Scope for growth and levels of contaminants in mussels from the Wash - Phase 2

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CONTENTS

Page

Figures

Tables

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GLOSSARY

CR	Clearance rate or suspension feeding rate
DoE	Department of the Environment
GC	Gas chromatography
GC-ECD	Gas chromatography - electron capture detection
GC-FID	Gas chromatography - flame ionisation detection
GC-MS	Gas chromatography - mass spectrometry
HPLC	High performance liquid chromatography
NR	Neutral red retention in blood cells
NRA	National Rivers Authority
PAH	Polyaromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PML	Plymouth Marine Laboratory
SFG	Scope for growth

EXECUTIVE SUMMARY

● The Wash embayment is an important area both in terms of nature conservation (RAMSAR site and Special Protection Area) and commercial fisheries (mussels and cockles). However, during the past decade many studies, particularly those concerning assessment of shellfish stocks, bird populations and pollution impact, have highlighted possible environmental problems in the area.

● The objectives of this study were: i) To quantify the degree of pollution impact in the Wash by measuring the scope for growth (SFG) of mussels (*Mytilus edulis*) collected from nine sites:- Stiffkey, Brancaster, Hunstanton, Pandora Sand, Great Ouse - Barrier Wall, Gat Sand, Roger Sand, Witham - Tabs Head, and Holy Island (an established 'clean reference' site); ii) To analyse the concentration of specific organic contaminants in the mussel tissues (i.e. aromatic hydrocarbons); iii) To provide a toxicological interpretation of the tissue residue data based on established relationships between tissue concentrations of aromatic hydrocarbons and the stress response SFG; iv) To extract, fractionate, and toxicity test fractions derived from tissues of Pandora Sand and Holy Island mussels; and v) To analyse the most toxic fractions by GC and GC-MS.

● Sampling and measurement of mussels was carried out in July 1995. The results of this study show that the SFG values of mussels living in the Wash are significantly lower than mussels inhabiting open coastal sites receiving minimal contamination. This reduction in SFG is primarily due to inhibitory effects on the ciliary feeding rate.

● Mussels from sites near the mouth of the two major rivers entering the Wash (Great Ouse and Witham) had the lowest SFG values and were the most stressed (i.e. Barrier Wall, Pandora Sand and Tabs Head). SFG measurements of mussel populations along the eastern coast of the Wash showed a gradual improvement in water quality (i.e. increase in SFG) from the Great Ouse - Barrier Wall, Hunstanton, Brancaster to Stiffkey. Mussels living in the central part of the Wash (e.g. Gat Sand and Roger Sand) had intermediate SFG values between those severely stressed populations living near the mouths of Great Ouse and Witham rivers and those on the north Norfolk coast.
The wider ecological consequences of low SFG may include reduced egg / larval quality, poor recruitment and growth, all of which will hinder recovery and sustainability of resources when combined with any possible over-fishing of mussel stocks.

● The SFG data for July 1995 were consistent with the results recorded in a pilot Wash study in July 1994 and an earlier North Sea mussels programme (July 1990).

● The aromatic hydrocarbon data for July 1995 were also in close agreement with the earlier Wash study (July 1994) and the North Sea mussels survey (July 1990). Measured values are approximately 5-fold higher than background levels in open ocean waters (e.g. Shetland Islands), but 50-fold less than the levels typical of the major urbanised / industrialised UK estuaries. Toxicological interpretation of the hydrocarbon concentrations in the mussels indicates that these levels can explain a significant but a relatively small proportion of the recorded decline in SFG.

● Mussel tissues from Pandora Sand and Holy Island were extracted / fractionated and the fractions were toxicity tested using two assays (clearance rate of small mussels and neutral

red retention by mussel blood cells). These results confirmed the 1994 pilot study and indicated that mussels from both Pandora Sand and Holy Island contain a complex and physicochemically diverse mixture of 'potentially toxic' chemicals (biogenic and anthropogenic) and that these were more evident in mussels from Pandora. Selected fractions have been analysed by HPLC, GC-FID and GC-ECD. These demonstrate the presence of higher levels of some compounds in the Pandora fractions compared with Holy Island. Attempts to confirm the identity of these compounds by GC-MS met with only limited success due to the low levels of the individual chemicals present, and in some cases to their likely polar and involatile nature rendering them unsuitable for direct GC-MS analysis.

● The spatial changes in both SFG and hydrocarbon contaminant levels in mussels from the Wash sites reflect the hydrographic data and the input of contaminants via the Great Ouse and the Witham. The output of a hydrodynamic model illustrates that water enters the Wash along the central channel and circulates away from the central region (Gat Sand) and out along the north-western and south-eastern coastlines. Therefore any contaminants entering the Wash via the Great Ouse will be transported out, along the north Norfolk coastline in the following sequence (Barrier Wall > Pandora Sand > Hunstanton > Brancaster > Stiffkey).

● The SFG results and the complexity of the tissue residue chemistry data are not inconsistent with the hypothesis that the adverse effects are related to the complex sewage inputs via the major rivers entering the Wash. This is supported by evidence from the literature which indicates that sewage inputs can have significant adverse effects on the SFG of mussels.

● Recommendations for further work are outlined. This includes using SFG to examine the possible improvement and recovery in environmental quality following the upgrading of a sewage treatment works on the Great Ouse in the autumn of 1996; as well as the further refinement and application of the important investigative approach to 'Environmental Diagnostics' which involves tissue extraction / fractionation / toxicity testing / chemical analysis.

Keywords:- Wash, Mussels, *Mytilus edulis*, Pollution, Contaminants, Hydrocarbons, Sewage.

1. INTRODUCTION

1.1 The Wash system

The Wash embayment with its four main tributary estuaries, the Witham, Welland, Nene and Great Ouse, forms the largest estuarine system in the UK draining a catchment area of 15,650 km^2, about 12% of the area of England. The surface area of the Wash between Gibraltar Point and Hunstanton is about 700 km^2 at high water on a spring tide, 50% of which is exposed as sandbanks and intertidal mudflats at low water.

The Wash is a site of international and national importance for nature conservation and was declared a RAMSAR site and Special Protection Area (SPA) in 1988. It is an area which supports substantial coastal wetlands, migratory and breeding bird populations, seal populations and several commercial fisheries, including important mussel and cockle fisheries.

1.2 Environmental quality

For more than a decade there has been a general decline in the shellfish populations of the Wash, which has largely been attributed to poor recruitment and over-fishing (P. Dare, unpublished data; Walker and Palmer, 1990; NRA, 1994). This has led to the creation of reserves, within which the collection of mussels and cockles is prohibited, thus encouraging the recovery of depleted stocks. In addition, the reduction in shellfish populations has been accompanied by a decline in bird populations, particularly of the oystercatcher, which feeds on mussels and cockles (Goss-Custard, unpublished data).

These observations are supported by quantitative data on sublethal stress effects in mussels from sites along >1000 km of UK North Sea coastline (Widdows, et al. 1995a). The North Sea study identified major estuaries / regions, as well as specific sites, that were significantly stressed by pollutants. One such area was the Humber - Wash region where mussels were found to have significantly reduced scope for growth. The tissue residue analysis of Wash mussels confirmed that the reduced scope for growth was not due to high levels of industrial contaminants (e.g. PAHs and PCBs), but other contaminants including sewage related chemicals and agrochemicals were not analysed and their relative importance as pollutants not established.

1.3 The use of mussels for assessing environmental quality

Mussels are widely used as 'sentinel' organisms for monitoring spatial and temporal changes in chemical contamination of the marine environment. Bivalves, and particularly mussels, have several important attributes:-

i) They have a widespread distribution and are dominant members of coastal and estuarine communities;

ii) They are sedentary and are therefore better than mobile species as integrators of chemical contaminants in a given area;

iii) They are suspension feeders that pump large volumes of water (several litres per hour) and bioconcentrate metals and organic contaminants in their tissues with minimal metabolic transformation.

However, in order to establish whether the accumulated chemical contaminants are inducing deleterious effects, or whether all potential toxicants are being analysed, it is necessary to combine chemical analyses of body tissues with appropriate measures of biological impact.

Growth provides one of the most sensitive measures of stress in an organism, since growth represents an integration of major physiological responses and specifically the balance between processes of energy acquisition (feeding and digestion) and energy expenditure (metabolism and excretion). Each of these physiological responses can be converted into measures of energy flow ($J\ g^{-1}h^{-1}$) and alterations in the amount of energy available for growth and reproduction (termed scope for growth) can be quantified by means of the energy budget. Therefore scope for growth (SFG) provides an instantaneous measure of the energy status of an animal, which can range from maximum positive values under optimal conditions, declining to negative values when the animal is severely stressed and utilising body reserves.

Recent advances in environmental toxicology involving the close coupling of the sensitive stress response (scope for growth - SFG) and contaminant levels in the tissues of mussels (*Mytilus edulis*) has provided a powerful and cost-effective method of assessing environmental pollution with rapid feedback of information to environmental managers (Widdows and Donkin, 1992; Widdows et al. 1995a,b). This approach not only enables a rapid detection and quantification of the biological effects of pollution, but also provides a means of identifying the cause(s) through toxicological interpretation of the tissue residue chemistry using an ever increasing toxicological database. SFG has been shown to be consistently one of the most sensitive and informative biological stress effects measurements in environmental pollution studies in the UK, Europe, USA, India, Asia and New Zealand.

In a recent North Sea study (Widdows et al. 1995a), the approach has been successfully extended and applied over a large spatial scale of >1000km of UK North Sea coastline (collaborative study between PML and MAFF Burnham). SFG measurements were able to detect and quantify changes in environmental quality, as well as identify the cause(s) of pollution through the use of QSARs (Quantitative Structure-Activity Relationships) and established cause-effect relationships (i.e. between concentration of contaminants in mussel tissues and the SFG response).

The North Sea mussels study identified major estuaries / regions, as well as specific sites, that were significantly stressed by pollutants. This included the Humber - Wash region where mussels had significantly reduced SFG values, although only c. 50% of the decline in SFG could be explained by the chemical contaminants analysed. These earlier findings were then confirmed in a 'follow-up' pilot study carried out for the Anglian NRA in July 1994 (Widdows et al. 1995c). The 1994 pilot study demonstrated that mussels living in the Wash had lower SFG values than those living on relatively uncontaminated open coastal sites in the NE of England (Holy Island). Lowest SFG values were found in mussels from the inner part of the Wash at Pandora Sand, near the mouth of the Great Ouse, and at Hunstanton. Selected chemical contaminants were also analysed (e.g. aromatic hydrocarbons, nonylphenols and silver). Subsequent toxicological interpretation of the levels found in the mussel tissues revealed that bioaccumulated aromatic hydrocarbons could only explain a relatively small proportion of the recorded decline in SFG, and there was no evidence of a significant accumulation of Ag or nonylphenols. Spatial changes in both SFG and hydrocarbon contaminant levels in mussels reflected the hydrodynamics of the Wash and indicated that the Great Ouse was one likely source of contaminant inputs. The second phase of the pilot

study was concerned with developing a new 'environmental diagnostic' approach that could provide a means of extending these findings and identifying 'unknown toxicants' present in the environment and accumulated by mussels.

The primary objectives of the 1995 Wash study were:-

1) To quantify spatial changes in environmental quality in the Wash in terms of the scope for growth of mussels. Such information will not only highlight those areas most severely stressed by contaminants but also indicate those areas which are least stressed and are therefore most suitable for shellfish growing and relaying of bivalve spat.

2) To determine the concentration of aromatic hydrocarbons in the mussel tissues (a widespread and major environmental contaminant) and to provide a toxicological interpretation of the tissue residue data based on established tissue concentration - response relationships.

3) To provide biological effects data that will help identify sites where the observed effects on SFG are significantly greater than predicted from the available tissue residue chemistry data.

4) To apply a tissue extraction / fractionation / toxicity testing approach to help identify the nature of 'unknown toxicants' and to apply a range of analytical techniques (e.g. GC-ECD, GC-FID, GC-MS) to identify potentially toxic compounds in the toxic fractions.

2. MATERIALS AND METHODS

In this study, procedures for sampling mussels, measurement of scope for growth and analysis of aromatic hydrocarbons in the tissues were identical to those adopted in the North Sea mussels programme (DoE Contract, Widdows et al. 1995a).

Ten days prior to the sampling of mussels, high quality seawater was collected offshore from the Eddystone (SW England) and placed in an aquarium ($3m^3$ total volume) where it was recirculated, filtered and mantained at 15°C.

Mussels (*Mytilus edulis*) of standard size (4cm shell length) were collected from eight sites in the Wash (Table 2.1; Fig. 2.1) and from an established clean reference site at Holy Island (Widdows et al. 1995a).

Two hundred mussels were sampled from each site at low water spring tides. They were immediately packed in polystyrene insulated containers with four frozen ice-packs (two at the bottom and two at the top) and thick absorbent material (disposable nappies) providing insulation between the mussels and the ice-packs. The containers were sealed and transported overnight to the PML via Datapost. Mussels were held air exposed and cool (c. 5°C) for a period of 20h before unpacking, recording the temperature and re-immersing in Eddystone filtered seawater (EFSW).

'Physiology' mussels (n=16) were cleaned of epibionts and sediment, placed in the aquarium and allowed 24h to recover from aerial exposure / transportation before measurement of their physiological responses, such as clearance rate (= feeding rate), food absorption efficiency, respiration rate (= energy expenditure) and scope for growth, as well as shell length and dry tissue weight. Physiological responses were determined under 'standard' conditions (15°C, 33 ppt and an algal cell concentration of 10×10^3 *Isochrysis galbana* cells ml^{-1} or 0.34mg L^{-1}). 'Organic chemistry' mussels (n=50) were cleaned and depurated in EFSW for 1 h. All mussels were frozen and stored at -25°C until analysed for chemical contaminants.

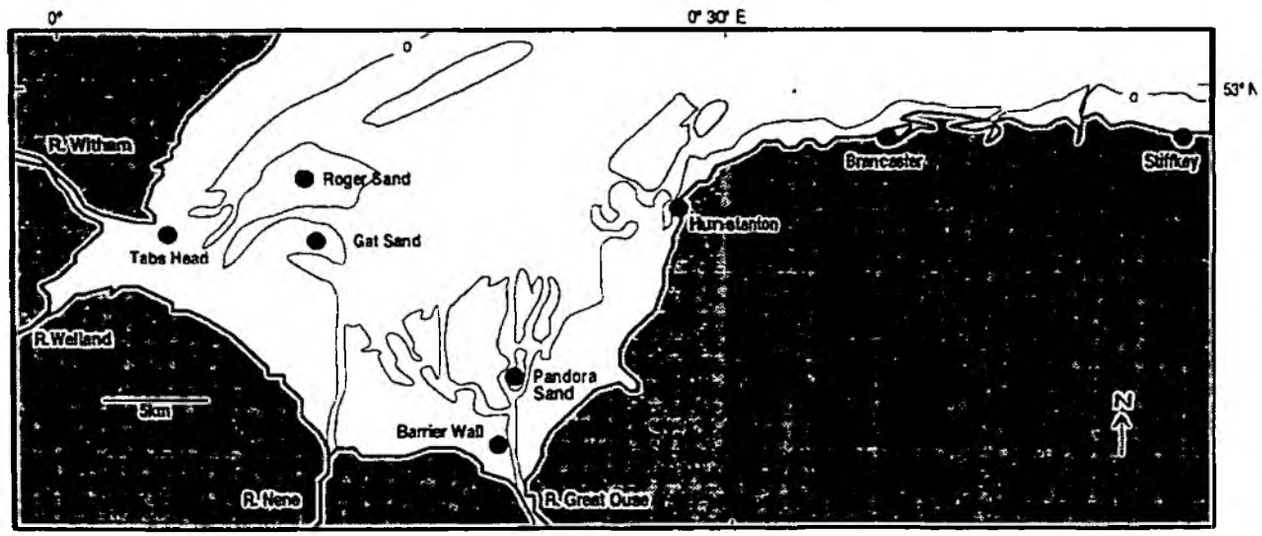

Figure 2.1 Map showing location of sampling sites within the Wash.

2.1. Measurement of physiological responses and scope for growth.

Clearance rate, or the volume of water cleared of suspended particles h^{-1}, by individual mussels was measured in a open-flow system with a flow rate of 170 ml h^{-1}. The removal of algal cells (*Isochrysis galbana*) by the mussels was determined by means of an electronic particle counter (Coulter Counter). Food absorption efficiency was measured by the ratio method (Conover, 1966) which compared the proportion of organic to inorganic matter in the faeces with that in the algal food. Respiration rates by individual mussels were measured simultaneously in closed glass respirometers by monitoring the rate of decline in the partial pressure of oxygen using Radiometer oxygen sensors connected to Strathkelvin oxygen meters. The mass-specific physiological rates were converted into energy equivalents and used in the balanced energy equation to calculate scope for growth, or the energy available for growth and reproduction. For further details of methodology see Widdows (1993).

2.2. Chemical Analysis: Polyaromatic hydrocarbons

Two groups of ten mussels from each site were dissected immediately following the 1 h depuration. The resulting tissues were bulked into two solvent-cleaned glass jars with foiled-lined screw caps and stored frozen (-25°C). In the first stage of the analytical procedure the tissues were thawed, homogenised at 0°C, and 6 g sub-samples extracted by saponification / steam distillation into iso-hexane. The distillates were analysed for 2 and 3 ringed aromatic hydrocarbons by means of high performance liquid chromatography (HPLC) on an amino-cyano column (Whatman Partisil-5 PAC, 250 x 2mm) eluted with iso-hexane at a flow rate of 0.6 ml min^{-1} (Donkin and Evans, 1984). Each distillate was analysed twice on HPLC and analysis repeated if the results were outside the range of ± 5%. The 2 and 3 ring aromatic hydrocarbons were quantified by reference to 2,3-dimethylnaphthalene and 1-methylphenanthrene respectively.

2.3. Tissue Chemical Residue Fractionation and Toxicology

The procedures used were a modification of the method described by Castaño et al. (1994) who tested the toxicity of fractionated extracts from fish and mussels which had been exposed to the effluent from a fish-processing factory. Their method was an extension of well established procedures (used by the US EPA) for identifying the toxic component(s) in complex industrial effluents (Burkhard et al., 1991).

2.3.1. Extraction of mussel tissue

Tissues homogenates of mussels from Holy Island and Pandora Sand were stored at c. -17 ° C in glass jars following collection and initial analysis for hydrocarbons in the summer of 1995. They were thawed, re-homogenised in an ice-bath and a 20 g aliquot of wet tissue taken.

The tissue samples were extracted at room temperature with solvents using procedures similar to those commonly applied in multi-residue analytical protocols (Holland & Malcolm, 1992). Tissue (20g) was homogenised over ice with 40 ml of acetone (HPLC grade). The acetone was filtered off using a sinter-glass funnel to which suction was applied. The suction was released and the tissue on the filter mixed with 10 ml of acetone which was then removed by suction. This procedure was repeated with a further 10 ml aliquot of acetone. The tissue was then removed from the sinter and re-homogenised with 20 ml of

Table 2.1 Sampling sites for mussels (*Mytilus edulis*).

Site	Date collected	Temperature °C	Salinity (psu)	Dissolved oxygen (%)	Location: GPS Position
Holy Island (ref.)	31st July 1995	15.2	34.1	114	[55° 40.094N 000° 48.140W]
Roger Sand	2nd August 1995	-	33.5	92	[52° 57.312N 000° 10.608E]
Witham Tabs Head	3rd August 1995	21.7	33.5	85	[52° 56.245N 000° 05.264E]
Gat Sand	10th July 1995	23.5	32.1	98	[52° 55.551N 000° 10.700E]
Great Ouse Barrier Wall	20th July 1995	20.5	29.4	70	[52° 49.397N 000° 21.219E]
Pandora Sand	1st August 1995	21.2	32.9	76	[52° 51.889N 000° 10.608E]
Hunstanton	13th July 1995	21.1	31.5	91	[52° 57.263N 000° 29.561E]
Brancaster	12th July 1995	21.0	31.2	140	[52° 58.531N 000° 40.953E]
Stiffkey	11th July 1995	17.2	29.8	-	[52° 57.825N 000° 58.127E]

HPLC grade dichloromethane which was then filtered off in the sinter-glass funnel. The tissue on the filter was rinsed with mixing using 3 x 10 ml aliquots of dichloromethane.

The acetone and dichloromethane extracts were combined in a separating funnel, 0.6g of solvent cleaned Analar sodium chloride added and the funnel swirled gently. The bottom dichloromethane layer was removed then the remaining aqueous layer re-extracted with 3 x 10 ml of dichloromethane. The solvent extracts were combined and reduced to a volume of 0.5 ml by a combination of rotary evaporation and evaporation under a stream of nitrogen. The solvent was replaced by methanol by adding 0.5 ml of methanol to the sample, then evaporating to the original volume of 0.5 ml under a stream of nitrogen. This procedure was then repeated to ensure that the dichloromethane had been removed.

This extraction procedure was a modified version of that used in the 1994 Wash mussel study (DFR-01/578/3/A). In 1994, dichloromethane extracts from the tissues were kept separate but no time was available for fractionation and toxicity testing. By combining both acetone and dichloromethane extracts, the chemicals present in both were fractionated and toxicity tested in a single step. Two replicate extracts were prepared from the 1995 samples. The first was fractionated and toxicity tested (see below) within a few days of preparation, but the second was stored in dichloromethane/acetone (5 ml) at c.a.-17°C in the dark for 2 months prior to final evaporation, methanol transfer and fractionation.

2.3.2. HPLC fractionation of dichloromethane extracts

300 µl of extract in methanol was injected onto a 250 x 4.5 mm HPLC column fitted with a guard cartridge. Both column and cartridge were packed with 5 µm ODS silica. The column was eluted with a methanol / water solvent system as recommended for fractionation / toxicology experiments by Burkhard et al. (1991).

The column elution conditions were modified during the course of this study. The conditions used to fractionate the first Holy Island / Pandora sample extract pair were identical to those employed in the 1994 study and were as follows. The column was eluted with a solvent gradient (flow rate 1 ml min^{-1}) beginning with 30% (v/v) methanol in water for the first 5 min increasing to 90% methanol at 29 min and 100% at 40 min. Pure methanol elution was continued until 46 min then the solvent composition returned to starting values by 50 min. Elution of light absorbing components was monitored with a dual channel u.v. detector set at 220 and 254 nm. Fractions were collected at 5 min intervals from the 5 min preceding sample injection (solvent blank) then throughout the solvent programme. The collected fractions were divided into a 2 ml aliquot for the neutral red assay and 3 ml for the mussel feeding rate assay.

Due to the high viscosity of the concentrated samples, the HPLC system suffered high back-pressures at the start of the run which could have adversely influenced chromatographic repeatability. To minimise this problem, for the second fractionation run (carried out with the remaining 200 µl of the methanol solution of the first tissue extract) a reduced solvent flow rate of 0.5 ml min^{-1} was utilised. The fractions collected were again of 5 ml volume; overall run time and programme rate of change were modified to extend the solvent gradient over the full period of sample collection.

This procedure was partially successful, so for the final fractionation experiment (with the second tissue extract), the flow rate was further reduced to 0.4 ml min^{-1}. Observation of the chromatograms from the earlier fractionation experiments showed that peaks were continuing

to be eluted as the solvent programme started to return to a more aqueous composition at the end of the run. In order to recover these peaks, 100% methanol composition was maintained until the final fraction was collected. The solvent programme used for this final fractionation experiment was as follows (flow rate 0.4 ml min^{-1}): 30% (v/v) methanol in water for the first 12.5 min, 90% methanol by 70.1 min, 100% methanol by 96.4 min and retained until 150 min then return to start conditions by 160 min.

Between each fractionation run, at least two chromatographic cycles were run following injection of a pure methanol-water mixture. If any peaks which could not be attributed to the solvent were observed following the second run, the clean solvent cycle was repeated.

2.3.3. Toxicity testing of fractions

2.3.3.1 Feeding rate of mussels

Some procedural modifications were introduced during the course of this series of experiments, although for the first set of fractions prepared from the first tissue extract, the same procedure as used in the 1994-95 series of experiments was adopted. This is described in the following paragraph.

Mussels ranging in shell length from 7.5 to 10 mm were collected from Whitsand in Cornwall. The 3 ml fractions were reduced in volume by evaporation under a stream of nitrogen. Evaporation of the aqueous fractions was continued until all the methanol was removed. The pure methanol fractions were reduced to 0.5 ml. The fractions were then transferred to 160 ml of filtered seawater (filter pore size of 0.45 µm). Ten mussels were then exposed individually for 24 h to 15 ml of seawater solution contained in scintillation vials. Following exposure, 0.5 ml of a culture of the unicellular alga *Isochrysis galbana* was added to each vial to give a final concentration of 12000 cells ml^{-1}. After precisely 10 min, the liquid was poured off into another vial and the number of algae present measured using an electronic particle counter (Coulter Counter). By comparing counts with those of controls without mussels, the feeding rate of the mussels exposed to the HPLC fractions could be calculated.
This procedure measures the rate of clearance of algal particles from the water column and is generally termed the clearance rate (CR).

In an attempt to increase the sensitivity of the procedure by increasing the exposure concentration, the fractions from the second experiment were made up to a final volume of 110 ml in filtered seawater and 10 ml of the resulting solution added to the individual mussels in the vials. After 24 h, algae were added to each vial in 5 ml of filtered seawater to achieve the 15 ml volume required for accurate counting. Also, for fractions 7 to 11, following the 24 h exposure period, the seawater / toxicant mixture was poured off then 10 ml of clean filtered seawater added to each vial to allow the mussels to purge themselves of any particulate material present in the toxicant mixture. After exactly 10 min, this water was poured off and replaced by another 10 ml of filtered seawater and the clearance rate procedure continued as described above.

For the final fractionation experiment carried out on the second tissue extract, the CR of the mussels exposed to fractions 1 to 6 was measured as previously, then the animals exposed to clean water in the same way as the mussels exposed to fractions 7 to 11 and the CR determined again. For all fractions, the volume of seawater added to the mussels at the washing step was reduced to 5 ml and the washing time to 5 min.

2.3.3.2 Dye retention in lysosomes of mussel blood cells

The 2 ml fractions were evaporated under a stream of nitrogen. When all the methanol had been removed from the aqueous fractions, 200 µl of dimethylsulphoxide (DMSO) was added and the volume of the fractions made up to 1 ml in high purity water. The pure methanol fractions were evaporated to approximately 200 µl, 200 µl of DMSO added, then the methanol evaporated off. The final volume was adjusted to 200 µl of DMSO as necessary. DMSO is of very low toxicity to this assay.

The neutral red dye retention assay was carried out on mussel blood cells as described by Lowe et al., (1995a, 1995b). Each assay was replicated using blood cells from 10 mussels. Aliquots of the concentrated fractions were added to the suspension of blood cells; 10 µl from the aqueous fractions and 2 µl from the more concentrated methanol fractions.

2.3.4. Chemical analysis of toxic fractions

Selected concentrated fractions prepared for the neutral red retention assay were analysed by gas chromatography (GC). An initial comparative screening procedure between the Holy Island and Pandora fractions was carried out using flame ionisation and electron capture detection (FID and ECD). For FID analysis, all fractions were injected directly into the GC. Because high concentrations of DMSO interfered with the ECD, the fractions in pure DMSO were extracted into 200 µl of ethyl acetate, or methyl *tertiary*-butyl ether, dried over sodium sulphate and the solvent volume readjusted to 200 µl before analysis. The GC analyses were carried out on Hewlett Packard 5890 chromatographs under the following conditions. Fused silica DB-5 capillary columns (30 m x 0.32 mm i.d.) were programmed from 40°C to 100°C at 70°C min^{-1}, then to 300°C at 5°C min^{-1}, a temperature which was held for 10 min. The carrier gas was helium and injection volumes 1 µl using an on-column injector.

Identification of selected peaks was carried out by GC-Mass Spectrometry (GC-MS) on a Hewlett Packard 5972 Series Mass selective Detector connected to a HP 5890 GC. The GC column was a 30 m x 0.25 mm i.d. fused silica capillary which was programmed at 20°C/min between 70° and 175°C, at 0.8°C min^{-1} between 175° and 181°C, at 3°C min^{-1} between 181° and 280°C, then held isothermally for 2 min. Total run time was 48.75 min. In order to better resolve the peaks in some samples, a programme covering the same temperature range at an incremental rate of 5°C min^{-1} was used.

3. RESULTS AND DISCUSSION

3.1. Biological Effects - Physiological Responses and Scope for Growth

The major physiological responses which form components of the energy budget including the integrated response scope for growth (SFG) of mussels from sites in the Wash and from the reference site at Holy Island are presented in Table 3.1 and Figure 3.1. Significant differences among sites were recorded in the clearance rate, respiration rate, food absorption efficiency and SFG of mussels.

The clearance rates (=feeding rates) were significantly reduced ($P<0.01$) at the inner sites in the Wash (Tabs Head, Barrier Wall and Pandora Sand) and gradually increased along the north Norfolk coast out to Stiffkey. The clearance rate at this outermost site was more than twice the rate at the innermost Wash sites. Those sites in the central-western part of the Wash (Gat Sand and Roger Sand) had intermediate clearance rates that were significantly ($P<0.05$) higher than at Hunstanton but less than Brancaster and Stiffkey. The clearance rates at the outer sites of Stiffkey and Brancaster were significantly lower ($P<0.05$) than the rates at the Holy Island reference sites, but this difference was not marked.

Respiration rates of the mussels followed a trend similar to the clearance rate and were significantly lower ($P<0.05$) in the western and inner part of the Wash and were highest along the eastern coastline.

Food absorption efficiencies were more variable than normal, probably due to some technical problems with the collection and ashing of samples. This component of the energy budget and SFG is not usually responsive to pollution impact and therefore the variation seen is not considered to be environmentally significant. Consequently, scope for growth has been calculated using both the recorded absorption efficiency at each site and the overall mean absorption efficiency of 0.71, and it is the latter which is considered to be more appropriate. In fact, there is very little difference between the two calculated values and the ranking of the various sites.

Scope for growth provides an integration of these physiological energetic responses and an overall assessment of the performance of the mussels both within and outwith the Wash. The SFG of mussels from the Wash sites largely reflect the changes recorded in the clearance rate, with the minimum SFG values (c. 5.6 to 6.2 J g^{-1} h^{-1}) at the inner Wash sites near the mouths of the River Witham (Tabs Head) and the Great Ouse (Barrier Wall and Pandora Sand). While mussels at Hunstanton are only slightly better with SFG values of 7.2 J g^{-1} h^{-1}, those sites in the central-western part have significantly higher SFG values of 13.9 to 15.1 J g^{-1} h^{-1}. However, all these mussel populations living in the Wash have markedly reduced SFG values compared to the two outer sites on the north Norfolk coast (17.6 to 19.5 J g^{-1} h^{-1}) which reflect a healthier condition, although even these were slightly less (but statistically significant at $P<0.05$) than the Holy Island reference site (26.2 J g^{-1} h^{-1}). Such SFG values above 20 J g^{-1} h^{-1} are typical of areas reflecting minimum levels of environmental contamination and therefore minimum stress (i.e. near optimal growth potential).

The scope for growth of mussels measured in July 1995 are in close agreement and not significantly different from those measured in July 1990 (North Sea Mussels Study; Widdows et al. 1995a) and July 1994 (NRA Wash Pilot Study; DFR-01/578/3/A), highlighting the consistency of the results.

It is important to note that previous experimental studies have demonstrated that the physiological responses of mussels (e.g. clearance rate, respiration and SFG) are maintained remarkably constant over a wide range of natural environmental variables (e.g. constant and fluctuating temperatures from 6 to >20°C; Widdows 1973, 1976; constant and fluctuating salinity from 19 to >33psu, suspended particulate concentration from <1 to >50 mg l^{-1}; Widdows et al. 1979, Kiørboe et al. 1980; dissolved oxygen levels above 50% air saturation, Bayne, 1976; and the duration of air exposure, Widdows and Shick, 1985). Furthermore, the physiological responses are measured under 'standardised' laboratory conditions. Consequently, any significant changes in physiological responses will reflect the stress induced by toxicants accumulated in their body tissues.

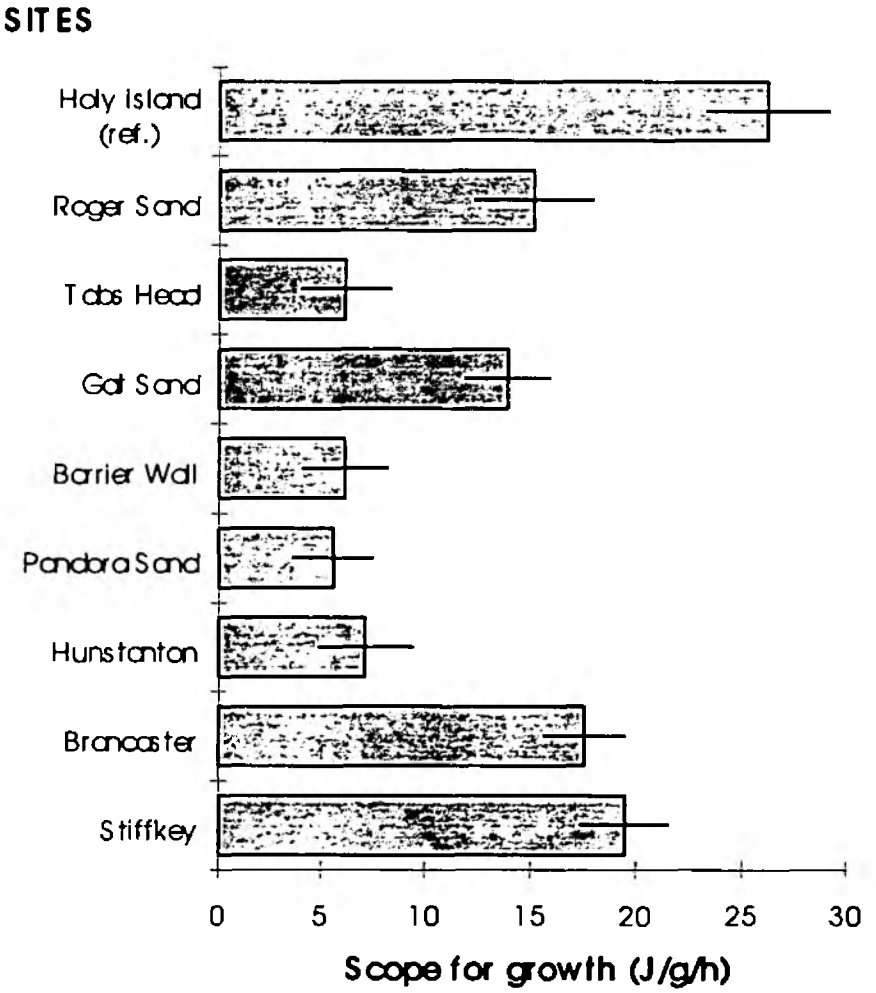

Figure 3.1. Scope for growth of mussels (*Mytilus edulis*) sampled from the Wash in July 1995. (Mean ± 95% C.I.).

Table 3.1 Physiological responses and the integrated scope for growth of mussels (*Mytilus edulis*). Mean ± 95% C.I.; n=16.

Date:- July 1995

Sites	Clearance rate ($l\ g^{-1}\ h^{-1}$)	Respiration rate ($\mu mol\ O_2\ g^{-1}\ h^{-1}$)	Absorption efficiency (mean =0.71)	Scope for Growth ($J\ g^{-1}\ h^{-1}$)	Scope for Growth (based on mean absorption efficiency) ($J\ g^{-1}\ h^{-1}$)
Holy Island (ref.)	5.02±0.45	14.61±1.36	0.75±0.04	28.01±3.10	**26.16±2.94**
Roger Sand	3.19±0.42	12.62±1.28	0.80±0.03	17.76±3.16	**15.11±2.82**
Tabs Head	1.74±0.32	11.43±1.01	0.69±0.04	5.85±2.08	**6.17±2.14**
Gat Sand	3.33±0.34	17.29±1.80	0.57±0.80	9.57±1.58	**13.86±2.00**
Barrier Wall	1.81±0.29	12.51±1.14	0.76±0.06	6.96±2.20	**6.12±2.04**
Pandora Sand	1.82±0.32	13.90±2.00	0.71±0.12	5.57±1.96	**5.57±1.96**
Hunstanton	2.20±0.39	15.78±1.22	0.74±0.04	7.75±2.38	**7.15±2.26**
Brancaster	3.81±0.35	16.01±2.10	0.64±0.12	15.12±1.72	**17.57±1.92**
Stiffkey	4.16±0.36	16.81±0.80	0.70±0.06	19.11±2.18	**19.49±2.10**

3.2. Bioaccumulation of Contaminants: Polyaromatic hydrocarbons

Hydrocarbon contamination was monitored in terms of two and three ring aromatic hydrocarbons because:- i) these groups represent a major toxic component of bioaccumulated petroleum hydrocarbons, ii) the method is sensitive and quantitative, and iii) the results are comparable with previous monitoring programmes. The results presented in Table 3.2 indicate that mussels from the eight Wash sites and the Holy Island reference site were only moderately contaminated by aromatic hydrocarbons. However, the hydrocarbon concentrations show that those mussels living near the river inputs of the Great Ouse and Witham (i.e. Barrier Wall and Tabs Head) have the highest tissues levels (e.g. 2.7 $\mu g\ g^{-1}$ dry wt. of 2 + 3 ring aromatic hydrocarbons). Pandora Sand and Roger Sand show intermediate hydrocarbon levels (1.4 $\mu g\ g^{-1}$ dry wt. of 2 + 3 ring aromatic hydrocarbons) and the concentrations decline to 0.7 $\mu g\ g^{-1}$ dry wt. of 2 + 3 ring aromatic hydrocarbons at Stiffkey. The hydrocarbon concentrations in Stiffkey mussels appear similar or slightly lower than Holy Island and reflect their greater distance from sources and inputs. Such values are typical of open coastal sites removed from industrial and urban developments.

The hydrocarbon concentrations for July 1995 are consistent with the values recorded in previous studies (July 1990 and July 1994), although the slightly lower values may reflect the exceptionally dry summer of 1995 and the reduced river flows.

Table 3.2 Concentration of 2 & 3 ring aromatic hydrocarbons in the body tissues of *Mytilus edulis* (2 pools of 5 individuals; Mean ± semi-range). Date:- July 1995

Site	2 & 3 ring aromatic hydrocarbons ($\mu g\ g^{-1}$ dry wt)
Holy Island (ref)	0.91±0.16
Roger Sand	1.48±0.11
Tabs Head	2.70±0.20
Gat Sand	0.91±0.02
Barrier Wall	2.73±0.28
Pandora Sand	1.40±0.20
Hunstanton	0.93±0.01
Brancaster	0.85±0.00
Stiffkey	0.68±0.11

3.3. Tissue Chemical Residue Fractionation and Toxicology

3.3.1 Toxicity of fractions

The feeding rate (or clearance rate) bioassay of the initial fractionation of the first extract showed that only fraction 6 had significantly different toxicity ($P<0.05$) between the two sites. Pandora F6 caused a reduction in CR to 2% of the control value. F6 from Holy Island also caused a substantial, though less severe reduction in CR to 16% of the control. Fractions 7 to 11 could not be satisfactorily assayed due to the presence of particulate material believed to derive from water insoluble components in the fractions. The CR assay procedure was therefore modified (see Materials and Methods section 2.3) to include a rinse step for these fractions, and the fractionation and assay procedure repeated using the 200 µl of the original extract remaining. This modification proved successful as can be seen from the results shown in Fig. 3.2.

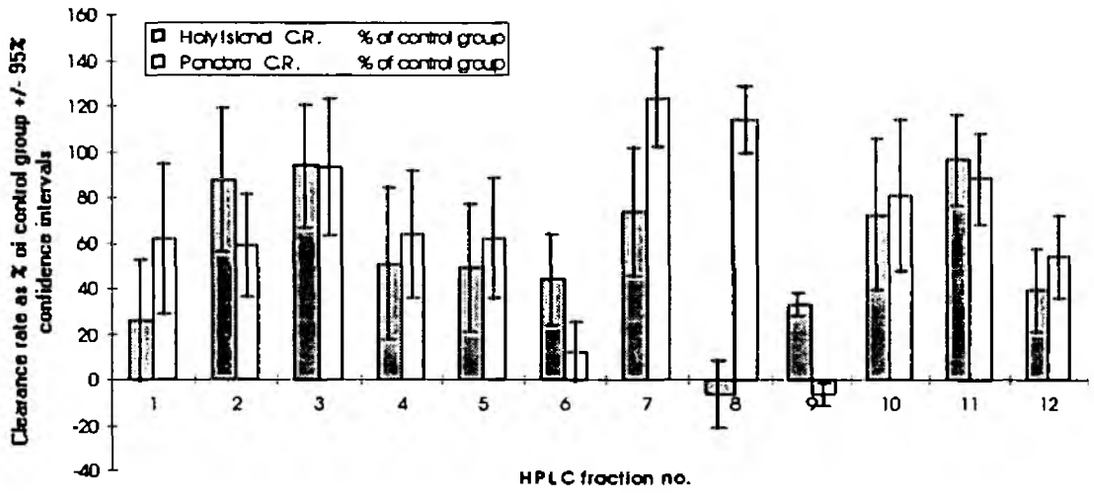

Figure 3.2. Mussel clearance rate (=feeding rate) assay to test toxicity of tissue fractions of mussels (*Mytilus edulis*) from Holy Island and Pandora Sand. July 1995 samples. (Mean ± 95% C.I.).

Again, differences between the toxicity of pairs of fractions from the two sites were often not significant. The lack of significant differences was in part due to high variability of the data caused by the 'condition' of mussels during the 'quiescent' period in autumn / early winter, before the onset of gametogenesis. Nevertheless, F6 was again considerably more toxic in Pandora than Holy Island animals. F9 Pandora was also more toxic than F9 HI. However, the reverse order was apparent in fractions 7 and 8, with HI the more toxic. F8 HI and F9 Pandora were extremely toxic to the CR assay.

Perhaps the most striking aspect of this data set, which followed a trend already observed in the first fractionation / bioassay run with this extract, was the increase in the CR of both Pandora and Holy Island assays from F1 to F3, followed by a decline to F6. This suggests that either there are common forms of contamination at both stations, or that the effects on

CR observed are strongly influenced by the presence of naturally occurring biochemicals extracted from the mussels. The CR protocol for the final fractionation / bioassay experiment was modified in order to reduce the impact of biochemicals.

The neutral red (NR) retention time data for the initial fractionation experiment with the first extract are shown in Fig. 3.3. Fractions were selected for this cytotoxicity assay based on perceived need to confirm the 1995 CR data, and both the CR and NR data from 1994. With this assay, the Pandora fractions 1, 2, 6, 7, 10, and 12 were shown to be clearly more toxic than the equivalent Holy Island fractions. The reverse order was apparent for fractions 3 and 11 and no significant difference was observed between the toxicity of fractions 4 from the two sites. The only concordance between the data for the two bioassay methods, CR and NR is the relatively elevated toxicity of Pandora F6 and the absence of any difference between the two fractions 4. However, there is also an indication from the CR data that Pandora F2 may have elevated toxicity.

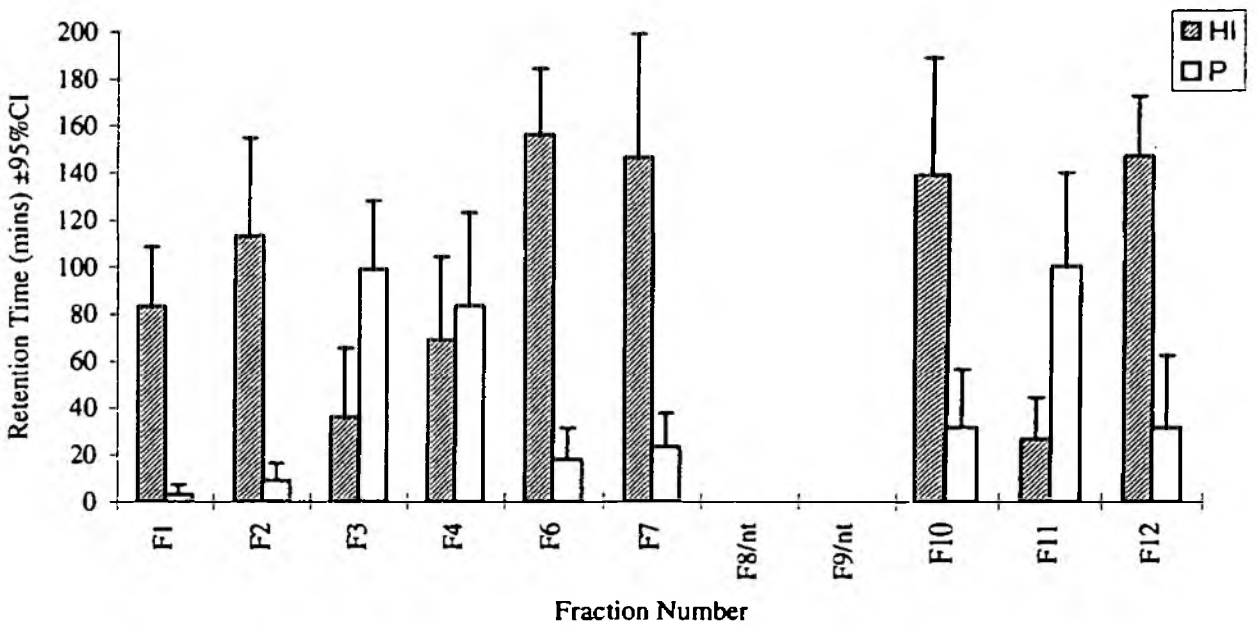

Figure 3.3. Use of neutral red retention in blood cell lysosomes of *Mytilus edulis* to assess toxicity in selected tissue fractions of mussels from Holy Island (HI - reference site) and Pandora Sand (P). July 1995 samples. (Mean ± 95% C.I.; nt = not tested).

The CR data for the fractionation experiment on the second extract (stored in freezer for 2 months) is shown in Fig. 3.4. The method used to derive this data set included a step in which the mussels used to test *all fractions* were rinsed for 5 min in 5 ml of water before the final assay. The only clear toxicity differences were for F7, where the Holy Island fraction was severely toxic in marked contrast to the Pandora fraction. Both F8 fractions were very toxic. Although differences were not significantly different at the $P<0.05$ level, fractions 1, 2, 3, 4 and 10 were consistently more toxic in the tissues extracted from Pandora mussels compared to Holy Island mussels.

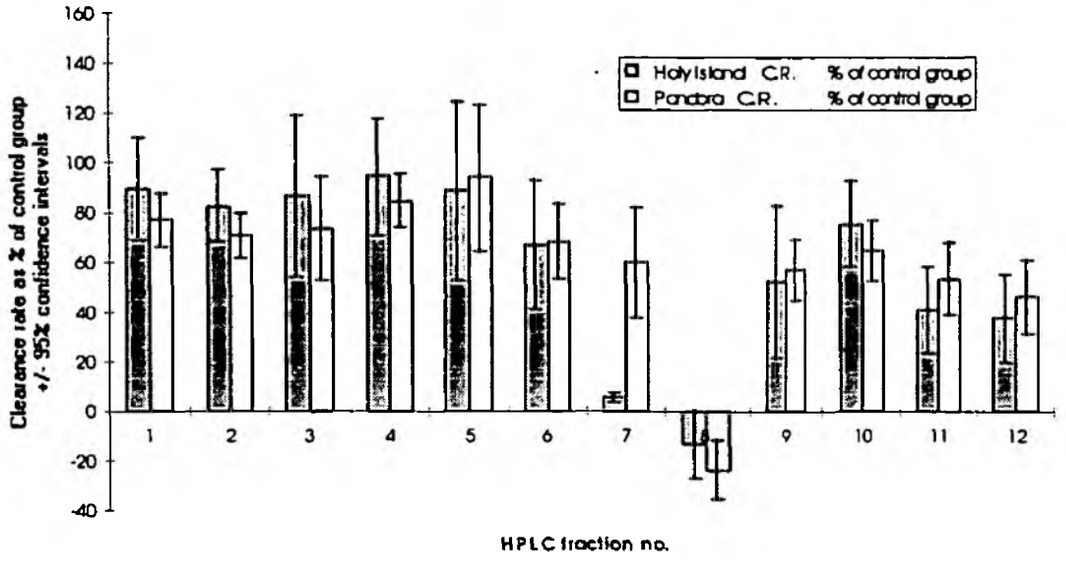

Figure 3.4. Mussel clearance rate (=feeding rate) assay to test toxicity of tissue fractions of mussels (*Mytilus edulis*) from Holy Island and Pandora Sand. July 1995 samples. (Mean ± 95% C.I.).

A small number of fractions were selected for NR retention assay. As for the NR data from the initial fractionation experiment, fractions 2, 6, and 12 obtained from Pandora mussels were more toxic than those from Holy Island mussels, but the differences in the last experiment were much less marked ($P>0.05$; Fig. 3.5). F3 Pandora was also more toxic than Holy Island, though as in the previous fractionation experiment, the CR of the Holy Island animals was also substantially reduced.

The NR data are supportive of the CR data in this final fractionation experiment for fractions 2 and 3 indicating elevated levels of toxicants in Pandora mussels and both assays indicate that F7 Holy Island may contain some toxic substances.

The three fractionation experiments carried out over a period of two months in 1995/96 are difficult to compare in precise terms since modifications were introduced with the objective of improving on perceived shortcomings of the techniques highlighted during use. However some common features and trends can be observed.

The NR retention assay and the CR assay do not always respond in the same way to toxicants. For example, F3 on 3 occasions produced substantial reductions in NR retention but this fraction never had a severe effect on CR. Also, the CR of mussels exposed for 5 min to clean water following 24 h exposure to tissue fractions, increased very rapidly. This may indicate that the mussels respond adversely to the "taste" of the extracts and recover rapidly when these predominantly water soluble components are washed away, leaving a residual effect due to accumulated contaminants. Clearly, isolated blood cells, without a nervous system, do not have a "taste response".

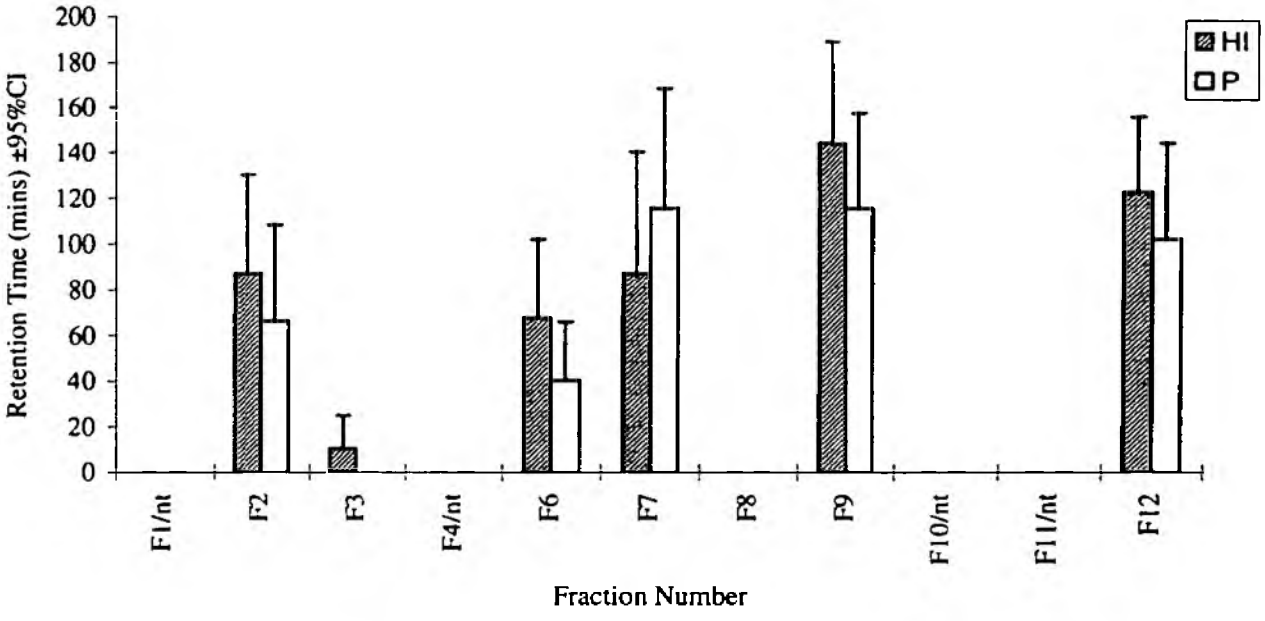

Figure 3.5. Use of neutral red retention in blood cell lysosomes of *Mytilus edulis* to assess toxicity in selected tissue fractions of mussels from Holy Island (HI - reference site) and Pandora Sand (P). July 1995 samples. (Mean ± 95% C.I.; nt = not tested).

However, both procedures detected the presence of highly toxic chemicals in both Holy Island and Pandora mussels eluting in the region fractions 7 to 9 (depending on the HPLC programme). The fact that very large changes in toxicity can occur from fraction to fraction means that maintaining a high degree of chromatographic repeatability will be essential for this comparative toxicological assay to succeed in this region of the chromatogram. A further aspect of this study which shows some evidence of a lower than desirable level of repeatability is the observation that the second extract seemed, based primarily on the NR data, to be less toxic than the first. This could be a consequence of storage of the extract for a month resulting in loss of unstable contaminants, or could be due to a seasonal change in the sensitivity of the mussel blood cells. These aspects require further investigation.

Despite these experimental difficulties, taken together the data from the three fractionation experiments indicate that several fractions from Pandora mussels spread over the entire range of the HPLC programme exhibited elevated toxicity relative to the comparable Holy Island fractions. This observation indicates the presence of many organic toxicants from hydrophilic to very hydrophobic. Thus it is likely that the stressed condition of Pandora mussels compared to those from Holy Island is due to the combined effects of many contaminants, each only present at low levels. There is no evidence for the presence of a small number of very toxic compounds. This conclusion is in accord with that reached at the end of the 1994/5 study and perhaps is not surprising considering the complex mixture of contaminants likely to be entering the Wash via, for example, sewage inputs.

3.4. Chemical analysis of toxic fractions

Fractions were analysed in Holy Island (HI) / Pandora Sand (PS) pairs such that direct comparison could be made between chromatograms of the same fraction injected sequentially into the GC system. Analysis was restricted to paired fractions where the Pandora fraction had enhanced toxicity. Data obtained with either bioassay and from any of the three fractionation experiments was considered in choosing fractions for analysis. In practice, application of this criterion resulted in the majority of the fractions being analysed. Most effort was directed towards analysis of the products from the final extraction and fractionation experiment, though selected fractions from the first fractionation experiment were used to confirm results. Unless otherwise stated, the discussion below refers to results from the final fractionation experiment. GC-FID, GC-ECD and GC-MS were used to investigate the compositional differences between the fractions derived from the two sites.

GC-FID Data. No significant peaks, other than those that could be attributed to the solvents used, were detected in the FID chromatograms for fractions 1, 2, 3, 4 and 6 derived from either HI or Pandora mussels. This contrasts with the HPLC chromatograms obtained at the fractionation step, in which these early eluting fractions from Pandora mussels contained many peaks detected at 220 nm which were not present in the equivalent fractions derived from the HI mussels. These HPLC chromatograms provide a screening method but are not able to identify or quantify the compounds detected. The compounds detected by HPLC were therefore either present at too low concentrations for GC-FID detection or were too polar or too involatile to pass through the GC column.

The GC-FID chromatogram for F9 HI had a major peak group at retention time between 30 and 40 min. These peaks are largely biogenic compounds, characteristic of mussels from any location. They were also present in Pandora F9, though at lower concentrations, probably reflecting different food sources at this site. Pandora F9 chromtograms also exhibited a major peak group within the retention time window of 18 to 30 min. Analysis of F8 samples showed that these peaks, which were also biogenic, were a carry over from F8. This same carry over had not occurred for the HI samples.

Fraction 10 from both sites gave chromatograms with a series of small peaks, though some additional peaks were present in the Pandora samples. Fractions 12 both contained substantial amounts of biogenic compounds with retention times greater than 30 min, but there was no evidence for elevated levels of any other components in the Pandora samples.

The FID analysis therefore did not reveal any substantial differences between the fractions derived from mussels from the two sites; and provided no clear evidence for enhanced contamination at Pandora.

GC-ECD data. The chromatograms for the F1's did not provide any evidence for greater contamination of Pandora mussels. In contrast there were substantial differences between the F2 chromatograms from each site, with major peaks present in the Pandora samples which were not present in HI samples. Little difference was apparent between the F3 chromatograms but the F4 Pandora chromatogram contained a significant peak at 30 min retention time which was not present in the HI F4. Similarly, F6 Pandora contained some small peaks not present in F6 HI.

Fractions 9, 10 and 12 were in pure DMSO which interfered with the electron capture detector. To reduce this interference, these samples from the final fractionation experiment were extracted into ethyl acetate. This procedure was only partially successful, as the DMSO signal obliterated the first 10 to 15 min of the GC runs, permitting analysis of only the less volatile compounds. However, there were substantial differences between the F9 chromatograms derived from the two sites, the Pandora fraction giving rise to many more and larger peaks than the HI fraction. The F10 Pandora fraction was also more contaminated than that from HI, though the differences were less than for F9. There was no significant difference between the F12 chromatograms derived from Pandora and Holy Island mussels.

The ECD data therefore demonstrate the presence of higher levels of some compounds in the Pandora fractions than in those derived from HI mussels.

GC-MS data. These analyses were focused on fractions for which GC-FID and GC-ECD had raised the possibility of differences between the two sites.
F2's. The main peaks identified by MS in these fractions were common to both sites and of biogenic origin. There was no evidence for enhanced contamination at Pandora.
Pand F4. The only peaks which could be reliably identified were of biogenic origin (e.g. long chain alcohols and alkenes), probably natural constituents of the mussel.
F9's. The total ion current chromatogram showed that more peaks were present in the F9 from Pandora than the F9 from Holy Island. The largest of these was the biogenic compound hexadecanoic acid. Butylated hydroxytoluene was also identified with high reliability (96% MS fit) in Pandora F9 but was not detected in any Holy Island fractions in the 1995 survey. This is an antioxidant with widespread use in plastics etc. so could be a laboratory contaminant, though its absence from other fractions suggests not. No other non-biogenic compounds could be reliably identified due to the low levels present.
F10's. GC-MS was unable to reliably detect any difference in anthropogenic contamination in these two fractions: the total ion current chromatograms looked similar due to the presence of biogenic compounds.

4. SYNTHESIS AND INTERPRETATION

The scope for growth values of mussels living in the inner part of the Wash (Barrier Wall, Pandora Sand, Hunstanton, Tabs Head) are significantly lower than mussels inhabiting open coastal sites receiving minimal contamination (Brancaster and Stiffkey). The reduction in SFG is primarily due to inhibitory effects on ciliary feeding rate. The inner mussel sites appear to be severely stressed (i.e. c. 5 J g^{-1} h^{-1}) and this is likely to contribute to the poor performance of the mussel populations in many parts of the Wash. The SFG values in the central region (Gat Sand and Roger Sand) are slightly higher and reflect better sites for mussel cultivation and relaying of spat. The possible wider ecological consequences of low SFG are reduced egg / larval quality, poor recruitment and growth which will hinder recovery and sustainability of resources when combined with any possible over-fishing of shellfish stocks.

The SFG data for July 1995 (present contract) are consistent with the results recorded in the earlier North Sea mussels programme (July 1990; Widdows et al. 1995a). The mean SFG values (J g^{-1} h^{-1} ± 95% C.I.) for mussels from the three sites measured in the three surveys are:-

1990	Holy Island = 22.1 ± 3.1	Gat Sand = 11.6 ± 3.3	Hunstanton = 4.1 ± 2.4
1994	Holy Island = 27.5 ± 3.6	Gat Sand = 17.2 ± 2.2	Hunstanton = 7.5 ± 1.5
1995	Holy Island = 26.2 ± 2.9	Gat Sand = 13.9 ± 2.0	Hunstanton = 7.2 ± 2.3

The aromatic hydrocarbon data are also in close agreement with the earlier North Sea mussels survey. The mean aromatic hydrocarbon values (µg g^{-1} dry wt) for mussels from the three sites are:

1990	Holy Island = 1.21	Gat Sand = 1.25	Hunstanton = 1.18
1994	Holy Island = 1.12 ± 0.1	Gat Sand = 1.12 ± 0.1	Hunstanton = 1.19 ± 0.03
1995	Holy Island = 0.91 ± 0.2	Gat Sand = 0.91 ± 0.1	Hunstanton = 0.93 ± 0.01

Such values are approximately 5-fold higher than background levels in open ocean waters (e.g. Shetland Islands), but 50-fold less than the levels typical of harbours and the major urbanised / industrialised UK estuaries (Widdows et al. 1995a).

Toxicological interpretation of the hydrocarbon concentrations in the mussels from the various Wash sites can explain a significant but variable proportion of the observed reduction in SFG. The size of the 'unexplained component' is greatest at the inner Wash sites, particularly Tabs Head, Barrier Wall, Pandora Sand and Hunstanton. For example, the size of the 'unexplained component' or the difference between the observed SFG and that predicted, based on the measured hydrocarbon concentration in the mussels, is as follows:-

Roger Sand = 2.4 J g^{-1} h^{-1};	Tabs Head = 8.8 J g^{-1} h^{-1};	Gat Sand = 6.1 J g^{-1} h^{-1};
Barrier Wall = 8.9 J g^{-1} h^{-1};	Pandora Sand = 12.4 J g^{-1} h^{-1};	Hunstanton = 12.8 J g^{-1} h^{-1};
Brancaster = 2.4 J g^{-1} h^{-1};	Stiffkey = 0.5 J g^{-1} h^{-1}.	

When the difference is within the typical 95% C.I. of approximately 2.5 J g^{-1} h^{-1}, then a mussel population is not considered to have significant 'unexplained component' indicative of the presence of 'unknown toxicants'. Consequently, the quantitative toxicological interpretation of SFG results indicate that there are no major 'unknown toxicants' present at sites such as Roger Sand, Brancaster and Stiffkey.

The quantitative toxicological interpretation of SFG and hydrocarbon levels at sites such as Tabs Head, Barrier Wall, Pandora Sand and Hunstanton suggest the presence of significant levels of 'unidentified toxicants' in the mussel tissues which cannot be explained by the present tissues residue chemistry data.

The spatial changes in both the SFG and hydrocarbon contaminant levels in mussels fron the central, western and eastern part of the Wash reflect the hydrodynamic data and the main input of contaminants via the Great Ouse and Witham. The output of a hydrodynamic model developed by Dr. Rose Wood at the PML illustrates the residual transport within the Wash region (Fig. 4.1). Water from the North Sea enters the Wash along the central channel and circulates away from the central region of Wisbech and Gat Sand and out along the eastern coast to Hunstanton and out along the north Norfolk coastline (Brancaster and Stiffkey) where it will mix with the North Sea.

The mussel tissue extraction, fractionation and toxicity testing experiments were less reproducible than expected. The reasons for this variability require further investigation. However, where highly toxic chemicals were present, as in fractions 7 to 9 (depending on the HPLC programme) from the two sites investigated, both bioassays gave consistent data. Furthermore, there was a clear trend towards higher toxicity in fractions derived from Pandora mussels. This was particularly marked for the neutral red retention data obtained from the first fractionation-experiment, where the data were closely comparable with those obtained in the 1994/95 study. As in 1994/95, the results suggest that Pandora mussels are stressed by a complex mixture of physicochemically diverse organic contaminants, each making a small but significant and probably additive contribution to the overall toxic load.

HPLC and GC analysis was supportive of the conclusion derived from the fractionation / toxicity testing experiments. The most severely toxic fractions (7 to 9 depending on the HPLC programme) were present in both Holy Island and Pandora extracts, suggesting that they may be of biogenic origin, and not necessarily freely mobile and toxicologically available within the intact organism. GC-FID chromatograms did demonstrate the presence of substantial quantities of biogenic compounds in these fractions. HPLC and GC-ECD analyses pointed to higher levels of contamination at Pandora but these observations could not be reliably confirmed by GC-MS due to a combination of the high polarity and perhaps involatility of some contaminants, rendering them unsuitable for direct GC-MS analysis, and due to the low concentration at which many of the compounds occurred. The pattern of biogenic compounds present was different between the two sites reflecting different food sources.

The conclusion that riverine inputs from the urban and agricultural catchment of the Wash are likely to contaminate mussels with a complex mixture of toxic chemicals which are not routinely analysed in chemical monitoring programmes is supported by recent PML analyses of dichloromethane extracts of water samples taken from the Humber estuary and the lower Trent and Ouse rivers discharging into the Humber (T.W. Fileman, pers. comm. 1996). A diverse range of organic contaminants was detected, most clearly of anthropogenic origin, but none of "Red List" status. The chemicals present varied with sampling station reflecting localised (largely industrial) inputs. These data suggest that the toxicology of such waters would be the consequence of the combined effects of many compounds, each present at low concentrations.

Why no tox.
tests on STW
effluents, mixer waters
+ Wash water ?

The biological and chemical results are therefore consistent with the hydrodynamics and the hypothesis that the adverse effects on SFG are related to complex and as yet unidentified mixtures of contaminants entering the Wash from many sources via the major rivers. At some sites, inputs from sewage may form a contribution to the complex mixture of contaminants. Previous toxicological studies (Butler et al., 1990) have shown that the dissolved phase of sewage (from Shieldhall treatment works in Glasgow) contains toxicants which inhibit the SFG of mussels and this stress response is an order of magnitude more sensitive than the mussel embryo bioassay. Furthermore, field studies have demonstrated a significant improvement in the SFG of mussels following the upgrading of a sewage treatment plant in Narragansett Bay (Nelson, 1990).

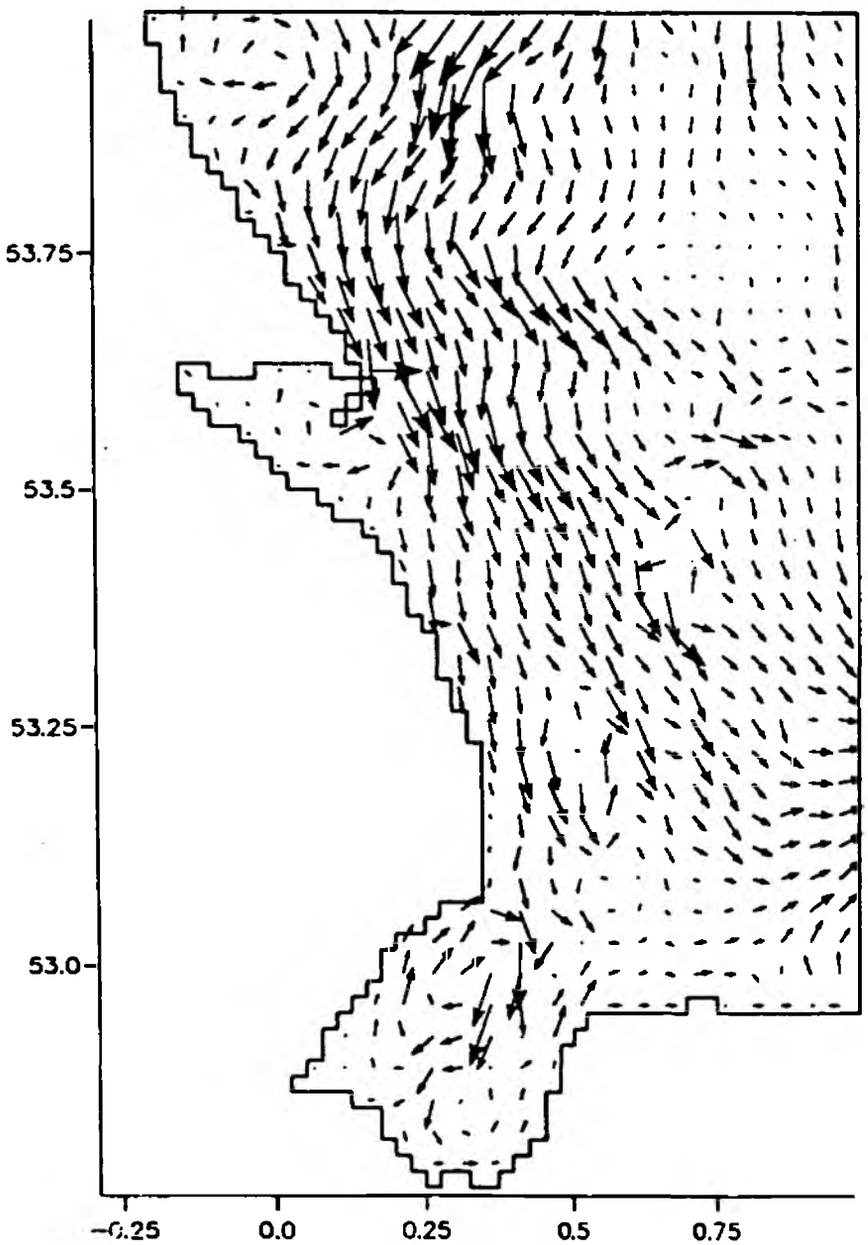

Figure 4.1 Residual transport in the Humber - Wash region of the North Sea (unpublished data from R.G. Wood, PML).

5. RECOMMENDATIONS FOR FURTHER WORK

The results of this study indicate the possibility of continuing environmental problems in the Wash region. The main objectives of ongoing and future studies are:-

- To compare the recorded SFG values with the measurement of tissue growth rates of native and transplanted mussels at two sites (Pandora Sand and Stiffkey).

- To establish the extent to which the SFG of transplanted mussels respond to the increase (Stiffkey > Pandora) and decrease (Pandora > Stiffkey) in contaminant levels in their immediate environment. [Objectives 1 and 2 are under investigation and should be completed in October 1996].

- To investigate the degree and time-course of any possible recovery in SFG of mussels following the upgrading of a sewage treatment works on the Great Ouse in the autumn of 1996. [It has been agreed that Objective 3 will be investigated in August 1997].

- To investigate the contribution that the river Nene makes to poor water quality in the Wash by deploying caged mussels near its mouth and determining SFG. – we know - Nene estuary work.

- To compare the SFG data from mussels deployed near the mouths of the 4 main rivers with Environment Agency assessments of water quality in the lower reaches of these rivers. Are the two data sets linked in a consistent way?

- The fractionation / toxicity testing approach adopted in this study was derived, with considerable modification, from a published study. It has succeeded in demonstrating the presence of enhanced levels of toxic chemicals in physiologically stressed mussels. Of particular importance is the observation that chemicals not readily analysed by direct GC analysis may be important. However, it is clear that considerable further development will be required before these fractionation / toxicity testing procedures can be routinely applied. The following topics need further investigation:- not needed.

  i). Factors influencing the repeatability with which toxicants are extracted and fractionated.

  ii). Investigation of the use of a more sequential approach in which, for example, the toxicity of the whole extract (i.e. including all the extractable contaminants) is tested. This approach could also become a vehicle with which to test the efficiency of different extraction procedures. This is R+D OI. not

  iii). Investigation of other fractionation procedures. Two disadvantages of the procedure used are apparent. Firstly, biogenic compounds probably interfered with the toxicity assessment of some fractions. The presence of high levels of lipids caused solubilisation problems, possibly reducing the bioavailability of the toxicants, and some biogenic components appeared to be highly toxic. There is clearly a challenge to remove biogenics without loosing anthropogenic toxicants. Gel permeation chromatography is likely to achieve some of these objectives so merits further study. The second disadvantage of reverse phase HPLC as a fractionation procedure is that some contaminant classes which could be considered to act toxicologically together and may have a single source (e.g. aromatic hydrocarbons, PCBs), are spread throughout the chromatogram resulting in the individual compounds perhaps being diluted to levels below the toxicity and chemistry detection thresholds. Some form of

chemical class based separation such as normal phase HPLC or gel permeation chromatography may provide data which are easier to interpret.
iv). Improvements in bioassays. There is a need to investigate ways of improving the sensitivity and selectivity of the small mussel CR assay and to investigate the role and choice of solvent carriers for both CR and NR assays. Comparative evaluation of a rapid assay such as Microtox should be carried out with the objective of increasing sample throughput, though this assay may lack the selectivity, sensitivity and relevance of the mussel based assays. It must be a long-term aim to adapt this entire field / laboratory procedure to organisms with different, more selective toxicant sensitivities than mussels (e.g. to some neurotoxic pesticides).
v). The analytical chemistry associated with this toxicity driven approach is particularly challenging. Polar fractions raise particular problems and may require derivatisation before GC and GC-MS, use of HPLC-MS, use of unusual GC columns and / or application of ion chromatography. Recent developments in GC have facilitated the identification of organic amines derived from sewage in marine environments (Fitzsimons, et al., 1995); QSARs would predict that these compound would be toxic.

● The toxicity/fractionation approach and the literature indicates that the difference between the health of Pandora and Holy Island mussels may be a consequence of the combined effects of many contaminants, individually present at low concentrations. The chemical analysis of fractions was designed to directly link chemistry and biological effects but represents a compromise from the point of view of analytical chemistry. Samples are more dilute than is desirable, solvents are used which are not ideal for chromatography and levels of background contamination are higher than is desirable because of the number of handling steps involved. For this reason it is recommended that a direct chemical analysis should be carried out on Pandora and Holy Island mussels using a larger quantity of mussel tissue and optimising analytical protocols for maximum sensitivity. Note should be taken of the results from the fractionation/toxicology experiments that suggest that compounds of a wide polarity range may be important. Difficulties of identification should be reduced for compounds which can be analysed by GC by using an element selective detector atomic emission detector in combination with GC-MS.

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