Generation of biodegradability data on petroleum constituents using enhanced tests (OECD Guideline 301 F)

In 2020, Concawe began an experimental programme to assess the ability of petroleum constituents to degrade in the environment. This article provides an overview of the results of the programme, which will aid in the development of new strategies for overcoming the difficulties of testing hydrocarbons for biodegradability in the environment.

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Biodegradation assessment

The assessment of biodegradation (the breakdown of chemicals by microbes in the environment) is a major step in the environmental risk assessment of chemicals. Indeed, substances that are eliminated quickly from the environment pose very low risk, while those that can remain for longer periods (days, months or even decades in the most extreme cases) have a much higher potential to build up in the environment, reach living organisms and cause toxic effects. For this reason, persistence assessment is of great importance under European Union chemicals legislation, and is the first step for identifying a substance as PBT/vPvB (Persistent, Bioaccumulative and Toxic/very Persistent and very Bioaccumulative), a category of Substances of Very High Concern under REACH.

In general, the assessment of persistence starts with cheap, fast and stringent screening tests, and gradually progresses to more complex and time-consuming analysis, if needed. The most frequent starting point for testing biodegradability is the ready biodegradability test (RBT), such as the OECD 301 series.[1] In an RBT, a test substance is mixed with a microbial inoculum, typically samples from a wastewater treatment plant, and the breakdown of that substance is monitored over time. Briefly, a substance is considered readily biodegradable if it reaches 60–70% removal in 28 days. These tests do not provide an exact estimation of how long a substance will remain in the environment, but are so stringent that a 'pass' level in a reliable RBT test is normally considered sufficient to conclude that a substance is 'not persistent' in any environmental compartment. The reverse, however, does not apply, i.e. if a substance is found to be 'not readily biodegradable', this does not allow us to conclude that it will persist in the environment, and would warrant further, more complex testing.

For PBT assessment, petroleum substances, which are comprised of hundreds to thousands of different chemicals, are not assessed at the level of the whole substance but by its chemical constituents. Although a lot of data exist on the environmental biodegradation of petroleum substances, tests on individual hydrocarbon constituents following one of the 301 Guidelines are less frequent, and any conclusions based on testing data generated according to these Guidelines are easier to accept for regulatory bodies. Based on the European regulator's stated intention to evaluate triaromatic PAHs for PBT, Concawe started a project in 2020 aimed at generating ready biodegradability data on a number of triaromatic PAHs. These new data can inform the environmental assessment of petroleum substances.

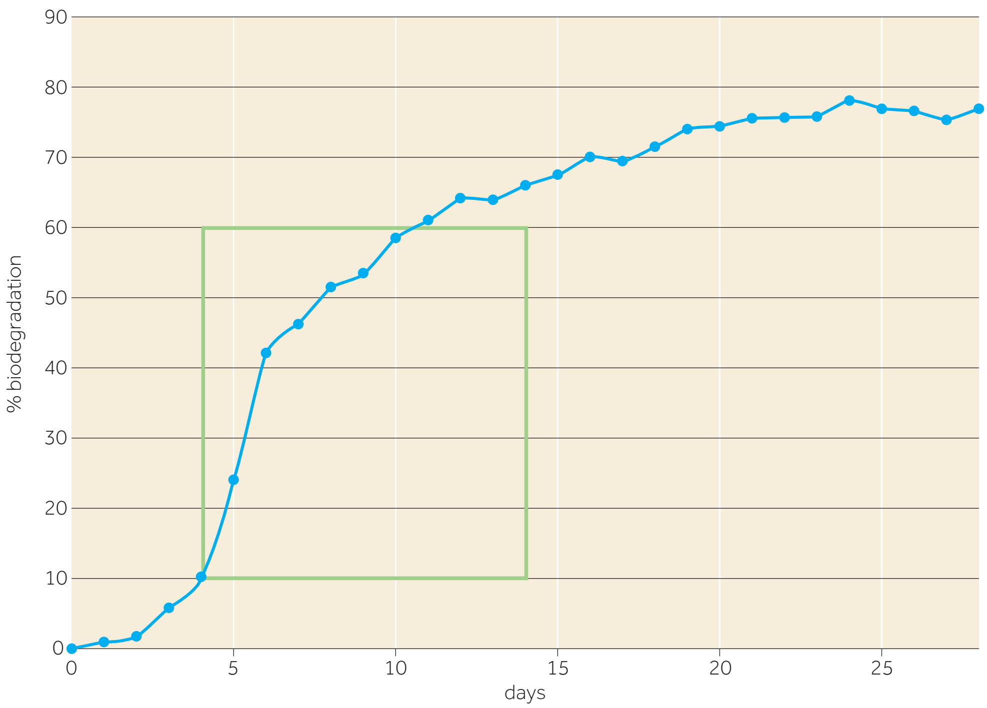
Ready biodegradability tests

RBTs involve incubation of a test substance with a microbial inoculum that is expected to be biodiverse, frequently from waste-water treatment plants, while monitoring the mineralisation of the test substance, which is the complete breakdown of the substance to water and CO_2.

RBTs are thus used to identify those substances that will mineralise quickly and rapidly in the environment (i.e. will not persist). Specifically, a chemical which achieves '≥ 70% biodegradation measured as DOC[1] removal (OECD Test Guidelines 301 A, 301 E and 306) or ≥ 60% biodegradation measured as $ThCO_2$[2] (OECD Test Guideline 301 B) or ThOD[3] (OECD Test Guidelines 301 C, 301 D, 301 F, 306 and 310)' within a 10-day window are designated as readily biodegradable.

Figure 1: Example of biodegradation with time in a 301 F test for a readily biodegradable substance

Note: the green box is the 10-day window within which 60% biodegradation must take place in order for the substance to be considered readily biodegradable.



[1] DOC = dissolved organic carbon

[2] $ThCO_2$ = theoretical amount of carbon dioxide

[3] ThOD = theoretical oxygen demand

The OECD Guidelines state that a readily biodegradable chemical can be assumed to undergo rapid and ultimate biodegradation under most environmental conditions (revised introduction to the *OECD Guidelines for the Testing of Chemicals*, Section 3, 2006). Within the context of the REACH standard information requirements, for mono-constituent substances, higher tier fate information on degradation in water, sediment and soil, such as the degradation rate of the substance and its degradation products, can be waived if the substance is readily biodegradable.[2] Furthermore, for persistence in the context of PBT/vPvB under REACH, additional information can be utilised from ready biodegradation tests, to reach a conclusion of 'not persistent'. Such additional information can be obtained through the waiving of the 10-day window, and extension of the test to 60 days.

Bioavailability improvement methods

Apart from the design of the test used to determine ready biodegradability, it also has to be born in mind that the determination of the biodegradability of a substance can be more challenging due to the inherent properties of the substance. Chemicals displaying high values in their physicochemical properties are harder to test in any kind of setting. For example, a highly volatile chemical will tend to escape from the test system, and a particularly adsorptive chemical may attach itself to the walls of a test vessel, etc. In the case of PAHs, one of the issues is that they are highly insoluble in water. These properties make it likely that, in a test performed in aqueous media, the microbial population that is supposed to biodegrade the test substance will be unable to access the substance from the media (the water). This is what is commonly known as a 'bioavailablity limitation', and in practice means that a chemical which would not persist in the environment appears as failing the ready biodegradability test.

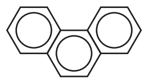
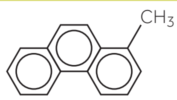
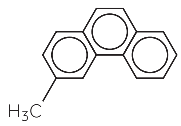
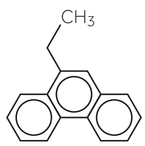
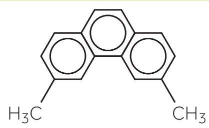
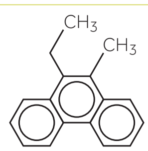
To correct this experimental artefact, the 301 Guideline was modified to include the possibility of applying a 'bioavailability improvement method' (BIM). BIMs increase the possibility that a microbe will be able to access the chemical in the test media, and therefore degrade it, thus allowing a better assessment of its intrinsic biodegradability. Concawe has already applied several such methods for PAHs in the past.[3]

A further possibility allowed for the regulatory assessment of persistence is to extend the incubation time of an RBT, which is normally 28 days, to 60 days.

Constituents tested

Six hydrocarbons were tested in this study. All of them can be obtained commercially, can appear in petroleum substances, and have physico-chemical properties that make them difficult to test (see Table 1).

Table 1: Identities of the hydrocarbons tested

Substance name	CAS number [a]	Structure
Phenanthrene	85-01-8	
1-methylphenanthrene	832-69-9	
3-methylphenanthrene	832-71-3	
9-ethylphenanthrene	3674-75-7	
3,6-dimethylphenanthrene	1576-67-6	
9-ethyl-10-methylphenanthrene	17024-02-1	

[a] Chemical Abstract Service registry number

The six substances were tested according to an OECD protocol, specifically the 301 F test. This Guideline is suitable for poorly soluble and adsorptive substances, and Concawe has obtained good results in the past when applying it to different cyclic hydrocarbons. Briefly, the substances were incubated in aqueous media with sewage from an urban wastewater treatment plant as inoculum (i.e. a source of microorganisms that will act as degraders of the chemicals present in the water). The sewage was sampled at several different days, mixed, and then blended to provide a homogeneous inoculum, with a variety of different microorganisms. The consumption of oxygen was evaluated with a manometric respirometer, and the pH was controlled daily throughout the test. The degradation of the test substance was calculated daily from the oxygen consumption using the equation provided in the 301 Guideline.

As mentioned above, since bioavailability limitations were very likely for these hydrocarbons, two of the BIMs allowed by the 301 Guideline were used: silicone oil and silicon dioxide (SiO_2). Thus, each hydrocarbon was tested:

- with no extra BIM;
- with SiO_2, in the form of silica gel; and
- with silicone oil.

Each of these treatments was applied to three different bottles, and incubated as indicated above. The overall biodegradation was calculated as the average value for the three bottles.

The tests would normally run for 28 days; however, in some cases it was decided to extend the duration to 60 days. The REACH guidance allows the extension of the test duration for the assessment of persistence in case a substance suffers from a bioavailability constraint, and in this case the time extension is considered as a type of BIM.

Any substance reaching a biodegradation level > 60% within 28 days, and with the degradation happening in a 10-day period (the so called '10 day window') can be considered as readily biodegradable, and thus disappearing very quickly from the environment. If the biodegradation reaches > 60% in 60 days, but does not meet the 10-day window, the substance is not readily biodegradable, but can still safely be considered as not persistent.

Results and discussion

For most of the hydrocarbons tested, significant biodegradation was observed during the first 28 days, which is the standard duration of the test. The results indicate that phenanthrene, 1-methylphenanthrene and 3,6-dimethylphenanthrene can clearly be considered as not persistent, since the level of biodegradation obtained was > 60% during the 28-day period with at least one of the BIMs, as displayed in Table 2.

Table 2: results of the OECD 301 tests according to the BIM used and incubation time (28 or 60 days)

Note: not all the tests were run up to 60 days.

	% biodegradation (average)					
	Direct addition (no BIM)		Silicone oil		SiO_2	
Substance name	**28 days**	**60 days**	**28 days**	**60 days**	**28 days**	**60 days**
Phenanthrene	60	N/A	77	N/A	75	N/A
1-methylphenanthrene	48	N/A	64	100	69	76
3-methylphenanthrene	56	58	46	51	46	41
3,6-dimethylphenanthrene	13	N/A	10	N/A	64	75
9-ethylphenanthrene	7	N/A	0	N/A	7	N/A
9-ethyl-10-methylphenanthrene	6	N/A	0	N/A	0	N/A

The degradation of 3-methylphenanthrene shows a less clear conclusion. Although the average biodegradation values are less than the required 60%, significant biodegradation is observed. Regarding the interpretation of the results, the 301 Guideline states that, *'Because of the stringency of the methods, low values do not necessarily mean that the test substance is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.'* Thus, even if 3-methylphenanthrene cannot be flagged as readily biodegradable based on these results, it seems likely that further, more complex testing would show a result of non-persistence.

Two of the hydrocarbons — 9-ethylphenanthrene and 9-ethyl-10-methylphenanthrene — showed hardly any degradation, even with the use of a BIM and the prolonged test duration. As mentioned above, the OECD 301 Guideline states that this is not a definitive indication of non-biodegradability under environmental conditions. These two constituents are the heaviest and most lipophilic tested in this programme, so it is not surprising that they show the lowest level of biodegradation. However, it is not possible to ascertain from these data whether this is due to actual persistence, or to very high bioavailability limitations which could not be overcome even with the use of BIMs.

It is worth mentioning that the use of BIMs enables a significant improvement in the meaningfulness of the 301 tests. As observed from the table above, the biodegradation improves when silicone oil or SiO_2 are used with the incubation, thanks to the enhanced bioavailability provided by these substances. In this case, overcoming the bioavailability limitations (i.e. ensuring that the microbes can access the test substance in order to degrade it) leads to a clear conclusion that 1-methylphenanthrene and 3,6-dimethylphenanthrene are not persistent (> 60 % in 28 days). Without the BIMs, these two substances would artificially appear as being more persistent than they would actually be in the environment.

A further conclusion is that the most suitable BIM seems to depend on the actual substance tested, which was not expected considering how closely related all of them are. For instance, both silicone oil and SiO_2 were effective when used with phenanthrene and 1-methylphenanthrene. However, in the case of 3,6-dimethylphenanthrene a noticeable difference is observed between BIMs, in that this substance is readily biodegradable when tested with SiO_2 but degrades very little if tested with silicone oil. Finally, one can observe how the BIMs had barely any effect on the degradation of 3-methylphenanthrene.

With regard to the extension of the test duration, although some differences are observed between the degradation values at 28 and 60 days, this does not change the conclusion of the tests in any case, so it is perhaps a less effective technique for overcoming bioavailability problems.

Conclusion

Concawe's experimental programme has enabled the generation of reliable results concerning the ability of PAHs to degrade in the environment, and the development of new strategies for overcoming the difficulties of testing hydrocarbons. The results of the 301 F tests described above have been submitted to ECHA's Petroleum and Coal stream Substances (PetCo) working group (in charge of the regulatory approach for petroleum substances), and will enable support for the lack of environmental concern for some hydrocarbon blocks commonly appearing in Concawe's substances. The learnings of this project will be applied in future environmental testing strategies.

References

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3. Hughes, C. B., Brown, D. M., Camenzuli, L., Redman, A. D., Arey, J. S., Vione, D., Wang, N. and Vaiopoulou, E. (2020). 'Can a chemical be both readily biodegradable AND very persistent (vP)? Weight-of-evidence determination demonstrates that phenanthrene is not persistent in the environment.' In *Environmental Sciences Europe*, Vol. 32, Article No. 148. https://doi.org/10.1186/s12302-020-00427-1