

Increased Zooplankton Behavioral Stress in Response to Short-Term Exposure to Hydrocarbon Contamination

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Abstract: The swimming behavior of the calanoid copepod *Centropages hamatus* is used as a very sensitive real-time screening tool to assess hydrocarbon contamination in marine waters. A behavioral stress index based on the complexity of swimming sequences is suggested as a potential tool to critically assess behavioral responses to natural and anthropogenic forcing in the marine environment.

INTRODUCTION

Massive crude oil spills such as *Torey Canyon* (1967), *Amoco Cadiz* (1978), *Ixtoc-I* (1979-80), *Exxon Valdez* (1989), *Sea Empress* (1996), *Erika* (1999) and *Prestige* (2002) are a major source of polycyclic aromatic hydrocarbons (PAHs) in estuarine and coastal waters [1]. However, leaking from ships, petroleum transport, refining, intentional spills, are more pernicious, but equally important, sources of PAHs in the ocean [1-2], especially in coastal and shelf waters [3-6].

The effects of PAHs contamination on marine planktonic organisms have been studied extensively in the laboratory and in the field, and a variety of reactive changes have been found in relation to incidental oil spills for a range of plankton species, manifested as alterations of biomass, abundance, and ecophysiological effects. Mesocosm experiments have yielded important information on the direct toxic effects on phyto- and zooplankton assemblages as well as the indirect effects on food-web interactions [7-11]. In these studies, artificial oil addition often increased the abundance of bacterioplankton and phagotrophic protists such as choanoflagellates and tintinnid ciliates, suggesting an enhanced carbon transfer from bacterial production to higher-order organisms in the microbial loop. Concurrently, the presence of oil readily depresses growth and photosynthesis of phytoplankton in pure cultures and natural aquatic environments [12-13], and most zooplankton in acute and chronic exposure experiments are highly vulnerable to hydrocarbons [14]. To date, the few studies regarding sub-lethal effects of hydrocarbons on copepods show, however, a very variable scenario depending on the chemicals used, their concentrations, and time of exposure, including anomalous metabolism [15-16], decreased or inhibited feeding [17-20], increased mortality [21], reduction in egg production [22], hatching rates [19], clutch size [23].

At low concentration and for short time exposure, hydrocarbons did not have any significant effect on feeding and egg production [17-19,24]. Decreases in egg production are observed, however, after long exposures to low hydrocarbon

concentrations [22], indicating detrimental cumulative effects unidentifiable under short-term incubations. While PAHs concentrations can reach dramatic concentrations up to 600 $\mu\text{g l}^{-1}$ in the North Sea and Sargasso Sea [25-26] and 10980 $\mu\text{g l}^{-1}$ in the Adriatic Sea [27], the 'natural' concentrations of PAHs typically range between 1 and 100 $\mu\text{g l}^{-1}$ [4,6,28-29]. The ability to assess rapidly any increase in the background concentration of PAHs related to e.g. incidental, localized oil spills is then critical to anticipate their pernicious cumulative effects on copepod biology and ecology.

Since swimming and feeding are intertwined in most copepod species, any disruption of copepod swimming is predicted to have detrimental consequences to copepod biology and ecology. Measurements of an organism's behavior following contaminant exposure may then provide a better understanding of the likely environmental consequences of toxic contamination than lethal effects, especially if behavior is disrupted at environmentally-realistic toxicant concentrations. Despite the few attempts to use the swimming behavior of the freshwater cladoceran *Daphnia* sp. as an indicator of exposure to toxic chemicals [30-34] and the increasing use of animals as an integrated measure of sub-lethal pollutant effects [35], similar information for marine invertebrates is still very limited [36]. In particular, no attempts have been made to assess the effects of hydrocarbons on copepod behavior, despite an impressive body of literature devoted to their behavioral ecology [37-40].

In this context, we assessed the potential for copepod swimming behavior to be used as an index of anthropogenic pollution. In particular, the effects of short-term exposure to 'natural' and extreme naphthalene (the most abundant hydrocarbon dissolved in oil contaminated waters) concentration are presented and analyzed.

ZOOPLANKTON COLLECTION AND ACCLIMATION

Copepods were collected in the coastal waters of the eastern English Channel using a WP2 net (200- μm mesh size) with close cod end horizontally towed between 0 to 5 m. Specimens were gently diluted in 30-litre isotherm tanks using *in situ* seawater ($S = 34$ PSU) and transported to the laboratory, where *Centropages hamatus* adult females were sorted by pipette under a dissecting microscope and acclimated for 12 hours in a 2-litre beaker containing fresh *in situ*

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seawater vacuum filtered through Whatman GF/C glass-fiber filters (porosity 0.45 μ m) prior to the behavioral experiments. All subsequent handling of animals was done at 18°C in a temperature controlled room.

TOXICITY TEST

The polycyclic aromatic hydrocarbon tested was the naphthalene as it has been widely used in toxicological assays involving copepods [17,22,24,41-42] and is one of the most abundant hydrocarbons dissolved in oil contaminated waters [41]. Naphthalene (96% purity, Sigma-Aldrich, St Louis, USA) stock solutions were prepared using acetone as a carrier (HPLC grade, 0.5 ml l⁻¹). Naphthalene stock solutions were transferred into the behavioral container and diluted with GF/C filtered (porosity 0.45 μ m) *in situ* seawater at 50, 100, 500, 1000, 2500, 5000 and 10000 μ g l⁻¹ to assess the effect of 'natural' (1-100 μ g l⁻¹; [3-6]) and extreme (up to 10980 μ g l⁻¹; [27]) PAHs concentrations encountered in the ocean. In addition to the toxicity experiments, two controls were considered with (0.5 ml l⁻¹ in GF/C filtered *in situ* seawater) and without (GF/C filtered *in situ* seawater) acetone to assess the potential effect of acetone on *C. hamatus* swimming behavior.

BEHAVIORAL OBSERVATIONS AND IMAGE ANALYSIS

Experiments were conducted for each naphthalene concentration with adult females in a temperature controlled dark room (18°C) to assess their behavioral properties in relation to short-term exposure to naphthalene contamination. Prior to each experiment, ten females 1.31 \pm 0.05 mm (cephalothorax length, mean \pm SD) were randomly selected from the females stock, transferred in the experimental filming set-up filled up (a cubical 3.375-l glass container, 15 \times 15 \times 15 cm) with the test solutions and allowed to acclimatize for 10 min [39]. All the experiments were conducted with aerated (100% air saturation) fresh *in situ* seawater (salinity $S = 34$ PSU and pH = 7.5), in the dark and at night to avoid any potential behavioral artifact related to endogenous swimming rhythms [39]. The three-dimensional trajectories of the copepod were recorded at a rate of 25 frame s⁻¹ using two orthogonal, synchronized infrared digital cameras (DV Sony DCR-PC120E) facing the experimental container. The various components of the set-up were adjusted so that the copepods were adequately resolved and in focus. To avoid any bias related to phototropism, the only light source was provided by six arrays of 72 infrared light emitting diodes (LEDs), each mounted on a printed circuit board about the size of a business card (i.e. 9.3 cm long and 4.9 cm wide) connected to a 12 volts DC power supply. For each experimental condition, 10 individual females were recorded swimming for 20 min, after which valid video clips were identified for analysis. Valid video clips consisted of pathways in which the animals were swimming freely, at least two body lengths away from any chamber's walls or the surface of the water. Between 8 and 20 swimming paths were considered for each female. Duration of individual observations varied, and swimming paths ranged between 39 s and 3 min. Selected video clips were captured (DVgate Plus, 25 frames s⁻¹) as AVI movies and converted into QuickTime TM movies (QuickTime Pro), after which the x , y and z coordinates of swimming pathways were automatically ex-

tracted and subsequently combined into a 3D picture using LabTrack software (DiMedia, Kvistgård, Denmark). The time step was always 0.04 s, and output sequences of (x, y, z) coordinates were subsequently used to characterize the motility.

BEHAVIORAL ANALYSIS

Free-swimming *Centropages hamatus* pattern typically exhibits swimming bouts separated by breaking events during which they eventually sink tail pointed upward. As a consequence, the impact of naphthalene on the swimming behavior of *C. hamatus* was assessed through potential changes in (i) swimming speed (mm s⁻¹), sinking speed (mm s⁻¹), and (ii) percentage of time allocated to swimming (%).

By analogy with self-organized critical systems that build-up stress and then release the stress in intermittent pulses, the level of stress arising from each experimental conditions was described by a power-law that states that the cumulative probability distribution function of move duration T greater than a determined duration t follows [43-44] $P(t \leq T) = kt^{-\phi}$ where k is a constant and ϕ the scaling exponent describing the distribution. The exponent ϕ should decline under stress [45-46], and would be referred to as a 'stress exponent' hereafter. The scaling exponent ϕ was estimated as the slope of $N(t \leq T)$ versus t in log-log plots. Linear regression on the log-transformed data was used instead of nonlinear regression on the raw data as the residual error will be distributed as a quadratic and the minimum error is not guaranteed. This is not the case with nonlinear regression [43]. Finally, because an objective criterion is needed for deciding upon the appropriate range of t values to be included in the regression, we used $N(t \leq T)$ and t values which maximized the coefficient of determination and minimized the total sum of the squared residuals for the regression [43].

The distribution of the behavioral parameters were significantly non-Normal (Kolmogorov-Smirnov test, $p < 0.01$). Non-parametric statistics were then used throughout this work. Multiple comparisons between experimental conditions were conducted using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test was used to identify distinct groups of measurements [47].

NAPHTHALENE CONTAMINATION AND SWIMMING BEHAVIOR

C. hamatus adult females typically swam in helical loops (Fig. 1), and no differences were visually perceptible between uncontaminated control seawater (Fig. 1a) and naphthalene contaminated seawater (Fig. 1b). The swimming speed (6.51 \pm 0.04 mm), the sinking speed (1.52 \pm 0.05 mm) and the time spent swimming (66 \pm 2%) were not significantly different for the control experiments with and without acetone ($p > 0.05$). No significant differences in swimming and sinking speeds were found between *C. hamatus* considered in uncontaminated and naphthalene contaminated seawater ($p > 0.05$). The time spent swimming exhibits, however, a significant linear increase ($p < 0.01$) with naphthalene concentration from 66 to 81%. This indicates an increase in swimming activity under conditions of naphthalene contamination, and is consistent with previous observations showing an increased in the foraging activity of *C. hamatus* individuals

exposed to micro-scale turbulence [48-49], a ubiquitous natural stressor of the marine environment.

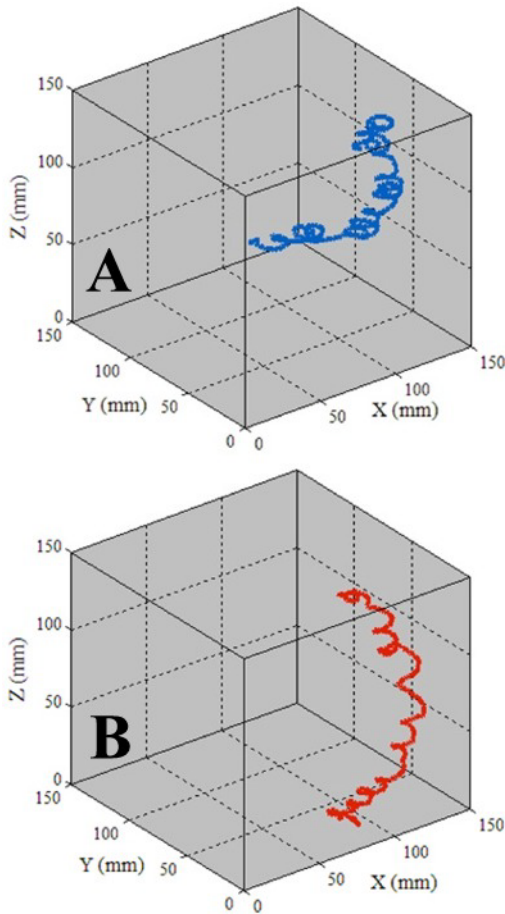


Fig. (1). Examples of the swimming behavior of the calanoid copepod *C. hamatus* in uncontaminated seawater (A) and in naphthalene contaminated ($1000 \mu\text{g l}^{-1}$) seawater (B).

The stress exponent ϕ did not significantly differ between the control experiments conducted with and without acetone (F-test, $p > 0.05$). In contrast, the values of ϕ were significantly different for uncontaminated and naphthalene contaminated seawater ($p < 0.05$, Fig. 2). While ϕ values were consistently significantly smaller ($p < 0.05$) for the naphthalene contaminated seawater than for the uncontaminated controls, they exhibit a highly significant ($p < 0.01$) power-law decay with naphthalene concentrations C as $\phi = 1.94C^{0.11}$ (Fig. 3). This clearly indicates an increase in behavioral stress with increasing naphthalene concentration.

BEHAVIORAL COMPLEXITY AND STRESS

In response to stress, organisms typically increase their metabolic rate and energy intake [50-51] and perform a series of compensatory responses to improve the probability of survival, involving the endocrine system and behavior [52]. As a consequence, a stressed animal will reduce the complexity of behavioral display [53-55]. The subsequent change

in pattern distribution of behavior sequences is likely to be related to the underlying dynamics of physiological components and can be used to detect the level of stress under different environmental conditions. This is consistent with the observed change in the complexity of behavior sequence with naphthalene concentration that illustrates the influence of stress on temporal patterns. The fact that fluctuations of behavior sequences tended to be more complex under non-stressful situations (i.e. no naphthalene or low naphthalene concentration) can be thought as an adaptive strategy that allows the organisms to exchange more information with the surroundings. During stressful situations (i.e. high naphthalene concentrations) there is not enough energy available and the complexity of behavior sequences are reduced [56-57].

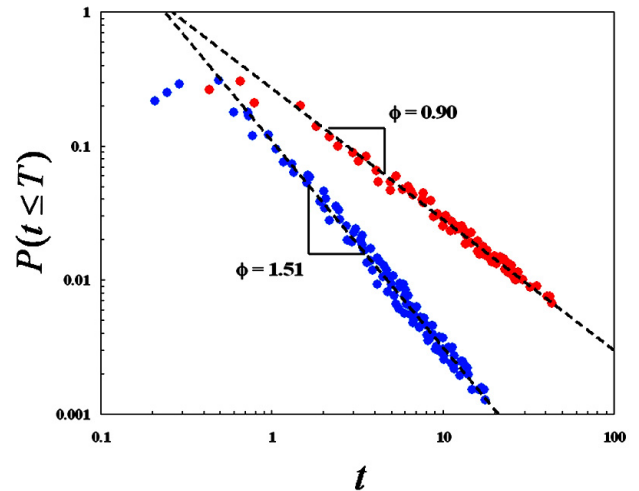


Fig. (2). Cumulative probability distribution function $P(t \leq T)$ shown as a function of move duration t (sec) for uncontaminated control seawater (blue dots) and naphthalene contaminated seawater (red dots).

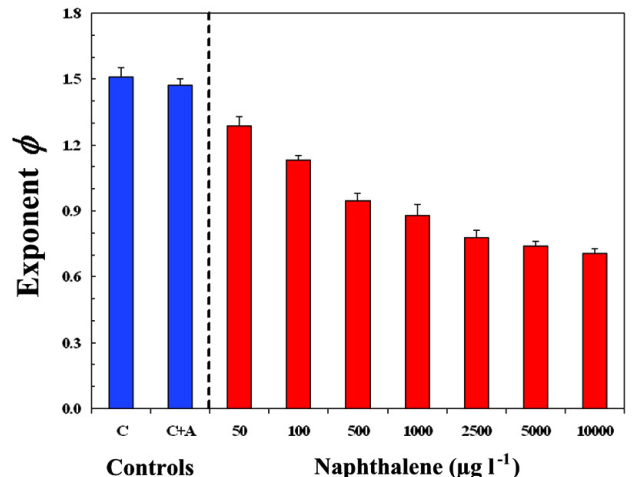


Fig. (3). The stress exponents ϕ estimated from the uncontaminated control experiments conducted without (C) and with (C+A) acetone, and the seawater contaminated with increasing naphthalene concentrations.

In addition, the scaling property of the cumulative probability distribution function of move duration T ($P(t \leq T) = kt^\phi$, see Fig. 3) is indicative of an underlying fractal structure

in the time series of behavioral sequences. Behavioral time series, though they often appear erratic, reveal $1/f$ like spectra; i.e., they are fractal-like because they display self-similar (scale invariant) fluctuations over a wide range of time scales [45,57]. Long-range correlation in biological systems is adaptive because it serves as an organizing principle for highly complex, nonlinear processes and it avoids restricting the functional response of an organism to highly periodic behavior [58]. For example, $1/f$ temporal fluctuations are found in the heart rate of healthy individuals [59], respiratory intervals in animals [60] and neuronal discharges during sleep [61]. The time series of interbeat intervals in healthy subjects have more complex fluctuations than patients with severe cardiac disease [58,62]. Long-range correlations have been also observed in the stride interval of human gait [63-64]. From primates to fish, fractal dimension can detect impairments in the behavior sequences of individuals under stress, indicating its value in early stress assessment [45,53-55,65].

In summary, fractal-like fluctuations in behavioral sequences reflect changes in environmental conditions. Animals acquire information about the resources in their surroundings by foraging, but this behavior uses metabolic energy and increases the risk of predation. The fractal organization of motion behavior optimizes the cost-benefit as demonstrated by comparing random and fractal search efficiency [66] and explains why locomotor activity does not occur randomly in time, but rather fractal-like [67]. A direct consequence of the observed sharp decay in the behavioral complexity of the copepod *C. hamatus* with increasing naphthalene concentration (Fig. 3) is a likely decrease in their foraging efficiency, hence their ingestion rates. This is consistent with the observed decreased or inhibited feeding [17-20], increased mortality [21], reduction in egg production [22], hatching rates [19] and clutch size [23] after exposure to PAHs.

The modifications of *C. hamatus* swimming sequences are also likely to affect the ability to detect potential sexual partners and to modify predation risk. As these modifications were clearly observed even at the lowest concentration tested (see Fig. 3), it is believed that the bottom-up effects of PAH pollutions on the whole planktonic ecosystems may be critical well below lethal concentrations. This is particularly relevant in estuarine, coastal and shelf waters where a variety of localized and obscure anthropogenic pollutions might ultimately be much more harmful for the ecosystem than the massive, though still relatively rare, crude oil spills. While this issue is well beyond the scope of the present work, future studies should thoroughly investigate the potential impact of PAH-induced behavioral modification on the structure and function of the planktonic food chain.

DISRUPTION OF SWIMMING AS AN INDICATOR OF ENVIRONMENTAL CONTAMINATION

Several behavioral responses have been used to assess toxicant effects on various test organisms, including distance traveled, travel velocity, frequency of direction change, time spent active, response to light and tail-flip escape response [68-69]. Swimming speed is perhaps the most frequently used 'behavioral' measure of an aquatic organism's physiological status [70-71]. In particular, many studies of swim-

ming behavior have focused on swimming capacity or ability and forced swimming. However, most of these studies have been made using teleost fish [72-74]. Similar information for aquatic invertebrates is limited.

Mysids have been frequently used in laboratory toxicity tests [75-76] but, despite a large literature on their swimming behavior [77-78], there are only a few published data on the effects of toxicant exposure on mysid swimming. For instance, the swimming ability and orientation of the *Neomysis integer* were disrupted after 7 day exposure to heavy metals [79] and pesticides [80]. While these results stress the advantages of behavioral toxicity tests for predicting the influence of pollutants and support the use of swimming behavior disruption as a sensitive end-point for detecting pollutant exposure in this species, the duration of pollutant exposure is not compatible with a rapid assessment of any increase in the background concentration of pollutants. Rapid (1 to 3-h) toxicity screening tests with the freshwater flea *Daphnia magna* using motion analysis systems were reported recently [33-34]. The immobility index EC_{50} (i.e. the contaminant concentration needed to inhibit swimming activity in 50% of the test individuals) revealed synergistic, antagonistic, or additive interactions existed between heavy metals and agricultural chemicals [81], while the fractal structure of *D. magna* swimming trajectories was related to the concentration of toxic chemicals and the exposure time [34].

Although acute toxicity tests provide a simple index of contaminant toxicity for regulatory authorities, they provide little information on the effects of toxicants in the natural environment, where concentrations of individual toxic contaminants are generally much lower than the LC_{50} (median lethal concentration) values estimated in the laboratory [82]. This is not the case for the toxicity assay performed in the present work which is very sensitive to naphthalene contamination, even at very low concentration (Fig. 3). In addition, the results presented here represent the first application of zooplankton behavior to the assessment of marine waters contamination, and improve previous behavior-based approaches in several ways. First, the short exposure time (20 min) allows a quasi-real time assessment of water quality. Second, the method used here to assess behavioral stress is free of the potentially fatal pitfalls identified for behavioral fractal analysis. The limited range of scales available in most behavioral studies can then erroneously lead to identify the result of underlying randomness as a fractal pattern [83-84], while non-fractal models such as Correlated Random Walk models can typically exhibit a fractal signature [85-86] referred to as 'apparent fractality' [87-88]. Finally, it is stressed that contrary to the numerical methods related to fractals and multifractals our approach does not require any specific numerical skills and can conveniently be used to compare the results from other studies using standard statistical tests.

CONCLUSIONS

The results presented in this study suggest that the change in the complexity of behavioral sequence of free-swimming copepods could be utilized as a sub-lethal stress indicator in toxicity tests and could become a very simple and rapid toxicity screening method. Given the advantages of the experimental simplicity, short exposure time, and low

concentration for detection, the behavioral analysis introduced here is especially useful for comparing swimming behavior, which could be taken as an endpoint for an acute bioassay. In particular, it is stressed that the measurements of an organism's behavior following contaminant exposure may provide a better understanding of the likely environmental consequences of toxic contamination than lethal effects, especially if behavior is disrupted at environmentally-realistic toxicant concentrations.

The efficiency of the behavioral approach introduced here has been specifically illustrated to investigate the level of stress of copepods exposed to a wide range of naphthalene concentration. It is stressed, however, that this method could conveniently be used to assess relative changes in the behavioral complexity of zooplanktonic organisms in a wide range of ecologically relevant situation related to e.g. the quality and the quantity of food, and the presence of mates and predators. In particular, as previously demonstrated for several fractal and multifractal measure of behavioral [39,89] and environmental [90-91] complexity, the differences between the values of the exponents ϕ observed for a given species or environment under stressful and non-stressful conditions might be more informative on the related behavioral changes rather than the absolute values of ϕ . While the structure of sequential behavior patterns has been successfully applied to assess stress in a variety of terrestrial and aquatic vertebrates [45-46,53-57], further studies are still needed to generalize their use to aquatic invertebrates.

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