by its high relative abundance in the USDA-ARS sample (19% of the total sequences).

Finally, the phylum *Armatimonadetes* showed a significant enrichment during the batch stage with a relative abundance increasing from 0.9% (B0) to 25.2% (B4). Conversely, the continuous stage promoted its disappearance with relative occurrence decreasing from 26.7% (C0) to 1.5% (C3). Recently identified organisms related to the phylum *Armatimonadetes* have been found in different natural environments and freshwater sediments (H. Tamaki *et al.*, 2001; H. Yin *et al.*, 2015).

The highly specific conditions applied to the biomass during this study (i.e., mild temperature, lack of oxygen, absence of organic compounds in the feeding, simultaneous exposure to ammonium and nitrite, and prevention to light exposure, among others) represent a significant shift in comparison to

the environmental conditions existing in the inoculum's sampling site (municipal WWTP), and governed the evolution and selection of the microbial community. As a consequence, the microbial community of the seeding activated sludge underwent strong composition rearrangement. Initially composed primarily of *Proteobacteria*, *Bacteroidetes*, *Chloroflexi* and *Actinobacteria*, with only 4.9% of *Planctomycetes* and undetected anammox microbial group, it ended up with about 70% of anammox Candidatus *Brocadia sinica*, 8% of *Rhodocyclaceae* family (class □-*Proteobacteria*), 7.4% of *Chloroflexi* (with 6.8% of *Anaerolineae*) and 7.5% of *Chlorobi* (with 7.1% of *Ignavibacteriaceae*). This final microbial community composition is somehow different from the one of the USDA-ARS anammox reactor which is dominated by the *Chloroflexi* (37.2%), the *Chlorobi* (20.6%), the *Proteobacteria* (20.4%) and the *Planctomycetes* (12.3%). Interestingly, both anammox reactors enriched in the same *Rhodocyclaceae* and *Ignavibacteriaceae* species but in different anammox Candidatus *Brocadia* species.

Indeed, high selective pressure through restrictive environmental trophic conditions has been widely reported to decrease microbial diversity and to favor the appearance of dominant species fitting the metabolic requirements, as expected in this study for the anammox bacteria (R. Connan *et al.* 2016; M.C.M.S. Costa *et al.*, 2014; E. Isanta *et al.*, 2015). On the other hand, when comparing the diversity indices for the anammox biomass here produced with those of the anammox biomass coming from the USDA-ARS laboratory, higher values were clearly found for the external sample (Table 20) although both biomasses were obtained from a continuous upflow reactor running during a long period (> 1 year) under very similar stable conditions. In this regard, M.C.M.S. Costa *et al.* (2014) reported that despite the long-term application of similar operational conditions in anammox reactors, differences may still exist in the microbial communities according to the biomass seeding source. The particular procedure followed for initial biomass enrichment, which was different in both cases (R. Connan *et al.*, 2016; M.B. Vanotti *et al.*, 2011), may also impact on the microbial community. Finally, the hydraulic performance of both reactors (afecting substrate concentration and biomass gradients within the reactor) may also play a role in such distinct diversities.

4. Conclusions

An enrichment procedure was set up which enabled the development of an anammox microbial community able to sustain an anammox N-conversion rate of 1166 ±118 mg N/L/d starting from an activated sludge having no detectable anammox activity and anammox microbial group. The procedure involved a first batch stage with increasing NO_2 influent concentration from 25 to 150 mg NO_2^--N/L and a subsequent continuous upflow column reactor stage with polyester non-woven support where the NLR was further increased from 111 to 1551 mg N/L/d. The overall procedure

selected for a microbial community composed of 70% of the anammox Candidatus *Brocadia sinica* species. Other microbial groups belong to the *Rhodocyclaceae* family (class *β-Proteobacteria*), the *Chloroflexi* and the *Chlorobi*. This microbial community differs from the one of the USDA-ARS anammox reactor who enriched essentially Candidatus *Brocadia fulgida* species.

Acknowledgements

This work was supported by Rennes Métropole [*allocation d'installation scientifique ref. 14C0509*]. The authors are grateful to Chrystelle Bureau for technical assistance with microbial analyses and to the master's student Marina Moya-Espinosa for help with the operation of the upflow reactor. The external USDA-ARS sample was provided to Irstea through the *Material Transfer Agreement (MTA) no. 10598*.

Conflict of interest

The authors have declared no conflict of interest.

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CHAPITRE 5 : Application du procédé anammox au traitement de fraction liquide d'effluent de digestion anaérobie

CHAPTER 5: Application of the anammox process to the treatment of anaerobic digester supernatant

Autotrophic nitrogen removal performance in one- and two-stage systems using the SBR technology

Romain Connan [a], Marina Moya-Espinosa [a], Gilbert Bridoux [b], Patrick Dabert [a], Fabrice Béline [a], Albert Magrí [a,*]

[a] IRSTEA, UR OPAALE, 17 Avenue de Cucillé-CS 64427, F-35044 Rennes, France

[b] SAUR, Recherche et développement, 2 rue de la Bresle, F-78201 Maurepas, France

Abstract

Advances in research and technological development in the field of wastewater treatment encourage the implementation of engineered autotrophic nitrogen removal (ANR) systems based on the coupling of partial nitritation (PN) and anaerobic ammonium oxidation (anammox). Both processes can be conducted in the same reactor under limited aeration (i.e., one-stage system) or, alternatively, in two independent dedicated reactors (i.e., two-stage system). In this investigation, and under a comparative approach, we successfully applied both configurations using the sequencing batch reactor (SBR) technology. Processed wastewater was municipal sewage sludge digester supernatant (~1 g NH_4^+-N/L). Eventually, supernatant pretreatment was considered through struvite precipitation. Performance of both ANR systems was assessed according to the N-loading rate (NLR), supernatant dilution, and phosphate concentration in terms of N-conversion efficiency (NCE) and nitrous oxide (N_2O) emission. For the one-stage system, feasible NLR was 0.5 g N/L/d (with NCE of approximately 90%). For the two-stage system, feasible NLRs were 1.3 g N/L/d in the PN-SBR and 0.6 g N/L/d (with NCE of approximately 90%) in the anammox-SBR. Supernatant dilution and phosphate precipitation favored process performance for both configurations. Concerning N_2O emission, measured values were 1.51% N-loaded for the one-stage system and 1.34% + 1.00% N-loaded for the two-stage system. Thus, according to these findings, higher N-loads were processed in the two-stage system but resulting in higher N_2O emissions.

Keywords

Partial nitritation; Anaerobic ammonium oxidation; Sequencing batch reactor; Sewage sludge digester supernatant; Nitrous oxide emissions

1 - Introduction

Effluents such as biogas digester supernatants, landfill leachates, and some industrial waste streams usually contain high nitrogen (N) concentrations (e.g., 0.5-5.0 g N/L), mostly as ammonium (NH_4^+), but low readily biodegradable organic carbon (C) concentrations (J.P. Sheets *et al.*, 2015; S.W.H. Van Hulle *et al.*, 2010). For these effluents, such low C/N ratio will conditionate treatment alternatives eventually applicable to reduce the nitrogen content. Conventional bioprocesses of nitrification-denitrification (N-DN) converting ammonium to dinitrogen gas (N_2) are inefficient in these scenarios as addition of an external biodegradable organic source is needed to provide appropriate nitrogen conversion (C. Hellinga *et al.*, 1998; A. Magrí and X. Flotats, 2008).

The feasibility of the completely autotrophic nitrogen removal (ANR) pathway based on the anaerobic ammonium oxidation (anammox) (Mulder *et al.*, 1995) offers new treatment alternatives. The metabolism of the anammox bacteria is based on the oxidation of the ammonium into dinitrogen gas under anoxic conditions through various intermediary metabolites (e.g., nitric oxide (NO) and hydrazine (N_2H_4)) using nitrite (NO_2^-) as final electron acceptor (Eq. 9) (M. Strous *et al.*, 1998; J.G. Kuenen, 2008). A partial nitritation (PN) of the influent ammonium is previously required in order to achieve the characteristic stoichiometric coefficients for the anammox reaction; this is 1.00:1.32 (NH_4^+:NO_2^-) which is equivalent to 57% ammonium oxidation (Eq. 10) (A. Magrí *et al.*, 2012). Since the complete nitrification reaction is usually led by two different microbial groups; i.e. nitritation ($NH_4^+ \rightarrow NO_2^-$) by ammonium-oxidizing bacteria (AOB) and nitratation ($NO_2^- \rightarrow NO_3^-$) by nitrite-oxidizing bacteria (NOB), activity of this second bacterial group must be repressed to avoid production of nitrate (NO_3^-). Several methods have been reported to achieve such repression: (i) the combination of mesophilic temperature (~35ºC) and low solids retention time (C. Hellinga *et al.*, 1998), (ii) high free nitrous acid (HNO_2) concentration (Y. Zhou *et al.*, 2011), (iii) high free ammonia (NH_3) concentration (A. Magrí *et al.*, 2012), and (iv) low dissolved oxygen (DO) concentration (I. Jubany *et al.*, 2009).

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (9)$$

$$NH_4^+ + 0.86\ O_2 \rightarrow 0.57\ NO_2^- + 0.43\ NH_4^+ + 0.58\ H_2O + 1.14\ H^+ \quad (10)$$

The coupled PN-anammox process can be carried out either in separated dedicated reactors (i.e., two-stage process) (U. van Dongen *et al.*, 2001; W.R.L. van der Star *et al.*, 2007; Dosta *et al.*, 2015) or concomitantly in the same reactor (i.e., single-stage process) (Eq. 11) (K. Furukawa *et al.*, 2006; M.J. Kampschreur *et al.*, 2009a). Jaroszynski and Oleszkiewicz (2011) reported, for the two-

stage systems, higher risk of instability due to high nitrogen concentrations within the reactors but very high potential for optimization and process intensification. Otherwise, the same authors described the one-stage systems as more simple in configuration but with high process control needs due to the complex interaction between microbial populations (aerobic and anaerobic ammonium-oxidizers, nitrite-oxidizers and heterotrophs). Indeed, the microorganisms coexisting in such systems may tend to the self-organization in specific structures forming biofilms or granules where conditions are more favorable for their development. Comparative studies in this framework may help to contrast strong and weak points for both configurations.

$$NH_4^+ + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O \quad (11)$$

Interest of PN-anammox in comparison to N-DN mainly relay on three different aspects; (i) lower aeration requirements for nitrification which can represent savings of ~60% of the total energy consumption; (ii) no requirements of a carbon source for heterotrophic denitrification; and (iii) lower sludge production (~90% reduction) due to this is a completely autotrophic process (E.I.P. Volcke, 2006; S.W.H. Van Hulle et al., 2010). In addition, the ANR is also an interesting alternative when considering energy consumption during processing in comparison to other physicochemical treatments based on nutrient recovery, especially when ammonium concentration falls below 2.0 g NH_4^+-N/L (A. Magrí *et al.*, 2013). The first full-scale PN-anammox installation was started-up in Rotterdam in 2003 (W.R.L. van der Star *et al.*, 2007) and currently more than 100 full-scale installations are running worldwide (referred to year 2014) (S. Lackner *et al.*, 2015).

Anammox microorganisms are known to exhibit a long doubling time depending on the environmental and operational conditions applied; this is usually between 2.1 and 11 days (at ~30ºC) equivalent to a maximum specific growth rate of 0.065-0.334 d^{-1} (M. Strous *et al.*, 1998; T. Lotti *et al.*, 2015). Because of this slow growth rate, and the specialized metabolism, the anammox systems are particularly sensible to perturbations and toxicity events. A wide range of molecules are reported as eventual inhibitors for the anammox process (e.g., nitrite, organic matter, phosphorous, etc.) (R.-C. Jin *et al.*, 2012). In order to improve anammox systems treatment capacities, inhibitions events must be prevented and concentration thresholds must be carefully defined. Additional particularities arise from the biomass structure as both biofilm and granules act as protective environments, increasing tolerance to external inhibitory compounds by limiting the mass transfer (C. Picioreanu *et al.*, 1998). Toxicity assessments are commonly carried out in vials running under batch mode, considering short-term exposure of the anammox biomass to the limiting compound under study and measuring the resulting specific activity (A. Dapena-Mora *et al*, 2007; J.M. Carvajal-Arroyo *et al.*, 2013). This is a useful technique to perform screenings for an eventual toxic compound considering a wide range of

concentrations, or even for a combination of compounds at different concentrations looking for synergistic effects (G.-F. Yang and R-C. Jin, 2012). However, inhibition assessment through short-term exposure tests may not reflect the real long-term impact of an eventual toxic compound at a given concentration. In this regard, uncertainty will be reduced and reliability increased by extending time of exposure or conducting long-term assays (T. Lotti *et al.*, 2012; I. Fernández *et al.,* 2012).

Conventional biological nitrogen removal treatments are eventual sources of greenhouse gases such as NO and nitrous oxide (N_2O), which are produced as intermediate products of the nitrification and denitrification reactions (G. Tallec *et al.*; 2006; A. Alinsafi *et al.,* 2008). ANR systems have also been reported as N_2O producers although such compound is not produced in the anammox metabolic pathway (F. Gori *et al.*, 2011). Thus, N_2O emissions in ANR systems can be related to the activity of both nitrifying and denitrifying microorganisms (S. Okabe *et al.*, 2011; A. Rodriguez-Caballero and M. Pijuan, 2013). Emissions are known to be positively impacted by (i) high nitrite concentration, (ii) acidic pH, ,and (iii) low DO concentration (A. Alinsafi *et al.,* 2008; M.J. Kampschreur *et al.*, 2009b; S.Okabe *et al.*, 2011). In addition, chemical side-reactions may also result in significant N_2O emissions (Kampschreur *et al.*, 2011). Usually, the single-stage systems are reported as those with lower emissions (M. Ali and S. Okabe, 2016).

The aim of this study is provide insights in the comparison of one- and two-stage ANR systems regarding process performance, and particularly focusing on the associated N_2O emissions under regular and transient operation. The sequencing batch reactor (SBR) technology was used for concept implementation. Owing to the high dissolved phosphorus concentrations in the processed sewage sludge digester supernatant, a pretreatment by struvite precipitation was considered. Inhibitory effects of phosphorus in the ANR was assessed considering both short-term batches and long-term continuous conditions.

2. Material and methods

2.1. Sludge sources

The nitrifying sludge used for conducting PN was enriched from activated sludge collected in the municipal wastewater treatment plant from Liffré (France). Such facility performed N-removal by conventional N-DN by applying intermittent aeration. The anammox sludge was obtained from the continuous upflow reactor packed with a support material and fed with mineral media described in Chapter 2 of this dissertation (Figure 31). Anammox bacterial species was identified as Candidatus *Brocadia sinica*.

2.2. Set-up of the ANR systems

Both ANR configurations were implemented in 3.5 L jacketed glass reactors (Trallero & Schlee, Spain) (Figure 30). Such reactors were operated under batch mode and controlled using a home-made software in LabView v14.0 (National Instruments, USA) through a PLC (Agilent 34970A, USA). Process temperature was controlled at 35ºC using a water heating circulator (Julabo EH-13, Germany). Biomass suspension was achieved by means of a mechanical mixing device (IKA RW20, Germany) equipped with an impeller. Liquid was pumped in and out of the reactor using a peristaltic pump (Heidolph PD 5001, Germany). For both the PN reactor and the one-stage reactor air was supplied through an air pump (KNF N811KN.18, France), a flow meter, and porous stones located at the bottom of the reactor. Concerning the PN reactor air was supplied at a flow rate of 2000 mL/min and antifoam polymer (Strucktol SB2113, Germany) was added at 0.02% V/V. Aeration in the one stage-system was initially provided at a variable flow rate according to a setpoint for the DO of 0.07-0. 18 mg O_2/L; secondly through an alternate pattern of aerated and anoxic phases. Online monitoring was performed using a DO probe (Hamilton VisiFerm DO Arc 120, Switzerland) and a pH probe (Hamilton Polilyte Plus Arc 120). For the anammox reactor, no air was supplied. Instead, synthetic N_2 was blown continuously in the headspace of the anaerobic reactor (gas flow rate: 150-200 mL/min) to prevent air from entering the reactor and allowing online gas measurement. Online monitoring only consisted in using a pH probe (Hamilton Polilyte Plus Arc 120). An additional peristaltic pump was used for controlling the pH (range: 7.3-7.4) within the reactor by acid dosage (HCl 10% (v/v)).

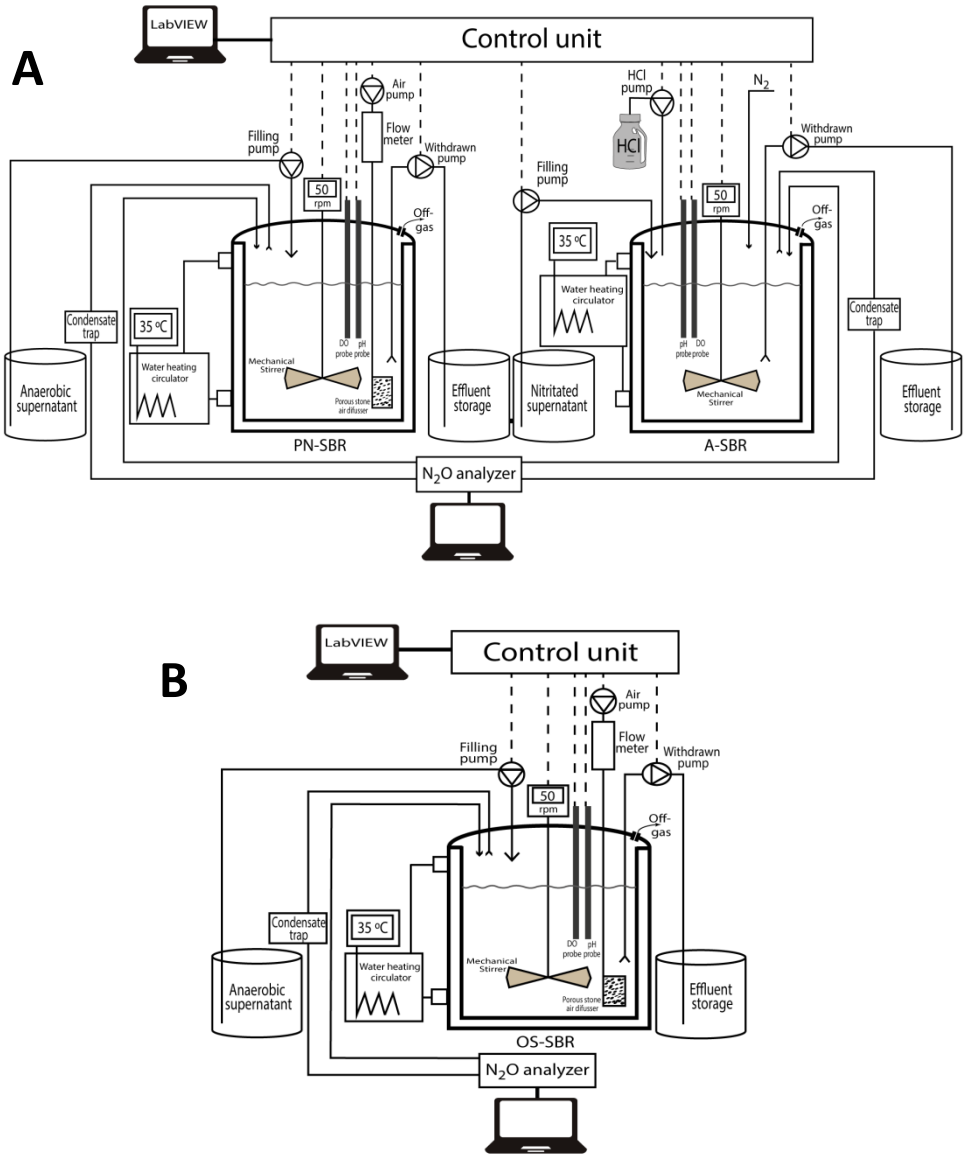

Figure 30: Set-up for the two- (A) and one-stage (B) ANR system. For the two-stage ANR system PN and anammox reactors were coupled in series.

2.3. Start-up and operation of the ANR systems

2.2.1. Two-stage system

The PN reactor was initially inoculated with 3.54 g volatile solids (VS) of activated sludge. The operational sequence applied consisted in cycles lasting 8 h (total: 480 min). During the first 450 min aeration was kept constant and punctual feeding events lasting 1 min were repeated every 45 min (10 feeding events per cycle). Last 30 min of the cycle included 20 min for biomass settling and 10 min for effluent withdrawal. The anammox reactor was inoculated with 6.87 g VS of enriched anammox sludge coming from the upflow reactor. Initial specific nitrogen conversion rate (sNCR) measured in batch after biomass harvesting was 969 mg N/g VS/d under NO_2^-/NH_4^+ reaction molar

ratio of 1.32. The operational sequence applied consisted in cycles lasting 8 h and divided into periods of continuous feeding plus mixing (430 min), only mixing (20 min), settling (20 min) and withdrawal (10 min), similarly as previously described by Magrí *et al.* (2012).

2.2.2. One-stage system

The one-stage system was inoculated using 3.43 g VS of active nitrifying sludge coming from the PN reactor included in the two-stage system and 2.22 g VS of enriched anammox sludge coming from the upflow reactor. The operational sequence applied consisted in cycles of 8 h**.** Feeding was performed continuously during the first 450 min and air supply was active as long as DO should be controlled under a threshold value. Subsequently, sludge was allowed to settle during 20 min and the clarified effluent was withdrawn during the last 10 min of the cycle.

2.3. Characteristics of the sewage sludge digester supernatant

Both process configurations were tested using biogas digester supernatants coming from the same municipal wastewater treatment plant (Liffré, France). The supernatant is obtained after anaerobically digesting dewatered activated sludge produced during secondary treatment as well as those greases separated during wastewater pretreatment. Liquid-solid separation is achieved onsite by centrifugation using polymer (FLOPAM EM 640 LOB) at a rate of 0.05% (v/v with respect to raw influent sludge). Characteristics of such supernatants are summarized Table 21. Typical features for this kind of effluents including high ammonium content (~1 g N/L) and low biodegradable organic-C content (~0.5 g COD/L mostly in non-biodegradable form), among others, were corroborated. When needed, phosphorus (P) was precipitated as struvite (i.e., magnesium-ammonium-phosphate, $MgNH_4PO_4 \cdot 6H_2O$) by adding $MgO_{(s)}$ at a molar ratio of 1:1 (Mg:P) (K.S Le Corre *et al.*, 2009). The process was conducted in a 10 L jacketed glass tank at 10°C during 2.5 h under low mechanical agitation.

Tableau 21: Characteristics of the sewage sludge digester supernatants used in this study.

		Sewage sludge digester supernatant	
	Unit	One-stage	Two-stage
pH		8.45	8.10
EC	dS/m	7.4	8.7
Alkalinity ($CaCO_3$)	mg/L	3275	4173
COD	mg O_2/L	510	510 ± 43.6
BOD_5		72	n.c.
NH_4^+-N	mg/L	1024 ±27	1177 ± 7.4
NO_2^--N	mg/L	0.4 ±0.0	0.0
NO_3^--N	mg/L	0.0	0.1 ±0.0
PO_4^{3-}-P	mg/L	260 ±4	351 ± 19
Na^+	mg/L	120 ±5	136 ±8
K^+	mg/L	400 ±2	626 ±3
Ca^{2+}	mg/L	30.0 ±0.1	9.6 ±1.3
Mg^{2+}	mg/L	5.8 ±0.0	4.5 ±0.9
Total-Fe	mg/L	2.2	2.6
Cl^-	mg/L	161 ±2.3	208 ±4.1
S^{2-}	mg/L	< 0.1	< 0.1
SO_4^{2-}-S	mg/L	5.6 ±0.3	4.4 ±0.04

Abbreviations: EC, electrical conductivity, COD, chemical oxygen demand, BOD_5, Biological oxygen demand at 5 days; n.c.: non characterized

2.5. Chemical Analysis

Digester supernatants were analyzed according to the *Standard Methods for the Examination of Water and Wastewater* (APHA *et al.*, 2005). Total solids (TS) were measured after sample drying to constant weight at 105°C and volatile solids (VS) were measured after further ignition in muffle furnace at 550°C. The pH was measured using a portable meter (WTW pH 197i, Germany) and electrical conductivity referred to 25ºC (EC) was measured using a conductivity probe (Hamilton Conducell 4USF PG-120, Switzerland). Alkalinity was measured by acid titration to a final pH of 4.50. Total Inorganic Carbon (TIC) was measured using the sequential titration method proposed by Moosbrugger *et al.* (1993). Chemical oxygen demand (COD) was determined through the reflux colorimetric method and 5 days biodegradable oxygen demand (BOD_5) was determined through biological incubation. Cations including ammonium (NH_4^+), sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}), as well as anions including nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), chloride (Cl^-), and sulfate (SO_4^{2-}) were all measured using ion chromatography (850 Professional IC, Metrohm, Switzerland). Total iron and sulfide (S^{2-}) were measured by inductively coupled plasma mass spectrometry (ICP-MS) and potentiometry, respectively. Outgoing N_2O in the off-gas was measured continuously during all the experimental period using an online gas analyzer (ABB URAS26 EL3020, Germany).

2.4. Short-term phosphorus inhibition test

Effect of short-term exposure of anammox biomass to phosphate was assessed in a batch test conducted using glass bottles (working volume: 250 mL) containing anammox biomass and mineral medium. A constant amount of biomass harvested from the upflow reactor (70 mg VS) was exposed to a range of phosphate concentrations (0-850 mg PO_4^{3-}-P/L) using mineral medium prepared according to R. Connan *et al.* (2016). Initial nitrogen concentration within the bottles was 60 mg NH_4^+-N/L and 60 mg NO_2^--N/L. The amount of phosphorus added to a bottle impacted on the pH (and final value was recorded). N_2 flushing was used to displace air in the bottles headspace. All bottles were sealed with a rubber stopper plus an aluminum cap and placed in an incubator shaker (KS4000i control, IKA, Germany) at 150 rpm, 35ºC, and in dark conditions. Liquid samples were collected using syringes (without opening the bottles) at regular time intervals and filtered using 0.45 µm polypropylene membrane filters before chemical analysis. The test lasted 6 h and was run in triplicates. Finally, the anammox activity was assessed according to linear regression analysis to describe nitrogen conversion.

3. Results and discussion

3.1. Digester supernatant pretreatment

Struvite precipitation was considered as eventual pretreatment because of the particularly high phosphate concentration in the supernatant (260-351 mg PO_4^{3-}-P/L). Such high values could be justified by the low Mg^{2+} and Ca^{2+} contents in the supernatant, which would limit further crystallization of phosphate salts despite the favorable pH values (pH > 8.10) (K.S. Le Corre *et al.*, 2009). According to the aforementioned pretreatment, the phosphorus concentration in the supernatant was decreased to 19-30 mg PO_4^{3-}-P/L during phases I to III for both ANR configurations. This can be considered as acceptable and non-limiting phosphate content for the process (A. Dapena-Mora *et al.*, 2007; Y. Jeanningros *et al.*, 2010). Additionally, the dosage of MgO to the supernatant affected on its properties according to two aspects: (i) the rise-up of the pH, and (ii) a partial depletion of NH_4^+. Such rising in the pH was moderate, without exceeding the value of 8.5 at the end of the crystallization period. Keeping low temperature during crystallization is a key point for both enabling precipitation and avoiding ammonia volatilization (K.S. Le Corre *et al.*, 2009). Moreover, the small rise in pH will also reduce risk of negative impact on subsequent ANR, especially concerning the one-stage configuration. In this regard, the pH has been identified as a significant parameter during anammox (J.M. Carvajal-Arroyo *et al.*, 2013). On the other hand, NH_4^+ depletion due to precipitation is well aligned with the overall treatment aim of decreasing the nitrogen content in the digester

supernatant. In this research, from 7.6% to 9.0% of the total ammonium content in the supernatant was depleted through the pretreatment based on precipitation.

3.2. Performance of the one-stage system

The one-stage system runs during 52 days. Four different operational phases can be identified during this timelapse: (*Phase I*) Start-up and increase of the nitrogen loading rate (NLR) applied using half-diluted and P-depleted supernatant [0-18 days]; (*Phase II*) Increase of the NLR applied using undiluted and P-depleted supernatant [19-29 days]; (*Phase III*) Recovery after a toxicity event using half-diluted and P-depleted supernatant [31-42 days]; and (Phase IV) Performance using diluted digester supernatant with P concentration adjusted at 300 mg PO_4^{3-}-P/L [43-52 days]. The overall performances achieved are summarized in Table 22.

During *Phase I* the one-stage system behaved well and the NLR applied was increased from 258 to 468 mg N/L/d by shortening the hydraulic residence time (HRT) from 1.81 to 1.01 days. During the first days of operation (0-16 days) aeration was provided to the system according to a DO concentration threshold in the liquid bulk (i.e., air was continuously provided into the reactor as long as the concentration of DO remained below a maximum value). Such control strategy led to intermittent aeration and low DO concentrations in the reactor (which prevented exposure of anammox microorganisms to oxic conditions). During these days the set-point for the DO was tuned from 0.07 to 0.19 mg O_2/L. However, by the end of the *Phase I* the reactor became unstable as the DO concentration remained below the control threshold even though considering continuous aeration, leading to NO_2^- accumulation in the effluent. To avoid lack of oxygen in the reactor, the aeration control was upgraded at day 16 shifting from variable-length oxic periods to fixed-length anoxic periods (4 min) and considering similar air flow rate between 0.4 and 0.5 L/min. According to this modification, the NRL could be successfully fixed at ~400 mg N/L/d without observing nitrite accumulation. Overall, aeration was active from 11% to 54% of the reaction time (i.e., 7.5 h/cycle) depending on the aeration strategy and the NLR applied. During *Phase II*, the supernatant fed into the system was not diluted. The system behaved well during the initial days at NLRs higher than 650 mg N/L/d. However, at day 24 both ammonium and nitrite start accumulating. Oxygen consumption and system performance progressively decreased until day 29 (i.e., the N-conversion efficiency (NCE) dropped from 90.7% to 68.3%). *Phase III* started at day 31 by operating the reactor similarly as in *Phase I* using again diluted supernatant. During this phase the system recovered stable behavior as NLR was kept stable then slightly increased from 400 to 574 mg N/L/d with a concomitant increase of NRE from 64.1 to 73.6%. Meanwhile, the biomass aggregates harvested from the upflow reactor had broken into smaller particles with granular shape and darker color, which was attributable to the

colonization of the external layer of the granules by nitrifying and heterotrophic microorganisms (S. Bagchi *et al.*, 2016). Besides, brownish activated sludge coexisted with the granules in the liquid bulk (Figure 31). During *Phase IV* the phosphate content in the diluted supernatant was adjusted to 300 mg PO_4^{3-}-P (similar P concentration than in the undiluted supernatant). Under this new conditions, the process performance collapsed rapidly and the applied NLR must be decreased from 468 to 135 mg NH_4^+-N/L/d in 9 days to maintain process efficiency and avoid nitrite accumulation. The specific anammox activity in the one-stage system was measured all over the experimental period values are shown in Figure 32a. An intensive anammox activity assessment on synthetic substrate, at low and high phosphate concentrations exposure were performed during this period, after introducing the new substrate and waiting for 3xHRT days. Initially, the specific anammox activity underwent a drop of 39.0% (i.e., from 154 to 94 mg NH^+_4-N/g VS/d) between inoculation and end of *phase III*. Then switching from low to high phosphate concentrations provoked a successive decrease of 58.5% in activity (i.e., from 94 to 39 mg NH^+_4-N/g VS/d). However, no significant evolution of the specific ammonium conversion rate was observed after 6 days of continuous reactor performance. Thus, average drop of activity after high phosphate concentration exposure was 55.7±6.7%.

An additional topic of concern related to the treatment of the digester supernatant is the low iron content measured (~2.5 mg Fe/L). Iron is a key element as constituent of the heme cytochrome involved in the anammox metabolism for energy production (J.G. Kuenen, 2008) and low concentrations may negatively affect the process rates. Y. Liu and B.-J Ni (2015) reported optimal concentrations between 3 and 6 mg Fe(II)/L, approximately. However, the iron finally needed for the biomass growth will also be related to the N content in the influent. In this regard, the aforementioned authors worked with total concentrations of 100 mg N/L (50% ammonium + 50% nitrite) and the content in the supernatants used in this study was tenfold higher. Finally, another topic of concern is the use of a cationic polymer in the centrifuge used for dewatering the digested sludge. Currently, no quantitative information was found in the scientific literature regarding toxicity by presence of a cationic polymer. In spite of this fact, a recent study showed the irreversible negative impact of anionic surfactants on anammox biomass activity (S. Qiao *et al.*, 2016).

Tableau 22: Overall performances of the one-stage ANR system.

▼ Parameter / Phase ►		I	II	III	IV
Timespan (days)		0-18	19-29	31-42	43-52
Supernatant dilution factor		2	1	2	2
Influent PO_4^{3-}-P (mg/L)		19-30	19-30	19-30	296-302
Influent (mg N/L)	NH_4^+	433-492	929-985	414-458	419-514
	NO_2^-	0.1-1.4	1.4-6.0	0.2-1.8	0.2-6.1
	NO_3^-	0.3-2.1	0.5-5.5	2.0-2.4	1.2-2.5
Effluent (mg N/L)	NH_4^+	2.4-36.9	29.0-234.3	350-72.8	110-4.8
	NO_2^-	2.1-21.7	11.3-48.2	11.7-22.2	3.6-50.2
	NO_3^-	8.2-41.2	46.7-66.6	12.2-34.7	20.1-28.0
HRT (d)		1.81-1.01	1.51-1.29	1.09-0.79	0.96-3.11
NLR (mg N/L/d)		258-468	695-719	403-574	468-135
NRR (mg N/L/d)		199-392	631-491	249-451	348-108.4
NRE (%)		92.0-89.1	90.7-68.3	64.1-73.6	74.5-80.3
TS content in the reactor (g)		12.6-12.6	16.2	n.c.	16.9-17.2
VS content in the reactor (g)		5.7-7.5	9.5	9.3	9.6-9.2
N_2O emitted (% N_2O-N/N-loaded)		0.57 (0.1-2.4)	0.78 (0.3-1.4)	1.51 (0.6-2.8)	4.32 (1.5-9.9)
N_2O emitted (% N_2O-N/N-removed)		0.81 (0.2-3.7)	1.16 (0.3-2.1)	2.06 (0.9-3.9)	5.86 (2.5-12.3)

Abbreviations: HRT, hydraulic residence time; NLR, nitrogen loading rate; NRR, nitrogen removal rate; NRE, nitrogen removal efficiency; TS, total solids; VS, volatile solids; n.c., not characterized.
Values into brackets represent registered extreme values within the time period.

3.2. Performance of the two-stage system

3.2.1. Partial nitritation stage

The start-up of the dedicated PN reactor was achieved after almost one month of operation using half-diluted supernatant and progressively increasing the NLR up to 500 mg N/L/d. Influent dilution was stopped at this point without limiting nitritation. Subsequently, the PN stage was tested during 2 months under three nominal NLRs; i.e. 500, 1000, and 1300 mg N/L/d (Table 23). Although no nitrate was produced, the desired 57% oxidation of the ammonium into nitrite (i.e 1.32 g NO_2^--N /g NH_4^+-N) was not achieved, and the NO_2^--N/NH_4^+-N ratio experimentally obtained was slightly below 1.0, which stands for deviations not lower than 25% with respect to 1.32 (reported values in Table 23 ranged from 0.84 to 0.96). Such deviation could be justified because of the low alkalinity content in the digester supernatant (4.07 g $CaCO_3$/g NH_4^+-N are theoretically required for 57% oxidation of ammonium into nitrite according to Magrí *et al.* (2012) but only 3.54 g $CaCO_3$/g NH_4^+-N were available according to Table 21) and the effective consumption by the AOB (which may depend on the NLR applied and the pH in the reactor liquid bulk, among other factors). Free ammonia (FA) and free nitrous acid (FNA) concentrations in the reactor were calculated as detailed by Anthonisen *et al.* (1976) according to the temperature, pH, and total ammonium and nitrite concentrations,

respectively. Values given by these authors as inhibitory to NOB were 0.08 to 0.8 mg FA-N/L and 0.06 to 0.83 mg FNA-N/L. Equivalent inhibitory values for the AOB were reported from 8.2 to 123.5 mg FA-N/L. Thus, the high concentrations of the unionized species FA and FNA were main responsibles for the selective inhibition of the NOB despite the continuous aeration (Table 23).

Tableau 23: Overall performances of the PN reactor under a two-stage ANR configuration.

▼ Parameter / Condition ►	I	II	III
Time (after end of the start-up)	49	56	63
pH (range in the cycle)[a]	6.25-6.41	5.91-6.41	5.79-6.65
FA-N (end of the cycle) (mg/L)	1.4	0.5	0.4
FNA-N (end of the cycle) (mg/L)	0.6	1.3	1.9
HRT (day)	2.44	1.11	0.86
NLR (mg/L/d)	450	1024	1373
NPR (mg/L/d)	259	527	639
NO_2^- effluent (% total N)	48.2	48.9	45.7
NO_3^- effluent (% total N)	0.3	0.3	0.2
NO_2^--N/NH_4^+-N ratio effluent	0.94	0.96	0.84
NO_3^--N/NH_4^+-N ratio effluent	0.01	0.01	0.00
N_2O emitted (% N_2O-N/N-loaded)	1.34	1.13	0.88
N_2O emitted (% N_2O-N/NP)	2.33	2.20	1.89
N_2O-N (mg/d)	14.0	29.4	39.3

Abbreviations: FA, free ammonia; FNA, free nitrous acid; HRT, hydraulic residence time; NLR, nitrogen loading rate; NP, nitrite produced; NPR; nitrite production rate.

[a] Given values represent registered extreme values.

3.2.2. Anammox stage

Similarly to the one-stage system, the dedicated anammox reactor runs during 39 days after inoculation. Four different operational phases can be identified during this time: (*Phase I*) Start-up and increase of the NLR applied using half-diluted and P-depleted supernatant [0-6 days]; (*Phase II*) Increase of the NLR applied using undiluted and P-depleted supernatant [7-17 days]; (*Phase III*) Recovery after a toxicity event using half-diluted and P-depleted supernatant [18-29 days]; and (*Phase IV*) Performance using half-diluted supernatant with P concentration adjusted at 300 mg PO_4^{3-}-P/L [30-39 days]. The overall performances achieved are shown in Table 24.

During *Phase I* the system behaved correctly and the NLR was increased from 572 to 668 mg N/L/d by shortening the HRT from 0.91 to 0.78 days. During *Phase II*, the supernatant was no longer diluted. Under such conditions, the system behaved well during the 3 following days at a NLR of about 830 mg N/L/d but, at day 10 both ammonium and nitrite start accumulating. Subsequently, the

NLR was decreased up to 508 mg N/L/d but, in spite of this fact, the system performance fell progressively until day 17 (i.e., the NRE decreased from 92.8% to 83.8%). *Phase III* started at day 18. The reactor was fed similarly to *Phase I* using half-diluted supernatant. During this period the system performance was enhanced with NLR increased from 437 to 623 mg N/L/d and the NRE increased from 87.0 to 90.3 % assuring system recovery. Similarly to the one-stage system, the biofilm extract from the upflow reactor broke into smaller reddish particles with overall granular shape once inoculated into the SBR (Figure 31). Finally, during *Phase IV* phosphate content was adjusted at 300 mg PO_4^{3-}-P/L to recreate the real concentration found in the digester supernatant. Process performance decreased during all this period and the NLR was decreased from 674 to 511 mg N/L/d in 9 days to maintain process efficiency and avoid nitrite accumulation. Anammox activity during this period was assessed similarly to the case of the one-stage system (Figure 32b). Specific anammox activity underwent a drop of 60.5% between initial batch on synthetic substrate and end of *phase III*. Furthermore a successive drop in activity of 48.9% (from 164.9 to 86.3 mg NH_4^+-N/g VS/d) was measured when switching from low to high phosphate concentration However subsequently, no further significant loss of activity was detected. Thus, average drop of activity after high phosphate concentration exposure is about 51.1±2.5%.

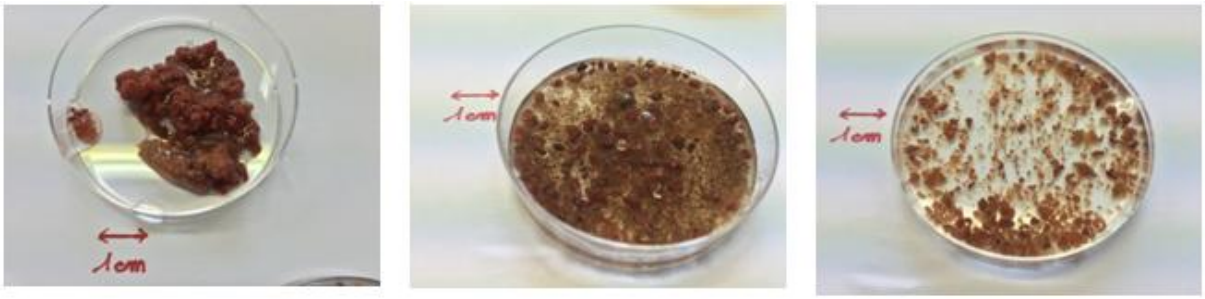

Figure 31: Left: Anammox biomass extracted from the continuous upflow column reactor. Middle: Mixture of reddish anammox granular biomass and nitrifying sludge in the one-stage system (day 36). Right: Anammox granular biomass in the two-stage system (day 23).

Tableau 24: Overall performances of the anammox reactor under a two-stage ANR configuration.

▼ Parameter / Phase ►		I	II	III	IV
Timespan (days)		0-6	7-17	18-29	30-39
Supernatant dilution factor		2	1	2	2
Influent PO_4^{3-}-P		12-31			287-301
Influent (mg N/L)	NH_4^+	245-250	437-472	215-276	311-339
	NO_2^-	268-298	417-499	211-265	264-306
	NO_3^-	2.0	2.4	2.4	2.4
Effluent (mg N/L)	NH_4^+	25.6-37.3	58.9-121.7	27.4-62.8	114.1-144.0
	NO_2^-	0.9-5.9	1.8-123.9	0.2-7.5	20.0-40.6
	NO_3^-	25.9-36.8	31.8-66.6	29.9-40.1	18.4-35.7
Effluent NO_2^--N/NH_4^+-N ratio		1.22 (1.11-1.28)	1.23 (1.15-1.31)	1.23 (1.13-1.42)	1.38 (1.15-1.50)
HRT (d)		0.91-0.78	1.14-1.75	1.40-0.83	0.77-1.21
NLR (mg N/L/d)		572-668	837-508	437-623	674-511
NRR (mg N/L/d)		502-574	729-405	310-527	560-358
NRE (%)		94.8-91.7	92.8-83.8	87.0-90.7	89.8-75.7
TS content in the reactor (g/L)		4.77 ±0.36	5.58 ±0.25	4.31 ±0.09	5.42 ±0.46
VS content in the reactor (g/L)		2.15 ±0.36	2.38 ±0.23	2.34 ±0.13	2.65±0.25
N_2O emitted (% N_2O-N/N-loaded)		n.c.[a]	2.3 (1.1-5.5)	1.0 (0.5-2.4)	3.2 (1.2-4.6)
N_2O emitted (% N_2O-N/N-removed)		n.c.[a]	4.5 (1.3-18.3)	1.2 (0.5-3.0)	4.6 (1.5-7.1)

Abbreviations: HRT, hydraulic residence time; NLR, nitrogen loading rate; NRR, nitrogen removal rate; NRE, nitrogen removal efficiency; TS, total solids; VS, volatile solids; n.c., not characterized.

Values into brackets represent extremes in the time period.

[a] Data unavailable because of analyzer failure.

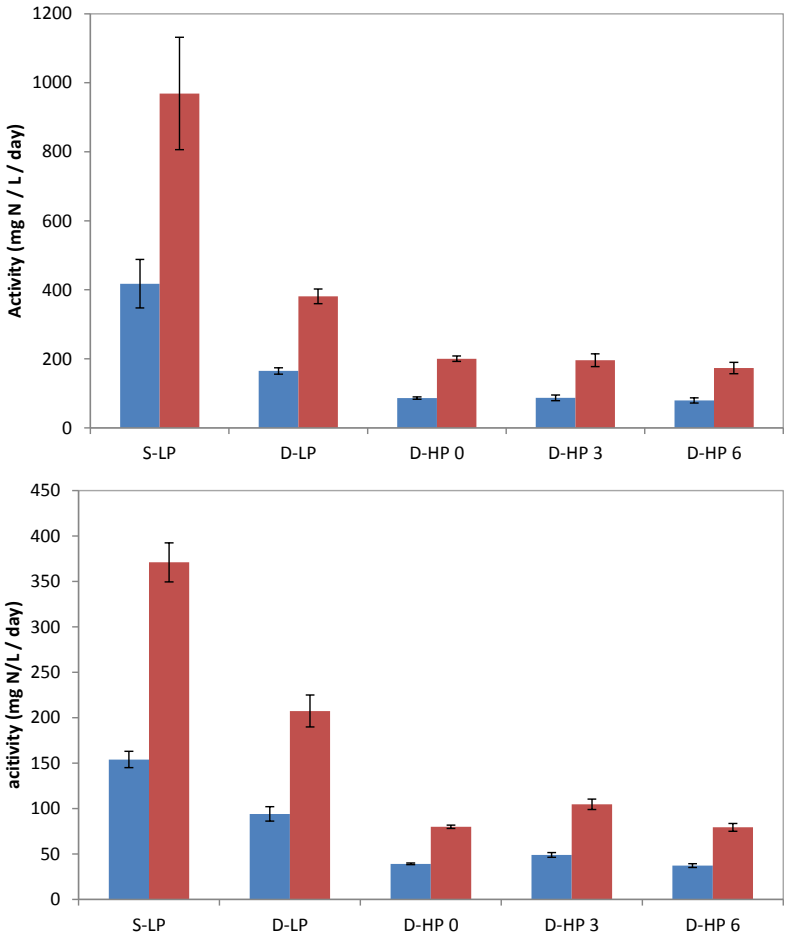

Figure 32: Anammox activity in the one-stage system under high phosphorus concentrations (during Phase IV) assessed through the ammonium conversion rate (blue) and the total nitrogen removal rate (red). Abbreviations: S, synthetic media; D, digester supernatant; LP, low phosphorus; HP, high phosphorus (300 mg PO_4^{3-}-P/L); numbers stand for time exposure in days after introducing the new substrate and waiting for 3xHRT days.

3.3. N_2O emissions

For both configurations, in those reactors with anammox activity, the N_2O emissions were related to the accumulation of nitrite, as previously reported elsewhere (M.J. Kampschreur *et al.*, 2009a). In addition, although during the inhibitory conditions applied during *Phase IV* the nitrite accumulation was avoided by reducing the NLR applied, the relative amount of N_2O emitted increased sharply, with average values of 4.32% N-loaded for the one-stage system and 3.2% N-loaded for the anammox dedicated reactor. For comparison purposes, equivalent average values during *Phase III* were 1.51% and 1.00% N-loaded, respectively (Tables 22 and 24). Additionally for the two-stage system, the amount of N_2O-N emitted in the PN reactor rose according to the NLR applied from 14.0 to 39.3 mg N_2O-N/d, which is equivalent to 0.88%-1.34% N-loaded.

According to the current knowledge, N_2O does not take part in the anammox metabolism (J.G. Kuenen, 2008; S. Okabe *et al.*, 2011). However, it is produced as intermediate metabolite during both nitrification and heterotrophic denitrification (J.L. Campos *et al.*, 2016). Presence of heterotrophic microorganisms is expected in both reactors with anammox activity growing on cellular decay materials and/or remaining biodegradable organic-C from the supernatant (T. Kindaichi *et al.*, 2012; M. Ruscalleda *et al.*, 2012). In the anammox dedicated reactor, this observation is strengthened by the evolution of the NO_2^--N/NH_4^+-N reaction ratio, which evolved from 1.30 to 1.50 at the end of *Phase IV*. Additionally, the production of NO_3^- in *Phases III* and *IV* dropped from 7.1% to 5.1% N-loaded. Such deviated ratios (under anammox biomass inhibitory conditions) could imply that the denitrification process was increasingly taking place during this period due to a higher decay of the anammox biomass. Thus, denitrification would be probably the process responsible for the increased N_2O emissions already described. Chemical-side reactions resulting in N_2O emission are also feasible under presence of reduced iron (M.J. Kampschreur *et al*., 2011), but this is not the case for this research (Fe content in the supernatant was < 3 mg/L).

3.4. Short-term phosphate inhibition test

Results obtained from the short-term (5 h) batch test carried out to assess effect of exposing anammox biomass to phosphate concentrations ranging from 0 to 850 mg PO_4^{3-}-P/L clearly showed that, after an initial plateau in the biological activity response up to concentrations of about 300 mg PO_4^{3-}-P/L, high concentrations clearly resulted in a decreased activity (Figure 33). However, since the pH within the bottles used to conduct the test was dependent on the amount of phosphate added, a synergistic effect between phosphate and pH could exist during the test (J.M. Carvajal-Arroyo *et al.*, 2013). Under these conditions, half maximal inhibitory concentration for phosphate (IC_{50}) was inferred as ~650 mg PO_4^{3-}-P/L which is similar to the values found elsewhere; e.g., A. Dapena-Mora

et al. (2007) reported an IC_{50} = 620 mg PO_4^{3-}-P/L working with Ca. *Kuenenia* sp. and J.M. Carvajal-Arroyo *et al.* (2013) reported an IC_{50} = 784 mg PO_4^{3-}-P/L working with Ca. *Brocadia* sp. Rencently, no inhibitory effect for phosphate has been found in an continuous-flow anammox granular system after gradually increasing the phosphate concentration up to 500 mg PO_4^{3-}-P/L (Z.-Z. Zhang *et al.*, 2016). Such high concentration slightly affected the specific anammox activity, hardly impacted the heme c content, remarkably decreased the extracellular polymeric substances production and significantly stimulated the dehydrogenase activity of anammox granules. In this context, novel anammox granules with a hydroxyapatite core were cultivated, which possessed excellent settleability, huge granule diameter and superior mechanical strength. Otherwise, concentrations higher than 1550 mg PO_4^{3-}-P/L induced the cytoplasm leakage (Z.-Z. Zhang *et al.*, 2016). In this research, the aforementioned sensibility to phosphate concentrations of 350 mg PO_4^{3-}-P/L detected in both ANR systems operated in continuous could be explained by the relative unmaturity and small size of the anammox granules formed after introducing the biomass harvested from the anammox upflow reactor into the perfectly-mixed SBRs.

Short-term incubation without phosphorus supply did no result in a decreased activity. It is well known that anammox aggregates contain phosphate and other ions in cristalised form (C. Trigo *et al.*, 2006) which could become available during the short-term test. However, long-term exposure to phosphorus scarcity conditions has been observed to lead to unstable anammox biomass culture (Chapter 4).

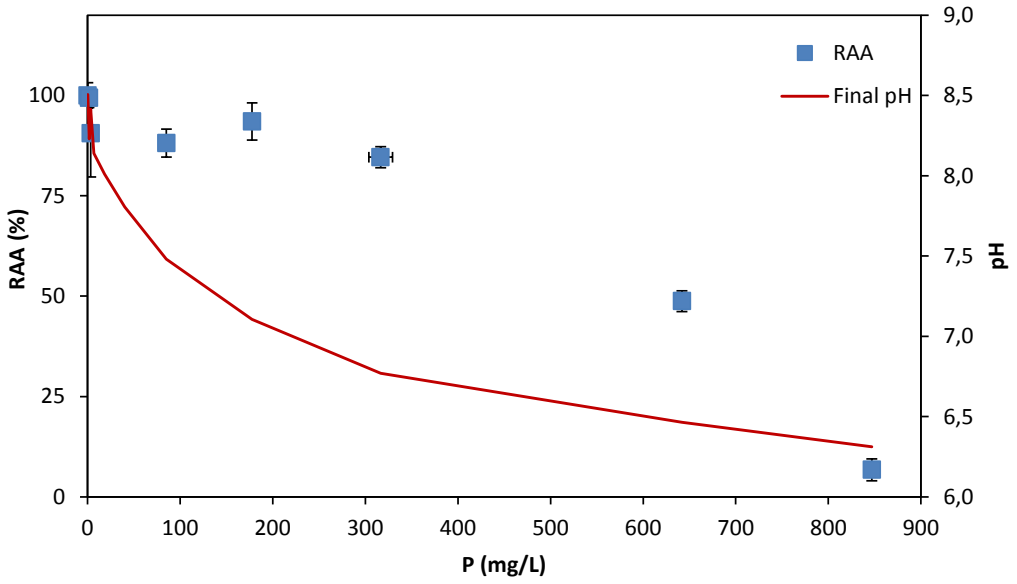

Figure 33: Short-term effect of the phosphorus concentration in the anammox biomass activity assessed through batch tests (in duplicates). Final pH is also reported. Abbreviations: RAA, relative anammox activity.

4. Conclusions

ANR by coupling PN and anammox was investigated in one-stage and two-stage systems using the SBR technology. The study focused on the treatment of sewage sludge digester supernatant. Main results concerning overall process performance, N_2O emission, and effect of phosphate on the anammox process as eventual inhibitory compound were discussed.

- Supernatant dilution and pretreatment through phosphate precipitation as struvite favored process performance for both configurations.
- For the one-stage system, feasible NLR was 0.5 g N/L/d (with NCE of approximately 90%), whereas for the two-stage system, feasible NLRs were 1.3 g N/L/d in the PN-SBR and 0.6 g N/L/d (with NCE of approximately 90%) in the anammox-SBR.
- Measured values for the N_2O emissions were 1.51% N-loaded in the one-stage system and 1.34% + 1.00% N-loaded in the two-stage system.
- According to the aforementioned findings, higher N-loads were processed in the two-stage system but resulting in higher N_2O emissions. Analysis of the microbial communities coexisting within the three reactors is currently in progress.
- This research focused on the start-up and operation of both configurations for a short time period (i.e., not longer than 2 months). During this time, several experimental conditions were tested. Further comparative analysis under stable operation would help to better characterize optimal conditions to be applied in order to maximize treatment capacity of both systems.

Acknowledgements

This work was supported by Rennes Métropole through the *allocation d'installation scientifique* ref. 14C0509. Sylvie Picard and Patricia Saint Cast are acknowledged for their assistance with chemical analysis. Fabrice Guiziou is acknowledge for his technical assistance with online gas measurement.

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Chapitre 6 : Conclusions et perspectives

Chapter 6. Overall conclusions and further works

The state of the art and the bibliometric analysis performed within this work exposed the widespread interest invested in the anammox process over the world and the concomitant industrial development associated with an important increase of research. Within this framework, the research unit OPAALE of Irstea - Rennes has been working for a long time on anaerobic digestion of livestock and municipal waste on one hand, and nutrient treatment and recovery on the other hand. About 4 years ago, the research unit decided to investigate the feasibility of using the anammox process to treat digestate supernatant. The beginning of this research investigation corresponded to the work performed within this PhD which was a new topic for the laboratory and required combining the main skills of the team: process engineering, experimental simulation and microbiology. The work carried out during this PhD allowed assessing various complementary aspects of the anammox process: its strategic interest, its practical value and its academic interest.

The bibliometric analysis revealed itself a power tool to investigate actual trends in a specific field of research. Instead of focusing on the anammox process, the case study targeted nutrient management and provided a larger overview integrating processes involved in similar aims. A well known fact, but particularly impressive when highlighted, is the evolution of the documentary volume production that is not only switching from one thematic to another but has its proper rate which is growing exponentially. Even if this trend is closely related to the fast pace of actual research, it also resides in the global importance nowadays of getting rid of pollution spreading and reducing treatment costs. In this context, the anammox process progressively took more importance in both academic research and industrial development since its discovery. Regarding the specific case of France, development of the anammox thematic at industrial scale is inexistent and few academic works are reported so far. This observation strongly contrasts with other European countries like Spain or the Netherlands whose highly invested in the development of anammox. Even if disparities exist all over the world, there is a clear global interest in pushing forward set-up of new installations and increasing knowledge about the process. Fully autotrophic nitrogen removal is on his way to represent a real paradigm shift in wastewater treatment in the following years bringing cost efficient and eco-friendly solutions.

As the anammox topic was initiated for the first time in our laboratory, the development of our own anammox inoculum cultivation through enrichment was chosen as the first step in order to develop laboratory scale processes, biomolecular tools and experience while producing original results.

First step targeting anammox enrichment has been achieved applying a batch methodology. Compared to continuous enrichment, batch mode gives the opportunity to test various inoculum

seeding sludge and operational conditions. In this sense batch mode strategy suits very well research purposes. It requires less equipment compared to continuous reactors and doesn't need any specific monitoring; it implies less time invested in daily operations. Finally, the methodology described in chapter 3 led to highly active anammox biomass up to 222 ± 2 mg NH_4^+-N/L/day (TNCR: 560 mg N/L/d) after a 4 months period. Surprisingly this time lap was enough to develop millimetric granules in one of the six inoculums (I1 – MMBR denitrification unit). However a very significant disparity in both time required for anammox activity appearance and final nutrient removal rate (NRR) after 4 months enrichment was observed from 0 to 92 day and 21 ± 1 to 118 ± 1 mg N/g VS/d, respectively. In this sense, batch approaches testing several seeding sludge represent a security as more responding inoculums can be selected to pursue further enrichment more effectively than simply starting from continuous enrichment. Due to this important disparity in inoculum responses, identification of clear characteristics for their selection is of crucial importance.

Previous works reported in literature some molecular approaches to help targeting this objective. Among them quantitative polymerase chain reaction (q-PCR) to evaluate anammox abundance and equitability in microbial community are the most reported (Y. Tao *et.al.*, 2013). Surprisingly both of them proved to be inefficient predicting neither time before activity appearance nor final activity for the six seeding sludge used in our study. However q-PCR data allowed assessing different enrichment behaviors (i) decrease in anammox populations followed by quick enrichment, (ii) lag phase of stagnation (up to 3 months) and sudden rapid enrichment. Trying to explain these behaviors, an interesting observation was the correlation of the VS content in raw inoculums environment and the lag phase duration before anammox activity appearance. In fact except for one inoculum, a linear correlation between those two parameters could be fitted as higher VS concentration triggered longer lag phase. Several hypotheses can be drawn regarding this negative impact of organic matter on anammox development. It could be an inhibitor or/and a source of competition with denitrifying microorganisms. In the first case, a dormancy strategy could take place avoiding unfavorable environment and waiting for better environmental conditions. In the second case, the fast growing heterotrophic denitrifiers could consume the NO_2 substrate and prevent the growth of anammox. Assuming both scenarios, a proportional dose-response effect, like the one we saw, would make sense. To go further in the understanding of this inhibition or competition effect, specific experiments should be done, including a better characterization of the organic matter composition. Actually, the sampled environment used as inoculums were coming from sediments or urban or pig slurry activated sludge. It implies important variability in the organic nature of the compounds present. Information regarding biodegradability (i.e., BOD_5 - Biological oxygen demand over 5 days), deeper characterization of organic matter fractions and/or identification of toxic

molecules as antibiotics could provide wilder insights regarding both lag phase and final NRR developed.

In definitive, the observed relation between inoculum VS content and lag of anammox development was informative since a previous study draw attention on not overloading the amount of seeding sludge as too much VS was suspected to handicap initial anammox growth. Some work even recommended to wash-out the sludge biomass as a favorable "early" methodology to fasten initial enrichment in continuous systems. Actually, the batch enrichment results presented in this manuscript are part of a second trial that followed a first unsuccessful one. A previous 4 months batch experiment had been initially realized without any anammox activity development. In this first trial, three seeding sludge had been tested (i) municipal WWTP –denitrifying pool sludge (ii) pig slurry treatment plant – partially aerated activated sludge (iii) river sediments. The only difference with the successful trial was the absence of the 15 days pre-treatment phase where denitrification was promoted through regular nitrate additions. Unfortunately sampling locations of the seeding sludge were different for the second trial preventing any direct comparison. But based on the 100% success of the second trial, regardless the seeding sludge sampling environment, this pre-treatment step is highly suspected to have shortened the enrichment initial lag phase. This observation reinforces the hypotheses regarding the effect of organic content on anammox dormancy or inhibition previously enounced. Maybe in the future use of transcriptomic tools will provide further insights in anammox response to organic matter.

Depending on the kind of inputs fed to anaerobic digestion an important variability of digester supernatants organic content is observed. Short trials have been realized performing partial nitritation (PN) of agro-industrial digester supernatants incorporating mainly industrial food waste and pig manure (Saint Gilles du Mené, France). The supernatant possessed a relatively high BOD_5 about 3.8 g O_2/L which is much higher than values found in the municipal WWTP (Liffré, France) digester supernatant treated in experiments related in chapter 5. Considering only the PN step switching from low to high BOD_5 influent, the system underwent important changes in sludge aspect, foam formation, greenhouse gas emissions (GHG) and nitritation performance. Impact on overall performance and sludge aspect suggested that the microbial community underwent shifts under transient organic loads affecting the process. Moreover significant impact was measured on N_2O emissions, probably due to the impact of organic load on both denitrification relative activity and oxygen depletion. Undoubtedly organic load also affected the anammox process, in this sense better characterization of organic molecules impact on anammox populations and microbial community may help improving both process stability and overall performance regarding N removal and GHG emissions.

After enrichment period, the choice of an anaerobic packed bed up-flow reactor was made as a breeding system for anammox sludge. Inoculation of the reactor was made with the more active sludge (I1) coming from the previous batch enrichment. Inoculation was realized after 5 months batch enrichment. This time period was considered but unplanned as waiting for reactor readiness was necessary. Since I1 developed small granules and significant NRR after 3 months, the reactor inoculation could have been done earlier. The initial conditions provided with the feeding media accidently deprived from any phosphorous source (as explicated in chapter 3) led to a delay in anammox growth. Anyway short recovery of the system was observed after phosphorus supplementation and no specific observation was made on either microbial community structure or selected anammox genera or specie. *Ca. Brocadia sinica* was selected from the beginning of batch enrichment and remained dominant in the anaerobic packed bed up-flow reactor. The use of a non-woven polyester as carrier material was proven to be an efficient way to retain biomass within the system. However the flow regime assessment revealed that the system was not a perfect plug-flow reactor as HRT for a given particle entering the system was highly variable. The reason for axial mixing is suspected to be due to a combined mixing effect of gas production and preferential flows. The NLR applied in this study was way below the system maximum capacity as biomass only partially recovered the carrier total surface and large parts of the working volume remained available for biomass growth. This failure assuring plug-flow regime could lower the system efficiency as part of the influent traveled faster within the reactor preventing further increase in NLR. However, NLR up to 1577 mg N/L/d was attained. Another study with a similar up-flow anammox system considered a different kind of carrier material more homogeneous within the working volume forcing liquid flow to travel through thin biomass layers (Y. Ren *et.al.*, 2014). No liquid flow assessment was presented within this work, but it may lead to less turbulence and closer plug-flow conditions.

Despite the long period (i.e., 5 months) considered during batch enrichment and subsequent phosphorous depleted initial condition during up-flow continuous cultivation, a highly enriched anammox biomass was ready to be used for any purpose only after a 7 months period. This time course could have been even made shorter without any waiting period. It demonstrates the feasibility of obtaining highly active anammox biomass from scratch within a relatively short time period.

Microbial analysis using NGS methodology revealed that both batch enrichment and continuous up-flow culture conducted to the development of the same anammox specie represented by *Ca. Brocadia sinica.* Compared to other genera, *Ca. Brocadia* are reported to be r-strategist microorganisms which is consistent to the conditions applied during both enrichment phases. Microbial communities have been compared to the ones found in a very similar packed-bed up-flow

reactor (ARS-USDA). Despite variations in cultivation conditions, especially in term of temperature (i.e., 30°C instead of 35°C), both system communities enriched in some closely related families like *Rhodocyclaceae* denitrifiers¸ filamentous *Chloroflexi*, and the heterotrophic anaerobe *Ignavibacteriaceae* (*Chlorobi*). All these microbial groups may participate in anammox bacteria enrichment by building a protective biofilm from oxygen and organic matter. However the anammox specie enriched in the ARS-USDA system is *Ca. brocadia fulgida* implying that the variation in temperature, or other enrichment conditions, may have favored one species instead of the other.

One remarkable result of the environmental screening realized looking for anammox inoculum is the important ubiquity and co-occurrence of various anammox genera in the sampled environments. One sampling location even exhibited 4 of the 6 known anammox genera. All six treatment plants sampled were proven to possess anammox bacteria within their sludge. It constitutes no prove and cannot be generalized at every locations, but regarding these specific results, anammox microorganisms seem to be widely present in man-made nitrogen rich and partially oxygenated environments. Moreover anammox communities were proven to be highly versatile as the initial dominant genus is not systematically the one finally enriched. This bring to light that anammox organisms could be more ubiquitous than initially expected. The hard work enriching them due to their low growing rate may have led to a wrong general idea regarding their rarity in both natural and entropized ecosystems even if their abundance remains low. In this sense the NGS techniques used all along this work provided an exciting way to explore microbial communities and even anammox diversity despite their low initial abundance. In this sense the combined nested PCR and NGS approach used within this work, selecting for *Planctomycetes*, allowed to lower enough the detection threshold to permit deep visualization of anammox communities.

Concerning microbial ecology, isolation of anammox bacteria as pure culture has not realized yet. It could be because we do not completely know their requirement; because, of a lack of effort and interest as their metabolic process is widely understood now; or because they need to grow closely related to other microorganisms. The data obtained in this work point out a few microbial groups (*Rhodocyclaceae*¸ *Chloroflexi*, and *Ignavibacteriaceae*) but are clearly insufficient in order to draw any conclusion or hypothesis on microbial synergy or antagonism between anammox and these microbial populations. Crossing DATA based on the statistical analysis of process parameters, medium composition and microbial community's structures of numerous highly enriched anammox systems could be a way to better identify redundant co-enriched microorganisms.

The packed-bed up-flow reactor was proven to be a really efficient tool to breed large amounts of highly enriched anammox sludge (up to 60% purity). This anammox sludge was actually

further used to start both single- and two-stages SBRs anammox reactors. The use of a biofilm shape biomass as inoculum was not an important handicap as shear fragmentation and further granulation process was realized within a really short period of time. During this period biomass retention in the system was efficient and flotation events were minor. The main advantage of using pre-enriched sludge is undoubtedly the short start-up and initial high activity found to be higher than 400 and 500 mg N/L/d within the first two weeks of operation. Despite the good response of the system and the prompt increase in system activity, moving from half diluted to pure digestate represented in this case a non-feasible limitation. Actually a first attempt treating the same partially nitrified digestate was realized earlier during a 2 months period in a dedicated anammox SBR. This time, the treated digestate was kept half diluted and no pre-treatment on phosphate was considered (i.e., PO_4^{3-}-P about 140-180 mg P/L). Despite half dilution NLR not higher that 500 mg/L/d was attainted and system efficiency progressively decreased over the operational period leading to unstable conditions, high N_2O emissions and nitrate depletion in the effluent. At this point phosphate was pointed out as responsible of unstable operation explaining why phosphorous crystallization was considered in second trial presented in chapter 5. However it appeared that even a decrease of P content to more common values (i.e., 10-30 mg PO_4^{3-}-P/L) didn't allow moving to undiluted digestate. It implies that another factor was not clearly identified despite its implication. Suspicion is now placed on both soluble iron and polymer contents within the digestate. Total iron analysis revealed that iron load per gram of nitrogen were similar to the one applied during up-flow cultivation. However this value may not reflect iron bio-disponibility as it can be present as precipitate especially at high phosphate concentrations. Otherwise, interesting work could be done regarding sensitivity to the various kind of polymers used in dewatering sludge steps and as antifoams. In our case antifoam (Strucktol SB-2113) and a surfactant (FLOPAM EM 640 LOB) were used during PN and liquid/solid separation respectively. Until recently literature was totally deprived from information related to anammox sensibility to surfactants. This can be explained by the variety of molecules found on the market. S. Qiao *et al.* (2016) demonstrated that anionic polymers provoked no harm to cellular integrity but block catabolic enzymes at high concentrations. They also evaluate different anionic surfactant families and identified one less harmful for anammox activity. This kind of approaches could be extended to other surfactant families providing precious insights selecting more appropriate molecules and increasing system performance and stability for both research and industrial purposes.

Biomass inhibition is always represented by the sum of inhibitory and deficiency effects. Use of P precipitation gave us a great possibility to compare anammox specific activity in continuous conditions at both low and high concentrations (i.e., 10-30 and 300 mg P/L). The transition from low

to high concentration triggered a highly significant decrease in anammox activity of about 48.9% and 55.7% in both one- and two-stages respectively. Recently Z.-Z. Zhang *et al.* (2016) has proven that granular anammox biomass in a UASB reactor exhibited no sensibility to phosphate concentrations up to 500 mg P/L. However they report great changes in exopolymeric substances and increase in granules diameter. P concentration was gradually increased during 200 days and anammox biomass seemed to adapt itself to this conditions shift. In this sense the high sensitivity to phosphate in our system may comes from the relative biomass unmaturity and especially the lack of time given to anammox bacteria to adapt themselves. A progressive exposure approache, similar to the one applied to nitrite supply described in chapter 3, could provide deeper information concerning relative sensibility during system start-up and could be extented to the surfactant toxicity issues. Moreover comparison between short time exposure and continuous test revealed great disparities in term of inhibition effects. The IC_{50} determined from the batch activity test was about 713.9 mg P/L and was close to other values reported in literature. This variation compared to continuous conditions evaluation illustrate the fact than short term exposure and acute behaviors may deviate from long term biomass reactions. In our case a clear underestimation of phosphate inhibition potential was observed. However in other cases a surestimation could be made as the short period of time considered may not allow biomass to react and adapt itself facing new conditions. If bottle batch assessment provide an easy way to measure wide scales of concentrations or conditions they tend to poorly describe biological reactions on long term periods; which is actually the purpose in many cases and the most valuable information.

Résumé

Ce travail de thèse est focalisé sur l'étude d'un bioprocédé, nommé anammox pour « anaerobic ammonium oxydation », basé sur le métabolisme d'une bactérie éponyme découverte au début des années 90'. Ce métabolisme singulier est retrouvé parmi 6 grands genres bactériens appartenant tous au *Plantomycetales*. Source d'énergie pour la bactérie il repose sur la bioconversion de l'ammonium (NH_4^+) en diazote (N_2) en utilisant le nitrite (NO_2^-) comme accepteur final d'électrons. Le diazote étant un gaz inerte, le procédé anammox constitue un procédé épuratoire qui peut être appliqué à différents types d'effluents ou eaux usées et particulièrement à la fraction liquide des digestats de méthanisation comme il est fait état durant ces travaux.

L'état de l'art ainsi que l'analyse bibliométrique réalisée dans le cadre de ce travail ont mis en évidence l'effort investi et l'intérêt porté au processus anammox dans le monde de la recherche académique et du développement industriel. Dans ce cadre, l'unité de recherche OPAALE d' Irstea - Rennes travaille depuis longtemps d'une part, sur la digestion anaérobie des effluents municipaux ainsi que d'élevages, et d'autre part, sur le traitement et la gestion des nutriments qui en sont issus. Il y a environ 4 ans, l'unité de recherche a décidé d'étudier la faisabilité d'utiliser le procédé anammox pour traiter la fraction liquide des digestats. Le début de ce thème de recherche correspondait aux travaux réalisés au sein ce doctorat qui constituait un nouveau sujet pour le laboratoire et nécessitait de combiner les principales compétences de l'équipe: ingénierie des procédés, simulation expérimentale et microbiologie. Les travaux menés au cours de cette thèse ont permis d'évaluer différents aspects complémentaires du processus anammox: son intérêt stratégique, sa valeur pratique et son intérêt académique.

L'analyse bibliométrique s'est révélée un outil puissant pour étudier les tendances actuelles et passées dans un domaine de recherche spécifique. Au lieu de se concentrer sur le processus anammox, l'étude de ce cas a ciblé la gestion des nutriments et a fourni une vue d'ensemble plus large intégrant les processus impliqués dans des objectifs similaires. Il est fait état à la fois de la production documentaire dans le milieu académique, sous forme de publications scientifiques ainsi que dans le domaine industriel, sous forme de brevets. Un fait bien connu, mais particulièrement impressionnant lorsqu'il est mis en évidence, est l'évolution de la production documentaire en volume qui ne se contente pas de passer d'une thématique à une autre au fil des années mais qui croît de façon exponentielle. Même si cette tendance est étroitement liée au rythme accéléré de la recherche ces dernières années, elle réside également dans l'importance mondiale où se situe aujourd'hui la nécessité de se réduire la propagation et l'impact des pollutions tout en réduisant les coûts de traitement. Dans ce contexte, le processus anammox a progressivement pris de plus en plus d'importance dans la recherche académique ainsi que dans le développement industriel depuis sa découverte. En ce qui concerne le cas particulier de la France, le développement d'anammox à l'échelle industrielle est inexistant et peu de travaux académiques sont rapportés jusqu'à présent. Cette observation contraste fortement avec d'autres pays européens, comme l'Espagne ou

les Pays-Bas, qui ont investi massivement dans le développement d'anammox. Même si des disparités existent dans le monde entier, il existe un intérêt mondial qui se manifeste pour la mise en place de nouvelles installations et une meilleure connaissance du processus. Les voies d'élimination autotrophique de l'azote sont en passe de représenter un véritable changement de paradigme dans le traitement des eaux usées dans les années à venir en apportant des solutions rentables et plus respectueuses de l'environnement.

Comme cette thématique de recherche a été initiée pour la première fois dans notre laboratoire, le développement de notre propre culture de bactérie anammox était nécessaire. Initialement ceci à été entrepris par un enrichissement en batch et constitue l'étape préalable à la mise en place de bioréacteurs à l'échelle de laboratoire, des outils biomoléculaires et de l'expérience tout en produisant des résultats originaux.

La première étape d'enrichissement en bactéries anammox a été réalisé en appliquant une méthodologie dite en batch. Par rapport à l'enrichissement en continu, le mode discontinu, ou en batch, donne l'occasion de tester divers inoculum et conditions opérationnelles. Dans ce sens, la stratégie en batch convient très bien à la recherche dans le sens où de nombreuse variabilités peuvent être testées. De plus, cette approche nécessite moins d'équipement que des réacteurs en continus et n'a pas besoin de surveillance journalière ; cela implique moins de temps investi dans les opérations quotidiennes. Enfin, la méthodologie décrite dans le chapitre 2, plaçant la concentration en nitrite comme facteurs primordial dans le processus précoce d'enrichissement, a conduit à un enrichissement en bactéries anammox très actif allant jusqu'à 222 ± 2 mg $NH4^{+}$-N/L/jour (TNCR: 560 mg N/L/d) après seulement une période de 4 mois. Étonnamment, cette période de temps a été suffisante pour développer des granules anammox millimétriques dans l'un des six inocula (unité de dénitrification I1 - MMBR). Cependant, une disparité très importante est apparu concernant le temps requis pour l'apparition de l'activité anammox et de son taux final après 4 mois d'enrichissement respectivement 0 à 92 jours et de 21 ± 1 à 118 ± 1 mg N/g VS/jour. Cependant une exposition précoce à de trop fortes concentrations en nitrites mène à une impossibilité d'enrichir la bactérie. En ce sens, l'approche en batch permettant de tester plusieurs inocula représentent une sécurité, car des inocula plus adaptés peuvent être secondairement sélectionnés pour poursuivre l'enrichissement en conditions continues. Parallèlement une méthodologie concernant des outils de biologie moléculaire sont venus apporter une vision plus fine à propos des modifications subies par les populations microbiennes et plus spécifiquement anammox en fonction des conditions tout au long de la période d'enrichissement. Il a été montré que même si les différences entre les populations microbiennes des différents inocula tendent à s'estompées au cours de l'enrichissement. Cependant malgré l'hétérogénéité des inocula uniquement les conditions opératoires marquent la faisabilité de l'enrichissement ainsi que le déterminisme du genre anammox sélectionné.

Après cette période de pré-enrichissement, le choix d'un réacteur à flux ascendant de type piston a été effectué en tant que système de culture pour les boues anammox. L'inoculation du

réacteur a été effectuée avec la boue (I1) la plus active provenant de l'enrichissement en batch. L'inoculation a été réalisée après 5 mois de pré-enrichissement. Puisque I1 a développé de petits granules et des NRR significatifs après 3 mois, l'inoculation du réacteur aurait pu être faite plus tôt. Un matériau porteur en polyester non-tissé à été utilisé afin de fournir un support matériel aux biofilm bactérien et ainsi promouvoir la rétention à l'intérieur du système. *Ca. Brocadia sinica* a été sélectionné à partir du début de l'enrichissement en batch et est restée l'espèce anammox dominante dans le bioréacteur. Même si utilisation d'un matériau support s'est avérée être un moyen efficace de retenir la biomasse dans le système, l'évaluation du régime d'écoulement a révélé que le système n'était pas un réacteur de type piston. Ceci est probablement dû à un mélange axial provoqué par la production biologique de gaz (diazote) et des écoulements préférentiels. La charge en azote appliquée dans cette étude était bien en deçà de la capacité maximale du système car la biomasse n'occupait que partiellement la surface totale du support et une grande partie du volume de travail est restée disponible pour la croissance de la biomasse. Cependant cette défaillance à assurer un écoulement en piston est susceptible de réduire l'efficacité du système, empêchant ainsi l'augmentation de la charge en azote (NLR) appliquée. Il a été possible d'obtenir un NLR allant jusqu'à 1577 mg N/L/jour. Une amélioration du régime d'écoulement sous différente configurations de matériaux supports reste nécessaire afin d'apporter moins turbulence et à des conditions d'écoulement plus uniformes.

Ces travaux ont démontré la faisabilité d'obtenir une biomasse d'anammox hautement enrichie étant prête à être utilisée à n'importe quel but après une période possiblement inférieure à 7 mois. Bien que relativement, long ce laps de temps reste relativement court du fait du faible taux de doublement de la bactérie et de l'inconvénient de démarrer à partir d'inocula non enrichis.

Les méthodologies de séquençage de nouvelle génération (NGS) ont démontré leur potentialité pour l'exploration des populations microbiennes, apportant des données précieuses à toutes les étapes de l'étude. Par rapport à d'autres genres anammox, les *Ca. Brocadia* sont rapportées comme étant des microorganismes r-stratège (favorisant le taux de croissance à l'affinité au substrat) ce qui est cohérent vis-à-vis des conditions non limitantes appliquées pendant les deux phases d'enrichissement. Les communautés microbiennes ont été comparées à celles trouvées dans un réacteur anammox similaire. En dépit des variations dans les conditions de culture, en particulier en terme de température (30 ° C au lieu de 35 ° C), les deux communautés microbiennes enrichies dans ces systèmes demeurent étroitement liées. On y retrouve les mêmes groupes bactériens dominants comme *Rhodocyclaceae* (bactéries dénitrifiantes), *Chloroflexi*, et des anaérobies hétérotrophes *Ignavibacteriaceae (Chlorobi).* Tous ces groupes microbiens peuvent participer à l'enrichissement des bactéries anammox en construisant un biofilm protecteur à partir de l'oxygène et de la matière organique.

Les boues d'anammox ainsi obtenues on été par la suite utilisées pour inoculer des réacteurs de nitrification partielle (NP) - anammox séquentiel (SBR) en une et en deux étapes afin de réaliser le

traitement de la fraction liquide d'effluents de digestion anaérobie. L'utilisation d'une biomasse sous forme de biofilm comme inoculum ne représente pas un handicap important car la fragmentation par la turbulence hydraulique a conduit à une granulation rapide de la biomasse anammox pendant les premières semaines. Pendant cette période, la rétention de la biomasse dans le système était efficace et les événements de flottaison mineurs. Le principal avantage de l'utilisation de boues pré-enrichies est sans doute le démarrage à court terme et la haute activité initiale allant de 400 à 500 mg N/L/jour dans les deux premières semaines de fonctionnement. Malgré la bonne réponse initiale des deux configurations et l'augmentation rapide de l'activité biologique des systèmes, le passage d'un effluent dilué (1 :2) à un digestat pur représentait dans ce cas une limite infranchissable quelque soit la configuration considérée. Le prétraitement du digestat à travers l'abaissement de la concentration en phosphates (PO_4^{2-}) s'est avéré insuffisant pour apporter des conditions tolérables pour les systèmes. Cela implique qu'un autre facteur n'a pas été clairement identifié malgré son implication. Le soupçon est placé à la fois sur le fer soluble et les contenus en polymères du digestat. L'analyse du fer total a révélé que la charge en fer par gramme d'azote était semblable à celle appliquée pendant la culture préalable. Cependant, cette valeur peut ne pas refléter la biodisponibilité du fer car il peut être présent en tant que précipité, en particulier à des concentrations élevées en phosphate. De plus, un travail intéressant pourrait être réalisé en ce qui concerne la sensibilité aux différents types de polymères utilisés dans les étapes de déshydratation des boues et comme anti-mousse dans les stations d'épuration. Dans notre cas, l'antimousse (Strucktol SB-2113) et un tensioactif (FLOPAM EM 640 LOB) ont été utilisés respectivement pendant la séparation liquide/solide et la NP. Jusqu'à récemment, la littérature était totalement dépourvue d'informations liées à la sensibilité des bactéries anammox aux tensioactifs. Cela peut s'expliquer par la variété des molécules trouvées sur le marché. Il a été très récemment démontré que les polymères anioniques provoque aucun dommage à l'intégrité cellulaire, mais bloquent les enzymes cataboliques à des concentrations élevées, démontrant que davantage d'informations sur la toxicité des surfactants revêt une importance cruciale afin d'augmenter la stabilité et les performances des installations de traitement anammox.

L'inhibition de la biomasse est toujours représentée par la somme des effets inhibiteurs et des carences. L'utilisation de la précipitation P a permis de comparer l'activité spécifique de l'anammox dans des conditions continues à des concentrations faibles et élevées (10-30 contre 300 mg P/L). Le passage d'une concentration faible à une concentration élevée a entraîné une diminution très significative de l'activité anammox d'environ 48,9% et 55,7%, respectivement dans les procédés en une et en deux étapes. D'autres études ont démontré que des biomasses anammox sont capable d'opérer à des concentrations en phosphate allant jusqu'à 500 mg de P/L sans pour autant perdre en activité. Cependant, ils rapportent d'importants changements structurels au niveau de l'organisation du granule. Ceci donnerait d'avantage d'importance à des phénomènes d'acclimatation progressive de la biomasse anammox démontrant leur capacité à s'adapter à des changements de conditions. En ce sens, la haute sensibilité au phosphate dans notre système pourrait provenir du caractère

immature de la biomasse utilisée et du manque de temps accordé aux bactéries anammox pour s'adapter au conditions plus défavorables que représente le traitement des effluent de digestion anaérobie . Une approche approfondie de l'exposition, semblable à celle appliquée à l'alimentation en nitrite décrite au chapitre 3, pourrait fournir des informations plus approfondies concernant la sensibilité relative lors du démarrage des systèmes et pourrait être étendue aux problèmes de toxicité du phosphate comme ceux des tensioactifs. En outre, la comparaison entre une exposition de courte durée et un test continu a révélé de grandes disparités en termes d'effets inhibiteur. La valeur d'IC_{50} déterminée à partir du test d'activité en batch est de 713,9 mg P-PO_4^{2-}/L et est semblable à d'autres valeurs rapportées dans la littérature. Cette variation par rapport à l'évaluation en conditions continues illustre le fait que l'exposition à court terme et les comportements de toxicité aigu peuvent différer fortement des réactions de la biomasse à long terme. Dans notre cas, une nette sous-estimation du potentiel d'inhibiteur du phosphate a été observée. Cependant, dans d'autres cas, une surestimation pourrait être faite, car la courte période de temps considérée, peut, ne pas permettre à la biomasse de réagir et de s'adapter face à de nouvelles conditions. Si l'évaluation en batch fournit un moyen facile de mesurer de larges échelles de concentrations ou de conditions, elles tendent à décrire aléatoirement les réactions biologiques sur le long terme ; restant en définitive la finalité recherchée et l'information la plus précieuse.

Summary

This work investigates the feasibility of fast implementation of the biological anaerobic ammonium oxidation (anammox) process for the treatment of anaerobic digester supernatants processing municipal sewage sludge. The framework is divided into three main parts (i) enrichment of anammox biomass in batch mode from various inoculums, (ii) implementation of a continuous pack-bed reactor for further enrichment of anammox bacteria, (iii) start-up of laboratory scale reactors for the treatment of biogas digester supernatants.

This manuscript begins with a description of the current state of art regarding the autotrophic nitrogen removal (ANR) based on the anammox process. It is focused on physiological and engineering aspects of the anammox process to better understand both possibilities and limitations for the implementation of anammox treatment reactors. Then, a bibliometric analysis provides an overview on both academic research and industrial patents related to the field of nutrients management from anaerobic digester supernatants. It turned into a useful tool to summarize current trends, and to contextualize application of anammox. An exponential growth in documentary productivity was confirmed during the last 20 years. In this context, the anammox process has progressively been gaining importance since its discovery.

As the anammox topic was initiated for the first time in our laboratory, obtaining own anammox inoculum through enrichment was chosen as the first step of the thesis. Such approach would help to develop new laboratory-scale implementation, biomolecular tools and experience on the process while producing original results.

First step targeting anammox enrichment has been achieved by applying a batch methodology. Batch mode gave the opportunity to test several inoculum seeding sludge and operational conditions at the same time. Finally, the methodology proposed led to highly active anammox enrichment able to anaerobically oxidize up to 222 ±2 mg NH_4^+-N/L/day after a 4 months period. This time span was long enough to result in the development of tiny red granules. After the enrichment period, a continuous packed-bed up-flow reactor was chosen as breeding system for culturing anammox biomass. The use of a non-woven polyester as carrier material resulted in an efficient strategy to retain the biomass within the reactor. Under these conditions, a targeted nitrogen loading rate (NLR) of 1551 mg N/L/d was applied resulting in conversion rates of 1183 ±100 mg N/L/d. The upflow reactor run in the laboratory for longer than 400 days providing excess sludge for further experimental research in dedicated ANR systems. Microbial analysis using next-generation sequencing revealed that both batch and continuous stages conducted to the enrichment of the anammox species *Ca. Brocadia sinica.* The anammox sludge produced in the packed-bed upflow reactor was subsequently used to start two different ANR implementations considering single- and two-stage configurations and using the sequencing batch reactor (SBR) technology. Performance of both ANR systems was assessed according to the NLR, supernatant dilution, and phosphate concentration in terms of N-conversion efficiency (NCE) and nitrous oxide (N_2O) emission.

Keywords: autotrophic nitrogen removal; anammox; enrichment; cultivation; SBR; phosphorous; NGS; q-PCR; nitrite