NITROUS OXIDE AND MICROBIAL SPECIATION: A META-GENOMIC SURVEY OF NEW ZEALAND WASTEWATER TREATMENT PLANTS

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ABSTRACT

Nitrous oxide (N_2O), a potent greenhouse gas and ozone-depleting compound, can be emitted from biological nitrogen removal (BNR) processes. Emissions have been reported to range from <1% - 25% of the influent nitrogen. Nitrous oxide reductase (*nosZ*), a key enzyme of the denitrification pathway, is the only known biological sink of N_2O. Denitrification is a modular pathway resulting in distinct denitrifying guilds: complete denitrifiers, incomplete denitrifiers and nitrous oxide sinks. The high variability in reported N_2O emissions may result from variation in the denitrifying microbial populations between studies.

This study presents a meta-genomic survey of three New Zealand WWTP denitrifying microbial populations. Data was mined for 16s rDNA and the functional denitrifying genes *nirK*, *nirS*, *nosZ* Clade I and *nosZ* CladeII. 16s analysis shows WWTPs to have a phyla profile typical of WWTPs and that they differ from one another at the genus level. Functional marker analysis shows a similar distribution of denitrifying genes across the WWTPs surveyed, with high *nirK*:*nirS* and low *nosZ* CladeII:*nosZ* Clade I ratios, indicative of a genetic potential for N_2O production.

KEYWORDS

Meta-genomics, Nitrous oxide, biological nitrogen removal, net metabolism, functional genes, *nirK*, *nirS*, *nosZ*

1 INTRODUCTION

Nitrous oxide (N_2O) is a potent greenhouse gas (GHG) and ozone depleting compound (ODC) (Ravishankara et al., 2009) produced by wastewater treatment plants (WWTPs) during biological nitrogen removal (BNR) (Foley et al., 2010; Wunderlin et al., 2012). N_2O emitted from WWTPs is highly variable, and reported values range from 0-25% influent-N for full scale WWTPs and 0-95% influent-N for lab scale systems (Kampschreur et al., 2009). It has been hypothesized that the variability in observed N_2O emissions between studies with similar experimental conditions is due to differences in the microbial consortia used (Lu and Chandran, 2010).

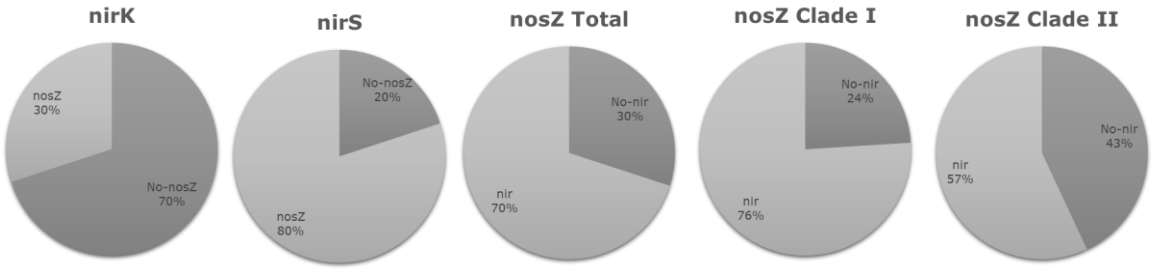
The primary determinant of N_2O production, accumulation and subsequent emission from any microbial consortia is the population's genetic potential. Nitrous oxide reductase (*nosZ*) is the only known sink of N_2O (enzyme known to consume N_2O) in the earth's troposphere (Richardson et al., 2009). This unique enzyme is found in a wide range of phylogenetically diverse bacterial and archaeal denitrifiers. Denitrification is an anaerobic respiratory process in which nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to nitric oxide (NO), N_2O and N_2 in a series of enzymatic steps (Figure 1). Denitrification is a modular pathway, meaning that organisms may not always contain the full set of genes, thus only performs a subset within the pathway (Graf et al., 2014). Due to the modularity of denitrification, organisms can be divided into three main groups: complete denitrifiers containing genes *nir – nos*, incomplete denitrifiers (approximately one third of denitrifiers lack the *nosZ* and are thereby genetically obliged to terminate denitrification at N_2O (Philippot et al., 2011)), and N_2O sinks - bacteria carrying *nosZ* but lacking the preceding denitrifying genes (Zumft & Kroneck, 2007; Sanford et al., 2012; Jones et al., 2014; Orellana et al., 2014). A causal link between the genetic potential of a microbial population and its nitrous oxide emissions has already been established. The introduction of a nitrous oxide-producing strain to the soil increased N_2O production, accumulation and emission (Philippot et al., 2011), however the level of emission is mediated by the ratio of N_2O producers to N_2O sinks in the soil (Jones et al., 2014).

Figure 1: Denitrification Reduction pathway:

The distribution of co-occurrence of denitrifying genes across taxa is non-random, with specific combinations of genes being more prevalent than others. For example, of all organisms with a sequenced or draft genome publically available and containing *nirK*, 70% lack *nosZ*, while for organisms containing *nirS* only 20% lack *nosZ* (Figure 2). The ratio of *nirK*:*nirS* and *nosZ clade II*:*nosZ clade I* acts as a good marker for the proportion of the denitrifying population that are N_2O producers (incomplete denitrifiers) or N_2O sinks respectively. These metric have support in the literature, with a positive correlation between *nirK* or *nirS* abundance to the total denitrification activity (N2 +N_2O) (Cuhel et al., 2010; & Wang et al., 2013). The ratio of *nosZ clade II:nosZ clade I* is an indicator of the proportion of N_2O sink organisms. Negative N_2O fluxes have been reported in field measurements (Chapuis-Lardy et al., 2007 & Wu et al., 2013), and it has recently been shown that the proportion of *nosZ Clade II* can explain observed N_2O sink capacity (Jones et al., 2013).

WWTPs differ in their microbial consortia geographically (Denecke et al., 2012; Hu et al., 2012; Wang et al., 2012). It is currently unclear if the patterns of co-occurrence observed from the fully sequenced and draft genome surveys are conserved in environmental samples and across WWTPs. While many studies have been conducted to determine the microbial speciation of WWTPs, few have focused on denitrifying organisms and their gene distributions. The aim of this study was to investigate the distribution of the denitrifying functional gene markers, *nirK*, *nirS*, and *nosZ* (*Clade I* (typical) and *Clade II* (atypical)) in a meta-genomic survey of three New Zealand WWTPs, and to determine their genetic potential for N_2O production.

Figure 2: Co-occurrence distribution of denitrifying genes:



2 METHODS AND MATERIALS

2.1 SAMPLE COLLECTION AND DNA EXTRACTION

Samples were collected from the return activated sludge line of three BNR treatment plants. All samples were shipped over night, and the DNA was extracted the next day. DNA was extracted from 1 g of centrifuged sludge with PowerSoil® DNA Isolation Kit as per manufacturer's instructions.

2.2 ILLUMINA SEQUENCING AND BIOINFROMATICS ANALYSIS

DNA samples (10 ug each) were subject to high-throughput sequencing using Illumina Hiseq 2500 with v4 chemistry. Nine Thruplex DNA-seq libraries were constructed as per manufacturer's instructions and run on one lane with 2x125 paired end sequencing.

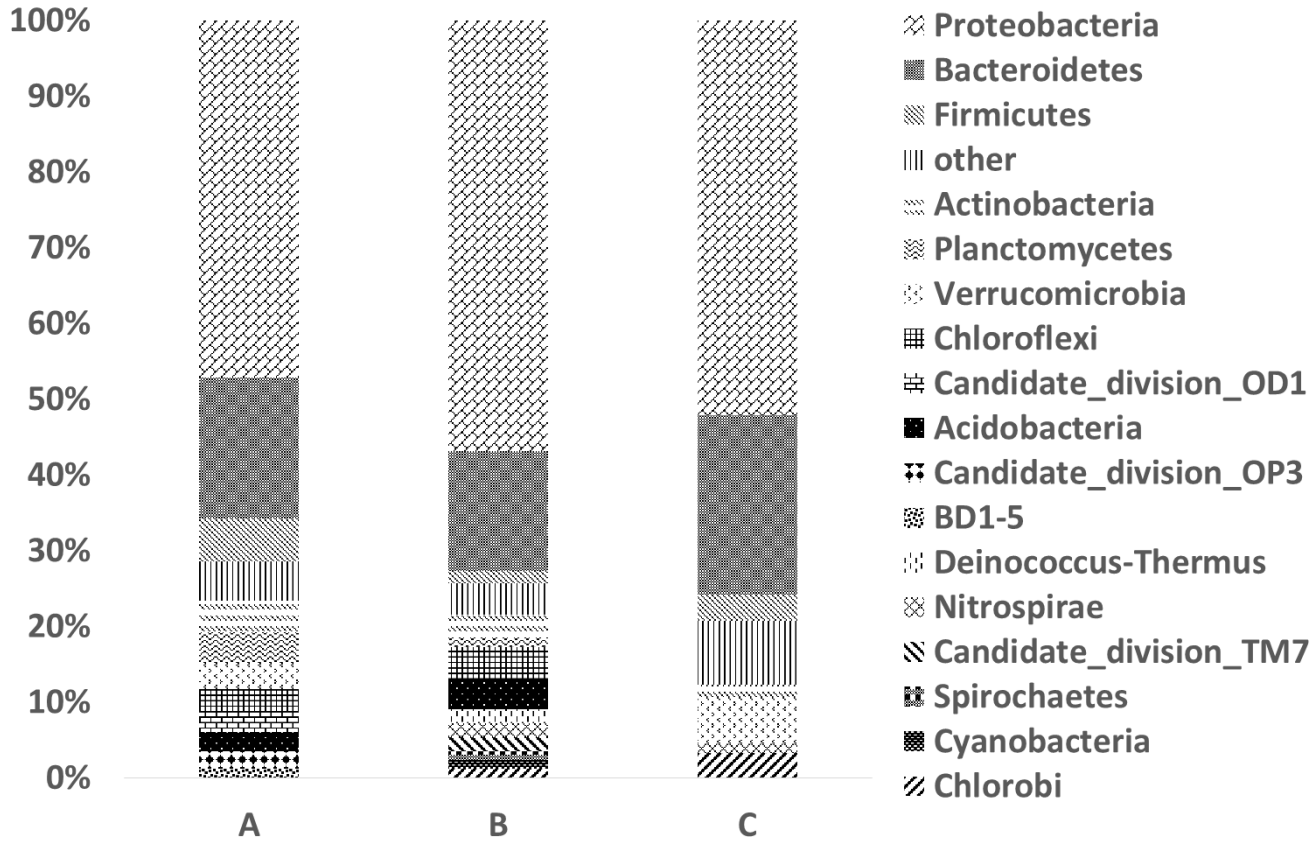
Read quality was assessed using FastQC (Bioinformatics B., 2016), and each sample was assembled individually using IDBA-UD (Peng et al., 2012) with default settings. Coverage was calculated for every contig longer than 1 kb. An index for each genome was created using Bowtie2 (Lagmead & Salzber., 2012). Reads were mapped to each index, and depth of coverage was calculated using Samtools (Li et al., 2009). The Silva (Quast et al., 2013) ribosomal database was BLASTed (Camacho et al., 2009) against the contigs produced by each assembly and de-replicated using vsreach (Rognes & Mahé, 2015). Open taxonomic units generated had a shared identity cut-off of 97%. Quantification of functional gene abundances followed the methods outlined by Wang et al (2014).

3 RESULTS AND DISCUSSION

3.1 16S RDNA

All WWTPs surveyed have a typical WWTP speciation and have major phyla similar to those reported in the literature (Denecke et al., 2012; Hu et al., 2012; Wang et al., 2012). The major phyla for treatment plant A included *Proteobacteria*, 47.17%, *Bacterodities*, 18.62% and *Firmicutes*, 5.65%. The major phyla for treatment plant B included *Proteobacteria*, 57.55%, *Bacterodities*, 16.03% and *Actinobacteria*, 3.07%. The major phyla for treatment plant C included *Proteobacteria*, 53.88%, *Bacterodities*, 24.50% and *Verrucomicrobia*, 6.36% (figure 3). The major phyla for A and B are similar to those reported in the literature, where populations are normally dominated by *Proteobacteria*, followed by *Bacterodities*, and then either *Firmicutes*, *Actinobacteria*, or *Acidobacteria* (Denecke et al., 2012; Hu et al., 2012; Wang et al., 2012). *Verrucomicrobia* has not yet been reported as a major phyla. Of the *Proteobacteria*, A was dominated by *Gama Proteobacteria*, 18.34%, followed by *Beta Proteobacteria*, 18.34%; B was dominated by *Beta Proteobacteria, 42.43%* followed by *Delta Proteobacteria* 10.29%; C was dominated by Beta *Proteobacteria*, 33.96%, followed by Gamma Proteobacteria 8.94%. Cluster analysis at the Genus level showed that, other than clustering at the family *Rhodocyclacae*, surveyed plants differ markedly (Figure 4).

Figure 3: Co-occurrence distribution of denitrifying genes:



3.2 FUNCTIONAL GENE MARKER COUNTS

WWTPs surveyed showed differing absolute values in gene counts but exhibited similar patterns of functional gene distribution (Table 1 and Figure 5). All WWTPs had a high count of denitrifying genes in comparison with the 16s rDNA count. While it is not possible to determine the exact proportion of denitrifiers from this data, this comparison functions as a good indicator of the portion of the total with denitrifying genes. A had the highest count of denitrifying genes to 16s, with the values being approximately equal, while denitrifying gene count was 66% and 37.96% of the 16s count for B and C respectively. All WWTPs also showed a higher proportion of *nosZ* gene to *nir* genes with *nir* gene making up only 81.48%, 88.19% and 61.62% of *nosZ* counts for A, B and C respectively. This pattern differs from previous reports. Typically, *nosZ* gene counts match those of *nirK* and *nirS*, while *nirK* and *nirS* are present in similar proportions with *nirS* values being higher than *nirK* (Wang et al., 2014, Ducey 2010). Unfortunately *nosZ Clade I*, and *nosZ Clade II* distributions are not yet widely investigated, so no comparison to other WWTPs can be made.

The surveyed WWTPs show a distribution of denitrifying functional genes, which indicates that each plant has the genetic potential for nitrous oxide production. Using *nirK*:*nirS* ratio as an indicator of the proportion of N_2O producers in a population, and the *nosZ Clade II*:*nosZ Clade I* ratio as an indicator of N_2O sinks, one can take the ratio of Producers:Sinks ((*nirK*:*nirS*):(*nosZ Clade II*:*nosZ Clade I*)) as an indication of a population's genetic potential for N_2O production. All surveyed WWTPs have a high *nirK;nirS* ratio and a low nosZ II:nosZ I ratio, indicating that a high proportion of the population are N_2O producers while a low proportion are N_2O sinks, indicating the genetic potential for net positive N_2O production, subsequent accumulation and emission (Table 2, and Figure 6).

Figure 4: Heat map of major Genera (with relative abundance over 1%):

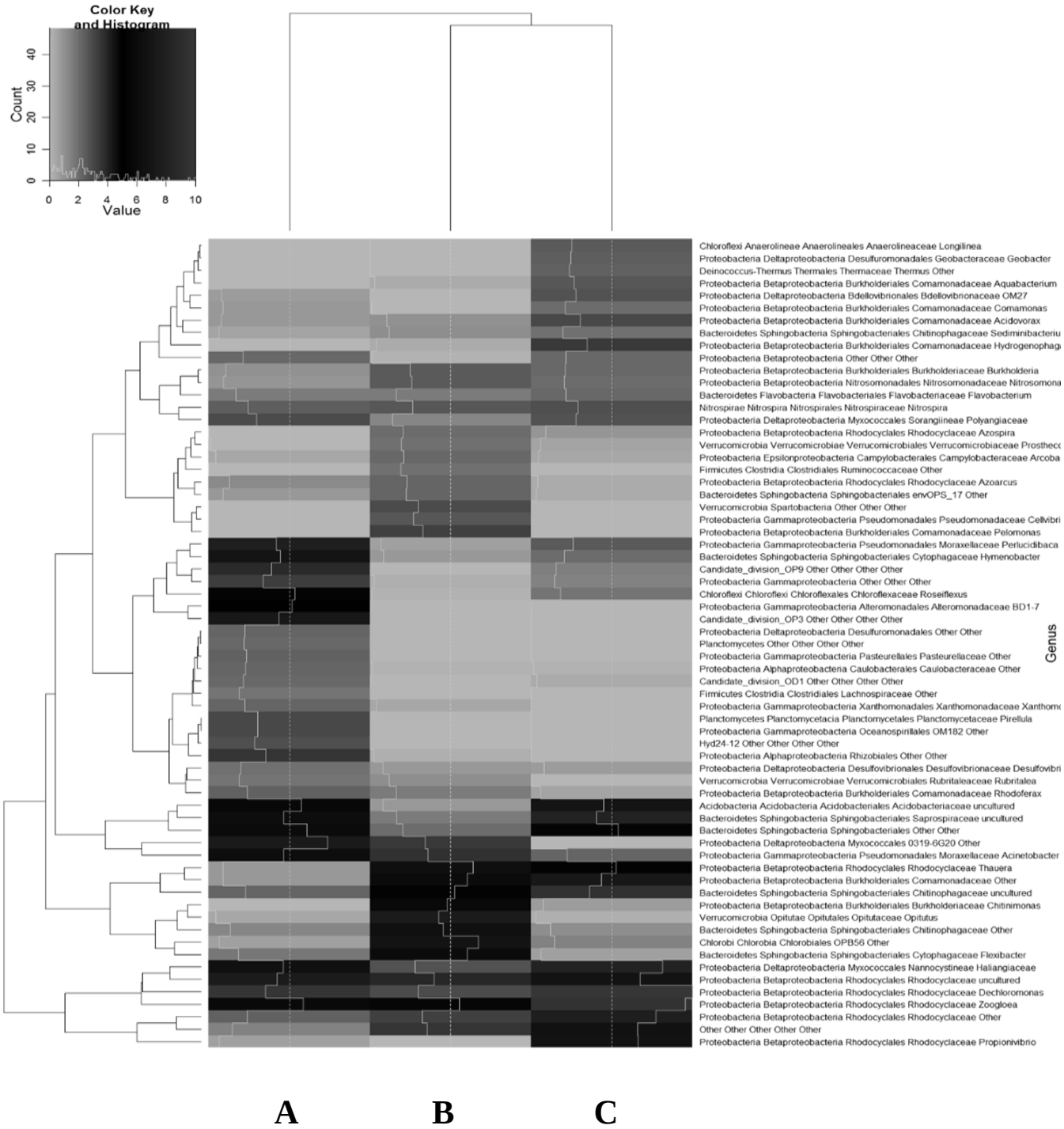


Table 1: Denitrifier and 16s counts

	nirK	nirS	total nir	nosZ Clade I	nosZ Clade II	Total nosZ	total denit	16s
A	3204.83	282.67	3487.50	3169.83	1110.50	4280.33	7767.83	7775.83
B	1496.33	87.33	1583.67	1097.83	697.83	1795.67	3379.33	5114.50
C	717.67	42.17	759.83	991.83	241.17	1233.00	1992.83	5249.17

Figure 5: Denitrifying Functional marker and 16s gene counts:

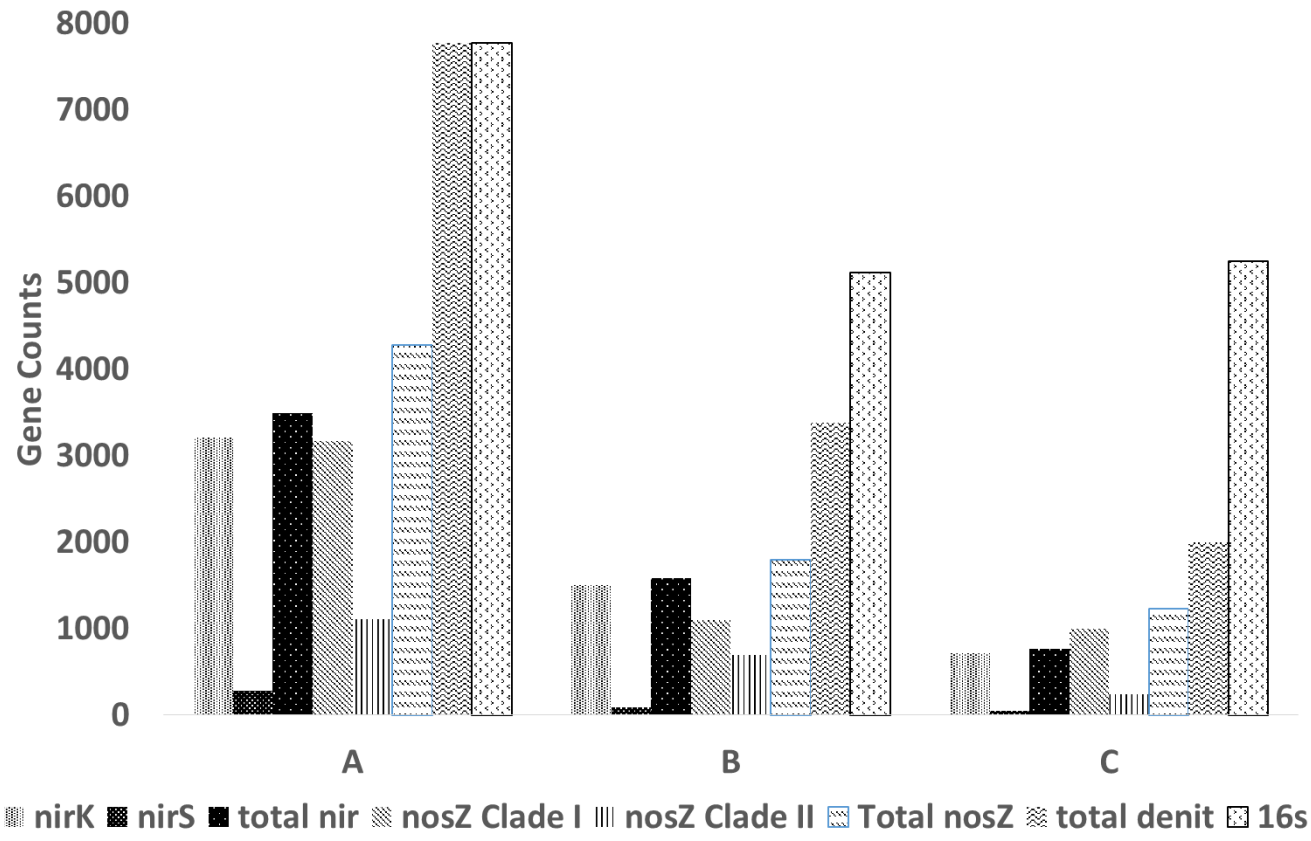
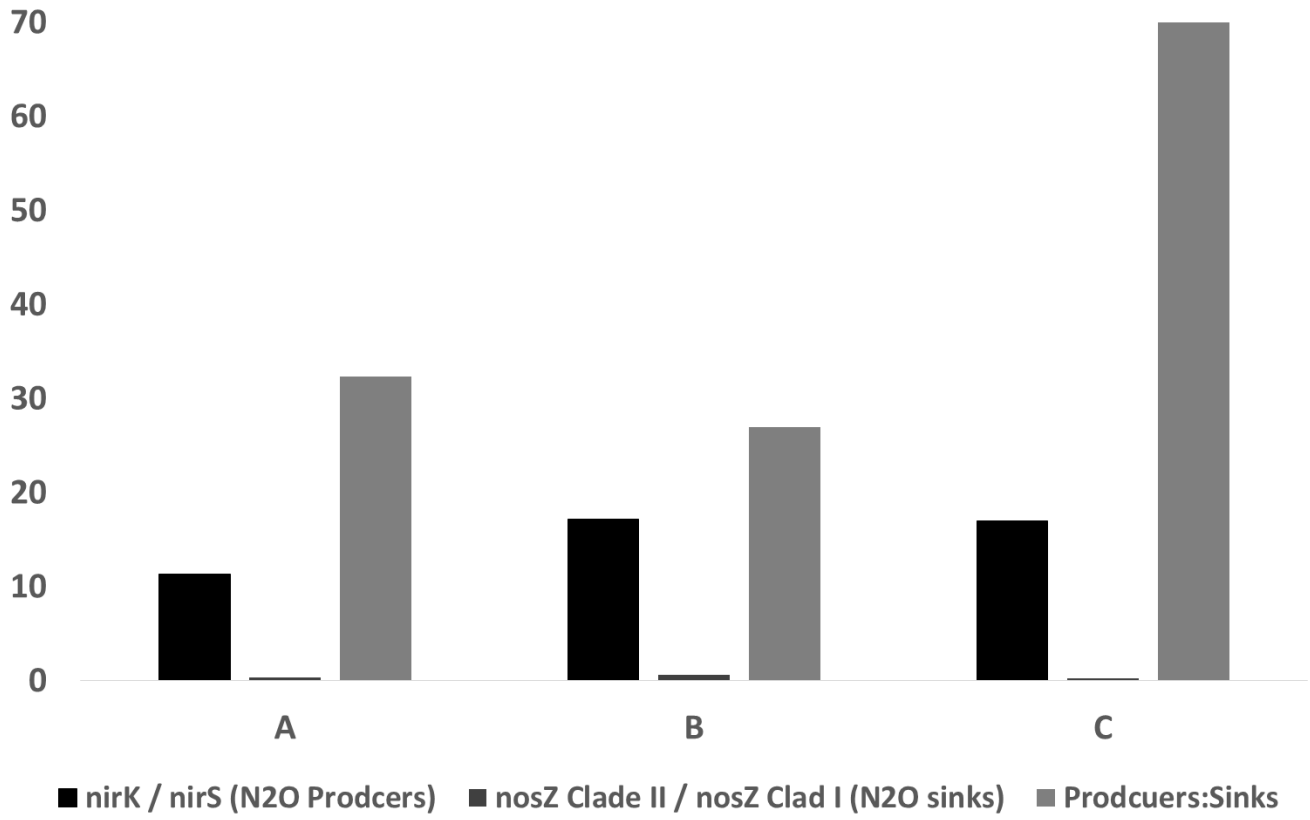


Table 2: Functional marker ratios, indicative of N_2O producers (nirK:nirS) and N_2O consumer (nosZ cladeI:nosZ clade II)

	nirK / nirS (N_2O Prodcers)	nosZ Clade II / nosZ Clad I (N_2O sinks)	Prodcuers:Sinks
A	11.34	0.35	32.36
B	17.13	0.64	26.95
C	17.02	0.24	70.00

Figure 6: Functional marker ratios, indicative of N_2O producers (nirK:nirS) and N_2O consumer (nosZ cladeI:nosZ clade II)



4 CONCLUSIONS

Surveyed WWTPs show similar patterns of speciation to the literature at the phyla level, but are markedly different in their relative abundances at the genus level. There appears to be conservation in the distribution of denitrifying genes between the treatment plants, however this distribution differs from other reports of denitrifying gene distributions. WWTPs surveyed exhibit the genetic potential for net positive N_2O production, accumulation and emission as evident by their high *nirK*:*nirS* ration and low *nosZ* Clade II:*nosZ* clade I ratio suggesting a high proportion of N2O producers and a low proportion of N2O sink organisms in the denitrifying population.

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