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# Nitrogen oxidation consortia dynamics influence the performance of full-scale rotating biological contactors

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ABSTRACT

Ammonia oxidising microorganisms (AOM) play an important role in ammonia removal in wastewater treatment works (WWTW) including rotating biological contactors (RBCs). Environmental factors within RBCs are known to impact the performance of key AOM, such that only some operational RBCs have shown ability for elevated ammonia removal. In this work, long-term treatment performance of seven full-scale RBC systems along with the structure and abundance of the ammonia oxidising bacteria (AOB) and archaea (AOA) communities within microbial biofilms were examined. Long term data showed the dominance of AOB in most RBCs, although two RBCs had demonstrable shift toward an AOA dominated AOM community. Next Generation Sequencing of the 16S rRNA gene revealed diverse evolutionary ancestry of AOB from RBC biofilms while nitrite-oxidising bacteria (NOBs) were similar to reference databases. AOA were more abundant in the biofilms subject to lower organic loading and higher oxygen concentration found at the distal end of RBC systems. Modelling revealed a distinct nitrogen cycling community present within high performing RBCs, linked to efficient control of RBC process variables (retention time, organic loading and oxygen concentration). We present a novel template for enhancing the resilience of RBC systems through microbial community analysis which can guide future strategies for more effective ammonia removal. To best of the author's knowledge, this is the first comparative study reporting the use of next generation sequencing data on microbial biofilms from RBCs to inform effluent quality of small WWTW.

## 1. Introduction

The removal of ammonia remains essential for purification of wastewater prior to discharge which is difficult to achieve at small wastewater treatment works (WWTWs) (e.g. < 2000–10,000 population equivalents) due to conflicting requirements for low energy and maintenance, land, capital expenditure and need for decentralised rural networks compared to conventional activated sludge processes (ASP) (Dutta et al., 2007; Hassard et al., 2015). Rotating biological contactors (RBC) are one technology which is commonly used within this small works context and are maintained based on decades of observed relationships between performance and operating parameters. However, differences underlying RBCs which perform well and RBCs which do not perform well are poorly understood, particularly for ammonia removal (Cortez et al., 2008; Hassard et al., 2018). A deeper analysis of the dynamics of RBC microbial communities and performance with respect to ammonia removal has the potential to highlight unprecedented insight into the performance of these biofilm reactors.

Ammonia-oxidizing bacteria (AOB) and ammonia oxidising archaea (AOA), which together are known as ammonia oxidising microorganisms (AOM) along with nitrite-oxidizing bacteria (NOB) are involved in nitrification processes (Bollmann et al., 2014; Daims et al., 2015). These chemolithoautotrophs obtain energy for growth by oxidizing either ammonia to nitrite (AOM) or nitrite to nitrate (NOM) using oxygen as a terminal electron acceptor. The AOM and NOB do not normally oxidize both substrates simultaneously. Complete nitrification is also possible by some members of the genus *Nitrospira,* however these complete ammonia oxidizers (Comammox) do not tend to dominate the nitrogen cycle within secondary treatments within WWTWs (Daims et al., 2015; Fitzgerald et al., 2015; Van Kessel et al., 2015). Recently comammox

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bacteria have shown to be abundant in tertiary RBC biofilms (i.e. low ammonia concentration), however their role in governing performance remains unknown particularly in highly loaded RBCs (Spasov et al., 2019). In other nitrifying organisms the separation of the two nitrification steps in different groups of organisms leads to process coupling and co-aggregation of AOM with NOB into reciprocal feeding nitrifying consortia harnessed for secondary wastewater treatment (Wijeyekoon et al., 2004).

Both the AOB and AOA possess the *amoA* gene which codes for the alpha-subunit of ammonia monooxygenase protein (AMO), which catalyses ammonia oxidation (Könneke et al. 2005) and the dominant pathway in conventional WWTW. This gene therefore was used to ascertain whether abundance of such organisms does or does not correlate with nitrification activity and therefore performance (Hassard et al., 2015). Since both AOB and AOA are responsible for ammonium removal in WWTWs, it was important to understand which environmental factors / process conditions influence the AOB and AOA abundance and performance of the RBCs.

Here, we focus on environmental factors which play important roles in maintaining stable nitrification as (i) AOM are sensitive to changes in their environment such as fluctuations in pH, salinity, ammonia concentration, light, oxygen and the presence of toxic or inhibitory compounds present within wastewater. (ii) AOA can outcompete AOB especially in low ammonia loading environments (e.g. distal through process) due to higher specific yield despite slower growth rate (You et al., 2009). Finally, (iii) AOB dominate over AOA when ammonia loading rates are high and the substrate-influx limits the growth within a spatial clustering of biomass which is common within microbial granules, aggregates or biofilms due to their higher specific growth rates (Wijeyekoon et al., 2004).

In this study we consider acclimation to substrate and oxygen limited concentrations would (1) lead to reduced biofilm ammonia removal rates and (2) result in a permanent change to the reactor community reflected in the distribution of AOA and AOB within biofilm reactors. These questions are studied through analysis of AOB and AOA ecology in seven full-scale RBC systems using high throughput sequencing of 16S rRNA gene and quantitative polymerase chain reaction (qPCR) of the AOB and AOA *amoA* genes and phylogenetic analysis. The insights into the microbial community dynamics in RBC systems will help to guide optimisation strategies for more effective ammonia removal. To our knowledge, this is the first study to compare the microbial ammonia oxidising communities across several different RBCs operating at small WWTW.

## 2. Materials and methods

### 2.1. RBC biofilm sampling

Biofilm was sampled from seven small WWTWs in the Midlands area of the United Kingdom (Table 1). At each WWTW, a 36 $cm^2$ circular area of biofilm was sampled from the surface of the individual RBC disks using a sterile disposable plastic spatula and microcentrifuge tube. The media could not be removed; therefore this represented a surface sample of biofilm. Subsequently the biofilm samples were transported on ice to the laboratory. Biofilm was sampled approximately twice monthly over a period of one year in two phases between November 2014 and February 2015, and August 2015 and November 2015 (Table S1). Samples were collected every two weeks whilst the RBC was temporarily stationary. During this period the RBC was operated under increasing load of municipal settled wastewater from 12 $m^3$ $day^{-1}$ to 38 $m^3$ $day^{-1}$. Access hatches enabled samples to be taken from the front and rear of the two biozones from the pilot scale RBC located WWTW 1 (Fig. 1A). Biofilm samples were taken from WWTW 2 – 7 over a period of 1 month between November 2015 and December 2015, access hatches permitted samples to be taken from the front and / or the rear of the RBC biozone (Fig. 1B). Sampling of front and rear of RBC was undertaken to understand if any differences between the biofilm was evident during the dynamic reactor conditions. Sampling of biofilm from WWTW 1 – 7 was undertaken in an identical way and details are provided within Table S1. The weight of the wet sample was recorded, the samples were homogenised prior to being snap frozen in liquid nitrogen and stored at − 80 °C. Samples storage occurred within 4 h of sampling from the RBC. Rotor speed was fixed at 2.4 rpm at each RBC. This led to differences in the media tip speed due to different RBC media diameter between different WWTW. Fixed rotor rpm (and variable tip speed) is typical for most water utilities due to complexity of operating variable rpm RBCs and mechanical issues which can arise. The media submergence was set at 38% for all the RBCs studied. No wastewater or solids recycle was undertaken at any of the WWTW studied. Therefore, denitrification is not expected to any significant degree. Ammonia oxidation assays were performed according to standard methods (details provided within Supplementary Material).

**Table 1**
Attributes of the 7 full-scale WWTWs from which biofilm samples were taken and analysed.

| | Design PE | Dry Weather Flow ($m^3$ $d^{-1}$) | Number of separate RBCs[#] |
|---|---|---|---|
| WWTW 1 | 50 | 12–38 | 1 |
| WWTW 2 | 438 | 147 | 1 |
| WWTW 3 | 572 | 223 | 2 |
| WWTW 4 | 602 | 230 | 3 |
| WWTW 5 | 463 | 140 | 3 |
| WWTW 6 | 167 | 45 | 1 |
| WWTW 7 | 11 | < 5 | 1 |

*DWF = Flow following 5 consecutive days of no rainfall; # RBCs can be situated in series or parallel; PE = unit per capita loading sum from industrial household pollution.

### 2.2. DNA extraction

Total nucleic acids were extracted from 0.3/0.0025 g (wet weight/dry weight [VSS]) biofilm samples using the Powersoil DNA isolation kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions and the DNA were re-suspended in 50 μL of molecular grade water (Ultrapure, Sigma, UK). These isolates were stored at − 80 °C until further analysis. Procedural blanks were included throughout.

### 2.3. qPCR of AOB and AOA amoA genes

qPCR for *amoA* genes of AOB and AOA were performed on a CFX96 Real-Time Detection System (Bio-Rad, USA). The *amoA* gene of AOB was amplified using the primers amoA-1F and amoA-2R (Rotthauwe et al. 1997). The *amoA* gene fragment of AOA was amplified using the primers CrenamoA23F and CrenamoA616R (Tourna et al. 2008). Full details of primers and sequences are provided in Table S2. For both AOB and AOA *amoA* genes, each 15 μL reaction mixture contained 1 μL of DNA template, 7.5 μL of 2 × SensiFAST SYBR No-Rox Kit (Bioline, UK), and 200 nM concentration of each primer. A dissociation curve analysis was performed at the end of each reaction to verify amplification of a single PCR product. The qPCR conditions for both AOA and AOB reactions were set as follows: initial denaturation for 3 min at 95 °C, followed by 40 cycles consisting of 95 °C for 5 s, then 60 °C for 30 s. Negative controls were performed with UltraPure Distilled water as the template. The samples were quantified against the corresponding standard curve using CFX Manager version 2.0 software (Bio-Rad). All purified DNA from each sample were subjected to duplicate qPCR assays.

DNA standards of known quantity were created using a dilution series $10^{-1}$–$10^{-7}$ of previously extracted *amoA* clones obtained from the PCR amplification of sediment DNA extracts (obtained from

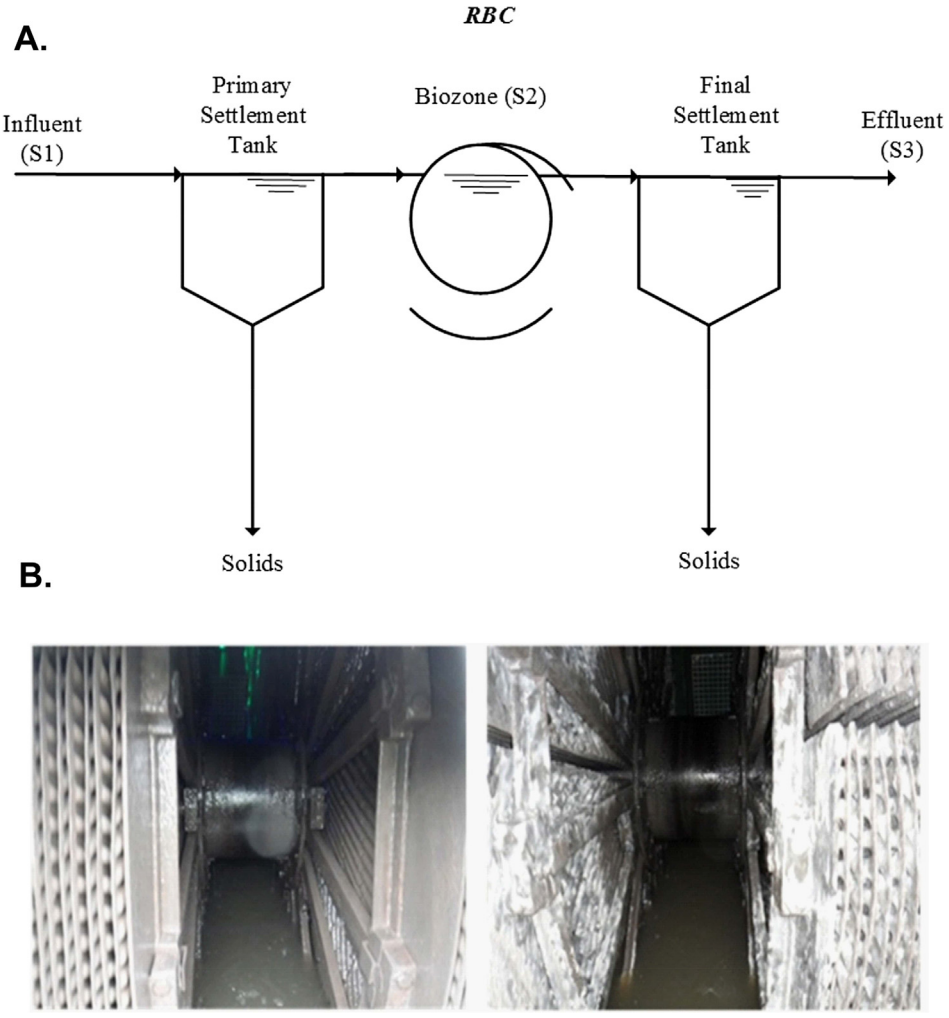


**Fig. 1. (A)** Schematic of WWTWs 1 – 7, the RBCs consist of a primary settlement tank, plastic disk me dia and final settlement tank. RBCs design differ in size and number of disks according to population equivalent. Sampling points are presented in brackets for wastewater quality analysis (S1&3) and biomass analysis (S2). **(B)** Pilot scale RBC – WWTW 1. RBC biozone at each WWTW was split into two zones and separated by a baffle, each zone has two distinct media plates (front and rear) within each zone.

University of Essex). The abundance of gene copies for standards was calculated by assuming a molecular mass of 660 Da for double-stranded DNA using the following formula: gene abundance = $6.023 \times 10^{23}$ (copies $mol^{-1}$) × standard concentration (g $ml^{-1}$)/molecular mass (g $mol^{-1}$). The DNA standards were diluted over a range from $10^2$ to $10^7$ target genes $ml^{-1}$ for both AOB *amoA* and AOA *amoA*. Standards, samples, and no-template controls (NTC) were amplified in duplicate with each primer set. The standard curve correlation coefficient ($R^2$) and amplification efficiencies (%) for the AOB were 0.981 and 95% respectively and for AOA were 0.997, 96% respectively. If $C_Q$ values between duplicate sample reactions exceeded 1 then data was discarded, and nucleic acids extraction and qPCR analysis was repeated on similar biofilm samples taken during the same sampling event. The qPCR data was normalised as gene copies per gram of wet biofilm ($GC.g^{-1}$ biofilm).

### 2.4. Sequencing of bacterial and archaeal 16S rRNA gene

For bacterial 16S rRNA gene libraries, primers Bakt_341F and Bakt_805R were applied (Herlemann et al., 2011). For archaeal 16S rRNA gene libraries, primers 344F (Raskin et al., 1994) and 915R (Stahl and Amann, 1991) were applied. All primers were flanked with the Illumina Nextera overhang sequences on the 5́ end Forward overhang: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; Reverse overhang: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. Samples were PCR amplified with a Veriti 96-Well Thermal Recycler (Applied Biosystems, UK) using the following conditions: initial denaturation for 3 min at 95 °C, followed by 28 cycles consisting of 95 °C for 30 s, then 55 °C for 30 s, and 72 °C for 30 s and finally 72 °C for 5 mins. Each reaction mixture contained 1 μL of DNA template, 12.5 μL of 2 × RedTaq Ready Mix (Sigma, UK), and a 5 μL of each primer (1 μM). Following the PCR amplification, the amplicons were cleaned using AMPure XP beads in conjunction with a SPRI super magnetic plate (Beckman Coulter, UK). Nextera XT identification indexes (Illumina, UK) and flow cell adapters were then applied to the 16S rRNA amplicons by PCR, using the same conditions, but limited to 8-cycles. Each reaction contained 5 μL of 16S rRNA amplicon product, 25 μL of 2 × RedTaq Ready Mix (Sigma, UK), and a 5 μL of each index primer. The PCR products were cleaned using the AMPure XP beads in conjunction with a SPRI super magnetic plate (Beckman Coulter, UK). A 1:10 dilution of PCR samples was the used to quantify the products using the PicoGreen (ThermoFisher, UK) assay and fluorescence was measured using FLUOstar Omega microplate reader (BMG, UK) before pooling the amplicon in equimolar concentrations. The final pooled 16S rRNA amplicon library was sequenced by The Earlham Institute, Norwich, UK) using a MiSeq Reagent Kit v3 (600-cycle) on an Illumina MiSeq instrument.

### 2.5. Sequence processing

Sequence reads were quality trimmed using Sickle (Joshi and Fass, 2011), error corrected within SPAdes (Nurk et al. 2013) using the Bayes Hammer algorithm (Nikolenko et al. 2013). Pair-end assembly was performed with PEAR (Zhang et al., 2014) within PANDASeq (Masella et al. 2012) with a minimum 50 base pair (bp) overlap for bacterial sequences. Archaeal amplicons were analysed on the forward read only as the amplicons were too long to overlap. The quality filtered (all sequences below 200 bp were removed) and error corrected sequences were then depreplicated, sorted by their abundance and operational taxonomic unit (OTU) centroids were picked using VSEARCH (Rognes et al., 2016) at the 97% level. Singleton and doubleton OTUs were removed, along with all chimeric sequences using reference-based chimera checking with UCHIME (Edgar et al., 2011) against the 16S rRNA RDP (Ribosomal Database Project). The number of sequences obtained for each group is presented within Table S3. Rarefaction analysis was performed to ensure sufficient observations were made to estimate sequence quantity that was measured by sampling and sequencing. Bacterial rarefaction curves were compared at a sampling effort of 7000 sequences to maximise the number of samples which could be used within the study (Fig. S1). Asymptotic accumulation curves were found indicating that the amplicon libraries represented dominant individuals within the RBC biofilm communities. Taxonomy assignment was performed with the RDP Classifier (Wang et al., 2007). 16S rRNA sequences of bacterial AOM obtained from this study were deposited to the GenBank Database under the accession numbers: MN326757- MN326769. Archaeon sequencing did not have even coverage across samples evidenced by rarefaction analysis (data not shown) therefore this data was not analysed further.

### 2.6. Phylogenetic analyses

16S rRNA gene sequences of AOM (AOB, NOB from this study and complete nitrification organisms from database inclusive) were aligned using MUSCLE (Edgar, 2004). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.2798 was constructed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (values over 70 shown) (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. The analysis involved 62 nucleotide sequences. There was a total of 435 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

### 2.7. Wastewater analysis

Performance and operating condition data were measured on a weekly basis, the flow rate to each of the RBCs studied was dependent on WWTW loading rates and was not controlled as part of this study. The substrate loading rate (feeding rate) was normalized to surface area of discs (total including wetted and non-wetted surface) and volume of wastewater per time, also referred to as disc loading rate. The following wastewater constituents were considered soluble and total chemical oxygen demand (sCOD and tCOD) concentrations (a measurement of how much soluble or total organic material is in wastewater), 5 days biochemical oxygen demand concentrations (a measure of degradation potential and oxygen consumption over 5 days – $BOD_5$), total suspended solids (TSS) and ammonia ($NH_4$-N) concentrations of water. The TSS was measured gravimetrically and the $NH_4$-N was measured using cell tests. The water pH and temperature were also recorded. Together, this dataset yielding a wide range of substrate / RBC removal performance values and relative percentage of the nitrogen oxidation pathway compared with the total removal of carbonaceous compounds. The hydraulic retention time (HRT) was calculated based on the wetted reactor volume/average dry weather flow rate (unit day). All chemical wastewater analysis was undertaken according to standard methods in APHA-AWWA-WEF (2012). Wastewater characteristics are presented within Table S4.

### 2.8. Statistical analysis

Multivariate distant based linear model (DistLM) were constructed to assess the effect of different reactor physicochemical features on the AOM abundance and overall microbial community composition. First, AOM data were $\log_{10} + 1$ transformed and environmental variables were normalised. Subsequently, a resemblance matrix based on Bray-Curtis similarity index was compiled using a step-wise forward procedure with 9999 permutations and Euclidian distance for physicochemical feature (PERMANOVA + for PRIMER). BEST analysis was used to distinguish most suitable characteristics for predictive analysis, by maximising a rank correlation between their respective resemblance matrices. Assumptions required for DistLM were met. Correlations between predictor variables was assessed and variables with autocorrelations $R > 0.8$ were excluded from the analysis, by taking the most suitable variable forward for the model construction. A canonical analysis of principal components was undertaken by constraining the community data and using it to predict positions along an 'X axis' using the multivariate data cloud. A Welch's *t*-test was undertaken using SPSS v25 (IBM, USA) to test for significance of different in AOM abundance from qPCR data between different RBC biofilm.

## 3. Results and discussion

### 3.1. Ammonia removal capacity of the 7 WWTW

Ammonia concentrations in the WWTW 1 and 2 were similar at 28.8 mg $L^{-1}$ (WWTW 1) and 33.2 mg $L^{-1}$ (WWTW 2) on average. WWTW 1 had higher organic load, represented by a COD of 546 mg $L^{-1}$ compared to 463 mg $L^{-1}$ for WWTW 2. Generally the wastewater treated by WWTW 2 was more biodegradable evidenced by a COD:$BOD_5$ of 3.1 compared to 4.8 at WWTW 2. WWTW 2 had RBCs which had 2.2 fold greater ammonia loading to the front of the RBC biozone than WWTW 1 (data not shown). The wastewater quality of WWTW 4–7 were not sampled frequently as part of this research programme and thus inferences were not made about the performance of these biofilm reactors. WWTW 1–3 were sampled for influent and effluent quality for a period of 7.5 years. WWTW 1 and 2 had water sampled weekly for influent, within reactor (biozone sample) and effluent for a period of 6 months. The biozone sample represented a composite of three samples taken interspaced within the biozone. Temperature strongly influenced the ammonia removal performance of RBCs. A decline in performance from a peak of 85.1% ammonia removal (WWTW 1) occurred at a temperature of 17.5 °C compared to a low of 68% ammonia removal (WWTW 2) which occurred at a temperature of 8 °C (Fig. 2A). Effluent ammonia concentrations were relatively stable over the 7.5 years sampling with characteristic elevated ammonia levels in effluent which typically occurred in winter months (notably WWTW 1). Average ammonia levels were 4.4, 0.36 and 1.61 mg $L^{-1}$ for WWTW 1, 2 and 3 respectively (Fig. 2B). Inter-stage sampling of WWTW 1 revealed intermediate removals across the RBC with higher concentrations than the final effluent (Fig. 2C). Superior performance occurred within WWTW 2 with indistinguishable concentrations within the biozone and the effluent quality which was attributed to the higher loading rates and nitrification activity of these biofilm (data not shown).

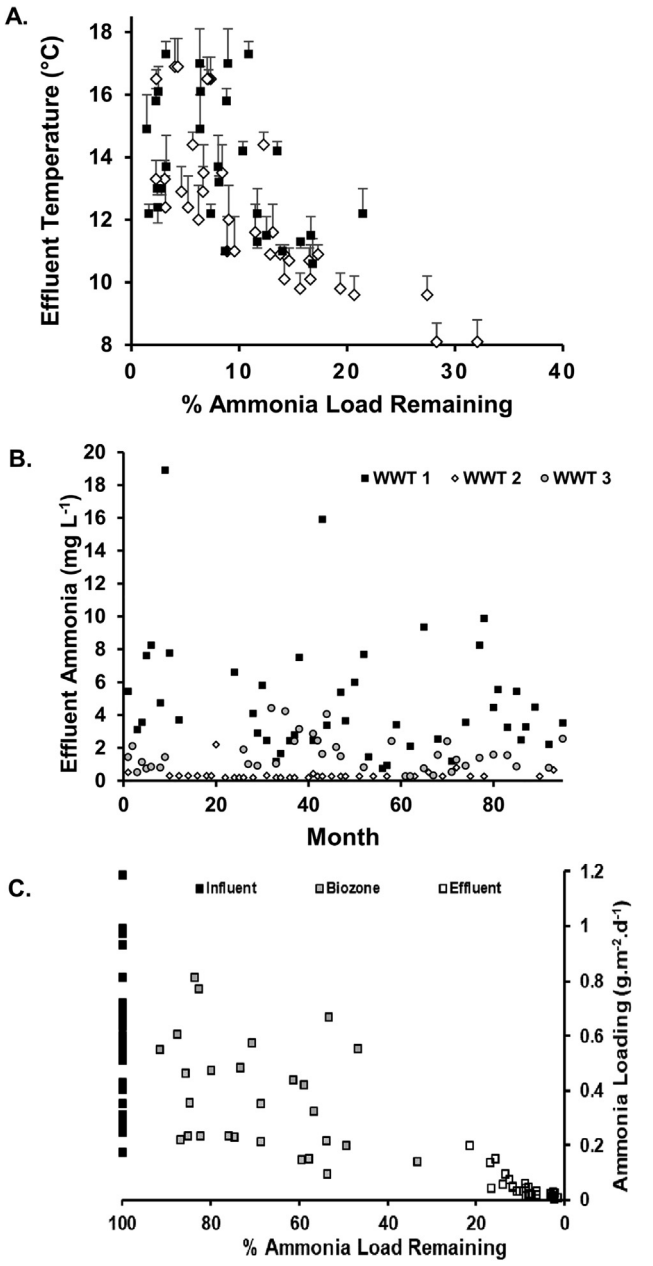


**Fig. 2. A** Impact of wastewater temperature (°C) on the ammonia removal % of influent ammonia load remaining in the effluent. Squares represent data from WWTW 1 (n = 30) and diamonds represent data from WWTW 2 (n = 38); **B** ammonia concentrations in the final effluent of WWTW 1–3 over 90 months (n = 175). Molecular biology sampling events occurred from months 54 to 90; **C** The ammonia nitrogen load for WWTW 1 showing the removal of ammonia load in the RBC (biozone) and effluent loading.

### *3.2. AOA and AOB abundance and distribution in RBC biofilm*

In this work, we use qPCR to assess the abundance of key functional groups of AOB and AOA and Next Generation Sequencing of the 16S rRNA gene to assess the diversity of bacterial AOM present within RBC biofilms across 7 operational WWTW. Biofilm samples were collected over a 1-month period from seven full-scale RBCs. Performance data was collected weekly (approx.) for a period of 1 year. A total of 24 biofilm samples were taken from RBCs in triplicate and analysed by qPCR and next generation sequencing of 16 s rRNA amplicons 72 samples were analysed from WWTW 1 and a further 15 samples from WWTW 2–7 (see Materials and Methods section).

The mean AOA *amoA* gene copy number was between $3.8 \times 10^4$ (GC. $g^{-1}$ biofilm) in WWTW 7 and $1.9 \times 10^8$ GC.$g^{-1}$ in WWTW 6. For AOB, the mean amoA gene abundance was between $2.6 \times 10^4$ to $2.6 \times 10^8$ (GC. $g^{-1}$ biofilm) in the same WWTW. WWTW 6 exhibited the highest abundance of both AOA and AOB in contrast to WWTW 7 which had the lowest abundance of both AOA and AOB (Fig. 3A). The ammonia effluent concentration was similar between WWTW 6 and 7, despite nine-fold difference in dry weather flow between the two WWTW (Table 2). WWTW 7 received a very low flow ($< 5$ $m^3$ $day^{-1}$) and furthermore this flow was intermittent. Therefore, the AOM in WWTW 7 was subjected to frequent drying / wetting periods and ammonia starvation, which could contribute to the observed low total *amoA* abundance (AOB + AOA). WWTW 4 and 6 ranked as first and second in terms of lowest ammonia effluent concentration (circumstantial evidence of high nitrification activity), these two sites exhibited significantly higher AOA gene abundance with an average of $1.3 \times 10^7$ and $1.9 \times 10^8$ GC. $g^{-1}$ biofilm respectively, compared to an overall average of $2.6 \times 10^5$ for WWTW 1, 2, 3, 5 and 7 (Fig. 3A). Welch's unequal variances t-tests revealed that AOB significantly varied across WWTW ($t_{Welch} = 4.6$, $p < 0.001$) whereas AOA was similar on average between WTWW ($t_{Welch} = 2.8$, $p > 0.05$; Fig. 3A). Post hoc test revealed that most of this difference was attributed to differences between WTWW 6 and the other WWTW which were similar (Games-Howell test, $p < 0.001$). Dominance in terms of abundance of each group of AOM in each RBC was tested. In most WWTW there was no significant difference between the AOB and AOA abundance. A Welch's *t*-test showed that WWTW 1 and 2 were dominated by AOA (WTWW 1, $t_{Welch} = 8.1$, $p = 0.05$) and (WTWW 2, $t_{Welch} = 2.3$, $p < 0.001$). In contrast, WWTW 4 was dominated by AOB (WTWW 4, $t_{Welch} = 3.5$, $p < 0.001$)

The WWTW with the highest ammonia effluent concentration ($17.4 \pm 10.8$ mg $L^{-1}$) had the lowest AOA:AOB ratios (0.034) in terms of *amoA* gene abundance (Table 2). WWTW 4 exhibited the lowest ammonia effluent concentration ($0.29 \pm 0.4$ mg $L^{-1}$) and was associated with the highest AOA:AOB ratio (38.7). WWTW 7 and 6 had the next highest AOA:AOB ratios (1.451 and 0.75, respectively) and these sites also had the third and second lowest average ammonia effluent concentration ($2.4 \pm 4.5$ and $2.6 \pm 2.5$ mg $L^{-1}$, respectively) (Table 2). This suggested that a high AOA:AOB ratio corresponds to better ammonia effluent quality and therefore AOMs could represent bio-sentinels of reactor performance. The suitability of this is discussed in more detail in subsequent sections.

The high ammonia and oxygen affinities (Stahl and de la Torre, 2012; Pan et al., 2016), may lead to the AOA dominance that has been reported in domestic WWTWs in China (Bai et al., 2012), municipal WWTWs in Thailand (Kayee et al., 2011; Limpiyakorn et al., 2011), industrial WWTWs in Europe (Mußmann et al., 2011), and a RBC treating municipal wastewater in Canada (Sauder et al., 2012). In contrast, other studies found that the abundance of AOB is greater than AOA in municipal WWTWs in the USA (Wells et al., 2009), a lab-scale WWTWs in China (Jin et al., 2012), industrial WWTW in China (Bai et al., 2012). This study reports that abundance varies according to the conditions within individual WWTWs, however AOB abundance usually outweighs AOA abundance in optimal conditions where excessive load is not present and oxygen availability is sufficient. Similar biomass activities were found between two intensively sampled RBCs (Fig. S2). However different AOM abundance was found between RBCs resulting in approx. 2.5 fold greater specific ammonia oxidation activity (Fig. S3). Differences in specific ammonia oxidising activity could account for the observed ammonia treatment performance at each WWTW (Fig. 2). Yao and Peng (2017) has reviewed the per cell AOB activity which ranged from 1.1 to 123 fmol-N. $cell^{-1}h^{-1}$ which is a similar order the the per GC AOB activity reported here (Fig. S3).

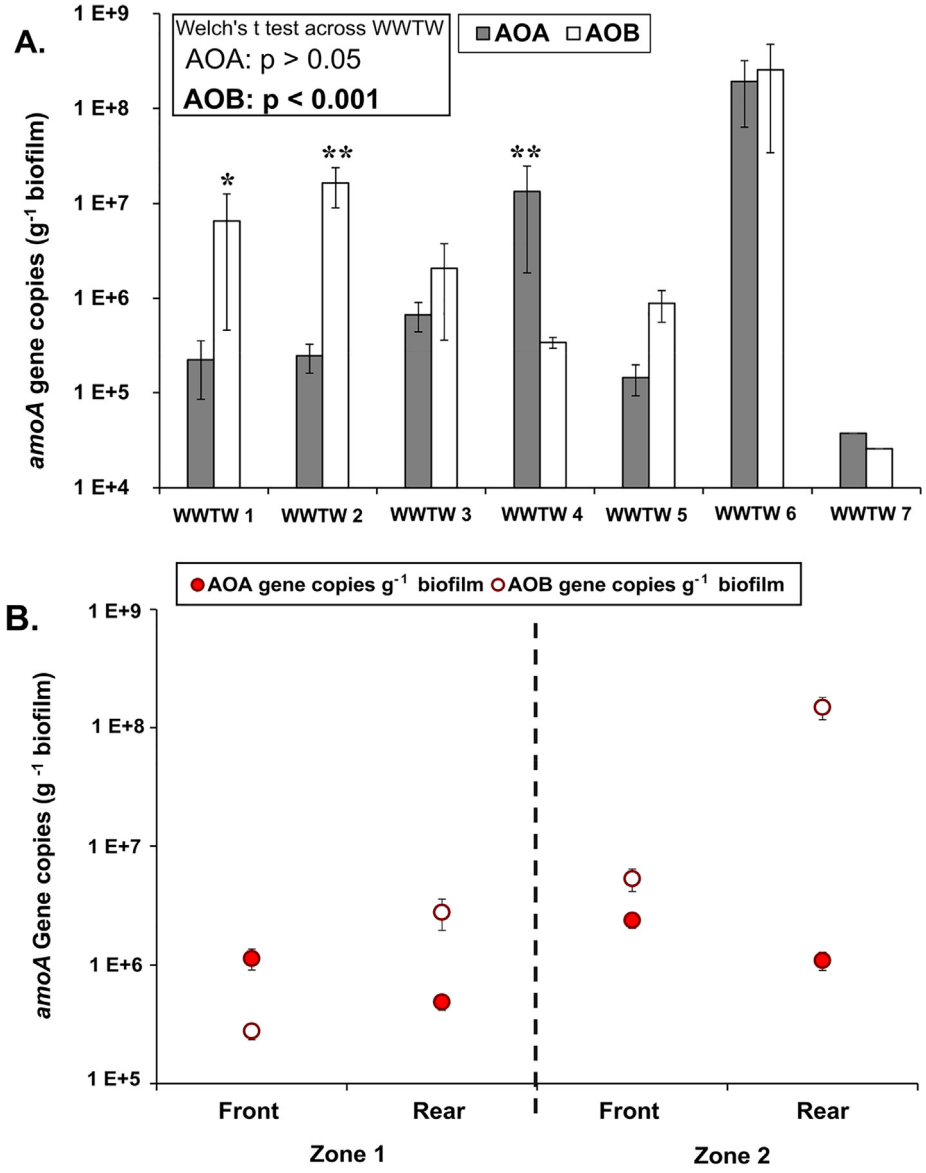


**Fig. 3. A.** *amoA* gene copies $g^{-1}$ biofilm (mean ± standard deviation) for AOA and AOB. The significance of the difference between AOA and AOB within each WWTW was tested by Welch's *t*-test and results are presented above each bar $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p > 0.05$ = blank. **B.** *amoA* gene copies $g^{-1}$ biofilm (mean ± standard deviation) averaged across front and rear of two zones in WWTW which were separated by a baffle.

**Table 2**
Ammonia removal capacity of the 7 WWTW 10 months prior biofilm samples collection.

| | DWF* flow ($m^3$ $day^{-1}$) | $NH_4$-N effluent (mg $L^{-1}$) | $NH_4$-N effluent standard deviation | $NH_4$-N effluent sample # (n) | Ratio of AOA and AOB |
|---|---|---|---|---|---|
| WWTW 1# | 12–38 | 17.4 | 10.8 | 28 | 0.034 |
| WWTW 2 | 147 | N/A | N/A | N/A | 0.015 |
| WWTW 3 | 223 | 3.42 | 3.3 | 11 | 0.325 |
| WWTW 4 | 230 | 0.29 | 0.4 | 13 | 38.698 |
| WWTW 5 | 140 | 2.8 | 3.9 | 40 | 0.163 |
| WWTW 6 | 45 | 2.4 | 2.5 | 44 | 0.75 |
| WWTW 7 | < 5 | 2.6 | 4.5 | 10 | 1.451 |

* DWF = Dry Weather Flow; N/A = no data available.
# data is prior to final sample taken, however samples were taken over a period of a year.

### 3.3. Impact of operating conditions on the ammonia oxidising consortia

All RBC biozones in this study consisted of at least 4 zones, with zone 2 and 3 separated by a baffle. This spatial separation of wastewater and biofilm is thought to enhance performance by selecting for specialist organisms adapted to wastewater in each biozone (Hassard et al., 2018; Petrie et al., 2014). Access hatches at WWTW 1 enabled samples to be taken at each zone (see Materials and Methods section), therefore this WWTW was selected for detailed sampling of biofilm and reactor physicochemical character. Mean AOB *amoA* gene abundance steadily increases from $2.77 \times 10^5$ to $1.48 \times 10^8$ GC. $g^{-1}$ biofilm from the front of zone 1 to the rear of zone 2 (Fig. 3B). On the other hand, AOA *amoA* gene abundance per gram of biofilm was similar throughout the process, with mean gene abundance ranging from $4.89 \times 10^5$ to $2.39 \times 10^6$ (Fig. 3B). The difference between AOA and AOB was significant in WWTW 1 ($t_{Welch,}$ $t = 23.4$, $p < 0.001$; Fig. 3B) with most of the difference attributed to the rear of zone 2 both within and between AOA and AOB (Games-Howell, $p < 0.001$). This suggests that either the AOA population was more resilient to fluctuating load and oxygen

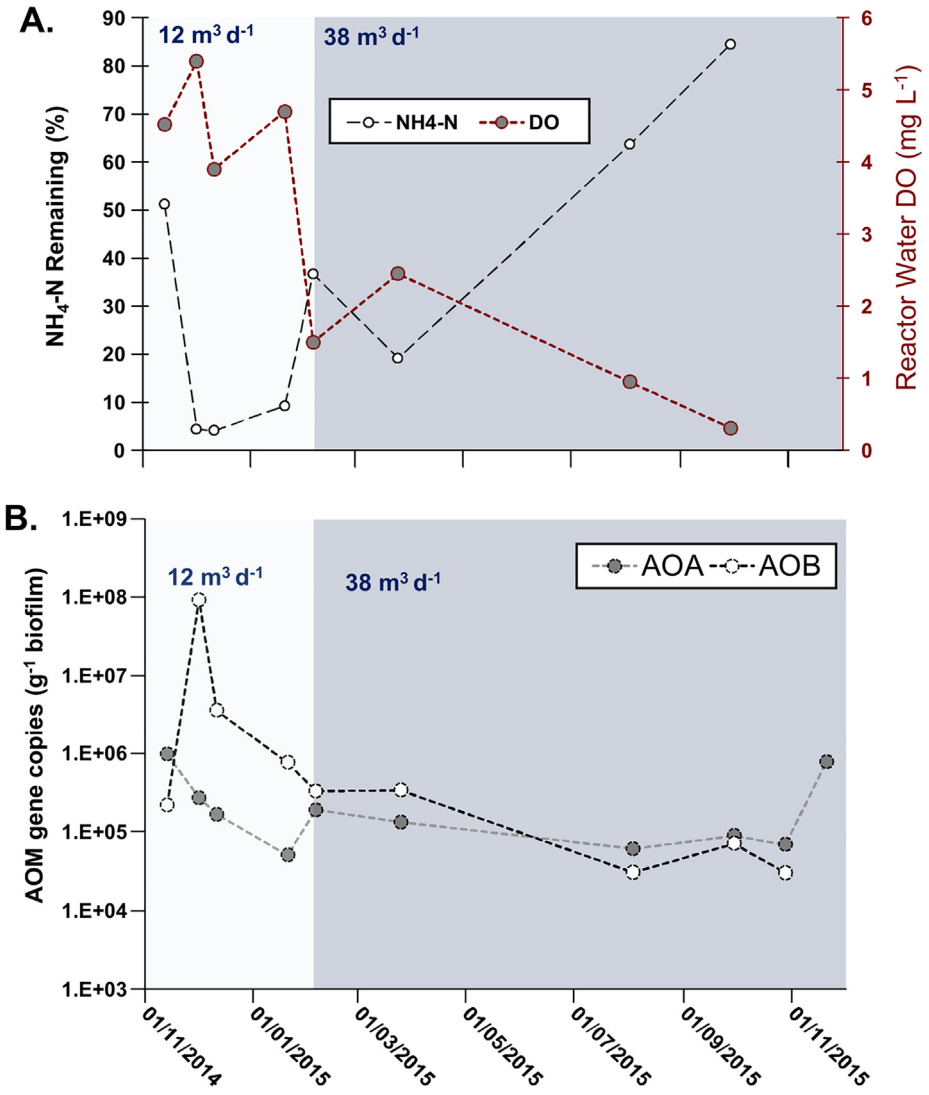


**Fig. 4. (A)** The ammonia removal performance and dissolved oxygen concentration in the RBC bulk liquid (n = 8 samples per sampling event). **(B)** The mean *amoA* bacterial and archaeal gene abundance (copies per gram of biofilm) within biofilm taken from the front of zone 2 of the RBC biofilm (WWTW 1) over a 1-year period.

availability, or that the AOB are readily outcompeted under lower HRTs. This finding is interesting as it indicated that AOA maintain activity in conditions that normally reduce AOB abundance. Also, as AOA have greater oxygen affinity than AOB, they can fill oxygen-limited niches within the deep layers of the biofilms and maintain a stable population where there is less competition. In contrast, AOB become more competitive in low loading conditions towards the distal end of the RBC biozone (zone 2 front and rear, Fig. 3B) where $BOD_5$ concentration is at its lowest and dissolved oxygen is at its greatest.

Biofilm was sampled on a monthly basis from the front of zone 2 of WWTW 1 in two sampling phases (see materials and methods). The first phase WWTW 1 had 12 $m^3 d^{-1}$ flowrate into the RBC. During this phase the RBC was nitrifying well (between 96 and 80% $NH_4$-N removed in December 2014 and January 2015) as the RBC was acclimatising and steady state was reached in December 2014. In the second phase, the flow was 38 $m^3$ $day^{-1}$ and the RBC appeared to heavily be loaded for nitrification as removal rates of 65 and 15% was achieved from March to October 2015 respectively (Fig. 4A). The bulk water dissolved oxygen concentration in phase 1 exceeded 4 mg $L^{-1}$ which diminished to < 2.5 mg $L^{-1}$ during the increased organic loading which occurred in phase 2. High ammonia removal in phase 1 correlated with the peak in AOB *amoA* with $9.2 \times 10^7$ GC.$g^{-1}$. This 1–2 orders of magnitude greater than the AOA which was between $6.1 \times 10^4$ and $9.8 \times 10^5$ *amoA* GC.$g^{-1}$ biofilm irrespective of wastewater loading (Fig. 4B). This agrees with previous studies which have shown that in most WWTW, AOA do not contribute in a significant way to overall ammonia removal performance (Fitzgerald et al. 2015).

The *amoA* gene is involved in the process of microbial ammonia oxidation (Tourna et al. 2008) and therefore can provide a useful indicator of ammonia oxidation dynamics in RBCs. In controlled lab-scale bioreactors variable AOB abundance has been shown in reactors with equivalent performance (Ahn et al. 2008) which could be explained by large variability in specific activity of AOB which was reviewed in Yao and Peng (2017). Reported specific AOB activity ranged from 1.1 to 123 fmol-N. $cell^{-1}h^{-1}$ but had an average of 23 fmol-N. $cell^{-1}h^{-1}$. However it is considered that the greater the number of AOM the higher the nitrification potential of a wastewater system (Almstrand et al. 2011). Whilst other studies have suggested limited correlation between nitrification rates and either AOA or AOB activity (Santoro et al., 2010). Despite the apparent lack of correlation, resilience of AOA to adverse conditions has been noted, compared to AOB, in a study where 50 mg $L^{-1}$ of silver nano-particles had a significant and detrimental effect on AOB *amoA*, but limited impact on AOA *amoA* gene abundance (Beddow et al., 2017). Differences could, in part, be due to differences in the ammonia monooxygenase (AMO) protein (e.g. active site for ammonia oxidation) between AOA and AOB species (Walker et al., 2010).

**Table 3**
Response variables are AOM abundance ($log_{10}$ + 1 mean *amoA* abundance). Predictor variables include $BOD_5$, COD and $NH_4$-N loading, percentage removal and load removed and temperature (water temperature). Model created from WWTW1 data only.

| Variable | Pseudo-F | p | Proportion of variation |
|---|---|---|---|
| HRT (d) | 6.15 | **0.002** | 0.32 |
| Loading of $NH_4$-N (kg $m^3$ $d^{-1}$) | 3.62 | **0.024** | 0.22 |
| Load of $BOD_5$ (kg $m^3$ $d^{-1}$) | 4.41 | **0.02** | 0.25 |
| Load removed $BOD_5$ (kg $m^3$ $d^{-1}$) | 3.37 | **0.05** | 0.21 |
| $NH_4$-N removal % | 2.71 | **0.05** | 0.17 |
| Temperature | 2.42 | 0.07 | 0.15 |

Contribution to total ammonia removal is a balance between diversity, abundance and activity of ammonia oxidisers which ultimately contribute to the nitrogen oxidation processes in wastewaters (Egli et al., 2003). Here we revealed which AOB species are found in RBCs and how the abundance of AOA and AOB relates to full-scale RBCs performance. Ultimately microbial community dynamics in RBC systems can be used as a tool to engineer more resilient ammonia oxidation in small WWTW.

Overall the data indicate that bacteria dominated at WWTW 1. However, in situations where load was high and dissolved oxygen was low, for example, at the beginning of the process or in high flow of 38 $m^3$ $day^{-1}$, archaea are competitive and may even play the dominant role. AOB appear to be more sensitive to changes in environmental conditions, with the *amoA* gene abundance increasing in low load, high dissolved oxygen conditions. DistLM revealed that a combination of HRT, organics loading, removal rate of $NH_4$-N and temperature were important factors governing the ammonia oxidizing community variability in WWTW 1 (Table 3). The HRT was the most important reactor variable (DBLM, Pseudo-F = 6.2, proportion = 0.32, p = 0.002) governing the AOM ecology suggesting this important process condition should be optimized for robust ammonia removal in RBCs (Table 3). Together the $NH_4$-N and $BOD_5$ loading rates accounted for ~ 25% each of the abundance of ammonia oxidizing community ($p < 0.05$). Whereas, the $NH_4$-N % removal accounted for 17% in the variability of the ammonia oxidizing community (DBLM, Pseudo-F = 2.71, proportion = 0.17p < 0.05), demonstrating the role of AOM and reactor performance (Figs. 5 and 6). Similarly, the role of $BOD_5$ removal was responsible for 21% of the variability in AOM (DBLM, Pseudo-F = 3.37, proportion = 0.21, $p < 0.05$) reinforcing previous studies which show the competition linked spatial localization between heterotrophic organisms and AOM. Modelling revealed a distinct nitrogen cycling community present within high performing RBCs operating at small WWTW, linked to efficient control of RBC process variables (mainly retention time, organic loading effects) and environmental variables e.g. temperature.

The ability of AOA to outcompete AOB at low ammonia concentrations is partly due to their superior ammonia affinity ($K_m$), reported as being 0.02 mg N $L^{-1}$ which is 100-fold lower than reported for AOB (Pan et al., 2016; Prosser and Nicol 2012). As well as higher ammonia affinity, AOA also possess higher oxygen affinity than AOB (Stahl and de la Torre, 2012; Mußmann et al., 2011). Consequently, AOA have been shown to exhibit higher activity than AOB in municipal WWTWs under DO of < 0.1 mg $L^{-1}$ (You et al., 2009; Fitzgerald et al., 2015). A similar trend of increased importance to nitrogen cycling and therefore wastewater reactor performance has been demonstrated for single step complete nitrification organisms (Daims et al., 2015). In contrast, another study showed that AOA only represent 8% of the AOM and have lower activity compared to AOB across 52 conventional WWWTs (Mußmann et al., 2011). The differing affinities for ammonia and oxygen, along with competition from heterotrophs can explain the dynamic state of ammonia oxidising communities and the ecological niche they occupy within biofilm (Pan et al., 2016).

### 3.4. Archaeal and bacterial community diversity

Phylogenetic analysis of sequences obtained by next generation sequencing showed that the AOB had diverse evolutionary ancestry from RBC biofilms and were similar to themselves and to reference OTUs. AOB-otu 586 was 97.2% similar to an isolate from a groundwater aquifer. Unsurprisingly, a very high similarity of 99.57% occurred between an isolate from a down-flow biofilm reactor and AOB-otu 887. In contrast, NOBs from RBC biofilm had a similar heritage with two distinct branches evident. NOBs was very similar (99.87%) to an uncultured *Nitrospira* sp. clone isolated from a submerged aerated biological filter (Fig. 5). These organisms were present on a separate branch to other NOBs and contained *Nitrospira*-like OTUs and separately, *Nitrobacter*-like organisms. High similarity was observed between RBC NOBs and database OTUs. For example, NOB-NOB-OTU 27 had a 99.34% similarity to a *Candidatus Nitrospira defluvii* and a clone isolated from a conventional WWTW. Previous studies also reported *Nitrososphaera* genus to be dominant in WWTWs (Kayee et al., 2011; Limpiyakorn et al., 2011; Mußmann et al., 2011; Park et al., 2006; Gao et al., 2014). *Nitrososphaera* members are slow growing and their ammonia oxidation activity (1.4 fmol $cell^{-1}$ $day^{-1}$) is less than other AOAs including which ranged between 2.5 and 12.8 fmol $cell^{-1}$ $day^{-1}$) (Li et al., 2016). Therefore, *Nitrososphaera* members are significant beneficiaries of the protective biofilm structure where they can exploit niches of low ammonia concentration (Hassard et al., 2018). Microbiome studies may help elucidate complex interactions between key functional groups (e.g. AOM and heterotrophs) and their impact on the nitrogen cycle which governs the performance of biofilm systems.

## 4. Conclusions

Some operational RBCs have shown ability for elevated ammonia removal. In this work, long-term treatment performance of seven full-scale RBC systems along with the structure and abundance of the ammonia oxidising bacteria (AOB) and archaea (AOA) communities within microbial biofilms were examined. The 90 month study revealed the following new insights into small biofilm based wastewater treatment processes:

- Long term data showed the dominance of AOB in most RBCs, although two RBCs had demonstrable shift toward an AOA dominated AOM community. Similar biomass activities were found between two intensively sampled RBCs. However different AOM abundance was found resulting in approx. 2.5 fold greater specific ammonia oxidation activity. This was thought to be a principal reason behind differences in observed ammonia treatment performance at each WWTW.
- Next Generation Sequencing of the 16S rRNA gene revealed a few new OTUs of AOM from RBC biofilms which were for the most part similar to reference databases. A
- AOA were more abundant in the biofilms subject to lower organic loading and higher oxygen concentration found at the distal end of RBC systems. Modelling revealed a distinct nitrogen cycling community present within high performing RBCs, linked to efficient control of RBC process variables (retention time, organic loading and oxygen concentration).
- We show that distinct AOM community exist between different RBC reactors operating at different WWTW. Nitrogen cycling organisms were present in all RBCs but differed in a significant manner between the RBCs.
- The study of fundamental process conditions (notably HRT, organic loading and $NH_4$-N removal rate) suggested that these acted as determinants governing variability in AOM.
- The major issue remains to be solved in future studies, how to increase the specific ammonia oxidation activity at small WWTW which are often treating dilute wastewater, are subject to shock

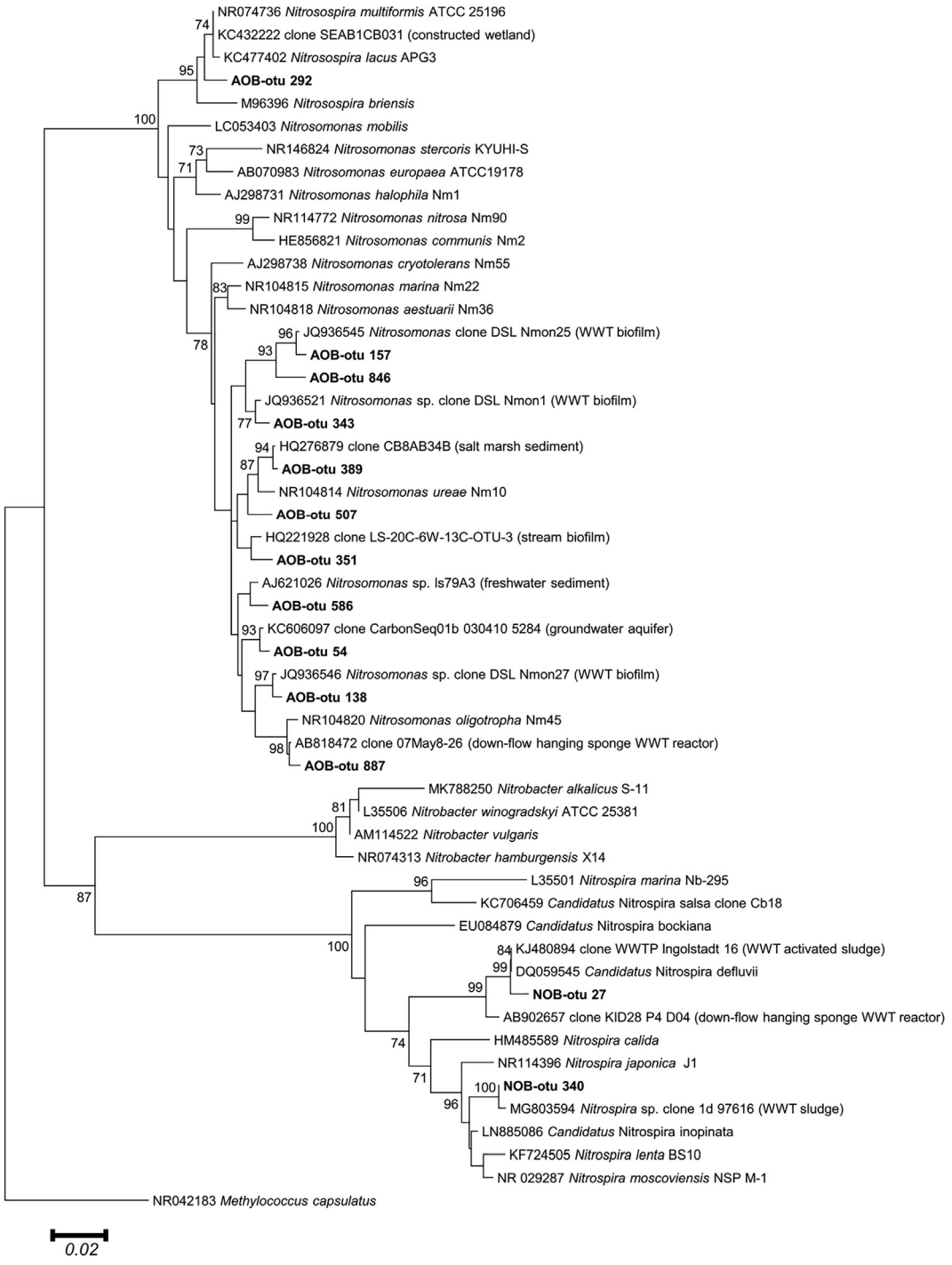


**Fig. 5.** Phylogenetic tree constructed from 16S rRNA sequences from nitrogen cycle organisms and comparative strains by Neighbour-Joining method. Scale bar denotes number of base substitutions per site. Reference strains were downloaded from GenBank and labelled with their accession number followed by Genus, species, metadata (if known). OTUs generated from this study are presented in bold.

loading, low temperatures, are for the most part remotely operated. Continued focus is recommended on stabilising ammonia loading rate to increase the resilience of ammonia oxidising microorganisms.

## 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.

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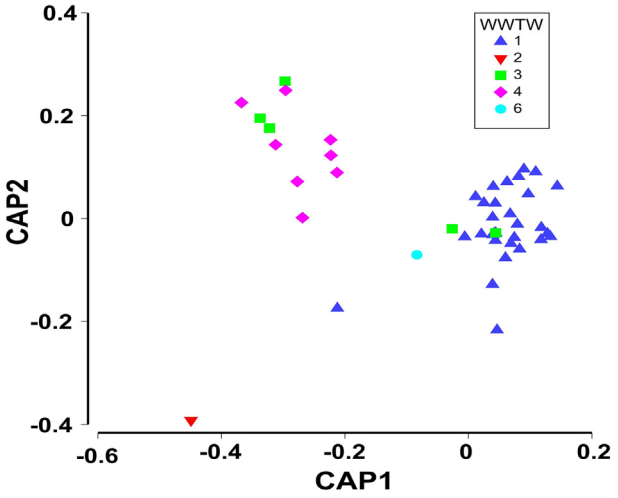


**Fig. 6. (A)** Principal coordinate ordination analysis of AOM community (AOA, AOB) and NOB across different RBCs (WWTW 1,2,3 4, 6). Constrained ordination using Canonical analysis of principal components (CAP) for five of the seven RBC reactors studied. Other RBCs excluded to meet assumptions of statistical method.

## Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105354.

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# Nitrogen oxidation consortia dynamics influence the performance of full-scale rotating biological contactors

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