CEFAS
The Centre for Environment, Fisheries & Aquaculture Science

Research on shellfish cultivation

A synopsis of research funded by the Department for Environment, Food and Rural Affairs (Defra) between 1990 and 2003

I. Laing, D.N. Lees, D.J. Page and K. Henshilwood

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LOWESTOFT
2004

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PART I – HUSBANDRY

*"**Husbandry supplieth all things necessary for food**"*

Edmund Spenser (1552–1599)

AN HISTORICAL PERSPECTIVE

Government funding of research in support of the development of the UK shellfish cultivation industry has a long history. For husbandry techniques, it began during the summer months of the early 1920s, when the mussel purification tanks at the CEFAS (then MAFF) Conwy Laboratory in North Wales were used for experiments in oyster breeding. The aim was to produce seed native oysters to restock the depleted traditional fisheries in England and Wales. The methods used were based on collecting the seed on oyster spatting tiles - roof tiles painted with lime wash.

Around 1935, indoor facilities in a greenhouse and other buildings were built alongside the tanks. In this way oyster culture moved into a more controlled environment and shellfish hatchery techniques were developed. It was soon realised that an on-growing area was needed, and a stretch of foreshore in the Menai Strait was acquired. This was used for field trials with hatchery reared oysters and gradually the idea of restocking oyster beds gave way to the prospect of shellfish cultivation on the lower shore.
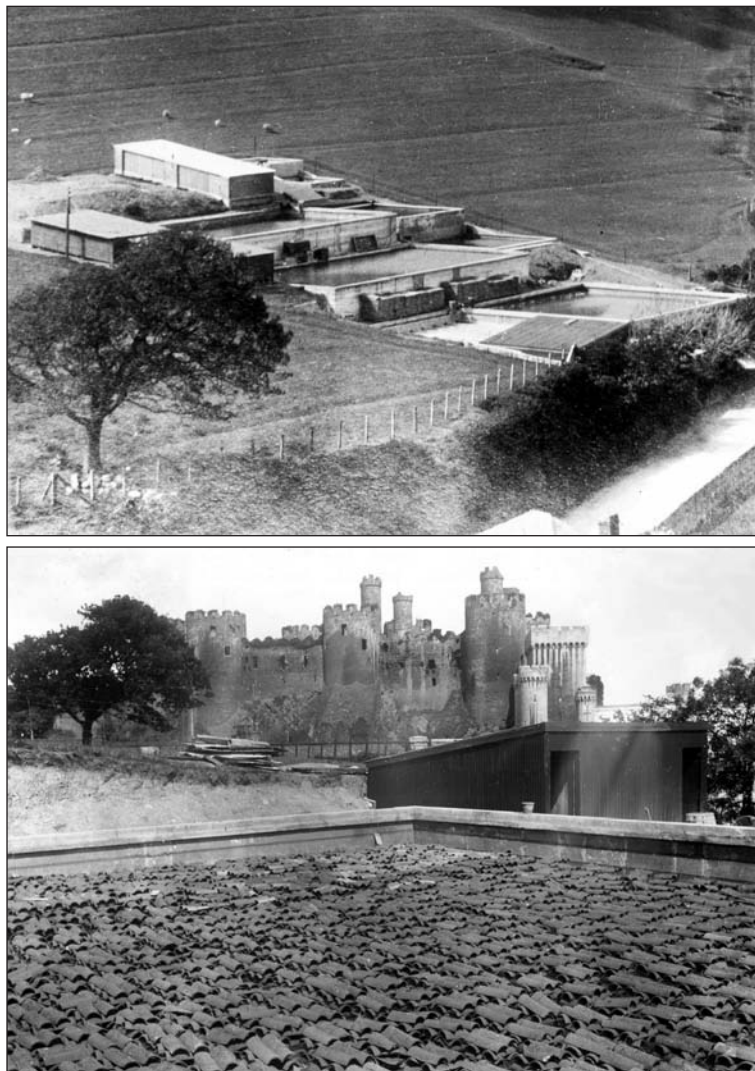

Figure 1. The CEFAS (then MAFF) Conwy Laboratory in 1935, showing the original mussel purification tanks and new buildings for the oyster spat work. The close–up view shows oyster spatting tiles in the tanks.

Development of techniques for broodstock conditioning, rearing bivalve mollusc larvae, settling them and growing the resulting spat to a size suitable for transfer to the sea progressed rapidly in the 1950s and 1960s and the range of species successfully spawned and reared in the hatchery increased. Species that were introduced through quarantine facilities and then tested included the New Zealand Bluff oyster, the Chilean oyster, the Pacific oyster, the Portuguese oyster, the American hard shell clam, the European palourde, and the Chilean mussel. Of these, the Pacific oyster, introduced from Canada and the USA, has achieved commercial prominence as a cultured species. It is now the mainstay of the UK oyster farming industry.

Hatchery techniques were developed to pilot commercial scale and commercial success was achieved by transferring the hatchery rearing technology from Conwy to newly developing private ventures from 1965 onwards. This close association with the shellfish cultivation industry continued over the years. Work at Conwy concentrated on developing and refining hatchery and nursery cultivation technology and included nutritional, physiological and genetic studies. Extensive on-growing trials were also carried out.

From the late 1970s to the early 1980s, techniques for culturing lobsters to market size in controlled environment systems were also developed. This was in response to concerns of increasing pressure on lobster stocks and lobster habitats within our coastal waters. The feasibility of increasing natural stocks in British waters by the release of hatchery-reared juveniles could now be re-investigated. Large numbers of juvenile lobsters were reared at Conwy. Prior to release they were tagged, which provided the means for monitoring their survival in the wild, and, in due course, assessing the impact that these animals have on the exploited stock. The results were the first to provide quantitative evidence that released juveniles survived to legal size in substantial numbers, contributed to fishermen's catches and to the natural breeding stock. This effort continues through the establishment of the National lobster hatchery in Padstow, Cornwall.

During the period covered by this report, lobster research programmes at Conwy turned to investigating the behaviour and habitat requirements of lobsters and the prospects for creating new habitat in artificial reef structures in support of the cultivation work described above. These studies are not presented here, but the results are available in other publications and scientific papers.

Total farmed shellfish production in the UK in the early 1990s stood at around 5-6,000 tonnes. In the last ten years, in line with a general worldwide trend, this has risen to almost 18,000 tonnes, with a value of about £13 million. There is still potential for further expansion of the bivalve cultivation industry in the UK. The number of sites available is not in itself a limiting factor, and growers can protect their right to manage and fish their cultivated stock by obtaining a Several Order or by gaining a lease on an existing Several Order. Aquaculture is set to continue to grow into the 21st century, underpinned by some of the Defra funded research carried out in the past and described below.
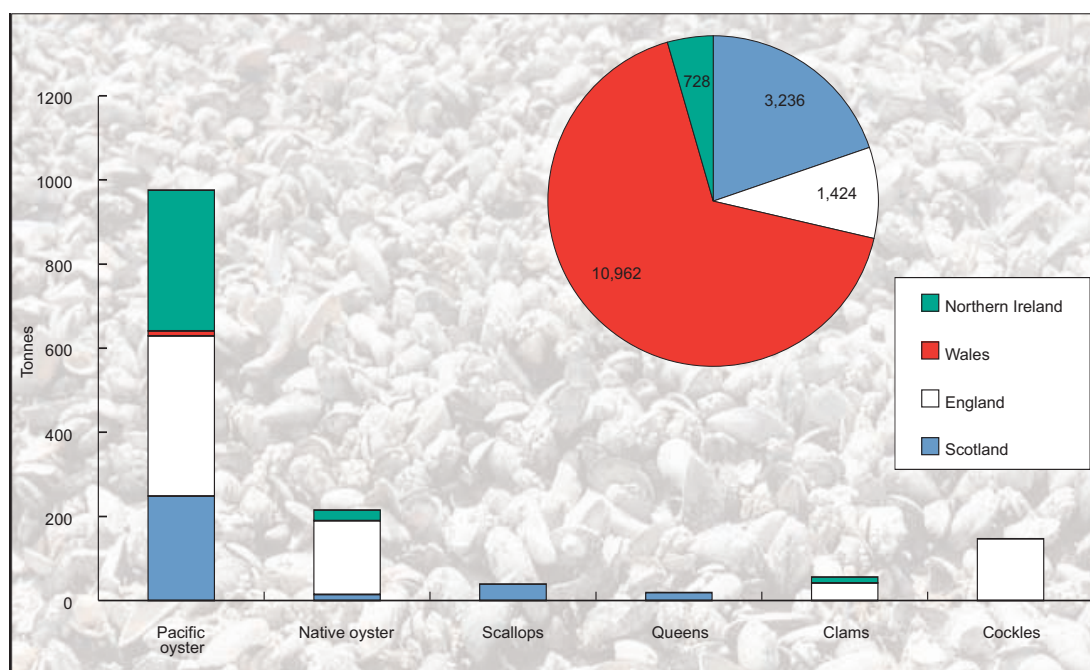
Figure 2. *Shellfish production in the UK (tonnes). Mussels are shown separately ~ they are by far the most important species by weight.*

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1. HATCHERY AND NURSERY PRODUCTION OF OYSTERS AND CLAMS

> The projects addressed in this section are:
>
> **FC0206**   Nutritional and environmental factors affecting recruitment of bivalves (CEFAS)
>
> **FC0909**   Preserved microalgae as an alternative diet in aquaculture (University of Wales Bangor)

1.1   Background

The Pacific oyster, *Crassostrea gigas*, a bivalve mollusc species originally from the Far East, is now regularly produced in UK hatcheries for on-growing in many estuaries, bays and lochs around our coastline. About 1,000 tonnes of this species are currently produced annually. The native flat oyster species, *Ostrea edulis*, is also important due to its higher market value. However, as a cultivated species, hatchery-reared flat oysters attract less attention at present because of their susceptibility to the disease *Bonamia*sis, which was first recorded in the UK in 1982. This problem is being
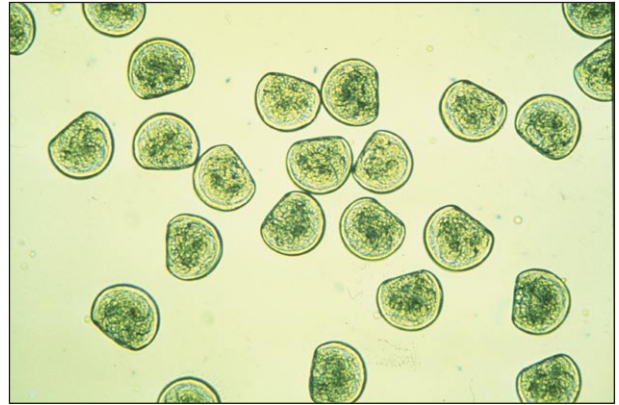
Figure 3.   Stages in Pacific oyster cultivation. *Adult pacific oysters (top left) are brought into the hatchery and conditioned to spawn by feeding at elevated temperatures. Fertilised eggs develop into 80 μm D-larvae in 24 hrs (top right). These grow rapidly, taking about 12 days to reach 250-300 μm at which stage they are ready to attach to a substrate (metamorphosis) when they become spat (or juveniles). These are reared in upwelling hatchery and nursery (bottom left) systems until they are ready to plant out in the sea. They are then normally grown in bags supported on trestles on the foreshore (bottom right).*

addressed through the Native Oyster Biodiversity Action Plan and some research associated with this programme is covered later (Chapter 5). About 150 tonnes of native oysters are farmed annually, with a further 1,000 tonnes per annum coming from managed fisheries. This compares with annual landings of about 2,000 tonnes in the early 1900s.

There are two clam species of commercial interest in the UK. The palourde, or carpet-shell clam (*Tapes decussatus*) is a commercially valuable bivalve mollusc native to Europe, occurring along Atlantic coasts from the British Isles to as far south as Morocco and Senegal and into the Mediterranean. The Manila clam (*Tapes philippinarum*) is a native of the Indo-Pacific coastal seas and has been introduced deliberately into Europe, as a hatchery broodstock, because of its value for commercial cultivation. It was introduced to the UK in 1980. Production of these species is currently relatively small, at about 50-60 tonnes per year, although there is also a managed Manila clam fishery in Poole harbour based on clams that have become naturalised there. Production is around 200 tonnes per year.

Studies undertaken within research contract FC0206 were designed to answer various questions in relation to cultivation of the species described above. These included the diet provided to the broodstock to promote maximum fecundity, the type and amount of food available at metamorphosis, to ensure success at this stage, and the tolerance of juveniles to low temperatures. These studies, as well as providing practical information of use to the commercial hatchery and nursery grower, are also relevant to recruitment where there are natural populations of the cultivated species.

Fresh algae culture is fed to all stages in the hatchery production of bivalve molluscs. It is important to provide the correct species for each stage of the life cycle (broodstock, larvae, juveniles) to promote vigorous and healthy growth. Commercial hatcheries need to produce large quantities of this marine microalgae as food for bivalve production. It has been estimated that the cost of producing algae for rearing bivalves in hatcheries is, on average, 30% of the total cost. It is clearly important to provide foods that are utilised efficiently and promote rapid growth of the animals. Investigations into these aspects were carried out as part of research project FC0206.

The amount of food provided in bivalve mollusc hatcheries is also important. Too little will mean lower growth rates and so higher rearing costs and too much will lead to waste. Maintaining a supply of fresh algae culture to match the demands of the animals can be tricky, and this has led to a desire to find some way of preserving algae such that the cells retain their

nutritional and physical characteristics. It would then be possible to increase the efficiency from current algae culture production methods by maintaining systems at optimum capacity and preserving and storing any excess culture for later use, as required. It would also be possible to exploit the seasonal production from extensive culture systems to a greater degree than at present. A preserved diet has the potential to increase profitability, as algal culture represents a significant hatchery cost. The potential of three techniques (concentration by centrifugation and storage at low temperature, spray-drying and freeze-drying) were assessed as methods for preserving algae (FC0909). Freeze-drying was found to be unsuitable. The nutritional value of algae preserved by the other two methods was compared with fresh, live algae diets for bivalve molluscs as well as for zooplankton used as food for fish larvae.

1.2 Diets

1.2.1 Microalgae

The correct diet is of crucial importance for efficient cultivation of bivalve molluscs. The nutritional value of several different species of algae was assessed. Pacific oyster larvae smaller than 150 µm (shell length) did not perform well with diatom diets, other than *Chaetoceros calcitrans*. In total, five diatom diets were evaluated (*C. calcitrans*, *C. neogracilis*, *C. ceratosporum*, *Thalassiosira pseudonana*, and *Skeletonema costatum*). They all contained high levels of PUFAs and all were suitable for clam larvae (from first feeding) and for larger oyster larvae. The two species of highest nutritional value are *C. calcitrans* and *T. pseudonana*.

It was shown that higher cell concentrations and greater cell volumes of the alga food species *Tetraselmis suecica* can be achieved by culturing in medium with additional nitrate. However, the food value of these cells to bivalves is lower than for cells grown in the normal medium, so this process is not recommended. Gross biochemical composition of the cells produced is apparently similar for both media. The enhanced levels of nitrate used in the algae culture medium did not in itself affect spat growth rates.

Several newly available algae species became available during the course of these studies and these were evaluated. The flagellate *Rhodomonas* (=*Rhinomonas*) was shown to be potentially valuable and was successfully used in nutritional studies within the scallop programme (Section 2.2). *Chaetoceros ceratosporum* was found to be an excellent food for bivalve juveniles, particularly Pacific oysters. *Nannochloropsis oculata*, although high in PUFAs, is a poor food for bivalve larvae, due to its low digestibility.

1.2.2 Replacement diets

Replacement diets are potentially cheaper to produce and more convenient to use than live algae. Some work was devoted to evaluating microencapsulated diets, an experimental manipulated yeast diet (produced by Artemia Systems, Belgium) and the spray-dried preparations of heterotrophically grown *Tetraselmis suecica*, supplied by Cellsys, a Cambridge biotechnology company. Microencapsulated diets were found to be of very limited food value. Oyster larvae and spat and clam larvae were unable to utilise the yeast diet, although clam spat showed some, very slow, growth with this diet. No further work was carried out with these products.

More promising were the studies with the spray-dried algae. Manila clam juveniles were reared through the hatchery with this product as a 100% diet. Mixtures of the dried diet with 10% to 20% of live algae gave fastest growth. Rearing of Pacific oysters required a higher (30% to 50%) supplement of live algae and so products of this type could be used as supplements when supplies of fresh live algae were limited for any reason.

Live diets remain the product of choice and so methods for concentrating and preserving seven species of microalgae (T-ISO, *Pavlova lutheri*, *Tetraselmis suecica*, *Chaetoceros ceratosporum*, *C. calcitrans*, *Rhinomonas reticulata* and *Nannochloropsis oculata*) were evaluated. All algae food species concentrated well when centrifuged at speeds of 3500 rpm, with excellent recovery rates. Storage life of the pastes at 4-6°C varies with species. Some algae, e.g. T-ISO and *Chaetoceros* sp. begin to smell 'off' after two to three days and T-ISO smells strongly even after 24 hours. This can be improved by bubbling the concentrated algae overnight, instead of holding it in a fridge. Concentrated pastes stored at 4 -6°C have a much shorter shelf life than other preservation methods, with an average of about 2 weeks.

It was found that freezing (at -20°C) can preserve cells of some species, and protocols were developed for this method. *Tetraselmis suecica* (» 60% cells recovered), T-ISO (» 45% cells) and *Nannochloropsis oculata* (100% cells) could all be preserved by freezing. For other species, a soup-like substance with very few intact cells resulted. This situation might be improved with the use of cryo-protectants, which were not investigated in this study.
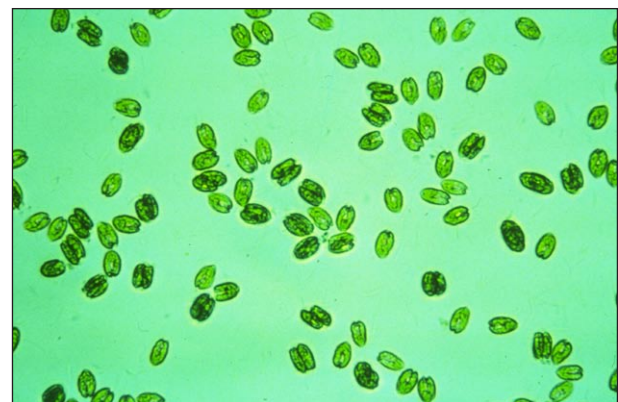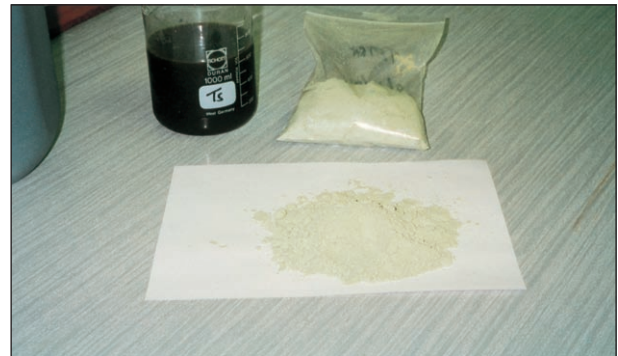

Figure 4. Preserving algae by spray-drying. *The marine unicellular alga Tetraselmis suecica can be spray-dried to a powder. It can then be stored and re-hydrated as required for use as a supplement to feed bivalve mollusc juveniles.*

Table 1. Preservation methods for a range of algae species. *The ease with which various algae can be preserved and their value as a food varies widely depending on species.*

Algae species tested	Preservation methods		
	Short term (as live cells)	Long term (as killed cells)	
	Cold storage (+4°C)	Frozen pastes (-20°C)	Spray-dried
Nannochloropsis occulata	√√√√	*****	ΔΔΔΔ
Tetraselmis suecica	√√√	****	ΔΔΔΔ
Rhinomonas reticulata	√√	-	ΔΔΔ
T-ISO	-	**	ΔΔ
Pavlova lutheri	-	*	Δ
Chaetoceros calcitrans	√	-	-
Chaetoceros cerastoporum	√	-	-
Pond water (mixed sp.)	√√√	****	Not tested

Key: a dash means unsuitable; otherwise, the more symbols, the better.

Spray drying of the pastes in a laboratory-scale dryer was also investigated. The resultant powder after spray-drying contains 5-7% of algae by dry weight, the rest of the powder comprising of salts, and with 55-71% recovery in terms of total algae dry weight from the original paste. The best method for re-hydration of the cells was found to be by adding the spray-dried powder to filtered seawater and blending for around 20 seconds in a domestic blender. This aided the breaking up of clumps of cells, especially for the larger species. When examining the re-hydrated cells under a light microscope, some physical changes could be seen. In *Chaetoceros* sp. the cells appeared very shrunken and shrivelled with many damaged cells. With T-ISO, *P. lutheri*, and *Rhinomonas reticulata* the cells appeared shrunken, although the majority remained intact. *T. suecica* was less affected and *N. oculata* cells did not appear to be any different after spray drying. Unfortunately the higher food value species tend to be less suitable for preservation.

There appeared to be a decrease in total carbohydrate content of the cells on spray drying. Also, spray drying generally reduces the essential PUFA (20:5w3 and 22:6w3) content of the cells. This is especially severe for the diatoms. It would explain why food value of dried algae is broadly similar (and poor) irrespective of species (see Table). With the other algae tested the 20:5w3 seems to be more easily lost on drying than the 22:6w3. There was no significant loss of cell numbers of spray dried or frozen species over time (18 weeks). With frozen algae, biochemical changes differed between species. For example, T-ISO retained 22:6w3 whereas *N. oculata* did not.

Feeding trials were carried out using Pacific oysters and king scallops. In the short-term (cold storage of pastes) nutritional value of algae is retained. Preservation for longer-term storage (freezing or spray drying) gives an immediate decline in food value. This is probably associated with the loss of essential long chain fatty acids.

Oyster larvae fed dried diets gave very little growth and showed high mortalities. Spat fed preserved diets, in three-week trials, survived and increased in organic weight, but at only 20% of the rate with the live algae control. Mixed live:preserved diets gave intermediate results, and supplements of preserved algae to lower rations of live algae significantly improved growth of spat. An experimental concentrated paste diet (heterotrophically-grown *Cyclotella cryptica* supplied by Liverpool John Moores University) was slightly more successful, with oyster growth rates of 50% of that with a live diet.

1.3 Juveniles (spat)

Information was obtained on optimum diets during metamorphosis of oysters, to ensure reliable and efficient production of high quality juveniles. The amount of food required by larvae was found to decrease at metamorphosis but very little spatfall was obtained if no food was present. Larvae can survive for 2 to 3 days without food, by utilising their lipid reserves. Maximum spatfall is given by a ration of at least 0.4 µg (organic weight of algae) per larva per day for native oysters and 0.6 µg per larva per day for Pacific oysters. With this amount of food per larva available, spatfall can occur at food concentrations as low as 0.035 mg (organic weight of algae) per litre. During normal development, native oyster larvae accumulate lipid reserves, and larvae with higher reserves are more competent to settle than those with lower reserves. Pacific oyster larvae accumulate less total lipid, which accounts for their higher food requirement during spatfall. The nutritional value of the algae provided during spatfall is also important, diets high in polyunsaturated fatty acids (PUFAs) give greater spatfall.

It was shown that diets which increase carbohydrate reserves in clam juveniles help to protect these juveniles against the effects of nutritive stress. When no food is available to these juveniles they utilise less

of their total organic reserves and they also grow faster when the food supply is restored. This is particularly important for clams that are about to be planted out, as availability of food in the natural environment is variable and less than that supplied during the hatchery phase.

Where winter temperatures are low, native palourdes were shown to be less suitable for commercial cultivation than the introduced Manila clam, as the former are less able to tolerate these conditions than the latter and small juveniles are less likely to survive severe winters, following planting out. Below 9°C palourdes cannot ingest a sufficient ration to maintain their body weight and reserves are utilised, whereas Manila clams are capable of growing at 6°C.

With oysters, small juvenile native oysters were better adapted to low temperature than Pacific oysters. Unfed native oysters lose less dry weight and maintain a higher condition index at 6°C and 9°C, and more juveniles survive at 3°C, than Pacific oysters. The stress of low temperature in the presence and absence of algae food cells induces changes in metabolic activity and in the lipid and fatty acid composition of the bivalves. For example, in all species the (n-6) PUFAs in the membrane lipids showed higher values in juveniles kept under conditions in which organic weight decreased compared with in animals that grew. The proportion of total PUFAs in the membrane lipids tended to be higher in bivalve species with a greater degree of cold tolerance. These indicators can be used both to assess the potential survival and performance of various species of cultivated bivalve molluscs at sites where winter temperatures are known and to assess

the degree of stress imposed on cultivated bivalves by environmental conditions at particular sites. Clams preferentially utilised carbohydrate as an energy reserve. In oysters, native oysters utilised lipid and Pacific oysters protein. These biochemical indicators are also useful for assessing performance in relation to environmental conditions.

The production rate of juveniles in relation to biomass (stocking density) and ration (live, dried and mixed algae diets) was evaluated. Rations greater than 0.2 g (dry weight of food) per g (live weight of animals) per week do not give a proportional increase in production rate i.e. doubling the ration gives only a 15-45% increase in production. An increase in stocking density above 0.2 g (live weight) of juveniles per litre does not give any increase in biomass production, and efficiency of production is lower as more food is needed to maintain the same ration.

1.4   Broodstock

Information was obtained on optimum diets for conditioning oyster and clam broodstock to ensure reliable and efficient production of high quality eggs and sperm. The food ration (dry weight of algae per unit dry weight of bivalve meat) required by bivalve mollusc (oyster and clam) broodstock to produce the greatest number of good quality eggs or viable sperm was found to be 6% per day. With this ration, broodstock fed algae diets of high nutritional value (usually ones rich in PUFAs) accumulate higher lipid reserves and the females produce more eggs. Spray-dried *Tetraselmis* (see 1.2.2) is a cheaper and more convenient diet to use than live algae. It
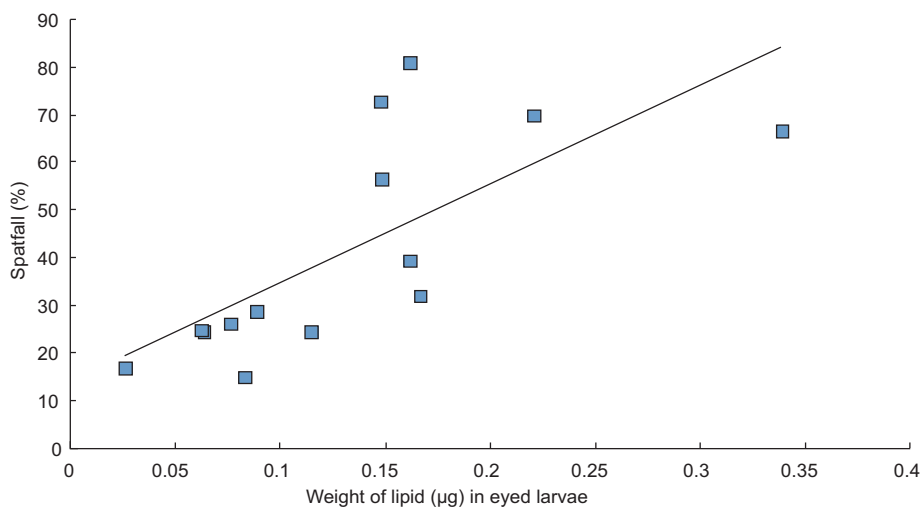

Figure 5.   The effect on spatfall of the amount of lipid in the larvae. *The proportion of Pacific oyster larvae successfully completing metamorphosis and settling as spat depends on the lipid reserves in the larvae.*

Figure 6. Broodstock conditioning experiments.
*Diet rations are metered into the seawater
supply by the peristaltic pumps.*

Figure 7. A Manila clam spawning.

was found to be an adequate replacement diet for broodstock conditioning, but more eggs are produced if supplements (30%) of live algae are added. The food value of the live algae supplements is in the order: *Isochrysis galbana* > *Skeletonema costatum* > *Dunaliella tertiolecta*. It was found that complete conditioning of Manila clam broodstock requires at least 40-48 days at 20-22°C.

The lipid content of Manila clam eggs is about 7-8 ng per egg, irrespective of the type of algae diet supplied to the broodstock. However, the PUFA content of the eggs reflects that of the diet. That is, if the broodstock diet contains high levels of a particular PUFA then the eggs will also have high levels of that same PUFA. For native oysters, the amount of lipid in larvae at release can be up to 25% higher when broodstock are fed diets of high nutritional value (those rich in PUFAs). The ability to boost the lipid and PUFA content at this early stage of development by feeding the appropriate algae diets to broodstock is important, as high lipid and PUFA content give better subsequent performance.

Eggs with a high PUFA content utilise less of their lipid during development from fertilised egg to D-larva. This is particularly noticeable if the eggs need to be treated in any way, for example induction of triploidy. The D-larvae with higher lipid reserves then have a greater tolerance to stress. For example, clam larvae that developed from eggs released by broodstock fed on *Dunaliella* (both alga and therefore eggs low in PUFAs) were less tolerant to stress than those from broodstock animals fed on food species such as *Isochrysis* or *Skeletonema*, which are high in PUFAs.

Large quantities of lipid are used by native oysters as they develop from fertilised egg to pre-release larva and larvae released from broodstock fed high PUFA diets (and so high in lipid) survived better than larvae that were low in lipid as a consequence of the broodstock being fed diets low in PUFAs.

1.5 Uptake of the results on hatchery and nursery production of oysters and clams

An understanding has been gained of the effects of various diets on the physiology and biochemistry of commercially valuable bivalve mollusc species, forming a useful basis for extrapolating results to other cultivated and new bivalve species and for planning further studies.

The potential of algae replacement diets has been identified and evaluated. Since this study was completed, preserved algae diets, both chilled and dried, have become commercially available and there has been significant uptake by the fish farming industry. The results from the Defra funded work have contributed to developments in this field.

We have shown that preserved diets cannot fully replace live algae in shellfish hatcheries but have some application as supplemental diets and could prove beneficial when the supply of algae within a hatchery

is interrupted for any reason. It is of note that there is considerable potential for preserved algae diet products in fish hatcheries.

A considerable amount of practical information on how diets affect growth and survival of commercially valuable bivalve mollusc species at the various stages involved in their cultivation under different environmental conditions has been obtained. Much of this is relevant to efficient operation of bivalve hatcheries and will help to ensure that reliable quantities of seed are available for the development of the bivalve culture industry. For juveniles in hatcheries, a simple model has been developed, relating growth rate of juveniles and food cost to economy of spat production under various rearing conditions. The results obtained will help promote and encourage the development of aquaculture and new aquaculture technologies. Some of the information is also relevant to environmental impact and the potential for recruitment of introduced species, where there is concern from conservation groups that these species may spawn in the wild.

1.6 Publications arising from this research

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2.   HATCHERY AND NURSERY PRODUCTION OF SCALLOPS

> The projects addressed in this section are:
>
> **FC0202**   The hatchery production of king scallop seed (CEFAS)
>
> **FC1001**   Transport of seed scallops - *Pecten maximus* (University of Wales Bangor)
>
> **FC1002**   Nursery cultivation of king scallops (CEFAS)

2.1   Background

UK hatcheries have traditionally supplied seed of various oyster and clam species to support the on-growing industries. Cultivation of scallops, *Pecten maximus*, which is mainly undertaken in Scotland, particularly on the west coast, has been entirely dependent on wild-caught seed. The vagaries of nature make this a rather hit and miss process, and as scallops are such a high value species research programmes to develop techniques for producing spat in hatcheries, to guarantee a reliable supply of good quality seed, were established (FC0202, FC1002). On-growing trials have shown that the quality of hatchery reared spat and seed collected from the wild is similar, in terms of both growth rate and viability.

Methods for transporting seed scallops from collection areas or hatcheries to on-growing sites had, in the past, often resulted in high mortality. This mortality would have arisen when scallops became exposed to stressful conditions. It would be preferable for all scallops to be transported in seawater, rather than in air, under moist conditions, since they would not have to cope with prolonged periods of stressful aerial emersion. However, owing to the greater logistical problems and higher costs of transporting large volumes of seawater scallops are typically transported, with variable success, in air under cool and moist conditions. The research project FC1001 undertook to investigate and evaluate the constraints within this process.

2.2   Seed production

In November and February of each year, adult scallops were obtained from SFIA, Ardtoe and later from a commercial scallop grower. In the hatchery, the scallops took approximately 9-11 weeks to become sexually mature. Increasing seawater temperature and food availability were the more important factors controlling gonad maturation; changing the photoperiod had no significant effect on egg production. The optimum diet ration was 3% of the dry meat weight of the scallops in dry weight of microalgae per day. This was more effective than higher rations of 6% and 9%. The best diet for producing high numbers of eggs with a high hatch rate was a mixture of *Tetraselmis*, *Rhinomonas* and T-ISO.

Figure 8.   Obtaining eggs and sperm from adult scallops. *Cyclical temperature treatment in a water bath is used to induce conditioned scallops to spawn.*

The techniques and equipment that were used for rearing the scallop larvae were generally similar to those used for other bivalve species produced in commercial hatcheries. In brief, larvae were reared in polypropylene rearing vessels filled with filtered, heated, UV-treated seawater. Water was changed three times each week and food (selected microalgae species) was added daily to a predetermined level.

It was found to be extremely important to ensure that the temperature of the water in the culture bins did not exceed 17-18°C and that the salinity was always above 30 ppt. Compared with the larvae of other bivalve species, scallop larvae are extremely fragile and they have to be handled as little as possible. Taking them out of water, even very briefly to sieve them, for example, might kill them.

The most critical time during the rearing process was at metamorphosis. For scallop larvae to be competent

Figure 9. Up-welling cylinders containing scallops in a through-flow of seawater. Inset: measuring the growing juvenile scallops.

to successfully complete metamorphosis, their dietary requirements (in terms of species composition of the algae diet and the essential fatty acid content of the diet) were generally similar to larvae of other bivalve species reared commercially although actual rations required were much lower.

After metamorphosis, the actual growth rates of small juvenile scallops differed from those of oysters and clams although it must be recognised that the rearing temperature for scallops (17 ± 1°C) is 3-4°C lower than that usually used in commercial hatcheries for the other species. Scallop spat grew very slowly immediately after settlement and metamorphosis, at an average rate of about 0.07-0.09 mm per day, requiring about 20 days to grow from size at settlement (0.5 mm) to 2 mm. Excessive handling of spat up to this stage was inadvisable, but once they had reached this size they were less susceptible to handling stress. There is then a rapid increase in size-specific growth rate. After approximately 5-6 weeks, the fastest growing scallops reached 5 mm, the size at which they can be put into perforated plastic trays. At this size they are capable of an increase in shell height of up to 2 mm per week. Within another 6 weeks, some of the scallops reached 10-15 mm, when they were transferred into pearl nets for on growing.

The growth rates of 2-15 mm juveniles were compared on intensively cultured microalgae and on natural phytoplankton produced by fertilising outdoor tanks. Using algal diets typically grown in commercial hatcheries it was shown that nutritional value was in the order *Pavlova lutheri* > *Chaetoceros calcitrans* > *Rhinomonas reticulata* > T-ISO > *Tetraselmis suecica*. A mixture of the first two of these species

gave significantly faster growth rates than any other combination of species tested.

The higher food value of *Pavlova* compared with *Chaetoceros* was of interest since *Pavlova* has been shown to be of no more than moderate food value for many other bivalve species. The very low food value of *Tetraselmis* was also in contrast to other bivalves, for which it generally provides moderate value, particularly in diet mixtures. The relative food values of these three species suggest that king scallops have a requirement for the two highly unsaturated essential fatty acids EPA (20:5w3) and DHA (22:6w3). *Pavlova* is rich in both of these, *Chaetoceros* has a high 20:5w3 content and *Tetraselmis* contains small amounts of 20:5w3. The relative food value of *Pavlova* and T-ISO was also consistent with this idea. However, T-ISO contains a high concentration of only DHA and was a poor food as a single species diet, but promoted good growth when combined with species containing adequate amounts of EPA. Optimum filtration rates were found on a *Pavlova* diet but a decrease in filtration rate was observed at cell concentrations of 200 cells per µl of *Pavlova* or the equivalent of other species tested.

In outdoor nursery systems, the addition of inorganic fertilisers to the seawater, to encourage the growth of algal food cells, is often carried out, as it is well established that this can enhance the performance of bivalves and reduce costs associated with intensive culture of algae. Growth rates of scallop spat fed bloomed seawater were similar to those fed an intensively cultured algal diet of high nutritional value. Comparable growth rates of scallop spat kept in unfertilised natural seawater were very much lower. However, this food source is only available at

certain times of the year, and growth rates of algae in outdoor bloom tanks sufficient to provide algal food cell concentrations high enough for rearing scallops may only be achieved between mid June to mid August in the UK. Blooming of natural phytoplankton, although inexpensive and simple to manage, can also be unreliable. The apparently lower consumption of smaller-celled cultured species and the significantly lower consumption of the 2-5 μm size group from the bloomed algal ration further supported the idea that *P. maximus* is similar to other scallop species in not being able to efficiently utilise food cells in this size range.

The optimum temperature for intensive rearing of scallop spat in a nursery was 17-18°C. At this temperature, the spat grew well (increase of up to 0.21 mm shell height per day) while maintaining a high condition index. Slightly faster growth rates at higher temperatures were compromised by a decline in condition, which could affect the performance of the spat when they are planted out in the sea. Food requirements were also greater at higher temperatures. At 17°C, fastest growth was achieved on a ration of 0.15 g organic weight of algae per g live weight of spat per week. Higher rations than this were not consumed efficiently. There is probably no commercial advantage to be gained from rearing scallop spat in the nursery above 17-18°C. Growth at any temperature is limited by the amount of algae that the scallops can efficiently consume at that temperature.

Low technology nursery systems were developed and tested in the laboratory and at two commercial sites (see figure). The technique of 'remote settlement of larvae' was assessed at the same sites, with some encouraging results.

Settlement System A

Settlement System B
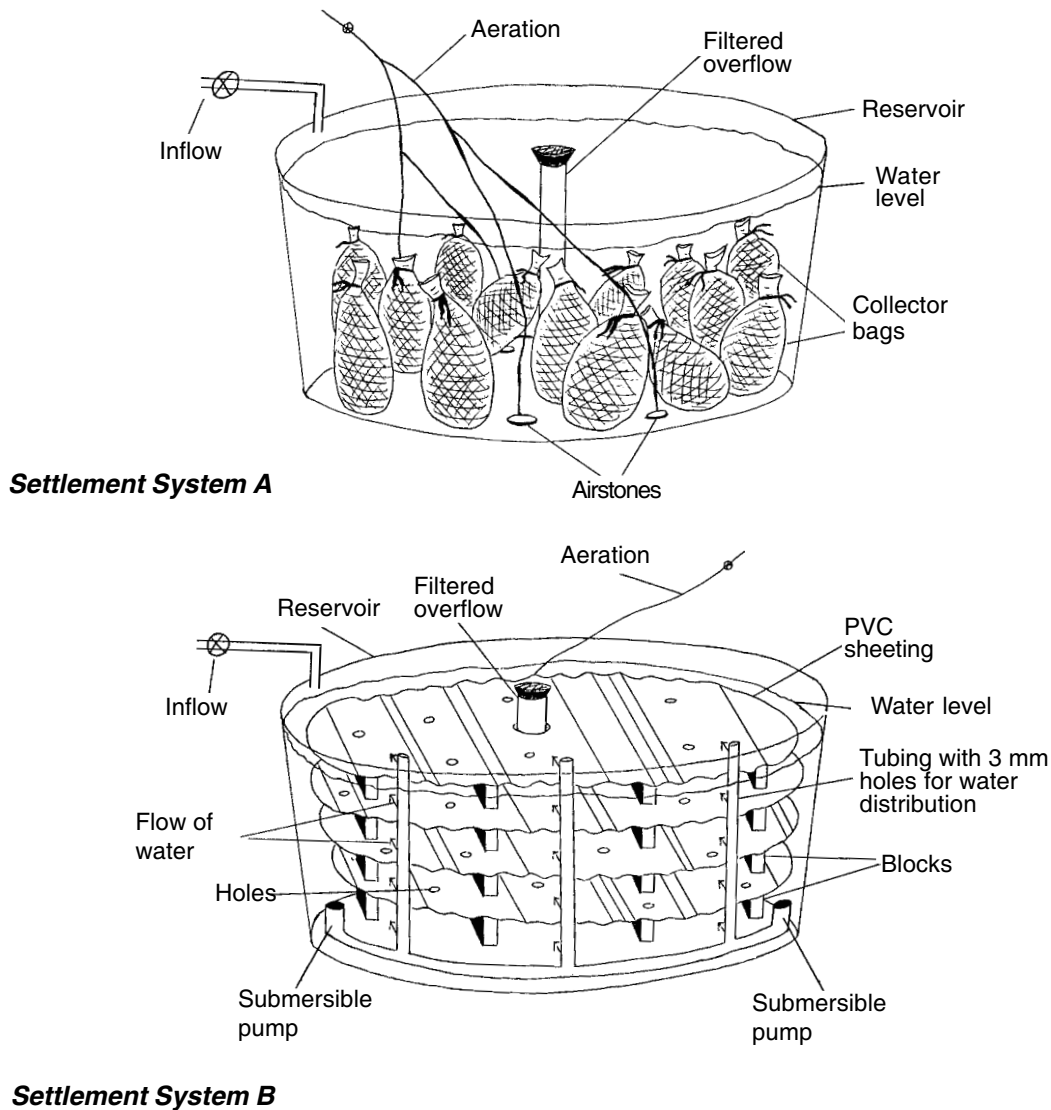
Figure 10. Comparison of systems for settling scallop larvae. *Early settlement trials concentrated on using onion bags as spat collectors, suspended in a 2000 litre seawater tank, simulating 'wild' conditions (System A). Only moderate numbers were settled, so an alternative system was designed and built (System B). This consisted of 6 layers of corrugated PVC sheeting separated by PVC pipe and included two submersible pumps to distribute sea water from the base of the tank to between each layer of sheeting. Trials showed that twice the number of spat settled in system B and that the grow-out period was reduced to one third compared with System A.*

2.3 Seed Transport

The source of seed scallops, either from a nursery or collection from the wild, may be remote from the on-growing site. For example, one of the established wild seed collection sites is off the west coast of Scotland and there is a Several Order on-growing site in the south of England.

The approach adopted for this investigation was to study commercial transports of seed scallops and to simulate in the laboratory the conditions in order to gain a clearer understanding of the problems facing the commercial scallop grower. There was regular contact with the industry enabling investigation of how the scallops were being handled and packed and over what distances they were being transported. Two fully commercial and three simulated transports of scallop seed were followed and using information from these investigations and also that supplied by the growers the commercial transport of scallops was studied under laboratory conditions. Small-scale experimental transports were conducted and survival, on-going growth and condition of these scallops then examined.

Simulated transports in air produced significant differences in the rate of shell growth after 12 hours aerial exposure and tissue weights were also affected. After more than 12 hours in air and two weeks back in water no significant increase in tissue weight was observed; scallops had effectively not grown, unlike those scallops exposed to air for less than 12 hours. In addition significant mortalities, with only a quarter of the scallops surviving, occurred if individuals were exposed to air for more than 12 hours.

Physiological changes were investigated in stressed scallops by examining the heart rate of individuals. Scallops were placed under a microscope and a bright light shone through the thin shell enabling the heartbeat of an individual to be counted. When a scallop was taken out of water the heart rate increased for the first 2-3 hours before dropping sharply to less than 10 beats per minute and in some cases stopping. Once back in water the heart often did not beat, or only beat slowly. After a period of time, directly related to the length of time the scallop had been out of water, the heart rate gradually increased and eventually returned to the baseline rate of 22 beats per minute.

Biochemical changes in scallop tissues during stress were investigated by examining changes in the levels of the energy reserve glycogen after periods of aerial exposure. In adult scallops a significant reduction in the amount of glycogen in the body was seen after aerial exposure. Reductions in seed scallops were also seen but these were not significant. Glycogen levels in seed scallops only constitute 2% of the total dry tissue weight.

Stress events were also detected in scallops in the external rings on the shell surface and internal bands in the shells. More clearly defined rings and more prominent bands were found with an increase in the time out of water.

Scallop behaviour was investigated as a way of measuring stress in seed scallops. Significant differences were found in the behaviour of scallops exposed to air for periods of time. Scallops out of water for more than 12 hours gaped widely and were
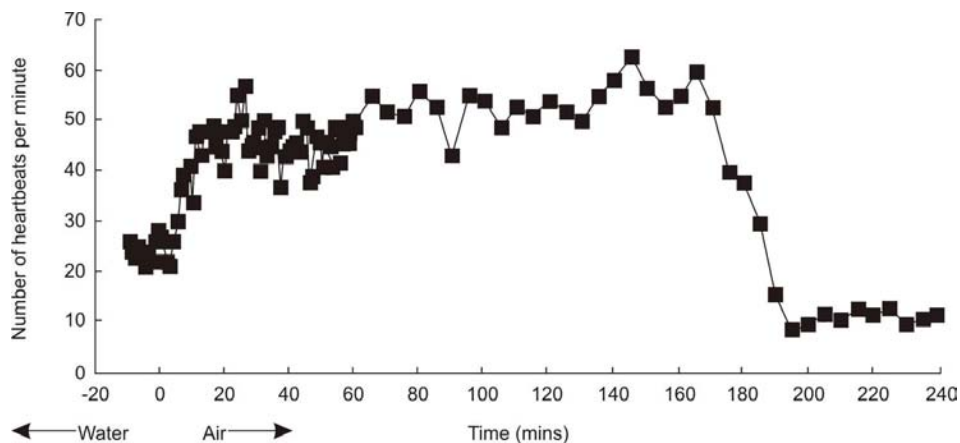
Figure 11. The effect of air exposure on scallop heartbeat rate. *The heartbeat of individual scallops (5-20 mm) was monitored during aerial exposure. Each scallop was transferred to a dish, containing seawater at constant temperature (12°C), placed beneath a binocular microscope and illuminated using a cold light source. The heartbeat could be easily viewed through the virtually transparent shell. Activity was recorded continuously (up to 6 hours) on videotape for later analysis. Initially baseline heart rates were established for a range of different sized scallops in water and in air and then the effect of different periods of aerial exposure was investigated.*

Figure 12. Investigating the transporting of scallops in a salmon well boat.
Above: Bagging up the scallops;
Right top: transporting them to a lorry for a short road journey; Middle: unloading into the hold of the well boat; Bottom: the bagged scallops distributed onto the floor of the hold, which was then flooded with seawater.

observed to be unresponsive to touch. However when returned into water after 24 hours they had recovered to a normal state. Differences were seen in their shell flapping behaviour. Normally when scallops are handled and briefly removed from water more than 60% of them flap their shells violently on return to the water. The percentage of scallops flapping reduces with the amount of time out of water. For example after 18 hours in air none of the scallops flap for the first 6 hours after return to seawater.

2.4 Uptake of the results on hatchery and nursery production of scallops

Culture technologies that may be successfully used for commercially rearing scallops have been developed. Scallops collected during the late winter and early spring months, outside their natural breeding season

when it is impossible to obtain ripe adults from the wild, may be conditioned to spawn, so that seed are ready for transfer into the sea during the summer and early autumn. This gives the seed a chance to grow and build up reserves before the onset of winter.

The transfer of technology was effected through collaborative field trials involving the industry. Although results of the 'remote settlement' technique were preliminary and will need further development to define the optimum conditions for transporting larvae and the time to move collectors from the nursery system into the natural environment, the industry representatives were very encouraged by the results and keen to participate in further studies. 'Remote settlement of larvae' would be a method of reducing the costs and risks of setting up commercial hatcheries for growing seed. Hatcheries could transport the 'eyed' larvae to individual growers for them to settle the

larvae on site, thereby reducing the need for the more expensive development of nurseries. It is highly likely that any development of scallop aquaculture in the UK will be based on a combination of techniques.

The work conducted on transport of seed scallops demonstrate unequivocally that if survival is not to be compromised they should be transported in air under moist conditions for no longer than 12 hours. The results of this work allow us to make predictions about the optimal conditions for transporting scallops in moist air and to identify a number of behavioral and physiological traits that can be used to predict the survival and assess the stress of transported scallop seed.

2.5 Publications arising from this research

LAING, I., 2000. Effect of temperature and ration on growth and condition of King scallop (*Pecten maximus*). Aquaculture 183: 325-334.

LAING, I., 2002. Effect of salinity on growth and survival of King scallops (*Pecten maximus*). Aquaculture 205: 171-181.

LAING, I., MILLICAN, P.F. AND EARL, N., 1999. Effect of sampling frequency on growth and survival of juvenile scallops (*Pecten maximus*). 12th International Pectinid Workshop, Bergen, Norway, May 1999, Book of Abstracts: 47-48.

LAING, I. AND PSIMOPOULOUS, A., 1998. Hatchery cultivation of King scallop (*Pecten maximus*) juveniles with cultured and bloomed algae diets. Aquaculture 169: 55-68.

LE QUESNE SYVRET, M., 1997. Some preliminary observations on factors affecting larval growth and metamorphosis of hatchery-reared King scallops (*Pecten maximus* Linnaeus). MSc thesis, University of Stirling, 83 pp.

MILLICAN, P.F. Hatchery culture of the King scallop (*Pecten maximus*), 1993. Poster presented at the Pectinid Workshop, Nanaimo, Canada & at World Aquaculture Society meeting, Torremolinos, Spain.

MILLICAN, P.F., 1997. Hatchery rearing of King scallop (*Pecten maximus*). CEFAS Laboratory Leaflet, Lowestoft, 40 pp.

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PSIMOPOULOU, A., 1997. Some factors influencing growth of hatchery reared juvenile king scallop *Pecten maximus* L. MSc thesis, Bangor University, 100 pp.

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UTTING, S.D. AND MILLICAN, P.F., 1998. The role of diet in the hatchery conditioning of *Pecten maximus*. 11th International Pectinid workshop, Baja La Paz, Mexico, 10-15 April 1998, Book of Abstracts: 31-31.

3. ON-GROWING AND RANCHING

The projects addressed in this section are:

FC0203 The environmental impact of clam cultivation (CEFAS)

FC0204 Ranching/stock enhancement trials with great scallop (*Pecten maximus*) (Seafish)

FC0212 Scallop seabed cultivation development (Seafish)

FC1004 Environmental requirements for successful scallop cultivation (CEFAS)

3.1 Background

When UK commercial bivalve hatcheries were established in the mid-1960s, the shellfish industry was slow to show interest in the cultivation of hatchery-reared clams (e.g. American hard shell clam, *Mercenaria mercenaria* and the native palourde or carpet-shell, *Tapes decussatus*). A modest change in attitude came about in the mid-1980s with the introduction of the non-native Manila clam, *Tapes philippinarum* which was readily available from commercial hatcheries and was a hardy and fast-growing species. It was introduced into the UK via the quarantine facilities at the CEFAS Conwy Laboratory, from where disease- and pest-free broodstock were given to the commercial hatcheries for rearing.

At about this time, nature conservationists began to show concern that the two non-native species of bivalves, the Manila clam and the Pacific oyster, *Crassostrea gigas*, which were cultivated widely in the British Isles, might pose a threat to the natural ecology of British coastal waters. Their concern lay with the prospect of these bivalves spawning and establishing a self-sustaining broodstock, or by competing with natural communities for space and food. The study described below (FC0203) was set up to answer some of the questions relating to whether clam cultivation has an effect on natural inter-tidal communities and whether these changes are reversed after harvesting.

Commercial cultivation of scallops is one of the more recent developments in bivalve mollusc aquaculture. Successful development of scallop culture techniques in Japan led to an upsurge of interest in the possibilities of cultivating king scallops (*Pecten maximus*) in European waters.

Scallop seed are initially raised in suspended cultivation, in pearl and lantern nets suspended from long-lines. Pearl nets are generally used for small (10-30 mm shell height) scallops and lantern nets for larger animals. However, it is very costly to use these suspended cultivation methods to raise scallops to market size. This is mainly due to the cost of the large number of nets or cages needed, to avoid crowding, and potential mortality, and to the cost of maintenance and labour charges over a long grow-out period, usually

Figure 13. *The stages in scallop on-growing.* *Juvenile scallops are first placed in fine-meshed pearl nets (top) and later reared in the larger, coarser mesh lantern nets (middle). The bottom photograph shows scallops growing on the seabed.*
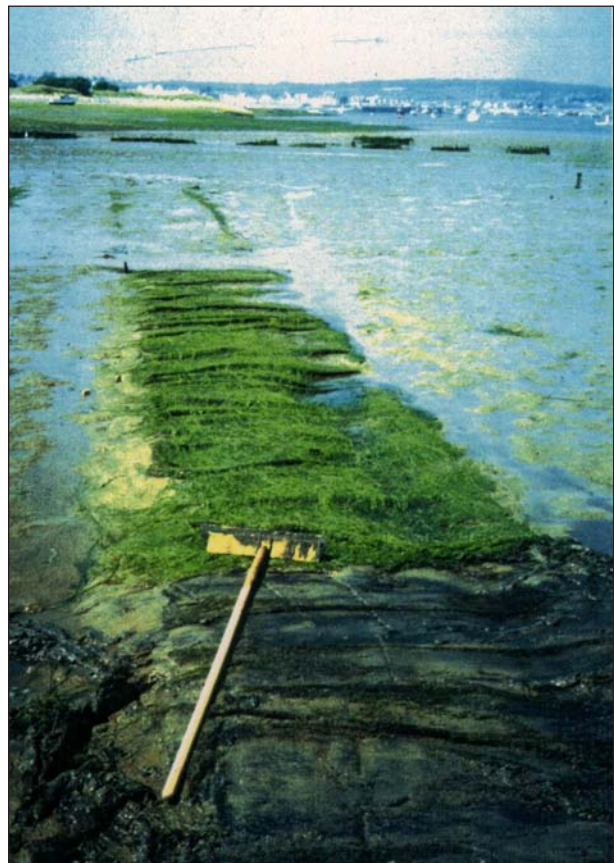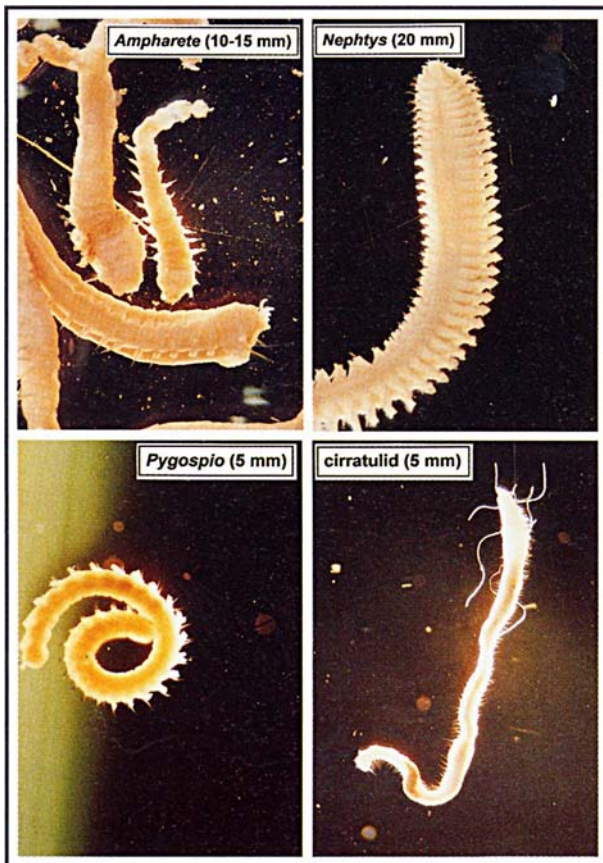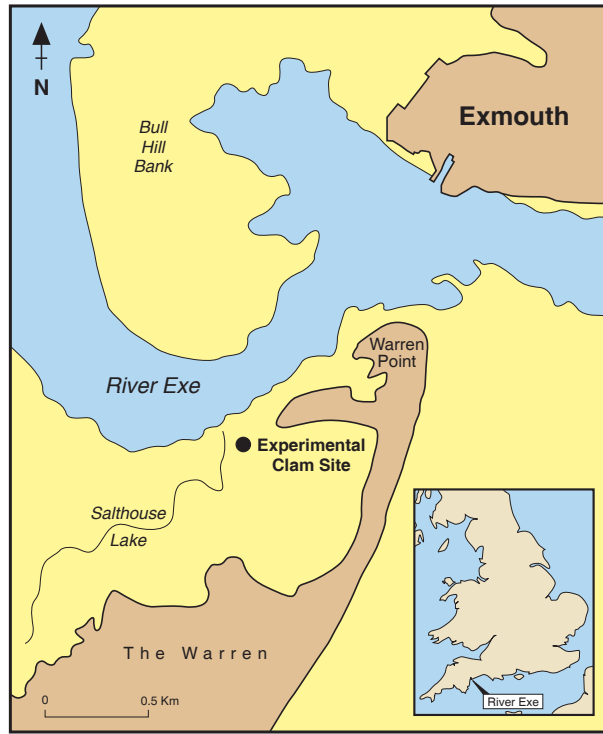
Figure 14. **The experiment on the environmental effect of clam cultivation in the River Exe, Devon.** *The map shows the location of the site. The photographs are of cleaning one of the plots with a squeegee broom and some of the commonly occurring worm species in the netted plots, with the approximate size of the specimens given.*

about 4 years. Growth rates of scallops in suspended cultivation, provided that they have plenty of space to themselves, are similar to those for animals on the seabed. However, studies have shown that wave action, and thus movement in the nets, can be a stress factor as the scallops grow. There can also be problems with fouling of the nets and bio-fouling of the shells with suspended cultivation methods. The above considerations have generally led farmers in the UK to cultivate scallops on the seabed, once they have reached a size (usually about 50-60 mm) at which they are less susceptible to predators such as crabs and starfish.

The success of seabed cultivation of scallops will depend on selection of appropriate sites. Some information was obtained on certain aspects of site selection, such as substrate, exposure and seabed area requirement, in relation to dispersal and density (FC0204, FC0212). Other environmental requirements of scallop spat, particularly temperature, food availability, water flow and salinity, needed to be determined in order to more fully establish the criteria for suitable sites and the research project FC1004 was funded, through the Aquaculture LINK initiative, to address these questions.

3.2 Environmental impact (clams)

The River Exe, Devon was selected as the site for cultivation of clams for the environmental impact experiment. The estuary has extensive sheltered areas that were already used for the commercial cultivation of mussels and Pacific oysters. The experiment incorporated four types of treatments plots. These were: clams with net covers; net covers only; control plots without clams or net covers; control plots without clams or net covers or human activity.

The netting, made from polypropylene, initially had 5 mm mesh apertures but after one year was replaced with netting with 10 mm mesh more suited to the size of the clams. Each plot was 10 m long x 1.5 m wide and was covered with a 2 m wide piece of netting buried at the edges to prevent escape of clams and predation by crabs.

The experiment, which lasted from 1991 to 1995, incorporated observations outside of the actual clam cultivation period (spring 1992 to winter 1994). These included pre-planting and post-harvest information and hence data on the recovery of the site. The plots were sampled and serviced at monthly intervals, when the nets were brushed free of accumulated silt and the green alga, *Enteromorpha*, which actively grew on them from May to October.

The presence of the netting and the green alga growing on it, irrespective of whether clams were present, caused an increase in sedimentation rate compared to the control plots. Thus, the netted plots became slightly humped in profile with their central areas elevated about 10 cm above surrounding levels. Associated with this, the sediment composition of the netted plots altered slightly, showing a slight increase in the proportion of silt. A small increase in organic content and chlorophyll breakdown products of the sediment was probably caused by the presence of the weed and the extra numbers of periwinkles grazing on it, and by the extra abundance of deposit-feeding worms beneath the nets.

The numbers of some worm species increased substantially beneath the netted plots irrespective of whether clams were present. This increase occurred within 6 months of net placement and persisted until the clams were harvested, 2½ years after seeding.

The clams reached marketable size (30 g) by November 1994. The plots were prepared for harvesting by removing the nets. The first harvesting was accomplished by hand-raking. This activity was immediately followed by the use of a commercial suction device to compare the effects of both methods on the other creatures in the sediment. Suction harvesting created an increased sediment load in the water, which dispersed to near background levels within 40 m of the device. Some of the suspended particles settled on the seabed as a thin veneer a few metres wide downstream of the harvester. The harvester left a trench about 10 cm deep.

The immediate effect of harvesting on species diversity and abundance differed between methods. Hand raking caused a reduction of 50% and suction dredging of 80-90%. Hand raking also disturbed the sediment to a lesser degree than suction harvesting. The latter activity left a trench, which took about 3-4 months to fill to background levels. By this time, the particle size compostion and the organic and pigment content of the sediment were similar between plots, indicating a return to the homogenous nature of the site seen before cultivation began. The animal community recovered slowly during the winter but by mid-summer natural recruitment had returned species diversity and abundance to the same levels as surrounding areas. That is, regeneration of species diversity and abundance, after harvesting in the winter, was completed by the following summer.

3.3 Scallops

Initial trials at the Seafish Marine Farming Unit at Ardtoe showed that seabed cultivation of scallops was a practical proposition and demonstrated the feasibility of setting up fenced seabed enclosure facilities for monitoring the performance of animals in replicated treatments. Much experience was gained that informed subsequent investigations. Some preliminary results on the effects of air exposure were developed more fully in later work on seed transport (Section 2.3).

Frequency of swimming and recessing behaviour of scallops was observed on four substrate types (mud, fine sand, coarse sand and maerl). Coarse sand was identified as the most suitable substrate for seeding scallops on the seabed, since scallops recessed more readily and swam less when on this substrate. However, other factors were also found to be influential. For example, scallops prefer to occupy areas of some water movement by being near to natural currents or tidal races.

The aim of the 4-year research project on 'Environmental requirements for successful scallop cultivation' (FC1004) was to determine the performance and tolerance limits of scallops, especially juveniles, within a range of environmental factors; in particular, factors that are important for site selection, such as sea water temperature and salinity, food availability and water flow rate. This information also serves for defining optimum conditions for rearing spat in the nursery, prior to planting out.

Continuous temperature loggers were deployed at two industry sites, Loch Fyne and Portland Harbour, and at Brixham, with the co-operation of the Devon Sea Fisheries Committee. Samples of seawater were collected from the sites on a weekly basis for the measurement of salinity, chlorophyll a, and particle number, weight and organic content. Samples were then taken over the next three years to show the seasonal and annual variation in temperature, salinity and food availability at these sites. Hatchery-produced juvenile scallops, 13 mm shell length, were put out in lantern nets at each of the field sites to monitor their growth and survival in relation to the environmental measurements made. The rates of growth and survival over specific ranges of temperature and food ration were determined in laboratory trials. Samples of juvenile scallops from these experiments were preserved for biological indicators of stress, measured at the Department of Oceanography, University of Southampton. Biological indicators of stress, such as the adenylic charge, can be used to determine when the scallops are reaching their limits of tolerance to environmental disturbance.

In Portland Harbour, a commercial seeding of 50 mm scallops was made on the seabed. These scallops were monitored for survival and time to reach market size. Data was collected on the resources (e.g. man hours, consumables) required to manage a seeding to harvest operation to be able to give potential new farmers an idea of the costs and risks involved in the seabed cultivation of scallops.

A summary of the results from the field and laboratory experiments is given below.

Temperature - King scallops did not grow at all below 6.5°C. While measurable, growth was minimal
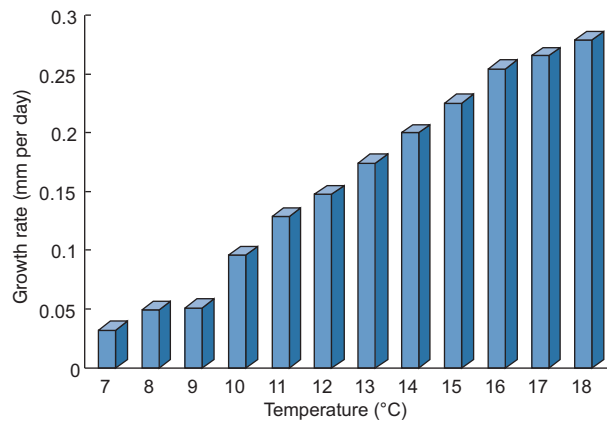

Figure 15. **Mean growth rate (increase in shell height, mm per day) of juvenile scallops in relation to temperature (°C) at the field sites.**
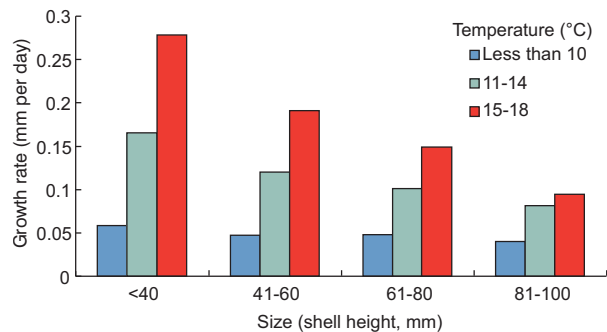
Figure 16. **Mean growth rate (increase in shell height, mm per day) of juvenile scallops in relation to size of scallops and temperature.**

between this temperature and 10°C. Studies measuring metabolic indicators of stress in scallops grown at a range of temperatures showed that this is the point above which the metabolism of the scallops changes from 'winter' to 'summer' mode. Growth rates increase with temperature above 10°C, and will continue to increase up to a maximum tested temperature of 23°C. However, at temperatures above 17-18°C the condition of the animals declines, and there is evidence that this is because these higher temperatures are also stressful. It follows that the best sites for scallop cultivation are those where temperature is between 10°C and 17°C for the maximum length of time. Growth rate at field sites was strongly correlated with temperature.

Salinity - An ambient salinity of 28 ppt or above is required for successful scallop cultivation. Scallops were usually able to tolerate short exposure (up to 6 hours per day for 3 days) to lower salinity (20 ppt) although there was always a short-term reduction in growth rate and sometimes a high mortality at lower temperatures.

Food (Quantity) - It seems that sufficient food was available in the water at all field sites for maximum growth in relation to temperature, especially as scallops can filter faster when there is less food available, so as to maintain an adequate ration. Filtration rates were suppressed at very high food cell concentrations, suggesting that areas with regular dense algae blooms are not suitable.

Food (Quality) - Filtration rate of scallops also varies with the type of algae available, being lower when species with a low nutritional value are present. Scallops are also less efficient at filtering smaller (2-5 μm) algae cells. Food quality is much more difficult to assess from water samples. Even where there was a sufficient quantity of food available, performance of scallops at the field sites sometimes varied from that expected from the prevailing temperature. These differences were attributed to the quality (nutritional value) of the algae species present in the seawater.

Water flow rate - A current velocity of 0.4-1.8 knots (0.2-0.9 metres per second) is most suitable for suspended culture systems. One knot is optimal for seabed culture, although up to 2 knots can be tolerated.

Other information on performance of cultivated king scallops was obtained during the course of the project. Seed at all UK sites reached a size (50-60 mm) at which they could be put out onto the seabed after 12-14 months. Survival at this stage was excellent (> 80%). The performance of hatchery-reared seed was similar to that of wild-caught seed in these respects, proving the quality of the former. It was found that mortality increases significantly if the scallops are kept any longer in lantern nets. Differences in growth rate due to temperature become less with increasing size of the scallops. As they approach market size they grow at a similar rate at all seawater temperatures above 10°C.

The combined wet weight of the edible parts, i.e. the adductor muscle and gonad (yield), from market size scallops increases with shell size such that a 20% increase, from 110 to 130 mm, gives a 70% increase in yield, from 30 to 50 g wet weight. There is therefore a balance between the additional costs of keeping scallops for a longer time against the benefits of a superior and more marketable product. The proportion of the edible part of the scallop that is composed of roe varies throughout the year. In spring the scallops will be ripe, with the roe comprising, on average, about 50% by weight of the total yield. Spawning occurs from late June/early July onwards, and by late summer (August/September) the roe will be, on average, just 10% of the yield. The size of the gonad is then restored gradually over winter, although the rate at which this happens will vary between individual scallops and between sites. It can be very slow at first, so that there may only be a small amount of roe in some scallops harvested for the Christmas market.

As part of the project an assessment was made of the resources required for rearing scallops from seeding to harvest, on a commercial scale, at a site on the south coast of England. Based on seeding 75,000 scallops per year, with 15% mortality and harvesting by diver collection, commencing after 3 years, the profit is 23% of costs. This is based on equipment purchase within the first 3 years, and so profit would be higher if based on depreciation costs over several years. Seafish has gathered further information on the costs associated with scallop cultivation for an economic modelling study. Results from this suggest that commercial operations are potentially viable, particularly as a diver collected scallop is a superior product to dredged scallops and can thus command a premium price.

3.4 Uptake of the results on on-growing and ranching

The information gained from the clam environmental effects experiment has enabled the preparations of guidelines for the management of clam cultivation in a more environmentally friendly way. It has helped counter the allegations frequently made by critics of the shellfish industry that cultivation in the sediment is a destructive activity.

The results from the scallop projects are of direct and immediate relevance to the commercial scallop cultivation industry. They give us an indication of the viability of scallops over a broad geographic range, typical of sites in the UK, for the future development of scallop aquaculture. The resources (e.g. man hours, consumables) required to manage a seeding to harvest operation have been evaluated, in order to be able to give potential new farmers an idea of the costs and risks involved in the seabed cultivation of scallops. Information on the effect of handling frequency at different times of the year on the performance of scallop spat will help farmers to decide how often to service and maintain their nets. The main output, a definitive guide on site selection criteria, available both as a printed publication and as an electronic document on the Internet, will be invaluable to everyone thinking of moving into scallop cultivation, with a view to increasing the chances of success. It should also act as a reference for existing cultivators and will hopefully assist them in site management and in forecasting performance of their stock, leading to increased efficiency of operation. This guide includes information from a parallel Defra-funded study on environmental conditions for depuration of scallops (FC1013 see Section 6.2). There was previously no information available on this and the work was carried out to ensure that Conditions of Approval could be set for scallop depuration plants, so that the emerging industry would not be constrained by adverse harvesting area classification (EU Directive 91/492).

The data generated by this work have also been integrated into the 'hyperbook' economic model of scallop cultivation developed by Seafish.

3.5 Publications arising from this research

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LAING, I., 2002. Scallop cultivation in the UK: a guide to site selection. CEFAS, Lowestoft, 26 pp.

LAING, I., 2002. Effect of salinity on growth and survival of king scallops (*Pecten maximus*). Aquaculture 205: 171-181.

LAING, I., 2003. King scallop cultivation. 14th International Pectinid Workshop, St. Petersburg, Florida, USA, 23-29 April 2003: 48-49.

LAING, I., 2004. Filtration of king scallops (*Pecten maximus*). Aquaculture (in press).

LAING, I., MILLICAN, P.F. AND EARL, N. H., 1999. Effect of sampling frequency on growth and survival of juvenile scallops (*Pecten maximus*). Poster and book of abstracts 12th International Pectinid Workshop, Bergen, Norway, May 1999.

LAING, I. AND UTTING, S.D., 2001. Cultivating king scallop (*Pecten maximus* L.) seed. Abstract of paper presented at the 13th International Pectinid Workshop, Coquimbo, Chile, 18-24 April 2001, pp 48-49.

MIKOLAJUNAS, J., 1996. Effect of exposure period upon survival and growth of juvenile scallops (*Pecten maximus*). Seafish Report No SR 477.

MIKOLAJUNAS, J., 1996. Swimming and recessing behaviour of scallops seeded onto a range of substrates. Seafish Report No SR 479.

NUGUES, M.M., KAISER, M.J. AND EDWARDS, D.B., 1996. Benthic community changes associated with intertidal oyster cultivation. Aquaculture Research 27: 913-924.

SLASKI, R.J., 2002. The scallop hyperbook. Sea Fish Industry Authority CD-ROM.

SPENCER, B. E., EDWARDS, D. B. AND MILLICAN, P. F., 1991. Cultivation of Manila clams. Lab. Leafl., MAFF Direct. Fish. Res., Lowestoft 65: 29 pp.

SPENCER, B. E., EDWARDS, D. B. AND MILLICAN, P. F., 1992. Protecting Manila clam (*Tapes philippinarum*) beds with plastic netting. Aquaculture 105: 251-268.

SPENCER, B. E., KAISER, M. J. AND EDWARDS, D. B., 1996. The effect of Manila clam cultivation on an intertidal benthic community: the early cultivation phase. Aquaculture Research 27: 261-276.

SPENCER, B. E., KAISER, M. J. AND EDWARDS, D. B., 1996. An experimental investigation of the effects of Manila clam cultivation: observations at the end of the cultivation phase. J. Applied Ecology 34: 444-452.

SPENCER, B.E., KAISER, M.J. AND EDWARDS, D.B., 1998. Intertidal clam harvesting: benthic community change and recovery. Aquaculture Research 29: 429-437.

SPENCER, B.E. AND 1996. Clam cultivation: Localised environmental effects. Results of an experiment in the River Exe, Devon (1991-1995). Handout, CEFAS Lowestoft, 10 pp.

UTTING, S.D., BURTON, C. AND LAING, I., 2003. Demonstration of Seafish hyperbooks on the cultivation of selected marine shellfish species. 14th International Pectinid Workshop, St. Petersburg, Florida, USA, 23-29 April 2003: 13.

4. GENETICS

The projects addressed in this section are:

FC0207 Production of sexually sterile bivalve molluscs (CEFAS)

FC0211 Tetraploid Pacific oysters (CEFAS)

FC0215 Enhancing growth and survival of hatchery produced scallops (CEFAS)

4.1 Background

Work on triploid shellfish, funded by MAFF, began with the Manila clam, because there were concerns from environmentalists that if these non-native bivalves were cultivated in the UK they might breed successfully and establish self-sustaining populations in the wild. Techniques that had been pioneered in the USA with oysters were applied to investigate the production of triploid seed of this species (FC0207).

Triploids are produced in the hatchery by treating normal eggs during early development, usually with a chemical, so that they retain an extra set of chromosomes. Because of this they are theoretically sterile.

Research on triploid shellfish then turned to another species, the Pacific oyster. These were originally introduced to the UK to fill the gap in the mid-year oyster market, when the quality of native oysters is poor, due to spawning. Pacific oysters need seawater temperatures higher than those normally found in the UK to spawn regularly. However, they will often show some development of the gonads. Because there is little gonad development in triploid oysters they usually retain a better meat quality in the summer months and do not go 'milky'. For the shellfish farmer, this means a better quality product at market. In all other respects they are identical to normal (diploid) oysters.

The aim was to produce batches of Pacific oyster seed that were all (100%) triploid. To attain this level of triploidy the chemical or other treatment (e.g. pressure or temperature shock treatment to the eggs) must be introduced when all eggs are at the same stage of development. As the eggs do not develop in perfect synchrony this technique will never be fully successful. Producing tetraploids, which have four sets of chromosomes, and crossing these with diploids may be the only way to guarantee 100% triploids, so an attempt was made to produce tetraploid oysters (FC0211).

Experiments to determine the heritability of growth in the Pacific oyster (*Crassostrea gigas*) were carried out at Conwy in the 1970s. Parents were selected to produce crosses of large animals and of small animals. The sizes of the progeny were measured.
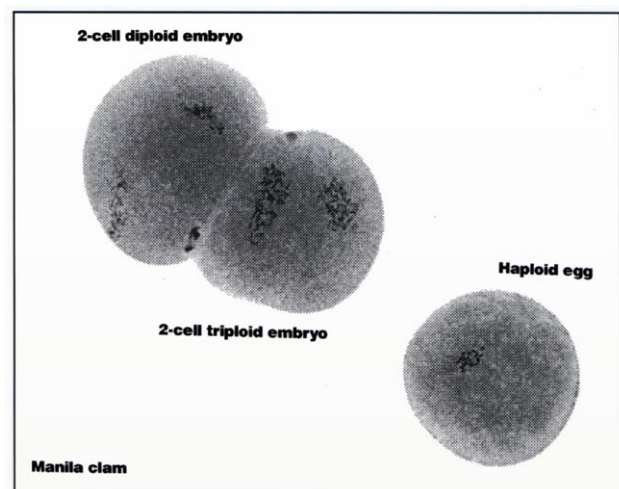

Figure 17. Chromosomes in Manila clam cells.

The heritability in these experiments was calculated to be about 30%. This suggested that under the conditions found at the site (Menai Strait) where the oysters were grown at the time, more than two thirds of the variation was due to environmental factors. However, the effects would be different in different years and at different sites, which highlights the problems associated with getting a meaningful result. This experimental method is further confounded by the fact that not all the progeny of a single cross could be retained. Random culling had to take place just to keep the numbers within manageable proportions with the consequence that some of the variation must inevitably be lost.

The question remained as to what extent the growth rate of shellfish will be related to their genetic variability (heterozygosity). There is some evidence to suggest that these are linked in Pacific oysters and a study (FC0215) was carried out to discover if this is the same in king scallops. Individual scallops, even progeny from the same adults that are reared under identical environmental conditions in the hatchery, grow at different rates. If growth rate (and survival) is correlated with heterozygosity, then it may be good practice to rear only the faster growers in the hatchery. Growth rates and genetic variability in scallops produced from crosses of a small number of parents in the hatchery and in scallops from a wild population were compared in this study.

4.2 Manipulation – polyploidy

Initially, several methods for assessing triploidy were compared. At the embryo stage, the number of chromosomes was counted. In seed and adults, measuring the diameter of nuclei from gill tissue cells was adopted as the standard procedure. Also, a biopsy technique was developed to measure nuclei in haemolymph removed from live animals. This technique was less reliable than measuring nuclei in gill tissue cells, but where results were obtained, in 8 out of 10 cases there was agreement between the two methods. By using the non-sacrificial biopsy technique, it is now possible to work specifically with triploid animals.

Triploid Manila clams were produced by treating eggs with the chemical cytochalasin B (CB). Routinely, 70-77% of treated eggs developed as triploids (when first measured at the embryo stage), with the highest level ever achieved being 83%. Mean survival to the D-larva stage from CB-treated eggs was relatively high, at 68% of the survival of untreated (control) eggs. When clams had reached the 8-15 mm seed stage, triploidy was measured again and ranged from 35-80%. In adults, the levels of triploidy were usually similar to those in the seed.

Growth rate, food consumption, respiration rate and biochemical composition of triploid larvae and juveniles were similar to those of control diploids. Differences only became apparent at the adult stage when triploid clams, held in the laboratory for 6-8 weeks, had significantly higher meat and glycogen
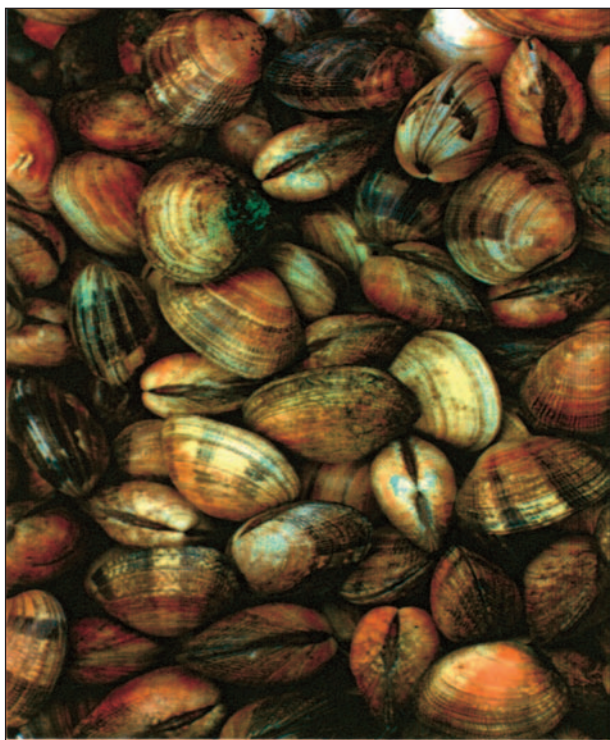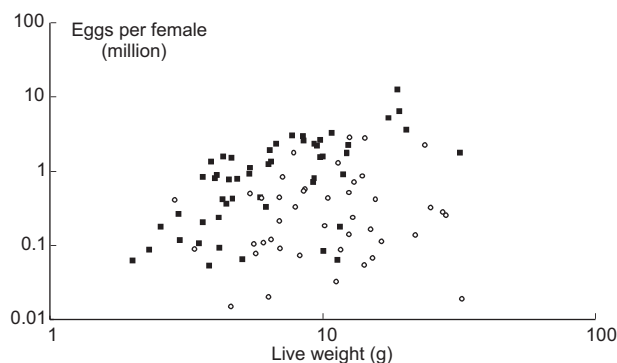
Figure 19. **The relationships between the live weight of adult Manila clams and the number of eggs (millions) spawned for diploid (solid squares) and triploid (open circles) females.** Note the log scale – considerably fewer eggs are produced by triploids.

contents than the diploids. The number of triploid clams that could be induced to spawn and the number of eggs they produced were significantly lower than for the diploids. Although eggs from triploid clams, when fertilised with diploid sperm, developed into D-larvae, larval viability (survival) was lower than for diploid larvae. When all of these factors were taken into account, the reproductive potential of triploids was estimated to be 12.5% of diploids. If all-triploid populations of clams were farmed then this would be further reduced, effectively to zero, as it was evident that very few triploid clams that spawned were male; out of a total of 45 clams that could be induced to spawn only one was a male.

It was found that the chemical 6-dimethylaminopurine (6-DMAP) was less effective than cytochalasin B for inducing polyploidy in batches of Pacific oyster larvae (measured as percentage success at the 2 or 4-cell embryo stage). Survival was variable between treatments. Although less effective than cytochalasin B, 6-DMAP will be an acceptable alternative if the shellfish industry is unable to use cytochalasin B in the future. The ability to use chemicals to produce triploid and/or tetraploid bivalves in the long term, and in particular the use of cytochalasin B, cannot be guaranteed.

Results of the samples of gill tissue from putative tetraploid Pacific oyster seed produced in 1996 that had been sent to Caen University for analysis by flow cytometry were disappointing. Less than 10% of the seed were tetraploid. No further work was carried out with this batch of seed.

In one of the batches of larvae that were produced in late 1996, 100% tetraploidy was recorded at the embryo stage, by counting the number of chromosomes in 2 or 4-cell embryos. The spat that resulted from this rearing were grown on to a size at which a small piece

Figure 18. **Manila clams** – triploids have a higher meat and glycogen content than diploids.

of the gill could be excised, taking great care to ensure that the animals remained alive. This was done by holding the oyster spat in a solution of 3.5% magnesium chloride solution so that the shell gaped and was open just enough for a small piece of gill to be removed. The diameter of nuclei in the gill tissue cells was measured under a binocular microscope. The diameter of the nuclei in tetraploid oysters is 1.26 times larger than in diploid oysters. Initial results on a batch of 30 animals that were screened showed that 56% of this batch was tetraploid, 36% triploid and the remainder diploid. Since tetraploidy was measured as 100% at the embryo stage and only 56% of spat were tetraploid, this raises the question as to whether reversion had occurred. In studies in the US, it has been shown that triploids revert back to the diploid state. Therefore, it is possible that tetraploids may exhibit reversion.

4.3 Natural stocks (scallops)

Scallops originating from the wild (Loch Moidart) and from the hatchery (Conwy) were grown at two sites: Loch Fyne, Scotland and Portland Harbour, Dorset. The scallops were initially measured monthly for eight months. The frequency of measurements was subsequently decreased after results from another project indicated that handling may be reducing growth. The heterozygosity (a measure of genetic variability) was assessed by looking at the allozymes (7 loci) of 20 individual scallops from each of the wild and the hatchery populations. No differences between the genetic variability of the wild and hatchery seed were detected with the analytical method used. There was 24.4% variability in the hatchery-produced scallops, compared with 25.7% in the wild stock.

With the development of more discriminate analytical techniques; the method for determining genetic variability was changed from the allozyme studies described above to a method involving measurements on mitochondrial DNA (mtDNA). This was accomplished by PCR-amplification of the mtDNA fragment, followed by digestion of this fragment with restriction enzymes (PCR-RFLP). The restriction enzymes were chosen from ones previously shown to produce polymorphic bands for scallops: The amplified fragments of 36 individuals originating from the hatchery and 36 individuals from the wild (both grown at Loch Fyne) were subjected to this restriction analysis.

The growth rate of both groups of hatchery scallops taken from Conwy was greater than that of the wild stock transferred from Loch Moidart to Loch Fyne. However, since handling has been shown to reduce growth, it is possible that during cultivation in the hatchery there was some degree of selection for those genotypes better suited to stresses incurred during handling. This had to be taken in consideration when looking at the results from the genetic analysis.

The results obtained for the hatchery and wild stocks grown at Loch Fyne were compared. The results were also compared with those previously made on other wild populations from the Isle of Man, Mulroy Bay and Plymouth (using the same analytical methods, including primers and restriction enzymes, but not part of this study). The level of genetic variation in the hatchery population was surprisingly low; 33 out of 36 individuals showed the same haplotype, and the values for mean nucleotide sequence diversity were consequently also low. Higher values for mean nucleotide sequence diversity in the wild stocks showed that there was greater genetic variation in these scallops.

So, although the allozyme study carried out at the beginning of this project (based on 20 individuals from each of two populations and 7 enzymes) failed to detect any differences in genetic variation between populations the analysis of mitochondrial DNA has revealed large differences in genetic diversity between the wild and the hatchery seed. This is most likely due to the generally higher resolution of studies carried out at the DNA level, rather than to profound changes in the haplotype composition of the populations over the time of the experiment. Furthermore, scallop mitochondrial DNA is, presumably, only maternally inherited, resulting in smaller effective gene flow, consequently bottleneck effects (as they occur when setting up a broodstock in a hatchery) are more severe than in nuclear DNA. The DNA-based methods generally offer higher resolution compared to allozymes.

The better performance of the hatchery population in this study indicates that reduced genetic diversity does not necessarily lead to reduced fitness (and growth) in scallops. The predominant haplotype in the hatchery samples, which may have been selected for during rearing, is apparently not linked to growth rate, as seven individuals from the wild population that share this haplotype did not grow particularly well (mean length/height = 66.5/61.7 mm), compared to the hatchery stock (70.5/66.1 mm).

4.4 Uptake of the results on genetics

Methods for producing triploid clams and oysters were successfully developed and the technology transferred to the industry. Commercial UK shellfish hatcheries can now produce triploid seed as and when required. However, the production of 100% triploid batches of seed will need the establishment of a tetraploid broodstock.

It has been shown that the polyploidy induction techniques developed for Pacific oysters, and originally for Manila clams at Conwy, are also appropriate for scallops and so probably for other commercially important bivalve species as well.

Triploid scallops are likely to have a better quality adductor muscle (in terms of taste and size) than the normal diploid. The technique of producing triploid scallop larvae was demonstrated but the animals were never reared beyond the hatchery stage. This is an aspect that could be considered during the further development of scallop aquaculture for improving the final market product and for potentially reducing the time to reach market size.

We already have evidence for the decline in variability in the Pacific oysters raised commercially in the UK, which came from a small population of parent stock. These have lost all the variation at the locus that codes for the enzyme glucose phosphate isomerase, which may be found in the natural Pacific oysters of Japan and indeed in the oysters in France that originated from a considerably larger gene pool. What we do not know is the hidden effects of reduced variation and inbreeding. A plethora of inbreeding deficiencies has been observed which have to be weighed against the advantages of selection. The work described here brings us a step nearer to deciding whether we can enhance scallop production by genetic selection.

The negative effects that handling and transportation may have on the growth rate of scallops warrants further studies, particularly as it is quite likely that there has been some selection in hatchery reared stocks for those more able to tolerate the handling stresses. In future projects, the handling stress could be reduced by conducting a single measurement at the end of the study or by sub-grouping the scallops to minimise the stress when taking samples during the course of the experiment. It may also be more appropriate to avoid hatchery-reared stocks; wild populations taken from an open-sea location could be compared to populations likely to demonstrate low levels of heterozygosity, such as those originating from an enclosed sea loch. In addition, non-invasive sampling (tentacle-clippings) to establish the genotypes of all individuals at the beginning of the experiment would not only allow the level of heterozygosity to be determined early in the project, but also determination of the genotypes of animals which have died or been lost during the course of the experiment.

Results from such trials would be of value for providing advice to the scallop aquaculture industry on the best practice for rearing seed in commercial scallop hatcheries to obtain genetically competent seed. That is, remove potential bottlenecks in broodstock selection and breeding programmes and ensure the viability of scallops in relation to their genetic heterozygosity. The protection of the genetic integrity of wild scallop stocks in areas of scallop aquaculture through the use of hatchery seed that have haplotype diversities comparable to the wild population could also be accomplished.

4.5 Publications arising from this research

ALLEN, JR S. K., SHPIGEL, M., UTTING, S.D. AND SPENCER, B.E., 1994. Incidental production of tetraploid Manila clams, *Tapes philippinarum* (Adams and Reeve). Aquaculture 128: 13-19.

CHILD A.R. AND WATKINS, H.P., 1994. A simple method to identify molluscan bivalves by the measurement of cell nucleus diameter. Aquaculture 125: 199-204.

HEIPEL, D.A., BISHOP, J.D.D. AND BRAND, A.R., 1999. Mitochondrial DNA variation among open-sea and enclosed populations of the scallop *Pecten maximus* in western Britain. J. Mar. Biol. Assoc. U.K. 79: 687-695.

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LAING I. AND UTTING, S.D., 1994. The physiology and biochemistry of diploid and triploid Manila clam (*Tapes philippinarum* Adams & Reeve) larvae and juveniles. J. Exp. Mar. Biol. Ecol. 184: 159-169.

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UTTING, S.D., MILLICAN, P.F. AND LAING I., 1996. The breeding potential and biochemical composition of triploid Manila clams (*Tapes philippinarum* Adams & Reeve). Aquaculture Research, 27: 573-580.

5. STRESS AND DISEASE

> The projects addressed in this section are:
>
> **FC0502** Factors affecting virulence and spread of *Bonamia* (CEFAS)
>
> **FC0504** Application of immunological and other biological water quality indicators in coastal waters (University of Southampton)
>
> **FC0926** Evaluation of factors affecting native oyster stock regeneration (University of Southampton)
>
> **FC1101** Quantitative evaluation of bivalve disease susceptibility and resistance (University of Southampton)
>
> **FC1121** *Ostrea*/Size/Age, Physiological Stress and Resistance to *Bonamia ostreae* (University of Southampton)

5.1 Background

The serious socio-economic consequences of shellfish disease are best exemplified by the devastation wrought by outbreaks of the disease Bonamiasis in native oyster stocks and highlight the need for fundamental evaluations of the disease susceptibility or resistance of commercially exploited species in British waters.

Bonamiasis is caused by the parasitic organism *Bonamia ostreae*. It was first recognised in Europe in Brittany in 1979 and first confirmed in England in 1982. The disease spread through movement of infected stock and it now occurs in many of the major oyster producing areas of the south and east coast of England. It is the most serious disease currently affecting molluscs in Europe and is the only significant disease present in oyster stocks in the UK. Defra has funded research into means of understanding the effects of the disease on the oysters in order to minimise its impact on cultivated oyster stocks. This work has concentrated in the areas of husbandry (FC0502 and FC1121) and immunology (FC0504 and FC1101).

The scope of the immunological studies also encompassed other aspects of stress. The synergistic effects of combinations of stressors within the normal ranges of environmental variables and common marine pollutants may affect the ability of shellfish to resist opportunistic pathogens. The degree of damage to immunological functions is often the determining factor that controls survival, since, for example, pollutant mortality can be caused by opportunistic infections rather than by acute intoxication. This can help us understand the apparently irregular and capricious patterns of outbreaks of Bonamiasis and other bivalve diseases, as well as the phenomenon of 'resistant' stocks and survivors in areas of high mortality.

The native oyster is one of the 19 individual named marine species in the UK Biodiversity Action Plan. The Native Oyster Species Action Plan (NOSAP) has identified numerous probable causes for the oyster's
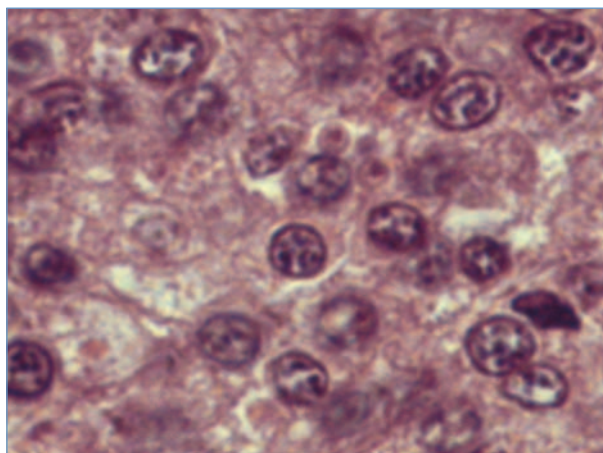
Figure 20. Oyster tissue infected with Bonamia.
Examination of stained tissue sections is the recommended method for screening for Bonamia. A transversal 'steak' is cut through the soft tissues, immediately placed in fixative and then stained with haematoxylin–eosin (H&E). Bonamia cells (2–5 µm diameter in size) occur within the haemocytes or freely in connective tissue or sinuses of the gill, gut or mantle epithelium.

decline in abundance, both from man-made and natural causes. They include, in addition to the effects of Bonamiasis, pests (American oyster drill and Slipper limpet), over-fishing, harsh winters (1947 and 1963) and poor spawning and spat-fall. The published oyster action plan has proposed numerous steps that can be taken to help improve the state of the stocks and revitalise fisheries. These proposals are supported by research and monitoring on Bonamiasis in relation to cultch and the interaction of oysters with Slipper limpets (FC0926).

5.2 Husbandry

Project FC0502 had limited resources allocated to it and thus was conducted on an opportunist basis. Three samples, each of 300 oysters, *Ostrea edulis,* of approximately the same age, originating from two

Essex Oyster and Seafood Company oyster beds and trestles at low water, Goldhanger Creek, River Blackwater, Essex.
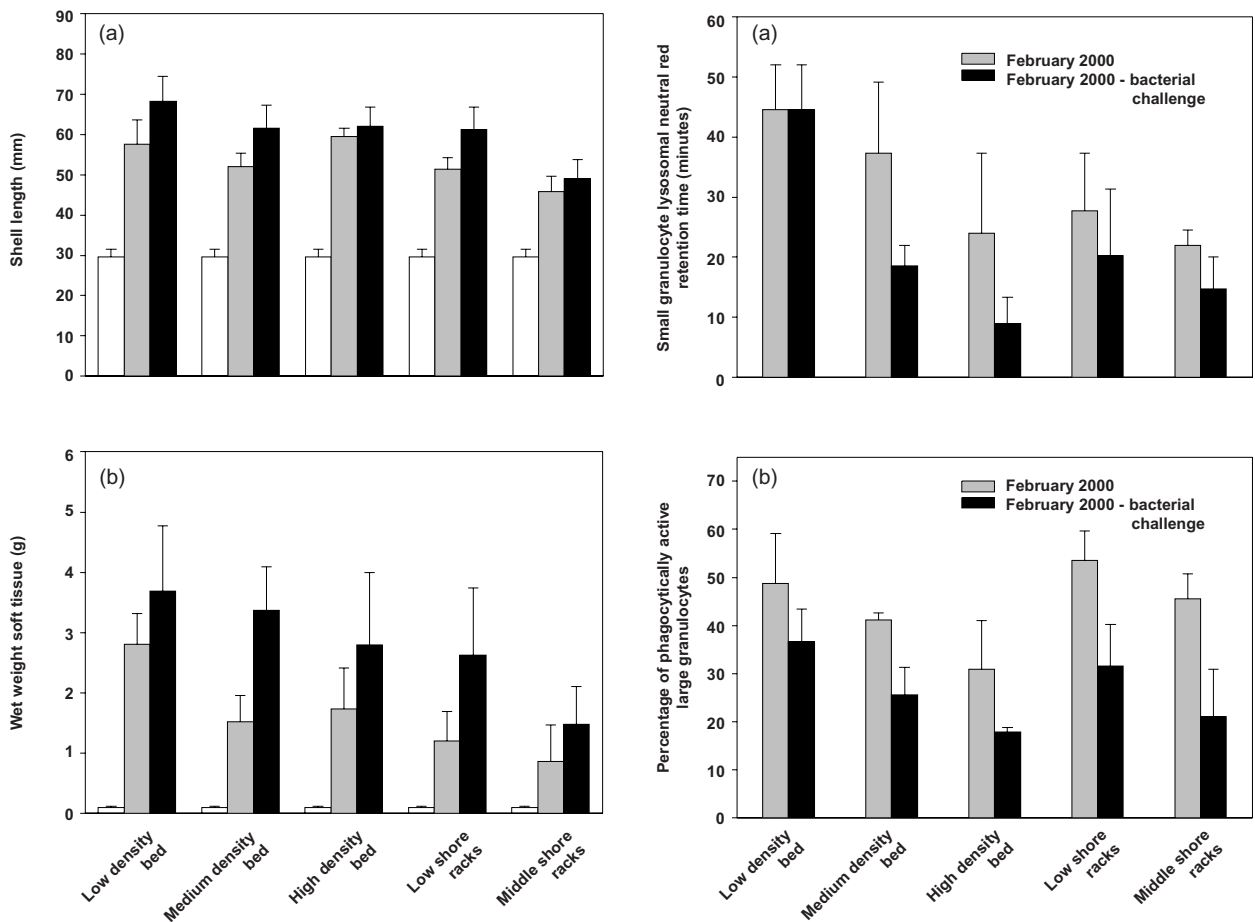
(a) Shell length (mm) and (b) wet weight soft tissue (g) of Ostrea edulis laid subtidally at three densities and in bag and rack culture at mid and low shore. Clear bar = 1998 initial laying, grey bar = February 1999 subsample, black bar = February 2000 subsample.

(a) Small granulocyte lysosomal neutral red retention time (minutes) and (b) percentage of phagocytically active large granulocytes of Ostrea edulis laid subtidally at three densities and in bag and rack culture at mid and low shore.

Figure 21. Field trials together with laboratory analysis of samples have demonstrated the benefits of cultivating native oysters at low intensity on the ground in areas affected by Bonamia.

separate hatchery-reared stocks (Conwy, Wales, and Loch Sween, Scotland), and a natural stock, dredged from the Solent, were laid down on a bed in the River Helford, Cornwall, known to be infected by *Bonamia ostreae*. Oysters from each group were sampled at regular intervals over a period of 18 months in order to investigate and compare seasonal trends in infection.

Results revealed that *Bonamia* infection was slow to develop, but led to mortalities in the Solent oysters. Ultimately all the Solent oysters became infected, compared with 74% for Conwy oysters, and 52% for Loch Sween oysters. Pathological changes in the 3 groups followed the same pattern of severity. Any apparent resistance of the Loch Sween oysters was at least partially attributable to their ability to sustain an effective and active generalised immune response to pathogen challenge. However, it would appear that the different groups of oysters were physiological races rather than genetically distinct populations. The poorer physiological state of the Solent oysters was most likely due to the fact that they are adapted for faster growth and earlier maturity compared with the Scottish stocks. This project should also be seen as an indicator that stocks of *O. edulis*, for whatever reason, can vary in their immune response to *Bonamia* infection.

The aim of project FC1121 was to quantify the efficacy of long-established methods of native flat oyster culture in minimising the incidence of disease, particularly *Bonamia*-related mortality. The approach was based on previous laboratory studies at Southampton University that showed that there is a link between disease susceptibility and physiological stress caused by handling and overcrowding.

Hatchery-produced native oyster spat were laid sub-tidally directly on the bed of the river Blackwater at three densities (10, 20 and 30 oysters per square metre). As an additional comparison spat were also placed in bags on racks on the mid and low shore. Both the traditional laying and the bag culture groups of oysters were sampled every two months and their disease susceptibility, health and growth assessed.

The success of the low intensity approach to the cultivation of native oysters was unambiguous. Growth was quite clearly inversely proportional to laying densities in the sub-tidal groups and in bag and rack culture groups on the mid and low shore, where the degree of tidal exposure markedly affected growth. All groups reached, or were approaching minimum legal sizes in the third year of growth on the ground, and the
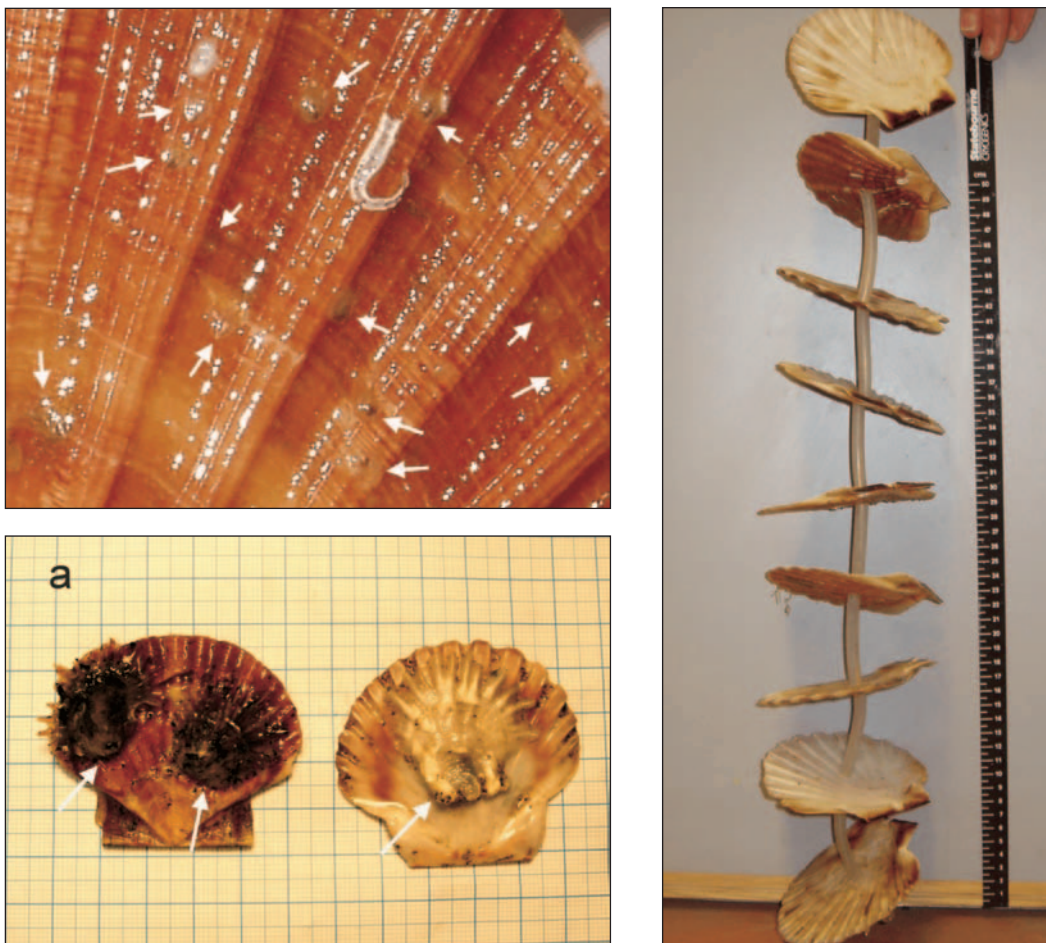

Figure 22. Oysters were successfully settled onto scallop shells. *These subsequently grew well, also showing a strong immune response.*

oysters were of a marketable quality. This is a relatively short growing period for this species. There was no indication of infection by *Bonamia* using histological examination and there has been no evidence of increased disease-related mortality. This is particularly significant, as monitoring of the trial beds continued through the reproductive phase when significant mortality might have been expected. In the previous 10 years this species had not grown well using more intensive culture methods and had not exceeded 32 mm shell size before significant mortalities had intervened.

Greater differences between groups were detected when measures of immunocompetence were used. Whilst this is not an obvious factor when considering the commercial viability of a culture method the ability to minimise losses to disease, it is a prime determinant of commercial viability. At low density the cellular defence mechanisms of the oysters was able to cope with an acute pathogen challenge without difficulty but this capability was diminished by increasing stock densities, and by bag culture combined with tidal exposure.

A further project (FC0926) is in progress in which the effects of post-settlement culture of native oysters on and off a range of cultch materials (broken and suspended scallop shells, cockle shells and weathered Slipper limpet shells) at different oyster densities and in the presence of Slipper limpets also at a range of densities will be evaluated.

Initial results suggest that there seems to be no appreciable differences in survival and growth on the different cultch types used or any apparent preference for surface type i.e. nacreous or outer shell surfaces. It was noted that growth of animals settled on suspended scallop shells was slower than that of animals in stronger flow conditions.

There is some preliminary indication that Slipper limpets can act as a stressor and that oysters settled onto Slipper limpet shell may be at an immunological disadvantage. This work has also again highlighted the advantages of lower densities in respect of lower levels of stress.

5.3 Immune response/ immunocompetency

The specific aims of project FC0504 were to define the baseline physiological, metabolic and immunological responses of two commercially exploited species, *Crassostrea gigas* and *Ostrea edulis*, to environmental variables. In quantifying the responses of these species to natural and anthropogenic stressors emphasis was placed on immunological and disease susceptibility aspects.

Measurement of the responses of whole animals using the scope for growth (SFG) index showed there to be a seasonal switch in the physiology of *C. gigas* at about 15°C which is most pronounced in the salinity range l6-25 ppt. This supports the findings of a similar mechanism in *O. edulis* and the existence of 'winter' and 'summer' physiological states. The optimum conditions for the maintenance of *C. gigas* are at the upper range of temperature (20°C to 25°C) with estuarine salinity conditions (19 - 25 ppt). These conditions are slightly different from those described for *O. edulis*, and reflect the different environmental niche occupied by the Pacific oyster. These findings are supported by results of the adenylate concentrations and adenylate energy charge metabolic indices of stress studies. The stability of indicators of metabolic activity such as pyruvate, lactate and succinate concentrations over a wide range of temperature and salinity conditions show that *C. gigas* can tolerate and to some extent adapt to environmental extremes more readily than *O. edulis*. The lactate:succinate ratio proved to be a simple and reliable index of metabolic stress and clearly showed that 15°C and 25 ppt were the most favoured conditions for *C. gigas*. The metabolic stability of *C. gigas* was also well illustrated by the maintenance of cell volume and production of the haemolymph microcidal agents hydrogen peroxide and lysozyme at high temperatures and low salinities, in conditions that would compromise the immunological activity of *O. edulis*.

The field study of *O. edulis* highlighted the influence of reproductive stress on energy partitioning and immunocompetence. The development of bacterial challenge methods to assess immunocompetence validated those indices that were most efficient in providing information on disease susceptibility and water quality. These were, in particular, cell motility measures and haemolymph hydrogen peroxide and lysozyme concentrations.

Project FC1101 investigated three bivalve species commercially exploited in British water, namely the native flat oyster *Ostrea edulis*, the Pacific oyster *Crassostrea gigas* and the scallop *Pecten maximus*, with respect to the immunological effects of bacterial challenge with the non-specific pathogen *Listonella* (*Vibrio*) *anguillarum*. The host-pathogen interactions were studied by quantification of host immunocompetence after acclimation to a matrix of temperature and/or salinity conditions representing the normal ranges experienced in British coastal waters and estuaries.

The suite of assay methods included differential haemocyte counts, haemocyte size, motility, phagocytic capability and the novel application of the neutral red retention assay to haemocytes to quantify lysosomal

stability and function. Material from these assays was also examined histologically. In addition to the main set of assays an additional set of preliminary experiments were introduced to test if the cellular immunity of *O. edulis* and *P. maximus* could be enhanced by stimulation with very low levels of pathogen or cell-free homogenates of healthy animals of the same species, the latter stimulant was included to test if there was inter-cellular signaling.

The interactions between the two ostreid species and *L. anguillarum* clearly show that the maintenance of animals at less than full-strength seawater (28-32 ppt) reduces the growth of the bacterial pathogen without seriously affecting the immunocompetence and general well-being of the bivalve hosts.

The study also produced the first comprehensive and quantitative set of data describing the environmental modulation of the main component of the immune system of *P. maximus*. These data have revealed that this species switches between 'winter' and 'summer' physiological states, similar to those observed in ostreids. The identification of this switching has important implications for current commercial practices as it was found that a cold shock from moving rapidly from the 'summer' to the 'winter' state caused major mortality, showing that excessive cooling in transit should be avoided as much as overheating.

The *P. maximus* studies also provide, for the first time in an invertebrate, results that are entirely consistent with Matzinger's 'Danger Hypothesis' for vertebrate immune systems. This hypothesis considers the cellular components of an immune system to be a resting or quiescent state until stimulated into activity by intercellular signals released by cells damaged by injury or initial contact with a pathogen.

The neutral red retention assay method was successfully applied to bivalve haemocytes for the first time, although its use as a specific tool to investigate bacterial infection seems to be limited. Its potential as an immunological indicator lies in the detection of hydrocarbon contamination and consequent damage to haemocytes.

5.4   Uptake of the results on stress and disease

Overall, the husbandry work described here has provided, for the first time, quantitative information that is needed to inform any management strategy seeking to balance the opposing requirements of maximising yield against greater product quality and minimising losses to disease.

It has been shown that native oysters will grow vigorously with minimal mortality when cultivated at low intensity on the ground. The success of these traditional methods, as opposed to the contemporary high intensity cultivation practices used for the Pacific oyster, during the experimental trial was sufficient to encourage the industry participant to consider investment in full-scale commercial implementation at a similar site. Indications are that if sufficient quantities of good quality seed can be produced in hatcheries, the production of native oysters could be significantly increased. It should also be noted that this study has also verified the anecdotal view that native oysters do not grow well in bag culture.

It follows that the application of husbandry methods that reduce stressors may make it possible to grow flat oysters again in British waters on a commercial scale. Work is still ongoing with the ultimate goal of validating husbandry methods that can be used to encourage the re-emergence of the native oyster industry, so that presently derelict lays can be brought back into use and provide a viable source of income for a greater number of fishermen and growers. One of the fundamentally important aspects of this work was the close collaboration between the industrial partners and the research team. The considerable experience and enthusiasm of the industrial partners guided the experimental work throughout the course of the project and ensured that the results will be of practical use to the industry.

The fact that oyster spat will settle on all types of shell surfaces with good subsequent survival is a simple and very clear result that will be of great use for the future. Some types of shell, which are known to attract oyster larvae and to work well as collectors, such as cockle and mussel shell are unlikely to remain in place in exposed sites such as the Solent oyster grounds. These results show that scallop shell can be used with equal effectiveness, as this is more likely to remain in place.

Project FC0504 defined the baseline ecophysiological responses of two commercially exploited oyster species. Ultimately, these data can be used in a predictive mode to assess either the suitability of new sites for cultivation or the effects of environmental change in existing habitats. The characterisation of responses to natural environmental variability is also an indispensable pre-requisite for future studies of the effects of anthropogenic stressors. The application of biochemical indices to quantify metabolic changes associated with external physical and chemical conditions has not only quantified stress responses at the biochemical level of organization but has also defined the internal milieu that modulates immunological responses.

The most important discovery of study FC1101 has been the insights provided by the series of immunostimulation experiments. The results obtained using *P. maximus* have important implications for the current understanding of the nature of immune systems

and the linkage between invertebrate and vertebrate systems, as well as practical aquacultural applications. The practical benefit of the observations on the effect of salinity will be in the management of hatcheries. Where vibriosis is a major threat it could be partly mitigated without recourse to costly water treatment or undesirable usage of antibiotics by the simple expedient of maintaining slightly reduced salinity conditions.

5.5 Publications arising from this research

HAUTON, C., HAWKINS, L.E. AND HUTCHINSON, S., 1998. The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). Comp. Biochem. Physiol. B 119(4): 619-623.

HAUTON, C., HAWKINS, L.E. AND HUTCHINSON, S., 2001. Response of haemocyte lysosomes to bacterial inoculation in the oysters *Ostrea edulis* L. and *Crassostrea gigas* (Thunberg) and the scallop *Pecten maximus* (L.) Fish Shell. Immunol. 11: 143-153.

HAUTON, C., HAWKINS, L.E. AND HUTCHINSON, S., 2000. The effects of salinity on the interaction between a pathogen (*Listonella anguillarum*) and components of a host (*Ostrea edulis*) immune system. Comp. Biochem. Physiol. B 127: 203-212.

HAWKINS, L.E., HUTCHINSON, S., BATTEN, S.D., PATERSON, G.L.J., LAMBSHEAD, P.J.D. AND RICE, A.L, 1992. A method for the enzymatic determination of glycogen in chemically preserved material. Limnol. Oceanogr. 37(8): 1784-1786.

HUTCHINSON, S. AND HAWKINS, L.E., 1992. Quantification of the physiological responses of the European flat oyster *Ostrea edulis* L. to temperature and salinity. J. Molluscan Studies 58: 215-226.

PART II - WATER QUALITY

"The general committee of the oyster trade…demanded nothing less than immediate legislation to prohibit altogether the discharge of sewage into rivers, estuaries or even the sea itself"

The Times, 6 January 1903

AN HISTORICAL PERSPECTIVE

In the early 1900s, many of the sewers and drains that emptied onto the foreshore in Emsworth Harbour, Hampshire were re-laid by the local council. Oyster farming had become established in nearby Langstone Harbour since 1819 and by 1900 there were many oyster fishermen in Emsworth, all of who owned their own beds (or ponds, as they were known). One of the oystermen constructed a number of new ponds close to the new outflow and seeded them with a considerable quantity of young oysters. In November 1902 these oysters were served at a local mayoral banquet. Not surprisingly, some of the shellfish had been contaminated by the foreshore outflow, and several of the diners, including the Dean of Winchester, died of typhoid. The shellfish industry collapsed almost overnight.

Various measures were taken to address this problem and some early investigations into removal of faecal contamination from shellfish were carried out. In 1913 the local Corporation in Conwy, North Wales, constructed the first mussel purification tanks. Mussels from the estuary were brought in by fishermen before marketing and, for a small fee, were purified of contamination by sewage bacteria. The initial method adopted proved to be unreliable in operation and, in 1918, the tanks were taken over by the then Board of Agriculture and Fisheries.

Research was started to develop a more effective method for mussel and oyster purification, and this work was taken forward during the 1920s and 1930s. Concurrently, surveys on the degree of sewage pollution of shellfish beds, and the development of reliable methods of bacteriological analysis of commercial shellfish were carried out. By 1928 the Conwy purification tanks were operating efficiently. These mussel purification tanks were used each winter (September - April) for 80 years, until the 1970s, when closure was enforced by new EC hygiene regulations. Today, harvested shellfish are purified in modern, purpose-built plant both here and throughout the UK, in line with standards laid down in EU legislation.

Figure 23. Bagging up purified mussels in the Conwy tanks.

Under certain conditions some species of unicellular algae can produce compounds that may be toxic to humans. Bivalve molluscs feeding on the algae may accumulate these toxic compounds to relatively high concentrations. The compounds may be passed further up the marine food chain to crustacea, etc., feeding on the bivalves. These other food animals may therefore also present a potential risk to human health. The toxins are generally very stable to heat and other processing operations.

The toxins that cause Paralytic Shellfish Poisoning (PSP) are known to have been present in shellfish on either side of the English-Scottish border on the east coast for several centuries. The major outbreak of PSP in 1968 extended from the estuaries of the Humber to the Forth and seriously affected 85 people who had eaten infected mussels. Thereafter, east coast shellfish were regularly tested for the presence of PSP, using a bioassay in which extracts of shellfish tissue were injected into mice. Potentially harmful levels of poison were detected in most years but exceptionally high shellfish toxicities were not detected again until 1990. Systematic monitoring for Diarrhetic Shellfish Poisoning (DSP), which was perceived to be much less of a problem, did not start until the early 1990s. A monitoring programme for the algae responsible for the presence of these toxins in shellfish, to comply with requirements in the EU Shellfish Hygiene Directive (91/492/EEC), started in 1995.
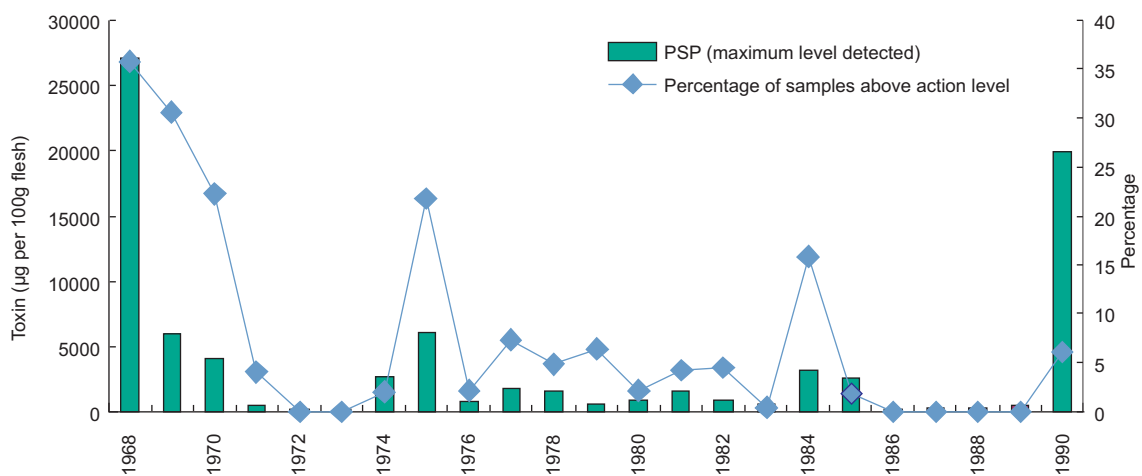

Figure 24. A summary of results from the PSP toxin monitoring programme of mussels sampled from the east coast of Northern England and Southern Scotland from 1968 to 1990.

6. BACTERIOLOGICAL CONTAMINANTS

The projects addressed in this section are:

FC0714 Depuration techniques for oysters and clams (Seafish)

FC0732 Re-appraisal of existing operating criteria for purification of bivalve molluscs in the UK (Seafish)

FC1005 An assessment of the impact of different types of sewage treatment on the contamination of shell fisheries (CEFAS)

FC1013 Requirements for depuration of scallops (*Pecten maximus*) (CEFAS)

6.1 Background

Classification of shellfish harvesting waters

*Filter-feeding bivalve molluscs such as oysters, clams, mussels and cockles can concentrate microorganisms, including pathogens, from contaminated waters. Consumption of shellfish contaminated by human sewage may then result in illness such as viral gastro-enteritis or infectious hepatitis. Under the EC Shellfish Hygiene Directive (91/492/EEC) controls are placed on the commercial harvesting of shellfish from such areas in order to reduce the risk of infection. Shellfish beds must be classified according to the faecal coliform (or Escherichia coli) levels of the bivalve flesh. The classification category then dictates what, if any, action must be taken before the bivalves are sold for human consumption. Bivalves harvested from **Grade A** areas (all samples less than 230 E. coli per 100g flesh) may be marketed live without further treatment, those from **Grade B** areas (less than 4,600 E. coli per 100g flesh in 90% of samples) require depuration (purification) or relaying, while those from **Grade C** areas (all samples less than 60,000 E. coli per 100g flesh) must be re-laid for several months in areas of A or B classification to allow time for them to reach an acceptable bacterial standard before further treatment or marketing. For shellfish sold as a processed product heat treatment (cooking) by an approved process is permitted for bivalves harvested from both Grade B and Grade C areas. Bivalves in areas classified as **Prohibited** (above 60,000 E. coli per 100g flesh) cannot be harvested under any circumstances.*

Improved knowledge of depuration requirements and an increasing range of species being traded commercially and requiring depuration led to a re-appraisal of the current recommendations (FC0714) for seawater dissolved oxygen and temperature in depuration systems. This was carried forward under project FC0732.

For scallops, there was no information previously available on depuration, and other MAFF/Defra funded studies (FC0202) had shown that cultivated scallops might accumulate levels of contamination above those that would allow the marketing of live animals without depuration (end-product standard of 230 *E.coli*/100g of shellfish flesh; EU Directive 91/492). It would therefore not have been possible to set Conditions of Approval (CoA) for this process. This was a significant constraint on development of the emerging scallop cultivation industry and so project FC1013 was designed to address this problem.

Reduction of contamination of shellfish harvesting areas from sewage by improved treatment should result in a reduction of the risk of illness and should also increase the commercial potential of these areas. However, predicting the effect of change in sewage treatment type on the extent of contamination of nearby shellfisheries is more difficult than predicting the effect on water quality. Project FC1005 was undertaken to assess the impact of different types of sewage treatment on the hygiene status of shellfisheries using monitoring information collected over a two year period.

6.2. Depuration

The main types of purification system currently in use in the UK are shallow tank, multi-layer tank, vertical stack and bulk bin systems. With the exception of the bulk bin system, which is designed to accommodate a deep layer of mussels, these systems should be suitable for all species of bivalve mollusc. In practice,

Figure 25. A vertical stack depuration system. *The molluscs are held in purpose designed, solid sided containers which are either directly stacked or supported above one another in a frame over a sump tank. Seawater is drawn from the sump up to one end of the top container of the stack. It passes along the container and then cascades down from the other end into the container below and so on until it returns eventually to the sump.*

however, the shallow tank, multi-layer and bulk bin systems are readily scaled up to suit the needs of high volume mussel fisheries, whereas the generally smaller vertical stack systems tend to be used for lower volume oyster and clam fisheries and particularly where there is a need for the purification system to serve also for immersed storage after purification. With the assistance of Seafish, multi-layer systems (medium or large size), the bulk bin system, the improved vertical stack system and a small shallow tank system have been developed as standard designs. These systems have been extensively tested in a wide range of conditions. They are manufactured to a specification and can be purchased ready-made as a complete package of tank, UV and plumbing.

A series of trials were undertaken over an 18-month period to determine optimum seawater temperatures and dissolved oxygen levels for mussels (*Mytilus edulis*), Pacific oysters (*Crassostrea gigas*) and native oysters (*Ostrea edulis*), cockles (*Cerastoderma edulis*) and Manila clams (*Tapes philippinarum*), all species that are depurated commercially.

In carrying this out, alternative techniques to the more traditional use of bacteriological analysis were used to establish the physiological response of bivalve molluscs to varying conditions. These techniques were the monitoring of ammonia excretion, consumption of dissolved oxygen and uptake of a neutral red dye.

The activity of the molluscs was found to vary greatly with both seawater temperature and dissolved oxygen level, with each species of mollusc responding differently. The activity of the molluscs increased with the level of dissolved oxygen up to full saturation. The minimum level for effective functioning of the molluscs varies with species and it was recommended that for Pacific and native oysters and Manila clams that consideration could be given to increasing it to say 75% saturation. The activity of the molluscs increased with seawater temperature but with evidence of optimum temperatures of 15°C for Native Oysters and 17-18°C for Manila clams. There is a substantial gain to be made through operating at these higher temperatures rather than at the minimum temperatures specified for purification. There may also be scope for operating at somewhat higher temperatures than those currently recommended but practical problems, particularly oxygen depletion, would be encountered in many purification systems. Oysters were found to have a very low rate of activity in comparison to other species and this raised the question of whether the standard 42-hour purification period is adequate.

For king scallops (*Pecten maximus*), controlled depuration experiments were carried out on contaminated diver-collected market sized cultivated stock at the CEFAS Weymouth laboratory, using UV-treated water. Initial experiments demonstrated that it was possible to depurate scallops using standard methodology.

Further experiments evaluated the environmental conditions necessary for the successful depuration of scallops. Following these experiments, recommendations for depuration conditions were that:

- Only natural seawater may be used.

- Salinity must be maintained at 30 psu or above.

- Temperature must be maintained at 10°C or above.

- Purification must commence within 10 hours of harvesting.

- Scallops may be loaded up to a maximum of 2 layers high with the cup side shell down.

- Scallops must be prevented from escaping from baskets during depuration. Any method used to contain shellfish in baskets must not interfere with their ability to open and filter.

- Scallop to water ratios should not be any less than 1:12.

A laboratory scale depuration system with a tray of scallops.

Increased scallop loading causes them to escape onto the base of the tank.

The E. coli analysis is being carried out showing the most-probable number (MPN) method.

Figure 26. Scallop depuration experiments. *Information from these experiments has allowed Conditions of Approval to be set for this species.*

6.3 Impact of sewage treatment

Project FC1005 involved the manipulation and statistical analysis of *Escherichia coli* data in conjunction with the evaluation of information on sewage discharges. The manipulation of data was undertaken using a Geographical Information System (GIS) (Arcview 2.1, ESRI Inc). A comparison was made of the effect of crude, primary, secondary and tertiary treated discharges on the *E.coli* concentrations at routine monitoring points within 2 kilometres of discharges of each type. The geometric mean *E.coli* concentrations were obtained for each set of monitoring point data and were used for evaluation of the effect of the discharges.

The results obtained from linear multiple regression analyses show that a significant proportion of the variation in average *E.coli* concentrations in shellfish is explained by size of discharge and discharge-monitoring point distance. The remainder of the variation may be due to such factors as average concentration of *E.coli* in the discharge, influence of other sources of contamination and local hydrographic,

bathymetric and topographic effects. Many of these effects will be local in nature and may exhibit limited variability. The results from some area-specific studies supported this contention.
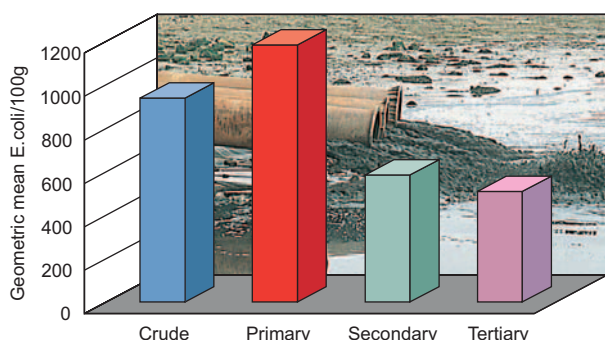

Figure 27. *Effect of sewage treatment level on the E. coli content of bivalve molluscs in the vicinity of the discharge.*

Concentrations of *E.coli* in shellfish taken from monitoring points in the vicinity of secondary-treated discharges were markedly lower than in those from monitoring points in the vicinity of crude or primary-treated discharges. The *E.coli* concentrations in shellfish from points in the vicinity of primary discharges were slightly higher than in those from points in the vicinity of crude discharges. There was little apparent beneficial effect of tertiary treatment for shellfisheries, possibly because of the impact of other sources of pollution. A number of laboratory experiments and studies on classification data have shown that *E.coli* is concentrated to different extents by the different species of shellfish, and this was evident in the analysis using GIS, with mussels being generally more contaminated than oysters, which in turn were more contaminated than clams.

6.4 Uptake of the results on bacteriological contaminants

For the maximum protection of public health it is important to optimise the depuration process to ensure that the greatest possible removal of biological contaminants is achieved. For this, Conditions of Approval are set that highlight the areas essential for control. Work undertaken by Seafish, such as that conducted in Projects FC0714 and FC0732, have enabled appropriate Conditions to be established. Seafish has also developed additional features of the process, concerning general good practice, and further information is available (see publications list) on this, including species-specific manuals on the operation of purification systems. Based on the experience of these studies more recent work, funded by the Food Standards Agency, has established the conditions for depuration of cockles and razor shells.

The CEFAS work on depuration of scallops (FC1013) has allowed recommended Conditions of Approval for depuration systems to be set for this species. This will be of great benefit to any continued expansion of the scallop cultivation industry in the UK.

Project FC1005 demonstrated the value of using a GIS in a relatively simplistic approach to the assessment of the impact of sewage discharges on the extent of contamination of shellfisheries. The models obtained in the study will contribute to the practical assessment of schemes and will provide the basis for the development of further improved models intended to predict more closely the impact of proposed sewage discharge schemes. It was realised that these would need to include the effects of multiple sources of pollution and variation due to the presence of different shellfish species.

6.5 Publications arising from this research

BOULTER, M., 1993. Summary Report into Oyster and Clam Purification Criteria. Seafish publication SR427.

BOULTER, M., 1994. The Development of a Bulk Bin, Deep Layer, Down Welling, Mussel Purification System. Seafish publication SR456.

BOULTER, M. AND DENTON, J.W., 1991. Development of a Small Scale Vertical Stack Oyster Purification Plant with Repeated Re-Use of Artificial Seawater. Seafish publication SR459.

BOULTER, M. AND WILSON, P., 1994. An Assessment of Optimum Environmental Criteria for the Successful Depuration of *Ostrea edulis*, *Mercenaria mercenaria*, *Tapes decussatus*, *Tapes philippinarum*, *Cerastoderma edule* and *Mytilus edulis* in the UK. Seafish publication SR510.

BOULTER, M. AND WILSON, P., 1998. The use of Physiological Assessment Techniques for determining the relative activity rates of bivalve shellfish during simulated depuration. Seafish publication SR520.

BOULTER, M. AND WILSON, P., 1998. Ultra Violet Light Sterilization of Seawater with Reference to Shellfish Depuration Tanks. Seafish publication SR511

DORÉ, W.J., FARTHING, J. AND LAING, I., 2003. Depuration conditions for great scallops (*Pecten maximus*). Journal of Shellfish Research 22: 409-414.

LAING, I., 2002. Scallop cultivation in the UK: a guide to site selection. CEFAS, Lowestoft, 26 pp

LART, W., WOOD, P., DENTON, J.W. AND WILSON, P., 1994. Biological Criteria for the Depuration of Pacific Oyster. Seafish publication SR433.

LEE R.J. AND GLOVER R.J.O., 1998. Evaluation of the impact of different sewage traetment processes on shellfishery pollution using a Geographic Information System (GIS). Wat. Sci.Tech. 38: 12.

WILSON, P., 1998. Mussel Purification - Repeated Re-use of Artificial Seawater in the Purification Process. Seafish publication SR339.

Seafish have also published further advisory documents including:

Guidelines
Guidance on Procedures to Minimise Risks to Food Safety in Bivalve Mollusc Purification.

Guidelines for the Facilities and Equipment Required for Handling Bivalve Molluscs from Harvesting through to Distribution to Retail Outlets.

Purification System Operating Manuals
Operating Manual for the Medium Scale Multi-Layer System (95/31/FT).

Operating Manual for the Vertical Stack System (95/32/FT).

Operating Manual for the Large Scale Multi-Layer System (95/33/FT).

Operating Manual for the Small Scale Shallow Tank System (95/34/FT).

Operating Manual for the Bulk Bin System for Mussels (95/35/FT).

General Operating Manual for Purification Systems of Non-Standard Design (95/36/FT).

Data Sheet
The Use of Artificial Seawater in Mollusc Purification (1994/25/FT).

7. VIRAL CONTAMINATION

The projects addressed in this section are:

FC0703   Studies of viruses in shellfish in relation to public health (CEFAS)

FC0706   Use of coliphage to assess the influences of re-laying and depuration on contamination of molluscan shellfish with viruses (PHLS)

FC0717   A study to evaluate the feasibility of using coliphage as hygienic indicators in molluscan shellfish (PHLS)

FC0727   Role and fate of micro-organisms in bivalve molluscs with reference to bacteria and viruses (PML)

FC0729   Studies on application of enterovirus RT-PCR to environmentally-contaminated shellfish and shellfish-growing waters (University of Surrey)

FC0733   Studies on the removal of human pathogenic viruses from molluscan shellfish during depuration (University of Surrey)

FC1009   Feasibility study for methods to distinguish between human and animal faecal contamination in shellfisheries (CEFAS)

FC0738   Detection and removal of human viral pathogens in bivalve shellfish (CEFAS)

FC1134   Provision of sandwich students: Shellfish quality problems caused by organic waste pollution of harvesting areas (University of Surrey)

7.1 Background

Infectious human disease acquired from the consumption of bivalve molluscan shellfish (such as oysters and mussels) is an internationally recognised problem and occurs because of the filter-feeding nature of these species. Human pathogens derived from sewage contamination of their growing waters are concentrated and retained. In the EU, Directive 91/492 stipulates legal controls that specify sanitary standards reliant on the traditional bacterial indicators of faecal contamination (*E.coli* and the faecal coliforms). However, in Europe and elsewhere shellfish associated outbreaks are predominantly caused by virus infections. Norovirus (NV) previously known as Norwalk-like viruses (NLV), predominate with a smaller proportion of cases caused by Hepatitis A virus (HAV). Unfortunately the specified sanitary controls for shellfish, based on bacterial faecal pollution indicators, do not perform well for viruses. Thus many outbreaks of infectious viral disease have been associated with shellfish fully compliant with the legal bacteriological standards.

To address these problems, Defra commissioned a programme of research in this area, concentrating on: the development of methods for detection of enteric viruses in shellfish; the evaluation of potential alternative faecal indicator organisms; and the improvement of commercial purification (depuration) procedures to better eliminate enteric viruses. Responsibility for this research was transferred to the

Food Standards Agency with the formation of this body in April 2000.

Defra has continued to support research in this area through contract FC1134. This has provided, over the period of the last five years, eight sandwich course students from the University of Surrey who have contributed to the development of methods for the detection and enumeration of pathogenic viruses and indicator organisms in shellfish, with some additional work on the removal of these during depuration.

The current *E.coli*/faecal coliforms indicators do not distinguish between human and animal sources of pollution. Consequently a shellfishery may receive a detrimental classification when impacted by agricultural run-off, even in the absence of human faecal contamination. Such shellfish are generally considered less likely to pose a health risk than shellfish that are contaminated by human sewage. Clearly a method for distinguishing between human and animal pollution would be of benefit in such circumstances both for assessing the potential health risk and for the development of appropriate management strategies for controlling contamination. This issue was addressed in Project FC1009.

7.2 Identification

Usually, phages in water or shellfish samples are enumerated by counting the number of plaques produced in a lawn of an *E.coli* strain. Both somatic
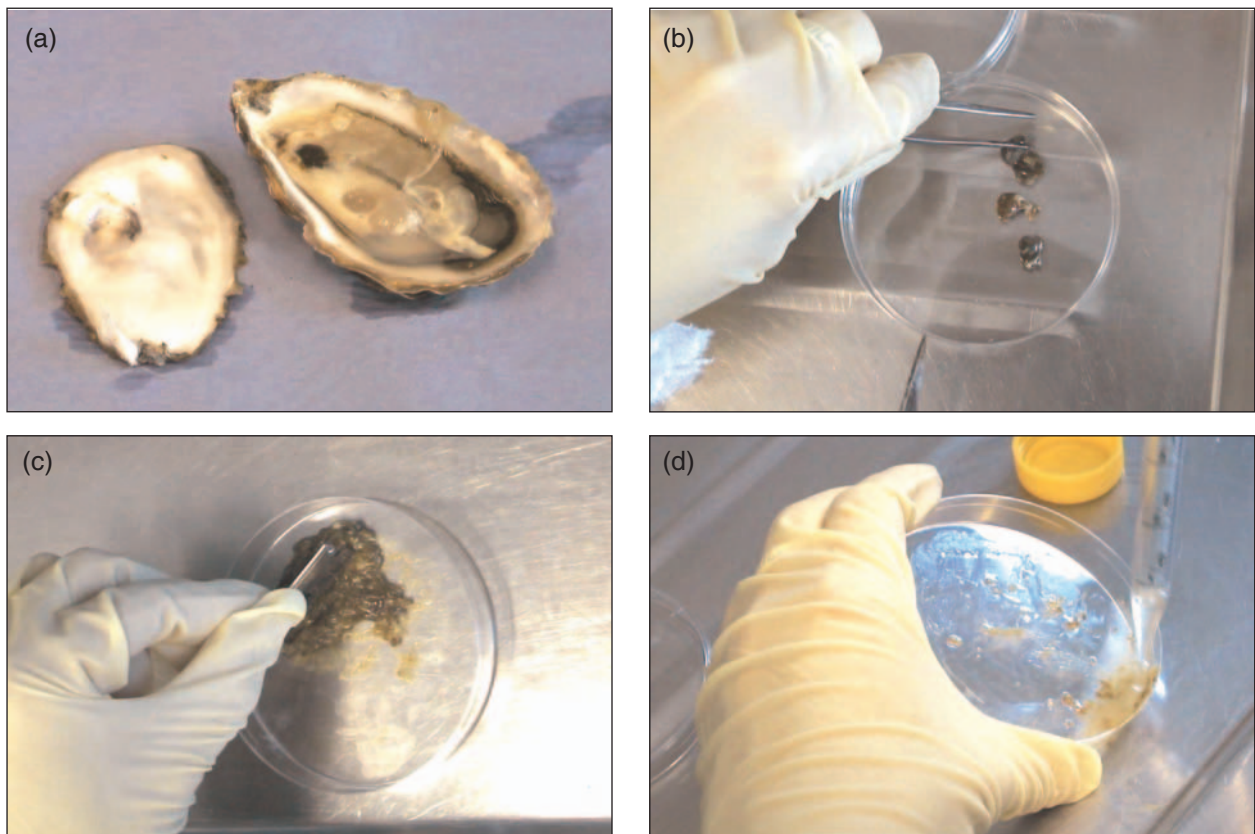
Figure 28. Extracting oyster tissue for virus detection. *The hepatopancreas or digestive gland (a) is the major site of virus concentration in oysters and is used as the tissue of choice for virus detection. The hepatopancreas is dissected from the other tissues of the oyster (b). It is then finely chopped with a razor blade (c) and diluted in peptone water (d) before centrifugation.*

and F+ bacteriophages will contribute to the result. There is also a highly selective method with which the counts originate only from F+ bacteriophages. Conventional methods for the isolation of enterovirus rely on time consuming and cumbersome methods such as tissue culture. These methods are not rapid and only 65% to 70% of different viral serotypes will show cytopathic effects in normal cell lines. Importantly enteric pathogens such as the NVs and Hepatitis A virus are either non-culturable or difficult to culture. Both of these viruses are important causes of human infections through shellfish consumption. Polymerase Chain Reaction (PCR) technique provides the sensitivity requested for the identification of low amounts of viral nucleic acid.

Initially, a reverse transcription (RT-) polymerase chain reaction (PCR) method for the detection and quantification of small amounts of human enteric virus within shellfish was developed. As shellfish had proved to be an inhibitory material for RT-PCR, whether these shellfish are environmentally contaminated or laboratory contaminated, it was necessary to establish a sensitive and specific RT-PCR method. A method used for shellfish processing and nucleic acid extraction was developed to overcome these inhibitory effects.
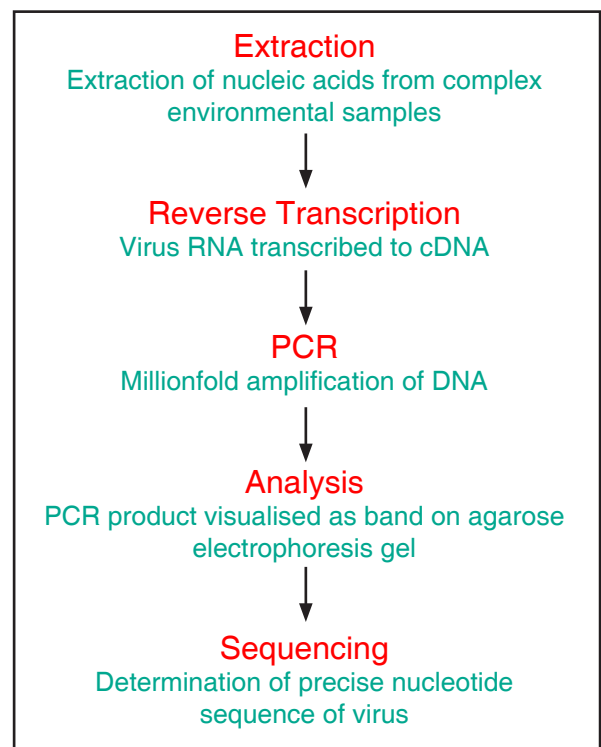

Extraction
Extraction of nucleic acids from complex environmental samples

↓

Reverse Transcription
Virus RNA transcribed to cDNA

↓

PCR
Millionfold amplification of DNA

↓

Analysis
PCR product visualised as band on agarose electrophoresis gel

↓

Sequencing
Determination of precise nucleotide sequence of virus

Figure 29. The steps in the method for identifying viruses by RT-PCR.

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However, amplification of the target DNA in this study exhibited some artifact bands in gel analysis suggesting the presence of undesired non-specific products. The specificity of the reaction was increased and non-specific bands largely eliminated by the use of an alternative polymerase enzyme. This work allowed RT-PCR based methods to be successfully applied to the detection of enteroviruses (EVs), Norovirus and Hepatitis A virus in shellfish extracts. This represented the first ever demonstration of the detection in shellfish of the Small Round Structured Viruses (SRSVs) and related viruses causing human gastro-enteritis.

Figure 31. *Viral pathogen detection using molecular biology techniques (PCR).*

Figure 30. *Isan automatic thermal cycler used for Polymerase Chain Reaction PCR - for amplification of DNA.*

The PCR technique cannot adequately quantify viral genome. This is problematical for epidemiological studies and for studies on virus behaviour during shellfish treatment processes. To address these problems, the improved enterovirus reaction conditions were further developed for quantifying enterovirus. A competitive nested RT- PCR procedure for the detection and quantification of enterovirus in shellfish was developed. The method relies on the use of a competitive RNA standard, differing from viral genome by an internal 20 base pair deletion that is co-amplified with the target RNA. The internal standard was developed by cloning restriction-deleted fragments of the *5' non-translated region of the poliovirus genome into a baculovirus transfer vector. RNA was then extracted from recombinant baculovirus infected *Spodoptera* cells. The competitive standard was seeded into shellfish samples following extraction and nucleic acid purification and prior to nested RT-PCR. Secondary nested primer sets were fluorescently labelled for accurate quantification of products. Seeding experiments showed the method to be both sensitive and reproducible for the determination of enterovirus RNA in oysters.
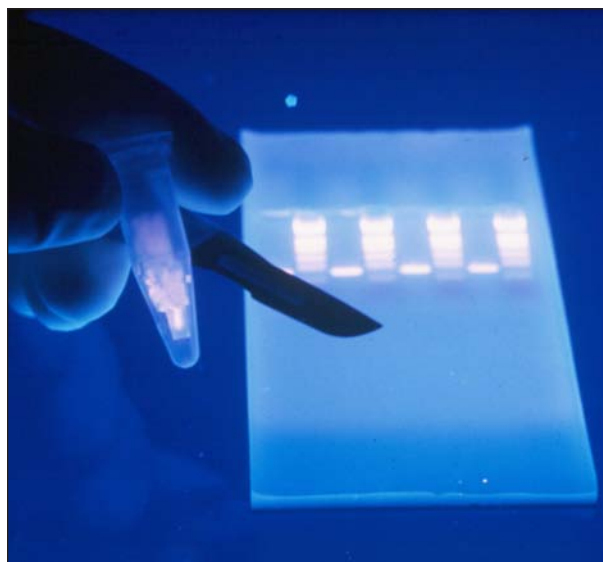
These newly developed PCR methods were used to directly assess the removal rates, during depuration, for the potential virus indicator (F+ bacteriophage), and for the standard bacterial indicator (*E.coli*). Removal rates of other potential virus indicators, such as enterovirus and somatic bacteriophage, were also investigated to allow comparison with the findings of other workers (see Section 7.4.1).

Furthermore, Southern Blotting was successfully applied after PCR to increase sensitivity and specificity of detection of enteroviruses from bivalve molluscan shellfish. Southern blotting following PCR was used to assay a range of environmental and commercially depurated shellfish (both winter and summer) to assess the prevalence of enteroviruses. Enterovirus was detected in 92% of environmental, 72% of winter depurated and 28% of summer depurated shellfish. These results were compared with other indicators of sewage pollution (*E.coli* and F+ bacteriophage).

Following the success of a single round RT-PCR for the detection of Norovirus in environmental shellfish, the sensitivity was further increased by the development of a broadly reactive nested RT-PCR. As the newly developed nested RT-PCR was found to be sensitive and specific for detection of both NV Genogroups in shellfish this facilitated sequencing of positive amplicons enabling the confirmation of RT-PCR results and the strain typing of the NLVs detected. Some problems were experienced with direct sequencing, and so protocols were established for cloning NLV positive amplicons prior to sequencing. These methods performed well for characterisation of mixed NLV strains contaminating shellfish, something that was found to be a common occurrence.

This work has thus resulted in the successful development of cloning and sequencing methods for detection and characterisation of NLVs in shellfish. The ability to sequence virus is a powerful tool for use in molecular epidemiological investigation of shellfish associated outbreaks. Recent research has focussed on the development of a quantitive TaqMan assay. TaqMan chemistry is very well standardised and has significant advantages of speed, robustness, and labour costs. Significantly it incorporates a probe confirmation step into a one-tube single stage assay. This is a major advantage over current approaches for result reliability. Real-time PCR is also capable of providing quantitative data which may prove significant for the interpretation of field data for NVs in shellfish. To date the TaqMan assay has been used in depuration studies, and on the removal of pathogens during sewage treatment.

7.3 Indicators

7.3.1 Evaluation of candidates

It is now well established that shellfish associated with outbreaks of NLV gastro-enteritis are normally fully compliant with the *E.coli* standards required by Directive 91/492. In the UK a major factor influencing this is the relative ease of removal of *E.coli* during commercial processing (depuration) compared with the difficulty of eliminating enteric viruses. A major factor may be the significantly different size, genetic composition, and resistance to environmental degradation, of *E.coli* (a bacterial indicator) compared to enteric viruses. FRNA bacteriophage has been proposed as an alternative indicator organism that is much more similar in size and properties to enteric viruses than is *E.coli*. Studies on the potential of FRNA bacteriophage, as a candidate 'viral' indicator, in commercial shellfish harvesting areas and in depurated shellfish sold ready for consumption have therefore been funded.

Studies on the distribution of FRNA bacteriophage showed that this organism was widely prevalent in commercial shellfish harvesting areas in the UK with average levels approximately 3 fold greater than *E. coli*. Interestingly a strong seasonal effect was noted with levels of FRNA bacteriophage particularly elevated during winter months. This effect was not seen for *E.coli*. It is interesting to note that elevated levels of FRNA bacteriophage during the winter months concur with the period of higher risk for shellfish associated viral gastro-enteritis. It is possible that shellfish related biological factors could similarly contribute both to the observed higher levels of FRNA bacteriophage contaminant concentrations, and retention of viral pathogens, during the winter months. Significantly, FRNA bacteriophage in shellfish from Category B sites was regularly detected at levels that would not be easily removed by conventional depuration, the usual treatment used for such shellfish.

Figure 32. FRNA Bacteriophage sampling.

In a two-year study FRNA bacteriophage content in market ready oysters was compared with both *E. coli* and NLV contamination, with the pollution status of each site, and with the officially reported gastroenteric illness incidents associated with products from each site. The inadequacy of *E.coli* was confirmed during this study with all samples meeting the EU end-product standard of <230 *E.coli* per 100 g shellfish despite oysters from two of the sites being implicated in outbreaks of viral gastro-enteritis. By comparison the frequency and degree of FRNA bacteriophage contamination was highly associated with the consumer health risk from enteric viruses as judged by both the degree of harvesting area pollution, the NLV content of shellfish and the association of products with reported gastroenteric illness incidents. Furthermore detection of FRNA bacteriophage in marketed oysters showed a clear seasonal trend with levels markedly elevated during winter months. This is consistent with oyster-associated gastro-enteritis in the UK, which occurs predominantly during winter months. Thus FRNA bacteriophage content in market ready oysters reflected the high-risk period for contamination with enteric viruses further suggesting their possible utility as a 'viral' indicator.

It should however be noted that, in addition to FRNA bacteriophage, other alternative viral indicators have been proposed. These include bacteriophage of the obligate anaerobe *Bacteroides fragilis*, somatic bacteriophage, faecal streptococci, human adenovirus, and human enteroviruses. The results of further

investigation into these alternative indicators, funded by the FSA, have confirmed that FRNA bacteriophage shows the most promise for routine applications. In practice, monitoring for both *E.coli* and FRNA bacteriophage in shellfish can be more informative than either indicator alone. High levels of FRNA bacteriophage, particularly in depurated shellfish, serve as a warning sign that the products are susceptible to viral contamination.

Little was known, however, about the epidemiology of the phages and some work was devoted to the examination of sewage effluent, faecal and environmental samples in an attempt to better understand the impact of sewage treatment and the behaviour of phage in the sewage effluent. Samples obtained both before and after treatment were examined for phage before and after storage. The results demonstrated that both F + and somatic phage are present in the vast majority of raw and treated sewage samples. Full treatment significantly (>90%) reduced phage levels but did not eliminate the particles. It was not possible to definitively identify the source of the phages in sewage although they were isolated from a variety of domestic animals and environmental samples. Both particles were absent, however, from the majority of human faecal samples tested.

Both F + and somatic phage were capable of prolonged survival in water at refrigeration and ambient temperatures. In some experiments, somatic phage could multiply in sewage effluent at 20°C provided that cells of the host bacterium were added. F + phage did not multiply in any experiments.

7.3.2   Source discrimination

The primary objective of Project FC1009 was to evaluate the potential of several published microbiological approaches for distinguishing between human (sewage) and animal (agricultural run-off) contamination of molluscan shellfisheries. Work during this one year feasibility study focused on establishing methods for these indicators at CEFAS, conducting initial work to verify their specificity, adapting the methods for use with shellfish samples and conducting initial field trials.

In general all methods tested gave positive results. However it was noticeable that bacteriophage of *Bacteroides fragilis* were present in much lower numbers than the other indicators examined. The inability to detect reasonable levels of *B. fragilis* bacteriophage, even in highly polluted samples, would have serious limitations on the use of this method. An additional problem was that the precise degree of specificity of this host as a measure of human contamination was unclear. From this study it was concluded that this methodology did not appear to be particularly useful because of the low sensitivity of the assay.

Unlike *B. fragilis* bacteriophage, FRNA bacteriophage was present in high numbers in the human effluents and polluted shellfish samples. Typing of FRNA phage plaques from effluent and shellfish samples gave encouraging results with phage types largely conforming to the pattern expected from the effluent source. In addition shellfish samples from sites associated with recent outbreaks of gastro-enteritis were also dominated by plaques of a human type. This work indicates that this methodology could well be useful for typing the origin of contamination in shellfisheries. However, many of the animal origin effluent samples tested were negative for FRNA bacteriophage limiting the ability of this feasibility study to confirm the specificity of each of the phage types for animal or human sources.

Like FRNA bacteriophage, adenovirus was also detected by PCR in the majority of human effluents and polluted shellfish samples tested. Adenovirus was not detected in any of the animal origin effluent samples. This suggests that the PCR conditions and extraction procedures established in this study for adenovirus are suitable for environmental samples. It also suggests that adenovirus is sufficiently prevalent in environmental samples to be a promising indicator. Detection of adenovirus in the majority of polluted shellfish tested suggests that they were impacted by effluents of human origin. However this would require further confirmation of the specificity of the adenovirus primers used for human strains, through the analysis of more sources and species of animal waste.
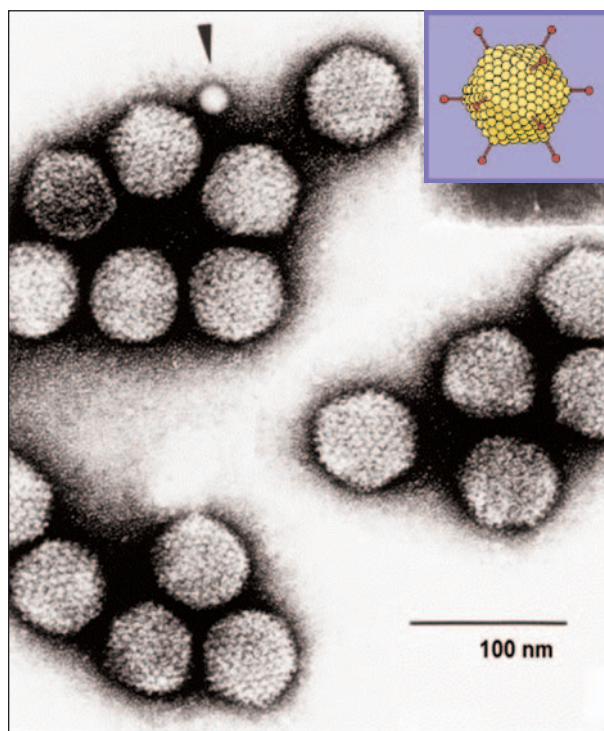

Figure 33.   Adenoviruses. *Adenoviruses are non-enveloped icosahedral particles. Enteric adenoviruses (serotypes 40 and 41) are common in sewage and in environmental samples polluted with human effluent.*

In summary, this study showed that both FRNA bacteriophage typing and adenovirus detection by PCR show considerable promise as a means of distinguishing between human and animal contamination of shellfisheries and the methods should be further evaluated. Low levels of bacteriophage of *Bacteroides fragilis* in polluted samples, and uncertainly about the specificity of the host currently employed, suggest that this system is of limited value for detection of human specific pollution.

7.4 Processes for removal

7.4.1 Depuration

The progress in PCR detection methods for hepatitis A virus and for the Small Round Structured Viruses (SRSVs) causing human gastroenteritis described above (Section 7.2), and the successful application of these methods to molluscan shellfish, has made significant progress in directly assessing the removal rates, during depuration, of the human enteroviruses feasible for the first time. These findings can then be compared with the removal rates for the potential virus indicator, F+ bacteriophage, and for the standard bacterial indicator *E.coli*. This is provided that care is taken to ensure that laboratory experiments are representative of field conditions by using realistic contamination systems for shellfish and by using standardised depuration tank design and operating parameters.

In one study, the effects both of different rations and enhanced temperature on the gut passage times (GPTs) of C^{14}-labelled algal organics and C^{14}-labelled phage in Pacific oysters were compared. There was no significant difference between the average GPTs of 5-10 h for algal organics and phage. Results suggested that GPT was faster both at the higher temperature and at the higher ration. Higher proportions of phage> *E.coli* > algal organics were found in a secondary pulse of egested faecal matter that is thought to derive from the products of intracellular digestion within tubules of the 'digestive gland'. Also, the average efficiency of organic absorption was significantly lower for phage than for *E.coli*. Different GPTs were recorded whereby depuration was quicker and/or less variable for algal organics> *E.coli* >phage.

In another study, oysters, following environmental contamination near a sewage outfall, were depurated under experimental conditions investigating the effect of depuration temperature, algal feeding, salinity, dissolved oxygen and system design. The major parameter determining the rate of viral removal in oysters was found to be depuration temperature with maximum rates of elimination occurring at 20°C. Elimination rates at elevated temperatures for viruses were found to be constant regardless of the harvesting area temperature (range 4-18°C) or season. These studies suggest that the ability of depuration
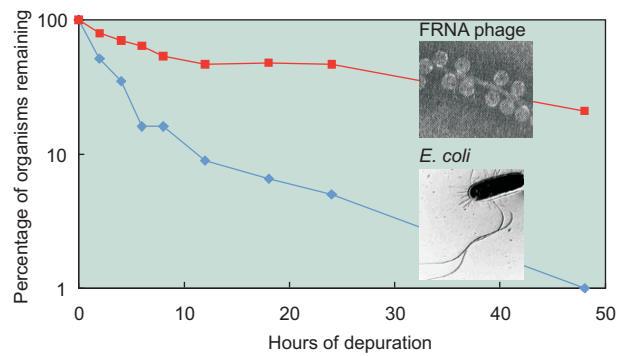

Figure 34. Elimination of E. coli and FRNA bacteriophage from oysters during depuration.

to achieve removal of enteric viruses, as judged by reduction of FRNA bacteriophage to low or absent levels, will be primarily dependant on the initial contaminant level, the temperature of depuration and the duration of depuration. Using the established rates of elimination for each temperature (8, 14 and 20°C) it was shown that it is possible to predict the time required to reduce any given initial level of FRNA bacteriophage to a low level (<100 pfu per 100 g shellfish). These predications suggested that during the winter months more than 50% of shellfish harvested from category B areas would have required depuration for extended periods to remove FRNA bacteriophage even at elevated temperatures. It should be noted that shellfish depuration protocols currently commonly used require depuration for 36 to 42 hours at minimum temperatures of 5-8°C. These studies suggest that such depuration protocols would be unlikely to clear FRNA bacteriophage from the majority of shellfish harvested from category B areas. Levels of 1000 pfu/100g FRNA bacteriophage (levels frequently observed in category B harvesting areas) would require more than 9 days depuration at 8°C to be eliminated. By comparison the same level of FRNA bacteriophage could be eliminated in about 4 days depuration at 20°C.

7.4.2 Natural (relaying)

The relative inefficiency of depuration as a means of reducing the viral load in shellfish focused attention on other procedures. The most important of these appeared to be re-laying in water demonstrated to be less polluted than that in the growing area. Re-laying can bring about a rapid reduction in the levels of bacterial contamination, but less was known about its impact on viruses. The feasibility of using naturally occurring coliphage as models for the removal of potentially pathogenic viruses during re-laying of *C. gigas*, the Pacific oyster was examined. This study was the first to use naturally contaminated Pacific oysters and to compare the behavior of two coliphages, somatic and F+ phage, both of which can be enumerated using simple, inexpensive, standard microbiological techniques.

Figure 35. Field trials to study natural depuration.
After contamination at a polluted site, Pacific oysters were held in bags on metal frames at various re-laying areas in the southwest and natural reduction in E. coli and phage concentrations followed.

The studies led to the adoption of standard techniques for the examination of oysters. Briefly, samples were homogenised in a blender for 45 seconds. Homogenates were centrifuged at 1,000 x g for 15 minutes with 1 ml of the supernatant, or dilutions thereof, being used for phage estimation. For somatic phage, pour plates containing 12 ml agar were used. These were incubated at 30°C for 18 hours before plaques were counted.

The uptake of either phages or *E.coli* by oysters exposed to sewage showed essentially the same kinetics irrespective of season or length of exposure to sewage effluent. Uptake was rapid and levels of both the bacterium and the phages in oyster tissues reached a maximum approximately 1-2 days after placing at the outfall. There was little different between these levels and those in oysters exposed for 25 weeks.

Using a variety of re-laying sites, the results demonstrated distinct differences in the speed with which the two phages were removed from oysters. Numbers of F+ phage declined rapidly, while removal of somatic phage was measurably slower. For example, in oysters placed in commercially used seawater ponds, F+ phage reached undetectable levels within 2-3 weeks, whereas somatic phage could still be detected five weeks after re-laying. In all experiments, *E.coli* levels fell more rapidly than those of either phage, although in some trials rates of removal were close to those of F+ phage

A further study was undertaken to investigate virus removal during extended relaying (4 to 6 weeks) combined with depuration. The results suggested that extended relaying combined with depuration could be effective for removal of viruses but that, like depuration, this was critically dependant on seawater temperature and initial contamination levels. The
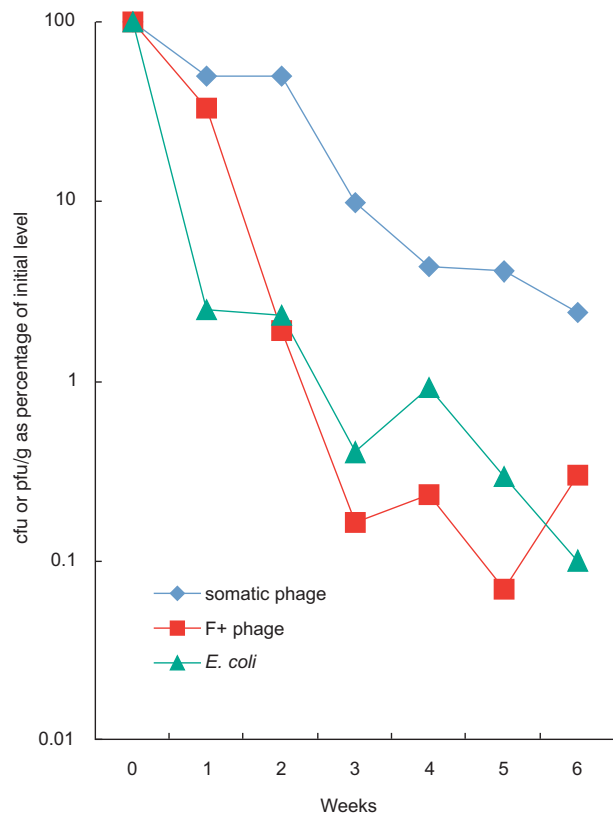

Figure 36. Removal of phage and E. coli from contaminated Pacific oysters during re-laying in May/June.

results also suggested that species-specific effects might be important. The results demonstrate that for effective virus removal during relaying it would be important to set minimum seawater temperatures during which relaying can proceed.

7.5 Uptake of the results on viruses

A significant first step towards developing virus detection techniques to aid development of procedures for reducing the human health risk from molluscan shellfish consumption was made in this suite of studies. This work has been taken forward in further projects funded by the FSA.

Project FC1009 showed that the methodologies examined, and in particular the FRNA bacteriophage and adenovirus methods, have potential as indicator organisms and as a means of distinguishing human and animal contamination in shellfisheries. Further development and evaluation of these methods was recommended and this work has been successfully carried forward in subsequent FSA-funded studies. The improved methods will ultimately allow the development of procedures for better assessment of the potential health risks associated with shellfish originating from harvesting areas where no clear source of human contamination can be identified. Furthermore the ability to determine the source of

faecal contamination in a shellfishery will allow more precise targeting of the management action needed to control that pollution source.

A quantitative competitive RT-PCR method for detection and enumeration of human enteroviruses in molluscan shellfish such as oysters was developed, as a means to establish, under laboratory and field conditions, the true extent of virus removal during shellfish depuration as currently practiced commercially. This is in support of a general government commitment to improve the public health status of molluscan shellfish and to provide a scientifically based control framework within which the shellfish industry can continue to trade.

The characteristics of FRNA bacteriophage removal during depuration are now well characterised and reliable predictive equations have been developed. A working hypothesis has been formulated suggesting that depuration systems capable of removing FRNA bacteriophage during winter months should provide significantly enhanced levels of consumer protection against human enteric viruses.

Results from these studies have assisted further research on the removal kinetics of human enteric viruses from molluscan shellfish. They have also helped in the determination of virus load in samples associated with outbreaks or in polluted harvesting areas.

7.6 Publications arising from this research

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8. CHEMICAL CONTAMINATION

The projects addressed in this section are:

FC0735 The significance to human health of the chemical contamination of commercial shellfish (CEFAS/University of Portsmouth)

FC1012 Assessment of Toxicity Identification techniques for the elucidation of bivalve shellfish hatchery & nursery production (CEFAS)

8.1 Background

Bivalve molluscs sold commercially must originate from production areas designated under the Shellfish Hygiene Directive 91/492/EEC, which are classified according to the level of *E.coli* present in shellfish samples from each area (see Section 6). In addition to the above criteria, when marketed for consumption, live molluscan shellfish must meet a number of end product standards. These include a requirement that the shellfish must not contain toxic or objectionable compounds such as, trace metals, organochlorine compounds, hydrocarbons and polycyclic aromatic hydrocarbons (PAH), in such quantities that the calculated dietary intake exceeds the permissible daily intake.

A study of commercial molluscs was carried out under Project FC0735 to provide information that would help the UK to meet its commitments under the above EU legislation.

Ever since the successful development of commercially viable techniques for the hatchery production of bivalves, the industry has been plagued by episodic events that lead to catastrophic failure of batches of larvae and spat. Obviously, constant failure would have huge economic impact on home and foreign markets; however, the problem seems to be largely affecting production seasonally during June through to September. There is also some evidence to suggest that these events are becoming more frequent and widespread. A previous, EU-funded, investigation established that the seasonal problems encountered by bivalve shellfish hatcheries are related to poor water quality. However, this programme did not investigate this in depth and did not provide answers to the cause of the problem and how the industry could mitigate the effects. Similar problems, frequently encountered in effluent management practices, are addressed by applying Toxicity Identification Evaluation (TIE) techniques. These allow the cost effective toxicity characterisation of a sample by using a bioassay directed fractionation approach. Toxic samples are simplified in complexity and the toxic component isolated by toxicity testing.

8.2 Contamination

Approximately 200 samples of bivalve molluscs were collected annually, over three years, from commercial harvesting areas around England and Wales. Samples included four species of molluscs, mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*), native oysters (*Ostrea edulis*) and Pacific oysters (*Crassostrea gigas*). In year 1 the flesh of these animals was analysed for metals. The same samples were analysed for hydrocarbons and PAHs in year 2 of the programme and organochlorines in year 3.

For heavy metals, Nitric acid digests of bulked whole tissue were analysed using a combination of atomic absorption spectrophotometry (for copper, iron, and zinc), atomic fluorescence spectrometry (for mercury) and inductively coupled plasma-mass spectrometry (for silver, arsenic, cadmium, cromium, nickel, lead and selenium).
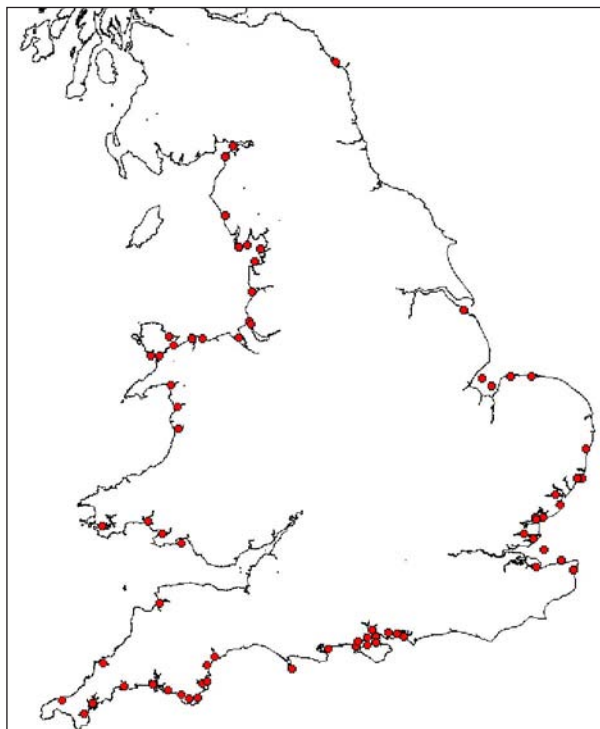

Figure 37. Designated bivalve mollusc production areas in England and Wales (at September 2003).

Variations in the levels of uptake of different metals both within and between species were evident. Cockles contained the highest concentrations of nickel and iron and the lowest concentrations of cadmium, copper and zinc. Oysters contained the highest concentrations of cadmium, copper and zinc and the lowest concentrations of lead and nickel, whilst mussel contained the highest concentrations of lead and arsenic.

The highest concentrations of metals were recorded in samples from areas subject to industrial discharges and emissions, or from highly mineralised areas. In all samples, the concentrations of metals were below existing standards/guidelines for shellfish in England and Wales and over 95% were below new limits for lead and cadmium in molluscs, as proposed by the EU. Estimated total dietary intakes of metals, for high-level consumers of molluscs, were below the Permitted Maximum Tolerable Daily Intakes established by the Joint FAO/WHO Expert Committee on Food Additives.

In the second year, samples were analysed for a suite of nineteen polycyclic aromatic hydrocarbons (PAH) and groups. There was some evidence of differences in the uptake of PAH between species, with cockles generally containing lower concentrations of PAH than oysters and mussels. Relatively high concentrations (>400 µg kg^{-1}) were recorded in approximately 15% of the samples and included samples from each of the species selected for study. The ranges of concentration for a sum of the PAH determined in the various shellfish tested were very wide, at (in µg kg^{-1} wet weight): 26 to 492 (cockles); 40 to 2,847 (mussels); 63 to 1,592 (native oysters); 14 to 942 (Pacific oysters).

As might reasonably be expected, some of the highest concentrations were recorded in areas associated with oil refineries/terminals, petrochemical industries, heavy shipping, power stations and other major sources of PAH inputs. The results indicate that the concentrations of PAH in bivalve molluscs around England and Wales are sufficiently high that shellfish may be an important dietary source of PAH for those who consume them, particularly for the extreme consumer.

In year 3, following n-hexane Soxhlet extraction of bulked whole shellfish tissue from the samples, residues were determined for a suite of 7 pesticide residues and 25 individual chlorinated biphenyls (CBs) by gas liquid chromatography, using electron capture detection after alumina and silica gel clean-up and separation.

Figure 38. Inductively coupled plasma-mass spectrometry (ICP-MS) was used to analyse for certain metals.

The concentrations of pesticide residues recorded in all samples were very low, with the majority at or below the limit of detection (0.001 mg per kg). The maximum concentration recorded was 0.033 mg per kg wet weight for TDE (one of the major metabolites of DDT) in mussels from the Humber.

The maximum estimated dietary intake for each pesticide residue, from molluscs alone, for high-level consumers of molluscs, accounted for less than 2% of the Acceptable Daily Intakes (ADIs) or Provisional Tolerable Daily Intakes (PTDIs), established by the Joint Meeting on Pesticide Residues. Concentrations of CBs were also generally very low.

8.3. Investigations into mortality

An oyster larval bioassay was developed and applied to a TIE characterisation of Guernsey Sea Farms (GSF) hatchery water during a period of high larval mortality. In this assay, the growth of larvae in the sample water is compared to growth in reference seawater. This growth is then measured by image analysis and results compiled by a sophisticated computer program. The TIE indicated that conventional organic contaminants (e.g. pesticides), cationic metals and volatile compounds were not responsible. The TIE also showed that filtration could reduce the level of larval mortalities. Since the TIE suggested that contaminants were not responsible for the seasonal mortalities the occurrence of other vectors (e.g. bacteria, viruses and disease) were investigated.

Pathological screening for disease revealed that no known disease was evident in the broodstock or larvae. A virological study (as part of the EU VINO programme) suggested that viruses were not the cause of the observed mortalities. A bacteriological study showed that the healthy broodstock, water and algal samples were low in bacteria typical of a shellfish hatchery environment. Those bacteria isolated in high numbers from affected batches of larvae were not particularly pathogenic to larval oysters and it was concluded that bacteria were not the primary cause of mortalities at the hatchery.

Histopathological examination of the larvae, using transmission electron microscopy (TEM) identified a number of spherical bodies in the stomach of affected larvae with observations that correlate to a condition described as 'Digestive Tract Impaction' (DTI) of oysters. The material impacting the gut of oysters was identified as *Isochrysis* algae originating from algal feed. Integrated experiments assessing the occurrence of DTI and high larval mortality were conducted using different combinations of water (filtered and unfiltered), algae from GSF and Seasalter (SS) and larvae from SS. Data from this experiment showed that GSF water was affecting larval survival and that filtering the water reduced larval mortality. DTI was observed in all but one of the samples that were fed algae from GSF. Growth did not seem to be affected by the presence of the DTI and it was not possible to ascertain whether it affected mortality. Another experiment assessing the possible contribution to poor larval survival from algae using different combinations of water and algae from GSF and SS showed that rearing larvae in water from SS or GSF with algae only originating from SS, did not result in high mortalities. Larval survival was particularly low in combinations that used GSF water and GSF mixed or GSF algae (*Pavlova lutheri*).

Identification of algae species in samples collected from GSF showed the presence of a species known to produce domoic acid, an Amnesic Shellfish Poison (ASP). The cell counts were below a level at which any action to safeguard human health would be necessary, but their presence is not suggested as ideal in an oyster hatchery. Water samples collected following this discovery tested negative for the presence of domoic acid.

In addition to the above activities, copper was shown to be present at low concentrations in water and algae samples provided by GSF. Mussels placed near the intake pipe and at a 'clean' location off the island were analysed for metals, TBT, PAHs and PCBs. All data show very low concentrations of these contaminants accumulated in the mussels placed at the GSF intake
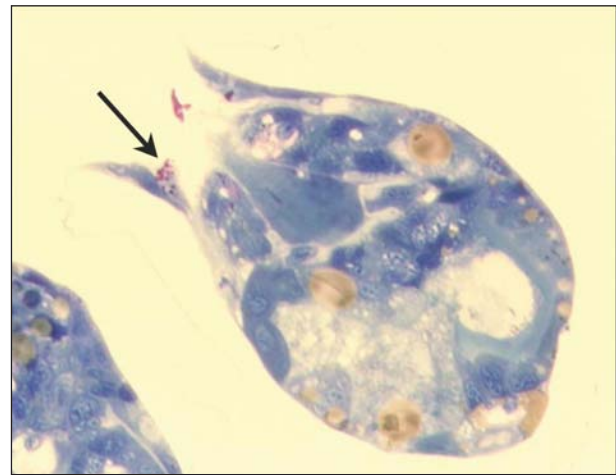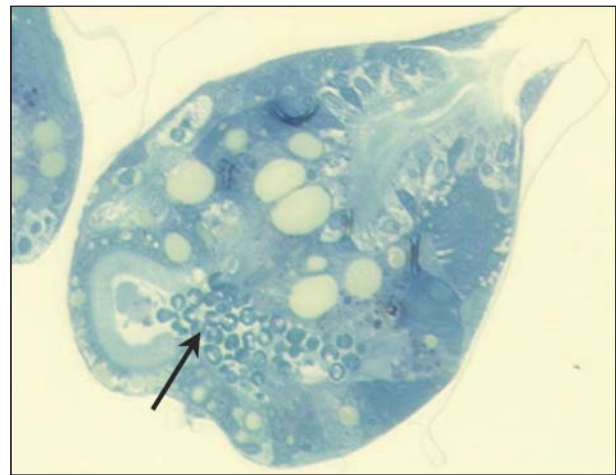

Figure 39. Digestive Tract Impaction of bivalve larvae. *Numerous Isochrysis cells can be seen in the dilated stomach of an oyster (above) and in the velum of a clam (below).*

and confirmed the results of the TIE that common anthropogenic contaminants are not responsible for the seasonal larval mortality events.

8.4. Uptake of the results on chemical contamination

The results from the contaminants analyses have provided a spatial picture of the levels of these chemicals in commercial bivalve molluscs. In general, concentrations of chemical contaminants in commercial bivalves are relatively low. However, the data also indicate that in some harvesting areas concentrations of some chemical contaminants can be relatively high, particularly at certain times of the year, and Tolerable Daily Intakes (TDIs) could be exceeded by extreme consumption of molluscs from these areas during these periods.

The data from project FC0735 provides an indication of the concentrations of chemical contaminants that might be expected to occur in different species in the various harvesting areas. These data together with additional information regarding, for example, time of sampling and species cultivated can be used to assess the likely risk, for each harvesting area, of the concentration of a chemical contaminant, reaching levels in the molluscs whereby TDIs could be exceeded. It was proposed that this risk-based approach could be used to form the basis for determining the frequency of sampling required in each area.

It has been shown that common chemical contaminants (e.g. organic compounds, metals and volatile compounds), viruses or bacterial were not responsible for observed mortalities in a commercial shellfish hatchery. Digestive tract impaction (DTI), a condition known to affect larval oysters, was linked with high incidences of larval mortality. However, the cause of DTI is unclear as is its role in the mortality events that have been recorded. The seasonal recurrence of this condition suggests that the cause might be linked to natural processes such as phytoplankton blooms.

8.5 Publications arising from this research

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LAW, R.J., THAIN, J.E., KIRBY, M.F., ALLEN, Y.T., LYONS, B.P., KELLY, C.A., HAWORTH, S., DYRYNDA, E.A., DYRYNDA, P.E.J., HARVEY, J.S., PAGE, S., NICHOLSON, M.D. AND LEONARD, D.R.P., 1998. The impact of the *SEA EMPRESS* oil spill on fish and shellfish. In: The *SEA EMPRESS* Oil Spill: Proceedings of the *SEA EMPRESS* Conference held in Cardiff, UK 11-13 February 1998. R. Edwards and H. Sime (Editors). Chartered Institute of Water and Environmental Management, London pp 109-136.

9. ALGAL TOXINS

The projects addressed in this section are:

FC0736 Development of reliable and specific analytical methods and rapid assay procedures for phycotoxins (algal toxins) - Diarrhetic Shellfish Poisoning (DSP) (CSL)

FC0737 Development of reliable & specific analytical methods & rapid assay procedures for phycotoxins-PSP (CSL)

FC0739 Development of "*in-vitro*" bioassays for detections of PSP and related toxins. (CEFAS)

FC0741 Development of reliable and specific analytical methods and rapid assay procedures for phycotoxins – PSP (FRS)

FC0742 Development of reliable and specific analytical methods and rapid assay procedures for phycotoxins – DSP (FRS)

9.1 Background

Algal Toxins

There are four recognized groups of illnesses associated with algal toxins: Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP) and Amnesic Shellfish Poisoning (ASP). NSP is not known in the UK.

PSP *is associated with algae of the genera Alexandrium, Gymnodinium and Pyrodinium. The illness comprises numbness in the mouth and fingertips followed by impaired muscle coordination. Respiratory distress and paralysis can occur and this may be fatal. The major toxin is saxitoxin but there are a large number of other toxic derivatives of the saxitoxin molecule that may be involved. Algae of the genera Dinophysis and Prorocentrum produce **DSP** toxins. Diarrhetic shellfish poisoning (DSP) is a gastrointestinal disease caused by ingestion of shellfish contaminated by toxic dinoflagellates. Human consumption of shellfish containing DSP toxins exceeding certain levels results in diarrhea, nausea, vomiting and abdominal pain starting 30 min to a few hours after ingestion. Complete recovery occurs within three days. The major toxins are okadaic acid and dinophysis toxins (1, 2 and 3), although various others are known.*

ASP *is caused by domoic acid produced by marine diatoms of the genus Pseudonitzschia. Symptoms include vomiting, diarrhoea, abdominal cramps and loss of short-term memory. The effects on memory may persist.*
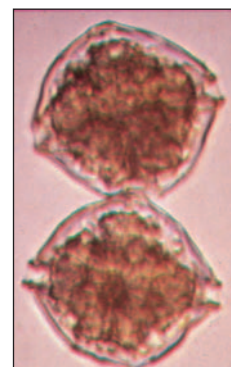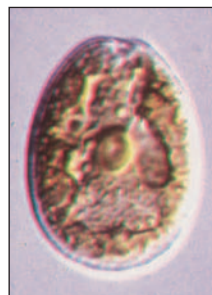

Figure 40.   Toxic algae. Shellfish can become toxic after ingesting toxin-producing algae. Some of the algae responsible are, from left to right: Dinophysis, Prorocentrum (both DSP) and Alexandrium (PSP).

Defra-funded research on algae toxins, now transferred to the Food Standards Agency, focused on finding alternative methods to the mouse bioassay. Much of the initial work was carried out at the Central Science Laboratory Torry Research Station. Following the closure of this laboratory in 1996 the responsibility and funding for this work was transferred to the Scottish Office Marine Laboratory, now the Fisheries Research Services Marine Laboratory, Aberdeen.

It is a statutory requirement under the terms of Directive 91/492/EEC that shellfish harvesting areas are monitored periodically for the possible presence of toxin-producing plankton and for the presence of biotoxins in live molluscs. The standard *in vivo* (mouse) bioassay is routinely employed for this monitoring and there is a clear need to replace this assay with a non-animal but cost-effective alternative. For example, an '*in-vitro*' cell bioassay would be ethically much more acceptable and could reduce costs by as much as 85-90%.

9.2   Alternative detection methods

9.2.1   PSP

The aim of project FC 0739 was to develop an '*in-vitro*' cell bioassay that can be used for routine screening of shellfish samples for Paralytic Shellfish Poison (PSP) toxins. The most promising alternative method to the mouse assay developed for detection of PSP toxins is a tissue culture bioassay based on the opposing pharmacological activities of compounds that have specific effects on the sodium channels of mouse neuroblastoma cells (N2a bioassay). The cells swell and eventually lyse upon exposure to veratridine, a sodium-channel activator, which, when added together with ouabain, enhances sodium ion influx. In the presence of PSP toxins, which block the sodium channels, the action of ouabain and veratridine is inhibited and the cells remain morphologically normal. In the N2a bioassay, the fraction of the cells protected in this way is in direct proportion to the concentration of PSP toxins in the extract. In the previous 5 years workers in Canada and the USA had developed modified versions of the N2a bioassay, which have included automated end-point determination methods.

Trials of the Canadian modification of the bioassay were started at the CEFAS Weymouth laboratory in 1993 (FC0739). The N2a cells were found to be robust and easy to maintain. However, the reproducibility of the assay was poor and further automation was successfully introduced to improve this aspect of the assay. The results of parallel tests during the 1994 monitoring programme, carried out in collaboration with the Food Science Laboratory at Torry (FC 0737), were very encouraging and a high correlation was found between the in-vivo and N2a bioassays when testing samples containing concentrations of PSP toxins below the action level (400 mouse units). This indicated that the N2a bioassay had potential as a 'negative' screening method. Parallel testing was continued in 1995 and 1996 and further improvements in assay precision were made when the neutral red end-point determination method, developed in Aberdeen, was adopted and further modifications in sample application techniques and cell culture conditions were introduced.

PSP extract storage experiments had suggested that storage might not influence the toxicity of the sample as much as was first thought, particularly over the short term. In trials, toxicity levels were maintained longest in samples frozen at -85°C or stored at 4°C in lyophilised form. Acid extracts from a number of species of molluscs and crustaceans were also tested and uncontaminated samples were shown not to produce false toxicity in the N2a bioassay.

A number of problems were experienced with culture of the N2a cells during 1996 and into 1997 and the main effort was switched to improving cell viability and growth rate, particularly after retrieval of cultures from cryo-storage. Modifications to the culture medium successfully overcame these problems and the time between sub-cultures of the neuroblastoma stock cells was also extended to enable less labour intensive maintenance of the cells with savings in wastage and cost.

The N2a assay was then standardised with regard to optimum cell seeding density, cell passage number (age) and titration of essential assay reagents. However, even after rigorous standardisation there was still variation in the quality of the standard curves prepared using saxitoxin from commercial sources. A semi-quantitative N2a bioassay was then tested for possible use as a high-throughput negative screen and then further developed to enable the estimation of toxin levels in all PSP samples tested with similar or better precision than the mouse bioassay. The method was developed to the stage where the semi-quantitative neuroblastoma bioassay gave results comparable with those of the mouse bioassay. However, it was recommended that results close to the regulatory limit should be confirmed by the *in-vivo* (mouse) bioassay for monitoring purposes.

9.2.2   DSP

Parallel studies were carried out on finding alternative analytical methods for the detection of DSP toxins. At the time, these focussed on detection using High Peformance Liquid Chromatography (HPLC). Some studies were also carried out on the colorimetric protein

phosphatase 2A (PP2A) inhibition assay. It was shown that both the HPLC methods and the PP2A assay could be used as analytical tools for quantifying DSP toxins, giving repeatable results. The detection limit of these methods was lower than in the mouse bioassay. However, these approaches eventually proved to be of limited usefulness and they have been superseded, in more recently funded FSA studies, by the development of LC-MS techniques. These entail a combination of liquid chromatography and mass spectrometry, and have been shown to be a powerful and extremely sensitive tool for the detection and quantification of toxins in both shellfish and plankton, at part-per-billion levels.

9.3 Uptake of the results on algal toxins

In Canada, Jellet Biotek have developed a commercial test kit called the Jellet Rapid Test for PSP (formerly MIST Alert[TM]), which utilises immuno-chromatography in a dipstick format. These kits can, within 20 minutes, indicate whether PSP or ASP toxins are present (positive/negative) in shellfish extracts, although they do not allow the levels of the toxins to be quantified. The UK Food Standards Agency commissioned Fisheries Research Services, Aberdeen to investigate whether Jellet Rapid Tests might be

suitable for use in UK monitoring programmes and assess its applicability at shellfish farms and processors as a shellfish harvest management tool and end-product test. Results from these trials suggest that the Jellet Rapid Test for PSP may be used as a screen to eliminate negative and low PSP toxicity shellfish samples from routine monitoring programmes prior to quantitative analysis of PSP toxin positive samples. In viewing its ease of use, the kit may have a role as a quick harvest and end product test.

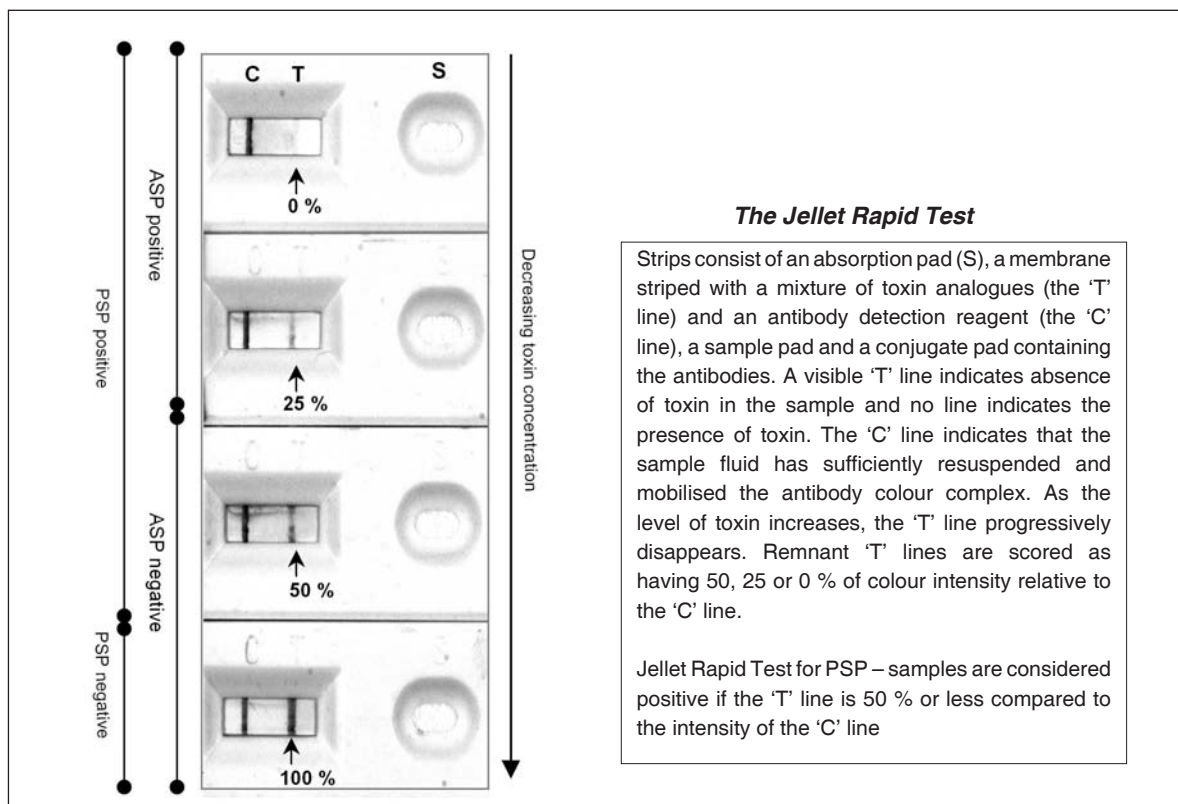*Figure 42. A shellfish processor using the Jellet Rapid Test.*

The Jellet Rapid Test

Strips consist of an absorption pad (S), a membrane striped with a mixture of toxin analogues (the 'T' line) and an antibody detection reagent (the 'C' line), a sample pad and a conjugate pad containing the antibodies. A visible 'T' line indicates absence of toxin in the sample and no line indicates the presence of toxin. The 'C' line indicates that the sample fluid has sufficiently resuspended and mobilised the antibody colour complex. As the level of toxin increases, the 'T' line progressively disappears. Remnant 'T' lines are scored as having 50, 25 or 0 % of colour intensity relative to the 'C' line.

Jellet Rapid Test for PSP – samples are considered positive if the 'T' line is 50 % or less compared to the intensity of the 'C' line

Figure 41. The Jellet Rapid Test. This has been evaluated as an alternative detection method for PSP toxins.

If official validation of these bioassay kits is successful then they could become an internationally recognised alternative to the *in-vivo* (mouse) bioassay and could be used, at the very least, as a relatively inexpensive negative screen. It is probable that the mouse assay could be replaced with an *in vitro* bioassay of comparable precision and that a fully quantitative bioassay would no longer be necessary for routine monitoring applications.

9.4 Publications arising from this research

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SMITH, E.A., GRANT, F., FERGUSON, C.M.J. AND GALLACHER, S., 2001. Biotransformations of Paralytic Shellfish Toxins by Bacteria Isolated from Bivalve Molluscs. Appl. Environ. Microbiol. 67: 2345-2353.

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TOBE, K., FERGUSON, C., KELLY, M., GALLACHER, S. AND MEDLIN, L.K., 2001. Seasonal occurrence at a Scottish PSP monitoring site of purportedly toxic bacteria originally isolated from the toxic dinoflagellate genus Alexandrium. Eur. J. Phycol. 36: 243-256.

ANNEX 1. Registry of shellfish cultivation research projects funded by MAFF/Defra, 1990-2003

Code	Project Title	Contractor	Start	End	Total cost	Summary Objectives
FC0202	The hatchery production of king scallop seed	CEFAS- Conwy	Apr-91	Mar-96	£753,454	Investigate factors that control larval growth leading to reliable hatchery production and a better assessment of natural recruitment variability.
FC0203	The environmental impact of clam cultivation	CEFAS- Conwy	Apr-90	Mar-96	£668,093	Assess the localised impact of intensive clam cultivation on the inter-tidal ecosystem and develop guidelines for environmentally acceptable methods of intensive clam cultivation.
FC0204	Ranching/stock enhancement trials with great scallop (*Pecten maximus*)	SFIA- Ardtoe	Apr-92	Mar-95	£355,674	Assess the feasibility of seabed cultivation of king scallop by investigating promising techniques and determining the expected yield from commercial ranching and stock enhancement.
FC0206	Nutritional and environmental factors affecting recruitment of bivalves	CEFAS- Conwy	Apr-92	Mar-95	£169,903	Assess how different diets of microalgae and associated environmental factors affect the growth, reproductive development and survival of bivalve molluscs.
FC0207	Production of sexually sterile bivalve molluscs	CEFAS- Conwy	Apr-92	Mar-95	£133,184	Assess the potential for producing a sterile seed of non-indigenous bivalves by genetic manipulation, develop reliable sterile spat production methods and monitor the viability of adults produced.
FC0211	Tetraploid Pacific oysters	CEFAS- Conwy	Apr-95	Mar-98	£246,216	Assess the potential of producing Pacific oyster (*Crassostrea gigas*) tetraploid broodstock that can be crossbred with diploid animals to produce sterile all-triploid seed.
FC0212	Scallop seabed cultivation development.	SFIA- Ardtoe	Jun-95	Mar-96	£30,000	Ensure more successful seabed cultivation by assessing the effect of different site substrates on the ability of seeded scallop to establish themselves.
FC0215	Enhancing growth and survival of hatchery produced scallops.	CEFAS- Conwy	Apr-96	Mar-98	£88,513	Assess whether the growth and survival of hatchery reared scallops is influenced by genetic variability.
FC0502	Factors affecting virulence and spread of Bonamia	CEFAS - Wey	Apr-90	Mar-93	£23,698	Investigate alternative methods of oyster husbandry and recommend handling practices which impart maximum resistance to Bonamia infection.
FC0504	Application of immunological and other biological water quality indicators in coastal waters	University of Southampton	Jul-90	Jun-93	£160,856	Examine the immunological response and disease susceptibility of bivalves, to natural and anthropogenic stresses in order to establish optimal relaying conditions.
FC0703	Studies of viruses in shellfish in relation to public health	CEFAS- Weymouth	Apr-90	Mar-95	£1,133,792	Develop standard virological methods to detect indicator viruses and important viral pathogens e.g. hepatitis A and use these to determine the kinetics of uptake and depuration of human pathogenic viruses.
FC0706	Use of coliphage to assess the influences of re-laying and depuration on contamination of molluscan shellfish with viruses	PHLS	Mar-90	Feb-92	£33,600	Assess the use of the coliphage E.coli as an indicator of the contamination of molluscan shellfish with enteric viruses and use this method to monitor the effects of relaying Pacific oysters and the efficacy of depuration.

Code	Project Title	Contractor	Start	End	Total cost	Summary Objectives
FC0714	Depuration techniques for oysters and clams	SFIA- Ardtoe	Jun-91	Jun-92	£73,400	Identify the most appropriate operating criteria for depuration of oysters (*C. gigas*) and clams by examining factors such as temperature, water flow, density of stacking etc. and produce a standard specification for medium and small scale depuration plants.
FC0717	A study to evaluate the feasibility of using coliphage as hygienic indicators in molluscan shellfish	PHLS	Jul-93	Jun-95	£53,646	Investigate the ecology and growth and survival pattern of two coliphages in shellfish, their growing waters and sources of pollution (sewage), with a view to using the coliphages as indicators for enteric viruses in shellfish.
FC0727	Role and fate of micro-organisms in bivalve molluscs with reference to bacteria and viruses	NERC-Plymouth Marine Laboratory	Oct-94	Sep-97	£159,700	Examine and compare the various methods by which shellfish process bacteria and viruses (including removal by feeding, partial digestion and egestion of faeces) in order to estimate the differing rates of microbial pollutant removal during purification.
FC0729	Studies on application of enterovirus RT-PCR to environmentally-contaminated shellfish and shellfish-growing waters	University of Surrey	Oct-94	Sep-96	£18,650	Investigate the application of a newly-developed technique for the identification of the human pathogenic viruses hepatitis A and SRSVs in molluscan shellfish-growing waters.
FC0732	Re-appraisal of existing operating criteria for purification of bivalve molluscs in the UK	SFIA	Apr-95	Mar-97	£46,312	Re-assess and confirm the optimum conditions for depurating bivalve molluscs, including seawater temperature, dissolved oxygen levels, light and dark, and disturbance.
FC0733	Studies on the removal of human pathogenic viruses from molluscan shellfish during depuration	University of Surrey	Nov-95	Dec-97	£113,583	Provide information on the extent of virus removal under prevailing commercial depuration practices.
FC0735	The significance to human health of the chemical contamination of commercial shellfish	CEFAS / University of Portsmouth	Oct-95	Oct-98	£40,239	Analyse samples of shellfish from commercial beds and determine the concentration of a range of chemical contaminants. Assess these concentrations in terms of a possible risk to the health of human consumers.
FC0736	Development of reliable and specific analytical methods and rapid assay procedures for phycotoxins (algal toxins) - diarrhetic shellfish poisoning (DSP)	CSL-TORRY	Apr-96	Sep-96	£26,250	Develop a range of analytical methods and assays to replace the mouse bioassay test for DSP, including chromatography, electrophoresis and immunoassay.
FC0737	Development of reliable & specific analytical methods & rapid assay procedures for phycotoxins-PSP	CSL-TORRY	Apr-96	Sep-96	£26,250	Investigate and validate suitable methods to replace the mouse bioassay test for PSP including immunoassay, chromatography, tissue culture and sodium probes.
FC0738	Detection and removal of human viral pathogens in bivalve shellfish	CEFAS-Weymouth	Apr-95	Mar-98	£974,933	Further develop viral detection for bivalve molluscs especially for small round structured viruses (SRVs) report on the value of viral indicatores and suggest factors that will reduce or remove human viral pathogens in these molluscs.

ANNEX 1.   continued

Code	Project Title	Contractor	Start	End	Total cost	Summary Objectives
FC0739	Development of "in-vitro" bioassays for detections of PSP and related toxins.	CEFAS-Weymouth	Mar-96	Mar-98	£87,095	Develop an '*in-vitro*' cell bioassay that can be used for routine screening of shellfish samples for Paralytic Shellfish Poison (PSP) toxins.
FC0741	Development of reliable and specific analytical methods and rapid assay procedures for phycotoxins – PSP	FRS	Sep-96	Mar-97	£40,062	Assess alternative assay procedures for PSP toxins using ELISA, tissue culture and HPLC and to compare these with the mouse bioassay. Also, to characterise PSP toxins in UK shellfish and develop a competitive binding assay for PSP.
FC0742	Development of reliable and specific analytical methods and rapid assay procedures for phycotoxins – DSP	FRS	Sep-96	Mar-97	£33,993	Develop a range of analytical methods and assays to replace the mouse bioassay test for DSP, including chromatography, electrophoresis and immunoassay.
FC0909	Preserved microalgae as an alternative diet in aquaculture (LINK ALG02)	University of Wales, Bangor	Sep-97	Aug-00	£132,252	Develop a suitable replacement preserved microalgae as an alternative to live algae as a feed for bivalve molluscs and zooplankton, including its nutritional value and storage life.
FC0926	Evaluation of factors affecting native oyster stock regeneration	University of Southampton	Dec-02	Nov-04	£45,000	To quantify the effects of commonly used cultch materials and competition with the slipper limpet (*Crepidula fornicata*), on the retention and growth of recently settled native flat oysters (*Ostrea edulis*) grown at different densities.
FC1001	Transport of seed scallops - *Pecten maximus*	University of Wales, Bangor	Feb-96	Jan-99	£47,944	Define the optimal conditions for seed survival in transport systems by investigating the physiological and biochemical changes that occur as a result of environmental stress.
FC1002	Nursery cultivation of King scallops	CEFAS- Conwy	Apr-96	Mar-00	£488,679	To improve the survival rate of hatchery-reared scallops by: 1) assessing their biological requirements at metamorphosis and during the early juvenile stages, and 2) developing reliable nursery rearing techniques for juvenile scallops. Also, to determine whether there is a need for the depuration of farmed scallops (New target from 09/1997).
FC1004	Environmental requirements for successful scallop cultivation (LINK SHL22)	CEFAS- Conwy	Oct-98	Sep-02	£374,405	Through laboratory and field trials, identify important criteria for selecting sites for scallop cultivation, including temperature, salinity and food availability, undertake an economic appraisal of a commercial operation and prepare a cultivation guide.
FC1005	An assessment of the impact of different types of sewage treatment on the contamination of shell fisheries.	CEFAS-Weymouth	Oct-97	Mar-00	£68,621	Use a Geographical Information System (GIS) to evaluate the effects of contamination from different types of sewage discharges and overflows on shellfish harvesting areas.
FC1009	Feasibility study for methods to distinguish between human and animal faecal contamination in shellfisheries.	CEFAS-Weymouth	Dec-98	Mar-99	£50,695	Assess the potential of three methodologies that could be used to distinguish between human and animal sources of faecal pollution in shellfish.

Code	Project Title	Contractor	Start	End	Total cost	Summary Objectives
FC1012	Assessment of Toxicity Identification techniques for the elucidation of bivalve shellfish hatchery & nursery production	CEFAS	Jun-00	May-03	£90,720	Use Toxicity Identification Evaluation (TIE) techniques to isolate and identify the causal substances responsible for unexplained seasonal and toxic events and poor water quality, which can result in closure or failure of shellfish farming sites.
FC1013	Requirements for depuration of scallops (*Pecten maximus*)	CEFAS	Nov-00	Dec-01	£58,023	Determine the environmental requirements for successful depuration of scallops (*Pecten maximus*) with the production of a report, for DEFRA, identifying critical minimum acceptable conditions for depuration and a manual for industry providing advice on effective depuration methods.
FC1101	Quantitative evaluation of bivalve disease susceptibility and resistance.	University of Southampton	Aug-95	Nov-98	£27,764	Examine susceptibility and resistance of the main commercially exploited species of bivalves (including native oysters, Pacific oysters and scallops) to *Bonamia* and other diseases, including differences between shellfish stocks and the effect of changing husbandry practices.
FC1121	Ostrea/Size/Age, Physiological Stress and Resistance to *Bonamia ostreae* (LINKSHL09)	University of Southampton	Sep-97	Aug-01	£149,075	Using quantitative measures of stress in oysters, evaluate how new husbandry methods may reduce their susceptibility to disease, especially to *Bonamia ostreae*.
FC1134	Provision of sandwich students: Shellfish quality problems caused by organic waste pollution of harvesting areas.	University of Surrey	Oct-99	Sep-04	£128,956	Through the provision of sandwich student support a) develop methods for the quantification of small round structured viruses in shellfish and b) assess the feasibility of using bacteriophages as specific indicators of human viral contamination in shellfisheries.
	Total value of all projects				**£7,153,230**	

ANNEX 2.   List of illustrations

CEFAS

The Centre for Environment,
Fisheries & Aquaculture Science

Research on shellfish cultivation

Research on shellfish cultivation

A synopsis of research funded by the Department for Environment, Food and Rural Affairs (Defra) between 1990 and 2003

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