Anaerobic Bioassay Test: Development of an Operational Method for Landfill Co-disposal

Technical Report
P252

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R&D Technical Report P252

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Statement of use
Controlled co-disposal to landfill is considered the best practicable environmental option (BPEO) for several types of industrial waste. The development of a test method provides for an understanding of whether a particular waste loading is acceptable at a given landfill site. Co-disposal has been a successful and relied upon method of landfill disposal in the UK. The forthcoming EC Landfill Directive will effectively abolish co-disposal as a future waste management option but the findings of this research will have applicability to existing co-disposal landfill sites in the UK for use by operators and regulators.

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EXECUTIVE SUMMARY

Background

When carried out according to best practice, controlled co-disposal to landfill is considered the best practicable environmental option (BPEO) for several types of industrial waste. Co-disposal must not impair the operation of the landfill as a bioreactor, so there is a need for a scientifically-based test to illustrate that a particular industrial waste loading is acceptable.

Methane production is one of the most sensitive indicators of the waste degradation process in landfill. Two tests based on methane inhibition were developed previously at the Centre for Applied Microbiological Research (CAMR) for the Department of the Environment (R&D Report No. CWM 107/94). The CAMR Anaerobic Bioassay Test for Solid wastes (S-ABT) added test material to a reactor containing landfill material and measuring methane inhibition over several months. The CAMR ABT for Liquid wastes (L-ABT) employed the principle in a liquid-phase culture. These were considered impractical for application in non-specialist site laboratories, but the L-ABT system showed potential for development into a practical acceptability test.

Objectives

The aim of this project was to produce a refined and simplified Standard Operating Procedure (SOP), based on the CAMR L-ABT. The objectives were:

- to replicate the CAMR test, to check reproducibility and provide a benchmark;
- to develop a simplified, faster, less expensive SOP derived from the CAMR method;
- to test the practicality of the SOP in the laboratories of Shanks and McEwan Ltd (SME).

The modifications were regularly monitored and reviewed by a group of industrial advisors (IAG) drawn from landfill operating companies. Major steps were made by AEA, reviewed by the IAG then tested by SME to check the reproducibility of the modified test, examine some real candidate wastes, and allow final practical refinements to be made.

Main Findings

The new SOP has been simplified in several ways:

- The seed culture is derived from landfill instead of sewage sludge;
- Household waste and yeast extract replace individual trace elements, soluble carbon and vitamins;
- Reagent solutions are boiled rather than filtered or autoclaved;
- The test is prepared in one step, rather than three;
- An acute inhibition value can be obtained in 7 days.

The method was evaluated with trichloroethylene (TCE), cadmium (Cd) and phenol as the main test toxins. For Cd and phenol a 50% inhibition value (IC_{50}) could be obtained at 7 days, but the value changed over time, probably due to adaptation by organisms, and

adsorption or degradation of toxins. The complete inhibition value (IC_{100}) proved unambiguous, giving results in 7 days and remaining stable thereafter. The new operational method (O-ABT) and the L-ABT gave the same IC_{100}, so simplification did not compromise the test. The IC_{100} for TCE was significantly different, probably due to the use of household waste in the O-ABT.

Tests showed excellent reproducibility (standard deviation <10%). At ambient laboratory temperatures the rate of gas production was reduced, but the IC_{100} value was the same as at 35°C (for phenol). Four real co-disposal wastes are also reported. One showed no inhibition at up to 1% v/v in the test system (the highest concentration tested), while three others gave unambiguous IC_{100} values.

Capital costs of implementation will be relatively low; a few hundred pounds for an incubator, seed culture, test bottles and gas manifolds. It is assumed that a gas chromatograph will already be in place. Normal laboratory staff can be taught easily to perform the test. Running costs depend on which version of the method is accepted; we recommend the acute 7-day test as the simplest and most robust and useful version, which is also the least expensive.

The data must be interpreted by reference to a known toxin tested in parallel with the unknown material; we used phenol. The test will take account of the synergistic or antagonistic effects of ill-defined or unknown components in a manner that is not feasible using specific chemical analysis. Measuring acute inhibition of undiluted test material at 7 days could be used to quickly and inexpensively identify and allow co-disposal of benign materials, and identify those requiring further examination. The same test system, used to test dilutions of the material, could then be used to determine an acceptable loading rate for more toxic materials.

With a doubling dilution series the nearest one can approach the true toxicity value is approximately ±50%. More closely spaced dilutions would give a more accurate answer and require a smaller safety margin on interpretation, but at greater cost. The test may prove site-specific, as it uses substrate and seed from the target site. Comparison of several sites is now required to assess the importance of this factor and validate the method. Until comparisons have been made the test can only be used by operators as an indicator for the order of magnitude of toxic effects, and absolute threshold values of the IC_{100} should not be set.

Conclusions

1. A version of the liquid-based anaerobic bioassay test has been developed which is sufficiently simplified and robust to implement in a landfill operator's laboratory.
2. The modified test gives results quickly; the optimum incubation time is 7-14 days.
3. The most reliable and practical end-point is complete inhibition of methanogenesis.
4. The test shows good reproducibility (of the order ±5%-10% on triplicates).
5. The test gives good comparative information but does not give absolute results (it is a useful tool for landfill operators, but is not validated for use by regulators).
6. The test 'fails safe'. The liquid system probably under-estimates the toxicity which an operational landfill would be able to tolerate.
7. The test measures total toxicity where it is unknown or cannot be predicted from the chemical composition because of unknown synergism or antagonism.

8. The test result can be expressed as 'phenol-equivalents' and used to (a) check whether a waste is toxic or not, (b) provide relative toxicity information to determine co-disposal rates for various toxic wastes.

KEY WORDS

Landfill; bioassay test; waste loading; co-disposal; methane.

ABBREVIATIONS

ABT	Anaerobic Bioassay Test
AEA	AEA Technology
BOD	Biochemical Oxygen Demand
BPEO	Best Practicable Environmental Option
CAMR	Centre for Applied Microbiological Research
COD	Chemical Oxygen Demand
DoE	Department of the Environment
GC	Gas Chromatograph
IAG	Industrial Advisory Group
L-ABT	Anaerobic Bioassay Test for Liquid wastes (the CAMR test)
O-ABT	Operational Anaerobic Bioassay Test
S-ABT	Anaerobic Bioassay Test for Solid wastes
SME	Shanks and McEwan
SOP	Standard Operating Procedure
TCE	Trichloroethylene

1 INTRODUCTION

This work was commissioned by Wastes Technical Division of the Department of the Environment under R&D Contract No. EPG 1/7/048, which transferred to the Environment Agency as R&D Project P1-251. The work was undertaken by AEA Technology over the period December 1995 to October 1996.

1.1 Landfill Co-Disposal

In the United Kingdom, most household waste is disposed of to landfill. Co-disposal of a restricted number of industrial wastes with the decomposing household waste has been common practice in the UK for many years. The Department of the Environment supports the views of the waste disposal industry that controlled co-disposal is a safe practice and is the best practicable environmental option (BPEO) for several types of industrial wastes. Guidelines on site location, engineering, operation and waste inputs for co-disposal were given in draft Waste Management Paper 26F [(1)].

It is important that co-disposal does not impair the operation of the landfill as a bioreactor. In particular the microbial population in the landfill must continue to:

- convert a high proportion of the degradable organic solids in the waste to gas,
- degrade or deactivate potentially toxic organic compounds in co-disposed wastes,
- maintain conditions which retain heavy metals and other non-degraded toxins within the mass of solid waste.

Research sponsored by Department of the Environment since 1973 (Knox, report no. CWM 07/89) has demonstrated that, at the recommended loading rates, the toxic components of industrial wastes can be degraded and immobilised in decomposing refuse. Furthermore, it is observed that landfills in which industrial wastes are co-disposed continue to generate substantial amounts of methane and do not suffer major changes in leachate composition.

There is a need for a scientifically based test to illustrate that a particular industrial waste has an acceptable toxic load when offered for co-disposal with domestic waste in the UK. Being able to identify wastes which needed alternative treatment or dilution before co-disposal would be in the interests of both operators and regulators, to:

- prevent risk of inhibition of microbial stabilisation of waste;
- reduce long-term liability due to unacceptable leachate quality;
- avoid disruption of landfill gas production; and
- illustrate a scientific basis to safe practice for co-disposal.

Anaerobic Bioassay Tests

Methane production is the most important indicator of the overall condition of a methanogenic consortium, such as that in an efficiently operating landfill bioreactor. Methane generation is a suitable end point for a bioassay of toxicity because:

- it indicates a complete and uninterrupted biochemical degradation pathway;

- methanogens are among the most sensitive organisms in landfill;
- methane is unreactive and relatively insoluble, so methane generated accurately reflects microbial activity.

A test which measures the inhibition of methane production gives a direct bioassay of all the toxic effects which a waste may have upon the anaerobic microbial population. Two tests based on this principle were developed by the Centre for Applied Microbiological Research (CAMR) under contract to the Department of the Environment [2].

The Solid ABT (S-ABT) developed by CAMR involves adding industrial waste to a reactor containing landfill material and measuring inhibition of in-situ methane generation over a period of months. This system models landfill quite well, and can take account of protective effects from sorption, adaptation of the population and biodegradation or detoxification of the inhibitory material. However, it is expensive and time-consuming, and requires specialist facilities and staff.

The Liquid ABT (L-ABT) developed by CAMR employs an aqueous-phase culture in small bottles. This arrangement is less realistic and gives potentially less information about protective mechanisms within solid wastes, but should be more reproducible and gives a result faster than the solid system. However, it still takes too long to be of practical day to day use to operators, and is unnecessarily complex. The CAMR tests are excellent research tools, but were not designed-for and are not appropriate to operation in landfill site laboratories.

The Industrial Advisory Group (IAG) felt that the methodology described in draft WMP 26F was impractical for widespread application in non-specialist laboratories serving sites with the normal pattern of waste delivery, i.e. wastes requiring urgent disposal, and not able to be stored for months. To be more practicable as a potential acceptability test for co-disposal they considered it preferable that:

- There is one unified test protocol which can be used for all wastes as presented:
- The most serious acute adverse effects of a toxic component are detectable in a relatively short time:
- There are no grounds for the waste producer to dispute the validity of the test if a waste is rejected:
- The test is consistent between sites, influenced only by local site conditions:
- The test employs equipment and skills which already exist or can be readily incorporated into an established on-site analytical laboratory:
- The actual cost of testing is less than the potential cost (long-term and short-term) of adverse effects on the landfill behaviour caused by toxic materials not identified by current vigilance and waste analysis:

1.2 The Need for a Bioassay

The draft of Waste Management Paper 26F on co-disposal describes a hierarchy of test procedures dealing with the acceptance of wastes for co-disposal:

- Level 1: Comprehensive Characterisation

- Level 2: Compliance Testing
- Level 3: On-site Verification.

Presently these rely on physical, chemical and visual tests. However, an operator needs to know quickly whether a waste presented for co-disposal can be accepted or whether it is likely to have any adverse effects on the health of the target landfill. 'Health' is taken to mean a landfill in which methane production is high, indicating the presence of a complete degradative microbiological population.

Many wastes for co-disposal are complex and/or incompletely defined chemically, so an assessment of the likely impacts based on chemistry alone may be misleading. The only practical way to assess the many possible synergistic or antagonistic interactions is therefore to measure directly the overall net toxicity in a methanogenic biological test system.

In the context of biological testing, characterisation tests aim to replicate environmental conditions to:

- understand the fundamental processes;
- cover a full range of conditions;
- give information on other factors (e.g. biodegradation, detoxification, adaptation, sorption).

Tests such as the CAMR S-ABT fall into this category, and Agency commissioned R&D work by Minton, Treharne and Davies is currently in progress to develop and refine such a test. This type of test can in principle give absolute values for toxic loads and actual inhibitory effects. However, these tests currently:

- take a long time to reach completion;
- need specialist equipment and staff;
- are expensive to perform.

The validity of S-ABT as a model of in-situ landfill conditions is questionable, since even large microcosms (of the order 1 m^3) still do not represent the large-scale heterogeneities known to be present in landfill. They will probably be limited to a few tests for strategic uses such as assessing common waste streams, compiling a national database of toxicities, or in fundamental studies of biodegradation.

Compliance tests are intended to look for changes in the basic characteristics of the waste and so are essentially acceptance tests. These will normally use standard conditions to give a reproducible but relative value, which must be related to an independent benchmark. The tests must be performed at the receiving landfill site laboratory, and must:

- be simple, quick and inexpensive;
- be standardised and easy to run in large numbers;
- employ standards which relate to other tests (e.g. chemical analysis).

By their nature, acceptance tests will give relative values so that benign materials can be accepted and potentially problematic materials can be identified quickly. The test:

- will not give absolute values;
- cannot indicate any protection factors present in-situ;
- gives little information on long term effects (adsorption, biodegradation, adaptation).

It was suggested that the L-ABT system of CAMR could form the basis for a compliance/acceptability test, but would need significant simplification if it were to be successfully applied in existing laboratory facilities at typical landfill operations.

The test would be used in conjunction with other data. Operational tests and information are already used to guide co-disposal, so the ABT would be considered in conjunction with:

- individual analyses to identify inorganic and organic toxins;
- guidance on acceptance in draft WMP 26F;
- experience with similar wastes.

However, biological assays are inherently variable, so it is recommended that an ABT is not used as the only regulatory tool. Other sources of information, e.g. chemistry, will still be essential to assist regulators in setting waste input limits.

1.3 The Present Study

The Department of the Environment placed a contract with AEA Technology (AEA) to adapt the L-ABT developed by CAMR. Since April 1996 this contract has been funded by the Environment Agency. The aim of the project is to refine the existing Standard Operating Procedure (SOP) by:

(a) replicating the CAMR test to check reproducibility and provide a benchmark comparison;
(b) assessing a simplified, quicker, less expensive procedure derived from the CAMR method;
(c) testing this revised SOP in the laboratories of a typical landfill operator.

The operational testing has been undertaken by staff at Shanks and McEwan (SME), AEA's partners in this project. An advisory group drawn from the industry has been formed to provide input at critical points, to ensure that the final test would take account of a broad cross-section of the industry's needs.

Membership of the Industrial Advisory Group (IAG) is given in Appendix 1. The first consultation point was the selection of the toxins to be tested. The second consultation point was a round-table discussion on the format of an interim test procedure to be evaluated by Shanks and McEwan (SME). Finally the group met to consider the outcome of the operational tests by SME and advise on the potential application of the resulting SOP.

This report consists of four sections:

1. The introduction to the study.
2. A description of the experimental work undertaken to revise and test the simplified ABT.
3. A discussion of the relevance of the data to the testing of co-disposal wastes

4. Recommendations for the use of the test and further development.

Appendices contain details of:

1. The Industrial Advisory Group meetings.
2. Experimental procedures followed in the development work.
3. The recommended Standard Operating Procedure for an Operational ABT method.

2 METHOD DEVELOPMENT

2.1 General Principles

It was agreed that the operational system should retain the fundamental principles of the CAMR L-ABT, i.e. the inhibition of methane production by a standardised anaerobic culture in liquid medium as the main measurable parameter. The L-ABT method had been validated against a wide range of potential toxins. The aim of the development work was to adapt and simplify the fundamental concept to make the test faster, simpler, and less costly to perform. During the development programme, the approach was to add known amounts of toxin at different dilutions to identical anaerobic cultures in order to plot a dose/response curve. When the test is used in practice, the dilutions of known toxin are used as a calibration against which to assess a candidate waste.

The objectives of this part of the project were:

- to replicate the CAMR L-ABT as closely as possible, to check reproducibility;
- to carry out a simplified operational method (O-ABT-1) as proposed;
- to compare the L-ABT with the results of AEA's O-ABT-1;
- to carry out further simplifications (O-ABT-2 and -3) in order to determine the minimum cost and effort giving an acceptably reliable and reproducible test.

The L-ABT methodology was followed as closely as possible. However, two changes were agreed in the protocol at the beginning of the project.

1. **Different source of methanogens**. CAMR used an inoculum of sewage sludge; this study used methanogens derived from healthily-gassing municipal landfill. It was felt that this type of culture was more representative of the site of concern. In addition, we have found the landfill waste culture easier to maintain and so more suitable for use in a non-specialist laboratory. The source of our microbial culture was household waste, estimated to be 10 years old, excavated from the Calvert landfill. Fresh material was used to establish the seed culture and the bulk material has been shredded and stored in plastic bags. These solids were used to feed the inoculum culture and were added to the test bottles as a source of nutrients.

2. **Different method of expressing inhibition.** CAMR looked for complete inhibition on a linear scale, but a dose-response curve may have an indeterminate head and foot. This could make estimation of the 100% inhibitory concentration (IC_{100}) potentially problematic. We therefore also examined interpretation of results according to the IC_{50} - that concentration of toxin causing 50% inhibition of methane production (analogous to the LD_{50} used for comparing toxicity in higher organisms). A logarithmic-linear plot normally gives a relatively linear relationship in mid-range, from which a half-inhibition value may be reliably determined. Some multiple of either the IC_{50} or IC_{100} concentration might then be selected as the acceptance criterion.

Details of the equipment, reagent and method used in replicating the L-ABT method and in simplifying the O-ABT method are given in Appendix 2. The CAMR method was followed

except for the two fundamental changes noted above. The revised methods are referred to as O-ABT, Versions 1, 2 and 3.

2.2 Choice of Toxins

The aims of this project were to:

- provide limited validation of the CAMR method (on three different toxins);
- simplify and develop the test, hence only a limited number of toxins were tested.

Three toxins representative of different groups were used in method development:

- a heavy metal (cadmium, as the soluble nitrate),
- a water-soluble organic (phenol) and
- a water-insoluble chlorinated compound (trichloroethylene, in acetone).

These were materials studied in the CAMR project, allowing comparison of results.

The concentrations of the species used in the current work were chosen to span the inhibitory concentrations reported in the CAMR development work. The Industrial Advisory Group members agreed to the choice of toxins and the concentration ranges to be studied.

It was recognised that toxins will have a wide range of characteristics and the three chosen in this study have properties which might influence the availability of the material in a culture. For instance, cadmium has a very insoluble sulphide and so any free sulphide produced from sulphate reducing bacteria may lower the concentration of cadmium in solution. It was hoped that using mature waste would avoid any variability caused by sulphide precipitation. Phenols and cadmium ions may be strongly adsorbed to organic material and so the concentration in free solution may be lower than anticipated.

Water-immiscible solvents such as TCE are difficult to administer in small amounts and a co-solvent was used by CAMR to aid accurate addition to the medium. The solvent may itself be either inhibitory or be a potential carbon source. We chose initially to use acetone as the co-solvent, reported by CAMR to be neutral in effect. An alternative solvent, methanol, was shown by CAMR to be stimulatory to gas production. The later studies at SME used water saturated with TCE. Any immiscible liquid will not be dispersed easily and evaporation of solvent may occur during preparation of samples. Care was taken to avoid these effects.

2.3 Simplification of the ABT Methodology

The main features of the assay system are described in Appendix 2. The specialist equipment requirements are:

- an inoculum culture;
- reagents containing essential nutrients;
- a system for purging the test bottles with inert gas (nitrogen);

- an array of test bottles into which nutrient solutions, the toxin and the microbial inoculum are placed;
- a system for collecting and measuring gas volumes;
- a gas chromatography system.

As far as possible AEA excluded equipment which would only be found in a specialist microbiology laboratory (e.g. anaerobic cabinet, autoclave, jacketed fermenters).

The CAMR method relied on fresh sewage sludge, which is freely available but:

- requires a period of adaptation in the test system before bottles have to be re-opened and toxin added;
- is probably not an ideal source of landfill-adapted methanogens;
- has to be used quickly, requiring in practice regular collection and transport from the nearest sewage works.

The AEA philosophy was to initiate and maintain a culture of more appropriate landfill-derived organisms, and to keep the culture under test-like conditions. The culture was therefore ready for use at any time, as required, eliminating:

- the bottle re-opening step;
- delays awaiting adaptation; and
- the need to collect fresh sludge.

Inoculum cultures were set up using sewage sludge taken from a local wastewater treatment plant at Didcot, in an attempt to replicate the CAMR method. These cultures were slow to become methanogenic and steady state gas production rates were low. After consultation with the DoE and CAMR it was agreed not to proceed with this inoculum.

In parallel an inoculum culture was established using aged waste freshly-excavated from the Calvert site in Buckinghamshire. This rapidly became methanogenic and gas production rates remained high for several months. Aliquots of this well-mixed suspension were removed to inoculate test bottles. Irrespective of whether material removed was used for tests, sufficient suspension was taken out to allow a total of 500 ml of new 10% w/v suspension to be added to the vessel each week. This rate of feeding maintained healthy gas production for at least 4 months while minimising significant accumulation of either fermentable carbon or recalcitrant material. The AEA microbial inoculum was maintained in a simple bioreactor comprising a glass vessel (5 litre capacity) from which a fixed volume (500 ml) was regularly withdrawn (weekly) and replaced with 500 ml of fresh medium containing freshly-milled household waste. This was incubated in a water bath, held at 35°C in a fume cupboard, and gas was collected over acidified water in an inverted burette for volume/quality measurement. The inoculum was fed on a weekly basis with sorted and milled household waste from Calvert landfill site, initially suspended in a minimum salts solution.

The tests were performed in serum bottles (nominally "125 ml", actually 155-160 ml) which can be sealed with a butyl rubber septum. Thin rubber septa are preferred so as to facilitate multiple sampling with the gas syringe. In the operational system 2g (wet weight) of aged

waste was added to the liquid medium (100 ml) to provide both nutrients and some solid surface akin to a landfill (though in no way intended to be as comprehensive a simulation as the S-ABT method). The household waste came from an older section (8-12 years) of Calvert landfill site, and had been hand-sorted to remove non-putrescibles, then ball-milled to (a) reduce the mean particle size to < 1 mm, (b) thoroughly homogenise the material. Results show that this gave very reproducible gas production, although it is not possible to say how representative this was of other landfill sites, or indeed of other parts of Calvert.

The original CAMR work in a solid-free culture used a completely defined anaerobic medium to ensure that fermentable carbon and essential elements were present. The addition of landfill solids to the AEA Technology test system provides an alternative and more realistic source of carbon and inorganic nutrients: as a result much a simpler liquid medium could be considered. Difficult reagents such as selenium (a scheduled poison) and vitamins (which are expensive and unstable) were therefore omitted. The simplified medium for the initial trials (Appendix 2) was an adaptation of a medium which had been used successfully in the Biological Methane Potential tests, originally developed by Biotal[3] and modified by AEA[4].

The main analytical parameter measured is the amount of methane in the headspace of the bottle. This is determined by measuring:

- the total volume of gas when the headspace has been equilibrated with atmospheric pressure; and then
- analysing the proportion of methane using a gas chromatograph (GC).

Inhibition by a toxin was judged by comparison with a positive control bottle containing microbial inoculum and all reagents except the toxin. The experimental conditions were each tested in triplicate, so each data point shown on subsequent figures is the mean of three points. For uninhibited positive control tests there is usually less than a 10% spread on the data. For inhibited cultures there may be more scatter. The choice of the ratio of liquid:headspace in the test bottles was guided by previous experience.

There is approximately 60 ml of headspace above the liquid so that production of 60 ml of new gas (about 30 ml of methane) will increase the pressure in the bottles to 2 atmospheres. Regular sampling was adopted to ensure that no bottle reached a failure pressure, thought to be at least 5 atmospheres (no bottles failed). In principle however there is some risk of explosion, so goggles and gloves are recommended when handling active bottles.

The gas production from a test bottle containing the standard inoculum and simplified nutrients and no toxins is illustrated in Figure 1. This shows linear production rates for methane over about 30 days, with some slowing down as substrate depletes.

These tests use a mixed microbial population in a heterogeneous medium and so the effects of toxins on methane production will be complex. Some of these effects will be seen in the dose response curve, normally at concentrations of toxin just below those which cause inhibition. For instance sub-lethal doses of toxin may become neutral or even stimulatory in effect. Figure 2 illustrates the effect of 1000 ppm of phenol on methane generation under the standard O-ABT conditions. The greatest impact on gas production is in the first week of incubation. Subsequently, gas generation rates increase, which may be attributed either to

adaptation of the microbial population and the proliferation of more tolerant sub-populations, or to adsorption/detoxification processes.

This effect may also occur with the solvent used for water-insoluble toxins. Figure 3 illustrates the effect of acetone on the test system - initially neutral in effect (as reported by CAMR) the population again appears to adapt, finding acetone stimulatory in the medium-term. This solvent was used to disperse one of the toxins, TCE, in the initial tests and the non-linear response illustrates the difficulty which might be encountered in interpreting results from mixed wastes containing both toxins and potential nutrients.

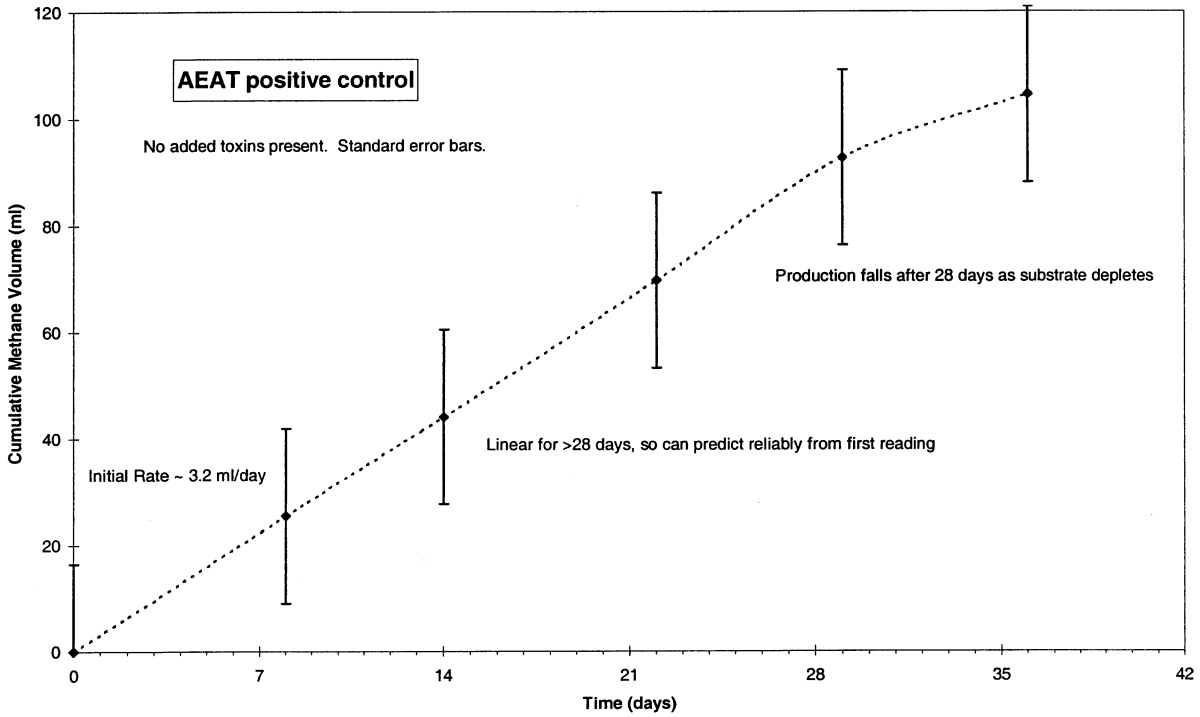

Figure 1 Positive control, AEA method

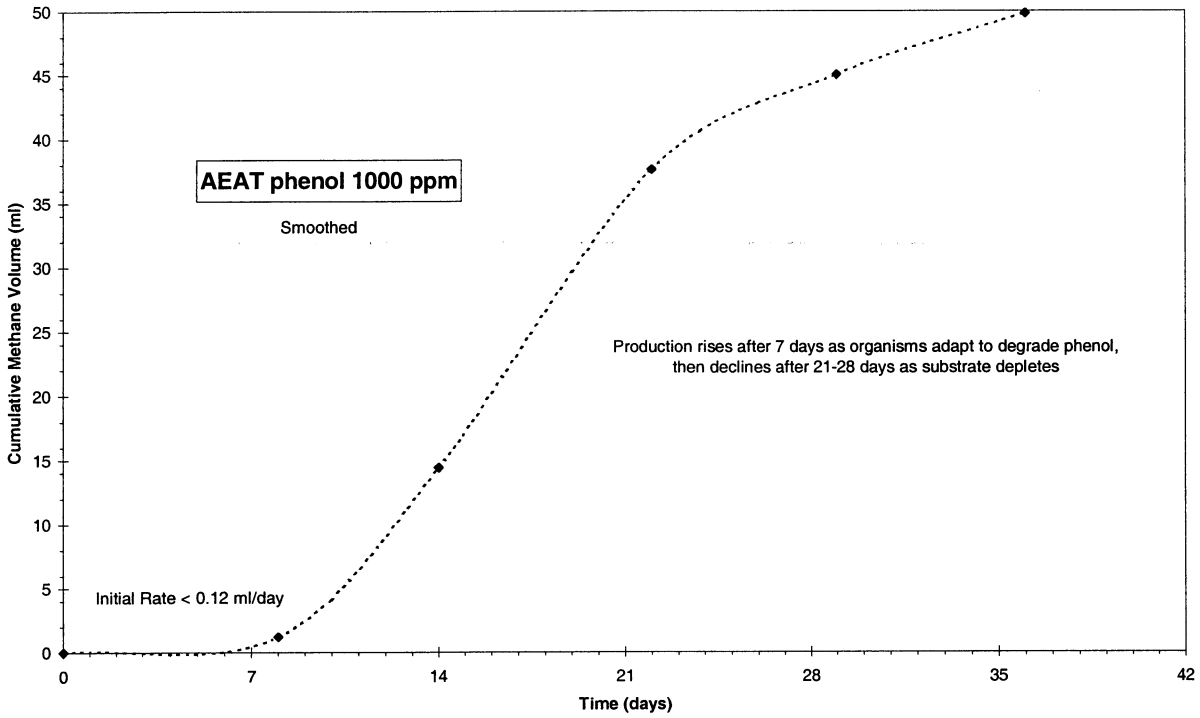

Figure 2 Phenol 1000 ppm, AEA method

In the short-term, inhibition increases with the amount of toxin added. The dose-response curve is exponential in character (see Figure 4) and in the early stages of the culture (e.g. at 8 days) it may be difficult to estimate the concentration which results in total inhibition (the IC_{100}). For this reason we examined:

(a) use of a logarithmic-linear (log-linear) plot, to straighten out the dose-response curve, and
(b) the concept of the half inhibition value (IC_{50}), particularly when measuring acute effects after a few days incubation.

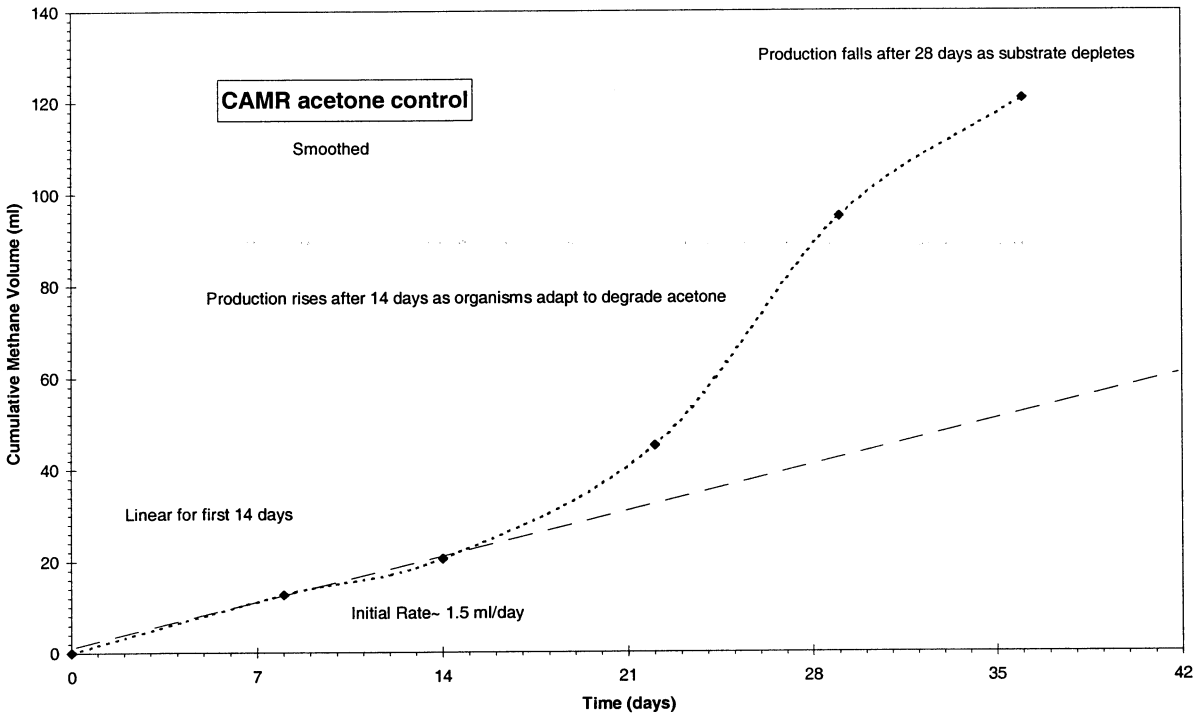

Figure 3 **Acetone control, CAMR method**

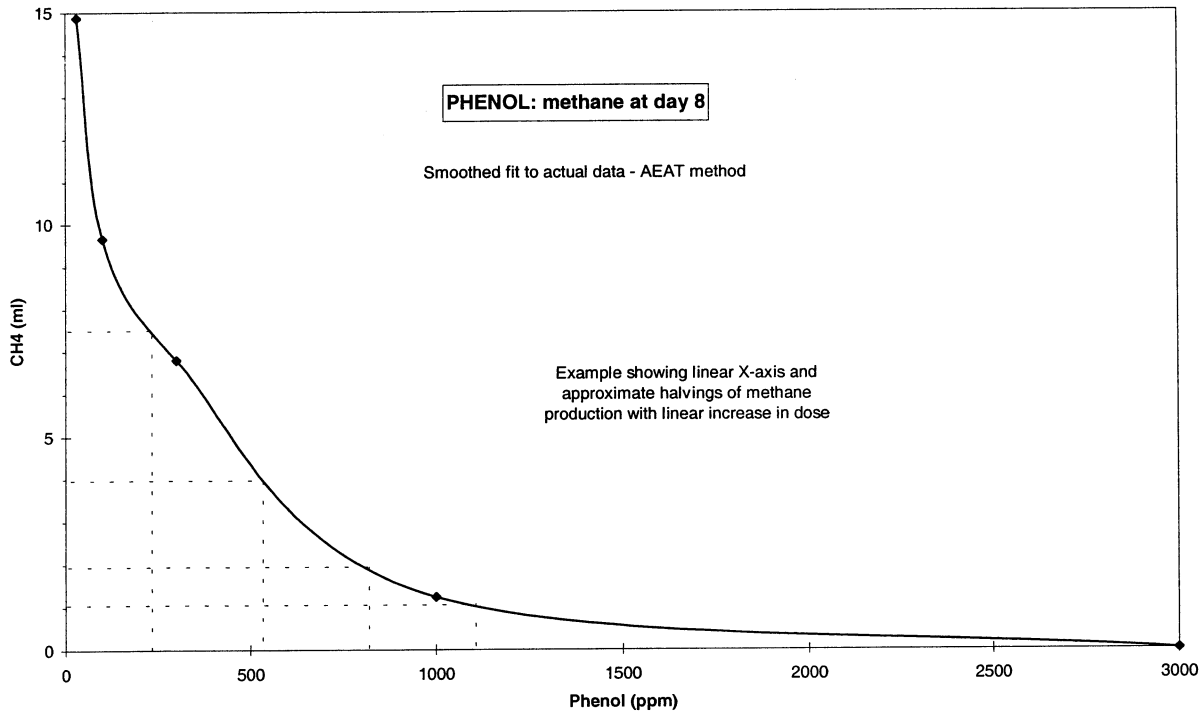

Figure 4 **Phenol by AEA method, linear-linear plot**

For higher organisms, the dose-response relationship is usually a function of the logarithm of dose, so it is convenient to use a log-lin graph. This also appears to be true of the effects of many toxins on methanogenesis, so the log-lin plot has generally been used in this study. The

log-lin plot is usually sigmoidal, giving a linear response in mid-range, and hence allowing reliable estimation of the 50% inhibition point.

The gas chromatograph (GC) was operated primarily to detect methane. Carbon dioxide is retained longer on the GC column and was ignored so as not to slow the analysis of multiple samples. Avoiding the use of carbon dioxide as a monitoring parameter makes it unnecessary to allow for the fact that many organisms, not just methanogens, produce CO_2 and that the solubility of this gas is high and pH-dependent. Small quantities of oxygen have been detected in the tests but this is probably a feature of the sampling technique. Higher concentrations would indicate leakage, but as the medium used contains a Redox-dependent dye (Resazurin, see Appendix 2) any serious leak will immediately be visible on inspection of the bottle. Hence, GC estimation of oxygen is unnecessary. Hydrogen was detected in some bottles, particularly in those close to inhibitory levels of toxin. This is consistent with the probability that acetogens, which generate hydrogen, are less susceptible to toxins than the hydrogen-consuming methanogens, and confirms the suitability of methanogens as the most sensitive indicator of toxicity. Hydrogen estimation will not be required in routine practice.

2.4 Comparison of L-ABT and O-ABT Version 1

The initial experiments compared the main changes proposed to simplify the test:

- a 'one-step' test using pre-adapted organisms;
- reliance on landfill-derived solids for carbon and trace nutrients;
- simplification in preparation and composition of the defined media;
- measurement of gas after the first week to identify acute effects;
- comparison of old (IC100) and new (IC50) end-points;
- new methods of interpretation;
- measurement of gas after several weeks to identify longer-term effects.

The CAMR L-ABT method and AEA O-ABT Version 1 method were run in parallel. The dilution range and mid-value tested were chosen to replicate the CAMR method and bracket their reported inhibition value. The solid added to the O-ABT method was 20 g/l (wet weight) of well-mixed, finely-divided household waste, from a landfill not used for co-disposal (Calvert). Figures 5-9 show the dose-response curves for the three toxins as:

(a) the methane yield with dose at 8 days,
(b) the cumulative methane yield with dose to day 79.

Phenol

Figure 5 illustrates the data from the original CAMR method and the first version of the simplified method after 8 days exposure to phenol. Both methods gave an IC_{100} between 1000 and 3000 ppm, confirming the comparability of the methods. The L-ABT method gave a non-linear response curve with an IC_{50} ~1100 ppm. The O-ABT-1 method was more sensitive and gave a more linear dose-response curve, with an IC_{50} ~50 ppm.

The longer-term incubation (79 days) in the presence of phenol (Figure 6) gave results which were comparable, with an IC_{100} between 1000 and 3000 ppm. The log-linear presentation facilitated making this judgement with more confidence.

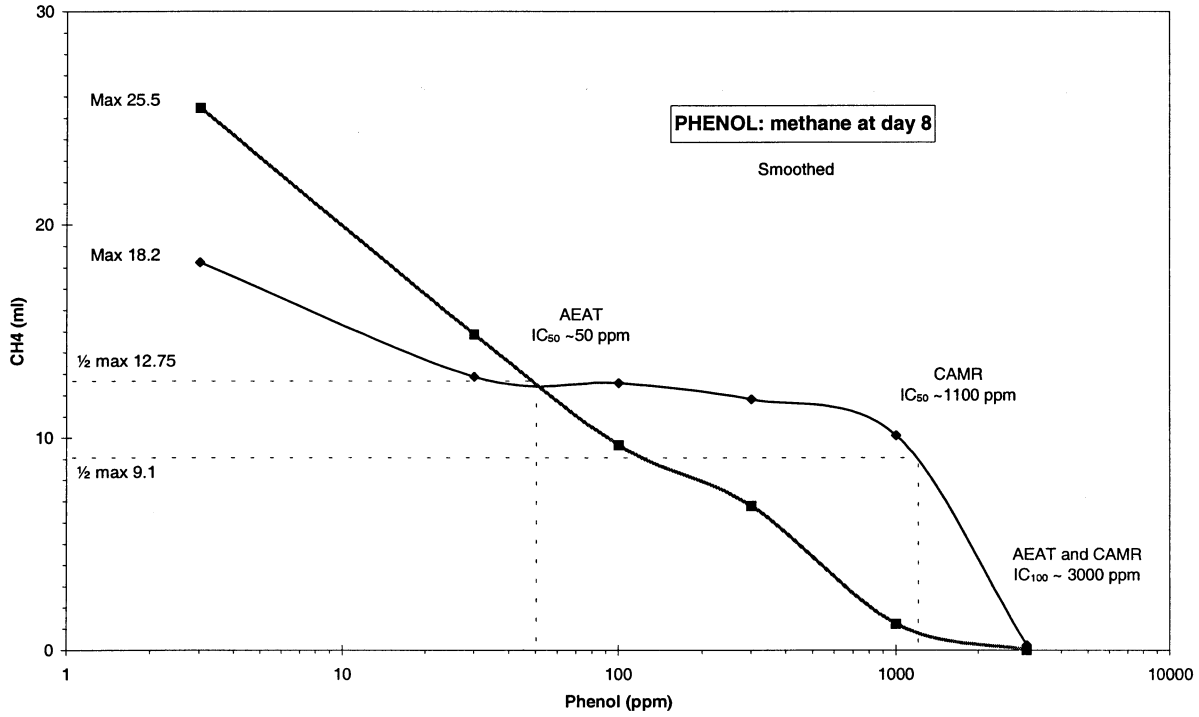

Figure 5 Phenol, 8 days incubation

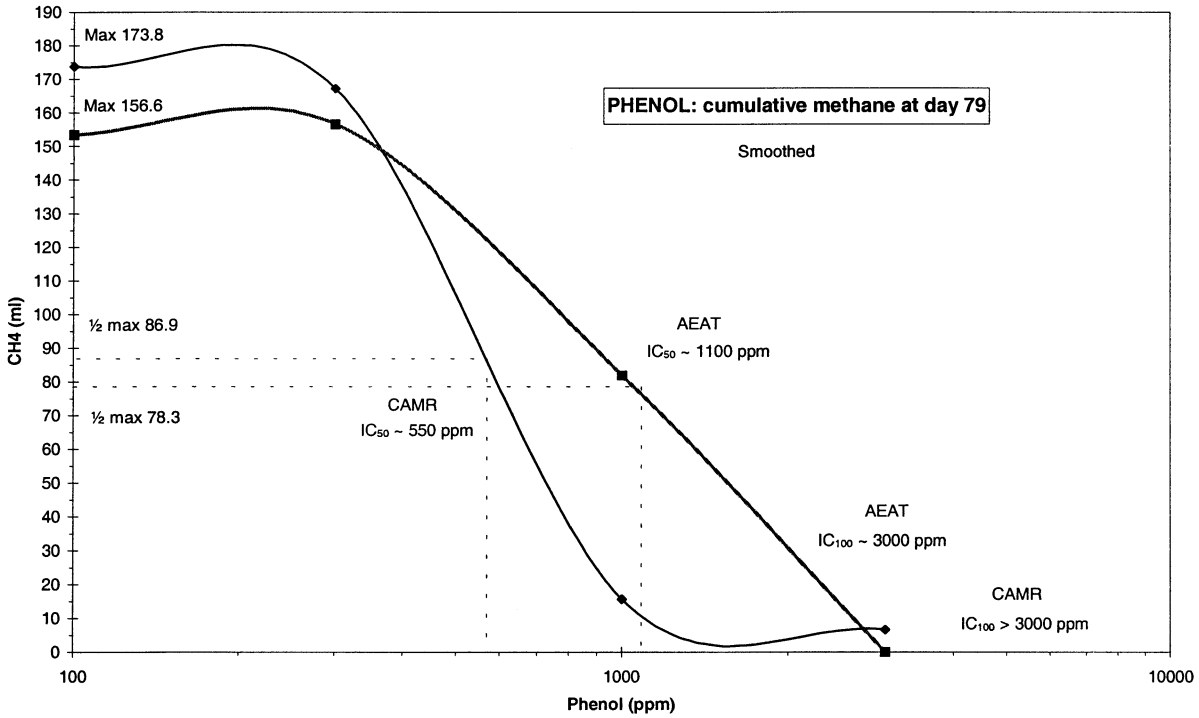

Figure 6 Phenol, 79 days incubation

The total inhibition end-point was the same as observed at 8 days incubation, i.e. those cultures which were initially completely inhibited did not adapt later to the phenol. However, the 50% inhibition point gave an IC_{50} of ~550 ppm by L-ABT and ~1100 ppm by O-ABT. This was a significant increase over the IC_{50} value at 8 days, indicating possibly recovery and adaptation of microbes at sub-lethal doses, or adsorption and detoxification of the phenol at sub-lethal phenol concentrations. The highest IC_{50} value had been achieved by 28 days and did not change markedly after this period.

For the dilution series used (steps of 30, 100, 300, 1000, 3000 ppm), 550 ppm and 1100 ppm are indistinguishable, and so the two methodologies were judged to have given similar results. In bioassays such as this, results must be at least two dilutions apart to be regarded as statistically different. For more accurate estimates, more closely-spaced dilutions would be required.

Cadmium

Inhibition by cadmium after 8 days and 79 days gave a similar pattern to phenol for the O-ABT method (Figures 7 & 8), although the dose-response curve was not linear at 8 days. Both methods gave an IC_{100} between 300 and 1000 ppm at 8 days, confirming comparability, and this result was stable, i.e. remained the same up to 79 days. The IC_{50} by the new method was readily determined: ~65 ppm at 8 days and ~550 ppm at 79 days, compared with an IC_{50} of ~300 ppm from the original CAMR report. This value had actually stabilised at the higher value after about 28 days incubation. As was observed for phenol, the IC_{50} had shifted over time, suggesting either adaptation of the microbes or reduction in bioavailability of the toxin. The more consistent data from this O-ABT method may be connected with the use of the landfill solids which provide some buffering capacity in the system.

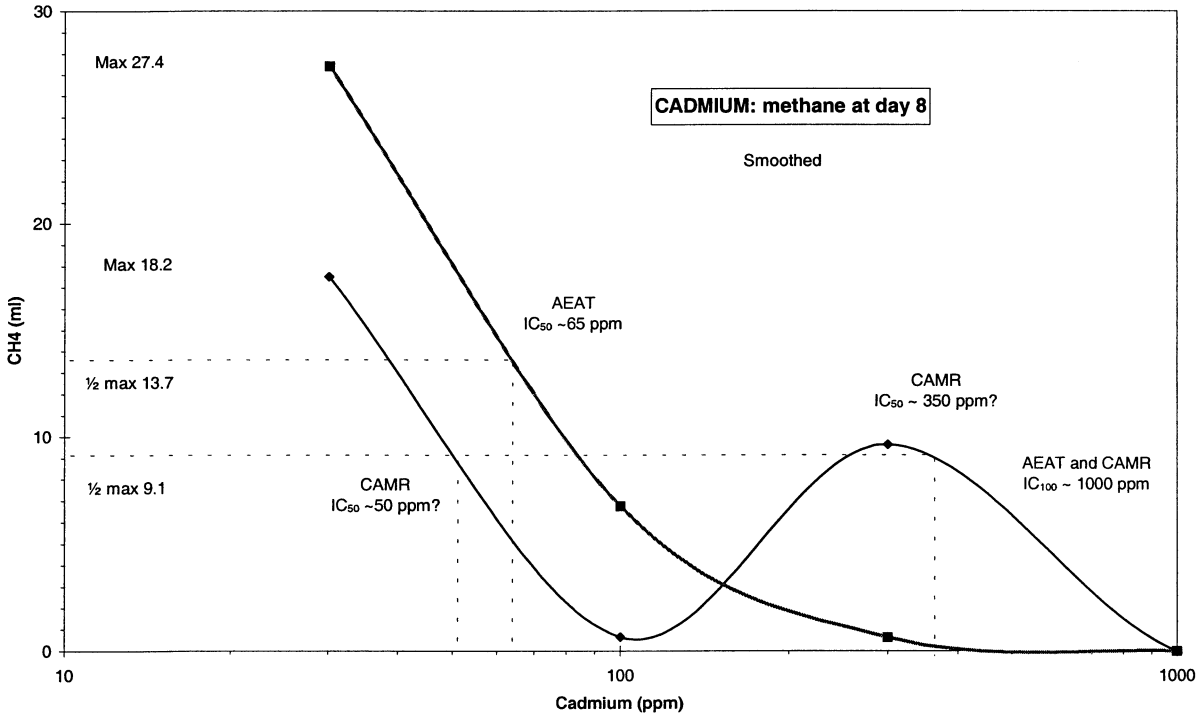

Figure 7 Cadmium, 8 days incubation

Incubation of these vessels continued for more than 5 months. There was a variable lag-phase at sub-lethal concentrations, during which a proportion of methanogens may have died (and so produced no gas) while some survived and adapted (to eventually degrade all available carbon, giving a similar cumulative volume of gas to that of the toxin-free controls). This non-linear behaviour meant that a final yield could not be predicted from the trend in methane production when toxins were present. If a total cumulative methane value were required, it would be necessary to incubate to completion to obtain this value.

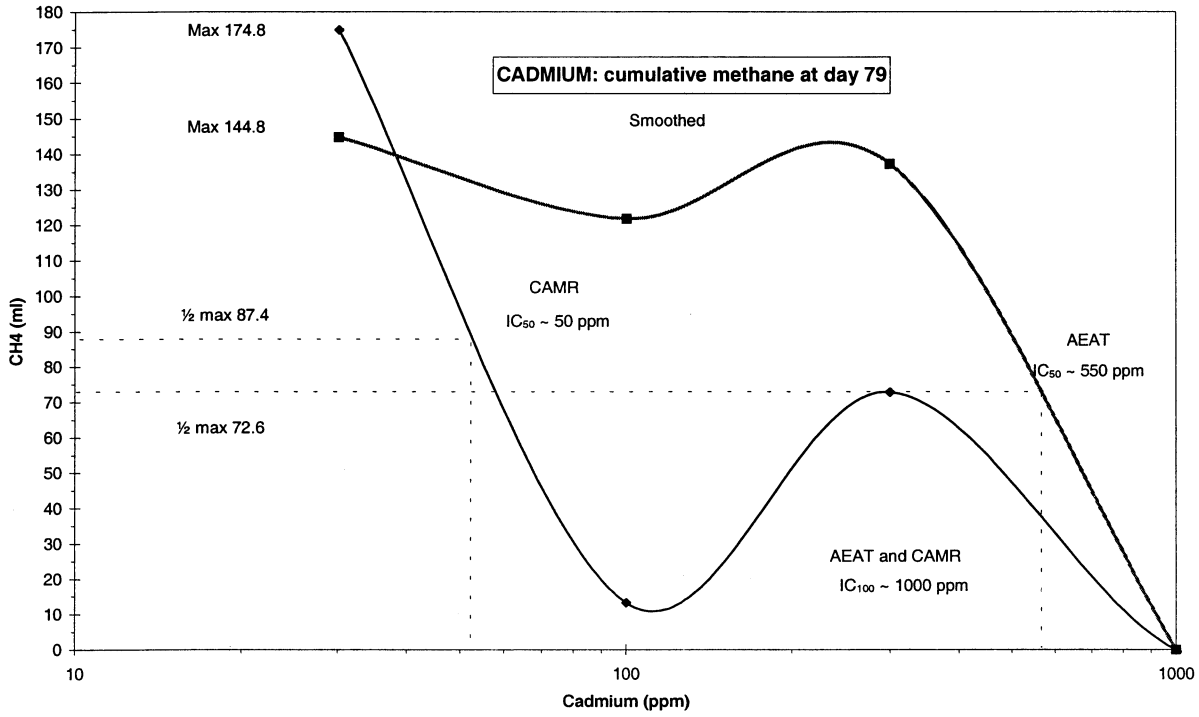

Figure 8 Cadmium, 79 days incubation

Trichloroethylene

The inhibition study using TCE gave a rather different result from that reported by CAMR (complete inhibition at ~1 ppm TCE). In the O-ABT-1 method TCE was not inhibitory even at the highest concentration used (10 ppm - see Figure 9). Gas production actually increased slightly with TCE dose, and this may be a result of:

1. organisms metabolising the co-solvent (acetone) used to disperse and dilute the TCE in the medium. An acetone-only control yielded more methane than the positive control. There is probably no solvent for non-polar compounds which can be guaranteed non-degradable.
2. organisms degrading TCE itself. The O-ABT used a landfill-derived inoculum whereas the CAMR study used sewage-derived cultures. The landfill culture certainly contained species which had been exposed to chlorinated xenobiotics, which are universally found in landfilled household waste, and would therefore be expected to tolerate or even degrade TCE.

The O-ABT test therefore proved less sensitive than the L-ABT test for TCE. This demonstrates the importance of using landfill-derived organisms.

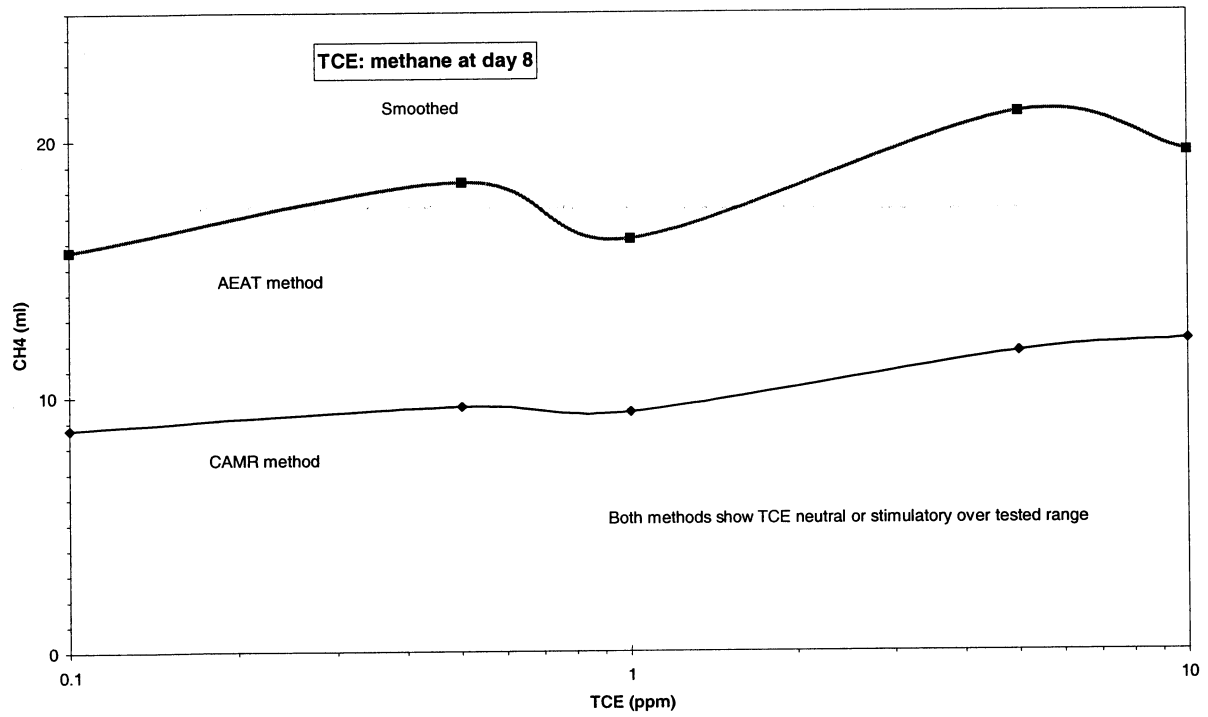

Figure 9 Trichloroethylene at 8 days

Methane production by these cultures was continued for 155 days (22 weeks) incubation, by which time methane production was very slow. The cumulative results after various incubation times are summarised in Figures 10-13 below.

Unchallenged positive control bottles (i.e. no toxin) are not shown, because a zero value of toxin concentration cannot be plotted on the logarithmic axes. These all showed high methane production, of the order 25 ml of methane (40 ml of total gas at over 50% methane) over the first 7 days, and tending towards a final yield of the order of 500 ml methane by day 155.

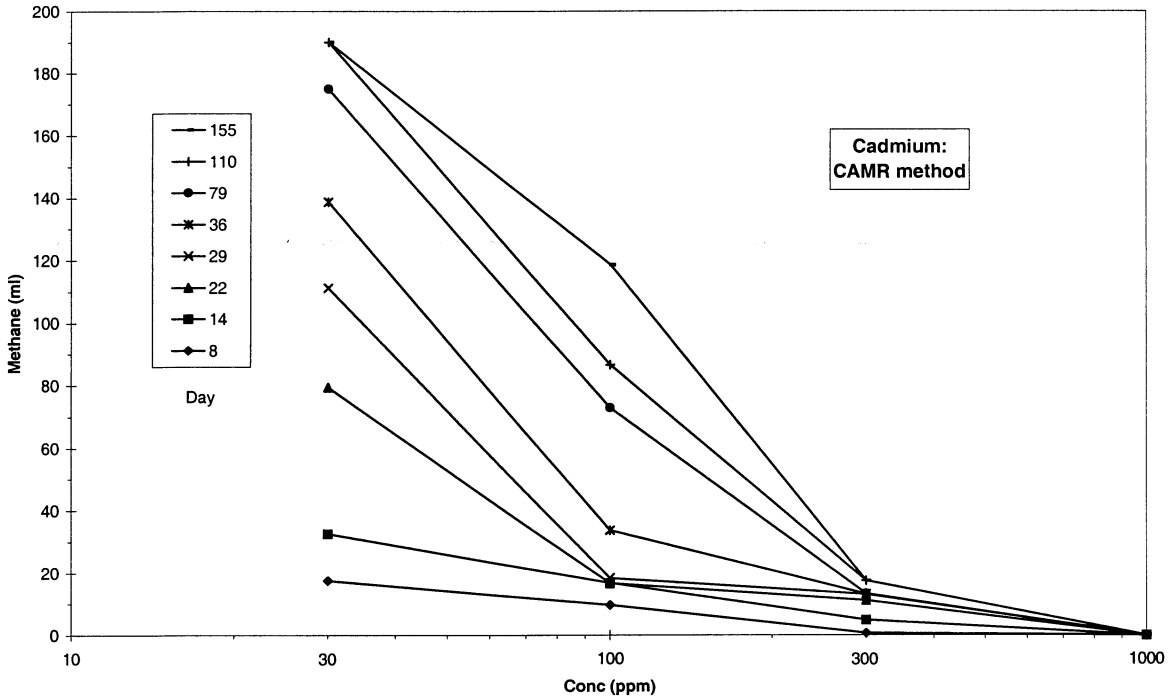

Figure 10 **Cadmium to 155 days, CAMR method IC_{100} 300-1000 ppm**

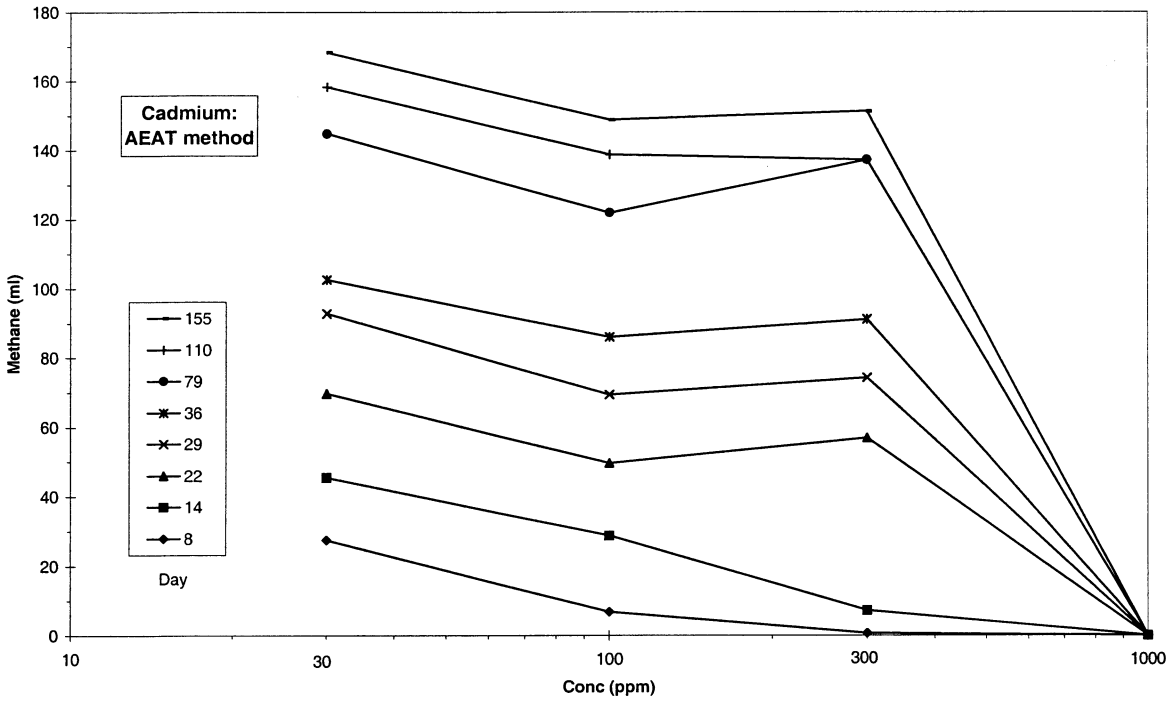

Figure 11 **Cadmium to 155 days, AEAT method IC_{100} 300-1000 ppm**

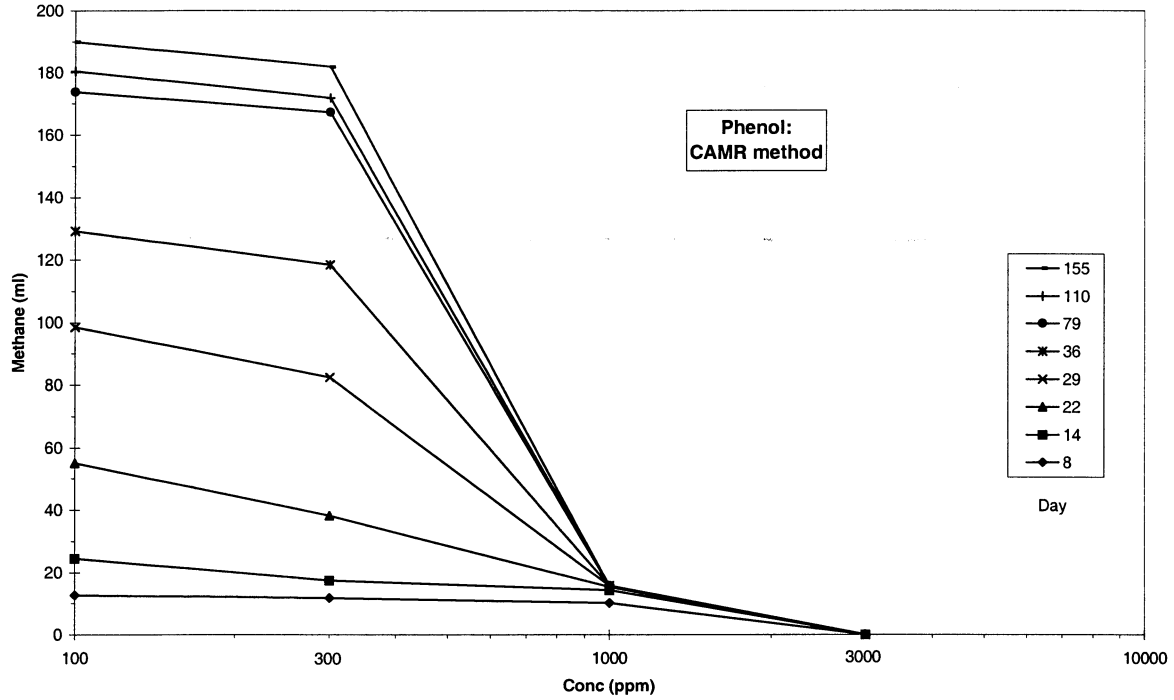

Figure 12 Phenol to 155 days, CAMR method IC_{100} 1000-3000 ppm

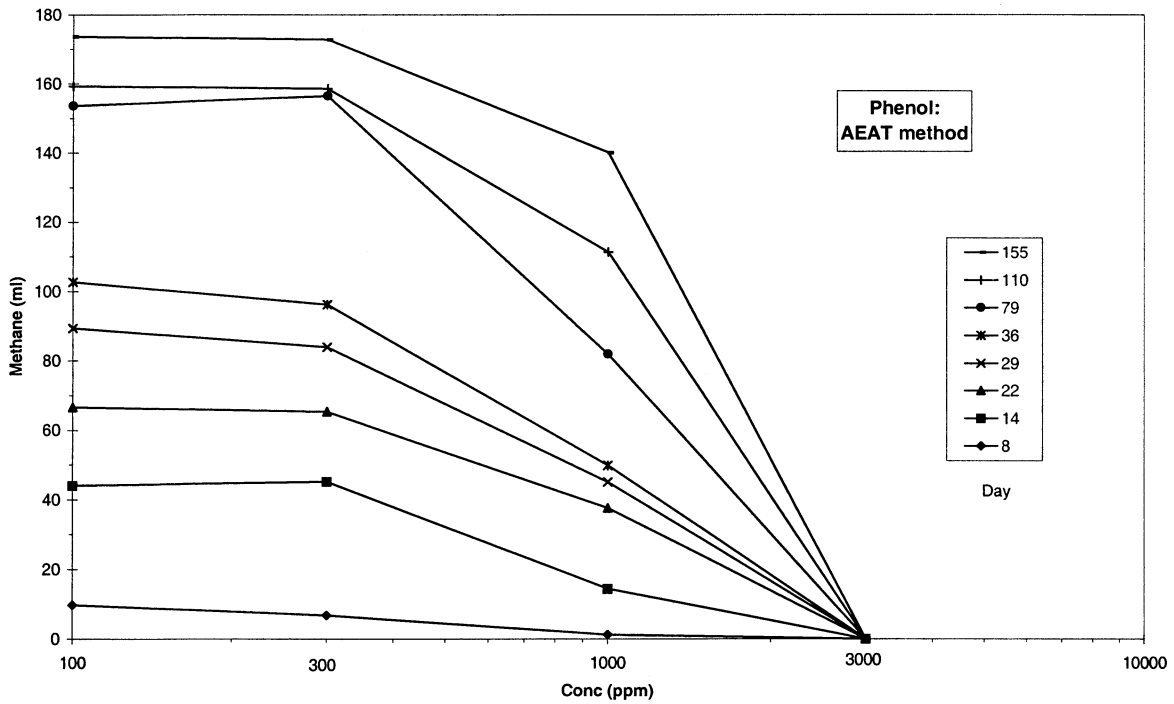

Figure 13 Phenol to 155 days, AEAT method IC_{100} 1000-3000 ppm

The figures presented show the cumulative dose-response curve on each sampling day. The IC_{50} (50% inhibitory concentration point) has not explicitly been marked as this drifts upwards over time for all the tests. This assessment of the data has highlighted the potential weakness of using the IC_{50} value to rapidly classify a waste, and leads AEA to recommend that the IC_{100} (complete inhibition - estimated by the first dilution to show no methane) be used as the most practical end-point for an acceptability test.

In the tests performed at AEA, the IC_{100} at 155 days was in every case precisely the same as that determined at just 7-8 days, suggesting that a rapid and reliable assessment of acceptability could be made using a one-week acute toxicity test. Furthermore, the IC_{100} as determined by the simplified L-ABT method and the original CAMR L-ABT method agreed perfectly, suggesting that the simplifications had no negative impact on any critical aspects of the test.

At the completion of this test, the opportunity was taken to assess the distribution of the toxin in two different dilutions of the cadmium test system. Cadmium can be chemically transformed but cannot be degraded, so the full inventory must be conserved in the test bottle. Samples of the liquid and solid phases were analysed by ICP-AES (Integrated Coupled Plasma Atomic Emission Spectrometry - Table 1).

Table 1 O-ABT version 1 - cadmium analysis at day 155

Sample	Cd added (mg/100ml)	Actual Cd found (mg/100ml) triplicates	Recovery (%)
Supernatant	30	*0.020*	*0.07%*
Solids		*7.90*	*26%*
Supernatant	10	*0.002*	*0.007%*
Solids		*2.43*	*24%*

Values shown are means of triplicate determinations

Of the cadmium added, less than 0.1% remained in solution at day 155, and approximately 25% was recovered in the filtered solids. The balance, approximately 75%, had presumably adsorbed to the glassware and bung. Cadmium is known to adsorb strongly to many materials. These observations illustrate the importance of knowing the way in which toxins partition in landfill waste before attempting to use bioassay data to advise on loading rates. In this instance the results suggest that the O-ABT should not be used for long-term toxicity assessment, since most of the cadmium becomes biologically unavailable over time.

Summary

The IC_{50} value was defined as that concentration at which methane yield was 50% of the uninhibited maximum, as determined from the smoothed interpolation of the dose-response curve - so was expressed as a single value. This value proved to be time-dependent. The IC_{100} was defined as that concentration causing complete inhibition of methane production, which was between the last dilution showing any methane and the first to show no methane - so was expressed either as a range or, more simply, as the first dilution showing complete inhibition.

These were largely time-independent, i.e. once a test bottle was completely inhibited, methane production rarely recovered.

The overall conclusion from these initial tests was that in the short-term the simplified method gave similar toxicity results to the original method developed at CAMR. Tables 2 and 3 summarise the data. The IC_{100} value was stable and reliable in as little as 8 days, and the IC_{50} was both quantitatively more sensitive (in as little as 8 days) and qualitatively useful in indicating adaptation potential (in as little as 28 days).

Table 2 Summary of IC_{50} inhibition values obtained in comparison of simplified O-ABT method and original CAMR L-ABT method

Method	AEAT	CAMR	AEAT	CAMR
Toxin	IC_{50} 7-day test (ppm)		IC_{50} 60-day test	
TCE	n	n	n	N
Phenol	50 [30-100]	1500 [1000-3000]	1000 [300-3000]	500 [300-1000]
Cadmium	50 [30-100]	i	500 [300-1000]	50 [30-100]

n = not inhibitory in tested range
i = indeterminate (bimodal)
[brackets] show range of value (± one dilution)

Table 3 Summary of IC_{100} inhibition values obtained in comparison of simplified AEA method with original CAMR method

Method	AEAT	CAMR	AEAT	CAMR
Toxin	IC_{100} 7-days (ppm)		IC_{100} 155-days	
TCE	N	n	n	n
Phenol	1000-3000	1000-3000	300-1000	300-1000
Cadmium	300-1000	300-1000	300-1000	300-1000

AEA and CAMR methods compare very closely
IC_{100} value is stable from 7 days onwards
n = not inhibitory in tested range

2.5 Further Simplification of the O-ABT

In the second batch of testing:

- the reproducibility of the short-term test was checked by repeating O-ABT Version 1; and
- further simplifications were introduced, notably dispensing with some of the more expensive and potentially hazardous or less biologically-important trace elements.

Many trace elements are required only in vanishingly small quantities. In a fully-defined laboratory system they must be expressly added to be sure they are present, but in the O-ABT

it was expected that the added milled waste would supply most if not all trace element requirements. Only the cadmium test was repeated. This version is termed O-ABT Version 2.

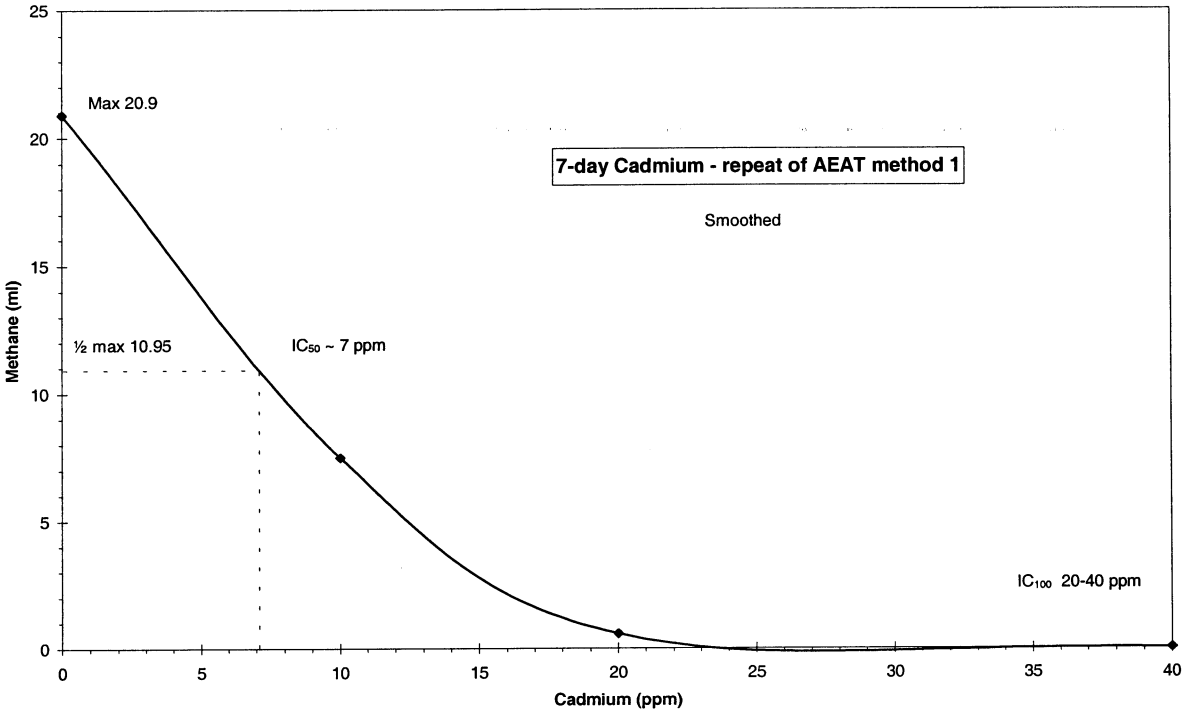

Figure 14 Cadmium, first repeat, 7 days (linear scale required - see text)

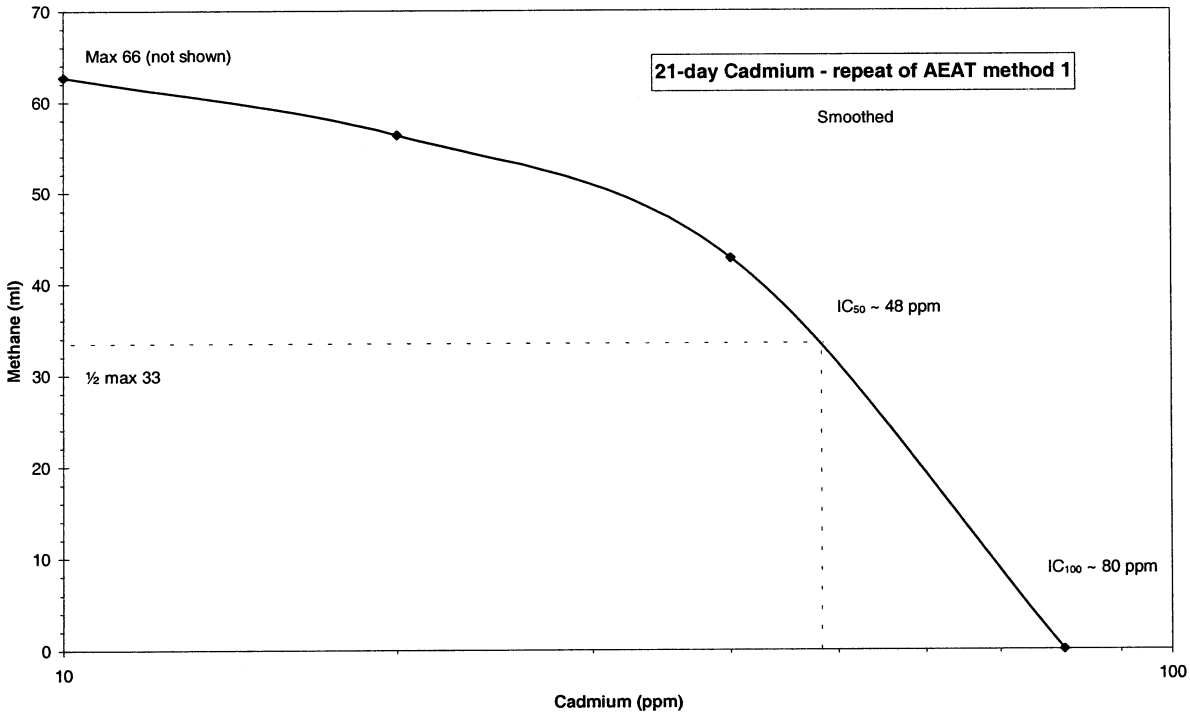

Figure 15 Cadmium, first repeat, 21 days

The repeat of Version 1 gave a rather lower IC_{50} value (~7 ppm) at 7 days (Figure 14) than the previous experiment had done at 8 days (~50 ppm). In addition it did not give the expected logarithmic response (note that Figure 10 is a linear-linear plot) and there was an indeterminate IC_{100} (estimated at 20-40 ppm). At 21 days (Figure 15) the IC_{50} had increased to ~48 ppm, and the IC_{100} had stabilised at ~80 ppm.

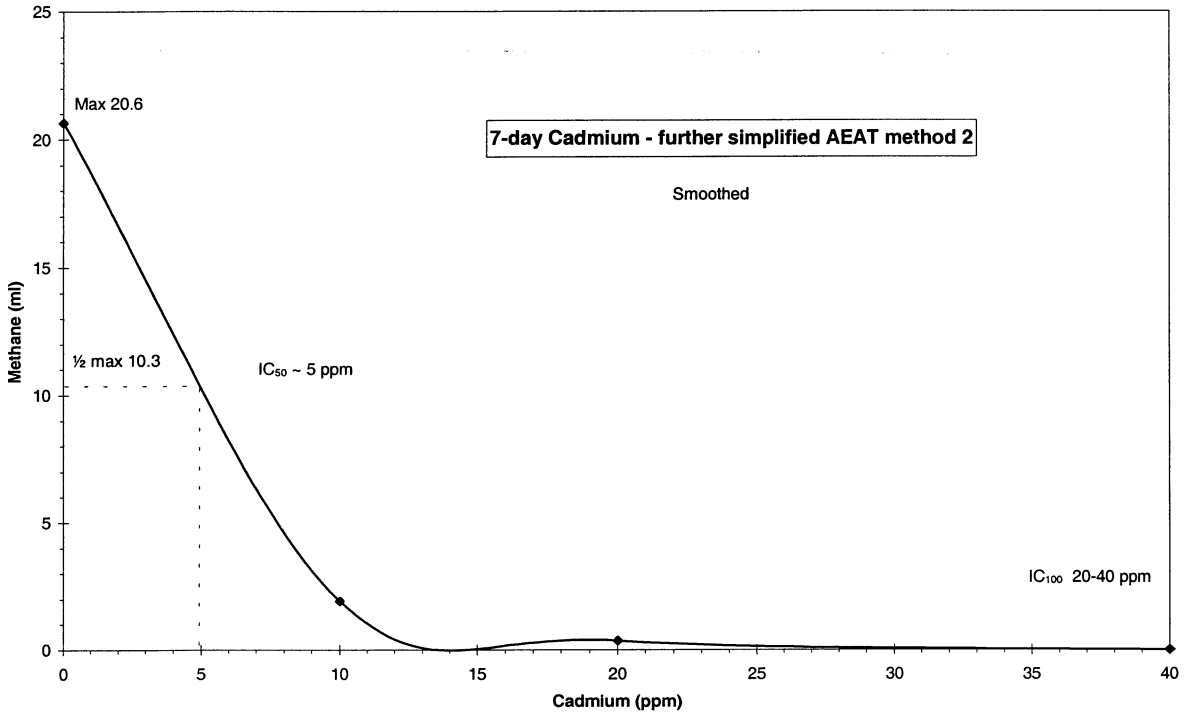

Figure 16 Cadmium, second repeat, 7 days

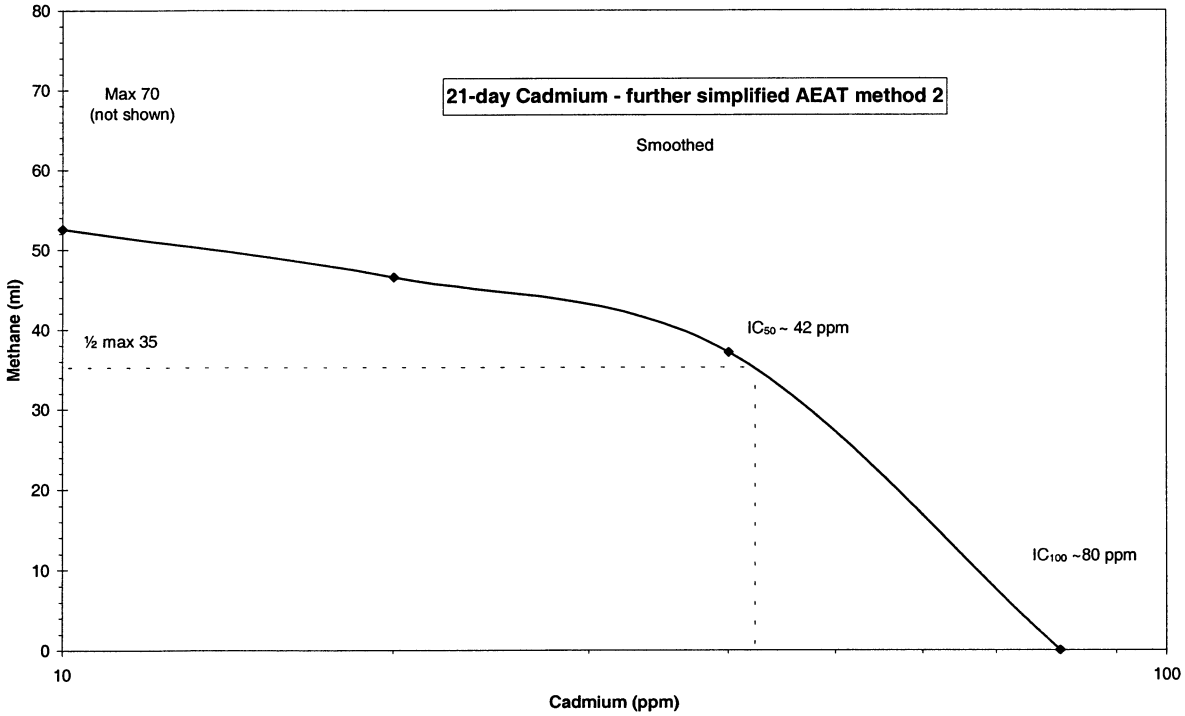

Figure 17 Cadmium, second repeat, 21 days

Inhibition by cadmium using simplified Version 2 gave an IC_{50} value of only ~5 ppm at 7 days (Figure 16), not statistically significantly different from the Version 1 repeat, but possibly indicating even greater sensitivity in the absence of certain trace elements. At 21 days the greater inhibition was still apparent, with an IC_{50} value estimated at ~42 ppm (Figure 17). Note that these small differences are not significant considering the doubling dilution steps employed. The IC_{100} stabilised at ~80 ppm, identical to that determined by O-ABT-1, suggesting that further simplification had not materially changed the sensitivity of the test.

Acidification of the cultures was possibly the cause of this instance of increased sensitivity of both the IC_{50} and the IC_{100}. A check of both sets of test bottles after incubation revealed a pH of ~5.5 ± 0.3. The work book had no record of final pH checking and adjustment of media prior to use, suggesting that this step may have been inadvertently omitted. At this pH methanogens would be directly inhibited, and metals would be highly mobile in solution, enhancing toxicity. In subsequent work particular care was taken in adjusting the pH of seed, nutrient and toxin solutions to pH 7.0 ± 0.1 just before use.

The seed culture was demonstrably gassing just prior to use, but it is possible that the seed culture was sub-optimal at the time of use (see discussion of SME trials). Biological systems are inherently variable, because the total biomass, balance of species numbers and rates of activity all change constantly. Use of, and comparison with, an appropriate defined standard toxin such as phenol may partially resolve this problem (see section 4. Discussion).

2.6 O-ABT Version 3

The cadmium test was re-run using the conditions of Version 1 but taking care:

- to adjust pH of all solutions and suspensions to 7.0 ± 0.1.
- to use more closely-spaced dilutions of cadmium (0, 5, 10, 20, 40, 80, 160 and 320 ppm) in order to achieve a more accurate IC_{50}.

In addition to positive controls, the test series was started at a lower cadmium dilution, to ensure that some tests would show relatively uninhibited gas production that could be plotted on a logarithmic axis.

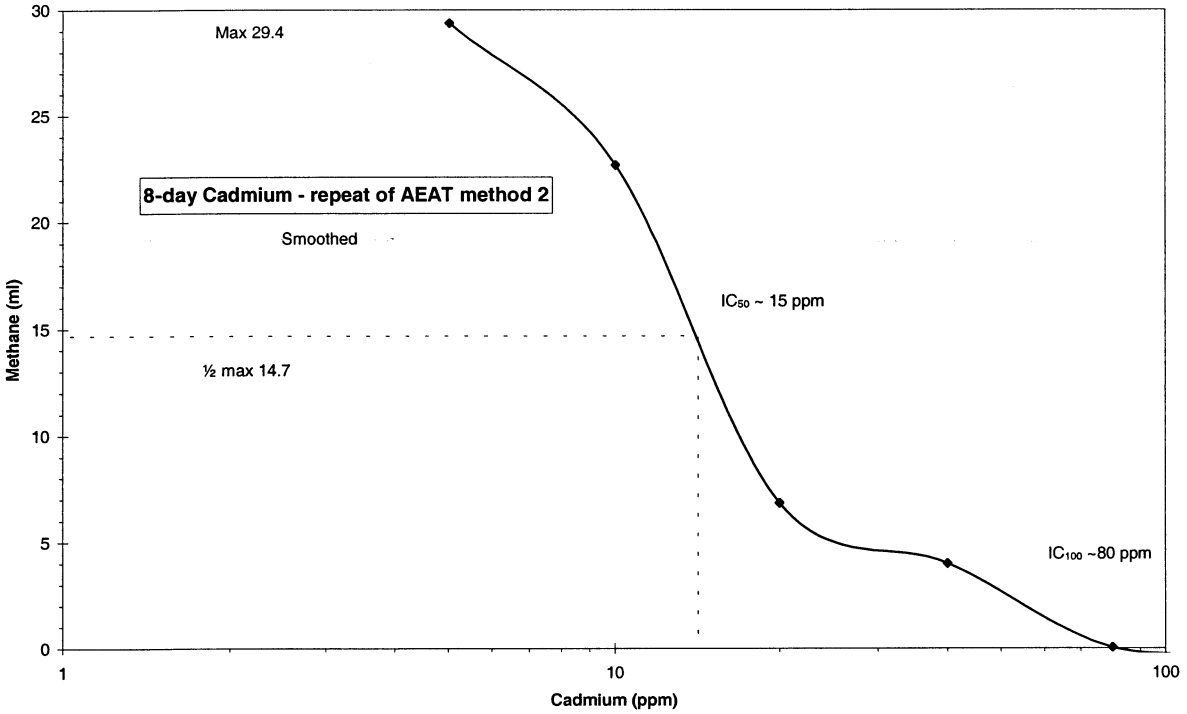

Figure 18 pH-corrected Cadmium, 8 days

Methane inhibition by cadmium at 8 days (Figure 18) gave an IC_{50} of ~15 ppm, certainly between 10 and 20 ppm. At 30 days (Figure 19) the Version 3 run gave an IC_{50} of ~60 ppm (between 40 and 80 ppm). The IC_{100} was ~80 ppm, and was stable from 8 days onward.

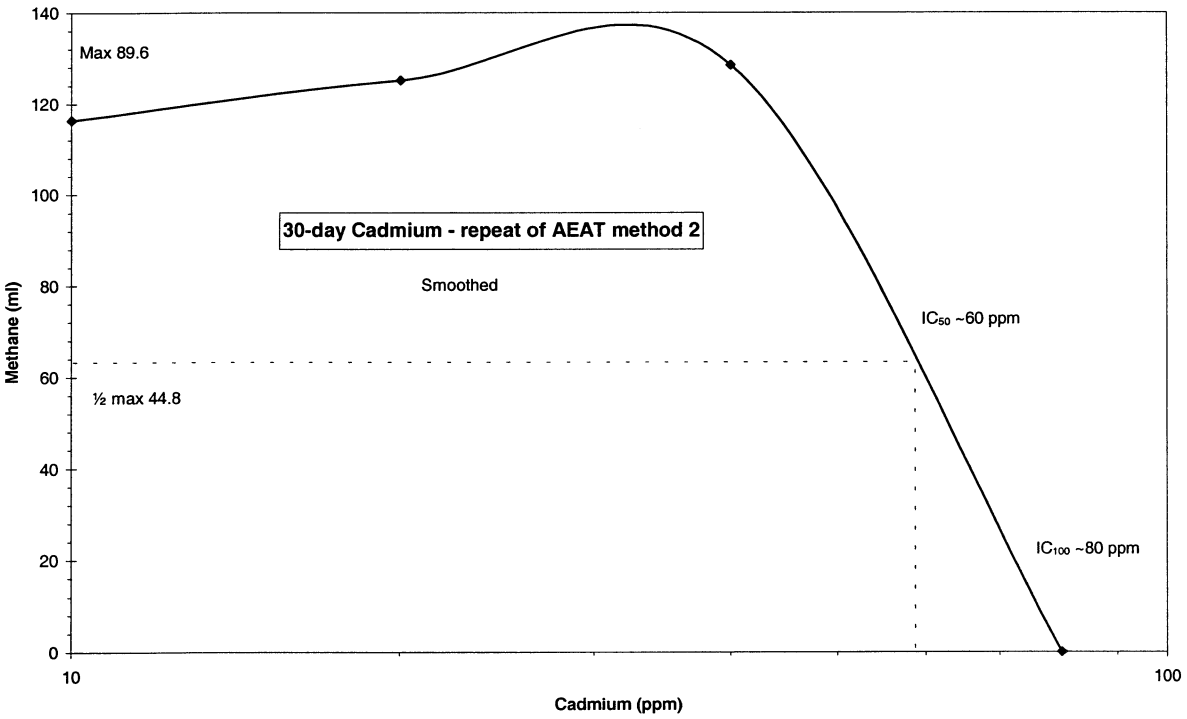

Figure 19 pH-corrected Cadmium, 30 days

These tests showed that the cultures were less sensitive to cadmium than the preceding low pH test as judged by the IC_{50}, but the complete inhibition value, the IC_{100}, was the same as the second run, and was significantly lower than the original comparative run. Correcting the pH therefore changed the IC_{50} but not the IC_{100}.

A drift in the sensitivity of the seed culture would probably explain this trend. As a guide, it is considered good laboratory practice for a new culture to be incubated for up to five hydraulic retention times before stability can be assured. At the rate of 10% per week change, it would take up to a year for the culture to fully stabilise. However, our seed culture was gassing healthily from the first week, but began to show erratic gas production by month 4 or 5. Since the feedstock comprised milled solid waste (containing a high proportion of recalcitrant/inert materials), prolonged incubation would cause these to accumulate. In addition, other recalcitrant or even toxic end-products may have been accumulating. The seed had been incubating for about 3 months when used for versions 2 and 3. Regular renewal of the seed culture is probably the simplest and most cost-effective solution (see section 4).

3 METHOD EVALUATION

The results and experience gained in developing a simplified ABT were used to write a preliminary Standard Operating Procedure. The Industrial Advisors discussed this procedure and a draft Standard Operating Procedure (SOP) was agreed. This formed the basis of the method evaluation conducted by SME at their Kempston laboratory. A final version of this SOP, incorporating later recommendations, is attached as Appendix 3.

The staff at Kempston had no specific training in microbiological techniques and the laboratory was not specially designed or fitted for testing anaerobic micro-organisms. The laboratory staff had observed the work at AEA and were given advice by a trained microbiologist but the bulk of the work was guided by the written draft SOP.

The principle objectives of this part of the work were:

- to examine the practical implementation of the method in an operator's laboratory;
- to examine the reproducibility of the method in a non-specialist laboratory;
- to examine the effects of temperature change;
- to test both pure toxins and candidate co-disposal wastes;
- to identify and address any further practical improvements;
- to identify any changes in the draft SOP and revise accordingly.

3.1 Adaptation of the Draft SOP

In the light of SME comments the draft Standard Operating Procedure was altered to facilitate work in the operator's laboratory. These modifications are outlined in Appendix 4, and include:

- correction of pH once, just prior to use (avoiding time-consuming multiple adjustment);
- avoiding prolonged boiling of the media;
- modifying the sampling device used to remove gas samples from the bottles;
- modifying the way toxins were added to the samples (to deal with the viscosity of phenol solutions and the low solubility of TCE).

3.2 Performance of the Seed Culture

The management of the seed culture was the only significant problem encountered in the operator's laboratory. An initial 10 litre culture was set up in April 1996 using 2.5 litres of the AEA seed (at a nominal 10% solids content), added to 7.5 litres of the recommended nutrient solution, and 50 grams of sorted and milled household waste from AEA. This gave a nominal solids content of 7.5%. This culture was soon producing some 100 - 200 ml of gas per day. The SOP required that 500 ml of this culture was replaced each week with 500 ml of the nutrient solution and 50 grams (wet weight) of milled household waste, thus allowing the solids content of the culture to gradually build up to ~10%. The rate of gas generation gradually increased, so that by the end of June total gas output was recorded at more than one litre per day. However, by August (after 4-5 months operation) gas production gradually became more erratic.

Most solids were noted floating on the top of the liquid, presumably due to trapped gas bubbles. This might have led to a reduction in the contact between the anaerobic bacteria (which adhere to the solids), and the available substrates (mainly in solution). The cultures were not mechanically stirred and regular shaking dispersed the solids temporarily although the problem recurred. In an attempt to minimise this problem the solids content in the vessel were progressively reduced to 5% by the addition of 500 ml of nutrient and 25 grams of household waste each week (from week 10 onwards). As a result gas production was lower but less erratic (Figure 20).

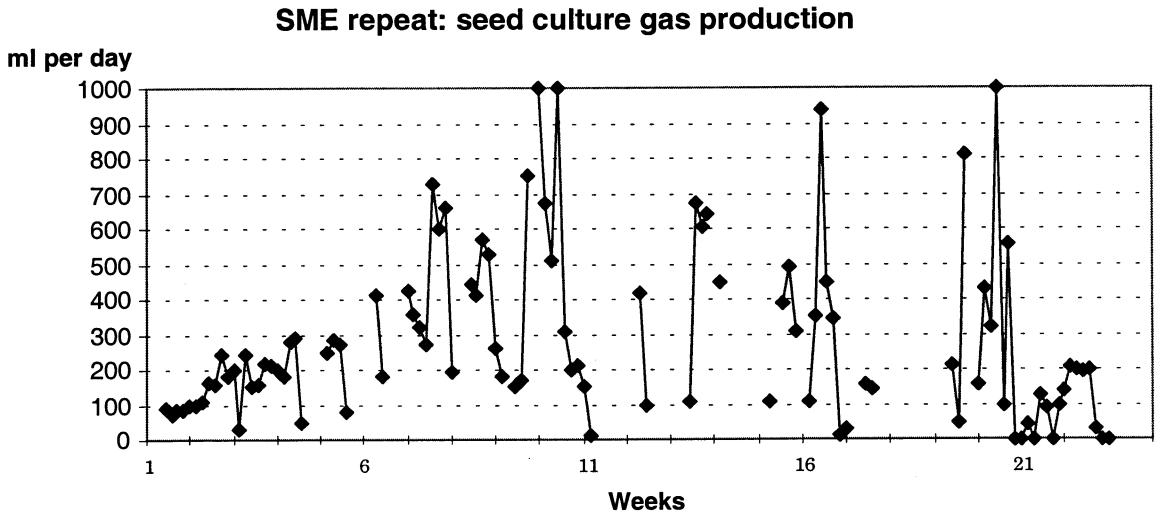

Figure 20 SME repeat; seed culture gas production over time

The pH of the liquor discarded each week remained in the range 7.0 - 7.3, so souring was not occurring. More sophisticated methods for maintaining seed cultures are possible (e.g. cultures used for the Biological Methane Potential tests by Minton, Treharne and Davies (personal communication). However, these require more routine attention, and at present it appears to be practical and inexpensive to establish a fresh culture at quarterly (no greater than 4 month) intervals. This aspect of the SOP should however be reviewed in the light of ongoing experience.

A second seed culture was set up at the end of July using a new batch of aged household waste. The household waste was prepared by taking solid waste collected from an 8-10 year old section of Calvert landfill site. It was sorted by hand to remove all visible non-putrescibles (plastic, metal, glass) then shredded with an industrial food processor to obtain a finely divided solid. Approximately 1 kg of this material was prepared and stored in a refrigerator. Storage as a dried powdered material was considered, but drying and loss of volatile compounds would probably have significantly reduced the bioavailable carbon content. This material was used to initiate and maintain a reactor running at ~5% solids. The seed culture was soon producing gas, generation being somewhat erratic from day to day, but on average yielding 125 ml per day volumes of gas.

3.3 Results from Application of Operational ABT

Nine tests were set up by SME over a period of 3 months. The first three tests were intended to replicate the tests performed at AEA using TCE, phenol and cadmium as the toxins, and were incubated at 35°C. A month later the phenol test was repeated at 35°C, and also at room temperature (20-30°C). The final tests were with four example liquid industrial wastes, all tested at 35°C. Tests on the four candidate liquid co-disposal wastes were conducted using the new seed cultures, standardised by running a further phenol test in parallel.

Phenol

The initial phenol test gave an IC_{100} of 2560-5120 ppm phenol, which was very similar to the value obtained by AEA in their tests, and did not change after week 1 (see Figure 21). The 10 to 80 ppm levels of phenol gave gas volumes very similar to that of the blank, so after 5 weeks analysis of these bottles was discontinued.

As the experiment progressed, at the concentrations of toxin just below the IC_{100} there was an enhanced volume of methane when compared to the blank bottles. This was presumably due to degradation of the phenol itself (i.e. a higher amount of organic carbon in these bottles). This is similar to the effect noted with TCE by AEA. The observation of phenol-degrading organisms in landfill is not particularly surprising.

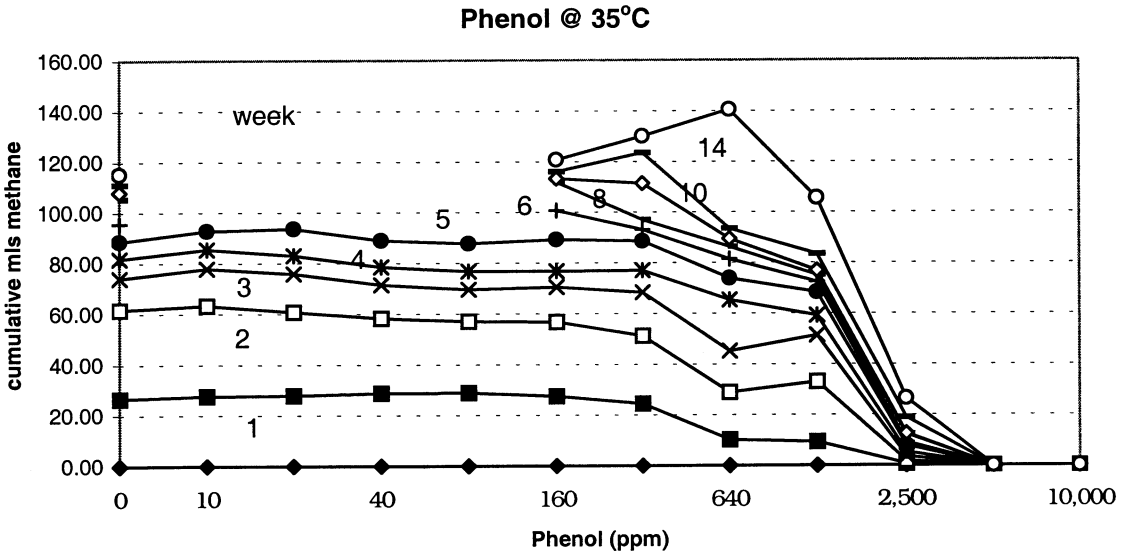

Figure 21 SME repeat, phenol at 35°C

Cadmium

The highest level of cadmium tested was 1000 ppm (see Figure 22). This eventually produced a few ml of methane after 3 months, which we discounted in the light of the earlier study showing loss of Cd by adsorption over time. The value for the IC_{100} was therefore very similar to that found by AEA in the preliminary work. Analysis of the lower concentrations was discontinued after 5 weeks and the frequency of testing was reduced to every fourth week once the pattern of inhibition was established.

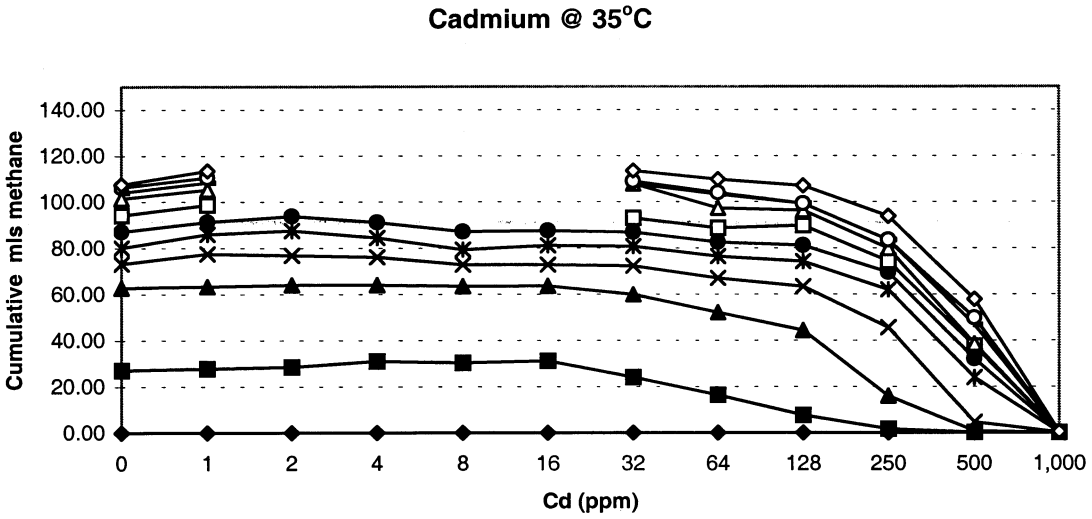

Figure 22 SME repeat, Cadmium at 35°C

Trichloroethylene

The highest level of TCE tested was 640 ppm w/v, a high concentration relative to the reported CAMR inhibition value of 1 ppm. This was chosen in the light of non-inhibition at up to 10 ppm found in the AEA tests, to be sure of finding the IC_{100}. The amount of methane produced by the 80 to 320 ppm TCE tests was fairly small, giving a much flatter pattern to this area of the graph, and an overall sigmoid shape (see Figure 23). After 4 weeks even at 640 ppm the tests had produced small volumes (< 1 ml) of methane.

This may relate to the low water solubility of TCE, which would naturally limit the concentration of TCE actually experienced by the organisms, allowing survival, then adaptation and growth, of the tolerant or degrading organisms. If this occurs with other insoluble toxins then the liquid-based test may prove inappropriate for these. However, by adhering firmly to the 7-day interpretation, a clear IC_{100} of 320-640 ppm was determined, which in practice would safely over-estimate the toxicity in-situ.

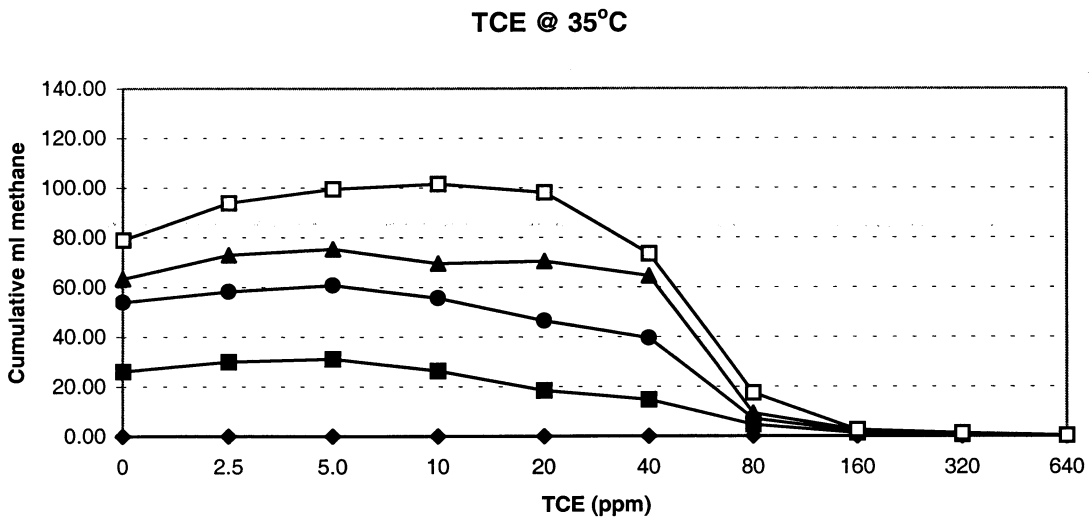

Figure 23 SME repeat, TCE at 35°C

The appearance of the test mixture was a good indication of whether the bottles were producing methane. The tests that were producing methane were black in colour, presumably due to production of sulphides by sulphate-reducing bacteria, whereas inactive tests were always paler in colour. With experience one could probably estimate the IC_{100} fairly accurately by eye, although we cannot recommend this.

In these tests the value for IC_{50} was not easy to establish, as it changed with time. This was illustrated in the first phenol test (Figure 21), where after 2 weeks the IC_{50} would have been below 640 ppm phenol, but by week 14 the IC_{50} was well above the 1250 ppm toxin concentration. By contrast, the IC_{100} was usually unambiguous.

3.4 Evaluating Critical Factors

3.4.1 Temperature

The Industrial Advisory Group (IAG) recommended that the effects of temperature on the ABT were evaluated, since this parameter might be difficult to control in some laboratories. The IAG wondered in particular whether an incubator was strictly required.

The O-ABT using phenol was repeated at 35°C and at room temperature. Although methane production was slower at room temperature, both experiments showed a similar pattern of inhibition and the same IC_{100} (Figures 24 and 25). Initially no methane was produced at 2500 ppm phenol at 35°C, giving an IC_{100} of 1280-2560 ppm, but by 3-4 weeks as the bacteria became acclimatised to the toxin, or as it adsorbed/detoxified, measurable amounts of methane were produced at this concentration. These figures suggest that it may take a month or more before a stable IC_{100} can be obtained, but week 1 or 2 is probably optimum for acute toxicity. The results were virtually identical to the first run (see Figure 21).

The gas production from the phenol bottles incubated at room temperature (approximate mean 25°C, and varying from <20°C to ~30°C, during the test period) was much lower than at 35°C. Over the 7 weeks of incubation they produced approximately half of the volume of gas compared to those incubated at 35°C. However, the methane concentration and cumulative methane production trends suggested that the end result would eventually be the same as those incubated at 35°C. Gas production at week 1 was so low that it was difficult to measure accurately, but by week 2 a clear IC_{100} was determined. Most significantly, precisely the same IC_{100} was estimated as at 35°C, with the bottles containing 5280 ppm toxin producing no gas, and those containing 2560 ppm phenol producing just a trace of gas. So, although the test conducted at room temperature gave the same IC_{100} of 2560-5280 ppm phenol, at low and variable temperatures a longer incubation was required before an assured value was reached.

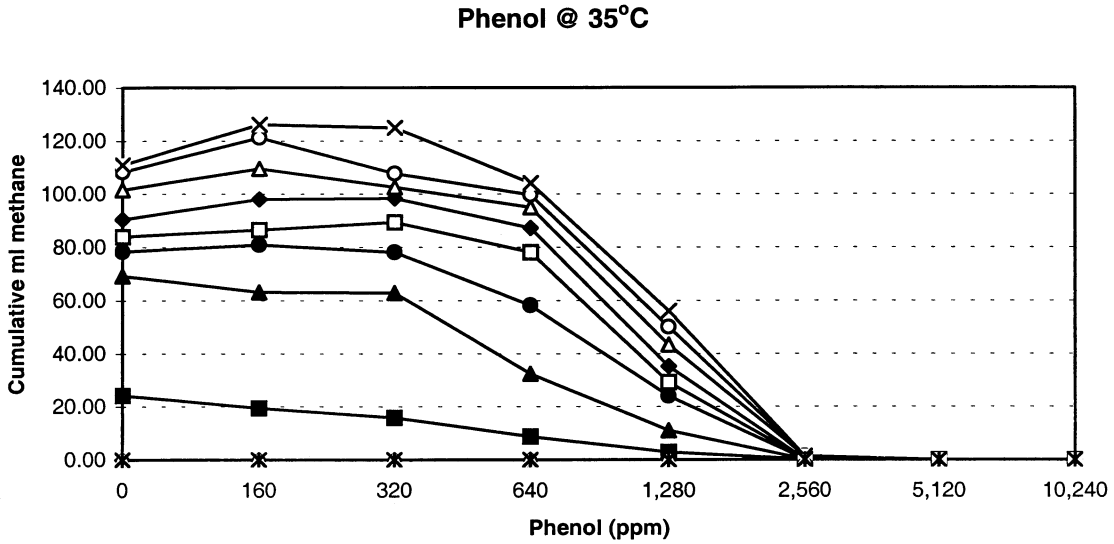

Figure 24 SME second repeat of phenol at 35°C

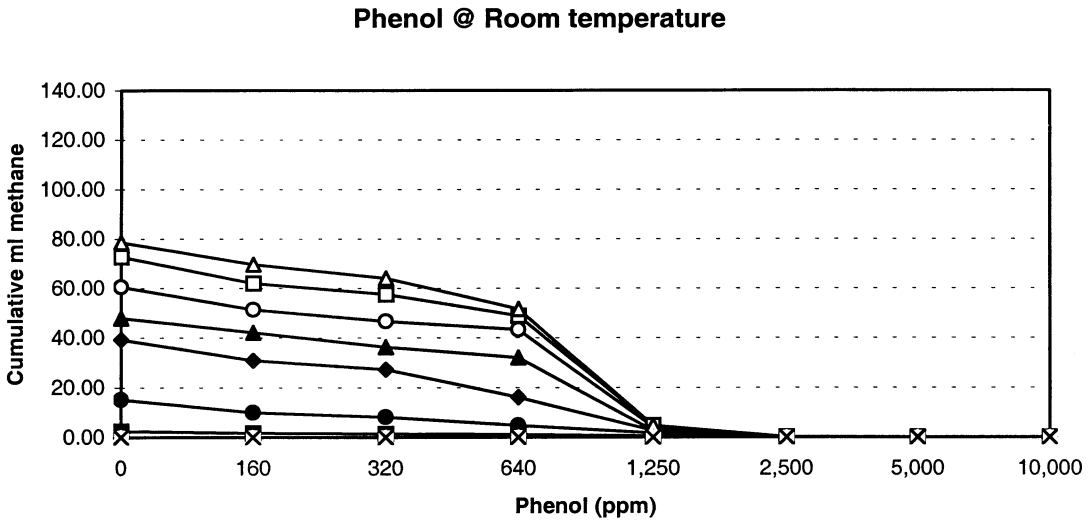

Figure 25 Repeat of phenol at ambient temperatures

3.4.2 Reproducibility

Figure 26 shows the mean of triplicate cumulative methane volume produced by the blank bottles for:

- 4 sets run in parallel with the SME repeat of phenol, Cd and TCE (labelled a-d);
- 3 sets run in parallel with the temperature test (e & f at 35°C, g at ambient).

The first 4 tests run at 35°C, a-d, showed very high reproducibility. The second sets at 35°C, e & f, showed slightly lower but not statistically different yields, perhaps an early sign of the seed culture losing viability. The mean yields at any time interval were very similar, and the standard deviation of each week's figures was extremely good, with a standard deviation of about ±5% of the mean methane production. The ambient test, g, showed a lag phase with slower gas production over the first week, but then recovered to a large extent after the second week. The lag in g may also have been associated with deterioration of the seed culture as noted above, since there was also some evidence of a lag in f, the last test run at 35°C.

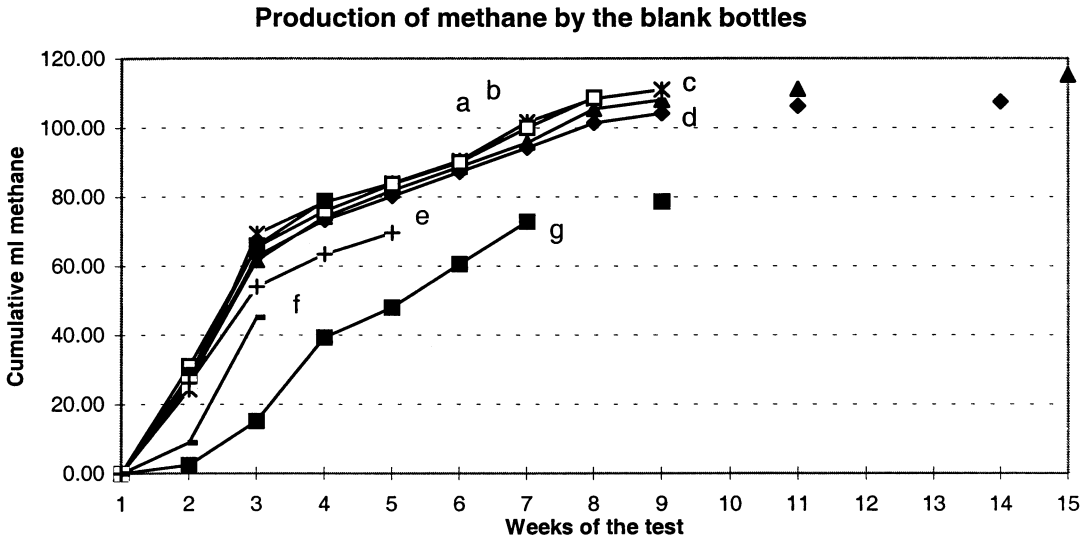

Figure 26 SME repeats; toxin-free control blanks

3.4.3 Further simplifications

In the original draft SOP individual nutrient solutions and seed were adjusted to pH 7.0 ± 0.1 then combined and checked again just before use, the usual practice in laboratories handling pure cultures in defined media. SME asked if it was possible to simplify this in order to save time and effort.

After some experimentation it was found that combining nutrient solutions without causing precipitation was possible, simplifying dispensing of reagents and allowing a single final pH check and adjustment just prior to use.

3.4.4 Final pH checks

It was impractical to check the pH of the solution after the addition of the toxin, or during the test programme. However, it was thought possible that the acidity of phenol at 10,000 ppm could have altered the pH of the solution in the bottles sufficiently to alter the apparent IC_{100} found. To see whether this effect was significant, as each phenol test was completed the pH of the solution in the bottles was measured. The pH was found to be within the acceptable range for methanogenic activity in all sub-IC_{100} bottles, suggesting that any acid effects due to phenol were adequately buffered.

In some bottles at or above the IC_{100} the pH had fallen. Given earlier AEA observations of hydrogen accumulation near the IC_{100}, indicating continued hydrogen production in the absence of methanogenesis, this fall in pH was probably a result of the continued activity of acid-producing organisms in the absence of active acid-consuming methanogens. It was therefore concluded that the test reagents plus added waste provided sufficient buffering of any effects from the toxin itself.

3.5 Comparison of Results from AEA and SME Laboratories

The reproducibility of the test between laboratories was acceptable for both phenol and cadmium (Table 4). The value of the IC_{100} for phenol was consistent between replicate tests and at the two different temperatures employed (i.e. 35°C and room temperature). An IC_{100} for TCE was established at 320-640 ppm. The reproducibility of the methane production in the blank bottles was good, but if the seed culture was used for more than four months the gas yields began to fall and variability began to increase.

Table 4 Summary of 7-day toxicity data from the O-ABT conducted in both laboratories

Toxin	AEA IC_{100} (ppm)	SME IC_{100} (ppm)
TCE	n [>10]	320-640
Phenol	1000-3000	2560-5120
Cadmium	300-1000	512-1024

n = not determined

3.6 Application to Real Wastes

Four liquid industrial wastes, typical of those sent to landfill operator's laboratories, were tested by SME, using the revised SOP.

The first sample (designated SN 8793) was an effluent with a Chemical Oxygen Demand (COD) of 310,000 ppm, and containing 11,000 ppm cyanide (Figure 27). This showed an IC_{100} of 10,000 ppm, (i.e. a 1% concentration of whole waste; 1 ml of the waste added to a bottle containing 100 ml of nutrient and seed). At concentrations of the waste just below this value enhanced production of methane was observed, presumably due to bacteria degrading components of the waste (and hence producing more methane than the control).

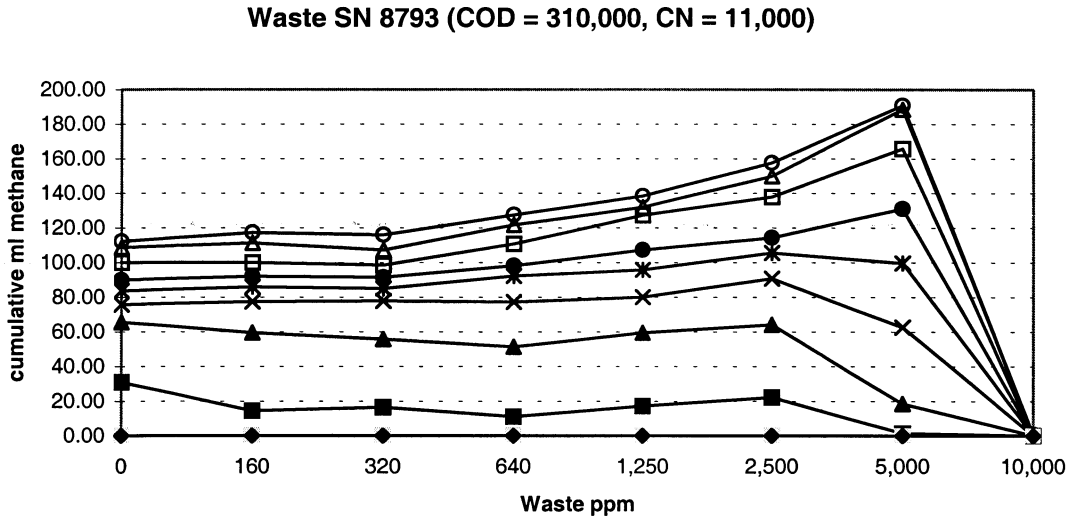

Figure 27 Test waste SN 8793

The second effluent tested (SN 9570) was from a pharmaceutical manufacturer and had a COD of 110,000 ppm. At the maximum concentration tested (1%) it had no effect on the amount of methane produced, either positive or negative, so the test was discontinued after 3 weeks (Figure 28).

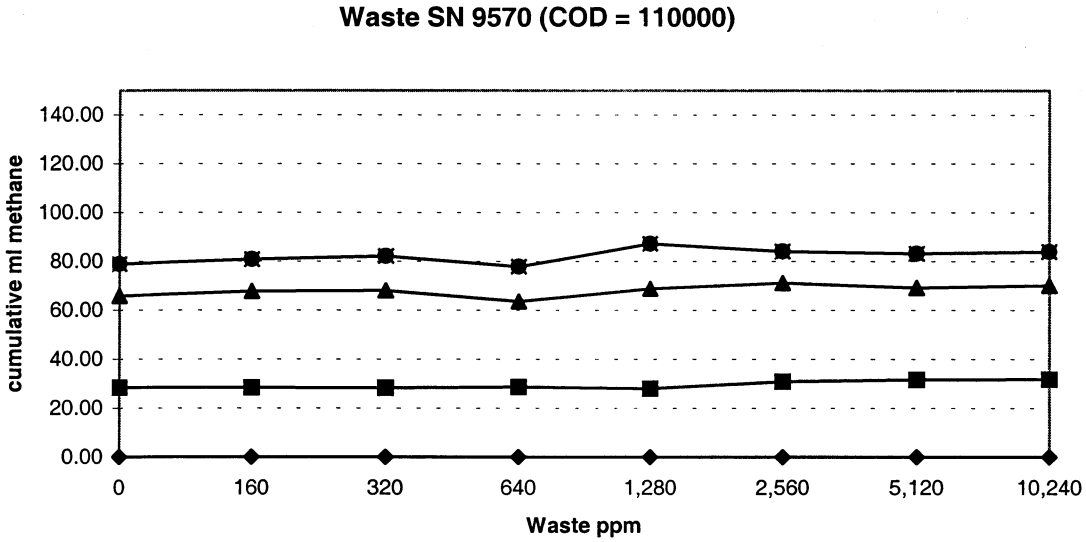

Figure 28 Test waste SN 9570

Figure 29 shows results to date from tests on a third liquid waste, designated SN 9438, which is ongoing. The data available suggests a sigmoid curve similar to that seen for TCE (see Figure 23). The IC_{100} at 2 weeks was 5000-10,000 ppm, and may eventually be assigned in excess of 10,000 ppm, i.e. it is relatively non-toxic. Erratic gas production was observed from the seed culture used at that time, but the effects of this cannot be determined. A phenol standard run in parallel would possibly have been useful.

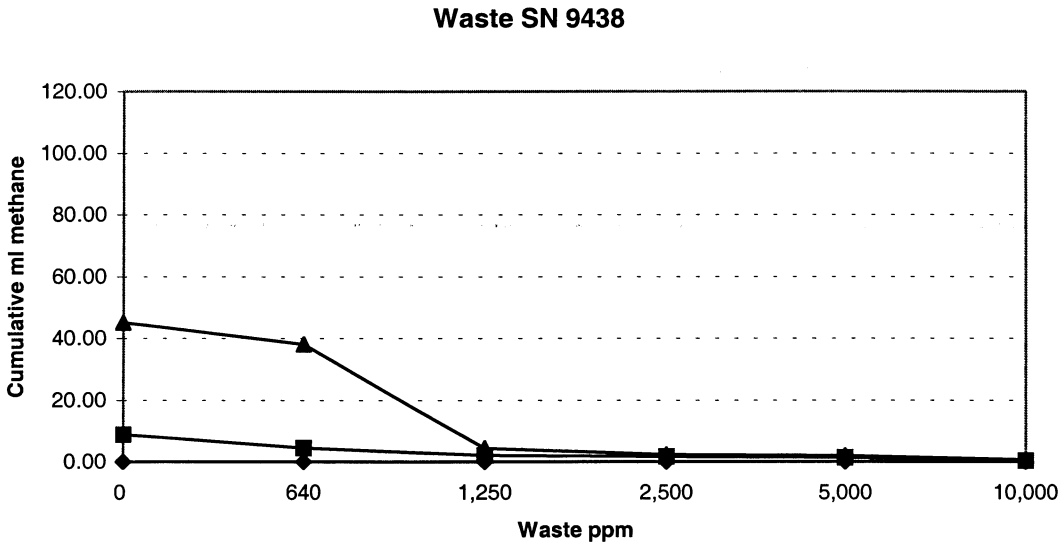

Figure 29 Test waste SN 9438

The fourth industrial waste (designated SN 9514) was a neutralised phenol sulphonic acid, with a COD of 15,000 ppm. This waste showed an initial inhibition of the gas production at 10,000 ppm. At 2 weeks 1 - 2 ml methane were produced at this dilution, again indicating that the IC_{100} could be in excess of 10,000 ppm (Figure 30).

NB: Testing concentrations greater than 10,000 ppm (i.e. 1%; 1 ml of waste in 100 ml of test medium) is not recommended, because this would probably dilute nutrients and exhaust buffer capacity significantly.

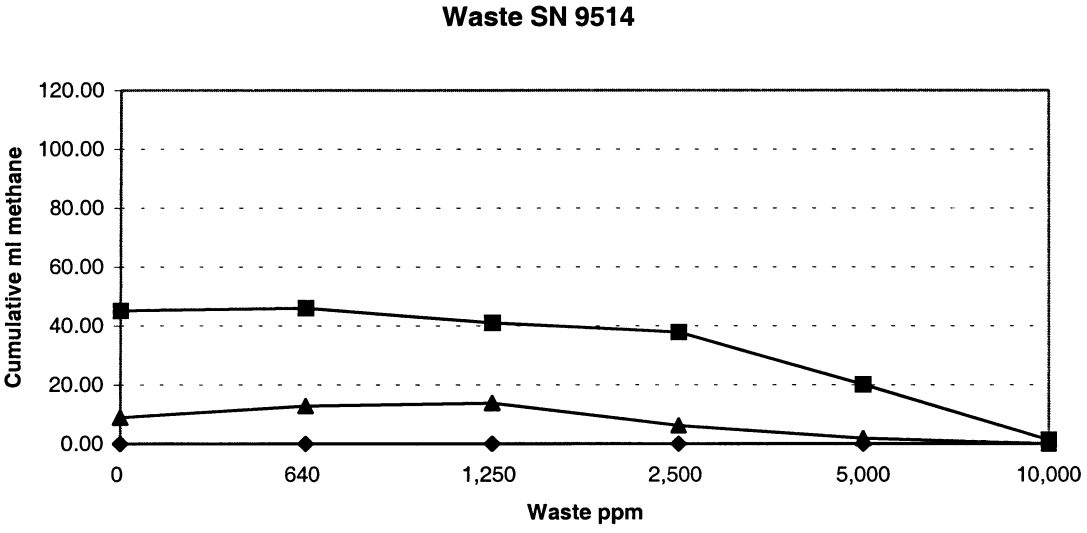

Figure 30 Test waste SN 9514

3.7 Theoretical and Actual Test Efficiency

Over the course of test development the positive control test system (containing no toxin) was repeated more than seven times, each in triplicate. This gave sufficient replication to examine the mean methane generation curve; the plot of cumulative methane yield over time (Figure 31). Visually this appears to have 3 distinct phases of production:

- a phase of high gas generation rate (days 0-14);
- a period of lower but steady gas production (days 14-49);
- a slow approach to exhaustion (day 49 onwards).

These phases are not statistically distinguishable, but the plot approximates closely to the classical exponential curve, which rises fast initially then exponentially approaches the final yield (at infinite time).

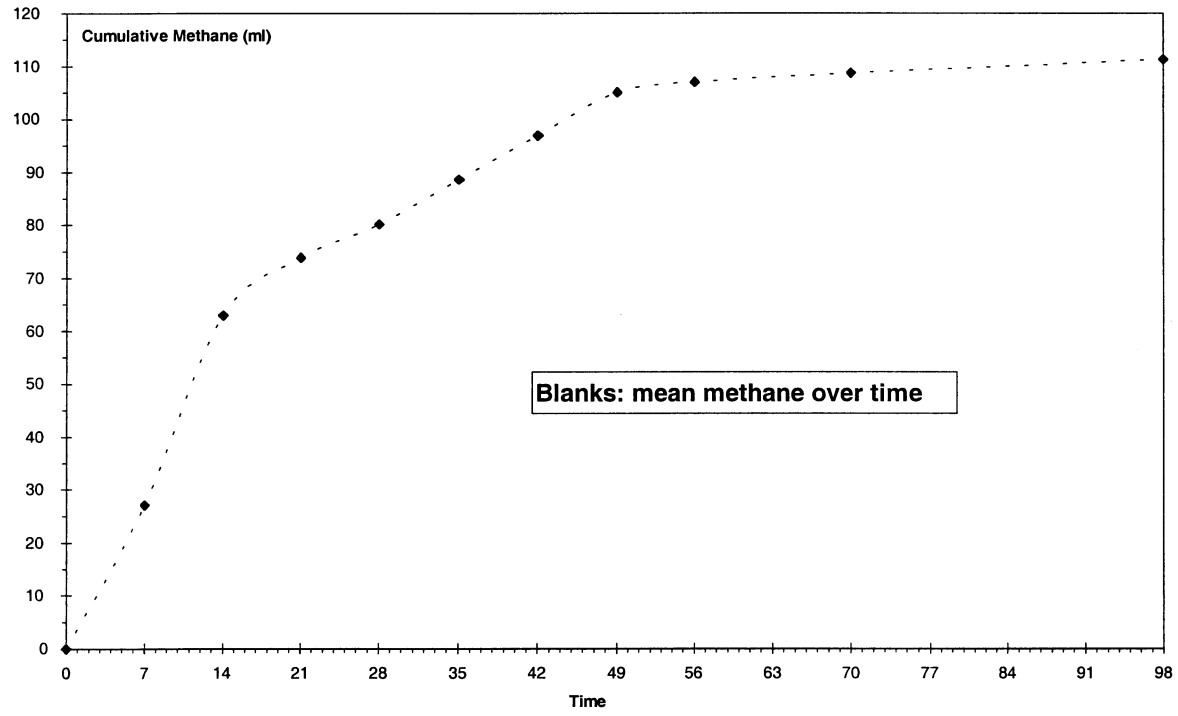

Figure 31 Cumulative methane yield over time (mean of 21 controls)

To simplify interpretation the exponential curve can be transformed into a straight line, either by plotting reciprocals of methane and time (the double-reciprocal plot), or by plotting cumulative methane yield against methane production rate (the Eadie-Hofstee plot, preferred for irregularly-spaced measurements, Figure 32).

The Eadie-Hofstee plot has several features of interest:

- the correlation coefficient of methane yield against methane production rate is good ($R^2 = 0.9377$);

- the initial (day 0) rate of methane production can be determined from the Y intercept (7.82 ml per 2 g of added waste per day, equivalent to 3.9 ml $CH_4.g^{-1}$.day);

- the expected final yield can be determined from the extrapolated X intercept (136.2 ml per 2 g waste, equivalent to 68 ml $CH_4.g^{-1}$).

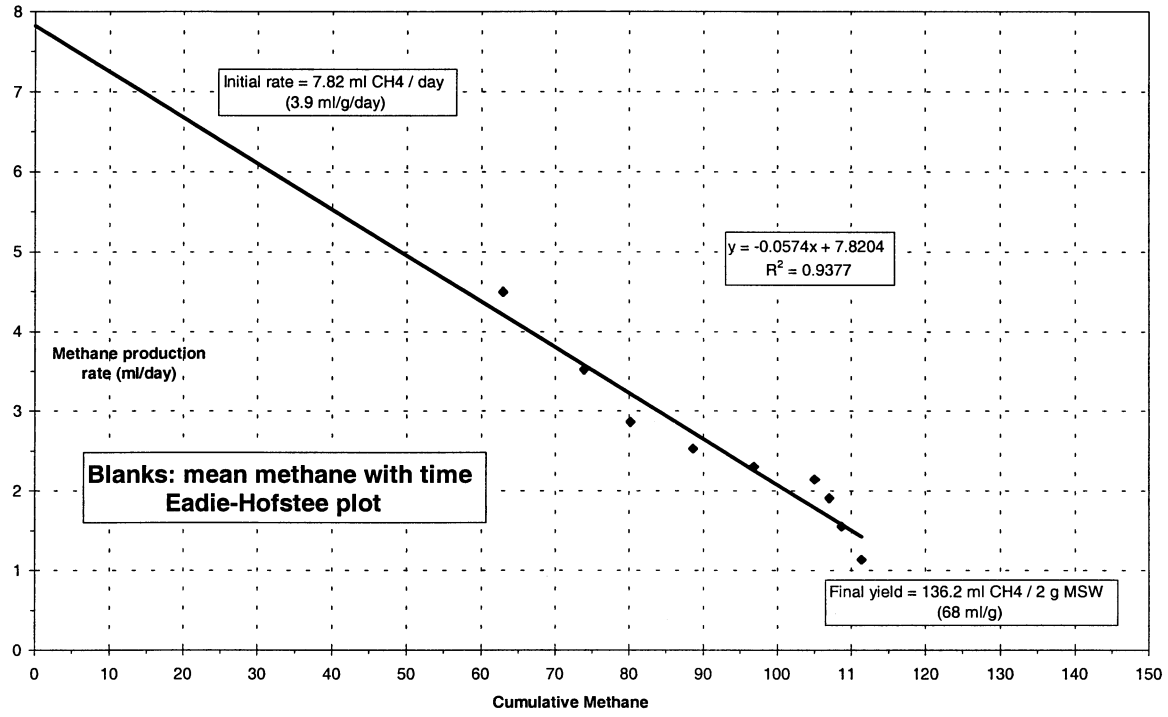

Figure 32 Cumulative methane over time; Eadie-Hofstee plot

The predicted final methane yield was low. Pure cellulose should yield more than 400 ml/g, but a figure nearer 200 ml/g is considered more typical for fresh household waste. The material used for the test was partially-degraded waste from a part of Calvert landfill site placed at least 8 years previously, so would be expected to give a lower yield, although it had been sorted to remove visible non-putrescibles.

This highlights the inherent unpredictability of landfilled waste, and may make the use of landfilled waste as a feedstock questionable. In practice it may prove necessary to check batches of waste before use to ensure that they still contain adequate degradable carbon concentrations. Alternatively a synthetic feedstock could be devised (see section 4).

4 DISCUSSION

4.1 Outcome of simplification study

The feasibility of making both technical and procedural changes to the L-ABT system was investigated. In general it was found possible to simplify the test without altering the fundamental principles or outcome of the method. The major technical changes were to use:

- a seed culture derived from landfilled household waste;
- addition of shredded household waste to the liquid in the test bottle;
- omission of some trace elements and all individual vitamins from the medium.

The most significant procedural changes were:

- boiling reagent solutions rather than requiring absolute sterility;
- combining all the reagent preparation and operations into one step;
- the use of doubling dilution techniques to achieve greater accuracy;
- the use of logarithmic-linear plots to improve interpretation;
- the introduction of an IC_{50} value to aid interpretation, for that concentration of toxin which halves the methane production rate;
- simplified determination of gas quality (only methane concentration is required);
- measurement of an 'acute toxicity' value (IC_{50} or IC_{100}) as early as 7 days;
- comparison of an 'acute' (7-day) and an 'adapted' (28 days+) value.

4.1.1 Technical changes

Source of seed culture

The use of landfill solids and a natural consortium of anaerobic organisms derived from the same landfill made the test in principle more representative of the landfill environment into which materials will be disposed. The use of aged landfill to provide a functioning consortium of micro-organisms was suggested in order to make the test more relevant. This proved to have other important benefits, in that:

- these heterogeneous cultures appeared to be more robust and easy to grow and maintain in the laboratory than the liquid suspension cultures derived from sewage; and
- use of a pre-adapted culture allowed one-step testing when required, with no appreciable delay due to a lag-phase.

It should prove convenient for operators to set up cultures using material taken from the site of a proposed disposal. Although this may make it difficult for different operators to compare results, it will ensure that the results they get will relate to their particular site. Wider testing of the method will show whether site to site variability is a real problem.

Aged waste from a particular location in an operational site is probably a more reliable and reproducible source of microbes than locally-obtained sewage sludge, and so is more likely to

provide a stable population when the seed culture need to be re-established. Furthermore, sewage probably does not contain representative methanogenic species. The difference of at least 2 orders of magnitude in IC_{100} for TCE between the sewage-derived L-ABT and landfill-derived O-ABT may be evidence of different species sensitivity, or may be the result of the presence of landfill solids.

Maintaining landfill-derived organisms on solid waste in the defined inorganic test medium also ensured that organisms were pre-adapted to test conditions, so that tests could be performed in one step at short notice. However, the landfill-derived seed culture appeared to lose activity at 4 to 5 months. In this study the seed was first used for tests at 2-4 weeks after establishment, and was stable for at least 3 months. The simplest approach may therefore be to re-establish a fresh culture every 3 months or so.

Alternatively one could examine use of a synthetically formulated 'waste', to try and avoid accumulation of recalcitrant or toxic materials. Minton, Treharne and Davies have apparently had some success with this strategy for a Biological Methane Potential test (personal communication). This may be feasible for the ABT, either in the seed culture or in the test itself, although we would caution that any move away from use of real landfilled waste materials may make the test less representative.

Composition of heterogeneous media

The addition of a small amount of landfill solids to the liquid test system probably served several purposes:

- it provided fermentable carbon in a realistic form;
- it provided a range of inorganic trace nutrients; and
- it created a micro-environment in which surface-dependent processes (such as biofilm-formation and toxin adsorption) could occur.

The first of these factors was verified by the results. In principle the 2 g waste per 100 ml medium per test should have provided approximately the same available carbon as the soluble substrates added to the CAMR version, and in practice the methane yields were similar.

The second factor was not tested rigorously, but empirically appeared to be the case. The simplified O-ABT gave very similar IC_{100} values for phenol and cadmium as the carefully defined medium used by CAMR in their L-ABT, while the final methane yield from toxin-free blanks was very similar to that obtained by AEA under optimised conditions[2]. This has had a direct advantage in allowing reduction of the range of individual vitamins and salts which need to be included, allowing exclusion of a number of expensive or toxic compounds without affecting the test outcome.

On the precautionary principle, CAMR had used a complete defined anaerobic medium to maintain the fastidious liquid-phase culture derived from sewage. It was anticipated that the rather more robust landfill culture would tolerate a less comprehensive range of defined medium constituents. Expensive chemicals or inconvenient scheduled poisons such as selenium were therefore omitted from O-ABT-1, and it subsequently appeared that the

organisms may tolerate complete omission of the trace element solution when household waste solids are present.

Any advantage derived from the presence of a solid surface during the tests was unverified. However, the proposed format using solids suspended in liquid offers the potential advantage of having a liquid to disperse reagents and carry specified nutrients while also providing some solid surfaces with which both organisms and toxins can interact. Although this arrangement does not truly simulate the conditions in the landfill, it is more representative than the liquid-only system, as the solids probably provide some buffering and protection to the microbial population.

The optimum ratio of solids to liquids was not specifically determined, but could be important. One could determine the significance of these factors by:

- adding sewage organisms to sterilised landfill solids; and
- using landfill organisms in the absence of solids.

4.1.2 Procedural changes

Media sterility

An important criterion for the practicability of the test is how easily it can be performed in a routine landfill laboratory. Dedicated microbiological laboratories have autoclaves and sterile areas, and staff trained in aseptic techniques. The L-ABT methodology was devised in this specialist environment and in the simplification process we have tried to remove non-essential features.

Given the source of the inoculum and the further deliberate addition of wet household waste solids there seemed no need to take precautions to exclude minor sources of organisms, dust etc. The main concern was to prevent cross-contamination between old and new test bottles, for example in case a toxin-tolerating methanogenic population developed. Consequently, it has been possible to simply boil reagent solutions to kill any methanogens or other non-sporing contaminants, and then use them immediately. This avoided expensive and time-consuming autoclaving or micro-filtration, and at the same time achieved partial de-gassing of the solutions.

However, if in practice it is more convenient for an operator to pre-prepare and store solutions, for example if tests are performed infrequently, sterilisation may still be needed to prevent deterioration. We consider boiling to be the minimum requirement, since in principle without it contamination with toxin-resistant methanogens or other undesirable organisms could occur.

The inoculum culture also seems sufficiently robust such that no special precautions were required to limit contamination from other organisms when removing or adding suspensions. Although it was possible that the observed loss of activity over time was the result of contamination, for example with out-competing contaminant organisms, replacement with a fresh culture every 3 months would solve the problem economically. Therefore no special

aseptic precautions are required, and the vessel can simply be opened and closed normally when adding feedstock or withdrawing old culture.

The main factor was to operate quickly, in order to minimise exposure to oxygen as far as possible, rather than to exclude contaminating organisms. The seed culture seemed very tolerant of exposure, no doubt because of the small surface area exposed to oxygen diffusion and the large volume of biomass. The test bottles however contained much less active biomass and have much higher exposure to atmosphere during preparation, so require more active oxygen exclusion. We therefore employed and recommend the use of nitrogen-sparging of media prior to sealing test bottles.

Minimising operations required

The CAMR L-ABT method allowed the cultures to adjust after each change in conditions or addition of reagent. This minimised the risk of the culture failing and maximised the gas yield, but required a 3-step process which was labour-intensive, presented several opportunities for error, and took a long time both to set up and to give results. In the O-ABT we adapted the preparation and used pre-adapted seed to allow all the additions to be made in one operation, and at short notice.

End-point

The ABT has been adapted to:

- use doubling dilutions to span the range of required concentrations, giving a more accurate answer, and
- measure the concentration which gives half inhibition of methane production, as well as complete inhibition.

The accuracy of the ABT depends to a large degree on the spacing of dilutions employed. The between-bottle variability is low, of the order ±5%, but because the interval between dilutions was large, so was the error on the estimated IC_{100}. We expressed the IC_{100} in the form "between x and y ppm", where x was the highest concentration showing methane, and y was the lowest concentration showing no methane; for the original 10-fold dilutions this meant that y=10x, while for doubling dilutions y=2x.

The true value lies somewhere in the range of x to y. With the repeated tests on phenol, for example, the true value seemed to lie close to 2560 ppm - because in some repeats the extinction point was below this value, in others above. A practical interpretation of this is that a difference of one dilution should not be regarded as significant and therefore one cannot regard another result as being 'different' until it is at least two dilutions away, i.e. for doubling dilutions a quarter of, or four times greater than, the initial value. Even with a perfectly reproducible system this must be the case, so the easiest way to improve accuracy would be to decrease the interval between dilutions. However, this would increase the cost of the test. An optimum cost-benefit for the ABT has yet to be determined, and cannot be attempted until the industry and regulators have agreed on an acceptable level of accuracy.

The estimation of an IC_{50} value in principle should have given a clearer end-point than trying to measure the IC_{100}. This is because complex living systems usually show a sigmoid dose-response curve to toxins, with indeterminate head and foot but linear mid-section. However, in practice, the IC_{100} as used by CAMR gave quite clear results in most circumstances, and under the modified AEA protocol the IC_{100} gave a stable reliable result at as early as 7 days. Only TCE showed the anticipated sigmoid response, for which the IC_{100} alone might therefore underestimate the sub-lethal but inhibitory effects at lower concentrations. The IC_{100} is therefore probably the best method for routine use by operators, to screen samples quickly for acceptability, not least because it's determination is straightforward and unambiguous.

The IC_{50} incorporates information from more data points so is perhaps more useful for scientific comparisons and research purposes, such as comparing the 7-day 'acute' and 28-day 'adapted' values. It may also prove to be the more appropriate method for interpreting follow-up detailed tests by operators, or for use by independent quality assurance laboratories.

Care must be exercised in interpreting the IC_{50} values in the same way as that used for measuring toxicity in higher animals. In an LD_{50} test half the animals are killed. In the ABT test the microbial population may be either killed quickly (at high concentrations) or merely inhibited (at sub-lethal concentrations). This will also depend on whether the toxin in question is bactericidal (lethal) or merely bacteriostatic (preventing activity, but non-lethal). In the latter case, the organisms may thrive again once the toxin has become adsorbed or degraded, such that gas production could eventually rise to the same or higher levels (if degradable) than in the uninhibited culture.

The IC_{50} value will therefore vary with the time over which the estimate is made, eventually approaching the IC_{100}. This was clearly observed on several occasions. For operators this is an unnecessary complication, but for researchers it could prove a useful tool.

This further emphasises that the ABT is a comparative test, which although incapable of absolute measurements, is capable of useful relative measures, including measuring adaptation over time and identifying synergistic or antagonistic reactions which could not be predicted by chemical analyses. However, it would be unsafe to use the inhibition values from the test to directly estimate absolute values for inhibition in the landfill. Absolute calibration of the test will require data from solids-based testing, and possibly from real landfill. Ongoing work on a related solids-based test by Minton, Treharne and Davies has for this reason been linked to our work, and in due course they will be reporting on the same test toxins.

Meanwhile the best way to standardise results is probably by comparison to a known defined toxin tested in parallel, such as phenol (see section 4.2.3. Interpretation).

Acute and 'adapted' toxicity measurement

In the simplified version of the test, the culture proved sensitive to additional inhibitory influences during the first 7 days of the incubation (e.g. variation in seed culture 'health'), but the IC_{100} seemed to be stable and reliable as early as 7 days, at least for cadmium and phenol. However, some of the industrial wastes tested by SME seemed to give a clearer result at 14 days. Measuring total inhibition at about 7 days to give an acute toxicity value could be used to quickly screen out the clearly benign materials (i.e. completely non-toxic at 1%) from those

requiring closer examination, and continuing incubation to 14 days for confirmation in borderline cases.

Since the short-term test was always very sensitive, the IC_{50} value obtained in the 7-day acute test will be significantly lower than the IC_{100}, perhaps lower than current co-disposal practice deems reasonable. Operators would be concerned that the results of an acute O-ABT might be interpreted as suggesting that a particular material would have an adverse effect, even though in situ in the receiving landfill there would probably be no discernible impact.

The IC_{50} inhibition value at 28 days may give a better indication of the impact of toxins after the microbial population has adapted to the initial impact, but the apparent toxicity will still probably be higher than the long-run effect in the real landfill, if only because of the much higher proportion of absorptive solids in situ. This 28 day 'adapted' value - once calibrated against solids-based tests - could however be of more relevance to landfill operations, since it would not include very short-term effects (which may be irrelevant) in the context of waste disposal.

It may therefore be possible to categorise materials into those which showed no effect at 7 days (the acute test), and could be passed for co-disposal, and those which showed an acute effect at seven days. This latter category could then be further subdivided into:

(a) wastes which when loaded undiluted, but with care taken over rate of addition, would present no risk to the landfill;
(b) wastes which could not be accepted without either further treatment or dilution;
(c) wastes too toxic to be accepted.

This system would depend on pre-determined agreed trigger values of the ABT. These could, in principle, be determined in carefully controlled field trials, although little information can be obtained in the context of this work to predict the efficacy of this.

The change from 'acute' to 'adapted' IC_{50} inhibition value (e.g. comparing at 7 and 28 days) might also prove be a measure of the relative importance of adaptation and deactivation for a particular toxic mixture. This will require confirmation by comparison with more realistic solids-based test systems.

Scale of the assay

The present study used an array of over 50 bottles for each test. In operational situations, the unknown material would probably need to be tested alongside the most relevant benchmark material (e.g. phenol or cadmium). Hence, the full O-ABT method would demand 100 bottles and at least 100 gas measurements to obtain a single 7-day IC_{100} value (200 measurements if repeated at day 14). In practice however, once the performance of a system had been established, the benchmark material might only be required as an occasional quality check (e.g. weekly) to ensure that no significant changes had occurred.

It is envisaged that the test material could be initially be screened undiluted, at 1 ml in 100 ml of medium, using a simple one-off test (in triplicate) to gain a pass/fail result in 7 days, rather than as a series of dilutions. Only if this screening test proved toxic would a dilution series be

required. Hence the operator's wish for a simpler protocol could possibly be developed involving no more than 3 bottles for each test material, plus a positive control, and giving an acceptance result in as little as 7 days.

Gas measurement

Measurement of methane alone seems to be adequate to provide a useful result. Multiple estimations of the gas in each bottle is a time-consuming part of the test which is not strictly necessary. Increasing the speed with which the GC analysis is performed is possible (e.g. by injecting directly into a Flame Ionisation Detector, with no column), but may not necessarily decrease analytical time requirements unless the time required for measuring the volume and collecting the sample can also be reduced.

The pre-screening version of the ABT could in principle be further simplified to develop a sacrificial test, by simply recording the presence or absence of methane at 7 days in a system challenged with undiluted waste - eliminating both the need for gas volume measurement and, possibly, the need for Gas Chromatography (portable apparatus commonly used by landfill operators for methane monitoring may prove adequate).

Comparison of theoretical and actual methane yield in positive controls showed a large disparity, even given the age of the waste used as substrate. Therefore even after sorting to remove visible non-putrescibles, the milled material was still relatively high in inert material. This could partly explain the erratic and failing gas production in the seed culture observed after 4-5 months; inert material by definition would accumulate in the system. This could be avoided by using a fully-degradable substrate, e.g. paper, but the seed culture would then be less representative of landfill, and the organism numbers and species not necessarily those desired.

4.1.3 Scope of use

The simplified O-ABT method was evaluated by AEA with cadmium and phenol as the main test toxins. Most data are available on phenol, since SME also used this as their internal standard. In the present system, trichloroethylene was not considered a suitable reference material because of it's low water solubility and high volatility. Either cadmium or phenol are relatively predictable toxins which are easy to dispense and could be used as a benchmark against which an unknown material could be evaluated. The data from the simplified O-ABT method are fairly consistent with the results obtained from the CAMR L-ABT method (Table 4). However, comparison of AEA and CAMR results from the work with TCE illustrates that the inhibitory effects of a particular compound may differ depending on the source of the microbial consortium used in the test.

The O-ABT method could either be more directly related to the likely performance of the target landfill (for example, by stipulating that seed organisms and waste feedstock are taken from the target site) or standardised for universal use (by using standardised feedstocks and reference organisms) - but not for both. Using substrate (solid waste) and seed organisms from the target landfill should give more relevant results for the particular operator, but results cannot then be compared with other landfills. A standardised test, perhaps using a defined substrate and independently maintained seed from a common and controlled source, would

allow compilation of a national database and inter-site comparison, but might reduce the relevance of the test to a particular site. Since there appear to be roles for both, it may now be necessary to develop two versions of the test - a local (for daily use by landfill operators) and a standardised (for use by regulators).

The results obtained show that the principle of the test is satisfactory but also highlight that an assay based on biological systems is inherently less precise than chemical analysis. In particular, although the reproducibility within each triplicate is good (typically ±5%), the state of the seed culture seems to be important to the absolute values obtained.

The effect of the spacing of the dilution series has already been discussed. In principle, to decide on a co-disposal loading rate, the toxicity of any waste could be compared to a standard toxin such as phenol in the ABT. However, expression of toxicity in terms of 'phenol-equivalent' or similar, because of the inherent limitation of accuracy due to the dilution spacing, would require a two-dilution safety margin. For example, using doubling dilutions, one should allow no co-disposed waste to exceed one quarter of it's phenol-equivalent. More closely-spaced dilutions would give a more accurate answer and confidently allow a smaller safety margin, but at greater cost.

The O-ABT at present is site-specific and requires validation across a range of situations. Using waste and organisms derived from the target disposal site will in due course probably show results varying from site to site in tolerance to a particular toxin. If this variability proves to be small then perhaps the test may be universally applied, and site to site comparisons may be made. If the variability proves to be high, then one must either abandon hopes of a national database and of site to site comparisons, or develop a truly universal and standardised method.

Development of a central, nationally standardised method is possible. It would require a common source of organisms (probably based on freeze-dried standard cultures) and degradable waste (perhaps using a synthetic waste-like substrate). Development of a standardisable yet effective consortium would be a major task, since many interacting species are required for complete waste degradation, and many more are probably required before the consortium would respond realistically to toxins. There have been previous attempts to develop a standardised synthetic household waste which might be drawn on, but application to the ABT would also require proof that this material behaved reasonably like landfilled waste in terms of interaction with toxins. Even where standardised materials were used there could still be a drift in the sensitivity of the consortium during culture under the slightly differing artificial conditions likely in different laboratories. These problems are significant but may not be insurmountable, and in principle a universally applicable test, broadly analogous to other biological tests such as the Biochemical Oxygen Demand (BOD), could be developed.

Our conclusion was that the O-ABT should currently only be used as an indicator for the order of magnitude of toxic effects, and that absolute blanket threshold values of the IC_{100} cannot yet be set. Inter-site comparisons are now required to validate the method before wider use, but the method itself should require little or no further modification before implementation by operators as a routine monitoring tool. It's use in regulation is more questionable; we believe the test may prove to very site-specific in it's current form, will certainly require wider comparative trials to demonstrate it's reproducibility, and may require further development to

produce a standard test for global use. The Industrial Advisory Group was particularly concerned that this operational test should not be used for regulatory purposes. The IAG thought that basing regulation on an inherently variable and local condition-dependent bioassay was neither necessary nor desirable, since individual toxins can be measured more accurately and more reproducibly by specific chemical assays.

The operational system developed here does not intentionally measure the biodegradation of organic toxins, another perhaps unrelated requirement of operators in the draft Waste Management Paper 26F. Biodegradability is a general characteristic of a compound which is best measured under standardised (probably solids-based) test conditions rather than monitored in a site-specific operational test, and for which a national database could eventually be assembled. However, although beyond the current scope of this work, the new test method has shown evidence of:

- adaptation (e.g. the increasing IC_{50} concentration over time), and
- biodegradation (e.g. enhanced methane production at sub-lethal doses of some organic materials).

These effects can apparently be accurately measured in the O-ABT, which could therefore prove to be of use in adaptation studies or in biodegradability testing.

4.2 Application of the Test in Practice

4.2.1 Scope for use

The O-ABT described here is best suited to use by operators to assist in decision-making for the co-disposal of industrial wastes. Operators already test wastes offered for co-disposal, and have other information on the waste in question, for example:

- the source and nature of the process producing the waste;
- chemical analysis (on specific analytes, not always a complete picture);
- physical and other data, e.g. pH, water content.

The operator will also have relevant site-specific information, such as the loading rates and cumulative burden of other similar and different waste streams, the rate of addition of other wastes, and the volume/age of the target landfilled domestic wastes.

In practice the results of the O-ABT will be part of, and augment, this overall picture. The O-ABT cannot stand alone. For co-disposed wastes containing a single well-characterised toxin the O-ABT will add little and may be unnecessary, but for new and complex mixed wastes - with unpredictable synergistic or antagonistic interactions - the O-ABT could prove to be very valuable.

Chemistry is often incomplete, because analysts cannot routinely check for every known compound, so are guided by their expectations. The O-ABT could also be used to cross-check that the toxicity is close to that expected from chemical analyses, perhaps thereby providing the only indication that the chemistry is incomplete. Further chemistry could then be

performed to identify and quantitate the unknown toxins, or more pragmatically and inexpensively the loading rate could be based on the O-ABT rather than the chemistry.

The method described here has so far only been applied to liquid wastes. Slurried solids should not present a great problem, but solid wastes could present difficulties, particularly where the waste is heterogeneous at a macroscopic scale. This is a problem already encountered by operators when attempting to take representative samples from such wastes, and the same principles apply. The best principle is probably to take large primary samples and then homogenise these to take smaller sub-samples. Another possibility may be to leach the waste in a standard way, and test the leachate. These issues are best addressed when validating the method in operator's laboratories.

4.2.2 Assay format

After discussion within AEA, then with SME and other members of the IAG, the following format is suggested.

The test could be used in two stages:

1. A simple qualitative 7-day, 3-bottle test, giving either a 'safe' or 'toxic' answer for the undiluted waste stream at low cost.
2. For materials designated 'toxic' after stage 1, determination of the IC_{100} in a dilution series, comparing against a standard such as phenol, reading the preliminary result at 7 days and confirming at 14 days.

The test is best seen as a 'worst case' acute toxicity test. Many toxic materials either degrade or are lost to adsorption in the longer term, rendering results from prolonged incubation questionable and potentially misleading. As a 7- or 14-day test based on total inhibition of methanogenesis, it gives a clear result, showing reasonable sensitivity and reproducibility.

4.2.3 Interpretation of assay

The O-ABT is not a simulation of an operational landfill. Although most of the factors affecting methane production will apply in the test bottle, the system is artificial, and some factors will clearly be different. For example, even at the microscopic level the O-ABT takes little account of the realistic distribution of toxins between solid and liquid phases.

At larger scale it becomes more unrealistic still. For example, it does not and cannot take account of full-scale factors such as the zone of influence of a co-disposed waste in a landfill. Eventually it may be possible to calibrate the test against solids-based laboratory tests and/or full-scale landfill studies, but until then the O-ABT can only give relative, comparative information.

In the absence of absolute data the O-ABT must be expressed in relative terms, and one way to do this is by reference to well-characterised benchmarks, e.g. to express the result in relation to a standard toxin such as phenol, expressed as 'phenol-equivalents'. The test could then be used most simply to ensure that no loading rate for a new toxic waste stream ever

exceeded that already permitted for phenol. Expression as 'phenol-equivalents' could also assist long-term landfill management, for example:

- to estimate the cumulative toxicity in a site accepting many toxic streams over many years;
- to determine maximum acceptable loading rates for biodegradable toxins;
- to determine net synergistic/antagonistic effects in common complex mixtures; and
- underpin development of a national database of toxicity values for common mixed toxic streams.

Toxins will generally be adsorbed or detoxified in association with the solids component in a landfill. The O-ABT has a much higher liquid:solid ratio and so might be expected to over-estimate toxicity (although this remains to be proven). In addition, a full-scale landfill would be expected to show other effects not modelled in the O-ABT, for example longer-term recolonisation of acutely intoxicated parts of the site. Minton, Treharne and Davies are currently involved in developing a solids-based test which it is hoped will address these and related issues.

In general, since a landfill would offer more opportunities for adsorption of toxins, and offer relatively isolated micro-niches of protected methanogens, the test must err on the side of environmental safety. That is, actual toxic effects in-situ will be less severe than seen in the O-ABT. Furthermore, since the 7 day test measures acute toxicity, the safety margin is increased, because processes such as detoxification and adsorption would in practice allow greater concentrations of most toxins to be safely co-disposed long-term. However, until the method has been calibrated and validated, the test will be unsuitable for setting nationally-applied loading rates.

In terms of relative comparisons the test is already useful. It is understood that SME have already run the test on several liquid wastes for their local Environment Agency office. In it's simplest form the test could be used to test any unknown waste for toxicity to anaerobic systems. For landfill the simplest application is to test a waste at the anticipated loading rate, to give a straightforward qualitative pass or fail answer.

In its present format we recommend that no more than 1 ml of liquid waste per 100 ml test medium is used. In a full-scale landfill, loading rates will often exceed this at the point of addition, and over a limited volume of household waste in the vicinity, so if a more absolute interpretation of the O-ABT is required, field work will be necessary to calibrate its response against that observed in-situ.

4.3 Practical & Financial Aspects of Operational ABT

The test as described in the SOP is still relatively time-consuming, with a typical standard or toxic waste plus controls requiring approximately 4 hours to set up, then 2-3 hours per week for monitoring. At the peak of activity in this study SME had 8 test batches running in parallel, demanding 3 man-days per week.

Capital costs will vary. Assuming major items such as the gas chromatograph are already in place, the costs of a suitable incubator, glassware and shredder are probably no more than £1,000. Purchase of a GC solely for this test would not be cost-effective, so operators without

a GC would have to submit the full test to a laboratory set up to do the work. However, it may prove possible to do the simple 7-day qualitative test using methane-monitoring equipment common to landfill sites.

On-going costs comprise staff time, media, consumable equipment and standards (toxins for the test and gases for the GC). For a single test waste at a time this equates to approximately £400 per week, but economies of scale apply - with 8 batches running in parallel, the cost fell to approximately £125 per test waste per week.

Some training would be required. The essential knowledge can be imparted in one day or less, but implementation in practice for staff with no previous microbiological experience would initially require close supervision, tailing off as experience grows. In this respect SME were perhaps not typical; their laboratory manager already had a good grasp of microbiological principles, and did not require explanation of fundamentals. Greater initial training and ongoing supervisory effort during start-up must be anticipated with less experienced staff.

Phenol-equivalent interpretation

The recommended ABT Standard Operating Procedure requires that a standard is always run in parallel. Phenol is suggested as it is a reference compound for chemical analyses, and already has a legally-defined and reasonably well-founded loading rate. A heavy metal such as cadmium could be used as well for toxic streams in which inorganic materials were dominant. Results can therefore be expressed in 'phenol-equivalents' or similar, by expressing the phenol IC_{100} as a ratio to the test waste IC_{100}.

A strict interpretation of the IC_{100} gives a range, since the true value usually falls between two dilutions; the last showing some methane and the first showing complete inhibition. To simplify interpretation we suggest that the working IC_{100} is taken to be the lowest concentration of waste causing complete inhibition.

To give some examples:

- in the present study, phenol gave an IC_{100} of approximately 3000 ppm;
- cadmium gave an IC_{100} of approximately 1000 ppm. Cadmium therefore had a 'phenol-equivalent' of 3000/1000 = 3.0, i.e. was approximately 3 times more toxic than phenol;
- TCE gave an IC_{100} of 640 ppm, so TCE had a 'phenol-equivalent' of 3000/640 = 4.7, i.e. was approximately 5 times more toxic than phenol.

Note the careful use of 'approximate'. As previously discussed, results must be at least two dilutions apart to be regarded as significantly different. For doubling dilutions this implies a factor of 4. Therefore TCE probably is significantly more toxic than phenol, but the apparently 3-fold higher toxicity for cadmium may not be significant.

If this approach is adopted, it may expose any inconsistencies in the current chemistry-based regulatory limits. Given the lack of absolute calibration and inherent margins of error in the O-ABT, the safest course would be to adopt the lower loading value when chemistry and microbiology differ significantly. We must emphasise that care is required because the IC_{100}

values are only accurate to ± one dilution, but the principle appears to be sound. For known pure toxins existing loading rates could be retained, or the ABT rate taken (whichever is safer, i.e. lower). In the case of uncharacterised or complex waste streams this approach could be used to rationally set a safe loading rate.

In the absence of better information on long-term effects, it could also possibly be used to safely set the total acceptable toxic load entering a landfill over it's lifetime, for example by summing the 'phenol-equivalents' of all co-disposed wastes entering that site. This might however impose unduly harsh limitations on co-disposal, since the ABT result makes little allowance for protective phenomena such as adsorption and biodegradation of toxins.

Comparison of ABT with other measures

Although the O-ABT requires wider testing and validation, it is interesting to provisionally compare our results with other published information on the three test toxins:

Material	AEA O-ABT IC_{100} (ppm)	[1] CAMR L-ABT (ppm)	[2] WMP 26F	[3] Operators (ppm)	[4] Acute rat LD_{50} (ppm)
Phenol	~3000	~1000	5 ppm/day	~200	317
Cadmium	~1000	~1000	10 ppm	~200	300
TCE	~640	~100	'ABT'	-	3670

[1] From CAMR report Tables 1, 3 and 11 [(2)].
[2] From draft WMP 26F Table 5.1 (note - 'ABT' recommended for TCE)
[3] From IAG, current typical working maxima
[4] From National Chemical Emergency Centre database

A lower concentration implies a higher toxicity. Phenol and cadmium are given similar toxicity by all sources, although the precise values differ slightly. Given that we expect the acute 7-day O-ABT to be more sensitive than landfill in-situ, both the draft WMP 26F guidance and current operational practice can be seen to be erring on the side of safety. If a similar pattern is found for other toxins then it may be possible to add a generous safety margin to interpretation of the O-ABT IC_{100} value without unduly affecting operational practice.

TCE is more interesting; it showed the highest toxicity of the three test toxins in both our system and CAMR's, but has the lowest toxicity in acute tests on rats. Clearly the mode of action of TCE differs from the other toxins, which might also explain the different dose-response curve seen for TCE in our experiments. However, the O-ABT gave a clear result.

The O-ABT is not suitable as a gate acceptance test. Even in revised form it takes at least 7 days to produce a result, so it is not practical to keep lorries waiting or even to unload and store consignments of waste while results are awaited. The O-ABT may prove to be very site-specific, while the ideal compliance test would be standardised and applicable globally. Such a test could possibly be developed, but more work is required to do so. A site-specific test could be of use to regulators in setting loading rates for a particular site, but if this were implemented it must be clearly understood that the rates so derived cannot be applied to any other sites.

In principle one could perhaps devise a more rapid test, in which for example an actively gassing system was challenged with waste and the effects monitored. However, we feel the test is a major advance on previous tests taking one to three months (or more), and is now capable of giving results in a time-frame similar to other widely-accepted tests such as the Biochemical Oxygen Demand test.

The O-ABT should prove to be a useful acceptability test. Used in simple 'pass or fail' screening mode to test the actual loading rate being employed for a particular waste, it would in 7 days provide either a 'pass', giving extra confidence that no harm could result, or 'fail', alerting the operator to either discontinue co-disposal of the material, or to pay closer attention to further batches of the same material. For such 'fail' materials it could also be used in extended (serial dilution) mode to decide on a lower, safe loading rate.

Related ongoing work by Minton, Treharne and Davies is examining and developing a solids-based test in parallel. They have agreed to test the same standard toxins as we did, over at least the same range of concentrations, so that our results can be compared. The solids-based system should in principle be much more representative of landfill (although this must be demonstrated). We expect to find a solids-based system tolerant of much higher concentrations of toxin, but it is hoped that this effect will prove to be constant and predictable, so that a correction factor can be applied to the O-ABT results to predict effects in the solids-based system (and hence in landfill).

5 CONCLUSIONS

1. A version of the liquid-based anaerobic bioassay test has been developed which accurately reproduces the scientifically rigorous method published by CAMR[3]. The revised method is sufficiently simplified and robust to implement in a landfill operator's laboratory.

2. The modified test gives results quickly; the optimum incubation time is 7-14 days. Longer incubation may be required (up to 8 weeks) to produce a stable inhibitory value, but processes such as adsorption and biodegradation in the system render this result questionable.

3. The most reliable and practical end-point is complete inhibition of methanogenesis: the IC_{100}. The concentration causing 50% inhibition (the IC_{50}) is more sensitive, and changes over time as toxins adsorb or degrade; this may be a useful research tool, but would only add difficulty in a practical on-site test.

4. The test shows good reproducibility (of the order ± 5% to 10% between triplicate tests). The result is however limited by the doubling dilution series used; more closely-spaced dilutions would give a more accurate answer, but at greater cost.

5. The test gives good comparative information. Calibration against results from solids-based tests is underway, and validation in operational landfills will be required in future. It is therefore a useful additional tool for landfill operators.

6. The test probably 'fails safe' using the IC_{100} as the end-point. The liquid system is less adsorptive and less buffered than an operational landfill, so should over-estimate the acute toxicity (to be confirmed by comparison with the solids-based test).

7. The test measures total toxicity. In complex mixed waste streams the overall toxicity cannot be predicted from the chemical composition because of unknown synergism or antagonism. It is therefore an important additional test.

8. The test may be used in several ways:

   - To provide a simple qualitative 'yes' or 'no' answer to the question 'is this waste toxic at the loading rate envisaged?' If there is no inhibition at the maximum concentration to be experienced in the landfill, material can be confidently co-disposed;

   - Where a material shows some toxicity, to quantitatively determine the inhibitory concentration, allowing appropriate adjustment to loading rates;

   - To cross-check conventional chemical analyses. If the measured toxicity is orders of magnitude higher than that predicted from the chemistry, the chemistry is probably incomplete, and more thorough chemical analysis may be required;

- To provide comparative toxicity information. Phenol was the 'de facto' standard used here, although cadmium might be more appropriate for inorganic waste streams. New toxic streams could be expressed in terms of their 'phenol-equivalent', to estimate the maximum permissible loading-rate for that particular stream, and the total toxic burden introduced into a given landfill over it's operational life;

6 RECOMMENDATIONS

Three types of activity are suggested:

1. Validation of the test.
2. Further development of the test.
3. Interpretation and application of the test in practice.

1. **Validation**. So far the test has only been applied by one operator, to one source of landfilled waste on one site. Wider testing is now required:

(a) To examine the general acceptability of the Standard Operating Procedure. Shanks and McEwan are now happy with the test as appended. However, the SME laboratory and personnel may not be typical, so wider application, perhaps to members of the Industrial Advisory Group, would help to identify any further implementation problems.

(b) To measure the effects of other landfilled wastes on seed culture and test performance. Solid wastes from other sites may not behave the same way as Calvert waste, potentially giving rise to differences in seed culture behaviour or even test performance. This would tend to make the test more site specific.

(c) Blind ring-testing should be performed, submitting to several operators a number of centrally-prepared toxic materials (both pure standards and real toxic wastes), in order to establish the true reproducibility of the method. Only one material should be fully identified; we suggest phenol, so that the suggested standardised method of interpretation based on 'phenol equivalents' can also be tested.

2. **Development.** Although we believe the ABT is now fit for purpose, and should require little if any further development as an acceptability test, it could also be used as the baseline from which to develop further tests:

(a) Further simplification, aiming to produce an even faster yet robust 'pass or fail' test. Possibilities include a simpler qualitative 'presence or absence' method for methane detection, or a colour-based method for (black) sulphide detection. In principle in an actively methanogenic culture one could challenge and obtain toxic effects almost immediately, but it is not clear how these could be measured.

(b) If ring-testing shows great site-to-site variability then it may be worthwhile trying to develop a standard version of the test, probably based on organisms from culture collections or maintained in a central laboratory, and a standardised or synthetic 'solid waste'.

(c) The observed changes in IC_{50} over time suggests that there may be applications for the test in adaptability testing, which would be useful to estimate the degree of importance of adaptation to toxins in landfill. Adaptation implies higher acceptable

loading rates, but this would also need to be related to long-term leachate quality.

(d) The observed extra methane yield with some organic materials suggests that there may be applications for the test in biodegradability testing. This is important both in assessment of the acceptable lifetime loading for a particular compound (which may be infinite if totally degraded), and by implication may help identify compounds and define acceptable loading rates for materials which can be biologically treated in this fashion.

3. **Implementation**. In practice there is still some work to be done in interpretation:

(a) How does the test relate to solids-based results and to real in-situ effects? A comparison with the results of Minton, Treharne and Davies' test should be made, and this in turn must be correlated with either literature or experimental observations of methanogenesis in landfill exposed to toxins.

(b) Are the results reproducible? If the test proves to be reproducible form site to site - or can be adapted to make it so - then collection of the data and production of regional or a national database would be useful, to collect statistics and document co-disposal, and to form a national information resource.

7 REFERENCES

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APPENDIX 1

DISCUSSIONS OF INDUSTRIAL ADVISORY GROUP

Martin Meadows *	ETSU on behalf of the Environment Agency
Rob Kate *	Cleanaway
Terry Andrews	Cleanaway
Nygel Thomas	Cleanaway
Andrew Maule	CAMR
Patrick Pointer *	Shanks & McEwan
John Stoddard *	Shanks & McEwan
Andy Childs *	Shanks & McEwan
Peter Clarricoats *	Cory Environmental
Alistair Brace *	Cory Environmental
Robert Hamilton	Caird
Theresa Davies	BFI Wastecare
Carolyn Stone	Biffa
Martyn Jarvis	Biffa
Steve Roscoe	Grundon
Chris Harries	Minton, Treharne & Davies.

* attended both group Meetings

Recommendations of the Industrial advisors

The technical details of this study were presented to the Industrial Advisory Group. A draft protocol for the O-ABT method (Appendix 3) was discussed. In general they endorsed the simplified protocol and agreed to the points listed below.

a. Using landfill inoculum and adding landfill solids was an important improvement. The issue of the source of the landfill material to be used by all operators was unresolved but it seems likely that the material from the proposed disposal site would be best. Nevertheless a general material should also be available, at least for occasional users, and it would be prudent to adopt standard materials for any test to be used by regulators. ***Decision: continue to use locally-derived landfill materials as seed and feedstock.***

b. The amount of solids in the test bottle could be further optimised to increase gas yield and the liquid:solid:headspace ratios could be changed. The gas production should be adjusted to make best use of the standard bottle size. ***Decision: the present arrangement gives an acceptable result so leave unchanged.***

c. The use of landfill solids and omission of many reagents is an improvement. The main cost is associated with making up the solutions rather than material costs. Getting rid of one whole reagent preparation (e.g. solution C) is more important than removing more individual chemicals. Making the seed culture medium and the test medium the same would save manpower/costs and further reduce stress on the organisms when transferred to

test vessels. Further major simplification may be risky. ***Decision: omit Trace Element Solution C, and make the media the same for inoculum and test. Be cautious over further changes.***

d. The need for de-gassing of the media with nitrogen has been questioned but in the opinion of AEA, CAMR and SME, it would be unsafe to omit this stage. ***Decision: retain nitrogen gassing of bottles.***

e. The present medium has several costly reducing agents and there is scope to change these and perhaps limit to one cheaper agent. Costs of reagents may be less important than time taken making-up and equilibrating solutions so changes here are low priority. ***Decision: fall back to one of the existing reducing agents (cysteine hydrochloride).***

f. The time running the GC is the greatest single use of manpower, even after simplification to estimating methane only. Measuring gas pressure or volume alone is insufficient but there is no need to do a full analysis; only the methane is of routine interest. Running with a empty column and just using the FID output would be fast and simple. The method of estimating gas volume and taking gas samples would be left to the discretion of the lab; i.e. the SOP should not be prescriptive (we favour the rising bubble meniscus and a three-way tap on a syringe, CAMR recommend use of a simple glass gas syringe). Volume measurement will be the next slowest step if direct FID is successful. In routine use sites with a high throughput should probably consider automated volume/quality measurement systems, which already exist. ***Decision: Identify good practice for manual gas sampling during tests at SME.***

g. The measurement of inhibition at seven days is useful in that the IC_{50} test is very sensitive at this early stage and permits rapid safe acceptance of benign wastes (in this respect it fails safe). However, it gives a much lower inhibition value than the IC_{100} test and so should probably not be promoted as the end-point of the test. The concentration at which the culture is irrecoverably killed can also be determined at 7 days, from the IC_{100}. It is an important value for the operator and would probably be the value used in discussion about loading rates etc. The need to have about 50 bottles for a standard curve and 50 bottles for the unknown is a concern to operators (particularly on space). A less comprehensive calibration curve (just to illustrate that the culture was performing correctly) and perhaps an undiluted sample of unknown waste may be appropriate initially, if the total inhibition approach is adopted. ***Decision: continue to interpret both the IC_{50} and IC_{100}, at both seven days (acute) and 28-days (adapted); write the protocol making decisions based on the IC_{100} value at it's earliest stable value.***

h. The format of the final SOP is not crucial since each operator will write it in their company style. ***Decision: continue to use the present format.***

i. There may be better ways of managing the inoculum so that a representative amount of suspension can be removed. However, the present system works and no specific plans for improvements were thought justified. ***Decision: continue with present inoculum management method but be aware of potential to improve.***

Priority for Operational Testing of the method

The Industrial Advisors recommended that SME give priority to two issues in the next stage of testing at Kempston.

a. ***Practicality of running the test at ambient temperature.*** Since it will be a comparative test (unknown against known toxin) this should be satisfactory and would make major savings in lab space (i.e. there would be no need for an incubator for the bottles or a waterbath for the inoculum). However, since we are now into warmer weather, running at ambient will not realistically test for winter ambient, which can be substantially lower than summer in typical on-site laboratory accommodation.

b. ***Practicality of adding real wastes to the test bottle.*** Sample representivity will need to be considered, though the strategy may be no different than that adopted in other analytical methods. Adding liquid wastes should be straightforward and so initial tests need to use these. Based on the figures in draft WMP 26 more than half co-disposed material is solid. The serum bottles are physically too small for samples of some of these wastes, which may also present substantial problems in taking representative sub-samples or making dilutions. Large bottles could not be run in the numbers envisaged in the present protocol (50 bottles). SME need to try a range of real wastes and the scope of this limitation needs to be assessed.

APPENDIX 2

OUTLINE OF METHODOLOGY USED FOR L-ABT AND O-ABT VERSION 1 (DEVELOPMENTAL)

Similar ingredients for the salts solutions are used in both the L-ABT and O-ABT methods.

The protocol was;

1. Make up solutions as described in the CAMR SOP. (Na_2SeO_3 and $AlCl_3$ have been omitted; individual vitamin-addition has been omitted, yeast extract has been increased).

Minerals Solution A [g/l] - K_2HPO_4 2.5

Minerals Solution B [g/l] - KH_2PO_4 2.5, $(NH_4)_2SO_4$ 5, NaCl 5, $MgSO_4$ 1, $CaCl_2$ 1

Trace Elements Solution C [mg/l] - $MnCl_2$ 100, $FeSO_4$ 150, $CoCl_2$ 100, $ZnSO_4$ 100, Na_2MoO_4 10, $CuSO_4$ 25, $NiCl_2$ 20, H_3BO_3 10, Na_2WO_4 10. EDTA was not added (not essential if used immediately). Once a simplified standard solution has been agreed and stock solutions are to be kept for any length of time, it may be necessary to add EDTA again (500 mg/l) to prevent loss of metals by adsorption to glass during storage.

2. These should be brought to the boil to ensure a degree of sterilisation (absolute sterility is not seen as essential for the test, but boiling prolongs shelf-life) and to reduce the concentration of dissolved oxygen. After boiling the solutions should be cooled to < 35°C before use, or sealed and stored at 4°C.

3. Make up whole medium as follows:

Component (make up to 1 litre)	O-ABT	L-ABT
Minerals Solution *A* (ml)	100	100
Minerals Solution *B* (ml)	100	100
Vitamin solution (ml)	-	10
Resazurin (0.1%, ml)	1	1
Trace Element Solution *C* (ml)	10	10
$FeCl_2$ (g)	0.1	0.1
Cysteine HCl (g)	0.3	0.3
Yeast extract (g)	1	1
$NaHCO_3$ (g)	-	3
Na acetate (g)	-	2.5
Na formate (g)	-	2.5
Tryptone* (g)	-	2
Dithiothreitol (g)	-	0.4
2- mercaptoethylsulphonate (g)	0.5	-
Tri-sodium citrate (g)	0.3	-

* Biotal reference to tryptose assumed to be in error, substitute tryptone or trypticase instead.

In setting up the trials the aim has been to keep things as simple as possible. The emphasis is to protect the seed culture from aerobic conditions as much as possible. It is therefore added last to the tests that have been thoroughly mixed.

Each trial bottle should contain 90ml liquid medium as described above. 57 bottles per test type are required so about 5.2 litres of medium is needed for a complete run. Prior to setting up the trials the whole medium should be stored at 35°C.

4. The tests should then be set up using the reagents defined earlier, and toxins added. These should be added as the raw material to the final concentration described (TCE should be added in acetone as shown below the table). The amounts added to each bottle should be as shown below.

Compound	Amount added to each trial				
Cadmium Nitrate (final conc)	3mg (30ppm)	10mg (100ppm)	30mg (300ppm)	100mg (1000ppm)	300mg (3000ppm)
TCE* (final conc)	[1]10μg (0.1ppm)	[2]50μg (0.5ppm)	[3]100μg (1ppm)	[4]500μg (5ppm)	[5]1000μg (10ppm)
Phenol (final conc)	3mg (30ppm)	10mg (100ppm)	30mg (300ppm)	100mg (1000ppm)	300mg (3000ppm)

All bottles should be prepared in triplicate

* TCE can be stored as a stock solution of 1g/10ml acetone at 4°C and serially diluted in acetone as required. Ensure all bottles receive a total of 100μl of acetone, and also prepare an acetone-only control triplicate.

1 = 100μl of 10^{-4}g/ml stock solution.
2 = 50μl of 10^{-3}g/ml stock solution + 50μl plain acetone.
3 = 100μl of 10^{-3}g/ml stock solution.
4 = 50μl of 10^{-2}g/ml stock solution + 50μl plain acetone.
5 = 100μl of 10^{-2}g/ml stock solution.

The inoculum does not require filtering of the gross solid matter out (as per the CAMR method), since any fermentable solids carried over into the test will be the same for all bottles, and small compared to available carbon from the added 2g waste. The inoculum should however be kept gassed with nitrogen before use, to minimise the toxic exposure to oxygen.

The household waste feedstock is prepared by hand-sorting freshly-excavated methanogenic waste to remove non-putrescibles (metal, glass, plastic), ball-milled to < 1 mm, then stored frozen until required.

The sequence of addition to the bottles is as follows:

a) add 2g milled waste per bottle for the modified method;
b) add 90 ml medium and swirl to mix;
c) add 10 ml seed culture and swirl to mix;
c) degas for 5 minutes by bubbling nitrogen through the bottles;
d) add toxic material, swirl to mix, insert and clamp seal, and incubate.

For the first trial we tested three toxins in triplicate at each of 5 dilutions, a total of 45 test bottles.

Additionally we set up:

- triplicate 'positive control' vessels, i.e. controls with no toxin;
- 'solvent control' vessels - as above but with 100ml acetone, to allow subsequent correction of the TCE results;
- 'inoculum-only control' - seed culture but no added shredded waste, to check that the contribution of degradable matter in the seed was negligible;
- 'blanks' - no waste and no inoculum, to ensure that sterility had been achieved and that there were no significant non-biological effects.

In total we therefore required 57 bottles for each trial.

Bottles were then incubated at 37°C, and the total gas volume and percentage methane generated were determined weekly.

APPENDIX 3

STANDARD OPERATING PROCEDURE: REVISED O-ABT METHOD

TITLE:	ANAEROBIC BIOTOXICITY TEST (ABT)	PREPARED BY	J Stoddart LABORATORY MANAGER
LOCATION	CENTRAL LABORATORY	EDITED/APPROVED BY	Barry Croft AEA Technology

1. INSTRUMENTATION AND APPARATUS

1.1 A 5 litre Quickfit culture vessel (Quickfit PV5L) fitted with a 100 mm i.d. flange joint, a reactor lid (Quickfit MAF2/2 or similar), a flask clip (JC 100F) and a means of collecting the gas generated in the vessel, for example, by displacing water from an inverted 1 litre measuring cylinder.

1.2 Syringe needles No 1, 21 gauge, 38 mm long , fitted with a Luer Lock

1.3 125 ml Wheaton Bottles (Pierce & Warriner Cat no 12995)

1.4 Neoprene septa (Pierce & Warriner Cat no 13233)

1.5 20 mm Aluminium seals (Pierce & Warriner Cat no 13214)

1.6 Syringe stopcock (Aldrich Cat no Z18.212.5), adapted to couple up to the existing gas sampling loop on the GC.

1.7 Bubble flow meter, GC type 50 ml capacity.

1.8 50 ml glass syringe fitted with a Luer lock

1.9 A gas chromatograph equipped with a 4.0 metre Porapaq N column, 0.25 ml motorised sample loop and a thermal conductivity cell.

1.10 20 mm Seal crimper and 20 mm Seal Decrimper (Aldrich Cat No Z11/427/8 & Z29/216/8)

Note: Trade names are used for purposes of identification only and their use does not imply endorsement by the Environment Agency, AEA Technology or Shanks and McEwan.

2. RISK ASSESSMENT

NB The bacteriological risk assessment has been made by Barry Croft of AEA Technology.

2.1 The main hazard associated with this technique is assumed to be biological. The chemical hazard is confined to the preparation of the reagents and the small amount of toxins involved. Observe the normal handling requirements for these.

2.2 Since the seed culture and test vessels contain organisms derived from soil and household waste, there is a potential biological hazard, which we assess as no more than Group 2 (*see Categorisation of pathogens, according to hazard and categories of containment. Advisory Committee on Dangerous Pathogens HMSO).*

2.3 This assumes that only aged household waste is used - if fresh household waste is used, or material landfilled for less than a year, there may be a potential for more serious Group 3 or Group 4 obligate human pathogens. Such materials are therefore not recommended. Since the material is anaerobic, no special precautions against aerobic fungi or protozoa are required (i.e. a safety cabinet is not required).

NB *A group 1 organism is defined as "an organism that is most unlikely to cause human disease". A group 2 organism is defined as "an organism that may cause human disease and which might be a hazard to laboratory workers but is unlikely to spread to the community. Laboratory exposure rarely produces infection and effective prophylaxis or effective treatment is usually available".*

2.4 The biological risks are essentially similar to those associated with soil or sewage. The most serious risk is probably tetanus or gangrene (via cuts) and precautions against ingestion and inhalation should also be observed. Only nominated staff should work with the material, who should be free of compromising infection, should not be taking immunosuppressant drugs, and should have current tetanus immunisation. Typhoid immunisation is not strictly required, but would be prudent. Gloves should be worn constantly, and any cuts or abrasions treated and reported, however minor. A paper mask and goggles should be worn whenever vessels are opened. Hands must be washed thoroughly with disinfectant soap after all operations. A strict "no eating, drinking or smoking" rule must be observed.

2.5 The main physical hazard is of fire or explosion if methane is allowed to accumulate in confined spaces. The volumes generated in test bottles are small and contained so present little hazard, but the seed culture generates significant volumes of both flammable and asphyxiant gases, so requires careful handling.

3. REAGENTS (General laboratory grade unless otherwise stated)

3.1 Refuse Fermentation Media

Weigh 2.5 g $(NH_4)_2SO_4$, 2.28 g $K_2HPO_4.3H_2O$, 1.36 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ into a 5 litre volumetric flask. Add 5 ml 0.1% Resazurin (cell culture grade), 0.5 g $CaCO_3$, 0.5 g $Fe_2Cl.4H_2O$, 5.0 g yeast extract and 2.5 g cysteine HCl. Add water (distilled) until the volume is nearly 5 litres and stir using a magnetic follower. Adjust to within the range pH 7.0 to 7.5 with a Na_2CO_3 solution. Bring to the boil and cool rapidly under running water. make up to five litres. Use within 4 hours, discard any of the solution not used within that time.

NB It is recommended that this solution is brought to the boil to sterilise the solution against bacteria that may become tolerant to any toxin that may be used in the test.

3.2 Mature Waste
Some mature solid waste is collected in a large sealed plastic container. Mature in this context means household waste that has been in place for at least 3 years but probably less than 20 years - such that it has been partially but not completely degraded. Storage under anaerobic conditions is not essential provided the material is handled promptly. The material is sorted by hand to remove non-putrescibles (plastic, wood, stones and metal) leaving largely degradable matter. This material is chopped to a small particle size in an Industrial type food processor. The material is then stored in an air tight plastic container in a deep freeze (at approximately -20°C) until needed.

3.3 0.1% Resazurin
Dissolve 0.10g Resazurin (cell culture grade) in water and dilute to 100 ml

3.4 500 ppm Sodium metabisulphite solution
Weigh 1.0g $Na_2S_2O_5$ into a 2 litre volumetric flask and make up to the mark.

3.5 Calibration gas
A calibration gas containing approximately 60% CH_4. The balance of the gas can be made up with CO_2, N_2, & O_2. The gas should have a certificate of analysis.

4. METHOD

NB *Any dispensing equipment to be used in the methods below should be thoroughly washed after use and then sterilised by soaking overnight in 500 ppm sodium metabisulphite solution.*

4.1 Preparation of the Anaerobic Seed

To a 5 litre reactor vessel add some 500 ml "seed" from an active anaerobic seed culture and then make the volume of the liquid to nearly 5 litres with the nutrient solution (3.1). For every 1 litre of medium add 50 gm of household waste (mature solid waste). Seal the vessel with the lid, well greasing the flange and any conical joints. Purge with nitrogen and then incubate at 35°C. Manually shake the vessel daily to disperse the solids within the liquor. Collect the gas produced by displacement of water and record the daily volume of gas produced.

Each week decant 250 ml of the well stirred liquor from the reactor vessel and add 250 ml of fresh medium (see 3.1) and 12.5 g of milled household waste, and purge with nitrogen. Return the vessel to the incubator.

When the "seed" is required for a biotoxicity test then decant the required amount of liquor from the reactor vessel and filter until free of suspended solids. Replace the liquor with Reagent 3.1 and household waste as above. It is recommended that the "seed" should be taken from a starving culture, i.e. a culture that has not been fed for some days

NB *Under the conditions described above, a 5 litre anaerobic digestor can be expected to generate 100 - 1000 ml of gas per day.*

4.2 The Biotoxicity Test

4.2.1 Mark each Wheaton bottle with a unique reference number, and then if required, record the dry weight and the weight of each bottle brim full of water to find the capacity of each bottle.

4.2.2 Weigh 2.0 +/- 0.1 g of milled household waste into each Wheaton bottle, and then add the required amount of toxin (see note below). Add 90 ml of the nutrient and 10 ml of the "seed liquor" by means of a 30 ml & 10 ml automatic dispenser.

4.2.3 Purge each bottle with nitrogen, and then ensure that the lip of each bottle is free of solid material before inserting a neoprene septum, and then crimp on an aluminium cap. Incubate the bottle at 35°C.

NB *Each level of toxin should be prepared in triplicate; a blank of three bottles without the toxin should be run with every test.*

4.2.5 Level of toxin to be used.
If a bottle is to contain 100 ppm of a toxin, then the amount of toxin needed to inoculate each of three bottles is 100 ml x 100 μg/ml toxin; i.e. 10 mg. Therefore 1.0 ml of a 10 mg/ml toxin solution should be added to each of those bottles.

A factor of no more than 2 should be used between each level of toxin i.e. 10 ppm, 20 ppm, 40 ppm, 80 ppm etc. The maximum level of

toxin chosen should be at least a factor of 10 above the expected toxic concentration, up to a maximum of 1 gram toxin in 100 ml test bottle (equivalent to 10 000 ppm of whole toxic waste), and at least a factor of 10 below the expected toxic concentration.

4.3 Measurement of the gas volume and percentage methane.

4.3.1 The gas chromatograph is initially calibrated against a certified calibration gas (refer to SME Work Instruction 12.1), with the exception that the instrument is only calibrated against methane.

4.3.2 The gas loop of the GC is connected up to the syringe valve and the bubble flow meter such that the gas passes through the gas loop before its volume is measured in the flow gauge.

4.3.3 Thoroughly wet the inside surface of the bubble flow meter to minimise the bubbles prematurely bursting and then adjust a bubble with the glass syringe so that the bubble sits on the zero mark of the gauge. Empty the syringe through the needle, and whilst holding the syringe tightly closed insert the needle through the septum into the bottle. Vent the excess gas in the bottle slowly through the gas loop into the flow gauge and measure the volume of gas produced. Inject the gas into the gas chromatograph and measure the gas volume.

Use a worksheet to record against the bottle number, the volume of gas produced, the methane content of the gas, and the GC data file.

4.3.4 The test can be continued until the total amount of methane produced has levelled off. Initially a measurement of the methane is made weekly but in the later stages of the test the measurement can be made every two or three weeks. The conclusion of the test is when no measurable amount of methane has been produced.

4.3.4 Transfer the data to an Excel spreadsheet, and use the spreadsheet to calculate the amount of methane that has been produced since the last reading, subtracting the amount of methane remaining in the headspace of the bottle from the previous reading. Assume that the void in each bottle is 60 ml.

Plot the mean cumulative amount of methane produced each week for each level of toxin against the concentration of the toxin in the bottle. The toxic level of the toxin is the amount of toxin that completely suppresses the production of methane.

5. PRECISION

5.1 The gas volume can be measured to an accuracy of less than 0.5 ml, and the methane to less than 1%, giving a accuracy of the methane produced of less than 0.01 ml methane. To achieve higher accuracy than this would involve measuring the capacity of each bottle before use, and then calculating the void left in each bottle after it has been filled. This figure would then be used in the calculations rather than the assuming that the void is 60 ml.

5.2 The method however is basically a biological method and a wide variation in the amount of methane produced can be expected between bottles. As all tests are carried out in triplicate, this error is somewhat reduced, but a probable variation of +/- 20% from replicate tests may be expected.

Interpretation

For the 7-day test:

1. Multiply the total gas volume (generated + headspace) by the methane concentration (if using %, divide by 100), to give the total methane yield in ml for each bottle (see Example 1). For the specified bottles a headspace of 60 ml can be assumed. Calculate the mean for each triplicate set. The standard deviation of triplicates can also be calculated, to ensure reasonable reproducibility; in toxin-free controls expect results within ±10%, with more variability between bottles likely as toxins near inhibitory levels.

2. Plot a graph of methane yield (ml) against $\log_{10}$ of toxin concentration (ppm) for each dilution used. This should ideally be an S-shaped curve, with a fairly linear mid-range.

3. The toxin-free control cannot be plotted (zero will not plot on a logarithmic axis), but should be marked on the vertical axis.

a) Half of the toxin-free control yield is the 50% inhibition value; where this value intercepts the dose-response curve defines the IC_{50} concentration.
b) The IC_{100} in practice usually falls between dilutions, but for interpretative purposes is the lowest concentration showing zero methane production.

Example 1, Day 7 readings:

Measured volume	25 ml	
Methane	30 %	
Headspace volume	60 ml	
Total gas volume =	60 + 25	= 85 ml
∴ Methane volume =	30/100 x 85	= 25.5 ml

For the long-term test:

1. Calculate the total methane yield for each bottle from the sum of the incremental yields over the 28 day (up to and exceeding 3 months for final yield) monitoring period. Note that after day 7, subsequent samplings for volume and quality will require application of a

correction factor for (a) residual methane and (b) removal of the previous GC sample (depending on sample volume - if following the specified method see Example 2). This can be automated on a spreadsheet.

2. IC_{50} and IC_{100} as for 7-day test.

Example 2, Day 7 to Day 14 methane production:

total methane present at day 14 ...		
Day 14 measured volume	20 ml	
Day 14 methane concentration	45 %	
Headspace volume	60 ml	
Total gas volume	60 + 20	= 80 ml
∴ Methane volume present =	45/100 x 80	= 36 ml
less residual methane present at day 7 ...		
Day 7 methane concentration as above	30 %	
Residual gas volume after sampling	53 ml (assumes 7 ml taken for GC)	
∴ Residual methane volume =	30/100 x 53	= 15.9 ml
total at day 14 minus residual methane from day 7		
Day 7 to Day 14 methane production =	36 - 15.9	= 20.1 ml
∴ True cumulative 14 day methane =	25.5 + 20.1	= 45.6 ml

1st draft - JAS 24/9/96
2nd draft - BCC 30/5/97

APPENDIX 4

SUMMARY OF MODIFICATIONS MADE TO THE INITIAL PROCEDURE

During the evaluation period, the operator's laboratory made a number of minor changes to the protocol discussed at the Industrial Advisors meeting. These changes have been incorporated in the SOP given in Appendix 3.

1. In the original draft SOP, nutrient Solution "A" containing ammonium sulphate, sodium phosphates & magnesium sulphate, was adjusted to pH 7.0 - 7.5 and was then used to prepare solution "B", which required further adjustment to bring the pH back to pH 7.0 - 7.5. In the operator's method solution "A" was used without pH adjustment to prepare solution "B".

2. The solution were brought to the boil and then cooled. This should normally kill sufficient of the organisms present to avoid irreproducibility of tests but it was agreed that any solution left at the end of the day was discarded.

To measure the volume and composition of gas produced, an Aldrich syringe stopcock was adapted so that the side arm had a 1/16" Swagelok thread. This enabled connection of the side arm of the stopcock directly to the inlet port of the automatic injection valve on a gas chromatograph, using a very short length of 1/16" OD s/s tubing. The outlet from the valve was then connected to a modified 100 ml gas burette graduated to 0.2 ml, which acted as a conventional bubble flow meter. In this way the GC gas sample loop can be filled at the same time as the volume of gas is measured. Gas samples were only taken from bottles where gas volumes had increased.

The actual preparation of the bottles at the SME laboratory was straightforward, with one exception, the addition of the toxin to each bottle. The preparation of the nutrient solution and the subsequent dispensing of the nutrient and "seed" into each bottle was carried out using 30 ml & 10 ml dispensers. The bottles were purged prior to capping by a manifold system rigged up with 1/8" OD tubing, "T" pieces and 6 lengths of 1/16" OD HPLC grade capillary tubing.

A set of 24 bottles, i.e. seven dilutions of toxin and a blank, all carried out in triplicate, took about 4 hours of a technician's time to set up. The subsequent weekly measurement of the volume of gas produced and the methane content of each bottle then took another 3 hours. The configuration of the gas chromatograph used was a 4 metre Porapaq N column at 100°C and 20 ml/min helium, giving a run-time of 5 minutes. Many other GC configurations would be acceptable; this time could have been reduced by using a shorter column, because there was more than adequate resolution between the three peaks found. All that is required is a total methane concentration. Another way of reducing the analysis time would have been by

repetitive injections into the gas chromatograph without a column in place, simply using the FI detector.

As referred to above, the only minor difficulty SME had in the preparation of the bottles was in the introduction of the toxin into each bottle. In the initial tests with phenol, the lower levels of phenol were added as a 1.0% or 10% phenol solution in water using a pipettor. In the bottles with the highest concentration of toxin, the phenol was added directly as the solid. For example the 10,000 ppm phenol bottle was prepared by adding 1.0 gram of solid phenol. Phenol, by its very nature, will adhere to any weighing implement, and the quantitative transfer of all the solid phenol into the bottle could not be guaranteed. The subsequent tests with phenol were set up by accounting for the weight of the bottle and then weighing the required amount of toxin directly into the bottle.

With the very volatile trichloroethylene, some caution had to be observed when preparing these bottles. Trichloroethylene does have a limited solubility in water and SME decided to make use of this property by preparing a 1000 ppm w/v solution of TCE in water for the lower dilutions of toxin, rather than the TCE solution in methanol or acetone used previously. This eliminated the need to run solvent blanks. The higher concentrations of TCE were made by using a micro syringe to inject the required volume of TCE directly into each bottle. To minimise loss of solvent, the household waste, nutrient and "seed" were all added to the bottles before the solvent. Once the TCE had been added to each bottle, the bottle was immediately purged with nitrogen for a few seconds only, to remove oxygen from the headspace, and then capped.

Anaerobic Bioassay Test: Development of an Operational Method for Landfill Co-disposal

R&D Technical Report P252

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Statement of use
Controlled co-disposal to landfill is considered the best practicable environmental option (BPEO) for several types of industrial waste. The development of a test method provides for an understanding of whether a particular waste loading is acceptable at a given landfill site. Co-disposal has been a successful and relied upon method of landfill disposal in the UK. The forthcoming EC Landfill Directive will effectively abolish co-disposal as a future waste management option but the findings of this research will have applicability to existing co-disposal landfill sites in the UK for use by operators and regulators.

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EXECUTIVE SUMMARY

Background

When carried out according to best practice, controlled co-disposal to landfill is considered the best practicable environmental option (BPEO) for several types of industrial waste. Co-disposal must not impair the operation of the landfill as a bioreactor, so there is a need for a scientifically-based test to illustrate that a particular industrial waste loading is acceptable.

Methane production is one of the most sensitive indicators of the waste degradation process in landfill. Two tests based on methane inhibition were developed previously at the Centre for Applied Microbiological Research (CAMR) for the Department of the Environment (R&D Report No. CWM 107/94). The CAMR Anaerobic Bioassay Test for Solid wastes (S-ABT) added test material to a reactor containing landfill material and measuring methane inhibition over several months. The CAMR ABT for Liquid wastes (L-ABT) employed the principle in a liquid-phase culture. These were considered impractical for application in non-specialist site laboratories, but the L-ABT system showed potential for development into a practical acceptability test.

Objectives

The aim of this project was to produce a refined and simplified Standard Operating Procedure (SOP), based on the CAMR L-ABT. The objectives were:

- to replicate the CAMR test, to check reproducibility and provide a benchmark;
- to develop a simplified, faster, less expensive SOP derived from the CAMR method;
- to test the practicality of the SOP in the laboratories of Shanks and McEwan Ltd (SME).

The modifications were regularly monitored and reviewed by a group of industrial advisors (IAG) drawn from landfill operating companies. Major steps were made by AEA, reviewed by the IAG then tested by SME to check the reproducibility of the modified test, examine some real candidate wastes, and allow final practical refinements to be made.

Main Findings

The new SOP has been simplified in several ways:

- The seed culture is derived from landfill instead of sewage sludge;
- Household waste and yeast extract replace individual trace elements, soluble carbon and vitamins;
- Reagent solutions are boiled rather than filtered or autoclaved;
- The test is prepared in one step, rather than three;
- An acute inhibition value can be obtained in 7 days.

The method was evaluated with trichloroethylene (TCE), cadmium (Cd) and phenol as the main test toxins. For Cd and phenol a 50% inhibition value (IC_{50}) could be obtained at 7 days,

but the value changed over time, probably due to adaptation by organisms, and adsorption or degradation of toxins. The complete inhibition value (IC_{100}) proved unambiguous, giving results in 7 days and remaining stable thereafter. The new operational method (O-ABT) and the L-ABT gave the same IC_{100}, so simplification did not compromise the test. The IC_{100} for TCE was significantly different, probably due to the use of household waste in the O-ABT.

Tests showed excellent reproducibility (standard deviation <10%). At ambient laboratory temperatures the rate of gas production was reduced, but the IC_{100} value was the same as at 35°C (for phenol). Four real co-disposal wastes are also reported. One showed no inhibition at up to 1% v/v in the test system (the highest concentration tested), while three others gave unambiguous IC_{100} values.

Capital costs of implementation will be relatively low; a few hundred pounds for an incubator, seed culture, test bottles and gas manifolds. It is assumed that a gas chromatograph will already be in place. Normal laboratory staff can be taught easily to perform the test. Running costs depend on which version of the method is accepted; we recommend the acute 7-day test as the simplest and most robust and useful version, which is also the least expensive.

The data must be interpreted by reference to a known toxin tested in parallel with the unknown material; we used phenol. The test will take account of the synergistic or antagonistic effects of ill-defined or unknown components in a manner that is not feasible using specific chemical analysis. Measuring acute inhibition of undiluted test material at 7 days could be used to quickly and inexpensively identify and allow co-disposal of benign materials, and identify those requiring further examination. The same test system, used to test dilutions of the material, could then be used to determine an acceptable loading rate for more toxic materials.

With a doubling dilution series the nearest one can approach the true toxicity value is approximately ±50%. More closely spaced dilutions would give a more accurate answer and require a smaller safety margin on interpretation, but at greater cost. The test may prove site-specific, as it uses substrate and seed from the target site. Comparison of several sites is now required to assess the importance of this factor and validate the method. Until comparisons have been made the test can only be used by operators as an indicator for the order of magnitude of toxic effects, and absolute threshold values of the IC_{100} should not be set.

Conclusions

1. A version of the liquid-based anaerobic bioassay test has been developed which is sufficiently simplified and robust to implement in a landfill operator's laboratory.
2. The modified test gives results quickly; the optimum incubation time is 7-14 days.
3. The most reliable and practical end-point is complete inhibition of methanogenesis.
4. The test shows good reproducibility (of the order ±5%-10% on triplicates).
5. The test gives good comparative information but does not give absolute results (it is a useful tool for landfill operators, but is not validated for use by regulators).
6. The test 'fails safe'. The liquid system probably under-estimates the toxicity which an operational landfill would be able to tolerate.

7. The test measures total toxicity where it is unknown or cannot be predicted from the chemical composition because of unknown synergism or antagonism.
8. The test result can be expressed as 'phenol-equivalents' and used to (a) check whether a waste is toxic or not, (b) provide relative toxicity information to determine co-disposal rates for various toxic wastes.

KEY WORDS

Landfill; bioassay test; waste loading; co-disposal; methane.

GLOSSARY OF TERMS

ABT	Anaerobic Bioassay Test
AEA	AEA Technology
BOD	Biochemical Oxygen Demand
BPEO	Best Practicable Environmental Option
CAMR	Centre for Applied Microbiological Research
COD	Chemical Oxygen Demand
DoE	Department of the Environment
GC	Gas Chromatograph
IAG	Industrial Advisory Group
L-ABT	Anaerobic Bioassay Test for Liquid wastes (the CAMR test)
O-ABT	Operational Anaerobic Bioassay Test
S-ABT	Anaerobic Bioassay Test for Solid wastes
SME	Shanks and McEwan
SOP	Standard Operating Procedure
TCE	Trichloroethylene

1 INTRODUCTION

This work was commissioned by Wastes Technical Division of the Department of the Environment under R&D Contract No. EPG 1/7/048, which transferred to the Environment Agency as R&D Project P1-251. The work was undertaken by AEA Technology over the period December 1995 to October 1996.

1.1 Landfill Co-Disposal

In the United Kingdom, most household waste is disposed of to landfill. Co-disposal of a restricted number of industrial wastes with the decomposing household waste has been common practice in the UK for many years. The Department of the Environment supports the views of the waste disposal industry that controlled co-disposal is a safe practice and is the best practicable environmental option (BPEO) for several types of industrial wastes. Guidelines on site location, engineering, operation and waste inputs for co-disposal were given in draft Waste Management Paper 26F [1].

It is important that co-disposal does not impair the operation of the landfill as a bioreactor. In particular the microbial population in the landfill must continue to:

- convert a high proportion of the degradable organic solids in the waste to gas,
- degrade or deactivate potentially toxic organic compounds in co-disposed wastes,
- maintain conditions which retain heavy metals and other non-degraded toxins within the mass of solid waste.

Research sponsored by Department of the Environment since 1973 (Knox, report no. CWM 07/89) has demonstrated that, at the recommended loading rates, the toxic components of industrial wastes can be degraded and immobilised in decomposing refuse. Furthermore, it is observed that landfills in which industrial wastes are co-disposed continue to generate substantial amounts of methane and do not suffer major changes in leachate composition.

There is a need for a scientifically based test to illustrate that a particular industrial waste has an acceptable toxic load when offered for co-disposal with domestic waste in the UK. Being able to identify wastes which needed alternative treatment or dilution before co-disposal would be in the interests of both operators and regulators, to:

- prevent risk of inhibition of microbial stabilisation of waste;
- reduce long-term liability due to unacceptable leachate quality;
- avoid disruption of landfill gas production; and
- illustrate a scientific basis to safe practice for co-disposal.

Anaerobic Bioassay Tests

Methane production is the most important indicator of the overall condition of a methanogenic consortium, such as that in an efficiently operating landfill bioreactor. Methane generation is a suitable end point for a bioassay of toxicity because:

- it indicates a complete and uninterrupted biochemical degradation pathway;

- methanogens are among the most sensitive organisms in landfill;
- methane is unreactive and relatively insoluble, so methane generated accurately reflects microbial activity.

A test which measures the inhibition of methane production gives a direct bioassay of all the toxic effects which a waste may have upon the anaerobic microbial population. Two tests based on this principle were developed by the Centre for Applied Microbiological Research (CAMR) under contract to the Department of the Environment [2].

The Solid ABT (S-ABT) developed by CAMR involves adding industrial waste to a reactor containing landfill material and measuring inhibition of in-situ methane generation over a period of months. This system models landfill quite well, and can take account of protective effects from sorption, adaptation of the population and biodegradation or detoxification of the inhibitory material. However, it is expensive and time-consuming, and requires specialist facilities and staff.

The Liquid ABT (L-ABT) developed by CAMR employs an aqueous-phase culture in small bottles. This arrangement is less realistic and gives potentially less information about protective mechanisms within solid wastes, but should be more reproducible and gives a result faster than the solid system. However, it still takes too long to be of practical day to day use to operators, and is unnecessarily complex. The CAMR tests are excellent research tools, but were not designed-for and are not appropriate to operation in landfill site laboratories.

The Industrial Advisory Group (IAG) felt that the methodology described in draft WMP 26F was impractical for widespread application in non-specialist laboratories serving sites with the normal pattern of waste delivery, i.e. wastes requiring urgent disposal, and not able to be stored for months. To be more practicable as a potential acceptability test for co-disposal they considered it preferable that:

- There is one unified test protocol which can be used for all wastes as presented:
- The most serious acute adverse effects of a toxic component are detectable in a relatively short time:
- There are no grounds for the waste producer to dispute the validity of the test if a waste is rejected:
- The test is consistent between sites, influenced only by local site conditions:
- The test employs equipment and skills which already exist or can be readily incorporated into an established on-site analytical laboratory:
- The actual cost of testing is less than the potential cost (long-term and short-term) of adverse effects on the landfill behaviour caused by toxic materials not identified by current vigilance and waste analysis:

1.2 The Need for a Bioassay

The draft of Waste Management Paper 26F on co-disposal describes a hierarchy of test procedures dealing with the acceptance of wastes for co-disposal:

- Level 1: Comprehensive Characterisation

- Level 2: Compliance Testing
- Level 3: On-site Verification.

Presently these rely on physical, chemical and visual tests. However, an operator needs to know quickly whether a waste presented for co-disposal can be accepted or whether it is likely to have any adverse effects on the health of the target landfill. 'Health' is taken to mean a landfill in which methane production is high, indicating the presence of a complete degradative microbiological population.

Many wastes for co-disposal are complex and/or incompletely defined chemically, so an assessment of the likely impacts based on chemistry alone may be misleading. The only practical way to assess the many possible synergistic or antagonistic interactions is therefore to measure directly the overall net toxicity in a methanogenic biological test system.

In the context of biological testing, characterisation tests aim to replicate environmental conditions to:

- understand the fundamental processes;
- cover a full range of conditions;
- give information on other factors (e.g. biodegradation, detoxification, adaptation, sorption).

Tests such as the CAMR S-ABT fall into this category, and Agency commissioned R&D work by Minton, Treharne and Davies is currently in progress to develop and refine such a test. This type of test can in principle give absolute values for toxic loads and actual inhibitory effects. However, these tests currently:

- take a long time to reach completion;
- need specialist equipment and staff;
- are expensive to perform.

The validity of S-ABT as a model of in-situ landfill conditions is questionable, since even large microcosms (of the order 1 m^3) still do not represent the large-scale heterogeneities known to be present in landfill. They will probably be limited to a few tests for strategic uses such as assessing common waste streams, compiling a national database of toxicities, or in fundamental studies of biodegradation.

Compliance tests are intended to look for changes in the basic characteristics of the waste and so are essentially acceptance tests. These will normally use standard conditions to give a reproducible but relative value, which must be related to an independent benchmark. The tests must be performed at the receiving landfill site laboratory, and must:

- be simple, quick and inexpensive;
- be standardised and easy to run in large numbers;
- employ standards which relate to other tests (e.g. chemical analysis).

By their nature, acceptance tests will give relative values so that benign materials can be accepted and potentially problematic materials can be identified quickly. The test:

- will not give absolute values;
- cannot indicate any protection factors present in-situ;
- gives little information on long term effects (adsorption, biodegradation, adaptation).

It was suggested that the L-ABT system of CAMR could form the basis for a compliance/acceptability test, but would need significant simplification if it were to be successfully applied in existing laboratory facilities at typical landfill operations.

The test would be used in conjunction with other data. Operational tests and information are already used to guide co-disposal, so the ABT would be considered in conjunction with:

- individual analyses to identify inorganic and organic toxins;
- guidance on acceptance in draft WMP 26F;
- experience with similar wastes.

However, biological assays are inherently variable, so it is recommended that an ABT is not used as the only regulatory tool. Other sources of information, e.g. chemistry, will still be essential to assist regulators in setting waste input limits.

1.3 The Present Study

The Department of the Environment placed a contract with AEA Technology (AEA) to adapt the L-ABT developed by CAMR. Since April 1996 this contract has been funded by the Environment Agency. The aim of the project is to refine the existing Standard Operating Procedure (SOP) by:

(a) replicating the CAMR test to check reproducibility and provide a benchmark comparison;
(b) assessing a simplified, quicker, less expensive procedure derived from the CAMR method;
(c) testing this revised SOP in the laboratories of a typical landfill operator.

The operational testing has been undertaken by staff at Shanks and McEwan (SME), AEA's partners in this project. An advisory group drawn from the industry has been formed to provide input at critical points, to ensure that the final test would take account of a broad cross-section of the industry's needs.

Membership of the Industrial Advisory Group (IAG) is given in Appendix 1. The first consultation point was the selection of the toxins to be tested. The second consultation point was a round-table discussion on the format of an interim test procedure to be evaluated by Shanks and McEwan (SME). Finally the group met to consider the outcome of the operational tests by SME and advise on the potential application of the resulting SOP.

This report consists of four sections:

1. The introduction to the study.
2. A description of the experimental work undertaken to revise and test the simplified ABT.

3. A discussion of the relevance of the data to the testing of co-disposal wastes
4. Recommendations for the use of the test and further development.

Appendices contain details of:

1. The Industrial Advisory Group meetings.
2. Experimental procedures followed in the development work.
3. The recommended Standard Operating Procedure for an Operational ABT method.

2 METHOD DEVELOPMENT

2.1 General Principles

It was agreed that the operational system should retain the fundamental principles of the CAMR L-ABT, i.e. the inhibition of methane production by a standardised anaerobic culture in liquid medium as the main measurable parameter. The L-ABT method had been validated against a wide range of potential toxins. The aim of the development work was to adapt and simplify the fundamental concept to make the test faster, simpler, and less costly to perform. During the development programme, the approach was to add known amounts of toxin at different dilutions to identical anaerobic cultures in order to plot a dose/response curve. When the test is used in practice, the dilutions of known toxin are used as a calibration against which to assess a candidate waste.

The objectives of this part of the project were:

- to replicate the CAMR L-ABT as closely as possible, to check reproducibility;
- to carry out a simplified operational method (O-ABT-1) as proposed;
- to compare the L-ABT with the results of AEA's O-ABT-1;
- to carry out further simplifications (O-ABT-2 and -3) in order to determine the minimum cost and effort giving an acceptably reliable and reproducible test.

The L-ABT methodology was followed as closely as possible. However, two changes were agreed in the protocol at the beginning of the project.

1. **Different source of methanogens**. CAMR used an inoculum of sewage sludge; this study used methanogens derived from healthily-gassing municipal landfill. It was felt that this type of culture was more representative of the site of concern. In addition, we have found the landfill waste culture easier to maintain and so more suitable for use in a non-specialist laboratory. The source of our microbial culture was household waste, estimated to be 10 years old, excavated from the Calvert landfill. Fresh material was used to establish the seed culture and the bulk material has been shredded and stored in plastic bags. These solids were used to feed the inoculum culture and were added to the test bottles as a source of nutrients.

2. **Different method of expressing inhibition.** CAMR looked for complete inhibition on a linear scale, but a dose-response curve may have an indeterminate head and foot. This could make estimation of the 100% inhibitory concentration (IC_{100}) potentially problematic. We therefore also examined interpretation of results according to the IC_{50} - that concentration of toxin causing 50% inhibition of methane production (analogous to the LD_{50} used for comparing toxicity in higher organisms). A logarithmic-linear plot normally gives a relatively linear relationship in mid-range, from which a half-inhibition value may be reliably determined. Some multiple of either the IC_{50} or IC_{100} concentration might then be selected as the acceptance criterion.

Details of the equipment, reagent and method used in replicating the L-ABT method and in simplifying the O-ABT method are given in Appendix 2. The CAMR method was followed

except for the two fundamental changes noted above. The revised methods are referred to as O-ABT, Versions 1, 2 and 3.

2.2 Choice of Toxins

The aims of this project were to:

- provide limited validation of the CAMR method (on three different toxins);
- simplify and develop the test, hence only a limited number of toxins were tested.

Three toxins representative of different groups were used in method development:

- a heavy metal (cadmium, as the soluble nitrate),
- a water-soluble organic (phenol) and
- a water-insoluble chlorinated compound (trichloroethylene, in acetone).

These were materials studied in the CAMR project, allowing comparison of results.

The concentrations of the species used in the current work were chosen to span the inhibitory concentrations reported in the CAMR development work. The Industrial Advisory Group members agreed to the choice of toxins and the concentration ranges to be studied.

It was recognised that toxins will have a wide range of characteristics and the three chosen in this study have properties which might influence the availability of the material in a culture. For instance, cadmium has a very insoluble sulphide and so any free sulphide produced from sulphate reducing bacteria may lower the concentration of cadmium in solution. It was hoped that using mature waste would avoid any variability caused by sulphide precipitation. Phenols and cadmium ions may be strongly adsorbed to organic material and so the concentration in free solution may be lower than anticipated.

Water-immiscible solvents such as TCE are difficult to administer in small amounts and a co-solvent was used by CAMR to aid accurate addition to the medium. The solvent may itself be either inhibitory or be a potential carbon source. We chose initially to use acetone as the co-solvent, reported by CAMR to be neutral in effect. An alternative solvent, methanol, was shown by CAMR to be stimulatory to gas production. The later studies at SME used water saturated with TCE. Any immiscible liquid will not be dispersed easily and evaporation of solvent may occur during preparation of samples. Care was taken to avoid these effects.

2.3 Simplification of the ABT Methodology

The main features of the assay system are described in Appendix 2. The specialist equipment requirements are:

- an inoculum culture;
- reagents containing essential nutrients;
- a system for purging the test bottles with inert gas (nitrogen);

- an array of test bottles into which nutrient solutions, the toxin and the microbial inoculum are placed;
- a system for collecting and measuring gas volumes;
- a gas chromatography system.

As far as possible AEA excluded equipment which would only be found in a specialist microbiology laboratory (e.g. anaerobic cabinet, autoclave, jacketed fermenters).

The CAMR method relied on fresh sewage sludge, which is freely available but:

- requires a period of adaptation in the test system before bottles have to be re-opened and toxin added;
- is probably not an ideal source of landfill-adapted methanogens;
- has to be used quickly, requiring in practice regular collection and transport from the nearest sewage works.

The AEA philosophy was to initiate and maintain a culture of more appropriate landfill-derived organisms, and to keep the culture under test-like conditions. The culture was therefore ready for use at any time, as required, eliminating:

- the bottle re-opening step;
- delays awaiting adaptation; and
- the need to collect fresh sludge.

Inoculum cultures were set up using sewage sludge taken from a local wastewater treatment plant at Didcot, in an attempt to replicate the CAMR method. These cultures were slow to become methanogenic and steady state gas production rates were low. After consultation with the DoE and CAMR it was agreed not to proceed with this inoculum.

In parallel an inoculum culture was established using aged waste freshly-excavated from the Calvert site in Buckinghamshire. This rapidly became methanogenic and gas production rates remained high for several months. Aliquots of this well-mixed suspension were removed to inoculate test bottles. Irrespective of whether material removed was used for tests, sufficient suspension was taken out to allow a total of 500 ml of new 10% w/v suspension to be added to the vessel each week. This rate of feeding maintained healthy gas production for at least 4 months while minimising significant accumulation of either fermentable carbon or recalcitrant material. The AEA microbial inoculum was maintained in a simple bioreactor comprising a glass vessel (5 litre capacity) from which a fixed volume (500 ml) was regularly withdrawn (weekly) and replaced with 500 ml of fresh medium containing freshly-milled household waste. This was incubated in a water bath, held at 35°C in a fume cupboard, and gas was collected over acidified water in an inverted burette for volume/quality measurement. The inoculum was fed on a weekly basis with sorted and milled household waste from Calvert landfill site, initially suspended in a minimum salts solution.

The tests were performed in serum bottles (nominally "125 ml", actually 155-160 ml) which can be sealed with a butyl rubber septum. Thin rubber septa are preferred so as to facilitate multiple sampling with the gas syringe. In the operational system 2g (wet weight) of aged

waste was added to the liquid medium (100 ml) to provide both nutrients and some solid surface akin to a landfill (though in no way intended to be as comprehensive a simulation as the S-ABT method). The household waste came from an older section (8-12 years) of Calvert landfill site, and had been hand-sorted to remove non-putrescibles, then ball-milled to (a) reduce the mean particle size to < 1 mm, (b) thoroughly homogenise the material. Results show that this gave very reproducible gas production, although it is not possible to say how representative this was of other landfill sites, or indeed of other parts of Calvert.

The original CAMR work in a solid-free culture used a completely defined anaerobic medium to ensure that fermentable carbon and essential elements were present. The addition of landfill solids to the AEA Technology test system provides an alternative and more realistic source of carbon and inorganic nutrients: as a result much a simpler liquid medium could be considered. Difficult reagents such as selenium (a scheduled poison) and vitamins (which are expensive and unstable) were therefore omitted. The simplified medium for the initial trials (Appendix 2) was an adaptation of a medium which had been used successfully in the Biological Methane Potential tests, originally developed by Biotal[3] and modified by AEA[4].

The main analytical parameter measured is the amount of methane in the headspace of the bottle. This is determined by measuring:

- the total volume of gas when the headspace has been equilibrated with atmospheric pressure; and then
- analysing the proportion of methane using a gas chromatograph (GC).

Inhibition by a toxin was judged by comparison with a positive control bottle containing microbial inoculum and all reagents except the toxin. The experimental conditions were each tested in triplicate, so each data point shown on subsequent figures is the mean of three points. For uninhibited positive control tests there is usually less than a 10% spread on the data. For inhibited cultures there may be more scatter. The choice of the ratio of liquid:headspace in the test bottles was guided by previous experience.

There is approximately 60 ml of headspace above the liquid so that production of 60 ml of new gas (about 30 ml of methane) will increase the pressure in the bottles to 2 atmospheres. Regular sampling was adopted to ensure that no bottle reached a failure pressure, thought to be at least 5 atmospheres (no bottles failed). In principle however there is some risk of explosion, so goggles and gloves are recommended when handling active bottles.

The gas production from a test bottle containing the standard inoculum and simplified nutrients and no toxins is illustrated in Figure 1. This shows linear production rates for methane over about 30 days, with some slowing down as substrate depletes.

These tests use a mixed microbial population in a heterogeneous medium and so the effects of toxins on methane production will be complex. Some of these effects will be seen in the dose response curve, normally at concentrations of toxin just below those which cause inhibition. For instance sub-lethal doses of toxin may become neutral or even stimulatory in effect. Figure 2 illustrates the effect of 1000 ppm of phenol on methane generation under the standard O-ABT conditions. The greatest impact on gas production is in the first week of

incubation. Subsequently, gas generation rates increase, which may be attributed either to adaptation of the microbial population and the proliferation of more tolerant sub-populations, or to adsorption/detoxification processes.

This effect may also occur with the solvent used for water-insoluble toxins. Figure 3 illustrates the effect of acetone on the test system - initially neutral in effect (as reported by CAMR) the population again appears to adapt, finding acetone stimulatory in the medium-term. This solvent was used to disperse one of the toxins, TCE, in the initial tests and the non-linear response illustrates the difficulty which might be encountered in interpreting results from mixed wastes containing both toxins and potential nutrients.

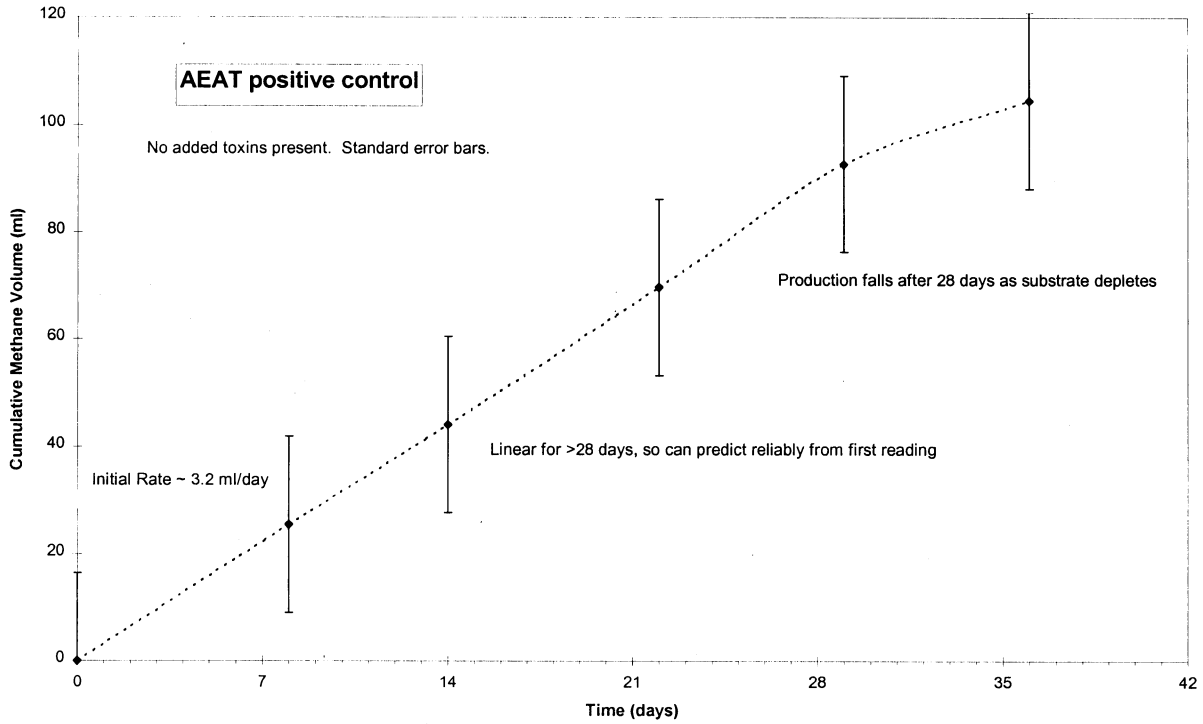

Figure 1 Positive control, AEA method

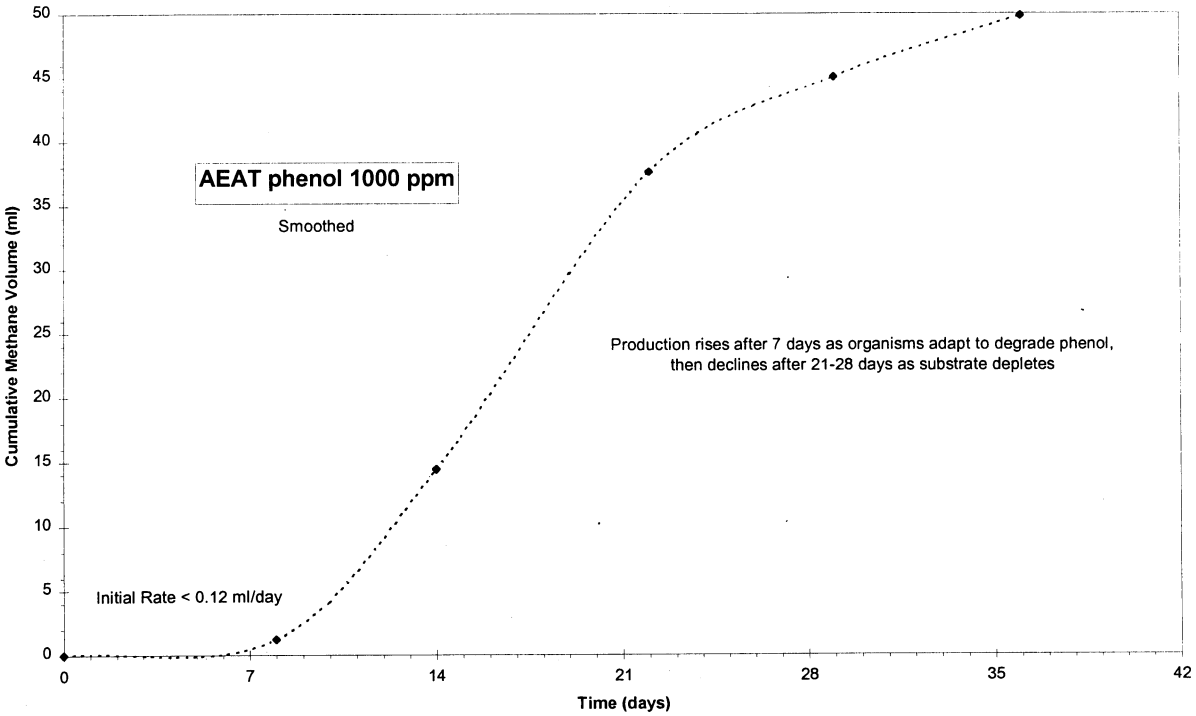

Figure 2 Phenol 1000 ppm, AEA method

In the short-term, inhibition increases with the amount of toxin added. The dose-response curve is exponential in character (see Figure 4) and in the early stages of the culture (e.g. at 8 days) it may be difficult to estimate the concentration which results in total inhibition (the IC_{100}). For this reason we examined:

(a) use of a logarithmic-linear (log-linear) plot, to straighten out the dose-response curve, and
(b) the concept of the half inhibition value (IC_{50}), particularly when measuring acute effects after a few days incubation.

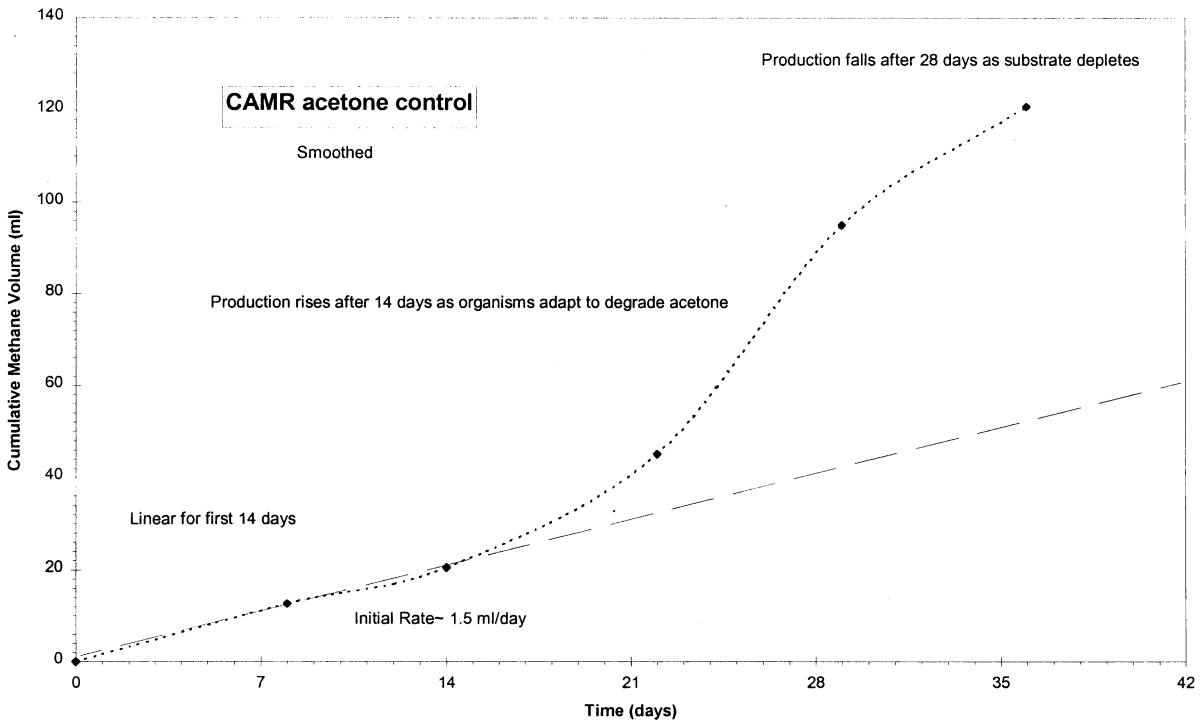

Figure 3 Acetone control, CAMR method

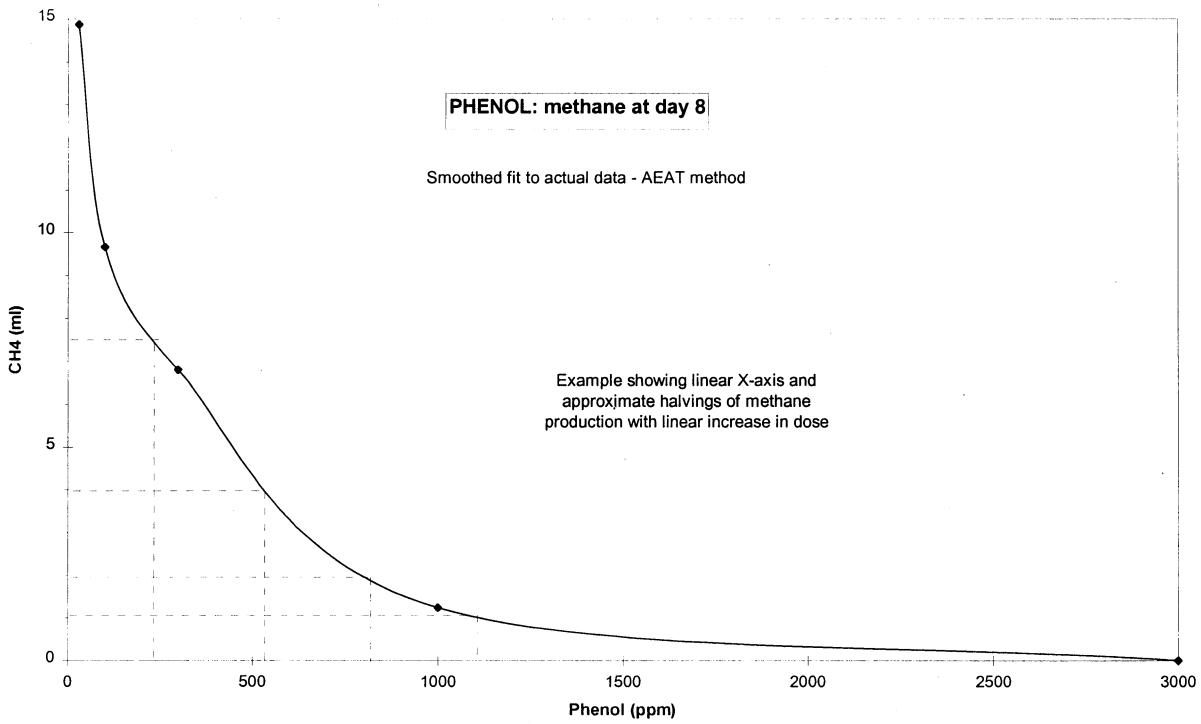

Figure 4 Phenol by AEA method, linear-linear plot

For higher organisms, the dose-response relationship is usually a function of the logarithm of dose, so it is convenient to use a log-lin graph. This also appears to be true of the effects of many toxins on methanogenesis, so the log-lin plot has generally been used in this study. The

log-lin plot is usually sigmoidal, giving a linear response in mid-range, and hence allowing reliable estimation of the 50% inhibition point.

The gas chromatograph (GC) was operated primarily to detect methane. Carbon dioxide is retained longer on the GC column and was ignored so as not to slow the analysis of multiple samples. Avoiding the use of carbon dioxide as a monitoring parameter makes it unnecessary to allow for the fact that many organisms, not just methanogens, produce CO_2 and that the solubility of this gas is high and pH-dependent. Small quantities of oxygen have been detected in the tests but this is probably a feature of the sampling technique. Higher concentrations would indicate leakage, but as the medium used contains a Redox-dependent dye (Resazurin, see Appendix 2) any serious leak will immediately be visible on inspection of the bottle. Hence, GC estimation of oxygen is unnecessary. Hydrogen was detected in some bottles, particularly in those close to inhibitory levels of toxin. This is consistent with the probability that acetogens, which generate hydrogen, are less susceptible to toxins than the hydrogen-consuming methanogens, and confirms the suitability of methanogens as the most sensitive indicator of toxicity. Hydrogen estimation will not be required in routine practice.

2.4 Comparison of L-ABT and O-ABT Version 1

The initial experiments compared the main changes proposed to simplify the test:

- a 'one-step' test using pre-adapted organisms;
- reliance on landfill-derived solids for carbon and trace nutrients;
- simplification in preparation and composition of the defined media;
- measurement of gas after the first week to identify acute effects;
- comparison of old (IC100) and new (IC50) end-points;
- new methods of interpretation;
- measurement of gas after several weeks to identify longer-term effects.

The CAMR L-ABT method and AEA O-ABT Version 1 method were run in parallel. The dilution range and mid-value tested were chosen to replicate the CAMR method and bracket their reported inhibition value. The solid added to the O-ABT method was 20 g/l (wet weight) of well-mixed, finely-divided household waste, from a landfill not used for co-disposal (Calvert). Figures 5-9 show the dose-response curves for the three toxins as:

(a) the methane yield with dose at 8 days,
(b) the cumulative methane yield with dose to day 79.

Phenol

Figure 5 illustrates the data from the original CAMR method and the first version of the simplified method after 8 days exposure to phenol. Both methods gave an IC_{100} between 1000 and 3000 ppm, confirming the comparability of the methods. The L-ABT method gave a non-linear response curve with an IC_{50} ~1100 ppm. The O-ABT-1 method was more sensitive and gave a more linear dose-response curve, with an IC_{50} ~50 ppm.

The longer-term incubation (79 days) in the presence of phenol (Figure 6) gave results which were comparable, with an IC_{100} between 1000 and 3000 ppm. The log-linear presentation facilitated making this judgement with more confidence.

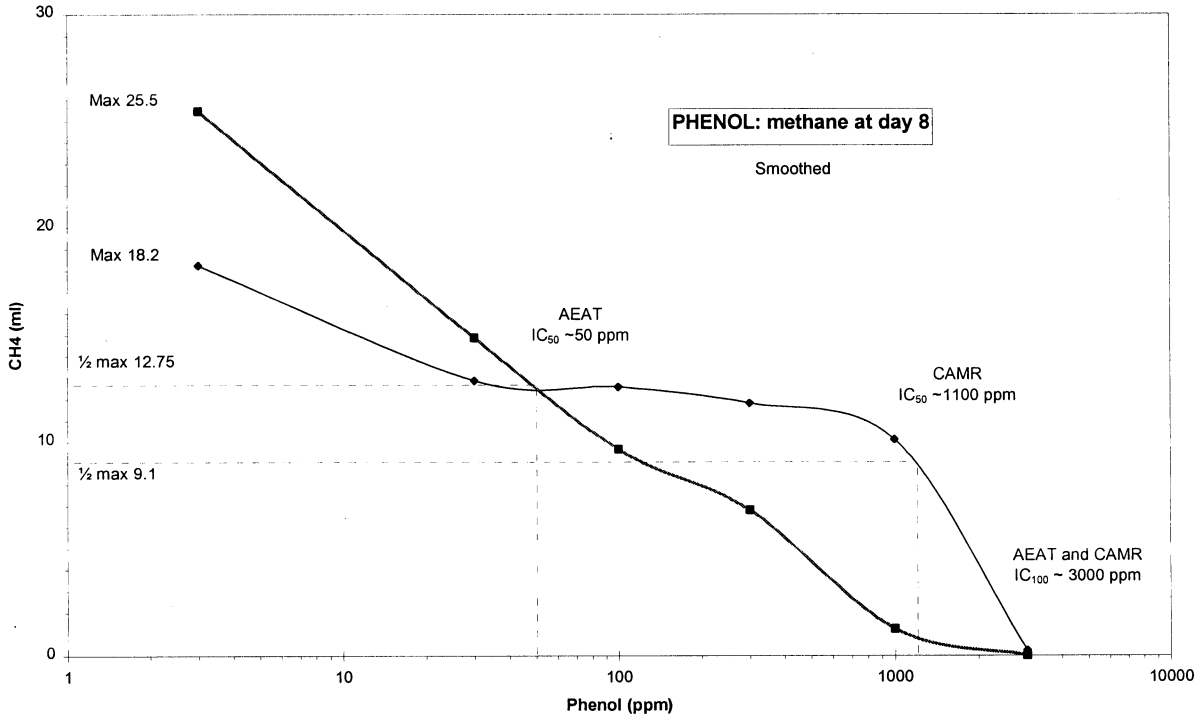

Figure 5 Phenol, 8 days incubation

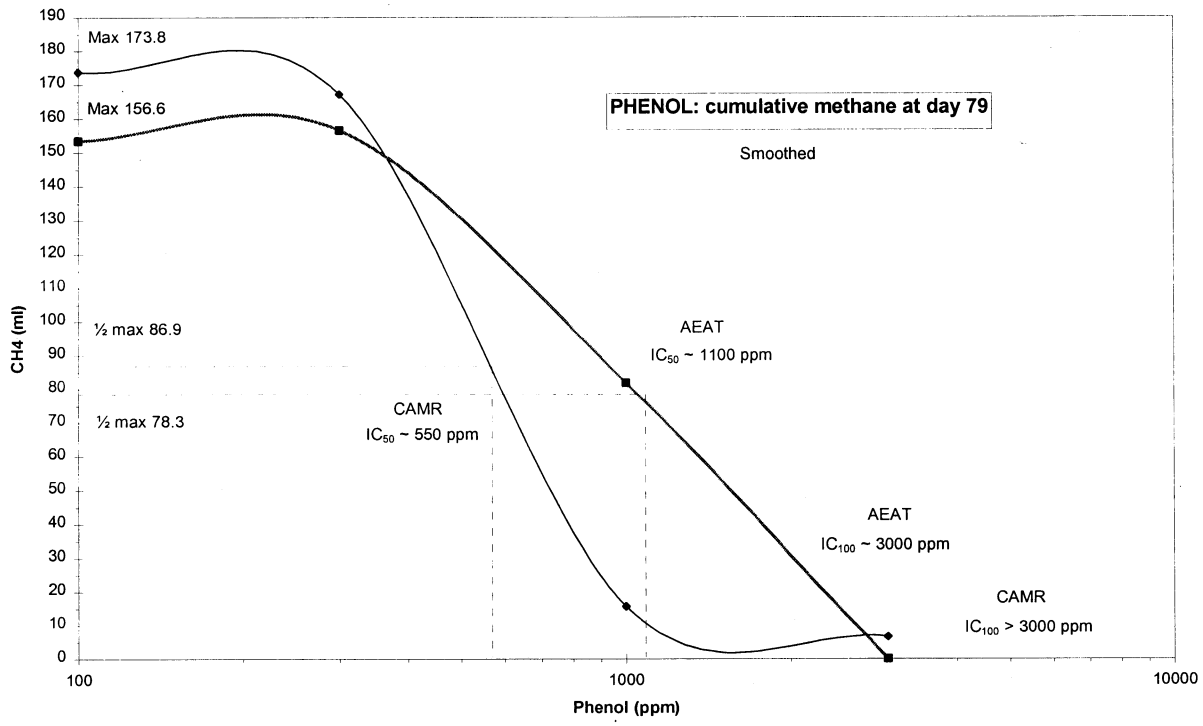

Figure 6 Phenol, 79 days incubation

The total inhibition end-point was the same as observed at 8 days incubation, i.e. those cultures which were initially completely inhibited did not adapt later to the phenol. However, the 50% inhibition point gave an IC_{50} of ~550 ppm by L-ABT and ~1100 ppm by O-ABT. This was a significant increase over the IC_{50} value at 8 days, indicating possibly recovery and adaptation of microbes at sub-lethal doses, or adsorption and detoxification of the phenol at sub-lethal phenol concentrations. The highest IC_{50} value had been achieved by 28 days and did not change markedly after this period.

For the dilution series used (steps of 30, 100, 300, 1000, 3000 ppm), 550 ppm and 1100 ppm are indistinguishable, and so the two methodologies were judged to have given similar results. In bioassays such as this, results must be at least two dilutions apart to be regarded as statistically different. For more accurate estimates, more closely-spaced dilutions would be required.

Cadmium

Inhibition by cadmium after 8 days and 79 days gave a similar pattern to phenol for the O-ABT method (Figures 7 & 8), although the dose-response curve was not linear at 8 days. Both methods gave an IC_{100} between 300 and 1000 ppm at 8 days, confirming comparability, and this result was stable, i.e. remained the same up to 79 days. The IC_{50} by the new method was readily determined: ~65 ppm at 8 days and ~550 ppm at 79 days, compared with an IC_{50} of ~300 ppm from the original CAMR report. This value had actually stabilised at the higher value after about 28 days incubation. As was observed for phenol, the IC_{50} had shifted over time, suggesting either adaptation of the microbes or reduction in bioavailability of the toxin. The more consistent data from this O-ABT method may be connected with the use of the landfill solids which provide some buffering capacity in the system.

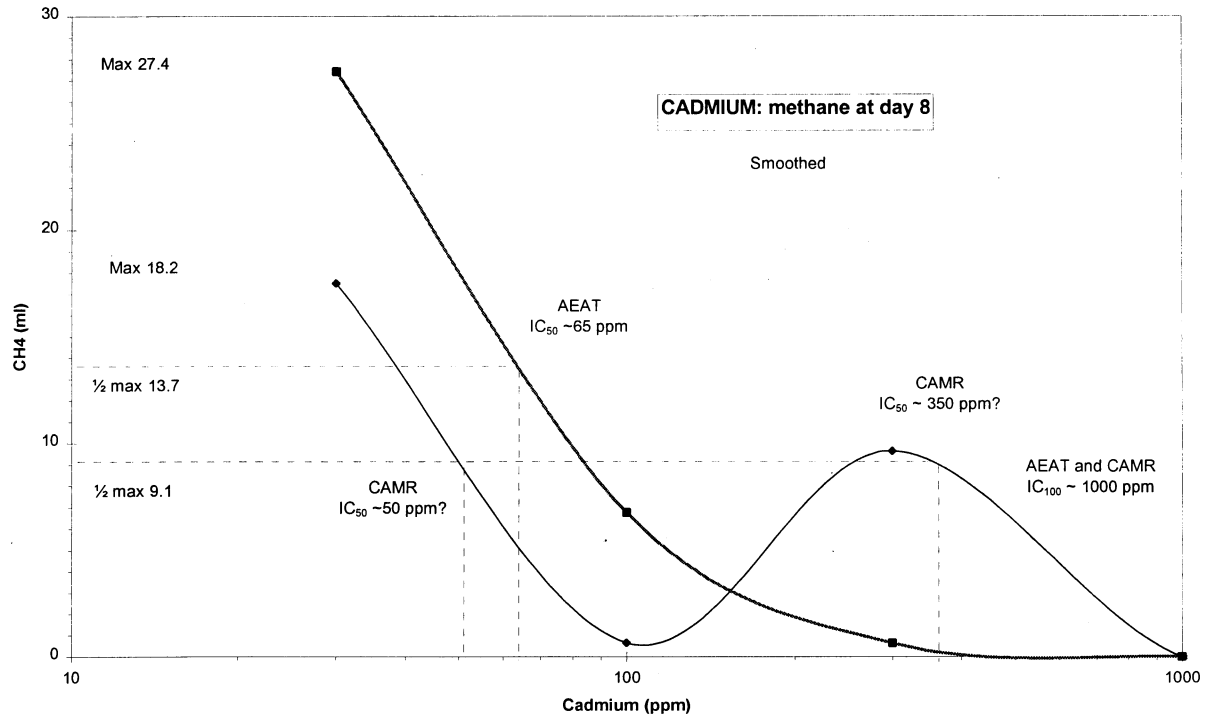

Figure 7 Cadmium, 8 days incubation

Incubation of these vessels continued for more than 5 months. There was a variable lag-phase at sub-lethal concentrations, during which a proportion of methanogens may have died (and so produced no gas) while some survived and adapted (to eventually degrade all available carbon, giving a similar cumulative volume of gas to that of the toxin-free controls). This non-linear behaviour meant that a final yield could not be predicted from the trend in methane production when toxins were present. If a total cumulative methane value were required, it would be necessary to incubate to completion to obtain this value.

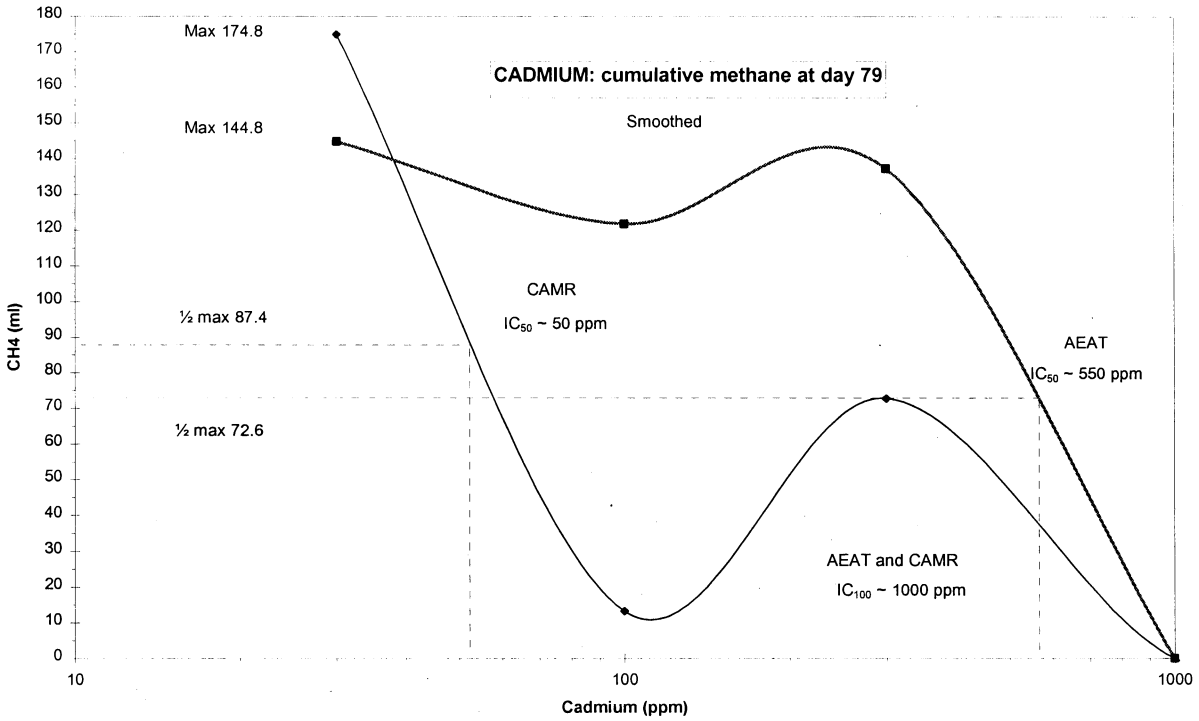

Figure 8 Cadmium, 79 days incubation

Trichloroethylene

The inhibition study using TCE gave a rather different result from that reported by CAMR (complete inhibition at ~1 ppm TCE). In the O-ABT-1 method TCE was not inhibitory even at the highest concentration used (10 ppm - see Figure 9). Gas production actually increased slightly with TCE dose, and this may be a result of:

1. organisms metabolising the co-solvent (acetone) used to disperse and dilute the TCE in the medium. An acetone-only control yielded more methane than the positive control. There is probably no solvent for non-polar compounds which can be guaranteed non-degradable.
2. organisms degrading TCE itself. The O-ABT used a landfill-derived inoculum whereas the CAMR study used sewage-derived cultures. The landfill culture certainly contained species which had been exposed to chlorinated xenobiotics, which are universally found in landfilled household waste, and would therefore be expected to tolerate or even degrade TCE.

The O-ABT test therefore proved less sensitive than the L-ABT test for TCE. This demonstrates the importance of using landfill-derived organisms.

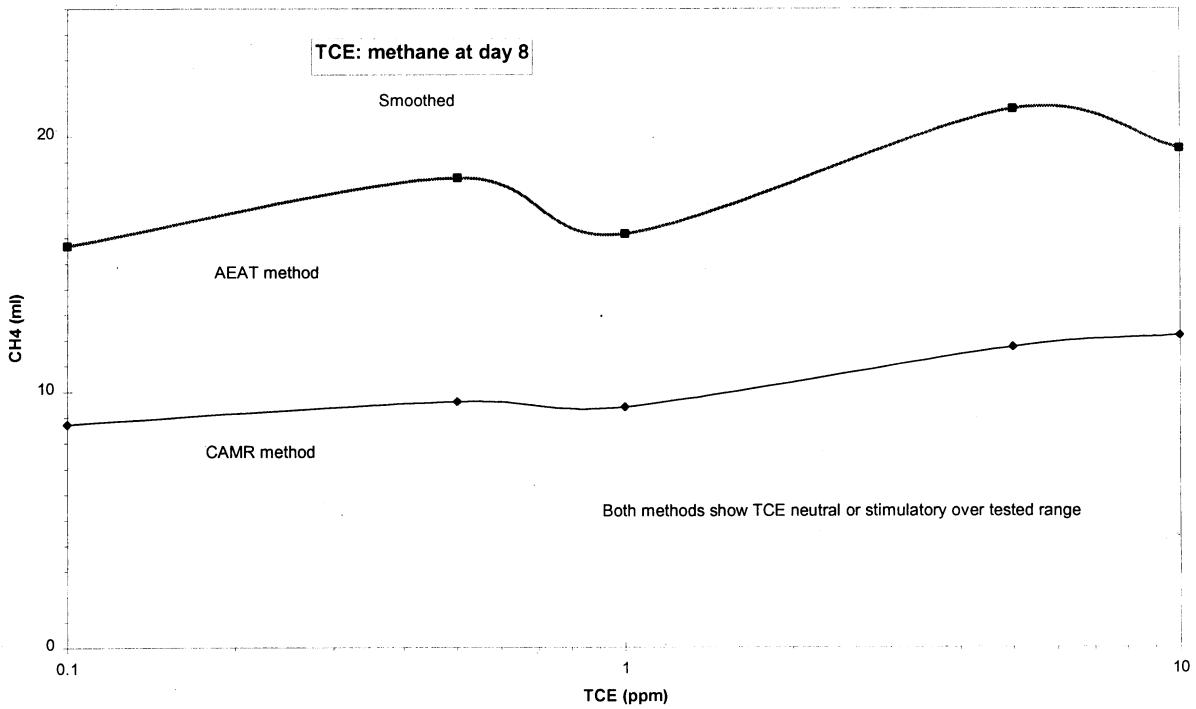

Figure 9 Trichloroethylene at 8 days

Methane production by these cultures was continued for 155 days (22 weeks) incubation, by which time methane production was very slow. The cumulative results after various incubation times are summarised in Figures 10-13 below.

Unchallenged positive control bottles (i.e. no toxin) are not shown, because a zero value of toxin concentration cannot be plotted on the logarithmic axes. These all showed high methane production, of the order 25 ml of methane (40 ml of total gas at over 50% methane) over the first 7 days, and tending towards a final yield of the order of 500 ml methane by day 155.

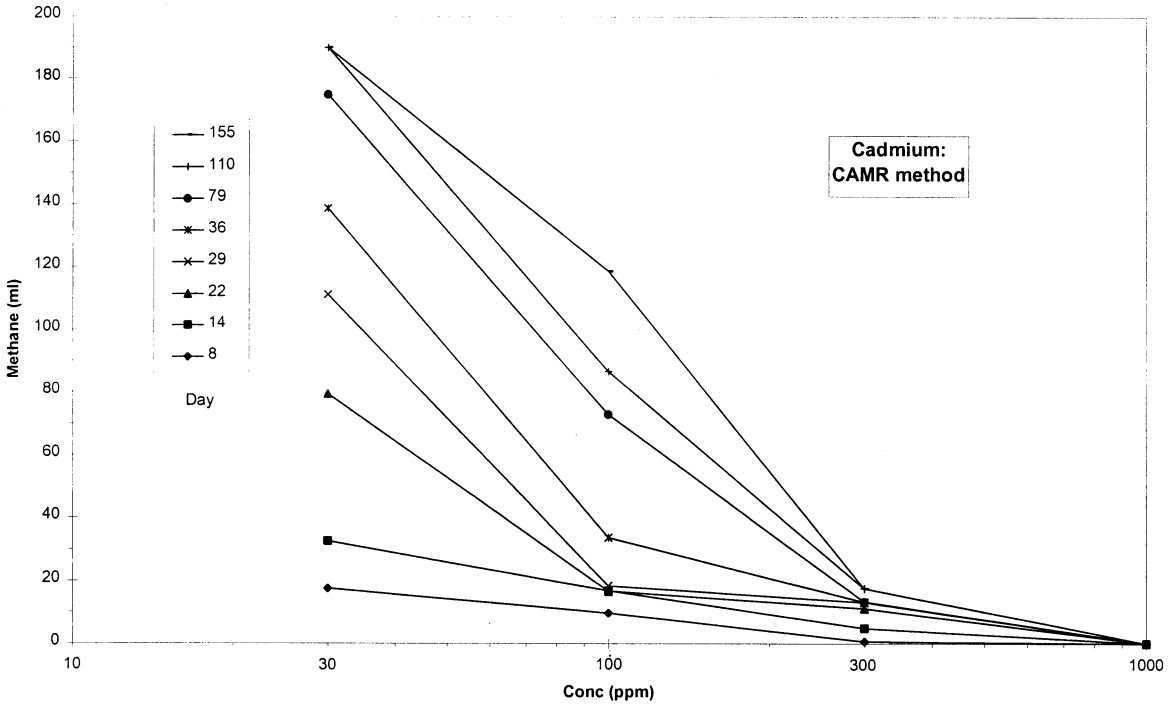

Figure 10 **Cadmium to 155 days, CAMR method IC_{100} 300-1000 ppm**

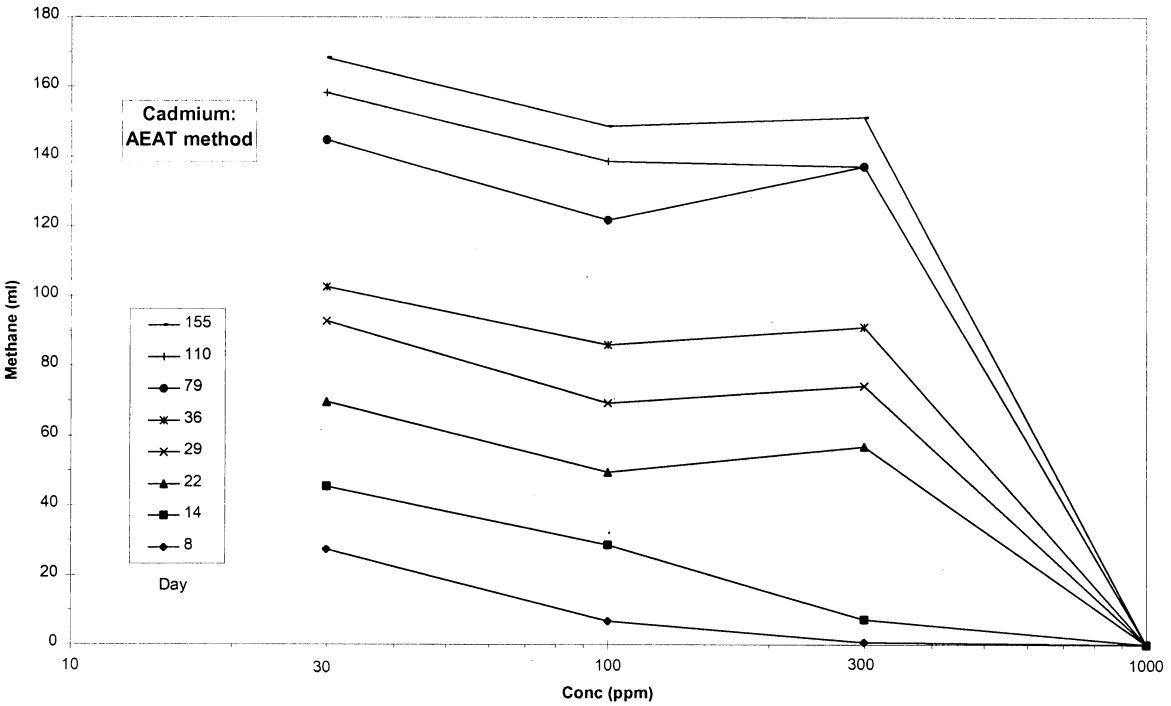

Figure 11 **Cadmium to 155 days, AEAT method IC_{100} 300-1000 ppm**

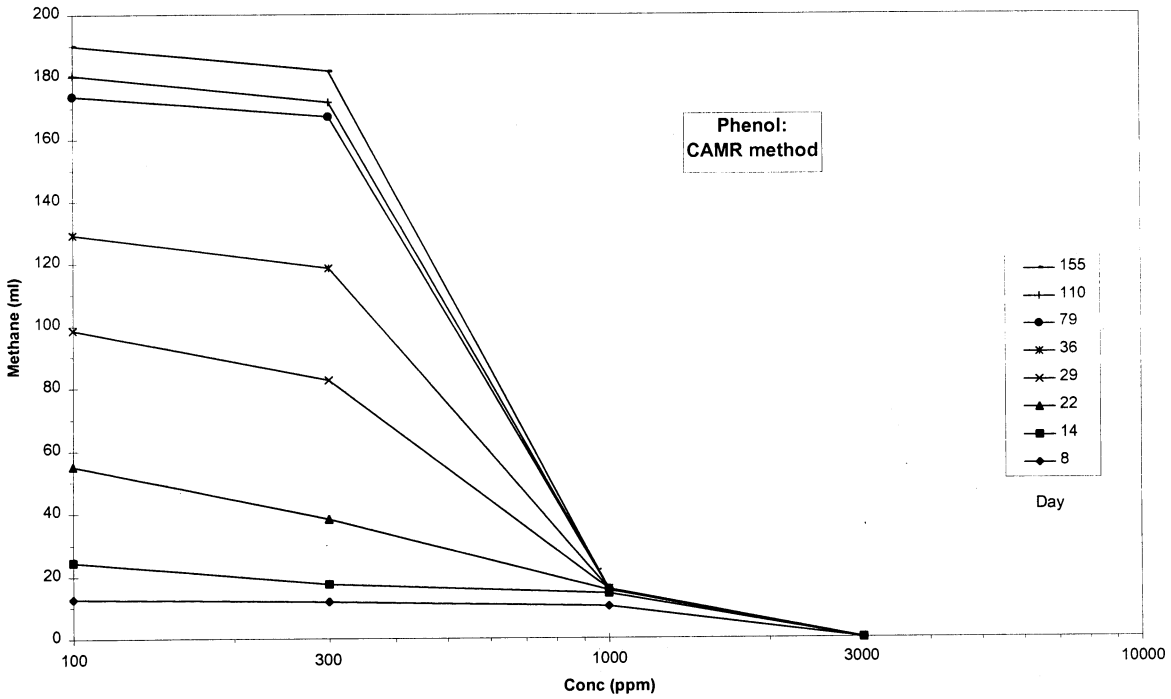

Figure 12 **Phenol to 155 days, CAMR method IC_{100} 1000-3000 ppm**

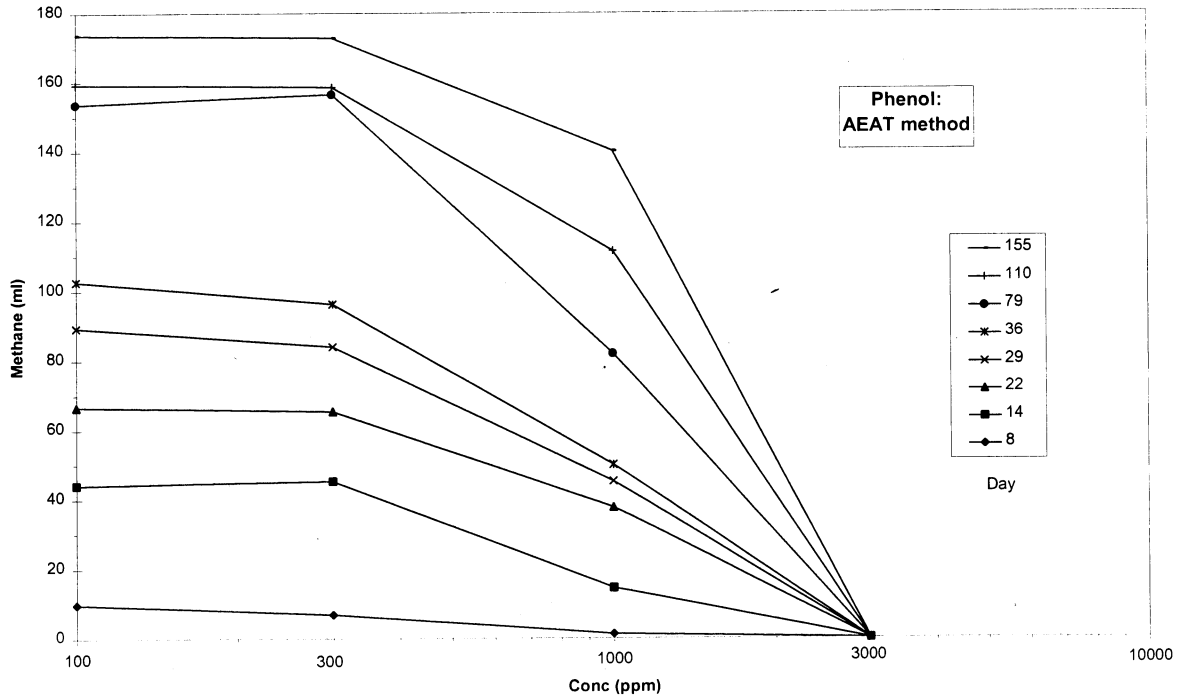

Figure 13 **Phenol to 155 days, AEAT method IC_{100} 1000-3000 ppm**

The figures presented show the cumulative dose-response curve on each sampling day. The IC_{50} (50% inhibitory concentration point) has not explicitly been marked as this drifts upwards over time for all the tests. This assessment of the data has highlighted the potential weakness of using the IC_{50} value to rapidly classify a waste, and leads AEA to recommend that the IC_{100} (complete inhibition - estimated by the first dilution to show no methane) be used as the most practical end-point for an acceptability test.

In the tests performed at AEA, the IC_{100} at 155 days was in every case precisely the same as that determined at just 7-8 days, suggesting that a rapid and reliable assessment of acceptability could be made using a one-week acute toxicity test. Furthermore, the IC_{100} as determined by the simplified L-ABT method and the original CAMR L-ABT method agreed perfectly, suggesting that the simplifications had no negative impact on any critical aspects of the test.

At the completion of this test, the opportunity was taken to assess the distribution of the toxin in two different dilutions of the cadmium test system. Cadmium can be chemically transformed but cannot be degraded, so the full inventory must be conserved in the test bottle. Samples of the liquid and solid phases were analysed by ICP-AES (Integrated Coupled Plasma Atomic Emission Spectrometry - Table 1).

Table 1 O-ABT version 1 - cadmium analysis at day 155

Sample	Cd added (mg/100ml)	Actual Cd found (mg/100ml) triplicates	Recovery (%)
Supernatant	30	*0.020*	*0.07%*
Solids		*7.90*	*26%*
Supernatant	10	*0.002*	*0.007%*
Solids		*2.43*	*24%*

Values shown are means of triplicate determinations

Of the cadmium added, less than 0.1% remained in solution at day 155, and approximately 25% was recovered in the filtered solids. The balance, approximately 75%, had presumably adsorbed to the glassware and bung. Cadmium is known to adsorb strongly to many materials. These observations illustrate the importance of knowing the way in which toxins partition in landfill waste before attempting to use bioassay data to advise on loading rates. In this instance the results suggest that the O-ABT should not be used for long-term toxicity assessment, since most of the cadmium becomes biologically unavailable over time.

Summary

The IC_{50} value was defined as that concentration at which methane yield was 50% of the uninhibited maximum, as determined from the smoothed interpolation of the dose-response curve - so was expressed as a single value. This value proved to be time-dependent. The IC_{100} was defined as that concentration causing complete inhibition of methane production, which was between the last dilution showing any methane and the first to show no methane - so was expressed either as a range or, more simply, as the first dilution showing complete inhibition.

These were largely time-independent, i.e. once a test bottle was completely inhibited, methane production rarely recovered.

The overall conclusion from these initial tests was that in the short-term the simplified method gave similar toxicity results to the original method developed at CAMR. Tables 2 and 3 summarise the data. The IC_{100} value was stable and reliable in as little as 8 days, and the IC_{50} was both quantitatively more sensitive (in as little as 8 days) and qualitatively useful in indicating adaptation potential (in as little as 28 days).

Table 2 Summary of IC_{50} inhibition values obtained in comparison of simplified O-ABT method and original CAMR L-ABT method

Method	AEAT	CAMR	AEAT	CAMR
Toxin	IC_{50} 7-day test (ppm)		IC_{50} 60-day test	
TCE	n	n	n	N
Phenol	50 [30-100]	1500 [1000-3000]	1000 [300-3000]	500 [300-1000]
Cadmium	50 [30-100]	i	500 [300-1000]	50 [30-100]

n = not inhibitory in tested range
i = indeterminate (bimodal)
[brackets] show range of value (± one dilution)

Table 3 Summary of IC_{100} inhibition values obtained in comparison of simplified AEA method with original CAMR method

Method	AEAT	CAMR	AEAT	CAMR
Toxin	IC_{100} 7-days (ppm)		IC_{100} 155-days	
TCE	N	n	n	n
Phenol	1000-3000	1000-3000	300-1000	300-1000
Cadmium	300-1000	300-1000	300-1000	300-1000

AEA and CAMR methods compare very closely
IC_{100} value is stable from 7 days onwards
n = not inhibitory in tested range

2.5 Further Simplification of the O-ABT

In the second batch of testing:

- the reproducibility of the short-term test was checked by repeating O-ABT Version 1; and
- further simplifications were introduced, notably dispensing with some of the more expensive and potentially hazardous or less biologically-important trace elements.

Many trace elements are required only in vanishingly small quantities. In a fully-defined laboratory system they must be expressly added to be sure they are present, but in the O-ABT

it was expected that the added milled waste would supply most if not all trace element requirements. Only the cadmium test was repeated. This version is termed O-ABT Version 2.

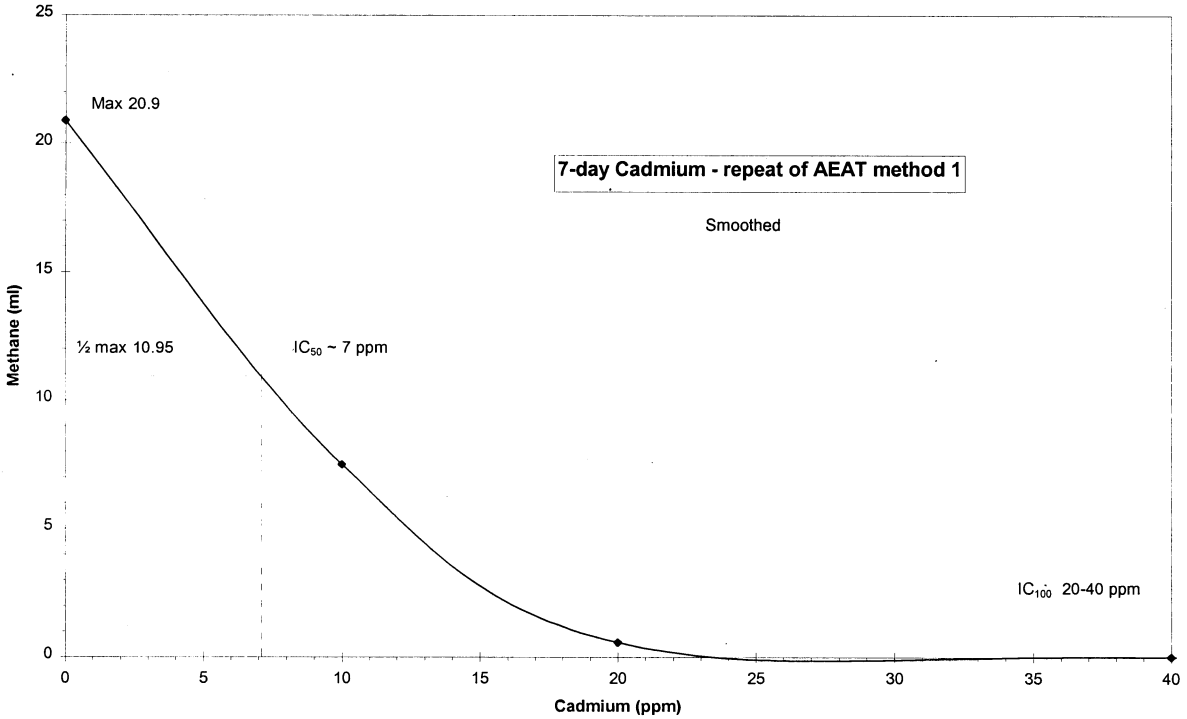

Figure 14 Cadmium, first repeat, 7 days (linear scale required - see text)

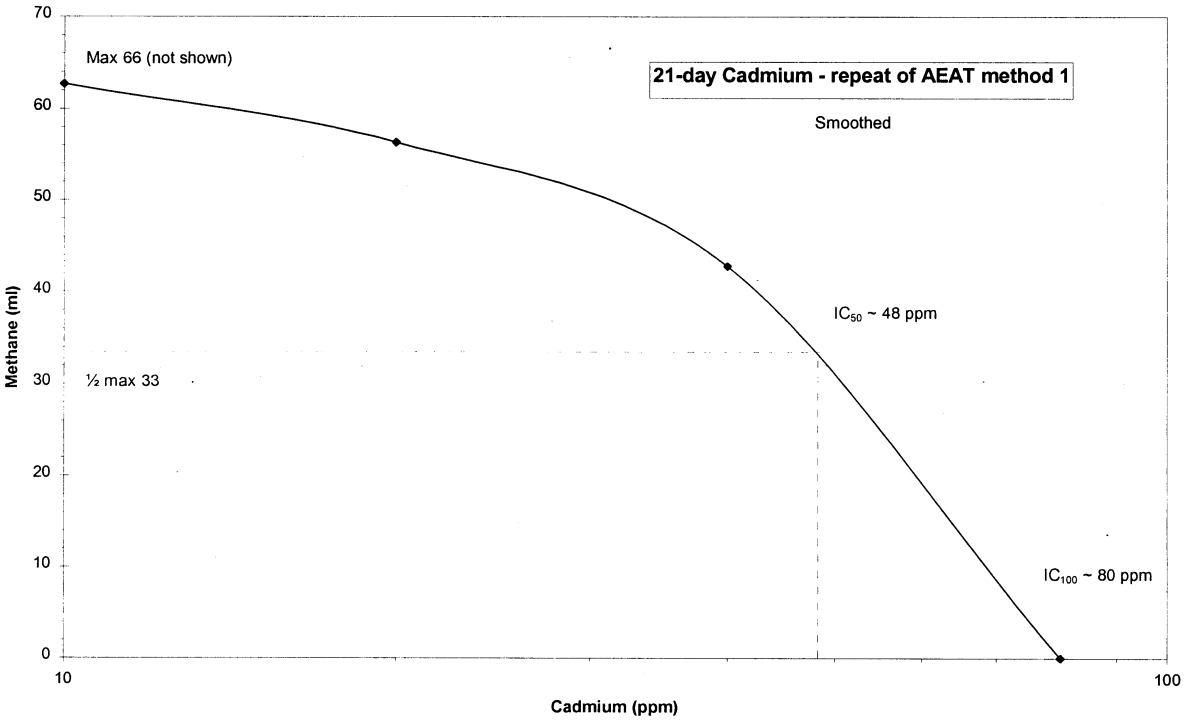

Figure 15 Cadmium, first repeat, 21 days

The repeat of Version 1 gave a rather lower IC_{50} value (~7 ppm) at 7 days (Figure 14) than the previous experiment had done at 8 days (~50 ppm). In addition it did not give the expected logarithmic response (note that Figure 10 is a linear-linear plot) and there was an indeterminate IC_{100} (estimated at 20-40 ppm). At 21 days (Figure 15) the IC_{50} had increased to ~48 ppm, and the IC_{100} had stabilised at ~80 ppm.

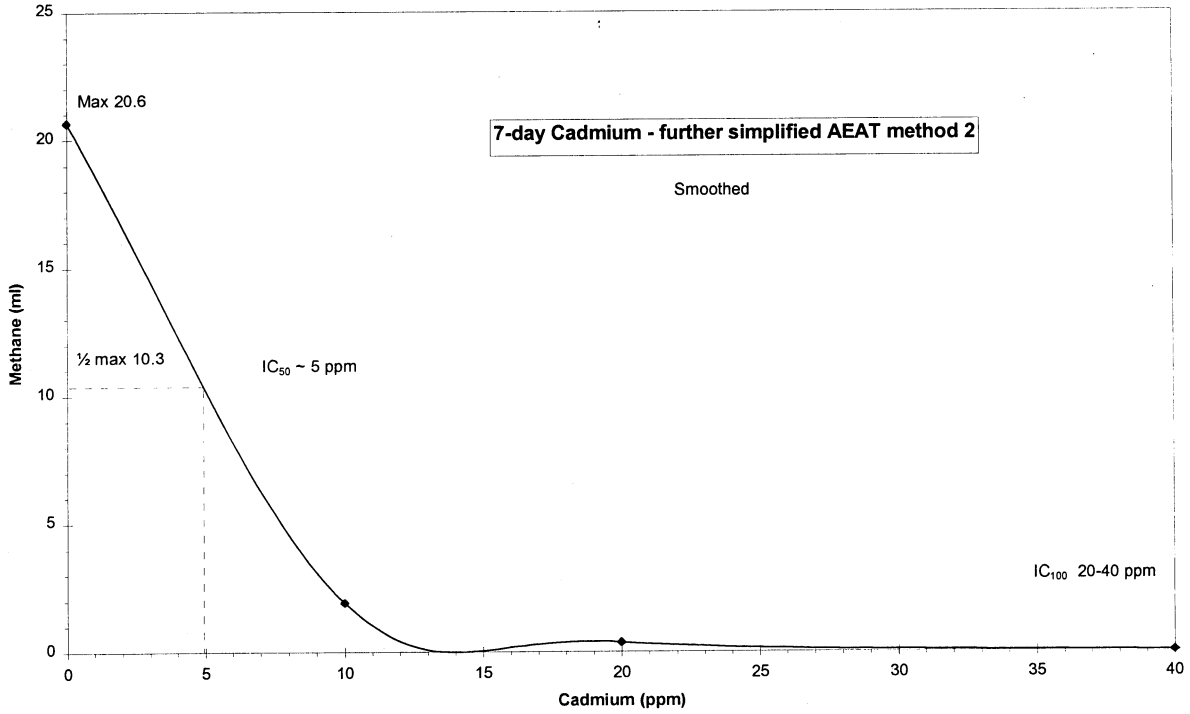

Figure 16 Cadmium, second repeat, 7 days

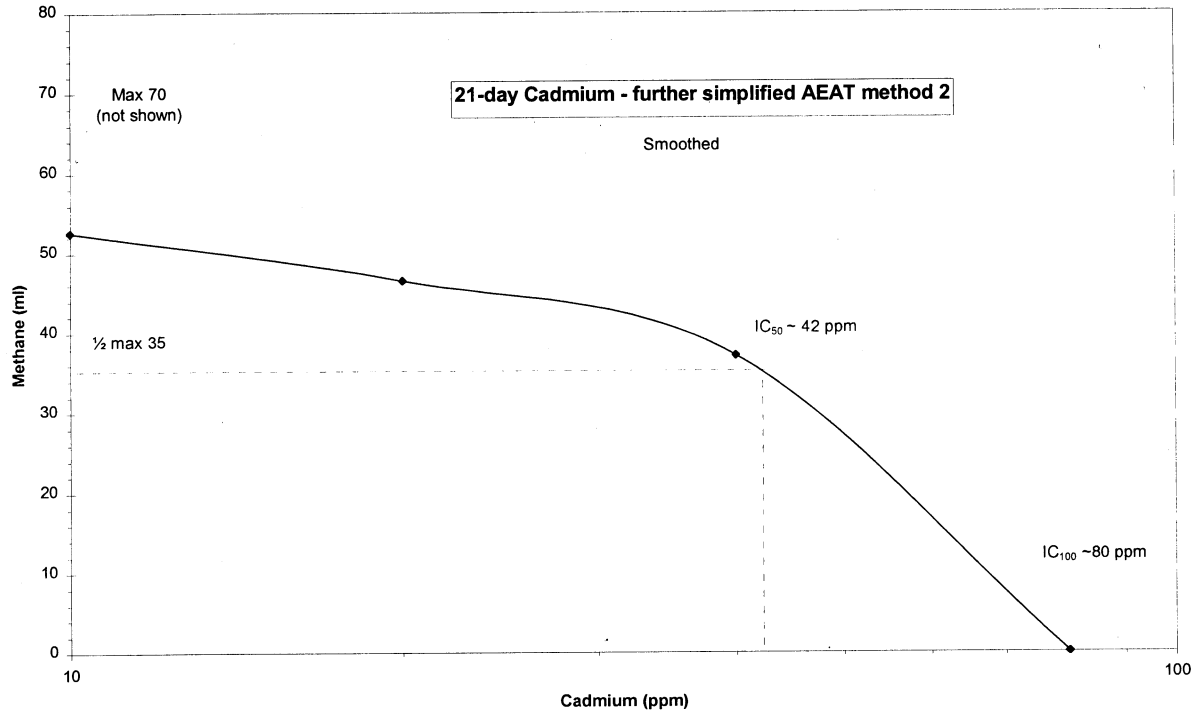

Figure 17 Cadmium, second repeat, 21 days

Inhibition by cadmium using simplified Version 2 gave an IC_{50} value of only ~5 ppm at 7 days (Figure 16), not statistically significantly different from the Version 1 repeat, but possibly indicating even greater sensitivity in the absence of certain trace elements. At 21 days the greater inhibition was still apparent, with an IC_{50} value estimated at ~42 ppm (Figure 17). Note that these small differences are not significant considering the doubling dilution steps employed. The IC_{100} stabilised at ~80 ppm, identical to that determined by O-ABT-1, suggesting that further simplification had not materially changed the sensitivity of the test.

Acidification of the cultures was possibly the cause of this instance of increased sensitivity of both the IC_{50} and the IC_{100}. A check of both sets of test bottles after incubation revealed a pH of ~5.5 ± 0.3. The work book had no record of final pH checking and adjustment of media prior to use, suggesting that this step may have been inadvertently omitted. At this pH methanogens would be directly inhibited, and metals would be highly mobile in solution, enhancing toxicity. In subsequent work particular care was taken in adjusting the pH of seed, nutrient and toxin solutions to pH 7.0 ± 0.1 just before use.

The seed culture was demonstrably gassing just prior to use, but it is possible that the seed culture was sub-optimal at the time of use (see discussion of SME trials). Biological systems are inherently variable, because the total biomass, balance of species numbers and rates of activity all change constantly. Use of, and comparison with, an appropriate defined standard toxin such as phenol may partially resolve this problem (see section 4. Discussion).

2.6 O-ABT Version 3

The cadmium test was re-run using the conditions of Version 1 but taking care:

- to adjust pH of all solutions and suspensions to 7.0 ± 0.1.
- to use more closely-spaced dilutions of cadmium (0, 5, 10, 20, 40, 80, 160 and 320 ppm) in order to achieve a more accurate IC_{50}.

In addition to positive controls, the test series was started at a lower cadmium dilution, to ensure that some tests would show relatively uninhibited gas production that could be plotted on a logarithmic axis.

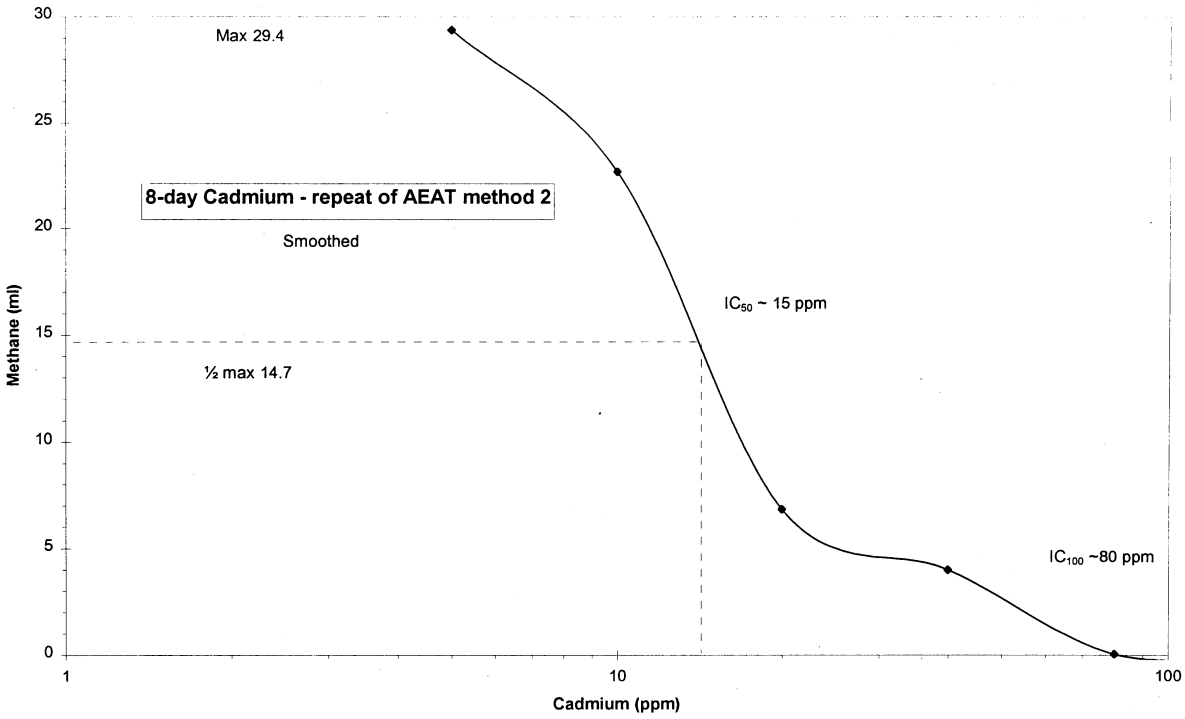

Figure 18 pH-corrected Cadmium, 8 days

Methane inhibition by cadmium at 8 days (Figure 18) gave an IC_{50} of ~15 ppm, certainly between 10 and 20 ppm. At 30 days (Figure 19) the Version 3 run gave an IC_{50} of ~60 ppm (between 40 and 80 ppm). The IC_{100} was ~80 ppm, and was stable from 8 days onward.

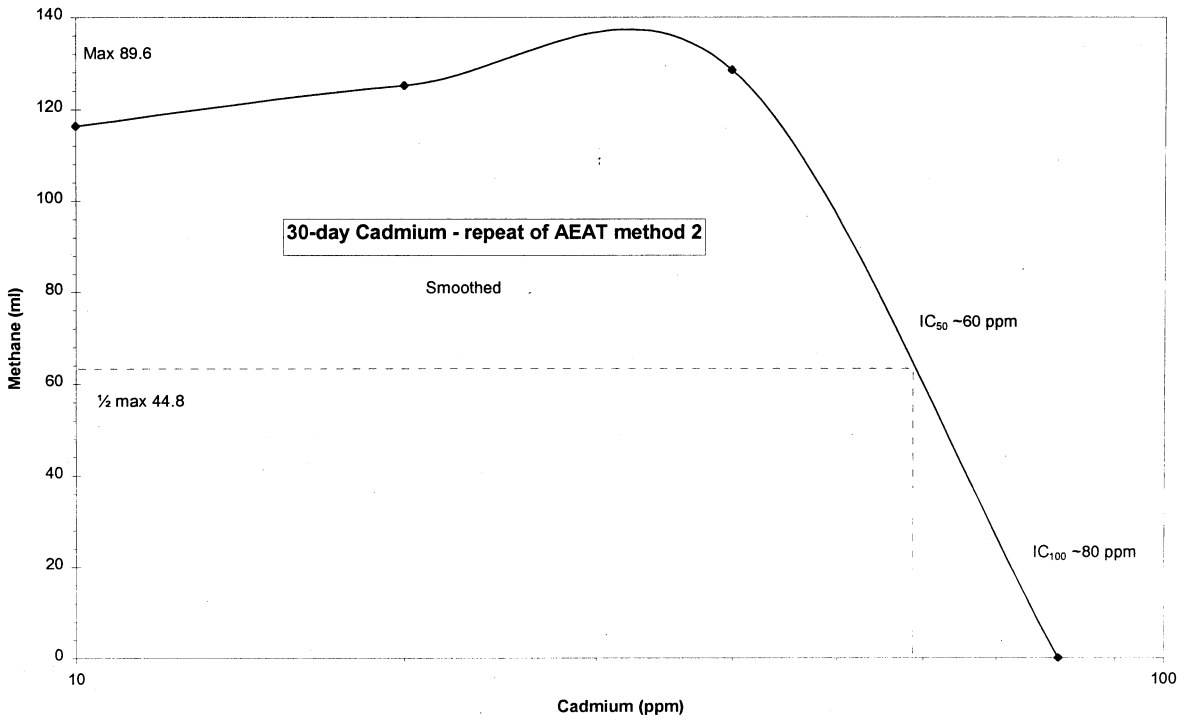

Figure 19 pH-corrected Cadmium, 30 days

These tests showed that the cultures were less sensitive to cadmium than the preceding low pH test as judged by the IC_{50}, but the complete inhibition value, the IC_{100}, was the same as the second run, and was significantly lower than the original comparative run. Correcting the pH therefore changed the IC_{50} but not the IC_{100}.

A drift in the sensitivity of the seed culture would probably explain this trend. As a guide, it is considered good laboratory practice for a new culture to be incubated for up to five hydraulic retention times before stability can be assured. At the rate of 10% per week change, it would take up to a year for the culture to fully stabilise. However, our seed culture was gassing healthily from the first week, but began to show erratic gas production by month 4 or 5. Since the feedstock comprised milled solid waste (containing a high proportion of recalcitrant/inert materials), prolonged incubation would cause these to accumulate. In addition, other recalcitrant or even toxic end-products may have been accumulating. The seed had been incubating for about 3 months when used for versions 2 and 3. Regular renewal of the seed culture is probably the simplest and most cost-effective solution (see section 4).

3 METHOD EVALUATION

The results and experience gained in developing a simplified ABT were used to write a preliminary Standard Operating Procedure. The Industrial Advisors discussed this procedure and a draft Standard Operating Procedure (SOP) was agreed. This formed the basis of the method evaluation conducted by SME at their Kempston laboratory. A final version of this SOP, incorporating later recommendations, is attached as Appendix 3.

The staff at Kempston had no specific training in microbiological techniques and the laboratory was not specially designed or fitted for testing anaerobic micro-organisms. The laboratory staff had observed the work at AEA and were given advice by a trained microbiologist but the bulk of the work was guided by the written draft SOP.

The principle objectives of this part of the work were:

- to examine the practical implementation of the method in an operator's laboratory;
- to examine the reproducibility of the method in a non-specialist laboratory;
- to examine the effects of temperature change;
- to test both pure toxins and candidate co-disposal wastes;
- to identify and address any further practical improvements;
- to identify any changes in the draft SOP and revise accordingly.

3.1 Adaptation of the Draft SOP

In the light of SME comments the draft Standard Operating Procedure was altered to facilitate work in the operator's laboratory. These modifications are outlined in Appendix 4, and include:

- correction of pH once, just prior to use (avoiding time-consuming multiple adjustment);
- avoiding prolonged boiling of the media;
- modifying the sampling device used to remove gas samples from the bottles;
- modifying the way toxins were added to the samples (to deal with the viscosity of phenol solutions and the low solubility of TCE).

3.2 Performance of the Seed Culture

The management of the seed culture was the only significant problem encountered in the operator's laboratory. An initial 10 litre culture was set up in April 1996 using 2.5 litres of the AEA seed (at a nominal 10% solids content), added to 7.5 litres of the recommended nutrient solution, and 50 grams of sorted and milled household waste from AEA. This gave a nominal solids content of 7.5%. This culture was soon producing some 100 - 200 ml of gas per day. The SOP required that 500 ml of this culture was replaced each week with 500 ml of the nutrient solution and 50 grams (wet weight) of milled household waste, thus allowing the solids content of the culture to gradually build up to ~10%. The rate of gas generation gradually increased, so that by the end of June total gas output was recorded at more than one

litre per day. However, by August (after 4-5 months operation) gas production gradually became more erratic.

Most solids were noted floating on the top of the liquid, presumably due to trapped gas bubbles. This might have led to a reduction in the contact between the anaerobic bacteria (which adhere to the solids), and the available substrates (mainly in solution). The cultures were not mechanically stirred and regular shaking dispersed the solids temporarily although the problem recurred. In an attempt to minimise this problem the solids content in the vessel were progressively reduced to 5% by the addition of 500 ml of nutrient and 25 grams of household waste each week (from week 10 onwards). As a result gas production was lower but less erratic (Figure 20).

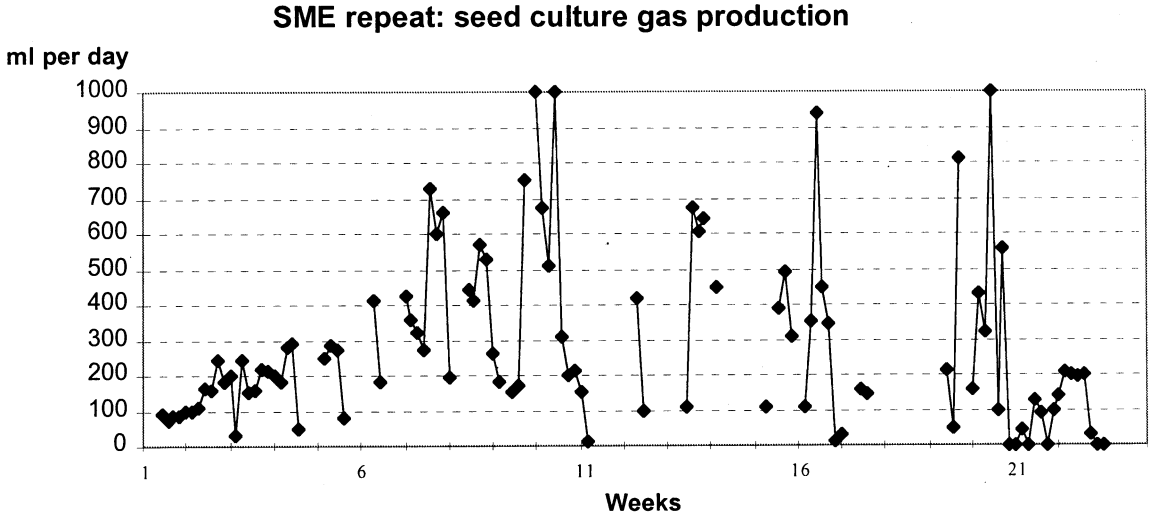

Figure 20 SME repeat; seed culture gas production over time

The pH of the liquor discarded each week remained in the range 7.0 - 7.3, so souring was not occurring. More sophisticated methods for maintaining seed cultures are possible (e.g. cultures used for the Biological Methane Potential tests by Minton, Treharne and Davies (personal communication). However, these require more routine attention, and at present it appears to be practical and inexpensive to establish a fresh culture at quarterly (no greater than 4 month) intervals. This aspect of the SOP should however be reviewed in the light of ongoing experience.

A second seed culture was set up at the end of July using a new batch of aged household waste. The household waste was prepared by taking solid waste collected from an 8-10 year old section of Calvert landfill site. It was sorted by hand to remove all visible non-putrescibles (plastic, metal, glass) then shredded with an industrial food processor to obtain a finely divided solid. Approximately 1 kg of this material was prepared and stored in a refrigerator. Storage as a dried powdered material was considered, but drying and loss of volatile compounds would probably have significantly reduced the bioavailable carbon content. This material was used to initiate and maintain a reactor running at ~5% solids. The

seed culture was soon producing gas, generation being somewhat erratic from day to day, but on average yielding 125 ml per day volumes of gas.

3.3 Results from Application of Operational ABT

Nine tests were set up by SME over a period of 3 months. The first three tests were intended to replicate the tests performed at AEA using TCE, phenol and cadmium as the toxins, and were incubated at 35°C. A month later the phenol test was repeated at 35°C, and also at room temperature (20-30°C). The final tests were with four example liquid industrial wastes, all tested at 35°C. Tests on the four candidate liquid co-disposal wastes were conducted using the new seed cultures, standardised by running a further phenol test in parallel.

Phenol

The initial phenol test gave an IC_{100} of 2560-5120 ppm phenol, which was very similar to the value obtained by AEA in their tests, and did not change after week 1 (see Figure 21). The 10 to 80 ppm levels of phenol gave gas volumes very similar to that of the blank, so after 5 weeks analysis of these bottles was discontinued.

As the experiment progressed, at the concentrations of toxin just below the IC_{100} there was an enhanced volume of methane when compared to the blank bottles. This was presumably due to degradation of the phenol itself (i.e. a higher amount of organic carbon in these bottles). This is similar to the effect noted with TCE by AEA. The observation of phenol-degrading organisms in landfill is not particularly surprising.

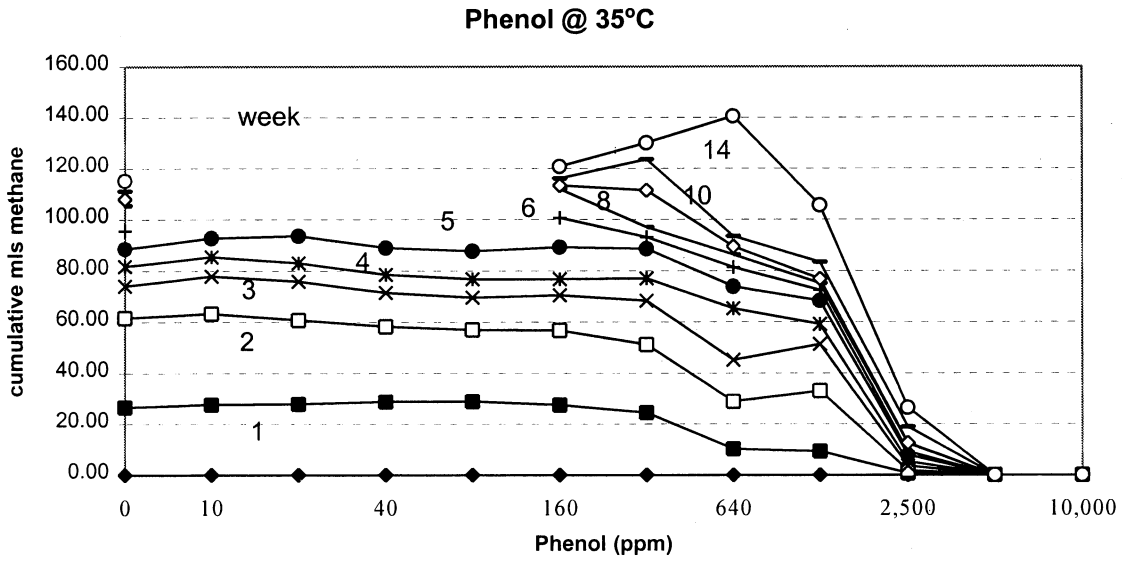

Figure 21 SME repeat, phenol at 35°C

Cadmium

The highest level of cadmium tested was 1000 ppm (see Figure 22). This eventually produced a few ml of methane after 3 months, which we discounted in the light of the earlier study showing loss of Cd by adsorption over time. The value for the IC_{100} was therefore very

similar to that found by AEA in the preliminary work. Analysis of the lower concentrations was discontinued after 5 weeks and the frequency of testing was reduced to every fourth week once the pattern of inhibition was established.

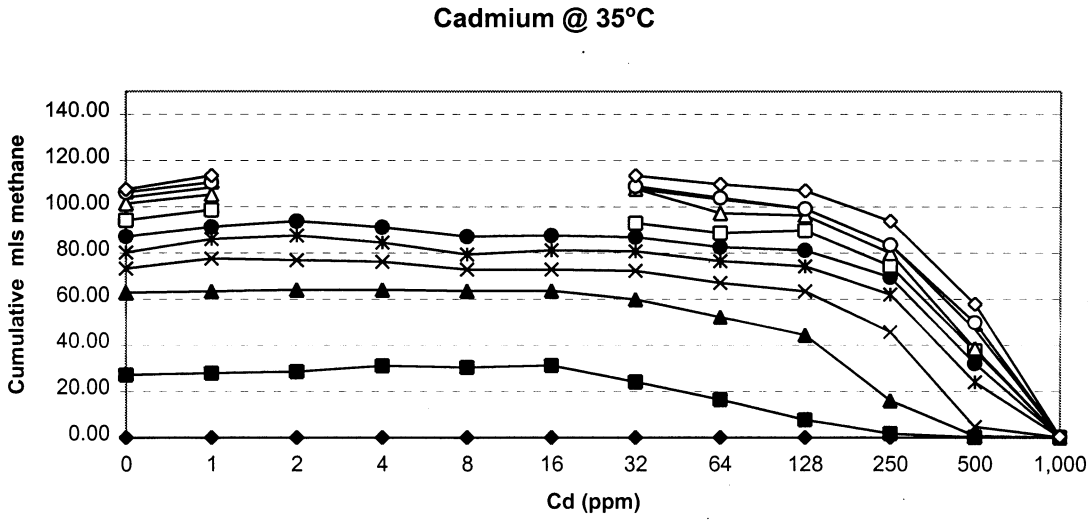

Figure 22 SME repeat, Cadmium at 35°C

Trichloroethylene

The highest level of TCE tested was 640 ppm w/v, a high concentration relative to the reported CAMR inhibition value of 1 ppm. This was chosen in the light of non-inhibition at up to 10 ppm found in the AEA tests, to be sure of finding the IC_{100}. The amount of methane produced by the 80 to 320 ppm TCE tests was fairly small, giving a much flatter pattern to this area of the graph, and an overall sigmoid shape (see Figure 23). After 4 weeks even at 640 ppm the tests had produced small volumes (< 1 ml) of methane.

This may relate to the low water solubility of TCE, which would naturally limit the concentration of TCE actually experienced by the organisms, allowing survival, then adaptation and growth, of the tolerant or degrading organisms. If this occurs with other insoluble toxins then the liquid-based test may prove inappropriate for these. However, by adhering firmly to the 7-day interpretation, a clear IC_{100} of 320-640 ppm was determined, which in practice would safely over-estimate the toxicity in-situ.

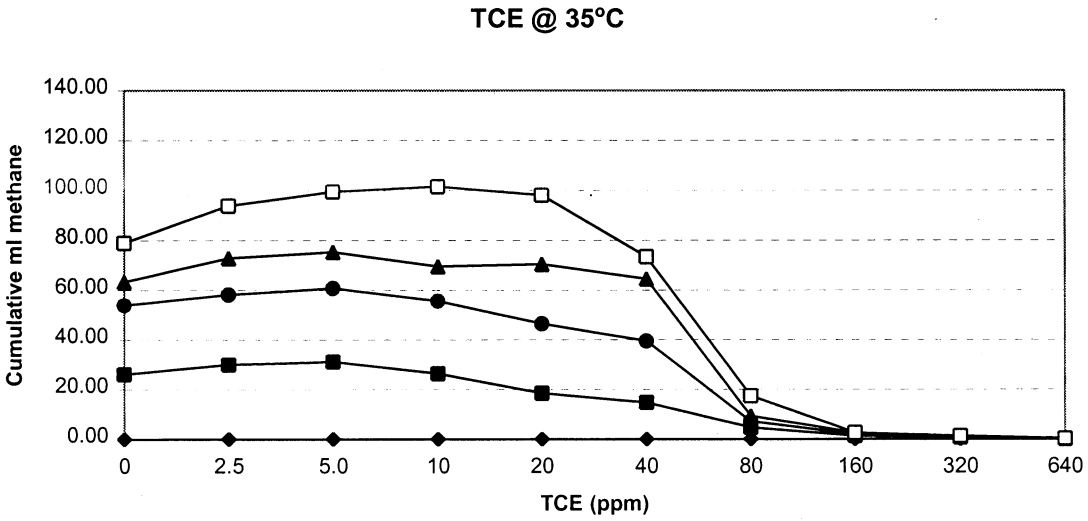

Figure 23 SME repeat, TCE at 35°C

The appearance of the test mixture was a good indication of whether the bottles were producing methane. The tests that were producing methane were black in colour, presumably due to production of sulphides by sulphate-reducing bacteria, whereas inactive tests were always paler in colour. With experience one could probably estimate the IC_{100} fairly accurately by eye, although we cannot recommend this.

In these tests the value for IC_{50} was not easy to establish, as it changed with time. This was illustrated in the first phenol test (Figure 21), where after 2 weeks the IC_{50} would have been below 640 ppm phenol, but by week 14 the IC_{50} was well above the 1250 ppm toxin concentration. By contrast, the IC_{100} was usually unambiguous.

3.4 Evaluating Critical Factors

3.4.1 Temperature

The Industrial Advisory Group (IAG) recommended that the effects of temperature on the ABT were evaluated, since this parameter might be difficult to control in some laboratories. The IAG wondered in particular whether an incubator was strictly required.

The O-ABT using phenol was repeated at 35°C and at room temperature. Although methane production was slower at room temperature, both experiments showed a similar pattern of inhibition and the same IC_{100} (Figures 24 and 25). Initially no methane was produced at 2500 ppm phenol at 35°C, giving an IC_{100} of 1280-2560 ppm, but by 3-4 weeks as the bacteria became acclimatised to the toxin, or as it adsorbed/detoxified, measurable amounts of methane were produced at this concentration. These figures suggest that it may take a month or more before a stable IC_{100} can be obtained, but week 1 or 2 is probably optimum for acute toxicity. The results were virtually identical to the first run (see Figure 21).

The gas production from the phenol bottles incubated at room temperature (approximate mean 25°C, and varying from <20°C to ~30°C, during the test period) was much lower than at 35°C. Over the 7 weeks of incubation they produced approximately half of the volume of gas compared to those incubated at 35°C. However, the methane concentration and cumulative methane production trends suggested that the end result would eventually be the same as those incubated at 35°C. Gas production at week 1 was so low that it was difficult to measure accurately, but by week 2 a clear IC_{100} was determined. Most significantly, precisely the same IC_{100} was estimated as at 35°C, with the bottles containing 5280 ppm toxin producing no gas, and those containing 2560 ppm phenol producing just a trace of gas. So, although the test conducted at room temperature gave the same IC_{100} of 2560-5280 ppm phenol, at low and variable temperatures a longer incubation was required before an assured value was reached.

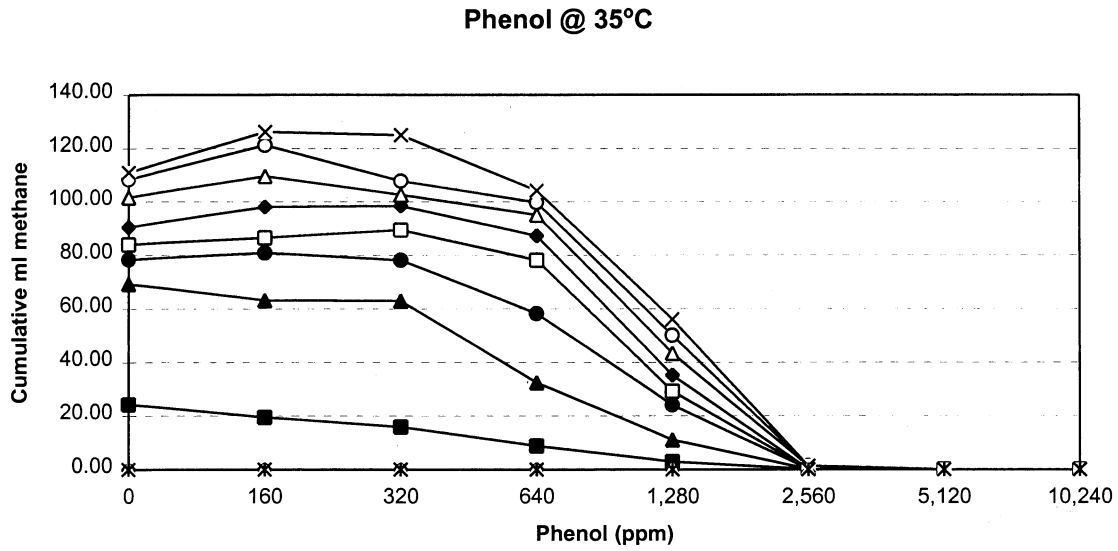

Figure 24 **SME second repeat of phenol at 35°C**

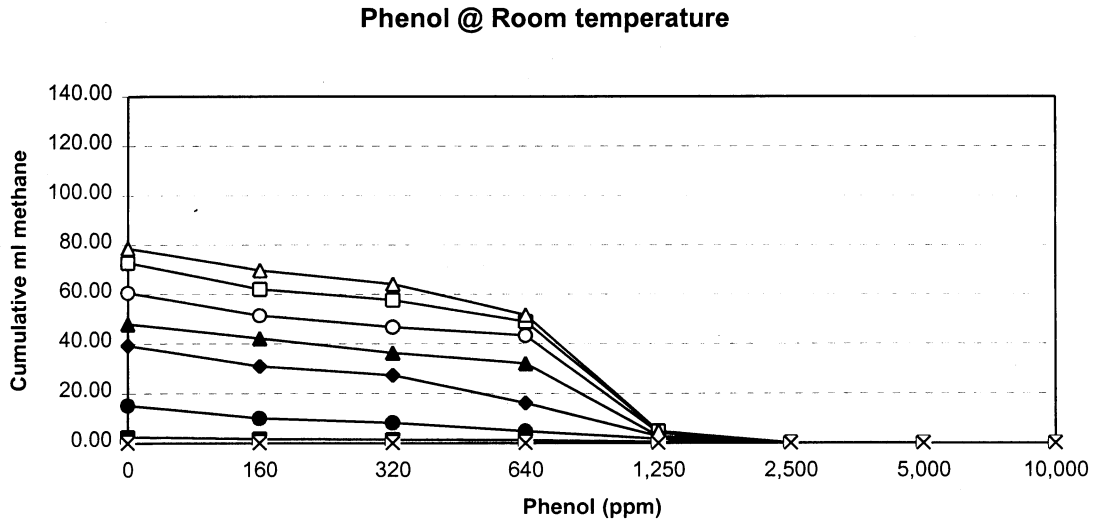

Figure 25 **Repeat of phenol at ambient temperatures**

3.4.2 Reproducibility

Figure 26 shows the mean of triplicate cumulative methane volume produced by the blank bottles for:

- 4 sets run in parallel with the SME repeat of phenol, Cd and TCE (labelled a-d);
- 3 sets run in parallel with the temperature test (e & f at 35°C, g at ambient).

The first 4 tests run at 35°C, a-d, showed very high reproducibility. The second sets at 35°C, e & f, showed slightly lower but not statistically different yields, perhaps an early sign of the seed culture losing viability. The mean yields at any time interval were very similar, and the standard deviation of each week's figures was extremely good, with a standard deviation of about ±5% of the mean methane production. The ambient test, g, showed a lag phase with slower gas production over the first week, but then recovered to a large extent after the second week. The lag in g may also have been associated with deterioration of the seed culture as noted above, since there was also some evidence of a lag in f, the last test run at 35°C.

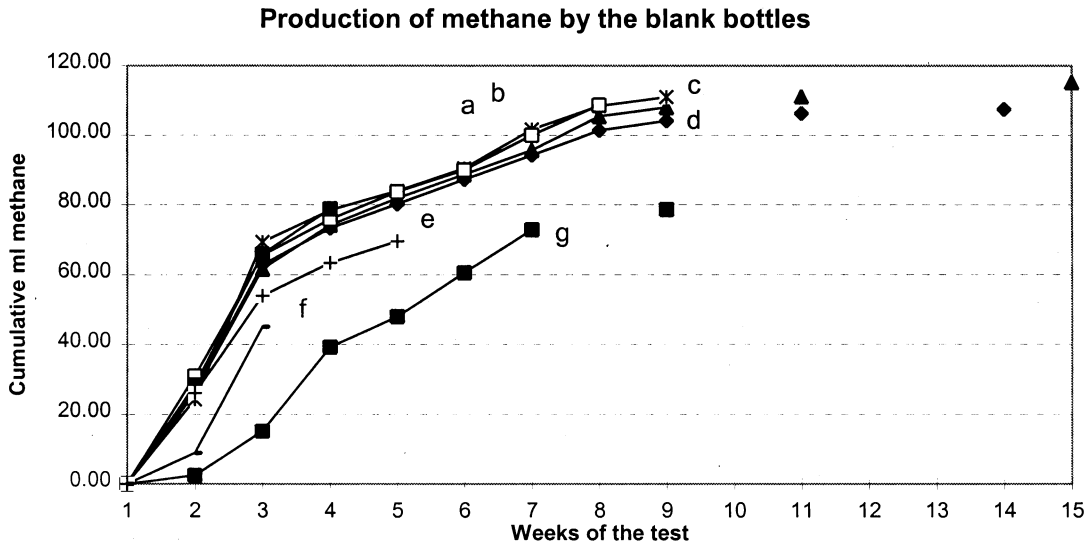

Figure 26 SME repeats; toxin-free control blanks

3.4.3 Further simplifications

In the original draft SOP individual nutrient solutions and seed were adjusted to pH 7.0 ± 0.1 then combined and checked again just before use, the usual practice in laboratories handling pure cultures in defined media. SME asked if it was possible to simplify this in order to save time and effort.

After some experimentation it was found that combining nutrient solutions without causing precipitation was possible, simplifying dispensing of reagents and allowing a single final pH check and adjustment just prior to use.

3.4.4 Final pH checks

It was impractical to check the pH of the solution after the addition of the toxin, or during the test programme. However, it was thought possible that the acidity of phenol at 10,000 ppm could have altered the pH of the solution in the bottles sufficiently to alter the apparent IC_{100} found. To see whether this effect was significant, as each phenol test was completed the pH of the solution in the bottles was measured. The pH was found to be within the acceptable range for methanogenic activity in all sub-IC_{100} bottles, suggesting that any acid effects due to phenol were adequately buffered.

In some bottles at or above the IC_{100} the pH had fallen. Given earlier AEA observations of hydrogen accumulation near the IC_{100}, indicating continued hydrogen production in the absence of methanogenesis, this fall in pH was probably a result of the continued activity of acid-producing organisms in the absence of active acid-consuming methanogens. It was therefore concluded that the test reagents plus added waste provided sufficient buffering of any effects from the toxin itself.

3.5 Comparison of Results from AEA and SME Laboratories

The reproducibility of the test between laboratories was acceptable for both phenol and cadmium (Table 4). The value of the IC_{100} for phenol was consistent between replicate tests and at the two different temperatures employed (i.e. 35°C and room temperature). An IC_{100} for TCE was established at 320-640 ppm. The reproducibility of the methane production in the blank bottles was good, but if the seed culture was used for more than four months the gas yields began to fall and variability began to increase.

Table 4 Summary of 7-day toxicity data from the O-ABT conducted in both laboratories

Toxin	AEA IC_{100} (ppm)	SME IC_{100} (ppm)
TCE	n [>10]	320-640
Phenol	1000-3000	2560-5120
Cadmium	300-1000	512-1024

n = not determined

3.6 Application to Real Wastes

Four liquid industrial wastes, typical of those sent to landfill operator's laboratories, were tested by SME, using the revised SOP.

The first sample (designated SN 8793) was an effluent with a Chemical Oxygen Demand (COD) of 310,000 ppm, and containing 11,000 ppm cyanide (Figure 27). This showed an IC_{100} of 10,000 ppm, (i.e. a 1% concentration of whole waste; 1 ml of the waste added to a bottle containing 100 ml of nutrient and seed). At concentrations of the waste just below this value enhanced production of methane was observed, presumably due to bacteria degrading components of the waste (and hence producing more methane than the control).

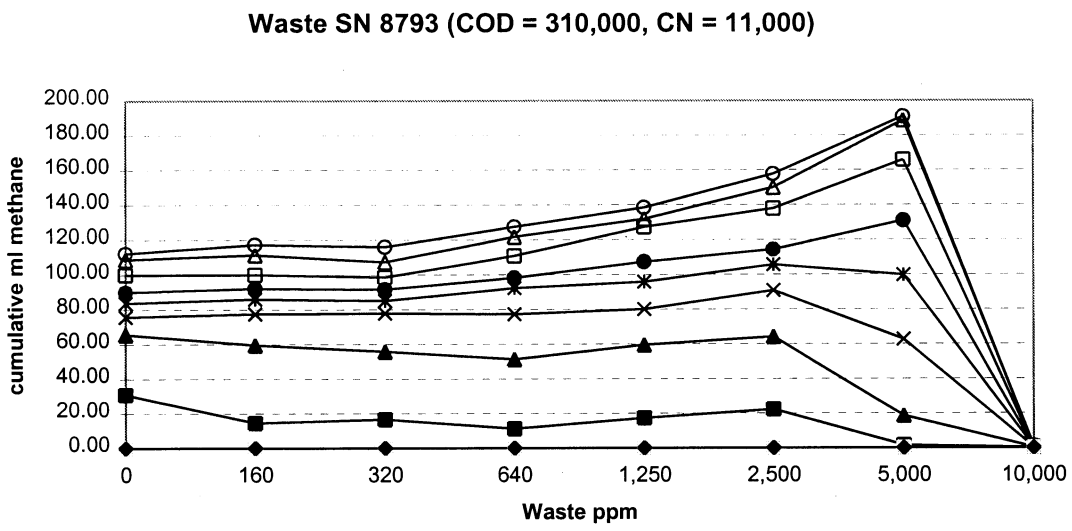

Figure 27 Test waste SN 8793

The second effluent tested (SN 9570) was from a pharmaceutical manufacturer and had a COD of 110,000 ppm. At the maximum concentration tested (1%) it had no effect on the amount of methane produced, either positive or negative, so the test was discontinued after 3 weeks (Figure 28).

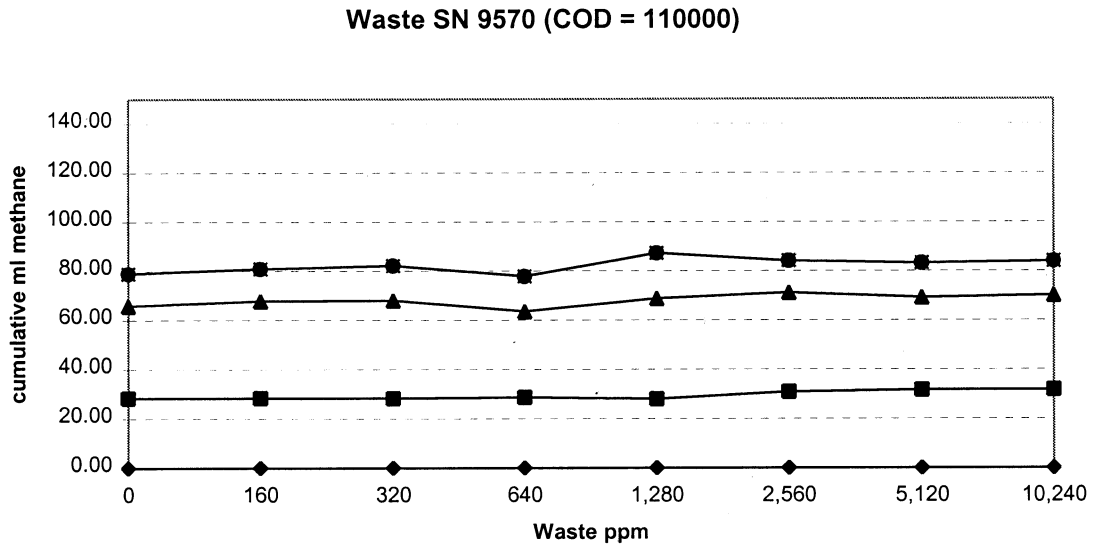

Figure 28 Test waste SN 9570

Figure 29 shows results to date from tests on a third liquid waste, designated SN 9438, which is ongoing. The data available suggests a sigmoid curve similar to that seen for TCE (see Figure 23). The IC_{100} at 2 weeks was 5000-10,000 ppm, and may eventually be assigned in excess of 10,000 ppm, i.e. it is relatively non-toxic. Erratic gas production was observed from the seed culture used at that time, but the effects of this cannot be determined. A phenol standard run in parallel would possibly have been useful.

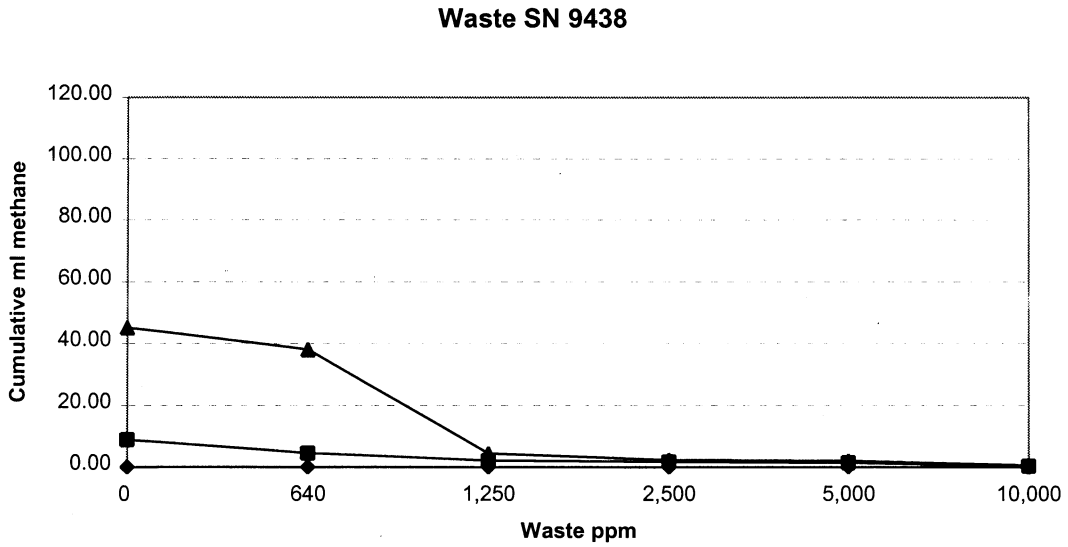

Figure 29 Test waste SN 9438

The fourth industrial waste (designated SN 9514) was a neutralised phenol sulphonic acid, with a COD of 15,000 ppm. This waste showed an initial inhibition of the gas production at 10,000 ppm. At 2 weeks 1 - 2 ml methane were produced at this dilution, again indicating that the IC_{100} could be in excess of 10,000 ppm (Figure 30).

NB: Testing concentrations greater than 10,000 ppm (i.e. 1%; 1 ml of waste in 100 ml of test medium) is not recommended, because this would probably dilute nutrients and exhaust buffer capacity significantly.

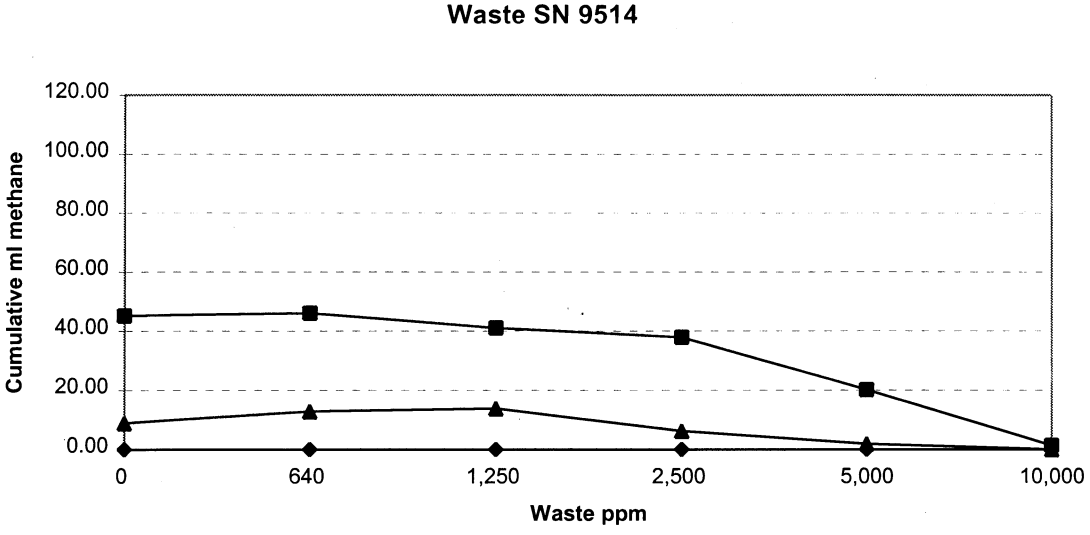

Figure 30 Test waste SN 9514

3.7 Theoretical and Actual Test Efficiency

Over the course of test development the positive control test system (containing no toxin) was repeated more than seven times, each in triplicate. This gave sufficient replication to examine the mean methane generation curve; the plot of cumulative methane yield over time (Figure 31). Visually this appears to have 3 distinct phases of production:

- a phase of high gas generation rate (days 0-14);
- a period of lower but steady gas production (days 14-49);
- a slow approach to exhaustion (day 49 onwards).

These phases are not statistically distinguishable, but the plot approximates closely to the classical exponential curve, which rises fast initially then exponentially approaches the final yield (at infinite time).

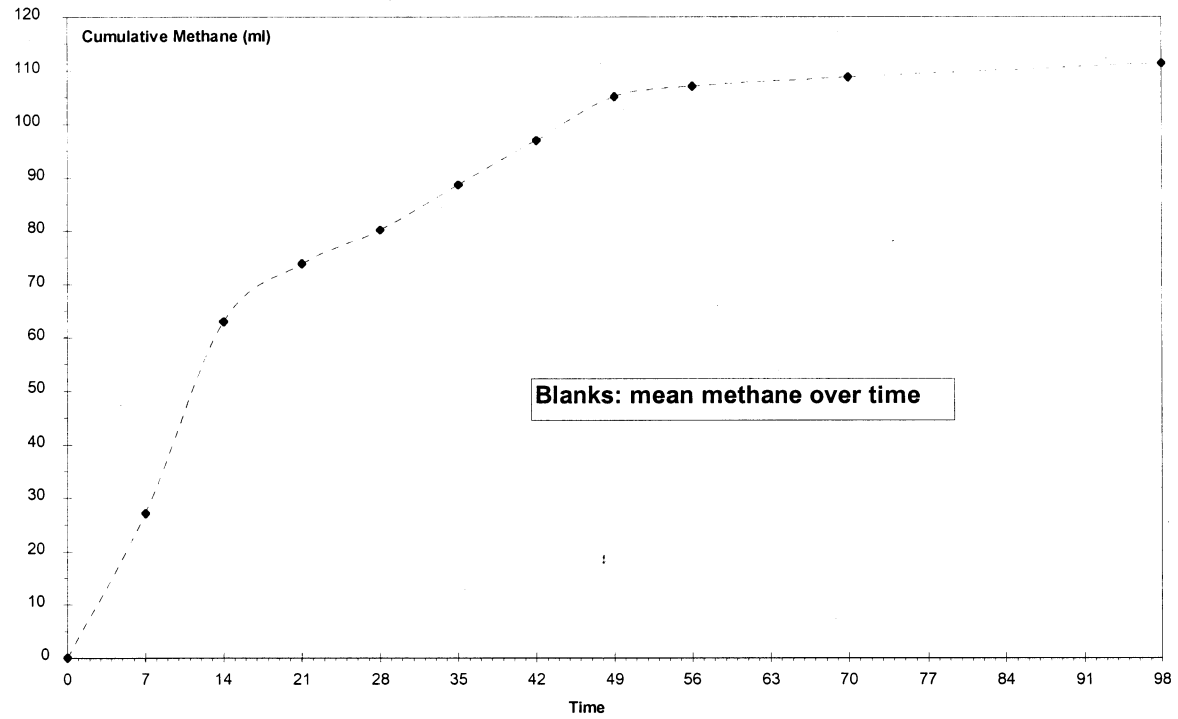

Figure 31 Cumulative methane yield over time (mean of 21 controls)

To simplify interpretation the exponential curve can be transformed into a straight line, either by plotting reciprocals of methane and time (the double-reciprocal plot), or by plotting cumulative methane yield against methane production rate (the Eadie-Hofstee plot, preferred for irregularly-spaced measurements, Figure 32).

The Eadie-Hofstee plot has several features of interest:

- the correlation coefficient of methane yield against methane production rate is good ($R^2 = 0.9377$);

- the initial (day 0) rate of methane production can be determined from the Y intercept (7.82 ml per 2 g of added waste per day, equivalent to 3.9 ml $CH_4.g^{-1}.day$);

- the expected final yield can be determined from the extrapolated X intercept (136.2 ml per 2 g waste, equivalent to 68 ml $CH_4.g^{-1}$).

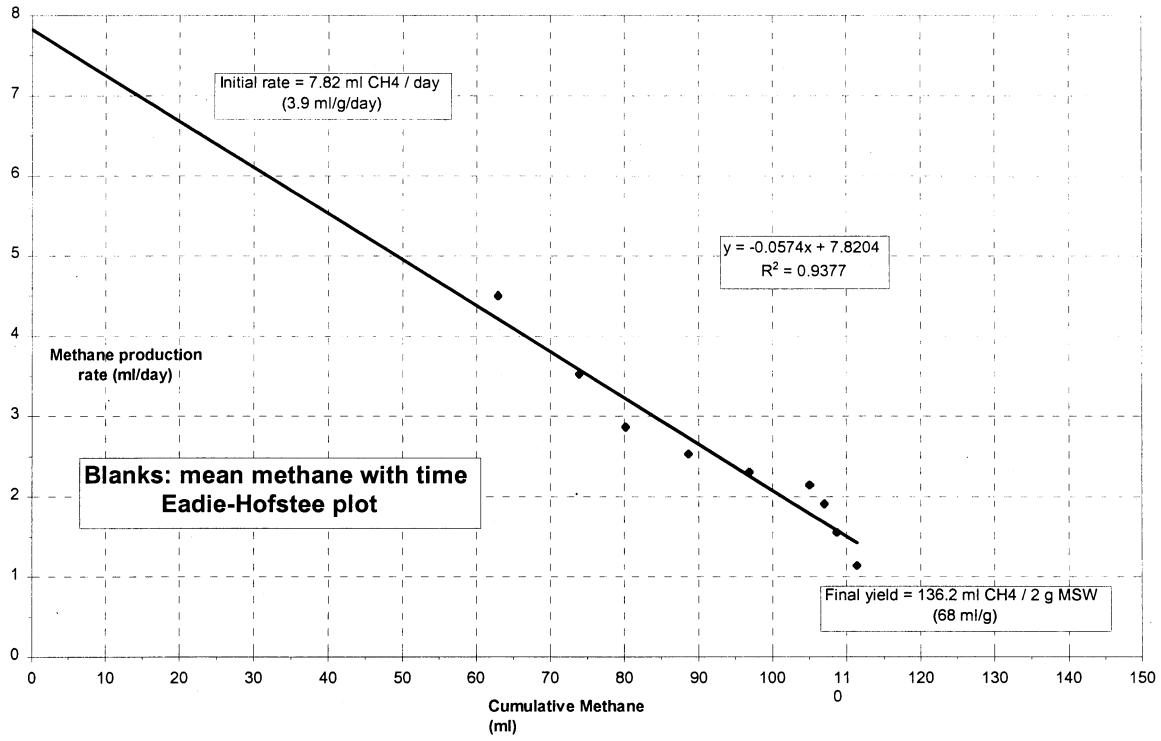

Figure 32 Cumulative methane over time; Eadie-Hofstee plot

The predicted final methane yield was low. Pure cellulose should yield more than 400 ml/g, but a figure nearer 200 ml/g is considered more typical for fresh household waste. The material used for the test was partially-degraded waste from a part of Calvert landfill site placed at least 8 years previously, so would be expected to give a lower yield, although it had been sorted to remove visible non-putrescibles.

This highlights the inherent unpredictability of landfilled waste, and may make the use of landfilled waste as a feedstock questionable. In practice it may prove necessary to check batches of waste before use to ensure that they still contain adequate degradable carbon concentrations. Alternatively a synthetic feedstock could be devised (see section 4).

4 DISCUSSION

4.1 Outcome of simplification study

The feasibility of making both technical and procedural changes to the L-ABT system was investigated. In general it was found possible to simplify the test without altering the fundamental principles or outcome of the method. The major technical changes were to use:

- a seed culture derived from landfilled household waste;
- addition of shredded household waste to the liquid in the test bottle;
- omission of some trace elements and all individual vitamins from the medium.

The most significant procedural changes were:

- boiling reagent solutions rather than requiring absolute sterility;
- combining all the reagent preparation and operations into one step;
- the use of doubling dilution techniques to achieve greater accuracy;
- the use of logarithmic-linear plots to improve interpretation;
- the introduction of an IC_{50} value to aid interpretation, for that concentration of toxin which halves the methane production rate;
- simplified determination of gas quality (only methane concentration is required);
- measurement of an 'acute toxicity' value (IC_{50} or IC_{100}) as early as 7 days;
- comparison of an 'acute' (7-day) and an 'adapted' (28 days+) value.

4.1.1 Technical changes

Source of seed culture

The use of landfill solids and a natural consortium of anaerobic organisms derived from the same landfill made the test in principle more representative of the landfill environment into which materials will be disposed. The use of aged landfill to provide a functioning consortium of micro-organisms was suggested in order to make the test more relevant. This proved to have other important benefits, in that:

- these heterogeneous cultures appeared to be more robust and easy to grow and maintain in the laboratory than the liquid suspension cultures derived from sewage; and
- use of a pre-adapted culture allowed one-step testing when required, with no appreciable delay due to a lag-phase.

It should prove convenient for operators to set up cultures using material taken from the site of a proposed disposal. Although this may make it difficult for different operators to compare results, it will ensure that the results they get will relate to their particular site. Wider testing of the method will show whether site to site variability is a real problem.

Aged waste from a particular location in an operational site is probably a more reliable and reproducible source of microbes than locally-obtained sewage sludge, and so is more likely to provide a stable population when the seed culture need to be re-established. Furthermore, sewage probably does not contain representative methanogenic species. The difference of at least 2 orders of magnitude in IC_{100} for TCE between the sewage-derived L-ABT and landfill-derived O-ABT may be evidence of different species sensitivity, or may be the result of the presence of landfill solids.

Maintaining landfill-derived organisms on solid waste in the defined inorganic test medium also ensured that organisms were pre-adapted to test conditions, so that tests could be performed in one step at short notice. However, the landfill-derived seed culture appeared to lose activity at 4 to 5 months. In this study the seed was first used for tests at 2-4 weeks after establishment, and was stable for at least 3 months. The simplest approach may therefore be to re-establish a fresh culture every 3 months or so.

Alternatively one could examine use of a synthetically formulated 'waste', to try and avoid accumulation of recalcitrant or toxic materials. Minton, Treharne and Davies have apparently had some success with this strategy for a Biological Methane Potential test (personal communication). This may be feasible for the ABT, either in the seed culture or in the test itself, although we would caution that any move away from use of real landfilled waste materials may make the test less representative.

Composition of heterogeneous media

The addition of a small amount of landfill solids to the liquid test system probably served several purposes:

- it provided fermentable carbon in a realistic form;
- it provided a range of inorganic trace nutrients; and
- it created a micro-environment in which surface-dependent processes (such as biofilm-formation and toxin adsorption) could occur.

The first of these factors was verified by the results. In principle the 2 g waste per 100 ml medium per test should have provided approximately the same available carbon as the soluble substrates added to the CAMR version, and in practice the methane yields were similar.

The second factor was not tested rigorously, but empirically appeared to be the case. The simplified O-ABT gave very similar IC_{100} values for phenol and cadmium as the carefully defined medium used by CAMR in their L-ABT, while the final methane yield from toxin-free blanks was very similar to that obtained by AEA under optimised conditions[2]. This has had a direct advantage in allowing reduction of the range of individual vitamins and salts which need to be included, allowing exclusion of a number of expensive or toxic compounds without affecting the test outcome.

On the precautionary principle, CAMR had used a complete defined anaerobic medium to maintain the fastidious liquid-phase culture derived from sewage. It was anticipated that the rather more robust landfill culture would tolerate a less comprehensive range of defined

medium constituents. Expensive chemicals or inconvenient scheduled poisons such as selenium were therefore omitted from O-ABT-1, and it subsequently appeared that the organisms may tolerate complete omission of the trace element solution when household waste solids are present.

Any advantage derived from the presence of a solid surface during the tests was unverified. However, the proposed format using solids suspended in liquid offers the potential advantage of having a liquid to disperse reagents and carry specified nutrients while also providing some solid surfaces with which both organisms and toxins can interact. Although this arrangement does not truly simulate the conditions in the landfill, it is more representative than the liquid-only system, as the solids probably provide some buffering and protection to the microbial population.

The optimum ratio of solids to liquids was not specifically determined, but could be important. One could determine the significance of these factors by:

- adding sewage organisms to sterilised landfill solids; and
- using landfill organisms in the absence of solids.

4.1.2 Procedural changes

Media sterility

An important criterion for the practicability of the test is how easily it can be performed in a routine landfill laboratory. Dedicated microbiological laboratories have autoclaves and sterile areas, and staff trained in aseptic techniques. The L-ABT methodology was devised in this specialist environment and in the simplification process we have tried to remove non-essential features.

Given the source of the inoculum and the further deliberate addition of wet household waste solids there seemed no need to take precautions to exclude minor sources of organisms, dust etc. The main concern was to prevent cross-contamination between old and new test bottles, for example in case a toxin-tolerating methanogenic population developed. Consequently, it has been possible to simply boil reagent solutions to kill any methanogens or other non-sporing contaminants, and then use them immediately. This avoided expensive and time-consuming autoclaving or micro-filtration, and at the same time achieved partial de-gassing of the solutions.

However, if in practice it is more convenient for an operator to pre-prepare and store solutions, for example if tests are performed infrequently, sterilisation may still be needed to prevent deterioration. We consider boiling to be the minimum requirement, since in principle without it contamination with toxin-resistant methanogens or other undesirable organisms could occur.

The inoculum culture also seems sufficiently robust such that no special precautions were required to limit contamination from other organisms when removing or adding suspensions. Although it was possible that the observed loss of activity over time was the result of

contamination, for example with out-competing contaminant organisms, replacement with a fresh culture every 3 months would solve the problem economically. Therefore no special aseptic precautions are required, and the vessel can simply be opened and closed normally when adding feedstock or withdrawing old culture.

The main factor was to operate quickly, in order to minimise exposure to oxygen as far as possible, rather than to exclude contaminating organisms. The seed culture seemed very tolerant of exposure, no doubt because of the small surface area exposed to oxygen diffusion and the large volume of biomass. The test bottles however contained much less active biomass and have much higher exposure to atmosphere during preparation, so require more active oxygen exclusion. We therefore employed and recommend the use of nitrogen-sparging of media prior to sealing test bottles.

Minimising operations required

The CAMR L-ABT method allowed the cultures to adjust after each change in conditions or addition of reagent. This minimised the risk of the culture failing and maximised the gas yield, but required a 3-step process which was labour-intensive, presented several opportunities for error, and took a long time both to set up and to give results. In the O-ABT we adapted the preparation and used pre-adapted seed to allow all the additions to be made in one operation, and at short notice.

End-point

The ABT has been adapted to:

- use doubling dilutions to span the range of required concentrations, giving a more accurate answer, and
- measure the concentration which gives half inhibition of methane production, as well as complete inhibition.

The accuracy of the ABT depends to a large degree on the spacing of dilutions employed. The between-bottle variability is low, of the order ±5%, but because the interval between dilutions was large, so was the error on the estimated IC_{100}. We expressed the IC_{100} in the form "between x and y ppm", where x was the highest concentration showing methane, and y was the lowest concentration showing no methane; for the original 10-fold dilutions this meant that y=10x, while for doubling dilutions y=2x.

The true value lies somewhere in the range of x to y. With the repeated tests on phenol, for example, the true value seemed to lie close to 2560 ppm - because in some repeats the extinction point was below this value, in others above. A practical interpretation of this is that a difference of one dilution should not be regarded as significant and therefore one cannot regard another result as being 'different' until it is at least two dilutions away, i.e. for doubling dilutions a quarter of, or four times greater than, the initial value. Even with a perfectly reproducible system this must be the case, so the easiest way to improve accuracy would be to decrease the interval between dilutions. However, this would increase the cost of

the test. An optimum cost-benefit for the ABT has yet to be determined, and cannot be attempted until the industry and regulators have agreed on an acceptable level of accuracy.

The estimation of an IC_{50} value in principle should have given a clearer end-point than trying to measure the IC_{100}. This is because complex living systems usually show a sigmoid dose-response curve to toxins, with indeterminate head and foot but linear mid-section. However, in practice, the IC_{100} as used by CAMR gave quite clear results in most circumstances, and under the modified AEA protocol the IC_{100} gave a stable reliable result at as early as 7 days. Only TCE showed the anticipated sigmoid response, for which the IC_{100} alone might therefore underestimate the sub-lethal but inhibitory effects at lower concentrations. The IC_{100} is therefore probably the best method for routine use by operators, to screen samples quickly for acceptability, not least because it's determination is straightforward and unambiguous.

The IC_{50} incorporates information from more data points so is perhaps more useful for scientific comparisons and research purposes, such as comparing the 7-day 'acute' and 28-day 'adapted' values. It may also prove to be the more appropriate method for interpreting follow-up detailed tests by operators, or for use by independent quality assurance laboratories.

Care must be exercised in interpreting the IC_{50} values in the same way as that used for measuring toxicity in higher animals. In an LD_{50} test half the animals are killed. In the ABT test the microbial population may be either killed quickly (at high concentrations) or merely inhibited (at sub-lethal concentrations). This will also depend on whether the toxin in question is bactericidal (lethal) or merely bacteriostatic (preventing activity, but non-lethal). In the latter case, the organisms may thrive again once the toxin has become adsorbed or degraded, such that gas production could eventually rise to the same or higher levels (if degradable) than in the uninhibited culture.

The IC_{50} value will therefore vary with the time over which the estimate is made, eventually approaching the IC_{100}. This was clearly observed on several occasions. For operators this is an unnecessary complication, but for researchers it could prove a useful tool.

This further emphasises that the ABT is a comparative test, which although incapable of absolute measurements, is capable of useful relative measures, including measuring adaptation over time and identifying synergistic or antagonistic reactions which could not be predicted by chemical analyses. However, it would be unsafe to use the inhibition values from the test to directly estimate absolute values for inhibition in the landfill. Absolute calibration of the test will require data from solids-based testing, and possibly from real landfill. Ongoing work on a related solids-based test by Minton, Treharne and Davies has for this reason been linked to our work, and in due course they will be reporting on the same test toxins.

Meanwhile the best way to standardise results is probably by comparison to a known defined toxin tested in parallel, such as phenol (see section 4.2.3. Interpretation).

Acute and 'adapted' toxicity measurement

In the simplified version of the test, the culture proved sensitive to additional inhibitory influences during the first 7 days of the incubation (e.g. variation in seed culture 'health'), but the IC_{100} seemed to be stable and reliable as early as 7 days, at least for cadmium and phenol. However, some of the industrial wastes tested by SME seemed to give a clearer result at 14 days. Measuring total inhibition at about 7 days to give an acute toxicity value could be used to quickly screen out the clearly benign materials (i.e. completely non-toxic at 1%) from those requiring closer examination, and continuing incubation to 14 days for confirmation in borderline cases.

Since the short-term test was always very sensitive, the IC_{50} value obtained in the 7-day acute test will be significantly lower than the IC_{100}, perhaps lower than current co-disposal practice deems reasonable. Operators would be concerned that the results of an acute O-ABT might be interpreted as suggesting that a particular material would have an adverse effect, even though in situ in the receiving landfill there would probably be no discernible impact.

The IC_{50} inhibition value at 28 days may give a better indication of the impact of toxins after the microbial population has adapted to the initial impact, but the apparent toxicity will still probably be higher than the long-run effect in the real landfill, if only because of the much higher proportion of absorptive solids in situ. This 28 day 'adapted' value - once calibrated against solids-based tests - could however be of more relevance to landfill operations, since it would not include very short-term effects (which may be irrelevant) in the context of waste disposal.

It may therefore be possible to categorise materials into those which showed no effect at 7 days (the acute test), and could be passed for co-disposal, and those which showed an acute effect at seven days. This latter category could then be further subdivided into:

(a) wastes which when loaded undiluted, but with care taken over rate of addition, would present no risk to the landfill;
(b) wastes which could not be accepted without either further treatment or dilution;
(c) wastes too toxic to be accepted.

This system would depend on pre-determined agreed trigger values of the ABT. These could, in principle, be determined in carefully controlled field trials, although little information can be obtained in the context of this work to predict the efficacy of this.

The change from 'acute' to 'adapted' IC_{50} inhibition value (e.g. comparing at 7 and 28 days) might also prove be a measure of the relative importance of adaptation and deactivation for a particular toxic mixture. This will require confirmation by comparison with more realistic solids-based test systems.

Scale of the assay

The present study used an array of over 50 bottles for each test. In operational situations, the unknown material would probably need to be tested alongside the most relevant benchmark

material (e.g. phenol or cadmium). Hence, the full O-ABT method would demand 100 bottles and at least 100 gas measurements to obtain a single 7-day IC_{100} value (200 measurements if repeated at day 14). In practice however, once the performance of a system had been established, the benchmark material might only be required as an occasional quality check (e.g. weekly) to ensure that no significant changes had occurred.

It is envisaged that the test material could be initially be screened undiluted, at 1 ml in 100 ml of medium, using a simple one-off test (in triplicate) to gain a pass/fail result in 7 days, rather than as a series of dilutions. Only if this screening test proved toxic would a dilution series be required. Hence the operator's wish for a simpler protocol could possibly be developed involving no more than 3 bottles for each test material, plus a positive control, and giving an acceptance result in as little as 7 days.

Gas measurement

Measurement of methane alone seems to be adequate to provide a useful result. Multiple estimations of the gas in each bottle is a time-consuming part of the test which is not strictly necessary. Increasing the speed with which the GC analysis is performed is possible (e.g. by injecting directly into a Flame Ionisation Detector, with no column), but may not necessarily decrease analytical time requirements unless the time required for measuring the volume and collecting the sample can also be reduced.

The pre-screening version of the ABT could in principle be further simplified to develop a sacrificial test, by simply recording the presence or absence of methane at 7 days in a system challenged with undiluted waste - eliminating both the need for gas volume measurement and, possibly, the need for Gas Chromatography (portable apparatus commonly used by landfill operators for methane monitoring may prove adequate).

Comparison of theoretical and actual methane yield in positive controls showed a large disparity, even given the age of the waste used as substrate. Therefore even after sorting to remove visible non-putrescibles, the milled material was still relatively high in inert material. This could partly explain the erratic and failing gas production in the seed culture observed after 4-5 months; inert material by definition would accumulate in the system. This could be avoided by using a fully-degradable substrate, e.g. paper, but the seed culture would then be less representative of landfill, and the organism numbers and species not necessarily those desired.

4.1.3 Scope of use

The simplified O-ABT method was evaluated by AEA with cadmium and phenol as the main test toxins. Most data are available on phenol, since SME also used this as their internal standard. In the present system, trichloroethylene was not considered a suitable reference material because of it's low water solubility and high volatility. Either cadmium or phenol are relatively predictable toxins which are easy to dispense and could be used as a benchmark against which an unknown material could be evaluated. The data from the simplified O-ABT method are fairly consistent with the results obtained from the CAMR L-ABT method (Table 4). However, comparison of AEA and CAMR results from the work with TCE illustrates that

the inhibitory effects of a particular compound may differ depending on the source of the microbial consortium used in the test.

The O-ABT method could either be more directly related to the likely performance of the target landfill (for example, by stipulating that seed organisms and waste feedstock are taken from the target site) or standardised for universal use (by using standardised feedstocks and reference organisms) - but not for both. Using substrate (solid waste) and seed organisms from the target landfill should give more relevant results for the particular operator, but results cannot then be compared with other landfills. A standardised test, perhaps using a defined substrate and independently maintained seed from a common and controlled source, would allow compilation of a national database and inter-site comparison, but might reduce the relevance of the test to a particular site. Since there appear to be roles for both, it may now be necessary to develop two versions of the test - a local (for daily use by landfill operators) and a standardised (for use by regulators).

The results obtained show that the principle of the test is satisfactory but also highlight that an assay based on biological systems is inherently less precise than chemical analysis. In particular, although the reproducibility within each triplicate is good (typically ±5%), the state of the seed culture seems to be important to the absolute values obtained.

The effect of the spacing of the dilution series has already been discussed. In principle, to decide on a co-disposal loading rate, the toxicity of any waste could be compared to a standard toxin such as phenol in the ABT. However, expression of toxicity in terms of 'phenol-equivalent' or similar, because of the inherent limitation of accuracy due to the dilution spacing, would require a two-dilution safety margin. For example, using doubling dilutions, one should allow no co-disposed waste to exceed one quarter of it's phenol-equivalent. More closely-spaced dilutions would give a more accurate answer and confidently allow a smaller safety margin, but at greater cost.

The O-ABT at present is site-specific and requires validation across a range of situations. Using waste and organisms derived from the target disposal site will in due course probably show results varying from site to site in tolerance to a particular toxin. If this variability proves to be small then perhaps the test may be universally applied, and site to site comparisons may be made. If the variability proves to be high, then one must either abandon hopes of a national database and of site to site comparisons, or develop a truly universal and standardised method.

Development of a central, nationally standardised method is possible. It would require a common source of organisms (probably based on freeze-dried standard cultures) and degradable waste (perhaps using a synthetic waste-like substrate). Development of a standardisable yet effective consortium would be a major task, since many interacting species are required for complete waste degradation, and many more are probably required before the consortium would respond realistically to toxins. There have been previous attempts to develop a standardised synthetic household waste which might be drawn on, but application to the ABT would also require proof that this material behaved reasonably like landfilled waste in terms of interaction with toxins. Even where standardised materials were used there could still be a drift in the sensitivity of the consortium during culture under the slightly differing

artificial conditions likely in different laboratories. These problems are significant but may not be insurmountable, and in principle a universally applicable test, broadly analogous to other biological tests such as the Biochemical Oxygen Demand (BOD), could be developed.

Our conclusion was that the O-ABT should currently only be used as an indicator for the order of magnitude of toxic effects, and that absolute blanket threshold values of the IC_{100} cannot yet be set. Inter-site comparisons are now required to validate the method before wider use, but the method itself should require little or no further modification before implementation by operators as a routine monitoring tool. It's use in regulation is more questionable; we believe the test may prove to very site-specific in it's current form, will certainly require wider comparative trials to demonstrate it's reproducibility, and may require further development to produce a standard test for global use. The Industrial Advisory Group was particularly concerned that this operational test should not be used for regulatory purposes. The IAG thought that basing regulation on an inherently variable and local condition-dependent bioassay was neither necessary nor desirable, since individual toxins can be measured more accurately and more reproducibly by specific chemical assays.

The operational system developed here does not intentionally measure the biodegradation of organic toxins, another perhaps unrelated requirement of operators in the draft Waste Management Paper 26F. Biodegradability is a general characteristic of a compound which is best measured under standardised (probably solids-based) test conditions rather than monitored in a site-specific operational test, and for which a national database could eventually be assembled. However, although beyond the current scope of this work, the new test method has shown evidence of:

- adaptation (e.g. the increasing IC_{50} concentration over time), and
- biodegradation (e.g. enhanced methane production at sub-lethal doses of some organic materials).

These effects can apparently be accurately measured in the O-ABT, which could therefore prove to be of use in adaptation studies or in biodegradability testing.

4.2 Application of the Test in Practice

4.2.1 Scope for use

The O-ABT described here is best suited to use by operators to assist in decision-making for the co-disposal of industrial wastes. Operators already test wastes offered for co-disposal, and have other information on the waste in question, for example:

- the source and nature of the process producing the waste;
- chemical analysis (on specific analytes, not always a complete picture);
- physical and other data, e.g. pH, water content.

The operator will also have relevant site-specific information, such as the loading rates and cumulative burden of other similar and different waste streams, the rate of addition of other wastes, and the volume/age of the target landfilled domestic wastes.

In practice the results of the O-ABT will be part of, and augment, this overall picture. The O-ABT cannot stand alone. For co-disposed wastes containing a single well-characterised toxin the O-ABT will add little and may be unnecessary, but for new and complex mixed wastes - with unpredictable synergistic or antagonistic interactions - the O-ABT could prove to be very valuable.

Chemistry is often incomplete, because analysts cannot routinely check for every known compound, so are guided by their expectations. The O-ABT could also be used to cross-check that the toxicity is close to that expected from chemical analyses, perhaps thereby providing the only indication that the chemistry is incomplete. Further chemistry could then be performed to identify and quantitate the unknown toxins, or more pragmatically and inexpensively the loading rate could be based on the O-ABT rather than the chemistry.

The method described here has so far only been applied to liquid wastes. Slurried solids should not present a great problem, but solid wastes could present difficulties, particularly where the waste is heterogeneous at a macroscopic scale. This is a problem already encountered by operators when attempting to take representative samples from such wastes, and the same principles apply. The best principle is probably to take large primary samples and then homogenise these to take smaller sub-samples. Another possibility may be to leach the waste in a standard way, and test the leachate. These issues are best addressed when validating the method in operator's laboratories.

4.2.2 Assay format

After discussion within AEA, then with SME and other members of the IAG, the following format is suggested.

The test could be used in two stages:

1. A simple qualitative 7-day, 3-bottle test, giving either a 'safe' or 'toxic' answer for the undiluted waste stream at low cost.
2. For materials designated 'toxic' after stage 1, determination of the IC_{100} in a dilution series, comparing against a standard such as phenol, reading the preliminary result at 7 days and confirming at 14 days.

The test is best seen as a 'worst case' acute toxicity test. Many toxic materials either degrade or are lost to adsorption in the longer term, rendering results from prolonged incubation questionable and potentially misleading. As a 7- or 14-day test based on total inhibition of methanogenesis, it gives a clear result, showing reasonable sensitivity and reproducibility.

4.2.3 Interpretation of assay

The O-ABT is not a simulation of an operational landfill. Although most of the factors affecting methane production will apply in the test bottle, the system is artificial, and some factors will clearly be different. For example, even at the microscopic level the O-ABT takes little account of the realistic distribution of toxins between solid and liquid phases.

At larger scale it becomes more unrealistic still. For example, it does not and cannot take account of full-scale factors such as the zone of influence of a co-disposed waste in a landfill. Eventually it may be possible to calibrate the test against solids-based laboratory tests and/or full-scale landfill studies, but until then the O-ABT can only give relative, comparative information.

In the absence of absolute data the O-ABT must be expressed in relative terms, and one way to do this is by reference to well-characterised benchmarks, e.g. to express the result in relation to a standard toxin such as phenol, expressed as 'phenol-equivalents'. The test could then be used most simply to ensure that no loading rate for a new toxic waste stream ever exceeded that already permitted for phenol. Expression as 'phenol-equivalents' could also assist long-term landfill management, for example:

- to estimate the cumulative toxicity in a site accepting many toxic streams over many years;
- to determine maximum acceptable loading rates for biodegradable toxins;
- to determine net synergistic/antagonistic effects in common complex mixtures; and
- underpin development of a national database of toxicity values for common mixed toxic streams.

Toxins will generally be adsorbed or detoxified in association with the solids component in a landfill. The O-ABT has a much higher liquid:solid ratio and so might be expected to over-estimate toxicity (although this remains to be proven). In addition, a full-scale landfill would be expected to show other effects not modelled in the O-ABT, for example longer-term recolonisation of acutely intoxicated parts of the site. Minton, Treharne and Davies are currently involved in developing a solids-based test which it is hoped will address these and related issues.

In general, since a landfill would offer more opportunities for adsorption of toxins, and offer relatively isolated micro-niches of protected methanogens, the test must err on the side of environmental safety. That is, actual toxic effects in-situ will be less severe than seen in the O-ABT. Furthermore, since the 7 day test measures acute toxicity, the safety margin is increased, because processes such as detoxification and adsorption would in practice allow greater concentrations of most toxins to be safely co-disposed long-term. However, until the method has been calibrated and validated, the test will be unsuitable for setting nationally-applied loading rates.

In terms of relative comparisons the test is already useful. It is understood that SME have already run the test on several liquid wastes for their local Environment Agency office. In it's simplest form the test could be used to test any unknown waste for toxicity to anaerobic

systems. For landfill the simplest application is to test a waste at the anticipated loading rate, to give a straightforward qualitative pass or fail answer.

In its present format we recommend that no more than 1 ml of liquid waste per 100 ml test medium is used. In a full-scale landfill, loading rates will often exceed this at the point of addition, and over a limited volume of household waste in the vicinity, so if a more absolute interpretation of the O-ABT is required, field work will be necessary to calibrate its response against that observed in-situ.

4.3 Practical & Financial Aspects of Operational ABT

The test as described in the SOP is still relatively time-consuming, with a typical standard or toxic waste plus controls requiring approximately 4 hours to set up, then 2-3 hours per week for monitoring. At the peak of activity in this study SME had 8 test batches running in parallel, demanding 3 man-days per week.

Capital costs will vary. Assuming major items such as the gas chromatograph are already in place, the costs of a suitable incubator, glassware and shredder are probably no more than £1,000. Purchase of a GC solely for this test would not be cost-effective, so operators without a GC would have to submit the full test to a laboratory set up to do the work. However, it may prove possible to do the simple 7-day qualitative test using methane-monitoring equipment common to landfill sites.

On-going costs comprise staff time, media, consumable equipment and standards (toxins for the test and gases for the GC). For a single test waste at a time this equates to approximately £400 per week, but economies of scale apply - with 8 batches running in parallel, the cost fell to approximately £125 per test waste per week.

Some training would be required. The essential knowledge can be imparted in one day or less, but implementation in practice for staff with no previous microbiological experience would initially require close supervision, tailing off as experience grows. In this respect SME were perhaps not typical; their laboratory manager already had a good grasp of microbiological principles, and did not require explanation of fundamentals. Greater initial training and ongoing supervisory effort during start-up must be anticipated with less experienced staff.

Phenol-equivalent interpretation

The recommended ABT Standard Operating Procedure requires that a standard is always run in parallel. Phenol is suggested as it is a reference compound for chemical analyses, and already has a legally-defined and reasonably well-founded loading rate. A heavy metal such as cadmium could be used as well for toxic streams in which inorganic materials were dominant. Results can therefore be expressed in 'phenol-equivalents' or similar, by expressing the phenol IC_{100} as a ratio to the test waste IC_{100}.

A strict interpretation of the IC_{100} gives a range, since the true value usually falls between two dilutions; the last showing some methane and the first showing complete inhibition. To

simplify interpretation we suggest that the working IC_{100} is taken to be the lowest concentration of waste causing complete inhibition.

To give some examples:

- in the present study, phenol gave an IC_{100} of approximately 3000 ppm;
- cadmium gave an IC_{100} of approximately 1000 ppm. Cadmium therefore had a 'phenol-equivalent' of 3000/1000 = 3.0, i.e. was approximately 3 times more toxic than phenol;
- TCE gave an IC_{100} of 640 ppm, so TCE had a 'phenol-equivalent' of 3000/640 = 4.7, i.e. was approximately 5 times more toxic than phenol.

Note the careful use of 'approximate'. As previously discussed, results must be at least two dilutions apart to be regarded as significantly different. For doubling dilutions this implies a factor of 4. Therefore TCE probably is significantly more toxic than phenol, but the apparently 3-fold higher toxicity for cadmium may not be significant.

If this approach is adopted, it may expose any inconsistencies in the current chemistry-based regulatory limits. Given the lack of absolute calibration and inherent margins of error in the O-ABT, the safest course would be to adopt the lower loading value when chemistry and microbiology differ significantly. We must emphasise that care is required because the IC_{100} values are only accurate to ± one dilution, but the principle appears to be sound. For known pure toxins existing loading rates could be retained, or the ABT rate taken (whichever is safer, i.e. lower). In the case of uncharacterised or complex waste streams this approach could be used to rationally set a safe loading rate.

In the absence of better information on long-term effects, it could also possibly be used to safely set the total acceptable toxic load entering a landfill over it's lifetime, for example by summing the 'phenol-equivalents' of all co-disposed wastes entering that site. This might however impose unduly harsh limitations on co-disposal, since the ABT result makes little allowance for protective phenomena such as adsorption and biodegradation of toxins.

Comparison of ABT with other measures

Although the O-ABT requires wider testing and validation, it is interesting to provisionally compare our results with other published information on the three test toxins:

Material	AEA O-ABT IC_{100} (ppm)	[1] CAMR L-ABT (ppm)	[2] WMP 26F	[3] Operators (ppm)	[4] Acute rat LD_{50} (ppm)
Phenol	~3000	~1000	5 ppm/day	~200	317
Cadmium	~1000	~1000	10 ppm	~200	300
TCE	~640	~100	'ABT'	-	3670

[1] From CAMR report Tables 1, 3 and 11 [(2)].
[2] From draft WMP 26F Table 5.1 (note - 'ABT' recommended for TCE)
[3] From IAG, current typical working maxima
[4] From National Chemical Emergency Centre database

A lower concentration implies a higher toxicity. Phenol and cadmium are given similar toxicity by all sources, although the precise values differ slightly. Given that we expect the acute 7-day O-ABT to be more sensitive than landfill in-situ, both the draft WMP 26F guidance and current operational practice can be seen to be erring on the side of safety. If a similar pattern is found for other toxins then it may be possible to add a generous safety margin to interpretation of the O-ABT IC_{100} value without unduly affecting operational practice.

TCE is more interesting; it showed the highest toxicity of the three test toxins in both our system and CAMR's, but has the lowest toxicity in acute tests on rats. Clearly the mode of action of TCE differs from the other toxins, which might also explain the different dose-response curve seen for TCE in our experiments. However, the O-ABT gave a clear result.

The O-ABT is not suitable as a gate acceptance test. Even in revised form it takes at least 7 days to produce a result, so it is not practical to keep lorries waiting or even to unload and store consignments of waste while results are awaited. The O-ABT may prove to be very site-specific, while the ideal compliance test would be standardised and applicable globally. Such a test could possibly be developed, but more work is required to do so. A site-specific test could be of use to regulators in setting loading rates for a particular site, but if this were implemented it must be clearly understood that the rates so derived cannot be applied to any other sites.

In principle one could perhaps devise a more rapid test, in which for example an actively gassing system was challenged with waste and the effects monitored. However, we feel the test is a major advance on previous tests taking one to three months (or more), and is now capable of giving results in a time-frame similar to other widely-accepted tests such as the Biochemical Oxygen Demand test.

The O-ABT should prove to be a useful acceptability test. Used in simple 'pass or fail' screening mode to test the actual loading rate being employed for a particular waste, it would in 7 days provide either a 'pass', giving extra confidence that no harm could result, or 'fail', alerting the operator to either discontinue co-disposal of the material, or to pay closer attention to further batches of the same material. For such 'fail' materials it could also be used in extended (serial dilution) mode to decide on a lower, safe loading rate.

Related ongoing work by Minton, Treharne and Davies is examining and developing a solids-based test in parallel. They have agreed to test the same standard toxins as we did, over at least the same range of concentrations, so that our results can be compared. The solids-based system should in principle be much more representative of landfill (although this must be demonstrated). We expect to find a solids-based system tolerant of much higher concentrations of toxin, but it is hoped that this effect will prove to be constant and predictable, so that a correction factor can be applied to the O-ABT results to predict effects in the solids-based system (and hence in landfill).

5 CONCLUSIONS

1. A version of the liquid-based anaerobic bioassay test has been developed which accurately reproduces the scientifically rigorous method published by CAMR[3]. The revised method is sufficiently simplified and robust to implement in a landfill operator's laboratory.

2. The modified test gives results quickly; the optimum incubation time is 7-14 days. Longer incubation may be required (up to 8 weeks) to produce a stable inhibitory value, but processes such as adsorption and biodegradation in the system render this result questionable.

3. The most reliable and practical end-point is complete inhibition of methanogenesis: the IC_{100}. The concentration causing 50% inhibition (the IC_{50}) is more sensitive, and changes over time as toxins adsorb or degrade; this may be a useful research tool, but would only add difficulty in a practical on-site test.

4. The test shows good reproducibility (of the order ± 5% to 10% between triplicate tests). The result is however limited by the doubling dilution series used; more closely-spaced dilutions would give a more accurate answer, but at greater cost.

5. The test gives good comparative information. Calibration against results from solids-based tests is underway, and validation in operational landfills will be required in future. It is therefore a useful additional tool for landfill operators.

6. The test probably 'fails safe' using the IC_{100} as the end-point. The liquid system is less adsorptive and less buffered than an operational landfill, so should over-estimate the acute toxicity (to be confirmed by comparison with the solids-based test).

7. The test measures total toxicity. In complex mixed waste streams the overall toxicity cannot be predicted from the chemical composition because of unknown synergism or antagonism. It is therefore an important additional test.

8. The test may be used in several ways:

   - To provide a simple qualitative 'yes' or 'no' answer to the question 'is this waste toxic at the loading rate envisaged?' If there is no inhibition at the maximum concentration to be experienced in the landfill, material can be confidently co-disposed;

   - Where a material shows some toxicity, to quantitatively determine the inhibitory concentration, allowing appropriate adjustment to loading rates;

   - To cross-check conventional chemical analyses. If the measured toxicity is orders of magnitude higher than that predicted from the chemistry, the chemistry is probably incomplete, and more thorough chemical analysis may be required;

- To provide comparative toxicity information. Phenol was the 'de facto' standard used here, although cadmium might be more appropriate for inorganic waste streams. New toxic streams could be expressed in terms of their 'phenol-equivalent', to estimate the maximum permissible loading-rate for that particular stream, and the total toxic burden introduced into a given landfill over it's operational life;

6 RECOMMENDATIONS

Three types of activity are suggested:

1. Validation of the test.
2. Further development of the test.
3. Interpretation and application of the test in practice.

1. **Validation**. So far the test has only been applied by one operator, to one source of landfilled waste on one site. Wider testing is now required:

   (a) To examine the general acceptability of the Standard Operating Procedure. Shanks and McEwan are now happy with the test as appended. However, the SME laboratory and personnel may not be typical, so wider application, perhaps to members of the Industrial Advisory Group, would help to identify any further implementation problems.

   (b) To measure the effects of other landfilled wastes on seed culture and test performance. Solid wastes from other sites may not behave the same way as Calvert waste, potentially giving rise to differences in seed culture behaviour or even test performance. This would tend to make the test more site specific.

   (c) Blind ring-testing should be performed, submitting to several operators a number of centrally-prepared toxic materials (both pure standards and real toxic wastes), in order to establish the true reproducibility of the method. Only one material should be fully identified; we suggest phenol, so that the suggested standardised method of interpretation based on 'phenol equivalents' can also be tested.

2. **Development.** Although we believe the ABT is now fit for purpose, and should require little if any further development as an acceptability test, it could also be used as the baseline from which to develop further tests:

   (a) Further simplification, aiming to produce an even faster yet robust 'pass or fail' test. Possibilities include a simpler qualitative 'presence or absence' method for methane detection, or a colour-based method for (black) sulphide detection. In principle in an actively methanogenic culture one could challenge and obtain toxic effects almost immediately, but it is not clear how these could be measured.

   (b) If ring-testing shows great site-to-site variability then it may be worthwhile trying to develop a standard version of the test, probably based on organisms from culture collections or maintained in a central laboratory, and a standardised or synthetic 'solid waste'.

   (c) The observed changes in IC_{50} over time suggests that there may be applications for the test in adaptability testing, which would be useful to estimate the degree of importance of adaptation to toxins in landfill. Adaptation implies higher acceptable

loading rates, but this would also need to be related to long-term leachate quality.

(d) The observed extra methane yield with some organic materials suggests that there may be applications for the test in biodegradability testing. This is important both in assessment of the acceptable lifetime loading for a particular compound (which may be infinite if totally degraded), and by implication may help identify compounds and define acceptable loading rates for materials which can be biologically treated in this fashion.

3. **Implementation**. In practice there is still some work to be done in interpretation:

(a) How does the test relate to solids-based results and to real in-situ effects? A comparison with the results of Minton, Treharne and Davies' test should be made, and this in turn must be correlated with either literature or experimental observations of methanogenesis in landfill exposed to toxins.

(b) Are the results reproducible? If the test proves to be reproducible form site to site - or can be adapted to make it so - then collection of the data and production of regional or a national database would be useful, to collect statistics and document co-disposal, and to form a national information resource.

7 REFERENCES

1. Department of the Environment (1994) *Waste management paper no. 26F: landfill co-disposal.* A draft for consultation.

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3. Biotal Ltd (1992) *Further development of landfill assessment methods: a potential gas yield and gas production rate test.* Department of the Environment report no. CWM 090/92

4. Croft BC and Campbell DJV (1994) *Assessment of a test for Biological Methane Potential.* Department of the Environment report no. CWM 103/94 (AEAT plc)

APPENDIX 1

DISCUSSIONS OF INDUSTRIAL ADVISORY GROUP

Martin Meadows *	ETSU on behalf of the Environment Agency
Rob Kate *	Cleanaway
Terry Andrews	Cleanaway
Nygel Thomas	Cleanaway
Andrew Maule	CAMR
Patrick Pointer *	Shanks & McEwan
John Stoddard *	Shanks & McEwan
Andy Childs *	Shanks & McEwan
Peter Clarricoats *	Cory Environmental
Alistair Brace *	Cory Environmental
Robert Hamilton	Caird
Theresa Davies	BFI Wastecare
Carolyn Stone	Biffa
Martyn Jarvis	Biffa
Steve Roscoe	Grundon
Chris Harries	Minton, Treharne & Davies.

* attended both group Meetings

Recommendations of the Industrial advisors

The technical details of this study were presented to the Industrial Advisory Group. A draft protocol for the O-ABT method (Appendix 3) was discussed. In general they endorsed the simplified protocol and agreed to the points listed below.

a. Using landfill inoculum and adding landfill solids was an important improvement. The issue of the source of the landfill material to be used by all operators was unresolved but it seems likely that the material from the proposed disposal site would be best. Nevertheless a general material should also be available, at least for occasional users, and it would be prudent to adopt standard materials for any test to be used by regulators. ***Decision: continue to use locally-derived landfill materials as seed and feedstock.***

b. The amount of solids in the test bottle could be further optimised to increase gas yield and the liquid:solid:headspace ratios could be changed. The gas production should be adjusted to make best use of the standard bottle size. ***Decision: the present arrangement gives an acceptable result so leave unchanged.***

c. The use of landfill solids and omission of many reagents is an improvement. The main cost is associated with making up the solutions rather than material costs. Getting rid of one whole reagent preparation (e.g. solution C) is more important than removing more individual chemicals. Making the seed culture medium and the test medium the same

would save manpower/costs and further reduce stress on the organisms when transferred to test vessels. Further major simplification may be risky. ***Decision: omit Trace Element Solution C, and make the media the same for inoculum and test. Be cautious over further changes.***

d. The need for de-gassing of the media with nitrogen has been questioned but in the opinion of AEA, CAMR and SME, it would be unsafe to omit this stage. ***Decision: retain nitrogen gassing of bottles.***

e. The present medium has several costly reducing agents and there is scope to change these and perhaps limit to one cheaper agent. Costs of reagents may be less important than time taken making-up and equilibrating solutions so changes here are low priority. ***Decision: fall back to one of the existing reducing agents (cysteine hydrochloride).***

f. The time running the GC is the greatest single use of manpower, even after simplification to estimating methane only. Measuring gas pressure or volume alone is insufficient but there is no need to do a full analysis; only the methane is of routine interest. Running with a empty column and just using the FID output would be fast and simple. The method of estimating gas volume and taking gas samples would be left to the discretion of the lab; i.e. the SOP should not be prescriptive (we favour the rising bubble meniscus and a three-way tap on a syringe, CAMR recommend use of a simple glass gas syringe). Volume measurement will be the next slowest step if direct FID is successful. In routine use sites with a high throughput should probably consider automated volume/quality measurement systems, which already exist. ***Decision: Identify good practice for manual gas sampling during tests at SME.***

g. The measurement of inhibition at seven days is useful in that the IC_{50} test is very sensitive at this early stage and permits rapid safe acceptance of benign wastes (in this respect it fails safe). However, it gives a much lower inhibition value than the IC_{100} test and so should probably not be promoted as the end-point of the test. The concentration at which the culture is irrecoverably killed can also be determined at 7 days, from the IC_{100}. It is an important value for the operator and would probably be the value used in discussion about loading rates etc. The need to have about 50 bottles for a standard curve and 50 bottles for the unknown is a concern to operators (particularly on space). A less comprehensive calibration curve (just to illustrate that the culture was performing correctly) and perhaps an undiluted sample of unknown waste may be appropriate initially, if the total inhibition approach is adopted. ***Decision: continue to interpret both the IC_{50} and IC_{100}, at both seven days (acute) and 28-days (adapted); write the protocol making decisions based on the IC_{100} value at it's earliest stable value.***

h. The format of the final SOP is not crucial since each operator will write it in their company style. ***Decision: continue to use the present format.***

i. There may be better ways of managing the inoculum so that a representative amount of suspension can be removed. However, the present system works and no specific plans for improvements were thought justified. ***Decision: continue with present inoculum management method but be aware of potential to improve.***

Priority for Operational Testing of the method

The Industrial Advisors recommended that SME give priority to two issues in the next stage of testing at Kempston.

a. ***Practicality of running the test at ambient temperature.*** Since it will be a comparative test (unknown against known toxin) this should be satisfactory and would make major savings in lab space (i.e. there would be no need for an incubator for the bottles or a waterbath for the inoculum). However, since we are now into warmer weather, running at ambient will not realistically test for winter ambient, which can be substantially lower than summer in typical on-site laboratory accommodation.

b. ***Practicality of adding real wastes to the test bottle.*** Sample representivity will need to be considered, though the strategy may be no different than that adopted in other analytical methods. Adding liquid wastes should be straightforward and so initial tests need to use these. Based on the figures in draft WMP 26 more than half co-disposed material is solid. The serum bottles are physically too small for samples of some of these wastes, which may also present substantial problems in taking representative sub-samples or making dilutions. Large bottles could not be run in the numbers envisaged in the present protocol (50 bottles). SME need to try a range of real wastes and the scope of this limitation needs to be assessed.

APPENDIX 2

OUTLINE OF METHODOLOGY USED FOR L-ABT AND O-ABT VERSION 1 (DEVELOPMENTAL)

Similar ingredients for the salts solutions are used in both the L-ABT and O-ABT methods.

The protocol was;

1. Make up solutions as described in the CAMR SOP. (Na_2SeO_3 and $AlCl_3$ have been omitted; individual vitamin-addition has been omitted, yeast extract has been increased).

Minerals Solution A [g/l] - K_2HPO_4 2.5

Minerals Solution B [g/l] - KH_2PO_4 2.5, $(NH_4)_2SO_4$ 5, NaCl 5, $MgSO_4$ 1, $CaCl_2$ 1

Trace Elements Solution C [mg/l] - $MnCl_2$ 100, $FeSO_4$ 150, $CoCl_2$ 100, $ZnSO_4$ 100, Na_2MoO_4 10, $CuSO_4$ 25, $NiCl_2$ 20, H_3BO_3 10, Na_2WO_4 10. EDTA was not added (not essential if used immediately). Once a simplified standard solution has been agreed and stock solutions are to be kept for any length of time, it may be necessary to add EDTA again (500 mg/l) to prevent loss of metals by adsorption to glass during storage.

2. These should be brought to the boil to ensure a degree of sterilisation (absolute sterility is not seen as essential for the test, but boiling prolongs shelf-life) and to reduce the concentration of dissolved oxygen. After boiling the solutions should be cooled to < 35°C before use, or sealed and stored at 4°C.

3. Make up whole medium as follows:

Component (make up to 1 litre)	O-ABT	L-ABT
Minerals Solution *A* (ml)	100	100
Minerals Solution *B* (ml)	100	100
Vitamin solution (ml)	-	10
Resazurin (0.1%, ml)	1	1
Trace Element Solution *C* (ml)	10	10
$FeCl_2$ (g)	0.1	0.1
Cysteine HCl (g)	0.3	0.3
Yeast extract (g)	1	1
$NaHCO_3$ (g)	-	3
Na acetate (g)	-	2.5
Na formate (g)	-	2.5
Tryptone* (g)	-	2
Dithiothreitol (g)	-	0.4
2- mercaptoethylsulphonate (g)	0.5	-
Tri-sodium citrate (g)	0.3	-

* Biotal reference to tryptose assumed to be in error, substitute tryptone or trypticase instead.

In setting up the trials the aim has been to keep things as simple as possible. The emphasis is to protect the seed culture from aerobic conditions as much as possible. It is therefore added last to the tests that have been thoroughly mixed.

Each trial bottle should contain 90ml liquid medium as described above. 57 bottles per test type are required so about 5.2 litres of medium is needed for a complete run. Prior to setting up the trials the whole medium should be stored at 35°C.

4. The tests should then be set up using the reagents defined earlier, and toxins added. These should be added as the raw material to the final concentration described (TCE should be added in acetone as shown below the table). The amounts added to each bottle should be as shown below.

Compound	Amount added to each trial				
Cadmium Nitrate (final conc)	3mg (30ppm)	10mg (100ppm)	30mg (300ppm)	100mg (1000ppm)	300mg (3000ppm)
TCE* (final conc)	[1]10µg (0.1ppm)	[2]50µg (0.5ppm)	[3]100µg (1ppm)	[4]500µg (5ppm)	[5]1000µg (10ppm)
Phenol (final conc)	3mg (30ppm)	10mg (100ppm)	30mg (300ppm)	100mg (1000ppm)	300mg (3000ppm)

All bottles should be prepared in triplicate

* TCE can be stored as a stock solution of 1g/10ml acetone at 4°C and serially diluted in acetone as required. Ensure all bottles receive a total of 100µl of acetone, and also prepare an acetone-only control triplicate.

1 = 100µl of 10^{-4}g/ml stock solution.
2 = 50µl of 10^{-3}g/ml stock solution + 50µl plain acetone.
3 = 100µl of 10^{-3}g/ml stock solution.
4 = 50µl of 10^{-2}g/ml stock solution + 50µl plain acetone.
5 = 100µl of 10^{-2}g/ml stock solution.

The inoculum does not require filtering of the gross solid matter out (as per the CAMR method), since any fermentable solids carried over into the test will be the same for all bottles, and small compared to available carbon from the added 2g waste. The inoculum should however be kept gassed with nitrogen before use, to minimise the toxic exposure to oxygen.

The household waste feedstock is prepared by hand-sorting freshly-excavated methanogenic waste to remove non-putrescibles (metal, glass, plastic), ball-milled to < 1 mm, then stored frozen until required.

The sequence of addition to the bottles is as follows:

a) add 2g milled waste per bottle for the modified method;
b) add 90 ml medium and swirl to mix;
c) add 10 ml seed culture and swirl to mix;
c) degas for 5 minutes by bubbling nitrogen through the bottles;
d) add toxic material, swirl to mix, insert and clamp seal, and incubate.

For the first trial we tested three toxins in triplicate at each of 5 dilutions, a total of 45 test bottles.

Additionally we set up:

- triplicate 'positive control' vessels, i.e. controls with no toxin;
- 'solvent control' vessels - as above but with 100ml acetone, to allow subsequent correction of the TCE results;
- 'inoculum-only control' - seed culture but no added shredded waste, to check that the contribution of degradable matter in the seed was negligible;
- 'blanks' - no waste and no inoculum, to ensure that sterility had been achieved and that there were no significant non-biological effects.

In total we therefore required 57 bottles for each trial.

Bottles were then incubated at 37°C, and the total gas volume and percentage methane generated were determined weekly.

APPENDIX 3

STANDARD OPERATING PROCEDURE: REVISED O-ABT METHOD

TITLE:	ANAEROBIC BIOTOXICITY TEST (ABT)	PREPARED BY	J Stoddart LABORATORY MANAGER
LOCATION	CENTRAL LABORATORY	EDITED/APPROVED BY	Barry Croft AEA Technology

1. INSTRUMENTATION AND APPARATUS

1.1 A 5 litre Quickfit culture vessel (Quickfit PV5L) fitted with a 100 mm i.d. flange joint, a reactor lid (Quickfit MAF2/2 or similar), a flask clip (JC 100F) and a means of collecting the gas generated in the vessel, for example, by displacing water from an inverted 1 litre measuring cylinder.

1.2 Syringe needles No 1, 21 gauge, 38 mm long , fitted with a Luer Lock

1.3 125 ml Wheaton Bottles (Pierce & Warriner Cat no 12995)

1.4 Neoprene septa (Pierce & Warriner Cat no 13233)

1.5 20 mm Aluminium seals (Pierce & Warriner Cat no 13214)

1.6 Syringe stopcock (Aldrich Cat no Z18.212.5), adapted to couple up to the existing gas sampling loop on the GC.

1.7 Bubble flow meter, GC type 50 ml capacity.

1.8 50 ml glass syringe fitted with a Luer lock

1.9 A gas chromatograph equipped with a 4.0 metre Porapaq N column, 0.25 ml motorised sample loop and a thermal conductivity cell.

1.10 20 mm Seal crimper and 20 mm Seal Decrimper (Aldrich Cat No Z11/427/8 & Z29/216/8)

Note: Trade names are used for purposes of identification only and their use does not imply endorsement by the Environment Agency, AEA Technology or Shanks and McEwan.

2. RISK ASSESSMENT

NB The bacteriological risk assessment has been made by Barry Croft of AEA Technology.

2.1 The main hazard associated with this technique is assumed to be biological. The chemical hazard is confined to the preparation of the reagents and the small amount of toxins involved. Observe the normal handling requirements for these.

2.2 Since the seed culture and test vessels contain organisms derived from soil and household waste, there is a potential biological hazard, which we assess as no more than Group 2 (*see Categorisation of pathogens, according to hazard and categories of containment. Advisory Committee on Dangerous Pathogens HMSO).*

2.3 This assumes that only aged household waste is used - if fresh household waste is used, or material landfilled for less than a year, there may be a potential for more serious Group 3 or Group 4 obligate human pathogens. Such materials are therefore not recommended. Since the material is anaerobic, no special precautions against aerobic fungi or protozoa are required (i.e. a safety cabinet is not required).

NB *A group 1 organism is defined as "an organism that is most unlikely to cause human disease". A group 2 organism is defined as "an organism that may cause human disease and which might be a hazard to laboratory workers but is unlikely to spread to the community. Laboratory exposure rarely produces infection and effective prophylaxis or effective treatment is usually available".*

2.4 The biological risks are essentially similar to those associated with soil or sewage. The most serious risk is probably tetanus or gangrene (via cuts) and precautions against ingestion and inhalation should also be observed. Only nominated staff should work with the material, who should be free of compromising infection, should not be taking immunosuppressant drugs, and should have current tetanus immunisation. Typhoid immunisation is not strictly required, but would be prudent. Gloves should be worn constantly, and any cuts or abrasions treated and reported, however minor. A paper mask and goggles should be worn whenever vessels are opened. Hands must be washed thoroughly with disinfectant soap after all operations. A strict "no eating, drinking or smoking" rule must be observed.

2.5 The main physical hazard is of fire or explosion if methane is allowed to accumulate in confined spaces. The volumes generated in test bottles are small and contained so present little hazard, but the seed culture generates significant volumes of both flammable and asphyxiant gases, so requires careful handling.

3. REAGENTS (General laboratory grade unless otherwise stated)

3.1 Refuse Fermentation Media

Weigh 2.5 g $(NH_4)_2SO_4$, 2.28 g $K_2HPO_4.3H_2O$, 1.36 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ into a 5 litre volumetric flask. Add 5 ml 0.1% Resazurin (cell culture grade), 0.5 g $CaCO_3$, 0.5 g $Fe_2Cl.4H_2O$, 5.0 g yeast extract and 2.5 g cysteine HCl. Add water (distilled) until the volume is nearly 5 litres and stir using a magnetic follower. Adjust to within the range pH 7.0 to 7.5 with a Na_2CO_3 solution. Bring to the boil and cool rapidly under running water. make up to five litres. Use within 4 hours, discard any of the solution not used within that time.

NB It is recommended that this solution is brought to the boil to sterilise the solution against bacteria that may become tolerant to any toxin that may be used in the test.

3.2 Mature Waste

Some mature solid waste is collected in a large sealed plastic container. Mature in this context means household waste that has been in place for at least 3 years but probably less than 20 years - such that it has been partially but not completely degraded. Storage under anaerobic conditions is not essential provided the material is handled promptly. The material is sorted by hand to remove non-putrescibles (plastic, wood, stones and metal) leaving largely degradable matter. This material is chopped to a small particle size in an Industrial type food processor. The material is then stored in an air tight plastic container in a deep freeze (at approximately -20°C) until needed.

3.3 0.1% Resazurin

Dissolve 0.10g Resazurin (cell culture grade) in water and dilute to 100 ml

3.4 500 ppm Sodium metabisulphite solution

Weigh 1.0g $Na_2S_2O_5$ into a 2 litre volumetric flask and make up to the mark.

3.5 Calibration gas

A calibration gas containing approximately 60% CH_4. The balance of the gas can be made up with CO_2, N_2, & O_2. The gas should have a certificate of analysis.

4. METHOD

NB *Any dispensing equipment to be used in the methods below should be thoroughly washed after use and then sterilised by soaking overnight in 500 ppm sodium metabisulphite solution.*

4.1 Preparation of the Anaerobic Seed

To a 5 litre reactor vessel add some 500 ml "seed" from an active anaerobic seed culture and then make the volume of the liquid to nearly 5 litres with the nutrient solution (3.1). For every 1 litre of medium add 50 gm of household waste (mature solid waste). Seal the vessel with the lid, well greasing the flange and any conical joints. Purge with nitrogen and then incubate at 35°C. Manually shake the vessel daily to disperse the solids within the liquor. Collect the gas produced by displacement of water and record the daily volume of gas produced.

Each week decant 250 ml of the well stirred liquor from the reactor vessel and add 250 ml of fresh medium (see 3.1) and 12.5 g of milled household waste, and purge with nitrogen. Return the vessel to the incubator.

When the "seed" is required for a biotoxicity test then decant the required amount of liquor from the reactor vessel and filter until free of suspended solids. Replace the liquor with Reagent 3.1 and household waste as above. It is recommended that the "seed" should be taken from a starving culture, i.e. a culture that has not been fed for some days

NB *Under the conditions described above, a 5 litre anaerobic digestor can be expected to generate 100 - 1000 ml of gas per day.*

4.2 The Biotoxicity Test

4.2.1 Mark each Wheaton bottle with a unique reference number, and then if required, record the dry weight and the weight of each bottle brim full of water to find the capacity of each bottle.

4.2.2 Weigh 2.0 +/- 0.1 g of milled household waste into each Wheaton bottle, and then add the required amount of toxin (see note below). Add 90 ml of the nutrient and 10 ml of the "seed liquor" by means of a 30 ml & 10 ml automatic dispenser.

4.2.3 Purge each bottle with nitrogen, and then ensure that the lip of each bottle is free of solid material before inserting a neoprene septum, and then crimp on an aluminium cap. Incubate the bottle at 35°C.

NB *Each level of toxin should be prepared in triplicate; a blank of three bottles without the toxin should be run with every test.*

4.2.5 Level of toxin to be used.
If a bottle is to contain 100 ppm of a toxin, then the amount of toxin needed to inoculate each of three bottles is 100 ml x 100 µg/ml toxin; i.e. 10 mg. Therefore 1.0 ml of a 10 mg/ml toxin solution should be added to each of those bottles.

A factor of no more than 2 should be used between each level of toxin i.e. 10 ppm, 20 ppm, 40 ppm, 80 ppm etc. The maximum level of toxin chosen should be at least a factor of 10 above the expected toxic concentration, up to a maximum of 1 gram toxin in 100 ml test bottle (equivalent to 10 000 ppm of whole toxic waste), and at least a factor of 10 below the expected toxic concentration.

4.3 Measurement of the gas volume and percentage methane.

4.3.1 The gas chromatograph is initially calibrated against a certified calibration gas (refer to SME Work Instruction 12.1), with the exception that the instrument is only calibrated against methane.

4.3.2 The gas loop of the GC is connected up to the syringe valve and the bubble flow meter such that the gas passes through the gas loop before its volume is measured in the flow gauge.

4.3.3 Thoroughly wet the inside surface of the bubble flow meter to minimise the bubbles prematurely bursting and then adjust a bubble with the glass syringe so that the bubble sits on the zero mark of the gauge. Empty the syringe through the needle, and whilst holding the syringe tightly closed insert the needle through the septum into the bottle. Vent the excess gas in the bottle slowly through the gas loop into the flow gauge and measure the volume of gas produced. Inject the gas into the gas chromatograph and measure the gas volume.

Use a worksheet to record against the bottle number, the volume of gas produced, the methane content of the gas, and the GC data file.

4.3.4 The test can be continued until the total amount of methane produced has levelled off. Initially a measurement of the methane is made weekly but in the later stages of the test the measurement can be made every two or three weeks. The conclusion of the test is when no measurable amount of methane has been produced.

4.3.4 Transfer the data to an Excel spreadsheet, and use the spreadsheet to calculate the amount of methane that has been produced since the last reading, subtracting the amount of methane remaining in the headspace of the bottle from the previous reading. Assume that the void in each bottle is 60 ml.

Plot the mean cumulative amount of methane produced each week for each level of toxin against the concentration of the toxin in the bottle. The toxic level of the toxin is the amount of toxin that completely suppresses the production of methane.

5. PRECISION

5.1 The gas volume can be measured to an accuracy of less than 0.5 ml, and the methane to less than 1%, giving a accuracy of the methane produced of less than 0.01 ml methane. To achieve higher accuracy than this would involve measuring the capacity of each bottle before use, and then calculating the void left in each bottle after it has been filled. This figure would then be used in the calculations rather than the assuming that the void is 60 ml.

5.2 The method however is basically a biological method and a wide variation in the amount of methane produced can be expected between bottles. As all tests are carried out in triplicate, this error is somewhat reduced, but a probable variation of +/- 20% from replicate tests may be expected.

Interpretation

For the 7-day test:

1. Multiply the total gas volume (generated + headspace) by the methane concentration (if using %, divide by 100), to give the total methane yield in ml for each bottle (see Example 1). For the specified bottles a headspace of 60 ml can be assumed. Calculate the mean for each triplicate set. The standard deviation of triplicates can also be calculated, to ensure reasonable reproducibility; in toxin-free controls expect results within ±10%, with more variability between bottles likely as toxins near inhibitory levels.

2. Plot a graph of methane yield (ml) against $\log_{10}$ of toxin concentration (ppm) for each dilution used. This should ideally be an S-shaped curve, with a fairly linear mid-range.

3. The toxin-free control cannot be plotted (zero will not plot on a logarithmic axis), but should be marked on the vertical axis.

a) Half of the toxin-free control yield is the 50% inhibition value; where this value intercepts the dose-response curve defines the IC_{50} concentration.
b) The IC_{100} in practice usually falls between dilutions, but for interpretative purposes is the lowest concentration showing zero methane production.

Example 1, Day 7 readings:

Measured volume	25 ml	
Methane	30 %	
Headspace volume	60 ml	
Total gas volume =	60 + 25	= 85 ml
∴ Methane volume =	30/100 x 85	= 25.5 ml

For the long-term test:

1. Calculate the total methane yield for each bottle from the sum of the incremental yields over the 28 day (up to and exceeding 3 months for final yield) monitoring period. Note

that after day 7, subsequent samplings for volume and quality will require application of a correction factor for (a) residual methane and (b) removal of the previous GC sample (depending on sample volume - if following the specified method see Example 2). This can be automated on a spreadsheet.

2. IC_{50} and IC_{100} as for 7-day test.

Example 2, Day 7 to Day 14 methane production:

total methane present at day 14 ...		
Day 14 measured volume	20 ml	
Day 14 methane concentration	45 %	
Headspace volume	60 ml	
Total gas volume	60 + 20	= 80 ml
∴ Methane volume present =	45/100 x 80	= 36 ml
less residual methane present at day 7 ...		
Day 7 methane concentration as above	30 %	
Residual gas volume after sampling	53 ml (assumes 7 ml taken for GC)	
∴ Residual methane volume =	30/100 x 53	= 15.9 ml
total at day 14 minus residual methane from day 7		
Day 7 to Day 14 methane production =	36 - 15.9	= 20.1 ml
∴ True cumulative 14 day methane =	25.5 + 20.1	= 45.6 ml

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2nd draft - BCC 30/5/97

APPENDIX 4

SUMMARY OF MODIFICATIONS MADE TO THE INITIAL PROCEDURE

During the evaluation period, the operator's laboratory made a number of minor changes to the protocol discussed at the Industrial Advisors meeting. These changes have been incorporated in the SOP given in Appendix 3.

1. In the original draft SOP, nutrient Solution "A" containing ammonium sulphate, sodium phosphates & magnesium sulphate, was adjusted to pH 7.0 - 7.5 and was then used to prepare solution "B", which required further adjustment to bring the pH back to pH 7.0 - 7.5. In the operator's method solution "A" was used without pH adjustment to prepare solution "B".

2. The solution were brought to the boil and then cooled. This should normally kill sufficient of the organisms present to avoid irreproducibility of tests but it was agreed that any solution left at the end of the day was discarded.

To measure the volume and composition of gas produced, an Aldrich syringe stopcock was adapted so that the side arm had a 1/16" Swagelok thread. This enabled connection of the side arm of the stopcock directly to the inlet port of the automatic injection valve on a gas chromatograph, using a very short length of 1/16" OD s/s tubing. The outlet from the valve was then connected to a modified 100 ml gas burette graduated to 0.2 ml, which acted as a conventional bubble flow meter. In this way the GC gas sample loop can be filled at the same time as the volume of gas is measured. Gas samples were only taken from bottles where gas volumes had increased.

The actual preparation of the bottles at the SME laboratory was straightforward, with one exception, the addition of the toxin to each bottle. The preparation of the nutrient solution and the subsequent dispensing of the nutrient and "seed" into each bottle was carried out using 30 ml & 10 ml dispensers. The bottles were purged prior to capping by a manifold system rigged up with 1/8" OD tubing, "T" pieces and 6 lengths of 1/16" OD HPLC grade capillary tubing.

A set of 24 bottles, i.e. seven dilutions of toxin and a blank, all carried out in triplicate, took about 4 hours of a technician's time to set up. The subsequent weekly measurement of the volume of gas produced and the methane content of each bottle then took another 3 hours. The configuration of the gas chromatograph used was a 4 metre Porapaq N column at 100°C and 20 ml/min helium, giving a run-time of 5 minutes. Many other GC configurations would be acceptable; this time could have been reduced by using a shorter column, because there was more than adequate resolution between the three peaks found. All that is required is a total methane concentration. Another way of reducing the analysis time would have been by

repetitive injections into the gas chromatograph without a column in place, simply using the FI detector.

As referred to above, the only minor difficulty SME had in the preparation of the bottles was in the introduction of the toxin into each bottle. In the initial tests with phenol, the lower levels of phenol were added as a 1.0% or 10% phenol solution in water using a pipettor. In the bottles with the highest concentration of toxin, the phenol was added directly as the solid. For example the 10,000 ppm phenol bottle was prepared by adding 1.0 gram of solid phenol. Phenol, by its very nature, will adhere to any weighing implement, and the quantitative transfer of all the solid phenol into the bottle could not be guaranteed. The subsequent tests with phenol were set up by accounting for the weight of the bottle and then weighing the required amount of toxin directly into the bottle.

With the very volatile trichloroethylene, some caution had to be observed when preparing these bottles. Trichloroethylene does have a limited solubility in water and SME decided to make use of this property by preparing a 1000 ppm w/v solution of TCE in water for the lower dilutions of toxin, rather than the TCE solution in methanol or acetone used previously. This eliminated the need to run solvent blanks. The higher concentrations of TCE were made by using a micro syringe to inject the required volume of TCE directly into each bottle. To minimise loss of solvent, the household waste, nutrient and "seed" were all added to the bottles before the solvent. Once the TCE had been added to each bottle, the bottle was immediately purged with nitrogen for a few seconds only, to remove oxygen from the headspace, and then capped.