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Observations on a bacterial disease in laboratory-cultured larvae of the European flat oyster *Ostrea edulis* L.

by

M. M. Helm and Mrs F. M. Smith
Shellfish Culture Unit, Fisheries Experiment Station, Conway

It is a common experience in laboratories and hatchery units culturing species of bivalves on a continuous production basis that there are times when substantial mortalities of larvae and juveniles occur. Research groups in the U.S.A. have reported incidences of fungal infection (Loosanoff and Davis, 1963) and mass mortalities caused by bacteria (Guillard, 1959; Tubiash, Chanley and Leifson, 1965) among the larvae of a variety of bivalve species.

Following the occurrence of a suspected bacterial disease in July 1968 at Conway, the 1969 season was interrupted in the summer by similar mortalities. This paper briefly reports the work carried out during the 1969 outbreak to isolate the causative organism, to determine a method of control and to attempt to find the route by which the organism entered the hatchery.

METHODS AND RESULTS

1. Experiments to determine the cause of mortalities

Moribund *Ostrea edulis* larvae from a recently collapsed culture were macerated with sterile sea water in an autoclaved tissue grinder, and large particles were removed from the resulting suspension with a sterile 32 μm nylon mesh screen. Samples of this extract were sterilized either by boiling for 10 minutes or by membrane filtration (pore size 0.45 μm).

10 ml aliquots of untreated and sterilized extract were added to duplicate one litre hard-glass beakers, each containing approximately 400 larvae in filtered, ultra-violet sterilized sea water, treated with the standard antibiotic mixture used at this laboratory of 50 i.u. Penicillin and 50 mg of Streptomycin sulphate per millilitre (Walne, 1966). The cultures were fed with a mixture of *Isochrysis galbana* Parke and *Tetraselmis suecica* (Kylin) Butch. (Walne and Spencer, 1968). Two broods of larvae from stock of different origins were tested simultaneously. Each beaker was covered with a plastic lid and was aerated for the duration of the experiment.

The results of this experiment (Table 1) show that the untreated extract caused a complete mortality of both batches of larvae before an increase in size was apparent. Membrane-filtered and boiled extracts had no effect on growth. These data suggest that mortalities were caused by pathogenic micro-organisms which the antibiotic mixture used failed to control. The possibility that a virus or a toxin was implicated in the deaths was precluded because a membrane-filtered extract was non-toxic. There was no evidence of fungal growth. Microscopic examination of moribund larvae showed that they were surrounded by swarms of motile bacteria. This feature has been reported by Tubiash et al. (1965) as characteristic of the disease they termed bacillary necrosis. No cross infection occurred between beakers in this experiment despite their close proximity in a water bath.

Table 1 The growth and survival of two broods of larvae when exposed to untreated extract of moribund larvae and samples of extract sterilized by boiling and membrane filtration. Initial size of both broods of larvae was 174 μm. Brood A was cultured for 2 days and Brood B for 4 days

Treatment	Final size of larvae (μm)		Mortality
	Brood A	Brood B	
Control	186	223	Negligible
Untreated extract	174	174	100%
Boiled extract	185	223	Negligible
Membrane-filtered extract	183	222	Negligible

A further experiment repeating the above procedure showed that an untreated extract of moribund larvae refrigerated for 48 hours failed to transmit the disease. Tubiash et al. (1965) also observed that refrigerated pathogens isolated from diseased larvae were no longer toxic. Isolated strains of bacteria have also lost their pathogenicity during a 6 month period of culture.

2. Isolation of pathogens

Streaked agar plates of a macerate of moribund larvae were prepared. Three formulations of agar were tested: ZoBell medium, ZoBell medium with sterile oyster flesh extract, and yeast extract nutrient broth. All culture media were made up with sterile sea water and autoclaved at a pressure of 1.03 kg per sq. cm for 15 minutes. The plates were incubated

at 25°C for 48 hours, after which time a wide selection of colonies from each plate were subcultured into nutrient broth for a further period of incubation. A Gram-stain preparation of each isolate was examined by phase-contrast microscopy and the morphology of each noted.

A simple bioassay technique was used to determine the effects on larvae of the various isolates. One hundred larvae were cultured in sterile sea water in a series of 30 millilitre test tubes fitted with aluminium caps. A small loop of nutrient broth from each of 29 bacterial isolates was added to duplicate test tubes. Controls with sterile nutrient broth and others without the addition of broth completed the series.

Within six hours of inoculation larvae in five pairs of tubes had lost motility. After 24 hours the experiment was concluded and the larvae were examined microscopically. Larvae with severely necrotized tissues were observed in the ten tubes mentioned above, indicating that the five isolates were pathogenic. Healthy larvae were recovered from the remaining tubes.

Many of the isolates which proved to be non-pathogenic were similar in appearance to the pathogens on agar plates, in nutrient broth and microscopically. The pathogens were all Gram-negative rods. Four of the isolates were motile and one not. Table 2 shows the culture characteristics of the five disease-causing isolates.

Further experiments confirmed the pathogenicity of the five isolates but one (C 27) appeared to be less virulent, causing lower mortalities and symptoms in some respects unlike those seen in previously infected cultures. The larvae of *Ostrea edulis* and *Crassostrea gigas* Thunberg were equally susceptible to the disease, but attempts to induce an infection in *Ostrea edulis* juveniles failed.

3. Symptoms of the disease

The first sign that larvae were affected by the pathogens was the cessation of normal swimming activity. Larvae lay on the bottom of the culture vessel, many with the velum and rudimentary larval foot extended. The cilia of the velum continued to beat feebly and occasional larvae moved about the bottom with a cart-wheeling motion. Within two hours of the loss of motility detached cilia and complete vela were swimming free in the water. The soft tissues were progressively becoming more granular in appearance and complete necrosis was evident in as little as eight hours after the development of the first symptoms. Moribund larvae were invaded secondarily with ciliates and colourless flagellates.

4. Control of pathogens

The sensitivity of the five pathogenic isolates to a wide range of antibiotics was tested with Oxoid 'Multodisks' on agar pour plates. Two non-pathogenic isolates, one similar in characteristics to C12 and the other to C1, C2 and C26 were included as controls. The controls were designated CA and CB respectively.

The results of the tests concluded after 48 hours of incubation at 25°C are shown in Table 3. Controls A and B were inhibited by a greater range of antibiotics than any of the pathogens. C1, C2 and C26 responded in a similar manner to the antibiotics tested. Oleandomycin was ineffective in the control of isolate C12 but inhibited the growth of C1, C2 and C26. Isolate C27 was inhibited by Streptomycin and Novobiocin, which failed to control the other pathogens.

Four antibiotics (Chloramphenicol, Colistin, Erythromycin and Polymixin B) inhibited the growth of all the bacterial isolates tested and may prove effective in the control of future infections. Each of these has been tested with oyster larvae at this laboratory but not during a period affected by disease.

Colistin and Erythromycin have a beneficial effect on the growth of *Ostrea edulis* larvae at concentrations of 2.5-10 ppm and 20-100 ppm respectively. Agricultural grade Chloramphenicol progressively depresses growth at concentrations above 5 ppm (Walne, 1966) and Polymixin B reduces the growth potential of larvae at all concentrations.

A further series of experiments was made in 1969, in duplicate one litre beakers, to test the effect of various concentrations of pure grade Chloramphenicol on the growth of larvae during a 96 hour period. Colomycin (10 ppm) and a mixture of 5 ppm Chloramphenicol and 5 ppm Colomycin were also tested. The standard antibiotic mixture of Penicillin and Streptomycin, and a pair of cultures without the addition of antibiotics, were included as controls.

The growth of cultures treated with Chloramphenicol at concentrations of 2.5, 5 and 10 ppm was equal to that of the controls, but growth was progressively depressed at higher concentrations. Colomycin caused a slight reduction in growth, but the mixture with Chloramphenicol enhanced the final size of larvae.

These antibiotics have not been tested in large-scale cultures at the time of disease at Conway, but their value in controlling pathogenic bacteria in a hatchery unit will probably be limited by their expense.

5. Source of pathogens

Attempts were made to determine the route by which the pathogens entered the larval cultures. Following the initial collapses of cultures the hatchery was closed for two days for disinfection, together with the culture vessels and handling equipment. From that time strict hygiene precautions were enforced so that each large-scale culture was maintained in isolation. Each culture vessel was provided with a set of equipment for routine handling and they were sterilized in a strong hypochlorite solution after use. The culture vessels were covered with PVC lids with small holes drilled to take aerator tubes. All hatchery fittings, including the walls and floor, were thoroughly washed with a sterilizing solution after each day's operation. As soon as diseased larvae were observed in vessels, those cultures were sterilized with hypochlorite before disposal.

Despite these precautions larvae continued to die within six days of setting up cultures in the hatchery, but the fact that some cultures survived for as long as six days suggested that the disease was being transmitted from vessel to vessel, perhaps by lapses in hygiene.

Three further experiments were set up at intervals in our 75 litre vessels, and larvae ~~were~~ dip-sampled from the cultures were set up in isolation in another part of the laboratory in one litre beakers. Diseased larvae were observed in both the large-scale culture and in beakers in the first experiment. In the subsequent experiments larvae died in the hatchery but not in the isolated beakers. On the first occasion larvae were obtained from a stock of oysters which had been held in the laboratory for six weeks during a period when the colonial alga Phaeocystis pouchetti (Hariot) Lagerh bloomed in the River Conway. These oysters received a continuous flow of sea water pumped from the river while conditioning in the laboratory tanks. All of the previously disease-stricken batches of larvae had been liberated by stock which were also in contact with the Phaeocystis water, but by the time of liberation the bloom had declined. Larvae used in the two further experiments were stripped from brooding oysters opened at the laboratory's oysterage in the Menai Straits.

These results suggested that the disease may have been linked with stock oysters which had been exposed to Phaeocystis water. To confirm this hypothesis stripped, disease-free larvae were suspended in PVC cylinders with a nylon mesh base in 16 conditioning tanks. Two of these tanks were controls without stock oysters, and four had stock oysters

from the River Colne which had been brought into the tank room after the decline of the Phaeocystis bloom. The remaining ten tanks all contained stock which had been in the tank room throughout the Phaeocystis bloom.

The following day it was found that the larvae in five of the ten tanks containing the Phaeocystis-affected stock had suffered a high mortality. The larvae in the remaining 11 tanks were healthy.

6. Identification of pathogens

Sub-cultures of the five pathogen cultures were sent to Dr G. Hobbs at the Torry Research Station, Aberdeen. It was found that the five toxic organisms all belonged to Pseudomonas group IV (i.e. no action on glucose). Tests showed that a serological approach to identification was unsatisfactory (pers. comm.).

DISCUSSION

The disease experienced at Conway in 1969 is apparently similar to that which has been reported by Tubiash et al. (1965). The bacilli found to be pathogenic by this research group were of the type commonly classified either as Aeromonas sp or Vibrio sp. Guillard (1959) had previously identified bivalve larval pathogens as Pseudomonas sp and Vibrio sp. These bacteria are not host specific and by their swarming behaviour in sea water can be free living.

Evidence suggests that the disease in 1968 and 1969 may have been associated with a bloom of Phaeocystis. Bivalves held in laboratory tanks are inevitably subject to various unnatural stresses, and the additional stress imposed by the turbid, mucoid water during these blooms may be sufficient to weaken their resistance to latent pathogens so that these bacteria are able to proliferate. Tubiash et al. (1965) showed that adult bivalves exposed to massive doses of pathogens ingested the test bacteria without ill effect. These pathogens may be established as members of the normal bacterial flora of adult bivalves but are held in check by vigorous individuals. Unfavourable conditions may encourage their proliferation and liberation into the surrounding sea water, to be ingested by larvae released by brood oysters. This hypothesis is highly speculative and further work is required before it can be confirmed.

The disease is extremely infectious and, despite reasonable precautions, is readily transmitted between large-scale culture vessels in the hatchery. Several potentially valuable antibiotics are available which will effectively control the pathogens. Chloramphenicol controls

the pathogens isolated in the U.S.A. at a concentration which *Ostrea edulis* larvae will tolerate, namely 10 ppm. Its value in commercial hatchery units will, however, have to be carefully weighed against its expense.

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Table 2 The culture characteristics and morphology of the five pathogenic bacteria isolated from diseased oyster larvae

Characteristics	Pathogen				
	C1	C2	C12	C26	C27
Colour of colony on ZoBell's medium	Cream	Cream	White	Cream	Yellow
Shape of colony	Round	Round	Round	Round	Round
Edge of colony	Smooth	Smooth	Smooth	Smooth	Smooth
Elevation of colony	Convex	Convex	Convex	Convex	Convex
Transparency of colony	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency of colony	Mucoid	Mucoid	Brittle ~~Mucoid~~	Mucoid	Mucoid
Appearance in nutrient broth	Slightly turbid Pellicle	Turbid, some sediment Small pellicle	Sediment	Turbid, some sediment Small pellicle	Slightly turbid
Gram stain	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods

Table 3 The sensitivity of 5 pathogenic and 2 non-pathogenic bacterial isolates to a range of antibiotics

Antibiotic	Pathogenic isolates					Non-pathogenic isolates	
	C1	C2	C12	C26	C27	CA	CB
Ampicillin	0	0	0	0	0	0	0
Aureomycin	0	0	0	0	0	+	+
Bacitracin	0	0	0	0	0	0	0
Chloramphenicol	+	+	+	+	+	+	+
Cloxacillin	0	0	0	0	0	0	0
Colistin	+	+	+	+	+	+	+
Erythromycin	+	+	+	+	+	+	+
Furazolidone	+	+	+	+	0	+	+
Fusidic acid	0	0	0	0	0	0	+
Kanamycin	0	0	0	0	0	0	0
Methicillin	0	0	0	0	0	0	0
Neomycin	+	+	+	0	+	+	+
Nitrofurantoin	0	+	+	+	+	+	+
Novobiocin	0	0	0	0	+	+	+
Oleandomycin	+	+	0	+	0	0	0
Penicillin G	0	0	0	0	0	0	0
Polymixin B	+	+	+	+	+	+	+
Streptomycin	0	0	0	0	+	0	0
Sulphafurazole	0	0	0	0	0	+	+
Terramycin	0	0	0	0	0	+	+
Tetracyclin	0	0	0	0	0	+	+

Symbols: 0 = no inhibition of bacterial growth
+ = inhibition of bacterial growth