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# High Throughput Biodegradation-Screening Test To Prioritize and Evaluate Chemical Biodegradability

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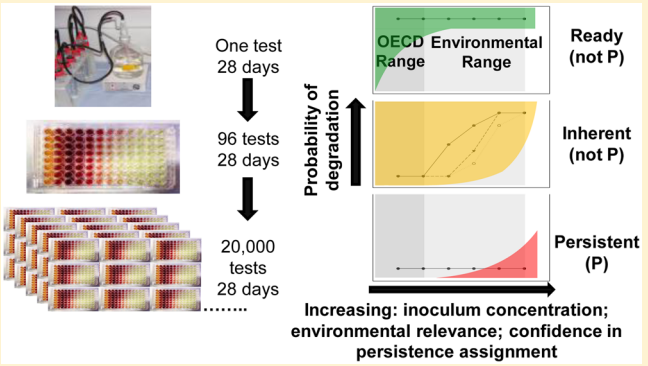
Supporting Information

**ABSTRACT:** Comprehensive assessment of environmental biodegradability of pollutants is limited by the use of low throughput systems. These are epitomized by the Organisation for Economic Cooperation and Development (OECD) Ready Biodegradability Tests (RBTs), where one sample from an environment may be used to assess a chemical's ability to readily biodegrade or persist universally in that environment. This neglects the considerable spatial and temporal microbial variation inherent in any environment. Inaccurate designations of biodegradability or persistence can occur as a result. RBTs are central in assessing the biodegradation fate of chemicals and inferring exposure concentrations in environmental risk assessments. We developed a colorimetric assay for the reliable quantification of suitable aromatic compounds in a high throughput biodegradation screening test (HT-BST). The HT-BST accurately differentiated and prioritized a range of structurally diverse aromatic compounds on the basis of their assigned relative biodegradabilities and quantitative structure−activity relationship (QSAR) model outputs. Approximately 20 000 individual biodegradation tests were performed, returning analogous results to conventional RBTs. The effect of substituent group structure and position on biodegradation potential demonstrated a significant correlation ($P < 0.05$) with Hammett's constant for substituents on position 3 of the phenol ring. The HT-BST may facilitate the rapid screening of 100 000 chemicals reportedly manufactured in Europe and reduce the need for higher-tier fate and effects tests.




## INTRODUCTION

Microbial biodegradation, which can determine the fate and burden of anthropogenic organic chemicals in the environment, is an important but poorly predictable process. It is one of the few processes that can transform potentially toxic chemicals into less harmful products and can lead to their complete mineralization into carbon dioxide, water, and inorganic nutrients and ions. Comprehensive assessment of biodegradation in the environment is limited by the use of low throughput "one sample-one test" systems that neglect the considerable structural and functional diversity inherent in natural microbial communities. Such tests are epitomized by the OECD (Organisation for Economic Co-operation and Development) 301 Ready Biodegradability Tests (RBTs)[1] that assess aerobic biodegradation in aqueous media, for which an inoculum of activated sludge is widely used.[2] They are at the forefront of international regulatory screening of manufactured chemicals, which number 100 000-plus in Europe alone.[3] The hazards of up to 90% of these chemicals are unknown.[4] The European registration, evaluation, authorization, and restriction of chemicals (REACH) directive was therefore introduced to streamline chemical legislation[5] and advocates the prioritization of chemicals based on hazards, including their ability to persist in the environment. The first tier of persistence assessments involves biodegradation screening tests (BST) such as RBTs. Indeed, unlike other biodegradation tests, RBTs are central to chemical hazard classification, labeling and packaging (e.g., persistent, bioaccumulatory, and toxic, PBT; very persistent and very bioaccumulative, vPvB regimes), environmental risk assessment (chemical safety assessment), and persistency assessments (e.g., in PBT).[6] While the legislative regimen has been streamlined, the tests have not. Not only are RBTs low throughput, but they also lack replication and are notoriously

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highly variable[7−10] and unreliable, mainly due to the environmentally unrealistic low inoculum concentrations and high substance concentration used (see ref 11 for a review). The ability of a chemical to biodegrade or persist in a global environment containing billions of cells, from thousands of species, is often concluded on the basis of as few as one diluted inoculum sample from a single environmental compartment. Consequently, it is estimated that 20−80% of fails reported in RBTs may be false negatives, which occur due to the low inoculum concentrations stipulated within standard RBTs, and result in unnecessary, costly, higher-tier assessment of chemicals that are amenable to biodegradation.[11,12] These shortcomings severely limit the quick and accurate identification of persistent chemicals from the thousands of new and existing chemicals being registered under REACH.

Despite the relatively low cost of persistence tests and guidance to perform such tests first,[13] improving the throughput of bioaccumulation and toxicity tests has received more attention[14] principally toward the reduction of animal testing (e.g., the replacement, reduction, and refinement 3 Rs framework[15,16]). However, failure to improve the scientific basis for persistence screening may result in unnecessary animal testing for bioaccumulatory and toxic (BT) or very bioaccumulative (vB) characteristics, when persistence has been falsely assigned. In RBTs, aerobic biodegradation is monitored using either nonspecific (oxygen demand, carbon dioxide evolution, or dissolved organic carbon, DOC) or more specific chemical analyses (detection of parent chemical concentrations using high performance liquid chromatography, HPLC, and/or mass spectrometry, MS), which measure so-called ultimate (i.e., complete mineralization) or primary (parent chemical disappearance) biodegradation, respectively. Innovations in sensor technologies are now allowing the high throughput measurement of ultimate biodegradation through oxygen uptake measurements.[17] However, many of the analyses above are unsuited for development into a high throughput format, especially for primary biodegradation.

A high throughput biodegradation screening test in a 96-well plate format has recently been developed to evaluate primary 4-nitrophenol biodegradation, which changes from yellow to colorless when degraded.[18] This assay has been used to demonstrate that inoculum concentrations and preparation methods used in international RBTs lead to highly variable unreliable tests and that tests with more environmentally relevant inoculum concentrations improve their reliability.[10] The frequency or probability of biodegradation it provides could be a useful measure to evaluate chemical fate[10,19] and can be used to estimate most probable number (MPN) counts of specific pollutant-degrading microorganisms. Degradation of 4-nitrophenol represents a special case due to its color and high molar extinction coefficient and is therefore not typical of many other chemicals.

Many common pharmaceuticals, personal care and hygiene products, pesticides, and petrochemical derivatives contain chemicals with heterocyclic and aromatic carbon-ring structures that result in high electron densities. Such electron dense aromatic and heterocyclic compounds are very stable but amenable to substitution reactions, facilitating their interaction with an array of substances resulting in colored products.[20] Aromatic compounds, including phenols, can form stable, colored azo dyes with high molar extinction coefficients in visible wavelengths, through electrophilic substitution with diazonium salts. The color and strength of the azo coupling reaction is dependent upon the number and type of substituent groups which alter the structure and optical properties of the azo compound.[21] The spectrophotometric absorbance of the dye can be related to the concentration of the parent compound remaining in a biodegradation test. This principle had been used for the detection of phenolic metabolites produced from *N*-methylcarbamate hydrolysis (e.g., carbofuran phenol) in soils using 4-nitrobenezediazonium tetrafluoroborate (4-NBTFB).[22] The exact number of chemicals amenable to these reactions is unclear. Inspection of the current water framework directive (WFD)[23] and United States Environmental Protection Agency (USEPA)[24] priority pollutant lists indicate that approximately two-thirds and half of the named compounds, respectively, are aromatic and may be amenable to this, or a similar, azo-dye reaction. Additionally, there are alternative colorimetric assays available,[25] and others can be developed.

In this study, we aimed to evaluate the principle that a highly replicated test format could be used to screen and prioritize chemicals based on their relative biodegradabilities analogous to the OECD RBTs. We developed a high throughput biodegradation screening test (HT-BST) capable of calculating the probability of biodegradation ($P_{exp}$) for a range of aromatic compounds, based on the detection of their azo-dye products following reaction with 4-NBTFB under alkaline conditions. The ability of the HT-BST to differentiate the relative biodegradabilities of a set of structurally diverse aromatic compounds was assessed by comparing its results with reported biodegradabilities and the probability of biodegradation ($P_{QSAR}$) obtained from commonly used quantitative structure−activity relationship models.[26] In addition, the effect of substituent group structure and position on the biodegradation of substituted phenols was evaluated.

## EXPERIMENTAL SECTION

### Colorimetric Determination of Aromatic Compounds.

Ten different structurally related compounds were selected and tested for their ability to react with 4-NBTFB, forming an azo-dye (Table 1). These chemicals were selected to represent a diversity of structures to demonstrate the potential utility of the assay as a screening and prioritization tool (Table S1a). 4-NBTFB (50 $\mu$L, 1 mg/mL prepared in 10% acetone) was added to 96-well plates containing 100 $\mu$L of the test compound (10 mg C/L) in each well; after 2 min, NaOH (50 $\mu$L, 1N) was added to intensify the reaction and the absorbance was measured. Peak absorbance was determined by measuring absorbance at wavelengths from 200 to 900 nm at 10 nm intervals, for the azo-dye product of each chemical compound. Standard curves were produced for each azo dye product using known concentrations of each compound (Figure S1). The reliability of the assay was measured using the coefficient of variation of repeated absorbance values at $\lambda_{max}$ for each standard chemical (Table S2). The accuracy of the method was assessed for 4-chlorophenol using HPLC against known standards. 200 $\mu$L samples were diluted 1:4 and placed in an autoinjector (SIL-10A, Schimadzu, UK). Samples were run on a liquid chromatograph (LC-10ALvp, Schimadzu, UK) fitted with a degasser (DGU-14A) and a UV−vis variable wavelength detector (SPD-10A, Schimadzu, UK) set at 280 nm. The separation was done on an Envirosep-PP priority pollutant column (125 × 4.6 mm, with a guard column (30 × 4.6 mm), Phenomenex, UK) that was heated to 45 °C in a column oven (CTO-GA column oven, Schimadzu, UK). Samples were run at


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Table 1. Summary of Chemical Properties for the Compounds Used To Evaluate the 4-NBTFB Colorimetric Assay[a]

| name (abbreviation) | CAS numbers | log $K_{OW}$ | boiling point (°C) | solubility (mg/L) | optimal test wavelength (nm) | probability of degradation[b] ($10^9$ cells/mL) | MPN of specific degraders ($mL^{-1}$) | Biowin probability values | | biodegradation classification (see Table S3) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Biowin1 | Biowin2 | |
| phenol (P) | 108−95−2 | 1.46 | 182 | $8.28 \times 10^4$ | 365 | 1.00 | $5.56 \pm 0.34 \times 10^3$ | 0.9466 | 0.9876 | readily (60−100% in 4−6 days) |
| 4-chlorophenol (4-CP) | 106−48−9 | 2.39 | 220 | $2.40 \times 10^4$ | 335 | 1.00 | $2.95 \pm 0.18 \times 10^2$ | 0.6197 | 0.5191 | inherently (>70−100% in 1−20 days) |
| 2-amino-1-naphthalene sulfonic acid (2-A-1-NSA) | 81−16−3 | −1.16 | 180 | 4100 | 395 | 0.10 | $1.89 \pm 0.11 \times 10^{-1}$ | 0.1837 | 0.0432 | not readily (6−24% in 14 days) |
| 4-amino-5-hydroxy-2, 7-naphthalene sulfonic acid (4-A-5-H, 2,7-NSA) | 5460−09−3 | −2.33 | n/a | n/a | 545 | 1.00 | $3.26 \pm 0.19 \times 10^2$ | 0.0301 | 0.0101 | not readily (0−8% in 14−29 days) |
| 1-amino-2-naphthol-4 sulfonic acid (1-A-2-N-4 SA) | 116−63−2 | −1.39 | n/a | n/a | 515 | 0.14 | $2.60 \pm 0.16$ | 0.2919 | 0.0820 | no data, low probability of degradation[c] |
| benzene sulfinic acid (BSA) | 98−11−3 | −1.17 | 84 | n/a | 335 | 0.15 | $3.84 \pm 0.23$ | 0.808 | 0.9421 | inherently (13−54% in 28 days) |
| 2-naphthol (2-N) | 135−19−3 | 2.7 | 285 | 755 | 470 | 1.00 | $4.88 \pm 0.09 \times 10^2$ | 0.7947 | 0.8665 | inherently[d] (28−100% in 4−14 days) |
| 4-nitrophenol (4-NP) | 100−02−7 | 1.91 | 279 | $1.16 \times 10^4$ | 365 | 1.00 | $4.55 \pm 0.28 \times 10^2$ | 0.4921 | 0.3620 | variable (0−100% in 7−28 days) |
| 4-hydroxybenzoic acid (4-HBA) | 99−96−7 | 1.58 | 214.5 | 5000 | 395 | 1.00 | $5.56 \pm 0.34 \times 10^3$ | 0.9745 | 0.9876 | readily (76.5% in 4 days) |
| 4-hydroquinone (4-HQ) | 123−31−9 | 0.59 | 287 | $7.20 \times 10^4$ | 380 | 1.00 | $5.56 \pm 0.34 \times 10^3$ | 0.9267 | 0.9631 | readily (70% in 14 days) |

[a]Data sourced from http://chem.sis.nlm.nih.gov. [b]Probability of degradation at environmentally relevant cell concentration determined by total cell counts. [c]From QSAR predictions. [d]Based on data with low reliability.

a flow rate of 0.5 mL/min with a mixture of 70% Eluent A and 30% Eluent B. Eluent A was HPLC grade water with 1% acetonitrile. Eluent B was HPLC grade methanol with 1% acetonitrile. Analysis was performed using Class-VP5 software (Schimadzu, UK).

**High Throughput-Biodegradation Screening Test (HT-BST).** 96-well plate HT-BSTs were set up with 50 $\mu$L of activated sludge (AS) inoculum (from Tudhoe Mill wastewater treatment plant in the North East of England), serially diluted to give a final concentration range of $10^4$−$10^9$ cells/mL, encompassing the concentrations used in standard OECD RBTs[1] and the concentrations encountered in a typical AS treatment plant ($10^8$−$10^9$ cells/mL) proposed for enhanced tests,[6] supplemented with 250 $\mu$L of mineral medium[1] containing the respective chemical at a test concentration of 10 mg C/L, typical of OECD RBTs.[1] The assay was performed with AS, as this is the inoculum typically used in OECD RBTs.[1,2] Control plates were included alongside each test: biotic controls containing glucose (500 mg/L) in the mineral medium and abiotic controls containing $HgCl_2$ (100 mg/L) in the chemical medium. HT-BSTs were incubated in the dark at 30 °C[27] for 28 days; this duration is typical for OECD RBTs, and the temperature used reflects previous 96-well plate biodegradation work.[27] A whole plate was used for each inoculum dilution (i.e., 96 replicates each). Plates were then centrifuged at 3000 rpm for 3 min in a plate centrifuge (CL30, ThermoFisher Scientific, Waltham, MA, USA), and 100 $\mu$L of supernatant was transferred to a new 96-well plate, to prevent the interference of solid particles. The 4-NBTFB assay was performed as described above. Regression analysis of standard curves was used to determine the absorbance at which 70% reduction in parent compound concentration had occurred, which would equate to DOC removal if full mineralization occurred; this was deemed to represent a pass in HT-BST (based on the more stringent pass threshold of 70% reduction required in OECD RBTs,[1] i.e., 60% ThOD or $ThCO_2$ production or 70% DOC reduction).

**Effect of Substituent Group on the Biodegradation of Phenols.** Twenty-five compounds containing the following monosubstituent groups, $-NO_2$ (nitro), −Cl (chloro), −OH (hydroxyl), $-CH_3$ (methyl), $-OCH_3$ (methoxyl), −COOH (carboxyl), −Br (bromo), and −F (fluoro), at 2 (*ortho*), 3 (*meta*), and 4 (*para*) positions were selected (Tables S1b and S2) to investigate the effect of changing the position (isomers) and type of functional group substitution (congeners) on biodegradation outcome. This group of chemicals, including isomers and congeners, was selected to provide an additional validation of the HT-BST by demonstrating its potential utility to represent existing QSARs, using chemicals which fall within the same applicability domain. A defined domain of applicability is one of the five principles by which QSARs are validated. HT-BSTs were performed and analyzed as detailed above. NaOH addition, used to intensify the reaction in the colorimetric detection of aromatic compounds, led to the formation of a precipitate, which caused erratic absorbance measurements for the 24 substituted phenols tested and thus was not used in these tests.

**Biodegradation Potential.** The results of the HT-BST were used to estimate the probability (frequency) of biodegradation at different inoculum concentrations for a given chemical. The probability of biodegradation can also be usefully represented by the specific degrader abundance determined by the most probable number (MPN) approach.



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MPNs were calculated from the HT-BSTs using Thomas' approximate MPN formula (eq 1),[20] taking into consideration the number of wells where biodegradation occurred, the volume in the wells where biodegradation was not observed, and the total volume of all wells. A high probability of biodegradation at a low inoculum concentration equates to high MPN values and vice versa. MPNs were therefore used as a proxy for the probability of biodegradation of the phenolic compounds as a measure of biodegradation potential.

Adaptation of Thomas' approximate MPN formula as an indication of approximate MPN in HT-BST:

$$\text{MPN/mL} = \frac{\text{number of positive wells}}{\sqrt{(\text{volume in negative wells} \times \text{volume in all wells})}} \quad (1)$$

**Biodegradation Data, Chemical Structure Constants, and Statistics.** The assigned biodegradability classifications for the ten structurally diverse chemicals were obtained from data submitted for regulatory assessment, accessed via eChemPortal.[28] The assignment and provenance of the biodegradation data is based on curated information submitted to the European Chemicals Agency (ECHA) from chemical registration documents, where possible (Table S3). The probability of biodegradation was also estimated for all chemicals in the study using the EPISuite biodegradation estimation models, Biowin1 and -2, which are based on quantitative structure-biodegradability relationships using a group contribution method.[26] The probabilities of biodegradation obtained from the HT-BSTs are different to those determined by the EPISuite software, and the HT-BSTs biodegradation probabilities are referred to as $P_{exp}$. $P_{exp}$ values correspond to an end-point assay after 28 days, and the output from the EPISuite software corresponds to probabilities based on whether the chemical can degrade rapidly and within a specific time frame (hours, days, weeks, months, or recalcitrant), which are used to predict Ready Biodegradability in the software.[26] The probability of biodegradation from EPISuite estimates are referred to as $P_{QSAR}$.

Hammett constants ($\sigma$) were used to assess the relationship between biodegradation potential and structure in the 25 monosubstituted phenols. Hammett constants are empirical parameters that quantitatively describe the relative electron-donating or withdrawing properties of substituent groups and are obtained from thermodynamic or kinetic parameters for a specified reference reaction. They have previously been used to predict both abiotic and biotic reaction rates.[29,30] Linear regression, correlations, and analysis of variance (ANOVA) were carried out using Minitab v16 (State College, Pennsylvania, USA).

## RESULTS

**Evaluation of the 4-NBTFB Colorimetric Assay.** All ten chemicals tested gave a pronounced optimum peak of maximum absorbance between 335 and 545 nm. Differences in structural complexity, and thus conjugated distance, were typically associated with a bathochromic shift, i.e., an increased absorbance wavelength of the azo dye.[21] The median coefficient of variation of replicate absorbance measurements for all chemicals was low (6%, $n$ = 8, Table S4), demonstrating that the 4-NBTFB assay was reproducible. The detection limit for the assay, based on the standard curves (Figure S1), was defined as the point at which the spectrophotometric standard curve was no longer linear. This limit of detection varied between chemicals, but standard curves for all compounds were linear down to 2 mg $L^{-1}$ C (Figure S1), with linearity exhibited as low as 1 mg $L^{-1}$ for select chemicals. The assay was able to resolve biodegradation of greater than 70% for all chemicals (i.e., detection limit was less than 3 mg $CL^{-1}$ for all chemicals), which is based on the more stringent 70% reduction required to pass an OECD RBT.[1] The chemical concentrations determined using the 4-NBTFB colorimetric assay were not significantly different from those obtained by HPLC when used to quantify the parent compound in two HT-BSTs showing partial degradation (ANOVA; $P$ = 0.992 and $P$ = 0.560). Biotic controls showed complete degradation of glucose, and no biodegradation was observed in abiotic control plates treated with $HgCl_2$ (data not shown).

**Evaluation of the HT-BST.** The test chemicals were employed as sole carbon sources in a HT-BST akin to an RBT. After 28 days, the colorimetric assay was performed and wells showing greater than 70% removal of parent compound were scored as positive. The ratio of positive wells was displayed as a probability of biodegradation (Figure 1). The chemicals clustered into three distinct groups that largely matched the assigned, or reported, biodegradabilities based on data from the eChemPortal database,[28] derived largely from RBTs (Table 1, Figure 1). The three groups were as follows: (i) those which showed a 100% probability of degradation at all inoculum concentrations and are classified as readily biodegradable (phenol, P; 4-hydroxybenzoic acid, 4-HBA; 4-hydroquinone, 4-HQ; Figure 1a); (ii) those that only showed any probability of biodegradation at the highest inoculum concentration and are classified as not readily biodegradable (2-amino-1-naphthalene sulfonic acid, 2-A-1-NSA; 1-amino-2-naphthalene-4-sulfonic acid, 1-A-2-N-4, SA; 4-amino-5-hydroxy-2,7-naphthalene sulfonic acid, 4-A-5-H, 2,7-NSA; Figure 1c); (iii) those that showed a range of probabilities at intermediate and high inoculum concentrations, depending on the inoculum concentration, and are classified as inherently biodegradable (4-nitrophenol, 4-NP; 4-chlorophenol, 4-CP; benzenesulfonic acid, BSA) or showed variable biodegradation (2-napththol, 2-N; Figure 1b). The probability of biodegradation ($P_{exp}$) was higher for monoringed structures than for multiple-ringed structures. The HT-BST correctly classified 80% of the chemicals tested with respect to their biodegradabilities and in terms of distinguishing readily and inherently biodegradable from not readily biodegradable chemicals at environmentally relevant AS concentrations ($10^8$–$10^9$ cells/mL; Figure 1). The HT-BST showed the potential for inherently biodegradable compounds to have a low probability of biodegradation at low OECD inoculum concentrations (Figure 1b): as the outcome of a BST, this can be termed a false negative. The probability of biodegradation increased, and conversely, the likelihood of reporting false negatives decreased for inherently biodegradable compounds, as the inoculum increased to environmentally relevant concentrations. The likelihood of observing a false negative was related to the statistical power of the test. If we consider the power to be 1, type II error, and type II error to be the likelihood of accepting a test fail when we know the chemical is classified as inherently biodegradable, the statistical power of the test increased from 0 at OECD inoculum concentrations ($10^4$–$10^5$ cells/mL) to 1 at higher inoculum concentrations ($10^8$–$10^9$ cells/mL) for the inherently bio-


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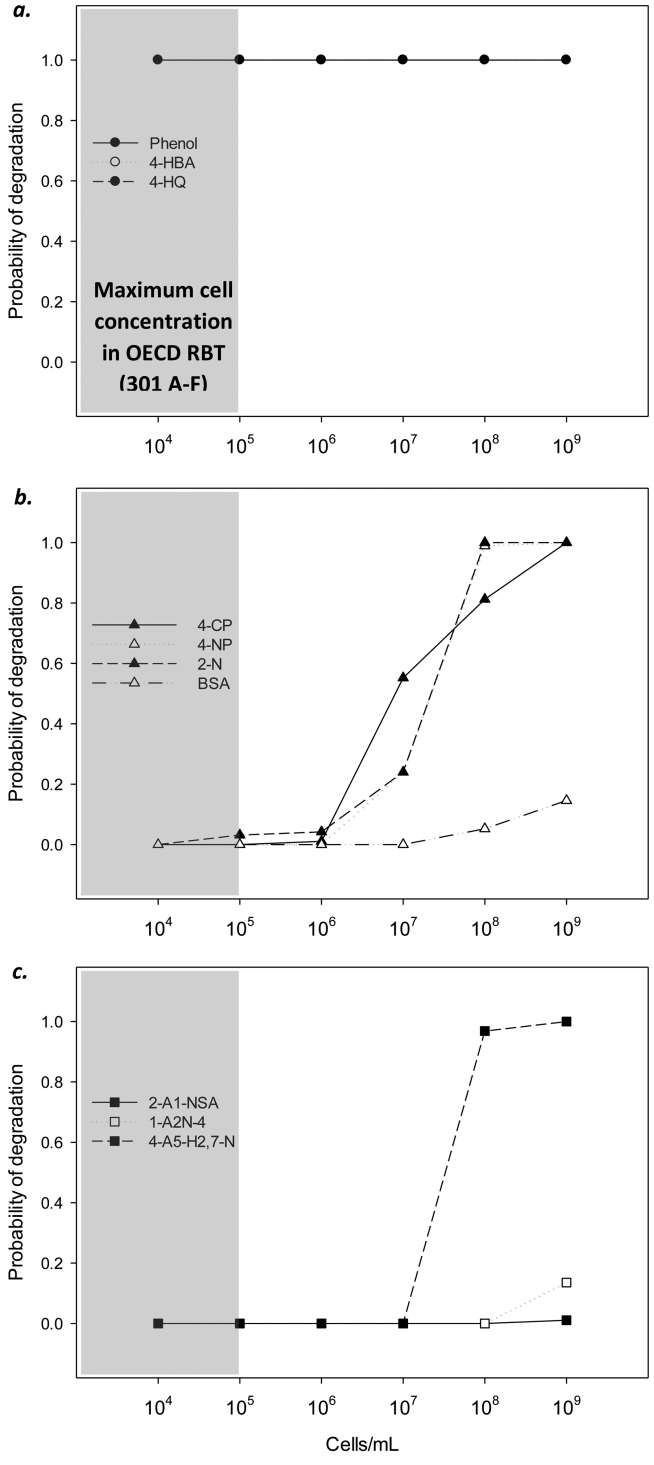


**Figure 1.** Probability of degradation for the selected chemicals at different activated sludge inoculum concentrations, displayed as cells/mL. Chemicals are clustered into 3 groups: (a) readily biodegradable; (b) inherently biodegradable or variable reported biodegradation; (c) not readily biodegradable. These assessments were based on regulatory data accessed via eChem portal.[28] The standard OECD 301 series inoculum concentrations are marked on the plot by the shaded area.

degradable compounds 4-NP, 4-CP, and 2-N (Figure 1b). It has been suggested that this type of probabilistic approach would be useful in assessing biodegradation and persistence of chemicals.[19] The HT-BST produced a false negative for one inherently biodegradable compound (benzenesulfonic acid) at environmentally relevant AS concentrations ($10^8$–$10^9$ cells/mL). The HT-BST also showed a high probability of degradation for 4-A-5-H, 2,7-NSA at environmentally relevant inoculum concentrations ($10^8$–$10^9$ cells/mL; Figure 1c), although it was classified as not readily biodegradable according to regulatory documents accessed via eChemPortal.[28] This could be regarded as a false positive. However, the study (an inherent biodegradability test) reported within the ECHA registration dossier for this chemical is highlighted as being of low reliability. Biodegradation is often a snapshot, with one assessment using one inoculum given to represent the biodegradation potential of a chemical, when actually it is recognized that biodegradation potential may change with time.[31] It is also possible therefore that the HT-BST provides a more accurate characterization of the biodegradability of 4-A-5-H, 2,7-NSA or that environmental microbial communities may have acclimated to degrade this chemical since it was previously tested (e.g., Itrich et al.[31]). However, further analysis would be required to verify this.

**Structure-Biodegradability Relationships in Monosubstituted Phenols.** Monosubstituted phenols containing hydroxyl and carboxyl substitutions were more readily degraded than phenols with other substituents as seen by MPN counts (Figure 2). Halogen and methoxy group substitutions were

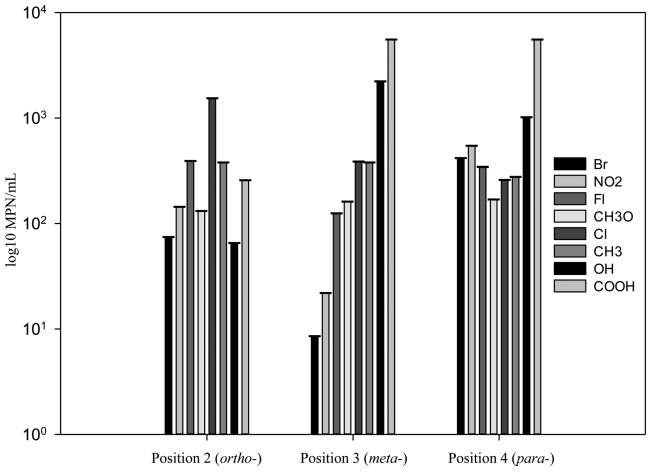


**Figure 2.** Most probable number (MPN) estimates of specific degrader abundance for phenolic compounds derived from the number of wells exhibiting a positive biodegradation outcome, volume contained within negative wells, and the total volume contained within all wells. Error bars indicate the standard deviation, which was low for all compounds.

least biodegradable in the 28 day test. Phenol substitutions on positions 2 (*ortho*-) and 4 (*para*-) showed much less variation in probability of biodegradation than those on position 3 (*meta*-). Overall there were no significant effects of substituent group or position on biodegradation potential (as measured by mean MPN counts; nested ANOVA, $P = 0.135$), although the majority of the variance (76%) in the data was accounted for by the position of the substituent. The effect of group substituent on biodegradation potential (as measured by MPN) was only observed in position 3 (*meta*-position) where there was a significant strong positive correlation in the rank order with Biowin2 $P_{QSAR}$ data (Spearman's $\rho = 0.643$, $P < 0.1$), which was marginally insignificant with Biowin1 $P_{QSAR}$ data (Spearman's $\rho = 0.619$, $P = 0.102$). The sequential order of biodegradation potential by substituent group was carboxylic acid (COOH) >


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hydroxyl (OH) > methyl ($CH_3$) = chloro (Cl) > methoxy ($CH_3O$) = fluoro (F) > nitro ($NO_2$) > bromo (Br) for MPN data, which was similar to COOH > OH > $CH_3$ > $CH_3O$ > Cl > $NO_2$ > Br > F for Biowin2 data, where COOH has the highest potential.

Regression analysis of the biodegradation potential (expressed as MPN counts) showed a weak but significant correlation with the substituent Hammett constant ($R^2$ = 0.19, $P$ < 0.05). When broken down into the different positions, there was a significant negative correlation ($R^2$ = 0.58, $P$ < 0.05) between the biodegradation potential and the Hammett constant for the substituent groups in position 3 (*meta*-position) on the ring (Figure 3). There was no significant correlation for positions 2 and 4 unlike those determined in previous studies for specific biodegradation rate.[30,32]

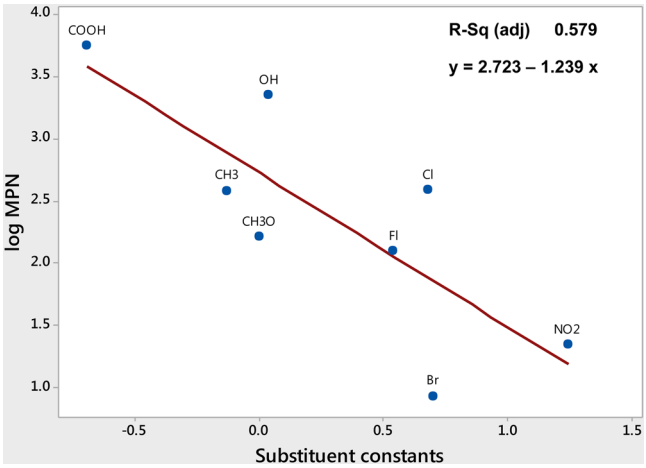


**Figure 3.** Regression analysis showing a strong and significant negative correlation ($R^2$ = 0.58, $P$ < 0.05) between the biodegradation potential (log MPN (/mL) and the Hammett constant for the substituent groups in position 3 (*meta*-position).

## DISCUSSION

**Proof of Principle for HT-BSTs.** The HT-BST described here, using a single activated sludge source of inoculum, has been shown to correctly prioritize a group of structurally diverse aromatic chemicals based on their previous biodegradability classifications using conventional RBT test criteria (i.e., ability to aerobically biodegrade in an aqueous medium). The HT-BST also allowed us to investigate the effect of substituent group on biodegradability of the 25 monosubstituted phenols, which compared well to biodegradability from QSAR estimates and other experimental studies.

The HT-BST has high throughput and replication. We typically used a 96-well plate per dilution of inoculum per chemical, resulting in approximately 20 000 individual biodegradation-screening tests during the course of this research at a fraction of the cost and time of a standard RBT (Table S5), in addition to addressing the labor and time restrictions associated with conventional analyses.[33] This method is amenable to automation using liquid handling robots, unlike existing RBTs, which may further streamline the process. The format described in the present study could also be adapted to include multiple chemicals, multiple inocula concentrations, and/or multiple inocula from the same or different environmental sources on one plate; indeed, during the standard curve production, each column (i.e., 8 wells) represented a different chemical concentration. It opens up the possibility of explicitly investigating the variability of chemical biodegradation both spatially and temporally in relation to differences in microbial communities. The colorimetric detection of specific chemicals was accurate and reproducible; concentrations were statistically indistinguishable from those quantified by HPLC in HT-BSTs showing primary degradation. The method had a low median coefficient of variation, and the method detection limits were suitable for the current end-point thresholds used for RBTs. At environmentally relevant inoculum concentrations, the assay effectively screened for potential environmental persistence, correctly characterizing eight out of the ten selected chemicals, based on their biodegradation potential. False negatives were more frequently observed at low inoculum concentrations. It should be noted that the total number of bacteria present in typical standard OECD RBTs (301 A–C, 301 F) might sometimes reach the total number present at the highest concentrations used in the HT-BST ($\sim 10^8$) even though the concentrations may be different. This is due to the test volumes used (up to L in RBTs; hundreds of $\mu$L in HT-BST). However, the ratio of chemical to inoculum concentration, i.e., the food to microorganism ratio, was the same in the HT-BST as that used in OECD RBTs for the range of OECD inocula concentrations used. Further, we have shown previously that the variation in biodegradation at these lower concentrations is extremely high, which in RBTs is further exacerbated by a drastic reduction in the bacterial diversity of inocula preparation compared to the original activated sludge samples.[10]

Other recent studies have also investigated high throughput miniaturized biodegradation tests based on measuring oxygen uptake as a proxy for ultimate biodegradation of two readily and one inherently biodegradable chemical.[17] These tests report similar limits of detection and substrate concentration ranges as those reported here. Unlike the current HT-BST, those based on oxygen uptake appear to scale differently to conventional RBTs; the biodegradation end-point achieved the same values but in a different amount of time, which may complicate cross-validation. The current HT-BST gave analogous results to conventional RBTs within the same time frame, although it measured primary biodegradation after a particular time and not over time. Using plates that were sacrificially sampled at different time points would rectify this. Nevertheless, both high throughput methods would complement each other at a cost comparable to current RBTs, thereby offering the opportunity to measure both primary and ultimate biodegradation in the same test. The assessment of primary biodegradation, as described in the present study, does not consider the potential formation of dead end byproducts, which may or may not be toxic. The HT-BST described is a relatively simple screening test and must be considered as a tool in a broader intelligent testing strategy, as is current practice in REACH.[9,10,13,34] To assess the potential formation of dead-end byproducts, the assay could be coupled with analytical chemistry, or the biodegradation of a rapidly degradable compound after the end of a study could be performed as a toxicity assessment.

**Regulatory Implications.** The prioritization of chemicals based on hazards including persistency, bioaccumulation, and toxicity (collectively termed PBT)[6] has been advocated since the introduction of REACH in 2007. Of these, persistence/biodegradation represents one of the greatest uncertainties and most sensitive parameters in chemical exposure models.[35]


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Persistence is also a critical parameter in risk assessment: half-life and $K_d$ are two important factors governing predicted environmental concentrations (PECs) in various media. There has been a significant drive toward increasing throughput and improving the reliability of bioaccumulation and toxicity tests,[16] with a view to ultimately reduce the overall number of required tests, as part of the replacement, reduction, and refinement (3Rs) framework for humane animal research.[15] Developing methods to effectively prioritize chemical persistence in the environment has received comparatively little attention, despite the need for it being highlighted in REACH documentation.[6] Persistence is probably the cheapest and most straightforward of the hazards to characterize. Guidance recommends that chemicals should undergo persistence assessments before bioaccumulation and toxicity assessments to avoid unnecessary animal tests, and the latter should be conducted when a chemical is assigned as potentially persistent, dependent upon the chemical's properties.[36] However, it has been estimated that of the many chemicals which fail RBTs, up to 80% may be false negatives,[12] thereby resulting in unnecessary bioaccumulation and toxicity tests. We estimate that accurate persistence assessment would save upward of 600 fish (Table S6) and approximately $75K for every chemical reliably screened out earlier in the testing process.[37] At the current rate of failure, this equates to savings in the order of millions of fish and hundreds of millions of dollars.

Such tests could also help fulfill the criteria espoused for green chemistry, i.e., process optimization and informed design to minimize exposure of the environment to hazardous substances.[38] The accurate characterization of chemicals liable to persist in the environment at an early stage of development has two benefits: (i) a cost-benefit opportunity to remove those chemicals which are unlikely to receive regulatory acceptance at an earlier stage of product development and (ii) prioritization of those chemicals which are designed to have a degree of persistence in order to perform effectively (e.g., certain pharmaceuticals which need to remain stable until they reach their intended point of action) for more intensive testing to determine their overall risk to the environment.

**Biodegradability and Structure Analysis.** High throughput biodegradation tests may also enable the generation of extensive and reliable biodegradation half-life data that could be used to develop and validate *in silico* QSARs.[39,40] The use of QSAR methods in a regulatory capacity is at present fundamentally undermined by a lack of reliable and robust experimental data. We used the assay to demonstrate that there was a strong and statistically significant correlation with Hammett's constant for substituents on position 3 (*meta*) of the phenol, indicating that biodegradability of phenols is influenced by the electrophilicity of the substituent group at this position. There was a similar but weaker relationship of the substituents on positions 2 (*ortho*) and 4 (*para*). This indicates that electronic effects on the 3 position are more pronounced than the 2 and 4 positions, which are adjacent and opposite to the C1 hydroxyl group. It is interesting to note that, for selected compounds, those with greater delta Gibbs free energy, for rate limiting reactions, had higher probabilities of degradation (4-HBA, phenol, 4-nitrophenol, and 4-chlorophenol: −251.97, −246.11, −245.61, and −207.39 kCal/mol, respectively). There was thus a general trend for increased biodegradability as the electrophilicity of the substituted group decreased. The rank order of the effect of substituent groups agrees noticeably with previous work by Pitter, who showed a correlation between the Hammett constant and specific rates of degradation for phenolic compounds.[30] Therefore, although the HT-BST is an end point, not a rate assay, it can be used as a good indicator of relative biodegradability.

**Future Applications.** The following limitations of the assay warrant further investigation: (i) the assay is only applicable to chemicals which can react with 4-NBTFB, although there are potential alternative colorimetric assays[25] which would benefit from further investigation; (ii) degradation products may interact with 4-NBTFB to form an azo-dye which may result in a false-negative assessment (although there was no evidence that this occurred); (iii) the limit of detection is suitable for current standard OECD test concentrations and pass thresholds (i.e., the test has the capacity to resolve 70% degradation given a starting chemical concentration of 10 mg C $L^{-1}$); however, it may not be sufficiently sensitive for investigations into environmentally relevant chemical concentrations in the ng to $\mu$g $L^{-1}$ range.[41] The assay is intended as an alternative to existing screening tests and is thus also limited by some of the general constraints of standard OECD RBTs. For example, this assay would not be suitable for volatile compounds or those with high log $K_{ow}$ and, in its current format, would not be suitable for accounting for cometabolic processes. Modifications to existing screening studies proposed within REACH can also be applied to HT-BSTs to address some of these issues; however, some compounds would still be problematic, as they are for existing screening studies. Similarly, potential inferences such as high DOC levels potentially impact OECD RBTs. There is a range of options currently prescribed within the guidelines to circumvent these issues, such as aging the inocula or washing to reduce background DOC levels.[1] The efficacy of these options could also be assessed for the HT-BST described. Modifications to the assay may allow the assessment of biodegradation under a range of different testing conditions, including the assessment of anaerobic biodegradability. Overall, the HT-BST proposed here promises to be a useful tool in the prioritization of persistent chemicals and for investigating the rules that govern the biodegradation rates of chemicals toward establishing reliable *in silico* methods of prioritization.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b00806.

Plot of absorbance against chemical concentration for chemicals used to evaluate the 4-NBTFB colorimetric assay; RBT data for 4-nitrophenol and 4-fluorophenol; chemical structures and properties; chemical persistence classification sources and summary; table of coefficients of variation for replicate absorbance measurements for chemicals used to evaluate the 4-NBTFB colorimetric assay; a relative cost comparison between HT-BST and RBT; estimated fish requirements for B and T assessments (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

| | |
|---|---|
| 1-A-2-N-4 SA | 1-amino-2-naphthalene-4-sulfonic acid |
| 2-A-1-NSA | 2-amino-1-naphthalene sulfonic acid |
| 2-N | 2-naphthol |
| 3-Rs | replacement, reduction, and refinement framework |
| 4-A-5-H | 2,7-NSA,4-amino-5-hydroxy-2,7-naphthalene sulfonic acid |
| 4-CP | 4-chlorophenol |
| 4-HBA | 4-hydroxybenzoic acid |
| 4-HQ | 4-hydroquinone |
| 4-NBTFB | 4-nitrobenzenediazonium tetrafluoroborate |
| 4-NP | 4-nitrophenol |
| AS | activated sludge |
| BSA | benzene sulphinic acid |
| BST | biodegradation screening test |
| DOC | dissolved organic carbon |
| HPLC | high performance liquid chromatography |
| HT-BST | high throughput biodegradation screening test |
| MPN | most probable number |
| MS | mass spectrometry |
| OECD | Organisation for Economic Co-operation and Development |
| P | phenol |
| PBT | persistent, bioaccumulatory, and toxic |
| $P_{exp}$ | experimentally derived probability of biodegradation |
| $P_{QSAR}$ | QSAR derived probability of biodegradation |
| QSAR | quantitative structure—activity relationship |
| RBT | ready biodegradability test |
| REACH | registration, evaluation, authorization, and restriction of chemicals |
| vPvB | very persistent and very bioaccumulative |

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