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ScienceDirect
Environmental Pollution 153 (2008) 492–496
ENVIRONMENTAL POLLUTION
www.elsevier.com/locate/envpol

Short communication

Flow cytometric measurement of the clearance rate in the blue mussel *Mytilus edulis* and the development of a new individual exposure system for aquatic immunotoxicological studies

Matthieu B. Duchemin [a,b,*], Nathalie Wessel [a], Michel Fournier [b,c], Michel Auffret [a]

[a] *Laboratoire des Sciences de l'Environnement Marin, UMR CNRS 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, place Nicolas Copernic, 29280 Plouzane, France*
[b] *INRS—Institut Armand-Frappier, 245 Hymus, Pointe-Claire, QC 9R 1G6, Canada*
[c] *Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, 310 Allée des Ursulines, Rimouski, QC G5L 3A1, Canada*

Received 17 January 2007; received in revised form 5 August 2007; accepted 8 August 2007

Test-tube mussels offer a new exposure design for immunotoxicology.

Abstract

Animals in poor health condition are not relevant biological models. The current study focused on the use of the clearance rate of *Mytilus edulis* to assess the gross physiological condition of individuals maintained in stressful experimental conditions. This approach was developed in a new, highly controlled experimental exposure device designed to investigate individual responses in aquatic ecotoxicological studies. Both clearance rate values and immune parameters analysis indicated that the health condition of mussels kept in 50 ml tubes for 24 h or 48 h was not altered compared to controls, while most parameters were depressed after 72 h. Moreover, this study confirms the relevance of flow cytometric for the measurement of clearance rate compared to techniques utilizing microscopy. Current results prompted us to perform further 24 h chemical exposure using this ‘‘in tubo’’ device.
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Keywords: Aquatic pollution; Individual exposure; Clearance rate; *Mytilus edulis*; Immune parameters

1. Introduction

Knowledge of individual behaviour of physiological endpoints is a must in any biological studies. New experimental set-up development has resulted in the individual confinement of mussels suitable for field studies (Salazar and Salazar, 1995) in order to characterize effects of paper mill effluents (Salazar and Salazar, 1997). It is widely accepted in experimental sciences that an animal in poor health is not a relevant biological model. Therefore the use of cellular biomarkers to study toxic effects of chemicals requires individual information on the gross physiological condition of contaminated organisms. Bivalves are filter feeders and are therefore inclined to accumulate dissolved toxicants or particle-adsorbed toxicants when filtering the water column (Shumway et al., 1985; Page and Widdows, 1991; Sobral and Widdows, 1997; Denis et al., 1999). Due to this ecological feature and to its geographical distribution, the blue mussel *Mytilus edulis* has been used as a model for many marine ecotoxicological studies in the Mediterranean and all North Atlantic coasts. Furthermore endpoints of immunotoxic evidences may be assessed individually in this species (Auffret, 2005) although high inter-individual variability is still an issue (Galloway and Depledge, 2001). In bivalve molluscs, the clearance rate, defined as the volume

* Corresponding author. Laboratoire des Sciences de l'Environnement Marin, UMR CNRS 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, place Nicolas Copernic, 29280 Plouzane, France. Tel.: +33 (0)2 98 49 86 77; fax: +33 (0)2 98 49 86 45.
E-mail address: matthieu.duchemin@univ-brest.fr (M.B. Duchemin).

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doi:10.1016/j.envpol.2007.08.017

of water cleared from suspended particles per unit of time (Riisgård, 2001a), is naturally related to the condition index and energy devoted to growth (Denis et al., 1999; Kesarcodi-Watson et al., 2001a,b) and also responds to chemical stress (Smaal et al., 1991; Widdows et al., 1995; Blackmore and Wang, 2003). Thus it represents a good non invasive endpoint of the gross physiological condition and it can be assessed through direct or indirect methods (Riisgård, 2001a; Petersen et al., 2004).

Flow cytometry is mainly devoted to cell analysis and is widely used in immunology of vertebrates and invertebrates, especially in bivalves (Auffret, 2005) and in phytoplankton research, using the natural fluorescence of phytopigments under blue LASER excitation (Phinney and Cucci, 1989; Platt, 1989; Toepel et al., 2004; Rutten et al., 2005; Bouman et al., 2006). It also offers the advantage of performing metabolic analyses of microalgae however microscopic techniques or the use of cell counters are usually preferred for cell counting (Dorsey et al., 1989).

An original protocol using standardized conditions, and with minimum handling of mussels was developed which involved keeping mussels in small volume units, i.e. 50 ml polypropylene tubes. This protocol (i) allowed for an individual high standardized experimental design and (ii) enabled the assessment of the gross physiological condition using the clearance rate of each exposed individual. Like all experimental conditions these artificial conditions were presumed to be stressful for the mussels, especially since these molluscs are known to filter up to 3 L kg^{-1} h^{-1} of water (Riisgård et al., 2003). The purpose of the current study was therefore to investigate the gross physiological condition of mussels and the immune endpoints under these conditions, using flow cytometry techniques.

2. Materials and methods

2.1. Animal husbandry

For all trials, mussels, *Mytilus edulis* (45–50 mm) were collected on the natural rocky bed of the Pointe du Diable (Plouzane, France) and were acclimated in open water tanks, fed continuously with Isochrysis (T-iso strain). This strain is commonly used for bivalve feeding and has been grown through standardized procedures and optimized culture media for many years in our laboratory.

2.2. Trial 1: Development of clearance rate as an endpoint

Trial 1 focused on the development of the use of the clearance rate to assess the gross physiological condition of constrained mussels. Five mussels were immersed individually in 50 ml Falcon® tubes filled with 30 ml of four algal suspensions (2.4×10^6 cells ml^{-1}, 1.6×10^6 cells ml^{-1}, 0.8×10^6 cells ml^{-1} and 0.4×10^6 cells ml^{-1}). These concentrations were obtained from serial dilutions with filtered seawater. Algal concentration was assessed after 0 min, 10 min, 20 min and 40 min. The seawater temperature during this trial was 15 °C. The clearance rate was measured with an indirect method of assessing the initial and final concentration of an algal solution (Riisgård, 2001a) according to classical algal cell counting (Utermöhl, 1958). Briefly, 100 µl of Lugol's solution (I_2, 5%; KI 10%, seawater) was added to 900 µl of the sampled solution. Iodine (I_2) colours and makes the algal cells heavier to help sedimentation and counting. Cell counting was performed under microscope using a Malassez counting chamber at 250×.

2.3. Trial 2: Temporal changes of the clearance rate and immune parameters

Trial 2 focused on temporal changes of the clearance rate and on immune parameters while mussels were kept in 50 ml tubes. Mussels were placed individually in 50 ml Falcon tubes filled with 30 ml of filtered seawater. The tubes were placed in a temperature-controlled room at 15 °C, the local ambient temperature of seawater at that time of the year (July). The temperature in a tube filled with cleared seawater was checked throughout the experiment. After 0 h, 24 h, 48 h and 72 h, 30 ml of a solution of 1.6×10^6 cells ml^{-1} of T-iso replaced the seawater. The clearance rate of five mussels per group was then measured as described above. Haemolymph was then immediately withdrawn from the adductor muscle to assess haemocyte mortality, and phagocytic index with a flow cytometer (Facscalibur®, Becton–Dickinson) and CellQuest® software (Becton–Dickinson) as described in Auffret et al. (2006).

2.4. Trial 3: Methods comparison for algal cells counting

To compare cell counting with a microscope versus flow cytometer (Facscalibur), three serial dilutions of a 1.6×10^6 cells ml^{-1} algae solution in filtered seawater (1/2, 1/4, 1/8) were used. Algal concentration was then measured with the microscope as formerly described and algal cells were plotted with CellQuest software according to their size and red fluorescence with a controlled-flow cytometer.

2.5. Data analysis

Data analysis was performed with Statistica v7.1 (Statsoft, France). Normality was tested by the Kolmogorov–Smirnoff test and ANOVA was performed in order to test the effect on immune parameters of mussels' passed time in tubes (Sokal and Rohlf, 1995).

3. Results and discussion

In the first trial, algal concentration decreased linearly during the very first minutes of incubation. Seawater was cleared of 90% of the algae within 20 min when initial concentrations were above 0.8×10^6 cells ml^{-1}. Below that concentration, more than 90% of cells were cleared within 10 min (Fig. 1). In the second trial, immediately after being placed in the

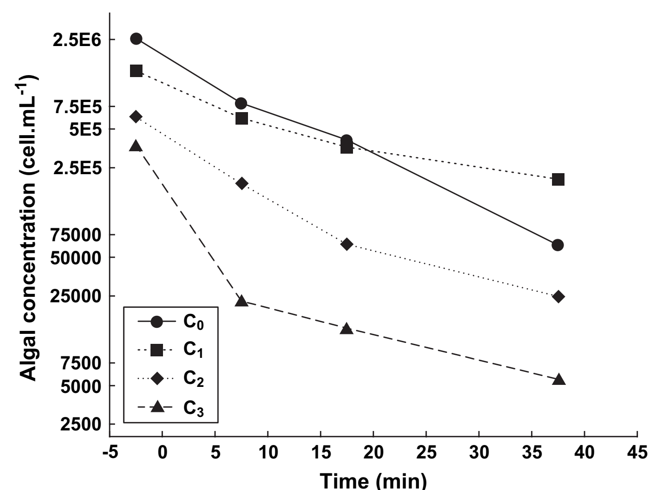

Fig. 1. Algal reduction of 30 ml of four different initial concentrations of T-iso in tubes filled in each with a mussel and measured with a cell counting chamber (Malassez). Each point is the mean of algal concentration of five tubes.

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