MARINE POLLUTION
PATHOGENIC MICRO-ORGANISMS
IN SHELLFISH

Report to the

WATER RESEARCH COMMISSION

by

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FRD-WRC Joint Venture Project 1990-1994
Marine Pollution: Pathogenic Micro-organisms in Shellfish

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EXECUTIVE SUMMARY

The project was initiated because infectious diseases are the most important concern about the quality of shellfish intended for human consumption. Various developments in South Africa increase the risk of marine pollution and raise concern about the safety of natural and mariculture shellfish supplies. The fundamental objective of the project was, therefore, to gather information, formulate guidelines, and establish expertise in support of local and national endeavours aimed at sustainable utilisation of valuable marine food resources.

Literature on infections associated with the consumption of sewage contaminated shellfish in many parts of the world has been reviewed. As a result of the risk of infection, the shellfish market is extremely sensitive and any indication of contamination may be considered sufficient to reject supplies or close down industries with devastating economic implications. South Africa has a substantial market for shellfish, both in terms of a commercial industry, and private collection by the general public. The literature also revealed that specifications and strategies to control the safety of shellfish in South Africa are in need of revision and updating, particularly with regard to viruses which are responsible for the great majority of infections transmitted by shellfish.

Against this background methods for the recovery of selected viruses, faecal bacteria and phages from shellfish meat have been evaluated and optimised. A practical procedure with high efficiency for the recovery of viruses and indicators has been established. The procedure is based on homogenisation of shellfish meat in a pH 8,5 glycine-saline buffer followed by direct analysis of the homogenate for bacteria and phages, and centrifugation for analysis of the supernatant for viruses using cell culture propagation or molecular techniques. Evaluation in tests on shellfish collected at selected sites along the coast confirmed that the procedure was suitable for research on viruses in shellfish and related marine environments, as well as routine monitoring of the quality of shellfish supplies.

The new procedures recommended for microbiological analysis of shellfish were applied in tests on representative samples of commercial supplies on the local seafood market. The results revealed that sometimes shellfish exceeded quality limits recommended for faecal bacteria and phages. These findings indicate a potential health risk, and call for more detailed investigation of the quality of local shellfish supplies. The results may warrant revision of quality specifications and quality control strategies.

The depuration of selected faecal bacteria, human viruses and phages by seeded oysters has been investigated by using tanks of seawater under controlled laboratory conditions. Viruses and phages were still detected after five days in the oysters, while faecal bacteria were not detected for longer than two days. These findings imply that commonly used depuration procedures based on the retention of shellfish for one or two days in clean water and tests for faecal bacteria to monitor depuration are not reliable. The results show that somatic and F-RNA (male-specific) coliphages are more reliable indicators of viral depuration than faecal bacteria such as coliforms

and streptococci. In research on reasons for the differential release of micro-organisms by shellfish, valuable technology and expertise has been established, but it was not possible to find conclusive evidence about the possible penetration of shellfish tissue by viruses and phages.

Studies on sources of faecal pollution were carried out at a major mariculture site in Saldanha Bay. Indications of faecal contamination were detected in some samples of mariculture mussels. The results suggest that wastewater discharged into the Bay was a potential source of faecal contamination. However, it would also seem that seabird droppings may account for at least some of the faecal organisms isolated from mussels. Droppings from various seabird species on the mussel rafts contained high counts of faecal coliforms, faecal streptococci, somatic coliphages and F-RNA coliphages, but obviously no human viruses. This implies that identification of the source of faecal indicators may prove important in quality control at mariculture sites and natural harvesting grounds. Methods for distinguishing between faecal pollution of human and seabird origin have, therefore, been investigated. Evidence has been presented that *Bacteroides fragilis* HSP40 phages which are highly specific for human excreta could be used. Indications are that sorbitol fermenting bifidobacteria and certain types of F-RNA coliphages which are specific for human excreta could also be included in a battery of indicators for distinguishing between faecal pollution of human and animal origin.

Despite numerous anecdotal reports on gastroenteritis associated with the consumption of shellfish harvested from natural beds, efforts to find cases for epidemiological confirmation and identification of the pathogens involved were not successful. In one outbreak of gastroenteritis at Grootbrak, small round structured gastroenteritis viruses (SRSVs) were detected in some of 18 patient stools, but epidemiological data eventually suggested that a supply of contaminated curried fish sold by a street vendor was the most likely source of food poisoning. Stool specimens from a number of additional gastroenteritis patients were obtained, but in no case was it possible to establish epidemiologically meaningful association with the consumption of shellfish. The study was successful in so far as that it offered an opportunity to develop technology for the first molecular detection of SRSVs in gastroenteritis stools in South Africa. The infrastructure for early detection of infections related to the consumption of shellfish, the recording of epidemiological details, and collection of patient specimens, would have to be improved for meaningful studies on infections associated with the consumption of shellfish.

Results obtained in this study and recommendations in the international literature were used to formulate a protocol for the microbiological analysis of shellfish, and to recommended limits for the microbiological quality of shellfish intended for human consumption. The methods are practical, simple, reliable and inexpensive, and can be carried out by microbiological laboratories with basic facilities and expertise. The recommended limits are realistic and within reach of most commercial mariculture shellfish suppliers, as well as naturally occurring shellfish in unpolluted marine environments.

Information generated by this project was included in the preparation of the national documents entitled *Water Quality Guidelines for the South African Coastal Marine Environment*, Volume 2: *Recreational Use*, and Volume 3: *Mariculture*, which are due to be published by the Department of Water Affairs and Forestry shortly. Needs for further research and technology development have been identified. These include attractive possibilities for the molecular detection of viruses in shellfish which are not detectable by conventional methods, and improvement of quality monitoring protocols. The project made a valuable contribution to the development of technology and expertise, as well as capacity building, in a field of national importance.

RESEARCH OUTPUTS

1. Publications

Grabow W O K (1991) Human viruses in water. Water, Sewage & Effluent 11, 16-21.

Grabow W O K, Puttergill D L and Bosch A (1992) Propagation of adeno-virus types 40 and 41 in the PLC/PRF/5 primary liver carcinoma cell line. Journal of Virological Methods 37, 201-208.

Grabow W O K, De Villiers J C and Schildhauer C (1992) Comparison of selected methods for the enumeration of fecal coliforms and *Escherichia coli* in shellfish. Applied and Environmental Microbiology 58, 3203-3204.

Grabow W O K, Puttergill D L and Bosch A (1993) Plaque assay for adenovirus type 41 using the PLC/PRF/5 liver cell line. Water Science and Technology 27, 321-327.

Grabow W O K (1993) Pathogenic organisms and indicators of faecal pollution. Proceedings: International Seminar on the Disinfection of Water and Wastewater in Developing Countries, Belo Horizonte, Brazil, 26-28 April. S471a Anais: Associacao Brasileira de Engenharia Sanitaria e Ambiental da Escola de Engenharia da UFMG, Belo Horizonte, Brasil. pp 17-31.

Taylor M B, Schildhauer C I, Parker S, Grabow W O K, Jiang X, Estes M K and Cubitt W D (1993) Two successive outbreaks of SRSV associated gastroenteritis in South Africa. Journal of Medical Virology 41, 18-23.

Grabow W O K (1993) Feature article: On the trail of the elusive virus. Water Quality International 1993/4, 22-24.

Grabow W O K, Neubrech T E, Holtzhausen C S and Jofre J (1995) *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Water Science and Technology 31, 223-230.

Jagals P, Grabow W O K and De Villiers J C (1995) Evaluation of indicators for assessment of human and animal faecal pollution of surface run-off. Water Science and Technology 31, 235-241.

Marx F E, Taylor M B and Grabow W O K (1995) Optimization of a PCR method for the detection of astrovirus type 1 in environmental samples. Water Science and Technology 31, 359-362.

Wolfaardt M, Moe C L and Grabow W O K (1995) Detection of small round structured viruses in clinical and environmental samples by polymerase chain reaction. Water Science and Technology 31, 375-382.

Wolfaardt M, Taylor M B, Grabow W O K, Cubitt W D and Xi J (1995) Molecular characterisation of small round structured viruses associated with gastroenteritis in South Africa. Journal of Medical Virology (in press).

Grabow W O K, Taylor M B and Webber L M (1995) Hepatitis E virus in South Africa (submitted).

Grabow W O K (1995) Member of team working on: Water Quality Guidelines for the South African Coastal Marine Environment. Volume 2: Recreational Use. Volume 3: Mariculture. Department of Water Affairs and Forestry, Pretoria.

2. Conferences (Domestic)

Grabow W O K (1990) Health risks associated with shellfish. Oral paper: Oceans '90, 7th National Oceanographic Conference, San Lameer, Natal, 25-29 June.

Grabow W O K (1991) Virological dangers of sewage effluents. Public Health Seminar, Southern Cape Public Advisory Board, Civic Centre, George, 19 June.

Puttergill D L and Grabow W O K (1991) Propagation of adenovirus types 40 and 41 in the PLC/PRF/5 human liver cell line. 31st Annual Congress of the Federation of South African Societies of Pathology, Warmbaths Overvaal, 1-3 July.

Van der Veen A, De Villiers J C, Molefe A M and Grabow W O K (1991) The microbiological quality of commercially marketed shellfish. Poster: 31st Annual Congress of the Federation of South African Societies of Pathology, Warmbaths Overvaal, 1-3 July.

Grabow W O K (1991) Human viruses in Water. Monthly Meeting, Water Institute of Southern Africa, Johannesburg, 17 October.

Holtzhausen C S, Jofre J and Grabow W O K (1992) *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Seventh Biennial Congress, South African Society for Microbiology, University of the Orange Free State, Bloemfontein, 30 March to 1 April.

Van der Veen A and Grabow W O K (1992) An evaluation of methods for the isolation of viruses and bacteriophages from shellfish. Poster: Faculty Day, Faculty of Medicine, University of Pretoria, 22 July.

Grabow W O K, Favorov M O, Taylor M B and Fields H A (1993) Seroprevalence of hepatitis E in 782 selected South African individuals. Oral paper: Berg-en-Dal International Virology Congress, 22-25 August.

Wolfaardt M and Grabow W O K (1993) The detection of enteroviruses by means of PCR and gene probe techniques. Oral paper: Berg-en-Dal International Virology Congress, 22-25 August.

Van der Veen A and Grabow W O K (1993) Accumulation of human viruses by oysters. Oral paper: Southern African Marine Science Symposium on Marine Science for a Sustainable Future, Club Mykonos, Saldanha Bay, 17-22 October.

Botma K L and Grabow W O K (1995) Research on the modification of cell culture sensitivity for the detection of enteric viruses. Oral paper: International Congress on the Impact of Viral Infections in the Developing World, Johannesburg, South Africa, 9-14 July.

Grabow W O K, De Villiers J C, Erasmus B, Erasmus D and Engelbrecht L (1995) Isolation and typing of cytopathogenic viruses in wastewater effluents from an informal settlement. Oral paper: International Congress on the Impact of Viral Infections in the Developing World, Johannesburg, South Africa, 9-14 July.

Marx F E, Taylor M B, Wolfaardt M and Grabow W O K (1995) A comparison of two primer pairs for the PCR detection of astrovirus type 1 in environmental samples. Oral paper: International Congress on the Impact of Viral Infections in the Developing World, Johannesburg, South Africa, 9-14 July.

Potgieter N and Grabow W O K (1995) Comparison of seven cell culture types for the isolation of enteric viruses. Poster: International Congress on the Impact of Viral Infections in the Developing World, Johannesburg, South Africa, 9-14 July.

Wolfaardt M, Taylor M B, Grabow W O K, Cubitt W D and Jiang X (1995) Molecular characterisation of small round structured viruses associated with gastroenteritis in South Africa. Oral paper: International Congress on the Impact of Viral Infections in the Developing World, Johannesburg, South Africa, 9-14 July.

Grabow W O K (1995) Waterborne transmission of hepatitis E. 7th International Symposium on Microbial Ecology, Santos, Brazil, 27 Aug - 1 Sept 1995.

3. Conferences (Abroad)

Idema G K, Bateman B W, Kfir R and Grabow W O K (1990) A comparison of methods for the isolation of a wide range of viruses from shellfish. Verbal paper: International Symposium on Health-Related Water Microbiology, Tübingen, West Germany, 1-6 April.

Grabow W O K, De Villiers J C and Prinsloo N (1990) An assessment of methods for the microbiological analysis of shellfish. Poster: International Symposium on Health-Related Water Microbiology, Tübingen, West Germany, 1-6 April.

Grabow W O K, Puttergill D L and Bosch A (1992) Plaque assay for adenovirus type 41 using the PLC/PRF/5 liver cell line. International Symposium on Health-Related Water Microbiology, Washington Convention Centre, Washington DC, USA, 24-30 May.

Taylor M B, Cubitt W D, Jiang X, Estes M K and Grabow W O K (1993) Gastroenteritis outbreaks in South Africa: SRSVs implicated. Poster: International Congress of Virology, Glasgow, Scotland, 8-13 August.

Grabow W O K, Neubrech T E, Holtzhausen C S and Jofre J (1994) *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Oral paper: International Symposium on Health-Related Water Microbiology, Budapest, Hungary, 25-30 July.

Jagals P, Grabow W O K and De Villiers J C (1994) Evaluation of indicators for assessment of human and animal faecal pollution of surface run-off. Oral paper: International Symposium on Health-Related Water Microbiology, Budapest, Hungary, 25-30 July.

Wolfaardt M, Moe C L, Grabow W O K and Marx F (1994) Detection of small round structured viruses by enzymatic amplification. Oral paper: International Symposium on Health-Related Water Microbiology, Budapest, Hungary, 25-30 July.

Marx F E, Taylor M B and Grabow W O K (1994) Optimization of a PCR method for the detection of astrovirus type 1 in environmental samples. Poster: International Symposium on Health-Related Water Microbiology, Budapest, Hungary, 25-30 July.

4. Visits to laboratories abroad

Visits to various universities and research establishments included a sabbatical visit of 3 months in 1994 by W O K Grabow to the Institute for Hygiene, University of Tübingen, Germany, for joint research on the recovery and detection of enteroviruses.

5. Visitors from abroad

The following visited the Department for research and training:
Prof A Bosch, University of Barcelona, Spain
Prof J Jofre, University of Barcelona, Spain
Dr W David Cubitt, Institute of Child Health, London, UK
Dr Christine L Moe, Centers for Disease Control, Atlanta, USA
Prof Xi and Dr Winnie Jiang, Eastern Virginia Medical School, Norfolk, Virginia, USA
Prof Mark D Sobsey, University of North Carolina, Chapel Hill, USA

6. Post-graduate students

6.1. Completed

Botma, Miss KL	BSc(Hons)
Du Plessis, Miss HS	BSc(Hons)
Neubrech, Miss T	BSc(Hons)
Potgieter, Miss N	BSc(Hons)
Urquhart, Miss L	BSc(Hons)
Holtzhausen, Miss CS	MSc

6.2. Submitted

Potgieter, Miss N	MSc
Van der Veen, Miss A	MSc

6.3. Registered

Botma, Miss KL	MSc
Marx, Mr FE	PhD
Wolfaardt, Mrs M	PhD

1. GENERAL INTRODUCTION AND OBJECTIVES

The project was initiated in view of reports from many parts of the world on infections associated with the consumption of contaminated shellfish. Although the mortality of most of these infections is low, they have far-reaching public health and socio-economic implications, affecting particularly the seafood, holiday and tourist industries.

Infectious pathogens represent the single most important concern about the quality of shellfish intended for human consumption. Examples of infections include an outbreak in 1988 in Shanghai involving some 300 000 cases of hepatitis A and 25 000 cases of viral gastroenteritis caused by the consumption of clams harvested from a sewage polluted bay. The public health impact of these infections is aggravated by secondary infections typical of enteric viral diseases. Reasons for the frequent transmission of viruses by shellfish are poorly understood, as are reasons for the failure of commonly used faecal bacteria such as coliforms, to indicate the virological safety of shellfish. Lack of information is largely because the majority of viruses concerned are not detectable by conventional techniques.

Health risks imply that the entire success of harvesting, cultivating, and marketing shellfish depends entirely upon quality in terms of infectious diseases as well as hygienic and aesthetic considerations related to pollution. Shellfish industries have been closed down with disastrous financial implications for as little as indications of products not conforming to the stringent international quality specifications imposed on shellfish intended for human consumption. According to unconfirmed reports, South Africa already has problems with exporting shellfish to at least some international markets due to a reputation of not being able to meet hygienic quality requirements.

Various developments in South Africa raise reason for concern about marine pollution. These include massive population growth country-wide, and particularly in many coastal regions. This situation is aggravated by virtually uncontrolled urbanisation, including squatter situations, which heavily precipitate in and around major seaports. These developments, together with growing agricultural and industrial activities along the coast, result in escalating loads of wastewater

being discharged to the sea. The large volumes of stormwater run-off from densely populated low-income settlements with poor hygiene and sanitation is of particular concern. Nationwide concerns about the health and aesthetic impact of marine pollution is reflected by regular outcries in public news media and increasing pressures exerted by environmental protection campaigns.

The fundamental objective of the project was to gather information required for local and national endeavours aimed at sustainable utilisation, control and protection of valuable marine resources. This includes the seafood industry as well as the general public who use the marine environment for recreation and as a source of food. Basic goals of the project were:

1.1. Assess and quantify the health risk associated with exposure to polluted marine environments, through the consumption of shellfish.

1.2. Determine sources of human faecal pollution in seawater and in shellfish, as well as factors affecting their variability.

1.3. Determine factors affecting the survival of selected indicator organisms and pathogens.

1.4. Conduct further research into the development of technology for the evaluation of selected indicator organisms and pathogens for health risk assessment.

1.5. Develop appropriate quality criteria for South Africa.

2. LITERATURE REVIEW

2.1. ENTERIC VIRUSES

2.1.1. Classification and general properties

The group of viruses generally referred to as human enteric viruses includes more than 140 different types of viruses (Gerba, 1988; Christensen, 1989; Madeley, 1989; West, 1991). Enteric viruses are grouped together because all of them primarily infect and replicate in the epithelial cells of the gastrointestinal tract and are excreted in stool (Clarke and Chang, 1959; Madeley, 1979; Melnick and Gerba, 1982; Ramia, 1985; West, 1991). Enteric viruses are excreted in numbers as high as 10^{11} particles/g of faeces in the case of adenoviruses and rotaviruses (Smith and Gerba, 1982; Christensen, 1989).

The group comprises viruses which differ widely with regard to structure, morphology and composition. For instance, it includes single- and double-stranded RNA viruses, double-stranded DNA viruses and apparently also single-stranded DNA viruses (White and Fenner, 1986; Madeley, 1989). One common feature, however, is that the great majority are without membranes, and most of them are exceptionally resistant to unfavourable environmental conditions. This is necessary to pass through the hostile environment of the stomach for infecting the host cells in the small gut (White and Fenner, 1986; Hall, 1989). Viruses with membranes and less resistant viruses are inactivated by the acidic and proteolytic activities in the stomach. Exceptional resistance is also required for successful transmission by the faecal-oral route (Madeley, 1989).

The present classification of known enteric viruses is as follows:

A. Gastroenteritis viruses

1. Reoviridae

1.1. Reovirus

1.2. Rotavirus

2. Adenoviridae

   Enteric adenoviruses types 40, 41, 32

3. "Small Round Structured Viruses" (SRSVs)

3.1. Caliciviridae

3.1.1. Norwalk = SRSV UK2 = Christmas (SA)

3.1.2. Snow Mountain = SRSV 4

3.1.3. Hawaii (USA) = Barnet (UK) = SRSV UK3 = Congress (SA)

3.1.4. Taunton

3.2. Astroviridae

3.2.1. Astrovirus

3.2.2. Marin County

3.3. Unclassified SRSVs

3.3.1. With names eg Montgomery County

3.3.2. Without names

4. "Small Round Viruses" (SRVs)

4.1. Parvo-like eg Wollan

4.2. Entero-like eg Cockle

4.3. SRVs without names

5. Enteroviridae

5.1. Coxsackievirus types A and B

6. Coronaviridae

7. Toroviridae eg Breda

8. Picobirnaviridae

B. Hepatitis viruses

1. Enteroviridae

1.1. Hepatitis A virus

2. Caliciviridae

2.1. Hepatitis E virus

Further details on enteric viruses, the diseases they cause and their mode of transmission have been described (Rao *et al*, 1986; Gerba, 1988; Christensen, 1991; IAWPRC Study Group, 1991; West, 1991; Hedberg and Osterholm, 1993; Van der Veen, 1995).

Although enteric viruses are primarily associated with faecal-oral transmission, evidence is accumulating that respiratory transfer may play a more important role than previously thought.

This is because many enteric viruses undergo primary replication in the epithelial cells of the oropharynx, which forms part of the gastrointestinal tract. These viruses are, therefore, detectable in saliva and sputum, which is well known for enteric viruses such as polio and rota (Fenner and White, 1986). The presence of enteric viruses in saliva and sputum implies that transfer by inhalation, ingestion or other contact with saliva, sputum or vomit of infected persons can be expected. Evidence for the airborne transmission of rota, Norwalk and viruses related to Snow Mountain agent have now been presented (Tyrrell and Kapikian, 1982; Sawyer *et al*, 1988; Ho *et al*, 1989; Zheng *et al*; 1991).

An important feature of enteric viruses is that many of them are not readily detectable by conventional cell culture propagation. Research on these viruses is, therefore, limited to tests in which special techniques are used. These tests include electron microscopy (EM) which has played a major role in research on enteric viruses. However EM is expensive, cumbersome and has low sensitivity (Christensen, 1989). Immunological tests have been developed for rota, enteric adeno and hepatitis A viruses, but these tests for viral antigens have low sensitivity (Metcalf *et al*, 1988). Experiments with human volunteers have been used in research on viruses such as Norwalk (Greenberg *et al*, 1981). Alternative possibilities for research on fastidious enteric viruses have recently been introduced by molecular techniques based on the detection of viral nucleic acid (Metcalf *et al*, 1988). Gene probe and polymerase chain reaction procedures are currently being developed for the sensitive and accurate detection of the nucleic acid of many enteric viruses, including Norwalk and hepatitis E, for which practical techniques were not previously available (Christensen, 1989; Cherfas, 1990; West, 1991; Goswami *et al*, 1993).

2.1.2. Public health significance

A comprehensive literature survey showed that increasing attention is being given to the contamination of water and food by human enteric viruses. Since the turn of the century it has well been recognized that there is a significant risk of contracting infectious disease from the ingestion of sewage-contaminated water and food. The widespread application of bacterial indicators of faecal pollution and modern disinfection treatment processes for water and food have served a most valuable purpose in controlling the spread of many enteric disease (Grabow, 1987a). Enteric viruses, however, have been shown to be less effectively removed or inactivated

than bacteria by many treatment processes, especially by disinfection (Sproul, 1976; Bitton, 1980; Feachem *et al,* 1982; Melnick and Gerba, 1982; Wanke and Geurrant, 1987).

In terms of general incidence of various types of infections, enteric infections rank second only to respiratory infections (Kapikian *et al,* 1980). Worldwide, acute gastroenteritis and its associated dehydration afflicts almost 500 million children annually (Tolia and Dubois, 1985). In underdeveloped and developing nations acute gastroenteritis, is the leading cause of death in children under the age of 4 years (Tolia and Dubois, 1985; Grabow, 1987a). Viruses play a major role in the aetiology of these infections. For instance, rotaviruses are the most common known cause of viral gastroenteritis in infants and young children, with the fastidious enteric adenoviruses often the second most common (Uhnoo and Svensson, 1986). Rotaviruses are also the most important single cause of infantile death in the world, with an estimated more than 5 million deaths mainly in developing countries (Tolia and Dubois, 1985). In the case of enteroviruses such as polio, coxsackie and hepatitis A viruses, the great majority of infections in children are asymptomatic. The number of infections which result in clinical disease, increases with increasing age (Jawetz *et al,* 1991).

There is a wealth of literature on the occurrence of enteric viruses in water and food. In 1954 a sewage-contaminated drinking water supply in Delhi, India, caused an outbreak of hepatitis E with an estimated 40 000 cases (Wong *et al,* 1980). Outbreaks of viral infections associated with the consumption of shellfish include an outbreak caused by Norwalk viruses in 1978 in Australia, with infections in some 2 000 people (Murphy *et al,* 1979; Grohmann *et al,* 1980). In 1988 the consumption of contaminated hairy clams in Shanghai, China, resulted in a recorded outbreak of hepatitis A with 293 000 cases and 32 deaths (Halliday *et al,* 1991).

Data on mollusc-borne disease outbreaks recorded for ten-year intervals by the UK Communicable Disease Surveillance Centre, confirm the predominant role of viruses, and reveal an increasing incidence during the last two decades (Sockett *et al,* 1986). Since 1983 the recorded incidence of these disease outbreaks has continued to escalate (Pain, 1986; Sockett *et al,* 1986). Similar trends are being reported from other parts of the world (Gerba, 1988). The increasing incidence of recorded outbreaks is ascribed to increasing marine pollution, growing demand for seafoods, and improving epidemiological surveillance techniques (Grabow, 1987a; 1987b).

2.1.3. Survival in the environment

Although enteric viruses would not appear to survive as long in marine water as in freshwater, they are still capable of prolonged survival in this environment. In laboratory studies, enteric viruses have been reported to survive from 2 to 130 days in seawater, and they generally survive longer than coliform bacteria in these environments (Melnick and Gerba, 1980; Gerba, 1988; Grabow, 1989). Bosch and Shields (1987) suggest that hepatitis A viruses may survive longer in seawater than other enteric viruses. Shellfish afford better opportunity than most foods for contraction of hepatitis A because in some instances contamination is continuous or recurrent, so newly collected samples may be representative of those eaten a month earlier (Cliver, 1991).

Vaughn and Metcalf (1975) and Kott *et al* (1978) showed that the survival of poliovirus and coxsackie B-3 was shorter than that of coliphages. When f2 coliphage and polio 1 were kept together in different environments, the poliovirus was no longer detectable after 100 days while phage f2 survived even after 300 days (Kott *et al,* 1978). Numbers of coxsackie B-3 viruses in estuarine waters during the fall-winter period were four times those of the spring-summer period (Vaughn and Metcalf, 1975). According to Joyce and Wiesner (1967), coliphage T2 did not survive as long as enteroviruses in farm pond water. The coliphage never survived longer than 14 days when the host was present but poliovirus was viable after 84 days at 20°-25°C and after 90 days at 4°C, and the coxsackievirus and echovirus were viable after 13 weeks at both temperatures. This is probably because the vaccine strain of poliovirus is shed in large numbers by vaccinated persons and is relatively stable to environmental stress (Bitton, 1980). The peak incidence of the shedding of rotavirus in the United Kingdom (UK) occurs between December and March (Sellwood, 1992). Coxsackievirus B and echo serotypes circulate in the UK population in largest numbers during the late summer and autumn (Sellwood, 1992). Goyal *et al* (1984) were able to isolate enteroviruses from sediment and blue crabs at a deepsea sludge dumpsite off the Philadelphia coast, 18 months after sludge disposal had stopped. Calculations based on survival curves indicate that viable viruses would be present at the site for several years (Gerba, 1988).

The survival of pathogens in the environment is subject to a large number of variables, including the following (Kapuscinski and Mitchell, 1980; Gerba, 1988; Grabow, 1989; Garcia-Lara *et al,* 1991):

Temperature: Pathogens survive longer at lower temperatures.

Seawater conditions: Most of the pathogens concerned do not survive as long in seawater as in fresh water.

Osmotic shock: Cytoplasmic membranes may rupture upon sudden exposure to seawater. Viruses, which do not have a cytoplasmic membrane, are affected to a lesser extent than bacteria.

Microbial antagonism: Certain marine organisms produce compounds which inactivate pathogens, including enteric viruses.

Sunlight: Ultraviolet light inactivates micro-organisms. Survival is, therefore, longer in deeper waters or in bottom sediments than at the surface.

Adsorption to solids and sediments: Adsorption to solids protects pathogens against inactivation. Counts of viruses have been found to be 10 to 100 000 times higher in marine sediments than in overlaying waters.

Ingestion by molluscs: Micro-organisms survive longer in the gastrointestinal tract and tissues of molluscs than in seawater environments.

Type of micro-organism: For reasons such as differences in structure and composition, the survival of various micro-organisms differs considerably. Viruses are generally more resistant than vegetative bacteria because of their simple structure.

Dilution: When sewage is discharged into the sea, numbers of pathogens may be reduced to levels below the minimum infectious dose. However, shellfish may accumulate low numbers of pathogens from polluted water.

Survival in seafoods: Pathogenic micro-organisms can survive for extended periods of time in raw or processed seafoods. Seafood may become contaminated during handling, transporting or after processing. Under favourable conditions certain bacteria such as *Bacillus* species,

certain salmonellae and coagulase-positive staphylococci may multiply in raw or processed seafoods. Human viruses cannot multiply in the marine environment or in shellfish.

In view of factors such as the following it is virtually impossible to predict the incidence and survival of viruses in wastes, water, food and other environments contaminated by human excreta:

1. Wide variety and large number of factors which affect the survival of viruses in the environment.
2. Seasonal variation in numbers of viruses in sewage.
3. Epidemiology of viral infections. Viruses are excreted only by infected individuals, this implies that during outbreaks of viral diseases viruses are present in sewage in exceptionally large numbers.

2.2. SHELLFISH

2.2.1. Classification and general properties

The collective term "shellfish" comprises both crustacean and molluscan species. Commercially important crustacea include lobsters, crabs, shrimps, prawns and crawfish. Molluscs are shellfish that have shells and are divided into two groups: gastropods such as whelks and winkles which have a whorled shell and bivalves of the class Pelecypoda, with two hinged shells such as cockles, oysters and mussels (Gerba and Goyal, 1978). Commercially important bivalves are (Table 2.1): oysters (*Ostrea edulis, Crassostrea gigas*), mussels (*Mytilus edulis, Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), and hard shell clams (*Mya mercenaria, Mercenaria mercenaria*) (Sockett *et al*, 1985; Pain, 1986; West, 1989a). The following molluscan shellfish are of commercial importance in South Africa: mussels (*Mytilus galloprovincialis, Mytilus edulis, Choromytilus meridionalis, Donax serra, Perna perna*), oysters (*Ostrea edulis, Crassostrea gigas, Striostrea margaritacea*) and clams (*Tapes philippinarum*) (Safriel and Bruton, 1984; Brown, 1987; van Erkom Schurink and Griffiths, 1990; Hecht and Britz, 1992). All these molluscan shellfish are harvested from estuarine and nearshore coastal waters where exposure to sewage contamination is likely (Pain, 1986; Brown, 1987; Grabow, 1989; West, 1989a).

Table 2.1: Main species of shellfish currently used in mariculture

Species	1991 South Africa Production	1987 World Production
Oysters:		
1. *Crassostrea gigas*	650*	997 155
Mussels:		
2. *Perna perna*	negligible	1 029 354
3. *Choromytilus meridionalis*	710	
4. *Mytilus galloprovincialis*	1 314	
Clams:		
5. *Tapes philippinarum*	15	1 213
Threshold species		
Abalone:		
1. *Haliotis (middae)*	-	-

Abstracted from Hecht and Britz (1992). All production figures in metric tonnes.

* Representing a total of 7,7 million oysters; - No data available

Bivalve molluscs such as mussels, oysters and clams obtain their food by filter feeding which permits them to selectively ingest small particles of organic matter sieved from large volumes of seawater (Fig. 2.1) (Jorgensen, 1966; Fleet, 1978). The potential food particles, swept onto gill structure surfaces are collected, sorted and transported with the help of mucus and ciliary action to the mouth region. The mucus sheathes or strands are secreted continuously by the shellfish as they pump water (Jorgensen, 1966; Di Girolamo *et al,* 1977). The chemical composition of the mucus is essentially similar in all the bivalve species (Soda *et al,* 1938). The accumulated material is then swept via ciliary action into the mouth where sorting takes place. Particles accepted as food are passed into the stomach where the digestion processes begin.

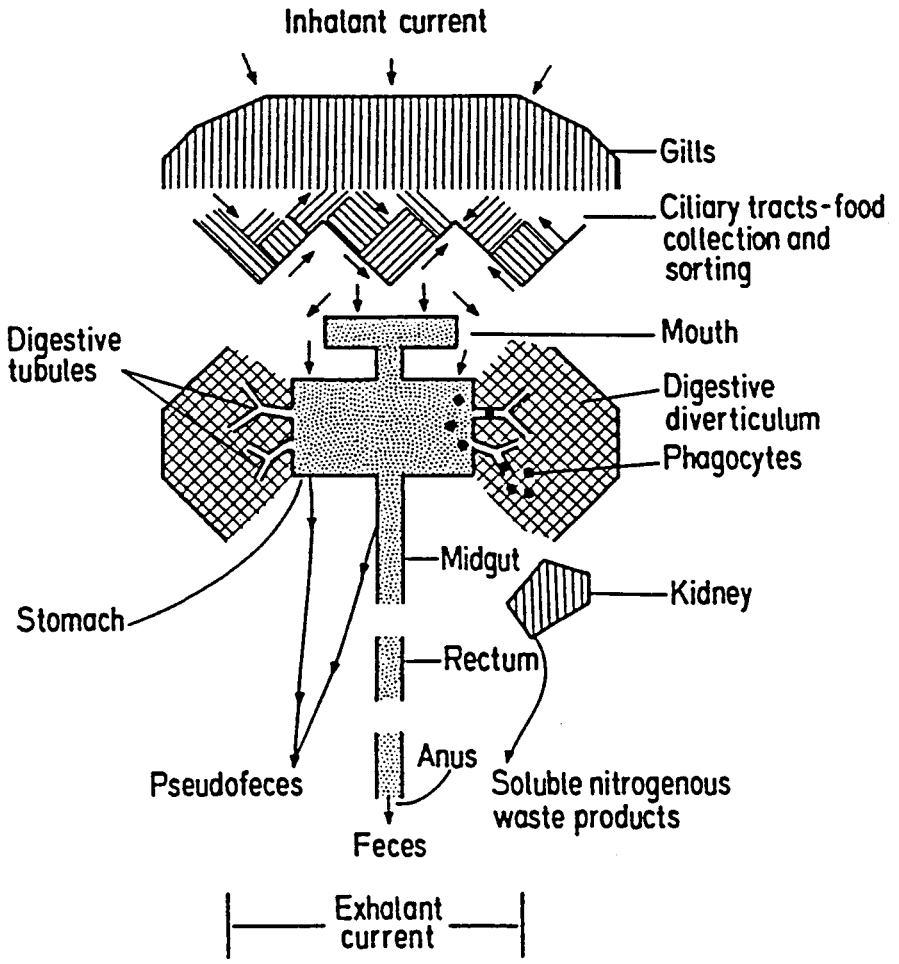


Figure 2.1: A schematic representation of shellfish structures involved in feeding, digestion, and waste elimination processes (Adapted from Metcalf, 1978)

Ingested food then passes through a digestion system and the great majority of pathogenic micro-organisms recovered from polluted seawater accumulates in the digestive gland. Digestion may also be carried on, through phagocyte intervention, in digestive diverticula tubules or tissues. Indications are that bacteria, which are relatively large, remain confined to the digestive tract, but that viruses, which are much smaller than bacteria, may penetrate tissues and are also detectable in, for instance, mussel tissue (Liu *et al,* 1966). One possible explanation of this is that the viruses in the mollusc's digestive system are removed by cells called haemocytes and then migrate across epithelial barriers into the haemolymph and other tissues (Pain, 1986; West, 1986). Another explanation is an irreversible virus binding to secreted mucus, followed by ingestion (Di Girolamo *et al*, 1977; Metcalf *et al*, 1979). The excretion of digested food takes place along rejection paths to the exterior, where it is eliminated as pseudofaeces (Galtsoff, 1964; Jorgensen, 1966). Solid wastes are eliminated in the faeces, and soluble nitrogenous

wastes are excreted through nephridial structures. Keuh and Chan (1985) showed that most of the heterotrophic bacteria and coliform organisms are concentrated in the gut, mostly in the stomach. Some authors (Leung *et al,* 1975) believe that the digestive diverticulum harbours the greatest numbers of pathogens, but despite its large size, the diverticulum accounts for only a small portion of the bacterial population in the oyster. Digestion and lysozymes may account for reduction in bacterial numbers (Birbeck and McHenry, 1982).

Molluscs can be intermediate hosts for a number of nematode (*Angiostrongylus cantonensis*) and trematode (*Paragominus westermani*) as well as other human parasitic infestations in the Far East and Pacific, but will not be discussed because they have not yet been associated with illness transmitted by bivalve mollusc in South Africa and other parts of the world (Earampamoorthy and Koff, 1975; Ahmed, 1991; Fang and Guerrant, 1991).

Human enteric viruses do not replicate in shellfish but by their way of feeding shellfish may accumulate viruses to levels 100 times as high as in the surrounding seawater (Fleet, 1978; Ellender *et al,* 1980; Feachem *et al,* 1982). Enriquez *et al* (1992) showed a 100-fold increase in hepatitis A virus titres relative to the surrounding waters. Similarly Mitchell *et al* (1966) found that after one hour of exposure to seawater containing polioviruses, numbers of the viruses in the meat of oysters were 27 times higher than in the water. Other studies revealed 10- to 180-fold increases in virus numbers (Hoff and Becker, 1969).

The interesting feature of accumulating and retaining for some time micro-organisms from the surrounding seawater, renders molluscan shellfish valuable monitoring tools. For instance, their accumulation of micro-organisms which are of faecal origin implies that they can be used to monitor sewage pollution of marine environments, and oysters have indeed successfully been used for this purpose (Coetzee, 1962; Ayers *et al,* 1978; Enriques *et al,* 1992). In addition, their ability to concentrate viruses from seawater, can also be used to monitor seawater for the presence of low levels of viruses (Enriques *et al,* 1992).

Infectious diseases are more frequently transmitted by shellfish such as oysters which are usually eaten raw or lightly cooked, than by mussels which are generally more intensively cooked (Goyal *et al,* 1979; Pain, 1986; Gerba, 1988).

2.2.2. Demand for shellfish as a seafood

"He was a bold man that first ate an oyster" said Jonathan Swift. Bivalve molluscs have been a source of food for man since prehistoric time. It is said that the Roman emperor Vitellius has eaten 1 000 oysters at a single sitting and Henry IV had 400 oysters before dinner (Noble, 1990). Charles Dickens made it the main food for the poor and destitute in his book "Pickwick Papers" (West, 1989a). But not everybody in the history was thrilled by eating raw oysters. Voltaire wrote in a letter "I might eat some too provided they are roasted; I feel there is something barbarious in eating such a pretty little animal raw" (William and Warner, 1987). But how nutritious are oysters? One hundred grams of eastern raw oysters (five to eight oysters) yield about 66 calories of which 8,4 g are protein, 3,4 g carbohydrate and 1,8 g are fat (Venkataramaiah and Kempton, 1975; Pennington and Church, 1980).

The bivalve molluscan industry has grown rapidly in recent years. The production value for bivalve molluscan shellfish in 1988 was £570 000 in Scotland, £160 000 in Northern Ireland, and in England and Wales it was approximately £1,1 million, that is more than 72 000 tonnes (Pain, 1986; West, 1989a). In South Africa the local and export market for bivalve molluscs (particularly to the Far East and Europe) has almost unlimited potential. In Natal, licensed collectors alone consumed some 106 tonnes of the brown mussel (*Perna perna*) in 1979. This grew to an approximate 318 tonnes in 1986, and the demand increased by an annual rate of 39% (Grabow, 1987a).

The latest estimates indicated a 10-fold increase in mariculture production in South Africa over the period 1985 to 1991 (Hecht and Britz, 1992). Molluscan shellfish constituted more than 95% of the total mariculture production in 1991. The market potential for the molluscan shellfish industry is also reflected by the increase in the number of active mariculture producers from four in 1985 to 13 in 1991 (Hecht and Britz, 1992). The total mariculture production increased from 250 tonnes in 1985 to just over 2 700 tonnes in 1991 (Fig. 2.2). The bulk of the current production is made up of mussels, representing 75,5% of the total (Hecht and Britz, 1992). These figures which illustrate the demand for molluscan shellfish in South Africa, exclude the collection of shellfish by private individuals and commercial enterprises from natural resources on which no meaningful figures are available. Estimates suggest that this harvesting of natural resources must be quite substantial (Grabow, 1987a).

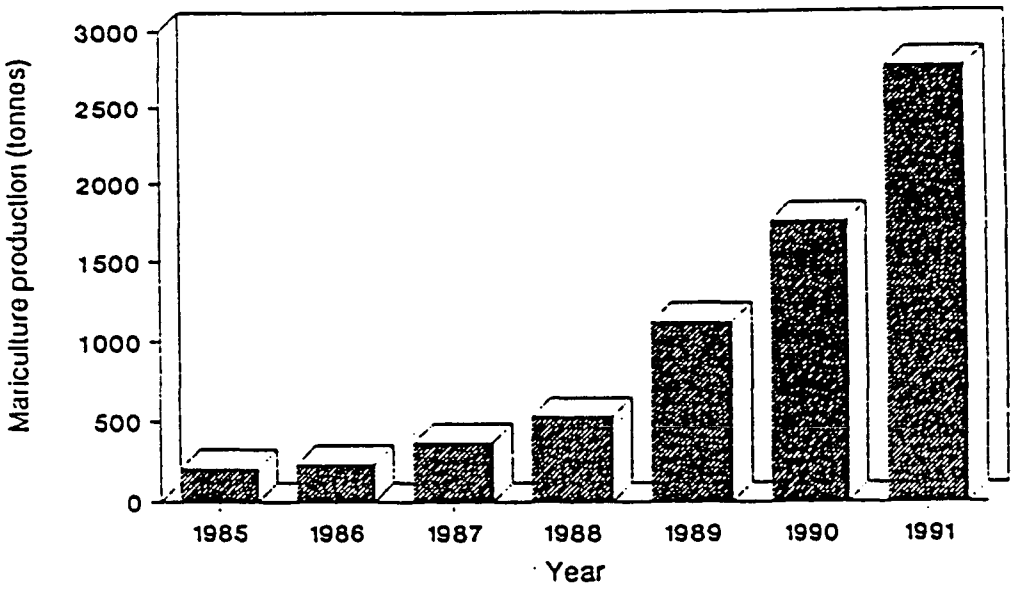


Figure 2.2: South African mariculture production, 1985 - 1991 (Adapted from Hecht and Britz, 1992)

Despite the long-standing history as a popular seafood, one of the most important barriers to the worldwide utilisation of shellfish is the lack of widespread consumer confidence resulting from outbreaks of gastroenteritis and other diseases associated with the consumption of shellfish (Pain, 1986; West, 1986). It is, therefore, essential to improve methods of cultivation, depuration, quality control, quality monitoring and marketing to encourage and support the expansion of the mariculture industry (Sockett *et al,* 1985; Grabow, 1987a; West 1989a; 1991).

2.3. INFECTIOUS DISEASES TRANSMITTED BY SHELLFISH

Theoretically any enteric viruses or other enteric pathogens may be transmitted by shellfish (Melnick and Gerba, 1980). Epidemiological confirmation of infections contracted by the consumption of shellfish is not easy, particularly when small numbers of people are infected (Gerba, 1988; Grabow, 1989). In addition, the identification of viral causes is particularly difficult because the great majority of viruses are not detectable by conventional methods. Despite all this, sound evidence has been presented for the transmission of a wide variety of pathogens by shellfish, including the following (Presnell *et al,* 1967; Lawson, 1970; WHO, 1974; Earampamoorthy *et al,* 1975; Brown and Dorn, 1977; Levin, 1978; Kane and Blacklow, 1979; Beuchat, 1982; Gunn *et al,* 1982; Gill *et al,* 1983; Harris *et al,* 1983; Kai *et al,* 1983;

Downey and Clarke, 1984; Fraiser and Koburger, 1984; Richards, 1987; Wanke *et al,* 1987; Appleton, 1990; Cubitt, 1991; Fang *et al,* 1991; Dillon and Patel; 1992; Baker, 1993; Balayan 1993):

2.3.1. Bacteria

a. *Salmonella* species including *S typhi*
b. *Vibrio* species including *V cholerae*
c. *Shigella* species
d. *Campylobacter* species
e. *Plesiomonas shigelloides*
f. *Clostridium botulinum*
g. *Escherichia coli*
h. *Aeromonas hydrophila*
i. *Bacillus* species
j. *Listeria monocytogenes*

2.3.2. Viruses

a. Caliciviruses (Norwalk virus and others)
b. Astrovirus
c. Hepatitis A virus
d. Hepatitis E virus

2.3.1. Bacteria

The consumption of raw shellfish has on occasions resulted in infections with autochthonous or free-living aquatic bacteria of the family Vibrionaceae, which includes the genera *Aeromonas, Plesiomonas* and *Vibrio* (West, 1989b,c). Epidemiological studies have established *Vibrio parahaemolyticus* as a worldwide agent of gastroenteritis. This pathogen and related species such as *Vibrio cholerae* 01, non-01 *Vibrio cholerae, Vibrio mimicus, Vibrio fluvialis, Vibrio hollisae* and *Vibrio vulnificus* have been isolated from seafoods (Kaneko and Colwell, 1975; Twedt *et al,* 1981; Joseph *et al,* 1983; Molitoris *et al,* 1985; Morris and Black, 1985; Kelly and Dan Stroh, 1988; DePaolo *et al,* 1988; West, 1989b,c; Anonymous, 1990; DePaola *et al,* 1990; Klontz, 1990). *Vibrio vulnificus* infection more often follows eating raw oysters. In general, the illness caused by the latter infection lasts for one to three days. Immunosuppressed persons and people with liver diseases and haemochroma-tosis should be cautioned against eating raw oysters because they have been shown to be at greater risk for complications after exposure to *Vibrio vulnificus* (Morris and Black, 1985; Bernardeschi *et al,* 1988; Klontz, 1990; Koening *et*

al, 1991; Centers for Disease Control, 1993). *Vibrio cholerae* 01 illness has been associated with eating crabs, shrimp and raw oysters harvested along the Gulf Coast of the United States (Blake *et al,* 1980). The CDC (1989) reported three outbreaks of *Vibrio cholerae* 01 involving 16 cases between 1978 and 1987 (Ahmed, 1991).

Most outbreaks of bacterial infections occur as a result of secondary contamination due to improper procedures during storage, preparation, and serving of the food in the home or in restaurants or other foodservice establishments (Phillips and Peeler, 1972; Wentz *et al,* 1985; Saddik *et al,* 1985). Careful handling and serving is, therefore, essential. Cooking and serving hot is still an excellent protection against most food-borne pathogens (Liston, 1989; Nightingale, 1990; Stammen *et al,* 1990).

According to Mosely (1974) the first reported epidemic of infections associated with the consumption of oysters was an outbreak of typhoid fever reported in France as early as 1816. Similar outbreaks of shellfish-associated typhoid were also described from other parts of the world at the turn of the century (Sooper, 1905).

2.3.2. Viruses

The first documented outbreak of an epidemic caused by a viral agent was in 1956 in Sweden, with the outbreak of 629 cases of hepatitis A caused by the consumption of raw oysters (Lindberg-Braman, 1956; Roos, 1956). The most common symptoms were fever, nausea and pain in the right hypochondrium. Similar outbreaks have since been reported (Table 2.2) in the United States and elsewhere, and outbreaks of gastroenteritis caused by shellfish consumption appear to be on the rise (Dougherty *et al,* 1962; Mason *et al,* 1962; Communicable Disease Centre, 1964; Grady, 1968; Ruddy *et al,* 1969; Richards, 1985; Sockett *et al,* 1985; Pain, 1986; Gerba, 1988; Grabow, 1989). The largest outbreak of viral disease epidemiologically confirmed to have been caused by the consumption of shellfish occurred as recently as 1988 in China. This outbreak was caused by the consumption of hairy clams harvested from a sewage-polluted bay (Xiao Miao Hong) near Shanghai. Some 25 000 cases of gastroenteritis, most of which evidently of viral aetiology, and close to 300 000 cases of type A hepatitis (over 4% of the population) with 32 deaths were recorded in Shanghai and epidemiologically related to the

consumption of the shellfish (Wang *et al,* 1990; Halliday *et al,* 1991). The risk of contracting hepatitis A was calculated to be 95,96% (Jianxiang *et al,* 1988). Another outbreak in 1988 saw 61 persons ill with hepatitis A, in 5 states in the USA after eating contaminated oysters (Desenclos *et al,* 1991). Although Australia (Au) antigen, a marker of type B hepatitis virus, was detected in clams, the hepatitis B virus itself has never been isolated from shellfish and the transmission of hepatitis B has never yet been associated with the consumption of shellfish (Kater *et al,* 1969; Mahoney *et al,* 1974). However, hepatitis E is like hepatitis A typically transmitted by the faecal-oral route and six reports have implicated shellfish as a source of hepatitis E infections (Caredda *et al,* 1981; 1985; 1988; Alter *et al,* 1982; Bamber *et al,* 1983; Tornè, 1988).

The first documented shellfish-associated epidemic caused by Norwalk virus took place in 1978 and involved 2000 Australians (Murphy *et al,* 1979). Similar outbreaks of Norwalk virus gastroenteritis were subsequently reported from other parts of the world (Chalmers and McMillan, 1995). For instance, during 1982 there were 103 documented outbreaks of Norwalk virus gastroenteritis in New York state involving 1017 people (Morse *et al,* 1986).

During the period 1941 - 1983, a total of 108 disease outbreaks associated with the consumption of molluscan shellfish was recorded in England and Wales; 65 of these were of unknown cause and 32 were due to viral infections. One of these outbreaks, a cockle-associated outbreak, occurred in several locations in the south of England in the winter of 1976 - 1977; the cockles were harvested from sewage-polluted waters of the Thames estuary (Appleton and Pereira, 1977). There has been a considerable increase in the number of outbreaks recorded since 1981, possibly reflecting trends such as increasing pollution of marine environments, a growth in the popularity of seafood, and increased surveillance of outbreaks in England and Wales (Sockett *et al,* 1985). A total of 32 outbreaks was recorded in the 3-year period 1981 - 1983, compared to about 30 in the previous 20 years. Within this period the number of cockle-associated outbreaks declined whereas those associated with oysters, increased. This may have resulted from a more rigorous application of cooking procedures for cockles since the major incident in 1977. This has not prevented incidents of hepatitis A infections of which seven outbreaks have been recorded during the period 1981 - 1985 (Sockett *et al,* 1985).

Some 81% of outbreaks associated with molluscan species recorded between 1941 and 1970 in England and Wales were of unknown cause (Fig 2.3). However, the clinical symptoms and application of recently developed electron microscopic techniques for the detection of viruses, suggest that the majority of these infections were probably caused by viruses, mostly small round viruses (SRV) (Sekine *et al,* 1989). Since about the late 1950's there has been a dramatic decrease in the number of shellfish-associated diseases caused by bacteria in England and Wales (Sockett *et al,* 1985), as well as in the United States (Richards, 1987). No outbreaks of bacterial diseases have been reported from 1965 to 1985 in England and Wales (Sockett *et al,* 1985). This has been ascribed to success in the implementation of depuration, decontamination and other regulations to improve the sanitary quality of shellfish marketed for human consumption (Sockett *et al,* 1985). During the same period, however, there has been a drastic increase in the incidence of viral disease outbreaks associated with shellfish, which was ascribed to increased pollution of shellfish harvesting grounds, viruses which are more resistant than indicator bacteria to decontamination processes, and viruses which are less readily released by shellfish during depuration than bacteria (Sockett *et al,* 1985; West, 1986; 1989a; Appleton *et al,* 1988; Gerba, 1988; Enriquez *et al,* 1992; see also Section 2.6). This explanation is supported by outbreaks epidemiologically confirmed to have been caused by shellfish which went through prescribed depuration procedures and met specifications for bacterial indicators of sanitary quality including coliform bacteria (Gill *et al,* 1983; Sockett *et al,* 1985; Heller *et al,* 1986; Chalmers and McMillan, 1995). Similar trends in the relative incidence of shellfish-related disease caused by viruses and bacteria have been reported from other parts of the world (Grohmann *et al,* 1981; Richards, 1985). It is important to recognise that, among the cases of shellfish-associated gastroenteritis reported in the United States in the past 50 years, over 75% were reported since 1980 (Richards, 1987). Seafood-borne illness reported by the CDC in the 10-year period 1978 - 1987 totalled 558 outbreaks involving 5 980 cases (Ahmed, 1991).

Table 2.2 lists some typical examples of viral disease outbreaks associated with shellfish. Apparently the transmission of rotavirus by shellfish has not yet been described (Ahmed, 1991). This may be because rotavirus infection is primarily a disease of infants (Christensen, 1989) and shellfish are eaten predominantly by adults.

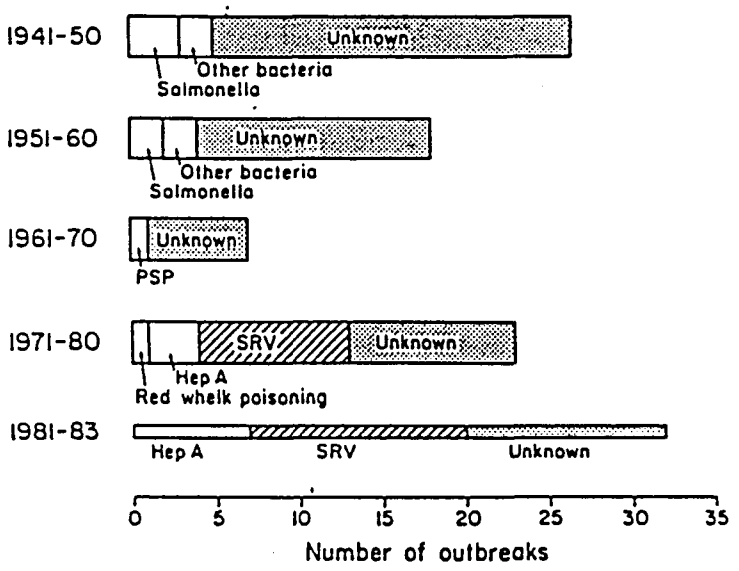


Figure 2.3: Aetiology of outbreaks of infectious diseases associated with molluscan shellfish in England and Wales from 1941 - 1983. Types of shellfish are unspecified for the period from 1941 to 1960. Small round structured (SRV) categories of viruses include parvovirus and Norwalk groups of viruses (Adapted from Sockett *et al*, 1985)

Table 2.2: Some examples of viral disease outbreaks epidemiologically associated with the consumption of shellfish

Year	Source	Place	Number of cases	Virus
1973	Oysters	Houston	263	Hepatitis A[1]
1976	Mussels	Australia	7	Hepatitis A[1]
1978	Oysters	Australia	2 000	Norwalk[1]
1978	Mussels	England	41	Hepatitis A[1]
1979	Shellfish	Maryland	33	Hepatitis E[6]
1980	Oysters	England	6	Norwalk[1]
1980	Oysters	Florida	7	Hepatitis A[1]
1982	Clams	Philippines	150	Norwalk - like, Hepatitis A[1]
1982	Oysters	Japan	225	Hepatitis A[3]
1983	Oysters	London	181	Small round structured viruses[2]
1984	Cockles	New York	322	Hepatitis A[1]
1985	Oysters	England	16	Norwalk[4]
1988	Clams	China	292 301	Hepatitis A[5]

References:

1. Verber (1984) 2. Gill *et al* (1983) 3. Fujiyama *et al* (1985)
4. Heller *et al* (1985) 5. Wang *et al* (1990); Halliday *et al* (1991)
6. Alter *et al* (1982)

2.3.3. Protozoan parasites

The cysts and oocysts of protozoan parasites such as *Cryptosporidium* and *Giardia* are transmitted by the faecal-oral route and often cause gastrointestinal infections in humans (West, 1991). *Cryptosporidium* is now recognized as an important cause of gastroenteritis in man (Tzipori, 1989). The oocysts of this parasite have frequently been detected in wastewater and may, therefore, also occur in sewage-polluted coastal waters (West, 1991). As far as could be established, however, the transmission of *Cryptosporidium* species has not yet been associated with the consumption of seafood.

The protozoan parasite *Giardia lamblia* causes a form of gastrointestinal illness known as giardiasis (Lin, 1985). This protozoan is associated with freshwater-borne epidemics in countries like the United States (Craun, 1977; 1979; West, 1991; Eastaugh and Sheperd, 1992). In the United Kingdom, waterborne giardiasis is far less common (Galbraith *et al,* 1987; Casemore, 1991). Very little is documented about food-borne giardiasis. Only one outbreak, caused by the consumption of contaminated salmon, has been reported (Osterholm *et al,* 1981; Eastaugh and Sheperd, 1992).

Although infections by *Giardia, Cryptosporidium* and other protozoan parasites are common in South Africa, there is no information on infections associated with water or food. As far as could be established, detection of the cysts and oocysts of these parasites in shellfish has not yet been reported anywhere in the world.

Shellfish may act as vectors for certain trematodes. Over 40 species of trematodes belonging to 11 genera share the following epidemiological characteristics: their first intermediate hosts are molluscs, their second hosts are fish or crustaceans, and their final hosts are humans or animals eating raw fish (Sheperd and Eastaugh, 1992). Vogel (1933) described a human infection of *Himasthla meuhlensi* associated with the consumption of clams. The clams were eaten raw, which was followed by gastrointestinal disturbances.

Migrant larvae of several nematode genera may be ingested together with infected fish, molluscs, or crustaceans when these are consumed raw or not adequately salted, pickled, smoked, or cooked (Anonymous, 1975; Ruitenberg *et al,* 1979).

An extensive outbreak of eosinophilic meningitis caused by the parasite *Angiostrongylus cantonensis* was recognized on Ponape in the Caroline Islands in 1948. Although the source of the outbreak could not be determined, it possibly was caused by the consumption of aquatic and amphibious crabs, or certain bivalve molluscs (Rosen *et al,* 1967).

Although there is no conclusive evidence that the known protozoan parasites of marine fish or shellfish are infective to man, it has been suggested that shellfish may serve as a potential vehicle for the transmission of certain free-living soil and fresh water amoebae, including *Naegleria, Hartmannella,* and *Acanthamoeba,* which can cause meningitis (Cheng, 1973).

2.3.4. Other diseases

Apart from diseases caused by pathogenic micro-organisms, various other diseases may also be contracted by the consumption of shellfish. Among these are red whelk poisoning and paralytic shellfish poisoning (Brown and Dorn, 1977; Sockett *et al,* 1985; Brown, 1987; Grabow, 1989; Bates 1993). Red whelk poisoning follows ingestion of the salivary glands of the red whelk *Neptunea antiqua* which contains the toxin tetramine. Paralytic shellfish poisoning is caused by toxins known as saxitoxin, produced by marine dinoflagellates of the genus *Gonyaulax,* a form of plankton on which the molluscs feed (Levin, 1978; Anonymous, 1988; Noble, 1990; Fang and Guerrant, 1991; Eastaugh and Sheperd, 1992). From 1927 to 1985 there were 505 cases of paralytic shellfish poisoning in California with 32 deaths, after the consumption of contaminated mussels, oysters or scallops (Noble, 1990).

Other hazardous compounds which may potentially be transmitted by shellfish include toxic metals, pesticides, hydrocarbon residues, and radioactive wastes (Brown and Dorn, 1977; Richards, 1987; Wanke *et al,* 1987; Edwards, 1988; Fang *et al,* 1991).

2.3.5. Epidemiological data

Assessment of the incidence of disease associated with marine pollution is complicated by various factors. For instance, infected people may subsequently transmit the disease to any number of others by personal contact or in other ways not directly related to marine pollution, and detection of such secondary spread is extremely difficult. Despite the difficulties of

epidemiological detection, secondary and even tertiary transmission has been clearly confirmed in various outbreaks (Morens *et al,* 1979; Gunn *et al,* 1980; Heun *et al,* 1987; Reid *et al,* 1988). These findings illustrate the need for ongoing revision of quality criteria as new methods for the detection of various pathogens, and new information on the epidemiology and infection risks of related diseases, becomes available (Grabow, 1987a; Gerba, 1988).

2.4. QUALITY GUIDELINES FOR SHELLFISH

In South Africa and other parts of the world, the importance of marine pollution is escalating dramatically as a result of the conflicting interests of the following and related claims on the marine environment (Grabow, 1987a; 1987b):

1. Rapid population and industrial growth in coastal areas.
2. Growing tourist, holiday and recreation industry.
3. Increasing demand for seafoods, locally and internationally.
4. Conservation of a unique nature heritage.
5. Convenient waste dumping site.

Due to the increasing incidence of shellfish-associated disease outbreaks, quality guidelines have been developed to ensure a microbial "safe" product and to assess related health risks (Grabow, 1989). Since it is difficult to establish meaningful correlations between the quality of seawater and seafood and the potential risk of infection, and since the interpretation of acceptable risks may vary extensively from one community to another, quality guidelines, recommendations and specifications for seawater and seafood vary considerably from one country or authority to another (Department of Health, 1973; EEC, 1976; Livingstone, 1976; Bitton, 1980; Cabelli *et al,* 1982; Lusher, 1984). The latest quality guidelines for seawater at shellfish harvesting grounds and for shellfish meat in South Africa and some other countries are presented in Tables 2.3 and 2.4, respectively.

Quality guidelines are generally based on limits for coliform bacteria which serve as indicators of faecal pollution (Shuval, 1986). The faecal coliform bacteria are commonly used as an indicator of the sanitary quality of seawater and shellfish, because faecal coliforms are normal inhabitants of the gastrointestinal tract of man and warm-blooded animals and are excreted in large numbers (Goyal and Gerba, 1978). Faecal coliform limits have been set for seawater used

Table 2.3: Recommended quality guidelines for seawater at shellfish harvesting grounds: Maximum acceptable levels (Adapted from Grabow, 1987b)

South Africa (Lusher, 1984)		
Faecal coliforms/100 ml	:	15 (50 % of samples)
United States of America (Bitton, 1980)		
Total coliforms/100 ml	:	70
	:	230 (90 % of samples)
Italy (Wood, 1976)		
Escherichia coli/100 ml	:	≤2 (90 % of samples)
WHO/UNEP (Helmer *et al*, 1991)		
Faecal coliforms/100ml	:	<10 (80% of samples)
Faecal coliforms/100ml	:	<100 (remaining 20% of samples)

for direct contact recreation (swimming, diving, surfing), for seawater at shellfish beds where shellfish are harvested, for human consumption, and for shellfish meat (Grabow, 1987a; 1987b; Grabow *et al*, 1989). These limits have been set at levels considered acceptable in terms of risk of infection. The World Health Organization and United Nations Environment Programme (WHO/UNEP) recommended that shellfish areas could be considered acceptable if the faecal coliform count in 100 g of shellfish flesh plus intervalvular fluid does not exceed 300 (WHO/UNEP, 1987). New standards for EEC countries (91/492/EEC) for the production and marketing of bivalve shellfish were published in September 1992 for implementation by 1 January 1993. Virus contamination of shellfish meat is now controlled through restrictions on harvesting areas. Shellfish are considered suitable for depuration by conventional methods when counts do not exceed 6 000 faecal coliforms or 4 600 *Escherichia coli* per 100 g of flesh, in 90% of samples tested by a five tube, three log dilution MPN test. When counts of faecal coliforms in shellfish flesh are in the range of 6 000 per 100 g, the shellfish must be relaid in clean, natural seawater for at least 2 months before depuration can be attempted. Above 60 000 faecal coliforms per 100 g of flesh, shellfish may not be harvested at all for human consumption even after depuration (Anonymous, 1992).

Table 2.4: Quality guidelines for shellfish meat intended for human consumption

1. South Africa (Department of Health, 1973)

Partly cooked or uncooked	
Salmonella species	not present
Shigella species	not present
Vibrio cholerae	not present
Coagulase-positive *Staphylococcus aureus*	10/g
Escherichia coli type 1	5/g
Total colony count (35°C/48 h)	not applicable
Cooked	
Salmonella species	not present
Shigella species	not present
Vibrio cholerae	not present
Coagulase-positive *Staphylococcus aureus*	10/g
Escherichia coli type 1	not present
Coliform organisms other than *Escherichia coli*	10/g
Total colony count (35°C/48 h)	100 000/g

2. United States (Wehr, 1978)

Uncooked	
Aerobic plate count	$5{,}0 \times 10^5$/g
Coliform	$2{,}4 \times 10^4$/g
Faecal coliform	230/g
Escherichia coli	not present
Salmonella	not present
Coagulase-positive *Staphylococcus aureus*	not present
Cooked	
Aerobic plate count	$1{,}0 \times 10^4$/g
Coliform	not present
Escherichia coli	not present
Salmonella	not present
Coagulase-positive *Staphylococcus aureus*	not present

3. WHO/UNEP (Helmer *et al*, 1991)

Faecal coliforms/g	0-2	sale permitted
Faecal coliforms/g	3-10	temporary prohibition of sale
Faecal coliforms/g	above 10	sale prohibited

Shortcomings in correlations between coliform counts and the risk of infection are illustrated by outbreaks of viral gastroenteritis caused by the consumption of shellfish supplies which (Gill *et al,* 1983; Sockett *et al,* 1985; Heller *et al,* 1986):

(a) Had been harvested from grounds where the seawater conforms to recommended faecal coliform limits.

(b) Had been depurated by specified procedures.

(c) In tests on the flesh had been found to have counts of faecal coliform bacteria within specification limits.

It is, therefore, necessary to continually revise quality guidelines as new information on the epidemiology and risk of infection becomes available (Grabow *et al,* 1989).

In view of the well established shortcomings of coliform bacteria (total coliforms, faecal coliforms or *Escherichia coli*) as indicators of faecal contamination of seawater/shellfish or of the safety of shellfish in terms of infectious diseases, inclusion of additional indicators and tests have been recommended in recent times. Among these are tests for enterococci or faecal streptococci which would seem to survive longer and are more closely related to infections associated at least with the recreational use of seawater (Helmer *et al,* 1991). Results on the incidence and behaviour in marine environments of bacteriophages which indicate faecal pollution are beginning to accumulate and indications are that these may also prove of value in the assessment of pollution and risks of infection (Simkova and Cervenka, 1981; Havelaar *et al,* 1986; Borrego *et al,* 1990; Cornax *et al,* 1991). Since viruses are involved in the great majority of infections associated with sewage-contaminated shellfish, direct testing of shellfish for viruses may prove essential (Grabow *et al,* 1989). With current progress in the development of practical techniques for the detection of viruses, this may soon become possible for routine monitoring purposes, at least in high risk situations (Grabow *et al,* 1989).

In view of the above considerations Grabow *et al* (1989) have recommended the guidelines summarised in Table 2.5.

Table 2.5: Recommended quality guidelines for seawater used for direct contact recreation and shellfish meat intended for human consumption (Grabow *et al,* 1989)

Seawater - direct contact recreation			
Faecal coliforms per 100 ml	:	100	(50% of samples)
Faecal streptococci per 100 ml	:	40	(50% of samples)
Somatic coliphages per 100 ml	:	50	(50% of samples)
Human enteric viruses per 10 l	:	0	(50% of samples)
Shellfish meat - human consumption			
Faecal coliforms per 100 g	:	500	(90% of samples)
Faecal streptococci per 100 g	:	200	(90% of samples)
Somatic coliphages per 100 g	:	10	(90% of samples)
Human enteric viruses per 100 g	:	0	(90% of samples)

Inadequacy of indicators to predict the microbiological quality of water and food has led to the implementation of the Hazard Analysis Critical Control Point system (HACCP), a more critical approach to control of microbiological hazards (Subcommittee on Microbiological Criteria *et al,* 1985; West, 1986). This system consists of (West, 1986):

(a) identification and assessment of hazards associated with growing, harvesting, processing, marketing and preparation

(b) determination of critical control points to control any identifiable hazard

(c) establishment of systems to monitor critical control points.

The HACCP concept aims to systematically ensure the production of wholesome shellfish to protect and enhance the reputation of this food (Subcommittee on Microbiological Criteria *et al,* 1985; West, 1986). The HACCP together with the development of practical and economic methods for assessment of the virological safety of shellfish, will help to decrease the risk of infections due to the consumption of shellfish (West, 1986).

Hazard Analysis assesses and identifies stages within a food preparation operation where undesirable micro-organisms can either enter the food, or fail to be eradicated by the appropriate treatment processes.

Critical Control Points are plant locations or processes which, if not correctly controlled, may lead to the survival of micro-organisms or the contamination of food (Subcommittee on Microbiological Criteria *et al,* 1985).

2.5. VALUE OF INDICATOR ORGANISMS

Indicator organisms are used for the relatively practical, simple, rapid and inexpensive assessment of the sanitary quality of food and water. Generally speaking these organisms typically occur in the faeces of humans and/or warm-blooded animals (Metcalf, 1978; Gerba *et al*, 1979; Metcalf, 1979; Bitton, 1980; Matches and Abeyta, 1983; Gerba, 1987). Their presence in food or water does, therefore, indicate faecal pollution which implies that enteric pathogens may also be present (Metcalf, 1978; Matches and Abyeta, 1983; Gerba, 1987). In other words, the absence of faecal indicators implies that the presence of enteric pathogens is unlikely (Hilton and Stotzky, 1973; Kott *et al*, 1978; Debartolomeis and Cabelli, 1991). In terms of this definition the requirements of an ideal indicator would include the following (Berg 1978; Stetler, 1984; Grabow, 1986):

(a) An indicator should always be present when pathogens are present and pathogens should always be absent when indicators are absent.

(b) The occurrence of indicator and pathogen should be in a constant ratio.

(c) Similarities in both persistence and growth characteristics of indicator and pathogen.

(d) Similarities in resistance to environmental factors and disinfectants.

(e) Levels of indicators in source of pollution should exceed levels of pathogens.

(f) The indicator should be non-pathogenic and the test for the indicator should not give false-positive results.

(g) The test for the indicator organism should be rapid, simple and economical and should be applicable to water and food.

Ideally, various other properties would be desirable, such as counts which are directly related to those of viruses.

The following indicators, each with its own advantages and disadvantages, have been used for assessment of the sanitary quality of shellfish (Metcalf, 1978; Scarpino, 1978; Goyal, 1983; Matches and Abeyta, 1983; Grabow, 1989; Grabow *et al*, 1989):

1. Total and faecal coliforms
2. *Escherichia coli*
3. Faecal streptococci
4. *Clostridium perfringes*
5. Bacteriophages

Each of the above indicators has certain indicator features which are useful for assessment of the sanitary quality of shellfish meat and seawater at harvesting grounds. However, evidence has been presented that commonly used indicators, notably coliforms, have shortcomings, particularly in terms of indicating the presence of viruses (Mossel, 1967; Silker and Gabis, 1976; Grabow *et al,* 1989). For instance, shellfish which went through prescribed depuration processes and met coliform limits have caused outbreaks of gastroenteritis and hepatitis A (Dismukes *et al,* 1969; Truman *et al,* 1987; Halliday *et al,* 1991; see also Chapter 2.3).

2.5.1. Total and faecal coliforms

The total coliform group is defined as bacteria which are aerobic or facultatively anaerobic, Gram-negative, nonspore-forming, rod-shaped and which ferment lactose with acid and gas production within 24 h at 35°C (Mundt, 1970; Metcalf, 1978). Faecal (thermotolerant) coliform bacteria refers to those members of total coliform bacteria which ferment lactose and produce indole at 44,5°C. Faecal coliforms generally consists of *E coli* and certain species of *Klebsiella* (American Public Health Association, 1992). The most important members of the group are *Escherichia coli, Klebsiella pneumonia, Enterobacter aerogenes* and *Citrobacter freundii* (Metcalf, 1978; Goyal, 1983; American Public Health Association, 1992). Although total and faecal coliforms serve a valuable purpose in assessment of the general sanitary and hygienic quality of shellfish and seawater, their indicator value certainly has shortcomings, particularly with regard to human viruses (Vaughn *et al,* 1983; Grabow *et al,* 1989). Outbreaks of gastroenteritis due to the consumption of raw shellfish do occur even though counts of coliforms in shellfish are within recommended limits (Berg *et al,* 1978; Gerba *et al,* 1979; Cole *et al,* 1986). Observations by Suñer and Piñol (1966), Goyal *et al* (1978; 1979) and Vaughn *et al* (1979) indicate that total and faecal coliforms are inadequate in predicting the presence of viruses since the survival of viruses in seawater is longer than that of coliforms (Lo *et al,* 1976; Goyal *et al,* 1978; Lucena *et al,* 1982; Grabow, 1986; O'Keefe and Green, 1989; Cornax *et al,* 1991). The ratios of faecal coliforms to viruses vary from 7 500:1 to 2 900:1 in raw sewage and from 14 000:1 to 7 600 000:1 in river water (IAWPRC Study Group, 1983). These values can be expected because coliforms are excreted by almost all persons in numbers of about 10^9/g of stool, while the excretion of viruses is limited to numbers of about 10^6/g stool during the period of infection. Numbers of viruses in sewage range from 0 - $2x10^3$/100ml (Grabow, 1986). At best total and faecal coliforms only indicate a probability of faecal pollution by man or warm-blooded animals (Metcalf, 1979). Due to their low persistence in the marine environment, these

microorganisms should not be used as the only indication of viral contamination (Gerba *et al,* 1979; LaBelle *et al,* 1980). Although the use of indicators has inadequately reduced the occurrence of shellfish associated diseases, relationships between coliform levels and the occurrence of enteric viruses in shellfish have not been established. Numerous studies involving the effectiveness of coliforms as indicators of enteric pathogens in shellfish found no statistically significant correlation between virus titers in shellfish meat and indicator bacteria levels in oysters or in the overlying waters (Gerba *et al,* 1979; Goyal *et al,* 1979; Vaughn *et al,* 1979; Ellender *et al,* 1980; Gerba *et al,* 1980; Marzouk *et al,* 1980; Vaughn *et al,* 1980; Carrick *et al,* 1981; Lucena *et al,* 1982; Goyal, 1983; Wait *et al,* 1983; Goyal *et al,* 1984). Macowiak *et al* (1976) state that under natural conditions shellfish eliminate bacteria and viruses differently and may retain certain enteroviruses for as long as two months after these organisms have disappeared from surrounding waters.

2.5.2. *Escherichia coli*

Escherichia coli is a highly specific indicator of faecal pollution because, in contrast to most other members of the total and faecal coliform groups, this organism cannot multiply in the environment. For all practical purposes *E coli* only multiplies in the gastrointestinal tract of man and warm-blooded animals, which implies that its presence reliably indicates faecal pollution (Brezenski and Russomanno, 1969; Gerba *et al,* 1979; Borrego *et al,* 1987). However, for reasons mentioned earlier, the indicator value of *E coli* also has shortcomings, particularly with regard to human viruses.

2.5.3. Faecal streptococci

Faecal streptococci are aerobic cocci that typically, but not exclusively, multiply in the intestinal tract of man and animals. This group includes *Streptococcus faecalis* and its two varieties *liquifaciens* and *zymogenes, S durans, S faecium, S bovis* and *S equinus.* This group is closely related to the group of streptococci known as enterococci (American Public Health Association, 1992). The use of faecal streptococci to predict the sanitary quality of live shellfish has been criticised because certain members of the group may multiply during storage of shellfish at temperatures of 11°C or higher (Wood, 1965). However, faecal streptococci have valuable indicator features. For instance, they tend to be more resistant than coliforms to unfavourable conditions in marine environments (Grabow, 1986). Furthermore, they are unable to multiply in sewage or natural water, and the faecal streptococci density in sea water is strongly related

to the incidence of swimming associated illness in bathers (Cabelli *et al,* 1982; Grabow *et al,* 1982; 1983; 1984; Payment *et al,* 1985). Martinez-Manzanares *et al* (1991) suggested that total coliforms and faecal streptococci could be the best indicators of the presence of pathogens at low levels of pollutions ($< 10^4$/100g total coliforms and $< 10^3$/100g faecal streptococci), and at high levels of pollution faecal streptococci, *E coli* and sulphite-reducing clostridia may be more appropriate indicators.

2.5.4. *Clostridium perfringens*

Clostridium perfringens is an obligate anaerobic bacterium. Like *E coli* it almost exclusively multiplies in the gastrointestinal tract of humans and warm-blooded animals. It is, therefore, like *E coli* a highly specific indicator of faecal pollution. The spores of *C perfringens* are extremely resistant to unfavourable environmental conditions; as far as is known, even more than the most resistant viruses (Metcalf, 1978; Goyal, 1983; Burger *et al,* 1984; Madden *et al,* 1986; Regan *et al,* 1993). This implies that *C perfringens* can be used to detect remote faecal pollution, and the absence of *C perfringens* is almost conclusive evidence that viruses will also be absent (Grabow, 1986). However, as a result of their extreme resistance, counts of *C perfringens* correlate poorly with those of viruses and other enteric organisms in water and food. In other words, *C perfringens* may still be detectable long after all other health-related organisms have been inactivated. This implies a shortcoming in that *C perfringens* may be an unnecessarily stringent indicator for routine monitoring of the safety of water and food (Grabow, 1986).

2.5.5. Bacteriophages

Bacteriophages (phages) are bacterial viruses which are ubiquitious in the environment and infect almost all groups of prokaryotic microbes (Metcalf, 1978; Goyal, 1983; Duckworth, 1987; Calender, 1988; IAWPRC Study Group, 1991; Grabow *et al,* 1993). They were discovered in human faeces by D'Herelle in 1917 (Merril, 1974). They closely resemble human viruses in size, structure, morphology and composition (Kott, 1981; Grabow *et al,* 1984; Grabow, 1986; Grabow *et al,* 1989). Phages and human viruses cannot replicate themselves. They infect specific host cells and through their nucleic acid instruct these cells to produce replicates of themselves. The host specificity of phages and human viruses implies that phages and human viruses can be associated with environments in which their host cells occur (Metcalf, 1978; Duckworth, 1987). The most important difference between phages and viruses is that phages have bacteria as hosts and viruses have mammalian cells as host (Scarpino, 1978; Grabow *et al,*

Coliphages (phages which typically infect *E coli*) are closely associated with wastewater pollution and they generally outnumber enteric viruses in water by a factor up to 1 000 or more (Grabow *et al,* 1984; Grabow and Coubrough, 1986; Grabow *et al,* 1993).

Phages used as indicators of the sanitary quality of food and water have the following attractive indicator features (Kott *et al,* 1974; Simkova and Cervenka, 1982; Grabow *et al,* 1986):

(a) Generally, phages are at least as resistant to environmental conditions as enteroviruses.

(b) Phages and enteroviruses are removed at comparable rates during treatment processes.

(c) Their behaviour in the environment resembles that of human enteric viruses.

(d) Generally phages such as somatic coliphages outnumber human viruses in sewage and sewage-polluted environments, which implies that if they are absent, human viruses are most likely also absent.

(e) Many phages are detectable by relatively practical, rapid and inexpensive methods whereas the detection of viruses is complex and expensive.

The following are examples of phages used as indicators of sanitary quality (Metcalf, 1978; Goyal, 1983; Grabow *et al,* 1986; Jofre *et al,* 1986; Grabow *et al,* 1989; 1993; IAWPRC Study Group, 1991):

2.5.5.1. Somatic coliphages

This term refers to a large group of phages which infect *E coli* and closely related bacteria. Coliphages can, therefore, to a reasonable extent be used as indicators of faecal pollution (IAWPRC Study Group, 1991). They are detectable by relatively simple methods which may yield results within 8 hours. Generally *E coli* C is used as host (Grabow *et al,* 1984; 1986; 1993). Coliphage counts may not always serve as reliable indicators of faecal pollution (Jofre *et al,* 1986) because they can also infect bacteria related to *E coli.* Failure to correlate the occurrence of viruses in marine waters and shellfish with indicator bacteria, the occurrence of viruses and coliphages in high numbers in waters which met bacteriological standards, and replication of bacteria and coliphages in marine waters during summer (Vaughn and Metcalf, 1974; Seeley and Primrose, 1980), raised concern about their indicator value (Gerba *et al,* 1979; Grabow *et al,* 1989; Cornax *et al,* 1991).

2.5.5.2. Male-specific coliphages

Male-specific (F-specific) phages cannot replicate at temperatures below 30°C because the adsorption site on the host, the F pili (sex fimbriae) are not formed below this temperature (Seeley and Primrose, 1982; Havelaar and Hogeboom, 1984; IAWPRC Study Group, 1991). This implies that they infect and replicate in the gastrointestinal tract of human and warm-blooded animals at 37°C, while chances for replication in the environment are extremely small (Seeley and Primrose, 1982; Havelaar and Hogeboom, 1984; Havelaar *et al,* 1986). These features make them highly specific for faecal pollution by man and warm-blooded animals. Male-specific phages, such as MS2, resemble human picornaviruses like polio, coxsackie and hepatitis A in size, structure, morphology and composition (Grabow, 1986). Male-specific coliphages are not as easily detectable as somatic coliphages (Seeley and Primrose, 1982; Havelaar and Hogeboom, 1984; Havelaar *et al,* 1986). The numbers of male-specific coliphages in water often tend to be relatively low, which casts doubt on their value as convenient indicators (Grabow *et al,* 1993).

2.5.5.3. *Bacteroides fragilis* phages

The indicator potential of bacteriophages of *Bacteroides* spp. was discovered by Jofre *et al* (1986) and Tartera and Jofre (1987). Bacteria of the *Bacteroides* group include *B fragilis, B distasonis, B thetaiotaomicrom,* and *B vulgatus* (Cato and Johnson, 1976). *Bacteroides* bacteria are among the most numerous bacteria in human faeces with numbers exceeding numbers of *E coli* by 100-fold (Finegold *et al,* 1983; Allsop and Stickler, 1984). *Bacteroides fragilis* is a normal inhabitant of the gastrointestinal tract of humans and warm-blooded animals (Jofre *et al,* 1986; Tartera and Jofre, 1987). *Bacteroides fragilis* is strictly anaerobic and strain HSP40 has the advantage that its phages are highly specific for human faecal pollution (Jofre *et al,* 1986; Tartera *et al,* 1988, 1989). In addition to the advantage of differentiating between human and animal faecal pollution, the behaviour and resistance of *B fragilis* HSP40 phages in the environment would seem to closely resemble that of human viruses (Tartera *et al,* 1989). However, the use of these phages as a universal indicator of faecal pollution would not appear advisable because they are present in low numbers in both sewage and in natural polluted waters, and because the detection of *B fragilis* phages is relatively complicated and requires the inclusion of recovery or enrichment procedures (Cornax *et al,* 1991).

2.5.6. Conclusions

Although phages have attractive features for assessment of the sanitary quality of shellfish, traditional indicators such as coliforms will probably always form part of the microbiological analysis of shellfish (Gerba, 1987).

In addition to commonly used indicators, other micro-organisms such as enteroviruses have been proposed as possible indicators of enteric pathogens because of the frequent isolation of these viruses from sewage-polluted waters (Coin *et al,* 1965). In countries where live attenuated poliovirus vaccine is administered to all infants three times during the first year of live, polioviruses could serve as an indicator (Goyal, 1983), but due to the inconsistent detection of poliovirus in countries like Israel, they were found unsuitable to serve as indicators of the virological quality of water (Katzenelson and Kedmi, 1979; Payment *et al,* 1979).

It would appear best to use combinations of indicators like coliforms, streptococci and bacteriophages for assessment of the presence or absence of enteric viruses in shellfish meat (Livingstone, 1976; Matches and Abeyta, 1983; Grabow, 1986). It must be emphasized, however, that no indicator can replace tests for enteroviruses themselves (Simkova and Cervenka, 1981). In view of bacterial indicator shortcomings and the need for further assessment of a bacteriophage indicator, it may be advisable to rather test shellfish for viruses themselves (Metcalf, 1978).

Since viruses are often transmitted by shellfish harvested form sewage-polluted marine environments (see Section 2.3), and technology for the direct virological analysis of shellfish has many shortcomings (see Section 2.7), research on practical methods for assessment of the virological safety of shellfish is essential. This research would include practical and reliable indicator systems, epidemiological data on viruses transmitted by shellfish, practical techniques for the virological analysis of shellfish, and the formulation of practical guidelines for the virological safety of shellfish.

2.6. DEPURATION AND DECONTAMINATION PROCEDURES

Details reviewed under Section 2.2.2 show that the market for molluscan shellfish is already extensive and rapidly growing in South Africa as in the rest of the world. Concern about the risk of infection associated with this seafood delicacy (see Section 2.3) has, therefore, become an issue with far-reaching public health and economic implications (Sockett *et al*, 1985; Grabow, 1989; West, 1989a). The risk of shellfish contamination increases as sewage pollution of many coastal areas increases, and is complicated by findings that conventional control measures which are adequate for bacterial pathogens and bacterial indicators of faecal pollution, are not good enough for viruses (see Sections 2.4 and 2.5).

Public health implications of shellfish consumption are based on concerns related to disease outbreaks from many parts of the world. Consumer confidence is, therefore, extremely sensitive and the slightest indication of a problem may immediately and completely terminate the utilisation of supplies concerned. Confidence is not restored by promises that the risk is most probably limited to a low mortality mild gastroenteritis. Any indication of infection is unacceptable to the consumer because the presence of any infectious agent confirms faecal pollution which is aesthetically unacceptable and implies that other pathogens with more serious complications may also be present. Economic implications may, for instance, result from safety testing programmes in which small random samples are tested from large supplies. If any one of these samples should fail the test, the entire supply is turned down with potentially enormous financial losses (Mitchell *et al*, 1966; Pain, 1986; Millard *et al*, 1987; PHLS Working Party, 1988).

In view of the above considerations it is important that shellfish supplies for the commercial market are safe, and that pollution of natural resources is limited. Basically the following three options are available to control the spread of disease by shellfish (WHO, 1974; Fleet, 1978; Willingham, 1982; West *et al*, 1985; West, 1986; Shuval, 1988; West, 1989a; Mallet *et al*, 1991):

1. Collection of shellfish from unpolluted sites.
2. Removal of pathogens from shellfish.
3. Inactivation of pathogens in shellfish meat.

These control measures are important and further details are as follows:

1. Collection of shellfish from unpolluted sites

Since shellfish accumulate pathogens from water in their environment, even low levels of pollution may constitute risks of significant contamination. Guidelines for the quality of seawater from which the harvesting of shellfish for human consumption is acceptable are, therefore, extremely tight (see Section 2.4). Particularly vulnerable are, for instance, members for the public, such as holiday makers, who walk along the beach and in good faith collect shellfish without knowledge of sewage pollution in the area.

2. Removal of pathogens

In practice this is mainly achieved by keeping shellfish harvested from polluted areas in unpolluted seawater under appropriate conditions and for suitable periods of time (Casagrande, 1978; Willingham, 1982; Board, 1983; West *et al,* 1985; Richards, 1988; Shuval, 1988). Success of the process depends on conditions which will promote normal feeding and filtration in the unpolluted water in order to release accumulated pathogens and to inactivate pathogens by metabolic processes. This is not easily achieved, because shellfish are sensitive to environmental stress and under suboptimal conditions they will not filter water which implies that the cleaning exercise is futile. There is no way of forcing or inducing shellfish into filter-feeding; they can only be nurtured into voluntary filter-feeding out of their own free will. In practice the mass dumping of shellfish in containers such as wire baskets in unpolluted seawater tends to create conditions which fail to promote normal feeding and metabolism. Success of the process furthermore very heavily depends upon the period of normal feeding and metabolism in the unpolluted water. As has been outlined in Section 2.3, all pathogens are not released at the same rate, and viruses take particularly long to be released, especially when accumulated in large numbers. It is, therefore, important to keep in mind that the time factor does not depend on the period for which the shellfish are kept in the unpolluted seawater, but the period of normal feeding and metabolism in the unpolluted water. After having been transferred under unnatural conditions, it may take a substantial period of time for shellfish to adapt to the new environment, settle down and start feeding (Sockett *et al,* 1985). In practice the removal of pathogens from contaminated shellfish is carried out mainly by one of the following approaches:

2.1. Relaying

This refers to the procedure in which shellfish harvested from polluted areas are transferred to unpolluted natural marine areas and kept there for appropriate periods of time in suitable

containers such as wire baskets. Removal and inactivation of pathogens released from contaminated shellfish to be purified, depends on natural die-off of pathogens in seawater. Obviously all these factors have to function optimally to ensure successful removal of pathogens. Relaying is not often applied because finding suitable sites becomes increasingly difficult due to escalating marine pollution, and transportation of shellfish to suitable areas is often expensive and labour intensive (Casagrande, 1978; Willingham, 1982; Board, 1983; West *et al,* 1985; Pain, 1986; Richards, 1988; Shuval, 1988; West, 1989a).

2.2. Depuration

Depuration refers to a process in which contaminated shellfish are kept in manmade containers, such as concrete ponds, with seawater for release of pathogens. The first facility of this kind on record was constructed in 1914 at Conway in North Wales (West, 1989a). Obviously the success of this process depends on a number of crucial factors. For instance, an artificial environment has to be created in which the shellfish feel comfortable and at home to the extent that they will proceed with normal filter-feeding and metabolism (Houser, 1965; Hamblet *et al,* 1969; Hurtley and Hammer-strom, 1971; Wood, 1976; Casagrande, 1978; Son and Fleet, 1980; Board, 1983; Pain, 1986; West, 1986; 1989a). This constitutes a challenge which may not always prove easy to accomplish in terms of, for instance, the environment, quality of seawater, temperature, turbidity, etc (Hamlet *et al,* 1969; Fleet, 1978). The importance of controlling depuration is illustrated by observations that *Vibrio vulnificus* bacteria multiply in oysters depurated at temperatures above 23°C with release of large numbers of these potential pathogens into the depuration water (Tamplin and Capers, 1992; Kaspar and Tamplin, 1993). This observation also emphasises the importance of decontaminations the water in conventional depuration systems based on the circulation of seawater in order to avoid recontamination of shellfish. This decontamination of circulating depuration water has challenges of its own because it has to be accomplish in a way which will not cause the shellfish to refrain from filter feeding. Water in depuration systems is generally expected to contain *E coli* counts of less than 1/ml (West, 1989a). Guidelines for depuration generally specify a minimum period of 36 - 48 h (Metcalf *et al,* 1979; West, 1989), but it is difficult to determine for how much of this time the shellfish were actually filter-feeding. However, evidence has been presented that viruses and phages may take much longer to be fully released (Sockett *et al,* 1985; Boher and Schwartzbrod, 1993; Humphrey and Martin, 1993). In South Africa there are no official guidelines or recommendations for any aspect of depuration. The following are examples of disinfectants used

for the decontamination of circulating seawater in depuration systems:

2.2.1. Chlorine

Seawater is decontaminated with hypochlorite and treated with sodium thiosulphate to remove residual chlorine which inhibits shellfish activity. This method is mainly used for less expensive shellfish such as mussels (Fleet, 1978; West *et al,* 1985; West, 1986; 1989a).

2.2.2. Ozone

Ozone (Burleson *et al,* 1975; Lawrence and Cappelli, 1977) is widely used in France. Ozone levels of 2 mg/l are required for seawater disinfection (Fleet, 1978). It is considered too expensive as an alternative to chlorination (Richards, 1988; West, 1989a) and oyster weakness and death have been reported on exposure to waters containing residual ozone (Wood, 1969).

Both chlorine and ozone can be very toxic to shellfish when mis-applied and are said by the connoisseurs to produce taint (West, 1986; 1989a).

2.2.3. Ultraviolet light

The application of ultraviolet light for decontaminating seawater in depuration systems was introduced in the UK in 1961 (Wood, 1961). This method of decontamination has important advantages for the particular purpose, and is widely applied (Wilingham, 1982; West *et al,* 1985; West, 1986; 1989a). As far as could be established it is the only method for decontamination used in South Africa. The most important advantages are that treatment does not change the quality of the water and does not introduce compounds such as chlorine residuals which may be objectionable to the shellfish. Operation is also easier than the careful control of disinfection and neutralisation required in the case of chlorination. However, even this system has frequently been observed to fail due to poor supervision and control (Pain, 1986; West, 1989a; Schwartzbrod, 1991). Ultraviolet light at a wavelength of 245 nm is recommended. This light has limited penetration power, and various devices are being used to irradiate a thin film of water. Even then disinfection efficiency heavily depends upon factors such as turbidity, flow rate, accumulation of dirt on the surface of the lamp, and the condition of the lamp (West, 1989a).

3. Inactivation of pathogens in shellfish meat

Infections can be prevented by inactivating pathogens in contaminated shellfish prior to consumption. This is not so easy because the flavour, taste and texture of the shellfish flesh is easily lost, to the extent that many prefer to consume shellfish, particularly oysters, raw (Liston, 1989). The following are examples of methods used for the disinfection of shellfish prior to consumption:

3.1. Heat treatment

This method is the most commonly used method of disinfection. Shellfish are often prepared by frying, stewing, steaming or cooking, which may inactivate pathogens. However, these preparation procedures are generally applied mildly or partially in order to retain as much as possible of the seafood flavour (Di Girolamo *et al,* 1970; PHLS Working Party, 1988; Liston, 1989; Nightingale, 1990). Furthermore, some pathogens, especially viruses, are surprisingly resistant to heat treatment, particularly when located deep inside in tissues (Di Girolamo *et al,* 1970). Experimental data indicate that an internal temperature of 98°C for 1 min is required to inactivate hepatitis A virus (West *et al,* 1985; Millard *et al,* 1987), and that viruses in shellfish meat can survive up to 30 min of steaming (Pain, 1986).

3.2. Gamma irradiation

The use of gamma radiation has been proposed as a potential means of eliminating pathogenic microorganisms from a variety of foods, including shellfish. However, various data indicate that viruses are more resistant to gamma radiation than other microorganisms such as bacteria. Higher doses of radiation would possibly prove more effective but it can cause organoleptic changes rendering the shellfish unpalatable (Liuzzo *et al,* 1967; Sullivan and Read, 1968; Sullivan *et al,* 1971; Di Girolamo *et al,* 1972; Stammen *et al,* 1990).

3.3. Freezing

Viruses are very stable in shellfish held in the frozen state (Lynt, 1966; Heidelbaugh and Giron, 1969; Di Girolamo *et al,* 1970). Results by Di Girolamo *et al* (1970) indicate that 91% of polioviruses were viable after 2 weeks of freezing, 40% were still viable at 6 weeks, and 10% still survived after 12 weeks of freezing. Lynt (1966) reported that poliovirus type 1 and coxsackievirus types B1 and B6 survived for 5 months in inoculated frozen foods held at -20°C.

3.4. Storage

Poliovirus in shellfish proved to be very stable under storage conditions. Di Girolamo *et al* (1970) reported that after 5 days of storage at 5°C, virus numbers were reduced by only 10% while after 15 days of storage virus numbers were reduced by 60%.

Gamma irradiation, freezing and storage are not widely used as methods for the decontamination of shellfish prior to consumption due to limited efficiency.

The following strategies for controlling the safety of shellfish supplies have been recommended (Anonymous, 1987; West, 1986; PHLS Working Party, 1988):

1.	Shellfish	:	improve heat processing
		:	investigate improved methods of depuration of oysters
		:	routine bacteriological examination
2.	Foodhandlers	:	re-emphasise personal hygiene
		:	reduce direct handling
		:	re-examine cross-contamination prevention practices
3.	In the event of an outbreak	:	discontinue high-risk foods
		:	decontaminate environment thoroughly and regularly

2.7. METHODS FOR THE MICROBIOLOGICAL ANALYSIS OF SHELLFISH

A wide variety of methods has been described for assessment of the hygienic quality of shellfish meat. The most important of these may be summarised as follows:

2.7.1. Bacteria

Methods generally recommended for bacteriological analysis include the following (Neufeld, 1985; Subcommittee on Microbiological Criteria, 1985; Grabow *et al,* 1992):

2.7.1.1. Heterotrophic plate count (standard plate count)

In this test, samples of shellfish meat suspensions are mixed with molten nutrient agar and poured into petri dishes for incubation at 37°C/48 h (Abeyta, 1983; Saddik *et al,* 1985). The test detects a wide variety of organisms, primarily bacteria, which give an indication of the general microbiological quality of shellfish (Grabow, 1986; 1990; Grabow *et al,* 1980). The heterotrophic plate count does not differentiate types of bacteria. The test is simple and inexpensive and yields results in a relatively short time. The heterotrophic plate count of shellfish intended for human consumption is generally expected not to exceed 100 000/g (Departement of Health, 1973; Grabow 1986; 1990; Grabow *et al,* 1980).

2.7.1.2. Total coliform bacteria

The term "coliform bacteria" refers to a vaguely defined group of Gram-negative bacteria (Mehlman, 1984; Grabow *et al,* 1991) (see Section 2.5). Total coliform bacteria in shellfish are best determined by spread plate procedures using M-Endo LES agar and incubation at 37°C/24 h (Grabow *et al,* 1991). This relatively simple and inexpensive test is primarily used for assessment of the general sanitary quality of shellfish.

The test has the following advantages:

(a) The test includes bacteria such as *Klebsiella* and *Aerobacter* which are more resistant than most pathogens.

(b) Isolates can be identified, and the presence of *Escherichia coli* renders almost conclusive evidence of faecal pollution.

2.7.1.3. Faecal coliform bacteria

These bacteria are primarily used for the assessment of faecal pollution in shellfish. Faecal coliforms are more closely associated with faecal pollution than total coliforms (see Section 2.5). Faecal coliforms in shellfish are best determined by spread plate procedures using M-FC agar and incubation at 44,5°C/24 h. This relatively simple and inexpensive test is widely used in routine monitoring of shellfish. It has the advantage that colonies can be identified (Grabow, 1990; Grabow *et al,* 1992).

2.7.1.4. *Escherichia coli*

Escherichia coli is a member of the faecal coliform group. It ferments lactose and produces indole at 44°C/24 h (American Public Health Association, 1992), and is a highly specific

indicator of faecal pollution (see Section 2.5). A variety of different methods and selective growth media has been described for enumerating *E coli* in shellfish meat. West and Coleman (1986) found that a most probable number (MPN) procedure using Minerals-Modified-Glutamate Broth (MMGB) yielded higher counts than pour and spread plate methods with MacConkey or Tryptone Soya agar (Motes *et al,* 1984). Humphrey and Gawler (1986) recommended an MPN technique using peptone water for the enrichment of *E coli*. Grabow *et al* (1991) reported that the MMGB MPN procedure yielded higher counts than a membrane filtration method in which shellfish homogenates were rendered filterable by digestion with trypsin and prefiltration. In a comparison of various methods the highest counts were obtained with a M-FC spread plate method for faecal coliforms followed by the identification of *E coli* using indole production at 44°C (Grabow *et al,* 1991).

2.7.1.5. Faecal streptococci/enterococci

This group of Gram-positive bacteria, many of which are of faecal origin, tends to be more resistant than coliform bacteria, particularly in marine environments, and are therefore often used for the assessment of the quality of shellfish meat (Grabow, 1986) (see Section 2.5). In a comparison of various procedures, Grabow *et al* (1991) obtained the best results with a spread-plate procedure using M-Enterococcus agar and incubation at 37°C.

2.7.1.6. *Staphylococcus aureus*

Staphylococcus aureus bacteria are part of the normal microbial flora associated with humans and to a lesser extent with animals. They are, therefore, sometimes used as indicators of pollution specifically related to humans (Charoenca and Fujioka, 1993). In addition, these Gram-positive bacteria are of health significance because they are often involved in secondary skin and related infections. In view of these features *S aureus* is sometimes included in combinations of indicators used for assessment of the hygienic quality of shellfish meat (Departement of Health, 1973; Grabow, 1986; 1990). Detection methods are generally based on cultivation using media with high salt concentrations for selection followed by identification using a test for DNAse activity which is highly specific for *S aureus*. Best results have apparently been obtained with the Baird-Parker agar medium for spread plate recovery of *S aureus* followed by purification on manitol salt phenol red agar and confirmation of manitol fermenting isolates by the DNAse test (Rayman *et al,* 1979; Tatini *et al,* 1984; Greenwood *et al,* 1985; SABS, 1989).

2.7.1.7. *Salmonella*

Salmonella bacteria include a number of species which cause intestinal infections. One of them is *S typhi*, which causes typhoid fever. These Gram-negative bacteria have a long history of transmission by shellfish (see Section 2.3) and are included in many guidelines for shellfish quality (see Section 2.4). A variety of methods are available for the detection of *Salmonella* (Lapage *et al*, 1979; SABS, 1989; Ministry of Agriculture, 1992), using agar media such as *Salmonella-Shigella* (SS), xylose lysine desoxycholate (XLD), bismuth sulphite (BS) and brilliant-green phenol-red lactose sucrose (BPLS) agar. Biochemical and serological tests are necessary for the confirmation of *Salmonella* and the identification of species (Poelma *et al*, 1984; Greenwood *et al*, 1985).

2.7.1.8. *Shigella*

These bacteria include highly pathogenic species, such as *S dysenteriae*, which causes dysentry. *Shigella* bacteria are generally transmitted by the faecal-oral route and may, therefore, potentially be transmitted by shellfish. Detection of *Shigella* in shellfish is usually done by an enrichment procedure followed by subculturing to SS agar, xylose lysine desoxycholate (XLD), bismuth sulphite (BS) and brilliant-green phenol-red lactose sucrose (BPLS) agar (Lapage *et al*, 1979; Hackney *et al*, 1980; Wait *et al*, 1983; Morris, 1984; SABS, 1989; Ministry of Agriculture, Britain, 1992).

2.7.1.9. *Clostridia*

One of the members of the group, *Clostridium perfringens*, is like *E coli* highly specific for faecal pollution because it almost exclusively multiplies in the gastro-intestinal tract of man and warm-blooded animals (see Section 2.5). *Clostridia* are generally detected by cultivation on differential re-inforced clostridium medium followed by subculturing suspect colonies for confirmation and identification of *C perfringens* (Harmon and Duncan, 1984; SABS, 1989).

2.7.1.10. *Vibrio*

Vibrios are motile, curved, rod-shaped, gram-negative bacteria (Koenig *et al*, 1991) and certain species are one of the major groups of bacteria found in the marine environment (Chan *et al*, 1989; West, 1989b). The genus also includes *V cholerae* which may cause cholera, *V parahaemolyticus* which may cause severe gastroenteritis (see Section 2.3), and *V vulnificus* which may cause life-threatening wound infections and septicaemias (Massad and Oliver, 1987).

Detection of *Vibrio* species in shellfish is generally done by primary enrichment in double strength vibrio enrichment medium (De Paola *et al,* 1987). Subculturing is usually done on thiosulphate-citrate-bile salt agar (TCBS), and the morphology and colour of the colonies provide useful information for purposes of identification (Furniss and Donovan, 1974; West *et al,* 1982; Colwell, 1984; Twedt *et al,* 1984; Farmer *et al,* 1985; Bryant *et al,* 1986). More recently, however, Massad and Oliver (1987) reported that a cellobiose-polymyxin B-colistin agar medium was more selective for *V cholerae* and *Vulnificus* (Miceli *et al,* 1993). A trypticase soy agar supplemented with sucrose, sodium chloride, bile salts, and triphenyl-tetrasolium chloride, proved suitable for the isolation of *V parahaemolyticus* and the differentiation of this organism from *V alginolyticus* and other bacteria (Kourany, 1983).

2.7.2. Protozoan parasites

Parasitic infections have rarely been associated with the consumption of shellfish and other seafoods (see Section 2.3). It would appear particularly surprising that transmission by shellfish of intestinal parasites such as *Cryptosporidium* and *Giardia*, which are frequently transmitted by sewage-polluted drinking water supplies (Casemore, 1991; Jakubowski *et al,* 1991), has apparently never yet been described. Theoretically, however, it would seem possible that the cysts, oocysts and even other stages of the life cycle of various parasites may, like pathogenic viruses and bacteria, also be transmitted by shellfish exposed to sewage-polluted seawater.

Although it may be argued that shellfish which conform to quality guidelines for indicators such as faecal bacteria, viruses and phages have never been associated with parasitic infections, the incidence and behaviour of these pathogens in marine environments differs widely from that of faecal bacteria. Commonly used bacterial indicators can, therefore, hardly be excepted to reliably indicate the presence of intestinal parasite cysts or oocysts.

Methods which have been described for the detection of parasitic cysts/oocysts in environmental samples, primarily water and wastewater, but also shellfish meat, include the following:

1. Immunofluorescent microscopy in which monoclonal antibodies coupled with a fluorescent brightener are used to facilitate the microscopic detection of cysts/oocysts (Rose, 1988; Rose *et al,* 1989; Smith *et al,* 1989; Anonymous, 1990). The monoclonal

antibody preparations are commercially available and highly specific for the cysts/oocysts of different parasites.

2. Molecular techniques in which the DNA of cysts/oocysts is detected by means of gene probes or the polymerase chain reaction (PCR) (Johnson *et al,* 1993).

The viability of cysts/oocysts is generally determined by:

1. *In vitro* excystation tests in which the release of sporozoites from oocysts is artificially induced by incubation in a medium containing bile salts (Current, 1986; Isaac-Renton *et al,* 1992).
2. Animal infectivity tests in which suitable animals such as mice are orally inoculated with cysts/oocysts and observed for completion of the parasite life cycle including the faecal excretion of new cysts/oocysts (Jarrol, 1988; DeRegnier *et al,* 1989; Isaac-Renton *et al,* 1992). Despite the disadvantages of cost and labour, *in vivo* excystation appears to be more useful than *in vitro* excystation for isolate retrieval.

2.7.3. Enteric viruses

There are four major considerations in the development of methods for the recovery of viruses from shellfish (Metcalf and Stiles, 1965; Vaughn and Metcalf, 1975; Sobsey *et al,* 1978; Vaughn *et al,* 1979; Richards *et al,* 1982; Gaillot *et al,* 1988):

1. The relative ease by which the experiment can be carried out without the need for expensive reagents or equipment.
2. The method must be sensitive enough to detect small numbers of virus from a few grams of shellfish.
3. The removal of toxic compounds associated with shellfish which may interfere with virus assay in cell cultures.
4. The recovery of representative members of viruses such as entero-, adeno-, and reoviruses associated with sewage pollution.

While no single method is able to fulfil the above criteria, methods developed over the past several years have greatly facilitated the recovery of viruses from shellfish. Most of these recovery methods are based on acid precipitation as described by Sobsey *et al* (1978), and the organic flocculation (OF) of Katzenelson *et al* (1976). Although good recoveries of enteric

viruses have been reported, both methods have some disadvantages such as fluctuations in pH which may compromise the viability of some viruses like human rotavirus, and the time it takes to perform each experiment (Vaughn *et al,* 1979; Speirs *et al,* 1987). Idema *et al* (1991) described a method in which shellfish meat is homogenised in pH 8,0 glycine-saline buffer. The suspension is centrifuged, and the supernatant tested for viruses. The efficiency of recovery for polio 1 was 91%.

The great majority of viruses of primary concern, such as Norwalk, astro and calici, are not detectable by conventional virological methods (Dahling and Wright, 1986; Anonymous, 1989; West, 1990; de Mesquita *et al,* 1991) and a new generation of methods based on nucleic acid detection (Jiang *et al,* 1986; Margolin *et al,* 1986; Gerba, 1988; De Leon and Gerba, 1991; Dubrou *et al,* 1991; Zhou *et al,* 1991) as well as the polymerase chain reaction (PCR) (Bej *et al,* 1990; Lampel *et al,* 1990; Hill *et al,* 1991; Atmar *et al,* 1993) will probably facilitate the detection of these viruses in shellfish. By using PCR it is theoretically possible to detect the nucleic acid of a single virus in environmental samples such as shellfish meat (Cherfas, 1990). However, PCR cannot distinguish between viable and non-viable viruses.

Techniques for the recovery of viruses from shellfish and subsequent detection are dealt with in more detail in Parts 4 and 5.

2.7.4. Phages

Phages are viruses which infect bacteria (IAWPRC Specialist Group, 1992). They have the following attractive features as indicators for human viruses:

1. Basically phages closely resemble human viruses in terms of size, morphology, structure, composition and behaviour in the environment.
2. Are detectable by simple, inexpensive and rapid techniques, and constitute no health risk.

Information on techniques for the recovery of phages from shellfish meat is limited. Procedures to isolate coliphages form shellfish involve blending of meats with a suitable diluent, then clarification of the sample by centrifugation (Kennedy *et al,* 1986). It may be desirable to treat the sample with chloroform in order to reduce the counts of contaminating bacteria which may

interfere with reading the phage assay plates (West and Wipat, 1988). Methods to detect coliphages usually involve the mixing of a sample of clarified material with a culture of a susceptible host bacterium, and suitable overlay medium, and pouring onto a solid agar surface (Kennedy *et al,* 1986; West and Wipat, 1988). Brodisch *et al* (1986) evaluated an adsorption-elution-flocculation procedure developed by Sobsey *et al* (1978) for the recovery of human viruses from shellfish. The average efficiency of recovery for two coliphages from shellfish was only 2%, possibly due to inactivation of the phages by exposure to high and low pH levels which form part of the procedure.

After recovery from shellfish meat phages are generally detected by phage assays (Adams 1959; Grabow *et al,* 1984; Debartolomeis and Cabelli, 1991). Phages may, however, also be detected by electron microscopy (EM) for specific purposes such as establishment of the morphology of the phages (Ackermann and Nguyen, 1983).

2.7.5. Other parameters

Various other tests are also included in the assessment of the suitability of shellfish for human consumption. These include:

Organoleptic examination: For the evaluation of quality attributes such as taste, colour and appearance.

pH changes: The pH of molluscs decreases during spoilage.

Indole: Indole is formed by the action of certain bacteria, such as *Proteus* spp and *E coli*, on the tryptophan present in oyster tissue (Subcommittee on Microbiological Criteria, 1985).

2.8. CONCLUSIONS

The transmission of infectious diseases by the consumption of contaminated shellfish such as oysters, mussels and clams has been described in many parts of the world. The exceptional tendency of these animals to transfer infections is due to their feeding by the filtration of large volumes of water, which results in the accumulation of pathogens in sewage-polluted sea water. The risk of infections is increased by the preference of many consumers to eat shellfish, particularly oysters, raw.

Although a wide variety of pathogenic micro-organisms may be involved, viruses are for various reasons responsible for the great majority of infections. The classical example is an outbreak in 1988 in China involving some 25 000 cases of viral gastroenteritis and 300 000 cases of hepatitis A due to the consumption of clams harvested from a sewage-polluted marine environment.

As a result of the risk of transmission of infectious diseases, microbiological safety is the most important concern about the quality of shellfish. The shellfish market is extremely sensitive in this regard, and any indication of faecal contamination or potential presence of pathogens is sufficient to discard supplies or close industries. The socio-economic impact of restrictions for the microbiological quality of harvesting grounds and shellfish supplies on the shellfish industry has, for instance, been described by Menon (1988).

The transmission of diseases by shellfish has not yet been recorded in any detail in South Africa. However, the risk of infection must exist as much as in other parts of the world because shellfish are as popular a seafood delicacy as elsewhere, and sewage pollution of marine environments does occur. Research on viruses and other pathogens in shellfish is, therefore, warranted in terms of public health, economic implications for the seafood industry, and details on the impact of marine pollution.

The literature review disclosed shortcomings in technology and information on issues such as methods for the recovery of viruses and related indicators from shellfish meat, the release of viruses and indicators by shellfish during depuration processes, and guidelines for the microbiological quality of shellfish intended for human consumption.

Presently available information does, therefore, reveal a need for research on a variety of relevant issues including the following:

a. Assessment and optimisation of techniques for the recovery of viruses and related indicators, notably phages, from shellfish meat.
b. Details on the release of viruses and related indicators by shellfish during depuration processes.
c. Information on the microbiological quality of naturally occurring shellfish and commercially marketed supplies of shellfish in South Africa.

d. Information required for recommendations on guidelines for the quality of shellfish intended for human consumption, as well as wastewater discharge to marine environments and depuration processes applied in the mariculture industry.

The primary objective of this study was to address some of the above issues, as outlined in broad perspective in Part 1.

3. SELECTION OF INDICATOR ORGANISMS AND METHODS FOR THEIR DETECTION

3.1. INTRODUCTION

In Part 2 it has been pointed out that shellfish harvested from sewage-polluted marine environments may contain a wide variety of pathogens. The pathogens of primary concern include many different viruses and bacteria. It would appear possible that even the eggs of helminthic parasites, and the cysts or oocysts of protozoan parasites, may potentially be transmitted by shellfish. The practical implication for quality assessment, and even more so for routine quality surveillance programmes, is that it is impossible to test shellfish for all the pathogens they may harbour. Many of the tests that would have to be carried out are complicated, expensive, labour-intensive and time-consuming. Even more important, however, is that practical test methods are not yet available for many of the pathogens concerned. Shortcomings in technology for quality testing applies in particular to viruses, which are most commonly transmitted by contaminated shellfish. Practical detection techniques are not yet available for the great majority of viruses concerned, and the detection of even those viruses for which techniques are available, is relatively complicated and expensive, and time and labour-intensive.

Assessment of the fitness of shellfish for human consumption is, therefore, generally based on indirect approaches. Indicator organisms, as described in Part 2, are primarily used for this purpose. Indirect quality assessment also includes approaches such as sanitary surveys of shellfish harvesting grounds for evidence of sewage pollution which may contaminate the shellfish.

In endeavours to find the most reliable and practical indicator, or combinations of indicators, a wide variety of indicator organisms, and methods for their detection has been described (see Part 2). In fact, the variety of indicators and methods for their detection is so wide that it would be impractical, and virtually impossible, to apply all or most of them in quality assessment or routine surveillance programmes. In terms of the requirements of indicators outlined in Part 2, each of the indicators described in the literature has its own advantages and disadvantages.

One of the fundamental objectives of this study was, therefore, to select a number of indicators and detection techniques which, according to available information, have the most promising features for practical quality assessment, and to evaluate these for routine application in research and surveillance programmes. A major goal of this study is to evaluate the reliability of individual indicators, or combination of indicators, for assessment of the presence of human viruses in shellfish. This Part summarises the indicators and techniques selected for this purpose, and the reasons for their selection. It should be noted that the study is restricted to human pathogens associated with sewage pollution. The study does not make provision for organisms which naturally occur in marine environments and may cause disease in humans, or toxins from naturally occurring marine organisms which sometimes contaminate shellfish. Neither does the study make provision for chemical compounds, such as heavy metals or organic compounds, with which marine environments are sometimes polluted to the extent that the safety of shellfish for human consumption may potentially be at risk.

3.2. INDICATORS AND METHODS SELECTED

3.2.1. Bacteria

The following bacterial indicators were selected:

1. Total coliforms
2. Faecal coliform bacteria
3. Faecal streptococci
4. *Escherichia coli*
5. *Clostridium perfringens*

Reasons for selecting these indicators, and the methods for their detection, are:

3.2.1.1. Total coliforms

Total coliforms are world-wide used as indicator of the general hygienic quality of water and food, and of potential sewage pollution (Grabow *et al,* 1989). Even though this vaguely defined group of bacteria has limited specificity for sewage pollution and seems to poorly correlate with the presence of viruses and more specific indicators of faecal pollution, it has been included because world-wide it forms part of most quality guidelines and specifications for shellfish meat and seawater at harvesting grounds (Part 2; Grabow *et al,* 1989).

The method selected for enumerating total coliforms is the spread plate technique using M-Endo Les Agar and incubation at 35 - 37°C for 24 h as described by Grabow *et al* (1989). In a detailed comparison of most probable number (MPN) procedures using minerals-modified-glutamate broth, membrane filtration procedures on trypsin-digested shellfish meat, and spread- and pour plate tests using homogenised but undigested shellfish meat, the M-Endo Les Agar spread plate method proved the most reliable, practical and inexpensive, and yielded results in the shortest time (Grabow *et al,* 1991; 1993).

3.2.1.2. Faecal coliform bacteria

Faecal coliforms are world-wide used as relatively specific indicators of faecal pollution of water and food (Grabow *et al,* 1989). Certain members of this group of bacteria may multiply in natural water environments, and all members of the group are generally less resistant to unfavourable conditions than viruses. In addition, these bacteria seem to have shortcomings for indicating the presence of viruses in sewage polluted water and shellfish meat (Grabow *et al,* 1989). The indicator was included because world-wide it forms part of many quality guidelines and specifications for sewage pollution of marine-environments, shellfish meat, and seawater at harvesting grounds (Part 2; Grabow *et al,* 1989).

The method selected for enumerating faecal coliforms is the spread plate technique using M-FC Agar without rosolic acid and incubation at 44°C $\pm$ 0,2°C for 24 h as described by Grabow *et al* (1989). In a detailed comparison of most probable number (MPN) procedures using minerals-modified-glutamate broth, membrane filtration procedures on trypsin-digested shellfish meat, spread- and pour plates using homogenised but undigested shellfish meat, and M-FC or mTEC agar, the M-FC Agar spread plate method proved the most reliable, practical and inexpensive, and yielded results in the shortest time (Grabow *et al,* 1991; 1993).

3.2.1.3. Faecal streptococci

Faecal streptococci are widely accepted as relatively specific indicators of faecal pollution of water and food (Grabow *et al,* 1991; Standard Methods, 1995). Certain members of this group of bacteria may multiply in natural water environments, and members of the group may at least under certain circumstances be less resistant to unfavourable environmental conditions than viruses. In addition, these bacteria seems to have shortcomings for indicating the presence of viruses in sewage polluted water and shellfish meat (Grabow *et al,* 1991). The indicator was

included because it forms part of some quality guidelines and specification for sewage pollution of marine environments, shellfish meat, and seawater at harvesting grounds (Part 2; Grabow *et al,* 1991). Faecal streptococci would also seem to be more resistant to unfavourable conditions than faecal coliforms, particularly in marine environments (Grabow *et al,* 1991).

The method selected for enumerating faecal streptococci is the spread plate technique using M-Enterococcus Agar and incubation at 35 - 37°C for 48 h as described by Grabow *et al* (1991). In a detailed comparison of membrane filtration on trypsin-digested shellfish meat, and spread- and pour plate tests using homogenised but undigested shellfish meat, incubation at 37°C or 44,5°C, and M-Enterococcus and M-E agar media, the M-Enterococcus Agar spread plate method and incubation at 35 - 37°C for 48 h proved the most reliable, practical and inexpensive, and yielded results in the shortest time (Grabow *et al,* 1991). The latter study on techniques for the enumeration of faecal streptococci was carried out as part of this project, but since it has been published in detail (Grabow *et al,* 1991), technical details are not included in this report.

3.2.1.4. *Escherichia coli*

Escherichia coli is a highly specific indicator of faecal pollution of water and food because it is not able to multiply in natural water environments or in shellfish (Grabow *et al,* 1992; Standard Methods, 1995). Generally speaking *E coli* is less resistant to unfavourable environmental conditions than viruses, which may to a large extent account for shortcomings as indicator for the presence of viruses in sewage polluted water and shellfish meat (Part 2; Grabow *et al,* 1992). The indicator was included because world-wide it forms part of many quality guidelines and specifications for sewage pollution of marine environments, shellfish meat, and seawater at harvesting grounds (Part 2; Grabow *et al,* 1992).

The method selected for enumerating *E coli* is the spread plate technique using M-FC Agar without rosolic acid and incubation at 44,5 $\pm$ 0,2°C for 24 h, followed by the purification of colonies and testing indole production at 44,5 $\pm$ 0,2°C as described by Grabow *et al* (1992). In a detailed comparison of most probable number (MPN) procedures using minerals-modified-glutamate broth, membrane filtration procedures on trypsin-digested shellfish meat, and M-FC or m-TEC agar, the above M-FC Agar spread plate method proved the most reliable, practical and inexpensive, and yielded results in the shortest time (Grabow *et al,* 1992). Since the evaluation of techniques for enumerating *E coli* and faecal coliforms in shellfish has been

published in detail (Grabow *et al*, 1992), technical details of the study are not duplicated in this report.

3.2.1.5. *Clostridium perfringens*

Clostridium perfringens is a highly specific indicator of faecal pollution of water and food because it is not able to multiply in natural water environments or in shellfish (Burger *et al*, 1984; Standard Methods, 1995). A major advantage of *C perfringens* is that its spores are more resistant to unfavourable conditions than any vegetative pathogenic bacteria and viruses tested to date. *Clostridium perfringens* is, therefore, often used as indicator of remote faecal pollution. The organism tends to be considered as too resistant for general purposes of quality assessment. In addition, test methods for *C perfringens* are not as simple and inexpensive as for coliform bacteria (Part 2; Burger *et al*, 1984). Despite attractive indicator features, *C perfringens* is rarely included in quality guidelines primarily as a result of the latter two disadvantages (Grabow *et al*, 1989). This indicator was nevertheless included, mainly for assessing its value as indicator for viruses.

The method selected for enumerating *C perfringens* is the membrane filtration method for water using lecithinase agar (Burger *et al*, 1984), or a spread plate technique using the same medium. In a detailed comparison of a number of growth media and test procedures Burger *et al* (1984) found that the above method was the most practical, reliable and inexpensive.

3.2.2. Bacteriophages

The use of bacteriophages (phages) as indicators of the hygienic quality of water and food is gaining ground, primarily because phages share properties with viruses in terms of structure, composition and size, because their survival in water and food resembles that of viruses to a closer extent than faecal bacteria, and because they are detectable by relatively simple, practical, inexpensive and rapid techniques (Part 2; IAWPRC Study Group, 1991; ISO, 1995a,b).

Indicator features of a wide variety of phages have been described in the literature (Grabow *et al*, 1993). After careful evaluation of available information, the following phages were selected for this study:

1. Somatic coliphages
2. Male-specific coliphages
3. *Bacteroides fragilis* HSP40 phages

Reasons for selecting these phages, and the methods for their detection, are:

3.2.2.1. Somatic coliphages

Somatic coliphages represent a large group of phages which infect various coliform bacteria. They do, therefore, occur in large numbers in sewage polluted water, and are associated with sewage pollution as are total coliform bacteria (Part 2; Grabow *et al,* 1993). Somatic coliphages are not specific for faecal pollution, because at least some of them may be replicated by host bacteria in suitable water and food environments. An outstanding feature of some somatic coliphages is that they are detectable by simple techniques. Together with other advantages, this group of phages is most commonly used in quality assessment and increasingly proposed as indicator for water and food (Part 2; IAWPRC Study Group, 1991; ISO, 1995a,b).

The method of choice for this study was a double agar layer plaque technique using *Escherichia coli* strain C (ATCC 13706) as host (Grabow and Coubrough, 1986; Grabow *et al*, 1993; ISO, 1995b). This method has been selected in a comparative study on various modifications of media and test procedures, and different host strains of *E coli.*

3.2.2.2. Male-specific coliphages

Male-specific (MS) coliphages represent a group of phages which infect those *E coli* bacteria which carry the fertility factor (F) which codes for the production of sex fimbriae on which the receptor sites for male-specific phages occur (Part 2; IAWPRC Study Group, 1991; Grabow *et al,* 1993; ISO, 1995a). These phages will, however, also infect other related bacteria which carry the *E coli* F factor (Debartolomeis and Cabelli, 1991). Since sex fimbriae are produced only at temperatures higher than about 30°C, MS phages are highly specific for faecal and sewage pollution. MS coliphages are not as easily detected as somatic coliphages, and their numbers are generally lower than those of somatic coliphages in water and food environments. Despite shortcomings, they have been included in this study, primarily because of their specificity for sewage pollution.

The method selected for this study is the double agar layer plaque technique (Grabow et al, 1993; ISO, 1995a), using *Salmonella* strain 3 Nal^r (F'lac::Tn5) WG49 as host. This procedure has been selected by an international working group of experts in the field, and is recommended as an international standard method. In some tests the same procedure was followed except that *E coli* strain HS(pFamp)R (Debartolomeis and Cabelli, 1991) was used as host.

3.2.2.3. *Bacteroides fragilis* HSP40 phages

Bacteroides fragilis HSP40 phages (phages which infect *B fragilis* strain HSP40) were included primarily because they are highly specific for human faecal pollution (Part 2; Grabow *et al*, 1993). Available information also suggests that they are more resistant than many viruses. These phages are, however, not as easily detected as coliphages, primarily because the host is a strict anaerobe and conditions of cultivation and maintenance of the host require carefully controlled conditions.

The method used in this study is the double agar layer technique described by Tartera *et al* (1987) using *B fragilis* HSP40 as host. No alternative techniques or modifications have as yet been described.

3.3. DISCUSSION

The indicators and methods for their detection which have been selected for this study include the most commonly used indicators as well as new indicators, particularly the phages, which may prove of value as individual indicators, or for inclusion in combinations of appropriate indicators, for assessment of faecal pollution of marine environments and contamination of shellfish meat.

Information on certain new detection methods was obtain too late for consideration in this study. This includes the MUG technique for the detection of *E coli* (ISO/CD 9308-3, 1993) and the MUD method for the detection of faecal streptococci (ISO/CD 7899-1, 1993). The application of these new methods in assessment of the sanitary quality of seawater and shellfish should be investigated.

No information is available on the extent to which the indicators included in this study may serve

to indicate the presence of the eggs of helminthic parasites, or the cysts or oocysts of protozoan parasites, in sewage polluted marine environments or shellfish meat. Although no meaningful evidence has yet been presented that these eggs, cysts or oocysts may be transmitted by shellfish, the value of indicators for the presence of these pathogens in shellfish meat should be investigated.

This study is also not intended to cast light on the value of the indicators used for pathogens such as *Leptospira monocytogenes*, which has recently caused lethal encephalitis in two children in New Zealand. Suspicion that the infection may have been associated with the consumption of mussels, led to the closing down of the New Zealand mussel industry, with devastating financial consequences (Baker, 1993).

4. SELECTION OF ENTERIC VIRUSES AND METHODS FOR THEIR DETECTION

4.1. INTRODUCTION

Literature on the wide variety of pathogens that may be transmitted by shellfish, and the prominent role of viruses among these pathogens, has been reviewed in Part 2. This study focuses on techniques for the recovery of viruses from shellfish meat, and the application of these techniques in research on the behaviour of the viruses in shellfish. In Part 2 it has also been pointed out that the great majority of the viruses concerned is not detectable by conventional techniques. This study is, therefore, limited to viruses which are readily detectable, and may serve as surrogates or indicators of the great majority of human viruses concerned.

Human viruses are primarily detected by the following methods:

4.1.1. Isolation

This basically refers to the propagation of viruses in cell cultures. In exceptional cases experimental animals are used, and in some studies on viruses not detectable by any other method, even human volunteers have been used (Ward *et al,* 1986; Guttman-Bass, 1987). Well established procedures are available for the propagation of viruses in cell culture. In laboratories with the appropriate facilities and expertise, these procedures are relatively simple and inexpensive. Important advantages include the relatively sensitive detection of viable viruses. The most important disadvantage is that most of the viruses concerned are not readily detectable, or not detectable at all, by presently available cell culture systems (Taylor *et al,* 1993). In addition, these procedures are time consuming and labour intensive.

The multiplication of viruses in cell cultures is generally detected by cytopathogenic effect (CPE) which is the result of visible damage caused to cells by replicating viruses. Viruses are generally enumerated by infecting dilutions of test samples into multiple series of tubes or wells in microtiter plates containing cell culture. The combination of tubes or wells in which a cytopathogenic effect develops, is then used to calculate the titre in the test sample using various statistical approaches, including the 50% tissue culture infectious dose (TCID50) principle, or a most probable number (MPN) calculation (Reed and Muench, 1938; Thomas, 1942; Grabow

and Nupen, 1981). Viruses may also be detected and quantitated by plaque assays, in which appropriate dilutions of test samples are inoculated onto layers of cell cultures in petri dishes of large well microtitre plates (Cromeans *et al,* 1987). These inoculated cell layers are covered with a layer of soft agar, and incubated. Each replicating virus then destroys its host cell, and the offspring spreads to neighbouring cells which are likewise destroyed, resulting in circular areas of cell destructing which are clearly visible, particularly after staining. These clear zones are known as plaques, and can be counted for direct counting of the number of viable viruses in the test sample. This method of enumeration is referred to as plaque assays (PA).

4.1.2. Direct detection of viruses

Direct detection of viruses generally refers to the detection of viruses by electron microscopy (EM) or techniques in which labelled antibodies directed at viral capsid proteins (antigens) are used to detect viruses. The latter immunological techniques include, for instance, enzyme-linked immunosorbent assays (ELISA) and latex agglutination (LA). Important advantages of EM and immunological techniques are that any viruses can be detected. EM is essential for determination of the size and morphology of viruses, and has the additional advantage that generally results are available in a relatively short time. Immunological techniques are relatively fast, inexpensive and simple. However, both EM and immunological techniques have the important disadvantage of not being able to distinguish between viable and non-viable viruses. In addition, they are not sensitive. The sensitivity of EM may be upgraded by, for instance, aggregation of viruses by specific antibodies, which is known as immune electron microscopy (IEM) (Taylor *et al,* 1993). Immunological techniques may also be upgraded by various procedures. Even with modification, the sensitivity is rarely below 10^5 virus particles per ml (Centers for Disease Control, 1990). However, Dahling *et al* (1993) report on commercial immunological techniques capable of detecting 10 virus particles per ml. Labelled antibodies may also be used to detect viral antigens in cell cultures when viruses undergo some replication and production of capsid components but fail to produce a cytopathogenic effect, as in the case of rotaviruses.

4.1.3. Serology

This approach generally refers to detection of a viral infection by the immunological response of the patient to the infection. In other words, infection of a patient is followed by the production of antibodies specifically directed against the virus which causes the infection, and

these antibodies can be detected by various serological techniques based on their binding with known viruses (Jawetz *et al*, 1991). Distinction can even be made between IgM and IgG antibodies, which gives an indication of a present infection or an infection in the past. Serological tests are routinely used for the diagnosis of viral infections in humans. Although these tests cannot be used to directly detect viruses in shellfish or other environments, their detection of antibodies against viruses confirms infection of the persons concerned with those viruses, which is evidence of the presence of the viruses. This approach has, for instance, been used to prove the endemic presence of the hepatitis E virus in South Africa even though the virus itself has never been isolated due to a lack of appropriate techniques (Grabow *et al*, 1994). Serological tests could also be used to identify aetiological agents in outbreaks (Taylor *et al*, 1993).

4.1.4. Detection of viral nucleic acid

Recently molecular techniques have been developed for the detection of viral nucleic acid. These are primarily based on the hybridisation of gene probes (pieces of nucleic acid with known nucleotide sequence, generally artificially synthesised) with viral nucleic acid (Le Guyader *et al*, 1993). These probes will only hybridise with matching nucleic acid, which implies that they are highly specific for the detection of viruses. Viral nucleic acid can also be detected by the polymerase chain reaction (PCR). This procedure refers to the use of highly specific primers which promote the artificial replication of selected stretches of viral nucleic acid under appropriate experimental conditions. The large quantities of replicated viral nucleic can be detected by separation and staining on electrophoresis gels, hybridization with specific probes, or nested PCR procedures (Durack *et al*, 1990). These techniques have the advantages of being highly specific and sensitive, and capable of detecting any virus for which the nucleotide sequence of appropriate stretches of the nucleic acid is known. According to Tsai *et al* (1993), certain molecular procedures may be 500 times more sensitive for polio and hepatitis A viruses than cell culture techniques. Molecular techniques require relatively advanced expertise and facilities. A major disadvantage of molecular techniques is that they cannot distinguish between viable and non-viable viruses. However, even though positive molecular techniques yield no evidence of infectivity, they prove the presence of viral nucleic acid which would imply that shellfish contain viable or non-viable viruses, or that they were exposed to an environment which contained viable or non-viable viruses. All of these possibilities indicate that shellfish had ingested viruses or viral nucleic acid, which indicates a risk of contamination of the shellfish

which is not acceptable even in the absence of evidence of the viability or infectivity of the viruses. Negative molecular tests would render valuable evidence of the absence of infectious viruses as well as no exposure to viruses or viral nucleic acid.

Information reviewed in Part 2 on viruses transmitted by shellfish, and information on viral detection techniques summarised above, were used to select the viruses and detection techniques described below.

4.2. ENTERIC VIRUSES AND METHODS FOR THEIR DETECTION

Cell culture propagation was used for the detection of viruses in this study because it offers a relatively practical approach to the detection of viruses, and has the important advantage of detecting viable viruses. The enumeration of viruses was carried out only by an MPN procedure based on CPE in microtitre plates because this procedure is economic, and relatively simple and reliable (Grabow *et al,* 1992). Although plaque assays have attractive features, they were not included because CPE in culture may be more sensitive and is less labour intensive (Grabow and Nupen, 1981). The decision to limit the detection of viruses to cell culture propagation, implies that only viruses detectable by this method could be included. The viruses, and cell cultures selected for their detection, are as follows:

4.2.1. Poliovirus type 1

This virus was selected because it is a typical member of the group of enteroviruses. Members of this group are often detected in waste water and shellfish harvested from sewage-polluted marine environments. Poliovirus is also important because attenuated strains are widely used for vaccination against poliomyelitis, and consequently vaccine strains can be expected in sewage-polluted environments. Available information suggests that poliovirus is at least as resistant to unfavourable conditions as most other enteroviruses, and probably also many other enteric viruses (Grabow *et al,* 1983). The vaccine strain Lsc-2ab of poliovirus (kindly supplied by the National Institute of Virology, Johannesburg) was used in this study because it readily grows in cell culture and does not constitute a risk of infection which may cause disease.

The Buffalo Green Monkey (BGM) line of monkey kidney cells was used because these cells are exceptionally susceptible to poliovirus (Schmidt *et al,* 1976; 1978; Grabow and Nupen, 1981),

because the cells easily grow in a variety of culture systems, and because they are widely used and readily available (Standard Methods, 1992). The cell line used in this study was obtained from the American Type Culture Collection (ATCC).

4.2.2. Rotavirus SA-11

Rotavirus is one of the most important causes of gastroenteritis morbidity and mortality in humans and animals (see Part 2). Rotaviruses are excreted in exceptionally high numbers by infected individuals, and they have been detected in a variety of polluted water environments (Menhert and Stewien, 1993). For these reasons, and because rotaviruses differ extensively from poliovirus in terms of structure, composition, and classification, their inclusion in this study was considered important. Their inclusion was considered important even though they have rarely been detected in shellfish, and even though rotavirus transmission has rarely if ever been associated with shellfish (Ahmed, 1991). Unfortunately wild-type human rotaviruses do not replicate with CPE in cell cultures (Aboudy *et al,* 1989). The closely related simian rotavirus SA-11 (SA = Simian Agent), originally isolated from a monkey in South Africa (Malherbe and Strickland-Cholmley, 1967), and now world-wide used as surrogate for human rotavirus, was selected for this study because it readily replicates with CPE in a variety of cell culture systems (Malherbe *et al,* 1963; Estes *et al,* 1979).

In this study the MA-104 monkey kidney cell line was used for the propagation of SA-11 because the virus grows exceptionally well in these cells, the cells are widely used for the propagation of rotaviruses, the cells are readily available, and they readily grow under a variety of laboratory conditions (Estes *et al,* 1979; Ward *et al,* 1984; Christensen, 1989). An ATCC cell line was used.

4.2.3. Adenovirus 40

Adenovirus types 40 and 41 (Ad40/41) are world-wide a major cause of gastroenteritis, primarily in infants (see Part 2). Infected individuals excrete these viruses in numbers of up to 10^{11} per gram of stool (Smith and Gerba, 1982; Christensen, 1989). They can, therefore, be expected in sewage. Information on these viruses in sewage, the environment, and in shellfish is extremely limited because they do not readily replicate with CPE in cell culture systems. For this reason these enteric strains of adenoviruses are known as the "fastidious adenoviruses".

Recently, however, practical cell culture techniques have been developed for the detection and titration of Ad40/41 (Grabow *et al,* 1992). Even though the transmission of these viruses has rarely if ever been associated with the consumption of shellfish (Part 2), they have been included because of their potential importance in shellfish, and because the cell culture techniques now available offer an ideal opportunity to study for the first time their behaviour in shellfish. Another important reason for their inclusion is that they are DNA viruses which in terms of structure and composition differ substantially form the RNA viruses used in this study. Adenovirus 40 strain Hovi-X (Kidd *et al,* 1984) was used as representative of Ad40/41 because its cultivation and titration using the PLC/PRF/5 cell line is well established (Grabow *et al,* 1992). The PLC/PRF/5 cell line, originally derived from primary human liver carcinoma (Macnab *et al,* 1981) has, therefore, been used for Ad40 in this study. An ATCC culture of the PLC/PRF/5 cell line has been used.

4.2.4. Hepatitis A

The hepatitis A virus (HAV) has often been associated with transmission by shellfish. One of the best known examples is the outbreak with some 300 000 cases associated in 1988 with the consumption of sewage-contaminated clams in Shanghai, China (see Part 2). Wild-type HAV does not readily replicate with CPE in presently available cell culture systems. However, cell culture adapted strains have been established which can readily be detected and enumerated in appropriate cells. In view of the major importance of HAV in shellfish, one of these culture-adapted strains was included in this study. HAV strain pHM-175 was used because its detection and tritiation in cell culture is well established and because its behaviour in the environment would appear to be very similar to that of the wild-type HAV (Cromeans *et al,* 1986, 1987). The virus, kindly supplied by prof MD Sobsey, was propagated and titrated on the FRhK-4R foetal rhesus monkey kidney cell line kindly supplied by Dr B Flehmig. This cell line proved exceptionally suitable for the propagation and titration of HAV (Flehmig *et al,* 1981).

4.3. DISCUSSION

Since it is practically impossible to include all viruses which may potentially be transmitted by shellfish, and various methods for their detection, in a single study of this nature, the investigation was limited to the viruses and detection methods discussed above. The viruses

concerned are representative of the wider group of enteric viruses often associated with shellfish transmission, and they also represent groups of viruses which differ in structure, composition and morphology, and which may, therefore, be expected to behave differently.

It seems logical that the evaluation of detection methods by means of the selected spectrum of viruses and detection methods should give a reliable indication of the efficiency of the detection methods concerned for subsequent application in research on other viruses using alternative detection techniques.

5. SELECTION OF METHODS FOR THE RECOVERY OF ENTERIC VIRUSES AND BACTERIOPHAGES FROM SHELLFISH

5.1. INTRODUCTION

A variety of methods has been described for the recovery of viruses (human viruses and bacteriophages) form shellfish meat (Metcalf and Stiles, 1965; Sobsey *et al,* 1978; Sobsey, 1984; Austin and Austin, 1989; West, 1989; Boher *et al,* 1991; Idema *et al,* 1991; Croci *et al,* 1993). The efficiency of recovery of these methods differs considerably for different viruses and phages, and they all have their own advantages and disadvantages in terms of complexity, labour and time requirements (Idema *et al,* 1991).

The first step in all recovery techniques is to homogenise specific quantities of shellfish meat in appropriate volumes of distilled water, saline or a buffer, generally by means of waring blender or sonnicator (Sobsey, 1984; Austin and Austin, 1989). The intravalvular fluid of the animals is usually included in the homogenate (Sobsey, 1984). The detection of viruses in these suspensions is approached in tow ways. In the first approach, referred to as the elution method, the suspensions are centrifuged to remove the meat, and viruses are then directly detected in the supernatant (Vaughn and Metcalf, 1975). This is a rapid and simple procedure but has the important disadvantage that a high percentage of viruses and phages are adsorbed to the tissue and removed by centrifugation (Idema *et al,* 1991). In the second approach, referred to as the precipitation method, relatively complicated and time consuming procedures are followed to first maximise virus adsorption to the meat at low pH. The meat with adsorbed viruses is them recovered form the suspension by centrifugation. This process is sometimes repeated twice (Sobsey *et al,* 1978), with the pH reduced to levels of 4,5 (Sobsey *et al,* 1978) or 3,5 (Richards *et al,* 1982). In some procedures recovery of meat is aided by the addition of a flocculant such as Cat-floc (Richards *et al,* 1982). The recovered meat with adsorbed viruses is then resuspended in a suitable buffer, such as 0,1M Na_2HPO_4, and the pH adjusted to 7,2 to 7,5 for the direct detection of viruses (Idema *et al,* 1991). Removal of meat by centrifugation as in the elution method, or washing of the meat as in the precipitation method, is required to remove compounds often present in shellfish meat which are toxic to cell cultures used for the detection of human viruses (Sobsey *et al,* 1978).

An efficiency of recovery (EOR) of 81% has been reported for poliovirus using the precipitation method (Sobsey *et al,* 1978; Idema *et al;* 1991). However, poliovirus is exceptionally resistant to the low pH levels involved (Sobsey *et al,* 1978; Vaughn *et al,* 1979; Wait and Sobsey, 1983), and the EOR for less resistant viruses was 3 - 6% for reovirus, 0,6 - 1,0% for the SA-11 simian rotavirus, and 2% for a somatic and male-specific coliphage (Brodisch *et al,* 1986; Idema *et al,* 1991). The following EOR values have been reported for various elution methods (Brodisch *et al,* 1986; Idema *et al,* 1991): poliovirus 8 - 91%; reovirus 33%, SA-11 virus 35%; MS2 male-specific coliphage 50% and V1 somatic coliphage 62%. Boher *et al* (1991) reported on an elution method for SA-11 using pH 9,0 borate buffer for preparing oyster and mussel homogenates. Although no data were given on the EOR, the borate buffer seems to have yielded satisfactory results.

Final preparations (with known wet mass of shellfish meat per volume of suspension) are usually decontaminated by chloroform treatment (Idema *et al,* 1991) after which human viruses are detected by cell culture propagation (Idema *et al,* 1991) and phages by double-agar-layer plaque assays (Grabow *et al,* 1993). Chloroform treatment would, however, seem to affect at least some phages because Brodisch *et al* (1986) reported higher EOR levels for phages in homogenates not exposed to chloroform.

This Part deals with research on the recovery of human viruses from shellfish meat in terms of human viruses and phages not previously tested, and a comparison of the efficiencies of precipitation and elution methods using different pH levels. The study is restricted to methods and procedures which in previous investigations have yielded the best results (Brodisch *et al,* 1986; Idema *et al,* 1991).

It should be noted that these tests on seeded shellfish meat homogenates may not exactly resemble conditions in which viruses and indicators are ingested by shellfish in sewage-polluted marine environments. However, this is the only way for quantitative comparison of the efficiency of recovery techniques. Any deviation from natural conditions of contamination will probably be a constant factor for all recovery techniques and have no meaningful impact on relative efficiencies. All previous studies on recovery techniques have been carried out in the same way. In addition, the results obtained on seeded shellfish meat in this Part, are confirmed by similar comparisons on shellfish which have ingested viruses and indicators in seawater tanks

under controlled laboratory conditions simulating natural conditions of uptake (see Part 7).

5.2. MATERIALS AND METHODS

5.2.1. Shellfish

Live or frozen black mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) (Brown, 1987) were purchased from a local commercial retail outlet. Meat was removed from the shells according to procedures described by Sobsey (1984), West and Coleman (1986) and Austin and Austin (1989). Meat and intravalvular fluid were pooled and a mass of 50 g was suspended in a buffer as described later. These test suspensions were seeded with both phages in combination with either the polio, SA-11 or Ad40 viruses. Seeded test suspensions were homogenised by means of ultra-turrax treatment (Ultra-Turrax T25, IKA Labortechnik) for 3 min at maximum speed selector setting (Grabow *et al,* 1991). The titre of seeded phages and viruses in these suspensions was about 10^6/ml of suspension.

5.2.2. Viruses and methods for detection

The following laboratory strains of viruses were used: A vaccine strain of poliovirus type 1 (National Institute for Virology), simian rotavirus SA-11 (ATCC) (Grabow *et al,* 1984) and enteric adenovirus type 40 (Ad40) (Grabow *et al,* 1992). Stock supplies of these viruses were cultivated and titrated by conventional procedures as described elsewhere (Grabow *et al,* 1984; 1992).

Aliquots of test suspension used for virus titration were decontaminated by adding 0,1 volume per volume (v/v) of chloroform, mixing by brief vortexing, allowing to stand for 10 min at room temperature, and centrifugation at 2000 rpm for 3 min (Grabow *et al,* 1986). The virus-containing supernatant was aseptically used for titration. Confluent monolayers of the following cells were used for titrating the viruses: The Buffalo Green Monkey Kidney (BGM) cell line (passage unknown, Highveld Biological) for the poliovirus, the PLC/PRF/5 cell line derived from a primary human liver carcinoma (Alexander *et al,* 1978; Oefinger *et al,* 1981) (passage 43 to 65, ATCC) for the Ad40 virus, and the MA-104 foetal rhesus monkey kidney cell line (passage 4 to 21, Highveld Biological) for the SA-11 rotavirus. Cells were cultured in Eagle's minimal essential medium (EMEM) (Highveld Biological) supplemented with 2% antibody-free foetal calf serum (FCS) (Delta Bioproducts), and 100 U of penicillin, 100 μg of streptomycin,

50 μg of neomycin, 292 μg of L-glutamine and 2 mg $NaHCO_3$ per 500 ml. Anti-PPLO agent (1%) (Gibco) was added to suppress mycoplasma growth in PLC/PRF/5 cultures. Cells were cultured in 75 cm^3 plastic culture flasks (Sterilin), and harvested when confluent by trypsinization using a 50:50 EDTA and 0,5% trypsin mixture (National Institute for Virology) as described by Grabow *et al* (1982, 1984). Each well in 96-well microtitre plates (CelCult) was seeded with 0,1 ml of the cell suspension. Cell monolayers were usually confluent after 24 h incubation at 37°C in a 5% CO_2 atmosphere. Cells were then washed with 0,1 ml of pH 7,2 Phosphate-Buffered Saline (PBS) per well for 30 min. Viruses were titrated by using 10-fold dilutions of test suspensions, 8 wells per dilution and an inoculum of 0,005 ml per well (Grabow *et al,* 1992). All tests included positive and negative controls. Plates were incubated at 37°C in a 5% CO_2 humidified atmosphere, and observed daily for cytopathic effect (CPE) (Grabow *et al,* 1992). Plates were incubated until the cell monolayers of negative controls underwent autolysis, which usually was after about 10 - 12 days. Wells inoculated with test samples in which cells developed CPE were recorded as positive. Positive wells usually showed CPE between 4 and 7 days. These results were used to calculate the most probable number (MPN) titre by means of a standard computer programme (Thomas, 1942; Grabow *et al,* 1992).

5.2.3. Phages and methods for detection

The following laboratory strains of phages were used: Somatic coliphage V1 (Grabow *et al,* 1984), and male-specific coliphage MS2 (Zinder, 1975; Grabow *et al,* 1993). Stock supplies of these phages were cultivated and titrated by conventional procedures as described by Grabow *et al* (1993).

Aliquots of centrifuged shellfish homogenates used for phage titration were not decontaminated by chloroform treatment as in the case of viruses. Test suspensions were directly used for counting phages by the following double-agar-layer plaque assays described in detail by Grabow *et al* (1993):

Somatic coliphage V1

A nalidixic acid-resistant mutant (WG5) (Havelaar and Hogeboom, 1983) of *Escherichia coli* strain C (ATCC 13706), kindly supplied by Dr A H Havelaar, was used as host. This host was cultured in Nutrient Broth (Difco). Bottom agar for double-agar-layer plaque assays were prepared as follows: Dissolve 10,0 g of Bacto Agar (Difco), 13,0 g of Tryptone (Difco), 8,0

g of NaCl (Holpro) and 1,5 g of Glucose (Sigma) in 100 ml of distilled water and autoclave, after which 330 μg/ml nalidixic acid (Sigma) was added, and 20 ml volumes poured into 90 mm diam petri dishes. These plates were stored at 4^{0}C for not longer than 10 days. Top agar was prepared just like bottom agar except that 7,0 g of agar was used instead of 10,0 g, and no nalidixic acid was added. The autoclaved top agar was dispensed in 2,6 ml volumes into sterile test tubes, and stored at 4^{0}C for not longer than 30 days.

Male-specific coliphage MS2

Escherichia coli strain HS(pFamp)R (Debartolomeis and Cabelli, 1991), kindly supplied by Prof V J Cabelli, was used as host for male-specific coliphage MS2 (Grabow *et al,* 1993). The host strain was cultivated in growth medium prepared by dissolving 10,0 g of Tryptone (Difco), 5,0 g of NaCl (Holpro) and 1,0 g of Dextrose (Sigma) in 1 000 ml of distilled water followed by autoclaving. Bottom agar for double-agar-layer plaques assays were prepared by dissolving 12,0 g of Bacto Agar (Difco), 10,0 g of Tryptone (Difco), 5,0 g of NaCl (Holpro) and 1,0 g of Dextrose (Sigma) in 1 000 ml of distilled water. After autoclaving the temperature was reduced to 50^{0}C when 20 ml of a stock solution of antibiotics was added. After mixing, 20 ml volumes of the medium were poured into 90 mm diam petri dishes which were stored at 4^{0}C for not longer than 10 days. The antibiotic stock solution was prepared by dissolving 150,0 mg each of Ampicillin (Sigma) and Streptomycin (Sigma) in 100 ml distilled water, which was decontaminated by filtration through a 22 μm pore size membrane (Millipore) and kept at 4^{0}C for not longer than a week. Top agar was prepared just like bottom agar except that 7,0 g of agar was used instead of 12,0 g and no antibiotic solution was added. The autoclaved top agar was dispensed in 2,5 ml volumes into sterile test tubes and stored at 4^{0}C for not more than 30 days.

Double-agar-layer plaque assays

The following procedure was used for both V1 and MS2 phages: Tubes of top agar were steamed to liquify and adjusted to 48°C in a thermostatic waterbath. A volume of 1,0 ml of test sample (or appropriate 10-fold dilutions in PBS) and 0,3 ml of a mid-log-phase 37°C culture of host strain were added to the 2,5 ml of top agar in a test tube, mixed by gentle swirling without producing bubbles, poured onto the bottom agar in a petri dish, and allowed to solidify. These plates were incubated upside down in a 37^{0}C circulating air incubator and after 18 - 24 h plaques were counted on plates which contained 30 - 100 plaques. All assays were carried out in

threefold and results (counts) expressed as the number of plaque forming units per volume (PFU/ml).

Autoclaving, disinfection and sterilisation

The following applies to all procedures in this report. Unless otherwise stated, all media were autoclaved in a conventional steam autoclave for 15 min at 121°C. Glassware (test tubes, flasks, etc) was sterilised for 60 min in a conventinal dry air oven at 160°C. Commercial supplies of sterile plastic petri dishes (Carbi, South Africa) were used.

5.2.4. Evaluation of recovery methods

In the evaluation of elution and precipitation methods, the following procedures based on those described by Sobsey *et al* (1978) and Idema *et al* (1991), respectively, were used:

Precipitation method

Seeded mussel and oyster samples were homogenised in 7x weight/volume (w/v) sterile distilled water and the pH adjusted to 4,5. Homologous aliquots of 230 ml suspension were centrifuged at 10 000 rpm for 45 min and the supernatant discarded. In comparative experiments the sediment was resuspended in 450 ml (7x w/v) glycine-saline buffer at pH levels of 7,5, 9,0 or 10,5. After adjusting the pH of the suspension to 7,5, 0,2% volume/volume (v/v) of a 0,1% Na_3PO_4 buffer was added, and the suspension centrifuged at 5 000 rpm for 30 min. The tissue containing sediment was discarded and the pH of the virus containing supernatant adjusted to 4,5 followed by centrifugation at 10 000 rpm for 45 min. The supernatant was discarded and the virus containing sediment resuspended in a 0,1 M Na_2HPO_4 buffer to a volume of 20 ml, and adjusted to pH 7,4. The suspension was then decontaminated with 0,1 v/v chloroform as described earlier followed by centrifugation at 10 000 rpm for 30 min. The supernatant was supplemented with 0,1 v/v of an antibiotic cocktail containing 3,0 mg penicillin, 5,0 μg streptomycin sulphate, 2,5 μg neomycin sulphate and 2500 U of mycostatin for further control of contamination. Viruses in these final test suspensions were enumerated in threefold using the techniques described earlier.

Elution method

In comparative tests seeded samples of mussel or oyster meat were suspended in 1x weight/volume (w/v) 0,05 M glycine - 0,15 M NaCl (glycine-NaCl) buffer (Sobsey *et al*, 1978)

at pH 7,0, 8,5 or 9,5, followed by homogenisation as described earlier and centrifugation at 5 000 rpm for 10 min. The tissue containing sediment was discarded. The pH of the virus containing supernatant was adjusted to 7,4, followed by decontamination with chloroform and supplementation with antibiotic cocktail for enumeration of viruses and phages as described for the precipitation method.

The efficiency of recovery (EOR) of test methods was calculated as a percentage of the counts of viruses and phages in the final concentrate, and the counts of viruses and phages seeded into test samples of oyster or mussel meat (Sobsey *et al,* 1978; Idema *et al,* 1991).

5.3. RESULTS

Results of comparative assays on seeded samples of oyster and mussel meat are presented in Tables 5.1 and 5.2, respectively. These results suggest that the EOR of the recovery procedures investigated does not differ significantly for oyster and mussel meat. In the case of the elution method the EOR was highest for all viruses and phages when the pH of the suspending buffer was 8,5. In the case of the precipitation method the EOR was not consistently higher for viruses at any pH level. The elution method yielded a maximum average EOR of 87,0% for poliovirus, and the precipitation method only 78,0%. The maximum average EOR for SA-11 and Ad40 viruses by means of the elution method was 33,0% and 25,0%, respectively, and by means of the precipitation method only 0,4% and 2,3%, respectively. The elution method yielded a maximum average EOR of 59,0% and 48,0% for the V1 and MS2 phages, respectively.

The precipitation method proved relatively labour intensive and took 4 h to complete. The elution method proved simple, required fewer buffers and reagents, and was completed in some 20 min. No problems were experienced with contamination or cell culture toxicity in any of the procedures.

Table 5.1: Efficiency of Recovery (EOR) of viruses and phages from oyster meat by means of precipitation and elution methods using 0,05 M -0,15 M glycine-saline buffer at various pH levels for suspending meat samples

Determinant	Percentage efficiency of recovery (EOR)					
	Precipitation method: pH of buffer			Elution method: pH of buffer		
	7,5	9,0	10,5	7,0	8,5	9,5
Poliovirus						
Range	66 - 81	64 - 79	62 - 76	75 - 86	81 - 92	81 - 87
SD	6,8	6,1	5,4	4,4	4,1	2,3
Median	80	75	68	84	88	85
Average	78,0	73,0	70,0	83,0	87,0	85,0
SA-11 virus						
Range	0,03 - 0,7	0,07 - 0,6	0,04 - 0,8	28 - 32	29 - 34	23 - 29
SD	0,3	0,2	0,01	1,5	2,1	2,4
Median	0,1	0,1	0,06	30	33	27
Average	0,4	0,2	0,08	30,0	33,0	27,0
Adenovirus 40						
Range	0,9 - 3,1	1,6 - 2,9	0,6 - 1,6	19 - 22	23 - 27	20 - 25
SD	0,8	0,5	0,3	1,1	1,5	1,8
Median	1,6	2,4	1,0	21	25	22
Average	1,7	2,3	1,2	20,0	25,0	22,0
Phage V1						
Range				53 - 58	55 - 61	52 - 56
SD	--	--	--	1,9	2,3	1,5
Median				56	59	54
Average				55,0	59,0	54,0
Phage MS2						
Range				42 - 47	44 - 51	39 - 43
SD	--	--	--	1,9	2,7	1,8
Median				44	49	42
Average				44,0	48,0	41,0

SD = Standard Deviation; each test was repeated 5 times (n = 5); -- = Not done

Table 5.2: Efficiency of Recovery (EOR) of viruses and phages from mussel meat by means of precipitation and elution methods using 0,05 M - 0,15 M glycine-saline buffer at various pH levels for suspending meat samples

Determinant	Percentage efficiency of recovery (EOR)					
	Precipitation method: pH of buffer			Elution method: pH of buffer		
	7,5	9,0	10,5	7,0	8,5	9,5
Poliovirus						
Range	69 - 85	65 - 78	67 - 75	78 - 88	81 - 93	79 - 88
SD	6,5	4,8	3,6	3,9	5,3	3,2
Median	73	73	69	82	86	85
Average	74,0	72,0	69,0	80,0	86,0	82,0
SA-11 virus						
Range	0,09 - 0,8	0,07 - 0,9	0,03 - 0,9	26 - 34	30 - 36	24 - 28
SD	0,3	0,3	0,02	3,3	2,4	1,4
Median	0,4	0,2	0,07	30	34	26
Average	0,2	0,3	0,06	29,0	31,0	26,0
Adenovirus 40						
Range	1,2 - 2,5	1,4 - 3,4	0,9 - 2,1	17 - 23	21 - 28	18 - 25
SD	0,5	0,7	0,5	2,5	2,7	2,5
Median	1,6	2,6	1,6	19	27	23
Average	1,6	2,5	1,4	19,0	26,0	23,0
Phage V1						
Range				52 - 55	57 - 63	50 - 57
SD	--	--	--	1,1	2,3	2,8
Median				54	60	54
Average				53,0	60,0	53,0
Phage MS2						
Range				43 - 47	45 - 50	37 - 45
SD	--	--	--	1,3	2,0	3,5
Median				44	47	40
Average				45,0	47,0	42,0

SD = Standard Deviation; each test was repeated 5 times (n = 5); -- = Not done

5.4. DISCUSSION

In the elution procedure as applied in the experiments described here, the quantities of wet mass shellfish meat tested were limited to 0,020 g for viruses (0,0025 g in 8 wells for undiluted 1xmass/volume homogenates), and 1,5 g for phages (0,5 g in each of 3 plates for the same undiluted homogenates). Tenfold dilutions contained an additional corresponding tenth of these quantities. In the precipitation procedure the quantities of meat tested were higher because homogenates consisted of 7xmass/volume. For purposes of comparison, all results were expressed as a calculated numbers per 10 g wet mass shellfish meat. The testing of these small quantities of shellfish meat was sufficient to enumerate viruses and phages for purposes of comparing the recovery procedures at the seeding levels of viruses and phages for the experimental purposes concerned. It is important to note that these enumeration procedures were used for purposes of comparing numbers in the evaluation of recovery procedures, and not for detecting the smallest possible numbers of viruses and phages in shellfish meat. The purpose of this investigation was to select the most efficient recovery procedure. The most efficient recovery procedure can then be used in a next step to detect the smallest possible numbers of viruses and phages. This could be approached by using larger quantities of shellfish concentrates in MPN microtitre plate assays for viruses, and plaque assays for phages. Sensitivity could be increased even further by inoculating large quantities of shellfish concentrates into flasks of cell cultures for the detection of viruses, and by using large volumes of homogenates in single agar layer plaque assays on large petri dishes for phages (Grabow and Coubrough, 1986). A variety of secondary concentration procedures could also be applied.

The objectives of the experiments described here were to compare recovery procedures, and not to develop techniques for detecting the smallest possible numbers of viruses and phages in shellfish. The numbers of wells used per dilution in microtitre pate assays for viruses were, therefore, limited to 8 which is a convenient number on standard 96-well microtitre plates and proved adequate for the purposes of comparison concerned. Although we have not seen literature on MPN assays for viruses in shellfish using more than 8 wells per dilution, more wells per dilution would obviously increase the accuracy of MPN assays. In the case of phage assays only 3 plates per dilution were used which is generally accepted as sufficient. We have not seen literature in which larger numbers of wells per dilution were used or recommended.

Poliovirus was included in this comparative study, as in most other similar studies, because it is a typical representative of the group of enteroviruses generally detected in relatively large number in sewage-polluted waters by cell culture propagation (Grabow *et al,* 1983; Goyal *et al,* 1984). In addition, it is relatively resistant to unfavourable conditions including pH extremes (Vaughn and Metcalf, 1975; Kott *et al,* 1978; Bitton, 1980), it is easy to detect and enumerate by cell culture propagation, and it is safe to work with in the laboratory (Fig. 5.1). The SA-11 simian rotavirus (Fig. 5.2) was included because it differs in various respects form poliovirus, and is closely related to the human rotavirus which is associated with faecal-oral transmission and is known to occur in sewage-polluted water environments (Menhert and Stewien, 1993). Differences include the SA-11 virus having a double-stranded RNA genome and poliovirus a single-stranded RNA genome, and the rotavirus is considerably larger with different capsid structure. The SA-11 rotavirus is not known to cause infection in humans (see Part 2.1). The Ad40 virus was included in the study because it is primarily associated with faecal-oral transmission and it differs from both poliovirus and SA-11 in having a double-stranded DNA genome and a considerably different morphology (see Part 2.1). This is the first report on experiments regarding the recovery of enteric adenoviruses from shellfish meat. The three viruses selected for this investigation do, therefore, represent a spectrum of viruses transmitted by the faecal-oral route, and which may occur in shellfish.

The two phages were likewise selected to represent different types of phages which may occur in sewage-polluted environments. The MS2 phage closely resembles poliovirus in terms of structure and composition, while V1 is a much larger tailed phage belonging to the group of *Siphoviridae* phages (Grabow, 1986; Demuth *et al,* 1993; Grabow *et al,* 1993).

In terms of efficiency, the elution method would clearly be the method of choice because EOR values for the polio, SA-11 and Ad40 viruses were 87,0%, 33,0%, and 25,0%, respectively, compared with 78,0%, 0,8% and 2,3%, respectively, for the precipitation method (Tables 5.1 and 5.2). The very large differences for SA-11 and Ad40 may be due to higher sensitivity of these viruses to pH 4,5 than poliovirus, or less efficient release of viruses adsorbed to shellfish tissue. In various tests on the precipitation procedure organisms were exposed for 60 min to pH levels ranging from 7,5 to 10,5, and also for 60 min to pH 4,5. In the elution procedure eventually selected test organisms were exposed to pH 8,5 for 15 min and during the rest of the recovery procedure the pH was about 7,4. No attempt was made to investigate reasons for the

differences in EOR in further detail because the precipitation procedure would clearly not be preferred to the elution method. Phages were not included in test on the precipitation method because Brodisch *et al* (1986) found that the method had an EOR of 2% for these phages. The elution method had an EOR of 59,0% and 48,0%, respectively, for the V1 and MS2 phages, which compares favourably with the EOR for viruses (Tables 5.1 and 5.2).
The average 78,0% EOR for poliovirus using the precipitation method is higher than the 46% reported by Sobsey *et al* (1978), but slightly lower than the 81% and 79% obtained by Idema *et al* (1991) using two similar methods in studies on mussel meat. These findings would seem to confirm that higher efficiencies by techniques based on the precipitation approach appear unlikely.

The maximum average EOR of 87,0% and 33,0% for poliovirus and SA-11 by the elution method (Tables 5.1 and 5.2) was slightly lower than the 91% and 35%, respectively, reported by Idema *et al* (1991) for a similar method applied to mussel meat. The reason for these marginal differences have not been investigated. It would appear unlikely that this may be due to differences such as the homogenisation of shellfish meat in pH 8,0 saline by Idema *et al* (1991), and in pH 8,5 glycine-NaCl buffer in this study. The latter buffer was preferred because it has been found highly efficient in the release of viruses adsorbed to a variety of surfaces and compounds (Sobsey *et al,* 1978; Vilagines *et al,* 1993).

The higher efficiency of the elution method, together with experience that it only take some 20 min to carry out compared to the 4 h for the precipitation procedure, and the requirement of fewer reagents and buffers, clearly identify the elution procedure as the method of choice for the recovery of viruses and phages form both oyster and mussel meat. The absence of problems with contamination or cell culture toxicity in the elution method, indicates that the time consuming and laborious precipitation procedure is not required for reasons in this regard. The selection of viruses and phages used in this study suggest that these findings are likely to apply to most viruses and phages associated with sewage pollution. However, it should be kept in mind that these experiments were restricted to selected laboratory strains of viruses and phages, and the results should be confirmed for a wider spectrum of viruses and phages, and naturally occurring viruses and phages in shellfish contaminated during feeding in sewage-polluted marine environments. Studies on naturally contaminated shellfish would strengthen the findings of the present study, even though Grabow *et al* (1991) found that shellfish meat seeded with laboratory

strains of viruses and bacteria were equally suitable for research on recovery and detection techniques than naturally contaminated shellfish.

6. ASSESSMENT OF THE QUALITY OF SHELLFISH SUPPLIES AND INCIDENCE OF HUMAN INFECTIONS

6.1. INTRODUCTION

In view of the potential health risks associated with the consumption of shellfish (see Part 2), the quality of shellfish in terms of faecal contamination and health-related micro-organisms has been investigated in many parts of the world. Data have been reported from, for instance, France (Denis, 1977; Jehl-Pietri *et al,* 1991), United States (Fugate *et al,* 1975; Baer *et al,* 1976; Forster *et al,* 1977; Abeyta, 1983; Cooke and Ruple, 1989), the Netherlands (Van den Broek *et al,* 1979), Indonesia (Molitoris *et al,* 1985), Britain (Ayers, 1975; Sockett *et al,* 1985; Chalmers and McMillan, 1995), Canada (Kelly and Dan Stroh, 1988), Vietnam (Neumann *et al,* 1972), Korea (Chun *et al,* 1974), Hong Kong (Chan *et al,* 1989), Iceland (Hartemink and Georgsson, 1991), Brazil (Sato *et al,* 1992), Italy (Bellelli and Leogrande, 1967), Bahrain (Qureshi *et al,* 1993) and South Africa (Grabow, 1989). Data obtained in these studies were used as a basis for the formulation of quality guidelines.

However, a wide variety of methods which differ in both the efficiency of recovery (EOR) of micro-organisms from shellfish meat (see Part 5), and in the sensitivity of detection of various micro-organisms (Grabow al, 1991) have been employed in these studies. This implies that the comparison of results, the assessment of the value of various indicators of faecal pollution or the potential presence of pathogens, and the uniform application of quality guidelines, are hardly possible. An additional important shortcoming of available information on the quality of shellfish meat is that the data are predominantly limited to faecal bacteria such as coliforms and streptococci with little details on human viruses and potentially valuable indicators such as phages.

Information on the quality of shellfish in terms of indicators of faecal contamination, pathogens (particularly viruses which are most frequently associated with infections contracted from shellfish), and potentially valuable indicators such as phages, is essential for the formulation of reliable, practical and cost-effective quality guidelines and safety surveillance programmes. As new technology and know-how become available, more details are gathered on the incidence and

behaviour of indicators and pathogens in shellfish. This ongoing process makes it possible to regularly update and modify quality guidelines, test methods, and surveillance programmes for improvement in terms of reliability and cost effectivity.

There is no meaningful information on the incidence of infections associated with the consumption of shellfish in South Africa. This may to large extent be due to good quality of commercial supplies and naturally occurring shellfish. However, the risks of infection must be the same as elsewhere in the world because parts of the coastline are exposed to sewage pollution (Grabow, 1989). Anecdotal data would also seem to indicate that infections have been associated with the consumption of shellfish collected by the general public at a number of coastal sites, but that these incidents had never been followed up and investigated in any detail. It would, therefore, seem that the lack of epidemiologically confirmed cases or outbreaks of diseases caused by shellfish may largely due to the absence of an infrastructure for the detection and epidemiological confirmation of such incidents.

This Part deals with the application of the recovery techniques selected, optimised and evaluated in Part 5, for assessment of the quality of randomly selected supplies of shellfish intended for human consumption in South Africa. Tests were carried out for a wide variety of faecal indicator bacteria, potential pathogens, three different groups of phages, and viruses detectable by cytopathogenic effect in the commonly used BGM cell line. This work provides information on the application in practice of a new recovery technique, and new details regarding the relative incidence of indicators and pathogens in shellfish. In addition, the results represent the first detailed account of the quality of commercially marketed shellfish in South Africa. An effort has also been made to find cases or outbreaks of disease which may have been caused by the consumption of contaminated shellfish.

6.2. MATERIALS AND METHODS

6.2.1. Shellfish, seawater and guano

Commercial supplies of shellfish

Forty two samples of shellfish were purchased at random from six commercial outlets in Pretoria over the period 1991-04-22 to 1992-09-15. The purpose for which the shellfish were purchased

was not disclosed to the suppliers. The test material included 29 samples of oysters (*Crassostrea gigas*), 7 samples of mussels (*Mytilus edilus*) and 6 samples of clams (*Mya arenaria*). Oysters were purchased as live or frozen supplies, but mussels and clams only as frozen supplies. Unless otherwise stated, all samples were processed by the elution procedure using pH 8,5 glycine-saline buffer as described in Part 5.

Mariculture supplies of shellfish

Thirteen samples of mussels (*Mytilus edilus*) were obtained from a commercial shellfish farm at Saldanha Bay (Fig. 6.1) where the mussels are cultivated on ropes hanging from rafts in the sea.

Seawater at a shellfish farm in Saldanha Bay

Samples of seawater (2 litres) were collected from selected sites at and near the shellfish farm. (Fig. 6.1).

Seabird droppings (guano)

Samples of droppings (about 10 g wet mass) from seabirds which often sit on the shellfish rafts were collected from the rafts as fresh as possible while they were still wet. Specimens were predominantly from cape cormorant (*Phalacrocorax capensis*), southern blackbacked gull (*Larus dominicanus*), Hartlaub's gull (*Larus hartlaubii*) and related species of seabirds.

Transportation of mariculture samples

Samples of mussels, seawater and bird droppings were aseptically collected into sterile nalgene containers immediately cooled to 4 - 10°C in cooler bags with freeze packs and transported at this temperature by air courier for arrival and analysis in the laboratory within 24 h of collection.

Preparation of test samples and basic procedures

Viruses, phages and bacteria were recovered from shellfish meat and guano by means of the elution procedure using 50% wet mass per volume pH 8,5 glycine-saline buffer homogenates as described in Part 5. Virus tests were carried out on centrifuged and chloroform decontaminated samples of homogenates. Tests for bacteria and phages were carried out on samples of homogenates prior to centrifugation and chloroform treatment. Tests for bacteria and

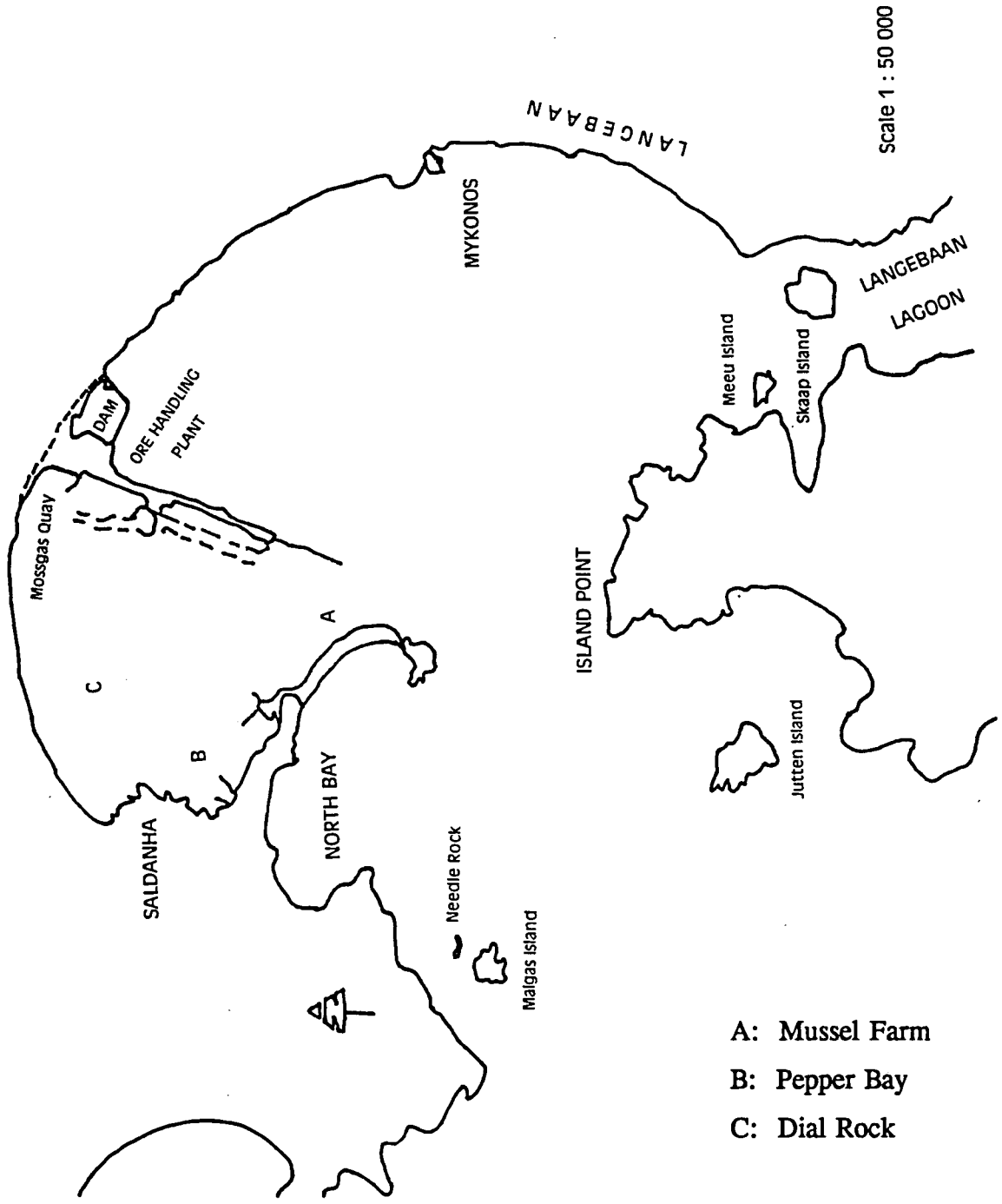


Figure 6.1: Map of Saldanha Bay showing location of mussel rafts and sampling points for seawater

phages were carried out within 30 min of adding shellfish meat or guano to the buffer, and the pH of these homogenates was not adjusted prior to testing. The pH of homogenate supernatants used for virus testing was adjusted to 7,4 immediately after centrifugation and prior to chloroform decontamination. In tests for low numbers of bacteria and phages, tests on dilutions were omitted and at least 5 plates were used for undiluted homogenates in each test. In doubtful cases tests were repeated using even more plates. Unless otherwise stated, seawater was tested for bacteria by a membrane filtration procedure (Grabow *et al,* 1992) using the same media as for the spread plate tests described below, and for phages by the plaque assays described below and in Part 5.

6.2.2. Human viruses

The BGM monkey kidney cell line was used for the detection and titration of viruses as described in Part 5.

6.2.3. Phages

Somatic and male-specific coliphages were enumerated by means of the plaque assays described in Part 5. Phages of *Bacteroides fragilis* strain HSP40 were enumerated by the plaque assay of Tartera *et al* (1992). The procedure, which was established under the personal supervision of Prof J Jofre, University of Barcelona, when he visited our laboratory, may be summarised as follows:

Growth medium

The following were dissolved in 1 000 ml of distilled water: Tryptone (Difco) 10,0 g; Beef Extract (Difco) 10,0 g; NaCl (Holpro) 5,0 g; Yeast Extract (Difco) 2,0 g; Cysteine HCl.$9H_2O$ (Sigma) 0,5 g; $CaCl_2$ (Merck) (0,5% in H_2O) 10,0 ml; $MgSO_4.7H_2O$ (Merck) (1,2% in H_2O) 10,0 ml. After autoclaving this medium was cooled down to 60°C and supplemented with filter-decontaminated 1 M glucose (Sigma); haemin (Sigma) (0,1% in 0,02% NaOH) 10,0 ml. The pH was then adjusted to 7,0 using concentrated HCl, followed by the addition of 100 mg of Kanamycin Sulphate (Sigma) and 7,5 mg of Vancomycin (Sigma). This growth medium for the host organism was stored at 4°C for not longer than 14 days.

Plaque assays

Double-layer-plaque assays for *B fragilis* HSP40 phages were basically carried out as described for coliphages in Part 5. Bottom Agar was prepared just like growth medium except that 12 g of Bacto agar was added prior to autoclaving and 1 M Na_2CO_3 (Merck) after autoclaving when the medium was cooled down to 60°C. Volumes of 20 ml bottom agar were poured into 90 mm petri dishes and stored at 4°C for not more than 4 days. Top agar was prepared just like bottom agar, except that only 7,0 g of agar was added. Volumes of 2,5 ml of top agar were dispensed into sterile tubes, stored at 4°C and used within 24 h. Plaque assay plates were incubated for 48 h at 37°C under strict anaerobic conditions using anaerobic jars (BBL GasPak Systems) and chemical deoxygenation (BBL GasPak Anaerobic System Envelope).

Host

Cultures of *B fragilis* HSP40 were kindly supplied by Prof Jofre. The host culture was streaked for single colonies on bottom agar plates which were incubated for 48 h as described for plaque assays. A typical Gram-negative colony was picked and inoculated into an appropriate volume of growth medium which was then incubated for 48 h as described for plaque assays. Subcultures of the host were made by transferring heavy inocula of typical colonies from plates to slopes of bottom agar in MacCartney bottles which were incubated for 48 h as described for plaque assays. These slants were stored at 4°C for not longer than 4 weeks.

6.2.4. Bacteriological tests

Unless otherwise stated, a spread plate procedure (Grabow *et al,* 1991) which may be summarised as follows was used: Leave 90 mm diam petri dishes (Carbi, South Africa) each containing 20 ml medium on the laboratory bench with lids removed for 3 h to dry; pipette 0,1 ml of shellfish meat homogenates or appropriate tenfold PBS dilutions onto these plates and evenly spread until dry by means of a smooth L-shaped 4 mm diam glass rod. The 3 cm long part of the rod used for spreading was decontaminated for each plate by dipping into 96% ethanol and burning dry. Plates were incubated upside down, and all colonies on plates containing 20 - 100 of the colonies concerned were counted. At least three plates were used for the undiluted homogenate and each dilution.

6.2.4.1. Standard plate count

The basic pour plate procedure as described for the heterotrophic plate count in Standard

Methods (1995), was used. The growth medium was prepared by adding the following to 1 000 ml of distilled water: Agar (Difco) 15,0 g; Tryptone (Difco) 5,0 g; Yeast Extract (Difco) 2,5 g; Glucose (Sigma) 1,0 g. The ingredients were dissolved by boiling, and the medium dispensed in 15 ml volumes into MacCartney bottles, autoclaved and stored at 4°C for not more than 4 weeks. Tests were carried out by steam dissolving the medium in bottles, cooling the medium down to 50°C, pipetting 1,0 ml of shellfish homogenate or dilutions, into petri dishes, adding one bottle of medium per plate, mixing by gentle swirling, and allowing to solidify. After incubation at 37°C for 48 h, all colonies were counted.

6.2.4.2. Total coliform bacteria

M-Endo LES Agar (Difco) was used (Grabow *et al,* 1992). Plates were incubated at 37°C ± 1°C for 24 h, after which typical brilliant green colonies with a metallic sheen were counted.

6.2.4.3. Faecal coliform count

M-FC Agar (Difco) without rosolic acid (Grabow *et al,* 1981; 1992) was used, plates were incubated at 44,5°C ± 0,2°C for 20 to 24 h in a water bath, and typical bright blue colonies were counted.

6.2.4.4. Enterococci

M-Enterococcus Agar (Difco) plates were incubated at 35-37°C for 48 h, and typical red to pink colonies were counted (Rippey *et al,* 1987; Grabow *et al,* 1991).

6.2.4.5. *Staphylococcus aureus*

Baird-Parker Agar (Difco) was used, plates were incubated at 37°C for 48 h, and typical *S aureus* colonies were counted (Tatini *et al,* 1984). These colonies were usually ≥ 1,5 mm in size, black to dark grey, smooth convex with entire margins and off-white edge, sometimes showing an opaque zone and/or a clear halo extending beyond the opaque zone. Representative numbers of typical colonies were picked and purified on the same medium for confirmation by the coagulase test (Tatini *et al,* 1984; SABS, 1989).

6.2.4.6. *Salmonella* species

Bismuth Sulphite (BS) Agar (Difco), *Salmonella Shigella* (SS) Agar (Difco), MacConkey's Agar (Difco), Brilliant-Green Phenol-Red Lactose Sucrose (BPLS) Agar (Difco) and Xylose Lysine

Desoxycholate (XLD) Agar (Difco) were used (Poelma *et al,* 1984), and after incubation at 37°C for 24 h, typical colonies were picked and purified on the same medium for confirmation by biochemical and serological tests (Poelma *et al,* 1984; Ministry of Agriculture, 1992). On XLD medium *Salmonella* colonies are usually pink, sometimes with black centres which may be so large as to give the entire colony an almost glossy black appearance. On BS agar *Salmonella* colonies are brown or black and sometimes the colonies have a metallic sheen, and on MacConkey's agar, colonies are transparent and colourless, sometimes with dark centres. Typical colonies on BPLS agar are pink and on SS agar colonies are opaque or transparent (Poelma *et al,* 1984; SABS, 1989).

6.2.4.7. *Shigella* species

Test samples (1,0 ml) were inoculated into 10 ml Gram-Negative (GN) Broth (Difco) and incubated at 37°C for 2 h for enrichment (Morris, 1984). Volumes of 0,1 ml were then streaked onto MacConkey's, XLD, SS and BPLS agar and incubated at 37°C for 24 h. Typical colonies on XLD agar appear as red or pink, opaque or transparent on MacConkey's and SS agar and pink on BPLS agar. Typical colonies were then picked and purified on the same medium for confirmation by biochemical tests (Morris, 1984; SABS, 1989).

6.2.4.8. *Vibrio* species

Test samples (1,0 ml) were added to 10 ml Alkaline Peptone Water (Oxoid) (West and Coleman, 1986) and incubated at 37°C for 6 - 8 h. Volumes of 0,1 ml were then streaked onto Thiosulphate-Citrate-Bile-Salts-Sucrose (TCBS) Agar (Difco) and incubated at 37°C for 24 h. Typical colonies were picked and purified on the same medium for biochemical identification. On TCBS agar typical colonies of *V cholerae* are large, smooth and yellow, and those of *V parahaemolyticus* round (2 - 3 mm diam) and green or blue. Colonies of *V alginolyticus,* which tend to interfere with other colonies, are larger than those of other *Vibrio* species, and yellow coloured (Twedt *et al,* 1984; SABS, 1989).

6.2.4.9. *Clostridium* species

A 2 ml sample of shellfish meat homogenate was kept in an 80°C waterbath for 2 min (Easterbrook and West, 1987; SABS, 1989). Spread plate counts were then carried out using Lecithin Agar plates (Difco). Plates were incubated anaerobically for 24 h at 37°C. *Clostridium*

perfringens hydrolyses egg yolk lecithin and produces an opaque halo around the black colonies. Cultures of presumptive black colonies were confirmed as *C perfringens* if they were nonmotile, reduce nitrate, ferment lactose and liquefy gelatin within 44 h and produce acid from raffinose (Harmon and Duncan, 1984; SABS, 1989).

6.2.5. Calculation of results

For purposes of convenient comparison in terms of recommended quality limits, all results were expressed as calculated numbers per 100 g wet mass of shellfish meat, 100 ml seawater and 1 g wet mass of guano. Thus, the detection limit for numbers in shellfish meat and guano in routine tests was:

Viruses: 0,005 ml undiluted test sample in each of 96 microtitre wells
= 0,48 ml total volume = 0,24 g wet mass shellfish meat,
ie, 1 virus per 0,24 g and 417 viruses per 100 g.
However, MPN calculations yield results of less than 1 virus per ml.

Phages: 1,0 ml undiluted test suspension in each of 5 plates
= 2,5 ml total volume = 1,25 g wet mass shellfish meat,
ie, 1 plaque per 1,25 g and count of 80 per 100 g.

Bacteria: 0,1 ml undiluted test suspension in each of 5 plates
= 0,5 ml total volume = 0,25 g wet mass shellfish meat,
ie, 1 colony per 0,25 g and count of 400 per 100 g.

Detection limits for seawater:

Phages: 1,0 ml seawater in each of 5 plates = 5,0 ml total volume
ie, 1 plaque per 5 ml and count of 20 per 100 ml.

Bacteria: Membrane filtration tests on up to 100 ml samples
ie, 1 colony per 100 ml and count of 1 per 100 ml.

6.2.6. Epidemiological studies

Arrangements were made with a medical practitioner at Grootbrak near George on the Cape East Coast for the collection of specimens and information from cases of disease which may be assosciated with the consumption of shellfish. A supply of specimen bottles for the immediate collection of stool and blood samples from suspect cases was made available, together with

questionnaires for clinical and epidemiological details. The project was kindly co-ordinated by the Regional Office of the Department of National Health and Population Development at George.

6.3. RESULTS

6.3.1. Commercially marketed shellfish

Results of tests are summarised in Table 6.1. Results are combined in one table because counts for various samples of live and frozen oysters, and samples of frozen mussels and clams, did not differ significantly. The following organisms were never detected in any of the samples: *Salmonella, Shigella, V cholerae* (*V alginolyticus* was detected in three samples of oysters), *Clostridium*, male-specific coliphages, *B fragilis* phages and enteric viruses.

The percentage of samples of commercial shellfish supplies which exceeded recommended guidelines (Department of Health, 1973; Lusher, 1984; Grabow *et al*, 1989) indicated in brackets for various quality indicators was as follows:

Standard plate count	(100 000/100 g)	11%
Faecal coliforms	(500/100 g)	23%
Enterococci	(200/100 g)	30%
S aureus	(1 000/100 g)	10%
Somatic coliphages	(10/100 g)	5%

6.3.2. Mariculture samples of shellfish

Results are summarised in Table 6.2. The following organisms were never detected in any of the samples: *S aureus, Salmonella, Shigella, Clostridium* and enteric viruses. A strain of *Vibrio* which had all biochemical characteristics of V *cholerae* was recovered from one sample of oyster meat. In agglutination tests, however, this isolate proved to be a non-pathogenic strain.

The percentages of 13 samples of mariculture mussels which exceeded guidelines (Department of Health, 1973; Lusher, 1984; Grabow *et al*, 1989) indicated in brackets for various quality indicators was as follows:

Standard plate count	(100 000/100 g)	53%
Faecal coliforms	(500/100 g)	38%
Enterococci	(200/100 g)	38%
V cholerae	(0/100 g)	8%
Somatic coliphages	(10/100 g)	54%

Male-specific coliphages were detected in 23% and *B fragilis* phages in 8% of samples.

6.3.3. Seawater samples

Results summarised in Table 6.2 show that counts of all organisms concerned were generally highest at B (Pepper Bay), lower at A (Mussel Farm) and lowest at C (Dial Rock). The average and median faecal coliform counts at A were 7 and 1 per 100 ml, respectively.

6.3.4. Seabird guano

Results for seabird guano collected from rafts at A (mussel farm) show very high counts for indicators of faecal pollution (Table 6.2). Outstanding features of the results are that *Bacteroides fragilis* HSP40 phages were never detected, and that the ratio of faecal coliforms to enterococci was more than one.

Table 6.1: Counts of indicator bacteria in commercially marketed shellfish

Determinand	Counts per 100 g of shellfish meat		
	Oysters (n = 29)	Mussels (n = 7)	Clams (n = 6)
Standard plate count			
Range	7 000 - $3,0x10^7$	20 600 - $1,2x10^8$	207 500 - $5,8x10^6$
SD	7844	448 921	21 071
Median	193 000	152 000	703 000
Average	656 400	$1,8x10^7$	$1,6x10^6$
Faecal coliforms			
Range	0 - 10 400	0 - 0	0 - 8 100
SD	27,2	0	31,8
Median	0	0	400
Average	1 000	0	1 600
Enterococci			
Range	0 - 9 100	0 - 600	0 - 5 100
SD	17,9	2,2	19,0
Median	0	0	1 300
Average	600	150	1 700
S aureus			
Range	0 - 3 300	0 - 1 000	0 - 600
SD	8,3	3,7	2,5
Median	100	0	200
Average	390	140	210

SD = Standard Deviation

Table 6.2: Counts of indicator bacteria and phages in seawater (A, B, C), mussels and seabird droppings at a mariculture site

Determinand	Counts per 100 g of mussel meat, 100 ml of seawater or 1 g of guano				
	A (n = 15)	B (n = 15)	C (n = 15)	Mussels (n = 13)	Guano (n = 6)
Standard plate count					
Range				$5{,}3\times10^{2}$ - $4{,}9\times10^{9}$	
SD	--	--	--	$1{,}6\times10^{7}$	--
Median				$4{,}7\times10^{5}$	
Average				$6{,}9\times10^{6}$	
Total coliform					
Range	0 - 17 000	0 - 53 000	0 - 1 300	0 - $9{,}8\times10^{6}$	$9{,}0\times10^{4}$ - $5{,}0\times10^{10}$
SD	4 403	14 058	360	$3{,}2\times10^{6}$	$8{,}1\times10^{9}$
Median	13	80	12	3 000	$1{,}5\times10^{9}$
Average	1 377	5 299	154	$1{,}2\times10^{6}$	$6{,}2\times10^{9}$
Faecal coliform					
Range	0 - 71	0 - 170	0 - 11	0 - $9{,}2\times10^{5}$	1 000 - $1{,}2\times10^{10}$
SD	18,0	59,7	2,9	255 002	$4{,}7\times10^{9}$
Median	1	16	1	0	$3{,}0\times10^{8}$
Average	7	43	2	71 307	$2{,}6\times10^{9}$
Enterococci					
Range	0 - 1 900	0 - 8 400	0 - 300	0 - $1{,}6\times10^{6}$	3 000 - $3{,}9\times10^{9}$
SD	488	2 153	103	459 705	$1{,}5\times10^{9}$
Median	3	19	3	30	$5{,}0\times10^{6}$
Average	138	623	50	143 055	$6{,}5\times10^{8}$
Somatic coliphages					
Range	0 - 66	0 - 133	0	2 700 - $4{,}9\times10^{9}$	13 - $1{,}2\times10^{7}$
SD	4,6	6,7	0	$1{,}3\times10^{9}$	$4{,}4\times10^{6}$
Median	0	0	0	0	$3{,}0\times10^{6}$
Average	12	18	0	$4{,}2\times10^{8}$	$3{,}7\times10^{6}$
MS coliphages					
Range	0 - 30	0 - 27	0	0 - $1{,}3\times10^{6}$	0 - $3{,}3\times10^{6}$
SD	0,5	1,2	0	360 526	$1{,}6\times10^{6}$
Median	0	0	0	0	240 600
Average	2	2	0	100 095	$1{,}1\times10^{6}$

SD = Standard Deviation, A = Mussel Farm, B = Pepper Bay, C = Dial Rock, mussels and guano were collected at A (see Fig 6.1)

6.3.5. Epidemiological studies

Only one outbreak of gastroenteritis among workers at a shoe factory was encountered. It was not possible to associate this outbreak with the consumption of shellfish, and eventually it appeared that the infections were caused by the consumption of curried fish sold by a street vendor. Stool specimens from 18 patients in the outbreak were analysed by molecular techniques and small round structured viruses (Norwalk-like) were detected in some of them (Wolfaardt *et al*, 1995a,b). Stool specimens from a number of additional individuals with gastroenteritis were also obtained. These were not investigated in any detail because no epidemiological association with the consumption of shellfish could be established, and in many cases materials and relevant information were insufficient for meaningful investigation.

6.4. DISCUSSION

6.4.1. Tests and detection limits

In the routine tests described in this Part, the quantities of wet mass of shellfish meat and guano tested were limited to 0,24 g for viruses, 1,25 g for phages, and 0,25 g for bacteria. The testing of these small quantities of shellfish meat was sufficient to enumerate viruses, phages and bacteria for purposes of evaluating the recommended pH 8,5 glycine-saline elution recovery procedure in practice. The sensitivity was also sufficient for assessment of the quality of shellfish in terms of recommended limits for bacteria and phages, and for assessment of faecal pollution of seawater at the mariculture site. All the quality guidelines referred to in this study are expressed in terms of calculated levels of organisms per 100 g, and it is not expected to literally analyse 100 g of shellfish meat for each organism. In order to avoid confusion and to increase the accuracy and reliability of quality guidelines, the specifications should include details on testing procedures and the minimum quantities of shellfish meat to be tested. This is one good reason for revising and updating current guidelines for the quality of shellfish meat.

As mentioned in Part 5, the intention of this study was not to detect the smallest possible number of viruses, phages and bacteria in shellfish meat. The purpose was to select the most efficient recovery procedure. A variety of established approaches can subsequently be applied to detect

the smallest possible numbers of organisms in the shellfish concentrates. Larger quantities of cell cultures could be inoculated with larger volumes of concentrates, and secondary concentration steps, molecular techniques, and qualitative presence-absence tests could be used. Similarly, a variety of tests could be used to detect smaller numbers of phages.

Seawater was not analysed for viruses because the intention was merely to assess faecal pollution at the mariculture site with regard to potential impact of wastewater discharge into the bay, and the contribution of seabird droppings to faecal bacteria and phages in seawater at the shellfish rafts. The inclusion of expensive, labour intensive and time consuming virus tests in these investigations was not considered justified. The membrane filtration counts with limit of 1 per 100 ml offered a practical and sensitive tool for quantitative assessment of total coliforms, faecal coliforms and faecal streptococci in the seawater. Routine tests for phages which were limited to 5 ml samples of seawater, were obviously less sensitive, but still yielded meaningful results. The very low counts in Table 6.2 were obtained by additional tests on larger volumes of seawater.

6.4.2. Test methods and quality guidelines

The recovery and detection techniques applied in tests on a variety of shellfish, seawater and guano samples proved suitable for routine purposes of quality assess-ment. No technical problems were encountered with the techniques. Colonies of bacteria and plaques of phages were clearly identifiable without interference by contaminants. In tests for cytopathogenic enteric viruses, shellfish meat preparations were not toxic to cell cultures, and contamination of cell cultures was rarely a problem. Together with data on recovery and detection techniques reviewed in Part 5 and in the introduction of this Part, and results recorded in Part 5, these observa-tions confirm that the pH 8,5 glycine-saline elution procedure is probably the most sensitive and practical presently available for shellfish quality assessment. These technique, could, therefore, form the basis of practical and reliable standards or specifications for assessment of the quality of shellfish meat. As mentioned earlier, a variety of procedures can subsequently be used to detect low numbers of viruses, phages and bacteria, as may be considered desirable for purposes of research or quality assessment. In terms of the public health and economic implications of pathogens in shellfish (see Part 2), continued research along these lines warrants high priority.

The wide range of efficiencies and sensitivities recorded in this and earlier evaluations of the many methods used for assessment of the microbiological quality and safety of shellfish, show that quality guidelines or standards without specifications for testing procedures are for all practical purposes useless. This implies that quality guidelines and specifications with no reference to recovery and detection methods, such as those in Department of Health (1973) and Lusher (1984), as well as specifications with outdated determinants and test methods such as SABS (1977), are in urgent need of revision and updating.

6.4.3. Commercial supplies of shellfish

The results recorded in this study, some of which in Table 6.1, are the first of this kind on the microbiological quality of randomly selected samples of shellfish commercially marketed in South Africa. The absence of detectable levels of *Vibrio cholerae,* cytopathogenic enteric viruses, species of *Salmonella, Shigella* and *Clostridium,* and male-specific coliphages and *Bacteroides fragilis* HSP40 phages, indicate acceptable quality at least in terms of these pathogens and indicators of faecal contamination. The detection of *Vibrio alginolyticus* in three samples of oysters is not altogether surprising because the organisms are natural inhabitants of the marine environment (Opal and Saxon, 1986), These organisms are not associated with enteric infections, but wound infections have been described (Opal and Saxon, 1986).

However, the finding that at least some recommended limits (Department of Health, 1973; SABS, 1977; Lusher, 1984; Grabow, 1989; Grabow *et al,* 1989) were exceeded for faecal coliforms in 23% of samples of commercially marketed shellfish, enteroccocci in 30% of samples, *Staphylococus aureus* in 10% of samples and somatic coliphages in 5% of samples, has the following implications for commercially marketed shellfish:

6.4.3.1. Safety of shellfish supplies

The presence of bacteria and phages associated with faecal pollution at levels considered unacceptable indicates the potential presence of enteric pathogens. These shellfish supplies may, therefore, be considered to constitute a potential health risk and not fit for commercial marketing.

6.4.3.2. Quality guidelines and specifications

Among questions in need of answers in this regard, is wether perhaps the recommended microbiological limits are too stringent, and whether perhaps shellfish of this quality do not constitute a meaningful health risk. Answers to these questions require epidemiological data on diseases transmitted by shellfish. At present no such data exist in South Africa, and there is no infrastructure or research projects from which such data may be forthcoming. Epidemiological data from other parts of the world show that the determinants and limits concerned have restricted relation to risk of infection, and that there is a need for alternative tests, notably the viruses associated with shellfish-borne infections (see Part 2). In the assessment of quality guidelines, attention should also be given to the sensitivity of test methods, because it may be possible that limits based on past test methods are unduly stringent for the more sensitive tests methods now available. Until more appropriate determinants, limits and tests have been developed, it would not appear unreasonable to expect that commercially marketed shellfish should, at least in terms of basic principles of hygienic and aesthetic considerations, meet the recommended limits for indicators of faecal contamination. This would not appear unreasonable since the results in Table 6.1 show that a high percentage of commercially marketed shellfish does indeed conform to the recommended limits.

6.4.3.3. Routine quality surveillance of commercially marketed shellfish

The finding that a substantial percentage of test samples exceeded basic quality limits, reveals a need for an investigation into routine quality surveillance protocols and specifications for quality monitoring (SABS, 1972) of commercially marketed shellfish supplies in South Africa.

6.4.3.4. Test methods for shellfish

Presently used test methods give no indication of the origin of indicators of faecal contamination. This may be important because at least some of these organism may multiply in shellfish supplies in transit or during storage (Stammen *et al,* 1990), which implies that the numbers are no longer related to levels of faecal contamination. In addition, as will be discussed later on, some of the indicators of faecal pollution may not originate from human excreta but from, for instance, seabirds, which may not constitute a meaningful risk of infection to humans (Jagals *et*

al, 1994). In the interest of realistic assessment of the quality and safety of shellfish supplies, it would, therefore, seem important to pay attention to test methods which supply more information on the origin and source of indicators of faecal pollution, and the actual levels of faecal pollution.

6.4.4. Mussels, seawater and seabird droppings at a mariculture site

The absence of detectable levels of cytopathogenic enteric viruses, *Staphylococcus aureus* and species of *Salmonella, Shigella* and *Clostridium*, indicates that the mussels concerned were of acceptable quality with regard to these pathogens and indicators. The detection of a non-pathogenic strain of *Vibrio cholerae* in one sample of mussels, has implications similar to those described above for the isolation of *V alginolyticus* from commercial supplies of shellfish.

However, the finding that limits recommended by the Department of Health (1973) and Grabow *et al* (1989) were exceeded for mussels collected at the mussel farm A (53% of samples for the standard plate count, 38% for faecal coliforms, 38% for enterococci, 8% for *V cholerae* and 54% for somatic coliphages), has implications for the mariculture industry similar to those described above for commercial supplies of shellfish. Another meaningful observation is that on one occasion seawater at the mussel farm exceeded faecal coliform limits recommended by Lusher *et al* (1984) for seawater at shellfish harvesting grounds. As discussed above for the commercial supplies of shellfish, these findings call for an investigation of microbiological quality guidelines, quality surveillance protocols, and test methods for the mariculture industry. Assessment of the limited information available for this study, suggests the following with regard to the origin of faecal pollution:

a) The presence of *B fragilis* HSP40 phages in one sample of mussels indicates faecal contamination of human origin (Grabow *et al,* 1994). This single detection of these phages would require confirmation for meaningful conclusions.

b) The relatively high counts of coliforms, enterococci, somatic coliphages and male-specific coliphages (Table 6.2) at station B (Pepper Bay), and lower counts at C (Dial Rock) and B (Mussel Farm), suggest that the presence of these organisms at A may originate from sewage discharged from the land to the sea near B. The possibility that indicators of faecal pollution in wastewater discharged into Saldanha Bay may reach the

mussel farm at point A would require confirmation, and detailed qualitative and quantitative analysis for assessment of the impact of wastewater discharge into Saldanha Bay on the comprehensive mariculture industry in the Bay.

c) The high counts of coliforms, enterococci, and somatic and male-specific coliphages in seabird droppings collected from shellfish rafts at the shellfish farm A, raise interesting and important questions about the presence of these organisms in the seawater and mussels at the rafts. It would appear possible that the faecal contamination of the seawater and mussels may, at least in part, be due to faecal pollution by seabirds.

6.4.5. Role of seabird droppings in quality guidelines

As mentioned above, seabird droppings, and possibly also excreta from other marine animals, may have a meaningful impact on conventional indicators of faecal pollution such as coliforms, streptococci and coliphages, in shellfish and seawater at mariculture sites and natural harvesting grounds. Since there would not seem to be evidence that excreta from seabirds or other marine animals may constitute a meaningful risk of infection for humans, it may prove advisable to distinguish between faecal pollution of human and animal origin in assessment of the quality of shellfish and seawater. Elucidation of questions in this regard would, among other things, require more detailed analysis of faecal organisms excreted by seabirds, quantities of excretion, survival of seabird faecal organisms in seawater, and the development of techniques for distinguishing between seabird and human faecal pollution. A point of interest is the observation that the ratio of faecal coliforms to enterococci was more than 1 in the seabird droppings (Table 6.1). A similar ratio has been reported for Canada geese and whistling swans (Hussong *et al*, 1979). The ratio for human faeces is also know to be more than 1, while that of most other animals tends to be considerably less than 1, a feature which is used to distinguish between faecal pollution of human and animal origin (Jagals *et al*, 1994).

It may prove advisable to include indicators which can more reliably distinguish between faecal pollution of human and animal (ie, seabird) origin. Indicators such as *B fragilis* HSP40 phages, and certain types of F-specific RNA coliphages which seem to be specific for human excreta, could play a role in this regard. Indicators such as the chemical compound coprostanol which

is highly specific for faeces (Tietz, 1976), or uric acid which is highly specific for sewage (Brown *et al,* 1982), could also be considered. Seabird droppings should also be investigated for the possible presence of seabird viruses which in detection systems such as cell culture propagation or PCR could be mistaken for human viruses. No information on enteric seabird viruses has been found in the literature.

6.4.6. Epidemiological studies

Grootbrak was selected as study area because unconfirmed reports had indicated that infections were frequently seen among the general public who consumed shellfish harvested from sites exposed to sewage pollution. The fact that the only outbreak of gastroenteritis encountered was probably caused by a supply of curried fish sold by a street vendor and not the consumption of shellfish, was disappointing. However, the failure to detect shellfish associated infections may again be due to shortcomings in the surveillance system. The collaborating medical practitioner who initially was very enthusiastic, left town shortly after initiation of the project, and after that communication virtually broke down. However, the molecular detetection of small round structured viruses (Norwalk-like) in some of the 18 patient stool specimens (Wolfaardt *et al,* 1995a,b) confirmed that expertise for the detection of these viruses often associated with shellfish (Atmar *et al,* 1995) had successfully been established. Follow-up studies would seem to be justified, and the experience gained in this first effort at finding shellfish-related infections should prove of value in future studies.

6.4.6. Conclusions

Evidence has been presented in this study that the pH 8,5 glycine-saline elution method is probably the most practical, sensitive and economical procedures presently available for the recovery of a variety of micro-organisms from shellfish. The method has successfully been applied in practice, and is ready for routine application. Application of the technique on randomly selected samples of commercial supplies of shellfish revealed that at least certain quality limits were exceeded in some samples, which calls for more detailed research on quality guidelines, routine quality surveillance programmes and test methods used in these programmes. The analysis of mussels, seawater and seabird droppings at a mussels farm in Saldanha Bay disclosed indications of faecal pollution. These findings raised questions about the origin of the

faecal contamination, and possibilities include wastewater discharged into the Bay, and droppings by seabirds, which frequent the shellfish rafts. Studies along these lines are important because faecal pollution would have a major impact on the extensive mariculture industry in Saldanha Bay. Although efforts to find cases of disease associated with the consumption of shellfish were not successful, the information gathered, experience gained and expertise established justify follow-up surveillance studies.

7. UPTAKE AND RELEASE OF VIRUSES AND INDICATORS BY SHELLFISH

7.1. INTRODUCTION

Faecal bacteria are generally used for assessment of the hygienic quality of shellfish, and most quality criteria are based on counts of these indicators in shellfish meat (see Part 2). However, evidence is accumulating that faecal bacteria are not fail-safe indicators for human viruses. This evidence is primarily based on epidemiological data which associate viral disease outbreaks with the consumption of shellfish which conform to specifications for faecal bacteria, as well as commercial depuration procedures (Sockett *et al,* 1986; Chalmers and McMillan, 1995). In addition, viruses have been detected in shellfish which meet faecal bacterial specifications. In shellfish analysed directly after collection from sewage polluted marine environments, counts of faecal bacteria are generally much higher than those of viruses detectable by conventional methods (Grabow, 1989). The presence of viruses in the absence of faecal bacteria does, therefore, imply that under circumstances such as those prevailing in depuration processes, faecal bacteria are released or inactivated by shellfish much faster than viruses. Longer survival of viruses than bacteria in shellfish meat during transport, storage, and decontamination by for instance cooking, may also play a role (Sockett *et al,* 1986; Grabow, 1989).

Better understanding of the uptake and release of viruses and commonly used bacterial indicators is essential for the formulation of guidelines for depuration processes, quality criteria, and test methods for the routine quality assessment of shellfish intended for human consumption. A number of studies aimed at finding answers to the many questions concerned, have been published. These studies primarily involved small-scale laboratory experiments in which the accumulation of selected organisms by shellfish in seeded water and subsequent release in clean water was investigated under optimal filtration conditions (Crovari, 1958; Mitchell *et al,* 1966; Duff, 1967; Hoff and Becker 1969; Canzonier, 1971; Di Girolamo *et al,* 1975; Hartland and Timoney, 1979; Son and Fleet, 1980; Sobsey *et al,* 1987; Richards, 1988; Power and Collins, 1989; De Mesquita *et al,* 1991; Enriquez *et al,* 1992; Boher and Schwartzbrod, 1993; Doré and Lees, 1995). Basically the results of these experiments confirm that at least some model viruses are released slower than conventionally used indicators such as coliform bacteria, and that shellfish treated according to generally accepted specifications for commercial depuration processes may conform to coliform limits but still contain infectious viruses.

Reasons for the slower reduction in counts of viruses (human viruses and phages) than faecal bacteria in a variety of bivalve molluscs during depuration have been investigated in a number of studies. Some workers suggested that differential elimination rates may be due to viruses being able to penetrate shellfish tissue from the digestive tract due to their small size, while the larger bacteria fail to do this (Canzonier, 1971; Di Girolamo *et al*, 1975). This theory was supported by a study in which *Crassostrea gigas* oysters were allowed to ingest ^{3}H-labelled cricket paralysis virus, an insect picornavirus. Autoradiographic methods were used to demonstrate the presence of the virus in the epithelial cells of the digestive diverticula tubules, mid-gut and connective tissues surrounding the digestive tract. Label was not apparent in the tissues of the gonads, gills, mantle, muscle, or labial palps. However, Doré and Lees (1995) failed to confirm these observation in a study using *Escherichia coli* and a male-specific phage as model bacterium and virus in naturally contaminated *Crassostrea gigas* oysters and *Mytilus edulis* mussels. Various tissues and organs were dissected and tested for the presence of *E coli* and the phage by conventional cultivation techniques. In situ molecular transcription studies also localised most bioaccumulated hepatitis A virus in the stomach and digestive diverticulum of *Crassostrea virginica* oysters and *Mercenaria mercenaria* hard-shell clams (Romalde *et al*, 1994).

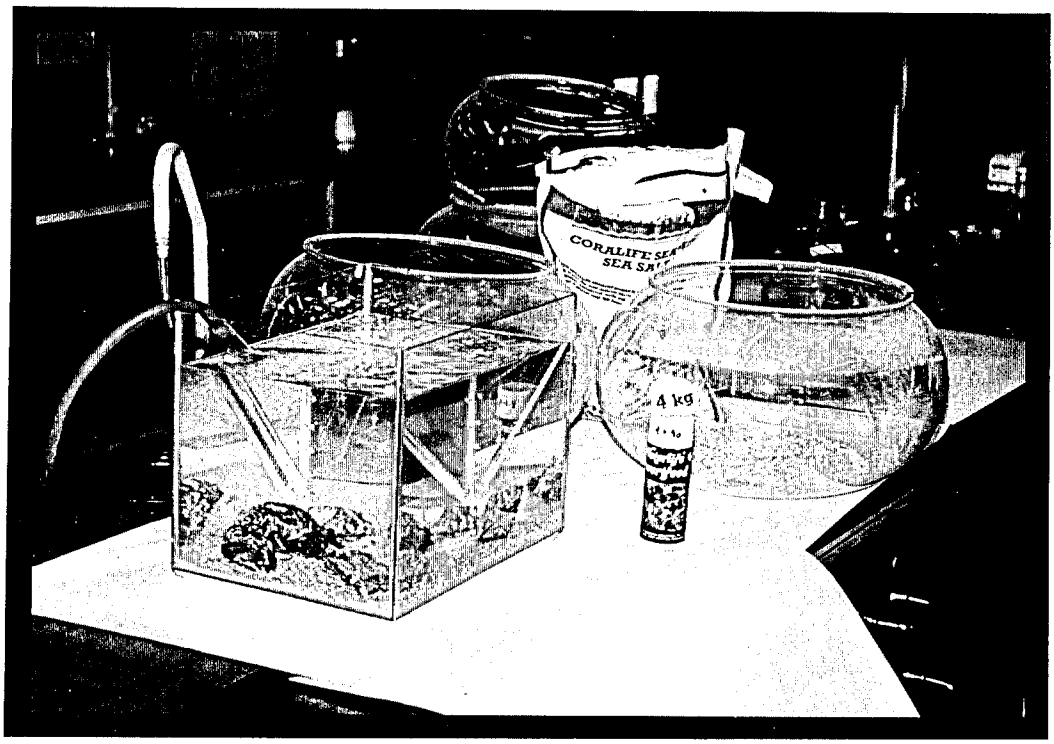
The objective of this study was to carry out similar experiments on shellfish, human viruses, indicators and experimental conditions not previously used. The intention was to assess the extent to which earlier findings apply to different shellfish obtained from different environments, and to viruses and indicators not previously studied. The phages included were considered of particular importance because they have attractive indicator features (see Parts 2 and 3). In addition, the opportunity was used to compare three buffers for the recovery of viruses and indicators. An attempt has also been made to investigate the penetration of shellfish tissue by viruses. The results were expected to supplement earlier findings, and to supply new information essential for the formulation of practical and reliable guidelines for depuration policies and techniques for assessment of the quality and safety of shellfish intended for human consumption.

7.2. MATERIALS AND METHODS

Shellfish depuration experiments

Oysters (*Crassostrea gigas*) were kindly supplied by Dr E Schumann (Department of Oceanography, University of Port Elizabeth). Medium size oysters were collected from ropes of floating rafts at a mariculture farm in Algoa Bay at Port Elizabeth on the East coast of South Africa. The oysters were directly packed into sealed containers and kept at 5-10°C during air transit (controlled pressure cabin) to reach our Pretoria laboratory within 8 h of collection from the sea. In the laboratory the oysters were immediately transferred carefully according to a step-by-step protocol limiting temperature and other environmental shocks, to glass storage aquaria for acclimatisation. Not more than 40 oysters were kept in a 40-L tank (Fig 7.1). Artificial seawater was used for storage and all experiments. The seawater (salinity at specific gravity of 1,020-1,021 and pH 8,3) was prepared from a commercial product (Coralife Seamix Salt, Superpet, South Africa) according to the instructions of the manufacturers. The storage tanks were aerated according to conventional procedures, and the water was pumped through a biological bed consisting of seashell chips and sand to control levels of ammonia, nitrates and other metabolites. Storage and experiments were carried out in a laboratory without temperature or air conditioning, in which the temperature of the water was 18-22°C. Oysters in storage and test aquaria were carefully observed, and only oysters which opened, seemed to filter water, and closed when touched with a glass rod, were used. Dead, gaping or seemingly inactive oysters were discarded immediately. Oysters were kept without feeding in storage tanks for 2-3 days.

Batches of 20 selected oysters were carefully transferred, under conditions of minimal shock and stress, to round test aquaria (26 cm diam, 23 cm high) (Fig 7.1) containing 5 L of seawater for exposure to test organisms under continuous aeration. After about 1 h when all test oysters had settled down and were pumping water, the water was seeded with 50 ml of inocula containing various combinations and quantities of viruses, phages and bacteria. Inocula were prepared as follows: Volumes of 10 ml of a maximum-level culture of *Tetraselmis* algae in seawater (kindly supplied by the Sea Fisheries Research Institute, Cape Town), and 1 ml each of appropriate suspensions of viruses, phages and faecal bacteria prepared as described in Parts 3 to 6, were added to 40 ml of seawater and gently stirred for about 15 min to allow adsorption of the test organisms to the algae. The inoculum was then added to the water in test aquaria and carefully stirred to obtain homogenous mixing without disturbing the oysters. A 20 ml sample of the



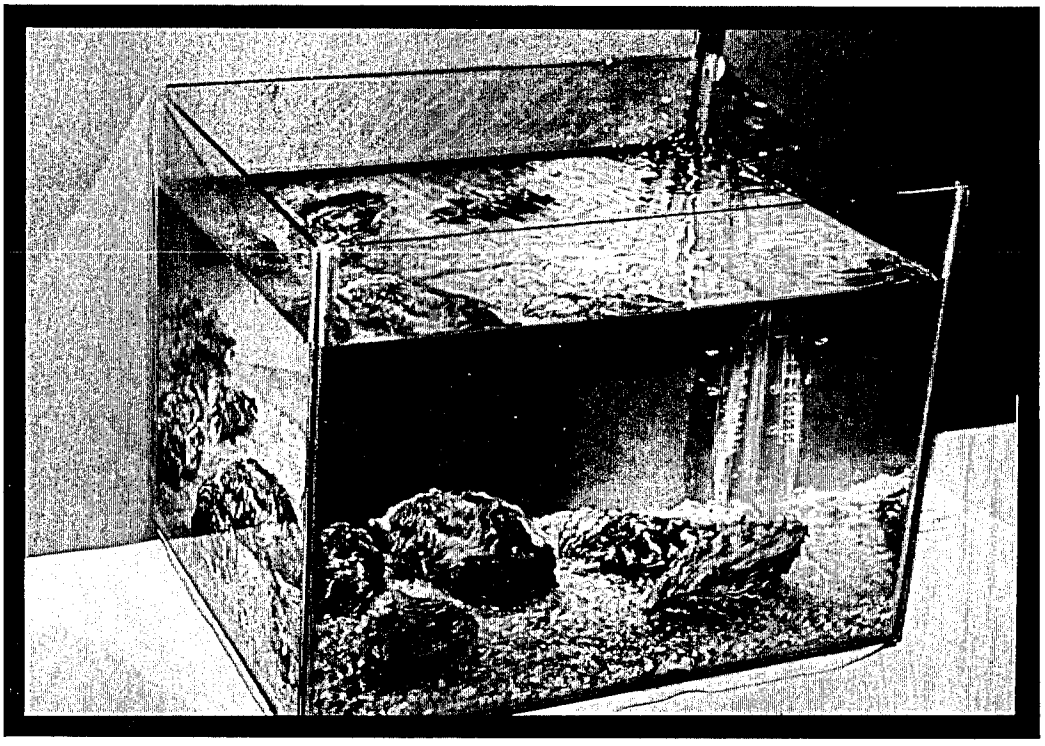


Figure 7.1: Oysters in 40-L square glass aquarium (bottom and top), round test aquaria, and commercial supplies of seawater salts and shellfish food (top)

water was then removed for enumerating test organisms in the water, and oysters were left undisturbed for 2 h to filter the seeded test water, which is more than the 30 min generally considered sufficient for seeding shellfish by ingestion during natural feeding (Hay and Scott, 1986; De Mesquita *et al*, 1991; Enriguez *et al*, 1992). Two oysters were removed for enumeration of organisms accumulated by the oysters during seeding. The rest of the oysters were then gently removed one by one from the seeding tank using scissor grabs and transferred to an aquarium with 8 L of clean water for depuration. After 24 h at least 2 more oysters were removed for analysis, and the remaining oysters were transferred to an aquarium with clean water for continued depuration. This procedure was repeated every 24 h for the duration of the experiments.

Microbiological analysis

The meat of at least two oysters collected at each time interval was pooled and processed according to the elution technique described in Part 5. Briefly, shellfish meat homogenates (50% w/v, ie, wet mass shellfish meat per volume of buffer) were prepared in parallel in pH 8,5 glycine-NaCl buffer (Part 5), pH 8,0 saline (0,85% NaCl) (Idema *et al*, 1991), or pH 9,2 borate buffer (Boher *et al*, 1991) using ultra-turrax homogenisation (Part 5). Human viruses were titrated by MPN assays in which 0,005 ml of shellfish meat homogenate and appropriate tenfold PBS dilutions were each inoculated into 8 wells of 96-well microtitre plates (Part 5).

Bacteria were enumerated by spreading 0,1 ml of the homogenate and dilutions each onto 3 plates of appropriate media, and phages by using 1,0 ml of homogenate and dilutions each in 3 plaque assays (Parts 5 and 6). With bacteria and phages, the numerical average of the three counts for the homogenate and each dilution were used to calculate the count in the homogenate. This implies that viruses were enumerated in counts per 0,0025 g of shellfish meat, bacteria in counts per 0,05 g of shellfish meat, and phages in counts per 0,5 g of shellfish meat. For purposes of comparison, and for presenting the results in tables easy to interpret, all counts were expressed as calculated counts per 10 g wet mass of shellfish meat. In the case of counts below the detection limits of the above procedures, additional test using larger quantities of shellfish meat were used to detect the presence of organisms.

Statistical analysis of results

The statistical significance of differences in counts was determined by means of Wilcoxon signed rank tests using the Statistix Version 4.0 computer programme.

Penetration of shellfish tissue by micro-organisms

Oysters were kept in a small aquarium with seawater seeded with poliovirus to a final titre of about $1x10^6$ per ml. Procedures were as described in detail for seeding aquaria above. After 24 h actively pumping oysters were removed and immediately processed for paraffin blocks or frozen for histopathological investigation of thin sections using conventional histopathological procedures (Bancroft et al, 1990). This period of exposure proved long enough for viruses to penetrate at least certain tissues of oysters (Hay and Scott, 1986). Biotin-strepavidine stained thin sections of freeze preparations yielded the best results. The histopathological investigations were kidly carried out by Prof IW Simson and staff of our Department of Anatomical Pathology.

7.3. RESULTS

Reduction in counts of micro-organisms during depuration

In two experiments the release of poliovirus and phages V1 and MS2, after exposure of oysters to high counts of the virus and phages in the absence of faecal bacteria, was compared over a period of 3 days. Unfortunately in both cases dilutions in poliovirus titrations were not high enough to obtain absolute counts. Higher dilutions used at later stages during the depuration process gave higher minimum counts but were still not high enough to yield absolute counts. The higher minimum counts at later stages in the depuration process do, therefore, not indicate an increase in counts of polioviruses. Nevertheless, the results (Tables 7.1 and 7.2) indicate decreases in counts of the phages. No decrease in counts of poliovirus is evident from the results. Table 7.1 indicates that phage V1 was depurated faster than phage MS2, and Table 7.2 indicates the opposite. Both experiments show that the oysters still contained high counts of poliovirus and at least one phage after 72 h. Glycine-NaCl homogenates yielded higher counts than borate homogenates in 10 phage titrations, and borate homogenates in 4 titrations.

In one experiment the depuration of oysters exposed to high numbers of poliovirus, phages V1 and MS2, and *Escherichia coli* and *Streptococcus faecalis*, was monitored over a period of 3 days. Unfortunately in the poliovirus titrations dilutions were again not high enough to obtain absolute counts. Nevertheless, the results in Table 7.3 show that counts of phage V1 decreased faster in the meat of the oysters than those of phage MS2. Counts of poliovirus would not appear to decrease as fast as those of the phages. In contrast to the poliovirus and phages, *E coli* and *S faecalis* were no longer detectable after 72 h, which has to be interpreted in terms of the much lower initial counts of the bacteria. Glycine-saline homogenates yielded the highest counts in 11 titrations of phages and bacteria, and saline homogenates in 2 titrations.

In two experiments the depuration of oysters exposed to high numbers of rotavirus SA-11, phages V1 and MS2, and *E coli* and *S faecalis*, was monitored over a period of 3 days (Tables 7.4 to 7.7). Taking into consideration the marginal differences in initial counts, the release of SA-11 and the two phages would not seem to differ significantly. Despite differences in initial counts, *E coli* and *S faecalis* would seem to have been released faster than SA-11 and the bacteria. Borate homogenates yielded higher counts than pH 8,5 glycine-saline homogenates in all titrations of SA-11. Glycine-saline homogenates yielded higher counts than borate homogenates in 16 titrations of phages and bacteria, while borate homogenates yielded the highest counts in 6 titrations, with no apparent differences for phages and bacteria.

In two experiments the depuration of oysters exposed to high numbers of SA-11, phages V1 and MS2, and *E coli* and *S faecalis*, was monitored over a period of 5 days. As in the previous two experiments, the release of SA-11 and the two phages would not seem to differ significantly (Tables 7.6 and 7.7). In contrast to SA-11 and the phages which were still detectable in substantial numbers after 5 days, the *E coli* and *S faecalis* bacteria were no longer detectable after 3 days. Despite differences in initial counts, these results clearly indicate that the bacteria were depurated substantially faster than SA-11 and the phages. Counts of SA-11 and phages were higher after 120 h than 72 h and 96 h. Borate homogenates yielded higher counts for SA-11 than glycine-saline in all three comparative titrations (Table 7.6). In the case of the phages and bacteria, glycine-saline homogenates yielded higher counts than borate homogenates in 5 titrations, while borate homogenates yielded the higher count in 1 titration. Glycine-saline homogenates yielded higher counts for SA-11 than saline homogenates in all three comparative titrations (Table 7.7). Glycine-saline homogenates yielded higher counts than saline

homogenates in two phage titrations, while saline homogenates yielded the higher count in three.

In three experiments the depuration of oysters exposed to high numbers of adenovirus type 40 (Ad40), phages V1 and MS2, and *E coli* and *S faecalis*, was monitored over a period of 5 days. As in the case of SA-11, the depuration of Ad40 would not seem to differ significantly from that of the two phages (Tables 7.8 and 7.10). In contrast to Ad40 and the phages which were still detectable after 5 days, the *E coli* and *S faecalis* bacteria were no longer detectable after 3 days. Despite differences in initial counts, these results indicate that the bacteria disappeared substantially faster than Ad40 and the phages. Glycine-saline homogenates yielded higher counts than borate homogenates in all six titrations of Ad40 (Tables 7.8 and 7.9). Glycine-saline homogenates yielded higher counts than borate homogenates in 8 titrations of phages and bacteria, while borate homogenates yielded the highest counts in 3 titrations, with no apparent differences for phages and bacteria. Glycine-saline homogenates yielded higher counts than saline homogenates in all three comparative titrations of Ad40, and all six assays of phages (Table 7.10). The reduction in counts of *E coli* and *S faecalis* would not seem to have differed significantly (Table 7.8).

In three experiments the depuration of oysters exposed to high numbers of hepatitis A virus (HAV), *Bacteroides fragilis* phage B40-8 (B40-8), and *E coli* and *S faecalis*, was monitored over a period of 5 days. In all three experiments HAV was still detectable in substantial numbers after 3 days (Tables 7.11 to 7.13). In one experiment phage B40-8, *E coli* and *S faecalis* were still detectable after two days (Table 7.11) and in two experiments only after one day (Tables 7.12 and 7.13). Taking into consideration the differences in numbers of organisms to which the oysters were exposed, counts of phage B40-8 would seem to have declined at a rate similar to that of the bacteria. These three experiments confirm the indications in the earlier experiments that faecal bacteria are depurated much faster than human viruses because in this case the shellfish were exposed to higher numbers of *E coli* and *S faecalis* than the virus, and yet the virus was detectable in high numbers much longer than the bacteria. Glycine-saline homogenates yielded higher counts than borate homogenates in all six comparative titrations of HAV (Tables 7.11 and 7.12). Glycine-saline homogenates yielded higher counts than borate homogenates in all 9 comparative titrations of phage B40-8 and bacteria. Glycine-saline homogenates yielded higher counts than saline homogenates in all three comparative titrations of HAV, and all three counts of phage B40-8, *E coli* and *S faecalis* (Table 7.13). As in

previous experiments, the reduction in counts of *E coli* and *S faecalis* would not seem to have differed significantly.

The depuration of test organisms is compared in Figure 1 which is based on data for typical average values and extrapolation among various tests. This presentation of results illustrates the rapid depuration of faecal bacteria, slower depuration of phages, and very slow depuration of human viruses. Results for poliovirus are not indicated because no absolute counts were obtained. However, the results in Tables 7.1 to 7.3 suggest that release is at least as slow as that of rotavirus SA-11, adenovirus type 40 and hepatitis A virus illustrated in Fig. 7.2.

Statistical analysis of differences in counts

In comparative results of tests on all viruses, phages and bacteria, pH 8,5 glycine-saline homogenates yielded the highest count in 51 comparative tests and pH 9,2 borate buffer in 17. Statistical analysis of individual paired tests yielded a two-tailed p value of 0,0056 which is well within the limit of 0,05 for statistical significance. Results for SA-11 rotavirus alone, show that the pH 9,2 borate buffer yielded the highest count in 10 comparative tests and pH 8,5 glycine-saline only in one. Statistical analysis of paired results yielded a p value of 0,0087, which confirmed the statistically significant superiority of the borate buffer compared to the glycine-saline buffer for the elution recovery of SA-11 rotavirus.

In comparative results of tests on all viruses, phages and bacteria, pH 8,5 glycine-saline homogenates yielded the highest count in 28 comparative tests and pH 8,0 saline in 5. Statistical analysis of individual paired results yielded a p value of 0,0017, which confirms the statistically significant superiority of the glycine-saline buffer over the saline buffer for the general recovery of viruses, phages and bacteria from shellfish meat by the elution procedure recommended in this study.

Penetration of shellfish tissue by micro-organisms

Preparation of thin sections of oysters was successfully accomplished and the digestive tract with epithelial cells and cilia, muscular tissue and fatty tissue were clearly seen in microscopic photographs (Fig 7.2). However, the detection of viruses was not clear enough for meaningful conclusions regarding the penetration of tissues.

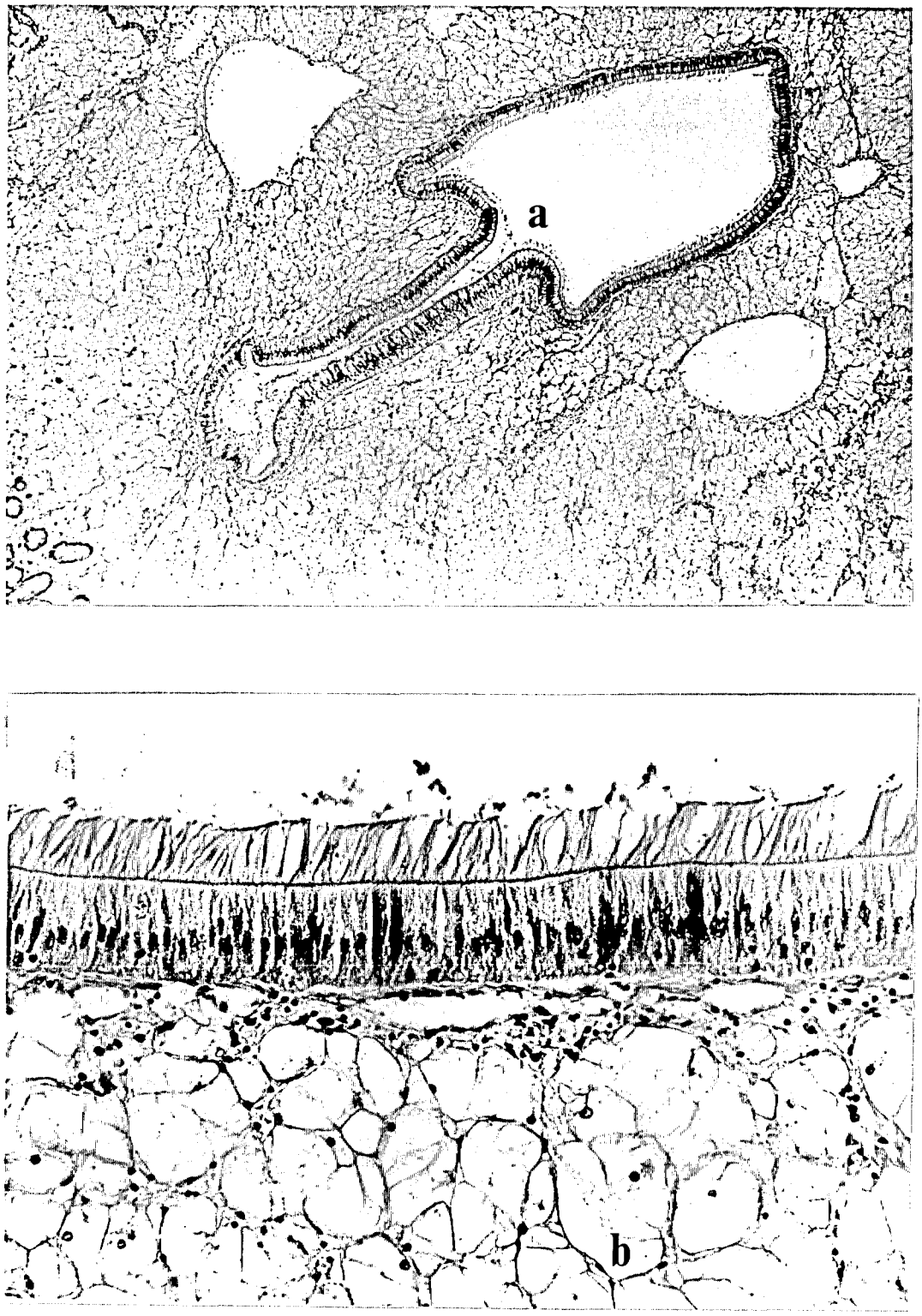


Figure 7.2: Microscopic thin sections of frozen oysters exposed to poliovirus showing the digestive tract with epithelial cell lining and cilia (a), and fatty tissue (b). The dark spots on the enlargement are cell nuclei

7.4. DISCUSSION

The testing of homogenates containing shellfish meat in quantities of 0,02 g for human viruses, 0,15 g for bacteria and 1,5 g for phages, with expression of results in calculated counts per 10 g of shellfish meat, does not adversely affect the basic objective of this study which was to compare the reduction in counts of various organisms during depuration. The reason is that the difference in quantities of shellfish meat analysed for each organism was the same in each test, which implies that the differences in quantities of shellfish meat analysed is a constant factor for all tests which does not affect the relative reduction in counts during depuration. The only implication of this procedure, which was used for purposes of convenience in recording results and avoiding decimal fractions for average counts, is that tests for phages were most sensitive and tests for viruses least sensitive. This implies that a zero count for viruses (which was never obtained) is less accurate than a zero count for phages and bacteria. In addition, a zero ("0") count does not necessarily imply the complete absence of the organisms concerned. Strictly speaking a zero count indicates that the organisms were present at levels not detectable in the quantity of shellfish meat analysed by the method used. However, even this is a constant factor because any count represents the number of organisms detected in the quantity of shellfish meat analysed by the method used.

The comparison of buffers for the general recovery of viruses, phages and bacteria from shellfish meat by the elution procedure, shows that pH 8,5 glycine-saline yielded statistically significant higher counts than pH 8,0 saline and pH 9,2 borate buffer for all organisms tested except SA-11 rotavirus. The borate buffer yielded statistically significant superior results for SA-11 rotavirus. Reasons for these differences are not clear. Possible factors which may play a role include the possibility that SA-11 rotavirus is more resistant to pH 9,2 than the other viruses, phages and bacteria tested. Borate buffer may, therefore, prove the buffer of choice for other viruses highly resistant to pH 9,2 not included in this study. The inclusion of glycine and the slightly higher pH level may explain the higher efficiency of the pH 8,5 glycine-saline buffer compared to the pH 8,0 saline buffer. Whatever the reasons, the results show that pH 8,5 glycine-saline would be the buffer of choice for the general recovery of viruses, phages and bacteria from oyster meat by means of the elution procedure recommended in this study.

The depuration data in Tables 7.1 to 7.13, and summary illustration in Fig.7.2 basically show

that counts of human viruses in shellfish meat declined at rates considerably slower than those of faecal bacteria. These results support and supplement earlier findings for other filter-feeding mollusca, viruses, faecal bacteria, experimental conditions and test methods (Sobsey *et al*, 1987; Boher and Schwartzbrod, 1993; Doré and Lees, 1995). The results also show that counts of phages V1 (large T5-like somatic DNA coliphage with complex structure including head, long flexible tail and tail fibres) and MS2 (small icosahedral RNA male-specific coliphage) declined during depuration at rates closer to those of viruses than those of faecal bacteria. The observation that counts of phages decline at a rate significantly slower than those of bacteria, is in agreement with results of similar studies on coliphage S-13 in the clam *Mercenaria mercenaria* (Canzonier, 1971), male-specific and somatic coliphages in the mussel *Mytilus edulis* (De Mesquita *et al*, 1991), and male-specific coliphages in *Crassostrea gigas* oysters and *Mytilus edulis* mussels (Doré and Lees, 1995). The finding that counts of *B fragilis* phage B40-8 (morphologically similar to V1) declined at about the same rate as those of *E coli* and *S faecalis*, which is faster than those of V1 and MS2 phages (Table 7.11 to 7.13), cannot be explained and should be investigated in further detail. The observation that *E coli* and *S faecalis* counts decreased at similar rates, is in agreement with findings of *E coli*, Group D faecal streptococci and spores of sulphite-reducing clostridia in the mussel *M edulis* (De Mesquita *et al*, 1991).

Depuration rates in Tables 7.1 to 7.13 and Fig. 7.2 should be compared with caution because initial counts of various organisms differed considerably in some cases. Organisms with high initial counts may possibly be detectable for longer than those with low initial counts. This may partly explain the relatively early disappearance of *B fragilis* phage B40-8 (Fig. 7.2).

Reasons for differences in the rate at which counts of human viruses, phages and faecal bacteria decline in the meat of shellfish during depuration are not clear. These differences may be due to differences in either, or a complex combination of, differences in the excretion of different organisms by shellfish, and their survival in the intestines of shellfish. According to Birbeck and McHenry (1982) bacteria, and particularly Gram-negative bacteria such as *E coli*, are highly susceptible to the enzymatic digestive processes of shellfish. Human viruses and many phages may be more resistant to these digestive processes than bacteria, but no evidence has yet been reported in this regard. Feeding would also seem to play a role. Enriques *et al* (1992) noted that in the presence of food, hepatitis A virus (HAV) was detectable in mussels for no longer than 7 days, but in the absence of algae, HAV was still detectable after 11 days under otherwise

similar conditions. However, Sobsey *et al* (1987) did not detect any difference in the reduction in counts of HAV and poliovirus in the meat of oysters depurated in the presence or absence of algae. Temperature may also play a role, since De Mesquita *et al* (1991) noted that *E coli* elimination in *M edulis* mussels was slower during depuration at 10°C than 5°C. Depuration would also seem to be considerably slower when mussels spawn, and in the UK the collection of mussels during the spawning season in summer months is not recommended (De Mesquita *et al*, 1991). Although some factors which may play a role have been identified, the fundamental reasons for differences in the depuration of various organisms remains poorly understood. Information in this regard is urgently required, because it has major implications for commercial depuration practices and policies regarding quality control and monitoring in the shellfish industry (De Mesquita *et al*, 1991).

Various factors should be taken into account in the interpretation of the results obtained in this and related studies carried out under controlled laboratory conditions (Metcalf, 1978; West, 1986; Richards, 1988). Among these are that depuration is almost certainly much more efficient than can be obtain in large scale commercial depuration systems and probably even relaying practices. On the other hand, depuration by disturbed shellfish in artificial seawater in a hostile laboratory environment is probably less efficient than by undisturbed shellfish in natural marine environments. Another important factor is that the numbers of viruses, phages and bacteria to which shellfish are exposed in many laboratory experiments is often much higher than typical for natural marine environments. The ideal objective for investigating the uptake and release of organisms by shellfish in the natural environment, would be to establish experimental laboratory conditions which resemble marine environments as closely as possible. This would include the numbers of organisms to which the shellfish are exposed. However, this is hardly possible in view of the complexity of conditions and microbial populations in marine environments. This would include micro-organisms which inactivate viruses (Toranzo *et al*, 1983), shellfish secretion and ingestion of mucus to which viruses attach which plays an important role in the uptake of viruses (Girolamo *et al*, 1977), survival of viruses in marine sediments and the role of sediments in shellfish intake of viruses (Smith *et al*, 1978), and the continuous exposure of shellfish for long periods to faecal organisms in sewage-polluted marine environments. In addition, the quality of seawater and sediments in sewage-polluted marine environments does not change rapidly, which implies that depuration in marine environments is to a large extent part of an ongoing process of uptake and depuration of faecal organisms.

The complexity of depuration, and research on the process, is illustrated by counts of SA-11 rotavirus (Table 7.6) and phages (Tables 7.2, 7.3, 7.4) which increased during depuration. These findings are interesting because the viruses and phages concerned cannot multiply in oysters. In attempts to explain the results it is important to keep in mind that different oysters were analysed after each cycle of depuration. It is not possible to analyse the uptake and release of micro-organisms by any one oyster because the oysters have to be killed and homogenised for each test. The increases of counts in these exceptional cases may be due to variation in the uptake and depuration of oysters removed for analysis after 24 h cycles of depuration. It is possible that some oysters failed to depurate during the preceding cycle of depuration, and that they therefore retained virtually unchanged counts for two cycles. In view of high initial counts of viruses and phages in some experiments, recontamination (uptake and accumulation of viruses and phages from the depuration water) may play a role since oysters transferred to tanks with clean water probably release substantial numbers of organisms into the water. However, each transfer to a tank with 8 L of clean water reduces the count of organisms that could be released by oysters by three logs, unless some oysters fail to depurate at the beginning and start to depurate during later cycles. Variation in the accuracy of testing may also play a role.

Whatever the reasons for the occasional apparent increase in counts of viruses and phages in oysters during depuration, the overall results and experimental conditions would seem to suggest that the same may also happen in natural environments, and particularly in commercial depuration systems. It is important to note that this phenomenon was never observed for faecal bacteria. In other words, in a batch of depurated shellfish, the great majority may have depurated to safe levels, but a number may still contain infectious levels of pathogens. In monitoring the safety of batches by testing random samples, the detection and removal of small numbers of shellfish which still contain infectious levels of pathogens is impossible.

The merit of research on conditions which more closely resemble those in natural marine environments and commercial depuration processes, is fully appreciated. However, this would require advanced, time-consuming and expensive research using sophisticated laboratory facilities. At least some of the research should ideally be carried out in natural marine environments and commercial depuration facilities. However, the objectives, time constraints and budget for this particular study did not make provision for entering the area of research in greater depth. The results disclose a need for further research. Relatively easy first-step

modifications which should be addressed in follow-up studies would include the exposure of shellfish to numbers of viruses and indicators more closely resembling those in natural marine environments. In terms of facilities for depuration studies, the present procedure of transferring shellfish at time intervals to clean water should be replaced by a continuous flow-through system with ultraviolet light decontamination of circulating water (De Mesquita *et al*, 1991; Doré and Lees, 1995). It may also be advisable to use shellfish contaminated under natural conditions in sewage-polluted seawater for depuration studies rather than shellfish exposed to seeded seawater in laboratory aquaria (Doré and Lees, 1995).

Despite shortcomings, this study has contributed meaningful new information and clearly outlined some important issues of shellfish depuration. This is the first study of its kind in South Africa using local oysters and research conditions. One important finding is that the depuration of three different viruses representative of enteric viruses typically associated with transmission by shellfish, is much slower than that of faecal bacteria commonly used for assessment of the hygienic quality of shellfish. The high counts of viruses in shellfish meat even after five days of depuration, indicate that the viruses were likely to persist in the shellfish for much longer. This is in agreement with earlier work in which HAV was detected in shellfish after 11 days following exposure to lower numbers than in this study (Enriques *et al*, 1992). Another important finding is that at least certain phages are depurated at rates similar to those of human viruses. The results of the comparison of three buffers for preparing shellfish meat homogenates for microbiological analysis makes a valuable contribution to technology for research and safety testing of shellfish.

Investigations aimed at finding reasons for the differences in reduction of counts of viruses and bacteria during depuration, were successful in so far as that procedures for the preparation of histological thin sections suitable for microscopic analysis have been established. Unfortunately techniques for the staining of viruses failed to meet requirements for conclusive evidence regarding the penetration of tissues outside of the digestive tract. However, the results obtained indicate that it should be possible to obtain valuable information by this approach. Suitable optimisation of virus detection procedures is a technical problem which should be not too difficult to solve. Histopathological analysis of shellfish may indeed prove more accurate and reliable than dissection of shellfish and microbiological analysis of individual organs and parts of shellfish for the presence of viruses and bacteria as carried out by Doré and Lees (1995).

The progress that has been made and the results obtained justify further resarch aimed at explaining the differential reduction in counts of viruses and bacteria during depuration.

The results of this study outline the need for revising depuration conditions and quality monitoring protocols. Faecal bacteria such as coliforms and streptococci clearly have serious shortcomings as indicators of the efficiency of depuration, mainly because their numbers decline much faster than those of viruses and phages during depuration, and have never shown signs of apparent increases in counts due to depuration failure or recontamination. Depuration procedures must ensure the safety of commercial shellfish supplies with regard to viruses, which are the most common cause of infections associated with the consumption of shellfish. The efficiency of conventional depuration systems in which masses of shellfish are kept in tanks for 24 h (Grabow, 1989; De Mesquita *et al*, 1991), is clearly questionable. There is no doubt that tests for faecal coliforms alone are no longer acceptable for monitoring the efficiency of depuration processes and the hygienic quality of shellfish intended for human consumption. Sound evidence is now available that at least appropriate tests for phages should be included in shellfish quality monitoring protocols. This has also been suggested by others (Chalmers and McMillan, 1995). Presently available evidence indicates that at least somatic coliphages should be included, and as far as possible also male-specific coliphages. Ideally tests for human viruses should also be included. However, these are still relatively expensive and require advanced facilities and expertise. They would, therefore, not be included in routine monitoring protocols. However, tests for human viruses are fully justified for certain purposes of detailed quality investigation. In addition, rapid progress is being made in the development of practical and economical technology for human viruses. This includes the development of practical and sensitive molecular techniques (Atmar *et al*, 1995). The inclusion of meaningful virus tests at appropriate frequency for particular purposes in quality monitoring protocols may, therefore, be justified in the not too distant future.

Table 7.1. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration							
	0 h		24 h		48 h		72 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
Polio 1	> 11 000	> 11 000	> 13 000	> 13 000	> 11 000	> 11 000	> 21 000	> 21 000
Phage V1	$5{,}2 \times 10^{7}$	$3{,}2 \times 10^{6}$	64 000	64 000	1 500	990	300	330
Phage MS2	$8{,}2 \times 10^{6}$	$8{,}0 \times 10^{6}$	$1{,}5 \times 10^{5}$	$1{,}3 \times 10^{5}$	24 000	51 000	1 300	510
E coli	--	--	--	--	--	--	--	--
S faecalis	--	--	--	--	--	--	--	--

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Poliovirus 1 = $3{,}4 \times 10^{8}$
Phage V1 = $1{,}3 \times 10^{6}$
Phage MS2 = $2{,}8 \times 10^{6}$
E coli = --
S faecalis = --
-- = Not done

Note: The higher minimum counts of poliovirus after 24 and 72 h than at 0 h are due to higher dilutions used in MPN assays at later times, and do not indicate an increase in counts of polioviruses. The indication "more than" (">") gives no indication of how much more it is than the minimum value.

Table 7.2. Depuration of oysters in laboratory tanks

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration							
	0 h		24 h		48 h		72 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
Polio 1	> 12 000	> 12 000	> 11 000	> 11 000	> 12 000	> 12 000	> 11 000	> 11 000
Phage V1	$3{,}1 \times 10^6$	$3{,}0 \times^6$	19 000	21 000	49 000	77 000	1 100	1 000
Phage MS2	$5{,}2 \times 10^6$	$2{,}0 \times 10^6$	96 000	60 000	2 300	1 000	0	0
E coli	--	--	--	--	--	--	--	--
S faecalis	--	--	--	--	--	--	--	--

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Poliovirus 1 = $3{,}3 \times 10^8$
Phage V1 = $1{,}25 \times 10^6$
Phage MS2 = $3{,}5 \times 10^5$
E coli = --
S faecalis = --
-- = Not done

Table 7.3. Depuration of oysters in laboratory tanks

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration							
	0 h		24 h		48 h		72 h	
	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl
Polio 1	> 11 000	> 11 000	> 11 000	> 11 000	> 11 000	> 11 000	> 26 000	> 26 000
Phage V1	$2{,}6\times10^{6}$	$1{,}4\times10^{6}$	1 800	610	2 200	1 300	2 000	1 200
Phage MS2	$2{,}3\times10^{6}$	$4{,}3\times10^{6}$	10 000	8 100	3 100	1 300	7 100	9 800
E coli	40 000	17 000	36 000	15 000	270	55	0	0
S faecalis	9 400	3 500	2 500	1 400	0	0	0	0

Glycine-NaCl pH 8,5 and Sterile-NaCl pH 8,5 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Poliovirus 1 = $3{,}6\times10^{8}$
Phage V1 = $3{,}2\times10^{6}$
Phage MS2 = $8{,}0\times10^{5}$
E coli = $9{,}6\times10^{3}$
S faecalis = $2{,}1\times10^{4}$

Note: The higher minimum counts of poliovirus after 24 and 72 h than at 0 h are due to higher dilutions used in MPN assays at later times, and do not indicate an increase in counts of polioviruses. The indication "more than" (">") gives no indication of how much more it is than the minimum value.

Table 7.4. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration							
	0 h		24 h		48 h		72 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
SA-11	$1,4x10^5$	$3,1x10^6$	29 000	89 000	6 300	44 000	250	5 700
Phage V1	$2,2x10^6$	$1,3x10^6$	1 300	630	2 900	1 600	1 800	1 200
Phage MS2	$2,8x10^6$	$4,5x10^6$	12 000	7 900	3 300	1 400	6 900	8 900
E coli	37 000	16 000	33 000	13 000	120	69	0	0
S faecalis	8 500	3 900	210	210	0	0	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

SA-11 = $2,3x10^7$
Phage V1 = $2,5x10^6$
Phage MS2 = $9,3x10^5$
E coli = 9 000
S faecalis = $3,3x10^4$

Table 7.5. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration							
	0 h		24 h		48 h		72 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
SA-11	$1{,}1 \times 10^5$	$2{,}0 \times 10^6$	13 000	90 000	5 200	3 200	390	6 800
Phage V1	$5{,}6 \times 10^6$	$2{,}9 \times 10^6$	17 000	17 000	600	5 10	710	480
Phage MS2	$8{,}9 \times 10^6$	$3{,}7 \times 10^6$	11 000	3 900	2 500	2 250	230	14
E coli	160	93	83	280	0	0	0	0
S faecalis	150	2 900	11	180	24	61	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

SA-11 = $2{,}1 \times 10^7$
Phage V1 = $1{,}3 \times 10^6$
Phage MS2 = $1{,}4 \times 10^6$
E coli = 800
S faecalis = 2 200

Table 7.6. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	72 h		96 h		120 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
SA-11	930	1 000	570	1 800	9 500	26 000
Phage V1	330	210	150	120	1 000	710
Phage MS2	470	280	150	160	590	470
E coli	0	0	0	0	0	0
S faecalis	0	0	0	0	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

SA-11 = $2{,}4 \times 10^7$
Phage V1 = $3{,}3 \times 10^6$
Phage MS2 = $1{,}4 \times 10^6$
E coli = 920
S faecalis = 4 400

Table 7.7. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	72 h		96 h		120 h	
	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl
SA-11	4 200	130	160	9	13	6
Phage V1	8 700	11 000	900	2 100	640	520
Phage MS2	$1,3x10^5$	$1,2x10^5$	91 000	91 000	140	530
E coli	0	0	0	0	0	0
S faecalis	0	0	0	0	0	0

Glycine-NaCl pH 8,5 and Sterile-NaCl pH 8,5 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

SA-11 = $2,3x10^7$
Phage V1 = $1,2x10^6$
Phage MS2 = $1,7x10^6$
E coli = 320
S faecalis = 6 300

Table 7.8. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	48 h		72 h		96 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
Adeno 40	35 000	30 000	9 600	71	56	21
Phage V1	6 300	5 600	380	360	39	52
Phage MS2	12 000	8 100	1 700	1 700	26	52
E coli	55	27	0	0	0	0
S faecalis	16	31	0	0	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Adeno 40 = $5{,}1 \times 10^6$
Phage V1 = $2{,}2 \times 10^6$
Phage MS2 = $3{,}0 \times 10^6$
E coli = 5 600
S faecalis = 3 500

Table 7.9. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	72 h		96 h		120 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
Adeno 40	5 300	3 100	2 600	1 100	5	2
Phage V1	900	800	420	330	6	6
Phage MS2	1 300	1 300	510	490	9	3
E coli	0	0	0	0	0	0
S faecalis	0	0	0	0	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Adeno 40 = $5{,}5 \times 10^6$
Phage V1 = $2{,}5 \times 10^6$
Phage MS2 = $3{,}6 \times 10^6$
E coli = 340
S faecalis = 520

Table 7.10. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	72 h		96 h		120 h	
	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl
Adeno 40	8 100	7 200	110	60	31	29
Phage V1	950	890	61	56	26	19
Phage MS2	1 500	900	92	76	13	9
E coli	0	0	0	0	0	0
S faecalis	0	0	0	0	0	0

Glycine-NaCl pH 8,5 and Sterile-NaCl pH 8,5 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Adeno 40 = $5,4 \times 10^4$
Phage V1 = $1,2 \times 10^6$
Phage MS2 = $2,9 \times 10^6$
E coli = 230
S faecalis = 950

Table 7.11. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	24 h		48 h		72 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
Hepatits A	5 300	5 100	4 100	3 900	2 600	2 400
B fragilis	330	270	70	60	0	0
E coli	290	200	110	103	0	0
S faecalis	730	700	220	205	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Hepatits A virus = 6 500
Bacteroides fragilis phage B40-8 = 4 300
E coli = 44 000
S faecalis = 66 000

Table 7.12. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	24 h		48 h		72 h	
	Glycine-NaCl	Borate	Glycine-NaCl	Borate	Glycine-NaCl	Borate
Hepatitis A	4 100	3 900	3 600	3 500	2 700	2 600
B fragilis	29	20	0	0	0	0
E coli	311	256	0	0	0	0
S faecalis	73	66	0	0	0	0

Glycine-NaCl pH 8,5 and Borate pH 9,2 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Hepatitis A virus = 5 900
Bacteroides fragilis phage B40-8 = 4 800
E coli = 61 000
S faecalis = 52 000

Table 7.13. Depuration of oysters in laboratory tanks.

Organism	Counts of organisms per 10 g wet oyster meat at time intervals of depuration					
	24 h		48 h		72 h	
	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl	Glycine-NaCl	NaCl
Hepatitis A	4 900	4 800	3 600	3 200	2 800	2 700
B fragilis	79	71	0	0	0	0
E coli	62	53	0	0	0	0
S faecalis	55	49	0	0	0	0

Glycine-NaCl pH 8,5 and Sterile-NaCl pH 8,5 buffers used for the recovery of organisms from oyster meat.

Counts/ml of organisms in seeded water to which oysters were exposed prior to depuration:

Hepatitis A virus = 5 600
Bacteroides fragilis phage B40-8 = 4 500
E coli = 45 000
S faecalis = 35 000

8. GENERAL DISCUSSION AND CONCLUSIONS

8.1. DISCUSSION AND CONCLUSIONS

The literature review contains details on the transmission of infectious diseases by molluscan shellfish in many parts of the world. An extreme example is the outbreak with some 300 000 cases of type A hepatitis and 25 000 cases of viral gastroenteritis in 1988 in China due to the consumption of shellfish harvested from a sewage polluted bay. The risk is constituted by the consumption of shellfish, often raw, which have accumulated pathogens during filter feeding in sewage polluted waters. Consequently the commercial and private markets for shellfish are extremely sensitive towards risks of infection, and any indication of potential contamination is sufficient to ban supplies or close down industries. The safety of shellfish with regard to pathogens is, therefore, of fundamental importance to the commercial shellfish industry as well as the general public who collect shellfish for private consumption.

South Africa has a substantial shellfish industry, and the general public collects large quantities of shellfish along the vast coastline for private consumption. Little information is available on the transmission of pathogens by shellfish in the country. However, the risks are similar to those in other parts of world, and the lack of evidence would seem to be due to the absence of an infrastructure for detecting and recording such infections. Compared to industrialised countries of the world, quality guidelines and control measures for the harvesting, cultivation and marketing of shellfish in South Africa would seem to be in need of revision and updating. In particular there are shortcomings with regard to human viruses, which cause the great majority of infections associated with the consumption of sewage contaminated shellfish (Part 2).

Against this background methods for the detection of viruses and related indicators in shellfish have been evaluated. Comparative tests were carried out on shellfish seeded with laboratory strains of viruses and indicators, as well as shellfish harvested from sewage polluted marine environments. Finally the methods have been used to investigate the survival and depuration of selected viruses, phages and faecal bacteria in oysters.

Data in the literature indicated that the following bacteria are generally considered as indicators of the virological safety of shellfish meat: Total and faecal coliform bacteria, *Escherichia coli*, faecal streptococci, and *Clostridium perfringens*. These bacteria are typically associated with faecal pollution or unacceptable hygienic conditions. More recently phages have been considered as indicators, primarily because some of them may resemble viruses more closely with regard to behaviour in the environment and in shellfish than faecal bacteria. Research along these lines focused on somatic coliphages and male-specific (F-RNA) coliphages, and *Bacteroides fragilis* HSP40 phages. According to the literature all these indicators have certain advantages and disadvantages for indicating the virological safety of shellfish and the efficiency of shellfish depuration. Some published data clearly demonstrate shortcomings of all these bacteria and phages as indicators for human viruses. In addition there is no information on the reliability of these indicators with regard to the cysts or oocysts of protozoan parasites, and pathogens such as *Leptospira monocytogenes*. This situation underlines shortcomings with regard to the reliability of indicators commonly used to asses the virological safety of shellfish and the efficiency of depuration processes. The best techniques available for these indicators were selected and optimised for research on their reliability with regard to human viruses (Part 3).

Details in the literature on infections associated with the consumption of contaminated shellfish were used to select model viruses for research on methods for the recovery of viruses from shellfish (Part 4). Since molecular detection techniques were not included in this study, the spectrum was limited to cytopathogenic viruses detectable by conventional cell culture propagation. One reason for not including molecular techniques was that the accurate quantisation of viruses and information on the viability of viruses were important for the objectives of the study, and molecular techniques do not meet requirements in this regard. Viruses eventually selected as representative of the widest possible spectrum of viruses were a vaccine strain of poliovirus, simian rotavirus SA 11, adenovirus type 40 and a cell culture adapted strain of hepatitis A virus (Part 4). Cell culture procedures based on a most probable number (MPN) microtitre plate assay were optimised for accurate quantisation of these viruses using appropriate cell cultures.

Studies on methods for the recovery of viruses and phages from shellfish were limited to comparative tests on seeded shellfish meat homogenates because earlier investigations had shown that efficiencies were similar for seeded shellfish meat and naturally contaminated shellfish. A practical and simple elution procedure based on the homogenisation of shellfish meat in a pH 8,5 glycine-saline buffer followed by centrifugation and enumeration of viruses and phages in the supernatant yielded higher efficiencies of recovery for a representative range of viruses and phages than more complicated and time consuming precipitation procedures. The efficiency of recovery of the elution procedure eventually selected for general use after evaluation of various buffers and elution conditions varied from 87% for poliovirus to 25% for adenovirus, and 59% and 48% for somatic and male-specific coliphages, respectively. In terms of information in the literature, this is the most efficient method described to date for recovery of the widest possible range of viruses and phages from shellfish meat. Various aspects of this work is new, including the local species of shellfish used, and details on the recovery of adenoviruses and phages from shellfish meat (Part 5). Details on recommended procedures and quality guidelines are described in Part 9. Although a substantial variety of viruses and phages has been used in the evaluation of the recovery procedure, the results show that the efficiency may differ considerably for different viruses and phages. It would, therefore, be advisable to assess the efficiency for representatives of viruses not included in this study, such as non-cytopathogenic viruses including calici and astroviruses.

In Part 6 evidence has been presented that the elution procedure for the recovery of viruses and phages is suitable for application in practice. Tests on oysters and mussels from selected sites along the coast yielded satisfactory results. Selected methods for the detection of viruses, faecal bacteria and phages also yielded satisfactory results in tests on samples of seawater and seabird droppings. These findings confirm that practical, reliable and inexpensive methods suitable for research and routine quality monitoring of viruses, faecal bacteria and phages in shellfish and related environments have been established. The results revealed a need for revision and updating of prevailing quality guidelines and specifications in South Africa with regard to determinands and methods to be used in quality control of shellfish.

In view of anecdotal reports from various sources about infectious diseases, mainly gastroenteritis, associated with the consumption of shellfish collected by the public at a number of sites along the coastline, the failure to find cases or outbreaks which could epidemiologically be confirmed to be caused by shellfish, is disappointing (Part 6). However, valuable experience has been gained and in future studies cases and outbreaks may be identified more readily. The detection of small round structured gastroenteritis viruses (SRSVs) in some of 18 stool specimens from a gastroenteritis outbreak at Grootbrak had valuable benefits, even though eventually it transpired that the outbreak was caused by contaminated curried fish sold by a street vendor. A major advantage was that it offered an opportunity to develop technology for the first detection of SRSVs by molecular techniques in South Africa. This technology is now available for application in future studies on outbreaks.

Application of the new methods for microbiological analysis of shellfish revealed that commercial supplies of shellfish sometimes exceeded recommended limits for faecal bacteria and phages (Part 6). These findings suggest that more stringent application of microbiological safety limits would result in the rejection of meaningful quantities of local commercial supplies of shellfish. This raises a number of questions regarding local shellfish supplies which warrant further investigation. For instance, do local commercial shellfish supplies constitute a meaningful health risk, are microbiological quality limits too stringent, and should the quality control of commercial shellfish supplies be tightened up? The observation that seabird droppings contain high levels of faecal indicator bacteria and coliphages, suggests that pollution from sources other than domestic sewage may have to be taken into account in quality guidelines for mariculture waters and shellfish. The reason is that seabirds and other marine life are not known to excrete human pathogens such as enteric viruses. The detection of faecal indicators in the vicinity of a major mariculture industry in Saldanha Bay raises questions about the risk of pollution from sewage discharged into the bay. Answers to these questions require further studies on the extent of sewage pollution of the bay, survival of indicators and pathogens in the environments concerned, and currents which may convey these organisms to the mariculture areas.

The most important findings of studies on the release of faecal indicators, phages and viruses by oysters in tanks under controlled laboratory conditions are that viruses were released much slower than bacterial indicators of faecal pollution generally used for assessment of the safety of shellfish and the efficiency of depuration processes (Part 7). Similar findings have been reported for other species of oysters in other parts of the world. Although high numbers of viruses were used in these experiments, the results clearly show that viruses may be present in shellfish long after faecal bacteria have reached undetectable levels. Even with high initial numbers, faecal bacteria were never detected for longer than two days, while viruses were detectable for five days. This has implications for generally accepted depuration guidelines based on depuration for one or two days and the absence of faecal bacteria. The results suggest that phages are more reliable indicators than faecal bacteria because phages were sometimes also detectable for as long as five days. However, some results suggest that even the numbers of phages declined more rapidly than those of viruses, which implies that their application as indicators for shellfish quality requires more detailed investigation.

Attempts to find answers for differences in the depuration of bacteria, viruses and phages by oysters using histopathological investigations of the penetration of shellfish tissue by test organisms were unfortunately not successfully completed in the available time. However, valuable experience has been gained and useful technology has been developed (Part 7). It would not seem too difficult to overcome the technical problems encountered in clearly visualising viruses in thin sections of freeze preparations of oysters exposed to test organisms. Valuable information may, therefore, be obtained in follow-up studies.

Although many questions remain unanswered, the study has given the first comprehensive overview of some fundamental issues regarding the microbiological quality of shellfish in the South African situation. Major accomplishments include the establishment of practical techniques with known levels of efficiency for research and routine monitoring of viruses, faecal bacteria and phages in shellfish, seawater and seabird droppings in South African marine environments. Observations that some commercial supplies of shellfish exceeded recommended quality limits for faecal

bacteria and phages, implies that quality control in the shellfish industry should be investigated in further detail. The experiments which demonstrated faster release of faecal bacteria than viruses during depuration in controlled laboratory conditions, suggest that specifications for commercial depuration procedures should be reviewed. The observations that phages more closely resemble the release of viruses during depuration than faecal bacteria, offers strong motivation for the inclusion of phages in quality control specifications for shellfish. The identification of seabird droppings as a potential source of indicators of faecal pollution at mariculture sites, suggests that more attention should perhaps be given to sources of faecal indicators at mariculture sites.

Perhaps the most important accomplishment of the study is that expertise and an infrastructure for research on the microbiological quality of shellfish in South Africa have been established, that the latest relevant information has been compiled, that potential problems and possible solutions have been disclosed, and that research needs have been identified.

8.2 FURTHER RESEARCH

Needs for further research may be summarised as follows:

1. Evaluation of the recommended procedure for the recovery of viruses from shellfish for viruses not included in this study. This would primarily concern non-cytopathogenic viruses such as hepatitis A and E, and calici-, rota- and astroviruses. Application of the recovery procedure in combination with detection techniques such as the polymerase chain reaction should be optimised and evaluated. This may eventually lead to the development of practical techniques more readily applicable for routine quality monitoring.

2. The value of phages as indicators for the virological quality of shellfish should be investigated in more detail. Results obtained so far suggest that phages may eventually play a major role in quality assessment of shellfish and related marine environments.

3. The microbiological quality of shellfish at selected sites along the coast and commercial supplies should be analysed in more detail. This information is essential for assessment of the safety of shellfish, and will supply information on indicators and viruses which is essential for the updating and improvement of limits and procedures for routine quality monitoring.

4. The release of viruses, phages and faecal bacteria during depuration procedures should be investigated in more detail. Experiments should ideally be carried out in continuous flow systems. The shellfish used in these experiments should be exposed to levels of indicators and viruses resembling those in natural environments. The results should be backed up by experiments on depuration of shellfish in natural marine environments and commercial depuration systems. This information is essential for the formulation of reliable guidelines for the depuration of shellfish.

5. Research on reasons for differences in depuration of bacteria, human viruses and phages by shellfish should be continued. A valuable basis for histopathological studies is available. The results should prove of value in the formulation of guidelines for shellfish quality and depuration procedures.

6. Sewage pollution of Saldanha Bay and its implications for the mariculture industry in the bay should be investigated in detail. The same may prove necessary for other mariculture sites. The study would yield valuable details on the survival of indicators and pathogens in seawater, and their movement in bodies of seawater. The results would supply information which is essential for the protection of the industry in Saldanha Bay. The results may also be extrapolated to other mariculture sites, and prove of value in the selection and evaluation of sites considered for mariculture.

7. The impact of sources of faecal pollution at mariculture sites such as bird droppings should be investigated in detail. The results may have implications for specifications for seawater and shellfish at mariculture sites. It may prove necessary to include methods for the distinction of faecal pollution of human and animal origin.

8. The application of shellfish as indicators for monitoring sewage pollution in marine environments should be optimised and evaluated in practice. The principle is not new, but presently available techniques for viruses and phages offer possibilities for substantially increasing the value of shellfish for monitoring sewage pollution.

9. A meaningful infrastructure for epidemiological monitoring of infections associated with the consumption of shellfish should be established. Details on such infections are essential for the formulation of practical and meaningful quality limits and quality control procedures.

10. The latest available information on microbiological technology, the quality of shellfish and epidemiological data should continually be fed into a system for ongoing revision and updating of quality specifications and quality control policies.

9. RECOMMENDED METHODS AND QUALITY LIMITS

9.1. Methods for microbiological analysis of shellfish

The following procedures recommended for the microbiological analysis of shellfish are based on results and literature recorded in Parts 3 to 7, which also contain technical details.

1. Preparation of test materials

1.1. Assessment of the microbiological quality of shellfish (oysters, mussels, etc) is generally carried out only on live, fresh or frozen animals (Speck, 1976; West and Coleman, 1986).

1.2. Select a representative number of animals typical of the supply under investigation.

1.3. Thoroughly clean and decontaminate outside shells of shellfish (West and Coleman, 1986; Austin and Austin, 1989).

1.4. Aseptically remove meat and intravalvular fluid from shellfish using sterile equipment, and pool meat and intravalvular fluid in a sterile beaker (West and Coleman, 1986; Austin and Austin, 1989).

1.5. A total mass of 50 g of shellfish meat plus intravalvular fluid is sufficient for comprehensive microbiological analysis. This quantity is recommended for convenience of microbiological analysis as well as meaningful representation of test animals from the supply under investigation (West and Coleman, 1986). However, for certain purposes and reasons smaller quantities may be acceptable.

1.6. Add 50 ml of pH 8,5 0,05 M glycine - 0,15 M NaCl (glycine-saline) buffer to 50 g of pooled shellfish meat including intravalvular fluid from test animals (West and Coleman, 1986; Parts 4 to 7), or use alternative equal ratios.

1.7. Homogenise suspension by ultra-turrax treatment (maximum speed for 3 min) (Part 5) or alternative procedure such as appropriate laboratory blender (Speck, 1976; West and Coleman, 1986). This suspension is referred to as "homogenate" and used for immediate microbiological analysis.

1.8. Reagents and growth media are prepared as described in Parts 4 to 7. Unless otherwise stated, conventional disposable sterile plastic petri dishes (90 mm diam) are used. Apart from the heterotrophic plate count, it is generally not necessary to test dilutions of test homogenates of shellfish intended for human consumption. Basically the same procedures with appropriate tenfold saline dilutions could be used for microbiological analysis of heavily contaminated shellfish.

2. Bacteria

See Part 6 for details on procedures and growth media. The minimum detection limit of the recommended tests is 2 organisms per gram of shellfish meat including intravalvular fluid.

2.1. Heterotrophic plate count

Prepare 4 pour plates using 1,0 ml homogenate and 15 ml of tryptone yeast extract agar per plate. Prepare similar plates using tenfold saline dilutions of the homogenate up to 10^4. Incubate 35-37°C/48h. Count all colonies.

2.2. Total coliforms

Spread 0,1 ml of homogenate onto each of 10 plates of M-Endo LES Agar.
Incubate 35-37°C/24h. Pick and purify typical brilliant green colonies with metallic sheen for confirmation and typing.

2.3. Faecal coliforms

Spread 0,1 ml of homogenate onto each of 10 plates of M-FC Agar without rosolic acid. Incubate 44,5°C/24h. Count typical blue colonies.

2.4. *Escherichia coli*

Pick and purify typical faecal coliform colonies, and test for *Escherichia coli* by indole production at 44,5°C/24h or alternative method.

2.5. Faecal streptococci (enterococci)

Spread 0,1 ml of homogenate onto each of 10 plates of M-Enterococcus Agar.
Incubate 35-37°C/48h. Count typical red to pink colonies.

2.6. *Staphylococcus aureus*

Spread 0,1 ml of homogenate onto each of 10 plates of Baird-Parker Agar.
Incubate 35-37°C/48h. Pick and purify typical colonies (black to dark grey, smooth convex, off-white edge, etc) and confirm by coagulase test.

2.7. *Salmonella* species

Spread 0,1 ml of homogenate onto each of 10 plates of Bismuth Sulphite (BS) Agar, *Salmonella Shigella* (SS) Agar, MacConkey's Agar, Brilliant-Green Phenol-Red Lactose Sucrose (BPLS) Agar and Xylose Lysine Desoxycholate (XLD) Agar. Incubate 37°C/24h. Pick and purify typical colonies for confirmation and typing by biochemical and serological tests.

2.8. *Shigella* species

Inoculate 1,0 ml of homogenate into 10 ml of Gram-Negative (GN) Broth. Incubate 37°C/2h for enrichment. Spread 0,1 ml onto 1 plate of each of the same plates used for *Salmonella* species. Pick and purify typical colonies for confirmation and typing by biochemical tests. This is a qualitative test.

2.9. *Vibrio* species

Inoculate 1,0 ml homogenate into 10 ml Alkaline Peptone Water. Incubate 37°C/6-8h for enrichment. Spread 0,1 ml onto a plate of Thiosulphate-Citrate-Bile-Salts-Sucrose Agar. Incubate 37°C/24h. Pick and purify typical colonies for confirmation and typing of *V cholerae*, *V parahaemolyticus*, *V alginolyticus* and other species. This is a qualitative test.

2.10. *Clostridium perfringens*

Inactivate interfering vegetative organisms by keeping 2 ml homogenate at 80°C for 2 min. Spread 0,1 ml of this homogenate onto each of 10 plates of Lecithin Agar. Incubate anaerobically 35-37°C/24h. Pick and purify typical colonies (black colonies with opaque halo) for confirmation by biochemical tests.

3. Phages

Use double agar layer plaque assays with 1,0 ml of homogenate in 2,5 top layer on each of 10 plates. The minimum detection limit of the recommended test procedure is less than 1 plaque forming unit per gram of shellfish meat including intravalvular fluid. Details on recommended procedures have been described in Parts 3 and 6.

3.1. Somatic coliphages

Use *Escherichia coli* strain C (WG4) as host. When heavy growth of organisms in the homogenate may interfere with the test, the nalidixic acid resistant host WG5 can be used in the presence of nalidixic acid. Incubate 35-37°C/20-24h. Count all plaques.

3.2. Male-specific (F-RNA) coliphages

Use the *Salmonella* F^+ WG49 host in logarithmic growth phase. Incubate 35-37°C/20-24h. Count all typical lysogenic plaques. Pick and purify plaques for confirmation of F-RNA phages by host specificity, electron microscopy, genetic hybridisation and neutralisation tests.

3.3. *Bacteroides fragilis* HSP40 phages

Use the host *B fragilis* HSP40. Incubate 35-37°C/48h under strict anaerobic conditions. Count all plaques.

4. Human viruses

Add 2,0 ml of chloroform to 20 ml of homogenate, mix by brief vortexing, allow to stand for 30 min at room temperature for decontamination, centrifuge at 2000rpm/3min to remove tissue and chloroform, recover virus-containing supernatant and aerate to remove traces of chloroform, and use for detection of viruses. Detect cytopathogenic viruses by inoculating 1,0 ml of supernatant into each of five 25 cm^2 flasks with monolayers of an appropriate cell culture. An evaluation of the susceptibility of a wide variety of cell cultures to cytopathogenic viruses in the environment indicated that best results would probably be obtained by parallel inoculation of each of five flask containing the BGM monkey kidney cell line, five flasks containing the PLC/PRF/5 human liver cell line, and five flasks containing primary vervet kidney cells. These are semi-qualitative tests with a minimum detection limit of 1 cell culture infectious dose per 2,5 g of shellfish meat including intravalvular fluid. When testing highly contaminated or seeded

shellfish quantitative assays may be carried out by using most probable number (MPN) microtitre plate assays. Technical details for cell culture detection of viruses have been described in Part 5. It is important to keep in mind that cell culture detection is limited to cytopathogenic viruses. The great majority of viruses associated with transmission by shellfish are not detectable by cell cultures. Molecular techniques are now being developed for the detection of many of these viruses (Atmar *et al*, 1995; Wolfaardt *et al*, 1995a,b).

9.2. Limits for microbiological quality of shellfish

The microbiological quality limits in Table 9.1 recommended for shellfish intended for human consumption are based on international guidelines, ongoing progress in technology and expertise, new information on risks of infection associated with the consumption of shellfish, and results of microbiological analysis of commercial, aquaculture and natural supplies of shellfish in South Africa (see Parts 2 to 7). According to presently available information the recommended limits are practical, reliable and within reach of the aquaculture industry, commercial suppliers and natural shellfish beds in marine environments not exposed to unacceptable levels of sewage pollution.

Table 9.1: Shellfish intended for human consumption: Recommended microbiological quality limits

Micro-organism	Count/g*
Heterotrophic plate count	100 000
Faecal coliforms	6
Faecal streptococci	2
Somatic coliphages	2
Male-specific (F-RNA coliphages)	1
Human viruses, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp, *Shigella* spp, *Vibrio* spp, *Clostridium perfringens*, *Bacteroides fragilis* HSP40 phages	0**

* Maximum allowable count per gram of shellfish meat including intravalvular fluid.
** Not detectable.

Test conditions and interpretation of results:

1. The maximum allowable counts apply to tests carried out according to procedures described here or methods of proven equal efficiency.

2. When the maximum allowable count for any organism is exceeded, another representative sample of shellfish from the same supply must be tested immediately for the widest possible range of organisms in the Table.

3. Faecal coliforms and somatic coliphages should be included in all tests on shellfish. When contamination is suspected, additional tests should be carried out for confirmation and assessment of the extent and type of contamination.

4. Microbiological analysis of shellfish should at all times be backed up by sanitary surveys. This would include assessment of sewage pollution of harvesting sites, depuration procedures, handling, transportation and storage. Under circumstances distinction between faecal pollution of human and animal (ie seabird) origin may be desirable.

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