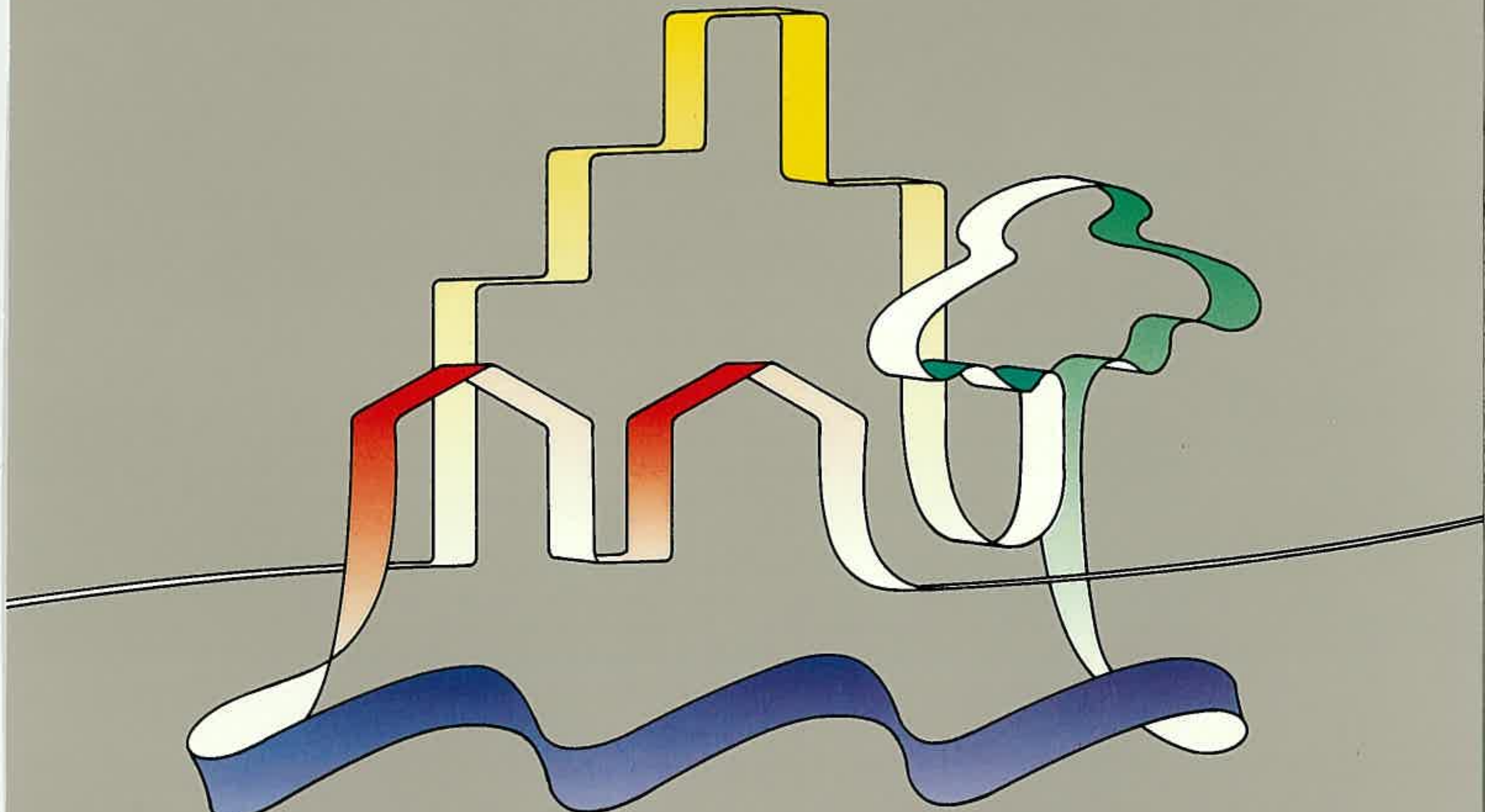
Urban Water Research Association of Australia

Tracer Studies using Bacteriophage to predict the Fate of Viruses in the Marine Community : Preliminary Assessments



Research Report No. 54

URBAN WATER RESEARCH ASSOCIATION OF AUSTRALIA

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Tracer Studies using Bacteriophage to predict the Fate of Viruses in the Marine Community : Preliminary Assessments

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FOREWORD

This report is based on UWRAA Research Project No SS-47: 'Tracer studies to predict the fate of viruses in the marine community' which was undertaken during the period February 1990 - July 1992. Organisational responsibility for the project was as follows:

Sponsoring Authority	:	Geelong and District Water Board, Victoria
Project Officers	:	Mr Ian M Lowther and Mr Peter H Ashton, Geelong and District Water Board
Research Agency	:	Department of Biological Sciences, Deakin University, Geelong
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SUMMARY

The fate of material disposed via ocean outfalls in the marine environment is important when determining the effectiveness and efficiency of an ocean outfall and the assimilative capacity of the marine environment, to which, the effluent is discharged.

Although routine monitoring and mathematical modelling can assist in predicting the fate of the disposed material, recently developed culturing and monitoring techniques for bacteriophage enable tracer studies to be carried out, which can greatly enhance the field verification of the fate of organisms in the real environment.

The bacteriophage of *Serratia marcescens*, a phage of *Bacillus sphaericus* isolated from compost, and two phages of *Eschericia coli* were successfully cultured under laboratory conditions. All phage grew well to a relatively high titre ($>10^9$ PFU mL^{-1}), and were easily counted using overlay techniques on solid media.

This research has successfully developed suitable methods for using non-pathogenic organisms similar in size to human enteric viruses, to simulate the fate of viruses in the marine environment and to track their presence along coastline beaches.

The methods adopted also allow relatively accurate calculations to be made on the initial dilution of marine outfalls in the surface waters above. The bacteriophage can also be tracked over a number of days to study the subsequent dilution and dispersion characteristics of the marine receiving waters, with time.

INTRODUCTION

The fate of material disposed in ocean outfalls is one of the most important factors to be considered in determining the efficiency of plant operation. Although routine monitoring and mathematical modelling can assist in predicting the fate of disposed material, tracer studies can greatly enhance the field verification of these efforts. This is particularly so in predicting the fate of enteric viruses in raw sewage in the marine environment.

Most tracers currently available (such as surface radar, floats, dyes and radioactive isotopes; Wimpenny, 1977) have inherent disadvantages which may prevent their use in certain environments, or inhibit their application on a large scale. For example, surface radar is expensive to establish and maintain, whilst floats, although inexpensive, only indicate water flow at a particular point. Dyes, which have been extensively used in tracing outfalls, are only suitable in waters of sufficiently high clarity to allow their detection, and may be rapidly degraded in sunlight. Handling, containment and monitoring of radioisotopes requires considerable experience, combined with expensive equipment.

In recent years, the use of micro-organisms has been seen as an inexpensive, yet practical alternative to the more traditional means of tracing the fate of material disposed *via* outfalls (Drury & Wheeler, 1982). Micro-organisms are relatively easily grown in laboratory culture, and distinguishing characteristics of organisms used as tracers, such as antibiotic resistance, pigmentation, or the need for specialised growth conditions, make them readily identifiable amidst what may be an enormous collection of organisms in field samples. The following criteria should be met by micro-organisms if they are to be used as tracers in aquatic environments (after Drury & Wheeler, 1982; Wimpenny *et al.*, 1972):

* *They should be non-pathogenic.*

* *They should not be present in the water body, or at least should be readily distinguishable from indigenous organisms. When it is desirable to use several tracers at once, they should be readily distinguishable from each other.*

* *They should be stable in the environment during the sampling period.*

- *They should not be affected by the water type in which the tracing exercise is to be performed.*

- *They should move with the water, and not be filtered, adsorbed or otherwise removed.*

- *They should be capable of being easily assayed in low concentrations using inexpensive methods.*

- *They should not interact with other organisms to produce changes in number, or changes in the organism's properties.*

In the past, yeasts, antibiotic resistant bacteria and bacterial spores have been widely used as tracers (Drury & Wheeler, 1980). However, some problems have been experienced in their use (see also Godfree, undated): for example, yeasts, because of their common occurrence in waters, have been found to be the least effective of the biological tracers. Antibiotic resistant bacteria require specialised media for their growth, and bacterial spores require germination before incubation. It has been suggested that bacterial viruses, known as "bacteriophage" or "phage" may offer a better alternative. The use of environmentally rare bacteriophage as tracers has many advantages, which may make them particularly suited to studies of ocean outfalls (Godfree, undated). These advantages include the following.

- *The detection of bacteriophage is highly sensitive, to levels as low as one plaque forming unit (PFU) per 100mL.*

- *The organisms can be rapidly assayed in the laboratory.*

- *Phage are highly selective in their host relationships, and the presence of other phage do not interfere with the assay.*

- *Phage grow to high titres (as much as 10^{14} mL^{-1}), and can thus be added to the effluent source in large numbers, making sensitive tracing an easier task.*

- *Phage have been shown to be non-infectious to living organisms apart from their host bacteria; therefore, they do not pose a public health risk and they do not pose a pollution threat to the environment into which they are added.*

*	*Bacteriophage have a buoyancy similar to water, and because of their size, they mimic the movement of enteroviruses in water.*

*	*Two or more phage, specific to different host bacterial species, can be used simultaneously as tracers without affecting each other.*

Several bacteriophage have been successfully used as semi-conservative tracers of water movement in both small- and large-scale exercises. These organisms are non-pathogenic, and the methods used in their cultivation are simple and rapid. The organisms have been used in clear, turbid or polluted waters, and are well suited to both qualitative labelling of potential or actual pollution, and to the quantitative description of water transit and dispersion (Drury & Wheeler, 1982). In addition, the organisms can be stored indefinitely in the laboratory, thus enabling tracer studies to be undertaken at short notice. The low cost of the surveys, and the speed and simplicity of the analyses, make the use of bacteriophage a practical and often preferable alternative to existing procedures.

Phage-tracer experiments have been conducted in a number of studies. For instance, phage of *Serratia marcescens* and *Staphylococcus aureus* have been used to index human enteric viruses in sewage (Carstens *et al.*, 1965; Weber-Schutt, 1965 in Godfree, undated) and commercially available strains of *Serratia marcescens* phage (NIOB 10645) have been evaluated as sewage tracers in a marine harbour (Drury and Wheeler, 1982). Purdy *et al.* (1986) assessed 5 phage of *Bacillus* sp. for their sewage tracing abilities in coastal outfalls,. and Sinton & Ching (1987) evaluated two bacteriophage, Phage 80 of *Staphylococcus aureus* and P2-like phage (FMWD1) of *Escherichia coli* as sewage tracers in a river system.

The principle objectives of the present project were to develop the use of suitable bacteriophage as tracers of sewage disposal operations currently carried out by the Geelong and District Water Board at Black Rock, Victoria, and to investigate the possible use of the organisms as an index of human virus survival and transport in locally disposed sewage.

BACTERIOPHAGE

Most work conducted with bacteriophage tracers has focused upon phage of *Serratia marcescens*, *Escherichia coli* (the so-called "coliphage"), and phage of the species *Bacillus*. In the course of this study, several phage were screened in the laboratory for their potential as tracers. The following were selected for further study:

1. Two phage of *Escherichia coli*, T4 and λ "small plaque" (λsm), obtained from the collection maintained by the Department of Biological Sciences, Deakin University (Charlton, 1990).
2. A phage of *Bacillus sphaericus* (named AF16), isolated from compost (Currie, 1990).
3. Bacteriophage NCIMB 10645, and its host bacterium, *Serratia marcescens* (NCIMB 10644), purchased from the National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland. This phage has previously been reported as having considerable potential as a microbial tracer of aqueous environments (Drury and Wheeler, 1982; D.J.H. Phillips, Acer Environmental, U.K., personal communication).

GROWTH AND ENUMERATION OF PHAGE

For the growth of *Escherichia coli* and its phages, and for *Bacillus sphaericus* and its phage, nutrient agar (Oxoid CM3) was prepared by adding 28g nutrient agar L^{-1} of distilled water in a sterile covered vessel, followed by autoclaving at 121°C and 100 kpa for 15 min per vessel. Unless otherwise specified, all sterilisation procedures in this project were carried out under these conditions. Agar was poured into 90 x 14mm sterile Petrie dishes, and allowed to set. Nutrient agar overlays were prepared from nutrient agar (Oxoid CM3) by adding 14g nutrient agar L^{-1} of distilled water. Approximately 5 mL of the resulting solution was then placed into sterile McCartney bottles, capped and autoclaved. When the overlays were required, they were autoclaved for 1min to melt the agar, and placed in a 45°C water bath to keep them molten.

Nutrient broth (Oxoid CM1) was prepared by adding 13g nutrient broth L^{-1} of distilled water in a sterile vessel; for small scale growth of phage, 9mL aliquots were distributed into McCartney bottles using a BSL Multivol-2000, and the bottles were capped and autoclaved. For larger-scale growth of phage, greater volumes were dispensed into appropriate-sized containers (e.g. 250mL Shott bottles). Isotonic saline solution was prepared from tablets (Oxoid BR53) according to the manufacturer's instructions, and 9mL aliquots were distributed into McCartney bottles, which were capped and autoclaved.

Stocks of phage were prepared by adding an appropriate phage inoculum (1% v/v) to a 3h log phase culture of host in nutrient broth; incubation was typically performed at 37^{o}C for a further 6h. Based upon predetermined burst times, it was assumed that all bacteria infected would lyse within this period. Cultures were then placed in a Clements GS100 centrifuge for 20min at 3,500 RCF (relative centrifugal force). Supernatents were decanted into suitably marked sterile McCartney bottles, along with 2 drops of chloroform to lyse any residual bacteria.

Phage were assayed in the following fashion. A 1mL aliquot of either homogeneous phage culture, infected bacterial culture or test sample was serially diluted to at least 10^{-8} in isotonic saline solution. Each 10-fold dilution was plated out using the pour plate (overlay) method. A 1mL aliquot was added to a nutrient agar overlay, along with 0.1mL of an overnight culture of the host bacterium. The overlay was gently mixed and poured onto a nutrient agar plate. Plates were incubated overnight, and plaques (zones of clearing in the host bacterial lawn, indicating growth of individual phage) were counted.

Serratia marcescens phage was grown under the conditions specified by Drury and Wheeler (1982).

TESTS FOR PHAGE PRESENCE IN ENVIRONMENTAL SAMPLES

In order to be effective tracers, phage should not be naturally present in the test environment. Phage presence was tested in a number of water types from various locations. Initially, trials were conducted in an artificial pond system on the campus of Deakin University, Geelong, where trials of the phage-tracer system were planned. Further trials were conducted using sewage from the Black Rock sewage treatment plant (BRSTP) and adjacent receiving waters.

Samples of sewage were collected by lowering a 5L plastic bucket at the base of the Archimedes screw pump (pre-treatment), and removing a sample; similar aliquots were removed from the entrance and exit of the grit tank after fine screening, as well as the shoreline adjacent to the disused intertidal outfall. Samples were transferred into a sterilised 4L Winchester flask immediately following collection and held at 4°C until analysis. Receiving water samples were obtained from a boat by lowering a sterile 2L container over the side. Sites sampled included: directly over the diffuser, 600m offshore from a point 1km west of the diffuser, 800m offshore from Point Flinders and 1.2km offshore from a site 3km west of the diffuser, near Point Impossible.

In the case of the coliphage, samples of Deakin pond water were assayed for susceptible phage by first centrifuging 9mL water samples at 3,500 RCF for 15min, and subsequently adding 1mL of supernatent and 0.1mL log phase host bacterium to nutrient agar overlays. After the overlays were poured and allowed to set, plates were incubated at 37°C overnight. This procedure was performed in quadruplicate. Assays of sewage and receiving waters were performed in a similar manner. In all cases, controls of phage only, host only and sample only were assayed. Results of the assays are shown in Table 1.

Table 1: Mean concentrations of coliphage (PFU mL^{-1}) in environmental test samples.

SAMPLE TYPE	PHAGE λsm	PHAGE T4
Deakin Pond Water	n.d.	n.d.
Sewage: *pre-treatment*	53	40
Sewage: *entry, grit tank*	45	20
Sewage: *exit, grit tank*	63	38
Old Intertidal Outfall	n.d.	n.d.
Over Diffuser	50	100
800m Offshore, Barwon Heads Bluff	n.d.	n.d.
1km West, 600m Offshore	n.d.	n.d.
3km West, 1.2km Offshore	n.d.	n.d.

n.d. = not detected.

Similar tests were conducted to identify the presence of *Bacillus sphaericus* and *Serratia marcescens* phage. In each case, none were detected in any environmental sample, and both of these phage were thought to be suitable for further assessment as tracers. Coliphage were not detected in Deakin pond water; nor were they found in receiving waters distant from the BRSTP. Low concentrations, in the range 20-100 PFU mL^{-1} were found, however, in samples of sewage and receiving waters directly above the diffuser, some 1.2 km offshore from the treatment complex. Coliphage such as λsm and T4, when used as tracers, would be added to an effluent stream in higher concentrations than those found in this study, and it was believed that they could still be successfully utilised as tracers at the BRSTP. Nonetheless, the other phage tested are more likely candidates for routine tracing studies of sewage effluent, as they are not naturally present. Coliphage may be more suited to other situations where they are not normally found as inhabitants.

SURVIVABILITY TESTS

A. Coliphage

A series of tests were undertaken to assess the survivability of potential tracer phage under conditions which may be experienced in receiving water environments. In the case of coliphage, three tests were conducted:

1. *Survival tests overnight at 16°C in nutrient broth; isotonic saline; 1.75% and 3.5% NaCl; and in a mixture of 2g slurry and 9mL nutrient both.*

The slurry used in these tests to simulate sediment and particulate matter in environmental samples consisted of 45% detritus, 45% mud and 10% water. To each duplicate sample, 1mL of either T4 or λsm phage stock was added, to produce a final concentration of 5.3×10^7 PFU mL^{-1}(λsm) and 6.9×10^7 PFU mL^{-1} (T4). Incubations were carried out at 16°C and duplicate 1mL samples of each experimental tube were assayed against the appropriate host bacterium following incubation. Results indicated a slight, but significant drop in phage numbers (Student's T Test, $p<0.05$) during the test: average survival of λsm was 1.2×10^7 PFU mL^{-1}, and for T4, 3.5×10^7 PFU mL^{-1}. A two-way analysis of variance revealed no significant differences between treatments. The success of these tests prompted further studies using a variety of environmental media.

2. *Survival tests over 14 days at 16°C in autoclaved seawater (collected from Eastern beach, Geelong); seawater and slurry; river water (obtained from the Barwon River); river water and slurry; Geelong tap water and a 50:50 mixture of tap water and seawater.*

The slurry used was of the same consistency as that detailed in (1) above, and 2 g slurry was added to 9mL of aqueous media in a McCartney bottle. A total of 56 replicates of each sample was made, and 28 of each were inoculated with either a 1mL aliquot of the phage T4 stock to produce a final concentration of 9.0×10^8 PFU mL^{-1}, or 1mL of phage λsm stock to produce a final concentration of 2.6×10^8 PFU mL^{-1}. Incubation was carried out at 16°C. Duplicate samples were removed every 24h for 14 days, and assayed against appropriate host bacteria. Assays for samples containing slurry were carried out on the supernatent alone, or following shaking of the containers in order to obtain an indication of phage bound to particles. Results of incubations after the 14 day period are shown in Table 2.

Results indicated a significant drop in phage λsm numbers after 14 days in all water types. A two way analysis of variance indicated a significant difference between the survival time in days and the various media tested. Largest decreases were observed in slurry-containing media. The results suggested that slurry (i.e. the presence of particulate organic matter) was more detrimental to phage viability than water type. Nonetheless, phage survival was the same order of magnitude (10^8) as the original stock, an extremely encouraging sign for a tracer which would be added to an effluent in relatively large numbers.

Similarly, phage T4 showed a significant decrease in numbers over the 14 day period, but again the decrease was less than an order of magnitude. For both phage types, the greatest decrease in viability occurred within the first 24h period. Again, results for both phage were encouraging, and further testing in sewage was carried out to determine if the phage would be suitable for use as a tracer in this medium.

Table 2: Mean phage survival (x10^8 PFU mL^{-1}) after 14 days at 16°C in various media. Assays of samples containing slurry were performed on the supernatent alone, and after mixing (see text).

SAMPLE TYPE	PHAGE λsm	PHAGE T4
Initial Phage Count	2.6	9.0
Seawater	2.0	8.75
Seawater & slurry	1.3	1.9
Stirred seawater & slurry	1.2	3.5
River water	2.3	5.5
River water & slurry	1.6	5.3
Stirred river water & slurry	1.0	8.4
Tap water	2.2	4.1
Tap water & seawater (50:50)	2.0	3.1

3. *Survival tests over 7 days at 13°, 16° and 22°C in post-settling tank sewage samples from the Black Rock Treatment Plant.*

Samples of sewage effluent were collected from the exit point of the grit tank at the BRSTP as previously described. Aliquots (10mL) were transferred into 84 sterile McCartney bottles, and to each of 42 bottles was added 1mL of phage T4 stock to produce a final concentration of 3.0x10^9 PFU mL^{-1}. To the remaining bottles, 1mL of phage lsm stock was added to produce a final concentration of 2.8x10^9 PFU mL^{-1}. A total of 14 bottles of each phage type was placed in water baths in a cold room at each temperature. After 24h, duplicate samples at each temperature were assayed against appropriate host bacteria. This procedure was repeated over 7 days. After day 2, an aliquot (50 mL) of chloroform was added to the assay samples to prevent background bacterial growth. Results are shown in Table 3.

Results indicated a significant decrease in phage T4 numbers at all temperatures during the test period. As was previously seen in tests involving other media, although the decrease in numbers was statistically significant total numbers remained high (in the order of 10^8 PFU mL^{-1}) which would be sufficient to permit tracing work in the environment. The situation with phage λsm, however, was enigmatic: it appeared that either background phage concentrations may interfere with the test, or that the phage multiplied whilst in the sewage effluent, indicating that the host bacterium may be present. The latter seems unlikely given the normal temperatures of incubation (37°C) for *Escherichia coli*. However, the results were sufficient for us to decide not to utilise phage λsm in future trials involving sewage effluents.

Table 3: Mean survival of phage in effluent from the Black Rock Sewage Treatment Plant held at three temperatures for 7 days. Initial concentrations of phage were: λsm, 2.8×10^9 PFU mL^{-1}; T4, 3.0×10^9 PFU mL^{-1}.

TEMPERATURE	PHAGE λsm ($\times 10^9$)	PHAGE T4 ($\times 10^8$)
13°C	5.9	2.1
16°C	12	2.8
22°C	9.3	6.5

B. Phage of Bacillus sphaericus

The survival of the phage of *Bacillus sphaericus* was tested in the following media: sterilised artificial seawater, unsterilised BRSTP effluent, and unsterilised seawater collected from above the diffuser offshore from the BRSTP. Aliquots of phage stock (0.1mL) were distributed into McCartney bottles containing 9.9mL of the appropriate water type, resulting in a final phage concentration of 2.5×10^6 PFU mL^{-1}. Incubation was carried out at 13°, 16° and 22°C. Over a period of 6 days, triplicate bottles from each temperature were assayed for surviving phage. Results are shown in Table 4.

A two-way analysis of variance, performed on the results from each water type, showed a significant difference in phage survival in the three test waters. As a general rule, temperatures of 16° and 22°C had a greater effect on phage survival. Between water samples and temperatures, there was little difference in phage survival during the first 24 hours, suggesting that optimal performance in tracing exercises should be achieved within this period.

Table 4: Mean survival of *Bacillus sphaericus* phage (x10^5 PFU mL^{-1}) over 6 days at three different temperatures in three types of water. Initial phage concentration was 2.5x10^6 PFU mL^{-1}.

WATER ORIGIN	TEMP °C	DAY1	DAY 3	DAY 6
Diffuser	13	9.5	7.0	3.1
	16	14.2	2.1	1.1
	22	10.6	1.7	1.0
Effluent	13	10.3	7.7	1.8
	16	10.5	3.5	0.6
	22	1.2	1.8	0.7
Seawater	13	10.8	9.2	0.9
	16	7.1	0.7	0.1
	22	5.5	2.2	0.7

C. Phage of Serratia marcescens

A study of the persistence of *Serratia marcescens* phage in the environment has been undertaken by Drury & Wheeler (1982). This investigation used samples of autoclaved river and seawater, as well as samples designed to simulate sewage works conditions (final sewage effluent, mixed sewage liquor and autoclaved mixed liquor). A summary of these results is shown in Table 5.

Drury & Wheeler (1982) noted that the *S. marcescens* phage seemed well suited for survival in sewage. Sunlight appears to decrease phage persistence, but the authors felt that this would not be a significant factor in relatively deep, or murky environments. Significantly, the study showed that adsorption of phage to particulates did not appear to result in removal, as was shown in comparisons between bacteriophage densities in surface samples of stirred and unstirred sewage. Lower numbers in mixed liquors were accounted for by possible protozoal grazing. Sunlight (or UV light exposure) appeared to lower phage survivability, but Drury & Wheeler (1982) felt that the minimum T_{90} value of over 30h in bright sunshine was likely to be extended considerably under "normal operating conditions". Indeed, the authors demonstrated this fact in a large-scale trial of the phage in Poole Harbour (U.K.), in which phage compared extremely well with another microbial tracer, *Bacillus subtilis* spores.

Table 5: Survival of *Serratia marcescens* phage in river water, seawater and sewage (after Drury & Wheeler, 1982). Sewage was incubated in the temperature range 10-14°C, pH 6.9-7.4.

SAMPLE TYPE	EXPERIMENTAL CONDITIONS	R_{90}(h)	T_{90}(h)
River water (10°C)	Bright sunlight		31.8
	Darkness		338
River water (20°C)	Bright sunlight		35.5
	Darkness		328.5
Seawater (10°C)	Bright sunlight		35.5
	Darkness		766.8
Seawater (20°C)	Bright sunlight		32.8
	Darkness		104.5
Sewage effluent	Sunlight (stirred)	31	
	Darkness (stirred)	49.2	
	Darkness (unstirred)	97	
Mixed sewage liquor	Darkness (stirred)	12.7	
	Darkness (unstirred)	126.9	
Sterile mixed liquor	Darkness (stirred)	58.2	

R_{90} = that time required for 90% removal or inactivation of the phage, computed by extrapolation of the regression line of log (PFU $100mL^{-1}$) on time of exposure.

T_{90} = that time required for a 90% reduction in phage titre due to inactivation, computed by extrapolation of the regression line of log PFU $100mL^{-1}$ on time of exposure

Drury & Wheeler (1982) concluded that phage of *Serratia marcescens* provided an excellent choice as semi-conservative microbial tracers of water movement. They believed that the phage could be used in small or large scale exercises, in clear, turbid or polluted waters. For these reasons, this phage was selected as a suitable candidate for future trials in Australian situations.

FIELD TRIALS OF PHAGE

A. *Small-Scale Trials*

Initial field trials of phage were conducted in a small pond system on the campus of Deakin University, Geelong. The ponds, which include a small fountain, are located in a central courtyard and contain water which is continuously recirculated. The upper-most pond is approximately 8 x 8m, and contains the fountain; it is connected *via* a spillway to a second, lower rectangular pond, which is approximately 20 x 4m. This pond is in turn connected by a spillway to a third, smaller (approximately 4 x 4m) pond; from here, water is pumped back to the uppermost pond. All ponds are approximately 600mm deep.

In the first trial conducted, bacteriophage T4 was grown in liquid culture in a 250mL Schott bottle. The resultant liquid was centrifuged, and the supernatent assayed (titre, 3.0×10^{10} PFU mL^{-1}). An aliquot of supernatent (100mL) was placed in a sterile 125mL Schott bottle, and its contents poured into to the uppermost pond; samples (10mL) were subsequently collected at fixed sites in all ponds every 5min for 1.5h. Phage were detected in these samples using the agar overlay technique previously outlined.

Phage were first detected at the sampling site in the upper-most pond 5min after introduction. Concentration peaked at this time at 5×10^4 PFU mL^{-1}. At the sampling site in the second pond, some 15m distant, phage were first detected 25min after introduction (3×10^4 PFU mL^{-1}); at the site in the third pond, maximum detection occurred at 45min (1×10^4 PFU mL^{-1}). The experiment indicated that phage movement could be sensitively traced through the ponds, and that there was considerable potential for larger-scale trials.

A further small-scale trial in the pond system, conducted in the same manner, was carried out using phage of *Serratia marcescens* (supernatent titre, 1.0×10^9 PFU mL^{-1}). Phage were again detected at the sampling site in the upper-most pond 5min after introduction; numbers of phage peaked at 2.0×10^4 PFU mL^{-1}. At the sampling site in the second pond, phage were first detected 35min after introduction (2.5×10^4 PFU mL^{-1}). In the third pond, detection occurred at 45min (1.5×10^4 PFU mL^{-1}). The experiment confirmed previous work of Drury & Wheeler (1982) regarding the potential of this phage as a tracer of water movement.

B. *Large-Scale Trial*

Following the success of the trials in the small pond system, a large scale, semi-quantitative trial was attempted at the Geelong and District Water Board's Black Rock sewage treatment plant utilising phage T4. The Geelong and District Water

Board (GDWB) is responsible for the collection, treatment and disposal of domestic and industrial wastewaters from the urban city of Geelong and surrounding coastal townships (see Figure 1). The region includes an area of approximately 160 km^2 and serves a population of 165,000 people, or about 87% of the total population of the district. Wastewater is currently discharged *via* a subtidal outfall over a 100m distance (diffuser), in an average depth of 15m. Prior to discharge, the effluent passes through a fine screening plant (0.5mm) which was designed to remove all floatable material, grit particles >0.2mm equivalent diameter, and visible floating oil and grease. The system discharges an approximate mean of 55 ML day^{-1}; during the time of the phage trial, discharge was at the rate of 400 L sec^{-1}.

This phase of the phage tracer research was undertaken with a number of key objectives in mind:

- *To enable an assessment and calculation of the initial dilution of the outfall surface waters;*

- *To gauge the subsequent dilution of the effluent plume away from the outfall surface waters;*

- *To track the phage in either direction (east or west) along the coastline over a number of days; and*

- *To assess whether the technique could prove useful in predicting the short-term fate (with respect to location) of organisms such as viruses in the marine environment.*

It was also decided that this trial should include dye tracing, in order to gauge the time taken for the phage to pass through the pipeline from the treatment plant to the diffuser some 1.2km offshore. The trial was initiated on the morning of 5 September, 1991; the weather was fine and sunny, with a light 5-10 knot northerly wind blowing, and a swell of 0.5-1.0m. Low tide occurred at 1:00 pm.

Phage were grown in liquid culture using the methods previously described. A total of 10L of phage (titre, 1.0x10^9 PFU mL^{-1}) was emptied into the drop structure after the grit tanks at the BRSTP at 9:35am. Personnel undertaking the phage dosing were not involved in any of the subsequent sampling or sample handling and analysis, thus eliminating any possibility of cross contamination. Five minutes prior to the dosing, approximately 2kg of sodium fluorescein dye was dosed in concentrated powdered form into the grit tank weir. The appearance of the dye offshore allowed personel in the boat to prepare for the initial sampling of the phage on the surface waters above the pipeline.

The dye was first observed in the open ocean at 10:50 am, forming an approximately 30m circular spread in an area midway between the end of the diffuser (1,200m from the shore) and the 1,100m mark above the pipeline (both points were marked by buoys). The iridescent dye plume was easily seen, and its spread over time was plotted by surveyors onshore. The plots (see Figure 2.) show that the plume spread in a circular fashion across the ocean surface for about 1.5 hours, until the influence of the changing tide caused a reduction in the rate of spread. Once the tide changed (after 1:30 pm), the plume quickly divided into two and began spreading in both easterly and westerly directions. The plume was barely visible after 3:00 pm.

Whilst surveyors onshore were tracking the plume, analysts in a boat offshore were taking surface samples at five locations every half hour for the first 1.5h, and in two locations thereafter. The first of the samples was always taken at the surface in the middle of the plume (location #1, see Table 6); the remaining samples were taken on the north, south, west and east boundaries of the plume as it spread over the ocean surface. The sampling protocol was established to allow calculation of phage dilution over time. The samples taken from the boat were collected in sterilised 500mL Shott bottles, which were carefully lowered from the side of the boat using a long pole. All samples were refrigerated prior to analysis. Samples were assayed (3x1mL subsamples) using the agar overlay technique previously described. Results of the analyses are shown in Table 6.

Table 6: T4 Phage Counts over the Black Rock Outfall in Bass Strait during the trial conducted on 5th September, 1991

Sample Number	Time Taken	Location	Phage Counts (PFU 100mL^{-1})
1	1030	1	0
2	1055	1	55300
3	1057	2	8900
4	1059	3	4300
5	1101	4	400
6	1103	5	1100
7	1130	1	600
8	1131	2	9000
9	1132	3	560
10	1133	4	5400
11	1134	5	4700
12	1200	1	200
13	1201	2	2500
14	1202	3	800
15	1203	4	5500
16	1204	5	600
17	1230	1	200
18	1231	2	1500
19	1232	3	200
20	1233	4	5700
21	1234	5	300
22	1330	4	9500
23	1332	5	8000
24	1430	4	200
25	1432	5	9700

Locations:		
	1.	*Centre of dye plume*
	2.	*North edge of plume*
	3.	*South edge of plume*
	4.	*West edge of plume*
	5.	*East edge of plume*

Figure 1.

Black Rock Sewerage Scheme And Surrounding Area

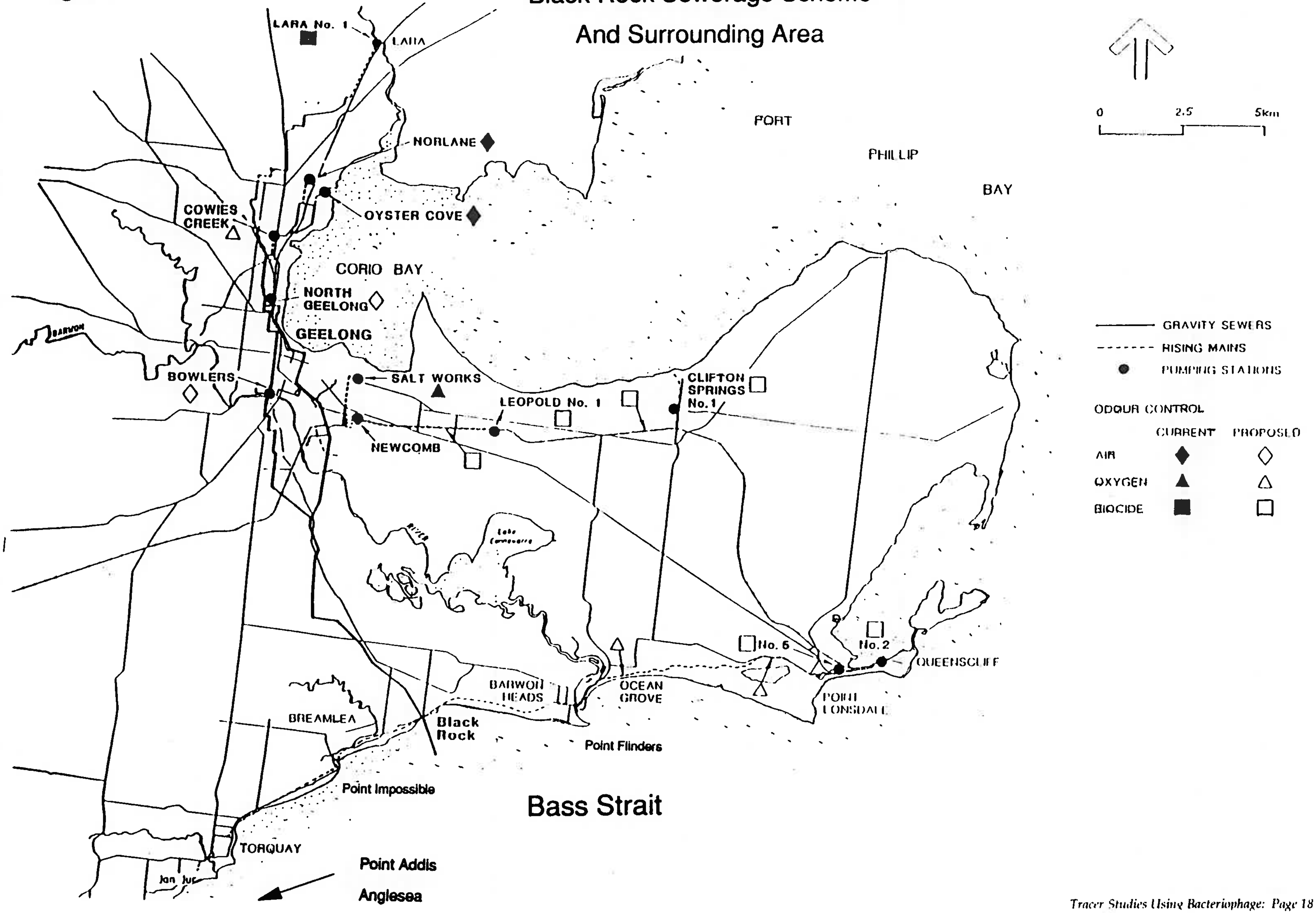
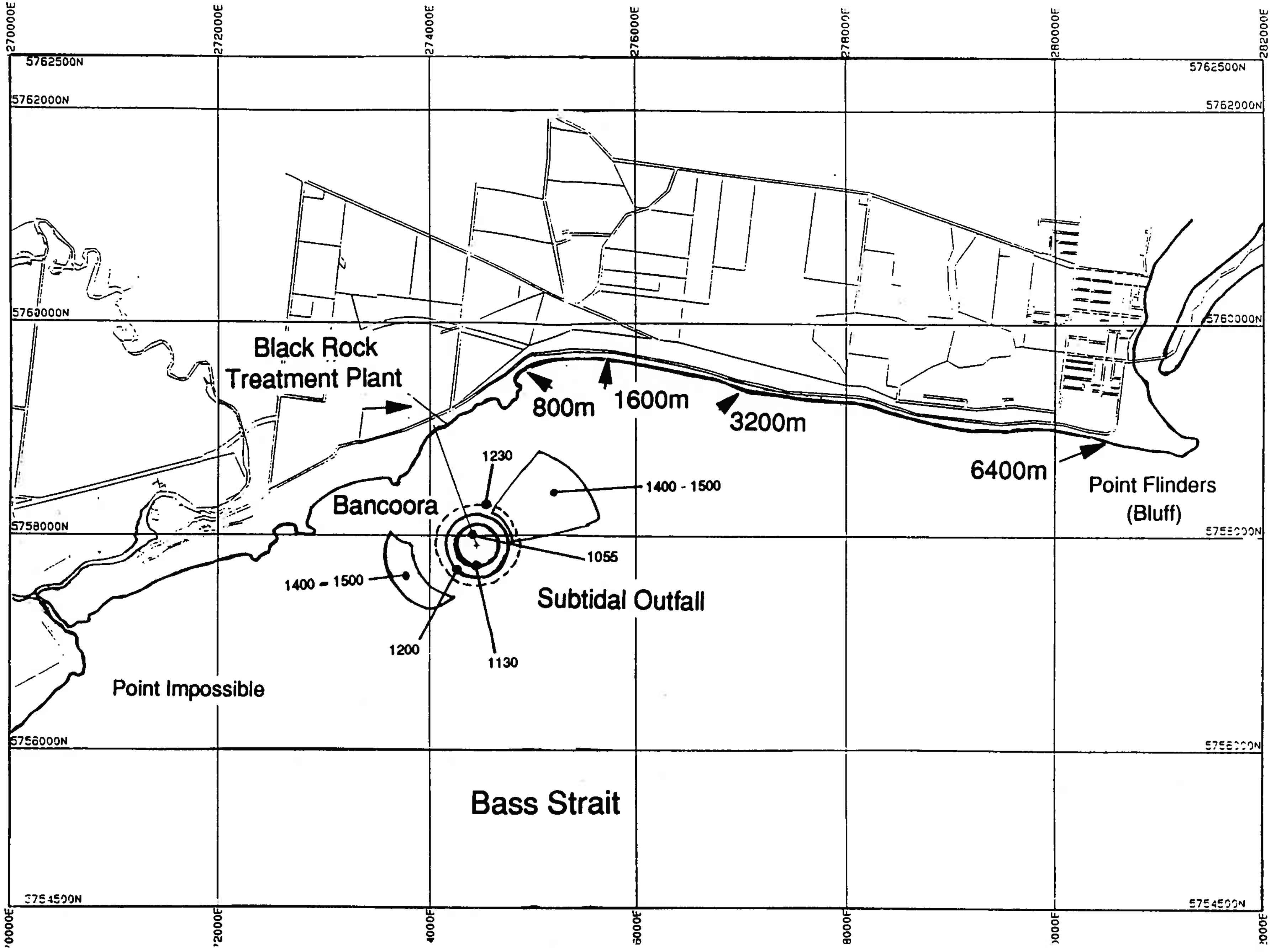


Figure 2.

Fluorescein Dye Spread During Phage Trial



In order to evaluate the longer-term fate of the phage, samples were taken at eight locations along the coastline on the 6th September, the day after dosing, and at eleven locations for the following five days (see Figures 1 & 2). This research concentrated upon tracking the phage along the coastline, and thus whether the phage persisted in the shoreline recreation waters, and could be used as an indicator of the fate of viruses in marine waters. The results of these analyses are shown in Table 7.

Table 7: Phage Counts in shoreline samples (PFU $100mL^{-1}$)

Sample Location	**Sampling Dates**					
	6/9/91	**7/9/91**	**8/9/91**	**9/9/91**	**10/9/91**	**11/9/91**
Point Addis	-	0	0	0	0	0
Torquay Beach	0	0	0	0	0	0
Point Impossible	0	0	0	0	0	0
Bancoora Beach	0	0	0	0	0	0
800m East	80	0	0	0	0	0
1600m East	100	0	0	0	0	0
3200m East	50	0	0	0	0	0
6400m East	0	0	0	0	0	0
Ocean Grove	0	0	0	0	0	0
Collendina	0	0	0	0	0	0
Point Lonsdale	-	0	0	0	0	0

N.B. *Conditions during the five day sampling period varied significantly, from northerly to southwesterly winds, and from fine sunny conditions to overcast with rain. Seas were slight over the entire period.*

Phage first appeared in the centre of the dye plume at 10:55 am, and closely mirrored the circular movement pattern of the dye for the period whilst it was visible. An estimate of the sewage dilution in the receiving waters above the diffuser was made, based upon phage counts in the area, using the following simple formula:

$$D = \frac{V.c.}{t.F.d_f.x}$$

where:

D = Initial dilution
V = volume of phage dosed (L)
c = concentration of phage used in dosing (PFU L^{-1})
t = dosing time (assumed to be 1sec)
F = plant flow (L sec^{-1})
d_f = dilution factor in drop structure & pipeline (assumed to be 500)
x = number of phage sampled at surface (PFU L^{-1})

Results indicated a better than 90:1 dilution at the surface above the pipeline. This value is in agreement with dilution studies undertaken at Black Rock under similar conditions using chemical tracers.

The results in Tables 6 and 7 can also be used to assess the rate of movement of the plume away from the outfall area, and subsequent surface dilution over time. Samples taken in the centre of the dye plume (location #1) were *always* taken in the same position and these results may be used to assess how quickly the phage disappeared. The initial counts of 5.5×10^5 PFU L^{-1} fall to 6.0×10^2 PFU L^{-1} within 30 minutes (i.e. approaching background levels; see tests for phage presence). The results shown in Table 7 indicate that phage were detected in only three locations along the coastline during the 6 days of sampling. This indicates that the ocean environment is very dynamic and relatively efficient in its dilution capacity. Further research in this area is required to accurately quantify phage dispersal, particularly if the technique is to be used to indicate the relative dispersal of human viruses contained in sewage effluent. Nonetheless, T4 phage are similar in size to enteroviruses, and hence likely to be good predictors of the fate of pathogenic viruses in the water column.

CONCLUSIONS

As a result of this project, a bacteriophage of *Serratia marcescens* from the NCIMB collection, a phage of *Bacillus sphaericus* isolated from compost, and two phage of *Escherichia coli* were successfully cultured under laboratory conditions. All phage grew well to a relatively high titre ($\geq 10^9$ PFU mL^{-1}), and were easily counted using standard overlay techniques on solid media.

Experiments were conducted on environmental samples to determine whether the selected phage occurred naturally in these waters. Low counts of coliphage were present in sewage samples, but neither of the other phage were found, indicating the potential of these organisms as tracers. In the case of the coliphage, indigenous numbers were low enough to permit the future use of the organisms as tracers.

Phage were tested for their survival over extended periods in several types of water samples, including sewage taken from Black Rock sewage treatment plant (BRSTP). All phage showed no serious decrease in viability over a 48 hour period (a duration long enough to undertake field experiments). Indeed, survival over longer periods was also high.

Small-scale field trials were conducted in a pond system on the campus of Deakin University. These trials indicated the relative ease with which the organisms could be used as tracers. A large scale trial at the BRSTP indicated the potential utility of phage in tracing water movements adjacent to an ocean outfall in a dynamic environment, particularly in relation to the fate of enteroviruses discharged in sewage.

We propose to develop, in the future, methods for the production of phage stocks to high titres (i.e. approximately 10^{14} PFU mL^{-1}). To date, we have prepared phage stocks by infection of bacterial hosts in aerated, shaken flasks. However, it is difficult to obtain yields greater than 10^{10} PFU mL^{-1} by this method. We have recently obtained protocols (from Acer Environmental, U.K.) for the rapid preparation of high yields of phage (i.e. up to 10^{14} PFU mL^{-1}), and methods for harvesting and storage of the phage. Implementation of these methods will allow:

(1) Preparation of high titre stocks, which would permit high rates of dilution during tracer studies;

(2) Storage for long periods, thus permitting tracer studies to be carried out with minimal delay.

We are also investigating improved methods for growing phage from samples taken during field studies. These methods involve the use of the bacterial host grown as a "lawn" on a filter pad, which would adsorb phage filtered from field samples allowing easier preparation and more efficient sample analysis following tracer studies (Purdy *et al.*, 1984). The net result of the studies, we believe, will be a more rapid, efficient and cost effective means of using bacteriophage as tracers in aquatic environments.

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