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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 in the last decades. Nitrate (NO_3^-) often reaches groundwater and causes significant degradation in groundwater quality. In an effort to address this issue, 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 >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, the reported hydraulic retention times and organic matter loading rate can be used to further improve the system performance to remove nitrate from groundwater.

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 <20,000 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 [5–7]. Nitrate (NO_3^-) often reaches groundwater due its high mobility in soil and causes significant degradation in groundwater quality [8–10]. 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

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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 [24–26]. 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 NirS and NirK 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 (NosZI). 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 *nirK*, *nirS*, and *nosZI* 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.

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

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 m 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 h), 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).

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 %

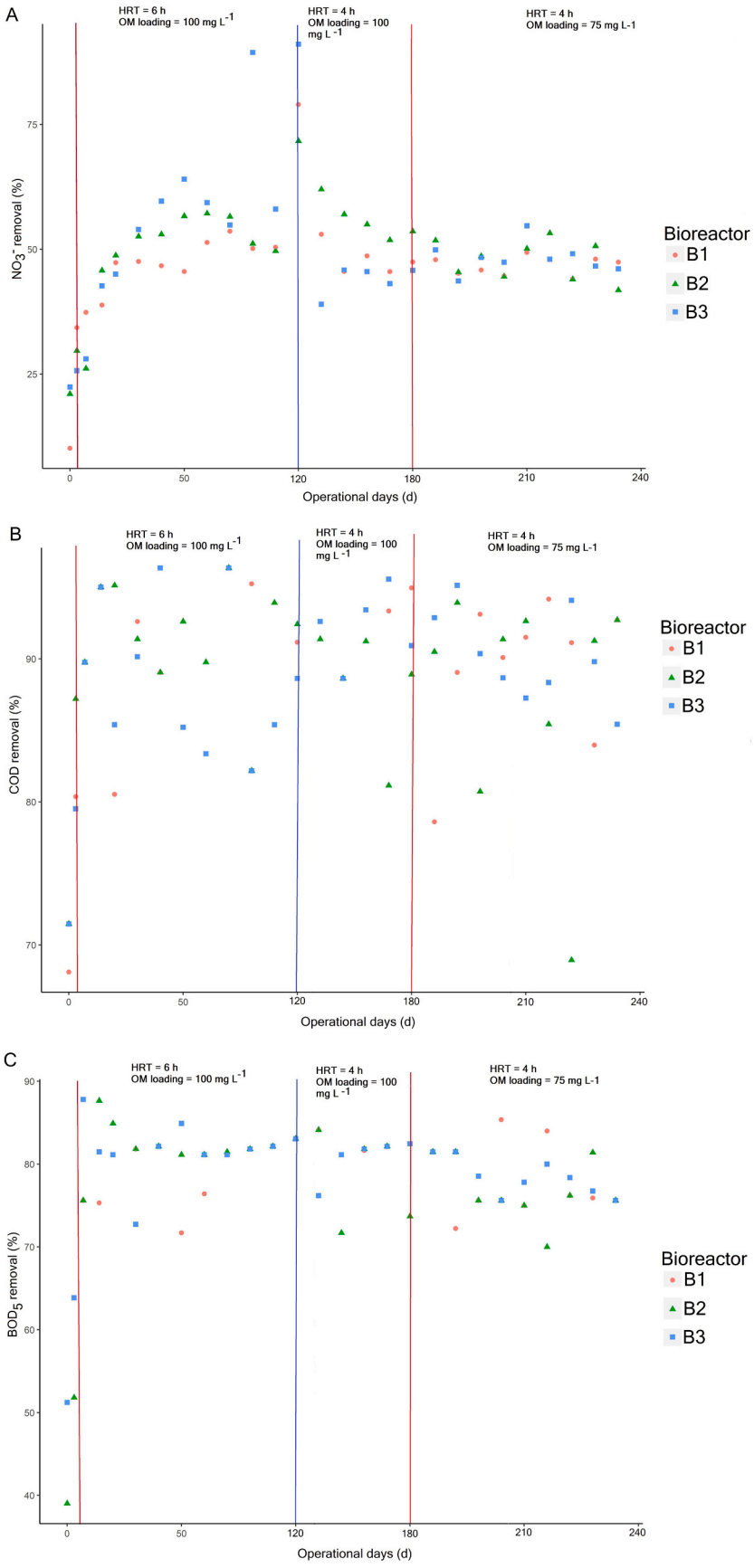

Fig. 1. Nitrate (A), COD (B), and BOD_5 (C) removal % during the experimental period. COD, chemical oxygen demand; BOD_5, biological oxygen demand.

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 °C 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. Quantification of denitrification genes

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 characterization of the denitrification 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 [42]. 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 [43] (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

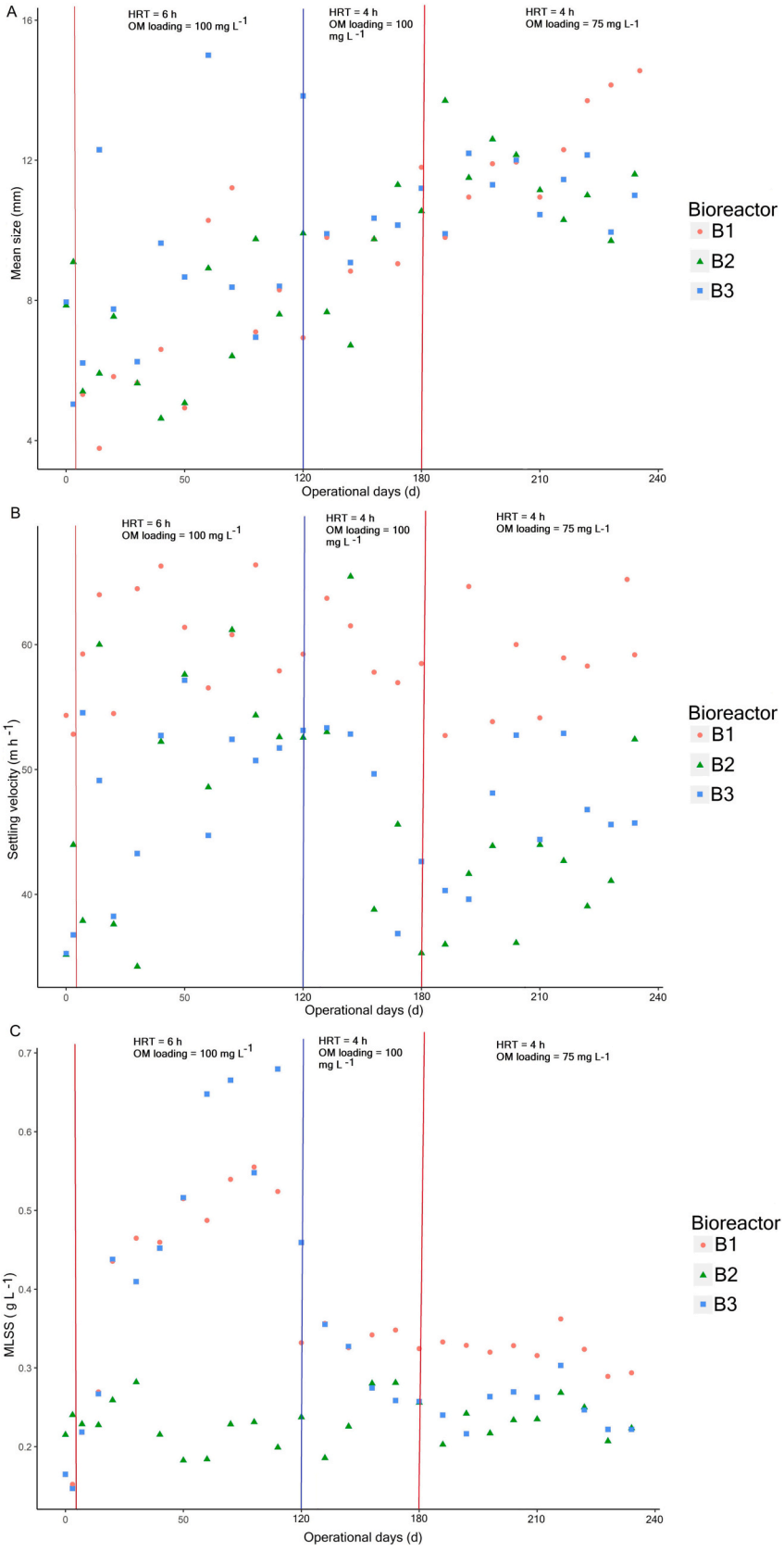

Fig. 2. Granule mean size (A), setting velocity of the granules (B), and MLSS concentration (C) during the experimental period. MLSS, mixed liquor suspended solids.

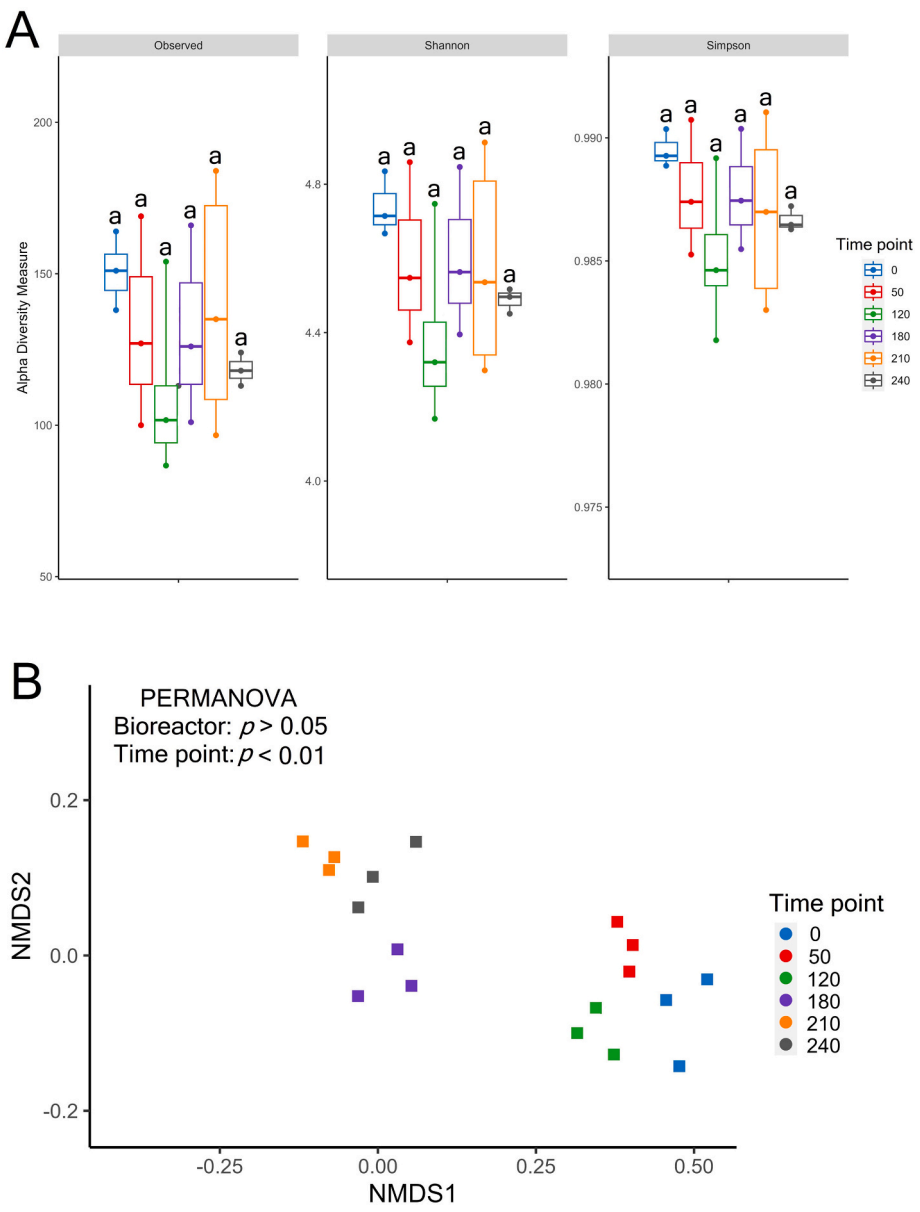

Fig. 3. (A) Number of ASVs, and values of Shannon, and Simpson diversity indices for the prokaryotic community. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed as means with standard error and are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

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 to the number of cycles and the amount of OM according to the characteristics of the water to be treated.

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 and 61 m h^{-1} in bioreactor 1 and between 42 and 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.

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

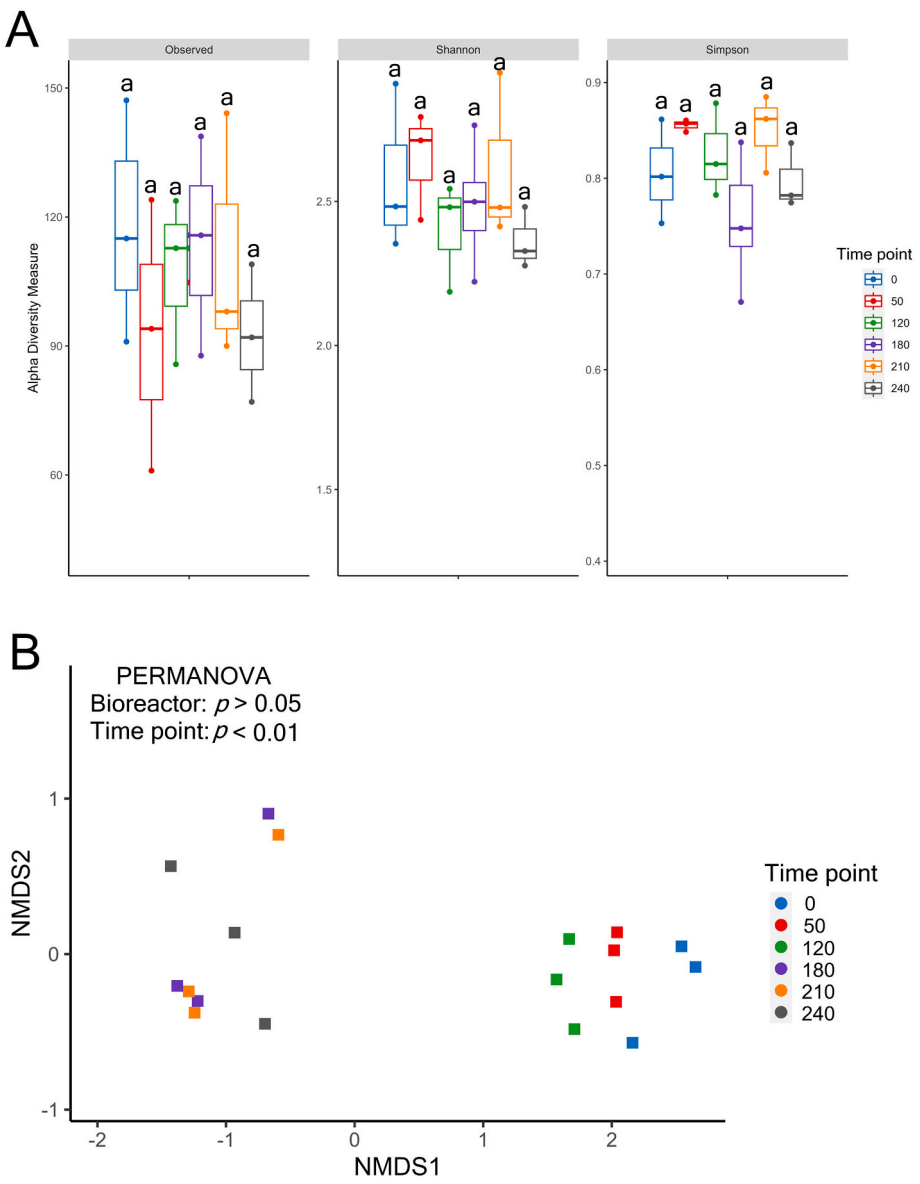

Fig. 4. (A) Number of ASVs, and values of Shannon, and Simpson diversity indices for the eukaryotic community. Different letters above the bars indicate significant differences between treatments and time points (Tukey's HSD, $p \leq 0.05$). Values are expressed as means with standard error and are the 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 $\leq$0.01 were considered significant.

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 h 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 *nosZI* 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 *nosZI*-type denitrifiers in the full-scale

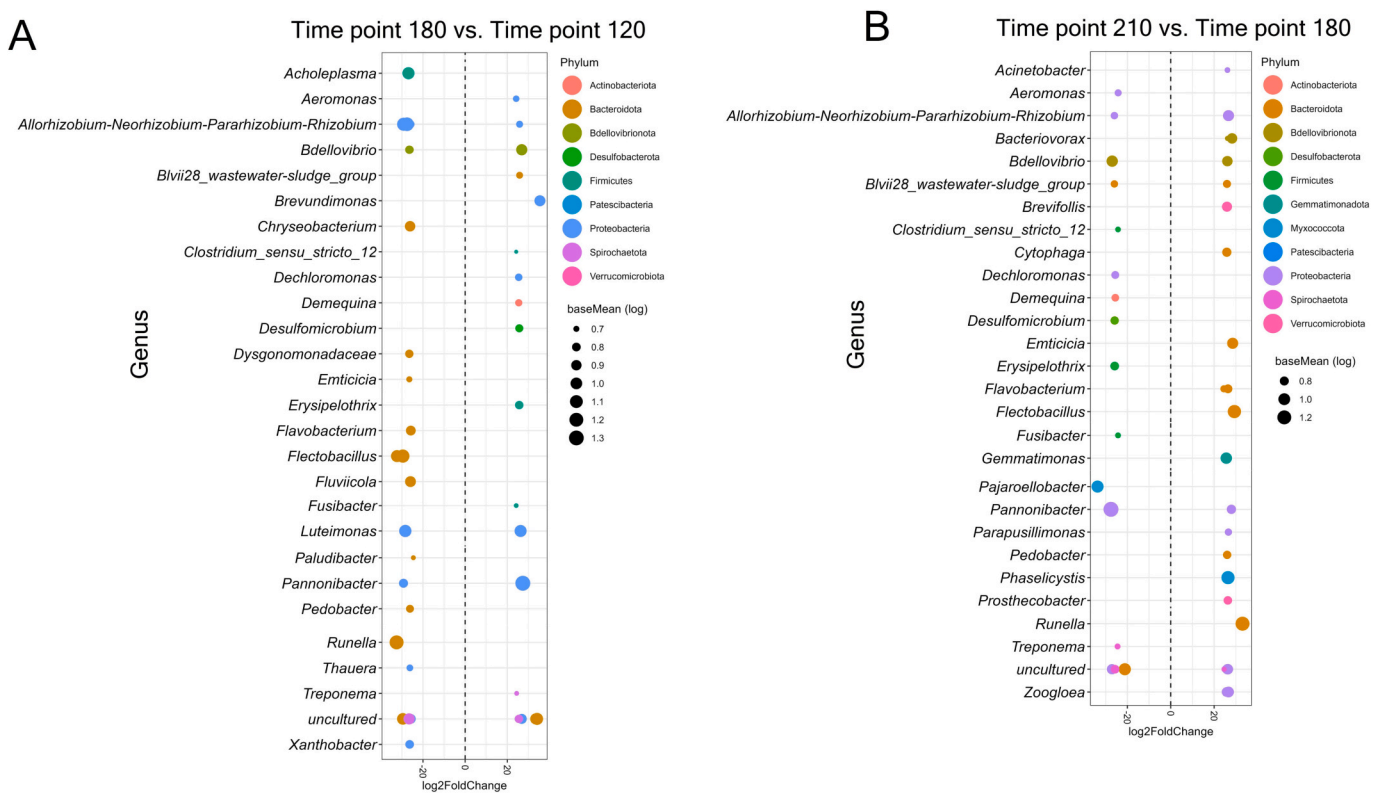

Fig. 5. Differential abundance ASVs at the genus taxonomic level between time points for the prokaryotic community. The fold change is shown on the X axis and genera are listed on the Y axis. Each colored dot represents an ASV that was identified by DESeq2 analysis as significantly differentially abundant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

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].

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

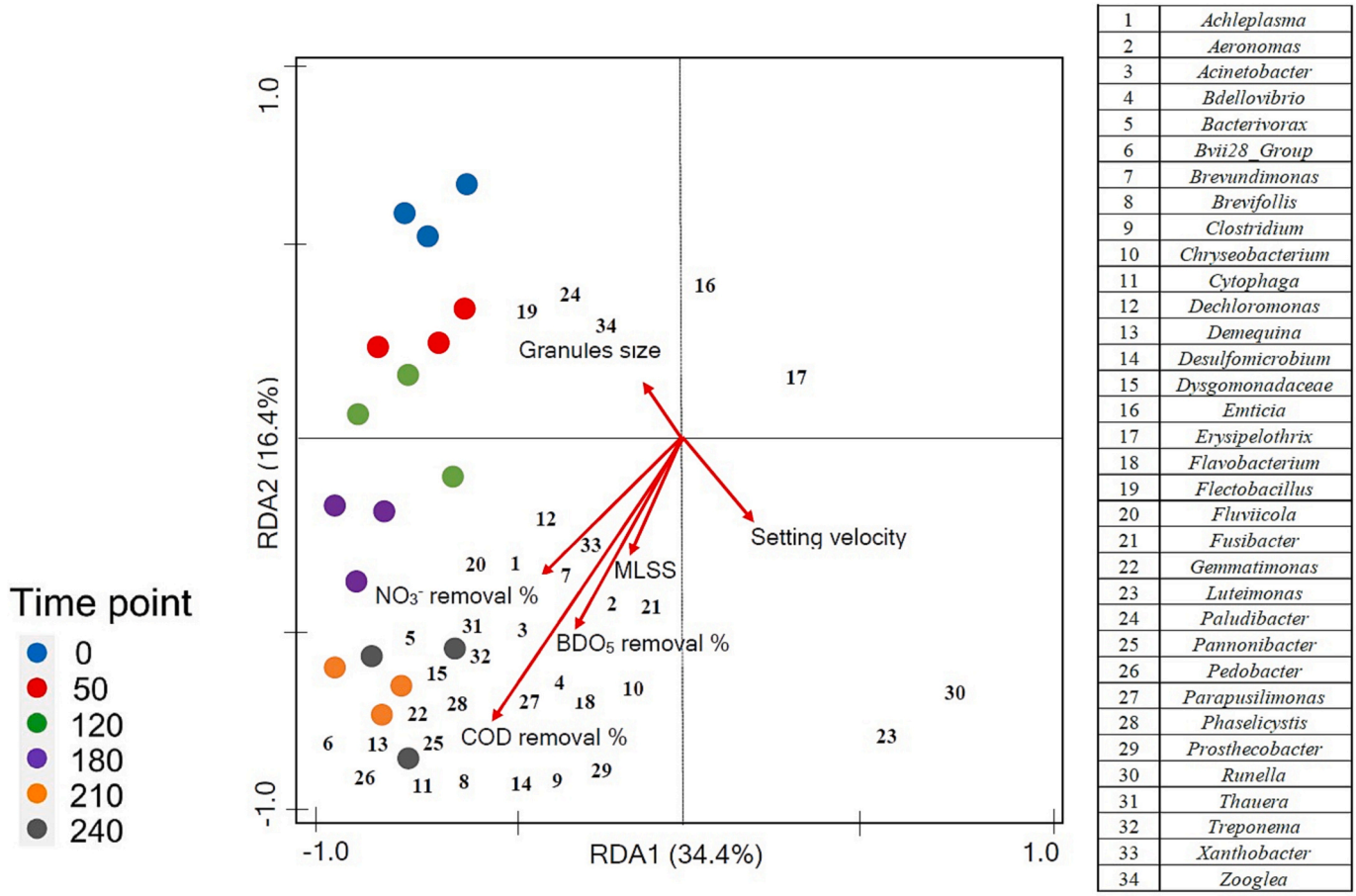

Fig. 6. RDA triplots relating differentially abundant prokaryotic taxa identified by DESeq2 analysis (Fig. 5) and physicochemical parameters of the system. COD removal %; BOD_5 removal %; NO_3^- removal; MLSS; mixed liquor suspended solids; Granules size; mean size of the granules; and Setting velocity;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

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).

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.

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; [44]) (Fig. 6). Among these genera, *Aeromonas*, *Acinetobacter*, *Brevundimonas*, *Dechloromonas*, *Prosthecobacter,* and *Thauera* are important denitrifiers in AGS systems [45–48]. 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.

CRediT authorship contribution statement

Miguel Hurtado-Martínez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Antonio Castellano-Hinojosa:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sarah L. Strauss:** Data curation, Investigation, Supervision, Validation, Writing – review & editing. **Jesús González-López:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing. **Alejandro González-Martínez:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2023.104601.

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