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 that 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 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 (Langbehn *et al.* 2020; Huang *et al.* 2016), 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).

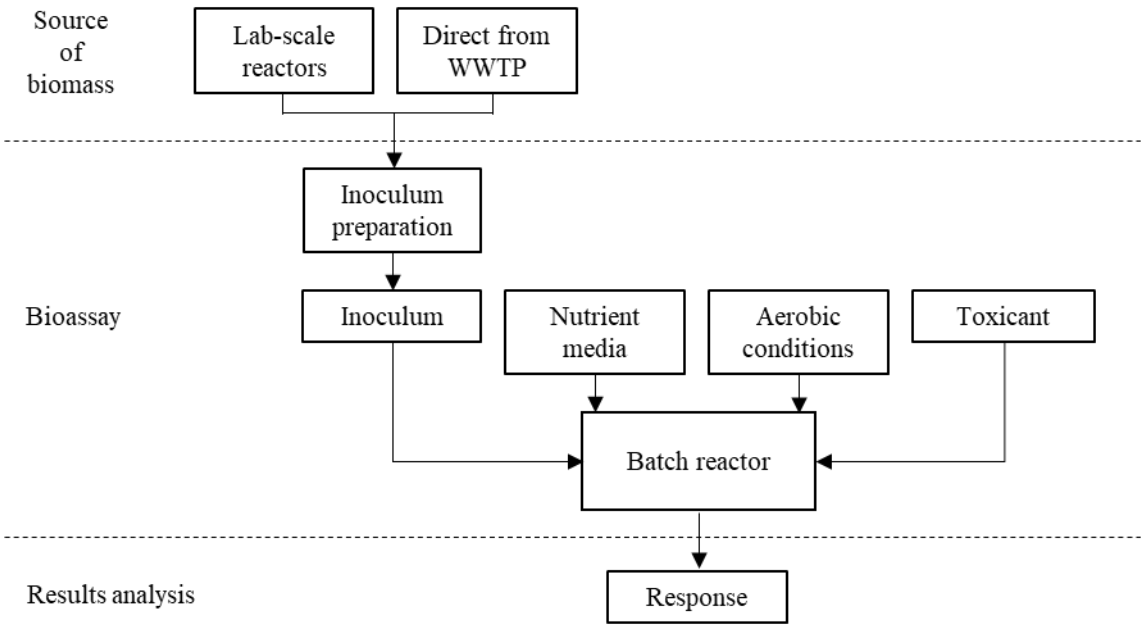

Fig. 1. An overall framework for nitrification batch assays.

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 (sealed Erlenmeyer flasks 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^+/g·MLVSS·h, and the yield nitrate production was about 0.95 mg NO_3^- produced/mg NH_4^+ 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 (supplementary data).

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

155 method using centrifugation at 10,000×*g* (Eppendorf, centrifuge model 5804 R) followed by media
156 settling, referred to in this study as Method 1 (see Fig. 2). The main objective of this method was the
157 maximum removal of oxidising compounds (nitrite and nitrate) in the culture suspension prior to
158 inoculation. We conducted further testing to determine the impact of this cleaning procedure on biomass
159 activity by comparing the inoculum performance with an optimised cleaning protocol (Method 2) (see
160 Fig. 2). Based on this, a series of batch reactors (n=3) were inoculated with biomass prepared with two
161 cleaning strategies (Fig. 2): a longer protocol with two cycles of centrifugation/setting (Method 1) and
162 an optimised version with one centrifugation cycle (Method 2). The operating conditions of the cleaning
163 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

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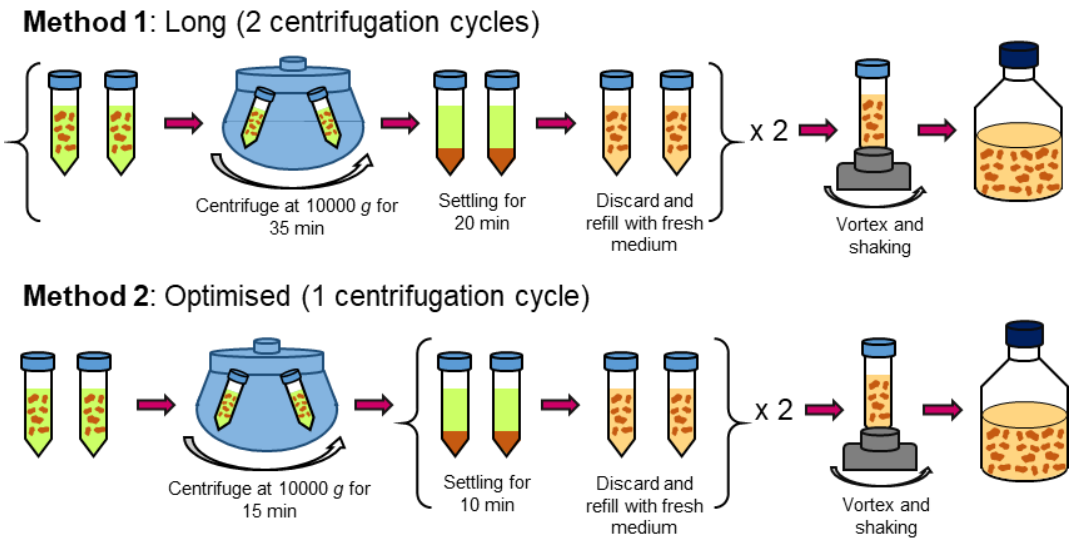


Fig. 2 Schematic of the two cleaning methods to prepare the inoculum.

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,

183 airflow was adjusted with an airflow meter (Brooks Instrument Model # MR3A12BVBN, USA). The
184 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

185

186 **2.2.3.Solvent test**

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

191 The effect of three conventional organic solvents: dimethyl sulfoxide (DMSO), acetone and ethanol, on
192 the nitrifying biomass was investigated. Each treatment was spiked with 0.1 ml of the solvent with a
193 final concentration of (0.03 % v/v). Testing conditions are shown in Table 2. All batch cultures were
194 cultivated at 20 °C for 56 hours, after which their performances were compared to the controls. The
195 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

196

197 **2.2.4. Biomass size inhibition test**

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

205

Table 4. Initial operating conditions for the biomass size inhibition test *. Values represent means and standard deviations (or range, in case of duplicates).

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})	43.9 ± 2.3	213.7 ± 4.9
Replicates	Triplicates	Triplicates

Duration (h)	24	24

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

206

207 **2.2.5.Nutrient Media inhibition test**

208 The enrichment of nitrifying biomass and all toxicity experiments reported in Lopez *et al.* (2021) and
209 this study were performed using the same nutrient media. The liquid medium was modified from
210 (Bollmann *et al.* 2011), containing HEPES as a buffering agent, basal salts, phosphate, trace metals and
211 $NaHCO_3$ as an inorganic carbon source. To evaluate whether the nutrient media composition affected
212 the microbial inhibition response, we conducted a series of toxicity tests comparing our experimental
213 test media with the media recommended in the ISO 9509 (2006) protocol. According to this
214 methodology, a solution with only $NaHCO_3$ should be sufficient to sustain the nitrification in short-
215 term assays without significant change of pH. The batch reactors were spiked either with ATU (0.1 mg
216 L^{-1}) or without. The responses were evaluated in terms of %inhibition compared with the control
217 cultures (sans ATU). All the treatments used $(NH_4)_2SO_4$ salt as a source of inorganic nitrogen. The
218 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

219

220 **2.3. Biomass and chemical analysis**

221 Biomass concentrations have been estimated by protein content and dry cell weight in nitrification
222 inhibition studies (Roh *et al.* 2009; Ben-Youssef *et al.* 2009; Dytczak *et al.* 2008). In the case of protein
223 analysis, tests were conducted to optimise protein extraction and quantification. The protein strategy
224 was selected considering the maximum protein yield from the combination extraction/assay method,
225 which resulted in freeze-thaw cycles and Micro BCA assay (see Fig. S3). The cell dry weight was
226 determined as total suspended solids (TSS), carried out according to the Standard Methods (APHA
227 1998). It is worth mentioning that although the nitrifying bacteria originated from activated sludge, the
228 inorganic suspended solids were removed during the cultivation process, leading to similar
229 measurements (see Table S3) between total suspended solids (TSS) and volatile suspended solids (VSS)
230 (He *et al.* 2013; Lopez *et al.* 2021). Analysis of nitrogen compounds was performed colourimetrically
231 as described in Lopez *et al.* (2021) using KoneLab Aqua 30 (Thermo Scientific, Aquarem 300; Clinical
232 Diagnostics Finland). No analytical interference from the test substances or matrix components was
233 found with any chemical analysis carried out in this study.

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

240 **2.4. Data analysis**

241 The responses were evaluated by comparing the concentration of nitrogen species, percentage of
242 ammonium consumed E, (mg NH_4^+-N consumed/g of initial NH_4^+-N × 100), and yield (Y, mg of NO_2^-
243 -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 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.

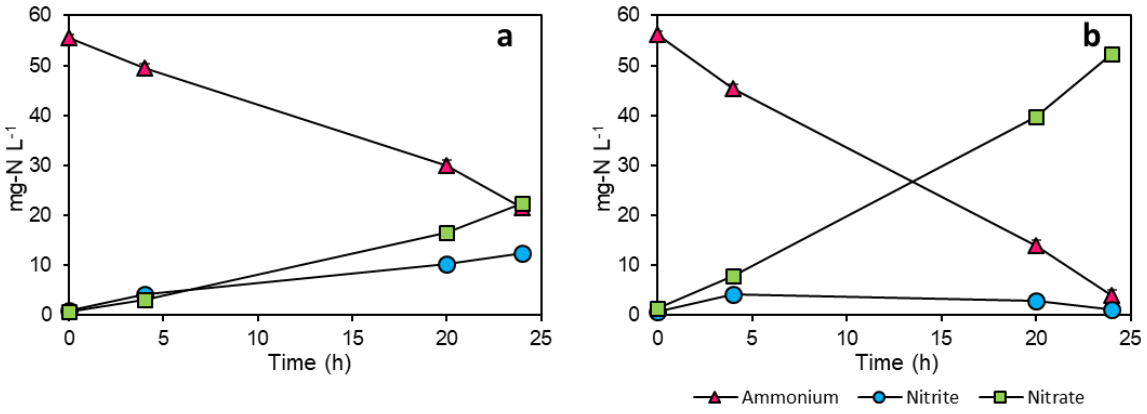


Fig. 3. Nitrification profiles following different washing procedures: a. Method 1 (long centrifugation). b. Method 2 (optimised, short centrifugation). Mean ± standard deviations (n=3).

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 ± 0.1 mg NO_3^--N produced/mg NH_4^+-N consumed) and nitrite accumulation (Y_{NO2^-} = 0.34 ± 0.1 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.

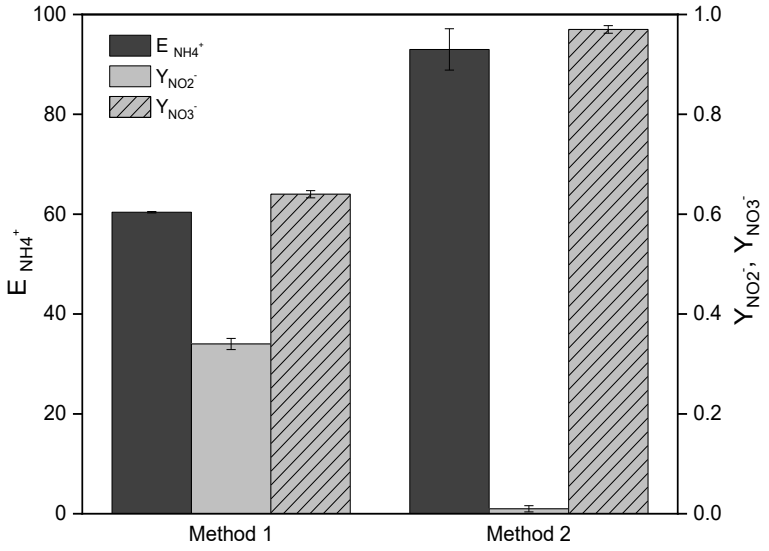


Fig. 4. Ammonium consumption efficiency, yields of nitrite and nitrate of the washing procedures. Method 1 and Method 2 (optimised). The bar represents Mean ± standard deviations (n=3).

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

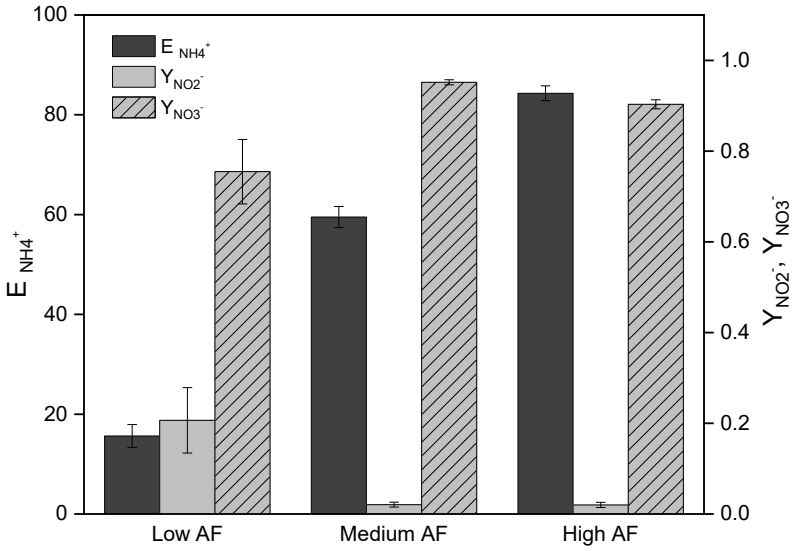
minimise the disturbance of AOB/NOB species in the inoculum and prevent poor performance during the toxicity assay.

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).



329

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

332 Concerning DO, the levels were maintained above 5 mg L^{-1} in the medium and high airflow reactors,
333 while the DO in the replicates with low airflow dropped to 4.3 mg L^{-1} after 24 h. Based on the
334 performance results (Fig. 5), incomplete nitrification (nitrite accumulation) was still observed by the
335 (low) aeration system even when the DO values at the end of all cultures were consistently higher than
336 4 mg L^{-1}.

337 Although complete nitrification can occur at lower DO values (Campos *et al.* 2007), the DO biomass
338 cultivation conditions have a significant impact on the capacity of nitrifiers to utilise oxygen, especially
339 in NOB populations. Because different microbial groups consume oxygen, the species with lower
340 oxygen affinities (high oxygen half-saturation K) may be affected during DO fluctuations. According
341 to many studies, NOB guilds usually present higher K values than AOB and heterotrophs, which are
342 more efficient in oxygen metabolism (Arnaldos *et al.*, 2015). Furthermore, differences in oxygen
343 affinity within NOB species have been observed, resulting in abundance changes or shift NOB
344 population (i.e. from *Nitrobacter*-like to *Nitrospira*-like) as a coping strategy to lower DO scenarios
345 (Liu and Wang 2015; Fan *et al.* 2017). In our work, the 16S-rRNA analysis in Lopez *et al.* (2021)
346 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)

366 and saturating the culture media by flowing air before testing. Another study from (Phan *et al.* 2020)
367 evaluated the short-term effect of Mn_2O_3 nanoparticles on nitrifying bacteria. These authors showed
368 that nitrification activity in the batch inhibition assays was significantly affected by DO with and
369 without aeration, resulting in the report of inhibition under low and high DO conditions. Other
370 modifications in the aeration system, such as bubble diffusers and DO-controlled devices, can enhance
371 oxygen supply in aerobic cultures. However, these solutions may substantially increase the research
372 cost and resources (Yao *et al.* 2021).

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

379

380 **3.3. Effect of Solvent on nitrification performance**

381 Assessing the toxicological effect of chemicals with poor aqueous solubility usually requires the use of
382 co-solvents as carriers. However, these substances may cause inhibition themselves, affecting the
383 response of the tested organisms. Due to this, the impact of common organic solvents (DMSO), acetone
384 and ethanol) on nitrification was studied in batch cultures. The changes of ammonium, nitrite and nitrate
385 concentrations using different solvents, including the unamended control after 24 h, are presented in
386 Fig. 6. According to the results, the ammonium removal was similar between all the treatments.
387 However, the cultures spiked with ethanol exhibited more discrepancies in the total inorganic N balance
388 (ammonium-N consumed versus NOx-N produced, > 68%) than the DMSO, acetone, and control
389 treatments difference less than 3%. Based on this, it was observed that ethanol significantly altered the
390 nitrification activity of the nitrifying consortium, even at lower concentrations (0.03% v/v). This

391 behaviour may be explained due to the possible growth of other bacteria (i.e., heterotrophs) that
392 consume organic substances as carbon sources (Du *et al.* 2003; Thomsen *et al.* 2007).

393 Concerning the other solvents, the Y_{NO3^-} of the DMSO and acetone cultures (0.88 and 0.86, respectively)
394 were similar to the control cultures (Y_{NO3^-}, 0.9). These results suggested that DMSO and acetone at
395 0.03% v/v may be used as solvents without affecting the overall nitrification performance in batch
396 assays with enriched nitrifying biomass. Furthermore, these results aligned with other studies with
397 similar nitrifying strains (Papadopoulou *et al.* 2020).

398 These findings show the importance of solvents as part of the experimental design. Besides solubility
399 with the toxicant, selecting the best solvent should evaluate both possible physicochemical and
400 microbial interaction in the batch assay. This is highly relevant in enriched biomass under autotrophic
401 conditions with ammonium as the sole energy source to suppress heterotrophic bacterial growth. There
402 are three critical aspects in working with microbial cultures to consider: establish the solvent
403 requirements in terms of concentration and exposure (Modrzyński *et al.* 2019), conduct solvent toxicity
404 tests for the specific bacterial communities, and finally evaluate the possibility of solvent as a substrate
405 source (Dyrda *et al.* 2019). Suppose the solvent pre-tests result in limited options. In that case, a
406 practical alternative might be adding the toxicant solution into empty batch reactors and allow the
407 solvent to evaporate before the toxicity tests (Men *et al.*, 2017; Dawas-Massalha *et al.*, 2014).

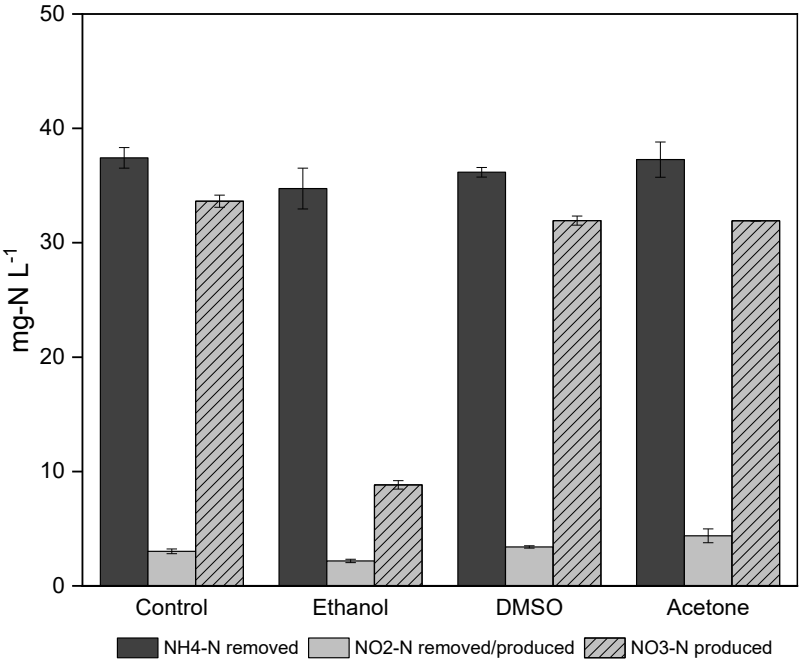


Fig. 6. Inorganic nitrogen variations using different solvents at 0.03 % (v/v). Bar represents Mean ± SD. (n=2)

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.

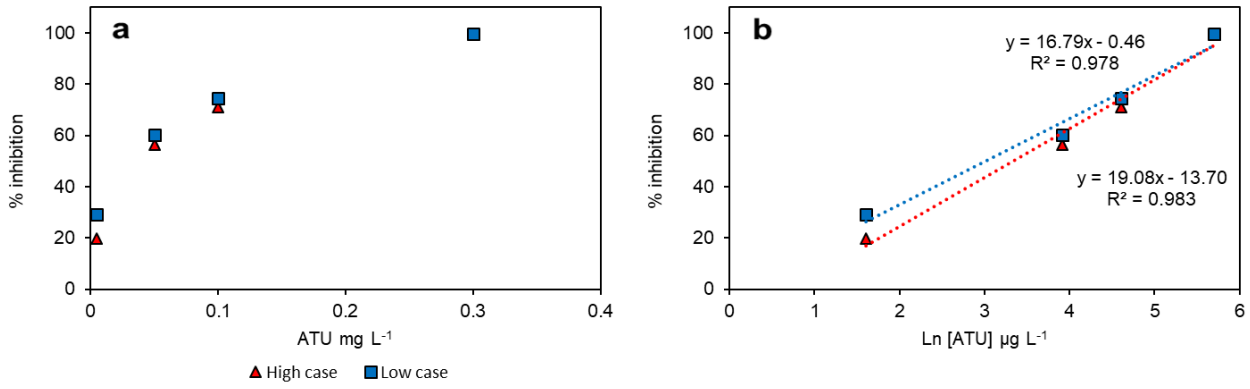


Fig. 7 inhibition level at different ATU concentrations. a. Inhibition %. b. Linear regression

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

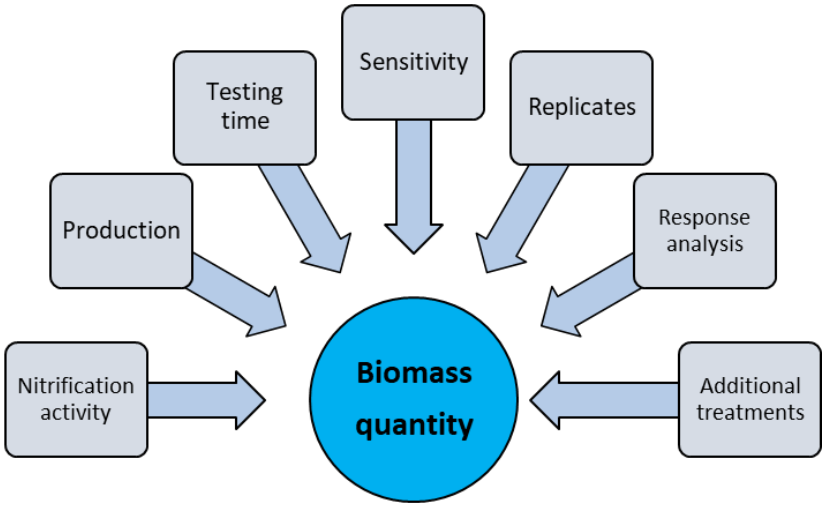
	low case	**High case**
Nitrification rate mg NH_4^+-N/ g SS·h	11.3 ± 4	9.9 ± 0.1
So/Xo (mg NH_4^+-N/mg SS)*	1.2 ± 0.1	0.3 ± 0.0

* 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

494 period (from hours to a few days) in balance with the sensitivity response against the toxic compound.
495 Regarding the quantification assay, biomass in nitrifying cultures is commonly expressed as total
496 protein and suspended solids VSS due to its relatively low cost and accessibility. However, the use of
497 these parameters may be problematic. A study from Liang *et al.* (2010) about the biomass analysis of
498 nitrifying biofilm and activated sludge confirmed that although proteins are the highest portion of the
499 VSS, the protein/VSS ratio is highly variable within the samples. According to these authors, these
500 discrepancies correlate to the efficiency of protein extraction and flocs in the biomass. Another
501 contributing factor is the high standard deviations reported in VSS measurements; a similar iissue has
502 been observed in our studies and highlighted by other authors (Lotti *et al.*, 2014). Despite these
503 variations, these parameters are still necessary to compare the specific nitrification activities among
504 published studies.



505

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

507 In summary, the review of previous methodologies and our own experience suggest that a suitable
508 amount of biomass should be determined by the specific research needs. Nevertheless, all the factors
509 discussed here are captured in Fig. 8, providing an overall picture of the common features that outline
510 the experimental design of nitrification inhibition bioassays. These guidelines may represent a start
511 point for many authors, supporting biomass optimisation strategies while working with challenging
512 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 73.8% and 72.3% 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	15.1 ± 0.2	54.6 ± 0.1	10.9 ± 0.1	41.3 ± 0.4
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 negatively 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) on reasonable request.

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

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Authors' contributions: Laboratory experiments, data analysis and first draft of the manuscript were performed by Carla Lopez. Charles Knapp contributed to the study's conception, commented and edited on previous versions of the manuscript.

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Evaluating acute toxicity in enriched nitrifying cultures: lessons learned

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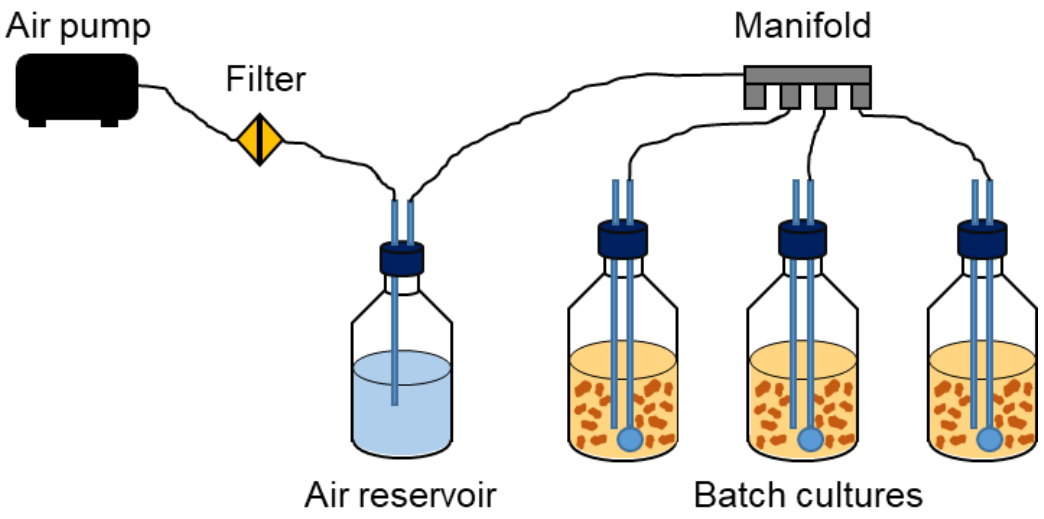
Supplementary information



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). In this study, two cellular lysis methods were tested: 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 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

Cell lysis via sonication was carried out according to Wood and Sørensen (2001). 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 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 (Thermo Scientific 23200) and Micro BCA (Thermo Scientific 23235). Details of the assay parameters are given in Table S2. The assay calibration curves were performed according to the manufactures' 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 S2. 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.

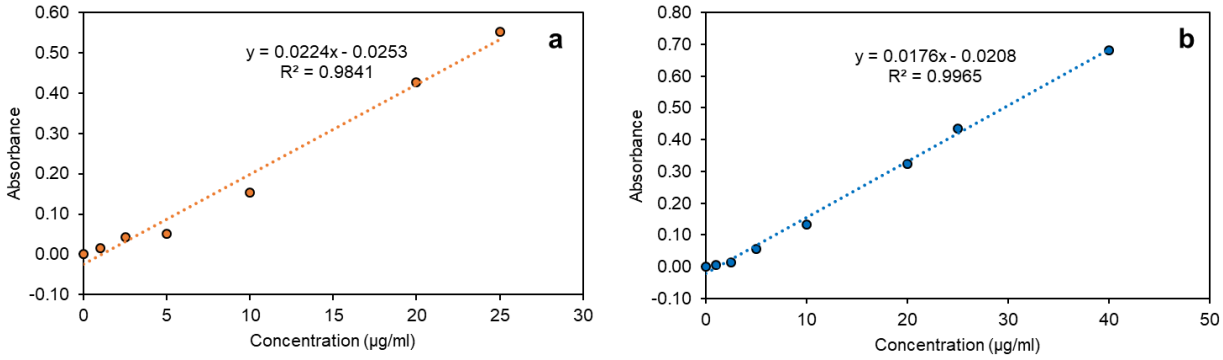


Fig. S2. Calibration curve a) Bradford b) Micro BCA. Values presented as mean (n=3)

Table S1. 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.

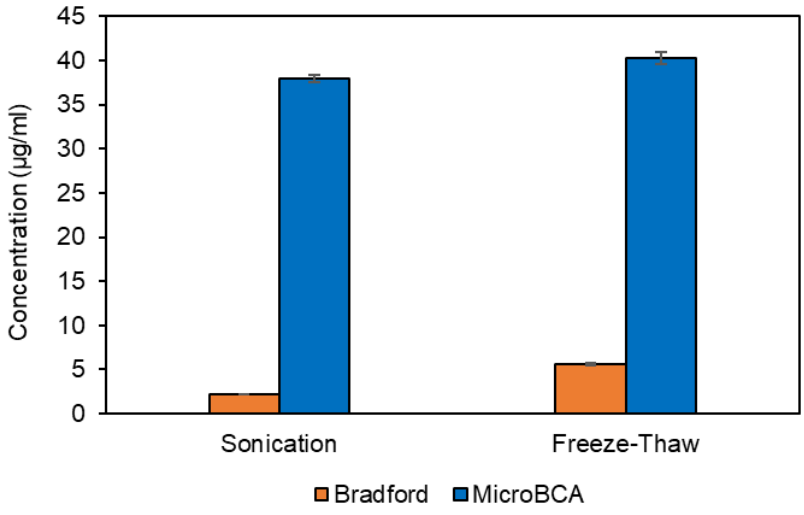


Fig. S3. Comparison between extraction/assay protocols. Values as mean ± SD (n=3)

Cell growth in the batch cultures

The cell growth of the microbial community was investigated in a separate test by measuring the change of protein 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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