Aquatic Toxicology 198 (2018) 49–62

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Evaluation of biomarkers in *Mytilus galloprovincialis* as an integrated measure of biofilm-membrane bioreactor (BF-MBR) system efficiency in mitigating the impact of oily wastewater discharge to marine environment: a microcosm approach

Cristina Pirrone[a], Federica Rossi[a], Simone Cappello[c], Marina Borgese[a], Giuseppe Mancini[b], Giovanni Bernardini[a,d], Rosalba Gornati[a,d,*]

[a] *Department of Biotechnology and Life Sciences, University of Insubria, Via Dunant 3, 21100, Varese, Italy*
[b] *Electric, Electronics and Computer Engineering Department, University of Catania, Viale Andrea Doria 6, 95125, Catania, Italy*
[c] *Institute for Coastal Marine Environment, National Research Center, Via San Raineri 86, 98122 Messina, Italy*
[d] *"The Protein Factory" Research Center, Politecnico di Milano, ICRM-CNR Milano and Università dell'Insubria, Via Mancinelli 7, 20131, Milano, Italy*

A R T I C L E I N F O

Keywords:
Laboratory-scale systems
Quantitative PCR
TEM
Pollution prevention
Biotechnological strategy

A B S T R A C T

The large volumes of oily wastewater discharged to marine environment cause heavy impacts on the coastal marine ecosystem. The selection of an appropriate technology to reduce these impacts should be based on the respect of the discharge limits and on the effective assessment and monitoring of its effects on biological organism preservation. To this aim, we set up a controlled microcosm-scale system to compare the effects of a treated and untreated oily wastewater discharge in which the restore process is performed through a Membrane Bio-Reactor. The system is completed by other three microcosms to control and isolate any possible concurrent effect on the *Mytilus galloprovincialis,* used as sentinel organism.

Mytilus galloprovincialis have been kept in all these microcosms, and then mRNA expression and morphology were evaluated on gills and digestive gland. The genes considered in this work are *Heat Shock Protein 70* and *Metallothionein 10*, involved in response to physicochemical sublethal stressors, *Superoxide dismutase 1, Catalase*, and *Cytochrome P450* involved in oxidative stress response. Our results evidenced a significant overexpression, both in gills and digestive gland, of *HSP70* in samples maintained in the microcosm receiving the untreated effluent, and of *MT10* in those animals kept in microcosm where the effluent was treated. Even though the mRNA modifications are considered "primary" and transient responses which do not always correspond to protein content, the study of these modifications can help to gain insights into the mechanisms of action of xenobiotic exposure. Morphological analysis suggested that, although different, depending on the microcosm, the most serious damages were found in the gill epithelium accompanied with severe haemocyte infiltration, whilst in digestive gland the tissue architecture alterations and the haemocyte infiltration were less pronounced. These observations suggest that the immune system was activated as a general response to stressful stimuli such as the presence of toxic compounds. Moreover, the results indicate that the treatment process is useful. In fact, samples derived from the microcosm receiving the treated effluent, even though presenting some signs of stress, seemed to partially recover the normal structure, although their mRNA expression indicated some cellular suffering.

1. Introduction

Environmental petrochemical contamination, arising from the exploration, production, refining, transport and storage of petroleum and its derivatives, poses a serious threat to marine ecosystems and human health (Fasulo et al., 2015; Maisano et al., 2016), especially in areas close to petroleum handling facilities such as harbours and refineries. The risk of exposure from oil transportation is mainly associated with routine operations related to slops, dirty ballast, sewage and bilge water management. Conversely, risks from the petrochemical production

Abbreviations: CAT, Catalase; CYP4Y1, Cytochrome P450; HSP70, Heat Shock Protein 70; MT10, Metallothionein 10; SOD1, Superoxide dismutase 1

* Corresponding author at: Dipartimento di Biotecnologie e Scienze della Vita, Università degli studi dell'Insubria, Via Dunant 3, 21100 Varese, Italy.
E-mail address: rosalba.gornati@uninsubria.it (R. Gornati).

https://doi.org/10.1016/j.aquatox.2018.02.018
Received 23 November 2017; Received in revised form 2 February 2018; Accepted 23 February 2018
Available online 24 February 2018

arise largely from continuous oily wastewater discharges along the coastline.

Most of these liquid waste streams are characterized by high salinity (mainly NaCl) but they are conventionally treated through physicochemical processes, as biological treatment that can be strongly inhibited by salts. As the costs of physicochemical treatments can be particularly high, alternative systems for the treatment of organic matter are nowadays increasingly the focus of research. In order to treat emulsified oily and salty wastewaters Mancini et al. (Mancini et al., 2016) proposed a chemical coagulation and sedimentation process followed by a granular activated carbon (GAC) filtration including the on-line bioregeneration of the exhaust carbons by the obligate marine hydrocarbonoclasticus bacterium (OHCB) *Alcanivorax borkumensis* SK2 to reduce the cost of GAC substitution. Several authors (Di Trapani et al., 2014; Sharghi et al., 2014; Di Bella et al., 2013; Jang et al., 2013; Sun et al., 2010) investigated the treatment of oily saline wastewaters through membrane bioreactor systems (MBR). Most of these studies, carried out using synthetic wastewater, showed high removal efficiencies and high biomass respiratory activity when subject to normal salinity. However, potential reduction in the removal efficiency and biomass activity was observed when the salinity increases. In addition, all of these studies measured the effects of the treatments only on the basis of chemical analysis and none focused on the effects on biological populations. Nevertheless, traditional concentration-limit approach, based on chemical investigations of pollutants in environmental matrices, may not be adequate to correctly evaluate the residual risks for ecosystem and human health after mitigation actions, since several site-specific conditions and uncontrolled process side-effects (e.g. by-products) may affect the pollutant bioavailability and, consequently, toxicity.

In the last decade, the scientific community has been particularly sensitive to these issues and several articles, reporting ecological effects of pollutants on organisms, have been published (Rossi et al., 2016; Oliva et al., 2015; Cappello et al., 2013; Libralato et al., 2013; Ciacci et al., 2012). Although these studies have dealt with the matter in different ways, all of these publications share the same animal model: the *Mytilus galloprovincialis*.

As reported by these authors this bivalve mollusc, due to its characteristics, is particularly appropriate for these studies and in recent papers, Rossi et al. (2016) and Maisano et al. (2016) reported the effects of toxic contaminants, particularly metals and oil, present in the Italian industrial areas of Augusta-Melilli-Priolo (Sicily) and Bagnoli (Naples, Campania) on caged *Mytilus galloprovincialis*. These results, even though interesting, may have been affected by several uncontrolled environmental variables. In this context, there is a clear need to set up controlled systems.

The effects of pollutants in the marine environment were usually measured in laboratory by traditional investigations on animal models and the experimental results were extrapolated to outdoor context. However, the greatest difficulty in the arrangement of this kind of laboratory experimentation lies in reproducing the environmental conditions. Consequently, the results are often biased. This constraint provided the impetus to set systems reproducing, as faithfully as possible, marine environment parameters (e.g. salinity, temperature, turbulences, sediment resuspension frequency and relevance).

When oil contaminations have to be investigated in laboratory, hydrocarbon concentrations are maintained in the system at a relatively constant level by regular replenishment of the Total Petroleum Hydrocarbons (TPH) or by the use of flow-through techniques. In recent studies, artificial simulation systems, such as microcosms and mesocosms, were successfully used to study both natural ecological processes and the effects of disturbance of oil contamination. The advantage of using such highly controlled laboratory-scale systems resides in the possibility to regulate most of the major ecological variables (chemical, physical or biological parameters) compared to those achievable in natural ecosystems. This possibility, alongside with their greater repeatability and reliability than those of natural environments makes microcosms and mesocosms very attractive (Cappello and Yakimov, 2010). For these reasons, their use is increasing and ranges from the evaluation of the effects of the dynamics of microbial populations in the marine environment to the analysis of diatom blooms (Cappello et al., 2015; Hassanshahian et al., 2014; Cappello et al., 2012; Cappello et al., 2007a, 2007b). Microcosms have also the capability to simulate the weathering processes of oil spills in marine environment such as photo-oxidation, evaporation and spreading on water surface, vertical penetration into shoreline matrices, and biodegradation. The evaluation of appropriate oily wastewater treatments requires to evaluate both the gross effectiveness of such techniques and the ecological impact of the response. Concerning these “scale system reactors”, such as micro- and mesocosms, where controlled experimental conditions can be maintained for prolonged time, increase the predictive capability of toxicity studies (Della Torre et al., 2012; Crisafi et al., 2011).

This study is aimed at evaluating the potential effectiveness of a biotechnological strategy in controlling and reducing the impact of marine oil pollution directly on the biological compartment; this poupose is achieved evaluating in the species *Mytilus galloprovincialis*the modifications in selected biomarkers.

We set up a series of 5 microcosms ensuring to control any potential effects: i) uncontaminated seawater, ii) seawater with only Commercial Diesel, iii) seawater with only dispersant BIOVERSAL HC, iv) seawater with hydrocarbons and BIOVERSAL HC (simulating the untreated oily wastewater discharge), and v) a system in which the same seawater as in microcosm iv has previously been treated by MBR (simulating the treated oily wastewater discharge). Samples of *Mytilus galloprovincialis* have been kept in all the microcosms to assess the effects of each different condition on gills and digestive gland morphology as well as the mRNA expression of a panel of genes (*Heat Shock Protein 70, Metallothionein 10, Superoxide dismutase 1, Catalase, Cytochrome P450*) involved in physicochemical and oxidative stress response.

2. Materials and methods

2.1. Experimental design

2.1.1. Microcosm setup

All experiments were carried out in the “Mesocosm Facility” of IAMC-CNR of Messina (Italy). As reported in Fig. 1, animals were housed in glass tanks (100 × 30 × 40 cm, volume 120 L) filled in continuous (8 L/h) flow of seawater (salinity 37–38‰) collected directly from the station “Mare Sicilia” (38°12.23′N, 15°33.10′E; Messina, Italy) by a pipeline from the sea, in order to ensure approximately two water turnovers daily (Cappello et al., 2015). Natural seawater was filtered through a 300-μm nylon mesh to remove large metazoans and detritus. To ensure a constant level of water, each microcosm was equipped with a relief valve connected to a vertical conduct (PVC-U pn10, 200 mm Ø) placed laterally in the tank to continuously discharge excess seawater. Water within each microcosm was continuously gently mixed with a pump (35 L/h) placed close to the entrance of each tank to provide more homogenous conditions within each microcosm. The measurement of pH and temperature, performed through a multiparametric probe Waterproof CyberScan PCD 650 (Eutech Instruments, The Netherlands), revealed values of 19.5–20.5 °C (daily temperature fluctuations not exceeded 1 °C) and approximatively constant pH values (around 7). The following five microcosms composed the experimental setup:

1) White (W): microcosm supplied only with seawater (uncontaminated system).
2) Black-1 (B1): this microcosm was conceived to simulate the effect of untreated oily wastewater discharged to the marine environment. In order to achieve this goal, the microcosm B1 was constantly supplied with a mixture of seawater, Commercial Diesel and