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

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1. Background

- Loss of soil organic matter in arable land has exacerbated, jeopardizing soil fertility and rising need for more inorganic fertilization.

→ Solution: soil application of **stabilized sewage sludge** (replenishes agricultural soil with organic matter and plant nutrients and is a rational solution for sludge's disposal).

- 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.
- Microbial communities are involved in nitrification and denitrification activities and are often used as reliable indicators of soil quality (Schloter et al., 2018).
- Clay minerals may stabilize sewage sludge, with the potential of preserving N. Determining nitrifiers and denitrifiers by isolating and cultivating is inaccurate.

→ Employment of molecular methods to examine the abundance and diversity of microorganisms implicated in nitrification and denitrification in various environments, such as sewage sludge or soil.

Purpose of this study

Quantification of the gene abundance of bacterial nitrifiers and denitrifiers in sewage sludge treated with clay minerals and biochar and in soil mixed with treated sewage sludge.

2. Materials and Methods

❖ Three clay minerals (**bentonite**, **vermiculite**, **zeolite**) and **biochar** as well as **Ca(OH)$_2$** 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) by qPCR targeting *amo*A gene-sequence.

✓ 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.

✓ 5 ng/μl DNA extract was used in all experiments.

✓ Each treatment: three biological and three technical replicates.

✓ SYBR-Green qPCR assays using primers described in **Table 1.**

✓ A negative control with no template was included for biological replicate.

Table 1. Target genes, qPCR primers and Ta conditions used in the herein study.

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

✓ The detection limits of the assays→ standard curves (serial dilutions of recombinant plasmids).

✓ Amplification efficiencies (*E*)

- ✓ equation $E = 10^{(-1/slope)}$ (Bustin 2000)
- ✓ expressed as percentages using the formula % = (E–1) × 100.

✓ **IBM SPSS Statistics** for Macintosh (V.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.

3. Results

- qPCR regarding 15% and 30% clay minerals, biochar and Ca(OH)$_2$→ significant differences in gene abundance (*ure*C, *nir*K, *nir*S, *amo*A-AOA, *amo*A-AOB) between treatments. 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 ($p<0.05$, data not shown).
- Relative abundance of **archaeal (AOA) *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 (AOB) *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**).
- 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**).

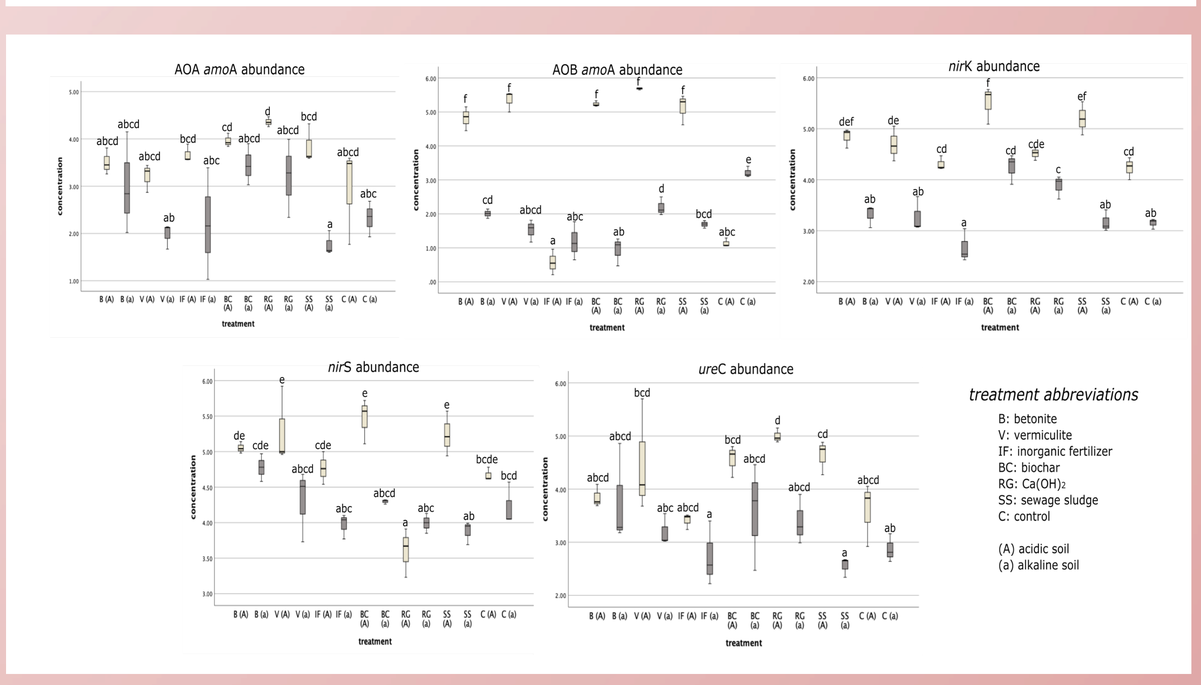

Figure 1. Gene abundance of *amo*A, *nir*S, *nir*K and *ure*C genes in soil treatments **with activated sewage sludge (SS) or sludge mixed with clay minerals [bentonite (B), vermiculite (V)], biochar (BC), Ca(OH)2 (RG) or inorganic fertilizer (IF). Soil samples were collected 15 days post equilibrium.** Acidic soil (A) is highlighted with light shade whereas alkaline soil (a) is depicted with darker shade. Statistically significant differences ($P<0.05$) were indicated by different letters. Error bars represent standard deviations of triplicate results of qPCR.

4. Conclusions

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 in soil with untreated or treated with clay minerals or biochar sludge. Further 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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