Paper presented at the
Second International
Conference on Molluscan
Shellfish Safety in Iloilo City,
Philippines, November 1997

Seafish Report No. SR511

November 1997

SEAFISH

The Sea Fish Industry Authority

Seafish Technology

The Use of Physiological Assessment Techniques for Determining the Relative Activity Rates of Bivalve Shellfish during Simulated Depuration

Paper presented at the Second International Conference on Molluscan Shellfish Safety in Iloilo City, Philippines, November 1997

Seafish Report No. SR511

Author: M. Boulter
P. Wilson
November 1997

SEAFISH

The Sea Fish Industry Authority

Seafish Technology

The Use of Physiological Assessment Techniques for Determining the Relative Activity Rates of Bivalve Shellfish during Simulated Depuration

Paper presented at the Second International Conference on Molluscan Shellfish Safety in Iloilo City, Philippines, November 1997

Seafish Report No. SR511

Authors: M. Boulter
P. Wilson

Summary

A series of trials were undertaken over an 18 month period to determine the relative activity rates for mussels (*Mytilus edulis*), Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*), cockles (*Cerastoderma edule*) and Manila clams (*Tapes philippinarum*), subjected to varying seawater temperatures and dissolved oxygen levels. All these species are currently commercially depurated in the UK. To achieve this, alternative techniques to the more traditional use of bacteriological analysis were used to establish the physiological response of bivalve molluscs to varying conditions. These were the monitoring of ammonia excretion, consumption of dissolved oxygen and uptake of a neutral red dye.

The monitoring of ammonia excretion correlated with dissolved oxygen consumption and these proved to be useful methods of obtaining information on the physiological response of bivalve molluscs subject to varying simulated depuration conditions. The information obtained could not have been achieved by bacteriological analysis. However the dye test, although already an established method, did not prove to be entirely satisfactory.

Overall the results found that both species of oyster were much less active than the other species, which may have implications for depuration systems. This work was funded by the UK, Ministry of Agriculture, Fisheries and Food (MAFF).

Table of Contents

1. The Food Safety Problem Associated with Bivalve Shellfish and the Current Control Mechanism

There is a known health risk associated with eating bivalve molluscs, especially those eaten raw or lightly cooked. Epidemiological evidence (Lees 1996, CEFAS 1997) suggests that in the UK and other developed countries, outbreaks are predominantly caused by small round structured viruses (SRSV's) of the Norwalk or Norwalk - like family which cause gastro-enteritis. Viral, not bacterial, infections are the predominant cause of infectious disease following shellfish consumption. The main species of shellfish implicated in England and Wales during recent years have been oysters.

This health risk is minimised and regulated by UK Food Safety Regulations (SI 1508 and SI 3164) which are derived from EU Directive 91/492/EEC. These Regulations require that bivalve molluscs taken from a category B classification area (the area most commonly found in England and Wales) must be treated by either controlled cooking, relaying or depuration. Depuration is the industry's most commonly preferred option.

The prescribed operating criteria for depuration systems are based upon seawater quality, temperature and flow and the loading density of the molluscs in the system. Much of the scientific research into these criteria in the UK was undertaken many years ago, at a time when only mussels and native oysters required depuration. Other species have been introduced and the existing criteria have been applied (as in the case of Pacific oysters) or criteria adopted from another country (as with clam species). To date, much of the scientific investigation of purification criteria has been made on the basis of bacterial reduction of indicator organisms. However, such bacteriological analysis usually gives only a positive or negative result. i.e. molluscs either depurated or they did not. It does not necessarily indicate how much better, one seawater temperature is, than another. Bacteriological analysis is also expensive and has the added uncertainty associated with the need to ensure high initial bacterial counts in the molluscs.

2. Activity Rate Trials using Physiological Assessment Methods

A series of trials were carried out to firstly, establish alternative physiological assessment methods for determining the activity rate of commercially harvested bivalve molluscs in simulated depuration conditions and then to compare the relative activity rates of five species of commercially harvested bivalve molluscs, at varying temperatures.

Three methods were used to assess the physiological response of bivalve molluscs. These were the measurement of ammonia excretion, dissolved oxygen (DO) consumption and the removal of dye from water. Ammonia excretion has been shown to correlate with faecal deposition in mussels (Hawkins et al, 1983), but this method has previously had only limited use in depuration investigations. In the context of this work, ammonia excretion is considered to be more an indicator of general metabolic activity. The consumption of DO represents respiration activity. The uptake of neutral red dye indicates filtration activity (Cole et al 1954).

3. Methodology and Equipment

A test unit supporting four standardised model depuration tanks was constructed for the trials. The mollusc test unit consisted of four identical test tanks, each with independent seawater circulation, aeration and temperature control systems, mounted on a bench as shown in Figure No. 1. Each tank consisted of a plastic Allibert type 12030 stack nest box of 30 litres capacity and effective internal working length of 650 mm, width 370 mm and depth of 110 mm. Seawater was drawn from one end of the tank to the other via a magnetically coupled pump. No spray bar was used, the water re-entering the tank below the water surface to prevent any re-oxygenation. Aeration was supplied instead by a solenoid air pump, feeding a porous tube aerator laid across the bottom of the tank at the water input end. Air supply was then controlled by an Oxyguard DO monitor, with a probe in the centre of each tank, which controlled the air flow to meet the required level of DO. Seawater could be heated by the use of thermostatically controlled titanium rod type heaters. A type 316 stainless steel immersion coil fitted to each tank provided chilling via a beer chilling unit, with a separate supply of water circulating through the chilling system.

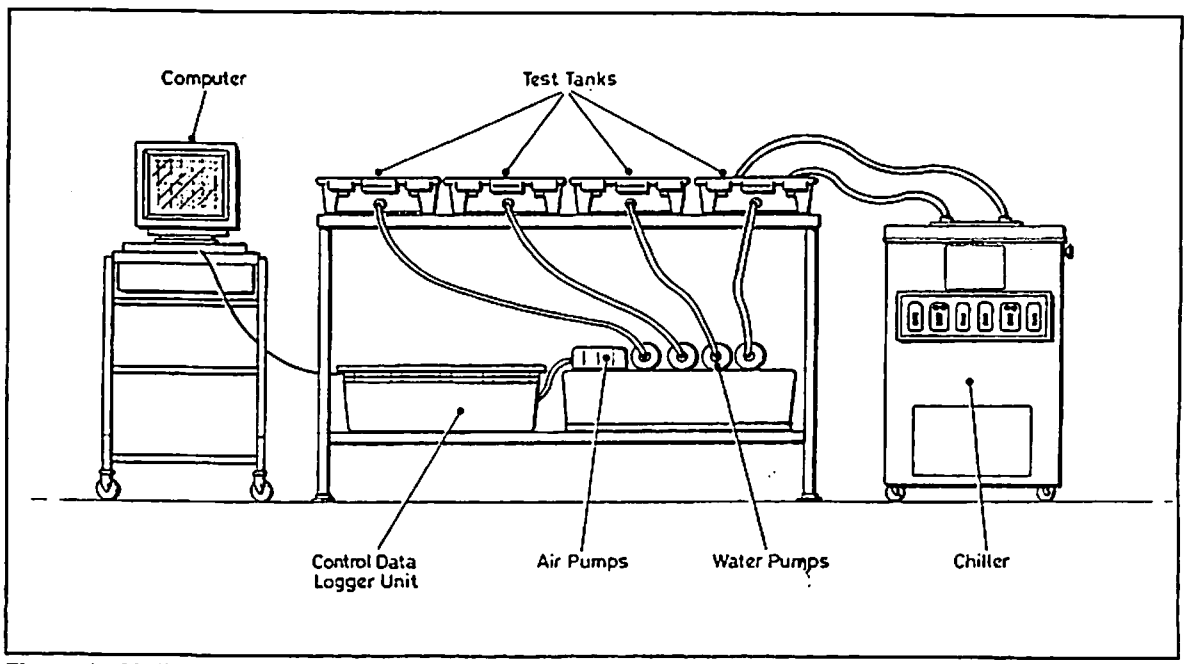


Figure 1 - Mollusc test unit

Each tank was filled with 24 litres of seawater at the required temperature and 5 kg (in-shell weight) of molluscs. An exception to this was made for cockles which because of their potentially high level of activity (Boulter et al, 1994) were limited to 2.5 kg. Each tank operated at a water flow rate of 10 litres/minute. Following trials with each batch of molluscs, some were shucked to obtain a meat yield. After each trial, the tank and its associated pipework was thoroughly cleaned and flushed through.

The accumulation of ammonia in the water was monitored using Dr. Lange type LCK 303 and LCK 317 test kits for ammonium nitrogen (NH_4N). Absorbency was measured on a HACH DR2000 spectrophotometer at a wavelength of 695 nm. The Dr. Lange kits were calibrated against ammonium nitrogen artificial seawater standards.

For each trial the seawater was tested prior to placing the molluscs in the tank. The molluscs were then immersed in the seawater. Temperature was allowed to settle at the required levels and DO was maintained at above the 80% saturation level. The seawater was tested after 24 hours and at the end of a simulated depuration period of 42-48 hours.

The approach to measurement of DO consumption was to load the tanks with molluscs and then run them for an extended period (at least overnight and normally 2 days) to stabilise the conditions and then to switch the air supply off. The single DO probe in each tank was then used to monitor the reduction in DO over a period of one hour. This was often used on completion of an ammonia monitoring trial. It was done at different seawater temperatures to obtain a relative measure of the variation in activity against temperature. DO was measured using a multi-probe Oxyguard DO meter connected to a data logger measuring in ppm.

The method used for dye removal was based upon monitoring the rate at which neutral red dye is removed from the water. The molluscs filter the dye out of the water and into their gills which stain red. 30 ml of a 1% solution of neutral red dye was mixed with 1 litre of seawater taken from the mollusc tank. This solution was then mixed with the rest of the water in the tank. The water in the tank was then sampled at half hourly intervals for the first 2 to 3 hours and then at hourly intervals up to 6 to 7 hours. Its absorbency was assessed at 450 nm, the test level for assessing turbidity in FTU's (formazin turbidity units), using a HACH DR/2000 Spectrophotometer. These trials were carried out on a similar basis to the oxygen consumption trials with the tanks running for an extended period to stabilise conditions before the dye was added.

Artificial seawater (ASW) made up to a seawater salinity of 30 parts per thousand (‰) was used throughout the trials. A standard pre-mix containing the five main constituent salts of seawater was used, obtained from Peacock Salts, Glasgow, Scotland. A fresh mix of ASW was used for every trial.

Five species of bivalve mollusc were obtained from commercial suppliers in the UK. These were: Cockles *(Cerastoderma edule)*, Manila Clams *(Tapes philippinarum)*, Mussels *(Mytilus edulis)*, Native Oysters *(Ostrea edulis)* and Pacific Oysters *(Crassostrea gigas)*. Care was taken to ensure that all molluscs used were hand gathered, freshly harvested and not subject to any unnecessary physical shock or delay in transport. On receipt at the laboratory, any gaping or damaged molluscs were discarded and any barnacle encrustation removed. Sufficient shellfish were usually obtained to carry out two or three consecutive trials sequences, within one week. They were held immersed until required in a 600 litre Seafish designed small scale depuration tank, which was used as a storage tank, at a temperature of 12° C. Molluscs were always conditioned in this holding tank for a minimum of one night

before trials to allow them to acclimatise to tank conditions and allow them to excrete any ammonia accumulated in their intra-valvular fluid as a result of dry storage during transportation to the laboratory. Molluscs for each trial were taken from the tank, debyssed if necessary, sorted to remove any further dead or damaged specimens, washed, weighed and then placed directly in the test unit. After each trial the particular molluscs used in that trial were disposed of and fresh molluscs used in the subsequent trial.

The trials were carried out over a period of 13 months between November 1995 and December 1996 and concentrated on each mollusc species in turn, as shown in Table 1 below.

Table 1 - Scheduling of trials and minimum depuration temperatures

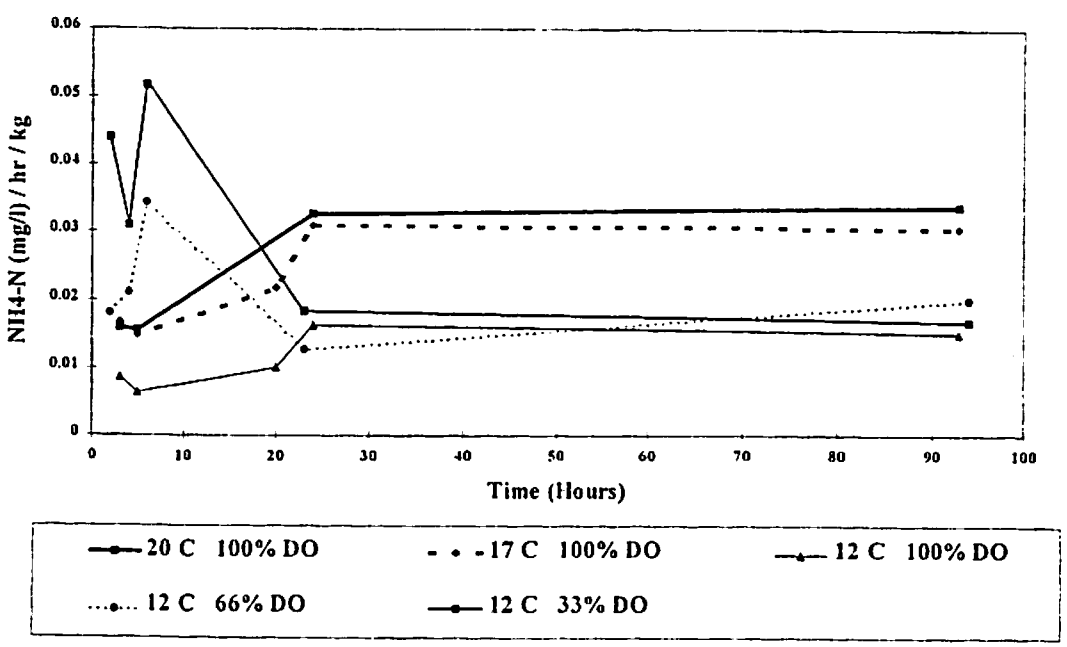
Species	Trials Period	No. of Trials	Average Meat Yields %	Minimum depuration temperature prescribed in the UK
Mussels (*Mytilus edulis*)	Nov 95 -Feb 96	60	25.0	5 °C
Pacific Oysters (*Crassostrea gigas*)	Mar 96 - June 96	60	5.8	8 °C
Manila Clams (*Tapes philippinarum*)	July 96	48	18.9	Not specified
Cockles (*Cerastoderma edulis*)	Oct 96 - Nov 96	49	21.6	7 °C
Native Oysters (*Ostrea edulis*)	Nov 96 - Dec 96	55	10.3	5 °C

4. Investigation of Ammonia Excretion as an Indicator of Mollusc Activity

In previous trials investigating the re-use of seawater (Allen et al, 1950, Wilson, 1989 and Boulter et al, 1991), the ammonia concentration in the water had been measured prior to and post depuration. This was done primarily to monitor the cumulative effect of water re-use, there being concern that beyond a certain level of ammonia concentration, mollusc activity would be inhibited. However, experience has shown that when molluscs are re-immersed in a tank after dry storage, their initial level of activity can vary considerably. If starved of oxygen they can initially be very active whereas if subject to physical or thermal shock they can exhibit little sign of activity at all. It was therefore thought prudent to measure ammonia excretion after having first given the molluscs time to settle down to the particular experimental conditions.

An initial series of trials was carried out with mussels, under varying conditions of seawater temperature and DO saturation. Ammonia excretion rates per hour (as ppm NH_4N) were measured during the first few hours of re-immersion and subsequently at 17-24 hrs and 92-95 hours. The results are shown in Figure No. 2. The results show that during the first few hours of re-immersion there is a considerable variation in ammonia excretion rate. This appears to stabilise after about 20 hours and remain consistent thereafter. On the basis of these trials it was therefore decided that ammonia samples would be taken after molluscs have been re-immersed for 24 hours.

Figure 2. Ammonia excretion rate of mussels per hour against immersion time.



5. Comparison of Mollusc Activity against Temperature in Simulated Depuration Conditions

The results are shown in Figure Nos. 3 - 6. Ammonia excretion rates against temperature, shown in Figure No. 3, indicate that Manila clams and cockles exhibit the most rapid increase in excretion rate with rising temperature, though Manila clams do not increase in activity until the temperature is above 7 °C. Mussels increase in activity at a slightly slower rate but show a similar pattern to the Manila clams, with activity not starting to rise until the temperature is greater than 9 °C. For native and Pacific oysters ammonia excretion activity was at very low levels but with a slight linear increase with rising temperature. What is most apparent is the large difference between the oyster species and the other three species. There is some narrowing of this gap when the data is corrected for meat yields, as shown in Figure No. 4, but oyster activity on the basis of ammonia excretion remains much lower than with the other three species.

Figure 3. Species comparison of ammonia excretion rates at different temperatures.

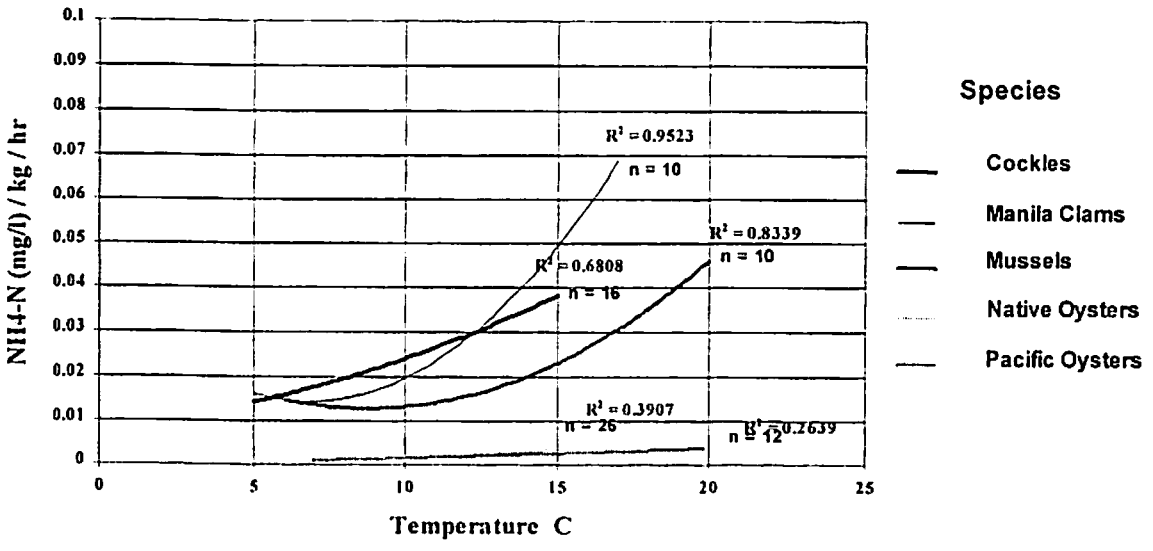


Figure 4. Species comparison of ammonia excretion rates at different temperatures corrected for meat yield.

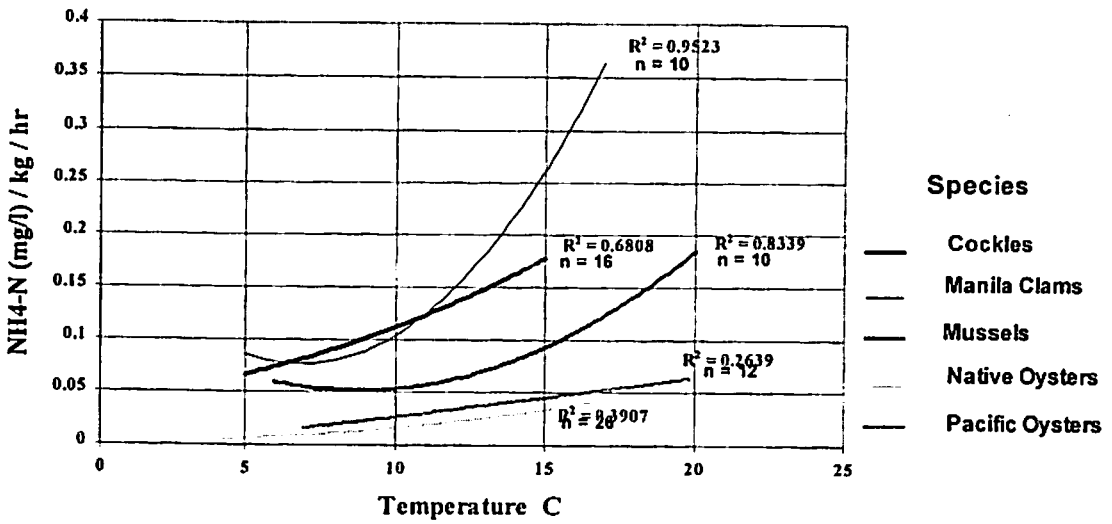


Figure 5. Species comparison of dissolved oxygen consumption rates over 1 hour, at different temperatures.

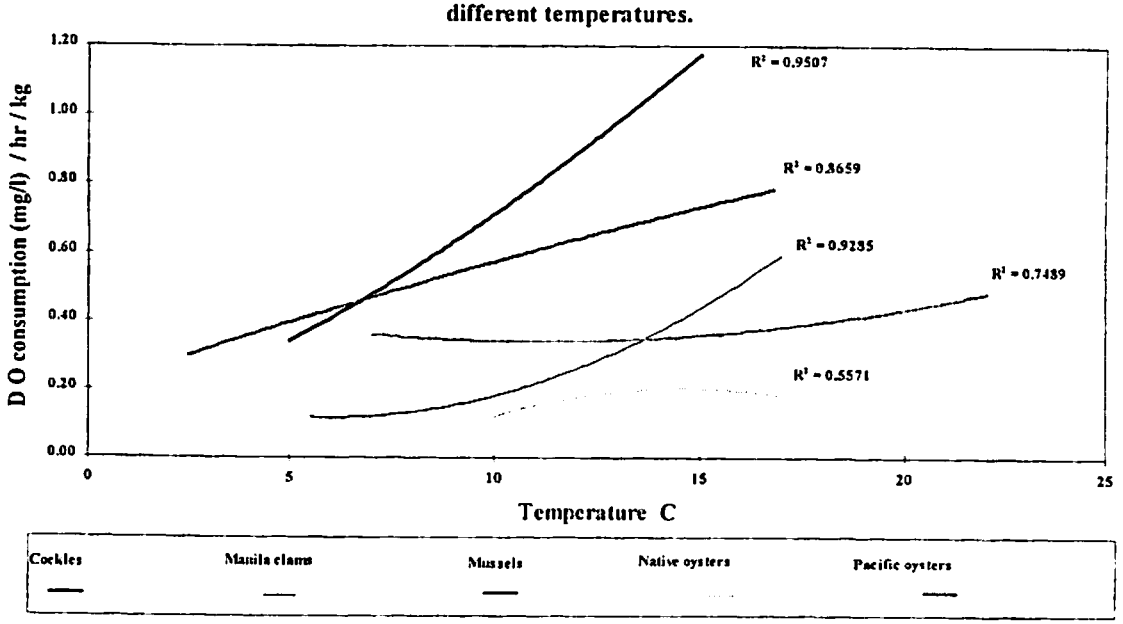
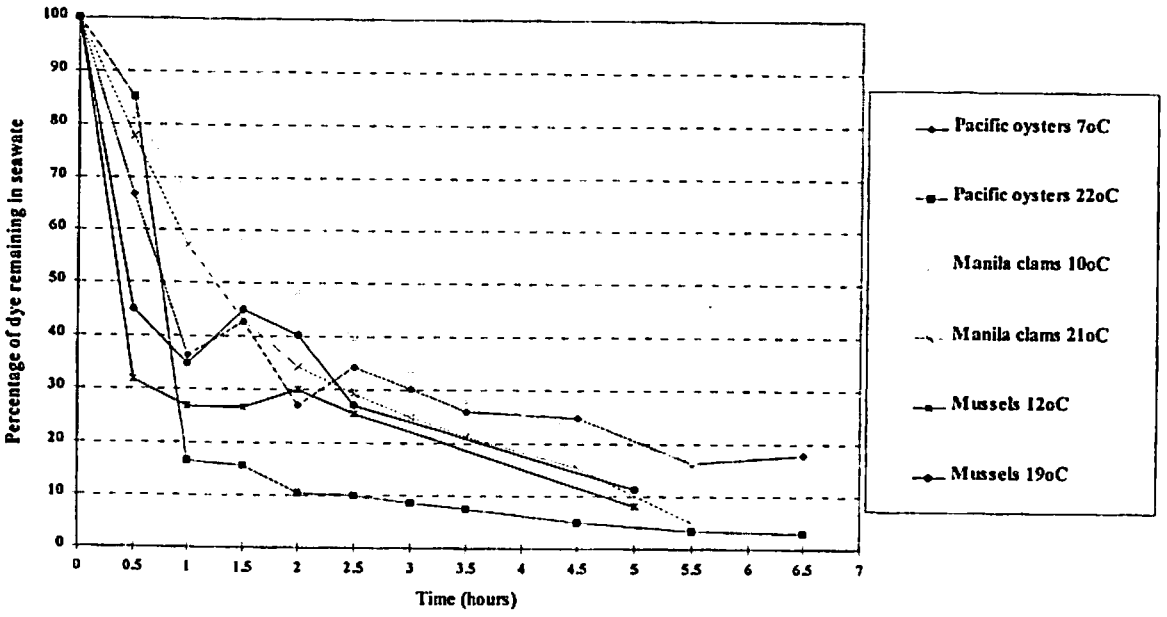


Figure 6. Comparison of dye removal rates by three species at different temperatures.



DO consumption rates against temperature, as shown in Figure No. 5, clearly show differences between the species. Cockles exhibit a rapid increase in oxygen consumption with temperature, as do mussels. Manila clams exhibit low oxygen consumption at the lower temperatures but consumption rapidly increases as temperature rises. At higher temperatures, all three species show higher oxygen consumption rates than oysters. Native oyster DO consumption is low and shows only a slight increase with temperature. Pacific oyster DO consumption although not so low also shows little increase as temperature rises.

Dye reduction data, Figure No. 6, showed little difference in dye removal rates between the species and little difference with temperatures in the range 5 - 20 °C.

5.1 Effectiveness of the Physiological Response Techniques

Ammonia Excretion

The technique appears to be useful and backs up observation of which species appear the most or least active in a depuration tank environment (personal observation and communication with depuration tank operators). The results for the oyster species showed lower significance levels, as can be seen by the R^2 values for the trend lines, (See Figure Nos. 3 and 4), indicating that there was more variance in ammonia excretion activity with these less active oyster species. Notwithstanding this, the results obtained compared quite well with those from the monitoring of DO levels. An advantage of the ammonia excretion technique was that it was generally run over 42 hours, which meant that the results related well to the depuration process. Unfortunately, numerous trials are

required for the technique to give any meaningful results which can be time consuming and costly. However, as a technique for measuring the physiological response of molluscs in varying immersed conditions, the measurement of ammonia excretion proved very useful. The information obtained could not have been readily achieved by bacteriological analysis.

Dissolved Oxygen Consumption

This technique was useful in that it could usually be run over only a few hours. The rate of reduction of DO at a particular temperature giving a good indication of mollusc activity rate. Oxygen consumption measured over one hour was a useful parallel to the measurement of ammonia excretion, giving some confirmatory and some further information on the physiological response of molluscs to varying conditions. In many cases, this technique was used following a particular ammonia excretion trial and so enabled direct comparison/confirmation.

Dye Removal

Although based on an already established method this technique did not prove entirely satisfactory for these trials. It is thought that due to being held in a simulated depuration environment, hence a no food environment, the shellfish reacted to the presence of the dye in the water and took it to be a food source, leading to a fairly standard filtration rate regardless of temperature or species. Dye trials have previously proved useful for determining the point of shellfish inactivity, such as when low temperature or salinity thresholds are being identified. The technique also suffers from the practical disadvantage that the dye has to be removed from the trials equipment after each use.

5.2 Relevance of this Work to Depuration Systems

The current UK stipulations for minimum depuration temperature (Table 1) are largely based on observation of whether or not the molluscs are functioning, rather than measurement of the level at which they are functioning. These results show that by operating at a water temperature of 15 °C rather than the stipulated minimum temperatures, the activity of the mollusc species studied would be at least double that at the stipulated minimum temperatures.

There is concern about the generally low level of activity of both species of oysters together with the associated facts, that they are often eaten without cooking and are implicated in the majority of recorded incidences of food poisoning, caused by bivalve molluscs in the UK.

6. Conclusions and Recommendations

The monitoring of ammonia excretion combined with dissolved oxygen consumption proved to be a useful method of obtaining information on the physiological response of bivalve molluscs to varying seawater conditions.

The activity of the molluscs increases with seawater temperatures over the range of temperatures investigated (up to about 20 °C). By operating at 15 °C the rate of activity of the molluscs is at least double that at the minimum temperatures currently specified in the UK for depuration system operation.

7. Literature Cited

1. ALLEN, L.A. et al. Repeated Re-use of Seawater as a Medium for the Functioning and Self Cleansing of Molluscan Shellfish. The Journal of Hygiene, Vol. 48, No. 4, December 1950.

2. ANON. Council Directive of 15 July 1991 laying down the Health Conditions for the Production and the Placing on the Market of Live Bivalve Molluscs. 91/492/EEC.

3. BOULTER, M. and DENTON, J.W. Repeated Re-use of Artificial Seawater in a Small-Scale Vertical Stack Oyster Purification Plant under Development by Seafish. Seafish Report No. 459, January 1991.

4. BOULTER, M., WILSON, P. and DENTON, J.W. Trials to Assess the Viability of Purifying Cockles. Seafish Report No. 443, May 1994.

5. COLE, H.A. and HEPPER, B.T. The Use of Neutral Red Solution for the Comparative Study of Filtration Rates of Lamellibranchs. Journal due Conseil 20, pp 107-203. 1954.

6. CEFAS Shellfish Depuration HACCP Study. Draft. March 1997.

7. HAWKINS, BAYNE and CLARKE. Co-ordinated Rhythms of Digestion, Absorption and Excretion in *Mytilus Edulis*. Marine Biology 74, pp 41-48. 1983.

8. LEES, D. Bivalve Molluscs and Public Health. MAFF Shellfish News. No 1. May 1996.

9. SI 1508. The Food Safety (Live Bivalve Molluscs) (Derogations) Regulations. 1992.

10. SI 3164. The Food Safety (Live Bivalve Molluscs and Other Shellfish) Regulations. 1992.

11. WILSON, P. Commercial Trial of a Multi-Layer Mussel Purification Tank with Re-use of Artificial Seawater. Seafish Report No. 355. March 1989.