Green Chemistry

PAPER

Biodegradability assessment of complex chemical mixtures using a carbon balance approach†

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The assessment of complex mixture biodegradability can be limited by technical issues and/or difficulties to rule on ready or inherent biodegradability. This work is composed of three different studies to introduce and improve Ultimately Transformed Organic Carbon (UTOC) as a quantification tool for biodegradation. The UTOC includes the inorganic carbon resulting from respiration and the carbon assimilated by microorganisms. The UTOC is correlated with dissolved organic carbon removal and can be a robust alternative for non-soluble substance evaluation. The UTOC was evaluated using a complex mixture of soluble and non-soluble substances and verified with an UVCB (vegetal extract). The advantages of UTOC are clear; it is an appropriate method to quantify the initial raw material converted into an inert product by the action of microorganisms and to determine the ready biodegradability of an unknown substance such as a vegetal extract.

Introduction

According to the United Nations (UN), substances are "chemical elements and their compounds in the natural state or obtained by any production process".[1] This definition has evolved according to different acts of regulation (see ESI Table 1†). Another category of substances is UVCB: Unknown or Variable composition, Complex reaction products or Biological materials such as crude oils or vegetal extracts.[2] In addition, there are "mixtures or solutions composed of two or more substances in which they do not react".

The methodology and metrology for biodegradability testing of well-defined substances were developed 40 years ago and standardized through OECD and ISO Guidelines. However, the chemical complexity of UVCB and mixtures leads to potential difficulties in quantifying their biodegradability (Table 1). Authors have described the biodegradation of minerals[3] and vegetal oils[4,5] using CEC L-33-T-82 and L-33-A-93 tests[6,7] and the biodegradation of UVCB can be measured using this strategy.[8] However, low extraction recovery during quantification (due to low solubility of the tested material) can lead to overestimation of biodegradation results.[9]

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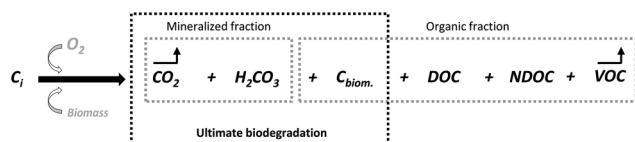†Electronic supplementary information (ESI) available. See DOI: 10.1039/C7GC03386A

Table 1 Possible strategies for the measurement of UVCB and/or non-soluble substance biodegradation. V, the measure is adapted; !, the metrology and/or methodology could inaccurately estimate biodegradability; DOC, dissolved organic carbon removal (*e.g.*, OECD 301A, 301C); BOD, biological oxygen demand (*e.g.*, OECD 301D, 301F); Min. (mineralization), CO_2 production in a closed bottle (*e.g.*, OECD 301B, 310); specific parameters, quantification of parent product removal using radiolabeled substances or analytical chemistry methods (*e.g.*, OECD 306–309)

			UVCB	Non-soluble
Primary biodegradation	CEC L-33-T-82		V	!
	CEC L-33-A-93		V	!
Ultimate biodegradation	Non-specific parameters	DOC	V	!
		BOD	!	V
		Min.	V	V
Specific parameters			!	!

Furthermore, interpretation of biodegradability is restricted to the measurement of primary (biotransformation) but not ultimate biodegradation (definitions provided in ESI Table 2†). Thus, the measurement of Dissolved Organic Carbon (DOC) removal can be used to determine the ultimate biodegradation of UVCB except for non-soluble substances. Specific measures that are easy to perform for a well-defined substance can be challenging for UVCB substances. The extraction step is subject to the limitations of the recovery process, and more important substances of unknown or variable composition are very difficult to characterize and quantify.

Respirometric tests such as the BOD method used by Swigert *et al.*[10] are another strategy for assessing UVCB bio-

degradability. Their interpretation is based on the ratio of BOD/ThOD (Theoretical Oxygen Demand, calculated from the elemental composition); alternatively, the Chemical Oxygen Demand (BOD/COD) could be used for UVCB biodegradability analysis.[11] However, biodegradation can be overestimated using the COD method,[12] especially when the solubility of the tested chemicals is low.[13] For UVCB and non-soluble materials, mineralization is quantified through the assessment of the CO_2 released during the test. This method requires only the measurement of the initial carbon quantity. Using the OECD 301B test, biodegradation assays of non-soluble materials and vegetal oils have been performed.[14,15] Even if the substance is volatile, the OECD 310 test with CO_2 measurement in a closed bottle can be used.[16] However, these two tests based on CO_2 evolution have limitations: OECD 301B is not adapted for volatile substances, and neither of them recommends quantification of the initial organic carbon converted by biomass.

In addition to these methodological limits, scientists must also determine ready or inherent biodegradability (definitions provided in ESI Table 2†). Beyond this classification, demonstration of ultimate biodegradation, and thus the absence of residues, is linked to the capacity to determine the carbon balance at the end of a test (Scheme 1).

Measuring the quantity of initial organic carbon converted into inert products (mineralized) and assimilated in biomass fractions appears to be a simple solution for quantification of ultimate biodegradation. By considering the remaining (non-mineralized or non-assimilated) fraction at the end of a test, classification of biodegradability can be assessed regardless of the complexity of the substance or mixture tested. Unexpectedly, the carbon balance approach has not been exploited; only 6 publications of more than 400 recommend this strategy[17–22] (web of science search using the keywords "biodegradation + carbon + balance", 2016). Furthermore, there are no guidelines dedicated to this strategy.

A new measurement entity has been created to establish a robust approach for determining the ready or inherent biodegradability of complex mixtures of chemicals: Ultimately Transformed Organic Carbon (UTOC). The UTOC measure includes the inorganic carbon from respiration and the carbon assimilated by microorganisms. Because these two types of carbon should be measurable regardless of their physico-chemical properties and the nature of the substance or mixture, the objectives of this work were to (i) define a methodology applicable to all types of substances and mixtures and (ii) propose an unequivocal approach to assess the ready biodegradability of complex chemical mixtures.

Results

Application of the UTOC method to a non-complex mixture of soluble chemicals

The first part is dedicated to the assessment of carbon balance accuracy and evaluation of UTOC relevance compared to the measurement of initial organic carbon removal. The methodology was assessed using three substances and their mixture in equal proportion (sodium benzoate (NaB), 4-nitrophenol (4-NP) and di-ethylene glycol (DEG)). The substances and their mixture are soluble and non-volatile to base the comparison on UTOC with the sole measurement of DOC removal. To overcome biodegradation variability due to microbiological diversity, these assays were inoculated with standardized biomass that was adapted to these three compounds. Depending on the biodegradation kinetics (see ESI Fig. 1†), the bioreactors were stopped, and the carbon concentration was measured in each compartment and was reported in percentages. The results are shown in Fig. 1A.

Regardless of the testing conditions, it can be observed that carbon at the end of the tests is distributed in both compartments. In every assay, DOC remains (min: 29.3 ± 3.1 (4-NP), max: 47.9 ± 0.7 (Mix)), and a major fraction has been mineralized (min: 33.1 ± 2.3 (Mix), max: 57.7 ± 5.8 (4-NP)) compared to that assimilated by the biomass (min: 9.2 ± 2.0 (4-NP), max: 28.5 ± 14.8 (DEG)). Low standard deviation values (except for the DEG biomass fraction due to a high biomass concentration measured in one bioreactor) demonstrate acceptable reproducibility to interpret recovery. The results are shown in Fig. 1B. With values approaching 100% for all assays (98.5 ± 0.2 (Mix) to 96.2 ± 0.7 (4-NP)), the methodology developed and the metrology used can be considered adequate for the investigation of the relevance of the UTOC compared to the measurement of DOC removal (Fig. 1C).

This table leads to a major observation: the % DOC removal and % UTOC are close to each other at the end of the test (min. difference: 52 ± 1 $vs.$ 50 ± 1 (Mix); max. difference: 70 ± 4 $vs.$ 66 ± 5 (4-NP)). The results appear to be logical regarding the physico-chemical properties of the evaluated substances (non-volatile and soluble) and the equation in Scheme 1 because the recovery reaches nearly 100%. The results demonstrate the feasibility of this type of approach using our protocol to assess the ultimate biodegradability of substances tested independently and in a non-complex soluble mixture. This validation step allowed for the formulation of the following hypothesis: if the DOC removal measurement can inaccurately estimate the biodegradability of non-soluble substances, interpretation of the UTOC should be an appropriate alternative. The next steps investigated the impact of the S_0 (sub-

Scheme 1 During aerobic biodegradation in a bioreactor, an initial quantity of carbon (C_i) is mineralized by microorganisms. The mineralized fraction of C_i equilibrates in two phases: gaseous (carbon dioxide) and liquid (carbonic acid). Some of the C_i is converted to biomass ($C_{biom.}$) and non-biodegraded carbon can be dissolved (DOC), not dissolved (NDOC) or volatilized (VOC), depending on the solubility and volatility of the parent and/or degradation products.
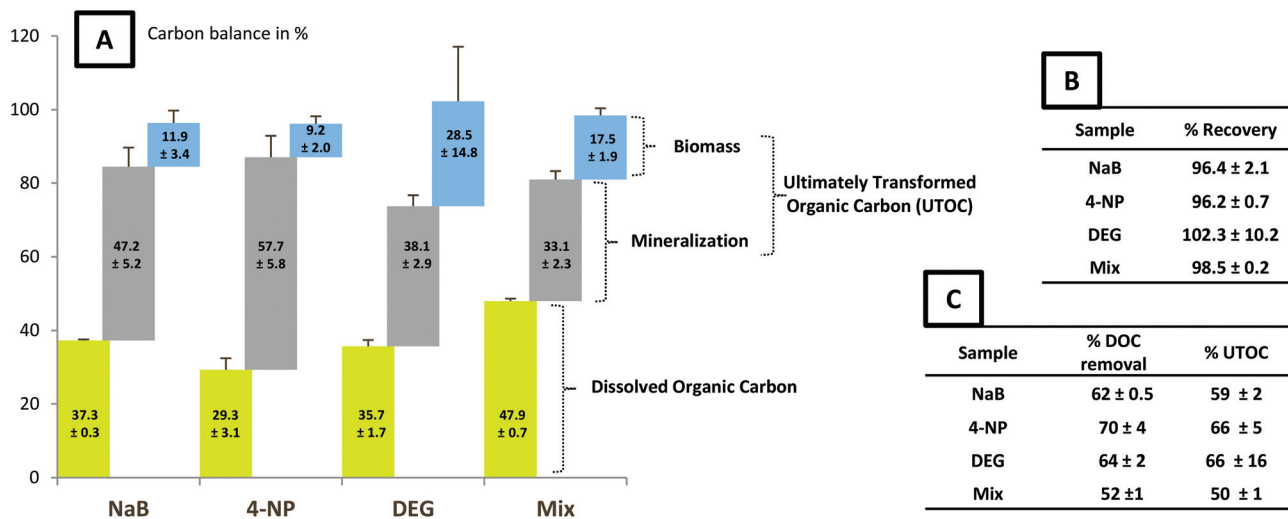
Fig. 1 (A) Duplicates of the biodegradation carbon balance of 3 substances (X_0 = 30 mg of carbon per liter (mgC l^{-1})): sodium benzoate (NaB), 4-nitrophenol (4-NP), di-ethylene glycol (DEG) and their mixture in equal proportion (33% × 3 × 30 = 90 mgC l^{-1}) and inoculated, S_0, at optical density (OD) = 0.033 ± 0.002 for the substances and OD = 0.1 ± 0.002 for their mixture to maintain the same S_0/X_0 ratio of 0.001. The carbon recovery and percentage of biodegradation based on DOC removal or the UTOC are shown in tables B and C.

stance)/X_0 (biomass) ratio. The results presented above were realized under the same S_0/X_0 ratio, and the literature has highlighted the variation in CO_2 production and biomass growth when the S_0/X_0 ratio evolves.

Application of the UTOC method to a complex mixture of non-soluble chemicals

The biodegradation of a mixture of 5 soluble and non-soluble substances emulsified in 76% water (cosmetic formula) was investigated. These 5 substances are typical ingredients in cosmetic formulas and were mixed at realistic concentrations (%): 8% isononyl isononanoate (ISO), 5% cetyl alcohol (CYA), 5% mineral oil (MIO) – registered as an UVCB for Variable Composition in the EINECS (European INventory of Existing Commercial chemical Substances) list – 5% glyceryl stearate (GST) – registered as an UVCB for Variable Composition in the EINECS list – and 1% phenoxyethanol (PHX) – the only substance that is soluble under the testing conditions. First, these substances were tested independently with bioreactors inoculated with activated sludge at different concentrations, giving an S_0 (mgC l^{-1})/X_0 (mgSS l^{-1}) of 10. Second, the emulsion of these substances was tested under two S_0/X_0 ratios: 30 and 3. Due to the low solubility of 4 substances under the testing conditions, the incubation was extended to 5 days (within a maximum of 28 days) after respiration activity stopped (ISO and CYA: 28 days; PHX: 20 days; and MIO and GST: 15 days). The carbon balance results are presented in Fig. 2A and B.

Considering only the substance results (Fig. 2A), it can be observed that carbon was quantified in each measured compartment except for the PHX duplicates, for which no carbon conversion into biomass was detected. The maximum carbon assimilated by biomass was observed for the CYA (12 ± 1%) assays. The proportion of initial carbon mineralized ranges

from 97 ± 18% (PHX) to 19 ± 3% (MIO). Interpretation of the mixture's carbon balance (Fig. 2B) is interesting because it is not the same mixure as that created in the first study (direct addition of several water-soluble substances, Fig. 1A). Here, the mixure is an emulsion of five substances. This process made the mixure miscible in the culture media, leading to a homogenate dispersion of the organic fraction in the bioreactors. Therefore, the miscibility of parent substances was highly increased in the mixture, explaining the higher percentages of carbon recovery achieved for the mixture (Mix 6: 96 ± 3%, Mix 60: 88 ± 4%) relative to that of individual non-soluble substances. Compared to the test results for individual non-soluble substances, the biomass assimilation (Mix 6: 23 ± 1%, Mix 60: 16 ± 2%) and mineralization (Mix 6: 70 ± 1%, Mix 60: 70 ± 1%) of the mixture are higher. These improved results for the mixture could be explained by an improvement in carbon bioavailability linked to the increased miscibility of its non-soluble components. Notably, the proportion of carbon measured in the biomass compartment was higher for the assays inoculated with less biomass (Mix 6: 23 ± 1% > Mix 60: 16 ± 2%), and the mineralization percentages were the same (70 ± 1%) for both high and low S_0/X_0 ratios.

The UTOC values were calculated to investigate the influence of mixing and are summarized in Fig. 2C. In this table, the percentage of UTOC is reported separately for each test and is expressed as the representative proportion of carbon in the mixture (framed columns) leading to the theoretical percentage of UTOC relative to the total quantity of carbon. There are four interesting observations. First, the percentage of UTOC is heterogeneous and widely dispersed depending on the substance tested (min: 23 ± 2% (MIO), max: 97 ± 18% (PHX)). This maximum was reached for PHX, the sole soluble substance. Second, the percentage of UTOC for all the non-
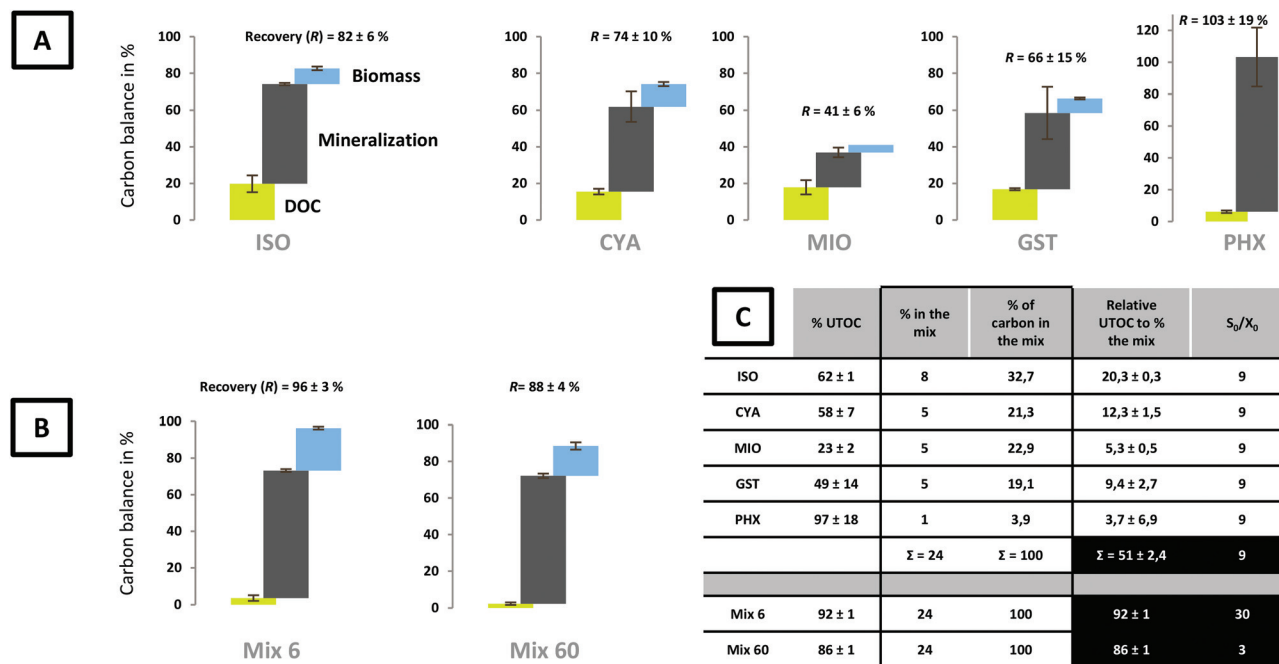
Fig. 2 (A) Biodegradation carbon balances of 5 independently tested substances, according to their relative proportions in the mixture: 8% isononyl isononanoate (ISO) – 80 mg l^{-1} (60 mgC l^{-1}), 5% cetyl alcohol (CYA) – 50 mg l^{-1} (39 mgC l^{-1}), 5% mineral oil (MIO) – 50 mg l^{-1} (42 mgC l^{-1}), 5% glyceryl stearate (GST) – 50 mg l^{-1} (35 mgC l^{-1}) and 1% phenoxyethanol (PHX) – 10 mg l^{-1} (7 mgC l^{-1}). The concentration of the inoculum was adapted to each carbon concentration to equalize the S_0/X_0 ratios. Duplicates containing ISO were inoculated at 6 mg l^{-1} of suspended solid (mgSS l^{-1}), CYA at 4 mgSS l^{-1}, MIO at 4 mgSS l^{-1}, GST at 3.5 mgSS l^{-1} and PHX at 0.7 mgSS l^{-1}. All assays had the same S_0 (mgC l^{-1})/X_0 (mgSS l^{-1}) ratio of 10. (B) Biodegradation carbon balance of the mixture – 183 mgC l^{-1} (the sum of the carbon corresponding to the relative concentration of each substance in the mixture) – inoculated at 6 (S_0/X_0 = 30) and 60 mgSS l^{-1} (S_0/X_0 = 3). (C) UTOC comparison between each substance and the mixture.

soluble substances tested is lower than the minimum percentage observed for the mixture (max: 62 ± 1% (ISO) < 86 ± 1% (Mix 60)). Third, considering the percentage of UTOC relative to the total quantity of carbon in the mixture, it can be observed that the sum of the UTOC generated by all the substances (51 ± 2.4%) is less than the minimum percentage observed for the mixture, independent of the S_0/X_0 ratio (% UTOC Mix 60 > the sum of relative percentage of UTOC of substances alone < % UTOC Mix 6). This observation confirms the increased bioavailability argument: emulsification of the mixture has a positive impact on biodegradation. Finally, Mix 6 (S_0/X_0 = 30) presents a higher proportion of UTOC than Mix 60 (S_0/X_0 = 3), 92 ± 1% and 86 ± 1%, respectively. Note that, in this case, the percentage of UTOC is only influenced by values measured in the biomass compartments: as described above, the percentage of mineralized carbon is the same for both high and low S_0/X_0 ratios.

During this study, the applicability of the UTOC concept was assessed using soluble and non-soluble UVCB and mono-constituent substances for comparison with their emulsion. The UTOC concept can be applied for all biodegradation tests and resulted in reliable assessments, contrary to (i) the DOC removal assessment, which was not relevant for 4 out of 5 substances and (ii) the mineralization assessment, which led to an underestimation of the ultimate biodegradation for all non-soluble substances tested.

Application of the UTOC method to an unknown mixture: a vegetal extract

The third part of this work was designed to verify that the UTOC concept is applicable to an UVCB substance (vegetal extract) of unknown composition. The strategy was to (i) quantify its carbon composition following a standard curve procedure and (ii) identify a suitable non-inhibitory concentration of the substance to be tested using activated sludge. The first task was realized by diluting different quantities of the water-soluble vegetal extract (powder) into culture media and measuring the carbon concentration using a TOC meter. Its high solubility in culture media allowed for the analysis of the carbon composition of the vegetal extract using a high concentration range. From 10 to 1000 mg l^{-1} (7 dilutions tested, results not shown), the linear coefficient was 0.999, indicating with confidence a carbon proportion of 48.9%. Other strategies could be investigated to quantify the carbon composition of a non-soluble UVCB substance, such as the direct injection of the substance into the TOC meter or use of a standard curve procedure with an adapted solvent, which was implemented due to the unknown composition of the tested substance. An inadequate concentration of the parent product can induce potential toxicity to the inoculated microorganisms, affecting their viability and/or inhibiting inoculum growth on the substrate and thus biodegradation. This potential toxicity has

been evaluated according to the OECD 209 test.[23] The results (not shown here) demonstrated that no inhibition of activated sludge was detectable from 10 to 1000 mg l^{-1} of vegetal extract. Next, the vegetal extract was tested in triplicate in biodegradation tests inoculated with activated sludge for 28 days. The online CO_2 sensor results are shown in Fig. 3A. Two replicates have the same observable trend and the final value of the third is slightly higher. The most important information is the time frame between the lag phase and the end of the log phase. This time window is less than 10 days. Even if the carbon balances were established after 28 days, it can be assumed that the biodegradation reaction has occurred in the initial days, which is less than 10 days. The carbon balances are shown in Fig. 3B.

The carbon recovery for each replicate is close to 100% (min: 92% (assay 1), max: 102% (assay 2)). Carbon was distributed in each compartment. The average DOC, mineralization and biomass percentages are 12 ± 3, 77 ± 9 and 8 ± 2%, respectively. Based on these results, the percentage of DOC removal was ≈88%, and the percentage of UTOC was ≈85%. In this case of a substance of unknown composition soluble in water, concordance between the DOC removal and UTOC was verified.

To integrate the UTOC concept in a weight-of-evidence approach to determine biodegradation, the vegetal extract evaluation study was extended to an ecotoxicological assessment. As a supplement to the activated sludge respiration inhibition test that is a part of the biodegradability assessment procedure, the purpose of this step was to reinforce the approach to determine ready biodegradability. Because approximately 12% of the organic carbon remained after 28 days, the acute toxicity of parent and biodegradation products was evaluated on micro- and macroorganisms using four normalized tests: ISO 10712 (*Pseudomonas putida* growth inhibition test), ISO 11348-3 (determination of the inhibitory effect on the light emission of *Vibrio fischeri* (luminescent bacteria test)), ISO 8692 (fresh water algal growth inhibition test with unicellular green algae) and ISO 6341 (determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)). For parent product acute toxicity, pure solutions of vegetal extracts in water were tested at 4 different concentrations: 10, 1, 0.1 and 0.01 g l^{-1}. For the biodegradation products, the culture media were sampled directly from the bioreactors of the biodegradation test, filtered, and tested. The results (Table 2) highlight the absence of acute toxicity to microorganisms of the parent and biodegradation products and acute toxicity to macroorganisms of the parent product and at the two highest concentrations (10 and 1 g l^{-1}). However, these toxic concentrations have no environmental relevance and markedly exceeded the regulatory threshold for considering substances to be hazardous for the aquatic environment (≤ 0.1 g l^{-1}).

Table 2 Ecotoxicological test results (percentage of inhibition) at T_0 (before biodegradation) and at T_{28} (after biodegradation, replicates (Rep.) 1, 2 and 3)

	T_0 (vegetal extract concentration, g l^{-1})				T_{28} (culture media)		
	10	1	0.1	0.01	Rep. 1	Rep. 2	Rep. 3
ISO 10712	0	0	0	0	0	0	0
ISO 11348-3[a]	NA[(a)]				0	0	0
ISO 8692	0	0	0	0	NA[(b)]		
ISO 6341[b]	100	80	0	0	NA[(c)]		

[a] Results at 30 min. [b] Results at 48 h. NA, not applicable; NA[(a)], emitted light is absorbed by the vegetal extract leading to false positive toxicity induced by the color of the solution; NA[(b)] and NA[(c)], addition of a sample from the bioreactor at T_{28} generates precipitate, making ISO media unsuitable for maintaining organism viability.
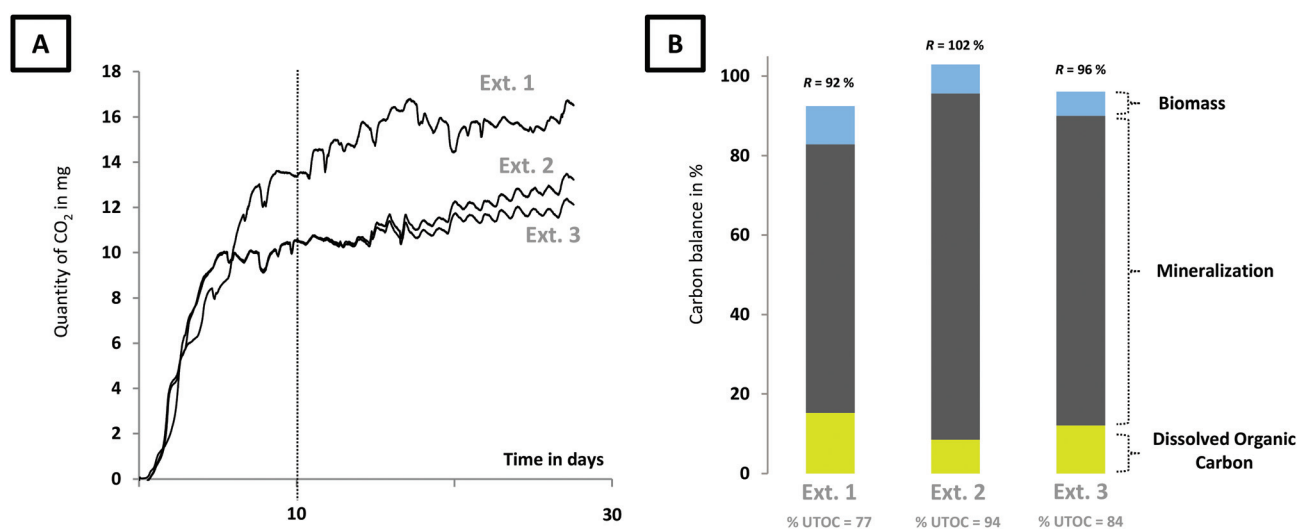

Fig. 3 (A) The quantity (mg) of CO_2 in the bioreactor atmosphere during a biodegradation test of the vegetal extract (Ext.) in triplicate (Ext. 1, 2 and 3). (B) Biodegradation carbon balances after 28 days for three assays of the vegetal extract (122 mg l^{-1} (59.6 mgC l^{-1})) in bioreactors inoculated with activated sludge (20 mgSS l^{-1}), $S_0/X_0 = 3$. R, carbon recovery.

Discussion

The materials and methods selected and the strategy designed to establish the carbon balance are subject to discussion. A large set of protocols have been published to study carbon transfer during microbiological reactions (as previously cited, more than 400 publications are referenced in the web of science with the keywords "biodegradation + carbon + balance"). In terms of carbon measurement for ready biodegradability assessment, the most commonly used are quantification of the DOC removal and/or mineralization and guidelines dedicated to these measures (*e.g.*, for DOC removal: OECD 301A/C tests; for mineralization: OECD 301B/OECD310 tests[11,24]). Technically, the methods recommended in these guidelines do not differ from those used in this work. For both DOC and dissolved inorganic carbon (DIC), sample filtration and, among others, utilization of a TOC meter have been shown to be appropriate.[25,26] The BlueSens technology (gas sensors) for atmospheric CO_2 measurement has been used for mineralization assessment.[27] The least-tried strategy, which is also not normalized for biodegradation tests, is the measurement of carbon in the biomass compartment. Authors have experimented with different materials and methods for this measure, such as analysis of the elemental composition of dried biomass (*e.g.*, standard test ATSM D5291), the biomass/protein ratio (determined using the Lowry method) or the relationship between the optical density and the biomass carbon content calibrated using a technique cited before.[21] The strategy designed in this study (sampling, centrifugation, dilution of the pellet in pure water, sonication and direct injection into the TOC meter) has resulted in robust results in preliminary studies with a pure culture when the carbon quantity was aligned with the optical density and number of cells during growth.

The referenced examples are summarized in Fig. 4 to confirm the reliability of this method using the biomass carbon content percentages measured from this work and those reported in the literature (Fig. 4). The simple technical design used to validate the UTOC concept has allowed for utilization of a minimum of analyzers, leading to a reduction in the incertitude of the results.

In the experiments establishing the UTOC proof of concept, no sorption events leading to an overestimation of carbon contained in the biomass were found for the substances assayed. In the case of appearance, as determined in some biodegradation tests,[28,29] one solution may be to incorporate the OPPTS 835.1110 guideline in the testing procedure to authorize or decline the designed methodology (this guideline describes a procedure for measuring the extent to which a chemical compound distributes itself between activated sludge as the sorbent and water as the solvent). Finally, for testing of high-density solid and/or viscous compounds, additional washing steps of the pellet should be included to enable good separation of the biomass from the liquid media and prevent parent product accumulation within the pellet.

The inoculation of adapted biomass in this initial study was designed to reduce microbiological variability (enabling a selection of specific degraders). This adaptation of the biomass was done to guarantee reproducible results and to focus the work both on the carbon balance accuracy and on the comparison of DOC removal with the UTOC for soluble substances.[47] However, adaptation could have radically modified the microorganism communities compared to those of a freshly sampled environmental inoculum, as recommended by standard test guidelines. Conversely, adaptation to the 3 different substances could have reduced diversity and thus the potential of the inoculum to totally metabolize these substances. This can explain why the biodegradability results obtained in our study did not systematically match the biodegradation percentages commonly found in the literature and reference databases such as ECHA, for which NaB, 4-NP and DEG are registered as readily biodegradable substances (DOC removal ≥ 70%, mineralization ≥ 60%)[43–45] and are used in the literature as a positive control.[30] Repetition of the experiments with non-adapted biomass could provide a second

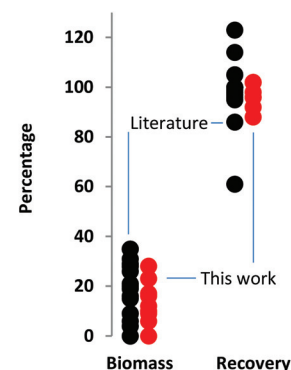| Reference	Biomass carbon content	Tested substances	Biomass (%)	Recovery (%)	n	
1	TOC meter	Bisphenol A / 4-HBA	19–26	98–100	2	
2	Optical Density	Glucose / Starch	20–35	nd	2	
3	Lowry method	Phenantrene	28–29	99–100	2	
4	Lowry method	PAH	16–35	86–105	11	
5	Elemental composition	Gasoline	31	97	1	
6	Elemental composition	Polymeric material	4–15	96–114	20	
7	Lowry method	Polymeric material	0–9	61–95	7	
8	Elemental composition	Bioplastics	6–21	100–123	9	
This work, first study		NaB / 4-NP / DEG	9–28	96–102	3	
		NaB + 4-NP + DEG	17	98	1	
This work, second study		ISO / CYA / MIO / GST / PHX	0–12	na	5	
		ISO + CYA + MIO + GST + PHX	16–23	88–96	2	
This work, third study		Vegetal extract	6–10	92–102	3	

Fig. 4 Comparison of min. and max. carbon converted into biomass and min. and max. carbon recovery values from 54 carbon balances with min. and max. values from this work. The percentages in the "biomass" and "recovery" columns are plotted in the graph on the right to visually interpret the difference in magnitude between these in the literature and in this work. References: 1 (Lobos *et al.*, 1992),[46] 2 (Raghavan *et al.*, 1993),[20] 3 (Bouchez *et al.*, 1995),[41] 4 (Bouchez *et al.*, 1996),[17] 5 (Solano-Serena *et al.*, 1999),[39] 6 (Calmon *et al.*, 1999),[42] 7 (Pagga *et al.*, 2001),[18] and 8 (Domenek *et al.*, 2004).[40] ND, not determined; NA, not applicable (due to non-soluble properties of tested substances).

interesting set of data in order to compare them to the results with adapted biomass and the literature. However; the adaptation strategy implemented does not interfere with the study of compartmentalization of carbon and the establishment of the proof of the UTOC concept. The same discussion and arguments can be applied to the interpretation of the influence of the NaB, 4-NP and DEG mixture. Even if the mixture, under the same S_0/X_0 ratio, appears to have a negative impact (% UTOC mix < % UTOC substances), the conclusion must be moderated without further investigation of the impact of adaptation on the initial microbiological diversity and behavior in bioreactors, among other factors.

The protocol used in the second study is closest to ready biodegradability (RB) test guidelines (fresh activated sludge inoculated at a concentration of <30 mgSS l^{-1}, substance tested at a concentration of <100 mgC l^{-1}). Except for PHX, comment on the proportions of DOC is not relevant, as these substances are not soluble. The carbon detected in the DOC compartments could be fractions of the parent product scattered in culture media and/or solubilized fractions of the parent product and/or soluble biodegradation products. For this reason, carbon recovery is subject to the same interpretation: from the moment that the insoluble parent product is added to the bioreactors, accurate sampling and dosing are impossible. The shortfalls in recovery could be attributed to this technical limitation, hence the necessity to demonstrat in the first study that the association of mineralization and biomass formation was conversely representative of DOC removal. In this second study, 4 out of 5 tested substances are known to be readily biodegradable: ISO, CYA, GST and PHX. MIO is the only substance categorized as inherently biodegradable and also has the lowest observed percentage of UTOC. Except for PHX, the others did not reach RB criteria (mineralization < 60%). However 2 substances (ISO and CYA) are really close to this pass level. The protocols used here could justify these gaps: (i) investigations have shown that registered results in databases (*e.g.*, ECHA) were obtained with higher concentrations of biomass (15–30 mgSS l^{-1}) relative to those used in this study (1–6 mgSS l^{-1}); and (ii) the geographical origin of the inoculum is different from that of other studies. These two parameters influence test results;[31,32] nevertheless, in our work, a clear differentiation was observed between RB and inherently biodegradable substances.

In order to investigate more precisely the strength of the UTOC concept, results of biodegradation tests realized with the cosmetic formula have been compared to those that would have been calculated according to the standardized methods OECD 301A and 301B/310. Indeed and as cited before, materials and methods implemented in this study are following recommendations of OECD 301 guidelines. Results are presented in Table 3.

For all non-soluble substances, the guideline 301A is not applicable. For PHX, the percentage of biodegradation is similar to 301B/310 and UTOC results. The results are in the same range as those published by the ECHA. Considering only the guidelines applicable for all the substances, the 301B/310

Table 3 Percentages of biodegradation according to the OECD 301A and OECD 301B/310 tests, UTOC concept and the results published by ECHA or US EPA. NA, not applicable

	301A based calculation	301B/310 based calculation	UTOC	Results published by ECHA or US EPA*
ISO	NA	54	62	61
CYA	NA	46	58	>60
MIO	NA	19	23	0–24*
GST	NA	41	49	95
PHX	94	97	97	90

The asterisk corresponds to the sole published result coming from US EPA whereas the other comes from ECHA.

standardized methods, all the results of non-soluble substances are lower than those obtained with the UTOC concept. It is also interesting to note that, except for the GST, UTOC results and ECHA/US EPA results are very similar. In addition, to provide an alternative to the method 301A for non-soluble substances, the UTOC concept makes the results more accurate and so close to the published results.

Emulsion has a clear positive impact on UTOC proportions. Such results can be explained by the bioavailability increase of poorly water-soluble components of the mixture due to the emulsion process and its incidence on biodegradability. This interpretation is well-described in the literature.[33–36] Finally, regarding the effect of the inoculum level, the results are concordant with the literature in terms of increased microorganism growth for assays inoculated at a low biomass level.[37]

Finally, the technical robustness and concordance between the results of the two initial studies and theory or the literature allowed for testing a substance of unknown composition. The results on a vegetal extract showed satisfying recovery percentages. In exploitation of CO_2, the online measurements allowed for defining a time window of less than 10 days, which is one of the requirements for reaching RB criteria. For DOC removal and mineralization, the RB criteria ($\geq 70\%$ and $\geq 60\%$, respectively) have been validated. Note that similarly to the strategy used to build Table 3, the result calculated with the 301A method for the vegetal extract is similar to the UTOC percentage (mean = 85 *vs.* 87%, respectively), while the result calculated with 301B/310 methods is lower (mean = 77%). It proofs again that (i) UTOC is a robust alternative to the 301A method for non-soluble substances and (ii) UTOC enhances the accuracy of the estimation of ultimately biodegraded materials compared to 301B/310 guidelines. Finally, the majority of ecotoxicological test results show an absence of toxicity for both the parent product and potential remaining residues at the end of the test. This global procedure could be related to a weight-of-evidence approach. A diagram summarizing our testing strategy for UVCB substances is presented in Fig. 5.

UVCB substances can be characterized by their elemental composition or their carbon content. In these two cases, the appropriate quantity of UVCB substances based on the carbon content can be determined to measure biodegradation. With specific standardized methods to evaluate the biosorption and
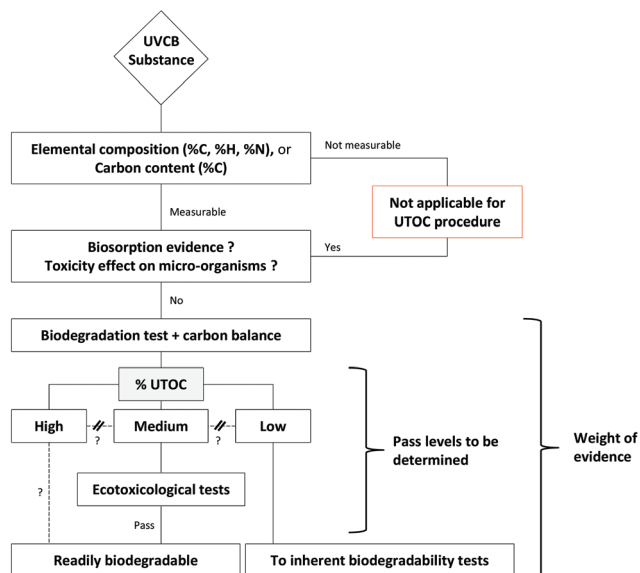
Fig. 5 The weight-of-evidence diagram designed to assess ready biodegradability of a complex mixture.

possible inhibition of activated sludge microbial activity, an initial diagnosis can be conducted to authorize the use of the UTOC concept (see "Not applicable for the UTOC procedure" in Fig. 5). After a biodegradation test, respiration kinetics acquisition and establishment of the carbon balance, pass levels for a ready biodegradability confirmation step with eco-toxicological tests can be defined (Fig. 5). These pass levels should be defined according to the UTOC to possibly bypass ecotoxicological tests in a weight-of-evidence approach. Established in 1979, the pass levels for classifying a substance as RB are 70% for COD removal and 60% for mineralization. The 10% difference between DOC removal and mineralization is justified by splitting of the initial DOC into two compartments: CO_2 and biomass.

The results obtained in this study showed that DOC removal and UTOC measures were equivalent. Thus, the pass level could be the same for both DOC removal and the UTOC. However, based on the nature of UVCB, which are complex mixtures of chemicals, a more stringent pass level should be applied to reduce the probability of recalcitrant residues or metabolites after the biodegradation event. An alternative to the 70% COD pass level should consider the arguments developed by Gerike concerning non-probable formation of C1 metabolites.[38]

Applied to NaB ($C_7H_5NaO_2$), a reduction of more than 6 carbons out of 7 eliminates the risk of recalcitrant residue formation. For the UTOC, the high level should be at least 86% to conclude on the absence of persistency. With the longest-molecule mineral oil, MIO ($C_{24}H_{51}$), this high pass level is evaluated at %UTOC > 96%. This solution appears convincing for well-defined mono-consistent substances, but questions arise for mixtures and UVCB. For mixtures, it could be considered to be an average of the carbon numbers of the mixed substances (first study: high pass level > 82%; second study: high level >

94%). In a more stringent approach, the longest carbon chain could be considered as a reference for precaution. According to these data, a high pass level set to 90% appears to be acceptable for UVCB substances. This would result in UTOC pass levels of high ≥ 90%, medium ≥ 70% and low < 70% for assessing ready biodegradation using the diagram in Fig. 5. Applied to the vegetal extract tested (Fig. 3 and Table 2), as all the arguments have been presented (plant natural origin, no ecotoxicity of the parent product, UTOC ≥ 90%, no bacterial ecotoxicity of remaining carbon), we could assume that this UVCB substance is readily biodegradable. Therefore, the major advantage of our testing strategy is the possibility of determining the ready biodegradability of UVCB substances.

Conclusion

The goal of this work has been to introduce a new entity for biodegradability assessment: the Ultimately Transformed Organic Carbon measure, which is the sum of test substance's carbon mineralized and the carbon converted to biomass. To achieve this, a testing strategy was developed to establish the carbon balance of the test substance. This strategy based on a weight-of-evidence approach was validated using a simple mixture of 3 substances tested with adapted biomass. It has been demonstrated that the UTOC is correlated with DOC removal and can be a robust alternative for assessing the ultimate biodegradability of non-soluble substances. Therefore, the advantages of integrating the UTOC for ready biodegradability assessment are substantial: regardless of the complexity of a test mixture, evaluation is reduced to a deductible fraction of the remaining carbon. Five substances mixed in a virtual cosmetic formula were tested. The approach highlighted the beneficial effects of an emulsion on the biodegradation of these substances. In addition, it is necessary to determine pass levels to differentiate unequivocally ready and inherent biodegradability, especially if the full composition of the test substance is unknown. For this reason, an investigation of the biodegradability and ecotoxicology assessment of a vegetal extract was performed. From these results, a weight-of-evidence approach was proposed to determine the ready biodegradability of complex mixtures. Based on the principle of reducing the probability of persistent parent products or generation of toxic by-products during biodegradation, the approach was reinforced with ecotoxicological tests using a weight of evidence approach for a moderate percentage of UTOC. This strategy coupled with the UTOC concept has been shown to provide a robust approach, and further research should focus on more complex substances (viscous or solid, absorbable, volatile).

Materials and methods

Bioreactors

The bioreactors used in this work are produced by BlueSens (BCPreFerm BlueSens gas sensor GmbH, Germany) (Fig. 6).
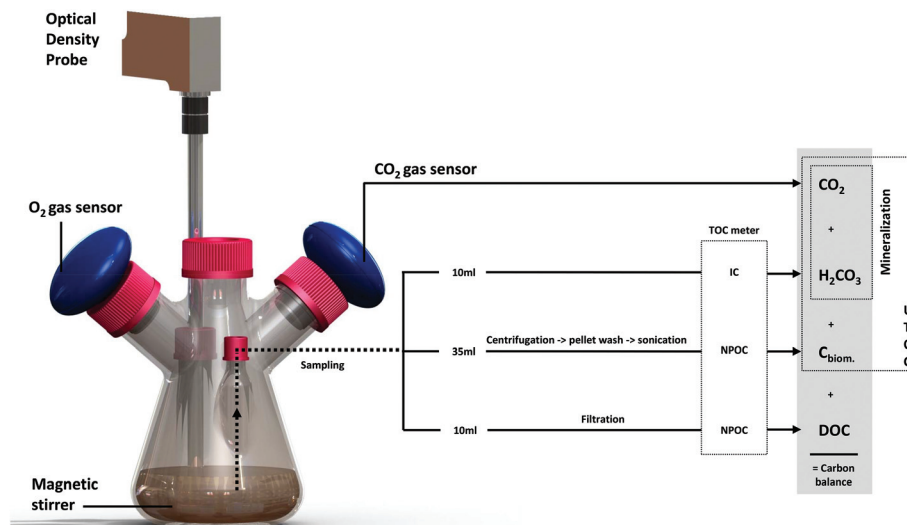
Fig. 6 A BCPreFerm BlueSens gas sensor bioreactor and description of the carbon balance protocol. The TOC meter IC mode corresponds to Inorganic Carbon measurement. The TOC meter NPOC mode corresponds to Non-Purgeable Organic Carbon measurement. Details of the carbon balance methodology are described in the materials and methods section. Details of carbon balance equations are described in Scheme 1.

Briefly, BCPreFerm consists of a closed 1.35 l flask connected with atmospheric CO_2 and O_2 gas sensors. The bioreactors were incubated at 22 ± 2 °C in the dark and coupled with a magnetic stirrer (250 rpm). The final culture media volume (mineral media recommended by OECD 301 guidelines) was 0.814 l for all the assays. As recommended by the 301 guidelines, evidence of hydrolysis and photolysis was confirmed as negative for all the substances. Finally, a bioreactor containing only the mineral media and the same microbial inoculum as those inoculated in the assay was implemented for all the experiments to control for the absence of endogenic respiration.

Chemicals

The substances used in the first study were obtained from Sigma (France) (sodium benzoate (CAS 532-32-1, >99%), di-ethylene glycol (111-46-6, >99%)) and Carlo Erba (4-nitrophenol (CAS 100-02-7, 99%)). The substances used in the second study, isononyl isononanoate (CAS 59219-71-5, Stéarinerie Dubois), cetyl alcohol (CAS 36653-82-4, Cognis), mineral oil (CAS 8042-47-5, Sonneborn), glyceryl stearate (CAS 31566-31-1, Ashland), phenoxyethanol (CAS 8042-47-5, Clariant), were obtained from the L'Oréal environmental research department. The emulsion was created according to the L'Oréal process. The substance used in the third study (vegetal extract) was obtained from the L'Oréal environmental research department.

Soluble substances were added to the bioreactors from a stock solution sterilized by filtration (0.20 μm cellulose acetate, Millipore) and diluted in the mineral media recommended by OECD 301 guidelines. Non-soluble substances were prepared by weighting or pipetting and directly added to the bioreactors containing the mineral media recommended by OECD 301 guidelines.

Microbial inoculum

Adapted biomass. For the first study, the inoculum was obtained from activated sludge sampled from the aerated tank of a municipal wastewater treatment plant. Directly after sampling, the sample was aerated using a magnetic stirrer at 250 rpm and incubated at 22 ± 2 °C. After 12 hours, 50 ml were transferred into 150 ml of mineral media containing NaB (10 mgC l^{-1}), 4-NP (10 mgC l^{-1}) and DEG (10 mgC l^{-1}) and incubated (250 rpm, 22 ± 2 °C). After one week, the suspended solids were collected by centrifugation (6400g, 5 min, RT) and re-incubated for one week in 200 ml mineral media (batch 0) containing NaB (30 mgC l^{-1}), 4-NP (30 mgC l^{-1}) and DEG (30 mgC l^{-1}). This operation was repeated twice. During the last week, 4 × 10 ml was sampled and diluted in 4 different batches: batch 1, NaB (30 mgC l^{-1}); batch 2, 4-NP (30 mgC l^{-1}); batch 3, DEG (30 mgC l^{-1}); and batch 4, Nab + 4-NP + DEG (30 + 30 + 30 mgC l^{-1}). DOC removal was measured daily with direct injection of the filtered sample (0.20 μm) into a TOC meter. After validation of 70% of DOC removal in batches 1, 2, 3 and 4, batch 0 was aliquoted and stored in glycerol (20%) at −20 °C. Before experimentation, a frozen aliquot was pre-cultured overnight (250 rpm, 22 ± 2 °C) in rich media (LB). After 12 hours, this pre-culture was centrifuged (6400g, 5 min, RT). The pellet was washed three times with 10^{-2} M $MgSO_4$. The optical density was measured at 620 nm and was adjusted depending on the test conditions.

Fresh biomass. For the second and the third studies, the inocula were obtained from activated sludge sampled from the aerated tank of a municipal wastewater treatment plant (85 000 eq. inhab.). Directly after this, the sample was aerated overnight using a magnetic stirrer at 250 rpm at 22 ± 2 °C. During aeration, 45 ml was sampled and centrifuged (6400g, 5 min, RT°), the pellet was washed three times with 10^{-2} M $MgSO_4$

and the suspended solid concentration was determined by drying 20 ml at 250 °C for 6 hours. At the end of overnight aeration, 45 ml was re-sampled and centrifuged, the pellet was washed using the same conditions as described above, and the bioreactors were inoculated based on the suspended solid concentration.

Carbon balance

Atmospheric CO_2 quantities were measured using BCP-CO_2 sensors calibrated according to the manufacturer's recommendations. Dissolved Inorganic Carbon quantities were measured by sampling 10 ml of mineral culture media and directly injecting into a TOC meter (TOC-VCSN, Shimadzu) with the following analytical parameters: acid addition (HCl 2N) = 20%, sparging time = 30 s, and injected volume = 50 μl. Dissolved Organic Carbon quantities were measured by sampling 10 ml of mineral culture media, filtering (0.20 μm, cellulose acetate, Millipore) and directly injecting into a TOC meter (TOC-VCSN, Shimadzu, France) with the following analytical parameters: acid addition (HCl 2N) = 20%, sparging time = 30 s, and injected volume = 50 μl. Carbon converted to biomass was measured by sampling 35 ml of mineral culture media, centrifuging (6400g, 5 min, RT) and eliminating the supernatant. The pellet was washed three times with 10^{-2} M $MgSO_4$ and resuspended in 35 ml of pure sterile water. This suspension was sonicated for 90 s (3500 kJ), vortexed and directly injected into a TOC meter (TOC-VCSN, Shimadzu) with the following analytical parameters: acid addition (HCl 2N) = 20%, sparging time = 30 s, and injected volume = 50 μl. Note that during the optimization step, an optical density probe was added to bioreactors to link the OD to the biomass carbon content. This strategy provided reliable results under basic conditions (a clear correlation between the OD and the initial carbon converted to biomass), but several difficulties and biases were highlighted: environmental inoculum (flocs and biofilm formation) and colored substances absorbing at the OD wavelength. With environmental inocula, formation of biofilm can occur. The solution has been to vortex the entire flask to break up flocs and homogenize biomass. This operation was performed when necessary prior to all the previous analyses. The TOC meter was calibrated before each assay according to the manufacturer's recommendations.

Ecotoxicological tests

ISO 10712. The *Pseudomonas putida* growth inhibition test was performed according to the International Standardization Organization Standard ISO 10712:1995. Briefly, a cryopreserved culture of *Pseudomonas putida* NCIB 9494 (stored at −80 °C in glycerol) was used for a growth inhibition test, evaluated using the change in the absorption of a bacterial culture during a 12-hour exposure, and measured using a SPARK 10 M spectrometer (Tecan, France) at 610 nm.

ISO 11348-3. Determination of the inhibitory effect of water samples on the light emission of *Aliivibrio fischeri* was performed according to the International Standardization Organization Standard ISO 11348-3:2007. Briefly, the method using freeze-dried bacteria was used with a cryopreserved culture of *Aliivibrio fischeri* NRRL B-11177 (stored at −80 °C in glycerol). Inhibition of the luminescence emitted by the bacterium was measured using a Lumistox luminometer (Lange, France) during a 30-minute exposure.

ISO 8692. Determination of the growth inhibition of unicellular green algae was performed according to the International Standardization Organization Standard ISO 8692:2012 and evaluated using ALGOTOXKIT F (MicroBioTests, Be). Briefly, growth inhibition of the algae *Selenastrum capricornutum* ATCC 22662 was measured using a Tristar LB 941 spectrometer (Berthold Technologies, France) at 670 nm during a 72-hour exposure.

ISO 6341. Determination of inhibition of the mobility (acute toxicity) of *Daphnia magna* Straus (Cladocera, Crustacea) was performed according to the International Standardization Organization Standard ISO 6341:2012 and evaluated using DAPHTOXKIT F (MicroBioTests, Belgium).

Conflicts of interest

There are no conflicts to declare.

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