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# Simultaneous removal of nitrate and pesticides from contaminated groundwater using aerobic granular biomass technology

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ABSTRACT

Aerobic Granular Biomass (AGB) technology is widely used for urban and industrial wastewater treatment, however, its application in groundwater remediation, is practically unknown. A mixture of carbendazim, simazine, and diuron were amended to the nitrate-polluted synthetic groundwater at increasing concentrations to validate the ability of technology to remove both kind of pollutants, pesticides and nitrate which are commonly found in the water resources. The nitrate removal was a success with values below 0.010 g·L$^{-1}$. The increased concentration of pesticides in the influent did not distort the pattern observed for pesticide removal. Carbendazim was almost completely eliminated, followed by simazine elimination, while diuron showed adsorption-desorption patterns during experimentation. The addition of pesticides had a drastic effect on the basal community conducted by proliferation of *Hyphomicrobium* and *Dokdonella*. The pesticide compounds had a negative effect on number of copies for fungal population, while archaeal population was unharmed, according to qPCR results. Denitrifying bacteria need 70 days as acclimatization period for achieving activity values as initial inoculum. The results obtained have shown for the first time the capacity of AGB system to treat groundwater polluted with nitrate and pesticide using low carbon load. Therefore, the results suggested the potential application of AGB technology for the purification of groundwater polluted with both nitrates and pesticides.

## 1. Introduction

Groundwater is the main freshwater resource for the global population, with approximately one third of the world relying on groundwater for drinking [1,2]. Groundwater is the most important source of water in low-income regions, as well as semi-arid and arid regions, and hence plays a critical role for the maintenance and development of human settlements [3]. It is also linked to drinking water for its use in daily activities in industrial, agricultural, and domestics sectors.

In many countries worldwide [3], groundwater reserves are being depleted rapidly due to the long-term overexploitation, but that is not the only issue [4,5]. Human activities such as the application of pesticides and fertilizers, urbanization, inappropriate disposal of solid wastes, or industrial activities have led to immense damage to our surface and groundwater resources [5]. Pollution caused by hydrocarbons, microplastics, emerging contaminant, pesticides, or heavy metals have increased in recent years by the agriculture and livestock activities [6]. [7] reported that 13 % of groundwater monitoring stations in Europe exceed nitrate limits described by the Drinking Water European Framework [8], but this environmental issue has become a pervasive scenario worldwide. This directive considers that values of nitrate in drinking water higher than 0.050 g L$^{-1}$ strongly affect a water body; the recommended limit for drinking water is 0.025 g L$^{-1}$. The highest levels of nitrate pollution in Europe had been monitored in Belgium, Denmark, Spain, and Cyprus, respectively, but many developing countries also report elevated levels of nitrates, as well as heavy metals and pesticides. Some European countries with nitrate pollution also register with high levels of pesticides in groundwater (>0.1 μg·L$^{-1}$) such as Germany or

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Spain, that together, with France and Italy, accounted for about two-thirds of the volume of total EU pesticide sales each year over the period between 2011 and 2020 [65].

Among others, simazine, diuron, and carbendazim are the pesticides most widely spread out in environmental ecosystems [9–11]. Simazine forms part of the triazine compounds detected in groundwater influenced by intensive chemical fertilization. Diuron is widely found at levels up to 500 $ng{\cdot}L^{-1}$ and carbendazim is a fungicide of wide spectrum. The European Union has set intervention thresholds for drinking water at 0.1 $\mu g{\cdot}L^{-1}$ for individual pesticides and 0.5 $\mu g{\cdot}L^{-1}$ for total pesticides [8].

The most widely implemented technologies for treating polluted groundwater are based on physical and chemical processes [12–17]. Some of these technologies are reverse osmosis, electrodialysis, distillation, chemical reduction, or ion exchange, and are competent approaches for removing nitrate and other contaminants such as pesticides, but the high operational cost, low selectivity, and production of secondary waste brine present a critical detriment for the implementation of these technologies. Nowadays, water technologies should follow the challenge to find 'the best-fit' technology to treat an environmental issue depending on economic, social, and environmental needs [18]. One of the main restrictions is the budged need to implement high-efficient technologies, as mentioned previously. However, biotechnological advances have promoted approaches to do easier the detection of groundwater pollution by several compounds [19]. In this sense, the promising modification in operational conditions for aerobic granular biomass technology to treat nitrate contaminated groundwater had been tested by Hurtado-Martinez et al. [20,21] and Muñoz-Palazon et al. [22]. However, it is unknown if this technology is capable of removing not only nitrate but also pesticides since both pollutants often appear together in these water bodies.

The design of Aerobic Granular Biomass (AGB) is based on the promotion of three-dimensional matrices formed with microorganisms embedded in a large amount of extracellular polymeric substances (EPS), which forced by the hydrodynamics of the system, forming a granular biofilm that allows for a gradient of oxygen and nutrients from the external layers to the internal layers of the granule. Furthermore, the high production of EPS by the microorganisms serves as a carbon source for the starvation stages. Since groundwater is known for its oligotrophic character, the high production of EPS allows the addition of carbon to be minimal. The conformation of the matrix allows for the presence of toxins to not alter the singular cells, since the biofilm acts as a barrier where the mass transfer behavior allows a progressive degradation by microorganisms that make up the biofilm. The comparison of AGB system versus physic-chemical technologies surpassed the advantages in both economic and environmental aspects, given by the absence the brine production, the lower operational cost and or lower footprint, among others [23,24].

The main goal of this research was to confirm if the AGB technology was able to carry out the nitrate and pesticides removal in a single reactor. Therefore, if this biological technology, which has previously been tested for the treatment of groundwater polluted with nitrates, could be also applied for the treatment of groundwater polluted with high concentration of nitrate ($> 0.050\ g{\cdot}L^{-1}$) and high concentrations of pesticides, situation frequently found in groundwater resources.

## 2. Materials and methods

### 2.1. Bioreactor design, start-up and operation

A sequential batch cylindrical reactor was started-up using 1.2 L of granular biomass from AGB-treated nitrate groundwater supplied with methanol as a carbon source. The reactor design was 90 cm in operational height (H), 7 cm in inner diameter (D) and total operational volume was 3.46 L. The air was supplied from the bottom of the reactor with an air flow of 2.0 $L{\cdot}min^{-1}$. The temperature, pH, and dissolved oxygen were monitored by Crison probes (Fig S1). The hydraulic retention time (HRT) was 5 h, the exchange volume was 50 % of volume and the cycle consisted of 3 min of settling, 3 min of effluent discard, 10 min of fill, 14 min in steady, and 120 min of aeration.

The influent was synthetic groundwater previously described by Hurtado-Martinez et al. [25]. Nitrates and pesticides were amended following previous studies of contaminated groundwater in areas with high agriculture applications in Spain. The synthetic groundwater was composed of mineral salts and distilled water comprised of 0.075 $g{\cdot}L^{-1}$ of $NO_3^-$ (added as $NaNO_3$), 0.1 $g{\cdot}L^{-1}$ of methanol, 0.040 $g{\cdot}L^{-1}$ of $K_2HPO_4$, 0.015 $g{\cdot}L^{-1}$ of $KH_2PO_4$, 0.005 $g{\cdot}L^{-1}$ of $MgSO_4$, and 0.015 $g{\cdot}L^{-1}$ of KCl [25]. The pesticide contamination was simulated reproducing the study performed by Belmonte Vega et al. [10], as it was corroborated by recent research [11,26]. The experiment was carried out for 225 days and distributed in four stages, during which a progressive increase in the pesticide's concentration was experimented. The stages included: blank stage (B) for 70 days, pesticides at low concentration (LC) for 50 days, pesticides at medium concentration (MC) for 50 days, and pesticides at high concentration (HC) for 50 days. In the low pesticide concentration stage (LC-S), groundwater was supplied with carbendazim (2 $\mu g{\cdot}L^{-1}$), simazine (1 $\mu g{\cdot}L^{-1}$), and diuron (1 $\mu g{\cdot}L^{-1}$). The medium pesticide concentration stage (MC-S) groundwater was amended with carbendazim (5 $\mu g{\cdot}L^{-1}$), simazine (2 $\mu g{\cdot}L^{-1}$), and diuron (3 $\mu g{\cdot}L^{-1}$). The high pesticide concentration stage (HC-S) groundwater was complemented with carbendazim (10 $\mu g{\cdot}L^{-1}$), simazine (5 $\mu g{\cdot}L^{-1}$), and diuron (5 $\mu g{\cdot}L^{-1}$). The pesticide concentrations for each stage are summarized in Supplementary Table S1.

### 2.2. Chemical removal performance and biomass characterization

The stability and evolution of the granular biomass were analysed in order to describe the mean size and decantation time. Granular size was measured using a scalimeter scale observed by a stereoscopic microscope and the settling velocity of the granules was measured using the protocol described by Muñoz-Palazon et al. [24]. Biomass concentration (MLSS) measurements in bioreactors were determined according to standard methods for the examination of water and wastewater [27].

The chemical oxygen demand (COD) and biological oxygen demand at day 5 ($BOD_5$) were determined by duplicate in the influent and effluent three times a week following the protocol described by APHA [28]. Samples of the influent and effluent were analysed for the evaluation of nitrogen ions concentration ($NO_3^-$ and $NO_2^-$) [28], conducted by ion chromatography (Metrohm). The dissolved oxygen (DO) and pH were measured daily using Crison probes within the reactor.

The pesticides samples pretreatment was filtered and concentrated on cartridges Oasis HLB 200 mg (Milford). Pesticide measurements were determinate by HPLC (WATER XEVO-TQS) tandem mass spectrometer (Waters Corporation, Milford, MA, USA) using with multiple reaction monitoring in positive electrospray ionization mode and capillary of 2.50 kV, water and acetonitrile 0.1% as solvent, with flow ramp rate 0.45 min and 10 μL of volume injection (Belmonte-Vega et al., 2005). Samples were analyzed in the Scientific Instrumentation Center, University of Granada.

An average mass balance of organic matter and nitrogen was calculated for influent and effluent samples following the equations described by Rodriguez-Sanchez et al. [29].

### 2.3. Samples collection, nucleic acid extraction, and cDNA retrotranscription

AGB biomass was collected at different operational days over control stages (CS) at day 0 and 70, LC-S of pesticides at days 71, 75, 90, and 119, MC-S at days 121, 125, 140, and 169, and HC-S of pesticides at days 171, 175, 190, and 219 for nucleic acids extractions (RNA and DNA). For extraction of genomic DNA, biological samples were submerged in sterile saline solutions, centrifuged at 5.000 r.p.m. for 30 min and kept at

− 20 °C. Then, nucleic acids of granular biomass pellets were extracted using the Fast Prep and the FastDNA Kit for soil (MP Biomedical, USA). For RNA extraction, samples were submerged in an RNA-protected (QIAGEN) and samples were kept at − 80 °C. Next, RNA extraction was done using the FastRNA Blue Kit (MP-Biomedical, USA) following the protocol manufacturer. DNA was digested and, the reverse transcription was carried out following the protocol described by Muñoz-Palazon et al. [30].

### 2.4. Next generation sequencing for total and metabolically active bacterial identification

The genomic DNA and retrotranscript RNA (cDNA) pools by duplicate were used for next generation sequencing using the platform MiSeq Illumina and Illumina MiSeq Reagent v3. The DNA and cDNA samples were amplified with the pair of primers: Bacteria807F-Bacteria1050R, for the amplification 16 S rRNA gene in *Bacteria* following the PCR conditions described by Muñoz-Palazon et al. [30].

### 2.5. Bioinformatics pipeline

The next generation sequencing raw data were treated using Mothur v1.47.0 software [32]. Firstly, paired-end reads were merged into a contigs, which were subjected to a quality analysis for removing sequencing artifacts with more than eight homopolymers and any ambiguous bases. Then, sequences were aligned against SILVA SEED 138 database with Needleman conditions to keep contigs starts and ends in the right primer set position. Chimeras were removed using VSEARCH algorithm with.elf-reference. The remaining contigs were classified using the SILVA nr v138 database filtered for the bacteria domain. The sequences were clustered within the operational taxonomic unit (OTU) groups with 97 % cutoff. The singleton OTUs were removed, and then taxonomic consensus for remaining OTUs were calculated [24].

### 2.6. Assessment on microbial abundance by qPCR assay

The number of copies of functional denitrifiers genes *(nosZ*, *norB* genes) and total archaeal and bacterial *16S rRNA* gene and fungal *18S rRNA* gene in the genomic DNA and retrotranscribed cDNA were estimated with qPCR analysis. qPCR was performed using SYBR Green qPCR in the Quant Studio3 system (ThermoFisher). The reaction mixture and the primer sets were done following the protocol described by Muñoz-Palazon et al. [30].

### 2.7. Diversity indices

The study of α-diversity of the genomic DNA and retrotranscribed cDNA sequencing data was made using the software PAST v4.09 considering the indices of Chao-1, Shannon-Wiener, Simpson, Pielou's evenness, and Equitability Index, which were calculated with a 97% confidence range by 999 bootstrap replications. Whittaker index was done to capture the β-diversity of all phylotypes among pairs of samples using the OTU table using the PAST v4.09 software.

### 2.8. Multivariate test analyses

Multivariate analyses were calculated to comprise the relationship between microorganisms in terms of absolute and relative to physic-chemical performance (including pesticides removal). First, a principal component analysis (PCA) was calculated by the zero-correction OTUs and centered log-ratio transformation of the OTU tables. Transformed OTUs tables were then used for singular value decomposition calculation; the results were represented through PCA plot using the PAST v4.09 software.

The non-parametric test PERMANOVA was used for testing the responses of the four stages in this research for the physic-chemical performance, quantification of target genes and pesticides removal differences among samples, and to envision the differences among samples and with the aim to interpret the PCA plot. PERMANOVA analysis was carried out using 9999 permutation and the Euclidean distance with PAST v4.09 software. In addition, normality and significance statistics were calculated for assumptions between stages using PERMANOVA.

Redundancy Analyses (RDA) were done to observe the linkage between total and metabolically active bacterial OTUs, biological samples over operational time and physicochemical parameters, pesticides removal, and the number of the copies of ribosomal and functional genes. The RDA were calculated with OTU tables computed by 499 unconstrained Monte-Carlo simulation run using the software CANOCO 4.5.

## 3. Results and discussion

### 3.1. Physic-chemical performance and biomass properties for long-term operation

The influent and effluent nitrate and its removal by AGB technology for treating contaminated groundwater by nitrate and pesticides are shown in Fig. 1. Nitrate concentration in the influent was controlled during the whole experiment close to 0.075 $g{\cdot}L^{-1}$of $NO_3$. During the adaptation and CS, the nitrate effluent concentration was below 0.005 $g{\cdot}L^{-1}$of $NO_3$, while nitrite was lower than 0.0002 $g{\cdot}L^{-1}$of $NO_2$ excepting values above in four eventual days. This event was only detected in the first period when biomass was in contact for the first time with pesticides, the pattern was not repeated during the subsequent stages. Once it was demonstrated that aerobic granular biomass was able to remove nitrate and did not accumulate nitrite over operational time, treated water reached quality standards for providing drinking water.

Once the system reactor was amended with pesticides under the lowest concentration (LCS stage), nitrate in the effluent was above 0.050 $g{\cdot}L^{-1}$ of $NO_3$. This data was detected only during the first 8 days in these conditions, afterwards, the system was gradually increased the nitrate removal ratio, achieving optimum values above 94 % (in the range of 0.005–0.012 $g{\cdot}L^{-1}$ of $NO_3$), and absence of nitrite. When pesticides concentration was increased in MCS stage, the removal ratio was slightly affected, although the nitrate concentration in the effluent was below 0.050 $g{\cdot}L^{-1}$of $NO_3$, suggesting the acclimatization of granules to the increasing concentration of pesticides in the influent. Thus, the results showed that aerobic granular biomass system treating polluted-groundwater with medium pesticides concentration was able to remove nitrate in the ranged of 78–89 %, which meant that values were below 0.025 $g{\cdot}L^{-1}$ of $NO_3$, meeting level recommended by the European Drinking water Framework. In this way, similar results were obtained when high pesticides concentration was added to the influent in HCS stage. Thus, during the first 20 days of HCS stage, the nitrate concentration in the effluent was higher than 0.020 $g{\cdot}L^{-1}$, reaching removal ratio performance of 25–61 % and observing nitrite accumulation. However, after this period, the nitrate values of treated effluent were lower than 0.025 $g{\cdot}L^{-1}$, and below 0.0005 $g{\cdot}L^{-1}$of $NO_2$ These results newly suggested an acclimatization process of the granular biomass in response to the increased pesticides concentration in the raw groundwater (Fig. 1).

The performance of $BOD_5$ and COD is shown in the Fig. 1. During all operational times, the $BOD_5$ removal rates were close to 100%, except during the first stage under pesticides treatment, following the same tendency of nitrate effluent concentration. This could be driven by the affection to the granular biomass by the presence of pesticides and consequently a worse performance. In fact, these periods are linked with high nitrate and nitrite concentration in the effluent during the LCS stage. The same pattern was detected on the COD removal performance, between operational days 93 to day 100, with a drop in terms of carbon removal. Although COD removal performance was also affected on the MCS stage, COD removal decreased from day 144 to day 150. For the

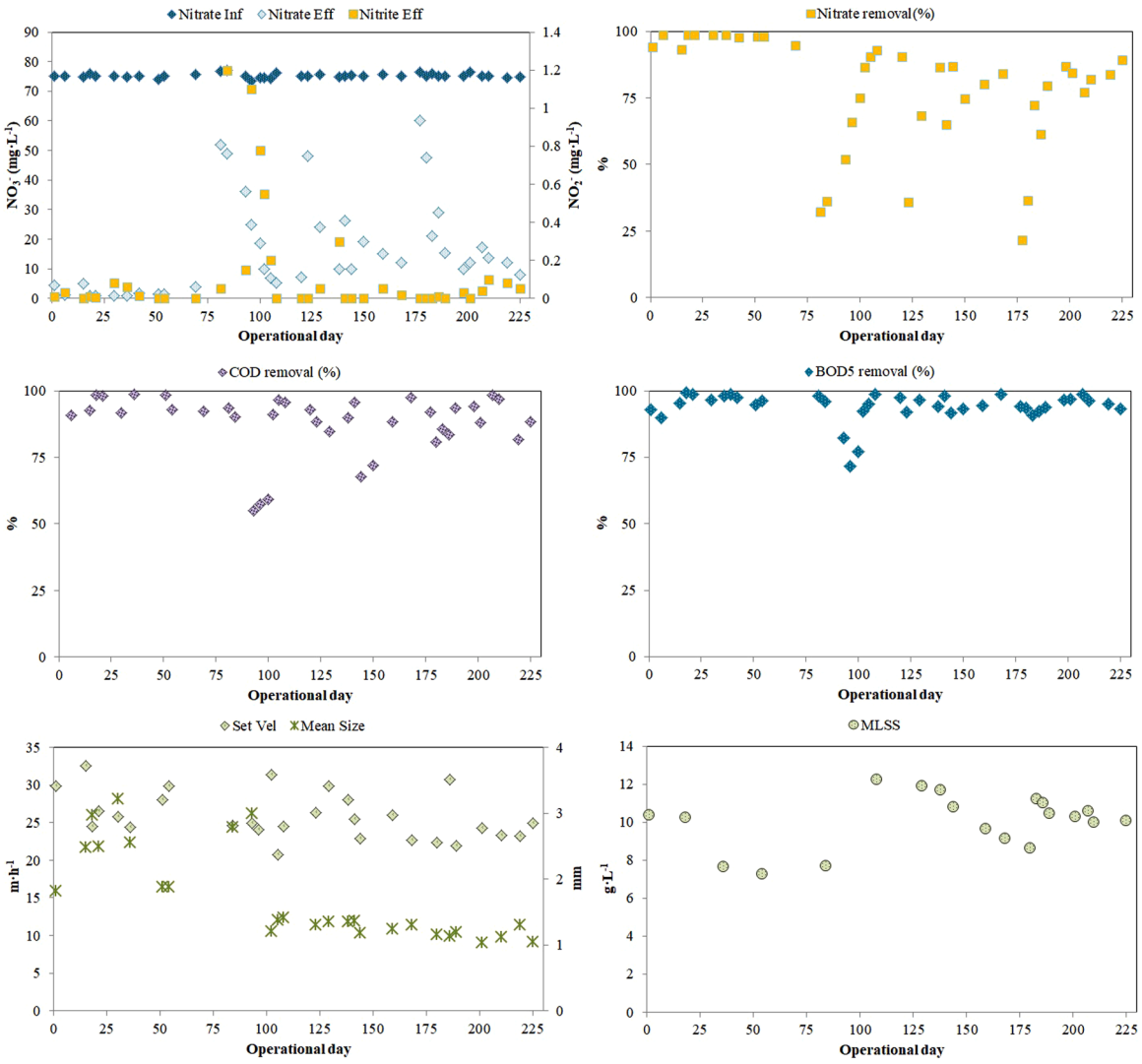


**Fig. 1.** Physic-chemical determination for concentration of nitrogen ions and total nitrogen removal (up), COD and BOD5 removal rates (middle), and settling ability, mean size and MLSS (down) of AGB system operated for 225 days.

rest of the time, values of COD removal were higher than 84 %. The COD and $BOD_5$ concentration in the influent and effluent of all stages is shown in Figure S2 in the supplementary material.

The granular stability over operational time was analyzed by the measurement of settling ability, mean granular size, and MLSS (Fig. 1). The granular biomass used as inoculum reflected average values of 29.82 ± 9.61 $m{\cdot}h^{-1}$ and 1.81 ± 1.07 mm for settling velocity and mean granular size, respectively. The size of the granular biomass was similar to other studies operated with groundwater, despite the low temperature. Muñoz-Palazon [24] reported values of granules ranging from 1.45 to 1.28 mm during the stabilization period treating groundwater, but with higher organic loading rate. During the CS stage, granular biomass employed as inoculum suffered strong modifications, possibly caused by the adaptation of biomass to the new conditions and operational design. At the beginning of the LCS stage, granules were larger than in the previous stage, suggesting that the presence of pesticides affecting the spatial conformation of granules (ranging from 2.78 ± 1.47–2.99 ± 1.79 mm). One theory suggests that disaggregation of the granules is a consequence of a small fraction of autophagy and the production of EPS under stressing conditions [33,34]. The granular size provides a different surface-volume ratio and aerobic zone volume fraction, contributing to diverse aerobic-anaerobic denitrification niches and microbial populations [35]. During the rest of the experimentation regardless of pesticides concentration amended, the patterns of granular size were extraordinarily stable in the range of 1.04 ± 0.35–1.41 ± 0.87 mm. Once the granular biomass is adapted to the stress conditions, such as toxic chemicals, the response is an increase of compactness because it is related to the robustness of the system against adverse compounds [34,36]. The settling velocity ranged from 20 to 33 $m{\cdot}h^{-1}$ during the whole experiment, but the trend showed how increased pesticides concentration had a slightly negative effect on the settle ability. It is remarkable to note that these values were only slightly lower than those analyzed in a full-scale plant treating groundwater [20]. The results of measurements of settling velocity and mean granular size in detail are shown in supplementary materials (Table S2).

MLSS was measured weekly and, during CS, the concentration was close to 10 $g{\cdot}L^{-1}$, while the presence of pesticides affected the biomass and hence the MLSS concentrations were below 7 $g{\cdot}L^{-1}$. Once the bioreactor was adapted to the pesticide's presence, the biomass growth was above 10 $g{\cdot}L^{-1}$. Finally, it could be observed that the HCS of pesticides reduced the granular biomass concentration, but only 7 days later, the system recovered their concentration despite pesticide concentration (Fig. 1).

The mass balance of AGB reactor is shown in Table 1. Related to nitrogen, the mass balance demonstrated the low load of nitrite in the effluent regardless of concentration and/or presence of pesticides. In

**Table 1**
Mass balance of nitrogen, COD and $BOD_5$, based on chemical performance of AGB technology. Aver: Average values; S.D: Standard Deviation.

| | Stage | Date | COD ($g \cdot d^{-1}$) | $BOD_5$ ($g \cdot d^{-1}$) | $COD_{nb}$ ($g \cdot d^{-1}$) | $CO_2$ gas COD ($g \cdot d^{-1}$) | $CO_2$ gas $BOD_5$ ($g \cdot d^{-1}$) | $CO_2$ gas $COD_{nb}$ ($g \cdot d^{-1}$) | TN ($gN \cdot d^{-1}$) | $NO_3$-N ($gN \cdot d^{-1}$) | $NO_2$-N ($gN \cdot d^{-1}$) | $N_{ous}$ gas-N ($gN \cdot d^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Influent | CS | 1–30 | 2507 | 1710 | 797 | 0.0 | 0.0 | 0.0 | 0.203 | 0.203 | 0.0 | 0.0 |
| | | 31–70 | 2643 | 1865 | 779 | 0.0 | 0.0 | 0.0 | 0.202.9 | 0.202.9 | 0.0 | 0.0 |
| | LCS | 71–100 | 2857 | 1661 | 1196 | 0.0 | 0.0 | 0.0 | 0.204.3 | 0.204.3 | 0.0 | 0.0 |
| | | 101–120 | 2855 | 1720 | 1135 | 0.0 | 0.0 | 0.0 | 0.203.3 | 0.203.3 | 0.0 | 0.0 |
| | MCS | 121–145 | 2808 | 1790 | 1018 | 0.0 | 0.0 | 0.0 | 0.203.6 | 0.203.6 | 0.0 | 0.0 |
| | | 146–170 | 2855 | 1720 | 1135 | 0.0 | 0.0 | 0.0 | 0.203.8 | 0.203.8 | 0.0 | 0.0 |
| | HCS | 171–200 | 2827 | 1620 | 1207 | 0.0 | 0.0 | 0.0 | 0.204.4 | 0.204.4 | 0.0 | 0.0 |
| | | 201–225 | 2667 | 1692 | 975 | 0.0 | 0.0 | 0.0 | 0.203.7 | 0.203.7 | 0.0 | 0.0 |
| | | Aver | 2719 | 1724 | 1030 | 0.0 | 0.0 | 0.0 | 0.203.7 | 0.203.7 | 0.0 | 0.0 |
| | | S.D. | 215 | 152 | 169 | 0.0 | 0.0 | 0.0 | 0.0019 | 0.0019 | 0.0 | 0.0 |
| Effluent | CS | 1–30 | 115 | 110 | 5 | 2384 | 1632 | 752 | 0.0061 | 0.006 | 0.0001 | 0.198 |
| | | 31–70 | 102 | 81 | 20 | 1973 | 1807 | 166 | 0.0053 | 0.005 | 0.0001 | 0.198 |
| | LCS | 71–100 | 721 | 403 | 318 | 2025 | 1425 | 600 | 0.1 | 0.097 | 0.0024 | 0.104 |
| | | 101–120 | 405 | 107 | 297 | 2450 | 1644 | 806 | 0.021 | 0.020 | 0.0009 | 0.182 |
| | MCS | 121–145 | 335 | 164 | 172 | 2433 | 1700 | 733 | 0.057 | 0.056 | 0.0002 | 0.146 |
| | | 146–170 | 405 | 108 | 297 | 2450 | 1644 | 806 | 0.041 | 0.041 | 0.0001 | 0.162 |
| | HCS | 171–200 | 296 | 171 | 125 | 2487 | 1514 | 972 | 0.082 | 0.082 | 0.0 | 0.121 |
| | | 201–225 | 255 | 950 | 160 | 2412 | 1625 | 787 | 0.034 | 0.034 | 0.0002 | 0.169 |
| | | Aver | 296 | 158 | 136 | 2323 | 1624 | 699 | 0.043 | 0.044 | 0.0005 | 0.158 |
| | | S.D. | 307 | 160 | 147 | 488 | 201 | 286 | 0.034 | 0.043 | 0.001 | 0.043 |

fact, only from operational day 71 to day 100 had slight consequences for the nitrite load in the effluent, with the highest values of 0.0024 $g \cdot L^{-1}$ of $NO_2$-N, while the rest of the experiment was in the range of 0.0001–0.0009 $g \cdot L^{-1}$ of $NO_2$-N.

Our results suggested that granular biomass suffered an adaptation process in response to increase of pesticides concentration that allowed to remove more efficiently both organic matter and nitrate, as previously reported Gu [19]. In consequence, it could be suggested that AGB systems allow the treatment of polluted groundwater with high nitrate and pesticides loads, and producing effluents with values of these pollutants below to the standard of European Drinking Framework for providing drinking water.

The removal of pesticides is shown in Fig. 2. In general, for all the studied pesticides, trends did not follow an analogous outline related to the pesticide's concentration in the raw water. Satisfactory results were determined for carbendazim during the low, medium, and high concentration stages. Carbendazim is not currently authorized to use in many areas, but it has been found in groundwater even after years of not being applied [11]. At the beginning of the LCS stage, the removal was 92–97 % during the first 5 days, suggesting that granules could have capacity to degrade, transform, or adsorb these compounds, but a few operational days later, the efficiency of removal dropped down to 70 %. The pattern indicated a possible process of bioadsorption and subsequent desorption when granules were saturated-unsaturated. Once the biomass was acclimated to these chemical mixtures, the removal ratio was close to 100 %. In the next stage (MCS), the increase of concentration affected the removal efficiency marginally, although the biological system was able to eliminate 60% of the influent load. Focusing on the HCS stage, the removal achieved efficiencies above 90 %, suggesting that previous adaptation mechanisms were developed and reinforced during the preceding phases, and then the biomass was able to resist and degrade the carbendazim, as reported [37]. These results were consistent with other studies based on the increased concentration of some toxic compounds in natural or engineering environments [36, 38]. Moreover, Parra et al. [18] described as bacterial conjugative plasmids could carry genes involved in pesticides biodegradation in very short times (5 min). The efficiency removal results related to simazine were very successful overall in the LCS stage, reaching values up to 90%, but the same pattern was observed under MCS stage, where the ratio of degradation decreased, possibly caused by a shock against mixture of carbendazim (5 $\mu g \cdot L^{-1}$), simazine (2 $\mu g \cdot L^{-1}$), and diuron (3 $\mu g \cdot L^{-1}$). The simazine removal was recorded with values until 65 % at the third stage (HCS). It is important to mention to simazine is currently banned, but it has been detected widespread in aquifers used for drinking water, and it is associated with its use in the past [9–11]. In the Fig. S4 (supplementary material) is shown influent and effluent concentrations of pesticides of LC, MC and HC stages.

The oscillation pattern observed in diuron removal rates over LCS, MCS, and HCS stages was intimately related to processes of bioaccumulation in the biomass, as reported by Chaumet et al. [39], who explained the behavior of this toxic compound in biofilms accompanied by diffusion mechanisms through EPS matrix. Equally, Chaumet et al. [39] described diuron accumulation in biofilms as nonlinear and remarked as the diffusion phenomenon was not linearly correlated with bioaccumulation, given the complex uptake mechanisms within the matrix biofilm. In this way, the efficiency removal from operational day 71 to the end of the operation was understandable because the ranges of diuron removal were at a minimum 5 % anda maximum 63 %. The oversaturation of diuron on the granular biomass surface could make the process of degradation practically null, while conditions of non-saturation of diuron biomass would allow granules to take up and carry out the uptake mechanisms with removal ratios close to 65 %, as it was observed in MCS stage. However, the smallest efficiency was found at days 121 and 171 because diuron is one of the most highly persistent and toxic herbicides, and consequently the results obtained from this research corroborate the demonstration given by Meephon et al. [40], where diuron was the most recalcitrant of the compounds. Although the European Parliament disavowed for specific goals for the use of diuron, there are several localizations where concentrations measured surpassed their respective annual average for Environmental Quality Standards set for water [11], caused by the long half-life of over 370 days in ecological environments [40].

The results of the study show that AGB systems can efficiently and jointly remove nitrates and pesticides present in groundwater, causing a process of adaptation of the granular biomass to the increase in pesticide concentrations. These facts are in our opinion of some importance since this technology has important environmental and economic advantages in relation to other physical technologies such as reverse osmosis [23, 24]. Therefore, using AGB technology, two of the most frequent contaminants in groundwater, such as nitrates and certain pesticides, could be removal on a real scale in a single reactor using a relatively simple and low-cost technology. Obviously, full-scale studies are necessary, as well as a stricter evaluation of costs.

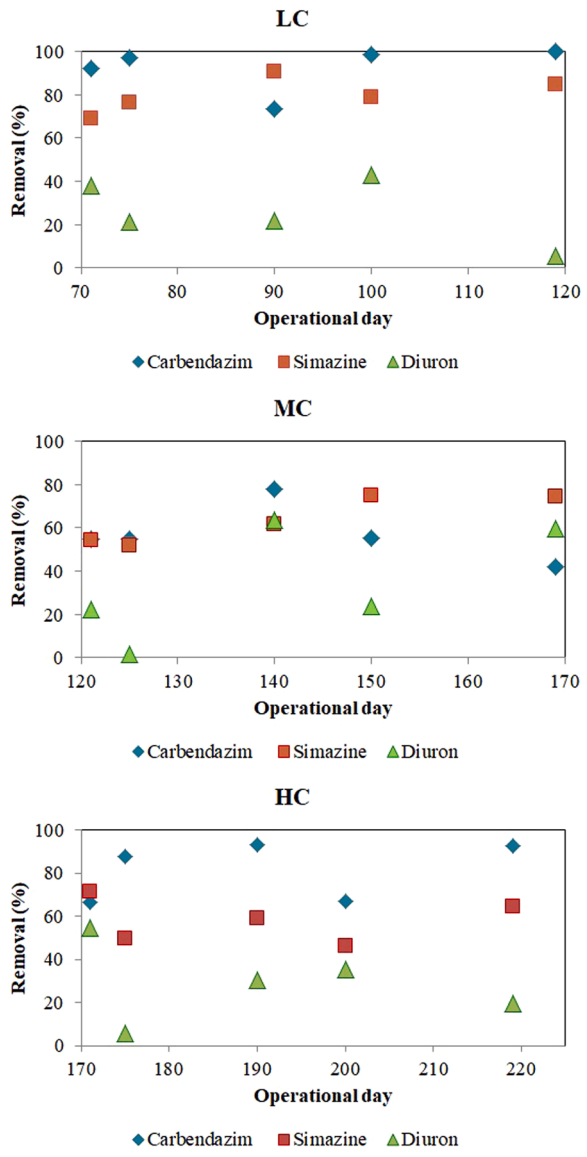


**Fig. 2.** Carbendazim, simazine and diuron removal ratio for LCS, MCS and HCS stages.

### 3.2. *Quantification of target genes*

Genomic DNA and retrotranscribed cDNA pools could elucidate the number of copies of genetic regions of total and metabolically active microbial populations conforming the granular biofilm using qPCR. This study has focused on the general microbial population (*Bacteria*, *Archaea* and *Fungi*) and denitrifiers dynamics.

A decrease in the number of copies of bacterial 16S rRNA from genomic DNA and retrotranscribed cDNA was detected during the first few operating days under pesticide pressure. At day 71, a more drastic effect on the active bacterial population, whose pattern kept stable during the LCS stage and half of MCS stage, $10^{11}$ copies of genes at day 0 and 70 drop below of $10^{11}$ for genomic DNA and from $10^{12}$ copies genes decreased to $10^{11}$ for retrotranscribed cDNA. Then, there was a one magnitude order decrease from inoculum samples to pesticides-adapted biomass samples. This drop was statistically significant ($p < 0.05$) for cDNA bacterial population, as well as for genomic DNA ($p > 0.05$). After this acclimatization period, the bacterial population increased the number of 16S rDNA copies even more than at the beginning of experimentation. The results suggested that the presence of toxic compounds promoted the growth of bacteria, possibly by the displacement of other domains. In fact, the magnitude orders were practically similar for DNA and cDNA. As reported by Tortosa et al. [41], the quantification by qPCR detected in DNA and cDNA represents a useful approach to understand the performance of populations.

The *norB* and *nosZ* genes were also analyzed by qPCR to quantify how affected the presence of pesticides to the denitrifiers bacteria were, as well as denitrification depletion. The *norB* gene plays a role to reduce nitric oxide to produce nitrous oxides, which is a potential greenhouse gas. The *nosZ* gene plays a role in the transformation of nitrous oxide to dinitrogen gas [42]. The control and maintenance of these genes in the biological system is indispensable, due to the biological water system contributing to the production of nitrous oxides, and subsequently affecting the carbon footprint and life cycle assessments. The AGB is a promising technology because it encourages the complete denitrification process [20]. Muñoz-Palazon et al. [43] demonstrated the efficiency by measurement of nitrous oxides production. The levels of total and expression denitrifying functional genes are shown in Supplementary Material Fig. S5. The *norB* copy number was increasing over operational time, with a positive correlation with higher pesticide concentration in the raw groundwater, from $10^{9}$ to $10^{12}$ gene copies·g$^{-1}$, almost a 3 magnitude order increase for the genomic DNA. While for retro-transcribed cDNA, the opposite pattern was found, because at the beginning of the experiment the number of copies was higher ($10^{10}$ gene copies·g$^{-1}$). For this, it seemed that the presence of pesticides in the long-term affected the functional activity and even decreased at a two magnitude order ($10^{8}$ gene copies·g$^{-1}$) for the metabolically active population. Next, the biomass systems recovered their high denitrifying activity with $10^{12}$ gene copies·g$^{-1}$ until the end of experimentation. This pattern suggested a clear affection by the presence of pesticides and posterior acclimatization of these microorganisms reflected a fast increase. Similarly, the metabolically active *nosZ* gene followed the same trend, with a strong depletion under the first shock-contact with pesticides; a later recovery achieved $10^{10}$ *nosZ* gene copies·g$^{-1}$. However, microorganisms with potential denitrifying activity measured by *nosZ* gene were kept over operational time, with the exception of the first day against the shock of pesticides. In conclusion, these results allowed the evaluation of the system and time needed for adaptation, but once microorganisms were acclimatized, the total denitrifying activity went back to normal and they are not affected as drastically by abiotic parameters such as environmental temperature [44]. With regard to the denitrifying population, RNA-based assays showed that not all denitrifiers, such as complete denitrifying microorganisms, had the optimal conditions to be active since the *nosZ* gene abundance was always greater than the transcripts of mentioned gene.

The average quantification of *16S rDNA* and *rRNA* archaeal gene copies were highly divergent, as the total number of *Archaea* increased by up to two orders of magnitude (from $10^{6}$ to $10^{8}$) while metabolically active *Archaea* reached $10^{11}$ gene copies·g$^{-1}$. These results may indicate that the *Archaea* had a role that could be linked to pesticide degradation as corroborated by Sharma et al. [45], or on the contrary to another task for the homeostasis of the community. Some authors described the archaeal community in the aquatic environment to shift in response to disturbance by pesticides, but usually the focus is on the dynamics of total archaeal populations [46,47], while in this research the focus is on active and total archaeal genes.

Finally, *18S rDNA* and *18S rRNA* of F*ungi* were quantified given the importance that the fungal population has on the biological water systems, and more specifically in granular systems [43,48,49]. The fungal population was sharply affected by the pesticides in the groundwater because the genomic DNA increased two magnitude orders, while the retrotranscribed cDNA decreased dramatically up to a minimum value of $10^{6}$ gene copies·g$^{-1}$. From the beginning, the number of copies of total and metabolically active fungi was diverse. This tendency could be explained because the numbers of diverse fungal phylotypes active in granular biomass were higher and essential for the granular stability. However, the presence of pesticides had a consequence in the fungi

phylotypes able to resist against toxic conditions. This is because carbendazim is a selective fungicide which can affect specific fungal taxa, but not all [50]. Therefore, the reduction of fungal activity in a 5 order of magnitude could be corroborated with the presence of this fungicide in the influent. Some hypothesis pointed out that fungi conform the initial core of the granule that later are colonized by bacteria acting as a bridge point [43]. However, in this case, in any of stage of operation, the granular conformation was compromised. This is observed in Fig. S4 of Supplementary Material, as the metabolically active fungal fraction could recapture their position in the community, although not as in the initial stadiums in the absence of pesticides.

The range of the numbers of copies of genomic DNA and retrotranscribed cDNA of bacteria and fungi genes were according to those reported in previously studies made on several biotechnological water processes [30,49,51]. However, earlier studies of *Archaea* did not focus on absolute quantification and could not indicate how it was driven by this domain.

### 3.3. Total and metabolically active bacterial community

The bacterial community dynamics were studied for identifying the total bacterial and metabolically active bacteria in the denitrification process by aerobic granular biomass technology. As reported by Emerson et al. [51], "with Erwin Schrödinger's quantum mechanics thought experiment, a dedicated assessment of living and/or dead microorganisms is usually a requirement to know whether members of microbial communities are alive or dead". The methods used for live/dead determinations and assessments of microbial activity can affect conclusions about both consortia in a biotechnological approach. The total bacterial community could represent a historical source of ecological niches, but it is the active microorganism's that describe the potential to grow and drive changes in the niches [51]. Also, the absolute quantification by qPCR of general and target genes is shown in the Supplementary material.

#### 3.3.1. Dynamics of the total bacterial populations

The total bacteria population was dominated by 24 OTUs with more than 1.00% of the total relative abundance, as shown in Fig. 3. The dynamics followed a clear pattern remarked by the absence and/or presence of pesticides in the raw groundwater. During the control stage, the bacterial OTUs did not modify their presence or their abundance because the inoculum proceedings from an AGB system treating nitrate polluted groundwater, and hence the microbial population was used for the operation conditions, carbon source, and denitrification process. The diversity was notable because several numbers of OTUs were represented by high relative abundance. Both on day 0 and day 70, the *Hyphomicrobium* genera (Otu01 and Otu04) was denoted by relative abundance higher than 25%, while Otu05 (4.91 ± 0.16), Otu06 (7.6 ± 0.017 %), and Otu10 (5.69 ± 0.19 %) affiliated with the *Hassallia* genus and *Comamonadaceae* and *Sapropiraceae* families, respectively, were highly represented. Moreover, a total of 6 OTUs had an abundance higher to 2.00 % of relative affiliated to phylotypes *Dokdonella*, *Devosia*, and *Pedomicrobium* genera and *Chitinophagaceae*, *Comamonadaceae*, and *Rhodobacteraceae* families. These families had been previously reported in microbial consortia given in aerobic granular for denitrification, with similar relative abundance [52], as well as *Dokdonella* being responsible for nitrite transformation and *Devosia* known for genomic plasticity by using wide a spectrum of substrates and a higher degree of adaptability [53,54].

The treatment of groundwater amended with pesticides entails a sharp change in bacterial community, possibly driven by the competence caused by the proliferation of bacteria more resistant and a competitive advantage against the pesticides. In this way, the encouragement most remarkable was driven by *Hyphomicrobium* and *Dokdonella* genera. *Hyphomicrobium* dominated the population dynamics over whole experimentation in LCS, MCS, and HCS, with more than 30.00 % of the total relative abundance. *Hyphomicrobium* is described as a common pesticide-degrading genus in soils and aquifers, and proliferates under different pesticide stresses [26]. This genus carries out nitrate reduction using methanol and other carbon sources under both aerobic and anaerobic conditions. The high relative abundance could be explained because *Hyphomicrobium* has competitive advantages, due to it performed aerobic and anaerobic denitrification metabolic pathways in the aerobic granular denitrifying biomass in the presence of pesticides compounds. This has been reported by Liu et al. [55] in biochemical technology for nitrate polluted groundwater. Likewise, *Dokdonella* is also detected in pesticide contaminated soils, but not in such high relative abundances as that detected in this research [26,56]. This genus

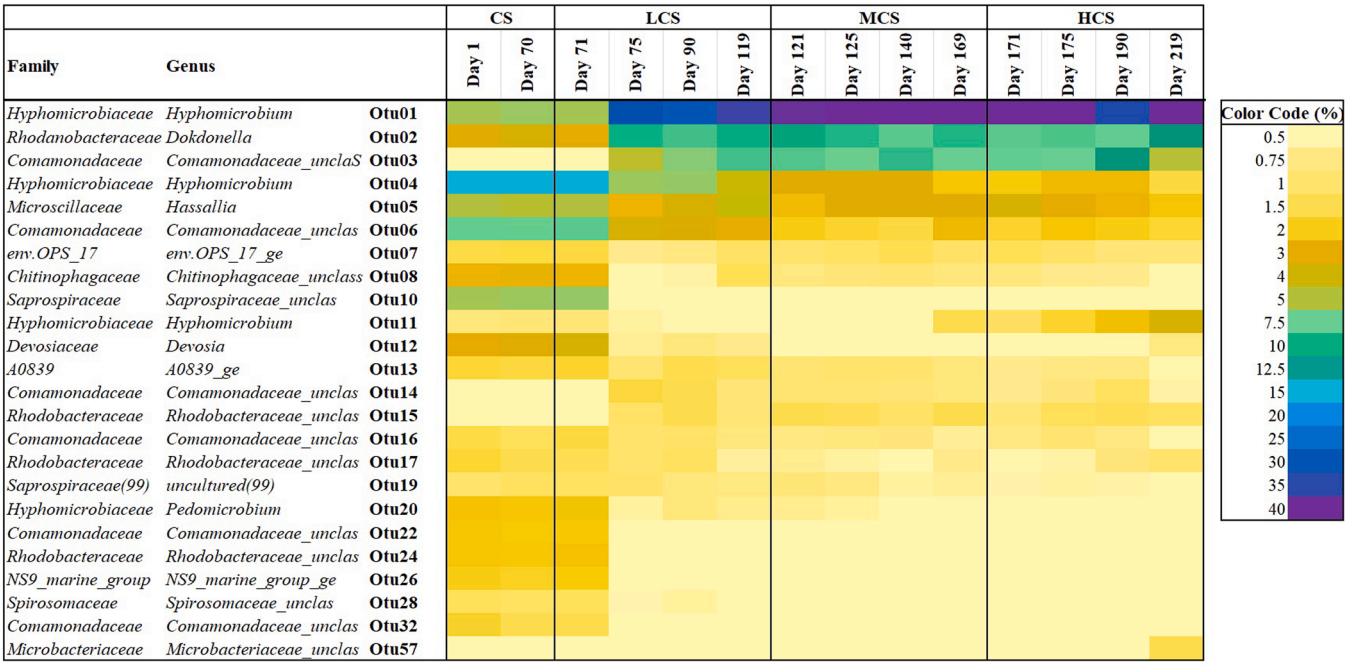


**Fig. 3.** Dynamics of total bacterial structure of dominant OTUs (>1.00 % of relative abundance) for CS, LCS, MCS, and HCS stages.

has been described as a degrader of pesticides and also involved in diuron biotransformation [56].

Other phylotypes proliferating under pesticide stress were the *Comamonadaceae* family, reaching 13 % of relative abundance. It is well known that *Comamonadaceae* is a potential denitrifying family and has also been previously described in aerobic granular denitrifying systems [20,22]. In addition, *Hassallia* was identified up to 5 % of relative abundance in CS and LCS stages. It is a cyanobacteria able to grow better and proliferate over other genera in biofilm systems; in fact, it used to be a key genus responsible for dissimilarities between suspended and environmental biofilm [57]. *Hassallia* is a phylotype producer of antifungal metabolisms but, although the role that it plays is still unknown [57], a possible hypothesis is the competition and subsequently displacement of fungal microorganisms that conform to the granule core [48,58].

A noticeable pattern marked by the proliferation of the Otu11 affiliated with *Hyphomicrobium* was observed at the end of the MCS stage, increasing until operational day 219, as well as the proliferation of a phylotype belonged to the *Rhodobacteraceae* family.

### 3.3.2. Dynamics of the active bacterial populations

For the active bacterial population, the number of total dominant OTUs (>1.00 %) was higher than the total bacterial population, because retrotranscribed cDNA showed 40 OTUs dominant in comparison with the 24 for genomic DNA (Fig. 4). This means that, despite the presence of bacterium phylotypes, the dominant activity is shared between greater numbers of OTUs. The microbial community composition obtained by DNA and cDNA sequencing differed because a metabolically active bacterium is usually recognized for being an intact cell and capable of duplication. While genomic DNA could persist 25 days in stream water [59]. Emerson et al. [51] described as "the live/dead protocols typically address one of the three aspects of microbial viability: (1) the existence of an intact, functional cell membrane, (2) the presence of cellular metabolism or energy, or (3) the possession of self-replicating DNA that can be transcribed into RNA".

The active bacterial population was interesting because there were great differences compared to other dominant phylotypes of total bacterial population studied by the retrotranscript of RNA and subsequent amplification. The results allow corroborating as it is a possible subestimate of the role of 'rare phylotypes' in the communities [60]. In this way, the stable granular population at day 0 and day 70 was highly linked with the presence of *Comamonadaceae* with the main representation of Otu31. This OTU was not dominant (1.0 % of relative abundance) in the genomic DNA population. Moreover, other OTUs affiliated with *Comamonadaceae* also played an active role such as Otu06, Otu10, and Otu11. *Hassallia* demonstrated a highlighted role in the absence of pesticides, as well as *Dokdonella* during the stage treating nitrate-contaminated groundwater, which have a role in the metabolic processes of nitrate reduction [56]. Other notable genera in the community of aerobic denitrifying granules in the first stage were *Methyloversatilis* and *Pseudomonas*, both taxa are intimately linked with denitrification metabolisms [20,21]. In addition, *Methyloversatilis* had been described in consortia with *Dokdonella*, *Hyphomicrobium*, and *Pseudomonas*, as main structural components of the bioremediation process in soils [56]. It is possible to find some synergistic metabolisms to enhance the performance of the reactors with these organisms [55, 61].

A similar tendency was observed under the pesticide stress on the active bacterial community because after one day of operation with pesticides, the most active OTU belonged to the *Comamonadaceae* family (Otu31) detected in CS and sharply decreased and the Otu01 affiliated to *Hyphomicrobium*, proliferated with relative abundance higher than 25 %. Undoubtedly, the sharp proliferation on this bacterium was also denoted in the total bacterial population because, in only 24 h, the increase of this taxon largely displaced the previously dominant population. Since systems began to treat pesticide compounds, several phylotypes of the *Comamonadaceae* family acquired a high relative abundance; the proliferation of Otu54 was more prominent at operational day 169, but its presence did not last over the experiment. Additionally, *Hassallia* had a high activity under pesticide stress overall in MCS and HCS stages. Unraveling the interactions between different bacterial phylotypes in granular biofilms is a challenge, particularly when biofilms are linked to several metabolic pathways in biotechnological processes. This is the main reason why microbe-microbe interactions have not been completely revealed yet in aerobic denitrifying granular sludge [62]. However, further research is needed to elucidate

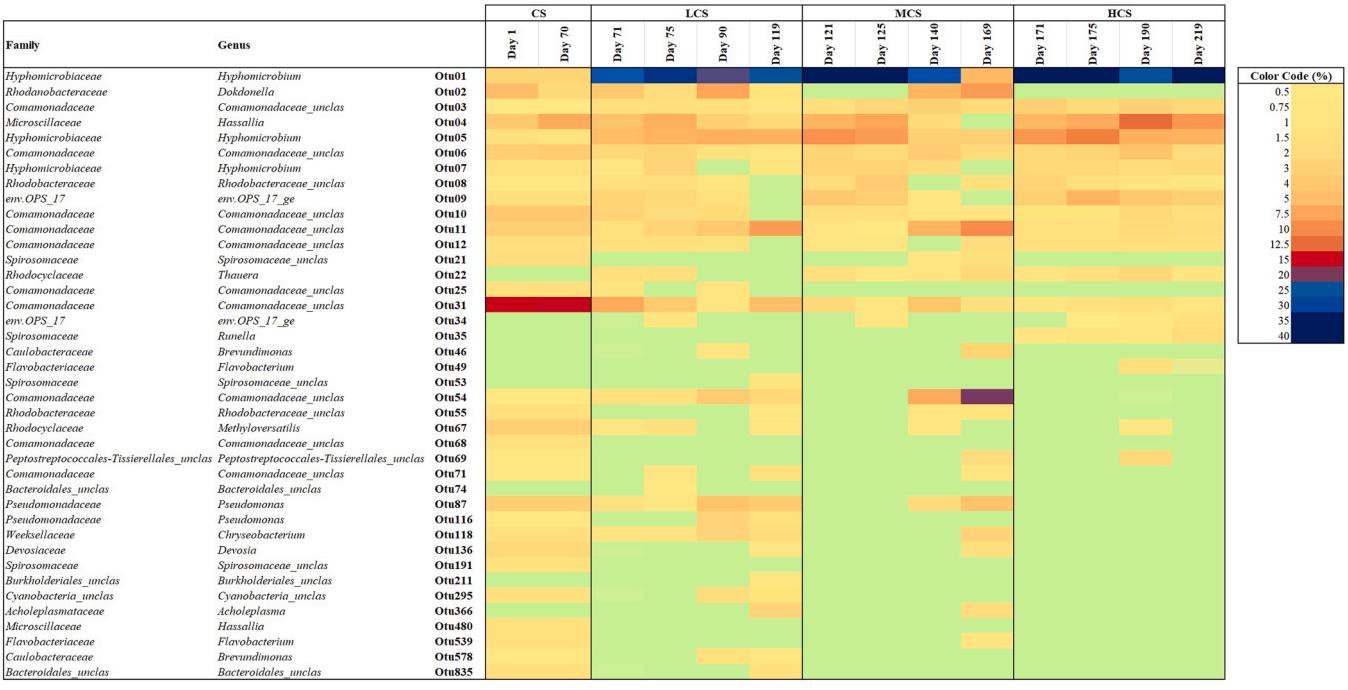


**Fig. 4.** Dynamics of metabolically active bacterial structure of dominant OTUs (>1.00 % of relative abundance) for CS, LCS, MCS, and HCS stages.

the distribution of metabolically active denitrifying microbial populations going on within the biofilms.

### 3.4. Diversity analysis indices

The main differences of the total bacterial community were found later in the treatment of pesticides related to the species richness measured by the Chao-1 index (Table 2). In the same way, even bigger differences in species richness were found in the metabolically active bacterial community. In addition, the RNA instability over time could be demonstrated due to the great variance, it was especially noticed in the MCS stage (96–6516). These results of species richness explained as despite the presence of total DNA belonging to a wide variety of taxa, the microorganisms that conduct functions and activities in the system are usually smaller, going from values index of thousands (genomic DNA) to hundreds (retrotranscribed cDNA), as it is represented in Table 2.

Statistical differences were found in the Shannon and Simpson diversity indices calculated for the different pesticide concentration stages. Hence, the contact of biomass with pesticides affected the evenness and dominance of the bacterial dynamics of granular biomass, but even more the active bacterial population. However, the diversity was not drastically modified by the treatment of pesticides, this fact was opposite to that described by Qi et al. [56]. In general terms, the values measured by Pielou's evenness and equitability indices showed higher evenness in active than in total bacterial populations, corroborating the data previously described in epigraph 3.4.2. Moreover, the evenness was higher in CS in the absence of pesticides; both DNA and RNA sequence data suffered depletion in terms of equitability, possibly caused by no competitive phylotypes in the adverse conditions.

The β-diversity results of total and metabolically active bacterial population are shown in Supplementary material.

### 3.5. Multivariate analyses

The PERMANOVA correlation analysis showed that most of the parameters were significantly correlated with the pesticide's concentration in the influent ($p < 0.05$) (Table S3). This behavior was especially notable for generic and functional active genes, where all of them revealed statistically significant differences with the concentration of pesticides ($p < 0.05$), as well as the total and denitrifiers total microorganisms ($p < 0.05$). The AGB technology also showed modifications in terms of nitrogen under each stage and in stages with-without pesticides as it is shown in Supplementary Table S3,S4 and S5.

The statically significant differences detected in the pesticide's removal ratio during the studied stages had a repercussion in simazine and carbendazim attenuation, while no difference was found for diuron. These results are comprehensive if the oscillating trend is evaluated given the diuron adsorption-desorption processes fostered by the biofilm [39].

Multivariate statistical analyses were calculated to understand the data integration obtained through PCA, PERMANOVA, and RDA analysis. While PCA allowed for the observation of similarity (in Supplementary material), PERMANOVA allowed for the explanation of the statistically significant parameters (in Supplementary material), and the RDA permitted to find the relationship between dominant OTUs (total and metabolically active) and the copy number of the genes, with physic-chemical performance and pesticides removal.

The RDA plot describes the active (Fig. 5a) and total (Fig. 5b) dominant OTUs with physic-chemical performance. A great number of OTUs were positively correlated with the denitrification process and nitrate removal, such as Otu71, Otu87, Otu116, and Otu578 affiliated with the *Comamonadaceae* family, *Pseudomonas* and *Brevundimonas* genus, as well as others more dominant in the community, such as Otu12 and Otu69. The high number of dominant OTUs showed that COD and $BOD_5$ removal were positively linked with Otu04, Otu06, and Otu07. Some of these phylotypes are reported as heterotrophic denitrifiers [54]. Moreover, granular properties related to size and settling velocity were negatively correlated with dissolved oxygen concentration and pH, although it was observed a stronger opposite relation in RDA of the active population than in total population (Fig. 5a and Fig. 5b). The major number of OTUs was positively correlated with nitrate removal, mean granular size, settling velocity, and COD removal, forming part of a cluster linked to the first days of operation. Next,opposite and weak linkage was found for biomass samples of the rest of experimentation, these results could be corroborated by the results given by α-diversity indices.

Regarding this last pattern, the studied population by means of genomic DNA followed the same trend, finding a greater number of OTUs linked to the initial days of operation. However, any OTU was related to nitrate, $BOD_5$, and COD removal. Conversely, higher clearance ratios were negatively correlated with the key days of the LCS and MCS phases, while it was positive for the operational days of the HCS. The dominant phylotypes, Otu01, Otu02, Otu03, and Otu15, were closely related to higher concentrations of biomass in the reactor, independently of the size and the ability to decant.

The RDA was also calculated to link the pesticides removal with the metabolically active and total dominant communities (Fig. 5c). Numerous OTUs were correlated with simazine and carbendazim removal, according to the pesticides removal efficiency. On the one hand, the strongest relation found for the carbendazim removal was with Otu71, Otu191, Otu480, followed by Otu12, Otu31, Otu74, Otu67, Otu68, and Otu74. On the other hand, simazine removal revealed a statistically significant result with the abundance of Otu06, Otu10, Otu21, and Otu25. Some genera of the *Comamonadaceae* family have

**Table 2**
Diversity indices for genomic DNA and retrotranscribed cDNA.

| | CS | LCS | | | | MCS | | | | HCS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 70 | Day 71 | Day 75 | Day 90 | Day 119 | Day 121 | Day 125 | Day 140 | Day 169 | Day 171 | Day 175 | Day 190 | Day 219 |
| | DNA | | | | | | | | | | | | | |
| Simpson | 0.7364 | 0.7346 | 0.7356 | 0.7177 | 0.7191 | 0.7117 | 0.6977 | 0.7048 | 0.6988 | 0.7025 | 0.6909 | 0.6986 | 0.7143 | 0.6984 |
| Shannon | 2.837 | 2.767 | 2.789 | 2.6835 | 2.689 | 2.523 | 2.342 | 2.491 | 2.3945 | 2.4055 | 2.3215 | 2.391 | 2.585 | 2.347 |
| Pielou's evenness | 0.0078 | 0.0109 | 0.0108 | 0.0061 | 0.0049 | 0.0048 | 0.0052 | 0.0046 | 0.0043 | 0.0103 | 0.0046 | 0.0052 | 0.0054 | 0.0093 |
| Equitability | 0.369 | 0.3799 | 0.381 | 0.3447 | 0.3353 | 0.3209 | 0.3079 | 0.3166 | 0.3056 | 0.3435 | 0.3013 | 0.3123 | 0.3311 | 0.334 |
| Chao-1 | 5866 | 4575 | 4735.5 | 7876.5 | 9044 | 9045.5 | 6921.5 | 8492.5 | 8242 | 5208 | 7934 | 7713.5 | 7811.5 | 4198.5 |
| | RNA | | | | | | | | | | | | | |
| Simpson | 0.7425 | 0.7292 | 0.7359 | 0.7211 | 0.732 | 0.7312 | 0.7018 | 0.701 | 0.7256 | 0.7343 | 0.6947 | 0.7083 | 0.7268 | 0.6982 |
| Shannon | 2.779 | 2.594 | 2.6865 | 2.4505 | 2.457 | 2.471 | 2.413 | 2.394 | 2.3865 | 2.36 | 2.326 | 2.407 | 2.5965 | 2.321 |
| Pielou's evenness | 0.1894 | 0.1029 | 0.1462 | 0.0938 | 0.2146 | 0.1914 | 0.0081 | 0.0052 | 0.1524 | 0.261 | 0.0063 | 0.0094 | 0.0195 | 0.0132 |
| Equitability | 0.6255 | 0.5328 | 0.5792 | 0.5078 | 0.6141 | 0.5991 | 0.3336 | 0.313 | 0.5591 | 0.6371 | 0.3134 | 0.3398 | 0.3961 | 0.3489 |
| Chao-1 | 178.6 | 321.2 | 249.9 | 548.55 | 143.2 | 174.8 | 4548.5 | 6516.5 | 233.8 | 96.61 | 6138.5 | 5179.5 | 3045 | 3214 |

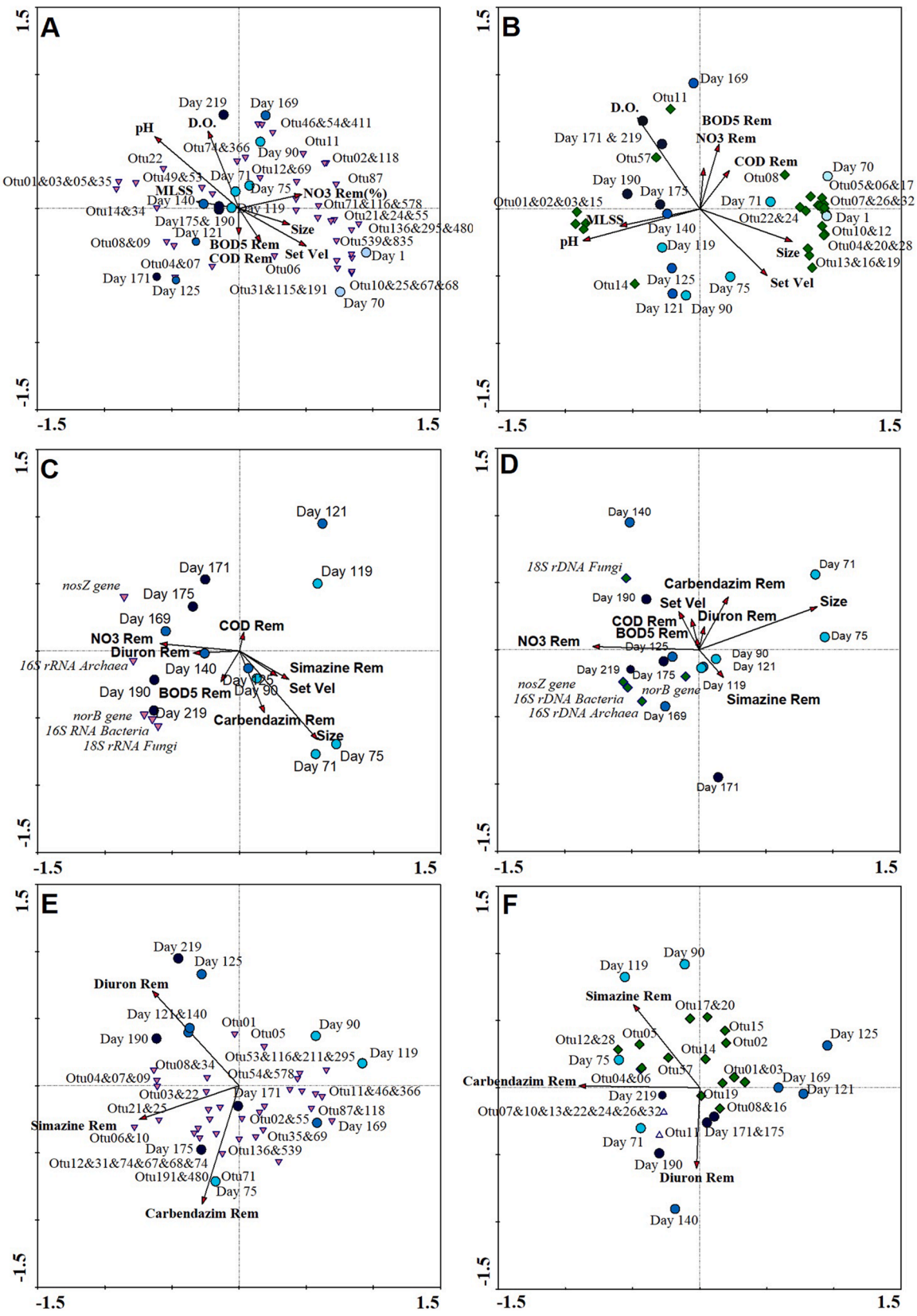


**Fig. 5.** RDA for active microbial community (left) and total bacterial community (right).

been found in systems contaminated with atrazine (or atrazine-derived compounds), this fact could be linked to a possible degradation mechanisms of simazine as reported Espín et al. [63]. Any phylotypes were positively correlated with diuron removal, but several negative relations were found with dominant OTUs that were metabolically active, some of them being linked with the carbendazim removal function. Analysing the RDA for total bacterial community and pesticides performance (Fig. 5d) could be observed as a similar trend because many phylotypes were correlated with diuron removal, while Otu04, Otu06, Otu12, Otu28, Otu5, and Otu57 (similar than in retrotranscribed cDNA) had a strong relation with the pesticide's removal.

RDA was calculated using the copies of active and total target genes (Figs. 5e and 5f). The RDA of active populations demonstrated that *Archaea* played a key role for diuron attenuation like earlier mentioned [45]. The absolute abundance of *nosZ* gene was positively linked with nitrate removal, but a weak correlation was found with the *norB* gene. The number of copies of *norB*, *16S rDNA* of *Bacteria*, and *18S rDNA* of *Fungi* were linked with the $BOD_5$ removal; results demonstrated high heterotrophic denitrification activity by bacteria. Additionally, *Fungi* had the ability to produce large quantities of enzymes and more of them related to carbohydrate oxidation. It has enormous biotechnological potential, for instance, with glucose oxidase [64]. The search for correlations with the number of total genes found in the samples does not reflect close relationships with the elimination of pesticides, nitrate, or organic matter.

## 4. Conclusions

Nitrate and nitrite concentration in polluted groundwater with nitrate and pesticides treated with aerobic granular biomass technology met the requirements of European Water Drinking framework. Carbendazim was removed up to 100 %, followed by simazine, while diuron demonstrated its recalcitrant nature. *Hyphomicrobium* led dominance in the metabolically active and total bacterial dynamics, and the *Comamonadaceae* family and *Hassallia* genus played a role in the decontamination. There was an overtaking of the *Archaea* against the *Fungi*, caused by the presence of the fungicide and the active role of *Archaea* to degrade pesticides. After a depletion episode of denitrifying bacteria, it was demonstrated how these microorganisms proliferated with adaptation mechanisms. AGB could be postulated as an efficient system for nitrate removal and attenuates the pesticides from the groundwater. For the first time, aerobic granular biomass technology was postulated to simultaneously treat nitrate and pesticides from polluted groundwater.

## CRediT authorship contribution statement

**Barbara Muñoz-Palazon**: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft preparation. **Miguel Hurtado-Martinez:** Methodology, Investigation. **Aurora Rosa-Masegosa**: Data curation, Formal analysis, Writing – original draft preparation. **Ramiro Vilchez-Vargas:** Methodology, Investigation, Writing – review & editing. **Alexander Link:** Funding acquisition, Investigation, Writing – review & editing. **Susanna Gorrasi:** Data curation, Writing – review & editing. **Jesus Gonzalez-Lopez:** Funding acquisition, Supervision, Project administration, Writing – review & editing. **Alejandro Gonzalez-Martinez:** Conceptualization, 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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.109527.

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