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Microbial performance and community resilience
Toxicological assessment of biological nitrification

A thesis presented for the Degree of
Doctor of Philosophy by

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Declaration

This thesis is the result of the author's original research. It has been prepared by the author and has not been previously submitted for examination which has led to the award of a degree.

‘Due acknowledgement must always be made of the use of any material contained in, or derived from, this thesis.

Signed:

Date:

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To my son

Abstract

Microbial communities play a vital role in the biogeochemical nitrogen cycle, supporting the transformation of nitrogen compounds in different environmental habitats and engineered facilities such as wastewater treatment plants (WWTPs). Traditional biological nitrification is a biochemical process carried out by two autotrophic microbial clades of nitrifiers: ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB). Together they perform the two-step ammonia oxidation to nitrate via nitrite. Although complete oxidation of ammonia can be performed by a single organism (Comammox), AOB/NOB guilds represent an essential microbial population for the removal of inorganic nitrogen compounds in conventional WWTPs. These important microbes are sensitive to the toxic action of many pollutants and operational changes, and their loss of activity can lead to nitrification failure in WWTP. As a result, many nutrients can be disposed into the environment with detrimental effects on the ecosystem and human health.

Over the years, there has been a growing concern about pharmaceuticals and personal care products (PPCPs) in wastewater streams. They comprise a vast number of chemicals, including antibiotics and commercial products. Some PPCPs have toxic potential for many organisms, including nitrifying bacteria. The main aim of this PhD was to assess the short-term effect of common PPCPs on the metabolic activity of enriched nitrifying cultures.

The first stage of the PhD research comprised the cultivation of AOB-NOB populations in batch reactors under selective growth conditions to obtain biomass for the toxicity bioassays. Two types of samples were used as sources of nitrifiers, WWTP sludge for no salt-adapted nitrifiers species and coastal marine sediments targeting halotolerant AOB-NOB communities. Subsequently, the toxicity experiments were carried out in short-batch tests to assess the individual toxic effect of selected PPCPs on nitrifiers.

The experimental findings presented in the thesis demonstrated that some of the tested PPCPs can negatively affect the metabolism of nitrifying populations in short-term exposures. In the WWTP cultures, the antimicrobial agent triclosan exhibited the most significant nitrification inhibition, reducing the overall oxidation process by 50 % (EC_{50}) with the lowest concentration of 89.1 μg L^{-1}. Within the antibiotics tested, colistin produced the highest nitrification inhibition, with an EC_{50} of 1 mg L^{-1}, with a more pronounced effect on AOB species compared to NOB strains. Other common antibiotics, ampicillin and ofloxacin, produced inhibition with EC_{50} values of 23.7 mg L^{-1} and 12.7 mg L^{-1}, respectively. Moderate inhibition was observed with the insect repellent ingredient DEET, causing less than 40% nitrification inhibition at 10 mg L^{-1}. On the other hand, the stimulant caffeine had no critical inhibitory effects on the activity of AOB/NOB species. Additionally, the acute toxicity bioassays performed with the salt-tolerant

nitrifying cultures showed that triclosan did not affect the oxidation activity up to the maximum concentration tested (0.1 mg L^{-1}) after 24 h of toxic exposure. Overall, the toxicant (PPCPs) concentrations causing nitrification inhibition presented in this thesis were considerably higher than relevant levels reported in environmental samples and WWTP streams. However, excessive human consumption of triclosan may lead to higher concentration in the sewage closer to the toxicity threshold found in the WWTP samples, posing a risk to nitrifying bacteria performance in biological wastewater treatments.

The research presented here highlights the importance of investigating pollutants' toxic effects on nitrifying populations. The experimental threshold responses can contribute to the AOB/NOB inhibition data, supporting mitigation strategies to maintain a stable nitrification process in WWTPs. In addition, this study captured practical recommendations associated with the lessons learned during the preparation of toxicity assays with enriched nitrifying cultures. This information can help future researchers develop high-quality testing protocols to continue understanding the toxic effect of pollutants in AOB/NOB communities.

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Introduction

1. Background

1.1. Autotrophic nitrification

Nitrogen is an essential element for all forms of life on Earth. This nutrient exists in multiple chemical forms and oxidation states in the environment, and it is provided to plants and animals through the nitrogen cycle (see Fig. 1) (Daims et al. 2016). In this cycle, microorganisms play a vital role in the transformation of nitrogen species, starting with the dinitrogen (N_2) fixation from the atmosphere into ammonia and subsequently assimilated into organic nitrogen as cell material (biomass) (Bock and Wagner 2013). Furthermore, the degradation of organic nitrogen produces ammonia, known as ammonification. Subsequently, ammonia/ammonium is converted to nitrate via nitrite in a two-step process (nitrification by two microbial guilds) or complete nitrification by a single organism called Comammox (Daims et al. 2016; Kuypers et al. 2018). After that, nitrate is assimilated by plants, transformed for anaerobic respiration and eventually converted to N_2 through denitrification or Anammox (anaerobic ammonium oxidation) (Bock and Wagner 2013)

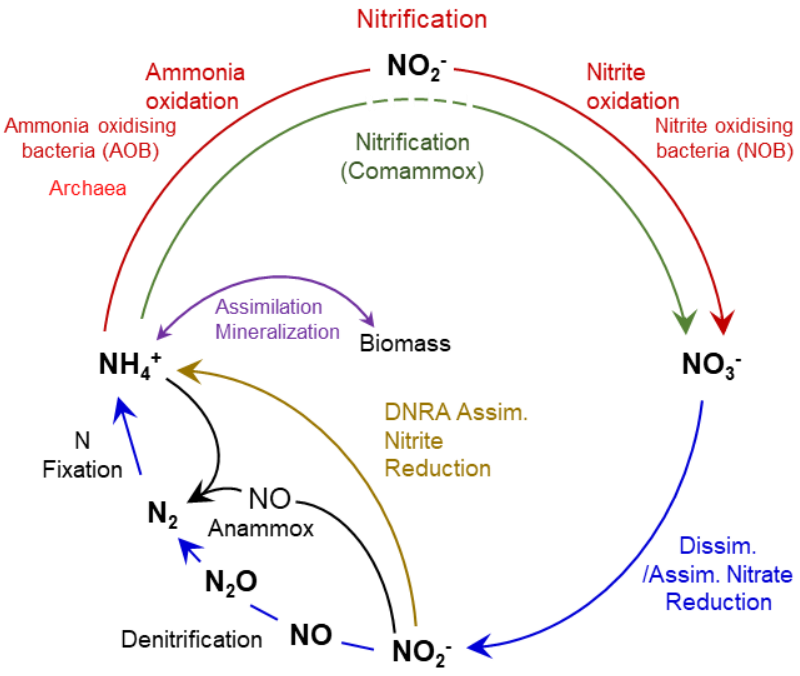

Fig. 1. Nitrogen cycle. Adapted from (Daims et al. 2016)

This thesis focuses on two physiological groups known as nitrifying bacteria (Madigan et al. 2000), which work in cooperation in the biological nitrification process, with relevance in their role in engineered facilities such as wastewater treatment plants (WWTPs) (Graham et al. 2007). In this process, the first step comprises the oxidation of ammonium/ammonia to nitrite carried out by the ammonia-oxidising bacteria (AOB), while the second step involves further oxidation of nitrite to nitrate by the nitrite-oxidising bacteria (NOB) (Koops and Pommerening-Röser 2001). The designated terminology for AOB is the prefix *Nitroso-*, while NOB species names start with *Nitro-* (Madigan et al. 2000). These two guilds are classified as chemolithoautotrophic organisms using inorganic carbon and nitrogen as the primary source of energy and typically obligate aerobes, requiring oxygen for metabolic activity (Bock and Wagner 2013). At the same time, novel ecophysiological characteristics and metabolic pathways in nitrifiers are being discovered thanks to innovative cultivation methodologies and the improvement of molecular techniques nowadays, expanding our understanding of these microbes and their role in the environment.

1.2. Why nitrifying bacteria?

AOB/NOB guilds are critical mediators in nitrogen transformations in many habitats and engineered facilities, and the prevalence of their metabolic activity is paramount to maintaining a healthy environment. In wastewater treatment plants (WWTPs), nitrifiers participate in removing nitrogenous compounds in wastewater, minimising the discharge of large amounts of nutrients to water bodies. Without their work, excessive release of nutrients can cause the death of all aquatic organisms due to overstimulation of algae growth and oxygen depletion. However, many nitrifying bacteria species are often reputed as sensitive bacteria in WWTP. Therefore, their performance could be affected by operational conditions and toxic chemicals in the wastewater (Pagga et al. 2006; Dytczak et al. 2008). Also, nitrifiers exhibit slow growth rates, with long doubling times ranging between 7 h -24 h for AOB (Cua and Stein 2011) and 12 h - 140 h for NOB species (Nowka et al. 2015a; He et al. 2021). Consequently, the loss of nitrifiers' activity produces nitrification failure in WWTPs, followed by long recovery periods (Kim et al. 2013). Susceptibility of nitrifiers to disturbance has been attributed to their low diversity (Egli et al. 2003; Siripong and Rittmann 2007; Figuerola and Erijman 2010; Zhang et al. 2019b) and fragile mutualism between synergistic AOB-NOB communities, leading to instability and disruption of the nitrification process (Graham et al. 2007). Nitrifiers are key functional groups in many ecosystems, including soils, sediments, and water bodies. Therefore, understanding the impact of contaminants on nitrifying populations is vital to prevent potentially damaging effects on the environment and engineered facilities. Lastly, the relevance of nitrifiers has been highlighted by many authors. For example, the publication from Curtis (2006) sends a clear message alerting the scientific community about the catastrophic consequences to all living creatures in the

possible scenario of nitrifiers' extinction. Surprisingly, toxicological data of AOB/NOB species remains relatively small, considering the significant number of toxic compounds released into the environment every day.

1.3. Description of nitrifying bacteria

This section briefly describes the AOB/NOB species, including nitrogen reactions, relevant ecophysiological characteristics, and preferred habitats, emphasising WWTP microbial communities.

1.3.1. Ammonia-oxidising bacteria (AOB)

1.3.1.1. Biochemistry of AOB

As mentioned previously, AOB carry out the first step of converting ammonia to nitrite, following the total reaction presented in Eq. 1 (Soliman and Eldyasti 2018). This process (usually referred to as nitritation) occurs in two reactions where hydroxylamine (NH_2OH) is formed as an intermediate product as Eq. 2. The enzyme responsible is the ammonia monooxygenase (AMO), an integral protein from the copper membrane monooxygenase (CuMMO) family (Lancaster et al. 2018). The AMO enzyme comprises three protein subunits encoded by *amoA*, *amoB*, and *amoC*, with *amoA* being the most common functional marker in ammonia oxidisers studies (Wang et al. 2020). Subsequently, hydroxylamine oxidation to nitrite is catalysed by a second enzyme, the hydroxylamine oxidoreductase (HAO), located in the periplasmic space (Eq. 3). (Madigan et al. 2000)

$$NH_3 + 1.5\,O_2 \rightarrow NO_2^- + H^+ + H_2O \quad (1)$$

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \rightarrow NH_2OH + H_2O \quad (2)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5\,H^+ + 4\,e^-$$
$$2\,H^+ + 0.5\,O_2 + 2\,e^- \rightarrow H_2O \quad (3)$$

1.3.1.2. Diversity of AOB

Based on the diversity classification in Koops and Pommerening-Röser (2001) and Purkhold et al. (2000), five AOB genera are included in the phylum *Proteobacteria*, which are generally Gram-negative bacteria. The AOB classification and species-level diversity are presented in Fig. 2. Most genera generally lie in the *β-Proteobacteria* subclass established as *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio*. The genus *Nitrosococcus* belongs to the *γ-Proteobacteria*, represented by two halophilic (salt-tolerant) species *Nitrosococcus oceani* and *Nitrosococcus halophilus*.

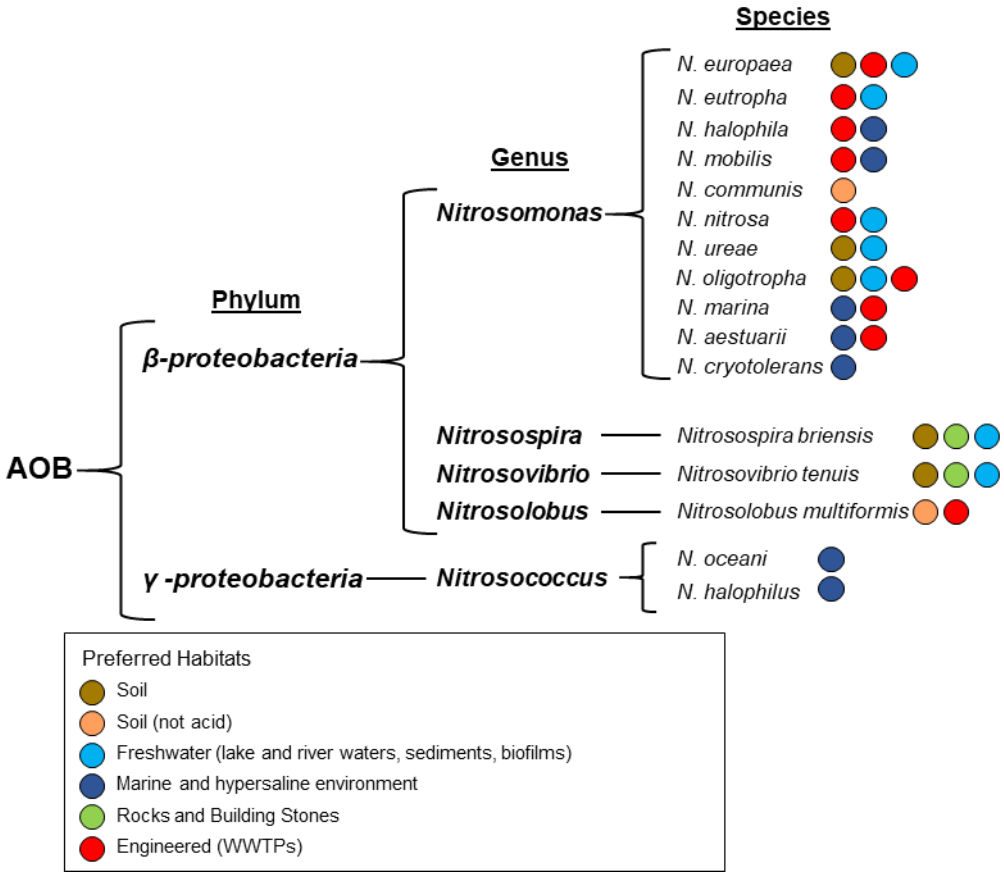

Fig. 2. Classification of AOB at species-level. Adapted from Koops and Pommerening-Röser (2001).

AOB were considered the primary species responsible for ammonia oxidation for many years. This statement, however, was broken by the discovery of ammonia-oxidising archaea (AOA; e.g., among those are representatives from Cren-, Eury- and Thaum-archaeota lineages) (DeLong 1992; Fuhrman and McCallum 1992) and anammox-associated Planctomycetes (Jetten et al. 2001). Nonetheless, the relative contributions of these microbial clades to ammonia conversion remain debated, and many still consider AOB dominant in WWTPs (Zheng et al., 2021)

1.3.1.3. Ecophysiology and Typical Habitats of AOB

AOB are ubiquitous in many natural habitats and engineered facilities, as shown in Fig. 2. The distribution patterns of AOB species are affected by different environmental conditions and operational parameters (bioreactors). Among these, ammonia concentration is one of the key drivers in the niche differentiation between AOB members, where strains with high substrate affinities (ability to utilise substrate) tend to dominate low concentration environments, while nitrogen-rich habits are preferred by species with low affinities (Lehtovirta-Morley 2018). *Nitrosomonas-like* organisms are the top AOB species found in WWTPs (Wu et al. 2019), possibly due to the broad substrate affinities represented in the *Nitrosomonas* lineages (Soliman and Eldyasti 2018). At the species level, *Nitrosomonas europaea/ Nitrosococcus mobilis/*

Nitrosomonas eutropha have been widely reported in bioreactors fed with high ammonia concentrations (Tan et al. 2008; Whang et al. 2009; Sui et al. 2014; Cho et al. 2016), whereas *Nitrosomonas ureae* and *oligotropha* were found dominant in systems at lower ammonia levels (Almstrand et al. 2011; Pan et al. 2018).

The occurrence of distinct AOB species can be correlated with temperature (Ducey et al. 2010). Most AOB isolates grow under mesophilic conditions, with an optimal temperature range between 25 – 30 °C (Grunditz and Dalhammar 2001). However, some AOB strains are capable of performing under low (psychrotrophic) and high (thermotolerant) temperature conditions. For instance, cold-adapted AOB species such as *Nitrosomonas cryotolerans*, isolated from marine arctic waters, can grow at temperatures as low as -5 °C (Rice et al. 2017), and thermotolerant AOB communities have been reported in hot springs samples with growth temperatures up to 55 °C (Lebedeva et al. 2005).

Salinity is another relevant factor that shapes AOB populations. While some AOB are sensitive to high salt levels, acclimation of moderate halophiles *Nitrosomonas*-like to salt fluctuations has been demonstrated in bioreactors (Moussa et al. 2006; Dorador et al. 2008; Ilgrande et al. 2018) and habitats with seasonal salt changes (Zhao et al. 2021). Within the *Nitrosomonas* clusters, *Nitrosomonas halophila* and *Nitrosomonas marina* are obligate halophilic strains reported in marine habitats (Koops et al. 2006), salty pond sediments (Baskaran et al. 2020) and high salinity WWTPs (Whang et al. 2009). The halotolerant strains in the Gammaproteobacterial AOB, *Nitrosococcus oceani* and *Nitrosococcus halophilus* have only been found in high salinity environments such as marine sediments (Dohra et al. 2019), ocean (Ward and O'Mullan 2002) and more recently, brackish biofilters (Hüpeden et al. 2020).

Oxygen availability can also affect the biodiversity and function of AOB species (Geets et al. 2006). It has been reported that a minimum dissolved oxygen concentration (DO) of 2.5 mg L^{-1} is required for adequate AOB performance (Cui et al. 2020). However, the adaptation of *Nitrosomonas* species to low oxygen has been observed in low-aeration reactors (DO < 0.5 mg L^{-1}) (Bellucci et al. 2011; Liu and Wang 2013), but the high viability among AOB's oxygen affinities published in the literature suggest that the oxygen requirements can vary in a case-to-case basis (Arnaldos et al. 2015).

1.3.2. Nitrite-oxidising bacteria (NOB)

1.3.2.1 Biochemistry of NOB

The nitrite-oxidising bacteria perform the second step of nitrification (nitratation), which is the conversion of nitrite to nitrate (see Eq. 4) (Madigan et al. 2000). The key enzyme is membrane-based nitrite oxidoreductase NXR, consisting of three subunits: alpha (*NxrA*), beta (*NxrB*) and gamma (*NxrC*) (Lücker et al. 2010). These subunits, especially *nxrA* and *nxrB* genes, have been

widely used as functional marker genes to detect and identify uncultured NOB species (Pester et al. 2014; Rani et al. 2017).

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \qquad (4)$$

1.3.2.2. Diversity of NOB

Since NOB are more challenging to grow in lab reactors, they are less studied than AOB (Daims et al. 2016). However, recent advances in molecular biology techniques, such as high-throughput DNA sequencing and novel sequencing capabilities (Ji et al. 2019), along with better cultivation/isolation methods (Fujitani et al. 2014; Nowka et al. 2015b), have contributed to access to genetic information in uncultured NOB populations and identify different clades within the single species.

The species-level diversity of NOB and preferred habitats is presented in Fig. 3. Nowadays, there are seven known NOB genera distributed in four bacterial phyla. Within the Proteobacteria phylum, three species are found: *Nitrobacter* (α-subgroup), *Nitrotoga* (β-subgroup) and *Nitrococcus* (γ-subgroup). In the Nitrospinae phylum group, *Nitrospina* and the uncultured *Candidatus Nitromaritima*. The last two correspond to *Nitrolancea* (Chloroflexi phylum) and *Nitrospira* in the Nitrospirae phylum (Daims et al. 2016)

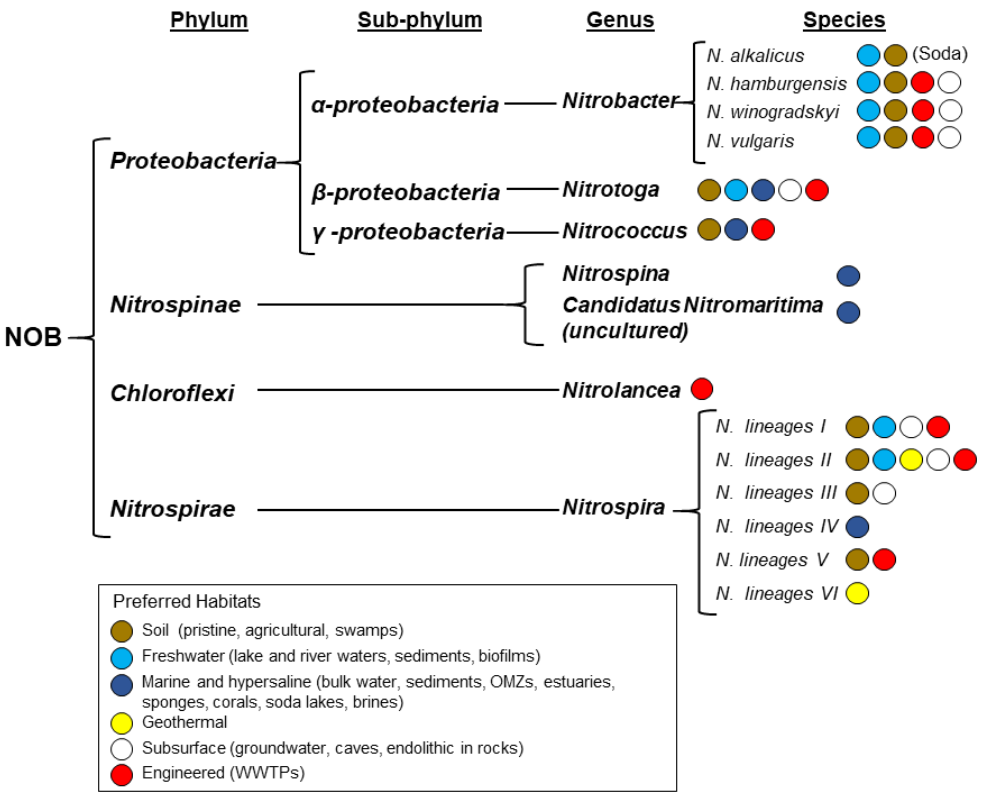

Fig. 3. Classification of NOB at species-level. Adapted from Daims et al. (2016)

Among NOB, *Nitrobacter* and *Nitrospira* are the most investigated species. The *Nitrobacter* genus subdivision contains the species *N. alkalicus*, *N. hamburgensis*, *N. winogradskyi* and *N. vulgaris* (Koops and Pommerening-Röser 2001; Nowka et al. 2015a). The most diverse NOB genus, *Nitrospira,* is divided into at least six sublineages (I *N. defluvii*, II (*Comammox, N. moscoviensis, N. japonica*), III *Nullarbor strains*, IV *N. Marina*, V *N. bockiana,* and VI N. *calida*) (Vijayan et al. 2020). Comammox is capable of complete ammonia oxidation in this group, recently discovered by two research groups (Daims et al. 2015; Van Kessel et al. 2015), changing the traditional statement that nitrification is labour divided process between two bacterial (AOB-NOB) guilds.

1.3.2.3. Ecophysiology and Typical Habitats of NOB

NOB are widely distributed in nature and WWTPs. However, the characterization of many NOB species and their biodiversity in the environment is still under study. According to the data analysis of activated sludge samples (~1200) collected from different WWTPs worldwide (Wu et al. 2019), *Nitrospira* is considered a core taxa in sludge bacterial communities. Within the *Nitrospira* group, lineages I and II are commonly reported in bacterial characterizations from these facilities (Gruber-Dorninger et al. 2015; Ji et al. 2019) and enrichment studies (Park et al. 2017; Ushiki et al. 2018; Spasov et al. 2020). However, it has been found that *Nitrotoga* species can become dominant in WWTPs operated at low temperatures in a range between 7°C - 16°C (Lücker et al. 2015).

Many studies investigating nitrifying diversity in WWTPs have reported that *Nitrobacter* and *Nitrospira* as the dominant species in NOB populations (Whang et al. 2009). Because NOB differ in their substrate affinity, nitrite level is one of the main factors determining dominance between *Nitrobacter* and *Nitrospira* species in the environment (Blackburne et al. 2007; Nowka et al. 2015a). In the literature, there is sufficient evidence supporting that the K/r hypothesis of K-strategies (slow growth under deprived conditions) and r-strategies (faster growers in substrate-rich environments) is applicable in the competition *Nitrospira* and *Nitrobacter* (Daims et al. 2016). Since *Nitrospira* species exhibit higher substrate and oxygen affinities than *Nitrobacter* strains, they dominate NOB populations under low DO (Liu and Wang 2013) and low nitrite conditions (Kim and Kim 2006). On the contrary, *Nitrobacter* will likely prevail under rich-substrate environments (Kim and Kim 2006).

Salinity can adversely influence the activity of non-adapted NOB species in WWTPs (Pronk et al. 2014). Regarding salt-tolerant species, some studies have reported that *Nitrospira* is the key NOB genus responsible for nitrite oxidation in high salinity reactors, such as moving bed biofilm reactors (MBBR) in aquaculture systems (Gonzalez-Silva et al. 2016; Gao et al. 2020) and brackish and marine (seawater) biofilters (Hüpeden et al. 2020). On the other hand, native NOB

strains from high-salt environmental samples (e.g., brackish shrimp ponds) have been successfully used as a source of halotolerant NOB inoculum in lab-scale bioreactors (Tangkitjawisut et al. 2016). This strategy has been applied in the bioaugmentation of bioreactors to improve nitrogen removal in systems receiving high salinity wastewater.

Low dissolved oxygen concentrations have a detrimental effect on NOB species, especially in *Nitrobacter* species (Martinez-Rabert et al. 2022). Moreover, NOB suppression can be suppressed through intermittent aeration, maintaining low DO in partial nitrification-anammox (PN/A) reactors, where nitrite accumulation is the preferred pathway for nitrogen removal (Peng and Zhu 2006; Xu et al. 2020).

1.4. State of the art of toxicological assessment of nitrifiers

Numerous authors recognise that the ecotoxicological assessment of sensitive species is paramount to preserving microorganisms' performance in the environment and engineered systems (Brandt et al. 2015). Due to their high sensitivity to a wide range of contaminants (Pagga et al. 2006), nitrifiers are common test microorganisms to evaluate the toxicity of pollutants in wastewater streams (Hassan et al. 2016). In WWTP research, ecotoxicity studies with nitrifiers tend to use more variable testing protocols with customised methods aligned to specific research objectives. While the standardisation seems far in ecotoxicology research, the lack of relevant details of experimental design frequently poses a constrain during the revision of published studies. Despite these challenges, most toxicological investigations are conceptualised following the "nut-shell schematic" path presented in Fig. 4, focused on studies involving nitrifying communities typically found in conventional WWTPs.

It is relevant to mention that putting the "eco" into toxicology (Chapman 2002) has been the main game-changer in the evolution of WWTP toxicity assessment over the years. As the key objective of these facilities is to meet water quality requirements, merging both toxicity and ecology disciplines has offered a deep understanding of how toxicants affect WWTP bioreactors performance linked to their bacterial community structure and the services they provide, resulting in better management strategies for more stable biological treatment processes (Daims et al. 2006).

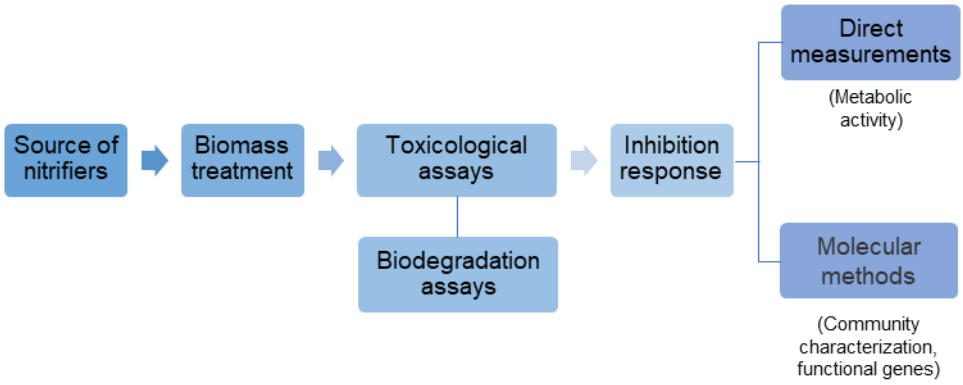

Fig. 4. Schematic of the experimental toxicological framework used in nitrifying populations from biological wastewater treatment systems. Adapted from Li et al. (2016).

1.4.1. Source of nitrifiers and biomass treatment

Regardless of the toxicological method applied, selecting the inoculum is the first step in any nitrifiers research. Overall, two biomass approaches to nitrifying testing are found, considering either individual species or the presence of other microbes as microbial communities. In the past, pure cultures were commonly used for toxicological studies, especially *Nitrosomonas* and *Nitrobacter* species, due to their ubiquitous presence in natural habitats and engineered ecosystems and relatively easy access to lab cultures (Dokianakis et al. 2004; Radniecki and Lauchnor 2011; Wang and Gunsch 2011). However, over the years, many authors have recognised that inhibition studies should evaluate the nitrifiers' performance as part of a complex bacterial population with a closer representation of the biomass found in WWTP bioreactors (Li et al. 2016; Brandt et al. 2015; Kapoor et al. 2018). One reason is the symbiotic cooperation between AOB and NOB species closely linked to achieving ammonia oxidation to nitrate via nitrite within a complex microbial population. In addition, individual species tend to be more sensitive to toxic compounds than multiple microbial communities (Jönsson et al. 2000; Langbehn et al. 2020). Therefore, most toxicity research nowadays employs a microbial consortium, testing the AOB/NOB behaviour and the interaction with other microorganisms. Although single-species toxicity tests are less common, they complement microbial community-based studies by providing useful information about direct inhibition mechanism in bacterial cells (Choi et al. 2008), detecting enzyme inactivation (Wright et al. 2020) or reference in the comparison of inhibition results (Langbehn et al. 2020).

In conventional WWTP studies, the type of biomass has been highly diverse. Experimental research frequently relies on laboratory cultures or enrichments because they are suitable for testing under well-controlled conditions. Activated sludge (AS) and environmental samples (e.g. for bioaugmentation) are familiar sources of nitrifiers. In AS biomass, nitrifying populations usually comprise a small fraction (< 10%) of the total bacterial community (Nielsen et al. 2004).

Due to this, nitrifiers abundances are frequently enriched through a selective cultivation under autotrophic conditions, resulting in a higher nitrification activity suitable for short-term assays (Katipoglu-Yazan et al. 2013; Du et al. 2016). General disadvantages with enriched nitrifying inoculum include the disparity between the bacterial populations grown in lab-scale reactors compared to those that are numerically dominant in WWTPs, considering that some AOB/NOB remain uncultured (Mehrani et al. 2020). Furthermore, changes in the coexisting communities could disrupt the stable AOB-NOB cooperation resulting in nitrite accumulation, which may cause additional effects in the culture, e.g., act as an inhibitor for AOB species (Limpiyakorn et al. 2007) or favouring NOB species with lower substrate affinities (Kim and Kim 2006). Because autotrophs' growth rate is considerably lower than that of the heterotrophs, nitrifying cultivations require lengthy incubation times (e.g., months to several years) (Zepeda et al. 2006; Bressan et al. 2013; Ramírez Muñoz et al. 2020; Wang et al. 2020). Despite all these constraints, enriched nitrifying bacteria continue as a common inoculum in many toxicological studies related to conventional nitrification process and natural habitats.

In recent years, there has been a growing interest in studying AOB/NOB strains in salt-adapted biomasses due to their capacity to thrive under high salinity conditions (Tan et al. 2019). Furthermore, the activity of salt-tolerant nitrifiers is significantly essential in the stable nitrogen removal for aquaculture systems (Ruiz et al. 2020) and many industrial effluents (e.g., tannery, petrochemicals, and textile facilities) (Srivastava et al. 2021). Despite this, toxicity studies with halotolerant nitrifiers inoculum are scarce in the literature.

Regarding biomass treatment, most samples used in lab-based toxicological studies are treated prior to the experimental phase. During this stage, some samples are maintained in the laboratory for adaptation to lab reactors (Kong et al. 2017a), cleaning and characterization purposes (Giordano et al. 2005). In addition, several toxicological studies are preceded by acclimation periods, which constitute an important treatment in the evaluation tolerance responses of nitrifiers. These studies usually investigate the adaptation capacity of AOB/NOB species to withstand high toxicant concentrations after pre-exposure periods (Du et al. 2016; Zou et al. 2020).

1.4.2. Toxicological assays methodologies

Multiple approaches have been taken to assess nitrification inhibition with AOB/NOB species. Traditionally, toxicological assessments have been performed using short-term batch assays. This format is a helpful screening tool for the preliminary identification of hazardous substances in wastewater streams. Several systematic short-term procedures are employed to evaluate the effect of toxic chemicals in WWTPs, such as respirometry assays ISO 8192 and nitrogen depletion assays ISO 9509 (Pagga et al. 2006). Advantages of these tests are low complexity and cost,

allowing simultaneous testing conditions, and covering a wide range of toxicant concentrations (Radniecki and Lauchnor 2011).

On the other hand, continuous lab reactors and pilot-scale facilities are closer to WWTP ecosystems; hence, they provide more realism in the experiments. Moreover, these systems are suitable for prolonged tests (chronic exposures) and the evaluation of additional variables like reactor configuration and operational parameters, microbial morphology and spatial patterns. Nonetheless, these studies can be expensive, laborious, and time-consuming, characterised by complex operations and a limited number of reactors. Moreover, these constraints bring other experimental challenges, such as restricted experimental conditions and low replication, considering that one control reactor (in the absence of the toxicant) is usually required for comparable performance results.

There are two common toxicological approaches regarding the duration and exposure patterns: acute and chronic. While acute toxicity represents the adverse effects from single or multiple exposures within a short period – usually within a few hours, chronic toxicity develops adverse effects that result from longer durations but often lower concentrations (Brandt et al. 2015; Roose-Amsaleg and Laverman 2016). A short-term test is preferred to evaluate acute effects, where the response of the microbes is reported for the action of pollutants in a shock exposure. Long-term studies are used for chronic tests where the microbial community are exposed (e.g., continuously or frequently) to the toxic compounds for months. Usually, tests are exposed to different concentrations with a gradual increase during experimentation.

The test substance's dosage and exposure levels are relevant aspects of toxicological bioassays. Most short-term studies introduce the toxicant into the culture at the start of the experiments, while continuous dosage (possibly in gradual increments) is applied in some chronic studies. On the other hand, the range of toxicant concentration is highly variable among scientific publications. Some authors evaluated therapeutic levels of the contaminants, while others employed high concentrations. Several inhibitions studies frequently cover low pollutant levels to evaluate the biomass biodegradation capacity, but it is vital to assess the exposure critically. In addition, knowing the compound’s fate is also important to note for exposure studies. Many chemicals are water-soluble and remain in the aqueous phase; however, they may have varied ranges of stability and metabolic transformation rates (Mitchell et al. 2014). Moreover, volatilization, chemical transformation, and bioaccumulation may affect inhibition impacts during the test. On the other hand, most publications investigate nitrification inhibition effects induced by individual substances. Although chemicals-mix studies are rare, some authors have evaluated the synergetic toxic effect of antibiotics cocktails (Meng et al. 2015) and heavy metal-antibiotics mixtures on AOB/NOB populations (Gao et al. 2021)

Other relevant parameters in toxicity studies include biomass concentration and physicochemical factors (e.g., pH, temperature, substrate levels, salinity, DO levels, sludge age, and organic compounds). While these variables are usually adjusted according to the research objectives, they significantly influence nitrifiers' performance in toxicological assays and affect, as a result, the outcome of the inhibition threshold (ISO 9509 2006). Unfortunately, many toxicity publications omit this information from the experimental description, making the comparison between studies a problematic task.

This thesis focused on the assessment of nitrification inhibition through acute toxicity tests. Further description of this type of bioassays is presented in this chapter, Section 1.4.5.

1.4.3. Toxicant biodegradation assays

Since biodegradation of some pharmaceuticals (at ug L^{-1} levels) has been positively associated with AOB cometabolic pathways, several nitrifiers studies nowadays combine toxicological and degradation experiments in a holistic assessment (Xu et al. 2016). To achieve this, the fate of the toxicant and possibly its metabolites is analysed during the exposure, evaluating not only the biotic (biodegradation by microorganisms) but abiotic degradation mechanism of the toxicant such as sorption, photolysis, hydrolysis and other transformation processes (Zhou et al. 2021). This approach requires the precise detection and quantification of the test substance through modern instrumentation tools. In nitrifiers studies, high-performance liquid chromatography coupled mass spectrometry (HPLC/MS) is one of the primary analytical technologies applied due to its high sensitivity and selectivity even at trace levels (ng L^{-1}) (Bian et al. 2020; Luan et al. 2020). Although advances in analytical testing have facilitated the integrated assessment of toxicant fate in nitrifying biomasses, the development of efficient chemical extraction and further method validation in complex matrices (e.g., culture media) brings significant additional challenges to the research.

1.4.4. Inhibition response

In WWTP research, most nitrifiers studies assess the impact of pollutants by measuring their metabolic activity directly through the chemical analysis of ammonia/ammonium and oxidising species (Xiao et al. 2015). These metabolic activities are frequently estimated based on endpoints, rates (Ramírez Muñoz et al. 2020) or through the Monod curve for calculation of kinetic constants (Bejarano-Ortiz et al. 2015). Other direct responses, such as oxygen uptake rate (OUR) by respirometric methods, are less common, usually targeting the respiration of aerobic microbes in activated sludge studies (Katipoglu-Yazan et al. 2018; Faria et al. 2021). However, from the ecotoxicity perspective, these tests cannot tell the whole story about nitrifiers' behaviour during exposure to toxic substances. Therefore, many ecotoxicity studies nowadays investigate the relationship between function and microbial community structure, frequently related to their

resistance and resilience capacity. These concepts are considered critical components in the system's overall robustness to withstand disturbances (Song et al. 2015).

In engineered biological facilities, resistance is the ability of bacterial populations to maintain their function against a disturbance, while resilience capacity is concerned with their ability to recover after a disturbance (Griffiths and Philippot 2013; Song et al. 2015). These two concepts are far more complex in the microbial ecology field. However, they have been adapted to the engineering field to understand the main factors driving microbial community stability in bioreactors. More importantly, knowing the resistance and resilience capacities of nitrifying populations is paramount, considering that their low biodiversity and slow growth make the operation and maintenance of nitrogen removal systems even more problematic.

An overview of the possible effects of toxic compounds on nitrifying bacteria is presented in Fig. 5. Given the difficulty of linking complex network communities to function, researchers usually focus on the specific responses of nitrifying populations. As shown in Fig. 5, both short and long-term exposures can change the bacterial function (e.g. nitrification rate) and community structure, including diversity, abundances and shift of species (Brandt et al. 2015). Overall, hypotheses testing community resistance are frequently formulated to understand how AOB/NOB populations resist toxicants linking metabolic activity and microbial composition. For instance, once exposed, shifts in community dynamics could overcome deficiencies among sensitive microorganisms. While this tends to impact structural composition (e.g., diversity indices), the functional performance of the system may remain less impacted (Kong et al. 2017b). On the other hand, resilience assessment studies investigate the temporal dynamics of nitrifying structure and recovery time during the toxicant exposure and post-disturbance, frequently evaluated long-term experiments (Luan et al. 2020; Zhang et al. 2020). Additionally, some scientific works explore new toxicity tolerance thresholds induced by previous toxicant exposures (e.g. acclimated biomass), where tolerant ones may substitute sensitive strains during adaptation periods (Du et al. 2016; Zou et al. 2020).

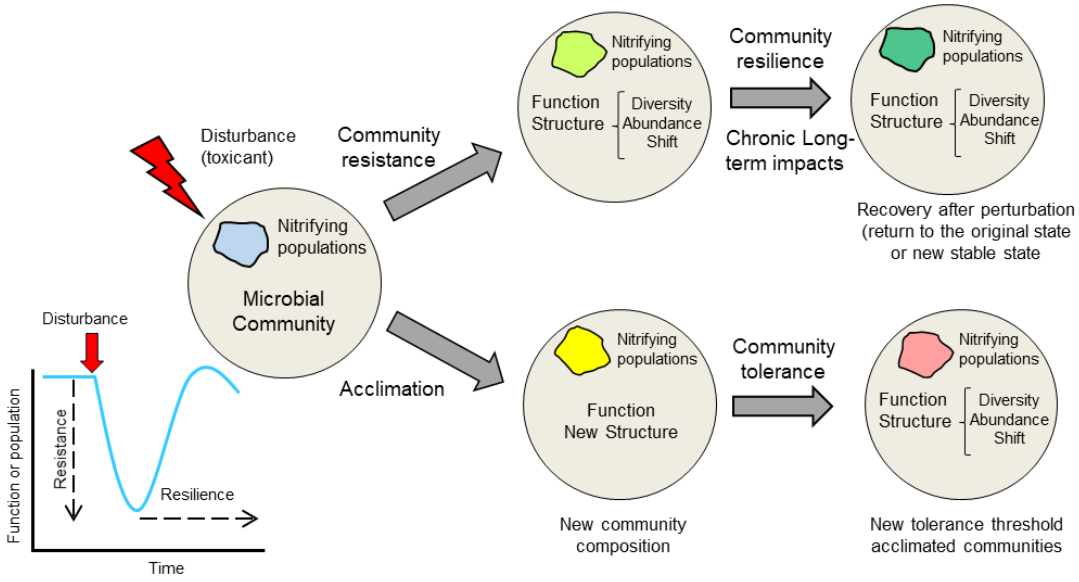

Fig. 5. Microbial community response to disturbance. Resistance and Resilience concepts (as engineering resilience). Adapted from (Griffiths and Philippot 2013)

Indeed, these ecotoxicity studies rely on the accurate and efficient characterization of bacterial communities. For many years, Polymerase Chain Reaction (PCR) and quantitative (qPCR) methods have been widely used in the identification of AOB-NOB communities, targeting the amplification of nucleic acid sequences of specific bacteria genes and/or specific functional genes (e.g., *amoA* gene in AOB). Other techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescence In Situ Hybridization (FISH) have been employed in the detection of microbial groups (Li et al. 2016). However, these methods often fail to accurately quantify bacteria abundances and identify nitrifiers strains at genus and species levels (Ge et al. 2015). Remarkably, advances in culture-independent molecular methods such as third-generation full-length 16S rRNA sequencing and "omics" analysis (e.g., metagenomic) have allowed better insight into nitrifiers in complex microbial communities at the strain level (Ge et al., 2015; Ji et al. 2019; Luan et al. 2020). With this high level of detail in community profiling, the research of nitrifiers has successfully moved into the "eco" toxicological field in the last few years, translating into more complex studies with a holistic perspective investigating the relationship between the toxicant, microbial community, and inhibition response.

1.4.5. Acute toxicity bioassays

In this thesis, the short-term batch test was selected to assess nitrifying bacteria's immediate (acute) response to shock toxic exposure, which remains a robust assay to detect compounds with antimicrobial properties on WWTP (Yuan et al. 2019). As discussed in Section 4.1, enriched nitrifying cultures are suitable for acute testing due to their high metabolic activity associated

with higher AOB/NOB abundances. Furthermore, from the literature, many acute studies follow a similar experimental set-up recommended for single AOB species (Bollmann et al. 2011) and the international standard for nitrification inhibition in activated sludge ISO 9509 (2006). Therefore, without a specific protocol for enriched nitrifying biomasses, we used the ISO 9509 (2006) guidelines as a basis of design for our experiments.

The principle of ISO protocol is used to calculate the metabolic activity of nitrifiers based on the chemical monitoring of ammoniac compound oxidation to products (i.e., nitrite and nitrate) in batch reactors. During the test, these cultures are spiked with different toxicant concentrations, including control reactors in the absence of the test substance. At the end of the experiment, the % inhibition values for each test concentration are calculated based on their metabolic activities divided by the control culture. Subsequently, these percentages are then plotted against toxicant concentration. Mainly the results can be determined at the linear section of the dose-response sigmoid curve (see Fig. 6) or analysed using a regression model (frequently plotted against log concentration (ISO 9509 2006). Finally, the degree of inhibition is calculated based on standardised toxicological indices such as EC_{50}, representing the effective toxicant concentration causing 50% of nitrification inhibition (see Fig. 6). If no effects are observed, the results can be reported as a No-Effect Concentration Level (NOEC), as the maximum test concentration that does not affect the bacteria (Halling-Sørensen 2001).

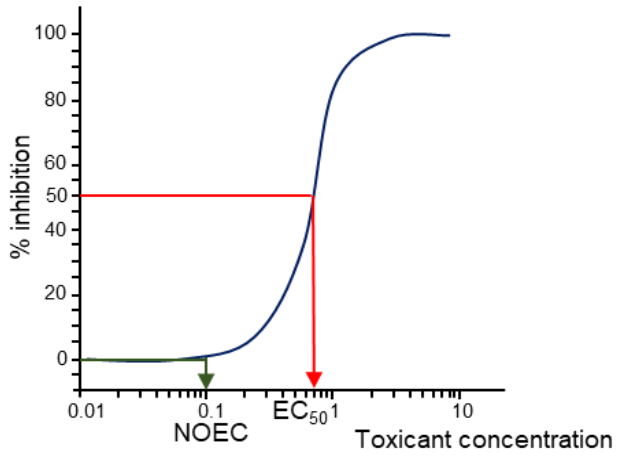

Fig 6. Calculation of EC_{50} based on % inhibition and toxicant concentrations. Adapted from (Gruiz et al. 2016)

While it is generally accepted that short-term batch assays are pretty straightforward tests compared to continuous reactors and pilot-scale studies, they should be carefully designed like any ecotoxicological study. Experimental design variables such as the establishment of baseline conditions and responses without exposure, appropriate controls, replicates, actual concentrations measurement, dose-response ranges and intervals, and confounding factors such as background conditions are some of the relevant aspects that should be considered in toxicological studies to ensure high-quality results (Harris et al. 2014). On top of these issues, working with nitrifying bacteria is challenging, considering their sensitivity and resistance to growing under lab conditions. In addition, biomass production is one of the significant obstacles in nitrifiers research, where low yields limit the amount of cells for follow-up studies (Daims et al. 2016). Also, investigators should be aware of additional factors that may alter the toxicity response of nitrifiers reflected in their oxidation reactions. Moreover, other nitrogen transformation processes may occur during the test, such as nitrogen assimilation, ammonia volatilisation and (occasionally) abiotic nitrification (e.g., Fe- and Mn-bearing minerals), affecting, as a result, the nitrogen balance in the culture (Bollmann et al. 2011).

1.5. Pollutants of interest

The presence of pollutants represents a crucial challenge toward maintaining stable AOB-NOB activities in WWTPs. These facilities are considered a significant point of source of pollutants. In the literature, almost a thousand papers are published every year reporting the detection of contaminants in domestic sewage, industrial wastewater and surrounding environments, making the data analysis significantly challenging. Instead, review publications are helpful sources in providing a sense of the most frequent chemicals and the range of concentrations (Kümmerer 2009; Tran et al. 2018; Dey et al. 2019; Rogowska et al. 2020)

Based on this information, pharmaceuticals and personal care products (PPCPs) are among the most common pollutants reported in water quality surveys. PPCPs comprise a broad group of natural and synthetic chemicals, including drugs used for medical treatment and active ingredients in many human consumption products (see fig. 7) (Dey et al. 2019). The sources of pharmaceutical compounds include the excretion of antimicrobials (and related compounds) and the improper disposal of prescriptions. In addition, there are increasing concerns about the clandestine production and disposal of active pharmaceutical ingredients, particularly antibiotics (Lübbert et al. 2017).

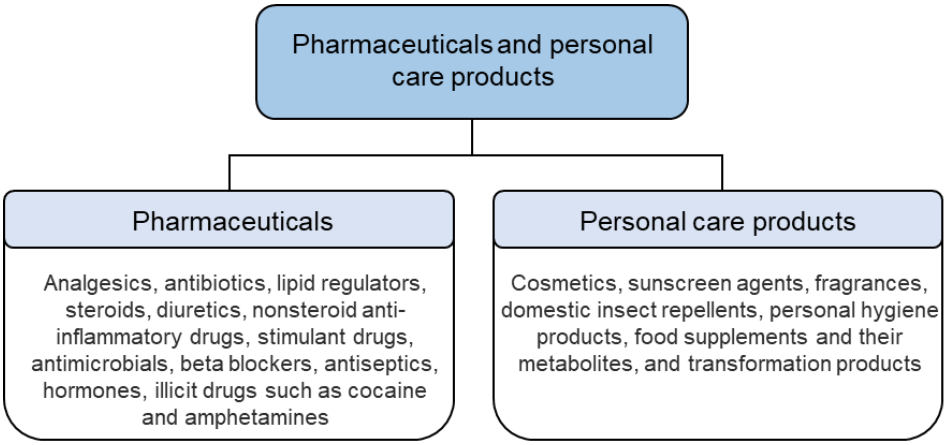

Fig. 7 Classification of pharmaceuticals and personal care products (PPCPs). Adapted from (Dey et al. 2019)

Within the PPCPs category, antibiotics, antivirals, analgesics/anti-inflammatories, nanoparticles, antimicrobials and stimulants are widely found in WWTPs, with concentrations ranging from ng L^{-1} to µg L^{-1} or even mg L^{-1} in hospital settings (Rogowska et al. 2020). These pharmaceuticals pose a risk to non-target microorganisms in the environment mainly due to their antibacterial properties and contribution to developing multiresistant-drugs bacteria (Brandt et al. 2015).

Many publications already highlight the detrimental effect of PPCPs on nitrifiers, which include antibiotics (Roose-Amsaleg and Laverman 2016), analgesics/anti-inflammatories (Park and Seungdae 2020), microplastics (Liu et al. 2019), nanoparticles (Kapoor et al. 2018) and antibacterial agents (e.g., Triclosan) (Zhang et al. 2020). These chemicals can exert their toxicity to AOB/NOB species through different modes of action. Mechanisms of inhibition in nitrifiers have been mainly associated with the interference of the AMO enzymatic activity, blocking the first step of nitrification by AOB species (Ruser and Schulz 2015). Some therapeutic pharmaceuticals can inhibit nitrifiers causing cell membrane damage (Wang and Gunsch 2011) and other typical inhibition mechanisms of antibiotics observed in target bacteria (Grenni et al. 2018). Other toxicants, such as nanoparticles, can damage the bacteria membrane, causing cell wall pitting (Choi et al. 2008).

Data of AOB/NOB inhibition has been reported with different classes of antibiotics such as macrolides (Gomez et al. 1996; Yu et al. 2019), fluoroquinolones (Dokianakis et al. 2004), tetracycline (Katipoglu-Yazan et al. 2015), sulphonamides (Huang et al. 2016; (Katipoglu-Yazan et al. 2018) and veterinary drugs (e.g., Colistin) (Bressan et al. 2013), among others. These studies are highly diverse, but some trends can be inferred while comparing their findings with caution. Overall, acute metabolic inhibition tests (like the ISO 9509) for toxicity screening has shown that AOB/NOB species may tolerate high concentration of antibiotics (even hundreds of mg L^{-1})

(Katsou et al. 2016; Langbehn et al. 2020). However, some may affect nitrifiers' activity at lower concentrations, requiring attention.

One example is the action of fluoroquinolones, a common antibiotic found in WWTPs. For instance, Dokianakis et al. (2004) used short-batch assays to assess the toxic effect of ofloxacin (OFX) on NOB pure cultures observing metabolic activity inhibition (reduction of ~ 40%) in a range of 6 -10 mg L^{-1} of OFX. In a more extended test (52 days) with activated sludge, Kong et al. (2017a) observed little effect on the NH_4^+-N removal efficiencies; however, fluctuations of AOB abundances were observed between 8-10 mg L^{-1} of OFX with full recovery at the end of the experiments. Similar responses were observed across different classes of fluoroquinolones, where the activity of nitrifiers was negatively affected within the same order of 5 -10 mg L^{-1} ciprofloxacin (Zhang et al. 2019a; Chen et al. 2021; Kim and Oh 2021), 4 -16 mg L^{-1} of levofloxacin (Hao et al. 2019), even with mixed antibiotics (1:1:1 of enrofloxacin, norfloxacin and ciprofloxacin up to 9 mg L^{-1}) (Meng et al. 2015).

Even though the toxicity thresholds of fluoroquinolones may be above the typical levels found in WWTPs (Tran et al. 2018) (except for hospitals or drug manufacturing effluents) (Guo et al. 2017), the microbial community assessments in these studies revealed the impact on abundances and diversity of nitrifier populations. From this perspective, these substances can significantly affect AOB abundances, correlated with the fluctuation in ammonium removal efficiency (Zhang et al. 2019). Furthermore, the work from Meng et al. (2015) reported that the fluoroquinolone-cocktail (1:1:1 of enrofloxacin, norfloxacin and ciprofloxacin up to 9 mg L^{-1}) produced a drastic shift in AOB species, from a *Nitrosomonas communis* lineage-dominated community to *N. oligotropha* lineage-dominated community. Regarding NOB populations, nitrite oxidation was negatively affected, possibly due to the decrease of NOB abundances reported in these studies (Kong et al. 2017a; Hao et al. 2019; Zhang et al. 2019a; Chen et al. 2021), in agreement with the early metabolic response reduction reported by Dokianakis et al. (2004) in NOB species.

On the other hand, while AOB/NOBs can tolerate the toxic action of some individual pollutants even at high concentrations, the combination with two or more contaminants may show detrimental synergetic effects. These effects are relevant for WWTPs ecosystems, where wastewater represents a collection of "nasty" elements and compounds in which microorganisms must carry out their biochemical reactions. For instance, the toxicity assessment from Du et al. (2015) reported a decrease in AOB/NOB activities (~10 % AOB and ~30 % NOB) from enriched nitrifying biomass in the presence of 20 mg L^{-1} azithromycin (AZM). However, the recent work from Gao et al. (2021) demonstrated a 70 % reduction in the ammonia removal rate using a mix solution of 1.0 mg L^{-1} Cu and 5 mg L^{-1} AZM in activated sludge. These findings suggest that the combined pollution of heavy metals and antibiotics has a more inhibitory effect on nitrifiers.

Other PPCPs of concern are the active antibacterial compounds found in cosmetics and other commercial products. Within this group, the antimicrobial agent Triclosan (TCS), commonly detected in wastewater, is a pollutant of concern for non-target microorganisms (Bedoux et al. 2012). In the study by Amariei et al. (2017), the short-term exposure of TCS produced a significant inhibition in the respirometric activity of activated sludge of $EC_{50} = 0.32 \pm 0.07$ mg L^{-1}, showing the detrimental effects on the aerobic microbial community present in activated sludge.

Overall, the number of toxicological tests on nitrifiers is still relatively small considering the broad spectrum of PCPPs, their metabolites products and other unknown chemicals present in wastewater. This group of chemicals continues as a relevant class of contaminants of emerging concern due to the increased use of pharmaceutics, such as antibiotics (Tran et al. 2018), antivirals (Slater et al. 2011) and antibacterial treatment of secondary infections (Singer et al. 2014) that may impact nutrient-removal performance in WWTP. Despite the significant increase in toxicity research over the years, the impact of many PPCPs on nitrifiers remains unexplored.

1.6. Research Aim and Objectives

The research presented in this PhD thesis investigated the short-term effect of individual pollutants on the metabolic activity of enriched nitrifying cultures. Two types of samples were used as sources of nitrifiers, WWTP sludge and coastal marine sediments targeting salt-tolerant species. Focusing on pharmaceuticals and personal care products, we decided to evaluate the impact of selected antibiotics (e.g., ampicillin, ofloxacin and colistin), a stimulant (caffeine) and personal-care ingredients (e.g., N,N-diethyl-m-toluamide (DEET) and Triclosan) on enriched nitrifying populations. Due to the limited amount of biomass harvested during the enrichment, short-term tests were conducted only in the presence of triclosan using salt-tolerant nitrifying consortia.

The research was partially funded by the Research Grants Council of the Hong Kong SAR, China (No. 18202116). The specific research objectives of this thesis were to:

- Cultivate and enrich nitrifying bacteria from WWTP samples and coastal marine sediments samples.
- Assess the effect of selected pollutants on the metabolism of enriched nitrifying bacteria from WWTP sludge using short-term batch assays.
- Assess the effect of selected pollutants on the metabolism of salt-tolerant nitrifying bacteria using short-term batch assays.
- Evaluate the experimental testing conditions employed in the toxicological assessment of nitrifying bacteria with short-term batch assays.

1.7. Thesis structure

The main body of this thesis consists of three self-contained chapters, each written to publish in peer-reviewed journals, with their introductions, material and methods, results, discussions, and conclusions. Each chapter also contains a preface section with a brief description of the paper, authors' roles and a bridging paragraph to link the individual chapters together. These chapters are preceded by the introduction and background (Chapter 1). A final Chapter contains a summary of the main conclusions and a proposal section highlighting future research. At the end of this thesis, the appendices section contains supplementary information for each Chapter. The chapters published (or intended for publication) are in the following order:

Chapter 2

Lopez C, Nnorom MA, Tsang YF, Knapp CW (2021) Pharmaceuticals and personal care products' (PPCPs) impact on enriched nitrifying cultures. *Environmental Science and Pollution Research* 28: 60968-60980. Status of the publication: Published. DOI: https://doi.org/10.1007/s11356-021-14696-7

Chapter 3

Lopez C, and Knapp CW (2022) Evaluating acute toxicity in enriched nitrifying cultures: Lessons learned. *Journal of Microbiological Methods* 192: 106377. Status of the publication: Published. DOI: https://doi.org/10.1016/j.mimet.2021.106377

Chapter 4

Lopez C, and Knapp CW (2022) Acute toxicity of triclosan on salt-tolerant on nitrifying cultures. Target journal - *Environmental Chemistry Letters*. Status of the publication: Under internal review by thesis supervisor (Dr Charles Knapp)

In addition, the supplementary information for each Chapter can be found in the appendices described below:

Supplementary information to Chapter 2: this section contains the supplementary information added in the published article, supplemental methodology including details of the cultivation methodology of nitrifying bacteria, batch toxicity assays and the standard operating procedures for the determination of ammonia and TON (nitrite and nitrate) using KONE analyser.

Supplementary information to Chapter 3: this section contains the supplementary information added in the published article.

Supplementary information to Chapter 4: this section contains supplemental methodology including details of the cultivation methodology of salt-tolerant nitrifying bacteria and batch toxicity assays.

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Chapter 2

Pharmaceuticals and personal care products' (PPCPs) impact on enriched nitrifying cultures

2.1. Preface

This chapter contains an original article published in the *Environmental Science and Pollution Research* in June 2021. This paper investigates the short-term effects of selected PPCPs on enriched nitrifying consortia, including antibiotics (ampicillin, ofloxacin and colistin), a stimulant (caffeine) and personal care ingredients (N,N-diethyl-m-toluamide (DEET) and Triclosan). C. Lopez Smitter is the principal author, responsible for all the toxicity experiments and nitrifiers' cultivation, samples collection, chemical analysis, data interpretation for all six toxicants and writing of the paper. Mac-Anthony Nnorom, as an MSc student, supported the laboratory experiments and data interpretation of the first three toxicants (ampicillin, caffeine and Triclosan). As project supervisors, C. Knapp and Yiu Fai Tsang contributed to the study's conception. All the authors provided support to review this manuscript before publication.

Additional information is presented in the appendix supplementary information to Chapter 2. This section contains the supplementary information added in the published article, supplemental methodology including details of the cultivation methodology of nitrifying bacteria, batch toxicity assays and the standard operating procedures for the determination of ammonia and TON (nitrite and nitrate) using KONE analyser.

Chapter 1 presented an overall description of the nitrifying bacteria, the main characteristics of toxicity assessment and the basis of design used to develop the testing protocol applied in this research. Furthermore, previous scientific studies reporting the impact of common PPCPs on AOB/NOB populations are discussed in the last section of Chapter 1. This information constitutes an introduction to understanding the following papers in Chapters 2, 3 and 4, highlighting the practical implications of working with nitrifying bacteria and implementing toxicity bioassays.

2.2 Journal paper

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Pharmaceuticals and Personal Care Products (PPCPs) Impact on Enriched Nitrifying Cultures

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Abstract

The impact of pharmaceutical and personal care products (PPCPs) on the performance of biological wastewater treatment plants (WWTPs) has been widely studied using whole-community approaches. These contaminants affect the capacity of microbial communities to transform nutrients; however, most have neither honed their examination on the nitrifying communities directly nor considered the impact on individual populations. In this study, six PPCPs commonly found in WWTPs, including a stimulant (caffeine), an antimicrobial agent (triclosan), an insect repellent ingredient (N,N-diethyl-m-toluamide (DEET)), and antibiotics

(ampicillin, colistin, and ofloxacin), were selected to assess their short-term toxic effect on enriched nitrifying cultures: *Nitrosomonas* sp. and *Nitrobacter* sp. The results showed that triclosan exhibited the greatest inhibition on nitrification with EC_{50} of 89.1 µg L^{-1}. From the selected antibiotics, colistin significantly affected the overall nitrification with the lowest EC_{50} of 1 mg L^{-1}, and a more pronounced inhibitory effect on ammonia-oxidizing bacteria (AOB) compared to nitrite-oxidizing bacteria (NOB). The EC_{50} of ampicillin and ofloxacin were 23.7 and 12.7 mg L^{-1}, respectively. Additionally, experimental data suggested that nitrifying bacteria were insensitive to the presence of caffeine. In the case of DEET, moderate inhibition of nitrification (< 40%) was observed at 10 mg L^{-1}. These findings contribute to the understanding of the response of nitrifying communities in presence of PPCPs, which play an essential role in biological nitrification in WWTPs. Knowing specific community responses helps develop mitigation measures to improve system resilience.

Keywords: PPCPs, nitrifying bacteria, nitrification inhibition, acute toxicity, nitrogen removal.

Introduction

Recently, the widespread presence of pharmaceutical and personal care products (PPCPs) in the environment has drawn the attention of the research community due to the potential adverse effects on ecosystems and human health (Kümmerer 2009; Tran et al. 2018). Studies on the occurrence and fate of PPCPs have shown that stimulants, antimicrobial agents, repellents, and antibiotics have frequently been detected in aquatic systems and engineered facilities such as wastewater treatment plants (WWTPs). These compounds, representing a wide range of human consumption products disposed and excreted into the sewage systems, pass through WWTPs and are discharged into the water bodies (Balakrishna et al. 2017; Yang et al. 2017).

Among the PPCPs, caffeine (CF) is one of the most abundant chemicals in WWTP samples, and its concentrations range from a few ng L^{-1} to µg L^{-1} (Luo et al. 2014; Tran et al. 2014; Balakrishna et al. 2017; Paíga et al. 2019). CF is an organic stimulant compound that is commonly added to beverages and other products. Although the literature shows that CF is highly biodegradable in biological WWTPs with removal efficiencies > 80 % (Sui et al. 2010; Dai et al. 2014; Tran et al. 2018), the increase in CF consumption worldwide may lead to higher amounts discharged to the water bodies, increasing the risk of exposure in the ecosystems (Quadra et al. 2020).

Other substances frequently detected in WWTPs include the antimicrobial agent: triclosan (TCS) and the active ingredient of insect repellent: N,N-diethyl-m-toluamide (DEET) (Liu and Wong 2013 ; Yang et al. 2017; Juksu et al. 2019). TCS concentrations have been reported in different influents of WWTPs worldwide, and the values are as high as 0.47 µg L^{-1} in China (Zheng et al.

2020), 86.1 µg L^{-1} in US (Kumar et al. 2010), and 17.6 µg L^{-1} in South Africa (Lehutso et al. 2017). In the case of DEET, the concentrations observed in WWTPs range from a few ng L^{-1} up to peak values as high as 15.8 µg L^{-1} in Europe (Merel and Snyder 2016) and 42.3 µg L^{-1} in USA during summer season (Mohapatra et al. 2016). Due to the variable removal efficiencies in WWTPs (Luo et al. 2014), TCS and DEET can be found in different environmental matrices, such as treated effluent, surface waters, waste sludge, and sediments (Ramaswamy et al. 2011; Dai et al. 2014; Zhao et al. 2010; Dsikowitzky et al. 2020).

One of the most concerning pharmaceuticals in WWTPs are antibiotic residues. These compounds treat different infectious diseases and their disposal into the environment toxicologically impacts non-target microorganisms in the ecosystems, develops antimicrobial resistance, and contaminates soils and water bodies (Kümmerer 2009). Studies have shown that many antibiotic compounds have often been excreted in urine and/or faeces, cleansed off bodies, or even disposed directly to sewers with minimal change after their administration (Marx et al. 2015); they are frequently detected in influent and effluent on WWTPs, suggesting a degree of persistence through treatment plants (Tran et al. 2016; Mutiyar and Mittal 2014; Leung et al. 2012). Survey-based studies indicate that conventional WWTPs generally do not efficiently remove antibiotics (Paíga et al. 2019)

According to the review of antimicrobial consumption by Robertson et al. (2019), β-lactams were the most commonly prescribed antibiotics worldwide in 2015. In this antibiotic class, ampicillin (AMP) has been widely used in human medicine and is considered highly degradable due to its unstable β-lactam ring structure (Watkinson et al. 2007). However, the chemical transformation of β-lactam antibiotics could vary depending on the matrix conditions (Mitchell et al. 2014), where in some cases AMP could still be detected even in treated effluent from WWTPs (Mutiyar and Mittal 2014).

Other predominant antibiotics in WWTPs are fluoroquinolones (Tran et al. 2018). Within this group, ofloxacin (OFX) is a second-generation antibiotic applied to treat urinary tract infections (King et al. 2000). Although restricted by WHO (Robertson et al. 2019), the presence of OFX continues in raw sewage and effluent in WWTPs (Brown et al. 2006; Dinh et al. 2017), reaching concentrations of 7.9 µg L^{-1} in Asia (Leung et al. 2012; Minh et al. 2009) and 8.6 µg L^{-1} in Europe (Dinh et al. 2017)

Another source of antibiotics in WWTPs are veterinary medicines (Kemper 2008). Under this application, colistin (CST) is a polymyxin antibiotic that is widely used in animal farms to treat Gram-negative infections (Liu et al. 2016; Kempf et al. 2016) and it has re-emerged as a "last-resort" antibiotic to target multidrug-resistant infections (Dagla et al. 2019). The occurrence of CST remains limited given that analytical methods for its quantification remain under

development for environmental samples (Song et al. 2020). However, the detection of CST on biological matrices (Dagla et al. 2019) and the presence of a CST resistome in bacteria from WWTPs (Hembach et al. 2017) suggest that CST may pose a risk to microbial communities.

The presence of PPCPs in WWTPs is crucial because they can adversely affect biological treatment processes; these systems rely on microbial communities to transform nutrients, such as nitrogen, to prevent aquatic eutrophication (Xiao et al. 2015). For example, biological nitrification, which is part of the nitrification-denitrification reaction sequence in WWTPs, involves a two-step process carried out by two autotrophic microorganisms, namely ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) (Koops and Pommerening-Röser 2001). Moreover, the performance of AOB-NOB communities can be disrupted due to their fragile mutualism (Graham et al. 2007; Knapp and Graham 2007), low phylogenetic diversity, slow growth characteristics and sensitivity to toxic chemicals (Li et al. 2016).

The role of nitrifiers on biological nitrogen removal is a critical process in wastewater treatment and their response against toxic chemicals is of great concern for the stability and performance of WWTPs (Xiao et al. 2015). The adverse effect of pharmaceuticals on nitrifying communities has been reported in wide range of conditions including short- and long-term exposure at different concentrations. The findings shown that these compounds can decrease nitrification rates, leading to poor ammonium removal efficiency and disruption of the AOB-NOB mutual cooperation, producing partial nitrification with nitrite accumulation. Other effects observed are the inhibition of enzymatic activities of AOB-NOB species with reduction of ammonia-monooxygenase (AMO) and nitrite-oxidoreductase (NOR) enzymes and the variation of the bacterial community composition, shifting their richness and diversity. (Kong et al. 2017; Yu et al. 2019; Li et al. 2020; Zhang et al. 2020).

Despite published data, toxicity assessments of the common PPCPs on AOB-NOB communities are limited and even unavailable in some cases. Most of the studies found in literature were performed with high concentrations of activated sludge as the biomass source, where the presence of more diverse microbial populations and high solid content could lead to varied inhibition results on nitrifying species (Lakshminarasimman et al. 2018; Armstrong et al. 2019; Zhang et al. 2020).

This study investigated the effect of selected PPCPs, including a stimulant (CF), personal care products (DEET and TCS), and antibiotics (AMP, OFX, and CST), on an enriched nitrifying community. Batch reactors were employed to assess the acute toxicity of these substances, where changes of ammonium, nitrite, and nitrate concentrations were measured to determine nitrification inhibition. Enriched nitrifying bacteria were selected as the inoculum with efforts to increase nitrification activity, control the presence of heterotrophic bacteria and remove solids

from activated sludge, which could alter nutrients, transform the toxic substances, and interfere with the interaction of nitrifying bacteria with the test substance (Zhang et al. 2020). The findings obtained in this study expand our understanding of the short-term effects of PPCP exposure on nitrifying bacteria, which could importantly prevent the failure of biological nitrogen removal systems in WWTPs.

Materials and Methods

Cultivation of nitrifying bacteria

Activated sludge, collected from a Sequencing Batch Reactor (SBR) in a WWTP in Scotland, was used as the source of nitrifying bacteria. The enrichment of nitrifiers was carried out in batch cultures according to the procedure described by Bollmann et al. (2011) and Radniecki and Lauchnor (2011). The nutrient media were modified from Bollmann et al. (2011) with the following chemical composition (g L^{-1}): 0.5 $(NH_4)_2SO_4$ as an inorganic nitrogen source; 0.585 NaCl, 0.054 KH_2PO_4, 0.147 $CaCl_2{\cdot}H_2O$; 0.075 KCl, 0.049 $MgSO_4{\cdot}7H_2O$, 0.5 $NaHCO_3$ as the inorganic carbon source, 7.21 HEPES as a buffer, and 1 ml of trace elements solution from Schmidt and Belser (1994). After autoclaving at 121°C for 20 min, the pH media was adjusted to 7.6 ± 0.2 (pH/conductivity meter Mettler Toledo, MPC 227, Switzerland) with 10M NaOH (sodium hydroxide solution, Fisher Scientific). The detailed preparation of the nutrient media is presented in the section supplemental methodology in the appendix supplementary information to Chapter 2.

In the first stage of enrichment, 1 g (wet mass) of activated sludge was inoculated into 100 ml Erlenmeyer flasks with media, following by a series of repeated transfers in fresh media to promote the growth of nitrifiers as explained by Bollmann et al. (2011). The procedure aimed to reduce the activated sludge flocs and particles that could interact or degrade the test substance, and minimise nitrogen assimilation by heterotrophic bacteria that could affect nitrification measurements (ISO 9509 2006; Chen et al. 2014). This process continued for three months, where AOB/NOB activities in each culture flask were evaluated through visible observation of ammonium disappearance using Nessler reagent (HACH, Germany) and spots test strips for nitrite and nitrate detection (Aquachek, HACH, Germany). (see section supplemental methodology in the appendix supplementary information to Chapter 2 for more detail).

Once the cultures presented stable activity, the bacterial broth was transferred to 2 L glass bottles (three lab-scale reactors in total) for further enrichment and provide sufficient inoculum for the toxicity tests. The air was supplied with an air pump (HDOM, Model HD-603, Shenzhen Hidom Electric Co., Ltd.) filtered with 0.2 μm sterilising-grade filter (Aervent™) to maintain the dissolved oxygen (DO) above 4 mg L^{-1} (DO meter, Eutech Instruments Pte Ltd., DO 6+ DO/Temp, Singapore). The reactors were operated at room temperature (20 °C - 27 °C) and were

periodically provided with $(NH_4)_2SO_4$ solution as substrate and NaOH solution (sodium hydroxide solution 10 M, Fisher Scientific) to maintain optimum pH (7.6 – 7.8). The reactors' working volume was 1.7 L and every two weeks, 1.2 L of supernatant was removed and replaced with the same volume of fresh nutrient medium to prevent excessive accumulation of by-products. (see section supplemental methodology in the appendix supplementary information to Chapter 2 for more detail)

Batch toxicity assays

We consulted the ISO 9509 (2006) protocols for the experiment design; it evaluates the exclusive capacity of nitrifiers to transform inorganic nitrogen into oxidation species, and it represents a more sensitive approach (Stasinakis et al. 2008; Yuan et al. 2019; Brandt et al. 2015). Study duration was extended up to 2-3 days in contrast to the few hours proposed by the protocol ISO 9509 (2006), to account for the relatively slow-growing populations (Radniecki and Lauchnor 2011). For each assay, the incubation was finalised before ammonium concentrations reached zero to avoid substrate limitation. (see section supplemental methodology in the appendix supplementary information to Chapter 2 for more detail)

An enriched nitrifying community was selected; it remains representative of the AOB-NOB communities present in WWTPs (Li et al. 2016) but minimises interference of ammonia-assimilation by excessive heterotrophic bacteria. The experimental conditions of the batch assays for the six PPCPs are summarised in Table 1. The tests were performed individually in the following order: CF, AMP, TCS, DEET, CST, and OFX. Batch cultures were undertaken in 500 mL glass bottles with 300-mL working volume. Each treatment was inoculated with 50 mL (equivalent to 337 ± 19 mg VSS L^{-1}) of bacterial suspension harvested from the 2-L (enriched stock) reactors. Due to the slow growth of nitrifying bacteria, the first three tests (CF, AMP and TCS) were run in duplicate to cover a broad range of concentrations. Eventually, sufficient quantities of biomass stock were generated to run the experiments in triplicates for the last three toxicants (DEET, CST and OFX). Similar nutrient media were prepared for the assays with lower initial ammonium concentration (<56 mg L^{-1} NH_4^+-N) as recommended by ISO 9509 (2006).

Before inoculation, the biomass was subjected to a cleaning procedure that involved centrifugation, settling, decanting, and resuspension to remove any remaining traces of oxidised products and minimise organic material, and ensure sufficient buffering capacity during the assays (Moussa et al. 2003; Salem et al. 2006). Initially, the nitrifying biomass used for the first three PPCPs (i.e., CF, AMP, and TCS) were centrifuged at 10000x g for 35 min, settled (20 min), decanted and refilled with new test media; this procedure was repeated twice. It was observed that longer centrifugation and settling time of the biomass led to significant nitrite accumulation

in the batch assays, thus the washing procedure for the last three PPCPs (i.e., DEET, CST, and OFX) was optimised by halving processing times.

Table 1. Initial operating conditions for the short-term batch assays.

Variable	CF	AMP	TCS	DEET	CST	OFX
NH_4^+-N (mg L^{-1})*	53.3 ±0.6	53.2 ±0.6	53.1 ±0.5	50.3 ±0.5	50.6 ±0.7	49.7 ±0.6
NO_2^--N (mg L^{-1})*	1.0 ±0.1	1.0 ±0.1	0.7 ±0.1	1.5 ±0.2	0.7 ±0.2	1.6 ±0.2
NO_3^--N (mg L^{-1})*	0.8 ±0.1	0.5 ±0.2	0.5 ±0.1	3.7 ±0.2	4.6 ±0.5	9.4 ±0.4
pH range	7.4 – 7.7	7.8	7. 7– 7.8	7.6 – 7.8	7.7	7.4 – 7.7
Temperature (°C)	20 - 22	20 - 22	22 - 27	20.5 - 22	21.5 - 22	21 - 22
DO (mg L^{-1})	> 5	> 5	> 5	> 5	> 5	> 5
Protein (mg L^{-1})	4.7	4.7	6.3	6.3	6.3	9.0
Replicates	Duplicates	Duplicates	Duplicates	Triplicates	Triplicates	Triplicates
Volume (mL)	300	300	300	300	300	300
Duration (h)	73	68	34	43	74	33

* Nitrogen values are represented by mean ± standard deviations

During experimentation, cultures were supplied with humidified air via aeration stones at the bottom of each bottle to maintain DO above 4 mg L^{-1}. pH was measured at the beginning and end of the incubation period. Temperature was recorded using a USB Temperature Data Logger (Lec, Easylog USB Version 7.6.0.0, Lascar Electronics Ltd.)

Biomass concentrations were estimated by their protein content using the Micro BCA Protein Assay kit (Thermo Scientific, USA), following their procedure. Volatile suspended solids (VSS) concentrations were determined according to Standard Methods (APHA 1998). All batch assays (per test substance) were inoculated with the same amount of biomass and substrate concentration, and the protein concentrations were 6.2 ± 1.6 mg L^{-1} (equivalent to 71 ± 5 mg VSS L^{-1}); changes in microbial protein content between beginning and end of the incubation period was negligible.

As an additional treatment 'control' and to compare activity responses of the consortium, reference inhibitor allylthiourea (ATU) was used at 0.3 mg L^{-1}, a selective ammonia monooxygenase inhibitor of AOB populations (Gwak et al. 2020) and nitrification. AOB are considered the limiting step of nitrification (ISO 9509 2006). This was used to verify whether ammonium disappearance was resultant of autotrophic nitrification.

Samples (4 ml) were collected during the incubation period, filtered with 0.45-µm cellulose filter and preserved following protocol BSI EN ISO 5667-3 (2018) for analysis of ammonium, nitrite, and nitrate. Unfiltered samples (2 ml) were collected for DNA and protein test and preserved at -80 °C until analysis.

The nitrification activity in the batch assays was measured by monitoring nitrogen species over time. The oxidised nitrogen (NO_X-N), the sum of NO_2^--N and NO_3^--N concentrations, obtained for the different treatments were used to calculate the inhibition percentages in Eq. (1). The values correspond to the measurements at the end of each experiment and reported as the mean value of triplicate or duplicate assays. These percentages were plotted as a function of the toxicant concentrations.

$$\%Inhibition = \frac{(NO_{X\ control} - NO_{X\ test})}{NO_{X\ control}} * 100 \quad (1)$$

$NO_{X\ test}$ and $NO_{X\ control}$ represented the concentrations of oxidised nitrogen (mg-N L^{-1}) in each batch reactor with the toxic substance, and the 'control' absent of the toxicant. All concentrations were adjusted by subtracting the initial concentration of nitrite or nitrate to record the variation of the oxidation species over the incubation period.

The substance concentration that decreases nitrification activity in comparison to the controls by 50% is defined as 50% effective concentration (EC_{50}). This value was estimated by interpolating the graph of inhibition percentage (Eq. 1) against the log-transformed toxicant concentration. The profile was adjusted to a linear or polynomial model, considering the best fit with a coefficient of determination (R^2) > 0.96 (ISO 9509 2006).

Test substances

Caffeine (> 95% purity), irgasan or triclosan (≥ 97 % purity), N,N-diethyl-3-methylbenzamide or DEET (> 97 % purity), ampicillin (ready-made solution, 100 mg/ml), ofloxacin (≥ 99 % purity), and colistin sulphate salt (≥ 15000 U mg^{-1}) were purchased from Sigma Aldrich. The range of concentrations selected for the batch assays are presented in Table 2. These concentrations were chosen to include a range commonly found in WWTPs based on the values reported in the literature for either WWTPs or previous inhibition studies (see Table 2); however, higher concentrations were included to evaluate whether target compounds would produce any response to nitrification performance (Pasquini et al. 2013).

Stock solutions were prepared on the same day of the assay, and Milli-Q water was used for the antibiotics and CF. Because TCS and DEET have poor solubility in water, the solutions were prepared with dimethyl sulphoxide (DMSO) as solvent (< 0.1 % v/v), and similar concentrations of DMSO were maintained in all treatments.

Table 2. PPCP concentrations tested in batch reactors

Class	**Substance**	**CAS number**	**Concentrations (mg L^{-1})**	**Reference**
Stimulant	CF	58-08-2	0.025, 0.115, 1, 10, 40, 90	(Gheorghe et al. 2016)
Antimicrobial	TCS	3380-34-5	0.01, 0.1, 0.3, 0.5, 1, 2	(Roh et al. 2009)
Insect repellent	DEET	134-62-3	0.02, 0.1, 1, 5, 10	(Aronson et al. 2012)
Antibiotics	AMP	69-52-3	0.5, 5, 50, 100, 175, 250	(Gomez et al. 1996)
	CST	1264-72-8	0.1, 1, 10, 100, 350	(Bressan et al. 2013)
	OFX	82419-36-1	0.01, 0.1, 1, 5, 10	(Dokianakis et al. 2004)

The fate of the substances was not analysed, but it was considered in the assays; reported half-lives were CF, a few hours (Dorival-García et al. 2013); OFX, >4 days (Dorival-García et al. 2013); DEET, days to weeks (Weeks et al. 2012; Lakshminarasimman et al. 2018); and partial biodegradation reported for TCS (Lakshminarasimman et al. 2018) and AMP (Ramírez Muñoz et al. 2020). Test bottles were covered with foil to prevent light exposure and possible photolysis (e.g., Bedoux et al. 2012); pH and temperature were balanced between microbial activity and compound stability (e.g., Mitchell et al. 2014; Li et al. 2003). Most previous studies used activated sludge with VSS quantities $10^2 – 10^3$ times higher than this study. Therefore, it is hoped that low biomass levels and autotrophic nature of the media in the assays minimised degradation of the tested chemicals.

Analytical methods

The concentrations of ammonium, nitrite, and nitrate were determined through colorimetric analysis using KoneLab Aqua 30 (Thermoscientific, Aquarem 300, Clinical Diagnostics Finland) according to the British Standard procedures BS ISO 15923-1 (2013). For the colorimetric analysis, pre-tests involved spiked controls to determine whether any interference by other compounds had any effect on assays. All the reagents were purchased from ThermoFisher. (the operating procedures followed for the determination of ammonia and total oxidising nitrogen (TON, nitrite and nitrate) are presented in the appendix supplementary information to Chapter 2)

DNA extraction and 16S-rRNA gene sequencing and analysis

Four samples were tested to analyse their microbial community structure. Two were collected from the stock bioreactors at the beginning of the experiments (S1 and S2) and the two other (S3 and S4) at the end of the testing period, after ~8 months of harvesting. All samples were stored at −80 °C in 2-ml tubes prior to the analysis.

DNA were extracted from biomass samples collected from the reactors using a QIAGEN DNeasy® Blood & Tissue Kit, according to the manufacturer instructions. The DNA quantity was estimated using a Spectrophotometer Microplate Epoch (Biotek Instruments, Inc., USA) and data collection and analysis software Gen5^{TM} V1.11.5 (Biotek Instruments, Inc., USA).

DNA sequencing was performed at Glasgow Polyomics (Glasgow, UK) using Illumina MiSeq platform, targeting the 16S-rRNA operon for taxonomy, using recommended primers by RDP-II Pipeline (Maidak et al. 2001; https://rdp.cme.msu.edu); and further, bioinformatic identification of microbial community was performed with QIIME2 version 2021.2 (Bolyen et al. 2019), with similar data analysis detailed by Al Ali et al (2020) (see section 16S-rRNA gene sequencing and taxonomy classification in the appendix supplementary information to Chapter 2).

Results and Discussion

During the pre-experimental enrichment process, ammonium conversion to nitrite increased rapidly and low nitrate production was observed (0.3 mg NO_3^--N /mg NH_4^+-N consumed), leading to nitrite build-up in the reactors. However, nitrite accumulation gradually decreased, reaching undetectable values after two months of operation. Subsequently, the reactors achieved a stable nitrification performance, maintaining an ammonium consumption rate between 11 - 20 mg NH_4^+-N/g MLVSS h and a nitrate production yield of 0.95 mg NO_3^--N/mg NH_4^+-N – i.e., 95% of ammonia disappearance was attributed to nitrification. These cultures were sustained in batch reactors and were the "stock" for subsequent assays.

Microbial community

The taxonomic classification derived from the 16S rRNA gene sequencing and analysis is illustrated in the Figure S1 (see section 16S-rRNA gene sequencing and taxonomy classification in the appendix supplementary information to Chapter 2). At phylum level, *Proteobacteria* were dominant in all analysed samples, accounting for 63–68% of the total bacterial population; followed by *Bacteroidetes*, 19% (S1-S2) to 30-32% (S3 and S4); *Chlorobi* 10% to 0.4-3% (S3 and 4, respectively); and the remaining bacteria represented <10% of sequences. These phylogenetic groups are representative of those in activated sludge (Johnston et al. 2019; Zhang et al. 2019) and enriched nitrifying cultures (Kapoor et al. 2016; Jeong and Bae 2021).

The 16S-rRNA phylo-taxonomic analysis recognised *Nitrosomona*s sp. (AOB) and *Nitrobacter* sp. (NOB) in the enriched community (see Table S2, section 16S-rRNA gene sequencing and taxonomy classification in the appendix supplementary information to Chapter 2). The relative abundance of *Nitrosomonas* sp. increased from 5.2% at the beginning to 5.7% at the end (after 8 months of cultivation) . In terms of *Nitrobacter* sp., they ranged 0.1% - 0.8% of the total microbial population. Notably, no *Nitrospira* sp., another possible NOB, were found. While these bacteria

are typically found in biological wastewater treatment systems, they tend to be more sensitive to environmental conditions (Graham et al. 2007; Knapp and Graham 2007). Further, the prevalence of *Nitrobacter* sp. over *Nitrospira* sp. exposed to higher nitrite levels has been demonstrated (Nogueira and Melo 2006; Nowka et al. 2015).

The final abundances of AOB and NOB species are contingent of the enrichment process; higher proportions of nitrifying bacteria could be grown with long periods of cultivation (Ye et al. 2011; Wang et al. 2019) and fully automatic controlled bioreactors (Yao et al. 2016). In comparison, here, the abundances of AOB ad NOB guilds were within the same order of magnitude to those with similar duration of enrichment (Stadler and Love 2016; Kwon et al. 2019; Jeong and Bae 2021). Additionally, nitrifying activity achieved complete removal of ammonium and nitrate production without nitrite accumulation over the period of enrichment.

Control Cultures

All experimental treatments were inoculated with similar nitrifying consortia, wherein batch reactors absent of the toxicant were used as controls. Fig. 1 shows the exemplary performance over time in the controls during the first (CF) and the last (OFX) assays; remaining information is presented in the appendix supplementary information to Chapter 2. All controls achieved microbial nitrification, reducing > 90% of the initial ammonium concentration. Moreover, the increase in nitrite and nitrate levels indicated the activities of AOB and NOB, respectively. However, a substantial amount of nitrite accumulated during the incubation, suggesting that the ammonium oxidation rate was higher than the nitrite oxidation rate. At the end of the experiment, nitrite levels were higher in the CF controls (Fig. 1a) than those in the OFX controls (Fig. 1b). This difference could be attributed to modification of culture-rinsing preparations between the two testing groups (as described in the Materials and Methods section); neither culture was exposed to any contaminant ("controls"). This effect has been highlighted by other authors (Moussa et al. 2003), and the reduction of oxygen level in the washed biomass could possibly have a detrimental effect on NOB activity (Peng and Zhu 2006).

Experimental conditions were consistent between assays (Table 1). However, temperatures during TCS test were aberrantly +5 °C (Table 1). While the change of temperature within this range can increase the activity of *Nitrosomonas* sp. (AOB species) and *Nitrobacter* sp. (NOB species) by 30% to 40% (Grunditz and Dalhammar 2001), it should be noted that all dose-response assays for each toxicant were conducted simultaneously and compared with controls to minimise collineating factors.

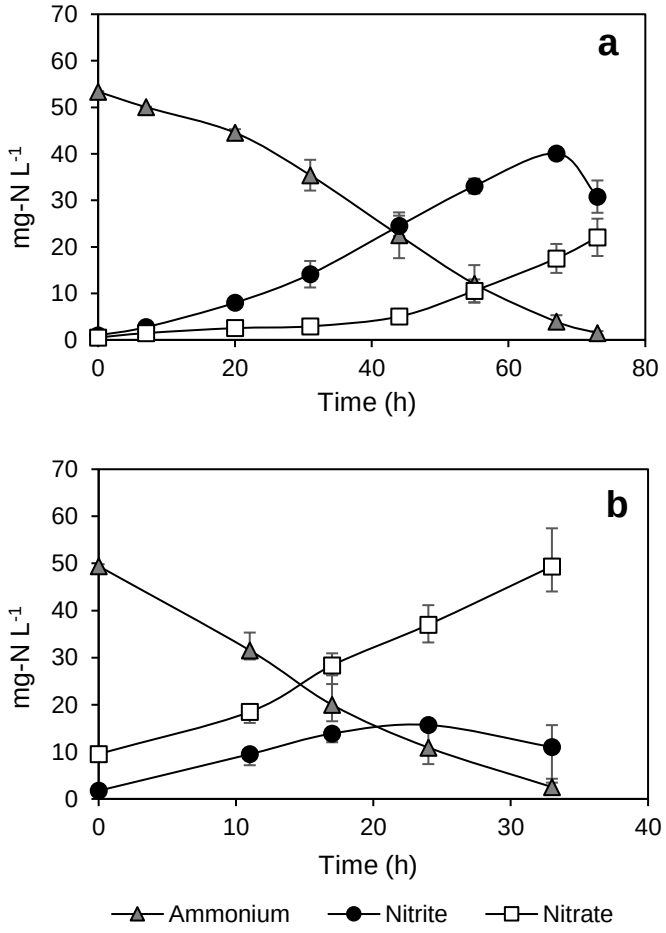

Fig 1. Nitrifying control cultures during the a) caffeine test and b) ofloxacin tests. Data points show average concentration ± maximum and minimum values.

Mass balance of nitrogen based on ammonium consumption, nitrite consumption/production, and nitrate production was monitored through the toxicological tests (see Table S1, appendix supplementary information to Chapter 2). Accountability of ammonia transformation to its oxidised products was within ±6% of expectation (mean 0.1%, ±2.6% standard deviation). Besides the slight variations from the chemical analyses, some differences may be due to ammonia volatilization or assimilation.

Effects of caffeine on nitrification activity

Nitrification activity was evaluated by monitoring the concentration of ammonium, and of the oxidation species nitrite and nitrate over time to assess the effects of CF on the nitrifying cultures. Fig. 2 shows the nitrification performance of the batch cultures for the different CF concentrations (Table 2), including that of the control culture and the reference inhibitor ATU. At the end of the experiment, 97.4% ± 0.65% of ammonium was consumed in all replicates and

the total oxidised compounds (controls and batch reactors spiked with CF) were 51.4 ± 1.0 mg L^{-1} NOx-N with a coefficient of variation less than 2%.

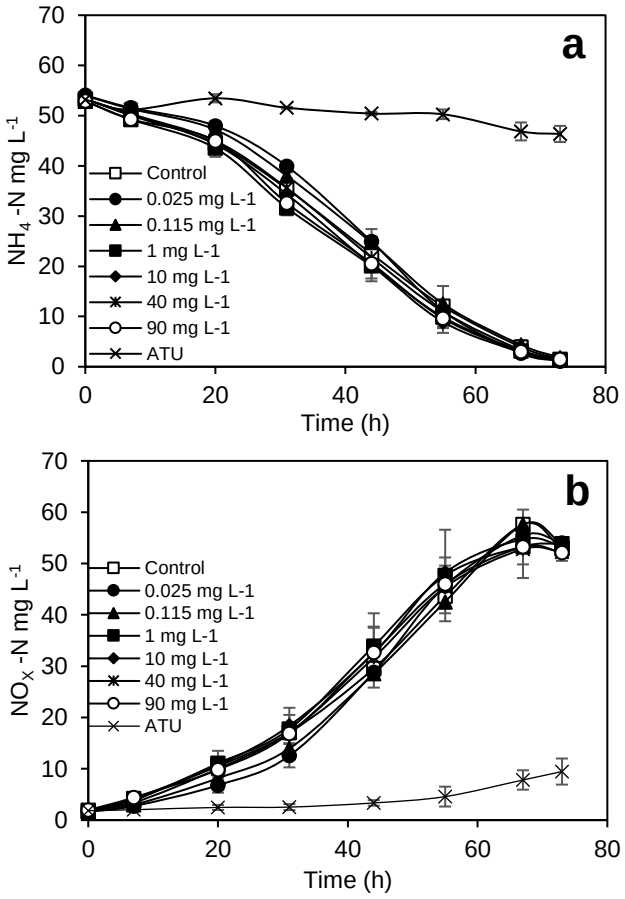

Fig 2. Nitrification profiles of cultures in presence of CF at different concentrations: a) NH_4^+-N and b) NOx-N (NO_2^- +NO_3^-). Data points show average concentration ± maximum and minimum values.

There is scarce information about CF impacts on nitrification; He et al. (2018) suggested nitrification was not impacted during a CF-biodegradation experiment. However, CF can affect other bacteria (Gheorghe et al. 2016). In summary, results indicated that CF did not considerably inhibit nitrification even at the highest concentration at 90 mg L^{-1}; neither %-inhibition nor EC_{50} was calculated.

Effects of triclosan and DEET on nitrification activity

The inhibition (%) at different TCS concentrations was estimated using Eq. 1, and the EC_{50} value was obtained from Fig. 3. There was no observable effect at the lowest concentration of 0.01 mg L^{-1} compared to the control treatments (Fig. 3a); however, a tenfold increase in TCS levels (0.1 mg L^{-1}) inhibited nitrification by >50%. Furthermore, inhibition was 72.3% at the highest concentration (2 mg L^{-1}). The EC_{50} value calculated for TCS was 89.1 µg L^{-1}, with the experimental data (Fig. 3b) adjusted to a second-degree polynomial regression model ($R^2 > 0.99$).

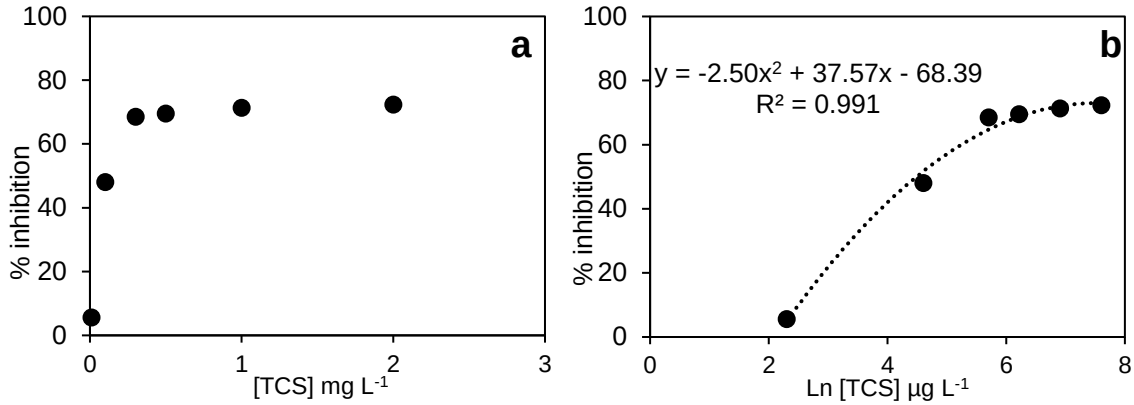

Fig. 3. Inhibition level at different concentrations of Triclosan a) TCS mg L^{-1} and b) Ln TCS µg L^{-1}

Similarly Roh et al. (2009) demonstrated that TCS at 2 mg L^{-1} reduced nitrite production to 70% by using batch reactors with pure AOB cultures as biomass, and Dokianakis et al. (2004) showed that the same concentration of TCS inhibited enriched NOB. However, neither aforementioned study jointly considered AOB-NOB as a community. Furthermore, almost all available data on TCS toxicity from other studies have been obtained from activated sludge. Amariei et al. (2017) reported EC_{50} value of 0.32 ±0.07 mg L^{-1} with 125 mg (TSS) L^{-1} via a respirometry assay, and via ammonia uptake rates (AUR), Stasinakis et al. (2008) estimated the EC_{50} value of 6.4 mg L^{-1} with 1100-1250 mg (VSS) L^{-1}.

Interestingly, all EC_{50} values obtained by the aforementioned studies were higher than the value obtained in the present work. These differences are attributed to the source and concentration of biomass used in the batch test, where lower biomass quantities could lead to lower toxicant tolerances. In this study, the biomass concentration was equivalent to 71 ± 5 mg VSS L^{-1}, lower than aforementioned studies due to limited amount of biomass available for the toxicity tests. Amariei et al. (2017), demonstrated that the EC_{50} values increased by 17-fold when increasing VSS from 125 mg L^{-1} to 1000 mg L^{-1}. Nevertheless, TCS had a detrimental effect on nitrification, with a considerable activity reduction from 10 µg L^{-1} to 100 µg L^{-1}. These results indicate that TCS may pose a risk on nitrifying bacteria under high peak loadings already reported in WWTPs (Kumar et al. 2010).

The impact of DEET on nitrification is shown in Fig. 4. DEET had a moderate effect on the nitrifying culture compared with the other PPCPs tested, with 38.7% inhibition at the highest concentration (10 mg L^{-1} DEET). Limited information on the acute toxicity of DEET is available in the literature. Most studies have been carried out on aquatic organisms (Costanzo et al. 2007; Seo et al. 2005), which found lower toxicity (EC_{50} > 100 mg L^{-1}) than the concentrations evaluated in the present work. Nevertheless, the batch test with DEET suggested that there is no significant toxicity (inhibition < 3%) at environmental levels reported in the literature (peak

values of 15.8 µg L^{-1} in Europe and 42.3 µg L^{-1} in the USA) (Merel and Snyder 2016; Mohapatra et al. 2016).

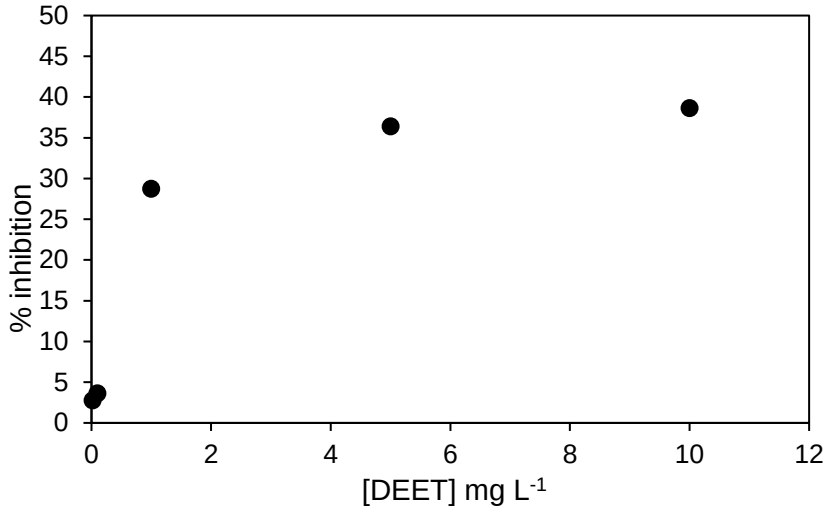

Fig 4. Inhibition percentage at different concentrations of DEET. Maximum nitrification inhibition was below 40%, thus the regression plot was not performed for EC_{50} estimation

Effects of antibiotics on nitrification activity

The %-inhibition results of AMP (ampicillin), OFX (ofloxacin), and CST (colistin) are presented in Fig. 5, along with the EC_{50}-estimation plots. In general, all antibiotics had a detrimental effect on nitrification within the range of concentrations tested.

By analysing Fig. 5b, the EC_{50} value of AMP was calculated at 23.7 mg L^{-1}, much lower than previous short-term studies with enriched nitrifying bacteria: 250 mg L^{-1} (Gomez et al. 1996) and 50 mg L^{-1} (Ramírez et al. 2020). The enhanced sensitivity of our assay can be attributed to lower biomass and degradation rates of AMP. Yu et al. (2019), with a long-term sequential batch reactor, demonstrated that 30 mg L^{-1} AMP had inhibited nitrification activity by 20% to 32%. Moreover, in this study, activities of ammonia-monooxygenase and nitrite-oxidoreductase declined with the increase in AMP concentration, demonstrating that this antibiotic affects the AOB and NOB.

The plot of OFX %-inhibition against the logarithm of the concentration in Fig. 5c was adjusted to a linear regression model, (R^2> 0.97), obtaining an EC_{50} value of 12.7 mg L^{-1} (Fig. 5d). As with DEET, limited data on the acute toxicity of OFX to nitrifying bacteria is found in the literature, and with mixed results. Dorival-García et al. (2013) reported OFX had no inhibitory effect on nitrifying activated sludge at 500 µg L^{-1}. When reported, again, EC_{50} values in activated sludge were considerably higher, e.g., 165 mg L^{-1} (Tobajas et al. 2016). However, the EC_{50} value

estimated in this study was within the range of OFX levels reported by Dokianakis et al. (2004) for enriched NOB culture: between 6 and 10 mg L^{-1}.

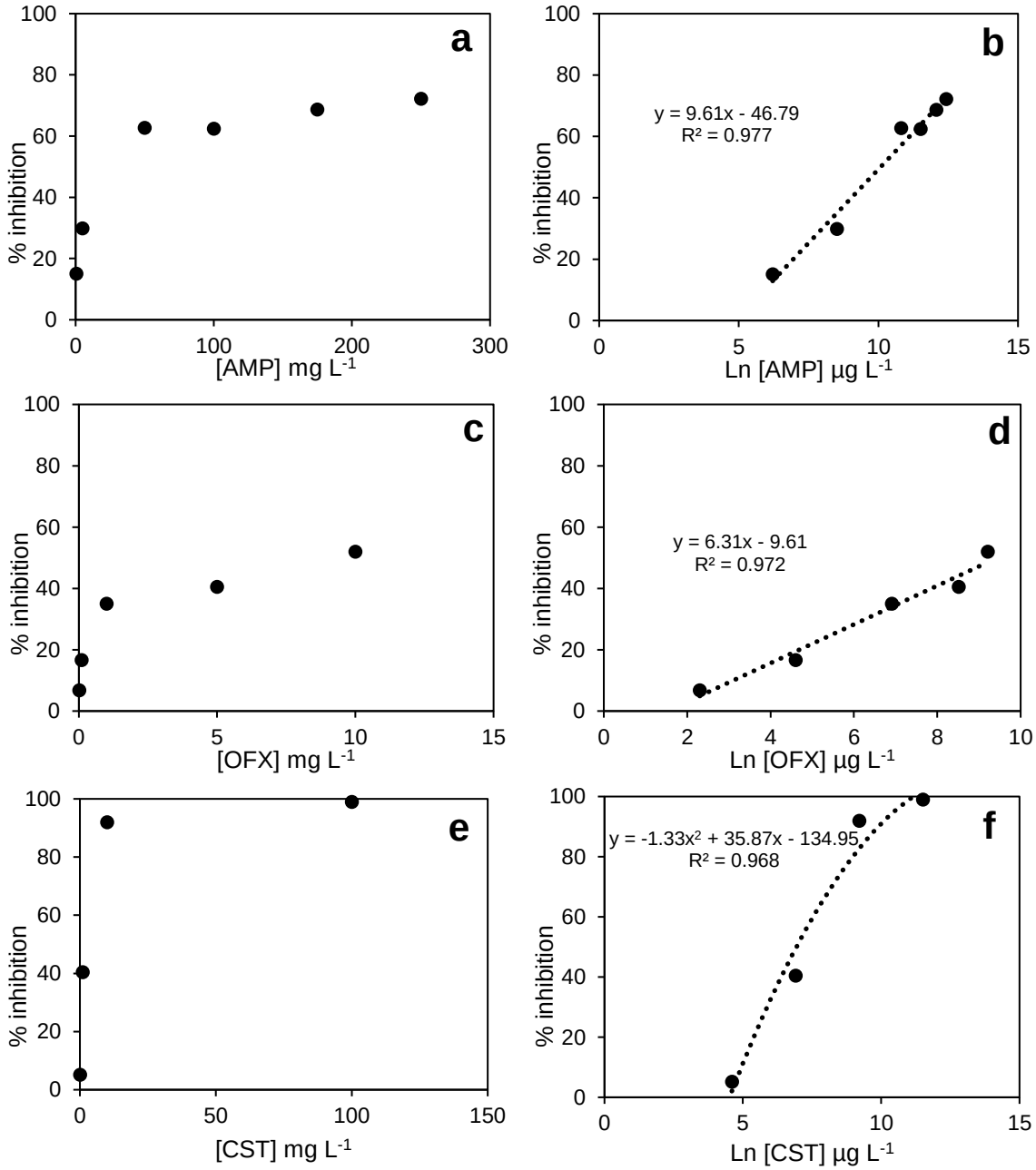

Fig. 5. Inhibition level at different antibiotic concentrations (a) and (b) ampicillin, (c) and (d) ofloxacin, (e) and (f) colistin.

Illustrated in Fig. 5f, 50% inhibition by CST was 1 mg L^{-1}. This estimation was about tenfold lower than the EC_{50} values reported by Bressan et al. (2013). The changes in the toxicity level

may be due to the composition of the CST solution; here, CST sulphate salt (polymyxin E ≥15,000 IU/mg, Sigma Aldrich) was used in the experiments. Bressan et al. (2013) tested two similar commercial CST formulations, one of which contained lactose as vehicle; they reported notable differences in mixed-microbial community tolerance between the two formulations (EC_{50} of 67 mg L^{-1} for CST versus EC_{50} of 10.8 mg L^{-1} for CST plus lactose), demonstrating that the composition of antibiotics could alter the response of the nitrifying bacteria.

Furthermore, Bressan et al. (2013) highlighted that nitrite oxidation was not affected by CST at their highest concentration (316 mg L^{-1}), suggesting that the inhibitory effect of CST was more pronounced on AOB than NOB. Given that NOB metabolism could be reduced due to lack of substrate availability, we spiked with 1000 mg L^{-1} sodium nitrite ($NaNO_2$) stock solution at the end of the incubation period to evaluate the response of NOB under the highest CST concentration (350 mg L^{-1}) and corroborate the results reported by Bressan et al. (2013). The considerable reduction of nitrite levels (~98%) and the increase in nitrate levels to 93.8 % demonstrated that CST had a low impact on NOB (Fig. 6). Moreover, the ammonium consumption remained low (< 5.5 %), showing the persistent inhibition of CST on AOB metabolism.

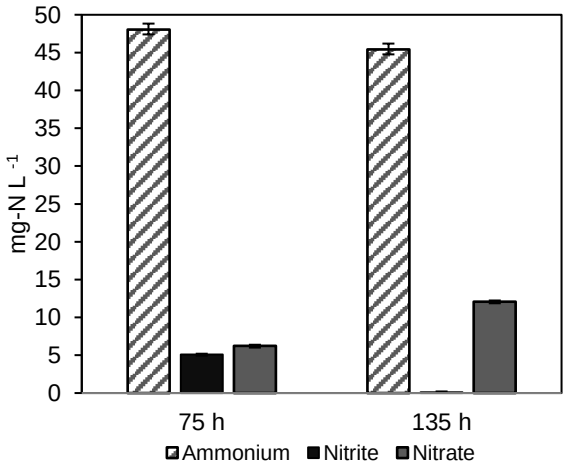

Fig 6. Change of ammonium, nitrite and nitrate levels after the incubation period at concentration of 350 mg L^{-1} colistin.

In general, most of the PPCPs tested in this study had an impact on the performance of nitrifiers with the exception of CF. The measurement of ammonium and oxidation products directly reflected the effects of each PPCP on AOB/NOB guilds compared to the control treatments. However, remarkable differences were observed with previous inhibition studies. Further research is required to determine how the concentration of nitrifying biomass can affect the toxicity tolerance against these contaminants. Additional experiments should be performed to evaluate other exposure scenarios, such as biomass acclimation, where the microbial

communities could show higher capacity to withstand a wide range of chemicals and exhibit possible synergistic effects of PPCP mixtures on nitrification activity.

In summary, the inhibition capacity (EC_{50}) of TCS, AMP, OFX, and CST in short exposure batch tests with nitrifying bacteria were 89.1 µg L^{-1}, 23.7 mg L^{-1}, 12.7 mg L^{-1} and 1 mg L^{-1}, respectively. The maximum inhibition in the presence of DEET was close to 40 % at 10 mg L^{-1}, whereas no remarkable effect was observed for CF in concentrations up to 90 mg L^{-1}. Among the PPCPs tested, TCS exhibited a more pronounced effect on nitrification activity at the concentrations above 0.01 mg L^{-1}. Although the TCS levels reported in the environment are rarely near the EC_{50} values estimated in this study, this antimicrobial agent is commonly detected in WWTPs worldwide at higher concentrations compared to other PPCPs of concern (Tran et al. 2018). The data suggested that TCS is still widely consumed despite the efforts of governmental agencies to restrict its application in numerous household and personal care products (Bedoux et al. 2012). Therefore, TCS usage should be revised to control its excessive consumption and further disposal in the sewage that may lead to its accumulation at higher concentrations in the environment, thereby posing a risk to non-target microorganisms.

The results of acute toxicity analysis indicated that the levels of AMP and OFX detected in the environment were too low to inhibit nitrifiers metabolism, considering that antibiotics often occur at concentrations from ng L^{-1} to µg L^{-1} (Kümmerer 2009). Moreover, further research is necessary to investigate the occurrence and fate of CST in different habitats and evaluate the risks of CST concentrations in the environment.

Conclusions

The toxicity of the most common PPCPs was investigated using short-term nitrification inhibition assays. Based on the 16S rRNA gene sequencing and analysis, *Nitrosomonas* sp. belonging to AOB and *Nitrobacter* sp. a common NOB, were identified in the nitrifying biomass along with other microbial groups typically found in activated sludge. The experimental results of acute toxicity to enriched nitrifying bacteria obtained in this study suggested that the most toxic chemical was TCS compared to the other 5 PPCPs, showing the lowest EC_{50} value of 89.1 µg L^{-1}. With regard to the antibiotics, CST exerted the highest toxicity to overall nitrification (EC_{50} value of 1 mg L^{-1}), with a more pronounced inhibition on AOB activity than on NOB activity. Results showed that the EC_{50} values estimated for AMP (23.7 mg L^{-1}) and OFX (12.7 mg L^{-1}) were considerably higher than environmentally relevant levels. CF had no remarkable inhibitory effects on nitrification performance. In the case of the insect repellent DEET exerted partial inhibitory effects on nitrifying bacteria below 40% at the highest concentration (10 mg L^{-1}).

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author Dr Charles W. Knapp (charles.knapp@strath.ac.uk) upon reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Methodology, material preparation, laboratory experiments, data collection and analysis were performed by CL for all six toxicants. MN supported the laboratory experiments and data interpretation of the first three toxicants. CK and YFT contributed to the study's conception. The first draft of the manuscript was written by CL and all authors commented and edited on previous versions of the manuscript. All authors read and approved the final manuscript.

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Chapter 3

Evaluating acute toxicity in enriched nitrifying cultures: lessons learned

3.1 Preface

This chapter presents an original article published in the *Journal of Microbiological Methods* in November 2021. This paper summarises the relevant aspects in the experimental design of toxicological batch assays using nitrifying cultures as biomass. Furthermore, the manuscript captures the lessons learned during the toxicological experiments with pharmaceuticals and personal care products presented in Chapter two of this thesis. C. Lopez Smitter is the principal author, responsible for all the experimental design, data analysis and interpretation and writing of the paper. C. Knapp, as project supervisor, provided support and assistance with reviewing the manuscript.

Additional information is available in the appendix supplementary information to Chapter 3. This section contains the supplementary information added in the published article, including supplemental methodologies and results.

Previous chapter 2 presented the findings on the short-term effects of selected PPCPs on nitrifying bacteria enriched from WWTP sludge. The experimental design for the toxicological assays was based on the international standard ISO 9509 (2006) Water Quality-Toxicity test for assessing the inhibition of nitrification of activated sludge microorganisms. Although this protocol provided essential guidelines for the development of the experiments, they are prepared for activated sludge biomass. For this reason, this Chapter contains practical recommendations to support further research in acute testing using nitrifying bacteria as a source of inoculum.

3.2 Journal paper

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Evaluating acute toxicity in enriched nitrifying cultures: lessons learned

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Abstract

Toxicological batch assays are essential to assess a compound's acute effect on microorganisms. This methodology is frequently employed to evaluate the effect of contaminants in sensitive microbial communities from wastewater treatment plants (WWTPs), such as autotrophic nitrifying populations. However, despite nitrifying batch assays being commonly mentioned in the literature, their experimental design criteria are rarely reported or overlooked. Here, we found slight deviations in culture preparations and conditions impacted bacterial community performance and could skew assay results.

From pre-experimental trials and experience, we determined how mishandling and treatment of cultures could affect nitrification activity. While media and biomass preparations are needed to establish baseline conditions (e.g., biomass washing), we found extensive centrifugation selectively destabilised nitrification activities. Further, it is paramount that the air supply is adjusted to minimise nitrite build-up in the culture and maintain suitable aeration levels without sparging ammonia. DMSO and acetone up to 0.03 % (v/v) were suitable organic solvents with minimal impact on nitrification activity. In the nitrification assays with allylthiourea (ATU), dilute cultures exhibited more significant inhibition than concentrated cultures. So there were biomass-related effects; however, these differences minimally impacted the EC_{50} values. Using different nutrient-media compositions had a minimal effect; however, switching mineral media for the toxicity test from the original cultivation media is not recommended because it reduced the original biomass nitrification capacity.

Our results demonstrated that these factors substantially impact the performance of the nitrifying inoculum used in acute bioassays, and consequently, affect the response of AOB-NOB

populations during the toxicant exposure. These are not highlighted in operation standards, and unfortunately, they can have significant consequential impacts on the determinations of toxicological endpoints. Moreover, the practical procedures tested here could support other authors in developing testing methodologies, adding quality checks in the experimental framework with minimal waste of time and resources.

Keywords: nitrification inhibition, nitrifying bacteria, AOB, NOB, acute toxicity

1. Introduction

Biological removal processes are fundamental in wastewater treatment plants (WWTPs) to control the release of excess nutrients into the environment. In particular, biological nitrification involves an aerobic process carried out by two lithoautotrophic clades of microorganisms: the ammonia-oxidising bacteria (AOB) and the nitrite-oxidising bacteria (NOB). These nitrifiers cooperatively transform nitrogen, where the AOB first oxidises ammonia to nitrite, which becomes subsequently oxidised to nitrate by the NOB (Koops and Pommerening-Röser 2001; Daims *et al.* 2016)

Nitrifiers are considered a sensitive community in activated sludge. The activity of AOB/NOB guilds in WWTPs could be severely impacted by environmental changes (Johnston *et al.* 2019; Sun *et al.* 2021), operating conditions (Tang and Chen 2015) and toxic compounds (Figuerola and Erijman 2010). Due to increased pollutants in wastewater, toxicological bioassays have become vital to assess the impact of these chemicals on nitrification activity, supporting the operational strategies and the functional stability of WWTPs (Xiao *et al.* 2015)

Traditionally, batch bioassays constitute a valuable screening tool for assessing microbial responses against acute (short-term) exposure to toxicants (Roose-Amsaleg and Laverman 2016). This methodology is relatively more accessible than continuous cultures, allowing the assessment of multiple conditions simultaneously (Radniecki and Lauchnor 2011). In nitrification inhibition studies, the enriched consortium is preferred over pure AOB/NOB isolates to better represent microbial diversity; further, they are easier to maintain (Li *et al.* 2016). Some authors employed samples with high nitrification activity directly from WWTPs (Li *et al.* 2020a; Velasco-Garduño *et al.* 2020), and others, more commonly, use nitrifying biomass enriched in lab-scale reactors under specific growth conditions seeded with activated sludge (Huang *et al.* 2016; Langbehn *et al.* 2020).

Although the experimental design reflects specific research objectives, inhibition assays conform to a similar framework (Fig. 1). Experiments involve a series of batch reactors with nutrient media under aerobic conditions that have been inoculated with a nitrifying population or community. The reactors are then spiked with multiple concentrations of a toxic substance and

incubated to assess biochemical responses. However, there are no specific standard conditions under which the nitrifying communities are enriched or cultivated, thus leaving opportunities for operational variability.

From Fig. 1, the source of biomass at the top of the chart highlights its relevance in the bioassay; the specific characteristics of the biomass could significantly affect the assay performance. The term "enriched nitrifying consortium" usually refers to enhancing nitrifiers populations under specific cultivation conditions, resulting in a targeted microbial structure but still with a broad spectrum of residual species in the biomass. Within the nitrifying bacterial communities, the operating conditions in lab-scale reactors are likely to favour certain members AOB/NOB species based on the ecophysiological differences such as substrate and oxygen affinity and their capacity to thrive under starvation periods (Koops and Pommerening-Röser 2001; Liu and Wang 2013; Daims *et al.* 2016; Sun *et al.* 2019). Because there is no standard limit for harvest periods or reactor configuration, biomass in inhibition studies will present a wide range of nitrification rates and different AOB/NOB abundances in the microbial consortium (Chen and LaPara 2008; Wang *et al.* 2019; Trejo-Castillo *et al.* 2021).

Moreover, nitrifying bacteria in these enrichments coexist with other microorganisms (i.e., heterotrophs), and their presence can interfere with the metabolic activity of nitrifiers. In some cases, lab-scale enrichment promotes the growth of AOB/NOB populations in the complete absence of organic substrates (Huang *et al.* 2016; Langbehn *et al*. 2020), while others enhance the fraction of nitrifiers under low C/N to sustain the heterotrophs in the culture (Katiglopu-Yazan *et al.* 2017). Other factors, e.g., flocs and cell aggregates, influence the distribution of nitrifiers in the inoculum (Manser *et al*., 2005; Fang *et al.*, 2009; Wang *et al.* 2012), affecting the mass transfer of substrates and oxygen. This can ultimately impact the microorganisms' metabolic interactions (Arnaldos *et al.* 2015), mutualistic cooperation (Graham *et al.* 2007; Knapp and Graham 2007) and competition for resources (Navada *et al.* 2020).

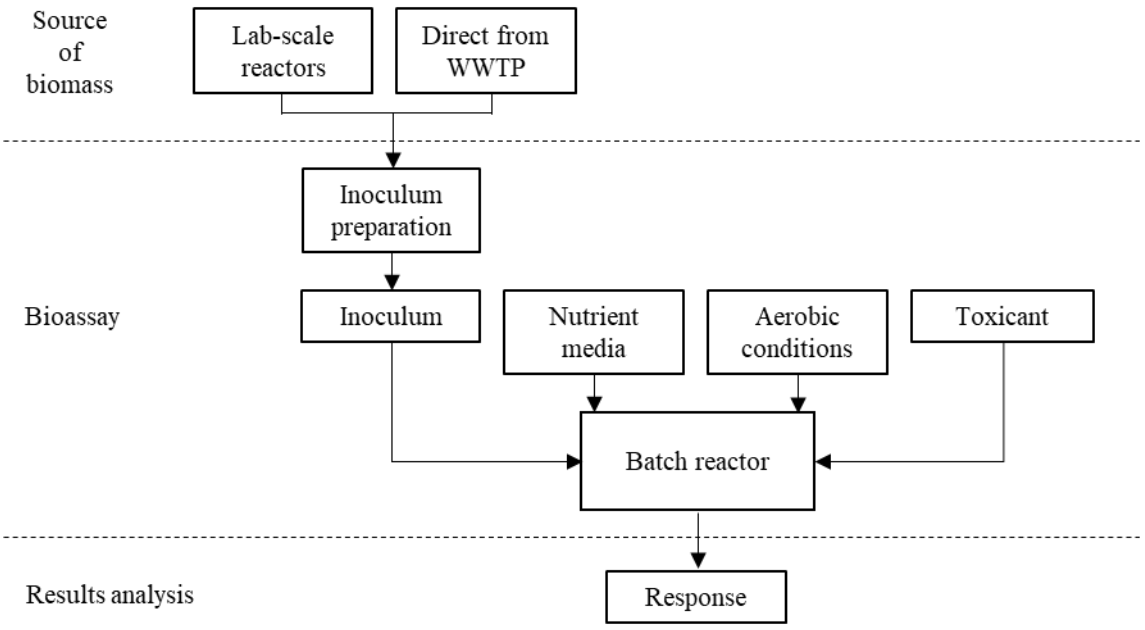

Fig. 1. An overall framework for nitrification batch assays.

Due to this, the experimental design should consider the specific characteristics of the nitrifying biomass to establish suitable conditions during toxicant exposure. Many testing parameters are well-established in the bioassays, such as pH, temperature, free ammonia, free nitrous acid and dissolved oxygen (DO) (Jiménez *et al.* 2012; Shanahan and Semmens 2015); others like biomass preparation, batch configuration and toxicant stock solution may not. This is highly relevant because these procedures can introduce small perturbations in the "new" batch environment where the inhibition assay takes place, resulting in transient behaviour in the microbial consortium, and likely, as a consequence, misleading inhibition responses (Chandran *et al.* 2008; Yuan *et al.* 2019). Standard protocols such as ISO 9509 (2006) for activated sludge and other pure cultures procedures (Radniecki and Lauchnor 2011) may show practical recommendations, but important parameters remain unclear for preparing nitrifying biomasses. These challenges were evident during the literature review in Lopez *et al*. (2021) for assessing the toxicity of pharmaceutical and personal care products (PPCPs) on nitrification performance. Among previous publications, it was observed that the design criteria and the rationale behind the bioassay arrangements were not reported, leading in some cases to unstable nitrification, even in the control cultures (Zepeda *et al.* 2006; Ramírez Muñoz *et al.* 2020; Velasco-Garduño *et al.* 2020).

In this context, we evaluated the effect of selected factors that could alter the performance of nitrifying bioassays, such as inoculum preparation, aerobic conditions adjustment, and organic solvents for toxicant dissolution. We also explored the impact of biomass quantity and nutrient media on inhibition response in the presence of allylthiourea (ATU), a standard reference

nitrification inhibitor (Tatari et al. 2017). The response of the different treatments was compared with the measurement of the substrate consumption and production of oxidation compounds in the batch cultures. We selected these factors due to the practical experience learned in Lopez *et al.* (2021). The assessment of testing parameters applied in nitrification studies is relevant considering the challenges faced with slow-growing and sensitive organisms. This work aims to understand the behaviour of mixed AOB-NOB cultures during the preparation of batch bioassays and establish the necessary adjustments to control the introduction of external factors frequently overlooked. This paper intends to supplement existing guidelines and could help scientists develop experimental protocols, optimise time-consuming procedures and improve test reliability with minimal alteration of the nitrifying biomass activity prior to the toxicity bioassay.

2. Materials and Methods

2.1. Source of nitrifying inoculum

An enriched nitrifying consortium was used as inoculum, which was cultivated in 2-L, lab-scale batch reactors (glass bottles with aeration) under autotrophic growth conditions to selectively enhance AOB/NOB populations. At the start of the cultivation period, the microbial consortium in the bioreactor grew as suspended free cells, forming dense clusters over time. However, the biomass eventually attached to the container walls, which was reduced periodically by cleaning the reactor; basically, the reactors were rinsed and replaced with 70% volume of fresh media. Further details of the reactor's operation and maintenance where the same biomass was collected were previously reported in Lopez *et al.* (2021). Samples collected from the cultivation batch reactors for 16S-rRNA sequencing and analysis (Lopez *et al.*, 2021), confirmed that the biomass phylogenetic groups were consistent with other analyses of microbial communities in activated sludge (Zhao *et al.* 2018). *Nitrosomonas* sp. and *Nitrobacter* sp. were identified as microorganisms responsible for autotrophic nitrification, with relative abundances of 5.7% and 0.8%, respectively (Lopez *et al.* 2021). Over the experimental period, the ammonium consumption rates ranged 11 – 20 mg NH_4^+-N/g·MLVSS·h, and the yield nitrate production was about 0.95 mg NO_3^--N produced/mg NH_4^+-N consumed. Under stable conditions, biomass was periodically withdrawn from the reactor and used in the short-term exposure assays.

2.2. Design and operation of the batch experiments

Based on the experiences in Lopez *et al.* (2021), several factors related to the toxicity tests were investigated through short-term batch assays. In the first set of experiments, three factors were assessed individually: inoculum cleaning procedure, aeration mode and organic solvents. After

that, two inhibition tests were performed using different biomass concentrations and liquid nutrient media. Further details are presented in the following sections. All testing assays were carried out under the same conditions described in Lopez *et al.* (2021). Briefly, 500-mL glass bottles with 300-mL working volume were used for the liquid batch cultures. This volume was based on sample requirements, such as type of analysis and frequency, and ensured that samples were never >10% of the initial volume. According to Bollmann et al. (2011), the basal media was prepared with the trace metal solution from Schmidt and Belser (1994) and $NaHCO_3$ as an inorganic carbon source. This nutrient media was also used in Lopez *et al.* (2021), prepared with an initial ammonium concentration of 56 mg/L to prevent free ammonia inhibition (ISO 9509 2006; Li *et al.* 2020b). The pH was adjusted using a pH/conductivity meter (Mettler Toledo, MPC 227, Switzerland), and the dissolved oxygen (DO) was measured with a DO meter (Eutech Instruments Pte Ltd., DO 6+ DO/Temp, Singapore).

For each study case, batch experiments were conducted in parallel (duplicates or triplicates) using the biomass withdrawn from the same parent reactor Lopez *et al.* (2021). A schematic of the batch assay configuration is presented in Fig. S1 (see appendix supplementary information to Chapter 3).

2.2.1. Inoculum cleaning test

Many assays require a rinse of the biomass to recondition the media to baseline levels and minimise any residual waste materials. In earlier stages of the experimental period, we developed a cleaning method using centrifugation at 10,000×*g* (Eppendorf, centrifuge model 5804 R) followed by media settling, referred to in this study as Method 1 (see Fig. 2). The main objective of this method was the maximum removal of oxidising compounds (nitrite and nitrate) in the culture suspension prior to inoculation. We conducted further testing to determine the impact of this cleaning procedure on biomass activity by comparing the inoculum performance with an optimised cleaning protocol (Method 2) (see Fig. 2). Based on this, a series of batch reactors (n=3) were inoculated with biomass prepared with two cleaning strategies (Fig. 2): a longer protocol with two cycles of centrifugation/setting (Method 1) and an optimised version with one centrifugation cycle (Method 2). The operating conditions of the cleaning test are presented in Table 1.

Table 1. Initial operating conditions for the short-term cleaning test. Values represent means and standard deviations (or range, in case of duplicates).

Test name	**Cleaning test (Method 1)**	**Cleaning test (Method 2)**
NH_4^+-N (mg L^{-1})	55.6 ± 0.6	56.3 ± 0.8
pH range	7.7 – 7.4	7.7 – 7.2
Temperature (°C)	19 - 20	19 - 20
DO (mg L^{-1})	> 5	> 5
Protein (mg L^{-1})	9.3 ± 0.4	10.0 ± 0.2
TSS (mg L^{-1})	143.7 ± 4.7	142.0 ± 3.0
Replicates	Triplicates	Triplicates
Duration (h)	24	24

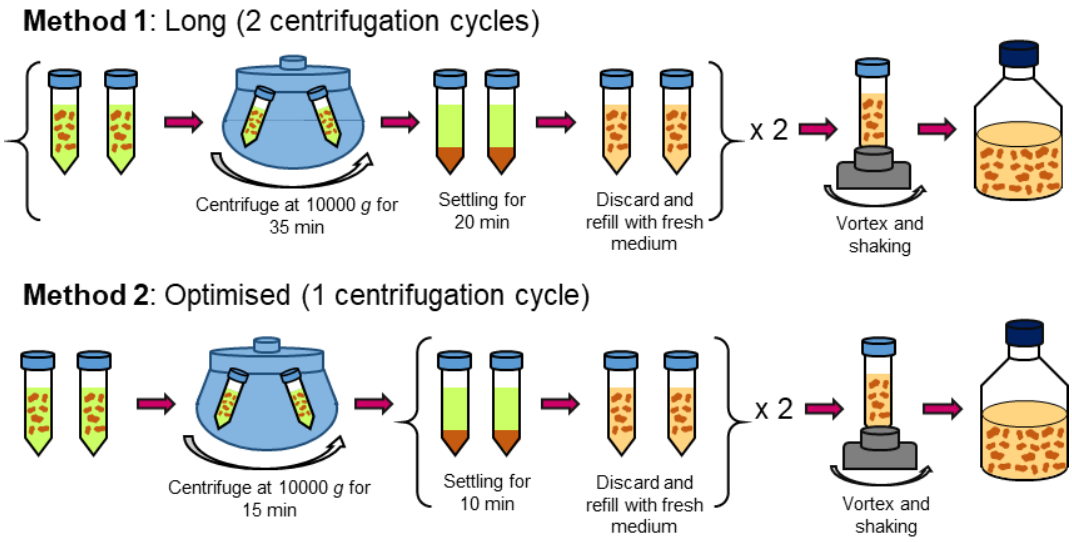

Fig. 2 Schematic of the two cleaning methods to prepare the inoculum.

2.2.2. Enforced aeration test

Previous batch tests (data not shown) demonstrated orbital shakers at 120 rpm result in low nitrification activity, providing insufficient aeration technique for the cultures. Due to this, enforced aeration was selected as the aeration strategy. Three airflows (AF) were tested to evaluate whether aeration was sufficient: low AF at 0.05 L min^{-1}, medium AF at 0.175 L min^{-1}, and high AF at 0.3 L min^{-1}. The air was supplied using airstones at the bottom of the bottles, connected to an air pump (HDOM, Model HD-603, Shenzhen Hidom Electric Co., Ltd.) and filtered with 0.2 µm sterilising-grade filter (Aervent™, Millipore, France). A reservoir with

sterile water was used to premoisten the air and minimise media evaporation (identified as "air reservoir" in the supplementary information). Because a direct DO sensor inside the batch reactors was unavailable (Dempsey 2011), the airflow was adjusted before the experiments to meet the DO criteria of 4 mg L^{-1} (ISO 9509 2006), and DO was measured at the start and end of the incubation period. Before use, the airstones were tested in terms of bubbling pattern, washed thoughly with deoinsed water and flushed with filtrated air in stelised batch bottles with deoinsed water (for 24 h prior testing), preventing airborne contamination into the culture. In addition, all the system was autoclaved before inoculation (Dempsey 2011). When the reactors were assembled, airflow was adjusted with an airflow meter (Brooks Instrument Model # MR3A12BVBN, USA). The test operating conditions are summarised in Table 2.

Table 2. Initial operating conditions for enforced aeration and solvent tests. Values represent means and standard deviations (or range, in case of duplicates).

Test name	**Enforced aeration test**	**Solvent test**
NH_4^+-N (mg L^{-1})	50.6 ± 0.4	53.6 ± 0.5
NO_2^--N (mg L^{-1})	0.7 ± 0.1	0.2 ± 0.0
NO_3^--N (mg L^{-1})	1.0 ± 0.1	0.5 ± 0.0
pH range	7.7 – 7.6	7.7 – 7.6
Temperature (°C)	19 - 20	22 - 24
DO (mg L^{-1})	5 – 4.3	> 5
Protein (mg L^{-1})	8.7 ± 0.4	6.1 ± 0.2
TSS (mg L^{-1})	122.2 ± 6.8	59.9 ± 2.1
Replicates	Triplicates	Duplicates
Duration (h)	24	56

2.2.3.Solvent test

Depending on their solubilities in water, toxicants may require an organic solvent for dissolution. Likewise, equal amounts of solvent must be added to each reactor to maintain comparable conditions regardless of toxicant concentration, and one must minimise the use and volume of solvent. However, it remained uncertain whether other toxicological effects existed from the solvents.

The effect of three conventional organic solvents: dimethyl sulfoxide (DMSO), acetone and ethanol, on the nitrifying biomass was investigated. Each treatment was spiked with 0.1 ml of the solvent with a final concentration of (0.03 % v/v). Testing conditions are shown in Table 2. All

batch cultures were cultivated for 56 h, after which their performances were compared to the controls. The description of the solvents is shown in Table 3.

Table 3. Solvent characteristics

Solvent	**Formula**	**MW**	**Grade**
Dimethyl sulfoxide (DMSO)	C_2H_6OS	78.13	>99.7%. Fisher Scientific
Acetone	C_3H_6O	58.08	>99.5%. Fisher Scientific
Ethanol	C_2H_6O	46.07	>99.5%. Fisher Scientific

2.2.4.Biomass size inhibition test

Researchers will often concentrate (or maximise) biomass to improve the detection resolution of any dose-related responses. However, it was hypothesised that elevated biomass levels may have reduced inhibition rates or require higher concentrations of a toxicant to get an equivalent effect. As such, the impact of biomass size on the inhibition response was evaluated by considering two inoculum concentrations, low (initial concentration) and high cases (5x concentrated amount) (Table 4). Allylthiourea (ATU), a standard reference inhibitor in nitrification toxicity assays (ISO 9509 2006), was used to spike the batch reactors at different final concentrations: 0, 0.005, 0.05, 0.1 and 0.3 mg L^{-1}.

Table 4. Initial operating conditions for the biomass size inhibition test *.

Test name	**Low case**	**High case**
NH_4^+-N (mg L^{-1})	56.9 ± 0.3	56.2 ± 0.4
NO_2^--N (mg L^{-1})	0.1 ± 0.0	0.6 ± 0.2
NO_3^--N (mg L^{-1})	0.3 ± 0.1	0.7 ± 0.4
pH range	7.6 – 7.5	7.7 – 7.3
Temperature (°C)	18 - 19	18 - 19
DO (mg L^{-1})	> 5	> 5
Protein (mg L^{-1})	4.2 ± 0.2	20.1 ± 0.4
TSS (mg L^{-1})	44.3 ± 2.3	213.7 ± 4.9
Replicates	Triplicates	Triplicates
Duration (h)	24	24

Values represent means and standard deviations (or range, in case of duplicates).

***** High case reactors contained five times the amount of biomass (protein or TSS) than the low case reactors

2.2.5.Nutrient Media inhibition test

The enrichment of nitrifying biomass and all toxicity experiments reported in Lopez *et al.* (2021) and this study were performed using the same nutrient media. The liquid medium was modified from (Bollmann *et al.* 2011), containing HEPES as a buffering agent, basal salts, phosphate, trace metals and $NaHCO_3$ as an inorganic carbon source. To evaluate whether the nutrient media composition affected the microbial inhibition response, we conducted a series of toxicity tests comparing our experimental test media with the media recommended in the ISO 9509 (2006) protocol. According to this methodology, a solution with only $NaHCO_3$ should be sufficient to sustain the nitrification in short-term assays without significant change of pH. The batch reactors were spiked either with ATU (0.1 mg L^{-1}) or without. The responses were evaluated in terms of %inhibition compared with the control cultures (sans ATU). All the treatments used $(NH_4)_2SO_4$ salt as a source of inorganic nitrogen. The testing conditions are summarised in Table 5.

Table 5. Initial operating conditions for the nutrient media inhibition test. (Mean values, with standard deviations in parentheses).

Test name	**HEPES medium**	**$NaHCO_3$ medium**
NH_4^+-N (mg L^{-1})	55.5 ± 1.8	54.8 ± 0.8
NO_2^--N (mg L^{-1})	0.5 ± 0.0	0.5 ± 0.0
NO_3^--N (mg L^{-1})	0.8 ± 0.0	0.9 ± 0.2
pH range	7.8 – 7.7	7.9 – 7.8
Temperature (°C)	18 - 19	18 - 19
DO (mg L^{-1})	> 5	> 5
Protein (mg L^{-1})	9.1 ± 0.1	9.1 ± 0.1
TSS (mg L^{-1})	123.2 ± 4.1	120.3 ± 2.4
Replicates	Duplicates	Duplicates
Duration (h)	24	24

2.3. Biomass and chemical analysis

Biomass concentrations have been estimated by protein content and dry cell weight in nitrification inhibition studies (Dytczak *et al*. 2008; Ben-Youssef *et al.* 2009; Roh *et al*. 2009). In the case of protein analysis, tests were conducted to optimise protein extraction and quantification. The protein strategy was selected considering the maximum protein yield from the combination extraction/assay method, which resulted in freeze-thaw cycles and Micro BCA assay (see Fig. S3 in section supplemental methodologies and results in the appendix supplementary information to Chapter 3). The cell dry weight was determined as total suspended

solids (TSS), carried out according to the Standard Methods (APHA 1998). It is worth mentioning that although the nitrifying bacteria originated from activated sludge, the inorganic suspended solids were removed during the cultivation process, leading to similar measurements (see Table S3) between total suspended solids (TSS) and volatile suspended solids (VSS) (He *et al.* 2013; Lopez *et al.* 2021). Analysis of nitrogen compounds was performed colourimetrically as described in Lopez *et al.* (2021) using KoneLab Aqua 30 (Thermo Scientific, Aquarem 300; Clinical Diagnostics Finland). No analytical interference from the test substances or matrix components was found with any chemical analysis carried out in this study. The detail of the operating procedures for the chemical analysis can be found in the appendix supplementary information to Chapter 2 of this thesis.

The biomass changes were evaluated to verify that cell growth was minimal over the experiments (Radniecki and Lauchnor 2011). These experiments (see Table S3 in section supplemental methodologies and results in the appendix supplementary information to Chapter 3) showed that ammonium and nitrite oxidation by nitrifiers occurred with a minimal increase of total protein (6.4 ± 0.0 %) over the incubation period (78h), with a biomass formation estimated in 0.01 ± 0.0 mg microbial protein/mg NH_4^+-N consumed. These results demonstrated that the process was mainly disassimilative with low cell growth. (Ramírez Muñoz *et al.* 2020; Trejo-Castillo *et al.* 2021).

2.4. Data analysis

The responses were evaluated by comparing the concentration of nitrogen species, percentage of ammonium consumed E, (mg NH_4^+-N consumed/g of initial NH_4^+-N × 100), and yield (Y, mg of NO_2^--N or NO_3^--N produced/mg of NH_4^+-N consumed]) at the end of the incubation. In addition, this approach facilitated the analysis of the stoichiometric mass balance in yields, considering the nitrogen transformation into oxidising species with minimal cell growth (Ramirez *et al.* 2020; Velasco-Garduño *et al.* 2020; Trejo-Castillo *et al.* 2021).

In the case of biomass size and nutrient media inhibition tests, we assessed nitrification performance by comparing the level of inhibition resulting from the toxicant exposure to unamended 'controls'. The %inhibition was determined as described in Lopez *et al.* (2021) using the following equation (1):

$$\%Inhibition = \frac{(NO_{X\ control} - NO_{X\ test})}{NO_{X\ control}} * 100 \quad (1)$$

Where $NO_{X\ control}$ and $NO_{X\ test}$ represented the changes of oxidised nitrogen species (NO_2^- + NO_3^-, mg-N L^{-1}) in the control cultures and the reactors with the 'test' substance, respectively. In addition, the concentration of the toxicant causing 50% inhibition (i.e. IC_{50}) in the two biomass

levels was estimated using the linear correlation between the inhibition percentage and the *log*-transformed toxicant concentration (ISO 9509 2006). All the experimental results were described as mean ±standard deviation from the replicates. Finally, statistical analyses were carried out using one-way ANOVA or the Student's *t*-test to determine whether the effect between the treatments statistically differed at a 95% confidence interval.

3. Results and discussion

Among researchers, nitrifying bacteria are complicated microorganisms due to their slow growth rate and sensitivity to different environmental conditions. When assessing them, these features pose a challenge, where biomass manipulation and inadequate experimental conditions could alter the testing outcome. Unfortunately, standardised procedures such as ISO 9509 (2006) do not state in detail the relevant steps to prevent introducing such errors. As such, we conducted a series of experiments to explore the effect of biomass cleaning procedures, airflow adjustments, testing media and biomass concentration on nitrification performance. The observations highlighted below could contribute to developing future protocols involving the evaluation of nitrifiers against toxic compounds.

3.1. Effect of cleaning procedure on nitrification performance

Biomass preparations often require a wash step to help reestablish baseline media conditions (e.g., removing accumulated oxidised nitrogen by-products); this involves settling, centrifugation, and replacing media. Without any specific guidance, the durations of settling and centrifugation were examined. "Method 1" involved longer centrifugation to remove oxidised N-species, where "Method 2" had a shorter, optimised time. The optimization of the cleaning procedure was carried out to minimise the impact of this process in the performance of nitrifiers.

The profiles of ammonium, nitrite and nitrate for the two cleaning methods are presented in Fig. 3. As can be seen, both batch cultures exhibited nitrite accumulation due to slower metabolic NOB activity caused by limited substrate (nitrite) at the start of the experiment (Martínez-Hernández *et al.* 2011). However, the Method 2 reactors (Fig. 3b) reached their nitrite peak (4.0 ± 0.3 mg L^{-1}) within the first five hours of incubation. From this point, the levels gradually decreased to near zero at the end of the incubation period. On the contrary, nitrite concentrations in the bottles treated with Method 1 continued to increase with a final level of 12.4 ± 0.3 mg L^{-1} at the end of monitoring.

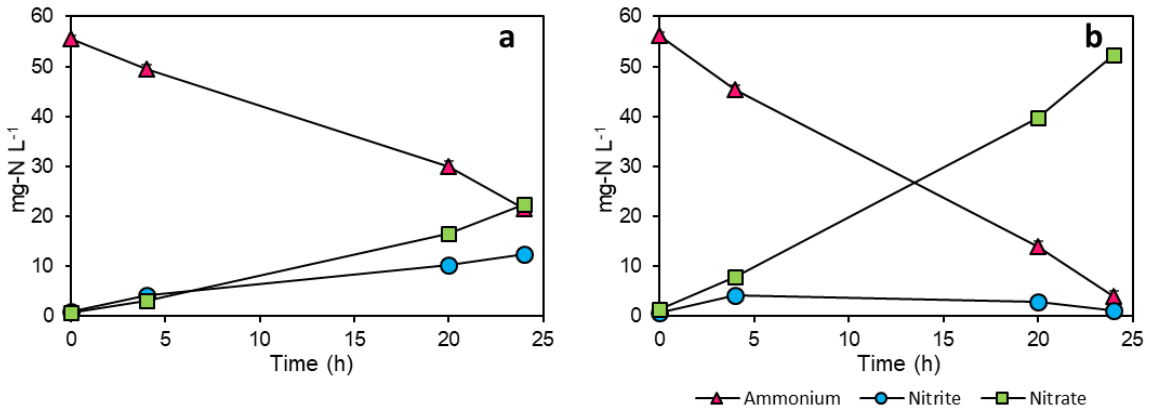

Fig. 3. Nitrification profiles following different washing procedures: a. Method 1 (long centrifugation). b. Method 2 (optimised, short centrifugation). Mean ± standard deviations (n=3).

Comparing the final batch reactors performance (Fig. 4), it is observed that the lengthy procedure (Method 1) dramatically impacted nitrification activity, based on the final nitrate yield (t-test $p < 0.05$) significantly. This extended protocol resulted in lower nitrate yields (Y_{NO3-} = 0.64 ± 0.01 mg NO_3^--N produced/mg NH_4^+-N consumed) and nitrite accumulation (Y_{NO2^-} = 0.34 ± 0.01 mg NO_2^--N produced/mg NH_4^+-N consumed) compared to the cultures with the optimised procedure (Method 2), where the Y_{NO3-} was closer to 1. In terms of ammonium consumption (E_{NH4+}), the removal efficiency in the Method 1 culture was 32.6% lower than Method 2 (93.0 ± 4.1%), suggesting that improper biomass cleaning has a detrimental effect in both AOB/NOB guilds, reflected in their unstable nitrification activity.

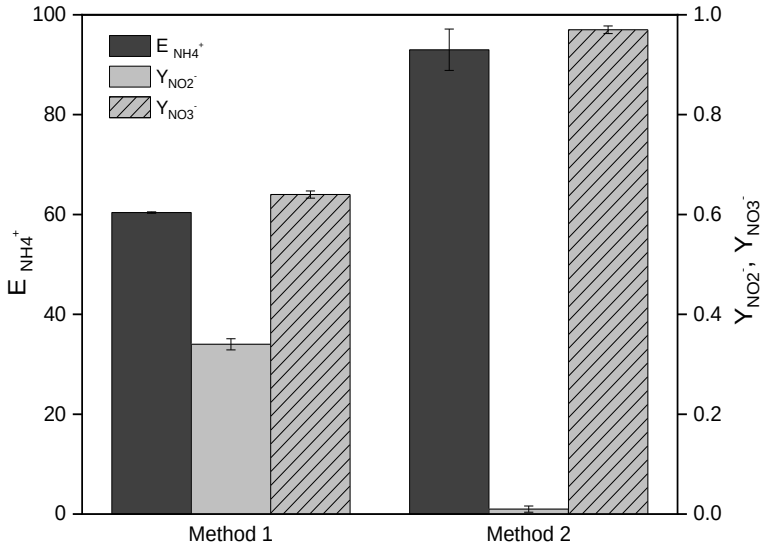

Fig. 4. Ammonium consumption efficiency, yields of nitrite and nitrate of the washing procedures. Method 1 and Method 2 (optimised). The bar represents Mean ± standard deviations (n=3).

Preparations of cell suspensions via similar procedures have been widely applied in research studies (Zepeda *et al.* 2006; Bian *et al.* 2020). Despite this, authors rarely demonstrate how the biomass rinsing protocol may have affected the performance of their nitrifying bacteria in batch experiments. For example, Moussa *et al.* (2003) reported that washing and re-suspending procedures in different buffer media affects nitrifiers activity. Another publication by Peterson *et al.* (2012) suggested that centrifugation produce bacterial cell damage due to pellet compaction. On the other hand, shorter cleaning procedures resulted in slightly higher oxidation products remaining in the inoculum, as observed in Lopez *et al.* (2021) and this study (Table 6; nitrite (t-test, p=0.32) and nitrate (t-test, p=0.004)). However, it is unlikely that these values have negatively impacted nitrifiers activity because they were less than the inhibitory nitrite and nitrate levels reported in the literature (Chandran and Smets 2000; Silva *et al.* 2011; Bollmann *et al.* 2011; Spieck and Lipski 2011). Nevertheless, the results in this study demonstrate that evaluating the impact of the cleaning methodology before testing is essential to minimise the disturbance of AOB/NOB species in the inoculum and prevent poor performance during the toxicity assay.

Table 6. Remaining oxidised N-species in the biomass suspension after the cleaning procedure. (Mean values, with standard deviation in parentheses)

Test name	**Method 1**	**Method 2**
NO_2^--N (mg L^{-1})	0.8 ± 0.0	0.7 ± 0.1
NO_3^--N (mg L^{-1})	0.7 ± 0.1	1.4 ± 0.1

3.2. Effect of airflow on nitrification performance

Nitrifying bacteria convert ammonia to nitrate in a two-step process under aerobic conditions. We evaluated how the air supply affected the AOB-NOB performance by monitoring the nitrification activity in batch cultures for 24 hours. Fig. 5 shows the final responses of the batch reactors at different airflow levels. As expected, the aeration mode significantly impacted nitrification performance, where the ammonium removal efficiency increased with the airflow (ANOVA, $p<0.005$). At low airflow (0.05 L min^{-1}), E_{NH4+} was 15.6 ± 2.3 % and the Y_{NO2^-} and Y_{NO3^-} were 0.21 ± 0.07 and 0.75 ± 0.07, respectively. This low performance may be attributed to small air bubbles production and poor mixing, creating oxygen-deprived zones in the full medium, reducing the mass oxygen transfer to the liquid phase and bioparticles (Dempsey 2011; Garcia-Ochoa *et al.* 2010; Yao *et al.* 2021). Furthermore, the spatial distribution and adhesion of the microorganisms in the bioparticles or cell clusters (Picioreanu *et al.* 2016) may also intervene in oxygen availability. For instance, the presence of floc and granules could increase the diffusional resistance in the oxygen transport and the cell clusters where nitrifiers bond with other

microbes, and in consequence, limiting the access to oxygen required by microbial communities for respiration (Larsen *et al.* 2008; Fang *et al.* 2009; Dempsey 2011). Based on this, although most activated sludge solids were removed from our harvesting batch reactors, cell aggregates in suspended cultures can still create substrate gradient within the microcolonies, reducing oxygen levels in the biomass (Picioreanu *et al.* 2016).

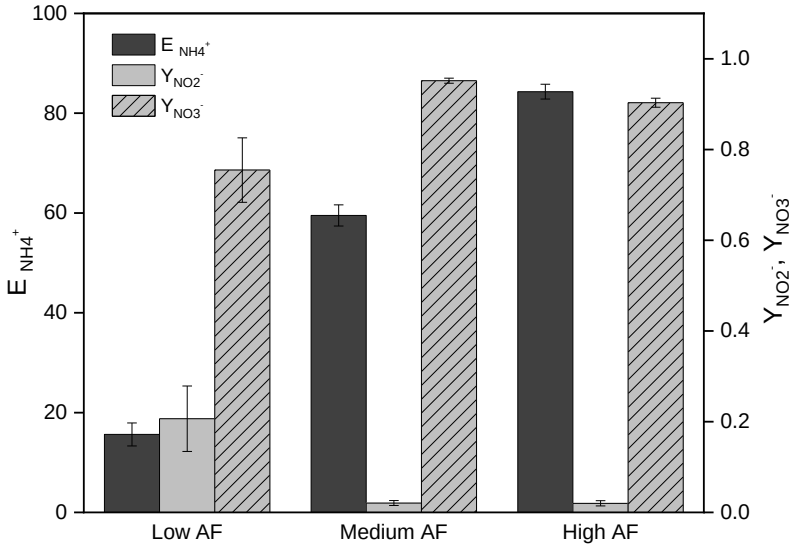

Fig. 5 Ammonium consumption efficiency, yields of nitrite and nitrite of the enforced aeration test (24 h incubation). The bar represents Mean ± SD. (n=3)

Concerning DO, the levels were maintained above 5 mg L^{-1} in the medium and high airflow reactors, while the DO in the replicates with low airflow dropped to 4.3 mg L^{-1} after 24 h. Based on the performance results (Fig. 5), incomplete nitrification (nitrite accumulation) was still observed by the (low) aeration system even when the DO values at the end of all cultures were consistently higher than 4 mg L^{-1}.

Although complete nitrification can occur at lower DO values (Campos *et al.* 2007), the DO biomass cultivation conditions have a significant impact on the capacity of nitrifiers to utilise oxygen, especially in NOB populations. Because different microbial groups consume oxygen, the species with lower oxygen affinities (high oxygen half-saturation K) may be affected during DO fluctuations. According to many studies, NOB guilds usually present higher K values than AOB and heterotrophs, which are more efficient in oxygen metabolism (Arnaldos *et al.*, 2015). Furthermore, differences in oxygen affinity within NOB species have been observed, resulting in abundance changes or shift NOB population (i.e. from *Nitrobacter*-like to *Nitrospira*-like) as a coping strategy to lower DO scenarios (Liu and Wang 2015; Fan *et al.* 2017). In our work, the 16S-rRNA analysis in Lopez *et al.* (2021) suggested that only *Nitrobacter* species were responsible for nitrite oxidation in the nitrifying biomass. As a consequence, nitrite as an

intermediate compound will likely occur due to low *Nitrobacte*r spp. activity in the cultures with inefficient aeration systems.

In the case of medium and high airflow conditions, both treatments presented high nitrate yields (0.95 ± 0.01 and 0.90 ± 0.01, medium and high, respectively), resulting in low nitrite concentrations at the end of the experiments, $Y_{NO2^-} < 0.02 \pm 0.01$. Ideally, excess aeration in the assays is preferred because higher nitrification rates will translate into shorter incubation periods. However, high airflow into the cultures may have some disadvantages. For instance, the nitrogen mass balance of the airflow test (Table 7) showed a higher difference between ammonium consumption and oxidised products (7.7 ± 0.5 %) in the high aeration case (t-test, p=0.0004); this is quite possibly due to ammonia stripping (Dempsey 2011; Bressan *et al.* 2013; Pulicharla *et al.* 2018). As a result, there was little nitrogen assimilation into biomass during this short time frame (see supplementary data). In addition, other problems were observed, such as media spillover and foaming. Ultimately, we selected aeration at medium airflow level (0.175 L min^{-1}) for batch experiments.

Table 7. Final nitrogen mass balance in the airflow test (24-hour incubation)

Airflow L min^{-1}	**DNH_4^+ (mg-N L^{-1})**	**DNO_X (NO_2^- + NO_3^-) (mg-N L^{-1})**	**Difference (%)·**
0.05	7.9 ± 1.1	7.6 ± 1.1	3.9 ± 0.2
0.175	30.0 ± 1.0	29.1 ± 0.8	2.8 ± 0.4
0.3	42.6 ± 0.6	39.3 ± 0.7	7.7 ± 0.5

· Difference estimated as (DNH_4^+-N - DNO_X-N)/ DNH_4^+-N·100

There are other examples in the literature of oxygen supply adjustments in batch inhibition assays with nitrifying biomass. For instance, (Kwon *et al.* 2019) evaluated the nitrifiers air requirements by estimating the oxygen transfer rates and nitrification efficiency under different shaking conditions (rpm) and saturating the culture media by flowing air before testing. Another study from (Phan *et al.* 2020) evaluated the short-term effect of Mn_2O_3 nanoparticles on nitrifying bacteria. These authors showed that nitrification activity in the batch inhibition assays was significantly affected by DO with and without aeration, resulting in the report of inhibition under low and high DO conditions. Other modifications in the aeration system, such as bubble diffusers and DO-controlled devices, can enhance oxygen supply in aerobic cultures. However, these solutions may substantially increase the research cost and resources (Yao *et al.* 2021).

Monitoring DO concentration during incubation is a common practice used in batch assays to verify that oxygen was not a limiting factor (ISO 9509 2006). However, factors such as low airflow, poor mixing and microbial structure in the biomass could significantly affect the oxygen transfer in the batch reactor (Arnaldos *et al.* 2015). For example, suppose online DO sensors and controlled air supply systems are unavailable, in that case, the specific biomass aeration requirements could be established by adjusting the air supply with nitrification performance tests prior to the toxicological bioassays.

3.3. Effect of Solvent on nitrification performance

Assessing the toxicological effect of chemicals with poor aqueous solubility usually requires the use of co-solvents as carriers. However, these substances may cause inhibition themselves, affecting the response of the tested organisms. Due to this, the impact of common organic solvents (DMSO, acetone and ethanol) on nitrification was studied in batch cultures. The changes of ammonium, nitrite and nitrate concentrations using different solvents, including the unamended control after 24 h, are presented in Fig. 6. According to the results, the ammonium removal was similar between all the treatments. However, the cultures spiked with ethanol exhibited more discrepancies in the total inorganic N balance (ammonium-N consumed versus NOx-N produced, > 68%) than the DMSO, acetone, and control treatments difference less than 3%. Based on this, it was observed that ethanol significantly altered the nitrification activity of the nitrifying consortium, even at lower concentrations (0.03% v/v). This behaviour may be explained due to the possible growth of other bacteria (i.e., heterotrophs) that consume organic substances as carbon sources (Du *et al.* 2003; Thomsen *et al.* 2007).

Concerning the other solvents, the Y_{NO3^-} of the DMSO and acetone cultures (0.88 and 0.86, respectively) were similar to the control cultures (Y_{NO3^-}, 0.9). These results suggested that DMSO and acetone at 0.03% v/v may be used as solvents without affecting the overall nitrification performance in batch assays with enriched nitrifying biomass. Furthermore, these results aligned with other studies with similar nitrifying strains (Papadopoulou *et al.* 2020).

These findings show the importance of solvents as part of the experimental design. Besides solubility with the toxicant, selecting the best solvent should evaluate both possible physicochemical and microbial interaction in the batch assay. This is highly relevant in enriched biomass under autotrophic conditions with ammonium as the sole energy source to suppress heterotrophic bacterial growth. There are three critical aspects in working with microbial cultures to consider: establish the solvent requirements in terms of concentration and exposure (Modrzyński *et al.* 2019), conduct solvent toxicity tests for the specific bacterial communities, and finally evaluate the possibility of solvent as a substrate source (Dyrda *et al.* 2019). Suppose

the solvent pre-tests result in limited options. In that case, a practical alternative might be adding the toxicant solution into empty batch reactors and allow the solvent to evaporate before the toxicity tests (Men *et al.*, 2017; Dawas-Massalha *et al.*, 2014).

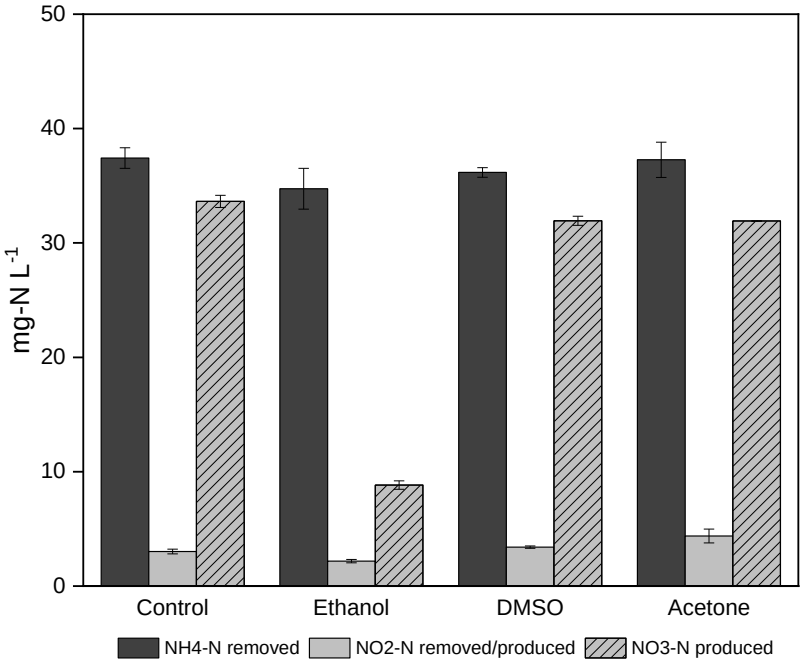

Fig. 6. Inorganic nitrogen variations using different solvents at 0.03 % (v/v). Bar represents Mean ± SD. (n=2)

3.4. Effect on biomass concentration on inhibition

In batch bioassays with liquid cultures, biomass is traditionally inoculated in bottles or flasks and diluted with nutrient media to a final concentration. However, the definition of bacterial suspension dilution and its further impact on the toxicant response is rarely evaluated in inhibition studies. Here, we compared the performance of two treatments (low and high biomass) in the presence of a well-known inhibitor. The inhibition percentage at different ATU concentrations after 24 h of incubation is shown in Fig. 7a. According to the results, the degree of inhibition increased with the ATU levels, following a dose-response pattern. In both cases, nitrification was strongly inactivated at the highest ATU concentration (0.3 mg L^{-1}). However, the sensitivity of the low biomass culture increased compared to the high case since the inhibition was 9% higher at the lowest 0.005 mg L^{-1} ATU. Based on this, we further explored the possible impact of these differences on the EC_{50} calculation. As a result, the linear regression plots from the %inhibition data (Fig. 7b) revealed that the EC_{50} values were similar between the low (0.02 mg L^{-1}) and high

case cultures (0.03 mg L^{-1}), suggesting that the differences in the inhibition response within this biomass range had a minimal effect on the final EC_{50} results.

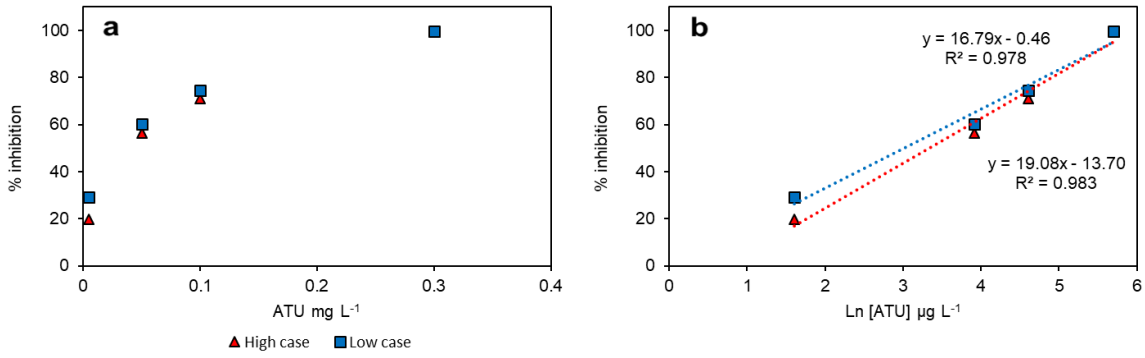

Fig. 7 inhibition level at different ATU concentrations. a. Inhibition %. b. Linear regression

These findings suggested that diluted cultures are more sensitive to the toxic compounds (higher inhibition responses) than concentrated experiments within the same type of biomass. Variation in toxicity response between different inoculum dilutions was consistent with other reports. For instance, Pagga *et al.* (2006) observed small changes in EC_{50} values while doubling the biomass concentration in the inhibition assessment of N-methylaniline in activated sludge. Moreover, Amariei *et al.* (2017) work with triclosan as a toxic agent showed that a higher biomass ratio among the cultures (16 times) could significantly differ in the inhibition response.

Comparing the ATU toxicity with other papers, the degree of inhibition reported is highly variable, depending on the biomass characteristics. For instance, the EC_{50} from our study (0.02 – 0.03 mg L^{-1}) is higher than the results reported in pure cultures (33 % inhibition at 0.025 mg L^{-1} ATU) (Grunditz and Dalhammar 2001) and low to those typical ranges reported in ISO 9509 for activated sludge (0.1 – 0.7 mg L^{-1}). As we mentioned before, these discrepancies show one of the significant challenges in nitrification bioassays while comparing different publications (Li *et al.*, 2016). Furthermore, characteristics such as the source of activated sludge, age, previous toxicant exposure, and culturing conditions can selectively favour a specific microbial consortia, resulting in a unique inoculum in a study (Dytczak *et al.* 2008; Xia *et al.* 2018; Zou *et al.* 2019). Thus, although standardised biomass seems unrealistic, evaluating the degree of inhibition through reference inhibitors (i.e. ATU) may help other researchers compare results in biomass sensitivity against other tests substances.

Regarding the biomass size, the amount of inoculum in enriched nitrifying bioassays vary from study to study, and its selection criteria are rarely reported. For activated sludge, the ISO 9509 (2006) recommends nitrification rates between of 2-6.5 mg-N/(VSS·h), which yields inoculum concentrations in the order of thousands of mg L^{-1} (VSS). The reviews from inhibition studies

with metals by (Li *et al.* 2016) and sulphide by (Bejarano Ortiz *et al.* 2013) suggested that similar biomass levels are used in experiments with nitrifiers. On the other hand, toxicological studies with enriched nitrifying cultures may allow lower inoculum quantities due to higher nitrification activities achieved during the cultivation period (Bejarano Ortiz *et al.* 2013; Giao *et al.* 2017). However, these optimisations should be carefully evaluated before the experimental phase. For further discussion of this point, we calculated the specific substrate uptake rates (mg NH_4^+-N/ g SS·h) in the control (low and high biomass case) cultures using the linear regression of ammonia profiles divided by the biomass (as SS) (Ramírez Muñoz *et al.* 2020) and the initial So/Xo ratio, where So is the initial substrate (ammonium) concentration and X_O is the initial biomass (Fang *et al.* 2009) The values from Table 8 showed that diluted cultures (low case) would result in higher So/Xo ratio (1.2 ± 0.1). According to many authors, a relatively high substrate could produce significant changes in the biomass from its original state, promoting the unwanted growth of other microbes (Spanjers *et al.* 1996; Chandran *et al.* 2008). Low (So/Xo) is preferred to prevent this issue, usually known as extant conditions, especially when kinetic analysis and respirometry technique for oxygen uptake are selected as testing protocols (Mainardis *et al.*, 2021). In our study, both So/Xo (Table 8) are considerably higher than other ratios found in the literature, such as 0.06 mg NH_4^+-N/ mg VSS used by (Phan *et al.* 2020) and 0.04 NH_4^+-N/ mg VSS in (Li *et al.* 2020a). However, the nominal cell growth and the nitrogen mass balance observed in Lopez *et al.* (2021) suggested that slow-growing nitrifying bacteria carried the ammonia oxidation with minimal interference of other microbial populations.

The low-case replicates exhibited slightly higher oxidation rates than the high-case treatments (Table 8). This behaviour could be explained considering the Monod curve model (Arnaldos *et al.*, 2015), where cultures with higher substrate concentrations may present faster growth. In our study, this difference in biomass represented a mild change in nitrification rates (within 14%) (Radniecki and Lauchnor 2011; Fang *et al.* 2009). Despite this low rate variation, understanding the impact of biomass adjustment in the bioassay is highly important, considering that these protocols are intended for short-term exposures. Based on the activity rates (Table 8), while the ammonium in the high-case test will be consumed within 24 hours, the lower-case requires four days to complete the ammonia oxidation, considering that some ammonium should remain at the end of the test for the prevention of substrate limitation (Radniecki and Lauchnor 2011; ISO 9509 2006).

Table 8. Performance of the control cultures in the biomass inhibition test

	low case	**High case**
Nitrification rate mg NH_4^+-N/ g SS·h	11.3 ± 1.0	9.9 ± 0.1
So/Xo (mg NH_4^+-N/mg SS)*	1.2 ± 0.1	0.3 ± 0.0

* SS= suspended solids TSS=VSS

Other relevant factors affecting the inoculum concentration is the number of treatments, replicates, toxicant concentration range and response analysis. For example, evaluating nitrification inhibition through the kinetic estimation of affinity constant (K) and maximum specific rates (μ_{max}) requires a series of experiments under different substrate concentrations for each toxicant level. This approach considerably increases the number of batch treatments, driving authors in some cases to reduce the amount of inoculum per culture, as it is observed in Bejarano-Ortiz *et al.* (2015) compared to other studies published within the same research group (Silva *et al.* 2011; Ramírez Muñoz *et al.* 2020). Additionally, when the fate of the toxic compound over the experimental period is included in the research objectives, more batch treatments are required to evaluate the biomass biodegradation and adsorption capacity. This approach has increased over the years, where many authors investigate the role of nitrifying communities in the co-metabolic degradation of contaminants with an emphasis on antibiotics (Du *et al.*, 2016) and other organic compounds (Silva *et al.* 2009; Trejo-Castillo *et al.* 2021)

In inhibition studies (Lopez *et al.*, 2021), the inoculum concentration was mainly driven by biomass formation per parent reactor as "master" culture to use as a standard inoculum within the replicates along with a suitable biomass range for the quantification assay. Thus, when biomass is a limiting factor, the inoculum could be adjusted to reasonable levels without significantly extending the incubation period (from hours to a few days) in balance with the sensitivity response against the toxic compound. Regarding the quantification assay, biomass in nitrifying cultures is commonly expressed as total protein and suspended solids VSS due to its relatively low cost and accessibility. However, the use of these parameters may be problematic. A study from Liang *et al.* (2010) about the biomass analysis of nitrifying biofilm and activated sludge confirmed that although proteins are the highest portion of the VSS, the protein/VSS ratio is highly variable within the samples. According to these authors, these discrepancies correlate to the efficiency of protein extraction and flocs in the biomass. Another contributing factor is the high standard deviations reported in VSS measurements; a similar issue has been observed in our studies and highlighted by other authors (Lotti *et al.*, 2014). Despite these variations, these parameters are still necessary to compare the specific nitrification activities among published studies.

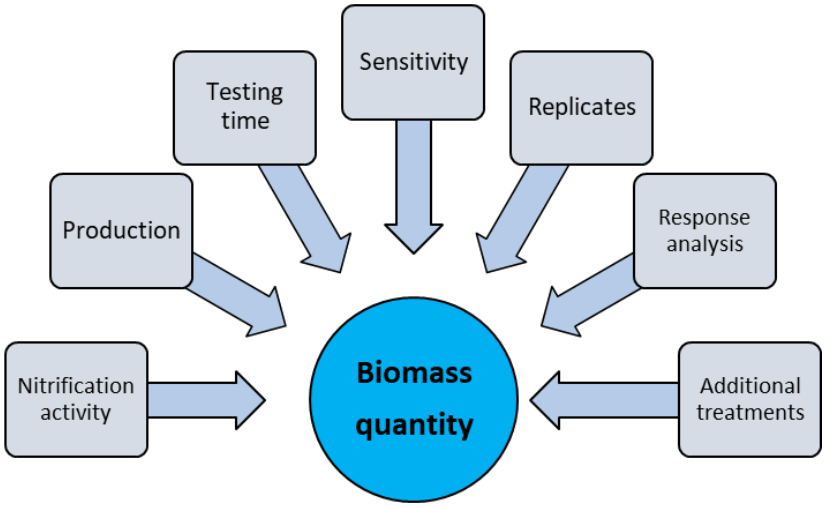

Fig 8. Common factors involved in the selection of biomass quantity for the toxicity batch assays

In summary, the review of previous methodologies and our own experience suggest that a suitable amount of biomass should be determined by the specific research needs. Nevertheless, all the factors discussed here are captured in Fig. 8, providing an overall picture of the common features that outline the experimental design of nitrification inhibition bioassays. These guidelines may represent a start point for many authors, supporting biomass optimisation strategies while working with challenging microorganisms such as nitrifying bacteria.

3.5. Effect of media on inhibition

Most bioassays studies with enriched nitrifying bacteria use autotrophic liquid media to promote the growth of AOB-NOB species. These media formulations are highly variable, usually implemented as a general methodology within the same research team. In the case of inhibition tests, the procedure ISO 9509 (2006) establishes a standard nutrient composition (Table 5, referred to as $NaHCO_3$ media) for the toxicological assessment of nitrifying activated sludge. Based on this, we investigated how the media composition could impact nitrification, using biomass cultured with a different growing media (Table 5, described as HEPES media). The results of the batch tests using these two nutrient media are presented in Table 9. In the absence of the reference inhibitor ATU, the ammonium consumption efficiency and oxidising products formed in the cultures with HEPES media were higher than the $NaHCO_3$ media after 24 h. As expected, the presence of 0.1 mg L^{-1} ATU reduced nitrification activity in both liquid media in respect to the control cultures. Similar pH changes were measured during the exposure, demonstrating that both media provided sufficient buffering to offset acidification caused by ammonia oxidation. The ATU inhibition (equation 1) was 72.3% and 73.7% for HEPES and $NaHCO_3$ media, respectively. These results show that although the lack of nutrients affected the overall nitrification activity, the media composition slightly changed the relative inhibition in the

toxicity assays. AOB/NOB species can grow in different media compositions if the culture is maintained at optimum pH levels (Koops *et al.*, 2006). However, using a different media from the one employed in the initial enrichment stage may not be recommended because it could lower the nitrifiers performance during the toxicity test.

Table 9. Results of the media inhibition test

Nutrient media	**HEPES medium**		**$NaHCO_3$ medium**	
	Control	**0.1 mg L ATU**	**Control**	**0.1 mg L ATU**
E NH_4^+ (%)	95.4 ± 1.1	28.7 ± 0.7	76.5 ± 0.2	20.6 ± 0.3
NO_x^--N (mg L^{-1}) produced	54.6 ± 0.1	15.1 ± 0.2	41.3 ± 0.4	10.9 ± 0.1
pH change	0.8	0.2	1.1	0.1
% inhibition		72.3 *		73.7 *

* From Equation 1.

4. Conclusion

Here, we evaluated multiple factors related to culture preparations that impact nitrification assays. The results demonstrated that long centrifugation/settling processes lead to unstable nitrification and low removal efficiencies. The enforced air test showed that the air supply should be adjusted to prevent nitrite build-up in the batch culture with minimum ammonia losses. From the sensitivity test of the nitrifying culture exposed to conventional organic solvents, no significant effect was observed in the nitrification activity with DMSO and acetone up to 0.03 % (v/v). The inhibition studies in the presence of ATU showed that diluted inoculum cultures might exhibit higher inhibition % compared to more concentrated cultures. However, these differences minimally impact the EC_{50} calculation in the high/low biomass ratio 5:1. Finally, the nutrient media test showed that relative inhibition % at 0.1 mg L^{-1} ATU is similar within the same liquid media composition. However, using different mineral media in the toxicity test from the original culturing media is not recommended because it could affect its nitrification capacity.

In conclusion, these results demonstrated that the biomass preparation, poor aeration, and inadequate solvent could alter the metabolic performance of nitrifying cultures and possibly, interfere with their tolerance toward toxic substances. Therefore, validation of these testing parameters should be considered in the experimental design when handling nitrifying cultures regardless of the specific research objectives. Furthermore, implementing these recommendations could support the development of acute batch assays protocols, enabling a more accurate evaluation of the nitrifying biomass, avoiding undesirable testing conditions such as incomplete nitrification, high variation in the replicates and biomass losses.

Declarations

Informed consent statement: consent was obtained from all the authors involved in the study. All authors read and approved the final manuscript.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author Dr. Charles W. Knapp (charles.knapp@strath.ac.uk) upon reasonable request.

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Chapter 4

Acute toxicity of triclosan on salt-tolerant nitrifying cultures

4.1. Preface

This chapter contains an original article for publication, targeting *Environmental Chemistry Letters*. This work investigated the acute effects of triclosan on an enriched halophilic nitrifying biomass. C. Lopez Smitter is the principal author, responsible for the bacteria cultivation, preparation of the toxicity experiments, execution of the bioassay along with the samples collection, chemical analysis, data interpretation and writing of the manuscript. C. Knapp, as project supervisor, contributed to the study's conception and provided support and assistance with reviewing the manuscript.

Additional information is presented in the appendix supplementary information Chapter 4. In this appendix, supplemental methodology was added to expand the details regarding the cultivation of salt-tolerant nitrifying bacteria and the batch toxicity assays.

Salinity can severely affect the performance of microbes in biological treatment. Due to this, the study of salt-tolerant nitrifiers has gained much attention for biological nitrogen removal systems receiving saline wastewater. Under high salt conditions, microbial communities shift as the salinity increases, where halotolerant ammonia and nitrite oxidizers become the dominant species in the biomass. Despite the importance of these microbes, the toxicological impact of contaminants on salt-tolerant nitrifying bacteria has not been extensively studied. In this work, we assessed the short-term effect of triclosan, a common antimicrobial agent detected in WWTPs, on halotolerant nitrifiers enriched from marine environmental samples. Although the work in this Chapter 4 was carried out using a different nitrifying population extracted from coastal marine samples, we applied the same recommendations captured in Chapter 3. In addition, relevant aspects observed during the cultivation of halophilic nitrifying bacteria are highlighted in the manuscript, including the comparison with other scientific publications from the literature working with similar salt-tolerant cultures.

4.2 Journal paper

Acute toxicity of triclosan on salt-tolerant nitrifying cultures

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Abstract

Salinity is a critical parameter for wastewater treatment plants (WWTPs) because it may have a detrimental effect on microbial communities in biological reactors, affecting nutrient removal efficiency. In biological nitrification, halophilic ammonia-oxidising bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are capable of metabolic activity and sustain nitrogen removal in highly saline systems. Despite their importance, acute (short-term) toxicological studies are rare among halophilic AOB/NOB communities. This work focused on the short-term effect of Triclosan, an antimicrobial chemical used in personal care products, on the metabolic activity of salt-tolerant nitrifying consortium enriched from marine sediments. After 10 months of cultivation, the AOB oxidation capacity in the biomass reached (7.1 mg-NH_4^+ $L^{-1}{\cdot}h^{-1}$ or 17 mg-NH_4^+ g-$SS^{-1}{\cdot}h^{-1}$) and the NOB activity exhibited a nitrate production of (3.5 mg NO_3^-N $L^{-1}{\cdot}h^{-1}$ or 8.4 mg NO_3^--N g-$SS^{-1}{\cdot}h^{-1}$). Due to the activity differences, nitrite accumulation was observed over the experimental period, indicating that NOB were more susceptible than AOB species. The 16S-rRNA gene sequence analysis resulted in *Nitrosomonas* sp. (abundances 1.9–2.3%) and *Nitrobacter* (abundances 1.4=1.8%) as dominate AOB and NOB species, respectively. The acute batch tests showed that Triclosan (up to 0.1 mg L-1) did not significantly affect nitrification activity after 24h of cultivation. Salt-tolerant nitrifying species can thrive under high salinity environments. Thus, these microbes are essential for the biological treatment technologies of high-salinity wastewater. However, the influence of other WWTP contaminants on halotolerant nitrifying communities is poorly understood. Therefore, this study can help the scientific community and WWTP operators to understand the response of halotolerant nitrifying communities against toxic chemicals. In addition, the challenges faced by using this type of inoculum in the toxicity tests are presented in this work as a reference for future toxicological research.

1. Introduction

Biological nitrification is an aerobic process where two microbial guilds cooperatively oxidise ammonia to nitrate. Frequently referred to as nitrifiers (Koops and Pommerening-Röser 2001; Graham *et al*. 2007), ammonia-oxidising bacteria (AOB) oxidise ammonia to nitrite, and subsequently, nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Nitrifiers species are ubiquitous in the environment, performing relevant transformations in the nitrogen cycle from natural habitat as well as engineered facilities such as wastewater treatment plants (WWTPs) (Martinez-Rabert *et al.* 2022)

Autotrophic nitrifiers are relatively slow-growing bacteria, and their growth is highly affected by different conditions (e.g., pH, temperature, dissolved oxygen (DO), salinity, nutrients levels and toxic chemicals) (Daims *et al*. 2016; Lehtovirta-Morley 2018). The impact of pollutants on nitrifying bacteria is of interest because these compounds can potentially inhibit nitrification, disrupting nitrogen removal systems in WWTPs. Guidelines for testing nitrification inhibition are established in the literature, where acute batch assays are typically applied to assess the short-term effect of pollutants on nitrifiers' activity (Brandt *et al*. 2015; Yuan *et al*. 2019). Under this approach, the enriched nitrifying inoculum is frequently used due to relatively higher nitrification activity that is suitable for metabolic assessment for hours or few days as described in our previous work on Chapter 2 (Lopez *et al*. 2021)and Chapter 3 (Lopez and Knapp 2022)

Salinity is another critical water parameter that affects the stability of biological reactors in WWTPs (He *et al*. 2017). High-salt content could cause operational problems, including poor sludge settleability, biofilm disruption, reduction of solid-liquid separation efficiency and, most importantly, alter the biomass microbial community structure (Moussa *et al*. 2006). Nowadays, many WWTPs received urban and industrial wastewater with high levels of salts (Beneduce *et al.* 2014) (Srivastava *et al*. 2021), reaching salinity levels of ~3% near seawater or even higher (hypersaline streams above 35g L^{-1}) (Wang *et al*. 2020), affecting the systems stability and nutrients removal performances.

In biological nitrification, salt is a discrimination factor among AOB/NOB populations (Koops and Pommerening-Röser 2001), and itself can inhibit the performance of non-adapted salt strains (Vendramel *et al.* 2011). Consequently, moderate halophilic, or halotolerant, bacteria become the dominant community in the biomass, leading to less biodiversity under salinity pressure (Moussa *et al*. 2006; Cortés-Lorenzo *et al*. 2015; Navada *et al.* 2020; Hüpeden *et al.* 2020). Due to this, it is recognised that salt-tolerant nitrifiers are critical to sustaining the nitrification process in saline wastewater, capable of growing and performing their function in a wide range of salt levels.

In the last decade, numerous studies have addressed strategies to improve nitrogen removal performance under salt conditions. Although nitrifiers could adapt (Bassin *et al*. 2012; Pronk *et*

al. 2014; Navada *et al.* 2019), adding halophilic nitrifiers remains the best solution for maintaining nutrient removal under saline conditions (Srivastava *et al.* 2021). These halophilic groups are capable of metabolic activity in wastewater with salt fluctuations (Gonzalez-Silva *et al.* 2016; Gonzalez-Silva *et al.* 2021) and even thrive under hypersaline conditions (Tan *et al.* 2019)

Despite the increased research around salt-tolerant nitrifiers species over the years, few studies have reported acute toxicological tests. (Sipos and Urakawa 2016; Chhetri *et al.* 2022). Since AOB/NOB guilds have prolonged generation times, it takes several months or years to obtain enough biomass with suitable nitrification activity for short-term assays (Koops and Pommerening-Röser 2001). Biomass limitation for toxicity assessment is even more critical in a saline environment due to the bioenergetic cost for nitrifiers to survive under high salt conditions (Gonzalez-Silva *et al.* 2016). On top of this constrain, halotolerant AOB/NOB co-cultures are more challenging because some NOB strains are unable to grow in laboratory bioreactors, leading to incomplete nitrification in the system (Daims *et al.* 2016)

One of the toxicants of concern is Triclosan (TCS), a broad-spectrum antibacterial agent in personal care products such as toothpaste and soaps. This chemical can persist in wastewaters; in some cases, it is not entirely biodegraded, reaching the environment through WWTPs effluents (Lv *et al.* 2014; Guerra *et al.* 2019; Komolafe *et al.* 2021). In a previous toxicity study performed in our laboratory with low salt nitrifying biomass, Triclosan caused high nitrification inhibition with concentrations above 0.01 mg L^{-1} (IC_{50} 86 µg L^{-1}) (Lopez *et al.* 2021).

This study aimed to conduct acute batch tests using salt-tolerant nitrifying biomass and assess the metabolic activity against a toxic chemical. We carried out the enrichment using marine sediments because they are a natural pool of halophilic nitrifiers relatively accessible in our laboratory with lower pathogenic microbial strains compared to WWTP sludge (Li *et al.* 2019; Gonzalez-Silva *et al.* 2021). For the inhibition batch assays, we spiked the cultures with Triclosan and evaluated the cultures' response by analysing the changes of nitrogen species over 24 hours. In addition, 16S-rRNA community analysis identified the AOB and NOB responsible for autotrophic nitrification. This paper aims to present the challenges associated with the cultivation of halophilic AOB/NOB co-cultures and understand the impact of toxic compounds on salt-tolerant nitrifiers.

2. Materials and methods

2.1. Cultivation of halophiles

Marine sediments (100 g) were collected from the west coast of Scotland (Prestwick Beach, Ayrshire 55°30'12"N 4°37'16"W), targeting the top 5-cm surface section of sediment and stored

at 4 °C before use. The salinity of the seawater was ~35 g L^{-1}, corresponding to a conductivity of 46.5 mS/cm at 20 C (pH/conductivity meter Mettler Toledo, MPC 227, Switzerland).

Initially, we followed the batch cultivation procedure described previously in (Lopez *et al*. 2021) to enhance the growth of nitrifiers under selective inorganic conditions. Briefly, 100-ml Erlenmeyer flasks (n=10) containing 50 ml of artificial seawater media were inoculated with 1 g of marine sediment (wet mass), covered with foil, and stirred at 100 -150 rpm in a shaker (Yellow line OS 2 basic, IKA®-WERKE, Germany). Next, the artificial seawater media was prepared by adding 26-30 g L^{-1} of sea salts (NutriSelect® Basic, Sigma Aldrich) to deionised water, with a conductivity of 33 ± 3 mS/cm at 20 C. Additional nutrients were added to the media according to (Bollmann *et al.* 2011) with the following composition (g L^{-1}): 0.5 $(NH_4)_2SO_4$; 0.585 NaCl, 0.054 KH_2PO_4, 0.147 $CaCl_2{\cdot}H_2O$; 0.075 KCl, 0.049 $MgSO_4{\cdot}7H_2O$, 7.21 HEPES as a buffering agent, and 1 ml of trace elements solution from Schmidt and Belser (1994). The solution was autoclaved for 20 min at 121 C, and after it cooled down to room temperature, $NaHCO_3$ (0.5 g L^{-1}) was added aseptically (filtered at 0.2 microns). Finally, the media was pH adjusted to 8.5 ± 0.2 using a 10M NaOH solution (sodium hydroxide solution, Fisher Scientific). In a first stage, the Erlenmeyer flasks were cultivated for six months at room temperature (20 -22 °C), replacing the nutrient media every 3 weeks. After that, the cultures showing nitrification activity were subsequently transferred into size 2-L glass bottles (two lab-scale reactors) for biomass enrichment (see the section supplemental methodology in the appendix supplementary information to Chapter 4 for more detail).

During the enrichment phase, aeration was continuously provided to the reactors with a rate of 0.2 L min^{-1} by an air pump (HDOM, Model HD-603, Shenzhen Hidom Electric Co., Ltd.) and filtered with a 0.2 μm sterilising-grade filter (Aervent™, Millipore, France). As a result, the dissolved oxygen (DO) concentration (Eutech Instruments Pte Ltd., DO 6+ DO/Temp, Singapore) and pH (pH/conductivity meter Mettler Toledo, MPC 227, Switzerland) were maintained above 5 mg L^{-1} and 8, respectively. The enrichment continued for ten months. Samples were regularly taken (every 2 or 3 weeks) to measure the ammonium, nitrite and nitrate levels in the cultures, estimate the AOB/NOB oxidation rates, and control the accumulation of oxidising species (see appendix supplementary information to Chapter 4 for more detail).

2.2. Toxicity experiments

The short-term effect of triclosan on the salt-tolerant nitrifying biomass was investigated using similar batch assays as described previously in Lopez *et al*. (2021) and Lopez and Knapp (2022). Briefly, 500-mL Duran bottles containing 250 mL of salt medium were inoculated with 50 ml of biomass extracted from the bench reactor, resulting in a biomass concentration ranging from 92.6 to 97.4 mg L^{-1} TSS (see table 1). After that, the cultures were spiked with TCS solution (%) using

DMSO as the solvent (final concentration of 0.01 and 0.1 mg L^{-1}). The lower and higher levels of Triclosan tested in this study were based on the inhibition response (Lopez *et al*. 2021) and values reported in the literature (Kumar *et al*. 2010). The treatments were established in triplicate, and additional bottles without the toxicant were incorporated into the experiments as controls. Finally, as a reference response, two replicates were spiked with ATU, a known nitrification inhibitor. During the experiments (24 h), samples were collected at 0h, +6h, +20 h and +24 h for chemical analysis of nitrogen species, pH, protein and TSS. The relevant parameters of the toxicity tests are given in table 1 (see the section batch toxicity assay with triclosan in the appendix supplementary information to Chapter 4 for more detail).

Table 1. Triclosan test. Values represent means and standard deviations (or range, in case of duplicates).

Parameters	**Control**	**Triclosan**	**ATU**
NH_4^+-N (mg L^{-1})	39.1 ± 0.6	39.4 ± 0.5	39.7 – 40.8
NO_2^--N (mg L^{-1})	1.9 ± 0.1	2.0 ± 0.1	1.8 – 2.0
NO_3^--N (mg L^{-1})	1.5 ± 0.2	1.5 ± 0.1	1.6 – 1.7
pH range	8.4 – 8.0	8.5 – 8.1	
Temperature (°C)	19 – 20	19 – 20	19 – 20
DO (mg L^{-1})	> 5	> 5	> 5
Protein (mg L^{-1})	7.4 ± 0.3	7.8 ± 0.4	7.1 – 7.8
TSS (mg L^{-1})	92.6 ± 2.3	95.6 ± 2.9	93.1 – 97.4
Concentration (mg L^{-1})	0	0.01 – 0.1	0.3
Replicates	Triplicate	Triplicate	Duplicate
Duration (h)	24	24	24

2.3. Chemical analysis

Ammonium and nitrite levels were measured colourimetrically using a discrete analyser (Model KoneLab Aqua 30, ThermoScientific, Aquarem 300, Clinical Diagnostics Finland) according to the British Standard procedures BS ISO 15923-1 (2013). The reagents were purchased from Thermofisher Scientific, UK. Ammonia detection is based on the modified phenol method (Berthelot reaction), where sodium salicylate substitutes phenol as a reagent. Nitrite analysis was carried out with the sulphanilamide method. Both chemical analyses may be affected by high salinity in the samples. However, these methods can be well-adapted by appropriate sample dilutions within the compound detection range, minimising the salt interference from the synthetic seawater media (Zhou and Boyd 2016; Stetson *et al.* 2019). Cuvette tests (HACH Lange LCK339, UK) were used to measure nitrate in the samples, detection range (0.23-13.5 mg L^{-1} NO_3-N) according to the UV-VIS-spectrophotometer manufacturer instructions (HACH, Model

DR 6000, Germany). This cuvette protocol is based on the dimethylphenol method, demonstrating high performance in samples with high chloride content (Ramaswami *et al*. 2017). For biomass quantification, protein content was determined using the Micro BCA Protein Assay kit (ThermoScientific, USA), following the manufacturer's protocol. Total suspended solids (TSS) were measured according to Standard Methods (APHA *et al*. 1998).

2.4. Calculations and data analysis

To monitor the AOB/NOB activity in the enriched biomass during the cultivation period, changes in oxidising species were measured for 24 h. This procedure was repeated over the ten-month incubation (approximately once every three months). The disappearance of ammonia over time was considered equivalent to the AOB activity rate, and the increase of nitrate levels due to the stoichiometric oxidation of nitrite was equivalent to the NOB activity rate in the culture (Spieck and Lipski 2011). For each activity test, linear regression was performed on the plot using NH_4^+-N or NO_3^--N concentration vs time.

The free ammonia (NH_3) concentration was calculated by Eq. (1) according to (Anthonisen et al. 1976), where Total nitrogen species TN-N was calculated as the sum of the NH_4^+-N, NO_2^--N and NO_3^--N concentrations (mg-N L^{-1}), pH and Temperature (T) as per table 1.

$$NH_3(mg\ N/L) = \frac{TN-Nx10^{pH}}{exp\left(\frac{6334}{273+T}\right)+10^{pH}} \quad \text{eq. 1}$$

For the toxicological batch test with Triclosan (TCS), we estimate the degree of inhibition using the same procedure applied in previous publications (Lopez *et al.* 2021; Lopez and Knapp 2022). We also compared the ammonia removal efficiency and the yields of nitrite and nitrate (Y, mg of NO_2^--N or NO_3^--N produced/mg of NH_4^+-N consumed]) at the end of the incubation between the batch cultures.

2.5. Microbial community analysis

Two samples were collected at the end of the experiments and stored at -80 C. First, DNA was extracted with the DNeasy UltraClean Microbial Kit from QIAGEN, following the manufacturer's protocol. After that, 16S-rRNA sequencing was carried out using Illumina MiSeq System, targeting V1 - V2 regions. Next, the raw sequences were processed using the QIIME2 pipeline (version 2021.2) (Bolyen *et al*. 2019) as previously described in Lopez *et al*. (2021). Finally, the resulted sequences were clustered into amplicon sequence variant (ASV) to analyse the community composition using the Silva138 database and report AOB and NOB abundances at the final stage of the enrichment.

3. Results

3.1. Performance of the Lab-scale reactors

Coastal marine sediments were used as the source of halophile nitrifying bacteria. Cultivation of nitrifiers is critical because enough biomass should be produced as a source of inoculum for the inhibition batch assays (Lopez and Knapp 2022). In our study, the main batch reactor was operated for more than 300 days (10 months) in the laboratory. The nitrogen levels over the experimental period are presented in Fig. S1 (see appendix supplementary information to Chapter 4), and the critical operational parameters are listed in Table 1. During the enrichment, the initial ammonia levels were maintained at 73.3 ± 22.5 mg L^{-1} NH_4^+-N, with pH above 7.3 and room temperature between 18.5 – 24 °C. Nitrite concentration increased with time as ammonia was converted through nitrification, exposing the biomass to high nitrite levels (54.0 ± 18.8 mg L^{-1} NO_2^--N). Despite this, nitrite oxidation was still observed due to the increased nitrate in the culture (228.3 ± 193.0 mg L^{-1} NO_3^--N). Except for a few days, the maximum nitrate levels reached 500 mg L^{-1} NO_3^--N, where the reactor was cleaned with fresh media to control nitrate accumulation beyond this level.

Table 2. Operating conditions of the salt-tolerant cultures

Parameters	**Values**
Initial NH_4^+-N (mg L^{-1}) *	73.3 ± 22.5
NO_2^--N (mg L^{-1}) levels*	54.0 ± 18.8
NO_3^--N (mg L^{-1}) levels*	228.3 ± 193.0
pH range	7.3 – 8.6
Room temperature (°C)	18.5 – 24
DO (mg L^{-1})	> 5
Protein (mg L^{-1}) range	24.5 ± 7.1
TSS (mg L^{-1}) range	310.9 ± 84.2
Replicates	triplicate

* Values represented as Mean ± SD

To evaluate the AOB/NOB performance, the oxidation rates were estimated by monitoring the nitrogen species for 24 h. The ammonia consumption and nitrate production over time was linearly fitted ($R^2 > 0.97$), which indicated that the two-steps nitrogen conversions followed the zero-order reaction kinetics. Nitrifier activities at the start of the cultivation are presented in Fig. 1. This procedure was applied every three months to evaluate oxidation capacity changes over the ten-month enrichment period (Fig 2). The results from the Fig. 1 show that the ammonia oxidation rate was 0.59 mg NH_4^+-N L^{-1} h^{-1} and the nitrite conversion rate to nitrate was 0.11 mg NO_3^--N L^{-1} h^{-1} at the beginning of the enrichment. Mass balances were performed, considering

the stoichiometric conversion of the nitrogen species. The differences below 5% confirm that autotrophic nitrification occurred in the batch reactor. Notably, the AOB oxidation rates were higher (five times) than NOB rates, leading to nitrite build-up in the culture.

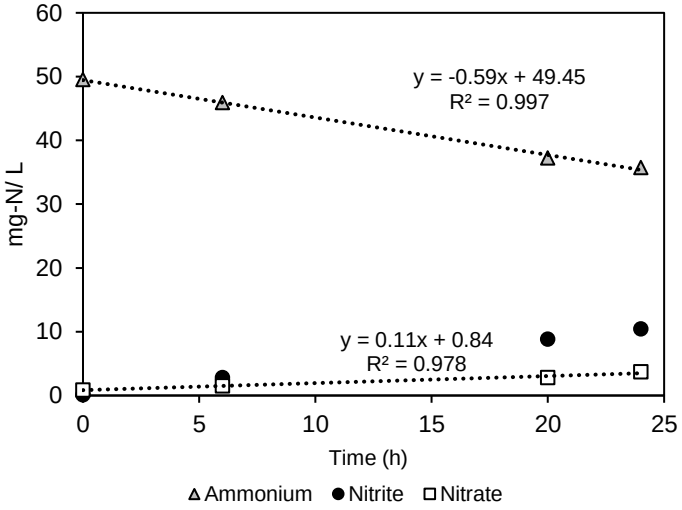

Fig 1. AOB and NOB activities at the beginning of the experimental period.

As shown in Fig 2, both AOB and NOB activities increased, reaching conversion rates of 17 mgNH_4^+-N g-SS^{-1} h^{-1} and 8.4 mg NO_3^--N g-$SS^{-1}h^{-1}$ after nine months of incubation. Although the difference between AOB/NOB activities was significantly reduced (twice), the unbalance persisted over time. All nitrogen species profiles revealed that nitrite accumulated in the reactors as long as ammonia nitrogen was present, and later on, only nitrate-nitrogen was present in the system.

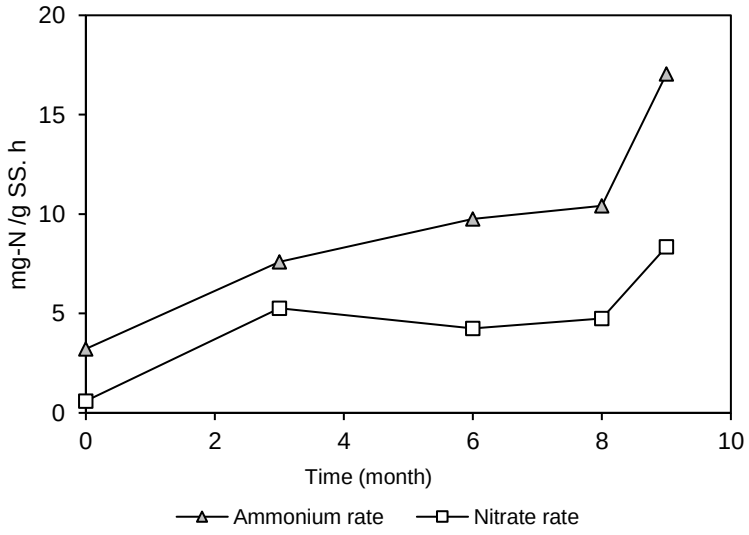

Fig. 2. Ammonia and nitrite oxidation rates during the nitrifying cultivation period.

The nitrification activity of enriched cultures can vary depending on the inoculum source, duration of the enrichment, type of reactor and operational conditions (Bassin *et al*. 2011; Lopez and Knapp 2022). These factors, together with the exposure to a wide range of salinity, make the comparison of nitrification performances across different publications challenging. Considering the incubation period, the ammonia oxidation rates in our reactor (7.1 mg NH_4^+-N L^{-1}. h^{-1} or 17 mgNH_4^+-N gSS^{-1}.h^{-1}) aligned with other halophiles cultures after eight months (Sánchez *et al*. 2005), 397 days (Cui *et al.* 2014; Cui *et al*. 2016) and 305 days of cultivation (Sudarno *et al*. 2010). However, all these studies employed different samples for enrichment. In the case of salt-tolerant microbes, popular sources are estuarine (Cui *et al*. 2014), marine sediments *(*Sudarno *et al.* 2010), industrial seafood samples (Antileo *et al.* 2002; Paungfoo *et al*. 2003), and more recently, intertidal wetland sediment (IWS) (Song *et al.* 2020; Zhang *et al.* 2022). These samples target AOB/NOB populations naturally exposed to high salinity and, in some cases, acclimatised to a rich nitrogen environment such as animal mature and aquaculture waste (Antileo *et al.* 2002).

Although halophilic nitrifiers are frequently found in these habitats (Huang *et al*. 2018; Santos *et al*. 2018; Zhao *et al*. 2019), the adaptation and further salt enrichment of AOB/NOB strains could be deficient under laboratory conditions. Furthermore, the evidence from the literature indicates that high salinity cultivation studies frequently report nitrite accumulation, suggesting that nitrite-oxidizing bacteria (NOB) are less likely to grow under lab-scale environments than AOB (Antileo *et al*. 2002; Cui *et al*. 2014; Gonzalez-Silva *et al*. 2016). However, the failure of NOB cultivation or unstable nitrite oxidation in nitrifiers co-cultures under halophilic conditions is still under debate. For instance, Cui *et al*. (2014) employed estuarine sediments as a source for nitrifying enrichment in a sequencing batch reactor SBR using commercial (synthetic) seawater. In this publication, nitrite resulted as the dominant end-product of nitrification after 140 days, and the accumulation persisted after 397 days in a follow-up study (Cui *et al*. 2016). These authors suggested that the poor NOB activity was due to the low abundance of NOB strains in the original sample. Another study by Sudarno *et al.* (2010), using coastal sediments in fixed-bed reactors (FBRs), attributed the nitrite accumulation due to pH control problems, biofilms detachment and an increase in ammonia concentration.

While the sensitivity of the NOB population to operational disturbances has been reported in the past (Knapp and Graham 2007; Campos *et al*. 2007), the resistance of NOB species to grow in saline cultures has been related to the marine medium. According to Koops and Pommerening-Röser (2001), using artificial seawater may lead to the failure of NOB when compared to natural seawater, even if sea salt is added into the media formulation. Other authors have validated this observation (e.g., Spieck and Lipski 2011). In many cases, natural seawater from the original natural sample is employed in the enrichment to favour the growth of native halophilic species. (Off *et al*. 2010; Kruse *et al*. 2013; Spieck *et al*. 2014)

Many studies have shown that attached-growth technologies are highly effective compared to dispersed (suspended) biomass reactors for saline wastewater (Lefebvre *et al*. 2005; Bassin *et al*. 2011; Srivastava *et al*. 2021). This type of reactor uses physical support where microorganisms can adhere, forming biofilms capable of holding nitrifying bacteria (Ruiz *et al.* 2020). The robust biofilm structure in these reactors allows for higher biomass retention with high nitrification capacities (Zhu *et al*. 2016; Ramaswami *et al*. 2019). Recent studies with membrane bioreactors (MBR) (Song *et al*. 2020) and moving bed biofilm reactor (MBBR) (Gonzalez-Silva *et al*. 2016; Zhang *et al*. 2022) have shown that bio-flocs can sustain the accumulation of slow-growing microorganisms such as nitrifiers. Thus, they result in a shorter acclimation with low nitrite accumulation and stable complete nitrification at the end of the enrichment period. Furthermore adding mature bio-flocs presents an effective technique to reduce even the cultivation period, accelerating the biofilm formation with halophilic bacteria (Gonzalez-Silva *et al*. 2016; Zhu *et al*. 2016; Liu *et al*. 2019). Because biomass production may constrain nitrifier studies (Lopez and Knapp 2022), these references may be relevant for toxicological studies because bio-flocs have successfully inoculated batches for acute exposure studies in high saline wastewater *(*Chhetri *et al*. 2022).

Regarding the NOB activity, the biomass in this study showed a conversion rate of nitrite to nitrate (3.5 mg NO_3^--N $L^{-1}.h^{-1}$ or 8.4 mg NO_3^--N g-$SS^{-1}.h^{-1}$) 50% below the ammonium consumption rate, with high nitrite peaks. Many factors could have intervened in the decoupling activity of AOB/NOB species during our enrichment. Firstly, the dynamics conditions of the batch reactor notably caused variability in the concentration of ammonia and oxidised by-products, affecting the nitrifying community from batch to batch. Furthermore, the accumulation of oxidising species in the culture media could significantly pressure NOB over AOB, especially in batch cultures. Many authors have reported that free ammonia (FA) inhibits the nitrification process, where NOB are far more sensitive than AOB species (Campos *et al*. 2007; Vadivelu *et al*. 2007). In our study, FA levels fluctuated from 4.7 to 41.8 mg L^{-1} (mean values, eq. 1), above the inhibition thresholds reported for NOB species (Zhang *et al*. 2018; Liu *et al*. 2019). Although adaptation to FA is possible among nitrifiers, this inhibition could lead to lower nitrite oxidation activities and even a shift of NOB communities as a coping mechanism. (Li *et al*. 2020)

Our research shows that a high substrate supply (73.3 ± 22.5 mg L^{-1} NH_4^+-N) was necessary to maximise biomass growth and nitrification activity required in short-term tests. (Lopez and Knapp 2022). This approach may be challenging with AOB/NOB co-cultures, where AOB species may adapt faster to cultivation conditions, leading to high nitrite production for NOB populations. In addition, a high concentration of nutrients in marine sediments may provide substrate at levels that are orders of magnitude greater than their natural ecosystem, posing a pressure for the nitrite oxidiser during the enrichment. Therefore, adapted biomass to high

nutrient conditions may be more suitable for acute toxicity studies, such as consortia from mature saline reactors in WWTPs. (Gonzalez-Silva *et al*. 2021; Zhang *et al*. 2022). Alternately, previous studies have reported other strategies to control unbalance of AOB and NOB activities, including a gradual increase of ammonia concentration in the co-culture or cultivation of AOB/NOB guilds in individual reactors. For example, (Haaijer *et al.* 2013) extracted biomass from the Dutch coastal North Sea, targeting the enrichment of marine nitrifiers. The authors started the incubation with less than 10 mg L^{-1} NH_4^+-N and subsequently increased the concentration to 140 ppm NH_4^+-N in seven months. This cultivation technique resulted in stable nitrification, but the final oxidation capacity remained at 1.2 mg -N L^{-1} per hour, significantly lower (6-fold) than the nitrifying biomass obtained in our study. Another research from Baskaran *et al.* (2020) used sediments samples from shrimp aquaculture ponds to enrich AOB and NOB in separate reactors fed with 70% less substrate (19 mg L^{-1} NH_4^+-N for AOB and 16 mg L^{-1} NO_2^--N for NOB). After six months, both cultures resulted in low oxidation capacity (< 0.1 mg-N L^{-1} per hour), which is unsuitable for acute testing. Despite the differences among AOB/NOB activities, our biomass reached high ammonia conversion capacity at similar levels obtained in our previous enrichment with low salt nitrifying biomass (Lopez *et al*. 2021).

3.2. 16S rRNA gene analysis results

The microbial community in the main reactor was analysed through 16S-rRNA gene sequencing, and the relative abundances of the two samples are illustrated in Fig 3. At the phylum level, *Proteobacteria* was the dominant phyla (38.6–40.1%), followed by *Bacteroidetes* (33.9–34.9%), *Chloroflexi* (10.5% in both samples), *Actinobacteria* (8.2–8.7%), *Planctomycetes* (4.9–6.8%) and others (below 2%). As mentioned previously, the cultivation conditions significantly influence the biomass nitrifying activity, and they are determining factors in the microbial population and community composition. Despite the differences, many studies reported *Proteobacteria* and *Bacteroidetes* as dominant species, including marine sediment inoculated into a sequencing batch biofilm reactor (SBBR) (Han *et al.* 2021), biofilters from marine aquaculture systems (Ruan *et al*. 2015), MBBR seeded with intertidal wetland sediment (IWS), and activated sludge (AS) (Zhang *et al*. 2022).

In the nitrifying community, the AOB guild was represented by *Nitrosomonas* sp., with an abundance of 1.9–2.3%. These AOB species belong to the family *Nitrosomonadaceae* of Betaproteobacteria phylum and the Nitrosospira and *Nitrosovibrio* genera (Arp *et al*. 2007). Most studies under high saline levels cited here reported *Nitrosomonas* clusters as the main AOB responsible for ammonia oxidation, demonstrating their adaptability to a wide range of salinity and cultured conditions. *Nitrobacter* was identified as the NOB species responsible for nitrite

oxidation, with an abundance range of 1.4–1.8%. Although *Nitrospira* is widely reported as the main NOB species in marine environments, this species was not found in our sequencing results. It is widely recognised that high nitrite levels played a crucial role in the culture's enrichment, favouring *Nitrobacter* species. (Daims *et al*. 2016; Jeong *et al*. 2018). In our study, nitrite levels (54.0 ± 18.8 mg L^{-1} NO_2^--N) influenced the prevalence of *Nitrobacter* in our reactors due to their low substrate affinity associated with rich-nitrite conditions (Daims *et al*. 2016). Other authors reported similar results in the past, working with cultures regardless of salinity levels. For instance, a previous nitrifying enrichment at low salinity conducted in our laboratory led to *Nitrobacter* as the main NOB species due to exposure to high nitrite levels in the earlier stages of the cultivation (Lopez *et al*. 2021). Another study by Tangkitjawisut *et al.* (2016) investigated the effects of nitrite concentrations on NOB communities in enrichments seeded with brackish shrimp pond sediment under saline seawater conditions. The results demonstrated that *Nitrobacter* outnumber *Nitrospira* with the increment of nitrite levels above 3 mg L^{-1} NO_2^--N after 90 days of cultivation. On the other hand, oxidation kinetics studies have revealed that halophilic NOB strains may present lower growth with high substrate affinity adapted to marine environments, where nitrite availability is relatively low compared to other terrestrial habitats (Jacob *et al*. 2017). Under this premise, the nitrite levels in our saline reactors may have affected the NOB oxidation capacity, exposing the salt-tolerant *Nitrobacter* strain beyond its tolerance substrate levels. In contrast, poor NOB performance was overcome in our previous study with a low salinity enrichment mentioned earlier (Lopez *et al*. 2021), where complete nitrification was achieved after two months of incubation. In addition to the substrate affinity, some *Nitrospira marina* will not grow with artificial seawater (Watson *et al*. 1986), and fluctuation in the reactor conditions may affect the survival of *Nitrospira* (Knapp and Graham 2007)

In general, the abundances of the AOB/NOB species were in a similar order to the values found in (Zhang *et al*. 2022), with nitrifiers abundances of *Nitrosomonas* (0.47%) and *Nitrobacter* (1.40%) and lower than values (by 10-fold) reported by (Gonzalez-Silva *et al*. 2016). The high abundance of nitrifiers spp. in the latter reference is attributed to the use of mature bio-flocs as a source of inocula, cultivated for more than five years prior to the experimental period. From the overall microbial composition perspective, nitrifiers species remained in a low proportion (abundance < 5%), whereas other microorganisms such as heterotrophs prevailed in the biomass culture even without organic carbon sources. This was consistent with most studies working with nitrifying enrichment (Cui *et al*. 2016; Gonzalez-Silva *et al*. 2016; Baskaran *et al*. 2020).

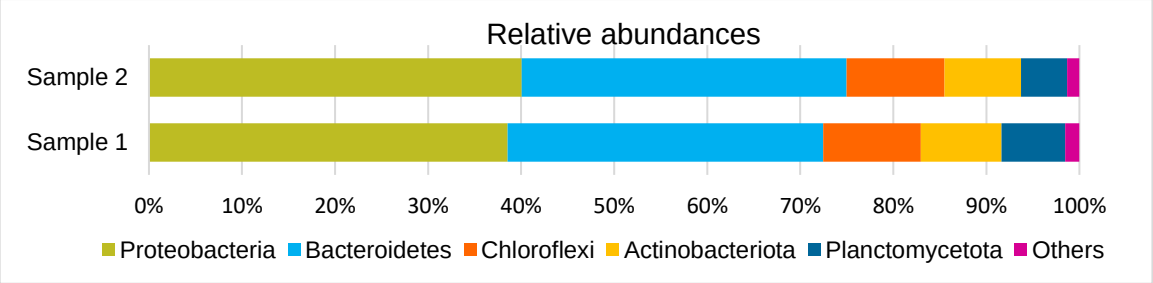

Fig. 3 Microbial community composition at the phylum level—from the main reactor.

3.3. Inhibition tests results

The impact of TCS on salt-tolerant nitrifying biomass was assessed using batch experiments over 24 hours of testing. The concentration of nitrogen species over time is illustrated in Fig. 4. The results showed that the ammonium depletion curves (Fig. 4a) followed a similar trend in all the treatments with the gradual increase of nitrite and nitrate levels (Fig. 4b), and no evident Triclosan inhibition was observed. As expected, ATU affected the nitrification activity resulting in low ammonia conversion (flatter curve in Fig 4a and 4b) compared with the rest of the cultures.

At the end of the experiments (Fig. 5), the average ammonium removal efficiency was 92.78 % in the reactors with 0.01 mg L^{-1} of triclosan, almost the same as the control (92.41%). The cultures spiked with 0.1 mg L^{-1} of Triclosan exhibited slightly lower ammonium removal efficiency (87.57%) than the controls. However, no significant differences were observed in the yields Y_{NO2} (ANOVA, $p < 0.005$) and Y_{NO3} (ANOVA, $p < 0.005$) between the cultures. All the reactors presented nitrite build-up in a range between 0.52 – 0.54 mg of NO_2^--N /mg of NH_4^+-N consumed due to the low NOB activity in the halophile nitrifying biomass as reported in Section 3.1.

Overall, the results suggested that the nitrification activity of the biomass was not affected by TCS up to 0.1 mg L^{-1}. Compared with the previous study (Lopez *et al*. 2021), TCS inhibited the biomass nitrification with an IC_{50} of 86 µg L^{-1}. In the literature, it has been reported that testing conditions (Pagga *et al*. 2006; Yuan *et al*. 2019) can significantly affect nitrifiers' response to toxic compounds. Despite that our work followed a similar methodology from (Lopez *et al*. 2021; Lopez and Knapp 2022), the addition of salt to mimic seawater levels may have hindered the toxicity capacity of TCS on the biomass. According to the study by (Aranami *et al*. 2007), salinity can greatly reduce the half-live of TCS, with a 50 % reduction in seawater matrices compared to freshwater. Therefore, it is possible that high salinity in the nutrient media (26 -30 g NaCl L^{-1}) enhanced the degradation of TCS during the test, hence, reducing its toxic effect on nitrifiers' activity. Another factor that can explain the differences in the inhibition response is the source of the testing biomass (ISO 9505 2006; Li *et al*. 2015). One example is the recent study from Chhetri *et al*. (2022), where the inhibition effect of different toxicants was evaluated with two biomasses

following the traditional and salt-adapted method #9509 for short-term nitrification tests. The results from this study reported that the bioassays with salt-tolerant biomasses exhibited greater toxicant tolerances (higher IC_{50} values) than low salinity biomasses in most of the toxic chemicals tested, suggesting that the origin of the inocula may contribute in the outcome to the toxicity response.

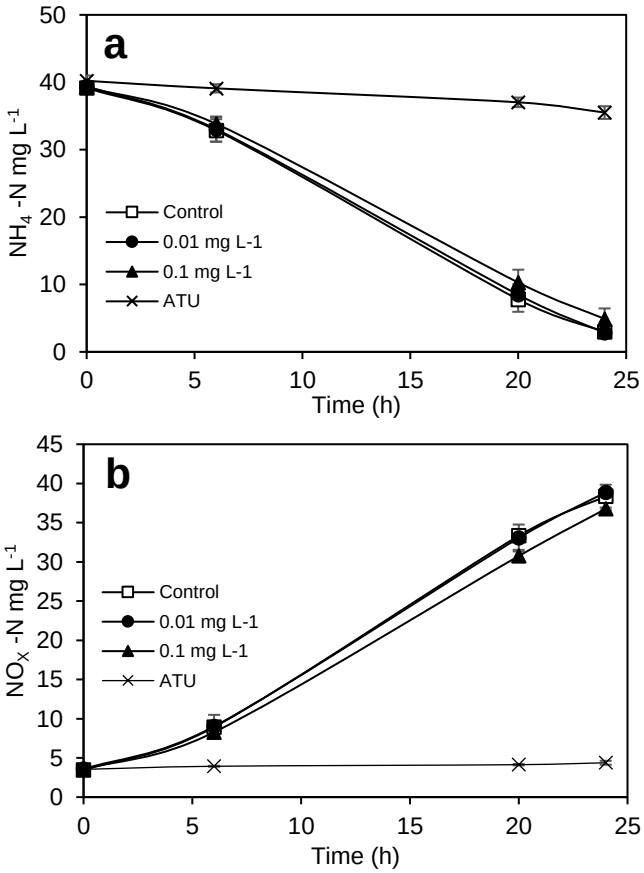

Fig. 4 Nitrogen species profiles. Inhibition test with Triclosan. a. NH_4^+-N and b. NO_X-N

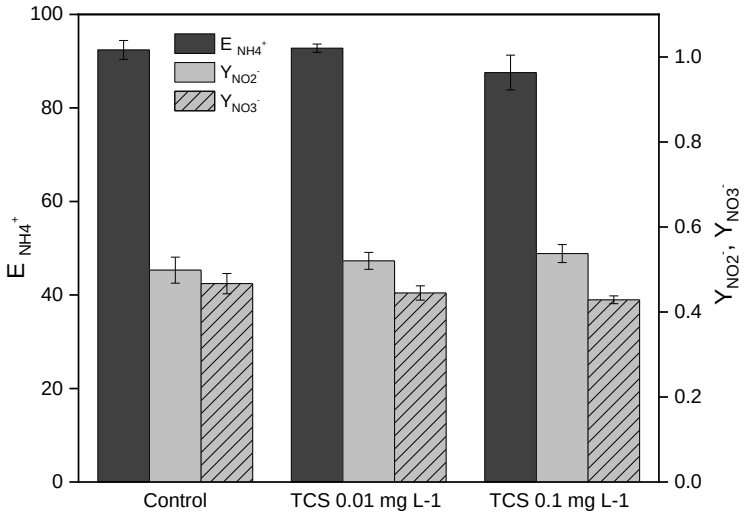

Fig 5. Ammonium consumption efficiency, Yields and concentrations of nitrite in the triclosan treatment (24 h incubation). The bar represents Mean ± SD. (n = 3).

4. Future studies

Halophilic nitrifying bacteria represent a vital consortium in the biological nitrogen removal in WWTPs under high salinity conditions. However, studies about the impact of toxic compounds on salt-tolerant nitrifiers are rare in the literature, possibly affected by the challenges related to the cultivation of slow-growing bacteria under laboratory conditions. In addition, many researchers recognise the laborious and time-consuming process of enriching biomass, extending the studies for years to achieve stable nitrification activity for bioassays lasting a few hours. These challenges seem even more critical with salt-tolerant AOB/NOB co-cultures, where the successful extraction and adaptation of halophilic strains depend on the history of samples exposed to rich-substrate and salinity environments.

On the other hand, the enrichment may have achieved high nitrification activity; however, biomass production is still a limitation (Daims *et al*. 2016; Sun *et al*. 2021). Because toxicological research relies on cultures, or enrichments, as a source for batch studies, future research should include improving cultivation strategies of halophilic nitrifiers for stable nitrification and maximised biomass production. In addition, the experimental method is required to redesign to optimise the biomass concentration and bottle sizes, including evaluating inoculum dilutions on the batch toxicity assays without affecting the test's repeatability and sensitivity.

5. Conclusion

This study investigated the impact of triclosan on halophilic nitrifying biomass using short-term exposure in batch cultures. Marine sediments were used as a source of salt-tolerant AOB/NOB species. After ten months of selective enrichment, the biomass oxidation capacity resulted in ammonia conversion of (7.1 mg N $L^{-1}.h^{-1}$ or 17.3 mg NH_4^+-N $gSS^{-1}.h^{-1}$) and nitrite to nitrate conversion of (3.5 mg NO_3^--N $L^{-1}.h^{-1}$ or 8.4 mg NO_3^--N $gSS^{-1}.h^{-1}$). The unbalance of AOB/NOB activities led to high nitrite accumulation in the medium, indicating that nitrite oxidizers are more resistant to growing in laboratory reactors than ammonia oxidisers. The 16S-rRNA gene sequencing analysis revealed that *Nitrosomonas* sp. (abundances 1.9–2.3%) represented the AOB population in the culture. In terms of NOB guilds, nitrite concentration was a strong selector between nitrite oxidisers in the biomass, resulting in Nitrobacter enrichment with abundances between 1.4–1.8%. The acute toxicological tests with Triclosan results indicated that nitrification activity of the halophilic biomass was not affected by this chemical up to 0.1 mg L^{-1} after 24 h of exposure. Due to the limited biomass production, further studies are required to improve the short-term test protocols for halophilic nitrifiers, including optimising the inoculum concentration and size of the batch bottles.

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CHAPTER 5

Conclusion and future work

1. Conclusions

This work investigated the short-term effect of individual PPCPs on the metabolic activity of two different nitrifying consortia, selectively enriched from WWTP sludge and marine sediments.

The specific research objectives of this work were outlined in Chapter 1:

- Cultivate and enrich nitrifying bacteria from WWTP samples and coastal marine sediments samples.
- Assess the effect of selected pollutants on the metabolism of enriched nitrifying bacteria from WWTP sludge using short-term batch assays.
- Assess the effect of selected pollutants on the metabolism of salt-tolerant nitrifying bacteria using short-term batch assays.
- Evaluate the experimental testing conditions employed in the toxicological assessment of nitrifying bacteria with short-term batch assays.

In this thesis, the experimental results were presented along with conclusions in each Chapter, as a self-contained manuscript. As a consequence, the main conclusions are only presented in brief here. This final Chapter summarises the key findings and the contribution of existing knowledge according to the specific research objectives listed above. The last section comprises the future work with recommendations for follow-up studies.

1.1 Cultivate and enrich nitrifying bacteria from WWTP samples and coastal marine sediments samples

1.1.1 Key findings

As described in Chapter 1, enriched nitrifying consortia were selected as inoculum for the acute toxicity bioassays. This biomass was obtained through the cultivation and further enrichment of AOB/NOB abundances in selective nutrient media using two sources: activated sludge from a WWTP and marine sediments.

The results in Chapter 2 showed that the nitrifying populations were successfully enhanced from the WWTP sample through the enrichment process, achieving stable nitrification performance with an ammonium consumption rate between 11 and 20 mg NH_4^+- N/g MLVSS h and a nitrate production yield of 0.95 mg NO_3^--N/mg after approximately six months of cultivation. In

addition, the 16S rRNA gene sequencing revealed that *Nitrosomonas sp.* (AOB) and *Nitrobacter sp.* (NOB) were the main nitrifying species in the enriched biomass.

From Chapter 4, coastal marine sediments were used as the source of halophile nitrifying bacteria. The results showed that both AOB and NOB metabolic activities increased, reaching nitrification rates up to 17 mgNH_4^+-N gSS^{-1} h^{-1} and 8.4 mg NO_3^--N gSS^{-1} h^{-1}, respectively, after nine months of incubation. However, the unbalance of AOB/NOB activities led to nitrite accumulation as an intermediate oxidising product, permanently observed during the enrichment. Similarly, the 16S rRNA gene sequencing identified *Nitrosomonas* sp. (AOB) and *Nitrobacter* sp. (NOB) as the key species responsible for autotrophic nitrification.

1.1.2 Contributions of existing knowledge

Nitrifiers are widely recognised as problematic bacteria among the scientific community, where their chemoautotrophic lifestyle is characterised by slow growth, low yields and frequent resistance to growth under laboratory conditions. Despite these challenges, here in this work, we demonstrated that it is possible to enrich nitrifiers species using lab-bench batch reactors at a relatively low cost, offering an affordable option to future researchers with limited lab resources and testing capabilities.

1.2 Assess the effect of selected pollutants on the metabolism of enriched nitrifying bacteria from WWTP sludge using short-term batch assays

1.2.1 Key findings

In this work, we evaluate the toxic effect of common PPCPs using short-term nitrification inhibition assays. The experimental results in Chapter 2 showed that the most toxic tested chemical was triclosan, with the lowest EC_{50} equal to 89.1 μg L^{-1}. Within the antibiotics group, colistin produced the highest nitrification inhibition, with an EC_{50} value of 1 mg L^{-1}, where NOB species were less impacted than AOB strains. The calculated EC_{50} values for ampicillin and ofloxacin were 23.7 mg L^{-1} and 12.7 mg L^{-1}, respectively. The insect repellent DEET caused less than 40% nitrification inhibition at the highest concentration of 10 mg L^{-1}. Lastly, caffeine had no critical inhibitory effects on the activity of AOB/NOB species.

1.2.2 Contributions of existing knowledge

Toxicological studies with nitrifying bacteria remain relatively low considering the significant contaminants found in wastewater streams. This study contributes to expanding AOB/NOB inhibition data, presenting an inhibition threshold that can be used as a reference for future research and developing better control strategies to prevent nitrification disruption in WWTPs.

1.3 Assess the effect of selected pollutants on the metabolism of salt-tolerant nitrifying bacteria using short-term batch assays

1.3.1 Key findings

The acute toxicity effect of triclosan was assessed in the halophilic nitrifying population. The findings presented in Chapter 4 indicated that this antimicrobial agent did not affect the nitrification activity up to 0.1 mg L^{-1} after 24 h of toxic exposure. In addition, the limited biomass production observed during the cultivation suggested that halophilic nitrifiers are more challenging to grow than no salt-adapted strains, especially NOB species.

1.3.2 Contributions of existing knowledge

To date, toxicity studies with halophilic nitrifiers biomasses are scarce. This work presents new inhibition data for salt-tolerant AOB/NOB species, representing an essential microbial population in nitrogen removal systems that treat high salinity wastewater. In addition, this research highlighted the challenges faced during the cultivation of halotolerant species, where stable nitrification is even more difficult to achieve in halophilic AOB-NOB co-cultures.

1.4 Evaluate the experimental testing conditions employed in the toxicological assessment of nitrifying bacteria with short-term batch assays

1.4.1 Key findings

In this study, we investigated the impact of multiple parameters on the short-term batch assays using enriched nitrifying bacteria as part of the experimental design. The findings from Chapter 3 demonstrated that the biomass cleaning procedure, poor aeration supply, and inadequate solvent and nutrient testing media could alter the metabolic activity of nitrifying cultures, affecting, as a consequence, the inhibition results.

1.4.2 Contributions of existing knowledge

The research work presented in Chapter 3 captures the lessons learned during the execution of the different toxicity experiments reported in Chapter 2. Moreover, the impact of multiple testing factors on nitrifiers' performance was assessed with experimental data, demonstrating the detrimental effects on the nitrifying inoculum. This scientific paper resulted in a summary of relevant guidelines for acute batch assays with nitrifying bacteria, helping researchers prepare for future toxicological experiments.

2. Future work

Nitrification inhibition assays have become a standard metric in microbiological toxicology studies. Their low diversity, highly recognised ecological role, straightforward and relatively simple performance assays, and sensitivity to environmental conditions are prevalent in ecotoxicology studies. The research presented in this thesis demonstrated the sensitivity of nitrifying populations to the shock exposure to some toxic compounds. This statement agrees with several toxicological assessments with nitrifiers published in the literature. Despite this, the impact of many pollutants on nitrifiers remains unexplored. Here, we offer some suggestions for future work:

- Better cultivation procedures. Biomass production is still one of the major bottlenecks in toxicity studies. The slow growth rates and low yields of nitrifiers make the enrichment and further culture maintenance significant labour-intensive. Therefore, future research should focus on improving culturing strategies and maximising biomass production with shorter cultivation periods.
- Despite the increase in research, the nature of pharmaceutical compounds and their metabolites remain untested. More fate studies and chemical analyses of pollutants are required. Some metabolites are more persistent, bioaccumulative, and toxic than the parent molecules. Chemical analysis of the toxicant remains crucial to confirm the presence of an active compound during the exposure. In addition, the measurement of the test substance provides a sense of the transformation or fate of the compound.
- Most toxicological studies with nitrifiers assess the impact of individual pollutants. However, more research is needed to test microbial activity in chemical mixtures, considering the complex chemical compositions found in WWTP effluents.
- In addition to the experimental design issues discussed above, a call is needed to better describe nitrifiers' activity in inhibition papers. In addition, the publications need to include standard features of AOB response for better comparison between studies. While there are published standards for nitrification assays, standardisation of approaches and methodologies facilitates the comparison of many studies, ultimately improving risk assessments.

Appendix

Supplementary information to Chapter 2

Table S1. Nitrogen mass balance

Substance	Concentrations (mg L^{-1})	DNH_4^+-N (mg-N L^{-1})	DNO_2^--N (mg-N L^{-1})	DNO_3^--N (mg-N L^{-1})	Difference (%)*
CF	0	51.88	29.80	21.53	1.05
	0.025	52.92	20.1	32.30	0.97
	0.115	52.12	30.91	21.21	0.07
	1	51.52	28.90	23.09	-0.91
	10	52.10	26.09	25.32	1.33
	40	51.92	27.22	23.04	3.19
	90	51.41	24.92	25.28	2.36
TCS	0	47.46	5.56	41.46	0.93
	0.01	43.59	7.24	37.15	-1.85
	0.1	24.06	6.59	17.88	-1.65
	0.3	14.34	6.95	7.88	-3.41
	0.5	14.67	6.92	7.45	2.03
	1	13.40	5.21	8.30	-0.89
	2	13.24	6.14	6.90	1.54
DEET	0	47.70	6.36	41.77	-0.91
	0.02	44.82	8.94	37.86	-4.43
	0.1	47.03	7.41	38.47	1.36
	1	35.24	14.71	19.60	2.65
	5	30.69	12.93	17.68	0.28
	10	29.49	13.80	15.73	-0.14
AMP	0	51.77	25.76	25.88	0.25
	0.5	43.94	21.89	22.00	0.11
	5	36.33	27.65	8.57	0.28
	50	19.43	13.02	6.24	0.88
	100	19.57	12.82	6.58	0.85
	175	15.89	10.49	5.70	-1.87
	250	14.41	9.87	4.49	0.33
CST	0	47.10	12.53	33.40	2.48
	0.1	44.83	13.50	30.08	2.77
	1	28.18	8.98	18.42	2.77
	10	7.61	-0.59	4.32	**
	100	3.49	-0.49	-0.02	**
	350	2.18	-0.51	0.24	**
OFX	0	46.90	9.27	39.82	-4.67
	0.01	43.88	9.95	35.81	-4.28
	0.1	41.37	13.36	27.55	1.12
	1	30.88	12.77	19.13	-3.34
	5	31.27	9.24	19.94	6.66
	10	22.36	9.48	14.10	-5.48

* Difference estimated as (DNH_4^+-N - DNO_X-N)/ DNH_4^+-N*100

** Value excluded due to low concentration of oxidising species.

16S-rRNA gene sequencing and taxonomy classification

The 16S-rRNA gene sequences analysis (V3 and V4 regions) was performed with QIIME2 (version 2021.2) with a similar workflow described by Al Ali et al. (2020). Briefly, the raw data were paired-end read merged and trimmed to remove primers, adapters sequences and low-quality reads, followed by denoising, chimaera removal, and de-replication. Then, the remaining sequences were classified taxonomically in terms of amplicon sequence variant (ASV) using the GreenGenes 16S-rRNA gene database available on the software website (www.qiime2.com). The results at the phylum level are summarized in Figure S1.

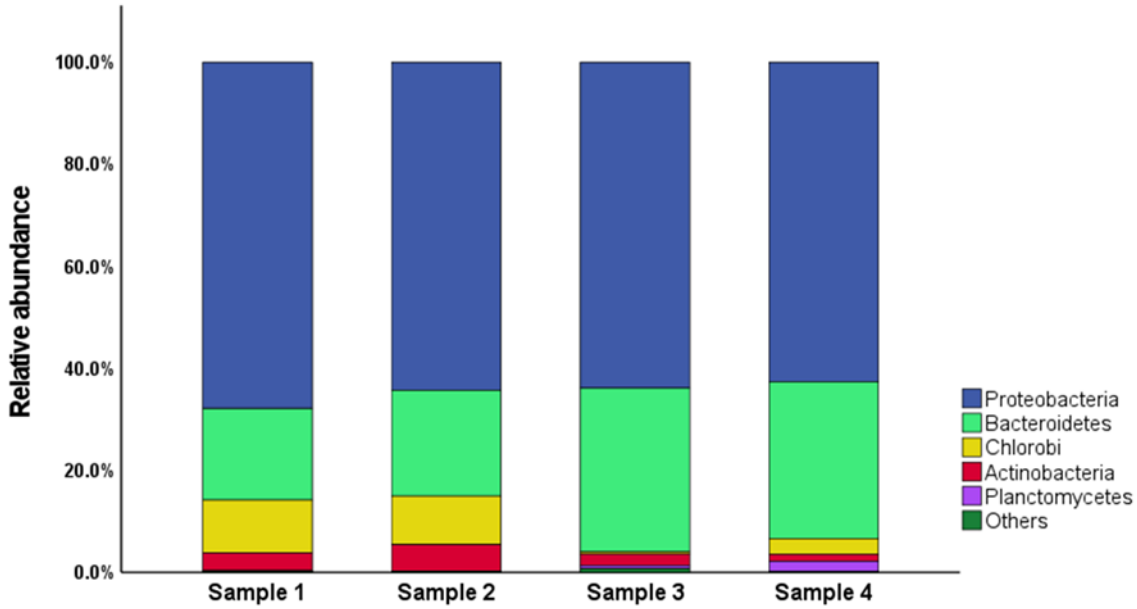

Fig. S1. Relative abundance of the total microbial community at phylum level.

Table S2. Relative abundance (total bacterial population) of nitrifying species

Nitrifying species	Average Abundance [%]			
	Sample 1	Sample 2	Sample 3	Sample 4
k__Bacteria; p__Proteobacteria; c__Betaproteobacteria;o__ Nitrosomonadales;f__Nitrosomonadaceae	4.35	4.23	5.76	5.71
k__Bacteria; p__Proteobacteria; c__Betaproteobacteria;o__ Nitrosomonadales;f__Nitrosomonadaceae;g__Nitrosomonas;	0.92	0.86		
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria;o__ Rhizobiales;f__Bradyrhizobiaceae;g__Nitrobacter;s__	0.83	0.79	0.14	0.36

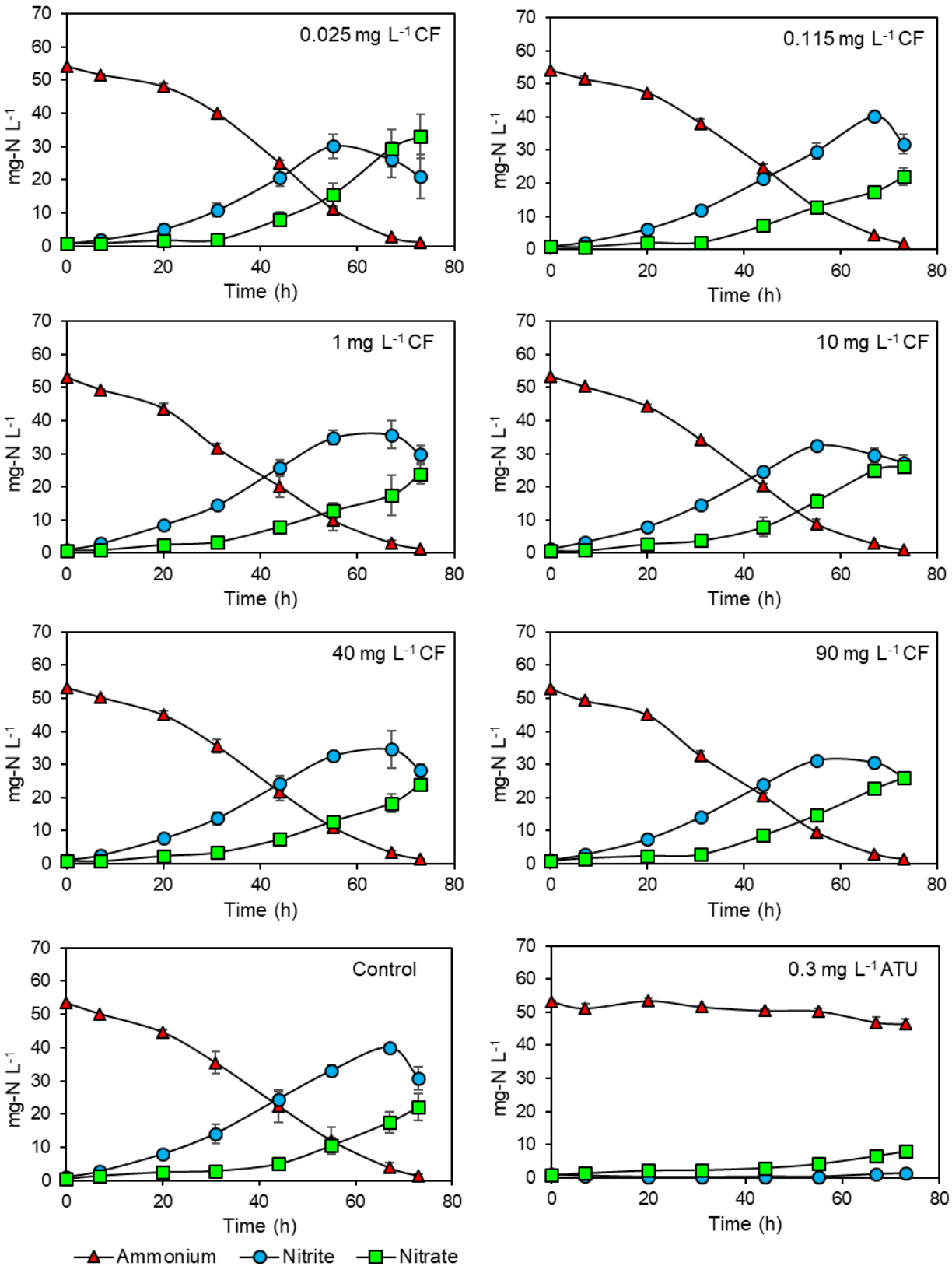

Fig. S2. Nitrification profile of nitrifying cultures in presence of caffeine (CF). Data points show average concentration ± highest and lowest values

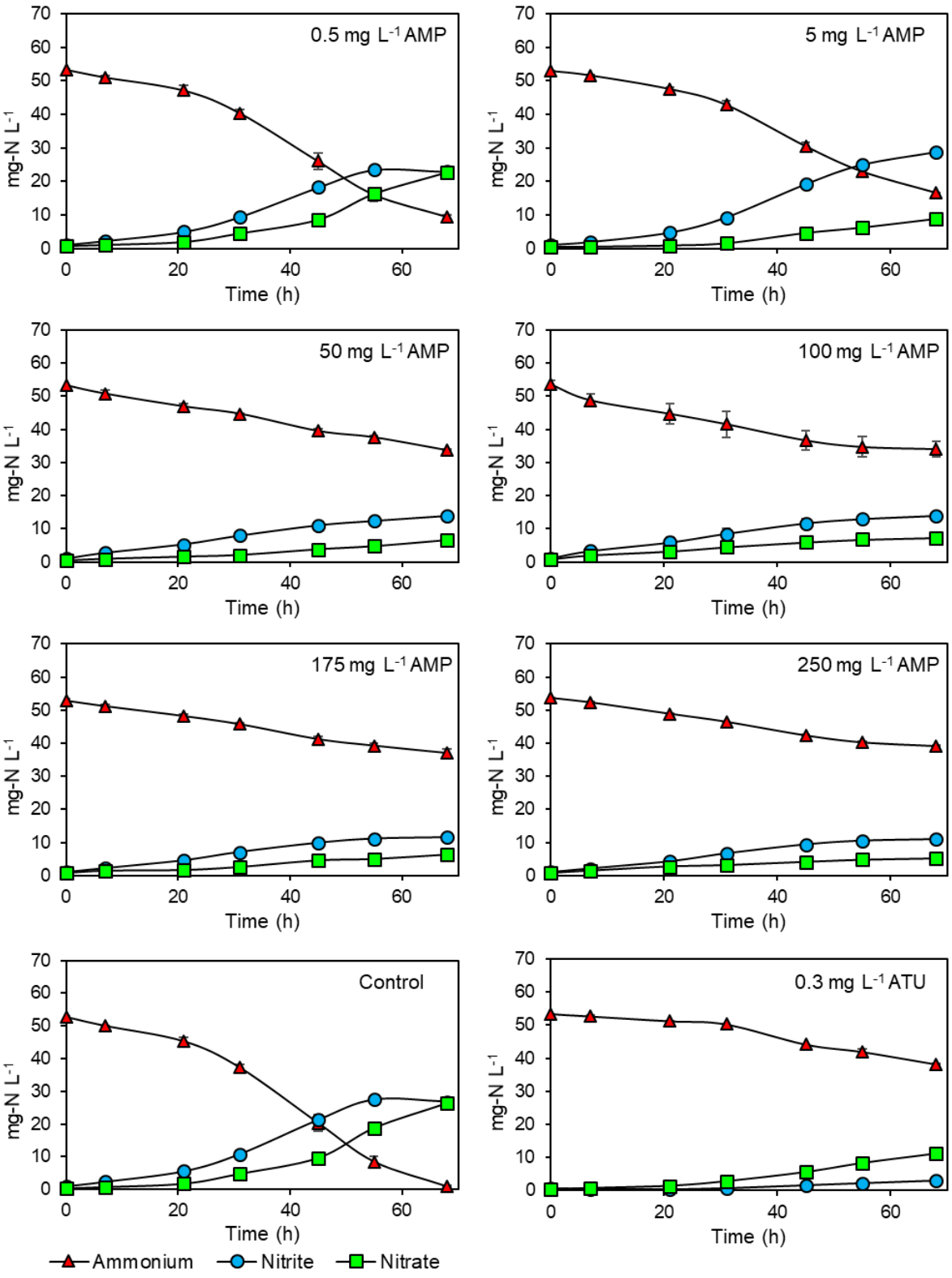

Fig. S3. Nitrification profile of nitrifying cultures in presence of ampicillin (AMP). Data points show average concentration ± highest and lowest values

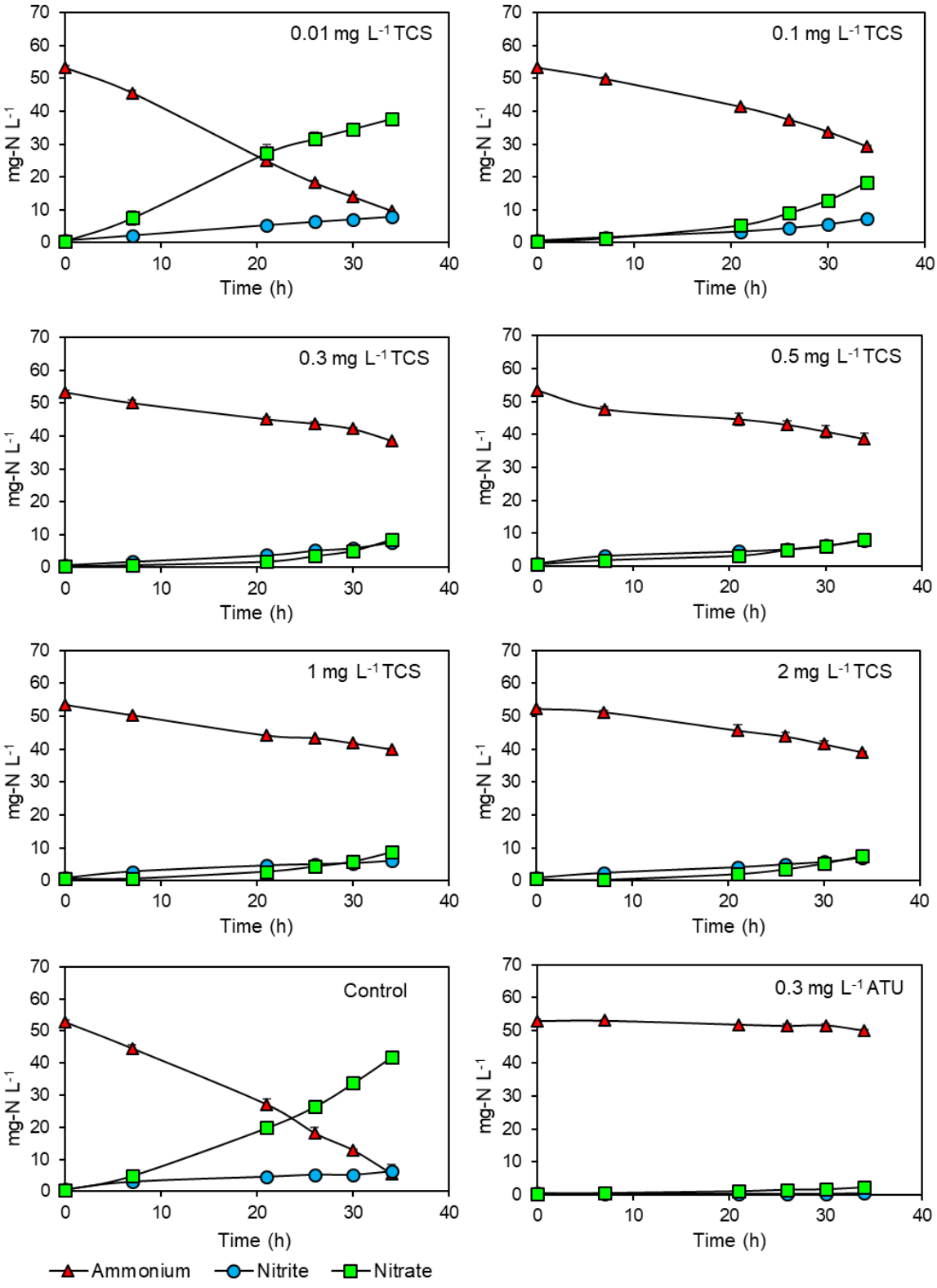

Fig. S4. Nitrification profile of nitrifying cultures in presence of triclosan (TCS). Data points show average concentration ± highest and lowest values

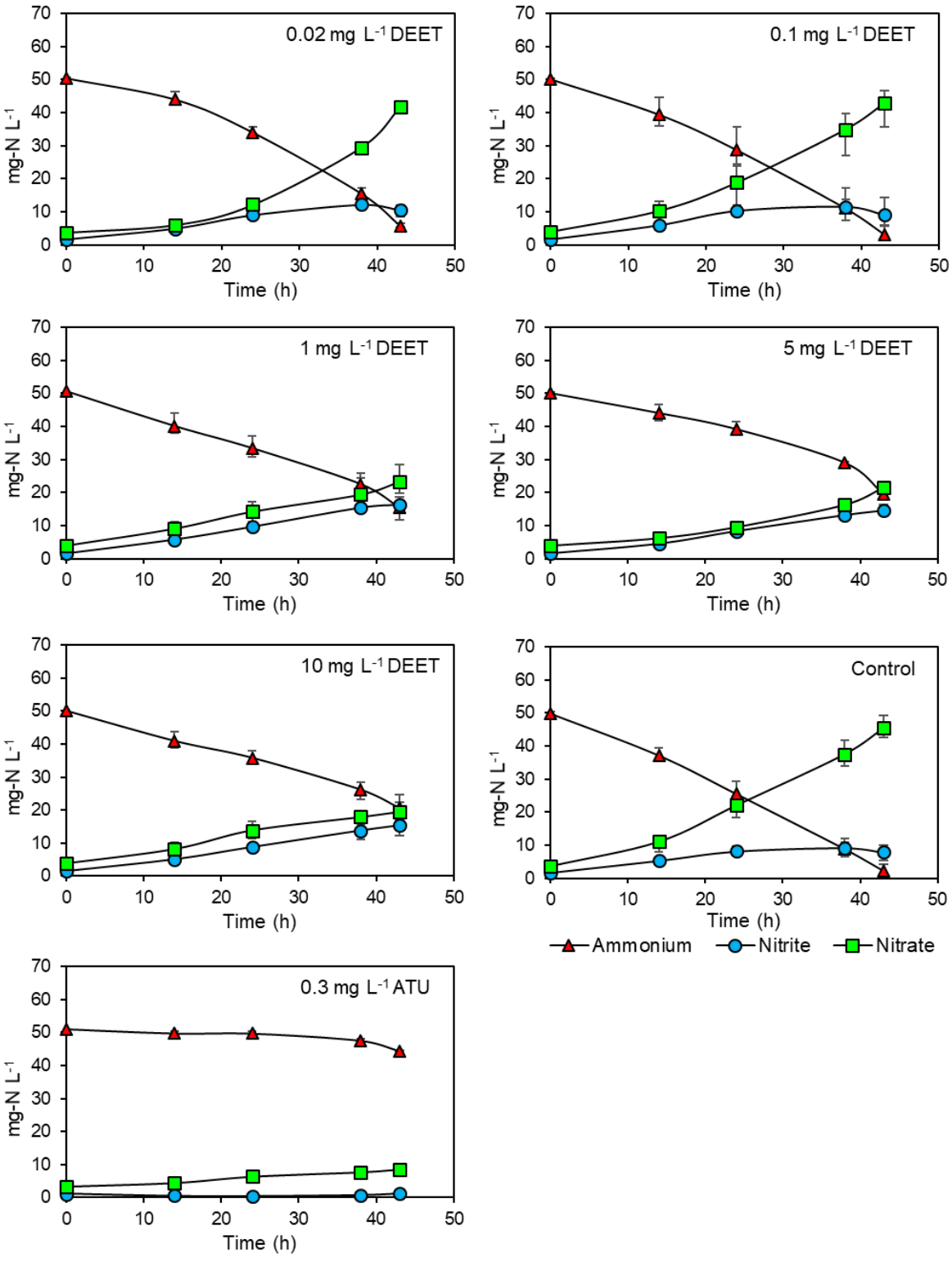

Fig. S5. Nitrification profile of nitrifying cultures in presence of DEET. Data points show average concentration ± highest and lowest values

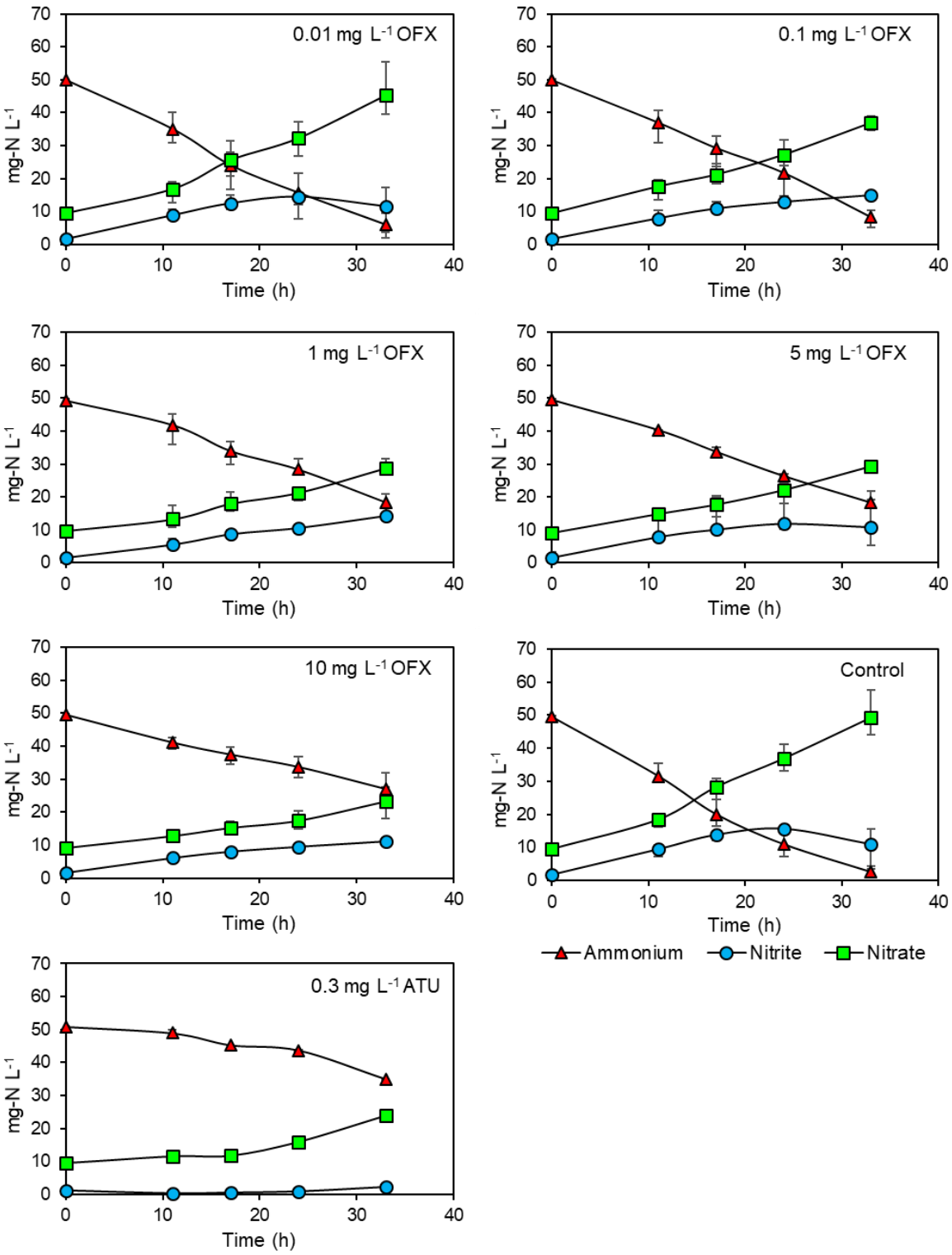

Fig. S6. Nitrification profile of nitrifying cultures in presence of ofloxacin (OFX). Data points show average concentration ± highest and lowest values

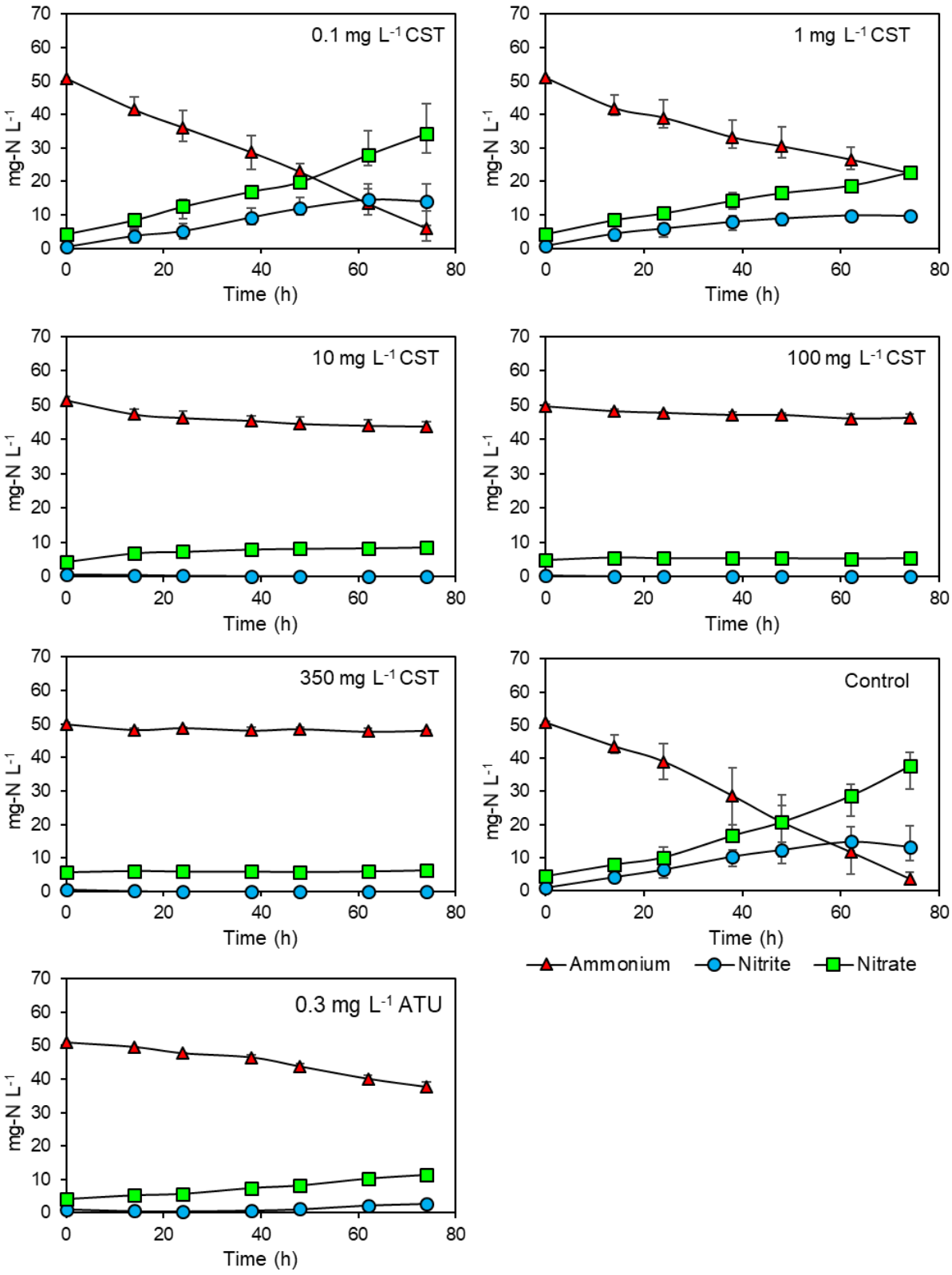

Fig. S7. Nitrification profile of nitrifying cultures in presence of colistin (CST). Data points show average concentration ± highest and lowest values

References

Al Ali AA, Naddeo V, Hasan SW, Yousef AF (2020) Correlation between bacterial community structure and performance efficiency of a full-scale wastewater treatment plant. J Water Process Eng. 37: 101472. https://doi.org/10.1016/j.jwpe.2020.101472

Supplemental methodology

Cultivation of nitrifying bacteria

The cultivation and enrichment of nitrifying bacteria were carried out following the batch cultivation methodology presented by Bollmann et al. (2011) and the recommendations suggested by Radniecki and Lauchnor (2011). The first step includes preparing of the nutrient media to sustain the growth of AOB/NOB bacteria. Glass bottles (1 L or 2 L volume capacity) with screw caps suitable for autoclaving were used for the liquid media. To prepare 1 L of media, dissolve the chemicals listed in table S3 one after the other in deionised water, except the KH_2PO_4 solution and the $NaHCO_3$. Next, autoclave the media and the KH_2PO_4 solution separately for 20 min (at 121 °C). After the solutions have cooled down to room temperature, add 10 ml of KH_2PO_4 solution and 0.5 g L^{-1} $NaHCO_3$ to the sterilised media (using the same media to dissolve the chemical and filtered with a 0.45 μm cellulose filter). Subsequently, adjust the pH to 7.6 ± 0.2 (pH/conductivity meter Mettler Toledo, MPC 227, Switzerland) with a 10 M NaOH solution (Fisher Scientific). The media can be stored at 4 °C up to one-week prior to use.

Table S3. Mineral growth media

Ingredients	Concentration
$(NH_4)_2SO_4$	0.5 g L^{-1}
NaCl	0.585 g L^{-1}
$CaCl_2 \cdot H_2O$	0.147 g L^{-1}
KCl	0.075 g L^{-1}
$MgSO_4 \cdot 7H_2O$	0.049 g L^{-1}
Trace metal solution	1 ml
HEPES Buffer	7.21 g L^{-1}
KH_2PO_4 solution	10 ml (5.45 g L^{-1})
$NaHCO_3$	0.5 g L^{-1}

The trace elements solution was prepared according to the ingredients listed by Schmidt and Belser (1994) for nitrifier media. To prepare 1 L of solution, dissolve the compounds of table S4 in deionised water (one after the other). After that, sterilise the solution by filtration (0.45 μm cellulose filter) and store in the dark at 4 °C. This solution can be used for several years.

Table S4. Trace metal solution

Ingredients	Concentration g L^{-1}
Na_2MoO_4-$2H_2O$	0.1
$CoCl_2$-$6H_2O$	0.002
$MnCl_2$	0.2
$ZnSO_4$-$7H_2O$	0.1
$CuSO_4$-$5H_2O$	0.02

Serial dilutions were used as the cultivation technique for the enrichment of nitrifying bacteria, as described by Bollmann et al. (2011). The description of the procedure is presented below. Initially, 1 g of the sample was inoculated in 100-ml Erlenmeyer containing 50 ml of sterile liquid nutrient media to harvest AOB/NOB guilds. The flasks (n=4) were sealed with cotton wool stoppers wrapped in aluminium foil and placed in a mechanical shaker at 120 rpm (Yellow line OS 2 basic, IKA®-WERKE, Germany) at room temperature. After 15 days, 5 ml of the cultured media was transferred into 45 ml of fresh media to remove the solids from the activated sludge. Subsequently, repeated transfers were carried out with a total of 10 cycles.

During the incubation, the growth of AOB/NOB in the cultures was monitored visually by using colorimetric reagents. In the case of AOB, the oxidation of ammonia was observed using Nessler reagent (HACH, Germany). This reagent (K_2HgI_4) reacts with the ammonia in the sample to form a yellow-brownish coloured compound, where the intensity of the colour is in direct proportion to the ammonia concentration. Weekly samples (1 ml filtered with a 0.45 μm cellulose filter) were taken to monitor ammonia using the 96-well plates format (250 uL sample/well) following the manufacturer instructions, where the change of colour (from yellow-brownish to light yellow) indicated that ammonia was consumed in the culture. In addition, test strips from (Aquachek, HACH, Germany) were used to observe nitrite concentration and nitrate production in the sample to monitor the NOB activity. These strips are paper-based tests with two reagent spots (one for nitrite and another one for nitrate) that reacts with these oxidising products by changing colour to pink. The range of detection is nitrite (0 - 3 mg L^{-1}) and nitrate (0 - 50 mg L^{-1}). Weekly samples (1 ml filtered with a 0.45 μm cellulose filter) were taken to monitor the nitrite/nitrate concentration following the manufacturer's instructions.

After 3 months (10 cycles of serial transfer), the cultures (n=2) showing stable nitrification activity (continuous ammonia consumption and nitrate production with no nitrite accumulation) were transferred to 2 L glass bottles for further enrichment. In this stage, the 2-L reactors (n=3) with a working volume of 1.7 L operated for 8 months at room temperature (20 °C - 27 °C) connected to air pumps to maintain the cultures aerated. The air was supplied with an Air pump

(HDOM, Model HD-603, SHENZHEN HIDOM ELECTRIC CO., LTD) filtered with 0.2 μm sterilizing grade filter (AERVENTTM) to maintain the dissolved oxygen (DO) above 4 mg L^{-1} (Dissolved Oxygen meter Eutech Instruments Pte Ltd., DO 6+ Dissolved Oxygen/Temp, Singapore). Weekly samples (4 ml filtered with 0.45 μm cellulose filter) were collected to monitor the concentrations of ammonia, nitrite and nitrate using colorimetric analysis with KoneLab Aqua 30 (Thermoscientific, Aquarem 300, Clinical Diagnostics Finland). The chemical analysis was carried out according to the BS ISO 15923 "Water quality — Determination of selected parameters by discrete analysis systems" for spectrophotometric analysis with automated discrete systems (KONE Analyser). A description of the colorimetric test is summarised in table S5. In addition, weekly samples (2 ml) were collected for pH analysis (pH/conductivity meter Mettler Toledo, MPC 227, Switzerland).

Table S5. Chemical analysis method through KoneLab Aqua 30 analyser

Colorimetric test	Method	Detection range
Ammonia	Modified phenol method (Bethelot reaction)	Low level: 0.1 - 1 mg L^{-1} as NH_4^+ High level: .75 - 20 mg L^{-1} as NH_4^+
Nitrite	sulphanilamide and N-1naphthylethylenediamine dihydrochloride method (pink azo-dye)	Low level: 0.1 - 2 mg L^{-1} as NO_2^- High level: 1 - 10 mg L^{-1} as NO_2^-
Nitrate	(TON) by the hydrazine reduction method	0.75 - 50 mg L^{-1} as NO_3^-

During the enrichment in the 2-L bench reactors, the media was periodically (weekly or by-weekly depending on chemical analysis results) fed with $(NH_4)_2SO_4$ stock solution as an inorganic source of nitrogen to maintain ammonium levels up to 106 mg L^{-1} NH_4^+-N and sustain the growth of AOB populations. Moreover, the AOB oxidation of ammonia to nitrite provided the substrate required for NOB species to grow. Additionally, NaOH solution (sodium hydroxide solution 10 M, Fisher Scientific) was added to maintain optimum pH (7.6 – 7.8). The reactors were cleaned periodically (by-weekly) to remove the excess oxidising products accumulated in the media. To clean the reactors, the 2-L glass bottles were placed in the safety cabinet and left for 20 min allowing the biomass to settle in the bottom of the bottles. After that, the liquid media was discarded (70 % of total volume) and replaced with fresh media.

The nitrification activity of the AOB-NOB co-cultures increased gradually over the period of enrichment in the lab scale reactors (8 months), with an accumulation of nitrite in the first two months of operation. Eventually, the reactors achieved stable nitrification with no nitrite as an

intermediate product with an ammonium consumption of between 11 - 20 mg NH_4^+-N/g MLVSS h and a nitrate production yield of 0.95 mg NO_3^--N/mg NH_4^+-N.

Batch toxicity assays

The acute toxicity experiments were designed following the guidelines of the ISO 9509 (2006) protocols, which is an international standard procedure to assess the inhibition of nitrification of activated sludge microorganisms. The principle of the test is to evaluate the nitrification activity in the presence of the toxicant using small-scale reactors under aerated conditions and compare its performance with the control in the absence of the test substance. In addition, extra test cultures are required to inhibit the nitrifying bacteria with a known reference inhibitor to verify that the oxidation process is carried out by the AOB-NOB community. The experimental set-up for the toxicity tests is described as follows:

500-ml glass bottles with screw caps were selected for the batch tests. The screw caps were perforated to accommodate three ports for sampling connection, airflow feed and breathing exit. The total working volume of the batch cultures was 300 ml to ensure at least 10 % of the volume for samples through the experiments. The sampling requirements per batch reactor are presented in table S6. During the experiments, the sample was collected from the sample port using a 20-ml sterile Luer-Lok syringe and transferred to sterile 20-ml centrifuge tubes (filtered if required). For the inhibition calculations, it is only required the concentration of ammonia, nitrite and nitrate at the beginning and at the end of the experiments. However, intermediate samples were collected over the experimental period to plot the nitrification profile against time, as shown in the Fig. S2 (Caffeine), Fig. S3 (Ampicillin), Fig. S4 (Triclosan), Fig. S5 (DEET), Fig. S6. (Ofloxacin) and Fig. S7. (Colistin) found in this supplementary information to Chapter 2.

Table S6. Sampling requirements for the toxicity tests per batch reactor

Analysis	Sample volume (ml)	Frequency	Replicates	Collection
Chemical analysis NH_4^+, NO_2^- and NO_3^-	4	At least four times	1	initial, final and intermediate points filtered 0.45 μm cellulose filter
pH	2	Twice	1	initial and final points filtered 0.45 μm cellulose filter
Protein	1	Once	3	Final point
TSS/VSS	20	Once	1	Final point

All the glassware was cleaned (soaked overnight with a 10% HCL solution) and sterilised at 121°C for 20 min to avoid contamination. An example of the preparation of the batch reactors is

presented in table S7 (Ofloxacin test). Initially, each batch reactor was filled with new media and the bacterial inoculum in the safety cabinet with the volume listed in table S7. Fresh medium was prepared the day before the test according to the procedure presented in the cultivation of nitrifying bacteria section (supplementary methodology - Chapter 2 tables S3 and S4) with the exception of the amount of ammonium. According to ISO 9509 (2006), the concentration of ammonium-N should not exceed 56 mg L^{-1} to prevent the ammonia assimilation by heterotrophic bacteria during the test. Due to this, the ammonium level in the nutrient media was adjusted to 0.264 g L^{-1} $(NH_4)_2SO_4$ (equivalent to 56 mg L^{-1} NH_4^+-N) for the short-term tests.

For the inoculum preparation, the biomass was extracted from the 2 L bench reactors, using one 2-L stock reactor per test substance. 50-ml sterile syringes were used to extract the biomass from the bottom of the stock reactor through the sampling port and transfer into 50-ml sterile centrifuge tubes. Before inoculation, the biomass was cleaned to remove the excess oxidising products accumulated during the harvesting. The cleaning procedure is explained in detail in Chapter 3 section 2.2.1 Inoculum cleaning test, where method 1: (long) was used on the first three substances (CF, AMP and TCS) and method 2: optimised was applied to the last three chemicals (DEET, CST and OFX). After cleaning, the inoculum was homogenised in a sterile bottle and filled with fresh media with enough volume to cover all the treatment cultures (50 ml bacterial suspension per bottle) (see table S7). The (50 ml) inoculum was transferred to each batch reactor using sterile centrifuge tubes.

Table S7. Example for preparation of the acute toxicity test- Ofloxacin

No. treatments	1	2	3	4	5	Control	ATU
Fresh medium (ml)	246	246	246	246	246	250	246
Inoculum (ml)	50	50	50	50	50	50	50
Toxicant stock solution (mg L^{-1})	0.8	8	80	400	800	0	24
Toxicant stock volume (ml)	3.8	3.8	3.8	3.8	3.8	0	3.8
Toxicant final concentration (mg L^{-1})	0.01	0.1	1	5	10	0	0.3
Total Volume (ml)/bottle	300	300	300	300	300	300	300
No. of bottles	3	3	3	3	3	3	2

During the experiment, the cultures were continuously aerated with humidified air through stones located at the bottom of each bottle. An example of the toxicity test (Ofloxacin) is shown in figure S8. The air supply was controlled with an air pump, maintaining the DO above 4 mg L^{-1} and the airflow in the cultures was adjusted to 0.175 L min^{-1} with an airflow meter (Brooks Instrument Model # MR3A12BVBN, USA). After all the batch reactors were assembled, each culture was

spiked with the toxicant according to the range of concentration selected for each test substance. Additionally, control reactors and cultures with reference inhibitor Allylthiourea (ATU) (see table S7 for Ofloxacin example) were incorporated into the experiments as per the recommendations of the ISO 9509 (2006)

In this work, the duration of the acute toxicity tests was extended up to 2-3 days in contrast to the few hours proposed by the protocol ISO 9509. The duration of the tests between the toxicant was affected by the limited biomass production in the harvested reactors, the nitrification activity of the biomass at the moment of the extraction, the cleaning procedure applied and other parameters such as temperature. All these factors had an impact on the duration of each experiment, where the shortest test was observed with Triclosan (34 h) and the most extended test was Colistin (74 h).

Fig. S8. Acute toxicity test set-up for Ofloxacin.

Standard Operation Procedure (SOP)

Determination of Ammonia by Colorimetric (Using Discrete Analyser Kone)

1. SUMMARY

The analysis of ammonia and ammonium ion content in water is based on the modified phenol method (Bethelot reaction), where Sodium Salicylate substitutes the phenol. (Zhou and Boyd, 2016). In this case, Ammonia in the sample reacts with hypochlorite, which is produced by hydrolysis of sodium dichloroisocyanurate to form monochloramine at alkaline conditions. This compound reacts with salicylate ions catalysed by sodium nitroprusside, forming a blue-coloured compound in proportion to the ammoniacal nitrogen concentration. Moreover, Trisodium Citrate is added to minimise the interference of cations (calcium and magnesium). Finally, the absorbance is measured spectrophotometrically at the wavelength of 660 nm.

This procedure was developed according to the BS ISO 15923 *"Water quality — Determination of selected parameters by discrete analysis systems"* for spectrophotometric analysis with automated discrete systems (KONE Analyser)

2. REQUIREMENTS

INSTRUMENTS/MATERIALS	REAGENTS
• KONE Analyzer • 4 ml KONE samples cuvettes • 2 ml KONE standards cuvettes • Multicell cuvettes KONE • KONE Segments • KONE Waste bag/Container • Volumetric flasks (Glass, 100ml) with suitable stoppers • Centrifuge tubes (15 ml) • Syringes Luer-Lok (10 or 20 ml) • Syringe filters (MF-Millipore MCE Membrane 0.45 µm)	• Ammonia 1 Reagent. Precision by Thermo Fisher Diagnostics • Ammonia 2 Reagent. Precision by Thermo Fisher Diagnostics • Ammonium Standard for IC. 1000 mg/l ± 4 mg/l. Sigma-Aldrich • Nano-pure-de-ionised water (n-DW) • Distillate water • Samples • Washing cleaner solution KONE

3. APPLICATION

- Sample type: drinking, ground, surface and wastewater. (Thermo Fisher Scientific ,2015)
- Sample pH range: 5 – 9. A pH correction is required for samples outside this range. (BS ISO 15923-1:2013)

- The relevant COSHH and Risk Assessment should be read before proceeding with this method.

4. ANALYTICAL RANGE

KONE discrete analyser has been programmed to analyse two ranges of concentrations according to the table below. The selection of the method will depend on the ammonia/ammonium concentration present in the samples. If the concentration is unknown, run the samples for the NH_4^+ Low level. The samples can be diluted manually. Also, the equipment can dilute the samples 10-fold during the analysis.

Table 1. Ammonia Analytical Range

Method	Range (mg L^{-1} as $NH4^+$)	KONE Method ID
NH_4^+ Low Level	up to 1	NH4
NH_4^+ High Level	0.75 - 20	NH4-HI

5. INTERFERENCE

- Magnesium and Calcium cations could interfere with the analysis. However, Trisodium Citrate is added to minimise interference.
- Primary amines could affect the reaction.
- Saline waters may require dilutions.
- Interference analysis should be assessed prior to the analysis (Matrix effect test)

6. SAMPLING AND SAMPLE PREPARATION

6.1. Transfer the sample into labelled 15ml centrifuge tubes using Syringe filters (0.45 µm) assembled into syringes.

Note 1: It is recommended to fill the sample tubes as much as possible to reduce the head space above the liquid and reduce ammonia evaporation. (Thermo Fisher Scientific, 2015)

6.2. Samples outside the suitable pH range (5 – 9) require pH adjustment.

6.3. Dilute the samples with Nano-pure-de-ionised water (n-DW) if it is required

6.4. Samples should be analysed as soon as possible after collection. Otherwise, samples could be stored at 5 ± 3 °C, for 14 days for Ammonium.

Note 2: For other compounds of interest such as Nitrate and Nitrite, samples could be stored at -20 °C for 8 days (BSI EN ISO 5667-3:2018)

6.5. Prepare a blank with water in the same way as the sample. (BS ISO 15923-1:2013)

6.6. Prepare the Control AQC Standard Solutions according to section 8.

7. PREPARATION OF CALIBRATION SOLUTIONS

7.1. Prepare an intermediate stock solution using the Ammonium Standard for IC (1000 mg L^{-1}) to a 100 ml volumetric flask and fill up to the mark with DW. (See table 2 for NH_4^+ low level)

7.2. Prepare the Calibration Standards using the intermediate stock solution (See table 2 for NH_4^+ low level)

7.3. In the case of analysis of NH_4^+ at high level, prepare the calibration standards according to table 3.

7.4. After preparation, transfer the solutions to labelled 15ml centrifuge tubes.

7.5. Storage the volumetric flask with the rest of the calibration solutions according to the stability instruction presented in tables 2 and 3.

Table 2. NH_4^+ Low level solutions

KONE Standard Code Position	NH4+, ppm	Stock solution, ppm	Flask Volume, ml	DF	Stock Volume, ml	Media	Stability
Intermediate	100	1000	100	10	10.000	n-DW	1 month at 5 ± 3 °C
S0	blank						
S6	0.1	100	100	1000	0.100		1 day
S10	0.25	100	100	400	0.250		1 week
S11	0.5	100	100	200	0.500		1 week
S12	0.75	100	100	133	0.750		1 week
S13	1	100	100	100	1.000		1 week

Table 3. NH_4^+ High Level solutions

KONE Standard Code Position	NH4+, ppm	Stock Solution, ppm	Flask Volume, ml	DF	Stock Volume, ml	Media	Stability
S0	blank					n-DW	
S6	20	1000	100	50	2.000		1 month at 5 ± 3 °C

8. PREPARATION OF CONTROL AQC STANDARD SOLUTIONS

8.1. Use the Ammonium Standard for IC (1000 mg L^{-1}) to prepare two independent (by another source) AQC Standard Solution considering the lower and the upper third of the calibrated working range (see table 4 for NH_4^+ low level and table 5 for NH_4^+ high level)

8.2. After preparation, transfer the solutions to labelled 15ml centrifuge tubes.

Table 4. NH_4^+ Low level AQC Standard Solutions

Description	Standard, ppm	Stock Solution, ppm	Flask Volume, ml	DF	Stock Volume, ml	Media	Stability
AQC Stock Intermediate	10	1000	100	100	1.000	n-DW	1 month at 4 C
High AQC	0.75	10	100	13	7.500		
Low AQC	0.1	10	100	100	1.000		1 day

Table 5. NH_4^+ High Level AQC Standard Solutions

Description	Standard, ppm	Stock Solution, ppm	Flask Volume, ml	DF	Stock Volume, ml	Media	Stability
High AQC	10	1000	100	100	1.000	n-DW	1 month at 4 C
Low AQC	0.75	10	100	13	7.500		1 day

9. KONE ANALYZER START UP

9.1. The KONE Analyser should be in "stand by" mode after the end of the working day. To start the system, press the "start up" button on the Main screen of the KONE software.

9.2. Ensure the KONE waste bin (with the waste bag) is in place.

9.3. Check the wastewater and the distilled water containers.

10. ANALYTICAL PROCEDURE

Before starting the analysis, fill out the form including the sample type, analysis type, date, samples ID and others if it is applicable (dilutions, pH).

10.1 Calibration

10.1.1 Transfer the calibration solutions to the 2 ml cuvettes.

10.1.2 Analyse the calibration solutions (section 7) according to the equipment manufacturer's instruction for the level of interest (low and/or high NH_4^+).

Note 3: Each calibration solution has a fixed position in the KONE equipment carrousel. Place the calibration solutions according to the analytical method range (NH4 or NH4-

HI). If the two methods have the same position for calibration solutions, they must be run separately)

10.1.3 Confirm the calibration curve before running the samples. The calibration is not accepted if the factor is out of the range, according to table 6. In addition, the minimum correlation coefficient is 0.995.

Table 6. Calibration criteria for KONE analyser

Method	Analytical Range	Calibration Factor
NH4	< 1 mg/L as NH_4^+	2.185 - 3.125
NH4-HI	0.75 - 20 mg/L as NH_4^+	16.877 – 24.449

Note 4: If the calibration is not carried out daily, verify the validity of the calibration by analysing the lower and the upper third from the standard calibration range. For NH_4^+ low level, run 0.1 and 0.75 mg L^{-1} NH_4^+. For high NH4 level, run 0.75 and 10 mg L^{-1} NH_4^+. The difference error between the measurements should be below 5 %.

10.1.4 Run the blank to check any carryover.

10.1.5 Run a calibration as a sample (4 ml cuvettes) and compare with the new calibration (0.75 mg L^{-1} NH_4^+ for low level) and (10 mg L^{-1} NH_4^+ for high level). The difference error between the measurements should be below 5 %. If the values are not satisfactory, identify the possible factors affecting the results and repeat the calibration.

Note 5: The sample matrix could interfere with the calibration, especially with samples from other applications such as liquid media for bacteria growth. It is recommended to carry out a matrix effect test by preparing the calibration solutions (see section 7) using the sample matrix considering other conditions like dilutions, pH)

Note 6: The minimum sample volume should be 1.2 - 1.5 ml. (Using 4 ml cuvettes)

10.2 System Quality check

10.2.1 After the calibration, transfer the AQC solutions to the 4 ml cuvette and place them in the segments (14 places/segment) and load them into the KONE Analyser. The AQC solutions for both low and high levels of ammonia are presented in section 8. The difference error between the measurements should be below 5 %.

10.2.2 Run the blank and matrix blank as samples. Verify if there is an effect on the results due to the sample matrix.

10.3 Sample Analysis

10.3.1 Transfer the samples into 4 ml cuvettes and place them in the segments (14 samples/segment).

10.3.2 Analyse the samples according to the manufacturer's instructions.

10.3.3 After 20 samples, analyse the first sample and one AQC (second AQC optional). The difference error between the measurements should be below 5 %.

10.3.4 Carry out a calibration Drift Check Standard (0.25 mg L^{-1} NH_4^+ for low level and 10 mg L^{-1} NH_4^+ for high level).

10.3.5 Continue with the second batch of samples (an example of the sequence can be found in appendix 1)

10.3.6 After completing the analysis, remove the samples, the reagents and place the washing solution in a 2 ml cuvette.

10.3.7 Press Stand by button on the Main screen.

10.3.8 After the cleaning process is completed, remove the washing solution.

10.4 Sample batch sequence

The samples were run in the KONE analyser following the sequence presented in the table below:

Table 7. Sequence example for batches of 20 samples

Phase	Solution/Sample	Comments
Calibration	Calibration solutions	Daily.
	Calibration Verification	If the calibration is not carried out, analyse the lower and the upper third solution from the standard calibration range.
	UPW	Check Carryover/Water Quality
	Old Calibration standard (optional)	Comparison between new and old calibration
System check	AQC 1	From independent source (Low level)
	AQC 2	From independent source (Third upper level)
	Blank	
	Matrix	(effect of the matrix in the analysis)
Samples 1st batch	Sample 1	First sample of the 1st batch
	Sample 20	Last sample of the 1st batch
	Sample 1. 1st batch	Quality Check
	AQC 2	From independent source
	Calibration Drift Check	End of each batch of samples
Samples 2nd batch	Sample 21	First sample of the 2nd batch
	Sample 40	Last sample of the 2nd batch
	Sample 21. 2nd batch	Quality Check
	AQC 2	From independent source
	Calibration Drift Check	use an standard (middle) or AQC
Samples 3rd batch	Sample 41	First sample of the 3rd batch
	Sample 61	Last sample of the 3rd batch
	Sample 41. 3rd batch	Quality Check
	AQC 2	From independent source
	Calibration Drift Check	End of each batch of samples
Closing KONE for Stand by	Washing Solution	Cleaning the system for stand by

11. TEST REPORT

The test report should include:

- The test method used
- Identification of the samples
- Date of the analysis
- Method detection range and MDL
- The analytical results consider that Ammonium can be expressed as N or as the relevant ion form. (see table below)

Table 8. Conversion factor

Parameter	Units	Conversion Factor	Converted units
Ammonia	mg L^{-1} N	1.286	mg/l NH_4
Ammonia	mg L^{-1} NH_4	0.7778	mg/l N

12. QUALITY MANAGEMENT

The Calibration is validated daily. If the calibration is not carried out, a calibration validity check is run to verify the performance. (BS ISO 15923-1:2013)

Two levels of quality control are used: AQC solutions (independent source) and the first sample of the batch is run in duplicate at the end to verify the results. Batch: 20 samples. An example of the sequence is presented in table 7.

13. QUANTIFICATION LIMIT MQL

The MQL from the manufacturer is presented in table 9. However, analysis of the MDL will be carried out in blanks (n=10) to verify the response.

Table 9. MQL (Thermo Fisher Scientific, 2015).

		NH_4^+ (mg L^{-1})	NH_4^+-N (mg L^{-1})
Method detection limits (mg/L)	LL	0.0006	0.0005
	HL	0.0021	0.0016

14. WASTE DISPOSAL & CLEANING

- Empty the analyser cuvette waste bin and wastewater daily.

- The cuvette waste will be placed in the area designated for the KONE waste in the fume cupboard.
- Place the reagents in the designated area.
- Use clean centrifuge tubes for sampling. (BS ISO 15923-1:2013)
- Volumetric flasks are soaked in 10 % v/v hydrochloric acid after use.

REFERENCES

EPA. (2001). Method 1690. Ammonia-N in water and biosolids by Automated Colorimetry with preliminary distillation.

BS ISO 15923-1:2013 Water quality — Determination of selected parameters by discrete analysis systems Part 1: Ammonium, nitrate, nitrite, chloride, orthophosphate, sulfate and silicate with photometric detection.

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https://assets.thermofisher.com/TFS-Assets/CMD/manuals/PI-D09161-DA-Ammonia-DIC-PID09161-EN.pdf [Accessed March 2019]

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Zhou, Li and Boyd, Claude E. (2016) Comparison of Nessler, phenate, salicylate and ion selective electrode procedures for determination of total ammonia nitrogen in aquaculture. Aquaculture, Vol.450, pp.187-193

Standard Operation Procedure (SOP)
DETERMINATION OF TOTAL OXIDISED NITROGEN (TON) AND NITRATE BY CALCULATION THROUGH COLORIMETRY (USING DISCRETE ANALYZER KONE)

1. SUMMARY

This procedure is based on the determination of the sum of nitrate and nitrite (TON) by the hydrazine reduction method (BS ISO 15923-1:2013). Nitrate reacts with hydrazine in alkaline conditions and it is transformed to Nitrite. After that, the total Nitrite in the sample is measured using the sulphanilamide and N-1naphthylethylenediamine dihydrochloride method (pink azo-dye) at acidic conditions. Finally, the absorbance is measured spectrophotometrically at the wavelength of 540 nm. By selecting the Nitrate method in the discrete analyser, the concentrations are presented in the form of Nitrite (as NO_2), TON and Nitrate (as NO_3) (by calculation).

This SOP was developed according to the BS ISO 15923 *"Water quality — Determination of selected parameters by discrete analysis systems"* for spectrophotometric analysis with automated discrete systems (KONE Analyser)

2. REQUIREMENTS

INSTRUMENTS/MATERIALS	REAGENTS
• KONE Analyzer • 4 ml KONE samples cuvettes • 2 ml KONE standards cuvettes • Multicell cuvettes KONE • KONE Segments • KONE Waste bag/Container • Volumetric flasks (Glass, 100ml) with suitable stoppers • Volumetric flasks (Glass, 20ml) with suitable stoppers • Centrifuge tubes (15 ml) • Syringes Luer-Lok (10 or 20 ml) • Syringe filters (MF-Millipore MCE Membrane 0.45 µm)	• TON 1 Reagent. Precision by Thermo Fisher Diagnostics. • TON 2 Reagent. Precision by Thermo Fisher Diagnostics. • TON 3 Reagent. Precision by Thermo Fisher Diagnostics. • Nitrate (as NO3) Standard for IC. 1000 mg/l ± 4 mg/l. Sigma-Aldrich. • Nitrite Standard (as NO2) for IC. 1000 mg/l ± 4 mg/l. Sigma-Aldrich. • Nano-pure-de-ionised water (n-DW) • Distillate water • Samples • Washing cleaner solution KONE by Thermo Scientific

3. APPLICATION

- Sample type: drinking, ground, surface and wastewater. (Thermo Fisher Scientific ,2015)
- The relevant COSHH and Risk Assessment should be read before proceeding with this method.

4. ANALYTICAL RANGE

KONE discrete analyser measured Nitrate by subtracting the Nitrite measured results from the TON value with an automated calculation. (Thermo Fisher Scientific Inc, 2018). The range of concentration is presented in the table below. It is important to mention that the equipment can dilute the samples 10-fold during the analysis in case it is required.

Table 1. Nitrite and Nitrate Analytical Range

Method	Range (mg L^{-1})	KONE Method ID
Nitrite	up to 2	NO2
Nitrate	1 - 50	NO3

5. INTERFERENCE

- Sulphide concentration ≥ 10 mg/L. (BS ISO 15923-1:2013)
- Chloride ≥ 100 mg/L. Saline waters may require dilutions. (BS ISO 15923-1:2013)
- For the Nitrite analysis, samples with high alkalinity ≥ 300 mg/L could require dilution.
- Free chlorine, chloramines, and high levels of polyphosphate, iron (III) or thiosulfate the analysis of Nitrite. (BS ISO 15923-1:2013)
- Interference analysis should be assessed prior to the analysis (Matrix effect test)

6. SAMPLING AND SAMPLE PREPARATION

6.1. Transfer the sample into labelled 15ml centrifuge tubes using Syringe filters (0.45 µm) assembled into syringes.

Note 1: It is recommended to fill the sample tubes as much as possible to reduce the head space above the liquid. (Thermo Fisher Scientific, 2015)

6.2. Dilute the samples manually with Nano-pure-de-ionised water (n-DW) if it is required.

6.3. Samples should be analysed as soon as possible after collection. Otherwise, samples could be stored at -20 °C, for 8 days for Nitrate and Nitrite. (BSI EN ISO 5667-3:2018)

6.4. Prepare a blank with water in the same way as the sample. (BS ISO 15923-1:2013)

6.5. Prepare the Control AQC Standard Solutions according to section 8.

7. PREPARATION OF CALIBRATION SOLUTIONS

7.1. Prepare an intermediate stock solution using the Nitrite Standard solution for IC (1000 mg L^{-1}) to a 100 ml volumetric flask and fill up to the mark with DW. (See table 2 for NO2 low level)

7.2. Prepare the Calibration Standards using the Nitrite intermediate stock solution using (100 mg L^{-1}) to a 100 ml volumetric flask and fill up to the mark with DW (See table 2)

7.3. In the case of analysis of NO3 level, prepare the calibration standards according to table 3.

7.4. After preparation, transfer the solutions to labelled 15ml centrifuge tubes.

7.5. Storage the volumetric flask with the rest of the calibration solutions according to the stability instruction presented in tables 2 and 3.

Table 2. NO_2 Low level

KONE Standard Code Position	NO2 ppm	Stock solution, ppm	Flask Volume, ml	DF	Stock Volume, ml	Media	Stability
Intermediate	100	1000	25	10	2.500	n-DW	3 months at 5 ± 3 °C
S0	blank						
S11	2	100	100	50	2.000		1 day

Table 3. NO_3 level

KONE Standard Code Position	NO3 ppm	Stock Solution, ppm	Flask Volume, ml	DF	Stock Volume, ml	Media	Stability
S0	blank					n-DW	
S12	50	1000	20	20	1.000		1 month at 5 ± 3 °C

8. PREPARATION OF CONTROL AQC STANDARD SOLUTIONS

8.1. Use the Nitrite and Nitrate Standard for IC (1000 mg L^{-1}) to prepare two independent (by another source) AQC Standard Solution considering the lower and the upper third of the calibrated working range (Table 2). AQC solutions can be prepared with a mixture of Nitrite and Nitrate defined by the user (even with ammonium if required).

9. KONE ANALYSER START UP

9.1. The KONE Analyser should be in "stand by" mode after the end of the operating day. To start the system, press the "start up" button on the Main screen of the KONE software.

9.2. Ensure the KONE waste bin (with the waste bag) is in place.

9.3. Check the wastewater and the distilled water containers.

10. ANALYTICAL PROCEDURE

Before starting the analysis, fill out the form including the sample type, analysis type, date, samples ID and others if it is applicable (dilutions, pH).

10.1 Calibration

10.1.1 Transfer the calibration solutions to the 2 ml cuvettes.

10.1.2 Analyse the calibration solutions (section 7) according to the equipment manufacturer's instruction for the level of interest.

Note 3: Each calibration solution has a fixed position in the KONE equipment carrousel except the NO_2 high level. Place the calibration solutions according to the analytical method range (for NO_2 low level and TON). If the two methods have the same position for calibration solutions, they must be run separately)

10.1.3 Confirm the calibration curve before running the samples. The calibration is not accepted if the factor is out of the range according to table 4. In addition, the minimum correlation coefficient is 0.995.

Table 4. Calibration criteria

Method	Analytical Range	Calibration Factor
NO2	< 2 mg/L as NO_2	1.3 - 1.6
NO3	0.75 - 50 mg/L as NO_3	50 – 55

Note 4: If the calibration is not carried out daily, verify the validity of the calibration by analysing the lower and the upper third from the standard calibration range. The difference error between the measurements should be below 5 %.

10.1.4 Run the blank to check any carryover.

10.1.5 Run a calibration as a sample (4 ml cuvettes) and compare it with the new calibration. The difference error between the measurements should be below 5 %. If the values are not satisfactory, identify the possible factors affecting the results and repeat the calibration.

Note 5: The sample matrix could interfere with the calibration especially with samples from other applications such as liquid media for bacteria growth. It is recommended to carry out a matrix effect test by preparing the calibration solutions (see section 7) using the sample matrix instead n-DW.

Note 6: The minimum sample volume should be 1.2 - 1.5 ml. (Using 4 ml cuvettes)

10.2 System Quality check

10.2.1 After the calibration, transfer the AQC solutions to the 4 ml cuvette and place them in the segments (14 places/segment) and load them into the KONE Analyser. The difference error between the measurements should be below 5 %.

10.2.2 Run the blank and matrix blank as samples. Verify if there is an effect on the results due to the sample matrix.

10.3 Sample Analysis

10.3.1 Transfer the samples into 4 ml cuvettes and place them in the segments (14 samples/segment).

10.3.2 Analyse the samples according to the manufacturer's instructions.

10.3.3 After 20 samples, analyse the first sample and one AQC (second AQC optional). The difference error between the measurements should be below 5 %.

10.3.4 Carry out a calibration Drift Check Standard.

10.3.5 Continue with the second batch of samples (an example of the sequence can be found in appendix 1)

10.3.6 After completing the analysis, remove the samples, the reagents and place the washing solution in a 2 ml cuvette.

10.3.7 Press Stand by button on the Main screen.

10.3.8 After the cleaning process is completed, remove the washing solution.

10.4 Sample batch sequence

The samples were run in the KONE analyser following the sequence presented in the table below:

Table 5. Sequence example for batches of 20 samples

Phase	Solution	Comments
Calibration	Calibration solutions	Daily.
	Calibration Verification	If the calibration is not carried out, analyse the lower and the upper third solution from the calibration standard range.
	UPW	Check Carryover/Water Quality
	Old Calibration standard (optional)	Comparison between new and old calibration
System check	AQC 1	From independent source (Low level)
	AQC 2	From independent source (Third upper level)
	Blank	
	Matrix	(effect of the matrix in the analysis)
Samples 1st batch	Sample 1	First sample of the 1st batch
	Sample 20	Last sample of the 1st batch
	Sample 1. 1st batch	Quality Check
	AQC 2	From independent source
	Calibration Drift Check	End of each batch of samples
Samples 2nd batch	Sample 21	First sample of the 2nd batch
	Sample 40	Last sample of the 2nd batch
	Sample 21. 2nd batch	Quality Check
	AQC 2	From independent source
	Calibration Drift Check	use an standard (middle) or AQC
Samples 3rd batch	Sample 41	First sample of the 3rd batch
	Sample 61	Last sample of the 3rd batch
	Sample 41. 3rd batch	Quality Check
	AQC 2	From independent source
	Calibration Drift Check	End of each batch of samples
Closing KONE for Stand by	Washing Solution	Cleaning the system for stand by

11. TEST REPORT

The test report should include:

- The test method used
- Identification of the samples
- Date of the analysis
- Method detection range and MDL
- The analytical results consider that Nitrite and Nitrate can be expressed as N or as the relevant ion form. (see table below)

Table 6. Conversion factor

Parameter	Conversion
Ammonium	NH_4-N × 1.29 = NH_4^+
Nitrite	NO_2-N × 3.28= NO_2^-
Nitrate	NO_3-N × 4.43 = NO_3^-

12. QUALITY MANAGEMENT

The calibration is validated daily. If the calibration is not carried out, a calibration validity check is run to verify the performance. (BS ISO 15923-1:2013)

Two levels of quality control are used: AQC solutions (independent source) and the first sample of the batch is run in duplicate at the end to verify the results. Batch: 20 samples. An example of the sequence is presented in the table 7.

13. QUANTIFICATION LIMIT MQL

The MQL from the manufacturer is presented in table 7. However, analysis of the MDL will be carried out in blanks (n=10) to verify the response.

Table 7. Minimum detection limit (MDL)

Chemical	Blank	Mean (mg L^{-1})	SD (mg L^{-1})	MDL (mg L^{-1})	Notes
Nitrite	n=3	0.06	0	> 0.1	60% recovery from 0.1 mg/L NO2
Nitrate	n=7	0.198	0.015	0.048	MDL=3.14*SD

SD: standard deviation

MDL: minimum detection level

14. HEALTH & SAFETY

Risk Assessment and COSHH should be carried out before the analysis.

Training is required before using the KONE discrete Analyser.

15. WASTE DISPOSAL & CLEANING

- Empty the analyser cuvette waste bin and wastewater daily.
- The cuvette waste will be placed in the area designated for the KONE waste in the fume cupboard.
- Place the reagents in the designated area.
- Use clean centrifuge tubes for sampling. (BS ISO 15923-1:2013)
- Volumetric flasks are soaked in 10 % v/v hydrochloric acid after use.

REFERENCES

BS ISO 15923-1:2013 Water quality — Determination of selected parameters by discrete analysis systems Part 1: Ammonium, nitrate, nitrite, chloride, orthophosphate, sulfate and silicate with photometric detection.

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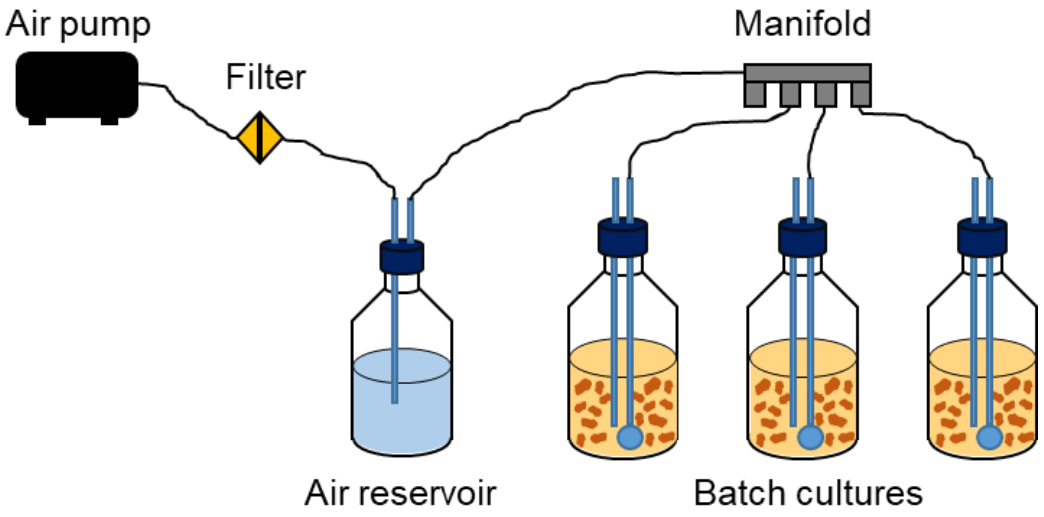
https://assets.thermofisher.com/TFS-Assets/CMD/manuals/PI-D09228-DA-TON-Hydrazine-Nitrate-PID09228-EN.pdf [Accessed April 2022]

BSI EN ISO 5667-3:2018 Water quality – Sampling. Part 3: Preservation and handling of water samples.

Thermo Fisher Scientific Inc (2018) Fast and accurate Thermo Scientific Gallery discrete industrial analyzers

Appendix

Supplementary information to Chapter 3



FigS1. Schematic of the short-term batch experiments

Supplemental Methodologies and Results

Biomass quantification

The amount of biomass was measured as total protein. This procedure requires two main steps: complete protein extraction from cells through lysis and further protein quantification (Cole et al. 2020). This study tested two cellular lysis methods: sonication and freeze-thaw cycles. For protein analysis, two commercial colourimetric kits were selected, Micro BCA (Thermo Scientific 23235) and Coomassie (Bradford) protein assay kit (Thermo Scientific 23200). Detailed descriptions of the methods are presented below. In addition, the extraction protocols were evaluated for protein recovery, and the final protocol was selected based on the highest protein yield from the combination of extraction and protein assays.

Samples (2ml) were withdrawn from the 2-L culture reactors in triplicate and distributed into sterile 2-ml tubes with screw caps on the same day as the tests. These methods were applied based on their relatively low cost, accessibility in our laboratory, sensitivity at low protein concentrations and suitability to 96-well plate format.

Protein extraction methods

According to Wood and Sørensen (2001), cell lysis via sonication was carried out. Briefly, the sample pellets were centrifuged at 13500 rpm for 5min (Eppendorf Centrifuge 5414 D, Germany) and resuspended in potassium phosphate buffer (50 mM, pH 7.8). Next, two-ml bacterial suspensions were sonicated (Sonicator Branson 2510, Bransonic, USA) (100W, 42 kHz) five

times for 20 s. Samples were kept on ice to prevent heating between cycles. After that, the sonicated suspension was centrifuged to remove the cell debris and stored at 4 C until ready for the protein assay.

The freeze and thaw lysis method submitted bacterial cells to freeze-thaw cycles from dry ice to a hot bath at 80°C (Grabski 2009). Initially, samples were centrifuged at maximum speed (13,200 rpm) for 5 min on a microcentrifuge (Eppendorf Centrifuge 5414 D, Germany). After that, the supernatant is discarded and replaced with sterile distilled water. Next, these samples were exposed to temperature shocks, cold (dry ice) and hot (water bath) for ten minutes each, vortexing the samples at the end of each cold-hot cycle. This procedure was repeated five times. Finally, the tubes were centrifuged at 13,200 rpm for 5 min to pellet the cell debris and transfer the supernatant with the extracted protein to a new sterile 2-ml graduated, skirted tubes with screw caps and stored at 4 C for protein analysis (Islam et al. 2017).

Determination of total protein

The amount of total protein was measured using two commercial kits: Coomassie Bradford (ThermoScientific 23200) and Micro BCA (Thermo Scientific 23235). Details of the assay parameters are given in Table S1. The assay calibration curves were performed according to the manufacturer's instructions. Both protein kits used the bovine serum albumin standard ampules, 2 mg/ml, as the calibration standard. The tests were conducted in sterile 96-well, flat-bottom microplates (Thermo Scientific) using a UV-VIS micro-spectrophotometer (Epoch Biotek, USA). All measurements were performed in triplicate. The limit of detection (LOD) was determined as 3 s/m, where "s" is the standard deviation of the lowest detectable concentration and "m" is the slope of the calibration curve (Pokhrel et al. 2020). Precision is reported as the % coefficient of variation (% CV) between the replicates. In addition, a culture media sample was added to the assay to verify whether residual liquid media from the cultures could interfere with the absorbance response. The difference with the blank (Milli-Q water) is reported as a % deviation of the blank. The analytical performances are summarised in Table S2, and calibration curves are presented in Fig. S2.

Table S1. Total Protein quantification assay parameters

	Micro BCA	**Bradford**
Incubation time	2 hours	10 min
Temperature	37 C*	Room temperature
Absorbance (nm)	562	595

* After incubation, cool the plate for 10 min at room temperature.

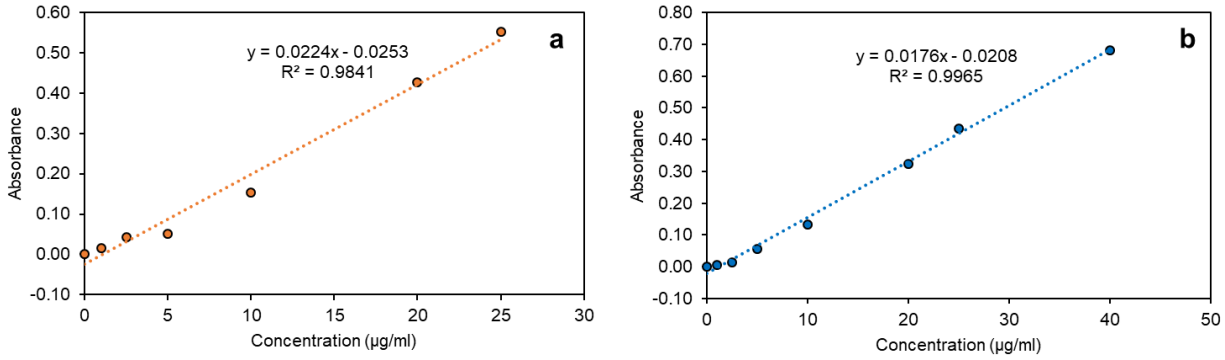


Fig. S2. Calibration curve a) Bradford b) Micro BCA. Values presented as mean (n=3)

Table S2. Performance of protein assays

	Micro BCA	**Bradford**
Lineal range (µg/ml)	2 - 40	1 - 25
%CV (n=3)	< 3 %	< 2 %
LOD (µg/ml)	0.2	0.4
% deviation blank	< 3 %	< 2 %

Comparison between extraction/assay protocols

After the protein was extracted through both cell lysis methods, samples were analysed with the commercial kits. The concentrations reported are shown in Fig. S3. As can be seen, the responses from the Bradford assay were low for both cell lysis procedures. Therefore, the highest total protein yield was obtained with the combination of Freeze-Thaw extraction with the Micro BCA assay.

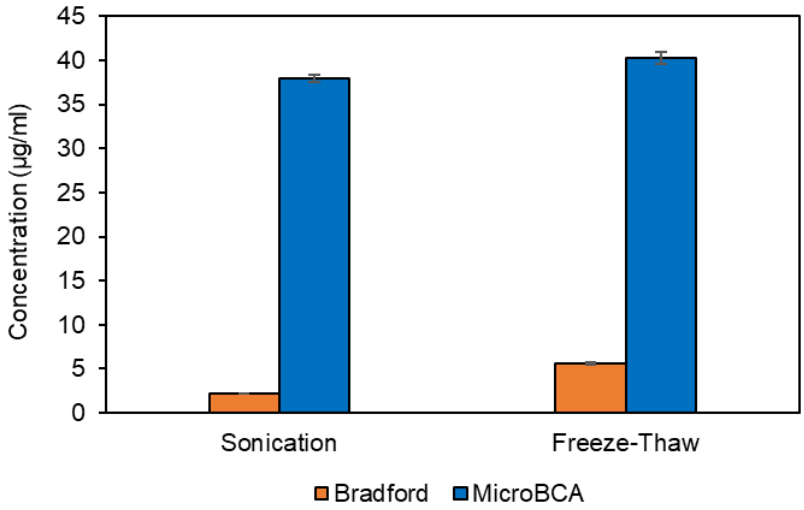


Fig. S3. Comparison between extraction/assay protocols. Values as mean ± SD (n=3)

Cell growth in the batch cultures

The cell growth of the microbial community was investigated in a separate test by measuring the protein change over time. The experimental configuration was similar to the tests conducted in this study, using three batch reactors working in parallel. For protein and nitrogen compounds, samples were collected in triplicate at the beginning and end of the incubation period (78 h). In addition, one sample (20 ml) was collected at the end for TSS (total suspended solids) and VSS (volatile suspended solids) analysis. The results are presented in Table S3.

Table S3. Results of the cell growth analysis in short-term batch assays

Parameter	**Reactor 1**	**Reactor 2**	**Reactor 3**
NH_4^+-N consumed (mg L^{-1})	38.4	39.5	42.4
NO_X^--N (mg L^{-1}) produced	37.2	38.9	40.9
Initial Protein (mg L^{-1})	9.1 ± 0.7	9.2 ± 0.7	8.9 ± 0.7
Final Protein (mg L^{-1})	9.7 ± 0.8	9.7 ± 0.7	9.5 ± 0.6
Final TSS (mg L^{-1})	101.0	104.0	112.1
Final VSS (mg L^{-1})	99.6	103.0	112.3

The results show that the protein increased slightly (6.4 ± 0.0 %) over the experimental period (78h), with a biomass formation of 0.01 ± 0.0 mg microbial protein/mg NH_4^+-N consumed.

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Appendix

Supplementary information to Chapter 4

Supplemental methodology

Cultivation of salt-tolerant nitrifying bacteria

The cultivation and enrichment of salt-tolerant nitrifying bacteria was performed following a similar procedure described in the Chapter 2 and the appendix supplementary information to Chapter 2 of this thesis. However, the preparation of the media was slightly different considering that halotolerant bacteria requires salt to grow. For 1 L of media, 26-30 g L^{-1} of sea salts (NutriSelect® Basic, Sigma Aldrich) was added to create an artificial seawater media to mimic marine environment conditions (conductivity of 33 ± 3 mS/cm at 20 °C). After the sea salts are dissolved, continue with the preparation of the media as described in the section of supplemental methodology in the appendix supplementary information to Chapter 2 of this thesis. After the media is prepared, the final pH was adjusted to 8.5 ± 0.2 (pH/conductivity meter Mettler Toledo, MPC 227, Switzerland) with a 10 M NaOH solution (Fisher Scientific) in the safety cabinet. The media can be stored at 4 °C up to one-week prior use.

Similarly, serial dilutions were employed to cultivate the salt-tolerant nitrifying bacteria as described previously in Chapter 2 and the appendix supplementary information to Chapter 2 of this thesis. However, the activity of the AOB/NOB species from the marine samples was lower compared to the WWTP samples used in Chapter 2 (by visual observation of colorimetric reagents), impacting the duration of the cultivation. In the first stage, 1 g of the marine sediments was inoculated in 100-ml Erlenmeyer containing 50 ml of artificial seawater media. Ten replicates were cultivated, transferring 5 ml of the cultured media into 45 ml new media every 3 weeks. This serial transfers were repeated in a period of 6 months, with a total of 9 cycles.

In the second stage (enrichment), the Erlenmeyer cultures (n=3) that showed stable nitrification activity was transferred to a 2 L glass bottles (1.7 L working volume) for biomass production. In this stage, the 2-L reactors (n=2) operated for 10 months at room temperature (20 °C - 27 °C) with similar installation described in Chapter 2 for the WWTP samples lab scale reactors. Samples were collected regularly to measure ammonia, nitrite and nitrate concentrations (every 2 or 3 weeks). For ammonia and nitrite analysis, 4 ml of sample was collected (filtered with a 0.45 μm cellulose filter) and analysed using colorimetric analysis with KoneLab Aqua 30 (Thermoscientific, Aquarem 300, Clinical Diagnostics Finland). In the case of nitrate, an additional sample (2 ml filtered with a 0.45 μm cellulose filter) was taken to measure nitrate in the samples using cuvette tests (HACH Lange LCK339, UK) suitable for seawater samples, detection range (0.23-13.5 mg L^{-1} NO_3-N) according to the UV-VIS-spectrophotometer manufacturer instructions (HACH, Model DR 6000, Germany). In addition, samples (2 ml) were collected for pH analysis (every 2 or 3 weeks) (pH/conductivity meter Mettler Toledo, MPC 227,

Switzerland) to maintain the pH (8.5 ± 0.2). Moreover, the performance of the lab reactors is presented in the Figure S1.

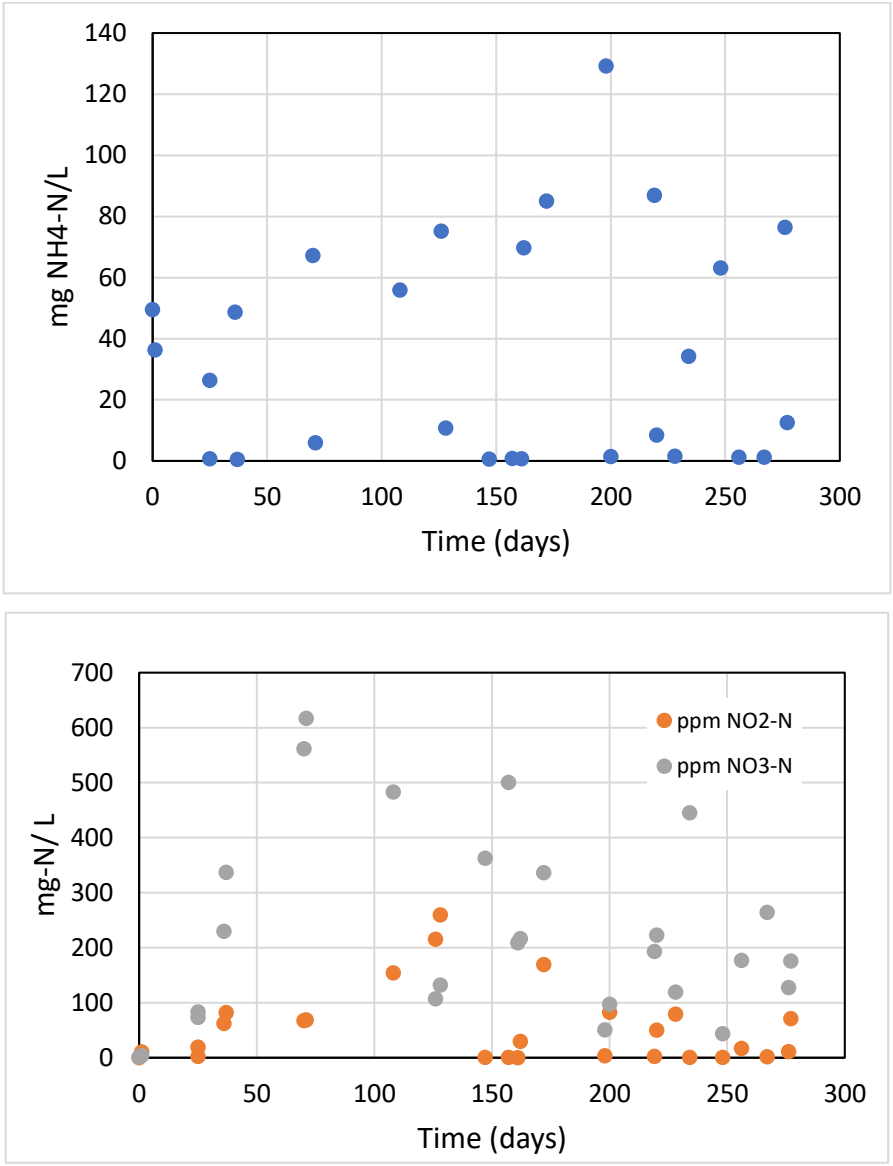

Figure S1. Ammonia, nitrite and nitrate levels over 300 days of enrichment.

Batch toxicity assay with triclosan

The short-term toxicity experiment with the salt-tolerant nitrifying bacteria was prepared with a similar procedure applied on the WWTP samples described in Chapter 2 and supplementary information to Chapter 2. Due to limited amount of biomass and time, the toxicological assessment of halotolerant nitrifiers was only carried out with Triclosan (TCS). The sampling requirements per batch reactor during the toxicity assay with TCS are presented in table S1.

Table S1. Sampling requirements for the toxicity tests per batch reactor-TCS test

Analysis	Sample volume (ml)	Frequency	Replicates	Collection
Chemical analysis of NH_4^+ and NO_2^-	4	Four times: 0h, +6h, +20h and +24h	1	filtered 0.45 µm cellulose filter
Chemical analysis of NO_3^-	2	Four times: 0h, +6h, +20h and +24h	1	filtered 0.45 µm cellulose filter
pH	2	Twice	1	initial and end points filtered 0.45 µm cellulose filter
Protein	1	Once	3	End point
TSS/VSS	20	Once	1	End point

Similarly, the assembly of the small bottles for the toxicity test was arranged as previously described on the appendix supplementary information to Chapter 2. The biomass preparation was carried out with the optimised cleaning procedure (Method 2) described on Chapter 3. All the treatments were prepared using the artificial seawater media applied for the cultivation of salt-tolerant nitrifying bacteria described in this supplemental methodology. Finally, the detail of the treatments prepared for the TCS toxicity test is shown in table S2

Table S2. Example for preparation of the acute toxicity test with TCS

No. treatments	1	2	Control	ATU
Fresh medium (ml)	250	250	250	250
Inoculum (ml)	50	50	50	50
Toxicant stock solution (mg L^{-1})	30	300	0	100
Toxicant stock volume (ml)	0.1	0.1	0	1
Toxicant final concentration (mg L^{-1})	0.01	0.1	0	0.3
Total Volume (ml)/bottle	300	300	300	300
No. of bottles	3	3	3	2