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# Are changes in the structure of nematode assemblages reliable indicators of moderate petroleum contamination?

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ABSTRACT

This study assesses through a multiple before-after-control-impact (MBACI) design the effects of diesel oil on the structure of nematode assemblages in unvegetated tidal flats of a subtropical estuary. Oil-exposed treatments were contrasted with controls for a duration of four successive days before and after an experimental spill in three distinct areas of the Paranaguá Estuarine Complex (Southern Brazil). No significant differences were observed in nematode total density, number of taxa and the overall assemblage structure between the control and impact treatments from before to after the experimental spill. This reinforces the idea that, despite being good indicators of environmental stress, free-living marine nematodes are able to tolerate low concentrations of hydrocarbons and to survive in moderately contaminated areas. We also show that robust experimental designs are useful to avoid confounding expected natural variability with the effects of a mild impact.



## 1. Introduction

Accidents involving oil spills such as the Torrey Canyon in England, Tampico Maru in the United States, Amoco Cadiz in France (Botello and Macko, 1982) and, more recently, the largest spill in history in the Gulf of Mexico, between April and July of 2010 (Mariano et al., 2011), have attracted the interest of the general public and scientists towards oil contamination of the oceans. Previous oil-spilling accidents in Brazil, such as those caused by the ships Norma and Vicuña, which released naphtha, methanol, diesel, and bunker in the Paranaguá Bay in 2001 and 2004, emphasize the need to assess the intensity and extent of damage caused by oil spills, as a first basis for monitoring and control measures.

Estuaries act as sinks for sediment and the associated pollutants from numerous human activities (Yang et al., 2006; Wang et al., 2012). Estuarine habitats are also considered more vulnerable to the impacts of oil spills because the confinement can favor the accumulation of hydrocarbons, mainly in intertidal vegetated areas (Sanz-Lázaro and Marín, 2009).

Oil effects on the benthic macrofauna have been extensively investigated through descriptive (Gómez Gesteira and Dauvin, 2000; Edgar et al., 2003; Zenetos et al., 2004; Andersen et al., 2008; Morales-Caselles et al., 2008; Ocon et al., 2008) and experimental approaches, both in the field (Faraco and Lana, 2003; Schratzberger et al., 2003; Lu and Wu, 2006; Egres et al., 2012) and laboratory (Bhattacharyya et al., 2003). However, few studies have experimentally investigated the effects of exposure to hydrocarbons on meiofaunal organisms (Fleeger and Chandler, 1983; Ansari and Ingole, 2002; Mahmoudi et al., 2005; Ansari et al., 2010; Beyrem et al., 2010; Boufahja et al., 2011).

Meiofaunal organisms in general and nematodes in particular are considered good indicators of contamination for their high abundance and diversity, short generation time, and direct benthic development (Fleeger and Chandler, 1983; Kennedy and Jacoby, 1999; Ansari et al., 2010). In addition, they are present in different sediment types, hydrodynamic conditions, and environments (Bongers and Ferris, 1999). Furthermore, their predominantly benthic life allows for direct contact with components dissolved in the interstitial water through their permeable cuticle (Warwick, 1981; Heip et al., 1985; Vranken and Heip, 1986; Bongers et al., 1991; Bongers and Ferris, 1999). Another advantage of using nematodes in environmental impact studies is the small sample volume necessary for routine studies, thereby allowing a large number of samples to be collected and, thus, ensuring statistical significance

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(Bongers and Ferris, 1999). In this context, the responses from nematode assemblages to environmental changes might provide stronger evidence of oil contamination than those obtained from other animals.

Assessments of the effects of oil spills on marine meiofauna are often contradictory and inconsistent. Overall responses seem to be dependent on the amount of oil spilled, environmental characteristics and target taxonomic groups (Fleeger and Chandler, 1983). Decreases in meiofaunal density and taxonomic richness have been repeatedly reported after experimental oil spills (Boucher, 1980; Danovaro et al., 1995; Mahmoudi et al., 2005) and exposure to sediments contaminated by mineral and synthetic lubricating oils (Beyrem et al., 2010). However, some meiofaunal taxa can be highly tolerant to contamination by hydrocarbons and positively respond to the experimental exposure (Fleeger and Chandler, 1983; Mahmoudi et al., 2005).

The analyses of impacts involving oil spills are often carried out after accidents and include descriptions of biological responses from plant and animal communities. Rarely, the pre-disturbance context is adequately known and inferences on the disturbance are made using a simple comparison between previously impacted locations and undisturbed control sites. Consequently, differences in the composition and structure of assemblages might simply reflect background variability preceding the spill (Underwood, 2000). Micro- and meso-scale experimental approaches are, therefore, more appropriate to establish a causal relationship between oil exposure and the biological responses (Glasby and Underwood, 1996). In this study, we investigated the effects of an experimental marine diesel spill on nematode assemblages using a multiple before–after control–impact (MBACI) design (Keough and Mapstone, 1997; Downes et al., 2002). We hypothesized that total density, number of taxa and overall structure of nematode assemblages living in oil-exposed areas would be significantly different from those in control areas, from before and after the experimental spill.

## 2. Materials and methods

### 2.1. Study area

The Paranaguá Estuarine Complex on the coast of Paraná State (48°25′W, 25°30′S) is formed by two main axes, the Paranaguá and Antonina Bays (east–west oriented) and the Laranjeiras and Pinheiros Bays (north–south oriented). This system comprises a diversity of estuarine and coastal ecosystems including coastal dunes, mangroves, salt marshes, rocky shores, and extensive tidal flats (Lana et al., 2001).

The Cotinga Channel (Fig. 1) is about 15 km long and receives freshwater input from the Maciel, Guaraguaçú, Correias, Almeidas, and Itiberê Rivers. Noernberg et al. (2006) classified this region as a sub-estuary based on its hydrographic and morphological features. This sub-estuary is composed of many meandering rivers with extensive floodplains, which favors the formation of unvegetated flats mainly through sediment delivery from tidal flows from east to west. Domestic effluents from the city of Paranaguá, where the municipal sewage is still discharged *in natura* in the estuary, reach the Cotinga Channel through the Itiberê River.

The tidal flats used in the experiment are located along the Cotinga Channel; the most internal area is near the mouth of the Guaraguaçú River, the intermediate area is near Rasa Island, and the most external area is near the mouth of the Maciel River (Fig. 1).

### 2.2. Experimental design and field procedures

We carried out an acute non-cumulative field experiment with the simulation of a single oil spill. Impacted treatments were contrasted with controls in three distinct areas over four successive sampling times, two before and two after the spill (Fig. 2). The MBACI design was used because it is logically capable of separating the effects of the experimental spill from the background environmental variation by using multiple controls and impacted areas (Keough and Mapstone, 1997). The temporal samplings, equally replicated at the times before and after the experimental spill, ensured the correct interpretation of interactions between time and space. The appropriate spatial and temporal replication ensures that the resulting estimates are reliable (Glasby and Underwood, 1996).

Experimental blocks were established in three areas along the Cotinga Channel. Each area included one experimental block corresponding to the impact treatment with the diesel spill and an undisturbed control. The control and impact blocks were established 40 m apart in each area and were positioned at similar tidal levels. Each block consisted of 12 1-$m^2$ plots with centralized experimental units of 0.35 × 0.35 m (Fig. 1). Plots were arranged in rows with a delimited pathway to avoid trampling and additional disturbances during sampling. Four of these 12 1-$m^2$ plots in each block were randomly assigned and actually used for sampling (Fig. 1).

The experiment was conducted during low tide, with the simulation of a single spill in early 2010, followed by the monitoring of biological responses between control and impact treatments in pre-established temporal scales two days before and two days after the oil exposure. In each centralized experimental unit of the impact treatment, 2500 ml of marine fuel oil, commercially named Marine Diesel Oil (MDO), was uniformly poured using a garden watering can. Maritime diesel oil is largely used as a fuel by small and medium vessels and in the auxiliary engines of large vessels. Marine fuel oil is produced by mixing of heavy oil fractions obtained by atmospheric distillation with fractions from secondary crude oil processing. The spilled oil was contained by wooden square artifacts properly allocated to prevent its dispersion and cross-contamination of the control treatments.

### 2.3. Biological sampling and processing

Four replicated cores were sampled for meiofaunal analyses from each randomly assigned treatment plot (control and impact), in the three unvegetated tidal flats during each of the four sampling times (1 and 2 days before and 1 and 2 days after the experimental oil spill) (Fig. 2). Meiofauna samples were taken using a corer 2.5 cm in diameter and 5 cm in height. Samples were processed according to the procedure proposed by Somerfield and Warwick (1996). Samples were first fixed in 4% formaldehyde and then were sieved through a 63-μm mesh. The retained material was separated using colloidal silica (Ludox TM 50) diluted to a specific gravity of 1.15 g $cm^{-3}$; this procedure was repeated three times. The final supernatant sample was transferred to a Dollfus plate, and 100 individuals (or all individuals if the total number was less than 100) were removed and diaphanized according to De Grisse (1969). Subsequently, permanent slides with approximately 10 individuals were assembled, and nematodes were counted and identified at the genus level under a stereomicroscope. The identification keys by Platt and Warwick (1983, 1988) and Warwick et al. (1998) were used. Finally, the total abundance of each species was calculated from the ratio between the frequency of each species among the 100 individuals and the total number in each sample.

Three sediment-replicated cores were collected from each treatment at each sampling time for chlorophyll-*a* and phaeopigment analyses; these samples were kept frozen until the analysis. Pigments were extracted from sediment samples with 10 ml of 100% acetone (Strickland and Parsons, 1972). The chlorophyll-*a* and phaeopigment concentrations were estimated using the equation described by Lorenzen (1967).