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Acute exposure to water-soluble fractions of marine diesel oil: Evaluation of apoptosis and oxidative stress in an ascidian

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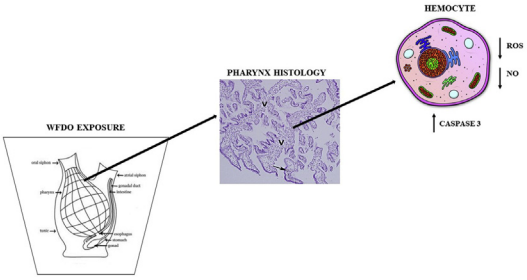
HIGHLIGHTS

- Water fraction diesel oil (WFDO) did not cause ascidian mortality.
- WFDO altered the ascidian siphon reflex.
- Nitric-oxide production was reduced in pharynx cells after acute WFDO exposure.
- Reactive oxygen species were reduced in pharynx cells after acute WFDO exposure.
- Catalase activity decreased after acute WFDO exposure.
- Pharynx cells reacted to WFDO by activating apoptotic pathways.

GRAPHICAL ABSTRACT



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ABSTRACT

To understand the mechanisms involved in organisms' responses to toxicity from oil pollution, we studied the effect of acute exposure (24 h) to the marine water-soluble fraction of diesel oil (WFDO) on the ascidian *Styela plicata*. We evaluated the mortality and behavior by means of the siphon reflex, and the response of blood cells (hemocytes) contained in the pharynx, by means of the production of nitric oxide (NO) and reactive oxygen species (ROS), in addition to the activity of the antioxidant enzyme catalase (CAT). We also correlated oxidative stress with the activation of apoptotic pathways. No mortality occurred 24 h after the ascidians were exposed to 5% and 10% marine WFDO; however, the siphon reflex, a behavioral test based on the time that the animals took to close their siphons, increased. We also observed an inflammatory response, as estimated by the increase in the number of hemocytes in the pharynx. NO and ROS production and CAT activity were reduced, whereas caspase-3, a signaling molecule involved in apoptosis, was activated. This suggests that in ascidians acutely exposed to oil, another mechanism can occur in addition to oxidative stress. Another possibility is that WFDO may directly interact with cellular macromolecules and activate caspase-3, independently of generating oxidative

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stress. The results showed that components of diesel oil affected a marine organism, which showed reduced ROS production in the pharynx cells, including hemocytes, and activation of apoptotic pathways.
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1. Introduction

Marine pollution, especially by xenobiotics, has increased in recent decades. Oils from industrial wastewater, watercraft and oil spills are some of the major ocean pollutants (Bhattacharjee and Fernando, 2008). Oil derivatives are poorly soluble but disperse easily in water, and contain many chemical compounds including heavy metals, monoaromatic hydrocarbons, phenols, nitrogen, sulfur, and, mainly, a mixture of polycyclic aromatic hydrocarbons (PAHs) (Saeed and Al-Mutairi, 1999; Santos et al., 2013). PAHs are the most toxic component found in the water-soluble oil fraction (Perez et al., 2010).

High concentrations of oil chemical compounds, similar to levels in the ocean after oil spills, can cause total mortality of marine organisms, although low concentrations usually do not (Cadiou et al., 2004; Claireaux et al., 2004; Milinkovitch et al., 2011). Marine filter-feeding animals exposed to oil and its fractions can accumulate toxic substances in their tissues (Ray et al., 2015) and produce reactive oxygen species (ROS) (Livingstone, 1991). The resulting imbalance between ROS formation and the activity of antioxidant enzymes, which eventually leads to oxidative stress (Valavanidis et al., 2006), has been suggested as the main mechanism involved in the cell death of marine organisms exposed to oil chemical compounds (Hannam et al., 2010). Nitric oxide (NO), also involved in oxidative stress, may activate signaling pathways that culminate in apoptosis, but has not been shown to be correlated with soluble oil fractions (Brüne, 2003; Comes et al., 2007).

Ascidians are sessile marine organisms and good indicators for marine pollution; however, their detoxification mechanisms activated by exposure to water-soluble oil are incompletely understood (Menin et al., 2008). Here, we studied the effects of acute exposure to low concentrations (5% and 10%) of a marine water-soluble fraction of diesel oil (WFDO) on the ascidian *Styela plicata*, and analyzed the mortality, behavior, and the production of NO and ROS and apoptosis in the pharynx, since this organ is involved in filter-feeding and is the part of the animal that first contacts the water.

2. Materials and methods

2.1. Animal collection

A total of 180 adults of *S. plicata* were collected from Praia Rasa, Armação de Búzios, Rio de Janeiro, Brazil, and maintained at 20 °C in an aerated aquarium with controlled photoperiod (12 h light/12 h dark), pH (approximately 8.0), and salinity (34 PSU), for seven days before the experimental procedures. The animals were fed with nauplii of *Artemia* (Sardet et al., 2011). The collection and maintenance of ascidians were approved by Sistema de Autorização e Informação em Biodiversidade (SISBIO), permit no. 60330-1.

2.2. Preparation of WFDO

Commercial marine diesel oil was obtained at a gas station. The WFDO was prepared following Singer et al. (2000). The sea water was obtained directly from the ocean, filtered (0.22 μm), and mixed with diesel oil in a proportion of 10:1 v/v for 24 h, in an amber-colored container. The resulting 100% WFDO was carefully separated from crude diesel oil using a decantation funnel, and then was diluted with sea water obtained directly from the ocean, to attain the experimental concentrations of 5% and 10% WFDO.

2.3. Exposure of ascidians to WFDO

The animals were separated into experimental groups: Group 1– Control animals, not exposed to WFDO; Group 2– Animals exposed to 5% WFDO; Group 3– Animals exposed to 10% WFDO.

After 24 h exposure to 5% and 10% WFDO, the animals were anesthetized with 10% menthol crystals dissolved in sea water. After 30 min, those that did not close their siphons after a touch were considered to be anesthetized. They were killed by dissection of the pharynx, and the blood cells (hemocytes) were isolated.

2.4. Stimulation siphon and mortality tests

Live ascidians (n = 9 from each group) were submitted to the siphon-stimulation test (SST) before (1 h before exposure to WFDO: 1 h) and after (0, 1, 5 and 24 h after exposure to WFDO). The SST consists of mechanical stimulation of the oral siphon, with no other type of disturbance (Medina et al., 2015). The SST was recorded using a Nikon Coolpix 1830 camera, and the time that the animals took to close their siphon was measured using the program Windows Live™ Movie Maker version 2011© (Microsoft Corp.). If an ascidian did not respond to the mechanical stimulation, it was presumed to be dead.

2.5. Preparation of pharynx for routine microscopy and immunofluorescence, and isolation of hemocytes for morphological observation

Pharynxes (n = 9 from each group) were dissected and fixed for 1 h in 4% paraformaldehyde prepared in artificial sea water (420 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$, 10 mM Hepes buffer, pH 8.0; ASW) and processed for embedding in Paraplast® (Sigma). The sections (5 μm) were stained with hematoxylin and eosin (H&E), and the morphology of the samples was observed under an Olympus DP71 light microscope. Other 5-μm sections were prepared for examination using immunofluorescence for apoptosis, with incubation overnight in the primary antibody, rabbit anti-activated antibody (Millipore, AB3623; 1:100 dilution), and then 1 h incubation with secondary anti-rabbit Alexa 546-conjugated antibody (Invitrogen, A11035; 1:5000 dilution). The antigens were unsuitable for pre-adsorption testing. For negative controls, the primary antibodies were omitted from the incubation steps. The images were obtained under the Olympus DP71 microscope.

To quantify the fluorescence per area from immunoreactions for activated caspase-3 in the pharynx of animals exposed and not exposed to WFDO, we observed 3 images from each of 3 animals, under 100× magnification. We analyzed the differences among the groups using the software Image J 1.5.

To obtain the hemocytes, the same ascidians used for routine histology of the pharynx were bled from incisions in their oral siphons before the pharynx was dissected. The hemocytes were loaded onto glass slides for 30 min, fixed with 4%

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