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Original Article

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Development of a high-throughput method for screening readily biodegradable chemicals

Aina C. Wennberg[1,*], Merete Grung[1], Malcolm Reid[1], and Adam Lillicrap[1]

[1]Norwegian Institute for Water Research, NIVA, Oslo, Norway

*Corresponding author: Aina C. Wennberg. Email: aina.charlotte.wennberg@niva.no

Abstract

Current standard test methods for assessing biodegradation of chemicals are laborious and not suited for high-throughput screening of chemicals because of both the required volume of the test medium and the limited possibility for automation of measurements of biodegradation. A high-throughput method (HTM) should be miniaturized, suitable for automation, and based on generic parameters that can indicate biodegradation of any chemical. The aim of this study was to develop an HTM based on bacterial proliferation (i.e., growth) as an indicator of biodegradation, measured by flow cytometry. Natural bacterial communities were exposed to reference chemicals in 96-well plates for up to 14 days at 19 °C and the results compared with parallel standard biodegradation screening tests for freshwater (Organisation for Economic Co-operation and Development [OECD] 301F) and seawater (OECD 306). Increased bacterial growth, compared with nonexposed inocula, was used as an indication of biodegradation. Sodium benzoate induced a significant growth response that corresponded to the biodegradation experiments in both freshwater and marine water. Aniline induced a lower frequency of significant growth compared with the frequency of positive biodegradation results, whereas caffeine induced a higher frequency and more rapid growth response compared with biodegradation results. This shows the potential for an HTM for biodegradation testing using bacterial growth.

Keywords: biodegradation, chemical regulation, growth response, miniaturized test, bacterial inoculum

Introduction

There is increased regulatory, societal, and media awareness of environmental pollution from persistent substances. Substitution of hazardous chemicals with safer and greener alternatives is part of the European Union (EU) strategy for a non-toxic environment and a circular economy (European Commission, 2019). Part of the definition of being safe and sustainable is that a chemical is not persistent, meaning that it will degrade in the environment. Also, consumers are becoming more aware of the need for products to be biodegradable, so there is a push to find biodegradable substances when developing or improving products. The Organisation for Economic Co-operation and Development (OECD) published a set of biodegradation screening tests (BSTs; OECD 301, 310, 306) in 1992 (OECD, 1992a, 1992b) that are still the recommended first tier for assessing the persistence of a chemical according to the EU regulation Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; European Chemicals Agency [ECHA], 2023). However, these tests are laborious and not suited for high-throughput screening of chemicals. Thus, this study herein proposes an approach for a high-throughput testing protocol for screening biodegradability.

A high-throughput method (HTM) should preferably be in a miniaturized system to reduce the laboratory footprint and use an indirect measurement of chemical biodegradation that is applicable to any type of organic test chemical and suitable for automation. Standard BSTs use the generic parameters dissolved oxygen (DO), respiration (production of carbon dioxide or pressure drop) or dissolved organic carbon (DOC; OECD, 1992a). However, these methods are difficult to implement in a miniaturized system for high throughput because they either require large sample volumes (i.e., for DOC analysis) or airtight systems (if measuring DO or carbon dioxide) with either internal sensors in individual vessels or sacrificial vessels to be sampled per timepoint.

In a BST, chemicals are inoculated as a sole source of carbon, and biodegradation is linked to the growth of bacteria (Alexander, 1981), meaning the chemical is used by the bacteria for proliferation. The pass criteria for a substance being considered completely degraded is 70% removal for methods measuring DOC and 60% reduction in DO compared with theoretical oxygen demand (ThOD) or 60% production of carbon dioxide compared with theoretical inorganic carbon (OECD, 1992a, 1992b). Thus, it is expected that for completely mineralized compounds, 60% of the carbon is mineralized to carbon dioxide via respiration and up to 40% can be incorporated into new biomass. Increase in bacterial biomass could be used as a sign of biodegradation if the test substance is the only carbon source used for growth and the change in bacterial numbers are compared with changes in a blank control. Flow cytometry (FCM) has been used to measure bacterial proliferation as an indicator of chemical biodegradation in at least three previous studies using mediums without background organic carbon: One study looked at biodegradation of

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sodium benzoate and six different fragrances. They found that 2 mg C/L was enough to detect a significant increase in bacterial biomass compared with the blank control without additional carbon. The tested bacterial communities were sampled from activated sludge and natural lake water (Lake Geneva; Czechowska et al., 2013). A similar study investigated kinetics and growth yields from pesticides using pure bacterial strain cultures (Helbling et al., 2014). A recent study assessed biodegradation of three readily biodegradable reference compounds, phenol, 1-octanol, and benzoate and three fragrances using a lake water bacterial community (Duygan et al., 2021).

The use of FCM methods for evaluating bacterial proliferation, as a generic method to measure biodegradation, is not new. However, all the previous studies used synthetic medium to avoid background organic carbon, and this necessitates pretreatments of inocula to harvest them from the natural environment, which might influence the diversity of the bacterial communities (Martin et al., 2018). In this study, we investigated the high-throughput potential of this method in a miniaturized system and with natural medium and inoculum without removing background carbon. Using 96-well plates as a miniaturized system for biodegradation testing has been evaluated previously where biodegradation was assessed either as a change/depletion in color of the test chemical (Martin et al., 2017a; Thouand et al., 1995) or in 24 well plates with built in noninvasive oxygen sensors (Cregut et al., 2014; Francois et al., 2016). Each of these methods has its limitations based on which type of chemicals to test (only chemicals with color) or scalability (need to invest in plates with built in DO sensors). The purpose of a suggested test setup with 96-well plates combined with FCM is the possibility to evaluate many chemicals simultaneously, for any type of water-soluble chemical without the need for establishing costly chemical analytical methods. In addition, information on how the bacterial communities respond after exposure to the test chemical is collected, that is, inhibition of growth.

The aim of this study was to explore the possibility of using FCM to measure bacterial growth as an indicator of biodegradation of organic chemicals in a miniaturized system for high-throughput analysis. Natural bacterial communities from an effluent from a wastewater treatment plant (WWTP), drinking water from a lake, and seawater were exposed to a set of three reference chemicals in 96-well plates for up to 14 days at 19 ± 2 °C. Increased cell count compared with nonexposed inocula was used as an indication of biodegradation.

Materials and methods

Overview of experiments

Five sets of experiments were performed with BSTs (OECD 301F or 306) paired with experiments using a 96-well plate format with FCM counts of bacteria using the same test medium and test chemicals (see Table 1). Inoculum sources were chosen to cover a wide range of microbial communities used in biodegradation testing, spanning from anthropogenic (WW effluent) to pristine (protected lake used for drinking water) freshwater communities and seawater for marine communities. The use of two different BSTs for comparison was because OECD 306 is the standard test for marine environments, whereas OECD 301 is the standard test for freshwater.

Test chemicals

Three degradable organic chemicals were used as test chemicals; the standard reference test substances aniline and sodium benzoate referred to in OECD 301 and 306 test guidelines, and caffeine, which is proposed as a new reference substance for the OECD 309 test guideline (Hakvåg et al., 2024). Details of the chemicals are listed in Table 2, including the ThOD calculated according to OECD 301 (OECD, 1992a) both with ($ThOD_{NO3}$) and without nitrification ($ThOD_{NH4}$). All chemicals were tested at a concentration corresponding to approximately 25 mg/L organic carbon in the well plates and OECD 301F tests, and at approximately 6 mg/L $ThOD_{NH4}$ in the OECD 306 tests.

Source of microbial inoculum and test media

Test media were prepared according to OECD 301F or OECD 306 test guidelines. The OECD 301F test media were prepared either with effluent from a municipal WWTP (VEAS Oslo, Norway) or freshwater from a relatively pristine lake (Elvåga Oslo, Norway [59.860 N, 10.9119 E]) used as a drinking water reservoir for the Oslo municipality. Effluent was collected on 2 days (08.02.2024 and 16.04.2024) and was placed in a refrigerator for 1 day after collection. The samples were then aerated at test temperature for 2 to 5 days before dilution with deionized water (10–50 ml per 1 L final volume) on the days of the experiments. Nutrient stock solutions were added according to OECD 301F (10 ml stock A, 1 ml stock B, C, and D per liter) and pH adjusted to between 7.3 to 7.5. Lake water was collected below the surface at approximately 50 cm depth approximately 1 to 2 m from the shore and brought directly to the laboratory within 1 hr (May 22, 2024). Water was filtered through a 10 μm nylon sieve to remove protozoa and large particles before adding nutrient stock solutions according to OECD 301F (10 ml stock A, 1 ml stock B, C, and D per liter). The pH was not adjusted (7.7) as it was only slightly out of the specified range of 7.4 ± 0.2.

The OECD 306 test media were prepared from seawater collected from a depth of 50 m from the Oslofjord (Drøbak, Norway [59.621 N, 10.6494 E],) on February 15, 2024, and June 11, 2024, and stored at test temperature with aeration until use (6 and 1 days, respectively). The seawater was also filtered through a

Table 1. Overview of pairs of experiments of standard Organisation for Economic Co-operation and Development (OECD) guideline tests (BST) and the new high-throughput test (HTM) with sampling times per test.

Experiment ID	Inoculum	OECD Test guideline	Sampling times BST	Sampling times HTM
Effluent 1	WW effluent (10 ml/L)	301F: OxiTop	Continuous 28 days	Day 0, 2, 5, 9, 14
Effluent 2	WW effluent (50 ml/L)	301F: OxiTop	Continuous 14 days	Day 0, 3, 5, 7, 14
Lake water[a]	Lake water	301F: OxiTop	Continuous 28 days	Day 0, 2, 7, 14
Seawater 1	Seawater	306: closed bottle	Day 0, 2, 9, 12, 21, 28	Day 0, 2, 7, 9, 12
Seawater 2	Seawater	306: closed bottle	Day 0, 5, 8, 14	Day 0, 1, 2, 5, 8, 12, 14[b]

Note. WW = wastewater.
[a] Only aniline and caffeine.
[b] Sampling for chemical analysis of caffeine on Day 14.

Table 2. Test chemicals, structure, supplier, purity, molecular weight (Mw), carbon content (%C), theoretical oxygen demand (ThOD) and test concentrations.

Test substance	Molecule formula	Supplier (catalogue no)	Purity (%)	Mw (g/mol)	%C	$ThOD_{NH4}$/$ThOD_{NO2}$ (mgO/mg)	Test conc. (mg/L)	Test conc. OECD 306 (mg/L)
Aniline	$C_6H_5NH_2$	Sigma Aldrich (242284)	≥99.5	93.13	77	2.41/3.09	31–34	2.5–2.6
Sodium benzoate	$C_7H_5NaO_2$	Sigma Aldrich (71300)	≥99.0	144.10	58	1.67/1.67	42–43	3.6
Caffeine	$C_8H_{10}N_4O_2$	Sigma Aldrich (PHR1009)	99.7 ± 0.8	194.19	49	1.07/2.39	22–51	5.5–5.6

Note: OECD = Organisation for Economic Co-operation and Development.

10 μm nylon sieve before adding nutrient stock solutions according to OECD 306 (1 ml stock A, B, C, and D per liter).

Standard BST methods

The OECD 306 closed bottle test method was used for seawater with a test concentration of 2.5 to 5 mg/L, corresponding to a $ThOD_{NH4}$ of 6 mg/L. Glass bottles (290 ml) with ground glass toppers were filled until overflow with test medium spiked with test substance stock solution and incubated submerged in a water bath. Dissolved oxygen was measured with an optical DO probe (SevenExcellence) in sacrificial bottles.

The OECD 301F respirometer method was used for media with effluent and freshwater from a lake. Test chemicals were added at the same concentration as in the well plates at approximately 25 mg C/L corresponding to 77, 71, and 54 mg/L $ThOD_{NH4}$ for aniline, sodium benzoate, and caffeine, respectively. OxiTop C heads (WTW) were used to measure pressure drop as evolved carbon dioxide was adsorbed to sodium hydroxide pellets. Respiration was measured continuously in sealed bottles throughout the incubation time. Test medium (250 ml) spiked with test chemical was added to amber glass bottles containing a magnetic bar and placed on a magnetic stirring platform (OxiTop Var 12) in a temperature-controlled room.

All BSTs were run with three replicates and incubated at 19 ± 2 °C. Results were reported as percentage of biodegradation and calculated as blank corrected decrease in DO compared with ThOD. The possibility of oxygen consumption due to nitrification must be considered for compounds that contain nitrogen, such as aniline and caffeine. No measurements of nitrite and nitrate were performed. However, when a two-phase degradation curve was observed or final oxygen consumption exceeded what would be expected without nitrification, full nitrification was assumed using $ThOD_{NO3}$ for calculations; otherwise, no nitrification was assumed (using $ThOD_{NH4}$). Indication of nitrification was only observed for caffeine in effluent samples and not for aniline. Biodegradation of the reference compounds aniline and sodium benzoate was compared with the validity criteria of readily biodegradation testing of more than 60% reduction in ThOD within 10 days from the point when the test substance had reached 10% degradation (OECD, 1992a).

Experimental design of HTM

A comprehensive pretest with exploration of different inoculant and different inoculum treatments and dilutions, different chemical concentrations, test chemicals, and incubation times is described in the online supplementary material. The pretest was done to investigate whether information that can be obtained on bacterial community using FCM counts during exposure to the test chemicals and was not supported by biodegradation testing to verify whether biodegradation had occurred. Thus, the results of the pretests are not described further in this article.

To allow for high throughput and automation, incubation of environmental inoculum with test chemicals was performed in (deep well) 96-well plates (Axygen, P-DW-20-C). A total of 1 ml inoculum was added to each well and spiked with 10 μl chemical stock solution and/or dilution water. One row with nine wells was used for each test chemical and blank control. The remaining wells per row were used as sterility controls or left empty. Plates were sealed with sterile sealing tape and shaken on a plate shaker at 1,000 rpm for 1 min before being placed in a light tight box in a climate-controlled room at 19 °C and incubated for 12 to 14 days. Before sampling, the plates were shaken using the same method to suspend bacteria in the water and the sealing tape removed during sampling and replaced afterwards. This also ensured replacement of the head space to reduce the risk of oxygen depletion. Sampling for FCM was carried out by pipetting 10 μl from the deep-well plate to a 96-well plate that was compatible with the FCM instrument; thus, the sampling volume was only 1% of the total well volume per sampling point.

Biological data analysis—FCM

Samples were stained with SYBRGreen I (SG; SYBRGreen I 10,000× concentrate in DMSO [Invitrogen, S7563]) for total cell count, and counter stained with propidium iodide (PI; Invitrogen, P1304MP) to enable cells with damaged membranes (i.e., dead cells) to be excluded (Nescerecka et al., 2016; Wennberg et al., 2022). However, as a low saline staining buffer might cause osmotic stress on seawater bacteria, which would enable PI to cross the cell membrane, PI counterstain was not used on the seawater samples; thus, total cell counts were reported for these samples. Analysis was run in Nunc flat bottom 96-well plates containing 10 μl undiluted or 1:10 diluted sample and 90 μl staining buffer consisting of phosphate-buffered saline and SG or SGPI (SG 10,000 diluted stock, PI at 6 μM final concentration), shaken for 1 min at 1,000 rpm and incubated for 10 min at 35 °C on a shaking heat plate (Thermal Shake lite, VWR) before immediate counting on the FCM. Bacterial cell counts were performed using a flow cytometer NovoCyte Advanteon (Agilent Technologies, Inc.) with excitation from a laser at 488 nm and detectors on filters 530/30 nm and 675/30 nm with a detection threshold set to >2,000 green fluorescence from filter 530/30 nm. Electronic gating was used for separating typical bacterial populations from background noise using the NovoExpress software (Agilent Technologies, Inc., 2023).

Caffeine analysis

Samples were diluted 5,000 times in MilliQ water and then analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an Acquity UPLC system (Waters) connected to a Xevo TQ-S mass spectrometer (Waters). Chromatography was done on an Acquity BEH C8 column (Waters) with a mobile phase gradient consisting of 5 mM ammonium formate with 0.005%

formic acid (Phase A) and 25% methanol in acetonitrile (Phase B). The LC-MS/MS transitions monitored were (m/z) 195 > 110 and 195 > 138 for caffeine.

Statistical analysis

NovoExpress software (Agilent Technologies, Inc., 2023) was used for processing FCM plots to generate bacterial count data that were exported to JMP statistical software (SAS Institute Inc., 2021) for statistical analysis and plotting graphs.

All cell counts were $\log_{10}$ transformed before further data processing. For each sampling point for each chemical, a test for normal distribution was performed on the nine datapoints by using the Andersson-Darling test where $p < 0.05$ was considered significant. The normal quantile (QQ) plot was inspected for the nine sampling points (out of a total of 96) that were not normally distributed. A QQ plot was used to identify and remove outliers defined as 1.5 times the interquantile range from the lower and upper quantiles. Datapoints defined as statistical outliers (17 of 855 datapoints) were removed from the dataset (see online supplementary material S1). For assessment of biodegradation, growth of exposed bacteria was compared with the blank controls by normalizing the individual counts to the mean of the blank controls for the same sampling time, and the relative difference was used for plotting the growth curves. Significant growth was defined by comparing the count from nine wells exposed to test chemicals with the nine wells of blank controls, using Dunnet's comparison of means and a significance level of $p < 0.05$.

Results

Sodium benzoate

Sodium benzoate exceeded 60% biodegradation between Days 3 to 9 of incubation with no measurable lag phase in all four BSTs (Figure 1A). The lake water experiment did not include sodium benzoate due to limited capacity. There was an increase in cell growth in the HTM experiments that corresponded to the biodegradation curves in the BSTs, with a significant increase in bacterial count compared with the blank on Days 1 to 3 (Figure 1B). However, the relative increase in bacteria count subsided rapidly in the experiments with effluent as the inoculum, whereas the high count relative to control was maintained for 14 days in the first seawater experiment. Intact cell count was used for all experiments except for seawater, where total cell count was used. Thus, it is possible that the maintained high counts in seawater were dead cells. In summary, an indication of biodegradation of sodium benzoate, based on bacterial proliferation, is feasible if counts are performed within the first 3 days after incubation but might be missed if the assessment is done after Day 5 if the growth has already started to decline.

Aniline

Aniline exceeded 60% biodegradation on Days 8 and 12 in both seawater BSTs (Figure 2A). The corresponding HTM experiments had at least one time point where the mean bacterial count was significantly higher than the blank controls (Figure 2B). For the first seawater experiment, only Day 2 had significant growth, whereas there was no significant difference of the means for the rest of the incubation period due to large variance between the wells. In the second seawater experiment, there were significantly higher bacterial counts compared with the blank control from Days 2 to 12, with the highest increase on Day 8, corresponding well with the biodegradation curves in the standard test.

For the Effluent 2 experiment, the BST passed 60% degradation on Day 9 for all replicates, whereas on Day 6, one replicate had no degradation. There was no timepoint when there was statistically significantly higher bacterial count when comparing means of the nine replicates in the corresponding HTM. However, there was variation between the wells, and it appeared that the increased bacterial count on Day 14 might be the beginning of an increasing trend; however, no more samples were taken in this experiment. The slower response in growth in the well plate compared with the biodegradation curve from the OECD 301F test might be because of the smaller test volume (1 ml compared with 250 ml), which results in fewer cells in the test (Martin et al., 2017b).

In two experiments, the lag phase of the BST was much longer than the others (7–18 days) and one and two replicates showed no degradation after 28 days. According to the validity criteria of OECD 301, this would mean these tests had failed due to a weak inoculum. These two experiments (Effluent 1 and lake water) also did not have any significant increase in cell counts compared with the blank control in the HTM experiments.

Thus, for the experiments with biodegradation of aniline, there was also growth when biodegradation occurred within the first 14 days of incubation, and a failure of the standard test (i.e., > 20% difference between replicates, or degradation after Day 14) would also mean a failure of the new method. However, for Effluent 2, the growth response was slower compared with the standard tests.

Caffeine

Caffeine was degraded within Day 14 in both BSTs using effluent inocula with a lag phase of 3 days (Figure 3A). In the Effluent 2 experiment, the biodegradation curve had two phases, with a rapid degradation from Days 3 to 4, a delay, and a second degradation from Days 7 to 12. The total oxygen consumption corresponded to 100% ThOD after full nitrification. Thus, the second part of the degradation occurring after Day 4 is probably related to nitrification that would not support growth. If the same process happened in the well plate, it would not be expected to give any increase in bacteria after Day 3, as nitrification is a catabolic reaction that would not generate biomass. The corresponding HTM experiments had a significant increase in bacterial counts compared with the blank control on Days 5 and 14 for Effluent 1 and only on Day 3 for Effluent 2 (Figure 3B).

There was only partial degradation in BSTs with seawater and lake water (Figure 3A) starting from Days 12 to 15 in at least one replicate per experiment. In Seawater 1, only one of three replicates had any degradation by Day 12 (32%; Figure 3A), whereas the corresponding HTM experiment had some individual wells in which the bacterial count was higher than the mean blank control on Days 7 and 9, whereas a significant difference to blank for all wells was only observed on Day 12 (Figure 3B). Also, for Seawater 2, only one replicate had biodegradation (39%) on Day 14, whereas the corresponding HTM experiment had significant growth on Days 2 and 5. Chemical analysis (LC-MS) of samples from the well plate on Day 14 indicated that caffeine had a partial primary degradation of between 70% to 90% from the nominal concentration (see online supplementary material). If the increase in bacterial count on Day 2 was due to the growth of caffeine degraders, it is surprising that caffeine was not completely degraded by Day 14, but this would need further investigation to verify the reason. For lake water, biodegradation reached 37% to 55% on Day 20, whereas the corresponding HTM had bacterial counts significantly lower than the blank on Day 2 but higher than the blank controls on Days 7 and 14. Unfortunately,

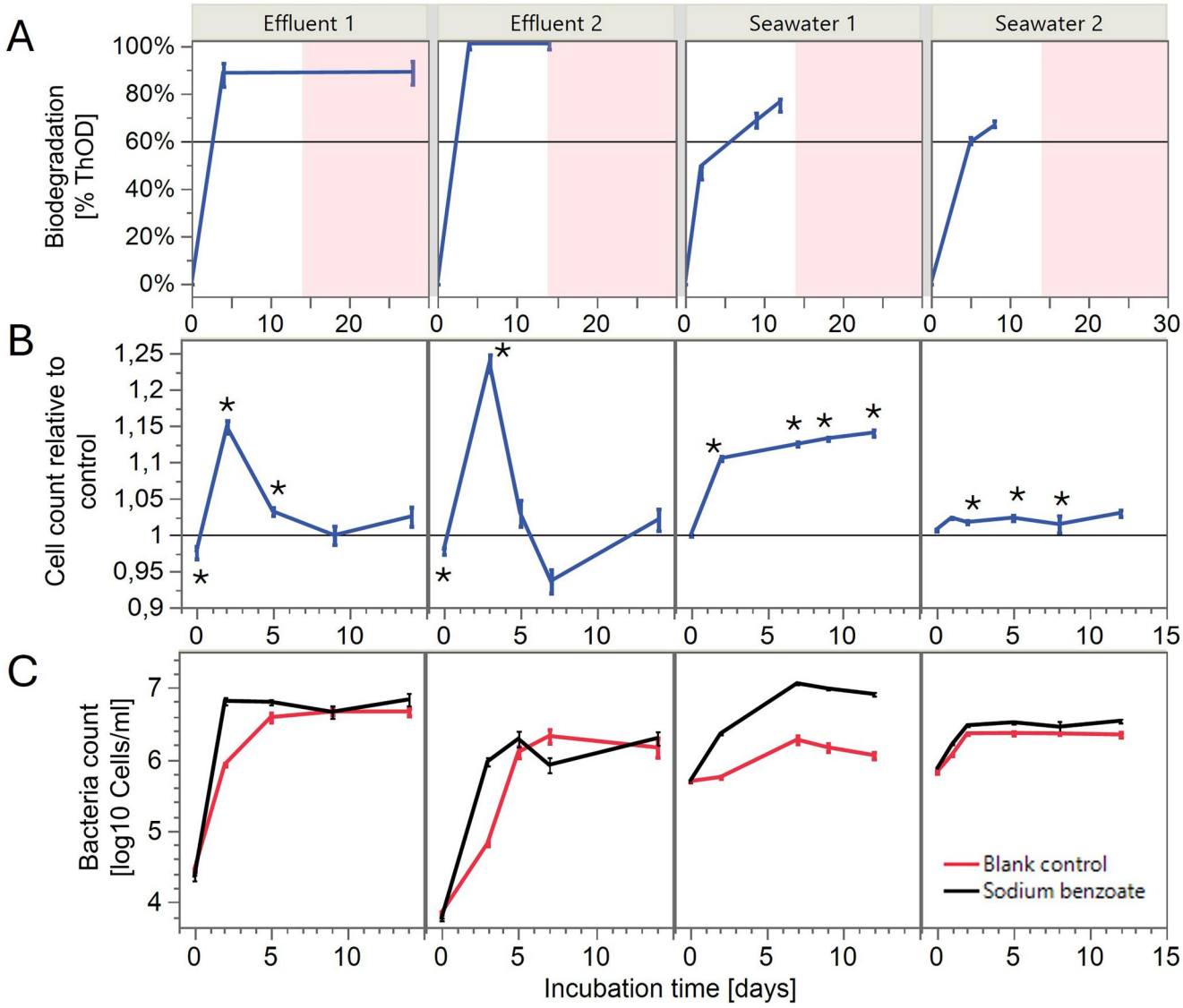

Figure 1. (A) Standard method: Biodegradation of sodium benzoate as a percentage of theoretical oxygen demand (ThOD). The line is drawn between the median of triplicate measurements with error bars representing the minimum and maximum value. The line at 60% represents the threshold for classifying a readily biodegradable substance. (B) New method: Bacterial growth, measured as $\log_{10}$ cell count relative to blank control for samples exposed to sodium benzoate. The line is drawn between the mean of nine samples with error bars representing the *SD*. Significant differences to the blanks are marked with *. The horizontal line at 1 represents equal to blank control. (C) New method: Mean and *SD* of bacterial count in blank controls and samples exposed to sodium benzoate.

samples were not taken for chemical analysis from the well plates to verify these results.

In summary, there was a good correlation of responses from BST and HTM for effluent inocula; however, the growth response was stronger (i.e., earlier and in more replicates) than what could be expected based on BST for seawater and lake water inocula.

Discussion

This HTM is not an alternative to a regulatory testing for biodegradability but could be used as a screening tool for comparing biodegradation potential between biodegradable substances. The growth response was not equal across different inocula or chemicals, so it cannot be used as a quantitative measure of biodegradation (i.e., percentage of mineralization or primary degradation). However, it gives new information on how different environmental communities respond to exposure to the chemicals, with the potential for discovering inhibition and to discriminate substances that give rise to high biomass production or other uses that are not yet foreseen. This study was not extensive enough to establish frequency of false-positive or false-negative results nor the applicability domains regarding chemical space and biodegradation rates. However, given the variability also in the standard biodegradation tests, variability in results related to inoculums is something that always needs to be considered when assessing persistence. A wider range of chemicals and concentrations were explored in the range finding study described in the online supplementary material, including different test concentrations.

In the interest of having an HTM, a well-plate format was chosen, although it is known that smaller volume gives more variability (Martin et al., 2017b). A deep well plate was chosen, with 1 ml medium instead of 150 µl as used by Thouand et al. (1995). It cannot be ruled out that oxygen depletion could be a factor for high substance concentrations and high background carbon, especially when a concentrated inoculum or nondiluted effluent is

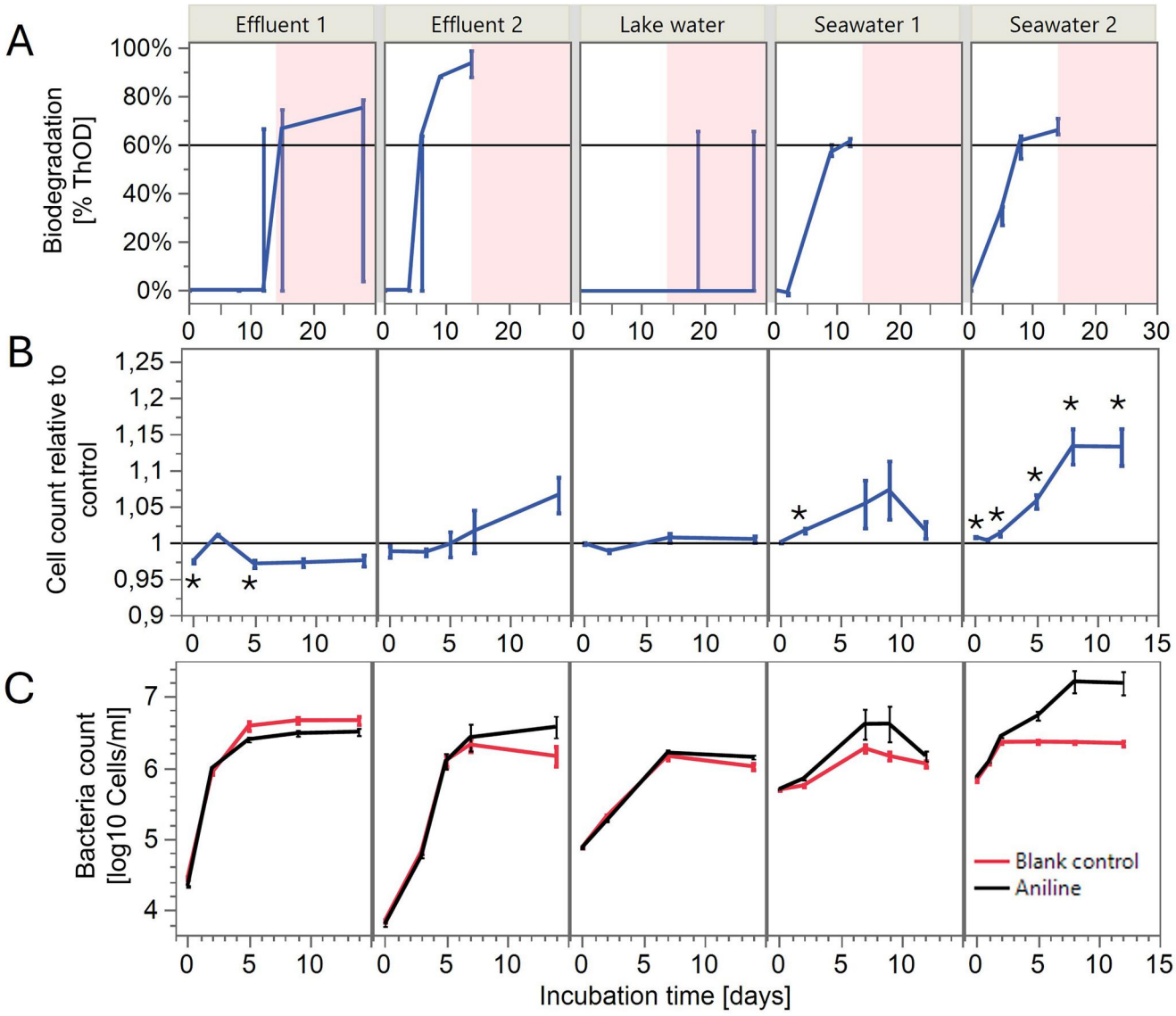

Figure 2. (A) Standard method: Biodegradation of aniline as a percentage of theoretical oxygen demand excluding nitrification ($ThOD_{NH4}$). The line is drawn between the median of triplicate measurements with error bars representing the minimum and maximum value. The line at 60% represents the threshold for readily biodegradable substance. The colored area is outside of the timeframe for the FCM counts. (B) New method: log_{10} cell count relative to blank control for samples exposed to aniline. The line is drawn between the mean of nine samples with error bars representing the *SD*. Significant differences to the blanks are marked with an *. The horizontal line at 1 represents equal to blank control. (C) New method: Mean and *SD* of bacterial count in blank controls and samples exposed to aniline.

used. However, frequent sampling with replacing the headspace would limit the chance of this. A high degree of growth in the blank control did not give less chance of significant increased growth from biodegradation for sodium benzoate and caffeine, as was apparent for the effluent samples where there was 2 log_{10} increase in the blank control. We suspected that there might be a maximum cell count for starting the test, but additional experiments performed in follow-up studies showed that this was not the case either. Thus, there is not enough data in these experiments to suggest parameters for validity criteria.

In summary, sodium benzoate had a growth response in HTM that corresponded with the biodegradation in BST in all experiments. Aniline had a lower frequency of growth compared with the frequency of positive biodegradation results, and caffeine had a higher frequency and more rapid growth response compared with BST biodegradation results. Most water sources with anthropogenic influence will have a bacterial community that is preexposed to caffeine, as this substance is so prominent in waste water that it can be used as a marker for waste water contamination of environmental waters (OECD, 1992a). Thus, it is not surprising that caffeine had higher and quicker degradation in the effluent inoculum compared with the natural water inocula (seawater and lake water). However, the fast significant growth in the HTM for these inocula is surprising and should be further investigated.

Depending on the diversity of the microbial community and the variation of abundance of the different species, the smaller the subvolume of inoculum the fewer species will be included in each volume and likely a variation of species in each subvolume. This is referred to as the microbial lottery (Gu et al., 2021) and is the reason why it has been proposed to increase test vessel volumes or concentrate the inoculum to improve screening tests (Martin et al., 2017b; Ott et al., 2020). This might explain the lack of growth response and likely lack of degradation of aniline in

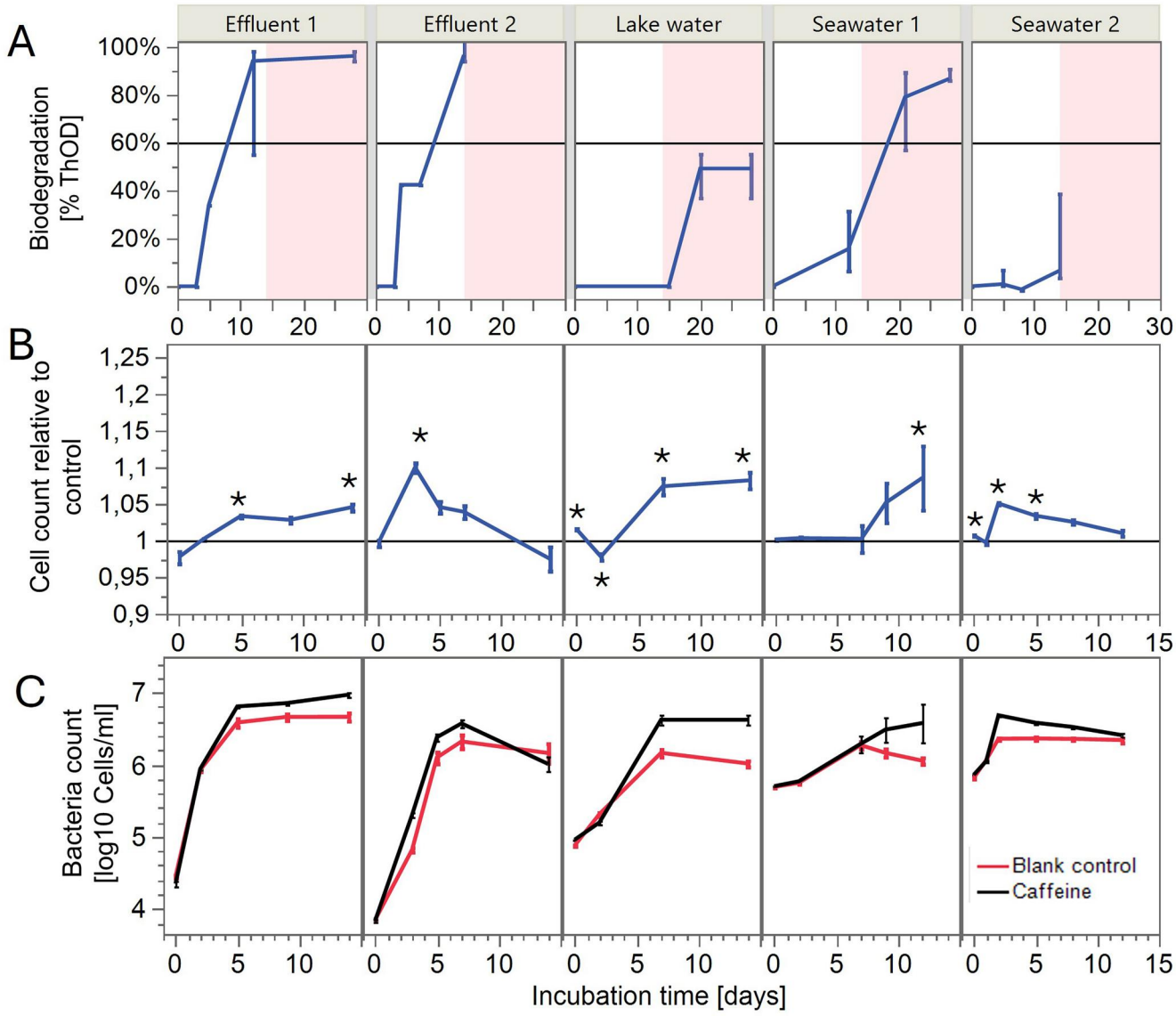

Figure 3. (A) Standard method: Biodegradation of caffeine as a percentage of theoretical oxygen demand (ThOD) including nitrification ($ThOD_{NO3}$) for effluent inoculum and excluding nitrification ($ThOD_{NH4}$) for seawater and lake water. The line is drawn between the median of triplicate measurements with error bars representing the minimum and maximum value. The line at 60% represents the threshold for readily biodegradable substances. The colored area is outside of the timeframe for the flow cytometry counts. (B) New method: Bacterial growth measured as $\log_{10}$ cell count relative to blank control for samples exposed to caffeine. The line is drawn between the mean of nine samples with error bars representing the *SD*. Significant differences to the blanks are marked with an *. The horizontal line at 1 represents equal to blank control. (C) New method: Mean and *SD* of bacterial count in blank controls and samples exposed to caffeine.

this miniaturized system even when there was degradation in the larger bottles. Both aniline (Vázquez-Rodríguez et al., 2007) and caffeine require specialist degraders that are often considered to be rare species (Wang et al., 2017). This is another reason that the fast growth from caffeine in seawater and lake water was surprising. Chemical analysis verified only partial primary degradation of caffeine in the second seawater experiment, and no analysis was done for lake water. Thus, there is a possibility that this represent false-positive results (i.e., growth that is not the results of metabolization of caffeine) that need further research.

The different responses of the reference substances in both the BST and HTM feeds into the debate on the suitability of the currently used reference substances for regulatory biodegradation testing (Davenport et al., 2022; Gu et al., 2021; Strotmann et al., 2023). The only test substance that was degraded in every experiment was sodium benzoate. The purpose of a reference substance as a positive control in a biodegradation test is to verify the experimental design, including the activity of the inoculum. However, sodium benzoate might degrade without added inoculum, according to the OECD 301 test guideline (OECD, 1992a). In a study by Gu et al. (2021) looking for a better alternative for the positive control to verify the activity of the inoculum, sodium benzoate was reported to be degraded even in sterile water, whereas aniline did not degrade without added inoculum (Gu et al., 2021). In the pretest (see online supplementary material), sodium benzoate resulted in significant growth even when diluting the inoculum 100 times, also indicating that the sodium benzoate degraders are ubiquitous. Thus, sodium benzoate should not be used as a verification of the viability or biodegradation potential of the inoculum but function as a method control for verifying the experimental set up and analytical method.

The previous studies using FCM for biodegradation evaluation all used synthetic medium to avoid background organic carbon (Czechowska et al., 2013; Duygan et al., 2021; Helbling et al., 2014). This study shows that significant growth from biodegradation can be observed even with natural medium and inoculum. However, the challenge with complex microbial communities is the unknown community dynamics that sometimes leads to a collapse of some subcommunities, as was seen as a decline in cell numbers in some of our experiments. The consequence are that the growth responses were temporary for some of the experiments, thus making it necessary to have sampling intervals adjusted to the expected biodegradation kinetics to capture the growth responses, with daily sampling for the more rapidly degrading substances such as sodium benzoate.

Only cell count information from the FCM analysis has been used in this study. However, the FCM also gives information on relative differences in size, morphology, and nucleic acid content that can be used to assess a "microbial fingerprint" and changes in the microbial community in the time course of a biodegradation test (Wennberg et al., 2022). Thus, this method has the potential to give more information on microbial inocula and responses of the bacteria to contact with test chemicals, including inhibition, as is described in the online supplementary material. The intact cell count as a proxy for live cells were used for the freshwater inocula was considered most relevant. In retrospect, and looking at the seawater counts where total cell count was used because of problems with osmotic stress during staining, it is possible that total cell count would give a better indication of total biomass produced from metabolizing the test substances.

This study has shown that it is feasible to measure the growth response of bacteria due to biodegradation in different natural media and inocula for the reference substances aniline and sodium benzoate in a HTM using FCM. Although this HTM offers a rapid screening for estimating biodegradation, further research is needed to explore the applicability of the method for a wider range of test chemicals. Further development of the method could include a comparison of intact and total cell count for biomass formation. A combination of cell counts with adenosine triphosphate analysis of total biomass in each well could also be tested to account for biofilm formation. A wider range of chemicals should also be tested to determine the chemical space of the applicability domain of the method.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contributions

Aina Charlotte Wennberg (Conceptualization, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Merete Grung (Conceptualization, Formal analysis, Writing—review & editing), Malcolm J. Reid (Conceptualization, Writing—review & editing), and Adam Lillicrap (Conceptualization, Writing—review & editing)

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Conflicts of interest

The authors declare no conflicts of interest.

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