THE MICROBIOLOGICAL QUALITY OF WATER

Edited by

DAVID W. SUTCLIFFE

Published by the Freshwater Biological Association

Invited papers from a specialised conference held in London
on 12-13 December 1995
by the
Freshwater Biological Association,
The Ferry House, Far Sawrey, Ambleside, Cumbria LA22 0LP
and
International Water Supply Association,
1 Queen Anne's Gate, London SW1H 9BT, UK

ISBN 0-900386-57-6

Detection of specific bacteria in water: implications of survival strategy

R. W. Pickup and G. Rhodes
Institute of Freshwater Ecology, Windermere Laboratory, Far Sawrey, Ambleside, Cumbria LA22 0LP, UK

It is widely recognised that conventional culture techniques may underestimate true viable bacterial numbers by several orders of magnitude. The basis of this discrepancy is that a culture in or on media of high nutrient concentration is highly selective (either through "nutrient shock" or failure to provide vital co-factors) and decreases apparent diversity; thus it is unrepresentative of the natural community. In addition, the non-culturable but viable state (NCBV) is a strategy adopted by some bacteria as a response to environmental stress. The basis for the non-culturable state is that cells placed in conditions present in the environment cannot be recultured but can be shown to maintain their viability. Consequently, these cells would not be detected by standard water quality techniques that are based on culture. In the case of pathogens, it may explain outbreaks of disease in populations that have not come into contact with the pathogen. However, the NCBV state is difficult to attribute, due to our failure to distinguish between NCBV and non-viable cells. This article will describe our experiences with the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* and the application of molecular techniques for its detection and physiological analysis.

Introduction

Certain species of Gram-negative bacteria enter a physiological state under low-nutrient conditions, in which they become non-culturable but viable (NCBV). This physiological change may be a protective mechanism influenced by a number of factors when the organism enters a "stressful" environment (Xu *et al.* 1982; Rozak & Colwell 1987; Rozak *et al.* 1987; Morita 1993). Bacteria capable of this change include *Escherichia coli*, *Vibrio cholerae* and other *Vibrio* species, *Shigella sonnei* and *S. flexneri*, *Salmonella enteriditis* and *Aeromonas salmonicida* (see Oliver 1993). The scientific basis for this state is that cells placed in low nutrient conditions cannot be recultured on the medium from which they were initially isolated or on other laboratory media. Cells in this state can be detected using acridine orange epifluorescence microscopy, which does not measure viability. However, the definition used to define this state, and difficulties with experimental interpretation, lead to the misrepresentation of bacteria that fail to be recovered in or on laboratory media. In some cases viability has been proved using techniques described in this article. For these the term NCBV can be used. If viability has not been shown unequivocally then this term is inappropriate; therefore we adopted the term non-culturable to describe these cells (Morgan *et al.* 1991, 1993). Unfortunately, proving viability may still not provide a definitive description of bacteria thought to be capable of entering the NCBV state. Non-culturability may be due to two other factors: (a) the cells retain limited viability but are injured to such a degree that recovery is not possible (i.e. they are dying), or (b) through our ignorance, recovery on artificial media is prohibited simply because optimal conditions are not provided (e.g. *Achromatium oxaliferums:* Head *et al.* 1995, 1996; Pickup 1995). Thus it is not clear whether the majority of bacteria in the environment are able

to adopt the NCBV strategy and, therefore, extensive physiological characterisation is necessary before the NCBV label is attached to a particular species.

Methods for determining NCBV strategies in bacteria

Oliver (1993) described a number of methods employed to determine the existence of the NCBV state which include direct viable counts (Kogure *et al.* 1979), detection of respiration using radioisotope-labelled substrates (e.g. Morgan *et al.* 1991) or redox dyes such as *p*-iodonitrotetrazolium violet (INT) or 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) (Rodriquez *et al.* 1992; Pyle *et al.* 1995), membrane potential dyes such as rhodamine 123 (Porter *et al.* 1995), examination of cellular morphology and nucleic acid retention (Morgan *et al.* 1993; Pickup *et al.* 1996). All the above possibilities are generally related directly to a direct count obtained by acridine orange staining linked to fluorescence microscopy (Jones & Simon 1975) to estimate the proportion of viable cells in a population (Fig. 1). Furthermore, polymerase chain reaction (PCR) amplification of DNA can be employed to detect the presence of non-culturable cells either by amplifying sequences specific to the target organism (Morgan *et al.* 1992, 1993; Pickup *et al.* 1996), or by introduced markers as a measure of genetic stability (e.g. *xylE* gene, Morgan *et al.* 1993). Cellular morphology and fluorescence through application of dye technologies permit rapid analyses of cells by flow cytometry, the principles of which are described elsewhere (Edwards *et al.* 1992; Pickup & Saunders 1990).

We have employed a range of physiological, molecular and flow cytometric methods to examine whether *Aeromonas salmonicida* becomes NCBV as part of its life-cycle strategy.

Experiences with *Aeromonas salmonicida* subsp. *salmonicida*

Aeromonas salmonicida subsp. *salmonicida* is the causative agent of furunculosis in fish (Popoff 1984). The possible existence of a NCBV state (Xu *et al.* 1982; Roszak *et al.* 1987; Nystrom *et al.* 1990) has been reported and disputed (Allen-Austin *et al.* 1984; Rose *et al.* 1990a,b; Morgan *et al.* 1993). The main controversy surrounds the ability of *A. salmonicida* to remain viable and survive within the plankton of lake water. We have shown that *A. salmonicida* cells, although non-culturable, maintain their cellular integrity, plasmid and genomic DNA, and RNA; in addition, changes in fatty acid profiles and cell size were detected (Morgan *et al.* 1991, 1992). Although it was confirmed that *A. salmonicida* had entered a NCBV state, resuscitation on a variety of media (including fish tissue) was not observed (Morgan *et al.* 1992, 1993). Therefore, the physiological state attained by the cells is such that pathogenicity may be lost as they attempt to survive.

The survival of *A. salmonicida* in sterile aquatic systems is generally predictable, with the organisms becoming unculturable after 2 weeks in saline water (Effendi & Austin 1991, 1994), after 1 to 3 weeks in sterile fresh water (Fig. 2; Morgan *et al.* 1993), and up to 10 days in lake water (Morgan *et al.* 1993), depending on strain differences and inoculum concentration. However, amendment of microcosms with tryptone soya broth extended the period of culturability, with concentrations above 0.01% permitting detection up to 25 days (Morgan *et al.* 1992). In addition, as the trophic status of the lake water became eutrophic, culturability was extended although considerable variability was observed during these studies (Morgan *et al.* 1992). Culturability was prolonged in marine microcosms in the presence of sediments and solid substrata (Michel & Dubois-Darnaudpeys 1980; Effendi & Austin 1991, 1994) and with the additional supply of tryptone (Sakai 1986).

We considered that it might be possible under certain circumstances to postpone or delay the onset of non-culturability through the addition of certain co-factors. It was observed that *A. salmonicida* required exogenous arginine and methionine when grown in a defined basal medium and this may reflect their relative amino acid requirement in protein synthesis (Nerland

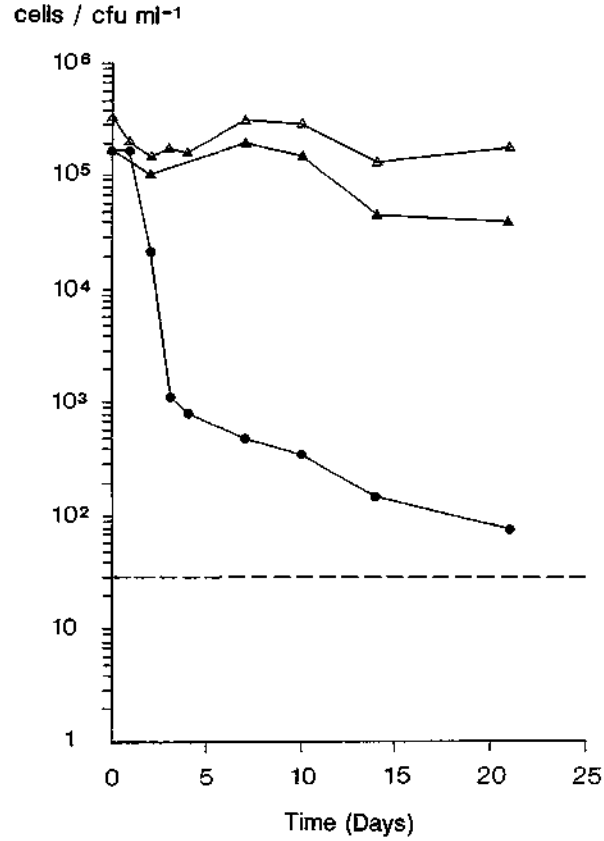

Figure 1. Survival of *Aeromonas salmonicida* in sterile lake water at 20°C, expressed as numbers of colony-forming units or cells per ml: CFU (●), rhodamine 123 viable cell count (▲), and acridine orange direct count (△). The horizontal broken line denotes the limit of detection of the plating method (Morgan *et al.* 1993).

et al. 1993). We found that the amendment of sterile freshwater microcosms with a combination of two amino acids (arginine and methionine) prevented *A. salmonicida* from becoming non-culturable or at least postponed the event during the experimental period. *A. salmonicida* MT432 becomes non-culturable but retains its viability in the freshwater environment (Morgan *et al.* 1991, 1992, 1993). Its survival, as judged by the ability to recover colony-forming units (CFU) in contained systems, is predictable. In general, CFU fall below the limit for detection after *ca.* 5 to 7 days. Similarly, marine isolates of *A. salmonicida* become undetectable within the same time period (Effendi & Austin 1994). In microcosm studies, many factors influence their recovery including inoculum concentration, temperature, the presence of invertebrates and other bacteria, available nutrients which can be linked to water quality, and the presence of sediments (Morgan *et al.* 1993; Effendi & Austin 1994). In marine systems, salinity exerts a considerable influence (Effendi & Austin 1994). Our previous studies showed that the addition of dilute nutrients enhanced the culturabilty of *A. salmonicida*, with colonies recoverable for more than 25 days at cell concentrations higher than in the original inoculum (Morgan *et al.* 1992). The trophic status of lake water (an indicator of the available nutrients from a variety of sources) affects *A. salmonicida*, with oligotrophic water increasing the rate of non-culturability (Morgan *et al.* 1992).

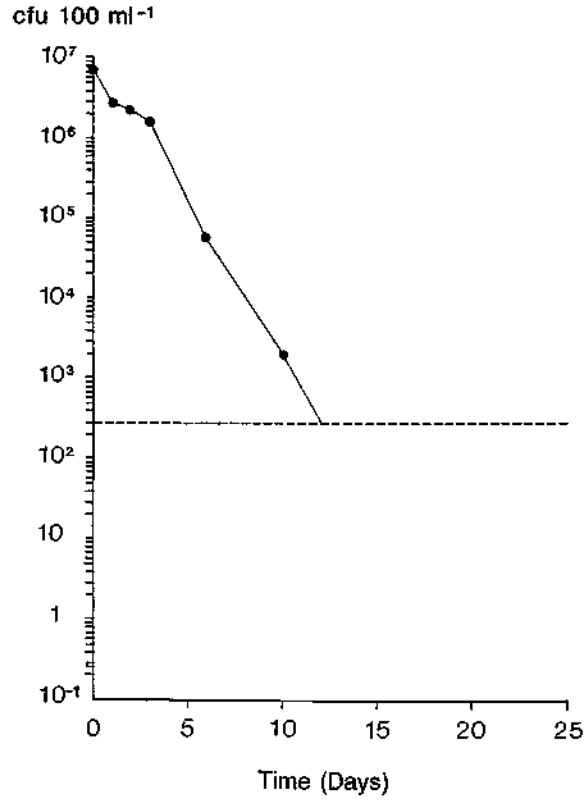

Figure 2. Typical survival of *Aeromonas salmonicida* in lake water at 20°C, determined by plate counts (tryptone soya agar) for numbers of colony-forming units per 100 ml. The horizontal broken line denotes the limit of detection of the plating method (Morgan *et al.* 1993).

We tested the effect of two amino acids, arginine and methionine, on culturability in amended sterile freshwater systems (Pickup *et al.* 1996). In the absence of any amino acid supplements the survival pattern over 60 days was no different to our previous studies over short time-courses of 25 days (Morgan *et al.* 1993), except for the occasional flask that showed a re-emergence of growth. However, these correlated with our ability to reculture *A. salmonicida* by reinoculation into tryptone soya broth from the experimental flasks. This is also further confirmation of the difficulties associated with assessing true non-culturability (Morgan *et al.* 1991). In these flasks, some cells were culturable but their numbers were maintained below our detection limit. However, the survival in microcosms receiving both amino acids was significantly enhanced. In these systems non-culturability was never attained. Single supplements of each amino acid enhanced survival to a lesser extent than the combined amendment.

As in all cases, we cannot resolve the question of whether all the cells in the control flasks remain viable whilst they are non-culturable. These cells are morphologically intact and contain genomic DNA and RNA, plasmid DNA and bound fatty acids. In addition, a proportion of the population fluoresce when challenged with the viability stain. Although the variability in fluorescence prevented meaningful interpretation, it did indicate that at least some of the cells remained viable (up to 50%). Comparison of these untreated cells with those amended with one or more amino acids shows that viability, at least in a proportion of cells, is maintained. No

significant difference was observed between the amended and unamended treatments. However, these were significantly different with respect to culturability (Pickup *et al.* 1996). Therefore, whether the unamended cells are truly dormant, or damaged to such a degree that regrowth is not possible, is difficult to determine. Common throughout was the universal physiological response of the cells, with a reduction in size and rounding up of cell shape (Pickup *et al.* 1996).

It is clear from these results that arginine and methionine prevented or at least postponed the entry into non-culturability. Although the different treatments created variable responses with respect to overall culturability, all cells showed an identical morphological response. Despite this "stress" response, cells amended with the two amino acids remained culturable. It would appear, therefore, that the reduction in size and morphological change cannot be taken as an indicator of non-culturability although it may be a significant step in that direction. Furthermore, the amended cells were significantly smaller than those that received no amendment, yet at that size (<1 μm) they remained culturable. It is apparent that both amended and non-amended cells undergo reductive cell division, i.e. the cells are smaller after each division. This phenomenon is well documented (e.g. Novitsky & Morita 1976; Amy & Morita 1983; James *et al.* 1995). The difference between the two treatments could be due to the amended cells undergoing more reductive cycles. The presence of larger cells in the amended population could not account for the numbers of cells that were culturable as judged by plating on to solid media (Pickup *et al.* 1996).

Environmental relevance

It is important to place these results in an environmental context. Our previous experiments in untreated lake water indicated that *A. salmonicida* shows no tenacity for survival and rapidly falls below the detection limit (Morgan *et al.* 1993). Despite this, if arginine and methionine were environmental stimulants, it would be unusual for *A. salmonicida* cells in the environment to enter regions where these factors were in elevated concentrations. However, the exception would be high nutrient sources (e.g. pelleted waste from fish farms or sewage effluent) where they would be contained in complex nutrient cocktails or in biofilms where they could scavenge the exudate from other cells. Therefore, it would appear that survival in the waterbody could be intrinsically linked to the chance encounter with a pulse of high nutrient content. This would lead to the supposition that the planktonic phase in the life cycle may not be of any major significance apart from being a route for dissemination. Results from Effendi & Austin (1994) and Kjelleberg *et al.* (1982, 1983) indicated that the presence of sediments enhances survival and that the source pool of infection might emerge from there. Sakai (1986) showed a direct correlation between cellular hydrophobicity (with negatively charged cells) and their attachment to positively charged particles, and positivity of the particles permitted, in turn, the utilisation of negatively charged humic acids and amino acids. In contrast, Enger & Thorsen (1992) reported that cells of *A. salmonicida* concentrate and are naturally enriched at the air/water interface within a lipid monolayer and are attracted there as a consequence of their hydrophobicity. Therefore general subsurface conditions, as crudely mimicked by these studies, show that *A. salmonicida* has the potential for survival in the freshwater environment. However, the conditions simulated here are far more likely to be encountered in areas of enrichment such as sediments and the air/water interface, where they would have some environmental significance.

Discussion

This article presents data showing that although we were able to determine that *Aeromonas salmonicida* can enter the NCBV state, and that entry can be postponed, the full ecological significance of this ability, or whether it remains pathogenic, remains unclear. This emphasises the point that in some cases there is a need to examine the ecology of the organism as well as an

ability to detect it in the environment. With obvious pathogens such as *Cryptosporidium* and *Giardia* (see other articles in this volume), sensitive and unambiguous direct detection is of paramount importance to the water supply industry. Rapid response is the primary requirement for the presence of a number of pathogens. However, it is possible that detailed ecological analyses may be required in the future for pathogens that may be involved in diseases but which are revealed only by long-term epidemiological studies exposing the clustering of cases. Their presence may not have an immediate effect on local or wider populations, and their numbers in the environment may lie below the level of detection for current methods, yet their long-term exposure to populations of aquatic organisms may result in the onset of disease or increase the susceptibility of populations to disease.

We wish to thank the Institute of Freshwater Ecology, the Freshwater Biological Association and the Natural Environment Research Council for support.

References

Allen-Austin, P., Austin, B. and Colwell, R. R. (1984). The survival of *Aeromonas salmonicida* in river water. *FEMS Microbiology Letters*, **2**, 246-146.

Amy, P. S. & Morita, R. Y. (1983). Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. *Applied & Environmental Microbiology*, **45**, 1109-1115.

Edwards, C., Porter, J., Saunders, J. R., Diaper, J. P. *et al.* (1992). Flow cytometry and microbiology. *Society for General Microbiology Quarterly*, **19**, 105-108.

Effendi, I. & Austin, B. (1991). Survival of the fish pathogen *Aeromonas salmonicida* in seawater. *FEMS Microbiology Letters*, **84**, 103-106.

Effendi, I. & Austin, B. (1994). Survival of the fish pathogen *Aeromonas salmonicida* in the marine environment. *Journal of Fish Diseases*, **17**, 375-385.

Enger, O. & Thorsen, B. K. (1992). Possible ecological implications of the high cell surface hydrophobicity of the fish pathogen *Aeromonas salmonicida*. *Canadian Journal of Microbiology*, **38**, 1048-1052.

Head, I. M., Gray, N. D., Pickup, R. W. & Jones, J. G. (1995). The biogeochemical role of *Achromatium oxaliferum*. In: *Organic Geochemistry: Developments and Applications to Energy, Climate, Environment & Human History. 17th International Meeting on Organic Geochemistry Donostia-San Sebastián, The Basque Country. Spain 4-8th September 1995* (eds J. O. Grimalt & C. Dorronsoro), pp. 895-898. A.I.G.O.A., San Sebastian, Spain.

Head, I. M., Gray, N. D., Clarke, K. J., Pickup, R. W. & Jones, J. G. (1996). The phylogenetic position and ultrastructure of the uncultured bacterium *Achromatium oxaliferum*. *Microbiology*, **142**, 2341-2354.

James, G. A., Korber, D. R., Caldwell, D. E. & Costerton, J. W. (1995). Digital analysis of growth and starvation responses of a surface-colonizing *Acinetobacter* sp. *Journal of Bacteriology*, **177**, 907-915.

Jones, J. G. & Simon, B. M. (1975). An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with respect to a new method of dyeing membrane filters. *Journal of Applied Bacteriology*, **39**, 317-329.

Kjelleberg, S., Humphrey, B. A. & Marshal, K. C. (1982). The effect of interfaces on small, starved marine bacteria. *Applied & Environmental Microbiology*, **43**, 1166-1172.

Kjelleberg, S., Humphrey, B. A. & Marshal, K. C. (1983). Initial phase of starvation and activity of bacteria at surfaces. *Applied & Environmental Microbiology*, **46**, 978-984.

Kogure, K., Simidu, U. & Taga, N. (1979). A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology*, **25**, 415-420.

Michel, C. & Dubois-Darnaudpeys, A. (1980). Persistence of the virulence of *Aeromonas salmonicida* strains kept in river sediments. *Annals of Recherches Veterinaires*, **11**, 375-380.

Morgan, J. A. W., Cranwell, P. A. & Pickup, R. W. (1991). The survival of *Aeromonas salmonicida* in lake water. *Applied & Environmental Microbiology*, **57**, 1777-1782.

Morgan, J. A. W., Rhodes, G. & Pickup, R. W. (1993). Survival of non-culturable *Aeromonas salmonicida* in lake water. *Applied & Environmental Microbiology*, **59**, 874-880.

Morgan, J. A. W., Clarke, K. J., Rhodes, G. & Pickup, R. W. (1992). Non-culturable *A. salmonicida* in lake water. *Microbial Releases*, **1**, 71-78.

Morita, R. Y. (1993). Bioavailabilty of energy and the starvation state. In *Starvation in Bacteria* (ed. S. Kjelleberg), pp. 1-24. Plenum Press, New York.

Nerland, A. H., Høgh, B. T., Olsen, A. B. & Jensen, H. B. (1993). *Aeromonas salmonicida* requires exogenous arginine and methionine for growth. *Journal of Fish Diseases*, **16**, 605-608.

Novitsky, J. A. & Morita, R. Y. (1976). Morphological characterization of small cells resulting from nutrient starvation in a psychrophillic marine *Vibrio*. *Applied & Environmental Microbiology*, **32**, 619-622.

Nystrom, T., Albertson, N. H. & Kjelleberg, S. (1990). Physiological and molecular adaptation to starvation and recovery by the marine *Vibrio* sp. S14. *FEMS Microbial Ecology*, **74**, 129-140.

Oliver, J. D. (1993). Formation of viable but non-culturable cells. In *Starvation in Bacteria* (ed. S. Kjelleberg), pp. 239-272. Plenum Press, New York.

Pickup, R. W. (1995). Sampling and detecting bacterial populations in natural environments. *Society for General Microbiology Symposium, Series* **52**, 298-315. Cambridge University Press.

Pickup, R. W. & Saunders, J. R. (1990). Detection of genetically engineered traits among bacteria in the environment. *Trends in Biotechnology*, **8**, 329-335.

Pickup, R. W., Rhodes, G., Cobban, R. J. & Clarke, K. J. (1996). Postponement of non-culturability in *Aeromonas salmonicida*. *Journal of Fish Diseases* **19**, 65-74.

Popoff, M. (1984). Genus III, *Aeromonas*. In *Bergey's Manual of Systematic Bacteriology* (eds N. R. Kreig & J. G. Holt), pp. 545-548. Williams & Wilkins, Baltimore.

Porter, J., Diaper, J., Edwards, C. & Pickup, R. W. (1995). Direct viability measurements of natural planktonic bacterial communities. *Applied & Environmental Microbiology*, **61**, 2783-2786.

Pyle, B. H., Broadaway, S. C. & McFeters, G. A. (1995). Factors affecting the determination of respiratory activity on the basis of cyanoditolyl tetrazolium chloride reduction with membrane filtration. *Applied & Environmental Microbiology*, **61**, 4304-4309.

Rodriguez, G. G., Phipps, D., Ishiguro, K. & Ridgway, H. F. (1992). Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Applied & Environmental Microbiology*, **58**, 1801-1808.

Rose, A. S., Ellis, A. E. & Munro, A. L. S. (1990a). Evidence against dormancy in the bacterial fish pathogen *Aeromonas salmonicida* subsp. *salmonicida*. *FEMS Microbiology Letters*, **68**, 105-108.

Rose, A. S., Ellis, A. E. & Munro, A. L. S. (1990b). The survival of *Aeromonas salmonicida* subsp. *salmonicida* in sea water. *Journal of Fish Diseases*, **13**, 205-214.

Rozak, D. B. & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews*, **51**, 365-379.

Rozak, D. B., Grimes, D. J. & Colwell, R. R. (1987). The viable but non-recoverable stage of *Salmonella enteridis* in aquatic systems. *Canadian Journal of Microbiology*, **30**, 334-338.

Sakai, D. K. (1986). Electrostatic mechanism of survival of virulent *Aeromonas salmonicida* strains in river water. *Applied & Environmental Microbiology*, **51**, 1343-1349.

Xu, H. S., Roberts, N., Singleton, F. L., Atwell, R. W. *et al.* (1982). Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in estuarine and marine environments. *Microbial Ecology*, **8**, 313-323.