

This document has been written by Thomas Eves as part of his Master thesis related to the University of Exeter (UK)



It has been carried out in the frame of the EU-Respil under EC-DG Environment grant agreement 07.030900/2006/448357/SUB/A3



For any request, please contact Thomas Eves teves@lyonsdavidson.co.uk or Thierry Baussant tb@iris.no

Phagocytosis in *Mytilus edulis* (blue mussels)

26/09/2008

Thomas Eves

University of Exeter

MSc – Marine and Freshwater Biology and Resource Management

Contents

1 – Abstract	- 2 -
2 – Introduction	- 4 -
2-1-1 Test chemicals - Aniline, Ethyl benzene and Cumene.....	- 8 -
2-1-2 Aniline	- 8 -
2-1-3 Ethyl benzene.....	- 9 -
2-1-4 Cumene	- 9 -
2-2 Blue/common/edible mussel - <i>Mytilus edulis</i>	- 10 -
2-2-1 mussel anatomy	- 10 -
2-2-2 Blue mussels historical importance.....	- 12 -
3 - Procedure / methodology	- 13 -
3-1 Taking mussel haemolymph sample	- 13 -
3-2 The two methods used to determine phagocytosis effects in <i>Mytilus edulis</i> haemolymph.....	- 14 -
3 -2-1 The Norwegian method for Phagocytosis.....	- 14 -
3 -2-2 The simplified French protocol to prepare measurement of hemocyte phagocytosis (Mussel) using a Guava flow cytometer (<i>Nelly Le Goic – Christophe Lambert - Michel Auffret – Thomas Eves (Stavanger Norway) April, 2008</i>).....	- 20 -
4 – Results	- 23 -
4 – 1 Norwegian method results	- 23 -
4 – 3 French method results.....	- 26 -
5 – Conclusion	- 28 -
6 – Discussion.....	- 29 -
7 -Bibliography	- 32 -
8 – Acknowledgments	- 41 -

1 – Abstract

With any anthropogenic activity there is the risk of accidents occurring. None make the media headlines more often than shipping accidents and the loss of toxic compounds such as polycyclic aromatic compounds (PAC). When a toxic cargo is lost into the marine environment, there are far reaching and unknown consequences on the effected marine life.

Due to these unseen and unknown affects, researchers have been working on a raft of biological marker (biomarker) tools to enable the rapid testing of possibly contaminated samples. These biomarkers should be able to detect if there is a negative effect post spill on a habitat, the range of impacts and the distance from the spill influenced. Biomarkers could also assist in planning mitigation where further investigations need to be carried out on the site, whilst it returns to as close to its pre-spill condition as is possible.

It is now widely seen that biomarkers could provide the scope to fill the gaps left by chemical investigations. Biomarkers might offer a way of assessing the effects of a chemical spill even though traditional chemical tests on the water cannot pick up readable traces of a spilled toxic substance.

The blue mussel *mytilus edulis* is a well used and proven tool in investigating impacts faced by organisms in the marine environment. Found on the foreshore and easily cared for in the laboratory, the ability to be bagged and placed in open water makes blue mussels an ideal sentinel for change.

From the exposures only significant results were seen with ethyl benzene at day three for control verses exposed. At this time all other days and for the chemical cumene and aniline the data is statistically insignificant.

2 – Introduction

In the past 50 years there have been 69 major accidents involving chemical tankers, container-carrying ships and ferries causing chemical and oil spills in the coastal waters of the European Union (EU). This has resulted in extensive environmental and economic impact (Baussant 2006). The waters of the EU are congested with shipping movements with the English Channel and North Sea accounting for 70% of the annual global maritime traffic (Baussant 2006) (Halldórsson, et al. 2008). With the melting of the polar icecap and the opening of the Northwest and Northeast polar passages to tanker shipping, it can be forecast that it will not be long till a spill event occurs in this relatively pristine environment (Halldórsson, et al. 2008).

The type of goods shipped varies considerably, however for this paper it is the chemicals that are moved that are the focus of attention. There is a wide range of chemicals transported on the seas around the EU regularly including acids e.g. hydrochloric, nitric, alcohols e.g. methanol, ethanol, vegetable and animal oils and organic compounds e.g. naphthalene, styrene, benzene (Baussant 2006). Generally chemical cargos are bulk shipped in large containers to reduce associated transport costs, but this has a negative result when an accident does occur when larger volumes of one or more chemicals are released within a short amount of time (Baussant 2006).

It is acknowledged that the marine environment is facing mounting pressures from toxic contaminant inputs, resulting in a range of effects from large-scale mortalities of

biota to subtle, but adverse physiological effects (Dyrynda, et al. 1998). These subtle effects include alterations in immunity (Grundy, Ratcliffe and Moore 1996), significant for the immune system defends an organism against pathogens, parasites, and transformed cells (Dyrynda, et al. 1998). The risks posed by toxic contaminants is of increased importance to regulatory agencies and the public, as these compounds pose a risk to human health through the tainting of foods and from directly being exposed to the toxin on the shore (Rogers 1995). It is now believed that damage caused by exposure to toxic contaminants is making organisms more susceptible to diseases through their weakened immune state (Pipe and Coles 1995) (Grundy, et al. 1996).

One year after the Erika oil tanker sank off the coast of Brittany in 1999 with a cargo of 20,000 tons of fuel oil onboard, the Levoli Sun sank off the coast of west France in the winter of 2000 (Bocquené 2004) (CEDRE 2006). The Levoli Sun had a cargo onboard consisting of 4000 tonnes of styrene, 1000 tonnes of methyl ethyl ketone and isopropyl alcohol which was spilled into the sea just 11 miles offshore (CEDRE 2006).

Traditionally the focus has been on the effects of oil spilled at sea; oil has a tendency to form an obvious slick which is easily observed by fouling of the shoreline. It was assumed that since over 70% of the Earth's surface is seawater, any chemical additions would soon be diluted, but in truth the aquatic environment just like the land can quickly become degraded (Sinnet 2007). When the Levoli Sun sank, the fate of its chemical cargo on the environment was unknown and the tests to monitor the environment post-spill focused on chemical concentration in the water and not how

the organisms were affected (Baussant 2006). It would be sensible, in conjunction with the chemical testing of seawater, to come up with a range of new tests that focus attention on the organisms affected as a biological indicator or “biomarker” (Sinet 2007). To do this, the sentinel species chosen would preferably need to be:-

- Easy to find in the waters of Northern Europe and easy to identify

- Easy to use in laboratory and field based experimental setups

- Inhabit a range of locations across the shore line

- Show a range of different feeding specialties

- Be easy to sample

- From a range of invertebrate phyla

(Galloway, Brown, et al. 2004)

With these criteria in mind, the blue mussel (*Mytilus edulis*) is an ideal organism to be used as a biomarker. It would be unfeasible to test comprehensively each individual species within a habitat, hence studies normally focus on key species, having different feeding and lifestyle specialism’s and occupying strategic trophic levels when carrying out environmental monitoring to gain an insight into the complete ecosystem (Galloway, Brown, et al. 2004). Blue mussels have a long history of use in scientific investigations making their physiology well known; they filter large volumes of water and in doing so accumulate both particulate (dispersed) and soluble fractions to a relatively high concentration within the body tissue (Baussant 2006). Blue mussels are easily maintained in a laboratory or caged in the field allowing investigations into short and long term effects from exposure to contamination (Baussant 2006).

In 1992 the Oslo and Paris (OSPAR) convention was established with the purpose of preventing and eradicating contamination of the marine environment (from dumping, land based, and offshore sources) and to conduct assessments into the quality of the marine environment (Stagg 1998) (Hagger, Jones and Leonard, et al. 2006).

OSPAR illustrates the need to insure comparability of data through a comprehensive quality assurance program to facilitate the use of biomarkers within international programs (Stagg 1998).

Environmental risk assessments should provide an unbiased stance on environmental health issues by combining results obtained from human and ecological risk assessments (Galloway, Biomarkers in environmental and human health risk assessment 2006). When combined, a more comprehensive assessment on the nature and levels of risk from a particular contaminant are achieved (Galloway 2006). A contaminates' source and the method of exposure might be the same for humans and other species if they exhibit comparable biological effects. By considering these together will improve the understanding of how different endpoints are reached post exposure (Galloway 2006). Risk assessments should be prepared in a standardised format and constructed using shared data by comparing results and communicating risk, so avoiding accepting risk to the environment that ultimately creates risk for human health or *vice versa* (Galloway 2006). The resulting environmental risk assessments will prove to be cost effective whilst also being ecologically relevant protecting both human and environmental health and possibly provide an early warning to risks previously unknown risks (Galloway 2006).

2-1-1 Test chemicals - Aniline, Ethyl benzene and Cumene

The chemicals Aniline, Ethyl benzene and Cumene have been selected as they represent a large volume of chemical movement every year (IRIS 2007). On average between 2002 and 2004 the tonnages of Aniline, Ethyl benzene and Cumene were moved through European waters were:-

Aniline (C ₆ H ₇ N)	359,429 tonnes
Ethyl benzene	130,524 tonnes
Cumene	35,000 tonnes

(IRIS 2007)

2-1-2 Aniline

Aniline is not known to have a natural source into the environment and all known sources arise through anthropogenic activities related to chemically-related pesticides (production, transport, storage, disposal, degradation and use) (Baussant 2006). Aniline is an aromatic amine that is used as an in-between product in the manufacturing of dyes and pigments as well as being a component in the manufacture of many other chemicals (Bhunja, Saha and Kaviraj 2003). It is a water soluble (34g l⁻¹ at 20°C and 35g l⁻¹ at 25°C) colourless oily liquid at room temperature (Bhunja, Saha and Kaviraj 2003). Aniline is considered not to be at risk of bioaccumulating and once in solution it has a short half life of a few weeks, but when 0.1mg/l of aniline is exposed to *Daphnia pulex* a lethal concentration for 50% of the population (LC₅₀) of 48 hours is recorded (Baussant 2006).

2-1-3 Ethyl benzene

Ethyl benzene is found naturally in crude oil and it is also found from the anthropogenic activities of refining oil products and the incomplete combustion of fuels (Baussant 2006). Ethyl benzene is a colourless liquid that is flammable and smells like petrol (ATSDR 2007). Found naturally in oil and coal tar, ethyl benzene is also found in manufactured products including inks, paints and pesticides (ATSDR 2007). A major use for ethyl benzene is as an intermediate compound in the manufacturing of styrene, which in turn is used to manufacture polystyrene (ATSDR 2007). Ethyl benzene has a LC₅₀ of 24 hours for *Daphnia magna* when exposed to 2.2mg/l and a LC₅₀ for Mysid shrimp 96 hours at 5.1mg/l (Baussant 2006). Ethyl benzene has a half life in seawater in winter (3-7°C) of 13 days, spring (8-16°C) of 20 days and in summer (20-22°C) 2.1 days (Baussant 2006) (Wakeham, Davis and Karas 1983).

2-1-4 Cumene

Cumene is a naturally present compound in crude oil, its anthropogenic route into the environment is from processed hydrocarbon fuels and refined product and from its use in the manufacturing of phenol and acetone (Baussant 2006). It is thought that there is a slight potential for Cumene to bioaccumulate in fish, cumene has a LC₅₀ of 24 hours for *Daphnia magna* when exposed to 4.8mg/l and a LC₅₀ for Mysid shrimp 96 hours at 1.2mg/l (Baussant 2006) (Livingstone, et al. 1990). In aerobic freshwaters, Williams recorded a half life of 2.5 days (no temperature provided) (Williams, Ziegenfuss and Lee 1993).

2-2 Blue/common/edible mussel - *Mytilus edulis*

(*Phylum Mollusca, Class Pelecypoda, Order Mytiloida, Family Mytilidae, Subfamily Mytilinae, Genus Mytilus, Species edulis*) (MarLIN 1997)

2-2-1 mussel anatomy

As its common name suggests *Mytilus edulis* is a blue-purple colour. The following features assist when identifying is a mussel is *Mytilus edulis*:-

Solid shell with a roughly triangular outline

Concentric lines on a smooth shell and lacking radiating ribs

Shell of purple, blue or sometimes brown in colour

Periostracum (outermost layer of shell) darker than shell, almost black, dark brown or olive in colour

Inside an empty shell pearl-white with a purple or dark blue border

Length of shell usually between 5-10 cm (some specimens never get above 2cm and others can attain 20cm making it possible to confuse them with the Mediterranean mussel (*Mytilus galloprovincialis*)

(MarLIN 1997) (Gouletquer 2004).

Mussels tend to occur in large high density communities that are commonly referred to as 'mussel beds', attached to one another by byssus, these threads are not secreted just to anchor the mussel in place against wave action, they are also used to trap predatory dogwhelks (*Nucella lapillus*) stopping them from feeding on

members of the colony (Farrell and Crowe 2007) (Moeser, Leba and Carrington 2006) (MarLIN 1997).

In order to anchor themselves in place with their byssus threads, mussels show a preference for rocky reefs and submerged or partly submerged structures, for example, pier supports and sea groins, managing to survive in high numbers in the high energy surf zone (MarLIN 1997) (Gouletquer 2004). Although they have a preference for a solid sea bed, aggregations can be found on shallow sandy areas exposed to low wave energy (MarLIN 1997). Typically to be found at depth above 10 meters, with the highest densities found around the low tide mark where the phytoplankton food supply is most concentrated (MarLIN 1997).

Blue mussels are widely distributed in European waters, extending from the White Sea, Russia as far as south as the Atlantic coast of Southern France (MarLIN 1997) (Gouletquer 2004). Blue mussels are able to exist over such a wide area because of their natural resilience allowing them to tolerate:-

Wide salinity fluctuations being euryhaline (the ability to adapt to a wide range of salinities), mussels are present in the brackish waters of the Baltic at a salinity of 4‰ – though growth rate drops at a salinity below 18‰.

Resist desiccation, by tapping water in their shells over their gills they can survive for extended periods of time out of the water

A wide temperature range of 5-20 °C by being eurythermal, mussels can also cope with freezing conditions for several months and have an upper temperature limit of around 29 °C

(MarLIN 1997) (Gouletquer 2004).

All of this allows the blue mussel to have a shoreline zonal range from subtidal up to high up the intertidal zone and from full salinity sea water to the low salinity of the estuary (MarLIN 1997) (Goulletquer 2004).

Members of the Mollusca Phylum, have an open haemolymph system containing a section containing heart, arteries and veins (see fig one – mussel anatomy), which is constantly exposed to altering environmental factors including any contaminants (Auffret and Oubella 1995) (R. Pipe, J. Coles and F. Carissan, et al. 1999). Within the open haemolymph system, there are a range of nonimmunoglobulin proteins that provide a defence against pathogens by identifying and eliminating them (St-Jean, Pelletie and Courtenay 2002).

2-2-2 Blue mussels historical importance

Mankind harvested mussels for food, bait and fertilizer for centuries; shells have been found in settlement sites dating back to 6000 B.C (Goulletquer 2004). Up until the 19th century all mussels were collected from natural beds, but today the rearing of mussels is an industrial process intensifying in the 1970's with technological advancements, blue mussels are now also reared outside their natural range in places like China (Goulletquer 2004). In the year 2000 the wild bed harvest was over 120,000 tonnes, but this was overshadowed by the aquacultured mussel harvest of over 210,000 tonnes (Goulletquer 2004). Most farmed mussels are harvested in less than 2 years although mussels have the ability to live up to 24 years (Goulletquer 2004).

With its ease of identification and handling, mussels have been used in marine experimentation for a long time, a search on sciencedirect.com using *mytilus edulis*

as the keyword brings back over 1500 papers. But there have been few studies into the immunocompetence in natural invertebrate populations, however (Dyrynda, et al. 1998) has shown there is significant difference in immunocompetence depending on whether mussels are collected from contaminated or clean reference sites (Dyrynda, et al. 1998).

3 - Procedure / methodology

3-1 Taking a mussel haemolymph sample

About 15 fresh mussels from the control and the exposure tanks are required to obtain 10 samples of good quality haemolymph.

To take the haemolymph sample from the posterior adductor mussel of *Mytilus edulis*, the bivalve is carefully opened using a sharp blade between anterior surfaces of *M edulis*. Once the halves of the shell are slightly separated the blade is held in position and the shell inverted with a firm shake to remove excess seawater from within the shell cavity. Once no more water readily comes out a 21 gauge needle is inserted just above where the byssal threads are produced (see fig 1 anatomy of *Mytilus edulis*), avoiding the mantle edge and gills the syringe is inserted into the firm flesh of the posterior adductor mussel.

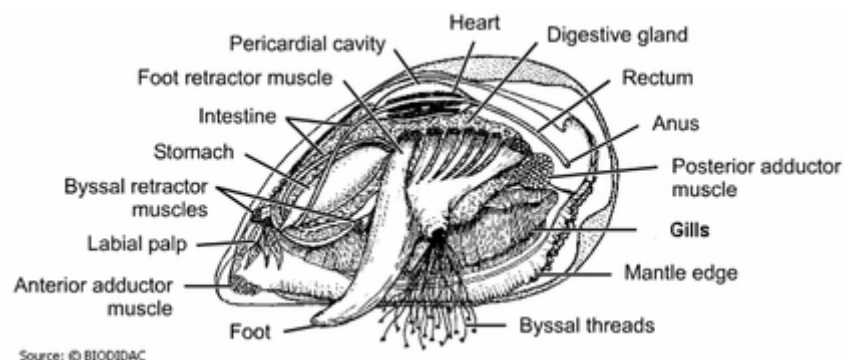


Fig 1 – Anatomy of *Mytilus edulis* (The Alfred Wegener Institute)

Without piercing the opposite side of the posterior adductor mussel the syringe plunger is gradually pulled back whilst rotating the needle slowly, stopping if a vacuum is created turning and testing to see if the blockage clears before drawing the plunger back further. Once 300 µl of haemolymph is collected the plunger is pushed in slightly to reduce the chance of collecting sea water and other debris on extraction and removed. The mussel shell is marked to allow further measurements to be taken as time permits and the haemolymph is then used for the investigation of phagocytosis.

3-2 The two methods used to determine phagocytosis effects in *Mytilus edulis* haemolymph

3 -2-1 The Norwegian method for Phagocytosis

From the neat haemolymph 20 µl is removed and added to an eppendorf tube which has been stored on ice containing 80 µl of Bakers formal, the solution is then gently vortexed and used during the incubation time to provide a sample of fixed cells for counting on the haemocytometer. As and when time permits 10 µl of cell count suspension is taken via a pipetter after the sample has been vortexed to a marked cell chamber to determine the samples cell count.

Once a sample has been fixed a further 200 µl sample of neat haemolymph is taken and pipetted into an eppendorf tube containing 200 µl of bivalve saline.

The saline hemocyte solution is then pipetted out on 50 µl quantities into four wells of the phagocytosis plate (chilled by placing on a freezer pack), providing quadruplicate replication. Before each sample is taken the mixture is gently flushed with the pipetter between each delivery.

This process is repeated for ten control mussels and for ten mussels from the exposure tank.

Once all the samples are taken and transferred to the 96 well plate, a plate cover is applied and then transferred to the refrigerator at 5°C for one hour incubation.

After one hour has past, the sample is taken from the fridge and inverted over a sink to remove excess fluid and any cells that have not attached to the plate bottom, at this stage no washing is carried out.

	1	2	3	4	5	6	7	8	9	10	11	12
a												
b												
c												
d												
e												
f												
g												
h												

Fig 2 – layout of the 96 well plate for the Norwegian method (a1-4 blank, h 1-4 negative control, b 1-4 through to g 1-4 standards in decreasing concentration and a/b 5-12 and c/h 5-8 phagocytosis exposure wells)

A 100 µl of Bakers formal is pipetted into the negative control wells (see fig 2) and 50 µl of stained zymosan solution is then added to each well (25×10^7

particles/ml) including the blank. The plate is incubated at room temperature for 30 minutes using a horizontal shaker set at a very slow speed, to maintain suspension of Zymosan without disturbing the cell layer and causing an uneven distribution of the particles.

Whilst the incubation period is taking place the zymosan standards (fig 3 – dilution factors) are made up during this period and any extra time spent continuing to complete the cell counts on the haemocytometer.

12.5x10 ⁷ particles	500 µl of stock A and add 500 µl of saline (B)
6.25x10 ⁷ particles	500 µl of stock B and add 500 µl of saline (C)
3.12x10 ⁷ particles	500 µl of stock C and add 500 µl of saline (D)
1.56x10 ⁷ particles	500 µl of stock D and add 500 µl of saline (E)
*0.78 x10 ⁷ particles	500 µl of stock E and add 500 µl of saline (F)
0.38x10 ⁷ particles	500 µl of stock F and add 500 µl of saline (G)
0.19x10 ⁷ particles	500 µl of stock G and add 500 µl of saline
* note that 0.78 std is not used on the plate.	

Fig 3 – The dilution factors used for the zymosan standards

After thirty minutes incubation 100 µl Bakers formal is slowly pipetted into each sample well. A multi pipetter was then used to remove as much excess fluid from

the plate as possible, after the fluid is pipetted out from all the wells four times with 200 µl of physiological saline introduced with great care (slow drips from pipette tips into centre of wells). Excess fluid removed from the first wash using pipette and by plate inversion for the remaining three.

100 µl of physiological saline was added to each sample well, the blanks and negative controls and 100 µl of each standard to the plate in correct position (fig 2 plate layout). 100 µl of acidified ethanol was added to each well and shaken for approximately 60 minutes at a high rpm rate on the plate shaker (continue to shake until stained particles are not visible in the standards).

After one hour the absorbance was read using a spectrophotometer (Lab systems Multi-scan RC, Ascent Software Version 2.6 Copyright 1996-2002 Thermo Lab systems Oy (Finland)) set to 550 nm

Solutions used in Norwegian protocol

(1) Physiological Saline

HEPES (4.77g)

Sodium chloride (25.46g)

Magnesium sulphate (anhydrous) (13.06g)

Potassium chloride (0.75g)

Calcium chloride (dihydrate) (1.47g)

Made up to 1 liter with distilled water

Adjust pH to 7.36 and store at 4°C, stable for 2 – 3 months.

NB: Physiological saline is used in the preparation of haemocytes; however, using the solution straight from the fridge will induce osmotic shock and stress the haemocytes. Allow the solution to adapt to room temperature before use.

(2) Acidified Ethanol (500 ml)

1% Acetic acid (5ml)

50% Ethanol (250ml)

Make up to 500 ml with distilled water.

Store at 4°C.

(3) Bakers formal for bivalves

10% formaldehyde (bought as a 40% solution) 100ml

2.5% NaCl 25gms

1% CaCl₂ 10g

Make up to 1l with dist H₂O

(4) Zymosan stained with neutral red

Zymosan solution is made by dying zymosan yeast particles with neutral red. This solution can be kept for up to 2 months when refrigerated.

1. Preparing Zymosan / neutral red solution

- a. Make up neutral red solution / PBS solution using 0.1g of neutral red dissolved in 50ml of phosphate buffer solution in a beaker.

- b. Add a complete container full of zymosan (1g) to the neutral red solution and shake well to ensure every zymosan particle is exposed.
- c. Heat fix by placing in a boiling water bath for 15 minutes
- d. Leave solution to stand to allow cooling. Once the solution has cooled place in a 50ml centrifuge tube.

2. Wash excess neutral red from solution

- a. Centrifuge solution at 1000rpm for 5minutes (this is centrifuge tube 1).
- b. Decant supernatant from centrifuge tube 1 into another 50ml centrifuge tube (this is centrifuge tube 2). Make both tubes up to 50ml with PBS and centrifuge @ 1000rpm for 5 minutes.
- c. Decant supernatant from tube 2 and dispose if clear. If cloudy put supernatant into a 3rd centrifuge tube and centrifuge again. Decant supernatant from tube 1 into tube 2 and top up both tubes with PBS. Centrifuge both tubes again @ 1000rpm for 5minutes. Repeat until supernatant in tube 1 becomes clear (approx 20-25 times).

3. Re-suspend solution at 50×10^7 particles / ml

- a. Re-suspend solution after centrifuging both tubes 1 and 2 and put suspension into a beaker.
- b. Make a 1/100 dilution in a micro-centrifuge tube by using 10 μ l of zymosan/neutral red solution and 990 μ l of PBS
- c. Use a haemocytometer to count the number of cells and adjust concentration.

3 -2-2 The simplified French protocol to prepare measurement of hemocyte phagocytosis (Mussel) using a Guava flow cytometer (*Nelly Le Goic – Christophe Lambert - Michel Auffret – Thomas Eves (Stavanger Norway) April, 2008*)

Before proceeding with a sample, a small amount of haemolymph is observed under the microscope to check the quality (e.g. absence of gametes or tissue debris). If the sample of haemolymph is deemed OK it was then passed through 80 µM mesh gauze, into a 1.5 mL micro-tube maintained on ice.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1											
B	E1											
C	C1a											
D	C1b											
E	C1c											
F	E1a											
G	E1b											
H	E1c										bead s	






-  **C1 to C10 :**
50µL HLPH control + 150 µL MIH
-  **E1 to E10 :**
50µL HLPH Exposed + 150 µL MIH
-  **C1a to C10c :**
50µL HLPH control + 25 µL beads solution + 125 µL MIH
-  **E1a to E10c :**
50µL HLPH exposed + 25 µL beads solution + 125 µL MIH
-  **Beads alone :**
25 µL beads solution + 125 µL MIH

Fig 4 – 96 wells micro-plate layout for the French method

A 96 well micro-plate is placed on an ice pack and 42 μL beads (Fluoresbrite micro spheres 2.5% solids-latex Yellow-Green 2.0 microns (Polysciences 18338)) and 4 mL moniodohistidine (MIH) (around 5.9×10^7 cells mL^{-1}) (Fig 4 - 96 wells micro-plate layout for the French method) is placed onto the cells of the 96 well micro-plate tissue culture treated, flat bottom (TPP 92696, Switzerland) filled with 50 μL formal 6% in seawater (4).

The plate is then sealed with well strip caps (polyethylene, Nalge nunc USA ref 430082) and stored in the dark at 4°C before sending it to Brest France via courier for analysis on GUAVA flow cytometer.

Buffer and chemicals

1) Beads: Fluoresbrite micro spheres 2.5% solids-latex Yellow-Green 2.0 microns (Polysciences 18338).

2) - MIH: *All is diluted in TRIS-HCL 0.05M*

- TRIS-HCl 0.05 M for MIH

1.21g Trizma base (121 MW) + 150 mL Distilled water

Adjust pH to 7.6 with 1N HCL

Complete at 200 mL with distilled water

Store at 4°C

- MIH – moniodohistidine

4.2 g NaCl

300 mg BSA

150 mg D-glucose

200 mg $\text{CaCl}_2, 2\text{H}_2\text{O}$

250 mg $\text{MgCl}_2, 6\text{H}_2\text{O}$

qsp 150 mL Tris-HCl 0.05 M, pH 7.6

Store at 4 °C

3) - SAAH (anti-aggregate solution for hemocytes):

Phosphate buffer 0.1 M, pH 7.4 (500 mL)

In a 1L beaker:

- Na_2HPO_4 (Sigma S 0876) : 5.75 g
- NaH_2PO_4 (Sigma S 0751): 1.14 g
- QSP distilled water: 500 mL

Control pH, normally around 7.4.

SAAH (500 mL)

In a 1L beaker:

- Dilute 12.5 g NaCl (Sigma S 9625) in 400 mL phosphate buffer 0.1 M, pH 7.4 (cf. above).
- Add 7.5 g EDTA (acid: Sigma E5134):
- adjust the pH to 7.4 with NaOH 10N

Transfer in a 500 mL calibrated flask.

Adjust to 500 mL with phosphate buffer 0.1 M, pH 7.4.

Storage: Filter sterilely at 0.2 μm (Stericup, 500 mL for ex.).

Store at 4 $^{\circ}\text{C}$.

- SAAH trypsin (100 mL)
 - 250 mg Trypsin (Sigma T4799)
 - QSP SAAH 100 mL

Refrigerate at 4 $^{\circ}\text{C}$.

4) - Formalin 6% in sea water

4 – Results

4 – 1 Norwegian method results

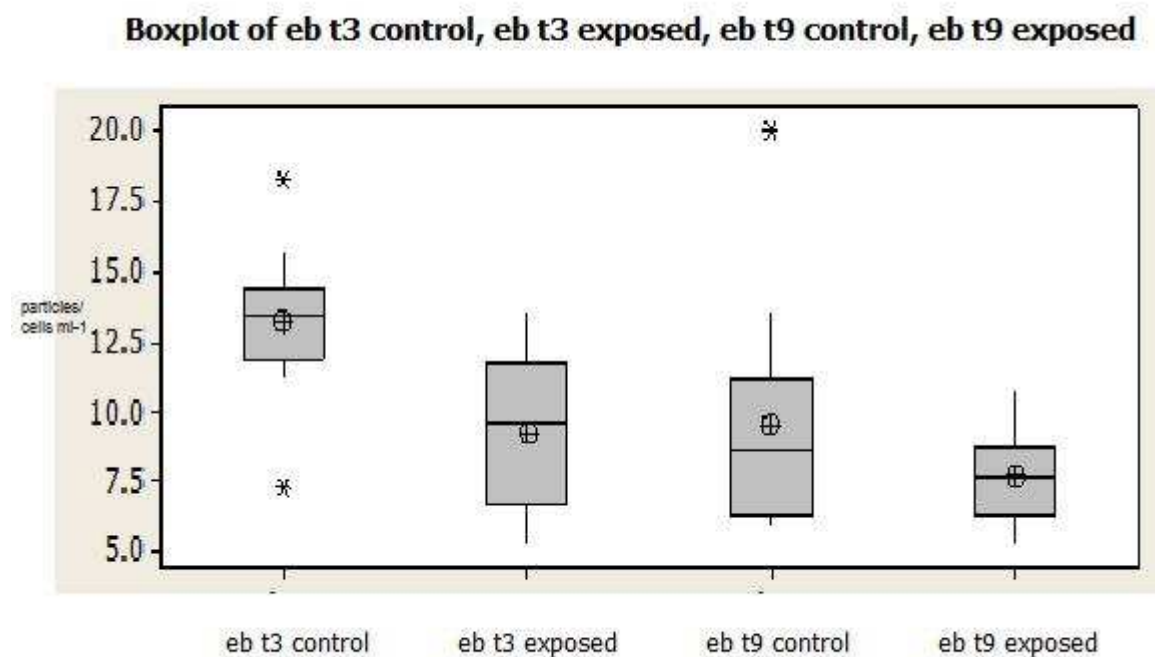


Fig 6 – Boxplot for ethyl benzene control and exposed at day three and day nine, with the mean marked on each boxplot and outliers shown

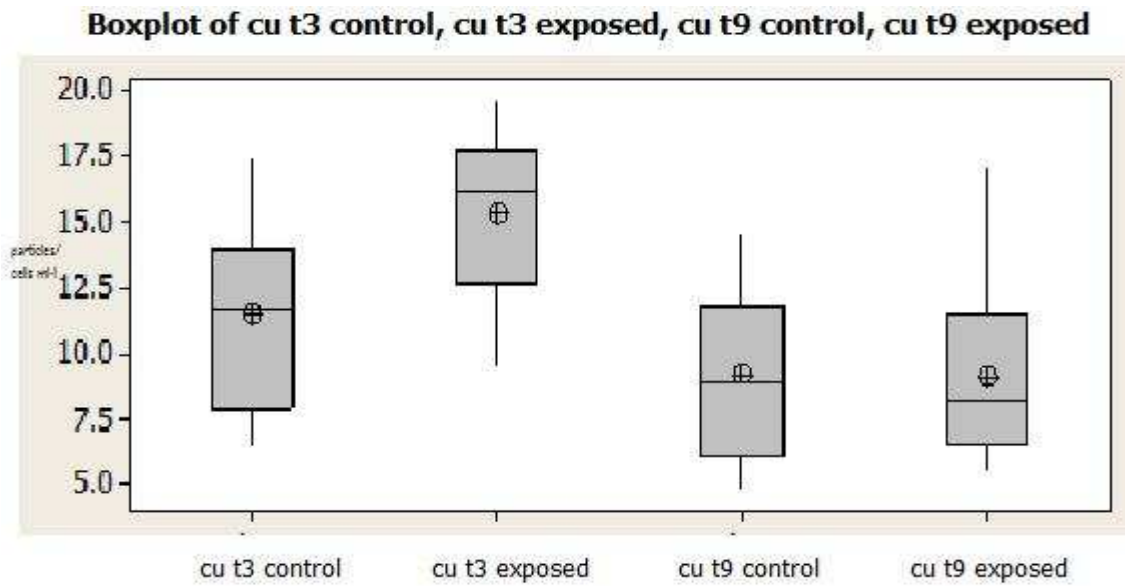


Fig 6 – Boxplot for Cumene control and exposed at day three and day nine, with the mean marked on each boxplot

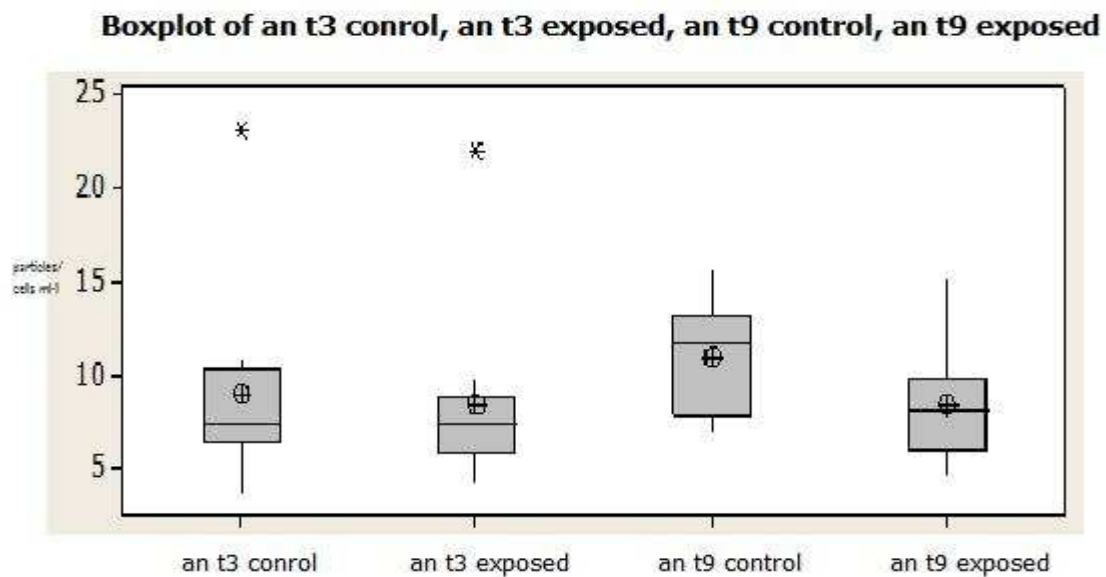


Fig 7 – Boxplot for aniline control and exposed at day three and day nine, with the mean marked on each boxplot and outliers shown

One way ANOVAs were carried out after any outliers had been removed. ANOVA's were undertaken of the control mussels to the exposed mussels at day three and nine and also ANOVA's were undertaken comparing control day three with day nine and the same with the exposed.

Significant differences were found between the number of particles phagocytised per cell during the Ethyl benzene assay for day three ($F_{1,17} = 13.77, P < 0.01$). The control cells had a mean particle count of 13 particles/cells ml⁻¹, whereas exposed cells had a 69% lower mean particle count of 9 particles/cells ml⁻¹.

Highly significant differences were found between the number of particles phagocytised per cell during the cumene assay for day three and day nine exposures ($F_{1,19} = 18.21, P < 0.001$). The day three exposed cells had a mean particle count of 15 particles/cells ml⁻¹, whereas day nine exposed cells had a 60% lower mean particle count of 9 particles/cells ml⁻¹.

Significant differences were found between the number of particles phagocytised per cell during the cumene assay for day three ($F_{1,19} = 7.20, P < 0.05$). The control cells had a mean particle count of 12 particles/cells ml⁻¹, whereas exposed cells had a 6% higher mean particle count of 15 particles/cells ml⁻¹.

Significant differences were found between the number of particles phagocytised per cell during the Aniline assay for day three and day nine controls ($F_{1,18} = 7.62$, $P < 0.05$). The day three control cells had a mean particle count of 7 particles/cells ml^{-1} , whereas day nine exposed cells had a 64% higher mean particle count of 11 particles/cells ml^{-1} .

4 – 3 French method results

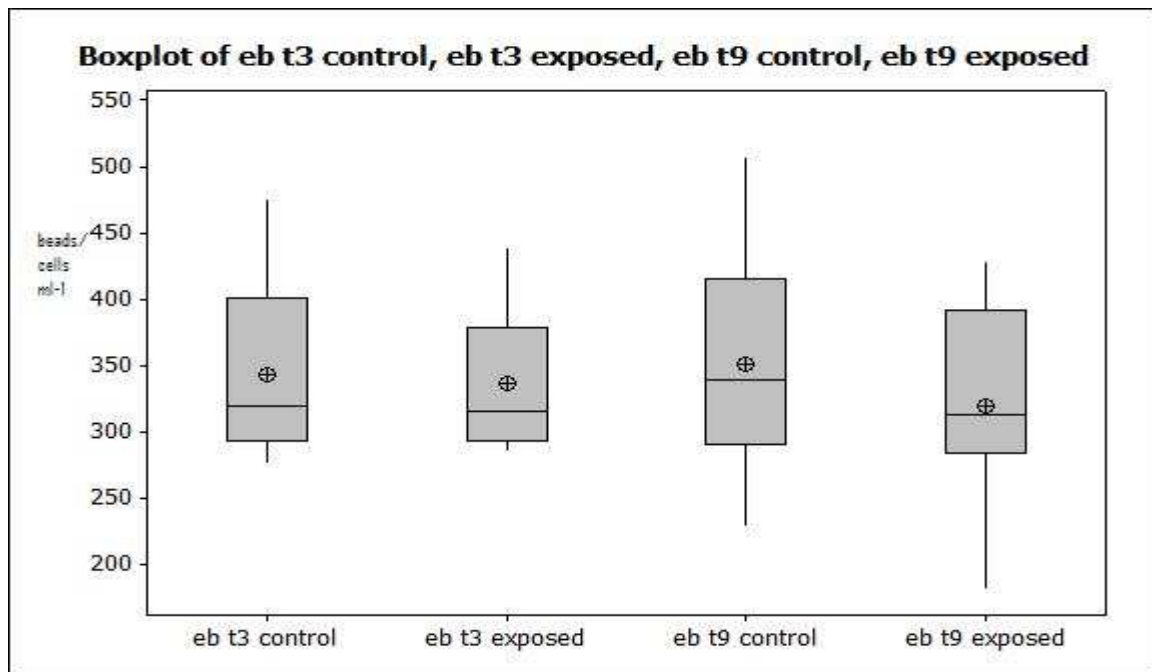


Fig 8 – Boxplot for Ethyl benzene control and exposed at day three and day nine, with the mean marked on each boxplot

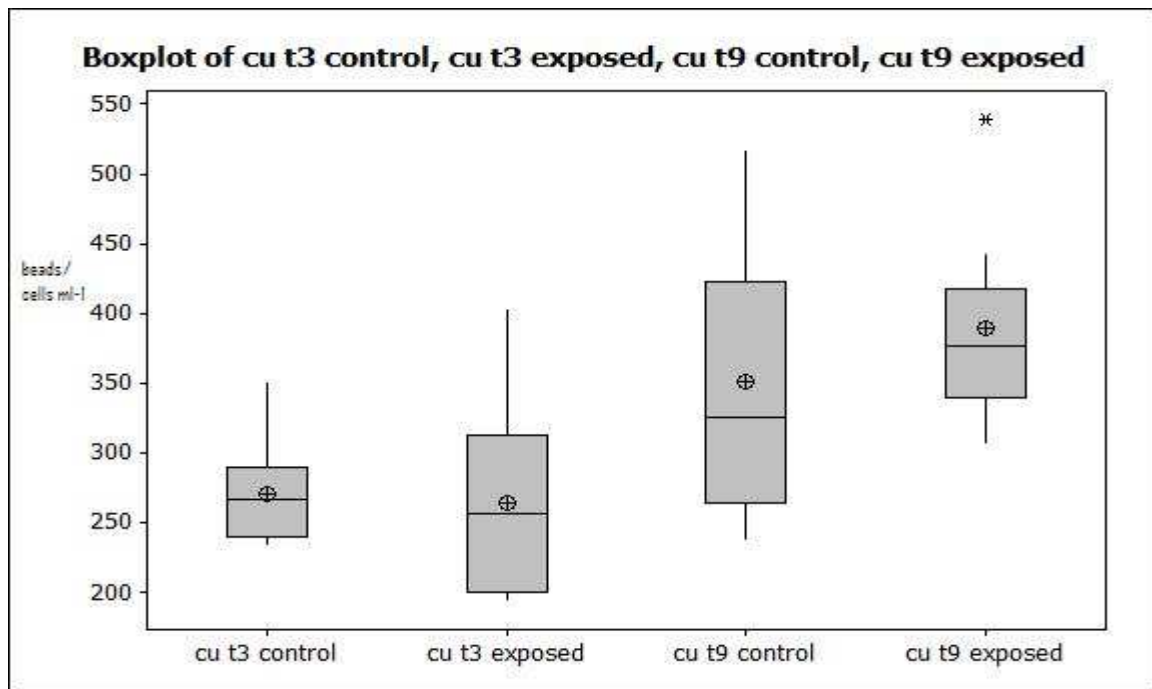


Fig 9 – Boxplot for Cumene control and exposed at day three and day nine, with the mean marked on each boxplot and outliers shown

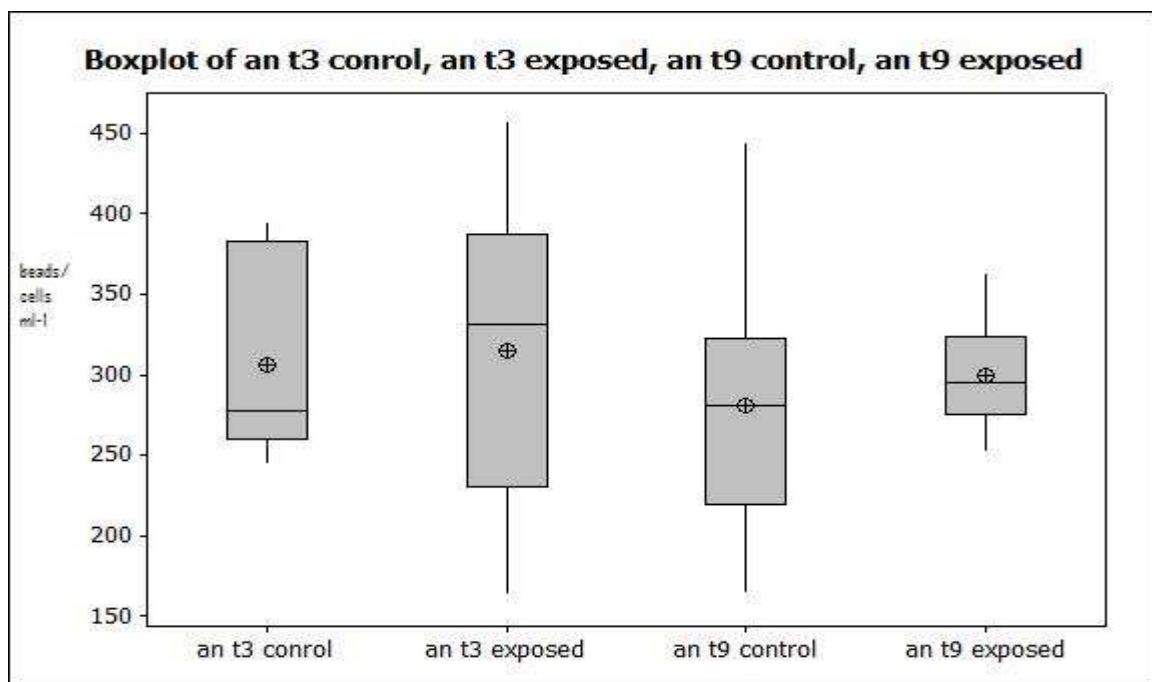


Fig 10 – Boxplot for Aniline control and exposed at day three and day nine, with the mean marked on each boxplot

One way ANOVAs were carried out after any outliers had been removed. ANOVA's were undertaken of the control mussels to the exposed mussels at day three and nine and also ANOVA's were undertaken comparing control day three with day nine and the same with the exposed.

Significant differences were found between the number of beads phagocytised per cell during the Cumene assay for day three and day nine controls ($F_{1,19} = 6.12$, $P < 0.05$). The day three control cells had a mean bead count of 271 beads/cells ml^{-1} , whereas day nine control cells had a 77% higher mean bead count of 351 beads/cells ml^{-1}

5 – Conclusion

From looking at the boxplot of results (Fig 5-7 chemical assay results at day three and nine using the Norwegian method) for the Norwegian assay, it can be observed that for each exposure there is little difference between the controls and the exposed means over the nine days. The same can be observed from the French exposure (Fig 8-10 chemical assay results at day three and nine using the French method).

A significant difference was observed for ethyl benzene on day three $P < 0.01$ when looking at the control verses exposed when using the Norwegian method. After running the data for ethyl benzene three days from the French assay through an ANOVA no significant result was observed.

For cumene assay when the exposed animals at day three and day nine were compared using a one way anova, a highly significant difference was observed between the number of particles phagocytised per cell ($P < 0.001$). This was unexpected and it shows there is something going on with these mussels that may or may not be caused by the exposure to cumene. The mean particle count dropped over the exposure and produced a highly significant result that was not observed in the data from the French assay.

Significant differences were found between the number of particles phagocytised per cell during the Aniline assay for day three and day nine controls ($P < 0.05$). This indicates something possibly happened to the batch of mussels whilst the experiment was running, it is not observed when the data from the French assay is run through a one way ANOVA.

6 – Discussion

From the results it is difficult to come to a firm conclusion at this time. Though the chemicals were observed to bring on spawning within 24 hours of the mussels being added to the exposure tank (the controls always spawned 48 hours after being added to the control tank). But from the statistical data it appears there is only evidence for significant effects from chemical exposure from ethyl benzene and even then it was only recorded at three days. When the other data sets provided by histological samples from CEFAS arrive, it will be easier to come to a firm conclusion. By carrying out further testing using bigger sample size up from ten mussels at each sample day to a minimum of thirty and over the whole of the study period as opposed to set days any changes will be easier to distinguish. This will especially be

true if the mussels have the ability to alter their immune state after getting acclimatised to the exposure of a chemical. Also if bigger sample collections had been carried out it may have possible to assess the possibility of calibrating the methods so that no matter the method adopted by a country, other countries using the alternative method will be possible to directly compare and contrast with data collected in their own waters using their preferred method.

It is not known if the time delay between creating the 96 well plates using the French method and the time the plates remained in storage and transit, before the reading of the plate on the Guava cytometer resulted in potential changes to the final result. A trial sample was sent through the courier system to make sure it arrived intact but this sample had a shorter time in storage before being retested on the Guava. Other unknowns potentially created by the French method, the samples of haemolymph are filtered through very fine mesh. There is the potential to lose some of the sample at this time and the stress created on the cells by passing through the mesh is thought to potentially lead to an increased rate of aggregation. With increased aggregates there is a reduced chance of the beads being ingested into those cells in the middle of a clump and they might also stick to the sides as opposed to passing into the cell.

There is the potential to use mussels as biomarkers when using the rate of phagocytosis observed when the haemolymph is exposed to zymosan or latex beads. The method is quick and simple compared to chemical testing and has the benefit of unlike chemical tests that show what's there at that moment, there is the ability to see historically if there has been an exposure that triggers immune

response. But before these tests can be extensively rolled out further laboratory and field trials must be carried out. This will insure the robustness of the methods and produce a method that should be easy for any one with limited laboratory training to carry out, reducing the costs currently associated with water quality testing using chemical testing.

7 -Bibliography

ATSDR. "ToxFAQs for Ethylbenzene." *Agency for Toxic Substances and Disease Registry (ATSDR)*. U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry (ATSDR). 26 10 2007.

<http://www.atsdr.cdc.gov/tfacts110.pdf> (accessed 7 24, 2008).

Auffret, Michel, and Radouane Oubella. "Cytological and Cytometric Analysis of Bivalve Mollusc Hemocytes." *Techniques in Fish Immunology* 4 (1995): 55-64.

Auffret, Michel, and Radouane Oubella. "Hemocyte Aggregation in the Oyster *Crassostrea gigas*: In Vitro Measurement and Experimental Modulation by Xenobiotics." *Comparative Biochemistry and Physiology* 118 (1997): 705-712.

Auffret, Michel, et al. "A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranean."

Ecotoxicology and Environmental Safety 63 (2006): 393–405.

Baussant, Thierry. *Respill - EU call for proposal*. international research institute Stavanger (IRIS). 7 11 2006.

[http://www.iris.no/Internet/respill.nsf/wvDocId/BD0E4C6021865D7FC1257292004EC48B/\\$file/EU2006proposal-REPSPIL-FINAL-Oct2006.pdf](http://www.iris.no/Internet/respill.nsf/wvDocId/BD0E4C6021865D7FC1257292004EC48B/$file/EU2006proposal-REPSPIL-FINAL-Oct2006.pdf) (accessed 7 23, 2008).

Besten, P. "Concepts for the Implementation of Biomarkers in Environmental Monitoring." *Marine Environmental Research* 46 (1998): 253-256.

Bhunja, F, N Saha, and A Kaviraj. "Effects of Aniline—An Aromatic Amine to Some Freshwater Organisms." *Ecotoxicology* 12 (2003): 397-404.

Bickham, John, Shabeg Sandhu, Paul Hebert, Lounes Chikhi, and Raghbir Athwal. "Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology." *Mutation Research* 463 (2000): 33-51.

Bocquené, Gilles. "Biological effects of the "Erika" oil spill on the common mussel (*Mytilus edulis*)." *Aquatic Living Resources* 17 (2004): 309–316.

Bowen, Robert, and Michael Depledge. "Rapid Assessment of Marine Pollution (RAMP)." *Marine Pollution Bulletin* 53 (2006): 631–639.

Brousseau, P, et al. "Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam *Mya arenaria* hemocytes following in-vitro exposure to heavy metals." *Toxicology* 142 (2000): 145–156.

Bussell, J., Gidman, E., Causton, D., Gwynn-Jones, D., Malham, S., Laurence, M., Reynolds, B., Seed, R. "Changes in the immune response and metabolic fingerprint of the mussel, *Mytilus edulis* (Linnaeus) in response to lowered salinity and physical stress." *Experimental marine biology and ecology*, 2008: 78-85.

Cajaraville, Miren, Maria Bebianno, Julian Blasco, Cinta Port, Carmen Sarasquete, and Aldo Viarengo. "The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach." *The Science of the Total Environment* 247 (2000): 295-311.

CEDRE. *Spills - Ievoli Sun*. 4 2006. <http://www.cedre.fr/uk/spill/ievoli/ievoli.htm> (accessed 7 23, 2008).

Chambers, J, J Boone, R Carr, and H Chambers. "Biomarkers as predictors in health and ecological risk assessment." *Human and Ecological Risk Assessment* 8 (2002): 165–176.

Dyrynda, Elisabeth, Richard Pipe, Gary Burt, and Norman Ratcliffe. "Modulations in the immune defences of mussels (*Mytilus edulis*) from contaminated sites in the UK." *Aquatic Toxicology* 42 (1998): 169–185.

Farrell, Edward, and Tasman Crowe. "The use of byssus threads by *Mytilus edulis* as an active defence against *Nucella lapillus*." *Marine Biological Association* 87 (2007): 559–564.

Gagne, F, T Burgeot, J Hellou, S St-Jean, É Farcy, and C Blaise. "Spatial variations in biomarkers of *Mytilus edulis* mussels at four polluted regions spanning the Northern Hemisphere." *Environmental Research* 107 (2008): 210-217.

Galloway, Tamara. "Biomarkers in environmental and human health risk assessment." *Marine Pollution Bulletin*, no. 53 (2006): 606-613.

Galloway, Tamara, et al. "A Multibiomarker Approach To Environmental Assessment." *Environmental Science Technology* 38 (2004): 1723-1731.

Galloway, Tamara, et al. "Ecosystem management bioindicators: the ECOMAN project – a multi-biomarker approach to ecosystem management." *Marine Environmental Research* 58 (2004): 233–237.

Galloway, Tamara, et al. "Rapid Assessment of Marine Pollution Using Multiple Biomarkers and Chemical Immunoassays." *Environmental Science Technology* 36 (2002): 2219–2226.

Gouletquer, P. *FAO - Fisheries & Aquaculture - culturedspecies - Mytilus edulis*. 1 1 2004. http://www.fao.org/fishery/culturedspecies/Mytilus_edulis (accessed 7 23, 2008).

Grundy, M.M, M.N Moore, S.M Howell, and N.A Ratcliffe. "Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*." *Aquatic Toxicology* 34 (1996): 273-290.

Grundy, Michelle, Norman Ratcliffe, and Michael Moore. "Immune Inhibition in Marine Mussels by Polycyclic Aromatic Hydrocarbons." *Marine Environmental Research* 42 (1996): 187-190.

Hagger, Josephine, Malcolm Jones, David Lowe, D.R. Paul Leonard, Richard Owen, and Tamara Galloway. "Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: A case study." *Marine Pollution Bulletin* 56 (2008): 1111–1118.

Hagger, Josephine, Malcolm Jones, Paul Leonard, Richard Owen, and Tamara Galloway. "Biomarkers and Integrated Environmental Risk Assessment: Are There More Questions Than Answers?" *Integrated Environmental Assessment and Management* 2 (2006): 312–329.

Hagger, Josephine, Michael Depledge, and Tamara Galloway. "Toxicity of tributyltin in the marine mollusc *Mytilus edulis*." *Marine Pollution Bulletin* 51 (2005): 811-816.

Halldórsson, Halldór, Maurizio De Pirro, Chiara Romano, Jörundur Svavarsson, and Gianluca Sarà. "Immediate biomarker responses to benzo[a]pyrene in polluted and unpolluted populations of the blue mussel (*Mytilus edulis* L.) at high-latitudes." *Environment International* 34 (2008): 483-489.

Handy, Richard, Tamara Galloway, and Michael Depledge. "A Proposal for the Use of Biomarkers for the Assessment of Chronic Pollution and in Regulatory Toxicology." *Ecotoxicology* 12 (2003): 331-343.

Harding, Joanne, Cyr Couturier, G Parsons, and Niel Ross. "Evaluation of the neutral red retention assay as a stress response indicator in cultivated mussels (mytilus spp.) in relation to seasonal and environmental conditions." *Shellfish research* 23 (2004): 745-751.

Hellou, J, and R.J Law. "Stress on stress response of wild mussels, *Mytilus edulis* and *Mytilus trossulus*, as an indicator of ecosystem health." *Environmental Pollution* 126 (2003): 407-416.

IRIS. "IRIS-RESPILL-Reports-Other Reports." *International Research Institute Stavanger (IRIS) - RESPILL - Reports*. 1 2 2007.

[http://www.iris.no/Internet/respill.nsf/wvDocId/DE882147CF1321C1C1257292004ED9DA/\\$file/TASK+ID+A-2verDraft2-F.pdf](http://www.iris.no/Internet/respill.nsf/wvDocId/DE882147CF1321C1C1257292004ED9DA/$file/TASK+ID+A-2verDraft2-F.pdf) (accessed 7 23, 2008).

Leinio, Sari, and Kari Lehtonen. "Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea." *Comparative Biochemistry and Physiology* 140 (2005): 408-421.

Livingstone, D, et al. "Oxyradical Production as a Pollution-Mediated Mechanism of Toxicity in the Common Mussel, *Mytilus edulis* L., and Other Molluscs." *Functional Ecology* 4 (1990): 415-424.

MarLIN. *MarLIN - Species - Mollusca - Mytilus edulis*. 1997.

<http://www.marlin.ac.uk/species/Mytilusedulis.htm> (accessed 7 23, 2008).

Moeser, Gretchen, Heather Leba, and Emily, Carrington. "Seasonal influence of wave action on thread production in *Mytilus edulis*." *Journal of Experimental Biology* 209 (2006): 881-890.

Pampanin, Daniela, Lorian Ballarin, Lucia Carotenuto, and Maria Marin. "Air exposure and functionality of *Chamelea gallina* haemocytes: effects on haematocrit, adhesion, phagocytosis and enzyme contents." *Comparative Biochemistry and Physiology* 131 (2002): 605–614.

Parry, H.E, and R.K Pipe. "Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*)." *Aquatic Toxicology* 69 (2004): 311-325.

Pipe, R.K, J.A Coles, F.M Carissan, and K Ramanathan. "Copper induced immunomodulation in the marine mussel, *Mytilus edulis*." *Aquatic Toxicology* 46 (1999): 43-54.

Pipe, R.K, J.A Coles, M.E Thomas, V.U Fossato, and A.L Pulsford. "Evidence for environmentally derived immunomodulation in mussels from the Venice Lagoon." *Aquatic Toxicology* 32 (1995): 59-73.

Pipe, Richard, and Jackie Coles. "Environmental contaminants influencing immune function in marine bivalve molluscs." *Fish & Shellfish Immunology* 5 (1995): 581-595.

Ranka, Jette, Kari Lehtonen, Jakob Strand, and Martha Laursen. "DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark." *Aquatic Toxicology* 84 (2007): 50-61.

Rickwood, Carrie, and Tamara Galloway. "Acetylcholinesterase inhibition as a biomarker of adverse effect A study of *Mytilus edulis* exposed to the priority pollutant chlorfenvinphos." *Aquatic Toxicology* 67 (2004): 45-56.

Rogers, Kim. "Biosensors for environmental applications." *Biosensors and Bioelectronics* 10 (1995): 533-541.

Sauvé, S, et al. "Phagocytic activity of marine and freshwater bivalves: in vitro exposure of hemocytes to metals (Ag, Cd, Hg and Zn)." *Aquatic Toxicology* 58 (2002): 189-200.

Schiedek, Doris, et al. "Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea." *Marine Pollution Bulletin* 53 (2006): 387-405.

Schlenk, Daniel. "Necessity of Defining Biomarkers for Use in Ecological Risk Assessments." *Marine Pollution Bulletin* 39 (1999): 48-53.

Sinet, Emilie. "international research institute stavanger (IRIS)." *IRIS-RESPILL-Reports-Other Reports*. 9 2007.

[http://www.iris.no/Internet/respill.nsf/wvDocId/DE882147CF1321C1C1257292004ED9DA/\\$file/LastVersionREPORT.pdf](http://www.iris.no/Internet/respill.nsf/wvDocId/DE882147CF1321C1C1257292004ED9DA/$file/LastVersionREPORT.pdf) (accessed 7 23, 2008).

Smolders, R, L Bervoets, W De Coen, and R Blust. "Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization." *Environmental Pollution* 129 (2004): 99-112.

Stagg, R. "The development of an international programme for monitoring the biological effects of contaminants in the OSPAR convention area." *Marine Environmental Research* 46 (1998): 307-313.

St-Jean, S, É Pelletie, and S Courtenay. "Hemocyte functions and bacterial clearance affected in vivo by TBT and DBT in the blue mussel *Mytilus edulis*." *Marine Ecology Progress Series* 236 (2002): 163-178.

Sundt, Rolf, et al. "The BEEP Stavanger Workshop: Mesocosm exposures." *Aquatic Toxicology* 78 (2006): 5-12.

The Alfred Wegener Institute. *The Alfred Wegener Institute*.

http://www.awi.de/de/forschung/neue_technologien/marine_aquaculture_maritime_technologies_and_iczm/research_themes/marine_aquaculture/candidate_species/blue_mussels/ (accessed 09 21, 2008).

Wakeham, Stuart, Alan Davis, and Jennifer Karas. "Mesocosm Experiments To Determine the Fate and Persistence of Volatile Organic Compounds in Coastal Seawater." *Environmental Science and Technology* 17 (1983): 611-617.

Wepener, V, L Bervoets, V Mubiana, and R Blust. "Metal exposure and biological responses in resident and transplanted blue mussels (*Mytilus edulis*) from the Scheldt estuary." *Marine Pollution Bulletin* 57 (2008): 624-631.

Williams, Richard, Scott Ziegenfuss, and Christopher Lee. "Aerobic biodegradation of CUMENE: AN ECOCORE STUDY." *Environmental Toxicology and Chemistry* 12 (1993): 485-492.

Wootton, E, E Dyrinda, R Pipe, and N Ratcliffe. "Comparisons of PAH-induced immunomodulation in three bivalve molluscs." *Aquatic Toxicology*, no. 65 (2003): 13-25.

Wootton, E.C, E.A Dyrinda, and N.A Ratcliffe. "Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*)." *Fish & Shellfish Immunology* 15 (2003): 195-210.

Wu, Rudolf, William Siu, and Paul Shin. "Induction, adaptation and recovery of biological responses: Implications for environmental monitoring." *Marine Pollution Bulletin* 51 (2005): 623–634.

8 – Acknowledgments

International Research Institute Stavanger (IRIS) Stavanger Norway

Thierry Baussant, Solveig Apeland, Nadia Aarab, Brit Fjone Godal, Emily Lyng,
Atle Nævdal, Shaw Bamber, Jan Fredrik Børseth

The laboratory of Sciences of the Marine Environment (LEMAR) Brest, France

Michel Auffret, Christophe Lambert, Nelly Le Goic

Centre of Documentation, Research and Experimentation on Accidental Water
Pollution (CEDRE) Brest, France

Anne Bado Nilles

University of Exeter, England

Tamara Galloway, Ceri Lewis, Christopher Pook

I have used the following in setting out the layout for the final report from the journal ecotoxicology as I felt it was a relevant publication to the subject matter covered.

The information below comes from their instructions list.

Ecotoxicology

Editor-in-Chief: Lee R. Shugart

ISSN: 0963-9292 (print version)

ISSN: 1573-3017 (electronic version)

Journal no. 10646

Springer US

Online version available

Text Formatting

Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or Math Type for equations.

Note: If you use Word 2007, do not create the equations with the default equation editor but use the Microsoft equation editor or Math Type instead.

Save your file in doc format. Do not submit docx files.

Word template

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes on the title page are not given reference symbols. Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data).

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

Citation

Cite references in the text by name and year in parentheses. Some examples:

Negotiation research spans many disciplines (Thompson 1990).

This result was later contradicted (Becker and Seligman 1996).

This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1993).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

Journal article

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. *N Engl J Med* 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med*. Doi:10.1007/s001090000086

Book

South J, Blass B (2001) *The future of modern genomics*. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) *The rise of modern genomics*, 3rd edn. Wiley, New York, pp 230-257

Online document

Doe J (1999) Title of subordinate document. In: *The dictionary of substances and their effects*. Royal Society of Chemistry. Available via DIALOG.

<http://www.rsc.org/dose/title of subordinate document>.

Accessed 15 Jan 1999

