UNIVERSIDAD DE GRANADA

Programa de Doctorado de Biología Fundamental y de Sistemas

Eliminación de Nitratos de las Aguas Subterráneas utilizando la tecnología Aeróbica Granular Secuencial

Departamento de microbiología

Instituto Universitario de Investigación del Agua

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Nitrate removal from groundwater using a sequential aerobic granular sludge technology

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TITULO: Eliminación de Nitratos de las Aguas Subterráneas utilizando la tecnología Aeróbica Granular Secuencial

Presentado por: Miguel Hurtado Martínez

Supervisado por: Prof. Dr. Jesús González López y Prof. Dr. Alejandro González Martínez

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Eliminación de Nitratos de las Aguas Subterráneas utilizando la tecnología Aeróbica Granular Secuencial

Memoria presentada por el Ldo.

Miguel Hurtado Martínez

para optar al título de Doctor con mención internacional

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Garantizamos, al firmar esta tesis doctoral que el trabajo ha sido realizado por el doctorando bajo la dirección de sus directores, y en la realización del trabajo se han respetado los derechos de los autores citados cuando han sido utilizados sus resultados en las publicaciones.

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Capítulos de Libros

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Congresos en los que se han presentado los resultados de esta tesis doctoral:

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Muñoz-Palazon, B., **Hurtado-Martinez, M**., Rodriguez-Sanchez, A., Gonzalez-Lopez, J., Gonzalez- Martinez, A., Operation of Aerobic Granular Systems

Based on Pure Cultures of Bacteria for the treatment of Nitrate Polluted Groundwater. (FEMS 2019) Gasgow (Scotland).

Hurtado-Martinez, M., (2019) Life Ecogranularwater technical seminar. Granada, Spain.

TABLE OF CONTENTS

FIGURE INDEX

Introduction and Materials and Methods

Chapter 1: Groundwater nitrate removal performance of selected *Pseudomonas* strains carrying *nosZ* gene in aerobic granular sequential batch reactors.

Supplementary material Chapter 2

Chapter 3. Application of full-scale aerobic granular sludge technology for removing nitrate from groundwater intended for human consumption.

Supplementary material Chapter 3

TABLE INDEX

Supplementary material Chapter 3

Summary

The dependence on groundwater for human consumption has increased worldwide over the last 50 years. Among the nutrient pollutants of concern, nitrate (NO_3^-) often reaches groundwater and causes significant degradation in groundwater quality. This dependence on groundwater has become even more important in the Mediterranean region due to increased desertification and global warming. In countries such as in Spain, 70% of the water resource demands of cities with less than 20000 inhabitants are supplied by groundwater.

The mail goal of our experimental work was development a new biological technology based on aerobic granular systems. In this sense, a full-scale water treatment plant using aerobic granular sludge (AGS) was built to remove NO_3^- from nitrate-polluted groundwater intended for human consumption, in order to achieve a complete nitrate removal with low economic cost and minimizing the environmental impact.

First of all (**Chapter 1**), four granular sequencing batch reactors (GSBRs) at lab-scale were inoculated with four denitrifying *Pseudomonas* strains carrying *nosZ* to study the process of granule formation, the operational conditions of the bioreactors, and the carbon concentration needed for nitrate removal. The selected *Pseudomonas* strains were *P. stutzeri* I1, *P. fluorescens* 376, *P. denitrificans* Z1, and *P. fluorescens* PSC26, previously reported as denitrifying microorganisms carrying the *nosZ* gene. *Pseudomonas denitrificans* Z1 produced fluffy, low-density granules, with a decantation speed below 10 m h^{-1}. However, *P. fluorescens* PSC26, *P. stutzeri* I1, and *P. fluorescens* 376 formed stable granules, with mean size from 7 to 15 mm, related to the strain and carbon concentration. *P. stutzeri* I1 and *P. fluorescens* 376 removed nitrates efficiently with a ratio in the range of 96%, depending on the source and concentration of organic matter. Therefore, the findings suggest that the inoculation of GSBR systems with denitrifying strains of *Pseudomonas* spp. containing the *nosZ* gene enables the formation of stable granules, the efficient removal of nitrate, and the transformation of

nitrate into nitrogen gas, a result of considerable environmental interest to avoid the generation of nitrous oxide.

In a second group of experiments (**Chapter 2**) a bioreactor was designed as a cylindrical sequential batch reactor (SBR), with a height of 3.52 m and a diameter of 0.49 m. The reactor was inoculated with 6 L of mature granules previously formed at lab-scale in the Water Research Institute (University of Granada) with a total volume of 660 L. In this research, a novel modification of aerobic granular sludge technology was developed for the treatment of nitrate-polluted groundwater, adding very low concentrations of a solution based on carbon and oligoelements in the groundwater to promote the growth of denitrifying microorganisms, avoiding expensive technologies to supply drinking water in small urban nuclei. The denitrification process was successfully reached at 0.15 g $C_2H_3NaO_2$ L^{-1}, meeting the Nitrate Directive of Europe for drinking water. The granular biomass was compact and dense with average values of mean size and settling velocity of 4.0 mm and 40 mh^{-1}, respectively. The prokaryotic and eukaryotic communities were studied by massive parallel sequencing techniques. The dominant prokaryotic phylotypes were related to influent composition, belonging to *Comamonadaceae*, *Rhizobiales*, *Acinetobacter* and *Pseudomonas*. The dominant eukaryotic phylotype was affiliated to *Haematococcus microalgae*. The diversity and evenness were high, regardless of influent composition. This study demonstrates support for the innovation of aerobic granular sludge technology application in terms of performance, operation, granular maturation and stability, as well as the role of denitrifying microorganisms to implement a low-cost, easy-to-use and maintain, environmental-friendly drinking water technology for rural populations.

Finally, in a third group of experiments (**Chapter 3**) a full-scale water treatment plant using aerobic granular sludge (AGS) technology was built, under real conditions, to remove NO_3^- from nitrate-polluted groundwater intended for human consumption in village Torre Cardela (Granada) in the South of Spain. The impact of changes in the operational conditions of hydraulic retention time (HRT) and organic matter loading (OML) rate on NO_3^- removal and overall system performance were examined. Variations in the abundance of the denitrification genes and the diversity and composition of the prokaryotic and eukaryotic communities in the granule microbiome

were studied. Regardless of the HRT, the AGS technology was successful in removing NO_3^- with removal rates greater than 50% with an optimal OML rate of 75 mg L^{-1}. Regardless of the HRT and OML rate, the organic matter removal rate was greater than 90%. No significant changes in the abundance of denitrification genes were detected during the experimental period. However, the composition of prokaryotic and eukaryotic communities was affected by changes in the HRT and OML rate. Specific prokaryotic taxa were identified as responsive to changes in operational parameters and their relative abundances were strongly linked to the removal of NO_3^-, confirming that the microbes are critical to the NO_3^- removal process.

The results obtained in this experimental work clearly demonstrate that the AGS technology can be successfully implemented to treat nitrate-polluted groundwater in rural villages to produce water of drinking quality.

Resumen

La dependencia de las aguas subterráneas para el consumo humano ha aumentado en todo el mundo en los últimos 50 años. Entre los nutrientes contaminantes preocupantes, el nitrato (NO_3^-) llega a menudo a las aguas subterráneas y provoca una degradación significativa de su calidad. Esta dependencia de las aguas subterráneas se ha hecho aún más importante en la región mediterránea debido al aumento de la desertificación y al calentamiento global. En países como España, el 70% de la demanda de recursos hídricos de las ciudades de menos de 20000 habitantes se abastece de aguas subterráneas.

El objetivo principal de nuestro trabajo experimental fue el desarrollo de una nueva tecnología biológica basada en sistemas granulares aerobios. En este sentido, se construyó una planta de tratamiento de agua a escala real utilizando lodos granulares aerobios (AGS) para eliminar NO_3^- de aguas subterráneas contaminadas con nitratos y destinadas al consumo humano, con el fin de conseguir una eliminación completa de nitratos con un bajo coste económico y minimizando el impacto ambiental.

En primer lugar (**Capítulo 1**), se inocularon cuatro reactores discontinuos secuenciales granulares (GSBR) a escala de laboratorio con cuatro cepas de *Pseudomonas* desnitrificantes portadoras de *nos*Z para estudiar el proceso de formación de gránulos, las condiciones operativas de los biorreactores y la concentración de carbono necesaria para la eliminación de nitratos. Las cepas de *Pseudomonas* seleccionadas fueron *P. stutzeri* I1, *P. fluorescens* 376, *P. denitrificans* Z1, y *P. fluorescens* PSC26, previamente reportadas como microorganismos desnitrificantes portadores del gen *nos*Z. *Pseudomonas denitrificans* Z1 produjo gránulos esponjosos y de baja densidad, con una velocidad de decantación inferior a 10 m h^{-1}. Sin embargo, *P. fluorescens* PSC26, *P. stutzeri* I1 y *P. fluorescens* 376 formaron gránulos estables, con un tamaño medio de 7 a 15 mm, relacionado con la cepa y la concentración de carbono. *P. stutzeri* I1 y *P. fluorescens* 376 eliminaron nitrato eficientemente con una proporción

en el rango del 96%, dependiendo de la fuente y concentración de materia orgánica. Por tanto, los resultados sugieren que la inoculación de sistemas GSBR con cepas desnitrificantes de *Pseudomonas* spp. que contienen el gen *nos*Z permite la formación de gránulos estables, la eliminación eficiente de nitrato y la transformación de nitrato en nitrógeno gas, un resultado de considerable interés medioambiental para evitar la generación de óxido nitroso.

En un segundo grupo de experimentos (**Capítulo 2**) se diseñó un biorreactor en forma de reactor secuencial (SBR) cilíndrico, con una altura de 3,52 m y un diámetro de 0,49 m. El reactor se inoculó con 6 L de gránulos maduros previamente formados a escala de laboratorio en el Instituto de Investigación del Agua (Universidad de Granada) con un volumen total de 660 L. En esta investigación se desarrolló una novedosa modificación de la tecnología de lodos granulares aerobios para el tratamiento de aguas subterráneas contaminadas con nitratos, añadiendo concentraciones muy bajas de una solución a base de carbono y oligoelementos en las aguas subterráneas para promover el crecimiento de microorganismos desnitrificantes, evitando costosas tecnologías de abastecimiento de agua potable en pequeños núcleos urbanos. El proceso de desnitrificación se alcanzó con éxito a 0,15 g $C_2H_3NaO_2$ L^{-1}, cumpliendo la Directiva Europea de Nitratos para agua potable. La biomasa granular era compacta y densa, con valores medios de tamaño medio y velocidad de sedimentación de 4,0 mm y 40 mh^{-1}, respectivamente. Las comunidades procariota y eucariota se estudiaron mediante técnicas de secuenciación masiva en paralelo. Los filotipos procarióticos dominantes estaban relacionados con la composición del influente y pertenecían a *Comamonadaceae, Rhizobiales, Acinetobacter y Pseudomonas*. El filotipo eucariota dominante estaba relacionado con las microalgas *Haematococcus.* La diversidad y la uniformidad fueron elevadas, independientemente de la composición del influente. Este estudio demuestra el apoyo a la innovación de la aplicación de la tecnología de lodos granulares aerobios en términos de rendimiento, funcionamiento, maduración granular y estabilidad, así como el papel de los microorganismos desnitrificantes para implementar una tecnología de agua potable de bajo coste, fácil de usar y mantener, y respetuosa con el medio ambiente para las poblaciones rurales.

Por último, en un tercer grupo de experimentos (**Capítulo 3**) se construyó, en condiciones reales, una planta de tratamiento de aguas a escala real utilizando la tecnología de lodos granulares aerobios (AGS) para eliminar NO_3^- de aguas subterráneas contaminadas con nitratos y destinadas al consumo humano en el municipio de Torre Cardela (Granada), en el sur de España. Se examinó el impacto de los cambios en las condiciones operativas del tiempo de retención hidráulica (TRH) y la tasa de carga de materia orgánica (OML) sobre la eliminación de NO_3^- y el rendimiento global del sistema. Se estudiaron las variaciones en la abundancia de los genes de desnitrificación y la diversidad y composición de las comunidades procariotas y eucariotas en el microbioma de los gránulos. Independientemente del TRH, la tecnología AGS tuvo éxito en la eliminación de NO_3^- con tasas de eliminación superiores al 50% con una tasa óptima de OML de 75 mg L^{-1}. Independientemente del TRH y de la tasa de OML, la tasa de eliminación de materia orgánica fue superior al 90%. No se detectaron cambios significativos en la abundancia de genes de desnitrificación durante el periodo experimental. Sin embargo, la composición de las comunidades procariotas y eucariotas se vio afectada por los cambios en el TRH y la tasa de OML. Se identificaron taxones procarióticos específicos que respondían a los cambios en los parámetros operativos y sus abundancias relativas estaban fuertemente relacionadas con la eliminación de NO_3^-, lo que confirma que los microbios son fundamentales para el proceso de eliminación de NO_3^-.

Los resultados obtenidos en este trabajo experimental demuestran claramente que la tecnología AGS puede aplicarse con éxito para tratar aguas subterráneas contaminadas por nitratos en aldeas rurales para producir agua de calidad potable.

1.GENERAL INTRODUCTION

1.General Introduction

Water is an essential element needed for the nature life and the human development. For this reason, water pollution is one of the main problems facing humanity. Water is a fundamental resource for the development of vital activities; and due to the exponential growth of the population, more and more of this resource is needed, and at the same time an increasing volume of polluted effluents is generated (Jia et al., 2020).

The increase in population in municipalities and cities means that an ever greater volume of resources is needed to supply them, including water. The lack or depletion of fresh surface water sources means that groundwater is used more frequently.

When drilling into the ground, two main types of wet zones can be found; In first place an upper zone, where the space not occupied by rock, is filled by water (accumulated in the pores) and gases. This zone is called unsaturated zone. In second place, there is a zone called saturated zone, in which all the pores of the ground are occupied by water. When this saturated zone coincides with rocks that allow high water transmission, capable of supplying large quantities of water, it is called an aquifer. There are two types of aquifers depending on their capacity to renew their water. Non-renewable aquifers are found in regions that currently have a drier climate than in the past. (For example, under the Sahara desert), this water can be considered as a non-renewable resource, since it comes from past times and currently there is no recharge. However, most aquifers are usually considered as a renewable resource, since the water that is extracted is replenished by the recharge areas of these aquifers and if they are exploited correctly, a balance can be maintained in the system (Bachmat, 2004).

Groundwater provides one third of the world's freshwater supply for domestic, agricultural and industrial activities. Therefore, groundwater is of vital importance for the development of the population. (Fillinger et al., 2021)

The occupation of the surface by population centers modifies the natural processes of groundwater recharge, since the infiltration zones are occupied, and the natural supply patterns are modified. At the same time, contaminated water flows are generated which, if not properly managed, end up infiltrating into the system, deteriorating it or even making it unfit for use (Haase et al., 2009; Kumar et al., 2018 ;Knoll et al., 2019).

The contaminants that enter the aquifer include nitrogenous compounds. These are generated in a multitude of anthropic processes such as the discharge of untreated wastewater and, to a large extent, due to the excessive use of fertilizers in agriculture. Most of the aquifer recharge areas are located in areas with significant agricultural activity, which means that excess fertilizer compounds, including nitrogen derivatives and pesticides, end up inside the aquifer, washed away by rainfall, as well as by the infiltration of polluted water (Vega et al., 2005).

In the same way that nitrogenous compounds others organic substances such as pesticides are very dangerous to health and the environment. Their uncontrolled use means that they end up infiltrating into the aquifer where they accumulate. However strict regulations to prevent the contamination of water with these substances, have been established in the European Union, such as Decision No. 2455/2001 of the European Parliament and of the Council (2001OJEC), which amends Directive 2000/60/EC (European Union, 1991).

Groundwater is threatened and needs protection to prevent its deterioration and depletion. In this sense, Directive 91/676/EEC establishes that values above 50 mg/L of nitrates are sufficient to classify water as heavily polluted, making it unfit for consumption. It recommends not to consume water whose nitrate value exceeds 25 mg/L. (Directive 91/676/EEC of 12 December 1991.)

The consumption of nitrate-contaminated water can cause significant health problems, such as certain cancers of the stomach and other parts of the digestive system in adults. It also generates the so-called "blue baby syndrome" in children, caused by a disorder in the red blood cells that causes a disease called methemoglobinemia, which is derived from an excessive consumption of nitrates. (Adimalla et al., 2021; Datta et al., 1997).

An increasing number of municipalities and cities around the world are using groundwater to supply their populations, due to the growing freshwater supply problems faced by mankind. However, the use of groundwater is especially relevant in regions with a high-water deficit. This is the case of countries located in arid or semi-arid climates and with seasonal rainfall that make certain months of the year the water deficit is very pronounced. Spain is one of these territories, since most of the country has a water deficit regime, with seasonal rainfall.

In Spain, twenty percent of the cities with more than twenty thousand inhabitants are supplied by groundwater for consumption, but this percentage rises to seventy percent when we look at the municipalities and cities with less than twenty thousand inhabitants (De Stefano et al., 2015).

Small villages in the Mediterranean area are facing major problems for their drinking water supply. The first problem they face is the lack of water resources at certain times of the year. In general terms the Mediterranean climate favors the occurrence of periods of drought that reduce their water resources; this, together with the fact that most of these municipalities are quite scattered throughout the territory, far from large freshwater reservoirs from which to draw their water supply, means that they are strongly dependent on groundwater, which becomes their only resource for supplying the population and for irrigation. In addition, the recharge areas of the aquifers from which they are supplied are usually occupied by crops, which, if not properly fertilized, cause nitrogen compounds and pesticides to leach into the aquifer. Consequently all these factors make these municipalities very vulnerable to nitrate contamination, with high levels of nitrates in their drinking water.

There are various technologies for the treatment of drinking water polluted with nitrates. The most commonly used are physicochemical technologies, such as reverse osmosis, ion exchange, electrodialysis or distillation systems, but there are also different biological alternatives for their removal, such as anoxic denitrification biofilter systems.

1.1Nitrate Removal System for Drinking Water.

Different systems are available for nitrate removal from drinking water. These systems include physicochemical and biological systems depending on the process used to remove nitrates from the water.

Physicochemical systems make use of physical techniques combined with different reagents to achieve nitrogen removal compounds. Biological systems, use the capacity of microorganisms to nitrogen removal compounds through their metabolic reactions.

1.1.1 Physicochemical systems for nitrate removal from drinking water

These systems use physicochemical processes for the elimination of contaminating compounds.

Reverse osmosis systems

Reverse osmosis systems (RO) can be used for the removal of different types of organic and inorganic pollutants such as nitrates, heavy metals and pesticides.

In RO technique, water is forced to pass through a semi-permeable membrane that allows water molecules to pass through while contaminants are prevented from passing through the membrane. This process requires pressure on the water to achieve this, as the water is forced to pass against the natural gradient. The pressure applied depends on several factors such as the ion charge of the water or the type of membrane being used. This process generates a concentrated solution of contaminants called brine or reject stream, which must be treated before being discharged into the environment.

Nitrate can be removed by RO systems applying pressures ranging from 2000 to 10000kPa depending on the type of membrane used (Malaeb & Ayoub, 2011). The membranes are composed of cellulose acetate, polyamides or composite materials. Energy consumption represents a very high percentage in the production of water by this method and can reach 50% of the total cost in a standard desalination plant (Molina & Casañas, 2010).

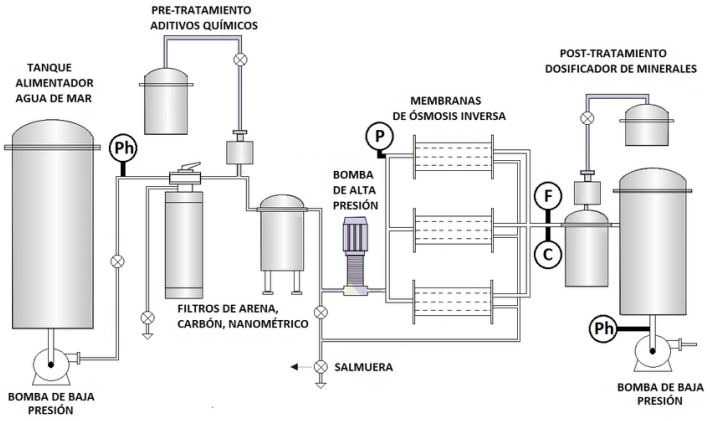


Figure 1: Reverse osmosis desalination plant. (Mar, 2014)

Ion-Exchange resins.

In this process (conventional ion exchange), a resin is used in which anion exchange takes place and is effective for the removal of nitrates from water (Mendow et al., 2017). The water must be pre-treated before passing through the system to remove suspended solids and compounds that can affect the nitrate removal (**Figure 2**). Moreover, the resin must be regenerated from time to time to avoid saturation of the resin (Samatya et al., 2006; Mendow et al., 2017). Therefore, the high cost of the reagents used in regeneration, together with the low ion selectivity of the technology, represent one of the most important problems of this technology.

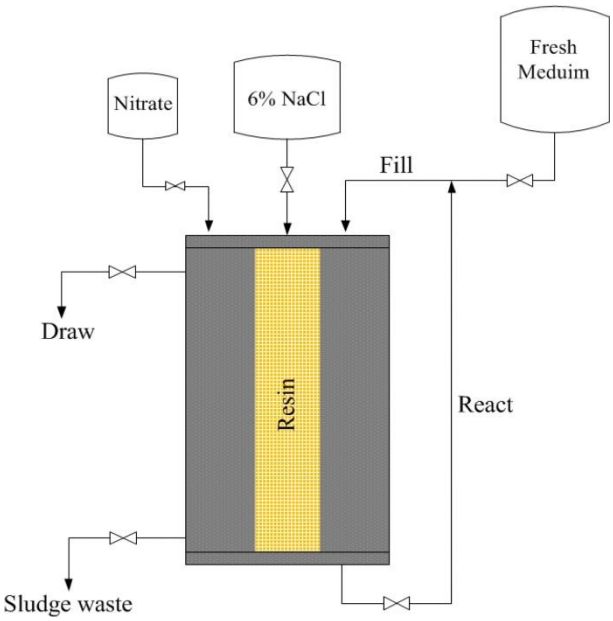


Figure 2: Ion exchange resin regeneration example system.(Ebrahimi & Roberts, 2016)

Electrodialysis.

Electrodialysis could be considered as a similar process that RO and consists of the physical separation of nitrate from the water by means of a series of membranes, which are subjected to an electric field to produce the mobility of the charged ions between the different poles of the electric field (Mohammadi et al., 2021). Obviuosly, electrodialysis technology generates a saturated solution (brine) that must be treated before being discharged into the environment (**Figure 3).**

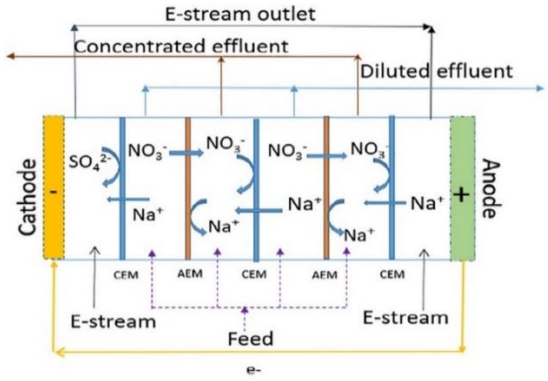


Figure 3: Diagram showing an electrodialysis unit with two compartments. (Mohammadi et al., 2021)

Membrane distillation.

Membrane distillation (MD) is a water ion separation process that simultaneously combines mass separation and heat through a hydrophobic membrane. In this process, water loaded with organic or inorganic comound passes through one side of a hydrophobic porous membrane, while a stream of cooler water flows through the other side of the membrane. The flux or mass transfer occurs due to the partial vapor pressure difference originating between the cold and hot sides of the membrane (Yarlagadda et al., 2011; Ren et al., 2018)

Membrane distillation process has the advantage that it can operate at much lower pressures than those required for RO process, with the consequent reduction in energy consumption, and it also has higher pollutant removal efficiencies than other systems. However, MD system present some disadvantages such as the production of brines with high loaded with pollutants that must be treated before being discharged into the environment (**Figure 4).**

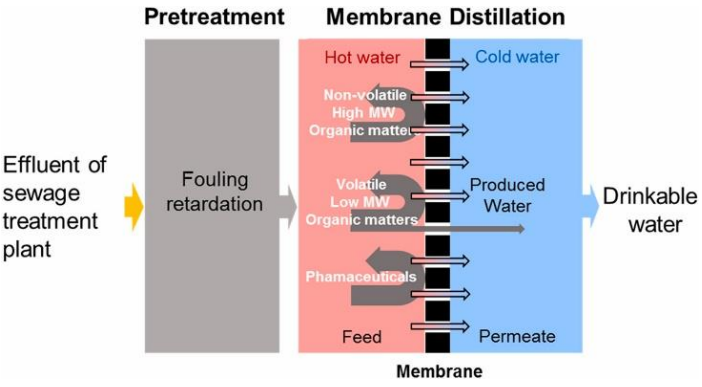


Figure 4: Example of membrane distillation process for wastewater treatment.(Jeong et al., 2021)

1.1.2 Biological systems for nitrate removal in waters.

Biological systems for nitrate removal from groundwater must be considered as emerging technologies. Specifically, biological technologies for nitrate removal are based on heterotrophic or autotrophic bacterial denitrification processes(Wang et al.,

2017).These microorganisms transform nitrate into dinitrogen by means of different metabolic pathways, although in nature this process is often carried out by bacteria that use nitrate as a final electron acceptor in their respiratory process (Soares, 2000). The autotrophic denitrification process is carried out by autotrophic denitrifying microorganisms, which use inorganic carbon compounds as substrate to carry out the process, in this relationship both the carbon source and the electron donor come from inorganic sources (Cecconet et al., 2018). However, heterotrophic denitrification process in contrast to the previous process requires an organic carbon source to be carried out. This carbon source can come from the water itself to be treated (in the case of wastewater treatment), or it must be added to the system (in the case of treating drinking water that does not contain organic matter).

Biological systems for nitrate removal must be considered more sustainable and environmentally friendlier than physicochemical technologies, since they do not generate any type of brein and present lower investment and operation costs, compared to other technologies such as RO and MD.

Within the biological systems for the treatment of drinking water and specifically used for nitrate removals, we can highlight:

In situ biological treatment

In situ biological systems for the treatment of groundwater polluted with nitrates, an enriching of denitrifying microorganisms present in the aquifer is required. Some of the microorganisms naturally present in the aquifer are capable of performing the denitrification process; the problem is that this process occurs at a very slow rate of elimination due to the scarcity of electron donors within the aquifer. For that reason, it is necessary the inoculation of the aquifer with denitrifying microorganisms and a carbon source, thus achieving good nitrate removal results. Different examples of this technology have been implemented with good results, although so far only at the level of experimental project (Margalef-Marti et al., 2019).

This technology has the advantage of being applied in the water supply itself, without the need for major structural works, but it is a technology in which the conditions inside the aquifer must be studied in detail, as well as the flow dynamics inside it, in order to achieve a homogeneous diffusion and a correct elimination process.

Denitrification using biofilm-fixed systems.

These technologies are based on the capacity of microbial growth adhered to a surface, originating complex polymicrobial aggregates capable of developing a wide diversity of metabolic functions, frequently by means of collaborative processes. In the specific case of the application of these biological systems to the treatment of water polluted with nitrates and pesticides, there are different technical alternatives that fundamentally influence the use of different support materials and bioreactor configuration. Thus, at present we can find a wide range of support materials on which abundant denitrifying microbial biomasses are developed, such as: sand, anthracite, activated carbon, calcium carbonate and plastic materials, among others. Fixed biofilm technologies can be divided into two groups: Moving Bed reactor and Submerged Biofilter.

1 Moving-bed biofilm bioreactors (MBBR)

These systems base their operation on the presence of support materials (carriers) that move freely inside the reactor, and on which a microbial biofilm develops. The carriers can be made of different materials such as polypropylene, polyethylene, polyurethane, activated carbon, etc. Generally, a material with a density lower than that of water is sought in order to achieve better movement inside the reactor. The system is aerated from the bottom and the water inlet is located at the top. These systems are commonly used in urban wastewater treatment plants (Gonzalez-Martinez et al., 2018; Rodriguez-Sanchez et al., 2020), although their use couuld be used in the treatment of groundwater polluted with nitrates.The systems can be

configured in different ways, aerated or non-aerated to achieve the desired objective (**Figure 5**).

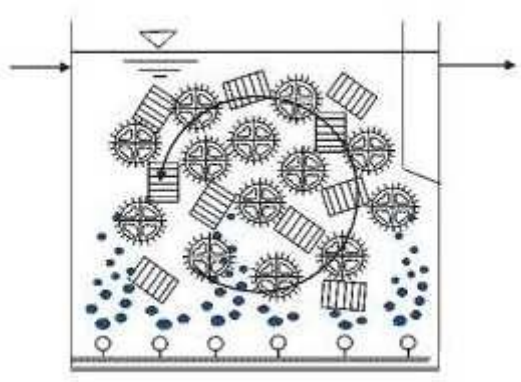


Figure 5: Diagram of an MBBR system with aeration at the bottom of the system

2. Submerged biofilters

This technology works with a fixed biofilm system attached to a carrier through which we pass the water stream to be purified. It is a simple technology, economical in its operating costs and certainly widespread in both wastewater treatment and drinking water. In short, it is a submerged biofilter that, under aerobic or anoxic conditions, allows good nutrient elimination results. (Ramos et al., 2007)

In the specific case of the application of these systems to the treatment of groundwater with high concentrations of nitrates, we can affirm that it is a well-consolidated technology with concrete examples of full-scale installations in Spain such as ETAP de Falset (Tarragona) and ETAP de Formiche Alto (Teruel).

1.2. Sequential Granular Aerobic Systems (AGS)

Sequential aerobic granular systems (AGS) consist of a special type of suspended biofilm in which the biomass is selected by settling time and hydrodynamic

forces, resulting in a compact granular biofilm (**Figure 6)**. This system was first described in the 1980s and was used for wastewater treatment. (de Sousa et al., 2018).

Figure 6 Aerobic granular biomass generated in a sequential bioreactor (AGS).

It is therefore clearly evident that the fundamental core of this technology is the ability of microorganisms to establish stable polymicrobial aggregates (granules) without the existence of a support material, which determines a very particular functionality of operation. The granule formation, for the moment, has been well analyzed in sequential reactors, although it is also true that some approximations have been carried out in continuous reactors. (Y. Li et al., 2020; D. Zhang et al., 2020; Long et al., 2015; Rosa-Masegosa et al., 2023)

The sequential granular aerobic systems are designed as a column type reactor, aerated at the bottom and with an outlet that will be placed according to the operational volume of the bioreactor, which can be at 50%, 60%, etc. (**Figure 7**). These systems operate discontinuously and in a series of phases. These phases consist of an aeration phase, a settling phase and an emptying step.

These stages are of vital importance for the proper functioning of the reactor, since they determine pollutan degradation and organic matter consumption. This

process occurs during the aeration stage, in which nutrient consumption occurs first, followed by a starvation period. This last period is of vital importance for the correct formation of the granular biomass (Adav et al., 2008). Once the aeration period is over the decantation of the granular biomass takes place proceeding to empty the reactor in an approximate volume of 50%.

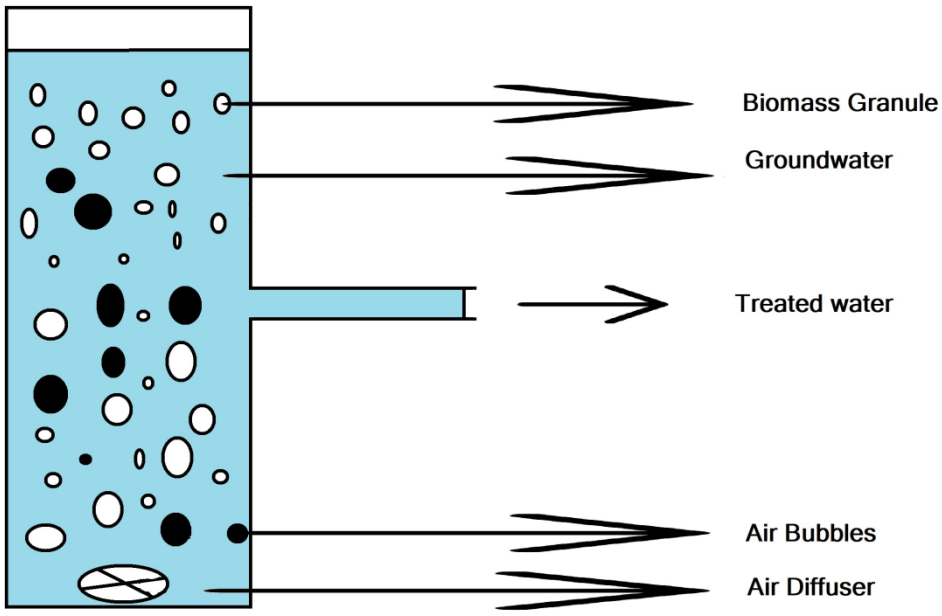


Figure 7: Generic schematic diagram showing a granular sequential aerobic bioreactor.

1.2.1. Aerobic granules

The aerobic granule formation is a complex mechanism in which different stages and factors can be established (**Figure 8**). There are different theories related to the initiation of the granulation process, (D. J. Lee et al., 2010; Nancharaiah & Kiran Kumar Reddy, 2018; de Graaff et al., 2019). It can be established that microorganisms in suspension, under certain hydraulic movement conditions, establish cell-cell surface

unions that originate multicellular formations that produce larger micro aggregates, in which there is a high production of exopolysaccharides that finally allows a greater degree of compaction. The hydrodynamic forces of the system and the van der Waals forces together with the negative surface charge that the microbial surfaces generally possess mean that the aggregates do not agglutinate with each other, maintaining their independence and growing individually according to the granule maturation process (Sarma et al., 2017)

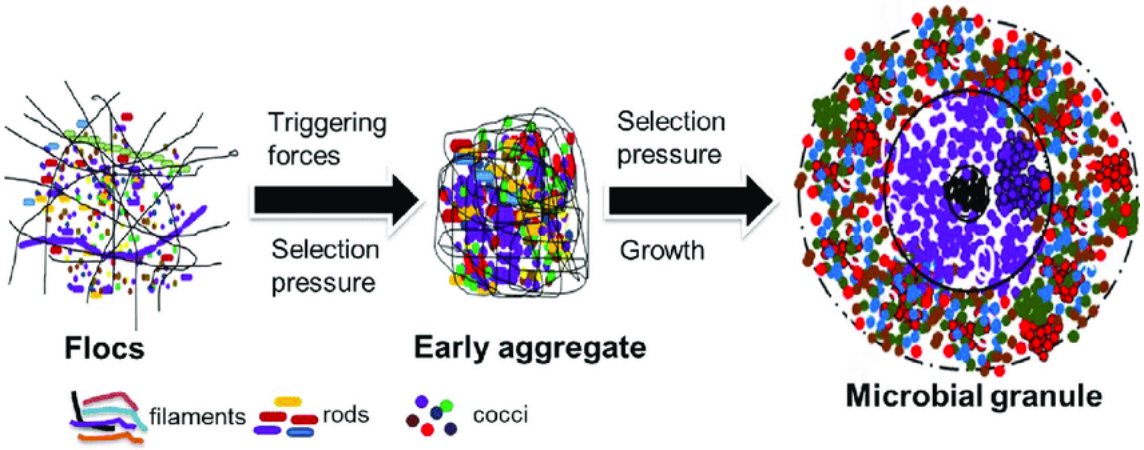


Figure 8: Mechanism of aerobic granular sludge formation in a sequential batch reactor. (Sarma et al., 2017; Lv et al., 2014).

The granulation process is affected by different environmental factors such as water composition, amount of dissolved oxygen, hydrodynamic forces of the system, feed regime, reactor configuration or hydraulic retention time. In this context different factors are involved in the granulation process such as organic matter concentration, C/N ratio and temperature.

The concentration of organic matter available to the microorganisms plays a fundamental role in the granulation process. Granule-forming microorganisms need a carbon source to survive. Therefore the application of aerobic granular systems in urban wastewater treatment, is not usually a problem, since it is in excess in the influent

to be treated, being available for microorganisms (Pronk et al., 2015). A period rich in organic matter, followed by a period of starvation (feast-famine), is necessary in the system to favor biomass aggregation. This period of starvation activates the surface forces of the microorganisms, achieving their aggregation and the consequent development of more compact granules (Pronk et al., 2015).

The problem is when AGS technology is used to remove certain compounds (i.e., nitrate) from effluents with very low concentrations of organic matter. In this case, it is necessary the addition of an external carbon source since the heterotrophic denitrification required the presence of certain amounts of organic carbon. Methanol, ethanol, or sodium acetate, among other, are external carbon sources that have been widely reported in AGS systems (Li et al., 2008),

The C/N ratio is also of special importance in the formation of granules and in the correct functioning of the technology. Normally this parameter is not a limiting factor in the treatment of urban wastewater, but it is of great importance when we treat oligotrophic effluents such as groundwater. In groundwater denitrification processes using AGS systems, it has been indicated that C/N values 1/1 would be the minimum capable of maintaining this biological activity, although it is evident that values 2/1 would allow more efficient activities (Chen et al., 2018).

Temperature plays an important role in the development of microbial communities in granules, and it has been observed that the formation of stable granules can originate in a wide range of temperatures, both psychrophilic and mesophilic (De Kreuk et al., 2005). For instance, different studies have corroborated an efficient granulation process at low temperatures (Muñoz-Palazon et al., 2018b; Gonzalez-Martinez et al., 2018)

The hydrodynamic force generated in the system is another important point to take into consider in the correct conformation of the granular aggregates, since it generates the appropriate shear forces for the biomass to be configured in granular aggregates (Wilén et al., 2018). Aeration speed between 2.4 and 3.2 cm s^{-1} have yielded the best results in the granulation process (Chen et al., 2007).

Particle selective pressure must be considered as another important factor for the formation of granular biomass in AGS systems, particularly to control the settling time in the early stages of granule formation (De Kreuk et al.,2005; Gonzalez-Martinez et al., 2018; Gonzalez-Martinez et al 2018) We cannot forget that granular biomass is compact; therefore, it has high settling velocities, which allows an easy separation of the microorganisms from the treated water. This characteristic makes these AGS systems interesting for the treatment of drinking water, among other applications in effluent treatment.

As a consequence of the compact structure of aerobic granular biomass, it is well known that microbial stratification occurs in response to the concentration of oxygen and nutrients (**Figure 9**). This determines the stratification of the microbial communities and consequently their metabolic activities.

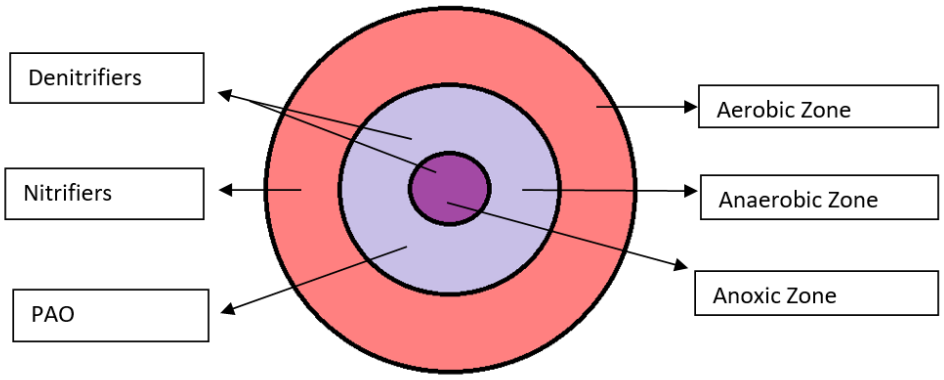


Figure 9: Distribution of microorganisms and nitrogen removal pathways in a granule.

If we observe the stratified structure of the granule we can establish three zones. An external aerobic zone made up of heterotrophic and nitrifying microorganisms, an intermediate zone where populations such as the PAO are located and finally an internal region of anoxic nature where the denitrifying populations would be located. Consequently, in this granular structure, we find very different metabolic

activities that largely represent a complex microbiome from a microbial and physiological point of view (Liu et al., 2015; Yuang and Gao, 2010)).

Consequently, the granular stratification is the key to the various processes of organic matter removal and the biotransformation of nitrogenous compounds, all of which occur simultaneously in the granule, without the need to modify the oxygenation conditions of the system (Chen et al., 2020).

1.2.2. Microbial diversity in aerobic granules

The microbial diversity in granular aerobic sludge is extraordinarily complex and greatly influenced by the different environmental factors where they develop. Thus, the granules contain multiple species from different microbial domains that interact with each other, sometimes positively and sometimes negatively through predation mechanisms (Jousset, 2012).

Within the bacterial community, *Thauera* and *Meganema* have been reported as filamentous organisms, which could be involved as granulation nuclei and therefore favor granule formation (Muñoz-Palazon et al., 2018a). *Zooglea* is known for its role in the process of floc formation through polymer production, and has been found in granular systems (Gonzalez-Martinez, Muñoz-Palazon, et al., 2018; A. J. Li et al., 2008). Also, have been reported microorganisms that participate in nitrogen compound removal such as *Acinetobacter* (Gonzalez-Martinez,et al., 2018 Muñoz-Palazon, et al., 2018; S. Yao et al., 2013).

Archaea are also present in granular systems, being reported the genera *Methanosaeta, Methanobacterium, Methanobrevibacter, Methanosphaera, Methanolinea, Methanospirillum, Thermoplasmata* and *Methanosaeta* (Muñoz-Palazon et al., 2018 a; J. Liu et al., 2017).

Fungi play an important role in the granule formation process; first, they act as a structure together with the filamentous microorganisms in the first stages of granule formation, acting as a support on which the rest of the microorganisms grow to achieve a compact granule. Within the granular systems, fungi have been reported, being one of

its maximum representatives *Trichosporon* (Muñoz-Palazon et al., 2018a; Gonzalez-Martinez et al., 2018) . It is a ubiquitous fungus present in many systems, and could play an important role acting as a support on which the rest of the microorganisms establish themselves and providing structure to the granule. *Scopularipsis, Tremellomycetes* and *Pleosporaceae* have also been reported (Liu et al., 2017; Wilén et al., 2018; Weber et al., 2007).

1.3. Design and Operation of Aerobic Granular Systems (AGS)

In the last decades the use and study of AGS systems has been extended due to the fact that they have important advantages in relation to traditional water treatment systems. Therefore, they present a reduction in operating costs of around 25% and up to 40% less energy costs (Adav et al., 2008). In addition, they require much less space than conventional treatment systems, due to the configuration of the granular biomass, since the removal of organic matter, nitrogen compounds and phosphorus compounds can be carried out in a single reactor, thus reducing the size of the treatment plants. However, AGS systems have been developed mainly for the treatment of urban and industrial wastewater, and there are no specific applications of this technology for drinking water. Likewise, these treatment systems have been implemented basically on sequential bioreactors; their use in continuous reactors is currently being investigated (Rosa-Masegosa et al., 2023).

1.3.1. Sequential granular aerobic bioreactors.

Design and optimal operating conditions are essential parameters for the correct functioning of an aerobic granular system in SBR configuration. Particularly a first design criterion is to establish a relationship between height and diameter of the bioreactor that allows the correct granulation of the biomass (Liu et al., 2005). In general, the granular bioreactors are designed with height/diameter ratios that allow aeration to create sufficient hydrodynamic force for the biomass to form granules. A

higher hydrodynamic force favors the development of dense granules and stimulates the production of exopolysaccharides, contributing to granule formation (Liu et al., 2005). This configuration improves the production of a circular movement in the system, which subjects the granules to hydraulic wear, causing the aggregates to organize in a circular morphology (Beun et al., 2002; Liu et al. 2005).

Several types of bioreactors (**Figure 10**) have been tested, with different height-diameter ratios. The height-diameter ratio combined with the percentage of water exchange in the reactor fundamentally determine the dynamics that occur inside the reactors (Liu et al., 2005).

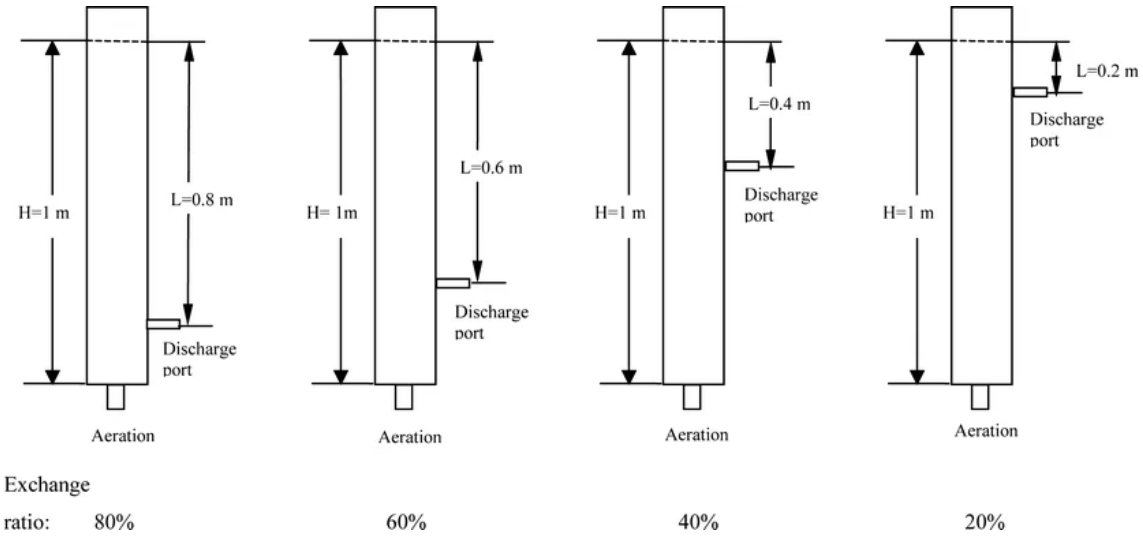


Figure 10: Schematic interpretation of exchange ratios in SBRs (Liu et al., 2005)

In general, although there is a wide range of bioreactor designs, granular sequential systems consist of column-type, cylindrical reactors aerated from the bottom of the bioreactor by fine bubbles. They are sequential systems that operate in a discontinuous cyclic manner. This means that the system is set up in a series of stages in a periodic manner (**Figure 11**), which we could describe in the following figure:

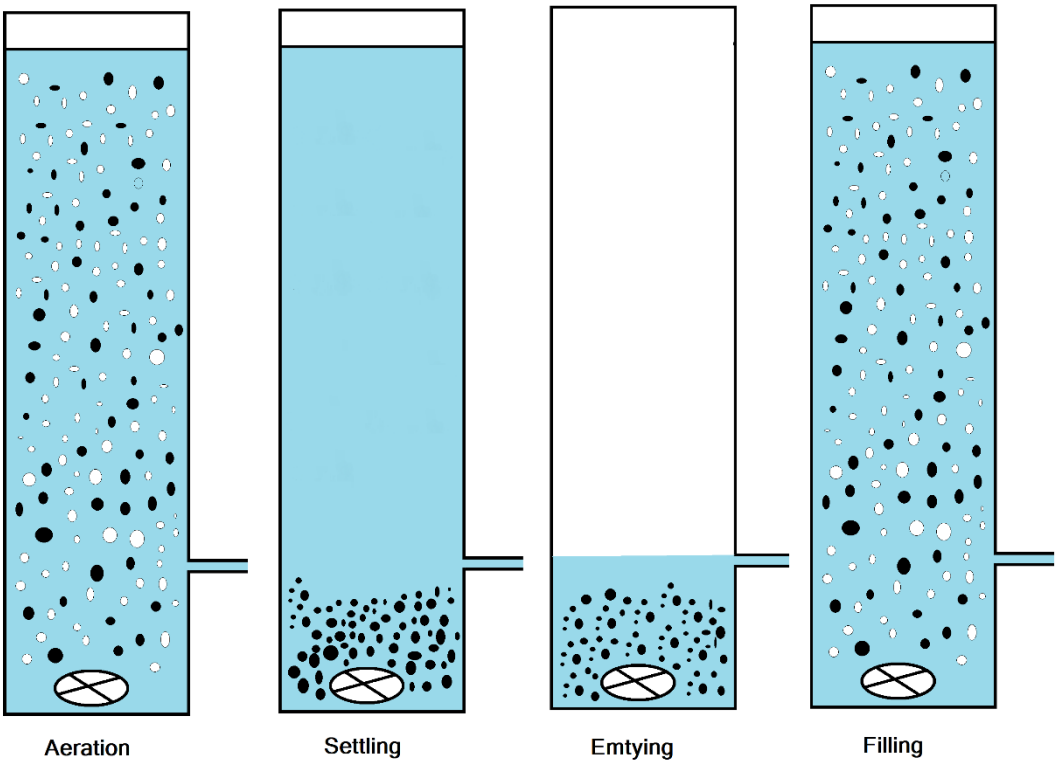


Figure 11: It shows the different stages of an SBR reactor, after the emptying stage the reactor is filled and starts a new aeration cycle.

- **Aeration Stage**. During this phase, the lower part of the bioreactor is aerated for a specific period of time. During this period, the bacterial biomass maintains contact with the water to be treated and with the air current, thus establishing adequate hydrodynamic force for the formation of the granules, as well as the most appropriate oxygenic conditions in the system. (Wang et al. 2006) indicated that during this phase of operation the maximum degradation of the pollutants present in the wastewater occurs, establishing at the same time first the consumption and degradation of the substrates present in the water, followed by a period in which there is no food available for the microorganisms. These periods of feeding and nutritional limitation of the biomass favor the development of dense and compact granules of great interest in AGS technology (Pronk et al., 2015).

- **Settling stage**: When the time stipulated in the operation cycle is finished, the aeration and the movement of the biomass is stopped in order to favor its settling, making a selection of the biomass according to its settling velocity. The selection of the biomass and the settling time is another fundamental factor in the granulation process, since it favors the subsequent elimination of the most light particles (filamentous biomass) from the system to the favor of the granular biomass, the latter having more substrate for its development (Gonzalez-Martinez et al., 2018). At this stage, a period of anoxia occurs in the biomass that favors the denitrification process.

- **Emptying stage**: After the selected settling time has elapsed, a certain volume of the bioreactor is removal. This volume of water that is exchanged in the bioreactor in each cycle is known as the exchange percentage, which is the treated water produced by the reactor in each cycle and replaced by new water to be treated. This percentage can change depending on the type of reactor or the process to be carried out in it. Low exchange percentages (between 40 and 20%) determine a large amount of suspended particles in the bioreactor that interfere with the correct development of the granules, the most appropriate exchange percentages being between 60 and 80% of the total volume of the reactor. This suggests that the exchange rate exerts a selective pressure on granule development (Liu et al., 2005).

The SBR operational cycle is based on the establishment of the most appropriate hydraulic retention time (HRT), so that optimal purification levels can be obtained according to the characteristics of the effluent to be treated. Mainly for urban wastewater treatment systems, the HRT can range from 3 to 12 hours (De Kreuk et al., 2005).

1.4. Nitrogen Removal in AGS Systems.

The removal of nitrogen compounds in sequential granular aerobic systems is a widely studied process (Muñoz-Palazon et al., 2021; Rodriguez-Sanchez et al., 2019; Sahinkaya et al., 2015) and largely influenced by the stratified structure of granular

biomass (**Figure 8**). As indicate above, during granule formation an oxygen gradient is established, which allows the outer layer to be aerobic, and the oxygen gradient decreases to form anoxic inner layers and a potentially anaerobic core (De Kreuk et al., 2007), this allows the simultaneous occurrence of denitrification and nitrification processes, which together with the assimilation of nitrogen compounds by the biomass, are the mechanisms that determine the nitrogen removal in the treated water (Adav et al., 2008).

1.4.1. Nitrification process.

In water treatment systems, as occurs mostly in nature, the dominant nitrification process is done by chemoautotrophic prokaryotes. Specifically, the nitrification process occurs mainly by two types of prokaryotes, the so-called ammonium-oxidizing bacteria or archaea (AOB and AOA, respectively) that transform ammonium to nitrite ($NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$) and the nitrite-oxidizing bacteria (NOB) that carry out the transformation of nitrite to nitrate ($NO_2^- + 0.5O_2 \rightarrow NO_3^-$).

Special reference should be made to the COMAMOX bacteria, which are able of performing the complete ammonia oxidation process to nitrate. Commamox bacteria are widely distributed in the environment and are abundant in terrestrial and aquatic ecosystems. Also have been identified in various water treatment systems including wastewater and drinking water treatment systems (Hu & He, 2017). This group grows simultaneously in the systems with AOB-AOA and NOB. Representative of this group is the genus *Nitrospira* (Manasa & Mehta, 2021).

In water treatment systems, a correct balance between the populations of AOB-AOA and NOB is of special importance, so that there is no environmental and nutritional competition between both physiological types, which can lead to metabolic inhibition processes and ultimately to a decrease in the efficiency of biotransformation of nitrogen compounds (Lawson & Lücker, 2018). To provide this metabolic balance, in the wastewater treatment plants, oxygen supply criteria must be established to

determine the correct functioning of the ammonium and nitrite oxidation processes. It is known that both processes occur optimally at different ambient oxygen concentrations. Specifically, the transformation of nitrite to nitrate requires less oxygen than the transformation of ammonium to nitrite (Lochmatter et al., 2014). In the case of aerobic granular systems, the existence of an oxygen concentration gradient in the granule favors the location of the nitrifying populations and their operational functionality in such a way that self-regulation and intermicrobial cooperation is determined in a simple way. Moreover, in AGS technology the population of NOB is usually lower than that of AOB as a consequence that denitrifying microorganisms can use both nitrite and ammonium as electron acceptors (Winkler et al., 2012).

1.4.2 Denitrification process

The denitrification process consists in the use of nitrate as terminal electron acceptor under anoxic conditions (Mohseni-Bandpi et al., 2013) and the transformation of this ion to dinatrogen. The most cited genera of denitrifying bacteria include: *Alcaligenes*, *Paracoccus*, *Pseudomonas*, *Thiobacillus*, *Rhizobium*, *Thiosphaera*, among others. Most denitrifying bacteria are heterotrophic, but some can grow autotrophically with hydrogen (H_2), carbon dioxide, reduced sulfur compounds, or reduced arsenic compounds (Haugen et al., 2002).

The process of nitrate reduction to molecular nitrogen consists of different successive stages catalyzed by different enzyme systems, in which different intermediate products appear.

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

Denitrification requires an oxidizable substrate as energy source, either organic or inorganic and therefore this process can be carried out by heterotrophic and autotrophic bacteria.

Denitrification process in wastewater treatment plants, can be carried out either in a pre-denitrification configuration or in a post-denitrification configuration, although

under post-denitrification condition the addition of an exogenous carbon source id needed. Logically, under pre-denitrification condition the organic matter content of the wastewater is normally enough to maintain this biological activity.

Under oligotrophic systems such as groundwater, heterotrophic denitrifying activity is often limited by low organic matter contents, and it is necessary the addition of organic matter (Costa et al., 2018). Obviously, the addition of an exogenous carbon source must be well controlled since an excess of organic matter can produce a contamination in the water, preventing its use for consumption, and generating an extra cost in the operation of the system due to this excess of added nutrients. It is therefore very important to maintain a balance between the minimum amount of organic matter added and the correct development of denitrifying microorganisms so that the nitrate removal process can be effective.

Denitrification consists of several stages, in which a series of reactions take place for the complete transformation of nitrate to molecular nitrogen, which is emitted to the atmosphere in the form of gas.

In the first place, the transformation of nitrate to nitrite occurs by the enzyme nitrate reductase (*Nar*), generating energy that is used by the microorganisms for their growth. This stage requires low oxygen pressures in the medium to take place. Subsequently, the enzyme nitrite reductase (*Nir*) comes into play, which reduces nitrite to nitric oxide, and as with the previous enzyme, this also requires low oxygen pressures for its correct functioning. Next, the enzyme nitric oxide reductase (*Nor*) comes into operation, which reduces nitric oxide to nitrous oxide. And in the last stage of the process, nitrous oxide is transformed into gaseous molecular nitrogen by the enzyme nitrous oxide reductase (*Nor*) (Bowles et al., 2012).

In aerobic granules, the microorganisms responsible for denitrification reactions are found in the internal parts of the granules, close to the nucleus. It is in these layers where the heterotrophic denitrification process takes place. Different bacterial genera have been reported to be involved in denitrification processes. The genus *Corynebacterium* has been reported as a ubiquitous microorganism in sequential

granular aerobic reactors (Muñoz-Palazon et al., 2020). The genus *Leucobacter* has also been reported in this type of system, related to granule formation processes due to its surface charges (Xie et al., 2010) The genera *Comamonadaceae* and *Pseudomonas* have also been reported to be involved in denitrification processes in AGS (Xu et al., 2018; J. Zhang et al., 2011).

Fungi play an important role in the granule formation process, first acting as a framework together with filamentous microorganisms in the primary stages of granule formation, acting as a support on which the rest of the microorganisms grow to achieve a compact granule (Muñoz-Palazon et al., 2020). Their denitrifying function has not been particularly studied, although they could potentially develop this activity.

1.5. Technical Applications of Aerobic Granular Systems.

Granular aerobic systems are an excellent alternative to conventional wastewater treatment systems. These systems have an important advantage, since due to the stratification of the microorganisms in the granule; they allow a complete oxidation of organic matter, as well as nitrogen and phosphorus. This characteristic makes them very interesting in comparison with a conventional treatment, allowing the simultaneous nitrification, denitrification and phosphorus removal processes, without multiple operation chambers (Pronk et al., 2015).

AGS wastewater treatment plants has been extensively applied at urban full scale since 2005, currently there are up to 70 plants using this technology under the commercial and patented name of Nereda.® (Guo et al., 2020) with lower investment and operational costs (Sarma et al., 2017).

In addition to the wide application that AGS technology has had in the last ten years for the treatment of urban wastewater, it would be important to highlight that granular systems have been shown to be especially effective in the biotransformation of phenolic compounds (Muñoz-Palazon et al., 2019) and emerging substances such as

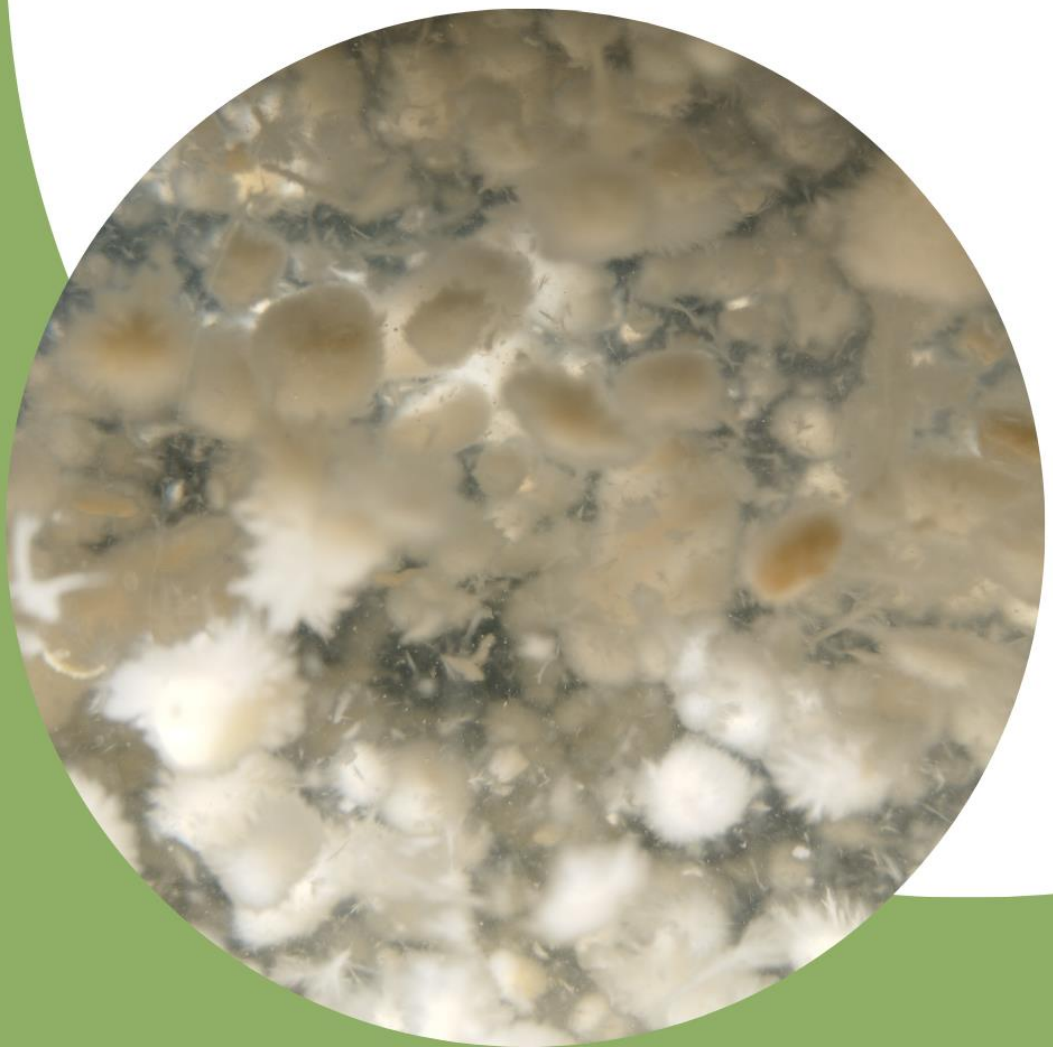
antibiotics (Muñoz-Palazon et al., 2021). Moreover, it is also well known that that granular biomass can very effectively remove heavy metals, therefore this technology can be used for the remediation of polluted effluents such as industrial wastewaters (Yao et al., 2008).

1.6. Potential Application of AGS to the Treatment of Groundwater Contaminated with Nitrates.

The use of granular sequential aerobic systems in the treatment of drinking water for human consumption has been little researched so far.

Groundwater has a series of characteristics that make it ideal for the application of Granular Aerobic technology. It has very low amounts of salts, it is a very oligotrophic medium, but it usually has high amounts of nitrate due to anthropogenic pollution processes. These characteristics make it ideal to be treated by a sequential granular aerobic system focused on the heterotrophic denitrification process. For this purpose, an external carbon source must be added to the water, and all of it must be consumed in the denitrification process. It is essential to calculate the correct amount of organic matter to be added, together with the contact time of the water with the biomass, since both factors must be calculated correctly. All these aspects raise a scientific challenge on how to implement this technology in a biological treatment plant for the elimination of nitrates in water for human consumption.

2.OBJECTIVES



2.Objectives

Groundwater contamination represents a serious problem for the world's population. A significant number of municipalities that use groundwater have problems with the concentration of nitrates as a consequence of the excessive use of nitrogen compounds in agriculture, as well as the uncontrolled discharge of polluted water. This means that these water resources contain nitrate levels higher than those established by EU regulations for human consumption.

The fundamental objective of our work has been to develop an aerobic granular technology configured as SBR at real scale, for the treatment of groundwater with high concentrations of nitrates that makes it possible to achieve a quality of drinking water that can be used for human consumption. For this purpose, a series of partial objectives have been established:

1. Study the granulation capacity and denitrification activiy of different denitrifying organisms carrying the *nos*Z gene to know the possible use of these microorganisms in the start-up of denitrification reactors.

2. Determine the most appropriate carbon source for the formation of stable granular biomass in SBR bioreactors and that at the same time allows the maximum levels of nitrate removal.

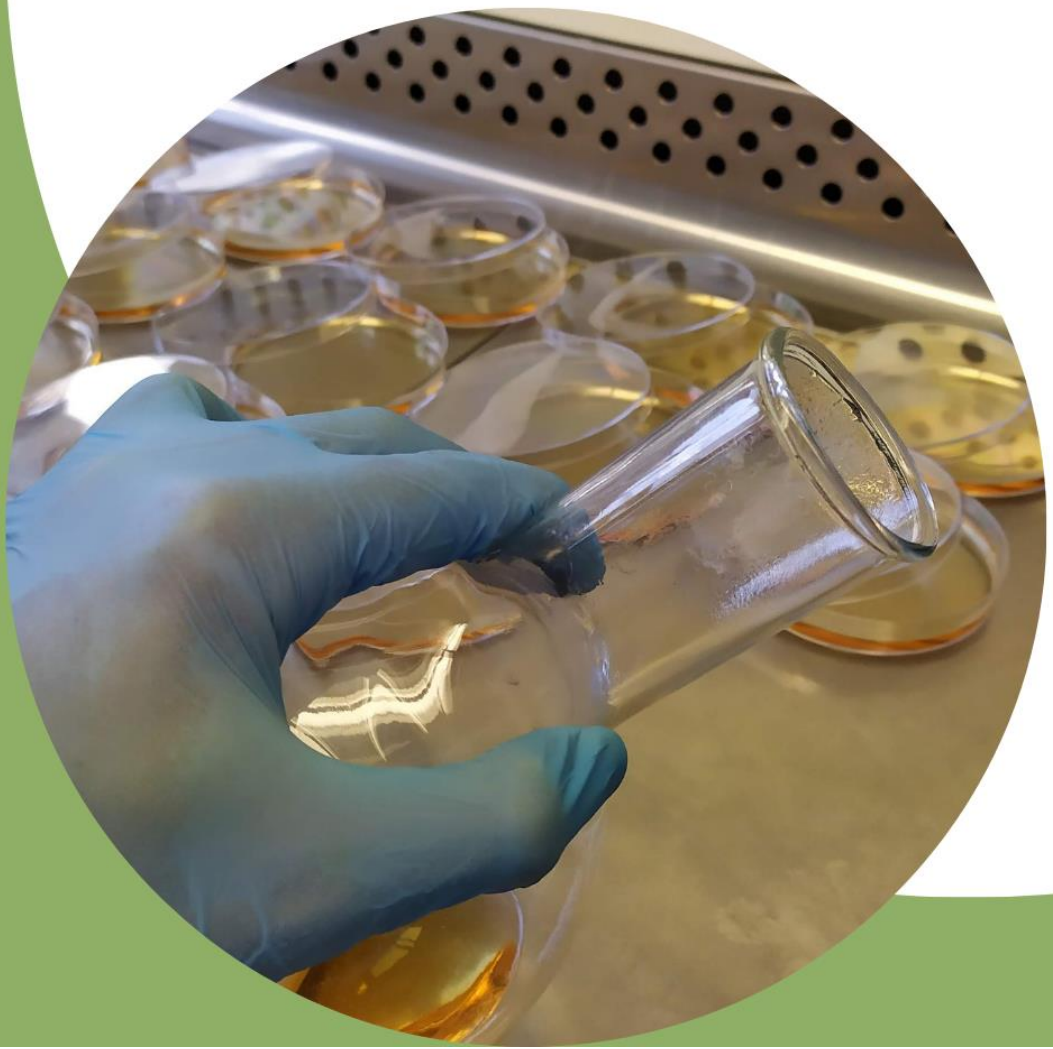
3. Selection of the best inoculation method for SBR full-scale bioreactors.

4. Design and construction of a full-scale SBR bioreactors with the capacity to treat nitrate-polluted groundwater for the supply of drinking water to the municipality of Torre Cardela (Granada, Spain)

5. Start-up of SBR bioreactors and study of the granular biomass growth process and nitrate removal capacity at real scale.

6. Design and construction of a full-scale plant for the treatment of groundwater polluted with nitrates, incorporating SBR aerobic granular systems, filtration and disinfection systems that make it possible to use the treated water as suitable for human consumption.

3.MATERIAL AND METHODS



3. Material and Methods

In the three chapters that make up this doctoral thesis, the materials and methods that have been used are described in detail. For this reason, this section does not describe the different methodologies used; referring the reader to each of the material and methods sections widely described in chapters 1, 2 and 3.

Chapter1 Groundwater nitrate removal performance of selected *Pseudomonas* strains carrying *nos*Z gene in aerobic granular sequential batch reactors.

Chapter 2. Biological nitrate removal from groundwater by an aerobic granular technology to supply drinking water at pilot-scale.

Chapter 3. Nitrate removal from groundwater for human consumption using a full-scale aerobic granular sludge technology.

4.CHAPTERS

4. Chapters

The three chapters that constitute the experimental part of this work have been carried out to respond to the previously established objectives. Specifically, chapter 1 responds to objectives 1 and 2. Chapter 2 has allowed us to achieve objectives 2 and 3. Finally, chapter 3 has made it possible to respond to objectives 4, 5 and 6. Next, we provide a detailed description of all the experimentation carried out and grouped in the aforementioned chapters.

Chapter 1.: Groundwater nitrate removal performance of selected *Pseudomonas* strains carrying *nos*Z gene in aerobic granular sequential batch beactors.

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Abstract

Four granular sequencing batch reactors (GSBRs) were inoculated with four denitrifying *Pseudomonas* strains carrying *nosZ* to study the process of granule formation, the operational conditions of the bioreactors, and the carbon concentration needed for nitrate removal. The selected *Pseudomonas* strains were *P. stutzeri* I1, *P. fluorescens* 376, *P. denitrificans* Z1, and *P. fluorescens* PSC26, previously reported as denitrifying microorganisms carrying the *nosZ* gene. *Pseudomonas denitrificans* Z1 produced fluffy, low-density granules, with a decantation speed below 10 m h^{-1}. However, *P. fluorescens* PSC26, *P. stutzeri* I1, and *P. fluorescens* 376 formed stable granules, with mean size from 7 to 15 mm, related to the strain and carbon concentration. *P. stutzeri* I1 and *P. fluorescens* 376 removed nitrate efficiently with a ratio in the range of 96%, depending on the source and concentration of organic matter. Therefore, the findings suggest that the inoculation of GSBR systems with denitrifying strains of *Pseudomonas* spp. containing the *nosZ* gene enables the formation of stable granules, the efficient removal of nitrate, and the transformation of nitrate into nitrogen gas, a result of considerable environmental interest to avoid the generation of nitrous oxide.

Keywords: *Pseudomonas*; granular biomass; sequential batch reactor; nitrate-polluted groundwater; heterotrophic denitrification.

1.Introduction

Nowadays, a large proportion of the world's population uses groundwater as a drinking water resource, so the quality of groundwater and the removal of any pollutants is of paramount importance worldwide. The increase in the use of chemical compounds such as fertilizers, pesticides, pharmaceuticals, and personal care products is

responsible for extensive groundwater pollution. Nitrate is one of the most widespread pollutants in groundwater due to the intensive use of fertilizers in agriculture, resulting in the joint release of nitrate and phosphate ions [1]. Excess nitrate can be released to aquatic environments because it is highly soluble and mobile, which affects the quality of water [2].

Some diseases, such as methemoglobinemia, thyroid disease, or neural tube defects, have been linked to the consumption of drinking water contaminated with nitrate [3]. Nitrite has been described as a carcinogenic agent [4,5]. Moreover, Brender et al. [6] demonstrated that high concentrations of nitrate could be associated with congenital malformations such as cleft palate, cleft lip, and deformities of the arm.

For these reasons, the European Water Framework [7] established the maximum nitrate concentration of 50 $mg \cdot L^{-1}$ for drinking water, even though the recommended nitrate concentration for drinking water is below 25 $mg \cdot L^{-1}$, assuming this value for high-quality drinking water. The areas most affected by nitrate pollution are designated as nitrate vulnerable zones and are subject to the mandatory Code of Good Agricultural Practice [8]. The results of compliance with this directive have been reflected in the trends in nitrate levels in some countries, such as the northern regions of Europe [5], while the Mediterranean countries have experienced major difficulties in achieving the quality standard due to the strong pressure linked with intensive agriculture [3]. To exemplify this difficulty, in 2012, 108 Spanish municipalities reported having an average of 3.5 $mg \cdot L^{-1}$ NO_3^- in the groundwater they used as a source of drinking water, with a concentration range of 0.4 to 66.8 $mg \cdot L^{-1}$ [9].

Given this background, the sanitary control of groundwater has become of special interest for governments. Therefore, in order to achieve the standard, several physical, chemical, and biological groundwater treatment technologies have been developed, amongst which we may highlight systems such as chemical reduction [10], electrodialysis [11], ion exchange [12], adsorption [13], reverse osmosis [14], biofilters [15], and aerobic granular technology [3]. Although ion exchange is a widely implemented technology for treating nitrate, when the resin is exhausted, the brine waste has negative environmental impacts [12]. Reverse osmosis and electrodialysis

systems have high initial and operational costs, which are translated into high economic and energy requirements [16], restricting their implementation in small populations.

Biological water treatment systems could be considered in general terms as environmentally friendly and cost-effective technologies because the processes are carried out by heterotrophic or autotrophic microorganisms. However, biological technologies could also present some problems derived from their operating conditions and the start-up or maintenance period of the bioreactors [17]. Several biological wastewater technologies have been developed using diverse strategies to reach a successful nitrate removal ratio, for instance, using external carbon sources in anoxic reactors [18]. For drinking water treatment, several technologies have been designed: some of them are autotrophic technologies such as sulfur- and hydrogen-based denitrification processes, others are heterotrophic technologies. The most widely implemented technologies use biofilters, membrane bioreactors, fluidized bed reactors, amongst others. These technologies provide advantages compared with physical or chemical technologies, mainly in terms of the economic and energetic aspects. However, the high hydraulic retention time directly affects the volume of groundwater treated and poses difficulties for cleaning the system, which can increase investment costs [15]. For these reasons, new low-cost and environmentally friendly technologies are gaining relevance, and in this context, a novel aerobic granular technology for treating nitrate-polluted groundwater was recently described [3].

Granules formed in the granular sequencing batch reactor (GSBR) system have a concentric structure of several layers, with different microbial populations between the layers; due to the restricted diffusion of oxygen and nutrients through the granule, the aerobic, anoxic, and anaerobic zones can be defined [19]. Aerobic microorganisms, such as ordinary heterotrophic organisms (OHOs), ammonium-oxidizing bacteria (AOBs), and nitrite-oxidizing bacteria (NOBs), grow in the outer layer of the granule. Polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) are located in the interlayer, and denitrifying microorganisms in the deeper layer of the granule [20]. Thus, granular microorganisms generate a constant gradient of nutrients used during the oxidation and reduction processes, allowing GSBR technology

to completely eliminate organic matter, nitrogen, and phosphorus in a single reactor [19].

The granular conformation is promoted by the circular and continuous motion produced in the sequential batch reactor (SBR). In addition, the production and content of extracellular polymeric substances accumulated in the microbial surface encourages attachment between cells [21]. The EPS promote the morphological structure, suggesting the role of EPS is key during the conformation of microbial aggregates and also in steady-state because it immobilizes the cells without any supporting material [22]. The system is based on the metabolism of different microorganisms, including denitrifying bacteria and specifically *Pseudomonas* strains, which may present the *nos*Z gene in their genomes. Nitrous oxide reductase is coded by the *nos*Z gene and catalyzes the conversion of nitrous oxide (N_2O) to dinitrogen gas (N_2) [22]. However, many denitrifying bacteria lack this enzyme, and when the denitrification process is performed by these *nos*Z-deficient bacteria, the nitrate is transformed into N_2O gas [23], which is well-known to be a potent greenhouse gas [24]. For instance, *Acidovorax facilis*, *Citrobacter diversus*, and *Enterobacter agglomerans* are only able to reduce NO_3^- to NO_2^-, without further reduction of NO_2^- to N_2 and cause greenhouse gas production [25]. Consequently, the removal of NO and N_2O in aerobic granular systems represents an additional advantage of this type of technology.

The main goal of this research was to optimize GSBR bioreactors at the laboratory scale using cultures of denitrifying strains of *Pseudomonas* carrying the *nos*Z gene as inocula to treat nitrate-polluted groundwater. To achieve this goal, the best operating conditions for each strain to form stable granules, nitrate removal capacity using different carbon sources, and the most appropriate C:N ratio to successfully remove nitrate were studied in order to obtain high-quality effluent that can be considered suitable for human consumption. Then, the knowledge of the denitrification process on aerobic granules using Pseudomonas culture would allow the implementation of GSBRs for contaminated groundwater treatment.

2. Materials and Methods

2.1. Design and Operating Conditions

Four sequential batch bioreactors were started using, as inoculum, 1 L of liquid (TSB medium) culture of four denitrifying strains of *Pseudomonas*, in which the presence of the *nos*Z gene had previously been identified: *P. stutzeri* var. mendocina (1I) and *P. fluorescens* PSC26 were provided by the Zaidin Experimental Station (CSIC-Granada), *P. fluorescens* 376 was provided by the Water Research Institute Culture Collection (University of Granada), and *P. denitrificans* Z1 was supplied by the National Collection of Industrial, Food, and Marine Bacteria (NCIMB) (NC012776) [26,27,28]. The bioreactor configuration was designed to be 50 cm long and 9 cm in diameter, with an operational volume of 2.5 L **(Figure 1**). The air flow (3.0 $L \cdot min^{-1}$) was supplied by fine bubbles at the bottom of the bioreactors through a spargers plate. The temperature was controlled to 25 °C. The dissolved oxygen and pH were monitored as 4.2 ± 0.4 mg O_2 L^{-1} and 7.4 ± 0.1, respectively, five times a week, using Crison probes.

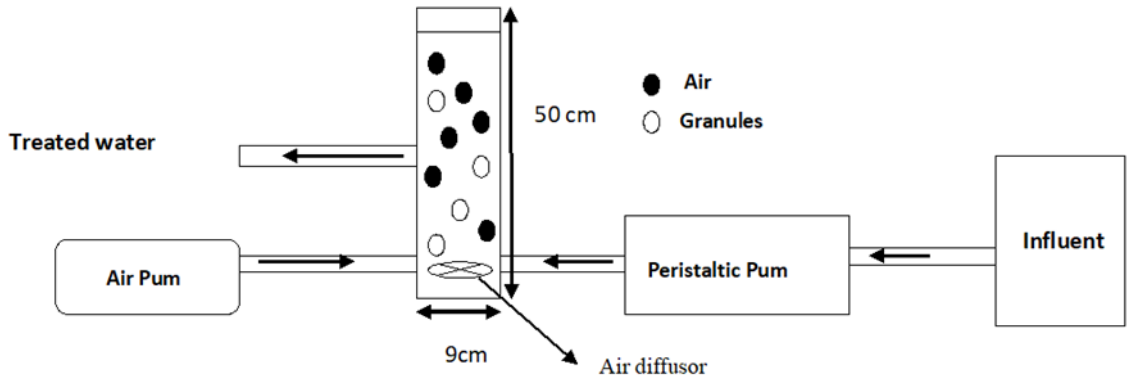

Figure 1. Schematic diagram of a sequencing batch bioreactor inoculated with cultures of denitrifying *Pseudomonas* strains.

Two types of experiment were set up in the sequential batch bioreactors. For the first experiment, the different bacterial strains were tested for the formation of granules using sodium acetate as the only carbon source. For the second experiment, two carbon sources (sodium acetate and methanol) were tested using the bacterial strains that formed granules (*P. stutzeri* PSC26 and *P. fluorescens* 376). The GSBR systems fed with sodium acetate were both operated for 180 days. In the case of *Pseudomonas denitrificans*, the bioreactor was operated for 30 days using sodium acetate because this strain was not able to form granules. For *P. stutzeri* PSC26 and *P. fluorescens 376*, which were fed with methanol as carbon source, the bioreactors were operated for 180 days.

The chemical composition of the synthetic groundwater used to feed the pilot plants consisted of mineral salts and a single source of carbon (sodium acetate in the first experiment or methanol in the second experiment). The mineral salts per liter of distilled water comprised 0.1 g $MgSO_4 \cdot 7H_2O$, 0.085 g K_2HPO_4, 0.04 g KCl, and 0.03 g KH_2PO_4. Different C: N (carbon/nitrogen) ratios were tested (**Table 1**) using acetate or methanol, with the aim of establishing the relationship most suitable for the granulation process and the nitrate removal capacity [29].

Table 1. Carbon (sodium acetate and methanol) and nitrate concentrations added to the synthetic groundwater used to feed aerobic granular pilot plants inoculated with denitrifying *Pseudomonas* strains

Operational Day	Sodium acetate	Sodium nitrate ($mg \cdot L^{-1}$)	Methanol
0-30	900	127	300
31-60	400	127	300
61-90	300	100	300
91-180	200	100	200

The sequential batch reactors cycle had a hydraulic retention time of 10 h, and the exchange volume was 60% of the operational volume reactor in each cycle. The stages consisted of: aeration 446 min, settling velocity 3 min, wash-out of 20 min, and feeding period 10 min. During the start-up phase, the settling time of the system decreased, so the settling time was established at 10 min during the first seven days of operation, whereas for days 8–14 of operation, the settling time was adjusted to 5 min. Finally, after day 15 of operation, the bioreactors were adjusted to 3 min of settling time to favor the development of the granular biomass [30].

2.2. Physical Determination

The granulation process and the stability of the systems were analyzed twice per week. Mean granule size was measured using representative samples during the aeration stage (n = 25) from each reactor. Measurements were taken using a millimeter scale observed by stereoscopic microscope. The settling velocity of the granules was also measured following the protocol described by Laguna et al. [31], slightly modified, using a 2 m glass column with a manual chronometer.

2.3. Chemical Determination

Duplicate samples from the influent and effluent were taken four times a week in order to evaluate the removal ratio performance. Chemical oxygen demand (COD) was measured following the standard spectrophotometric method described by APHA [32]. Biological oxygen demand at day 5 (BOD_5) was also measured using dark bottles and an OxiTop™ [3].

Effluent and influent samples were taken periodically from each of the reactors for the determination of nitrite and nitrate concentration. This determination was performed by ion chromatography (Metrohm) according to Gonzalez-Martinez et al. [33] with a Metrosep A Supp 4—250 anion column. A carbonate/bicarbonate solution was used as the eluent. Calibration curves for the ion chromatography were created at the beginning of each analysis.

2.4. Mass Balance of Pollutants for All Bioreactors during the Operational Period

Physicochemical performance data were used to calculate the amounts of daily contaminants processed by each bioreactor, which were inoculated with different denitrifying Pseudomonas strains in order to identify the system with the lowest carbon footprint and the most sustainable strain to develop the transformation into N_2 by denitrification and the carbon load.

For calculation of the mass balance of nitrogen, total nitrogen entering into the bioreactors, which was in the form of nitrate, was controlled; it was converted either into nitrite (NO_2^-) or nitrogenous gases (NO, N_2O, and N_2). The equations employed for these determinations were previously described by Rodriguez-Sanchez et al. [34]:

$$Qin\ TNin = Qout\ TNout + Nous\ gas$$

$$Qin\ (NH4+-Nin + NOx-Nin) = Qout\ (NH4+-Nout + NOx-Nout) + Nous\ gas$$

3. Results and Discussion

3.1. Granulation Processes and Bioreactor Start-Up

After the aerobic granular bioreactors were inoculated with the four selected denitrifying strains of *Pseudomonas* containing the *nos*Z gene, the granulation process was studied for the three strains between operational days 5 and 40 (**Figure 2**). *Pseudomonas denitrificans* strain Z1, instead of forming granules, produced floccular structures with a filamentous and irregular surface. *Pseudomonas denitrificans* was able to form a granular morphology but the granules were fluffy and irregular, with a rough surface. For this reason, given the low density and lack of compactness of the granules, this strain was excluded from further experiments.

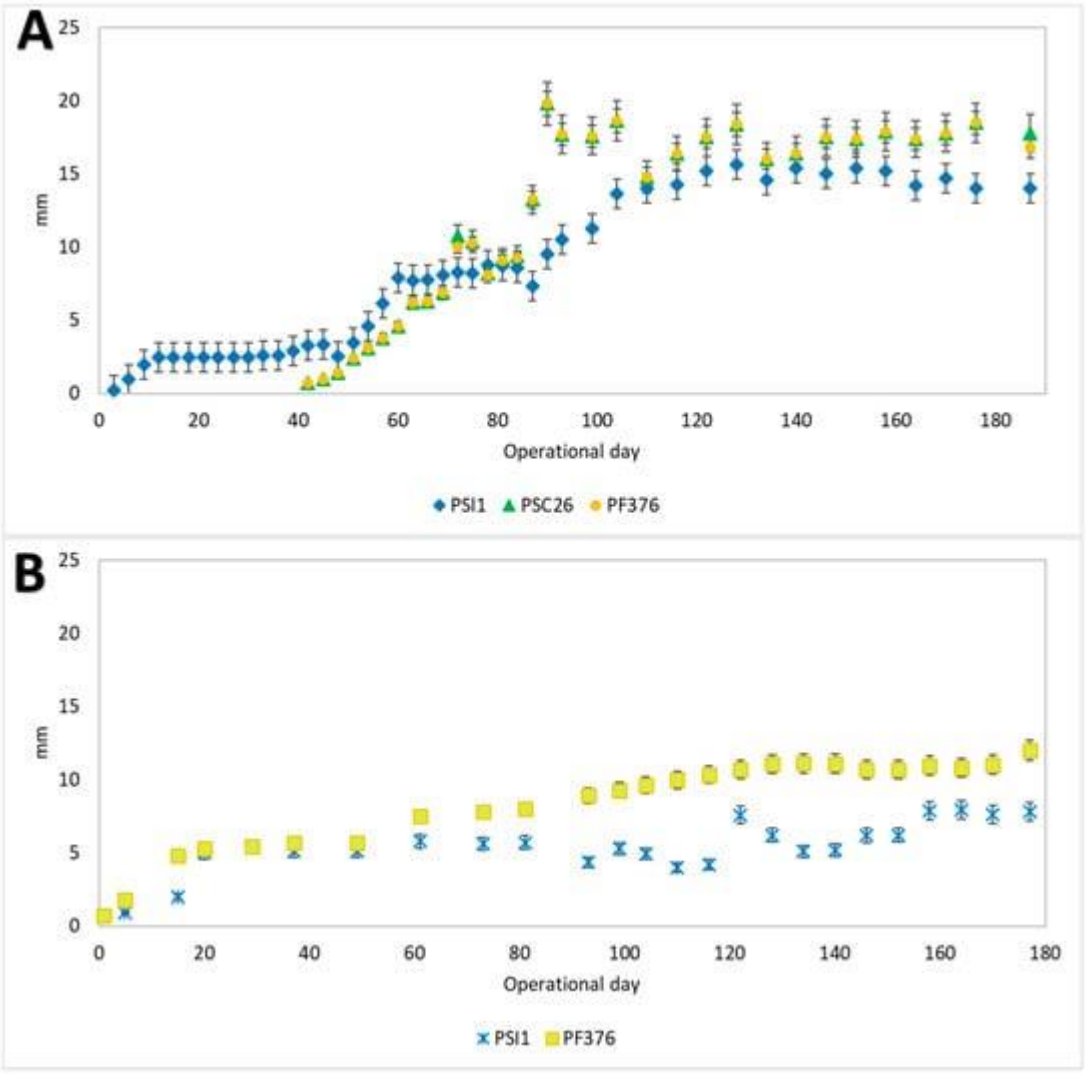

Figure 2. Mean size of granular biomass of *Pseudomonas fluorescens* PSC26 (PSC26), *Pseudomonas fluorescens* 376 (PF376), and *Pseudomonas stutzeri* I1 (PSI1) fed with sodium acetate (A) and methanol (B).

Unlike the P. *denitrificans* strain, the other strains tested in this study produced regular granules **(Figure 2**). *Pseudomonas stutzeri* I1 was the first strain to produce a core during granule formation (produced after five days of operation), with an average size of 2 mm. The granules formed were stable during 40 days of operation, showing the excellent granulation capacity of this bacterial strain under the operational

conditions applied in the experiment. After day 40 of operation, the mature granules formed by *P. stutzeri* I1 were highly adapted to the system, which was observed by the increase in the mean size over operational time, despite the reduction in carbon concentration. The granules were dense and compact, presenting a regular surface, regardless of their average mean size (**Figure 3A**)

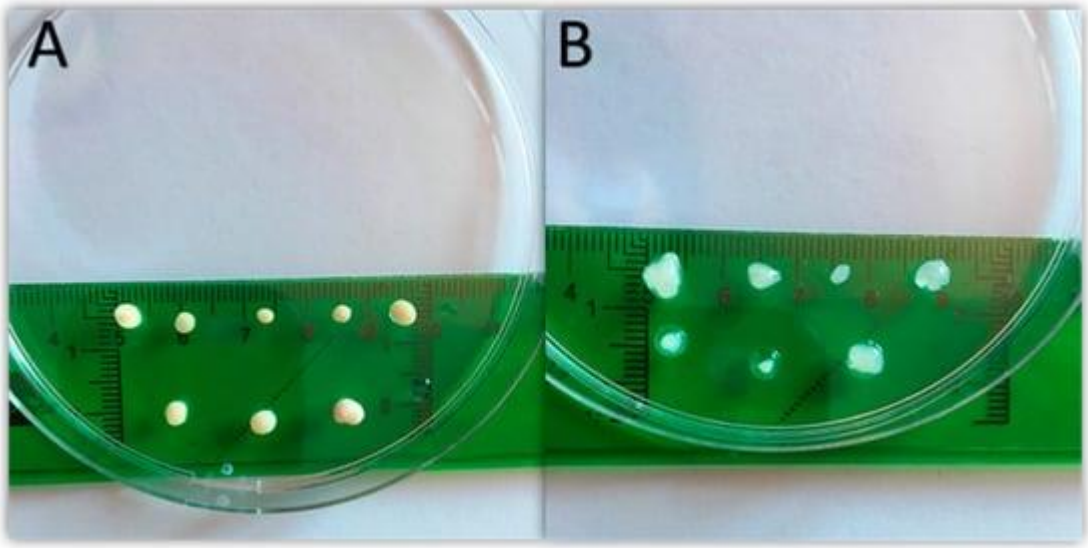

Figure 3. (A) Granules of *Pseudomonas stutzeri* at operational day 40; (B) granules of *Pseudomonas fluorescens* PSC26 at operational day 40.

Pseudomonas fluorescens strains PSC26 and 376 showed similar trends in terms of granular conformation and average size (**Figure 2**). The first granules were observed at operational day 40, (**Figure 3B**). Once the cores of the granules formed, the average diameter of the granule increased quickly, reaching 19 mm after 90 days in bioreactors inoculated with *P. fluorescens* strains. Notably, the trend was similar for all experiments under different concentrations of sodium acetate, and a mean size from 15 to 20 mm was observed at operational day 120. The granules presented a soft surface and a compact aspect throughout the experiment. A slightly higher density was

observed for granules produced by *P. fluorescens* strain 376 than those formed by the PSC26 strain.

In order to study the ability of the denitrifying strains to form granules in the presence of other carbon sources, two bioreactors were fed with synthetic groundwater supplemented with methanol as a carbon source. The bioreactors were either inoculated with *P. stutzeri* I1 or *P. fluorescences* 376, since these two strains showed the best capacity to remove nitrogen and produce stable granular biomass (**Figure 2**). The granulation results showed that both strains were able to produce granules that were very similar regardless of the carbon source available in the aerobic granular systems (**Figure 3**), although the results indicated that the range of granular size was smaller than that of the granules cultivated with sodium acetate. The results indicate that *P. fluorescens* 376 produces granules with larger diameters than the corresponding granules produced by *P. stutzeri* I1, with average sizes of 10 and 7 mm, respectively, after operational day 150 (**Figure 4**).

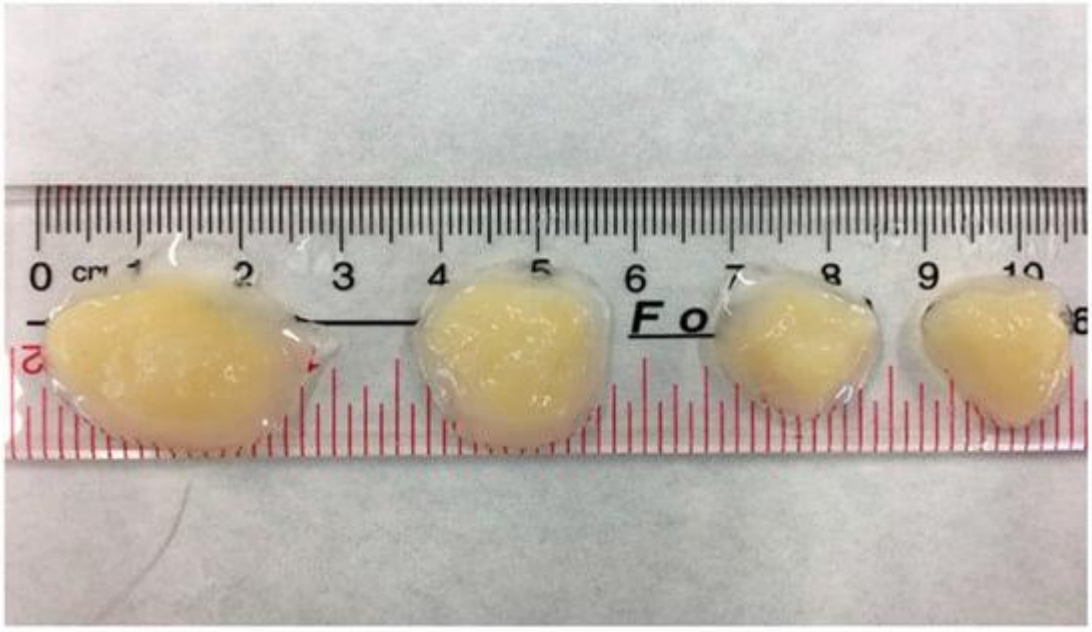

Figure 4. Mature granular biomass of *Pseudomonas fluorescens* 376 at operational day 150.

The settling velocity of the granules in the bioreactors fed with sodium acetate (**Figure 5A**) produced a decrease in decantation time, although after operational day 100, the average settling velocity of the granules did not change, regardless of the strain used. This rising trend was more pronounced for *P. stutzeri* I1 and *P. fluorescens* PSC26 than for *P. fluorescens* 376. The fastest production of granules occurred with *P. stutzeri* I1 inoculum, coinciding with the observation of the higher density and compactness of such granules. Similar settling velocities were detected when the bioreactor was inoculated with the two strains of *P. fluorescens* (PF376 and PSC26), but increased firmness conformation was observed when the bioreactor was inoculated with *P. fluorescens* 376 (**Figure 4**). *Pseudomonas stutzeri* I1 and *P. fluorescens* 376 were selected as the best strains for producing granules and for nitrate removal (Figure 5B). Once the best strains for the formation of granules and nitrate removal were determined, an alternative C source was used to feed the bioreactors (methanol). With the alternative carbon source, the opposite effect of using sodium acetate was observed, because the PSI1 inoculum produced granules with slower settling velocity (close to 25 $m \cdot h^{-1}$), and in the bioreactor inoculated with PF376, the granules produced presented velocities close to 60 $m \cdot h^{-1}$. This outcome could be caused by the strain's affinity and consumption advantage for the different organic carbon source [35]. These results suggest that, in general terms, the settling velocity for the granules is higher when sodium acetate is used as a carbon source compared with the same conditions but using methanol.

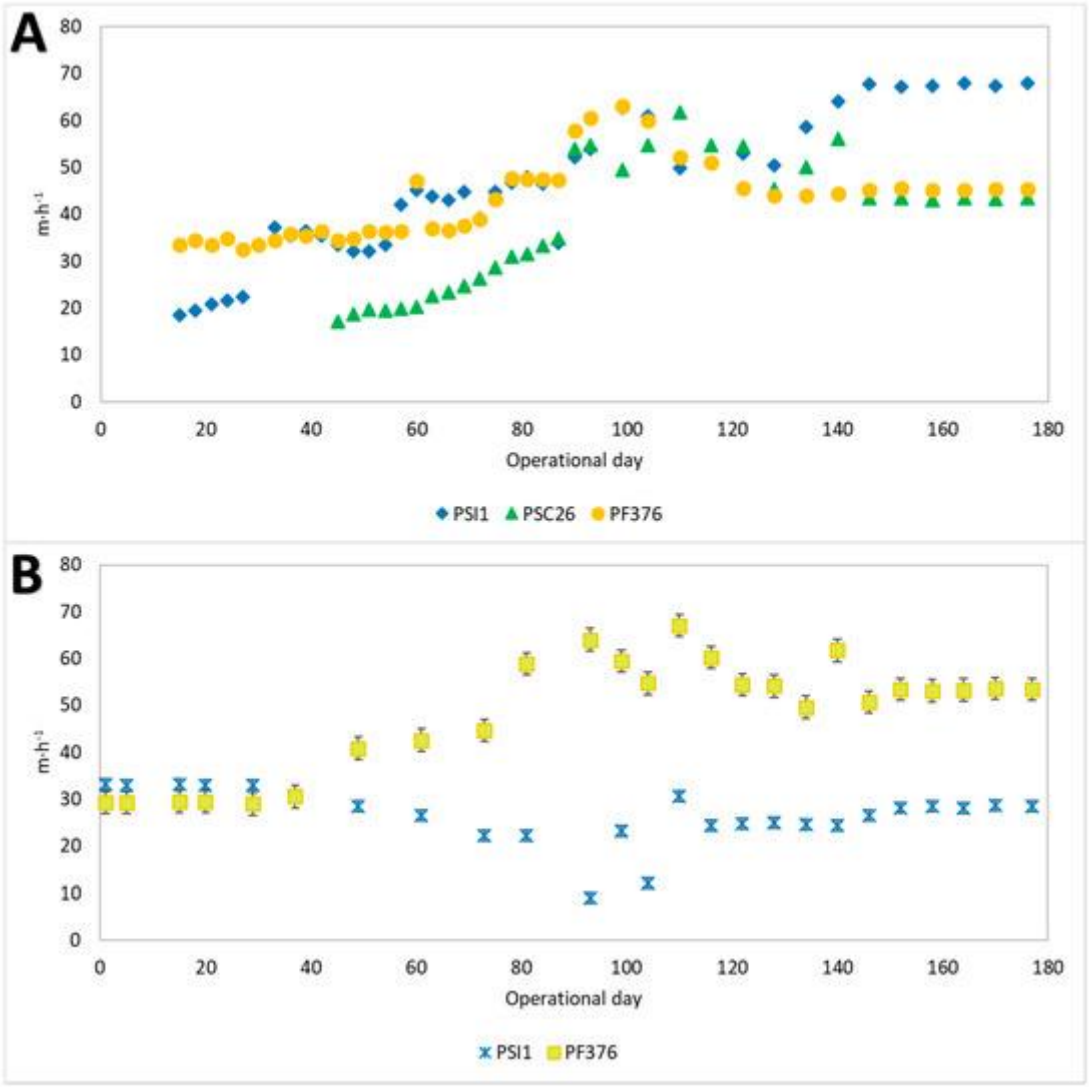

Figure 5. The settling velocity of granular biomass of *Pseudomonas fluorescens* PSC26 (PSC26), *Pseudomonas fluorescens* 376 (PF376), and *Pseudomonas stutzeri* I1 (PSI1) fed with sodium acetate (A) and methanol (B)

3.2. BOD_5 and COD Degradation

Organic matter removal was monitored based on the biological oxygen demand at day 5 (BOD_5). The BOD_5 removal ratio in the different bioreactors was strongly affected by changes attributed to the composition of the influent water. A sharp decrease

in BOD_5 was observed at operational day 35 for all the different conditions. However, at operational day 97, abrupt changes in the BOD_5 removal ratio were recorded (**Figure 6A**). In this respect, the most affected bioreactors were those inoculated with cultures of *P. stutzeri* strain and *P. fluorescens* strain 376.

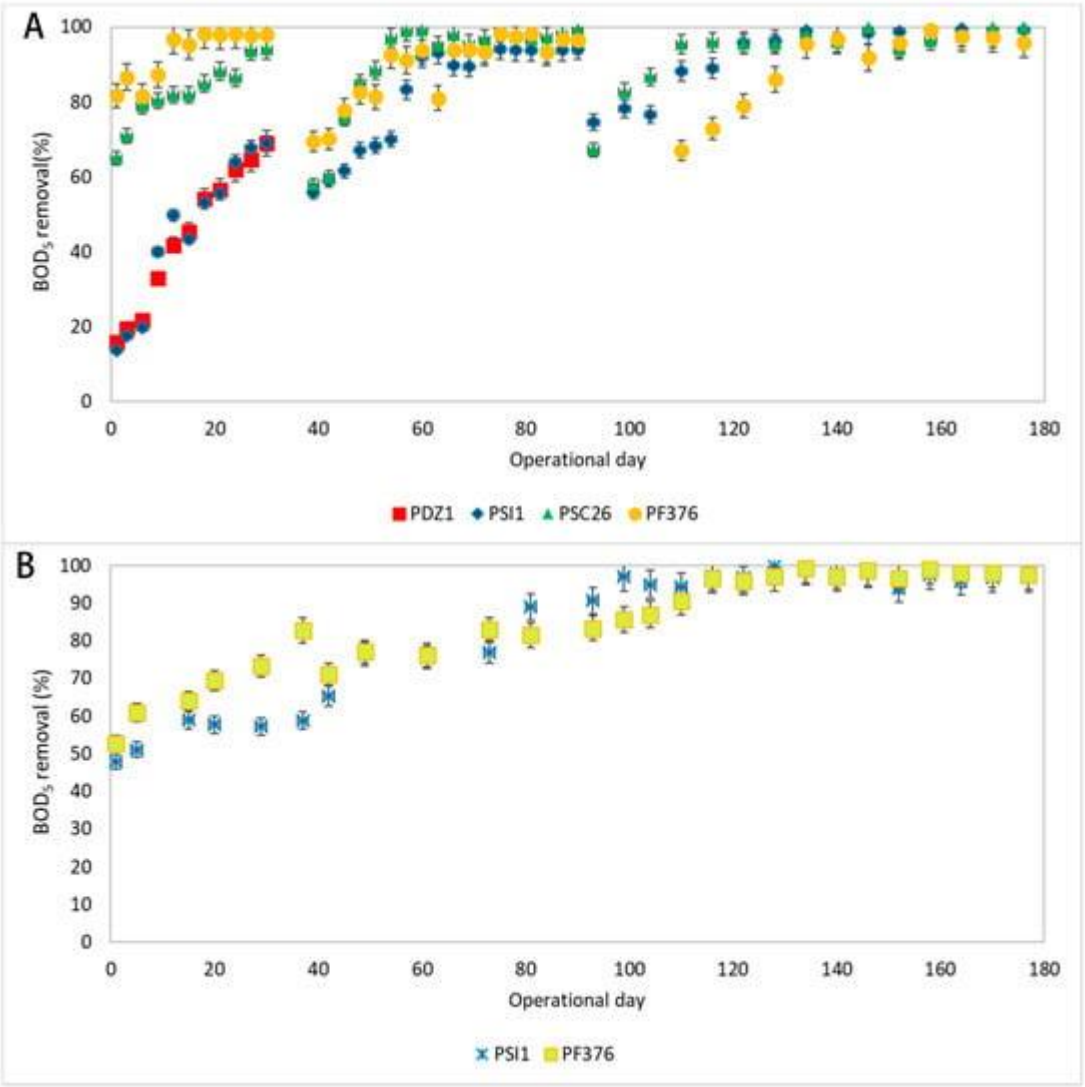

Figure 6. BOD_5 removal by granular biomass for *Pseudomonas fluorescens* PSC26 (PSC26), *Pseudomonas fluorescens* 376 (PF376), *Pseudomonas stutzeri* I1 (PSI1), and *Pseudomonas denitrificans* Z1 (PDZ1) fed with sodium acetate (A) and methanol (B)

When the GSBR systems were fed with methanol as a carbon source, the bioreactors inoculated with *P. stutzeri* and *P. fluorescens* 376 showed increased BOD_5 removal over the operational time (**Figure 6B**). These results suggest that methanol and acetate are excellent carbon sources for the formation of granules and nitrate removal. Regardless of the carbon source tested in the granular systems, the consumption of organic matter was practically 100% of the concentration added to the synthetic groundwater, suggesting that nitrate removal is limited by the concentration of organic matter added. Therefore, the data obtained confirm that after treatment, the generated effluents do not contain significant amounts of organic matter, which is important for their possible use for human consumption [36].

The removal efficiency based on COD followed a similar trend to the removal of BOD_5. Accordingly, changes in the COD responded to the carbon concentration in the influent (**Figure 7A**), with the highest COD removal observed in the bioreactor inoculated with *P. fluorescens* PSC26. This finding indicates that this microorganism needs a shorter period of adaptation to a new influent composition. The COD removal ratio observed in bioreactors inoculated with *P. denitrificans* increased quickly during the first days of operation, but this strain was discarded from the experiment as no granules formed in the presence of this microorganism. The COD removal observed when the systems were inoculated with *P. stutzeri* I1, *P. fluorescens* PSC26, and *P. fluorescens* followed a similar trend from operational day 60, with a removal ratio ranging from 75% to 98%. The highest removal ratios were reached at the end of operation, when the bioreactor operated under 200 $mg \cdot L^{-1}$ sodium acetate, because the available substrate for heterotrophic denitrifiers was reduced. However, the bioreactors fed with methanol showed an increased COD removal ratio during the first 40 days (**Figure 7B**). COD removal ratios higher than 90% were achieved at an earlier stage using methanol than when using sodium acetate with *P. stutzeri* I1 and *P. fluorescens* 376. Therefore, the average values in the steady-stable period ranged from 75% to 95% for reactors fed with sodium and up to 90% for reactors fed with methanol.

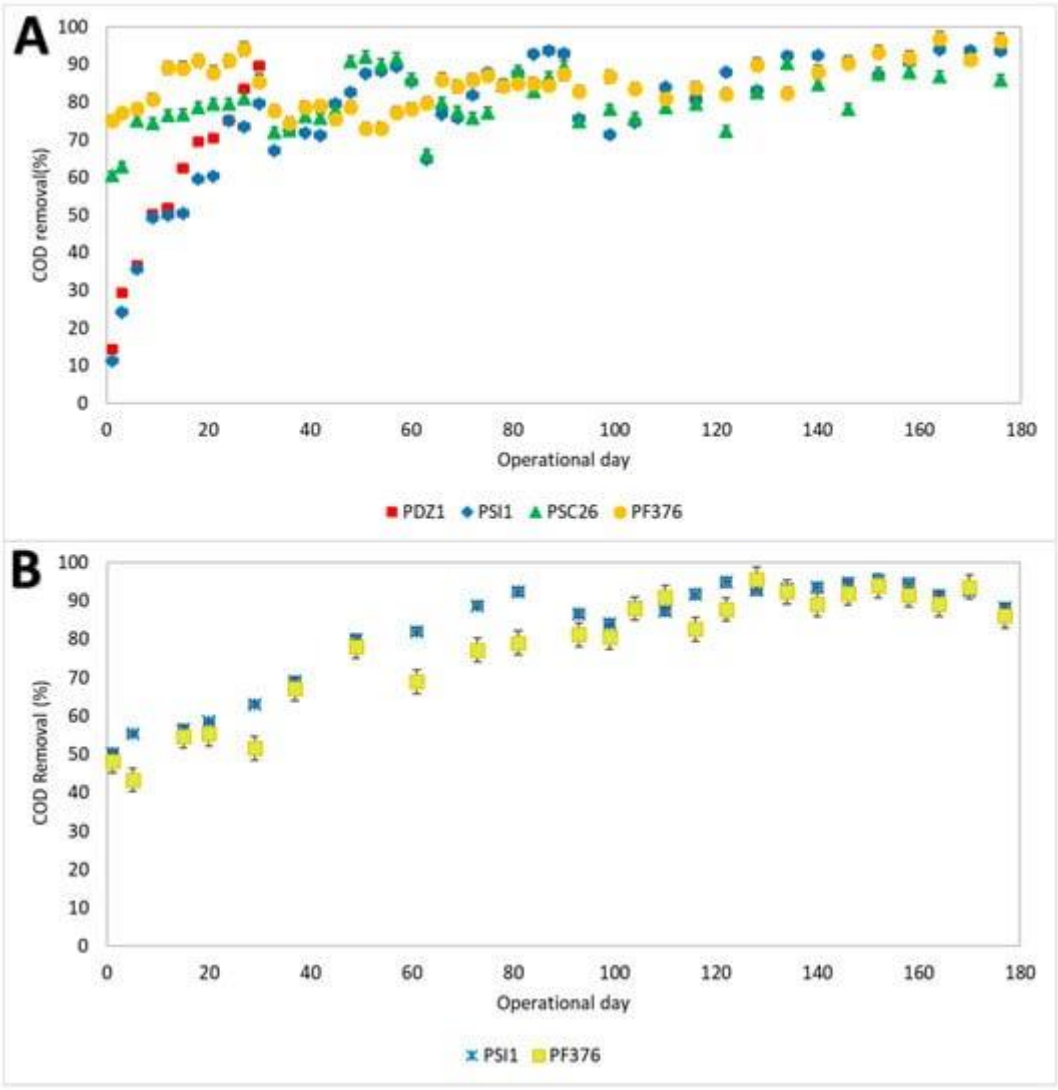

Figure 7. The COD by granular biomass for *Pseudomonas fluorescens* PSC26 (PSC26), *Pseudomonas fluorescens* 376 (PF376), *Pseudomonas stutzeri* I1 (PSI1), and *Pseudomonas denitrificans* Z1 (PDZ1) fed with sodium acetate (A) and methanol (B).

3.3. Nitrate Removal Performance

One of the main goals of this research was to assess the performance of the selected denitrifying strains in removing nitrate from groundwater within GSBR systems. The nitrogen removal capacity was studied to characterize the most efficient carbon source, and the C/N ratio was assessed to optimize the denitrification process in GSBR systems containing granular biomass produced by *Pseudomonas* strains. When

concentrations of sodium acetate below 400 $mg \cdot L^{-1}$ were used in the GSBRs, a remarkable reduction was observed in the system's capacity to remove nitrate from the groundwater. Nitrate removal was around 50% when 200 to 300 $mg \cdot L^{-1}$ sodium acetate was used, being even lower if the bioreactor was inoculated with *P. fluorescens*. It is therefore evident that a high C:N ratio is necessary for good nitrate removal performance when acetate is used as a carbon source (**Figure 8A**). These results confirm previous data published by Tian et al. [37], who observed that high nitrate removal capacity in biological systems was directly related to acetate concentration, indicating that a C:N ratio of approximately three to five was the most appropriate ratio to support the metabolism of heterotrophic denitrifiers [38]. Since the C:N ratio is crucial for nitrate removal from groundwater, the amount of carbon source required should be determined as a function of the level of nitrate pollution, as established by the European Water Framework [36]. Thus, the acetate concentration can be adjusted to account for the degree of groundwater contamination in the production of potable quality water. This biological system was successful in reducing the nitrate concentration from groundwater. For even higher nitrate concentration reduction, other methods based on physic-chemical processes have been proposed to reach removal values close to 90%, such as those reported by Eljamal et al. [39] and Mokete et al. [40] based on the use of nanoparticles.

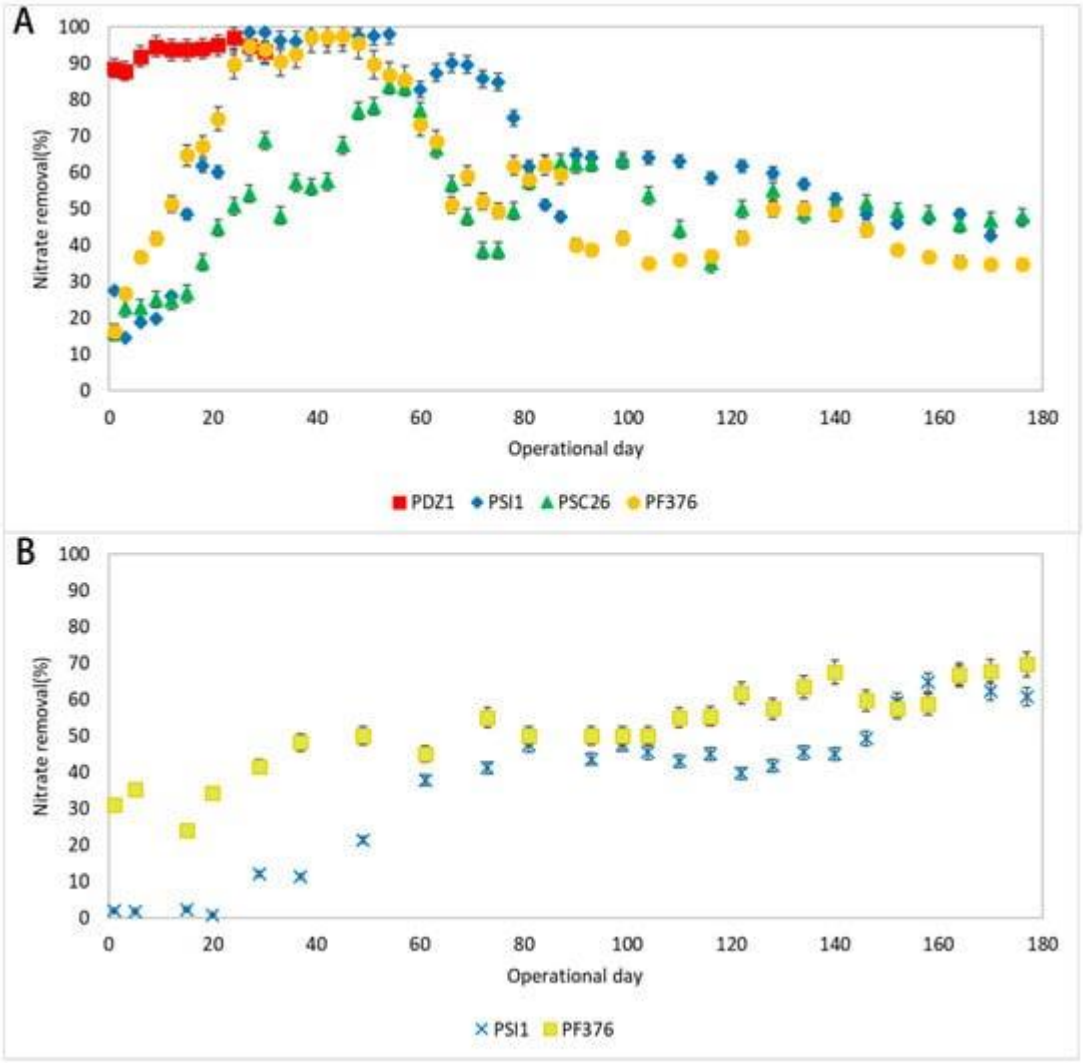

Figure 8. Nitrate removal ratio of granular biomass for *Pseudomonas fluorescens* PSC26 (PSC26), *Pseudomonas fluorescens* 376 (PF376), and *Pseudomonas stutzeri* I1 (PSI1) fed with sodium acetate (A) and methanol (B).

Some differences were observed when the GSBR systems were fed with methanol **(Figure 8B**). The addition of methanol at concentrations of 250 and 300 $mg \cdot L^{-1}$ resulted in efficient nitrate removal when the systems were inoculated with *P. stutzeri* and *P. fluorescens* strain 376, applying a C: N ratio close to two. In some cases, nitrate removal above 70% was observed, suggesting that denitrification occurs at lower

organic matter/nitrate ratios than when acetate provides the carbon source [41]. It is possible that these yields, as in the case of acetate, can be increased with the addition of larger amounts of methanol, but if we consider that the regulations established by the European Water Framework [36], in our opinion, it is clear that methanol is an appropriate carbon source for the removal of nitrates from groundwater. Moreover, *P. stutzeri* strains were reported as denitrifying microorganisms under aerobic conditions [42], supporting the results observed in this research.

3.4. Nitrogen Mass Balance over the Period of Operation

Based on our analysis of the nitrogen mass balance (**Table 2**), the bioreactor inoculated with *P. fluorescens* PSC26 produced the lowest conversion of nitrate to nitrogen gas, regardless of operation stage, with absolute amounts ranging from 29.00 to 66.00 mg-N·d^{-1}. In general, the most success was observed in the bioreactor with granules derived from *P. fluorescens* 376, followed by the bioreactor with granules derived from P. stutzeri I1. It follows that bioreactors inoculated with both strains separately presented a better transformation of nitrate to gas, and a C:N ratio close to 3–4, suggesting that this C:N ratio results in complete denitrification. This is supported by the findings and is corroborated by other authors working in wastewater treatment plants, such as Miyahara et al. [43] and Vacková et al. [44]. Those authors drew attention to the importance of aerobic denitrification in biotechnological processes in order to reduce the emission of greenhouse gases. However, lower concentrations of carbon in the influent resulted in the reduced production of gases in this study. Moreover, it is relevant to point out that the bioreactor operating with the same C:N ratio but using methanol as a carbon source promoted a more efficient reduction of nitrate than the bioreactor fed with acetate. Importantly, the generation of intermediate products from denitrification was not detected, as could be observed by the NO_2-N in the effluent.

Table 2.- Nitrogen mass balance expressed as mg-N L-1 during the different studies cases for *P. fluorescens* PSC26, *P.fluorescens* 376 and *P.stutzeri* (St dev*: standard deviation; rem perform: removal performance) *P.denitrificans* was not included due to did not achieve granulation

Strain		**Phase**	TNi_{in}	TN_{out}	NO_3-N_{in}	NO_3-Nout	NO_2-Nout	NO, N_2O, N_2 gas	Rem Perform (%)
P. fluorescens PSC26	NaAc:$NaNO_3$	900:127	130,75	88,32	130,75	88,27	0,05	42,44	32,45
		400:127	130,75	23,50	130,75	23,50	0,00	107,25	82,02
		300:100	102,95	53,28	102,95	53,25	0,03	49,67	48,25
		200:100	102,95	37,80	102,95	37,79	0,00	65,16	63,29
		Average	116,85	50,72	116,85	50,70	0,02	66,13	
		St dev.	16,05	27,85	16,05	27,83	0,02	29,00	
P. fluorescens 376	NaAc:$NaNO_3$	900:127	130,75	14,11	130,75	14,11	0,00	116,64	89,21
		400:127	130,75	20,31	130,75	20,31	0,00	110,44	84,46
		300:100	102,95	37,79	102,95	37,79	0,00	65,16	63,29
		200:100	102,95	44,50	102,95	44,50	0,00	58,45	56,78
	CH_3OH:$NaNO_3$	300:100	102,95	63,51	102,95	63,51	0,00	39,44	38,31
		200:100	102,95	35,59	102,95	35,59	0,00	67,36	65,43
		Average	112,22	35,97	112,22	35,97	0,00	76,25	

		St dev.	14,35	17,65	14,35	17,65	0,00	30,57	
P. stutzeri	$NaAc:NaNO_3$	900:127	130,75	4,39	130,75	4,34	0,05	126,36	96,64
		400:127	130,75	24,16	130,75	24,16	0,00	106,59	81,52
		300:100	102,95	49,74	102,95	49,73	0,00	53,22	51,69
		200:100	102,95	34,79	102,95	34,79	0,00	68,16	66,21
	$CH_3OH:_{NaNO3}$	300:100	102,95	34,79	102,95	34,79	0,00	68,16	66,21
		200:100	102,95	58,17	102,95	58,17	0,00	44,78	43,50
		Average	112,22	34,34	112,22	34,33	0,01	77,88	
		St dev.	13,10	17,35	13,10	17,36	0,02	29,06	

Although *P. denitrificans* Z1 was initially included in this study, the inability of this strain to form granules precluded its use as an inoculum in GSBR systems. Other strains of *P. denitrificans* could be tested in order to establish whether it is possible to use this denitrifying microorganism [29]. Our observations must be considered limited to the strains used in our study, although, based on nitrogen mass balance results, it appears that nitrate removal and the transformation of nitrate to dinitrogen gas could be easily modified by adjusting the concentration of the carbon source in the influent. Moreover, inoculation with denitrifying strains of *Pseudomonas* that are able to produce stable granules in GSBR systems can be considered an innovation in the treatment of groundwater contaminated with nitrate since the pollutant is removed and no nitrous oxide is released into the atmosphere as a result of this denitrification process. This process therefore represents an advance in the knowledge of biological denitrification systems driven by microorganisms, which encourages the complete reduction of nitrate to dinitrogen gas [45]. To the best of our knowledge, for the first time, this study describes the use of cultures of denitrifying strains containing the *nos*Z gene for the treatment of groundwater polluted with nitrates. Therefore, this study is important from a technical point of view, in addition to its environmental significance. A potential application of this technology is the addition of mature granules of *Pseudomonas* to AGS systems for treating polluted groundwater [1], with the aim of encouraging the presence of denitrifying bacteria carrying the *nos*Z gene.

4. Conclusions

The aerobic granular technology inoculated with denitrifying bacteria showed a success nitrate removal ratio, which depends on the carbon concentration added to the influent. The findings showed that denitrifying *Pseudomonas* strains are able to produce stable granules in GSBR systems. resulting in high nitrate removal. Moreover. the denitrification process can be efficiently performed with different carbon sources such as acetate and methanol. Therefore. inoculation and enrichment of GSBR systems with culture of selected denitrifying *Pseudomonas* strains carrying the *nos*Z gene may be considered as an innovative alternative in order to improve AGS technology. The results obtained about nitrate removal using identifying granular biomass provide deeper knowledge about the granular formation and denitrification process within granules in systems implemented for the treatment of polluted groundwater. Biological denitrification can be considered an environmentally friendly alternative since no greenhouse gases are produced during the denitrification process.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Chapter 2.: Biological nitrate removal from groundwater by an aerobic granular technology to supply drinking water at pilot-scale

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Abstract

Granular aerobic sludge systems have been a very efficient technology in urban and industrial wastewater treatment. In this research, a novel modification of aerobic granular sludge technology was developed for the treatment of nitrate-polluted groundwater, adding very low concentrations of a solution based on carbon and oligoelements in the groundwater to promote the growth of denitrifying microorganisms, avoiding expensive technologies to supply drinking water in small urban nuclei. The denitrification process was successfully reached at 0.15 g $C_2H_3NaO_2$ L^{-1}, meeting the Nitrate Directive of Europe for drinking water. The granular biomass was compact and dense with average values of mean size and settling velocity of 4.0 mm and 40 mh^{-1}, respectively. The prokaryotic and eukaryotic communities were studied by massive parallel sequencing techniques. The dominant prokaryotic phylotypes were related to influent composition, belonging to *Comamonadaceae, Rhizobiales*, *Acinetobacter* and *Pseudomonas*. The dominant eukaryotic phylotype was affiliated to *Haematococcus microalgae*. The diversity and evenness were high, regardless of influent composition. This study demonstrates support for the innovation of aerobic granular sludge technology application in terms of performance, operation, granular maturation and stability, as well as the role of denitrifying microorganisms to implement a low-cost, easy-to-use and maintain, environmental-friendly drinking water technology for rural populations.

Keywords: Groundwater; Biological treatment; Nitrate-polluted water; Drinking water; Aerobic granular denitrifying-sludge; Microbial community

1. Introduction

The growth of human settlements and economic activities has developed in the recharge areas for many aquifers, promoting the infiltration of water with different pollutants. Pollution in groundwater reservoirs is becoming progressively more dangerous with serious health and environmental problems worldwide. Water stress affects approximately two billion people across the globe, especially in arid and semiarid regions [1]. Groundwater is the main resource for drinking water in many countries and megacities. In Spain, groundwater resources supply 20 % of the total population demand for cities with more than 20,000 inhabitants; however, 70 % of urban supplies for towns with less than 20,000 inhabitants are supported by aquifers as their only freshwater source. In total, 35 % of the Spanish population drinks groundwater [2]. Between 70 and 80 % of the groundwater pumped in Spain is used for irrigation, similar to most developed countries in arid and semi-arid climates. In Spain, pumping satisfied 30 % of irrigated area; it reaches 20 % of the total volume of water for agriculture [3].

Nitrate pollution of groundwater originates in several ways, including point pollution (e.g. wastewater effluent and intensive farming) and diffuse contaminants (e.g. fertilizer and atmospheric deposition). However, nitrate pollution from agriculture applications (including the excess application of inorganic nitrogenous fertilizers and manures) had been considered the main cause of degradation to water quality in Europe [4].

The improvement of quality groundwater and reduction of water nitrate pollutant from agriculture source is ruled by the 91/676/EEC from the Council of the European Communities [5]. This Directive considers that values higher than 50 $mg\ L^{-1}$ of nitrate in freshwater strongly affect a water body; the recommended limit for drinking water is 25 $mg\ L^{-1}$ [6]. A higher nitrate concentration in drinking water supply can cause methemoglobinemia in children and cancer of the alimentary canal [7].

The groundwater treatment process had been developed to remove nitrate from aqueous solutions. Physical-chemical technologies such as reverse osmosis, electrodialysis, distillation, chemical reduction or ion exchange are efficient methods to remove nitrate from groundwater, but the high cost of operation and maintenance, low selectivity and production of secondary waste brine are disadvantages for the implementation of these technologies [8]. Other alternative methods for treating drinking water involve technologies based on the promotion of biological denitrification, either through heterotrophic or autotrophic microorganisms, which are some of the most extensively applied technologies for nitrate removal. Autotrophic denitrification is used through bioelectrochemical systems; elemental sulphur or pyrite are used as electron donors, which are carried out carbon-free. Unfortunately, the inorganic electron source needed for metabolism increases the operational cost; the diffusion of organic compounds and microbial products to the water are disadvantages [9]. Heterotrophic denitrifiers use bacteria metabolism in membrane bioreactors (MBRs) and biofilters, which are more economically and environmentally sustainable [9,10], because the fixed film maximises the surface area available for the development of biofilm on support media; however, the costs are based on biomass recirculation, and a high hydraulic retention time is required [11].

Because of these problems, the development of a new technology based on heterotrophic denitrifying microbial metabolism in granular biomass could be a promising technology for drinking water supply. In this way, the aerobic granular sludge technology for treating drinking water is based on the growth of denitrifying microorganisms in a three-dimensional aggregate matrix. Hydrodynamic shear force by continuous circulation stimulates the granular layer, allows for the mass transfer of oxygen and substrates, and provides places for aerobic, anoxic and anaerobic niches within granules. The continuous and circular is essential to get dense and compact granules, more than other parameters such as dissolved oxygen or design of reactor [12]. During the last decade, aerobic granular sludge technology had been a promising process for urban and industrial wastewater treatment because of the ability to remove carbon, nutrients and hazardous contaminant in only one chamber, with low foot-print and environmentally sustainable [13,14]. The granular sludge is an aggregation of

eukaryotic and prokaryotic microorganisms embedded in a three-dimensional matrix attached by extracellular polymeric substances, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs [[12], [13], [14]]. The employment of this technology for nitrate-polluted groundwater could be promoted by a carbon source addition at low concentration due to the auto-management of ecosystem within granules. The denitrification process could carry out by filamentous microorganisms belonging to fungi [15] and denitrifying bacteria; one of the most common phylotypes that carry out denitrification metabolism is the Pseudomonas genus [16,17], but there are also little-known archaeal metabolisms capable of denitrification [18]. The production of extracellular polymeric substances is the result of the excretions from quorum-sensing metabolism. Extracellular polymeric substances have an essential role in the structure and aggregation of granules, providing a denser and more compact configuration, as well as promoting the dissolved oxygen and nutrients gradients from the external layers to the core of the granules. The nitrate is removed by heterotrophic denitrifiers in the external layer, while in the internal layer, anaerobic and autotrophic nitrogen-metabolisms could be promoted due to anaerobic conditions. The aerobic granular sludge has a high resistance to adverse conditions (e.g. changing influent composition and toxins), a higher biomass retention (i.e. avoiding recycling of biomass) and a lower hydraulic retention time [19]. The influent composition and the operational parameter play key roles in the selection of microbial populations that conform the biomass and, subsequently, the performance process [20].

In this study, the development and optimisation of a novel alternative for treating drinking water based on aerobic granular sludge technology was tested. The evaluation characterised the strategies for shorter start-up periods, optimised the protocol to apply the lowest concentration of carbon source needed to remove a nitrate concentration higher than $25\ mg\ L^{-1}$ and determined the prokaryotic and eukaryotic microorganisms involved in the denitrification process and granular stability.

2. Materials and methods

2.1. Study area and groundwater characterization

The municipality of Torre-Cardela is located in the north-central area of Granada province (population: 760 inhabitants; Andalusia, Spain). The water supply service pumps water from the groundwater body of Calcarenitas de Torre-Cardela. This aquifer consists of calcarenites, sandy limestone, bioclast sandstone and marls, which are affected by faulting and bending. The resulting aquifer is fissured-karstic. The annual resource of this aquifer is 4.1 hm^3, as is described in Guadalquivir Hydrological Plan [21].

The water used for human consumption in Torre-Cardela is taken exclusively from the aquifer. The main pumping points are 'Pedrin' and 'Doña Marina', which are drilled wells of 170 m and 90 m deep, respectively.

To assess the hydrochemical characteristics and temporal fluctuation of groundwater, different water points were sampled throughout a year covering all the groundwater body (**Table 1**). In the main abstraction well during the monitoring period, the nitrate concentration ranged between 23 and 84 mg L^{-1}. The oscillations were a consequence of the combination of crop fertilisation cycles and rainfall events, with the characteristic flow of a fissured-karstic aquifer, which allows the excess nitrogen in the soil to percolate to the water table.

Table 1. Summary of hydrochemical data of the samplings carried out in the main abstraction well for drinking water supply.

[mg L^{-1}]	minimum	maximum	Average
Sulphates	31	60	43
Nitrates	23	84	47
Chloride	15	16	15
Bicarbonates	239	359	322
Carbonates	0	1.5	0.5
Calcium	81	117	94
Sodium	8.9	12	11
Potassium	0.4	1.3	0.9
Magnesium	11	33	28
Electrical conductivity (µS/cm)	628	699	650
Hardness	534	534	534
pH	7.4	7.9	7.5
Nitrites	0.03	0.04	0.03
Phosphates	Not detected		

2.2. Design of bioreactor, start-up strategy and operational conditions

This bioreactor was designed as a cylindrical sequential batch reactor (SBR), with a height of 3.52 m and a diameter of 0.49 m (**Figure S1**). The reactor was inoculated with 6 L of mature granules previously formed at lab-scale in the Water Research Institute (University of Granada), whose operational parameter and influent characteristics are shown in the **(Table S1)** and (**Table S2)**, respectively. The airflow was controlled and introduced in the reactor by a fine bubble from the bottom. The total volume of the reactor was 660 L, and it was built with two exchange out-flows at 50 % and 60 % of total volume. The cycles were operated for 3 h each; therefore, the hydraulic retention time was 6 h. The cycle consisted of 170 min of aeration, 3 min of decantation, 4 min of effluent discharge and 3 min of filling. During the first 15 days of operation, the settling time was modified according to the granule needs: 10 min for the first 3 days, 7 min during the next week, and 5 min in the following 5 days. The system operated at an environmental temperature ranging from 0 °C to 32 °C (summer, autumn and winter seasons) for 235 days. The temperature during the start-up (Stage A) was ranging daily from 27 to 32 °C (summer). Then, from operational day 38–159 (Stage B) the daily and seasonally temperature varied from 28 to 12 °C. From operational day 160–219 (Stage C and Stage D) the temperature was maximum 11 °C and minimum 0 °C. In the last stage of operation (Stage E), the temperature was ranging from 13 to 6 °C.

The reactor was operated using raw groundwater from the aquifer of Torre-Cardela (Granada, Spain). A supplementary composition was added to the raw groundwater using a peristaltic pump feed to the top of the bioreactor. Some oligoelements, ammonium (at the beginning) and carbon sources are essential to carry out the needs for microbial metabolisms during the denitrification process. The carbon source used for the bioreactor was sodium acetate since it has provided the best results in previous studies at lab-scale [22]. The ammonium chloride was added to improve the initial growth and stability of granules during the start-up stage, because previous lab-scale experiments corroborated that the start-up of aerobic granular sludge for nitrate

biodegradation using both nitrogen sources (ammonium and nitrate) were shorter, promoting denser and more regular surface of and also encouraging the compactness of granular biomass in order to achieve the diversification of anaerobic-anoxic niches in the interlayer and in the granules core where denitrification process takes place. In this sense, the ammonium concentration added was being reduced during operational time until this source was disabled at operational day 173.The details of the supplementary compounds are described in (**Table 2)**. The main goal was to find the lowest sodium acetate and mineral compounds concentration to reduce the cost of treatment and to obtain successful results under several ranges of carbon-nitrogen ratio (C: N) in the influent.

Table 2. Supplementary chemical compounds composition (g L^{-1}) added to raw groundwater.

Stage	Operational Day	$C_2H_3NaO_2$	NH_4Cl	$MgSO_4 \cdot 7H_2O$	K_2HPO_4	KH_2PO_4
A	0 to 37	0.4	0.250	0.100	0.085	0.030
B	38–159	0.2	0.098	0.025	0.021	0.008
C	160 to 172	0.2	0.024	0.018	0.015	0.005
D	173 to 219	0.2	0	0.018	0.015	0.005
E	220 to 234	0.15	0	0.010	0.009	0.003

The chemical oxygen demand (COD) concentration in the influent were 430 mg O_2 L^{-1} (Stage A), 180 mg O_2 L^{-1} (Stage B), 160 mg O_2 L^{-1} (Stage C and Stage D) and 130 mg O_2 L^{-1} (Stage E).

The concentration of ammonium (NH_4-N) in the Stage A was 825 mg NH_4−N L^{-1}, in the Stage B was 3234 mg NH_4-N L^{-1}, and finally in the Stage C the concentration

was 0. 792 mg NH_4-N L^{-1}. In the stage D and E any ammonium chloride was added (**Table 2**). The stages details are shown in the (**Table 2.)**

The C:N ratios were varying related to the nitrate concentration in the polluted groundwater, as well as, the changes done in the supplementary composition (generated by the decreased ammonium concentration and it absence). Thus, the C:N ratio at which the bioreactor operated is shown in (**Figure S2**).

2.3. Physical-chemical determination

Influent and effluent samples were taken periodically and transported at 4 °C to the laboratory. Chemical oxygen demand (COD) and biological oxygen demand (BOD_5) were measured following the protocols described by APHA [23]. Ion chromatography was performed in triplicate for the determination of inorganic nitrogen compounds (NO_3^-, NO_2^-, NH_4^+) using a Metrohm Ion Chromatograph. Mixed liquor suspended solids (MLSS) were measured weekly in triplicate to estimate the biomass concentration according to standard methods APHA [23]. The pH and dissolved oxygen were monitored using probes at 7.9 ± 0.3 and 6.7 ± 1.2 mg O_2 L^{-1}, respectively.

The granular biomass properties were characterised by mean size and settling velocity. The mean size was measured following the protocol described by Muñoz-Palazon et al. [13]. The settling ability was measured by the settling velocity in a 2-m column according to Gonzalez-Martinez et al. [24]

2.4. Sampling collection and nucleic acid extraction

The biomass samples were collected from the top of the bioreactor during the aeration period over operational time [13,14]. 300 mL of samples were taken by duplicate samples at operational Day 0, 38, 160, 173 and 220.

The samples were transported and transferred quickly to the laboratory at 4 °C. The samples were submerged in saline solution 0.9 % NaCl and centrifuged at 4500 rpm

for 15 min at room temperature. The supernatants were discarded and the pellet was kept at -20 °C.

The DNA pellets were extracted using the FastDNA SPIN Kit for Soil MP Biomedical following the protocol given by the manufacturers. The DNA pool extracts were kept at -20 °C and sent to Lopez-Neyra Laboratory (Granada, Spain) for subsequent Illumina MiSeq high throughput sequencing.

The pair of primers Bac357 (CCTACGGGAGGCAGCAG) – Bac806 (GGACTACHVGGGTWTCTAAT) was used for the amplification of the 16S rRNA prokaryotic gene [20] and the pair of EUK1391 (GTACACACCGCCCGTC) – EUKbr (ETGATCCTTCTGCAGGTTCACCTAC) was selected to amplify a region of eukaryotic 18S rRNA gene [20].

2.5. Absolute quantification of target genes involved in nitrogen cycle by qPCR assays.

The number of copies of bacterial 16S rRNA gene, *amo*A gene, *nor*B gene and *nos*Z gene were quantified using the nucleic acids using the extracted DNA by quantitative real-time PCR. The analysis was done using the QuantStudio™ 3 Real Time PCR Systems and the data was calculated used the own software QS3 (Applied Biosystems, ThermoFisher Scientific. The reaction mixture contained 0.125 µL of SYBER Green PCR, 0.125 µL of Taq Polymerase, 0.5 µL of dNTPs, 0.15 µL of forward and reverse primers at 10 µM, 1.5 µL of MgCl2, 2.5 µL of Buffer, 0.0625 µL of BSA, 17.88 µL of miliQ water and 2 µL of DNA template (1:20 diluted) [13]. The primers and annealing conditions for studied target genes was reported by Muñoz-Palazon et al. [13]. qPCR calibration curved were constructed with the aid of plasmid standards harboring inserts of the targeted genes. All calibration curves had a correlation coefficient $r2 > 0.95$ within the linear range of standard curve of $r2>0.990$.

2.6. Bioinformatic pipeline

Raw sequencing data from 16S rRNA Prokarya and 18S Eukarya rRNA genes were processed separately using Mothur software under the same conditions [25]. First, all paired-end reads were merged into contigs avoiding any ambiguous bases in the overlap region due to the differences between the paired-end reads [26]. Then, a quality trimming was done to remove any instance of ambiguous bases or more than eight homopolymers. The samples were aligned against full SiLVA database using Needleman conditions; subsequently, the sequences that start and end in the right position of forward and reverse primer positions were selected, which eliminated those that failed in these positions. The reads were then checked by searching and removing chimerical sequences using VSEARCH [27] through self-reference. The remaining contigs were taxonomically affiliated using the database of choice by the K-nearest-neighbour method and searching algorithm using a k-mer size of 8. The cluster of the contigs were calculated using the abundance-based greedy clustering method [28] and a cut-off for clustering into OTUs of 5% for 18S rRNA gene and 3% for 16S rRNA gene sequences. The sequences represented with more than 30.00 % in all samples or more than 5.00 % in at least one sample of each OTUs were not taxonomically affiliated to Eukarya phylotypes using SiLVA_nr and BLASTed against NCBI database.

The raw data from the massive parallel sequencing has been uploaded to Mendeley Data under the accession 10.17632/jvcsrbmv4v.1.

2.7. Ecological analysis of samples

To determine the differences of the most represented prokaryote and eukaryote phylotypes, heat maps of dominant OTUs were generated to account for all consensus phylotypes with at least >1% relative abundance in at least one of the biological samples.

The α-diversity was calculated using Past v3.14 software [29], the diversity indices of Chao-1, Shannon-Wiener, Simpson, Berger-Parker and Pielou's evenness

were computed with 95 % confidence range for 1000 bootstrap replications. The Morisita-Horn and Symmetric indices were used for the estimation of β-diversity among pairs of samples; these indices have been reported as the most robust to capture the diversity [30]. Both indices were calculated using the packages fossil, vegetarian and vegan, implemented in R-Project software.

The structure of OTUs was analyzed to observe which OTUs contributed more to dissimilarity among samples. This was calculated by SIMPER analysis, which was computed based on Bray-Curtis distance using PASTv3 software. The samples were clustered in 8 different groups, attending to influent carbon concentration (high, medium and low) and the presence or absence of ammonium in influent.

The reads of OTU table sequence samples were used for the calculation of the phylogenetic tree of biological samples. The phylogenetic tree was built by hierarchical clustering, under 9999 bootstrap replications following the Bray-Curtis model and using PAST software v3.4.

2.8. Compositional statistics

The reads generated through the bioinformatics procedure were taken for the similarity analysis, applying the principles of compositional statistics [31].

The principal component analysis was calculated by the values of OTUs zero-correction and centred log-ratio transformation of the OTU tables by the generation of 128 Monte-Carlo Dirichlet simulations. Transformed OTU tables were used for singular value decomposition calculation, which was represented through principal components analysis plot.

Differential abundance analysis was used to determine the differential OTUs in the microbial community, operated with or without ammonium and under different carbon concentration. Thus, the singular value decomposition was conducted by zero-correction and centred log-ratio transformation of OTU tables by generation of 128

Monte Carlo Dirichlet distributions. The expected effect size was calculated using ALDEx2 package implemented in R-project software [31].

2.9. Multivariate redundancy analysis

Multivariate redundancy analyses (RDA) were done to observe the relationship of prokaryotic and eukaryotic OTUs, biological samples, physical-chemical parameters and operational parameters for the start-up. The calculation of the RDAs was made by a 499 unconstrained Monte-Carlo simulation and run using the software CANOCO 4.5 for Windows. For calculation of linkages, the OTU maps and physical-chemical parameters were normalized under the logarithm model.

3. Results and discussion

3.1. Physical-chemical determination and start-up of granular sludge

At the start-up, the denitrifying granular sludge full-scale reactor was inoculated with 6 L of mature granular sludge from pilot-scale reactors. The biomass growth in the reactor was promoted by the addition of sodium acetate to encourage the proliferation of heterotrophic bacteria. The experimentation was based on the search for the minimum concentration of supplementary mineral medium added to groundwater in order to reach less than 25 mg L^{-1} of nitrate concentration in the effluent; this limit was established as the recommendable value for drinking water by European Nitrate Directive [6]. During the start-up period, the performance ratio of nitrate removal was lower, ranging from 20 % to 45 %, possibly due to the microbial adaptation process. However, the nitrate removal ratio from operational Day 114 (Stage B) was higher than 50 % (**Fig. 1**).

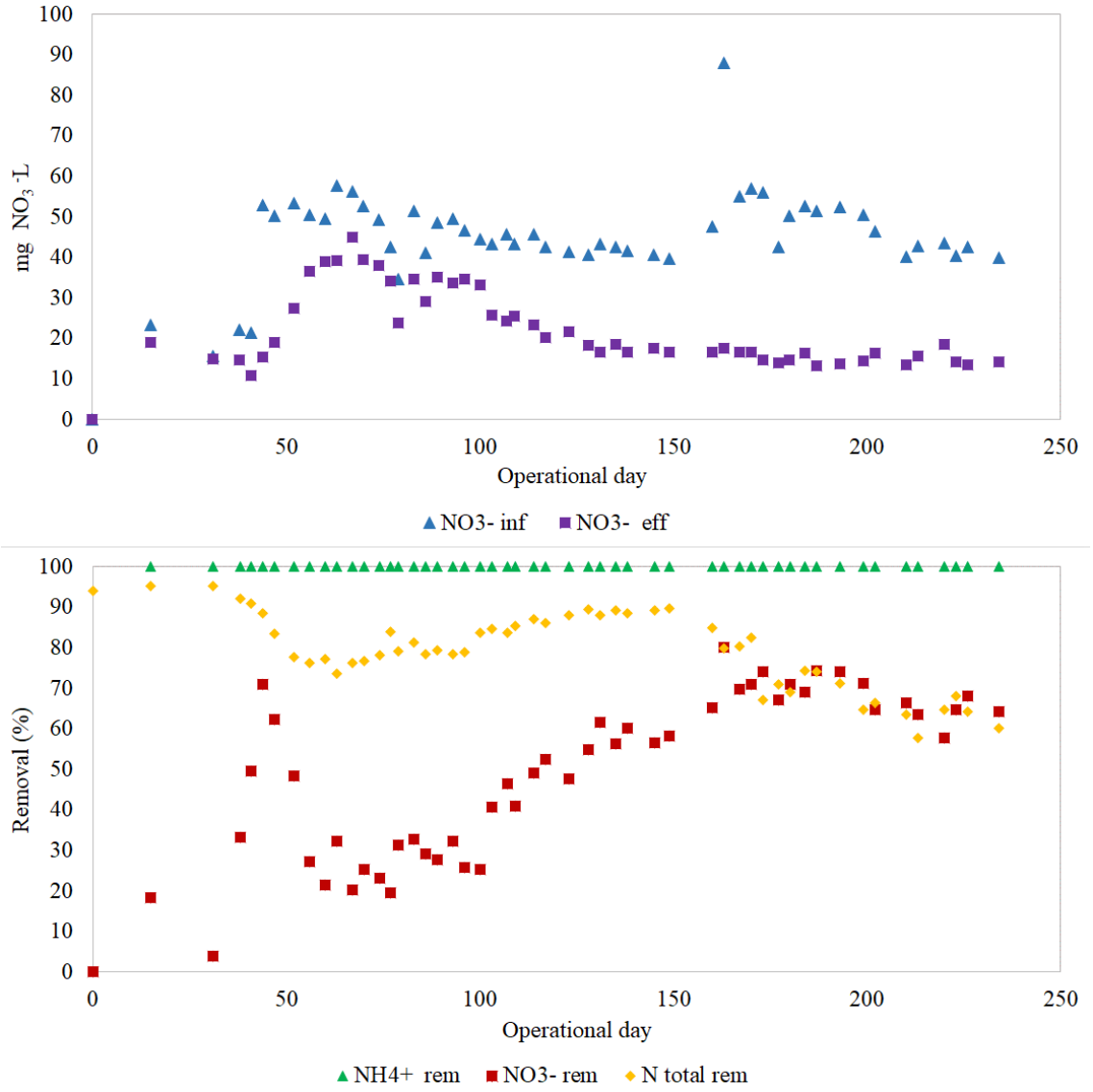


Fig. 1. Nitrate concentration (mg L^{-1}) in the influent and effluent (top); ammonium, nitrate and total nitrogen removal ratio (bottom). Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234).

During the stage C (until operational day 170), the groundwater treated in the bioreactor was supplemented with small amounts of ammonium (8–84 mg NH_4^+ L^{-1}), which was completely removed during the biological treatment. Thus, after groundwater treatment, the amount of ammonium in the effluent was undetectable. Aerobic granular

sludge has been reported to remove ammonium with high efficiency [13,14,24] so the presence of a low concentration of ammonium (8–84 mg NH_4^+ L^{-1}) could be removed easily by the granular biomass.

The nitrate concentration in the influent ranged from 15 to 80 mg L^{-1}. The nitrate origin was coming exclusively from groundwater. The nitrate in the effluent did not exceed 50 mg L^{-1}, which the European framework considers safe for drinking water, although during the first operational days (50–100), the values varied from 30 to 40 mg L^{-1}. These results could be related to lower performance during the adaptation and stabilisation of the granular biomass. From operational Day 100, the nitrate concentration in the effluent was lower than 25 mg L^{-1} (**Fig. 1**). Under stable conditions in the stage D (after 170 days of operation), the granular bioreactor very efficiently removed the nitrate concentration in the groundwater without supplementary addition of ammonia, suggesting that once the system was adapted to the strict denitrification condition, it was able to remove nitrates very efficiently from groundwater. Therefore, all stages reached stable values of nitrate concentration that were lower than limits for drinking water established by European Framework Water Commission; steady-stable values achieved the recommendable values (lower than 25 mg NO_3^- L^{-1}).

Organic matter was added to the influent groundwater as an essential compound to maintain heterotrophic denitrification; thus COD and BOD_5 were measured (**Figure S2**). The COD was stable throughout the operation, with removal values higher than 80 % during the experiment. The BOD_5 removal ratio achieved average values of 95 % during the exploitation period. Therefore, it can be considered that all sodium acetate was consumed by microorganisms while carrying out the denitrification process. These results indicated that the system efficiently removes nitrate with very low organic matter concentration (as sodium acetate) reaching C: N ratio close to 1:1.

The granular properties were monitored in the different stages of operation (**Fig. 2**). The granules used as inoculum had a mean size of 5 mm and settling velocity 30 m h^{-1}. The granular mean size suffered strong changes related to influent composition; during the first 47 days, the size ranged from 3 to 5 mm. This could be

explained by changes in operational parameters such as hydraulic stress and circulation. After the reduction of ammonium and sodium acetate concentrations in the influent, the mean size decreased from 7 mm to 3 mm. From operational Day 128, the granular biomass increased the size until it obtained average values of 6 mm, which then remained constant until the end of the experiment.

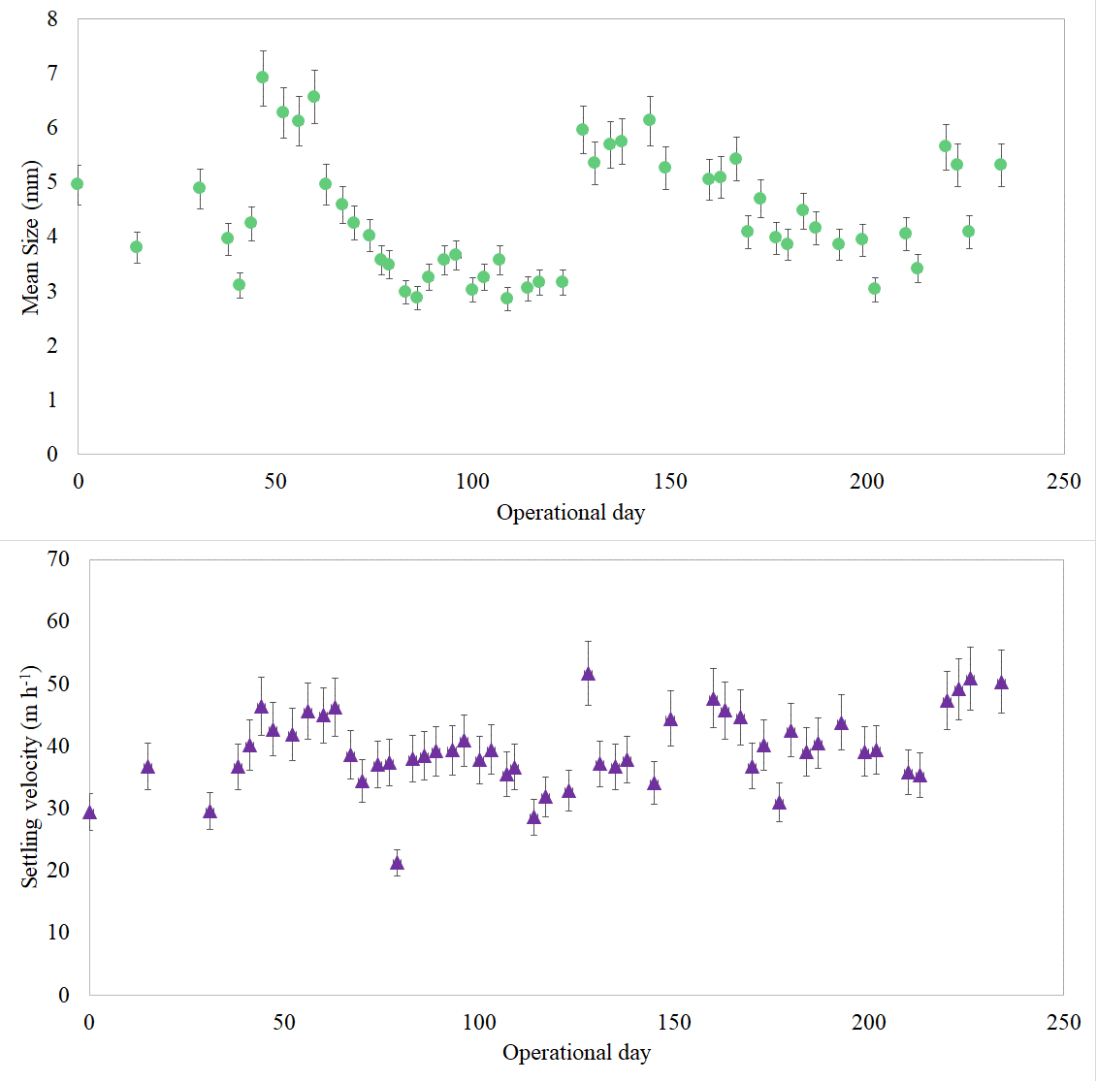


Fig. 2. Mean size (top) and settling velocity (bottom) of granular biomass during the start-up and operation period. Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234).

The settling velocity ranged from 30 to 40 m h^{-1} during the first 50 days of operation. After this period, a slight increase in the settling velocity was detected during the decrease of organic matter concentration; the settling velocity is related to the compactness and density of granules. The granules showed a more compact and stable granulation under feast-famine periods, which can explain these results. The periodic starvation had a strong effect on the surface charge and the granules became more hydrophobic [32]. From operational Day 100 until Day 250, the settling velocity was stable regardless influent composition, which ranged from 30 to 50 m h^{-1}; these results corroborate other aerobic granular sludge studies based on wastewater treatment [33].

The biomass concentration was measured by MLSS, the concentration showed an increasing trend throughout the experiment, regardless of the composition of the groundwater (**Figure S3**). The effect in the start-up stage was observed until operational Day 120, when the average values ranged from 500 to 1000 mg L^{-1}, but afterwards, the concentration increased due to the fast division of granules and the maturation of the systems. The highest values were found at the end of experimentation with approximately 2000 mg L^{-1}. It is possible that these values were low, which could be because the aerobic granular sludge system was designed for treating wastewater [33] rather than treating exclusively nitrate as the focus pollutant.

In order to obtain conclusions about the technology, it is relevant taken into account the influent characterization of the treated water, because related of the composition the optimal operational conditions and supplementary would be different. In the case the nitrate polluted groundwater between 50–100 mg NO_3^- L^{-1}, lower ratio C: N will conform smaller and denser granules, and the removal performance efficiency is high meet the drinking water European Water Framework.

In the case of higher concentration of nitrate polluted groundwater (> 120 mg NO_3^- L^{-1}), the C: N must to higher in order to get higher removal performance ratio, in these cases the size of the granules will be larger but the distance from external layer to core of the granules allow the existence of a strong gradient of oxygen and nutrients as occurs with low C: N. The optimal mode of operation will be totally related to the groundwater in-situ conditions, although slight guidelines could be given for instance:

C:N ratio ranging from minimum 1 to maximum 4, the hydraulic retention time could be shorter than 6 h depending of necessities, the up velocity of granules into bioreactor is very important to keep the density and conformation of them and finally the circular and continuous motion of the circulation of biomass is essential for long-term operation. In conclusion, the main advantages are the robustness and capacity to resist against diverse conditions and changes in the groundwater polluted composition (even in industrial effluent with high nitrate concentration), while that the main limitation is stablish the optimum C:N ratio depending of the necessities required for effluent quality, in order to save cost of supplementary compounds.

3.2. Ecological diversity analysis of prokaryotic and eukaryotic community structure in the granular biomass over operational time

The α-diversity analysis of granular biomass samples is shown in (**Table 3)**. The indices selected to observe the species richness, diversity and evenness were Chao-1, Shannon-Wiener, Simpson, Equitability and Pielou's evenness. For *Prokarya*, the Chao-1 index showed that the species richness reached the highest value during the operation with 0.2 g CH_3COONa L^{-1} and the lowest concentration of ammonium; this result suggests that ammonia oxidisers phylotypes were displaced and denitrifying microorganisms were promoted within granules. In terms of Pielou's evenness and equitability, the lowest values were for the scenario under the absence of ammonium in the influent (Day 173), possibly due to the proliferation and competition of other phylotypes than ammonium oxidisers this was corroborated by the observation in the species richness at operational Day 160. The diversity was kept stable during all operation, corroborating the values of Simpson and Shannon indices.

Table 3. Alpha diversity of Prokarya and Eukarya samples; Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234).

	Day 0	**Day 38**	**Day 160**	**Day 173**	**Day 220**
	Prokarya				
Simpson	0.7379	0.7334	07,363	0.7229	0.7365
Shannon-Wiener	2.507	2.324	2.456	2.332	2.535
Pielou's Evenness	0.02633	0.02392	0.02561	0.02163	0.02712
Equitability	0.4081	0.3837	0.4012	0.3782	0.4127
Chao-1	546.5	505.2	593.3	580.4	531.1
	Eukarya				
Simpson	0.8523	0.8749	0.8868	0.8381	0.9141
Shannon-Wiener	2.732	2.678	2.64	2.572	2.937
Pielou's Evenness	0.1348	0.147	0.0966	0.07154	0.109
Equitability	0.5769	0.5827	0.5304	0.4937	0.57
Chao-1	127.1	115.7	180.9	207.8	188.1

The diversity of the eukaryotic community followed the same trend as the prokaryotic community. The changes in diversity and evenness occurred in the biological sample after the ammonium withdrawal in the influent and the drop of carbon concentration. In general, both diversity and evenness increased, with the highest values at operational Day 220 (**Table 3**).

Overall, the values of the Morisita-Horn indices for prokaryotic communities showed high similarity in dominant phylotypes among operational Days 0 and 38, while greater differences were found at Days 173 and 220 (**Fig. 3**). The dominant eukaryotic phylotypes were similar in the granules, although differences were observed at low ammonium concentration in the influent. However, the eukaryotic communities were not affected by the organic matter concentration. Despite this trend, the eukaryotic community showed more similarities in dominant phylotypes than the prokaryotic community for a pair of samples.

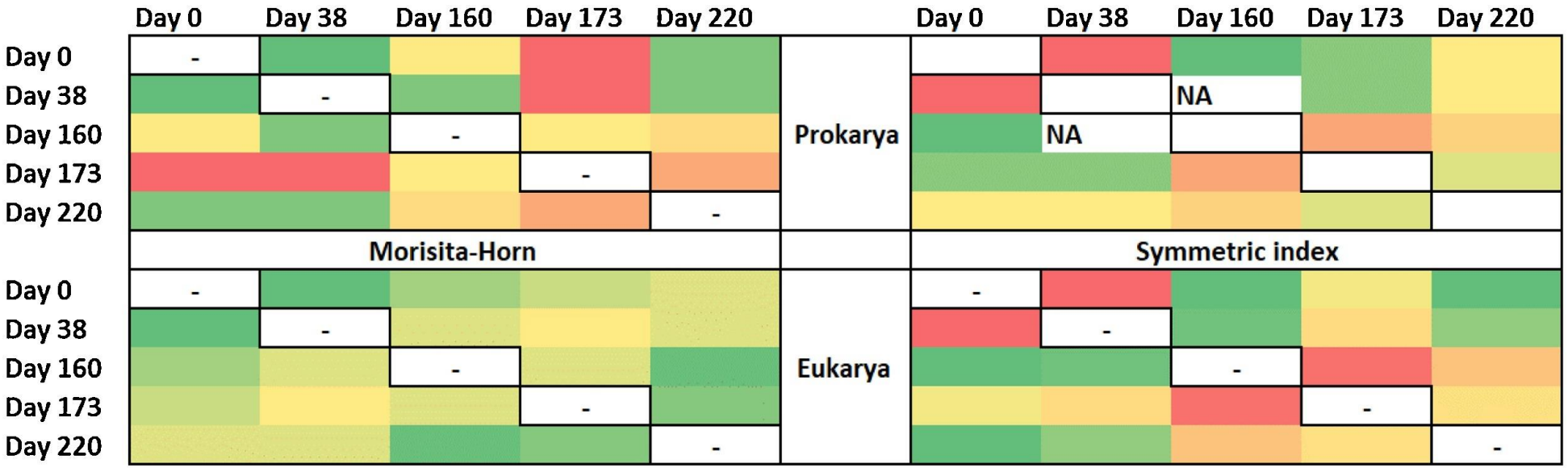


Fig. 3. β-diversity indices for pair of samples over operational time measured by Morisita-Horn (dominant phylotypes) and Symmetric index (rare phylotypes). Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234)

The symmetric index for both *Prokarya* and *Eukarya* sample pairs showed higher differences in rare phylotypes during the first stage and during the acclimatisation period without ammonium in the effluent.

3.3. qPCR of target bacterial 16S rRNA, *amo*A, *nor*B and *nos*Z genes

To quantify the target genes involved in the nitrogen cycle RT-PCR was conducted, which is shown in the (**Fig. 4)**. The number of bacterial 16S rRNA gene was kept stable during whole operation in the granular sludge with magnitude order ranging from 12 to 14. In this sense, this results suggest that once the denitrifying granular biomass is stable at lab scale the operation under real conditions, means nitrate-contaminated groundwater, the number of general bacteria is similar, because it was not registered any negative affection by changes in operational conditions and environment.

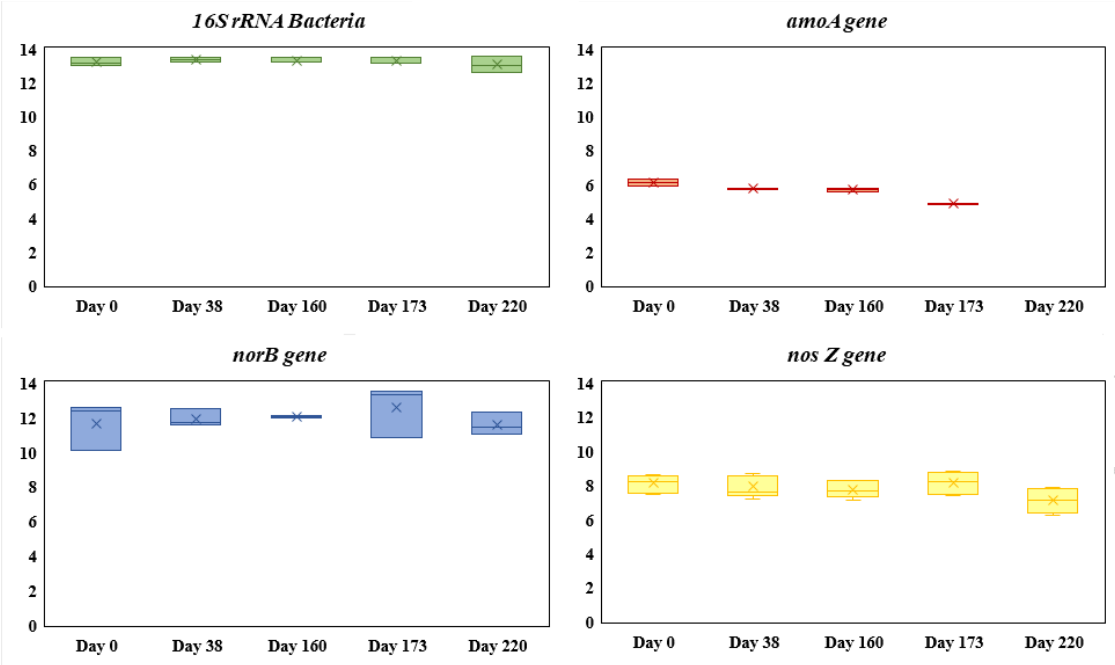


Fig. 4. Absolute quantification of nucleic acid of target genes of bacteria domain involved in the nitrogen cycle during the start-up and operation Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234).

The *amo*A gene was analysed to quantify the ammonia-oxidizing bacteria (AOB) during the experiment. This gene was evaluated because during the start-up of denitrifying bioreactor at real scale, the groundwater was enriched with ammonium chloride to promote the granular stability, conformation and density. In this sense, the number of ammonia-oxidizing bacteria was decreasing during the operation from magnitude order of 6 until 4 at operational day 173, however *amo*A gene was not detected in samples from operational day 220, possibly caused by the lack of ammonium source, which is specific for the growth of these microorganisms, which disappeared due to a competitive disadvantage against other microorganisms involved in the nitrogen cycle (specifically nitrate depollution). These results suggest that the decreasing ammonium chloride concentration in the first stages of operation, ranging from 0.250 to 0.024 between operational day 0 and 160. At operational day 173, the bacterial *amo*A gene was lowest registered, possibly caused by the absence of ammonium in the influent.

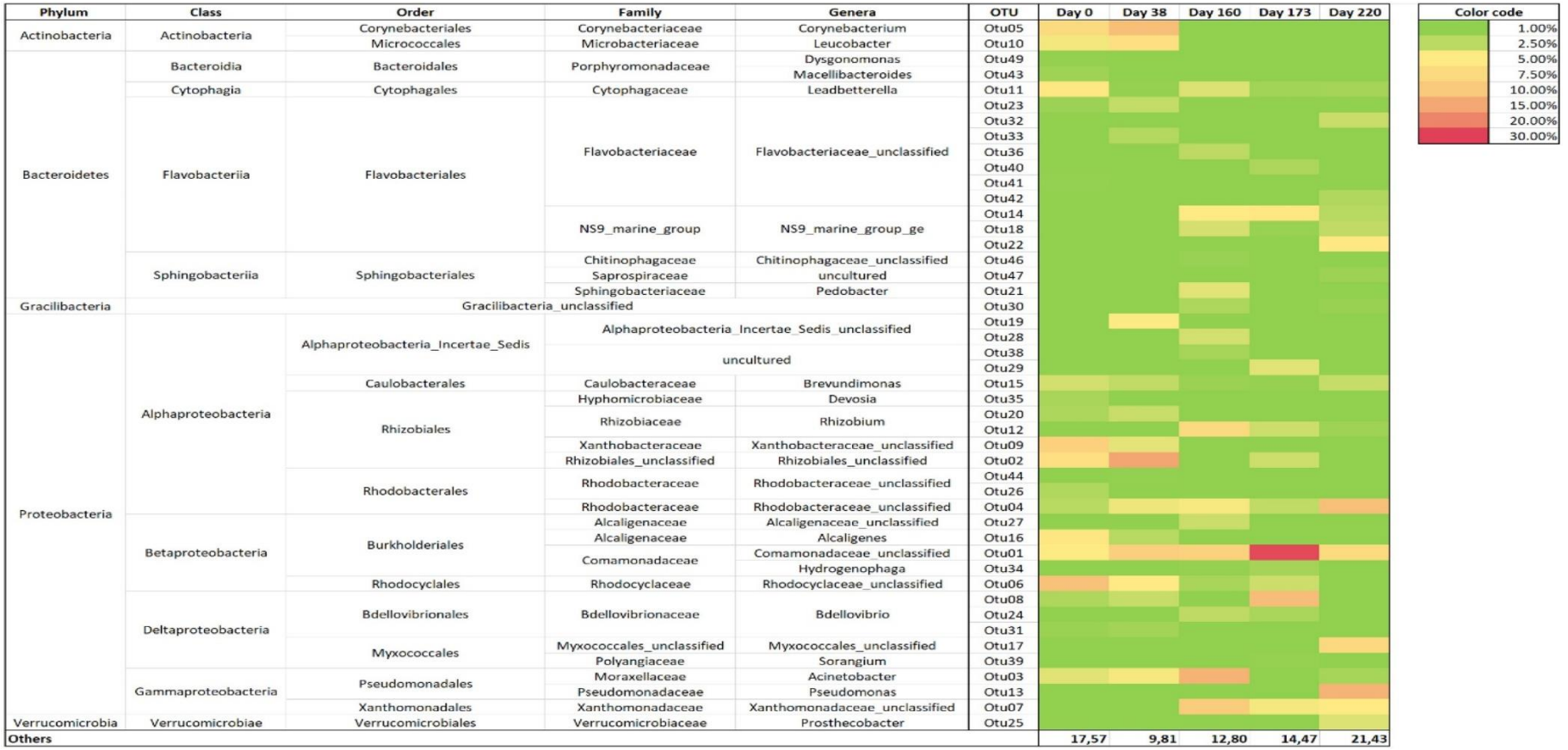
The NOx-reducer genes involved in the denitrification process studied were *nor*B and *nos*Z genes. In general terms, the number of *nor*B copies was 4 magnitude order higher than *nos*Z gene. Also, the abundance of *nor*B copies average was stable ranging from 11 to 14, which is in charge of the production of nitrous oxide. The number of *nos*Z gene did not show a clear pattern, because during the operation the copies value range from 7 to 9 regardless of nitrate concentration in the groundwater. The trend of denitrifying granular sludge for treating nitrate-polluted groundwater was opposite to the results obtained in several studies about granular biomass for treating wastewater [13,14]. These results allow confirming the nitrate removal by denitrification process carry out by denitrifying bacteria despite the presence of oxygen, because the granular biomass allows the coexistence of aerobic zone and anoxic-anaerobic zone in interlayer and in the core of the granules. In this mention position, the denitrification process take place in anaerobic conditions by denitrifying bacteria which containing *nor*B and *nos*Z genes, as it had been verified by the results obtained from absolute quantification. In addition, the results of qPCR corroborated the competitive advantage of denitrifiers microorganisms containing *nos*Z gene (nitrous oxide conversion manager into dinitrogen gas) in a denitrifying granular biomass biosystems

versus aerobic granular sludge systems for treating wastewater, which are clearly focus on ammonia oxidation.

3.4. Microbial community

3.4.1. Prokaryotic dynamics population

Forty-six OTUs of interest belonging to *Prokarya* were found among all massive parallel sequencing samples, with more than 1.00 % of relative abundance (Fig. 5). Each phylotype had different metabolic roles that allow them to proliferate in granular biomass for treating nitrate-contaminated groundwater. However, a high amount of these phylotypes were involved in the nitrogen cycle, such as the orders of *Rhizobiales, Rhodobacteriales, Burkholderiales, Rhodocyclales, Pseudomonadales* and *Xanthomonadales, among others.*



Phylum	Class	Order	Family	Genera	OTU	Day 0	Day 38	Day 160	Day 173	Day 220
Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium	Otu05					
		Micrococcales	Microbacteriaceae	Leucobacter	Otu10					
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas	Otu49					
				Macellibacteroides	Otu43					
	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	Otu11					
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacteriaceae_unclassified	Otu23					
					Otu32					
					Otu33					
					Otu36					
					Otu40					
					Otu41					
					Otu42					
			NS9_marine_group	NS9_marine_group_ge	Otu14					
					Otu18					
					Otu22					
	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Chitinophagaceae_unclassified	Otu46					
			Saprospiraceae	uncultured	Otu47					
			Sphingobacteriaceae	Pedobacter	Otu21					
Gracilibacteria	Gracilibacteria_unclassified				Otu30					
Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_Incertae_Sedis	Alphaproteobacteria_Incertae_Sedis_unclassified		Otu19					
					Otu28					
			uncultured		Otu38					
					Otu29					
		Caulobacterales	Caulobacteraceae	Brevundimonas	Otu15					
		Rhizobiales	Hyphomicrobiaceae	Devosia	Otu35					
			Rhizobiaceae	Rhizobium	Otu20					
					Otu12					
			Xanthobacteraceae	Xanthobacteraceae_unclassified	Otu09					
			Rhizobiales_unclassified	Rhizobiales_unclassified	Otu02					
		Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_unclassified	Otu44					
					Otu26					
			Rhodobacteraceae	Rhodobacteraceae_unclassified	Otu04					
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Alcaligenaceae_unclassified	Otu27					
			Alcaligenaceae	Alcaligenes	Otu16					
			Comamonadaceae	Comamonadaceae_unclassified	Otu01					
				Hydrogenophaga	Otu34					
		Rhodocyclales	Rhodocyclaceae	Rhodocyclaceae_unclassified	Otu06					
	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio	Otu08					
					Otu24					
					Otu31					
		Myxococcales	Myxococcales_unclassified	Myxococcales_unclassified	Otu17					
			Polyangiaceae	Sorangium	Otu39					
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	Otu03					
			Pseudomonadaceae	Pseudomonas	Otu13					
		Xanthomonadales	Xanthomonadaceae	Xanthomonadaceae_unclassified	Otu07					
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Prosthecobacter	Otu25					
Others						17,57	9,81	12,80	14,47	21,43

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Fig. 5. Heat Map >1% genera for the samples of prokaryotic communities during operational successive operational days.

The prokaryotic community in the granules used as inoculum was dominated by the genera *Corynebacterium*, *Leucobacter* and *Leadbetterella* and families Rhodocyclaceae and *Xanthobacteraceae*. The genus *Corynebacterium* has been reported as a ubiquitous phylotype in granular sludge reactors for treating wastewater [13,14]. This phylotype has been described in relation to an improvement of nitrogen removal in biological wastewater treatment, with a potential role to phosphate metabolism, which possess enzymatic mechanisms such as nitrate reductase and organic nitrogen hydrolase [13,14]. Also, *Leucobacter* genus has been found in biological granular sludge, which was described as highly hydrophobic and with negative superficial charges [34]. These characteristics are beneficial to compactness and density of granules due to the increased electrostatic attraction between surfaces; this allows a high association for the flocculation-granulation index and consequently improves the settling ability [24]. The families *Rhodocyclaceae* and *Xanthobacteraceae* are consumers of acetate in a system for treating nitrogen. More specifically, these families were found in combined partial-denitrification and anammox systems, where the nitrogen source was ammonia and nitrate [35]. Thus, these results suggest that both *Rhodocyclaceae* and *Xanthobacteraceae* families could be involved in process of partial-denitrification based on heterotrophic metabolisms [36]; at operational Day 0, the bioreactor was subjected to high sodium acetate and ammonia concentrations. The prokaryotic population at operational Day 38 was similar to the inoculum samples. Although, some variances were identified in the community such as the proliferation of *Comamonadaceae* family and *Rhizobiales* order; these phylotypes acquired more than 10.00 % of the relative abundance. Previous reports indicated that members of the family *Comamonadaceae* are capable of performing nitrification-denitrification [37], but they play a major role in denitrification processes in the presence of acetate as a carbon source, as was the start-up of denitrifying granular sludge reactor [38]. Moreover, *Comamonadaceae* has been identified in several microbial studies of granular sludge systems as dominant phylotype in mature granules [39,13,14]. The *Acinetobacter* genus was identified with 5.00 % of relative abundance; nitrogen removal

has been recognised as a role of this genus [40]. In addition, novel aerobic denitrification by heterotrophic Acinetobacter has been described [41].

The drop of ammonium concentration in the influent at Day 160 provoked changes in the prokaryotic community, as was observed by the displacement of dominant OTUs of previous stages. *Rhizobium*, NS9*_marine_group* and *Xanthomonadaceae* proliferated with relative abundances ranging from 5.00–10.00%. Moreover, *Comamonadaceae*, *Acinetobacter* and *Rhodobacteraceae* also increased their presence in the population.

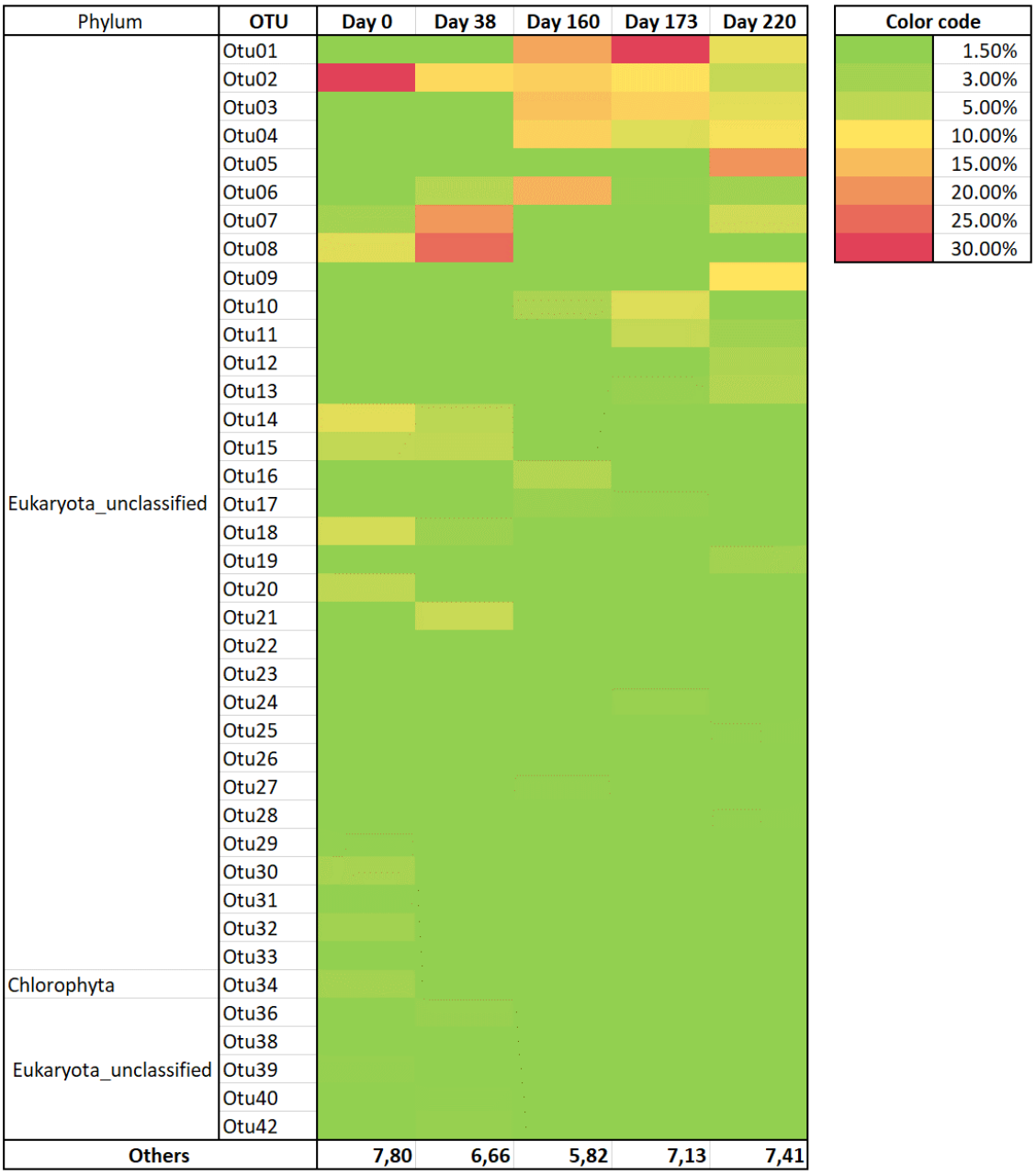
In the next stage, which had an absence of ammonium, *Comamonadaceae* family displaced several OTUs, acquiring great relevance in the system with approximately 30.00 % of relative abundance. In addition, genus *Bdellovibrio*, which has a possible role as a predator of pathogenic organisms. *Bdellovibrio* significantly reduces viability, decreases impact and alters the microbial composition in granules, suggesting that population changes could be encouraged by the proliferation of these phylotypes [42].

By the end of the operation under a low concentration of sodium acetate and the absence of ammonium in the influent, *Pseudomonas*, *Rhodobacteraceae* and *Myxococcales* increased their relative abundance in the community, although the diversity and evenness were very high. Biological nitrogen removal could be carried out by *Pseudomonas* genus, which converts nitrite and nitrate to N_2 gas, although, depending on the species, it could perform all steps or only some of them [43].

3.4.2. Eukaryotic community

The bioinformatic analysis using the full SiLVA database to match the eukaryotic community showed a lack a consistent taxonomic affiliation; exclusively one OTU was taxonomically classified as *Chlorophyta* (**Fig. 6**). However, this reveals the lack of knowledge about the diversity and ecological role of the Eukarya domain in biological water treatment. Muñoz-Palazón et al. [13,14] and Rodriguez-Sanchez et al. [20] used the pair of primers for Eukarya and obtained a high-level affiliation in

different wastewater technologies at the pilot-scale, but the affiliation of granular biomass treating nitrate-polluted groundwater was not successful. Thus, the representative sequence of dominant OTUs (with more than 30.00 % in all samples or more than 5.00 % in at least one samples) were BLASTed against NCBI database (Table 4). Otu01, Otu02 and Otu04 were BLASTed against the NCBI database and made a match with *Haematococcus lacustris*, a photosynthetic unicellular green microalga, known as a good producer of water-soluble sulphated polysaccharide; this function is being explored for the production and bioengineering of astaxanthin, a potent antioxidant used for industrial productive purposes [44]. These algae are cultivated in a photobioreactor for industrial applications [45]. The proliferation of *Haematococcus lacustris* could be promoted by the optimal influent and operational conditions, despite the low sunlight radiation, the bioreactor was located inside of a shed. Thus, the influent composition from groundwater and supplementary medium was optimal for algae growth.



Phylum	OTU	Day 0	Day 38	Day 160	Day 173	Day 220
	Otu01					
	Otu02					
	Otu03					
	Otu04					
	Otu05					
	Otu06					
	Otu07					
	Otu08					
	Otu09					
	Otu10					
	Otu11					
	Otu12					
	Otu13					
	Otu14					
	Otu15					
	Otu16					
Eukaryota_unclassified	Otu17					
	Otu18					
	Otu19					
	Otu20					
	Otu21					
	Otu22					
	Otu23					
	Otu24					
	Otu25					
	Otu26					
	Otu27					
	Otu28					
	Otu29					
	Otu30					
	Otu31					
	Otu32					
	Otu33					
Chlorophyta	Otu34					
	Otu36					
	Otu38					
Eukaryota_unclassified	Otu39					
	Otu40					
	Otu42					
Others		**7,80**	**6,66**	**5,82**	**7,13**	**7,41**

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Fig. 6. Heat Map >1.50 % for the samples of eukaryotic communities during operational successive operational days.

Table 4. BLAST of representative eukaryotic OTUs against the NCBI database.

OTU	Specie	Query Cover	Per. Ident	Accession
Otu01	*Haematococcus lacustris* 16S ribosomal RNA gene,	100 %	86.15	MF683078.1
Otu02	*Haematococcus lacustris* 16S ribosomal RNA gene	100 %	82.40	MF683078.1
Otu03	Fungal sp. isolate	100 %	90.13	MH5625410.1
Otu04	*Haematococcus lacustris* 16S ribosomal RNA gene,	100 %	84.38	MF683078.1
Otu05	*Haematococcus lacustris* 16S ribosomal RNA gene	100 %	84.16	MF683078.1
Otu06	*Trachelomonas* sp. Jilnal030207BT small subunit ribosomal RNA gene,	100 %	92.19	KT305105.1
Otu07	*Trachelomonas* sp. Jakeun052407A small subunit ribosomal RNA gene, p	100 %	87.23	KT305103.1
Otu08	*Massila* sp. 1MBG 16S rRNA gene, isolate 1MBG	100 %	90.13	AM286547.1
Otu10	Uncultured *Uromyces* sp. clone sw-xj69 16S ribosomal RNA gene,			

Also, during the operation, the proliferation of Otu03 was affiliated with a Fungal species, which increased its relative abundance to 12 % of the total relative abundance. The fungi play an essential role in granular biomass, as has been previously reported in several studies on the structural missions of union bridges and increase of surfaces to be colonised by bacteria [13,14].

Trachelomonas sp. was affiliated to Otu06 and Otu07; this is a unicellular microalgae with approximately 250 species that inhabit freshwater environments worldwide [46]. Therefore, the eukaryotic community in the aerobic granular biomass for treating nitrate-pollutant groundwater showed a widespread displacement of the fungal and metazoan microorganisms in water treatment, given by the proliferation of microalgae, even under very low sunlight radiation.

3.5. Analyses of similarity of biological samples

The contribution to the dissimilarity among different groups of samples for *Prokarya* and Eukarya OTUs is shown in (**Fig. 7)**. The SIMPER analysis performance for *Prokarya* showed that 33 OTUs contributed to dissimilarity with more than 1.50 % in at least one sample. The group of samples showed that the contribution correlated with ammonium and nitrate or exclusively nitrate as a nitrogen source; however, the samples were comparably related to high (0.4 g NaAc L^{-1}), medium (0.2 g NaAc L^{-1}) and low (0.1 g NaAc L^{-1}) sodium acetate concentrations.

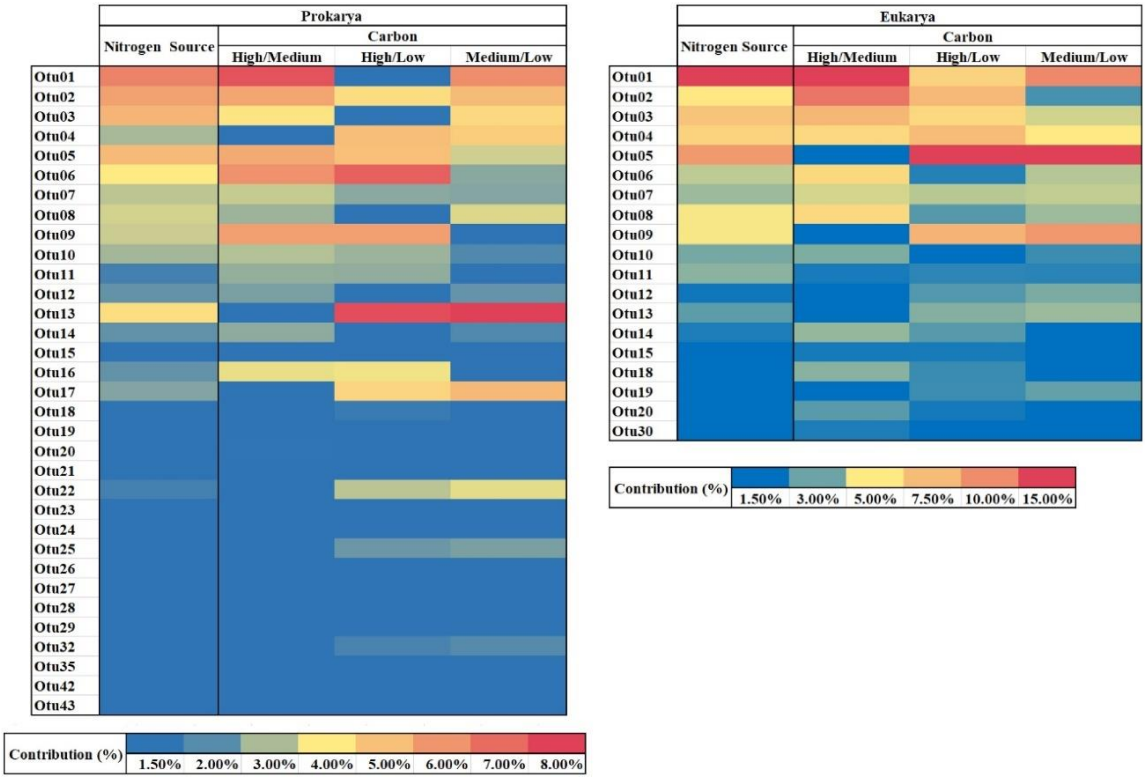

Fig. 7. Similarity percentage analysis of the each OTU contribution to dissimilarity between different conditions of operation.

Otu01, Otu02, Otu03, Otu05 and Otu13 taxonomically affiliated to *Comamonadaceae*, *Rhizobiales*, *Acinetobacter*, *Corynebacterium* and *Pseudomonas*, respectively, contributed to the dissimilarity between presence or absence of ammonium in the influent, with greater than 23.00 %. These results corroborated the prokaryotic structure community; for instance, the proliferation of *Pseudomonas* genus with only nitrate in the influent. Also, these phylotypes marked the greater differences in the prokaryotic community between different stages than with influent composition. Thus, nitrogen source, carbon concentration and the maturation of aerobic granular biomass were important for the community.

Also, there were not many OTUs that contributed to wide dissimilarities among carbon concentrations. However, the contribution of Otu01, Otu06 and Otu13 implied a high dissimilarity (>8.00 %); this representation was strongly marked in the dynamic populations.

The OTUs that showed differences between high and medium carbon concentration were Otu01, Otu02, Otu05 and Otu09, contributing more than 30.00 %. These results corroborate the community studies. In this way, all these OTUs except Otu09, had much higher representation in the reactor at the medium carbon concentration (0.2 g L^{-1}), while at high carbon concentration, the relative abundance of all dominant OTUs had high evenness. The same pattern followed the SIMPER analysis between the medium and low carbon concentration communities, showing a specialisation of the biomass within of bioreactor at the medium carbon concentration, which could due to the specialisation of phylotypes in mature granules. Otu013 also affiliated to *Pseudomonas*, which most contribute to dissimilarity.

The OTUs that contributed to the dissimilarity between the high and low carbon concentration were mainly Otu06 and Otu13. Otu13 affiliated to *Pseudomonas* exclusively proliferated at the low carbon concentration. This analysis allows hypothesising the role of *Pseudomonas* as a denitrifying bacteria in the presence of nitrate under a low carbon concentration (oligotrophic condition); thus it was the most well-known genera for carrying out the heterotrophic denitrification [16].

Eukarya members showed two clear patterns for the contribution to dissimilarity. First, Otu01, belonging to *Haematococcus lacustris*, contributed to both nitrogen source and high-medium carbon concentrations, this could be explained because of the proliferation of the green microalgae over operational time. At the beginning of the bioreactor operation, the abundance relative of Otu01 was ranged from 0.2 to 0.5 %. Moreover, Otu05 had higher importance for the differentiation of samples among nitrogen sources because the proliferation occurred when the ammonium was absent or in low concentration (8 mg NH_4^+ L^{-1}). Second, the contribution to the dissimilarity trend was found for high and medium concentrations, but not the low concentration of sodium acetate; the major contribution was from Otu05, affiliated to *Haematococcus lacustris*. Otu05 was promoted at the end of experimentation under a low sodium acetate concentration, the absence of ammonium and the maturation of granules in the system. This species could have competitive advantages over other phylotypes and could have even displaced Otu02.

The hierarchical clustering based on Bray Curtis similarity for *Prokarya* and *Eukarya* samples showed that for both domains, the samples at Day 0 and Day 38 were similar, while another cluster was observed for Days 160, 173 and 220 (Figure S4).

3.6. Analysis of differential abundance of OTUs and correlation of OTUs

The results of the expected effect size for the OTUs of *Prokarya* and *Eukarya* followed the same trend. The number of OTUs that were significantly related to the carbon concentration was higher than the number of OTUs that were significantly related to the nitrogen source (**Figure S5**). These results suggest that the microbial communities were more affected by the sodium acetate concentration, and although the presence of ammonium promoted significantly different OTUs, the effect was not as great in the communities. The expected effect size analysis demonstrated significant differences in the relative abundance of certain microorganisms that were favoured by influent composition in the granular maturation process.

For *Prokarya*, the singular decomposition value in principal component analysis (PCA) plot showed three clusters for operational Day 0 to Day 38 and Day 160 to Day 173; the largest distance was in Day 220 against the rest of samples (**Figure S6**). These results suggest that the *Prokarya* community was greatly modified by the low carbon concentration. Day 160 and Day 173 maintained a short distance, encouraging the hypothesis that the low ammonium concentration or absence of ammonium did not create great changes in the communities, and resulted in the displacement of few phylotypes. The singular decomposition value for the eukaryotic community showed the same trend (**Figure S5**). Therefore, the most important factor affecting the microbial community was the carbon concentration, as was corroborated by the contribution to dissimilarities by SIMPER analyses results.

3.7. Linkage of the microbial community, physical-chemical performance and operational conditions

The RDA supported the results of physical-chemical determinations with the microbial community during start-up and stable condition of the aerobic granular system (**Fig. 8**). For Prokarya, the sodium acetate concentration, nitrate removal and BOD_5 removal were strongly and positively correlated with Otu02, belonging to the *Rhizobiales* order. *Rhizobiales* is a phenotypically-diverse taxonomic group, represented by aerobic and heterotrophic phylotypes involved in the nitrogen cycle in soils and aquatic environments [47]. Also, the COD removal ratio and the influent ammonium concentration were correlated with start-up samples (Days 0–38); these stages had the highest nutrient supplementations. Also, strong correlations with ammonium were found with Otu05, Otu09, Otu10 and Otu16, taxonomically affiliated with *Corynebacterium*, *Xanthobacteraceae*, *Leucobacter* and *Alcaligenes*. All these phylotypes had been found previously in aerobic granular biomass systems, reported as granular forming bacteria [13,19,49]. *Alcaligenes* and *Corynebacterium* had also been reported as heterotrophic nitrifiers bacteria [48], which could play a role in ammonia oxidation. The mean size, settling velocity and MLSS were negatively correlated with the parameters previously described. This trend demonstrates that the granules matured

during the granular process, regardless of supplemented carbon or ammonium concentration. The mean size of granules was correlated to Otu13 and Otu47, affiliated to *Pseudomonas* and *Saprospiraceae*, as well as with Day 220 of bioreactor operation. *Pseudomonas* is an effective heterotrophic-denitrifying bacteria, which has been used in several denitrification technologies such as biofilters and microbial fuel cells [49,50]. The MLSS was linked with Otu18 and Otu30, a NS9*_marine_group* and *Gracilibacteria*, respectively, which possess unusual and diverse metabolisms [51]. Settling velocity was correlated with Otu07 affiliated with *Xanthomonadaceae*. The phylotypes of this family produce N-acyl-homoserine-lactone, a component of extracellular polymeric substances that possesses a relevant role in quorum-sensing activity to lead the microbial aggregation [52]. These characteristics could encourage the density and compactness of the granular structure, which provides faster settling velocity [53]. There was also a cluster composed of a many phylotypes including dominant OTUs such as Otu01, Otu12 and Otu14, belonging to *Comamonadaceae*, *Rhizobium* and NS9*_marine_group*, respectively, that was positively linked with operational Days 160 and 173; these OTUs were very abundant during these stages of operation.

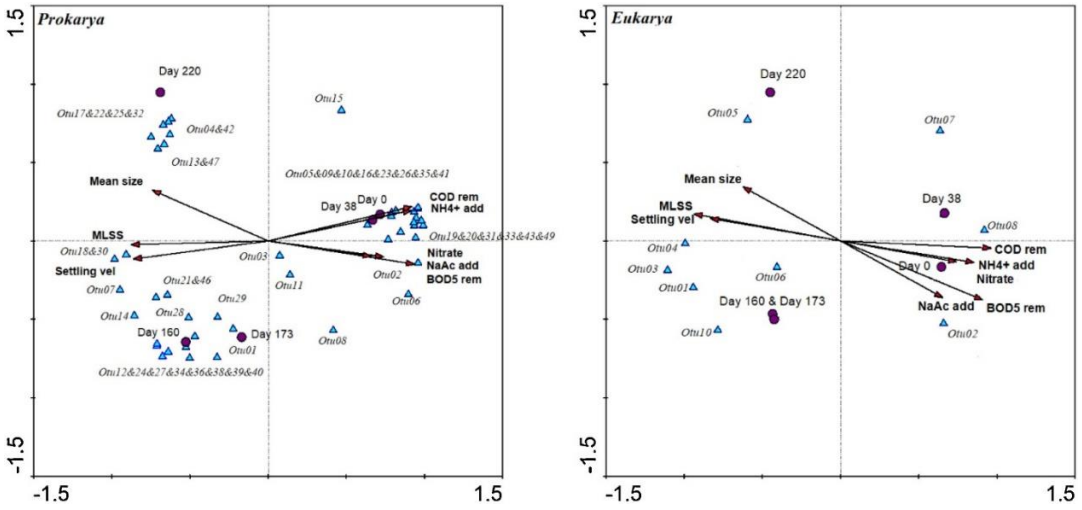

Fig. 8. Multivariate redundancy analysis linking the LOG -transformed results of physic-chemical analysis with the whole communities of *Prokarya* (left) and *Eukarya* (right) and with their dominant OTUs.

The multivariate redundancy analysis linked the dominant eukaryotic community with physical-chemical parameters and operational conditions (**Fig. 8**), showing a weak positive correlation of Otu01, Otu03, Otu4 and Otu05, affiliated with microalgae species and one fungus phylotype with increased of settling velocity, mean size and MLSS. In the same way, all these OTUs were negatively correlated with the nitrate concentration in the influent. However, Otu02, belonging to *Haematococcus* microalgae, was positively correlated with the influent nitrate concentration.

4. Conclusions

A pilot-scale denitrifying granular biomass reactor (SBR) was successfully built, started-up and operated to find the optimal operational conditions for nitrate removal under real conditions and amended with real nitrate-polluted groundwater. Under the lowest concentrations of carbon source (0.15 g NaAc L^{-1}) and mineral nutrients, the nitrate removal ratio of the granular system was greater than 70 %, and the nitrate concentration in the treated groundwater was always lower than recommended values for drinking water (25 mg NO_3^- L^{-1}). In addition, nitrite and ammonium were never detected in the effluent. Organic matter was removed with high efficiency (over 98.0 %). The microbial population was dynamics during the operation, although a clear proliferation of heterotrophic denitrifying bacteria was found, being the most dominant phylotypes belonged to *Rhodobacteraceae*, *Pseudomonas* and *Comamonas*. Eukaryotic community showed a great number of Otus affiliated to *Haematococcus microalgae* which had nitrogen source and environmental conditions to proliferate in this system. Therefore, granular biomass systems (SBR) can be an innovative and efficient technology for the treatment of nitrate-polluted groundwater under low-cost operating conditions and without any of brine.

Supplementary materials

Figure S1: Schematic diagram of granular sludge reactor at full-scale; **Figure S2**: C:N ratio during whole operation. Stage A (Day 0–37); Stage B (Day 38–159); Stage C (Day 160–172); Stage D (Day 173–219); Stage E (Day 220–234). **Figure S3**: Chemical and biological oxygen demand removal ratio in all stages of aerobic granular sludge operation;Stage A (Day 0–37); Stage B (Day 38–159); Stage C (Day 160–172); Stage D (Day 173–219); Stage E (Day 220–234). **Figure S4**: Mixed liquor suspended solids concentration during the operation; Stage A (Day 0–37); Stage B (Day 38–159); Stage C (Day 160–172); Stage D (Day 173–219); Stage E (Day 220–234);. **Figure S5**: Hicherical clustering calculated by Bray-Curtis algorithm for *Prokarya* and *Eukarya* samples; **Figure S6**.- Expected effect size for nitrogen source and sodium acetate concentration of the *Prokarya* (left) and *Eukarya* (right) domains; **Figure S7**: Principal component analysis for prokaryotic and eukaryotic samples.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Provincial de Granada) and the disposition of Municipal Water Worker of Torre-Cardela Town.

Appendix A. Supplementary data

The following is Supplementary data to this article:

Biological nitrate removal from groundwater by an aerobic granular technology to supply drinking water at pilot-scale

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Table S1. Operational conditions, physic-chemical performance and reactor design of AGS system used as inoculum

Parameter		Unit
Hydraulic retention time	6	h
Cycle	3	h
Volume exchange	50	%
Temperature	17 ± 4.2	ºC
D.O.	5.6 ± 1.2.	mg O_2 L^{-1}
Bubble	Fine	
Air introduced	3.0	L min^{-1}
Height reactor	90	cm
Diameter reactor	7	cm
COD removal	98	%
Nitrate removal	91	%
Mean size	1.15 ± 0.3	mm
Settling velocity	87 ± 11	m h^{-1}
MLSS	1.78	mg MLSS L^{-1}

Table S2. Synthetic medium composition of reactor used for the inoculation

$C_2H_3NaO_2$	NH_4^+Cl	NO_3Na	$MgSO_4 \cdot 7H_2O$	K_2HPO_4	KH_2PO_4
0.2 g L^{-1}	0.25 g L^{-1}	0.13 g L^{-1}	0.010 g L^{-1}	0.009 g L^{-1}	0.003 g L^{-1}

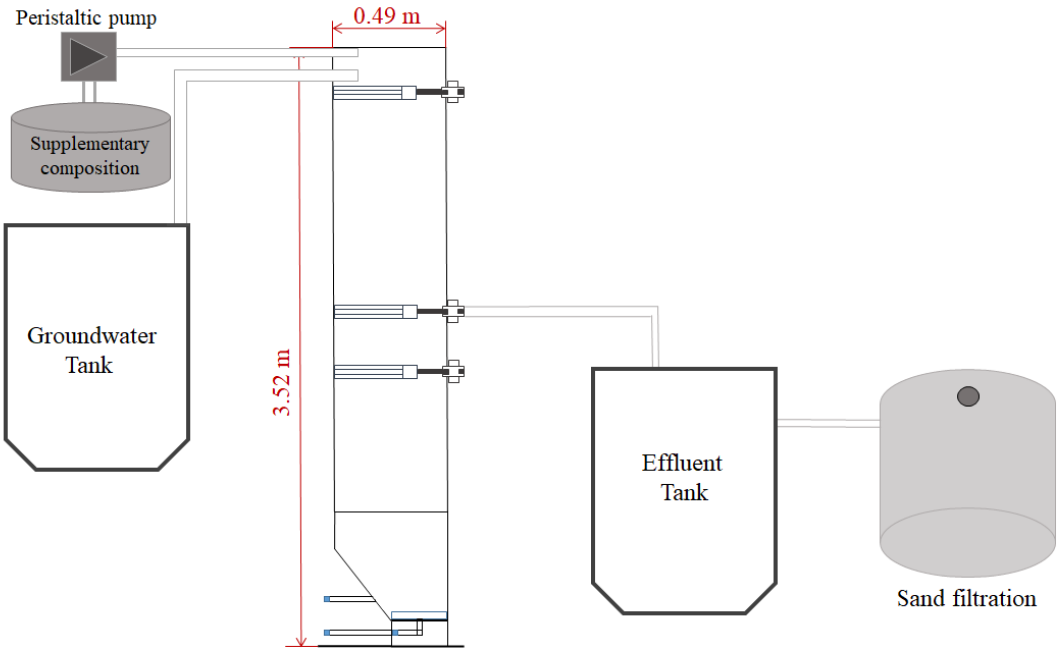

Figure S1- Schematic diagram of granular sludge reactor at full-scale.

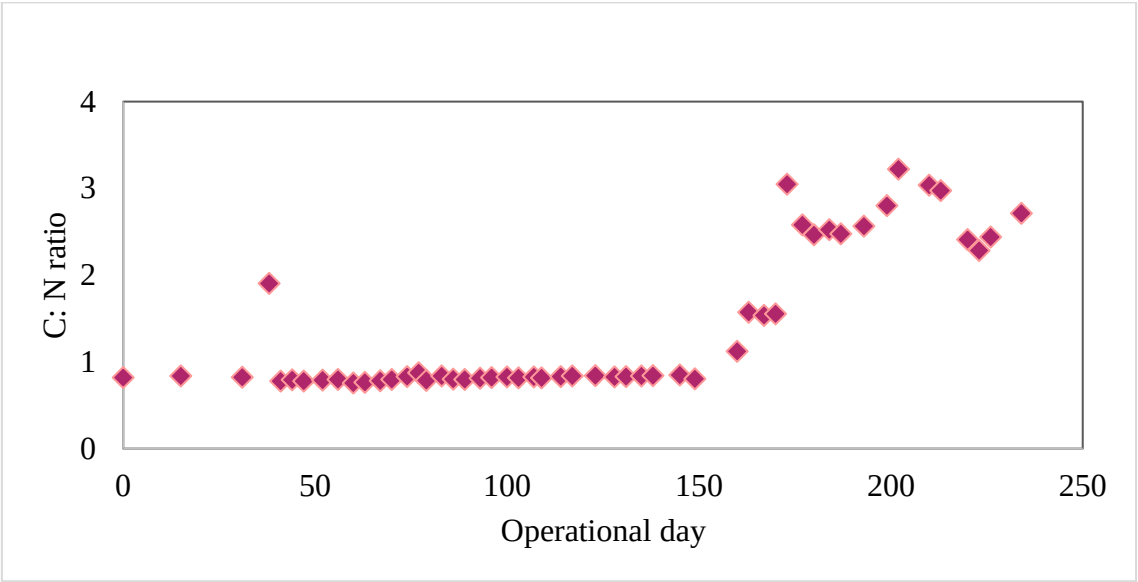

Figure S2.- C:N ratio during whole operation. Stage A (Day 0 to 37), Stage B (Day 38 to 159), Stage C (Day 160 to 172), Stage D (Day 173 to 219); Stage E (Day 220 to 234)

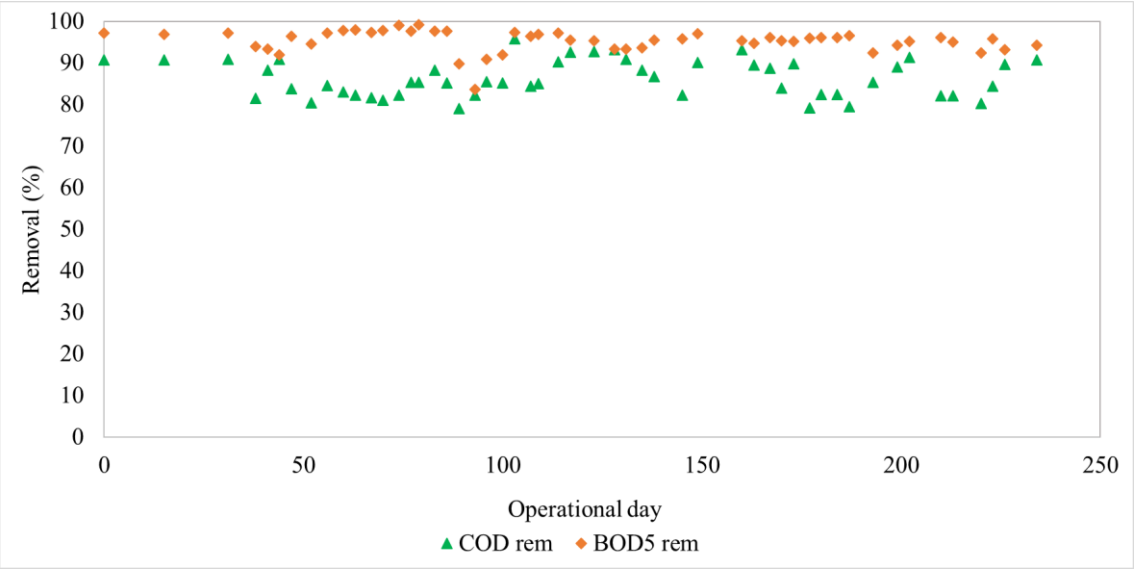

Figure S3- Chemical and biological oxygen demand removal ratio in all stages of aerobic granular sludge operation; Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234).

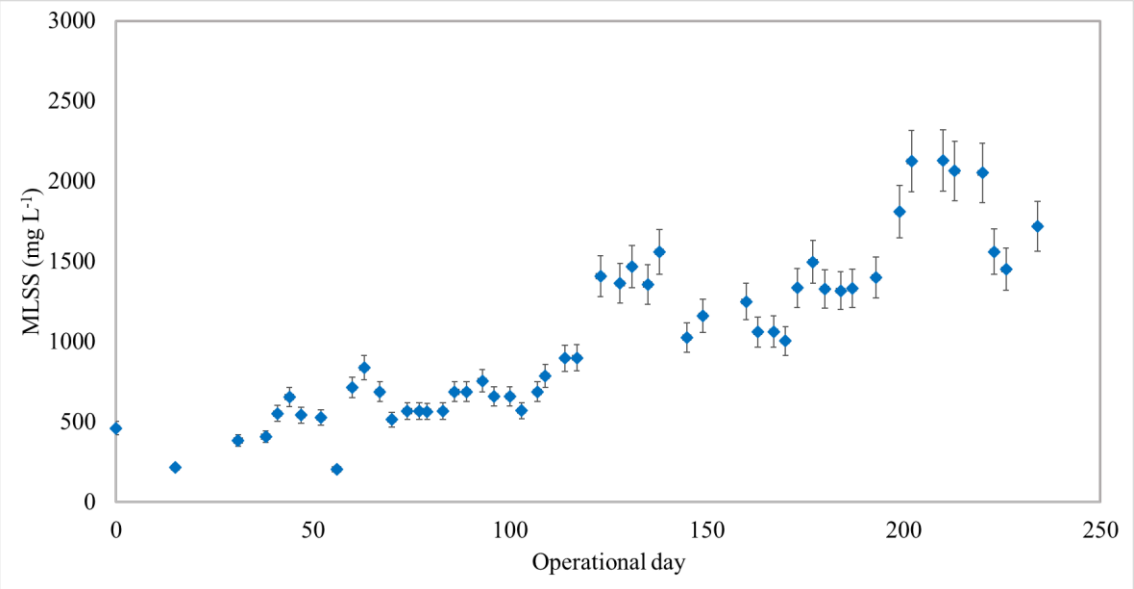

Figure S4- Mixed liquor suspended solids concentration during the operation; Stage A (Day 0 to 37); Stage B (Day 38 to 159); Stage C (Day 160 to 172); Stage D (Day 173 to 219); Stage E (Day 220 to 234).

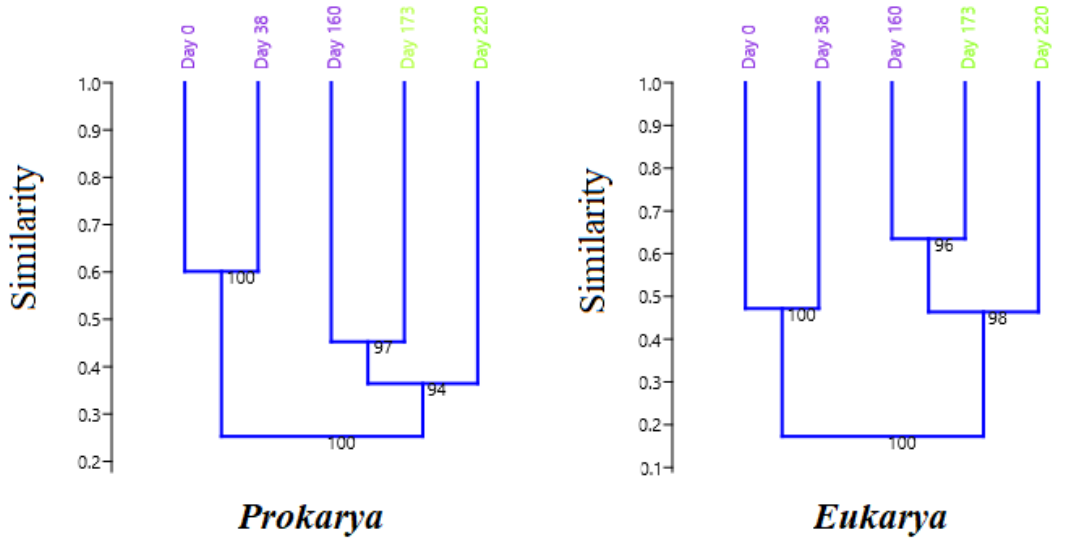

Figure S5- Hicherical clustering calculated by Bray-Curtis algorithm for *Prokarya* and *Eukarya* samples

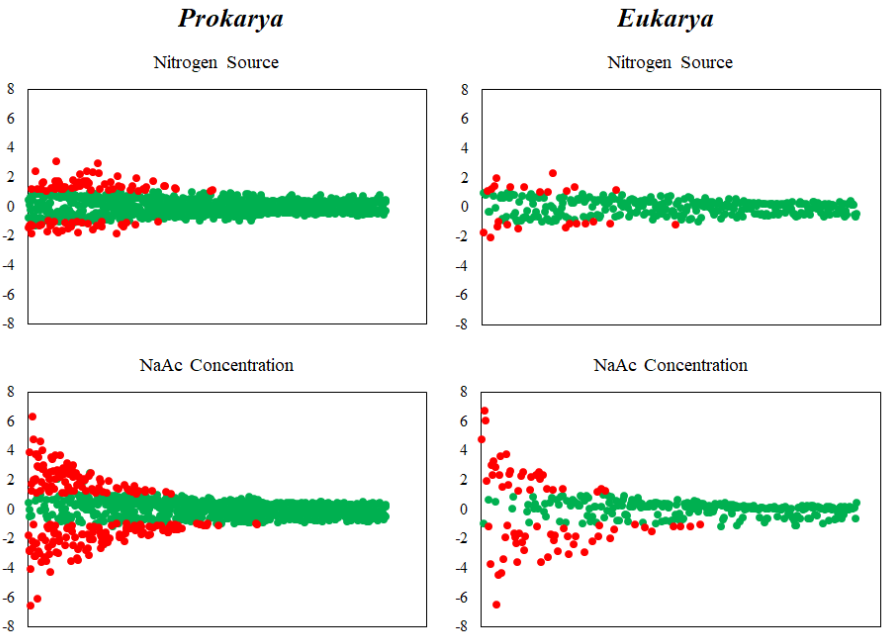

Figure S6.- Expected effect size for nitrogen source and sodium acetate concentration of the *Prokarya* (left) and *Eukarya* (right) domains**.**

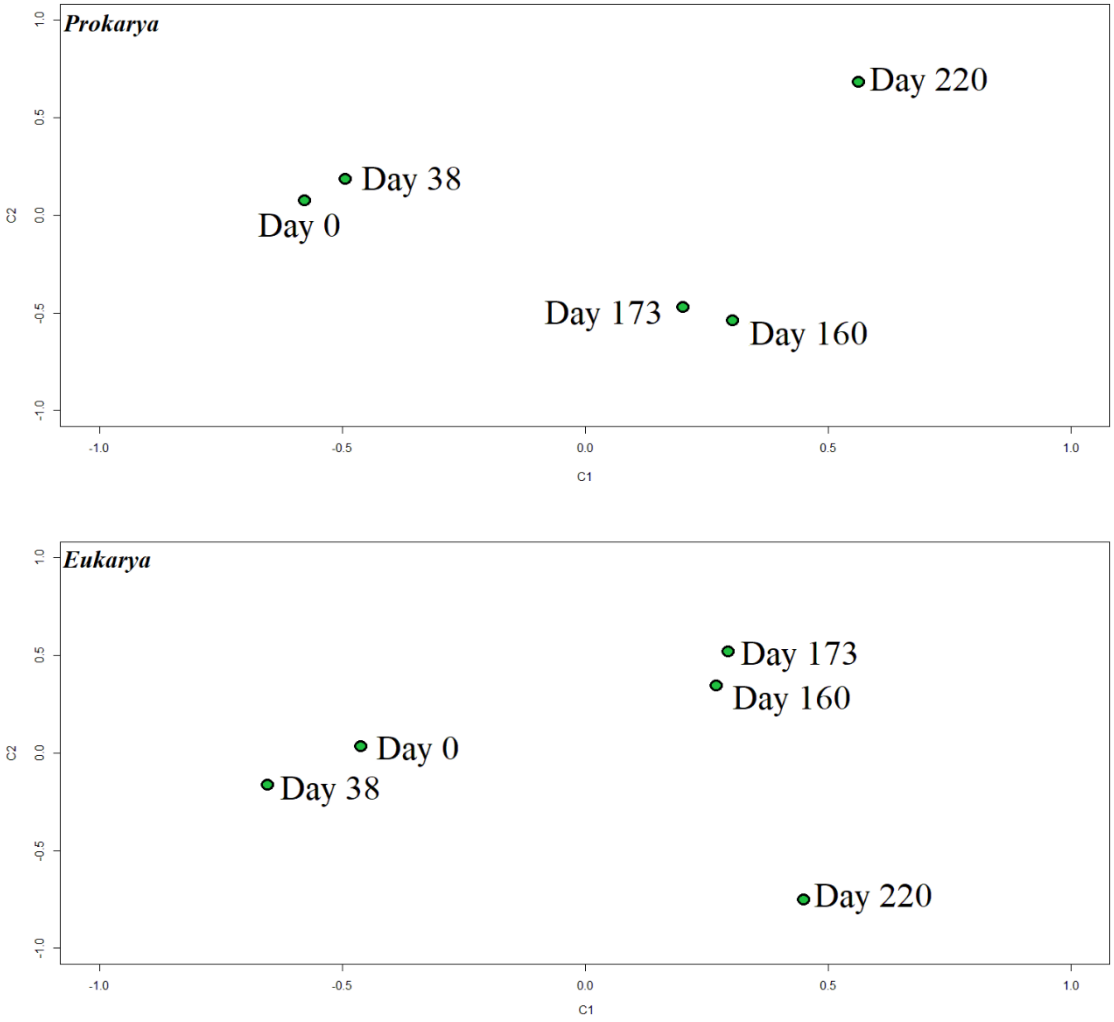

Figure S7- Principal component analysis for prokaryotic and eukaryotic samples

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Chapter 3.: Application of full-scale aerobic granular sludge technology for removing nitrate from groundwater intended for human consumption

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Abstract

The dependence on groundwater for human consumption has increased worldwide over the last decades. Nitrate (NO_3^-) often reaches groundwater and causes significant degradation in groundwater quality. A full-scale water treatment plant using aerobic granular sludge (AGS) technology was built to remove NO_3^- from nitrate-polluted groundwater intended for human consumption in a rural village. The impact of changes in the operational conditions of hydraulic retention time (HRT) and organic matter loading (OML) rate on NO_3^- removal, overall system performance, and the granule microbiome were studied. Regardless of the HRT, the AGS technology was successful in removing NO_3^- with removal rates greater than 50% with an optimal OML rate of 75 mg L^{-1}. No significant variations in the total abundance of any of the denitrification genes were observed. The composition of prokaryotic and eukaryotic communities was affected by changes in the HRT and OML rate. Specific prokaryotic taxa were identified as responsive to changes in operational parameters and their abundances were linked to the removal of NO_3^-, confirming that the microbes are critical to the NO_3^- removal process. This study demonstrates that the AGS technology can be successfully implemented to treat nitrate-polluted groundwater in rural villages to produce water of drinking quality. In addition, it is reported that the hydraulic retention times and organic matter loading rate can be used to improve the performance of the system to remove nitrate from groundwater.

Keywords: Drinking water; Nitrate-polluted water; Denitrification; Microbial communities; Full-scale plant

1. Introduction

The continued increase in the world population threatens water resources, resulting in serious global water scarcity and quality degradation problems. For example, the dependence on groundwater has quadrupled in the past 50 years worldwide [1]. This dependence on groundwater has become even more important in the Mediterranean region due to increased desertification and global warming [2]. In countries such as in Spain, 70% of the water resource demands of cities with less than 20000 inhabitants is supplied by groundwater [3].

Natural environments have been extensively and rapidly altered due to land use transformation and agricultural development over the last three decades [4]. For example, agricultural development has contributed to increased application of chemical nitrogen (N) fertilizers that can lead to several environmental problems including eutrophication [5], [6], [7]. Nitrate (NO_3^-) often reaches groundwater due its high mobility in soil and causes significant degradation in groundwater quality [8], [9], [10]. Consumption of water with excess NO_3^- is related to many health problems and diseases such as methemoglobinemia, thyroid disorder, and neural tube defects [11], [12]. In addition, NO_3^- is an element that can be transformed and when in the form of nitrite (NO_2^-) has the potential to form N-nitroso compounds (NOCs) such as N-nitrosamines that can induce cancer in the stomach and intestines [13]. Therefore, the concentration

of NO_3^- in drinking water is regulated by the Council Directive 91/676/EEC of the European Community [14] and has a recommended limit of 25 mg L^{-1}.

Multiple treatments for NO_3^- removal from water intended for human consumption have been developed over the last decades including chemical reduction [15], electrodialysis[16], [17], ion exchange[18], adsorption [19], reverse osmosis [20], biofilters [21], and aerobic granular technologies [22]. Physicochemical technologies are effective at removing contaminants in groundwater, but they have a high cost of operation and low selectivity in the removal of specific ions from groundwater [23]. As an alternative to physicochemical technologies for the treatment of polluted groundwater, a new biological technology has been developed that takes advantage of the capacity of aerobic granular sequential (AGS) systems [24],[25],[26]. This technology is based on the heterotrophic metabolism of microorganisms present in the system that form granular microbial aggregates using extracellular polymeric substances (EPS). The hydrodynamic forces inside the bioreactor are able to generate a stratification of the microorganisms inside the granular aggregates allowing the development of both aerobic and anaerobic conditions that favor nitrification (oxidation of ammonium to NO_3^-) and denitrification (sequential reduction of NO_3^- to dinitrogen) processes, respectively, for N transformation and removal [22].

Recently, we showed that the AGS technology can remove approximately 50% of NO_3^- from groundwater at a pilot-scale [27]. However, as groundwater is usually oligotrophic and contains very low carbon (C) content, the addition of a C source is necessary for granule formation and therefore NO_3^- removal. Optimization of AGS technologies to remove NO_3^- from groundwater remains necessary to achieve high

removal rates in treated water using a low organic matter loading (OML) rate for not only optimal biological NO_3^- removal but also minimal cost. The residence time of the water in the bioreactor, known as hydraulic retention time (HRT), is also a highly important operational parameter for correct optimization of OM and nutrient removal in AGS systems. In AGS operated in sequential batch reactor (SBR), the HRT can be up to 3 times shorter than in other biological systems[28]. However, it is not known how the HRT should be adjusted to improve NO_3^- removal in AGS systems treating contaminated groundwater.

The granular sludge in AGS systems is formed by prokaryotic and eukaryotic microorganisms present in the granular biomass. In particular, NO_3^- removal from wastewater is carried out by heterotrophic microorganisms known as denitrifiers under oxygen-limiting conditions. Biological denitrification takes place in a series of stages by enzymes encoded by denitrification genes. For example, the reduction of NO_2^- to nitric oxide (NO) is carried out by two NO_2^- reductase enzymes called *Nir*S and *Nir*K present in denitrifying organisms. The next step in the denitrification process is the reduction reaction of NO to nitrous oxide (N_2O), which is carried out by microorganisms containing the *norB* gene. Finally, the reduction reaction of nitrous oxide (N_2O) to nitrogen gas (N_2) is catalyzed by the periplasmic enzyme N_2O-reductase (*Nos*ZI). This step is particularly important to consider because it eliminates N_2O, a potent greenhouse gas, and generates N_2 that is not dangerous for the atmosphere[29]. Although the abundance, structure, and composition of microbial communities have been widely characterized in wastewater, those of groundwater contaminated with NO_3^- have been less explored. The study of the prokaryotic (bacteria and archaea) and eukaryotic (e.g.,

fungi and other fauna) granular microbiome can help understand treatment performance in biological wastewater treatment technologies such as granular sludge. For instance, the study of changes in the granule microbiome in AGS systems can help identify optimal operational conditions that favor specific microorganism involved in the removal of OM and NO_3^-[27].

In this study, a full-scale water treatment plant using aerobic granular technology was built and operated in the municipality of Torre-Cardela (Spain). This treatment plant was designed to remove NO_3^- from groundwater intended for human consumption. The impact of changes in the operational conditions of HRT and OML on NO_3^- removal and overall system performance (e.g., OM removal, granule size, biomass concentration) were examined. Variations in the total abundance of the *nir*K, *nir*S, and *nosZ*I denitrification genes, and the diversity and composition of prokaryotic and eukaryotic microorganims present in the granules were also examined and related to changes in physicochemical and performance parameters under different OML rates and HRTs.

2. Materials and methods

2.1. Study area and groundwater characteristics

The plant was located in the southeastern part of the Iberian Peninsula in the municipality of Torre-Cardela (Andalusia, Spain), with a population of 760 inhabitants. The water supply system operates by pumping water from the aquifer corresponding to the Torre-Cardela Calcarenites. The aquifer includes calcarenites, sandy limestones, bioclastic sandstones, and marls affected by a secondary porosity due to the high degree

of fissuring [22]. The result is a fissured-Karstic aquifer whose water is extracted by pumping from wells perforated at 170 and 90 meters deep. The aquifer can produce annual resources of 4.1 hm^3 of groundwater. The water from the aquifer is exclusively used for human consumption in the Torre-Cardela municipality.

Preliminary studies were carried out over a year to analyze the physicochemical characteristics of the groundwater as well as the fluctuations in NO_3^- concentrations over time. Nitrate levels fluctuated between 23 and 84 mg L^{-1} [22]. These oscillations in NO_3^- levels are the result of a combination of the characteristics of the aquifer which has very dynamic water flows due to its secondary porosity, together with the rain cycles, causing NO_3^- flushing in the recharge zones of the aquifer towards the groundwater level. The excessive use of nitrogen fertilizers during agricultural practices in the region is thought to be the main source of NO_3^- contamination.

2.2. Bioreactor design and operation

Three cylindrical sequential batch reactors (SBRs) were built with the following characteristics: Bioreactor "1" had a height of 3.52 m, a diameter of 0.49 m, and a volume of 660 L. Bioreactors "2" and "3" had heights of 3 m, diameters of 1 m, and volumes of 2160 L, and were built with two exchange out-flows at 50 % and 60 % of total volume (**Supplementary Figs. S1-S3**). Bioreactors 1, 2, and 3 were inoculated with 6 L of granular biomass taken from a denitrifying AGS system operated in a SBR, inoculated with activated sludge from the Baza wastewater treatment plant (WWTP) (Granada, España) that treats groundwater contaminated with NO_3^- [22]. The air flow was controlled at the facility and was introduced through the bottom of the bioreactors using a membrane diffuser that generated a small bubble size. The operation of the

system consisted of a series of three stages described in **Table 1** which included changes in the OML rate (150 to 75 mg L^{-1}) added to the system in the form of food grade sodium acetate, along with a reduction of the HRT (6 to 4 hours), to achieve satisfactory NO_3^- removal with a low amount of added OM. Each cycle consisted of 178 or 118 min of aeration (depending on the HRT), decantation for 2 min, effluent discharge for 4 min, and 3 min of filling. The reactor was directly fed with the groundwater used by the municipality of Torre-Cardela. The total duration of the experiment was 240 days. At the beginning of each operational cycle, the C source necessary for the biological denitrification process was added to the unprocessed groundwater together with a series of trace elements essential for the correct development and function of the granular biomass (**Supplementary Table S1**).

Table 1. Change in the operational parameters of the system.

Days	**Stage**	**HRT (h)**	**OM loading rate (mg L^{-1})**
0-14	Start-up	6	150
15-120	I	6	100
121-180	II	4	100
181-240	III	4	75

During the startup (first 15 days of operation), the settling time was adjusted to meet the needs of the granules based on previous experiments [22]: 10 min during the first 3 days, 7 min the following week, and 3 min during the last 5 days. The reactors

were operated using 60 % of their volume in each operating cycle to maximize plant efficiency based on previous experiments [27]. The filling and emptying of the bioreactors was done automatically in each cycle by installing level sensors connected to a control system that allowed the plant to operate automatically (**Supplementary Figs. S1-S3**). The system operated at room temperature which ranged from -3 to 32°C (**Supplementary Table S2**).

2.3. Determination of physico-chemical parameters

Influent and effluent samples were taken three times a week from each of the bioreactors. These samples were transported at 4 ºC to the laboratory for analysis. Samples of granular biomass were also collected at these time points. The concentration of NO_3^- in the influent and effluent of the bioreactors was determined using a Metrohm ion chromatograph. The parameters of chemical and the biological oxygen demand (COD and BOD_5, respectively) were measured using standard protocols [30]. The mixed liquor suspended solids (MLSS) were measured as a proxy of the biomass concentration every week in triplicate [30]. The pH and dissolved oxygen content were monitored daily by sensors placed inside the bioreactors and connected online to a data logging system. The mean size and settling velocity variation of the granular biomass were monitored periodically as describer earlier [31].

2.4. Control of treated water for human drinking water use

The treated water discharged from each bioreactor was homogenized in a 500 L tank (**Supplementary Fig. S1**) and filtered through a sand filter designed for drinking water. The filter filler used was a "Hi-Tech Filter Media" which is a high-tech glass for

drinking water filtration with Bureau Veritas S.L. certification that certifies the absence of contaminants, high purity, and anti-compaction. The specific composition of the filter is as follows: SiO_2 74%, Na_2O = 11%, CaO = 10%, MgO = 3%, Al_2O_3= 1%. (**Supplementary Fig. S1**). After filtration, 0.040 mL NaClO L^{-1} was added to the treated water. This level ensured that the treated water always contained the residual chlorine level required by Real Decreto 140/2003 of the Spanish regulations [32]. The treated water was analyzed weekly, and samples were sent to an authorized and accredited external lab to check for the 48 parameters regulated by the Real Decreto 140/2003 of the Spanish regulations for water used for human consumption (**Supplementary Table S3**).

2.5. Sampling for microbial analysis and extraction of nucleic acids

Granular biomass samples were taken from the top of the bioreactors during the aeration phase on operating days 0, 50, 120, 180, 210, and 240, as described earlier [31]. Samples were stored at 4°C for transport from the treatment plant to the laboratory and kept at -20°C until analysis. DNA was extracted from the resulting sediment using the Fast DNA SPIN kit for Soil (MP Biomedicals, USA). DNA extracts were kept at -20 °C until analysis.

2.6. Absolute quantification of genes involved in the nitrogen cycle by qPCR.

The total abundance of bacterial (16SB), archaeal (16SA), and fungal (18SF) communities, and *nirK*, *nirS*, and *nosZ*I denitrification genes were measured using quantitative PCR (qPCR) in a QuantStudio 3 thermocycler (Applied Biosystems, ThermoFisher Scientific) as described by Muñoz-Palazón et al. [31]. Primers, thermal

cycler conditions, and qPCR standards are shown in **Supplementary Table S4**. Ten-fold serial dilutions of plasmids harboring inserts of each of the target genes were used as standards for qPCR. The PCR efficiencies were in the range of 90-100% in all cases.

2.7. Microbial community analysis

DNA was sequenced using Illumina MiSeq at the Institute of López-Neyra (Granada, Spain). The prokaryotic 16S rRNA gene was sequenced using the primer pairs Bac357-Bac806 [33] and the eukaryotic 18S rRNA gene using the primer pairs EUK1391 – EUKbr [34]. The raw sequencing data were processed separately for the prokaryotic and eukaryotic communities using QIIME2 v2018 [35]. The reads were assembled and dereplicated using DADA2 [36] as described by Castellano-Hinojosa et al. [37]. The raw sequence data are available in the NCBI under BioProject number PRJNA1003521.

Sequencing analysis was done with R software version 4.2.0 (http://www.rproject.org/). Alpha (Shannon and Inverse Simpson) using the R package "vegan " v 2.5-2′ and "phyloseq" v1.24.0 [38], [39]. Data were normalized on a logarithmic basis to avoid increased error rates due to rarefaction [39]. The Non-metric multidimensional scaling (NMDS) analysis was used to study changes in the composition of the communities (beta diversity) using unweighted UniFrac distances. Significant differences in beta diversity between time points were examined using permutational analysis of variance (PERMANOVA). Taxa whose relative abundance changed significantly between time points were detected using the DESeq2 package [40].

2.8 Functional characteristics of the bacterial community

The ASV tables, which contained the ASVs of the bacterial taxa, were converted into BIOM files using the command "biom-convert" in QIIME2. Bacterial functions involved in the denitrification pathways were predicted using the PICRUSt2 tool [341]. Four denitrification enzymes (nitrate-, nitrite-, nitric oxide-, and nitrous oxide- reductase) were used as molecular markers to compare changes in denitrification activity between time points. For each denitrification enzyme, the relative abundance of orthologs (KO) assigned to denitrification gene products of the above-mentioned denitrification enzymes was compiled using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [36] (**Supplementary Table S5**).

2.9. Statistical analyses

All the statistical analyses were done using the R software version 4.2.0. Redundancy analysis (RDA) was performed to examine the relationship between the differentially abundant prokaryotic and eukaryotic ASVs with the different physicochemical parameters of the system (NO_3^- in the influent, NO_3^- in the effluent, NO_3^- removal %, BDO_5 removal %, COD removal %, mean size of granules, setting velocity of granules, and MLSS). The calculation was processed using CANOCO 4.5 software for Windows.

3. Results and discussion

3.1. Physicochemical and performance determinations

The concentration of NO_3^- in the influent varied between 41.7 and 54.1 mg L^{-1} during the experimental period (**Supplementary Fig. S4**). These results showed that the

concentration of NO_3^- in groundwater in Torre-Cardela municipality was close and/or above to the limit established by the Council Directive 91/676/EEC of the European Community for NO_3^- in drinking water. Therefore, the groundwater had to be treated for human consumption in this municipality.

Our results showed the implementation of a full-scale AGS technology was successful in removing NO_3^- (**Fig. 1A**). We found the percent NO_3^- removed gradually increased from 25% to approximately 50% from the start-up phase to day 50 of operation and remained stable until the end of the experimental period (**Fig. 1A**). Regardless of the HRT and OML rate, the COD (**Fig. 1B**) and BOD_5 (**Fig. 1C**) removal rates were >90% and 80%, respectively, during the experiment showing microorganisms rapidly consumed organic compounds. These results indicate the system was able to efficiently remove NO_3^- with very low OM concentration (≤ 100 mg L^{-1}) reaching a C:N ratio close to 1:1. The maintenance of a stable percentage of NO_3^- removal with a OML rate of 75 mg L^{-1} and 100 mg L^{-1} showed that the system was operating with excess OM at 100 mg L^{-1} of OML. It is important to note that the NO_3^- concentration in the influent decreased after 100 days of operation to values lower than 50 mg L^{-1}. This allowed an optimization of the bioreactors by decreasing the OML rate and HRT, allowing them to perform a greater number of cycles and to treat more water per day. This feature is important in biological water treatment such as aerobic granular technology because it allows adjustment to the number of cycles and the amount of OM according to the characteristics of the water to be treated.

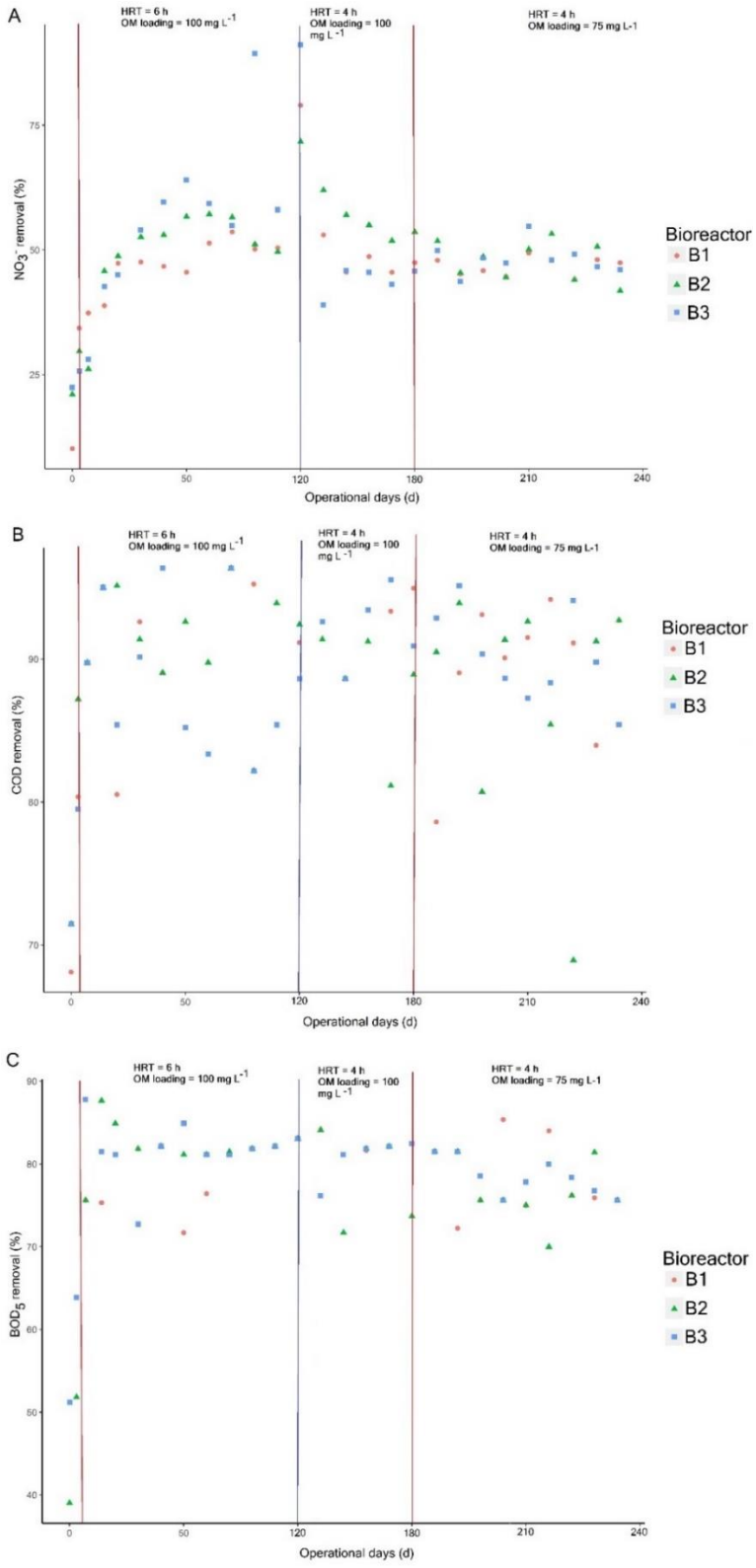

Fig. 1. Nitrate (A), COD (B), and BOD_5 (C) removal % during the experimental period. COD, chemical oxygen demand; BOD_5, biological oxygen demand

We found all bioreactors obtained similar sized granules and the biomass became larger as the granular biomass matured in the bioreactors. The granules used as inoculum had an average size of 6 mm and a settling velocity of 50 m h^{-1}. In all three bioreactors, an increase in granule size was observed from the start-up phase until day 150 where a stabilization of the size around 12 mm occurred and was maintained until the end of the experiment (**Fig. 2A**). This stabilization coincided with the change of OML rate from 100 mg L^{-1} to 75 mg L^{-1}. Variations in the HRT of the system did not impact the size of the granular biomass (**Fig. 2A**). Regardless of the HRT and OML rate, the settling velocity of the granules remained stable during the experimental period in all bioreactors, varying between 57-61 m h^{-1} in bioreactor 1 and between 42-51 m h^{-1} in bioreactors 2 and 3 (**Fig. 2B**). The lower settling velocities of the granular biomass of bioreactors 2 and 3 compared to bioreactor 1 may be due to the design of these bioreactors, which had different dimensions than bioreactor 1.

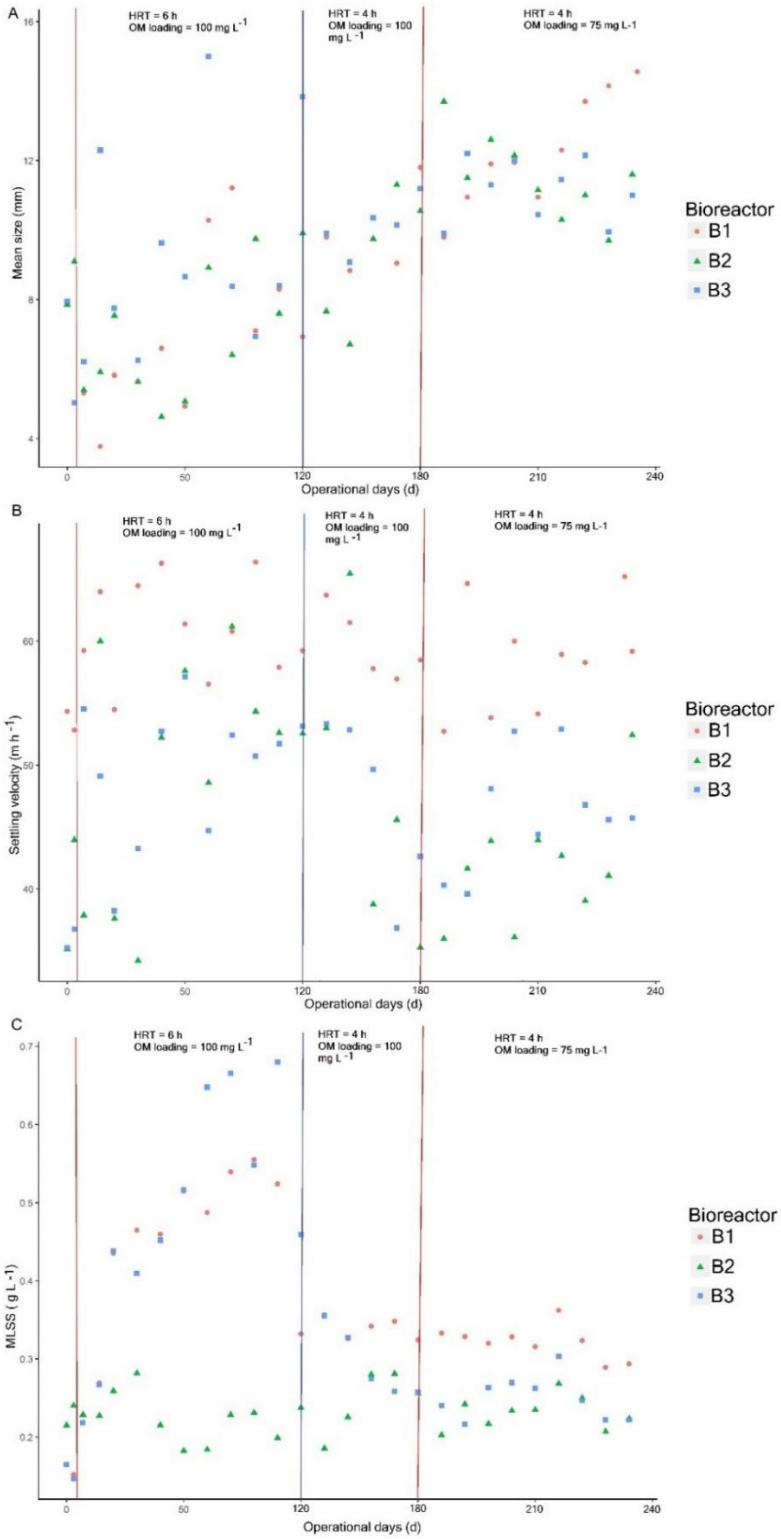

Fig. 2. Granule mean size (A), setting velocity of the granules (B), and MLSS concentration (C) during the experimental period. MLSS, mixed liquor suspended solids.

Gradual increases in the MLSS concentration were observed after inoculation of the bioreactors until day 100 of operation (**Fig. 2C**). The MLSS concentration was greater in bioreactors 1 and 3 compared to bioreactor 2 until day 110 of operation (**Fig. 2C**). The decrease in granular biomass after 110 days of operation likely occurred due to the higher number of cycles per day together with a lower amount of OM in the system. For instance, the reduction of the HRT from 6 to 4 hours decreased the MLSS concentration to values close to 3000 mg L^{-1} in the 3 bioreactors from day 110 of operation until the end of the experiment.

3.2. Analysis of treated water for human consumption

Based on the results of the physicochemical and microbiological analysis of the treated water leaving the facility, the quality of the treated water was safe for human consumption. Values of the 48 parameters regulated by the Real Decreto 140/2003 of the Spanish regulation were within the values required for drinking water intended for human consumption (**Supplementary Table S3**). Therefore, our results showed for the first time that a full-scale AGS system treating NO_3^--contaminated groundwater can not only be used to eliminate NO_3^- but to produce water that meets the quality standards for drinking water.

It is interesting to note that a previous study demonstrated that the full-scale AGS technology used in this study produces drinking water in a more environmentally friendly cost-effective way and with lower energy costs compared to reverse osmosis [41]

3.3. Abundance of microbial communities and denitrification genes

The effect of changes in HRT and OML rate on the total abundance of microbes and denitrification genes was assayed in the full-scale AGS system treating groundwater. No significant differences in the abundance of 16SB, 16SA, 18SF, and *nirK*, *nirS*, and *nosZ*I denitrification genes were detected between time points during the experimental period (**Supplementary Fig. S5**). These results showed that the small variations in HRT and OML rate in this study were not sufficient to alter the total abundance of bacteria, archaea, and fungi and denitrification genes which agrees with previous observations of aerobic granular systems fed with groundwater [22]. The lack of changes in the abundance of denitrification genes could be is not unexpected considering the percent NO_3^- removed remained stable during the experimental period. The presence of *nirK*-, *nirS*-, and *nosZ*I-type denitrifiers in the full-scale AGS system suggests there were oxygen-limiting conditions in the granular biomass that allowed complete denitrification of nitrate-contaminated groundwater. This has important environmental implications as it shows the ability of the AGS system to reduce the greenhouse gas N_2O to N_2 while treating groundwater contaminated with NO_3^-.

3.4. Changes in the diversity and composition of the prokaryotic and eukaryotic community and predicted denitrification functions

No significant changes in the values of the alpha diversity indices were detected during the experimental period both for the prokaryotic (**Fig. 3A**) and eukaryotic communities (**Fig. 4A**). However, the beta diversity analysis showed significant differences in the composition of the prokaryotic (**Fig. 4B**) and eukaryotic

communities (**Fig. 4B**). Together, these results showed it is not the diversity but the composition of microbial communities that was most responsive to changes in HRT and OML rates in this AGS system. This was further supported by stability of the prokaryotic (**Fig. 4B**) and eukaryotic (**Fig. 4B**) community compositions during the first 120 days of operation ($p > 0.05$) and the subsequent change in compositions when the HRT and OML rate were reduced from 6 h to 4 h and from 100 mg L^{-1} to 75 mg L^{-1}, respectively. Therefore, these results suggest that variations in OM availability and HRT drive changes in the composition of the granule microbiome in this full scale AGS system fed with groundwater, and that these changes can occur rapidly in the granule microbiome. Microorganisms in AGS systems are known to rapidly respond to alterations in nutrient availability which may explain our results [22], [28], [34].

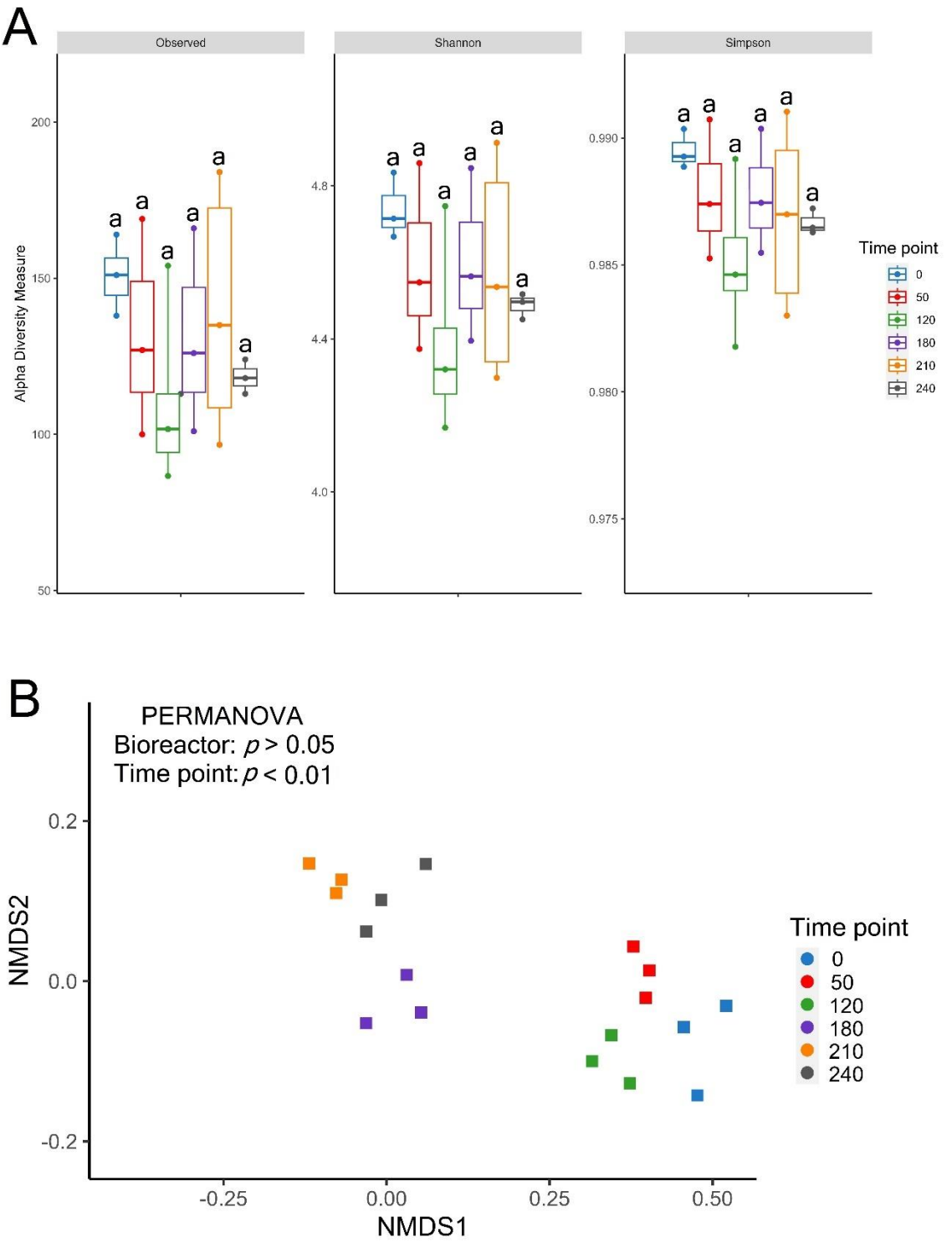

Fig. 3. (A) Number of ASVs, and values of Shannon, and Simpson diversity indices for the prokaryotic community. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed as mean with standard error

and are the average of Bioreactors 1, 2, and 3. (B) Non-metric multidimensional scaling (NMDS) plots on unweighted UniFrac distances for the prokaryotic community. Differences in community composition between treatments and time points were tested by permutational analysis of variance (PERMANOVA), and p values ≤ 0.01 were considered significant.

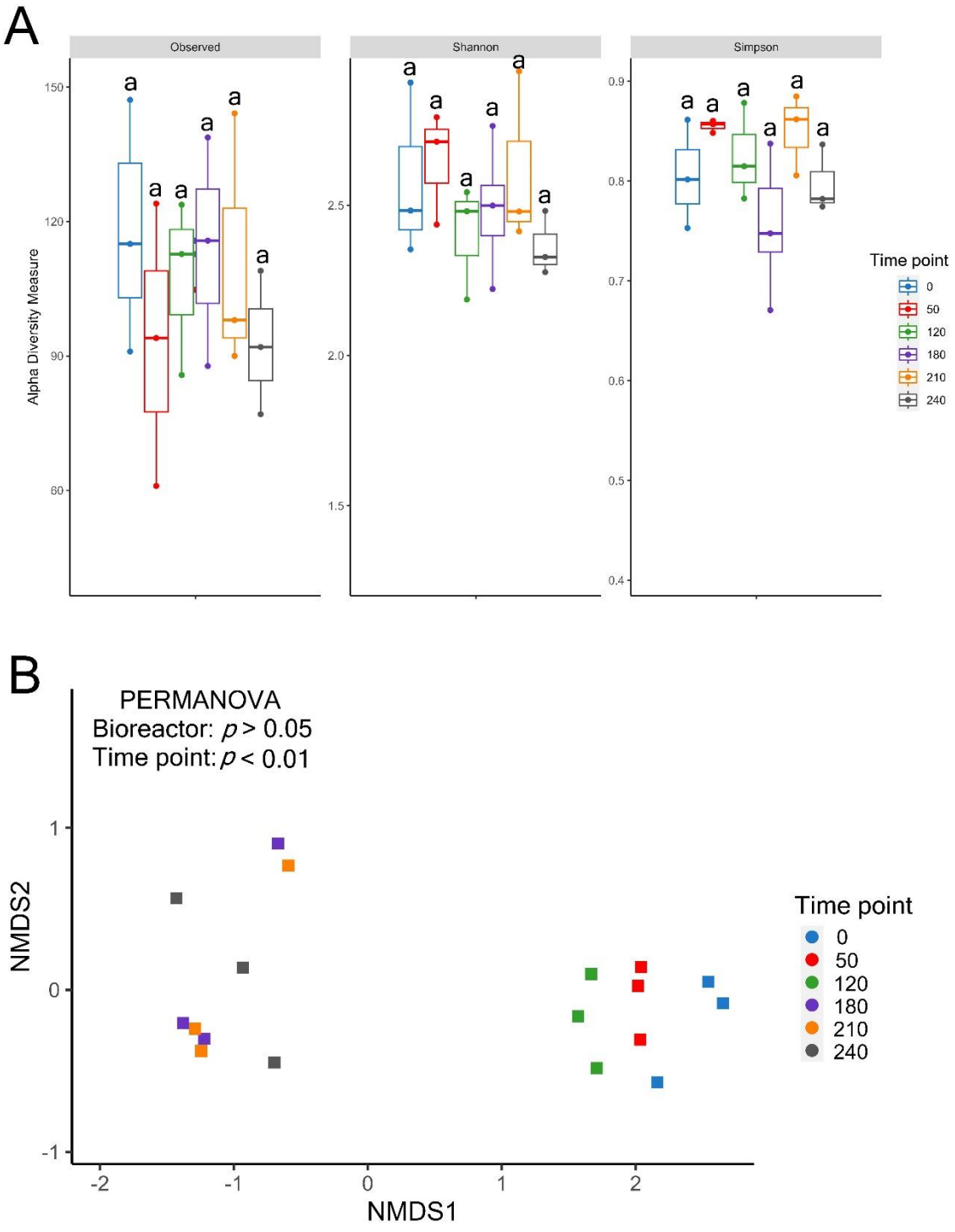

Fig. 4. (A) Number of ASVs, and values of Shannon, and Simpson diversity indices for the eukaryotic community. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed as mean with standard error and are the average of Bioreactors 1, 2, and 3. (B) Non-metric multidimensional scaling (NMDS) plots on unweighted UniFrac distances for the eukaryotic community. Differences in community composition between treatments and time points were tested by permutational analysis of variance (PERMANOVA), and p values ≤ 0.01 were considered significant.

For additional insights into the composition changes of the prokaryotic and eukaryotic communities, we identified the relative abundance of phyla and families with at least 1% relative abundance in the granule microbiome. On average, Proteobacteria (44.2%) and Bacteroidota (22.4%) were the most abundant bacterial phyla across all time points and their relative abundance remained stable during the experimental period (**Supplementary Fig. S6A**). However, other bacterial phyla such as Firmicutes and Bdellovibrionota were more responsive to changes in HRT and OML rate particularly on days 180 and 210 of operation when the HRT and OML rate were reduced from 6 h to 4 h and from 100 mg L^{-1} to 75 mg $L^{-}1$, respectively (**Supplementary Fig. S6A**). This was further supported by the identification of changes in the relative abundance of prokaryotic families with at least 1% of relative abundance in the granule microbiome, particularly by day 180 and 210 of operation (**Supplementary Fig. S6B**). The eukaryotic microbiome was dominated by Ascomycota during the first 120 days of operation (**Supplementary Fig. S7A**). However, Basidiomycota, Cercozoa, and Rotifera were the most abundant eukaryotic phyla on days 180, 210, and 240 of operation (**Supplementary Fig. S7A**). Similar to the prokaryotic community, the reduction of the HRT and OML rate after 180 days of operation induced changes in the relative abundance of eukaryotic families (**Supplementary Fig. S7B**).

The study of the granule microbiome also showed that the HRT and OML rate did not significantly impact the relative abundance of predicted KEGG pathways involved in any of the denitrification steps (**Supplementary Fig. S8**). These results agree with those of the absolute abundance of denitrification genes detected by qPCR and showed that the abundance and functionality of microorganisms involved in denitrification remained stable during the experimental period.

3.5. Differentially abundant prokaryotic taxa and their relationship to changes in operational conditions.

Significantly enriched and depleted prokaryotic ASVs between time points were identified at the genus taxonomic level (**Fig. 5**). There were no significant differences in the relative abundance of eukaryotic genera between any of the time points. Differentially abundant prokaryotic taxa were detected only between the time points 180 vs. 120 and 210 vs. 180 when the HRT and OML rate were reduced from 6 to 4 h and from 100 to 75 mg L^{-1}, respectively (**Supplementary Fig. S6A**) (**Fig. 5**). In general, we found prokaryotic ASVs were both enriched and depleted within the same genera suggesting HRT and OML rate effects on the prokaryotic microbiome may be species-specific. However, we detected specific genera whose relative abundance significantly increased on day 180 compared to day 120 (e.g., *Aeromonas*, *Brevundimonas*, *Clostridium*, *Dechloromonas*, *Demequina*, *Desulfomicrobium*, *Erysipelothrix*, and *Treponema*) thus suggesting ASVs assigned to these genera may be favored at lower HRT (**Fig. 5A**). Other genera such as *Acholeplasma*, *Chyseobacterium*, *Emticia*, *Flavobacterium*, *Fluvicola*, *Paludibacter*, *Pedobacter*, *Thauera*, and *Xanthobacter* were depleted with lower HRT (**Fig. 5A**). Decreases in the OML rate on

day 210 compared to day 180 favored increases in the relative abundance of ASVs assigned to 13 different genera (e.g., *Acinetobacter*, *Cytophaga*, *Flectobacillus*, *Gemmatimonas*, and *Zooglea*) (**Fig. 5B**). ASVs belonging to 9 different genera such as *Aeromonas*, *Desulfomicrobium*, *Dechloromonas*, and *Fusibacter* were depleted when OML was reduced from 100 to 75 mg L^{-1} (**Fig. 5B**).

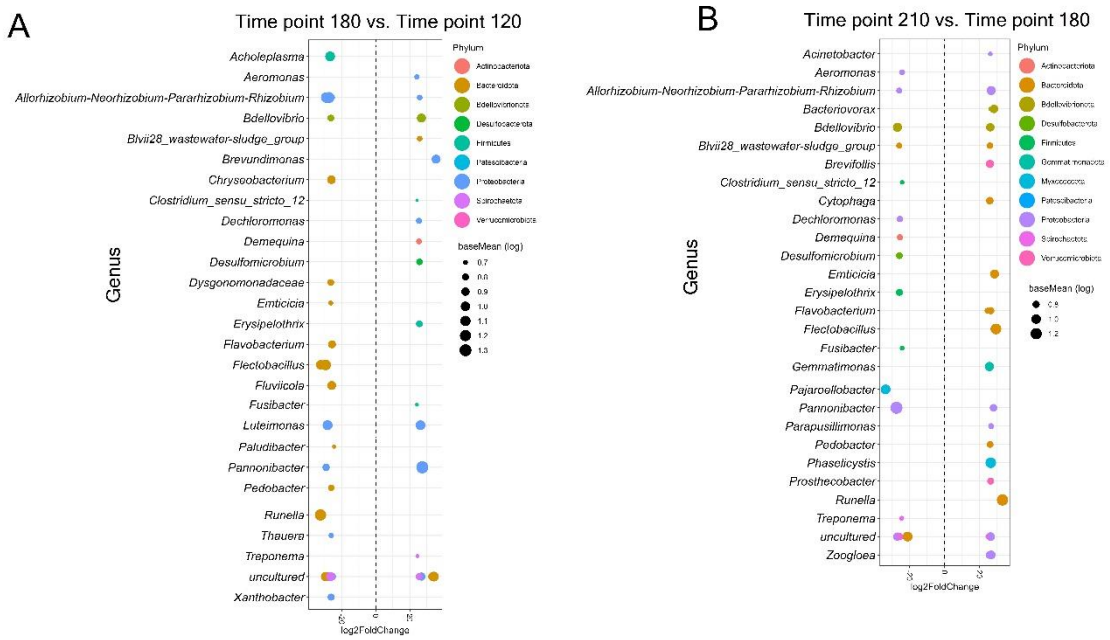

Fig. 5. Differential abundance ASVs at the genus taxonomic level between time points for the prokaryotic community. The fold change is shown on the X axis and genera are listed on the Y axis. Each colored dot represents an ASV that was identified by DESeq2 analysis as significantly differentially abundant between treated and non-treated soils ($p \leq 0.05$).

Although we found different prokaryotic genera were enriched between the time points 180 vs. 120 and 210 vs. 180, an RDA analyses showed these genera mainly grouped with NO_3^-, COD, and BDO_5, percent removal, and MLSS (**Fig. 6**). These results suggest different taxa were involved in the removal of N and C compounds when

the HRT and OML rate were reduced from 6 to 4 h and from 100 to 75 mg L^{-1}, respectively. Despite these changes in the granule microbiome, the performance of the system remained stable during the experimental period, thus showing the plasticity of the prokaryotic microbiome to adapt to different operational conditions.

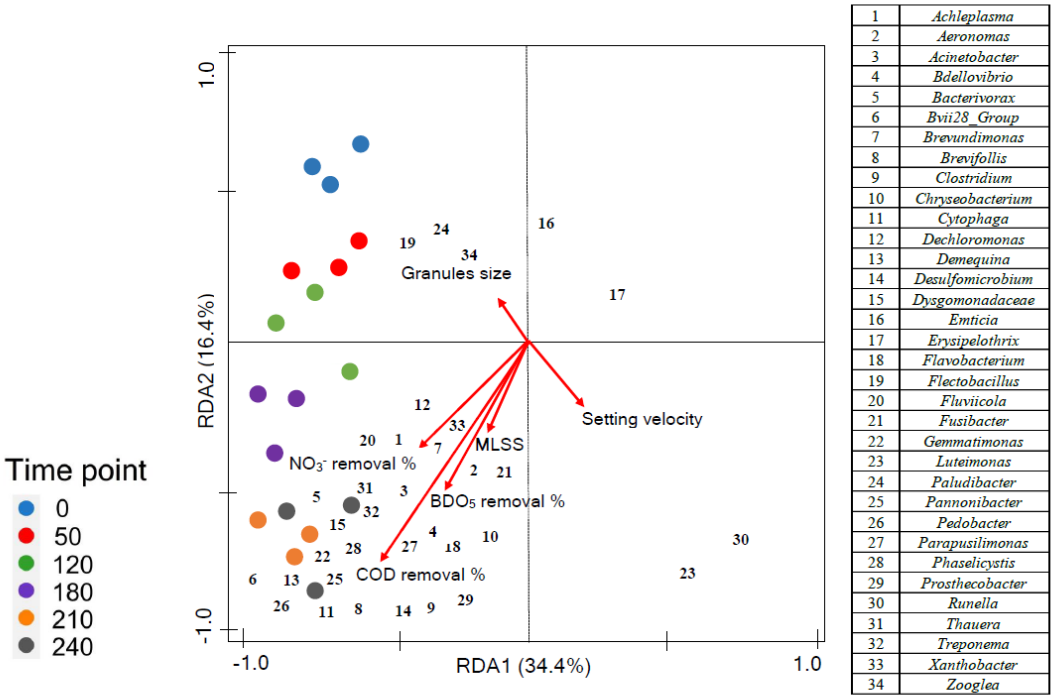

Fig. 6. RDA triplots relating differentially abundant prokaryotic taxa identified by DESeq2 analysis (**Figs. 5**) and physicochemical parameters of the system. COD removal %; BOD_5 removal %; NO_3^- removal; MLSS; mixed liquor suspended solids; Granules size; mean size of the granules; and Setting velocity; setting velocity of the granules.

We detected a diverse group of prokaryotic genera that were linked to removal of NO_3^- and organic compounds thus showing an AGS system treating nitrate-polluted groundwater can hold taxa with the same or similar functions (that is functional

redundancy; [40]) (**Fig. 6**). Among these genera, *Aeromonas*, *Acinetobacter*, *Brevundimonas*, *Dechloromonas*, *Prosthecobacter*, and *Thauera* are important denitrifiers in AGS systems [45], [46], [47], [48]. Changes in the relative abundance of other bacterial genera such as *Pedobacter*, *Fluviicola*, *Thauera,* and *Xanthobacter* have been previously linked to the degradation of organic compounds in AGS systems [9], [28], [49].

We found the genera *Zooglea*, *Flectobacillus,* and *Paludibacter* were related to changes in granule size (Fig. 6). The floc-forming bacteria *Zooglea* is key for activated sludge floc formation in AGS systems [50], [51]. Filamentous bacteria such as *Flectobacillus* have also been previously detected in AGS systems and associated with granule formation [52]. Members of *Paludibacter* are facultative anaerobes or anaerobes that may play important roles in aerobic granulation process in AGS systems [53], [54].

4. Conclusions

This study has important practical implications as it shows aerobic granular sludge technology can be successfully implemented to treat nitrate-polluted groundwater in a rural village of south Spain to produce water of drinking quality. We found the AGS plant was successful at removing nitrate with a minimal input of organic matter, thus showing this technology can achieve not only optimal biological nitrate removal but also at a minimal cost. We also found the hydraulic retention times and organic matter loading rate can be used to improve the performance of the system to remove nitrate thus illustrating how AGS technology can be optimized to remove varying concentrations of nitrate present in the groundwater. The abundance of denitrifiers remained stable in these AGS systems fed with nitrate-contaminated groundwater indicating that microbes with the ability to remove nitrate can adapt to contrasting hydraulic retention times and organic matter loading rates. While there were changes in the composition of the prokaryotic microbiome, these were related to variations in nitrate and organic matter removal and again illustrated the ability of the prokaryotic microbiome to adapt to different operational conditions. Microbes are the key drivers of nitrate removal in AGS systems fed with nitrate-contaminated groundwater, as specific prokaryotic taxa in the granular microbiome were related to nitrate and organic matter removal. Future studies should examine the scale-up of AGS technology so they can be implemented not only in rural villages but larger municipalities by increasing the size and/or the number of the bioreactors. In addition, it

should be explored whether other type of AGS systems (e.g., continuous-flow) could improve treatment performance of groundwater contaminated with nitrate.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Application of Full-Scale Aerobic Granular Sludge Technology for Removing Nitrate from Groundwater Intended for Human Consumption

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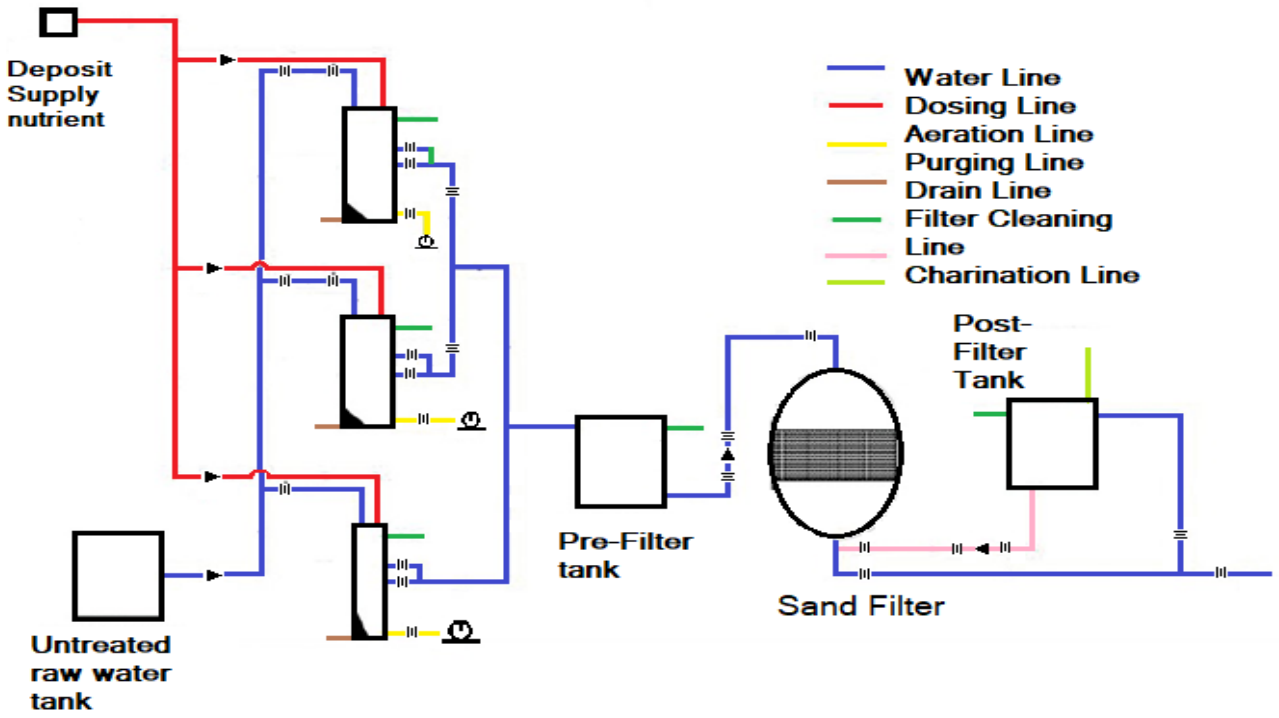
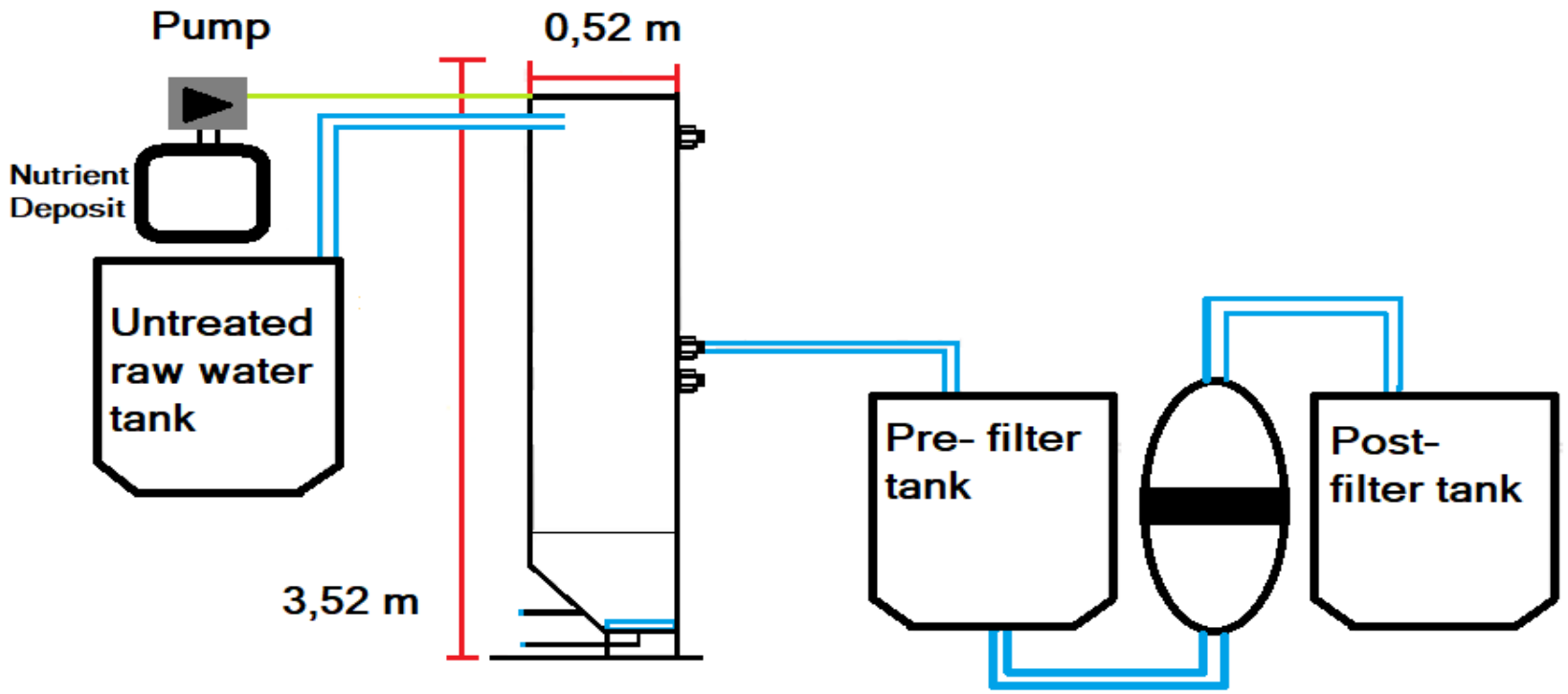
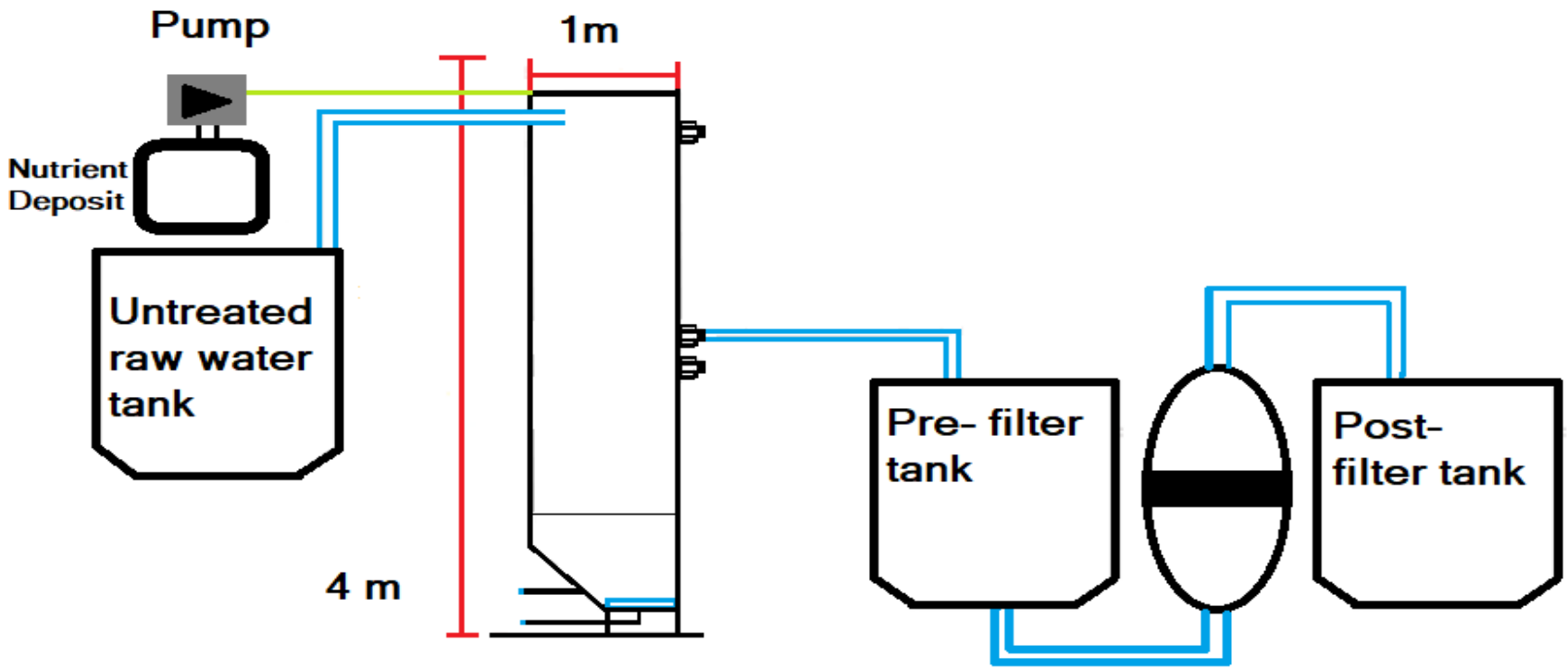


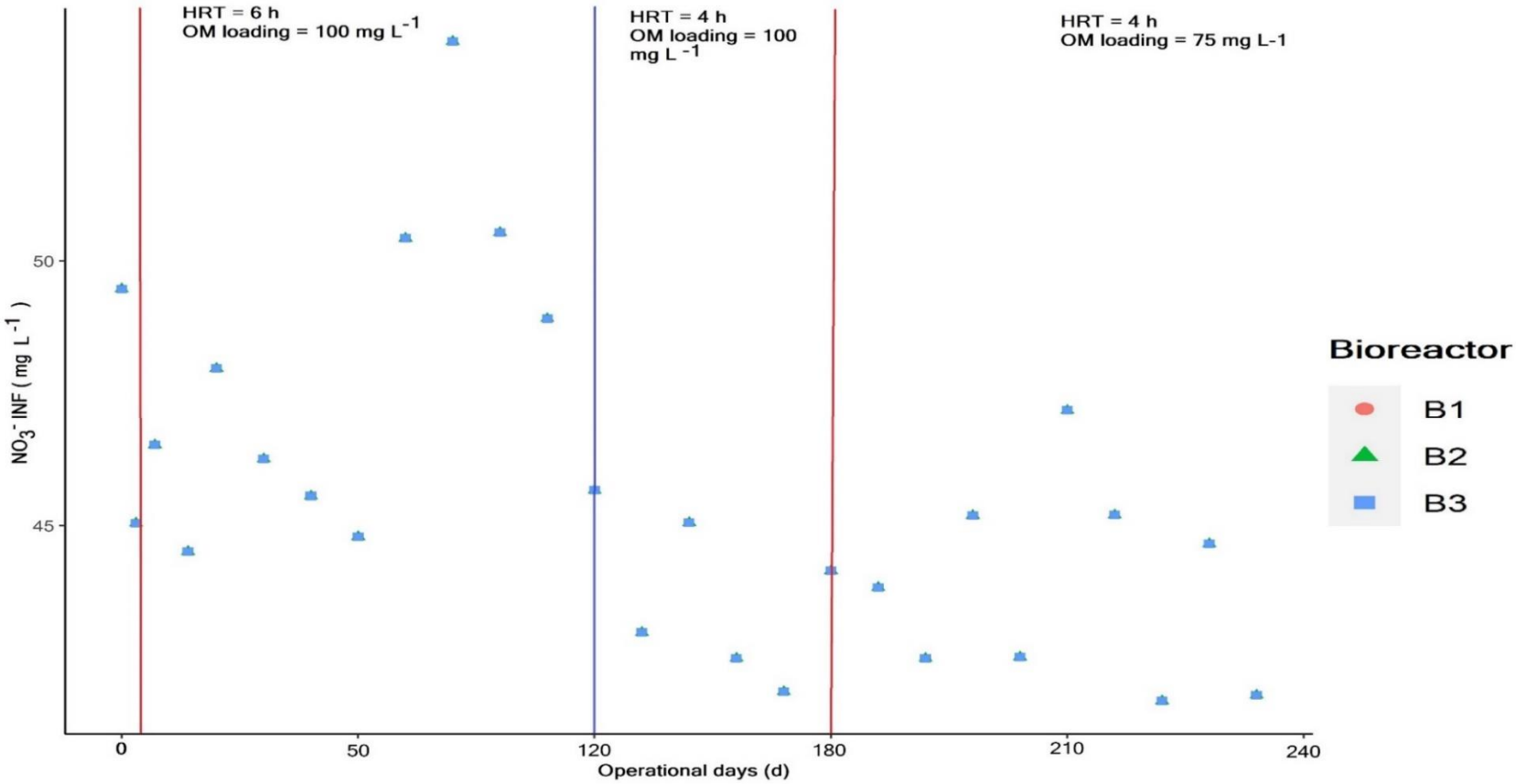
Supplementary Fig. S1. General scheme of the installation.



Supplementary Fig. S2. General diagram of bioreactor 1.



Supplementary Fig. S3. General scheme of bioreactors 2 and 3.



Supplementary Fig. S4. Variation of the nitrate concentration in the influent during the experimental period.

Supplementary Table S1. Composition of the nutritional supplement added to groundwater.

Reactive	**Start-up (mg L^{-1})**	**Stage I (mg L^{-1})**	**Stage II (mg L^{-1})**	**Stage III (mg L^{-1})**
Anhydrous sodium acetate ($C_2H_3NaO_2$)	150	100	100	75
Magnesium sulfate heptahydrate ($MgSO_4*7H_2O$)	7	7	7	7
Dipotassium hydrogen phosphate (K_2HPO_4)	7.2	7.2	7.2	7.2
Monopotassium hydrogen phosphate (KH_2PO_4)	2.1	2.1	2.1	2.1
Potassium chloride (KCL)	3.1	3.1	3.1	3.1

Supplementary Table S2. Annual average temperature recorded in the municipality of Torre-Cardela.

	Jan.	**Feb.**	**Mar.**	**Apr.**	**May.**	**Jun.**	**Jul.**	**Ago.**	**Sept.**	**Oct.**	**Nov.**	**Dic.**
Maximum	9 ºC	11 ºC	15 ºC	17 ºC	22 ºC	28 ºC	32 ºC	31 ºC	26 ºC	19 ºC	13 ºC	10 ºC
Temp.	2 ºC	4 ºC	7 ºC	10 ºC	14 ºC	20 ºC	23 ºC	23 ºC	18 ºC	12 ºC	7 ºC	3 ºC
Minimum	-3 ºC	-1 ºC	1 ºC	3 ºC	7 ºC	11 ºC	14 ºC	14 ºC	10 ºC	6 ºC	1 ºC	-2 ºC

Supplementary Table S3 Parameters analyzed in the water treated at the facility that guarantee its use as water for human consumption.

DETERMINATIONS "IN SITU"						
Parameter	**Method**	**LIM.QUANT**	**RD 140/2003**	**Result**	**Standard error**	**Units**
Total chlorine "in situ"	-	-	-	0.6	-	mg/L Cl2
Free residual chlorine "in situ	-	-	-	0.6	-	mg/L Cl2
In situ combined chlorine	-	-	2.0 mg/L Cl2	0	-	mg/L Cl2
Temperature "in situ	-	-	-	9	-	ºC
LABORATORY RESULTS						
Parameter	**Method**	**LIM.CUANT**	**RD 140/2003**	**Result**	**Standard error**	**Units**
Microbiological parameters						
Escherichia coli	UNE-EN ISO 9308-1	-	0 UFC/100ml	0	-	UFC/100ml
Enterococos	UNE-EN ISO 7899-2	-	0 UFC/100ml	0	-	UFC/100ml
Clostridium perfringens	UNE-EN ISO 14189	-	0 UFC/100ml	0	-	UFC/100ml
Chemical parameters						
Nitrates	CI/002-a	0,50 mg/L	50 mg/L	34	±7	mg/L

Nitrites	COL/007-a	0,010 mg/L	0.1 mg/L	<0.010	-	mg/L
Fluoride	CI/002-a	0,015 mg/L	1.5 mg/L	0.24	±0.02	mg/L
Total Cyanides	EA/019-a	12 µg/L	50 µg/L	<12	-	µg/L
Antimony	ICP-MS/002-a	1,0 µg/L	5.0 µg/L	<1,0	-	µg/L
Arsenic	ICP-MS/002-a	1,0 µg/L	10 µg/L	<1,0	-	µg/L
Selenium	ICP-MS/002-a	1,0 µg/L	10 µg/L	3.8	±0.5	µg/L
Boron	ICP-MS/002-a	0,010 mg/L	1.0 mg/L	0.043	±0.006	mg/L
Cadmium	ICP-MS/002-a	1,0 µg/L	5.0 µg/L	<1.0	-	µg/L
Copper	ICP-MS/002-a	0,010 mg/L	2.0 mg/L	<0.010	-	mg/L
Chromium	ICP-MS/002-a	5,0 µg/L	50 µg/L	<5.0	-	µg/L
Mercury	ICP-MS/002-a	0,10 µg/L	1.0 µg/L	<0.10	-	µg/L
Nickel	ICP-MS/002-a	1,0 µg/L	20 µg/L	1	±0.1	µg/L
Lead	ICP-MS/002-a	1,0 µg/L	10 µg/L	<1.0	-	µg/L
Benzo(a) Pyrene	CGM/019-a	0,003 µg/L	0.010 µg/L	<0.003	-	µg/L
HPA	CGM/019-a	0,012 µg/L	0.100 µg/L	<0.012	-	µg/L
Benzo (b) Fluoranthene	CGM/019-a	0,003 µg/L	-	<0.003	-	µg/L
Benzo (k) Fluoranthene	CGM/019-a	0,003 µg/L	-	<0.003	-	µg/L
Benzo (g,h,i) Perylene	CGM/019-a	0,003 µg/L	-	<0.003	-	µg/L

Indeno (1,2,3,c,d) Pyrene	CGM/019-a	0,003 µg/L	-	<0.003	-	µg/L
1,2-Dichloroethane	CGM/024-a	0,30 µg/L	3.0 µg/L	<0.30	-	µg/L
Benzene	CGM/024-a	0,30 µg/L	1.0 µg/L	<0.30	-	µg/L
Tri +Tetrachloroethylene	CGM/024-a	1,0 µg/L	10 µg/L	<1.0	-	µg/L
Trichloroethylene	CGM/024-a	0,5 µg/L	-	<0.5	-	µg/L
Tetrachloroethylene	CGM/024-a	0,5 µg/L	-	<0.5	-	µg/L
Trihalomethanes	CGM/024-a	4 µg/L	100 µg/L	11	±3	µg/L
Chloroform	CGM/024-a	1,0 µg/L	-	1,7	±0.4	µg/L
Dichlorobromomethane	CGM/024-a	1,0 µg/L	-	3	±0.8	µg/L
Dibromochloromethane	CGM/024-a	1,0 µg/L	-	4	±1	µg/L
Bromoform	CGM/024-a	1,0 µg/L	-	2.6	±0.6	µg/L
Pesticides	-	-	0.50 µg/L	<0.50	-	µg/L
Organochlorine pesticides						
Trifluralin	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
a-HCH	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Hexachlorobenzene	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
b-HCH	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Lindane	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L

d-HCH	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Heptachlor	CGM/019-a	0,010 µg/L	0.03 µg/L	<0.010 -	µg/L
Aldrin	CGM/019-a	0,010 µg/L	0.03 µg/L	<0.010 -	µg/L
Heptachlor epoxide (isomer B)	CGM/019-a	0,010 µg/L	0.03 µg/L	<0.010 -	µg/L
Endosulfan 1	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Dieldrin	CGM/019-a	0,010 µg/L	0.03 µg/L	<0.010 -	µg/L
p,p-DDE	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Endrin	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Endosulfan 2	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
p,p-DDD	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Oxyfluorfen	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Endosulfan sulfate	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
p,p-DDT	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Organophosphorus pesticides					
Dichlorfention	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Phenchlorphos	CGM/019-a	0,010 µg/L	0,10 µg/L	<0.010 -	µg/L
Fenitrothion	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L
Ethyl-Parathion	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010 -	µg/L

Chlorpyrifos	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Methyl-Bromophos	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Ethyl-Bromophos	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Chlorfenvinphos	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Tetrachlorvinphos	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Methidathion	CGM/019-a	0,010 µg/L	0.10 µg/L	<0.010	-	µg/L
Nitrogen pesticides						
Simazine	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Atrazine	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Triethazine	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Terbuthylazine	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Amethrin	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Promethrin	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Terbutryn	CGM/019-a	0,020 µg/L	0.10 µg/L	<0.020	-	µg/L
Indicator parameters						
Odor at 25ºC	ORG/006	1 Ind. dil.	3	1	-	Ind. dil.
Flavor at 25ºC	ORG/006	1 Ind. dil.	3	1	-	Ind. dil.
Color	EA/002-a	3.0 mg/L	15 mg/L	9.7	±1.0	mg/L

Turbidity	NF/001-a	0.30 UNF	1 UNF	1	±0.1	UNF
pH	EL/002-a	4.0 Unidad pH	9,5 Unidad pH	8.5	±0.2	Unidad pH
Conductivity at 20ºC	EL/001-a	10.0 µS/cm	2 500 µS/cm	669	±53	µS/cm
Ammonium	COL/007-a	0.050 mg/L	0.50 mg/L	<0,050	-	mg/L
Chlorides	CI/002-a	0.50 mg/L	250 mg/L	24	±3	mg/L
Sodium	ICP-MS/002-a	1.0 mg/L	200 mg/L	29	±4	mg/L
Sulfates	CI/002-a	0.50 mg/L	250 mg/L	42	±6	mg/L
Oxidability	UNE-EN ISO 8467	0.50 mg/L	5.0 mg/L	<0,50	-	mg/L
Aluminum	ICP-MS/002-a	10 µg/L	200 µg/L	<10	-	µg/L
Iron	ICP-MS/002-a	5.0 µg/L	200 µg/L	<5.0	-	µg/L
Manganese	ICP-MS/002-a	5.0 µg/L	50 µg/L	<5.0	-	µg/L
Total coliforms	UNE-EN ISO 9308-1		0 UFC/100ml	0	-	UFC/100ml
Colony count at 22ºC	UNE-EN ISO 6222/1999		100 UFC/ml	11	-	UFC/ml
Langelier Index	CALCU/001-n	-3	0,5	1,2	-	-

Supplementary Table S4. Primers used for quantification of total abundance of Bacteria and Archaea (16SB and 16SA, respectively), Fungi (18S) and denitrifiers (*nir*K, *nir*S, *nos*ZI) by qPCR (A). Bacterial and archaeal strains used for the generation of qPCR standards are also included. qPCR conditions for quantification of each of the target genes (B).

A.

-	Primer sequence (5´-3´)	Target gene	Strains	Reference
EUB338F	ACTCCTACGGGAGGCAGCAG	16S rRNA Bacteria	*Streptomyces* sp. ME01	Lane et al. (1991)
EUB518R	ATTACCGCGGCTGCTGG			Muyzer et al. (1993)
Arch931F	AGGAATTGGCGGGGGAGCA	16S rRNA Archaea	*Candidatus* nitrosopumilus	Einen et al. (2008)
ArchM1100R	BGGGTCTCGCTCGTTRCC			
FungiQuant-F	GSWCTATCCCCAKCACGA	18S rRNA Fungi	*Candida albicans* ATCC 10231	Liu et al. (2012)
FungiQuant-R	GGRAAACTCACCAGGTCCAG			
nirK876F	ATYGGCGGVAYGGCGA	*nirK*	*Ensifer meliloti* 1021	Henry et al. (2004)
nirK1040R	GCCTCGATCAGRTTRTGGTT			
nirS4QF	AACGYSAAGGARACSGG	*nirS*	*Pseudomonas fluorescens* C7R12	Throbäck et al. (2004)
nirS6QR	GASTTCGGRTGSGTCTTSAYGAA			
nosZ1840F	CGCRACGGCAASAAGGTSMSSGT	*nosZ*I	*Bradyrhizobium*	Henry et al. (2006)

nosZ2090R	CAKRTGCAKSGCRTGGCAGA A	*diazoefficiens* USDA110

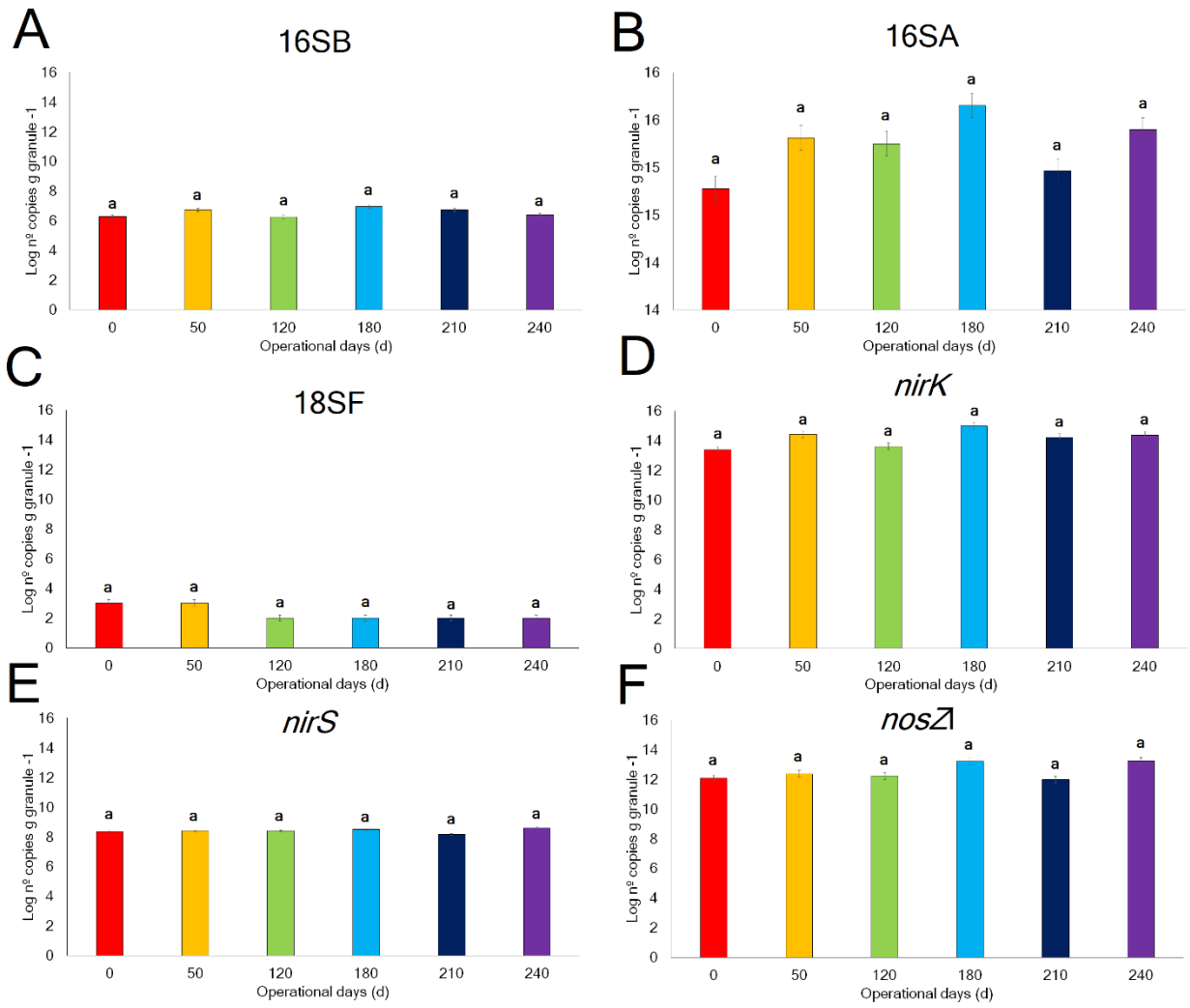
B.

	16SB	16SA	18SF	*nirK*, *nirS*	*nos*ZI
Stage 1: 1 cycle	10 min at 95 ºC	10 min at 95 ºC	3 min at 95 ºC	10 min at 95 ºC	10 min at 95 ºC
Stage 2: *nirK*, *nirS* and *nosZ*I: 6 cycles, with 1 ºC decrease per cycle				15s at 95 ºC	15s at 95 ºC
				30s at 63 ºC	30s at 65 ºC
				30s at 72 ºC	30s at 72 ºC
Stage 3: 35 cycles	15s at 95 ºC	30s at 95 ºC	30s at 94 ºC	15s at 95 ºC	15s at 95 ºC
	30s at 60ºC	30s at 60 ºC	30s at 62 ºC	30s at 58 ºC	30s at 60 ºC
	30s at 72 ºC	30s at 72 ºC	45s at 72 ºC	30s at 72 ºC	30s at 72 ºC
Stage 4: 1 cycle	10 min at 72 ºC	10 min at 72 ºC	10 min at 72 ºC	10 min at 72 ºC	10 min at 72 ºC

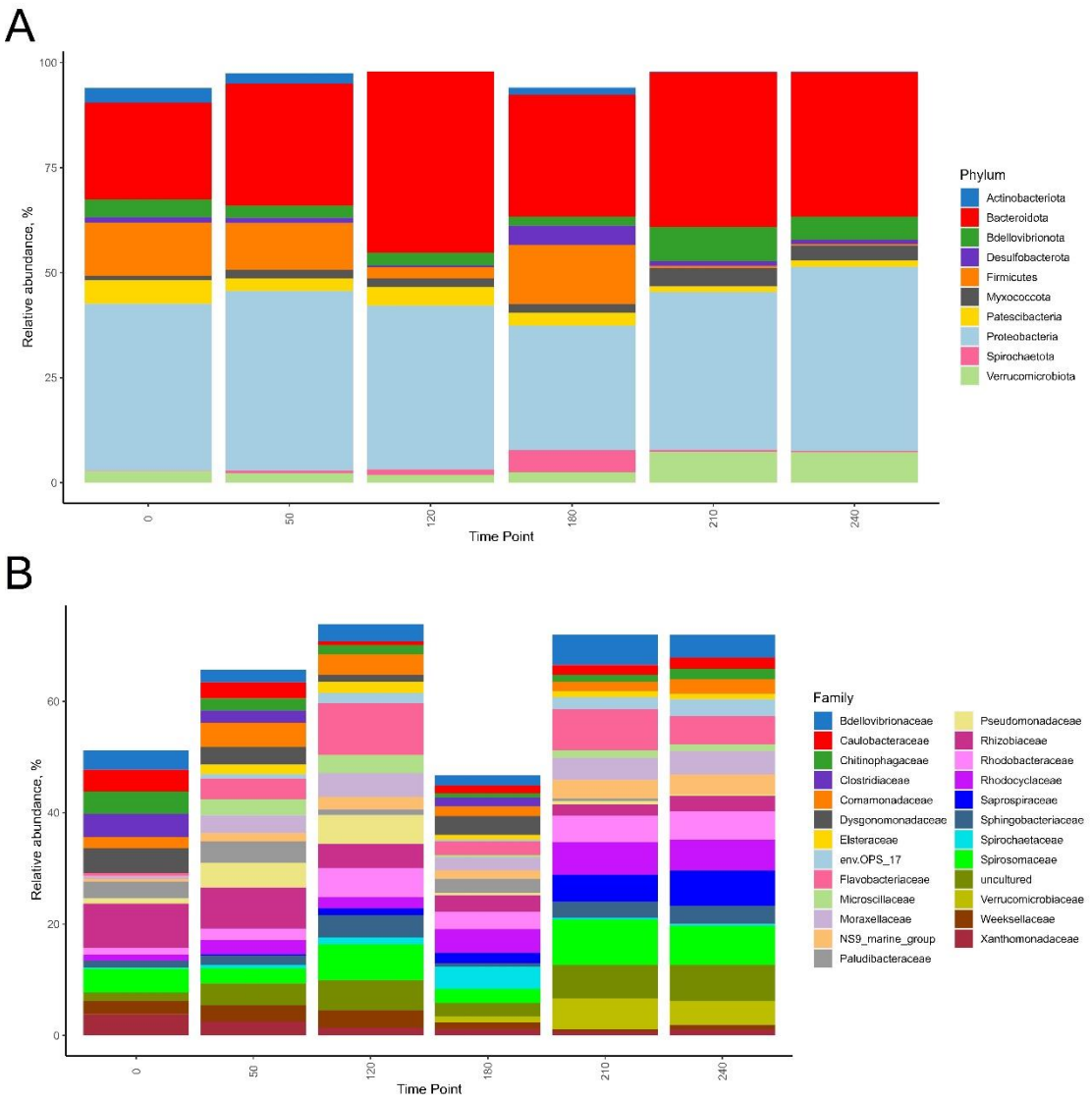
Supplementary Table S5. KOs considered per denitrification enzyme using the KEGG database.

Pathway	KOs number	Gene product
Nitrate reductase (NO_3- to NO_2-)		
	K00360	nasB; assimilatory nitrate reductase electron transfer subunit [EC:1.7.99.-]
	K00367	narB; ferredoxin-nitrate reductase [EC:1.7.7.2]
	K00370	narG, narZ, nxrA; nitrate reductase / nitrite oxidoreductase, alpha subunit [EC:1.7.5.1 1.7.99.-]
	K00371	narH, narY, nxrB; nitrate reductase / nitrite oxidoreductase, beta subunit [EC:1.7.5.1 1.7.99.-]
	K00372	nasC, nasA; assimilatory nitrate reductase catalytic subunit [EC:1.7.99.-]
	K00373	narJ, narW; nitrate reductase molybdenum cofactor assembly chaperone NarJ/NarW
	K00374	narI, narV; nitrate reductase gamma subunit [EC:1.7.5.1 1.7.99.-]
	K02567	napA; nitrate reductase (cytochrome) [EC:1.9.6.1]
	K02568	napB; nitrate reductase (cytochrome), electron transfer subunit
	K02570	napD; periplasmic nitrate reductase NapD
	K02571	napE; periplasmic nitrate reductase NapE
	K10534	NR; nitrate reductase (NAD(P)H) [EC:1.7.1.1 1.7.1.2 1.7.1.3]
Nitrite reductase (NO_2- to NO)		
	K00361	nasB; nitrite reductase [NAD(P)H] [EC:1.7.1.4]
	K00362	nirB; nitrite reductase (NADH) large subunit [EC:1.7.1.15]
	K00363	nirD; nitrite reductase (NADH) small subunit [EC:1.7.1.15]
	K00366	nirA; ferredoxin-nitrite reductase [EC:1.7.7.1]
	K00368	nirK; nitrite reductase (NO-forming) [EC:1.7.2.1]
	K00370	narG, narZ, nxrA; nitrate reductase / nitrite oxidoreductase, alpha subunit [EC:1.7.5.1 1.7.99.-]
	K00371	narH, narY, nxrB; nitrate reductase / nitrite oxidoreductase, beta subunit [EC:1.7.5.1 1.7.99.-]
	K03385	nrfA; nitrite reductase (cytochrome c-552) [EC:1.7.2.2]
	K04017	nrfF; formate-dependent nitrite reductase complex subunit NrfF
	K04018	nrfG; formate-dependent nitrite reductase complex subunit NrfG
	K15864	nirS; nitrite reductase (NO-forming) / hydroxylamine reductase [EC:1.7.2.1 1.7.99.1]
	K15876	nrfH; cytochrome c nitrite reductase small subunit
	K17877	NIT-6; nitrite reductase (NAD(P)H) [EC:1.7.1.4
	K19343	nirI; NosR/NirI family transcriptional regulator, nitrite reductase regulator

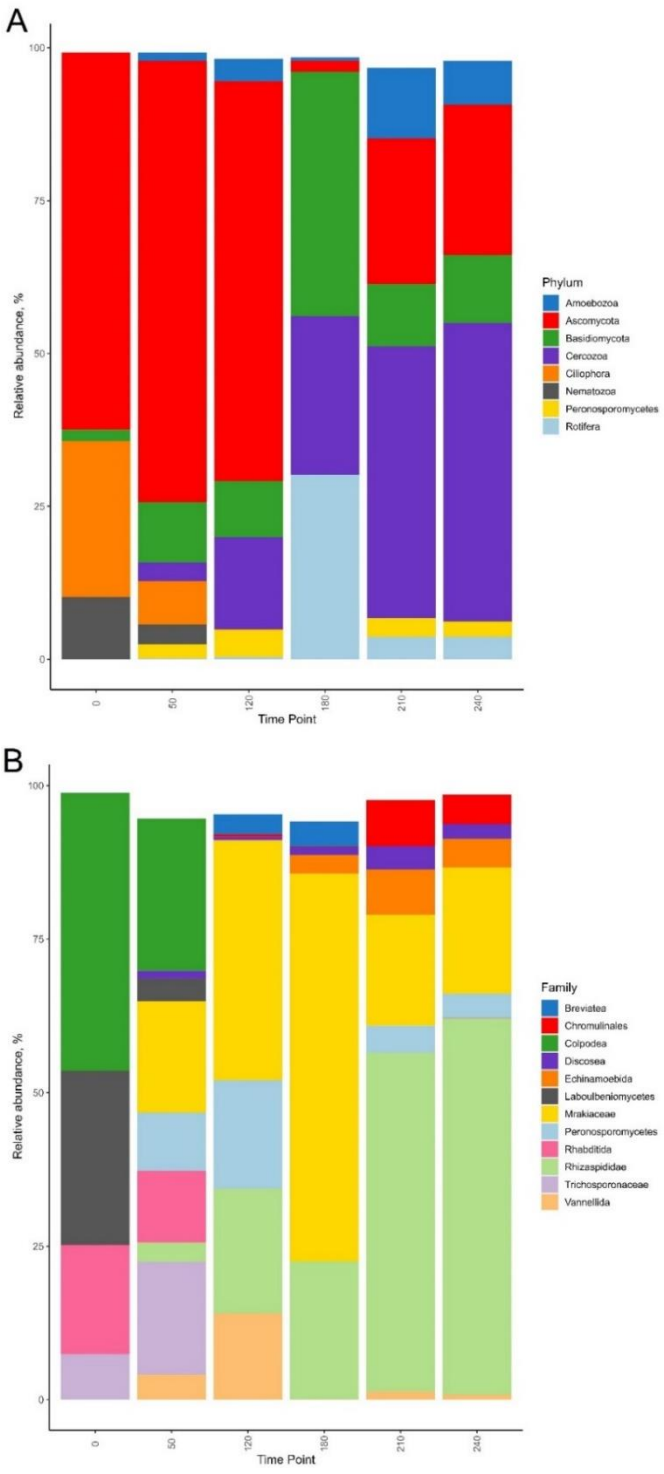
	K26138	nasE; nitrite reductase [NAD(P)H] small subunit [EC:1.7.1.4]
	K26139	nasD, nasB; nitrite reductase [NAD(P)H] large subunit [EC:1.7.1.4]
Nitric oxide reductase (NO to N_2O)		
	K02164	norE; nitric oxide reductase NorE protein
	K02305	norC; nitric oxide reductase subunit C
	K02448	norD; nitric oxide reductase NorD protein
	K04561	norB; nitric oxide reductase subunit B [EC:1.7.2.5]
	K04747	norF; nitric oxide reductase NorF protein
	K04748	norQ; nitric oxide reductase NorQ protein
	K12264	norV; anaerobic nitric oxide reductase flavorubredoxin
	K12265	norW; nitric oxide reductase FlRd-NAD(+) reductase [EC:1.18.1.-]
	K12266	norR; anaerobic nitric oxide reductase transcription regulator
	K15877	CYP55; fungal nitric oxide reductase [EC:1.7.1.14]
Nitrous oxide reductase (N_2O to N_2)		
	K00376	nosZ; nitrous-oxide reductase [EC:1.7.2.4]
	K19339	nosR; NosR/NirI family transcriptional regulator, nitrous oxide reductase regulator



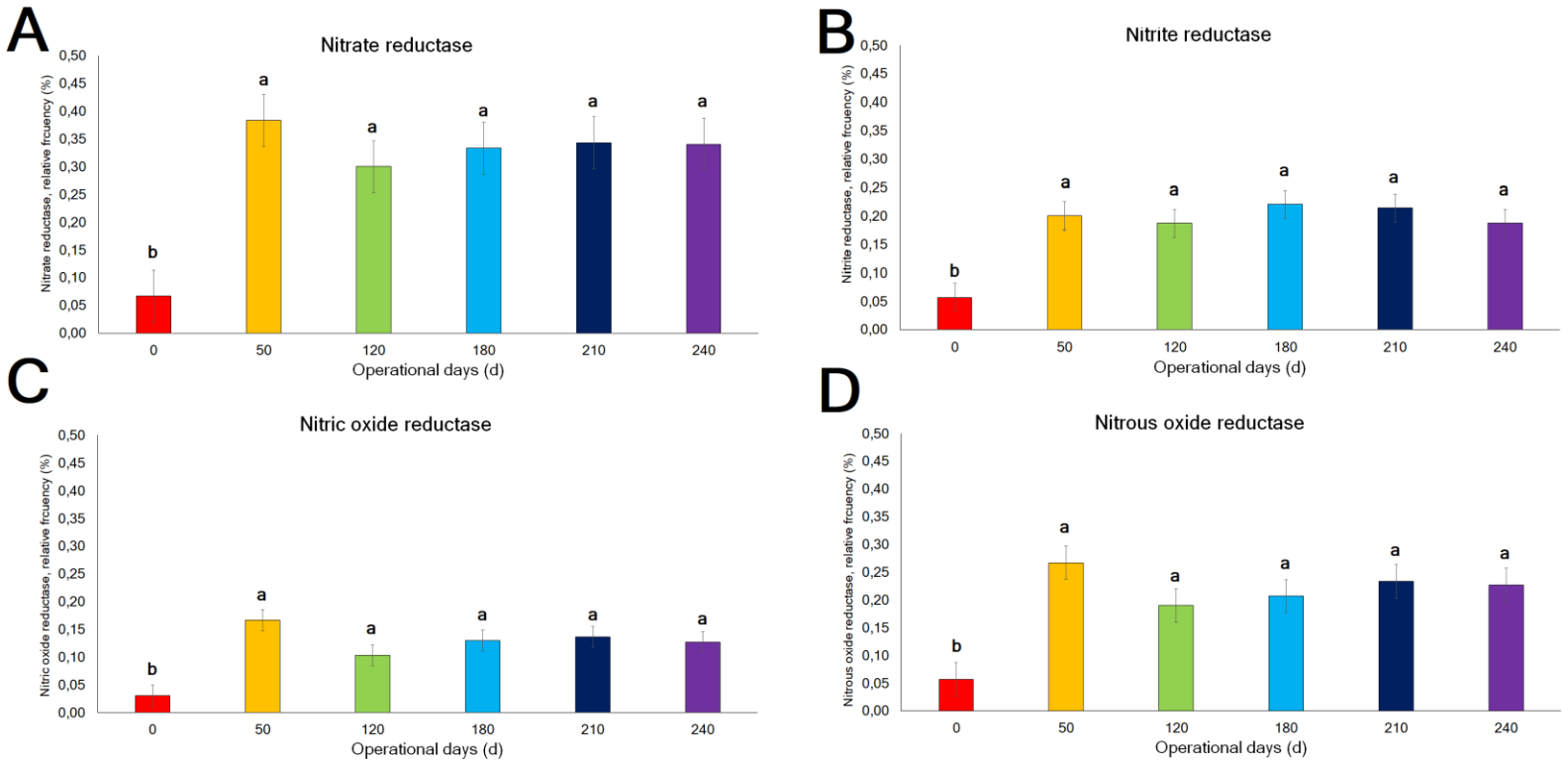
Supplementary Fig. S5. Total abundance of 16SB (A), 16SA (B), 18SF (C) communities, and *nirK* (D), *nirS* (E), and *nosZ*I denitrification genes during the experimental period. Bars marked with the same letter are not significantly different ($p < 0.05$). Values are expressed as mean with standard error.



Supplementary Fig. S6. Relative abundance of prokaryotic ASVs at the phylum (A) and family (B) taxonomic levels during the experimental period. Phyla and families with at least 1% of relative abundance are shown.



Supplementary Fig. S7. Relative abundance of eukaryotic ASVs at the phylum (A) and family (B) taxonomic levels during the experimental period. Phyla and families with at least 1% of relative abundance are shown.



Supplementary Fig. S8. Changes in the relative frequency (%) of predicted denitrification functions during the experimental period. Bars marked with the same letter are not significantly different ($p < 0.05$). Values are expressed as mean with standard error. The KEGG pathway categories and KOs considered are shown in Supplementary Table S4.

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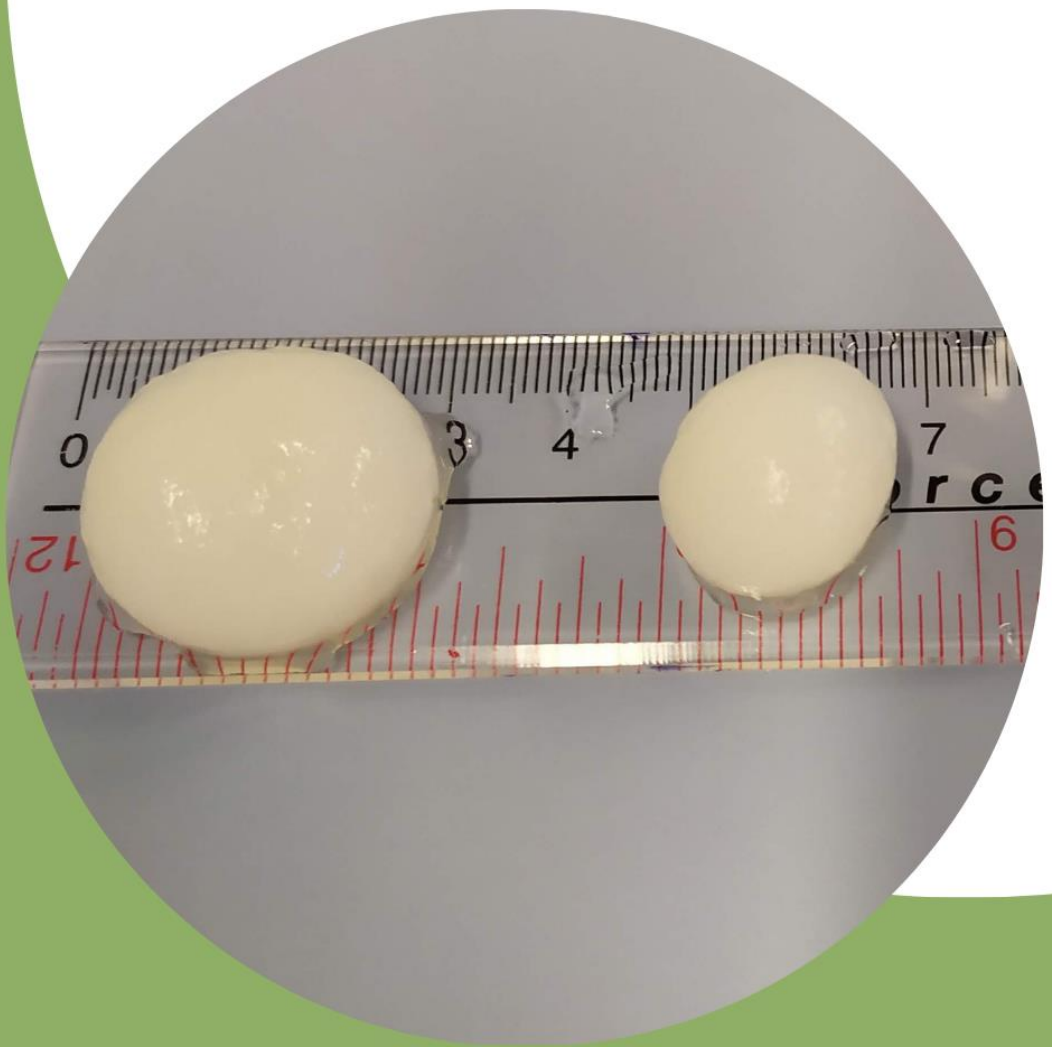
5.GENERAL DISCUSSION



5. General Discussion

Water used for human consumption is exposed to a wide range of polluting substances of both organic and inorganic origin multitude of contaminants. These must be removed to make it fit for human consumption. One of the main water resources for human supply is groundwater, which is oftenly nitrate polluted. This study proposes an alternative solution to the existing problem of nitrogen compounds in groundwater for human consumption.

There are a multitude of physicochemical processes that completely removal nitrates from water, However, these systems, regardless of their high economic cost and brine generation represent an important environmental problem. This is why biological denitrification is an environmentally friendly alternative with low operating costs.

In this study, we have demonstrated the capacity of an AGS system for the denitrification of groundwater. First of all carrying out laboratory-scale experiments to finally design and build a real plant that would allow the supply of drinking water to a town (Torre-Cardela, Granada), with all the technical and health guarantees. Although the capacity of sequential granular aerobic systems for nitrate removal in different effluents, including groundwater, has been previously described (Muñoz-Palazon et al., 2023) but so far, they have not been put into practice in a real and fully operational installation.

One of the main goals of this research was to assess the performance of the selected *Pseudomonas* denitrifying strains in removing nitrate from groundwater within aerobic granular systems. The nitrogen removal capacity was studied to characterize the most efficient carbon source, and the C/N ratio was assessed to optimize the denitrification process in aerobic systems containing granular biomass produced by *Pseudomonas* strains. When concentrations of sodium acetate below 400 $mg \cdot L^{-1}$ were used in the GSBRs, a remarkable reduction was observed in the system's capacity to

remove nitrate from the groundwater. Nitrate removal was around 50% when 200 to 300 $mg \cdot L^{-1}$ sodium acetate was used, being even lower if the bioreactor was inoculated with *P. fluorescens*. It is therefore evident that a high C:N ratio is necessary for good nitrate removal performance when acetate is used as a carbon source. These results confirm previous data published by (Tian et al. 2020), who observed that high nitrate removal capacity in biological systems was directly related to acetate concentration, indicating that a C:N ratio of approximately three to five was the most appropriate ratio to support the metabolism of heterotrophic denitrifiers (Eljamal et al., 2006). Since the C:N ratio is crucial for nitrate removal from groundwater, the amount of carbon source required should be determined as a function of the level of nitrate pollution, as established by the European Water Framework (2000). (Knoll et al., 2019). Thus, the acetate concentration can be adjusted to account for the degree of groundwater contamination in the production of potable quality water. This biological system was successful in reducing the nitrate concentration from groundwater. For even higher nitrate concentration reduction, other methods based on physic-chemical processes have been proposed to reach removal values close to 90%, such as those reported by (Eljamal et al., 2006 ; Mokete et al. 2016) based on the use of nanoparticles.

Some differences can be observed when the GSBR systems are fed with methanol. The addition of methanol at concentrations of 250 and 300 $mg \cdot L^{-1}$ resulted in efficient nitrate removal when the systems are inoculated with *P. stutzeri* and *P. fluorescens* strain 376, applying a C: N ratio close to two. In some cases, nitrate removal above 70% was observed, suggesting that denitrification occurs at lower organic matter/nitrate ratios than when acetate provides the carbon source (Yang et al., 2012; C. Li et al., 2015). It is possible that these yields, as in the case of acetate, can be increased with the addition of larger amounts of methanol, but if we consider that the regulations established by the European Water Framework (2000), in our opinion, it is clear that methanol is an appropriate carbon source for the removal of nitrates from groundwater. Moreover, *P. stutzeri* strains were reported as denitrifying microorganisms under aerobic conditions (Sun et al., 2017), supporting the results observed in this research.

Our results showed that denitrification process can be efficiently performed with different carbon sources such as acetate and methanol. Therefore. inoculation and enrichment of GSBR systems with culture of selected denitrifying strains carrying the *nos*Z gene may be considered as an innovative alternative in order to improve AGS technology. Moreove, our data shown that the trend of denitrifying granular sludge for treating nitrate-polluted groundwater was opposite to the results obtained in several studies about granular biomass for treating wastewater (Muñoz-Palazon et al., 2019 and 2020). These results allow confirming the nitrate removal by denitrification process carry out by denitrifying bacteria despite the presence of oxygen, because the granular biomass allows the coexistence of aerobic zone and anoxic-anaerobic zone in interlayer and in the core of the granules. In this mention position, the denitrification process take place in anaerobic conditions by denitrifying bacteria which containing *nor*B and *nos*Z genes, as it had been verified by the results obtained from absolute quantification. In addition, the results of qPCR corroborated the competitive advantage of denitrifiers microorganisms containing *nos*Z gene (nitrous oxide conversion manager into dinitrogen gas) in a denitrifying granular biomass biosystems versus aerobic granular sludge systems for treating wastewater, which are clearly focus on ammonia oxidation.

In order to obtain conclusions about the technology at real scale, it is relevant taken into account the influent characterization of the treated water, because related of the composition the optimal operational conditions and supplementary would be different. In the case the nitrate polluted groundwater between 50–100 mg NO_3^- L^{-1}, lower ratio C: N will conform smaller and denser granules, and the removal performance efficiency is high meet the drinking water European Water Framework (2000). In the case of higher concentration of nitrate polluted groundwater (> 120 mg NO_3^- L^{-1}), the C: N must to higher in order to get higher removal performance ratio, in these cases the size of the granules will be larger but the distance from external layer to core of the granules allow the existence of a strong gradient of oxygen and nutrients as occurs with low C: N. The optimal mode of operation will be totally related to the groundwater in-situ conditions, although slight guidelines could be given for instance: C:N ratio ranging from minimum 1 to maximum 4, the hydraulic retention time could be shorter than 6 h depending of necessities, the up velocity of granules into bioreactor is very important to

keep the density and conformation of them and finally the circular and continuous motion of the circulation of biomass is essential for long-term operation. In conclusion, the main advantages are the robustness and capacity to resist against diverse conditions and changes in the groundwater polluted composition (even in industrial effluent with high nitrate concentration), while that the main limitation is stablish the optimum C:N ratio depending of the necessities required for effluent quality, in order to save cost of supplementary compounds.

The granular biomass formedin AGS reactor treated groundwater was compact and dense with average values of mean size and settling velocity of 4.0 mm and 40 mh^{-1}, respectively. The prokaryotic and eukaryotic communities is certainly diversa, although the dominant prokaryotic phylotypes are related to influent composition, belonging to *Comamonadaceae, Rhizobiales*, *Acinetobacter* and *Pseudomonas*. The dominant eukaryotic phylotype was affiliated to *Haematococcus microalgae.*

Our data suggest that under real conditions and amended with real nitrate-polluted groundwater and lowest concentrations of carbon source (0.15 g NaAc L^{-1}) and mineral nutrients, the nitrate removal ratio of the AGS system was greater than 70 %, and the nitrate concentration in the treated groundwater was always lower than recommended values for drinking water (25 mg NO_3^- L^{-1}). In addition, nitrite and ammonium were never detected in the effluent. Organic matter was removed with high efficiency (over 98.0 %). The microbial population was dynamics during the operation, although a clear proliferation of heterotrophic denitrifying bacteria was found, being the most dominant phylotypes belonged to *Rhodobacteraceae*, *Pseudomonas* and *Comamonas*. Eukaryotic community showed a great number of Otus affiliated to *Haematococcus microalgae* which had nitrogen source and environmental conditions to proliferate in this system. Therefore, granular biomass systems (SBR) can be an innovative and efficient technology for the treatment of nitrate-polluted groundwater under low-cost operating conditions and without any of brine.

Our results showed that the implementation of a full-scale AGS technology includind a filtration and disinfection units was successful in removing NO_3^-. We found the percent NO_3^- removed gradually increased from 25% to more than 50% from the

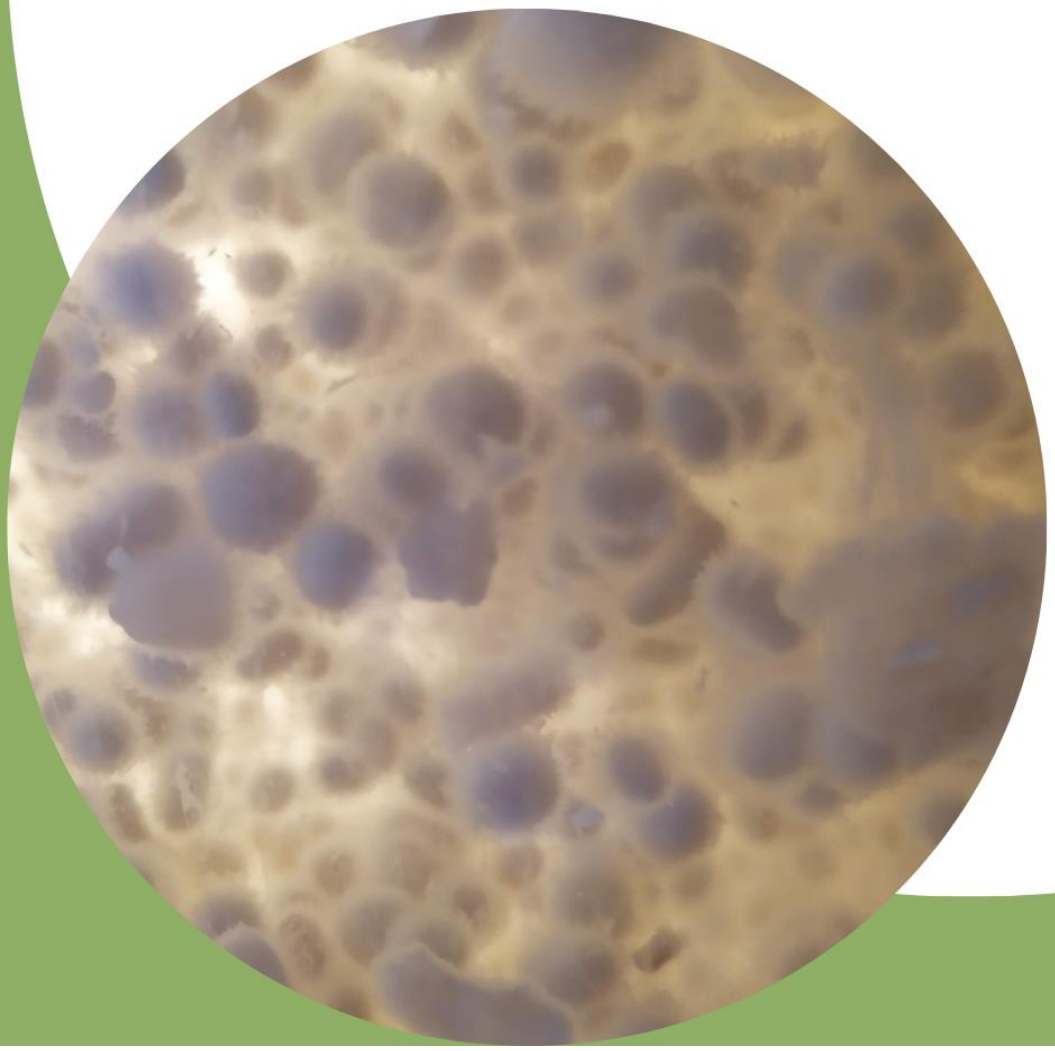
start-up phase and remained stable afterwards until the end of the experimental period. Regardless of the HRT and OML rate, the COD and BOD_5 removal rates were greater than 90% and 80%, respectively, during the experiment showing microorganisms rapidly consumed organic compounds. These results indicate the system was able to efficiently remove NO_3^- with very low OM concentration ($\leq$ 100 mg L^{-1}) reaching a C:N ratio close to 1:1. The maintenance of a stable percentage of NO_3^- removal with a OML rate of 75 mg L^{-1} and 100 mg L^{-1} showed that the system was operating with excess OM at 100 mg L^{-1} of OML. It is important to note that the NO_3^- concentration in the influent decreased after 100 days of operation to values lower than 50 mg L^{-1}. This allowed an optimization of the bioreactors by decreasing the OML rate and HRT, allowing them to perform a greater number of cycles and to treat more water per day. This feature is important in biological water treatment such as aerobic granular technology because it allows adjustment of the number of cycles and the amount of OM according to the characteristics of the water to be treated. Moreover, the stability in the abundance of denitrification genes agrees with the percent of NO_3^- removed during normal operation of the AGS system. Together, these results suggest biomass in this full-scale AGS system created oxygen-limiting conditions allowing for denitrification of nitrate-contaminated groundwater. The presence of *nir*K, *nir*S, and *nos*ZI-type denitrifiers in the microbiome of the granules suggest that microorganisms with the ability to perform complete denitrification also reduced the release of the greenhouse gas N_2O while treating the contaminated groundwater.

Our results showed that the quality of the treated water was safe for human consumption. Anlysis of 48 parameters regulated by the Real Decreto 140/2003 of the Spanish regulation were within the values required for drinking water intended for human consumption. Therefore, our results showed, for the first time, that a full-scale AGS system treating NO_3^- contaminated groundwater can be not only used to eliminate NO_3^- but to produce water that meets the quality standards to be used as drinking water.

Our study clearly confirms that aerobic granular sludge technology can be successfully implemented to treat nitrate-polluted groundwater to produce water of drinking quality. We found that AGS plants are successful at removing nitrate with a

minimal input of organic matter, thus showing this technology can achieve not only optimal biological nitrate removal but also at a minimal cost. We also report that the HRT and OM loading rate can be used to improve the performance of the technology to remove nitrate thus illustrating how AGS systems can be optimized to remove varying concentrations of nitrate present in the groundwater. The abundance of denitrifiers remained stable in these AGS systems fed with nitrate-polluted groundwater indicating that microbes with the ability to remove nitrate can adapt to contrasting HRT and OM loading rates. While there were changes in the composition of the prokaryotic microbiome, these were related to variations in nitrate and organic matter removal and again illustrated the ability of the prokaryotic microbiome to adapt to different operational conditions. Microbes are the key drivers of nitrate removal in AGS fed with nitrate-polluted groundwater, as specific prokaryotic taxa in the granular microbiome were related to nitrate and organic matter removal.

6.GENERAL CONCLUSIONS



6.General Conclusions

The main conclusions that can be obtained from our study could be stated as:

1. Denitrification process can be efficiently performed in AGS systems with different carbon sources such as acetate and methanol. Moreover, the inoculation and enrichment of GSBR systems with culture of selected denitrifying strains carrying the *nos*Z gene may be considered as an innovative alternative in order to improve AGS technology.

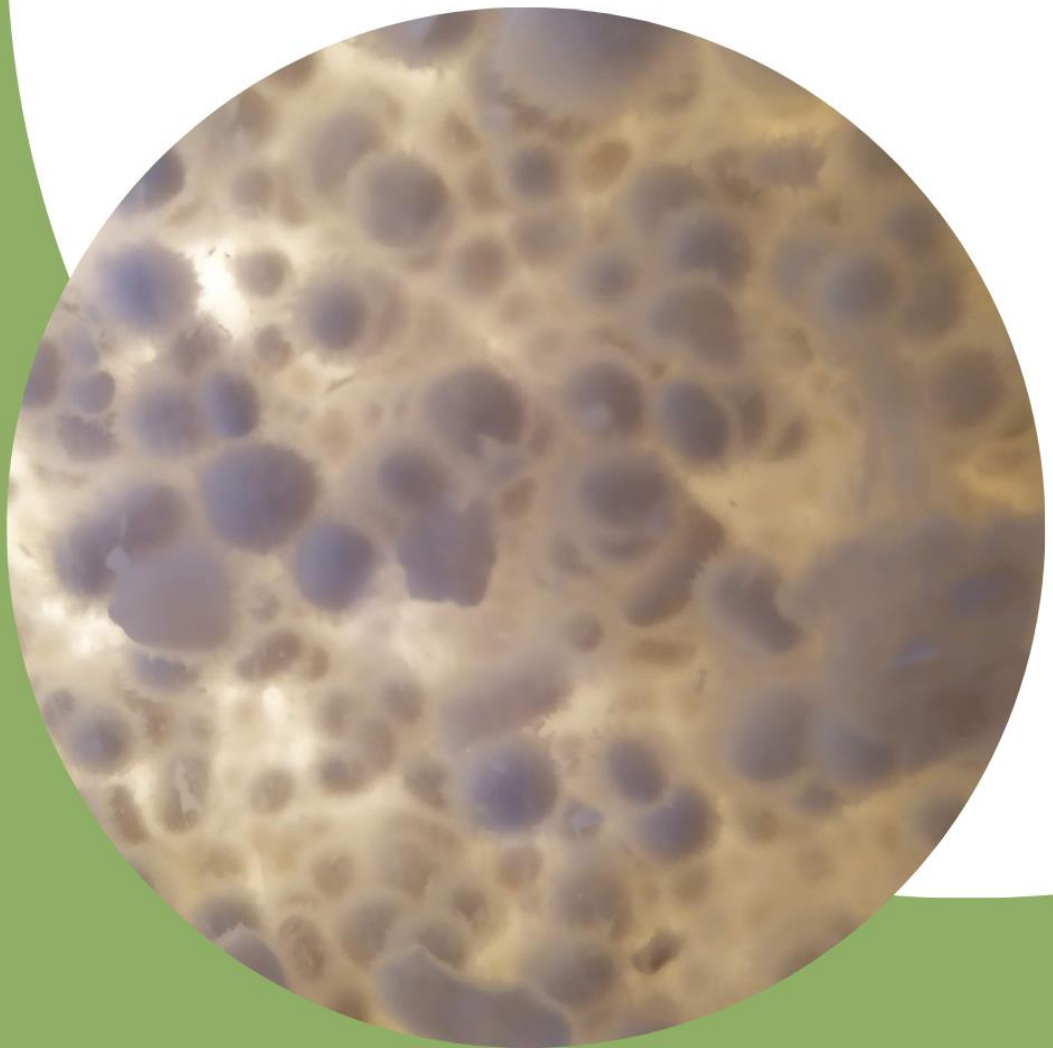
2. Denitrification process carry out by denitrifying bacteria in AGS systems despite the presence of oxygen, because the granular biomass allows the coexistence of aerobic zone and anoxic-anaerobic zone in interlayer and in the core of the granules.

3. At real conditions with nitrate-polluted groundwater and lowest concentrations of carbon source, nitrate removal ratio of the AGS system was greater than 70 %, and the nitrate concentration in the treated groundwater was always lower than 25 mg NO_3^- L^{-1}. Therefore, granular biomass systems (SBR) can be an innovative and efficient technology for the treatment of nitrate-polluted groundwater under low-cost operating conditions and without brine production.

4. The implementation of AGS systems at full-scale including a filtration and disinfection units was able to efficiently remove NO_3^- with very low organic matter concentration ($\leq$ 100 mg L^{-1}) reaching a C:N ratio close to 1:1.

5. Aerobic granular sludge system treatment nitrate-polluted groundwater produce water that meets the quality standards to be used as drinking water according to the Real Decreto 140/2003 of the Spanish normative.

7.CONCLUSIONES GENRALES



7. Conclusiones generales

Las principales conclusiones que pueden obtenerse de nuestro estudio podrían enunciarse así:

1. El proceso de desnitrificación puede llevarse a cabo eficientemente en sistemas AGS con diferentes fuentes de carbono como el acetato y el metanol. Además, la inoculación y enriquecimiento de sistemas GSBR con cultivos de cepas desnitrificantes seleccionadas portadoras del gen *nos*Z, puede considerarse como una alternativa innovadora para mejorar la tecnología AGS

2. El proceso de desnitrificación se lleva a cabo por bacterias desnitrificantes en sistemas AGS a pesar de la presencia de oxígeno, ya que la biomasa granular permite la coexistencia de zona aeróbica y zona anóxica-anaeróbica en la capa intermedia y en el núcleo de los gránulos.

3. En condiciones reales con aguas subterráneas contaminadas por nitratos y concentraciones mínimas de fuente de carbono, el ratio de eliminación de nitratos del sistema AGS fue superior al 70 %, y la concentración de nitratos en las aguas subterráneas tratadas fue siempre inferior a 25 mg NO_3^- L^{-1}. Por lo tanto, los sistemas de biomasa granular (SBR) pueden ser una tecnología innovadora y eficiente para el tratamiento de aguas subterráneas contaminadas con nitratos en condiciones de operación de bajo coste y sin producción de salmuera.

4. La implementación de sistemas AGS a escala real incluyendo una unidad de filtración y otra de desinfección fue capaz de eliminar eficazmente NO_3^- con muy baja concentración de materia orgánica ($\leq$ 100 mg L^{-1}) alcanzando una relación C:N cercana a 1:1.

5. El sistema de tratamiento aeróbico de lodos granulares de aguas subterráneas contaminadas con nitratos produce agua que cumple las normas de calidad para ser utilizada como agua potable de acuerdo con el Real Decreto 140/2003 de la normativa española

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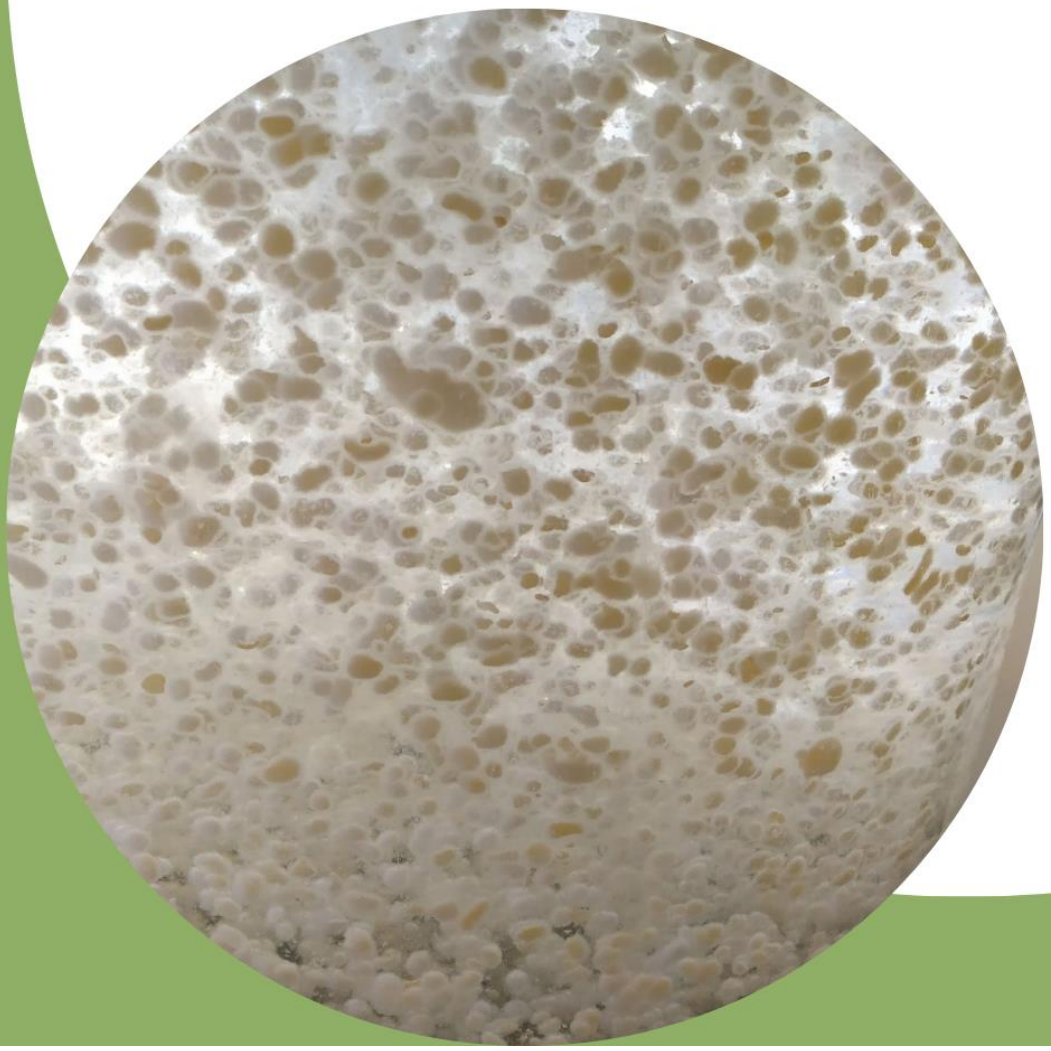

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Annex Patent P202230988

DESCRIPCIÓN

Procedimiento de obtención de biomasa granular acondicionada para sistemas oligotróficos, biomasa y biopelícula obtenida

Campo de la invención

La presente invención se encuadra dentro del campo de tratamiento de agua, específicamente en la producción de biomasa granular acondicionada para el tratamiento de sistemas oligotróficos.

Estado de la técnica

Según la Organización Mundial de la Salud (OMS, 1985), la ingesta de agua con alto contenido de nitratos y nitrito pueden causar efectos nocivos para la salud. Los nitratos no causan efectos dañinos por ellos mismos; sin embargo, los nitratos se reducen a nitritos durante el metabolismo humano, que son capaces de oxidar la hemoglobina, produciendo un aumento de los niveles de metahemoglobina que reduce la capacidad de la sangre para transportar oxígeno (Wright et al., 1999). Los nitritos también pueden reaccionar con las aminas, sustancias obtenidas del metabolismo de las proteínas, produciendo nitrosaminas, que son agentes potencialmente cancerígenos (Van Maanen, 1996), así como pesticidas.

Es muy común encontrar en las aguas subterráneas la presencia de compuestos contaminantes debido a la filtración a través del suelo. Por ejemplo, en la UE, el agua subterránea es un recurso estratégico, especialmente para el suministro urbano e industrial. En el sur de Europa, el papel de los acuíferos es aún más relevante, ya que el agua subterránea es un recurso estratégico durante las sequías. En Europa, los

fertilizantes minerales representan casi el 50% de los aportes de nitrógeno a los suelos agrícolas y al consumo de nitratos. La contaminación de las masas de agua por nitratos y otros contaminantes se debe principalmente al uso inadecuado o excesivo de fertilizantes y productos fitosanitarios en la agricultura.

El resultado de estos procesos es la superación del valor límite en agua en aproximadamente un tercio de las aguas subterráneas de las cuales se tiene información actualmente disponible (EEA, 2003).

Del mismo modo, la aplicación extensiva de plaguicidas representa un problema emergente que afecta de manera más significativa a los países europeos con escasas precipitaciones, como en los países mediterráneos.

Como ejemplo, se puede describir la situación de España. España tiene alrededor de 25 millones de hectáreas de uso agrícola, por lo que, miles de toneladas de productos fitosanitarios son utilizados cada lo año, lo que produce altos niveles de nitratos y de pesticidas, en las aguas subterráneas, de las áreas agrícolas (Köck-Schulmeyer et al. 2014).

El primer instrumento utilizado para abordar el problema de la contaminación de las aguas subterráneas fue la publicación de la Directiva de Nitratos (91/676 / CEE) relativa a la protección de las aguas contra la contaminación causada por nitratos de fuentes agrícolas. La presente Directiva obligaba a los Estados miembros a designar a los nitratos vulnerables zonas. En España, se han identificado 8 millones de hectáreas como zonas "vulnerables" a la contaminación por nitratos (MAGRAMA, 2012). En 1998, se publicó la Directiva sobre agua potable (98/83 / CE) con el objetivo de

proteger salud de los efectos adversos de cualquier contaminación del agua y también establecer un máximo permitido concentración de nitrato de 50 mg / l. La incorporación de esta Directiva a la legislación española se convirtió en vigente en el RD (140/2003), de 7 de febrero, por el que se establecen criterios sanitarios para la calidad del agua para se estableció el consumo.

Hoy en día, las tecnologías más utilizadas para la eliminación de estos nutrientes, para el suministro de agua, son las tecnologías de intercambio iónico, la tecnología de ósmosis inversa (OI) y los sistemas de tratamientos biológico bajo configuración de filtro sumergido.

Estos métodos tienen una eficiencia similar, por lo que la elección del tratamiento más adecuado se centra en otras características, como la producción de residuos y el consumo de energía (Panyor & Fabiani, 1996, Schoeman & Steyn, 2003). Los métodos de intercambio iónico y OI se utilizan con frecuencia, aunque estos sistemas generan grandes cantidades de agua de aguas de rechazo que requieren una gestión compleja y costosa. Mientras que los sistemas de filtro sumergido requieren de una inversión inicial muy grande por la utilización de soportes, así como, la necesidad de hacer limpiezas periódicas para evitar su colmatación.

Sin embargo, los avances actuales determinan que estos sistemas de tratamiento convencionales deban de ser innovados o sustituidos por nuevas alternativas biotecnológicas más eficientes y medioambientalmente menos impactantes

Por ello, durante la última década, los investigadores y las empresas del sector del agua, han mostrado un gran interés en el tratamiento de aguas residuales domésticas e industriales mediante la utilización de tecnologías basadas en sistemas granulares aeróbicos. La granulación aeróbica permite una mayor retención de biomasa, así como una mejor capacidad de sedimentación y resistencia a cambios bruscos en el influente y a compuestos tóxicos, en comparación con los sistemas biológicos convencionales de fango activo.

La tecnología biológica granular representa una importante innovación en el tratamiento para suministro de agua ya que, a diferencia de otros sistemas biológicos convencionales, la tecnología granular aeróbica solo necesita un biorreactor sin flujo de reciclaje, lo que en gran medida reduce los costos económicos.

El sistema granular permite la operación a 15-20 g/L de sólidos, que es mayor que el máximo de 3-5 g/L permitido en los sistemas biológicos convencionales (González-Gil Holliger, 2014). Esto lleva a menores volúmenes de reactor y, por lo tanto, ahorros económicos. Además, la biomasa granular se deposita con una velocidad >10 m/h, 10 veces más rápido que las tecnologías convencionales (Zhou et al., 2014).

Por otro lado, se ha confirmado que cada ciclo de los sistemas granulares aeróbicos necesita un tiempo de retención hidráulico (TRH) tres veces menor para lograr la misma eficiencia de eliminación de materia orgánica y nutrientes con respecto a otros sistemas (Lotito et al., 2014).

Todo esto muestra una clara ventaja económica por parte de los sistemas granulares aeróbicos.

Además, la granulación aeróbica permite una mayor retención de biomasa, así como una mejor capacidad de sedimentación y resistencia a cambios bruscos en el influente y a compuestos tóxicos, en comparación con el fango activo formado por flóculos. La estructura estratificada de los gránulos aeróbicos permite conseguir zonas aeróbicas y anaeróbicas dentro de la biomasa. Esto permite realizar múltiples procesos biológicos al mismo tiempo como por ejemplo los procesos de nitrificación, desnitrificación, eliminación de fosforo y materia orgánica, los cuales se pueden realizar de manera simultánea en un solo biorreactor bajo condiciones de aireación.

La eliminación de compuestos, por ejemplo, derivados del nitrógeno o del fósforo, especialmente en las aguas subterráneas, siempre ha sido considerada como procesos de elevada complejidad. El principal problema radica en la formación del granulo, ya que se requiere relaciones C/N adecuadas para que sea factible el proceso de granulación. De forma general, de acuerdo con las soluciones actuales, la formación del granulo requiere relaciones C/N comprendidas en el intervalo entre 5 – 12,5.

Sin embargo, en algunas situaciones, como los ambientes oligotróficos, por ejemplo, el agua subterránea, presenta una relación C/N inferior, por lo que la granulación no se ve favorecida de una forma espontánea.

La biomasa granular existente actualmente está pensada para el tratamiento de efluentes con altas concentraciones de materia orgánica y baja de nitrógeno por lo que

no alcanzan rendimientos óptimos de eliminación ni de formación granular al tratar aquellos efluentes oligotróficos, es decir, con baja carga de nutrientes. Por tanto, los sistemas de biomasa granular aeróbica actuales, al tratar efluentes oligotróficos, presentan importantes limitaciones:

- Dificultad en la formación del gránulo
- Necesidad de alta carga orgánica
- Largos periodos de puesta en marcha
- Degradación y descomposición de los gránulos
- Imposibilidad de tratar efluentes con poca concentración de nutrientes.
- Dificultad de obtener un tamaño granular óptimo para realizar procesos anaeróbicos
- Formación de biomasa granular con poca capacidad de decantación

Con el objeto de superar las limitaciones reportadas en el estado del arte en cuanto a los sistemas de biomasa granular para el tratamiento de aguas, por ejemplo, aguas subterráneas, se propone una biomasa granular aeróbica desnitrificante para dicho fin y su proceso de producción.

Descripción de la invención

A diferencia con otras soluciones actuales empleadas para el tratamiento de agua, la presente invención describe una biomasa granular desnitrificante para el tratamiento de aguas, especialmente acondicionada para sistemas oligotróficos, contaminadas con nitratos u otras sustancias como, por ejemplo, plaguicidas.

Esta biomasa granular puede formar una biopelícula desnitrificante, con la capacidad de adaptación y de operación con efluente de aguas, incluso subterráneas, para su uso en agua potable.

La biopelícula de biomasa granular está formada por gránulos. Cada gránulo está compuesto por una gran diversidad microbiana, tales como bacterias heterótrofas aeróbicas, bacterias acumuladoras de fosfato (PAO) y bacterias desnitrificantes. Estos microorganismos crecen en diferentes lugares dentro de la biomasa granular en base a las condiciones operacionales del biorreactor y a la compactación conseguida en la biomasa granular. Dado que las condiciones de desarrollo de cada uno de los microorganismos existentes en la biomasa son específicas, éstos no suelen mezclarse entre ellos, y da lugar a una biomasa granular estratificada en capas.

En este sentido, dentro de la biomasa granular se obtendrán condiciones aeróbicas (superior a 2 mg O2/L), condiciones con microaerofilia (en torno a 2 mg O2/L), y condiciones anóxicas o anaerobias. Esto permitirá obtener las condiciones óptimas para la realización de los procesos metabólicos de las diferentes poblaciones microbianas. De este modo, se podrá realizar la eliminación de los contaminantes existentes en el agua.

Por tanto, según la presente invención, la biomasa granular desnitrificante obtenida por el presente procedimiento comprende:

- Zona anaeróbica: zona interna del granulo, donde se encuentran las bacteriasdesnitrificantes,

- Zona microaerofilica: donde se encuentran las bacterias acumuladoras de fosfato
- Zona aeróbica: donde se encuentran bacterias heterotróficas formadoras de exopolisacaridos.

Por ello, en el interior de la biomasa granular, se encuentran microorganismos anaeróbicos, como las bacterias desnitrificantes. Estos microorganismos heterótrofos permiten reducir el nitrato a nitrógeno molecular. En las zonas intermedias de la biomasa granular se localizan las bacterias acumuladoras de fosfato, las cuales permiten retirar el fosfato de las aguas residuales mediante la alternancia de procesos aeróbicos y anaeróbicos. En el exterior, se disponen los organismos aeróbicos, tales como las bacterias heterotróficas aeróbicas, que son las encargadas de la formación de exopolisacáridos para la compactación granular.

De manera preferente, la zona interior, especialmente la zona anaerobia se verá beneficiada por las condiciones de operación, por lo que dicha zona presentará un mayor espesor en comparación con la zona aeróbica.

La configuración presentada por los gránulos de la biomasa aeróbica desnitrificante permite realizar procesos aeróbicos en la superficie del gránulo y, gracias a su morfología compacta con un tamaño granular, que oscila en el intervalo de 5 a 50 mm, preferiblemente entre 5 y 35 mm de diámetro y una velocidad de decantación superior a 10 m/h, preferiblemente entre 15 – 140 m/h, da lugar en el interior de los gránulos a procesos anaerobios.

El espesor de cada una de las zonas viene determinado por las condiciones operacionales durante el procedimiento de formación de la biomasa granular, tales como la aireación o la relación C/N, lo que permite interaccionar en el desarrollo de los microorganismos presentes en cada una de las zonas.

En una operación con elevada aireación, la presencia de oxígeno será elevada, y el espesor de la zona aeróbica se verá incrementado. Así, en una realización preferente de la presente invención, es la zona anaeróbica la que presenta un espesor mayor al resto de las zonas, al

tratarse de una biomasa destinada para el tratamiento de aguas con baja capacidad de nutrientes. De este modo, es preferible que el desarrollo de los microorganismos que forman dicha capa anaeróbica se vea favorecido a través de la limitación del aporte de oxígeno durante la etapa de granulación de la biomasa.

En un aspecto adicional de la invención se describe el procedimiento de producción de biomasa granular. Come se ha mencionado anteriormente, el procedimiento de formación permite definir la configuración de la biomasa granular obtenida.

Este procedimiento permite desarrollar las condiciones idóneas de Tiempo de retención hidráulico, pH, concentración de materia orgánica, tiempo de aireación, etc. para el desarrollo óptimo de una biomasa granular. El presente procedimiento de producción permite seleccionar las poblaciones microbianas necesarias para la producción de biomasa granular aeróbica con alta actividad desnitrificante que puede ser usada como inoculante para la rápida puesta en marcha de

biorreactores a escala real que puedan ser utilizados para la potabilización de aguas subterráneas.

Por tanto, las condiciones empleadas permiten poder obtener dentro del granulo zonas aeróbicas y microaerófilos muy finas y una zona anaeróbica mucho más gruesa, necesarias para poder llevar a cabo el proceso de desnitrificación, hasta en ambientes más exigentes como los sistemas oligotróficos. En otras palabras, el procedimiento de obtención de biomasa granular resulta especialmente importante ya que, debido a las bajas concentraciones de nutrientes en las aguas a tratar (por ejemplo, aguas subterráneas), se hace imprescindible la inoculación externa de una biomasa granular específica en los biorreactores, ya que el proceso de granulación directo resulta inviable al no tener suficiente concentración de microorganismos en las aguas subterráneas.

Por tanto, el procedimiento de formación de biomasa granular acondicionada para el tratamiento de sistemas oligotróficos comprende las siguientes etapas:

- Inoculación de biomasa que comprende microorganismos en un medio acuoso
- Granulación de biomasa inoculada
- Relación C/N entre 1 y 5, preferiblemente entre 1 y 3
- Entrada de aire en el tanque del biorreactor con una concentración de oxígeno en agua entre 3 - 10 mg O_2/L,
- Tiempos de Retención Hidráulica (RTH): 1 – 24 h,
- pH: 5,5 – 9,5, y

- Temperatura: 3 y 30ºC

- Formacion de la biomasa granular en el tanque del biorreactor,

- Separación biomasa granular.

A diferencia de otros procesos de formación de biomasa granular aeróbica, el procedimiento según la presente invención permite generar una biomasa granular en condiciones de baja carga orgánica, que posteriormente puede dar lugar a una biopelícula, acondicionada para ser empleada en tratamiento con baja carga orgánica. Esto hace que se pueda emplear esta solución, por ejemplo, en situaciones como las aguas subterráneas contaminadas con nitratos, donde la presencia de nutrientes es baja. Como se comentó anteriormente, la formación tradicional de la biomasa granular requiere relaciones C/N adecuadas, como son el intervalo entre 5–12,5, para que sea factible el proceso de granulación. Sin embargo, en algunas situaciones, como los ambientes oligotróficos, por ejemplo, una corriente de agua subterránea donde la relación C/N es 1 – 5, preferentemente 1 – 3. Bajo estas condiciones, la granulación no se ve favorecida de forma espontánea al carecer de la relación adecuada de C/N, luego el tratamiento de este tipo de agua presenta elevadas dificultades.

Para poder solventar este problema, en primer lugar, se requiere la inoculación selectiva del biorreactor con biomasa. Es decir, se lleva a cabo la adicción de un fango con bacterias y otros microorganismos como arqueas y hongos, provenientes de un medio externo, que permite tener un numero de microorganismos suficiente para acelerar el proceso de granulación. Posteriormente dicho fango es sometido a unas condiciones operacionales que permiten la formación de biomasa granular y su acondicionamiento a las condiciones oligotróficas desnitrificantes de estos ambientes, donde la relación C/N es baja.

Las condiciones operacionales establecidas para la producción masiva de biomasa granular aeróbica desnitrificante se encuentran favorecida por un proceso de decantación previo al vaciado, etapa por la cual la biomasa granular se prepara para la separación posterior del medio acuoso; y al movimiento circular dentro del biorreactor, sin que ello determine perdidas de biomasa. Dicho proceso de decantación se produce una vez alcanzado el rendimiento óptimo de eliminación de nutrientes mediante la detención del proceso de aireación. De esta manera, al parar la aireación, se produce la decantación de la biomasa granular al fondo del biorreactor. Esta etapa impide que se produzca la perdida de la biomasa granular durante el proceso de vaciado del agua tratada.

El movimiento circular se produce por las condiciones establecidas de aireación, las cuales crean un proceso de turbulencias continuas en el agua. Este efecto se traduce en un movimiento de la biomasa continuo, lo que permite obtener una mayor compactación de la biomasa flocular y da lugar a la biomasa granular deseada.

A diferencia de los procesos actuales, el proceso de granulación se lleva a cabo bajo condiciones de oligotrofia con una relación de C/N baja, C/N = 1 – 5, preferentemente entre 1-3, como ocurre en las aguas subterráneas. Además, se han establecido las condiciones ambientales requeridas para que bajo esas condiciones de baja concentración de nutrientes las poblaciones microbianas puedan producir los exopolisacáridos necesarios para la estabilidad del granulo, así como, para la eliminación de los contaminantes presentes en el agua.

Este procedimiento da lugar a la obtención de una biomasa granular que permite tratar todo tipo de contaminantes sin necesidad de utilizar otro biorreactor

distinto como ocurre con otros sistemas biológicos actuales. Además, gracias a la biomasa granular producida según la presente invención, se logra operar bajo condiciones con baja concentración de nutrientes sin degradarse, creando las estructuras compactas que necesita la biomasa granular, para poder realizar los procesos aeróbicos y anaeróbicos, para su óptimo rendimiento.

La capacidad de actuar bajo condiciones aerobias y anaerobias consigue mantener procesos de granulación estables a largo plazo, con unos rendimientos óptimos, incluso, bajo condiciones oligotróficas, donde la relación de C/N se encuentra entre 1 – 3 y las actuales soluciones no son operativas. Así, el rendimiento alcanzado por esta tecnología llega a niveles de eliminación de nitrógeno del 100%, además de alcanzar rendimientos del 70% en la eliminación de fosfato y del 100% de materia orgánica.

De este modo, el agua subterránea, una vez tratada, puede ser abastecida a poblaciones humanas como agua potable. Por tanto, el empleo de la biomasa granular o la biopelícula obtenida según la presente invención resulta especialmente importante para su aplicación real en los biorreactores con biomasa granular desnitrificante para el tratamiento de una corriente acuosa oligotrófica. Dicho empleo se traduce en la mejora de la puesta en marcha de los biorreactores, incrementando el rendimiento de estos equipos.

En las figuras, se muestran los siguientes elementos:

1. Zona aeróbica
2. Zona microaerofílica
3. Núcleo desnitrificante

A lo largo de la descripción y las reivindicaciones la palabra "comprende" y sus variantes no pretenden excluir otras características técnicas, componentes o pasos. Además, la palabra "comprende" incluye el caso "consiste en". Para los expertos en la materia, otros objetos, ventajas y características de la invención se desprenderán en parte de la descripción y en parte de la práctica de la invención. Los siguientes ejemplos y dibujos se proporcionan a modo de ilustración, y no se pretende que sean limitativos de la presente invención. Además, la presente invención cubre todas las posibles combinaciones de realizaciones particulares y preferidas aquí indicadas.

Breve descripción de las figuras

La Figura 1 muestra un esquema de una realización de la biomasa granular desnitrificante.

La Figura 2 muestra la evolución del tamaño del diámetro (D) granular, en mm, de las partículas de biomasa obtenidas a lo largo del tiempo (t), en días.

Descripción detallada de la invención

La presente invención describe biomasa granular desnitrificante para el tratamiento de aguas. En un segundo aspecto de la presente invención, la agrupación de un conjunto de

biomasa granular da lugar a una biopelícula desnitrificante. Tanto la biomasa granular como la biopelícula pueden ser empleada para el tratamiento de una corriente acuosa oligotrófica.

La Figura 1 muestra un esquema de una realización de la biomasa granular desnitrificante. Como se aprecia en dicha Figura 1, los microorganismos del gránulo están distribuidos en dos zonas: una zona interior y una zona exterior. En el interior, se encuentran los microorganismos anaeróbicos, como las bacterias desnitrificantes y las bacterias acumuladoras de fosfato. Estas segundas se encuentran localizadas en una zona microaerófila, donde la concentración de oxígeno todavía existe, si bien es baja. La zona microaerófila es una zona, por tanto, de enlace entre la zona anaeróbica del núcleo del gránulo, y la zona aeróbica. En su conjunto, estos microorganismos permiten eliminar o reducir el nitrógeno y el fosfato presentes en sistemas oligotróficos, por ejemplo, las aguas subterráneas. En el exterior, se disponen los organismos aeróbicos, tales como bacterias nitrificantes y heterotróficas.

Así, en una realización preferente de la presente invención, se pueden distinguir múltiples capas dentro la biomasa granular:

- Zona anaeróbica: zona interna del granulo, donde se encuentran las bacterias desnitrificantes,
- Zona microaerofilica: donde se encuentran las bacterias acumuladoras de fosfato.
- Zona aeróbica: donde se encuentran bacterias heterotróficas formadoras de exopolisacaridos.

Los microorganismos de la zona aeróbica son los encargados de formar los exopolisacaridos que permiten la compactación del granulo. Ademas, durante este proceso consumen oxigeno reduciendo, o incluso evitando, que dicho compuesto llegue a las zonas internas del granulo.

Los gránulos, con un tamaño entre 5 – 35 mm de diámetro y una velocidad de decantación superior a 10 m/h, preferiblemente entre 15 - 140 m/h, alcanzan valores de concentración de biomasa entre 0,3 – 10 g/L en un reactor de tratamiento de aguas. Estas características, sumado a la disposición estratificada de los microorganismos dentro de la biomasa granular desnitrificante, permite realizar procesos aeróbicos en la superficie del gránulo y, gracias a la morfología compacta, en el interior de los gránulos se pueden producir procesos anaerobios incluso en condiciones oligotróficas del sistema a tratar.

Paralelamente, se describe un proceso de producción de biomasa granular acondicionada para sistemas oligotróficos. Este procedimiento permite desarrollar las condiciones idóneas para poder seleccionar las poblaciones microbianas necesarias para la producción de biomasa granular aeróbica con alta actividad desnitrificante que puede ser usada como inoculante para la rápida puesta en marcha de biorreactores a escala real que puedan ser utilizados para la potabilización de aguas subterráneas.

Este procedimiento de producción de biomasa granular desnitrificante, resulta especialmente importante ya que, debido a las bajas concentraciones de nutrientes en las aguas oligotróficas, como por ejemplo las aguas subterráneas, contaminadas con nitratos, se hace imprescindible la inoculación externa de los biorreactores con biomasa, para poder acelerar el proceso de granulación en dichos sistemas.

Como se comentó anteriormente, la formación del granulo requiere relaciones adecuadas C/N, de 5-12, para que sea factible el proceso de granulación de manera espontánea. Sin embargo, en algunas situaciones, como los ambientes oligotróficos, por ejemplo, el agua subterránea, no favorecen la granulación de una forma espontánea al carecer de una relación adecuada de C/N.

A diferencia de otros procesos de formación de biomasa granular aeróbica en el proceso de la presente invención no se requiere condiciones de alta carga orgánica. Esto hace que la biomasa granular obtenida según la presente invención deba estar acondicionada previamente para su empleo posterior en sistema oligotróficos, por ejemplo, en situaciones como las aguas subterráneas contaminadas con nitratos, donde la presencia de nutrientes es baja. Es decir, a pesar de existir otras soluciones de biomasa granular, no se ha descrito una biomasa granular acondicionada para sistemas oligotróficos, donde las soluciones actuales presentan numerosas limitaciones en su aplicación.

Así, el procedimiento de obtención de biomasa granular acondicionada para sistemas oligotróficos comprende las siguientes etapas:

a) Inoculación de biomasa que comprende microorganismos en un medio acuoso; es decir, una adición selectiva de ciertos microorganismos en los biorreactores.
b) Granulación de la biomasa inoculada;
c) Formación de los gránulos de biomasa; y
d) Separación de la biomasa granular del medio acuoso

A diferencias con los procesos descritos actualmente, la granulación de los microorganismos que forman la biomasa se lleva a cabo en un medio acuoso con una carga orgánica con una relación C/N entre 1 y 5, siendo preferente la relación 1 – 3. La modificación de las condiciones de operación supone una modificación relevante en la estructura resultante de la biomasa granular. Por tanto, para la obtención de gránulos adecuados, de manera adicional a la correcta relación C/N, el medio acuoso donde se han inoculado los microorganismos debe comprender ciertos nutrientes y condiciones específicas, que dependerán de los microorganismos empleados para la formación de la biomasa granular.

La temperatura para la formación de la biomasa granular se encuentra en el intervalo 3 – 30 ºC, y el pH entre 5,5 y 9,5, de modo que los microorganismos pueden desarrollarse en el dicho medio acuoso.

Adicionalmente, los microorganismos que forman la biomasa se ven favorecidos en presencia de otros nutrientes que ayudan en la formación de los exopolisacáridos necesarios para la estabilización del gránulo y la obtención de una velocidad de decantación mínima que permita su empleo posterior en un sistema oligotrófico. Estos nutrientes pueden o no estar incluidos en el medio acuoso de manera inicial, por lo que puede requerirse la adición externa de otros compuestos tales como $MgSO_4*7H_2O$, K_2HPO_4, KH_2PO_4 o KCl.

Un ejemplo de estos nutrientes puede verse en la Tabla 1.

Tabla 1. Concentración de nutrientes adicionados a un agua subterránea con contaminación por nitrato/L (50-1000 mg/L) para la obtención de gránulos

Componentes	Concentración de nutrientes mg/L
Acetato sódico ($C_2H_3NaO_2$)	25 – 500
$MgSO_4*7H_2O$	3 – 30
K_2HPO_4	3 – 30
KH_2PO_4	0,1 - 10
KCl	1 – 20

El resultado obtenido por la presencia de estos nutrientes en el medio acuoso es el crecimiento de la biomasa hasta la formación de gránulos. Estos gránulos se encuentran en constante movimiento debido a la aireación, preferentemente ascendente, existente en el biorreactor.

Por su parte, la biomasa granular obtenida en la etapa c) del presente procedimiento, requiere ser separada del medio acuoso para un posterior uso. Dicha separación se lleva a cabo mediante el vaciado del tanque. De este modo, la biomasa granular se queda retenida en el tanque, mientras que el medio acuoso se extrae. El vaciado del tanque puede verse favorecido por una etapa de decantación previa al vaciado del tanque.

Esta decantación permite que la biomasa granular que presente una cierta velocidad de decantación descienda a la parte inferior del biorreactor. De este modo, se puede lograr una biomasa granular con una velocidad de decantación superior a 10

m/h, siendo preferente aquella biomasa con una velocidad de decantación en el intervalo comprendido entre 15 y 140 m/h.

Las condiciones operacionales establecidas para la producción masiva de biomasa granular aeróbica desnitrificante se encuentran favorecida por un movimiento circular dentro del biorreactor, sin que ello determine perdidas de biomasa. Este proceso de movimiento circular se produce por las condiciones establecidas de aireación. En una realización preferente, dicha aireación presenta una configuración ascendente y somete a la biomasa a un movimiento permanente ascenderse obligando a la biomasa a compactarse dentro del sistema lo que permite obtener una mayor densidad de la biomasa formando gránulos. En este sentido, la formación de los gránulos de la biomasa granular se lleva a cabo con un tiempo de retención hidráulico entre 1 – 24 h.

Por tanto, el desarrollo de una biomasa granular tal y como la descrita en la presente invención permite eliminar todo tipo de contaminantes sin necesidad de utilizar otro biorreactor distinto como ocurre con otros sistemas biológicos y, además, operar bajo condiciones con baja concentración de nutrientes sin degradarse, al crear estructuras de biomasa compactas, con un tamaño entre 3 - 35mm de diámetro y una velocidad de decantación entre 15 - 140 m/h.

La capacidad de actuar bajo condiciones aerobias y anaerobias consigue mantener procesos de granulación estables a largo plazo, incluso bajo condiciones oligotróficas, con unos rendimientos óptimos, de hasta un 100% de eliminación del nitrógeno.

La biomasa granular producida está constituida por poblaciones microbianas desnitrificantes, *tales como las bacterias Holophagaceae, Gemmatimonadaceae, Trichococcus Bifidobacterium y Candidatus Microthrix*, junto con las arqueas y hongos *Trichosporonaceae* y *Methanospirillum*, las cuales son necesarias para el tratamiento de efluentes de aguas subterráneas. Al tratarse de un conjunto de microorganismos, tales *como Holophagaceae, Gemmatimonadaceae* y *Trichococcus* presentan una mayor capacidad de crecimiento. Estos microorganismos aportan una mayor compactación y estabilidad, al crear exopolisacáridos lo que se traduce en una mayor estabilidad del gránulo formado según la presente invención.

De este modo, el empleo de una biomasa granular como la descrita en la presente invención, logra obtener un corriente de agua tratada que puede ser abastecida a poblaciones humanas como agua potable.

Ejemplo – Tamaño y velocidad de decantación de la biomasa obtenida

Se construyeron dos biorreactores granulares aeróbicos secuenciales uno de 660L (Ejemplo E1) y uno de 2.163L (Ejemplo E2) de capacidad. El biorreactor se alimentó con agua subterránea real contaminada, con una concentración de 85-100 mg/L de nitrato a temperatura ambiente. En este sentido, con el objetivo de poder obtener la biomasa granular desnitrificante, se inoculó con fango activo proveniente de un reactor biológico de una EDAR. Posteriormente, se adicionó al agua subterránea, justo en la entrada del biorreactor, una serie de nutrientes como la mostrada en la Tabla 2.

Tabla 2. Composición de los nutrientes añadidos al agua residual empleada para la obtención de gránulos

	Componentes				
Ejemplos	Acetato sódico ($C_2H_3NaO_2$) mg/L	$MgSO_4*7H_2O$ mg/L	K_2HPO_4 mg/L	KH_2PO_4 mg/L	KCl mg/L
E1	100	7,0	7,2	2,1	3,1
E2	150	14,0	14,4	4,2	6,2

En estas condiciones, se llevó a cabo la granulación de la biomasa con una carga orgánica C/N de 1 para para el Ejemplo 1 y de 2 para el ejemplo 2. a una temperatura de 15-20 ºC, un pH de 7-8 y con una concentración de oxígeno en agua de 6 mg O_2/L durante un Tiempo de Retención Hidráulica (RTH) de 6 h.

Como se aprecia en la Figura 2, donde se muestra el tamaño del diámetro (D) granular, en mm de las partículas de biomasa formada, frente al tiempo (t), en días; se observó como la formación de la biomasa granular se estabiliza a lo largo del tiempo en ambos biorreactores. De este modo, se obtiene un tamaño granular superior a 15 mm en ambos ejemplos, una decantación superior a 90 m/h y alcanzando valores de eliminación de nitrógeno y de materia orgánica superiores al 80% (Tabla 3).

Tabla 3. Parámetros físico-químicos obtenidos en la biomasa granular desnitrificante.

Ejemplos	Diámetr(D) granular	Velocidad de Decantación (m/h)	Rendimiento de Materia Orgánica (%)	Rendimiento Nitrógeno Total (%)
E1	25,1	110	100	80
E2	16,8	90	99	100

FIGURAS

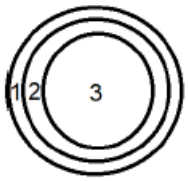

Fig. 1

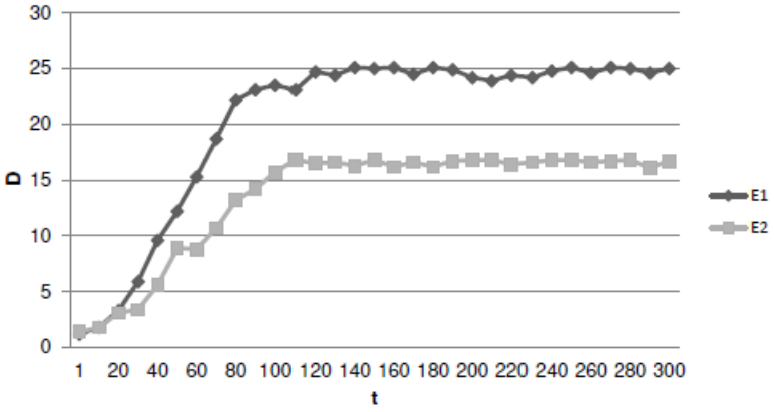

Fig. 2

RESUMEN

Procedimiento de obtención de biomasa granular acondicionada para sistemas oligotróficos que comprende las siguientes etapas: a) Inoculación de biomasa que comprende microorganismos en un medio acuoso aireado; b) Granulación de la biomasa inoculada; c) Formación de biomasa granular; d) Separación de la biomasa granular del medio acuoso; caracterizado por que la granulación de la biomasa se realiza en un medio acuoso con una carga orgánica C/N en el intervalo 1 – 5, a una temperatura entre 3 y 30 ºC, un pH entre 5,5 y 9,5 y con una concentración de oxígeno en agua entre 3 – 10 mg O_2/L durante un Tiempo de Retención Hidráulica entre 1 – 24 h; biomasa obtenida por dicho procedimiento y biopelícula que la comprende.