USE OF REFERENCE SUBSTANCES IN LABORATORY BIODEGRADATION TESTING

Summary

In laboratory biodegradation testing, reference substances are used to prove that the bacterial inoculum used (typically micro-organisms taken from a municipal waste water treatment plant) is alive and sufficiently active for use in the test. Suitable reference substances are typically chemicals of simple structure, low molecular weight and with well-known biodegradation characteristics such as aniline, sodium acetate, sodium benzoate, glucose etc. Systematic evaluation has shown that the bacterial inoculum is sufficiently active when the pass level is reached after a period of 14 days with these simple reference substances. For more complex chemical substances, including surfactants, it is generally recognised that a longer period of up to 28 days is appropriate to prove ultimate biodegradation in these tests systems.

Biodegradation is the process whereby organic (ie carbon-containing matter) is decomposed by the action of micro-organisms present in the environment which utilise the carbon as food; both to sustain life and to grow the bacterial population. If a substance is capable of undergoing *biodegradation*, it is said to be *biodegradable.*

In the natural environment the process of biodegradation is ubiquitous; involving very many varieties of bacteria, fungi, yeasts and other micro-organisms and operating under an enormous variety of environmental conditions. It is, of course, difficult from a practical perspective to carry out experiments under controlled and reproducible conditions in the natural world and laboratory tests have therefore been developed to demonstrate the potential for chemical substances to biodegrade in the environment. Many of these methods have been developed as International Standards and some of these are incorporated into EU Law for chemicals control and into the Detergents Regulation.

The procedures and operating conditions in these laboratory tests are very precisely defined but there are some significant differences from the situation likely to obtain in the environment. Typically, the test chemical alone is incubated in an aqueous medium containing only mineral salts and a small quantity of micro-organisms; generally taken from a municipal waste water treatment plant.

- The composition of micro-organisms present in any waste water treatment plant will vary from time to time and will obviously be different from one plant to another. This mixture of micro-organisms, known as the bacterial inoculum, is a critical and sensitive feature of the test since it has to be taken from the plant, contained and transported to the test laboratory; where it may be stored for some time before use.

- All the laboratory test procedures require a reference or positive control substance to be run alongside the test substance in order to demonstrate that

the micro-organisms in the inoculum are alive and sufficiently active. These reference substances are typically simple, highly water soluble, chemical structures which is known to biodegrade very easily; such as aniline; sodium benzoate; sodium acetate or glucose. In the so-called "mineralisation" tests, the test is considered invalid if the reference or positive control fails to reach the threshold 60% carbon dioxide production or oxygen consumption level within a prescribed time; typically 14 days. This is less than the 28 days allowed for the test substance, since it is recognised that these reference materials are very easily degraded by a wide range of micro-organisms.

- A relatively high concentration of the test chemical is required to ensure that the carbon dioxide produced, the oxygen consumed or the soluble organic carbon removed during the biodegradation of the test chemical can be analysed adequately. This high concentration is often greater than would be expected in the natural environment and may have an inhibitory or toxic effect on some of the micro-organisms; thereby slowing down the rate at which biodegradation takes place. In some cases, with substances of limited water solubility, not all of the material will be in solution in the early stages of the biodegradation process and this will also delay achievement of the pass level.

- In the laboratory, the test substance is the sole source of carbon available to the micro-organisms so that only those which can metabolise the substance directly will be capable of degrading it and growing. In the natural environment, other sources of carbon are likely to be present as well; allowing co-metabolism to take place in which a wider variety of organisms may become involved in the biodegradation process. The simple substances used as references are capable of being degraded by very many types of micro-organisms and it is to be expected that the test substance may take longer to reach the 60% mineralisation threshold than the easily biodegradable positive control.

For many chemical substances, including detergent surfactants, biodegradation of the molecule is a sequential or step-wise process. The first step is cleavage of the parent structure into fragments; often of quite different chemical structure. This will require presence or growth of a sufficient concentration of those particular micro-organisms with specific functionality to cleave the parent molecule. Before further degradation of the fragments can proceed, sufficient concentrations of other micro-organisms with the necessary functionality to degrade these structures must be generated. This is a much more complicated process than that applying to the simple reference substances and, once again, leads to the expectation that the test substance would take longer to reach the 60% mineralisation threshold than the positive control.

All of these considerations have been taken into account in the design of the standard test methods and the prescription of a 28-day period for the test substance to reach the threshold pass level. It is widely recognised that surfactants which pass the "mineralisation" tests prescribed by the Detergents Regulation would be expected to undergo fast and virtually complete ultimate biodegradation in the natural aquatic environment.