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Testing the biodegradability of priority and emerging contaminants as a mixture

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Abstract

Currently about 110,000 chemical substances are present in the European market. The fate of most of those chemicals in the environment is not known. However, biodegradability of those chemicals should be tested before they are registered to the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) System. Current protocols offered by The Organisation for Economic Co-operation and Development (OECD) for testing the biodegradability of the chemicals are limited mainly due to they are low throughput and do not reflect real-world conditions. In OECD protocols, the biodegradability of a single chemical is tested. However, many chemicals coexist in the environment. In addition, experiments are set at a very high initial chemical concentration that is not expected in the environment. Both limitations are due to the lack of an analytical method which can measure multiple compounds simultaneously at very low concentrations. In this study, we coupled OECD 314 Simulation Tests to Assess the Biodegradability of Chemicals Discharged in Wastewater protocol with a powerful liquid chromatography mass spectrometry with scheduled multiple reaction monitoring and tested the biodegradability of 32 priority substances and chemicals with emerging concern. Only seven chemicals were degraded in the test within 28 days. The biodegradation half-lives of those degradable chemicals ranged between 0.6 to 18 days. Acetaminophen was degraded the fastest whereas biodegradation of sulfamethoxazole took longer than the rest of the biodegradable chemicals tested. The novel methodology described here can be applied to test biodegradability of different chemicals as a mixture and adopted as a standard protocol.

Keywords: biodegradability, biodegradation testing, chemicals of emerging concern, priority substances

1. INTRODUCTION

Surfactants, flame retardants, pharmaceuticals and personal care products, gasoline additives and their degradation products, biocides, polar pesticides and their degradation products and various endocrine disrupting compounds are frequently detected both in w astewater and surface water. Since their concetration are at microgram per liter levels, they are generally attributed as micropollutants. Some of those micropollutants are listed as “priority substances (PS)” by European Union (EU) states and the United States of America (USA), and some of them are classified as “contaminants of emerging concern (CEC)”. Overall, there are about 1050 chemicals classified as PS and CEC and they are frequently detected in the environment where they threaten both ecology and human health [1].

Microbial biotransformation and mineralization are two main mechanisms controlling the fate of micropollutants in the environment. In most of the cases, a toxic pollutant is converted to a non-toxic compound or mineralized to inorganic molecules such as carbon dioxide and ammonium. On the other hand, biotransformation products of some micropollutants are more toxic than the parent compound. Presence of micropollutants in aquatic environment has a number of negative effects, including short-term and long-term toxicity, endocrine disrupting effects and antibiotic resistance of microorganisms [2, 3]. Therefore, assessment of biodegradability is important for

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predicting the behaviour and impact of a chemical substance in the environment [4].

OECD offers several protocols for testing the biodegradability of a chemical substance. Those protocols are divided into three: (1) Ready biodegradability (Test no: 301A-F and 310) [5], (2) Inherent biodegradability (Test no: 302A-C) [6] and (3) Simulation biodegradability tests (Test no: 314A-E) [7]. In "Ready biodegradability tests (RBT)", the test substance serves as the sole carbon and energy source; thus it is a growth- or primary-substrate. Relatively low concentration of biomass is used to avoid excessive adsorption of the test substance as well as to limit release of carbon due to endogenous decay. The test substance is not measured during the course of the experiment, but non-specific parameters such as dissolved organic carbon (DOC), oxygen uptake and carbon dioxide are measured to verify biodegradation. Complete removal of DOC is indicative of ultimate biodegradation. However, concentration of the test substance should be high enough to be measured by the mentioned non-specific methods. As a result, the initial concentration of a test substance in the screening test is generally kept at a very high level i.e 2-100 mg/L which is above environmentally relevant concentrations. As an alternative to RBTs, "simulation tests" are performed. In simulation tests, the initial concentration of the test substance ranges between 1 µg/L and 100 µg/L which is low enough to ensure that the biodegradation kinetics obtained in the test reflect those expected in the environment being simulated as well as higher than the limit of detection of the instrument used for the measurement of the chemical or the by-products. Biodegradation is measured either by radiolabeling techniques or using specific instrumental methods. Shortcomings of RBT like low biomass density, high initial test substance concentration and indirect measurement of biodegradation by non-specific parameters like DOC are eliminated in simulation tests [8]. On the other hand, biodegradation of a single chemical is tested in simulation tests which makes it a low-throughput testing method.

Relatively recently, two high-throughput methods have been developed for testing biodegradability of chemical substances. One offers assessing compound biodegradation at 1-2 mg C/L by measuring microbial community growth over time with direct cell counting with flow cytometry [4]. The other uses microwell plates for testing biodegradation of multiple compounds individually at 10 mg C/L. However, the latter method is applicable only for compounds containing aromatic groups since it uses a coloring agent which reacts with the aromatic ring [9].

Wastewater and surface water are complex matrix which contain many micropollutants together at very low concentrations. The biodegradability tests described are limited to mimic the real conditions in the environment since the initial concentrations of chemicals are too high and they are performed for only one chemical at a time. Here, we described a methodology to test biodegradability of multiple chemicals simultaneously at 10 µg/L. This method couples a conventional shake flask test with a powerful liquid chromatography-mass spectrometry method that employs scheduled multiple reaction monitoring (LC-MS/sMRM). Testing biodegradation of multiple chemicals simultaneously with a novel LC-MS methods described below is performed for the first time in this study. The method described here can be used as an alternative for the other protocols offered by OECD.

2. MATERIALS AND METHODS

2.1. Source of Inoculum

Two samples were taken from Luleburgaz Wastewater Treatment Plant which is a biological nutrient removal system treating domestic wastewater using staged aeration. The capacity of the plant is about 20,000 m^3/day. About 100 mL of sample taken from the aeration tank was diluted with 900 mL secondary effluent in 2-L glass bottle. The reactor was operated fed-batch with 7-days solids retention time and fed with 0.250 g glucose and 0.250 g tryptone three times a week. In each time, secondary effluent was used to replace the liquid taken out of the reactor to maintain the solid retentation time. The reactor was aerated continuously while agitated on a magnetic stirrer. The content in the reactor was used as the

inoculum for the biodegradability test described below.

2.2. Chemicals and reagents

In this study, we tested biodegradability of 32 organic chemicals that have been listed either as CECs by NORMAN Network or PS in Water Framework Directive of European Commission. Those chemicals included: (1) antibiotics such as amoxicillin, azithromycin, ciprofloxacin, erythromycin, norfloxacin, ofloxacin, sulfamethoxazole; (2) industrial chemicals such as diphenylamine, hexadecyl benzyl dimethyl ammonium, drometrizole, 8-hydroxyquinoline; (3) pharmaceuticals and personal care products such as paracetamol, N,N-Diethyl-m-toluamide, galaxolide, gamma-methyl ionone, tonalide and (4) agricultural chemicals including prochloraz, linuton, alachlor, atrazine, isoproturon, simazine, triazophos, chlorfenvinphos, chlorpyrifos, dichlorvos, pyriproxifen, quinoxyfen, aclonifen, terbutryn, piperonylbutoxide. The water-octanol partitioning coefficient (logP) of these chemicals ranges between -1.03 and 6.23. These chemicals were purchased from Sigma Aldrich Chemical Company at their highest purity. The stock solutions of these chemicals were prepared in methanol at 1000 ppm and stored at -20 °C. Methanol and water used in sample preparation as well as in chromatography were obtained from Merck Chemicals at MS grade.

2.3. Biodegradation experiments

Biodegradation experiments were performed according to the modified OECD Test No 314B: Simulation Tests to Assess the Biodegradability of Chemicals Discharged in Wastewater. Experiment was performed in 250-mL amber Erlenmeyer flasks with screw caps. About 5 mL of sample taken from the biological reactor described above was diluted with 45 mL of salt medium composed of 85 mg/L KH_2PO_4, 217.5 mg/L K_2HPO_4, 334.0 mg/L $Na_2HPO_4.2H_2O$, 5.0 mg/L NH_4Cl, 36.4 mg/L $CaCl_2.2H_2O$, 22.5 mg/L $MgSO_4.7H_2O$ and 0.25 mg/L $FeCl_2.6H_2O$. Then, a mixture of test substances was added in to the flask. The initial concentration of each substance in the flask was 10 µg/L. A flask without inoculum was served as a control. All flasks were agitated at 100 rpm and incubated at 22 ºC for 28 days. Samples taken from the flasks at specified time intervals were mixed with an equal volume of methanol, centrifuged at 10,000 rpm and filtered through regenerated cellulose filter having 0.22 µm pore size. Chemicals were measured using liquid chromatography with mass spectrometry as described below.

2.4. Analytical Methods

An AB SCIEX QTrap 4500 linear ion trap tandem mass analyzer system coupled with Eksigent Ekspert UltraLC 110 ultra-high performance liquid chromatography (UHPLC) unit was used in quantification of the chemicals used in this study. UHPLC was equipped with Phenomenex Kinetex C18 2.6µ column (50 x 3 mm). Mobile phases included water (A) and methanol (B) buffered with 0.1% formic acid. A gradient elution was performed for separation of the analytes (Table 1). The flow rate was constant at 0.5 mL/min and the column was kept at 40 ºC. Overall run time was 14 minutes. All of the analytes were detected within 8 minutes. Scheduled multiple reaction monitoring was performed for detection and quantification of the chemicals. Optimized MRM parameters were scanned at the determined retention times of the chemicals during the gradient elution (Table 2). This method was used for the first time in this study for the measurement of aforementioned chemicals simultaneously.

Table 1. Details of gradient elution applied for the separation of chemicals in UHPLC

TIME	A (%)	B (%)
0:00:01	0	100
0:01:30	0	100
0:02:00	50	50
0:04:00	50	50
0:08:00	100	0
0:14:00	100	0

3. RESULTS AND DISCUSSION

Biodegradation test lasted 28 days. At the end of the incubation only 7 out of 32 chemicals tested were degraded above 60 percent (Figure 1). Those chemicals were acetaminophen, benzyl dimethyl

hexadecyl ammonium, diphenylamine, g-methylionene, pyriproxyfen, sulfamethoxazole and tonalide. The biodegradation extent of those chemicals were above 80% at the end of the incubation. Biodegradation rate and the corresponding half-life ($t_{1/2}$) of the chemicals were calculated by fitting first order degradation kinetics equation to the experimental data obtained during 28 days (Equation 1) using Sigma Plot v10.2 Software.

$$\frac{C}{C_0} = e^{-kt} \quad (1)$$

where C is the concentration of the chemical (µg/L) at time t (d), C_0 is the initial concentration, k is the biodegradation rate constant (d^{-1}).

Table 2. Optimum sMRM parameters of chemicals used in identification and quantification

Compound	RT	DP	Q1	$Q3_1$	CE_1	$Q3_2$	CE_2
8-Hydroxyquinoline	0.48	61	146.0	128	33	74.9	57
Acetaminophen	2.62	56	152.0	110	23	65	41
Aclonifen	6.26	61	265.0	248	25	182.1	39
Alachlor	5.94	46	270.2	238	13	162.1	29
Amoxicillin	0.49	52	366.1	348.9	13	113.9	27
Atrazine	4.02	21	216.1	174.1	25	103.9	41
Azitromycin	2.85	86	749.5	591.3	43	82.8	89
Benzyldimethyl hexadecylammonium	6.67	101	360.4	90.9	71	268.1	33
Chlorfenvinphos	6.37	66	358.9	155.1	19	169.9	59
Chlorpyrifos	7.18	56	349.9	96.8	55	197.9	35
Ciprofloxacin	2.72	76	332.2	314	29	230.9	51
Dichlorvos	3.69	71	221.0	108.9	25	127	25
Diphenylamine	5.73	71	170.0	93	37	92.4	27
Diuron	4.15	61	233.0	71.9	41	159.9	37
Drometrizole	7.01	76	226.1	119.9	25	107	27
Erythromycin	3.66	21	734.5	158.1	39	83	89
g-Methylionone	6.89	41	207.1	111	17	69	27
Galaxolide	7.55	91	257.1	227.1	41	115	99
Isoproturon	4.06	66	207.1	71.9	37	165.1	21
Linuron	5.26	61	249.0	159.9	27	132.9	47
N,N-Diethyl-m-toluamide	4.07	71	192.1	118.9	25	90.9	43
Norfloxacin	2.71	71	320.2	302	29	231	55
Ofloxacin	2.69	46	362.2	318	27	261	37
Piperonylbutoxide	7.02	16	356.2	177	17	118.9	47
Prochloraz	6.08	26	376.0	307.8	17	265.8	23
Pyriproxifen	7.11	51	322.1	95.9	23	185.1	31
Quinoxyfen	7.11	111	307.9	197	45	162	65
Simazine	3.83	41	202.1	103.9	35	124	25
Sulfamethoxazole	2.90	46	254.0	91.9	35	107.8	35
Terbutryn	4.54	61	242.0	186	27	68	63
Tonalide	7.55	71	259.1	175.1	25	147.1	37
Triazophos	5.82	66	314.0	161.9	27	119	51

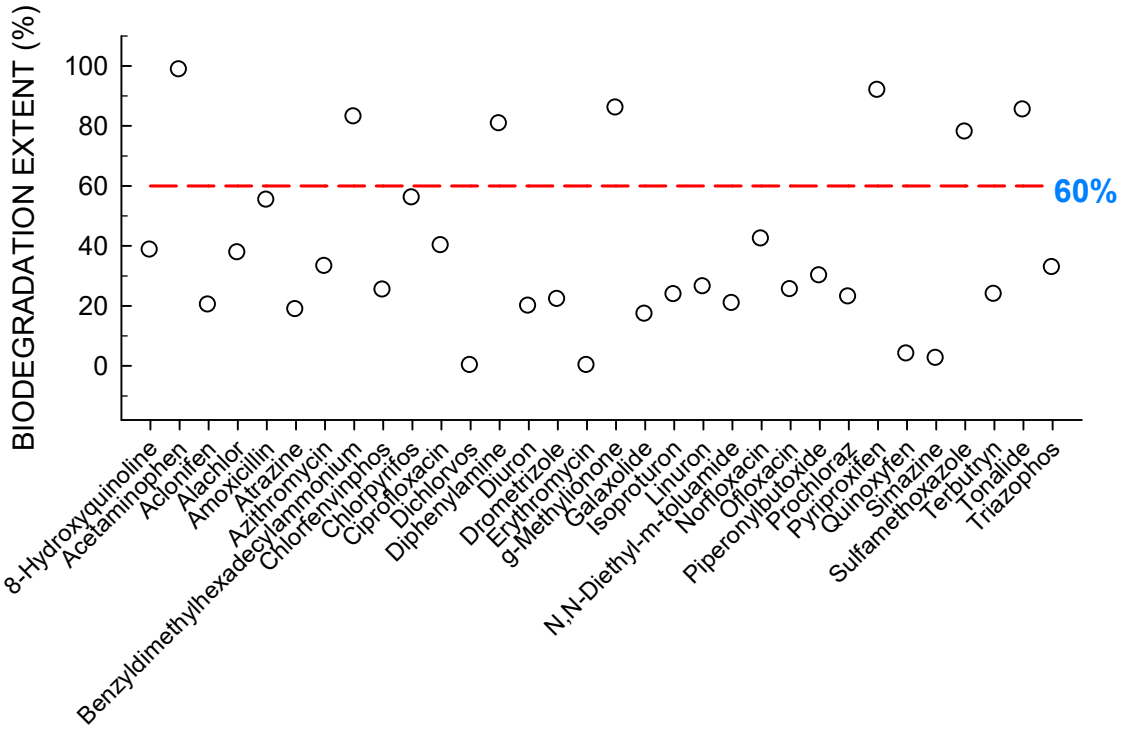

Figure 1. Biodegradation extent of chemicals

Acetaminophen, which is an active ingredient of analgesic drugs, was completely degraded within 5 days (Figure 2A). Its degradation rate and corresponding half-life were 1.24 d^{-1} and 0.56 d (Table 3). Up to date several microorganisms that can degrade acetaminophen have been isolated [10, 11]. The main mechanism of the degradation is deacetylation which converts acetaminophen to p-aminophenol [12]. *p*-aminophenol is more toxic than the parent compound.

Diphenylamine is mainly used in the production of dyes, pesticides, pharmaceuticals, and photographic chemicals and as stabilizer for explosives. In addition, it is formed as a reaction byproduct during aniline production [13]. About 80% of dipenylamine amended into the test flasks were utilized within 28 days (Figure 2B). Half-life of diphenylamine was 12 days which suggests that this chemical is not readily biodegradable (Table 3). On the contrary, half-life of diphenylamine was reported as 0.54 days in experiments performed with cultures of *Pseudomonas cepacia*, *Pseudomonas putida*, *Pseudomonas resinovorans* and mixed activated sludge [14]. Diphenylamine biotransformation starts with integration of two hydroxyl groups to one benzyl group by an action of a novel dioxygenase enzyme. Reaction results in formation of aniline and catechol which further mineralized to carbon dioxide and ammonium. Relatively recently *Burkholderia* sp. strain JS667 and *Ralstonia* sp. JS668 which can utilize diphenylamine as both carbon and nitrogen source were isolated [13].The half-life that was reported in the literature is almost twenty times less than the value reported in this study. Difference may be related to the use of pure culture instead of a mixed microbial consortium, since the population of a degrader may be less in the mixed activated sludge community compared to the pure culture of the degrader.

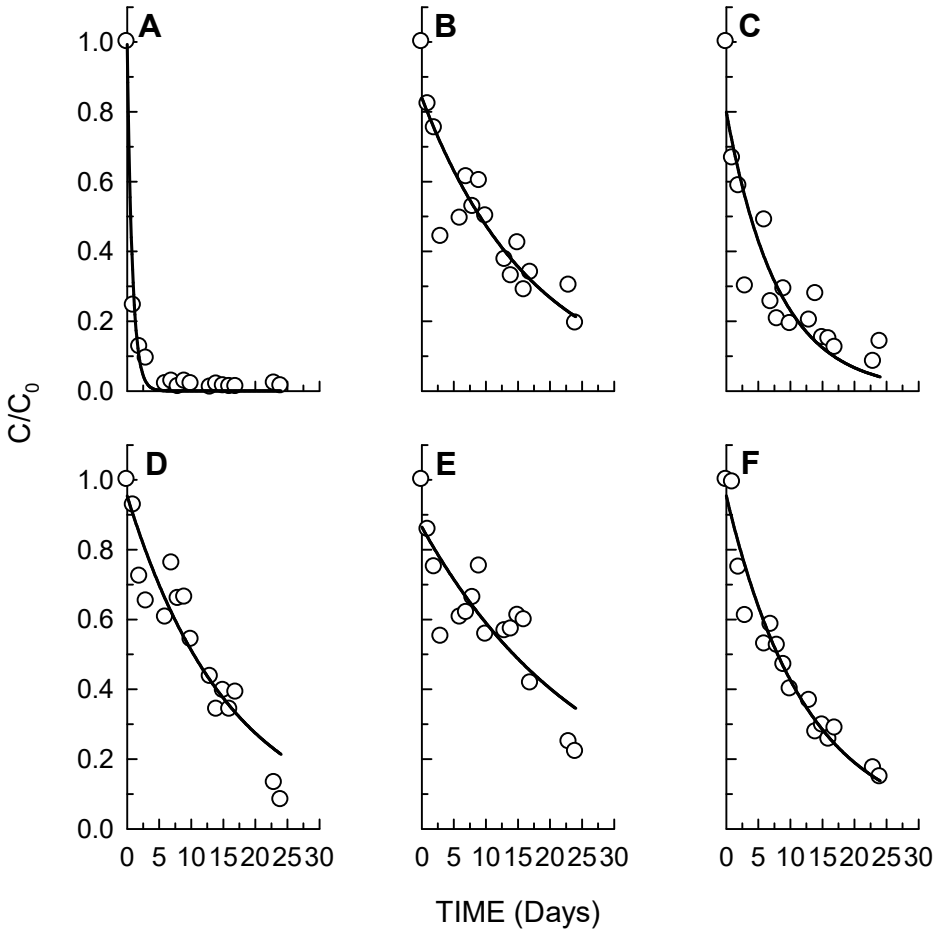

Figure 2. Biodegradation profile of (A) Acetaminophen; (B) Diphenylamine; (C) g-Methylionene; (D) Pyriproxyfen; (E) Sulfamethoxazole and (F) Tonalide

g-Methylionene, which is a fragrance, was degraded rapidly. Over 80% of g-methylionene was utilized within 5 days (Figure 2C). The half-life of this chemical was 5.6 days (Table 3). Yamazaki et al. (1988) showed that g-methylionene was degraded by cis/trans hydroxylation by *Aspergillus niger* [15]. Optimum biodegradation period by that microorganism was 3-4 days which was similar to the value reported in this study.

Pyriproxyfen, which is a pyridine based pesticide, was completely degraded within 28 days (Figure 2D). Its half-life was calculated as 11 days (Table 3). Similarly, Fathulla (1993) reported a half-life of 23 days for pyriproxyfen [16]. During the biodegradation of pyriproxyfen, two major by-products i.e. 4-(4'-hydroxyphenoxy)phenyl-2-(2-pyridyloxy)-propyl ether and 2-(2-pyridyloxy)propionic acid were identified [16].

Sulfamethoxazole is one of the most frequently detected antibiotic in wastewater and surface water. Biodegradation of sulfamethoxazole by mixed cultures or pure strains have been reported [17, 18]. In our study, 80% of sulfamethoxazole were degraded within 25 days with a corresponding half-life of 18 days (Figure 2E and Table 3). The half-life of sulfamethoxazole was the highest among the other chemicals that were degraded, this probably due to its antimicrobial effect although its test concentration was low. Sulfamethoxazole degradation is initiated by hydroxylation of the carbon atom attached to the sulfonyl group (ipso-hydroxylation). The most common by-products detected include 3-amino-5-methylisoxazole, and benzoquinone-imine [19, 20]. The latter is then transformed to 4-aminophenol.

Tonalide is a musk that is extensively used in fragrances. Above 80% of the tonalide was

degraded in 25 days with a half-life of 9 days (Figure 2F and Table 3). Half-lives between 1 and 20 days were reported for tonalide in activated sludge and surface waters. Broad range of half-lives reported may be due to the difference in the population size of the degraders in inoculum used in those studies.

On the contrary, concentration of the rest of the chemicals that are not biodegradable such as quinoxyfen (Figure 3A) and simazine (Figure 3B) did not change during the incubation period. Although there is no evidence for the biodegradation of quinoxyfen; simazine, which is a triazine pesticide, is known to be utilized as carbon and nitrogen source by microorganisms [21-24]. This suggest that not every inoculum contains degraders that can utilize a certain chemical.

Table 3. Biodegradation rate constants and half-lives of six biodegradable chemicals tested in this study

Compound	k (d^{-1})	$t_{1/2}$ (days)	R^2
Acetaminophen	1.24	0.56	0.99
Diphenylamine	0.0572	12.12	0.80
Methyl ionene	0.1241	5.59	0.81
Pyriproxyfen	0.0623	11.13	0.89
Sulfamethoxazole	0.0382	18.15	0.72
Tonalide	0.0807	8.59	0.95

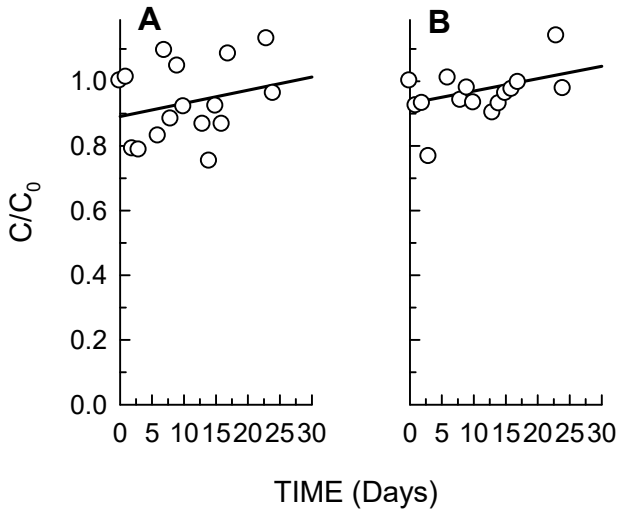

Figure 3. Biodegradation profile of (A) Quinoxyfen and (B) Simazine

4. CONCLUSIONS

In this study, we showed that conventional biodegradation tests may be enhanced by coupling mass spectrometry and become more sound and representative for testing biodegradation of chemicals. This is the first study that tested the biodegradation of many compound simultaneously at such low concentrations. Succesfull results promise that this methodology can be used as an alternative to current standart OECD biodegradation protocols. However, in order to synchronize the output of high-throughput biodegradation experiments, we also need to include genomics to understand the microbial community structure of inocula used in different experiments.

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