# Gene abundance of bacterial nitrifiers and denitrifiers in sewage sludge treated with clay minerals and biochar

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Globally, the loss of soil organic matter in arable land has exacerbated, endangering soil fertility and rising need for food. Application of treated and/or digested sewage sludge as soil amendment provides an appealing alternative solution as it replenishes agricultural soil with organic matter and plant nutrients, such as nitrogen and phosphorus. Nitrogen (N) cycling processes in soil are of crucial importance as various microbial N transformations take place and regulate the availability of N for plants. Moreover, these microbial communities are involved in nitrification and denitrification activities and are often used as reliable indicators of soil quality (Schloter et al., 2018). Due to technological challenges in isolating and cultivating bacterial nitrifiers and denitrifiers in laboratory conditions, molecular techniques have been rapidly employed and used extensively to examine the abundance and diversity of microorganisms implicated in nitrification and denitrification in various environments, such as sewage sludge or soil.

Accordingly, in the herein study, used three clay minerals (bentonite, vermiculite, zeolite) and biochar as well as $Ca(OH)_2$, which were added to dewatered sewage sludge at 15% and 30% rates, in order to quantify the gene abundance of ammonia oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The abundance of these microorganisms (AOA and AOB) was determined by qPCR targeting *amo*A gene-sequence encoding for an ammonia monooxygenase specific to each domain. Additionally, the abundance of denitrifying bacteria was measured by quantitative PCR (qPCR) of gene encoding copper and cytochrome-cd1-containing nitrite reductase (*nir*K and *nir*S, respectively), whereas the abundance of bacterial *ure*C gene, that codes for one of three subunits in the urease enzyme was also quantified.

DNA was extracted from 0.1 g soil using the HigherPurity™ Soil DNA Isolation kit (Canvax Reagents, Spain), quantified in a nanophotometer and for subsequent qPCR analysis, 5 ng/μl were used in all experiments. Each treatment involved three biological and three technical replicates. SYBR-Green qPCR assays were developed in a Strategene Mx3005P qPCR System in a 15-μl reaction mix containing 7,5 μl of 2x qPCR mix (Luna® Universal qPCR Master Mix, New England Biolabs), 0.2 μM each primer set and 1 μl of DNA, under the following thermocycling conditions: an initial step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at specific temperatures for each primer set (Table 1) for 30 s and extension at 60°C for 60 s, with a final melting curve cycle of 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. A negative control with no template was included for biological replicate.

Table 1. qPCR primers used in the herein study.

| Target | Primer name | Primer sequence | Annealing temperature (ºC) | Reference |
|---|---|---|---|---|
| AOA *amo*A | Arc-AmoA-F | STAATGGTCTGGCTTAGACG | 53 | Francis et al., 2005 |
| | Arc-AmoA-R | GCGGCCATCCATCTGTATG | | |
| AOB *amo*A | amoA-1F | GGGGTTTCTACTGGTGGT | 57 | Rotthauwe et al., 1997 |
| | amoA-2R | CCCCTCKGSAAAGCCTTCTTC | | |
| *nir*K | nirK876 | ATYGGCGGVCAYGGCGA | 58 | Henry et al., 2004 |
| | nirK1040 | GCCTCGATCAGRTTRTGGTT | | |
| *nir*S | nirSR-3dcm | GASTTCGGRTGSGTCTTSAYGAA | 60 | Throbäck et al., 2004 |
| | nirSC-d3aFm | AACGYSAAGGARACSGG | | |
| *ure*C | ureC-F | AAGSTSCACGAGGACTGGGG | 60 | Collier et al., 1999 |
| | ureC-R | AGGTGGTGGCASACCATSAGCAT | | |

The detection limits of the assays were determined by using standard curves, developed based on serial dilutions of recombinant plasmids containing the genes of interest Amplification efficiencies (*E*) of targeted genes were estimated from the standard curves by the equation $E = 10^{(-1/slope)}$ (Bustin 2000) and expressed as percentages using the formula $\% = (E–1) \times 100$. IBM SPSS Statistics for Macintosh, version 29 was used for statistical analysis. One-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) ($P<0.05$) was employed to compare the mean values of gene abundance in each treatment.

In a next experimental step, untreated sludge or sludge treated with 15% addition of the aforementioned materials (except for zeolite) were added to two soils (one acidic, pH = 5.0 and one alkaline, pH = 8.2) at 2% rate, in three replications, and left for equilibration with periodic wetting and air-drying. At 15d post equilibration,

samples of the soil treatments were collected and processed for DNA extraction and qPCR, according to the protocols described above.

Results from qPCR regarding 15% and 30% clay minerals, biochar and $Ca(OH)_2$ revealed statistically significant differences between treatments ($p<0.05$, data not shown). A similar scenario was also observed when untreated sludge or treated sludge with 15% addition of the materials was added to the two soils, at 2% rate. More specifically, qPCR analysis showed that the relative abundance of archaeal *amo*A gene did not vary significantly in soil mixtures with clay minerals or biochar compared with the soils without any treatment (Figure 1). The abundance of bacterial *amo*A gene was significantly higher ($P<0.05$) in mixtures of the acid soil with bentonite, vermiculite, biochar, whereas for *nir*K gene biochar mixtures were higher, relative to the untreated soils (Figure 1). Finally, the addition of treated sludge with the clay minerals or biochar in the soils did not change the abundance of *nir*S and *ure*C gene when compared with acidic or alkaline soil (controls), whereas $Ca(OH)_2$ in acidic soil resulted in the most drastic increase in *nir*S and *ure*C abundance (Figure 1).

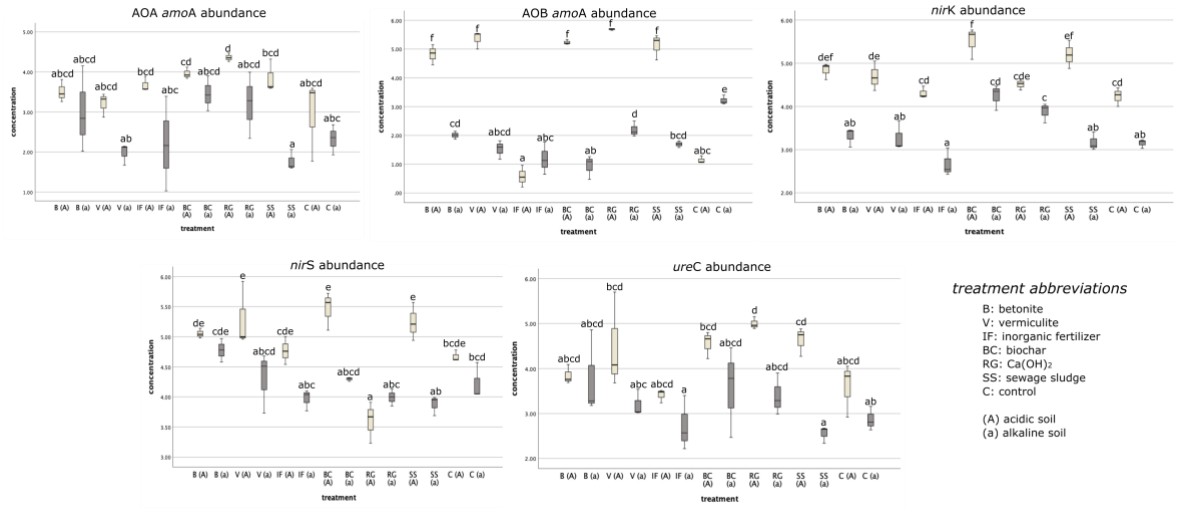


**Figure 1.** Gene abundance of *amo*A, *nir*S, *nir*K and *ure*C genes in soil treatments with activated sewage sludge (SS) or sludge mixed with clay minerals [bentonite (B), vermiculite (V)], biochar (BC), $Ca(OH)_2$ (RG) or inorganic fertilizer (IF). Soil samples were collected 15 days post equilibrium. Acidic soil (A) is highlighted with light shade whereas alkaline soil (a) is depicted with darker shade. Statistically significant differences ($P<0.05$) as calculated by one-way ANOVA were indicated by different letters. Error bars represent standard deviations of triplicate results of qPCR.

Overall, in this experiment, we quantified specific marker genes known to encode enzymes in the pathways of interest. The results characterize the genetic potential of the microbial communities to perform nitrification or denitrification is soil with untreated or treated with clay minerals or biochar sludge. Future work is in progress regarding the investigation of the microbial communities by high throughput sequencing of the 16S ribosomal RNA gene (16S rRNA) and to correlate the results from both methods.


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