Aquatic Toxicology 201 (2018) 109–118

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Proteomic response of gill microsomes of *Crassostrea brasiliana* exposed to diesel fuel water-accommodated fraction

Gabrielle do Amaral e Silva Müller[a], Karim Hahn Lüchmann[b], Guilherme Razzera[a], Guilherme Toledo-Silva[a], Maria João Bebianno[a,c], Maria Risoleta Freire Marques[a], Afonso Celso Dias Bainy[a,*]

[a] *Laboratory of Biomarkers of Aquatic Contamination and Immunochemistry – LABCAI, Biochemistry Department, Federal University of Santa Catarina, Florianópolis 88040-900, Brazil*

[b] *Laboratory of Biochemistry and Molecular Biology – LBBM, Fishery Engineering Department, Santa Catarina State University, Laguna, 88790-000, Brazil*

[c] *Centre of Marine and Environmental Research (CIMA), University of Algarve, Campus de Gambelas, 8000-139 Faro, Portugal*

ARTICLE INFO

Keywords:
Diesel oil
Ecotoxicoproteomics
Microsome
Crassostrea brasiliana
Endoplasmic reticulum

ABSTRACT

Diesel fuel water-accommodated fraction (diesel-WAF) is a complex mixture of organic compounds that may cause harmful effects to marine invertebrates. Expression of microsomal proteins can be changed by oil exposure, causing functional alterations in endoplasmic reticulum (ER). The aim of this study was to investigate changes in protein expression signatures in microsomes of oysterl *Crassostrea brasiliana* (=*C.gasar*) gill after exposure to 10% diesel-WAF for 24 and 72 h. Protein expression signatures of gills of oysters exposed to diesel-WAF were compared to those of unexposed oysters using two–dimensional electrophoresis (2-DE) to identify differentially expressed proteins. A total of 458 protein spots with molecular weights between 30–75 kDa were detected by 2-DE in six replicates of exposed oyster proteomes compared to unexposed ones. Fourteen differentially expressed proteins (six up-regulated and eight down-regulated) were identified. They are: proteins related to xenobiotic biotransformation (cytochrome P450 6 A, NADPH-cytochrome P450 reductase); cytoskeleton (α-tubulin, β-tubulin, gelsolin); processing and degradation of proteins pathways (thioredoxin domain-containing protein E3 ubiquitin-protein ligase MIB2); involved in the biosynthesis of glycolipids and glycoproteins (beta-1,3-galactosyltransferase 1); associated with stress responses (glutamate receptor 4 and 14-3-3 protein zeta, corticotropin-releasing factor-binding protein); plasmalogen biosynthesis (fatty acyl-CoA reductase 1), and sodium-and chloride-dependent glycine transporter 2 and glyoxylate reductase/hydroxypyruvate reductase. Different patterns of protein responses were observed between 24 and 72 h-exposed groups. Expression pattern of microsomal proteins provided a first insight on the potential diesel-WAF effects at protein level in microsomal fraction of oyster gills and indicated new potential biomarkers of exposure and effect. The present work can be a basis for future ecotoxicological studies in oysters aiming to elucidate the molecular mechanisms behind diesel-WAF toxicity and for environmental monitoring programs.

1. Introduction

Due to the increased oil demand worldwide, about three billion tons of petroleum and gas were transported in the oceans in 2016 (UNCTAD/RMT, 2017). Associated with this activity, a number of accidents related to oil exploration, production and transport have occurred in the last decades, leading to the exposure of aquatic organisms to petrochemical compounds (Anisuddin et al., 2005; Bejarano et al., 2006; ITOPF, 2018). Diesel oil is the main source of energy for industry, transportation, agricultural and commercial purposes (Brasil, 2007, 2012). Thus, there is an urgent need to predict and evaluate short-term toxic effects of crude oil and its by-products in aquatic organisms.

Bivalves are ecologically and economically important in coastal ecosystems and aquaculture areas. Understanding the bivalve responses and their susceptibility to contaminants became an important issue (Cajaraville et al., 2000; Mi et al., 2005; Gomes et al., 2013, 2014) since they are sessile, filter-feeders, abundant, show wide geographical distribution and can bioconcentrate contaminants (Melwani et al., 2014). The mangrove oyster *Crassostrea brasiliana* (sin. *Crassostrea gasar*, Lazoski et al., 2011) can be found along the Brazilian coast (Galvão et al., 2013) and can be exposed to a wide range of direct and indirect anthropogenic impacts. Recent data indicate that diesel fuel water-

* Corresponding Author.
E-mail address: afonso.bainy@ufsc.br (A.C.D. Bainy).

https://doi.org/10.1016/j.aquatox.2018.06.001
Received 3 October 2017; Received in revised form 28 May 2018; Accepted 4 June 2018
Available online 06 June 2018
0166-445X/

accommodated fraction (diesel-WAF) induces enzymatic and molecular changes in *C. brasiliana* (Lüchmann et al., 2011, 2012). However, there is a need to search for additional biomarkers, at protein level, in order to get more powerful end-points which could reflect the responses to exposure (Dowling and Sheehan, 2006; Sanchez et al., 2011; Ge et al., 2013).

Proteomics is a powerful tool to get a holistic view of protein expression signatures (PES), overcoming the traditional disadvantages of single biomarker analysis (Liu and Wang, 2012; Sanchez et al., 2011) and has been used to assess disturbances in the metabolic pathways, and identify protein changes induced by stressors (Chen et al., 2016). This technique allows a rapid qualitative and quantitative analysis to identify protein expression changes and provides a robust platform for subsequent identification of specific proteins (Sheehan, 2006). In bivalves, proteomic approaches were used to investigate changes in PES in *Mytilus edulis* exposed to crude oil (Mi et al., 2007), *Crassostrea gigas* exposed to urban sewage (Flores-Nunes et al., 2015), *Mytillus galloprovincialis* exposed to metal nanomaterials (Gomes et al., 2014) and to a mixture of contaminants (Mi et al., 2005), and in *Crassostrea hongkongensis* and *Crassostrea virginica* exposed to metals (Liu and Wang, 2012; Meiller and Bradley, 2002). These assessments were carried out using two-dimensional gel electrophoresis (2-DE). However, this methodology is biased, since bivalves and other non-model animals often lack genome or protein sequences in the databases. However, sequences identified in the *Crassostrea gigas* genome (Zhang et al., 2012) are helping in the protein identification in *C. brasiliana*.

Proteomes of subcellular compartments show some advantages over traditional approaches which use entire cells because they allow a more detailed analysis of a particular organelle and its biological functions (Jung et al., 2000; Wong and Adeli, 2009). Microsomes are the most active membranous structure involved in the synthesis and secretion of proteins and play a vital role in the biotransformation of many stressors (Wong and Adeli, 2009). Subcellular analyses showed that oil can induce morphological and functional changes in the endoplasmic reticulum (ER) (Bach and Baker, 1991). PES changes were identified in the microsomal content of mussel tissues (*Mytilus* sp.) exposed to organic compounds (Jonsson et al., 2006a), of rats exposed to silicon dioxide (Tananova et al., 2014) and to hydrocarbon squalene (Ramírez-Torres et al., 2012), and pathogenic fungus *Aspergillus fumigatus* challenged with metals (Moloney et al., 2016).

In this work, a proteomic approach based on 2-DE coupled with mass spectrometry (MS) was applied to assess microsomal protein changes in gills of the oyster *C. brasiliana* exposed to diesel-WAF for 24 and 72 h. This study is an addition to previous research carried out by our research group, which evaluated the bioaccumulation of polycyclic aromatic hydrocarbons and their effects on oxidative stress-related enzymes in oysters exposed to 2.5, 5, 10, 20% diesel-WAF for 96 h (Lüchmann et al., 2011). Likewise, transcriptional changes in gills of *C. brasiliana* exposed to 10% WAF for 24 h were evaluated (Lüchmann et al., 2012). The results obtained here showed differences in PES that contribute to elucidating the effects of exposure to diesel-WAF in this species and identify potential new biomarkers to assess diesel toxicity in bivalves.

2. Materials and methods

2.1. Diesel-WAF preparation

Diesel-WAF was prepared according to Singer et al. (2000) with minor modifications. Briefly, one part (1 L) of fresh diesel fuel was diluted with nine parts (9 L) of 0.45 μm-filtered seawater (salinity 25) in a sealed 14 L glass flask protected from light, in order to minimize evaporation and degradation of the fuel components. The diesel-water mixture was stirred for 23 h with a homogenizer Glass-Col at 1600 rpm at a constant temperature of 21 °C. After settling for 1 h, the aqueous phase was collected. A dilution of WAF (10%) was prepared by adding the appropriate amount of the stock solution to the filtered seawater (1:9, v:v). This diesel-WAF concentration was chosen based on a previous results obtained in *Crassostrea brasiliana* exposed to 2.5, 5, 10 and 20% (Lüchmann et al., 2011).

2.2. Exposure experiment

Mangrove oysters, *C. brasiliana*, of similar shell size (6–8 cm) were obtained from an oyster farm at Sambaqui beach (Marine Mollusc Laboratory/Federal University of Santa Catarina, UFSC) in Florianópolis, southern Brazil. After collection, the animals were immediately transported to the laboratory, where they were held in aerated 0.45 μm-filtered seawater maintained at constant temperature (21 °C) and salinity (25 ppt), and fed twice a day on microalgae *Chaetoceros muelleri* and *Isochrysis sp.* at a density of 3.3×10^6 cells mL^{-1} and 2.2×10^6 cells mL^{-1}, respectively. Oysters were acclimated for 7 days and water was changed daily prior to the chemical exposure. After the acclimation period, groups of 30 oysters were exposed to 10% diesel-WAF for 24 and 72 h in 40 L glass tanks (one animal per 1 L) kept at 21 °C and at a salinity of 25 ppt. The exposure was carried out in duplicate. A control group was kept without the addition of diesel-WAF. Oysters were not fed during the exposure period to prevent accumulation of diesel-WAF via food. After the exposure periods, five unexposed and exposed oysters were collected, dissected and the gills were immediately excised, frozen individually in liquid nitrogen, and stored at −80 °C until further analysis.

2.3. Sample preparation

Gills of five oysters from control and exposed groups weighing 100 mg each were homogenized in 4 volumes of (w:v) chilled buffer (20 mM Tris-HCl pH 7.6 containing 0.5 M sucrose, 0.15 M KCl, 0.1 mM PMSF and 1 μL/mL cocktail of protease – GE Healthcare) using the homogenizer Tissue-Tearor™. Homogenates of control and exposed gills were centrifuged separately at 10,000 x g for 20 min at 4 °C. The supernatant was submitted to an ultracentrifugation at 100,000 x g for 1 h at 4 °C to obtain the microsomal fraction. Microsomal pellet was resuspended in chilled buffer (20 mM Tris pH 7.6 containing 20% glycerol and 0.1 M EDTA) (Jonsson et al., 2006a, 2006b). Two independent protein extractions from each pool (control and exposed) were carried out in triplicate to assess protein levels. The microsomal proteins were quantified with 2-D Quant Kit (GE healthcare) according to the protocol described by the manufacturer using bovine serum albumin (BSA) as a standard (Bradford, 1976). Samples were stored at −80 °C until further proteomics analysis.

2.4. Two-Dimensional gel electrophoresis (2-DE)

2-DE was carried out in both control and exposed groups. 300 μg of microsomal proteins were re-suspended in rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 0.5% IPG buffer pH 4–7 (GE Healthcare), 40 mM 1,4-dithiothreitol (DTT), 0.002% (w/v) bromophenol blue and 0.5% Triton X-100) (Jonsson et al., 2006b).

The hydrophobic structure of the microsomal fraction (Amar-Costesec et al., 1974) makes protein solubilization difficult (Mi et al., 2005). In order to optimize 2-DE resolution, several technical adjustments regarding protein extraction, solubilization and setup of electrophoretic parameters were performed. Three different protein extraction methods were tested, based on acetone (Gorg, 2004), TCA/acetone (adapted from Westermeier and Naven, 2002) and 2-DE Clean Up Kit (GE Heathcare). Proteins were loaded on 13 cm IPG strips (ImmobilineDrystrip Gels - IPG strips - GE Healthcare) of pH 4–7 and 3–10 and passively rehydrated at room temperature in IPGphor strip holder (GE Healthcare). Isoelectric focusing (IEF) was run in Ettan IPGphor III system (GE Healthcare) at 20 °C with total maximum current of 50 μA/