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A modified nitrification inhibition test for high-salinity wastewater

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ABSTRACT

Nitrification inhibition is a standard test for industry wastewater received by wastewater treatment plants (WWTPs). Standard tests such as ISO9509 cannot differentiate the effect of toxicity from relevant chemicals and the temporary inhibition of bioactivity that is induced by a change in osmotic pressure, which occurs if a sample has a high salt concentration. This, however, is unrealistic, as salt does not result in nitrification inhibition on real WWTPs, as their nitrifiers adapt to salt concentration in the same way that nitrification occurs in the oceans. To provide a more realistic inhibition test, we cultured nitrifiers on biofilm carriers in wastewater constantly high in salt content and fortified the salinity of the tested samples to the salt concentration used for culturing, thus eliminating the salt effect. The salt tolerance test was compared with the existing ISO9509 method on statistical uncertainty with results for the common type of salty wastewater from oil extraction and a solution of single chemicals relevant to this wastewater. The nitrification inhibitions of formaldehyde and methanol were similar between the salt-adapted method and the ISO9509 method, while lower inhibition at biocide tetrakis (hydroxymethyl)phosphonium sulphate (THPS) was observed with the salt adapted method. The new methods standard deviation of nitrification inhibition around the 50% inhibition level was below 3%. Thus the method is well suited to test nitrification inhibition in salty water samples as both repeatability and sensitivity are similar or better than the ISO9509 method.

1. Introduction

Wastewater contains pollutants such as pathogens, nutrients and oxygen-demanding substances, which, if they left untreated and discharged, can compromise the water quality of receiving water bodies [1,2]. Wastewater treatment plants (WWTPs) are facilities that combine various physical, chemical and biological processes able to treat and remove pollutants from such waters [3,4]. Among many pollutants present in wastewater, ammonium is an oxygen-demanding nutrient that consumes oxygen up to 4.57 g O_2/g NH_4-N and is toxic to aquatic microorganisms [5]. The treatment of ammonium is mandatory, and it is removed in the biological stage of WWTPs by the nitrification process [2,4]. Nitrification is a two-step biological process where in the first step, the ammonium oxidizing bacteria (AOB) oxidize the ammonium (NH_4-N) to nitrite (NO_2-N) (equation (1)). At the second step, nitrite oxidizing bacteria (NOB), oxidize the generated nitrite into nitrate (NO_3-N) (equation (2)), which is the end-product of nitrification process [2,6,7].

$$2NH_4^+ + 3O_2 \xrightarrow{AOB} 2NO_2^- + 4H^+ + 2H_2O \quad (1)$$

$$2NO_2^- + O_2 \xrightarrow{NOB} 2NO_3^- \quad (2)$$

Complete nitrification is of major importance, since an incomplete process can lead to the accumulation of nitrite, which is a toxic intermediate [8,9]. Nitrifying bacteria are sensitive to chemical inhibition such as a range of organic and inorganic compounds, heavy metals, and variable and high levels of salinity [5,10]. Fluctuation in salinity inhibit the nitrification process [11] and NOB are sensitive on change in the salinity and inhibited by fluctuation in the salinity [12]. Chen et al.[13], showed that slow adaptation of heterotrophic nitrification on high salinity wastewater could be possible. They are also intolerant to low oxygen concentrations and very sensitive to pH [5,14]. The optimal growth of AOB and NOB has been reported in the pH ranges 7.4–8.2 and

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Table 1
Nitrification inhibition (%) of different dilutions of produced water observed inter-and intra-day.

Inhibition% Dilution	Day	Replicate 1	Replicate 2	Replicate 3
300 mL/L	1	97%	97%	99%
	2	98%	98%	99%
	3	96%	97%	97%
150 mL/L	1	97%	95%	96%
	2	95%	93%	97%
	3	93%	95%	93%
10 mL/L	1	26%	25%	28%
	2	26%	24%	20%
	3	25%	22%	19%
1 mL/L	1	2%	1%	4%
	2	7%	0%	1%
	3	4%	1%	3%

7.5–8.3, respectively [15–18]. Additionally, nitrifying bacteria are slow-growing, due to their autotrophic and chemolithotrophic nature [5,7,14]. In practice, this means that nitrification process can take some time to recover from a potential shock load [19].

It is important to assess WWTP influent for harmful substances that may inhibit the nitrification process. Nitrification inhibition tests such as ISO9509 or the modified ISO9509 method 3:2004, which is common in Denmark, assess the short-term inhibitory effects of wastewater on nitrifying bacteria in activated sludge over an exposure period, with and without the investigated wastewater (ISO 9509, 2006).

Sectors such as the food-processing, nuclear, aquaculture, leather manufacturing, petroleum and natural gas extraction industries produce high-salinity wastewater effluent [20–25]. High organic content, possible harmful substances and high saline concentrations can inhibit nitrification in a receiving treatment plant [1,19,26,27]. Existing nitrification inhibition tests based on activated sludge cannot distinguish toxicity between salt and a toxicant in a sample, which is unrealistic when seeking to regulate highly saline wastewater treated in WWTPs with sufficient volumes to dilute salts to harmless levels, or at least to even out the influents so the treated water maintains a stable salt concentration.

In this work, we described a new method to test nitrification inhibition based on salt-adapted nitrifying bacteria grown on as a suspended biofilm in a high-salt artificial medium. The advantage of this method is that the nitrifying bacteria are able to withstand saline levels that would normally inhibit conventional activated sludge used in existing WWTP nitrification inhibition tests. The aim of this paper was to validate performance on real samples and with specific chemicals and to compare the nitrification inhibition measured with this new salt-adapted nitrification inhibition test with the standard method, namely ISO 9509.

2. Materials and methods

2.1. Reagents

Reagent-grade ammonium sulphate, sodium bicarbonate, sodium chloride, potassium phosphate, methanol, formaldehyde, tetrakis (hydroxymethyl)phosphonium sulphate (THPS), ethanoltriazine and 2-mercaptoethanol were purchased from Sigma-Aldrich (Brønby, Denmark). Sea salt was purchased from Aquarium Systems (Dordrecht, Netherlands). All stock solutions were prepared using ultrapure water provided from the PURELAB Flex dispenser (Veolia, France).

2.2. Sample preservation

Produced water samples taken from various oil production units were received in bottles without headspace and transported and maintained at 4 °C until analysis was initiated. They consisted of salty formation water with natural oil-derived organic substances and different production chemical substances such as formaldehyde, methanol, THPS, ethanoltriazine, 2-mercaptoethanol, etc.

2.3. Salt-adapted nitrification reactor

A laboratory-scale bioreactor was established for the cultivation of salt-adapted nitrifying bacteria in the Department of Environmental Engineering at the Technical University of Denmark. The bioreactor was initiated by adding activated sludge obtained from Lundtofte WWTP, Lyngby, Denmark, 1,000 carriers (Z400) provided by AnoxKaldnes AB, Sweden, and saline water with a concentration of 40 g NaCl/L, which corresponds to a conductivity of 50.5 (±1) mS/cm at 21 °C. To overcome the problem of chemical precipitation, two feed tanks were used to supply the bioreactor. One contained the ammonium stock solution along with bicarbonate and sodium chloride to maintain alkalinity and salinity in the reactor, respectively. The other tank contained seawater together with trace chemicals to enhance the growth of the nitrifying culture [28]. Additionally, a settler was connected in the bioreactor's effluent in order to recirculate the generated sludge.

The reactor was operated at a constant hydraulic retention time of 6.25 d. pH was controlled in the optimal range between 7.4 and 7.8 by adjusting the alkalinity of the ammonium stock solution. Oxygen in the reactors was constantly maintained above 4 mg/L to ensure complete nitrification. At least once a month, 200 mL of activated sludge (MLSS 3.0 ± 1.0 g/L) from different WWTPs was added to the reactor to enhance variety in the nitrifying culture.

2.4. Salt-adapted nitrification inhibition experiment

Conical reactors with a capacity of approx. 1 L were used for the nitrification inhibition experiments. Aeration was carried out from the bottom of the reactors (aeration intensity approximately 600–800 mL air/min), thereby ensuring a homogeneous mixing of the carriers. The tests were performed in 2–3 replicates prepared for each test substance, control and reference inhibitor. Different dilutions of test substances, an ISO medium consisting of ammonium sulphate and sodium bicarbonate, distilled water and up-concentrated saltwater were added to each reactor until a final volume of 500 mL was reached. The latter two were used in selected volumes in order to achieve a final sea salt concentration of 40 g/L (50.5 mS/cm at 21 °C), i.e. salinity similar to the nitrification reactor. Fifty Z400 carriers were added into a 500 mL reactor in order to achieve satisfactory nitrification rates, which ranged between 0.10 and 0.25 mg of N/L min. Samples were taken after 0, 120, 180 and 240 min in order to analyse oxidised nitrogen (NO_2-N, NO_3-N, NH_4-N) levels. Samples were filtered with 0.2 µm pore size syringe filters and stored in the refrigerator until initiation of the analysis – and no more than two days after completing experiment. pH, oxygen and conductivity were monitored in all replicates within the first 10 min of initiating the test and at 60, 120, 180 and 240 min. Initial pH was maintained around 7.6, while conductivity was 50.5 mS ± 0.5. Oxygen was monitored throughout the experiment and maintained above 4 mg/L in order to ensure complete nitrification. After the experiment was completed, the water was discharged and carriers were rinsed well with saline water (40 g/L sea salt) before being placed back into the nitrification reactor.

2.5. Analytical methods

The simultaneous analyses of NO_2-N, NO_3-N, NH_4-N from the water samples were carried out with the auto-sampler San^{++}, SKALAR. THPS was quantified with iodine titration by using the Tolcide PS chemical test kit, #4–8776-01, LaMotte. Formaldehyde was measured with a formaldehyde reagent test set, 3–500 µg/L CH2O, #2257700, Hach Lange. Methanol was quantified by using GC–MS. The quantitative determination of total suspended solids (TSS) was carried out following the standardised method (APHA, 2005). Measurements of oxygen,

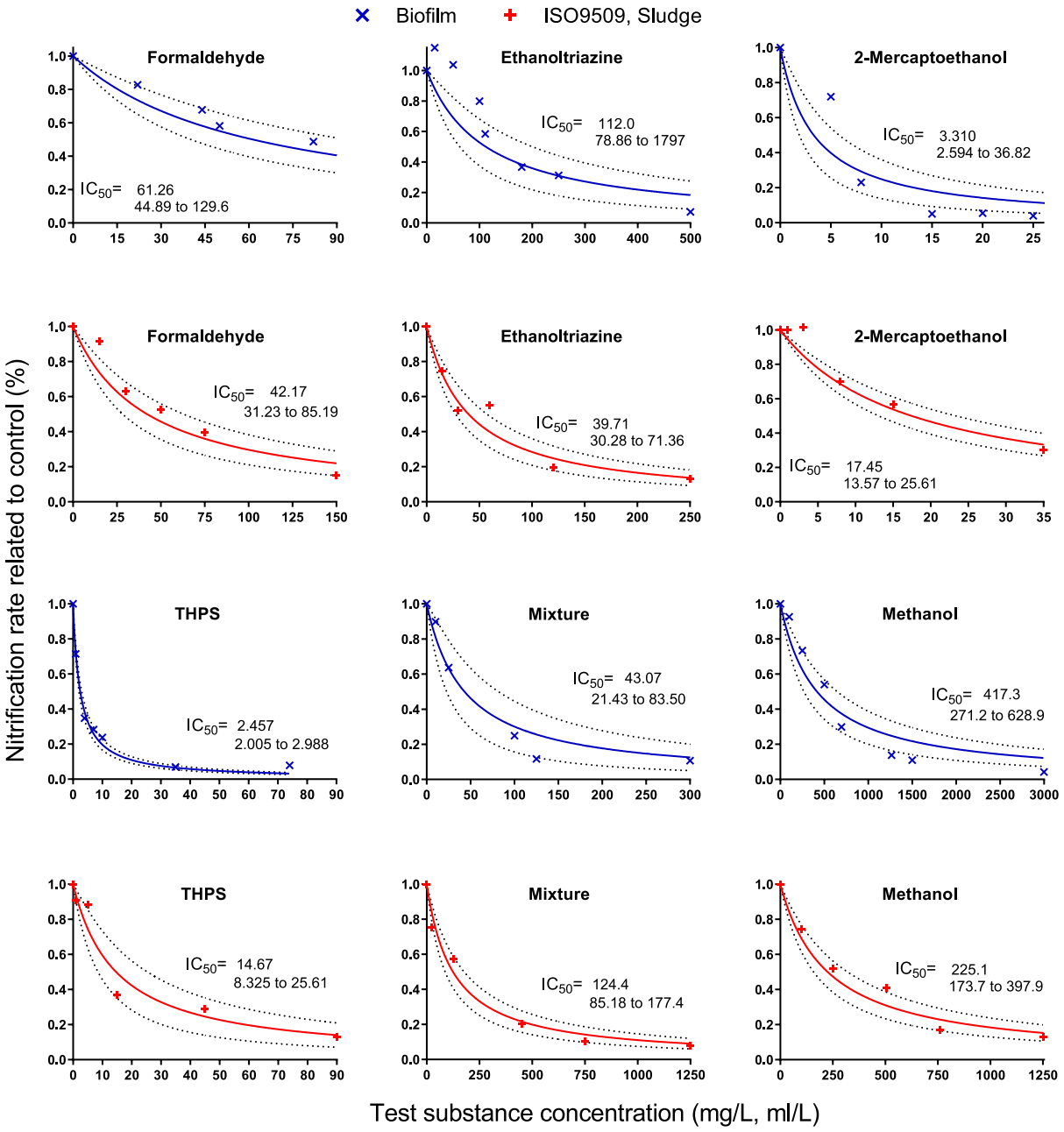

Fig. 1. Dose-response curve for methanol, formaldehyde, THPS, ethanoltriazine and 2-Mercaptoethanol as well as a mixture of chemicals (methanol, formaldehyde & THPS), obtained from nitrification inhibition experiments using the salt-adapted method and the ISO9509 method. Activated sludge from Lundtofte WWTP was used for the ISO9509 method. A blue 'x' symbol represents results from the salt-adapted method, and a red '+' symbol represents results from the ISO 9509 method.

conductivity and pH in the batch reactors were conducted using WTW electrodes (WTW GmbH, Germany).

2.6. Data treatment/Statistical analysis

The estimation of the nitrification inhibition was carried out based on the nitrification rates of the control and the tested samples as follows:

$$Inhibition = 1 - \frac{k_{sample}}{k_{control}} \quad (3)$$

where,

- Inhibition (%) is the inhibition percentage
- k_{sample} is the ammonium degradation rate constant of the sample
- $k_{control}$ is the ammonium degradation rate constant of the control

By plotting a percentage inhibition graph against the logarithm to the concentration of test substance and interpolate, the median inhibition concentration IC_{50} was estimated.

Statistical analysis for the results generated from the different treatments was conducted by using a one-way ANOVA with Tukey's Multiple Comparison Test (95% confidence level) and a T-Test using Graphpad prism 8.0. Treatments were grouped according to significant differences ($P < 0.05$). The repeatability and reproducibility of the salt-adapted nitrification inhibition test method were calculated using the ISO 5725–2 method.

Table 2
Median inhibition concentration (IC_{50}) of chemicals and the mixture of chemicals, obtained from nitrification inhibition tests with the salt-adapted method and the ISO9509 method. The test with ISO9509 was conducted with sludge from Lundtofte WWTP. The 95% confidence interval is presented in parenthesis.

Compound	IC_{50} Salt-adapted method	ISO9509	Significant ($\alpha = 0.05$), p
Formaldehyde (mg/L)	61 (39.7–93.2)	42 (28–61)	No, 16%
Methanol (mg/L)	417 (271–629)	225 (153–312)	No, 7%
THPS (mg/L)	2.5 (2.0–2.9)	15 (8–27)	Yes, 0.4%
Mixture (mL/L)	43 (21–84)	124 (82–177)	Yes, 0.2%
Ethanoltriazine (mg/L)	112 (60–190)	40 (27–56)	No, 5%
2-Mercaptoethanol (mg/L)	3.3 (1.5–5.5)	17 (13–23)	Yes,0.00074%

3. Results and discussion

3.1. Statistical variation of salt-adapted test method results applied to a sample

The nitrification inhibition test was conducted in triplicate on three different days on subsamples of a produced water sample, in order to test inter- and intra-day repetition. On each day, a new bottle of produced water, stored on ice, was used to analyse each dilution level three times. Samples were diluted to different level to achieve different level of inhibition e.g. close to no, median and complete inhibition to calculate the IC_{50} with narrow 95% confidence interval in dose–response curve. Nitrification inhibition test dilutions covered the entire range of outputs from the test, with almost complete nitrification inhibition observed at dilutions of 300 mL/L and 150 mL/L, while dilution at 10 mL/L illustrated typical regulation thresholds of 20 or 30. A dilution of 1 mL/L showed almost no inhibition, whilst the 10 mL/L sample averaged 24% inhibition (Table 1).

To test whether nitrification inhibition observed within the replicates and intra-day was statistically significant, a two-way analysis of variance (ANOVA) test was performed. For the statistical significance analysis, samples with dilutions of 1 mL/L and 300 mL/L were excluded, since they were very close to no and complete inhibition, respectively. ISO method 5725–2 was used to test the repeatability and reproducibility of the same experiment/test performed in different laboratories, using the equation:

$s_R^2 = s_L^2 + s_r^2$ (equation (2))

where s_R^2 is the estimate of the reproducibility variance, s_L^2 is the estimate of laboratory variance and s_r^2 is the arithmetic mean of the repeatability variance. In this study, only one laboratory conducted a nitrification inhibition experiment; however, it was conducted inter-and intra-day. The standard deviations of the salt-adapted test method (s_M) were 1.62% and 2.98% for the 150 mL/L and 10 mL/L dilutions, respectively. The standard errors of mean (S.EM) were 0.54% and 0.99% for 150 mL/L and 10 mL/L dilutions, respectively. A statistical summary of the nitrification inhibition of 10 mL/L and 150 mL/L diluted samples, and detailed results of the two-way ANOVA test, is presented in the supporting information to this article (SI; Figure S1 and Figure S2). In both cases, the nitrification inhibition results do not vary significantly inter- or intra-day.

3.2. Inter- and intra-day reproducibility of the salt-adapted test method on a single chemical at IC_{50}

The inter- and intra-day reproducibility of the nitrification inhibition test using the salt-adapted test method was further assessed for a solution of single toxic chemical. Biologically treated produced water without inhibitory effects was used to dilute formaldehyde for this experiment. Since the bioavailability and sensitivity of microorganisms are affected by all the water components, testing in authentic biologically treated produced water provides realism in terms of the results. Formaldehyde was selected from among a selection of different chemicals relevant to oil well-produced water, since it is stable in terms of concentration changes due to degradation or evaporation and does not interfere with the colorimetric analysis of ammonium.

To achieve 50% inhibition of nitrification, the concentration of formaldehyde that correspond to the IC_{50} (61 mg/L) was spiked to diluted biological treated produced water. Establishing IC_{50} for different relevant chemicals is addressed in the following section. For inter-day repeatability, five sample replicates were tested in the nitrification inhibition experiment, which was repeated four times under the same conditions, in order to verify the intra-day reproducibility of the results. The inhibition percentages of all the replicates were quite similar, with 23 out of the 25 samples in the 41–49% inhibition range.

The reproducibility of the inter- and intra-day results was analysed with a two-way ANOVA, and a statistical summary is presented in the supporting information (Figure S3). Inter-day variation on relation to nitrification inhibition was not significant, albeit intra-day variations were significant but low. The standard deviation (s_M) of the salt-adapted method on the nitrification inhibition of formaldehyde-spiked biologically treated produced water was 2.97%, and the standard error on mean ($S.E_M$) was 0.6%.

3.3. Comparison of the test methods' sensitivity to chemicals

Nitrification inhibitions for methanol, formaldehyde, THPS, ethanoltriazine, 2-mercaptoethanol and a mixture of methanol, formaldehyde and THPS were tested with the salt-adapted method and the

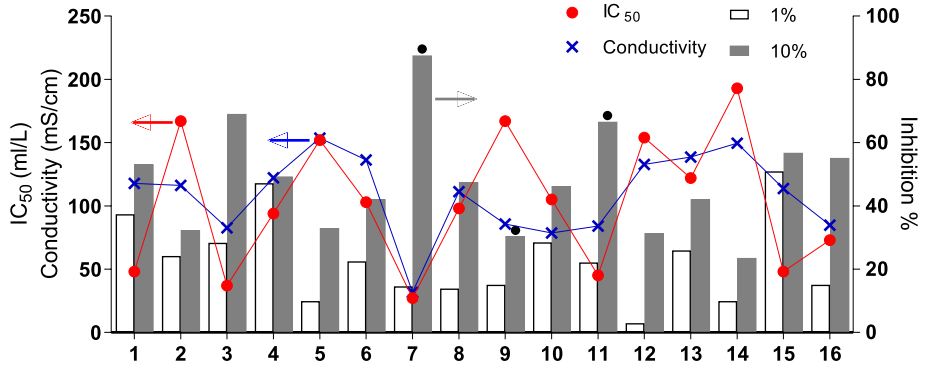

Fig. 2. Nitrification inhibition of 16 water samples collected on different days. Each sample was diluted to 1% and 10% in sea saltwater. The red closed symbol represents the median inhibition concentration (IC50), while the blue cross symbol represents the samples' conductivity. Bar represents the inhibition % of samples diluted to 1% and 10%. A black dot on top of a bar represents the inhibition of the sample with a visibly high oil content.

ISO9509 method. For the ISO9509 method, activated sludge was taken from Lundtofte WWTP, the nitrification rate for which was 3.1 mg N/(g SS h). The chemicals were dissolved in biologically treated produced water with undetectable inhibition, when testing with salt-adapted method and ultrapure water was used for the ISO9509 method, which was diluted with an ISO medium consisting of ammonium sulphate and sodium bicarbonate. Among the five chemicals and the single mixture of chemicals tested, THPS was toxic to nitrifying bacteria, followed by the chemical mixture for both the salt-adapted method and the ISO9509 method (Fig. 1, Table 2).

To verify whether or not the IC_{50} values of chemicals acquired from the salt-adapted method and the ISO9509 method were statistically significant, a *t*-test was done. The statistical analysis, presented in Table 2, shows that differences in the IC_{50} of formaldehyde, methanol and ethanoltriazine between the two methods were not significant. For THPS, the mixture of chemicals and 2-mercaptoethanol, IC_{50} values obtained from the two methods varied to a greater degree – a possible reason for which could be due to the bio-sorption of the chemicals into the sludge. For regulators to accept the use of the modified method to control the quality of salty wastewater it is important that the sensitivity for toxic chemicals is at least as good as the standard method. Thereby ensuring that replacing the method will not result in less ability to detect nitrification inhibition in water and therefore not reduce the protection from reducing the efficient functioning of WWTPs.

3.4. Nitrification inhibition test on actual samples

Sixteen frozen produced water samples were obtained from weekly samples taken from the same point inside a produced water treatment facility, and a nitrification inhibition test was conducted using the salt-adapted method. The samples' pH levels ranged from 6.6 to 7.7, and their conductivity ranged from 83 mS/cm to 154 mS/cm. Three samples were black, i.e. they contained suspended raw oil, likely due to incorrect sampling and the fact that measuring conductivity in the sample collected on day 7 was difficult, due to its high oil content.

Nitrification inhibition of 16 water samples were conducted at dilutions of 1% and 10% in sea saltwater. Results from nitrification inhibition and median inhibition concentration (IC_{50}) is presented in Fig. 2. Nitrification inhibition for the 1% dilution samples varied from 2.9% (the day 12 sample) to 51% (day 15). For the 10% dilution samples, these figures varied from 24% (day 14) to 88% (day 7), as illustrated in Fig. 2.

The sample collected on day 7 showed the highest inhibition effect and the lowest inhibition concentration of 26 mL/L; this sample also appeared to have a high oil content.

Overall, nitrification inhibition for all the samples was significant, and the bacteria were not affected by the salt concentration and were quite similar between samples. This could suggest that inhibition – to a large degree – can be caused by biocides, which are likely added in constant concentrations, while oil components will probably vary significantly between oil wells.

It is important to screen regularly if industrial wastewater inhibit the nitrification process of receiving WWTP by conducting nitrification inhibition test. Industries such as food-processing, aquaculture, leather manufacturing, petroleum and natural gas extraction produce high-salinity effluent that can inhibit nitrifying bacteria at receiving treatment plants. Currently, the existing nitrification inhibition test, namely the ISO 9509 method, cannot identify toxicity caused by salt or another toxicant in a sample, since both salt and toxicants present in water samples are toxic to the bacterial communities found in activated sludge. However, the salt-adapted nitrification inhibition test introduced can make this differentiation, and thus it is well-suited to replace existing nitrification tests for highly saline water samples.

4. Conclusion

A nitrification inhibition experiment using a salt-adapted method well suited to amend the ISO9509 method for high-salinity water samples, since both the repeatability and sensitivity of the former are similar to or better than the latter. Produced water tested in relation to inter- and intra-day repetition showed almost complete nitrification inhibition for 300 mL/L and 150 mL/L dilutions, i.e. an average of 24% inhibition for a 10 mL/L dilution, and almost no inhibition for a 1 mL/L dilution. Statistical analysis revealed that the nitrification inhibition results do not vary significantly inter-and intra-day. The IC_{50} values of formaldehyde, methanol and ethanoltriazine between the salt-adapted method and ISO9509 were not significantly different, either. For THPS, a mixture of chemicals and 2-mercaptoethanol, IC_{50} values obtained from the two methods varied to a greater degree, a possible reason for which could be the bio-sorption of the chemicals into the sludge. However, the lower inhibition concentration of THPS was observed with the salt-adapted method, therefore making the method more sensitive to THPS and thus able to detect it at lower concentrations. This salt-tolerant method for testing the nitrification inhibition of salty wastewater from the food-processing, aquaculture, leather manufacturing, petroleum and natural gas extraction industries is not affected by high salt concentration. Thus, how much toxicity relevant chemical contribute can be identified on an individual basis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2021.132460.

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