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AEROMONADS IN POTABLE WATER (EM 9015)

Final report to the Department of the Environment

DoE 3072-(P)

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Author: R A Pitchers

Contract Manager: P Jago

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Any enquiries relating to this report should be referred to the Contract Manager at the following address:

WRc plc, Henley Road, Medmenham, PO Box 16, Marlow, Buckinghamshire SL7 2HD. Telephone: Henley (0491) 571531

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GLOSSARY

Anaerobe	-	an organism which has the ability to grow in the absence of oxygen. A facultative anaerobe is an organism which can grow in the presence or absence of oxygen.
Anaerogenic	-	refers to an organism which does not produce gas from a given substrate.
Background flora	-	growth of organisms on a selective isolation medium other than the organism of interest.
Biofilm	-	a heterogeneous assemblage of micro-organisms adhered to a surface and usually surrounded by exopolymeric material.
Heterotroph	-	an organism which uses organic compounds for most or all of its carbon requirements.
Mesophile	-	an organism whose optimum temperature for growth lies generally within the range 15 - 45 °C.
Opportunistic pathogens	-	organisms which are normally free living or are normal inhabitants of a host but may adopt a pathogenic role under certain conditions, e.g. when the defence mechanism of the host becomes impaired.
Photoautotrophic	-	refers to an organism which uses carbon dioxide for most or all of its carbon requirement and also uses light as the primary source of energy for metabolism and growth.
Psycrophile	-	an organism which grows optimally at or below 15 °C and which has an upper limit of growth around 20 °C.

PREFACE

Recently consideration has been given to components of the heterotrophic bacterial population, in particular aeromonads but also pseudomonads, in drinking water as potential opportunistic pathogens but also as indicators of regrowth potential. Initially, effort was directed at developing suitable methods for the isolation and identification of aeromonads. A survey was undertaken of water treatment plants found aeromonads were present during the treatment process but were absent following final disinfection. In the distribution system aeromonads and pseudomonads were recovered, in highest numbers, at the dead ends of distribution systems where chlorine concentrations were low and residence times high.

This final report contains the work undertaken to the Department of the Environment with respect to a contract (PECD 7.7.235) placed with WRc in May 1987 to conduct a study of Microbial growth on materials in contact with water and in distribution and is specifically related to objectives 3 to 5. The final report covering the first two objectives was submitted separately.

AEROMONADS IN POTABLE WATER

R A PITCHERS (WRc plc)

SUMMARY

Recently concern has been expressed over the recovery of aeromonads from potable water supplies as certain strains are known to be potentially pathogenic. Similarly, pseudomonads, and in particular the fluorescent group, are considered as potential opportunistic pathogens. This study was initiated to examine the occurrence and significance of these bacteria in water distribution systems.

The main emphasis of the study was the examination of the significance of aeromonads. Initially an evaluation of suitable media to recover aeromonads was undertaken and ampicillin dextrin agar was selected for all routine investigations. Confirmation of the identity of aeromonads required a diagnostic test to distinguish them from vibrios. The reaction to the antibiotic 0/129 was used but did not prove satisfactory as some aeromonads were recovered which were sensitive at concentrations to which they would be expected to be resistant.

A survey of aeromonads in potable water distribution systems was conducted which revealed that aeromonads were found in the highest numbers at the dead ends of the network. In such environments the aeromonad population was responding to low chlorine concentrations and long residence times. Experiments conducted in support of this study demonstrated that aeromonads were susceptible to the effects of disinfection and that they were capable of growth in distribution system waters once the chlorine had been neutralised. A similar pattern in the occurrence of pseudomonads was found. Comparison of the total heterotrophic plate count and recovery of aeromonads and pseudomonads revealed that these bacteria comprised a low proportion of the total bacterial population.

This study was funded by the Department of the Environment and carried out under the supervision of the Drinking Water Inspectorate whose permission to publish is gratefully acknowledged.

This report can be obtained from the WRc Publication Unit,
Tel: (0491) 571531, priced £25

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1. INTRODUCTION

The microbiological quality of drinking water has primarily focused on the occurrence of coliforms. This is because contamination by sewage or human or animal excrement is the greatest risk associated with water supplies. Standards for microbiological quality are therefore based on bacterial indicators of faecal contamination of the water such as coliform bacteria and *E. coli*. Another index of the microbiological quality of water is the standard plate count (SPC) or heterotrophic bacteria plate count (HPC). The value of this test lies not in determining the absolute numbers of bacteria which develop at the two incubation temperatures used (22 °C and 37 °C) but in detecting a significant change in the relative size of one or both of these populations. Any significant change in the populations can be indicative of a problem requiring further investigation. Although there are no statutory limits for the SPC the Water Supply (Water Quality) Regulations 1989 require no significant increase in concentrations of heterotrophic bacteria in drinking water over that normally observed.

Water treatment plants and drinking water distribution systems are complex ecosystems where many factors will operate to influence the bacterial population. Of particular significance would be the selection pressures exerted by nutrient availability and levels of disinfectant (Maul *et al* 1991). Nutrients arise from many sources including the water, the primary materials of construction, pipe jointing materials and contamination in the form of ingress. The recognition that materials of construction can contribute nutrients to the distribution system has caused testing methods to be developed to identify the most suitable pipe and sealant materials for use. Although these testing and approval systems have played a significant role in improving distribution system design and construction there is a demonstrable need to develop more rigorous standards of testing so that materials which are more microbiologically inert can be identified.

One aspect of testing which is currently not considered is the capacity of disinfectants to leach carbon from materials in a form which is biodegradable. This biodegradable organic carbon called AOC (assimilable organic carbon) is recognised as a potentially important cause of bacterial regrowth and some

water treatment systems have been optimised for its removal. The significance of AOC leached from materials of construction has received less attention particularly that liberated by short or long term contact with disinfectants. However, increased focus in recent years on microbiological quality in distribution and the accumulation of biofilms has brought renewed interest in materials of construction and specific components of the heterotrophic population as indicators of regrowth potential, particularly bacteria belonging to the genera *Aeromonas* and *Pseudomonas* (Gibbs and Hayes 1989). Both genera are frequently found in a wide variety of aquatic habitats where their versatile metabolism allows them to compete successfully for available nutrients. Additionally, concern has been expressed that aeromonads are causative agents of gastrointestinal disease, although the link between the presence of aeromonads in drinking water and the outbreak of waterborne illness has not been established (Section 2.5). Also, it has been reported that aeromonads can interfere with coliform determinations as they will produce similar colonies on the selective media (Edge and Finch 1986).

Thus, this study was initiated to monitor the presence of aeromonads during water treatment and in distribution and to identify the extent to which environmental factors affect the aeromonad population. Additionally, the scope of this study was extended to examine the occurrence of pseudomonads in distribution systems.

The following set of objectives were established for this contract:

1. To determine the effects of various water disinfectants upon the release of AOC from materials in contact with water.

2. To assess the significance of this work on the existing Water Bye-laws Advisory Service (WBAS) fittings testing procedure.

3. To assess the significance of *Aeromonas* spp. and anaerogenic coliform organisms in distribution by study of their regrowth potential.

4. To assess the ability of aeromonads and anaerogenic coliforms to colonise different materials of construction.

5. To extend the aeromonad survey and investigate the occurrence and significance of pseudomonads in distribution systems.

This report describes work carried out between April 1987 and March 1992 in connection with the Department of the Environment research contract 'Microbial Growth in Contact with Water and in Distribution' (PECD 7/7/235). The studies on the effects of exposure of disinfectants on plumbing materials (objectives 1 and 2) have already been concluded. This work has been published separately (WRc Report : DoE 2853 (P), Jago 1992) and is available from the WRc Publications Unit.

The other three objectives are addressed in this report. As part of the study a literature survey on aeromonads was undertaken (Section 2). Initially, as there are no standard methods for the isolation of aeromonads an evaluation of available procedures for their isolation and identification was carried out (Section 3). A series of experiments were conducted to determine the effect of selected environmental factors on the growth of aeromonads (Section 4). In support of these experiments the occurrence of aeromonads during water treatment and in distribution was examined (Section 5). In addition, consideration was given to the occurrence of Pseudomonads in distribution (Section 6).

2. LITERATURE REVIEW

2.1 Introduction

In support of the experimental programme for this project a literature review was undertaken. The aim was to examine the current state of knowledge concerning the significance of aeromonads in drinking water. As the genus *Aeromonas* has not been properly defined it was first necessary to examine its taxonomic status (Section 2.2). On a practical basis consideration was given to the available methods for the isolation and identification of aeromonads (Section 2.3). To identify those factors that affected the growth of aeromonads the review examined both their occurrence in natural waters, where most of the studies have been conducted, and specifically in drinking water

distribution systems (Section 2.4). Finally, the review investigated the role of aeromonads as potential pathogens (Section 2.5).

2.2 Taxonomy of aeromonads

The genus *Aeromonas* was first proposed by Kluyver and Van Neil (1936) to accommodate those microorganisms, similar to enteric bacteria, which were associated with freshwater environments and which, in motile forms, had a polar flagella. The current definition of the genus also includes the characteristics of being; Gram negative, facultative anaerobic bacteria which are oxidase and catalase positive and breakdown carbohydrates fermentatively (Kreig and Holt 1984). At present the genus is designated to the family *Vibrionaceae*. However, molecular genetic evidence, based on 5S and 16S ribosomal ribonucleic acid sequence analysis and DNA hybridisation patterns has indicated that aeromonads were sufficiently different from other members of the family to warrant the creation of a separate family, the *Aeromonadaceae* (Colwell *et al* 1986).

Within the genus a clear distinction exists between the psychrophilic non-motile and the mesophilic motile aeromonads (Kreig and Holt 1984). The former group are clustered around the single species *Aeromonas salmonicida*. This group has an optimum growth temperature of 22-25 °C with a minimum and maximum growth temperature of 5 °C and 35 °C respectively. An additional non-motile species *A. media* has recently been described (Allen *et al* 1983). The classification of the mesophilic motile aeromonads to species level has not yet been equivocally established. The group has an optimum growth temperature of 28 °C with minimum and maximum growth temperatures of 5 °C and 38-41 °C. It is this group which are of most importance in the context of this review.

The previous classification of the motile aeromonads was proposed by Schubert (1967a, 1967b and 1968) in which the group was divided into *A. hydrophila* with three sub-species and *A. punctata* with two sub-species. A subsequent revision of the genus was undertaken by Popoff and Veron (1976). Extensive biochemical analysis produced three distinct patterns and the motile group was divided into *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* biovar *anaerogenes* and a new

species *A. sobria* was proposed. In subsequent studies (Popoff *et al* 1981), comparison of DNA hybridisation patterns indicated that *A. hydrophila* biovar *anaerogenes* represented a separate species and was renamed as *A. caviae*. Their investigation (Popoff *et al* 1981) also indicated that additional DNA hybridisation groups existed within these species, with three in *A. hydrophila*, two in *A. caviae* and at least two in *A. sobria*, but these groups could not be distinguished on the basis of specific biochemical reactions. Recent work conducted at the Centres for Disease Control (Atlanta, USA) has confirmed the existence of these DNA hybridisation groups and identified two aeromonad strains that differed, by a single biochemical reaction, from the currently recognised species. *A. veronii* was proposed to represent those strains that produced a positive ornithine decarboxylase reaction (Hickman-Brenner *et al* 1987) and *A. schubertii* to represent those strains that were mannitol negative (Hickman-Brenner *et al* 1988).

Until the nomenclature of the genus has been established this review will adopt the classification proposed in the ninth edition of Bergeys Manual of Determinative Bacteriology (Kreig and Holt 1984) which recognises the three motile aeromonad species proposed by Popoff and Veron (1976).

2.3 Isolation and identification

2.3.1 Isolation media

Aeromonads are not fastidious in their nutritional requirements and will readily grow on a wide range of nutrient agars. Also, aeromonads can grow on selective media used for the isolation of coliform bacteria, where they produce colonies of a similar morphology because they share a common fermentative metabolism. This is a particular problem on selective media for coliforms containing lactose which leads to the possibility of aeromonads causing false coliform counts (Neilson 1978 and Edge and Finch 1986). However, the ability to ferment lactose appears not to be species specific but is variable within each species. Janda (1985) determined the percentage lactose fermentation within each of the species as 34% in *A. hydrophila*, 11% in *A. sobria* and 68% in

A. caviae. However, LeChevallier *et al* (1982) failed to detect any lactose fermenting strains of *A. sobria* in a drinking water distribution system.

Numerous selective media have been developed specifically for the isolation of aeromonads in mixed microbial communities from a variety of locations including aquatic, clinical and food environments (see Joseph *et al* 1988). Many of the selective media developed for clinical and food applications have also been evaluated for the recovery of aeromonads from environmental sources (Millership and Chattopadhyay 1985, Havelaar *et al* 1987, Arcos *et al* 1988, Gray and Stickler 1989 and Knochel 1989). Varying degrees of success were reported and probably reflected not only the inherent ability of each medium to recover aeromonads satisfactorily but also their ability to suppress the background flora present at each location.

However, media have been developed especially for the isolation of aeromonads from aquatic environments (Table 2.1). In the majority of these media selectivity is achieved by the specificity of aeromonads to metabolise fermentatively the following: starch; its intermediate oligosaccharide, dextrin; or its principal disaccharide component, maltose. A variety of sugar alcohols (inositol, mannitol and sorbitol), a disaccharide sugar (trehalose), a pentose sugar (xylose) and glycogen, the animal polysaccharide equivalent to starch have also been used.

The source of carbohydrate used in the medium can have a significant effect on the expression of colony characteristics. Havelaar and Vonk (1988) evaluated a range of dextrins, obtained from different manufacturers, to prepare ampicillin dextrin agar (ADA). It was found that the rate of fermentation varied between sources of dextrin and only those products supplied by Merck and Difco were suitable. As different methods are used to manufacture dextrin its composition and quality will vary between suppliers (Cowan 1974).

To enhance the selectivity of the isolation media antibiotics are additionally incorporated to suppress the background flora. To specifically inhibit the growth of the *Enterobacteriaceae*, ampicillin is commonly used at a range of concentrations from 5-30 mg/l (Table 2.1). This would be particularly important where dextrin is used as the selective carbohydrate because bacteria

Table 2.1 Selective media used to recover aeromonads from aquatic environments

Isolation agar	Selective agent	Inhibitory agent (1)	Indicator	Colony characteristics	Incubation conditions	Reference
Rimler-schotts agar	lysine ornithine maltose	citrate novobiocin (5) deoxycholate	BTB	yellow	35 °C 24 hours	Schotts and Rimler 1973
MacConkey (Trehalose substituted for lactose)	trehalose	crystal violet bile salts	PR	yellow	35 °C 24 hours	Kaper *et al* 1981
Peptone-beef extract glycogen agar	glycogen	sodium dodecyl sulphate	BTB	yellow with yellow halo	25-30 °C 18-24 hours	McCoy and Pilcher 1974
mA agar	trehalose mannitol	ampicillin (20) deoxycholate ethanol	BTB	yellow on trehalose and yellow on mannitol	35 °C 20 hours 2-3 hours	Rippey and Cabelli 1979
Ampicillin-dextrin agar	dextrin	ampicillin (10) deoxycholate	BTB	yellow	30 °C 18 hours	Havelaar *et al* 1987
GSP agar	starch	penicillin (100 000 iu)	PR	yellow	25 °C	Von Kleiwein 1969
SGAP agar	starch glucose	penicillin (100 000 iu) ampicillin (20)	PR	yellow	28 °C	Huget and Ribas 1990
MIX agar (2)	xylose inositol	ampicillin (20) bile salts citrate	BTB	blue-green	30 °C 18 hours 35 °C 24 hours	Cunliffe and Adcock 1989

Table 2.1 continued

Isolation agar	Selective agent	Inhibitory agent (1)	Indicator	Colony characteristics	Incubation conditions	Reference
Dextrin-fuschin Sulphite agar	dextrin	none	F	red	30 °C 24 hours	Schubert 1989
Aeromonas agar	lysine arginine inositol lactose sorbitol xylose	ampicillin (5)	BTB TB	dark green-opaque with dark centre	30-35 °C 24 hours	Oxoid Manual 1991

Key
BTB - bromothymol blue
PR - phenol red
TB - thymol blue
F - fuschin

Notes
(1) The figure quoted in parentheses indicates an antibiotic concentration in mg/l.
(2) With this technique the conditions refer to anaerobic, in an atmosphere of nitrogen, followed aerobic incubation.

from the genera *Klebsiella* and *Enterobacter* are also capable of fermenting this substrate (Schubert 1987). Alternatively, the use of penicillin-G has been described (Von Kielwein 1969). However, it has been found that this antibiotic was not sufficiently selective for aeromonads and the efficacy of the medium was further improved by the incorporation of ampicillin (Huget and Ribas 1990).

However, these media have not been found to be entirely selective for aeromonads and allowed the growth of other organisms, of which pseudomonads predominated. This has led to modifications in the isolation technique and incubation conditions to improve recovery of aeromonads. A frequently adopted method is incubation under anaerobic conditions as aeromonads are facultatively anaerobic and can grow in the absence of oxygen. The background flora, in comparison, tended to be composed of aerobic non-fermenting bacteria which would not grow under these conditions. Millership and Chattopadhyay (1985) reported that incubation under totally anaerobic conditions gave improved recoveries of aeromonads. However, Havelaar *et al* (1987) found that when aeromonads were incubated in the absence of oxygen, their ability to ferment the carbohydrate was reduced and therefore colonies did not develop satisfactorily. As a consequence of the impaired ability of aeromonads to ferment under total anaerobic conditions, Cunliffe and Adcock (1989) used a combination of both anaerobic and aerobic incubation. Schubert (1987) created sufficiently anaerobic conditions by overlaying the isolation plate with molten medium which inhibited growth of the aerobic background flora.

Additionally, because the genera *Aeromonas* and *Vibrio* are closely related the majority of media selective for aeromonads will also allow the growth of vibrios. This is usually considered to be a particular problem in estuarine and marine environments and involves principally the group F vibrios (Nakano *et al* 1990 and Kaper *et al* 1981). However, this could also be a problem in other aquatic environments as the distribution of vibrios is not confined to saline waters since freshwaters are known to support a vibrio population (Lee 1990). Growth of vibrios can be prevented by incorporating a specific antibiotic into the selective medium, usually referred to as vibriostatic agent O/129. Havelaar *et al* (1987) reported the addition of this agent at a concentration of 50 mg/l successfully inhibited the growth of vibrios. An alternative approach was taken by Huget and Ribas (1990) who recommended that a

medium without sodium chloride would prevent growth of vibrios, since all vibrios, with the exception of *V. chlorae*, required it for optimal growth.

2.3.2 Identification systems

Unfortunately, to confirm the identity of presumptive aeromonad isolates, additional tests must be applied after selection and purification because most media do permit the growth of other bacteria. Aeromonads can be identified by determination of their response to a series of biochemical test reactions. Such schemes are based on the reactions proposed by Popoff and are given in the ninth edition of Bergeys Manual of Determinative Bacteriology (Kreig and Holt 1984). Cunliffe and Adcock (1989) and Monfort and Baleux (1990) have both proposed schemes for the identification of aeromonads to species level. Although the use of a relatively large number of tests would allow more reliable identification this approach would not lend itself to routine applications where numerous isolates required screening.

To meet these requirements a system was developed in which the number of differential tests had been reduced to the minimum level to still permit speciation (Medema, personal communication). The differentiation of presumptive isolates to species level is based upon four biochemical test reactions. Isolates that were positive for the oxidase reaction and possessed a fermentative metabolism in Hugh and Leifsons medium (Cowan 1974) were confirmed as the genus *Aeromonas*. Two additional tests were used to determine speciation (Table 2.2).

Table 2.2 Classification of aeromonads to species level

Aeromonad species	Biochemical test reaction	
	Gas from glucose	Esculin hydrolysis
A. hydrophila	positive	positive
A. sobria	positive	negative
A. caviae	negative	positive
unknown	negative	negative

Commercial systems have been used to identify aeromonads. Most commonly used are either the API 20 E (LeChevallier *et al* 1982, Burke *et al* 1984a and Gray and Stickler 1989) or the API 20 NE (Arcos *et al* 1988 and Huget and Ribas 1990). The API 20 E primarily recognises bacteria from the family *Enterobacteriaceae* but does include *A. hydrophila*. The API 20 NE is designed specifically for non-enteric bacteria and recognises all three current species in the motile aeromonad group. However, an evaluation of both these systems found they were unable to identify aeromonads satisfactorily (McGarey and Wrona 1989). Both systems misidentified *A. caviae* as a species of *Vibrio*. Additionally, the API 20 NE had variable identification rates for *A. hydrophila* and *A. sobria* of 40 per cent and 60 per cent respectively. With the API 20 E all isolates of *A. hydrophila* were correctly identified.

All systems to identify aeromonads require the performance of an oxidase test on the isolate. It has been shown that aeromonads growing on a medium containing a carbohydrate yielded a false negative reaction if the test was performed directly on the colony (Hunt *et al* 1981). The oxidase test reaction was inhibited by acid produced during fermentative metabolism. Thus, before the test was undertaken, it was necessary to first subculture all isolates onto nutrient agar, which does not contain a fermentable carbohydrate substrate.

2.4 Occurrence of aeromonads in aquatic environments

2.4.1 Natural waters

Aeromonads are common aquatic bacteria and have been isolated from diverse environments ranging from freshwater to marine ecosystems. They have been isolated from waters of a high salinity although it has not been established whether they represent a truly indigenous marine population (Abeyta and Wekell 1988). Although, Hazen *et al* (1978) recovered aeromonads from marine waters with salinities up to 100 o/oo, their occurrence was considered to represent survival of the bacterium following input from freshwater sources. Similarly, Nakano *et al* (1990) found that aeromonads were capable of surviving in marine waters with salinities of 22-33 o/oo.

They have been found in various types of freshwater. Hazen *et al* (1978) isolated *A. hydrophila* from a diverse selection of waters with temperatures between 4 and 45 °C and over a pH range of 5.2 to 9.8. Additionally, aeromonads are commonly associated with polluted waters through the input of sewage effluent (Araujo *et al* 1989 and Monfort and Baleux 1990). Schubert (1990) suggested that a ratio of anaerogenic to non-anaerogenic aeromonads could be used as an index of water quality as different species were found in natural waters compared with sewage effluent.

Factors affecting the growth of aeromonads

Numerous studies have been undertaken to identify the factors affecting the growth of aeromonads in natural waters. Hazen *et al* (1978) surveyed a number of natural freshwater environments where a good correlation was found between numbers of aeromonads and the conductivity of the water. It was suggested that some unmeasured water quality parameter, which varied proportionally with conductivity, was affecting the aeromonad population. Studies by Rippey and Cabelli (1985) indicated that the numbers of aeromonads were related to the trophic status of the water as measured by dissolved reactive phosphorus, chlorophyll *a* and Secchi depth. These were parameters of primarily photoautotrophic (algal) biomass. This was confirmed by growth studies conducted in filtered, autoclaved natural waters from a range of lakes inoculated with an aeromonad population which indicated that *A. hydrophila* responded directly to levels of phosphorus and the Secchi depth of the water.

Hazen and Esch (1983) suggested that levels of phytoplankton directly influenced the aeromonad population. A distribution pattern was found where the maximum aeromonad population paralleled the seasonal changes in phytoplankton density. The effect of the phytoplankton was to increase the dissolved oxygen concentration, total phosphorus and total organic carbon. Monfort and Baleux (1990) also considered that aeromonads were responding directly to the influence of algae. It was suggested that either the aeromonads were responding to specific nutrients released by the algae or that they were more resistant to bacterial toxins produced by the algae. However, Nakano *et al* (1990) observed an inverse relationship between chlorophyll *a* and

the aeromonad population in some locations. Their study suggested that the density of the aeromonads was not linked to a single factor but was related to the cumulative effect of several physicochemical parameters.

It has also been observed that aeromonads are associated with decomposing aquatic macrophytes. Hazen (1979) recovered high numbers of aeromonads from waters in which *Myriophyllum spicatum* (asian millfoil) was decaying. The bacteria were able to degrade the macromolecules released from the rotting vegetation.

Of the various physical factors affecting the aeromonad population, temperature appears to be significant. Fliermans *et al* (1977) found that the highest numbers occurred during the Spring. Similarly, studies on the seasonal abundance of *A. hydrophila* in the Anacostia River (Washington, USA) showed that temperature was the predominant factor controlling the population (Seidler *et al* 1980). The numbers of aeromonads in the top and bottom waters as well as in the sediment exhibited a seasonal fluctuation. Counts increased during periods when the water temperature was elevated and reached a maximum in waters above 20 °C. Further studies on this location by Cavari *et al* (1981) also showed a seasonal distribution with peaks occurring during the summer months. Experiments conducted in the laboratory showed that aeromonads were unable to grow in waters at 4 °C, because at this temperature they were not able to produce the energy required to maintain a viable population.

2.4.2 Water distribution systems

Aeromonads are frequently isolated from water in distribution systems and have been recovered from both chlorinated and unchlorinated waters. According to van der Kooij (1988) early work in Holland recognised the potential problems of aeromonads in drinking water. The health authorities set water quality standards in which the maximum acceptable concentration of aeromonads in water leaving a treatment works should not exceed 20 CFU/100 ml and in distribution 50 CFU/100 ml. Improved techniques for the detection of aeromonads resulted in an increase of the limit to 200 CFU/100 ml for water in distribution.

In locations where aeromonads have been recovered they constitute only a small fraction of the total microbial population. An investigation by LeChevallier *et al* (1982) reported that aeromonads contributed to approximately 10 per cent of the total bacterial population in chlorinated water. However, in unchlorinated water aeromonads accounted for an increased proportion of 21 per cent. Other studies have generally found this value to be lower. In an extensive survey of the bacterial population in a distribution system aeromonads comprised between 0.9 and 2.4 per cent of the total bacterial population (Clark *et al* 1982). Also, van der Kooij (1988) found that aeromonads only represented 0.2 per cent of the total microbial population. Similarly, Knochel and Jeppesen (1990) recovered aeromonads at concentrations of between 1-40 CFU/100 ml which represented approximately 10 percent of the total bacterial population.

Factors affecting the growth of aeromonads in distribution

In treated water it appears that an interaction between temperature and levels of chlorine is significant in controlling the aeromonad population. Burke *et al* (1984b) monitored aeromonad populations in a metropolitan drinking water supply over one year. They found that when the concentration of chlorine was below 0.3 mg/l a seasonal variation in the population occurred and when temperatures were above 14.5 °C the count increased. Conversely, they found that temperature had no effect on the growth of aeromonads when the total available chlorine concentration was above 0.3 mg/l.

In other distribution systems the occurrence of aeromonads has only been considered as a function of seasonality. Millership and Chattopadhyay (1985) reported isolation rates of 25 per cent in summer and 7.0 per cent in winter from a chlorinated water supply. Similarly, during a one year survey of a distribution system, aeromonads were more frequently isolated during warmer months when the temperature of the water ranged between 15 to 20 °C (LeChevallier *et al* 1982). Conversely, Mascher *et al* (1988) failed to find any seasonal correlation in occurrence of aeromonads with numbers of less than 40/100 ml being recovered throughout the year.

The quality of the source water has also been shown to affect the aeromonad population in the distribution system. Edge and Finch (1986) recovered much lower numbers of aeromonads in distribution systems where the water was derived from a groundwater source rather than a surface water source. Studies by van der Kooij and Hinjen (1988) suggested that the nutritional versatility of aeromonads provided them with a selective advantage over the competing heterotrophic population. It was found that an aeromonad strain M800 had a high substrate affinity for mixtures of amino acids and long chain fatty acids. Thus, it was inferred that they were able to compete successfully with other members of the microbial community for these compounds even at low substrate concentrations. Further investigations demonstrated that aeromonads were capable of utilising the plumbing materials used in distribution system pipework (van der Kooij 1988). Soft soap, which is used as a lubricant in pipe joints, was capable of promoting the growth of aeromonads at low substrate concentrations. He suggested that these organisms were found in distribution systems where they could utilise the exopolymeric breakdown products of microorganisms in the biofilm. Additionally, this could represent a survival mechanism because bacteria inhabiting biofilms have been found to possess a greater resistance to chlorination (LeChevallier *et al* 1988).

2.5 Significance to health of aeromonads in potable water

The specific role of aeromonads as causative agents in human disease has not been conclusively established. Primary and secondary wound infections have been reported to arise through injuries sustained in waters with a high aeromonad count (Kaper *et al* 1981 and Abeyta and Wekell 1988). They have also been isolated from invasive diseases such as septicemia (Janda and Duffey 1988).

Gastroenteritis is by far the most common form of infection caused by aeromonads. Aeromonads have been isolated from diarrhoeal stools even when no other recognised enteric pathogen has been recovered, although the isolation rates from infected individuals are typically low (Janda and Duffey 1988). Altwegg (1985) recovered aeromonads from less than 2% of patients with diarrhoeal symptoms. A range of virulence factors that might be involved in

pathogenesis have been identified. These include the production of exotoxins, including enterotoxins and hemolysins, cytotoxins and the ability to adhere and invade Hep-2 cells (Thomas *et al* 1990). However, human challenge experiments with strains possessing one or more of these virulence factors have failed to produce diarrhoeal symptoms in healthy individuals.

As the occurrence of aeromonads is widespread their transmission to humans can be through a number of routes, the two most significant vectors being food and water. Aeromonads have been frequently isolated from a wide range of food products (Palumbo *et al* 1985, Nishikawa and Kishi 1988 and Knochel and Jeppesen 1990). Kirov *et al* (1990) reported a strong correlation in the possession of virulence factors between food and clinical aeromonad isolates.

With respect to transmission via drinking water there is no firm evidence to implicate the role of aeromonads as causative agents of gastroenteritis. In a limited epidemiological study Burke *et al* (1984a) did find a relationship between the abundance of aeromonads in a distribution system and the number of reported outbreaks of diarrhoea. However, this was a non-randomised study and would not include all patients possessing gastroenteritis. Other workers have isolated aeromonads from distribution systems and have considered them as potential pathogens because they have demonstrated that some of these strains were capable of producing certain virulence factors in *in vitro* assays. LeChevallier *et al* (1982) recovered only *A. sobria* from 27% of samples in a drinking water network, of these he found that over 80% were cytotoxic but that none were enterotoxic. Burke *et al* (1984c) monitored an unchlorinated domestic water supply and found that 61% and 66% of the aeromonads produced enterotoxin and hemolysin respectively. Mascher *et al* (1988) isolated aeromonads from distribution network in which 50% of the strains were capable of producing enterotoxins.

It would appear that differences exist in the distribution of biochemical and virulence factors between aeromonads isolated from environmental and clinical sources. At the biotype level Burke *et al* (1984a) reported that 58.5% and 39.4% of environmental isolates compared to 15.0% and 6.8% of faecal origin were able to ferment arabinose and salicin respectively. Similarly, there were differences in the distribution of virulence factors between the two groups of

isolates. Both sets of isolates demonstrated enterotoxic activity at 91.2% and 70.2% for those of clinical and environmental origin respectively. However, over 70% of drinking water strains exhibited a non-pathogenic haemoglutination pattern compared with 10% of faecal origin.

2.6 Discussion

Before investigations can be undertaken on the occurrence of aeromonads consideration needs to be given to techniques for their isolation and identification. It would appear that a number of factors operate to make this process difficult. In the first instance, classification of the motile aeromonad group has not been fully established and it seems likely that additional species exist. However, until classification of the group has been resolved then isolates should be designated to one of the three currently recognised species.

Although aeromonads are easy to recover from aquatic environments the majority of selective media do not give a satisfactory performance. Because of the similarity between aeromonads and vibrios both will be recovered on most isolation media. As vibrios are likely to occur in all waters, possibly including potable water, then incorporation of the vibriostatic agent 0/129 into the isolation medium to prevent their growth should be considered for all applications. Also, the majority of selective media are not able to prevent the growth of aerobic non-fermenting bacteria which can, through their excessive growth, obscure the aeromonad colonies. This problem can be suppressed by alterations to the incubation conditions to create a more anaerobic environment.

Also, identification is made more difficult by the lack of a suitable confirmation system for use in routine applications where large numbers of isolates require screening. Unless vibrios are prevented from growing on the isolation medium it is essential that any identification system should be able to discriminate aeromonads from vibrios. Again the vibriostatic agent could be employed as an additional diagnostic feature. The use of a series of specific biochemical tests appears to offer the best alternative to the commercial API

identification system, which does not appear to be able to discriminate satisfactorily between aeromonads and vibrios. Other commercial identification systems do exist, but it is not known how well they function.

In natural waters the densities of aeromonads appear to show a seasonal distribution and it has been suggested that the aeromonad population was responding to water temperature. However, it is difficult to establish a clear association with temperature as other factors would also be likely to exhibit a seasonal variation. Indeed other studies have shown that high numbers of aeromonads corresponded with peaks in phytoplankton populations and also the presence of decomposing vegetation. Thus, aeromonads could be selectively responding to specific changes in the composition of the water through selective utilisation of the macromolecular components released from these organisms. In potable water production where a surface derived source water is used nutrients could be passed into distribution to allow for selective growth of aeromonads. To detect any specific response associated with seasonal availability of nutrients it would be useful to determine if a shift occurs in the proportion of aeromonads to the total bacterial population.
The majority of studies on potable water systems have tended to consider the occurrence of aeromonads as a function of one or two parameters of which the effects of chlorine concentration and temperature are most often examined. It would appear that aeromonads are only recovered when the total available chlorine concentration is low. In support of this, laboratory studies have shown that aeromonads have a lower resistance to disinfection than other Gram negative bacteria (Knochel 1990 and Medema *et al* 1990). When disinfection is not adequate the aeromonad population will respond to such influences as temperature, retention time and nutrient availability. Because of the complexity of distribution systems the numbers of aeromonads present at any one location will be a function of an interaction of all of these parameters if not more.

The most common association between aeromonads and pathogenicity is as causative agents of gastroenteritis although this remains to be conclusively established. Therefore, it is not known if the presence of aeromonads in drinking water will present a threat to public health. However, evidence from laboratory studies has indicated that a proportion of the population of

aeromonads from drinking water do possess a range of virulence factors. It would be useful to conduct epidemiological studies to determine if aeromonads are of significance to public health.

2.7 Conclusions

1. The classification of the motile aeromonad group has not been unequivocally established. In most cases classification to genus level would be appropriate.

2. The selective media for recovery of aeromonads do not perform entirely satisfactorily. Modification of the incubation conditions to create an anaerobic environment will suppress the background flora.

3. Identification of isolates would be better achieved by using a series of specific biochemical tests in preference to the commercially available API system. In routine application the number of tests can be reduced but it is essential to have a diagnostic reaction to discriminate aeromonads from vibrios.

4. In natural waters the numbers of aeromonads exhibit seasonal variations which could be linked to temperature and selective utilisation of algal and macrophyte components.

5. In potable waters aeromonads respond to an interaction between a number of factors including chlorine concentration, temperature and residence time.

6. The link between the presence of aeromonads in drinking water and the outbreak of waterborne gastroenteritis has not been established.

3. ISOLATION AND IDENTIFICATION OF AEROMONADS

3.1 Evaluation of isolation media

3.1.1 Introduction

Aeromonads are nutritionally diverse bacteria and can be recovered on a variety of non-selective media. However, a number of selective media have been developed specifically for the recovery of aeromonads from a wide variety of habitats (Section 2.3). Work conducted by Havelaar *et al* (1987) evaluated a range of media for the isolation of aeromonads from drinking water. Although none of the media investigated were found to perform completely satisfactorily a combination of two was developed. This medium, referred to as ampicillin dextrin agar (ADA), (Appendix A) was found to be highly selective for aeromonads; their growth producing characteristic yellow colonies. This medium has been used extensively by drinking water laboratories throughout the Netherlands.

In our study the suitability of ADA was evaluated for the detection and enumeration of aeromonads. As a comparison a commercially available isolation medium, Aeromonas Medium supplied by Unipath, was also investigated.

3.1.2 Methods

For this series of experiments aeromonads were obtained from various sources including:

- a pure culture strain of *A. hydrophila* grown overnight in nutrient broth, obtained from the National Collection of Typed Cultures (NCTC 9240).
- a natural source of aeromonads derived from River Thames water and lagoon water at Medmenham.

- samples from a distribution system known to support an aeromonad population

In all cases the water samples were processed by membrane filtration. The inoculated filters were placed on the surface of the isolation medium incubated at 30 °C for up to 48 hours.

3.1.3 Results and discussion

On ampicillin dextrin agar the pure culture of *A. hydrophila* produced characteristic bright yellow colonies. On Aeromonas medium aeromonads produce dark green opaque colonies with a dark green centre. Comparison of the efficacy of isolating natural populations of aeromonads from freshwater sources revealed that that the recovery of aeromonads from both habitats was slightly higher on the ampicillin dextrin agar (Table 3.1).

Table 3.1 Comparison of two media to recover aeromonads from natural environments

Medium employed	Numbers of aeromonads recovered	
	Lagoon water (CFU/100 ml × 10^2)	Thames river water (CFU/100 ml × 10^2)
Ampicillin-dextrin agar	197	293
Aeromonas medium	110	260

With populations of aeromonads derived from a drinking water distribution system, the colonies that developed on ampicillin-dextrin medium revealed a diversity in morphology and colour which was dependent upon the length of incubation. These aeromonads produced yellow colonies which were visible after

24 hours incubation. However, incubation of up to 48 hours resulted in the colonies changing colour to various shades of orange, pale brown and green. This observation was not found by Havelaar *et al* (1987) who indicated that aeromonads remained yellow throughout the incubation period. However, Knochel (1989) noticed that with prolonged incubations aeromonad colonies reverted from yellow to blue when this medium was evaluated, the result of the use of an inferior source of dextrin. In our study aeromonad isolation was initially performed using dextrin supplied by Sigma. This dextrin had a dull brown colour indicating that it was relatively unrefined. When this dextrin was incorporated into the medium a diverse range of colony types was observed at 24 hours none of which were typically characteristic of aeromonads. Subsequently a 'white' dextrin (grade not defined) obtained from Merck was used in the preparation of the medium which produced enhanced aeromonad colony expression and yellow colonies were observed by 24 hours.

During routine investigations it was found that aeromonads were not the only bacteria capable of growth on this medium. After 24 hours small pale blue colonies, identified as *Pseudomonas* sp., were observed. This organism was readily distinguishable from the aeromonad colonies by its colour. However, at 48 hours, a diverse range of colonies was found belonging principally to the genus *Pseudomonas* including; *P. putida* (green/brown), *P. luteola* (blue/green), *P. fluorescens* (orange/brown) and *P. paucimobilis* (orange/yellow). Additionally, small orange colonies, identified as *Flavobacterium* spp., were also found. Havelaar *et al* (1987) found that prolonged incubation yielded bright yellow colonies which interfered with the enumeration of aeromonads as they were indistinguishable from the dextrin fermenting aeromonads. These were produced by either strictly aerobic non-fermenting bacteria or occasional pseudomonads.

Considering the loss of colony colour after 24 hours combined with an emergence of a considerable background flora following prolonged incubation which made colony discrimination difficult, the aeromonad count was determined after 24 hours. Havelaar *et al* (1987) similarly concluded that a 24 hour incubation was the most suitable as aeromonads could not be visually differentiated from other bacteria after this time.

3.2 Identification of aeromonads

3.2.1 Comparison of two systems for identification

The principal of a selective isolation medium is that it will only allow the growth of the organism of interest. Unfortunately, most media are not entirely selective for individual genera and additional diagnostic tests must be applied after selection and purification of isolates before their identity can be confirmed. The tests for confirmation are typically based upon the response to various biochemical tests. Commercial systems have been developed, which exploit a range of biochemical tests, enabling many different genera of bacteria to be identified. One such database of biochemical test responses has been developed by API Laboratories for identification of Gram negative bacteria.

Alternative identification schemes have been proposed which are specific to aeromonads. One such scheme has been developed by workers at RIVM Bilthoven (Holland), which is based on four standard biochemical tests, and is routinely used for the confirmation of presumptive aeromonads recovered on ampicillin dextrin medium (Medema, personnel communication). To classify the isolates to genus level two biochemical tests, Hugh and Leifson's oxidation-fermentation test with glucose as the substrate and the oxidase reaction are employed. Cultures which possessed a fermentative metabolism and were additionally oxidase positive were considered to be aeromonads. To classify the confirmed aeromonads to species level two further tests of gas from glucose and esculin hydrolysis are employed. The procedures used for all the biochemical tests are given in Appendix C. The differentiation to species level on the basis these tests is given in Table 2.2 but has been reproduced here for convenience (Table 3.2).

Table 3.2 Differentiation of aeromonads to species level using two biochemical tests

Aeromonad Species	Biochemical test reaction	
	gas from glucose	esculin hydrolysis
A. hydrophila	positive	positive
A. sobria	positive	negative
A. caviae	negative	positive
unknown	negative	negative

It was therefore decided to carry out a comparison of the RIVM and API systems to determine the suitability of each as confirmation procedures. In studying the occurrence of aeromonads it was essential that the bacteria were identified correctly, although it was not considered necessary to take this to species level.

3.2.2 Method

Source waters containing aeromonads were obtained from either the River Thames at Medmenham or from a distribution system. A representative number of colonies which exhibited the typical characteristics of aeromonads were subcultured onto nutrient agar. This first step was essential as one of the biochemical tests, determination of the oxidase reaction, can only be undertaken on a medium that does not contain a fermentable substrate.

The API 20 NE system was inoculated and interpreted according to the manufacturers instructions. The biochemical tests used in the RIVM system are standard procedures and were performed according to the methods of Cowan (1974).

3.2.3 Results and discussion

A comparison of the identification profiles with isolates obtained from River Thames water is given in Table 3.3.

Table 3.3 Comparison of identification of isolates recovered from River Thames water

Identification Result	
API 20 NE	RIVM tests
acceptable identification of the genus *Aeromonas* *Aeromonas sobria* (74.2) *Aeromonas hydrophila* (14.5)	*Aeromonas sobria*
low discrimination between *Vibrio parahaemolyticus* (45.9) *Aeromonas hydrophila* (28.3) *Aeromonas sobria* (22.3)	*Aeromonas caviae*
low discrimination between *Vibrio fluvialis* (70.1) *Aeromonas hydrophila* (28.2)	*Aeromonas hydrophila*
low discrimination between *Vibrio parahaemolyticus* (46.4) *Aeromonas hydrophila* (43.8)	*Aeromonas* spp.
acceptable identification *Vibrio parahaemolyticus* (83.0)	*Aeromonas* spp.
low discrimination between *Aeromonas hydrophila* (56.9) *Vibrio parahaemolyticus* (29.8)	*Aeromonas* spp.
low discrimination between *Vibrio fluvialis* (70.1) *Aeromonas hydrophila* (28.2)	*Aeromonas caviae*

The API 20NE system was not able to satisfactorily characterise all the isolates recovered from River Thames water. Some isolates produced identification profiles that were neither typical for *V. fluvialis* or *A. hydrophila*. Similarly, the RIVM system was unable to identify the majority of these isolates to one of the three currently recognised species. Those bacteria identified as *Aeromonas* spp. with the RIVM system tended to correspond

to those isolates where a low discrimination between *A. hydrophila* and *V. parahaemolyticus* was obtained with the API 20NE system. It seems unlikely that these isolates were *V. parahaemolyticus* as this is a truly marine organism (Kreig and Holt 1984).

With isolates obtained from the distribution system the API 20NE results were generally more precise in their definition although with some cultures confusion with identification between aeromonads and vibrios existed (Table 3.4). A much better agreement between the two systems emerged, particularly with identification of *A. sobria*. This would be expected as both systems utilise esculin hydrolysis as the principal diagnostic feature between species of aeromonads. *A. sobria* does not produce a positive response whereas *A. hydrophila* and *V. fluvialis* do.

Table 3.4 Comparison of identification methods with isolates obtained from a distribution system

	Identification Result	
	API 20 NE	RIVM tests
a)	distribution sample 1	
	good identification *Aeromonas hydrophila* (98.7)	*A. hydrophila*
	good identification *Aeromonas hydrophila* (98.7)	*A. hydrophila*
	good identification *aeromonas hydrophila* (98.7)	*A. hydrophila*
	good identification *Aeromonas hydrophila* (98.7)	*A. hydrophila*
	very good identification of the genus *Aeromonas* *Aeromonas hydrophila* (78.6) *Aeromonas sobria* (20.7)	*A. hydrophila*
	acceptable identification *Aeromonas sobria* (80.2)	*A. sobria*
	low discrimination between *Vibrio fluvialis* (49.2) *Aeromonas hydrophila* (48.4)	*A. hydrophila*
	low discrimination between *Vibrio fluvialis* (70.1) *Aeromonas hydrophila* (28.2)	*A. hydrophila*
	low discrimination between *Vibrio fluvialis* (70.1) *Aeromonas hydrophila* (28.2)	*A. hydrophila*
b)	distribution system sample 2	
	Very good identification *Aeromonas sobria* (99.6)	*A. sobria*
	Very good identification *Aeromonas sobria* (99.6)	*A. sobria*
	Acceptable identification *Aeromonas sobria* (80.2)	*A. sobria*

Table 3.4 continued

Identification Result	
API 20 NE	RIVM tests
acceptable identification *Aeromonas sobria* (80.2)	*A. sobria*
c) distribution system sample 3	
very good identification *Aeromonas sobria* (99.6)	*A. sobria*
very good identification *Aeromonas hydrophila* (98.7)	*A. hydrophila*
very good identification *Aeromonas hydrophila* (98.7)	*A. hydrophila*
acceptable identification *Aeromonas sobria* (80.2)	*A. sobria*
low discrimination between *Vibrio fluvialis* (70.1) *Aeromonas hydrophila* (28.2)	*A. hydrophila*

A comparison of the two systems has revealed that for the identification of aeromonads neither performs entirely satisfactorily. Confusion existed over the discrimination between aeromonads and vibrios. This observation was surprising since Havelaar *et al* (1987) reported that vibrios would not interfere with aeromonad isolation from non-saline waters. However, because vibrios share similar nutritional requirements with the aeromonads they will grow on aeromonad isolation media. Although the vibrios, with the exception of *V. chloreae*, do have an absolute requirement for sodium chloride for optimal growth certain species are known to inhabitat riverine environments (Lee 1990).

McGarey and Wrona (1989) reported that the API system was not suitable for the identification of aeromonads. An evaluation of both the API 20 E and API 20 NE found that *A. caviae* was frequently identified as a species of *Vibrio*. Additionally, the API 20 NE had variable identification rates for *A. hydrophila*

and *A. sobria* of 40% and 60% respectively. With the API 20 E all isolates of *A. hydrophila* were correctly identified. Thus, the organisms assigned in this study to various species of *Vibrio* could represent *A. caviae*. This would render such a system impractical if both organisms were present in the same sample. With the RIVM tests the occurrence of vibrios would also present a problem as there no diagnostic tests to discriminate between the two genera.

When adopting a confirmation procedure for routine analysis consideration needs to be given not only to accuracy but also to cost and time. The API system worked out more expensive per test than the equivalent RIVM system. Additionally, although the API system requires no preparation of tests actual inoculation and interpretation can be relatively time consuming. In contrast although the biochemical tests do require preparation of media it can be obtained ready formulated and the tests are simple to inoculate and easy to interpret. It is recommended that the RIVM system offers the better alternative particularly if the additional test to distinguish aeromonads from vibrios is also included.

3.3 Discrimination between aeromonads and vibrios

In our study two systems failed to confirm satisfactorily the identity of presumptive aeromonad isolates (Section 3.2). To overcome this problem the use of a specific diagnostic test to distinguish aeromonads from vibrios was investigated. This test, based on the reaction to an antibiotic 2,4-diamino-6,7-diisopropylpteridine, referred to as 0/129, has been reported to be able to discriminate aeromonads from vibrios (Lee *et al* 1978). By determination of the minimum inhibitory concentrations of 0/129 it was found that aeromonads were resistant at concentrations equivalent to or greater than 320 µg/l whereas vibrios were sensitive to concentrations of 50 µg/l. For routine diagnostic applications discs, impregnated with the antibiotic at a concentration of 150 µg were available.

When this test was applied during screening of isolates recovered from the aeromonad survey certain bacteria were recovered which exhibited the characteristics of *Aeromonas hydrophila* in both the API 20 NE system and the

RIVM system but were found sensitive to the antibiotic 0/129 at 150 µg/l, suggesting that these isolates were vibrios. To confirm the identify of these isolates one of these strains was submitted to the National Collection of Industrial and Marine Bacteria (NCIMB) for formal identification. It was confirmed that the isolate was a strain of *A. hydrophila* which was unusual in that it was unable to produce gas from glucose or ferment salicin.

Thus, in our study the vibriostatic agent 0/129 was not found to be satisfactory for confirming the identity of aeromonads recovered from the distribution system. Therefore, the use of the RIVM tests and the API system will not be practicable even if the vibriostatic test is also employed. However, the RIVM and API systems are not the only ones available for identification of bacteria. Other commercial systems are available which might allow more reliable identification. However, as the isolate, submitted to NCIMB and isolated from the distribution system, also possessed other atypical biochemical responses for *Aeromonas hydrophila* it could similarly interfere with other commercial identification systems.

3.4 Modifications to the isolation technique

Ampicillin-dextrin-agar (ADA), was found to allow the growth of other bacteria which interfered with the enumeration of aeromonads. Although the antibiotic ampicillin was incorporated into the agar its activity is limited to suppressing the growth of the *Enterobacteriaceae* (Want and Millership 1990). In samples from a distribution system overgrowth by pseudomonads and acinetobacters occurred, particularly if incubation was extended beyond 24 hours (DoE Report 2653-M).

One characteristic feature of these interfering bacteria is that they are generally aerobic in their metabolism. Aeromonads, in comparison, are facultatively anaerobic and are capable of growth in the absence of oxygen. This characteristic has been used by some workers as an additional selective mechanism for isolating aeromonads. Cunliffe and Adcock (1989) excluded oxygen by incubating the isolation plates in an atmosphere of nitrogen. However, Havelaar *et al* (1987) found that aeromonads did not exhibit sufficient

fermentation, under totally anaerobic conditions, to allow adequate differentiation on the diagnostic medium. Schubert (1987) used a dextrin based medium in which the membrane was placed on the isolation agar and covered with more molten isolation agar. Sufficient anaerobic conditions were produced to allow satisfactory growth of aeromonads whilst also inhibiting the proliferation of the aerobic flora.

It was decided therefore to investigate whether this agar overlay technique could be used with ADA to aid the isolation of aeromonads from distribution systems.

3.4.1 Method

A distribution system sample was obtained from a network where aeromonads had previously been isolated. Ampicillin dextrin agar (ADA) was used as the isolation agar. The sample was processed by standard membrane filtration techniques. The filter was placed on the agar surface either the normal way up or inverted. A volume (10 ml) of molten agar, held in a water bath at 45 °C, was poured onto the plates to cover the filter to an equivalent depth as the solidified agar. All plates were incubated, inverted, at 30 °C.

3.4.2 Results

Overlaying the plates with agar did not affect adversely the recovery of aeromonads compared to the normal method of incubation, with similar numbers being obtained for each method (Table 3.5). The overlay technique completely inhibited the growth of the background flora. The appearance of aeromonad colonies on these plates was characterised as small orange/yellow colonies surrounded by yellow zone. With prolonged incubation, in excess of 24 hours, gas formation occurred but it did not interfere with colony enumeration.

Table 3.5 Recovery of aeromonads with different isolation techniques

Isolation Technique	Incubation time (hours) 18	24	48
(a) distribution system sample - CFU/100 ml volume			
upright filter	51	57	(1)
upright filter agar overlay	45	68	(1)
inverted filter agar overlay	48	58	(1)
(b) distribution system sample, CFU/10 ml volume			
upright filter	6	7	(2)
upright filter agar overlay	6	10	10
inverted filter agar overlay	10	14	17

[(1) confluent growth prevented enumeration. (2) overgrowth by aerobic flora obscured aeromonad colonies.]

Although this method proved useful in improving the detection of aeromonads with this particular sample, its efficacy would need to be established with other samples. The technique should however be considered as an alternative to the standard incubation technique to increase the specificity of the selective agar for the detection of aeromonads.

3.5 Demonstration of the oxidase reaction

Both of the identification systems previously described (Section 3.2) have one diagnostic reaction in common; the demonstration of the oxidase reaction. For an isolate to be considered an aeromonad it should produce a positive reaction in this test. However, it has been found that if the test is performed on

aeromonads growing on a medium containing a carbohydrate source they will yield a negative oxidase reaction (Hunt *et al* 1981). The reason for this is that aeromonads metabolise carbohydrates fermentatively to produce acid which reduces the pH and inhibits the oxidase reaction.

To eliminate false negative reactions arising from colonies growing on amplicillin dextrin agar each isolate needed to be subcultured onto a medium such as nutrient agar which contained a non-fermentable substrate. This increased the time taken to confirm presumptive isolates.

As the oxidase reaction appeared to be dependent upon pH a study was conducted to determine if a positive reaction could be produced if the pH of the colony was reversed.

3.5.1 Method

A water sample from a distribution system was processed by membrane filtration, using ampicillin dextrin agar as the isolation medium. A membrane filter from an isolation plate which supported colonies representative of aeromonads and also included a background flora was transferred to a pad saturated with phosphate buffer (pH 8.0) (Appendix B). After a contact time of five minutes the membrane filter was transferred to another pad saturated with oxidase reagent. Colonies taking on a characteristic purple colour indicated a positive reaction.

3.5.2 Results

The performance of this method was found to be variable between filters from different isolation plates. If the reaction was positive it was for all the yellow colonies on a membrane whilst all yellow colonies on other membranes did not produce a positive reaction. Because the metabolism of the background flora was non-fermentative its oxidase reaction was unaffected by growth on the isolation agar. If their growth was dominant a strong oxidase reaction was

produced which obscured that of the aeromonads. Thus, this modification of the technique was not found to be suitable.

3.6 Development of a confirmation media

For confirmation of presumptive isolates the colony from the isolation plate is usually inoculated directly into the identification system. Because of the inherent inability of aeromonads to produce a positive oxidase reaction when growing on ampicillin dextrin agar it has been necessary to first subculture onto nutrient agar before identification (Section 2.3). However, as the isolation medium supported the growth of other bacteria, particularly pseudomonads, pure subcultures were not always obtained. With the non-selective agars colonies can not be readily distinguished. In some cases complete overgrowth of pseudomonads had occurred which produced false negative results when confirmation was undertaken.

An investigation was conducted therefore to determine whether contamination of presumptive isolates could be detected by subculturing onto a differential medium. It was decided to develop further this procedure with the aim of producing a single step procedure which, if it incorporated sufficient diagnostic tests, would allow confirmation of presumptive isolates. It has been shown amongst the Gram negative bacteria that starch fermentation is generally confined to aeromonads and vibrios (Palumbo *et al* 1985). It was therefore decided to investigate starch agars as a basis for a differential procedure.

3.6.1 Method

Soluble starch (Analar grade, Merck) at concentrations from 5 to 20 g/l was incorporated into a basal medium of either yeast extract agar or a mineral salts solution supplemented with glucose (0.1 g/l) (Appendix B). To detect acid produced by fermentation a pH indicator, phenol red (18.0 ml/l of a 2% w/v solution) was incorporated into the agar. Hydrolysis of starch was detected by flooding the plates with 1/3 strength Lugol's iodine. The presence of a clear zone around a colony indicated utilisation of starch.

Isolates, previously identified as aeromonads, were spread over the agar surface. The plates were incubated at 30 °C for 24-48 hours.

3.6.2 Results

Better colony growth was obtained on the basal medium containing yeast extract agar compared to the mineral salt solution supplemented with glucose. Therefore, use of the latter medium was abandoned. Fermentative growth on the starch medium by aeromonads gave rise to yellow colonies in which a clear zone was produced with Lugol's iodine. However, high concentrations of starch (20 g/l) were necessary for satisfactory expression of colony colour. Growth of aeromonads at low starch concentrations produced pink colonies which indicated that fermentation was not occurring.

Although this medium appeared suitable its reliability will require further evaluation. Additionally, it is unable to discriminate between aeromonads and vibrios. However, the vibriostatic agent 0/129 could be incorporated into the agar as an additional diagnostic feature to differentiate between the two genera.

4. FACTORS AFFECTING THE GROWTH OF AEROMONADS

4.1 Effect of chlorine concentration

4.1.1 Effect on a pure culture strain of *A. hydrophila*

Introduction

In a water distribution system maintenance of a chlorine residual is important in controlling the bacterial population. Therefore, a series of experiments

were conducted to investigate the disinfection resistance of aeromonads. In this experiment a pure culture of an environmental strain of *A. hydrophila* was used which had previously been isolated from a water distribution system.

Method

A pure culture strain of *A. hydrophila* inoculated into distilled water was exposed to sodium hypochlorite at a concentration sufficient to produce a residual of 1.0 mg/l free chlorine at the beginning of the test. Samples were taken after a range of contact times and analysed to determine the numbers of aeromonads.

A different chlorination procedure was also carried out using a membrane filtration technique to assess the sensitivity of *A. hydrophila* to chlorine. The pure culture was diluted so that approximately 100 bacteria would be present in the sample and this was filtered through a standard membrane. A volume (10 ml) of sodium hypochlorite solution was either filtered through immediately, or held in contact with the membrane surface for a five minute contact time before being filtered through. Chlorine concentrations were varied from 0.1 to 1.0 mg/l. The membranes were placed on pads soaked in ampicillin dextrin broth and incubated for 48 hours at 30 °C.

Results

From a starting concentration, of the pure culture strain, of between 30-50 cfu/ml there were no survivors after a contact period of only five seconds. This suggests that the pure culture was not resistant to 1.0 mg/l chlorine.

No survivors were observed using the membrane contact method confirming that this strain of *A. hydrophila* was not resistant to chlorine above a concentration of 0.1 mg/l. Experiments using chlorine concentrations below 0.1 mg/l were not attempted since the spectrophotometric method currently in use is not sensitive enough to measure such low chlorine concentrations (Department of the Environment 1981).

4.1.2 Effect of chlorine on natural populations of aeromonads

Introduction

As aeromonads are known to grow within distribution systems in which the water has been treated with chlorine, it is possible that some strains would be resistant at low disinfectant concentrations. Previous work using pure cultures of *A. hydrophila* showed that the strains tested were very sensitive to chlorine (Section 4.1.1). Therefore, to simulate more closely what happens to natural populations of aeromonads when they come in contact with chlorine during water treatment it was planned to study the effects of chlorine on mixed natural populations of aeromonads.

Methods

In all experiments glassware was prepared and reagents were used so as to prevent their reaction with chlorine. Glassware was prepared by overnight contact with chlorine solution (10 mg l^{-1}) and thoroughly rinsing, before use, with double distilled water. The reagents sodium hypochlorite and monochloramine were prepared freshly each day.

In initial experiments natural populations of aeromonads were derived from River Thames water. In subsequent experiments an aeromonad population was obtained from a distribution system.

Two different methods were used to expose the bacteria to the disinfectant. A membrane filter procedure was used as described in Section 4.1.1. Untreated control membranes, exposed only to the sample and rinse water, were prepared along with the chlorine-treated membranes. An alternative procedure was used in which the water sample (1 l) was incubated for a range of time periods at 20 °C after dosing with various concentrations of applied free chlorine and monochloramine. At specified times samples were withdrawn, neutralised with sodium thiosulphate and analysed for aeromonads.

Results

When using the membrane filtration procedure the majority of aeromonads in River Thames water were found to be sensitive to free chlorine (0.5 mg/l) as nearly 96% of the total population did not survive a ten second contact period. However, a small percentage of the aeromonad population did survive contact periods of ten seconds with free chlorine concentrations of up to 5.0 mg/l. In contrast, the aeromonad population was found to be 100% resistant to monochloramine concentrations of up to 10 mg/l with similar contact periods.

Using the alternative disinfection procedure the majority of the aeromonad population did not survive one minute exposure to free chlorine. However, some survivors were still detected after a contact period of one hour. Monochloramine exhibited a less rapid inactivation rate taking 30 minutes to reduce the population by 99%. Even after a 24 hour contact period aeromonads (<0.02%) were still detected.

Chlorination of raw source waters similarly revealed that a small percentage of the aeromonad population was capable of surviving disinfection. Contact with less than 1.0 mg/l of free chlorine resulted in the vast majority (>99.5%) of the cells becoming unculturable. However, a very small percentage (<0.5%) of the original number of aeromonads were still viable after a ten second contact period with 8.5 mg/l free chlorine.

4.2 Effect of temperature on the growth of aeromonads

Samples of water taken from a treatment works and the River Thames were analysed for their ability to support the growth of aeromonads at two different temperatures. All samples were filter sterilised using a 0.22 µm membrane and dispensed as 100 ml volumes. Four different strains, obtained from a collection of environmental isolates held at the WRc, were used to inoculate the flasks. Each flask received the same volume of inoculum of each strain but the cell concentration varied between strains. Two sets of flasks were maintained on orbital shakers (200 rpm), one at 10 °C the other at 20 °C. Growth was measured daily by ATP determinations using luminometry (Stanfield

and Jago 1989) and a maximum growth potential was derived for each strain at the two temperatures.

It was found that the aeromonads were capable of growth at both temperatures in the water obtained from the treatment plant, in some cases maximum growth occurred at the lower temperature. However, the growth of the heterotrophic population at 10 °C was considerably reduced compared to that at 20 °C (Table 4.1).

Table 4.1 The effect of temperature on the growth of aeromonads and the heterotrophic population

Aeromonad strain	Maximum ATP concentration (ng/l)	
	10 °C	20 °C
A1	3 472	1 904
A4	352	352
A8	1 756	1 315
A11	1 842	1 454
heterotrophic population	42	1 473

Other investigations on the effects of temperature in pure culture studies found that at 4 °C the viable aeromonad cell count was less than at 25 °C, over a 48 hour period (Cavari *et al* 1981). The lower temperature was found to depress the substrate uptake mechanism so that the bacteria were not able to compete with the more psychrotolerant bacteria. In our study variable results were found with River Thames water which could have occurred from contamination of the filtered river water due to ineffective sterilization of the sample by membrane filtration. Rippey and Cabelli (1985) found it necessary to autoclave the filtered samples to prevent the growth of filterable bacteria when undertaking pure culture studies.

4.3 Effect of chlorine residual

If aeromonads were affected by chlorine then they should be capable of growth in water leaving the treatment works if the chlorine had been neutralised. It was therefore decided to examine the growth of aeromonads in samples from a selected distribution system in water as it left the works where it had a residual of 0.5 mg l^{-1} and from a dead end where it had a residual of typically less than 0.1 mg l^{-1}.

Methods

A water sample was collected from a treatment works immediately prior to entering distribution. A sample was also collected from a dead end known to support an aeromonad population. The chlorine in both samples was neutralised by the addition of sodium thiosulphate.

The samples were filtered through a polycarbonate membrane (0.22 µm pore size) and volumes (900 ml) were collected. An inoculum (100 ml) of the unfiltered dead end water was added to each bottle. Additionally, duplicate samples were inoculated with 100 ml of a pure culture of *A. hydrophila* previously isolated from this distribution system.

Samples were stored at 20 °C for seven days. The numbers of aeromonads were determined by membrane filtration using ampicillin dextrin agar. The total heterotrophic population was enumerated by spread plates on R2A medium.

Results

Both the water entering distribution and from the dead end were capable of supporting the growth of a pure culture of *A. hydrophila* (Table 4.2). However, the total microbial population greatly exceeded the aeromonad concentration because the filtration technique used in preparing the water did not achieve complete sterility.

Table 4.2 Growth of *A. hydrophila* in two waters from the beginning and end of a distribution system

Time (days)	Aeromonad Count beginning (CFU/ml)	Aeromonad Count end (CFU/ml)	Heterotrophic Plate Count beginning (CFU/ml)	Heterotrophic Plate Count end (CFU/ml)
0	10	10	10	10
3	7 100	45	26 800	500
7	116 000	440	605 000	385 000

Using the inoculum from the dead end both waters failed to show much of an increase in numbers of aeromonads (Table 4.3). An increase would have been difficult to detect because the inoculum possessed a low aeromonad count of less than 10 CFU/ml. In both samples however, there was an increase in the heterotrophic population.

Table 4.3 Growth of aeromonads, with an inoculum obtained from a distribution system, in two waters from the beginning and end of a distribution system

Time (days)	Aeromonad Count beginning (CFU/ml)	Aeromonad Count end (CFU/ml)	Heterotrophic Plate Count beginning (CFU/ml)	Heterotrophic Plate Count end (CFU/ml)
0	<1	<1	840	810
3	(1)	(1)	727 500	54 000
7	5	3	193 250	565 000

[(1) count not determined]

5. AEROMONADS IN WATER DISTRIBUTION SYSTEMS

5.1 Survey of aeromonads at a water treatment plant and in distribution

The aim of this survey was to isolate aeromonads from various distribution systems and to identify those factors which may have influenced their growth and distribution. A letter was written to four water companies requesting their co-operation in supplying water samples from areas where aeromonads were known, or were likely, to occur.

A response was obtained from two water companies and water samples from two treatment works and their respective distribution areas have been obtained.

5.1.1 Methods

Water samples for bacteriological analysis were collected in accordance with standard methods (Anon 1983). All samples were transported to the laboratory at 4 °C. Aeromonads were recovered by membrane filtration using ampicillin dextrin medium (Section 3.1). The heterotrophic plate count was undertaken on nutrient agar with incubation at 25 °C for seven days.

In addition the assimilable organic carbon was determined using the method developed at WRc (Stanfield and Jago 1989). Also, a wide range of inorganic determinands were measured.

5.1.3 Results

Sampling visits were undertaken to two treatment works on two successive occasions (Tables 5.1/5.2 and Tables 5.3/5.4 respectively). The distribution system samples were obtained from consumer taps designated by the water company. Aeromonads were found in the source waters at both sites. Water treatment was very effective at reducing the numbers of aeromonads such that none were found (per 100 ml) in the final water produced from one works, although a low number (1-4/100 ml) were found in the final water at the other treatment works.

Table 5.1 Effect of water treatment on microbiological and chemical quality of water at a treatment works and in distribution

Sample name	AOC (µg/l)	TOC (µg/l)	Aeromonads (cfu/100 ml)	Coliforms Presumptive (cfu/100 ml)	Coliforms *E. coli* (cfu/100 ml)
Raw	426	6 540	40 000	13	1
Softened	422	5 560	0	0	0
Filter influent	230*	5 580	37	0	0
Filter effluent	451	5 840	0	0	0
Clarified	693	6 980	20 000	90	0
Softened	821	5 850	30	0	0
Filter effluent	853*	5 580	ND	ND	ND
Works final	315	5 580	1	0	0
Distribution	372	4 340	0	0	0
Distribution	306	5 490	20	9	0

ND not determined
* Had not reached maximum, possible residual chlorine effect

The numbers of aeromonads isolated from the distribution samples were variable, ranging from zero to concentrations higher than that found in the untreated source water. Of the parameters that were measured it was found that a relationship appeared to exist between the numbers of aeromonads, and the residence times and concentrations of free chlorine within the system. The numbers of aeromonads appeared to be independent of all the other chemical parameters measured.

5.2 Further survey of a single treatment works and distribution system

The initial survey of the two sites had indicated that residence time and free chlorine were important factors in influencing the aeromonad population. Thus, one of the treatment works and its distribution system were chosen for a more detailed investigation. Additionally, to detect any seasonal changes in the

Table 5.2 Effect of water treatment on the microbiological and chemical quality of water

	Raw water	Softened	Filter Effluent	Clarified	Softened	Filter Influent	Filter Effluent	Works Final
Aeromonads (per 100 ml)	4 925	32 300	0	8 675	295	1 045	4.5	4
7 day plate count (per 100 ml)	152 000	315 000	93 200	320 000	206 000	10 000	2 000	50 000
E. coli (per 100 ml)								
Presumptive	1	0	0	6	0	0	0	0
Confirmed	0	0	0	0	0	0	0	0
Free chlorine residual (mg/l)	0	0.42	0.70	0	0.50	0.49	1.30	0.64
Alkalinity (mg/l CaCO3)	240.6	ND	ND	ND	ND	ND	ND	136.7
Orthophosphate (mg/l P)	0.68	ND	ND	ND	ND	ND	ND	0.18
Nitrate (mg/l N)	2.70	ND	ND	ND	ND	ND	ND	2.64
Nitrite (mg/l N)	0.052	ND	ND	ND	ND	ND	ND	<0.001
Ammonia (µg/l N)	70.3	ND	ND	ND	ND	ND	ND	171
TOC (µg/l)	5 700	ND	ND	ND	ND	ND	ND	5 600

ND Not determined

Table 5.3 Effect of water treatment and distribution system on microbiological and chemical quality of water

	Raw Water	Settled Water	Mixed Filtrate	Works Final	Distribution samples 6	8	9	10	11
Aeromonads (CFU per 100 ml)	29 200	91 700	872	0	0	1477	30 000	12 900	323
7 day plate count (CFU per 100 ml)	285 000	1 740 000	555 000	0	47 800	352 000	467 000	79 000	144 000
E. coli (per 100 ml)									
Presumptive	4	0	11	0	0	0	0	0	0
Confirmed	0	0	0	0	0	0	0	0	0
Free chlorine residual (mg/l)	0	0.24	1.1	0.18	0.04	0.04	0.01	0.04	0.02
Alkalinity (mg/l CaCO3)	235	148	219	208	208	246	208	208	208
Orthophosphate (mg/l P)	0.89	0.10	0.08	0.90	0.89	0.88	0.87	0.77	0.89
Chloride (mg/l)	60.9	58.4	59.3	64.2	59.8	59.5	59.6	62.9	64.5
Sulphate (mg/l)	147	155	156	160	156	155	155	160	159
Nitrate (mg/l N)	3.40	3.41	3.40	3.49	3.62	3.68	3.72	3.65	3.61
Nitrite (mg/l N)	0.06	<0.01	<0.01	<0.01	0.06	<0.01	<0.01	<0.01	<0.01
Ammonia (µg/l N)	28	12	<12	24	16	12	<12	<12	28
TOC (µg/l)	6 400	5 800	4 500	5 400	5 300	5 100	4 600	4 500	4 800
AOC (µg/l)	600	202	440	103	333	277	284	273	390
Temperature (°C)	ND	ND	ND	ND	18.2	19.6	18.1	18.7	18.5
Diss. oxygen (%)	ND	ND	ND	ND	81.7	81.8	86.0	75.9	83.1
pH	ND	ND	ND	ND	7.6	7.6	7.6	7.7	7.7
Residence time (hours)	ND	ND	ND	ND	34	50	60	41	41

ND not determined

Table 5.4 Effect of water treatment and distribution system on the microbiological and chemical quality of water

	Raw Water	Works Final	Distribution system samples 1	2	3	5	6	8	9	10	11
Aeromonads (CFU per 100 ml)	11 000	0	0	0	0	145	6	462	10 400	135	6 050
7 day plate count (CFU per 100 ml)	9 200 000	10 500	3 000	17 000	25 500	14 000	280 000	115 000	480 000	76 500	108 000
E. coli (per 100 ml)											
Presumptive	TNTC	0	0	0	0	0	0	0	0	0	0
Confirmed	ND	0	0	0	0	0	0	0	0	0	0
Free chlorine residual (mg/l)	0	ND	0.06	0.11	0.04	0	0.04	0	0	0.02	0.01
Total chlorine (mg/l)	0	0.7	0.30	0.27	0.25	0.02	0.22	0.16	0.07	0.21	0.12
Alkalinity (mg/l CaCO3)	252	222	215	217	208	202	219	221	218	221	220
Orthophosphate (mg/l P)	0.94	0.94	0.93	0.94	0.94	0.07	0.93	0.92	0.92	0.92	0.88
Chloride (mg/l)	60.3	62.7	62.4	62.8	62.9	59.9	63.8	63.8	64.4	63.5	62.8
Sulphate (mg/l)	142	164	165	165	164	161	164	163	166	165	165
Nitrate (mg/l N)	0.369	2.81	2.80	2.90	2.90	0.038	3.00	3.00	3.00	3.00	3.00
Nitrite (mg/l N)	0.034	<0.01	<0.01	0.011	0.029	<0.01	0.019	<0.01	<0.01	<0.01	<0.01
Ammonia (µg/l N)	47	<20	27	40	27	<20	<20	<20	<20	<20	22
TOC (µg/l)	6 200	5 100	5 000	5 700	4 700	3 100	4 600	4 300	4 000	4 300	4 100
AOC (µg/l)	148	167	224	446	188	178	160	159	121	160	124
pH	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.7	7.6	7.7	7.7
Residence time (hours)	ND	ND	23	26	30	94	34	50	60	41	41

ND not determined
TNTC too numerous to count

aeromonad population the monitoring programme was conducted over several months.

5.2.1 Method

Description of the treatment works

Water for treatment is abstracted from a river and pumped to a storage reservoir where the residence time is up to 12 months. As water is withdrawn from the reservoir a chlorine dose of 1.0 mg/l is applied at the draw off point. Additional chlorination is applied to achieve a free chlorine residual of 0.3 mg/l prior to clarification. Flocculation is undertaken by the addition of ferric sulphate (4 mg/l) and polyelectrolyte (0.12 mg/l) and further processed by filtration through beds of granular activated carbon (Chemviron TL 830). The final water is chlorinated to produce a free chlorine residual of 0.8 mg/l with a nominal residence time of six hours. After trimming of the free chlorine residual ammonia is dosed to combine with the remaining free chlorine to form monochloramine and reduce the free chlorine residual to less than 0.2 mg/l.

During the summer of 1990 a programme of phosphate stripping, by precipitation with iron salts, was conducted at the reservoir in an attempt to combat the problem of algal growth. This involved the addition of ferric sulphate into the raw water pipeline.

Sample collection

Samples were collected directly from the water treatment plant. For the distribution system survey, samples were obtained from individual households by employees at the treatment works and was at a different location from those sampled on the previous occasions. A description of the samples is given in Table 5.5.

Table 5.5 Source of water monitored for aeromonads

Sample	Description	Residence time (hours)
W1	raw, unprocessed reservoir water	--
W2	raw, chlorinated water	--
W3	mixed filtered, prior to final chlorination	--
W4	chlorinated final	--
D1	direct from the works	2-3
D2	direct from the works	2
D3	via one service tower	8
D4	via one service reservoir	14-20
D5	via one service reservoir	16
D6	via one service reservoir	26-27

Bacteriological analysis

Samples were collected and analysed as described in Section 5.1. However, in this and subsequent analyses in our study, the heterotrophic plate count was determined using R2A medium (Reasoner and Geldreich 1985).

5.2.3 Results

The numbers of aeromonads at the treatment works and in distribution were monitored from May to October (Table 5.6). A distinct pattern was found in the distribution of aeromonads. The raw, reservoir water supported the largest population which was considerably reduced when chlorinated following abstraction. During treatment numbers increased but not to the levels encountered in the raw water. Final chlorination of the water reduced the numbers of aeromonads to below the limits of detection.

Samples from the distribution network, with the exception of that from sampling point D3, generally did not support an aeromonad population. However, specific episodes occurred with samples from points D4, D5 and D6 in which aeromonads were isolated on single occasions. It was only with samples from sampling

Table 5.6 Survey of aeromonads at a water treatment plant and in distribution

	Aeromonad count (log10 CFU/100 ml)																
	May	June				July				August				Sept		Oct	
	24	05	12	19	26	03	10	17	24	08	14	21	29	04	11	10	17
W1	-	-	-	-	-	-	-	-	-	4.85	5.79	-	4.45	-	-	-	-
W2	3.99	-	0	1.85	0	-	1.0	0	(1)	(1)	-	-	2.65	2.24	1.60	2.63	1.30
W3	-	3.66	4.03	3.28	3.79	4.60	4.08	4.06	3.00	4.59	4.73	3.93	3.85	3.95	3.69	3.17	3.19
W4	-		0	0	(1)	0	1.11	0	0	0	0	0	0	0	0	0	0
D1	2.45	-	-	-	0	0	(1)	0	0	0	-	-	-	0	0	0	0
D2	-	(1)	-	-	0	0	-	0	0	0	-	0	-	0	0	0	0
D3	-	3.00	-	2.83	2.41	3.45	2.72	2.74	2.67	-	-	3.34	3.58	2.24	3.22	2.77	3.00
D4	1.92	1.90	-	-	0	0	(1)	0	0	0	-	0	0	0	0	0	0
D5	0.70	2.23	-	0	0	0	0	0	0	0	-	1.69	0	0	0	0	0
D6	1.49	1.65	-	0	0	0	0	0	0	0	0	3.18	0	0	-	0	0

(-) = not determined
(1) = <10 cfu/100 ml

point D3 that a consistently high aeromonad count was found. This sampling point was in a dead end of the distribution network where flow would be expected to be low.

5.3 Extension of the aeromonad survey

5.3.1 Survey along a single section

Further survey work was conducted to establish if aeromonads were associated with dead ends. The specific section of the network which consistently yielded a high aeromonad count (D3) was chosen for a more detailed investigation.

Method

Water from the treatment works is pumped to a water tower and enters the distribution network from which sample D3 was collected. Samples were collected at various points along this section from the water tower to the end of the system.

The analysis of the water samples was conducted as described in Section 5.2.

Results

Two successive analyses of the network were undertaken in late October (Table 5.7) and early November (Table 5.8). In the first analysis aeromonads were either absent or present in low numbers in samples P1-P3. Samples from the rest of the system, where the water entered the dead-end section of the network, contained high numbers of aeromonads. The highest counts were found at the extremities of the network. On the second visit the number of aeromonads were considerably lower than were found previously for samples P1 to P7, although similar levels of aeromonads were found in samples P8 and P9. Between these visits there had been three mains breaks in this section of the

network which would have been flushed on each occasion and therefore reduced the bacterial numbers.

Table 5.7 Survey of aeromonads in a distribution system during October

Sample	Aeromonad count (log10 cfu/100 ml)	Chlorine concentration (mg/l)	
		Free residual	Total available
P1	0	0.06	0.47
P2	0	0.02	0.42
P3	(1)	0.01	0.36
P4	2.95	0.01	0.18
P5	1.87	0.07	0.24
P6	4.36	0.06	0.30
P7	3.18	0.29	0.57
P8	4.65	0.05	0.16
P9	4.88	0.07	0.16

(1) <10 CFU/100 ml

Table 5.8 Survey of aeromonads in a distribution system during November

Sample	Aeromonad count (log10cfu/100 ml)	Chlorine concentration (mg/l)	
		Free residual	Total available
P1	0	0.03	0.56
P2	0	0.06	0.46
P3	0	0.02	0.47
P4	(1)	0.01	0.19
P5	(1)	0.01	0.21
P6	1.28	0.01	0.13
P7	(1)	0.08	0.28
P8	4.57	0.00	0.10
P9	4.24	0.03	0.14

(1)= <10 CFU/100 ml

Once the water entered the dead-end section of the system the total available chlorine concentration was lower than in the trunk main, with the exception of P7 on the first visit. This anomaly was probably caused by inadequate flushing of the sampling point, following sterilisation using sodium hypochlorite solution, which resulted in a residue of chlorine entering the sample.

5.3.2 Comparison of different dead ends

The survey of the distribution system reported previously had initially only examined one particular section of the network (Section 5.2). Aeromonads were confined to the dead end sections of the system where the concentration of chlorine was low. It was decided to expand this survey to other distribution systems to determine if aeromonads were only found in dead ends.

Failure to detect aeromonads in some of the samples in the November sampling programme could have occurred because the cells were stressed and did not recover on the isolation medium. Alternatively, they could be present at concentrations below the limits of detection with the isolation technique of membrane filtration. Thus, an additional set of samples would be collected from all sampling points and stored for seven days before analysis, with the aim of allowing either for recovery or growth of aeromonads if they were present.

Method

Four different sections of the distribution system were identified for analysis. All received water from the same treatment works but each section was isolated from the other. Samples were obtained from both the dead end and its associated trunk main. Each sample was collected in accordance with the methods given in Report 71 (Anon 1983). One set of samples was analysed immediately whilst an identical set were stored for seven days at 20 °C before analysis.

The bacteriological analysis was undertaken as described in Section 5.2.

Results

In the March visit aeromonads were confined to the dead ends of distribution systems but were only recovered in two locations and at relatively low numbers (Table 5.9). Dead end 2 corresponded to the previously reported location (W3) where a relatively high aeromonad population had been found (Section 5.3). This survey was conducted during March when the water temperature was low and so the numbers would not be expected to be as high. Additionally, because the water company recognised that this particular section possessed an aeromonad problem remedial action was undertaken between the sampling visits to control the population. Although swabbing with disinfectant and flushing were used aeromonads still persisted in the system.

The heterotrophic plate count was always greater at the dead end than in the corresponding trunk main, with the highest count at dead end 4 (Table 5.9). Where aeromonads were recovered they comprised a low proportion of the total microbial population, being 3.8% and 0.23% at dead ends 1 and 2 respectively.

Table 5.9 Occurrence of aeromonads in a distribution system, during March

Location	Chlorine concentration		Aeromonad Count (CFU/100 ml)		Heterotrophic plate count (CFU/ml x 100)	
	Free residual	Total available	Day 0	Day 7	Day 0	Day 7
Mains 1	0.21	0.35	0	0	30	10 000
Dead end 1	0.00	0.03	47	85	1 250	115 000
Mains 2	0.08	0.27	0	0	180	202 000
Dead end 2	0.00	0.00	32	41	14 200	19 600
Mains 3	0.01	0.18	0	0	70	237 000
Dead end 3	0.09	0.20	0	0	370	(1)
Dead end 4	0.00	0.19	0	0	75 300	420 000

(1) count not determined

In both locations where aeromonads were recovered the free residual and total available chlorine concentrations were low. Previous sampling in this network indicated that aeromonads were found when the total available chlorine in the

dead end was below 0.3 mg/l (Section 5.3.1). However, two dead end locations did not support an aeromonad population even though the total chlorine concentration was below this value.

After seven days storage no aeromonads were recovered where previously none were detected. In those samples where aeromomads had been detected their numbers had increased by 81% and 18% for dead ends 1 and 2 respectively. There was however, a much greater increase in the total microbial population particularly with the mains samples.

Two further visits were made to these location during the summer months. During August aeromonads were recovered from all locations including the mains sections of the network (Table 5.10). The numbers (1-3.5/100 ml) in the mains sections were, however, much lower than at the corresponding dead end (280-625/100 ml). The water temperature and pH remained relatively constant throughout each section. No sample was obtained from one of the dead ends as the hydrant could not be located because of overgrowth by vegetation.

Table 5.10 Occurrence of aeromonads in a distribution system, during August

Location	Chlorine Free (mg/l)	Chlorine Total (mg/l)	Temperature oC	pH	Aeromonad count (CFU/100 ml)	Heterotrophic plate count (CFU/ml)
Mains 1	0.09	0.25	18.9	7.37	3.5	1 195
Dead end 1(a)	nd	nd	nd	nd	nd	nd
Mains 2	0.17	0.32	18.9	7.40	1	391
Dead end 2	0.03	0.09	18.5	7.35	625	14 820
Mains 3	0.11	0.26	18.8	7.54	2.5	694
Dead end 3	0.04	0.20	19.4	7.55	280	11 210
Dead end 4	0.02	0.11	20.5	7.23	575	2 800

[nd = not determined]

On the second visit in September a similar pattern was observed in the occurrence of aeromonads (Table 5.11). Only one mains sample supported an

aeromonad population but at a low concentration (2 CFU/100 ml). Similarly, as with the visit in August, there was a higher aeromonad population at each of the dead ends (90-1225 CFU/100 ml) compared to the mains sections of the network. The water temperature and pH remained constant throughout each system and was similar to the previous visit.

Table 5.11 Occurrence of aeromonads in a distribution system during September

Location	Chlorine Free (mg/l)	Chlorine Total (mg/l)	Temperature oC	pH	Aeromonad count (CFU/100 ml)	Heterotrophic plate count (CFU/ml)
Works final	nd	nd	nd	nd	0	nd
Mains 1	0.12	0.26	19.2	7.36	2	1 295
Dead end 1	0.03	0.04	15.4	7.36	820	4 250
Mains 2	0.17	0.32	18.7	7.36	0	195
Dead end 2	0.09	0.13	18.8	7.32	1 225	20 400
Mains 3	nd	nd	18.4	7.58	0	3 000
Dead end 3	nd	nd	18.4	7.45	90	35 250
Dead end 4	nd	nd	19.0	7.33	300	36 000

nd = not determined

6. PSEUDOMONADS IN DISTRIBUTION

6.1 Introduction

The genus *Pseudomonas* comprise a very diverse group of bacteria and are frequently recovered from distribution systems (LeChevallier *et al* 1980, Clark *et al* 1982 and Gibbs and Hayes 1989). They are considered to possess a nutritional diversity which enables them to exploit a wide range of ecosystems.

To isolate these bacteria from water numerous experimental media have been employed. The majority have been specifically for the recovery of *Pseudomonas aeruginosa* (Sands and Rovira 1970, Brodsky and Nixon 1974, Brodsky and Ciebin 1978, Gould *et al* 1985, Havelaar *et al* 1985, Havelaar and During 1986, Alonso *et al* 1989 and Keeven and DeCicco 1989). Also, Report 71 describes a medium

specifically for the the recovery of *P. aeruginosa* (Anon 1983). However, the scope of this project was to investigate the occurrence of the total population of pseudomonads and there are media available from commercial sources for this purpose. To allow this study to progress it was decided to use media from such a source to avoid having to establish the efficacy of experimental media. Thus in conjunction with the aeromonad sampling programme described in Section 4, samples were also collected and analysed for pseudomonads.

6.2 Method

The series of dead end sections on the distribution system network reported on previously were analysed for the occurrence of pseudomonads. Although all received water from the same treatment works each was an isolated system. Four such sections of the network were chosen.

Samples were obtained from both the dead end and its associated mains supply. Each sample was collected in accordance with the methods given in Report 71 (Anon 1983). Samples were transported to the laboratory and analysed immediately. Samples were processed by membrane filtration.

To determine the total population of pseudomonads two different media were used. One medium, GSP agar developed by Von Kielwein (1969), was obtained from Merck. On this medium pseudomonads are able to utilise glutamate selectively. The pseudomonad colonies are detected by incorporation of an indicator, phenol red, which produces a purple colour in response to metabolism of glutamate. Incubation was for three days at 25 °C.

In contrast pseudomonas agar base, available from Unipath, is formulated so that by the addition of an appropriate antibiotic supplement the medium becomes selective for either the total pseudomonad population or *P. aeruginosa* specifically. For the total pseudomonad population the supplement contains; cetrimide (5.0 mg), fucidin (5.0 mg) and cephaloridine (25.0 mg). The supplement for *P. aeruginosa* (CN) contains cetrimide (100 mg) and sodium nalidixate (7.5 mg) for each 500 ml of medium. With both these media plates were incubated at 25 °C for two days.

To confirm presumptive isolates of *P. aeruginosa* representative colonies were subcultured onto milk cetrimide agar (Appendix A) and incubated at 42 °C for 24 hours following the methods described in Report 71 (Anon 1983). On this medium a positive identification is shown by hydrolysis of the casein and production of a blue green pigment - pyocyanin and/or a fluorescent pigment - fluorescein. With the isolates recovered on the media for general pseudomonads no other confirmation method was available other than using the API 20 NE system.

6.3 Results and discussion

Two visits were made to the distribution system, one during August (Table 6.1) and the other in September (Table 6.2). The total pseudomonad count with both agars revealed that the highest concentrations of pseudomonads were found at the dead end locations of the network. In some instances low numbers (10 or less per 100 ml) were found in the mains sections. The counts from GSP and pseudomonas CFC agar did not always agree. As with the aeromonads the total pseudomonad population was a low percentage of the total bacterial population in the distribution system.

It would appear that no bacterial species other than pseudomonads were found with Pseudomonas CFC medium, although only a representative of each colony type was confirmed. However, GSP agar also allowed the growth of aeromonads, which produced yellow colonies, and tended to make counting of pseudomonad colonies on this agar difficult.

No isolates that were recovered on Pseudomonas CN agar confirmed as *P. aeruginosa* on milk cetrimide agar on either visit to this distribution system. Colonies on this medium were identified as *P. fluorescens*.

7. OVERALL DISCUSSION

It is vitally important in any investigation on the occurrence and distribution of a bacterium that suitable methods for its isolation and identification are

Table 6.1 Occurrence of pseudomonads in a distribution system, during August

Location	Chlorine Free (mg/l)	Chlorine Total (mg/l)	Temperature oC	pH	Total Pseudomonad count (CFU/100 ml) GSP	Total Pseudomonad count (CFU/100 ml) CFC	*P. aeruginosa* (CFU/100ml)	Aeromonad count (CFU/100 ml)	Heterotrophic plate count (CFU/ml)
Mains 1	0.09	0.25	18.9	7.37	0	0	0	3.5	1 195
Dead end 1(a)	nd	nd	nd	nd	nd	nd	nd	nd	nd
Mains 2	0.17	0.32	18.9	7.40	10	0	0	1	391
Dead end 2	0.03	0.09	18.5	7.35	6 700	8 000	0	625	14 820
Mains 3	0.11	0.26	18.8	7.54	0	0	0	2.5	694
Dead end 3	0.04	0.20	19.4	7.55	1 850	5 800	0	280	11 210
Dead end 4	0.02	0.11	20.5	7.23	500	40	0	575	2 800

nd = not determined

Table 6.2 Occurrence of pseudomonads in a distribution system, during September

Location	Chlorine Free (mg/l)	Chlorine Total (mg/l)	Temperature oC	pH	Total count GSP	Pseudomonad (CFU/100 ml) CFC	*P. aeruginosa* (CFU/100 ml)	Aeromonad count (CFU/100 ml)	Heterotrophic plate count (CFU/ml)
Works final	nd	nd	nd	nd	1	0	0	0	nd
Mains 1	0.12	0.26	19.2	7.36	3	4.5	0	2	1 295
Dead end 1	0.03	0.04	15.4	7.36	15	3	0	820	4 250
Mains 2	0.17	0.32	18.7	7.36	100	15	0	0	195
Dead end 2	0.09	0.13	18.8	7.32	360	1 950	0	1 225	20 400
Mains 3	nd	nd	18.4	7.58	0	1	0	0	3 000
Dead end 3	nd	nd	18.4	7.45	2 500	2 050	0	90	35 250
Dead end 4	nd	nd	19.0	7.33	500	550	0	300	36 000

nd = not determined

available. This was particularly important in our study as aeromonads were found to comprise a low percentage of the total microbial population and therefore the potential for interference from other bacteria was high. The isolation medium, ampicillin dextrin agar (ADA), was found to be satisfactory for the recovery of aeromonads providing the length of incubation was limited to 24 hours. This allowed sufficient time for the aeromonad colonies to develop but prevented overgrowth by the predominately aerobic background flora. Alterations to the isolation technique, especially overlaying with agar, improved the specificity of the medium by restricting the growth of the background flora.

A major problem in our study has been to establish a satisfactory method to confirm the identity of presumptive aeromonads. Modifications to the isolation procedure, to improve specificity, would be useful in reducing the additional biochemical tests required for confirmation. However, confusion existed as to whether the isolates belonged to the genus *Aeromonas* or *Vibrio*. The API system proved unsuitable because species from both genera were capable of producing identical profiles. The system developed by RIVM did not incorporate a test to distinguish between the two genera as it was assumed that vibrios did not present a problem in freshwater environments. However, certain *Vibrio* species are known to occur in freshwaters (Lee 1990) and their occurrence in distribution system waters could be expected. Therefore, it was necessary to introduce an extra confirmatory procedure based on the reaction to the antibiotic 0/129. This test is routinely employed as a diagnostic test to separate species from the two genera. However, it was surprising to find, and indeed it was necessary to confirm by an independent laboratory, that some isolates which were sensitive to this antibiotic at concentrations to which they have been reported to be resistant were aeromonads. This phenomenon was not investigated further but would warrant consideration during any investigation into aeromonad confirmation.

The survey of a water treatment plant and its distribution system revealed that aeromonads occurred in highest concentrations at the dead ends of distribution systems. At these locations the total available chlorine concentration was below 0.3 mg/l. This figure was above that reported by Edge and Finch (1986) who monitored a distribution system in the same region where it was found that

aeromonads were only present at total available chlorine concentrations below 0.2 mg/l. However, an investigation by Burke *et al* (1984b) concluded that total available chlorine concentrations above 0.3 mg/l controlled the aeromonad population.

The laboratory experiments, on the disinfection resistance of aeromonads, conducted in support of this project found that the majority of the natural population of aeromonads were sensitive to chlorine. However, a small percentage of aeromonads were capable of surviving the disinfection regime. Thus, the occurrence of aeromonads at dead ends could be the result of growth of these survivors. No studies were conducted specifically to examine the disinfection resistance of aeromonads at dead ends. Studies conducted by Medema *et al* (1990) reported that aeromonads have a low resistance to chlorine. Therefore, it seems likely that aeromonads proliferate at dead ends as a result of low chlorine concentrations. In addition, our laboratory experiments revealed that aeromonads were capable of growing in distribution system waters after the residual chlorine had been neutralised.

A similar pattern of distribution was also found for the pseudomonads in which the highest numbers were found at the dead ends of distribution systems. Although, these bacteria did not receive the same consideration as aeromonads it seems likely that they were similarly affected by chlorine.

The survey of a series of dead ends revealed a distinct seasonal pattern in the occurrence of aeromonads. Higher numbers of aeromonads were recovered during August and September compared to March, possibly because of increased water temperature. Other studies have reported that the aeromonad population responds to water temperature. Burke *et al* (1984b) monitored the aeromonad population in a metropolitan water supply over one year and reported that a seasonal variation occurred only when the chlorine concentration was below 0.3 mg/l. Millership and Chattopadhyay (1985) found isolation rates for aeromonads from a chlorinated water supply of 25 percent in the summer and only 7 percent in the winter. LeChevallier *et al* (1982) also stated that aeromonads were more frequently isolated when the water temperature ranged between 15 and 20 °C. However, it was found in our laboratory experiments that aeromonads grew in water at 10 °C. This temperature would be higher than that encountered

during winter in a distribution system. Cavari *et al* (1981) demonstrated that aeromonads were not able to survive in waters at 4 °C. Alternatively, the aeromonad population could have been responding to another factor which varies seasonally but which was not identified in this study. The total bacterial population was greater during the summer months with the exception of one dead end. At this location high volumes of sediment were collected during the visit in March which could have interfered with the enumeration of aeromonads by membrane filtration by preventing normal development of the colonies. The heterotrophic bacterial population is determined using a small volume (0.1 ml) of sample under which conditions the sediment would not have such a profound effect on colony enumeration.

It emerged from the study that aeromonads and pseudomonads comprised a low percentage of the total microbial population and increases in their numbers corresponded to an increase in the heterotrophic population. Thus, the higher bacterial loading in the mains section on the sampling visits in summer would increase the opportunity for the recovery of aeromonads. Other investigations have found that these bacteria can comprise a higher proportion of the total bacterial population. Clark *et al* (1982) reported that aeromonads contributed between 0.9 and 2.1 percent of the total heterotrophic population. Knochel and Jeppeson (1989) found that aeromonads accounted for 10 percent of bacterial population. In a study by LeChevallier *et al* (1982) aeromonads also comprised 10 per cent of the bacteria in a chlorinated water supply and reached 21 per cent of the total population in unchlorinated water. In a distribution system monitored by Gibbs and Hayes (1989) the predominant bacteria were identified as belonging to the genera *Pseudomonas, Alcalagenes* and *Aeromonas*.

In the above studies the various authors found that aeromonads and pseudomonads were not the only bacteria recovered. Potable water supplies were found to support a diverse bacterial population comprising many different genera. Our investigation did not investigate the significance of the other component genera but it is recommended that consideration should be given to these bacteria.

Our investigations also indicate that both aeromonads and pseudomonads are found in a low proportion of the microbial population in distribution systems.

This would make them unreliable indicators of regrowth potential. It appears that these bacteria respond as a function of the total bacterial population. This study did not establish which bacteria make the major contribution to the total bacterial population. An alternative approach suggested by Reasoner *et al* (1989) was to monitor the occurrence of pigmented bacteria during water treatment and in distribution. A change in the ratios of these bacteria was used to indicate a change in water quality.

8. CONCLUSIONS

1. Ampicillin dextrin agar was suitable for recovery of aeromonads provided incubation was not extended beyond 24 hours.

2. There is a lack of a satisfactory confirmation procedure particularly regarding discrimination between aeromonads and vibrios.

3. Aeromonads present during water treatment are inactivated at the disinfection stage.

4. The highest numbers of aeromonads and pseudomonads were found at dead ends of distribution systems, where chlorine concentrations were low and residence time was high.

5. Aeromonads and pseudomonads comprised a low and variable percentage of the total bacterial population which would make them unreliable indicators of regrowth potential.

6. As aeromonads and pseudomonads comprise a low proportion of the total heterotrophic population, further work should consider the other component genera of the heterotrophic bacterial population in drinking water.

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APPENDIX A - MEDIA FORMULATION

AMPICILLIN DEXTRIN AGAR

A medium for the selective isolation of aeromonads

Basal medium:

tryptose	5.0 g
dextrin (1)	10.0 g
yeast extract	2.0 g
sodium chloride	3.0 g
potassium chloride	2.0 g
magnesium sulphate($7H_2O$)	0.2 g
iron III chloride($6H_2O$)	0.1 g
Distilled Water	1000 ml

Combine all the ingredients and dissolve by stirring; do not heat. Add 8.0 ml of a bromothymol blue stock solution (see note A) and adjust the pH to 8.0. Dispense the suspension as 200 ml volumes and add 2.0 g of agar. Boil to dissolve and autoclave at 121 °C for 15 minutes. If the medium is to be used immediately cool to 45 °C in a water bath and add 2.0 ml of filter-sterilised (0.22 µm filter) ampicillin deoxycholate solution (see note B). Alternatively, the basal medium can be stored for up to one month at 4 °C and melted before use. Dispense into petri dishes and dry the plates before use.

(1) the source of dextrin is important (see Section 3.1.3)

Note A Preparation of bromothymol blue stock solution

stock solution concentration = 1.0 % w/v

Add 10 ml of 5.0 M NaOH to 1.0 g of bromothymol blue. Dilute to 100 ml with distilled water.

Note B Preparation of ampicillin-deoxycholate stock solution

Add 0.1 g of ampicillin to 10 ml of distilled water, dissolve by swirling. Add 1.0 ml of the antibiotic solution to 9.0 ml of distilled water containing 0.1 g of sodium deoxycholate solution, which had been dissolved by stirring.

References

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MILK CETRIMIDE AGAR

A medium developed by Brown and Foster (1970) for the confirmation of *Pseudomonas aeruginosa*. The formulation is taken from Anon (1983).

Yeast extract broth:

yeast extract	0.75 g
peptone	2.50 g
sodium chloride	1.25 g
distilled water	250 ml

Dissolve the ingredients and adjust the pH to 7.2 - 7.4. Autoclave at 115 °C for ten minutes.

Final medium:

skimmed milk powder (1)	100 g
yeast extract broth	250 ml
agar	15 g
Cetrimide (cetyltrimethyl-ammonium bromide	0.3 g
distilled water	750 ml

Add the cetrimide and agar to the yeast extract broth and steam to dissolve the agar. Mix the skimmed milk powder and distilled water by stirring. Autoclave both solutions separately at 121 °C for five minutes and remove them promptly from the autoclave after the autoclave cycle has been completed in order to prevent caramelization of the lactose in the milk. Cool to 50-55 °C, mix both components aseptically and pour into petri dishes. Store the plates at 4 °C for not longer than four weeks in sealed containers to prevent drying.

(1) Thermophile-free skimmed milk powder for bacteriological purposes is available commercially.

References

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APPENDIX B - REAGENTS

PHOSPHATE BUFFER

Stock solutions (0.2M)

A - sodium dihydrogen orthophosphate [NaH_2PO_4]
(27.8 g in 1000 ml)

B - sodium hydrogen orthophosphate [Na_2HPO_4]
(53.7 g 0f $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$)

For a buffer solution of pH 8.0 mix 5.3 ml of A with 94.7 ml of B and dilute, with distilled water, to a total volume of 200 ml.

MINERAL SALTS SOLUTION

di-potassium hydrogen orthophosphate [K_2HPO_4]	7.0 g
potassium dihydrogen orthophosphate [KH_2PO4]	3.0 g
ammonium sulphate [$(NH_4)_2SO_4$]	1.0 g
magnesium sulphate [$MgSO_4$]	0.1 g

Reference

Davies, D.G. and McFeters, G.A. (1988) Growth and comparative physiology of *Klebsiella oxytoca* attached to granular activated carbon particles and in liquid media. *Microbial Ecology*, **15**, 165-175

APPENDIX C - BIOCHEMICAL TESTS

OXIDASE REACTION

reagent:

tetramethyl-p-phenylenediamine dihydrochloride 1.0 percent w/v in distilled water.

The reagent should be colourless and stored in a glass stoppered bottle, protected from light, at 4 °C. The solution should not be used if it becomes deep blue. Experience in our laboratory has shown that it is best to use this reagent fresh for each set of tests.

On a piece of filter paper on a glass slide add 2-3 drops of the test solution; do not allow the drops to dry on the paper. Remove the test organism, grown a medium free from a fermentable substrate, using a platinum loop (not nichrome) or glass rod and smear across the surface of the impregnated paper. A positive reaction is shown by the development of a purple colour within ten seconds.

THE OXIDATION-FERMENTATION (O/F) TEST

To determine whether the bacterium utilises carbohydrates by oxidation or fermentation. The O/F test is undertaken by growing the bacterium in two tubes of Hugh and Leifson's (1935) medium; in which the medium in one tube is covered with paraffin oil after inoculation. Oxidisers show show acid production in the open tube only, whereas fermenters show acid in the paraffin oil covered tube and, starting at the bottom, in the open tube. The usual sugar employed in the medium is glucose and was used in our confirmation protocol.

Basal medium:

a commercially available formulation of the basal medium is available from Merck.

Prepare the basal medium according to the directions and when cooled to 50 °C add a sterile solution of glucose aseptically to achieve a final concentration of 1.0 percent w/v. Mix and distribute aseptically in 10 ml volumes into sterile tubes of not more than 16 mm diameter.

Inoculate duplicate tubes by stabbing with a straight wire. To one of the tubes add a layer of paraffin oil to a depth of about 1.0 cm. Incubate the tubes at 30 °C and examine after 24 hours.

Result	open tube	sealed tube
oxidation	yellow	green/blue
fermentation	yellow	yellow
no action	green/blue	green/blue

GAS FROM GLUCOSE

To determine whether the bacterium utilises glucose with the production of gas.

Basal medium:

Andrades peptone water is used as the basal medium and is available as a commercial preparation from Unipath. Add a sterile glucose solution to achieve a final concentration of 1.0 g l^{-1}.

Inoculate a tube of the medium which contains a Durham tube. Examine after 24 hours for the production of acid (yellow colour) and gas (bubble visible in Durham tube).

Esculin hydrolysis

To determine whether the bacterium possess the ability to hydrolyse esculin (6,7-dihydroxycoumarin-6-glucoside). This reagent is available from the Sigma Chemical Company.

Broth:

esculin	1.0 g
ferric citrate	0.5 g
peptone water	1000 ml

Dissolve the esculin and iron salt in the peptone water and sterilize at 115 °C for 15 minutes.

Inoculate the broth with the test organism and examine for blackened broth after 24 hours.

ANTIBIOTIC SENSITIVITY

The sensitivity to the vibriostatic agent (0/129) is used to distinguish between aeromonads and vibrios. Vibrios have been found to be sensitive whilst aeromonads are resistant. However, observations in our laboratory have found strains of Aeromonas hydrophila to be sensitive to 0/129 (see Section 3.3 for a full discussion).

A suspension of the test organism in prepared in a sodium chloride solution (1.5 percent w/v) and spread over the surface of a nutrient agar plate. Three antibiotic discs (150 µg), supplied by Unipath, are placed on the surface of the agar along with a control disc which has not been impregnated with the antibiotic and the plates are incubated at 30 °C for 24 hours. A clearing zone, in excess of that observed with the control disc, is taken to indicate sensitivity.