Virus in sewage, polluted water and molluscan shellfish: A critical assessment of coliphage as a possible indicator organism

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CONTAINS PULLOUTS

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ABSTRACT

This thesis begins with a comprehensive review of bacterial indicator systems and, by drawing on published material related to the transmission of enteric viral disease in the marine environment, shows that bacterial indicators are inadequate for assessing risks from viruses. Literature in respect of coliphages has also been reviewed as an introduction to the experimental section of this present study, which looks at some of the factors influencing bacteria, viruses and phage in sewage and the marine environment.

This study has thrown new light on the survival of coliphage and bacteria and has assisted in showing for the first time the interrelationship between factors thought to contribute to viral inactivation. It is evident that adsorption is the major factor involved and that this applies to bacteria and coliphage as well as viruses. Other factors such as salinity, temperature and pH may exert an influence by increasing or decreasing adsorption processes.

It is concluded that there are potentially serious limitations in the use of DNA T-type phages as virus models and that, while coliphages clearly have useful applications, the use of the single-stranded RNA coliphages is to be preferred. As indicators of risk from viral pathogens coliphage do not conform reliably to the criteria demanded of an indicator. However, they may be usefully applied in conjunction with the continued use of an accepted bacterial indicator such as *E. coli*. Ultimately the best indicators for viruses may be the viruses themselves.

It is apparent from the review of literature and experimental work undertaken for this present study that many questions remain unanswered. Suggestions have been made as to some priorities for future studies which it is envisaged would make a significant contribution to our knowledge of viruses in the marine environment.

Explanatory notes of terms symbols and abbreviations used in the text

ASW	Artificial sea water
PFU	Plaque-forming unit (tissue or cell culture)
$*TCID_{50}$	Tissue culture infective dose ∿ 50% of cell cultures showing virus activity. May be prefixed at * by dilution used
T_{90}	Time taken to achieve 90% reduction in original count or titre
u/v	Ultra-violet (light). Normally referring to germicidal wavelength around 2540 Å
↓	Arrow on symbols used on figures. Indicates that count below that indicated but not necessarily equal to 0. Usually marks the limit of sensitivity of technique employed

1. INTRODUCTION

In England and Wales there are no statutory standards for the quality of sewage effluents discharged into tidal waters and therefore some may fall well below the standard of similar discharges to fresh waters, which are covered by the Clean Rivers Act. Estuarine waters which are the major centre of shellfish production (the most important species exploited being oysters, cockles, mussels and clams) may be especially affected. Apart from the biological and physical effects of sewage on these organisms, e.g. smothering, reduction in growth or reproductive ability, they are all filter feeders and so effectively remove suspended particulate matter from their environment. In areas subjected to sewage pollution this particulate material may include large numbers of bacteria of faecal origin which will also be ingested by filter-feeding molluscs. Not only will bivalve molluscs remove bacteria, but, more important, they have the ability to concentrate them far in excess of the levels found in the surrounding water. WOOD (1965) has demonstrated that at 16°C the European flat oyster (*Ostrea edulis*) may concentrate up to six times the number of faecal bacteria present in the surrounding water.

The bacteria contributed to the estuarine environment by sewage will consist largely of non-pathogenic types, members of the coliform group (particularly *Escherichia coli*); streptococci (Lancefield group D); Pseudomonads, etc. but may also include pathogenic types such as the Salmonellae, Shigellae and Clostridia. ALLEN, BROOKS & WILLIAMS (1949) showed that a coarsely settled effluent containing 2.9×10^4 faecal coli/ml still contained some 4.9×10^3 faecal coli/ml after receiving full sewage treatment. Therefore even in areas where both shellfish and amenity interests may be involved and effluents are subject to lagooning or other forms of tertiary treatment, large numbers of bacteria will still be liberated in the discharge. It has been tentatively estimated that

the probable ratio of pathogens such as *Salmonella* to *E. coli* in a 'typical' effluent may be in the order of 1:10 000. The risk is evident when one considers that some large outfalls may discharge in excess of 10 million gallons per day (MGD) of effluent into tidal waters.

In the early years of this century cases of typhoid and paratyphoid transmitted by the consumption of polluted shellfish were not uncommon and in fact have occurred sporadically even as recently as the early 1950s. However, just as there are no statutory standards for effluent discharges, there are no statutory standards for the sanitary quality of molluscan shellfish intended for human consumption. Public health and medical authorities have long been aware of the dangers of shellfish-transmitted disease and HOUSTON (1904) made some early practical proposals to remedy the situation. The Fishmonger's Company, the controlling authority for the shellfish and wet fish market at Billingsgate, London, have also been prominent in attempts to achieve a worthwhile standard. Even so it was not until 1951 that the Fishmonger's Company test of KNOTT (1951) was introduced, followed by the recommendations of SHERWOOD & SCOTT THOMPSON (1953) who compared KNOTT's method with the direct count roll-tube method of CLEGG & SHERWOOD (1947), and published information enabling a direct comparison of the various criteria in current use.

Since pathogens are greatly outnumbered in sewage by faecal organisms such as coliform bacteria, sanitary standards have generally been orientated towards assessing degree of faecal contamination by using these more numerous and easily enumerated 'indicators' of pollution levels. The inference being that if normal faecal coli are present in a given sample, it could also contain pathogens, albeit in smaller numbers. Conversely the absence of faecal organisms indicates that pathogens are also probably absent. Apart from the impracticability of

looking for pathogens routinely, the appearance of infection from typhoid for example might be as much as two weeks after contamination had occurred. In such a case suspicion would be aroused more on epidemiological grounds than as a result of bacteriological enquiry. Simpler, routine observations on *E. coli* levels, for example, would reveal that a particular source of samples was subject to sewage pollution, possibly containing pathogens, and thus giving a wider margin of safety. All the bacterial indicators in common use have limitations and these will be reviewed, but all were selected primarily as indicators of the possible presence of bacterial pathogens such as *Salmonella*. However, it has become increasingly evident in recent years that sewage also contains a variety of viruses, including known pathogens such as poliovirus, other enteroviruses and the agent(s) of infectious hepatitis. These may pose the same potential public health problems as bacterial pathogens since they are accumulated by shellfish and may be present in recreational waters yet cannot be reasonably assessed by use of accepted indicators or standards based on such indicators. The most dramatic demonstration of such a risk was the infectious hepatitis outbreak in India in 1955 when an estimated 30 000 to 50 000 people were affected by drinking contaminated water. The water concerned had been treated to a standard judged to be satisfactory by accepted methods, including bacteriological testing, and yet proved to be the vehicle of infection.

The object of this presentation is to propose a possible indicator for assessing viral risks and to examine its feasibility both from the author's experimental work and the published work of others. To add perspective to the proposal, current bacterial indicators will be reviewed, together with a broad survey of virus in sewage, polluted waters and shellfish. The science of virology has made rapid advances but the degree of sophistication and technology required for environmental

monitoring is likely to remain beyond the reach of all but a few highly specialized centres for some time to come and there exists a pressing need for a rapid routine 'virus indicator'. As a bacterial virus, coliphage combines similarity to animal virus with the ease of enumeration normally associated with the bacterial indicators. Potentially, at least, it is a strong candidate for consideration as a virus indicator in this field and is the indicator proposed in this present assessment.

2. REVIEW OF BACTERIAL INDICATORS OF FAECAL POLLUTION

2.1 Coliform group

Organisms of this group are the most widely used indices, not only in the United Kingdom but also in Scandinavia, Europe and the USA. The term generally refers to gram-negative, oxidase-negative, non-sporing rods capable of growing aerobically on an agar medium containing bile salts and able to ferment lactose within 48 hours at 37°C with the production of both acid and gas. Further tests to differentiate *Escherichia coli* from other coliform organisms are used in many European countries including the United Kingdom but North American standards for both shellfish and waters are based on coliforms only. Although this is current practice in the USA, recognition has been given to the merits of using either *E. coli* or, less specifically, faecal coli and it is likely that these will be adopted in preference to coliforms in the near future. *E. coli* will ferment lactose at both 37°C and 44°C, produces indole from tryptophan, is capable of utilizing sodium citrate as its sole source of carbon, is incapable of producing acetyl methyl carbinol (Voges Proskauer or VP test) and gives a positive methyl red reaction.

E. coli dies off quite quickly in sea water, therefore high counts generally indicate heavy and recent pollution, low counts, light, remote or recently past pollution. There is no satisfactory method of determining whether the *E. coli* isolated is of animal or human origin but its presence can be regarded as indicative of potentially dangerous pollution since the faeces of birds, rodents and domestic animals may contain *Salmonella* group organisms, including *Salmonella paratyphi* B. Other members of the coliform group may be of faecal origin but may also originate in soil washings or decaying vegetation. Interpretation of coliform data is therefore at best speculative and of value, generally, only when taken with simultaneous observations of *E. coli* levels.

The main advantages of both *E. coli* and the coliform group are the ease and rapidity with which they can be enumerated and identified, vital factors in the routine examination of samples where matters of public health are involved. *E. coli* has a similar mortality rate in shellfish and waters to that of pathogens such as *Salmonella* (KELLY & ARCISZ, 1954) and thus is an important indicator for risks of this type. Coliforms are of less value in this respect because of their more varied origins and greater resistance than many bacterial pathogens. SLANETZ *et al.* (1968) isolated *Salmonella* from both seawater and shellfish samples in areas which met the current US coliform standard for approved waters. In recent years, with improvements in methods of shellfish purification and sanitation generally, diseases such as typhoid have become less important in countries such as the United Kingdom and the USA and attention here has switched to the possibility that shellfish may act as vectors of viruses. Evidence (which will be reviewed later) suggests that viral particles are more resistant to sewage treatment and exposure to the marine environment than bacterial pathogens. This in turn has led to the suggestion that *E. coli* is not particularly suited as an indicator of this type of risk. Additionally there have been reports of instances where *E. coli* failed to act even as an indicator of bacterial pollution. SELIGMAN & REITLER (1965) cite a case of enteritis from well water, samples of which yielded several types of *Salmonella* but no or few *E. coli*. A similar case from California where an epidemic of *Salmonella typhimurium* was attributed to a water supply which yielded coliform counts in the order of 2 per 100 ml and less is cited by ROSS, CAMPBELL & ONGERTH (1966). A more recent paper by SAVAGE & HANES (1971) on the toxicity of sea water to coliform bacteria showed that under certain conditions (i.e. when the biological oxygen demand (BOD) of water was between 1 and 10 mg/litre) the coliform count

was not reduced and could not therefore be used as an indicator to assess risks from pathogens which were not similarly affected. This confirmed the earlier findings of SLANETZ & BARTLEY (1965) that coliforms and *E. coli* may even increase in waters enriched with sewage.

2.2 Faecal streptococci (Lancefield Group D)

This group includes a number of species found in both human and animal intestines, particularly *Streptococcus faecalis* and *S. faecium*. The former species is found in some animals but is generally more abundant in man and may therefore be of considerable value in detecting and confirming faecal pollution in circumstances where *E. coli* are absent but other, less specific, coliforms are present. Although faecal streptococci are generally less numerous in sewage than the coliform group they do not multiply in water and may survive longer than *E. coli* (BURMAN, 1961). There is a considerable body of evidence to show that they are more resistant to a wide range of conditions (see DEIBEL (1964) for a review) including marginal chlorination and may therefore be more suited as indicators of virus pollution. In broad terms many authors have suggested that faecal streptococci and faecal coli are better indicators than coliform bacteria (DUTKA, 1973; SLANETZ, BARTLEY & STANLEY, 1968) but DUTKA & BELL (1973) demonstrated that *Salmonella* were present in waters judged satisfactory by bacterial indicators including faecal streptococci. In applied situations the faecal streptococci or enterococci are widely favoured as indicators in the USA but since coliforms are used basically for routine purposes in that country this does little other than demonstrate some lack of confidence in standards and criteria based on coliforms alone.

There is however little evidence to suggest exactly how significant numbers of faecal streptococci are in relation to virus pollution and

more basic work is required particularly on methods of enumeration before this can be adequately assessed. WOOD (1965) demonstrated that faecal streptococci apparently multiply in shellfish stored above 11°C, which would virtually invalidate them as indicators for routine purposes. Other major disadvantages are the widespread distribution of these organisms (COOPER & RAMADAN, 1955; SMITH, 1965) and evidence which suggests that faecal streptococci may be isolated in areas far removed from any source of pollution (MUNDT, 1964). The use of a specific strain *Streptococcus faecalis (sensu strictu)* as an indicator may be more advantageous than the enumeration of faecal streptococci as a whole in that it may at least differentiate between human and animal sources (COOPER & RAMADAN, 1955).

Recent trends in the USA have suggested a useful role for faecal streptococci by comparing the ratio of these organisms to faecal coliforms to differentiate between human and non-human pollution sources. FEACHEM (1975) suggested that initially high faecal coli (FC):faecal streptococci (FS) values which fell with increased distance from source indicated human contamination. Initially low ratios which subsequently rose suggested a non-human faecal source. Observations on waste discharges and receiving waters by GELDREICH (1976) reported that human sources were characterized by FC:FS ratios of 4:1 or higher and those of other warm-blooded animals 0.6:1 or less.

2.3 Clostridium welchii (syn. Cl. perfringens)

The spores of this organism survive for much longer than many vegetative organisms and are usually resistant to chlorination. Its main value as an indicator lies in its extended survival since it can be used to demonstrate pollution on some past occasion when examination for *E. coli* and other indicators may prove negative. In this way it

may give valuable warning of pollution before it reaches the potentially dangerous levels that can be demonstrated by other bacterial indices. More widespread use as an indicator would need the support of a vast amount of data on distribution, enumeration, survival, etc., which as yet does not exist, and may possibly not warrant extensive investigation. BONDE (1962, 1966) however attaches great significance to the presence of *Cl. welchii* although he used a technique including vegetative cells and spores of this organism, and a method of counting more precise than the standard coliform count. BONDE considered that *Cl. welchii* did not multiply in the aquatic environment, an advantage possibly over some of the coliform group, but this view is questionable (WILLIS, 1957). Under the conditions studied, however, BONDE's technique did demonstrate a background pollution which could be readily distinguished from subsequent faecal pollution imposed upon it.

2.4 Pseudomonas and Aeromonas species

Pseudomonads are widespread in nature but the only strictly pathogenic species to man, *Pseudomonas aeruginosa*, is abundant in sewage and has been encountered in water samples when *E. coli* were low or undetectable (REITLER & SELIGMAN, 1957). The organism's greater resistance to chlorination than most coliforms suggests that it may be a better indicator for viruses but it cannot be considered indicative of faecal pollution in the same way as *E. coli*. CLARK & VLASSOFF (1973) advocated the use of other indicators, particularly the fluorescent Aeromonads and Pseudomonads in conjunction with faecal coliforms and DUTKA (1973) also includes *Ps. aeruginosa* as a good indicator of potential health hazards from sewage pollution. INHORN *et al.* (1973) however evaluated *Ps. aeruginosa* as an indicator of bathing beach water quality and concluded that it was not sensitive enough.

Aeromonas spp. have been considered as indicators but may be present in greater numbers than coliforms and exhibit a poor correlation with sanitary quality (ARTEMOVA, 1971). Similar observations were reported by BRUNNER (1970) who concluded that high levels of *Aeromonas* spp. in surface waters and fish ponds were due to multiplication in nutrient-rich wastes, unrelated to faecal pollution.

2.5 *Other bacterial indices*

Staphylococcus aureus was evaluated and rejected by INHORN (1973) due to lack of sensitivity but suggested as useful in judging bacterial quality of swimming pools by PALMQUIST & JANKOW (1973). The direct use of *Salmonella* as indicators of water quality was advocated by CHERRY *et al.* (1972) who demonstrated that these organisms were widely distributed and simply enumerated using a combination of swab and fluorescent antibody techniques.

JAMIESON *et al.* (1976) compared the survival of a number of pathogenic microorganisms in sea water and on the results concluded that the yeast *Candida albicans*, which survived longer in sea water than all commonly-occurring pathogens, might be an ideal long-term indicator of faecal pollution.

2.6 *Summary*

The widespread use and reliance for many years on the coliform group of bacteria, and in particular *E. coli*, as indicators of faecal pollution stresses the importance of this group. Furthermore, despite the suggested replacement by other bacterial indicators which have been briefly reviewed above, *E. coli* is still the most widely-used indicator organism. Other bacteria such as the faecal streptococci and *Clostridium welchii* have certain particular advantages over *E. coli* but are rarely used as indicators without simultaneous reference to

estimates of *E. coli*. Certainly the water or public health bacteriologist has yet to find a more useful tool in demonstrating possible hazards which might arise from sewage pollution of waters or shellfish and after some 50 years of research one might reasonably conclude that they are unlikely to do so. Whatever the discussion about relative merits of one bacterium against another as an indicator of faecal pollution there appears to be almost unilateral agreement that coliforms alone are unsatisfactory.

However, there still remains the suspicion that the use of *E. coli* either alone or in conjunction with other bacterial indicators may be unsuitable for assessing risks from viruses because of their widely differing characteristics and survival patterns in sea water. Much of the published work on the destruction of *E. coli* in sea water which has been reviewed by BERNARD (1970) reports the effects of factors which have little effect on known viruses, although the degree of susceptibility may be expected to vary from one type of virus to another.

Thus the microbiologist concerned with faecal pollution is in the position of using bacterial indicators for assessing faecal contamination knowing they may have marked potential shortcomings in relation to levels of virus but accepting they are proven for bacterial pathogens of faecal origin. It has often been suggested that the rapid test for *E. coli* does at least confirm faecal pollution so one might expect by inference, though not necessarily find, virus to be present also. Having thus located pollution *per se*, specific tests for the presence of viruses may then be undertaken. However, as will be apparent from later sections where virus will be examined in detail, greater survival of certain viruses may result in their recovery long after *E. coli* have disappeared (METCALF, WALLIS & MELNICK, 1974).

3. VIRAL POLLUTION OF FAECAL ORIGIN

On reviewing the rather scant literature on this subject the reader is constantly reminded that one of the major problems is developing techniques sensitive enough to recover and identify viruses. It therefore follows that until our knowledge is more comprehensive we cannot hope to evaluate fully the real problem which may exist from faecal pollution. Much of the information on disease or risk of disease from viral agents in polluted waters is based on epidemiological evidence, either proven or suggested. The problem of factual assessment is further complicated by the suggestion that many viruses, particularly the adenoviruses and picornaviruses are non-specific and may produce widely different clinical effects or indeed no observable effect at all (see Table 1). GOLDFIELD (1967) concludes that it is therefore no accident that the two viruses most readily accredited with causing disease are poliomyelitis and infectious hepatitis, each of which is recognized by a uniform clinical syndrome.

Worthy of mention at this stage are the virus-linked episodes of gastroenteritis and diarrhoea in which no recognized pathogen can be incriminated (McDERMOTT, 1975). MOSLEY (1967) reviews the problem briefly and states that this type of illness represents 62% of all epidemics of waterborne disease, and 72% of all cases reported (data for the USA). Various workers have shown a virus-like transmissible agent as being incriminated in this type of non-specific illness (GORDON *et al.*, 1949; JORDAN *et al.*, 1953; REIMANN, 1963) and such an agent may possibly be responsible for non-specific illness resulting from the consumption of oysters (AYRES, 1971). A review of the virus problem in sewage, polluted waters and shellfish is presented to lay the foundations for the consideration of coliphage as an indicator; the subject of this investigation.

Table 1 Human enteric viruses and diseases associated with them (from GRABOW, 1968; VARMA *et al.*, 1974)

Virus sub-group	Number of types	Diseases caused*
Poliovirus	3	Paralytic poliomyelitis, aseptic meningitis
Coxsackie virus Group A	25	Herpangina, aseptic meningitis
Group B	6	Pleurodynia (Bornholm disease), aseptic meningitis, acute infantile myocarditis
Echovirus	30	Aseptic meningitis, rash and fever, diarrhoeal disease, respiratory illness
Infectious hepatitis	1(?)	Infectious hepatitis
Reovirus	3	Fever, respiratory infections, diarrhoea
Adenovirus	33	Respiratory and eye infections

*In some cases infection with these disease agents may be so mild that it is mistaken for a slight cold. On the other hand it may be as severe as paralytic poliomyelitis.

3.1 Virus in sewage

Theoretically any human virus that is excreted in faeces may be transmitted through water and shellfish that is contaminated by faecal matter. About one hundred identifiable enteric viruses have been demonstrated in human faeces and these may give rise to a variety of diseases (Table 1). MALHERBE & STRICKLAND-CHOLMLEY (1967) estimated that more than 70 serologically-distinct human enteric viruses can occur in sewage and to these can be added animal viruses from farm and abattoir wastes.

To quote from NUPEN & STANDER (1973) "a fundamental objective is to solve the growing problem of water pollution and keep waters clean. To do this pollution must be tackled at source and not allowed to spread

throughout the environment by discharge into waterways." The authors regarded tertiary treatment of sewage as a '*sine qua non*' when dealing with polluted waters. However, it is unfortunately a fact that relatively few discharges into coastal and estuarine areas are subject to tertiary treatment and many receive, in effect, no treatment at all other than maceration or coarse screening. Since it is precisely in such areas that our most important molluscan fisheries are located, a knowledge of the virus status in sewage is of prime importance in any assessment of problems connected with shellfish-transmitted viral disease. Much of the interest in this latter field of research is centred in the USA and it is therefore not surprising that considerable attention has been given in that country to the whole sphere of 'virus in sewage'.

Poliovirus, coxsackie virus, echovirus and other relatively thermostable viruses such as reovirus and adenovirus can be readily recovered from sewage, particularly during outbreaks of disease in the population. CHIN *et al.* (1967) showed that during an epidemic of polio in Iowa, USA frequency of recovery of the causative agent in sewage varied from 9.1 to 100% and was related to the attack rate reported in the communities served by the various sewers sampled. During the 13 months following this epidemic 21% of 213 sewage samples from the area affected contained enteroviruses and a third of these were identified as poliovirus. Studies in Sweden by LUND & HEDSTROM (1967) demonstrated that coxsackie virus could be isolated from sewage prior to their appearance in epidemics. RIORDAN (1962) and GELFAND *et al.* (1962) raised the point that during oral polio vaccine campaigns the virus can be demonstrated in sewage some 2 to 3 months after administration of the vaccine to the population. Numerous other reports of enteric viruses in sewage have appeared (CLARKE *et al.*, 1951; MELNICK *et al.*, 1954; KELLY *et al.*,1957;

BLOOM *et al.*, 1959; GRAVELLE & CHIN, 1961; WILEY *et al.*, 1962; LAPINLEIMU & PENTTINEN, 1963; LAMB *et al.*, 1964). One of the few reports of observations on sewage in Britain (SELLWOOD & DADSWELL, 1975) has shown that although sewage treatment reduces virus content a significant number of swabs from final effluent were positive for virus.

It is recognized however that the frequency of isolation of many types of virus is related to the sensitivity of the technique used and one may therefore postulate increasing frequency of isolation as techniques are refined. This may lead us to suggest that the full extent of the problem reviewed by CLARKE & KABLER (1964) may be greater than they suggested. Having demonstrated the obvious hazard posed by the presence of virus in sewage some research has been directed towards the development of treatment methods which may remove or at least reduce the virus content of sewage. There have been relatively few field experiments reported but BLOOM *et al.* (1959) published the results of enteric virus isolations from 214 samples representing various stages of sewage treatment in a plant at East Lansing, Michigan (Table 2). This demonstrated

Table 2 Isolations of enteric viruses from various locations in a sewage plant (from BLOOM *et al.*, 1959)

Sampling location	% of samples positive
Influent	32.6
Raw sludge	38.1
Primary trade effluent	23.8
Activated sludge tank	11.1
Activated sludge return	6.7
Final settling tank	5.3
Final, unchlorinated effluent	9.8

a progressive decline in the percentage of positive virus isolations through the stages of treatment, with a particularly large reduction after activated sludge treatment. The latter observation agrees with the findings of KELLY *et al.* (1961) and ENGLAND *et al.* (1967) in other field studies. WEBER-SCHUTT *et al.* (1969) considered that the activated sludge process was more effective in removing viruses because of adsorption on to solids in suspension and various workers have shown that the virus reduction capacity of activated sludge treatment may exceed 90% (KELLY & SANDERSON, 1959; SAFFERMAN & MORRIS, 1976).

SPROUL *et al.* (1967) considered that virus inactivation in sewage treatment may be due to a number of mechanisms including:

1. loss of protective protein coat by enzymatic action;
2. denaturation of the surface protein coat;
3. loss of structural integrity due to pH effects;
4. alterations of the nucleic acid core or surface protein by oxidants and inorganic toxicants;
5. adsorption to various surfaces.

These workers considered that adsorption was the major factor involved in virus removal and there is other evidence to support it as the predominant mechanism (KOLLINS, 1966; De MICHELE, 1974; MALINA Jr. *et al.*, 1974). The activated sludge process produces an adsorption area relative to sludge volume in excess of that produced by more conventional systems and this may account for the greater efficiency of the process in virus removal. KELLY *et al.* (1961) isolated strains of bacteria with antiviral activity from sludge and demonstrated that aeration of the sludge was essential for virus removal, suggesting that biological agents were responsible. Studies by MALHERBE & STRICKLAND-CHOLMLEY (1967) failed to demonstrate active removal of

poliovirus and reovirus by algae in sewage oxidation ponds and suggested that photoinactivation by sunlight at the surface was responsible for the observed decline in enteric virus. Raw sewage appears to exert a protective effect on virus survival even though it contains very large numbers of bacteria. Adsorption and removal by protozoa were favoured by BORNEFF (1967) as the most likely factors involved but varying susceptibility of different virus types may implicate a few or all of the factors suggested. Evidence presented in a number of papers suggests that reovirus may be particularly resistant to sewage treatment and the factors involved in virus reduction since it is usually the last one detected after disinfection by chlorine etc.

Although forms of tertiary treatment may be relatively very efficient in removing viruses some problems still remain. The removal of virus from sewage via adsorption on to particulate matter may, in practical terms, mean a transfer of virus from water to sludge rather than inactivation of virus and it is therefore necessary to study virus content of sludges (LUND & RØNNE, 1973). Work by PALFI (1973) and SPROUL (1973) on virus inactivation during anaerobic sludge digestion showed that reduction was a function of, and even primarily caused by, temperature and retention times used. The latter author concluded that digested sludge must be considered as a material containing pathogenic viruses. LUND (1971) demonstrated that most of the virus in sludge was bound to particles but that virus binding capacity varied with climatic conditions. The author also showed that the virus particles removed by flocs (either natural or chemical) were not inactivated and care must therefore be exercised in sludge disposal.

Even if enterovirus numbers in sludge or effluent are reduced by over 90% a potential hazard may still exist in the small numbers which remain. Disinfection may produce a virus-free effluent but the

susceptibility of viruses varies as does the effectiveness of the commonly used disinfecting agents. Additionally the presence of disinfectants such as chlorine may pose environmental problems when an effluent is discharged to a river or estuary. The minimal infective dose of virus for man by the oral route may be as low as 1 plaque forming unit (PFU) (PLOTKIN & KATZ, 1967). Clearly therefore there are considerable technological problems to overcome before the high standard of treatment necessary to cope with this order of risk is achieved.

To summarize the effect of sewage treatment on virus, it appears to be that levels are significantly reduced, particularly by secondary and tertiary treatment. However, low levels of virus may still be detectable in the final effluent and also in the sludge after treatment. The suggested minimal infective dose of 1 PFU and the possibility of concentration by estuarine bivalve molluscs support the contention that a truly virus-free effluent is desirable. Use of chemical flocculation or disinfection techniques could provide a possible solution but some disinfectants (e.g. chlorine) could be environmentally damaging and present techniques of virus detection are not sensitive enough to confirm complete virus removal.

3.2 Virus in polluted waters

In the previous section on sewage it was concluded that domestic sewage was the chief source of viruses found in polluted waters. The density of virus in sewage may vary considerably; CLARKE & KABLER, (1964) have estimated that it is in the region of 500 units/100 ml of sewage but this may be reduced to very low levels by sewage treatment. Given that some viruses may have a minimum infective dose level as low as 1 PFU, continuous discharge of even a treated effluent may contribute substantial numbers of virus to receiving waters. The fate of these

Figure 1. Survival of enteric viruses in estuary water during winter. (after METCALF & STILES., 1967)

Figure 2. Survival of enteric viruses in estuary water during summer. (after METCALF & STILES, 1967)

Table 3 Suggested viral standards for various waters

Type of water	Standard	Volume of sample to be examined	Reference
Drinking	< 1.0 PFU/litre	10 litres	WHO, 1970
Drinking and food preparation	Not more than one infectious unit	100 gallons	MELNICK, 1971
Bathing/recreation	"	10 gallons	
Disinfected, renovated, potable	Absence of virus in 100 gallons	100 gallons minimum	BERG, 1971

particles is of considerable importance, particularly when the receiving waters are utilized for recreation, abstraction for drinking purposes, or, as in marine situations, the cultivation of molluscan bivalve shellfish. Reference to suggested standards for various waters shown in Table 3 emphasizes just how much importance is attached to the presence of virus even in very small numbers. When one considers that bacteriological standards for potable water in the UK are based on absence of *E. coli* in 100 ml of sample it can be appreciated that detection of virus, in itself not a simple task, poses considerable technological problems when sample volumes of 100 gallons or more are necessary.

The work of METCALF & STILES (1967) showed that survival of enteric virus in estuary waters is dependent on temperature, pollution levels and type of virus involved. Data for survival of poliovirus 1, coxsackie B-3 and echovirus 6 (Figures 1 and 2) showed that survival of these viruses was prolonged in estuarine waters at low temperatures, e.g. poliovirus, winter 42 days, summer 23 days; coxsackie virus, winter 56 days, summer 32 days. The authors observed a similarity between laboratory survival of virus at 37°C and field survival at 16°C suggesting that survival was not particularly related to temperature in estuarine conditions. They further suggested that virus mortality was due to an interaction between viruses and soluble by-products of the biotic flora and fauna. Such a theory could explain extended virus survival in winter when biotic activity is reduced. CLARKE *et al.* (1962) and METCALF & STILES (1967) have also demonstrated prolonged virus survival in grossly polluted waters (Figure 3) and in clean water but generally reduced in moderately polluted water.

Two papers with contrasting results have dealt with the survival of poliovirus in sea water and included studies on the effects of

salinity. AKIN *et al.* (1976) measured the loss of infectivity (LOI) and demonstrated that LOI had no explanation apart from true virus inactivation and concluded that the specific component(s) responsible were yet to be ascertained. As salinity increased so the LOI increased; maximum survival being observed in distilled water. In contrast, LO *et al.* (1976) demonstrated that temperature was more important and concluded that on published evidence available the only consistent finding on virus survival in waters is a direct relationship between virus stability and temperature, where the higher the temperature the greater the rate of inactivation. LO *et al.* also showed, as many earlier authors have, that stability of different viruses varies and their findings agreed with those of METCALF & STILES (1967) that coxsackie was more stable than echovirus; polio being the least stable of those tested.

MATOSSIAN & GARABEDIAN (1967) found that microorganisms and particulate matter had no effect on inactivation of poliovirus. In contrast, SHUVAL *et al.* (1971), also using poliovirus in sea water, could not demonstrate anti-viral activity in the absence of marine bacteria. Studies in Scandinavia on the virus inactivity capacity (VIC) of sea water have shown that this may be associated with marine bacteria (MAGNUSSON *et al.*, 1967). GUNDERSEN *et al.* (1967) isolated a bacterium resembling *Vibrio marinus* which could restore the VIC capacity of sea water (removed by heating and filtration) when added at a concentration of 1×10^5 cells. Support for a biotic factor was presented by LYCKE *et al.* (1965) who showed that heating sea water at 45°C for one hour removed the VIC it had previously contained. These authors also reported that proteins and amino-acids were important; detailed work with glycine showed that increasing concentrations of this amino-acid inhibited the VIC component, possibly pointing towards a reaction between VIC factors in sea water and amino groups in viral proteins.

Virus titer $\log TCID_{50}$/specimen

Repeated isolations of enterovirus have been made from waters some distance from sources of pollution: METCALF & STILES (1968b) isolated enterovirus four miles from the nearest source. Studies such as this give a vivid indication of the transmission potential of polluted waters.

Information is still limited about low levels of virus and their importance as infectious agents. As techniques become more sophisticated we can expect to begin to understand the complex relationship between enterovirus in sewage, polluted waters and shellfish. The current position may still be summed up by quoting from METCALF & STILES (1968a) "whatever doubts one may have about the suitability of coliform indices to reflect enterovirus presence, it is necessary to remember that virus isolation methods are tedious, demanding and expensive. These have been, and remain, formidable technical barriers to the adoption of a virus testing programme for water."

3.3 Virus in shellfish

The background relating to transmission of bacterial pathogens by the consumption of raw shellfish has already been discussed and mention has been made of the long history of this type of association. It was not until 1956, however, that the problem of virus transmission really came to the fore when the first documented cases of infectious hepatitis from shellfish appeared in Sweden (ROOS, 1956; CHRISTENSON, 1956). During the first two months of 1956 no fewer than 627 cases of the disease occurred in oyster-eaters in the Stockholm/Gothenburg districts of Sweden. Epidemiological enquiries reported by CHRISTENSON (1956) revealed that prior to the outbreak two cases of infectious hepatitis had occurred, one in a workman at the fish marketing depot and another in a sailor at Gothenburg. The local sewers carrying faeces of both of these individuals discharged into the harbour at Havstenssund where

oysters were stored in suspended baskets prior to sale.

Shellfish were not recognized as vectors of infectious hepatitis in the United States until 1961 when two epidemics occurred. One caused by raw oysters produced 84 cases (DOUGHERTY & ALTMAN, 1962) and another caused by raw clams produced an estimated 485 cases in the north-eastern states (MASON & McLEAN, 1962). Two further epidemics were reported from this area in 1964 involving some 316 cases altogether and, as with the Swedish outbreak, a positive link between sewage pollution and epidemic shellfish vectored infectious hepatitis. At this point the distinction should be drawn between the epidemic cases reported above and a number of endemic cases which have been documented. In these cases people who had eaten shellfish at a time and place where epidemic shellfish associated infectious hepatitis had not occurred became infected. The problem is further complicated by the fact that although this disease has been clinically recognized for many years there is still no method to detect or cultivate the causative agent(s) outside its natural human host (KISSLING, 1967).

More recently other cases of infectious hepatitis have been reported following the consumption of clams (DISMUKES *et al.*, 1969 RATZAN et al 1969) and mussels (BEGG, 1975) where the problem first appeared to be one of gastroenteritis affecting a number of people; a few of which subsequently developed infectious hepatitis. This is of particular interest because problems of non-specific illness arising from the consumption of oysters (AYRES, 1975a) and cockles (AYRES, 1977a) may, in the light of recent investigations, have a possible virus aetiology (AYRES, 1977a) and be associated with sewage contamination of shellfish. An historical and world-wide review of diseases transmitted by foods contaminated by waste water shows that typhoid, infectious hepatitis, fascioliasis and cholera are most frequently transmitted by contaminated foods

(BRYAN, 1977). In the outbreaks recorded by BRYAN the largest number were associated with shellfish as can be seen from the figures reproduced below. Details of the recorded outbreaks where shellfish were the vehicle of infection show that infectious hepatitis and typhoid are particularly common but gastroenteritis and cholera have also been reported. Apart from infectious hepatitis there have been no confirmed cases of viral transmission to the consumer via shellfish. Various workers have, however, looked at the virus content of bivalve molluscs taken from areas subject to sewage pollution and used enteroviruses such as polio in controlled field and laboratory experiments to study the uptake, retention and excretion of viruses by shellfish. More detailed reference is made to this work later.

Table 4 Incidents of disease transmission by waste-water contaminated foods (from BRYAN, 1977)

Food vehicle	Number of outbreaks
Shellfish	28
Watercress	10
Fish	3
Shrimp	1
Vegetables	21
Fruit	4

Since no specific causative agent for infectious hepatitis has been isolated METCALF & STILES (1968a) used enteroviruses which are of similar size and have an alimentary mode of dissemination for their studies. KELLY (1957) demonstrated that this group could be routinely isolated from sewage and GELFAND (1961) states that in north temperate zones they are particularly numerous during the period July-October. The enterovirus group includes forms pathogenic to man so knowledge of their occurrence and significance in estuarine waters and shellfish was also useful. Another report of this four-year programme (METCALF &

STILES, 1968b) showed that various other viruses had been isolated during the study including coxsackie B, echo- and reovirus types, all potentially capable of causing illness in man. This confirmed earlier evidence reported by the same authors (METCALF & STILES, 1965) illustrating the presence of such potential pathogens in a polluted estuary. Mention should be made again of the difficulty in relating the presence of virus types other than some of the better known ones such as poliomyelitis to any known disease or recognized clinical syndrome. In the studies by METCALF & STILES (1965) evidence was presented which showed that oysters had accumulated poliovirus within two days of its appearance in effluents a quarter of a mile away, and perhaps more significant, evidence which showed retention of virus for some time after it had ceased to appear in sewage or the overlying water. As one might expect, a seasonal influence on virus levels in shellfish was apparent and this appeared to be related to the physiological responses of the oysters to water temperature. Like all living organisms the metabolic and feeding activity of the oyster declines with falling water temperature and this had a profound effect on viral status. METCALF & STILES (1965) found that if oysters were unpolluted by virus at the beginning of 'dormancy' they remained free of virus until activity was resumed. Conversely, if they were polluted by virus then levels remained unchanged throughout the period of 'dormancy' and under such conditions oysters served as vehicles for prolonged virus survival, an important observation from a public health viewpoint. HAUSER (1964) concluded that, because of the apparent widespread pollution of sea water and oysters, waste-water treatment methods in the USA may be considered unable to solve problems of sewage pollution in shellfish-growing areas and there is little doubt that this also applies in Europe and elsewhere.

A study carried out by DENIS (1973) in France showed that 10% of 840 oysters and 20% of 120 mussels examined were positive for coxsackie virus group A. Strain A-16 was most frequently isolated. It could be shown also that sporadic infections associated with consumption of shellfish could be traced back to coxsackie A virus on the basis of similarity of the symptoms observed and those normally associated with coxsackie A infections from other sources.

To date no positive evidence of virus transmission by molluscan shellfish in the United Kingdom has been demonstrated, nor indeed have any reports on virus in oysters or polluted sea water been produced. It should be added that this is largely a reflection of the inadequate attention which this field of research has received and it would be unwise to presume from the lack of published data that the problem is any less important here than in the USA or elsewhere. The apparent lack of problems in the UK is possibly allied in some way to the widespread use of purification methods for shellfish (WOOD, 1969); methods which are not in general commercial use in the USA. To put the matter in perspective however two main points should be taken into account:

(i) Molluscan shellfish production in the USA dwarfs the figures for production in the UK and therefore, assuming the incidence of viral illness from consumption of shellfish is related to the production (= consumption), it is possibly no accident that shellfish-transmitted viral disease such as infectious hepatitis has not been reported in the UK. On this basis, since we are unlikely to so increase our level of production in the foreseeable future, it has been felt that we need not consider virus in shellfish as a problem. This would obviously be a short-sighted view and where matters of public health are concerned even a remote risk should be adequate reason for vigilance.

(ii) Because we are able to purify molluscan shellfish and render them free of faecal pollution it is standard practice in the UK to take shellfish from areas known to be polluted, treat them and offer them for sale. In the USA, however, controlling sanitary standards for shellfish are based on the coliform content of the waters where the shellfish are harvested. Areas shown to be polluted are closed for commercial production and, since purification as practised in the UK is not generally adopted, marketed shellfish should originate from pollution-free areas. The various reports of infectious hepatitis to which reference has been made do, however, conclude that the shellfish incriminated came from areas which were grossly polluted by sewage.

An important point has arisen recently following an outbreak of oyster-associated hepatitis in Louisiana (MACKOWIAK *et al.*, 1976). Oysters implicated in the outbreak were traced to approved growing areas. Due to flooding and consequent faecal pollution, the areas concerned had been temporarily closed for the harvesting of shellfish until bacteriological examination showed that faecal contamination was no longer identifiable and 'approval' was reinstated. This was some 1½ to 2 months after the initial closure and the cases of infectious hepatitis which followed suggest that the agent(s) responsible remained viable in the shellfish during this period. This supports evidence that under natural conditions shellfish eliminate viruses and bacteria differently and may even retain enterovirus long after bacteria have gone. Further mention will be made of this in a later section (4.5).

In the USA shellfish authorities have conceded in the last few years that it may be more realistic to admit that the problem of pollution encroachment in estuary waters has exceeded the possible remedial

measures available and it may therefore soon be impractical to close areas with shellfish potential on evidence of water pollution alone. Although purification has been used in a small way since 1925 attention is now increasingly directed towards its adoption as routine in commercial shellfish handling. At present the American health and shellfish authorities are concerned with a few minor biological and engineering problems to ensure a reliable and economic system and will probably implement commercial usage of such systems at any time now. British experience with purification originates with the report of DODGSON (1928) on mussel cleansing and various reports since (BAIRD, 1954; REYNOLDS, 1956; WOOD, 1961), but it should be remembered that the impetus was the fear of shellfish-transmitted typhoid. By comparison, interest in purification in the USA has resulted primarily from concern about the transmission of viruses by shellfish. While we have purification systems operational and now question whether these will also cope with any virus pollution, research in the USA has asked these questions first. It is therefore not surprising that a number of papers on the mechanics of viral uptake and elimination by oysters and other shellfish have originated from the USA.

MITCHELL *et al.* (1966) reported that the oyster *Crassostrea virginica* could accumulate enterovirus to a level sixty times that existing in the surrounding water, and it is this ability to concentrate which is fundamental in understanding the role shellfish may play as vectors of viral disease. HOFF & BECKER (1968) working with polio virus obtained accumulation factors up to 180x in studies with Olympia oysters and Manilla clams. HEDSTRÖM & LYCKE (1963) and LIU *et al.* (1966b) also working with polio virus obtained similar results but, as CANZONIER (1971) remarks, all such data were obtained by dosing shellfish with single high titre doses of virus, not strictly comparable

with the expected pattern of natural accumulation. In nature it would be reasonable to suppose that virus levels in shellfish are usually the result of the animal's ability to concentrate very low levels of virus present in the environment. The studies reported by LIU *et al.* (1967), HEDSTRÖM & LYCKE (1964), CROVARI (1958) and ATWOOD *et al.* (1964) are in general agreement and show that viral uptake by shellfish occurs rapidly so that maximum levels are reached within a few hours. High levels are maintained as long as sufficient virus is present in the water but when numbers in the water decrease there is a corresponding decrease in the viral content of the shellfish. LIU *et al.* (1967) also showed that after 96 hours' purification polio virus levels in the hard clam (*Mercenaria mercenaria*) had been reduced from $\log_{10}$ 3.4 PFU/ml to undetectable levels (see Figure 4), but other workers, notably METCALF & STILES (1968a), showed that with high initial levels of coxsackie B-3 oysters held between 4.5°C and 10°C still carried quite high numbers of virus after 29 days (Table 5). They do, however, stress that good purification was achieved at temperatures near 20°C, even with initially high virus titres, in 3 to 6 days. An important point raised here by METCALF & STILES (1968a) is that reduction of virus to undetectable levels (see LIU *et al.* (1967) above) should not infer elimination since it is believed possible to have virus in numbers sufficient to produce human infection and yet be undetectable in the laboratory.

Experimental purification systems in the USA generally use what is known as the Kelly-Purdy ultra-violet (u/v) sterilizer which uses 15 x 30 watt u/v tubes side by side under which the tank water is circulated. Similar systems in the UK rarely utilize more than 2 x 30 watt u/v sources but it should be remembered that the scale of operation in the USA means large capacity tanks both in terms of water and shellfish content and thus a requirement for proportionately greater

Figure 4. Depuration of the meat and liquor of Northern Quahogs (*Mercenaria mercenaria*) contaminated in running seawater with poliovirus 1. (after Liu *et al.*, 1967)

u/v output. HILL *et al.* (1969) demonstrated reductions of waterborne poliovirus in excess of 99% within 15 seconds' treatment by a Kelly-Purdy type unit (Table 6) and, although no similar work has been carried out with the system described by WOOD (1961) as used in the UK, the makers of the TUV tubes used here state that susceptibility of viruses is comparable to that of bacteria (0.5-200 mw seconds/cm^2).

Table 5 Influence of water temperature and enterovirus numbers upon depuration in estuary waters (coxsackie B-3)* (from METCALF & STILES, 1968a)

Days	Temperature (°C)					
	4.5 to 10		10 to 14		15 to 20	
	Enterovirus (numbers)					
	High	Low	High	Low	High	Low
0	5 536	207	4 817	418	9 694	506
1	-	-	-	-	1 958	45
2	1 137	82	-	-	268	0
3	-	-	944	58	0	33
4	-	-	-	-	26	0
5	-	-	105	41	0	0
6	681	71	-	-	-	-
7	47	23	35	0	-	-
8	-	-	46	0	0	0
9	80	28	0	0	0	0
10	-	-	-	-		
11	0	0	0	0		
13	42	32	-	-		
15	27	0	-	-		
17	-	-	0	0		
19	-	-	0	0		
21	20	0	-	-		
23	0	0	-	-		
25	-	-	0	0		
27	12	0				
29	31	0				

*measured in plaque forming unit (PFU) values.

Some of the apparent discrepancies in virus removal by purification methods may be due to the action of virus uptake from low concentrations in water over extended periods. The uptake of high initial titres of virus seems to result in rapid elimination in much the same way that

faecal bacteria are eliminated. Exposure to low levels over extended periods may result in retention quite independent of the physiological activity of the animals. Studies by LIU *et al.* (1966), LIU *et al.* (1967) and SERAICHEKAS *et al.* (1968) show that small numbers of virus may be sequestered in shellfish tissues and apparent elimination may be due solely to inactivation of virus in the animals. Since the stability of viruses under such conditions has not yet been determined, and if the sequestered particles constitute an infectious dose, then further controls will need to be implemented before purification can be considered wholly acceptable as a means of eliminating virus from polluted shellfish.

Table 6 Poliovirus multiplicities of flowing u/v-treated sea water in the Kelly-Purdy experiments (from HILL *et al.*, 1969)

Heat sterilization or cooking is often used as an alternative to purification in the processing of shellfish for human consumption but the efficiency or otherwise of such methods is judged primarily by the use of bacterial indices. The cockle poisoning incident described by AYRES (1977) suggested that assumptions based on such indices may be less than adequate where virus contamination may be present. BRYAN & HUFF (1973) discussed the problem of infectious hepatitis arising from the consumption of clams and concluded that cases generally arose from raw or inadequately cooked clams. Although the animals would open in one minute after immersion in boiling water it might be as much as 4 to 6 minutes before their internal temperature reached the ambient temperature (KOFF & SEAR., 1967). The minimum period required for inactivation of infectious hepatitis agent has not been established but boiling water for 20 minutes will inactivate hepatitis A virus, and KRUGMAN *et al.* (1970) demonstrated that one minute at 98°C destroyed the MS-1 strain of hepatitis A.

3.4 Relationship to bacterial indices of faecal pollution

The most commonly used bacterial indices of faecal pollution have been reviewed in an earlier section (2.1 to 2.5) and it has been noted that the main disadvantage with most, if not all, is the apparent lack of correlation between numbers of faecal bacterial indicators and numbers of virus, due to differing mortality rates in sea water etc. However, since either the coliforms as a group or *Escherichia coli* specifically are the foundation on which sanitary standards for water and shellfish are based, attention has been given to the relative distribution and survival of these organisms compared with that of viruses.

The small percentage of sewage samples yielding viruses indicates that they normally occur at low densities in sewage, much lower than

either coliforms or faecal streptococci (see MACK *et al.*, 1958). It should be added that while most humans excrete coliforms and faecal streptococci in faeces the excretion of enteric viruses in healthy individuals is largely confined to children under the age of fifteen (RAMOS-ALVAREZ & SABIN, 1956). CLARKE *et al.* (1962) calculated that the relative enteric virus density to coliform density in human faeces is in the order of 1 to 65 000. If we assume that the density of streptococci in faeces is about one-third that of coliforms (KENNER *et al.*, 1960) then we have a calculated virus to faecal streptococci ratio of 1 to 195 000. More recently, CLARKE & KABLER (1964) have calculated enteric virus to coliform ratios of about 1 to 92 000 for sewage and about 1 to 50 000 for polluted surface water.

In assessing the value of accepted bacterial indicators as indicative of enteric virus in polluted waters the relative survival times need to be evaluated. Preliminary data presented by CLARKE *et al.* (1962) are summarized in Table 7. The average coliform density of the areas studied in ascending order was Little Miami River (54/ml); Ohio River (197/ml); sewage (208 000/ml). The largest survival of viruses was observed in the 'cleanest' area and in the raw sewage with reduced survival in the moderately polluted area (see section 3.2). Observations on enteric bacteria, however, showed that survival time was related to degree of pollution, survival being greatest in the most polluted sample area. It was suggested that additional nutrients could account for extended bacterial survival in the more polluted waters. In Little Miami River the viruses, except coxsackie A-9, survived almost twice as long as any of the bacteria. In Ohio River, however, all the bacteria except *E. coli* survived longer than any viruses. In sewage, viruses except coxsackie A-9 appeared to be hardier than the bacteria.

Table 7 Average time in days for 99.9% reduction in original titre of indicated microorganisms at three temperatures (from CLARKE *et al.*, 1962)

GILCREAS & KELLY (1955) in reviewing the problem of using coliforms as indices of viral pollution asked two questions: (1) how do the survival of viruses and coliforms compare under different conditions? (2) whether the presence or absence of members of the coliform group can be used without reservation to indicate the biological quality of water? A previous publication by GILCREAS & KELLY (1954) indicated that under certain conditions of water treatment the coliform index could be taken to indicate freedom from virus pollution. Experiments reported by these authors in 1955 (Figures 5 and 6) showed that between 8°C and 10°C (Figure 5) virus showed greater survival than *E. coli* but that storage between 20°C and 30°C (Figure 6) resulted in reproduction of the *E. coli* and greater survival than viruses tested. Field studies indicated that when the coliform content of a sewage sample was less than 13 000/ml (by most probable number technique) no viruses were isolated. GILCREAS & KELLY (1955) concluded that the coliform index was generally a valid measure of pollution, bacterial and viral, and a reliable indication of efficiency of sewage treatment. The coliform index should, however, be interpreted with discretion in the light of known survival rates for enteric viruses. Experiments on the effect of chlorine (Figure 7) showed that a higher concentration was required to kill coxsackie viruses than that required to kill *E. coli*. The authors therefore stress that complete, efficient water treatment is necessary to remove enteric virus pollution problems; marginal treatment may reduce coliforms to acceptable levels but still leave viruses. Later work by NICHOLS & KOEPPE (1961) concluded that the absence of coliforms did not necessarily imply an absence of virus and this may reflect the improvements in techniques of virus isolation during the six years separating their study from that of GILCREAS & KELLY (1955). Further evidence of this is provided in the paper by

Figure 5. Storage of viruses and coliform bacteria in water at 8 - 10 °C (after GILCREAS & KELLY 1955)

Figure 6. Storage of viruses and coliform bacteria in water at 20 - 30°C (after GILCREAS & KELLY, 1955)

METCALF & STILES (1968a) who demonstrated that although enteroviruses were not entirely unaffected in a primary treatment plant with chlorination of waste waters, coliforms and Salmonellae were drastically reduced.

A comparative study on the persistence of the bacterium *E. coli* and echovirus 6 in sea water has been reported by WON & ROSS (1973). The authors showed that the addition of low concentrations of organics, including faeces, enhanced the survival of *E. coli* at temperatures of 3°C to 5°C and became growth-promoting at 22°C, resulting in a 40x increase and viability persisting for eighteen weeks. Added organics did not enhance virus survival although initial inactivation was greater at 22°C than at 3°C to 5°C. FANNIN *et al.* (1977) compared coliforms and coliphages as indicators of airborne contamination from waste treatment plants and concluded that coliforms were unreliable as indicators of airborne viral contamination.

Data presented by MITCHELL *et al.* (1966) on the relative uptake and elimination of poliovirus and *E. coli* by the eastern oyster *Crassostrea virginica* demonstrated that uptake and elimination of virus paralleled that of *E. coli* (Figures 8 and 9).

Attention should be drawn, however, to the findings of LIU *et al.* (1966b, 1967), reported in section 3.3, that some virus may be sequestered in the shellfish tissues, a phenomenon not so far described for *E. coli* and therefore may raise a question mark over the significance of the findings reported by MITCHELL *et al.* (1966). CANZONIER (1971) comments that elimination of virus parallels that of *E. coli* when the shellfish are polluted with high virus titres. He suggests that the mechanisms of uptake and elimination with virus titres of < 100 PFU/ml are different from those when shellfish contain > 1 000 PFU/ml, and only in the latter case does it parallel bacterial elimination.

Very little data appear in the literature regarding field comparisons of coliforms and viruses in shellfish but METCALF & STILES (1968a) presented evidence to show that shellfish and waters conforming to coliform-based standards still contain enteroviruses. Confirmation of the apparent failure of coliform standards to prevent problems of viral disease is given by the outbreak of infectious hepatitis reviewed by MACKOWIAK *et al.* (1976). One must at the present time consider that any evidence of virus presents a potential problem, otherwise two questions are raised again: (1) what level of virus constitutes a hazard to human health? (2) what virological standards can be applied to shellfish and shellfish-growing waters? The answers to either question are yet to be provided so we must consider any virus a potential problem in the same way as we regard evidence of faecal bacterial contamination as a possible threat of pathogens such as *Salmonella*.

In summary, it seems reasonable to conclude that there are doubts about the relationships between virus levels and coliforms but until an alternative is found standards must be based on bacterial indices, stressing the need for extreme caution in interpretation of results so obtained.

Figure 7. Survival of virus and coliforms after chlorination. (after GILCREAS & KELLY, 1955).

Figure 8. Uptake by oysters in seawater containing 300 PFU's/ml of poliovirus (after MITCHELL et al., 1966)

Figure 9. Elimination of poliovirus and *E. coli* in a flow-through water system. (after MITCHELL *et al.*, 1966)

4. BACTERIOPHAGE

It was almost sixty years ago that d'Herelle (1926) introduced the term bacteriophage to describe the filterable agents which TWORT (1915) had reported and which had the ability to lyse bacterial cells. Initial research was directed towards the possibility of using these agents, or 'corpuscles' as they were then called, as bactericidal agents to combat infections in man and animals. The results were negative but led to the development of the idea that bacteriophage studies could be used as a model to study host-virus interactions. The results of this and subsequent work led eventually to a series of important discoveries in virology and microbial genetics such that the bacteriophages have received more attention than any other group of viruses. Applications in respect of research into microbial genetics account for much of this work in recent years but phages have other uses such as the typing of potentially pathogenic bacteria, e.g. staphylococci (see BLAIR & WILLIAMS, 1961).

However, with the growing realization that certain viruses associated with sewage pollution may present a public health problem, together with doubts about the validity of bacterial indicators in assessing such risks, a few workers have turned their attention to bacteriophages as virus models and potential viral indicators. This would appear to be a logical development since bacterial and animal viruses have many similar physical, chemical and biological properties (ADAMS, 1959) but the bacteriophages may be more easily and rapidly cultured, using relatively simple and economic techniques, and, as one would conclude, are more indicative of the role and fate of enteroviruses than any bacterial indicators so far examined. Although sewage may be expected to contain a variety of different phages in keeping with the variety of bacteria it also contains [ARNOLD, 1925] numbers of phages may also be expected to be related

to numbers of host bacteria. The interest in coliform bacteria has already been dealt with and mention made of the vast numbers of these organisms in sewage and polluted waters and shellfish. The coliform group have a number of specific phages, common in sewage and easily enumerated, presenting the researcher with a useful tool to investigate the importance of bacteriophage as a possible indicator. Much of what follows will deal with coliphage specifically, reflecting the majority interest in these phages as opposed to those of other bacteria such as the *Salmonella* or Pseudomonads.

Coliphages particularly have been widely favoured as virus models and include DNA-containing tailed phages of the T type and small spherical RNA phages such as MS2. The RNA phages have many similarities with enteroviruses such as polio and are frequently used as models, e.g. in disinfection studies the phage f2 is generally more resistant to chlorination than poliovirus but is a particle of the same size and shape (CRAMER *et al.*, 1976).

4.1 Coliphage in sewage

Sewage is frequently used as a source of coliphage for further study but, although the occurrence of phage is recognized and utilized in this way, rarely have studies been directed towards determining the numbers and types of phage which may occur. Attention has been focussed on seeding sewage with coliphage as a model to study the efficiency of various treatment processes, including disinfection, on the reduction or removal of enteroviruses.

Limited work has been orientated towards an appraisal of coliphage as a factor involved in the observed reduction of coliforms during sewage treatment. WARE & MELLON (1956) showed that the ratio of phage-resistant to phage-sensitive organisms in sewage decreased from 1 to 1.3

in the crude effluent to 1 to 4.5 in the effluent. Addition of a host *E. coli* resulted in no significant change in the numbers of bacteriophage and the authors concluded that no evidence had been found to support the hypothesis that phage were responsible for decline of coliforms. Similar studies by BECKWITH & ROSE (1930) indicated that although phages isolated from sewage were quite capable of lysing coliforms isolated from the same samples it was unlikely that this occurred under natural conditions when *E. coli* and coliforms are not in a growth phase susceptible to phage attack. Further studies by CALABRO *et al.* (1972) concluded that although phage may play a role in lysing specific pathogens they were unlikely to serve as biological control agents for coliforms. A paper by KOTT (1966a) describes the use of sewage as a source of coliphage but is concerned with methodology rather than coliphage content as such.

One of the few published studies on the types of coliphage occurring in sewage (DHILLON *et al.*, 1970) concerns the distribution of coliphage in sewage taken at 11 sites representing urban and rural sewage in Hong Kong. Urban samples were slightly richer in phage than rural samples. Of 72 purified isolates studied, 50% were able to grow on three host *E. coli* strains (K12F$^+$, K12F$^-$ and B) and were represented at 10 of the 11 sites sampled. Only one strain appeared to be of a temperate nature and the authors concluded that virulent phages are far more widespread in nature as free virions than the virions of temperate phages. It was suggested that temperate phages are predominantly present as prophage in host cells. Counts of coliphage obtained varied from 0.036×10^3 to 15.9×10^3 PFU/ml. An interesting result of serological investigations reported in the same paper was the finding that cylindrical single stranded DNA phages (e.g. AE2 and M13) produced turbid plaques in contrast to the clear phages produced by the spherical

single stranded RNA phages (e.g. MS2). Extensive distribution of male specific RNA and DNA coliphages suggested that host cells must be quite widespread in nature but of 700 *E. coli* isolated from two sites none was lysed by phage MS2. GILCREAS & KELLY (1955) followed the seasonal variation of coliforms and *E. coli* B phage in two sewage plants and found they exhibited no consistent pattern of fluctuation throughout the year. Bacteriophage content of the sewage samples varied by a factor of 100 and bore no relation to numbers of coxsackie viruses which were also sampled.

Considerable variations in the indigenous coliphage population of primary effluent, ranging from 5×10^4/ml to more than 8×10^5 PFU/ml were reported by SAFFERMAN & MORRIS (1976). A single peak of coliphage was demonstrated at 4 pm in the afternoon. The ratio of indigenous phage to enterovirus was given at 3300:1 but no direct relationship between phage and enterovirus was observed, although similar, wide variations in levels of enterovirus were recorded during 24 hour sampling. SHUVAL (1970) reported levels of enterovirus ranging from 5 PFU to more than 11 000 PFU/ml. From published figures by CLARKE *et al.* (1964) and others it is apparent that coliphage in sewage may exceed enterovirus by a factor of 100 to 1000x depending on the treatment process used.

KOTT *et al.* (1974) reported a number of results obtained from investigations into the use of bacteriophages as viral pollution indicators and quote the following ratios of phage to enteric virus:

Flood water	1:1	to	1 000:1
Waste water	100 000	to	1
Trickling filter effluent	Winter		10 000:1
	Spring		100 000:1
	Summer, Autumn		10 000:1

Oxidation pond effluent	Winter	1 000:1
	Spring	10 000:1
	Summer)	1 000:1
	Autumn)	

Chlorination experiments on experimental oxidation pond effluent showed that coliphages were more resistant than poliovirus. The coliphage f2 was very resistant (in agreement with the findings of CRAMER *et al.* (1976)) and MS2 slightly less so. Levels of chlorination which killed off all coliforms (8 mg/litre) hardly affected coliphages.

Commenting on the possible use of coliphage as an indicator of virus in sewage BERG (1962) pointed out that coliphage would not always reflect changes in virus levels because virus may occur in faeces from 0 - 100% frequency depending on season, population, age, etc. In raw waste water the ratio of phage to virus may increase temporarily, therefore if coliphage increased and it was applied as a criterion of effective treatment all effluents would fail. He suggested that a comparison of the survival of indigenous coliphage and poliovirus in all types of waste-water treatment systems would be useful. The presence or absence of host organisms for coliphage in treatment systems has an influence on the coliphage survival whereas enteric virus seems less likely to enter a host-parasite interaction.

RANGANATHAN *et al.* (1974) reported very different survival patterns between coliphage and poliovirus in activated sludge systems. In contrast, GUY & McIVER(1977) using a pilot-scale treatment plant demonstrated that phage and enterovirus removal rates were similar and suggested that phage could be used to indicate virus removal. Incomplete removal of coliphage could indicate incomplete removal of enterovirus with possible resulting health hazards.

Reference has been made earlier to the work of FANNIN *et al.* (1977) in respect of the use of coliforms as indicators of viral presence or risk. However, these authors also evaluated coliphage as a possible indicator of airborne viral contamination from waste-water treatment facilities. They concluded that coliphage would be more acceptable and found that it was generally stable at high relative humidity levels. The mean virus concentration was estimated to be 3.6 to 3.7 logs lower than that of coliphage and the authors estimated that to isolate one virus unit they would have to sample between 15 and 16 x 10^3 m^3 of air; coliphage being more numerous greatly simplified the procedure once small samples were required.

LUND (1969) has queried the use of bacteriophage to model virus in experimental or sewage treatment systems because the T phages, equipped with specific functional structures for the process of adsorption and penetration into the host cell, may well attach to particulate matter in the same way. If this is so it may be counterbalanced to some extent by the greater susceptibility of such phages to mechanical damage. SORBER *et al.* (1972), comparing the effects of the reverse osmosis and ultra filtration processes for sewage treatment, used both coliphage T_2 and Poliovirus I. The authors concluded that the different results they obtained may have been due to tail damage of the T_2 phage particles. Poliovirus being a spherical particle with multiple receptor sites was potentially less likely to suffer random damage.

CARSTENS (1963) reviewed the possible uses of bacteriophage in sewage purification and quoted the work of COETZEE (pers. comm.) who suggested that the phage of *Serratia marcescens* could be used as an indicator of die-off of human viruses in sewage water, rivers and sea. This phage was suggested because the bacterium does not normally occur in sewage and therefore if added to any of the systems mentioned it could

not multiply due to absence of host cell. An expansion of this earlier work followed (CARSTENS *et al.*, 1965) and the conclusion at the end of a lengthy and detailed investigation was that *Serratia marcescens* phage could be used as an indicator of virus survival in sewage and water treatment. The main reservation expressed was the lack of agreement between this investigation and other published work, particularly in respect of the effects of chlorine on various viruses.

4.2 Coliphage in polluted waters

Coliphages have been shown to be present in sewage in numbers greater than corresponding levels of enteroviruses, and appear to be removed during sewage treatment at a rate and in a manner comparable to many enteroviruses but may be more resistant to disinfection procedures such as chlorination. Public health concern is however focussed on the fate of enteroviruses once released into the aquatic environment and it follows therefore that studies should proceed to understand the fate of coliphage in water if we are to make a balanced assessment of their potential as virus models or indices of viral contamination.

In studying the possible factors affecting survival of *E. coli* in sea water CARLUCCI & PRAMER (1960b) looked at coliphage and studied its survival in various waters. Their results (Table 8) showed that coliphage was rapidly inactivated in untreated sea water but persisted in filter sterilized and, even more so, in autoclaved sea water. A similar effect has been noted for many bacteria and some viruses (see section 3.2 and LYCKE *et al.*, 1965), and although no satisfactory explanation has been proposed it would appear that heating possibly breaks down a biotically produced inhibitory substance, whilst filtration removes the agent(s) responsible but leaves inhibitory substances. If this is so one may perhaps postulate that what was said about virus in polluted

water may be equally applicable to phage, i.e. extended survival in 'clean' and grossly polluted waters.

Table 8 Persistence of coliphage in various waters (from CARLUCCI & PRAMER, 1960b)

As far as the author is aware only two studies concerned solely with coliphage in polluted waters have appeared in the literature (SUÑER & PIÑOL, 1967; KOTT, 1966b) and both may be said to represent completely opposite views; particularly on the relationship between coliphage and coliform bacteria (see later section 4.4). KOTT (1966b) expressed the view that the fate of T bacteriophages (coliphages) in sea water was unknown probably because of poor methods for evaluation of small numbers. The method used by CARLUCCI & PRAMER (1960b) was a qualitative estimation only for small numbers of phage in large volumes of sea water. KOTT's study showed that there was a progressive decline in coliphage with increasing remoteness from the source of sewage pollution and this was supported by the findings of SUÑER & PIÑOL (1967). Whether this was due to mortality or dilution or a combination of the two is not clear but one would expect both factors to be important. GUELIN (1948) outlined a procedure for detection of phage in water and noted that they were more numerous in human than animal faeces. In addition there appeared a positive correlation between numbers of phage and the degree of sewage pollution. BUTTIAUX (1951) held the view that isolation of phage specific to pathogenic bacteria in water was a

useful indication of faecal pollution. He stated that, on the basis of 80 000 water samples, when bacteriophages were isolated as the sole indication of contamination later samples from the same sources invariably showed the presence of *E. coli*, faecal streptococci or *Clostridium perfringens (welchii)*.

In the discussion of SUÑER & PIÑOL's paper when it was presented at a meeting in Munich in 1966, T. ARDELEAN commented on the widespread use of bacteriophage for studies of polluted waters in Roumania. He remarked that of 5 000 water samples examined between 40 and 96% contained coliphages and 10 to 68% contained *Salmonella typhi* phages. High values of phage were encountered in the months of December, March, April and November and lower numbers in July, August and September.

In the field situation, VAUGHN & METCALF (1975) examined the practicality of using a coliphage indicator system for human enterovirus in polluted waters. The study included shellfish and the authors found that while coliphage could be isolated from oysters throughout the period March to October, enteroviruses were only isolated during the June to September period. Additionally, 13 of 21 enteric virus isolations were made from samples yielding no coliphage though whether this applied to all samples examined or those of shellfish only is not clear from the paper. Variation in choice of host *E. coli* strains produced different isolation rates of coliphage but no strain of the three tested yielded consistently better results. Similar limitations on the accurate assessment of coliphage in aquatic systems have been noted by HILTON & STOTZKY (1973) and further, more detailed, studies are needed.

4.3 Coliphages in sediments

Much of the work on coliphages in relation to sediments has been orientated towards the use of phage as a model to study the fate of

viruses in soils and not directly concerned with the marine environment. CARLSON *et al.* (1968) reported that up to 99% adsorption of added phage T_2 occurred on common clay materials such as kaolinite, montmorillonite and illite in concentrations of cations that occur in natural waters at pH 7. Virus adsorption to montmorillonite was reputed to occur fairly uniformly over the pH range 3.5 to 9.5 by SCHAUB *et al.* (1974). Natural clays are normally a mixture of clay types and organic matter and virus adsorption to these may be lower (GERBA & SCHAIBERGER, 1975). Significant reduction in the inactivation of coliphage T_7 in sea water plus 75 $\mu g/ml^{-1}$ colloidal montmorillonite was reported by BITTON & MITCHELL (1974). The authors concluded that protection was affected by adsorption of viruses on to colloidal surfaces but that this may not have rendered phage inactive. COOKSON & NORTH (1967) using activated carbon demonstrated that adsorbed virus did not lose its infective ability and CARLSON *et al.* (1968) also reported that adsorption was reversible and dependent on concentration and type of cation present. In sea water, the ionic strength is high and the report of ROPER & MARSHALL (1974) showing how desorption could occur if the electrolyte concentration was diluted below a critical level could explain in part the effects of salinity reviewed earlier. Clay minerals may also absorb lytic enzymes or antiviral toxins produced by antagonistic microflora and as such adsorption to sediment or particulate matter may contribute to some effects formerly attributed to other factors. Failure of virus-coated particulates to sediment has been postulated as a factor contributing to the mobility of virus in water (METCALF *et al.*, 1974). As a word of caution, considerable differences between the behaviour of coliphage and enteroviruses in soils have been reported (LEFLER & KOTT, 1974; GERBA *et al.*, 1975) which may negate the use of coliphage as a model or indicator in such situations.

4.4 Coliphage in shellfish

In the introduction mention was made of the role of shellfish in the transmission of infectious hepatitis and of the concern about potential transmission of human enteroviruses. In view of this established and potential risk it is not surprising that numerous studies have been reported of investigations into the uptake and removal of viruses by shellfish (HEDSTROM & LYCKE, 1963; LIU *et al.*, 1966a,b; MITCHELL *et al.*, 1966; HOFF & BECKER, 1968). In some studies accumulation of virus has not exceeded or even reached the concentration present in ambient waters, but accumulation factors of up to 180x have been reported for poliovirus (HOFF & BECKER, 1968). Studies using coliphage S-13 (CANZONIER, 1971) and the hard clam (*Mercenaria mercenaria*) have yielded accumulation factors from 0.3 to over 1 500 times for some individual animals. CANZONIER (1971) commented that the apparently greater accumulation rates obtained, compared with those of other workers using polio (e.g. MITCHELL *et al.*, 1966), was probably due to two factors: (1) low exposure levels, 1-8 PFU/ml compared with 40-1 000 PFU/ml in experiments by other workers, and (2) high stability and recoverability of coliphage S-13 compared with poliovirus. The author also remarks that there may be considerable variation in the kinetics of uptake of different phages which may pose problems in the application of data obtained from phage experiments to possible hazards arising from the uptake of enteroviruses. Experiments with S-13 and a larger, *Staphylococcus aureus* phage showed that the latter phage was not readily accumulated by clams but was rapidly eliminated by them and quickly inactivated in seawater controls. Laboratory studies by VAUGHN & METCALF (1975) showed that accumulation of coliphage T_7 was 5 to 30 times greater than that of coxsackie B-3.

It has been stated or implied that viruses are eliminated from shellfish by direct physiological activity of the animals but CANZONIER (1971) suggested that accumulation of low titres of S-13 phage over long periods could result in virus retention independent of physiological activity. Similar results were reported by SERAICHEKAS *et al.* (1968) working with poliovirus. The uptake of high initial titres of virus seems to result in rapid elimination in much the same way that faecal bacteria are eliminated, but prolonged exposure to low titres of virus may result in retention. What loss of virus is observed may be due solely to inactivation of virus in the animals.

KOTT & GLOYNA (1965) looked at the bacteriophage content of various samples of commercial shellfish and found that levels were generally very low and proportionately similar to those of coliforms (see Table 9). Laboratory studies by HOFF & JAKUBOWSKI (1966) on development of methods for phage enumeration looked at recovery of phage from shellfish but only used them as test models for experiments they performed.

4.5 Relationship to bacterial indices

The majority of the published work on coliphage and other phages is concerned with their relationship (if any) with the widely used bacterial indicators such as *E. coli* and coliforms. As previously mentioned in section 4.3, KOTT & GLOYNA showed that counts of *E. coli* phage in shellfish were very low compared with those of coliforms (see Table 9) but no field data for comparative levels in shellfish have been published elsewhere.

Comparative data for polluted waters and sewage are more readily available however. GILCREAS & KELLY (1955) presented data for seasonal variations in coliform/phage levels in effluent from two sewage treatment plants and found that both exhibited patternless fluctuations

apparently unrelated either to each other or to the season. The authors also made a number of comparisons between coliphage, *E. coli*/coliforms and other viruses. To test the risks of pollution from effluent seepage through soil the experiment they devised showed that coliphage was reduced to a lesser degree than either *E. coli* or coxsackie and Theiler viruses. Comparative survival rates determined for these various organisms (see section 3.4, Figures 5 and 6) showed that phage was less persistent in water at 8-10°C than other viruses tested but at least 4x more persistent than *E. coli*. Conversely, *E. coli* multiplied at 20-30°C and survived in much greater proportions than either phage or viruses tested. When the same organisms were stored in sewage, mortality of coliphage decreased but *E. coli* increased at a greater rate than when held in water at the same temperature.

Table 9 Count of coliforms and *E. coli* bacteriophage in commercial market oysters (from KOTT & GLOYNA, 1965)

The papers of KOTT (1966b) and SUÑER & PIÑOL (1967) contain much information on the relative abundance of *E. coli*/coliforms and coliphage at various distances from sources of pollution. KOTT (1966b) found that the general pattern of rate of decay of coliform bacteria was equal to the rate of die-off or mortality of coliphage at stations sampled. In presenting his data on probability graphs KOTT demonstrated that the ratio

between numbers of coliforms to phage showed some decrease with increasing distance from a sewage outfall. His results may be summarized as follows:

Station 1 5 metres from pollution source
phage/coliform ratio 1:100

Station 3 150 metres from pollution source
phage/coliform ratio 1:10

Station 4 250 metres from pollution source
phage/coliform ratio 1:16.

The paper by SUÑER & PIÑOL (1967) looked at samples up to 10 000 metres from the coast and found that numbers of *E. coli* and coliphages were essentially the same, i.e. although both decreased the ratio of one to another remained at approximately 1:1. Several criticisms of these findings were made when the paper was presented at Munich in 1966. Few people present at the meeting apparently accepted this 1:1 ratio as a true picture and suggested that the methods used, particularly the receptor strain of *E. coli*, were suspect and perhaps some multiplication of phage had occurred.

One point raised by SUÑER & PIÑOL (1967) was that when bacteriophage was absent from a sample *E. coli* was also absent and vice versa, suggesting, if both were present in a 1:1 ratio, that they disappeared from sea water at approximately the same rate. In this context CARSTENS (1963) quotes a personal communication from COETZEE (1961) about isolations of *Salmonella typhi* and its phage from river water. At the Pretoria sewage works samples were positive for *S. typhi* and its phage; 2½ miles away both *S. typhi* and phage were still present; 4½ miles from the sewage works only phage was present and further down the river still neither *S. typhi* nor phage were present. Human viruses that might have been present at the works could be assumed to have

disappeared before the last sampling point.

Various papers looking at the possible relationships between coliphage and bacterial indicators (particularly coliforms) have concluded that there is no consistent relationship between the two (FANNIN *et al.*, 1977; BERRY & NOTON, 1976; HILTON & STOTZKY, 1973). An interesting paper by KENARD & VALENTINE (1974) is in complete contrast. These authors demonstrated a consistent ratio of phage to faecal coliforms of 0.7 to 1 regardless of the level of contamination in polluted waters (over the range 0.1 to over 6 000/ml). They concluded that phage and coliform numbers were related and although both were related to precipitation, river depth and date sampled, none of these factors affected the observed ratio.

SMEDBERG & CANNON (1976) proposed that the cyanophage CPP-1, a non-pathogenic blue green algal virus, would make an ideal indicator since although its presence in sewage paralleled that of coliforms it was more resistant to chlorination.

In summary, the evidence generally points towards bacteriophage, be it coliphage, *S. typhi* or similar, being far more resistant to exposure to sea water (and therefore more available for uptake by shellfish) than *E. coli* or the bacterial pathogens. The following section accepts that this being so the possibility of using coliphages, the most abundant and easily cultured phages of faecal origin, as an indicator of faecal pollution, is worthy of investigation. It would be presumptious to suggest that this should replace *E. coli* and other bacterial indices but reasonable to hope that coliphage could provide important supplementary information on possible risks from enteric viruses.

There is little doubt that the science of virology will continue to advance rapidly and in the foreseeable future it will be possible

for many laboratories to make routine examinations of sewage, waters and shellfish for viruses. For many laboratories, however, particularly in developing countries, lack of experience and facilities will not permit such development. In these circumstances an alternative indicator which could be rapidly and economically applied with limited laboratory facilities is required but it must be equated with the indicator system already in use.

The review section has examined why *E. coli* is preferred of the possible bacterial indicators and so any assessment of an alternative, more applicable to virus problems, must be made in parallel with an examination of *E. coli*. In the experimental work that follows current knowledge of the fate of *E. coli* in the aquatic environment has been re-examined in a series of experiments designed to make direct comparisons between *E. coli* and coliphage. These studies have been designed to follow the transmission route of enteroviruses, i.e. sewage, water, sediments, shellfish.

5. EXPERIMENTAL WORK

5.1 Overall objectives

In order to make a rational assessment of published work the earlier sections dealing with virus and bacteriophage have been broadly subdivided into sections on sewage, polluted waters and shellfish. Where applicable additional sections dealing with relationships to bacterial indices have also been included and in summarizing the material available a number of avenues of research have been revealed. In making a critical appraisal of the literature certain topics appeared worthy of further enquiry and others appear to have received little or no attention in published work. The objectives of the experimental work described have been set within the same broad framework adopted earlier, i.e. sewage, polluted waters and shellfish, with subdivision of each section into the various avenues explored.

Some aspects of published work have been repeated for comparative purposes, or extended to yield more useful and critical data, while other topics investigated include omissions from any published work known to the present author. As a general comment, it may be said that there are few investigations to date which have attempted a critical appraisal of any indicator, bacterial or viral, in a variety of materials, and it is hoped therefore that the investigations described here could, at least in part, help to remedy that situation and make some contribution to what is considered an important field of study.

5.2 Methods for estimating bacteria, phage, and total plate counts

Details of media used are given in Appendix 1.

5.2.1 Estimates of *Escherichia coli* and coliform bacteria

When the investigations were started the modified roll-tube technique of REYNOLDS & WOOD (1956) was adopted for the enumeration of

E. coli at 44°C and coliforms at 37°C since it is used as a standard method by the Ministry of Agriculture, Fisheries and Food and many laboratories of the Public Health Laboratory Service in the UK. However, as other aspects of the work developed it became apparent that other methods were more suitable in some cases, particularly because of practical considerations such as the number of tubes required and time taken to complete a test. The application of these techniques will be referred to under relevant headings in the sections 5.3 onwards as appropriate, but the details of each method are summarized below.

Roll-tube technique (REYNOLDS & WOOD, 1956) The method involved preparing serial decimal dilutions of samples in 0.1% (w/v) peptone water diluent. One ml aliquots of sample were then inoculated into a roll tube containing 4 ml of MacConkey Agar No. 3 (Oxoid). Normally 10 roll tubes were inoculated in this way from each sample material, or dilutions thereof as appropriate, 5 incubated at 44°C for 24 hours and 5 at 37°C for 24 hours. Counts of red, lactose fermenting colonies were taken as estimates of the viable *E. coli* and coliform counts respectively and results expressed as the mean/ml of sample examined (with appropriate corrections for dilutions where necessary). This method was used primarily for the examination of shellfish samples taken from natural sources, and in some initial work on sewage.

Multiple tube or Most Probable Number (MPN) This was performed in MacConkey Broth, dispensed into capped 20 x 150 mm test tubes, fitted with inverted Durham tubes. Normally 4 rows, each of 5 tubes were inoculated, the first row with 1 ml of sample and subsequent rows with decimal dilutions of sample, i.e. Neat, 10^{-1} 10^{-2} 10^{-3} corresponding to 1, 0.1, 0.01 and 0.001 ml of sample. These tubes were incubated at 37°C for 24 hours, production of acid and gas recorded as positive and the

results for coliforms computed using MPN tables (Report 71, 1969). Estimates of *E. coli* were obtained by removing a loop of each positive broth into Brilliant Green Bile Broth at 44°C for 24 hours. Production of gas was recorded as positive and the MPN for *E. coli* computed by using MPN tables as before. Results for both coliforms and *E. coli* were expressed as the MPN per ml multiplied by the appropriate dilution factor. This method has advantages of detecting small numbers of bacteria in relatively large volumes (accommodated by the use of double strength broth) but has a very large sampling error. Confidence limits for the MPN are given in tables in International Standards for Drinking Water (WHO, 1963), but for the 15-tube method the upper limit may be between twice and three times the MPN and the lower limit between one-quarter and a third of the MPN. Although it is possible that actual values could lie outside these limits, the upper limit can, for practical purposes, be regarded as the maximum number of bacteria the sample might contain.

MILES and MISRA (drop count) method This method (MILES & MISRA, 1938) proved particularly useful in experimental work where high numbers of bacteria were expected and therefore where a number of dilutions were required. Drops of sample or dilutions of sample were inoculated on to the surface of MacConkey Agar plates from glass pasteur pipettes subsequently calibrated and delivering 0.02 ml (i.e. 1/50 ml) per drop. Wherever possible five drops of each sample or dilution, equivalent to 0.1 ml, were used to reduce sampling error. Plates inoculated in this way were left on the bench until the drops had been absorbed into the agar and then incubated for 24 hours at 37°C and 44°C for coliforms or *E. coli* as required. Counts of red, lactose fermenting colonies made under a low power binocular microscope were taken as estimates of viable

bacteria (coliforms or *E. coli*). Direct colony count methods were generally favoured because they have advantages of being reasonably specific (CLEGG & SHERWOOD, 1939), having a good yield, producing a result in 18-24 hours and being economic in time and labour.

Membrane filtration technique Large sample volumes can be most readily examined by the membrane filtration technique and high levels of sensitivity can be achieved without undue sacrifice of accuracy, since the method yields a direct colony count after incubation. The method is now increasingly used for the examination of water, yielding a rapid, reliable result and is suitable for examination of large numbers of samples at any one time. Some media and methods have been described elsewhere (DHSS, 1969) but in the present work membranes were incubated on an enriched teepol broth (MWB, 1967). The recommended teepol concentration was increased 10-fold from 0.4 to 4%. Earlier work by HALLS & AYRES (1974) had shown that the 4% concentration made the medium more selective for *E. coli* in the examination of seawater samples without reduction in yield. Sample volumes up to 100 ml were filtered through a 0.45 µm pore size 5 cm membrane and the filter placed on a pad impregnated with 4% enriched teepol broth. Incubation at 30°C for 4 hours to resuscitate any attenuated organisms was followed by 14-18 hours at 44°C ± 0.2°C in a water bath. Membranes and pads were incubated by placing them in purpose-made plastic trays in a weighted heavy gauge polythene bag. After incubation, yellow lactose fermenting colonies were taken as *E. coli* type I. (Characterization of such colonies showed some 82% to be *E. coli* type I and a further 11% Irregular type I (HALLS & AYRES, 1974).) The method is unsuitable for the examination of shellfish due to the high suspended matter content of this material which clogs membranes.

5.2.2 Estimates of coliphage

Initial experiments in the laboratory suggested that plaque counting methods for phage were of limited use on experimental material partly due to the relatively small sample volumes which could be examined and often because plaques were masked by overgrowth of bacteria from the samples (e.g. sewage). The MPN multiple tube technique described by KOTT (1966a) was adopted in all early experiments with sewage using a phage assay broth of the following composition: beef extract 3 g; peptone 5 g; sodium chloride 5 g; magnesium sulphate 0.2 g; manganese sulphate 0.05 g; and distilled water to 1 000 ml (autoclaved at 15 psi for 15 minutes).

Each of 5 tubes containing 10 ml of double strength broth was inoculated with 10 ml of sewage sample. A second set of 5 tubes containing 10 ml of single strength broth was inoculated with 1 ml of sample, and a third set with 1 ml of a 1:10 dilution. Where applicable the 10 ml inocula were omitted and a set using 1 ml of a 1:100 dilution used instead (i.e. 1, 0.1, 0.01 ml). To each tube was added 0.1 ml of an overnight *E. coli* B culture (approximately 10^7 cells), mixed thoroughly and incubated at 37°C for 24 hours. For confirmation of phage loopfuls of each MPN broth were placed on the surface of nutrient agar plates, surface seeded with *E. coli* B. Phage could be detected after 6 hours' incubation at 37°C. Incubation of plates at 20°C and 30°C was evaluated but although the plaques were very distinct they took longer to produce and yielded lower counts than similar plates incubated at 37°C. The MPN method obviously had advantages for the enumeration of overall numbers of coliphage, e.g. in water as used by KOTT (1966a), because of the sample volumes which could be accommodated. However, MPN methods as discussed in 5.2.1 may give estimates which lie within a wide range of the true values. It was considered that more

accurate and direct methods were required for some aspects of the planned investigations and also that the tube method would impose considerable practical and logistic problems when large numbers of samples needed to be examined.

For the majority of work described here a modified overlay technique (ADAMS, 1959) was applied. Nutrient agar (NA) plates were poured and dried to remove excessive moisture. A small bijou bottle containing 2.5 ml of a soft agar was melted and held at 47°C. Just prior to use, 0.15 ml of an overnight *E. coli* host culture was added, plus 0.1 ml of sample inoculum, mixing them together by rotating the bottle rapidly between the hands. This was poured rapidly on to the surface of a prepared NA plate and left to cool and gel. Plates prepared in this way were inverted and incubated at 37°C overnight prior to plaque counting.

Concentrated phage suspensions were prepared for experimental use by adopting the same procedure and selecting those plates producing confluent plaques, i.e. complete lysis of the host cells. Two ml of phosphate buffered saline were pipetted on to the surface of such plates and incubated with occasional gentle agitation for 1 hour at 37°C. The liquid was then pipetted off and treated with a few drops of chloroform to kill any bacterial cells before centrifugation and collection of the aqueous phase. In this way high yields of phage, often in the range 10^{10} to 10^{12}/ml were obtained. All phage suspensions were stored at 4°C for future use.

5.3 Sewage

5.3.1 Introduction, objectives and methods

From a public health viewpoint, sewage is the start as it were for exploring existing and possible problems in polluted waters and ultimately in shellfish. It was noted in an earlier section (3.1) that domestic sewage effluent can be recognized as the major source of pathogens (both bacterial and viral) of human origin which may be detected in marine situations. It is pertinent therefore to examine sewage in some detail in order to determine some baseline information on which any subsequent enquiry can be constructed. To obtain maximum benefit from the sampling effort much of the work to be described below was designed to incorporate a number of facets of investigation together. One of the first priorities was to look at the natural distribution of coliphage in sewage in relation both to numbers of bacteria (*E. coli* and total plate count) and the time of year to determine any seasonal differences.

A local sewage treatment works was adopted for sampling and the effects of various stages of the sewage treatment process on both phage and *E. coli* numbers on a seasonal basis were studied. With a view to considering in some detail the survival of these organisms in sea water certain of the sewage samples were retained after preliminary examination in order to study survival patterns in pre-discharge situations. In summary, the investigations with sewage were broadly divided under the following headings, each closely interrelated:

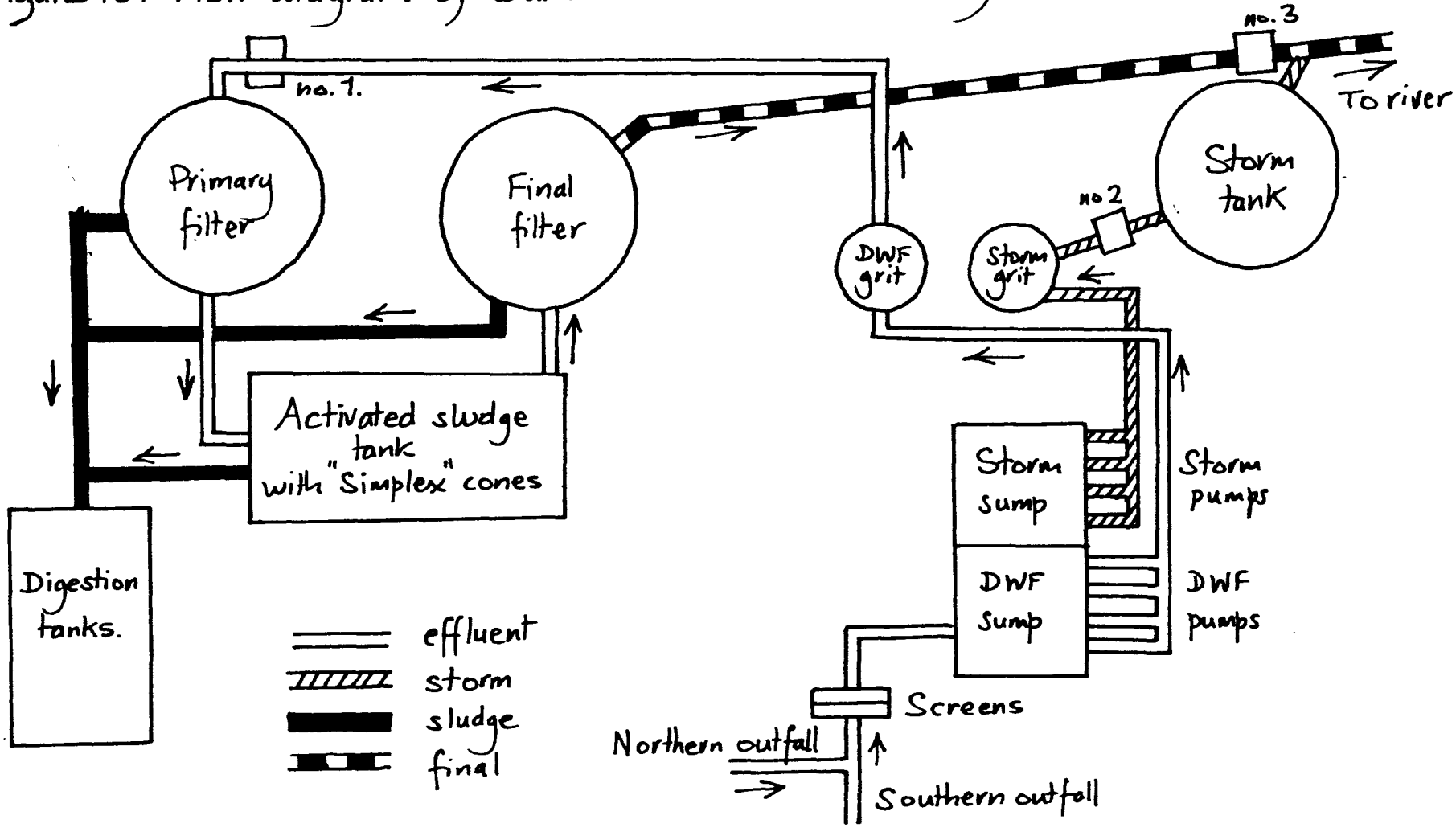
1. Effect of sewage treatment on coliphage.
2. Seasonal distribution of coliphage in sewage.
3. Relationship between coliphage, *E. coli* and total plate count.
4. Survival of coliphage in stored effluent.

5.3.1.1 Effect of sewage treatment on coliphage

Potentially one of the most useful applications of coliphage as an indicator of enterovirus is in sewage itself since this is the primary vehicle whereby viruses are distributed to sea water, sediments, and ultimately to shellfish. The effectiveness of sewage treatment prior to effluent discharge is vital in removing, or at least reducing, this risk. In an earlier section published work showed that, while conventional sewage treatment methods remove a substantial part of the enterovirus loading, sterilization by some means is necessary to produce a virus-free effluent. In practice this is rarely achievable and it is important to have some measure of the potential risk which remains in the effluent. Published work, reviewed earlier, confirms that of the conventional treatment processes in current use those based on the activated sludge principal are particularly effective for virus removal. It was fortunate that the local sewage works used for these tests was of this type. While it was not possible to make estimates of enterovirus levels, the objective was to make a detailed comparison between coliphage and *E. coli* and then try to correlate this with published virological data.

To determine the effects of sewage treatment on coliphage and *E. coli* weekly samples of effluent were taken at various points throughout the treatment works, which is shown in Figure 10 and also described in the Appendices (Appendix 2). Samples were taken with a polythene cup mounted in a wire holder to form a dip sampler and decanted from this into sterile 250 ml glass medical flat bottles. The dip sampler was rinsed in the effluent being sampled at each stage to avoid one sample influencing another. Samples were returned to the laboratory and examined within an hour of collection. *E. coli* were estimated by roll-tube method and phage using the soft agar overlay method with

Figure 10. Flow diagram of Burnham-on-Crouch Sewage Treatment Works.



E. coli B (NCIB 9484) host (methods detailed in section 5.2). Sampling points were as follows: (1) raw influent; (2) grit trap; (3) primary filter; (4) trough (activated sludge tank); (5) final filter; (6) final effluent, and each was examined at weekly intervals for one year to fulfil requirements of 5.3.1.2.

5.3.1.2 Seasonal distribution of coliphage in sewage

While it was clearly important to study the effects of sewage treatment as a process it was considered equally important to look at factors which might influence the overall performance of the treatment works under study. Published work has shown that within the entero-virus group as a whole particular virus types may be seasonally predominant in sewage. Limited work on coliphage in sewage has suggested similar seasonal and even daily variation in the indigenous coliphage population. In this current study it was not practical to sample very frequently without prejudice to either aspects of the planned investigation and so this work was combined with the objective stated in 5.3.1.1, by increasing the sampling frequency and total time span beyond that required by objective 5.3.1.1 alone.

In terms of methodology, sampling etc. was performed exactly as described under 5.3.1.1. Sampling was continued throughout the year in order to examine any seasonal trends, both in overall performance and in the individual treatment stages.

5.3.1.3 Relationship between coliphage, *E. coli* and total plate count

A few of the studies concerned with the survival of virus in sea water have examined the influence of total bacterial plate count and possible role of bacteria in producing anti-viral compounds or exhibiting anti-viral properties. No such studies seem to have been documented with sewage although there are some data on total plate counts of sewage in

experimental or pilot-scale treatment plants. In conjunction with the work described in sections 5.3.1.1 and 5.3.1.2 studies of phage, *E. coli* and total plate counts were proposed over a limited period to evaluate what, if any, association might be found between them.

Over a four-month period from December to March the weekly samples taken for objectives 5.3.1.1 and 5.3.1.2 were also examined for total viable plate counts of bacteria. Using the Miles and Misra drop-counting technique, estimates were made on each of the six samples representing stages of sewage treatment at weekly intervals. Plates of Casitone Glycerol Yeast Extract Agar (CGYE) and Nutrient Agar (NA) were inoculated in triplicate with appropriate serial dilutions of sample and incubated at 20, 30 and 37°C. Colony counts were made at daily intervals under a low power binocular microscope until no further increase in count was observed.

5.3.1.4 Survival of coliphage in stored primary effluent

Although activated sludge treatment is probably the most efficient conventional sewage treatment process for virus removal the solution of one problem (i.e. removal of virus from the liquor phase) creates another problem. Much of the virus so removed ends up in the sludge which then has to be safely disposed of either by incineration, dumping at sea or by land disposal as a fertilizer. Clearly the disposal to sea or land may pose additional health hazards. Ideally it would have been desirable to examine sludge at intervals but because of operational difficulties regular access to sludge from the works under study was not possible. However, in the treatment plant the bulk of sludge produced is derived from the primary settlement/filtration stage and from the activated sludge tank itself. To obtain data on potential survival of coliphage (in comparison with *E. coli*) in sludge, the samples of

primary effluent (i.e. before settlement) were used. In all these samples suspended solids accounted for over 33% of the settled volume and while not strictly comparable with sludge samples they offered a useful alternative for study.

Samples of primary effluent taken as part of the studies reported under sections 5.3.1.1 to 5.3.1.2 were retained after initial examination and stored in 250 ml (8 oz) glass medical 'flats', in a dark refrigerator at 8°C. Examination for *E. coli* and 'total' coliphage was carried out at intervals using the methods described earlier. *E. coli* B (NCIB 9484) was used as the host bacterium for plaque assay of coliphage. Sampling was carried out by shaking the bottle thoroughly to resuspend settled solids and a small aliquot (usually 5 ml) removed for examination. This was continued at intervals until such time as numbers of phage and *E. coli* had declined below the limits of detection by the method used, or in the case of phage until it was no longer feasible to continue sampling. An average of three samples per month, a total of 39 in a year, were stored and repeatedly sampled in this way. Since sampling from some bottles continued for over a year wherever possible all bottles stored at any one time were examined simultaneously, usually on the same day rather than on a fixed time interval.

5.3.2 Results

5.3.2.1 Effects of sewage treatment

As a preliminary study a complete series of samples was taken from the sewage works and sampling points referred to in 5.3.1.1. These samples were examined for *E. coli*/coliform content by the MacConkey Broth MPN method, using Brilliant Green Bile Broth at 44°C for confirmation of *E. coli*. The MPN method of KOTT (1966a) was used for estimates of coliphage as described in 'Methods' (5.3.1). The data obtained are

shown plotted in Figure 11 together with data for virus obtained by BLOOM *et al.* (1959) in a sewage plant of similar type to that sampled in the present investigation. The reduction of *E. coli* and coliphage was similar to that reported for enterovirus. In detail, a small reduction in numbers of *E. coli* and phage occurred between the inlet to the works and the primary filter. At the activated sludge stage a tenfold reduction in both *E. coli* and coliphage was observed and the decline continued through to the final filter. Final values showed that from raw to fully treated effluent there was a tenfold reduction in numbers of coliforms, a 40-fold reduction in *E. coli* and a 40-fold reduction in coliphage. With a single test like this it was considered desirable to repeat the investigation over a long period to accommodate any possible seasonal variation.

For regular sampling it was considered impractical to use MPN methods because of the equipment and manipulation required. For further work described below it was decided to use direct count methods, sampling every week at six sites throughout the sewage plant (see 5.3.1.1).

The complete results obtained from the sampling programme are given in the Appendices (Appendix 3) but have been summarized in Tables 10 and 11 by calculating the percentage of the original influent count at various stages of treatment. The results for *E. coli* (Table 10) and total coliphage (Table 11) show that there is a very wide variation in the results obtained both within the different treatment stages and between the stages themselves. The range of values and calculated means are presented in graphical form in Figure 12. Both *E. coli* and total coliphage estimates increased in the grit trap which is the only part of the process where active mechanical pumping occurs. It is likely that this procedure, which involves considerable agitation, breaks up faecal particles and liberates both *E. coli* and phage into

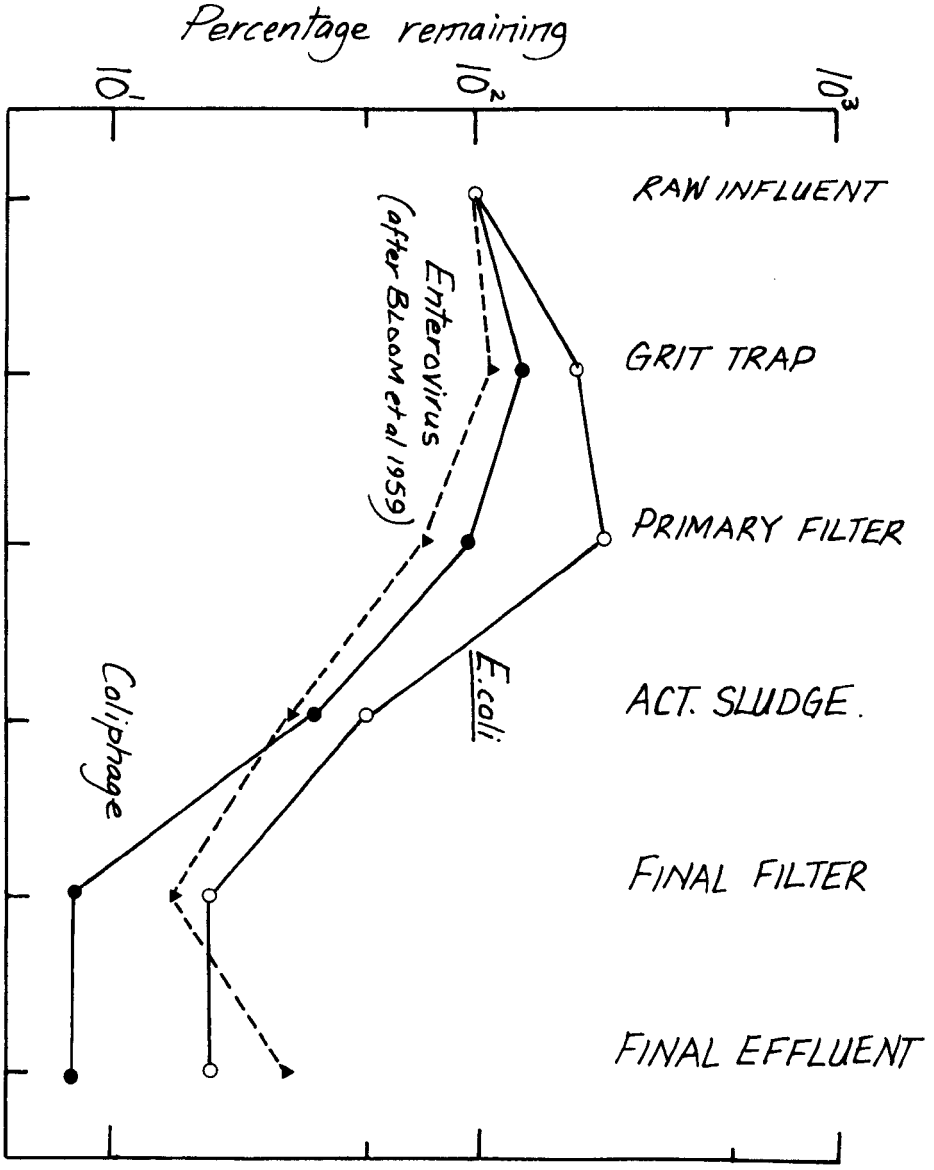


Figure 11. Effect of sewage treatment on E.coli, coliphage and enterovirus.

Table 10 Effect of sewage treatment on reduction of *E. coli* (expressed as a percentage of raw influent count remaining)

Date	Raw influent	Grit trap	Primary filter	Activated sludge tank	Final filter	Final effluent
1 Jun	100	-	-	-	-	76.19
7 "		468.75	284.38	22.19	7.81	10.63
15 "		91.46	8.54	6.59	0.1	0.07
21 "		62.5	31.25	10.0	0.21	0.44
27 "		-	-	-	-	-
4 Jul		175.56	186.67	72.33	13.11	9.56
11 "		54.17	58.33	22.5	0.58	0.67
19 "		354.84	209.68	37.1	2.9	1.94
27 "		80.91	136.66	2.45	1.55	1.82
2 Aug		73.85	92.31	30.0	6.38	4.54
11 "		138.3	163.83	23.4	18.72	1.72
18 "		57.81	-	6.25	1.17	1.36
24 "		46.15	38.46	138.46	0.15	1.15
31 "		160.47	165.12	111.63	20.0	19.53
7 Sep		108.0	128.0	20.8	2.2	1.52
14 "		106.59	109.89	67.03	6.26	6.04
21 "		83.33	100.0	62.5	3.17	4.42
5 Oct		357.14	250.0	23.57	8.21	6.79
11 "		-	-	-	-	-
19 "		263.89	202.78	94.44	69.44	38.89
24 "		100.0	150.54	39.78	39.78	18.28
15 Nov		66.0	65.33	18.00	10.0	4.2
25 "		118.18	63.64	25.45	0.16	0.13
30 "		33.33	20.83	29.17	3.19	2.78
6 Dec		-	-	-	-	-
13 "		138.99	111.11	11.67	4.56	0.89
20 "		123.08	70.77	13.85	0.38	1.08
3 Jan		122.22	88.89	33.33	1.94	1.94
10 "		166.67	91.67	29.17	0.67	1.08
17 "		127.17	103.26	51.09	0.41	0.57
24 "		235.71	107.14	23.57	1.93	1.57
31 "		100.0	37.22	15.56	1.11	2.17
7 Feb		85.71	47.62	13.81	0.17	0.36
14 "		109.09	60.91	70.0	0.79	8.82
21 "		88.89	72.22	16.67	0.51	1.56
28 "		105.88	70.59	70.59	2.12	1.94
7 Mar		92.11	50.0	17.37	27.89	0.02
14 "		57.14	36.67	14.29	0.13	0.15
21 "		128.57	85.71	46.43	0.11	0.19
28 "		125.0	162.5	58.75	2.75	1.88
4 Apr		114.29	57.14	33.33	0.52	1.19
11 "		66.67	39.58	21.67	0.21	0.09
25 "		94.12	58.82	-	3.82	4.41
1 May		83.33	43.33	66.67	1.58	2.92
9 "		51.61	75.81	16.13	2.58	4.68
15 "		45.63	34.38	28.13	2.31	1.75
22 "		100.0	94.74	14.74	0.43	0.01
Range of values						
Low		33.33	8.54	2.45	0.10	0.01
High		468.75	284.38	138.46	69.44	76.19
Mean		124.72	96.82	36.44	6.33	5.73

Table 11 Effect of sewage treatment on reduction of coliphage (expressed as a percentage of raw influent count remaining)

Date	Raw influent	Grit trap	Primary filter	Activated sludge tank	Final filter	Final effluent
23 May	100	496	1240	240	ND	112
31 "		209.7	8	0.23	0.17	0.05
1 Jun		225	52.8	4.72	1.11	0.83
7 "		289.6	130.17	5.17	4.3	3.45
15 "		184	128	6.0	2.0	4.0
21 "		196	538.5	103.8	26.9	42.3
27 "		-	-	-	-	-
4 Jul		45.7	325.7	140	40	14.3
11 "		310.2	257	90.8	2	7.14
19 "		39.6	226.42	33.96	33.96	22.64
27 "		25.68	93.24	45.95	14.86	12.16
2 Aug		350.32	124.2	1.27	1.91	1.27
11 "		109.36	90.64	9.15	2.77	1.91
18 "		89.89	19.55	11.01	17.98	4.83
24 "		76.71	153.42	19.18	8.22	5.48
31 "		78.9	300.92	11.01	12.84	15.6
7 Sep		304.55	636.36	134.09	43.18	22.73
14 "		22.84	67.38	10.49	2.47	1.23
21 "		142.86	121.43	28.57	< 3.57	< 3.57
5 Oct		143.66	164.79	21.13	< 2.82	2.82
11 "		-	-	-	-	-
19 "		167.91	350.75	17.16	29.1	30.97
24 "		94.38	130.34	32.58	16.85	11.24
15 Nov		231.58	384.21	73.68	31.58	52.63
25 "		36.36	190.91	36.36	36.36	36.36
30 "		21.43	67.86	57.14	10.71	14.29
6 Dec		63.81	132.38	118.1	18.1	24.76
13 "		50.67	52.67	29.33	12.67	12.0
20 "		71.43	440	25.71	11.43	20.0
3 Jan		238.46	86.15	11.54	< 0.38	0.77
10 "		222.22	37.56	8.0	0.89	0.22
17 "		106.76	39.86	14.19	< 0.68	1.35
24 "		735.29	121.57	56.86	< 0.98	< 0.98
31 "		150.94	284.91	10.38	3.77	14.15
7 Feb		-	-	-	-	-
14 "		108.21	261.19	62.31	1.12	55.6
21 "		62.74	35.85	8.96	< 0.47	1.42
28 "		287.27	140	27.27	3.64	3.64
7 Mar		500	282.14	7.14	12.5	64.29
14 "		37.5	262.5	75	2.5	20
21 "		206.35	131.75	39.68	0.79	1.59
28 "		24.44	135.56	266.67	7.78	11.11
4 Apr		141.67	18.06	4.17	0.28	0.28
11 "		89.29	39.29	30.36	3.57	1.79
25 "		200	1500	140	50	40
1 May		171.43	342.86	57.14	<14.29	28.57
9 "		-	-	-	-	-
16 "		66.67	166.67	44.44	50	33.33
22 "		721.65	75.26	10.31	1.55	2.58
Range of values						
Low		21.43	8.00	0.23	0.17	0.05
High		735.29	1500	266.67	50.0	112.0
Mean		181.09	230.86	48.46	<20.53	<16.94

the liquid phase of the effluent. Thereafter the works is entirely gravity-fed through the primary filter into the activated sludge tank and the *E. coli* levels exhibit a progressive decline from the grit trap through to the final effluent. Other than some retention in the pipework there is no further treatment after the final filter and not unexpectedly therefore there is little reduction between final filter and the discharged effluent. Total coliphage continued to increase until after the primary filter when levels dropped rapidly and progressively until discharge from the works. The initial increase may in part be due to mechanical disruption of sewage solids but between the grit trap and primary filter an increase of some two and a half times the raw influent count may indicate some phage multiplication within the system.

In general terms, it is interesting to compare these results in Figure 12 with those of the pilot experiment shown in Figure 11, which are remarkably similar.

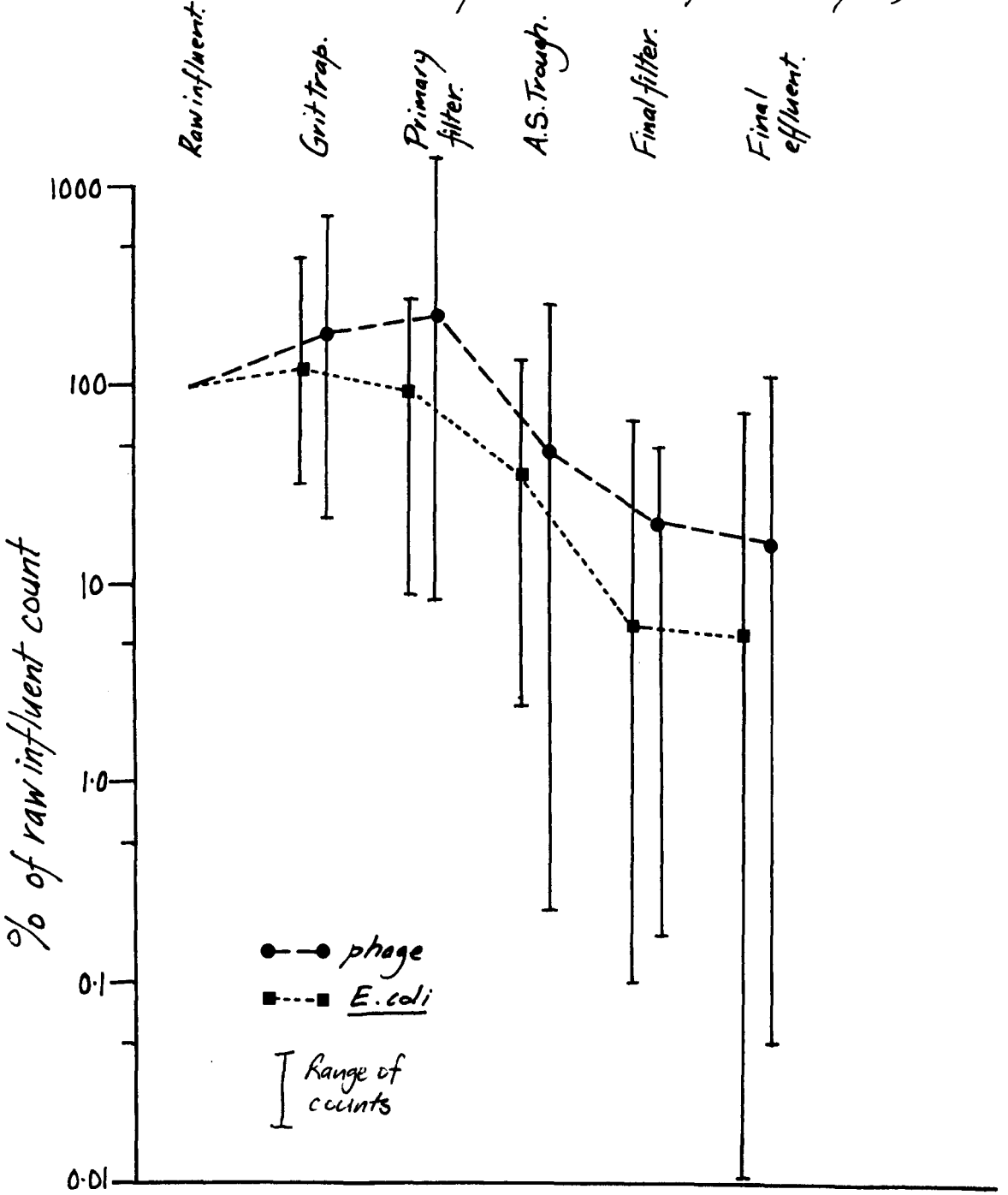
It was apparent from the results shown in Tables 10 and 11 that although both *E. coli* and coliphage were reduced by treatment a greater percentage of the initial phage input remained after treatment. In order to quantify this, the ratios of phage to *E. coli* at different treatment stages were calculated and are shown in Table 12.

Table 12 Effects of sewage treatment on the phage/*E. coli* ratio

	Ratio phage/*E. coli**
Raw influent	1 : 513
Grit trap	1 : 570
Primary filter	1 : 392
Activated sludge trough	1 : 508
Final filter	1 : 229
Final effluent	1 : 152

*based on the results of 47 weekly observations.

Figure 12. Effect of sewage treatment on coliphage and E.coli. (based on weekly observations for one year.)



From an initial ratio of one phage particle to 513 *E. coli* in raw influent sewage the phage to *E. coli* ratio in final effluent was 1:152. Of particular interest is the manner in which the ratio changes at different treatment stages which may be reflecting differential reduction of the organisms, i.e. different removal efficiencies of the treatment processes.

5.3.2.2 Seasonal distribution

Results of the weekly sampling of various treatment stages in the sewage works, included as Appendix 3, were subject to a preliminary appraisal. It was apparent from the data that counts of *E. coli* and coliphage varied in a random fashion at all stages of treatment so it was decided to take the raw influent and final effluent data as indicative of the changes which occurred. The *E. coli* data are shown plotted in Figure 13 over one year of sampling. Influent counts ranged from 1.3×10^4 to 6.4×10^5 but showed a tendency to be grouped around the mean, so fluctuations were generally small. In contrast, the effluent counts exhibited very wide fluctuations throughout the year which suggests that plant performance or efficiency is a key factor. Values for *E. coli* ranged from 2.5×10^1 to 4×10^4/ml. In the six months of the year from July to December influent and effluent levels followed the same trends suggesting that the performance of the works had stabilized after a more erratic performance in the early part of the year.

The coliphage data have been plotted in Figure 14 and although both influent and effluent levels vary considerably there is a good measure of agreement in the trends shown, particularly again during the period July-December. Influent levels ranged from > 5 to 1.25×10^5 PFU/ml and effluent levels from > 5 to 7.45×10^2 PFU/ml.

Figure 13. Seasonal variation in *E.coli* content of sewage

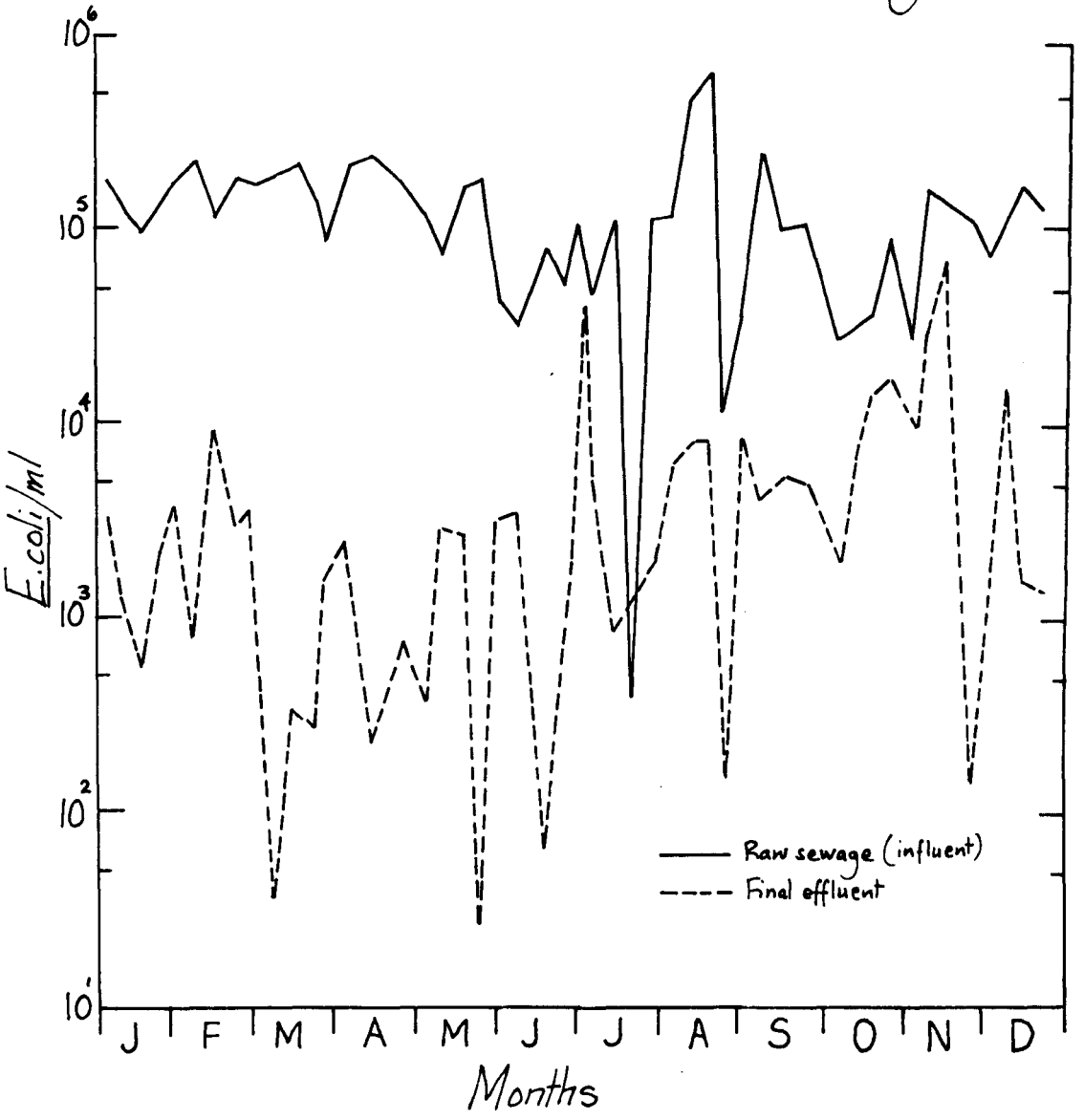
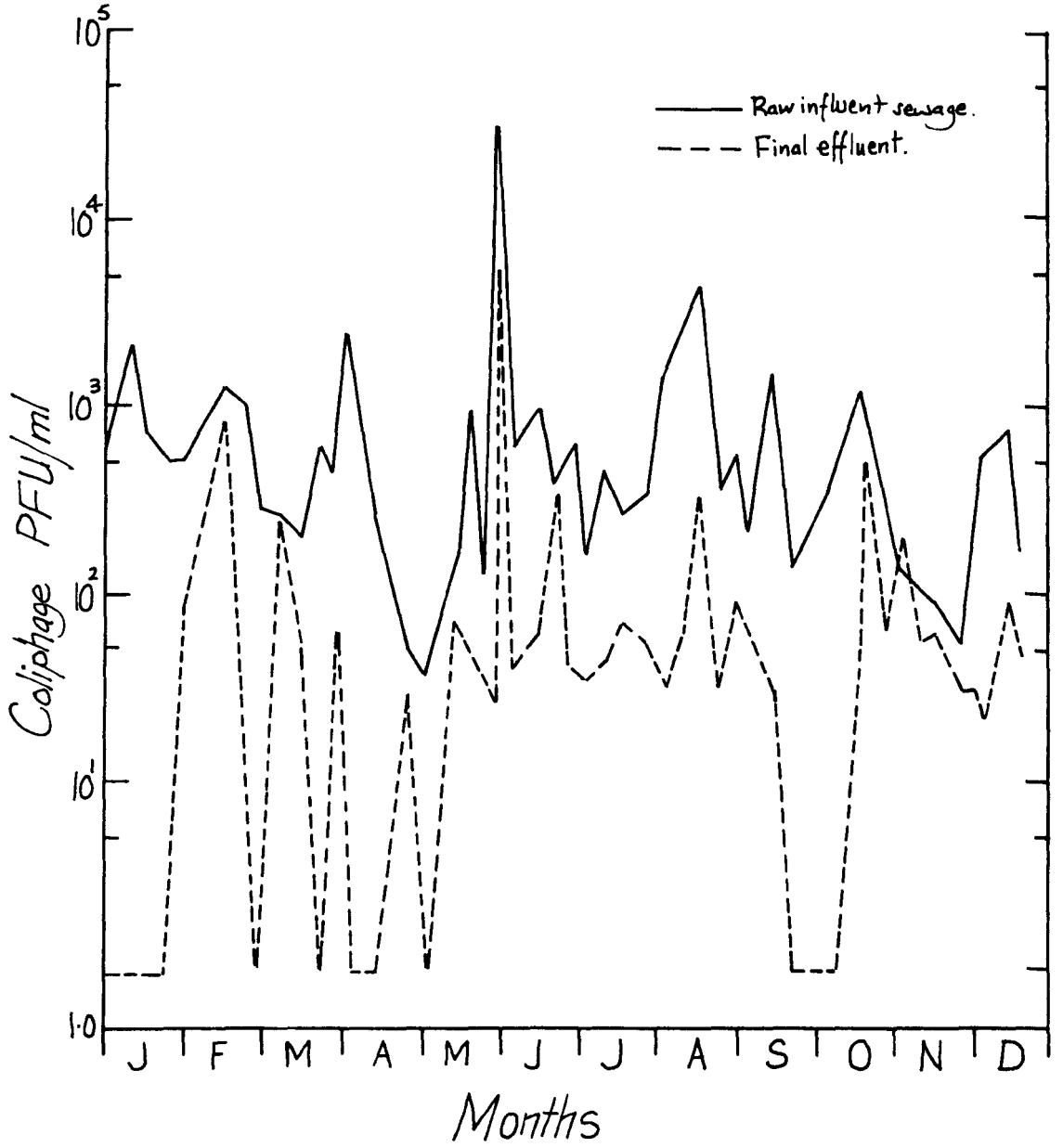


Figure 14. Seasonal variation in coliphage levels in sewage.



The sewage works at which the sampling was carried out did not unfortunately keep regular flow records or other data which could be used as an aid to interpretation of the results obtained. However, such limited dry weather flow (DWF) data as were available did not suggest any correlation with either *E. coli* or coliphage content at any stage of treatment, nor indeed at any particular time of year. Particular note was kept prior to sampling of any heavy rainfall since it was visualized that this would dilute the effluent and cause considerable flushing effects in the sewers serving the treatment works. Again, however, no such correlation appeared in the either abnormally wet or dry periods which were noted to occur during the year.

From discussions with the sewage works' manager it was revealed that as a newly commissioned works and one of a type not normally serving such a small population, considerable teething troubles had occurred. Low flows made stabilization of the activated sludge tank difficult and for the early part of the year its performance varied widely. In addition, the illegal introduction of solvents from local glass fibre boat building into the sewage system had poisoned the activated sludge. This occurred on at least two occasions in the early part of the year, during which time the system was either completely inactive or below its design performance.

5.3.2.3 Relationship between coliphage, *E. coli* and total plate count

From the year-long sampling programme reported for 5.3.2.1/ 5.3.2.2 a series of samples covering some five months was subject to analysis for total plate counts in addition to *E. coli* and coliphage. The full results are given in Appendices 4 to 10 but have been summarized by taking the range of values and means for each set of data. Table 13 shows results obtained for *E. coli* and total coliphage.

Table 13 *E. coli* and coliphage estimates on sewage samples examined for total counts

Sample point	*E. coli*			Coliphage		
	No. of observations	Range of observations count/ml	Mean/ml	No. of observations	Range of observations count/ml	Mean/ml
Raw influent	16	7.2×10^4 to 2.1×10^5	1.49×10^5	16	1.4×10^2 to 2.25×10^3	6.57×10^2
Grit trap	17	1.63×10^3 to 3.3×10^5	1.61×10^5	17	3×10^1 to 3.75×10^3	1.11×10^3
Primary filter	17	1.5×10^4 to 2×10^5	1.06×10^5	17	9.5×10^1 to 3.5×10^3	7.61×10^2
A.S. trough	17	1.8×10^4 to 1.2×10^5	4.52×10^4	16	4.5×10^1 $\times 1.2 \times 10^3$	2.68×10^2
Final filter	17	2.7×10^2 to 5.3×10^4	5.45×10^3	16	$<2.5 \times 9.5 \times 10^1$	$<2.42 \times 10^1$
Final effluent	17	3.3×10^1 to 1.5×10^4	2.95×10^3	16	<5.0 to 7.45×10^2	$<8.91 \times 10^1$

Total plate counts made at 20, 30 and 37°C on Casitone Glycerol Yeast Extract Agar (CGYE) are shown in Table 14 and observations made with Nutrient Agar (NA) in Table 15. In order to compare the results obtained *E. coli*, phage and total plate counts were graphed and are expressed in Figures 15 and 16. Total plate count observations made at 30°C were omitted from the graphs for the sake of clarity but follow closely the trends shown by 20°C and 37°C counts.

Plate counts at 20°C on both media were higher than counts made at 37°C but followed the same trends; there were no major differences between the results on CGYE and NA. There was an overall reduction in total plate count from influent to effluent, i.e. during treatment, but both media demonstrated an increase in count at the activated sludge stage. This is expected since the activated sludge is essentially a culture system of microorganisms being actively mixed and aerated. CGYE agar gave higher counts than NA at this stage which was again not unexpected as CGYE agar was developed for estimating bacteria in activated sludge processes.

E. coli and coliphage results were essentially as reported for an earlier section (5.3.2.1), a slight increase at the grit trap followed by a progressive decrease during subsequent treatment. The apparent increase in coliphage at the end of treatment is probably an artifact because some observations were below detectable levels and the means are accordingly 'less than' the values shown on the figures.

5.3.2.4 Survival of coliphage in stored effluent

The raw data obtained from the studies on stored primary effluent have been summarized in Table 16 to show the initial coliphage and *E. coli* content of the samples as collected from the treatment plant and the period, in days,

Table 14 Total plate counts of sewage on Casitone Glycerol Yeast-extract agar incubated at 20, 30 and 37°C

Sample point	Incubation temperature (°C)	No. of observations	Range of observations (TPC/ml)	Mean count/ml
Raw influent	20	17	2.9×10^6 to 1.9×10^7	6.3×10^6
	30	17	2×10^6 to 1.8×10^7	5.92×10^6
	37	17	9.0×10^5 to 3.0×10^6	1.47×10^6
Grit trap	20	17	1.2×10^6 to 1.9×10^7	6.54×10^6
	30	17	1.8×10^6 to 1.5×10^7	6.31×10^6
	37	17	1.2×10^6 to 3×10^6	1.82×10^6
Primary filter	20	17	2.6×10^6 to 1.1×10^7	4.34×10^6
	30	17	1.8×10^6 to 8×10^6	3.66×10^6
	37	17	5.7×10^5 to 2.9×10^6	1.49×10^6
A.S. trough	20	17	3.3×10^6 to 6.5×10^7	2.05×10^7
	30	17	2.4×10^6 to 3.6×10^7	1.57×10^7
	37	17	1.2×10^6 to 1.7×10^7	4.74×10^6
Final filter	20	16	5.5×10^3 to 1.8×10^7	1.45×10^6
	30	16	6.7×10^3 to 1.3×10^7	1.05×10^6
	37	17	2×10^3 to 2.4×10^6	2.65×10^5
Final effluent	20	16	1.6×10^4 to 3.9×10^6	7.91×10^5
	30	17	1.1×10^4 to 3.0×10^6	6.44×10^5
	37	17	5×10^3 to 1.8×10^6	2.5×10^5

Table 15 Total plate counts of sewage on Nutrient agar incubated at 20, 30 and 37°C

Sample point	Incubation temperature (°C)	No. of observations	Range of observations (TPC/ml)	Mean count/ml
Raw influent	20	24	3.4×10^6 to 3.3×10^7	1.03×10^7
	30	24	2.4×10^6 to 1.8×10^7	5.88×10^6
	37	24	1.3×10^6 to 6×10^6	2.61×10^6
Grit trap	20	24	3.9×10^6 to 7.2×10^7	1.46×10^7
	30	24	1.9×10^6 to 1.9×10^7	6.12×10^6
	37	24	1.5×10^6 to 4.7×10^6	2.47×10^6
Primary filter	20	24	2.7×10^6 to 2.1×10^7	6.32×10^6
	30	24	2.4×10^6 to 1.3×10^7	4.74×10^6
	37	24	1.6×10^6 to 8.2×10^6	2.62×10^6
A.S. trough	20	24	3.3×10^6 to 1.3×10^8	2.25×10^7
	30	24	3.1×10^6 to 3.5×10^7	1.23×10^7
	37	24	1.9×10^6 to 1.1×10^7	4.05×10^6
Final filter	20	23	1×10^4 to 6.2×10^6	6.29×10^5
	30	23	1.2×10^4 to 2.6×10^7	2.97×10^5
	37	24	3.2×10^3 to 4.6×10^6	2.85×10^5
Final effluent	20	24	2×10^4 to 6.6×10^6	8.92×10^5
	30	23	5.2×10^3 to 4.2×10^6	4.6×10^5
	37	24	5.0×10^3 to 2.5×10^6	3.52×10^5

Figure 15. The effect of sewage treatment on total plate counts on CGYE agar at 20 & 37°C, coliphage & E.coli

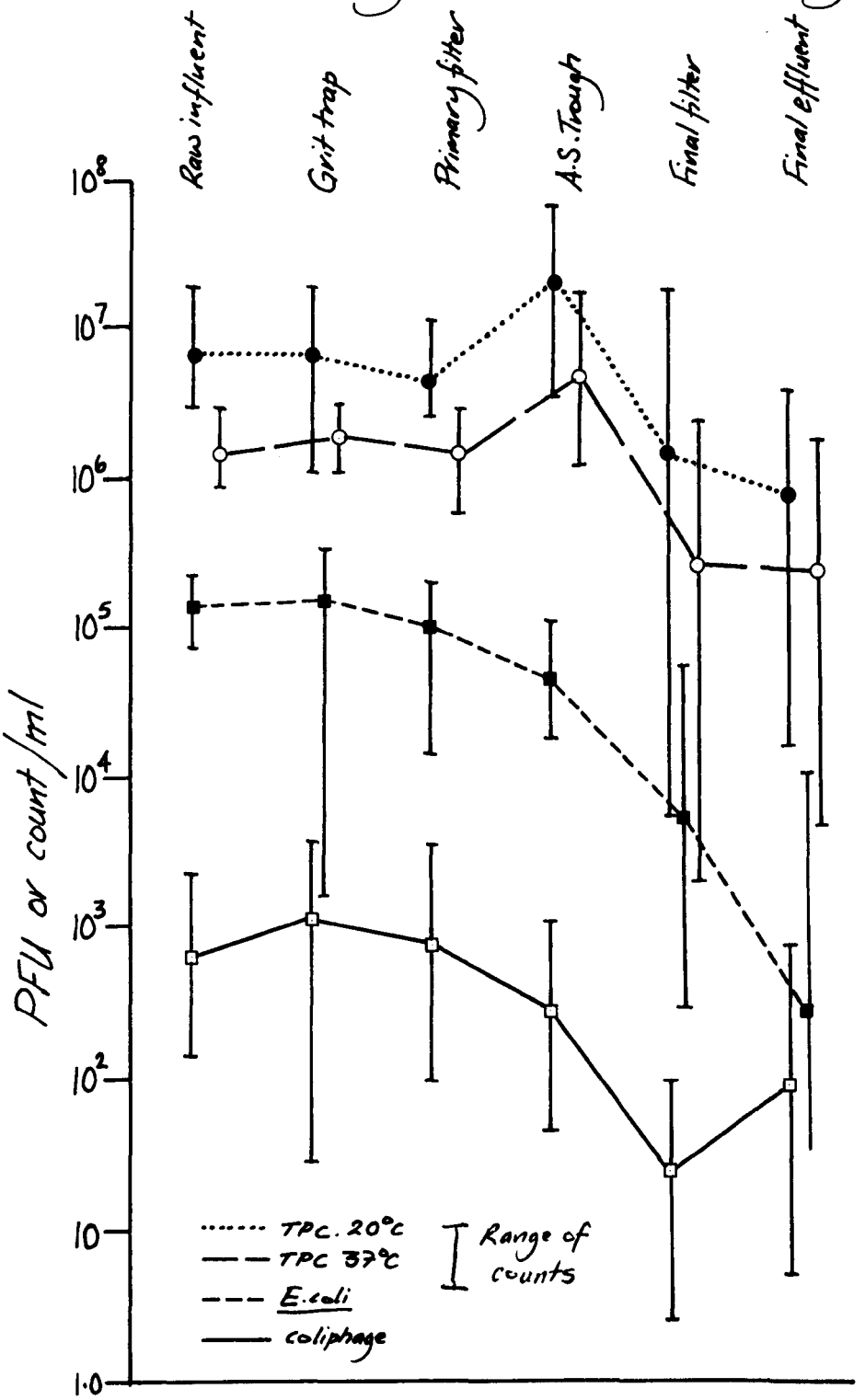
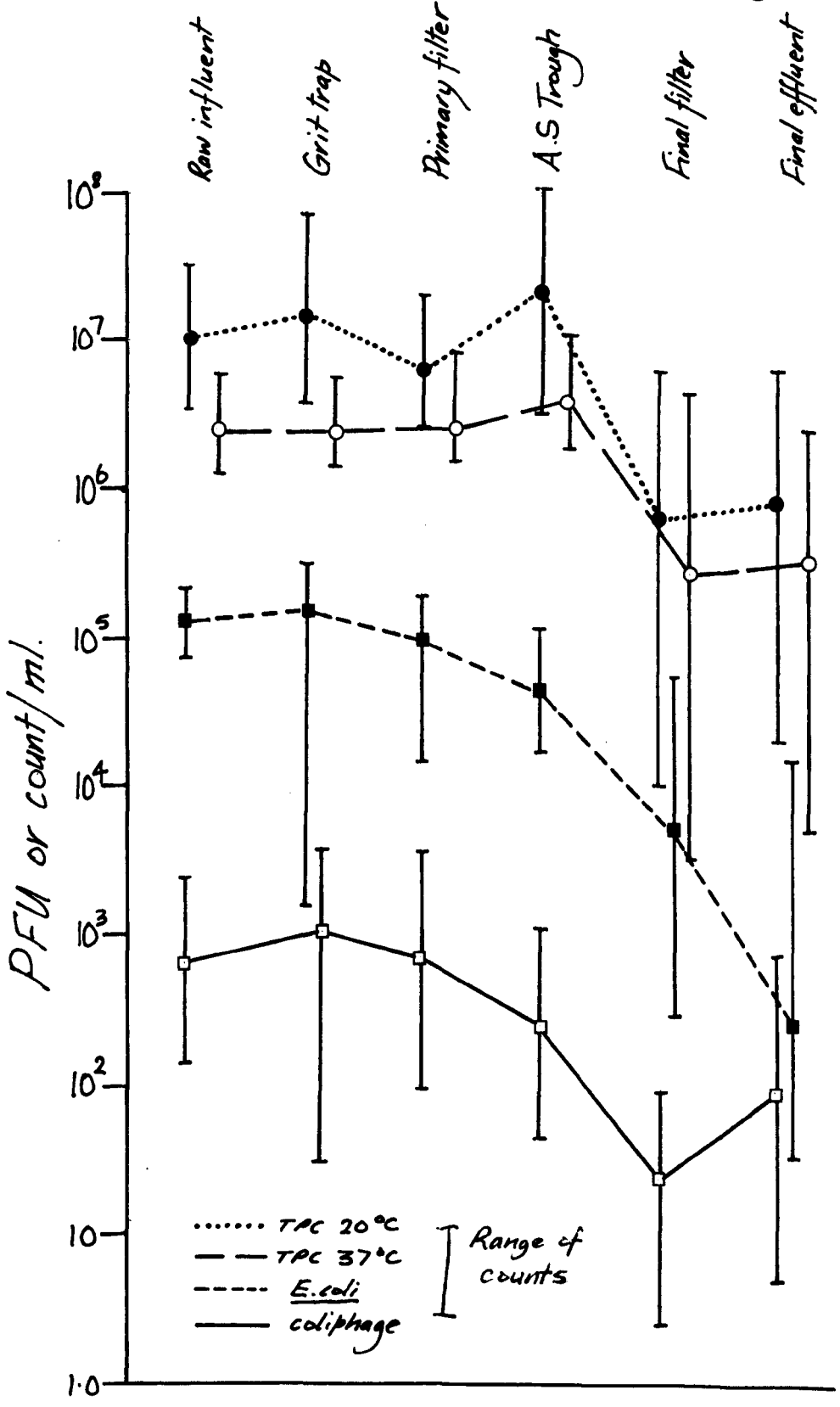


Figure 16. The effect of sewage treatment on total plate counts on N.A at 20 & 37°C, coliphage & E.coli



to achieve a reduction to < 5 PFU/ml coliphage and < 0.4/ml *E. coli* (the lower limits of detection by the respective techniques used). Initial phage levels ranged from 20 to 4 700 PFU/ml with a mean of 811 PFU/ml. *E. coli* levels ranged from 5×10^3/ml to 2×10^5/ml with a mean of 9.8×10^4/ml. Neither the survival of coliphage or *E. coli* is related to the initial count, i.e. long survival is not dictated by high initial levels. Both the original levels of phage and *E. coli* and their respective survival times vary in an apparently random fashion in samples taken at different times of year reflecting the observations noted earlier with respect to influent/effluent levels and the effects of various treatment stages. Survival times for coliphage ranged from 90 to > 384 days with a mean of > 196 days. Corresponding survival times for *E. coli* ranged from 50 to 131 days with a mean of 97 days.

5.3.3 Discussion

In the work described here with sewage the bacterium *E. coli* B (*NCIB 9484*) was used throughout as a host strain and estimates of coliphage made are referred to as total coliphage since this host is not specific for any one coliphage. However, it was recognized that it was a suitable host for the tailed phages T_1 to T_7 and an equivalent American Type Culture Collection (ATCC) strain of *E. coli* B, *ATCC 11303-1*, is also recognized as being a suitable host for 39 different coliphages. The nearest other multi-coliphage host is *E. coli* C (*ATCC 13709*) which only supports five coliphages (ATCC 1974). BELL (1976) demonstrated that the ATCC strain of *E. coli* B supported 15 times as many phage from domestic sewage as a faecal coliform host isolated from sewage and 200 times more than a host strain isolated from river water. This agrees well with the findings of DHILLON & DHILLON (1974)

Table 16 Survival of coliphage and *E. coli* in primary effluent

Date	Coliphage		*E. coli*	
	Initial count PFU/ml	Days storage to <5 PFU/ml	Initial count/ml	Days storage to <0.4/ml
3 Jan	563	155	1.6×10^5	99
10 "	845	244	1.1×10^5	105
17 "	295	246	9.5×10^4	85
24 "	620	106	1.5×10^5	50
31 "	1510	258	6.7×10^4	50
7 Feb	-	230	1×10^5	50
14 "	3500	384*	6.7×10^4	85
21 "	380	202	1.3×10^5	105
28 "	385	230	1.2×10^5	98
7 Mar	790	92	9.5×10^4	92
14 "	525	216	7.7×10^4	99
21 "	830	365†	1.2×10^5	99
28 "	610	365‡	1.3×10^5	99
4 Apr	655	174	1.2×10^5	148
11 "	115	363§	9.5×10^4	96
25 "	750	174	1×10^5	not tested
1 May	120	133	5.2×10^4	100
9 "	-	160	4.7×10^4	99
16 "	305	132	5.5×10^4	106
22 "	730	196	1.8×10^5	100
13 Jul	1255	100	7×10^4	100
27 "	345	105	1.5×10^5	75
2 Aug	1950	161	1.2×10^5	104
24 "	560	182	5×10^3	119
31 "	1640	98	7.1×10^4	98
14 Sep	1090	201	1×10^5	98
21 "	170	126	1.2×10^5	104
5 Oct	585	97	7×10^4	97
11 "	710	113	1.6×10^5	98
19 "	4700	336	7.3×10^4	75
24 "	580	310	1.4×10^5	100
2 Nov	460	175	1.5×10^4	131
9 "	20	196	1×10^5	98
15 "	365	176	9.8×10^4	106
23 "	105	126	7×10^4	126
30 "	95	229	1.5×10^4	103
6 Dec	695	140	7×10^4	113
13 "	395	90	2×10^5	71
20 "	770	253	9.2×10^4	99
mean value	811	>195.9	9.77×10^4	96.8 days

*635 PFU/ml remaining
†280 PFU/ml remaining
‡875 PFU/ml remaining
§ 50 PFU/ml remaining.

who reported that *E. coli* strains freshly isolated from natural sources were inefficient indicators of coliphage in sewage.

In this present study much of the sewage work had been completed or was underway before use of the RNA phage MS2 was introduced for laboratory-based experiments. However, a few trials were made with the MS2 host *E. coli (NCIB 9481)* and *E. coli* B (*NCIB 9484*) on some sewage samples and the results suggested that *E. coli* 9481 gave higher numbers of coliphage. GERBA *et al.* (1978) reporting trials in their own laboratory showed that the ATCC equivalent of NCIB 9481, ATCC 15597, yielded equivalent or higher numbers of phage isolates from sewage than did *E. coli* B. The work of DHILLON & DHILLON (1974) reported that F-specific RNA-containing phages (which includes MS2) were the most widely distributed in sewage and occurred in concentrations up to 1 000 PFU ml^{-1} in untreated sewage. If this is a general reflection of the situation elsewhere then the NCIB 9481 *E. coli* may indeed be a better host for future estimates of total coliphage in sewage.

Sections 5.2 and 5.3 detail how the effects of sewage treatment on phage and *E. coli* have been determined at various stages of treatment and on a regular basis over a long period. From an extensive survey of the literature it is apparent that no comparable studies have been made elsewhere. However, observations have been made which indicate changes in coliphage and coliform bacteria as a result of sewage treatment and how the efficiency of removal depends on the treatment method employed, e.g. trickling filter, activated sludge, oxidation ponds, etc. WARE & MELLON (1956) detected coliphage levels of 3.76×10^4/100 ml of sewage and calculated a coliphage to coliform ratio of 1:358. Similar studies by PRETORIUS (1962) recovered higher levels of coliphage, 2.65×10^5/100 ml from raw sewage and quoted a coliphage to faecal coliform ratio of 1:52. BELL (1976) took samples of raw sewage and lagoon effluent at

intervals over a period of four months from November to March. In the raw sewage coliphage levels ranged from 0.02 to 0.96 x 10^5/100 ml, and in the lagoon effluent from 0.13 to 0.49 x 10^5/100 ml. Coliphage to faecal coliform ratios calculated on the sample results were as follows: raw sewage ranged from 1:5 to 1:270 with a mean of 1:87; lagoon effluent ranged from 1:09 to 1:66 with a mean of 1:24. BURAS & KOTT (1969) in studies performed in Israel reported a ratio of coliphage to coliforms in raw sewage of 1:100 which was subsequently reduced by treatment to 1:10 in the plant effluent.

Results of the present study (5.3.2.1) for comparison suggest that although the coliphage levels are similar to those reported elsewhere, the ratio of phage to faecal coliforms is much higher than in published figures. This may be because other reports have concentrated on only a few samples where the faecal coliform count may have been lower than average.

DIAS & BHAT (1965) reported coliphage levels in raw sewage ranging from 1.8 x 10^3 to 9.6 x 10^5/100 ml and showed that coliphage did not increase during activated sludge treatment. Two hours' aeration reduced the influent coliphage tenfold but additional aeration and retention did not reduce this further. Others have also concluded that coliphages (and incidentally faecal coliforms) do not multiply during sewage treatment and furthermore are not responsible for the reduction of coliform bacteria observed during trickling filter treatment (WARE & MELLON, 1956) or in oxidation ponds (PRETORIUS, 1962).

Clearly it is the activated sludge process which is the most efficient part of the treatment system studied here, a result which agrees well with other published work. BERG (1973) considered that the activated sludge process was generally more effective at virus removal than other conventional biological treatments and removal efficiencies for

virus which exceed 90% have been reported (KELLY & SANDERSON, 1959; ENGLAND *et al.*, 1967; SAFFERMAN & MORRIS, 1976). SPROUL and his colleagues (SPROUL *et al.*, 1969) concluded that adsorption was the major factor involved and this is not surprising since the activated sludge process produces an enormous potential area for adsorption. An interesting paper by STAGG *et al.* (1978) describes attempts to differentiate between freely-suspended and solids-associated coliphage in sewage. A series of samples were taken at treatment plants using activated sludge or trickling filter and the results showed that phage concentrations in the trickling filter were always higher. Studies with f2 coliphage by SHERMAN *et al.* (1975) confirm that trickling filters have a low removal efficiency ranging from 9 to 18.9%. In the work of STAGG *et al.* (1978) the proportion of coliphage directly associated with solids ranged from 0.1 to 24%. Some 15% of the coliphage were actually embedded in the solids but the majority could be eluted out, suggesting that they were associated with solids by surface adsorption.

Studies using laboratory-scale activated sludge pilot systems have invariably given different results to studies on full-scale treatment plants. Experiments using poliovirus (BALLUZ *et al.*, 1977) and f2 coliphage (BALLUZ *et al.*, 1978) have shown that the distribution of these two different viruses in a model system is almost completely reversed. Percentage distribution of poliovirus between liquid and solid fractions of the liquor was 15:85 whereas the distribution for f2 phage was 84:16. A similar detailed study by RANGANATHAN *et al.* (1974) demonstrated that the percentage inactivation of *E. coli* B phage was lower than that of poliovirus in all the activated sludge systems studied. These authors also demonstrated that phage could replicate in the activated sludge component and unless sufficient time was allowed for flocculation to occur coliphage levels in the effluent would be higher than those in

the influent. Although no other references to phage replication in such systems have been found in this present study, if it generally occurs then it would invalidate coliphage as an indicator of enteric virus which does not multiply at all outside host cells.

The present study and some published work of others which has been referred to demonstrates that the input levels of coliphage vary widely as to the levels of enteric bacteria and viruses. As a result the ratios between coliphage and enteric bacteria/viruses also exhibit wide variation. In this present work it was hoped that regular sampling of treatment stages in the same treatment plant would demonstrate whether these variations in input levels and in the efficiency of treatment could be attributed to any seasonal factor. However, as discussed in section 5.3.2.2, variations in both coliphage and *E. coli* levels appeared to be random and although it was suspected that some anomalies arose because of reduced efficiency of the treatment during part of the study this could not be quantified.

From the literature the only comparable study was performed by GILCREAS & KELLY (1955) who over a six-month period examined the distribution of coliphage and coliforms entering two treatment plants at Albany and Colonie in New York State. Figure 17 is adapted from their paper and demonstrates that, as in the present study, the results show only a random patternless fluctuation and no apparent relationship between coliform bacteria and coliphage numbers. An important study by SAFFERMAN & MORRIS (1976) included a complete 24 h sampling of primary sewage effluent for coliphage. Phage levels over the 24 h period studied ranged from 5×10^4 to 5×10^5 per litre and showed a pronounced peak at 4 pm (1600 h). Repeat sampling two days later showed that this peak, although less pronounced, was still evident (Figure 18). The authors stated that data available to them did not support the

Figure 17. Coliform / coliphage content of raw sewage.
(after GILCREAS & KELLY 1955)

Figure 18. Coliphage assay of primary effluent. Washington Sewage Disposal Works. (after SAFFERMAN & MORRIS 1976)

contention that peak phage levels corresponded to excessive loading of the treatment works. SAFFERMAN & MORRIS (1976) also performed analyses for viruses other than coliphage and found that virus levels averaged 85 PFU per litre during the 24 h period. Compared with an average coliphage concentration of 284 230 PFU per litre this gave a phage to virus ratio of 3 300:1. The virus results also exhibited two peaks, one at 6 pm (1800 h) and one at 2 am (0200 h). Although the earlier peak corresponded broadly with peak coliphage concentrations no such correlation was evident with the 2 am peak in virus numbers.

Figure 19, adapted from the work of SAFFERMAN & MORRIS (1976), has been included here because it demonstrates a principle which appears to have been ignored in other studies, including those of the present author. Samples of primary sewage effluent from the activated sludge module were sampled over a 24 h period and examined for coliphage. Again a peak concentration of coliphage was detected at 4 pm (1600 h) and this was reflected in both primary and activated sludge effluent. What the authors did in their study was to allow for residence or detention time in the system and sampled the activated sludge module when they calculated that the effluent sampled at the primary stage had arrived. In this manner they were effectively sampling the same slug of sewage as it passed through the treatment process. Clearly, if the results they obtained are typical of what one might anticipate at other treatment plants, sequential sampling as performed in the present investigation would show variation in levels of phage etc. but would not necessarily demonstrate the true efficiency of treatment or indeed the influence of any seasonal factors.

A study reported by RAO *et al.* (1977) described virus sampling in activated sludge plants in India. These authors tried sampling at 2 h intervals for a 24 h period, for 5 h between 7 am (0700 h) and 12 noon

Figure 19. Coliphage assay. Washington Sewage Disposal Works (after SAFFERMAN & MORRIS 1976.)

(1200 h) and also repeated the 7 am till 12 noon sampling but deferred examination of samples until the day after collection. Their results showed that there was very little difference in the estimates of virus reduction whichever methods were used. Of interest in the study was the finding that virus removal efficiencies of the plant were reduced by some 50% during the monsoon season when flows through the works were greatly increased.

GELFAND (1961) showed that the concentration of viruses of human origin in sewage reaches a peak during late summer and early autumn. This seasonal trend reflects directly the high infection rates that occur; especially among children during the warmer months of the year. Estimates of the virus content of sewage vary, CLARKE *et al.* (1962) estimated 1 000 $TCID_{50}$ per 100 ml whereas KELLY & SANDERSON (1959) estimated 30 $TCID_{50}$ per 100 ml during cold months, increasing to 600 $TCID_{50}$ per 100 ml during warm months. These authors used the gauze pad method which appears to concentrate about fourfold (KELLY, 1957; BLOOM *et al.*, 1959), therefore the corrected figures vary from 7 $TCID_{50}$ to 150 $TCID_{50}$/100 ml.

During a polio epidemic in the USA CHIN *et al.* (1967) found that sewage contained between < 200 to 560 $TCID_{50}$/100 ml with an average value of 250 $TCID_{50}$ per 100 ml. From these data GRABOW (1968) postulated that the average number of enteric viruses in sewage ranges from 5 $TCID_{50}$/100 ml during winter to 600 $TCID_{50}$ in summer but stressed that the use of averages might in itself be fallacious. Highest virus levels occurred when a community was maximally seeded with viruses and at a time of day when the greatest number of people were contributing excreta to the sewage system. KELLY *et al.* (1957) demonstrated that the occurrence of coxsackie virus in sewage from a number of plants sampled over a four-year period varied widely, some types being dominant one year and

absent the next. It is likely that such fluctuations reflect epidemic infection in the community even though this may go unrecognized clinically (CLARKE & KABLER, 1964). MELNICK (1976) reported that sewage from communities of people in the lower socio-economic groups generally yielded more enterovirus than sewage from high socio-economic communities. This was confirmed by RUITER & FUJIOKA (1978) in some studies performed in Hawaii. These latter authors also carried out what they claimed to be the only known study to compute the total 24 h input of viruses from an ocean outfall using hourly or composite sampling. They observed hourly fluctuations and peak discharges around midday (1100 to 1400 h) and again in late evening (2000-2300 h). Virus concentrations ranging from 85 to 240 000 PFU per litre were computed to give an input of 8.5×10^{10} PFU over a 24 h period.

While there are obviously instances where the coliphage and virus levels in sewage show seasonal effects it is equally clear that this will vary from place to place and from time to time. Published work has suggested some seasonal variation in the enterovirus levels in sewage but this is not evident for coliphage or indeed faecal indicator bacteria as shown by the present study.

No publications which report studies on phage or virus and total plate counts in sewage have been located during this present study. However, KELLY *et al.* (1961) isolated pure cultures of a number of bacteria, including *Flavobacterium, Aerobacter, Klebsiella* and other coliforms from activated sludge which were capable of inactivating poliovirus under laboratory conditions. CLIVER & HERMANN (1972) examined six species of bacteria for antiviral activity and found only *Bacillus subtilis* and *Pseudomonas aeruginosa* to be positive. Experiments concluded that inactivation of virus was not due to adsorption to bacterial cells but to some product of the cells themselves. In the present study

total plate counts increased at the activated sludge stage of the process when *E. coli* and coliphage showed a significant decline. Bacteria are an essential part of the activated sludge floc and so an increase is not unexpected. However, whether they contribute to the decline of coliphage/*E. coli* merely by assisting floc formation and hence increasing available adsorption area, produce specific anti-phage substances, or a combination of the two is not clear. On balance it is likely that adsorption on to the floc is a major mechanism of removal and so bacteria may contribute to this indirectly.

Reference has been made earlier to the work of BELL (1976) which also includes a study on the faecal coliform/coliphage populations of stored sewage. This is the only study comparable to that performed by the present author and BELL's results have been adapted here as Figure 20. These results show that the faecal coliform to coliphage ratio fell from 87:1 to 1:1 in seven days at 20°C but that at a lower temperature (4°C) it took 28 days to achieve a 1:1 ratio. Unlike a simple count of faecal coliforms as a measure of faecal pollution the coliphage/coliform ratio is independent of dilution. Therefore because of differential persistence a high ratio of faecal coliform to coliphage could indicate recent contamination, a low ratio, less recent contamination. Some support for this type of hypothesis is furnished by the results of KOTT *et al.* (1971) who found a coliform/coliphage ratio of 100:1 in fresh faeces and the results of BELL (1960) who reported ratios of 87:1 for raw sewage, 4.2:1 for lagoon effluent and 0.15:1 for river water, that is to say a differential die-off between coliforms and coliphage over time. The results obtained in the present study were from primary effluent stored at 8°C but nevertheless it is evident that even *E. coli* can survive for very long periods as shown by the mean value of all the trials performed (96.8 days). It is also evident that coliphage

Figure 20. Ratio of faecal coliforms to total coliphage in stored sewage at 4° and 20°C. (after BELL. 1976)

survived for over a year in some samples and a minimum of three months in others. Since the phage estimates were of total coliphage it is possible that dominant phage types in the samples vary and this may account for the range of survival times recorded. Although primary effluent cannot be regarded as being comparable with sewage sludge the obvious potential of long-term survival evident in the present work seems to emphasize that unless sludges are properly treated before disposal a risk of virus transmission still remains. (WARD & ASHLEY, 1976)

5.4 Sea water

5.4.1 Introduction, objectives and methods

The stated objective of this thesis was to make a critical assessment of coliphage as an indicator of viral pollution and to compare phage with *E. coli*, the most widely used bacterial indicator. In demonstrating how the behaviour of phage compares with that of *E. coli* and to obtain data which might support the use of phage to replace or complement the use of *E. coli* it is necessary to examine what factors are known to affect survival of *E. coli* in sea water and how these may influence coliphage. The work of CARLUCCI & PRAMER (1960a,b) reports a detailed examination of factors affecting *E. coli* in sea water and this has been adopted in the present work as a basis for experimental studies with coliphage. All experiments were also carried out using *E. coli* to obtain comparative data. Inevitably some of the parameters or factors examined cannot be treated in isolation since they are closely, and often, interrelated, e.g. sunlight and temperature. However, for the purpose of clarity the 'method' and 'result' sections which follow are described separately and brought together in the discussion whence these relationships may be explored more concisely. Experimental work was carried out under the following headings:

1. Salinity and major ions in sea water;
2. pH;
3. Seasonal variation in phage/bacterial survival;
4. Biotic factors - effects of sterilization by autoclaving and filtration;
5. Sunlight and temperature;
6. Effect of u/v light;
7. Adsorption and sedimentation;
8. Effect of organics;
9. Effect of inorganics.

All experiments were carried out at least twice to check the reproducibility of the results. Details of the precise number of replicates are given in the results (5.4.2).

5.4.1.1 Salinity and the effect of major ions in sea water

In published work which has been reviewed in sections 3.2 and 4.2 considerable differences have been observed in the survival of coliphage, enterovirus and bacterial indicators in saline and non-saline waters. One of the major variables, particularly in estuarine water, is salinity or salt content due to the influence of freshwater run-off and rainfall. In oceanic conditions sea water has an almost constant salinity of $35^{o}/oo$ (HARVEY, 1955) but in coastal and estuarine areas the influx of fresh water may produce both a lowering of salinity and marked fluctuations over short periods of time. In addition, as has been noted earlier, on the basis of concentration, inorganic salts are potentially the most toxic substances in sea water and may affect bacterial survival by general osmotic effects or by specific ion toxicity; the latter factor may also be important in virus survival.

In order to evaluate the effects of salinity, and sea water itself, on the survival of coliphage and *E. coli* experiments were set up to examine particular aspects as follows:

I Comparison of survival in saline and non-saline waters;

II Survival in waters of various salinities, i.e. to cover a range of salinities from fresh water ($0^{o}/oo$) to estuarine sea water ($30^{o}/oo$);

III To determine the effects of the major component salts in sea water.

I Survival in saline and non-saline waters

As an initial experiment to study survival and to evaluate the duration of an experiment necessary to follow phage survival down to low levels various types of water were seeded with MS2 phage. Since the infective dose of some viruses may be as low as 1 PFU it was important to follow the fate of phage for as long as detectable numbers remained. A series of flasks containing 500 ml quantities of the following waters: distilled, deionized, tapwater and sea water were seeded with 0.5 ml of MS2 phage suspension and held at 15°C. The pH of each water was determined at the start and on completion of the experiment. Samples of water were removed aseptically from each flask at intervals and assayed for phage by the soft agar overlay technique described in section 5.2 using host *E. coli* NCIB 9481.

II Effect of salinity

Estuarine sea water was collected at high tide from the River Crouch, Essex and coarsely prefiltered through a Whatman No. 1 filter to remove large silt and organic particles. The salinity of the water was determined using the method and conversion graph given by WOOD & AYRES (1977). This water was then diluted with distilled water to give

a range of salinities from fresh water (0°/oo) to 30°/oo in 5°/oo steps, i.e. seven salinities in all, as follows:

Treatment	Sea water (ml)	Tapwater (ml)	Salinity (°/oo)
1	500	-	30
2	417	83	25
3	334	166	20
4	250	250	15
5	166	334	10
6	83	417	5
7	-	500	0

Each 500 ml flask was equilibrated at 15°C overnight and then inoculated with either MS2 phage suspension, phage + *E. coli* (NCTC 9481) or *E. coli* only. Counts were made at intervals using the agar overlay method for phage and Miles and Misra direct count for *E. coli* as described earlier. Counting was continued until the majority of treatments showed no demonstrable phage or *E. coli* remaining.

III Effect of major component salts (ions) in sea water

Artificial sea water (ASW) was prepared according to the formula given by WOOD & AYRES (1977) using commercial or technical grade salts as detailed below to give a sea water of approximately 30°/oo salinity.

Salt	g/litre of tapwater
Sodium chloride (NaCl)	23.51
Magnesium sulphate ($MgSO_4 7H_2O$)	5.74
Magnesium chloride ($MgCl_2 6H_2O$)	4.55
Flake calcium chloride ($CaCl_2 2H_2O$)	1.19
Potassium chloride (KCl)	0.56
Total	35.55 g

Further 500 ml quantities were prepared in deionized water to give approximately the same salinity but less one of the constituent salts. To avoid any unintentional osmotic effects by the omission of salts the salinity was brought to that of the full sea water (30°/oo) by the

addition of extra sodium chloride. NaCl was used since CARLUCCI & PRAMER (1960) reported no significant difference between the survival of *E. coli* in different seawater concentrations and solutions of NaCl of equivalent salinity.

In addition to the flask containing full artificial sea water the following were prepared:

(i) NaCl only - 16.0 g/500 ml gave a salinity of 28°/oo.

(ii) Artificial sea water (ASW) less $MgSO_4 7H_2O$

Formula less $MgSO_4 7H_2O$ gave a salinity of 24°/oo and the addition of 2.5 g NaCl raised this to 28°/oo.

(iii) ASW less $MgCl_2 6H_2O$

Formula less $MgCl_2 6H_2O$ gave a salinity of 26°/oo and the addition of 2 g NaCl raised this to 28.95°/oo.

(iv) ASW less $CaCl_2 2H_2O$

Formula less $CaCl_2 2H_2O$ gave a salinity of 26.6°/oo and the addition of 1.5 g NaCl raised this to 28.6°/oo.

(v) ASW less KCl

Formula less KCl gave a salinity of 27.1°/oo and the addition of 1 g NaCl raised this to 28.95°/oo.

All six flasks were sterilized at 121°C for 15 minutes and allowed to cool overnight before seeding with phage MS2 suspension (0.5 ml/500 ml water) and storage at 15°C. Counts were made at intervals using the soft agar overlay method and *E. coli* NCIB 9481 host.

On completion of the phage experiment, similar treatments were set up to compare survival of *E. coli* 9481. Each flask was inoculated with 1 ml of 10^{-2} dilution of *E. coli* culture and counted at intervals on MacConkey Agar No. 3 using the Miles and Misra method.

5.4.1.2 pH

The normal range of pH for sea water is between 7.5 and 8.5 and may be influenced by temperature, pressure and the photosynthetic and respiratory activities of microorganisms (HARVEY, 1955). The work of CARLUCCI & PRAMER (1960) demonstrated that death of *E. coli* in sea water was more rapid at alkaline pH but that survival in sea water was consistently greater than in NaCl solutions of equal salinity and pH. A similar, protective effect of the balance of salts in sea water has also been described by SPENCER (1957). Viruses and bacteria entering the marine environment from sewage outfalls or land drainage will be subject to changes in pH as well as salinity. Enteroviruses such as poliovirus are typically resistant to low pH (down to pH 3.0) and in assessing phage as a possible indicator it is therefore important to examine pH effects in sea water in comparison with *E. coli*. Experiments carried out by the present author (5.4.1.1.I) suggested a pH effect which warranted further study.

Preliminary experiments by the author followed the methods used by CARLUCCI & PRAMER (1960) and it was clear that these were unsuitable because stable pH values could not be obtained due to the strong buffering capacity of the sea water. The background to this work and subsequent experiments to find a more suitable method are described in the Appendices (Appendix 12). To overcome these problems heat sterilized tapwater was used to examine the effects of pH on survival of *E. coli* and coliphage MS2. The following treatments were set up using the formulae described in Appendix 12 to give stable pH values. All pH determinations were made initially and checked at intervals using an EIL model 23A direct reading pH meter.

Flask	1	pH 4
	2	pH 5
	3	pH 6
	4	pH 7
	5	pH 8
	6	pH 9
	7	pH 10

Replicate sets of flasks were set up, one inoculated with MS2 phage and the other with *E. coli* 9481. Each flask was mixed on a magnetic stirrer prior to sampling at intervals until bacteria/phage were undetectable.

5.4.1.3 Seasonal variation

In formulating the objectives for the experimental work to be carried out it was possible to isolate certain factors for individual attention, e.g. pH, temperature, etc., and indeed this was logistically desirable. However, it was appreciated that it was unlikely that any single factor acted in isolation and that survival of both bacteria and viruses in sea water was a complex situation involving many factors, some known, some unknown, acting in a variety of combinations and possibly quite differently in specific situations. Accordingly, the objective of this part of the investigation was to look at seasonal variation in general terms, i.e. are there seasonal differences in survival *per se* and can these subsequently be related to the specific factors examined individually?

From pilot experiments and data provided by published work it was apparent that coliphage could survive in sea water for periods in excess of 30 days. Since the intention of this part of the planned programme was also to look for possible seasonal changes in factors which affect coliphage it was necessary to limit experiments to a maximum duration of one month before commencing a new experiment. These tests were designated short-term mortality experiments to distinguish them from concurrent long-term tests designed to explore the effects of autoclaving and filtration on waters sampled seasonally. These latter tests are dealt with

in section 5.4.1.4.

Flask treatments were set up as follows using sea water from the River Crouch, Essex.

Flask code	Test water	Additions*
FA	500 ml autoclaved sea water	MS2 phage
FB	" " " " "	MS2 phage + *E. coli* 9481
FC	" " " " "	*E. coli* 9481 only
FD	" " natural sea water	MS2
FE	" " " " "	MS2 phage + *E. coli* 9481
FF	" " " " "	*E. coli* 9481 only

*MS2 phage 0.5 ml suspension/500 ml water; *E. coli* 9481 0.2 ml of 10^{-1} dilution overnight broth culture.

Each flask was prepared with a sterile polypropylene magnetic follower to permit stirring before samples were taken. Sampling was continued at intervals for a maximum of one month before a new series of flasks were set up and a fresh experiment commenced. All flasks were stored in the dark at a constant temperature of 15°C. Sample examination was performed by the Miles and Misra method for *E. coli* and the soft-agar overlay method for MS2 (host *E. coli* 9481).

5.4.1.4 Effects of sterilization

It has been noted in earlier sections that published work suggests that autoclaving sea water results in a considerable reduction in the anti-bacterial properties of such a medium and it has been postulated that similar effects may be observed with viruses. Components of the indigenous microflora of sea water (bacteria, phytoplankton) have also been shown to exhibit inhibitory effects for faecal bacteria and viruses. The mechanisms involved may include production of antibacterial/viral compounds, adsorption, competition, or presence of organic matter. In an attempt to elucidate the possible role of these mechanisms it was decided to compare survival in natural sea water with survival in water which had been sterilized by autoclaving and by membrane filtration.

Filtration through a 0.47 µm pore size cellulose acetate membrane was designed to remove the majority of bacteria and phytoplankton and much of the organic suspended material. Application of heat via autoclaving would kill all living organisms without removing organics (i.e. organic material already present plus the bacterial and phytoplankton cells). Additionally it would also demonstrate whether any heat-labile components significantly affected bacterial and viral survival.

Methods used in these experiments were essentially similar to those used in section 5.4.2.3 although it was intended that the tests should continue until phage levels had declined to undetectable levels, necessitating larger initial volumes of test waters. Experimental flasks were set up as follows:

Flask code	Test water	Additions*
F1	4 litres membrane-filtered sea water	MS2 phage
F2	" " tapwater	" "
F3	" " autoclaved sea water	" "
F4	" " natural sea water	" "
F5	" " " " "	MS2 + *E. coli* 9481
F6	" " " " "	*E. coli* 9481

*Additions MS2, 4 ml phage suspension; *E. coli* 9481 1.6 ml overnight culture.

The various flasks were designed to test different effects as described below.

F1 Natural sea water filtered through an Oxoid 0.45 µm filter to remove all particles including bacteria and phytoplankton which might exert some biotic influence, e.g. production of antiphage factors or removal of phage by adsorption to particulates and/or by direct competition.

F2 Mains tapwater to act as a non-saline control to the seawater treatments where salinity etc. was expected to vary during the year.

F3 Autoclaved natural sea water to kill any microorganisms and to act as a control for comparison with F1 and F4.

F4 Natural sea water to detect any long-term seasonal effects.

F5 Natural sea water with added phage host bacterial cells - comparison with F4 to see if presence of host influences phage survival.

F6 *E. coli* host only for direct comparison with phage treatment F4; examination of any common influences on survival.

Each flask was set up as in the short-term experiments (5.4.1.3) and sampled at intervals until phage declined to undetectable levels or until it became impractical to continue without prejudice to the setting up of new experiments.

5.4.1.5 Effect of sunlight and temperature

It has been demonstrated that temperature affects virus survival in water and similar observations have also been made for *E. coli* and other bacteria. Additionally, exposure to sunlight has been shown to exert a pronounced effect on the survival of coliform bacteria (REYNOLDS, 1965; GAMESON, SAXON, 1967) and possibly result in photodynamic inactivation of bacteriophage (BERRY & NOTON, 1976). In seasonal terms there will be a broad relationship between sunlight and temperature, e.g. mid-summer - high temperature, mid-winter - low temperature. The object of the experiments described here was to compare the survival of *E. coli* and MS2 phage over a range of temperatures and in light and dark conditions. For clarity and ease of performance the investigation was divided as follows:

I Sunlight.

II Temperature.

I Sunlight

For experiments to determine the effect of sunlight on the survival of *E. coli* and coliphage MS2 four 10 litre plastic aquarium tanks were each filled with 6 litres of natural sea water (settled to remove gross silt and suspended particulates). Two tanks were covered with a single layer of 500 gauge clear polythene sheeting and two were covered with a single layer of black polythene. Earlier experiments as to the suitability of various materials showed that of the materials tested clear polythene permitted maximum transmission of solar radiation (measured as u/v + infra-red). These experiments are described in detail in Appendix 13.

The tanks were placed on an elevated platform one metre above ground level so that they were exposed to full sunlight from 1030 h until late afternoon and allowed to equilibrate for three days in this position. At the start of the experiment 3.0 ml of MS2 phage suspension was added to one clear (light) tank and one dark treatment. Similarly 0.3 ml of overnight *E. coli* 9481 culture was added to two more tanks, one light and one dark treatment. The treatments were thoroughly mixed and sampled immediately for phage or *E. coli* as appropriate using methods described earlier. Further samples were taken at daily intervals where possible until no detectable phage or *E. coli* remained.

II Temperature

As will be noted in the results section it was observed that containers covered in black polythene maintained a more stable temperature than those covered in clear polythene. To examine more closely the difference of temperature it was therefore decided to run the experiment in the dark. 500 ml flasks were completely covered with black polythene

and 400 ml of natural sea water was added to each. Flasks were sealed with aluminium foil and left to equilibrate for three days at the following temperatures:

Flask	Temperature (°C)	Situation
1	8	Refrigerator
2	15	Controlled temperature room
3	20	Incubator
4	25	"
5	30	"
6	37	"
7	ambient range*	Outdoors in exposed position

*This treatment was exposed to ambient temperatures, i.e. variable through a 24 h cycle to evaluate the effect of temperature fluctuation. After equilibration 0.4 ml of stock MS2 suspension was added to each flask, mixed thoroughly and sampled immediately. Further samples were taken after 3 and 8 days' storage.

5.4.1.6 Effect of ultra-violet (u/v) light

It is current practice in England and Wales to measure the efficiency of newly constructed u/v shellfish purification plants by dosing the system with screened sewage effluent or *E. coli* culture and following the reduction in bacterial numbers during recirculation under a u/v light source. Although a limited amount of research has been carried out in the USA on the effects of u/v systems on survival of waterborne enterovirus the results cannot be extrapolated to the situation here for two reasons, (i) purification, or depuration as it is known in the USA, is mainly experimental and rarely used for commercial shellfish production, (ii) systems used in the USA employ multiple u/v sources of greater output and different design to those used here.

There is a tentative association between consumption of shellfish and incidents of non-bacterial gastroenteritis in England (see AYRES, 1975) and some doubt that u/v systems designed to remove faecal bacteria are equally effective at removing viral agents. Clearly therefore in

any assessment of coliphage as a potential indicator it is valuable to look at the survival of coliphage in a u/v shellfish purification system to evaluate its possible applications in testing these systems.

The sump tank of an experimental u/v shellfish purification plant as illustrated in Figure 21(a) was filled with 640 litres of sea water and dosed with a culture of MS2 phage. Two replicate experiments were performed using low and high phage titres (17 and 34 ml of suspension). The phage was mixed in the system by switching on the pump to circulate the water for 30 minutes. A start sample was taken prior to switching on the u/v lamp (Philips T.U.V. 30 watt) and further samples at intervals with the lamp on. Phage content of samples was estimated by the agar overlay method described earlier using the host *E. coli* K12 (NCIB 9481) with overnight incubation at 37°C.

5.4.1.7 Adsorption and sedimentation

Adsorption to particulate matter and subsequent sedimentation of particles has been shown to be a major factor in the removal of bacteria and viruses during sewage treatment. A similar process of adsorption and sedimentation can be postulated for the removal of bacteria and viruses from polluted water, although in water particulate matter may be primarily inorganic and generally present in relatively small amounts. Adsorption and sedimentation mechanisms have also been considered important in prolonging the survival of microorganisms beyond that demonstrated for those suspended in the water column. This creates in effect a 'pool' of bacteria and viruses, which by subsequent resuspension may repollute the overlying water.

To explore the effect of these processes on MS2 phage in comparison with *E. coli* bacteria a series of experiments was designed. By adding particulate matter in known amounts to aqueous suspensions of

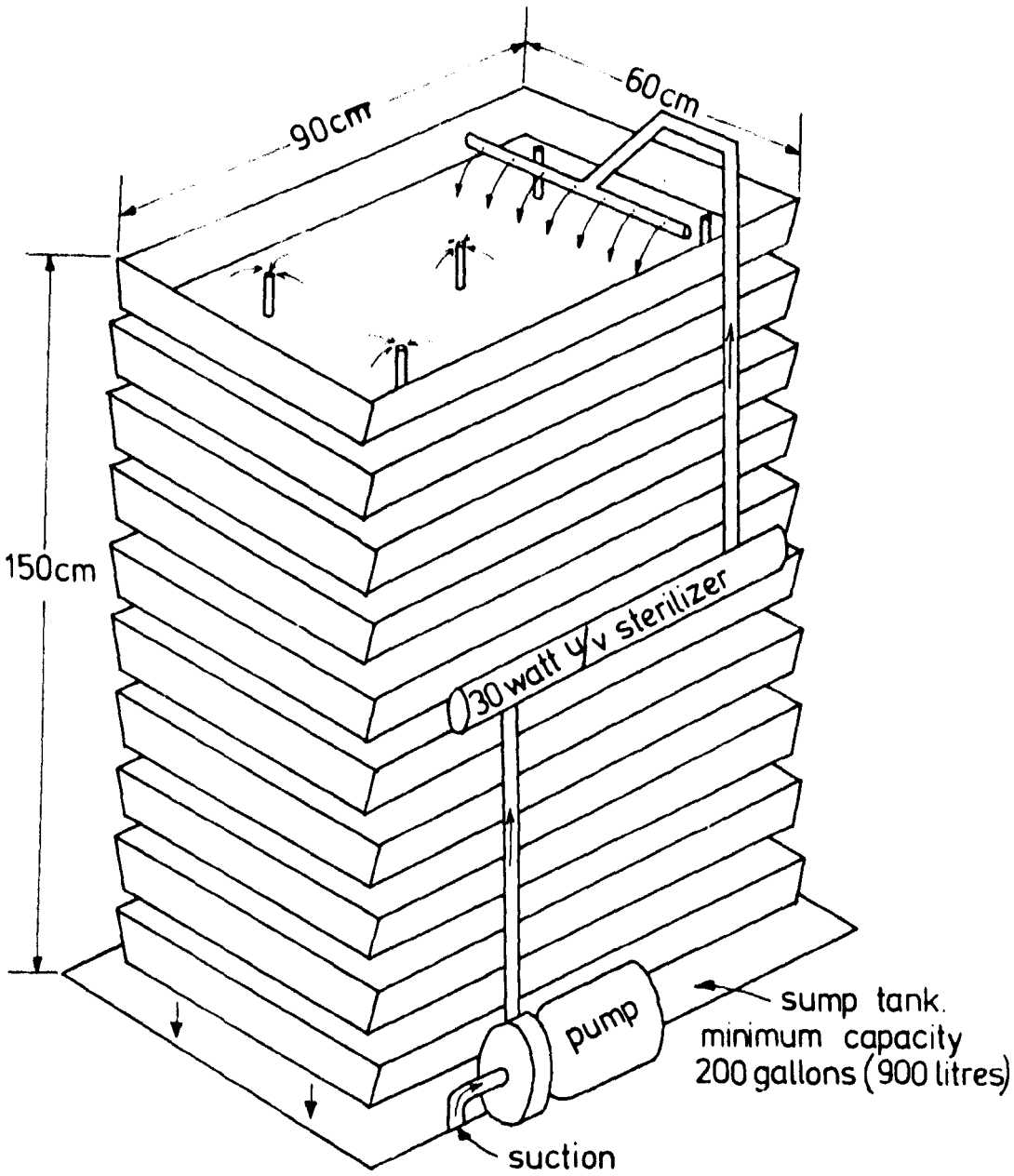


Figure 21A Diagram of high density purification system.
(details of framework omitted)

MS2 and *E. coli* (with controls lacking particulate material) and treating them by centrifugation, ultrasonics and shaking, the adsorption, sedimentation and resuspension effects could be studied. Although these processes are interrelated, for the purpose of clarity, the treatments are considered separately under 'methods' and 'results' and brought together in the discussion.

For these experiments five 500 ml flasks were set up, each containing 200 ml of natural sea water which had been previously filtered (Whatman No. 1) to remove coarse silt and organic particles. Measured quantities of Fullers earth (B.P.) were added to the flasks to give a range of concentrations 0, 1, 10, 50 and 100 ppm (w/v). To each flask was added 0.2 ml of a 10^{-4} dilution of MS2 phage suspension.

An initial sample was taken after mixing the flask contents thoroughly and the remainder divided into three equal parts which were treated as follows:

(i) centrifuged for 10 minutes at 2000 upm (MSE Magnum);

(ii) treated in an ultrasonics bath for 30 seconds;

(iii) shaken vigorously for 2 minutes by hand.

Coliphage estimates were made on treated samples using the soft-agar overlay method described.

To obtain comparative data for *E. coli* similar treatments were set up using inocula of 2.5×10^{-4} *E. coli* B culture instead of coliphage and estimates made by the Miles and Misra method.

5.4.1.8 The effect of organics

Although sea water has been shown to exhibit both bacteriocidal and virucidal properties there are antagonistic factors capable of lowering or even overcoming some of these properties. The addition of simple organic matter may substantially reduce the bacteriocidal power

of sea water (ORLOB, 1956; VACCARO *et al.*, 1950) but effects on the virucidal properties may not be evident (MATOSSIAN & GARABEDIAN, 1967). Similarly low concentrations of organic substances such as peptone and glucose have been shown to promote the survival of *E. coli* (CARLUCCI & PRAMER, 1960). In the present work the influence of both peptone and glucose at various concentrations on the survival of *E. coli* and coliphage MS2 was examined. Amino acids have also been mentioned in the literature as being capable of reversing or modifying the bacteriocidal and virucidal properties of sea water and cysteine was selected for experiments described here. JOHANNESSON (1957) demonstrated that low levels of cysteine greatly increased survival of *E. coli* in sea water possibly by reacting with halates which ordinarily result in the death of the bacteria. Halogens are known to have strong anti-viral properties and cysteine may therefore also reduce anti-viral properties of sea water.

To investigate the effects of glucose and peptone the concentrations used by CARLUCCI & PRAMER (1960) in studies with *E. coli* were used as supplements to flasks of sea water as follows:

Flask no.	
1	Sea water + phage MS2
2	Sea water + 1 ppm glucose + MS2
3	Sea water + 10 ppm glucose + MS2
4	Sea water + 100 ppm glucose + MS2
5	Sea water + 1 ppm peptone + MS2
6	Sea water + 10 ppm peptone + MS2
7	Sea water + 100 ppm peptone + MS2

All held in the dark at 15°C and mixed prior to examination. Experiments were repeated with *E. coli* 9481 using similar concentrations of glucose and peptone, and in all tests plate counts of total viable bacteria were made on Nutrient Agar incubated at 30°C since it was anticipated that one effect of the organic supplement would be to increase the bacterial count of the sea water used. Such an increase in

bacterial count could affect the survival of *E. coli* by direct competition for nutrients and survival of phage by production of anti-phage substances or other factors.

In designing the experiments with cysteine, the possible protective effect of this substance, acting by reducing heavy metal ion toxicity, was to be investigated. Autoclaving sea water may have a similar effect in reducing the toxicity of these ions so two series of experimental flasks were set up, one using raw sea water, and the other using autoclaved sea water. Flasks were treated thus:

Series A

Flask 1	Raw sea water only
2	Raw sea water + 1 ppm cysteine hydrochloride
3	Raw sea water + 10 ppm cysteine hydrochloride
4	Raw sea water + 100 ppm cysteine hydrochloride

Series B

Flask 1	Autoclaved sea water only
2	Autoclaved sea water + 1 ppm cysteine hydrochloride
3	Autoclaved sea water + 10 ppm cysteine hydrochloride
4	Autoclaved sea water + 100 ppm cysteine hydrochloride

Each series was repeated using either *E. coli* 9481 or MS2 phage as appropriate and counts made at intervals. Flasks were held in the dark at a constant 15°C and mixed thoroughly prior to sampling.

A series of 750 ml conical flasks were sterilized together with bungs and polypropylene covered magnetic stirrers. The object of the study was to examine the effects of polluted water on the survival of coliphage and it was considered that two major effects might be involved, either separately or combined. To separate biological effects, e.g. due to bacteria, from those due to the presence of extra organic matter, e.g. sewage solids, treatments were set up in duplicate using equal quantities of raw or sterilized effluent. Two flasks were set up, one with natural sea water, one with autoclaved sea water which were not dosed with effluent, to serve as control treatments. MS2

phage was added to every flask and the final list of treatments studied was as detailed below:

Flask no.	
1	Natural sea water
2	Autoclaved sea water (sterile)
3	Natural sea water + sterilized effluent to equal dose of 10 *E. coli*/ml
4	Natural sea water + sterilized effluent to equal dose of 100 *E. coli*/ml
5	Natural sea water + raw effluent to equal dose of 10 *E. coli*/ml
6	Natural sea water + raw effluent to equal dose of 100 *E. coli*/ml.

The experiment was repeated with two additional treatments using quantities of effluent, raw and sterilized, equal to a dose of 1000 *E. coli*/ml. By using 10, 100 and 1000 *E. coli*/ml the effects of light, moderate and heavily polluted waters on survival of phage could be studied.

5.4.1.9 Effect of inorganics

Deficiencies of nitrogen and phosphorus are known to limit the decomposition of organic matter and development of bacteria in sea water (ZOBELL, 1946; HARVEY, 1955). In accord with the work of CARLUCCI & PRAMER (1960) the influence of concentration of $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ on the survival of *E. coli* as described by them was repeated and coliphage was included for comparison. Levels of nitrogen and phosphate in sea water are of the order of 0.7 and 0.1 ppm (SVERDRUP *et al.*, 1942) but in estuarine situations where sewage is discharged these levels may be greatly exceeded and possibly contribute to extended bacterial survival. If survival and development of bacteria is enhanced then this may in turn exert an influence on survival of virus. Although viruses depend on host cells for multiplication the possibility that inorganics influence their survival cannot be ignored.

Two series of flasks were set up, one for tests with ammonium phosphate $((NH_4)_2HPO_4)$ and the other with ammonium sulphate $((NH_4)_2SO_4)$. In all, eight flasks were used for each series, four containing natural sea water and four containing autoclaved sea water. To each flask of 100 ml water 0.2 ml of a 10^{-2} dilution of overnight *E. coli* 9481 culture was added and mixed thoroughly. Flasks set up were designated as follows:

$(NH_4)_2HPO_4$ series

Flask code	Water	ppm $(NH_4)_2HPO_4$
P1	natural sea water	0
P2	" " "	1
P3	" " "	10
P4	" " "	100
P5	autoclaved sea water	0
P6	" " "	1
P7	" " "	10
P8	" " "	100

For the initial experiment counts were made daily but *E. coli* levels declined rapidly and a further experiment was performed with sampling twice daily. *E. coli* were obtained by the Miles and Misra method.

On completion of the tests with *E. coli* similar flasks were set up and experiments repeated using MS2 phage, counted by the soft-agar overlay method (host *E. coli* 9481).

5.4.2 Results

5.4.2.1 Salinity and the effect of major ions in sea water

5.4.2.1.I Survival in saline and non-saline waters

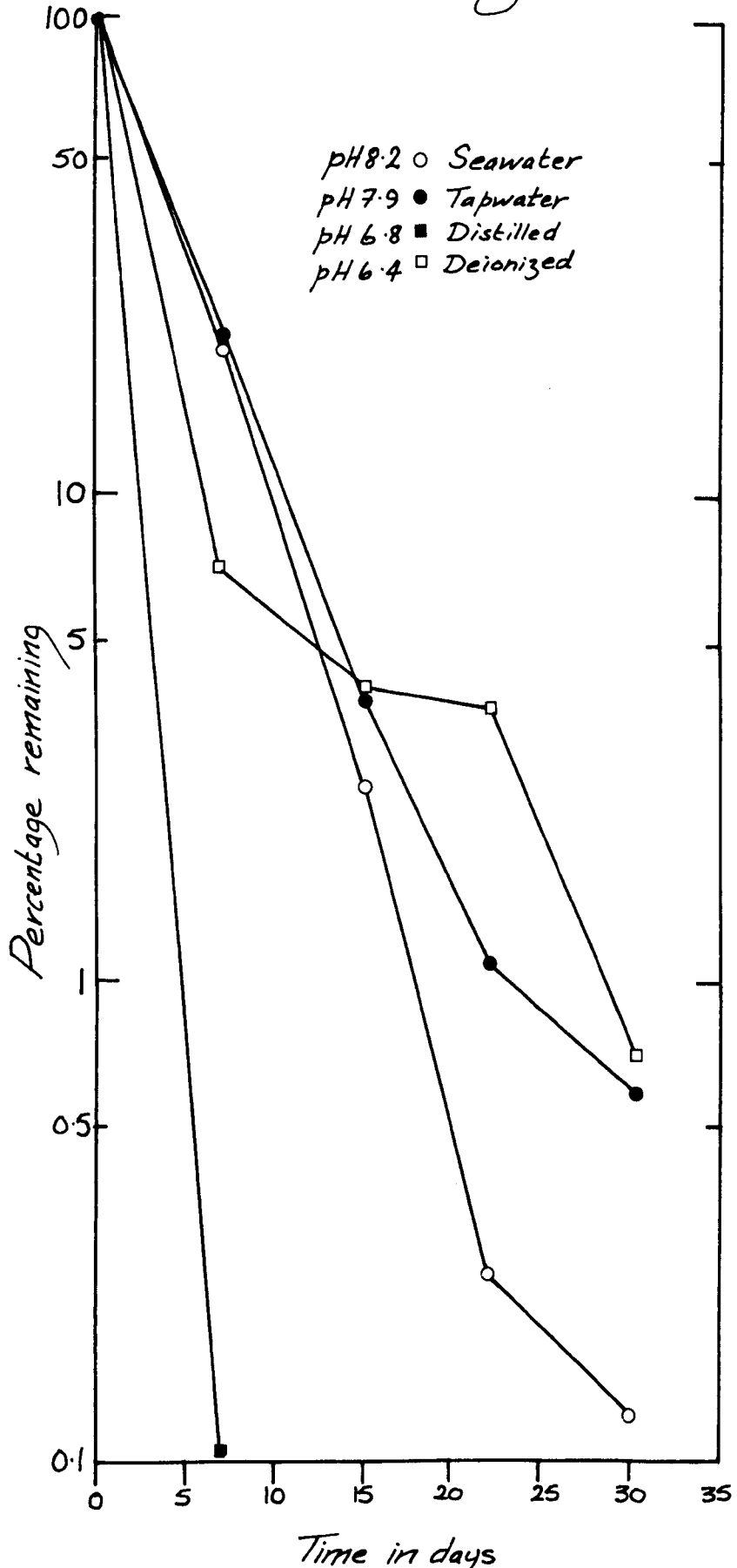
The results of two experiments to study the survival of phage MS2 in various waters were combined and are shown in Table 17 and Figure 21(b). The very rapid decline in the number of PFU in distilled water was in marked contrast to the survival of phage in the same water which had been deionized even though the pH values were similar (distilled, pH 6.8; deionized, pH 6.4). Sea water and tapwater gave similar

Table 17 Survival of phage MS2 in saline and non-saline waters

Time (days)	Treatment							
	Sea water		Tapwater		Distilled water		Deionized water	
	PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%
0	7.95×10^3	100	1.2×10^4	100	1.24×10^4	100	6.55×10^3	100
7	1.65×10^3	20.0	2.56×10^3	21.0	1.0×10^1	0.08	4.7×10^2	7.2
15	2.05×10^2	2.5	4.55×10^2	3.8	<5	< 0.04	2.55×10^2	3.9
22	2×10^1	0.25	1.35×10^2	1.1	-	-	2.75×10^2	4.2
30	1×10^1	0.125	7.5×10^1	0.6	-	-	5×10^1	0.7

*% remaining calculated from day 0 = 100%.

Figure 21b Survival of coliphage MS2 in waters of different types.



results (pH 8.2 and 7.9 respectively) although low levels of phage persisted longer in tapwater. In all treatments there was evidence of an initial mortality (inactivation) rate probably linked to pH. Although the survival rates varied, initial die-off was more rapid in the deionized/distilled treatments than in either sea water or tapwater at a higher pH. This suggests that initial effects are due to pH and that longer-term survival of phage is associated with some other factor(s), e.g. salinity as indicated here.

5.4.2.1.II Effect of salinity

The results of replicate experiments with MS2 phage in waters over a range of salinity from $0^{o}/oo$ (fresh water) to $30^{o}/oo$ (estuarine sea water) have been combined and are shown in Table 18 and Figure 22. Minimum survival was observed at the extremes of the range, i.e. in fresh water and full estuarine salinity ($30^{o}/oo$) and maximum survival, albeit at low levels, at 5 and $10^{o}/oo$. Survival at intermediate salinities over the range 15 to $25^{o}/oo$ was similar in general trends although increasing salinity evidently resulted in a decrease in survival, i.e. survival in $15^{o}/oo$ > $20^{o}/oo$ > $25^{o}/oo$. Detectable phage persisted at $15^{o}/oo$ and $20^{o}/oo$ for > 77 days and at $25^{o}/oo$ for > 53 days.

The presence of added host, *E. coli* cells at a ratio of one cell per PFU of MS2 phage, was investigated in a single experiment (Table 19, Figure 23). This gave similar overall results to those described above, with poorest survival at extremes of salinity, $0^{o}/oo$ and $30^{o}/oo$. Although some differences in the survival rates of MS2 with and without added host cells was evident at intermediate salt concentrations (15 to $25^{o}/oo$), maximum survival was observed at 5 and $10^{o}/oo$ in both experiments indicating that added host cells did not confer any degree of protection independent of salinity effects.

Table 18 Effect of salinity on phage MS2

Days	Salinity (°/oo)													
	0		5		10		15		20		25		30	
	PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%
0	10 500	100	16 500	100	23 500	100	13 000	100	43 500	100	34 000	100	24 500	100
7	550	5.24	8 800	53.33	9 600	40.85	14 000	107.69	12 000	27.59	43 000	38.24	2 800	11.43
13	75	0.71	5 200	31.52	9 000	38.3	10 000	76.92	6 100	14.02	3 000	8.82	325	1.33
20	75	0.71	4 900	29.70	2 435	10.36	3 100	23.85	5 150	11.84	2 000	5.88	45	0.18
27	50	0.48	2 800	16.97	2 240	9.53	2 750	21.15	3 050	7.01	1 800	5.29	25	0.10
41	15	0.14	1 550	9.39	2 100	8.94	515	3.96	850	1.95	385	1.13	< 5	< 0.02
53	< 5	< 0.05	1 760	10.67	1 595	6.79	115	0.89	105	0.24	80	0.24	< 5	< 0.02
68	-	-	855	5.18	Discontinued in error		10	0.08	15	0.03	< 5	0.02	-	-
77	-	-	650	3.94			5	0.04	10	0.02	-	-	-	-
91	-	-	230	1.39			-	-	-	-	-	-	-	-

*% remaining calculated on day 0 = 100.

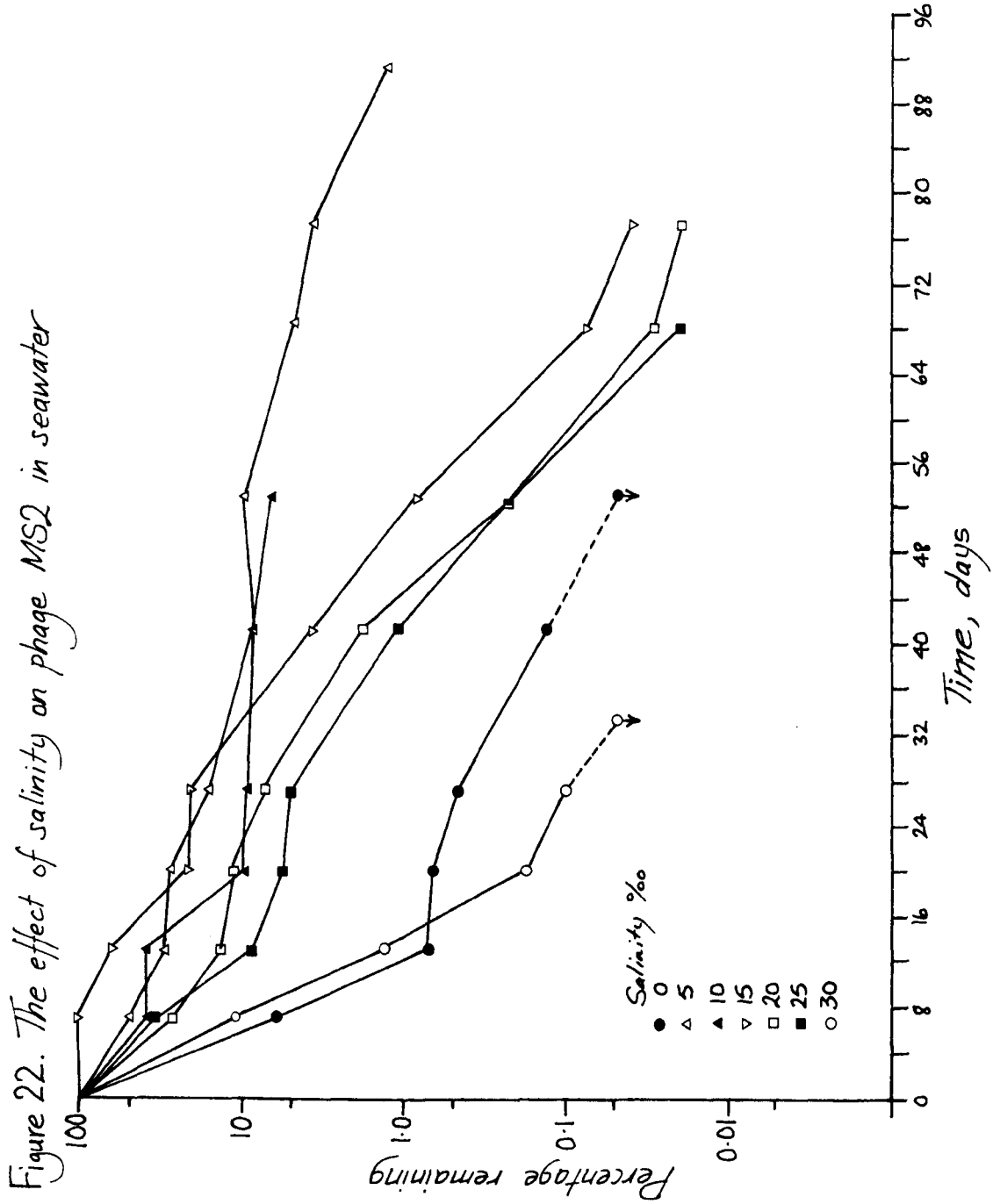


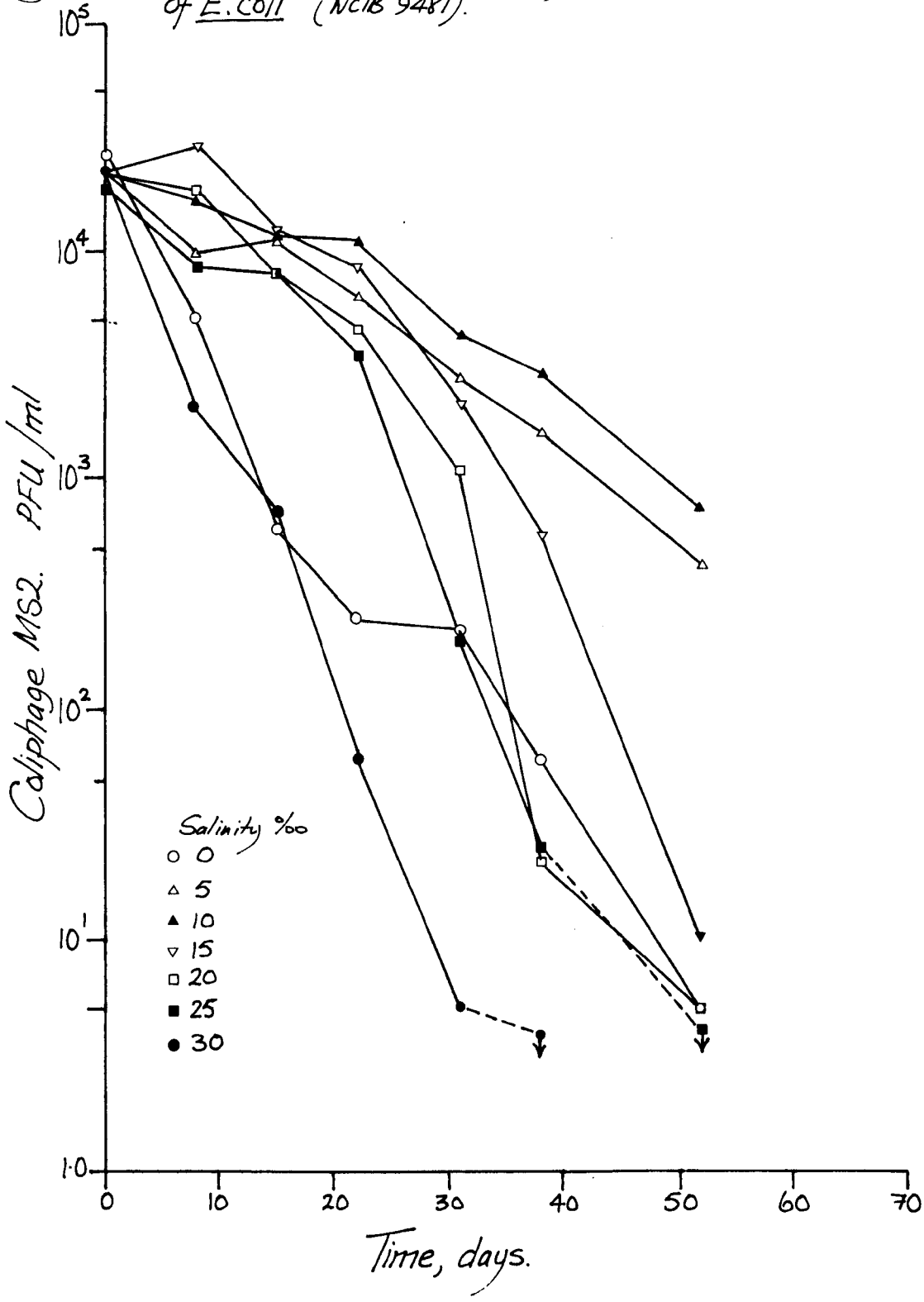
Figure 22. The effect of salinity on phage MS2 in seawater

Table 19 Effect of salinity on phage MS2 in the presence of host *E. coli* cells

Salinity (°/oo)	Days																	
	0		8		15		22		31		38		52		59		66	
	PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%
0	26 000	100	5 000	19.23	600	2.31	250	0.96	220	0.85	60	0.23	5	0.02	-	-	-	-
5	23 000	100	9 900	43.04	11 000	47.83	6 300	27.39	2 750	11.96	1 585	6.89	415	1.8	not done		50	0.21
10	23 000	100	17 000	73.91	13 000	56.52	11 150	48.48	4 300	18.7	2 850	12.39	755	3.28	120	0.52	130	0.57
15	22 000	100	29 000	131.82	12 000	54.55	8 650	39.32	2 200	10.0	585	2.66	10	0.05	-	-	-	-
20	21 000	100	18 000	85.71	7 900	37.62	4 600	21.9	1 100	5.24	215	1.02	5	0.28	-	-	-	-
25	18 000	100	8 500	47.22	8 000	44.44	3 450	19.17	200	1.11	25	0.14	<5	<0.03	-	-	-	-
30	22 000	100	2 000	9.09	700	3.18	60	0.27	5	0.02	-	-	-	-	-	-	-	-

*% remaining calculated from day 0 = 100%.

Figure. 23. The effect of salinity on phage MS2 in the presence of *E. coli* (NCIB 9481).



Results of a single experiment using *E. coli* 9481 alone are given in Table 20 and Figure 24, with an anticipated rapid mortality in contrast to that observed with phage. However, survival was poorest at the extremes of salinity (0 and 30°/oo) and better over the range 5 to 15°/oo, in good agreement with the phage, and phage plus host studies.

5.4.2.1.III Effect of major component salts (ions) in sea water

Because of the long duration of the first experiment under this heading it was not possible to repeat it. The results obtained from the single experiment to study the effects of omitting constituent salts on MS2 phage are shown in Figure 25 and Table 21. In the complete artificial sea water (ASW) and a NaCl solution of equivalent salinity 28 to 30% of the original phage inoculum was still remaining after 104 days when the experiment was terminated. The poorest survival (< 1% after 104 days) was in the treatment lacking $MgSO_4$ and the greatest survival in the treatment lacking $MgCl_2$ (55% remaining after 104 days), suggesting that the sulphate ion is important in phage survival. A comparison of the treatments lacking one or other of the chloride salts shows wide differences in survival, i.e. lacking $MgCl_2$, 55% left at 104 days; lacking $CaCl_2$, 45% left at 104 days; and lacking KCl, 30% left at 104 days. This would suggest that the potassium ion is also important in phage survival.

The experiments with *E. coli* showed poorest survival with the treatment lacking KCl but this was less apparent with the chlorides of magnesium and calcium. Overall the treatment lacking $CaCl_2$ showed the best survival, again suggesting that the potassium ion is important rather than the chloride. NaCl and complete sea water were also again similar. The mortality of *E. coli* is rapid and it would have been desirable to sample throughout the 24 h duration of the experiment (Table 22, Figure 26).

Table 20 Effect of salinity on *E. coli* 9481 (host for MS2)

Salinity (°/oo)	Days									
	0	1		2		3		7		
	Count/ml	%*	Count/ml	%	Count/ml	%	Count	%	Count	%
0	19 000	100	3 500	18.42	<15	<0.08	not tested			
5	20 000	100	13 000	65.0	1 050	5.25	250	1.25	<15	<0.075
10	19 000	100	15 000	78.95	950	5.0	15	0.08	-	-
15	23 000	100	10 000	43.48	900	3.91	35	0.15	<15	<0.07
20	21 000	100	6 000	28.57	115	0.55	<15	<0.07	-	-
25	20 000	100	1 700	8.5	115	0.58	<10	<0.05	-	-
30	19 000	100	1 500	7.89	15	0.08	not tested			

*%remaining calculated from day 0 = 100%.

Figure 24. The effect of salinity on the survival of E.coli

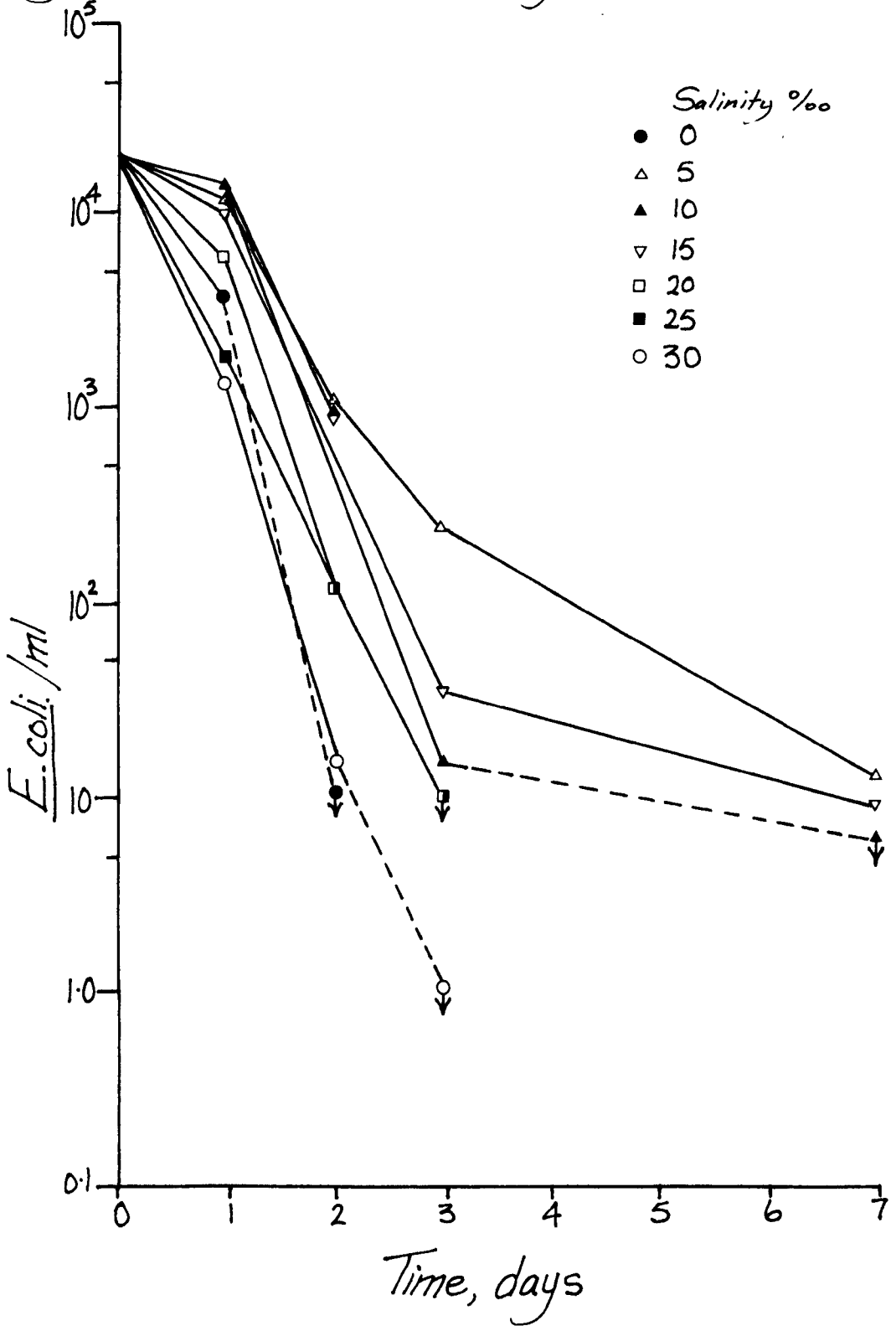


Figure 25. The effect of the major ions in seawater on the survival of coliphage MS2.

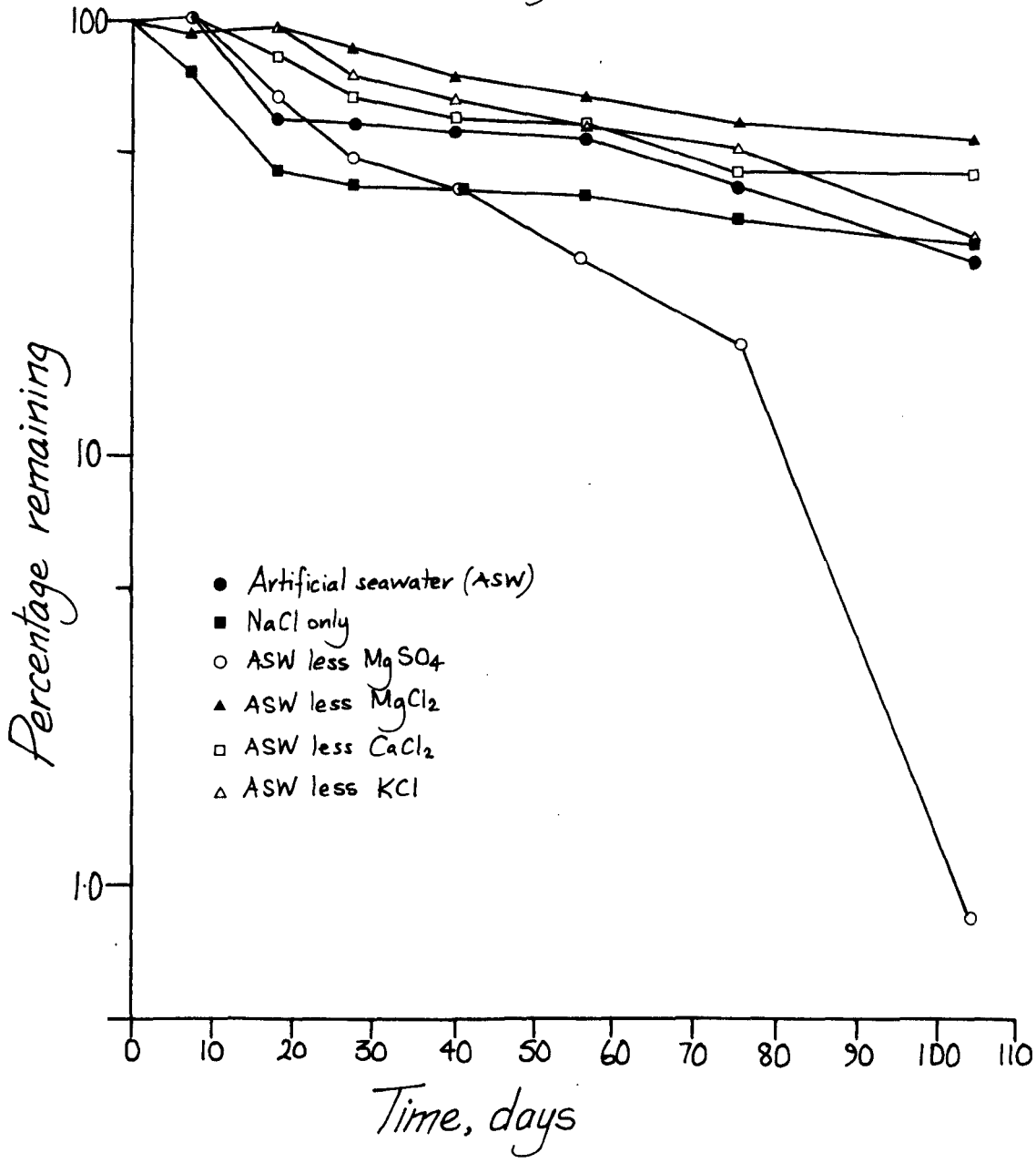


Table 21 Effect of major component salts on phage MS2 in sea water

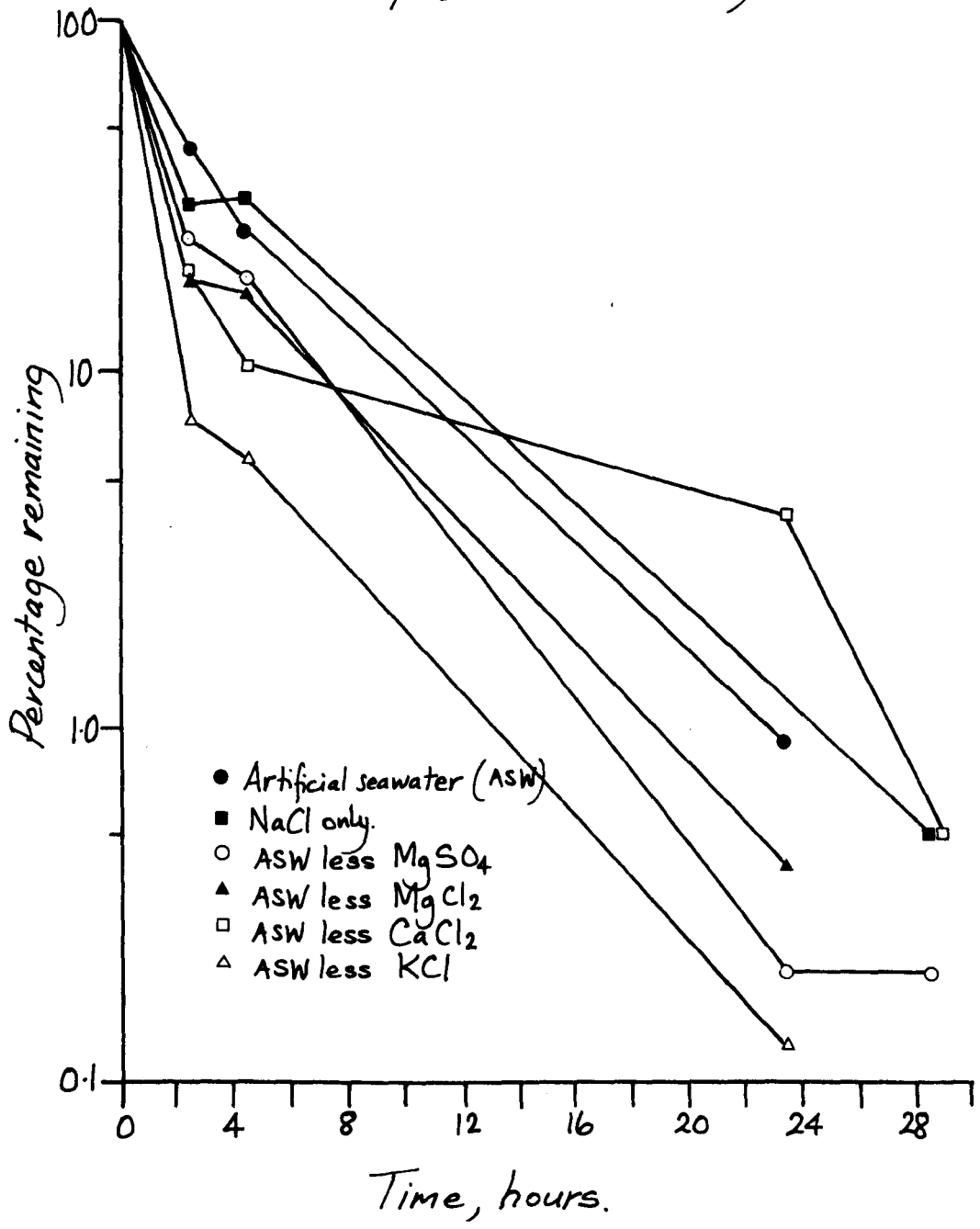
Time		Treatment					
		F1 Artificial sea water (ASW)	F2 NaCl only	F3 ASW less $MgSO_4$	F4 ASW less $MgCl_2$	F5 ASW less $CaCl_2$	F6 ASW less KCl
Start (0)	PFU/ml	1.96×10^4	1.89×10^4	1.98×10^4	1.38×10^4	1.48×10^4	1.47×10^4
	%	100	100	100	100	100	100
7 days	PFU/ml	2.2×10^4	1.25×10^4	2.19×10^4	1.34×10^4	1.76×10^4	1.64×10^4
	%	112.5	66.0	110.8	97.45	118.6	112.2
18 "	PFU/ml	1.17×10^4	8.35×10^3	1.28×10^4	1.27×10^4	1.26×10^4	1.36×10^4
	%	59.8	44.2	64.8	92.4	85.1	92.2
27 "	PFU/ml	1.13×10^4	8.2×10^3	9.25×10^3	1.19×10^4	1.01×10^5	1.09×10^4
	%	57.5	43.4	46.8	86.2	68.2	73.8
40 "	PFU/ml	1.11×10^4	7.6×10^3	8×10^3	1.03×10^4	8.75×10^3	9.3×10^3
	%	56.7	40.2	40.5	74.5	59.1	63.3
56 "	PFU/ml	9.85×10^3	7.35×10^3	5.55×10^3	9.25×10^3	8.6×10^3	8.5×10^3
	%	50.4	38.8	28.1	67.3	58.1	57.8
75 "	PFU/ml	7.95×10^3	6.9×10^3	3.55×10^3	8.05×10^3	6.7×10^3	7.35×10^3
	%	40.7	36.5	18.0	58.5	45.3	50.0
104 "	PFU/ml	5.55×10^3	5.88×10^3	1.7×10^2	7.45×10^3	6.7×10^3	4.45×10^3
	%	28.4	30.9	0.86	54.9	45.3	30.3

Table 22 Effect of major component salts on *E. coli* 9481 in sea water

Time	Treatment					
	F1 Artificial sea water (ASW)	F2 NaCl only	F3 ASW less $MgSO_4$	F4 ASW less $MgCl_2$	F5 ASW less $CaCl_2$	F6 ASW less KCl
Start (0)	100	100	100	100	100	100
2½ hours	43.4*	32.5	19.3	24.4	19.5	7.6
4½ "	25.5	30.3	16.2	18.6	10.3	5.8
23½ "	0.9	-	0.4	0.2	4.0	
28½ "		0.5		0.2	0.5	

*% remaining calculated on start (0 hours) = 100%.

Figure 26. The effect of the major ions in seawater on the survival of *E. coli* (NCIB 9481)



5.4.2.2 Effect of pH

The combined results of three experiments examining the effects of pH on MS2 phage are summarized in Table 23 and Figure 27. These experiments gave very clear evidence of a pH effect enlarging upon the suggestion raised in the results of section 5.4.2.1.I. The poorest survival was in the acid pH range and indeed the mortality of phage in pH range 1 to 4 was virtually instantaneous. The best survival was at pH 6.0 with 5% of the original inoculum still evident after 49 days. As pH was taken through neutral to the alkaline end of the scale mortality increased substantially, although extreme alkaline pHs (9 and 10) were less destructive to phage than the acid end of the range.

A number of experiments with *E. coli* failed because of rapid mortality but two were successfully completed and the combined results are shown in Table 24. As with the MS2 experiments, mortality of *E. coli* was virtually instantaneous at the extreme acid pHs 1 to 4. Best survival was observed at pHs 6 and 7. At pH 8 and above mortality was again very rapid and *E. coli* were undetectable after less than 3 h exposure.

5.4.2.3 Seasonal variation

A total of ten experiments was performed to cover a period of one calendar year and as a result a mass of data was obtained. In order to reduce these data to manageable proportions the results of the ten experiments with each treatment were graphed and from the graphs values of T_{90} were taken (i.e. time taken for 90% reduction of original count). The graphs are included in the appendices for information (Appendix 14) and the T_{90} values are presented in Table 25, together with the range of values obtained and the means. The results for treatments based on autoclaved sea water with and without added host *E. coli* gave similar

Table 23 Effect of pH on the phage MS2 in tapwater

Flask code	pH	Time (weeks)															
		0	1		2		3		4		5		6		7		
			PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	
A	1	1.5×10^1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
B	2	3×10^1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C	3	2×10^1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
D	4	7×10^2	1×10^1	0.14	-	-	-	-	-	-	-	-	-	-	-	-	
E	5	8.25×10^3	1.11×10^3	13.39	1.6×10^2	1.94	3.5×10^1	0.42	1×10^1	0.12	-	-	-	-	-	-	
F	6	9.25×10^3	5.1×10^3	54.59	5.1×10^3	54.59	3.9×10^3	42.16	3.55×10^3	38.38	3.45×10^3	37.3	2.05×10^3	22.16	4.55×10^2	4.92	
G	7	6.95×10^3	3.85×10^3	55.4	3.55×10^3	51.08	2.05×10^3	29.5	1.7×10^3	24.46	8×10^2	11.51	3.5×10^2	5.04	7×10^1	1.01	
H	8	8.25×10^3	2.19×10^3	26.55	1.57×10^3	19.03	1.03×10^3	12.42	6.55×10^2	7.94	1.15×10^2	1.39	1.35×10^2	1.64	1.5×10^1	0.18	
I	9	5.95×10^3	1.3×10^3	21.74	4.95×10^2	8.28	2.2×10^2	3.68	1.05×10^2	1.76	not done		5.5×10^1	0.92	-	-	
J	10	9.4×10^3	1.9×10^3	20.21	1.03×10^3	10.96	2.8×10^2	2.98	1.85×10^2	1.97	1.65×10^2	1.76	6.5×10^1	0.69	4×10^1	0.43	

*% remaining calculated from taking 0 week (start) as 100%.

Figure 27. Effect of pH on phage MS2 in tapwater.

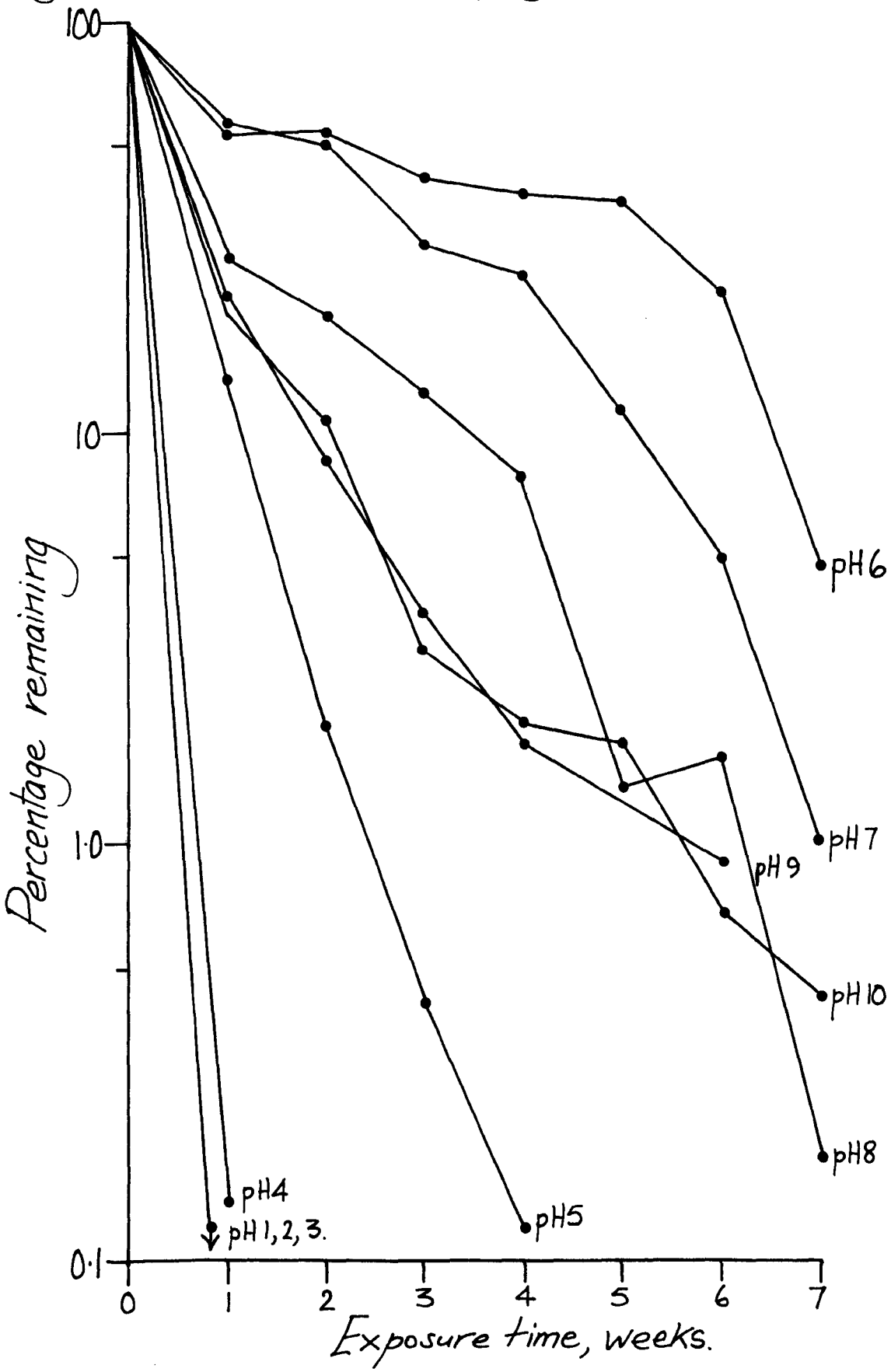


Table 24 Effect of pH on *E. coli* in tapwater

pH	Time						
	0	3 hours		6 hours		24 hours	
	Count/ml	Count/ml	%*	Count/ml	%	Count/ml	%
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	100	-	-	-	-	-	-
5	150	10	6.7	10	6.7	-	-
6	190	180	94.7	130	68.4	80	42.1
7	260	210	80.8	80	30.8	80	30.8
8	19	0	-	-	-	-	-
9	24	0	-	-	-	-	-
10	19	0	-	-	-	-	-

*% remaining calculated from 0 hours (start) as 100%.

Table 25 T_{90} values for MS2 phage in sea water (in days)

Experiment no.	Period	Treatment			
		Autoclaved sea water + MS2 phage	Autoclaved sea water + MS2 phage + *E. coli* 9481	Natural sea water + MS2 phage	Natural sea water + MS2 phage + *E. coli* 9481
1	28 Feb to 27 Mar	18.7	19.6	3.5	3.2
2	28 Mar to 18 Apr	13.4	15.5	9.5	9.2
3	25 Apr to 16 May	13.6	12.0	5.0	5.0
4	22 May to 12 Jun	18.6	8.5	7.2	3.2
5	19 Jun to 10 Jul	26.3	29.6	6.2	6.0
6	17 Jul to 31 Jul	16.0	17.4	4.8	3.8
7	13 Aug to 4 Sep	34.5	20.0	<2.0	<5.0
8	17 Sep to 8 Oct	13.5	8.5	5.2	7.4
9	16 Oct to 6 Nov	24.3	31.6	3.9	4.6
10	22 Nov to 12 Dec	28.3	27.0	10.9	11.2
Range (days)		13.4 to 34.5	8.5 to 29.6	<2.0 to 10.9	<5 to 11.2
Mean (days)		20.72	18.97	5.82	5.86

T_{90} values, and substantially higher than corresponding results with raw natural sea water. Within the different experiments the range of observations was considerably wider with autoclaved sea water than with natural sea water which may suggest that autoclaving affects some variable component of the water. Added host cells made no overall difference in survival in either autoclaved or raw sea water although there were individual experiments in the autoclaved sea water group where host cells appear to have substantially raised or lowered survival of phage. To examine any relationship which might be seasonal the T_{90} values of each treatment were graphed (Figures 28a and b). The good agreement between the plots with and without host cells suggests that the fluctuations observed are real ones and not due to experimental technique. It was not possible to make regular chlorophyll 'a' determinations as an indication of phytoplankton activity but the smallest T_{90} values in the autoclaved treatments correspond to the spring and autumn plankton blooms in the River Crouch where the water originated. The spring depression is also evident in the raw sea water treatments although the magnitude of the fluctuations is considerably less.

5.4.2.4 Effects of sterilization

Very apparent differences between survival of phage in autoclaved and raw sea water observed in section 5.4.2.3 and elsehere in this present work coupled with possible evidence of phytoplankton involvement (see 5.4.2.3) led to a series of 12 long-term experiments. A mass of data was collected during the year of observations and T_{90} values were calculated from graphed results as previously (5.4.2.3). These values are shown in Table 26, together with range of the observations made and calculated mean values for each set of experiments.

Figure 28a. T90 values for MS2 phage in seawater.

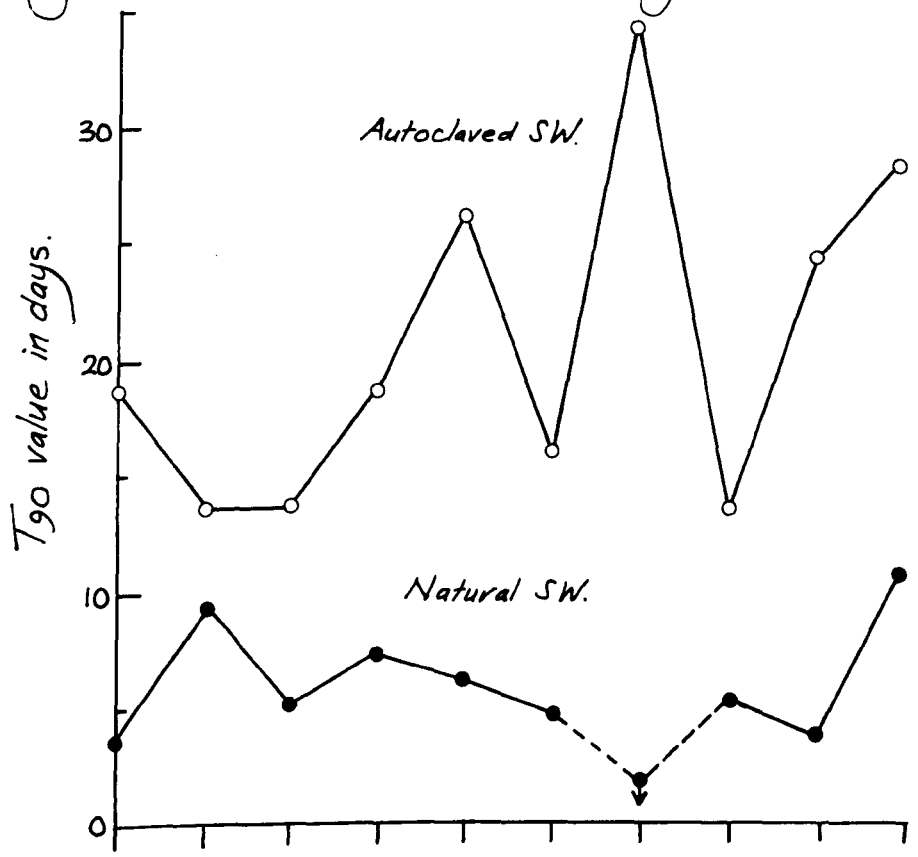


Figure 28b. T90 values for MS2 phage in seawater in the presence of E.coli (NCTB 9481).

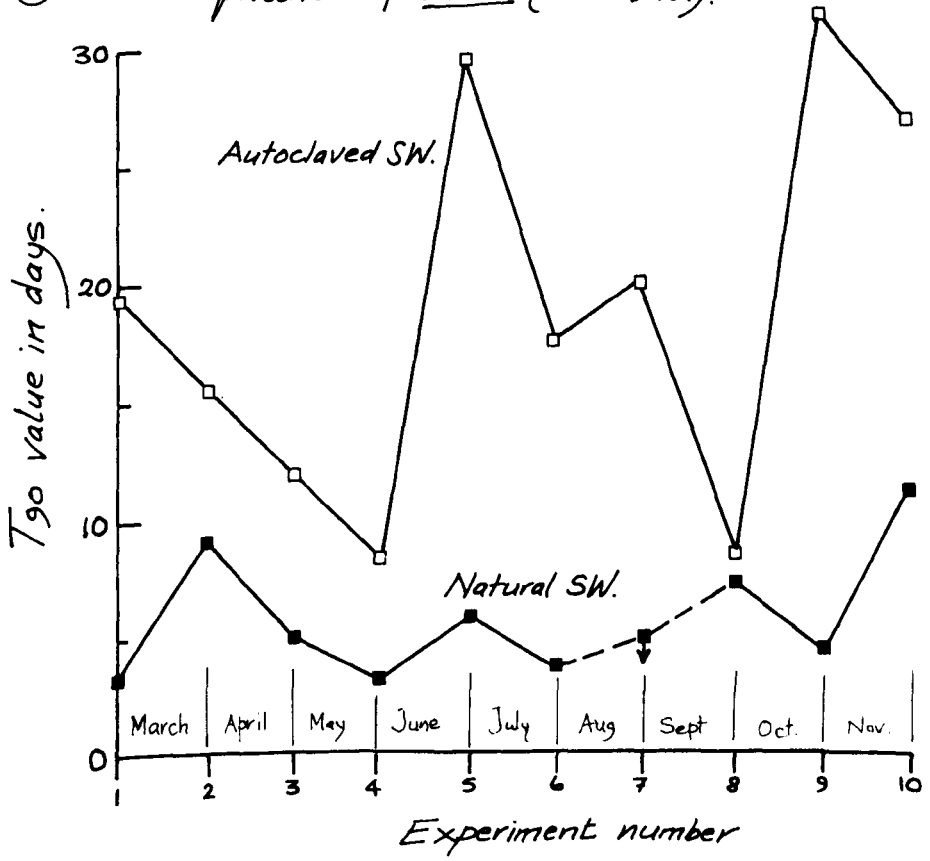


Table 26 T_{90} values for phage MS2 in various waters (in days)

Experiment no.	Period	Treatment				
		Tapwater + MS2 phage	Autoclaved sea water + MS2 phage	Natural sea water + MS2 phage	Natural sea water + MS2 phage + *E. coli* 9481	Membrane-filtered sea water + MS2 phage
1	18 Jan to 14 Mar	40.0	16.8	>63.0	17.5	8.4
2	17 Mar to 18 Jul	41.5	>65.0	15.0	23.5	13.5
3	8 Aug to 18 Aug	9.3	8.0	3.5	7.8	3.5
4	29 Nov to 14 Jan	9.0	14.5	10.3	5.2	20.1
5	29 Jan to 26 Mar	11.2	58.5	3.0	2.5	11.4
6	18 Apr to 4 Jun	7.7	24.5	12.8	7.0	22.0
7	5 Jun to 4 Jul	7.0	15.0	2.4	2.5	8.5
8	11 Jul to 2 Aug	9.8	21.0	5.5	4.9	15.5
9	13 Aug to 5 Sep	11.8	28.8	2.4	2.8	7.2
10	10 Sep to 10 Oct	18.0	14.5	6.5	7.5	14.0
11	16 Oct to 4 Nov	8.5	35.0	8.0	3.0	>35.0
12	28 Nov to 2 Jan	28.0	20.5	14.0	4.0	12.0
Range (days)		7.0 to 41.5	8.0 to >65.0	2.4 to >63	2.5 to 23.5	3.5 to >35.0
Mean (days)		16.87	26.8	12.2	7.29	14.31

Complete summaries of the data are given in Table 27. The tapwater was included as a non-saline control since results of section 5.2.1.I suggested that phage survived well in it. However, there was considerable variation in the T_{90} values with a range from 7 to 41.5 days. Enquiries addressed to the local water supply authority failed to provide any explanation such as variations in the treatment process, water source, chlorine content, etc. Possibly the differences reflect fluctuations in water quality for the abstraction source for which no data was available. As in previous experiments addition of host *E. coli* cells made no significant difference, tending if anything to reduce the T_{90} values slightly. Omission of the one high value (experiment 1) gives a mean of 7.58 for raw sea water and 7.29 for raw sea water + MS2.

Greater differences were apparent between the raw, autoclaved and membrane-filtered treatments and the individual T_{90} values were graphed for ease of interpretation (Figure 29). Of considerable interest was the similarity between results of raw and membrane-filtered treatments. An almost cyclical fluctuation in T_{90} values is apparent, with T_{90} values consistently higher for the membrane-filtered water. Although these experiments were of much longer duration than those described in section 5.4.2.3 the low T_{90} values for the autoclaved sea water again occur during the periods when phytoplankton should have been most abundant. Highest T_{90} values in the autoclaved treatment correspond to mid-summer and mid-winter periods when phytoplankton levels would be low.

5.4.2.5 Effects of sunlight and temperature

I Sunlight Results of the experiments conducted with *E. coli* 9481 and its phage MS2 are summarized in Figure 30. As expected, *E. coli* survival was better in dark conditions than in the light, taking three

Table 27 Long-term mortality experiments

Experiment no.	Date	Days storage	Coliphage MS2										*E. coli* 9481			
			F1		F2		F3		F4		F5		F5		F6	
			PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%
1	Commenced	0	3 400	100	15 000	100	7 000	100	8 650	100	8 100	100	6 800	100	6 100	100
	18 Jan	7	0	-	5 200	35	1 010	14	6 200	72	2 050	25	4	0.5	132	2.1
		14	0	-	4 450	30	760	11	4 700	54	1 055	13	0	-	4	0.07
		21	15	0.4	3 450	23	625	9	4 650	54	615	7.6	0	-	-	-
		28	0	-	2 900	19	265	4	3 600	42	495	6	-	-	-	-
		44	0	-	1 155	8.0	140	2	3 450	40	95	1.2	-	-	-	-
	ended	56	5	0.15	125	0.8	8	0.07	2 540	29	10	0.1	-	-	-	-
	14 Mar	63	0	-	60	0.4	0	-	945	11	0	-	-	-	-	-
2	Commenced	0	3 200	100	5 240	100	3 950	100	6 430	100	4 040	100	4 625	100	4 500	100
	17 Mar	8	750	23.0	3 000	57	3 780	95.7	2 700	42	1 960	49	0	-	0	-
		15	270	8.4	3 120	60	3 385	85.7	660	10	1 070	26	-	-	-	-
		22	105	3.3	2 500	48.0	3 240	82.0	105	1.6	735	18	-	-	-	-
		29	30	0.9	980	19.0	3 010	76.2	30	0.5	100	2.5	-	-	-	-
		43	10	0.3	470	9.0	2 980	75.4	0							
		50	0	-	355	6.8	2 620	66.3	0							
		56	0	-	205	4.0	1 140	28.9	0							
		64	0	-	150	2.9	1 060	26.8	0							
		71	0	-	125	2.4	1 045	26.5	0							
	ended	85	0	-	30	0.6	390	9.9	0							
	18 Jul	92	0	-	0	-	0	-	0							

note. Flek. = Flask number

Table 27 (cont.)

Experiment no.	Date	Days storage	Coliphage MS2										*E. coli* 9481			
			F1		F2		F3		F4		F5		F5		F6	
			PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%
3	Commenced	0	4 750	100	1 810	100	7 240	100	8 650	100	890	100	2 700	100	3 500	100
	8 Aug	7	not done													
	ended	10	0	-	160	8.5	400	5.5	10	0.1	50	5.6	not done			
	18 Aug															
4	Commenced	0	2 850	100	5 750	100	5 900	100	3 400	100	4 800	100	67 500	100	77 500	100
	29 Nov	7	1 650	57.9	140	24	1 780	30	1 435	42	280	5.8	-	-	-	-
		14	940	33	35	0.6	685	11.6	60	1.7	10	0.2	not done			
		21	270	9.5	20	0.3	145	2.4	0	-	-	-				
	ended	34	55	1.9	5	0.08	20	0.08	0	-	-	-				
	14 Jan	46	5	0.2	5	0.08	5	0.3	not done							
5	Commenced	0	12 700	100	15 100	100	11 600	100	14 100	100	14 100	100	55 000	100	60 000	100
	29 Jan	9	4 350	34	2 050	13.5	12 050	103.8	0	-	0	-				
		16	305	2.4	805	5.3	6 300	54.3	0	-	0	-				
		23	60	0.5	470	3	5 450	47	not done							
		30	10	0.08	200	1.3	2 600	22.4	not done							
		44	not done		135	0.9	1 915	16.5	not done							
	ended	56	not done		45	0.3	1 700	14.6	not done							
	26 Mar	70	not done		5	0.03	325	2.8	not done							

note. F1 etc = Flask number.

Table 27 (cont.)

Experiment no.	Date	Days storage	Coliphage MS2										*E. coli* 9481			
			F1		F2		F3		F4		F5		F5		F6	
			PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%
6	Commenced	0	4 150	100	1 020	100	3 450	100	3 500	100	4 100	100	27 000	100	27 000	100
	18 Apr	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7	3 800	91.5	95	9.3	2 550	74	410	11.7	450	11	5 000	18.5	0	-
		12	1 685	40.6	50	5	1 340	38.8	45	1.3	50	1.2	0	-	-	-
		21	555	13.4	30	3	590	17	0	-	———	———	not done	———	———	———
		28	90	2.2	10	0.98	215	6.1								
	ended	34	65	1.5	not done		130	3.7	———	———	———	not done	———	———	———	———
	4 Jun	47	5	0.1	not done		110	3.2	———	———	———	not done	———	———	———	———
7	Commenced	0	130 000	100	9 950	100	11 850	100	11 350	100	10 050	100	37 500	100	47 500	100
	5 Jun	7	3 100	23.8	850	8.5	1 900	16.0	-	-	5	0.05	0	-	0	-
		14	125	0.96	725	7.3	1 385	11.7								
	ended	21	60	0.46	185	1.9	525	4.4	———	———	———	not done	———	———	———	———
	4 Jul	28	5	0.04	105	1.1	130	1.1								
8	Commenced	0	6 100	100	205	100	6 500	100	5 700	100	3 750	100	14 000	100	17 000	100
	11 Jul	7	4 550	74.6	105	51.2	3 500	53.8	30	5.3	155	41	0	-	0	-
	ended	14	1 065	17.5	7.5	3.8	1 180	18.2	0							
	2 Aug	22	55	0.9	4.0	2.0	595	9.1	0		———	———	not done	———	———	———
9	Commenced	0	4 050	100	3 450	100	4 250	100	3 300	100	2 100	100	120 500	100	42 750	100
	13 Aug	7	405	10	600	17.4	3 300	77.6	0	-	10	0.5	0	-	0	-
	ended	14	30	0.74	130	3.8	980	23.1								
	5 Sep	20	5	0.1	65	1.9	930	21.9	———	———	———	not done	———	———	———	———

note. Flek = Flask number

Table 27 (cont.)

Experiment no.	Date	Days storage	Coliphage MS2										*E. coli* 9481			
			F1		F2		F3		F4		F5		F5		F6	
			PFU/ml	%*	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%
10	Commenced	0	3 550	100	1 250	100	2 400	100	3 100	100	1 550	100	47 750	100	29 000	100
	10 Sep	7	3 150	88.7	615	49.2	1 040	43.3	230	7.4	155	10	0	-	0	-
	ended	14	555	15.6	355	28.4	375	15.6	5	0.2	0	-	not done			
	10 Oct	20	0	-	70	5.6	25	1.0	-	-						
11	Commenced	0	3 250	100	1 950	100	1 850	100	2 250	100	2 950	100	26 750	100	43 750	100
	16 Oct	6	2 000	61.5	1 115	57.1	280	15.1	535	24	35	1.2	2 900	10.8	0	-
	ended	14	820	25.2	5	0.3	280	15.1	20	0.9	0	-	0	-	ND	
	4 Nov	20	785	24.1	ND		275	14.9	not done							
12	Commenced	0	12 700	100	9 500	100	17 500	100	13 550	100	13 700	100	2 275	100	13 250	100
	28 Nov	6	3 550	28	7 150	75.3	10 900	62.2	7 750	57.2	450	3.3	0	-	100	0.8
		13	890	7	2 850	30.0	5 000	28.6	1 555	11.5	45	0.3	-		0	-
		20	50	0.4	1 750	18.4	1 850	10.6	685	5	10	0.07	not done			
	ended	25	15	0.1	10 950	11.5	830	4.7	100	0.7	5	0.04				
	2 Jan	35	-	-	650	6.8	393	2.3	50	0.4	-	-				

*% remaining calculated on day 0 = 100%.

F1 Membrane-filtered sea water + MS2
F2 Tapwater + MS2
F3 Autoclaved sea water + MS2
F4 Natural sea water + MS2
F5 Natural sea water + MS2 + *E. coli* 9481
F6 Natural sea water + *E. coli* 9481.

note. Flekt = Flask number

Figure 29. Seasonal variation in T90 values for phage MS2 in raw, membrane filtered and autoclaved seawater.

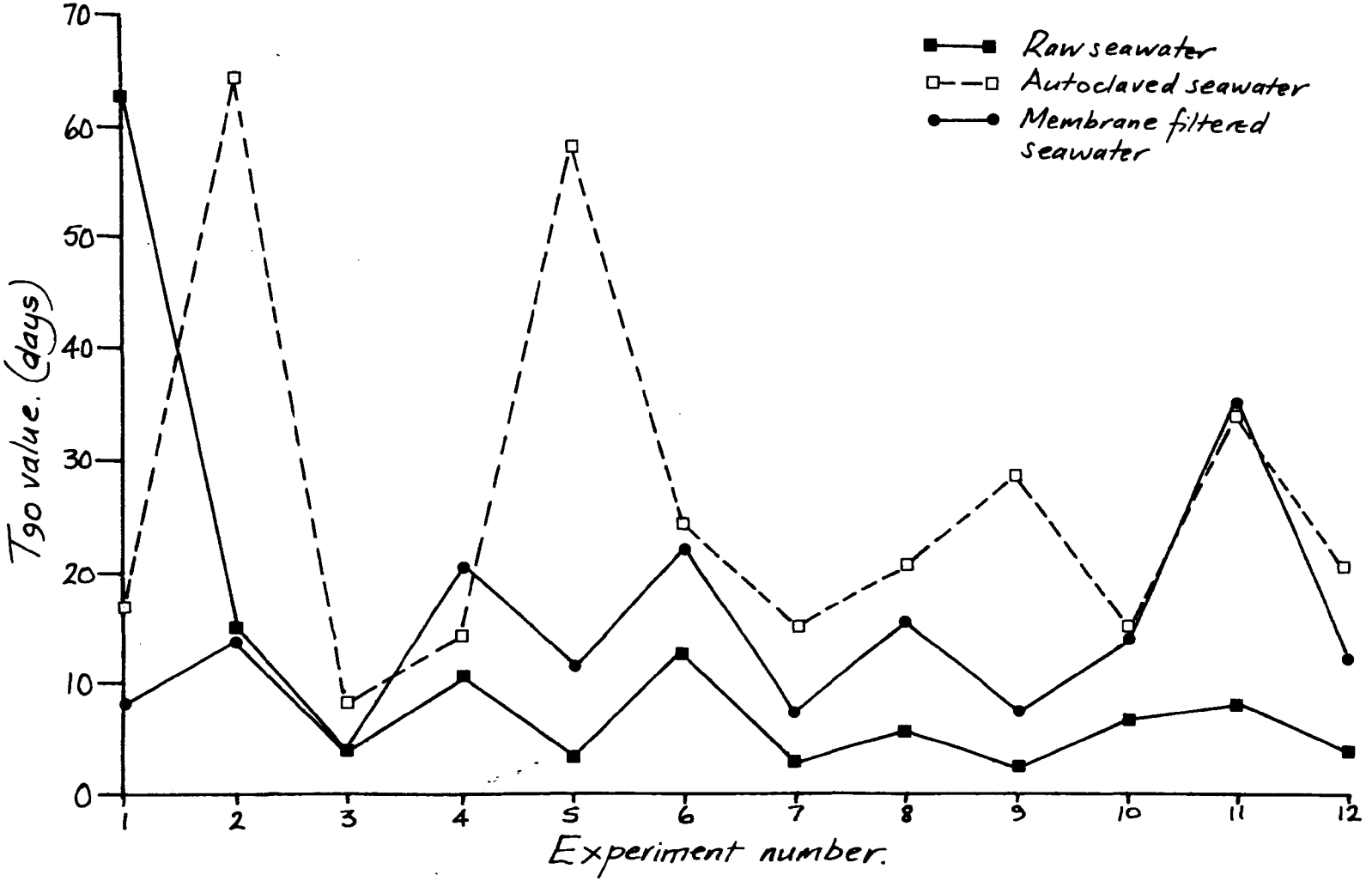
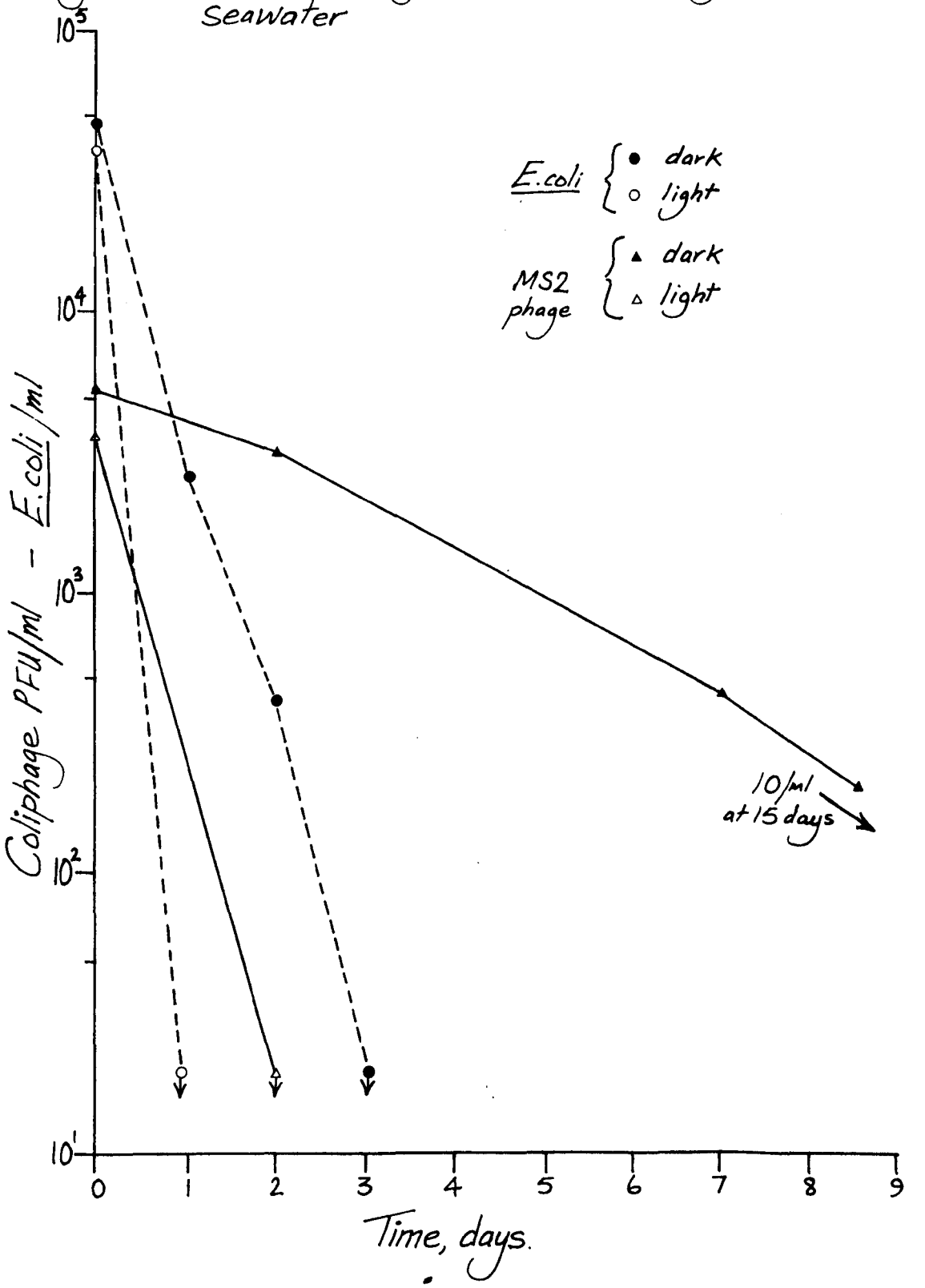


Figure 30. Effect of sunlight on *E.coli* and phage MS2 in seawater



times as long to decline to undetectable levels. Of particular interest however were the results obtained with MS2 phage when a similar clear difference emerged between survival in light and dark conditions. In the light treatment the initial inoculum had declined to undetectable levels, i.e. from 4×10^3 to < 5/ml in under 2 days. In contrast, a similar inoculum held in dark conditions still contained low numbers of phage (10/ml) after 15 days' exposure.

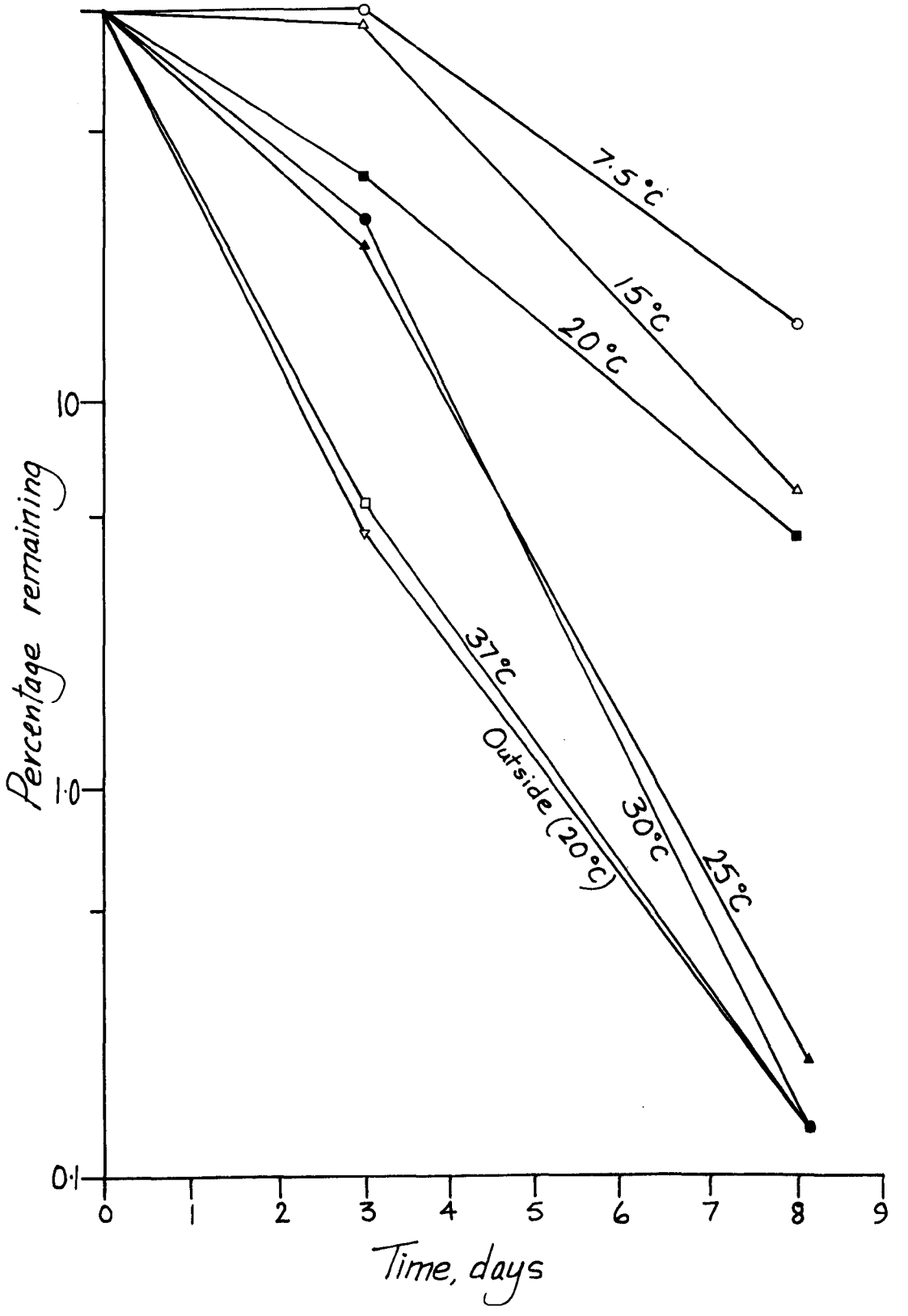
It was noted throughout the duration of the experiments that the temperature in the light treatments varied by 3 to 4°C, increasing during the day and falling again at night (i.e. in the dark). In contrast, the treatments held constantly in the dark remained relatively stable at 17 to 18°C.

II Temperature All treatments in this experiment were held in the dark in constant temperature conditions over the range selected. However, in view of the dramatic effect of light observed above (section 5.4.2.5.I) and a possible additional effect introduced by temperature variation in the light treatment, an additional 20°C (ambient) treatment was set up in conditions of daylight. The results of the experiment are shown in Table 28 and Figure 31 and indicate a marked temperature effect with decreasing survival as temperature increased, i.e. maximum survival at 7.5°C, minimum survival at 37°C. However, with exposure to light as an additional factor poorest survival of all was observed in the outdoor treatment. By comparing the two results for 20°C a light effect as observed in 5.4.2.5.I was very evident. This suggests that both light and temperature influence survival of coliphage and that when both factors are combined survival is less than that observed with either light or temperature considered separately, i.e. a synergistic effect.

Table 28 Effect of temperature on MS2 phage in sea water

Time (days)		Refrigerator 5.5-10°C	Holding temperature (°C)					Outside 14-23°C
			15	20	25	30	37	
0	Count	9 300	7 800	8 750	9 050	10 000	9 250	8 650
3	Count (PFU/ml)	10 200	7 600	3 300	2 300	2 950	500	400
	% remaining	109.6	97.4	37.7	25.4	29.5	5.4	4.6
8	Count (PFU/ml)	1 450	450	385	5	<5	<5	<5
	% remaining	13.6	5.8	4.4	0.05	<0.05	<0.05	<0.05

Figure 31. Effect of temperature on MS2 in seawater.



5.4.2.6 Effect of u/v light

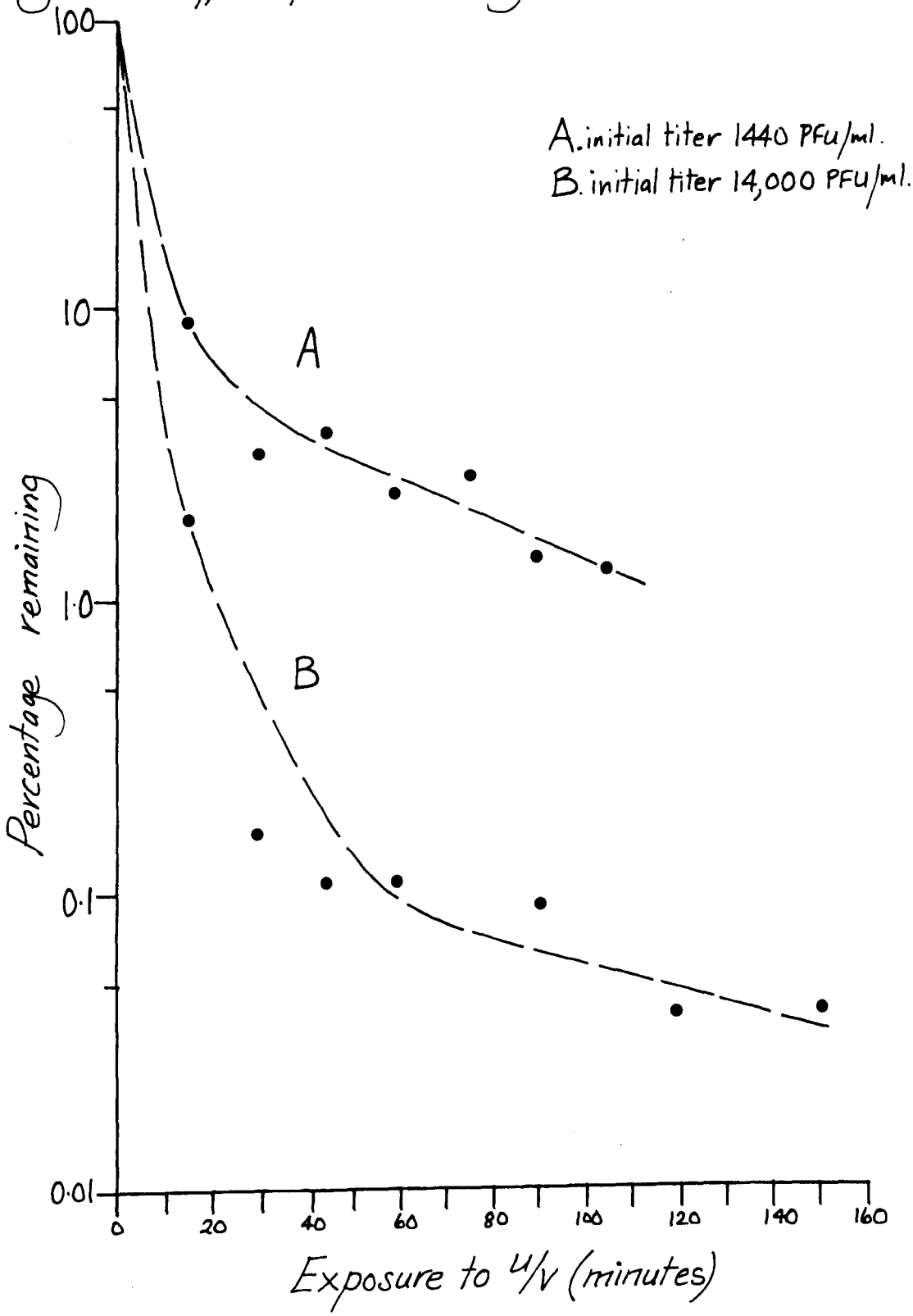
The results of experiments carried out using low and high titre doses of phage are shown in Table 29. It is interesting to note that although an effective reduction was achieved with both dosing rates the times for 99% reduction were very different. The data plotted on

Table 29 Effect of u/v light on MS2 phage in sea water

Experiment	Time (min)	Exposure to u/v (min)	Phage PFU/ml	% remaining
I	30	0	1 440	100
	45	15	130	9.0
	60	30	46	3.2
	75	45	54	3.8
	90	60	33	2.3
	105	75	39	2.7
	120	90	21	1.4
	135	105	20	1.3
II	30	0	14 000	100
	45	15	270	1.9
	60	30	22	0.16
	75	45	15	0.11
	90	60	15	0.11
	120	90	13	0.09
	150	120	6	0.04
	180	150	6	0.04
	240	210	1	0.007
	270	240	3	0.02
	300	270	3	0.02
	360	330	1	0.007
	390	360	<2	-
	410	380	<2	-

a graph (Figure 32) indicate that at high phage titre a 99% reduction was achieved within 20 minutes. At low phage titre (i.e. a tenth of the high dose) it took 120 minutes or six times as long to achieve a 99% reduction. Similar effects can be observed with *E. coli* cells and the differences in the mortality curves can be attributed to a 'one hit' effect of u/v. In a liquid containing a large number of bacteria or phage, exposure to u/v of a given volume of liquid per unit time will initially expose a high concentration of cells. A proportion of these

Figure 32. Effect of ultra-violet light on waterborne MS2.



will, dependent on the exposure time and dose of u/v irradiation, be killed or inactivated immediately. On each subsequent exposure to u/v further cells will be killed but since the relative numbers are decreasing the chances of being hit are reduced and this can be seen in the results where there is evidently a progressive flattening out of the mortality curves.

5.4.2.7 Adsorption and sedimentation

As a preliminary, an experiment using *E. coli* and Fullers Earth was set up and counted at intervals for up to 24 h. The results are shown in Table 30 and Figure 33. After 3 h exposure the percentage *E. coli* remaining was directly related to concentration of Fullers Earth, maximum survival being evident in the treatment without any Fullers Earth. Since all treatments were shaken before sampling the *E. coli* cells appear to have become attached to the particles and sedimented out. After 6 and 24 h exposure such a clear trend was not evident, due to the expected rapid mortality of *E. coli* in sea water.

Table 30 Effect of added Fullers Earth on *E. coli* in sea water

ppm Fullers Earth	Time (hours)						
	0	3		6		24	
	Count/ml	Count/ml	%*	Count/ml	%	Count/ml	%
0	2 090	910	43.5	650	31.1	40	1.91
1	1 940	730	37.62	650	33.5	20	1.03
10	2 110	710	33.6	470	22.27	10	0.47
50	1 820	388	21.3	467	25.66	10	0.55
100	1 850	300	16.2	80	4.32	-	

*%remaining calculated on 0 hours = 100%.

Table 31 and Figure 34 show the summarized results of experiments where the effects of shaking, ultrasonics and centrifugation were investigated with *E. coli*. Vigorous shaking showed increased recovery

Figure 33. Effect of Fullers Earth concentration and exposure time on the survival of E.coli in seawater.

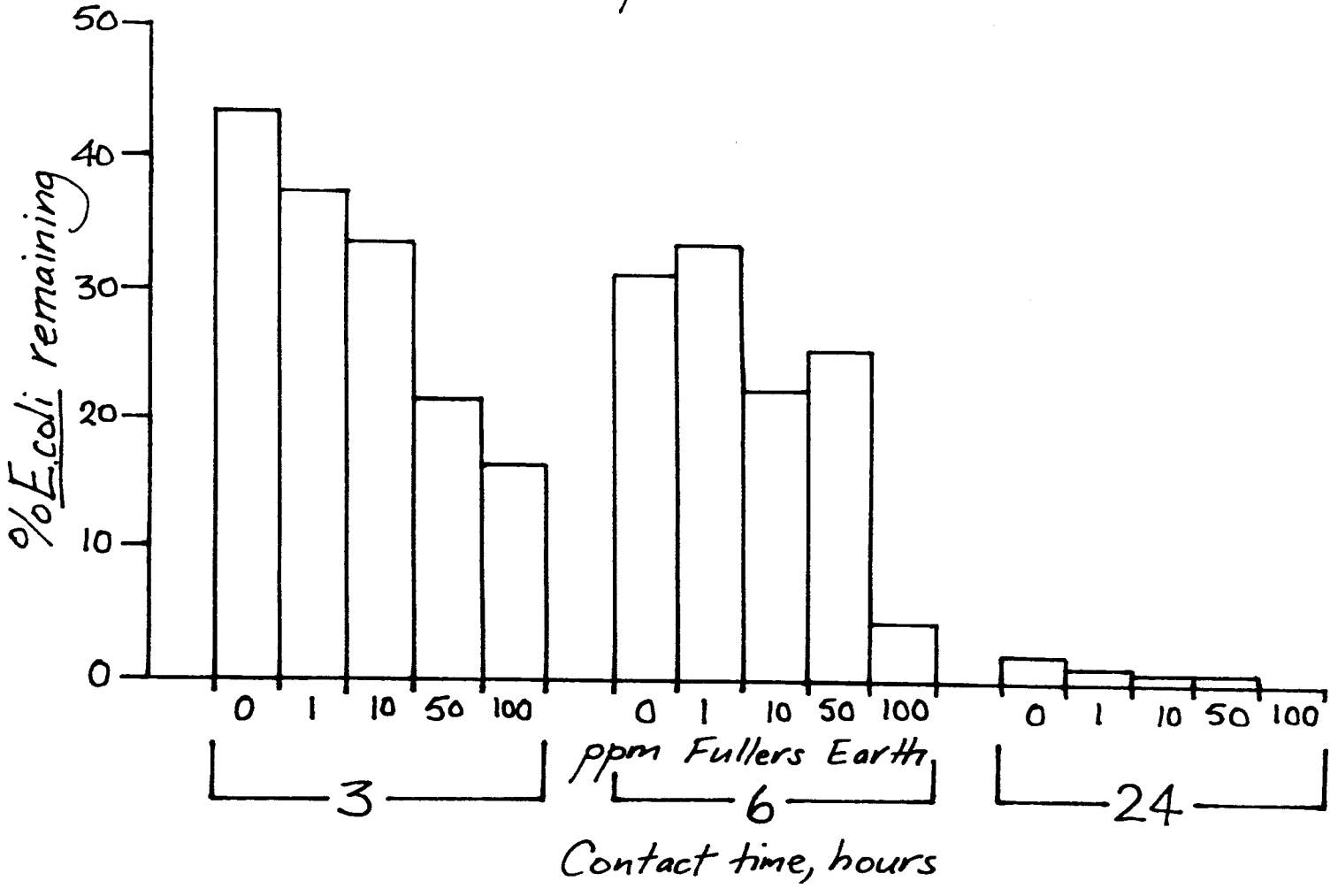


Table 31 Effect of subsequent treatment on *E. coli* in sea water in the presence of Fullers Earth

Treatment	ppm Fullers Earth	Count/ml		% remaining
		before	after	
Vigorous shaking	0	291	221	76.21
	1	253	224	88.54
	10	259	239	92.28
	50	267	280	104.87
	100	267	311	116.48
Ultrasonics	0	201	221	109.95
	1	240	211	87.92
	10	208	156	75.14
	50	228	139	60.96
	100	185	54	29.19
Centrifugation	0	197	142	72.08
	1	177	107	60.45
	10	136	69	50.74
	50	82	34	41.46
	100	148	42	28.38

as Fullers Earth concentration increased, i.e. *E. coli* associated with solids and resuspended by shaking. Unexpectedly the ultrasonics treatment did not appear to detach *E. coli* cells from suspended solids and recovery rate dropped dramatically as Fullers Earth concentration increased. Centrifugation in effect increases the natural slow sedimentation of solids and in the experiments performed this was shown as a gradual reduction in *E. coli* recovered. When the same experiments were repeated with phage MS2 very similar results were obtained (Table 32, Figure 35). Shaking increased recovery of phage as suspended solids increased. Ultrasonic effects seem to be random and unrelated to solids concentration. Centrifugation results were as for *E. coli* although it was of interest to note that almost 50% could not be recovered even from the treatment where no solids were added. A mortality of this degree is unlikely and reduction may have been due to suspended solids already present.

Figure 34. *Effect of Fullers Earth concentration and treatment on recovery of E.coli in seawater.*

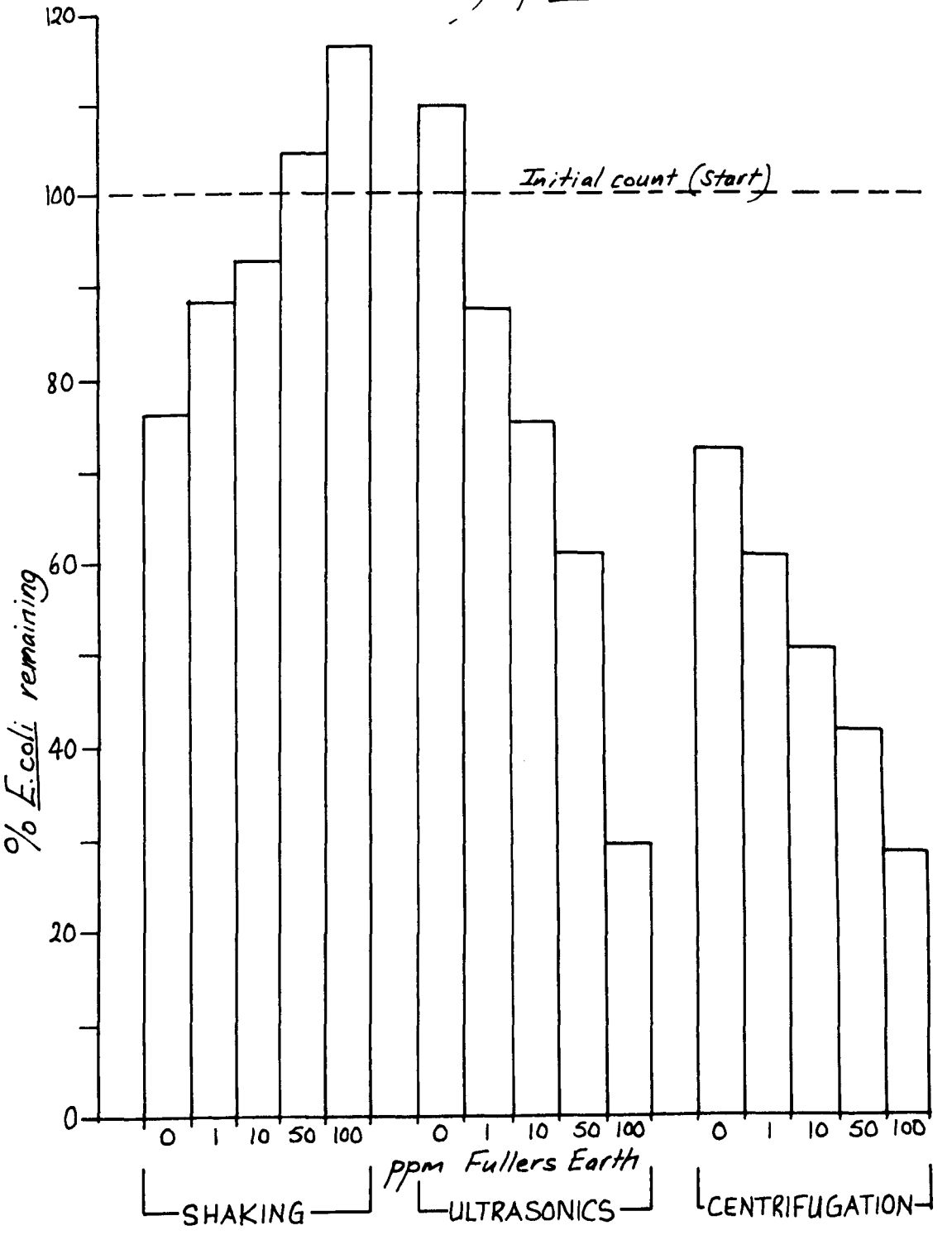


Table 32 Effects of subsequent treatment on MS2 phage in sea water in the presence of Fullers Earth

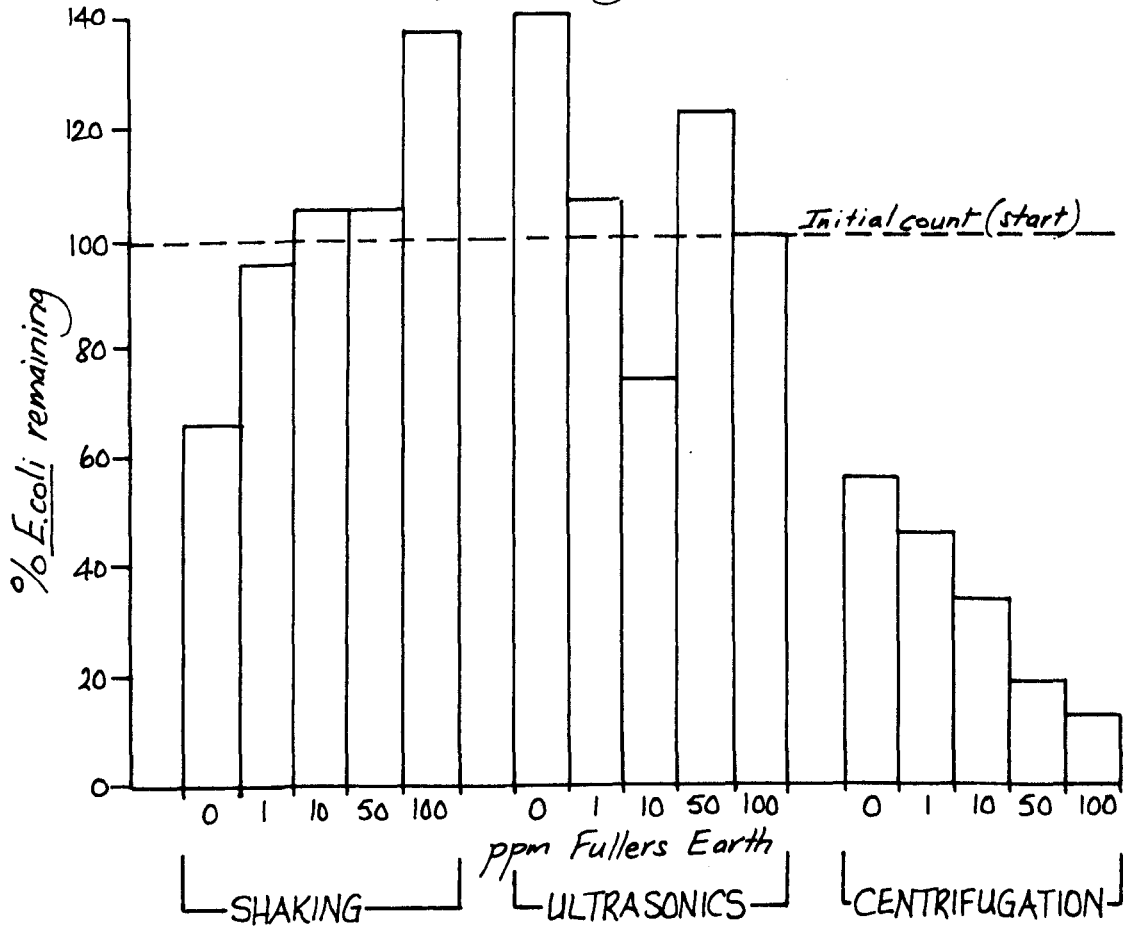
Treatment	ppm Fullers Earth	PFU/ml		% remaining
		before	after	
Vigorous shaking	0	1 170	760	64.96
	1	1 000	955	95.5
	10	825	780	105.77
	50	885	930	105.08
	100	805	1 105	137.37
Ultrasonics	0	1 170	940	140.3
	1	1 150	1 220	106.09
	10	1 170	855	73.08
	50	985	1 210	122.8
	100	600	600	100.0
Centrifugation	0	880	485	55.1
	1	1 055	475	45.02
	10	925	60	32.43
	50	835	31	18.56
	100	865	21	12.14

5.4.2.8 Effect of organics

As a preliminary to experiments using phage and *E. coli* the effects of added peptone and glucose on the total bacterial plate count of sea water was examined. The results obtained (Table 33) demonstrated that neither glucose nor peptone in concentrations up to 100 ppm substantially altered the general trends observed when these substances were omitted.

Results of two experiments carried out with MS2 phage are shown in Tables 34 and 35 and Figures 36/37 and 38/39. In the trials with glucose, superior survival was shown with 100 ppm glucose but results with lower concentrations of 1 and 10 ppm did not differ greatly from those obtained without glucose. The total plate count only increased in the 100 ppm glucose treatment suggesting an association with the survival of MS2 phage which was also better in this treatment. In the trials with peptone the 100 ppm concentration gave markedly better

Figure 35. Effect of Fullers Earth concentration and treatment on the recovery of phage MS2 in seawater.



survival than either the 1 or 10 ppm concentrations which differed little from that observed without peptone. Increases in total plate count were also only observed in the 100 ppm peptone treatment.

When the experiments were repeated with *E. coli*, glucose appeared to be marginally beneficial in reducing mortality though this was not apparently linked to glucose concentration (Table 36, Figure 40). In marked contrast the results obtained with peptone (Table 36, Figure 41) demonstrated that increasing concentrations of peptone promoted increased survival.

Results of the experiments using cysteine (as the hydrochloride) and MS2 phage are shown in Table 37. No protective effect related to cysteine concentration was observed although it was interesting to note that the superior survival in autoclaved sea water consistently observed in other sections was not evident here. On the contrary, raw seawater trials with added cysteine gave better survival than similar trials run with autoclaved water. Experiments with *E. coli* again showed no effect of cysteine related to concentration but superior survival was noted in the raw seawater trials (Table 38).

Table 33 Effect of glucose and peptone on the total plate counts of sea water

Treatment	Time (hours)							
	0	3	5	24	27	29	48	53
Sea water only	1.1×10^5	8.7×10^4	9.5×10^4	4.45×10^4	4.35×10^4	3×10^4	1.05×10^4	1.83×10^4
Sea water + 1 ppm glucose	1.2×10^5	9.25×10^4	9.75×10^4	3.25×10^4	3.25×10^4	2.38×10^4	1.5×10^4	1.13×10^4
Sea water + 10 ppm glucose	1.07×10^5	8.25×10^4	8×10^4	3.75×10^4	1.7×10^4	2.2×10^4	1.95×10^4	2.3×10^4
Sea water + 100 ppm glucose	1.3×10^5	1×10^5	8.75×10^4	3.7×10^5	2.4×10^4	3.1×10^4	2.03×10^4	2.48×10^4
Sea water + 1 ppm peptone	1.12×10^5	8.75×10^4	7.25×10^4	3.47×10^5	2.18×10^4	2.28×10^4	1.38×10^4	1.68×10^4
Sea water + 10 ppm peptone	1.1×10^5	8.25×10^4	7×10^4	6×10^5	5×10^4	4.5×10^4	1.8×10^4	1.48×10^4
Sea water + 100 ppm peptone	1.22×10^5	1.45×10^5	2×10^5	6.25×10^5	7.5×10^4	7.5×10^4	9.5×10^4	1.15×10^5

Table 34 Effect of glucose and peptone on phage MS2 and total plate counts in sea water

Treatment	Time (days)							
	0		12		20		27	
	Phage	TPC	Phage	TPC	Phage	TPC	Phage	TPC
Sea water only	5 051	2.45×10^4	<5	1.55×10^3	<5	1.15×10^3	-	1.13×10^3
Sea water + 1 ppm glucose	3 300	2.18×10^4	20	2.6×10^3	<5	6.5×10^2	-	3.5×10^2
Sea water + 10 ppm glucose	7 250	1.95×10^4	<5	3.53×10^3	<5	1.33×10^3	-	4.5×10^2
Sea water + 100 ppm glucose	7 550	2.35×10^4	475	4.95×10^5	105	2.88×10^5	25	7.25×10^4
Sea water + 1 ppm peptone	10 200	2.15×10^4	10	3.05×10^3	5	9.75×10^2	<5	5×10^2
Sea water + 10 ppm peptone	8 550	2.15×10^4	175	1.4×10^3	10	1.35×10^2	<5	5.25×10^2
Sea water + 100 ppm peptone	8 650	2.15×10^4	5 900	3.48×10^4	3 550	1.93×10^5	90	6.75×10^4

Table 35 Effect of glucose and peptone on phage MS2 and total plate counts in sea water

Treatment	Time (days)											
	0		7		14		21		28		35	
	Phage	TPC	Phage	TPC	Phage	TPC	Phage	TPC	Phage	TPC	Phage	TPC
Sea water only	5 450	1.38×10^4	385	1.7×10^3	100	7.3×10^2	25	1.65×10^3	<5	1.4×10^3	-	not tested
Sea water + 1 ppm glucose	3 600	1.45×10^4	445	3.78×10^3	50	9.5×10^2	<5	2.15×10^3	-	1.02×10^3	-	" "
Sea water + 10 ppm glucose	5 400	1.33×10^4	10	3.15×10^3	<5	2.12×10^3	-	2.58×10^3	-	2.5×10^2	-	" "
Sea water + 100 ppm glucose	4 600	1.5×10^4	1 980	2.52×10^4	420	1.12×10^5	10	2.72×10^3	<5	1.47×10^5	-	" "
Sea water + 1 ppm peptone	4 650	1.3×10^4	440	2.7×10^3	105	1.6×10^4	10	1.65×10^3	<5	2×10^2	-	" "
Sea water + 10 ppm peptone	5 150	1.45×10^4	295	1.77×10^3	40	6.5×10^2	<5	1.4×10^3	<5	5.5×10^2	-	" "
Sea water + 100 ppm peptone	4 700	1.88×10^4	2 585	3.4×10^4	2 500	2.9×10^4	1 185	9.0×10^3	245	3×10^3	25	1.95×10^3

Figure 36. Influence of glucose on phage MS2 and total plate count in seawater.

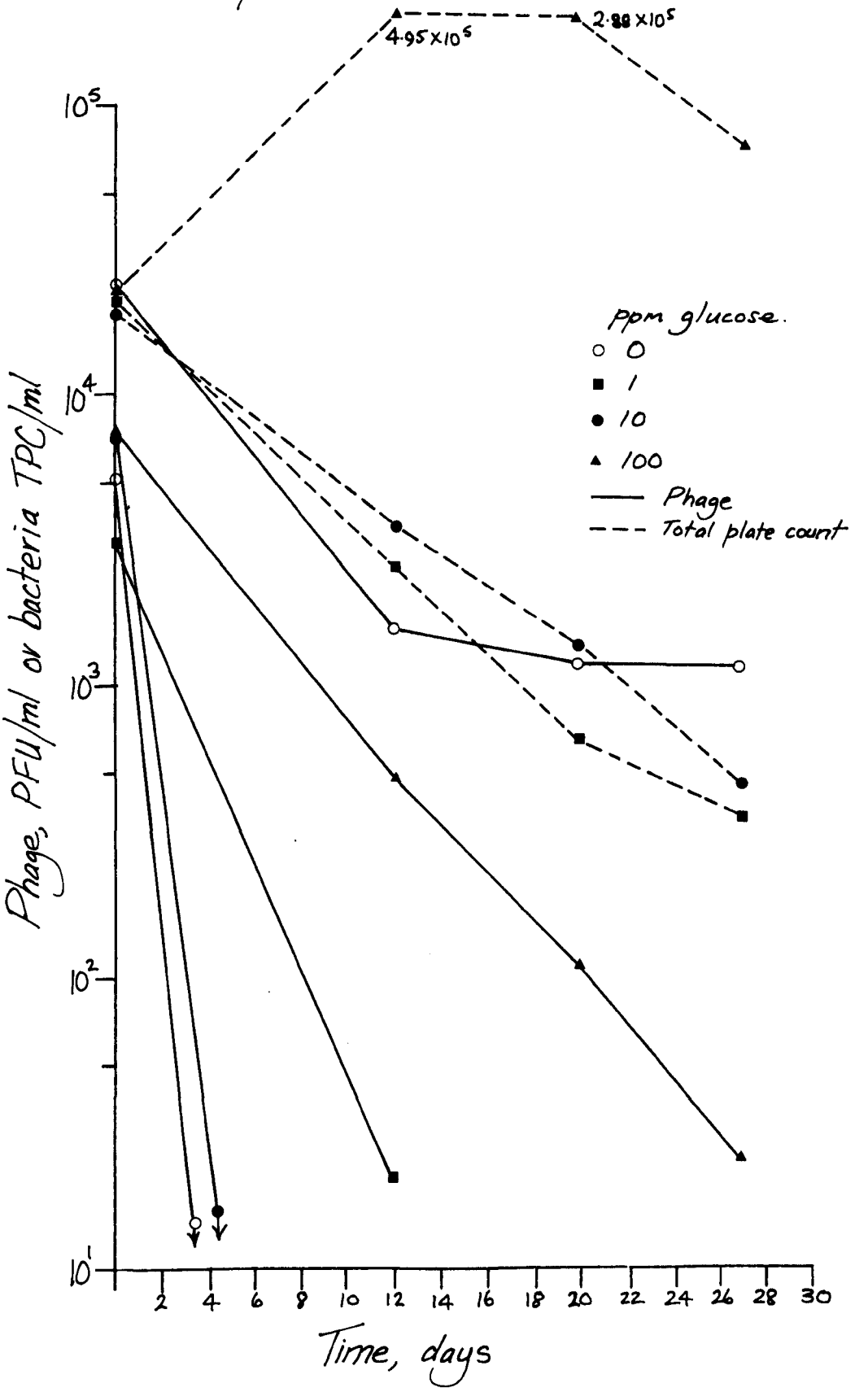


Figure 37. Influence of peptone on phage MS2 and total plate count in seawater.

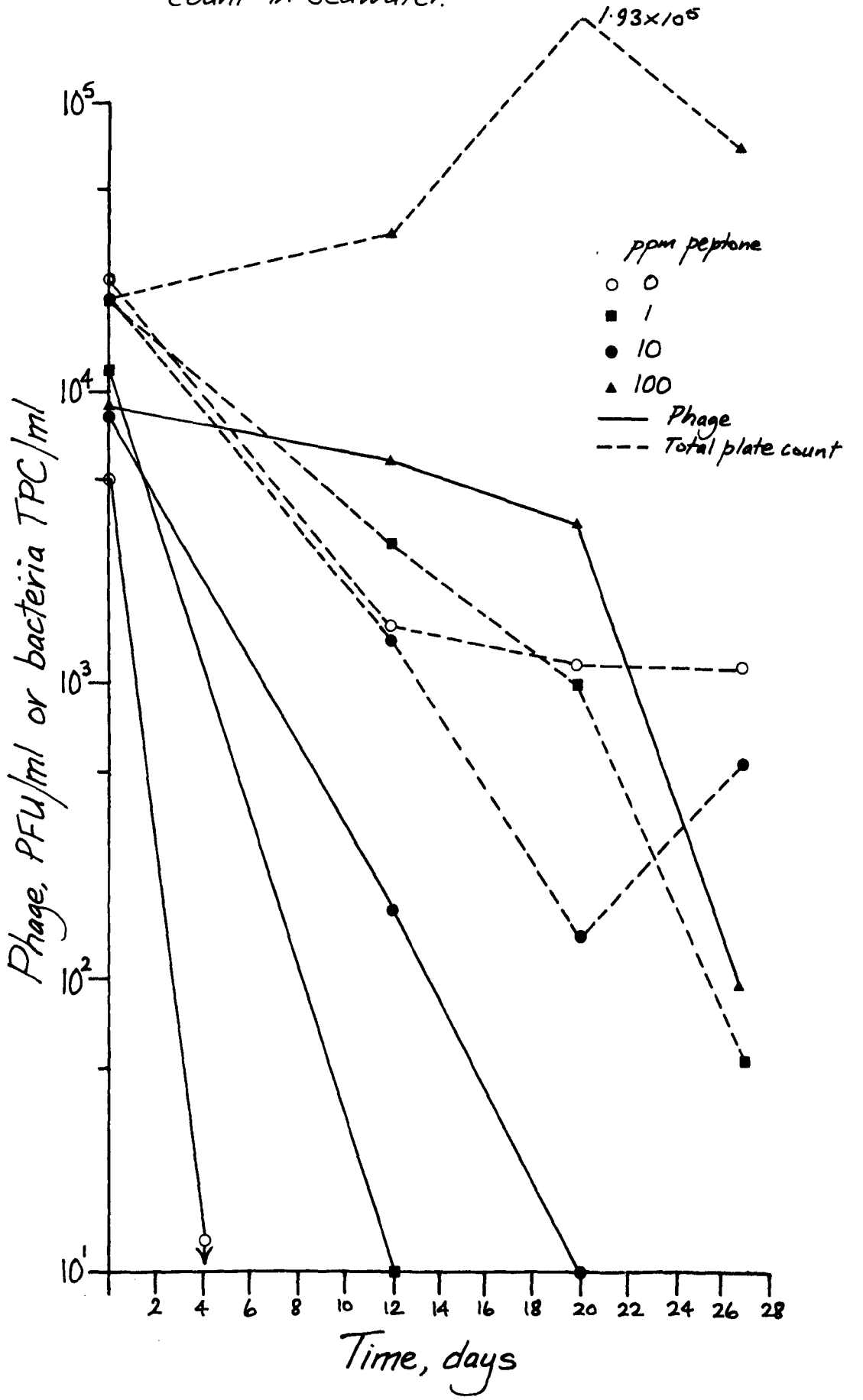


Figure 38. Influence of glucose on phage MS2 and total plate count in seawater.

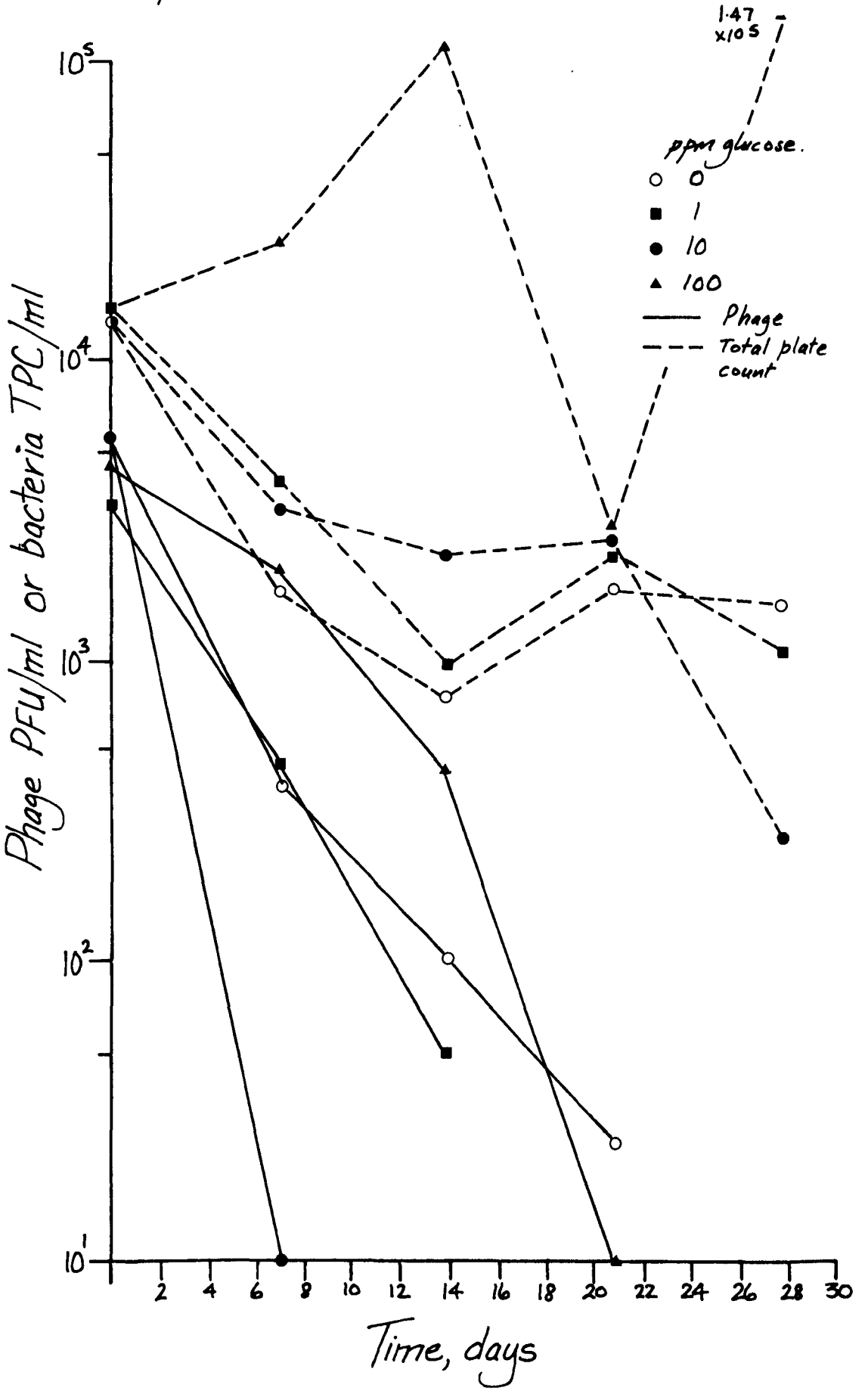


Figure 39. Influence of peptone on phage MS2 and total plate count in seawater.

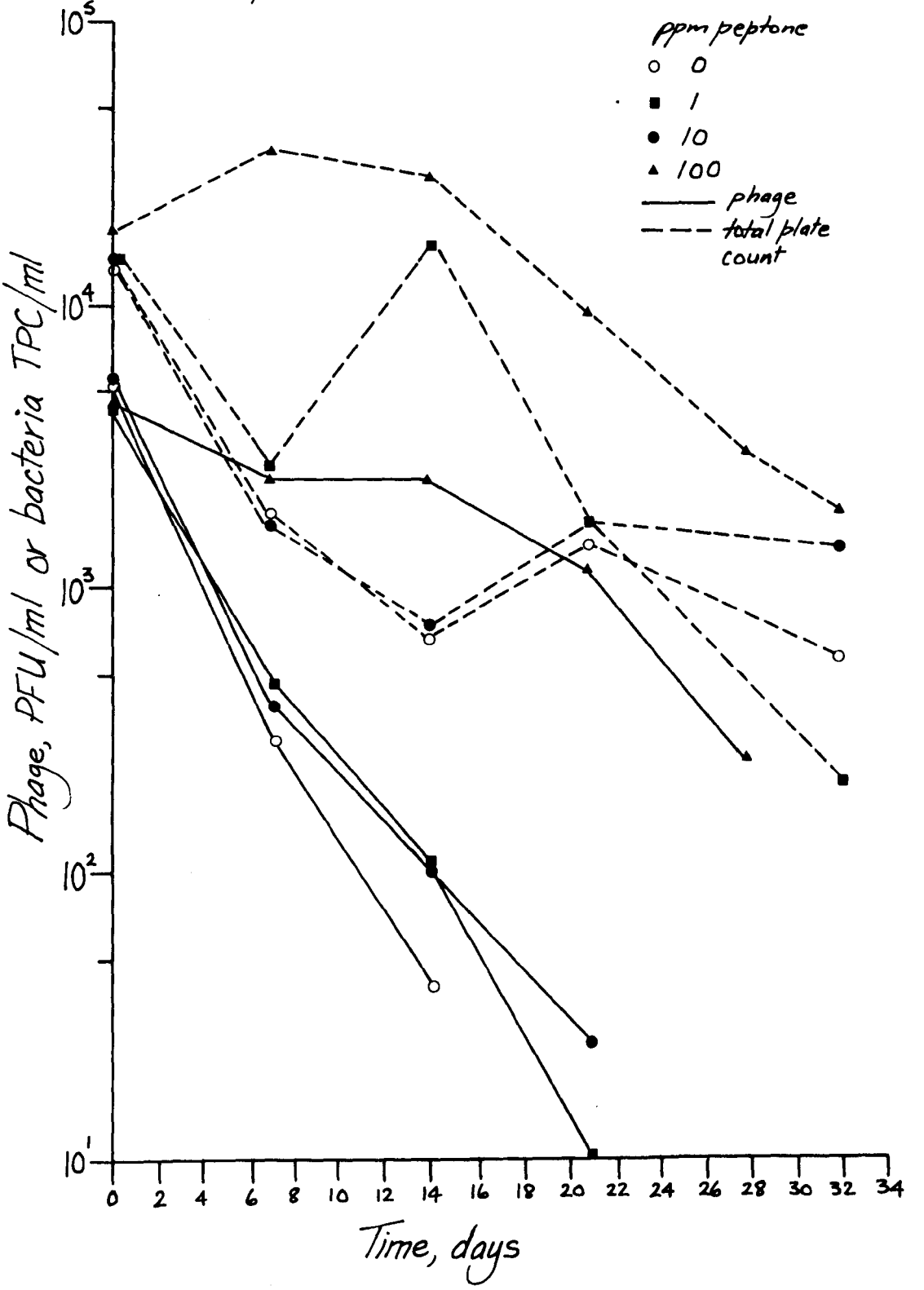


Table 36 Effect of glucose and peptone on *E. coli* 9481 in sea water

Treatment	Time (hours)							
	0	3	5	24	27	29	48	53
Sea water only	8.5×10^4	4.73×10^4	4.75×10^4	3.4×10^3	2.83×10^3	1.8×10^3	6.75×10^2	2.5×10^1
Sea water + 1 ppm glucose	9.75×10^4	5.23×10^4	4.78×10^4	6.15×10^3	4.15×10^3	2.95×10^3	1.73×10^3	2.25×10^2
Sea water + 10 ppm glucose	9.75×10^4	5.15×10^4	4.7×10^4	5.28×10^3	4.95×10^3	3.98×10^3	2.48×10^3	1.25×10^2
Sea water + 100 ppm glucose	1.22×10^4	4.78×10^4	4.13×10^4	5.48×10^3	4.75×10^3	3.48×10^3	1.83×10^3	1.75×10^2
Sea water + 1 ppm peptone	1.02×10^4	6.13×10^4	4.3×10^4	1.93×10^4	9.3×10^3	6.5×10^3	6.75×10^2	7.5×10^1
Sea water + 10 ppm peptone	9.25×10^4	5×10^4	4.5×10^4	3.45×10^4	2.3×10^4	1.85×10^4	6.35×10^3	1.73×10^3
Sea water + 100 ppm peptone	1.04×10^4	7.2×10^4	6.38×10^4	6.03×10^4	5.5×10^4	4.53×10^4	4.28×10^4	3.68×10^4

Figure 40. *Influence of glucose on the survival of* *E. coli* *in seawater.*

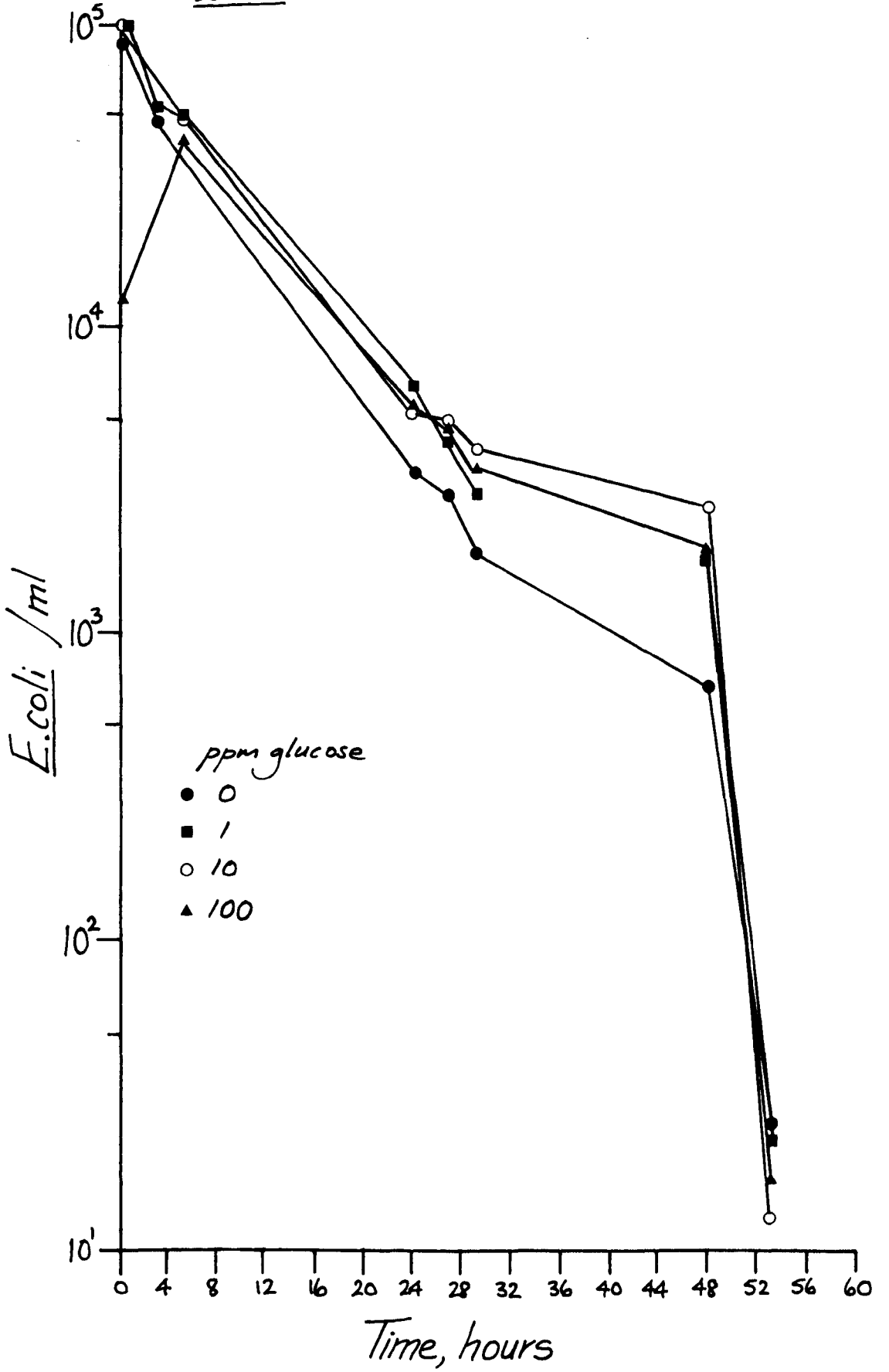


Figure. 41. *Influence of peptone on the survival of E. coli in seawater.*

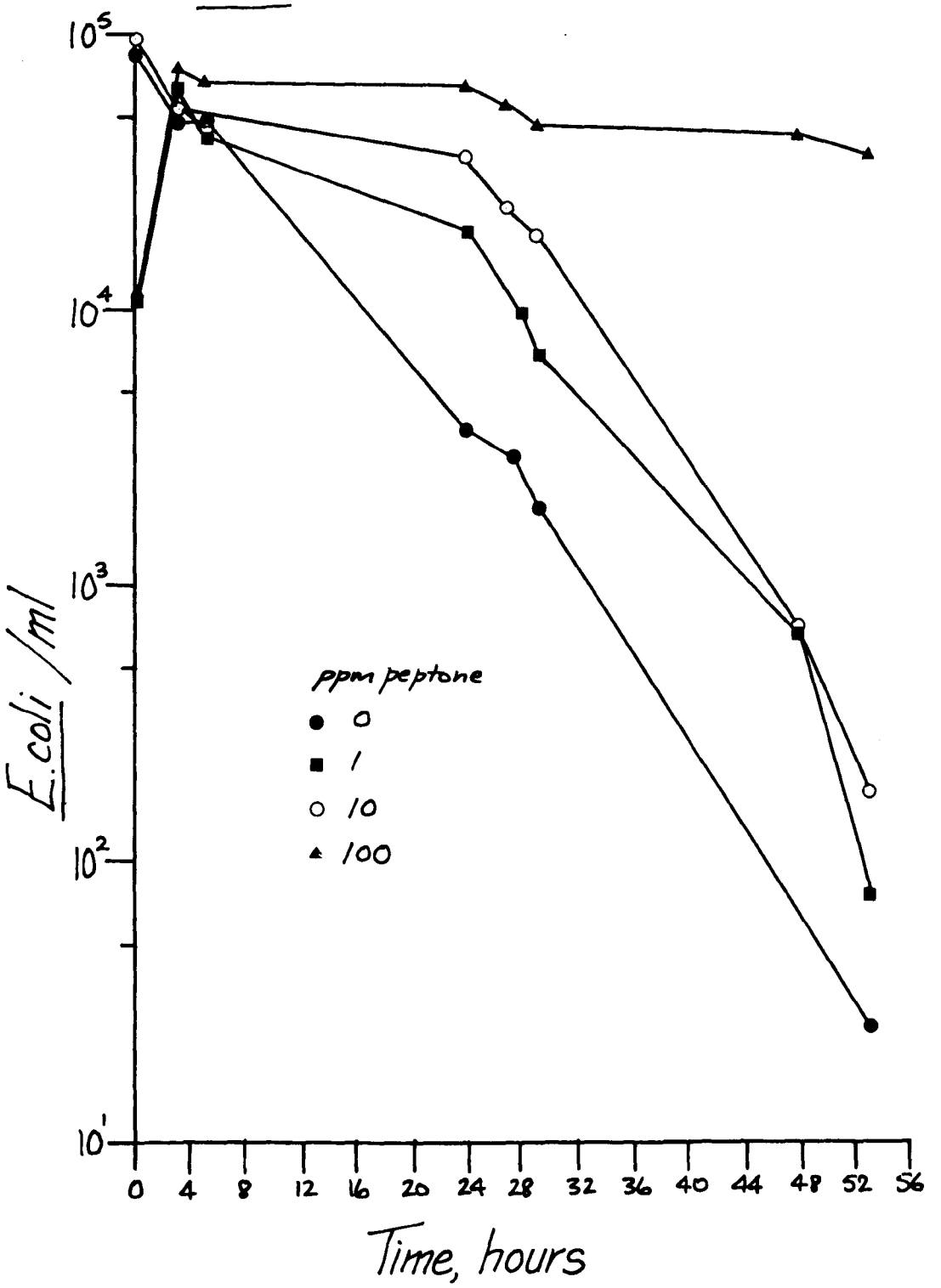


Table 37 Effects of cysteine on MS2 phage in autoclaved and raw sea water

Raw sea water, ppm cysteine (HCl)	0 day	7 days		14 days	
	PFU/ml	PFU/ml	%	PFU/ml	%*
0	1 680	155	9.2	25	1.49
1	1 200	95	7.9	40	3.33
10	850	795	93	<5	<0.59
100	415	<5	<1.2	-	-
Autoclaved sea water, ppm cysteine (HCl)					
0	1 150	100	8.7	15	1.3
1	1 415	15	1.06	<5	<0.35
10	800	100	12.5	35	4.38
100	460	20	4.35	<5	<1.09

*% remaining calculated on 0 days = 100%.

Table 38 Effects of cysteine on *E. coli* in autoclaved and raw sea water

Raw sea water, ppm cysteine (HCl)	0 hour	3 hours		6 hours		24 hours	
	E.coli/ ml	*E.coli*/ ml	%	*E.coli*/ ml	%	*E.coli*/ ml	%*
0	1 880	650	34.0	500	26.6	20	1.1
1	1 380	220	15.9	160	11.6	50	3.6
10	1 430	150	10.5	100	7.0	60	4.2
100	2 500	210	8.4	100	4.0	100	4.0
Autoclaved sea water, ppm cysteine (HCl)							
0	1 770	200	11.3	120	6.7	50	2.8
1	1 860	120	6.5	20	1.1	-	-
10	2 010	210	10.4	150	7.5	50	2.5
100	1 480	210	14.2	180	12.1	50	2.1

*% remaining calculated from 0 hours = 100%.

Two experiments carried out using sewage solids equivalent to doses of up to 100 *E. coli*/ml are shown in summary form in Table 39 and Figure 42. A useful comparison can be made here with the cysteine results reported above. In the sewage solids trial the best survival was demonstrated in the autoclaved seawater control without added sewage, 10% of the original phage inoculum remaining after 18 days. Added sewage either as sterile or raw effluent gave poorer survival figures than the raw natural seawater control and there was no apparent relationship with concentration of sewage added.

Additional experiments performed with doses of sewage equivalent to 1 000 *E. coli*/ml (Series II) are summarized in Table 40 and Figure 43. Survival in the autoclaved seawater control was again better than in any treatment containing either raw or sterile sewage. However, all treatments with sewage gave better results than the raw seawater control and survival was marginally better in those treatments containing the sterile effluent, i.e. solids only.

5.4.2.9 Effect of inorganics

In these experiments the effects of added inorganics, namely ammonium phosphate and ammonium sulphate, in both autoclaved and raw natural sea water were examined with *E. coli* and phage MS2. Two experiments were carried out for each test described and the results presented are means of the combined results of these experiments.

Results for *E. coli* in the presence of added $(NH_4)_2HPO_4$ are shown in Table 41. Although each treatment received the same initial inoculum of *E. coli* the actual counts obtained at the start of the experiments varied. This was particularly noticeable in the autoclaved sea water where increasing concentration of the phosphate appeared to lower the count obtained. Subsequent counts after 6 and 24 h were expressed

as a percentage of the original inoculum, and at 6 h survival was still better in the raw sea water and related directly to increasing phosphate concentration, i.e. survival increased as phosphate increased.

The treatments where $(NH_4)_2SO_4$ was used with *E. coli* are shown in Table 42. Results for autoclaved and natural sea water were in better agreement although survival was marginally better in the autoclaved sea water after 24 h exposure. The initial counts in autoclaved sea water indicated that survival was better in those treatments with increased sulphate. Decline of *E. coli* during the first 6 h exposure was substantially less with sulphate added than in comparable experiments with added phosphate.

Two experiments were performed with phage MS2 and added phosphate and the combined mean results are shown in Table 43. In agreement with earlier experiments survival in autoclaved sea water was superior to that observed in raw sea water. Raw sea water with added phosphate appeared to confer no protection for phage except in the first week of the experiment when survival was marginally better as phosphate concentration increased. Increasing phosphate concentration in the autoclaved sea water results in decreased phage survival so that autoclaved sea water + 100 ppm phosphate was only marginally better than some raw seawater treatments (Figure 44).

Experiments performed with phage MS2 in the presence of sulphate (Table 44) failed to demonstrate any beneficial influence of $(NH_4)_2SO_4$. Figure 45 shows that the differences observed were between autoclaved and raw sea water; survival being substantially better in the autoclaved water regardless of sulphate concentration.

Table 39 Effect of organics on phage MS2 in sea water, Series I

Flask no.	Treatment	Sewage equivalent*	Time (days)											
			0	6		7		13		21		28		
			PFU/ml	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	PFU/ml	%	
1	Raw sea water	-	5 150	-	-	2 150	41.75	300	5.83	55	1.07	35	0.68	
2	Autoclaved sea water	-	7 450	4 350	58.39	-	-	3 050	40.94	1 535	20.6	800	10.74	
3	Raw + sterile effluent	10	7 650	-	-	5 850	76.47	350	4.58	80	1.05	5	0.67	
4	Raw + sterile effluent	100	4 700	-	-	4 050	85.26	50	1.05	55	1.16	5	0.11	
5	Raw + raw effluent	10	8 600	-	-	6 700	77.91	250	2.91	45	0.52	15	0.17	
6	Raw + raw effluent	100	8 600	-	-	4 650	54.07	250	2.91	100	1.16	10	0.12	

*Sewage equivalent measured in terms of *E. coli* content, e.g. 100 = volume of sewage containing 100 *E. coli*.

Figure 42. Effect of organics on phage MS2 in seawater.

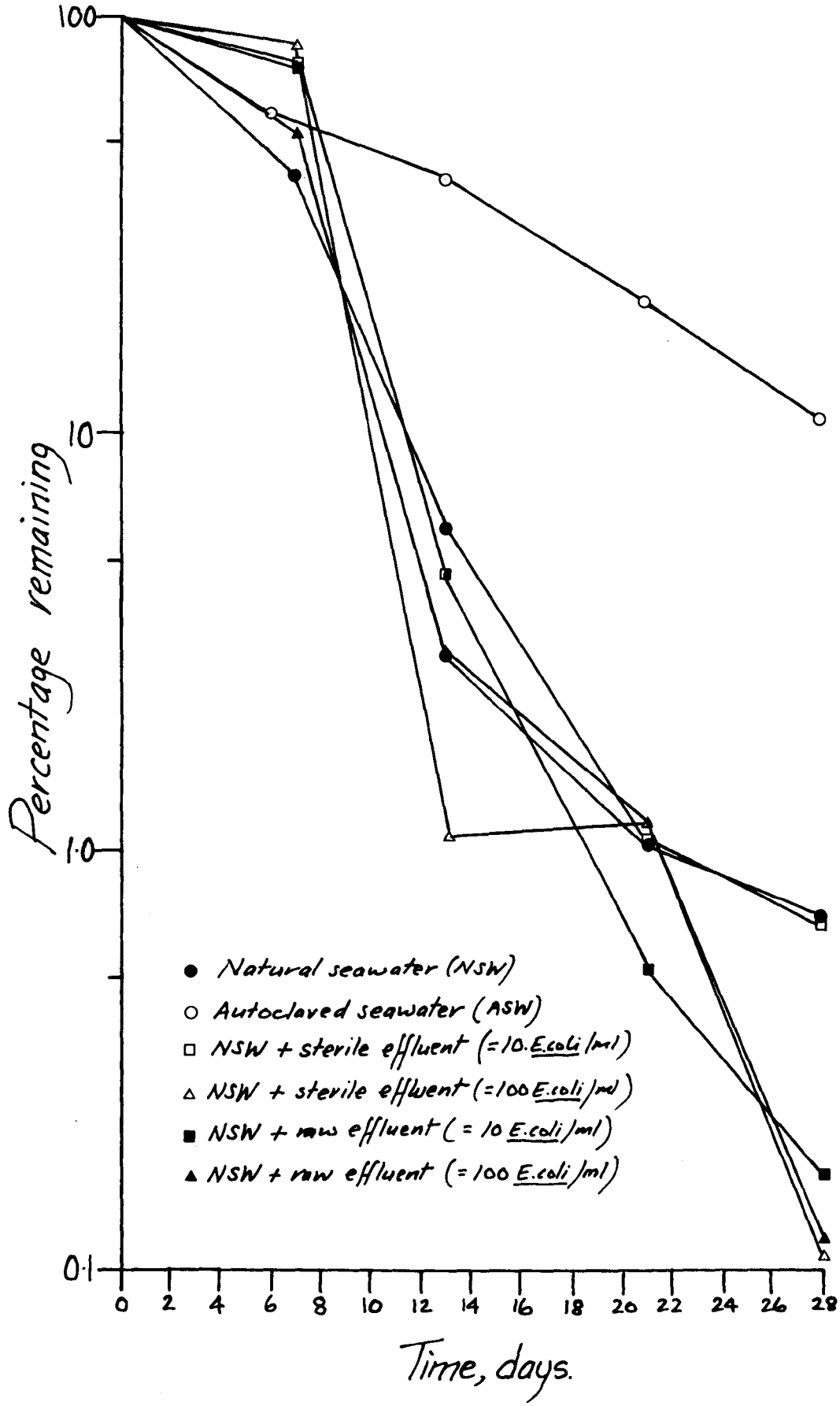


Table 40 Effect of organics on phage MS2 in sea water, Series II

Flask no.	Treatment	Sewage equivalent*	Time (days)				
			0	8		17	
			PFU/ml	PFU/ml	%	PFU/ml	%†
1	Raw sea water	-	4 100	550	13.41	<5	<0.12
2	Autoclaved sea water	-	5 500	2 400	43.64	640	11.64
3	Raw + sterile effluent	10	4 150	1 900	45.78	5	0.12
4	Raw + sterile effluent	100	4 650	1 250	26.88	55	1.18
5	Raw + sterile effluent	1 000	1 900	1 350	71.05	60	3.16
6	Raw + raw effluent	10	6 550	1 450	22.14	95	1.45
7	Raw + raw effluent	100	4 250	950	22.35	45	1.06
8	Raw + raw effluent	1 000	4 100	750	18.29	45	1.10

Table 40(a) Effect of organics on coliforms in flask treatments used above (Table 40)

Flask		Count/ml	Count/ml	%	Count/ml	%
6)		29.4	2.6	8.84	0.4	1.36
7)	details as above table	386	9.2	2.38	4.4	1.14
8)		2 600	35.6	1.37	3.4	1.13

*Sewage equivalent measured in terms of *E. coli* content, e.g. 100 = sewage volume containing 100 *E. coli*.
†% remaining calculated on day 0 = 100%.

Figure 43. Effect of organics on phage MS2 in seawater.

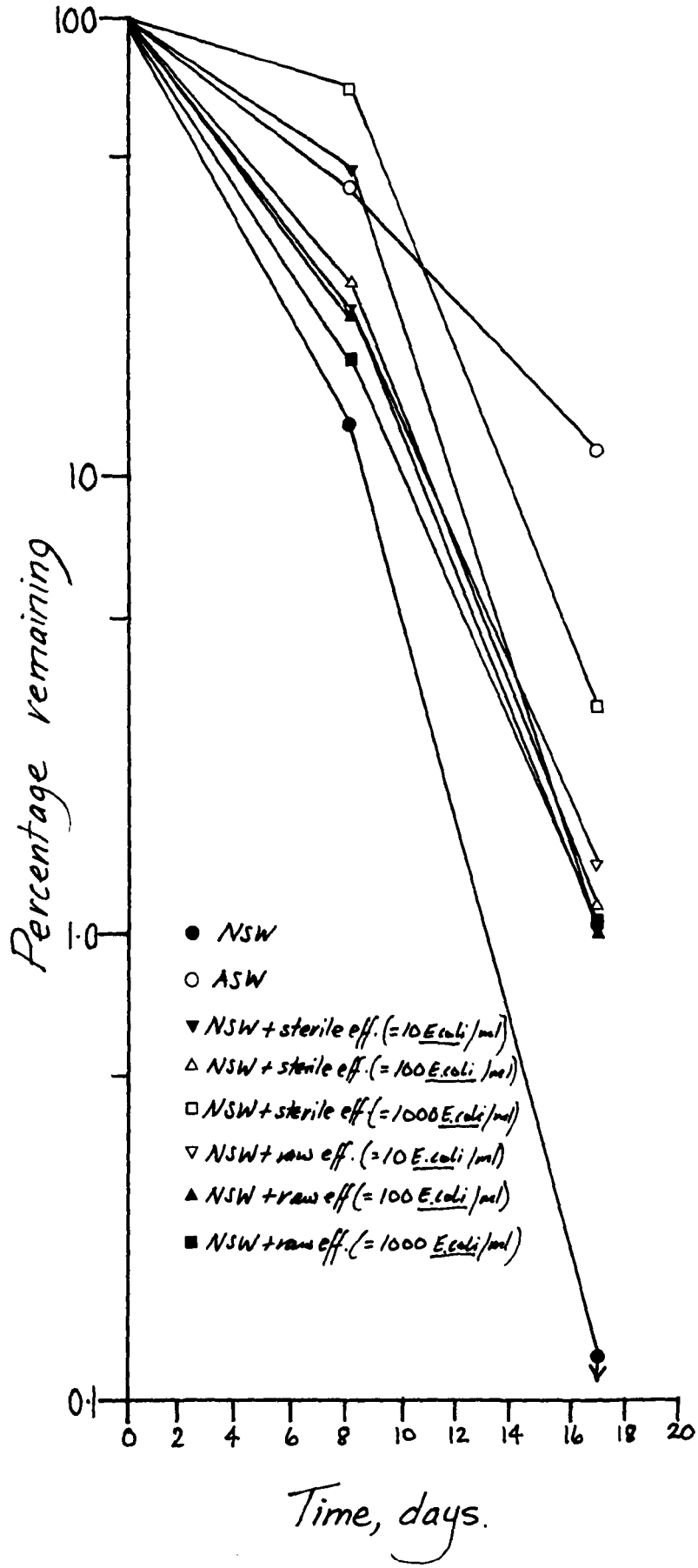


Table 41 The effect of $(NH_4)_2HPO_4$ on *E. coli* in raw and autoclaved sea water

Raw sea water ppm $(NH_4)_2HPO_4$	0 hour	6 hours		24 hours	
	E. coli/ml	*E. coli*/ml	%	*E. coli*/ml	%*
0	24 750	367	1.48	-	-
1	14 840	617	4.16	6	0.04
10	22 500	467	2.07	-	-
100	21 750	1 050	4.83	-	0.03
Autoclaved sea water ppm $(NH_4)_2HPO_4$					
0	19 000	67	0.35	-	-
1	12 330	117	0.95	-	-
10	8 170	134	1.63	-	-
100	9 840	300	3.05	19	0.19

*% remaining calculated on 0 hour = 100%.

Table 42 The effect of $(NH_4)_2SO_4$ on *E. coli* in raw and autoclaved sea water

Raw sea water ppm $(NH_4)_2SO_4$	0 hour	6 hours		24 hours	
	E. coli/ml	*E. coli*/ml	%	*E. coli*/ml	%*
0	24 000	6 834	28.48	30	0.125
1	15 500	2 133	13.76	>10	>0.06
10	29 330	4 500	15.34	120	0.41
100	20 830	4 400	21.12	10	0.048
Autoclaved sea water ppm $(NH_4)_2SO_4$					
0	15 500	4 400	28.39	70	0.45
1	20 830	3 316	15.92	130	0.62
10	27 660	3 583	12.95	40	0.14
100	26 750	4 133	15.45	30	0.11

*% remaining calculated on 0 hour = 100%.

Table 43 Effect of added $(NH_4)_2HPO_4$ on MS2 phage in sea water

Flask no.*	ppm $(NH_4)_2HPO_4$		Time (days)						
			0	6	12	20	26	34	41
1	0	Count PFU/ml	13 350	2 900	665	130	40	5	5
		% remaining	100	21.7	5.0	0.97	0.3	0.04	0.04
2	1	Count	7 950	1 750	430	195	80	60	35
		%	100	22.0	5.4	2.45	1.0	0.75	0.44
3	10	Count	8 650	3 450	720	170	50	10	<5
		%	100	39.9	8.3	1.96	0.58	0.12	-
4	100	Count	6 550	6 150	2 650	45	<5.0	<5	<5
		%	100	93.9	40.5	0.69	-	-	-
5	0	Count	5 900	6 550	7 050	5 000	1 650	1 515	1 375
		%	100	111.0	119.5	84.7	27.9	25.7	23.3
6	1	Count	7 050	5 950	5 200	3 800	2 250	1 490	1 300
		%	100	84.3	73.7	53.9	31.9	21.1	18.43
7	10	Count	10 000	4 600	3 250	1 900	2 350	985	145
		%	100	46.0	32.5	23.5	19.0	9.85	1.45
8	100	Count	8 050	2 900	665	290	105	50	5
		%	100	36.0	22.9	3.6	1.3	0.62	0.06

*Flasks 1-4 raw natural sea water, 5-8 autoclaved sea water.

Figure 44. Effect of $(NH_4)_2SO_4$ on MS2 in seawater

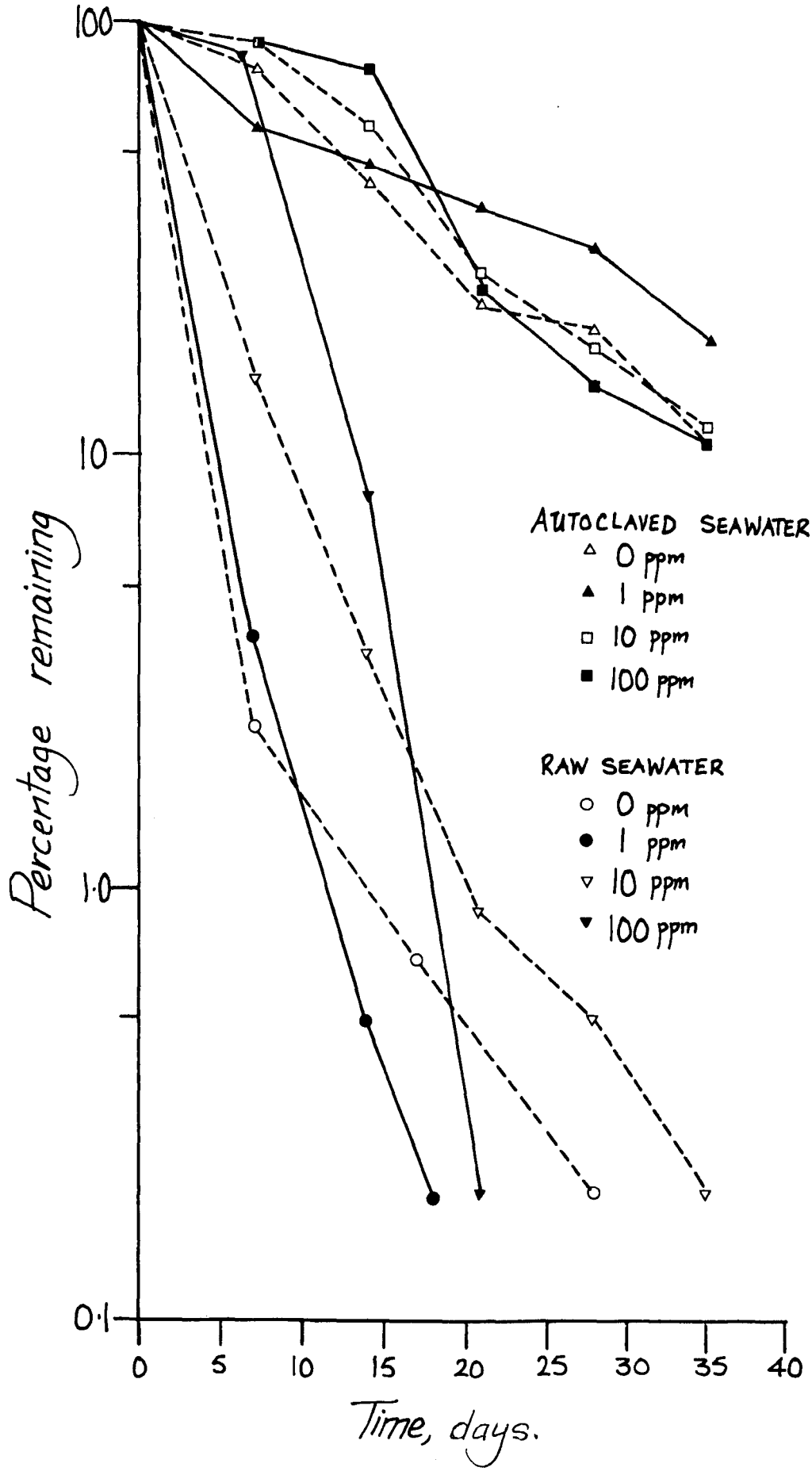
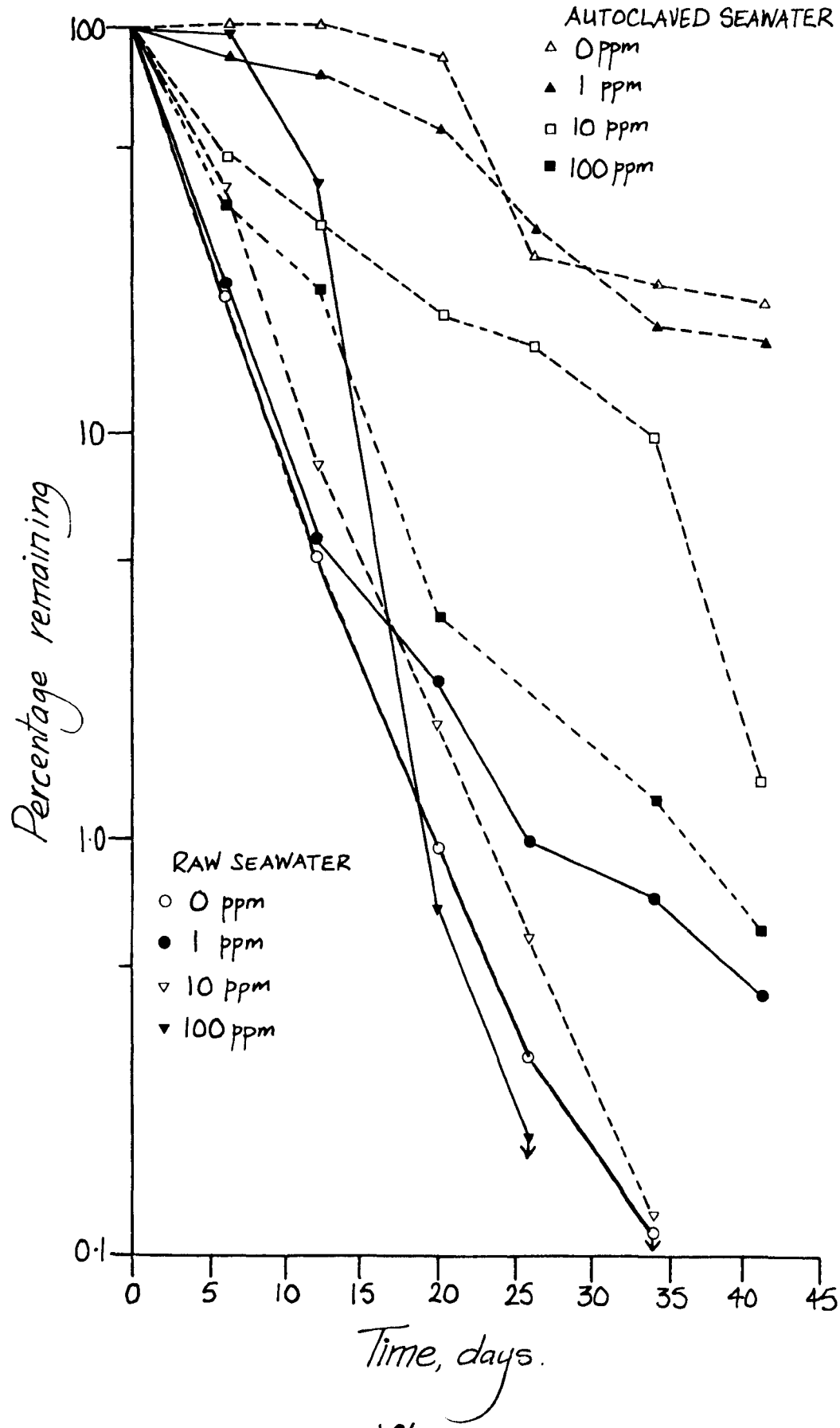


Table 44 Effect of added $(NH_4)_2SO_4$ on MS2 phage in sea water

Flask no.*	ppm $(NH_4)_2SO_4$		Time (days)					
			0	7	14	21	28	35
1	0	Count PFU/ml	4 050	100	35	20	-	-
		% remaining	100	2.4	0.86	0.5	-	-
2	1	Count	5 200	200	30	-	-	-
		%	100	3.8	0.6	-	-	-
3	10	Count	7 100	1 050	245	60	35	-
		%	100	14.8	3.5	0.84	0.5	-
4	100	Count	2 900	2 400	220	5	-	-
		%	100	82.8	7.9	0.2	-	-
5	0	Count	6 600	5 200	2 900	1 485	1 240	690
		%	100	78.8	43.9	22.5	18.8	10.5
6	1	Count	8 400	4 600	3 800	3 100	2 350	1 470
		%	100	54.8	45.2	36.9	29.4	17.5
7	10	Count	4 800	7 000	4 500	2 050	1 145	1 400
		%	100	87.5	56.3	25.6	23.9	17.5
8	100	Count	11 000	9 650	8 350	2 575	1 585	1 150
		%	100	87.7	75.9	23.4	14.4	10.5

*Flasks 1-4 raw natural sea water, 5-8 autoclaved sea water.

Figure 45. Effect of $(NH_4)_2HPO_4$ on MS2 in seawater.



5.4.3 Discussion

In the earlier discussion on sewage some of the important points which emerged were that coliphage, virus and bacterial numbers in sewage vary, sometimes seasonally but particularly with the degree of treatment which the effluent receives, and indeed type of treatment. Of the available sewage treatment processes which may be applied there seems to be almost unanimous agreement that the activated sludge process is the most effective. The key to the success of this type of treatment is adsorption of the virus on to solids which are subsequently removed as sludge. GERBA *et al.* (1978) have shown that the percentage of solids-associated virus may range from 3 to 100% and that higher concentrations were usually associated with the effluent from activated sludge treatment although overall turbidity and concentration of viruses was apparently low in comparison with trickling filter effluents. MOORE *et al.* (1975) demonstrated that the adsorption of some coliphages on to sewage solids was different to that of poliovirus which generally adsorbed to a greater extent under the same conditions. These authors also demonstrated that high pH enhanced adsorption although GERBA *et al.* (1978) reported the opposite and suggested that the use of autoclaved sewage by MOORE *et al.* could have altered the ability of coliphages to adsorb to solids. The quantity of solids-associated viruses in sewage effluent is very fundamental to a consideration of virus survival in water (GERBA & SCHAIBERGER, 1975) and it has been shown with coliphage (STAGG *et al.*, 1977) that a plant most effective in reducing the total coliphage population (i.e. activated sludge process) is also found to have the highest percentage increases of solids-associated viruses. Field data have indicated a similar protective effect for animal viruses (WELLINGS *et al.*, 1976). In general terms the efficiency of a sewage plant to removes viruses is closely related to the ability to remove

suspended solids (BALLUZ *et al.*, 1977) but colloidal particles less than 1 μm in diameter are less likely to be removed and large numbers of viruses may be associated with them. In summary therefore virus will be released in the effluents from sewage treatment works and in addition will be adsorbed on to solids, particularly small colloidal particles.

When one comes to consider the fate of viruses in the marine environment, therefore, it is as solids-associated particles and generally not as freely suspended virus particles.

Although it would be advantageous to consider the various factors which have been studied in the present work quite separately they are in fact closely interrelated. In preliminary experiments it was apparent that survival of coliphage MS2 in sea water was better than in either distilled or deionized water though not as good as in tapwater. Distilled and deionized water as used here were essentially free of any particulate material, whereas sea water and tapwater might be expected to contain both dissolved and particulate organics. Further experiments with both MS2 phage and *E. coli* showed that, regardless of any consideration of particulate matter as such, salinity itself had a profound effect on survival and that salinities within the range 5 to 10°/oo were apparently least destructive. The extremes, that is to say fresh water (0°/oo) and full salinity estuarine water (30°/oo), were associated with the poorest survival of both phage and *E. coli*. These experiments were carried out at 15°C and LO *et al.* (1976) working with poliovirus have shown that survival is better at 10°/oo salinity at this temperature. They have also demonstrated that at 4°C and 25°C a salinity of 10°/oo is not associated with maximum survival; at 4°C they suggested poliovirus were more stable at high salinity, at high temperatures (25°C) it was more stable at low salinities. Additionally, LO

et al. (1976) also showed that poliovirus was more labile in natural saline waters than in laboratory studies performed with artificial sea water. In contrast, BERRY & NOTON (1976) in studies with T_2 phage showed that inactivation was greater in natural waters than under laboratory conditions. GOYAL *et al.* (1978) studied survival of a number of different bacteria and viruses in sea water and obtained a negative correlation between salinity and survival of all the microbes tested except viruses. Experiments reported by AKIN *et al.* (1976) with poliovirus demonstrated that increasing salinity, under both laboratory and natural conditions, increased inactivation of the virus and that the best survival was in distilled water. GERBA *et al.* (1978) have suggested that one effect of salinity may be to increase or decrease the rate of elution of virus adsorbed to solids. Natural estuarine water eluted solids-associated virus to a greater degree than artificial sea water of the same salinity. For bacteria, BERNARD (1970) confirmed that mortality of *E. coli* is much more rapid in distilled water than in sea water.

One of the possible explanations for salinity effects on both bacteria and viruses is the presence of various salts or ions (both cations and anions). Experiments carried out in the present study suggest that the potassium ion is important in the survival of both phage and *E. coli*, and that for phage, magnesium sulphate ($MgSO_4$), particularly the sulphate ion, was necessary for survival. In this context an interesting paper by JOYCE & WEISER (1967) reported survival studies using bacteria and viruses in farm pond waters. They found that $MgSO_4$, and to a lesser extent $MgCl_2$, prolonged survival of viruses but that this effect was only operative at low temperature (4°C) where it effectively reversed earlier findings showing increased survival at 25°C and 37°C. Their conclusion was that $MgSO_4$ stabilized virus in contrast to

$FeCl_2$ which increased the disappearance of virus, possibly due to ferrous ion toxicity or acidity effects. In the present study, best phage survival was exhibited in those treatments lacking $MgCl_2$ whereas JOYCE & WEISER (1967) had shown this salt to be beneficial. However, they were working in fresh waters and at a lower pH and PETRILLI *et al.* (quoted by MELNICK, 1962) have suggested that a role of $MgCl_2$ may be to stabilize pH effects. MELNICK (1962) reported that divalent ions such as Ca^{2+} and Mg^{2+} stabilize enteroviruses at all temperatures between 4°C and 50°C whereas monovalent ions such as Na^+ and K^+ stabilize poliovirus at 50°C but inactivate it at 37°C. Working with the T phages, T_1 to T_7, KOTT *et al.* (1969) showed that NaCl, $MgSO_4$ and $CaCl_2$ had no significant effect at the concentrations normally found in sea water.

The apparent influence of metal cations has further implications with reference to the opening statements of this section dealing with adsorption. Viruses are charged colloidal particles which means they have the ability to adsorb to surfaces of suspended particulate matter and could also form aggregations (flocculation). Flocculation of viruses as hydrophilic biocolloids in the presence of cations (sea water) would be consistent with colloidal theory (STUMM & MORGAN, 1970), however AKIN *et al.* (1976) did not consider this to be the reason for viral inactivation in sea water. STUMM & MORGAN (1970) have also shown that the adsorption of hydrophilic colloids such as viruses depends primarily on the presence of cations but also involves the charge density and particle geometry of the adsorbent, pH of the solution, isoelectric point of the virus and the ionizable groups on virus and adsorbent. Most sewage viruses discharged into coastal waters are already attached to or incorporated in faecal particles (DUFF, 1970; LUND & RONNE, 1973; WELLINGS *et al.*, 1976) and this supports the predictions by BERG (1973a) that as much as 99% of viruses present in

coastal waters are adsorbed to colloidal and particulate matter. In terms of their influence on adsorptive processes, BITTON *et al.* (1976) suggest that on a concentration basis trivalent ions are more effective than divalent ions which in turn are more effective than monovalent ions.

According to these authors the increase in ionic strength leads to a reduction in the thickness of the double layer around particles and as a result they are bound together by attractive forces, e.g. London and Vander Waals forces. As pH decreases towards the isoelectric point of the virus then adsorption is also decreased because of changes in charge and ion concentration. Various authors have demonstrated that cation concentration is important for adsorption of viruses to clays, sand and other particulate materials (CARLSON *et al.*, 1968; COOKSON, 1969; LEFLER & KOTT, 1974; SCHAUB *et al.*, 1974). Adsorption seems to be a phenomenon shared by all viruses (including coliphage) and certainly also occurs with bacteria (BRISOU *et al.*, 1964) but it must be stressed that adsorption does not necessarily imply inactivation and adsorbed viruses may still be infectious (COOKSON & NORTH, 1967; MOORE *et al.*, 1975). It must also be remembered that adsorption is not a one-way process and may be reversed depending on cation type and concentration, changes in salinity and presence of dissolved organic matter which may compete with viruses for adsorption sites (CARLSON *et al.*, 1968; COOKSON & NORTH, 1967; ROPER & MARSHALL, 1974). The work of ROPER & MARSHALL (1974) showed that desorption of coliphage from marine sediment was moderate at high salinity, decreased with decreasing salinity and then increased rapidly at very low salt concentrations. This would explain the findings in the present study and others elsewhere that poorest survival is at the extremes of salinity (fresh water and full sea water). The studies of ROPER & MARSHALL (1974) and FILDES

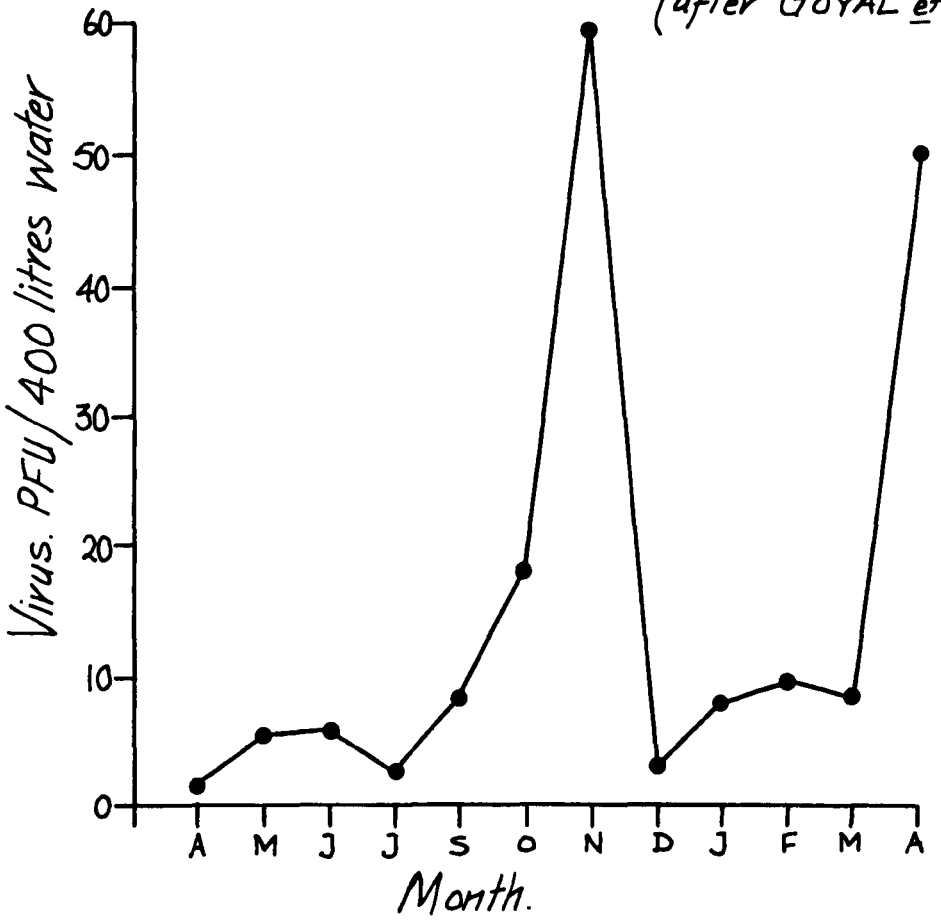
& KAY (1963) certainly support the premise that as salinity varies due to tides, rainfall, etc. then the rate of adsorption/desorption will vary also with the changing cation concentrations.

The results of the present study show that pH values between 6 and 7 are optimum for survival of both coliphage MS2 and *E. coli*. The normal pH of sea water is between 7.5 and 8.5 (HARVEY, 1957) and is influenced by pressure, temperature and respiratory and photosynthetic activities of microorganisms. Sea water itself contains cations in excess of the equivalent anions derived from strong acids. Titratable alkalinity is equivalent to the bicarbonate, carbonate and borate ions in the water and the contribution of other weak acids is insignificant. Results obtained in the present work with *E. coli* show rapid mortality at pH 8 and above and agrees with CARLUCCI & PRAMER (1960a) who demonstrated that death of *E. coli* was more rapid at alkaline than acid pHs. Studies by JOYCE & WEISER (1967) showed that pH over the range 5.1 to 8.8 did not significantly affect survival of poliovirus but they suggested that the effects were modified by the presence of bacteria. The presence of high bacterial numbers (20 000/ml) at lower pH favoured the disappearance of virus while low numbers of bacteria favoured survival in neutral or alkaline pH. Studies conducted by GOYAL *et al.* (1978) under field conditions included a multiple correlation analysis between physiochemical characteristics of water and various microbiological parameters. Data collected in respect of viruses showed a strong negative correlation with pH even though the pH range measured was small (pH 7.4 to 8.7). VAUGHN & RYTHER (1974) working with MS2 showed a sharp decline in phage survival above pH 8 which was not apparent in the presence of algae. In contrast, PARHAD & RAO (1974) working with *E. coli* demonstrated that growth of algae raised the pH and increased mortality of *E. coli*. THAYER & SPROUL (1966) have shown

that high pH values play a role in the destruction of viruses, particularly over pH 10. It is interesting to postulate that pH effects may be due to changes in ion concentration permitting desorption of absorbed virus or the failure of freely suspended virus to adsorb, thus making them vulnerable to inactivation. Clearly, however, further work would be necessary to examine whether this is in fact so.

Experimental work performed for the present study suggested some seasonal effects (as shown by a comparison of T_{90} values) and a possible link with phytoplankton abundance. Wider fluctuations were exhibited by the treatments based on autoclaved as opposed to raw sea water which indicates that autoclaving affected some variable component of the water itself. It is evident that many of the factors studied here such as salinity, temperature and pH are also seasonal and it may be anticipated that at times such factors will act singly, together, synergistically or even antagonistically. The suggested association between low T_{90} values for phage and the presence of phytoplankton may be linked to increased pH as the result of algal activity as discussed earlier. Figure 46 is adapted from GOYAL *et al.* (1978) and shows one of the few seasonal studies on virus occurrence in marine waters. These authors showed a high incidence of virus in the autumn and spring, the autumn peak coinciding with unusually heavy rainfall. BERG (1967) has observed that the highest concentration of viruses in waste water in temperate climates occurs during autumn. A very recent study by PAYMENT *et al.* (1979) looked at sewage samples over a 13-month period and isolated viruses from 47 of 53 samples. Polioviruses were found in 39 samples with maximum numbers occurring during August and September. What this study indicated, and which is of relevance to virus in water, is that polio seemed to be a good indicator of human faecal pollution in communities where live attenuated poliovaccine is

Figure 46. Monthly distribution of virus isolates from water.
(after GOYAL et al., 1978)



used and can be related to times of dosing the population. Although such an idea seems attractive KATZENELSON & KEDMI (1979) put up an equally convincing case for not using polio virus as an indicator in this way.

It is very apparent from the literature that one of the major unsolved puzzles in studies on phage, virus and bacterial survival is the effect of autoclaving on the destructive or inactivating properties of sea water. Throughout the present study when the effects of various factors have been evaluated the most striking differences have constantly been between 'survival' (no inactivation or true destruction) in autoclaved and raw sea water. Similar effects have been noted by other authors working with phage and poliovirus (CARLUCCI & PRAMER, 1960; SHUVAL *et al.*, 1971; PIETRI & BREITTMAYER, 1976; FUJIOKA *et al.*, 1978). JONES (1967) has reported that the autoclaving of sea water produces a small precipitate of aragonite which scavenges trace metals and MITCHELL & JANNASCH (1969) found that autoclaving greatly reduced the rapid viral inactivation they had observed in filtered sea water. Other observations suggest that proteins and amino acids may also reduce viral inactivation in sea water (LYCKE *et al.*, 1965) possibly by neutralizing or mopping up the toxic effects of heavy metals. The role of clays and other particulates in reducing viral inactivation may also be as adsorbents of metals. JONES (1964) found that similar effects to those observed from autoclaving could be obtained by adding organic chelating agents and suggested that the metals were only effective in their uncomplexed state. Reduction of trace metals by passage through a chelex column has also been shown to have beneficial effects on the survival of bacteria in sea water (GRAHAM & SIEBURTH, 1973). However, the exact nature of the effects, the metals involved and true significance in relation to survival under natural

conditions has yet to be established for either bacteria or viruses.

Whatever the reasons for the effects of autoclaving there is some evidence to suggest that its effect also varies depending on whether the water is natural or artificial sea water (ASW). This is of significance since many published reports base their laboratory studies on the use of ASW. BERRY & NOTON (1976) working with T_2 phage showed that autoclaving and filtration abolished the antiviral activity of ASW but only decreased such activity in natural sea water. SHUVAL (1978) reported a 99% reduction in poliovirus in sea water at 15°C for 6 days and only a 40% reduction over the same period in autoclaved water. In contrast to reports showing such extended survival in autoclaved water, reports of virus studies in fresh water (JOYCE & WEISER, 1965) and marine waters (AKIN *et al.*, 1976) failed to demonstrate any differences between autoclaved and raw waters.

Filtration also has an effect on virus survival and a filterable chemical agent has been suggested as a primary cause of virus inactivation in sea water (MATOSSIAN & GARABEDIAN, 1967). Filtration obviously removes suspended particulate matter and depending on the pore size of the filter this may include bacteria and colloids which can act as adsorption sites for viruses. In the present study results obtained with raw and membrane-filtered (MF) sea water followed similar trends although T_{90} values were consistently higher with the MF water. An interesting comparison can be made with corresponding T_{90} values for autoclaved sea water which are generally higher when T_{90} values for raw and MF sea water are low. This suggests that autoclaving exerts a different effect to that of filtration although AKIN *et al.* (1976) found that such treatments had similar effects on poliovirus survival in sea water.

Temperature has been cited as the most important factor affecting die-off rates of bacteria and viruses in sea water (ORLOB, 1956; PRIER & RILEY, 1967; LO *et al.*, 1976; DENIS *et al.*, 1977). With bacteria, low temperatures may induce slowing down of cell metabolism thereby reducing the destructive effects of sea water (VASCONCELOS & SWARTZ, 1976). Temperatures of 37°C represent the host temperatures for enteroviruses and it could be anticipated that unless temperatures rose above this level such viruses would remain active. However, there is no doubt that virus survival outside the host cell, e.g. in sea water, is influenced by temperature. BERRY & NOTON (1976) reported that 50% of T_2 phage still remained after 48 days at 4°C whereas only 0.003% remained after the same period at 14°C. Field data for virus survival also show extended survival in winter months when temperatures are low. SHUVAL (1978) and NIEMI (1976) found that virus survival in laboratory studies was influenced more by the season of water sampling than by the incubation temperature. This information all suggests that temperature may regulate virus inactivation in some way, rather than actually cause it. Possible mechanisms include the general slowing down of enzyme activity, biotoxicity or some other chemical-linked process.

It has been stressed at various points throughout this study that many factors are closely interrelated and this is certainly true when studying the effects of temperature and sunlight. Field data which show extended virus survival in cold winter months could well be attributed to less sun as well as low temperatures, and clearly the lack of sun contributes to low temperatures and vice versa. However, it has also been accepted for a long time that light in the ultra-violet and blue wave-lengths kills viruses and bacteria. Although the exact mechanisms are not understood they include direct adsorption of ultra-violet and near ultra-violet light and photooxidation (FOOTE, 1968;

SPIKES & LIVINGSTON, 1969; HILL *et al.*, 1970, 1971). There is quite a lot of data on mortality of coliform bacteria in sea water exposed to sunlight (GAMESON & SAXON, 1967; GAMESON & GOULD, 1975; BELLAIR *et al.*, 1977) but relatively little about viruses under similar conditions. Sunlight has been implicated in virus reduction in oxidation ponds (MALHERBE & STRICKLAND-CHOLMLEY, 1967; CARSTENS *et al.*, 1965) and on sewage-irrigated crops (KOTT & FISHELSON, 1974). The present study with *E. coli* and phage both confirms the published observations on coliform reduction referred to earlier and also demonstrates that phage are inactivated by sunlight. BERRY & NOTON (1976) showed that suspensions of T_2 phage were invariably inactivated more rapidly at or near the surface of the water than at greater depth. They concluded that although sunlight was not a major factor in inactivation it did have an enhancing effect. A recent paper by BITTON *et al.* (1979) has developed some of these ideas further using poliovirus, and Figure 47 is adapted from their work. These authors have shown that the rate of virus inactivation varies with depth, being greater at 2 and 4 inches below the surface than at 6 inches depth. Since light intensity decreases with depth below the water surface and ultra-violet particularly penetrates water very poorly, these results support a photo-inactivation theory. BITTON *et al.* (1979) also showed that a clay material, nontronite and blue green algae reduced inactivation rates of poliovirus in illuminated samples, probably by a shading effect. However, it is possible that photooxidation reactions occur as ZAFIRIOU (1977) has stated that a number of reactive oxidants can be formed photochemically in sea water. Naturally-occurring organic compounds such as lignins, fulvic and humic acids in surface waters (CHRISTMAN, 1970; SMART *et al.*, 1976) may act as sensitizers in photooxidate reactions. One site where such inactivation can occur in

Figure 47. Effect of solar radiation on survival of poliovirus 1 in water. (after BITTON et al., 1979)

bacteria is nucleic acid and since viruses also contain nucleic acids (e.g. phage MS2 contains RNA) the mechanism may be common to both bacteria and viruses. The experiments reported in the present study certainly confirm that a concentrated artificial source of u/v light has a rapid inactivating effect on water-borne phage MS2.

Frequent mention has already been made of the importance of adsorption in preventing virus inactivation. Experiments performed for the present study were relatively crude but demonstrated that both phage and *E. coli* readily adsorbed to Fullers Earth, used as a particulate material. The importance of particulates has been demonstrated by studies elsewhere which show decreased inactivation of coliphage when sea water is filtered (MITCHELL & JANNASCH, 1969; GERBA & SCHAIBERGER, 1975).

Experiments performed with organic supplements in this present study showed that high concentrations of glucose (100 ppm) increased the total bacterial count and also extended the survival of phage MS2. Similar concentrations of peptone had the same effect and also considerably extended the survival of *E. coli*. Autoclaving sea water kills the marine bacteria and as has been mentioned earlier also extends the survival of bacteria and viruses; such effects can be partially reversed by adding marine bacteria to autoclaved water (MAGNUSSON *et al.*, 1967; MITCHELL & JANNASCH, 1969; SHUVAL *et al.*, 1971). It has also been shown that viable counts of marine bacteria increase during the early stage of virus disappearance in sea water (MITCHELL & JANNASCH, 1969; SHUVAL *et al.*, 1971). A number of studies have identified specific bacteria with antiviral properties (GUNDERSEN *et al.*, 1967; MAGNUSSON *et al.*, 1967) and algal extracts have been shown to exhibit similar properties (EHRESMANN *et al.*, 1977; SOBSEY & COOPER, 1973). Protozoa have been implicated in coliform die-off (ENZINGER & COOPER,

1976) and as a major factor in the disappearance of *E. coli* in sea water (ROPER & MARSHALL, 1978). Little is known about the importance of bacterial inactivation of viruses under field conditions and KAPUSCINSKI & MITCHELL (pers. comm.) have stated that it is conceivable that effects observed are artifacts of containment, incubation temperatures, large virus inocula, high bacterial concentrations or the absence of dissolved trace metals. KIM (1974) has shown that the addition of nutrients increased virus survival possibly by some protective action. KLETTER *et al.* (1976) using phage T_2 showed that although a decrease in marine bacteria (prompted by pre-incubation of sea water) was accompanied by reduction in phage the addition of nutrient broth (Figure 48) reduced the inactivation of phage even though it also resulted in an increase in marine bacteria. The authors suggest that this apparent anomaly can be explained by the nutrient broth acting as a competitive inhibitor to proteolytic enzymes produced by marine bacteria. BITTON & MITCHELL (1974) showed that *E. coli* cells which could not be infected by the T_7 phage they used exerted a protective effect and extended phage survival in sea water. Bacteria can be regarded as biocolloids and in relation to what has been said earlier extended phage survival could be due to adsorption to bacterial cells.

Mention has been made earlier of the possible effects of heavy metals on bacterial and viral persistence in sea water and the role of some amino acids in reversing such effects. JONES (1964) suggested that cysteine acted in this way in reducing mortality of *E. coli*, work subsequently confirmed by CARLUCCI & PRAMER (1960) and SCARPINO & PRAMER (1962). In the present study no such beneficial effect of cysteine, related to concentration, was observed with either *E. coli* or phage MS2. A curious finding, however, was that the presence of cysteine in raw natural sea water gave better survival than autoclaved

Figure 48. The effect of nutrient broth on the coliphage inactivation capacity of an active marine bacterium (after KLETTER et al 1976)

sea water with cysteine. Whether this indicates that cysteine neutralizes some inactivating factor in raw sea water or reverses some process resulting from autoclaving is not clear. If autoclaving precipitated heavy metals and cysteine also removed them one would expect a synergistic effect and even greater survival in autoclaved water + cysteine.

In an earlier review section (3.2) mention was made of the paper by CLARKE *et al.* (1962) which showed that virus survival was greatest in clean waters and raw sewage and reduced in moderately polluted water. Survival of enteric bacteria was related to degree of pollution, being greatest in the most polluted area. The authors suggested that this was due to the additional nutrients present. METCALF & STILES (1967) have also demonstrated prolonged virus survival in grossly polluted waters and clean water. WON & ROSS (1973) showed that low concentrations of organics (including faeces) enhanced the survival of *E. coli* and even promoted its growth but did not enhance virus survival. Results of the present study failed to demonstrate any clear association between the amount of sewage added to sea water and phage or *E. coli* survival. It might be anticipated that sewage solids would form adsorption sites for viruses and bacteria and although this obviously occurs it is clearly much more complex than a mere presence or absence situation.

Although CARLUCCI & PRAMER (1960) had demonstrated that inorganic supplements, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$, had a beneficial effect on *E. coli* survival in sea water, results obtained in the present study failed to show any significant effect on either *E. coli* or phage. However, in what was essentially a negative result an interesting observation was made. When phosphate was added to autoclaved sea water a precipitate was formed, increasing as the dose of phosphate increased. Associated with this increase in phosphate and precipitation survival of phage showed a decrease and gave results only slightly better than

similar trials with natural sea water [see BRUNNER & SPROUL, 1970]. Undoubtedly the phage particles adsorbed to the precipitate and thus gave the indication of having disappeared from the liquid phase. As a final note for this discussion section this underlines a very important point, that removal of phage from aqueous suspension by adsorption on to solids does not necessarily imply destruction or even inactivation. Thus, although it is difficult to avoid the terms 'survival' or 'mortality' they should be interpreted with caution and considered within the context in which they are applied.

5.5 Shellfish

5.5.1 Objectives and methods

Much of the published work on viruses and phage in bivalve shellfish has been reviewed in earlier sections (3.3 and 4.4) and there have been few other contributions in this field. In many respects this reflects changes in policy in research in the USA where a reorganization of laboratory programmes and Government agencies has led to the closing of a number of specialist centres formally associated with work on shellfish. However, considerable effort has been expended over the years looking at virus in shellfish and there seemed only a few avenues open for original research. The three areas of research where it was felt some contribution could be made with the facilities and time available were as follows:

1. Comparison of *E. coli* and phage in naturally polluted shellfish.
2. Accumulation and survival of phage in shellfish.
3. Effect of heat processing on phage in shellfish.

It was stated in an earlier section (3.3) that to date no positive evidence of virus transmission by shellfish in the UK had been demonstrated. Although this is currently still true to a degree, there have been two incidents involving shellfish which implicate viruses as a causative agent of illness in the consumer. In late 1976, early 1977 a number of reports were received of gastroenteritis following consumption of cockles (*Cardium edule*) which appeared to involve an unknown viral agent (AYRES, 1977a). Subsequent investigations demonstrated a Parvovirus-like agent in faecal samples taken from some of those people affected (APPLETON & PEREIRA, 1977). The existence of such an agent causing shellfish-associated gastroenteritis had been postulated earlier by AYRES (1975a). No further problems were reported until early 1978 when an investigation of some cases of infectious hepatitis (Hepatitis A) revealed a common source association with the consumption of mussels (*Mytilus edulis*). Details of this outbreak have since been published (BOSTOCK *et al.*, 1979) and although the link with mussels is somewhat tenuous it is certainly more than circumstantial.

5.5.1.1 *E. coli* and coliphage in naturally polluted shellfish

Clearly it would be advantageous if an indicator could be found which assessed viral risks rather more reliably than the use of the bacterial indicator *E. coli*. As mentioned earlier, coliphage seems to be a promising candidate as an alternative or complementary indicator. However, little is known of the relative incidence of coliphage to *E. coli* in shellfish harvested from polluted sources and thus a primary objective was to look at samples of bivalve shellfish taken from areas subject to sewage contamination.

Previous work by the author (PAA) not associated with the present study had demonstrated a clear association between the degree of

shellfish pollution (as measured by the presence of *E. coli*) in a polluted area and water temperature, i.e. a seasonal effect (AYRES *et al.*, 1978). Accordingly, it was decided to examine shellfish samples taken in late summer (July to September) when levels of pollution were expected to be high. Because of logistic problems it was necessary to perform phage estimates on samples of shellfish taken for routine bacteriological analysis, prepared according to the methods of AYRES (1975b). Methods for phage and *E. coli* estimation were as described earlier (5.2.1 and 5.2.2).

Samples of the European flat oyster (*Ostrea edulis*) and the hard clam (*Mercenaria mercenaria*) consisting of 10 individual animals per sample were taken from populations of these animals situated at low water mark approximately 1 km below a sewage outfall and delivered to the laboratory within 2 hours of collection. On receipt the animals were thoroughly scrubbed under running tapwater to remove mud and epifauna from the exterior surfaces of the shells. The hinge ligaments of the animals were broken with a sterile oyster knife and tissues severed from the shells with a sterile scalpel prior to preparation of extracts for analysis. An additional set of samples of oysters was taken from various points along the River Roach, Essex at various distances away from a known source of pollution.

5.5.1.2 Accumulation and survival of phage in shellfish

Published work by other authors reviewed in section 4.4 has shown that shellfish accumulate phage by the same biological mechanism as they accumulate bacteria and viruses and often to very high levels. There seems to be differences in accumulation and retention of viruses depending on whether the experimental animals receive large single doses of virus or long-term (continuous) doses of low virus numbers.

It would have been desirable to adopt the long-term continuous low dose approach in the experimental work performed here since it is under these conditions that virus retention (and possible resulting virus problems to the consumer) can occur. However, it was considered that because of lack of sophisticated dosing facilities and continuous flow systems this type of approach could not be adopted on a critical basis. Accordingly it was decided to adopt the single dose approach using both *E. coli* and MS2 phage and to observe the effects of subsequent storage in air and in phage-free water on the phage content of test animals. In all four experiments were performed at 15°C with details as follows:

Expt 1 20 oysters in 20 litres water + 1.4 ml 10^{-2} dil of MS2 suspension. Water and oysters sampled at intervals for 24 h.

Expt 2 25 oysters in 20 litres water, otherwise as Expt 1 but sampled over a longer period and with 2 ml x 10^{-3} *E. coli* added.

Expt 3 50 oysters in 30 litres of water + 0.3 ml stock MS2 phage. Left to equilibriate for 24 h then samples stored in air at 10°C and water at 15°C for up to 6 days.

Expt 4 Two identical tanks set up, 30 oysters in 25 litres water. Tank A sampled regularly over 30 h; Tank B sampled less frequently but over a period of 5 days.

5.5.1.3 Effect of heat processing on phage in shellfish

The incidents of cockle poisoning mentioned earlier (AYRES, 1977a) were due to a combination of events but could probably have been avoided if the cockles had received sufficient heat processing. As the result of cockle problems, some attention was given to the

development of a more satisfactory cooking procedure and the resulting recommendations have been published (AYRES, 1979). This work showed that a brief period of heating to separate the cockle tissues from the shells followed by a four minute period of immersion in boiling water was sufficient to totally destroy *E. coli* and coliform bacteria and reduce total plate count by as much as 99%. One of the big question marks which still remains however is how effective would this process be at dealing with viruses?

For the experiments performed in this present study samples of cockles (*Cardium edule*) were hand-raked from the shore at Bradwell on the River Blackwater and at Burnham on the River Crouch, Essex. A half-gallon measure of cockles was placed in 20 litres of sea water with aeration and dosed with 1.5 ml of MS2 phage suspension. Cockles were left in contact with phage for varying periods depending on the particular experiment. At the end of the exposure period all cockles were removed from the water and split into five separate batches. One batch was taken as the raw sample and the others were placed in muslin net bags and immersed in boiling water for periods of up to four minutes. When cockles were immersed in boiling water the water temperature fell below 100°C so boiling times were taken from the resumption of boiling (i.e. 100°C) and not from the point of immersion. All samples of cockle tissue were diluted with an equal volume of 0.1% sterile peptone water after maceration and examined by the soft-agar overlay technique.

5.5.2 Results

5.5.2.1 *E. coli* and coliphage in naturally polluted shellfish

The results of the analysis of shellfish samples are shown in Table 45. Although counts of *E. coli* varied considerably over the range 0.2 to 135.0 per g of shellfish tissue they would generally have

Table 45 Coliphage and *E. coli* in shellfish from a polluted area

Month sampled	Shellfish	Phage PFU/g tissue	*E. coli*/g tissue (mean)
July	Oysters	< 10	< 1.0
"	"	< 10	0.2
August	"	10	79.0
"	"	10	135.0
September	Clams	< 10	40.0
"	Oysters	< 10	54.2
"	"	< 10	13.0
"	Clams	< 10	10.4

Table 46 Coliphage, *E. coli* and coliforms in oysters from the River Roach, Essex (sampled July)

Station number	Coliphage PFU/g	*E. coli* mean count/g	Coliforms mean count/g
1	12	< 0.2	9.2
2	3	0.4	3.4
3	6	1.0	2.0
4	0	1.2	4.8
5	0	5.6	12.6
6	3	6.6	12.4
7	0	32.0	34.2
8	6	48.8	54.4

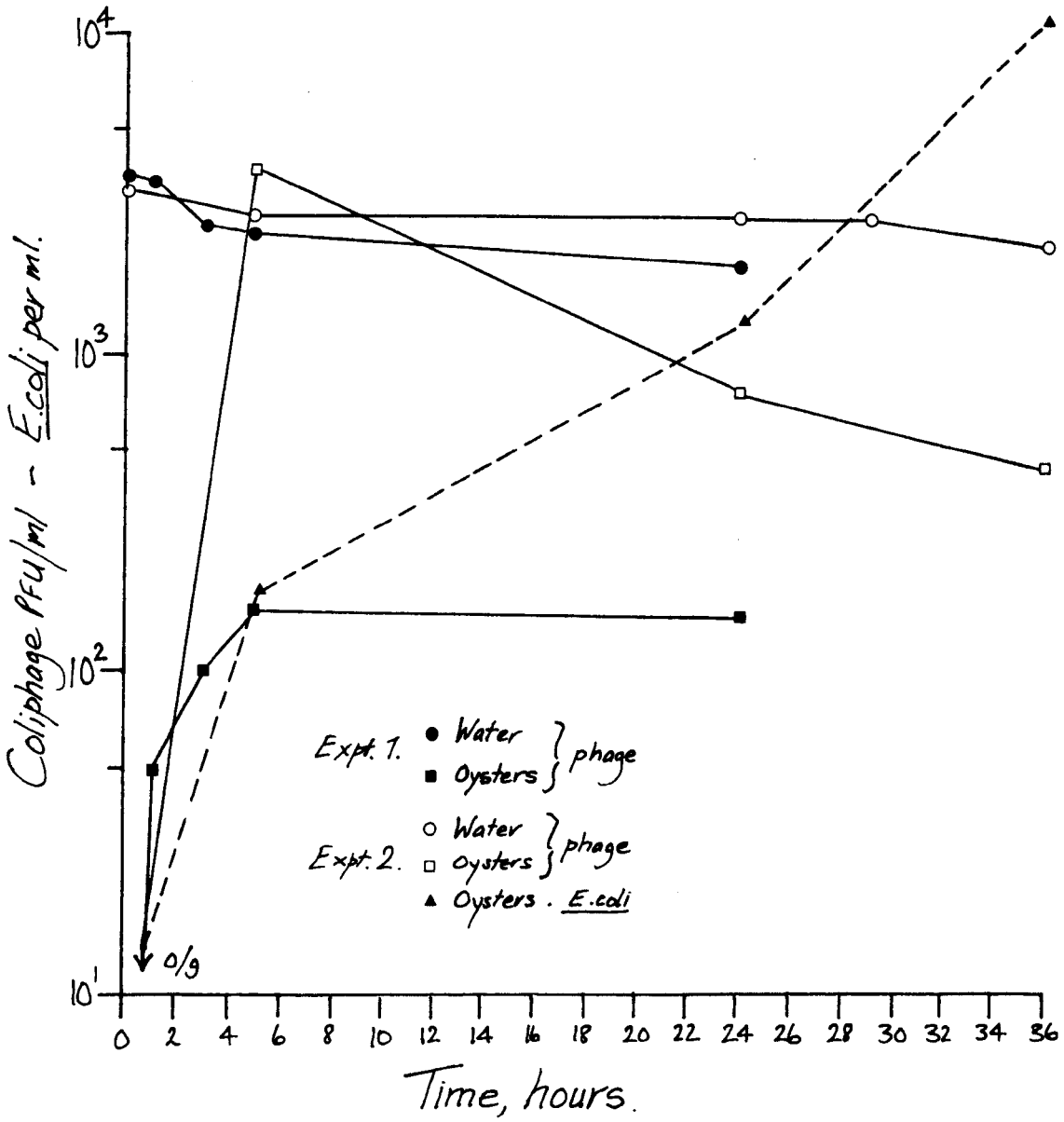
been considered unfit for direct sale by most health and market authorities. It was surprising therefore that only two of the samples were found to contain coliphage although these did coincide with the highest *E. coli* counts. However, it should be added that because of the fact that these samples were prepared primarily for bacteriological analysis the degree of dilution considerably reduced the sensitivity of the phage technique and those samples marked as < 10 may have contained single numbers of phage.

Samples taken as part of a sanitary survey of the River Roach were accordingly treated differently and phage estimates were made on neat shellfish tissue before being diluted for bacteriological analysis. The results of this survey are shown in Table 46. Station 1 represents the point of sampling farthest from the pollution source, a distance of approximately 10 km. Conversely, Station 8 represents the sampling point almost adjacent to the point of discharge. Clearly the *E. coli* results reflect the effect of distance, being highest near the discharge and lowest at a point remote from it. Coliforms show a similar pattern although since they are less specific some evidence of agricultural pollution, run-off from the land, etc. can be seen in the slight increase from Station 3 to Stations 2 and 1. It was interesting to note that coliphage levels have no relationship to numbers of either *E. coli* or coliforms. The variation in numbers could reflect residual pollution from effluent discharge on previous tides and/or some influence not associated with the pollution source, e.g. agricultural run-off.

5.5.2.2 Accumulation and survival of phage in shellfish

Results of experiments 1 and 2 are shown in Figure 49. In experiment 1 phage was accumulated rapidly by oysters for the first 5 h after

Figure 49. Accumulation of phage MS2 and E.coli by oysters at 15 °C (Expts. 1 and 2.)



which it levelled off and remained fairly constant up to 24 h. Counts of phage in water showed a decline while oysters were obviously accumulating phage and thereafter declined slowly. In experiment 2 phage levels in water showed a very gradual decline over 36 h whereas the oysters accumulated phage very rapidly in the first 4 h and thereafter began to eliminate them. It is interesting to compare these results with the accumulation of *E. coli* on the same time-scale. Oysters appeared to be continually accumulating *E. coli* over the entire 36 h period although physiologically it is likely that this represents a net gain over losses due to natural elimination by the oysters. In experiment 3 (Figure 50) the decline of phage levels in oysters stored in air at 10°C and water at 15°C were studied. Losses in water were initially rapid and this represents active elimination or excretion of phage. After 2 days, phage levels increased again before a further period of decline. This demonstrates elimination, re-accumulation and further elimination. In air at 10°C decline of phage was much slower and probably represents natural decay of phage in the animals, as contrasted with elimination and decay observed in those animals stored in water. Figure 51 shows the results of a longer-term experiment to look at uptake of phage. In tank A samples were taken at intervals over a 30 h period and in tank B less frequently over a period of 5 days. Counts of phage in the water exhibited considerable fluctuation but a general decrease over the 5-day period. Oysters exhibited rapid initial accumulation, then elimination/accumulation as seen in previous experiments. Over a 5-day period phage levels in oysters exhibited an overall decline.

5.5.2.3 Effect of heat processing

The results of the three experiments are shown in Table 47. Phage disappeared within a minute's boiling in the first experiment

Figure 50. Effect of storage in air and water on coliphage MS2 in oysters. (Expt. 3)

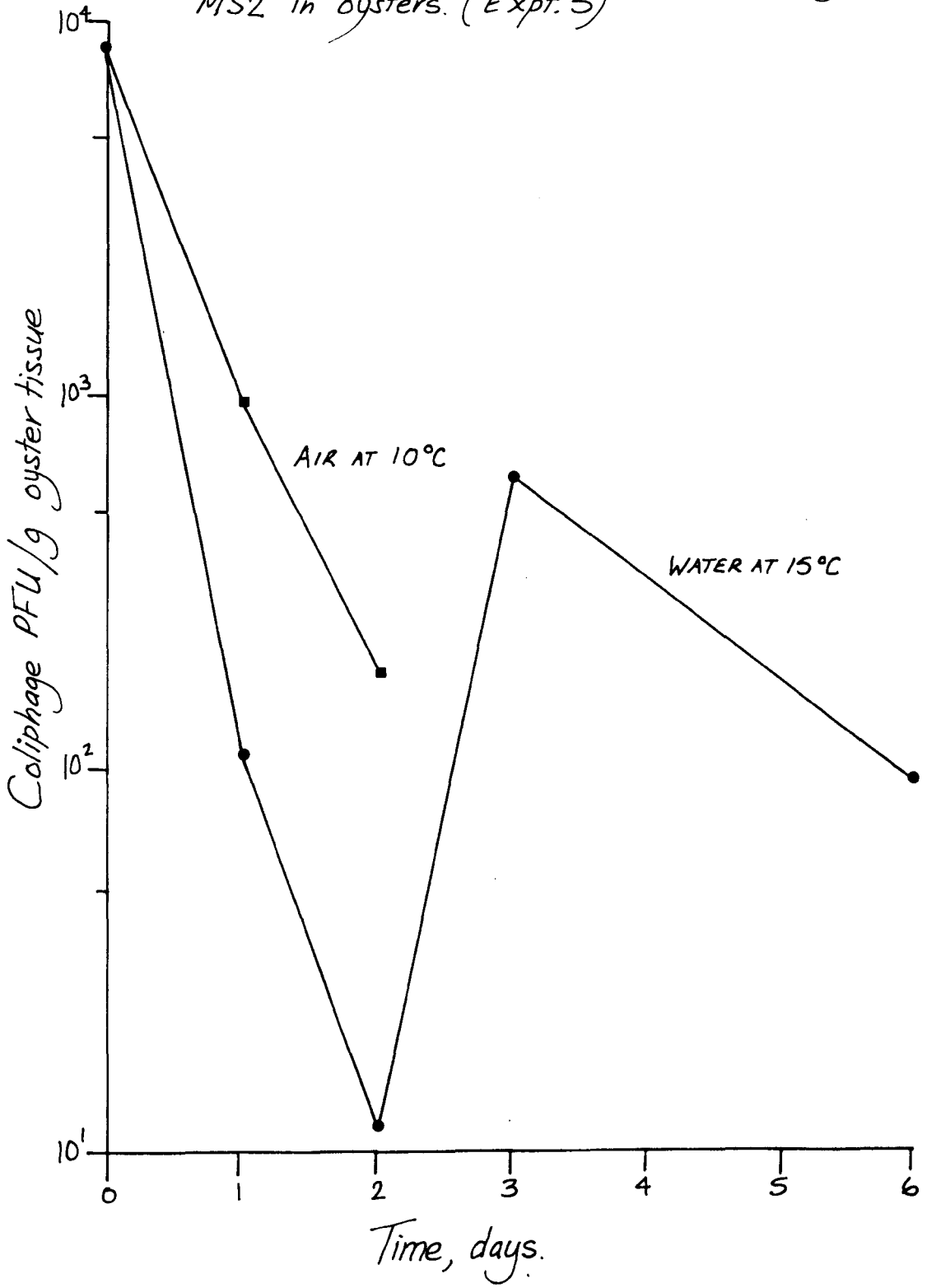
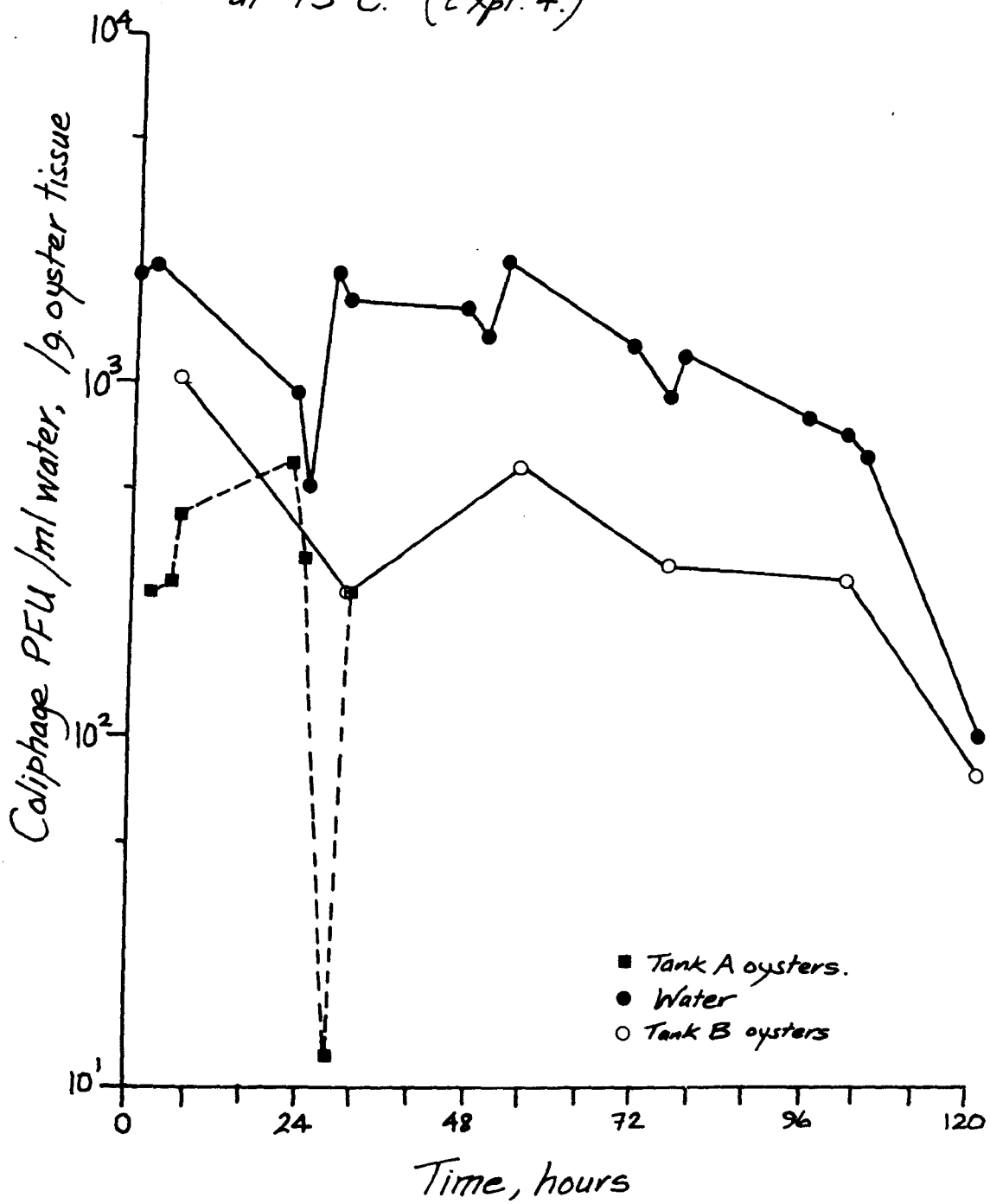


Figure 51. Accumulation of phage MS2 by oysters at 15°C. (Expt. 4.)



and when this was repeated with a higher dose of phage an equally rapid disappearance was observed. For experiment 3, boiling times of less than one minute were tried and an initial phage count of 8.75×10^4 was reduced to 5×10^2 in 15 seconds and to undetectable levels in 30 seconds. This compares very favourably with earlier results for *E. coli* and coliforms in cockles (AYRES, 1979) where a 100% kill was achieved within one minute's exposure to boiling water.

Table 47 Effect of boiling time on phage MS2 of cockles (*Cardium edule*)

Experiment number	Boiling time (minutes)				
	0	1	2	3	4
1	1.04×10^{2}*	0	0	0	0
2	2.06×10^4	0	0	0	0
	0	¼	½	¾	1
3	8.75×10^4	5×10^2	0	0	0

*Count in PFU/g cockle tissue.

5.5.3 Discussion

In the present study no relationship could be demonstrated between *E. coli* and coliphage in shellfish samples taken from sewage-polluted areas and this seems to be in good agreement with the results of studies performed elsewhere. Investigations on the Gulf Coast of the USA (PORTNOY *et al.*, 1975; MACKOWIAK *et al.*, 1976) and in Galveston Bay (FUGATE *et al.*, 1975) have shown that coliforms are an inadequate indicator of virological quality of shellfish. GOYAL *et al.* (1978) demonstrated that high concentrations of enteric virus could be identified in waters which met the current US standards for bacteriological quality of shellfish harvesting areas. METCALF & STILES (1968) clearly showed that widespread dissemination of viruses in estuary

water was followed by widespread appearance of the same viral types in shellfish and that viruses could be recovered from water meeting a standard of < 70 coliform median MPN/100 ml. More recent studies by VAUGHN & METCALF (1975) have suggested, however, that coliphage indicators of virus in shellfish are open to question. Oysters demonstrated a greater potential for the accumulation of coliphage than for enteroviruses when placed in sea water containing coliphage and coxsackie virus B3. Although the initial concentration of coxsackie B3 in water was five times that of the coliphage, oysters accumulated between five and thirty times more coliphage than coxsackie virus in a period of two hours' exposure. In field observations the same authors also found that 82% of coliphage isolations in water and shellfish samples were made without isolating enterovirus whereas over half the enterovirus isolations were made from samples with no coliphage activity. When VAUGHN & METCALF (1975) dosed oysters with coliphage and coxsackie virus and immersed them in estuarine waters at different seasons they produced some interesting results (Figure 52). These showed that coliphage survived in oysters for considerably longer than coxsackie virus B3 and that retention times were clearly associated with water temperature (season), being shortest in summer (14°C to 21°C) and longest in winter/spring (1°C to 11°C).

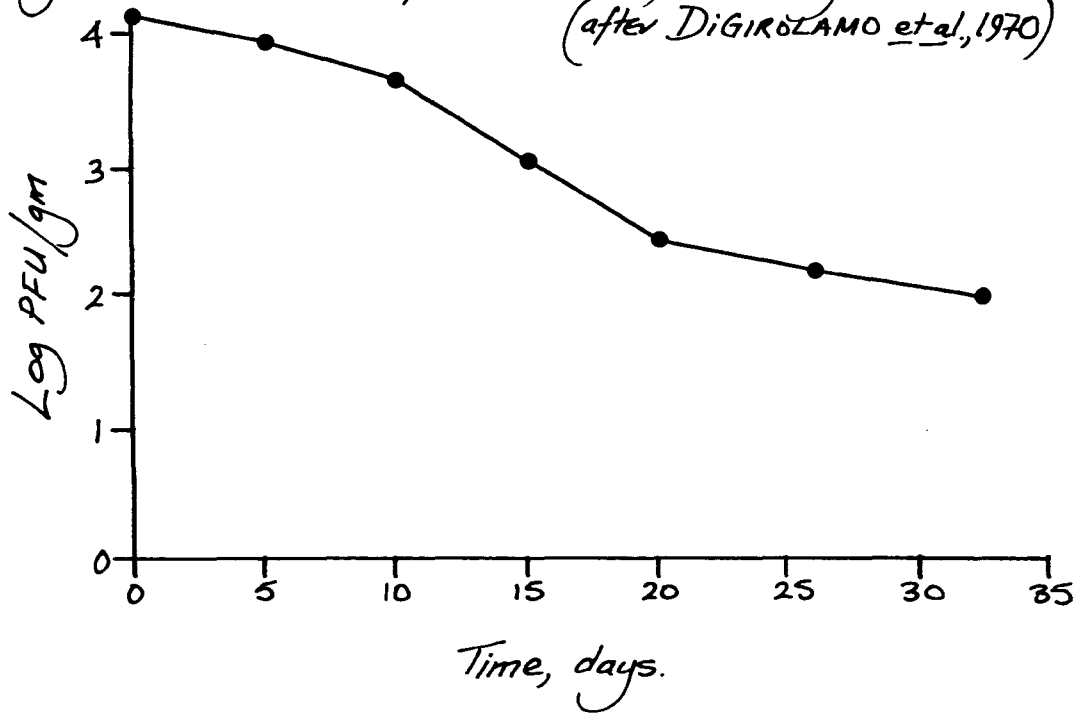
Results such as these serve to illustrate again the importance of oyster-feeding activity on the accumulation, retention and elimination of viruses. In the introduction to the present study mention was made of the influence of water temperature on activity of bivalve shellfish and the results of VAUGHN & METCALF (1975) show in effect that in warm water when oysters are active they eliminate virus, in cold waters they are virtually dormant and so retain virus. Experiments performed in this present study show that accumulation of phage MS2 is rapid but

Figure 52. Retention of coliphage and coxsackie virus B3 by oysters immersed in estuarine waters during spring, summer and winter months.
(after VAUGHN & METCALF, 1975)

that elimination and re-accumulation can occur as long as MS2 remains in the water. When oysters containing phage were transferred for storage in water without any initial phage present, most of the phage were eliminated in two days (Figure 50) but the phage now present in the water were available to be re-accumulated by the oysters. Air storage of oysters at 10°C led to a gradual decline in phage numbers although this is attributable to inactivation of the phage rather than elimination by physiological processes. This indicates that the results of VAUGHN & METCALF (1975) are also modified by direct temperature effects on the rate of virus inactivation in addition to the observed effects on virus elimination by shellfish. The importance of storage temperature can be illustrated by the work of DiGIROLAMO *et al.* (1970) who showed that poliovirus could survive for between 30 and 90 days in oysters stored at refrigeration temperatures. Figure 53 is adapted from their work and shows that at 5°C, 46% of virus remained after 10 days, and even after 30 days when the oysters were badly decomposed 13% of the virus was still viable. These studies also showed that survival of virus in the digestive area of oysters was 76% of the total virus content after 5 days at 5°C and 36% after 15 days. This illustrates that virus in the digestive gland are as resistant to cold as they are to the effects of heating reviewed earlier.

Detailed mention has been made elsewhere in this present study of the concentration and elimination rates of virus and phage by shellfish (sections 3.3 and 4.4) and it would be superfluous to reiterate that information here. However, it has also been stressed that the commercial purification of shellfish to eliminate bacterial and viral pathogens is a very important facet of the shellfish industry in the United Kingdom. Although the process is very effective for the elimination of bacterial pathogens studies have shown that

Figure 53. Survival of poliovirus in oysters refrigerated at 5°C.
(after DiGIROLAMO et al, 1970)



laboratory strains of enteroviruses contained in oysters and clams are reduced rapidly initially but may persist at low levels for several days (HEDSTROM & LYCKE, 1963; LIU *et al.*, 1967; METCALF & STILES, 1968; SERAICHEKAS *et al.*, 1968). Normal commercial purification of shellfish operates on a 36 to 48 h treatment period so possibilities of a virus residual raise potentially serious doubts about such processes.

Two recent papers which have not been referred to in earlier sections of this submission throw new light on the potential problem of viral depuration in shellfish purification systems. Studies by DIGIROLAMO *et al.* (1977) suggest that the bio-accumulation efficiency shown by oysters in relation to viruses involves an ionic-binding mechanism which causes the virus to attach to the shellfish tissues. Attachment of virus in this way was considered to occur through an irreversible binding of the virus to mucous excreted by the shellfish; mucous and virus being subsequently ingested by the shellfish. A very recent paper by METCALF *et al.* (1979) describes accumulation and elimination experiments with the soft shelled clam (*Mya arenaria*). The authors showed that virus uptake was greatest when the virus were associated with solids and when pollution levels in water were equivalent to or greater than those normally found in grossly polluted areas. Ingested viruses were largely concentrated in the hepatopancreas and siphon tissues and there was no evidence of irreversible sequestering which has been reported by others (DIGIROLAMO *et al.*, 1975; LIU *et al.*, 1967). The study by METCALF *et al.* (1979) also set out to recreate the type of situation most likely to occur in field conditions, that is to say, exposure of clams to low numbers of solids-associated virus, and showed that shellfish polluted under such conditions could be successfully purified. However, the authors also found that some individual clams did not function reliably and that these could

potentially render the whole process ineffective. Their conclusions were that although they were unable to measure the risk involved, their studies had shown that the greatest reduction in risk from viruses in shellfish would result from depuration (purification) of clams harvested from waters of good sanitary quality.

The results obtained from the heat processing experiments show that coliphage MS2 in cockles is rapidly inactivated at a rate comparable to that of the bacterium *E. coli*. Viruses are known to be inactivated by heat which causes coagulation and breakdown of virus protein coat. Double-stranded DNA phages of the T-type lose the ability to adsorb to cells and the nucleic acid is released but it is not known whether this occurs with RNA phages (such as MS2) or the single-stranded DNA phages (CLIVER & SALO, 1978).

Experiments by KRUGMAN *et al.* (1970) showed that the MS1 strain of hepatitis which resembles the classical type A or infectious hepatitis (KRUGMAN & GILES, 1970) was inactivated by boiling at 98°C for one minute. Experiments with human volunteers have shown that such an agent is still infectious after treatment at 56°C for 30 minutes (HAVENS, 1945). Few studies appear to have been conducted on the effect of heat on virus in foods but there are two papers dealing with shellfish. The first is a paper by DIGIROLAMO *et al.* (1970) who studied the survival of poliovirus in oysters subject to various forms of treatment. Samples of Pacific and Olympia oysters (*Crassostrea gigas* and *Ostrea lurida*) were contaminated with poliovirus and subjected to four types of heat processing commonly used in the preparation of these foods, stewing, frying, baking and steaming. The results obtained have been summarized in Figure 54 and show that although the effectiveness of the processes varied, 7 to 13% of the poliovirus still remained. It has been demonstrated that most of the virus in

Figure 54. Kinetics of poliovirus inactivation in oysters heat processed by four different methods. (after DiGiROLAMO et al., 1970)

contaminated oysters is found in the digestive tract (LINDBERG & BRAMAN, 1956; MASON & McLEAN, 1962) and clearly in the experiments of DIGIROLAMO *et al.* (1970) the processing times were insufficient to raise the internal temperature of the oysters high enough to inactivate viruses completely. In fact the authors themselves showed that it took 35 minutes' steaming to reach a temperature of 100°C in the digestive tract area of the oysters. Studies by KOFF & SEAR (1967) followed the internal temperature changes during the steaming of soft clams. Although the shells usually opened within the first minute it took between four and six minutes' steaming to raise the internal temperature of the clam tissues to 100°C. No correlation was found between weight (size) of the clams and time taken to reach 100°C, although it should be stated that they treated clams individually and not in bulk as would be the case commercially. Therefore their results might be regarded as somewhat understating the time required under commercial shellfish processing conditions. DISMUKES *et al.* (1969) in an investigation into gastroenteritis and infectious hepatitis arising from the consumption of clams showed that the attack rate for people eating raw clams was 44%, for those eating raw and steamed clams it was 53% and for those eating steamed clams only, 33%. This would suggest that some form of cooking does reduce the risk of infection and might explain why the majority of incidents reported in the literature are associated with the consumption of raw shellfish. Crustacea are not normally associated with such problems due to viruses but DIGIROLAMO *et al.* (1972) in studies with crabs contaminated with coliphage T_4 showed that the potential for virus transmission did exist. These authors demonstrated that although reduction of virus did occur between 2-5 and 20% remained after processing, depending on the processing time. This demonstrates that the efficiency of heat processing is a function

of both time and temperature. In addition, it should be noted that the rate of thermal inactivation of viruses is influenced by the medium in which they are held (WALLIS *et al.*, 1962; WALLIS & MELNICK, 1965; DIMMOCK, 1967).

5.6 Sediments

The marine disposal of sewage sludge is widely practised in the United Kingdom and some 8 million tons are dumped annually, primarily at eight dumping sites approved for this purpose. The dumping of such wastes is controlled by the Dumping at Sea (DAS) Act introduced in 1974 and in England and Wales the Ministry of Agriculture, Fisheries and Food (MAFF) is the licensing authority. MAFF's reponsibility in this respect is "to have regard to the need to protect the marine environment, and the living resources which it supports, from any adverse consequences of dumping...". In respect of sewage sludges field studies are made to determine the distribution and physical/chemical effects of the sludge on the water column and sediment in addition to biological effects and effects on fish and shellfish quality. At the present time, however, these studies do not look at the possible effects of introducing pathogenic bacteria and viruses.

Studies by GERBA *et al.* (1977) have shown that viruses may occur in sediments in concentrations from 10- to 10 000-fold greater than in the overlying water. Laboratory studies (GERBA & McLEOD, 1976; SMITH *et al.*, 1978) have also shown that viruses and faecal coliforms survive longer in sediments than in the water. The water-sediment interface is not a static system and under the influence of environmental factors and man's activities (e.g. dredging) sediments may be resuspended in the water column. This suggests that marine sediments may act as reservoirs of viral and bacterial pathogens, a situation which may be enhanced by the dumping of sewage sludge.

Some limited microbiological studies on sewage dumping grounds have been made with the objective of trying to plot the distribution of sewage solids on the bottom sediment (AYRES, 1977b). The technique has shown that high concentrations of *E. coli* and coliforms associated with dumping can be identified using methods developed for sample analysis at sea. Although no laboratory studies on the survival of phage in sediments were conducted as part of this present study, some of the field samples were brought back for examination. Samples of sediment were preserved by the addition of a few millilitres of chloroform to kill bacteria and stored at -20°C for a week. On arrival at the laboratory 1 g quantities of sediment were suspended in sterile 0.1% peptone water and assayed for coliphage using methods described earlier. Although one or two plaques were produced these were not from samples yielding high coliform or *E. coli* counts. The opportunity to repeat this work was not available and it is felt that the handling and storage of the samples prior to examination may well have been at fault. A study by GOYAL *et al.* (1978) has shown a high degree of correlation between enteric virus isolation and total coliform counts in bottom sediments so it would seem worth while to examine dumping site sediments at some future date.

6. SUMMARY AND CONCLUSIONS

One of the most serious limitations of the experimental work performed for, and described in this submission is the absence of practical data on enteric viruses. Ideally one would have wished to make a simultaneous appraisal of bacterial indicators such as *E. coli*, coliphage and the enteroviruses but apart from logistic considerations it is clearly beyond the scope of one individual to perform such a study. With this in mind, the present study has attempted to review available knowledge from published papers, and the author's personal experience, to show what has been done already; to make an original practical contribution in terms of the experimental studies described, and ultimately to draw this information together to make a rational and balanced appraisal of the current situation. In order to fulfil the objective of this study, to make a critical appraisal of coliphage as a possible indicator of enteric virus in sewage and the marine environment, it has been necessary to explore a number of scientific disciplines of which the author cannot claim to have comprehensive, or perhaps even adequate, knowledge. For possibly the first time, however, the available information as it relates to the chosen topic has been brought together for a single document.

Detailed discussions of the situation pertaining to virus, phage and indicator bacteria have been made at length earlier in this submission and the purpose of this final section is to reach some general conclusions and, in the light of these, make some recommendations as to priorities for future work.

A strong case can, and has been made, to show that the most widely applied bacterial indicators, particularly *E. coli*, are grossly inadequate to demonstrate risks from viral pathogens. The consensus of opinion voiced in published studies has been adequately confirmed

here and shows that the behaviour of *E. coli* in sewage, and particularly in the environment, bears little similarity to that of known enteroviruses. In situations of heavy pollution, as measured by bacterial indicator systems, it can be postulated that there is an existing risk from viruses as well as bacterial pathogens. However, in lightly polluted situations where even bacterial densities are low the possible hazard from viruses, potentially infectious in exceedingly small numbers, cannot be measured by such criteria. Factors such as the prolonged survival of enteroviruses in sewage and the marine environment, and very wide fluctuations both in the types and numbers of viruses which may be present, are further complications. The inadequacy of bacterial indicators can be postulated not only from the wealth of comparative studies available but also from their proven failure under field conditions where shellfish-transmitted viral disease has been linked with areas judged to be acceptable by bacterial indicator systems.

Although no work could be carried out on enteroviruses in this present study a comprehensive review of the literature has been attempted. Many investigations have produced apparently contradictory results and taking a broad view of these it is clear that many such problems arise from inherent difficulties in estimating viruses, due to techniques and to differences between the viruses themselves. There seems to be no 'typical' enterovirus yet identified which could fulfil the criteria demanded of an indicator, and good reason to believe that what applies, for example, to poliovirus does not automatically apply to other enteroviruses. This suggests that future studies should always attempt to compare a number of different enteroviruses; where this has been done in the past considerable variation in the behaviour of viruses has always been evident.

Turning to coliphage, the objective of this current appraisal, there seems little doubt from published work, and that performed here, that coliphage are a better indicator of risk from enteroviruses than bacterial indicators. Having said that, one must also ask whether being better than bacterial indicators is an adequate criterion for adoption as a virus indicator. BERG (1969), in a discussion of a paper by KOTT *et al.* (1969), stated that "reasonable proof is now required that coliphage are always present when viruses of human origin are present and in equal or greater numbers; proof is also required that coliphage are at least as resistant to the marine environment as the viruses of man." This was a fair summary of the situation in 1969, and now, ten years later, it is doubtful that reasonable proof has been forthcoming. However, considerable advances have been made, if only to show that there are many 'grey' areas in what otherwise might have been taken as a 'black' and 'white' situation. Coliphage in sewage seem to fulfil BERG's (1969) first criterion, they are always present when viruses are present and usually in greater numbers, though never less. Indeed, coliphage may be said to be ubiquitous in sewage whereas enteroviruses probably reflect the trends of incidence in the community which by their very nature are highly variable. Adapting the second criterion, that coliphage are at least as resistant as enteroviruses to sewage treatment, again this is fulfilled. Certainly the RNA phages, particularly f2, are very resistant and in disinfection studies survive for considerably longer than poliovirus. The finding that coliphage replicated in sewage would render them useless as indicators, and although replication has been demonstrated in a laboratory study, the evidence from field situations is contrary to this. On balance, results of the field studies seem to be correct in that it is unlikely the faecal bacteria in sewage are at a stage of

growth conducive to phage infection and replication.

Results from the present study and published papers also show that coliphage in sea water and shellfish are promising indicators. However, the criteria advanced by BERG, which have been cited earlier, cannot be met entirely nor with any certainty. Coliphage obviously survive for long periods in sea water and are comparable to many of the known enteroviruses in this respect. However, they have been absent when enteroviruses have been identified and if this is a common or widespread occurrence then coliphage could not be considered adequate. As virus models, coliphage have been successfully used in studies on both sea water and shellfish and their use has advanced our knowledge of the physical, chemical and biological factors involved in the ultimate fate of enteroviruses. The use of coliphage MS2 in this present study and the use of this and other coliphages in published studies has shown that adsorption is probably the most important factor involved in virus survival both in sewage and in the marine environment. It is clear that many other factors such as salinity, temperature and pH are also important but this submission demonstrates for the first time how all these factors could influence adsorption processes. At the same time it has been stressed that there are many variables to consider in making a critical appraisal such as that attempted here and there are likely to be many exceptions to what must in essence be a generalized account.

One of the problems which exists in using bacteriophage as a model of virus behaviour in any system, be it sewage, water, sediments or shellfish, is the choice of the phage itself. It should be apparent from the literature reviewed in this submission that the double-stranded DNA phages of the T_1-T_7 series are popular choices for many studies both in the field and in the laboratory. This is partly

because both the phage strains and the host bacteria strains are readily available, and also, because one study inevitably leads to another, it is common practice to adopt those strains used by earlier workers for comparative purposes. T-phages are equipped with highly specialized and complex structures for the process of adsorption and penetration of the host cell, so that phage DNA can enter. Bacteriophage T_7 for example has a head structure which contains the nucleic acid, a tail, tail plate, and tail fibres for attachment to the host bacterium. It is possible that the possession of specific structures for adsorption to host cells also means that T-phage are equally efficient at attaching to other particulate materials. Additionally, these highly specialized structures such as tail fibres render T-phages prone to mechanical damage which may prevent adsorption. Animal enteric viruses are icosahedral in shape, very much smaller than any T-phage, and attach themselves to the plasma membranes of mammalian cells by electrostatic means. Being icosahedral, or near spherical, in shape they usually have multiple receptor sites so are potentially less likely to suffer from random damage. Multiple receptor sites also mean that orientation of animal enteric virus to host cells is less important than it is for T-phages which may require face to face or tail to face orientation for successful attachment to host cells. Some of the single-stranded RNA phages such as f2, MS2 and Q beta attach to the sides of the pili or small projections which occur only on male (F+) strains of *E. coli*. RNA phages enter through the sides of these F pili and pass through the pilus down to the cell surfaces. RNA phages like MS2 are also icosahedral in shape, of about the same size as poliovirus for example, and like the animal enteric viruses are not so prone to the type of mechanical damage which can occur with the tailed T-phages. It is therefore apparent that on such

considerations as these, the RNA phages have the potential to be far more indicative of the fate and behaviour of enteric virus than any of the tailed T-phages so often used.

While it is obviously true that the choice of a particular phage model necessarily dictates the choice of the host bacterium which is to be used as a receptor strain, it is equally true that the choice of host bacterium will dictate the types of phage which are identified. This is particularly important in field investigations when one is concerned with the levels of phage naturally occurring in sewage or sea water for example. Adoption of an *E. coli* B host will permit the cultivation of over 30 different phages and is generally adopted as a 'universal host' for the T series phages. However, as has been stated here and earlier in the study, enumeration of single-stranded RNA phages such as MS2 requires the use of a male-specific (F+) strain of *E. coli* which is unsuitable for many T-type phages (AYRES, unpublished data). Evidence of the practical problems which are posed by the choice of host strain are well illustrated by the work of HILTON & STOTZKY (1973) and VAUGHN & METCALF (1975). Both studies showed quite clearly that the use of a number of different host strains revealed changes in the composition of the total coliphage population. In some instances a particular host strain would yield large numbers of coliphage and then later on would have implied that no phage was present. Just as the frequency of occurrence of enteroviruses varies so does the frequency of coliphage. Therefore one should be careful to note that the host selected for a study has limitations in terms of its susceptibility to types of coliphage which may be present in the environment studied.

Because of the limitations detailed above it is concluded from this present study that coliphage offer some possibilities as a

satisfactory model for enteroviruses in studies on the efficiency of sewage treatment, inactivation mechanisms and development of detection and concentration methods. However, even in these situations they must be regarded as a means to understanding the behaviour and fate of viruses and not an end in themselves. As indicators of viral pollution, many questions remain unanswered and although coliphage show promise there is insufficient evidence to demonstrate conclusively that they meet the criteria required of an indicator. It is the author's current view that the RNA phages such as MS2 are potentially the most useful of the known coliphages, particularly because of their similarity to enteroviruses. As indicators, coliphage present a useful indicator system to complement the routine use of bacterial indicators such as *E. coli* and the fact that they increase the margin of safety over use of bacterial indicators alone seems some justification for their adoption. Ultimately the actual viruses may be the only safe indicator system but until routine analysis for virus is achievable by relatively simple and rapid techniques, the development of criteria based jointly on coliphage and faecal coliforms (*E. coli*) offers a real alternative.

7. SUGGESTIONS FOR FUTURE WORK

This submission would not be complete as a critical appraisal unless some suggestions were forthcoming as to priorities for future work. Much has been achieved in a relatively short time but answers to the following are urgently required:

1. A critical comparison between DNA T-type phages and RNA phages such as MS2. Published work is divided between use of one or other type and comparisons of the data are therefore not yet possible.
2. Methods of analysis and identification of Hepatitis A virus and the agents of viral gastroenteritis. These are established risk areas in terms of proven transmission of viral disease in the marine environment and until it is possible to routinely cultivate the agents responsible the adequacy or otherwise of existing criteria cannot be assessed.
3. Attempts to find a phage indicator system which is more closely allied to the occurrence and distribution of enteroviruses.
4. To demonstrate in field conditions that any phage-based indicator system is superior to faecal/total coliform indicators for assessing virus risk.
5. Comparative studies between coliphage, bacterial indicators and enterovirus in all types of systems. Fragmented studies comparing combinations of any two of these have posed more questions than they have generally answered.
6. Evaluation of coliphage or other indicators for determining the fate of viruses in marine sediments, especially in relation to the marine disposal of sewage sludge.

7. A concerted effort to look at the possible associations between all the factors which are said to contribute to virus inactivation in sea water.

8. There is an urgent need for epidemiological investigations into the whole problem of viral disease transmission by shellfish. Studies by CABELLI *et al.* (1979) have shown that there is a problem associated with bathing in sewage polluted water. Although this had been postulated for many years a well designed and detailed epidemiological study was necessary to confirm it. Such a study would form an ideal model for similar studies on the association between consumption of shellfish and disease.

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10. APPENDICES

Appendix 1 Composition of media referred to in text

4% enriched Teepol broth

Peptone (Evans Medical Ltd)	40 g
Yeast extract (Oxoid)	6 g
Lactose	30 g
Teepol 610 (BDH)	40 ml
0.4% aqueous phenol red	50 ml
Distilled water	to 1000 ml

Autoclaved 121°C (15 lb/sq in) 15 minutes; final pH 7.4

MacConkey broth

Peptone (Oxoid L37)	20 g
Lactose	10 g
Bile salts (Oxoid L55)	5 g
Sodium chloride	5 g
Neutral red	0.075 g
Distilled water	1000 ml

Autoclaved 121°C (15 lb/sq. in) 15 minutes; final pH 7.4

MacConkey agar no. 3

Peptone	20 g
Lactose	10 g
Bile salts no. 3	1.5 g
Sodium chloride	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	15.0 g
Distilled water	1000 ml

Autoclaved 121°C (15 lb/sq in) 15 minutes; final pH 7.1

Note: This formulation was used for the preparation of plates for the Miles and Misra Technique. For roll tubes an extra 5 g per litre of agar was added to give a firmer gel.

Brilliant green bile broth

Peptone	10 g
Lactose	10 g
Oxbile (purified)	20 g
Brilliant green	0.0133 g
Distilled water	1000 ml

Autoclaved 121°C (15 lb/sq in) 15 minutes; final pH 7.4

Nutrient agar

'Lab-Lemco' beef extract	1 g
Yeast extract (Oxoid L20)	2 g
Peptone (Oxoid L37)	5 g
Sodium chloride	5 g
Agar	15 g
Distilled water	1000 ml

Autoclaved 121°C (15 lb/sq in) 15 minutes; final pH 7.4

The nutrient broth used routinely for propagation of host strains was of the above composition less the agar.

Casitone Glycerol Yeast Extract Agar (CGYE)

Casitone	5 g
Glycerol	5 g
Yeast extract	1 g
Agar (Oxoid no. 3)	13 g
Distilled water	1000 ml

Autoclaved 121°C (15 lb/sq in) 15 minutes; final pH 7.2

Appendix 2 Burnham-on-Crouch Sewage Treatment Works (see Figure 10)

This works is of the 'Simplex' aeration type, a relatively recent concept in sewage treatment and unusually situated in serving a town with a small population. The plant is situated to the east of the town at sea level some 50 yards from the River Crouch to which the final effluent is discharged. It is served by two outfall sewers; a 24 inch northern outfall serving the more rural areas of the district and a 15 inch southern outfall serving the town itself. Both sewers combine at the treatment works site into a 36 inch total outflow sewer.

The combined effluent is screened to remove large solids and debris and passes into a DWF sump. Four pumps take the effluent to the DWF grit trap where solid particles such as stones are settled and subsequently lifted out by compressed air for direct disposal. The effluent then passes by gravity to the PRIMARY tank fitted with a blade scraper where further settlement of solids occurs. Solids are drawn off from the bottom of the tank and the liquor passes to CHAMBER 1 and thence to the aeration tanks fitted with two 'Simplex' cones (large motor-driven rotors which aerate the liquor). Some liquor is returned continuously to the tank to continue the activated sludge formation but the bulk of it passes into the FINAL tank which is fitted with a chain scraper. Liquor then passes to CHAMBER 3, through a flap valve and 24 inch gravity sewer outfall to the River Crouch, discharging just below LWMOT.

At times of heavy rain, excess flow is diverted to the storm sump and lifted by four large capacity pumps to the storm grit trap (a larger version of the DWF one). This then flows via a 33 inch gravity feed main to CHAMBER 2 and on via a 22 inch feed to the storm tank, CHAMBER 3 and out to the River Crouch.

Appendix 3 The coliphage and E. coli content of sewage at various treatment stages

Sample date	Raw influent		Grit trap		Primary filter		Trough		Final filter		Final effluent	
	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*
25 May	1.25×10^{5}	ND	6.2×10^{2}	ND	1.55×10^{3}	ND	3×10^{2}	ND	ND	ND	1.4×10^{2}	ND
31 "	2.98×10^{4}	ND	6.25×10^{4}	ND	2.4×10^{3}	ND	7×10^{1}	ND	5×10^{1}	ND	1.5×10^{1}	ND
1 Jun	3.6×10^{3}	4.2×10^{4}	8.1×10^{3}	ND	1.9×10^{3}	ND	1.7×10^{2}	ND	4×10^{1}	ND	3×10^{1}	3.2×10^{3}
7 "	5.8×10^{2}	3.2×10^{4}	1.68×10^{3}	1.5×10^{5}	7.55×10^{2}	9.1×10^{4}	3×10^{1}	7.1×10^{3}	2.5×10^{1}	2.5×10^{3}	2×10^{1}	3.4×10^{3}
15 "	1×10^{3}	8.2×10^{4}	1.84×10^{3}	7.5×10^{4}	1.28×10^{3}	7×10^{3}	6×10^{1}	5.4×10^{3}	2×10^{1}	8×10^{1}	4×10^{1}	6×10^{1}
21 "	3.9×10^{2}	4.8×10^{4}	7.65×10^{2}	3×10^{4}	2.1×10^{3}	1.5×10^{4}	4.05×10^{2}	4.8×10^{3}	1.05×10^{2}	1×10^{2}	1.65×10^{2}	2.1×10^{2}
27 "	ND	ND	5.45×10^{2}	8.7×10^{4}	1.09×10^{3}	7.8×10^{4}	5.5×10^{1}	1.8×10^{4}	3×10^{1}	2.6×10^{3}	3×10^{1}	2×10^{3}
28 " *	6.7×10^{2}	1.1×10^{5}	-	-	2.8×10^{2}	3.4×10^{4}	2.5×10^{2}	1.4×10^{4}	-	-	2.95×10^{2}	4×10^{4}
4 Jul	1.75×10^{2}	4.5×10^{4}	8.0×10^{1}	7.9×10^{4}	5.7×10^{2}	8.4×10^{4}	2.45×10^{2}	3.3×10^{4}	7×10^{1}	5.9×10^{3}	2.5×10^{1}	4.3×10^{3}
11 "	4.9×10^{2}	1.2×10^{5}	1.52×10^{3}	6.5×10^{4}	1.26×10^{3}	7×10^{4}	4.45×10^{2}	2.7×10^{4}	1.0×10^{1}	6.9×10^{2}	3.5×10^{1}	8×10^{2}
19 "	2.65×10^{2}	6.2×10^{4}	2.2×10^{5}	1.05×10^{2}	1.3×10^{5}	6×10^{2}	2.3×10^{4}	9×10^{1}	1.8×10^{3}	9×10^{1}	6×10^{1}	1.2×10^{3}
27 "	3.7×10^{2}	1.1×10^{5}	9.5×10^{1}	8.9×10^{4}	3.45×10^{2}	1.5×10^{5}	1.7×10^{2}	2.7×10^{3}	5.5×10^{1}	1.7×10^{3}	4.5×10^{1}	2×10^{3}
2 Aug	1.57×10^{3}	1.3×10^{5}	5.5×10^{3}	9.6×10^{4}	1.95×10^{3}	1.2×10^{5}	2×10^{1}	3.9×10^{4}	3×10^{1}	8.3×10^{3}	2×10^{1}	5.9×10^{3}
11 "	2.35×10^{3}	4.7×10^{5}	2.57×10^{3}	6.5×10^{5}	2.13×10^{3}	7.7×10^{5}	2.15×10^{3}	1.1×10^{5}	6.5×10^{1}	8.8×10^{4}	4.5×10^{1}	8.1×10^{3}
15 "	4.45×10^{3}	6.4×10^{5}	4×10^{3}	3.7×10^{5}	8.7×10^{2}	ND	4.9×10^{2}	4×10^{4}	8×10^{2}	7.5×10^{3}	2.15×10^{2}	8.7×10^{3}
24 "	3.65×10^{2}	1.3×10^{4}	2.8×10^{2}	6×10^{3}	5.6×10^{2}	5×10^{3}	7×10^{1}	1.8×10^{4}	3×10^{1}	2×10^{1}	2×10^{1}	1.5×10^{2}
31 "	5.45×10^{2}	4.3×10^{4}	4.3×10^{2}	6.9×10^{4}	1.64×10^{3}	7.1×10^{4}	6×10^{1}	4.8×10^{4}	7×10^{1}	8.6×10^{3}	8.5×10^{1}	8.4×10^{3}
7 Sep	2.2×10^{2}	2.5×10^{5}	6.7×10^{2}	2.7×10^{5}	1.4×10^{3}	3.2×10^{5}	2.95×10^{2}	5.2×10^{4}	9.5×10^{1}	5.5×10^{3}	5×10^{1}	3.8×10^{3}
14 "	1.62×10^{3}	9.1×10^{4}	3.7×10^{2}	9.7×10^{4}	1.09×10^{3}	1×10^{5}	1.7×10^{2}	6.1×10^{4}	4×10^{1}	5.7×10^{3}	2×10^{1}	5.5×10^{3}
21 "	1.4×10^{2}	1.2×10^{5}	2×10^{2}	1×10^{5}	1.7×10^{2}	1.2×10^{5}	4×10^{1}	7.5×10^{4}	< 5	3.8×10^{3}	<5	5.3×10^{3}
5 Oct	3.55×10^{2}	2.8×10^{4}	5.1×10^{2}	1×10^{5}	5.85×10^{2}	7×10^{4}	7.5×10^{5}	6.6×10^{3}	<10	2.3×10^{3}	10	1.9×10^{3}
11 "	ND	ND	6.2×10^{2}	1.2×10^{5}	7.1×10^{2}	1.6×10^{5}	9.5×10^{1}	2.7×10^{4}	5.0×10^{1}	8×10^{3}	4×10^{1}	6.5×10^{3}
19 "	1.34×10^{3}	3.6×10^{4}	2.25×10^{3}	9.5×10^{4}	4.7×10^{3}	7.3×10^{4}	2.3×10^{2}	3.4×10^{4}	3.9×10^{2}	2.5×10^{4}	4.15×10^{2}	1.4×10^{4}
24 "	4.45×10^{2}	9.3×10^{4}	4.2×10^{2}	9.3×10^{4}	5.8×10^{2}	1.4×10^{5}	1.45×10^{2}	3.7×10^{4}	7.5×10^{1}	3.7×10^{4}	5×10^{1}	1.7×10^{4}
2 Nov†	1.35×10^{2}	2.8×10^{4}	4.6×10^{2}	1.5×10^{4}	4.7×10^{2}	1.7×10^{4}	1.5×10^{2}	3.3×10^{3}	4.1×10^{2}	1.6×10^{4}	1.1×10^{2}	1×10^{4}
9 " †	<5	1.7×10^{5}	2×10^{1}	1×10^{5}	2×10^{1}	1.1×10^{5}	8×10^{1}	4×10^{4}	1.5×10^{1}	3×10^{4}	4.5×10^{1}	3×10^{4}
15 "	9.5×10^{1}	1.5×10^{5}	2.2×10^{2}	4.9×10^{4}	3.65×10^{2}	9.8×10^{4}	7×10^{1}	2.7×10^{4}	3×10^{1}	1.5×10^{4}	5×10^{1}	6.3×10^{3}
25 "	5.5×10^{1}	1.1×10^{5}	2×10^{1}	1.3×10^{5}	1.05×10^{2}	7×10^{4}	2×10^{1}	2.8×10^{4}	2×10^{1}	1.8×10^{2}	2×10^{1}	1.4×10^{2}
30 "	1.4×10^{2}	7.2×10^{4}	3×10^{1}	2.4×10^{4}	9.5×10^{1}	1.5×10^{4}	8×10^{1}	2.1×10^{4}	1.5×10^{1}	2.3×10^{3}	2×10^{1}	2×10^{3}
6 Dec	5.25×10^{2}	ND	3.35×10^{2}	2×10^{5}	6.95×10^{2}	7×10^{4}	6.2×10^{2}	7.5×10^{4}	9.5×10^{1}	1.1×10^{4}	1.3×10^{2}	1.5×10^{4}
13 "	7.5×10^{2}	1.8×10^{5}	3.8×10^{2}	2.5×10^{5}	3.95×10^{2}	2×10^{5}	2.2×10^{2}	2.1×10^{4}	9.5×10^{1}	8.2×10^{3}	9×10^{1}	1.6×10^{3}
20 "	1.75×10^{2}	1.3×10^{5}	1.25×10^{2}	1.6×10^{5}	7.7×10^{2}	9.2×10^{4}	4.5×10^{1}	1.8×10^{4}	2×10^{1}	5×10^{2}	3.5×10^{1}	1.4×10^{3}
3 Jan	6.5×10^{2}	1.8×10^{5}	1.55×10^{3}	2.2×10^{5}	5.6×10^{2}	1.6×10^{5}	7.5×10^{1}	6×10^{4}	< 2.5	3.5×10^{3}	5	3.5×10^{3}
10 "	2.25×10^{3}	1.2×10^{5}	5×10^{3}	2×10^{5}	8.45×10^{2}	1.1×10^{5}	1.8×10^{2}	3.5×10^{4}	2×10^{1}	8×10^{2}	5	1.3×10^{3}

Appendix 3 (continued)

Sample date	Raw influent		Grit trap		Primary filter		Trough		Final filter		Final effluent	
	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*	Phage	*E. coli*
17 Jan	7.4×10^2	9.2×10^4	7.9×10^2	1.17×10^5	2.95×10^2	9.5×10^4	1.05×10^2	4.7×10^4	< 5	3.75×10^2	10	5.25×10^2
24 "	5.1×10^2	1.4×10^5	3.75×10^3	3.3×10^5	6.2×10^2	1.5×10^5	2.9×10^2	3.3×10^4	< 5	2.7×10^3	<5	2.2×10^3
31 "	5.3×10^2	1.8×10^5	8×10^2	1.8×10^5	1.51×10^3	6.7×10^4	5.5×10^1	2.8×10^4	2×10^1	2×10^3	7.5×10^1	3.9×10^3
7 Feb	ND	2.1×10^5	3.05×10^2	1.8×10^5	1.35×10^2	1.0×10^5	ND	2.9×10^4	ND	3.5×10^2	ND	7.5×10^2
14 "	1.34×10^3	1.1×10^5	1.45×10^3	1.2×10^5	3.5×10^3	6.7×10^4	8.35×10^2	7.7×10^4	1.5×10^1	8.7×10^2	7.45×10^2	9.7×10^3
21 "	1.06×10^3	1.8×10^5	6.65×10^2	1.6×10^5	3.8×10^2	1.3×10^5	9.5×10^1	3×10^4	< 5	9.2×10^2	1.5×10^1	2.8×10^3
28 "	2.75×10^2	1.7×10^5	7.9×10^2	1.8×10^5	3.85×10^2	1.2×10^5	7.5×10^1	1.2×10^5	10	3.6×10^3	10	3.3×10^3
7 Mar	2.8×10^2	1.9×10^5	1.4×10^3	1.75×10^5	7.9×10^2	9.5×10^4	2×10^1	3.3×10^4	3.5×10^1	5.3×10^4	1.8×10^2	3.3×10^1
14 "	2×10^2	2.1×10^5	7.5×10^1	1.2×10^5	5.25×10^2	7.7×10^4	1.5×10^2	3×10^4	5	2.7×10^2	4×10^1	3.2×10^2
21 "	6.3×10^2	1.4×10^5	1.3×10^3	1.8×10^5	8.3×10^2	1.2×10^5	2.5×10^2	6.5×10^4	5	1.5×10^2	10	2.7×10^2
28 "	4.5×10^2	8×10^4	1.1×10^2	1×10^5	6.1×10^2	1.3×10^5	1.2×10^3	4.7×10^4	3.5×10^1	2.2×10^3	5×10^1	1.5×10^3
4 Apr	3.6×10^3	2.1×10^5	5.1×10^3	2.4×10^5	6.5×10^2	1.2×10^5	1.5×10^2	7×10^4	10	1.1×10^3	10	2.5×10^3
11 "	2.8×10^2	2.4×10^5	1.5×10^2	1.6×10^5	1.1×10^2	9.5×10^4	8.5×10^1	5.2×10^4	10	5×10^2	5	2.2×10^2
25 "	5×10^1	1.7×10^5	1×10^2	1.6×10^5	7.5×10^2	1×10^5	7×10^1	4×10^4	2.5×10^1	6.5×10^3	2×10^1	7.5×10^3
1 May	35	1.2×10^5	60	1×10^5	1.2×10^2	5.2×10^4	20	8×10^4	< 5	1.9×10^3	10	3.5×10^3
9 "	-	6.2×10^4	-	3.2×10^4	-	4.7×10^4	-	1×10^4	-	1.6×10^3	-	2.9×10^3
16 "	1.8×10^2	1.6×10^5	1.2×10^2	7.3×10^4	3×10^2	5.5×10^4	8×10^1	4.5×10^4	9×10^1	3.7×10^3	6×10^1	2.8×10^3
22 "	9.7×10^2	1.9×10^5	7×10^3	1.9×10^5	7.3×10^2	1.8×10^5	1×10^2	2.8×10^4	1.5×10^1	8.2×10^2	2.5×10^1	2.5×10^1

*Gt Stambridge
†Rayleigh

Appendix 4a E. coli in sewage at various stages of treatment

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	7.2×10^4	2.4×10^4	1.5×10^4	2.1×10^4	2.3×10^3	2×10^3
6 Dec	ND	2×10^5	7×10^4	7.5×10^4	1.1×10^4	1.5×10^4
13 "	1.8×10^5	2.5×10^5	2×10^5	2.1×10^4	8.2×10^3	1.6×10^3
20 "	1.3×10^5	1.6×10^5	9.2×10^4	1.8×10^4	5×10^2	1.4×10^3
3 Jan	1.8×10^5	2.2×10^5	1.6×10^5	6×10^4	3.5×10^3	3.5×10^3
10 "	1.2×10^5	2×10^5	1.1×10^5	3.5×10^4	8×10^2	1.3×10^3
17 "	9.2×10^4	1.17×10^5	9.5×10^4	4.7×10^4	3.75×10^2	5.25×10^2
24 "	1.4×10^5	3.3×10^5	1.5×10^5	3.3×10^4	2.7×10^3	2.2×10^3
31 "	1.8×10^5	1.8×10^5	6.7×10^4	2.8×10^4	2×10^3	3.9×10^3
7 Feb	2.1×10^5	1.8×10^5	1×10^5	2.9×10^4	3.5×10^2	7.5×10^2
14 "	1.1×10^5	1.2×10^5	6.7×10^4	7.7×10^4	8.7×10^2	9.7×10^3
21 "	1.8×10^5	1.6×10^3	1.3×10^5	3×10^4	9.2×10^2	2.8×10^3
28 "	1.7×10^5	1.8×10^5	1.2×10^5	1.2×10^5	3.6×10^3	3.3×10^3
7 Mar	1.9×10^5	1.75×10^5	9.5×10^4	3.3×10^4	5.3×10^4	3.3×10^1
14 "	2.1×10^5	1.2×10^5	7.7×10^4	3×10^4	2.7×10^2	3.2×10^2
21 "	1.4×10^5	1.8×10^5	1.2×10^5	6.5×10^4	1.5×10^2	2.7×10^2
28 "	8×10^4	1×10^5	1.3×10^5	4.7×10^4	2.2×10^3	1.5×10^3
Range						
Low	7.2×10^4	1.6×10^3	1.5×10^4	1.8×10^4	2.7×10^2	3.3×10^1
High	2.1×10^5	3.3×10^5	2×10^5	1.2×10^5	5.3×10^4	1.5×10^4
Mean	1.49×10^5	1.61×10^5	1.06×10^5	4.52×10^4	5.45×10^3	2.95×10^3

Appendix 4b Coliphage in sewage at various stages of treatment

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	1.4×10^2	3×10^1	9.5×10^1	8×10^1	1.5×10^1	2×10^1
6 Dec	5.25×10^2	3.35×10^2	6.95×10^2	6.2×10^2	9.5×10^1	1.3×10^2
13 "	7.5×10^2	3.8×10^2	3.95×10^2	2.2×10^2	9.5×10^1	9×10^1
20 "	1.75×10^2	1.25×10^2	7.7×10^2	4.5×10^1	2×10^1	3.5×10^1
3 Jan	6.5×10^2	1.55×10^2	5.6×10^2	7.5×10^1	<2.5	5
10 "	2.25×10^3	5×10^3	8.45×10^2	1.8×10^2	2×10^1	5
17 "	7.4×10^2	7.9×10^2	2.95×10^2	1.05×10^2	<5	10
24 "	5.1×10^2	3.75×10^3	6.2×10^2	2.9×10^2	<5	<5
31 "	5.3×10^2	8×10^2	1.51×10^3	5.5×10^1	2×10^1	7.5×10^1
7 Feb	ND	3.05×10^2	1.35×10^2	ND	ND	ND
14 "	1.34×10^3	1.45×10^3	3.5×10^3	8.35×10^2	1.5×10^1	7.45×10^2
21 "	1.06×10^3	6.65×10^2	3.8×10^2	9.5×10^1	<5	1.5×10^1
28 "	2.75×10^2	7.9×10^2	3.85×10^2	7.5×10^1	10	10
7 Mar	2.8×10^2	1.4×10^3	7.9×10^2	2×10^1	3.5×10^1	1.8×10^2
14 "	2×10^2	7.5×10^1	5.25×10^2	1.5×10^2	5	4×10^1
21 "	6.3×10^2	1.3×10^3	8.3×10^2	2.5×10^2	5	10
28 "	4.5×10^2	1.1×10^2	6.1×10^2	1.2×10^3	3.5×10^1	5×10^1
Range						
Low	1.4×10^2	3×10^1	9.5×10^1	4.5×10^1	<2.5	<5.0
High	2.25×10^3	3.75×10^3	3.5×10^3	1.2×10^3	9.5×10^1	7.45×10^2
Mean	6.57×10^2	1.11×10^3	7.61×10^2	2.68×10^2	$<2.42 \times 10^1$	$<8.91 \times 10^1$

Appendix 5 Total counts in sewage: casitone/glycerol/yeast extract at 37°C

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	9 $\times10^5$	3 $\times10^6$	1 $\times10^6$	1.7 $\times10^7$	4 $\times10^5$	4.7$\times10^5$
6 Dec	1.4 $\times10^6$	2.3 $\times10^6$	1.6 $\times10^6$	1.2 $\times10^6$	1.1 $\times10^6$	1.8$\times10^6$
13 "	1.1 $\times10^6$	1.4 $\times10^6$	1.5 $\times10^6$	4.6 $\times10^6$	2.4 $\times10^6$	3.3$\times10^5$
20 "	1.7 $\times10^6$	1.6 $\times10^6$	2.9 $\times10^6$	7 $\times10^6$	7.7 $\times10^4$	9.7$\times10^4$
3 Jan	1.1 $\times10^6$	1.8 $\times10^6$	1.3 $\times10^6$	1.4 $\times10^7$	1 $\times10^5$	1.3$\times10^5$
10 "	3 $\times10^6$	1.5 $\times10^6$	2.4 $\times10^6$	4.3 $\times10^6$	3.1 $\times10^4$	5.5$\times10^4$
17 "	1.3 $\times10^6$	1.8 $\times10^6$	1.8 $\times10^6$	2.2 $\times10^6$	1.5 $\times10^4$	1.6$\times10^4$
24 "	1.1 $\times10^6$	3 $\times10^6$	1.1 $\times10^6$	2 $\times10^6$	9 $\times10^4$	5.2$\times10^4$
31 "	1.6 $\times10^6$	2 $\times10^6$	1 $\times10^6$	2.4 $\times10^6$	8 $\times10^4$	1.3$\times10^5$
7 Feb	1.3 $\times10^6$	1.5 $\times10^6$	1.6 $\times10^6$	2.5 $\times10^6$	1 $\times10^4$	1.8$\times10^4$
14 "	1.8 $\times10^6$	1.7 $\times10^6$	9.2 $\times10^5$	2.3 $\times10^6$	2.7 $\times10^4$	2.8$\times10^5$
21 "	2.7 $\times10^6$	1.7 $\times10^6$	1.1 $\times10^6$	2.1 $\times10^6$	1.1 $\times10^4$	4.5$\times10^4$
28 "	1.4 $\times10^6$	1.3 $\times10^6$	1.5 $\times10^6$	5.7 $\times10^6$	5.7 $\times10^4$	2.9$\times10^4$
7 Mar	1.1 $\times10^6$	1.9 $\times10^6$	5.7 $\times10^5$	1.8 $\times10^6$	7.7 $\times10^4$	7.7$\times10^5$
14 "	1.1 $\times10^6$	1.2 $\times10^6$	1.4 $\times10^6$	2.2 $\times10^6$	1 $\times10^4$	9.5$\times10^3$
21 "	1.3 $\times10^6$	1.5 $\times10^6$	1.9 $\times10^6$	2.3 $\times10^6$	2 $\times10^3$	5 $\times10^3$
28 Mar	1.1 $\times10^6$	1.8 $\times10^6$	1.7 $\times10^6$	7 $\times10^6$	2.6 $\times10^4$	2.1$\times10^4$
$\bar{x}$	1.47$\times10^6$	1.82$\times10^6$	1.49$\times10^6$	4.74$\times10^6$	2.65$\times10^5$	2.5$\times10^5$

Appendix 6 Total counts in sewage: nutrient agar at 37°C

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	1.6×10^6	2.2×10^6	3.3×10^6	8.7×10^6	3.5×10^5	$<2.5 \times 10^6$
6 Dec	2.3×10^6	3.6×10^6	1.6×10^6	2.1×10^6	9.2×10^5	2.2×10^6
13 "	4.1×10^6	2.4×10^6	3.5×10^6	5.7×10^6	4.6×10^6	3.5×10^5
20 "	6×10^6	2.2×10^6	3.5×10^6	4.8×10^6	8.5×10^4	9.7×10^4
3 Jan	1.5×10^6	2.9×10^6	1.7×10^6	1.1×10^7	1.2×10^5	1.5×10^5
10 "	3.8×10^6	3.1×10^6	3×10^6	5.6×10^6	3.7×10^4	1.1×10^5
17 "	2.1×10^6	2.2×10^6	2×10^6	5.6×10^6	1.6×10^4	1.8×10^4
24 "	1.8×10^6	4.7×10^6	1.9×10^6	3.5×10^6	8.2×10^4	7×10^4
31 "	2×10^6	2.9×10^6	1.5×10^6	2.5×10^6	6.5×10^4	1.7×10^5
7 Feb	1.9×10^6	1.8×10^6	2.1×10^6	3.5×10^6	1.5×10^4	2.7×10^4
14 "	2.3×10^6	2.1×10^6	2×10^6	3.7×10^6	3.7×10^4	4.8×10^5
21 "	5.4×10^6	1.7×10^6	1.6×10^6	2.6×10^6	1.2×10^4	5.3×10^4
28 "	1.7×10^6	2.3×10^6	1.8×10^6	4.6×10^6	4.3×10^4	5.2×10^4
7 Mar	1.6×10^6	3.5×10^6	2.7×10^6	1.9×10^6	1.4×10^4	1.3×10^6
14 "	1.3×10^6	1.5×10^6	1.6×10^6	2.7×10^6	1.4×10^4	1.4×10^4
21 "	1.8×10^6	2.1×10^6	2.7×10^6	2.8×10^6	3.2×10^3	1.7×10^4
28 "	4×10^6	1.6×10^6	2.1×10^6	2.2×10^6	3.1×10^4	3.3×10^4
4 Apr	2.3×10^6	2.2×10^6	4.3×10^6	3.5×10^6	1.4×10^4	1.1×10^5
11 "	3.3×10^6	2.4×10^6	2.6×10^6	3×10^6	5×10^3	5×10^3
25 "	2.1×10^6	2.8×10^6	2.4×10^6	4.8×10^6	1.1×10^5	2.1×10^5
1 May	2.2×10^6	2.7×10^6	2.1×10^6	4×10^6	4×10^4	1.4×10^5
9 "	1.2×10^6	2×10^6	8.2×10^6	2.1×10^6	5.9×10^4	1.4×10^5
16 "	3.2×10^6	2.8×10^6	2.5×10^6	3.5×10^6	1.6×10^5	1.9×10^5
22 "	3×10^6	1.5×10^6	2.1×10^6	2.9×10^6	2×10^4	2.3×10^4
$\bar{x}$	2.61×10^6	2.47×10^6	2.62×10^6	4.05×10^6	2.85×10^5	3.52×10^5

Appendix 7 Total counts in sewage: nutrient agar at 30°C

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	4×10^6	4.4×10^6	8.5×10^6	2.8×10^7	5×10^5	9×10^5
6 Dec	5.1×10^6	4.7×10^6	3.6×10^6	3.5×10^7	1.7×10^6	4.2×10^6
13 "	3.1×10^6	4.9×10^6	3.5×10^6	1.9×10^7	2.6×10^7	9.7×10^5
20 "	1.2×10^7	4.5×10^6	5.3×10^6	2.6×10^7	1.7×10^5	1.9×10^5
3 Jan	3.9×10^6	6.1×10^6	4.5×10^6	2.6×10^7	2.3×10^5	2.6×10^5
10 "	1.6×10^7	1.8×10^7	5.8×10^6	2×10^7	1.6×10^5	3.6×10^5
17 "	6.2×10^6	6.3×10^6	4.9×10^6	1.6×10^7	4.5×10^4	6.6×10^4
24 "	4×10^6	1.4×10^7	4×10^6	1.7×10^7	1.5×10^7*	2×10^5
31 "	4.9×10^6	4.7×10^6	3.4×10^6	5.1×10^6	1.7×10^5	3.6×10^5
7 Feb	4.7×10^6	4.1×10^6	4.8×10^6	5.2×10^6	1.9×10^4	1.6×10^5
14 "	4.8×10^6	4.6×10^6	3.6×10^6	7×10^6	1.2×10^5	1.1×10^6
21 "	1.8×10^7	4×10^6	3.7×10^6	5.3×10^6	2.4×10^4	1.4×10^5
28 "	4.3×10^6	4.4×10^6	2.8×10^6	1×10^7	1.3×10^5	1.6×10^5
7 Mar	3.2×10^6	1.9×10^7	3.5×10^6	3.6×10^6	3.1×10^4	-
14 "	2.4×10^6	3.7×10^6	2.4×10^6	4.7×10^6	1.6×10^4	3×10^4
21 "	3.7×10^6	4.4×10^6	3.9×10^6	4×10^6	1.2×10^4	5.2×10^3
28 "	6×10^6	4×10^6	3.4×10^6	8.2×10^6	9×10^4	7.7×10^4
4 Apr	4.4×10^6	4.6×10^6	6×10^6	1×10^7	3.8×10^4	3×10^5
11 "	8.5×10^6	5.5×10^6	7×10^6	1.2×10^7	2.3×10^4	2.4×10^4
25 "	5.5×10^6	5×10^6	3.6×10^6	1.2×10^7	2.3×10^5	3.4×10^5
1 May	4.4×10^6	4.4×10^6	3.8×10^6	1.1×10^7	1.1×10^5	2.7×10^5
9 "	3×10^6	1.9×10^6	1.3×10^7	3.1×10^6	1.4×10^5	2.2×10^5
16 "	3.5×10^6	4.7×10^6	4.3×10^6	4.9×10^6	2.5×10^5	2.1×10^5
22 "	5.6×10^6	4.9×10^6	4.4×10^6	3.1×10^6	3.2×10^4	3.3×10^4
$\bar{x}$	5.88×10^6	6.12×10^6	4.74×10^6	1.23×10^7	2.97×10^5	4.6×10^5

*not included.

Appendix 8 *Total counts in sewage: Casitone/glycerol/yeast extract agar at 30°C*

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	4.5×10^6	3.2×10^6	8×10^6	1.7×10^7	7×10^5	7×10^5
6 Dec	9.2×10^6	1×10^7	2.4×10^6	2.8×10^7	1.9×10^6	2.8×10^6
13 "	2×10^6	1.8×10^6	5.5×10^6	3.5×10^7	1.3×10^7	3×10^6
20 "	9.2×10^6	9.2×10^6	3.6×10^6	1.5×10^7	1.5×10^5	1.4×10^5
3 Jan	2.7×10^6	3.4×10^6	6×10^6	2.4×10^7	2.2×10^5	1.9×10^5
10 "	1.8×10^7	1.2×10^7	3.5×10^6	2.4×10^6	2.1×10^5	2.6×10^5
17 "	1×10^7	2.9×10^6	1.8×10^6	2.1×10^7	3.2×10^4	1×10^5
24 "	7.2×10^6	1.4×10^7	5.5×10^6	2×10^7	1.8×10^7*	1.4×10^5
31 "	3.5×10^6	3.8×10^6	3×10^6	5.6×10^6	1.6×10^5	2.8×10^5
7 Feb	3.5×10^6	3.9×10^6	3.5×10^6	1.8×10^7	1.9×10^4	1.7×10^5
14 "	3.5×10^6	3.4×10^6	2.1×10^6	3.6×10^7	1.9×10^5	1×10^6
21 "	1.3×10^7	3.7×10^6	2.7×10^6	1.1×10^7	1.5×10^4	1.2×10^5
28 "	3.2×10^6	3×10^6	2.7×10^6	9.7×10^6	9.2×10^4	1.3×10^5
7 Mar	2.4×10^6	1.5×10^7	2.6×10^6	3.4×10^6	1.6×10^4	1.8×10^6
14 "	2.4×10^6	8×10^6	2×10^6	8.7×10^6	1.6×10^4	1.9×10^4
21 "	3×10^6	6.5×10^6	2.3×10^6	4.7×10^6	6.7×10^3	1.1×10^4
28 "	3.4×10^6	3.5×10^6	5×10^6	7.7×10^6	1×10^5	8.2×10^4
$\bar{x}$	5.92×10^6	6.31×10^6	3.66×10^6	1.57×10^7	1.05×10^6	6.44×10^5

*not included.

Appendix 9 Total counts in sewage: Casitone/glycerol/yeast extract agar at 20°C

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	6.2×10^6	5.2×10^6	6.5×10^6	2.2×10^7	1.1×10^6	8.7×10^5
6 Dec	6.5×10^6	1.2×10^6	2.9×10^6	6.5×10^7	2.2×10^6	3.3×10^6
13 "	2.9×10^6	3×10^6	3×10^6	5×10^7	1.8×10^7	3.9×10^6
20 "	1.3×10^7	3.6×10^6	3.4×10^6	3.4×10^6	2.3×10^5	2.6×10^5
3 Jan	3.8×10^6	4.8×10^6	3.1×10^6	4.6×10^7	3.4×10^5	4.4×10^5
10 "	1.6×10^7	1.5×10^7	1.1×10^7	2.4×10^7	2.3×10^5	4×10^5
17 "	4.8×10^6	3.4×10^6	3.2×10^6	2.8×10^7	1.9×10^5	1.5×10^5
24 "	3.3×10^6	1.9×10^7	9×10^6	2.8×10^7	1.3×10^7*	8.7×10^5
31 "	4.4×10^6	5×10^6	3.6×10^6	6.5×10^6	2.6×10^5	3.1×10^5
7 Feb	4.4×10^6	5.4×10^6	4.6×10^6	5.1×10^6	3.5×10^4	1.6×10^5
14 "	4.8×10^6	5.4×10^6	3.6×10^6	3.5×10^7	2.8×10^5	1.6×10^6
21 "	1.9×10^7	5.2×10^6	3.9×10^6	4.5×10^6	2.7×10^4	1.4×10^5
28 "	4.1×10^6	4.6×10^6	3.6×10^6	1.3×10^7	1.3×10^5	1.2×10^5
7 Mar	3.3×10^6	1.7×10^7	2.6×10^6	3.3×10^6	2.6×10^4	-
14 "	3.4×10^6	4.6×10^6	3.2×10^6	3.6×10^6	2.7×10^4	2.6×10^4
21 "	3.9×10^6	4.6×10^6	3.2×10^6	7.5×10^6	5.5×10^3	1.6×10^4
28 "	3.3×10^6	4.2×10^6	3.4×10^6	4.4×10^6	1.1×10^5	9.7×10^4
$\bar{x}$	6.3×10^6	6.54×10^6	4.34×10^6	2.05×10^7	1.45×10^6	7.91×10^5

*not included - see experiment notes.

Appendix 10 Total counts in sewage: nutrient agar at 20°C

Date	Raw influent	Grit trap	Primary filter	Trough	Final filter	Final effluent
30 Nov	8.2×10^6	1.5×10^7	1.2×10^7	1.3×10^8	1.3×10^6	1.8×10^6
6 Dec	1.2×10^7	1.6×10^7	5.1×10^6	6×10^7	3.2×10^6	6.6×10^6
13 "	4.6×10^6	3.9×10^6	5.5×10^6	2.7×10^7	6.2×10^6	1.8×10^6
20 "	1.9×10^7	7.2×10^7	6.8×10^6	3.6×10^7	2.3×10^5	2.5×10^5
3 Jan	6.7×10^6	1.7×10^7	8.1×10^6	4×10^7	4.6×10^5	7.2×10^5
10 "	3.3×10^7	3×10^7	2.1×10^7	2.9×10^7	2.6×10^5	7.3×10^5
17 "	2×10^7	1.8×10^7	6.4×10^6	3×10^7	2.4×10^5	2.9×10^5
24 "	7.2×10^6	3.4×10^7	5.4×10^6	2.6×10^7	1.2×10^7*	3.8×10^5
31 "	7.5×10^6	9.8×10^6	5.3×10^6	7.9×10^6	2.8×10^5	5.3×10^5
7 Feb	8.8×10^6	7.5×10^6	5.4×10^6	3.3×10^6	5.1×10^4	2.6×10^5
14 "	7.6×10^6	5.4×10^6	5.1×10^6	1.4×10^7	3.4×10^5	2.6×10^6
21 "	1.9×10^7	6.3×10^6	5.3×10^6	7.2×10^6	2.2×10^5	1.5×10^5
28 "	5.1×10^6	5.8×10^6	2.7×10^6	1.6×10^7	2.5×10^5	2.2×10^5
7 Mar	7.8×10^6	1.7×10^7	5×10^6	4.2×10^6	3.6×10^4	2.7×10^6
14 "	3.7×10^6	5.1×10^6	3.1×10^6	4.9×10^6	3.2×10^4	3.3×10^4
21 "	4.5×10^6	4.4×10^6	4.1×10^6	9.7×10^6	1×10^4	2×10^4
28 "	7.7×10^6	1.2×10^7	4.5×10^6	6.4×10^6	1.6×10^5	2.4×10^5
4 Apr	5.7×10^6	6.7×10^6	5.3×10^6	1.1×10^7	4×10^4	4.2×10^5
11 "	1.9×10^7	1.3×10^7	8.7×10^6	1.5×10^7	1.4×10^5	1.4×10^5
25 "	7×10^6	2×10^7	6×10^6	1.5×10^7	3.4×10^5	4.6×10^5
1 May	9.7×10^6	5.8×10^6	4.4×10^6	1.7×10^7	1.7×10^5	2.8×10^5
9 "	3.4×10^6	-	-	-	1.9×10^5	3.1×10^5
16 "	6.1×10^6	5.7×10^6	5.1×10^6	5.2×10^6	2.7×10^5	3.4×10^5
22 "	1.4×10^7	5.1×10^6	5×10^6	3.7×10^6	4×10^4	1.3×10^5
$\bar{x}$	1.03×10^7	1.46×10^7	6.32×10^6	2.25×10^7	6.29×10^5	8.92×10^5

*24 Jan final filter not included in mean.

Appendix 12 The use of buffers in sea water to obtain a stable pH for experimental work

Estuarine sea water has a pH of 7.6 or higher and considerable buffering capacity. CARLUCCI & PRAMER (1960) in their experiments to examine the effect of pH on *E. coli* survival in sea water apparently altered the pH by the simple addition of NaOH (alkali) or HCl (acid) as required. This method of pH adjustment was tried by the present author and not unexpectedly it proved impossible to obtain stable pH values. When NaOH or HCl was added to sea water the pH returned within two days to around pH 7.6 from original values lying within the range of pH 3 and pH 10. It was hoped that this 'drifting' or instability could be overcome by the use of buffers in combination with NaOH or HCl to obtain a range of pH values. The following were set up:

pH required		Solutions added
1	25 ml 0.2 M KCl	+ 67 ml 0.2 M HCl
2	25 ml 0.2 M KCl	+ 6.5 ml 0.2 M HCl
3	50 ml 0.1 M $KHC_8H_4O_4$	+ 22.3 ml 0.1 M HCl
4	50 ml 0.1 M $KHC_8H_4O_4$	+ 0.1 ml 0.1 M HCl
5	50 ml 0.1 M $KHC_8H_4O_4$	+ 22.6 ml 0.1 M HCl
6	50 ml 0.1 M KH_2PO_4	+ 5.6 ml 0.1 M NaOH
7	50 ml 0.1 M KH_2PO_4	+ 29.1 ml 0.1 M NaOH
8	50 ml 0.025 M $Na_2B_4O_7$	+ 20.5 ml 0.1 M HCl
9	50 ml 0.025 M $Na_2B_4O_7$	+ 4.6 ml 0.1 M HCl
10	50 ml 0.05 M $NaHCO_3$	+ 10.7 ml 0.1 M NaOH

The pH of the solutions was checked and found to be correct using a pH meter. Working solutions using deionized water and sea water were then prepared, checked, left for five days and checked again. The pH solutions were found to give stable pH values over the range tested when prepared in deionized water. However, results for the pH solutions prepared in sea water varied considerably. The solutions for pH 3, 4, 5,

6 and 7 were fairly stable but after a few days began to drift towards pH 7.6. For the preparation of pH solutions at values of pH 1, 2, 8, 9 and 10 large volumes of buffer were required (as much as 50:50 buffer to sea water) and this resulted in drastic alterations to the salinity. An attempt to overcome the salinity problem by preparing the pH buffers in sea water failed as this resulted in the formation of heavy chemical precipitates.

Therefore it was concluded that the effects of pH could only be properly assessed by using non-saline waters and appropriate pH buffers and this procedure was adopted for the experiments described.

Appendix 13 Experiments to determine methods for use in investigations into effects of sunlight and temperature

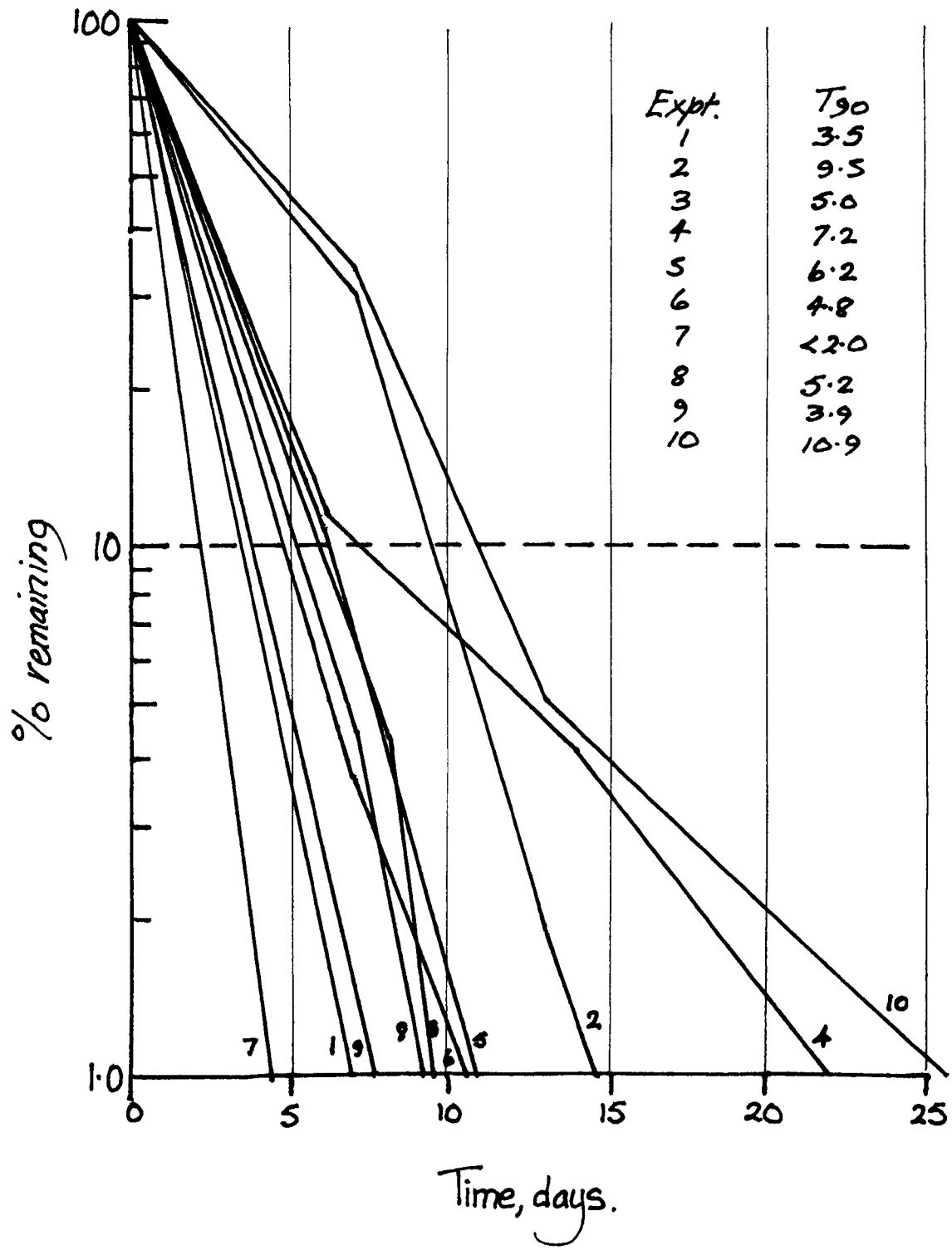
No solarimeter to measure solar radiation could be obtained and it was decided to base all measurements on ultra-violet and infra-red using a Blak-Ray J-225 ultra-violet meter. By using a contrast filter u/v can be absorbed and readings of infra-red (IR) taken. The object of these experiments was to find suitable container and covering materials for mortality experiments.

	u/v + IR	IR	u/v
Direct sunlight	46 (100%)	43 (93.5%)	3 (6.5%)
Perspex (3 mm thick)	40 (86.9)	38 (95)	2 (5)
Plate glass (5 mm thick)	32 (69.5)	29 (90.6)	3 (9.4)
Pyrex glass-beaker	35 (76.0)	30 (85.7)	5 (14.3)
Polythene 500 gauge	42 (91.3)	40 (95.3)	2 (4.7)
Polythene 150 gauge	40 (87.0)	38 (95.0)	2 (5.0)

As expected, only a small percentage of the measured radiation was u/v and this percentage was similar for all materials examined except Pyrex glass which has a high quartz content and therefore absorbs less u/v. The heavy gauge polythene allowed transmission of the most total radiation (91%) and this was adopted as a covering material for tank experiments operated outside.

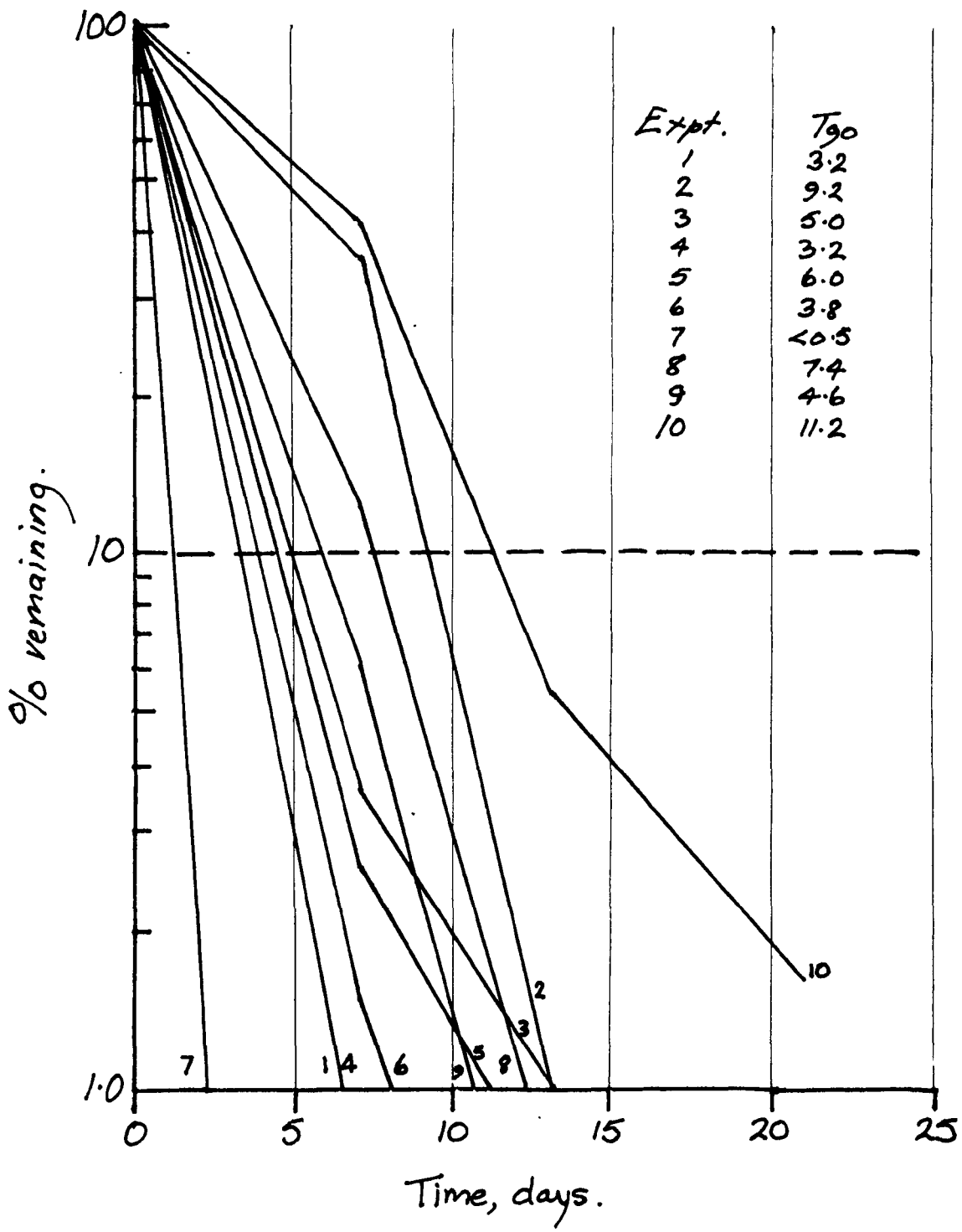
Appendix 14.

Short term mortality; natural seawater + MS2.



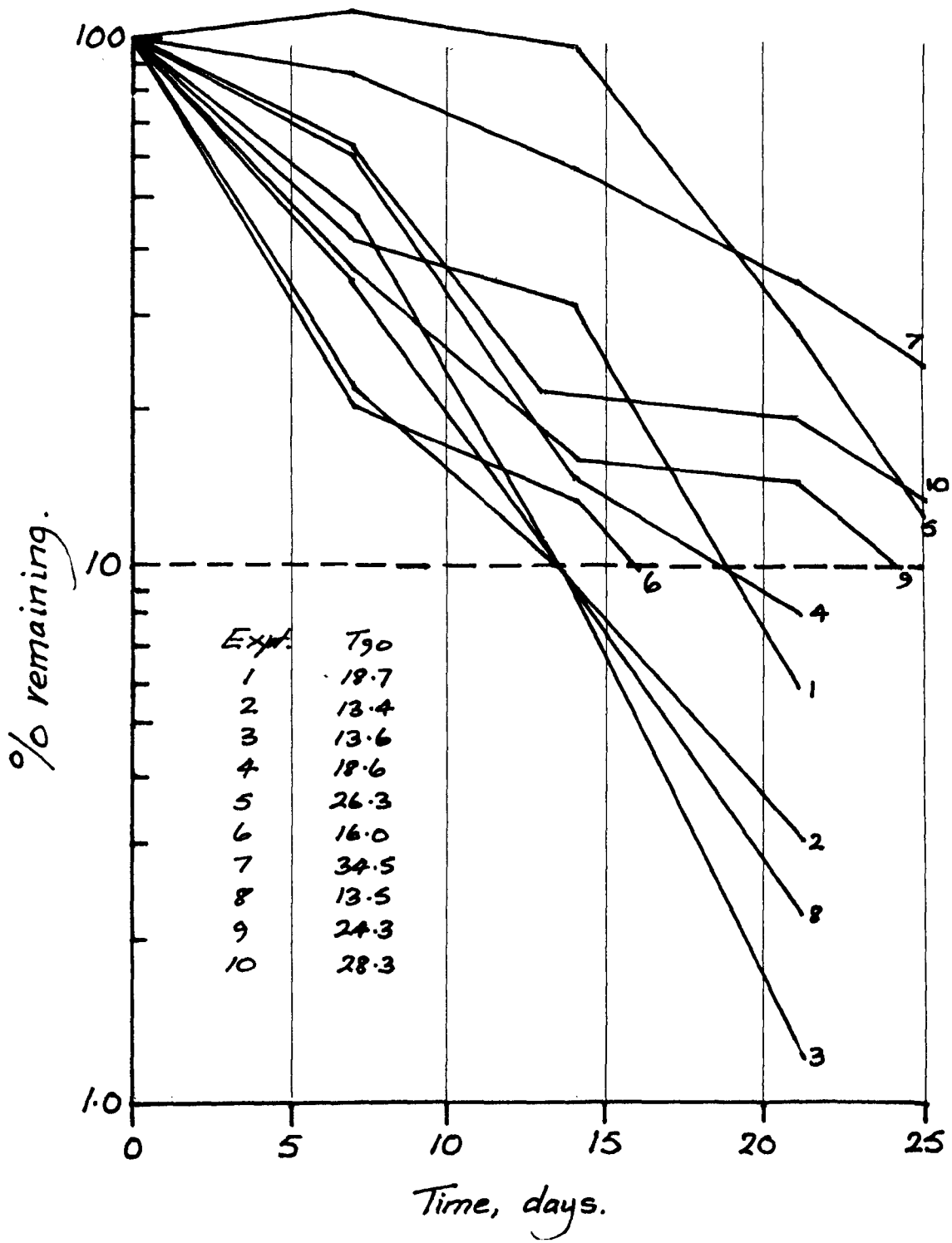
Appendix 14.

Short term mortality; natural sw + MS2 + E.coli.



Appendix 14.

Short term mortality; autoclaved seawater + MS2.



Appendix 14.

Short term mortality; autoclaved sw + MS2 + E.coli.