funding organisations:

EU DG-ENVIRONMENT:

project RESPIL: Response means to chemicals spilled at sea and environmental damage
grant agreement 07.030900/2006/448357/SUB/A3 (2007-2008)

Centre Français pour l'accueil et les échanges internationaux (EGIDE):

PHC AURORA, projet « Validation and harmonisation of methods for ecotoxicological assessment of marine ecosystems following spill " (2008-2009)

Methodological workshop
on immune diagnosis in ecosentinel mussels

Stavanger (No), April 2008

1. Management

scientific leadership:

Dr Michel AUFFRET (LEMAR, Brest, France)
Dr Thierry BAUSSANT (IRIS, Stavanger, Norway)

date and location:

April 5-10, 2008

IRIS Akvamilio
4068 Stavanger
Norway

funding

EU programme RESPIL
AURORA programme

2. Participants

IRIS

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CEDRE

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3. Aims and schedule

Assessment of environmental effects of chemical contaminants spilled at sea and impacting coastal ecosystems partially relies on ecotoxicological biomarkers measured in sentinel species, including bivalve molluscs. Among the now available panel of biomarkers, those related to the immune system have been demonstrated as efficient tools able to detect both short- and long term alterations in exposed individuals (Auffret, 2005[1]).

Methodologies for operational application of immunological biomarkers in pollution surveys are now used by an increasing number of laboratories worldwide. However, any comparison of results and further comprehensive analysis of data require the use of relevant protocols. Indeed, harmonization exercises for laboratory protocols among partners for assessing biological effects of chemical contaminants were included in the RESPIL project. Partner UBO has got a recognized expertise in bivalve mollusc immunology and especially, by introducing flow cytometry in 1999 as a performing methodology to obtain immune parameters of individuals used in ecotoxicological studies. This laboratory was recently equiped with a new flow cytometer partially purchased by the RESPIL project (see appendix 1 for full description of the analyser). This small-sized cytometer offers the opportunity to be moved out the laboratory (Fig. 1). In that context, a methodological workshop was co-organized by IRIS and LEMAR and held in IRIS from April 5-10, 2008. The schedule is detailed in appendix 2;

Figure 1: Bench-top, portable GUAVA EasyCyte Plus flow cytometer transported from LEMAR and temporarily operated in IRIS facilities in April 2008.

4. Material and methods

4.1. Experimental mussels

[1] Auffret M. 2005. Chapter 3: Bivalves as models for marine immunotoxicology. In Investigative immunotoxicology. H. Tryphonas, M. Fournier, B.R.Blakley, J.E.G Smits and P. Brousseau (eds). CRC Press, Boca Raton, pp. 29-48.

Adult blue mussels *Mytilus edulis* were obtained from a commercial source near Stavanger and acclimated in IRIS facilities. They were exposed for a week to a cocktail of heavy metals (table below) at environmentally relevant concentrations (contaminated coastal areas) (Fig. 2). Control individuals were maintained in seawater.

cocktail composition	
Zn	160 $mg.L^{-1}$
Cu	60 $mg.L^{-1}$
Cd	8 $mg.L^{-1}$
Pb	20 $mg.L^{-1}$

flow rates	
heavy metal solution	0.25 $mL.mn^{-1}$
seawater	1 $L.mn^{-1}$

Figure 2: Mussels were exposed in tanks dosed with selected contaminants in IRIS facilities

4.2. Immunological methods

see appendix 3 and 4

4.3. Analytical methods

flow cytometry (partner LEMAR)

Microplates used for the phagocytosis assay were processed in the GUAVA cytometer (transported from LEMAR to IRIS for the workshop) for fluorescence analysis. Parameters were calculated from fluorescence intensity distribution histograms to assess the phagocytic activity of hemocytes for each individual mussel:

microplate spectrofluorimetry (partner IRIS)

Microplates used for the phagocytosis assay were processed in a microplate reader for dye concentration measurement. Parameters were calculated to assess the phagocytic activity of hemocytes for each individual mussel.

5. Results

The phagocytosis activity of individual mussels was found moderately but not significantly reduced by exposure, either by flow cytometry or by spectrofluorimetry (Fig. 3-4).

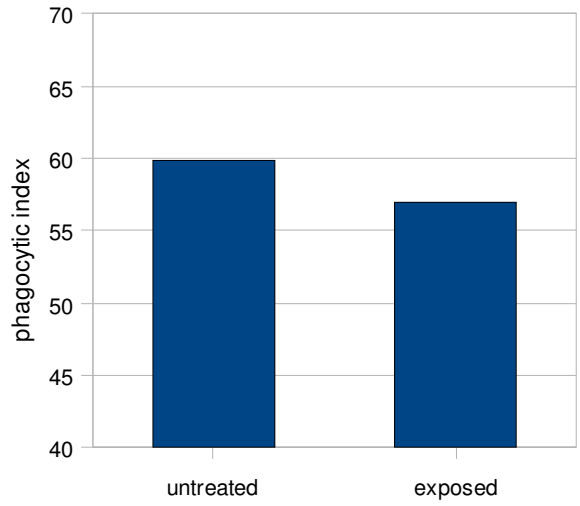

Figure 3: Comparison of phagocytic activity in fresh hemocytes collected from exposed or untreated mussels assayed by flow cytometry (LEMAR protocol).

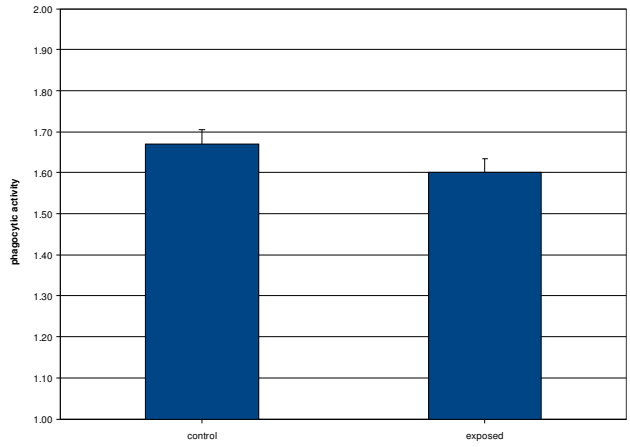

Figure 4: Comparison of phagocytic activity in fresh hemocytes collected from exposed or untreated mussels assayed with IRIS protocol (microplate reader).

To assess the effect of cell fixation by formaldehyde and subsequent transportation of microplates, the microplate was processed, kept refrigerated and analyzed *a posteriori* by flow cytometry (Guava) in LEMAR laboratory 3 weeks after the workshop. Data were found not significantly altered by this treatment (Fig. 5). This indicates that cells processed in a partner laboratory A could be analyzed by another laboratory B equiped with a cytometer.

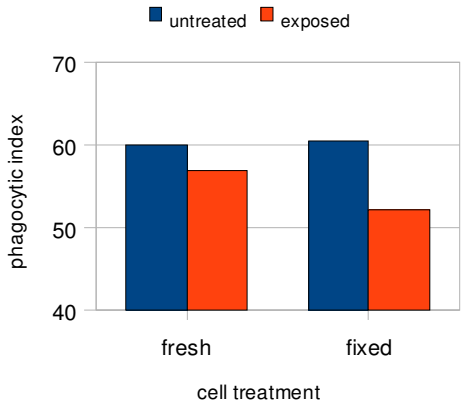

Figure 5: Effect of cell treatment after the assay (untreated or formalin-fixation) on the measurement of the phagocytic activity by flow cytometry. Untreated cells were analyzed at IRIS-Stavanger and fixed cells were analyzed at LEMAR-Brest after a 3-week storage.

6. Conclusions and recommendations

The main outputs of this workshop were:

1. Validated methods for immunological diagnosis were compared and found both relevant to assess potential effects of chemical contaminants in mussels.

2. By allowing a cell-by-cell analysis, the flow cytometer provided quantitative data within the entire hemocyte population to calculate a phagocytosis index.

3. By performing a rapid cell counting, the flow cytometer provided acute hemocyte counts. This measurement is a preliminary step for any phagocytosis assay.

The methods compared here had been previously validated and both provided acceptable results. However, one should consider that the methodology selected for laboratory analysis of biological samples may qualitatively affect the parameters or index finally obtained. For exemple, flow cytometry allowed the calculation of two index to characterize the phagocytic activity of hemocytes. In addition, the total time required for running assays indicate that flow cytometry should be systematically performed to allow comparison of data among experiments and experimental sites of ecotoxicological studies.

These results highlight the need for harmonization of laboratory methods among partners involved in concerted programmes.

APPENDIX 1

Technical information of the Guava flow cytometer

L'analyseur GUAVA un **microcytomètre de paillasse** fournissant une lecture automatisée à haut débit d'échantillons préparés dans ddifférents supports tels que des microplaques 96-puits ou des portoirs de microtubes 1,5 mL. Ce format flexible rendra le système utilisable à la fois pour le **développement d'essais en série** (par exemple: effets biologiques en réplicats en fonction de la dose de traitement) mais aussi la mise en œuvre d'**analyses en séries** (par exemple: mesure de paramètres biologiques dans des échantillons d'effectif important pour le suivi de populations). L'optique est simplifiée et comprend un laser bleu accompagné d'un système de détection avec un détecteur de type "scatter". Une caractéristique importante du système GUAVA est qu'il réalise des **prélèvements absolus** de petit volume. L'accompagnement informatique indispensable au pilotage et au traitement des données se résume à un ordinateur portable fourni avec le système. Des logiciels d'exploitation sont dédiés aux applications: numérations cellulaires, mortalité cellulaire, détection d'antigènes, apoptose, etc…

SPECIFICATIONS	Guava EasyCyte
Excitation	488 nm
Emission	525/30 nm 583/26 nm 680/30 nm
Light Scatter	Forward Side (optional)
Dimensions	13 in (33 cm) *h* 16 in (40.6 cm) *w* 22.5 in (57.2 cm) *d*
Weight	80 lbs (35.6 kg) w/laptop
Sample Format	0.5 mL & 1.5 mL Tubes 96-Well Plates

APPENDIX 2

Workshop Schedule

Monday, April 7

- arrival of LEMAR and CEDRE participants at IRIS
- briefing meeting with IRIS, LEMAR and CEDRE staffs
- unpackaging of GUAVA cytometer
- preliminary experiments on mussels and cytometer calibration

Thuesday, April 8

- immunological analysis of experimental mussels by microplate reader and cytometer

Wednesday, April 9

- analysis and computerization of data
- discussion on data
- packaging of GUAVA cytometer
- debriefing meeting with IRIS, LEMAR and CEDRE staffs

Thursday, April 10

- departure of LEMAR and CEDRE participants

APPENDIX 3

Protocol for measurement of phagocytosis by hemocyte from blue mussels using the Guava flow cytometer
laboratory: LEMAR

Animals

Blue mussels (n=15) will be collected in experimental tanks to obtain 10 hemolymph samples from each treatment.

Hemolymph

material: 1 mL syringes + adapted needle (maintained on ice).
After withdrawal in the adductor muscle sinus (mini 250 µL), a drop is observed under the microscope to check for absence of gametes or tissue debris
The needle is removed before transferring hemolymph through a 80 µM mesh into a 1.5 mL micro-tube maintained on ice.

Buffer and chemicals

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

2) Incubation medium (MIH)
All is diluted in TRIS-HCL 0.05M (see above!

- 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
  4.2 g NaCl
  300 mg BSA
  150 mg D-glucose
  200 mg $CaCl_2$, $2H_2O$
  250 mg $MgCl_2$, $6H_2O$
  qsp 150 mL Tris-HCl 0.05 M, pH 7.6
  Store at 4°C

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

Phosphate buffer 0.1 M, pH 7.4 (500 mL)

In a 1L becker:

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

Adjustl pH at 7.4

SAAH (500 mL)

In a 1L becker:

- 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): could last long !
- adjust the pH to 7.4 with NaOH 10N

Adjust the volume to 500 mL with phosphate buffer 0.1 M, pH 7.4 .
Filter sterically at 0.2 µm (Stericup, 500 mL for ex.) and store at 4°C.

SAAH trypsin (100 mL)

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

Store at 4°C.

4) Fixative
Formalin 6% in sea water

Phagocytosis assay

The assay is described for two treatments: control (C) and exposed (E)
All the following procedures must be performed on ice.

Beads (1) solution: 42 µL beads + 4 mL MIH (2) (around 5.9 x 107 billes.mL-1)

- distribute in a 96 wells micro-plate tissue culture treated, flat bottom (TPP 92696, Switzerland) according to the following schedule:

	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**										**beads**	

legend of the diagram:

50µL HLPH control + 150 µL MIH	50µL HLPH exposed + 25 µL beads solution + 125 µL MIH
50µL HLPH Exposed + 150 µL MIH	Beads alone : 25 µL beads solution + 125 µL MIH
50µL HLPH control + 25 µL beads solution + 125 µL MIH	

- mix on plate vortex for 10 s – 750 rpm
- Centrifugation 1000 rpm – 4°C – 10 min
- Incubation 4h00 at 15°C
- Remove the supernatant, add 150 µL SAAH Trypsin (3).
- Incubate 5 min at room temperature
- vortex 1 min – 900 rpm.
- Transfer in a 96 wells micro-plate (round bottom, BD Falcon, 353910) filled with 50 µL Formol 6% in seawater (4).
- Close safely the plate (well strip cap, polyethylene, Nalgen Nunc USA ref 430082)
- Store at 4°C in the dark until analysis on GUAVA flow cytometer.

Flow cytometry

Process the microplate in the GUAVA flow cytometer for yellow-green fluorescence analysis (FL1 PMT).

Two parameters are calculated from fluorescence intensity distribution histograms according to Auffret et al (2002[2]) to assess the phagocytic activity of hemocytes:

- phagocytic index: % of cells associated with 3 beads or more. This parameter will inform on the rate of immunocompetent cells among hemocyte populations.
- phagocytic capacity: mean (geometric mean) fluorescence intensity in the population of phagocytic cells. This parameter will inform on the immunocompetence degree within the phagocytic cell population.

[2] Auffret M., Mujdzic N., Corporeau C. and Moraga D. 2002. Xenobiotic-induced immunomodulation in the European flat oyster. *Marine Environmental Research* 54: 585-589

APPENDIX 4

Protocol for measurement of phagocytosis by hemocyte from blue mussels by microplate spectrophotometry
laboratory: IRIS

Animals

Select 10 mussels from tank and stand them in crushed ice within a polystyrene tray marked to identify each individual.
Measure length, height and width and wet weight and record in data sheet

Hemolymph

Take a minimum 300 ml haemolymph sample from each bivalve by carefully opening the valves with a sharp blade and inserting a 21 gauge needle connected to a 1ml syringe into the posterior adductor muscle.
Determine total cell counts.

Phagocytosis assay

Dilute haemolymph into an equal volume of bivalve saline.
Pipette 50 #l in quadruplicate into the base of marked sample wells of phagocytosis plate (include 4 samples for negative control).
Cover with plate sealer and transfer to 5 °C for 1 hour
After 60 minutes the phagocytosis plate is removed from the fridge and the
plate is inverted to remove excess fluid (no washes at this stage).
Add Bakers formol to the negative control wells.
Stained zymosan solution is added to each well including the blank
Incubate at room temperature for 30 mins using a horizontal shaker
After the 30 minutes has elapsed the celles are fixed with formol and washed
Add acidified ethanol to each well and shake
Read absorbance at 550 nm.