Universidad de Valladolid

PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA Y AMBIENTAL

TESIS DOCTORAL:

NOVEL BIOTECHNOLOGIES FOR NITROUS OXIDE ABATEMENT

Presentada por **Osvaldo David Frutos González** para optar al grado de Doctor por la Universidad de Valladolid

Dirigida por:

Raúl Muñoz Torre

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Memoria para optar al grado de Doctor

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Siendo tutores en la Universidad de Valladolid:

Raúl Muñoz Torre

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Certifican que:

OSVALDO DAVID FRUTOS GONZÁLEZ ha realizado bajo su dirección el trabajo "*Novel biotechnologies for nitrous oxide abatement*", en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente en la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, _____de _____2018

Fdo. Raúl Muñoz Torre

Fdo. Guillermo Quijano

Universidad de Valladolid

Reunido el tribunal que ha juzgado la Tesis Doctoral Titulada

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PRESIDENTE SECRETARIO VOCAL

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Resumen

La temperatura del planeta ha ido aumentando a una tasa sin precedentes a partir de la segunda mitad del siglo XIX, resultando en la aceleración de los efectos del cambio climático. A día de hoy, existen evidencias irrefutables que demuestran que el ser humano es el principal responsable del atípico aumento de la temperatura del planeta. En efecto, se ha encontrado una clara correlación entre el aumento de la concentración atmosférica de gases de efecto invernadero (GEI), los cuales tienen la capacidad de retener la radiación infrarroja emitida por la Tierra, y el aumento de la temperatura global del planeta. Los GEIs más importantes emitidos por el hombre son el dióxido de carbono (CO_2), el metano (CH_4), el óxido nitroso (N_2O) y los clorofluorocarbonos (CFCs). El N_2O representa cerca del 6,2 % del total de GEIs emitidos globalmente (en un horizonte temporal de 100 años), siendo la agricultura, la industria química y las estaciones depuradoras de aguas residuales (EDARs) las principales fuentes de emisiones de N_2O. Además, algunos autores han afirmado que el N_2O es el más importante destructor de ozono emitido en el presente siglo.

Tecnologías físico-químicas son utilizadas en la actualidad para el control de las emisiones de N_2O producidas por la industria. Sin embargo, estas requieren la utilización de agentes reductores, combustible fósil y catalizadores de alto costo, lo que resulta en altos costos de operación, elevado consumo energético e impactos ambientales secundarios. En este contexto, las tecnologías biológicas han surgido como una prometedora alternativa a los sistemas físico-químicos teniendo en cuenta su alta eficiencia y robustez para el tratamiento de una amplia gama de contaminantes atmosféricos a temperatura y presión ambiental. Además, las biotecnologías están caracterizadas por su rentabilidad, baja generación de impactos ambientales, ausencia de químicos necesarios para su operación y la generación de subproductos inocuos para el ambiente.

En la presente tesis, se llevó a cabo la evaluación sistemática del potencial de varias configuraciones de biorreactores con diferentes estrategias de operación enfocadas al tratamiento de N_2O originados en la industria química y EDARs.

En primer lugar, el tratamiento de emisiones de N_2O de EDARs fue evaluado en un biolavador compuesto de una columna de absorción empacada conectada a un tanque de agitación (STR, por sus siglas en inglés). El biolavador fue operado a distintas velocidades de recirculación del líquido manteniendo un tiempo de residencia del gas en el lecho vacío (TRLV) de 3 min siendo metanol el donador de electrones para la reducción de N_2O y para la mantención de condiciones anóxicas en el STR. La desnitrificación heterotrófica fue identificada como el principal mecanismo responsable de la remoción del N_2O absorbido en la columna empacada. La eficiencia de remoción (ER) de N_2O en este biolavador operado con metanol llegó a valores del 40 ± 1 %, bajo una velocidad de recirculación del líquido de 8 m h^{-1}. Posteriormente, un biolavador de similares características fue diseñado y operado para el tratamiento de emisiones de aire cargadas de N_2O utilizando agua residual domestica como fuente de donadores de electrones (para la reducción del N_2O y para mantener condiciones anóxicas en el STR) con el fin de reducir los costos operacionales asociados a la eliminación de N_2O con metanol. Dicho proceso logró una alta remoción de la materia orgánica (>90 %) del agua residual con una ER de N_2O de hasta 36 ± 3 % bajo un TRLV de 3 min. De igual manera, el aumento secuencial del TRLV desde 3 min hasta los 40 min resultó en un mejoramiento de la ER de N_2O de hasta el 92 %.

El crecimiento excesivo de microorganismos en la columna empacada limitó el potencial del biolavador como una tecnología alternativa para el control de N_2O en EDARs. En este sentido, la evaluación de la eliminación de N_2O originados en las EDARs fue realizada bajo condiciones aerobias y microaerobias en un sistema de difusión de lodos activos bajo concentraciones de oxígeno disuelto (OD) de 4,2 ± 0,5; 2,8 ± 0,3; 2,1 ± 0,1; y 1,1 ± 0,01 mg L^{-1}. Sin embargo, los resultados mostraron que solo una pequeña remoción (~5

%) fue obtenida bajo una concentración de OD de 2,1 ± 0,1 mg L^{-1}, mientras que en las demás condiciones estudiadas se detectó producción de N_2O.

La alta concentración de oxígeno presente en las emisiones típicas de las EDARs constituye una de las mayores limitaciones para cualquier sistema biológico de tratamiento de N_2O basado en la desnitrificación heterotrófica, debido a que el mismo debe ser eliminado antes de que el N_2O pueda ser reducido efectivamente. Por lo tanto, procesos biológicos aerobios alternativos para la eliminación de N_2O bajo condiciones de alto contenido de oxígeno son necesarios. En este contexto, la nitrificación de N_2O a nitrito y nitrato representa un mecanismo poco explorado pero termodinámicamente viable. Por tanto, dos experimentos fueron realizados de modo a explorar la viabilidad empírica de este mecanismo biológico. Primero, un biofiltro percolador inoculado con lodo activado fue operado por 3 meses con el continuo suministro de N_2O en aire, carbonatos y nutrientes necesarios para promover el crecimiento de una biopelícula nitrificante de N_2O. En segundo lugar, ensayos en lote fueron realizados por un periodo de 11 meses utilizando tres cultivos microbianos (lodos activos, bacterias nitrificantes y un cultivo anammox) bajo una atmosfera de 11400 ± 160 ppm_v de N_2O. Desafortunadamente, ninguno de los sistemas evaluados demostró evidencias significativas que puedan sostener la hipótesis de nitrificación de N_2O.

Ningún sistema biológico ha sido previamente evaluado para el tratamiento de emisiones de N_2O originados en la industria química a pesar de las características favorables que estas presentan en comparación a aquellas encontradas en las EDARs (bajo nivel de O_2 y alta concentración de N_2O). En este sentido, el potencial de unos biorreactores de columna de burbujeo (CB) y airlift (AL) fue evaluado para el tratamiento de N_2O de las emisiones de plantas de producción de ácido nítrico acoplado a la producción simultanea de poli(3-hidroxibutirato-co-3-hidroxivalerato) (PHBV), mediado por dos estrategias de limitación de nitrógeno utilizando metanol como fuente de carbono y donador de electrones. Un alto contenido celular de PHBV (38-64 %) fue

obtenido junto con eliminaciones de N_2O de ≈57 y ≈84 % en el AL y la CB respectivamente. Finalmente, la influencia de diferentes fuentes de carbono/electrones (glicerol, metanol y acetato-ácido acético) en la eliminación de N_2O acoplada a producción de PHBV y coenzima Q10 fue estudiada en la CB. Altas ERs de N_2O fueron obtenidas (81-91 %) en el biorreactor independientemente de las diferentes fuentes de carbono evaluadas. El contenido celular de PHBV se mantuvo en el rango de 23-53 % con un significativo aumento del contenido del homopolimero 3-hidroxivalerato (PHV) (23 %) en la composición del PHBV cuando glicerol fue utilizado como fuente de carbono y electrones. Así también, el contenido celular de la coenzima Q10 se mantuvo en el rango de 0,4 y 1 mg g^{-1}, siendo el acetato-ácido acético y el metanol los que indujeron el mayor contenido celular de la coenzima.

La baja transferencia de masa gas-líquido del N_2O (como resultado de la alta constante adimensional de Henry de este gas) fue sistemáticamente observada como una limitación fundamental de los procesos a lo largo de esta tesis, lo cual implica una necesidad de utilizar biorreactores de gran volumen (con el consecuente aumento de costos de inversión). En este sentido, se realizaron ensayos en lotes para evaluar la influencia de la adición de aceite de silicona al 10 y 30 % (utilizado como un vector de transferencia de masa) en la eliminación de N_2O por desnitrificación heterotrófica. El uso de una fase orgánica como el aceite de silicona en los llamados reactores bifásicos fomenta el mejoramiento de la transferencia de masa gas-líquido de contaminantes poco solubles en agua. Sin embargo, los resultados obtenidos en los ensayos en lotes demuestran que el aceite de silicona no ejerce ningún tipo de efecto beneficioso en la remoción del N_2O bajo las condiciones estudiadas.

Abstract

The global temperature of the planet has increased at unprecedentedly high rates since the second half of 19th century, which is mediating also a rapid climate change. Today, there is consistent scientific evidences to demonstrate that human activities are the main responsible of this atypical temperature increase. Indeed, a clear correlation has been found between the increase in the atmospheric concentration of greenhouse gases (GHGs), which have the capacity to retain the infrared radiation emitted by the Earth, and the increase in the global temperature of the planet. Carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and chlorofluorocarbons (CFCs) are the most important GHGs emitted by human activities. N_2O represents approximately 6.2 % of total GHGs emissions globally (on a 100 years horizon), with agriculture, chemical industry and wastewater treatment plants (WWTP) identified as the most important emissions sources of N_2O. In addition, some authors have recently claimed that N_2O is the most important ozone depleting substance emitted in this 21th century.

Nowadays, physical-chemical technologies are used for N_2O emission control in industry. However, the operation of these technologies requires the use of reducing agents, fuels and expensive catalysts, resulting in high operating costs, high-energy consumptions and environmental impacts. In this context, biological technologies have emerged as a promising alternative to physical-chemical technologies based on their high robustness and efficiency for the treatment of a wide range of atmospheric pollutants. Biotechnologies are characterized by their cost-efficiency and low environmental impacts as a result of their low energy consumption, absence of chemical requirements and innocuous final degradation products.

In the present thesis, a systematic evaluation of the potential of multiple innovative bioreactor configurations and operational strategies devoted to the treatment of N_2O emissions from chemical industry and WWTPs was conducted.

Firstly, the treatment of WWTP N_2O emissions was evaluated in a bioscrubber composed of a packed bed absorption column interconnected to a stirred tank bioreactor (STR). The bioscrubber was operated at increasing liquid recycling velocities and a gas empty bed residence time (EBRT) of 3 min using methanol as the electron donor for N_2O reduction and for the maintenance of anoxic conditions in the STR. Heterotrophic denitrification in the STR was identified as the main mechanism responsible of the removal of the N_2O absorbed in the packed column. The removal efficiencies (REs) of N_2O in this methanol supplemented bioscrubber reached up to 40 ± 1 % at a liquid recycling velocity of 8 m h^{-1}. A similar bioscrubber was designed and operated for the treatment of N_2O-laden air emissions using domestic wastewater as the electron donor source (for N_2O reduction and maintenance of anoxic conditions in the STR) to decrease the operating costs associated to N_2O abatement with methanol. The process supported a high organic matter removal (>90 %) concomitantly with N_2O REs of 36 ± 3 % at an EBRT of 3 min. In addition, the sequential increase of the EBRT from 3 min to 40 min supported an enhancement in the N_2O REs up to 92 %.

Biomass overgrowth in the packed bed limited the potential of bioscrubbers as a platform technology for N_2O control in WWTPs. In this context, the microaerobic and aerobic removal of N_2O from WWTPs emissions was assessed in an activated sludge diffusion system (ASD) under dissolved oxygen (DO) concentrations of 4.2 ± 0.5, 2.8 ± 0.3, 2.1 ± 0.1 and 1.1 ± 0.01 mg L^{-1}. Unfortunately, the results showed a slight (~5 %) N_2O removal only at a DO of 2.1 ± 0.1 mg L^{-1}, whereas N_2O production was observed under all operational conditions in the carbon removal-nitrification activated sludge system evaluated.

The high oxygen concentrations typically present in WWTPs N_2O emissions constitute the largest limitation of any N_2O abatement biotechnology based on heterotrophic denitrification, since massive amounts of O_2 need to be depleted in the anoxic tank before N_2O can be effectively reduced. Thus, alternative aerobic biological processes for

N_2O biodegradation are required. In this context, the nitrification of N_2O to nitrite or nitrate represents a thermodynamically feasible but poorly explored mechanism. Two independent experiments were conducted to evaluate the empirical feasibility of this biological mechanism. Firstly, a biotrickling filter inoculated with activated sludge was operated for 3 months with a continuous supply of N_2O-laden air, alkalinity and nutrients to promote the enrichment of a N_2O nitrifying biofilm. Secondly, batch assays were conducted for 11 months using three different microbial cultures (activated sludge, nitrifying bacteria enrichment and an anammox culture) under an 11400 ± 160 ppm_v N_2O headspace atmosphere. Unfortunately, none of the systems evaluated showed significant evidence to support the initial hypothesis of a potential N_2O nitrification.

No biological technology has ever been evaluated for the treatment of industrial N_2O emissions in spite of their favorable characteristics compared to WWTPs N_2O emissions (low O_2 and high N_2O levels). Thus, the potential of a bubble column (BCR) and an internal loop airlift (ALR) lab-scale bioreactors was evaluated for the abatement of N_2O from a nitric acid plant emission coupled with the simultaneous production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) under two nitrogen-limiting strategies using methanol as the sole carbon and electron donor source. A high PHBV cell content of 38-64 % was recorded together with N_2O REs of ≈57 and ≈84 % in the ALR and BCR, respectively. Finally, the influence of the carbon/electron donor (glycerol, methanol and acetate-acetic acid) on the abatement of N_2O coupled to the production of PHBV and coenzyme Q10 was evaluated in BCRs. Similar N_2O REs (81–91%) were observed in the BCRs regardless of the carbon/electron donor sources. The PHBV cell content ranged from 25 to 53 %, with a significant increase of the molar ratio of the homopolymer 3-hydroxyvalerate (23%) in the composition of PHBV when glycerol was used as a carbon/electron donor. On the other hand, the Q10 coenzyme cell content ranged from 0.4 to 1 mg g^{-1}, acetate-acetic acid and methanol induced highest Q10 cell contents.

The poor N_2O gas-liquid mass transfer (as a result of the high Henry`s law constant of N_2O) was consistently identified in this thesis as a key limitation entailing the need for high bioreactor volumes (and therefore high investment costs). In this context, batch assays were also conducted to investigate the influence of the addition of silicone oil (used here as N_2O mass transfer vector) at 10 and 30 % on the removal of N_2O based on heterotrophic denitrification with methanol. The use of organic phases such as silicone oil in the so-called two-phase partitioning bioreactors (TPPBs) has been previously shown to support an enhancement in the gas-liquid mass transfer of scarcely water-soluble gas pollutant. The results here obtained showed that silicone oil did not exert any significant enhancement in N_2O removal under the conditions tested.

List of publications

The following publications are presented as part of the current thesis. Four of them have been published in international journals indexed in the ISI Web of Knowledge (Articles I, II, V and VI). Articles III, IV and VII are unpublished manuscripts.

Article I. O.D. Frutos, I.A. Arvelo, R. Pérez, G. Quijano, R. Muñoz, *Continuous nitrous oxide abatement in a novel denitrifying off-gas bioscrubber*, Appl. Microbiol. Biotechnol. 99 (2015). doi:10.1007/s00253-014-6329-8.

Article II. O.D. Frutos, G. Quijano, R. Pérez, R. Muñoz, *Simultaneous biological nitrous oxide abatement and wastewater treatment in a denitrifying off-gas bioscrubber*, Chem. Eng. J. 288 (2016) 28–37. doi:10.1016/j.cej.2015.11.088.

Article III. O.D. Frutos, R. Lebrero, R. Muñoz, *N_2O nitrification feasibility studies*, unpublished manuscript.

Article IV. O.D. Frutos, D. Marin, R. Muñoz, *Influence of the dissolved oxygen concentration on the abatement and generation of N_2O in activated sludge diffusion systems*, unpublished manuscript.

Article V. O.D. Frutos, I. Cortes, S. Cantera, E. Arnaiz, R. Lebrero, R. Muñoz, *Nitrous oxide abatement coupled with biopolymer production as a model GHG biorefinery for cost-effective climate change mitigation*, Environ. Sci. Technol. 51 (2017). doi:10.1021/acs.est.7b00643.

Article VI. O.D. Frutos, G. Barriguín, R. Lebrero, R. Muñoz, *Assessing the influence of the carbon source on the abatement of industrial N_2O emissions coupled with the synthesis of added-value bioproducts*, Sci. Total Environ. 598 (2017) 765–771. doi:10.1016/j.scitotenv.2017.04.161.

Article VII. O.D. Frutos, O. Sanz, R. Muñoz, *Enhancement of N_2O mass transfer in two-liquid phase systems*, unpublished manuscript.

Contribution to the manuscripts included in the thesis

Article I. In this work, I was responsible for the design, start-up and operation of the experimental set-up, under the supervision of Dr. Raúl Muñoz. Ilan Arvelo collaborated in the monitoring of the bioreactor and batch assays. I evaluated the experimental results and prepared the manuscript under the supervision of Dr. Guillermo Quijano and Dr. Raúl Muñoz. Dr. Rebeca Pérez was responsible of the molecular biology analysis, where I contributed in the discussion section.

Article II. During the execution of this work, I was responsible of the design, start-up and operation of the experimental set-up, results evaluation and manuscript preparation with the collaboration of Dr. Guillermo Quijano and under the supervision of Dr. Raúl Muñoz. Dr. Rebeca Pérez was responsible of the molecular biology analysis, where I contributed in the results analysis and discussion.

Article III. In this research, I was in charge of the design, start-up, operation of the experimental set-up and results evaluation under the supervision of Dr. Raquel Lebrero and Dr. Raúl Muñoz. I prepared the manuscript under the supervision of Dr. Raúl Muñoz.

Article IV. During this research, I was in charge of the design, start-up and operation of the experimental set-up with the collaboration of David Marin. I was the responsible of results analysis and manuscript preparation under the supervision of Dr. Raúl Muñoz.

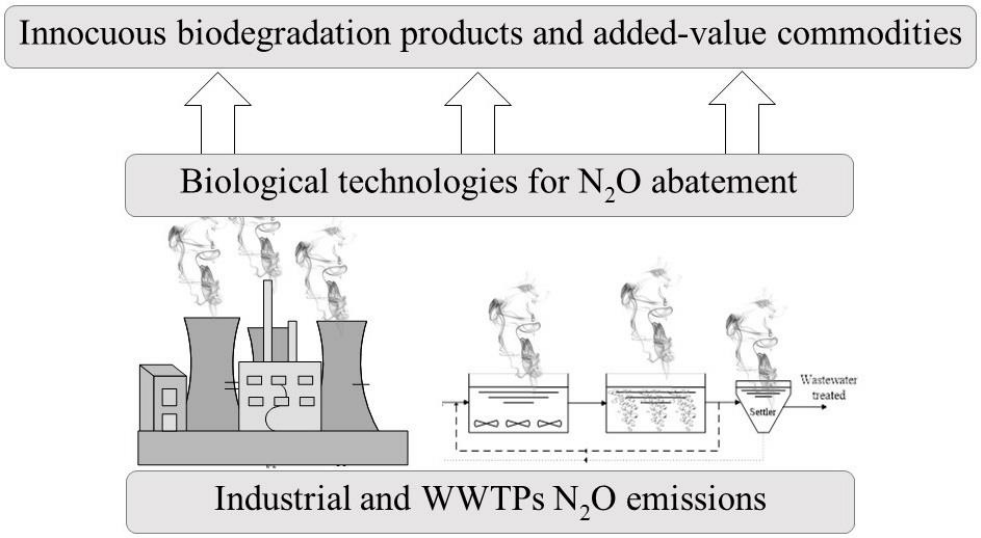
Article V. During the execution of this work, I was responsible of the design, start-up and operation of the experimental set-up, results evaluation and manuscript writing with the collaboration of Dr. Raquel Lebrero under the supervision of Dr. Raúl Muñoz. Irene Cortes performed part of the bioreactor monitoring. Esther Arnaiz collaborated on the

development of the analytical method for biopolymers analysis. Sara Cantera performed the microbiological analysis and associated results evaluation, where I contributed in the discussion.

Article VI. In this work, I was responsible of the design, start-up and operation of the experimental set-up, results evaluation and manuscript preparation with the collaboration of Dr. Raquel Lebrero under the supervision of Dr. Raúl Muñoz. Gonzalo Barriguín performed part of the bioreactor monitoring.

Article VII. During this research, I was in charge of the design of the experiment. Oscar Sanz performed the experiments under my co-supervision. I was the responsible of results analysis and manuscript preparation under the supervision of Dr. Raúl Muñoz.

Introduction



1. Overview of Climate Change

The consistent measurement of multiple atypical variations in the pattern of climate system and the apocalyptic predictions of its associated effects have ranked climate change as the top environmental problem faced by our planet nowadays [1]. Climate change is defined as the sum of long-term significant variations observed in the measurements of climate parameters. In this context, the Fifth Assessment Synthesis Report from the Intergovernmental Panel on Climate Change (IPCC) published in 2014 revealed the most recent evidences in climate change [1]. For instance, the Arctic sea-ice surface in September 2012 accounted for only 56 % of the minimum sea ice area in the period of 1981 to 2010 for that specific month [2]. Similarly, the Antarctic and Greenland ice layers have melted during the past decades at approximately twice the rate recorded in previous decades, which has induced a sea level increase of 1.28 mm $year^{-1}$ [3]. At this point, it should be stressed that the Antarctic and Greenland are the largest fresh water reservoirs on Earth, representing about 99 % of the global freshwater ice on Earth with an approximate ice volume of 32×10^6 km^3. The melting of the Antarctic and Greenland ice would represent a sea level rise of ≈ 66 meters [4]. However, one of the most consistent evidences of climate change is the average atmospheric and ocean temperature increase. Thus, the global temperature evolution observed since 1850 presented completely unusual patterns, which suggests that not only natural phenomena are altering the mean temperature of Earth (Figure 1.1). In this sense, the average temperature increase of 0.85 ºC in the period 1880-2012 represented an unprecedented increase rate compared to those encountered in the past millennia and pointed out towards anthropogenic activities as the main responsible [1,5]. Moreover, the National Aeronautics and Space Administration (NASA) of United States of America recently confirmed that 2016 was the warmest year ever recorded and the third consecutive year to set a new record of average temperature of the planet [6].

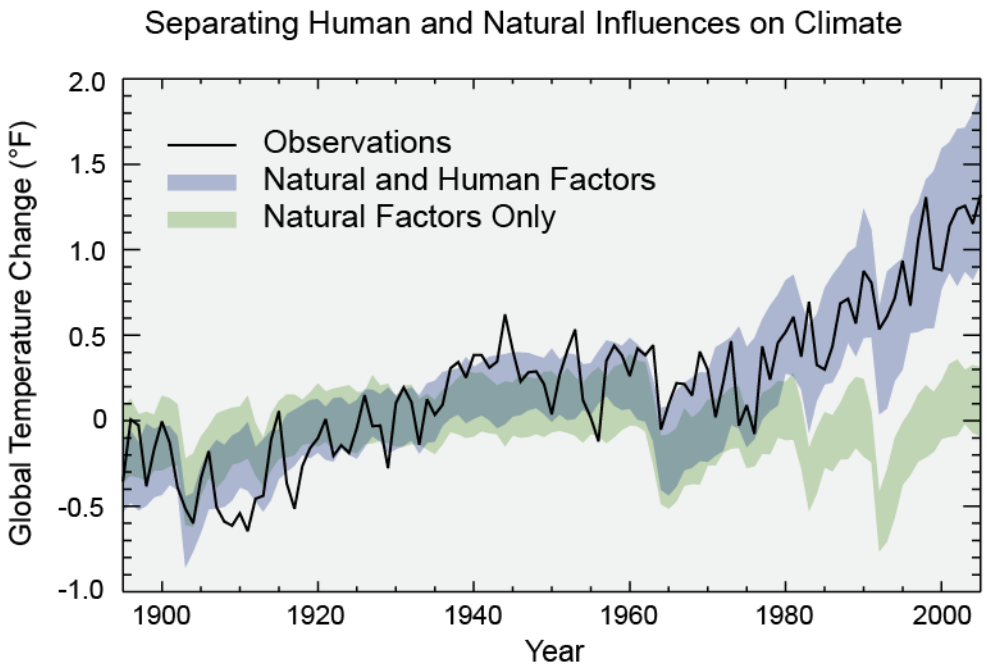


Figure 1. Time course of the global temperature changes. Source: US National Climate Assessment [7]

The Paris agreement, as a convention of the United Nations Framework Conference on Climate Change (UNFCCC) held on December 2015, targeted a maximum Earth temperature increase of 2 ºC above the levels recorded prior industrialization and further agreed to attempt to limit this increase to 1.5 ºC [8]. Schleussner and co-workers (2016) have forecasted that the rise of the global temperature to either 1.5 and 2 ºC will result in significantly different scenarios of extreme weather events, water availability, agricultural yields, sea level rise and risk of coral reef loss [9]. The same authors indicated that the Mediterranean water availability will be reduced by 9 and 17 %, and the dry spells will increase by 7 and 11 % under a global temperature increase of 1.5 and 2 ºC, respectively. Likewise, the sea level will rise by 35 or 50 cm under a mean temperature rise scenario of 1.5 and 2 ºC, respectively. Finally, agriculture in temperate regions will benefit from this increase in temperature, which on the other hand will jeopardize crop productivity in tropical regions.

1.1. Greenhouse Effect

The increase in the average temperature of the planet is nowadays attributed to the continuous increase in the concentration of the greenhouse gases (GHGs) mediated by anthropogenic activities. GHGs exhibit the capacity to retain the heat emitted from Earth as infrared radiation, which itself derived from solar radiation absorption by the Earth surface. This so-called greenhouse effect maintains an average temperate suitable for most species in the planet. In fact, the temperature of the planet would drop to -18 ºC during the night in the absence of GHGs in the atmosphere. The major GHGs emitted by anthropogenic activities nowadays are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and chlorofluorocarbons (CFCs). Their unique atmospheric lifetime and capacity to absorb infrared radiation entail a gas-specific global warming potential (GWP), which represents the estimated contribution of each GHG to global warming in a specific timeframe. Thus, CO_2 is used as a reference gas and assigned a GWP of 1 in 100 year scenario. CH_4 and N_2O have GWPs of 28 and 310, respectively, due to their higher capacity to absorb infrared radiation and persistence in the atmosphere [1]. In addition, N_2O constitutes the greatest O_3 depleting substance emitted in this 21th century [10].

Despite the major GHGs are mainly emitted by natural sources, the emissions from anthropogenic activities represent ~ 40 % of the global GHG inventory. Nowadays, there are sufficient scientific evidences to support that human activities after the industrial revolution have caused the increase in GHG atmospheric concentration responsible of the rise in the global temperature of the planet. As matter of fact, the IPCC have recently confirmed with a 95 % confidence that human activities are responsible of the observed global warming after the mid-20th century [11]. Measurements of the air trapped in the ice core of the Antarctica in the past showed a direct correlation between the changes in the average temperature of the planet and the atmospheric concentration of CO_2 over the time, the highest CO_2

concentration periods corresponding to the warmest periods (Figure 1.2). In the last century, the concentration of CO_2 has increased to almost 400 ppm_v, while CH_4 and N_2O levels have risen from 255 and 265 ppm_v to ~2000 and 320 ppm_v, respectively (Figure 1.2).

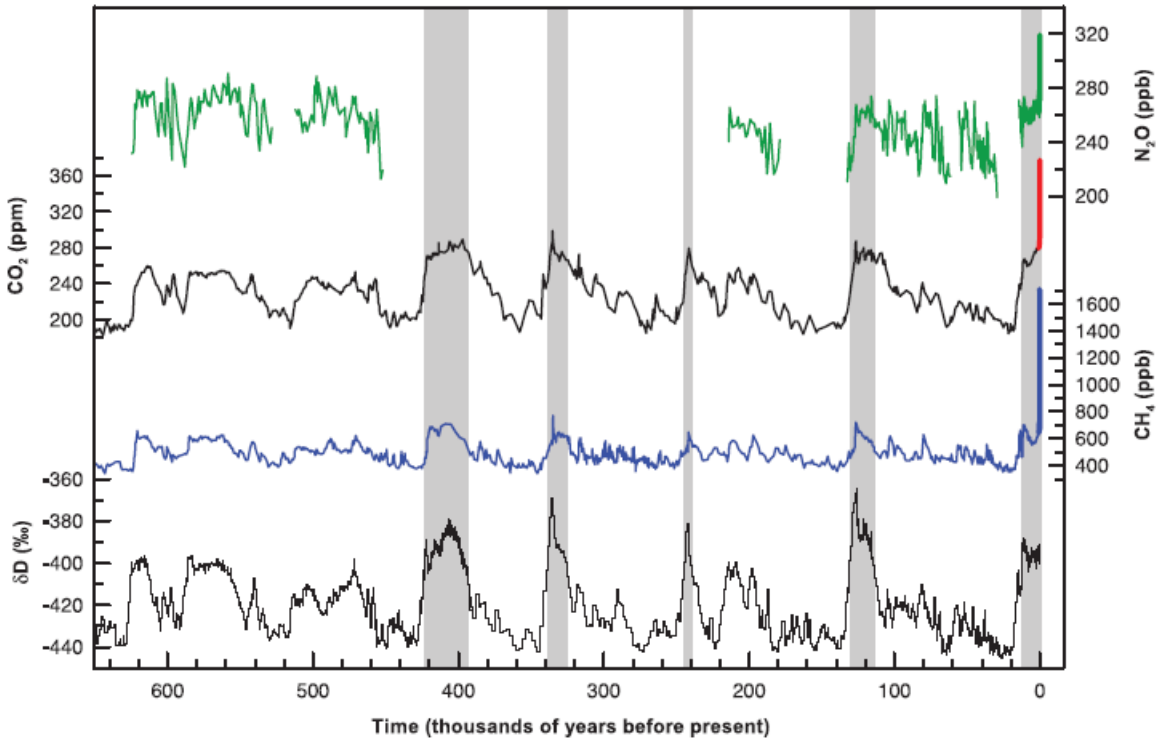


Figure 2. Atmospheric concentration of CO_2, CH_4 and N_2O trapped in ice core and in the past century. Grey zones indicate interglacial warm periods. Variations of deuterium (δD) in the Antarctic ice indicate an approximation of local temperature. Source: IPCC [12].

1.2. Greenhouse Gases Emission Sources

Anthropogenic GHG emissions have increased by 80 % since 1970. The increase in CO_2, CH_4 and N_2O levels during this period accounted for 90, 47 and 43 %, respectively [13]. By the year 2010, the contribution of these three GHGs to global warming represented 75, 16 and 6.2 %, respectively. The IPCC identified in the Fifth Assessment Report six key economic sectors responsible for GHGs emissions: Energy Systems; Transport; Buildings; Industry; Agriculture, Forestry and Other Land Use (AFOLU) and Waste [13]. The largest contributor to the total GHG emissions by 2010 was the Energy sector, with a share of approximately of 35

% and yearly emissions of 17 $GtCO_2eq$ [13]. CO_2 production from the combustion of fossil fuels represented the major source of GHG emissions [14]. Indeed, ~374 billion metric tonnes of carbon have been burned and released as CO_2 to the environment during fossil fuel combustion since 1745 [15]. CH_4 is also emitted during coal and gas extraction and transportation, while N_2O can be indirectly emitted from the combustion of coal and fuel-wood [14].

AFOLU represents the second major GHG emission source (12 $GtCO_2eq$), accounted for 20 to 24 % of total emissions at a global scale [13]. CO_2, CH_4 and N_2O in AFOLU are emitted from plant respiration, decomposition of biomass and soil organic matter, and combustion of dry crops. Interestingly, this sector constitutes a source and a sink of CO_2 simultaneously (deforestation *vs* afforestation). CO_2 generation by agriculture may be considered neutral as a result of the concomitant carbon fixation and oxidation during photosynthesis and respiration, respectively. On the other hand, CH_4 and N_2O are generated during the biological transformation of organic matter and nutrients involved in AFOLU. Hence, CH_4 is produced from enteric fermentation and anaerobic degradation of animal waste and organic matter in soils [16]. Finally, soil fertilization in agriculture constitutes the largest anthropogenic source of N_2O, which explains why many scientists have linked the atmospheric N_2O concentration increase to the need for food production [17]. N_2O generation occurs via denitrification when the nitrate present in soils gets reduced by microbial activity, the rate of N_2O generation increase during nitrogen fertilization [18]. The Environmental Protection Agency of United States (US-EPA) (2011) reported that agriculture represented 56 % of the total non-CO_2 GHG emitted worldwide by the year 2005 [19].

The industrial sector constitutes the third most important source of anthropogenic GHG emissions (accounting for 20 % of the GHG inventory), with emissions over 8.8 $GtCO_2eq$ by 2010 [13]. The GHGs emitted in this sector are related to the direct CO_2 emissions from

energy generation, indirect CO_2 emissions from electricity and heat production, non-CO_2 GHG, and direct emissions from waste/wastewater processes [20]. The largest GHG contributor in this sector is CO_2, with a share of 85 % followed by CH_4 (8.6 %), hydrofluorocarbons (3.5 %) and N_2O (2 %). Hence, N_2O is the third most important non-CO_2 GHG, nitric acid (HNO_3) and adipic acid ($C_6H_{10}O_4$) production being the major contributor to N_2O emissions in the industrial sector [19].

The transport sector, with yearly emissions of 7 $GtCO_2eq$, represents ~ 14.2 % of the global GHG emissions [13]. The emissions recorded in this sector have doubled since 1970, road vehicles being the major contributors to the emissions of CO_2 with 72 % of total transport emissions by 2010 [21]. The emissions from the building sector by 2010 reached 3.2 $GtCO_2eq$, which constitutes ~ 6.5 % of total GHG emitted globally [13]. Finally, the waste treatment sector, involving solid waste management and wastewater treatment processes, are an important source of CH_4 and N_2O emissions. This sector yearly contributes with 1446 $MtCO_2eq$ and represents ~ 3 % of the global GHG emission inventory [13]. CH_4, CO_2 and N_2O are mainly emitted from the disposal of solid waste and wastewater treatment via denitrification-nitrification, which account for 43 and 54 % of the GHG emission in this sector, respectively.

2. N_2O Emissions Sources

Anthropogenic emissions of N_2O represent approximately 40 % of the global emissions of this GHG and O_3 depleting substance. AFOLU, industry and waste treatment sectors rank among the top 3 anthropogenic N_2O sources. Indeed, soil fertilization in agriculture (AFOLU sector) constitutes, by far, the largest source of N_2O as a result of the microbial nitrogen transformations within the soil. Furthermore, chemical industries such as HNO_3 and $C_6H_{10}O_4$ production processes account for the largest share of N_2O emission within the industrial sector. On the other hand, wastewater treatment is the key player in N_2O generation within

the waste treatment sector. In this context, the implementation of abatement technologies to mitigate the emissions from diffusive sources such as agriculture is not technically feasible. Therefore, the studies conducted in this PhD Thesis were focused on the development of end-of-the-pipe biotechnologies targeting N_2O laden emissions from wastewater treatments processes and chemical industries.

A detailed description of the N_2O production pathways and the potential mechanisms for the mitigation of this GHG and O_3-depleting gas are presented in the next sections. In addition, the state of the art physical-chemical technologies used nowadays for end-of-the-pipe off-gas treatment in chemical industries are also revised along with an introduction to the potential and limitations of biotechnologies for N_2O emissions control.

2.1. N_2O Generation in Wastewater Treatment Processes

Wastewater treatment plants (WWTPs) are composed of physical, biological and chemical processes capable of removing the dissolved and suspended pollutants present in wastewater (Figure 1.3). Wastewater pre-treatment and primary treatment based on screens, grid separation, settling tanks, etc. initially remove large solids by means of physical processes. Primary treatment can also involve chemical processes based on the addition of reagents to promote the precipitation of suspended and colloidal solids, heavy metals, phosphorus, ammonium, and dissolved inorganic substances. The dissolved organic matter and nutrients are finally removed from wastewater via biological degradation, where activated sludge constitutes nowadays the most applied method in developed countries. The activated sludge process entails the use of aerobic microorganisms capable of bioconverting the organic and inorganic pollutants present in wastewater (i.e organic matter, nitrogen and phosphorous) into more innocuous end-products such as new microorganisms, CO_2, N_2 and H_2O. The microorganisms generated from wastewater

treatment are finally separated from the treated water by settling or flotation, which generates a water effluent free of solids and contamination.

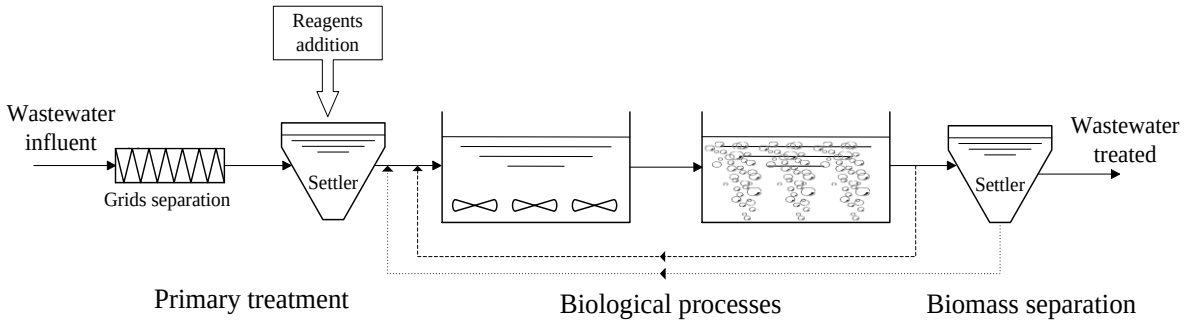


Figure 3. Typical WWTP configuration during domestic and industrial wastewater treatment.

Nitrogen removal in activated sludge processes is carried out via nitrogen assimilation into biomass for protein built-up and nitrogen reduction into N_2 using nitrogen nitrification followed by denitrification. The latter process is based on the oxidation of ammonia (nitrification) and the subsequent reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to molecular nitrogen (N_2) using biodegradable organic matter as electron donor (denitrification). Nitrogen in wastewater is found in the form of organic nitrogen (i.e aminoacids, aminosugars, and urea), ammonia (ionized and free), NO_3^- and NO_2^-. Organic nitrogen is rapidly converted to ammonia trough ammonification, a biological process conducted by the microbial communities present in wastewater and in the activated sludge tanks [22]. Both the ionized ammonia (NH_4^+) and the unionized or free ammonia (NH_3) remain in equilibrium in water, the share of NH_4^+ being greater under acid and neutral pH. During dissimilatory biological nitrogen removal in WWTPs, NH_3 is first oxidized to NO_2^- by aerobic chemoautotrophs microorganisms (named ammonia-oxidizing bacteria (AOB)) to obtain energy using O_2 as electron acceptor and inorganic carbon (CO_2) as a carbon source [23]. This NO_2^- is further oxidized to NO_3^- by the chemoautotrophs nitrite-oxidizing bacteria (NOB) (Figure 1.4). Hydroxylamine (NH_2OH) is an intermediate from NH_3 oxidation in the nitritation step (oxidation of NH_3 to NO_2^-) catalyzed by the enzyme ammonia

monooxygenase, while the further oxidation of NH_2OH to NO_2^- is catalyzed by the enzyme hydroxylamine oxidoreductase [24]. NH_2OH, together with NH_3 or H_2, can be used by AOB as energy source for nitrifier denitrification, which represents a shortcut to conventional nitrogen removal where NO_2^- is reduced to NO, N_2O and finally to N_2 under O_2 limitation (Figure 1.4) [25]. Nitrifier denitrification has been hypothesized as the responsible of great fraction of the N_2O generated in WWTPs [26,27]. In this context, AOB are known to possess the genes encoding the enzymes catalyzing NO_2^- and NO reductions. However, the genes required for the synthesis of the enzyme that catalyzes the reduction of N_2O to N_2 (nitrous oxide reductase) are missing, which suggests that the final reduction step during nitrifier denitrification yields N_2O rather than N_2 [23,28–30]. Other authors have hypothesized that N_2O is generated via chemical decomposition of the nitrosyl radical (NOH), which is an intermediate in the oxidation step of NH_2OH to NO_2^- [31,32].

On the other hand, N_2O is an intermediate during heterotrophic denitrification, where NO_3^- and NO_2^- are sequentially reduced to NO, N_2O and N_2 during organic matter oxidation in the absence of oxygen (anoxic conditions) (Figure 1.4). Most denitrifying bacteria use preferentially oxygen instead of nitrogen oxides due to the higher energy yield of aerobic oxidation. Thus, the variations in the dissolved oxygen levels during denitrification may result in the accumulation of N_2O in the mixed liquor, since the enzyme nitrous oxide reductase (N_2OR), a dimeric multicopper protein present in most denitrifiers that catalyzes the transfer of two electrons from the electron donor to N_2O, is inhibited in the presence of dissolved O_2 [33–35]. In addition, transient aerobic/anoxic conditions in the mixed liquor of the denitrification tank may result in the accumulation of N_2O since the synthesis of the enzyme N_2OR induced under anoxic condition is slower than that of other enzymes involved in the heterotrophic denitrification reduction steps [36].

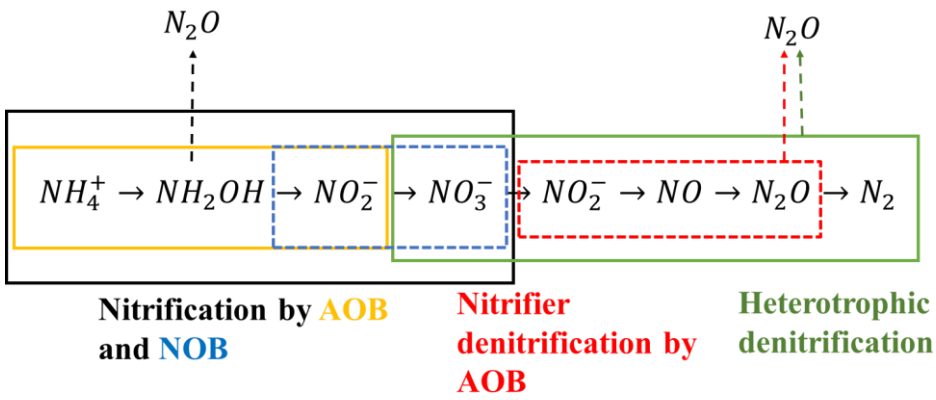


Figure 4. Biological pathway for N_2O formation during conventional wastewater treatment in denitrification-nitrification systems.

Dissolved oxygen is also one of the most important factors governing the generation of N_2O during nitrification. In this context, low dissolved O_2 concentration during nitrification may result in the production of N_2O via nitrifier denitrification by AOB. The COD/N ratio has been also identified as a key factor determining N_2O formation during wastewater treatment. Thus, low COD/N ratios induce N_2O generation owing to the limited availability of biodegradable organic carbon for heterotrophic denitrification [37]. High levels of NO_2^- concentration can also trigger the formation of N_2O during nitrification when AOBs reduce NO_2^- to N_2O under low O_2 levels and during denitrification by partially inhibiting N_2O reduction [26,38]. Advanced nitrogen removal processes such as nitritation-denitrification involve operational conditions that may also promote the emissions of N_2O [39]. This process represents a shortcut of nitrification as a result of the partial oxidation of NH_3 to NO_2^-, which is readily denitrified to reduce up to 25 % the oxygen consumption, 40 % the COD requirements and biomass production compared to conventional nitrification-denitrification. In this particular process, the low oxygen levels, the high NO_2^- concentration and the abundance of AOB bacteria represent the ideal conditions for N_2O generation. Indeed, the initial benefits derived from the reduced energy consumption during nitritation-denitrification can be jeopardized by the high GHG footprint of the process derived from N_2O emissions.

Nitrogen removal in WWTPs represents an estimated contribution of ≈ 10 % of the global share of anthropogenic N_2O emissions and can account for up to 26 % of the GHG footprint of the anthropogenic water cycle [40,41]. The N_2O emissions produced during wastewater treatment are typically expressed as a fraction of the nitrogen inlet load to the WWTP and can vary from 0 to 25 % [40,42]. This high variation from plant to plant shows that N_2O emissions are strongly affected by the particular design and operational conditions prevailing in the WWTP. Finally, the concentrations of N_2O present in the emissions from WWTP range from very low emission levels of 3 ppm_v to high levels of 200-600 ppm_v [43–47].

2.2. N_2O Emissions in Nitric and Adipic Acid Production Plants.

Nitric acid (HNO_3) production plants are the largest source of N_2O emission in the chemical industry, with annual emissions of ≈ 400 kt of N_2O [48]. HNO_3 is typically produced via two processes configurations namely weak and high-strength HNO_3 production, where low and high strength HNO_3 are manufactured, respectively. Weak HNO_3 production is conducted in three catalytic ammonia oxidation steps at high temperature. A catalytic convertor composed of platinum (90 %) and rhodium (10 %) is typically employed to oxidize ammonia with hot air to NO and water in a first stage. The reaction is highly exothermic and can reach temperatures ranging from 750 to 900 ºC. The NO produced in the first oxidation step is further transformed to NO_2 and N_2O_4 via oxidation with the residual oxygen present in the gas stream. The final step, carried out in an absorption tower, involves NO_2 absorption into water and the formation of HNO_3. The reactions involved during weak HNO_3 production are as follows [49].

$$4NH_3 + 5O_2 \rightarrow 4NO + 6H_2O \quad (1)$$

$$2NO + O_2 \rightarrow 2NO_2 \quad (2)$$

$$3NO_2 + H_2O \rightarrow 2HNO_3 + NO \quad (3)$$

The oxidation of ammonia to NO (first step) is the main contributor to N_2O generation, which depends on the operational temperature and pressure. The formation of NO_2 is a function of the combustion conditions, catalyst composition and age of the burner. Thus, the main product of the catalytic oxidation of NH_3 is NO at a high temperature and low pressure, while low temperatures and high pressures result in the catalytic oxidation of NH_3 to N_2O and N_2. However, the oxidation of ammonia is not the sole mechanism of N_2O formation during weak HNO_3 production. In this context, the reactions between unreacted ammonia and NO can also mediate the generation of N_2O as follows [48]:

$$NH_3 + 4NO \rightarrow 2.5N_2O + 1.5H_2O \quad (4)$$

$$NH_3 + NO + 0.75O_2 \rightarrow N_2O + 1.5H_2O \quad (5)$$

$$NH_3 + O_2 \rightarrow 0.5N_2O + 1.5H_2O \quad (6)$$

High-strength HNO_3 production involves an initial reaction of air and ammonia, whose products are condensed to produce weak HNO_3. The remaining NO, separated from the liquid HNO_3, is oxidized to NO_2 in two oxidation reactors. The vapors are compressed and cooled to form dinitrogen tetroxide, which reacts with O_2 under high pressure to form strong HNO_3 of 95-99 %. Typically, the emissions of N_2O from high-strength HNO_3 production range from 2 to 9 kg N_2O per tonne of nitric acid and up to 19 kg N_2O per tonne in the absence of N_2O emission control [50]. Finally, tail gas from HNO_3 production plants contain concentrations of NO_x ranging from 100 to 3500 ppm_v, 300 to 3500 ppm_v of N_2O, 1 to 4 % of O_2 and H_2O up to 2% in a N_2 basis [51].

The production of $C_6H_{10}O_4$, with yearly emissions of ≈ 100 kt of N_2O, represents another chemical industry with a key contribution to the global anthropogenic emissions of N_2O [48]. The absence of N_2O abatement technologies such as thermal and catalytic oxidation in

this industry before 1995 resulted in yearly emissions of ≈ 600 kt of N_2O. Adipic acid, used as a feedstock in the manufacture of nylon 6,6 polyamide, is produced from the conversion of cyclohexane to cyclohexanol (alcohol), cyclohexanone (ketone) or a mixture of both via oxidation. This ketone/alcohol mixture is further oxidized with HNO_3 to produce $C_6H_{10}O_4$, which is finally purified by crystallization [52]. The oxidation of cyclohexane is catalyzed by a cobalt, chromium and/or copper catalyst operated at moderate temperature and pressure, while copper and vanadium are used as a catalyst in the final reaction yielding adipic acid (at 45 to 55 %) (Equations 7 and 8). The use of HNO_3 in this second reaction results in the generation of large amounts of nitrogen oxides, which can represent a concentration of up to 20 % in the tail gas.

$$(Cyclohexanone) + 1.5HNO_3 \rightarrow Adipic\ Acid + 3/4\, N_2O + 3/4\, H_2O \quad (7)$$

$$(Cyclohexanol) + 2HNO_3 \rightarrow Adipic\ Acid + N_2O + 2H_2O \quad (8)$$

In fact, the IPCC 2006 inventory guidelines have set an emission factor of 300 kg of N_2O per tonne of $C_6H_{10}O_4$ produced in the absence of N_2O control unit [53]. In this context, while most NO formed in these reactions is easily recovered by water absorption, N_2O constitutes an undesired byproduct that requires the implementation of cost-effective end-of-the-pipe technologies.

3. N_2O Emissions Control

2.3. Mitigation Strategies to Reduce N_2O Formation and Emission

Several mitigation strategies to prevent N_2O formation during wastewater treatment have been proposed and tested both at laboratory and full-scale. The main strategies evaluated are based on the control of operational conditions to avoid sudden changes in dissolved oxygen levels, pH, nitrogen and carbon load, and solid retention time. For instance, an

increase in the carbon or nitrogen loads would induce a rapid decrease in the dissolved oxygen level in the nitrification tank, with the subsequent production of N_2O. In this context, the implementation of wastewater load control strategies by recycling the effluent of the plant to the headworks could eventually prevent transient spikes of nitrite, ammonia, nitric oxide, etc. and thus avoid their pernicious effects [54]. Furthermore, an adequate aeration control to prevent overaeration by optimizing the air bubble size could prevent the stripping of dissolved N_2O [55]. Overaeration in the nitrification tank indirectly increases the supply of dissolved oxygen to the denitrification tank (via internal recirculation), thus promoting N_2O formation by inhibition of the enzyme N_2OR. On the other hand, a precise control of the biodegradable organic carbon supply is crucial during denitrification, the supplementation of additional external carbon source representing a key strategy to prevent N_2O generation during denitrification. Finally, special attention should be payed to the availability of copper during wastewater treatment based on key role of this heavy metal on the activity of N_2OR. In this sense, Zhu et al. (2013) observed that the addition of this element to the denitrification tank increased the activity of the enzyme N_2OR, with an associated reduction in N_2O emissions by 50-73 % [56].

The strategies to control N_2O emissions during the production of HNO_3 are classified based on their position in the production process. Primary strategies pursue N_2O reduction during the ammonia oxidation step via optimization of the catalyst and oxidation conditions. Secondary strategies are based on the removal of N_2O by catalytic reduction between the NH_3 oxidation step and the absorption column [57]. Some companies (Yara, BASF, Johnson Matthey, and Hereaus) have developed catalysts that are placed in the NH_3 burner and by increasing the residence time of the gas, the N_2O is reduced to N_2 and O_2. Finally, tertiary strategies for N_2O control are based on end-of-the-pipe technologies upstream or downstream of the tail gas expansion unit. In this context, the kinetic of NH_3 oxidation and the operational conditions constitute key factors to control N_2O generation during HNO_3

production. Thus, an NH_3/air ratio of 1:9, high oxidation temperatures (>1173 K), low operating pressures (4-5 bar), good mixing of air and NH_3 and a periodic catalysts replacement (3-12 months) can support high HNO_3 production yields and avoid the generation of undesirable byproducts such as N_2O [48]. On the other hand, the replacement of the HNO_3 used in the oxidation of the alcohol/ketone mixture by chemicals that do not result in the formation of N_2O has been proposed as a mitigation strategy during $C_6H_{10}O_4$ production. In this sense, Soto et al. (1988) successfully replaced HNO_3 by an aqueous solution of hydrogen peroxide (30 %), resulting in a $C_6H_{10}O_4$ yield of 90 % with negligible concentrations of N_2O in the tail gas. Unfortunately, this process is not economically feasible due the currently high production costs of hydrogen peroxide [58]. The use of the N_2O-laden tail gas from $C_6H_{10}O_4$ production as a raw material in the production of phenol via benzene catalytic oxidation using ZSM-5 zeolites has been also proposed in order to close all chemical transformations [59]. Furthermore, promising results have been achieved during the biological production of $C_6H_{10}O_4$ via microbial engineering as an alternative to chemical production with petroleum-based precursors mediating N_2O generation. However, a recent review by Kruyer and Peralta-Yahya (2017) on the latest developments of microbial production of $C_6H_{10}O_4$ from renewable feedstocks (i.e lignocellulosic biomass, lipids and amino acids) highlighted that this approach is still not feasible due its high investment costs and low production yields [60].

2.4. End-of-the-pipe Technologies for N_2O Emissions Control.

The application of mitigation strategies to prevent the formation of N_2O is not always feasible or effective, and the implementation of end-of-the-pipe technologies is often required to minimize the environmental impact of N_2O emissions. Several physical-chemical technologies are currently applied to abate N_2O emissions in chemical industry driven by recent advances in catalyst design. Conversely, physical-chemical technologies have been

scarcely implemented for the abatement of N_2O emissions in WWTPs likely due to the high O_2 and water levels, and low N_2O concentrations. Likewise, the number of studies devoted to the assessment of biological technologies for the abatement of N_2O from chemical industry or WWTP is scarce despite the *a-priori* environmental and economic benefits. This section will critically review the state of the art physical-chemical and biological technologies available for the treatment of N_2O emissions, along with a comprehensive description of the biological pathways involved in N_2O biodegradation under aerobic and anaerobic conditions.

2.4.1. Physical-chemical End-of-pipe Technologies for Nitric and Adipic Acid Production plants

The physical-chemical technologies available nowadays for N_2O control derive from chemical engineering and are mainly based on thermal and catalytic pollutant destruction (Figure 1.5). The most widely implemented physical-chemical N_2O abatement technologies are:

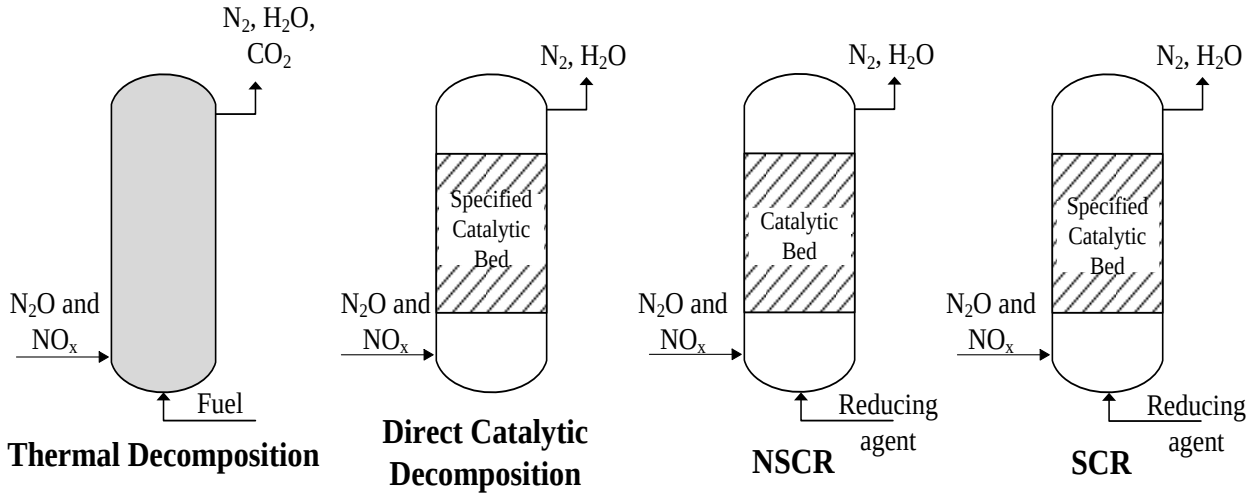


Figure 5. Physical-Chemical technologies for N_2O abatement

Thermal Decomposition

This technology consists of the combustion of N_2O at temperatures ranging from 1023 to 1273 K. This slightly exothermal reaction requires a continuous fuel supply in order to maintain combustion temperatures in the operating range. The combustion unit is often coupled to a heat exchanger to recover part of the energy from the exhaust gas in order to decrease its inherently high fuel expenses. In this context, DuPont developed a method based on the combustion of the N_2O-laden tail gas (57 %) from $C_6H_{10}O_4$ production plants with methane to support a thermal decomposition at 1126 K and exhaust N_2O concentrations of ≈ 200 ppm_v [61].

Direct Catalytic Decomposition

This technology, which entails the catalytic reduction of N_2O to N_2 and O_2, does not require an additional fuel for N_2O combustion. Catalytic N_2O decomposition constitutes the most attractive approach from an environmental and economic viewpoint based on its lower demand for additional fuel, its higher CO_2 footprint and the undesired hydrocarbon emissions from fuel slips compared to thermal decomposition. This catalytic N_2O destruction can be carried out using noble metal, metal oxide and zeolite based catalysts. Despite the merits of this technology, few industrial scale units are nowadays in operation as a result of the high cost and poor thermal stability of the catalysts, especially those based on noble metals [62]. The companies Süd-Chemie and Uhde developed a novel process named *EnviNOx*, which provides high N_2O removal efficiencies without additional chemical requirements during the treatment of high temperature tail gas from the HNO_3 production plants [49]. *EnviNOx* supports the removal of both N_2O and NO_x in a two-step process, where N_2O is firstly reduced to N_2 and O_2, and thereafter NO_x is reduced to water vapor and N_2 in a secondary catalytic bed via ammonia addition.

Non-Selective Catalytic Reduction (NSCR)

This technology was originally developed for the control of NO_x emissions in HNO_3 production plants using a reducing agent like CH_4, H_2 or other hydrocarbons at temperatures varying from 473 to 753 K (depending on the catalyst and fuel used). The catalysts used in NSCR are based on platinum, vanadium, titanium or iron oxide. NSCR requires a preheating of the tail gas when exhausts gases are at ambient temperature. Besides, the presence of high O_2 concentrations in the tail gas may also require a pre-combustion unit due to the extreme exothermic reactions caused by O_2 in the reduction unit, which will negatively affect the long-term performance of the catalysts. Industrial scale NSCR systems have been installed in multiple HNO_3 production plants over the U.S. with removal efficiencies for NO_x and N_2O averaging 90 and 70 %, respectively [48,49]. The Süd-Chemie and Uhde companies developed a variant of the *EnviNOx* process for the treatment of low temperature tail gas from HNO_3 production plants based on hydrocarbon addition to support the catalytic reduction of N_2O [49].

Selective Catalytic Reduction (SCR)

Selective catalytic reduction requires propane, natural gas, liquefied petroleum gas or ammonia as the reducing agent for N_2O destruction. Conventional catalysts are based on platinum oxide with noble metals like vanadium, molybdenum and tungsten as the active component for the reaction, although zeolites doped with metal oxides have supported a high N_2O removal performance with ammonium as the reducing agent [63]. The type of catalyst and reducing agent determines the ignition temperature of the system, which typically varies from 573 to 773 K. This technology is not suitable for the treatment of tail gas at low temperatures, which represents one of the major drawbacks together with the catalyst poisoning mediated by the high operating temperature, particulate clogging and inhibition.

2.4.2. Biological End-of-pipe Technologies

Biological technologies for the abatement of industrial gas pollutants such as H_2S, NH_3, odors, NO and VOCs have been successfully evaluated at laboratory, pilot and full scale for the past four decades [64,65]. These technologies have consistently shown a high robustness, cost efficiency and low environmental impacts as a result of their low energy consumption and innocuous final products. Indeed, biological processes do not entail the use of reducing agents, additional fuel and expensive catalysts, which typically results in high operating costs, high-energy consumption and environmental impacts (i.e uncombusted fuel emitted to the atmosphere and hazardous disposal of the spent catalysts). Therefore, the development of innovative biological alternatives to conventional physical-chemical technologies for the abatement of N_2O is necessary. This section focused on the potential mechanisms of N_2O biodegradation and the bioreactor configurations evaluated for the abatement of this GHG.

N_2O biodegradation can be hypothetically carried out via three biological mechanisms: nitrification, assimilation and denitrification. Nitrification of N_2O to NO_2^- or NO_3^- could be theoretically conducted by nitrifying bacteria under sufficient inorganic carbon and oxygen concentrations at an optimal pH based on the negative Gibbs free energy of the reactions involved in the oxidation of N_2O (Equations 9 and 10). Chapters 1 and 2 investigated these tentative mechanisms, which to the best of our knowledge has never been reported in literature.

$$N_2O + O_2 + H_2O \rightarrow 2NO_2^- + 2H^+ \left(\Delta G^{0'} = -87.4\ kJ\right) \quad (9)$$

$$N_2O + 2O_2 + H_2O \rightarrow 2NO_3^- + 2H^+ \left(\Delta G^{0'} = -15\ kJ\right) \quad (10)$$

The conversion of N_2O into organic nitrogen to form the building blocks for protein synthesis, using a similar pathway to microbial N_2 assimilation, has been also hypothesized

[66–68]. Unfortunately, the feasibility of this mechanism has not been empirically confirmed. Today, heterotrophic denitrification represents the only confirmed biological mechanism capable of degrading this GHG via reduction to N_2 using an electron donor such as organic matter. N_2O reduction to N_2 is a strongly exergonic reaction (Equation 11), which is conducted by the enzyme N_2OR [35].

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \ (\Delta G^{0'} = -341\ kJ) \quad (11)$$

The fact that N_2O denitrification occurs only in the absence of dissolved O_2 requires a costly depletion of the dissolved O_2 in the bioreactor (to maintain anoxic conditions) when treating the diluted N_2O emission from WWTPs. Very few biological systems for N_2O denitrification have been implemented for the abatement of N_2O emissions from WWTPs and chemical industries. In this context, the performance of conventional off-gas treatment bioreactors such as biofilters has been recently assessed at laboratory-scale. For instance, the abatement of N_2O from swine house pit exhaust gas was evaluated by Hood (2011) in a biofilter packed with compost and woodchips (30/70%) at a gas empty bed residence time (EBRT) of 7.6 s [69]. Low N_2O removal efficiencies (14 to 17%) were recorded at the low N_2O concentrations (0.38-0.69 ppm_v) present in the pig farm emissions. Likewise, Akdeniz et al. (2011) evaluated the performance of a biofilter packed with pine nuggets and lava rock at a gas EBRT of 5 s and a relative humidity of 90% treating the exhaust gases from a swine manure and wastewater storage pit [70]. A low N_2O removal efficiency (~ 0.7%) was also recorded at the low inlet concentrations (428 ± 22 ppb_v) present in the manure emissions, which was attributed to both the presence of O_2 and to the low gas EBRT. These factors hindered an efficient mass transfer of this poorly water-soluble gas pollutant (with a dimensionless Henry law constant of 1.6 at 25 ºC [71]) and its further biodegradation by the microbial community present in the biofilter. Desloover and co-workers (2011) evaluated the potential of a novel bioelectrochemical system with an autotrophic denitrifying biocathode for the removal of

N_2O [72]. Sodium acetate was initially used in this study as the electron donor and NO_3^- as the cathodic electron acceptor during the enrichment of a denitrifying culture, which was further used to completely denitrify N_2O in a second operational stage. In the view of the limited advances in the development of efficient biotechnologies for N_2O abatement, this Thesis evaluated the abatement of WWTPs N_2O emissions in an innovative bioscrubber using methanol as carbon source (Chapter 2). The N_2O-laden air emission was introduced at the bottom of a packed absorption column (2 L) operated counter-currently with a trickling mineral salt medium (MSM) pumped from an anoxic stirred tank reactor (STR) (3 L). The N_2O absorbed in the trickling MSM was then reduced to N_2 in the STR by an immobilized heterotrophic denitrifying community using methanol as the sole carbon and electron donor source. The packed bed column was operated at a gas EBRT of 3 min with increasing N_2O removal efficiencies (6 to 40 %) promoted by the increase of liquid recycling velocity (1 to 8 m h^{-1}) in the bioscrubber. Thereafter, a similar bioscrubber configuration was evaluated for the continuous N_2O abatement using domestic wastewater as the carbon and electron donor source as an operational strategy to reduce the overall operating cost (Chapter 3). The bioscrubber consisted of a packed bed absorption column coupled (2 L) to a fixed bed anoxic bioreactor (FBR) (two units of 3 and 7.5 L were evaluated) filled with polyurethane foam for the microbial growth immobilization. The N_2O abatement performance of the bioscrubber was assessed at different liquid recycling velocities (1 to 8 m h^{-1}) and gas EBRTs of 3, 6, 12, 18, 40 and 80 min. The system achieved N_2O removal efficiencies of up to 94 % with a consistent wastewater treatment performance. Finally, the influence of the dissolved oxygen concentration (1 to 4 mg L^{-1}) on the continuous abatement of N_2O in a 2.5 L activated sludge diffusion system (ASD) devoted to N_2O abatement during the treatment of domestic wastewater was evaluated (Chapter 4). In this experiment, the potential generation of N_2O mediated by the low dissolved O_2 concentrations during wastewater treatment was also monitored in the ASD system. The study showed a limited removal of N_2O in the ASD

regardless of the dissolved O_2 concentration set in the aerobic tank. However, an emission of this GHG during wastewater treatment was always observed, the highest N_2O emissions recorded at the lowest dissolved O_2 concentrations.

On the other hand, the biological treatment of N_2O emissions from nitric or adipic acid production plants has never been reported (to the best of our knowledge). In this context, the low O_2 and high N_2O concentrations in industrial emissions support the direct diffusion of N_2O emissions in denitrifying suspended cultures supplemented with an organic carbon source as the carbon source and electron donor. Thus, based on the nature of industrial N_2O emissions, innovative design and operational strategies can be evaluated for the co-production of added-value bioproducts coupled to the continuous removal of N_2O using a biorefinery approach aiming at increasing the cost-competitiveness of biotechnologies for GHG abatement. In this context, two 2.3 L biological systems, namely a bubble column and an airlift reactors, were evaluated for the continuous abatement of industrial N_2O emissions using methanol as a carbon source (Chapter 5). The co-production of biopolymers promoted by nitrogen limitation was investigated in both systems. The bioreactors were operated at a gas residence time of ~17 min and inoculated with a pure *Paracoccus denitrificans* culture. The results showed a high removal efficiency (~87 %) regardless of the bioreactor and a biopolymer accumulation ranging from 34 to 68 % on a biomass weight basis. Furthermore, the influence of different carbon sources/electron donor on the synthesis of biopolymers and the co-enzyme Q10 was evaluated in a bubble column bioreactor (Chapter 6). Methanol, acetate/acetic acid, and glycerol were used as the feedstock. Removal efficiencies ranging from 81 to 91 % were achieved concomitantly with a high accumulation of biopolymers promoted by a sustained feed/famine nitrogen supply strategy. In addition, the specific cell contents of co-enzyme Q10 ranged from 0.4 to 1mg g^{-1}.

Finally, the performance of off-gas treatment biotechnologies can be limited by the gas-liquid mass transfer during the treatment of scarcely soluble water pollutants such as N_2O. This limitation can be overcome in the so called two-phase partitioning bioreactors (TPPB), which are based on the addition of a biocompatible, non-miscible non-aqueous phase (NAP) with a high affinity for the target hydrophobic pollutant [73]. The presence of a NAP in the bioreactor supports an increase in the gas-aqueous concentration gradient and the gas-liquid interfacial area, with the subsequent increase in the pollutant mass transport to the microbial community in the aqueous phase (Figure 1.6).

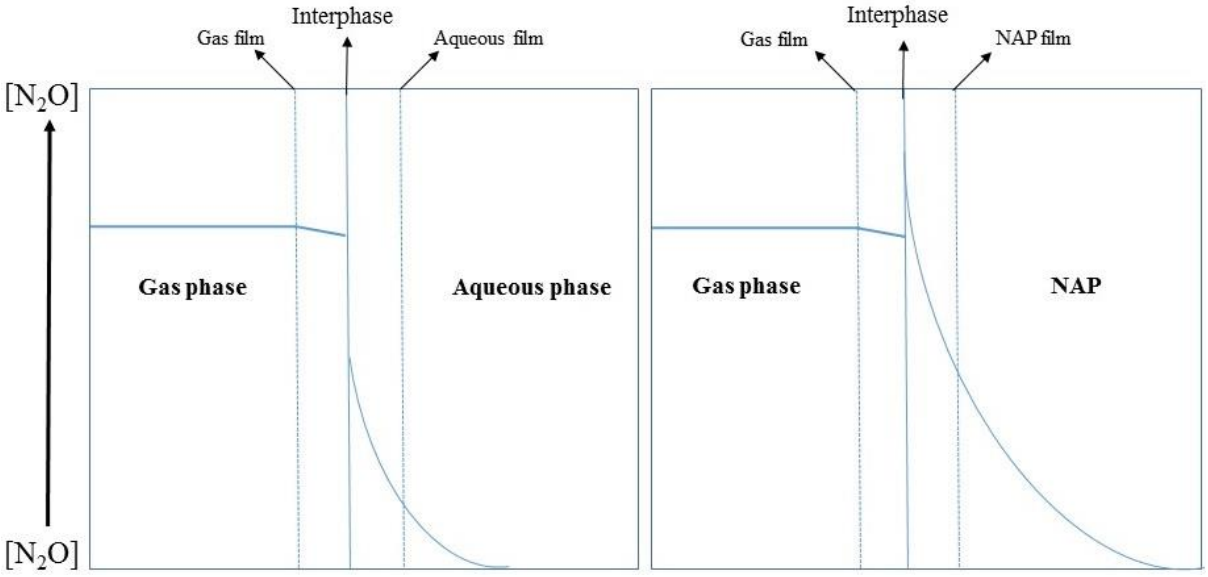


Figure 6. N_2O concentration gradient in a gas-aqueous system (left) and in a gas-NAP system (right).

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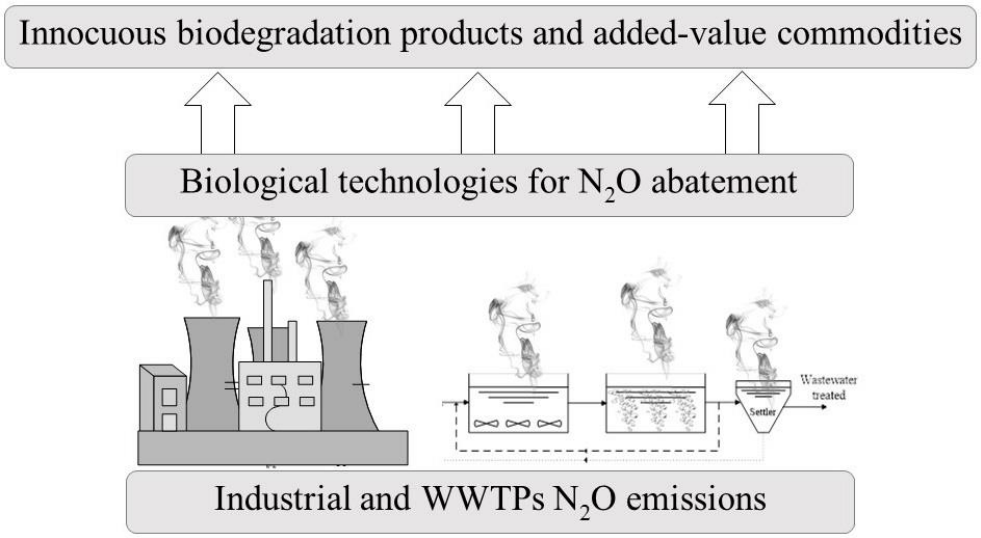
Aims and scope



Justification of the thesis

The rapid increase in the atmospheric GHGs concentrations over the past 50 years is the main responsible of the current climate change and its associated catastrophic consequences. Nitrous oxide, which has experienced a 43 % concentration increase since the pre-industrial era, represents one of the most important GHGs at a global scale. Furthermore, N_2O is considered the most important ozone depleting substance emitted in this century. Despite its environmental relevance, the number of technologies devoted to the treatment of N_2O emisions is scarce. The end-of-the-pipe N_2O abatement market is dominated by physical-chemical technologies, which entail high operating costs, high-energy consumptions and environmental impacts. On the other hand, biotechnologies have recently emerged as cost-effective methods for the control of industrial atmospheric pollution. However, none of the available bioreactor configuration has been tested, to the best of our knowledge, for the control of N_2O emissions. In this context, the development of cost-effective and environmentally friendly biotechnologies for N_2O emissions control requires an intensive research on both the microbiology underlying N_2O biodegradation and innovative high-mass transfer bioreactors under different emission scenarios.

Main objectives

The overall goal of this thesis was the assessment of the potential of novel bioreactors configurations for the treatment of N_2O emissions from WWTPs and HNO_3 production plants, along with the evaluation of mechanisms underlying N_2O biodegradation. Special attention was given to the generation of added-value bioproducts out of N_2O mitigation. More specifically, the individual objectives required to achieve this overall goal are:

1) Systematic evaluation of the performance of innovative biotechnologies for the treatment of N_2O-laden air emissions from WWTPs.
2) Evaluation of the potential mechanisms underlying N_2O biodegradation

3) Evaluation of novel biotechnologies for the continuous abatement of N_2O emissions from HNO_3 production plants coupled to the generation of added value byproducts under different carbon-energy sources and operational strategies.
4) Assessment of two-phase partitioning bioreactors (TPPBs) as a model technology to overcome mass transfer limitations of N_2O abatement biotechnologies.

Development of the thesis

In the current thesis, five innovative biological technologies were systematically investigated for the treatment of N_2O emissions from WWTPs and HNO_3 production plants. More specifically:

In order to fullfill the first objetive aforementioned, a bioscrubber was operated for the continuous abatement of N_2O-laden air emissions based on heterotrophic denitrification under several liquid recycling velocities (**Chapter 1**). Furthermore, the removal of N_2O coupled to domestic wastewater treatment was also assessed in an innovative bioscrubber under different gas retention times (**Chapter 2**). Finally, an activated sludge diffusion system was operated under aerobic and microaerobic conditions to investigate the potential removal of N_2O in WWTPs emissions (**Chapter 4**).

The second objective was accomplished in **Chapter 1** and **3**. First, the reduction of N_2O to N_2 based on heterotrophic denitrification using methanol as the carbon and electron donor source was investigated in gas-tight batch assays using activated sludge as the inculum. Secondly, the potential oxidation of N_2O to nitrate or nitrite was investigated in gas-tight batch assays (incubated 9 months) using multiple nitrifying inocula. Finally, a biotrickling filter inoculated with activated sludge was operated for three months under continuous supply of air, N_2O, NH_4^+ and $NaHCO_3$ to promote the enrichment of a N_2O nitrifying biofilm.

In **Chapter 5**, a bubble column and airlift bioreactors were operated for the continuous removal of N_2O from HNO_3 production plants coupled to the production of biopolymers under nitrogen limiting conditions using methanol as the carbon and electron donor source. Then, the influence of the type of carbon-energy source (methanol, glycerol and acetate/acetic acid) on both the N_2O removal performance and the co-production of biopolymer and coenzyme Q10 was also investigated in a buble column bioreactor (**Chapter 6)**. Chapter 5 and 6 fulfilled the third objective of this thesis.

Finally, **Chapter 7** adressed the fourth objective by evaluating the influence of silicon oil addition as a mass transfer vector for the enhacement of N_2O removal in denitrifying batch assays.

Continuous nitrous oxide abatement in a novel denitrifying off-gas bioscrubber

O.D. Frutos, I.A. Arvelo, R. Pérez, G. Quijano, R. Muñoz. Appl. Microbiol. Biotechnol. 2015, 99 (8) 3695–3706.

Chapter 1

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ENVIRONMENTAL BIOTECHNOLOGY

Continuous nitrous oxide abatement in a novel denitrifying off-gas bioscrubber

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Abstract The potential of a bioscrubber composed of a packed bed absorption column coupled to a stirred tank denitrification bioreactor (STR) was assessed for 95 days for the continuous abatement of a diluted air emission of N_2O at different liquid recycling velocities. N_2O removal efficiencies of up to 40±1 % were achieved at the highest recirculation velocity (8 m h^{-1}) at an empty bed residence time of 3 min using a synthetic air emission containing N_2O at 104±12 ppm_v. N_2O was absorbed in the packed bed column and further reduced in the STR at efficiencies >80 % using methanol as electron donor. The long-term operation of the bioscrubber suggested that the specialized N_2O degrading community established was not able to use N_2O as nitrogen source. Additional nitrification assays showed that the activated sludge used as inoculum was not capable of aerobically oxidizing N_2O to nitrate or nitrite, regardless of the inorganic carbon concentration tested. Denitrification assays confirmed the ability of non-acclimated activated sludge to readily denitrify N_2O at a specific rate of 3.9 mg N_2O g VSS h^{-1} using methanol as electron donor. This study constitutes, to the best of our knowledge, the first systematic assessment of the continuous abatement of N_2O in air emission. A characterization of the structure of the microbial population in the absorption column by DGGE-sequencing revealed a high microbial diversity and the presence of heterotrophic denitrifying methylotrophs.

Keywords Biofiltration · Denitrification · Greenhouse gas · N_2O · Wastewater treatment plants

Introduction

Nitrous oxide (N_2O) emissions represent a serious environmental concern worldwide due to their key role in global climate change and stratospheric ozone layer depletion. N_2O is both a major greenhouse gas (GHG) with a global warming potential 300 times higher than that of CO_2 and the most important O_3-depleting substance emitted in this twenty-first century, with an approximate yearly atmospheric concentration increase of 0.3 % (Ravishankara et al. 2009). N_2O emissions constitute 6 % of the total GHG emissions worldwide and account for 17.9 Tg N $year^{-1}$ (Intergovernmental Panel on Climate Change 2013). In the EU-27, N_2O is mainly emitted in agriculture (238 million tn CO_2-eq), livestock farming (23 million tn CO_2-eq), nitric and adipic acid production (13 million tn CO_2-eq), and waste treatment activities (12 million tn CO_2-eq) (European Environment Agency 2013). Concentrations ranging from 10 to 2000 mg N_2O m^{-3} (corresponding to 6 to 1112 ppm_v) are common in the emissions of these activities (Kampschreur et al. 2008; Xu et al. 2004). During wastewater treatment, N_2O is produced in biological nitrogen removal (BNR) processes, mainly in conventional nitrification-denitrification processes (Foley et al. 2010). Furthermore, some authors have reported the potential of N_2O generation during wastewater biofiltration (Garzón-Zúñiga et al. 2005; Melse and Mosquera 2014; Tallec et al. 2006), where N_2O emission was mainly associated to nitrification and denitrification processes. Even novel microbial nitrogen removal processes such as nitritation/anammox emit significant amounts of N_2O (Kampschreur et al. 2008). Today, the minimization and abatement of N_2O emissions is becoming

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one of the main challenges for wastewater treatment plants (WWTPs) operators worldwide (Kampschreur et al. 2009).

In spite of the relevance of this gas pollutant, the development of technologies for N_2O abatement has been scarce. Conventional physical/chemical technologies such as selective catalytic reduction, selective non-catalytic reduction, or thermal decomposition involve the consumption of costly and/or hazardous chemicals, the generation of secondary pollution, and both high operating costs and environmental impacts when treating large emission flow rates containing low concentrations of N_2O, which represents the most common real case scenario (Skalska et al. 2010). Biotechnologies, which are based on the biocatalytic action of specialized microorganisms, have been consistently shown as robust and efficient abatement technologies for the treatment of industrial volatile organic compounds (VOCs) and malodors, exhibiting lower operating costs and environmental impacts than their physical/chemical counterparts (Estrada et al. 2011). Thus, biological off-gas treatment technologies can become a low cost and environmentally friendly alternative for the abatement of N_2O emissions. Several metabolic pathways for N_2O biodegradation such as autotrophic nitrification, heterotrophic denitrification, or assimilation as the sole nitrogen source have been hypothesized (Vieten et al. 2007; Yamazaki et al. 2013; Zumft 1997). Unfortunately, little information is available in literature to fully support the occurrence of these potential biodegradation mechanisms, which has limited the development of cost-effective N_2O abatement biotechnologies. In this context, the few studies conducted on the biodegradation of N_2O were carried out in batch assays at high N_2O concentrations (ranging 39,000 to 62,000 ppm_v), and to the best of our knowledge, no single study on the continuous treatment of diluted N_2O-laden air emissions in bioreactors has been conducted to date (Apel and Turick 1993; Miyahara et al. 2010).

This work constitutes a proof of concept study of the continuous N_2O abatement under conditions typically found in off-gas emissions from WWTPs. The potential of nitrification, assimilation as N source for biomass growth, and heterotrophic denitrification of N_2O was investigated in a novel bioscrubber composed of a packed bed absorption column coupled to a denitrification tank using CH_3OH as electron donor. The influence of liquid recycling velocity on N_2O abatement was also evaluated.

Materials and methods

Chemicals and mineral salt medium

Synthetic gas mixtures of 1000 and 10000 ppm_v of N_2O in N_2 were purchased from Abelló Linde S.A. (Barcelona, Spain). The mineral salt medium (MSM) used in the experimentation was composed of (in g L^{-1}): $Na_2HPO_4{\cdot}12H_2O$ 6.15, KH_2PO_4 1.52, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCl_2$ 0.02, $NaHCO_3$ 4.9, NH_4Cl 1.8, and 1 mL L^{-1} of a trace element solution (containing per liter: EDTA 5.35 g, $FeCl_2{\cdot}4H_2O$ 2.49 g, $MnCl_2{\cdot}4H_2O$ 0.12 g, $CoCl_2{\cdot}6H_2O$ 0.03 g, $CuCl_2{\cdot}2H_2O$ 0.02 g, $NiCl_2{\cdot}6H_2O$ 0.03 g, Na_2MoO_4 0.03 g, $Na_2WO_4{\cdot}2H_2O$ 0.04 g). A similar nitrogen-free MSM was used during the batch nitrification assays and the first 30 days of bioscrubber operation. The pH of the mineral salt media used was 7.5. All chemicals were purchased from PANREAC with a purity of +99 % (Barcelona, Spain). CH_3OH was purchased from Carlo Erba Reagents (Milano, Italy) with a purity of 99.9 %.

Continuous N_2O biodegradation in a bioscrubber

A bioscrubber composed of a jacketed PVC absorption column packed with 2 L of 1 cm^3 polyurethane foam (PUF) cubes coupled to a 3 L jacketed stirred tank bioreactor (STR) (Afora S.A., Spain) filled with 1.5 L of kaldnes rings and magnetically agitated at 300 rpm was set up for the continuous abatement of a diluted air emission of N_2O for 95 days (Fig. 1). The stirred tank reactor was initially filled with 1.55 L of a nitrogen-free MSM and inoculated with 1 L of activated sludge from the WWTP of Valladolid (operating in a denitrification-nitrification configuration). The MSM was recirculated from the STR to the packed column using a pig tail nozzle located at the top of the column at a velocity (U_L) of 1 m h^{-1} from day 0 to 27, 3 m h^{-1} from day 27 to 72, 4.75 m h^{-1} from day 72 to 88 and 8 m h^{-1} from day 88 to 95. These sequential U_L increases were performed to evaluate the enhancement in bioscrubber performance at increasing U_L. Both the packed column and the STR were maintained at 30 °C. A syringe pump (Fusion 100, Chemyx Inc. USA) was employed to supply pure CH_3OH (electron donor for N_2O reduction) into the recycling MSM entering the STR. Fresh MSM (500 mL) was daily replaced from the STR to supply nutrients for microbial growth and to avoid the accumulation of potentially inhibitory metabolites. The pH in the STR was continuously monitored and maintained at 7.5–8 by addition of 1 M HCL. The dissolved oxygen concentration (DO) was also on-line monitored to ensure anoxic conditions in the STR. The gas and liquid phases in the absorption column were operated in countercurrent at a gas empty bed residence time (EBRT) of 3 min with a 104±12 ppm_v N_2O air emission obtained by mixing 540 mL min^{-1} of air (RESUN LP-60 air-pump, China) and 60 ml min^{-1} of a 1000 ppm_v N_2O calibrated gas mixture supplied using a mass flow controller (Aalborg, Denmark). Prior to inoculation, an abiotic test with MSM was performed for 4 days to assess any potential removal of N_2O by adsorption or photodegradation in the experimental set-up.

Liquid samples (70 mL) were periodically drawn from the inlet and outlet of the absorption column to determine the concentration of NO_2^-, NO_3^-, total organic carbon (TOC),

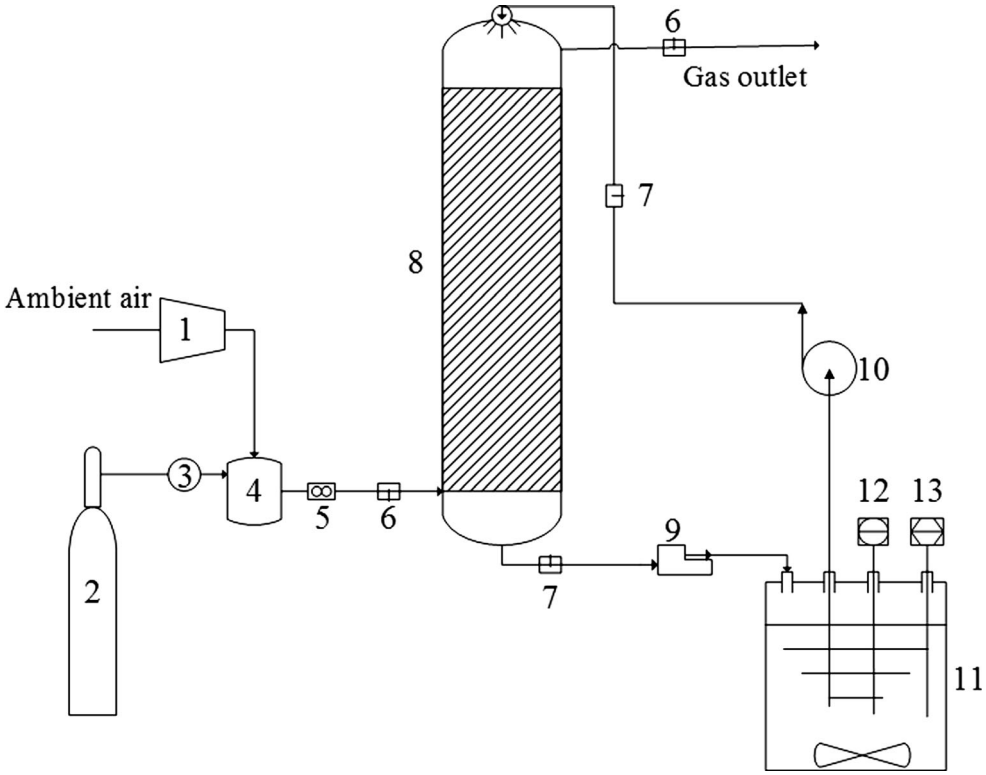

Fig. 1 Schematic of the bioscrubber experimental set-up. *1*) Air compressor. *2*) N_2O reservoir. *3*) Mass flow controller. *4*) Mixing chamber. *5*) Gas flowmeter. *6*) Gas sampling port. *7*) Liquid sample port. *8*) Absorption packed column. *9*) Methanol syringe pump. *10*) Liquid recycling pump. *11*) Stirred tank reactor. *12*) OD electrode. *13*) pH electrode

inorganic carbon (IC), and total nitrogen (TN). MSM was added every 2 days to compensate medium losses due to sampling and evaporation. The N_2O gas concentration at the inlet and outlet of the absorption column was determined every 2 days by GC-ECD. In addition, the dissolved N_2O concentration was measured by headspace GC-ECD at the inlet and outlet of the STR to assess the denitrification capacity of the system. All measurements were carried out in duplicate.

The NO_3^- and NO_2^- concentrations at the inlet and outlet of the packed column were monitored for 2 weeks at the end of bioscrubber operation in the absence of N_2O (1 week) and in the absence of both N_2O and ammonium (1 week) to elucidate the origin of the oxidized forms of nitrogen detected throughout bioscrubber operation.

Batch N_2O nitrification assay

A nitrification assay was carried out at different dissolved inorganic carbon concentrations to assess the feasibility of N_2O removal via nitrification. The experiment was conducted batchwise in 1.15 L glass serum bottles magnetically stirred at 650 rpm and 25 °C. The bottles were filled with 250 mL of nitrogen-free MSM, inoculated with fresh activated sludge (previously centrifuged at 10,000 rpm for 10 min and resuspended three times in nitrogen-free MSM) at a final concentration of 160 mg VSS L^{-1} under an air headspace containing 544±37 ppm_v of N_2O. The nitrogen-free MSM was supplemented with inorganic carbon at 25, 100, and 200 mg IC L^{-1}. A control test containing 200 mg IC L^{-1} and 25 mg N-NH_4^+ L^{-1} was used to assess both the nitrifying activity of the activated sludge and any potential influence of NH_4^+ on N_2O oxidation. An additional control test prepared as above described, but in the absence of biomass, was also carried out to rule out any potential abiotic N_2O removal. All tests were carried out in duplicate. The N_2O headspace concentration was periodically monitored by GC-ECD for 38 days, along with the initial and final NO_2^-/NO_3^- concentrations.

Batch N_2O denitrification assay

This assay was performed in order to assess the feasibility of N_2O biodegradation via denitrification. Batchwise tests were carried out in 1.15 L glass serum bottles magnetically agitated at 650 rpm under anoxic condition at 25 °C. The bottles were filled with 250 mL of deoxygenated carbonate-free MSM with 32 mg L^{-1} of CH_3OH under a N_2 headspace containing 10154 ±281 ppm_v of N_2O and inoculated with activated sludge (resuspended three times in carbonate-free MSM) at a final concentration of 160 mg VSS L^{-1}. Control tests prepared as above described under a N_2 atmosphere without N_2O were performed to assess the endogenous CO_2 production of the inoculum. An abiotic control test in the absence of biomass was also conducted to rule any potential abiotic N_2O removal. All tests were conducted in duplicate. The concentration of

N_2O, CO_2, and O_2 at the bottle headspace was periodically determined by GC-ECD and GC-TCD.

Analytical procedures

N_2O gas concentration was determined using a Bruker Scion 436 gas chromatograph (Palo Alto, USA) equipped with an Electron Capture Detector and a HS-Q packed column (1 m× 2 mm ID×3.18 mm OD) (Bruker, USA). Injector, detector, and oven temperatures were set at 100, 300, and 40 °C, respectively. Nitrogen was used as the carrier gas at 20 mL min^{-1}. External standards prepared in volumetric bulbs (Sigma-Aldrich, USA) were used for N_2O quantification.

Dissolved N_2O concentration was determined via headspace measurements using the above described GC-ECD. A 15-mL gas tight vial, closed with a butyl rubber stopper, sealed with an aluminum cap and containing 0.1 mL of H_2SO_4 to stop biological activity, was filled with 5 mL of N_2O-containing aqueous sample. The vial was immediately shaken vigorously and allowed to equilibrate for 1 h at 25 °C prior to the determination of the N_2O headspace concentration. The dissolved N_2O concentration was calculated as follows:

$$C_a = \left(C_G V_G + \frac{C_G}{H} V_L \right) / V_s \quad (1)$$

where C_a represents the total dissolved N_2O concentration in the aqueous sample (μg L^{-1}), C_G the N_2O headspace concentration (μg L^{-1}), V_G the volume of the headspace (L), V_L the total liquid phase volume considering the initial H_2SO_4 (L), V_S the volume of the aqueous sample (L), and H is the dimensionless Henry's Law constant of N_2O at 25 °C and 1 atm (H=2) (López et al. 2013).

TOC, IC, and TN concentrations were measured using a TOC-VCSH analyzer (Shimadzu, Tokyo, Japan) coupled with a total nitrogen chemiluminescence detection module (TNM-1, Shimadzu, Japan). NO_2^- and NO_3^- were measured in a UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan) according to the Standard Methods 4500-NO_2^- B and 4500-NO_3^- E, respectively (APHA 2005). Biomass concentration was estimated as volatile suspended solid concentration (VSS) measured according to Standard Methods (APHA 2005). The DO concentration was online monitored by a DO probe connected to a 4100e O_2 Transmitter (Metter Toledo, Undorf, Switzerland). The pH was recorded by a pH probe connected to a Black Stone BL 7916 pH controller (Hanna Instrument, Romania).

Molecular biology analysis

Biomass samples corresponding to the inoculum and to the biomass in the packed column of the bioscrubber at the end of the experimentation (day 95) were stored immediately at −20 °C to evaluate the richness and composition of the microbial community. The genomic DNA was extracted according to Lebrero et al. (2011). The PCR mixture (50 μL) was composed of 25 μL of BIOMIX ready-to-use 2× reaction mix (Bioline, Ecogen) containing reaction buffer, magnesium chloride, deoxynucleotide triphosphates (dNTPs), Taq polymerase and additives, 2 μL of the extracted DNA, PCR primers 968-F-GC and 1401-R (10 μM) (Sigma-Aldrich, St. Louis, MO, USA) for bacterial 16S rRNA gene amplification, and Milli-Q water up to a final volume of 50 μL. The PCR thermo-cycling program used was previously described in Lebrero et al. (2011).The DGGE analysis of the amplicons was performed with a D-Code Universal Mutation Detection System (Bio Rad Laboratories) using 8 % (*w*/*v*) polyacrylamide gel with a urea/formamide denaturing gradient from 45 to 65 %. The DGGE running conditions were applied according to Roest et al. (2005). The gels were stained with GelRed Nucleic Acid Gel Stain (biotium) for 1 h 30 min and the obtained DGGE patterns processed using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens- Latem, Belgium). After image normalization, bands were defined for each sample using the band search algorithm within the program. Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product–moment correlation coefficient (Häne et al. 1993). The peak heights in the densitometric curves were also used to determine the Shannon–Wiener diversity index (H).

The most relevant bands were excised from the DGGE gel in order to identify the bacteria present in the samples above described. The procedure was previously described in Lebrero et al. (2011). The taxonomic position of the sequenced DGGE bands was obtained using the RDP classifier tool (50 % confidence level) (Wang et al. 2007). The closest matches to each band were obtained using the BLAST search tool at the NCBI (National Centre for Biotechnology Information) (McGinnis and Madden 2004). Sequences were deposited in GenBank Data Library under accession numbers KM504999-KM505009.

Statistical treatment

Results from the continuous N_2O abatement experiment and the nitrification assays were evaluated using an analysis of variance (ANOVA) with a Fisher's least significant difference (LSD) test using a 95 % confidence level to assess any significant influence of the operational conditions tested on process parameters.

Results

Continuous N_2O biodegradation in the bioscrubber

No significant N_2O removal by adsorption or photolysis occurred in the abiotic removal test, as shown by the negligible

difference between inlet and outlet N_2O gas concentrations in the bioscrubber (<2 %), which confirmed that microbial degradation was the only mechanism responsible for N_2O removal during the entire experiment. Process operation during the first 27 days of experimentation using a nitrogen-free MSM and a U_L of 1 m h^{-1} was characterized by a steady state N_2O removal efficiency (RE) of 6±2 % (Fig. 2a). Dissolved N_2O removal by anoxic denitrification in the STR remained above 95 % (Fig. 2b), while DO concentration was maintained at 0 mg L^{-1} by the addition of 8.5 g of CH_3OH per cubic meter of liquid recycled. NO_2^- and NO_3^- concentrations during steady state operation at 1 m h^{-1} at the inlet of the absorption column were 0.017±0.03 mg N-NO_2^- L^{-1} and 0.066± 0.04 mg N-NO_3^- L^{-1}, respectively, which increased up to 0.023±0.01 mg N-NO_2^- L^{-1} and 0.115±0.07 mg N-NO_3^- L^{-1} across the packed bed, thus confirming the nitrifying capacity of the microbial community attached. However, this fact did not allow us to state that N_2O was oxidized to nitrite or nitrate, since residual trace levels of NH_4^+ from the MSM or from the ammonification of lysed organic biomass nitrogen were always present in the recycling broth. The steady state TN, TOC, and IC concentrations in the STR were 2±1, 22±8, and 31±20 mg L^{-1}, respectively.

The U_L was increased to 3 m h^{-1}, which mediated a rapid raise in the DO concentration in the STR up to 7.26 mg L^{-1} despite the increase in CH_3OH dosage to 9.32 g m^{-3} and in the TOC concentration of the recycling liquid from 22±8 mg L^{-1} to 500 mg L^{-1} by day 29 (data not shown). While the gas N_2O

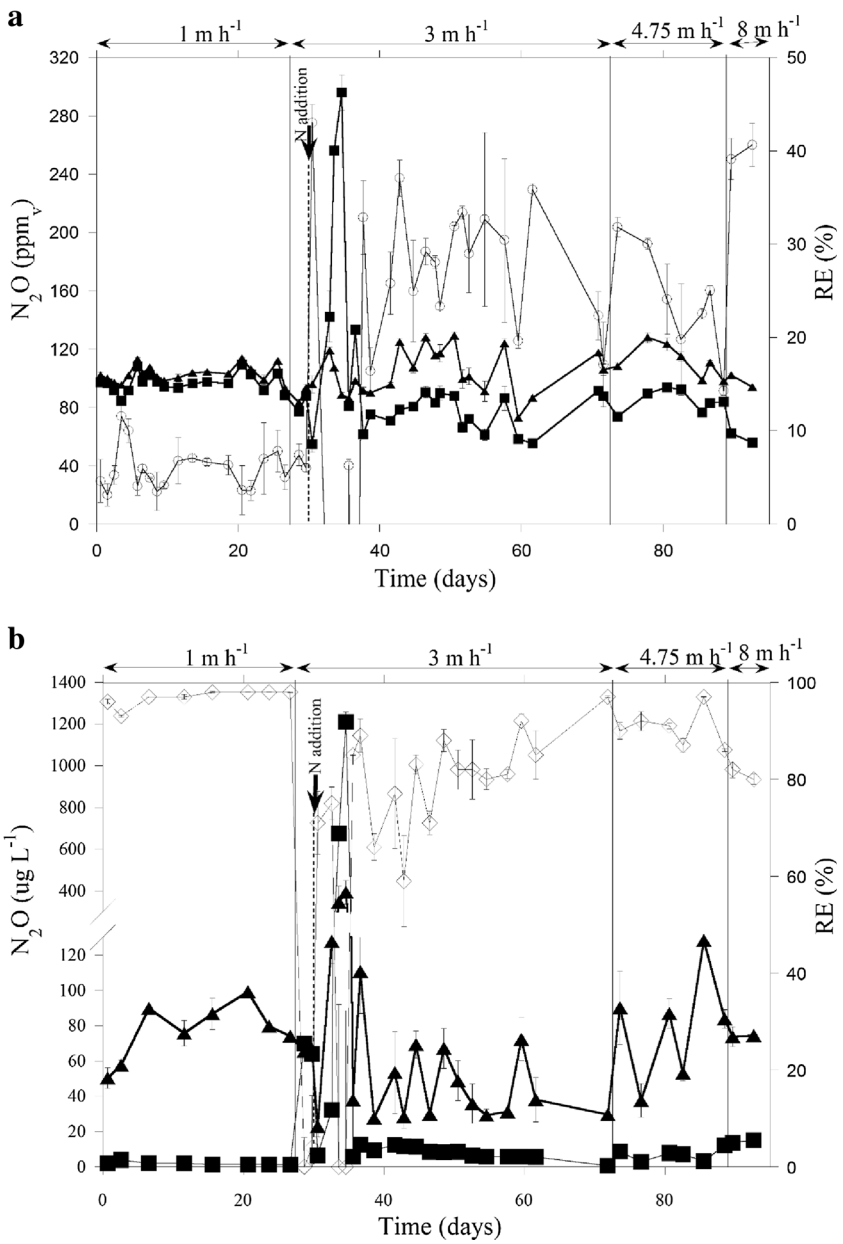

Fig. 2 Time course of **a** the inlet (*black triangle*) and outlet (*black square*) N_2O gas concentrations and removal efficiency (*white circle*) in the bioscrubber, and **b** inlet (black triangle) and outlet (*black square*) dissolved N_2O concentrations and denitrification removal efficiency (*white diamond*) in the STR. *Vertical bars* represent the standard deviation from duplicate measurements

RE remained similar to the previous operational stage until day 29, the dissolved N_2O removal in the STR was reduced to zero (Fig. 2b). The absence of oxygen consumption in the STR despite the presence of CH_3OH in the recycling media suggested the occurrence of nitrogen limitation in the bioscrubber.

Therefore, ammonium chloride was supplemented into the MSM daily replaced from day 30 onward, resulting in a rapid decrease in the DO concentration of the STR. The complete depletion in DO concentration within the first 14 h following nitrogen addition resulted in a gradual TOC concentration decrease to 12 mg L^{-1} by day 32 and in the restoration of the dissolved N_2O removal efficiency in the STR up to 71 %. However, the addition of NH_4^+ initially mediated a sudden increase of the gas N_2O outlet concentration up to 296 ppm_v (Fig. 2a) at day 34, resulting in a gas N_2O RE of −233 %. A production of dissolved N_2O was also observed in the STR, where inlet and outlet dissolved N_2O concentrations of 394 and 1211 μg L^{-1} were recorded by day 34, respectively (Fig. 2b). Likewise, a sudden increase in NO_3^- inlet and outlet concentrations in the absorption column up to 8.1 and 3.9 mg N-NO_3^- L^{-1}, respectively, and in NO_2^- inlet and outlet concentrations up to 3.0 and 0.8 mg N-NO_2^- L^{-1} were recorded by day 34 (Fig. 3a and b). A stable process operation with gas N_2O removal efficiencies of 27±8 % was achieved from day 36 to 71 (Fig. 2a). A gradual rise in the dissolved N_2O removal in the STR occurred also from day 36 up to a steady state value of 84±8 % (Fig. 2b). On the other hand, NO_3^- concentrations during steady state operation at 3 m h^{-1} at the inlet and outlet of the absorption column were 0.072±0.20 and 0.085±0.08 mg N-NO_3^- L^{-1}, while NO_2^- concentrations of 0.001±0.002 and 0.018±0.02 mg N-NO_2^- L^{-1} were also recorded at the inlet and outlet of the absorption column, respectively. The steady state TN, TOC, and IC concentrations were 1.3±0.5, 31.4±22.5 and 83.1±31.9 mg L^{-1}, respectively.

The increase in U_L up to 4.75 m h^{-1} carried out at day 72 did not entail a further increase in N_2O degradation based on the steady state REs recorded (24±6 %). The dissolved N_2O removal in the STR remained also constant at 91±4 % supported by the complete O_2 depletion mediated by CH_3OH dosage (7.33 g m^{-3}). The inlet and outlet NO_3^- concentrations in the absorption column were 0.029±0.02 and 0.051±0.02 mg N-NO_3^- L^{-1}, while inlet and outlet NO_2^- concentrations recorded were 0.005±0.004 and 0.036±0.04 mg N-NO_2^- L^{-1} (Fig. 3a, b). Concentrations of TN, TOC, and IC corresponding to the steady state at U_L of 4.75 m h^{-1} were 2.0±0.3, 27.0±3.6, and 116.2±8.7 mg L^{-1}, respectively.

Finally, process operation at a U_L of 8 m h^{-1} from day 88 onward entailed the highest N_2O REs of bioscrubber (40±1 %) (Fig. 2a). The dissolved N_2O removal decreased to 81±1 % despite of the absence of O_2 in the STR supported by a CH_3OH dosage of 6.12 g m^{-3}.

The ANOVA and LSD tests showed a significant influence of U_L on the N_2O removal efficiencies for U_L of 1, 3, and 8 m h^{-1}. However, no significant difference on bioscrubber N_2O abatement performance was recorded between 3 m h^{-1} and 4.75 m h^{-1}.

Overall, the NO_3^- and NO_2^- concentrations in the outlet of the absorption column were significantly higher than those recorded in the inlet, excluding the few days of process instability following NH_4^+ addition (Fig. 3a, b). Finally, the results from the ANOVA test of the production of NO_2^- and NO_3^- in the packed column showed not significance influence by the supply of N_2O or NH_4^+.

Batch N_2O nitrification assay

There was no significant variation of the N_2O headspace concentration over the 38 days of assay regardless of the IC concentrations tested or the presence of NH_4^+, the variations observed being attributed to the errors in the analytical procedure (Fig. 4). The highest final NO_3^- concentration (17.8±0.3 mg N-NO_3^- L^{-1}) was recorded in the tests supplemented with 200 mg IC L^{-1} and NH_4^+, where negligible concentrations of NH_4^+ and NO_2^- were detected at the end of the nitrification assay. The tests supplemented with 25, 100, and 200 mg IC L^{-1} showed significant differences in the final nitrate concentrations (5.2±0.1, 13.5±0.3, and 10.6±0.8 mg N-NO_3^- L^{-1}, respectively) and negligible NO_2^- concentrations. Neither NO_3^- nor NO_2^- were detected in the control test carried out in the absence of biomass.

Batch N_2O denitrification assay

The N_2O in the assays supplied with CH_3OH and activated sludge was completely depleted in 105 h at a specific N_2O consumption rate of 3.9 mg N_2O g VSS^{-1} h^{-1}. No abiotic removal of N_2O was recorded in the control test while the test conducted with activated sludge did not support any significant production of N_2O (Fig. 5a). On the other hand, N_2O removal was correlated with an increase in CO_2 production, which stabilized at 2451±93 ppm_v. The control test supplied with biomass in the absence of N_2O supported an increase in CO_2 concentration up to 1046±410 ppm_v, while no significant CO_2 generation was detected in the abiotic control test (Fig. 5b). No O_2 was detected in the headspace of the test bottles regardless of the conditions tested.

Bacterial population dynamics

The Shannon-Wiener diversity index takes into account both the number (richness) and the evenness of the species present in a microbial community, with typical values ranging from 1.5 to 3.5 (low and high species evenness and richness, respectively) (McDonald 2003). The activated sludge

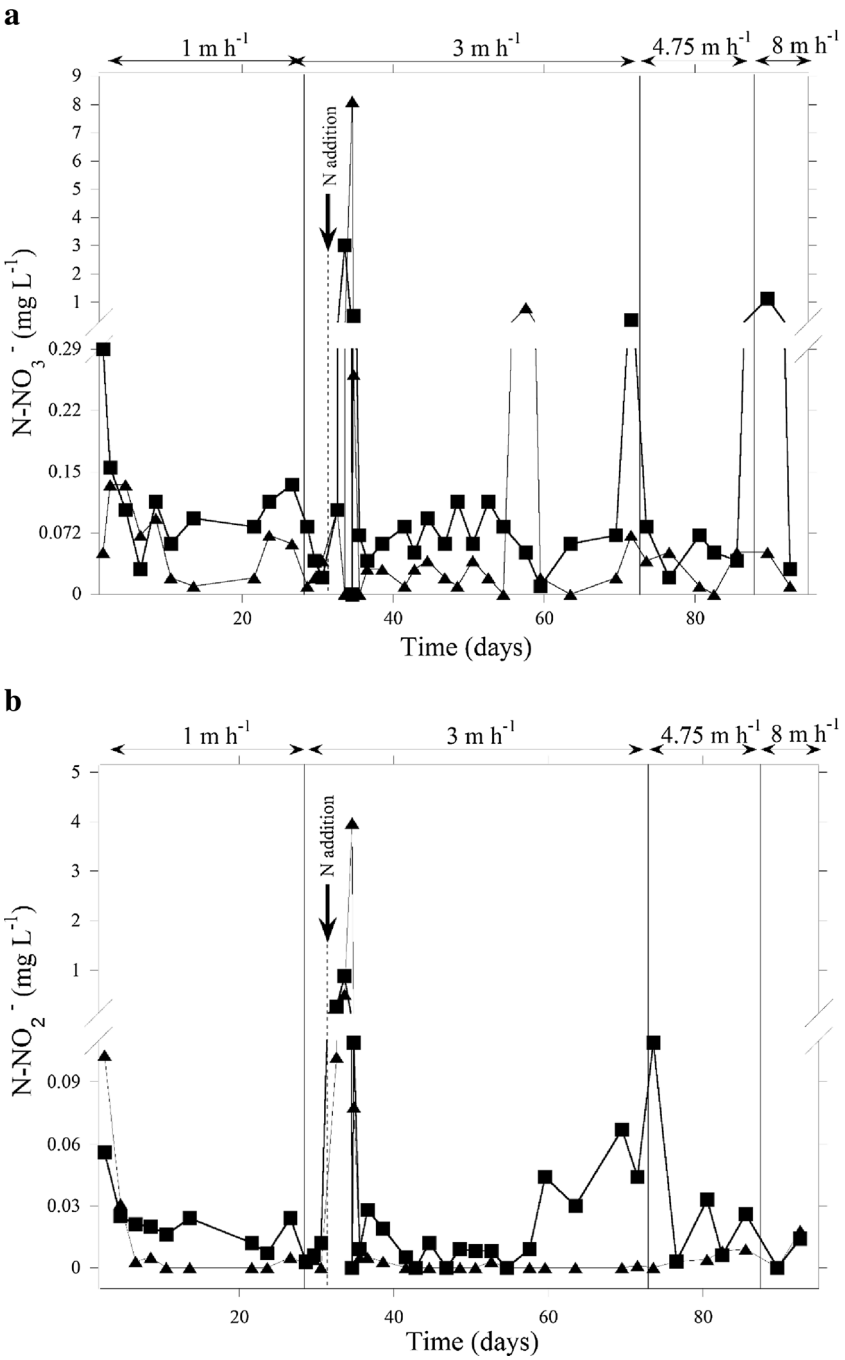

Fig. 3 Time course of the inlet (*black triangle*) and outlet (*black square*) nitrate (**a**) and nitrite (**b**) concentration in the packed bed absorption column. *Vertical bars* represent the standard deviation from duplicate measurements

inoculum sample exhibited a relatively low diversity index (2.9), which increased by the end of bioscrubber operation up to 3.4 (highlighting the high biodiversity of the community established in the system). The analysis of the Pearson similarity coefficients showed a low similarity (16.5 %) between the inoculum and the microbial community present in the absorption column at day 95 of experimentation. From the DGGE gel, 11 bands were sequenced (Fig. 6) and 5 different phyla were retrieved in the RDP database: *Actinobacteria* (3 bands), *Proteobacteria* (2 bands), *Lentisphaerae* (2 bands), *Verrucomicrobia* (2 bands), *Nitrospira* (1 band), while one band remained unclassified. The closest matches for every band (BLASTN) according to the NCBI database, together with its similarity percentages and sources of origin, are provided as supplementary material (Table S1).

Discussion

This study constitutes, to the best of our knowledge, the first systematic evaluation of the performance of a continuous

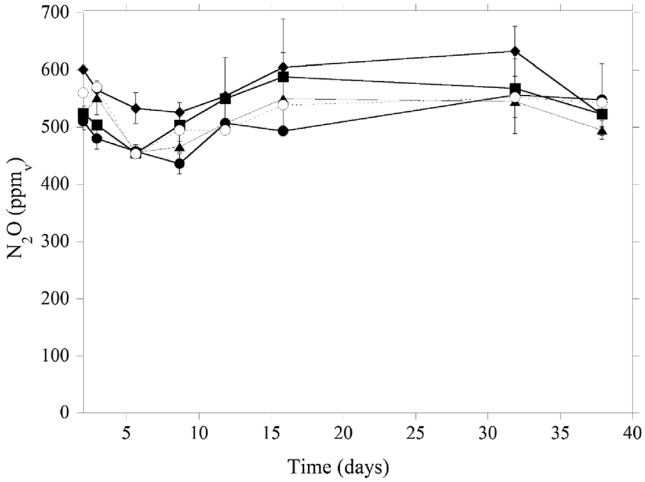

Fig. 4 Time course of the N_2O headspace concentrations during the nitrification assay carried out in the presence of 50 mg IC L^{-1} (*black circle*), 100 mg IC L^{-1} (*black square*), 200 mg IC L^{-1} (*black diamond*) 200 mg IC L^{-1} supplemented with 25 mg N-NH_4 L^{-1} (*black triangle*) and abiotic control tests (*white circle*). *Vertical bars* represent the standard deviation from duplicate measurements.

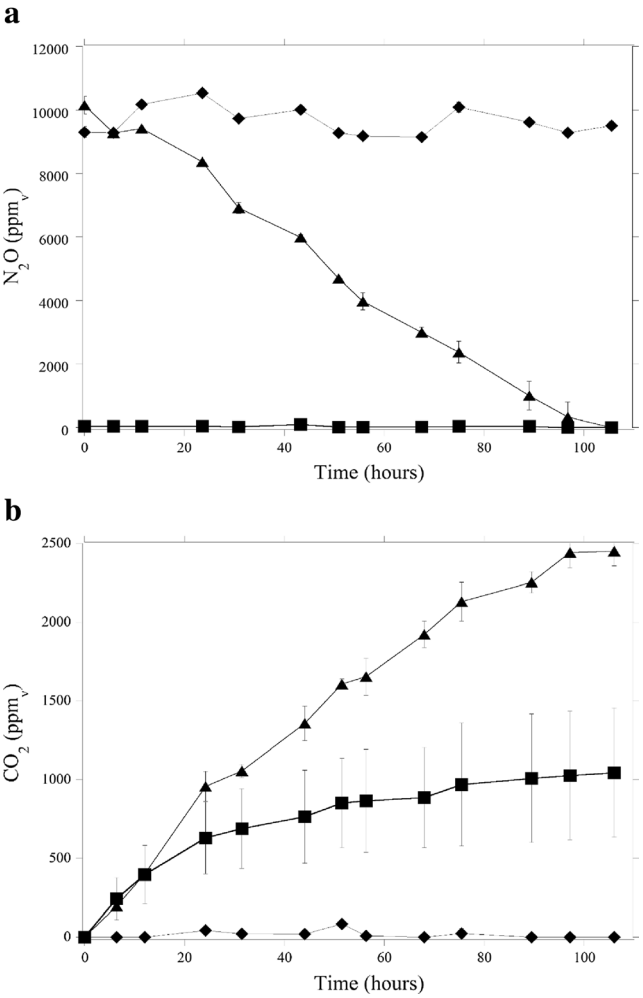

Fig. 5 Time course of **a** the N_2O headspace concentration in the denitrification tests supplied with N_2O and CH_3OH (*black triangle*), endogenous respiration tests in the absence of N_2O (*black square*) and abiotic control test (*black diamond*), and **b** CO_2 production in the denitrification tests supplied with N_2O and CH_3OH (*black triangle*), endogenous respiration tests in the absence of N_2O (*black square*) and abiotic control test (*black diamond*).*Vertical bars* represent the standard deviation from duplicate measurements

bioscrubber for the treatment of diluted air emissions of N_2O, targeting the abatement of this greenhouse under conditions typically found in emissions from WWTPs. The process was based on a preliminary absorption of N_2O in the liquid trickling in the packed bed column followed by its biological reduction via denitrification in the STR using CH_3OH as electron donor. The removal capacity of the experimental bioscrubber was likely limited by the low N_2O carrying capacity of the recycling MSM due the relatively low aqueous solubility of the N_2O (Henry law constant H=2). In this context, the higher the liquid recirculation velocity was, the higher the overall N_2O removal efficiencies in the bioscrubber were. In addition, no deterioration of N_2O denitrification in the STR was recorded in spite of the low hydraulic residence time in the tank (32 min at U_L of 1 m h^{-1} and 4 min at U_L of 8 m h^{-1}), which highlighted the rapid kinetics of the N_2O biodegradation mediated methanol oxidation.

Operation with a nitrogen-free MSM during the first 27 days of operation was devised to promote N_2O biodegradation by nitrification to NO_3^-/NO_2^- or biological N_2O assimilation (N_2O to organic nitrogen as a building block for protein formation) (Vieten et al. 2007). Unfortunately, these hypotheses were not confirmed by the results obtained in this work. In this context, Desloover et al. (2014) did not observe N_2O assimilation into microbial biomass in a study tracing ^{15}N labelled N_2O during anoxic incubation of *Pseudomonas stutzeri* using sodium acetate as electron donor. The low REs observed during process operation at a U_L of 1 m h^{-1} were likely due to the limited N_2O carrying capacity of the recycling solution. The nitrogen-deprived microbial community present in the STR under these particular operating conditions efficiently used the methanol supplied, thus maintaining low DO levels in the denitrification tank as a result of CH_3OH oxidation from maintenance purposes. However, the increase in MSM recirculation velocity to 3 m h^{-1} overcame the non-growth associated capacity of the microbial community present in the STR to oxidize CH_3OH and resulted in the accumulation of TOC and DO in the STR, which immediately stopped N_2O denitrification. The addition of NH_4^+ to the MSM confirmed the occurrence of a nitrogen limitation in the process and restored the denitrification capacity of the STR. The supplementation of the MSM with NH_4^+ also resulted in the initial accumulation of NH_4^+, which induced a period of process instability from days 33 to 36 (Figs. 2 and 3) characterized by the production of high concentrations of N_2O, nitrate, and nitrite in the STR.

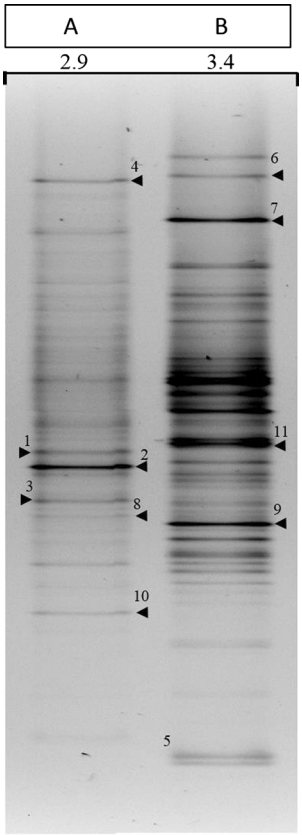

Fig. 6 Bacterial DGGE profiles. Sample names and Shannon diversity indices are indicated in the upper part of the gel: (*A*) inoculum sample, (*B*) bioscrubber end operation sample. The sequenced DGGE bands are indicated with an arrow (*black arrow*) and the corresponding number of each band

Despite the expected increase in the N_2O mass transfer when increasing U_L from 3 to 4.75 m h^{-1}, the observed accumulation of biomass in the packed bed of the absorption column likely reduced the superficial area of the packing material available for mass transport and consequently the N_2O absorbed by the recycling liquid. The highest removal efficiency in the bioscrubber (≈40 %) were achieved at the highest U_L (8 m h^{-1}) applied, which agrees with the previous studies where pollutant mass transfer from polluted gas emissions was enhanced at increasing liquid recycling velocities in biotrickling filters (Estrada et al. 2014; Estrada et al. 2013; Kim and Deshusses 2008). A collateral effect of the increase in liquid recycling velocity was the slight decrease in dissolved N_2O removal by denitrification in the STR at a U_L of 8 m h^{-1} as a result of both the reduction in the liquid residence time in the STR and the low biomass concentration accumulated in the kaldnes rings even after 3 months of operation (the heterotrophic biomass formed from methanol was retained in the PUF packing bed).

Conventional biotechnologies such as biofiltration, biotrickling filtration, or bioscrubbing have been rarely applied for N_2O abatement, with most of the studies on NO removal based either on autotrophic nitrification or on heterotrophic denitrification in O_2-free emissions (Bin et al. 2009; Chagnot et al. 1998; Jiang et al. 2009; Niu et al. 2014; Yang et al. 2007). In this context, Hood (2011) recorded gas N_2O REs ranging from 14 to 17 % in a biofilter packed with compost and woodchips (30/70 %) at an EBRT of 7.6 s and N_2O inlet concentrations of 0.38 to 0.69 ppm_v. Lower gas N_2O removal efficiencies (0.7 %) were supported by a biofilter packed with pine nuggets and lava rock operated at an EBRT of 5 s, a relative humidity of 90 %, and inlet N_2O concentrations of 428±22 ppb_v (Akdeniz et al. 2011). The work here conducted constitutes the first study of N_2O abatement from air emission carried out at relatively high loading rates (inlet N_2O concentration of 104±12 ppm_v).

The relatively high initial NO_3^- and NO_2^- concentrations recorded during process start-up could be attributed to the nitrification of lysis products from the activated sludge used as inoculum. Low NO_3^- and NO_2^- concentrations were however detected in the recycling liquid throughout almost the entire experimentation, except during the instability period following the commencement of NH_4^+ supplementation in the MSM, when concentrations up to 3.9 mg N-NO_2^- L^{-1} and 8.1 mg N-NO_3^- L^{-1} were recorded. These oxidized nitrogen forms detected throughout the entire experimentation could have originated from the nitrification of either the NH_4^+ introduced with MSM replacement, the N_2O fed to the bioscrubber, s or from the ammonified organic nitrogen released from the lysis of the biomass accumulated in the packed bed. The ANOVA carried out to the NO_3^- and NO_2^- concentrations in the recycling liquid exiting the packed bed absorption column in the absence of N_2O or N_2O and NH_4^+ (corresponding to the two last weeks of operation) ruled out any potential nitrification of the NH_4^+ daily supplied to the bioscrubber and of the N_2O present in the air emission.

The results obtained in the specific nitrification assays conducted confirmed the absence of N_2O nitrification under the conditions evaluated (Fig. 4). Based on the sufficient levels of dissolved oxygen and inorganic carbon and the optimum pH present in the cultivation broth of the assays, the absence of N_2O nitrification was likely due to the lack of specialized enzymes capable of transforming N_2O into NO_2^- or NO_3^- despite the reactions are thermodynamically favorable (Eqs. 2 and 3).

$$N_2O + 2O_2 + H_2O \rightarrow 2NO_3^- + 2H^+ (\Delta G^{\circ\prime} = -87.4\ \text{kJ}) \quad (2)$$

$$N_2O + O_2 + H_2O \rightarrow 2NO_2^- + 2H^+ (\Delta G^{\circ\prime} = -15\ \text{kJ}) \quad (3)$$

The ammonium nitrification capacity of the inoculum used in the nitrification assay was confirmed by the fact that all

ammonium added to the nitrification control test was totally depleted, which itself also supported the highest nitrate production recorded. The nitrate detected in the biotic tests not supplied with ammonium was likely due to the ammonification and further nitrification of the organic nitrogen released from the hydrolysis of the heterotrophic activated sludge over the 38 days of experimentation (since the tests were not supplied with any external carbon source).

The specific denitrification assays performed confirmed the ability of the microbial community to biologically reduce N_2O to N_2 via heterotrophic denitrification using CH_3OH as electron donor. The enzyme N_2O reductase (NOS), which completes the final reduction step in the denitrification pathway (Schmidt et al. 2004), is often considered the only enzyme able to perform N_2O reduction to N_2 (Zumft 1997). In our particular study, N_2O depletion was correlated with CO_2 production, which was significantly higher than the endogenous CO_2 production (test conducted with activated sludge and N_2 as the sole headspace gas). The following equation clearly show that N_2O reduction to N_2, a step involved in the denitrification pathway, is indeed a much more thermodynamically favorable process compared with the N_2O nitrification process.

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O (\Delta G^{\circ\prime} = -341\ \text{kJ}) \qquad (4)$$

Desloover et al. (2014) recently confirmed the occurrence of N_2O denitrification by successive enrichment of denitrifying activated sludge under anoxic conditions using sodium acetate as electron donor. The denitrifying microbial community enriched achieved a specific N_2O consumption rate of 78 mg N_2O g VSS^{-1} h^{-1} at an initial N_2O concentration of≈50,000 ppm_v. Likewise, the ability of nine bacterial species to reduce N_2O to N_2 under anaerobic conditions (N_2O in an He atmosphere) was assessed by Apel and Turick (1993) using trypticase soy broth as electron donor. The authors found that seven species were able to successfully consume N_2O, with *Pseudomonas denitrificans* and *Paracoccus denitrificans* exhibiting the best N_2O biodegradation performance after an acclimation period. These bacterial species supported, respectively, maximum specific N_2O consumption rates of 57.2 and 50.5 mg N_2O g VSS^{-1} h^{-1} under initials N_2O headspace concentrations of 22,000 and 39,000 ppm_v. These specific N_2O degradation rates reported in literature, which were 10–20 higher than the 3.9 mg N_2O g VSS^{-1} h^{-1} reported in our study, were likely supported by the previous acclimation of the sludge and the higher N_2O concentrations used in the assays.

The low similarity between the inoculum and the community established in the bioscrubber at the end of the experimentation (16.5 %) was likely due to the selective pressure of the organic carbon source used in the experimentation on microbial enrichment. The DGGE analysis of the inoculum and final bioscrubber community showed a typical microbial population structure of activated sludge, with bacteria from the *Actinobacteria*, *Verrucomicrobia*, and *Nitrospira* phyla (DGGE bands 1, 2, 3, 4, and 10), most of them in high abundance in the inoculum sample (Lebrero et al. 2013; Gonzalez-Gil and Holliger 2011). Stricter anaerobic *Victivallis*-like bacteria (DGGE bands 6 and 7) were found in the final bioscrubber community (Chaganti et al. 2012; van Passel et al. 2011), which were likely produced in the anoxic STR and retained in the PUF of the absorption packed column as a result of liquid recycling. Likewise, *Xanthomonadaceae*-related bacteria were found (DGGE bands 8) both in the inoculum and bioscrubber samples. Is this context, *Rhodanobacter* species, belonging to the *Xanthomonadaceae* family, are known to be able to carry out the full heterotrophic denitrification pathway (Green et al. 2012; Kostka et al. 2012). Likewise, *Methylocystacea*e-related species, capable of using methanol and methane as the sole carbon and energy source under aerobic/anoxic conditions and able to assimilate atmospheric nitrogen (Garrity 2005), were detected (DGGE bands 9) with high intensity in the final bioscrubber community. The denitrification capacity of *Methylocystacea*e was also confirmed by Dam et al. (2013), who reported that *Methylocystis*-like bacteria were able to produce N_2 from the metabolic action of plasmid-borne nitric oxide and nitrous oxide reductases. Osaka et al. (2008) also found *Methylocystacea*e-related species in an activated sludge involved in methane-dependent denitrification. Finally, it must be highlighted that one of the most abundant species in the bioscrubber by end of the experimentation (band 11) remained unclassified.

In brief, the proposed two-stage process based on N_2O gas-liquid absorption in a packed bed column coupled to a denitrification STR (supplied with methanol as the electron donor for N_2O reduction) efficiently removed N_2O in an air emission mimicking those found in WWTPs at unprecedentedly high N_2O loading rates. Higher REs were obtained at increasing the liquid recycling velocity, which suggests that the main process limitation was the N_2O carrying capacity of the recycling liquid. While, neither nitrification nor assimilation of N_2O occurred under the operational conditions evaluated, the feasibility of N_2O reduction coupled to methanol oxidation was demonstrated both in the continuous bioscrubber and in an additional batch denitrification assay. However, despite the promising results here obtained, more research is needed to overcome the limitations of this technology in terms of enhancement of the N_2O gas-liquid mass transfer and use of low-cost electron donors in order to develop more cost-efficient technologies for N_2O abatement with potential implementation in WWTPs.

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Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 2

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Simultaneous biological nitrous oxide abatement and wastewater treatment in a denitrifying off-gas bioscrubber

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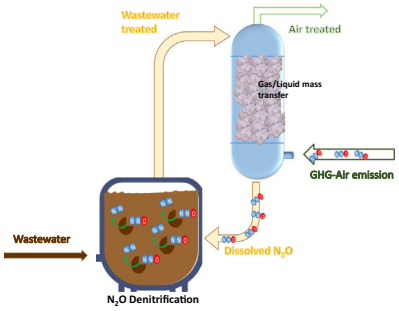
HIGHLIGHTS

- The potential of an innovative anoxic bioscrubber for N_2O abatement was evaluated.
- The simultaneous N_2O abatement and wastewater treatment was feasible.
- Higher N_2O removals supported by increasing liquid recycling velocities and EBRTs.
- N_2O removal efficiencies of 92% were achieved at an EBRT of 40 min.
- Efficient organic carbon removals (85–95%) from wastewater were recorded.

GRAPHICAL ABSTRACT



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ABSTRACT

The simultaneous treatment of N_2O-laden air emissions and domestic wastewater was assessed in a novel denitrifying bioscrubber composed of a packed bed absorption column interconnected to a fixed bed reactor (FBR). The influence of liquid recycling velocities (U_L) and gas empty bed residence times (EBRTs) in the absorption column on bioscrubber's performance was evaluated using synthetic wastewater (SW) and a 100 ± 8 ppm_v N_2O air emission. Steady state N_2O removal efficiencies of 36 ± 3% concomitant with SW total organic carbon removals of 91 ± 1% were achieved at an EBRT of 3 min and at the highest U_L tested (8 m h^{-1}). The removal of dissolved N_2O by heterotrophic denitrification in the FBR constituted the main N_2O biodegradation mechanism and limited the abatement of N_2O. While the supplementation of SW with Cu^{2+} (a cofactor of the N_2O reductase) did not result in an enhancement in N_2O reduction, the increase in FBR volume supported a higher N_2O removal. The increase in EBRT up to 40 min supported an enhancement in the gas N_2O removal of up to 92%. The DGGE-sequencing analysis of FBR microbial population revealed a high microbial diversity and the abundance of denitrifying bacteria capable of reducing N_2O to N_2.

1. Introduction

Nitrous oxide (N_2O) is one of the major greenhouse gases (GHG) emitted nowadays, which contributes to climate change with a 6.2% of the total GHG emissions due to its high global warming potential (≈300 times higher than that of CO_2) [1]. N_2O is also considered the most important O_3-depleting substance emitted in this XXI century [2]. In Europe, N_2O is mainly emitted from agriculture (268300 Gg of CO_2 eq), wastewater treatment processes (12299 Gg of CO_2 eq) and adipic and nitric acid production (9682 Gg of CO_2 eq) [3]. In wastewater treatment plants (WWTPs), N_2O is mainly produced during biological nitrogen removal, with nitrifier denitrification,

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heterotrophic denitrification and hydroxylamine oxidation as the main routes of N_2O production in activated sludge processes [4]. Some authors have also reported N_2O emissions during wastewater biofiltration [5,6], where N_2O production was mainly associated to nitrification and denitrification processes. Even new microbial nitrogen removal processes such as nitritation/anammox or SHARON emit significant amounts of N_2O [7,8].

Based on the renovated and more ambitious EU objective for the reduction of the European GHG emissions by 40% in 2030 (compared to 1990 levels) [9], the minimization of N_2O emissions from wastewater treatment has become one of the main challenges of WWTP operators in this XXI century. In this regard, physical/chemical technologies such as thermal decomposition, selective catalytic reduction and selective non-catalytic reduction, typically used for industrial NOx emission abatement, could be applied as end-of-the-pipe technologies in WWTPs. However, these technologies entail the consumption of costly and/or hazardous chemicals, process operation at high temperatures and the generation of secondary pollution, which results in high operating costs and environmental impacts [10]. On the other hand, biotechnologies have been consistently shown as an environmentally friendly and low cost alternative for off-gas treatment, which exhibit a robustness and efficiency comparable to that of their physical/chemical counterparts [11]. Unfortunately, despite some works on NO/NO_2 nitrification and denitrification have been carried out [12,13], the number of studies assessing the potential of biotechnologies for N_2O abatement is scarce. This GHG is an obligate intermediate during the anoxic nitrogen reduction ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), which up to date has been reported as the only biological N_2O removal mechanism. Therefore, the removal of N_2O from air emissions entails the need for bioreactor configurations involving a N_2O absorption step in water followed by a N_2O reduction step under anaerobic conditions. Bioscrubbers are two-stage systems that can support the above mentioned functionalities simultaneously: the contaminant (N_2O) is transferred from the polluted air emission to a liquid phase flowing counter currently in a packed column (absorption step). The absorption column is interconnected to a stirred tank reactor where the N_2O transferred to the liquid phase in the absorption step is biologically reduced to N_2 under anoxic conditions (biotransformation step). However, the maintenance of anaerobic conditions in the denitrification tank requires the external supply of a biodegradable carbon source (e.g. methanol) to biologically deplete all O_2 present in the N_2O-laden aqueous stream, with the subsequent increase in process operating costs [14]. Therefore, innovative operational strategies based on the use of free carbon sources such as wastewater in WWTPs must be developed in order to achieve cost-effective N_2O removal processes.

The aim of this work was to evaluate the feasibility of the simultaneous N_2O abatement and wastewater treatment in a lab-scale bioscrubber as a model technology for an integrated wastewater treatment. The influence of liquid recycling velocities and gas empty bed residence times on the removal of N_2O and wastewater treatment performance was also investigated.

2. Materials and methods

2.1. Chemicals and synthetic wastewater

A 40 L calibration gas mixture of 10,000 ppm_v of N_2O in N_2 was purchased from Abelló Linde S.A. (Barcelona, Spain). A modified synthetic wastewater (SW) from Bajaj, et al. [15] was used as a model urban wastewater with the following composition (in g L^{-1} of tap water): peptone 0.16, meat extract 0.11, urea 0.03, NaCl 0.007, $CaCl_2{\cdot}2H_2O$ 0.004, $MgSO_4{\cdot}7H_2O$ 0.002, K_2HPO_4 0.028, $CuCl_2{\cdot}2H_2O$ 50×10^{-6} and glucose 0.25. The final concentrations of total organic carbon (TOC), total nitrogen (TN) and PO_4^{3-} of the SW were 256.1 ± 22.7, 54.4 ± 2.9 and 11.7 ± 3.3 mg L^{-1}, respectively. All reagents were purchased from PANREAC with a purity of +99% (Barcelona, Spain). The biodegradability of the SW was experimentally determined in independent batch assays by monitoring the TOC and TN concentrations for 14 days in three 500 mL Erlenmeyer initially filled with 99 mL of sterilized SW and 1 mL of activated sludge from Valladolid WWTP (Spain). Two non-inoculated sterilized Erlenmeyer with 100 mL of SW were used as controls to elucidate any potential carbon or nitrogen abiotic removal.

2.2. Experimental set up

A lab-scale bioscrubber was set up for the continuous abatement of a diluted air emission of N_2O and the simultaneous treatment of SW for 140 days. The experimental system was composed of a N_2O absorption column made of PVC (8.3 cm of inner diameter, 53 cm height) and packed with 2 L of Kaldnes rings (High Density Polyethylene rings of 50% porosity, diameter = 0.9 cm, Evolution Aqua, United Kingdom) interconnected with a 3 L fixed bed bioreactor (FBR) (Afora S.A., Spain). The FBR was filled with 1 L of methylotrophs-containing polyurethane foam (PUF) cubes (1 cm^3) used in a previous experiment as the packed bed of an absorption column [14]. The FBR was constructed with a 0.55 L liquid distribution chamber located at the bottom of the tank and operated with magnetic stirring at 300 rpm (Fig. 1). The experimental set-up was located in an air-conditioned room at 25 °C. Prior to inoculation, an abiotic test was performed with tap water for 4 days in order to assess any potential removal of N_2O by adsorption or photodegradation in the experimental set-up.

2.3. Bioscrubber operation

The SW was introduced at the bottom of the FBR, where it mixed with the N_2O-laden recycling liquid from the absorption column, and was further recirculated from the top of the FBR to the top of the packed bed absorption column using a peristaltic pump (Watson Marlow, UK). The N_2O-laden air emission was introduced at the bottom of the absorption column flowing upwards counter currently with the recycling liquid. The synthetic N_2O-laden air inflow was obtained by mixing 660 mL min^{-1} of air and 6.7 mL min^{-1} of the 10,000 ppm_v N_2O calibration gas mixture using a mass flow controller (Aalborg, Denmark), resulting in a gas empty bed residence time (EBRT) in the absorption column of 3 min and a mean N_2O concentration of 100 ± 8 ppm_v, which correspond to typical off-gas emissions from WWTPs. The SW was supplied to the FBR at flow rates determined by the maintenance of anoxic conditions (targeting a dissolved oxygen concentration = 0 mg L^{-1}) in the FBR. No N_2O (0.05 ± 0.06 ppm_v corresponding to the atmospheric N_2O concentration) was supplied to the inlet air for the first 18 days of operation (stage I) in order to assess any potential N_2O generation in the system as a result of wastewater treatment. During stage I, the bioscrubber was operated with a SW flow rate of 3 ± 0.1 L d^{-1} and a liquid recycling velocity (U_L) of 1 m h^{-1}. Stage II (days 19–51) was characterized by process operation at a N_2O of 100 ± 7 ppm_v, U_L of 1 m h^{-1} and a SW flow rate of 4 ± 1 L d^{-1}. U_L was increased up to 4 m h^{-1} during stage III (days 52–83) concomitantly with an increase in SW flow rate to 19 ± 1 L d^{-1}, while maintaining the inlet N_2O concentration at 104 ± 11 ppm_v. The bioscrubber was operated from day 84 to 104 (stage IV) with a U_L of 8 m h^{-1}, a SW flow rate of 36 ± 4 L d^{-1} and at 95 ± 5 ppm_v of N_2O. Similar SW flow rates and U_L were maintained during stage V (days 105–118) while maintaining the N_2O concentration at 100 ± 5 ppm_v, which was

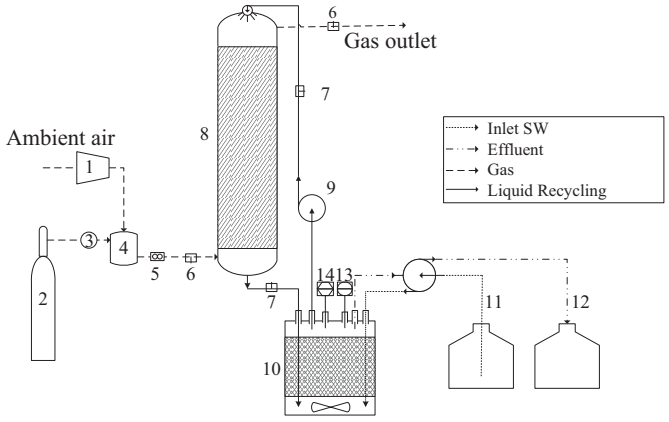

Fig. 1. Schematic of the bioscrubber set-up. (1) Air compressor (2) N_2O reservoir (3) Mass flow controller (4) Mixing chamber (5) Gas flowmeter (6) Gas sampling port (7) Liquid sampling port (8) Absorption packed bed column (9) Liquid recycling pump (10) Denitrifying fixed bed reactor (11) Synthetic wastewater reservoir (12) Effluent storage tank (13) DO electrode (14) pH electrode.

characterized by the supplementation to the SW of $CuCl_2{\cdot}2H_2O$ at 50 μg Cu^{2+} L^{-1} in order to assess the influence of copper (a cofactor of the nitrous oxide reductase) on N_2O degradation. Finally, the 3L-FBR volume was substituted by a new 7.5 L-FBR in stage VI (days 119–135) in order to enhance N_2O reduction under process operation at a U_L of 8 m h^{-1}, a SW flow rate of 38 ± 1 L d^{-1} and at a N_2O inlet concentration of 100 ± 5 ppm_v. The packed bed of the initial FBR, plus 4 L of new PUF cubes, constituted the packing medium of the 7.5 L FBR. From day 136 to 140, the EBRT was stepwisely increased to 6, 12, 18, 40 and 80 min under the operational conditions set in stage VI. A summary of the operational conditions evaluated in the bioscrubber is presented below (see Table 1).

Liquid samples from the SW at FBR inlet, and inlet and outlet of the absorption column were periodically drawn to determine the concentration of NO_2^- and NO_3^-. TOC, inorganic carbon (IC) and TN concentrations were also measured in the SW inlet and effluent of FBR. The N_2O and CO_2 gas concentrations were determined at the inlet and outlet of the absorption column. In addition, the aqueous N_2O concentration of the recycling liquid was measured by headspace method at the inlet and outlet of the FBR to assess the denitrification capacity of the system. All measurements were carried out three times a week. The biomass concentration as total suspended solids (TSS) and the dissolved reactive orthophosphate (PO_4^{-3}) concentration were also measured in the SW and bioscrubber effluent under steady state conditions.

2.4. Analytical procedures

The N_2O and CO_2 gas concentration were measured by GC-ECD and GC-TCD according to Frutos, et al. [14] and López, et al. [16], respectively. The aqueous N_2O, TOC, IC, TN and O_2 concentrations were determined following the methodology described in Frutos, et al. [14]. NO_2^-, NO_3^- and PO_4^{-3} were measured colorimetrically in a UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan) according the Standard Methods 4500-NO_2^- B, 4500-NO_3^- E and 4500-P C, respectively [17].

2.5. Molecular biology analysis

Samples of biomass from the methylotrophs-containing PUF used as inoculum and from the biomass entrapped in the PUF of the 7.5 L FBR at the end of the experimentation (day 140) were stored immediately at −20 °C to assess the diversity and composition of the microbial community. The genomic DNA was extracted according Lebrero, et al. [18]. The PCR mixture was composed of 25 μL of BIOMIX ready-to-use 2 × reaction mix (Bioline, Ecogen), 2 μL of the extracted DNA, 2 μL of the PCR primers 968-F-GC and 1401-R (10 μM) (Sigma–Aldrich, St. Louis, MO, USA) for bacterial 16S rRNA gene amplification, and Milli-Q water up to a final volume of 50 μL. The PCR thermo-cycling program used and the DGGE analysis was previously described in Lebrero, et al. [18]. The gels were stained with GelRed Nucleic Acid Gel Stain (biotium) for 1 h 30 min and the obtained DGGE patterns processed using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product–moment correlation coefficient [19]. The peak heights in the densitometric curves were also used to determine the Shannon–Wiener diversity index (H).

The most relevant bands were excised from the DGGE gel and the procedure was previously described in Lebrero, et al. [18]. The taxonomic position of the sequenced DGGE bands was obtained using the RDP classifier tool (50% confidence level) [20]. The closest matches to each band were obtained using the BLAST search tool at the NCBI (National Centre for Biotechnology Information) [21]. Sequences were deposited in GenBank Data Library under accession numbers KT200317–KT200331.

2.6. Statistical analysis

Results from the bioscrubber performance were evaluated using a parametric Student's t-test and Kruskal Wallis ANOVA non-parametric tests, both at 95% confidence level to assess any significant influence of the operational conditions tested on process parameters.

3. Results

No significant N_2O removal (<2%) by adsorption or photolysis was observed during the 4-days abiotic test. The average error in N_2O measurements by GC-ECD was 2.1% and therefore any potential N_2O degradation recorded during experimentation was attributed to microbial activity. On the other hand, the pH of the bioscrubbing solution during the 140 days of biotic operation remained roughly constant at 7.3 ± 0.2. During stage I (days 1–18), N_2O gas concentrations of 1 ± 1 ppm_v were recorded at the outlet of the absorption column in the absence of N_2O supply at a U_L of 1 m h^{-1} (Fig. 2a). The determination of the aqueous N_2O during this stage showed higher concentrations at the inlet (46 ± 16 μg N_2O L^{-1}) than at the outlet of the FBR (15 ± 4 μg N_2O L^{-1}) (Fig. 2b). With a SW flow rate of 3 ± 0.1 L d^{-1}, a steady state TOC and TN removal efficiencies of 90 ± 5% and 75 ± 15% were recorded, respectively (Fig. 3a and c). This TOC removal resulted in elimination capacities (ECs) of 184 ± 24 g C m^{-3} d^{-1} and CO_2 gas production rates of 138 ± 8 g C m^{-3} d^{-1} (Fig. 3b). Steady state NO_3^- concentrations in the inlet SW, effluent of the FBR and outlet of the absorption column accounted for 0.93 ± 0.17, 0.13 ± 0.05 and 0.55 ± 0.31 mg N L^{-1}, respectively (Fig. S1). Likewise, NO_2^- concentrations of 0.07 ± 0.06, 0.39 ± 0.20 and 1.27 ± 0.51 mg N L^{-1} were recorded in the SW, effluent of the FBR and outlet of the absorption column (Fig. S1).

Process operation at an inlet N_2O gas concentration of 100 ± 7 ppm_v and a U_L of 1 m h^{-1} during stage II (days 19–51) was characterized by a steady gas N_2O removal efficiency (RE) of 8 ± 3% (Fig. 2a). Under these particular conditions, an efficient dissolved N_2O removal by anoxic denitrification in the FBR was observed (65 ± 16%) (Fig. 2b). An increase in the TOC-RE up to 95 ± 3% was recorded, which corresponded to an EC of 327 ± 35 g C m^{-3} d^{-1} and a CO_2 production rate of 277 ± 43 g C m^{-3} d^{-1}. Likewise,

Table 1
Operational conditions under the different stages evaluated.

Stages	Operational conditions						
	N_2O concentration (ppm_v)	Operation days	U_L ($m\ h^{-1}$)	EBRT (min)	SW flow rate ($L\ d^{-1}$)	Addition of Cu^{2+} in the SW ($\mu g\ L^{-1}$)	FBR volume (L)
I	0.05 ± 0.06	0–18	1	3	3 ± 0.1	0	3
II	100 ± 7	19–51	1	3	4 ± 1	0	3
III	104 ± 11	52–83	4	3	19 ± 1	0	3
IV	95 ± 5	84–104	8	3	36 ± 4	0	3
V	100 ± 5	105–108	8	3	36 ± 4	50	3
VI	100 ± 5	119–135	8	3	38 ± 1	50	7.5

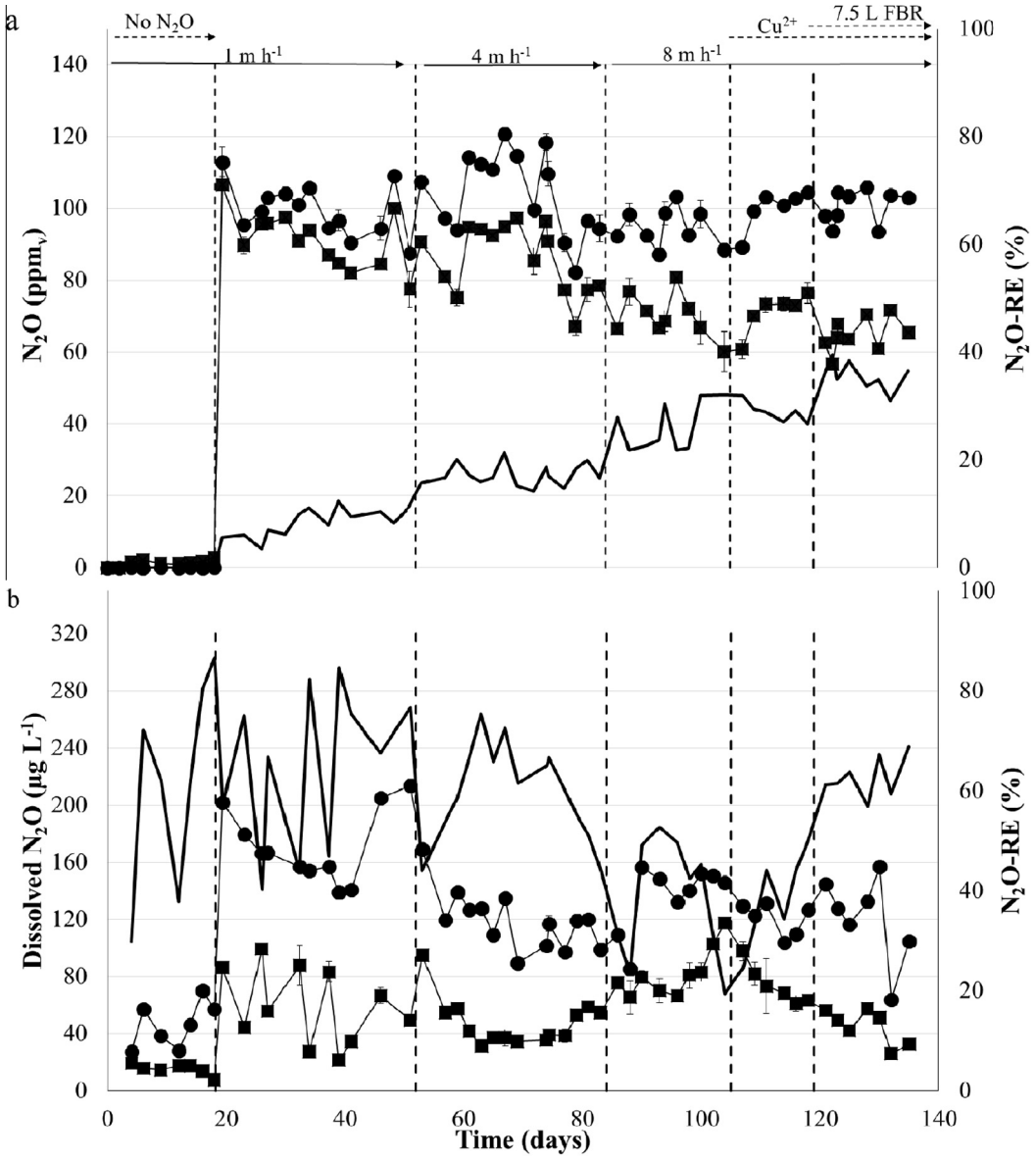

Fig. 2. Time course of the (a) inlet (●) and outlet (■) N_2O gas concentrations and N_2O removal efficiency (solid line) in the bioscrubber, and (b) inlet (●) and outlet (■) aqueous N_2O concentrations and N_2O removal efficiency (solid line) in the FBR. Vertical bars represent the standard deviation from duplicate measurements.

TN-RE during stage II reached 90 ± 2% (Fig. 3c). On the other hand, NO_3^- concentrations in the SW, effluent of the FBR and outlet of the absorption column remained constant during stage II at 0.92 ± 0.18, 0.46 ± 0.26 and 0.76 ± 0.35 mg N L^{-1}, respectively (Fig. S1). Likewise, NO_2^- concentrations of 0.04 ± 0.02, 0.37 ± 0.47 and 0.30 ± 0.40 mg N L^{-1} were recorded at the above referred sampling points (Fig. S1). Finally, PO_4^{3-} removal efficiency under steady state accounted only for 21%, which resulted in effluent concentrations of 7.3 mg P L^{-1} (Table S1). Negligible TSS concentrations were observed in the FBR effluent at the end of stage II (e.g. <0.1 mg L^{-1}).

From day 52 to 83 (stage III), the inlet N_2O gas concentration was maintained at 104 ± 11 and U_L was increased to 4 m h^{-1}. This entailed an enhancement in the gas N_2O degradation up to steady state REs of 17 ± 2% (Fig. 2a). During stage III, the reduction to N_2 of

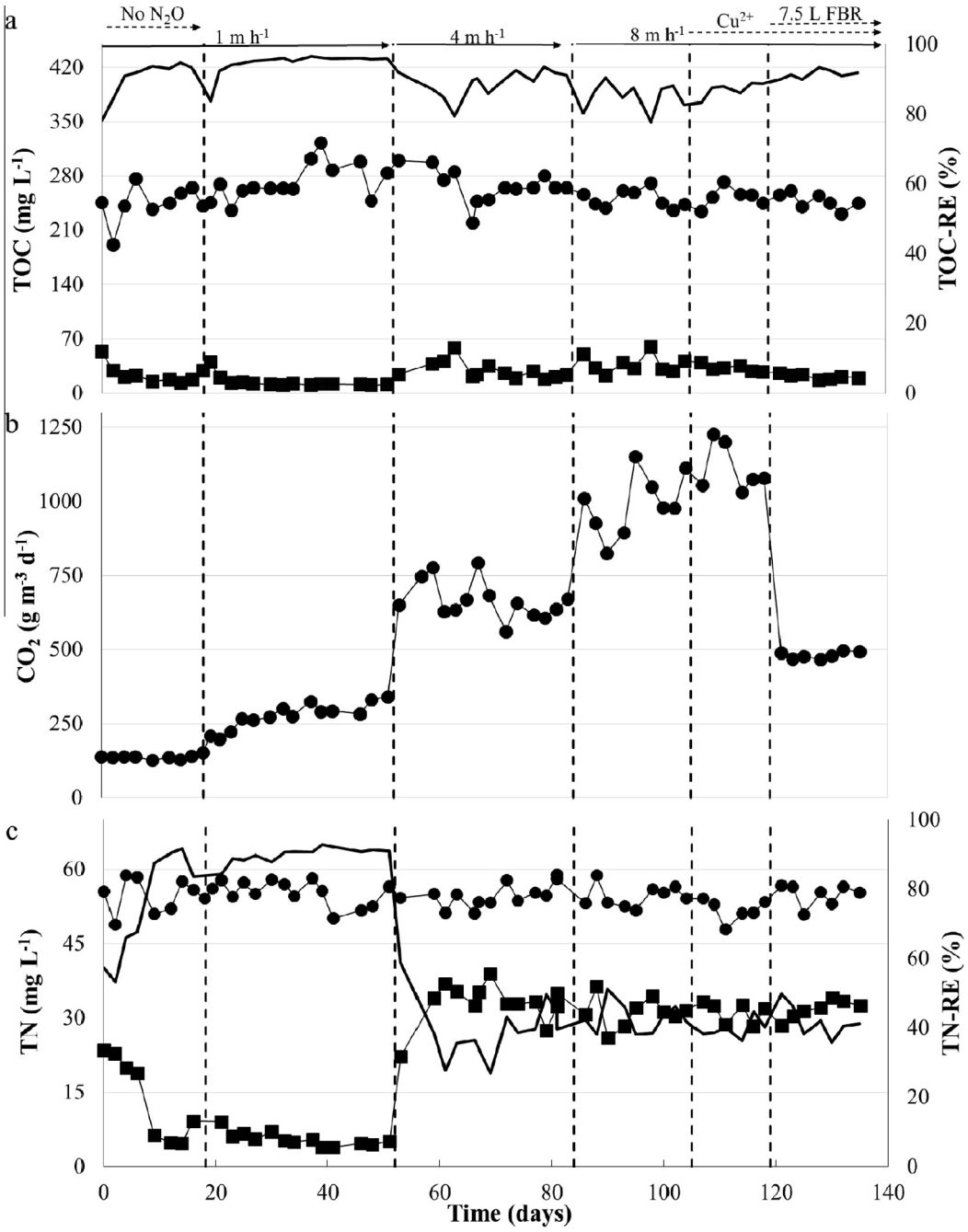

Fig. 3. Time course of the (a) inlet (●) and outlet (■) TOC concentrations and TOC removal efficiency (solid line) in the FBR; (b) CO_2 production rate (●); and (c) inlet (●) and outlet (■) TN concentrations and TN removal efficiency (solid line) in the FBR.

the aqueous N_2O in the FBR remained similar at 60 ± 10% despite the 4 times increase in U_L (Fig. 2b). However, a slight but significant decrease in the TOC removal efficiency was recorded (89 ± 4%) along with the increase in the SW flow rate to 19 ± 1 L d^{-1} required to deplete O_2 concentration in the FBR (Fig. 3a). This increase in the SW loading rate resulted in an increase in the TOC-EC up to 1503 ± 131 g C m^{-3} d^{-1} and in a CO_2 production rate of 667 ± 66 g C m^{-3} d^{-1} (Fig. 3b). Surprisingly, a severe decrease in the steady state TN removal efficiency to 40 ± 9% was observed (Fig. 3c). Under these particular conditions, the concentrations of NO_3^- in the SW, effluent of the FBR and outlet of the absorption column at steady state were 0.77 ± 0.27, 0.04 ± 0.08 and 0.04 ± 0.08 mg N L^{-1}, respectively, while NO_2^- concentrations were negligible (Fig. S1). Finally, despite the removal efficiency of PO_4^{3-} increased up to 49 ± 1%, the effluent concentration remained at 8.5 ± 1 mg P L^{-1} as a result of the increase in SW phosphorus concentration to 16.6 ± 0.1 mg P L^{-1}. The concentrations of TSS in the effluent during stage III remained constant at 0.08 ± 0.01 g L^{-1} (Table S1).

With an inlet N_2O gas concentration of 95 ± 5 ppm_v, a steady state gas N_2O RE of 26 ± 5% (Fig. 2a) was recorded along with the increase in U_L to 8 m h^{-1} in stage IV (days 84–104). A concomitant deterioration in the N_2O denitrification efficiency in the FBR to 38 ± 12% occurred (Fig. 2b). The increase in the SW flow rate up to 36 ± 4 L d^{-1} in order to maintain anoxic conditions in the FBR mediated a decrease in the TOC-RE (Fig. 3a) to 85 ± 4%, which

corresponded to an EC and a CO_2 production rate of 2599 ± 95 and 992 ± 103 g C m^{-3} d^{-1}, respectively (Fig. 3b). Nevertheless, TN removal showed no significant variation under steady state conditions during stage IV (43 ± 4%) (Fig. 3c). NO_3^- and NO_2^- were only detected in the SW at 0.46 ± 0.33 mg N L^{-1} and 0.07 ± 0.06 mg N L^{-1}, respectively (Fig. S1). The increase in SW flow rate resulted in PO_4^{3-} removal efficiencies of 52 ± 6% and in an increase in TSS effluent concentration up to 0.14 ± 0.04 g L^{-1} (Table S1).

During stage V, $CuCl_2{\cdot}2H_2O$ was added to the SW at a concentration of 50 μg L^{-1} from day 105, while the inlet N_2O gas concentration and U_L were maintained at 100 ± 5 ppm_v and 8 m h^{-1}, respectively. These conditions supported steady state gas N_2O REs of 29 ± 2% and N_2O denitrification efficiencies of 38 ± 9% in the FBR. The removal efficiencies of TOC and TN during stage V remained constant at 87 ± 2 and 40 ± 3%, respectively (Fig. 3a and c) in spite of the increase in SW flow rate to 36 ± 4 L d^{-1}. The CO_2 production rate and TOC-EC under steady state conditions were 1112 ± 82 g C m^{-3} d^{-1} and 2732 ± 179 g C m^{-3} d^{-1}, respectively (Fig. 3b). NO_3^- and NO_2^- were only detected in the SW at 0.20 ± 0.05 and 0.28 ± 0.12 mg N L^{-1} (Fig. S1), respectively. The PO_4^{3-} removal efficiency of the FBR during stage V reached 58 ± 13% and resulted in effluent concentrations of 3.7 ± 1 mg P L^{-1}. On the other hand, TSS concentrations in the SW and effluent were 0.04 ± 0.02 and 0.12 ± 0.04 g L^{-1}, respectively (Table S1).

Finally, the increase in the volume of FBR from 3 L to 7.5 L during stage VI (days 119–135) brought about a gas N_2O RE of 36 ± 3% and an increase in the removal of the aqueous N_2O up to 63 ± 4% in the FBR under similar conditions as those described in stage V. A TOC removal efficiency of 91 ± 1% was recorded under steady state operation (Fig. 3a) along with an EC of 1133 ± 51 g C m^{-3} d^{-1} at a SW flow rate of 38 ± 1 L d^{-1}. Likewise, a decrease of CO_2 production rate to 482 ± 12 g C m^{-3} d^{-1} occurred (Fig. 3b). The recorded TN-RE remained at 42 ± 5% (Fig. 3c). Negligible concentrations of NO_3^- and NO_2^- were recorded at the FBR effluent and at the outlet of the absorption column (Fig. S1), whereas the NO_3^- and NO_2^- concentrations in the SW were 0.32 ± 0.18 and 0.27 ± 0.20 mg N L^{-1}, respectively. PO_4^{3-}-REs remained constant at 57 ± 7%, with effluent concentrations of 4.3 ± 0.2 mg P L^{-1}. The TSS effluent concentration decreased to 0.06 ± 0.01 g L^{-1} in this last stage.

Process operation at an EBRT of 3 min, U_L of 8 m h^{-1} and an inlet N_2O gas concentration of 105 ± 8 ppm_v supported a gas N_2O-RE of 36 ± 0.2% along with dissolved N_2O and TOC removals of 69 ± 1 and 92%, respectively. The stepwise increase in gas EBRT in the absorption column resulted in a sequential enhancement in the gas N_2O-REs but in a deterioration in the removal efficiencies of aqueous N_2O and TOC (Fig. 4). Hence, the highest gas N_2O-RE (94 ± 0.2%) was achieved at an EBRT of 80 min along with TOC and dissolved N_2O removal efficiencies of 74% and 17 ± 2%, respectively (Fig. 4).

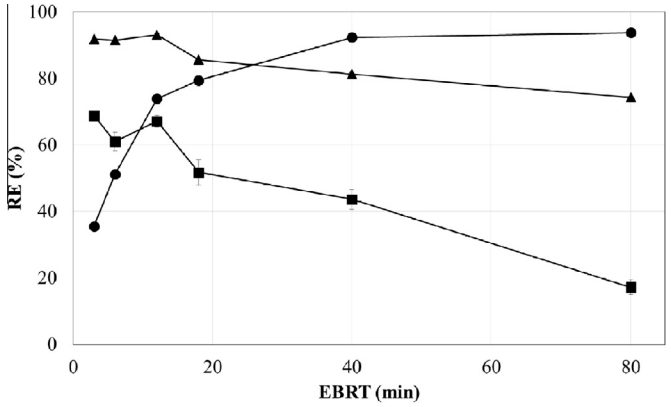

Fig. 4. Influence of the EBRT on the removal efficiencies of gas N_2O (●), aqueous N_2O (■) and total organic carbon (▲). Vertical bars represent the standard deviation from duplicate measurements.

The Shannon-Wiener diversity indices of the microbial communities present in the inoculum and at the end of the experimentation were 3.48 and 3.47, respectively (Fig. 5). The initial and final bacterial populations were analyzed using the Pearson similarity correlation coefficient in order to elucidate the structure of the bacterial communities in the FBR. A similarity value of 11.7% was obtained between the communities present at day 0 and day 140. From the DGGE gel, 15 bands were sequenced (Fig. 5) and 4 different phyla were identified from the RDP database: *Proteobacteria* (11 bands), *Firmicutes* (2 bands), *Lentisphaerae* (1 band) and *Cloacimonetes* (1 band). The closest matches for every band (BLASTN) according to the NCBI database, together with its similarity percentages and sources of origin, are provided as Supplementary material (Table S2). Furthermore, phylogenetic relationships between the bands sequenced from the DGGE gel of (a) the microbial community of the inoculum and (b) final operational stage and their closest relatives in Gen-Bank (obtained by the Blast search tool) were carried out (Fig. 6).

4. Discussion

This work demonstrated the feasibility of a simultaneous removal of N_2O from WWTP air emissions coupled to wastewater treatment using an innovative absorption unit-anoxic tank bioscrubber configuration. The proficient performance of a bioscrubber configuration treating a N_2O air emission using methanol as a carbon and electron donor source was demonstrated in a previous work [14]. However, the supply of such an expensive external carbon source at the rates required entails prohibitive operational costs. For this reason, the potential of wastewater (a free resource available in WWTPs) was evaluated as an alternative carbon and electron donor source to support a cost-effective N_2O removal in an innovative process that can also support satisfactory levels of wastewater treatment. N_2O removal was based on the sequential N_2O mass transfer from the gas to the recycling liquid in the packed bed column followed by N_2O reduction under anoxic conditions in the FBR using the organic matter present in the SW as electron donor. This two stage (absorption-anoxic biodegradation) strategy is crucial to maintain the anoxic conditions required for N_2O reduction to N_2. The single stage configuration for air pollution control in biofilters or biotrickling filters would not be able to support the reduction of N_2O from WWTP air emissions due to the inherent presence of oxygen. In fact, the sole degradation mechanism of N_2O was heterotrophic denitrification in the FBR, while no significant N_2O biodegradation was observed in the absorption column likely due the high O_2 levels in the air emission. The technology herein proposed could be implemented at large scale in anoxic tanks of WWTPs via interconnection to an external absorption column.

N_2O production was observed in the first operational stage (Fig. 2), which could be attributed to the oxidation of the hydroxylamine produced by the action of ammonium oxidizing bacteria in the absorption column [4]. The sequential increase in U_L mediated the enhancement in the gas N_2O RE as a result of the higher turbulence in the gas/liquid interface (which likely increased the N_2O mass transfer coefficients [22]) and the higher N_2O carry over capacity of the recycling liquid. Hence, the increase in U_L entailed a higher aqueous N_2O loading rate to the FBR since the dissolved N_2O concentration at the outlet of the absorption column was close to saturation regardless of the operational conditions. However, it is worth noting that this stepwise increase in U_L deteriorated the dissolved N_2O removal in the 3 L FBR likely due to its associated

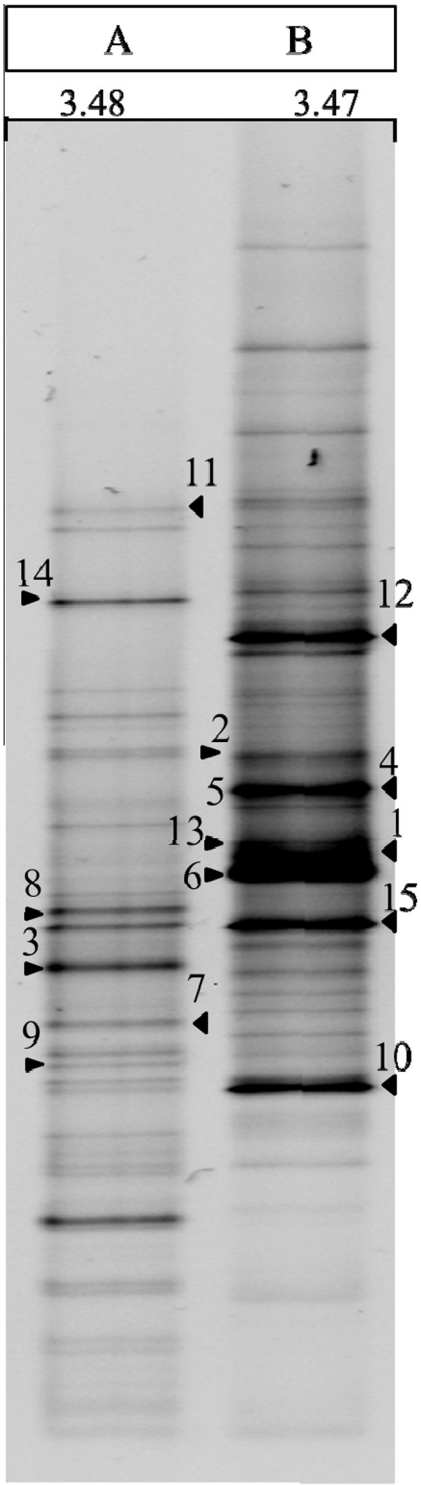

Fig. 5. Bacterial DGGE profiles. Sample names and Shannon diversity indices are indicated in the upper part of the gel: (A) inoculum sample, (B) FBR end operation sample. The sequenced DGGE bands are indicated with an arrow (►) and the corresponding number of each band.

decrease in the recycling liquid residence times from 32 min at a U_L of 1 m h^{-1} to 4 min at a U_L of 8 m h^{-1}. Thus, the dissolved N_2O removal efficiency dropped from 65 ± 16% in stage II to 38 ± 9% in stage V (Fig. 2b), corresponding to the lowest and highest U_L evaluated in the bioscrubber, respectively. This deterioration in the dissolved N_2O removal likely caused the low gas N_2O RE of 29 ± 2% recorded in stage V as a result of the reduced gas–liquid N_2O concentration gradient (Fig. 2a). In this context, the 3 L FBR was replaced by a 7.5 L FBR in stage VI in order to enhance the reduction in dissolved N_2O in the anoxic tank, which would allow operating the absorption column at a maximum concentration gradient. The higher recycling liquid residence time in the 7.5 L FBR (≈10 min) promoted an increase in the dissolved N_2O REs up to 63 ± 4% (Fig. 2b), which consequently resulted in the enhancement of the gas N_2O REs up to 36 ± 3% (Fig. 2a). Copper is a structural component of the nitrous oxide reductase, the enzyme supporting the final reduction step of N_2O to dinitrogen in the bacterial denitrification pathway [23]. In our particular study, Cu^{2+} supplementation to the SW from stage V onward did not induce any significant improvement in N_2O reduction in the anoxic tank. Conversely, Zhu, et al. [24] did observe a decrease in N_2O production by 55–73% following the addition of 50–100 μg Cu^{2+} L^{-1} in a 4 L anaerobic–aerobic–anoxic sequencing batch reactor treating municipal wastewater. Overall, the N_2O removal performance of this innovative bioscrubber configuration was likely limited by the poor mass transfer of this greenhouse gas from the air emission to the liquid phase in the absorption column due its low aqueous solubility (H = 1.6 at 25 °C [25]) and also due to the low biological denitrification efficiency of the dissolved N_2O mediated by the low residence time of the recycling liquid in the anoxic tank.

The wastewater treatment performance of the bioscrubber was characterized by high TOC removal efficiencies, similar to the maximum biodegradability (96%) of the SW used as a model wastewater (determined in an independent set of experiments). O_2, N_2O, NO_2^- and NO_3^- were simultaneously used as electron acceptors to support TOC oxidation in the FBR. A slight deterioration in TOC-RE was observed along with the sequential increase in SW flow rate to maintain the anoxic conditions needed for N_2O reduction in the FBR, which resulted in a decrease in the hydraulic retention time (HRT) of the wastewater in the bioscrubber (29, 19, 4, 2, 2 and 5 h for stages I, II, III, IV, V and VI, respectively). Thus, TOC effluent concentrations increased from 19 ± 11 mg C L^{-1} in stage II to 29 ± 11 and 31 ± 11 mg C L^{-1} in stages III and IV, respectively (Fig. 3a). These effluent TOC concentrations remained below the maximum discharge limits required by European legislation [26], which demands a BOD effluent concentration of 25 mg L^{-1} (≈70 mg C L^{-1} based on a typical BOD/TOC ratio of 0.35 for treated wastewater [27]). On the other hand, while Cu^{2+} supplementation did not influence the TOC-REs, the increase in the FBR volume in stage VI supported superior TOC-REs due to the HRT increase from 2 to 5 h. At this point it is important to highlight that a high TOC removal efficiency in the FBR is desirable to avoid biomass overgrowth in the absorption column, which punctually resulted in a reduction of the effective gas/liquid interfacial area and thus in a progressive deterioration of the N_2O mass transfer capacity of the packed bed. Despite TOC-REs slightly decreased with increasing SW flow rates, the EC of the bioscrubber increased from 327 ± 35 g C m^{-3} d^{-1} in stage I to 2732 ± 179 g C m^{-3} d^{-1} in stage V, which confirmed the potential for organic matter treatment of the technology here evaluated. Similarly, CO_2 production rates increased concomitantly with the increase in EC (Fig. 3b). Carbon mineralization ratios of ≈82% were recorded in the two first operational stages, while the sequential decrease in the HRT of the FBR resulted in a ≈53% of the carbon mineralization ratio.

Nitrogen assimilation into microbial biomass and, in a much lesser extent, ammonium nitrification in the absorption column coupled with denitrification in the anoxic tank of the produced NO_3^- and NO_2^- were the main processes governing TN removal in the bioscrubber. These mechanisms occurred simultaneously in the two first operational stages, where nitrification in the absorption column supported the high TN removals observed (Figs. 3c and S1). Furthermore, ammonia stripping in the absorption column cannot be ruled out since the wastewater fed into the FBR was

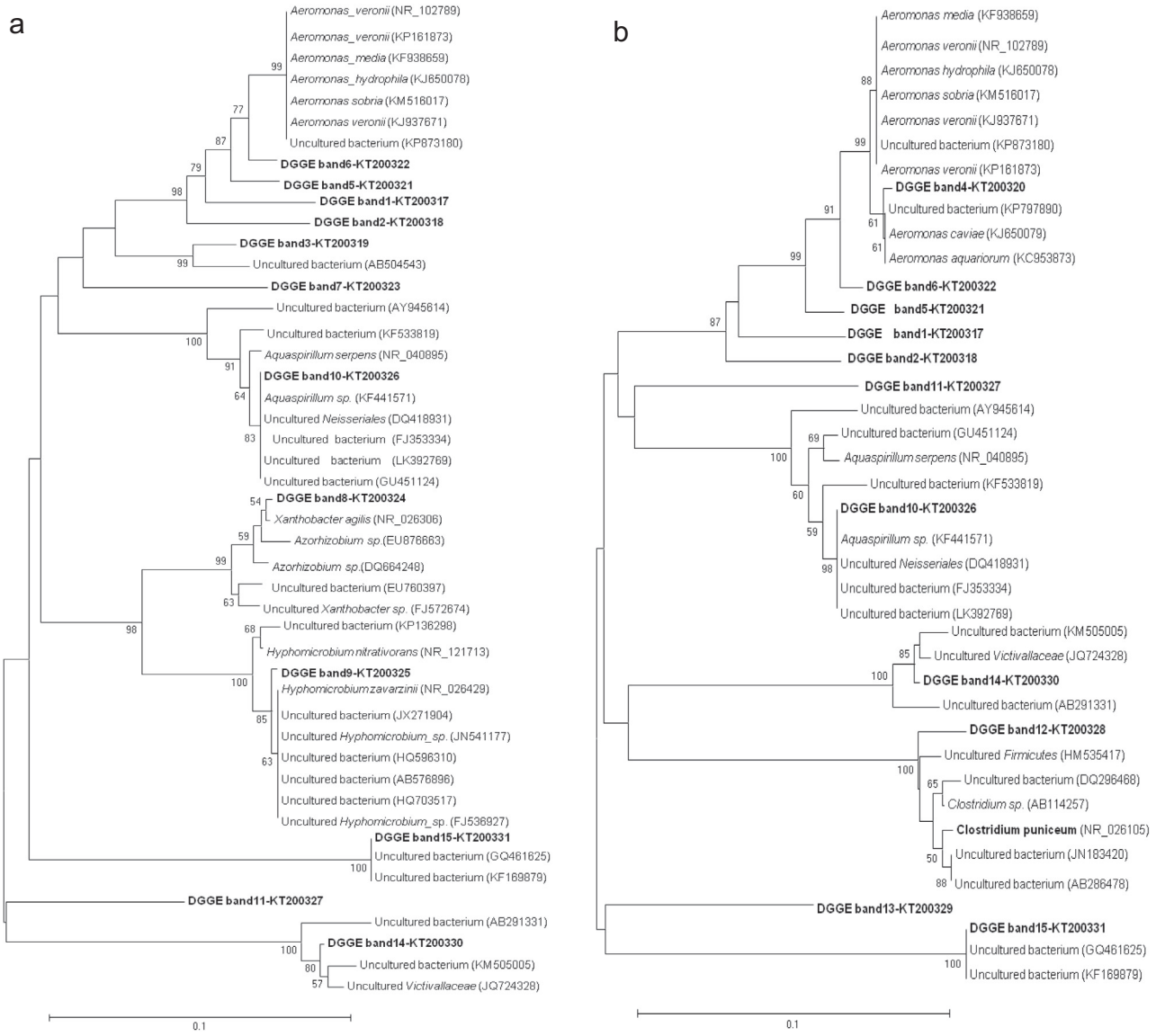

Fig. 6. Bacterial phylogenetic tree based on neighbor-joining analysis of 16S rRNA sequences from (a) the microbial community of the inoculum and (b) final operational stage and their closest relatives (similarity ⩾ 97%) in GenBank obtained by the Blast search tool. Accession numbers are indicated. Numbers on the nodes indicate bootstrap values of 50% and higher (1000 replicates). The scale bar indicates 10% sequence difference.

recycled at least 45 times/day through the absorption column during process operation at a HRT of ≈1 day and at a U_L of 1 m h^{-1}. The increase in U_L to 4 m h^{-1} lowered the TN-RE of the system, which remained at ≈40% from stage III onward (regardless of Cu^{2+} supplementation and FBR volume increase), matching the N requirement for microbial growth. Effluent TN concentrations of 14 ± 8 mg N L^{-1} and 6 ± 1 mg N L^{-1}, which complied with the EU regulatory effluent values of 15 mg N L^{-1} [26], were recorded in stages I and II, but remained above 30 mg N L^{-1} from stage III onward (Fig. 3c). The good denitrification performance of the FBR supported low effluent NO_2^- and NO_3^- concentrations throughout the entire experimentation (Fig. S1). Finally, phosphorus removal efficiencies remained at ≈50% over the entire experimental period (except during stage II), with effluent concentrations significantly higher than the maximum permissible concentrations established by EU legislation (2 mg P L^{-1}) (Table S1). The concentrations of TSS in the effluent increased over the time course of the experimentation (Table S1) likely due to the accumulation (and further detachment) of biomass in the PUF of the FBR and the higher shear stress mediated by the stepwise increase in liquid recycling. A reduction in the effluent TSS concentration was observed as a result of the FBR volume increase to 7.5 L (Table S1). Overall, despite the effluent concentrations of TN, phosphorus and TSS were often higher than those recommended by EU legislations for direct wastewater discharge, the technology here evaluated represents a cost effective pre-treatment unit, whose effluent can be returned back to the WWTP headworks or conducted to maturation ponds for tertiary treatment.

The stepwise increase in the EBRT of the air emission confirmed the potential of this innovative anoxic bioscrubber to simultaneously achieve high N_2O, organic matter and nutrient removal efficiencies (Fig. 4). A gradual increase in the gas N_2O RE at increasing EBRTs occurred, with process operation at 80 min of EBRT supporting the highest N_2O removal efficiencies. On the contrary, this increase in the EBRT induced a deterioration in the removal of both TOC and aqueous N_2O (Fig. 4), probably due to the decrease in the loading rate of electron acceptors (O_2 and N_2O) in the FBR, as confirmed by the lower aqueous N_2O concentrations at the FBR inlet at

increasing EBRTs (from 105 μg $N_2O\ L^{-1}$ at an EBRT of 3 min to 26 μg $N_2O\ L^{-1}$ at an EBRT 80 min). The in-situ generation of N_2O in the system prevented the complete abatement of N_2O. Thus, process operation at an EBRT of 40 min and a U_L of 8 m h^{-1} was here identified as the optimal operating conditions, supporting N_2O and TOC removals of 92% and 81%, respectively (Fig. 4). To the best of our knowledge, the REs here obtained under continuous operation were the highest so far reported in literature. In this context, Akdeniz, et al. [28] reported a 0.7% N_2O removal efficiency in a lava rock media biofilter inoculated with swine manure and compost, and operated at an EBRT of 5 s. Likewise, Hood, et al. [29] operated a biofilter composed of 70% compost and 30% wood chips to treat the exhaust air from a swine barn pit ventilation fan at an EBRT of 7.6 s, with N_2O removal efficiencies of 14–17% at an inlet concentration of ≈170 ppm_v. In our particular study, the high EBRTs required to achieve high N_2O REs would result in large bioscrubber volumes (with the subsequent increase in capital costs), which highlights the need for research on innovative cost-effective N_2O mass transfer enhancement strategies.

The Shannon-Wiener diversity index takes into account both the number (richness) and the evenness of the species (by evaluating and comparing the intensity of the bands), allowing to obtain semi-quantitative results from the DGGE analysis (Table S2). Typical values ranging from 1.5 to 3.5 correspond to low and high species evenness and richness, respectively [30]. Thus, the diversity indices of the inoculum and the community present in the FBR at the end of the experimental period showed a high species evenness and diversity (Fig. 5). However, a low similarity between both microbial communities was observed likely due to the different electron donor and carbon source used in this study (synthetic wastewater) compared with that used in the bioscrubber previously hosting the inoculum (methanol). Three families were the most abundant microbial communities (Table S2) in the inoculum sample: (i) the *Xanthomonadaceae* family (DGGE band 3, Figs. 5 and 6), with the capacity to carry out the full heterotrophic denitrification pathway [31], ii) the *Xanthobacteraceae* family (DGGE band 8, Figs. 5 and 6), with the genus *Xanthobacter* which is strictly aerobic and can grow chemoorganoheterotrophically in methanol [32], and iii) the stricter anaerobic *Victivallaceae* family (DGGE band 14, Figs. 5 and 6), with three uncultured species of the genus *Victivallis* [33]. Many species of the *Aeromonas* genus (DGGE bands 1–2 and 4–6, Figs. 5 and 6), which possesses the enzymatic machinery to denitrify N_2O under aerobic conditions, were observed in the final community present in the FBR (Table S2) at the end of the experimentation [34]. Furthermore, the abundance of denitrifiers *Aquaspirillum* related species (DGGE band 10, Figs. 5 and 6) in the FBR agreed with previous studies where these denitrifying *Betaproteobacteria* were found in municipal activated sludge [35]. Finally, the presence of *Clostridium sensu stricto* and *Candidatus cloacamonas* related bacteria (DGGE bands 12 and 14, respectively, Figs. 5 and 6) and anaerobic species from the order *Selenomonadales* (DGGE band 13, Figs. 5 and 6) suggested the occurrence of anaerobic niches in the FBR.

5. Conclusions

In brief, the simultaneous treatment of both N_2O-laden air emissions and wastewater was achieved in this innovative absorption unit-anoxic tank bioscrubber configuration. Higher gas N_2O REs were recorded at increasing liquid recycling velocities and gas EBRTs in the absorption column. The increase in liquid recycling velocity, which entailed an increase in the wastewater loading rate in order to maintain anoxic conditions in the FBR, resulted in a slight deterioration in the removal efficiencies of organic carbon and in the denitrification of N_2O. The increase in the HRT in the FBR enhanced the removal performance of N_2O and TOC. In our particular study, the N_2O abatement performance was mainly limited by the low denitrification activity in the FBR and the N_2O carrying capacity of the recycling liquid, which itself was restricted by the low aqueous N_2O solubility. Innovative design and operational strategies are therefore needed to overcome the gas–liquid N_2O mass transfer limitations identified in order to develop more cost efficient technologies for the abatement of N_2O.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2015.11.088.

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Simultaneous biological nitrous oxide abatement and wastewater treatment in a denitrifying off-gas bioscrubber

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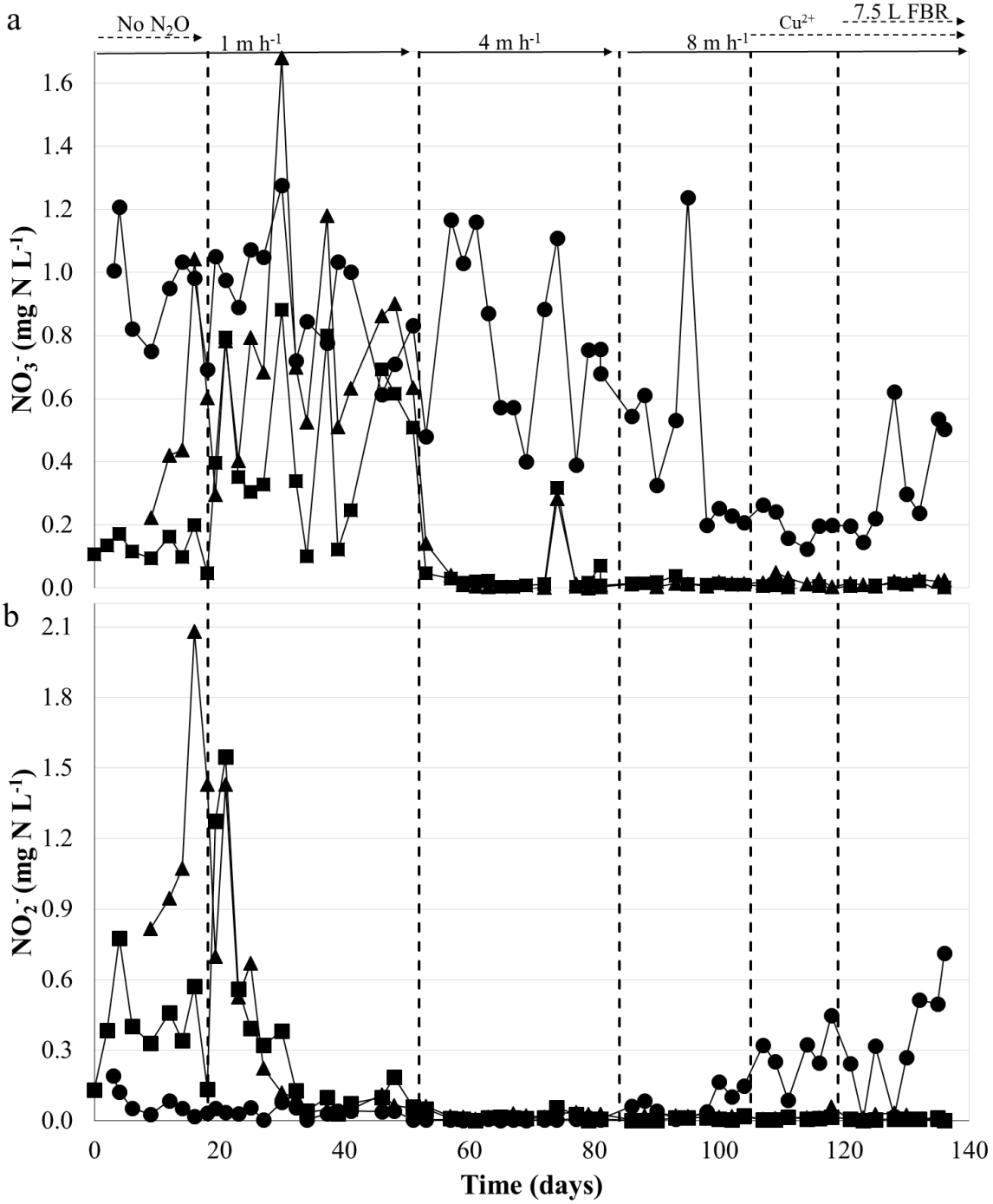

Fig. S1. Time course of (a) nitrate and (b) nitrite concentrations in the SW (●), effluent of the FBR (■) and outlet of the absorption column (▲).

Table S1. Total suspended solid and total PO_4^{3-} concentrations under steady state in the different operational stages evaluated.

Stages	PO_4^{3-} (mg P L^{-1})			TSS (g L^{-1})	
	SW	Effluent	RE (%)	SW	Effluent
II	9.3	7.3	21	0.01 ± 0.01	0
III	16.6 ± 1	8.5 ± 1	49 ± 1	0.02 ± 0.01	0.08 ± 0.01
IV	13.5 ± 5	6.6 ± 3	52 ± 6	0.06 ± 0.01	0.14 ± 0.04
V	8.8 ± 0.3	3.7 ± 1	58 ± 13	0.04 ± 0.02	0.12 ± 0.04
VI	9.9 ± 1	4.3 ± 0.2	57 ± 7	0.07 ± 0.04	0.06 ± 0.01

Table S2 RDP classification of the bacterial DGGE bands sequences and corresponding matches (Standard Nucleotide BLAST) using the NCBI database with indication of the similarity percentages and sources of origin. The relative abundance of each band in the samples was ranked by x, xx, xxx or xxxx

Taxonomic placement (50% confidence level)	Band n°	A	B	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
Phylum *Proteobacteria*	B1	x	xxxx	Uncultured *Aeromonas* sp. (GU356327)	92	South Shore wastewater treatment plant influent
				Aeromonas hydrophila (GQ407267)	92	Drinking water sources
Class *Gammaproteobacteria*	B2	x	xxx	Uncultured *Aeromonas* sp. (GU356122)	92	Jones Island wastewater treatment plant influent
				Aeromonas caviae (JF920474)	92	Wastewater treatment plant - raw wastewater
				Aeromonas caviae (KJ650079)	92	Synthetic-municipal wastewater
	B3	xxx		Uncultured bacterium(AB504543)	97	Methane oxidizing DHS reactor
				Uncultured *Xanthomonadaceae* (JF346068)	95	Biofilms grown on aluminium surfaces, exposed to water from a storm water canal for 3 and 6 hours, in a flow cell
Order *Aeromonadales* Family Aeromonadaceae Genus Aeromonas	B4		xxxx	Uncultured bacterium(KP797890)	97	Microalgae from HRAP treating diluted vinasse with wastewater treatment plant activated sludge
				Aeromonas caviae (KJ650079)	97	Synthetic-municipal wastewater
				Aeromonas aquariorum (KC953873)	97	Hypereutrophic water
	B5	x	xxx	Uncultured bacterium(KP873180)	97	Supercritical CO_2 enrichment
				Aeromonas veronii (KJ937671)	97	River water
				Aeromonas sobria (KM516017)	97	Culture collection
				Aeromonas hydrophil (KJ650078)	97	Synthetic-municipal wastewater
				Aeromonas media (KF938659)	97	Membrane bioreactor activated sludge
				Aeromonas veronii (NR_102789)	97	Culture collection
	B6	x	xxx	*Aeromonas veronii* (KP161873)	98	Rver sediment
				Aeromonas veronii (KJ937671)	98	River water
				Aeromonas sobria (KM516017)	98	Culture collection
				Aeromonas hydrophil (KJ650078)	98	Synthetic-municipal wastewater

Taxonomic placement (50% confidence level)	Band n°	A	B	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
				Aeromonas media (KF938659)	98	Membrane bioreactor activated sludge
				Aeromonas veronii (NR_102789)	98	Culture collection
Order *Xanthomonadales*						
Family *Xanthomonadaceae*	B7	xx		Uncultured bacterium(KM293030)	93	Sludge with earthworm
				Uncultured bacterium(KJ002525)	93	Aerobic activated sludge from wastewater treatment plant
				Uncultured bacterium (KJ399527)	92	Denitrifying biofilm
Class *Alphaproteobacteria*						
Order *Rhizobiales*						
Family *Xanthobacteraceae*						
Genus *Xanthobacter*	B8	xxx		*Xanthobacter agilis* (NR_026306)	99	Culture collection
				Azorhizobium sp. (EU876663)	98	Activated sludge collected from wastewater treatment system of a pesticide manufacturer
				Azorhizobium sp. (DQ664248)	97	Freshwater pond
				Uncultured bacterium (EU760397)	97	Water column
				Uncultured *Xanthobacter* sp. (FJ572674)	97	Fouled MF membrane from drinking water treatment plant
Family *Hyphomicrobiaceae*						
Genus *Hyphomicrobium*	B9	xx		Uncultured bacterium (JX271904)	99	Seed sludge
				Uncultured *Hyphomicrobium* sp. (JN541177)	99	Activated sludge from sewage treatment plant
				Uncultured bacterium (HQ596310)	99	Denitrification bioreactor
				Uncultured bacterium (AB576896)	99	Denitrifying PGE pellet samples
				Uncultured bacterium (HQ703517)	99	Denitrifying SBR with methanol
				Uncultured *Hyphomicrobium* sp. (FJ536927)	99	Waste-activated sludge from municipal waste water treatment plant
				Hyphomicrobium zavarzinii (NR_026429)	99	Culture collection
				Uncultured bacterium (KP136298)	98	Wastewater from high-rate denitrifying reactor
				Hyphomicrobium nitrativorans (NR_121713)	98	Denitrifying biofilm treating seawater
Class *Betaproteobacteria*						
Order *Neisseriales*						
Family *Neisseriaceae*						

Taxonomic placement (50% confidence level)	Band n°	A	B	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
Genus *Aquaspirillum*	B10	x	xxxx	*Aquaspirillum* sp. (KF441571)	99	Urgeirica mine, water and sediments
				Uncultured Neisseriales (DQ418931)	99	Coastal waters
				Uncultured bacterium (FJ353334)	99	Lake Charles Treatment Facility, raw sewage
				Uncultured bacterium (LK392769)	99	Activated sludge treating municipal wastewater
				Uncultured bacterium (GU451124)	98	Semi-aerobic and anaerobic landfill leachate
				Uncultured bacterium (KF533819)	97	Full-scale EBPR activated sludge plant
				Uncultured bacterium (AY945914)	97	Sludge-seeded bioreactor
				Aquaspirillum serpens (NR_040895)	97	Culture collection
Class *Deltaproteobacteria*	B11	x	xx	Uncultured *Desulfomonile* sp. (AB908725)	92	River sediment
				Uncultured bacterium (LK392795)	92	Activated sludge treating municipal wastewater
				Uncultured *Bdellovibrio* sp. (GU198955)	92	Granule biomass
Phylum *Firmicutes*						
Class *Clostridia*						
Order *Clostridiales*						
Family *Clostridiaceae 1*						
Genus *Clostridium sensu stricto*	B12		xxxx	Uncultured bacterium (JN183420)	98	Water sample
				Uncultured bacterium (AB286478)	98	Activated sludge
				Clostridium puniceum (NR_026105)	97	Culture collection
				Uncultured *Firmicutes* (HM535417)	97	Plant leaves in a freshwater environment
				Uncultured bacterium (DQ296468)	97	Lab-scale anaerobic biofilm reactor for quinoline degradation
				Clostridium sp. (AB114257)	97	Isolated from surface-sterilized plant root
Class *Negativicutes*						
Order *Selenomonadales*	B13		xxxx	Uncultured bacterium (HM749865)	96	EGSB reactor
				Bacterium enrichment (HQ602828)	96	Anaerobic and non-axenic culture, inoculated with sediment; culture enriched in benzene and AQDS
				Uncultured bacterium (HQ008114)	96	Laboratory scale EGSB hybrid bioreactors operated at 12 ºC
				Uncultured bacterium (GQ423795)	96	Anaerobic EGSB bioreactors treating a synthetic sewage-based wastewater at low temperatures < 15 ºC
Phylum *Lentisphaerae*						

Taxonomic placement (50% confidence level)	Band n°	A	B	Closest relatives in Blast Name (accession number)	Similarity (%)	Source of origin
Class *Lentisphaeria* Order *Victivallales* Family *Victivallaceae* Genus *Victivallis*	B14	xxx	xx	Uncultured bacterium (KM505005) Uncultured *Victivallaceae* (JQ724328) Uncultured bacterium (AB291331)	99 99 97	Biotrickling filter for nitrous oxide abatement Biofilm from electrode material in a microbial fuel cell Mesophilic UASB sludge granules
Phylum *Cloacimonetes* Genus *Candidatus cloacamonas*	B15	xxx	xxxx	Uncultured bacterium (GQ461625) Uncultured bacterium (KF169879)	100 99	Anaerobic dechlorinating culture Mesophilic anaerobic digester fed with brown water and food waste

N_2O nitrification feasibility studies

O.D. Frutos, R. Lebrero, R. Muñoz. (Unpublished manuscript)

Chapter 3

N_2O nitrification feasibility studies

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Abstract

The sole biological pathway for nitrous oxide (N_2O) degradation identified to date is anaerobic heterotrophic denitrification. Since most N_2O emissions are typically characterized by the presence of high O_2 concentrations, the occurrence of the anaerobic conditions required for N_2O reduction represents a limiting factor for the widespread implementation of biotechnologies as N_2O emission control strategy. Therefore, there is an urgent need for the identification of alternative mechanisms of N_2O biodegradation, aerobic N_2O oxidation to nitrate or nitrite being the most plausible one. This hypothesis is based on the thermodynamic feasibility of N_2O oxidation to nitrite and nitrate by nitrifying bacteria ($\Delta G^{o\prime}$= -87.4 and -15 kJ, respectively). Thus, two independent experiments were conducted to elucidate the feasibility of these pathways. Firstly, a 3 L biotrickling filter reactor inoculated with activated sludge was continuously operated with a synthetic emission of N_2O-laden air at a concentration of 157 ± 17 ppm_v and a gas empty bed residence time of 3 min. Secondly, gas-tight batch assays were conducted in 1.2 L bottles sealed with rubber septa under a 11400 ± 160 ppm_v of N_2O-air headspace. The bottles were inoculated with activated sludge, a consortium of ammonia oxidizing and nitrite oxidizing bacteria, and ANAMMOX cultures. The results recorded in both experiments did not show any significant evidence of biological removal of N_2O by nitrification despite the maintenance of optimal environmental conditions for nitrifying bacteria growth.

Introduction

The sole biological mechanism reported to date for the degradation of nitrous oxide (N_2O) is based on the dissimilatory reduction pathway of nitrate (NO_3^-) named heterotrophic denitrification [1]. This process occurs when facultative bacteria use NO_3^- as the electron acceptor for the biological oxidation of organic matter in the absence of oxygen (O_2). N_2O is an obligate intermediate in this reduction process and most denitrifying bacteria possess the enzyme that reduces N_2O to the harmless molecule of N_2 (namely N_2O reductase) [2]. Thus, anaerobic conditions (absence of O_2) are required for the activation of this metabolic pathway, which results in severe technical limitations when biological technologies are to be implemented for the treatment of N_2O emissions based on their high O_2 content [3].

In this context, alternative biological mechanisms for N_2O biodegradation are required. For instance, a pathway mediating N_2O assimilation to organic nitrogen to form the building blocks for proteins synthesis, similar to the N_2 assimilation process, has been theoretically proposed [4–6]. However, there is still ongoing debate about the feasibility of this pathway and further empirical evidences are needed. Likewise, the aerobic oxidation of N_2O to nitrate or nitrite constitutes also thermodynamically favorable processes. Indeed, the stoichiometric semi-reactions of N_2O oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) by nitrifying bacteria using O_2 as electron acceptor can be described by equations 1 and 2, respectively.

In this context, two independent experiments were conducted to assess the potential nitrification of N_2O in a biotrickling filter (BTF) reactor and gas-tight batch bioreactors using multiple inoculum sources.

$$\boldsymbol{N_2O + O_2 + H_2O \rightarrow 2NO_2^- + 2H^+ \left(\Delta G^{0'} = -87.4\ kJ\right)} \quad \textbf{(1)}$$

$$\boldsymbol{N_2O + 2O_2 + H_2O \rightarrow 2NO_3^- + 2H^+ \left(\Delta G^{0'} = -15\ kJ\right)} \quad \textbf{(2)}$$

Material and Methods

Chemicals and mineral salt medium

The mineral salt medium (MSM) used for the evaluation of the continuous N_2O nitrification in the BTF was composed of (in g L^{-1}): $Na_2HPO_4{\cdot}12H_2O$ 6.15, KH_2PO_4 1.52, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCl_2$ 0.02, $NaHCO_3$ 2, NH_4Cl 0.04, and 1 mL L^{-1} of a trace element solution (containing per liter: EDTA 5.35 g, $FeCl_2{\cdot}4H_2O$ 2.49 g, $MnCl_2{\cdot}4H_2O$ 0.12 g, $CoCl_2{\cdot}6H_2O$ 0.03 g, $CuCl_2{\cdot}2H_2O$ 0.02 g, $NiCl_2{\cdot}6H_2O$ 0.03 g, Na_2MoO_4 0.03 g, $Na_2WO_4{\cdot}2H_2O$ 0.04 g). A similar MSM was used in the batch

assays with NH_4Cl and $NaHCO_3$ at concentrations of 0.19 and 1.4 g L^{-1}, respectively. All chemicals were purchased from PANREAC with a purity of +99 % (Barcelona, Spain). The N_2O gas was provided by Abelló Linde S.A. (Barcelona, Spain) in 40 L cylinders with a concentration of ~50000 ppm_v in N_2.

Biotrickling filter set up and operation

The BTF operated for the continuous N_2O oxidation was composed of a 3 L (PVC) absorption column (filled with 2 L of polyurethane foam cubes of 1 cm^3) interconnected to a stirrer tank reactor (STR) of 7.5 L magnetically stirred at 300 rpm (Figure 1). The N_2O-laden air emission was obtained by mixing air with a stream of the 50000 ppm_v N_2O standard regulated by means of a mass flow controller (Aalborg, Denmark), which resulted in inlet N_2O concentrations of 157 ± 17 ppm_v (mimicking the emissions from wastewater treatment plants). The synthetic N_2O air emission was introduced at the bottom of the absorption column at a gas empty bed residence time of 3 min and counter currently with the MSM recycled from the STR at a trickling velocity of ~1 m h^{-1}. Fresh MSM was fed at the bottom of the STR at a flow rate of 0.6 L d^{-1}. The system was initially inoculated with 1 L of activated sludge from Valladolid wastewater treatment plant. The experiment was conducted at 25 ºC.

The BTF was operated for 90 days with a periodic monitoring of the N_2O and CO_2 gas concentrations at the inlet and outlet of the absorption column. Similarly, the aqueous N_2O, NO_2^- and NO_3^- concentrations from the recycling liquid were measured at the inlet and outlet of the STR. In addition, the concentrations of total nitrogen (TN) and $N\text{-}NH_4^+$ in the MSM and SRT effluent were periodically measured. The pH was periodically determined and adjusted at 7.5 by manual addition of HCl (1 M).

Batch experiments

This study assessed the potential nitrification of N_2O by three different NH_4^+ degrading inocula in gas-tight batch assays. The experiment was conducted in 1.2 L gas tight bottles using activated sludge from Valladolid wastewater treatment plant, nitrifying bacteria (ammonia oxidizing bacteria-AOB - and nitrite-oxidizing bacteria - NOB) and ANAMMOX cultures as inocula. The AOB-NOB and ANAMMOX cultures were kindly supplied by Dr. Kartik Chandran (Columbia University, NY, USA). The bottles were filled with 200 mL of modified MSM and inoculated with 5 mL of each culture. Then, the headspace was flushed out with fresh air and sealed with rubber septa. Finally, 200 mL of headspace air were replaced with 200 mL of the 50000 ppm_v N_2O standard, which resulted in an initial N_2O concentration of 11400 ± 160 ppm_v. All tests were performed in duplicate and

incubated at 25 ºC under magnetic agitation (200 rpm) for 330 days. The gas concentrations of N_2O, CO_2 and O_2 were periodically monitored by GC-ECD and GC-TCD.

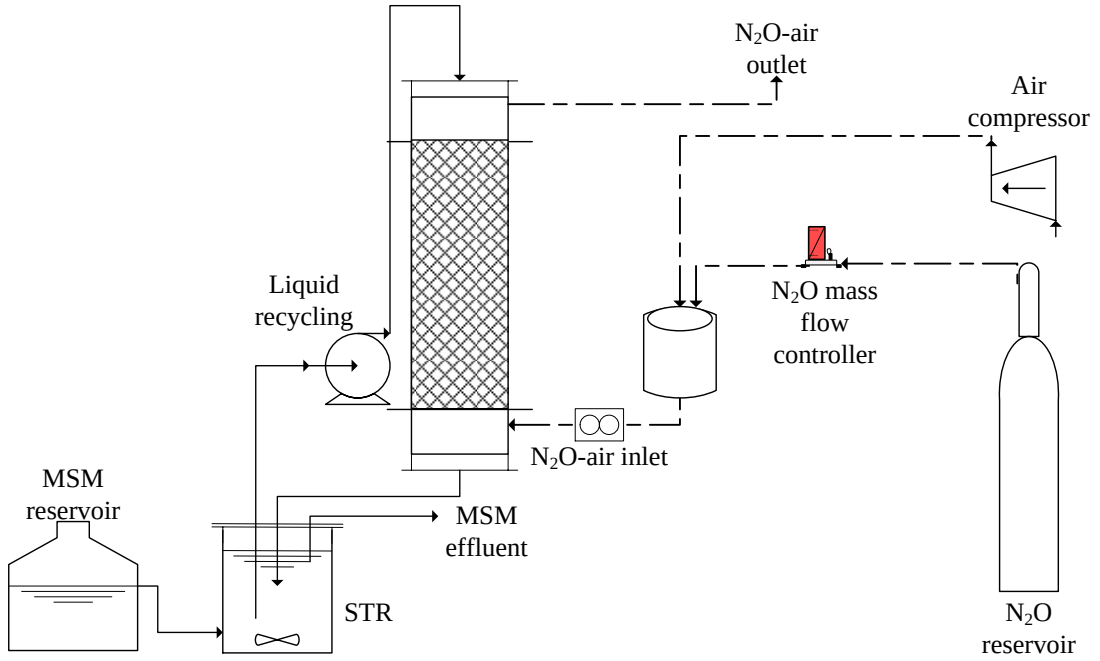

Figure 1. Scheme of the biotrickling filter set-up.

Analytical procedures

The CO_2 and O_2 gas concentrations were measured using a gas chromatograph equipped with a thermal conductivity detector (GC-TCD) following the procedure described by Lopez et al. [7]. Similarly, the gas and aqueous N_2O concentrations were determined in a gas chromatograph equipped with an electron capture detector according to the procedures of Frutos et al. [8]. The aqueous NO_2^- and NO_3^- concentrations were measured following the standard methods 4500-NO_2^- B and 4500-NO_3^- E, respectively [9]. The concentration of TN was determined using a TOC-VCSH analyzer (Shimadzu, Tokyo, Japan) coupled with a total nitrogen chemiluminescence detection module (TNM-1, Shimadzu, Japan). N-NH_4^+ concentration was monitored by means of an ammonium specific electrode Orion Dual Star (Thermo Scientific, The Netherlands).

Results and discussion

Continuous biotrickling filter for N_2O nitrification

No significant N_2O removal was observed in spite of the sufficient inorganic carbon and low NH_4^+ concentrations provided to promote the nitrification potential of the activated sludge present in the BTF. Indeed, no significant differences were recorded between the inlet and outlet gas concentrations of N_2O in the absorption column (Figure 2A). Similarly, the aqueous N_2O concentrations

recorded at inlet and outlet of the STR showed no removal of the N_2O absorbed in the packed column. However, a significant generation of N_2O was observed in the STR between days 48 and 63 (Figure 2B).

On the other hand, a complete removal of the NH_4^+ supplied with the MSM occurred as confirmed by the fact that NH_4^+ concentrations in the effluent of the STR remained below the detection limit of the instrumental methodology used (1 mg N L^{-1}). However, the fact that the inlet and outlet STR concentrations of NO_3^- remained at 0.04 ± 0.03 and 1.9 ± 0.6 mg N L^{-1}, respectively, suggest that only a small fraction of the NH_4^+ fed to the system was nitrified. Thus, NH_4^+ removal could have been caused by the stripping of NH_3 trough the absorption column at the relatively high pH prevailing in the system (8.3 ± 0.5) during most of the experimental period (despite the manual pH control). Surprisingly, TN concentrations in the outlet of the STR remained at 11 ± 3 mg L^{-1} along the 90 days of BTF operation, likely caused by the organic nitrogen released by cell breakdown. Finally, 29 ± 5 g m^{-3} d^{-1} of CO_2 was produced by both metabolic activity and the partial stripping of the carbonate supplied in the MSM.

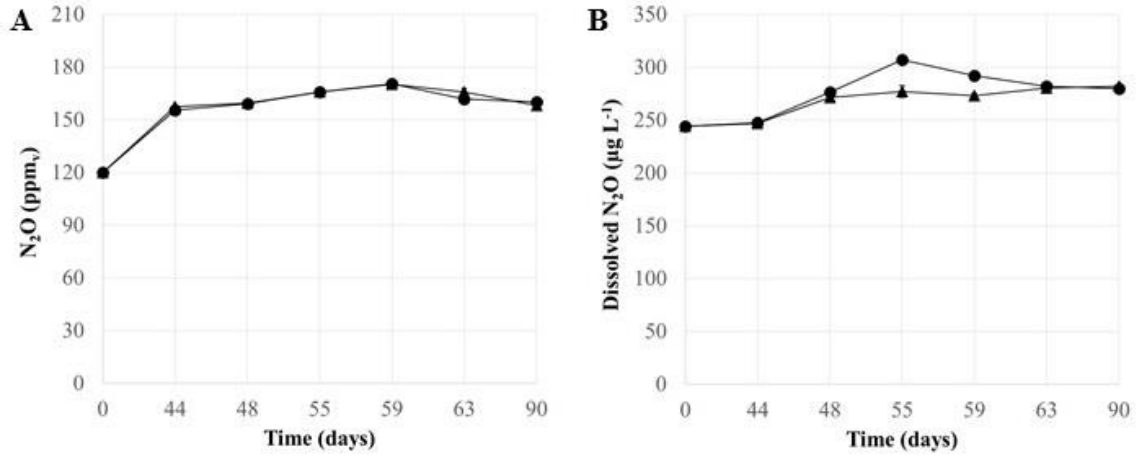

Figure 2. Time course of the inlet (▲) and outlet (●) N_2O gas concentrations in the absorption column (A), and inlet (▲) and outlet (●) N_2O aqueous concentrations of the STR (B).

Batch N_2O nitrification assays

The batch nitrification assays showed similar N_2O headspace concentrations patterns regardless of the inoculum tested. The concentrations initially increased from 11400 ± 160 ppm_v to levels ranging from 16000 to 18000 ppm_v up to day 115, the AOB-NOB and activated sludge systems exhibiting the highest and lowest N_2O concentrations recorded, respectively (Figure 3A). Similarly, the CO_2 headspace concentration increased from an initial concentration of 51 ± 7 g m^{-3} to a final concentration of 117 ± 15 g m^{-3} during the first 150 days of experiment (Figure

3B). This increase was likely due to the endogenous metabolic respiration of the cultures and to the partitioning of the inorganic carbon initially supplied in the MSM as HCO_3^-.

On the other hand, the oxygen present in the headspace of the systems was consumed by biological activity from 17 ± 0.2 % (v/v) to 10 ± 1 % (v/v) by the end of the experiment (Figure 3C). Interestingly, the N_2O concentration decreased to average value of 10693 ± 420 ppm_v from day 115 onwards in all systems. Nevertheless, no significant variations were observed in the CO_2 and O_2 concentrations in the same period of time, which suggested that this variation might not be due biological activity but to an error in the instrumental determination of N_2O (Figure 3).

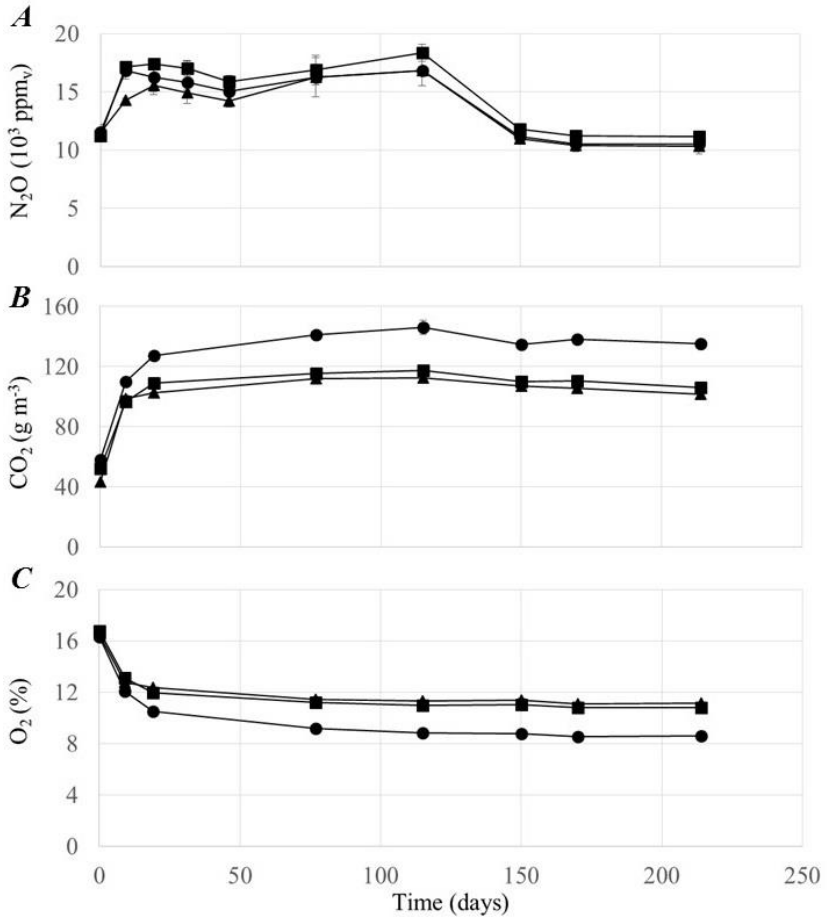

Figure 3. Time course of the N_2O (A), CO_2 (B) and O_2 (C) headspace concentrations in the bottles containing activated sludge (▲), AOB-NOB (■) and ANAMMOX (●) cultures.

In order to elucidate the mechanisms underlying the decrease in N_2O concentration observed from days 115 to 150, the biomass from each bottle was centrifuged and resuspended in fresh MSM (200 mL) with a fresh N_2O air headspace as above described. Additionally, an abiotic control bottle was prepared without biomass as above described. The headspace of the three

cultures and the control test was monitored for 116 days. The analysis of the headspace revealed higher CO_2 productions and O_2 consumptions in the tests inoculated with biomass than those recorded in the abiotic control test (Figure 4). The results also confirmed the absence of N_2O removal in spite of the biological activity observed in all assays inoculated with biomass (Figure 4A). The increase in N_2O concentration recorded in all tests was not attributed to metabolic activity since a similar trend was observed in the test without biomass (Figure 4A).

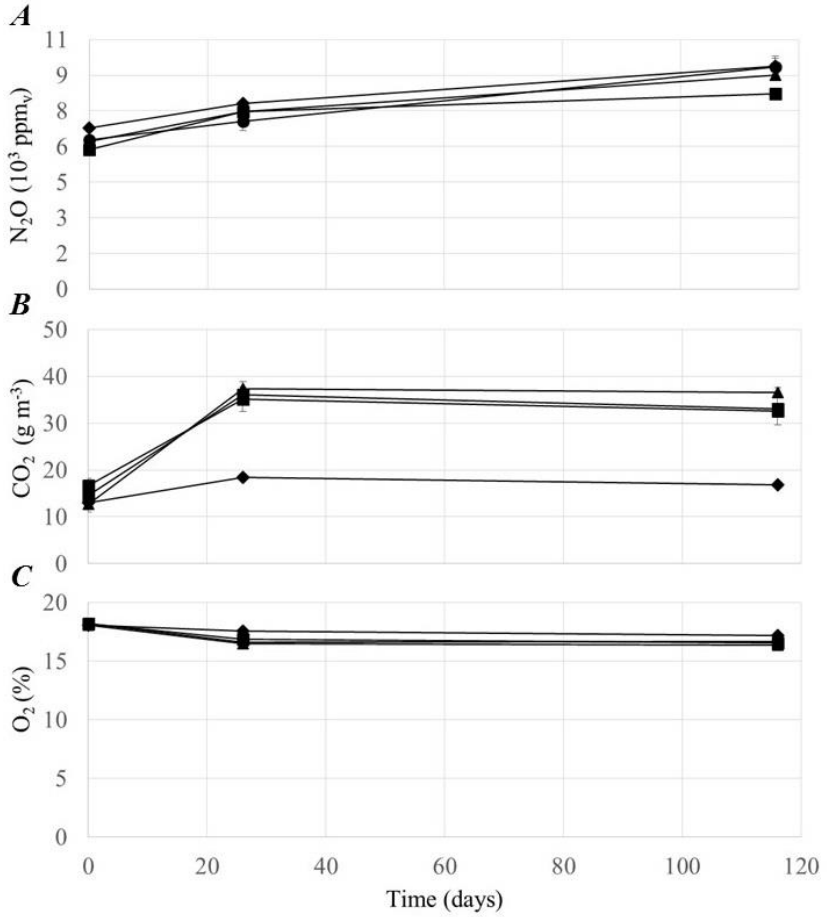

Figure 4. Time course of the N_2O (A), CO_2 (B) and O_2 (C) headspace concentrations in the abiotic control (♦) and the resuspended cultures of activated sludge (▲), AOB-NOB (■) and ANAMMOX (●).

Conclusions

This study assessed the potential nitrification of N_2O to NO_2^- or NO_3^- based in the thermodynamic feasibility of these reactions ($\Delta G^{o\prime}$= -87.4 and -15 kJ, respectively) in two independent series of experiments. First, a biotrickling filter inoculated with activated sludge was operated for a period of 90 days with a continuous supply of N_2O, O_2 and inorganic carbon. Secondly, batch assays

were performed with different NH_4^+ degrading inocula in gas-tight bottles at high N_2O concentrations under non-limiting concentrations of O_2, NH_4^+ and carbonate. The results did not show any significant evidence of biological removal of N_2O by nitrification. Further tests with NH_4^+ degrading inocula presenting a higher microbial diversity should be conducted to find microorganisms capable of oxidizing N_2O.

Acknowledgments

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Influence of the dissolved oxygen concentration on the abatement and generation of N_2O in activated sludge diffusion systems

O.D. Frutos, D. Fernández, R Muñoz. (Unpublished manuscript)

Chapter 4

Influence of the dissolved oxygen concentration on the abatement and generation of N_2O in activated sludge diffusion systems

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Abstract

Wastewater treatment plants (WWTPs) represent an important source of anthropogenic nitrous oxide (N_2O) emissions. Several operational strategies have been proposed to prevent the generation of this GHG in WWTPs in the last decade, but their effectiveness is never complete. In this context, the development of cost-effective end-of-the-pipe technologies for the control of N_2O emissions in WWTPs is necessary. This study was devoted to evaluate the potential of an activated sludge diffusion system (ASD) designed for carbon removal and nitrification for the in-situ abatement of WWTPs emissions of N_2O during domestic wastewater treatment. The performance of a lab-scale ASD aerated with a 71 ± 7 ppm_v N_2O air emission was assessed under dissolved oxygen (DO) concentrations of 4.2 ± 0.5, 2.8 ± 0.3, 2.1 ± 0.1 and 1.1 ± 0.01 mg L^{-1}. Furthermore, N_2O production under similar DO concentrations was also determined using N_2O-free aeration. The results showed a slight (~5 %) N_2O removal only at DOs of 2.1 ± 0.1 mg L^{-1}, whereas N_2O production was observed under all operational conditions, the highest production being recorded at DO concentrations of 4.2 ± 0.5 and 1.1 ± 0.01 mg L^{-1}. The ASD wastewater treatment performance was characterized by a high organic carbon removal (>94 %), nitrogen removals ranging from 10 to 24 % and a high nitrification capacity, which maintained NH_4^+ effluent concentrations below 4 mg L^{-1}. In brief, this study showed for the first time the limited capacity of ASD systems operated under microaerobic and aerobic conditions for the abatement of the N_2O-laden air emissions from WWTPs.

Introduction

Wastewater treatment is nowadays responsible of a major share of the anthropogenic nitrous oxide (N_2O) emissions from the Waste sector according to the Intergovernmental Panel on Climate Change (IPCC) [1]. The greenhouse gases (GHGs) emitted by wastewater treatment plants (WWTPs), mainly in the form of N_2O and CH_4, represent 54 % of the total GHG emissions from the waste sector [2]. Nitrous oxide constitutes the third most important GHG, with a share of 6.2 % of the total GHG emissions worldwide [2]. Furthermore, N_2O has been recently identified as the most important ozone depleting substance emitted in this 21th century [3].

Nitrous oxide is produced in WWTPs during the biological and chemical transformation of nitrogen in nitrification and denitrification processes [4,5]. During nitrification, the ionized and unionized forms of ammonia (NH_4^+ and NH_3) are oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) by nitrifying bacteria in a process that requires O_2 as the electron acceptor. Afterward, the NO_2^- and NO_3^- produced during nitrification are reduced sequentially to nitric oxide (NO), N_2O and finally to N_2 in the absence of oxygen using organic matter as the electron donor. Thus, denitrifying bacteria use these nitrogen oxides as the electron acceptor (instead of oxygen) during the oxidation of organic carbon in a process called heterotrophic denitrification. The generation of N_2O during these two biological processes is governed by environmental and operational parameters such as the dissolved oxygen level, C/N load ratio and NO_2^- concentration [6–8].

Several studies have recently elucidated potential mechanisms underlying the mitigation of N_2O emissions in WWTPs. Most of the strategies devoted to reduce N_2O emissions during wastewater treatment (WWT) are based on the control of the environmental and operational parameter in the water line. Unfortunately, these strategies are not always effective and end-of-the-pipe technologies should be additionally implemented for the abatement of N_2O emissions. In this context, physical-chemical technologies have been traditionally used as end-of-the-pipe strategies for N_2O abatement in industries. However, biotechnologies have recently emerged as a promising and environmentally friendly alternative for the treatment of the diluted N_2O emissions from WWTPs. For instance, an innovative two-stage bioscrubber was successfully operated with N_2O removal efficiencies ranging from 40 to 90 % [9,10]. However, the need for a constant supply of external organic matter (acting as a carbon source and electron donor), the excessive growth of biomass in the packed bed (causing the formation of

preferential channels and increased pressure drop) and the limited gas-liquid mass transfer of N_2O were identified as the main technical limitations of this innovative technology for the end-of-the-pipe abatement of N_2O in WWTPs. Therefore, there is an urgent need to develop new biological processes capable of overcoming the above mentioned limitations while providing satisfactory levels of N_2O abatement.

Activated sludge diffusion (ASD) systems have shown promising results for the treatment of odours emissions in WWTPs. Indeed, this technology exhibits a higher mass transfer potential than conventional off-gas treatment biotechnologies (biotrickling filters and biofilter), allows the direct use of the N_2O emission as air supply in the nitrification tank (which avoids the need for an external unit for gas treatment) and the cost of N_2O abatement would be negligible (since the operating cost is associated to WWT) [11,12]. Hence, activated sludge diffusion systems represent a promising technology to mitigate N_2O emissions in WWTPs, although no research has been conducted to date in this particular topic.

This study aimed at systematically evaluating the effect of dissolved oxygen (DO) concentrations on the performance of an activated sludge diffusion system (ASD) during the treatment of N_2O from a contaminated air emission in the WWTP context.

Materials and Methods

Chemicals

A standard of 50000 ppm_v of N_2O (40 L) in nitrogen used for the preparation of the model WWTP emission was purchased from Abello Linde S.A. (Barcelona, Spain). The ASD system was operated with a synthetic wastewater (SW) prepared with reagents purchased from PANREAC (Barcelona, Spain) (with a purity > 99 %).

Synthetic Wastewater and Inoculum

The SW was composed of (in g L^{-1}) peptone 0.16, meat extract 0.11, urea 0.03, NaCl 0.007, $CaCl_2{\cdot}2H_2O$ 0.004, $MgSO_4{\cdot}7H_2O$ 0.002, K_2HPO_4 0.028 and glucose 0.25. The concentration of total organic carbon (TOC) and total nitrogen (TN) of the SW was 230 ± 21 and 50 ± 3 mg L^{-1}, respectively. The bioreactor was inoculated with 1 L of settled activated sludge from the WWTP of Valladolid (Spain), operated in a denitrification-nitrification configuration.

Activated sludge diffusion system set-up and operation

The ASD system consisted of 3 L Biostat A bioreactor (Sartorius Stedim, Spain) coupled to a 1 L Imhoff-type settler. The SW was fed to the system using a Watson Marlow 102R peristaltic pump, while the mixed liquor was continuously pumped out using a Biostat A integrated pump

controlled by a water level sensor that maintained the reactor working volume at 2.5 L (Figure 1). The mixed liquor was conducted to the 1 L settler to achieve an almost complete biomass separation via sedimentation. The settled biomass was then recirculated to the bioreactor at a flow rate set at 50 % of the SW inlet flow rate (Figure 1). Biomass was purged in order to maintain a sludge retention time of 30 days. The N_2O-laden emission was obtained by mixing the 50000 ppm_v N_2O standard with air by means of a mass flow controller (Aalborg, Denmark), which resulted in an inlet N_2O concentration of 71 ± 7 ppm_v. The inlet gas was continuously supplied through a metallic gas diffuser (2 µm) at a flow rate of 250 mL min^{-1} in order to maintain a gas residence time of ~10 min. The pH of the system was automatically controlled by addition of HCl (1 M) with a Biostat A integrated pump activated by a pH sensor (Ceragel CPS71D) with a set point at 7 ± 0.05 (Figure 1). The DO concentration and temperature were measured using an Oximax H COS22Z probe. Finally, the temperature was maintained at 25 °C with a cold finger and a heater jacket (Figure 1).

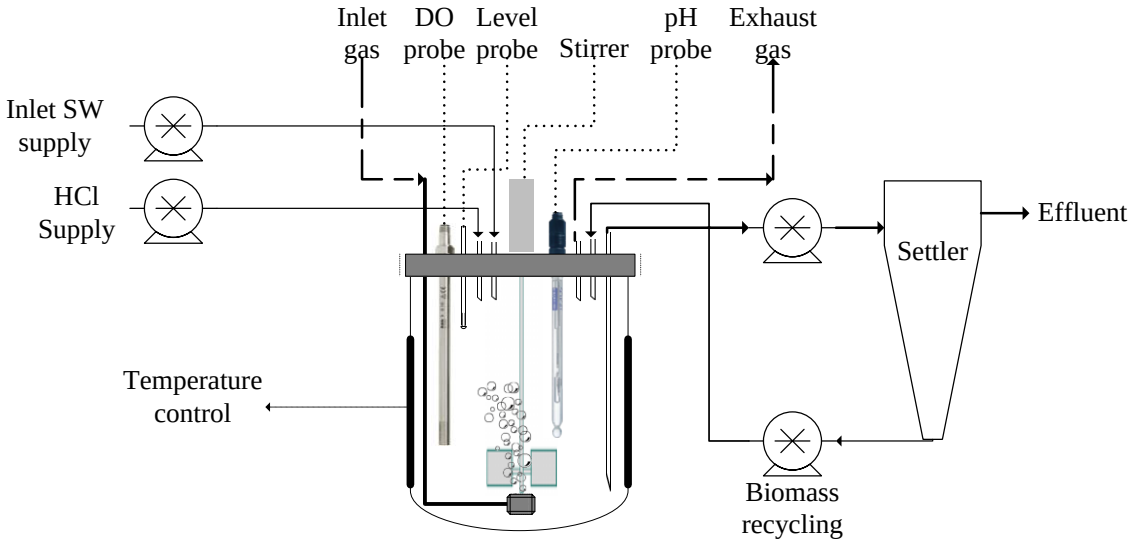

Figure 1. Scheme of the activated sludge diffusion system.

The N_2O abatement potential of the ASD system was evaluated under four different DO concentrations of 4.2 ± 0.5 (EI), 2.8 ± 0.3 (EII), 2.1 ± 0.1 (EIII) and 1.1 ± 0.01 mg L^{-1} (EIV) (Table 1). These DO levels were controlled by adjusting the SW inlet flow rate and the stirring rate (from 80 to 250 rpm) of the bioreactor. A control stage was conducted under each DO concentration by shutting off the N_2O supply (i.e N_2O-free air was used for bioreactor aeration) in order to evaluate the potential generation of N_2O during wastewater treatment (carbon removal and nitrification). The measurements taken under each operational stage

involved at least 10 days of steady state operation.

The gas concentrations of CO_2, O_2 and N_2O were daily monitored at the inlet and outlet of the ASD system. Liquid samples of the SW and the settler effluent were drawn three times per week to determine the NH_4^+, NO_2^-, NO_3^-, TOC, TN and total suspended solid (TSS) concentrations. Liquid sample of the settled biomass and bioreactor mixed liquor were also drawn to evaluate the TSS concentrations.

Table 1. Operational conditions of the ASD system

Experimental stage	*DO concentrations (mg L^{-1})*	*N_2O supply*	*ASD Carbon inlet load (mg m^{-3} d^{-1})*	*TSS (mg L^{-1})*	*Stirring speed (rpm)*
I	4.2 ± 0.5	No	328 ± 108	588 ± 195	230±12
		Yes		359 ± 123	226±14
II	2.8 ± 0.3	No	331 ± 23	1854 ± 210	232±11
		Yes		1615 ± 206	233±4
III	2.1 ± 0.1	No	352 ± 35	1800 ± 201	209±9
		Yes		2000 ± 385	194±9
IV	1.1 ± 0.01	No	363 ± 32	760 ± 400	182±16
		Yes		735 ± 104	159±26

Analytical procedures

The gas concentrations of CO_2 and O_2 were determined using a gas chromatograph equipped with a thermal conductivity detector (GC-TCD) according to Lopez et al. (2014) [13], whereas the gas N_2O concentrations were quantified in a gas chromatograph equipped with an electron capture detector (ECD) according to Frutos et al (2016) [14]. The concentrations of N-NO_2^- and N-NO_3^- were determined using a Waters HPLC equipped with an ion conductivity detector according to Akmirza et al. (2017) [15]. The TOC and TN concentrations were measured using a TOC-VCSH analyzer (Shimadzu, Tokyo, Japan) coupled with a total nitrogen chemiluminescence detection module (TNM-1, Shimadzu, Japan). The determination of TSS concentrations was performed according to standard methods [16]. N-NH_4^+ concentration was determined with an ammonium specific electrode Orion Dual Star (Thermo Scientific, The Netherlands).

Statistical data analysis

Analysis of variance (ANOVA) with a 95 % confidence level was performed to compare the inlet and outlet N_2O concentrations of the ASD system under all operational conditions evaluated.

Results and discussions

Influence of the DO on the emission and abatement of N_2O.

This study evaluated the potential of an ASD system for the abatement of N_2O-laden air emissions from WWTPs under four different DO levels. Figure 2 depicts the steady state inlet and outlet gas N_2O concentrations in the ASD system along the four operational stages evaluated. Thus, a slight removal (2.5 ± 5 %) was only observed in experiment III, where a DO concentration of 2.1 ± 0.1 mg L^{-1} was maintained. However, this removal was not statistically significant according to the ANOVA analysis conducted to the N_2O inlet and outlet concentrations.

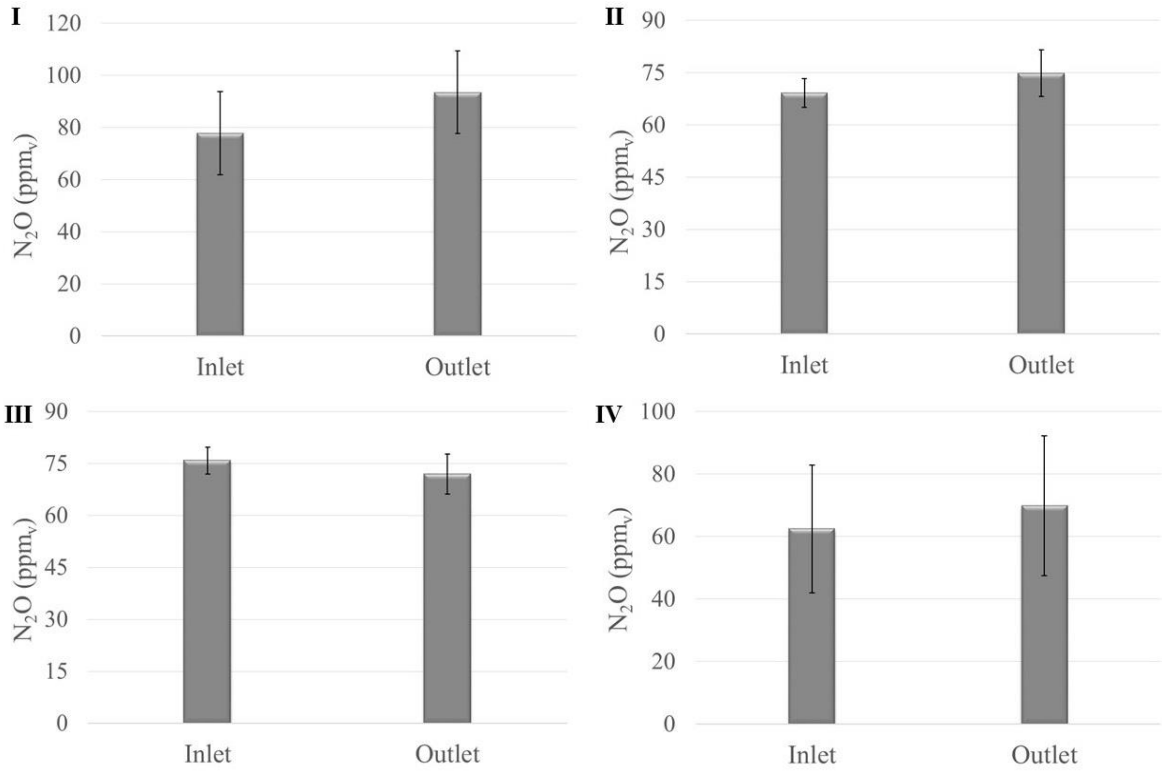

Figure 2. Average N_2O gas concentrations at the inlet and outlet of the ASD system during SW treatment under the four tested DO concentrations (experiments I to IV).

On the other hand, higher outlet average N_2O concentrations during SW treatment were recorded under DO concentrations of 4.2 ± 0.5, 2.8 ± 0.3 and 1.1 ± 0.01, which resulted in average N_2O productions ranging from 1.3 ± 0.8 to 3.6 ± 2.2 g m^{-3} d^{-1} (Table 2). However, the ANOVA analysis showed not significant differences between the inlet and outlet N_2O concentrations in the ASD in experiments I, III and IV. These results differed from those reported by Desloover et al. (2014), who demonstrated that a pure culture of *Pseudomonas stuzeri* was able to use O_2 and N_2O as electron acceptors simultaneously in batch assays using sodium acetate as carbon/electron donor under a headspace O_2 concentration of 5 and 10 % [17]. Those O_2 headspace concentrations corresponded to DO concentrations of 2 and 4 mg L^{-1} (25 ºC

and 1 atm), respectively, which are similar to those set in our ASD system in experiments I and III. In this context, a recent study conducted by Figueroa and coworkers (2016) showed a high inhibition of N_2O removal in batch tests under O_2 headspace concentrations higher than 2 % (corresponding to DO ~ 0.9 mg L^{-1}) [18], similar to the DO concentration tested in experiment IV (Table 2). Our study, and that from Figueroa et al. (2016), were conducted with fresh activated sludge without previous acclimation.

Process operation in the absence of N_2O supply in the aeration of the ASD system was also conducted at the four DO concentrations in order to assess the potential N_2O emissions. The results showed N_2O productions ranging from 0.1 to 1.4 g m^{-3} d^{-1} during SW treatment in this carbon removal-nitrification process (Table 2). These productions in the absence of N_2O supply could be estimated as a fraction of the inlet TN load to the bioreactor (N-N_2O/TN), which accounted for 0.04 to 0.5 % of the inlet nitrogen load (corresponding to 0.4 to 5.3 mg of N-N_2O per kg of influent TN).

Table 2. Influence of the DO concentration on N_2O production and the fraction of inlet TN emitted as N-N_2O

Experiments	*DO concentration (mg L^{-1})*	*N_2O supply*	*N_2O production (g m^{-3} d^{-1})*	*N-N_2O/TN ratio (%)*
I	4.2±0.5	No	1.3±0.3	0.4
		Yes	3.6±2.2	1.5
II	2.8±0.3	No	0.1±0.1	0.04
		Yes	1.3±0.8	0.5
III	2.1±0.1	No	0.5±0.3	0.2
		Yes	-	-
IV	1.1±0.01	No	1.4±0.6	0.5
		Yes	1.7±0.3	0.6

Interestingly, the results did not show a linear or direct correlation between the DO levels imposed and the N_2O production rates recorded in the ASD system. Hence, the lowest production occurred at a DO concentration of 2.1 ± 0.1 mg L^{-1}, whereas the highest productions were observed at DO of 4.2 ± 0.5 and 1.1 ± 0.01 mg L^{-1} (Table 2). Under low DO level, nitrifier denitrification could be the main responsible of N_2O generation [19,20]. During nitrifier denitrification, AOB use nitrite as the electron acceptor instead of oxygen, and reduce NO_2^- to nitric oxide and N_2O. Shaw et al. (2006) demonstrated that some species of AOB do not have the enzyme required to

catalyze N_2O reduction to N_2, which could cause the accumulation of N_2O as a final product of nitrifier denitrification [21]. Conversely, process operation at 2.8 ± 0.3 and 4.2 ± 0.5 mg L^{-1} could be considered completely aerobic, thus nitrifier denitrification must be discarded as a production mechanism. In this scenario, NH_4^+ nitrification and the oxidation of hydroxylamine to N_2O can be hypothesized as the main mechanisms underlying N_2O generation [22].

Influence of the DO concentration on wastewater treatment efficiency

High removal efficiencies of TOC (>94 %) were recorded regardless of the DO concentrations tested (Figure 3). However, the removal of TN ranged from 10 to 24 %, the lowest TN removals recorded at 4.2 mg L^{-1} likely due the low biomass concentrations prevailing in the bioreactor (359±123). The CO_2 produced from the oxidation of the organic matter present in the SW is also depicted in Figure 3. CO_2 productions ranged from 503 to 878 g m^{-3} d^{-1} and were closely correlated to the biomass concentrations in the mixed liquor.

The urea originally supplied in the SW was rapidly transformed to ammonia by enzymatic hydrolysis and thereafter oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) via nitrification regardless of the DO concentrations.

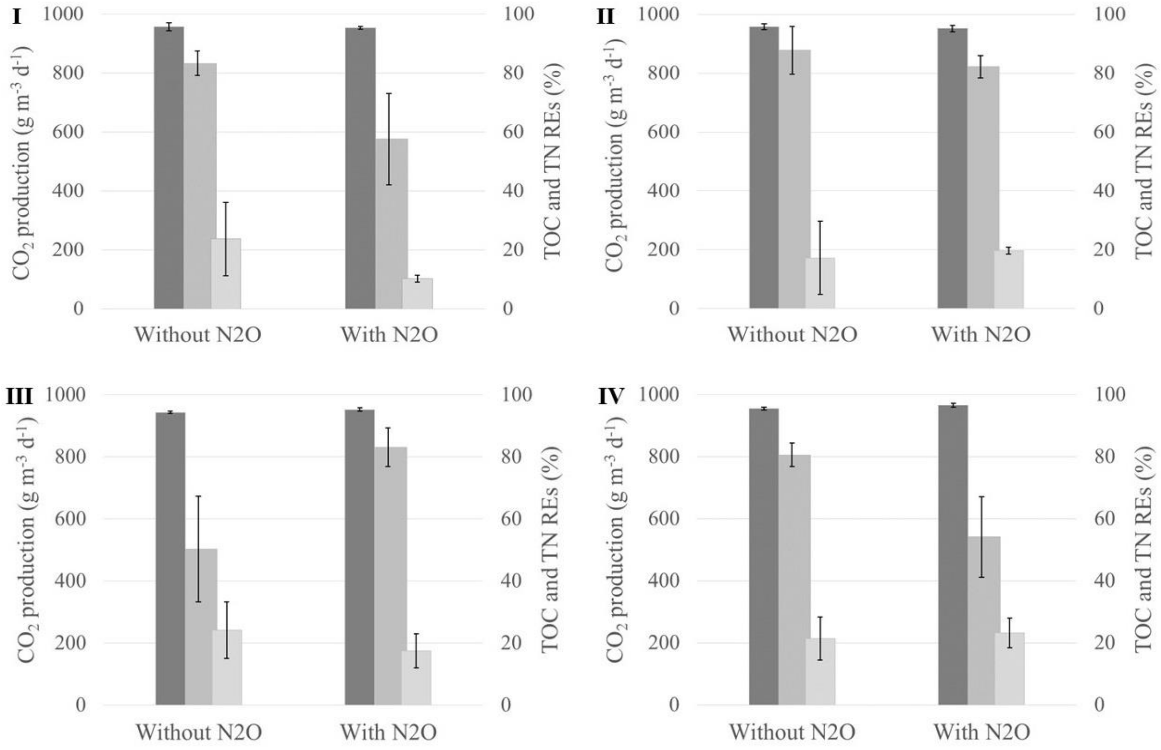

Figure 3. CO_2 productions (pale gray) and removal efficiencies of TOC (dark gray) and TN (light gray) in experiments I to IV.

Thus, $N\text{-}NO_3^-$ concentrations in the effluent ranged from 23 to 37 mg L^{-1}, while the concentrations of $N\text{-}NO_2^-$ ranged from 0.6 to 8.7 mg L^{-1} (Table 3), which revealed that 62 to 84 % of the influent nitrogen was nitrified. In this context, the low $N\text{-}NH_4^+$ concentrations recorded in the effluent confirmed the high nitrification activity of the activated sludge regardless of the DO concentrations (Table 3). NO_3^-/NO_2^- denitrification was not observed in the bioreactor, which suggested that N_2O generation occurred under nitrifying conditions. Finally, the comprehensive nitrogen mass balance calculation conducted in this study suggested that TN removal occurred likely due to biomass assimilation via synthesis of new cell material and ammonia stripping, the latter accounting for 3 to 14 % of the N disappearance.

Table 3. Steady state nitrate, nitrite and ammonia effluent concentrations

Experiments	*DO concentrations (mg L^{-1})*	*N_2O supply*	*$N\text{-}NO_2^-$ (mg L^{-1})*	*$N\text{-}NO_3^-$ (mg L^{-1})*	*$N\text{-}NH_4^+$ (mg L^{-1})*
I	4.2±0.5	No	8.7	23	2.3±1.2
		Yes	2.2	29	3.6±1.8
II	2.8±0.3	No	0.6±0.8	36±4	0.6±0.8
		Yes	2.5±1.3	35±4	0.7±0.8
III	2.1±0.1	No	2.6±1	37±5	1.1±0.8
		Yes	-	-	1.3±0.1
IV	1.1±0.01	No	6.7±7	28±4	1.7±1.1
		Yes	2.3	37	3.3±4.1

Conclusions

This study evaluated the influence of the dissolved oxygen concentration on the potential N_2O abatement in activated diffusion systems during the treatment of domestic wastewater. The results showed a slight (but not significant) removal of N_2O only in the experiment conducted at a DO concentration of 2.1 ± 0.1 mg L^{-1} and a net production of N_2O under the rest of the DO concentrations evaluated, which could be attributed to nitrifier denitrification and hydroxylamine oxidation. On the other hand, the activated sludge process showed an efficient organic carbon removal and a high nitrification activity, nitrate being the main species of nitrogen in the effluent. In brief, this study revealed for the first time the limited performance of ASDs during the treatment of N_2O-laden air emissions under microaerobic and aerobic conditions.

Acknowledgments

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Nitrous oxide abatement coupled with biopolymer production as a model GHG biorefinery for cost-effective climate change mitigation

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Chapter 5

Environmental Science & Technology

Article

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Nitrous Oxide Abatement Coupled with Biopolymer Production As a Model GHG Biorefinery for Cost-Effective Climate Change Mitigation

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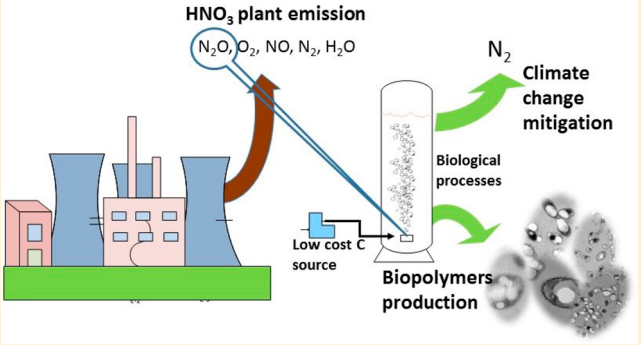
Supporting Information

ABSTRACT: N_2O represents ~6% of the global greenhouse gas emission inventory and the most important O_3-depleting substance emitted in this 21st century. Despite its environmental relevance, little attention has been given to cost-effective and environmentally friendly N_2O abatement methods. Here we examined, the potential of a bubble column (BCR) and an internal loop airlift (ALR) bioreactors of 2.3 L for the abatement of N_2O from a nitric acid plant emission. The process was based on the biological reduction of N_2O by *Paracoccus denitrificans* using methanol as a carbon/electron source. Two nitrogen limiting strategies were also tested for the coproduction of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) coupled with N_2O reduction. High N_2O removal efficiencies (REs) (≈87%) together with a low PHBV cell accumulation were observed in both bioreactors in excess of nitrogen. However, PHBV contents of 38−64% were recorded under N limiting conditions along with N_2O-REs of ≈57% and ≈84% in the ALR and BCR, respectively. Fluorescence in situ hybridization analyses showed that *P. denitrificans* was dominant (>50%) after 6 months of experimentation. The successful abatement of N_2O concomitant with PHBV accumulation confirmed the potential of integrating biorefinery concepts into biological gas treatment for a cost-effective GHG mitigation.



INTRODUCTION

The increasing global temperature rise and climate change reported by scientists has attracted the attention of the community and the policy makers during the past decade. Nowadays, there is now good evidence that these environmental problems are caused by the rapid accumulation of greenhouse gases (GHGs), whose concentrations are 45% higher than those prevailing in the preindustrial era.[1] Nitrous oxide (N_2O), the third most important GHG with a global warming potential 300 times higher than that of CO_2 (on a mol per mol basis) due to its high atmospheric persistence (150 years), accounts for 6.2% of the total GHG emissions globally. N_2O is also one of the main sources of stratospheric NO_x and is considered the most important ozone depleting substance emitted in this 21st century.[2] Agriculture is the principal source of anthropogenic N_2O emissions, followed by chemical industry and waste management processes. The production of nitric and adipic acid are the major N_2O source in industry, whose global emissions reach up to 400 Kton of N_2O per year.[3] A typical waste gas from nitric acid production plants is characterized by 100−3500 ppm_v of NO_x, 300−3500 ppm_v of N_2O, 1−4% of O_2 and 0.3−2% of H_2O (in a N_2 matrix).[4]

Several physical-chemical technologies such as nonselective catalytic reduction (NSCR) or catalytic decomposition have been applied as end-of-the-pipe strategies for the treatment of N_2O emissions from industrial sources.[5] However, these technologies entail the consumption of a reducing agent such as hydrocarbons or ammonia and require the preheating of the tail gas for N_2O destruction, resulting in a considerable energy consumption since nitric acid waste gas is typically emitted at ambient temperature.[6] Moreover, the environmental sustainability of NSCR technologies can also lead to production of CH_4, another atmospheric GHG, derived from an incomplete fuel combustion during the treatment of N_2O.[7]

Biological technologies have been shown to exhibit a high robustness, cost efficiency, and environmental friendliness for the treatment of industrial off-gases containing malodorous and volatile organic compounds.[8] In spite of their inherent advantages, no biological process has ever been evaluated for the abatement of N_2O emissions from nitric and adipic acid plants.[9,10] This GHG is an obligate intermediate in the reduction of NO_3^- and NO_2^- to N_2, which to the best of our knowledge is the only known biochemical mechanism for N_2O removal. Thus, since nitric and adipic acid emissions are mainly composed of N_2O, N_2 and trace levels of O_2,

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denitrification appears as an attractive alternative for the abatement of N_2O when a cheap source of organic carbon and electron donor is available for the growth of heterotrophic bacteria.[9,10] In this context, the economic viability of this process can be significantly improved by coupling the abatement of N_2O via denitrification with the production of added value bioproducts such as polyhydroxyalkanoates (PHA) biopolymers. These biobased chemicals, especially poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), share with conventional fossil-derived thermoplastics similar physical/chemical characteristics such as melting point, molecular weight and tensile strength.[11] PHAs also possess a rapid biodegradability in nature, which render them a perfect substitute of conventional fossil polymers. There are several denitrifying bacteria such as *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, and *Ralstonia eutropha*, capable of producing intracellular PHA as a carbon storage material in excess of organic carbon under nutrient limitation.[12,13] Therefore, an innovative GHG biorefinery could be engineered for the simultaneous abatement of N_2O and coproduction of PHAs in nitric and adipic acid plants in order to enhance the economic and environmental sustainability of N_2O abatement.

In this context, the performance of two low-cost and eco-friendly suspended-growth bioreactors, namely bubble column (BCR) and airlift (ALR), was evaluated for the treatment of a synthetic N_2O emission from nitric acid plant. The strain *Paracoccus denitrificans* (DSM 413) was used as a model denitrifying bacterium in the coproduction of the copolyester PHBV using methanol as a carbon-energy source under nitrogen sufficiency and two different nitrogen limiting strategies.

MATERIALS AND METHODS

Chemicals and Mineral Salt Medium. The mineral salt medium (MSM) used in the experimentation was composed of (g L^{-1}): $Na_2HPO_4{\cdot}12H_2O$ 6.16, KH_2PO_4 1.52, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCl_2$ 0.02, NH_4Cl 1.5, and 10 mL L^{-1} of a trace element solution containing (g L^{-1}): EDTA 0.5, $FeSO_4{\cdot}7H_2O$ 0.2, $ZnSO_4{\cdot}7H_2O$ 0.01, $MnCl_2{\cdot}4H_2O$ 0.003, H_3BO_3 0.03, $CoCl_2{\cdot}6H_2O$ 0.02, $CuCl_2{\cdot}2H_2O$ 0.001, $NiCl_2{\cdot}6H_2O$ 0.002, $NaMoO_4{\cdot}2H_2O$ 0.003. The final pH of the MSM was 7. All reagents, including methanol, were purchased from PANREAC with a purity of >99%. Benzoic acid (>99%) and PHBV standards were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). A 40 L calibrated gas cylinder of 50 000 ppm_v of N_2O in N_2 and 50 L industrial N_2 cylinder were purchased from Abelló Linde S.A. (Barcelona, Spain).

Microorganism Cultivation. The methylotrophic strain *Paracoccus denitrificans* (DSM 413) was purchased from DSMZ (Braunschweig, Germany). The bacterium was cultivated in sterilized 1 L E-flasks with 0.5 L of MSM with methanol (1% v/v) as the sole carbon and energy source under aerobic conditions for 3 weeks.

Experimental Set Up. A BCR of 42 cm of height (H) and 9 cm of inner diameter (ID), and an ALR of the same dimensions with a concentric draft tube (i.d. = 5.5 cm, H = 29.5 cm) located at 4 cm from the bottom of the reactor, were inoculated with 0.5 L of *P. denitrificans* inoculum and filled with MSM to a working volume of 2.3 L, resulting in an initial total suspended solid (TSS) concentration of 56 mg L^{-1} in both bioreactors. The synthetic nitric acid plant N_2O emission was obtained by mixing the calibrated mixture of N_2O (50 000 ppm_v), air from a compressor and pure N_2 using mass flow controllers (Aalborg, Denmark). The gas mixture resulted in BCR and ALR inlet N_2O gas concentrations of 3520 ± 290 and 3560 ± 300 ppm_v, respectively. The O_2 inlet gas concentration remained at 1.1 ± 0.1% in each bioreactor. Both the BCR and ALR were supplied with an inlet gas flow rate of 137 ± 8 and 140 ± 10 mL min^{-1}, respectively, which corresponded to a gas empty bed residence time (EBRT) of ≈17 min. Pure methanol (CH_3OH) was injected in the gas line by means of a syringe pump in a sample port filled with fiberglass wool to facilitate solvent evaporation. Methanol was selected as a carbon and energy source based on its empirically proven ability to support biopolymer accumulation in *P. denitrificans*, its low cost and extensive use in wastewater treatment plants for denitrification purposes.[14] The systems were operated in a controlled temperature room at 25 °C. A detailed diagram of the experimental setup can be found in Figure S1 (Supporting Information (SI)).

Operational Conditions. Three operational strategies, corresponding to Stages I, II, and III, were evaluated in both bioreactors under different MSM nitrogen concentrations in order to assess the feasibility of a simultaneous N_2O removal and PHBV cell accumulation. During the first 43 days of operation (Stage I) the bioreactors were maintained under nitrogen sufficiency to evaluate both the N_2O removal performance and the accumulation of PHBV under optimal cultivation conditions by supplying MSM with 396 mg N L^{-1} and 124 g C m^{-3} d^{-1} of CH_3OH. During Stage I, 300 mL of the cultivation broth was replaced by fresh MSM three times per week, which resulted in a dilution rate of ∼0.056 d^{-1} and an N inlet load of 22.1 g N m^{-3} d^{-1}. Stage II (days 44 to 127) was devised to promote the accumulation of intracellular PHBV at a CH_3OH inlet load of 93 g C m^{-3} d^{-1}, which guaranteed carbon availability. The N concentration in the MSM was reduced to 34 mg N L^{-1} during Stage II, with 300 mL of fresh MSM being replaced every 2 days. This resulted in a N inlet load of 2.2 g N m^{-3} d^{-1}, a dilution rate of 0.065 d^{-1} and nitrogen fast:famine cycles of 1d:1d. In Stage III (days 128−179), the nitrogen concentration in the MSM was increased to 68 mg N L^{-1} while decreasing the frequency of MSM replacement (300 mL) from 2 to 4 days at a CH_3OH inlet load of 108 g C m^{-3} d^{-1}. The dilution rate and N inlet load during Stage III was 0.033 d^{-1} and 2.2 g N m^{-3} d^{-1}. The strategy conducted in this last stage aimed at increasing the biomass concentration in the bioreactors and consequently the N_2O removal performance by simultaneously reducing and increasing the dilution rate and N-MSM concentration, respectively. A mass transfer test was carried out according to Cantera and co-workers[15] at the end of Stages II and III by increasing the N_2O inlet concentration from ≈3527 to ≈9058 in order to elucidate the limiting factor during N_2O reduction to N_2 under the experimental conditions evaluated.

Sampling and Analytical Procedures. The gas phase monitoring procedure entailed the periodical measurement of N_2O, CO_2, and O_2 gas concentrations at both inlet and outlet bioreactors sampling ports. The monitoring of the liquid phase involved the withdrawal of 300 mL of cultivation broth from each bioreactor in order to determine the dissolved total organic carbon (TOC), total nitrogen (TN), CH_3OH, TSS and PHBV concentrations. The dissolved oxygen concentration was measured in situ. In addition, 20 mL of the cultivation broth was centrifuged, wash with distilled water and dried at 105 °C

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for 24 h for the measurement of C, N, H, and S cell content at the end of each experimental conditions.

The N_2O and CO_2/O_2 gas concentrations were measured by GC-ECD and GC-TCD according to Frutos et al.[9] and Lopez et al.,[16] respectively. TOC and TN concentrations were measured using a TOC-VCSH analyzer (Shimadzu, Tokyo, Japan) coupled with a total nitrogen chemiluminescence detection module (TNM-1, Shimadzu, Japan). Dissolved CH_3OH concentration was determined in a GC-FID (Bruker 3900, Palo Alto, CA) equipped with a SupelcoWax (15 m × 0.25 mm × 0.25 μm) capillary column. GC-FID injector and detector temperatures were maintained at 200 and 250 °C, respectively. Nitrogen was used as the carrier gas at 1 mL min^{-1} and H_2 and air flows were fixed at 30 and 300 mL min^{-1}, respectively. N_2 was also used as the makeup gas at 25 mL min^{-1}. The determination of TSS concentration was performed according to standard methods.[17] The dissolved oxygen concentration was measured with a hand-held OXI 330i oximeter (WTW, Germany) while pH was periodically monitored using a pH/mV/°C meter (pH 510 Eutech Instruments, Nijkerk, The Netherlands). To quantify the PHBV concentration, 2 mL of the cultivation broth were centrifuged at 9000 rpm for 15 min and the biomass pellet obtained was processed according to Zuñiga and co-workers,[18] using chloroform as extraction solvent. The PHBV extracted was measured by GC-MS (Agilent Technologies: GC System 7820A MSD 5977E, Santa Clara, CA) equipped with a DB-wax column (30 m × 250 μm × 0.25 μm) with detector and injector temperatures of 250 °C and a split ratio of 1:10. The oven temperature was initially maintained at 40 °C for 5 min, increased at 10 °C min^{-1} up to 200 °C and maintained at this temperature for 5 min. The PHBV cell content was normalized as %PHBV = (g PHBV/g TSS) × 100. The analysis of C, N, H and S biomass content was conducted using a LECO CHNS-932 elemental analyzer.

Electron Microscopy Analysis. Cultivation broth samples of 1 mL were drawn in order to assess the presence of PHBV inside the cells by microscopy analysis at the end of Stage III. The samples were centrifuged at 4000 rpm and 4 °C for 5 min, to subsequently process the biomass pellets according to Bozzola.[19] The samples were then cut in thin slices by a microtome and contrasted according to Wendlandt and co-workers.[20] A TEM JEOL JEM-1011 electron microscope (Teknolab, Indonesia) equipped with an ES1000W Erlangshen CCD camera (Gatan, Germany) was used for the analysis.

Fluorescence in Situ Hybridization (FISH) Analysis. FISH analyses were conducted to evaluate the abundance of *P. denitrificans* in the culture broth during each operational stage since the systems were run under nonsterile conditions (similarly to the scenario envisaged for its large scale implementation). Aliquots of 250 uL of the cultivation broth from both bioreactors at the end of each operational stage were fixed in 4% (w/v) paraformaldehyde for 3 h, washed three times with phosphate-buffered saline (PBS) and then preserved in alcohol 96% (v/v). Aliquots of 10 μL of samples were placed on glass microscope slides and dehydrated with ethanol at 50%, 80%, and 96% (v/v). The probes used were EUB338 I–II–FITC (for general bacteria)[21,22] and PAR651-Fam (specific for the genus *Paracoccus*).[23] Hybridization was carried out at 46 °C using formamide at 40% .[24] For quantitative FISH analysis, 16 images were randomly acquired from each well on the slides using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany). The relative biovolumes of the specific genus *Paracoccus* from the total bacteria (EUB338 I–II) were calculated using the commercial software DAIME and split into individual color channels before image segmentation.[25]

RESULTS AND DISCUSSION

Influence of Nitrogen Supplementation on N_2O Abatement. The two bioreactors exhibited a low and stable dissolved oxygen (DO) concentration during the entire experimentation (0.07 ± 0.1 mg L^{-1} in both bioreactors). Similarly, a stable pH of 6.8 ± 0.2 was recorded in both systems along the three operational stages. The N_2O REs reached a steady state 10 days after the startup of the bioreactors. Hence, steady state N_2O REs of 87 ± 3% were reached during Stage I in the BCR with inlet and outlet N_2O concentrations of 3380 ± 340 and 440 ± 74 ppm_v, respectively (Figure 1A). Similarly, the ALR supported steady state REs of 88 ± 2% with inlet and outlet N_2O concentrations of 3610 ± 340 and 420 ± 69 ppm_v, respectively (Figure 1B).

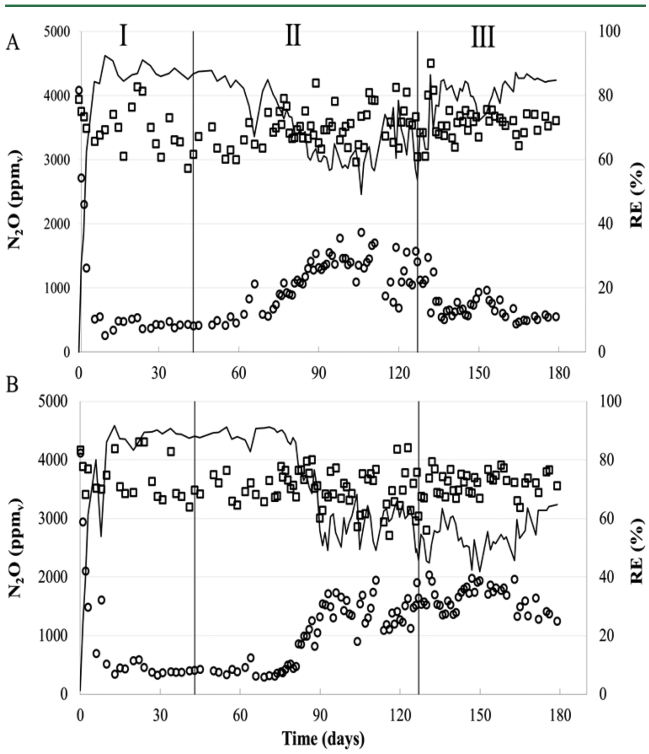

Figure 1. Time course of the inlet (□) and outlet (○) N_2O gas concentrations and removal efficiency (solid line) in the BCR (A) and ALR (B). Vertical lines indicate the different operation stages.

The CO_2 produced from the oxidation of CH_3OH during Stage I was correlated with the removal of N_2O, resulting in comparable CO_2 production rates of 85 ± 8 g C m^{-3} d^{-1} and 91 ± 8 g C m^{-3} d^{-1} in the BCR and ALR, respectively (Figure 2A). In this context, the ratio of C–CO_2 produced per C–CH_3OH consumed in this steady state accounted for 0.73 and 0.78 C–CO_2 C–CH_3OH^{-1} in the BCR and ALR, respectively. Biomass concentration, measured as TSS, reached stable values of 853 ± 76 and 856 ± 90 mg L^{-1} in BCR and ALR, respectively, after 20 days of operation (Figure 2B). These biomass concentrations entailed specific N_2O removal capacities of 0.52 ± 0.07 gN_2O $gTSS^{-1}$ d^{-1} in the BCR and 0.66 ± 0.08 gN_2O $gTSS^{-1}$ d^{-1} in the ALR. Likewise, the ratio of CO_2 produced per mol of N_2O reduced were similar in both

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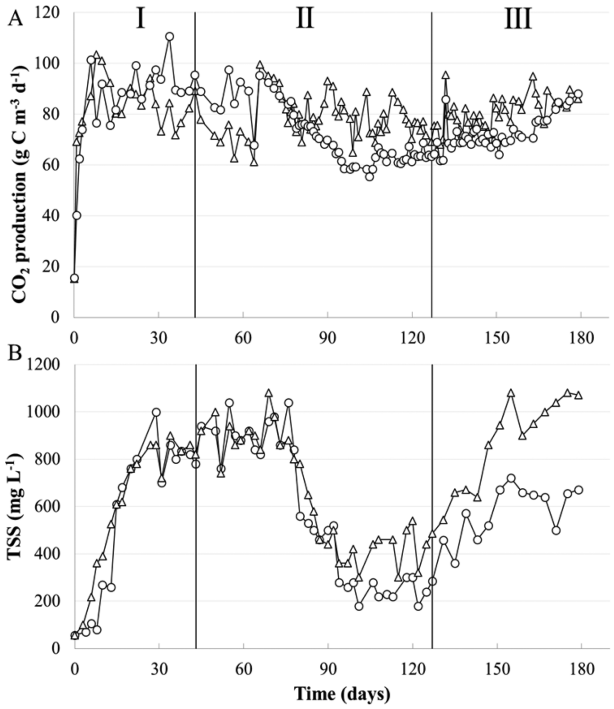

Figure 2. Time course of CO_2 production rates (A) and TSS concentrations (B) in the BCR (Δ) and ALR (○). Vertical lines indicate the different operation stages.

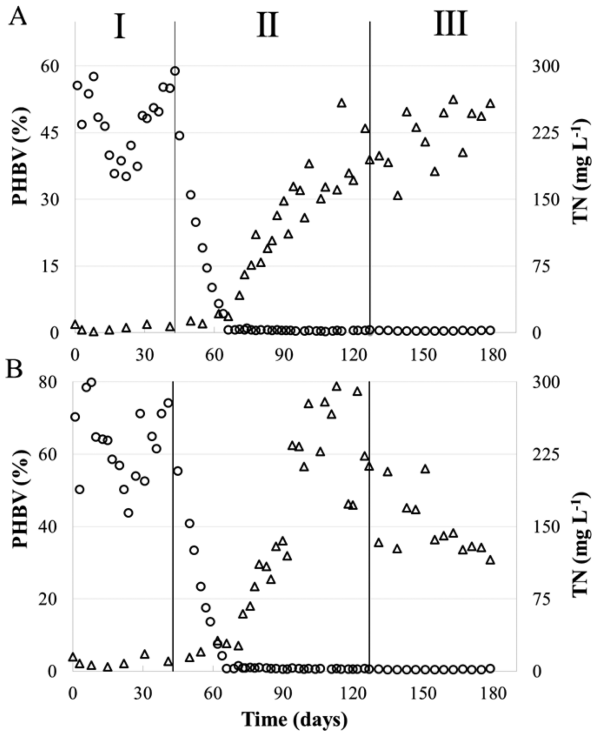

Figure 3. Time course of the PHBV cell content (Δ) and TN concentrations (○) in the BCR (A) and the ALR (B). Vertical lines indicate the different operation stages.

bioreactors, with average values of 0.7 gCO_2 gN_2O^{1-} in the BCR and 0.6 gCO_2 gN_2O^{1-} in the ALR.

The decrease in N supply rate from day 44 (Stage II) in order to achieve 1d:1d nitrogen fast-famine cycles resulted in a progressive reduction in the N concentration down to a complete depletion by day 66 in both bioreactors (Figure 3). Nitrogen depletion entailed a gradual deterioration in N_2O REs down to steady state values of 62 ± 7% in BCR and 58 ± 6% in ALR (Figure 1A). This significant decrease in N_2O REs was correlated to a concomitant reduction in biomass concentration as a result of the limited N availability. In this context, the TSS concentration decreased gradually to steady values of 422 ± 76 in the BCR and of 285 ± 99 mg L^{-1} in the ALR from day 94 (Figure 2B). Surprisingly, the microbial population in the ALR was more impacted by N deprivation than that present in the BCR. Furthermore, the presence of the internal draft tube in the ALR likely entailed higher gas bubble rising velocities compared to those in the BCR, and thus a lower N_2O mass transfer to the liquid phase that limited biomass growth. In addition, biofilm formation in the internal draft tube of the ALR could have led to an underestimation of the TSS concentrations. The reduction in biomass concentration and N_2O RE resulted in a concomitant decrease in the CO_2 production rate in the ALR (63 ± 3 g C m^{-3} d^{-1}) compared to CO_2 production rates of 78 ± 7 g C m^{-3} d^{-1} in the BCR (Figure 2A). This reduction resulted in an increase in the CO_2 produced per N_2O consumed ratio up to 0.83 and 0.75 gCO_2 gN_2O^{1-} in the BCR and ALR, respectively. Similarly, an increase in the CO_2 produced per CH_3OH consumed ratio was observed in both bioreactors (0.99 and 0.96 $C{-}CO_2$ $C{-}CH_3OH^{-1}$, respectively), which indicated that cell maintenance increased as a result of N deprivation. A mass transfer test was conducted at this point to assess the limiting factor in N_2O removal during Stage II. An increase in the N_2O inlet load by a factor of 2.4 ± 0.2 did not result in a concomitant increase in CO_2 production rate and N_2O elimination capacity (SI Figure S2). Hence, this test confirmed that both bioreactors were limited by microbial activity due to the low biomass concentration supported by the limited N supply imposed. Furthermore, the highest specific N_2O removal were recorded during Stage II, with average values of 0.76 ± 0.19 gN_2O $gTSS^{-1}$ d^{-1} in the BCR and 1.31 ± 0.34 gN_2O $gTSS^{-1}$ d^{-1} in the ALR.

The increase in N concentration by a factor of 2 along with the reduction in the dilution rate from 0.065 d^{-1} to 0.033 d^{-1} by day 127 (Stage III) supported an increase in biomass concentration to 1017 ± 71 mg TSS L^{-1} and 646 ± 64 mg TSS L^{-1} in the BCR and ALR, respectively. This entailed a concomitant increase of N_2O removal in the BCR up to steady REs of 84 ± 3% but similar N_2O-REs of 57 ± 7% were recorded in the ALR during Stage III (Figure,[1] Figure[2]). This increase in biomass concentration did support an increase in the CO_2 production rates up to 85 ± 5 and 78 ± 6 g C m^{-3} d^{-1} in the BCR and ALR, respectively. The CO_2 produced per N_2O consumed ratio showed a slight increase in the ALR (0.72 gCO_2 gN_2O^{1-}) and a decrease to 0.66 gCO_2 gN_2O^{1-} in the BCR. On the other hand, the CO_2 produced per CH_3OH consumed ratio decreased to 0.81 and 0.85 $C{-}CO_2$ $C{-}CH_3OH^{-1}$ in the BCR and ALR, respectively, due the enhanced biomass growth. In this context, the mass transfer test carried out at the end of Stage III revealed that both systems were limited by N_2O mass transfer. Thus, the increase in N_2O inlet load by a factor of 2.8 ± 0.1 promoted a rapid increase in the N_2O elimination capacity by a factor of 2.4 ± 0.1 in both reactors (SI Figures S3). Mass transfer limitations have been previously identified as the limiting step in a bioscrubber treating N_2O laden air emissions from wastewater treatment plants, where a gas EBRT of 40 min was needed in the

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adsorption column in order to obtain a satisfactory N_2O RE of 92%.[10] Indeed, previous studies have consistently shown gas−liquid mass transfer limitations in bioreactors devoted to the treatment of poorly water-soluble gas pollutants.[15,26] This mass transfer limitation could be also observed in the decrease of the specific N_2O removal capacity of both bioreactors, which resulted in average values of 0.47 ± 0.03 and 0.54 ± 0.1 gN_2O $gTSS^{-1}$ d^{-1} in the BCR and ALR, respectively. This limited N_2O mass transfer from the gas emission entails process operation at high EBRTs, thus resulting in large bioreactor volumes (= high investment costs). In this sense, the use of ceramic or polymer membrane gas diffusers with a pore size <1 μm and internal gas recycling strategies can significantly enhance the volumetric gas−liquid mass transfer coefficient (at the expenses of slightly higher operating costs).[27,28] Another potential alternative to overcome this mass transfer limitation is the addition to the bioreactor of an immiscible, nonvolatile, nontoxic and nonbiodegradable nonaqueous phase (NAP) with a high affinity for N_2O.[27,29] However, the selection of the optimum NAPs to enhance the mass transfer of N_2O in the so-called two-phase partitioning bioreactors has not been conducted yet.

This study evaluated the potential biological removal of N_2O along with the simultaneous accumulation of PHBV as a cost-effective alternative for industrial GHG emission mitigation. The bioreactors herein used have been consistently proven as low cost alternative technologies for the treatment of wastewaters and off-gases.[30−33] These bioreactor configurations are pneumatically agitated, resulting in low energy consumptions. Moreover, their simple construction (with no moving parts) and high gas−liquid mass transfer rates constitute also key advantages over their biological counterpart.[34−36] In addition, the selection of BCR and ALR configurations in this study was based on the need of a suspended culture to facilitate biomass harvesting and ensure a homogeneous methanol/nitrogen distribution. In our particular study, N_2O-REs of 80−90% were consistently achieved concomitantly with the coproduction of added value biopolymers (see section below), which were comparable with the N_2O abatement efficiencies of conventional physical/chemical technologies such as NSCR.[7] Decrease in the performance of N_2O removal was observed during Stage II due the low biomass concentrations, but was easily overcame with the strategy implemented in Stage III (same nutrient load at a higher inlet N concentration) maintaining a high biopolymer accumulation (Figures 1A and 3A). However, the gas EBRT (≈17 min) required to obtain high REs in the two bioreactor configurations evaluated would entail high bioreactor volumes.

PHBV Accumulation During N_2O Abatement. A low PHBV cell content was recorded during Stage I (1.9 ± 1.3% in the BCR and 2.6 ± 1.3% in the ALR) under TN concentrations in the cultivation broth of 238 ± 38 and 238 ± 40 mg N L^{-1} in the BCR and ALR, respectively. The dissolved CH_3OH concentrations in the BCR and ALR also remained constant during Stage I at 395 ± 20 and 367 ± 39 mg C L^{-1}, respectively.

N was completely depleted by day 66 in Stage II, which promoted the gradual increase in the PHBV cell content in both bioreactors (Figure 3). The nitrogen supply strategy evaluated during Stage II resulted in 24 h of nitrogen sufficiency after MSM renewal followed by 24 h under nitrogen limitation, where PHBV synthesis and accumulation was likely to occur. N-limitation induced a steady state PHBV cell content of 38 ± 7% in the BCR under TN and dissolved CH_3OH concentrations of 2.6 ± 0.5 mg N L^{-1} (Figure 3A) and 177 ± 28 mg C L^{-1}, respectively. The PHBV cell content recorded in the ALR was significantly higher than in the BCR, with average values of 64 ± 11% (Figure 3B) under steady TN concentrations of 2.8 ± 0.6 mg N L^{-1} and dissolved CH_3OH concentrations of 368 ± 39 mg C L^{-1}. The transmission electron micrographs depicted in Figure 4 confirmed the accumulation of PHBV as granules inside bacteria with a cell diameter ranging from 0.5 to 1 μm, which matched the cell size of *P. denitrificans*.[37]

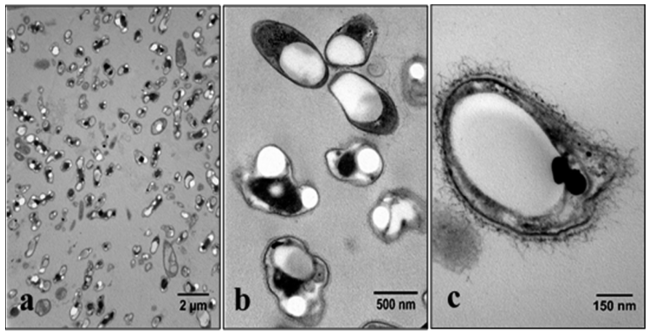

Figure 4. Transmission electron micrographs of cells containing PHBV in the BCR (a, c) and the ALR (b). Samples were drawn at the end of Stage III.

Process operation at a reduced dilution rate of 0.033 d^{-1} under similar N loads as Stage II mediated microbial cultivation with N sufficiency for 24 h followed by 3 days of N deprivation in Stage III. These operational conditions promoted an enhanced PHBV cell content in the BCR of 47 ± 5%, under steady TN and dissolved CH_3OH concentrations of 2.3 ± 0.3 mg N L^{-1} and 134 ± 23 mg C L^{-1}, respectively. However, the PHBV cell content recorded in the ALR decreased to 40 ± 8% in spite of the comparable TN concentration (1.9 ± 0.4 mg N L^{-1}) and dissolved CH_3OH concentrations (373 ± 72 mg C L^{-1}). Both bioreactors supported low PHBV productivities in excess of nitrogen (~0.8 g PHBV m^{-3} d^{-1}). These productivities increased in Stage II up to 10 g PHBV m^{-3} d^{-1} in both bioreactors. The highest PHBV productivities were recorded in the last stage of BCR operation (15 g PHBV m^{-3} d^{-1}), although PHBV productivity in the ALR decreased to 7 g PHBV m^{-3} d^{-1} in stage III. PHBV yield of 0.06 ± 0.02 gPHBV g^{-1}_{CH3OH} was observed in the ALR in Stage II, decreasing to average values of 0.03 ± 0.01 gPHBV g^{-1}_{CH3OH} during Stage III. The BCR supported comparable production yields of 0.05 ± 0.01 gPHBV g^{-1}_{CH3OH} and 0.06 ± 0.02 gPHBV g^{-1}_{CH3OH} in Stages II and III, respectively. The yields obtained were in agreement with previously reported PHBV yields ranging from 0.02 to 0.19 gPHBV g^{-1}_{CH3OH} using methanol as the carbon source.[38−40]

The GC-MS analysis of the copolymer PHBV showed a small share of 3-hydroxyvalerate (PHV) regardless of the operational conditions evaluated. PHV/PHBV molar ratios of 2.5 ± 0.9% and 2.9 ± 1.6% were recorded at Stage I in the BCR and the ALR, respectively. When the bioreactors were subjected to nutrient limitation during Stages II and III, this ratio decreased to 0.46 ± 0.2 and 0.29 ± 0.1 in the BCR, and to 0.35 ± 0.1 and 0.32 ± 0.2% in the ALR, respectively. Several authors have recorded similar results using methanol as the sole carbon and energy source under different nutrient limitation strategies.

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In this context, Ueda et al.[41] did not detect PHV in the PHBV copolymer accumulated in *P. denitrificans* when CH_3OH was used as the sole substrate (0.3% v/v). However, the PHV molar fraction increased up to 87% when *n*-amyl alcohol (0.25% v/v) was supplied together with CH_3OH (0.3% v/v) . Similarly, Yamane et al.[12] explored the role of the type of alcohols (methanol, ethanol, *n*-propanol, *n*-butanol, and *n*-pentanol) at a concentration of 0.1% (v/v) on the PHBV cell content in *P. denitrificans* under N limiting conditions. The results revealed no PHV accumulation when CH_3OH was the sole carbon source, which suggested that CH_3OH is not the most suitable carbon source when a high share of PHV is desired. In spite of the non-negligible cost associated with the purchase of the methanol (≈180 € ton^{-1})[14] required for biological N_2O reduction, this operating cost will be compensated by the fact that a large fraction of this methanol is converted into PHBV (with a market price of 4000−2000 € ton^{-1})

The analysis of the elemental cell composition (C, H, S, and N) carried out at the end of Stage I showed C and N cell contents of ≈44 and ≈11%, respectively (SI Table S1), which represented a C/N ratio of ≈4. This value was in agreement with the typical elemental composition for bacterial cells.[42] However, a significant reduction in N cell content was observed in the biomass from both bioreactors as a result of cell adaptation when N limiting strategies were implemented in Stages II and III. Thus, the C/N ratio recorded in the ALR and the BCR under nitrogen limitation increased to values ranging from 6.1 to 8.2. The likely decrease in protein cell content due to the limited N uptake also entailed a decrease in the S content of the microbial communities present in both bioreactors. A variation in the C cell content was not observed in spite of the accumulation of the biopolymer likely due to the similar elemental composition (C, H, and O) of PHBV and biomass.

FISH Analysis of the Microbial Population Structure. The FISH analysis revealed the variation of the abundance of the *P. denitrificans* along the entire operational period (SI Table S2). Both bioreactors showed a *P. denitrificans* abundance higher than 90% by the end of Stage I (Figure 5, SI Table S2).

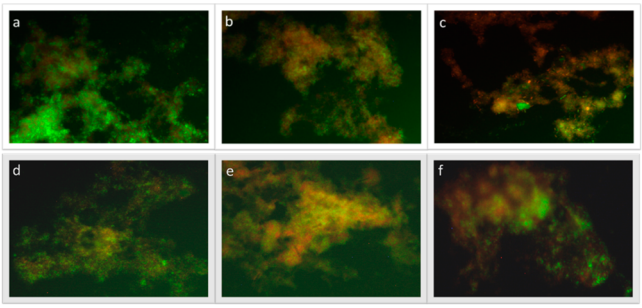

Figure 5. FISH micrographs of the microbial culture present at the end of the three operational stages evaluated in the ALR (a−c) and BCR (d−f). PAR651-fam (green) appears yellow due to a double hybridization with the EUB338 I−II- FITC probes (red).

At the end of Stage II (day 120), the abundance of the inoculated strain in the BCR and ALR slightly decreased to 88% and 86% (SI Table S2). These results confirmed that *P. denitrificans* was capable of growing and dominating the microbial culture under anoxic conditions using CH_3OH as the sole carbon/energy source and N_2O as electron acceptor. By the end of the experimentation (day 180), *P. denitrificans* remained dominant in both bioreactors (abundances >50%) (Figure 5c and f). In this context, the presence of others microbial strains capable of accumulating biopolymers may explain the maintenance of the PHBV cell content observed in Stage III despite the decrease in *P. denitrificans* abundance. Nonetheless, it is important to point out that FISH analysis could have slightly underestimated the abundances of *P. denitrificans* due to the foreground effect, which prevented the observation of the bacterial cells present in the backside of the flocs.

In summary, this work demonstrated the feasibility of the combined biological abatement of N_2O from industrial emissions and coproduction of PHBV. High N_2O-REs were recorded in spite of process operation under nitrogen limiting conditions. The nitrogen limiting strategies assessed in this study resulted in a high accumulation of PHBV by *P. denitrificans* using methanol and N_2O as the carbon/energy source and the electron acceptor, respectively. This study reports the first bioprocess for the active abatement of N_2O using a waste-to-value biorefinery approach.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b00643.

A schematic diagram of the operational setup depicted in Figure S1 as well as additional data obtained from the mass transfer tests conducted at the end of Stages II (Figure S2) and III (Figure S3). Furthermore, the results from the analysis of the elemental composition (Table S1) and FISH analysis (Table S2) of the biomass (PDF)

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Notes

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Nitrous oxide abatement coupled with biopolymer production as a model GHG biorefinery for cost-effective climate change mitigation

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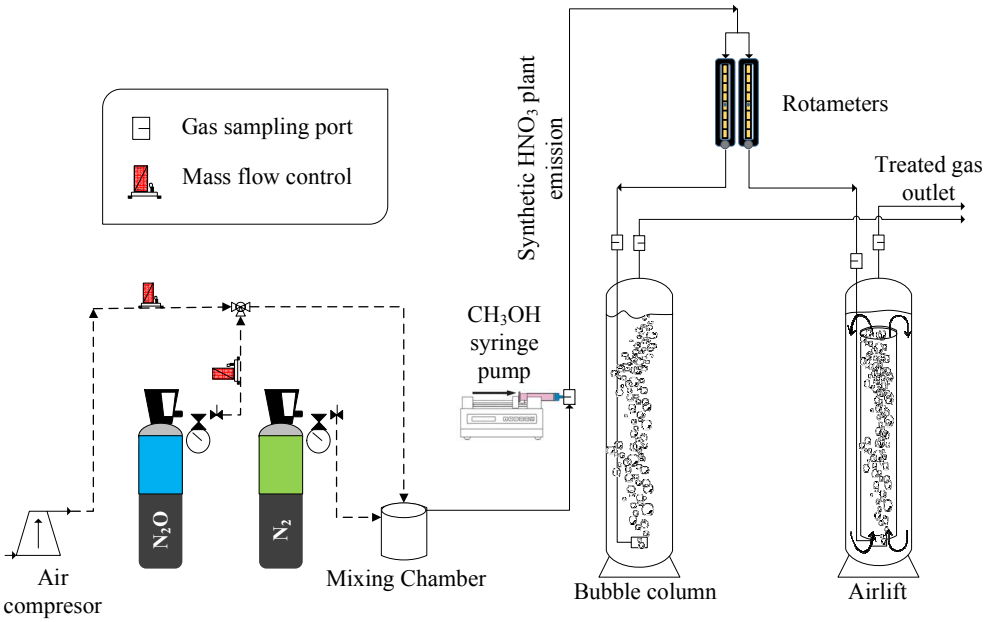

Figure S1. Schematic diagram of the BCR and the ALR.

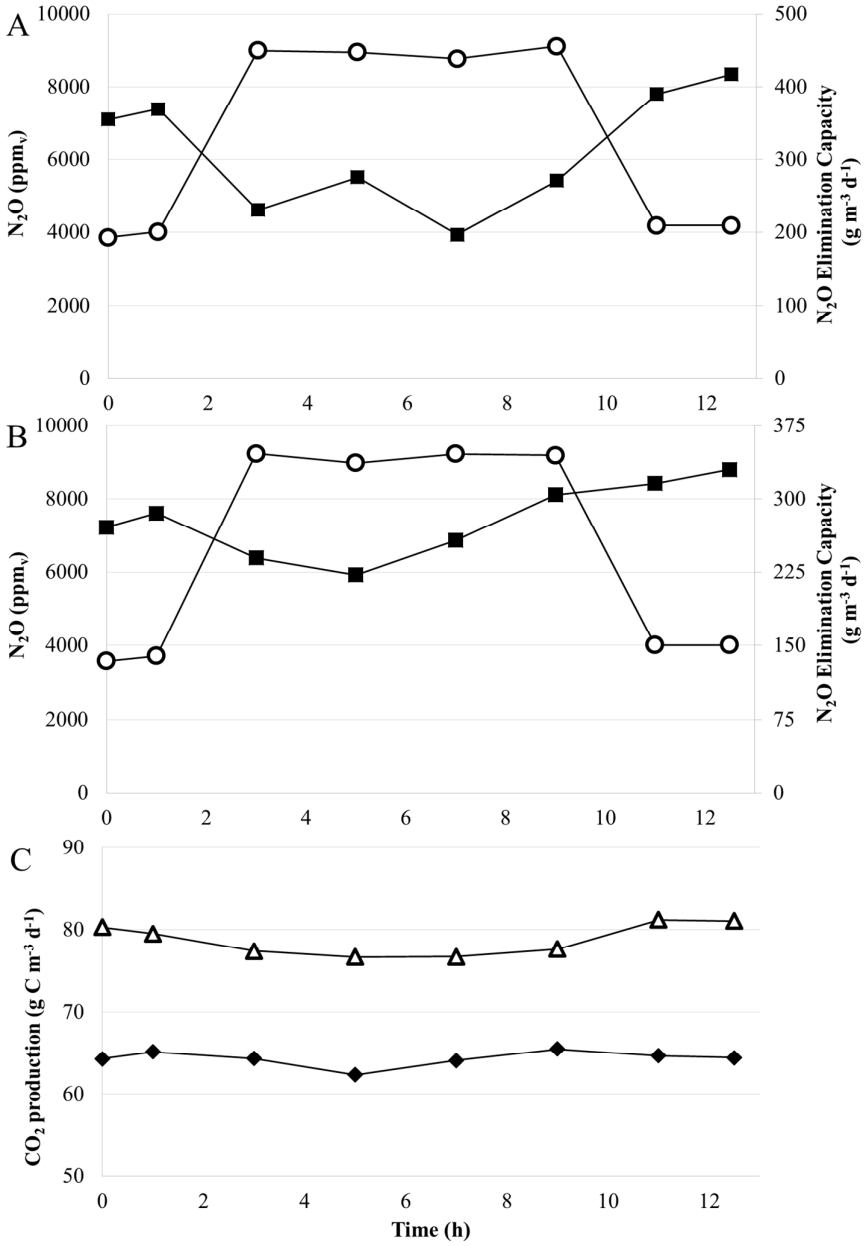

Figure S2. Time course of the inlet N_2O concentration (○) and N_2O elimination capacity (■) in the BCR (A) and ALR (B), and CO_2 production rate (C) in the BCR (Δ) and ALR (♦) during the mass transfer test carried out at the end of Stage II.

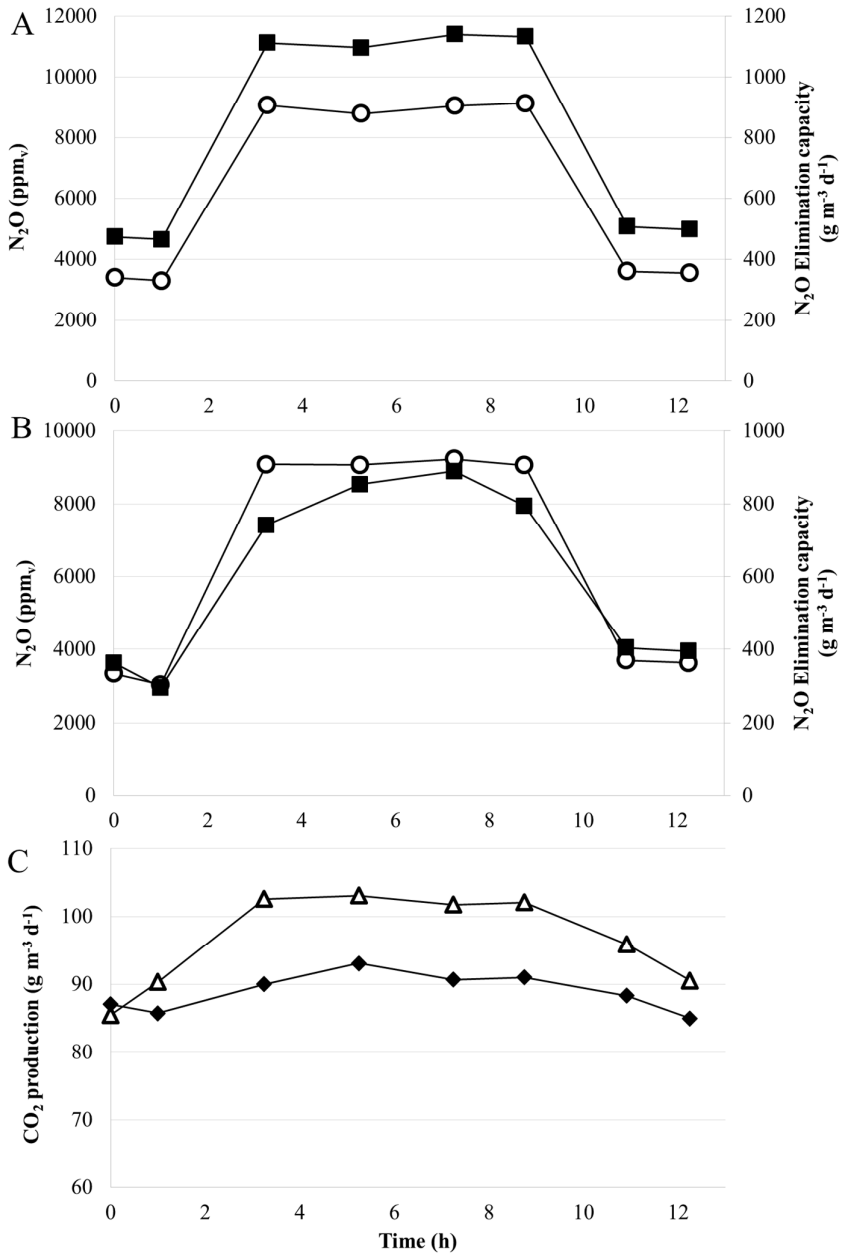

Figure S3. Time course of the inlet N_2O concentration (○) and N_2O elimination capacity (■) in the BCR (A) and the ALR (B), and CO_2 production rate (C) in the BCR (Δ) and ALR (♦) during the mass transfer test carried out at the end of Stage III.

Table S1. Elemental composition of the microbial cultures in the BCR and ALR.

Bubble Column Reactor				
Stages	**Carbon (%)**	**Hydrogen (%)**	**Sulphur (%)**	**Nitrogen (%)**
I	43.0	6.2	0.6	11.2
II	45.7	6.4	0.4	7.5
III	44.2	6.2	0.3	5.4
Airlift Reactor				
Stages	**Carbon (%)**	**Hydrogen (%)**	**Sulphur (%)**	**Nitrogen (%)**
I	44.4	6.4	0.6	11.1
II	42.2	6.1	0.4	5.9
III	38.0	5.6	0.4	5.4

Table S2. Abundance of *Paracoccus denitrificans* (PAR651-fam probe) relative to the total bacterial population (EUB338 I-II- FITC probes) in the BCR and ALR.

Bioreactors	**Stages**	***Paracoccus denitrificans* (%)**
ALR	I	90
	II	86
	III	58
BCR	I	95
	II	88
	III	53

Assessing the influence of the carbon source on the abatement of industrial N_2O emissions coupled with the synthesis of added-value bioproducts

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Chapter 6

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Assessing the influence of the carbon source on the abatement of industrial N_2O emissions coupled with the synthesis of added-value bioproducts

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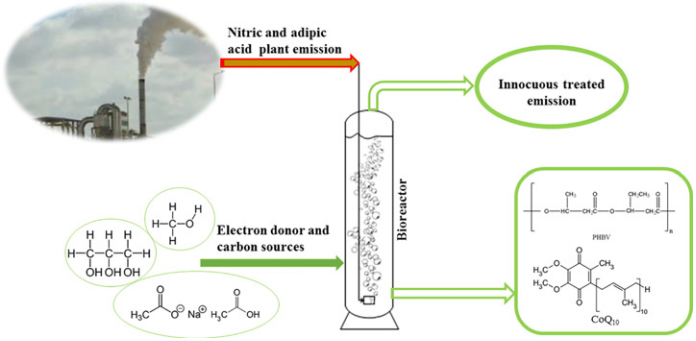
HIGHLIGHTS

- HNO_3 and $C_6H_{10}O_4$ production is the largest industrial source of N_2O emissions.
- Biotechnologies have emerged as an eco-friendly alternative for GHG treatment.
- Three C sources were assessed for N_2O bioconversion into added-value bioproducts.
- A high N_2O removal was obtained concomitantly with a high PHBV accumulation.
- The coenzyme CoQ_{10} was also synthetized during N_2O abatement.

GRAPHICAL ABSTRACT



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ABSTRACT

The continuous abatement of a synthetic N_2O emission from a nitric acid plant coupled with the simultaneously production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) copolymer and the coenzyme Q10 (CoQ_{10}) in a bubble column reactor (BCR) was tested using methanol, glycerol and a mixture of sodium acetate-acetic acid (Ac-HAc) as a carbon and electron donor source. The BCRs were inoculated with *Paracoccus denitrificans* and supplied with the carbon/electron donor at a loading rate of 139 g C m^{-3} d^{-1}. High N_2O removal efficiencies (81–91%) were achieved, with glycerol supporting the highest abatement. The PHBV cell content ranged from 25 to 53%, with highest accumulation in the culture obtained with methanol and Ac-HAc. However, the greatest PHBV productivities were observed in the BCRs operated with glycerol and Ac-HAc (21.7 and 33.5 g PHBV m^{-3} d^{-1}, respectively). Glycerol supply induced the highest molar ratio (23%) of the homopolymer 3-hydroxyvalerate in the composition of PHBV. In addition, the specific cell content of CoQ_{10} ranged from 0.4 to 1 mg g^{-1}. This work constitutes, to the best of our knowledge, the first study combining N_2O abatement with the simultaneous production of multiple bioproducts, which pave the way to the development of greenhouse gas biorefineries for climate change mitigation.

1. Introduction

The reduction of greenhouse gas (GHG) emissions has emerged as one of the most important environmental and economic challenges for the countries endorsing the recent Paris Agreement (COP21), which

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aimed at maintaining below 1.5 °C the global average temperature increase compared to the levels of the pre-industrial era (UNFCCC, 2015). In this context, the abatement of the emissions of nitrous oxide (N_2O), a potent GHG with a global warming potential 298 times higher than that of CO_2 and a contribution to the global GHG inventory of ~6.2%, will be mandatory in any future national strategy to mitigate climate change.

Anthropogenic N_2O in industry is mainly emitted during nitric and adipic acid production, which is responsible for the emission of approximately 500,000 tons of N_2O per year (Pérez-Ramírez et al., 2003). The control of these industrial emissions has been traditionally conducted by physical/chemical technologies, which are characterized by the utilization of costly catalysts (at high pressure and temperature) and reducing agents such as ammonia or hydrocarbons. In addition, physical/chemical processes entail pernicious environmental impacts such as secondary gas emissions, the generation of a toxic spent catalyst and a high energy consumption (Environmental Protection Agency, 2010). Conversely, biotechnologies based on the use of denitrifying bacteria capable of heterotrophically reducing N_2O to N_2 have recently emerged as a low-cost and eco-friendly alternative to conventional physical/chemical technologies (Frutos et al., 2015; Frutos et al., 2016a, 2016b). These biotechnologies consist of a two-stage process where N_2O is firstly transferred from the gas emission to an aqueous phase and thereafter reduced biologically using an external carbon/electron donor source. The purchase of this carbon/electron donor entails an increase in the operating cost of biotechnologies devoted to N_2O abatement, particularly when high levels of oxygen are present in the N_2O-laden emission and therefore a high organic loading rate is required to maintain anoxic conditions.

The biological transformation of GHGs such as CH_4 into added value biopolymers such as poly(3-hydroxybutyrate) (PHB) and the *co*polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) has emerged as a promising alternative to enhance the cost-competitiveness of biological off-gas treatment (Myung et al., 2016; Zúñiga et al., 2011). These biodegradable biopolymers, which are present as water insoluble inclusions, accumulate as energy reservoir in eubacteria and archaea under excess of carbon source and nutrient limitation (Rehm, 2003). PHBV exhibits similar characteristics to the fossil thermoplastics polypropylene and polyethylene in terms of molecular weight, tensile strength or even melting point (Khosravi-Darani et al., 2013). Furthermore, superior physical properties than PHB can be achieved when a high proportion of the homopolymer 3-hydroxyvalerate (PHV) is present in the PHBV copolymer (Khanna and Srivastava, 2005; Reddy et al., 2003). Likewise, the co-production during GHG treatment of the coenzyme Q10 (CoQ_{10}), with a market price of ~300 € kg^{-1}, could turn climate change mitigation into a profitable process (Wu and Tsai, 2013). CoQ_{10} is intensively used nowadays for the treatment of cancer and hypertension (Jeya et al., 2010), and as antiaging agent in cosmetics manufacture (Ernster and Dallner, 1995). Despite the economic and environmental advantages derived from the co-production of PHBV and CoQ_{10} coupled to N_2O abatement, the potential of this novel GHG abatement approach has been poorly explored.

The present study systematically evaluated the influence of the type of carbon source/electron donor (methanol, glycerol and acetate-acetic acid) on the abatement of N_2O from a nitric acid production plant coupled to the simultaneous production of PHBV and CoQ_{10} in a bubble column bioreactor using *Paracoccus denitrificans* as a model denitrifying microorganism.

2. Material and methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used was composed of (g L^{-1}): $Na_2HPO_4 \cdot 12H_2O$ 6.16, KH_2PO_4 1.52, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2$ 0.02, NH_4Cl 0.26 and 10 mL L^{-1} of a trace element solution containing (g L^{-1}): EDTA 0.5, $FeSO_4 \cdot 7H_2O$ 0.2, $ZnSO_4 \cdot 7H_2O$ 0.01, $MnCl_2 \cdot 4H_2O$ 0.003, H_3BO_3 0.03, $CoCl_2 \cdot 6H_2O$ 0.02, $CuCl_2 \cdot 2H_2O$ 0.001, $NiCl_2 \cdot 6H_2O$ 0.002, $NaMoO_4 \cdot 2H_2O$ 0.003. The pH of the MSM was adjusted to 7. All chemicals were purchased from PANREAC with purities >99%. PHBV (12% of PHV on a molar basis, equal to ~14% on a mass basis), benzoic acid, methanol (CH_3OH), glycerol ($C_3H_8O_3$), sodium acetate ($C_2H_3NaO_2$) and acetic acid (CH_3COOH) were obtained from Sigma-Aldrich® (Sigma-Aldrich, St. Louis, MO, USA). The 40 L calibrated gas cylinders of 50,000 ppm_v of N_2O in N_2 and 50 L industrial N_2 cylinders were purchased from Abelló Linde S.A. (Barcelona, Spain).

2.2. Experimental set-up and operational conditions

The influence of the type of carbon source/electron donor (methanol, glycerol and acetate-acetic acid) on the abatement of N_2O and co-production of PHBV and CoQ_{10} was assessed in three independent experiments in a 2.5 L glass bubble column bioreactor (BCR) treating a synthetic nitric acid plant emission. In each experimental run, the BCR was inoculated with 1 L of fresh *Paracoccus denitrificans* culture (Frutos et al., 2016a, 2016b) and filled up with MSM to a working volume of 2.3 L (Fig. 1). Fish analysis revealed that *P. denitrificans* was the most abundant microorganism in the culture used as inoculum. The initial biomass concentration in the bioreactors was 493 mg L^{-1}. Based on the typical composition of N_2O emissions from nitric acid production plants (Pérez-Ramírez et al., 2003), a synthetic off-gas was prepared by mixing the 50,000 ppm_v N_2O standard with pure N_2 and air at a final concentration of 3560 ± 360 ppm_v of N_2O, 1.1 ± 0.1% of O_2 and 98.5% of N_2. The synthetic N_2O emission was fed at the bottom of the BCR via a gas diffuser (2 μm) at a flow rate of 137 ± 7 mL min^{-1} (equivalent to ~0.06 vvm), thus resulting in a gas residence time (RT) of ~17 min. The low concentration of oxygen in the synthetic gas emission, along with its poor aqueous solubility, supported an effective anoxic reduction of N_2O during bioreactor operation. Based on previous experiences in a similar experimental set-up, aliquots of 300 mL of culture broth were exchanged every 4 days with fresh MSM. This strategy imposed a series of 1 day–3 days nitrogen feast-famine cycles under excess of carbon source. The experiments were conducted at 25 °C in a temperature-controlled room.

The experiment using CH_3OH as a carbon source/electron donor (BCR-A) was carried out for 65 days at a carbon loading rate of 139 g C m^{-3} d^{-1}. The experimental run using glycerol (BCR-B) was initially operated at a carbon loading rate of 139 g C m^{-3} d^{-1} for 40 days, which was increased to 209 g C m^{-3} d^{-1} for the last 25 days of operation to prevent carbon limitation in the process. The experiment with acetate (BCR-C) was performed with a 95%/5% (Cmol/Cmol) mixture of sodium acetate/acetic acid (Ac-HAc; pH 6.5) at a carbon loading rate of 139 g C m^{-3} d^{-1} for 75 days. BCR-C was operated without pH control for the first 34 days and at a pH of 7 afterwards via daily addition of HCl (37%).

The determination of the inlet and outlet gas concentrations of N_2O, CO_2 and O_2 was daily conducted by GC-ECD and GC-TCD according to Frutos et al. (2016a, 2016b). More specifically, N_2O gas concentration was determined using a Bruker Scion 436 gas chromatograph (Palo Alto, USA) equipped with an Electron Capture Detector and a HS-Q packed column (1 m × 2 mm ID × 3,18 mm OD) (Bruker, USA). Injector, detector and oven temperatures were set at 100 °C, 300 °C and 40 °C, respectively. Nitrogen was used as the carrier gas at 20 mL min^{-1}. External standards prepared in volumetric bulbs (Sigma–Aldrich, USA) were used for N_2O quantification. The N_2O removal efficiency (RE) was estimated as the fraction of the input N_2O removed in the bioreactors as follow $RE = ([N_2O_{inlet}] - [N_2O_{outlet}]) / [N_2O_{inlet}] \times 100$. The gas concentration of CH_3OH was determined by GC-FID every 4 days before MSM exchange. The total organic carbon (TOC), total nitrogen (TN) and inorganic carbon (IC) concentrations were measured every 4 days from the withdrawn culture broth. Similarly, volatile fatty acid (VFA) and glycerol in the culture broth was determined by GC-FID and HPLC-IR, respectively. Liquid

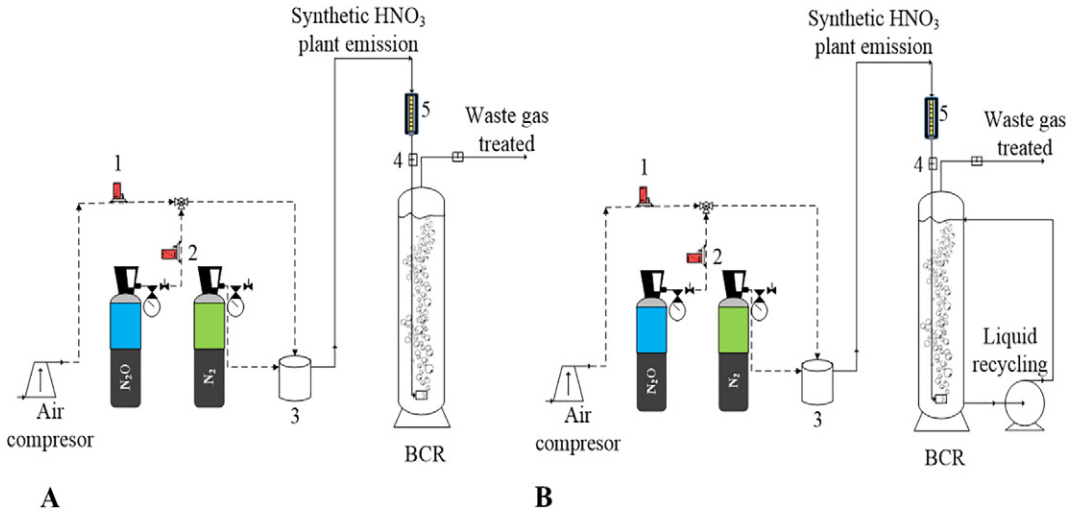

Fig. 1. Schemes of the BCR-A, BRC-B (A) and BCR-C (B) where: 1) air mass flow controller, 2) N_2O mass flow controller, 3) gas mixing chamber, 4) gas sampling port and 5) rotameter.

samples of 40 mL were drawn for the measurement of the concentrations of total suspended solid (TSS) and PHBV every 4 days, while 10 mL were used for CoQ_{10} determination at the end of each BCR operation. pH was determined every 4 days in each bioreactor, but daily measured during BCR-C operation with pH control.

2.3. Analytical procedures

The concentration of TOC, TN and IC was measured in a TOC-VCSH analyzer (Shimadzu, Tokyo, Japan) coupled with a TN chemiluminescence detection module (TNM-1, Shimadzu, Japan). The TSS concentration was determined according to Standard methods (APHA, 2005).

The GC-FID (Bruker 3900, Palo Alto, USA) used for CH_3OH determination was equipped with an Agilent HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column maintained at 50 °C for 0.6 min with a subsequence temperature ramp increase of 75 °C min^{-1} up to 200 °C. The injector and detector temperatures were maintained at 200 and 250 °C, respectively. Nitrogen was used as the carrier gas at 1 mL min^{-1} and make-up gas at 25 mL min^{-1}, while the flowrates of H_2 and air were set at 30 and 300 mL min^{-1}, respectively. Glycerol was determined in a Waters e2695 HPLC (Massachusetts, USA) equipped with a Waters 2414 refractive index detector using a Bio-Rad HPX-87H column at 50 °C and a 5 mM H_2SO_4 aqueous eluent at 0.6 mL min^{-1}. The concentration of dissolved VFA was determined by GC-FID following the procedure indicated in Alcántara et al. (2015). The analysis of PHBV involved the centrifugation of 2 mL of culture broth at 9000 rpm for 15 min and the processing of the biomass pellet according to Zúñiga et al. (2011). Then, the PHBV was extracted for 4 h at 100 °C using 2 mL of chloroform and quantified (using external standards) by GC–MS in a GC System 161 7820A MSD 5977E (Agilent Technologies, Santa Clara, USA) equipped with a DB-wax capillary column (30 m × 250 μm × 0.25 μm). The temperatures of the detector and injector were set at 250 °C, with a split ratio of 1:10. The oven temperature was initially maintained at 40 °C for 5 min, increased at 10 °C min^{-1} up to 200 °C and maintained for 5 min. The PHBV cell content was normalized as %PHBV = (g PHBV / g TSS) × 100. The PHV molar ratio of the PHBV copolymer was estimated based on the concentration of each homopolymer and the weight/M ratio of the standard as follow %PHV = (PHV/PHBV) × 100 × (12/14).

Similarly, the analysis of CoQ_{10} involved the centrifugation of 10 mL of culture broth and the lysis of the biomass pellets by addition of 0.5 mL of CelLytic™, vortexing and incubation in an ultrasonic bath for 30 min. CoQ_{10} was then extracted in a water bath with 2.5 mL of a propanol/hexane solution (3:5 v:v) at 40 °C (30 min). Finally, the organic phase was filtered (0.2 μm) and transferred to a 1 mL vial for analysis by HPLC-UV. The Waters e2695 HPLC was equipped with a Waters symmetry C18 column (3.5 μm × 3 mm × 100 mm) using a methanol/hexane (83:17 v:v) mobile phase at a flow rate of 0.6 mL min^{-1}. A UV detector (UV 2487) was used for CoQ_{10} determination at a wavelength of 275 nm. The final CoQ_{10} concentration was correlated to the TSS concentration and expressed as milligram of CoQ_{10} per gram of biomass (mg g^{-1}).

3. Results and discussion

3.1. Process performance with methanol

The use of methanol as a carbon source/electron donor supported average removal efficiencies (REs) of 87 ± 3% along the entire operational period (Fig. 2A). Steady CO_2 production rates of 283 ± 15 g m^{-3} d^{-1} were recorded from day 20 despite N_2O-REs remained constant from day 5 onward (Fig. 2B). This stabilization in CO_2 production occurred

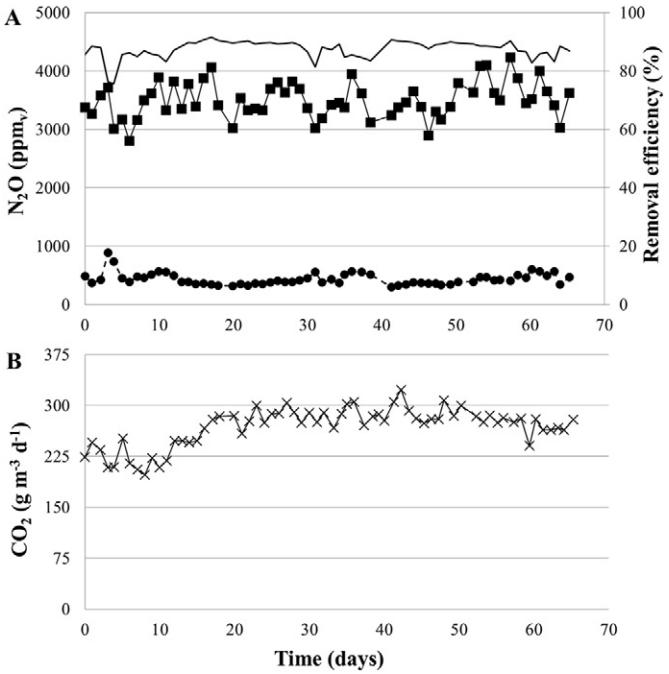

Fig. 2. Time course of the A) inlet (■) and outlet (●) N_2O concentrations and removal efficiency (solid line), and B) CO_2 production rates in the BCR supplied with methanol.

concomitantly with the stabilization in biomass concentration, which averaged 1086 ± 80 mg L^{-1} (Table 1). In this context, the specific N_2O elimination capacity supported by methanol under biomass steady state concentration was 0.46 ± 0.05 $gN_2O\ gTSS^{-1}\ d^{-1}$. No significant variation was however observed in the pH of culture broth, which remained constant at 6.8 ± 0.1 along the 65 days of operation.

The ratio of CO_2 produced per CH_3OH consumed averaged 0.83 ± 0.18 $gC\ gC^{-1}$ under steady state conditions, which indicates that most of the carbon supplied as methanol was used for energy production purposes. Likewise, the ratio of CO_2 produced per N_2O reduced to N_2 accounted for 0.54 ± 0.07 $gCO_2\ gN_2O^{-1}$. Part of the CH_3OH supplied accumulated as dissolved TOC in the culture broth (356 ± 54 mg L^{-1}, which represented ~29% of the methanol fed to the BCR-A), while the remaining methanol resulted in the formation of biomass and accumulation of PHBV to a cell content of 46 ± 5% under nitrogen limitation (Fig. 3). Nitrogen limitation was characterized by steady state TN concentrations of 3.2 ± 0.4 mg L^{-1} (Table 1). The PHBV cell content here recorded was higher than that reported by Yamane et al. (1996) using CH_3OH as the carbon source during *P. denitrificans* growth under nitrogen limitation and aerobic conditions (13.3%). Our study revealed a PHV molar ratio of 0.38 ± 0.25% in the PHBV copolymer, which agreed with the results reported in previous works using CH_3OH as a substrate (Ueda et al., 1992; Yamane et al., 1996). The PHBV productivity in BCR-A under steady state conditions averaged 16.3 gPHBV $m^{-3}\ d^{-1}$.

Despite no particular strategy was implemented to boost CoQ_{10} accumulation in the microbial culture, a specific cell content of 0.9 mg g^{-1} (associated to a productivity of 32 mg $m^{-3}\ d^{-1}$) was recorded in BCR-A (Table 1). Similar results were reported by Yoshida et al. (1998) assessing the performance of 34 bacterial strains previously characterized as CoQ_{10} producers. These authors found CoQ_{10} specific cell contents of 0.86 mg g^{-1} in *Paracoccus denitrificans* strain (ATCC19367) under aerobic conditions using cane molasses as a carbon source at a concentration of 5%. Likewise, a study assessing the effect of O_2 levels on the production of CoQ_{10} in *P. denitrificans* CCM 982 showed that the highest yield (1.2 mg g^{-1}) was obtained at an oxygen concentration of 2.5%, with a decrease in the coenzyme content to 0.43 mg g^{-1} when the O_2 levels increased to 21% (Kaplan et al., 1993). Recently, the CoQ_{10} biosynthesis capacity of a mutant strain of *P. denitrificans* (P-87) has been evaluated using the precursor parahydroxy benzoic acid (Tokdar et al., 2014). This study revealed a maximum specific cell content of 1.63 mg g^{-1}, which was only 1.8-fold higher than that obtained in our study with no particular optimization strategy.

3.2. Process performance with glycerol

The use of glycerol at a loading rate of 139 g C $m^{-3}\ d^{-1}$ in BCR-B supported a N_2O-RE of 89 ± 2% (Fig. 4A), which was similar to the removal efficiency supported by CH_3OH at a similar C loading rate. Glycerol, which was a more favorable substrate than CH_3OH from an energy viewpoint, promoted higher steady state biomass concentration (1638 ± 76 mg L^{-1}) than that attained with methanol after 10 days of bioreactor operation (Table 1). This higher biomass concentration resulted in greater steady state CO_2 productions of up to 306 ± 8 g $m^{-3}\ d^{-1}$ (Fig. 4B).

Process operation at a low glycerol loading rate resulted in a similar CO_2 produced per N_2O consumed ratio (0.59 ± 0.08 $gCO_2\ gN_2O^{-1}$) to that recorded in BCR-A, but a lower specific CO_2 production yield (0.58 ± 0.04 $gC\ gC^{-1}$). This lower respiration yield revealed that more carbon was devoted to the synthesis of new microbial cells compared to the use of methanol as a carbon source/electron donor. Likewise, the specific N_2O removal capacity of the culture decreased to 0.33 ± 0.03 $gN_2O\ gTSS^{-1}\ d^{-1}$ mediated by the higher biomass concentration and the limited mass transfer of N_2O from the gas emission. Indeed, the fact that similar N_2O removal efficiencies were recorded at higher *P. denitrificans* concentrations clearly showed that the process was limited by the mass transport of N_2O from the gas to the liquid phase rather than by biological activity. The low water solubility of N_2O (H = 1.6 at 25 °C (Sander, 2014)) resulted in a limited N_2O transfer from the gas to liquid phase, thus limiting the N_2O elimination capacity of the bioreactor. On the other hand, the dissolved TOC concentration in the culture broth stabilized at 75 ± 9 mg L^{-1} from day 10 to 40 (Table 1). Surprisingly, the analysis of the culture broth by HPLC revealed that this TOC did not correspond to residual glycerol and was likely due to the carbon released from cell lysis (cell debris). In this context, although the determination of the concentration of extracellular polymeric substances (EPS) was out of the scope of this study, the capability of *P. denitrificans* to produce EPS under certain conditions was previously reported and could have contributed to the residual TOC observed (Klueglein et al., 2014). In this scenario of glycerol limitation and high biomass concentration, a PHBV cell content of 31 ± 8% was recorded under nitrogen limitation (TN concentrations of 4.2 ± 0.5 mg L^{-1}). Despite PHBV accumulation under glycerol limitation was slightly lower than in BCR-A, a remarkable increase in the content of the PHV homopolymer was recorded (with a PHV/PHBV ratio of 10 ± 1% on a molar basis) in the first 40 days of BCR-B operation (Fig. 3). In addition, the productivity of PHBV was slightly higher (16.7 gPHBV $m^{-3}\ d^{-1}$) than that recorded in BCR-A.

The increase in glycerol loading rate to 209 g C $m^{-3}\ d^{-1}$ resulted in a slight increase in the N_2O-RE to 91 ± 3%, with a concomitant increase in the CO_2 production rates to 342 ± 15 g $m^{-3}\ d^{-1}$ (Fig. 4). Likewise, biomass achieved stable concentrations of 2707 ± 85 mg L^{-1} mediated by the higher glycerol loading rate (Table 1). However, the N_2O mass transfer to the culture broth remained similar to that recorded in the previous runs as a result of the low aqueous N_2O solubility and equal k_la (which was determined by the diffuser and bioreactor configuration). Thus, the limited N_2O mass transfer, together with the increase in biomass concentration in this operational stage, resulted in a decrease in the specific N_2O removal capacity to 0.17 ± 0.03 $gN_2O\ gTSS^{-1}\ d^{-1}$. These empirical findings finally supported the above-hypothesized N_2O mass transfer limitation. On the other hand, the ratio of CO_2 produced per glycerol consumed decreased to 0.47 ± 0.05 $gC\ gC^{-1}$, which confirmed that a higher proportion of the carbon present in glycerol was routed to the production of cell material. Glycerol has recently emerged as a cost-competitive substrate for the production of added-value bioproducts and represents an alternative to support the biological removal of N_2O as a result of the decreasing market price (approx. 0.12 $ per kg of crude glycerol) mediated by the increasing biodiesel market. Despite the fact that a higher glycerol loading rate entailed an increase in the dissolved TOC concentration in the system (331 ± 61 mg L^{-1}), the microbial culture did not accumulate a higher content of PHBV (25 ± 1%) (Fig. 3). However, the higher glycerol loading did result in an increase in the PHV/PHBV ratio, which achieved stable values

Table 1
Summary of the main process performance parameters during steady state for the three carbon source/electron donors evaluated in this study.

Bioreactors		TSS (mg L^{-1})	TOC (mg L^{-1})	TN (mg L^{-1})	CoQ10 (mg $gTSS^{-1}$)	CoQ10 productivity (mg $m^{-3}\ d^{-1}$)
BCR-A	–	1086 ± 80	356 ± 54	3.2 ± 0.4	0.9	32
BCR-B	Low load	1638 ± 76	75 ± 9	4.2 ± 0.5	–	–
	High load	2707 ± 85	331 ± 61	4.9 ± 0.5	0.4	33
BCR-C	No pH control	525 ± 119	55 ± 29	4.3 ± 1.0	1	18
	pH control	1940 ± 106	62 ± 4	4.5 ± 0.5	0.5	30

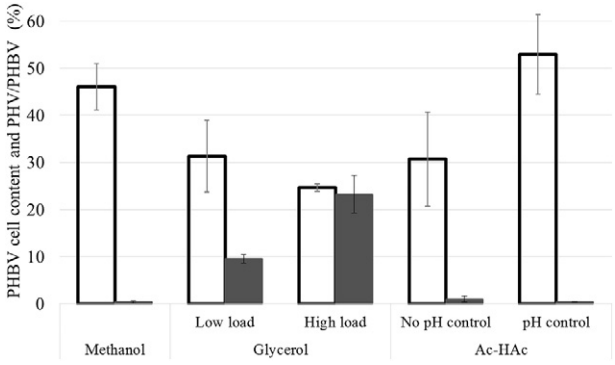

Fig. 3. Specific PHBV cell content (white column) and the homopolymer (PHV) molar ratio (gray column) of the cultures supplied with the three carbon sources evaluated.

of 23 ± 4% (Fig. 3). In this context, the physical-chemical properties of the PHBV copolymer enhance with the increase in the proportion of the homopolymer PHV (Bonartsev et al., 2007). Higher PHV/PHBV ratios confer greater copolymer characteristics such as a lower crystallinity and melting point, which expand the range of potential uses of PHBV (Eschenlauer et al., 1996). Typically, the production of PHBV with a high PHV monomer ratio has involved the use as precursors of costly co-substrates such as propionic acid, valeric acid, *n*-pentanol or other fatty acids (Shozui et al., 2010; Steinbüchel, 2001). Indeed, propionic acid is the most commonly used precursor in the commercial production of Biopol (PHBV) using glucose as the main carbon source. From a metabolic point of view, propionate is converted to propionyl-CoA, which generates a hydroxyvalerate monomer (HV) when condensed with acetyl-CoA (Dias et al., 2006). Finally, it must be highlighted that despite the fact that no precursors were used to promote PHV accumulation, the analysis of the culture broth by GC-FID revealed the presence of volatile fatty acids (in particular propionic acid), which may contribute to the synthesis of PHV. The production of these metabolites was likely induced by the anaerobic fermentation of the excess of glycerol not oxidized by the N_2O and O_2 transferred to the culture broth of BCR-B. Process operation with glycerol also resulted in a lower CoQ_{10} content of 0.4 mg g^{-1} but a higher productivity (33 g m^{-3} d^{-1}) compared to BCR-A as a result of the higher biomass productivity (Table 1). Bule and Singhal (2010) reported a specific cell content of 6.1 mg CoQ_{10} per gram of biomass in *Pseudomonas diminuta* using glycerol as a carbon source and an optimized operation by switching the aeration flow and agitation rates for the enhancement of coenzyme production.

3.3. Process performance with acetic acid

Process operation with Ac-HAc entailed a stable and high N_2O RE (~90%) and a rapid increase in the pH of the culture broth up to 9.3 by day 12 (Fig. 5A and S1). This increase in pH induced floc formation, which avoided a proper suspension of the culture broth and resulted in a sharp decrease in N_2O-RE from day 16 (Fig. 5A). Therefore, the liquid broth was continuously recycled from the bottom to the top of the BCR-C by a peristaltic pump at a flow rate of 200 mL min^{-1}. The pH under steady state remained at 9.4 ± 0.2, resulting in low biomass concentrations (525 ± 119 mg L^{-1}), N_2O REs (20 ± 7%) and CO_2 production rates (181 ± 28 g m^{-3} d^{-1}) (Fig. 5). This poor performance was clearly due to the limitation caused by the high pH prevailing in the culture both. In this sense, optimal pH values of 7.5–8 have been reported for *P. denitrificans* growth by Brenner et al. (2005). Similarly, Kučera et al. (1986) showed that the highest activity of the nitrous oxide reductase enzyme in *P. denitrificans* was obtained in a pH range of 8–9 using sodium succinate as the carbon source. This high pH increased the capacity of the aqueous mineral salt medium to accumulate HCO_3^- and CO_3^{2-}, thus contributing to underestimate the CO_2 stripped out from the bioreactor. This phenomenon was confirmed by the high inorganic carbon concentrations recorded in the culture broth (~520 mg C L^{-1}) compared to that recorded in BCR-A and B (~1.2 and ~0.5 mg C L^{-1}, respectively). On the other hand, the dissolved TOC concentration in the culture broth remained at 55 ± 29 mg C L^{-1} despite the pH-hindered biomass growth, which suggested that a significant fraction of the acetic acid was stripped out from the reactor. In this context, a fair estimation of the specific CO_2 yield was not possible in the absence of pH control.

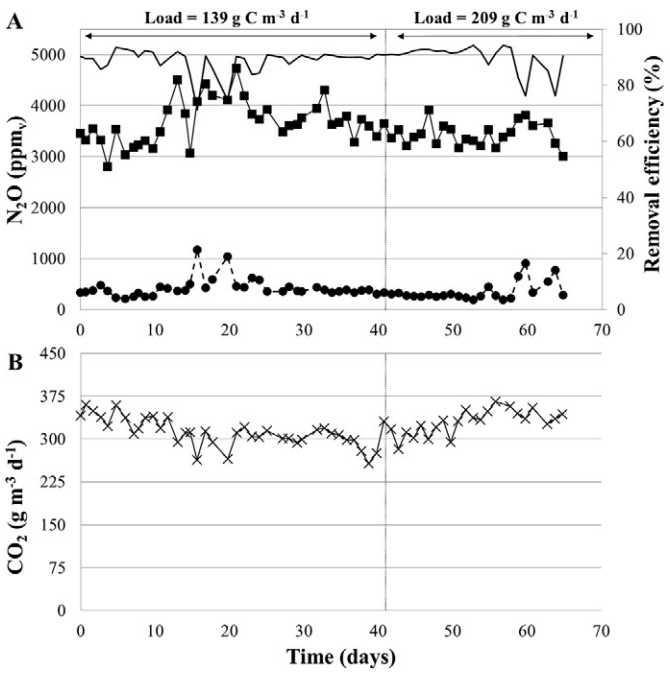

Fig. 4. Time course of the A) inlet (■) and outlet (●) N_2O concentrations and removal efficiency (solid line), and B) CO_2 production rates in the BCR supplied with glycerol. Two-way arrows indicate the carbon loading rates applied.

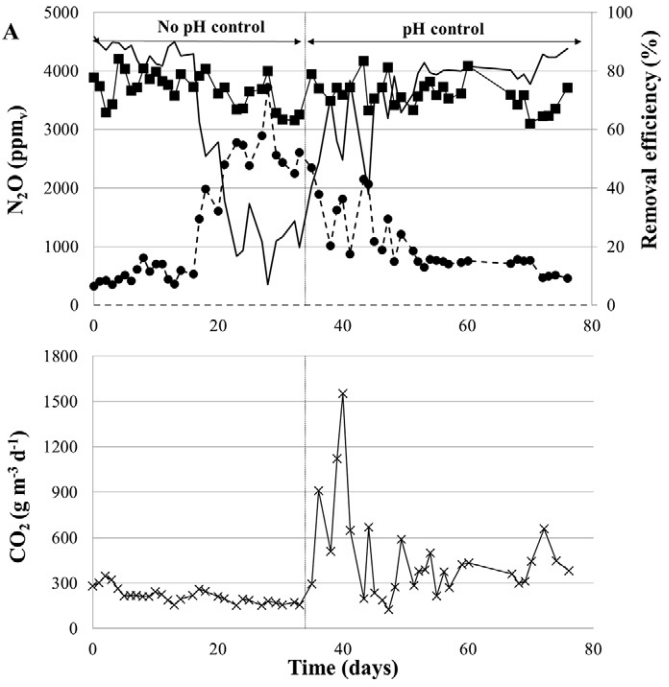

Fig. 5. Time course of the A) inlet (■) and outlet (●) N_2O concentrations and removal efficiency (solid line), and B) CO_2 production rates in the BCR supplied with Ac-HAc. Two-way arrows indicate the period with and without pH control.

The specific N_2O removal remained low at 0.17 ± 0.07 gN_2O $gTSS^{-1}$ d^{-1}, similar to that observed in BCR-B under high glycerol loading rates. Process operation with acetic acid and no pH control was characterized by a steady PHBV cell content of $31 \pm 10\%$ promoted by the low dissolved TN concentration (4.3 ± 1 mg L^{-1}) (Fig. 3). The PHBV productivity of the BCR-C was very low (5.3 gPHBV m^{-3} d^{-1}) due to the low biomass concentration. In addition, the PHV/PHBV ratio was ~1%, which was slightly higher than that achieved during process operation with CH_3OH, but much lower than that recorded using glycerol. Surprisingly, the highest specific CoQ_{10} cell content (1 mg g^{-1}) was recorded under this steady state without pH control. CoQ_{10} biosynthesis in bacteria starts with the formation of 4-hydroxybenzoate from chorismate via the shikimate pathway. Thereafter, 4-hydroxybenzoate undergoes a sequential ring modification by three hydroxylations alternating with three methylations, which results in the formation of ubiquinone (Meganathan, 2001). On the other hand, the synthesis of PHB starts with the condensation of two units of acetyl-CoA to produce acetoacetyl-CoA, which is further reduced to hydroxybutyrate monomer (HB) and then polymerized (Dias et al., 2006; Khosravi-Darani et al., 2013).

The pH of the BCR-C was daily reduced to ~7 from day 35 onward by the addition of HCl (Fig. S1), which promoted an increase in biomass concentration up to steady state values of 1940 ± 106 mg L^{-1} (Table 1). High and unstable CO_2 concentrations were observed following pH control (Fig. 5B). Thereafter, a stable CO_2 production of 361 ± 77 g m^{-3} d^{-1} was recorded from day 55. The increase in TSS concentration in BCR-C promoted an increase in N_2O-REs up to $81 \pm 3\%$ (Fig. 5A), which resulted in specific N_2O elimination capacities of 0.23 ± 0.03 gN_2O $gTSS^{-1}$ d^{-1}. These two parameters were lower than those recorded in BCR-A and BCR-B likely due to the poor availability of the dissolved electron donor for N_2O reduction as confirmed by the low dissolved TOC concentration (62 ± 4 mg L^{-1}) (Table 1). pH control resulted in a limited stripping of acetic acid as confirmed by GC-FID analyses. The estimated specific CO_2 production yield accounted for 0.74 ± 0.1 gC gC^{-1}, and pointed out that a large fraction of the carbon input was dedicated to the energy production for cell growth and maintenance.

The steady state PHBV cell content obtained during BCR-C operation under pH control averaged $53 \pm 8\%$ (Fig. 3). The biomass concentration increase mediated by pH control resulted in an enhanced biopolymer productivity of up to 33.5 gPHBV m^{-3} d^{-1}, which constitutes the highest recorded in this work. However, the homopolymer PHV represented only $0.4 \pm 0.1\%$ of the PHBV copolymer, a value comparable to that observed in the culture of BCR-A (Fig. 3). Lemos et al. (1998) assessed the production of PHBV in a phosphate-accumulating bacterial culture using acetate, propionate and butyrate. The highest PHBV cell content of ~17.5% (with a PHV/PHBV ratio of 24.75%) was obtained with acetate as the sole carbon source. Similarly, Jiang et al. (2011) reported a PHB accumulation >80% using acetate in a fed-batch culture dominated by *Plasticicumulans acidivorans*. Similarly to our results, acetate supported a low PHV synthesis, while propionate supplementation increased the PHV/PHBV ratio to 89%. Finally, the decrease in the specific cell content of CoQ_{10} to 0.5 mg g^{-1} when implementing pH control was counterbalanced by the increase in biomass which led to a CoQ_{10} productivity of 30 mg m^{-3} d^{-1} (Table 1).

The N_2O REs recorded in the three experimental runs (81 to 91%) are comparable to those supported by non-selective catalytic reduction methods, which is a common physical-chemical technology for the control of N_2O in nitric acid production plants (Environmental Protection Agency, 2010). However, the gas residence time in this technology is significantly lower than that in N_2O-abatement biotechnologies.

4. Conclusions

This study aimed at evaluating the feasibility of coupling the abatement of industrial emissions of N_2O to the co-production of commodities (PHBV) and high added value bio-products (CoQ_{10}) using different carbon sources/electron donors. High N_2O removal efficiency were achieved in the BCRs evaluated. However, further enhancement in N_2O removal and biomass productivity was limited by the mass transport of this poorly water soluble gas pollutant from the emission to the culture broth, regardless of the carbon source. Methanol and glycerol supported similar PHBV productivities under the same carbon loading rates, while the operation with acetate as a carbon source doubled its productivity. Glycerol supported the highest content of PHV in the composition of the PHBV copolymer likely due to the formation of propionic acid from glycerol fermentation. To the best of our knowledge, this study reported for the first time the co-production of the co-enzyme CoQ_{10} and the biopolymer PHBV by *P. denitrificans* during N_2O abatement, which paves the way to the development of GHG biorefineries as a cost-competitive tool for climate change mitigation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.04.161.

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Assessing the influence of the carbon source on the abatement of industrial N_2O emissions coupled with the synthesis of added-value bioproducts

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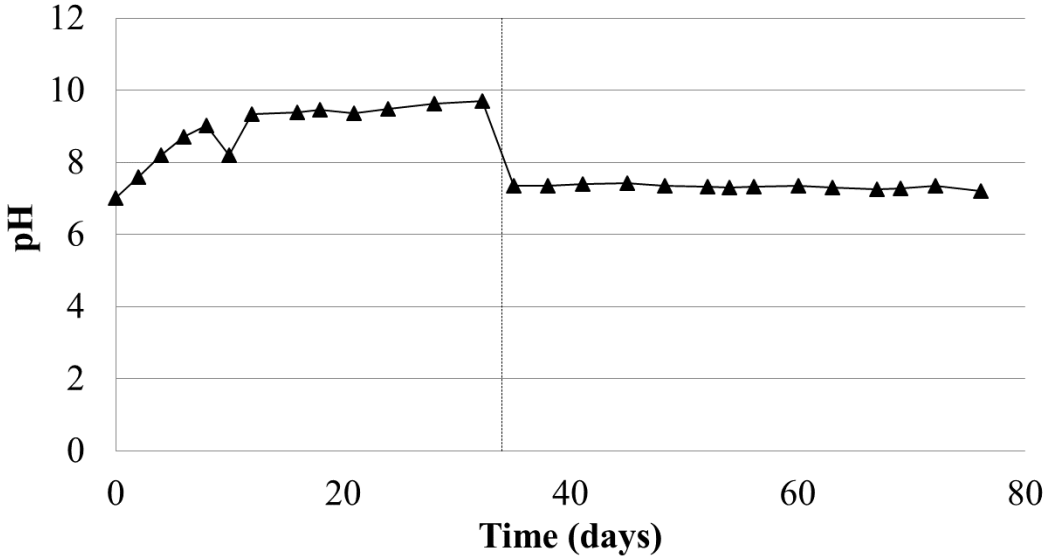

Figure S1. Time course of pH during BCR-C operation.

Enhancement of N_2O mass transfer in two-liquid phase systems

O.D. Frutos, O. Sanz Sevillano, and R. Muñoz. (Unpublished manuscript)

Chapter 7

Enhancement of N_2O mass transfer in two-liquid phase systems

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Abstract

Biological technologies have been successfully applied for the treatment of waste gases containing water-soluble pollutants. However, the treatment of gas pollutants with a low water-solubility often results in mass transfer limitations in biological systems. Recent studies have demonstrated that biological nitrous oxide (N_2O) removal systems typically show a poor process performance due to the low water-solubility of this GHG. In this sense, two-phase partitioning bioreactors (TPPBs) have emerged as an alternative to overcome pollutant mass transfer limitations. TPPBs are based on the utilization of an organic non-aqueous phase (NAP) with a high affinity for the gas pollutant that acts as a gas transfer vector in these bioreactors. Thus, silicon oil was here tested as a model NAP in order to evaluate the potential improvements in the mass transfer and subsequent biodegradation of N_2O. The experiment was conducted in batch assays using a *Paracoccus denitrificans* culture as inoculum with 10 and 30 % of silicon oil. In addition, a test without silicon oil was performed to serve as control. Unfortunately, no significant enhancement was observed in N_2O removal regardless of the fraction of silicon oil used.

Introduction

Biological off-gas treatment technologies have been successfully applied for the abatement of gas pollutants such as volatile organic compounds, H_2S, NH_3, etc. [1]. Scientific evidences have consistently shown the effectiveness and robustness of these technologies at lab, pilot and full scale. In addition, their environmental friendliness and low operation costs have increased the acceptance of biotechnologies versus conventional physical/chemical technologies [1,2]. However, the performance of biotechnologies is typically limited by the mass transfer of the pollutant from the gas to the liquid phase, which can be very low during the treatment of scarcely water-soluble pollutants (i.e those with high dimensionless Henry`s law constant) such as greenhouse gases.

This mass transfer limitation was previously observed in biotechnologies devoted to the treatment of nitrous oxide (N_2O) emissions (N_2O Henry`s law constant of 1.6 at 25 ºC [3]) [4–7]. Thus, innovative bioreactor configurations such as two-phase partitioning bioreactors (TPPB) should be tested as an alternative to overcome this limitation during N_2O emission control. TPPBs are based on the addition of an organic non-aqueous phase (liquid or solid) with greater affinity for the target gas pollutant than water, serving as a transfer vector of the pollutant to que aqueous phase [8]. Additional criteria for the selection of the optimum non-aqueous phase (NAP) are non-biodegradability, low cost and low toxicity and vapor pressure [9]. In this context, silicone oil constitutes the most popular NAP [10,11]. Unfortunately, the potential of TPPBs for the abatement of N_2O has never been evaluated.

The present study aimed to evaluate the potential enhancement of N_2O biodegradation via heterotrophic denitrification using silicone oil 200 cSt as the model NAP in gas-tight batch TPPBs.

Material and Methods

Chemicals and mineral salt medium

The mineral salt medium (MSM) used for the evaluation of N_2O biodegradation was prepared according to Frutos et al (2016) [12]. All chemicals required for MSM preparation and methanol (CH_3OH) were purchased from PANREAC (Barcelona, Spain) with a purity of +99 %. The N_2O gas was provided by Abelló Linde S.A. (Barcelona, Spain) in 40 L cylinders with a concentration of ~50000 ppm_v of N_2O in N_2. Silicone oil 200 cSt was obtained from Sigma–Aldrich (Madrid, Spain).

Experimental set up and procedure

Heterotrophic denitrification batch assays were conducted in 1.2 L sterile glass bottles aimed at evaluating the influence of silicon oil fraction on the removal rate of N_2O. The methylotrophic

strain *Paracoccus denitrificans* (DSM 413), purchased from DSMZ (Braunschweig, Germany), was used as inoculum, prior three weeks of aerobic acclimation in a sterile 500 mL e-flask supplied with MSM and methanol at a concentration of 1 g L^{-1}. Two silicon oil fractions of 10 and 30 % were evaluated in experiments with a final liquid volume of 200 mL. In addition, a control test was performed with 200 mL of sterile MSM in the absence of silicone oil. All bottles were inoculated with 10 mL of *P. denitrificans* culture, resulting in a final liquid volume of 210 mL and initial biomass concentrations of 210 mg L^{-1} of total suspended solid (TSS). The bottles were then sealed with rubber septa and plastic screw caps, and the air headspace was flushed out with the N_2O standard to ensure anoxic conditions, resulting in an initial N_2O concentration of 49760 ± 995 ppm_v. Finally, pure methanol was injected to the bottles to achieve an initial concentration of 1 g L^{-1}. All tests were performed in duplicate and incubated at 25 ºC under magnetic agitation (300 rpm) for 7 days. The gas concentrations of N_2O, CO_2 and O_2 were daily monitored by GC-ECD and GC-TCD.

Analytical procedures

The CO_2 and O_2 gas concentrations were measured using a gas chromatograph equipped with a thermal conductivity detector (GC-TCD) following the procedure described by Lopez et al. [13]. Similarly, the N_2O gas concentration was determined in a gas chromatograph equipped with an electron capture detector (GC-ECD) according to the procedure described by Frutos et al. [4]. TSS concentration was measured according to Standard Methods [14].

Results

The N_2O headspace concentrations showed similar consumption patterns regardless the presence of silicone oil, with a complete removal of N_2O after 7 days of experiment. These results confirmed the absence of a significant influence of the NAP on N_2O removal (Figure 1). Indeed, the specific N_2O consumption rate did not exhibit significant variations (0.279 ± 0.007, 0.290 ± 0.006 and 0.282 ± 0.011 mgN_2O $mgTSS^{-1}$ d^{-1} in the tests conducted with 10, 30 and 0 % of silicone oil, respectively).

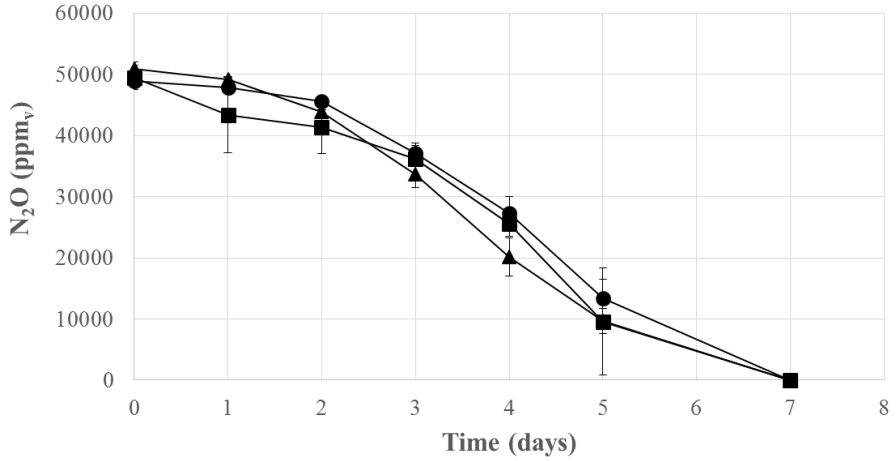

Figure 1. Time course of the N_2O headspace concentration in the control test (■), and tests supplied with 10 (●) and 30 % (▲) of silicon oil.

On the other hand, the test supplied with 30 % of silicon oil showed a slightly higher CO_2 generation (as a result of methanol oxidation with N_2O) than that recorded in the rests of the experimental conditions (Figure 2). In spite of the significantly higher CO_2 production rate observed, this could not be attributed to a higher N_2O consumption rate but to an enhanced mass transfer of the O_2 traces initially present in the headspace of the bottles.

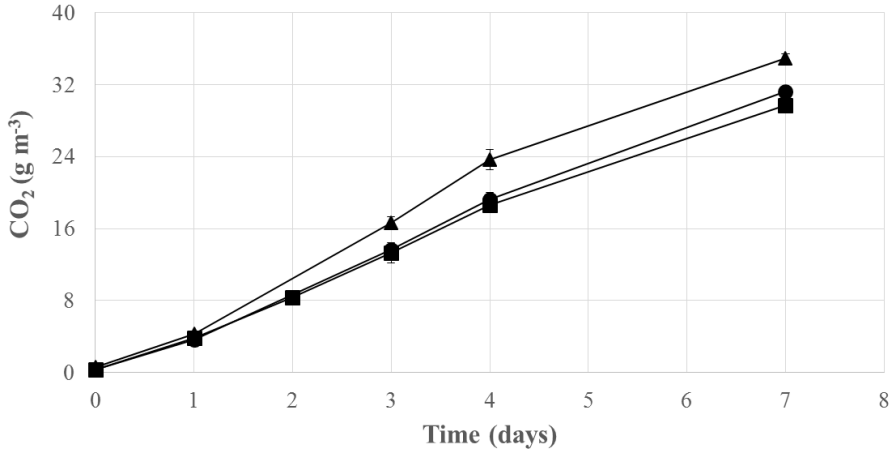

Figure 2. Time course of CO_2 headspace concentration in the control test (■), and tests supplied with 10 (●) and 30 % (▲) of silicon oil.

Conclusions

This study evaluated the influence of silicone oil on the removal rate of N_2O by a heterotrophic denitrifying culture in anoxic batch assays at silicon oil/aqueous ratios of 0, 10 and 30 %. The results showed similar N_2O removal rates at all NAP ratios tested, which ruled out any potential mass transfer enhancement of silicon oil. Further work should focus on

testing different NAPs with higher affinity for N_2O than silicon oil that could eventually increase the mass transfer of N_2O from the gas phase and its further biological removal.

Acknowledgments

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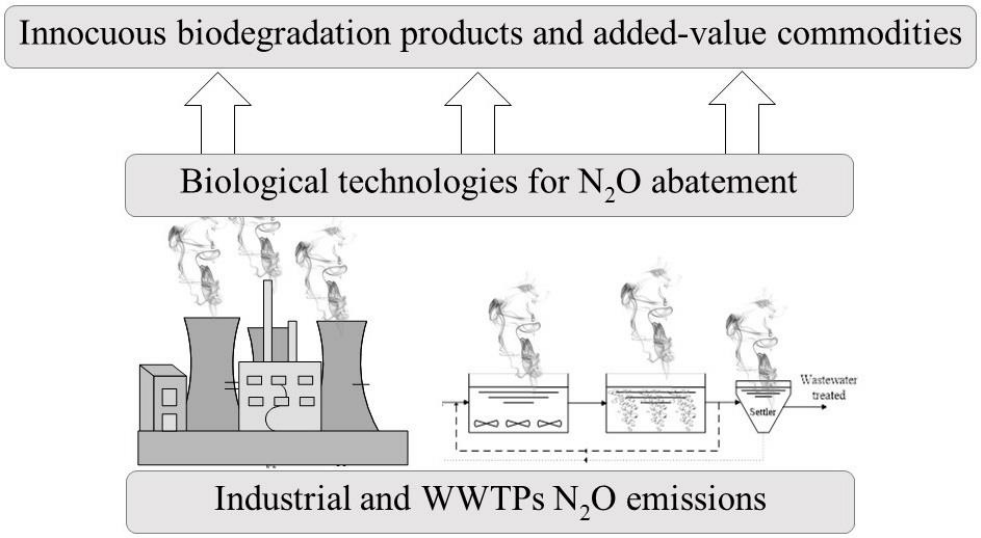
Conclusions and future work



Conclusions and future work

A battery of novel bioreactor configurations for the treatment of N_2O emissions from WWTPs and HNO_3 production plants, along with the mechanisms underlying the biodegradation of this GHG and the simultaneous synthesis of added-value byproducts was investigated in this thesis.

The results herein obtained showed, for the first time, the feasibility of an aerobic-anoxic bioscrubber for the abatement of N_2O-air emissions from WWTPs using methanol as the carbon and energy source. Heterotrophic denitrification was identified as the main biodegradation mechanism in the bioscrubber (**Chapter 1**). Furthermore, the simultaneous removal of N_2O and domestic wastewater treatment was also confirmed with consistent removals of organic matter and N_2O. The increase in the EBRT up to 40 min resulted is satisfactory N_2O removal efficiencies, while increasing liquid recycling velocities also enhanced the removal of this GHG (**Chapter 2**). However, the bioscrubber operation was always mass transfer limited due the poor water-solubility of N_2O. Likewise, the excessive biomass accumulation in the packed absorption column also jeopardized the long-term abatement of N_2O in WWTP emissions. The data here obtained revealed that the large bioscrubber volumes required for a satisfactory N_2O removal will restrict the scale-up of this technology. In addition, the high oxygen concentrations in WWTPs emissions compared to those of N_2O entail a massive supply of organic matter to ensure an effective reduction of N_2O in the anoxic tank.

The performance of an ASD system, which is known for its high mass transfer potential and absence of packed bed clogging, was investigated for the continuous removal of N_2O under aerobic and microaerobic conditions (**Chapter 4**). Unfortunately, the removal of N_2O was negligible in the range of dissolved oxygen concentrations evaluated.

The biological degradation mechanisms of N_2O were studied in **Chapters 1** and **3**. The heterotrophic denitrification of N_2O using methanol as a carbon-energy source was confirmed in anaerobic batch assays, whereas no nitrification of N_2O to nitrite and nitrate

was recorded in a continuous biotrickling filter or in aerobic batch assays operated for extended periods of time.

The abatement of industrial N_2O emissions coupled to the synthesis of added-value byproducts was demonstrated in a BCR and ALR bioreactors under multiple carbon-energy sources and operational strategies. Hence, high N_2O removals were achieved in both bioreactors with a high cell content of the copolymer PHBV promoted by nitrogen limitation using methanol (**Chapter 5**). The use of glycerol as the carbon-energy source resulted in the improvement of the biopolymer characteristics due to an increase of the homopolymer PHV content in the PHBV copolymer, whereas the bioreactor operated with acetate-acetic acid as the carbon-energy source supported the highest biopolymer productivity (**Chapter 6**). To the best of our knowledge, the study conducted in **Chapter 5** and **6** reported for the first time the feasibility of co-producing the coenzyme Q10 and the biopolymer PHBV during N_2O abatement. However, the low biomass concentrations prevailing in all experiments resulted in low PHBV productivities when compared with industrial biopolymer production from conventional substrates (i.e. glucose). In this context, the gas-liquid mass transfer limitations observed during the operation of these systems will likely limit the scale-up of this technology.

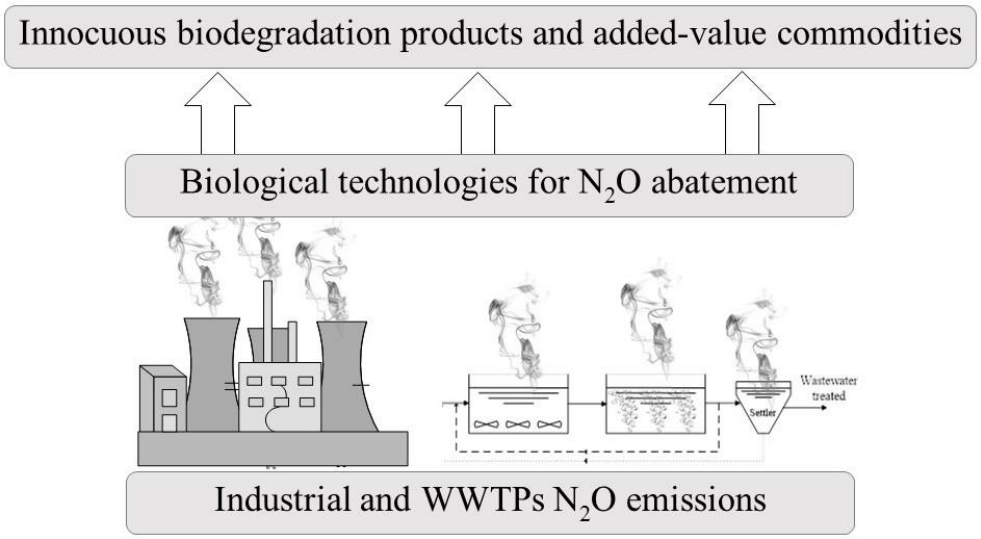
Finally, **Chapter 7** assessed the potential improvement of N_2O mass transfer by addition of silicon oil in batch experiments under heterotrophic denitrification. However, the results ruled out any potential mass transfer enhancement mediated by the presence of this vector in the bioprocess.

In spite of the significant advances carried out in this thesis towards the development of novel biotechnologies for N_2O emissions control, further studies are needed in order to overcome the mass transfer limitations typically encountered in bioreactors. Moreover, a further process optimization is also required to enhance biopolymer productivity, whereas the identification of low cost carbon-energy sources remains a key challenge for the cost-effective abatement of N_2O. Finally, the identification of strains capable of

utilizing N_2O under aerobic or microaerobic conditions, and to oxidize this GHG to nitrate or nitrate, will support the development of novel and more effective bioreactor configurations. In brief, further research on biological N_2O abatement should focus on:

- The evaluation of novel membrane bioreactors with a high specificity for N_2O, which may significantly enhance the selective mass transfer of N_2O while preventing O_2 diffusion (and therefore the need to deplete it).
- Testing different NAPs with higher affinity for N_2O than silicone oil, which could eventually overcome the mass transfer limitations of bioscrubbers, BCR and ALRs.
- The identification of low cost and readily available carbon and energy sources such as urban and agroindustrial wastewaters that could cost-effectively support the biological abatement of N_2O coupled to biopolymers synthesis. Moreover, the elucidation of the influence of O_2 concentrations, microbial strain and type of nutrient limitation on PHBV accumulation will provide further insights towards the development of GHG biorefineries.
- The enrichment of N_2O nitrifiers or microaerobic denitrifiers could be addressed by evaluating alternative inoculum sources with a high microbial diversity.
- The pretreatment of N_2O-air emissions with UV radiation could result in a viable alternative for the aerobic abatement of N_2O since nitrous oxide breakdown results in the formation of NO and N, which could eventually be treated aerobically in conventional nitrifying bioreactors such as biotrickling filters.

About the author



Biography

Osvaldo David Frutos González (Asunción, Paraguay 1988) started his studies in Environmental Engineering in 2006 at the National University of Asuncion (Paraguay). After finishing his studies in December 2010, he was hired as a technical assistant at the Paraguayan National Secretary of Housing, where he worked on the evaluation of contructions impacts. By the end of 2011, Osvaldo started his Master degree in Environmental Science and Technology at the University of A Coruña (Spain) through a scholarship awarded by the Spanish Agency for International Development Cooperation. During his Master studies at A Coruña, he collaborated at the Environmental Engineering Research Group headed by Dr. Carmen Veiga and Dr. Christian Kennes. Back to Paraguay in Agoust 2012, he joined the National University of Asuncion as research assistant up to November 2013, when Osvaldo was awarded with an Erasmus Mundus PhD scholarship to conduct his doctoral studies at the University of Valladolid (Spain). Thus, Osvaldo joined the VOC & Microalgae Research Group headed by Associate Professor Raúl Muñoz in the Environmental Technology Research Group from the Department of Chemical Engineering and Environmental Technology.

The PhD thesis of Osvaldo was focused on the development of novel biological technologies for the treatment of N_2O emissions, with special attention to the synthesis of commodities and high added-value products out of N_2O mitigation.

By the end of 2016, Osvaldo returned to Paraguay and joined again the National University of Asuncion as research assistant, where he is currently involved in teaching at the Environmental Engineering degree and collaborates in research projects funded by the National Council of Science and Technology from Paraguay.

Publications in international journals

1. O.D. Frutos, I.A. Arvelo, R. Pérez, G. Quijano, R. Muñoz, *Continuous nitrous oxide abatement in a novel denitrifying off-gas bioscrubber*, Appl. Microbiol. Biotechnol. 99 (**2015**) 3695–3706. doi:10.1007/s00253-014-6329-8.

2. O.D. Frutos, G. Quijano, R. Pérez, R. Muñoz, *Simultaneous biological nitrous oxide abatement and wastewater treatment in a denitrifying off-gas bioscrubber*, Chem. Eng. J. 288 (**2016**) 28–37. doi:10.1016/j.cej.2015.11.088.

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Book chapters

1. Jin Y, Guo L, Frutos OD, Veiga MC, Kennes C. Bioprocesses for the Removal of Nitrogen Oxides. In *Air Pollution Prevention and Control: Bioreactors and Bioenergy*. Kennes C. and Veig MC., Eds.; Wiley and Sons, **2013**; pp. 275-292; DOI: 10.1002/jctb.1260.

2. Cantera, S., Frutos, O.D., López, J.C., Lebrero, R., Muñoz, R. Technologies for the bio-conversion of GHGs into high added value products: Current state and future prospects. In *Carbon Footprint and the Industrial Life Cycle, From Urban Planning*

to Recycling; Álvarez-Fernández, R.; Zubelzu, S.; Martínez R., Eds.; Springer International Publishing, **2017**; pp. 359–388; DOI 10.1007/978-3-319-54984-2.

Contributions to conferences

1. Frutos, O.D., Pérez, R., Quijano, G., Muñoz, R. *Nitrous oxide abatement in a continuos denitrifying off-gas bioscrubber*. 6th international conference on Biotechniques for air pollution control, September **2015**, Gent, Belgium (Oral Presentation).

2. Frutos, O.D., Lebrero, R., Quijano, G., Marin D., Muñoz, R. *Nitrous oxide: global trends, mitigation and abatement.* 1st International Conference on Bioenergy & Climate Change: Towards a Sustainable Development, June **2016**, Soria, Spain (Oral Presentation).

3. Frutos, O.D., Cortez, I., Arnaiz, E., Lebrero, R., Muñoz, R. *Biological nitrous oxide abatement by paracoccus denitrificans in bubble column and airlift reactors*. 5st International Conference on Environmental Odour Monitoring and Control, September **2016**, Ischia, Italy (Oral Presentation).

Committees and Reviewer Experience

i. ***Member of the organizing committee*** of the NOVEDAR technical seminar: Characterization and Management of Odours and Greenhouse Gases in WWTPs, October **2015** (Valladolid, Spain).

ii. ***Reviewer*** for the 4th National Agrarian Congress. April **2017** (San Lorenzo, Paraguay).

Co-supervision

1. ***Master Thesis:*** Ilan Arvelo (Febraury 2014 – July 2014) '*Optimización de procesos biológicos para la eliminación de N_2O*', University of Valladolid (Spain).

2. ***Research project:*** Oscar Sanz (September 2015 – Febraury 2016) '*Condiciones óptimas para el crecimiento de paracoccus denitrificans en ecosistemas anaerobios*', University of Valladolid (Spain).

3. ***Master Thesis:*** Gonzalo Barriguín (Febraury 2016 – September 2017) '*Eliminación biológica de óxido nitroso y producción simultanea de biopolímeros*', University of Valladolid (Spain).

Teaching

1. **Fluids Treatment Technologies** (2017). Lecturer. Environmental Engineering Degree. National University of Asunción (Paraguay). 45 h.

2. **Bioremediation** (2017). Lecturer. Environmental Engineering Degree. National University of Asunción (Paraguay). 45 h.

Participation in Research & Development Projects

1. *Advanced biological processes for the abatement of the greenhouse gases CH_4 and N_2O: targeting the direct gas-cell mass transport and process microbiology*. Ministry of Economy, Industry and Competitiveness (2013-2016). Ref: CTQ2012-34949. P.I. Dr. Raúl Muñoz.

2. *Wastewater treatment by artifitial wetlands*. Paraguayan Council of Science and Technology (2017-2019), Ref: PINV15-458. P.I. Ing. Carlos Enciso.