The effect of temperature and NaCl concentration on the kinetic method of toxicity determination using *Vibrio fischeri*

Elsa Correia Faria and Richard D. Snook*

Department of Instrumentation and Analytical Science, UMIST, PO Box 88, Manchester, UK M60 1QD. E-mail: richard.snook@umist.ac.uk; Fax: ++44-(0)161-200-4911; Tel: ++44-(0)161-200-4893

Received 21st June 2004, Accepted 13th September 2004
First published as an Advance Article on the web 28th October 2004

In this paper the effect of temperature and NaCl concentration on the kinetic method of toxicity determination using *Vibrio fischeri* was studied for 50 ppm Zn²⁺. This work shows that both NaCl concentration and temperature affect the kinetics of toxicity as well as the luminescence of the bacteria, and hence these are important factors that need to be considered in the development of a miniaturised portable instrument. Furthermore, this work shows that the conditions for which the kinetic test was most sensitive, *i.e.* exhibited the greatest response, were 23 °C and 2% NaCl. However, at these conditions small variations in temperature and NaCl concentration could lead to great errors in the results. Thus 12.5 °C and 2% NaCl are preferred as at these conditions the obtained results are more robust. Although at the latter conditions the toxicity rate constant was found to be 5.5 times less than that for 23 °C, the value is comparable to that obtained for 15 °C. From the data available it was also found that the temperature dependence of the toxicity rate fits the Arrhenius equation, in a behaviour similar to that of simpler chemical reactions.

Introduction

The marine luminescent bacterium *Vibrio fischeri* is one of the most extensively employed test organisms for environmental and wastewater monitoring, and since Bulich¹ described the Microtox® test as a practical and reliable method for the monitoring of aquatic samples several other tests have arrived on the market. These include LUMISTox®,² Biotox™,³ and ToxAlert®,^{4,5} and like the Microtox® test these assays rely on the measurement of the percent inhibition in the light produced by bacteria caused by the addition of dilutions of the sample to the bacteria after an incubation period (usually 5, 15, or 30 min). However, when testing environmental water samples using *Vibrio fischeri* the presence of colour and/or turbidity, which are common characteristics of this type of sample, can cause interferences leading to erroneous results usually deeming the samples more toxic than they are.⁶

This problem, although more prominent, is not exclusively associated with wastewater. Even natural water, *e.g.* from upland catchment areas, can constitute a problem as it can be naturally yellow due to organic matter, particularly humic and fulvic acids which originate from humus, peat and decaying vegetation. These acids are similar to tannic acid from tea and are not harmful.^{7,8} Naturally occurring metallic ions like iron and manganese may also impart a red and black hue, respectively. Furthermore, plankton, weeds, and contamination from industrial or municipal effluents also contribute to the colour and turbidity of environmental water samples.⁷ Natural water can also contain suspended solids (SS) in the form of small particles of clay, silt, small organic and inorganic particles resulting from erosion and large numbers of micro-organisms.⁸

A novel method to determine toxicity using *Vibrio fischeri*, first described by Fielden *et al.*,⁹ was recently shown to overcome these limitations and to be applicable to coloured and turbid samples in the same way in which it is used to test clear samples without the need for any sample pre-treatment.¹⁰ This test is a kinetic one and relies on the determination of the rate at which a test sample reduces the light produced by the bacteria. The toxicity of a sample is evaluated either by the toxicity rate constant (k_1) or the time in which the sample

tested reduces by 50% the light produced by the bacteria (ET₅₀). The time in which the sample tested reduces by any chosen percentage x the light produced by the bacteria (ET _{x}) can also be determined. Furthermore, it was also found that blank correction was not necessary for a 10-min test.¹⁰

Because this method does not suffer from interferences from colour and turbidity, and does not need blank correction it has great potential to be developed into a simple miniaturised portable single-probe toxicity test. The drawback, which the authors had already drawn attention to, is that the method relies on a kinetic measurement and thus would be influenced by temperature. Good temperature control would therefore be of the utmost importance in the development of such a test. The effect of temperature, as well as the effect of NaCl concentration, on the kinetic method are the subject of the present paper.

Conditions such as catabolite repression, pH, osmotic strength, oxygen concentration, and iron concentration also influence the bioluminescence of *Vibrio fischeri*.¹¹ However, when testing toxicity with commercially available *Vibrio fischeri*, it is temperature and NaCl concentration that need to be controlled as all other conditions are either constant or characteristic of the sample, in which case their effect on the bioluminescence is part of the toxic response.

Although the bioluminescence of *Vibrio fischeri* has been found to be optimal at temperatures greater than 24 °C the toxicity tests using this organism are commonly performed at 15 °C.¹² This particular temperature is actually an arbitrary one selected purely on historical grounds as in the past the light produced by the bacteria was found to be difficult to stabilise at higher temperatures, and lower temperatures were technically difficult to achieve.^{6,13} In itself the value (15 °C) has little meaning outside its use in standard methods, and as with the commercially available instruments toxicity measurements using *Vibrio fischeri* may be carried out within the range between 10 °C and 30 °C. The test temperature needs to be quoted with the test result.^{6,13}

It must be noted that temperature not only affects the bioluminescence of the bacteria but also the toxicity of some compounds. Although many toxicants show little toxicity difference at lower or higher temperatures, the toxicity of some

chemicals is temperature dependent. Examples of compounds that exhibit this behaviour are heavy metals which tend to be more toxic at higher temperatures, and phenolic compounds which can show the opposite behaviour. It is not the aim of this paper to study thoroughly the effect of temperature on the toxic response of *Vibrio fischeri*, but to illustrate the effect of temperature on the toxicity rate constant and thus the importance of temperature control in the kinetic method. The study was therefore carried out only on ZnCl.

Sodium chloride concentration is also a very important factor that influences bacterial bioluminescence. *Vibrio fischeri* is a marine bacterium and thus is usually found in seawater containing approximately 3.4% NaCl. When NaCl concentration is lower than 0.5% the cells lyse, and when NaCl concentration is greater than 10% the light produced is low.⁶ *Vibrio fischeri* bioluminescence was however found to be optimal and stable between 1.8 and 3.5% NaCl in which range it does not change much.^{6,13} DIN and EN ISO 11348 standards recommend the use of 2% NaCl since this is convenient and enables effectively 100% bioluminescence. Hence, non-marine samples need to be adjusted to 2% NaCl and even if the sample originally contains a small amount of salt, as is the case with many wastewaters which can contain from 0.1 to 0.5% NaCl, little impact on the test will be seen using the common approach.¹³ Marine samples should be tested as they are without osmotic adjustment.

The effect of temperature and NaCl concentration on the kinetic method had not previously been reported and it was not known whether the two conditions had an interactive effect on the response of the bacteria. Here we show the effect of both factors (NaCl concentration and temperature) on the determination of the toxicity rate constant of a 50 parts per million (ppm) Zn²⁺ solution and optimise the method simultaneously for those factors.

Experimental

The experiments were carried out using the previously described in-house built prototype Toxos-3.⁹ The test temperatures were chosen in random order from 5, 10, 15, and 23 °C. For each temperature two vials of Microtox® reagent (AZUR Environmental Ltd, now Strategic Diagnostics Inc.) were reconstituted with 1 ml of reconstitution solution (AZUR Environmental Ltd, now Strategic Diagnostics Inc.) pre-cooled to 2 °C. Six aliquots of 330 ml of the reconstituted reagent were then diluted in random order (so as to avoid virtual trends due to variability in vials) with 20 ml NaCl solutions of concentrations from 0 to 5% (NaCl 99+%, Sigma-Aldrich Co. Ltd). 50 ppm Zn²⁺ solutions (ZnCl₂ anhydrous powder 99.999%, Sigma-Aldrich Co. Ltd) of different NaCl concentrations were then tested in triplicate at a 1 : 1 sample : bacterial reagent ratio in random order, to avoid virtual trends due to reagent ageing and variability in vials, using diluent and bacterial reagent of the same NaCl concentration. The light produced by the bacteria was measured for 10 min and the toxicity rate constant was determined without blank correction.

Results and discussion

The production of light by the bacteria is influenced by temperature and if it is not well controlled erroneous results can be obtained. In a 10-min test, when temperature is controlled, and the bacteria have been allowed to acclimatise to the test temperature before the test is carried out, the light produced by unchallenged bacteria increases slightly but not significantly, and the light produced by bacteria challenged with a toxic sample decays exponentially following first order kinetics, as is shown in Fig. 1 for 50 ppm Zn²⁺ in 2% NaCl tested at 15 °C (hollow data points). However, if the bacteria are not allowed to completely acclimatise to the test tempera-

Fig. 1 Light produced (LO) by unchallenged bacteria and bacteria challenged with 50 ppm Zn²⁺ at 2% NaCl when bacteria had acclimated to the test temperature of 15 °C (hollow data points), and when the bacteria had not acclimated to the test temperature (solid data points).

ture, the light produced by unchallenged bacteria increases steeply at first and then stabilises, while the light produced by the challenged bacteria, instead of decaying exponentially, first increases steeply and then decays exponentially, as is also shown in Fig. 1 (solid data points).

As well as affecting the light produced by the bacteria, temperature also affects the kinetics of toxicity. The effect of temperature on the toxicity rate constant of a 50 ppm Zn²⁺ solution is shown in Fig. 2 for NaCl concentrations 2, 3, 4 and 5%. The toxicity rate constants were determined without blank correction because, independently of test temperature and NaCl concentration, as long as the bacteria were allowed to acclimatise to the test temperature the light produced by unchallenged bacteria increased only negligibly (see Fig. 3). As expected, for NaCl concentrations of 0 and 1% the intensity of the light produced by the bacteria was too low to be detected as these concentrations are too low to protect the bacteria from osmotic pressure and thus the bacteria lyse in these solutions. However, unexpectedly, the light produced by bacteria in 5% NaCl was also too low to be detected (except at 23 °C), suggesting that this concentration was too high for the bacteria. The toxicity rate constant values obtained for the remaining conditions were determined using the 10-min data, except the ones obtained for NaCl concentrations of 2 and 3% at 23 °C, which were obtained using the first 200 seconds and 400 seconds of the data, respectively, because the light produced

Fig. 2 Effect of temperature (T) on the toxicity rate constant (k_1) for different sodium chloride concentrations ([NaCl]). Error bars are the 95% confidence interval.

Fig. 3 Light produced by *Vibrio fischeri* at different temperatures and NaCl concentrations.

by the challenged bacteria was too low to be detected after those times.

The results in Fig. 2 show that k_1 increases with temperature for all the NaCl concentrations. The increase is not so marked between 5 and 15 °C, but is very steep between 15 and 23 °C. Thus, temperature control is important in this latter interval in particular, as small variations in temperature could lead to large errors in the results.

From the same data, when the logarithm of the rate constant is plotted against $1/T$ for 2 and 3% NaCl (Fig. 4), a temperature dependence of the toxicity rate which fits the Arrhenius equation (eqn. (1)) can be observed. For 4% NaCl the toxicity rate did not follow the Arrhenius equation.

$$\ln k = \ln A' - \frac{E_a}{R} \frac{1}{T} \quad (1)$$

Where, k is the rate constant; A' is the pre-exponential term; E_a is the activation energy; R is the perfect gas constant; T is the absolute temperature.

Although it is not known if Zn^{2+} kills the bacteria or just inhibits their metabolism, this temperature dependence is in agreement with the common observation that the rate of killing of bacteria increases with temperature in accordance with the

Arrhenius equation, a similar behaviour to that of simpler chemical reactions.¹⁴

The effect of NaCl concentration on the toxicity rate constant is shown in Fig. 5 for 5, 10, 15 and 23 °C. These results show that the toxicity rate constant decreased with NaCl concentration, except for 5 °C. At this temperature k_1 did not vary significantly with NaCl concentration. For the remaining temperature conditions, 2% NaCl was found to be the concentration that produced the greatest response.

The effect of both temperature and NaCl concentration on the toxicity rate constant is shown in Fig. 6. This figure shows that the conditions for which the greatest response (greatest k_1) and therefore greatest sensitivity were obtained, are 2% NaCl at 23 °C. Although at these conditions k_1 is 5.5 times greater than k_1 obtained for 2% NaCl at 15 °C, at 23 °C small errors in temperature or NaCl concentration can generate great errors in the determination of k_1 . More robust and reproducible results are obtained when NaCl concentration is 2% and the temperature is 12.5 °C. At these conditions, although the sensitivity of the method is much less than that obtained at 23 °C, it is comparable to that obtained with the commonly used conditions, i.e. 15 °C and 2% NaCl. It is therefore considered that 2% NaCl and 12.5 °C are the best conditions to test the toxicity of Zn^{2+} using the kinetic method.

Fig. 4 Logarithm of the toxicity rate constant ($\ln k_1$) obtained for 2 and 3% NaCl versus $1/T$. Error bars are the 95% confidence interval.

Fig. 5 Effect of NaCl concentration ($[NaCl]$) on the toxicity rate constant (k_1) for different temperatures. Error bars are the 95% confidence interval.

Fig. 6 3D graph showing the effect of NaCl concentration ([NaCl]) and temperature (T) on the toxicity rate constant (k_1).

The work reported in this paper shows that NaCl concentration and temperature both affect the kinetics of toxicity as well as the luminescence of *Vibrio fischeri*, and that these parameters need to be controlled. NaCl concentration can easily be controlled, but temperature control is more difficult. Thus, although the 23 °C is the temperature at which the most sensitive results are obtained it is best to carry out the tests at 12.5 °C as small errors in temperature will not have such great impact on k_1 , and the test will thus be more reproducible.

Acknowledgements

The authors would like to thank the UMIST Graduate School and the Fundação para a Ciência e Tecnologia (PRAXIS XXI) for funding this work. The authors would also like to thank Dr Bernard Treves Brown for his invaluable help and advice.

References

- 1 A. A. Bulich, *Process Biochem.*, 1982, **17**, 45.
- 2 L. Guzzella, *Chemosphere*, 1998, **37**, 2895.
- 3 A. Kahru, *ATLA*, 1993, **21**, 219.
- 4 M. Farré, M.-J. Garcia, L. Tirapu, A. Ginebreda and D. Barceló, *Anal. Chim. Acta*, 2001, **427**, 181.
- 5 V. L. K. Jennings, M. H. Rayner-Brandes and D. J. Bird, *Water Res.*, 2001, **35**, 3448.
- 6 *Beckman Microtox™ System Operating Manual*, Beckman Instruments Inc., Microbics Corporations, Carlsbad, CA, 1982.
- 7 *APHA-AWWA-WEF, Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, Washington DC, 1998, 20th edn., ch. 2.
- 8 T. H. Y. Tebbutt, *Principles of Water Quality Control*, 5th edn., 1998, Butterworth-Heinemann, Oxford.
- 9 P. R. Fielden, T. McCreedy, R. D. Snook and B. J. Treves Brown, *Anal. Commun.*, 1996, **33**, 335.
- 10 E. Correia Faria, B. J. Treves Brown and R. D. Snook, *J. Environ. Monit.*, 2004, **6**, 97.
- 11 *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecology, Physiology, Isolation, Identification, Applications*, eds. A. Balows., H. G. Träper, M. Dworkin, W. Harder and K.-H. Schleifer, Springer Verlag, New York, 2nd edn., 1992, **Vol. 3**, ch. 25.
- 12 P. M. Fidopiastis, S. von Boletzky and E. G. Ruby, *J. Bacteriol.*, 1998, **180**, 59.
- 13 C. C. Chou and S. S. Que Hee, *J. Biolumin. Chemilumin.*, 1993, **8**, 39.
- 14 F. H. Johnson, H. Eyring and B. J. Storer, *The Theory of Rate Processes in Biology and Medicine*, Wiley, New York, 1974, pp. 461–471.