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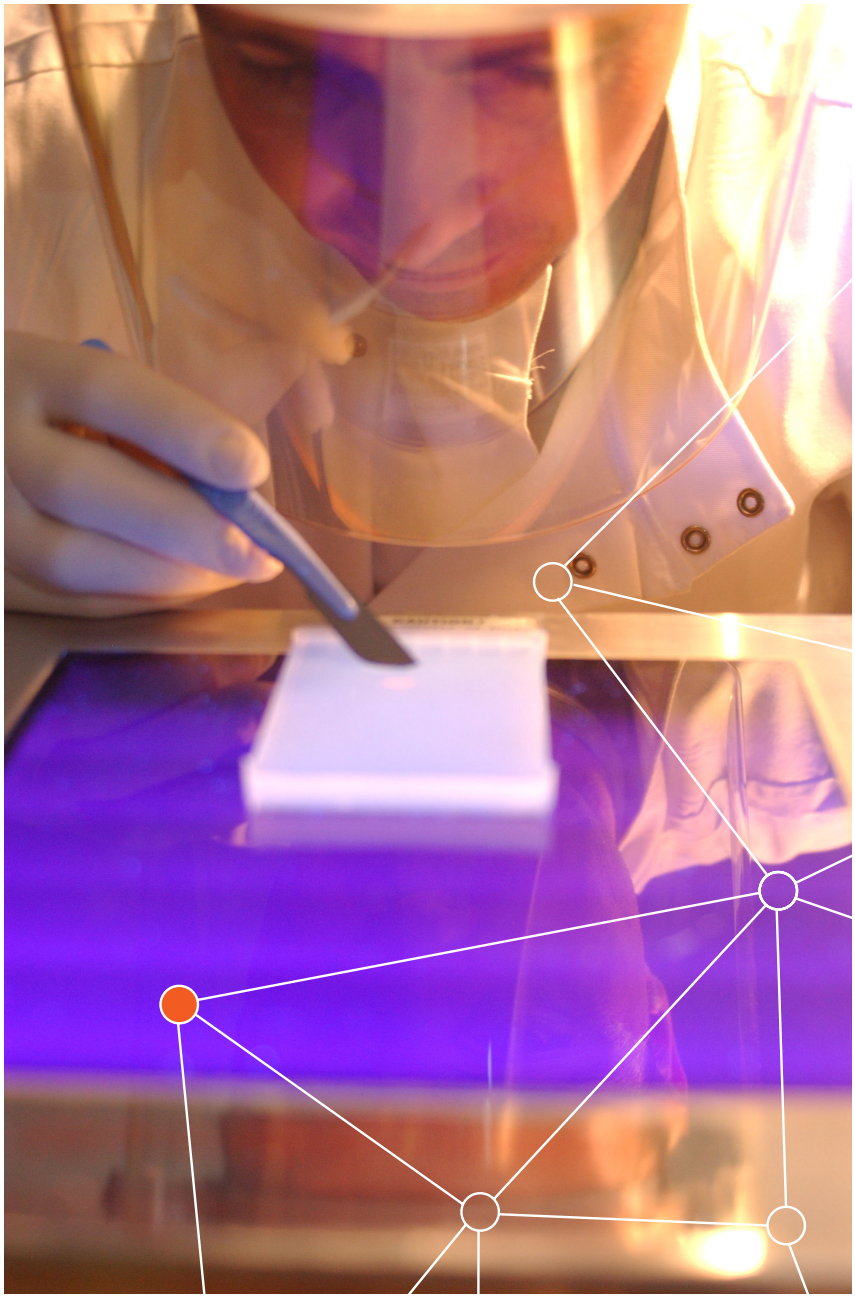

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Biological effects of contaminants: Stress on stress (SoS) response in mussels

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Correspondence about the details of any method or procedure should be directed to the author(s).

This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and demonstrated to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

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Contents

1 Introduction

1.1 The concept of stress on stress

It is well known that the physiological status of marine organisms changes when they are exposed to contaminants (Bayne *et al*., 1986; De Zwaan *et al*., 1995; Viarengo *et al*., 1995). One consequence is that the organism is less able to tolerate the natural fluctuations of environmental factors. Mussels can tolerate aerial exposure for many days but, under sustained aerial exposure, they will eventually die. The ability of mussels to keep valves closed and to resist aerial exposure relates to the amount of adenosine triphosphate (ATP) available to fuel the adductor muscle (De Zwaan and Mathiew, 1992). In mussels from contaminated sites, part of the metabolic energy is spent on detoxification processes, thus depleting the ATP needed for other physiological functions. The reduction of survival in air, or stress on stress (SoS) biomarker, is a simple and low-cost whole organism response that can show pollutant induced alterations in the organism's physiology that renders the animal more sensitive to further environmental changes.

Different studies have demonstrated the applicability of aerial survival as an early warning indicator of contaminant-induced stress. The effects of xenobiotics, including heavy metals, organometals, and organics, as well as contaminated field sediments, on invertebrate survival in air have been demonstrated (De Zwaan *et al*., 1996). Bivalve molluscs have been used in most studies, with marine mussels (*Mytilus* sp.*)* being the most common organism (Brooks *et al*., 2018; Eertman *et al*., 1995; Smaal *et al*., 1991; Veldhuizen-Tsoerkan *et al*., 1991; Thomas *et al.,* 1999; Petrovic *et al.,* 2004; Viarengo *et al*., 1995; Pampanin *et al.,* 2005; Labarta *et al.,* 2005; Gorbi *et al.,* 2008; Marcheselli *et al.,* 2011). Laboratory studies have been conducted to establish the relationships between toxicant concentrations in tissue and SoS. For example, it was demonstrated that short-term exposure to sublethal concentrations (less than μM) of pollutants such as Cu^{2+}, DMBA (9, 10-dimethyl 1, 2 benzanthracene), and Aroclor 1254 significantly reduced the capacity of mussels to survive in air (Viarengo *et al.,* 1995). This effect was markedly dose-dependent, and was strongly increased by pollutant mixtures, such as Cd and PCB 126 (Viarengo *et al.,* 1995; Eertman *et al.,* 1996). Clams exposed to high concentrations of 4-nonylphenol (3 mg NP l^{-1}) were also found to have a significant decreased ability to survive in air (Matozzo *et al*., 2003). A marked decrease in tolerance to aerial exposure has also been reported in mussels exposed to high concentrations of the anti-fouling biocide zinc pyrithione (ZnPT) (Marcheselli *et al*., 2011). Aerial exposure tolerance, as a monitoring tool, has been reported as better able to reflect smaller differences between mussels from sewage outfall sites and mussels from reference sites than other physiological measurements, such as byssal thread production (Moles and Hale, 2003). Survival in air measurements also appear to be a sensitive and statistically significant parameter for monitoring the effect of long-term exposure to crude oil (Thomas *et al*., 1999).

The SoS biomarker provides evidence of the effects of pollutants at the whole organism response level. It shows a typical dose-response curve, characterized by a continuous decrease of the parameter LT_{50} (the median survival time or the time (days) in which 50% of mussels have died) with increasing pollutant concentrations. However, in some experiments with low concentrations of contaminants a slight increase in LT_{50} has been observed, possibly due to a hormetic effect (Eertman *et al.,* 1995). The method for determining SoS in mussels is being applied routinely to both toxicant-exposed mussels in laboratory studies and to mussels collected in national monitoring programmes from polluted environments and along pollution gradients. The added value of SoS in

mussels is that this response measures the overall impact of multiple stressors on an organism. Thus, SoS responses can be quantitatively correlated to contaminant tissue concentrations, providing an integrated biological effect–chemical monitoring tool.

2 Methodology

2.1 Equipment

The measurement does not require sophisticated equipment and is low cost in terms of personnel required to undertake the work (see figures 1 and 2). The minimal equipment needed includes:

- thermo-insulated containers to transport mussels from field to laboratory;
- plastic containers for mussel storage in laboratory;
- incubator chamber (optional) if laboratory room temperature cannot be controlled (e.g. by using conditioned air system).

2.2 Field sampling

Bivalve molluscs can survive for a long time in air, but individuals stressed by pre-exposure to pollutants show greater mortality than controls or individuals collected from a reference location. Both caged and native mussels can be used to assess the SoS response. The size of individuals for survival profiles must be selected from frequency distributions of the whole population under study. The individuals must be of a size approximating to the mean shell length of the population. When mussels are collected from the intertidal zone, it is important to sample them when they are submerged (i.e. before they are uncovered as the tide recedes or, conversely, when they are covered as the tide comes in). Water and air temperatures at the time of sampling should not be extreme (i.e. when environmental temperatures are close to 0°C or above 25°C) as this may influence the measurement. As a supporting parameter, condition index (CI) should be measured (Hansson *et al*., 2017). Spawned-out mussels with a low CI tend to be weak and will die quickly when measured for SoS. If information on spawning state is not known, then do not undertake SoS during or immediately after the main spawning season.

Figure 1. Mussel shipment to assess stress on stress response. Mussel samples must be received in good condition with optimum temperature and humidity from field station to laboratory.

For spatial/temporal studies, the same size range should be selected (ideally 4–5 cm). Forty mussels are used for each determination of SoS. Once collected from the sampling site, mussels should be separated from each other by cutting the byssal threads carefully with scissors to avoid injuring the animals. Mussels should be placed into net bags and firmly wrapped to prevent them from opening. The mussel bags are placed

in an insulated container covered with a wet towel to maintain humidity and transported to the laboratory. If the mussels are transported for a long time, i.e. more than 1 hour, ice packs should be used to maintain temperature (5-10°C).

Field information that must be recorded includes:

- total number of animals sampled;
- date and time of sampling;
- sampling location and position (e.g. latitude and longitude);
- temperature, salinity, and dissolved oxygen of ambient seawater.

Table 1. Example of a stress on stress template for recording mortalities. Area: Murcia. Area code: MU. Salinity ambient water: 37‰. Sampling date (Time = 0): 06/06/2012. Temperature ambient water: 19˚C.

Date	Day	Dead mussels (size mm)											Alive	% Alive
6/6/11	0												40	100
7/6/11	1												40	100
8/6/11	2												40	100
9/6/11	3	43	41	48									37	93
10/6/11	4	41	39										35	88
11/6/11	5	38	36	42	38	40	44	37	41	39	41	40	24	60
12/6/11	6	36	35	37	40	35	38	35					17	43
13/6/11	7	37	35	38	37								13	33
14/6/11	8	38	39	36	41								9	23
15/6/11	9	37	36	39									6	15
16/6/11	10	37											5	13
17/6/11	11	35	38										3	8
18/6/11	12												3	8
19/6/11	13												3	8
20/6/11	14	43											2	5
21/6/11	15	39											1	3
22/6/11	16	37											0	0

2.3 Air exposure in laboratory

Upon arrival at the laboratory, 40 mussels are selected and placed into plastic containers over filter paper dampened with water (to achieve a continuous humidity of approximately 100%). The plastic containers are placed into an incubation chamber at a temperature of 18°C. The temperature to which the animals are exposed affects survival time, with lower temperatures resulting in higher LT_{50} values.

The mussels are inspected once every 24 hours daily, until 100% mortality is reached. This may take up to 25 days. Mussels are considered alive when they resist forcible valve separation. Animals are considered dead when the valves gape and external stimulus (squeezing of valves) does not produce any response. Dead mussels are removed from the plastic containers. The filter paper is replaced and the humidity chamber is cleaned daily.

2.4 Recording data

Mortality and size of dead mussels are recorded at the same time daily. The position of the plastic containers should be randomly changed each time mussels are inspected for mortality.

Figure 2. Inspection of mussels (recording data) and example of incubation chamber and plastic containers used to assess the stress on stress response in laboratory.

2.5 Calculation and presentation of stress on stress response

The median survival time ($LT_{50;}$ the time (days) when 50% of mussels are dead), must be recorded, along with the associated 95% confidence interval. LT_{50} can be estimated using the Kaplan–Meier test (Kaplan and Meier, 1958) as well as the trimmed Spearman–Karber method (α=0.005; Hamilton *et al.*, 1977). Survival curves are constructed to represent SoS results (see Figure. 3). They represent the percentage of mussels alive as a function of time (days) in each sample studied. The Wilcoxon X^2 and Gehan test (Pyke and Thompson, 1986) can be used to test if there are significant differences between survival curves of different mussel populations ($p < 0.05$).

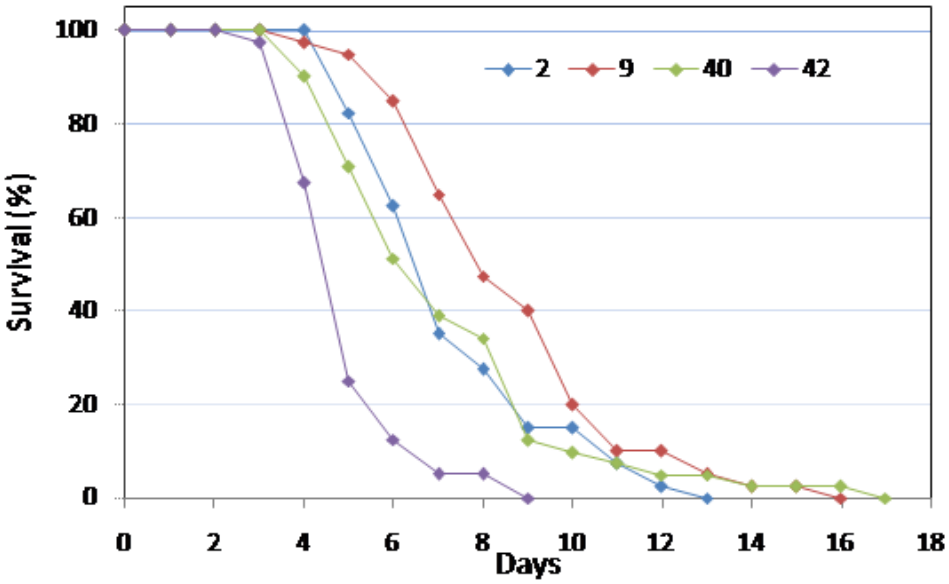

Figure 3. An example of the survival curves obtained from mussels from four different sampling sites. The sampling sites are labelled by station number 2, 9, 40 and 42.

3 Source of error

3.1 Quality assurance

LT_{50} values have been reported to show comparability with stress indices determined at the cellular level (Hellou and Law, 2003). Due to the simplicity of the method, data quality assurance has not been tested by national or international programmes and is not considered necessary.

3.2 Confounding factors

Biological responses that measure general physiological fitness, such as SoS, can be significantly influenced by the seasonal variations of environmental factors. It has been demonstrated that the tolerance of mussels to aerial exposure has a seasonal dependence, showing a high negative correlation with temperature (Eertman *et al.,* 1993; Petrovic *et al.,* 2004). Accordingly, the lowest survival in air of mussels is observed in the summer months when the highest seawater temperatures are recorded. The higher energetic demand triggered by temperature that occurs during gonadal development could also contribute to the lower survival time of mussels, as explained by Eertman *et al.* (1993). Thus, it seems that both factors may contribute to a reduced ability of mussels to survive in air.

Food availability does not seem to significantly influence the SoS response (Eertman *et al.,* 1993; Petrovic *et al.,* 2004). Furthermore, small mussels demonstrate a significantly greater tolerance to air exposure than large mussels (Thomas *et al*., 1999). To date, there is no evidence that suggests that there are differences in SoS response for different species of mussels (*Mytilus* spp.) or hybrids. Additionally, laboratory experiments did not provide any indication that the SoS response was affected (positively or negatively) by long term adaption to salinities as low as 23 ‰ (Eertman *et al.,* 1993).

4 Interpretation of results

For assessment purposes, LT_{50} (days) should be assessed against the developed BAC (background) and EAC (environmental) assessment criteria (Martínez-Gómez *et al.*, 2017). An LT_{50} shorter than the EAC level suggests that the organisms are severely stressed. An LT_{50} between the BAC and the EAC levels signifies that the organisms are stressed, but compensating. An LT_{50} higher than the BAC level indicates that the animals may be considered healthy.

Table 2: Background assessment levels (BAC) and environmental assessment criteria (EAC) for stress on stress measurements.

Days	BAC	EAC
LT_{50}	10	5

Background SoS responses may be as high as 18 days (*M. galloprovincialis,* size range 40–50 mm, Spanish dataset), 16 days (*Mytilus edulis,* size range 40-50 mm, UK dataset), and 13–14 days (*M. trossulus,* size range 30–35 mm) and 20–24 days (*M. trossulus*, size range 18–20 mm) (Martínez-Gómez *et al*., 2017; Thomas *et al.,* 1999).

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6 Abbreviations and technical terminology

Aroclor 1254	Commercial PCB mixture
ATP	adenosine triphosphate
BAC	background assessment levels
EAC	environmental assessment criteria
Cd	Cadmium
CI	condition index
Cu2+	Ionic form of copper (cupric form)
DMBA	9, 10-dimethyl 1, 2 benzanthracene
LT_{50}	the median survival time or the time (days) in which 50% of mussels are dead
NP	4-nonylphenol
PCB 126	Polychlorinated biphenyl 126
μM	Micro mole
SoS	stress on stress
Spearman–Karber method	α=0.005
ZnPT	zinc pyrithione

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