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Influence of Hydrocarbons Exposure on Survival, Growth and Condition of Juvenile Flatfish: A Mesocosm Experiment

Camille Gilliers¹, Guy Claireaux³, Robert Galois⁴, Véronique Loizeau⁵ and Olivier Le Pape^{2*}

¹ ANSES (French Agency for Food, Environmental and Occupational Health Safety)
254 avenue du Général Leclerc 94701 Maisons-Alfort, France

² Université Européenne de Bretagne, UMR 985 Agrocampus Ouest, Inra
« Ecologie & Santé des Ecosystèmes »,
E-mail: Olivier.Le.Pape@agrocampus-ouest.fr
Ecologie halieutique, Agrocampus Ouest, 65 rue de St Brieuc, CS 84215, 35042 Rennes,
France

³ Université Européenne de Bretagne, Campus de Brest, Laboratoire ORPHY,
6, Avenue Le Gorgeu, CS 93837, 29238 Brest Cedex 3, France

⁴ LIENSS, UMR 6250, Université de La Rochelle, Bâtiment Marie Curie, Avenue Michel
Crépeau, 17042 La Rochelle Cedex, France

⁵ Laboratoire Biogéochimie des Contaminants Organiques (LBCO),
Département Biogéochimie et Ecotoxicologie (BE), Ifremer, BP70 29280 Plouzané, France

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ABSTRACT Juveniles of numerous commercial marine flatfish species use coastal and estuarine habitats as nurseries. Hence, they are likely to be exposed to a number of anthropogenic stressors such as accidental and chronic exposure to chemical contaminants. Little is known about their response to such pollutants at the individual level and about the consequences on their population dynamics. Mesocosm experiments were conducted to determine whether short (24 h) but high exposure to petroleum hydrocarbons (1/1000 v: v water: fuel), similar to what happened after an oil spill on coastal areas, affects survival and biological (growth, body condition and lipid reserve) performances of juvenile common sole, which live on near shore and estuarine nursery grounds. Results demonstrated that this type of exposure significantly reduce survival, growth (size, recent otolith increment and body condition), and especially energy storage (triacylglycerol to free sterol ratio) of the juvenile fish on the medium-term (three months after the exposure). These medium-term consequences affect future recruitment of this long-lived species.

1. INTRODUCTION

Coastal and estuarine areas are under threat of chronic and accidental releases of a wide range of anthropogenic pollutants (Halpern et al. 2008). Among these pollutants, considerable attention has been given to petroleum hydrocarbons and in particular Polycyclic Aromatic Hydrocarbons (PAHs). PAHs are regarded as priority contaminants of terrestrial as well as marine ecosystems (that is, PAHs are listed in the OSPAR list of chemical for priority action) because of their mutagenic and carcinogenic activity. Direct effects of hydrocarbon exposure on adult fishes have been well documented in laboratory (Fletcher et al. 1982; Heintz et al.

2000; Payne and Fancey 1989) or in field studies, following a spill (Bue et al. 1998; Haensly et al. 1982) in polluted estuaries (Heintz 2007; Johnson et al. 1998) or in relation with oil platforms (Stagg et al. 1995). These effects include the induction of detoxification process, tissue alterations and functional abnormalities. However, little is known about the effects of chronic or accidental hydrocarbons exposure on juvenile stage (Peterson et al. 2003). The juvenile stages of a number of fish species settle in coastal and estuarine areas, especially flatfish (Gibson 1994; Le Pape et al. 2003; Meng et al. 2002). As strongly associated with the benthic environment, flatfish are exposed to hydrocarbons through direct contact with the sediment and ingestion of benthic prey.

The bays and estuaries of the western coasts of France provide essential nurseries for the juveniles of many fish species and especially a major commercial flatfish species: the common sole *Solea solea* (Dorel et al. 1991; Le Pape et

Address for correspondence:

Olivier Le Pape,
Agrocampus Ouest,
65 rue de St Brieuc, CS 84215, 35042 Rennes, France
Telephone: 00 33 2 23 48 55 31
Fax: 00 33 2 23 48 55 31
E-mail: olivier.le.pape@agrocampus-ouest.fr

al. 2003). However, the French national network for the observation of the chemical contamination of the coastal environment (RNO, French ministry of the Environment, see details and process on http://envlit.ifremer.fr/surveillance/contaminants_chimiques/presentation) has reported chronic contamination of many parts of these French coasts by hydrocarbon compounds (Beliaeff et al. 1998) and particularly PAHs. In addition, during the last decades these western coasts of France have been particularly affected by accidental oil spills such as Torrey Canyon 1967, Olympic Bravery 1976, Amoco Cadiz 1978, Erika 1999, Prestige 2002 (Saliot 2004). For instance, after the wreck of the oil tanker Erika, the western coasts of France showed high levels of 1-hydroxypyrene (that is, the predominant metabolite of pyrene in fish bile) in juvenile soles two months after the Erika sinking (Budzinski et al. 2004). This demonstrated the important exposure of juvenile soles to the Erika fuel-oil in French Atlantic nurseries.

Hence, the objective of this study was to determine if accidental short (266) exposure to PAHs at concentration occurring during an oil spill has negative effects on the medium-term (3 months) on the biological performances of flatfish juveniles. A mesocosm experiment was conducted and the biological performances between exposed and non-exposed juvenile sole were compared using five biological indicators: survival, average (size) and recent (otolith increment) growth, body condition and lipid content.

2. METHODOLOGY

2.1. Animal Collection

In the western coast of France, the juveniles of the common sole are concentrated in shallow coastal nursery grounds (Le Pape et al. 2003). The 0-group juveniles of common sole (that is, young of the year, few months since their metamorphosis (Dorel et al. 1991) are considered as benthic organisms: they feed on sediment with benthic preys and they spend their life in the top layers of the sediment, burying themselves almost all day long (Gibson 2005).

An experimental population of 630, 0-group juvenile soles (Table 1) aged from 4 to 5 months was sampled at night (7 - 8th of July 2002) using a shrimp otter trawl (20 mm stretched mesh) in the Vilaine Estuary (northern Bay of Biscay, France) and transported to the laboratory, 200

km away, in seawater tanks. Fifteen individuals died during the transport.

Table 1: Number of individuals monitored in the experiment and used in biological performances assessments. C: control specimens; E: exposed specimens; RecGr: Recent Growth (μm); RCI (mg): morphometric type relative condition indices; TAG: ST: triacylglycerol to free sterol ratios

Date	C	E
<i>Before Exposure</i>		
Catch 07&08-07-2002	630	
Exposure (day 1) 08-07-2002	293	298
<i>After Exposure</i>		
24 h (day 2) 09-07-2002	266	253
day 63 08-09-2002	30 removed	30 removed
day 109: 24-10-2002	128	68
<i>Analyse at Day 109</i>		
RecGr	30	25
RCI	44	34
TAG/ST	30	30

2.2. Laboratory Experimental Phase

This experiment is part of a wider project including several experiments on the common sole to determine the consequences on an oil spill after the wreck of the tanker Erika in France. Hence, some parts of the protocol and of the results of this study are based on the previous and published experiments (Claireaux et al. 2004; Budzinski et al. 2004; Tronczynski et al. 2004) from this wider project.

Holding of Fish

Upon arrival in the laboratory (day 1 (D1); Table 1), fish were measured (total length = 57 to 120 mm, mean = 80 mm), allocated at random to two sub-groups and held for 6 hours in flow-through seawater 400-litre tanks (50 cm depth; oxygen saturation, temperature = 19°C). The bottom of those tanks was covered with a 0.5 cm layer of fine sand, a natural substrate for young sole (Le Pape et al. 2003).— During that 6h-acclimation period, 24 individuals died.

Contamination Protocol and Exposure Phase

Fuel-oil No.2 (for a detailed comparison see Claireaux et al. 2004) was used to mimic as an Erika like oil spill.

The experimental protocol consisted of exposing one sub-group (n = 298) for 24 hours to the heavy fuel-oil No. 2, while a second sub-

group (n = 293) was used as control (Table 1). After stopping water inflow to the exposed sub-group rearing tank, fuel-oil was directly added at the surface of the water (1/1000 vol: vol water: fuel-oil). During this exposure period a gentle bubbling of air maintained optimal oxygenation conditions of the water column in the tanks. All through this time the fish remained buried in the sediment and were not in direct contact with the fuel-oil that floated at the surface. During this period, 27 and 45 individuals died in the control and exposed sub-groups, respectively (Table 1).

Levels of Contamination

The level of contamination in water was 50 ng.l⁻¹ of summed-PAHs from fuel-oil No.2 (the summed-PAHs are the following: phenanthrene, fluoranthene, pyrene, benz(a)anthracene, chrysene+triphenylene, benzo(b)fluoranthene, benzo(j)fluoranthene benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene + dibenz(a,c)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene) for a fuel to water ratio of 1/1000 (vol: vol; fuel: water) (Claireaux et al. 2004). Biodegradability of heavy fuel oil is considered to be low (Bordenave et al. 2004) and was not taken into consideration in this experiment using fresh fuel-oil N°2 to mimic consequences of an oil spill on shallow coastal waters.

2.3. Post-exposure Phase in Tidal Earthen Pond Mesocoms

Immediately following the laboratory exposure phase (day 2; Table 1), the two sub-groups (exposed and control) were transferred into two identical neighboring earthen tidal ponds for 105 days (Table 1).

The earthen tidal ponds (200 m², 1 m depth) were situated at the CREMA-L'Houmeau field site in the western coasts of France. The ponds were sole free before the experiment. These ponds were provided with natural food each incoming tide, while sets of standpipes and meshing prevented the fish from escaping. Preliminary studies and empirical observations have shown that the natural fauna present in these tidal ponds provides a carrying capacity corresponding to the nutritional needs of approximately 3kg of fish. Avian predation was pre-

vented by covering the ponds with fine-mesh netting. Pots were used to remove shore crabs *Carcinus maenas*. Water temperature and salinity were monitored daily. Water oxygenation was maintained by mechanical aerators.

During this post-exposure phase: 30 fish were sampled from each pond with a push net (on the day 63 (D63): September 4th 2002).

At the end of the post-exposure phase: all the surviving juveniles were finally collected after drawing down the water (on the day 109 (D109): October 24th Table 1).

The mobile and benthic macro fauna was sampled (sampling area 0.1 m², 20 cm depth, triplicates). Grab samples were sieved, and benthic fauna was sorted and extracted from sediment particles. Organisms were identified to the lowest possible taxonomic level, generally to the species level.

All fish that were removed from the ponds during (D63) and at the end of the experiment (D109) and were measured (total length, TL).

At the end of the experiment (Table 1), a first subsample of both exposed and control sub-groups was frozen for the analyses of otoliths and body conditions. A second subsample of both sub-groups (30 individuals for each group) was frozen for lipid composition analysis.

2.4. Evaluation of Biological Performances

Survival

Survival rate was calculated for both exposed and control sub-groups. The two subsamples of 30 individuals removed on September 24th (Table 1) were added to living specimens on October 24th for both control and exposed samples.

Growth rate in length (Gr)

Gr in length (cm) was calculated as the difference in mean length (\overline{TL}) on the total number of survivals (Table 1) in each sub-sample:

$$Gr = \frac{\overline{TL}_{t.end} - \overline{TL}_{t.start}}{t.end - t.start}$$

where *t.start* and *t.end* (in Julian calendar days, Table 1) were (1-63), (63-109) and (1-109).

Recent Growth of the Otolith: Recent Growth Index (RecGr)

RecGr (last 10 days; μm) was determined for each individual on a subsample of 55 fish

(30 from control and 25 from exposed subgroup; Table 1) by measuring the average width of the peripheral daily increments of the sagittal otoliths (distance between the margin of the otolith back to the 10th ring). Preparation and measurements of the otoliths are detailed (Gilliers et al. 2004). The width of the daily increments of an otolith can be used as an indicator of recent growth if a linear relationship between the length of the fish and the size of the otolith is found (Campana and Neilson 1985). This was demonstrated for 0-group common sole (Gilliers et al. 2006).

Body Condition: Relative Condition Index (RCI)

The relative condition index (mg) is the residual between body weight and predicted weight estimated from the length-weight relationship of the total sample (Blackwell et al. 2000). It was calculated on 78 fish individually weighted and measured (44 from control and 34 from exposed sample) using the equation:

$$RCI = \log(W) - \log(Wc)$$

Where W is the observed body weight and Wc is the computed body weight derived from the logged length-weight linear regression.

Lipid Content: TAG: ST Ratio

The triacylglycerol to free sterol ratio (*TAG:ST*) was chosen as the most relevant lipid-based condition index (Amara and Galois 2004; Fraser 1989). It was determined on the whole body (digestive tracks were removed) of 60 individuals from both control and exposed sub-samples (Table 1). Each specimen was first crushed and homogenized in distilled water, then freeze-dried before lipid analyses. The analytical procedure for lipid extraction (Häkanson et al. 1994) was followed: 100 mg of each lyophilized homogenates were extracted twice for 10 minutes in 3 ml of chloroform: methanol (1:2 then 2:1, v/v). A solution of NaCl (1%) was then added to each sample, which was left for decantation for 12 hours at 4°C. The chloroformic phase containing lipids was then stored at -20°C for analyses. Concentrated aliquots of the lipid extracts were deposited onto Chromarods SIII and total lipids were directly measured with an Iatroscan TH-10 (TLC-FID). The lipid concentration of each sample was assessed using a calibration curve fitted with a total lipid extract of

sole. Lipid classes were separated in 30 min on Chromarods using a mixture of hexane, diethyl ether and formic acid (85:15:0.05, v/v) (Fraser et al. 1985). Chromarods were individually calibrated using Sigma pure standards (Parrish and Ackman 1985). A Shimadzu CR3A was used for chromatogram integration and for the quantification of the triacylglycerols and free sterols.

2.5. Data Analysis

Survival

Two samples z-tests were used to compare survival rates between exposed and unexposed juvenile soles. These z tests were calculated as follow:

$$z = \frac{|Pe - Pc|}{\sqrt{\frac{pxq}{Ne0} + \frac{pxq}{Nc0}}}$$

Where Ne_0 and Nc_0 are the initial number of individuals in each sample (exposed and control), Pe and Pc , the respective proportion of survivals in these two samples ($Pe = Ne1/Ne0$, with $Ne1$, the final number of individual in the exposed sample), p is the theoretically equal proportion of survivals: $p = (Ne1 + Nc1)/(Ne0 + Nc0)$ and $q=1-p$. The value of z is compared with the distribution of a standardized Gaussian.

Other Biological Indicators: Gr, RecGr, RCI, TAG: ST

Student t test were used to compare growth rate in length of fish (Gr; cm), recent growth of otolith (RecGr, μm), body condition (RCI; mg) and lipid content (TAG:ST ratio) of the control and exposed fish. General linear models were additionally used to test if recent growth RecGr, body condition RCI and lipid content TAG:ST varied with fish length (Gr) and if this covariation impacted the response to exposure. Data distribution allowed using these parametric statistical methods without contra indications.

3. RESULTS

3.1. Living Conditions in Mesocosms

In terms of environmental conditions, temperature (from 18.3 in July to 14.4 in October) and salinity (from 35.4-35.7 in Summer to 34.4 in October) in the ponds were similar to near

Table 2: Comparisons (z test) of survival rates between control (C) and exposed (E) animals. N_0 and N_1 : respectively initial and final number of individuals, S survival rate

Period	C	E	z-test and p-values
08-07-2002 to 09-07-2002	$N_1 = 293$ $N_2 = 266$ $S = 0.977$	$N_1 = 298$ $N_2 = 253$ $S = 0.850$	2.18, p-value < 0.05
08-07-2002 to 24-10-2002	$N_1 = 293$ $N_2 = 188$ $S = 0.539$	$N_1 = 298$ $N_2 = 98$ $S = 0.329$	5.16, p-value < 10^{-6}
09-07-2002 to 24-10-2002	$N_1 = 266$ $N_2 = 158$ $S = 0.594$	$N_1 = 253$ $N_2 = 98$ $S = 0.387$	4.70, p-value < 10^{-6}

shore waters and no difference between the two ponds was observed. Faunistic inventory of the samples taken at various stages of the experiment in the earthen ponds showed that from July onward, sediments were colonized by polychaetes and bivalves, which constitute the preferred prey of juvenile sole. Mobile macro fauna was dominated by the shrimps *Palaemonetes varians* and *Crangon crangon*, which, at young stages, may have made a substantial contribution to the soles' diet. A total of 4.2 kg of macro fauna was obtained in average for each pond with similar species diversity and abundance. Except for Gobiidae, no other fish was found in the ponds and, thus, competition for food was presumably low.

3.2. Survival Rates

During the 24 h-laboratory exposure period, the exposed sub-group exhibited a lower survival rate (85 %) than the control sub-group (98 %). The same trend was observed for the 108 days long post-exposure phase in mesocosms where survival rates were 39 % for the exposed and 59 % for the control sub-group. These differences in survival rate were statistically significant (Table 2).

3.3. Growth Performances

After 62 days in the mesocosm ponds (Fig. 1), the exposed juveniles were significantly (p

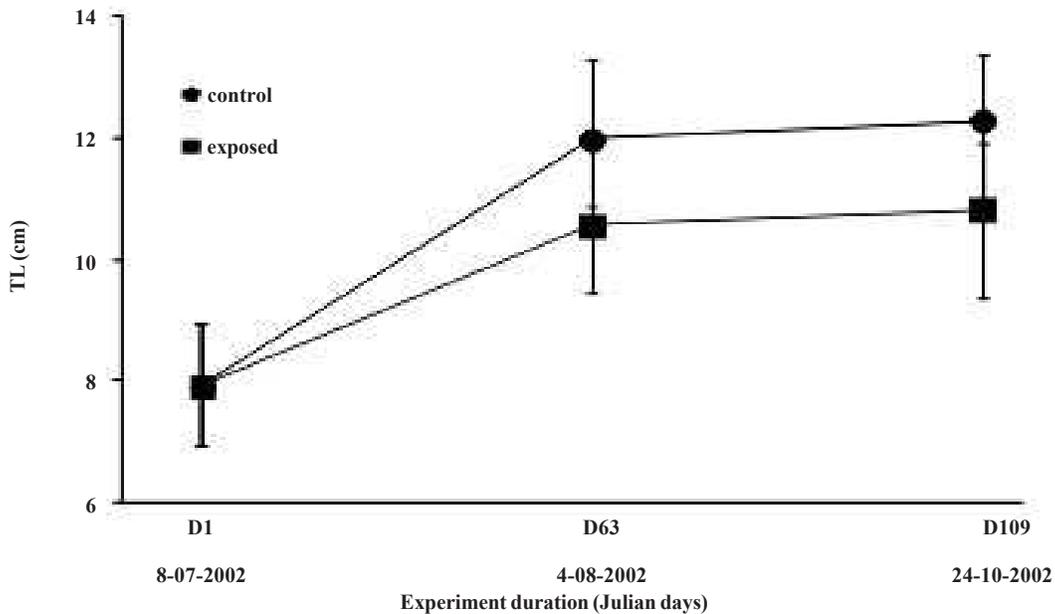


Fig 1. Evolution of total length TL (cm) (mean \pm standard deviation) in control and exposed 0-group sole juveniles during the monitoring phase in mesocosm

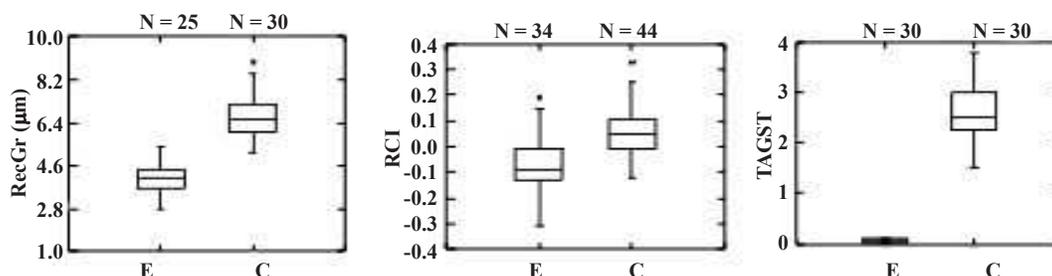


Fig. 2. Biological indicators (median, +/- SD; 95% confident interval and outliers) measured in control (C) and exposed (E) 0-group sole juveniles. *RecGr* (μm): Recent Growth; *RCI* (mg): morphometric condition; *TAG:ST*: triacylglycerol to free sterol ratio; n: number of individuals

< 0.01) smaller than the control juveniles: $TL_{\text{exposed}} = 10.57 \pm (\text{SD}=1.31 \text{ cm})$ and $TL_{\text{control}} = 12 (\text{SD}=1.17 \text{ cm})$. After 108 days, this difference persisted ($p < 0.01$) between exposed and control juveniles:

$TL_{\text{exposed}} = 10.82 \pm (\text{SD}=1.10 \text{ cm})$ and $TL_{\text{control}} = 12.30 \pm (\text{SD}=1.50 \text{ cm})$.

The corresponding calculated growth rate in term of length was:

- 0.45 for exposed and 0.64 $\text{mm}\cdot\text{day}^{-1}$ for control juveniles between day 1 and D63

- 0.054 for exposed and 0.070 $\text{mm}\cdot\text{day}^{-1}$ for control juveniles between D63 and D109.

3.4. Recent Growth and Condition Indices

Pearson correlation coefficient did not show significant relationship between these 3 indicators, recent growth, body condition and lipid content were thus analyzed independently. Recent growth and lipid content were size dependent but body condition was not (Table 3). All of these 3 indicators displayed a significant difference between exposed and control sub-groups, more than 3 months after the 24h oil exposure (Fig. 2) recent growth present a more than 30% decrease for the exposed fish with regard to the reference mesocosm, body condition demonstrated the weight deficit of exposed fish (negative values) and the most dramatic difference was observed for lipid content, exposed fish presenting highly depleted lipid reserves, with *TAG:ST* ratios close to zero.

General linear models showed that after removing the effect of length, the exposure to fuel-oil still significantly affected recent growth *RecGr*, body condition *RCI* and lipid content *TAG:ST* (Table 3).

Table 3: Results of general linear models used to test the effect of «length» (cm) and « treatment group » (Control or Exposed) on biological performances of 0-group sole juveniles *Solea solea*. *RecGr*: Recent Growth (μm); *RCI* (mg): morphometric condition; *TAG:ST*: triacylglycerol to free sterol ratio. n: number of individuals

Response variable	n	Covariate / factor	p-value
<i>RecGr</i>	55	length	0
		treatment group	0
<i>RCI</i>	78	length	0.99
		treatment group	0
<i>TAG:ST</i>	30	length	0
		treatment group	0

4. DISCUSSION

4.1. Contamination Levels and Exposure Routes

In this study, we attempted to mimic realistic condition of contamination on coastal and estuarine ecosystems after an oil spill, that is, a high but short exposure (24h) to PAHs.

During the contamination phase, water PAHs concentration was not measured but we used the same fuel-oil (Fuel-oil No.2); the same fuel to water ratio (1:1000 vol: vol fuel-oil: water) and the same protocol than in a previous experiment conducted (Claireaux et al. 2004). They reported a concentration of summed-PAHs of 50 $\text{ng}\cdot\text{l}^{-1}$ in the laboratory exposure phase. This is a relatively high contamination level in comparison, likely to be representative of the contamination during the first week after a coastal wreck. In addition, such PAH levels have been reported in the literature. For instance, Gustafson and Dickhut (1997) measured PAHs concentration in water as high as 20 to 65.7 $\text{ng}\cdot\text{l}^{-1}$ in the Chesapeake Bay (USA). Similarly, Maskaoui et al.

(2002) reported concentrations between 192 and 2651 ng.l⁻¹ in the Jiulong river estuary (China). This level of contamination is also realistic if one considers the local massive but short-term conditions found in shallow waters shortly after oil reaches a coastline (O'Clair et al. 1996).

Routes of exposure and the level of contamination of the different compartments (water column, sediments, suspended matter) are essential elements to take into account when assessing the level of risk from a contamination. As fish were not fed during exposure to fuel, we considered that dietary intake was negligible and that the most likely route of contamination was through dissolved compounds diffusing into the fish across epithelia. Considering the benthic life of juvenile soles that feed with benthic organisms and actively ingest the sediment when feeding, the contamination of these organisms may have been underestimated in our experiment that considered only the contamination via the soluble phase, albeit at high concentrations.

4.2. Reduced Biological Performances at Individual Level

“Performance” describes the morphological and functional integrity of the whole organism (Claireaux et al. 2004). The researchers considered that survival, growth, body condition and lipid content (energy storage) could properly reflect the biological performance of juvenile fish.

Early exposure to PAHs had both instantaneous and lasting effects on survival of juvenile sole. The exposed animals showed reduced survival rates compared with the control animals both right after the exposure phase and after the mesocosm phase (39% for exposed vs. 59% for control fish). Considering that this type of pollutant may induce tissue alterations, DNA alterations, and cancers (Cachot et al. 2006; Myers et al. 1990) the survival rate of contaminated fish is likely to be even more reduced on longer term, all along the life for *S. solea* (potentially 3 decades or even more).

Juvenile sole growth in the control pond followed well known patterns (Laffargue et al. 2007), with high growth rate in summer then low values between D63 and D109, characteristic of a growth stop related to both reduction in temperature at the end of the summer growing season for *Solea solea* (Amara et al. 2001)

and lower photoperiod action on hormonal levels (Laffargue et al. 2007). On the contrary, exposure to fuel-oil for as little as 24 hours had inhibited growth in flatfish juveniles. At the end of the experiment, lengths for exposed fish were about 12 % smaller (1.5 mm less) than for the unexposed fish. Exposed fish also presented reduced recent growth 3 months after the exposure. Similar insights were shown in previous studies. Kubin (1997) found a 15 % reduction in the growth of juvenile English sole exposed during six months to PAHs-contaminated sediments. Moles and Norcross (1998) also found lasting reduced growth in juveniles of 3 flatfish species exposed to PAHs-contaminated sediments or water for 1 to 3 months. Growth may be the most important factor in recruitment of fishes to the fisheries. A rapid growth and a larger size at the end of the growing season tend to improve survival due to reduced predation (Van der Veer et al. 1994) and reduced susceptibility to environmental stressors (Sogard 1997). There was no sign of food limitation in both tidal ponds and these mesocosms provide temperature and salinity similar to near shore waters. In addition growth rates of unexposed fish were similar to those reported *in situ* (Amara et al. 2001) or in previous experiments in tidal ponds (Laffargue et al. 2007). Since temperature and food are the two most important factors governing fish growth (Fonds 1979) the growth reduction of exposed fish in this study is likely to be related to their exposure to fuel oil and to impaired ability to allocate energy to growth. In a similar experiment, (Claireaux et al. 2004) have demonstrated that exposed sole displayed disrupted metabolism (reduced cellular adenylate content, increase of cytochrom C oxydase activity, reduced pumping capacity of the myocardium) and 20% of reduction in their aerobic metabolic scope (integrated measure of the energy resources).

Our results on body condition and lipid content strengthened the hypothesis of altered metabolic pathways of exposed juvenile soles. The exposed juveniles exhibit lower condition factors. As lipid reserve in control juvenile appeared especially high with regards to *in situ* measurements in adjacent coastal nurseries, the exposed juvenile presented and *TAG:ST* ratios dramatically lower than *in situ* observed minimum (Durieux et al. 2007). A similar, toxicant-induced starvation associated with reduced growth

and lipid content was also found in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) fed PAH-contaminated food (Meador et al. 2006).

4.3. Consequences for the Recruitment

The present study demonstrated that short but high PAHs exposure can reduce survival, growth, condition and energy storage on medium-term (3 months) of juvenile flatfish. 0-group flatfishes are vulnerable to contaminant exposures in their nursery habitats and negative effects on their biological performances may impact their recruitment to adulthood. For example, Gilliers et al. (2006b) reported reduced growth and low densities of juvenile sole in highly contaminated coastal and estuarine nursery areas. Similarly, the contamination of the nurseries in the Prince William Sound following the Exxon Valdez oil spill was associated with reduced growth in juvenile pink salmon populations and with a lower return of the adults to their native rivers (Wilette 1996).

It was hypothesized that the relative failure of the cohort present in the nursery ground at the time of the Erika oil spill was linked to this impaired ability for environmental adaptation (Claireaux and Davoodi 2010; Davoodi and Claireaux 2007). This study strengthened this view.

In conclusion, the contamination of the habitats of juvenile stages of fish could affect their growth and energy storage, their immediate and future survival, and, consequently, the success of the recruitment for the following years (Gibson 1994; Johnson et al. 1998; Wilette 1996) and on the long term (Jewett et al. 2002). As chronic exposure to low levels of PAHs can erode population size and resistance to extinction (Heintz 2007), brief but acute exposure can alter recruitment strength of many succeeding year classes.

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