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Effects of the water-soluble fraction of diesel oil (WSD) on the fertilization and development of a sea urchin (*Echinometra lucunter*)

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ABSTRACT

Considering the high number of accidents with diesel oil spills occurring in the marine ecosystem, toxicity tests aimed at assessing the effects of this pollutant on biota are necessary and urgent. Thus, the present study aimed to evaluate the toxicity of the soluble fraction of diesel oil (WSD) in the fertilization success of gametes and pluteu larvae of the sea urchin *Echinometra lucunter*. To do this, gametes and embryos were exposed to concentrations of 0% (control group), 0.5%, 1.5% and 2.5% of WSD. The fertilization success of exposed gametes and embryos were reduced significantly when compared to the control group in all tested concentrations. With this finding, it is evident that diesel oil can be significantly promoted in the early and adult life stages of a particular organism, and a better way of evaluating this toxicity is through the analysis of contaminant effects throughout the reproductive cycle of a species.

1. Introduction

Of all the problems caused by excess pollution in the marine environment, one of the most important is the contamination caused by oil and its derivatives (Simonato et al., 2008). Diesel oil is the primary fuel used in large and small fishing vessels, as well as in recreational boats, making it one of the most targeted petroleum derivatives responsible for the increase in the release of harmful organic compounds into the marine environment (Pacheco and Santos, 2001a). Diesel oil can promote severe disturbances in the ecosystem, as its water-soluble fractions (WSD) may become available to marine biota (Teles et al., 2003). This bioavailability can occur through the incrustation of the contaminant to the sediment and by the solubility of the hydrocarbons in water (Rodrigues et al., 2010).

Diesel oil is a complex mixture of chemical elements containing hundreds of harmful chemicals and a high concentration of mono-hydrocarbons (BTEX) and polyaromatic hydrocarbons (PAHs) that make it the most dangerous derivative (Pacheco and Santos, 2001a). In the soluble fraction of diesel oil (WSD), benzenes, toluene and xylenes (BTEX) are among the compounds most harmful to the environment due to their high solubility in water (i.e., directly related to their pollutant potential), their chronic toxicity and their exceptional ability to migrate. Additionally, BTEX can cause cancer when under chronic exposure conditions and can accumulate in the lipid compartments of the cell membrane, disrupting the physiological properties of the cell membrane (Peralta-Zamora et al., 2005; Tiburtius et al., 2005; Rodrigues et al., 2010). However, PAH compounds can induce reproductive failure and lead to immunological and behavioral changes (Sagerup et al., 2016). BTEX and PAHs are also known to promote mutagenic and genotoxic effects, which may lead to the induction of oxidative stress and even neoplasia (Nogueira et al., 2013).

The sea urchin *Echinometra lucunter* (Linnaeus, 1758) is of great ecological importance because it modifies the architecture of the ecosystem due to its bio-erosive capacity and contributes to the control of the community of benthic macroalgae (Lima et al., 2009). Its geographical distribution extends from the southern USA to the south of Brazil (Lima et al., 2009; Mariante et al., 2009). Many aspects prove that *E. lucunter* can be a useful indicator of environmental contamination. First, they are critical components of marine ecosystems (i.e., contributing 90% benthic biomass) (Brusca and Brusca, 1990). Second, they have an expanded outer epithelium, through which they can pick up substances dissolved in the environment, and their benthic habits may make them more susceptible to capturing pollutants adsorbed on marine sediments (Candia Carnevali, 2005). Third, they are second- or

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third-degree predators, and therefore are subject to biomagnification processes. Finally, their developmental biology is well characterized, and tests involving their gametes and embryos are already well studied for contaminant evaluation and environmental changes. These tests are easy to manipulate and have faster responses to address the problem (Cameron, 2002). For that reason, the aimed of the present study was to evaluate the toxicity of WSD in fertilization success of gametes and pluteus larvaes of *Echinometra lucunter* exposed directly to the contaminant.

2. Material and methods

2.1. Preparation of WSD

For the preparation of the WSD, commercial automotive diesel oil acquired in a fuel supply station was used. To obtain the WSD, one part of commercial diesel oil was added to four parts of seawater in a glass aquarium. The mixture was then exposed to intense sunlight for 6 h, simulating a diesel spill in tropical conditions (Nicodem et al., 1998). After that, the upper insoluble phase was withdrawn from the aquarium through a faucet located on the lower side of the aquarium to avoid any contact between the phases, and the remaining water phase was collected and diluted to 0.5%, 1.5% and 2.5% with filtered seawater.

2.2. Quantification of hydrocarbons from the different dilutions of WSD

A commercial laboratory performed the chemical analysis of the 0.5%, 1.5% and 2.5% WSD used in the toxicity bioassay. The BTEX analysis that followed employed a gas chromatograph (Perkin-Elmer Clarus® 500) with flame ionization detector (GC-FID) and headspace Turbomatrix HS-40 sampler (Perkin-Elmer) (Method 8015B-EPA). The PAHs were analyzed with a gas chromatograph (Perkin-Elmer Clarus® 500) with a mass spectrometer (GC-MS) (METHOD 8270D-EPA).

2.3. Collection of organisms

Echinometra lucunter sea urchins were collected on a rocky shore located on the north coast of the State of Espírito Santo - Brazil (20° 10'24.32 "S, 40° 11'11.89" W). For the gametes and embryos test, a total of 20 individuals were collected per month to have a considerable proportion of males and females (n = 5 males and n = 5 females) for the extraction of gametes as these individuals do not have sexual dimorphism. Sea urchins were collected by free diving. The collections were performed at the end of the crescent moon and beginning of the full moon of each month (reproductive period) and low tide period, according to the method proposed by Mariante et al. (2009), from March to December of 2016 to guarantee the success of gametes extraction, considering that in some months the animals do not release gametes due to some natural events as rain or a variation in the tide. In eight months of collections, we got success only in five months. After the collections, the sea urchins were kept in polyethylene tanks with a 60 L capacity (four animals per aquarium) and with constant aeration for a period of 24 h until the beginning of the experiments. The collected seawater was filtered and maintained retaining the appropriate values of salinity (35 UPS), temperature (25 °C) and pH (7.8–8.3) according to standard ABNT-NBR 15350 of test methods with sea urchins (Echinodermata: Echinoidea)/2006.

2.4. Obtaining gametes

For experimental with gametes and embryos, the induction and extraction of gametes were performed through the injection of 2.5 mL of a 0.5 M solution of potassium chloride (KCl) in the coelom cavity. After, the methodology proposed by standard ABNT-NBR 15350 of test methods with sea urchins (Echinodermata: Echinoidea)/2006 was followed.

2.5. Bioassay

For the gametes (fertilization) toxicity test, 0.5 mL of sperm liquid was diluted in 25 mL of seawater for activation. Next, 20 µL of this solution was added to test tubes (20 mL) containing 10 mL of the fractions 0.0% (control), 0.5%, 1.5% and 2.5% of WSD for a period of 20 min. After this exposure, a total of 300 oocytes (estimated according to standard ABNT-NBR) were added to the tests tubes containing the spermatozoon to promote fertilization. After 40 min, all test tubes were fixed with 0.5 mL of formaldehyde for fixation and subsequent observation of the fertilization rate. The percent fertilization of one hundred eggs was determined using an inverted microscope (40 × magnification) and a Sedgwick-Rafter camera. For the fertilization test, one spawning was carried out per month for eight months, and for each of the spawnings, the test was performed in quintuplicates. For the acceptability of the test results, it was necessary that the percentage of fertilized eggs in the control was greater than or equal to 80%.

For the toxicity test with embryos, the female gametes were added into a beaker containing 600 mL of seawater, and soon after, 2 mL of the already diluted and activated sperm solution was added to the solution containing the eggs. The solution was stirred for 3 min to promote fertilization and then allowed to stand for 2 h at the beginning of embryonic development. After this step, 100 µL of the egg solution was added to test tubes (20 mL) containing 10 mL of WSD concentrations of 0.5%, 1.5% and 2.5% and a control (0.0%). The experiments were kept in a refrigerated incubator B.O.D. (TE-371) with a controlled temperature of 26 °C and photoperiod of 12 h light and 12 h dark for approximately 36 h, the time required for the *E. lucunter* embryos to develop to the pluteus larval stage. After 36 h, all test tubes were fixed with 0.5 mL formaldehyde for evaluation of normal embryos (well characterized in a triangular shape and developed arms), and those that presented some morphological anomaly or retardation in their development (unstructured body and not developed and any primary stage of embryonic development). The embryos were evaluated using an inverted microscope (40 × magnification) and a Sedgwick-Rafter camera. For the embryo test, one spawning per month was also carried out for a total of eight months, and for each of the spawnings, the test was done in quintuplicate. For the acceptability of the test results, it was necessary that the percentage of well-developed pluteus larvae in the control was higher than or equal to 80%.

Both tests with gametes and embryos were carried out according to standard ABNT-NBR 15350.

2.6. Statistical analyses

The results concerning the fertilization and embryonic development tests were tested for their normality using the Shapiro-Wilk test (with $p < 0.001$) and represented by a linear regression using SigmaPlot version 12.5.

3. Results

3.1. Water chemical analyses

After quantifying hydrocarbons by gas chromatography, the concentrations of total BTEX and PAHs were obtained for all tested WSDs. The analysis indicated a higher concentration of PAHs than BTEX. Among the PAHs, naphthalene was the compound that presented the highest concentration of all the compounds found. Among BTEX, toluene was the compound with the highest concentration. The total BTEX was < 5.0 in 0.5%, 29.917 in 1.5% and 24.84 in 2.5%. The concentration of total PAHs was 2.21 in 0.5%, 51.80 in 1.5% and 145.13 in 2.5%. The concentrations of PAHs and BTEX in the WSD are summarized in Table 1.