

Assessing stimulation and inhibition of anaerobic lagoons 4C-105

Report prepared for the
Co-operative Research Centre for High Integrity Australian
Pork

By

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Executive Summary

Covered anaerobic lagoons are critical to achieve low carbon pork, however, potential failure of pond systems is a key issue, especially with a number of potential inhibitors in piggery flush effluent. Despite the importance of quantifying the influence of inhibitory compounds, there is currently no well-defined method to assess inhibition and experimental methods vary widely/are frequently very time-consuming and not workable. As a result, proper inhibition testing rarely forms part of anaerobic digestion studies, despite its usefulness and importance. To promote the widespread use of inhibition testing, the present study develops and validates a simplified and unified inhibition assay.

The detailed project approach and results are primarily defined in a draft journal paper. However, in summary, a simplified and unified inhibition test protocol was successfully defined and validated through extensive experimental test work. The targeted biological step was acetoclastic methanogenesis as it is typically most sensitive to inhibition and provides rapid inhibition testing. In the method development experiments, attention was given to important details of the unified method, including identification of the optimum carbon source and a preferred inoculum concentration. Measured specific methanogenic reaction rates covered a reasonable range of 0.005 (fully inhibited) to 0.16 (native inoculum, no inhibitor added) gCOD methane.gVS⁻¹ inoculum.day⁻¹, which indicated reasonable test outcomes. Results suggested that acetate (added as sodium acetate) was a better carbon source than acetic acid, and that an inoculum-to-acetic acid volatile solids ratio of five was preferred to not limit the availability of carbon and to not have conditions which are too dilute. Further, of relevance to concentrated biomass sources such as covered lagoon settled sludge, the results suggested that dilution of sludge samples down to 10 gVS.L⁻¹ is required before testing. This is to obtain reliable measurements by minimizing mass and gas transfer limitations. The method was tested on two very distinct inoculum sources, one being sludge from a covered piggery lagoon, and the other being conventional municipal wastewater anaerobic digester sludge. Testing was done with two well-known inhibitors, namely ammonia and sodium. The inhibition response was observed to vary slightly with the inoculum under trial and was found to be largely consistent between the simplified (minimum number of sampling events) and the full-test method (multiple sampling events). Significantly, in the simplified version of the test technique, as few as two sampling events over 1.5 days provided a reliable measure of inhibition. The test was also successfully applied to an antibiotic sulfathiazole and gave results in line with expectations. Further testing of the method on other inhibitors/stimulants has been deferred to the larger Pork CRC inhibition/stimulation project 4C-109.

The inhibition test method will form an integral part of the toolbox of analyses to be used in future anaerobic digestion studies including Pork CRC projects 4C-111 *Anaerobic treatment for emissions reduction from solid manure residues* and expanded inhibition and stimulation of anaerobic lagoons in Project 4C-109 *Enhanced methane production from pig manure in covered lagoons and digesters*. Further, through the Appended draft journal paper (a Methods paper), the inhibition assay will be more broadly promoted also outside the pork industry. Adoption of the test method by NATA accredited analytical test laboratories across Australia is expected to provide testing capabilities to support producers across the country that are undertaking biogas projects.

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1. Introduction

Covered anaerobic lagoons are critical to generation of low carbon pork from intensive production. However, potential failure of pond systems is a key issue, especially under the influence of inhibitors that enter the pig flush effluent. Additives such as antibiotics and other chemicals are of key concern and, in addition, lagoons will increasingly be exposed to co-digestion of more than just manure when co-products are used to augment feed and a portion ends up directly in the effluent treatment system. Further, microbial additives are being marketed that claim to substantially enhance pond performance. There is very little information on how to conduct tests that analyse whether a compound is likely to have a beneficial or negative impact. Existing test methods are considered to be poorly validated, require extended periods which are not workable, and are very expensive, mainly due to the high level of speciality expertise and time required. While more extensive test protocols may need to be maintained as an option to increase scientific rigour, there is also a market need for a low cost, relatively rapid test that can provide threshold analysis (use/don't use) rather than the very detailed results.

In view of appropriately quantifying the impact of inhibitors and/or enhancing agents, this project sought to define and validate, through extensive experimental work, an inhibition and activity test assay. Such a unified assay has not been previously reported, even in the broader anaerobic digestion community, and therefore also has broader implications for anaerobic digestion studies across other industries. Various test assay conditions were assessed through the project to fully define the assay method. To promote widespread adoption, a simplified and unified test assay was targeted with a cost potentially as low as \$250/test without compromising data quality.

2. Methodology

This section lists the original defined methodology as per the project proposal followed by key project outcomes achieved against each method step.

1. Method step as per original proposal: Definition of a test method and application to Pork Industry model compounds. A detailed inhibition and stimulation test method will be developed from existing protocols at UQ and UWA, specifically for the Pork Industry.

Outcomes at project completion: A test method was properly defined including consideration of detailed aspects which were developed through extensive experimental test work as per the attached Draft Journal paper. This method was then tested on two microbe sources (inoculums) and ammonia and sodium as two key potential inhibitors that are of relevance to the pork industry.

2. Method step as per original proposal: The test method is to be applied from the second year onwards, with recruitment of a PhD student to continue the project.

Outcomes at project completion: Pork CRC PhD student Shao Dong Yap is likely to use the test method extensively on Project 4C-111 to study inhibition during the digestion of spent ecoshelter bedding. The inhibition test method will also form an integral part of the toolbox of analyses to be used on the expanded inhibition and stimulation of anaerobic lagoons in Project 4C-109.

3. Method step as per original proposal: development of a low cost, widely applicable test method. (This is to provide a unified method that, unlike the existing methods available in the literature, is simple and effective for more general use.) This can then give relative performance over a wide range of concentrations, to provide a continuous reflection of how activity changes with concentration. A lower cost test that will test a single target compound at a single concentration will be developed. This will allow parallel test of a control, with a test at a known concentration, and will indicate using a threshold performance evaluation, whether the compound can be used at this concentration.

Outcomes at project completion: A simplified test method was developed and validated with two very distinct microbe sources (including sludge from a covered piggery lagoon and a conventional municipal wastewater anaerobic digester sludge). This simplified method is very practical and can be readily adopted by general analytical services laboratories across Australia, making it more readily available for use by piggeries that are taking up covered anaerobic lagoons. The simplest, yet robust form, involving testing of one sample with one inhibitor at a single designated concentration, can cost as little as AU\$250/sample, thus achieving this target cost objective.

4. Method step as per original proposal: The two tests will be developed and applied in the first year on a minimum of three model compounds. One known to be inhibitory, one thought to be stimulatory, and one with mixed properties. Producers will be consulted to select these model compounds, but there may be phosphorous, the second an antibiotic, and the third potassium. The test methods will be simultaneously developed and cross-verified between UQ and UWA using separate inoculums. As part of the analysis, impact on microbial population in the detailed test will be assessed through 16s DNA directed analysis. This will indicate whether specific microbial populations are impacted by the target compounds.

Outcomes at project completion: Producers were consulted early-on the project via the bioenergy support program, most notably including a live inhibition analysis and test series performed to troubleshoot the Rivalea Bungowanah effluent treatment systems. A literature review early on in the project revealed that the available test techniques were very poorly developed. As a result the present project had to undertake extensive additional experimentation to define robust conditions for the protocol. Attention had to be given to details such as selection of a preferred carbon source and the need for dilution of a microbe sample upon receipt in preparation for the test series. Due to this extensive additional experimental work, microbial population analysis was deferred to the more relevant in-depth 16s DNA directed analysis of the expanded inhibition project in 4C-109. This will indicate whether specific microbial populations are impacted by other targeted compounds that are less well-known than sodium and ammonia nitrogen, and will be used to assess the potential for engineering a robustness in the microbial community against inhibitors.

The project included two visits to the UWA to standardize their test techniques so that UWA and UQ methods align. UWA is planning on carrying out inhibition testing using the assay of the present project on Nomensin as a key suspected inhibitor of some ponds in WA. The UWA tests will contribute data to 4C-109, and depending on timing, perhaps to the journal paper of the present project before submission.

The inhibition assay was tested against two model/well-known inhibitors, namely ammonia nitrogen and sodium, which are particularly relevant at piggeries where effluent is recycled. The testing plan did deviate slightly from that detailed above, in that the literature review early on in the project (See Appendix 4 for evidence) indicated that sodium and ammonia would be better inhibitors on which to standardise the test protocol, instead of what was proposed above in the original proposal. This was because of the well-known inhibition profile of sodium and ammonia and the extent of background information on their action. This is noted, because sodium and ammonia do not represent stimulatory or mixed properties, such as potassium. Nevertheless, testing of other inhibitors/stimulants such as phosphorus, potassium and other antibiotics were deferred to and will be performed in the larger inhibition project 4C-109.

3. Outcomes

The detailed project results of experimental test work are primarily defined in the Appended draft journal paper to be submitted to a highly ranked peer reviewed journal.

However, in summary, a simplified and unified inhibition test protocol has been defined and validated through extensive experimental test work. The method development was performed on two very distinct inoculum sources, one being sludge from a covered piggery lagoon and the other being conventional municipal wastewater anaerobic digester sludge. Two well-known inhibitors were used, namely ammonia and sodium. Attention is given to important details of the unified method that had not been previously resolved by other studies in the available literature, including identification of the optimum carbon source and a preferred inoculum concentration. In a simplified version of the test technique, as few as three sampling events over 1.5 days provided a reliable measure of inhibition.

The inhibition test method will form an integral part of the toolbox of analyses to be used to properly assess anaerobic digestion of solid manure residues in Project 4C-111 *Anaerobic treatment for emissions reduction from solid manure residues* and expanded inhibition and stimulation of anaerobic lagoons in Project 4C-109 *Enhanced methane production from pig manure in covered lagoons and digesters*.

Further, through a draft journal paper (a Methods paper), the inhibition assay is likely to be more broadly adopted across other industries that research anaerobic digestion.

4. Application of Research and Conclusion

This project defined and validated, through extensive experimental work, a robust yet simple inhibition and activity test assay. The test assay is of a general nature, being potentially applicable across anaerobic digestion in many industries, including pork production. This is significant, because such a unified assay has not been previously reported, even in the broader anaerobic digestion community, and therefore the test technique developed in this project also has broader general implications for anaerobic digestion studies. Various test assay conditions were assessed through the project to fully define the assay method. To promote widespread adoption, a simplified and unified test assay was targeted with a cost potentially as low as \$250/test without compromising data quality.

It is expected that the test would work well for water soluble antibiotics (and indeed has been found for one such antibiotic to give results which were in line with expectations). The test would however require some modifications for non-water soluble antibiotics with potential addition of a third component solvent and parallel testing to confirm that the added third component does not give a false negative result due to its own inhibitory action.

The test should also work well with stimulant products and/or mixed property compounds, provided these influence the sensitive step aceticlastic methanogenesis.

The test technique is well-documented in this report/in the associated journal paper and can accordingly be promoted for adoption by NATA accredited test facilities across Australia.

It is highly recommended that the test assay development work carried out in this project be further validated through submission of the prepared journal paper to a high quality journal to go through the associated peer review.

5. Commercialization Pathways

To commercialise the inhibition assay, the following steps need to be taken by the nominated entities:

1. Peer review and publication of methods paper - The University of Queensland with Pork CRC endorsement.
2. Promotion at and feedback from international conferences - subsequent refinement of the assay - To be delivered by The University of Queensland with Pork CRC endorsement. An abstract has already been submitted to the Latin-American Anaerobic digestion conference in Habana Cuba 2014.
3. Promotion at and feedback from national conferences - subsequent refinement of the inhibition assay. - To be delivered by The University of Queensland with Pork CRC endorsement. An abstract will be submitted to Bioenergy Australia conference 2014.
4. Promotion of assay method to analytical laboratories associated with The University of Queensland and partner entities. These currently include Water Utilities/Municipal wastewater entities who have a natural need for the assay with anaerobic digester control and studies, and therefore will have internal drive to adopt the assay for internal purposes. One key issue identified to date is the general lack of robustness and capability of other labs around measurements of methane production.
5. Further refining the test method through use on on-going Pork CRC projects 4C-109 and 4C-111. Publishing and promoting of results in other peer reviewed outlets - The University of Queensland with Pork CRC endorsement.
6. Promotion of results to NATA accredited labs across Australia. The simple inhibition assay that was developed through the project, is able to be run during convenient daytime work hours, and so fits in well with normal logistics of typical analytical laboratories. Any uncertainty in results can also be well managed by statistical analysis of the test results, by replication and with a positive control test.

6. Limitations/Risks

The test method has been validated with two very distinct inoculum (microbe) sources, and may not be applicable to all inoculum sources (e.g. microbes that are present in spent eco-shelter bedding). However, residual risk is low, because the test conditions that were trialed during the project covered a wide range which is expected to be inclusive of the behavior of most of the microbe sources of relevance to anaerobic digestion in the pork industry.

The test method has been validated with two key inhibitors, but may not be applicable to all other inhibitors. However, residual risk is low, because on-going use of the test method in Projects 4C-109 and 4C-111 will further clarify/defend the general applicability of the assay to other inhibitors and stimulants of relevance to the pork industry.

7. Recommendations

- It is recommended that the draft journal paper be submitted for publication to a quality peer reviewed journal such as *Journal of Hazardous Materials*.
- It is recommended that the inhibition test method be adopted to value-add to research in Projects 4C-111 and 4C-109.
- It is recommended that the commercialization pathways proposed above be followed to encourage adoption of the inhibition test method. Also, the method should be promoted to NATA accredited analytical test laboratories across Australia, to encourage adoption and to create analytical service capability to producers across the country.

Appendix 1 - Proposal for PhD student project previously defined for CRC board consideration

Project did not continue, but 4C-111 acquired a PhD student and will use the inhibition assay. Project 4C-109 also captures a fair quantity of the work described in this PhD project proposal.

PhD/MPhil Research Project: Activity and Inhibition in Anaerobic Lagoons
Pork CRC Program 4.

University support: UQ/UWA

This is a PhD proposal for a project to be run in conjunction with existing UQ and UWA projects assessing fundamentals of microbial activity and inhibition in covered anaerobic lagoons and other anaerobic digester systems funded by the pork CRC.

Background and Motivation. The overall mechanisms of inhibition, the way in which different communities respond, robustness to inhibition, and recovery characteristics are not well understood. This project will use experimental platforms being developed at UQ and UWA to investigate fundamentals of inhibition and toxicity in anaerobic systems, and identify key characteristics which either make microbial populations more robust, or increase their capacity to recover. It is envisaged that the PhD project will focus on fundamentals while supporting projects will focus on applications.

Methodology and Areas of Application. The project will either be 2-year (M. Phil) or 3-year (PhD), with an M. Phil expandable to PhD if this better suits prospective candidates and the CRC goals. Infrastructure and supervision will be provided by hosting institutes and the higher level projects, while direct analytical support, consumables, and salary/salary top-up will be provided by the CRC:-

- (a) **Method development, investigation of inhibition mechanisms, and identification of community sensitivity.** The first year will focus on identifying inhibition thresholds for the key model compounds (biostatic and biocidal - at least 4 model compounds) on a number of different feed materials. It will also assess in further detail the cut-down methodology being developed for 4C-105. Community analysis will be used to assess biological response to inhibition of microbial populations. Appropriate models will be developed for biostatic and biocidal inhibition. **Deliverable: Identification of inhibition modes. Models for inhibition types. Determination of factors influencing community types**
- (b) **Engineering Robustness.** Continuous reactors will be operated with defined inhibitory compounds in a control-experiment format. Pyrosequencing will be done to assess the differential development of the community. Acclimatised and control communities will be subsequently tested for sensitivity to inhibitors in batch mode. **Deliverable: Identification of microbial factors determining robustness. End of M. Sc. Program.**
- (c) **Optimal operation.** Continuous reactors will be operated in control-experiment formats with alternative operating conditions (temperature will be the initial variable) to identify operating conditions that can promote community robustness. The community will not otherwise be acclimatised, but batch inhibition testing will be done on different inhibitors after steady state is achieved. **Deliverable: operational conditions that enhance robustness. End of PhD Program (1).**
- (d) **Option Functional analysis.** If the communities in (b) are convergent, an alternative to (c) may be to use advanced molecular techniques to identify the mechanism through which additional robustness is achieved. **Deliverable: Identification of factors that determine robustness. End of PhD Program (2).**

Appendix 2 - Protocol for short-cut inhibition test assay.

Glossary

Acetate	An organic acid of length 2 - CH_3COOH . A major metabolic intermediate.
Aceticlastic methanogenesis	The major process of conversion of acetate to methane and carbon-dioxide
Active compound	Where both basic and acidic forms of a compound exist, the form that is uncharged
Ammonia	$\text{NH}_3/\text{NH}_4^+$ Reduced form of nitrogen that is generally inhibitory
Anaerobic	Biological processes in the absence of oxygen, nitrate, or nitrite
Cellular homeostasis	The need for cells to maintain a specific intracellular range of salinity and pH
Control	A complement to an experiment that will have a defined response
Digestate	Material sampled from the contents or outlet of a digester
GC-TCD	Gas-chromatography thermal conductivity detector. Standard method for CH_4 and CO_2 analysis
HAc	Acetic acid
$\text{IC}_{50}/\text{KIC}_{50}$	The concentration at which a microbial population has 50% of their maximum, uninhibited rate
Inhibition	An impairment in function, generally reversible
LD50	The concentration at which 50% of a population has been killed
Method 169-03	The current method used in Melbourne for inhibition testing
Microcosm	A test that attempts to simulate a real world environment for biological tests
NaAc	Sodium acetate
NaCl	Sodium chloride (Salt)
Primary sludge	Material settled from raw sewage
Serum flasks	Glass flasks used to conduct batch tests
Specific methanogenic activity (SMA)	The rate at which methane is generated in the presence of excessive substrate (per gram biomass)
Sulfide	Reduced form of sulfur
Test selectivity	The extent to which the test evaluates the target property
Total compound	The total amount of acid+base where both are present
Toxicity	Generally lethal impact on microbes
UQ	University of Queensland
Waste activated sludge	Waste sludge from an activated sludge plant (feed to digester).

Background

Anaerobic processes

Anaerobic digestion is a multi-step process in which complex organics are converted sequentially to solubles, organic acids, and methane (Figure 1) (Batstone and Jensen 2010). During anaerobic degradation of organics, particulate material is fed as primary or waste activated sludges.

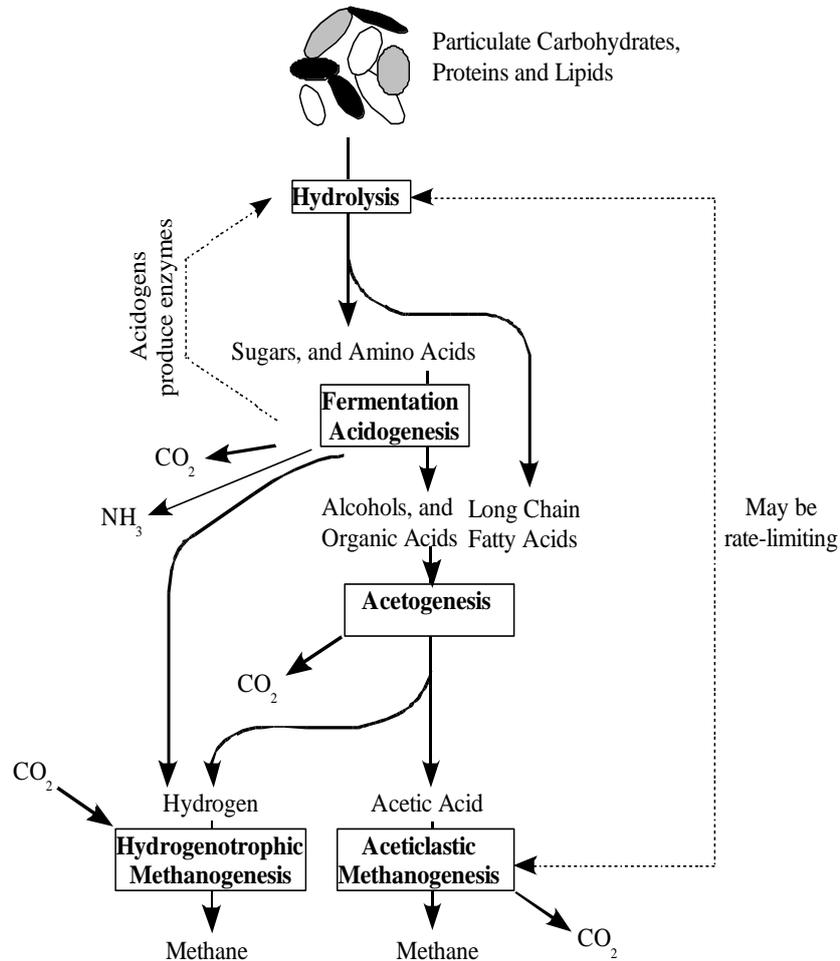


Figure 1: Sequential degradation of organics in anaerobic processes (Batstone and Jensen 2011).

Primary sewage sludge and activated sewage sludge are generally rate-limited by the first step, or hydrolysis, with primary sludges being slower to degrade, but degrading to a greater extent, and with activated sludges being more rapid to degrade, but degrading to a lower extent (Ge et al. 2009; Ge et al. 2011). This is why primary sludge can produce more gas, but still remain unstabilised at a nominal retention time of 15 days.

Digestion of primary and activated sludge has a number of goals (Batstone and Jensen 2010):-

- (a) Removal of putrescent material that will subsequently cause odours and attract flies and other pests.
- (b) Destruction of organics to reduce final bulk volume and improve dewaterability.
- (c) Production of methane (renewable energy) from the material destroyed in (a).
- (d) Destruction of pathogens.

The optimal conditions for addressing all treatment goals is a temperature of 37°C, and a retention time of 15-20 days (for activated and primary respectively). The higher temperature is required to enhance rates for all processes, which approximately double for each 10°C rise in temperature. The upper limit is fixed to 37°C to avoid increased ammonia inhibition at higher temperatures (Batstone and Jensen 2010).

Inhibition and Toxicity

Inhibition and toxicity are separate mechanisms which can be defined as follows (Speece 2008):-

Inhibition:- An impairment of function. Generally reversible. The inhibition coefficient ($K_{IC,50}$) is the inhibitor level at which the biological uptake rate is half the maximum.

Toxicity:- an adverse effect (not necessarily lethal) on bacterial metabolism. Generally irreversible. Toxicity is measured by the LD_{50} or median dose - dose which will kill half the population.

While design is based on the first step of hydrolysis, the processes of inhibition and toxicity have the largest impact on the final step of acetoclastic methanogenesis, conversion of acetate to methane. Approximately 50% of the carbon and 70% of the energy (COD) is said to flow through this final precursor.

In the case of inhibition, organic acids can accumulate, causing poor digester performance, or in more extreme cases, accumulation of acids, causing the reactor to sour (low pH causes its own inhibition, which generates a feedback mechanism). While mild inhibition cases may be recovered by reducing load, or pushing through the inhibition, severe cases require pH correction, load decrease, and may require other corrective actions.

In the case of toxicity, organic acids will again accumulate, but in extreme cases, methanogenesis will completely stop. This is not recoverable, the undigested material needs to be removed, and the digester reseeded.

Modes and compounds causing inhibition

Inhibition is commonly due to increases in compounds that disrupt cellular homeostasis, or otherwise increase the amount of energy needed for cellular

maintenance. These broadly include the following classes (from most to least common):-

1. Free acids or bases that disrupt homeostasis (organic acids, hydrogen sulfide, free ammonia)
2. Cations and anions (wide range, all with different IC₅₀ values)
3. Lumped factors (largely pH and temperature).

These are listed in the following table (together with approximate IC₅₀ values):-

Table 1: Common inhibitory compounds

Compound	IC ₅₀ (total compound) @ pH 7, 37°C	IC ₅₀ (active compound)	Condition at which inhibition occurs.
NH ₃ (ammonia)	2000 mgN L ⁻¹	14-30 mgN.L ⁻¹	High pH
H ₂ S (hydrogen sulfide)	100 mgS L ⁻¹	32-40 mgS.L ⁻¹	Neutral and low pH
HVFA (organic acids)	2000 mgHAc L ⁻¹	13 mg.L ⁻¹	Low pH
Sodium	5-30 gNa L ⁻¹	N/A	All
pH	6.5	N/A	Low
pH	8	N/A	High

The most important column is the second, which indicates the approximate indigester concentration for inhibition. The third column indicates the inhibition coefficient for the active inhibitor (normally free acid or base). The fourth indicates where inhibition commonly occurs. Many inhibitors are also stimulants, and reducing below the acclimatised level may cause a decrease in activity.

Modes and compounds causing toxicity

Toxicity is far rarer, and can have a wide range of modes. The most common though is adsorption of the compound onto the surface of the microbe, inhibiting membrane bound enzymes. Long chain fatty acids are probably the most common toxicant, operating via this mechanism (Hwu 1996). Other examples of toxicants include detergents, aldehydes, nitro-compounds, cyanide, azides, antibiotics and electrophiles (Batstone et al. 2002a; Speece 2008).

Once compounds adsorb or react with the cellular matrix, they are commonly, though not always deactivated.

Toxic compounds generally have no stimulating impact.

Analysis of inhibition and toxicity

The three tests that can be used for practical digester inhibition or toxicity are target compound testing, microbial population testing, and activity testing. The first two are generally only for specific toxicants or for forensic testing, and are not suitable for routine analysis of samples with unknown constituents.

Biochemical activity of the methanogens is tested by the specific methanogenic activity (SMA) (Angelidaki et al. 2007; Jensen and Batstone 2010). In this test, 1g/L acetate is added in a serum flask in triplicate, and the generation of methane measured over time. This results in a curve as shown below, which can be fitted against a line to give specific methanogenic activity (SMA):-

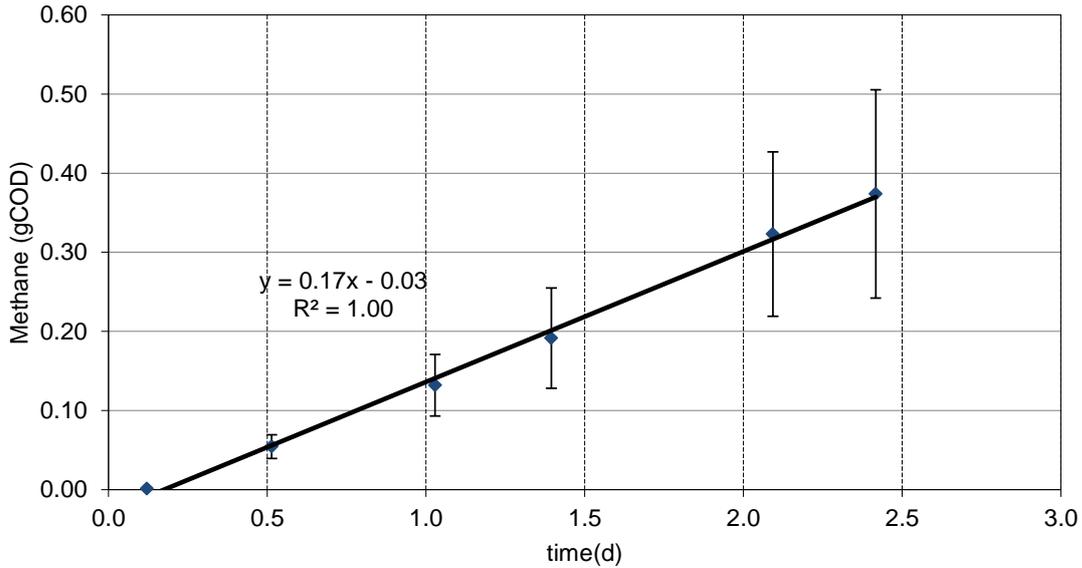


Figure Example SMA test showing SMA of $0.17 \text{ gCOD gVS}^{-1} \text{ d}^{-1}$

Gas production can be measured by either gas chromatography with a thermal conductivity detector (GC-TCD), by pressure accumulation, or by liquid displacement.

This can be then done against different dilutions of the target inhibitor or matrix (sample to given an inhibition curve):-

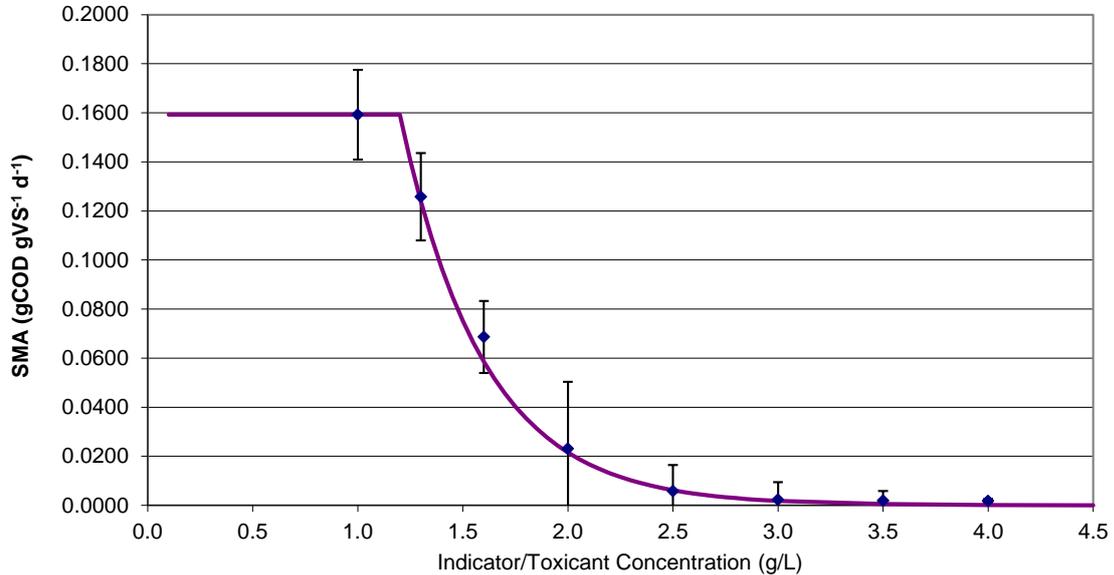


Figure Inhibition response of SMA to serial dilutions

This shows clearly an inhibitor response with a $K_{IC,50}$ of approximately 1.5 g L^{-1} .

Inhibition Tests

Goals of a typical anaerobic process inhibition test

A test methods need to identify whether a candidate matrix is likely to cause inhibition in lagoons when added at normal dilution levels. It needs to have the following characteristics:-

- (a) Provide a quantifiable level of inhibition at 5% addition rate
- (b) Have a realistic pass/fail measure of acceptance of inhibition impact
- (c) Provide repeatable results between different tests and laboratories
- (d) Provide some level of objective and inherent quality control
- (e) Be able to identify overall impact of matrix and/or unknown inhibitors
- (f) Have commercial pricing of <\$300/sample for a shortcut method
- (g) Commercial pricing on the order of \$5,000 for the full method (5-7 triplicate levels).

This project will develop and validate a shortcut method as an alternative to the full method by cross-validation.

Apparatus

- Determination of the specific methanogenic activity (SMA)
 1. 120 or 160 mL serum glass bottle, gas-tight septum and its corresponding screw cap or aluminium crimp (per sample).
 2. Precision balance capable of weighting 310 g with an accuracy of ± 0.01 g.
 3. 1 mL micropipette.
 4. Temperature-controlled incubator set at 35 ± 1 °C.
 5. Measurement biogas/methane production device:
 - Electronic or liquid manometer
 - Liquid displacement system
 - Low friction gas syringe
 6. Gas chromatograph for biogas production analysis, if required.
- Determination of inoculum total and volatile solids
 7. Analytical balance with an accuracy of ± 0.001 g.
 8. 50 mL crucibles.
 9. Drying oven set at 103 ± 2 °C
 10. Muffle furnace set at 550 °C.
 11. Desiccator with silica gel.

Reagents and materials

7. Deionised water.
8. Sodium acetate stock solution (200 g acetate L⁻¹). Dissolve 69.47 g of sodium acetate anhydrous into a 0.25-L volumetric flask. Mix well with a magnetic stirrer.
9. Inoculum dilution. Determine the VS concentration of the as-received inoculum and dilute it to reach a volatile solid concentration of 10 g VS L⁻¹ approximately.
10. Inoculum conditioning. The inoculum should be stored at a 35 °C temperature-control incubator for 20 - 30 hours before starting the experiment.
11. Determine the VS concentration and the inhibitor concentration of the diluted inoculum, since this is the value to be used for the specific methanogenic activity calculations.

Procedure

1. Add, by volume addition or weighting, the required amount of chemical needed to reach the desired concentration of inhibitor.
 2. Weight 99.0 ± 0.1 g of diluted inoculum (10 g VS L⁻¹) in a serum bottle.
 3. Add 1 mL of the sodium acetate stock solution.
 4. Swirl and measure the pH of the bottle content.
 5. Flush the headspace of the bottle with inert gas (e.g. 99.99% N₂, 80/20% N₂/CO₂, 99.99% He).
 6. Seal the bottles with a rubber stopper and fix it with a screw cap or aluminium crimp.
 7. Place the serum bottles at the temperature-controlled incubator set at mesophilic conditions.
 8. Measure the methane production at 0.5, 1.0 and 1.5 days, approximately, after the beginning of the assay (or at more frequent intervals if better statistics are desired).
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1. Plot the accumulated specific methane production in COD-basis (g COD g⁻¹ VS_{inoculum}) in the course of time. Remember that 350 mL of methane at standard conditions (0 °C, 1 bar) is 1 gram of COD.
 2. Determine the slope and uncertainty of the linear zone on the methane production curves (as shown in the figures above).

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