# The Malthus system for biocide efficacy testing against *Desulfovibrio desulfuricans*

**EE de Bruyn[1]*, E Croukamp[2] and TE Cloete[1]**
[1]*Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa*
[2]*Swift Micro Laboratories, Mayfield Park, Johannesburg 2001, South Africa*

## Abstract

Microbially influenced corrosion (MIC) makes an important contribution to corrosion in various industries. Considerable success has been achieved by the use of biocides. Little information for controlling MIC is, however, available on the effectivity of biocides against sulphate-reducing bacteria (SRB) due to the difficulties of culturing these organisms using conventional techniques. Conductance changes monitored using the Malthus system were evaluated as an alternative method of estimating numbers of *Desulfovibrio desulfuricans* for laboratory biocide evaluations. The correlation of $\log_{10}$ counts of *Desulfovibrio* cells in iron sulphite (IS)-medium using conventional techniques with detection times using the Malthus systems was highly significant (r = 0,974), indicating that the Malthus system can be used as an alternative method to conventional media for the enumeration of SRB. Growth studies of *Desulfovibrio* using the Malthus system were useful in the evaluation of biocides. A 56 % and a 100 % kill was obtained when using 60 and 200 mg/l quaternary ammonium compounds (QAC), respectively.

## Introduction

The importance of dissimilatory SRB in MIC has been widely recognised for many years. Whilst their role in the sulphur cycle is fundamental in maintaining our environment, the adverse economic consequences of their activities can be devastating in industrial processes. These bacteria can cause health hazards and corrosion of equipment and pipelines (Boivin and Costerton, 1991; Crombie et al., 1980; Ford and Mitchell, 1990; Hamilton, 1985). The detection and monitoring of SRB in industrial water systems as well as their control by making use of biocides are therefore important to the industry.

The use of biocides to control biofouling in industrial water systems is an accepted practice (Cloete et al., 1992). However, incorrect use of biocides gives rise to biofouling and resistance developement in bacteria (Brözel and Cloete, 1991). It is therefore essential to select the correct biocide or combinations and their respective concentrations for the organisms to be killed. There are a variety of techniques for determining the effectivity of biocides (Cloete et al., 1990; Hill et al., 1989). Little published information is, however, available on the effectivity of biocides against SRB (Sharma et al., 1987).

There are many culture media formulations available that can be used for enumerating SRB (Ferodak et al., 1987; Pankhurst, 1971; Pfennig et al., 1981; Postgate, 1984). The preparation of anaerobic media is difficult and laborious (Gaylarde and Cook, 1987). It has been recommended that media should be incubated for up to 28 d (Herbert and Gilbert, 1984). Alternative methods such as antibodies (Bobowski and Nedwell, 1987; Gaylarde and Cook, 1987; Odom et al., 1991), have a low sensitivity. The high cost involved in using nucleic acid probes (Amann et al., 1990; Amann et al., 1992) and antibodies limit their use in the industry as well as in routine evaluations of biocides in the laboratory. Because of the difficulties associated with the enumeration of SRB, biocide evaluations against SRB have been neglected in the past.

Electrical methods (conductance, impedance and capacitance) are established methods for monitoring microbial growth and estimating bacterial numbers (Richards et al., 1978). One such system (Malthus) is based on the automated monitoring of electrical conductance in growing bacterial cultures. Conductance is measured by the introduction of platinum electrodes in the medium and the application of a low frequency voltage. When conductance values increase beyond a threshold value, these are recorded by the system and displayed graphically. The change detected in conductance is due to the metabolism of the constituents of the culture medium by the organisms. The time lapse between inoculation and a noticeable change in conductance is termed the detection time. Detection time is inversely proportional to the logarithm of the number of viable organisms inoculated into the medium assayed so that the instrument can be used for determining bacterial numbers (Gibson, 1985). Gibson (1987) used conductance measurements (Malthus Instruments, LTD Stoke and Trent, UK) to detect the growth of *Clostridium botulinum* in a selective medium. This indicated that the Malthus system had successfully been used for enumerating bacteria using selective media.

Therefore the technique of monitoring conductance changes using the Malthus system was evaluated as an alternative method of estimating numbers of *Desulfovibrio desulfuricans* for laboratory biocide evaluations. Not all culture media may be appropriate for conductive measurements (Gibson, 1987). Iron sulphite-medium (Mara and Williams, 1970) was chosen for these experiments, since this medium yielded the highest numbers when counting pure cultures of *D. desulfuricans* in studies comparing this medium with other generally used culture media for SRB (De Bruyn and Cloete, 1993).

Quaternary ammonium compounds are generally used in water water cooling systems for the control of algae. It would therefore be of interest to know what the effect of QAC would be against SRB in these systems. This was used as a model compound for evaluating this technique.

## Materials and methods

### Test organism

*Desulfovibrio desulfuricans* subsp. *desulfuricans* (DSM 1924) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

*To whom all correspondence should be addressed.
*Received 27 November 1992; accepted in revised form 5 August 1993.*

### Culture medium

Six m*l* of IS-medium (Mara and Williams, 1970) was dispensed into Malthus tubes which were then autoclaved and used for conductance studies. Resazurin (1 m*l* of a 0,1 % w/v solution) was added to the medium as a redox-potential indicator. Titanium(III) citrate (Zehnder and Wuhrmann, 1976) was used as a reducing agent. Anaerobic tubes with 5 m*l* pre-reduced sterile IS-medium (Mara and Williams, 1970) with 1,65 % w/v agar was used for the enumeration of *Desulfovibrio* cells. The tubes were filled with a gas phase of 20 % $CO_2$, 10% $H_2$, balanced with $N_2$ and sealed with neoprene rubber stoppers and screw caps.

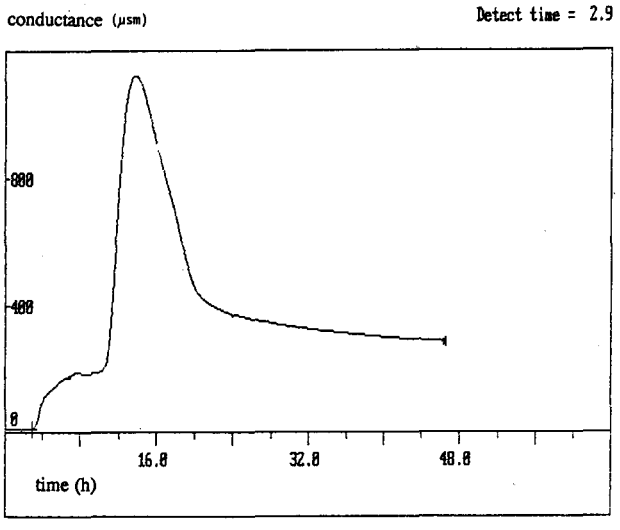
### Malthus calibration experiments

*D. desulfuricans* was precultured in IS-medium under anaerobic conditions at 30°C for 3 d. A primary dilution series ($10^{-1}$ to $10^{-9}$) was prepared from the culture. A secondary dilution series ($10^{-1}$ to $10^{-9}$) was prepared from each primary dilution. An 0,1 m*l* aliquot of each of the latter dilutions was inoculated into anaerobic tubes containing molten (45°C) IS-agar. After inoculation and preparation of agar roll tubes (Hungate, 1969) these were incubated at 30°C for 14 d. All the black colonies that developed after 14 d were counted as being *Desulfovibrio*. Subsequently, 23 Malthus tubes (the required amount for statistical analysis) were inoculated with 1 m*l* of the primary and secondary dilution series. Conductance readings at 30°C were recorded for up to 48 h, using the Malthus 2000 detection system (Swift Micro Laboratories (Pty) Ltd.). Detection times were defined as the time at which there was a noticeable change in conductance (Fig. 1). Detection times were then plotted against the conventional *Desulfovibrio* counts in agar roll tubes containing IS-agar. Statistical analysis of the results was performed using the Malthus statistics software version H2.02.01. (Malthus 2000, Swift Micro Laboratories).

### Biocide evaluations

All experimental work was carried out in triplicate. A *Desulfovibrio* culture was grown anaerobically in IS-medium (Mara and Williams, 1970). Initial numbers of this culture were determined by inoculating 6 Malthus tubes each, with 1 m*l* of the culture and monitoring detection time using the Malthus 2000 system. After determining the initial *Desulfovibrio* numbers a quaternary ammonium compound (QAC) was added to 3 of the respective tubes to give a final concentration of 20 mg/*l*, 40 mg/*l* and 200 mg/*l*, respectively. The other 3 tubes were used as controls. The Malthus tubes with culture and bactericides as well as Malthus tubes with culture alone (control) were incubated at 30°C for 6 h, after which the *Desulfovibrio* numbers were again determined. The initial *Desulfovibrio* numbers and the numbers after 6 h biocide exposure were used to calculate the % kill using the following equation:

100 - (survivor count/initial count x 100) = % kill




## Results

### Malthus calibration experiment

The relationship between detection time using the Malthus system and conventional enumeration ($log_{10}$/m*l*) of *Desulfovibrio* cells using IS-agar is shown in Fig. 2. Regression analysis of the number of viable cells ($log_{10}$/m*l*) against detection time using the Malthus system gave a regression line with a slope of -3,820 and a correlation coefficient of r = 0,974. This indicated a statistically significant correlation between detection time using the Malthus system and bacterial numbers in IS-agar using the roll tube method, indicating that the Malthus system could be used for the enumeration of pure cultures of *Desulfovibrio*.

### Biocide evaluations

The detection times and bacterial numbers (derived from the regression line) of the 6 h kill test of the different biocide concentrations are shown in Table 1. Biocide concentrations of 20 mg/*l* were not effective against *Desulfovibrio*, whereas a 56 % and a 100 % kill were obtained when using 60 and 200 mg/*l* biocide, respectively.

## Discussion

Distinct, easily measurable detection times using the Malthus system were obtained when using pure cultures of *D. desulfuricans* in IS-medium. There was a good correlation (r = 0,974) between *D. desulfuricans* numbers in IS-agar and detection time of *D. desulfuricans* which indicated that the Malthus system could be used to enumerate SRB.

*Desulfovibrio* cells were not killed when QAC was used at concentrations of 20 mg/*l*, whereas a 56 % and a 100 % kill was obtained when using 60 and 200 mg/*l* biocide, respectively. The Malthus system therefore proved useful in determining whether a particular biocide concentration would be effective or not against an SRB-strain or not and could be applied for the evaluation of different biocides. The procedures involved when using the Malthus system were less difficult and less laborious than when using anaerobic culture media for the enumeration of SRB because of the smaller volumes of media used than with standard methods. Samples could furthermore be inoculated directly into the system without the need for the preparation of serial dilutions. Biocide evaluations could be completed within 48 h when using the Malthus system as opposed to 14 d using conventional techniques.

***Figure 1***
*A typical plot of the conductance change over time (h) of* Desulfovibrio desulfuricans *in iron sulphite broth using the Malthus system*

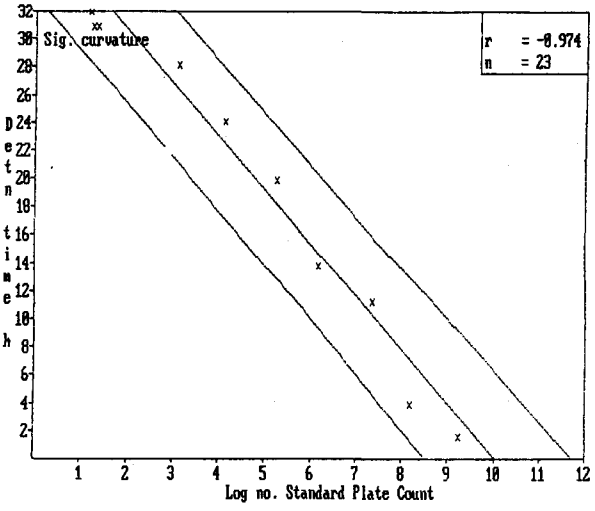



***Figure 2***
*Correlation between detection time using the Malthus system and numbers ($log_{10}$/m$\ell$) of* Desulfovibrio desulfuricans *determined in agar roll tubes containing iron sulphite medium*


## Acknowledgements

We thank Fatima Da Silva for technical assistance and Swift Micro Laboratories for supplying the Malthus 2000 detection system. This work was supported by a grant from the Water Research Commision of South Africa.


**TABLE 1**
**BIOCIDE EVALUATION USING THE MALTHUS SYSTEM**

| Biocide [ ] | Treatment | Detection time/h | Numbers /m$\ell$ | % Kill |
|---|---|---|---|---|
| 20 mg/$\ell$ | Initial numbers(0 h) | 3.8 | $2 \times 10^9$ | |
| | Control after 6 h | 1.7 | $6 \times 10^9$ | |
| | 6 h after biocide addition | 2.0 | $5 \times 10^9$ | 0 |
| 60 mg/$\ell$ | Initial numbers(0 h) | 0.4 | $9 \times 10^9$ | |
| | Control after 6 h | 0.4 | $9 \times 10^9$ | |
| | 6 h after biocide addition | 2.4 | $4 \times 10^9$ | 56 |
| 200 mg/$\ell$ | Initial numbers(0 h) | 3.1 | $3 \times 10^9$ | |
| | Control after 6 h | 2.0 | $5 \times 10^9$ | |
| | 6 h after biocide addition | >48 h | 0 | 100 |

## References


AMANN, RI, BINDER, BJ, OLSON, RJ, CHRISHOLM, SW, DEVEREUX, R and STAHL, DA (1990) Combination of 16S r RNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56** 1919-1925.

AMANN, RI, STROMLEY, J, DEVEREUX, R, KEY, R and STAHL, DA (1992) Molecular and microscopic identification of sulphate-reducing bacteria in multispecies biofilm. *Appl. Environ. Microbiol.* **58** 614-623.

BOBOWSKI, S and NEDWELL, DB (1987) A serological method, using a micro ELISA technique for detecting and enumerating sulphate-reducing bacteria. *Ind. Microbiol. Testing* 171-179.

BOIVIN, J and COSTERTON, JW. (1991) Biodeterioration of materials; biofilms and biodeterioration. In: Rossmoore, HW (ed.) *Biodeterioration and Biodegradation.* **8** 53-61. Elsevier Applied Science, New York.

BRÖZEL, VS and CLOETE, TE (1991) Fingerprinting of commercially available water treatment bactericides in South Africa. *Water SA* **17**(1) 57-65.

CLOETE, TE, BRÖZEL, VS and VON HOLY, A (1992) Practical aspects of biofouling control in industrial water systems. *Int. Biodegradation and Biofouling* **29** 299-341.

CLOETE, TE, BRÖZEL, VS and DA SILVA, E (1990) The use of bio-indicators for determining biocide concentrations in industrial water systems. *Water SA* **19** (4) 343-345.

CROMBIE, DJ, MOODY, GJ, and THOMAS, JDR (1980) Corrosion of iron by sulphate-reducing bacteria. *Chem. and Ind.* **21** 500-504.

DE BRUYN, EE and CLOETE, TE (1993) Media for the detection of sulphide-producing bacteria in industrial water systems. *J. Microbiol. Methods* **17/4** 261-271.

FERODAK, PM, SEMPLE, KM, and WESTLAKE, DWS (1987) A statistical comparison of two culturing methods for enumerating sulphate-reducing bacteria. *J. Microbiol. Methods* **7** 19-27.

FORD, T and MITCHELL, R (1990) The ecology of microbial corrosion. In: Marshall, KC (ed.) *Advances of Microbial Ecology.* **11** 231-262. Plenum Press, New York.

GAYLARDE, C and COOK, P. ELISA (1987) Techniques for the detection of sulphate-reducing bacteria. In: Grange, JM, Fox, A and Morgan, NC (eds.) *Immunological Techniques in Microbiology.* **24** 231-244. Blackwell Scientific Publications, Oxford.

GIBSON, DM (1985) Predicting the shelf life of packaged fish from conductance measurements. *J. Appl. Microbiol.* **58** 465-469.

GIBSON, AM (1987) Use of conductance measurements to detect growth of *Clostridium botulinum* in a selective medium. *Lett. Appl. Microbiol.* **8** 19-21.

HAMILTON, WA (1985) Sulphate-reducing bacteria and anaerobic corrosion. *Ann. Rev. Microbiol.* **39** 195-217.

HERBERT, JE and GILBERT, PD. (1984) Isolation and growth of sulphate-reducing bacteria. In: Grainger, JM and Lynch, JM (eds.) *Microbiological Methods for Environmental Biotechnology.* 235-257. Academic Press, Inc., New York.

HILL, EC, HILL, GC and ROBBINS, DA (1989) An informative practical strategy for preventing spoilage and improving preservation using a simple assay for biocides and preservatives. *Int. Biodeterior.* **25** 245-252.

HUNGATE, RE. (1969) A roll tube method for cultivation of strict anaerobes. In: Norris, JR and Ribbons, DW (eds.) *Methods in Microbiology.* 117-132. Academic Press, Inc., New York.

MARA, DD and WILLIAMS, DJA (1970) The evaluation of media used to enumerate sulphate-reducing bacteria. *J. Appl. Bacteriol.* **33** 543-552.

ODOM, JM, JESSIE, K, KNODEL, E and EMPTAGE, M (1991) Immunological cross-reactivity of adenosine-5'-phosphosulphate reductase from sulphate-reducing and sulphide-oxidizing bacteria. *Appl. Environ. Microbiol.* **57** 727-733.

PANKHURST, ES. (1971) The isolation and enumeration of sulphate-reducing bacteria. In: Shapton, DA and Bond, GG (eds.) *Isolation of Anaerobes*. 223-240. Academic Press, New York.

PFENNIG, N, WIDDEL, F and TRUPER, HG. (1981) The dissimilatory sulphate-reducing bacteria. In: Starr, MP, Stolp, H, Truper, HG, Balows, HG and Schlegel, HG (eds.) *The Prokaryotes*. 929-940. Springer-Verlag, Berlin.

POSTGATE, JR (1984) *The Sulphate-reducing Bacteria* (2nd edn.). Cambridge University Press, Cambridge.

RICHARDS, JCS, JASON, AC, HORNS, G, GIBSON, DM and CHRISTIC, RH (1978) Electronic measurement of bacterial growth. *J. Phys. Sci. Instrum.* **11** 560-567.

SHARMA, AP, BATTERSBURY, NS and STEWARD, DJ (1987) Techniques for the evaluation of biocide activity against sulphate-reducing bacteria. *Pharmaceutical and Environmental Industries*. Blackwell Scientific Publications, USA.

ZEHNDER, ABJ and WUHRMANN, K (1976) Titanium(III) citrate as nontoxic oxidation-reduction buffering system for the culturing of obligate anaerobes. *Science* **195** 1165-1166.