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# Hydrocarbon Contamination and the Swimming Behavior of the Estuarine Copepod *Eurytemora affinis*

Laurent Seuront

<sup>1</sup>School of Biological Sciences, Flinders University,

<sup>2</sup>South Australian Research and Development Institute, Aquatic Sciences,

<sup>3</sup>National Center of Scientific Research, UMR LOG 8187,

<sup>4</sup>Center for Polymer Studies, Department of Physics, Boston University,

<sup>1,2</sup>Australia

<sup>3</sup>France

<sup>4</sup>USA

## 1. Introduction

Rivers and estuaries have allowed and sustained human settlement and related activities for thousands of years (e.g. Morhange et al., 2005). These water bodies have been used for activities such as irrigation, industry, transportation, fisheries, tourism and the related development of industrial and recreational facilities, and have subsequently been polluted by waste discharges, intentional and accidental spills, urban, industrial and agricultural runoffs (McLusky and Elliott, 2004; Shannon et al., 2011). These various sources of pollution all carry a wide range of inorganic and organic pollutants such as polycyclic aromatic and monoaromatic hydrocarbons (Vane et al., 2011), heavy metals (Jose et al., 2011), radioactive compounds (Villa et al., 2011) and a range of pharmaceuticals and pesticides (Arias et al., 2011; Munaron et al., 2011) that accumulate in water, sediment and living organisms (Whaltham et al., 2011; Schnitzer et al., 2011; Barua et al., 2011). Consequences have been observed from the molecular to the ecosystem level through e.g. immunological, cellular, reproductive and developmental impairments, and teratogenic effects that have widely been reported for a wide range of aquatic species (Noaksson et al., 2005; Thompson et al., 2007; Galante-Oliveira et al., 2009; Yang et al., 2010).

Changes in motion behavior, as a response to exposure to organic or inorganic pollutants, have been observed in a range of aquatic invertebrates such as *Artemia salina* (Venkateswara Rao et al., 2007), *Balanus amphitrite* (Faimali et al., 2002, 2006; Amsler et al., 2006), *Brachionus calyciflorus* (Janssen et al., 1994; Charoy et al., 1995; Charoy & Janssen, 1999), *Chironomus* sp. (Gerhardt & Janssens de Bisthoven, 1995; Janssens de Bisthoven et al., 2004), *Choroterpes picteti* (Macedo-Sousa et al., 2008), *Corophium volutator* (Kirkpatrick et al., 2006a; Kienle & Gerhardt, 2008), *Crangonyx pseudogracilis* (Kirkpatrick et al., 2006b), *Daphnia magna* (Bailleul & Blust, 1999; Shimizu et al., 2002; Understeiner et al., 2003; Goto & Hiromi, 2003; Gerhardt et al., 2005; Ren et al., 2007, 2008; Ren & Wang, 2010; Duquesne & Küster, 2010),

*Echinogammarus meridionalis* (Macedo-Sousa et al., 2007, 2008), *Gammarus fossarum* (Xuereb et al., 2009a,b), *Gammarus pulex* (Gerhardt, 1995; Gerhardt et al., 2007), *Hippolyte inermis* (Untersteiner et al., 2005), *Hydropsyche pellucidula* (Macedo-Sousa et al., 2008), but still rarely on copepods (Sullivan et al., 1983; Seuront & Leterme, 2007; Seuront, 2010a,b, 2011a,b; Cailleaud et al., 2011). Despite the central role played by copepods in aquatic ecosystems (Schmitz, 2008; Matthews et al., 2011), the amount of work devoted to copepod chemoreception (e.g. Doall et al., 1998; Bagøien & Kiørboe, 2005; Goetze & Kiørboe, 2008; Yen et al., 2011), and recent evidence for copepods to modify their swimming behavior in response to exposure to hydrocarbon compounds (Seuront & Leterme, 2007; Seuront, 2010a,b, 2011a,b) and 4-nonylphenol and nonylphenol-ethoxy-acetic-acid (Cailleaud et al., 2011), little is still known on the potential for chemical contaminants to affect copepod swimming behavior.

The quantitative assessment of changes in copepod swimming behavior is, however, critical as it is the main driver of encounter rate probability (Visser, 2007; Kiørboe, 2008; Seuront, 2011a), which in turn controls key processes such as mating and feeding rates, and predator avoidance, hence individual fitness and population dynamics. As a consequence, behavioral changes may be used as important indicators for ecosystem health. While they are driven by biochemical processes, they also reflect the fitness of individual organisms as well as potential consequences at the population level, such as altered abundance of a species in an ecosystem. Behavioral responses to water contamination, or more generally changes in water properties, have been shown to be a sensitive non-invasive sub-lethal end-point with short-response times for toxicity bioassays (e.g. Cailleaud et al., 2011; Seuront, 2011a,b), compared to community-related measures which require changes in species composition before an impact is detected. In ecotoxicology, behavioural approaches allow for repeated measures and time-dependent data analysis, and have the advantage of being of similar sensitivity and efficiency than biochemical and physiological responses (Gerhardt, 2007, 2011), and more sensitive than mortality responses (Garaventa et al., 2010).

In the drastic instance of oil spills that particularly threaten coastal waters (Varela et al., 2006; Lee et al., 2009; Rumney et al., 2011), previous studies have shown acute and deleterious effects in both meroplanktonic (Fisher & Foss, 1993; Epstein et al., 2000; Shafir et al., 2003) and holoplanktonic organisms (Samain et al., 1980; Jernelov et al., 1981; Cowles & Remillard, 1983a,b; Guzmán del Prío et al., 1986; Tawfiq & Olsen, 1993; Pavillon et al., 2002; Chen & Denison, 2011), which ultimately lead to biomass decrease and structure change at the community level. The identification and assessment of water contamination is, however, far more complex in situations where zooplankton communities are exposed to sub-lethal concentrations of the water-soluble fraction of pollutants that affect zooplankton physiology, feeding and fecundity as most marine bioassays still rely on exposure times of 24 to 48-h to determine the concentration of a test chemical at which 50% of neonates die or are immobilized; or the number of individuals that died (e.g. Barata et al. 2002; Calbet et al. 2007).

In this context, the objectives of this chapter were (i) to assess the ability of *E. affinis* adult males and females to detect and avoid patches of contaminated water, (ii) to evaluate whether their three-dimensional swimming behavior is affected by hydrocarbon contamination of estuarine waters and (iii) to illustrate the ability of fractal analysis (i.e. the fractal properties of three-dimensional swimming paths and the cumulative probability

distribution function of move lengths) to detect the stress potentially induced by hydrocarbon contamination. Note that a specific attention has been given to low concentrations of the water-soluble fraction of diesel oil (0.01%, 0.1% and 1%), that are shown to be well below the lethal concentration for *E. affinis*, to assess the impact of a chronic exposure to low concentrations of petroleum hydrocarbons.

## 2. Methods

### 2.1 Study species, sampling and acclimatization

*Eurytemora affinis* is one of the most abundant zooplankton species in the brackish part of Northern Hemisphere estuaries, usually localized around the Maximum Turbidity Zone (Soetaert & Van Rijswijk, 1993), and plays a significant role in estuarine food webs as an important food supply for many fishes, shrimps and mysids (Fockedeey & Mees, 1999). *E. affinis* individuals were collected from the Seine estuary using a WP2 net (200- $\mu$ m mesh size) at a temperature of 15°C in the low salinity zone (S=4 PSU) at low tide near the 'Pont de Normandie' (49°28'26N, 0°27'47W). Specimens were gently diluted in 30-litre isotherm tanks using *in situ* estuarine water and transported to the laboratory where adult males and both non-ovigerous and ovigerous females were immediately sorted by pipette under a dissecting microscope, and kept separately in 20-liter aquaria filled with filtered (Whatman GF/C glass-fibre filters, porosity 0.45 $\mu$ m) *in situ* estuarine water for 24-h until the behavioral experiments took place.

### 2.2 Hydrocarbon contaminant: The water-soluble fraction of diesel oil

The product considered as a potential contaminant of coastal waters was commercial diesel fuel oil. The water-soluble fraction of commercial diesel oil (WSF) was prepared stirring 1.8 l of filtered *in situ* seawater (Whatman GF/C filters) with 0.2 l of commercial diesel fuel oil for 2 h at 100g. The mixed solution was allowed to stand for 24 h without stirring to separate the oil layer from the oil-saturated water. WSF stock solutions were siphoned into autoclaved, acid-rinsed glass containers and diluted with uncontaminated seawater at 'high' (1%), 'medium' (0.1%) and 'low' (0.01%) concentrations. The water-soluble fraction of oil and their derivatives products contain a mixture of polycyclic aromatic hydrocarbons (PAH), monoaromatic hydrocarbons often referred to as BTEX (benzene, toluene, ethylbenzene and xylenes), phenols and heterocyclic compounds, containing nitrogen and sulphur (Saeed & Al-Mutairi, 1999; Elordui-Zapatarietxe et al., 2008; Rodrigues et al., 2010).

Technical limitations hampered the assessment of the precise chemical nature of the WSF stock solutions. The range of WSF concentrations used in the present work has, however, specifically been chosen to investigate the sub-lethal effects related to natural background concentrations of pollutants (Ohwada et al., 2003; Hashim, 2010). More specifically, among those compounds, BTEX are the main class of hydrocarbons found in WSF (Carls & Rice, 1990; Saeed & Al-Mutairi, 1999), and naphthalene is one of the most abundant polycyclic aromatic hydrocarbons dissolved in oil contaminated waters (Corner et al., 1976) and has been widely used in toxicological assays (Corner et al., 1976; Berdugo et al., 1977; Harris et al., 1977; Calbet et al., 2007). BTEX and naphthalene concentrations are respectively in the range 450-35000  $\mu$ g l<sup>-1</sup> and 30-26000  $\mu$ g l<sup>-1</sup> in 100% water-soluble fraction (Saeed & Al-Mutairi, 1999; Rodrigues et al., 2010). The 'high' (1%), 'medium' (0.1%) and 'low' (0.01%)

concentrations used in the present work hence correspond to concentrations in the range 4.5-350  $\mu\text{g l}^{-1}$ , 0.45-35  $\mu\text{g l}^{-1}$  and 0.045-3.5  $\mu\text{g l}^{-1}$  for BTEX, and 0.3-260  $\mu\text{g l}^{-1}$ , 0.03-26  $\mu\text{g l}^{-1}$  and 0.003-2.6  $\mu\text{g l}^{-1}$  for naphthalene.

### 2.3 Acute responses (mortality and narcosis) to WSF contamination

The water-soluble fraction concentrations considered here are well below the lethal concentrations observed for a range of copepod species (Barata et al., 2002; Calbet et al., 2007; Seuront & Leterme, 2007; Seuront, 2011a). As to my knowledge, no information is available on the effects of WSF on *Eurytemora affinis*, toxicity assays were conducted to assess the acute responses (mortality and narcosis) produced by the water-soluble fraction of commercial diesel oil. Acute responses (mortality and narcosis) to WSF of the copepod *E. affinis* were investigated by 24-h and 48-h incubations at 15°C (12/12 light/dark cycle) in contaminated 0.2  $\mu\text{m}$  filtered estuarine water. Groups of ten adult *E. affinis* males and non-ovigerous females were placed separately in 1-liter Pyrex glass bottles (Schott) sealed with a Teflon screw lid and filled with the appropriate test solution. The effect of the water-soluble fraction of diesel oil on *E. affinis* mortality and narcosis was inferred for a range of concentrations (50, 25, 10, 5, 1, 0.1 and 0.01%). Each treatment was triplicated, and triplicate uncontaminated control bottles were used to assess baseline mortality. At the end of the incubations, copepods were sieved through a 200  $\mu\text{m}$  nylon mesh, washed with filtered estuarine water, and transferred into Petri dishes, where their activity was monitored using a stereomicroscope. To discriminate mortality from narcotization, copepods were examined after a period of 4-h in uncontaminated filtered estuarine water to assess the degree of recovery (Berdugo et al., 1977; Calbet et al., 2007). The lethal concentration  $LC50$  (concentration at which 50% of the specimens died) was subsequently estimated from the nonlinear allosteric decay of the survival rate  $S$  following:

$$S = S_{\max} LC50^{\alpha} / (C_{\text{WSF}}^{\alpha} + LC50^{\alpha}) \quad (1)$$

where  $S_{\max}$  is the maximum survival rate (%),  $C_{\text{WSF}}$  the experimental WSF concentration (50%, 25%, 10%, 5%, 1%, 0.1% and 0.01%) and  $\alpha$  a fitting parameter (Barata et al., 2002).

The survival responses of *E. affinis* males and non-ovigerous females were highly significantly fitted by Eq. (1) (Fig. 1). Specifically, after a 24-h exposure, the survival rates of both males and non-ovigerous females (Fig. 1a,b) were very high, with  $LC50_{\text{male}} = 18.9\%$  and  $LC50_{\text{female}} = 20.0\%$ . In contrast, after a 48-h exposure, the survival rates of both males and females decay much faster with increasing WSF concentration (Fig. 1c,d), with  $LC50_{\text{male}} = 6.5\%$  and  $LC50_{\text{female}} = 7.5\%$ . No significant differences were found in  $LC50$  between males and females ( $P > 0.05$ ). In addition, no narcotic effects were observed; the mortality and narcosis were very similar, i.e.  $LC50_{\text{mort}} = 6.5\%$  and  $LC50_{\text{narc}} = 6.3\%$  for males, and  $LC50_{\text{mort}} = 7.5\%$  and  $LC50_{\text{narc}} = 7.7\%$  for females. Note that the lethal concentration  $LC50$  estimated here are substantially higher than the  $LC50$  obtained for *Temora longicornis* adult females after 24-h ( $LC50_{24} = 1.5\%$ ) and 48-h ( $LC50_{48} = 1.3\%$ ) toxicity assays conducted over the same range of WSF concentrations; see Seuront (2011a), his Figure 1. While further work is needed to infer the origin of the observed differences in the lethal concentration of *E. affinis* and *T. longicornis*, it is likely to be related to the highly

polluted nature of the estuarine environment where *E. affinis* proliferate. The behavioral properties of ovigerous females are not considered in the present work. It is nevertheless stressed that in contrast to adult males and non-ovigerous females, a clear narcotic effect was observed, with  $LC50_{mort} = 25.2\%$  and  $LC50_{narc} = 12.0\%$  after a 24-h exposure and  $LC50_{mort} = 8.1\%$  and  $LC50_{narc} = 4.0\%$  after a 48-h exposure. The behavioral experiments described below were conducted with the same WSF stock solutions than the above-mentioned toxicity assays for mortality and narcosis.

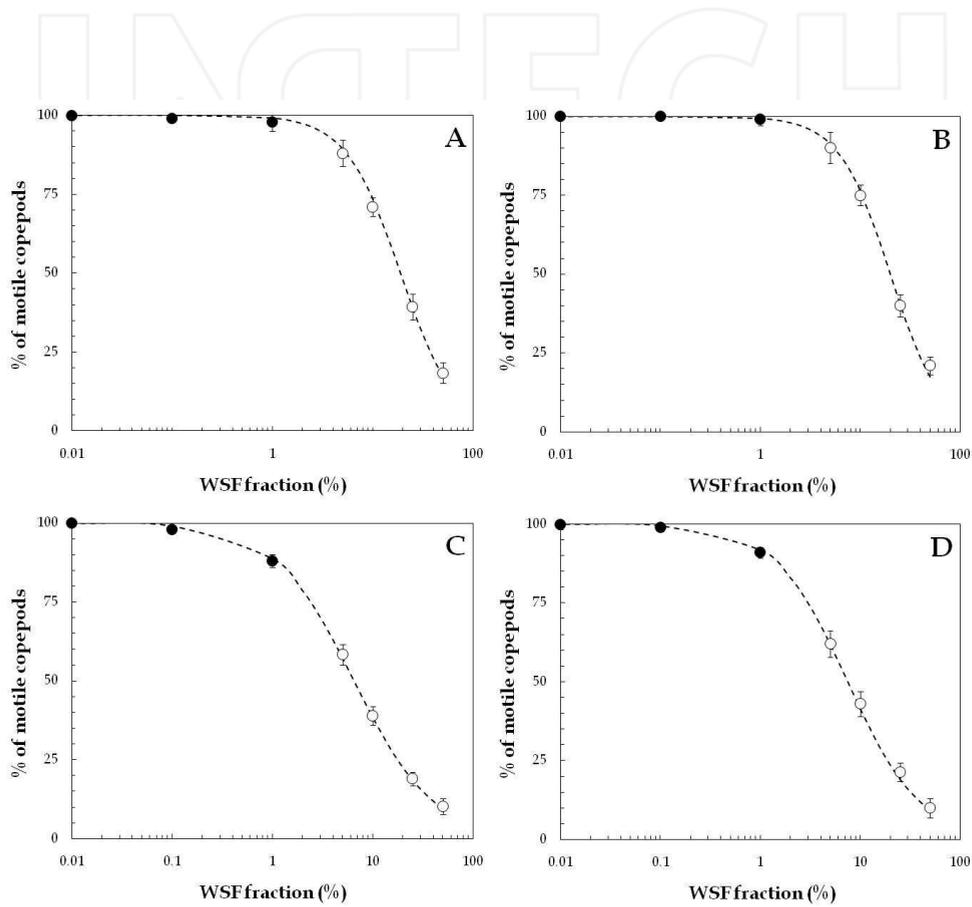


Fig. 1. Survival response of *Eurytemora affinis* adult males (A,C) and non-ovigerous females (B,D) to a range of WSF concentrations (50%, 25%, 10%, 5%, 1%, 0.1% and 0.01%) over 24-h (A, B) and 48-h (C,D) toxicity assays. The dashed lines are the best fits of the allosteric decay model, see Eq. (1). Error bars are standard deviations. The black dots correspond to the WSF concentrations used in the behavioral experiments.

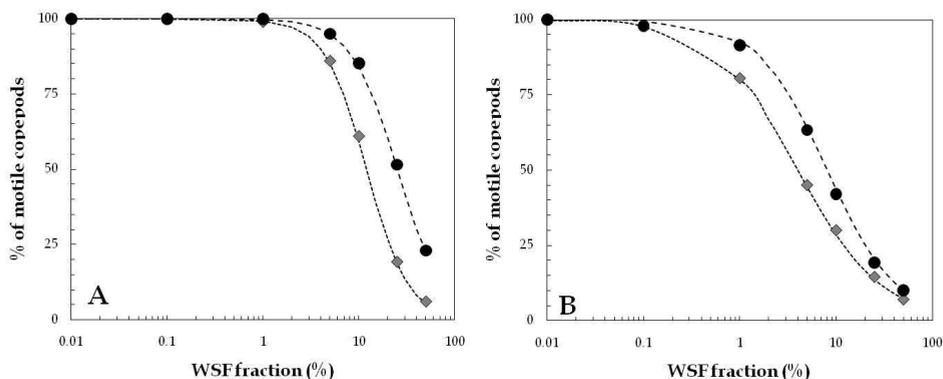


Fig. 2. Survival (black dots) and narcotization (grey diamonds) responses of *Eurytemora affinis* ovigerous females to a range of WSF concentrations (50%, 25%, 10%, 5%, 1%, 0.1% and 0.01%) over 24-h (A) and 48-h (B) toxicity assays. The dashed lines are the best fits of the allosteric decay model, see Eq. (1).

## 2.4 Behavioral experiments

Two behavioral assays were developed to assess the impact of the water soluble fraction of diesel oil on the swimming behavior of *Eurytemora affinis* adult males and non-ovigerous females. The first one was based on the ability of *E. affinis* to detect and avoid local patches of contaminated water. The second behavioral assay evaluates whether the three-dimensional swimming behavior of *E. affinis* is affected by contaminated water and demonstrates that fractal analyses based on either the geometric properties of their swimming paths or the cumulative probability distribution of their move lengths can detect the stress potentially induced by hydrocarbon contamination. Prosome lengths of males and females used during the behavioral experiments respectively ranged between 0.83 and 0.86 mm, and 0.87 and 0.92 mm.

### 2.4.1 Patch avoidance experiments

The first behavioral assay developed in the present work is based on the ability of *E. affinis* adult males and non-ovigerous females to avoid a patch of WSF contaminated estuarine water. Behavioral experiments were conducted in a 3.375-liter (15 × 15 × 15 cm) glass chamber. Patches of contaminated water were created in the centre of the chamber as a contaminant point source by dispensing contaminated fluid from a fine pipette (Eppendorf) to flow down into a 5-mm diameter permeable sphere (20- $\mu$ m pore size) that was suspended in the experimental container. Patches were created through the slow injection (<0.5 ml min<sup>-1</sup>) of contaminated water in the porous sphere. Prior to the behavioral experiments, the amount of contaminated water to be injected to create patches of different diameters (i.e. 1, 2, 4, 5.5 and 7 cm) was determined through the injection of fluorescein-stained GF/C filtered and autoclaved estuarine water into the experimental container. Note that the patches created were spherical and characterized by an isotropic distribution of contaminant across and a non-significant increase in size due to molecular diffusion for behavioral experiments lasting up to a hour; see Seuront (2010a) for more details.

For each WSF treatment, behavioral experiments were conducted in triplicate for each patch size (1, 2, 4, 5.5 and 7 cm), and an equivalent amount of uncontaminated estuarine water was injected into the experimental chambers for control observations (Seuront, 2010a). Preliminary experiments conducted with and without the injecting porous sphere did not exhibit any significant difference in the occurrence of *E. affinis* males and non-ovigerous females in the middle of the experimental chamber ( $\chi^2$ -test,  $P > 0.05$ ); this is consistent with previous observations conducted on *E. affinis* non-ovigerous adult females and *Temora longicornis* adult females (Seuront, 2010a). Before each behavioral experiment, 30 individuals of either *E. affinis* non-ovigerous adult females or adult males were transferred to the experimental filming set-up, filled with uncontaminated estuarine water and the corresponding contaminated patch, and allowed to acclimatize for 15 min (Seuront, 2006, 2010a). A new group of 30 individuals was used for each treatment.

#### **2.4.2 Behavioral response to hydrocarbon contaminated estuarine water**

These experiments were specifically designed to study the detailed properties of the swimming behavior of *E. affinis* adult males and non-ovigerous females under conditions of increasing WSF contaminations, and in the absence of the local gradients related to point source contamination. These experiments were also conducted in a 3.375-liter ( $15 \times 15 \times 15$  cm) glass chamber at 5 PSU under control conditions (uncontaminated estuarine water) and under conditions of contamination by the water-soluble fraction of diesel oil (WSF). For each treatment, the water was contaminated with 'high' (1%), 'medium' (0.1%) and 'low' (0.01%) WSF concentrations. Before each behavioral experiment, 30 individuals of either *E. affinis* non-ovigerous adult females or adult males were transferred into the experimental vessel filled with control or WSF contaminated estuarine water, and allowed to acclimatize for 15 min (Seuront, 2006). A new group of 30 individuals was used for each treatment.

#### **2.5 Behavioral observations and image analysis**

All behavioral experiments were replicated three times. WSF stock and working solutions were prepared 24 h before the behavioral experiments took place. The control experiments and the treatments were randomized, as well as the replicates of each experimental condition. The experimental chamber was rinsed with acetone, GF/C filtered estuarine water and distilled water, and allowed to dry between trials to remove any chemical compound. Prior to each experiment, the experimental individuals were transferred in a filming set-up filled up with uncontaminated seawater and the corresponding treatments, and were allowed to acclimatize for 15 min (Seuront, 2006). All experimental individuals were used only once, and no narcosis or mortality was ever observed on any of the tested individuals. The three-dimensional trajectories of *E. affinis* adult males and non-ovigerous females were recorded at a rate of 25 frames  $s^{-1}$  using two orthogonally oriented and synchronized infrared digital cameras (DV Sony DCR-PC120E) facing the experimental chamber. 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  $\times$  4.9 cm wide) connected to a 12-V DC power supply, provided the only light source from the bottom of the chamber. The cameras overlooked the experimental chamber from the side, and the various components of the set-up were adjusted so that the copepods were adequately resolved and in focus. The

two cameras represented the  $x$ - $z$  and the  $y$ - $z$  planes of the experimental chamber; 3-D swimming paths were obtained by combining information from the 2-D views. All the experiments were conducted in a temperature-controlled room at 18°C in the dark and at night to avoid any potential behavioral artifact related to the diel cycle of the copepods (Seuront, 2011b). Selected video clips were captured (DVgate Plus) as MPEG movies and converted into QuickTime TM movies (QuickTime Pro), after which the  $x$ ,  $y$  and  $z$  coordinates of swimming pathways were automatically extracted 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 motion behavior.

## 2.6 Behavioral analysis

### 2.6.1 Swimming speed

The swimming speed  $v$  (mm s<sup>-1</sup>) over consecutive tracking intervals was estimated as  $v = f \times d_t$ , where  $f$  is the sampling rate of the camera ( $f = 25$  frame s<sup>-1</sup>), and  $d_t$  the distance (mm) between two points in a three-dimensional space. The distance  $d_t$  (mm) was computed from the  $(x, y, z)$  coordinates as  $d_t = [(x_{t+1} - x_t)^2 + (y_{t+1} - y_t)^2 + (z_{t+1} - z_t)^2]^{1/2}$ , where  $(x_t, y_t, z_t)$  and  $(x_{t+1}, y_{t+1}, z_{t+1})$  are the positions of a copepod at time  $t$  and  $t + 1$ , respectively. Average swimming speed and their standard deviations were measured over the duration of each individual track.

### 2.6.2 Patch avoidance and patch escape behavior

The avoidance response of *E. affinis* adult males and non-ovigerous females to patches of contaminated water was expressed by both their patch avoidance and escape behaviors, i.e. the percentage of individuals that respectively avoided contaminated patches and escaped patches after entering them (Seuront, 2010a). Avoidance was identified as a sharp change in the direction of travel or the combination of a sharp change in swimming direction and an increase in swimming speed. Patch avoidance behavior was quantified by the distance  $d_a$  at which individuals avoided contaminated patches, the avoidance turning angle  $\alpha_{i,j}$  and avoidance velocity  $u_{i,j}$  for a contaminated patch of diameter  $i$  ( $i = 1, 2, 4, 5.5$  and  $7$  cm) and concentration  $j$  ( $j = 0.01\%$ ,  $0.1\%$  or  $1\%$ ). Patch escape behavior was quantified by the escape turning angle  $\beta_{i,j}$  and escape velocity  $v_{i,j}$  for a contaminated patch of diameter  $i$  and concentration  $j$ . The significance of turning angles  $\alpha_{i,j}$  and  $\beta_{i,j}$ , and velocities  $u_{i,j}$  and  $v_{i,j}$  was assessed inferring if  $\bar{\alpha}_{i,c} = \alpha_{i,j}$  and  $\bar{\beta}_{i,c} = \beta_{i,j}$ , and if  $\bar{u}_{i,c} = u_{i,j}$  and  $\bar{v}_{i,c} = v_{i,j}$ , where  $\bar{\alpha}_{i,c}$  and  $\bar{\beta}_{i,c}$  and  $\bar{u}_{i,c}$  and  $\bar{v}_{i,c}$  are the mean turning angles and swimming speed estimated outside and inside uncontaminated control patches of diameter  $i$ . Note that no significant differences were observed between  $\bar{\alpha}_{i,c}$  and  $\bar{\beta}_{i,c}$  (Wilcoxon-Mann-Whitney  $U$ -test,  $P > 0.05$ ) and between  $\bar{u}_{i,c}$  and  $\bar{v}_{i,c}$  (Wilcoxon-Mann-Whitney  $U$ -test,  $P > 0.05$ ), i.e.  $\bar{\alpha}_{i,c} = \bar{\beta}_{i,c}$  and  $\bar{u}_{i,c} = \bar{v}_{i,c}$ . The turning angle  $\theta_t$  between two successive moves was defined as  $\theta_t = 180 - 180\theta / \pi$ , where  $\theta = \arccos(\bar{A}\bar{B} / \|A\|\|B\|)$ ,  $\bar{A}$  and  $\bar{B}$  the vectors between the locations  $X_1(x_t, y_t, z_t)$  and  $X_2(x_{t+1}, y_{t+1}, z_{t+1})$  and between locations  $X_2(x_{t+1}, y_{t+1}, z_{t+1})$  and  $X_3(x_{t+2}, y_{t+2}, z_{t+2})$ , and  $\|A\|$  and  $\|B\|$  the lengths of the vectors  $\bar{A}$  and  $\bar{B}$ .

### 2.6.3 Patch entrance rate and proportional residence time

The avoidance of contaminated patches was further quantified by the patch entrance rate and the proportional residence time. The patch entrance rate is the ratio  $F_{i,j} = 100N_{i,j} / N_{i,c}$ , where  $N_{i,j}$  is the number of individuals that encountered a contaminated patch of diameter  $i$  and concentration  $j$  during the 30-min behavioural experiment, and  $N_{i,c}$  is the number of individuals that entered an uncontaminated patch of diameter  $i$  during the 30-min control experiments. Finally, the patch residence time was estimated as the time individual copepods spent in uncontaminated and contaminated patches. The proportional residence time (PRT<sub>*i,j*</sub>) is the ratio of the time spent in a contaminated patch of diameter  $i$  and concentration  $j$  to the total time in spent in an uncontaminated patch of diameter  $i$ .

### 2.6.4 Complexity of swimming paths

The complexity of swimming paths was assessed using fractal analysis. In contrast to standard behavioral metrics such as turning angle and net-to-gross displacement ratio (NGDR), fractal analysis and the related fractal dimension  $D$  have the desirable properties to be independent of measurement scale and to be very sensitive to subtle behavioral changes that may be undetectable to other behavioral variables (Seuront & Leterme, 2007; Seuront et al., 2004a,b; Seuront & Vincent, 2008; Seuront, 2010b, 2011b). Fractal analysis has been applied to describe the complexity of zooplankton and ichthyoplankton swimming paths (Coughlin et al., 1992; Bundy et al., 1993; Dowling et al., 2000; Seuront et al., 2004a,b,c; Uttieri et al., 2005, 2007, 2008; Seuront, 2006, 2010b, 2011a,b; Seuront & Vincent, 2008; Ziarek et al., 2011). The fractal dimensions of *E. affinis* swimming paths were estimated using two different, but conceptually similar, methods to ensure the reliability of fractal dimension estimates; see e.g. Fielding (1992) and Hastings and Sugihara (1993), and Seuront (2010b) for a review.

The box dimension method relies on the “ $l$  cover” of the object, i.e. the number of boxes of length  $l$  required to cover the object. A more practical alternative is to superimpose a regular grid of boxes of length  $l$  on the object and count the number of boxes occupied by a subset of the object. This procedure is repeated using different values of  $l$ . The volume occupied by a swimming path is then estimated using a series of counting boxes spanning a range of volumes down to some small fraction of the entire volume. The number of occupied boxes increases with decreasing box size, leading to the following power-law relationship:

$$N(l) \propto l^{-D_b} \quad (2)$$

where  $l$  is the box size,  $N(l)$  is the number of boxes occupied by the swimming path, and  $D_b$  is the box fractal dimension. The fractal dimension  $D_b$  is estimated from the slope of the linear trend of the log-log plot of  $N(l)$  versus  $l$ .

The mass dimension method counts the number of pixels occupied by an object in cubes ( $\delta \times \delta$ ) sampling windows as  $N_o(\delta)$ . The mass  $m(\delta)$  of occupied pixels is then defined as:

$$m(\delta) = \frac{N_O(\delta)}{N_T(\delta)} \quad (3)$$

where  $N_O(\delta)$  and  $N_T(\delta)$  are the number of occupied pixels and the total number of pixels within an observation window of size  $\delta$ . These computations are repeated for various values of  $\delta$ , and the mass dimension  $D_m$  is defined as:

$$m(\delta) \propto \delta^{D_m} \quad (4)$$

The fractal dimension  $D_m$  is estimated from the slope of the linear trend of the log-log plot of  $m(\delta)$  versus  $\delta$ . Practically, the mass  $m(\delta)$  can be estimated using cubes of increasing size  $\delta$  starting from the centre of the experimental domain (Seuront, 2010b). Note that increasing in the box size  $l$  (Eq. 2) and  $\delta$  (Eq. 4) may result in exclusion of a greater proportion of pixels along the periphery of the domain. Under an assumption of three-dimensional isotropy, this issue can be circumvented applying a toroidal edge correction (Seuront, 2010c). However, to avoid potential biases related to both the anisotropy of the swimming paths and the initial position of the overlying three-dimensional grid of orthogonal boxes, for each box size  $l$  and  $\delta$  the grid was rotated in  $5^\circ$  increments from  $\alpha = 0$  to  $\alpha = 45^\circ$  in the  $x-y$  plane and from  $\beta = 0$  to  $\beta = 45^\circ$  in the  $x-z$  plane. The resulting distributions of fractal dimensions  $D_b$  and  $D_m$  were averaged, and the resulting dimension  $\bar{D}_b$  and  $\bar{D}_m$  used to characterize the complexity of a swimming path.

The appropriate range of scales  $l$  (Eq. 2) and  $\delta$  (Eq. 4) to include in the regression analyses was chosen following the  $R^2$ -SSR criterion (Seuront et al., 2004a). Briefly, I consider a regression window of varying width ranging from a minimum of 5 data points to the entire data set. The windows are slid along the entire data set at the smallest available increments, with the whole procedure iterated  $n - 4$  times, where  $n$  is the total number of available data points. Within each window and for each width, we estimated the coefficient of determination ( $r^2$ ) and the sum of the squared residuals for the regression. I subsequently used the values of  $l$  (Eq. 2) and  $\delta$  (Eq. 4), which maximized the coefficient of determination and minimized the total sum of the squared residuals (Seuront et al., 2004a), to define the scaling range and to estimate the related dimensions  $D_b$  and  $D_m$ . Note that  $D_b$  and  $D_m$  are bounded between 1 for a linear swimming path, and 2 for a path so complex that it fills the whole space available.

### 2.6.5 Complexity of instantaneous successive displacements

By analogy with a self-organized critical system that builds up stress and then releases the stress in intermittent pulses (Seuront & Spilmont, 2002), the level of stress arising from each experimental condition was described by a power law, which states that the cumulative probability distribution function of move length  $L$  greater than a determined length  $l$  follows (Seuront & Leterme, 2007; Seuront, 2011b):

$$N(l \leq L) \propto l^{-\phi} \quad (5)$$

where the move lengths  $L$  correspond to the distances travelled by *E. affinis* individuals every 0.04 s and  $\phi$  a scaling exponent (referred to as a 'stress exponent' hereafter) describing the distribution. The exponent  $\phi$  is estimated as the slope of  $N(l \leq L)$  vs.  $l$  in log-log plots, and has been shown to decrease under stressful conditions for both vertebrates (Alados et al., 1996; Seuront & Cribb, 2011) and invertebrates (Seuront & Leterme, 2007; Seuront, 2011b). It is stressed, however, that Eq. (5) differs from the power law previously used to identify Lévy flights in a range of marine organisms including microzooplankton (Bartumeus et al., 2003), gastropods (Seuront et al., 2007) and fish (Sims et al., 2008; Humphries et al., 2010):

$$P(l_d = l) \propto l^{-\mu} \quad (6)$$

where  $l_d$  is the displacement length,  $l$  a threshold value, and  $\mu$  ( $1 < \mu \leq 3$ ) characterizes the power law behavior of the tail of the distribution. The move lengths  $L$  (Eq. (5)) differ from the flight path lengths  $l_d$  (Eq. (6)) which are defined as sequences of straight-line movements between the points at which significant changes in direction occurred; a significant change in direction is considered when the direction of the current flight segment (joining two successive recorded positions) and the direction of the previous flight segment is more than  $90^\circ$  (Bartumeus et al., 2005; Reynolds et al., 2007).

## 2.7 Statistical analyses

The distribution of the behavioral parameters was significantly non-normal (Kolmogorov-Smirnov test,  $P < 0.01$ ), even after  $\log_{10}$  or square-root transformations. Non-parametric statistics were then used throughout this work. Comparisons between behavioral parameters inside and outside patches were conducted using the Wilcoxon-Mann-Whitney  $U$ -test (WMW test; Zar, 2010). The effects of the size of uncontaminated control patches were compared using the Kruskal-Wallis test (KW test; Zar, 2010). The effects of contaminated patch size and concentration were investigated using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (SRH test; Sokal & Rohlf, 1995). Appropriate multiple comparison procedures were subsequently used to test for differences between patch diameter and patch concentration. Multiple comparisons between WSF treatments 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 (Zar, 2010). Correlation between variables was investigated using Kendall's coefficient of rank correlation,  $\tau$  (Kendall & Stuart, 1966).

## 3. Behavioral response to point sources of hydrocarbon contamination

### 3.1 Patch avoidance and patch escape

The swimming paths of *Eurytemora affinis* adult males and non-ovigerous females were not affected by uncontaminated control patches (Fig. 3a,b). However, both males and females consistently avoided contaminated patches (Fig. 3c,d; Fig. 4), irrespective of patch size. The distance  $d_a$  at which individuals exhibited patch avoidance was not affected by patch size ( $P > 0.05$ ) or patch concentration ( $P > 0.05$ ), but were significantly smaller ( $P < 0.05$ ) for *E. affinis* adult males ( $1.7 \pm 0.1$  mm,  $\bar{x} \pm SD$ ) than for non-ovigerous females ( $2.2 \pm 0.2$  mm).

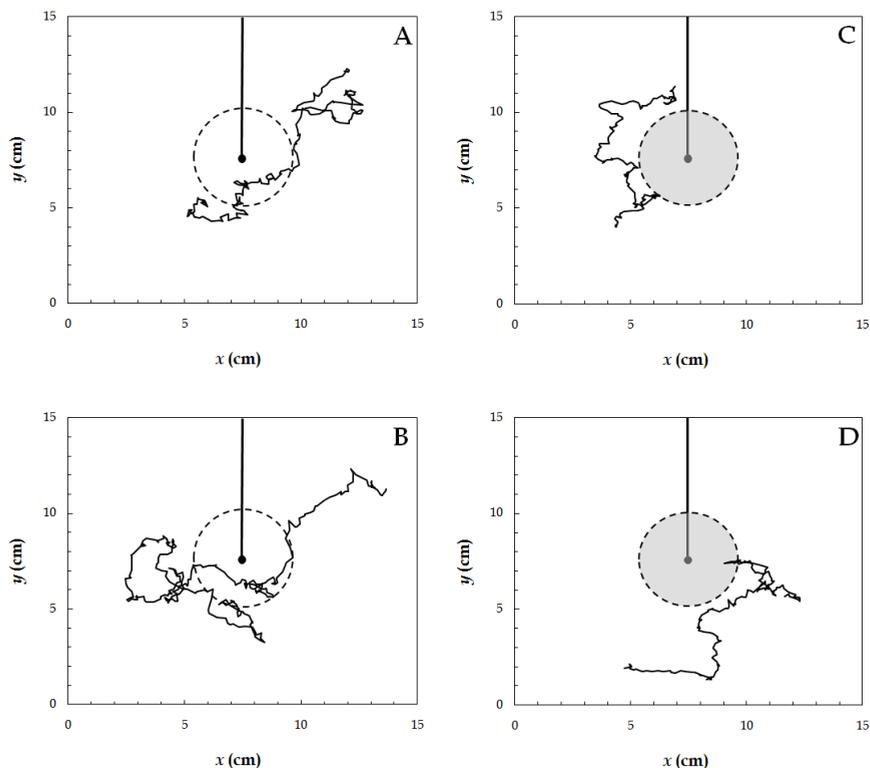


Fig. 3. Two-dimensional projections of three-dimensional swimming paths of *Eurytemora affinis* adult males (a,c) and non-ovigerous females (b,d) in response to uncontaminated (a,b) and contaminated patches (c,d) 4 cm in diameter. Contaminated patches correspond to 0.01%, 0.1% and 1% dilutions of the water-soluble fraction of diesel oil in filtered estuarine water, while uncontaminated patches were created using uncontaminated GF/C filtered and autoclaved estuarine water.

No avoidance and no escape behaviors were observed for the different sizes of uncontaminated patches (Fig. 3a,b; Fig. 4). The percentages of individuals that exhibited a patch avoidance behaviour (Fig. 3c,d) did not significantly differ with patch sizes and concentrations ( $P > 0.05$ ) for both males ( $97.1 \pm 2.3\%$ ) and females ( $95.2 \pm 2.1\%$ ). The avoidance turning angle  $\alpha_{i,j}$  and avoidance velocity  $u_{i,j}$  did not differ with the size or the concentration of the contaminated patches ( $P > 0.05$ ) for males ( $\alpha_{i,j} = 68.1 \pm 3.2^\circ$  and  $u_{i,j} = 4.9 \pm 0.9 \text{ mm s}^{-1}$ ) and females ( $\alpha_{i,j} = 65.2 \pm 2.3^\circ$  and  $u_{i,j} = 8.5 \pm 0.6 \text{ mm s}^{-1}$ ). No significant differences were found in avoidance turning angle  $\alpha_{i,j}$  between males and females ( $P > 0.05$ ). In contrast, avoidance velocity  $u_{i,j}$  was significantly higher for females than for males ( $P < 0.05$ ). The avoidance turning angle and velocity were significantly higher ( $P < 0.01$ ) than the mean turning angle  $\bar{\alpha}_{i,c}$  and swimming speed  $\bar{u}_{i,c}$  estimated for uncontaminated patches, i.e.  $\bar{\alpha}_{i,c} = 36.7 \pm 1.2^\circ$  and  $\bar{u}_{i,c} = 2.2 \pm 0.5 \text{ mm s}^{-1}$  for males and  $\bar{\alpha}_{i,c} = 37.3 \pm 1.2^\circ$  and  $\bar{u}_{i,c} = 1.7 \pm 0.3 \text{ mm s}^{-1}$  for females.

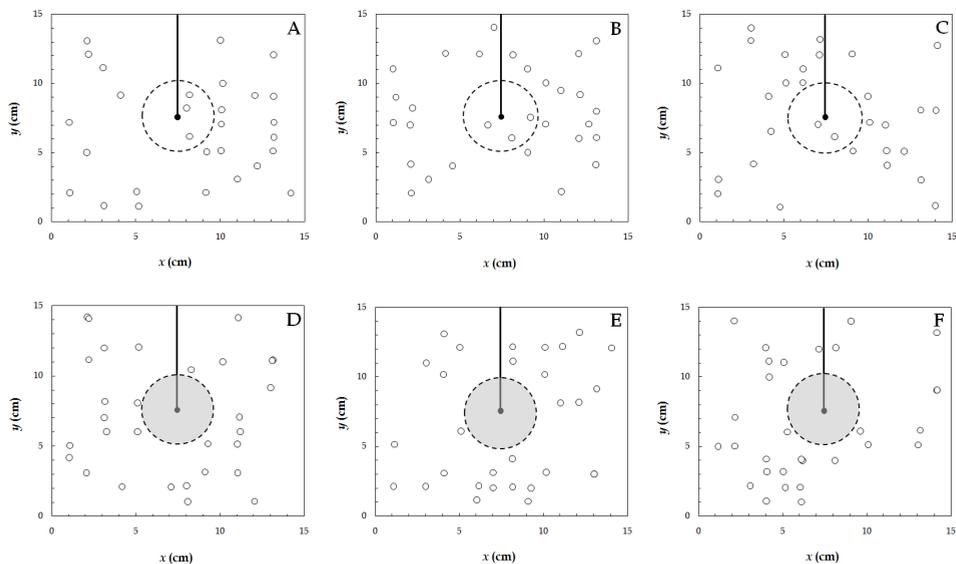


Fig. 4. Two-dimensional snapshots of the position of 30 *Eurytemora affinis* adult males (open dots) in relation to the position of an uncontaminated patch (a-c) and a patch contaminated with the water-soluble fraction of diesel oil at a concentration of 0.1% (d-f). Both patches were 4 cm in diameter.

The escape turning angle  $\beta_{i,j}$  did not differ with the size or the concentration of the contaminated patches ( $P > 0.05$ ) for males ( $\beta_{i,j} = 70.1 \pm 3.3^\circ$ ) and females ( $\beta_{i,j} = 68.4 \pm 2.1^\circ$ ). In contrast, the escape velocity  $v_{i,j}$  significantly increased ( $P < 0.05$ ) with WSF concentration for both males and females, with  $v_{i,j} = 4.7 \pm 1.1 \text{ mm s}^{-1}$ ,  $v_{i,j} = 5.4 \pm 1.2 \text{ mm s}^{-1}$  and  $v_{i,j} = 6.5 \pm 0.9 \text{ mm s}^{-1}$  for males and  $v_{i,j} = 8.2 \pm 1.0 \text{ mm s}^{-1}$ ,  $v_{i,j} = 8.8 \pm 1.1 \text{ mm s}^{-1}$  and  $v_{i,j} = 9.8 \pm 1.1 \text{ mm s}^{-1}$  for females for estuarine water contaminated at 'low' (0.01%), 'medium' (0.1%) and 'high' (1%) concentrations of the soluble-fraction of diesel oil. The escape turning angle  $\beta_{i,j}$  and escape velocity  $v_{i,j}$  were significantly higher ( $P < 0.01$ ) than the mean turning angle  $\bar{\beta}_{i,c}$  and swimming speed  $\bar{v}_{i,c}$  estimated inside uncontaminated patches, i.e.  $\bar{\beta}_{i,c} = 37.4 \pm 1.4^\circ$  and  $\bar{v}_{i,c} = 2.1 \pm 0.4 \text{ mm s}^{-1}$  for males and  $\bar{\beta}_{i,c} = 37.6 \pm 1.3^\circ$  and  $\bar{v}_{i,c} = 1.8 \pm 0.4 \text{ mm s}^{-1}$  for females. No significant differences were found in avoidance turning angle  $\beta_{i,j}$  between males and females ( $P > 0.05$ ). The avoidance velocity  $v_{i,j}$  was, however, significantly higher for females than for males ( $P < 0.05$ ).

Despite the dependence of both the avoidance and escape velocities  $u_{i,j}$  and  $v_{i,j}$  to the concentration of the soluble-fraction of diesel oil, the smaller avoidance distance, avoidance velocity and escape velocity observed for males suggest that male sensory abilities may be less acute than female ones. The behavioral responses of both males and females nevertheless converge towards an adaptation to avoid and escape WSF contaminated patches, hence minimize the exposure time to a source of contamination.

### 3.2 Entrance rate in uncontaminated vs. hydrocarbon-contaminated patches

The percentage of adult males entering contaminated patches (Fig. 5a) was highly significantly affected by patch concentration ( $P < 0.01$ ), but not patch size ( $P > 0.05$ ), leading to  $F_{i,1} = 12.3 \pm 0.6$  %,  $F_{i,0.1} = 21.7 \pm 1.2$  % and  $F_{i,0.01} = 28.7 \pm 1.5$  %. In contrast, the ratio  $F_{i,j}$  was not affected by patch size ( $P > 0.05$ ) and patch concentration ( $P > 0.05$ ) for non-ovigerous females (Fig. 5b), with  $F_{i,1} = 10.3 \pm 0.6$  %,  $F_{i,0.1} = 10.2 \pm 1.3$  % and

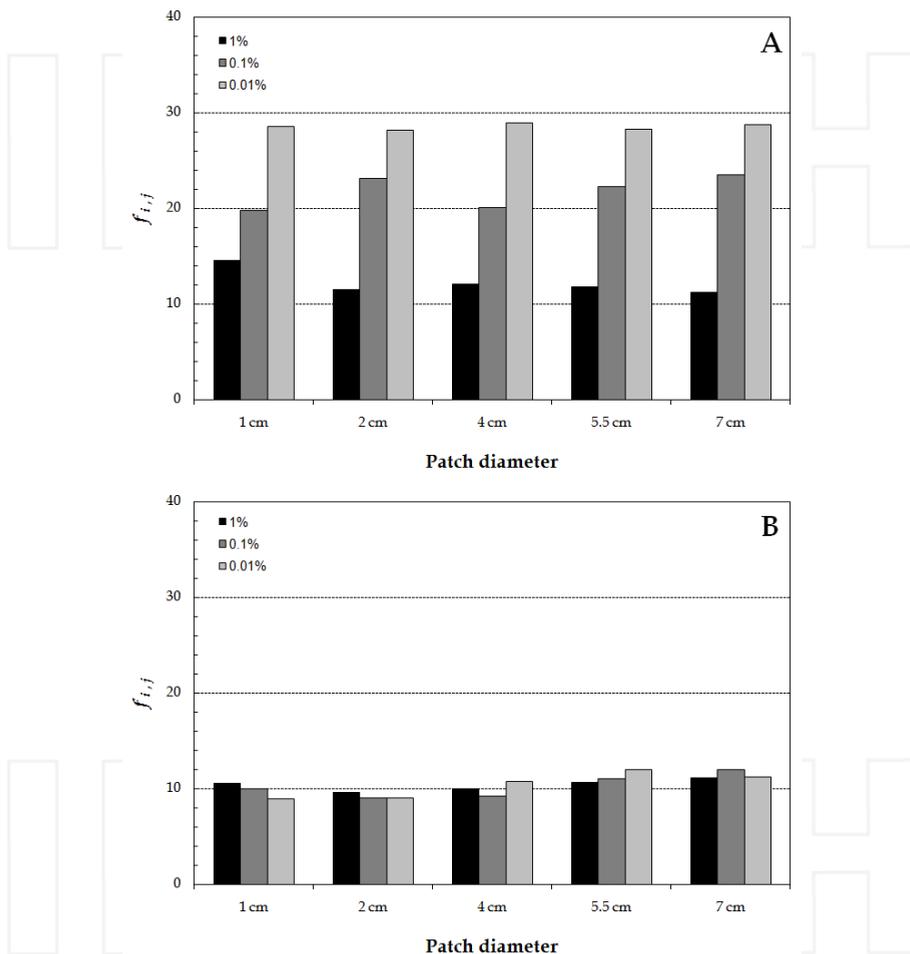


Fig. 5. The patch entrance rate  $F_{i,j}$  between the number of *Eurytemora affinis* individuals that entered a contaminated patch of diameter  $i$  and concentration  $j$  and the number of individuals that entered an uncontaminated patch of diameter  $i$ , shown for adult males (a) and non-ovigerous females (b). Contaminated patches correspond to 0.01%, 0.1% and 1% dilutions of the water-soluble fraction of diesel oil in filtered estuarine water, while uncontaminated patches were created using uncontaminated GF/C filtered and autoclaved estuarine water.

$F_{i,0.01} = 10.4 \pm 1.4$  %. Note that for each patch size, the percentage of adult males entering contaminated patches significantly decreases with patch concentration ( $P < 0.01$ ). This is consistent with the significant exponential increase observed in  $F_{ij}$  with decreasing contaminant concentration in *Temora longicornis* adult females (Seuront, 2010a). These results suggest that

- i. *E. affinis* non-ovigerous females may have comparable sensory abilities irrespective of the concentrations of the contaminant, whereas males seem to identify more efficiently high-density contaminated patches than low-density ones, and
- ii. the ability of copepods to detect patches contaminated with the soluble-fraction of diesel oil (hence with polycyclic aromatic and monoaromatic hydrocarbons, and their derived products), is likely to be both species- and sex-dependent.

The differences observed between male and female chemosensory abilities are consistent with the role played by chemoreception in males foraging ecology (e.g. Kjørboe et al., 2005; Bagøien & Kjørboe, 2005; Goetze & Kjørboe, 2008; Seuront, 2011a). Further work is, however, needed to generalize these results to a representative range of copepod species, and to assess the potential differences that may exist between the chemosensory abilities of different sexes and development stages.

### 3.3 Residence time in uncontaminated vs. hydrocarbon-contaminated patches

The residence time of both *E. affinis* males and non-ovigerous females in uncontaminated control patches significantly increased with the size of the patch ( $P < 0.05$ ) and ranged from 8 to 47 s for patches 1 cm and 7 cm in diameter (Fig. 6). The residence time significantly decreased in contaminated patches for both males and females ( $P < 0.05$ ), and does not exhibit any significant change with patch size ( $P > 0.05$ ). The residence time observed for males significantly decrease with increasing concentration of the water-soluble fraction of diesel oil, i.e. 0.83 s, 0.70 s and 0.60 s for patches contaminated at 0.01, 0.1 and 1% levels, respectively. In contrast, females residence time (0.34 s, 0.34 s and 0.33 s at WSF concentration of 0.01, 0.1 and 1%) did not significantly vary with the level of water contamination, but were consistently significantly shorter than male ones ( $P < 0.05$ ).

These results are specified by the proportional residence time  $PRT_{ij}$  (Fig. 7).  $PRT_{ij}$  ranged from 1.6% to 8.8%, 1.3% to 7.2% and 1.2% to 6.3% for adult males, and from 0.6% to 3.1%, 0.5% to 3.1% and 0.6% to 3.6% for non-ovigerous females, at WSF concentration of 0.01, 0.1 and 1%.  $PRT_{ij}$  significantly decreased with the size of patches for both males and females for each WSF concentration ( $P < 0.05$ ), and was significantly higher for males at each WSF concentration ( $P < 0.05$ ).

These observations are consistent with the hypothesis that *E. affinis* non-ovigerous females may have comparable sensory abilities irrespective of the concentrations of the contaminant, hence exhibit a 'on-off' behavioral response that leads them to escape a source of contamination, irrespective of the concentration of the contaminant, and ultimately lead them to minimize the exposure time to the contaminant. In contrast, males have a modulated behavioral response that is *sensu stricto* less efficient than female's behavior; it nevertheless also leads to minimize the exposure time to a contaminant through a density-dependent response. Ultimately, these behavioral changes are likely to avoid a stress-related

reduction in individual fitness, which might in turn affect the whole zooplankton community.

Sex-specific response to hydrocarbon contamination and behavioral alterations for concentrations of the water-soluble fraction of diesel oil ranging from 0.01 to 1% have also been observed in another common calanoid copepod, *Temora longicornis* (Seuront, 2011a). This demonstrates the very acute chemosensory abilities of both *E. affinis* and *T. longicornis*, and generalizes previous work showing behavioral changes elicited by a variety of chemical cues (Katona, 1973; Doall et al. 1998; Weissburg et al., 1998; Yen et al., 1998; Woodson et al., 2007, 2008; Seuront, 2010a, b, 2011a; Cailleaud et al., 2011).

In this context, the next section investigate the behavioral stress that may be induced in the swimming behavior of *E. affinis* adult males and non-ovigerous females by the diffuse hydrocarbon contamination that is likely to follow any point-source contamination in estuarine waters.

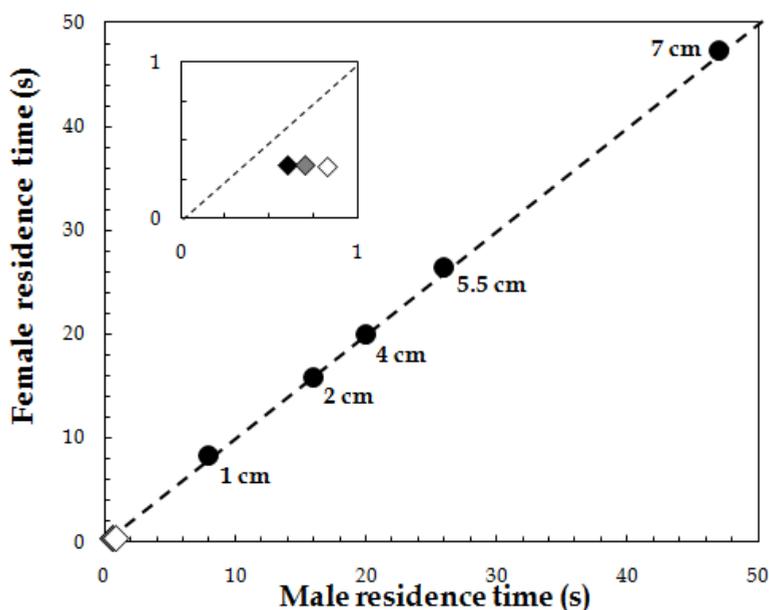


Fig. 6. Residence time of *E. affinis* adult males and non-ovigerous females in uncontaminated control patches of increasing diameter (black dots) and in patches contaminated with the water-soluble fraction of diesel oil (insert) at 0.01% (white diamond), 0.1% (grey diamond) and 1% (black diamond).

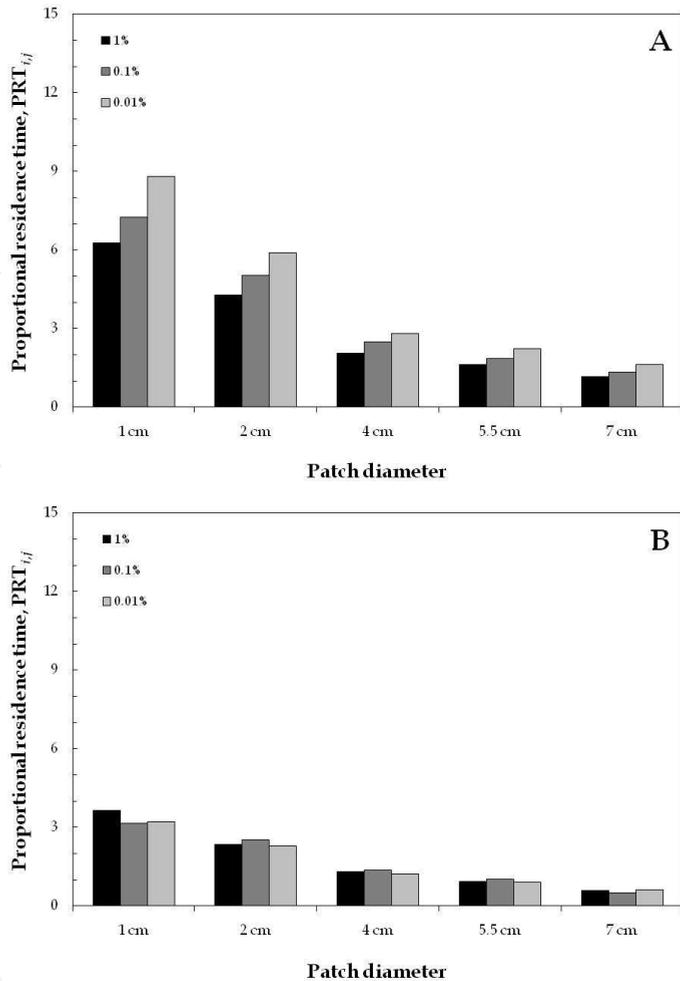


Fig. 7. Proportional residence time of *E. affinis* adult males (A) and non-ovigerous females (B) in patches of increasing diameter contaminated with the water-soluble fraction of diesel oil at 0.01% (black), 0.1% (dark grey) and 1% (light grey). The proportional residence time ( $PRT_{i,j}$ ) is the ratio of the time spent in a contaminated patch of diameter  $i$  and concentration  $j$  to the total time spent in an uncontaminated patch of diameter  $i$ .

#### 4. Behavioral response to a diffuse hydrocarbon contamination

##### 4.1 Swimming speed and turning angle in hydrocarbon contaminated water

Four kind of swimming behaviors were further considered to quantify the swimming behaviour of males and non-ovigerous females (Seuront, 2011a): (i) cruising, in which their rostro-caudal body axes were aligned with the direction of motion, whether they were swimming up, down or horizontally, (ii) hovering, i.e. swimming upward at low speed,

often with a horizontal component, with the rostro-caudal body axis oriented upward (Doall et al., 1998), (iii) passive sinking, i.e. downward vertical motion, with tail down, and (iv) breaking, in which they remain motionless, with their rostro-caudal axes oriented upward. Each behavioural activity was quantified in terms of time allocation percentage for each category of organisms.

In control, non-contaminated estuarine water, the swimming speeds of *E. affinis* adult males ( $u_{m,c} = 2.3 \pm 0.4 \text{ mm s}^{-1}$ ) and non-ovigerous females ( $u_{f,c} = 1.8 \pm 0.6 \text{ mm s}^{-1}$ ) were not significantly different ( $P > 0.05$ ), and did not significantly differ from the swimming speed estimated in uncontaminated patches (i.e.  $\bar{u}_{i,c} = 2.2 \pm 0.5 \text{ mm s}^{-1}$  for males and  $\bar{u}_{i,c} = 1.7 \pm 0.3 \text{ mm s}^{-1}$  for females). Similarly, males and females did not exhibit any significant changes in their turning angle ( $\alpha_{m,c} = 38.6 \pm 1.6^\circ$  and  $\alpha_{f,c} = 39.2 \pm 1.4^\circ$ ), and were not significantly different from the turning angle  $\bar{\alpha}_{i,c}$  estimated in uncontaminated patches, i.e.  $\bar{\alpha}_{i,c} = 36.7 \pm 1.2^\circ$  for males and  $\bar{\alpha}_{i,c} = 37.3 \pm 1.2^\circ$  for females. The swimming speeds and turning angles of both males and females significantly differ between experimental conditions, were significantly smaller in contaminated water, and significantly decreased with the concentration of the water-soluble fraction of diesel oil considered ( $P < 0.05$ ; Fig. 8). In contrast, sinking speed did not significantly differ between males ( $v_m = 0.5 \pm 0.1 \text{ mm s}^{-1}$ ) and females ( $v_f = 0.6 \pm 0.1 \text{ mm s}^{-1}$ ) in non-contaminated and contaminated estuarine waters ( $P > 0.05$ ), and no significant differences were observed between control and WSF treatments ( $P > 0.05$ ).

Two previous behavioural studies of *E. affinis* (Michalec et al., 2010; Cailleaud et al., 2011) considered sinking as “a swimming speed between 1 and 8 mm/sec and a direction straight towards the bottom, when the copepod is not swimming but sinks slowly due to the influence of gravity”. This is highly questionable, as well as the results of the rather convoluted subsequent analyses, as the sinking speed reported for *E. affinis* falls in the range 0.4–0.8 mm s<sup>-1</sup> (Seuront, 2006; present work). In addition, previous experiments conducted on various calanoid copepod species have reported sinking velocities typically ranging from 0.3 to 2.5 mm s<sup>-1</sup>; see e.g. Tiselius & Jonsson (1990), and Weissman et al. (1993). Typical sinking speed obtained for anaesthetised (hence likely to be the fastest ones in the absence of any appendage movement) *E. affinis* range from 0.4 to 0.7 mm s<sup>-1</sup> for adult males (cephalothorax length, 0.81 to 0.83 mm) and 0.5 to 1.2 mm s<sup>-1</sup> for non-ovigerous females (0.84 to 0.86 mm); Seuront, unpublished data. Those values are consistent with the passive sinking velocities reported for *E. affinis* (Seuront, 2006; present work) and other species (Tiselius & Jonsson, 1990; Weissman et al., 1993) where copepods were moving downwards, with their tail down, but also with copepod carcasses (Frangoulis et al., 2011). The slight differences observed between sinking velocities of non-anaesthetised (Seuront, 2006; present work) and anaesthetised *E. affinis* suggest that the influence of gravity was partially counterbalanced by the motion of feeding appendages, which is consistent with the intrinsic link existing between swimming and feeding behaviours in calanoid copepods (e.g. van Duren & Videler, 1995). However, the much higher velocities reported by Michalec et al. (2010) for *E. affinis* ‘sinking’ may rather suggest that the resolution of their camera, located 50 cm away from a 5 × 5 × 6 cm tank was not good enough to distinguish individuals actually sinking with their tail down from individuals actively swimming downwards.

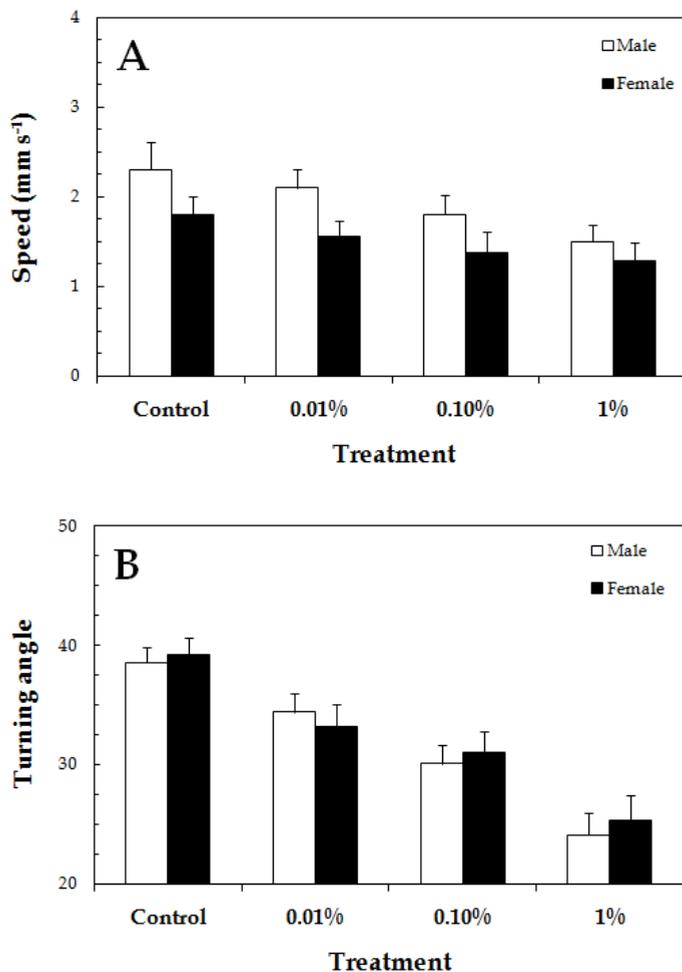


Fig. 8. Swimming speed (A) and turning angle (B) of *E. affinis* adult males and non-ovigerous females (B) in uncontaminated estuarine water and in estuarine water contaminated with the water-soluble fraction of diesel oil at 0.01%, 0.1% and 1%. The error bars are standard deviations.

Besides, both studies (Michalek et al., 2010; Cailleaud et al., 2011) report mean prosome lengths of respectively 0.85 mm and 0.95 mm for *E. affinis* males and females, which are consistent with the size of *E. affinis* individuals investigated previously (i.e. 0.81 to 0.83 mm for males and 0.84 to 0.87 for females; Seuront, 2006) and in the present work (0.83 to 0.86 mm for males and 0.87 to 0.92 for females). For sinking velocities of copepods of similar size to range between 1 and 8 mm s<sup>-1</sup> as reported in Michalek et al. (2010) and Cailleaud et al. (2011), their density need to vary by a factor of 8 or to violate Stokes law which would both be unprecedented in the zooplankton literature.

Males and non-ovigerous females exhibited very comparable swimming paths (see Fig. 3a,b). The swimming activity of *E. affinis* is, however, clearly sex-dependent (Fig. 9). In uncontaminated estuarine water, males spend significantly more time cruising (76.3%) than hovering (16.7%), sinking (2.6%) and breaking (4.4%). In contrast, non-ovigerous females spend most of their time cruising (51%) and sinking (36.3%), with significantly less time spent sinking (7.4%) and breaking (5.4%). Under increasing condition of WSF contamination, both males and females decrease their swimming activity with a decrease in the time allocated to cruising (Fig. 9).

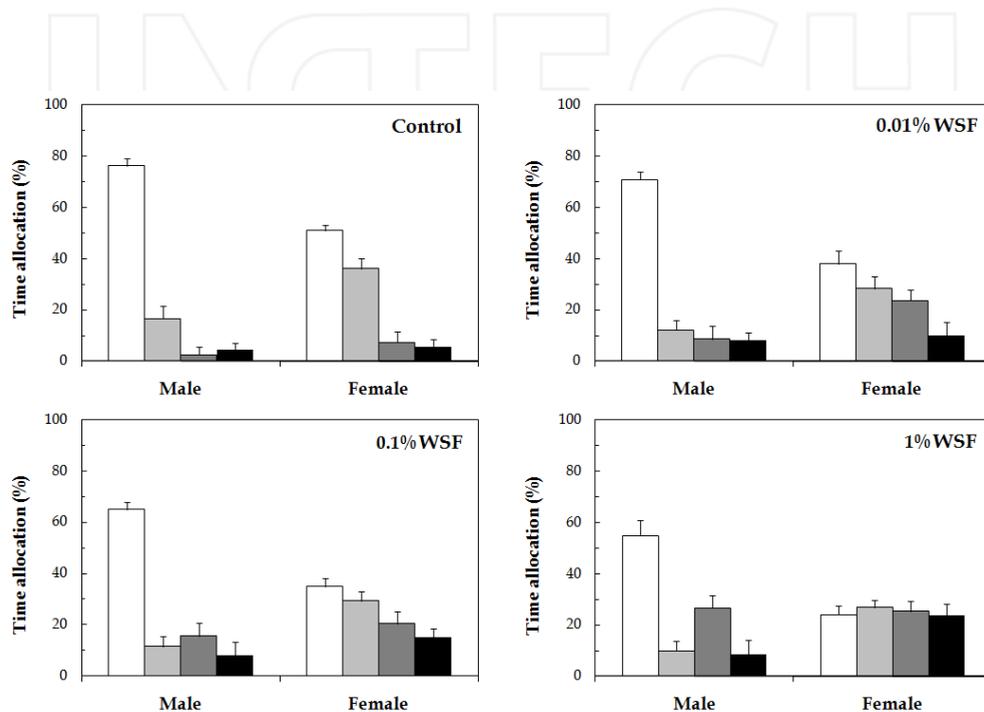


Fig. 9. Fraction of time allocated by *E. affinis* adult males and non-ovigerous females to cruising (motion with rostro-caudal body axis aligned with the direction of motion, whether they were swimming up, down or horizontally; white), hovering (upward motion at low speed, often with a horizontal component; light grey), sinking (downward vertical motion, with tail down; dark grey) and breaking (no motion, with rostro-caudal axis oriented upward; black) in control uncontaminated estuarine water and estuarine water contaminated with the water-soluble fraction of diesel oil at 0.01%, 0.1% and 1%. The error bars are standard deviations.

More specifically, while the time allocated to cruising significantly decreased from uncontaminated to contaminated waters, it remained the main behavioural activity for

males in WSF contaminated water (Fig. 9). The time allocated to hovering slightly decreased with increasing WSF concentration, while sinking and breaking increased with increasing WSF concentration. In contrast, the time allocated by females to cruising and hovering significantly decreased from uncontaminated to contaminated water (Fig. 9); however, cruising and hovering respectively significantly decreased and did not significantly change with increasing WSF concentration. Sinking and breaking increased with increasing WSF concentration, leading to an even time allocation between cruising, hovering, sinking and breaking in water contaminated with WSF at 1% (Fig. 9).

The decrease in the swimming speed and swimming activity reported here of both adult males and non-ovigerous female swimming speed with increasing WSF concentration *a priori* contrasts with recent observations conducted on *E. affinis* adult males and females, which significantly increased their swimming speed following an exposure to  $2 \mu\text{g l}^{-1}$  of 4-nonylphenol and nonylphenol-ethoxy-acetic-acid (Cailleaud et al., 2011). However, in this work (Cailleaud et al., 2011) the swimming behavior of *E. affinis* was recorded from the same individuals before and after the injection of 15  $\mu\text{l}$  of test solution. As such, the behavioral observations conducted in contaminated water are more likely to result from the exposure to a gradient than a background concentration of nonylphenols, and/or to the stress response induced by the injection of the contaminant in uncontaminated water. This is consistent with *T. longicornis* and *E. affinis* males and females escaping at high velocities when reaching patches of WSF contaminated seawater (Seuront, 2010a; present work) and to the decrease in swimming speed observed in *T. longicornis* males and females under conditions of increasing WSF contamination (Seuront, 2011a), hence with distinct behavioral reactions following an exposure to a background concentration of contaminants and a gradient of contaminants.

To specify this, we compared the swimming speed of *E. affinis* males and females recorded over 3 successive 5-min intervals (i.e. 0-5 min, 5-10 min and 10-15 min) during the 15-min acclimation phase, with the swimming velocity recorded during the 30-min behavioral experiment *per se*. Note that no significant differences were found in the swimming speeds recorded over the 6 successive 5-min intervals available from the 30-min behavioral experiments ( $P > 0.05$ ). This resulted in comparing swimming speeds between 4 time intervals, i.e. 0-5 min, 5-10 min, 10-15 min and 15-45 min (Fig. 10). *E. affinis* swimming speed did not significantly differ between the four temporal categories under conditions of uncontaminated water. In contrast, under conditions of WSF contamination, the swimming speed observed during the acclimation phase were significantly higher ( $P < 0.05$ ) than those recorded during the behavioral experiment (Fig. 10). This effect increased with increasing WSF concentrations, and the the increase in swimming speed observed during the first 5-min interval (0-5 min) under WSF contamination leads to a decrease in swimming speed that ultimately converges towards the values observed during the 30-min behavioral experiment for both males and females; the higher the WSF concentration, the higher the swimming velocity during the acclimation phase (Fig. 10). This suggests that the claimed increase in *E. affinis* swimming speed in the presence of sub-lethal concentrations of nonylphenols (Cailleaud et al., 2011) may reflect a stress reaction related to the changes in water properties induced by the injection of nonylphenols rather than the actual effect of nonylphenols on behavior.

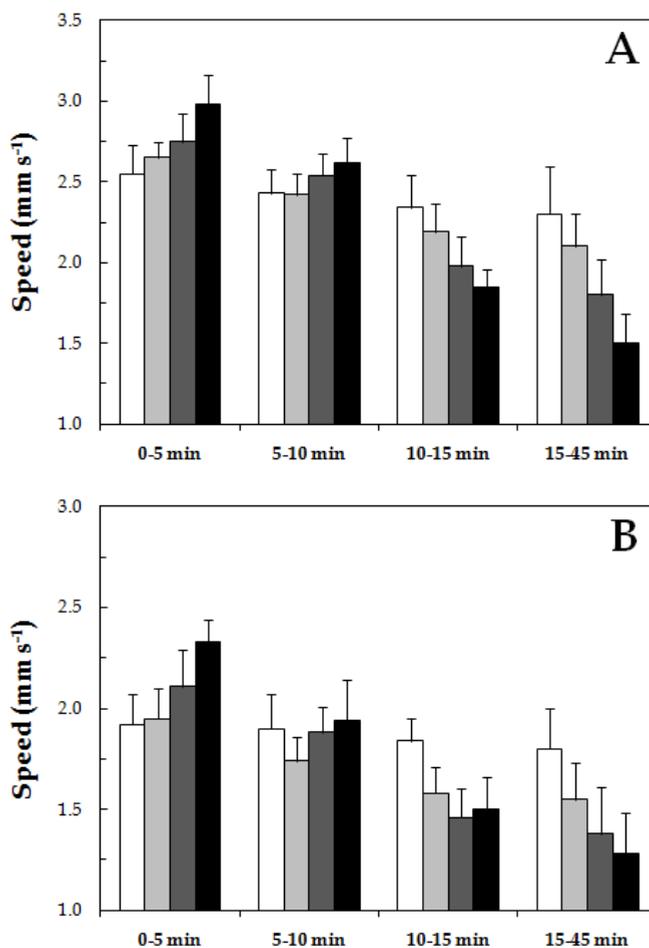


Fig. 10. Swimming speed of *E. affinis* adult males (A) and non-ovigerous females (B) in uncontaminated water (white) and in estuarine water contaminated with the water-soluble fraction of diesel oil at 0.01% (light grey), 0.1% (dark grey) and 1% (black), expressed as a function of the time elapsed since the start of behavioral experiments, that include acclimation (0-5 min, 5-10 min and 10-15 min) and experimental phase (15-45 min). The error bars are standard deviations.

Note, however, that the behavioral responses previously observed in zooplankton following water contamination range from hypoactivity to hyperactivity, depending on the species, sex, concentration and nature of the contaminant, and exposure time. For instance, *Daphnia magna* decreased their swimming speed after several days of exposure to cadmium (Baillieul & Blust, 1999) and a 9-h exposure to copper at 30 mg l<sup>-1</sup> (Untersteiner et al., 2003). In contrast, no changes were recorded following a 3-h and 24-h exposures to copper at 0.01 mg ml<sup>-1</sup> (Shimizu et al., 2002) and 5 mg l<sup>-1</sup> (Untersteiner et al., 2003), and an increase in

swimming speed occurred after a 24-h exposure to methyl-paraoxon at 0.7 mg l<sup>-1</sup> (Duquesne & Küster, 2010). Similar results were obtained on the larvae of the cirriped *Balanus Amphitrite* (Faimali et al., 2006), the crustacean *Artemia* sp. and the rotifer *Brachionus plicatilis* (Garaventa et al., 2010) exposed to a range of chemical pollutants (i.e. antifouling biocides, neurotoxic pesticides, and heavy metals). In copepod ecotoxicology, *E. affinis* nauplii respond to sub-lethal copper concentration by successive phases of hyperactivity and hypoactivity, whose magnitude is season-dependent (Sullivan et al., 1983). In much shorter (30-min to 1-h) bioassays, exposure to sub-lethal hydrocarbon concentrations, leads to decrease the swimming speed of *T. longicornis* females (Seuront, 2011a) and both *E. affinis* males and females (present work). In contrast, no significant changes were observed in *Centropages hamatus* females (Seuront & Leterme, 2007; Seuront, 2010b) and *T. longicornis* males (Seuront, 2011a).

As stressed above from the patch avoidance response of *E. affinis* males and females, these observations imply that behavioral responses to pollution based on the analysis of swimming speed alteration are highly variable. As a consequence, and even if alterations in swimming speed have been detected at toxic compound concentrations well below LC50 values for a range of invertebrates (Avila et al., 2010; Ihara et al., 2010; Garaventa et al., 2010; Cailleaud et al., 2011; Seuront, 2011a, present work), its claimed use as a non-specific behavioral end-point in marine ecotoxicology and environmental monitoring program (Faimali et al., 2006; Garaventa et al., 2010) cannot be warranted. A practical alternative based on the intrinsic fractal nature of behavioral properties and their subsequent modification under stressful conditions, applied in both invertebrate (Seuront & Leterme, 2007; Seuront, 2010b, 2011b) and vertebrate (Escós et al., 1995; Alados et al., 1996; Alados & Huffman, 2000; María et al., 2004; Seuront & Cribb, 2011), including humans (e.g. Togo & Yamamoto, 2000; Goldberger et al., 2002; West & Scaffeta, 2003), is provided in the next two sections.

#### 4.2 Swimming path complexity in hydrocarbon contaminated water

The fractal dimensions  $\bar{D}_b$  and  $\bar{D}_m$  estimated from *E. affinis* males and females swimming paths were not significantly different ( $P > 0.05$ ). This is in accordance with the theoretical formulation  $D_b = D_m$  derived from Eqs. (2) and (4); see Seuront (2010b) for further details and theoretical developments. The fractal dimension  $D$ , i.e.  $D = (D_b + D_m)/2$  was hence used hereafter to characterize the complexity of *E. affinis* swimming paths.

In uncontaminated estuarine water, male fractal dimensions ( $D = 1.43 \pm 0.11$ ) were significantly higher ( $P < 0.05$ ) than female ones ( $D = 1.31 \pm 0.08$ ). This difference is consistent with the results obtained from *E. affinis* males ( $D = 1.23 \pm 0.01$ ) and females ( $D = 1.20 \pm 0.02$ ) in GF/C filtered estuarine water at 5 PSU (Seuront, 2006), and from *T. longicornis* swimming paths observed in GF/C filtered coastal waters (i.e.  $D = 1.32 \pm 0.02$  for males and  $D = 1.27 \pm 0.02$  for females; Seuront, 2011a). In contrast, the swimming paths of *Oncaea venusta* males and females were characterized by non-significantly different fractal dimensions, i.e.  $D = 1.14 \pm 0.06$  for males and  $D = 1.15 \pm 0.06$  for females, investigated in natural seawater (Seuront et al., 2004b). The differences reported above between *T. longicornis* and *E. affinis*, and *O. venusta* may, however, be due to the absence and presence of cues in their respective experimental set-ups. In the absence of cues, the different fractal

dimensions estimated from male and female swimming paths in both *T. longicornis* and *E. affinis* may be related to intrinsic (i.e. innate) differences in male and female foraging strategies. Male fractal dimensions are significantly higher than female ones, hence males are engaged in more intensive foraging strategies than females, which is consistent with the reported behavior of males in the presence of female cues (e.g. Doall et al., 1998; Nihongi et al., 2004; Bagøien & Kjørboe, 2005; Goetze & Kjørboe, 2008; Yen et al., 2011). The similar complexity of the swimming paths of *O. venusta* males and females in natural seawater may be related to a common, hence sex-independent, adaptive behavioral strategy developed in response to the range of chemical cues (from e.g. preys, conspecifics and predators) that are likely to be present in their experimental containers. This is consistent with the fractal dimensions of *T. longicornis* adult females observed in natural seawater range, however, between  $D = 1.18 \pm 0.04$  and  $D = 1.82 \pm 0.05$  depending on the nature and abundance of the phytoplankton community and seawater viscosity (Seuront & Vincent, 2008). More generally, the fractal dimensions estimated from *E. affinis* female swimming paths observed in uncontaminated estuarine water are in the range of values observed for females of different calanoid species investigated using the same experimental protocol than the present work, i.e.  $D = 1.25 \pm 0.02$  for *Acartia clausi*,  $D = 1.37 \pm 0.03$  for *Centropages typicus*,  $D = 1.27 \pm 0.02$  for *Pseudocalanus elongatus*,  $D = 1.42 \pm 0.03$  for *Paracalanus parvus*, and  $D = 1.25 \pm 0.03$  for *T. longicornis* (Seuront, 2011b).

Males and females fractal dimensions both significantly decrease ( $P > 0.05$ ) with WSF concentration (Fig. 11a). The observed decrease is comparable to the decrease observed in *T. longicornis* males and female fractal dimensions under the same conditions of WSF contamination (Seuront, 2011a). In addition, the relative changes observed in *E. affinis* male and female fractal dimensions under control and WSF contamination range respectively from 3.5 to 11.9% and 3.8 to 11.5% in estuarine water contaminated at 0.01% and 1%. These rates are also similar to those observed in *T. longicornis* males (2.4 to 9.5%) and females (3.0 to 8.3%; Seuront, 2011a), suggesting a sex-independent response to WSF contamination. The swimming path complexity of adult females of 5 species of calanoid copepods did not change, however, under varying experimental light regimes (Seuront, 2011b). This divergence in the response of calanoid copepods to distinct sources of stress may indicate that the impact of stressful conditions on the fractal properties of movement behavior may be dependent on the nature of the stressor itself. The resolution of this specific issue is, however, far beyond the objectives of the present work.

In contrast, the fractal dimensions reported for *Daphnia* sp. swimming paths seem to consistently increase under stress. The fractal dimensions of *Daphnia magna* and *Daphnia pulicaria* swimming paths respectively increased in water contaminated by copper, organophosphorus (Dichlorvos) and carbamate (Propoxur) (Shimizu et al., 2002), and following a 1-min exposure to turbulence (Seuront et al., 2004c). Some of the fractal dimensions estimated for *Daphnia magna* swimming paths under conditions of chemical contaminations are, however, higher than the upper theoretical limit  $D = 2$  (see Shimizu et al., (2002), their figures 4 and 5), which questions the relevance of their results. More generally, the application of fractals to cladocerans behavioral ecology and ecotoxicology is still far too limited (Shimizu et al., 2002; Seuront et al., 2004a,c,d; Uttieri et al., 2005; Ziarek et al., 2011) to allow reliable conclusions.

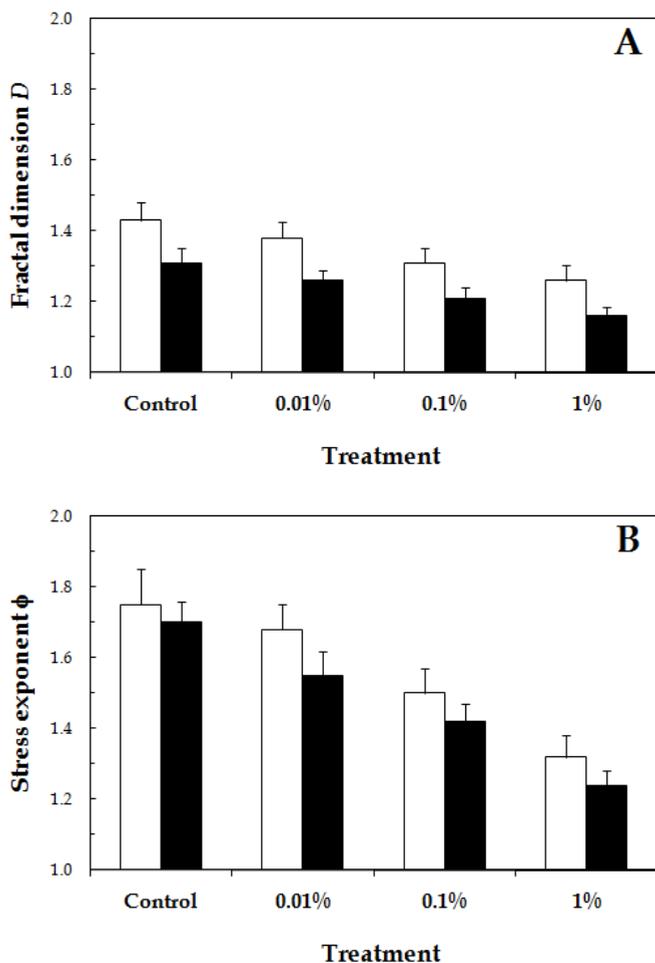


Fig. 11. The fractal dimension  $D$  (A) and the stress index  $\phi$  (B) estimated from swimming paths of *E. affinis* adult males (white) and non-ovigerous females (black) in control uncontaminated estuarine water and in estuarine water contaminated with the water-soluble fraction of diesel oil at 0.01%, 0.1% and 1%. The error bars are standard deviations.

In contrast to the nature of alterations in swimming speed (i.e. hypoactivity vs. hyperactivity) which are likely to be sex- and species-specific as discussed above, the alterations observed in the fractal dimension of copepod swimming paths under conditions of hydrocarbon contamination seem to be both sex- and species-independent. Fractal dimensions are hence likely to provide a very sensitive and robust behavioral end-point in marine ecotoxicology and environmental monitoring program. Further work is nevertheless needed to confirm and generalize to other copepod species the congruent results obtained from *T. longicornis* (Seuront, 2011a) and *E. affinis* (present work) swimming behavior under conditions of hydrocarbon contamination.

### 4.3 Complexity of instantaneous displacements in hydrocarbon contaminated water

Log-log plots of  $N(l \leq L)$  vs.  $l$  (not shown) were highly significantly linear ( $P < 0.01$ ) and characterized by coefficients of determination  $r^2$  consistently higher than 0.99 for both males and females. This shows that the cumulative probability distribution of move lengths  $L$  is compatible with a power-law behavior (see Eq. (5)), hence an underlying fractal structure. A major consequence of this fractal structure is that the statistical distribution of *E. affinis* swimming speed is far from Gaussian. As such, comparisons of experiments with different durations using mean values of standard behavioral metrics (e.g. swimming speed) that have a fractal structure are unlikely to be meaningful, because those mean values intrinsically depend on the duration of the experiment.

The stress exponent  $\phi$  did not significantly differ between males ( $\phi = 1.75 \pm 0.11$ ) and females ( $\phi = 1.71 \pm 0.06$ ) in control experiments conducted in uncontaminated estuarine water (Fig. 11b). This suggests that the fractal structure of move lengths is similar for *E. affinis* males and females in the absence of any experimental stressors. In contrast, while the values of  $\phi$  significantly decreased with increasing WSF concentrations for both males and females, they were consistently significantly ( $P < 0.05$ ) lower for females than males at each WSF concentration (Fig. 11b). This indicates that WSF has a stronger disruptive effect on the swimming behavior of females, and specifies the conclusions obtained from the fractal dimension of male and female swimming paths (Fig. 11a).

The values found here for *E. affinis* in the absence of hydrocarbon stress are consistent with previous values reported for a range of calanoid copepods in the absence of any stressors, i.e.  $\phi = 1.51 \pm 0.04$  in *Centropages hamatus* (Seuront & Leterme, 2007), and  $\phi = 1.81 \pm 0.05$  in *Acartia clausi*,  $\phi = 1.74 \pm 0.04$  in *Paracalanus parvus*,  $\phi = 1.71 \pm 0.04$  in *Centropages typicus*,  $\phi = 1.69 \pm 0.05$  in *Temora longicornis*, and  $\phi = 1.61 \pm 0.04$  in *Pseudocalanus elongatus* (Seuront, 2011b). The observed inter-species variability in the values of  $\phi$  nevertheless suggests that the value of the exponent  $\phi$ , hence the fractal structure of move lengths, are species-specific. However, the 7 species of calanoid copepods investigated in the literature using Eq. (5) consistently show a decrease in the related stress exponent under stressful conditions, whatever the source of stress may be (Seuront & Leterme, 2007; Seuront, 2010b, 2011b; present work). More specifically, the relative decrease observed here in the exponent  $\phi$  were 4.0%, 14.3% and 24.6% for males, and 8.8%, 16.5% and 27.1% for females at WSF concentrations of 0.01%, 0.1% and 1%. This suggests that the behavioral stress induced by WSF contamination of estuarine water at 0.1% and 1% is similar to the stress observed in other calanoid copepods during behavioral experiments conducted in the dark during daylight hours and in the light at night, with relative decrease in  $\phi$  ranging from 10.9% in *P. elongatus* and 27.9% in *T. longicornis*; see Seuront (2010b) for further details. As a consequence, even if the magnitude of the changes observed in the exponent  $\phi$  between control experiments and under stressful conditions is likely to be both sex- and species-specific, it is critical that  $\phi$  consistently decreases under stressful conditions.

It is finally stressed, that the relative decrease observed in the exponent  $\phi$  in Spanish ibex (*Capra pyrenaica*) parasited by the arthropod *Sarcoptes scabiei*s (21.4%; Alados et al., 1996) and bottlenose dolphin (*Tursiops aduncus*) under various conditions of boat presence and traffic (8.7 to 31.5%; Seuront & Cribb, 2011) is also consistent with those reported for copepods (Seuront & Leterme, 2007; Seuront, 2010b, 2011b; present work). This may indicate that the

differences between the values of the exponent  $\phi$  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  $\phi$ , as previously shown for several fractal and multifractal measures of behavioral and environmental complexity (Seuront, 2004, 2005, 2010b; Seuront et al., 2004a,b). Similar approaches, based on the structure of sequential behavior patterns (e.g. moving versus non-moving) have also been successfully applied to assess stress in a variety of terrestrial and aquatic vertebrates (Quenettes & Desportes, 1992; Carlstead et al., 1993; Escós et al., 1995; Alados et al., 1996; Alados & Weber, 1999; Alados & Huffman, 2000; María et al., 2004), and in the copepod *Centropages hamatus* under conditions of naphthalene contamination (Seuront & Leterme, 2007), and have consistently shown a decrease in the fractal complexity of behavioral display under stressful conditions.

## 5. Conclusion

This work investigated the ability of the estuarine copepod *Eurytemora affinis* to detect and subsequently avoid point-source contamination by non-lethal doses of the water-soluble fraction (WSF) of diesel oil, and their behavioral response to the diffuse contamination that is likely to follow any point-source contamination.

Both adult males and females have the ability to detect, consistently avoid and eventually escape localized patches of WSF contaminated estuarine water. They also exhibit a range of behavioral changes in contaminated estuarine water, i.e. a decrease in swimming speed and turning angle, a decrease in the fractal complexity of their swimming paths and a decrease in the fractal complexity of their successive displacements. The present work and published behavioral responses to pollution based on the analysis of swimming speed alteration indicate that the behavioral responses previously observed in zooplankton following water contamination range from hypoactivity to hyperactivity, depending on the species, sex, concentration and nature of the contaminant, and exposure time. As such, it is stressed that even if alterations in swimming speed have been detected at toxic compound concentrations well below LC50 values for a range of invertebrates (e.g. Avila et al., 2010; Ihara et al., 2010; Garaventa et al., 2010; Cailleaud et al., 2011; Seuront, 2011a), its claimed use as a non-specific behavioral end-point in marine ecotoxicology and environmental monitoring program (Faimali et al., 2006; Garaventa et al., 2010) cannot be warranted.

A practical alternative is based on the intrinsic fractal nature of behavioral properties and their subsequent modification under stressful conditions. Both the fractal properties of swimming paths and instantaneous displacements exhibit a fractal complexity that, in sharp contrast to swimming speed alterations, is consistently decreasing under conditions of water contamination by the water-soluble fraction of diesel oil. A major consequence of the fractal properties observed in *E. affinis* swimming pattern in particular, but also in a range of invertebrates and vertebrates (e.g. Sims et al., 2008; Humphries et al., 2010), is their departure from Gaussianity. The use of the mean values of standard behavioral metrics such as swimming speed is hence unlikely to be meaningful, because they intrinsically depend on the duration of the experiment; see Seuront, 2010b for more discussion.

This generalizes and specifies previous claims that behavioral responses seem to be of similar sensitivity and efficiency as biochemical and physiological responses, thus allowing

the field of behavioral ecotoxicology to expand (Dell, 2002). The observed changes in the fractal properties of swimming behavior indeed occur for very low contaminant concentration, and consistently converge towards a decrease in behavioral complexity under stressful conditions. As such, the use of fractal analysis is recommended in invertebrate ecotoxicology as a sensitive, non-invasive and robust behavioral sub-lethal end-point end-point with short-response times for toxicity bioassays, in particular as it is very sensitive to subtle behavioral changes that may be undetectable to other behavioral variables.

The application of fractals to crustaceans behavioral ecology in general (Coughlin et al., 1982; Bundy et al., 1983; Seuront, 2006; Seuront et al., 2004a-d; Uttieri et al., 2005, 2007, 2008; Seuront & Vincent, 2008; Dur et al., 2010, 2011a,b; Ziarek et al., 2011), and to crustacean ecotoxicology in particular (Shimizu et al., 2002; Seuront, 2010a,b, 2011a,b; present work) is, however, still in its infancy. Further work is needed to entangle the fractal complexity of behavioral properties and to generalize the use of fractal-based approaches to stress assessment in marine invertebrates.

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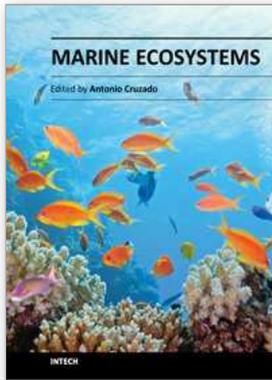
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## **Marine Ecosystems**

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Marine ecosystems, a very wide topic, includes many different processes, groups of organisms and geographical peculiarities. The objective of this book is to present various topics of great importance for understanding the marine ecosystems, what they are, how they work and how we can model them in order to forecast their behaviour under changing conditions. They have been thoroughly reviewed and accepted for publication. The chapters cover aspects such as: Threats to ultraoligotrophic marine ecosystems (Ch. 1); Modelling the pelagic ecosystem dynamics: the NW Mediterranean (Ch. 2); The marine ecosystem of the Sub-antarctic, Prince Edward Islands (Ch. 3); Meiofauna as a tool for marine ecosystem biomonitoring (Ch. 4); Chemical interactions in Antarctic marine benthic ecosystems (Ch. 5); An Interdisciplinary Approach on Erosion Mitigation for Coral Reef Protection- A Case Study from the Eastern Caribbean (Ch. 6); A revisit to the evolution and ecophysiology of the Labyrinthulomycetes (Ch. 7); Seabed mapping and marine spatial planning: a case-study from a Swedish marine protected area (Ch. 8); Management strategies to limit the impact of bottom trawling on VMEs in the High Seas of the SW Atlantic (Ch. 9); Hydrocarbon contamination and the swimming behavior of the estuarine copepod *Eurytemora affinis* (Ch. 10), and Interactions between marine ecosystems and tourism on the Adriatic and Mediterranean (Ch. 11).

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### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821