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Evaluation of the toxicity of the *Deepwater Horizon* oil and associated dispersant on early life stages of the ecologically and economically important Eastern oyster, *Crassostrea virginica*

Thèse soutenue le 23 Janvier 2015
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Avant-propos

Ce travail de thèse s'est déroulé en cotutelle entre la Florida Gulf Coast University (FGCU) et l'UBO/IUEM, sous la codirection d'Aswani Volety et de René Robert. Cette thèse s'est effectuée en grande partie au sein de la station expérimentale de la Vester Marine Field Station mais également dans les laboratoires du Pr. Aswani Volety à la FGCU aux Etats-Unis. Ce travail de recherche s'est porté sur l'étude de la toxicité du pétrole brut relâché lors de la marée noire du *Deepwater Horizon* (DWH) et du dispersant associé (Corexit 9500A®) sur les premiers stades de vie de l'huitre Américaine, *Crassostrea virginica.* Plusieurs études similaires menées par des universités et laboratoires américains, utilisant différents organismes tests (crevettes, crabes, poissons…), sont en cours de déroulement et s'inscrivent dans le cadre de l'étude d'impact environnementale de la marée noire DWH (Natural Resource Damage Assessment, NRDA) mené par l'organisation gouvernementale National Oceanic and Atmospheric Administration (NOAA). Ce travail de thèse a donc été financé par des fonds fournis pour le NRDA et ce projet est sous-traité par le cabinet de consultant indépendant, Stratus Consulting Inc basé à Boulder aux Etats-Unis. En outre, les travaux réalisés dans le cadre de cette thèse ont bénéficié du soutien de l'Université de Bretagne Occidentale de Brest et du « Laboratoire d'Excellence » LabexMer (ANR-10-LABX-19), cofinancé par une bourse du gouvernement français sous le programme « Investissements d'Avenir ».

Cette thèse comprend en premier lieu une introduction générale qui propose une mise en contexte de la marée noire DWH d'avril 2010, ainsi qu'un état des connaissances sur l'utilisation de l'huitre en écotoxicologie. En second lieu, trois chapitres sous forme d'articles scientifiques détailleront les résultats mis en évidence lors de ce travail. Enfin, une discussion générale terminera le présent ouvrage. En raison du caractère juridique de cette étude dont les résultats pourront potentiellement être utilisés lors du procès environnemental entre l'Etat Américain et la compagnie British Petroleum (BP), cette thèse bénéficie d'un statut de confidentialité et aucun des articles présentés dans cette thèse n'a pu encore être publié. Néanmoins, certaines données de cette thèse ont fait l'objet de communications orales et d'affiches lors de congrès nationaux et internationaux (cf Appendix 2).

Remerciements

Je tiens à exprimer mes remerciements à toutes les personnes qui ont contribué et participé, à titre professionnel ou personnel, à la réalisation de cette thèse.

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Enfin, je souhaite bien entendu remercier toute ma famille, ma belle-famille et mes amis pour leur soutien. Bien évidemment mes remerciements les plus spéciaux se dirigent vers ma moitié, mon épouse Anne, qui m'a suivi en Floride, encouragé et soutenu durant cette épopée, à qui je dédie bien sur cette thèse.

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Abstract

The explosion of the *Deepwater Horizon* (DWH) oil drilling rig on 20th April 2010 resulted in the largest oil-related environmental disaster in U.S history with an unprecedented amount of oil (779,000 t) and gas discharged continuously in the Gulf of Mexico, over a period of 3 months. As a response, 8 million L of chemical dispersants (mainly Corexit 9500A®) were applied on surface to dissipate the slicks, and injected directly at the well head (3 million L) at 1500 m depths. The oil spill coincided with the spawning and recruitment season of the ecologically and commercially important Eastern oyster, *Crassostrea virginica*. Due to its biological characteristics (sessile, filter-feeding, ubiquity, "free" spawner), oysters have been employed as a model species in ecotoxicology and for monitoring the environment. However, information on the toxicity of crude oil (HEWAF), dispersed oil (CEWAF) and dispersant alone (Corexit 9500A®) on early developmental stages of *C. virginica* are limited.

The aim of this study was to i) determine the lethal and sublethal effects of acute and chronic exposure to surface-collected DWH oil and/or Corexit 9500A® on various life stages of oysters, ii) examine the mode of toxicity of oil-associated PAHs (dissolved or particulate) and dispersant on sensitive physiological processes, and iii) establish whether there is a relationship between results collected in the laboratory and field data collected during the NRDA sampling program.

Our results indicated that oil and/or dispersant adversely affected reproduction and early development of *C. virginica*, with dispersed oil and dispersant having generally the highest impacts. Furthermore, sublethal effects such as inhibition of larval growth, settlement success or filtration rates were observed at environmentally realistic concentrations of tPAHs or DOSS. These results suggest that oil spills and the use of dispersant as a spill response, especially at the time of oyster spawning season, could affect oyster recruitment and ultimately oyster populations in affected regions. Besides, in order to assess more precisely the environmental impact of an oil spill and the fate of its constituents, meaningful endpoints other than lethality should be selected.

Keywords: Deepwater Horizon oil, dispersant, CEWAF, sublethal, oyster, larvae, toxicity

Résumé

L'explosion du forage et de la plate-forme pétrolière *Deepwater Horizon* (DWH) le 20 Avril 2010 a entrainé la plus importante catastrophe pétrolière de l'histoire des Etats-Unis, avec des quantités sans précédent de pétrole (779,000 T) et de gaz relâchées continuellement dans le Golfe du Mexique durant près de 3 mois. En retour et comme moyen de réponse, 8 million L de dispersant chimique (principalement Corexit 9500A®) furent utilisés pour disperser les nappes, dont 2.9 million L furent injectés directement à la tête du puit à 1500 m de profondeur. La marée noire coïncida avec la saison de ponte et de recrutement de l'huitre américaine *Crassostrea virginica*, une espèce à haute valeur écologique et commerciale dans le Golfe. En raison de ces caractéristiques biologiques (sédentaire, espèce filtreuse, répartition géographique, ponte et fécondation externe), les huitres ont été utilisées comme organisme modèle en écotoxicologie. Néanmoins, il existe très peu de données disponibles sur la toxicité du pétrole brute (HEWAF), du pétrole dispersé (CEWAF) ou du dispersant sur les jeunes stades de vie de *C. virginica*.

L'objectif de ce travail de thèse fut 1) de déterminer les effets létaux et sublétaux d'expositions aigues et chroniques à du pétrole DWH et/ou du Corexit 9500A® sur différents stades de développement des jeunes huitres, 2) d'examiner les mécanismes de toxicité des HAP (dissouts ou particulaires) issus du pétrole et du dispersant sur des processus physiologiques sensibles, et 3) d'établir si les résultats obtenus en laboratoire correspondent aux valeurs recueillies sur le terrain lors du programme NRDA.

Nos résultats ont démontré que le pétrole et/ou le dispersant pouvaient affecter la reproduction et le développement embryonnaire et larvaire de *C. virginica*, et que le pétrole dispersé et le dispersant induisaient en général le plus d'impact. En outre, des effets sublétaux tels que des inhibitions de croissance larvaire, de fixation ou de filtration furent observés à des niveaux d'HAP et de DOSS mesurés dans l'environnement. Ces résultats suggèrent que le pétrole et l'utilisation de dispersant, en particulier lors de la saison de ponte de l'huître, pourraient affecter son recrutement et impacter la ressource de façon délétère dans des régions touchées par une marée noire. Par ailleurs, des critères biologiques plus sensibles que la mortalité devraient être choisis afin d'estimer plus précisément l'impact environnemental des hydrocarbures et le devenir de ses constituants.

Mots-clés : pétrole Deepwater Horizon, dispersant, sublétaux, CEWAF, huitre, larves, toxicité.

I. GENERAL INTRODUCTION

I. General Introduction

1. The Gulf of Mexico

1.1 Geography

The Gulf of Mexico is the ninth largest body of water in the world. Bordered by the U.S, Mexico and Cuba, the Gulf is a Mediterranean-like basin connected to the Caribbean Sea via the Yucatan Strait and with the Atlantic Ocean by the Straits of Florida (Tunnell, 2009). The Gulf occupies a surface area of approximately 1.5 million km^2, and it has a maximum depth of ≈ 4,000 m (Darnell and Defenbaugh 1990); the U.S. side of the Gulf spans 2,700 km across 5 states. A total of 33 major rivers draining from 31 states lead to an extensive freshwater input to the Gulf, totaling approximately 10.6×10^{11} m^3 per year. Eighty-five percent of this flow is drainage from 3.8 million km^2, equivalent to 41% of the United States (NOS/NOAA 2008). The Mississippi River provides 65% of the inflow, delivering extensive sediment loads and contaminants (including excess nutrients) resulting in a major hypoxic zone stretching along the Texas-Louisiana coast (Mitsch et al., 2001; Rabalais et al., 2002). Due to the nutrient-rich sediment deposits, the northern coastline of the Gulf is rich with bays, inlets, and marshland. Wetland areas along the Gulf cover around 2.02 million ha (NOAA).

1.2 The Gulf of Mexico ecosystem

Biologically, the shallow waters of the northern Gulf are categorized as warm temperate (Tunnell, 2009). Ecologically, these waters are some of the most highly productive coastal waters in the world and include important habitats, such as barrier islands, tidal flats, coastal wetlands and marshes, oyster reefs, seagrass meadows, and open bay bottoms (NOS/NOAA 2008). These important habitats provide many ecological functions, such as feeding grounds, nursery grounds, nutrient sources, and structure to hide from predators. Over 90% of all commercially and recreationally important species depend on these estuarine and coastal habitats at some stage in their life cycle (Gunter, 1967): this is a strong evidence of their significant ecological value. The north-central Gulf of Mexico is sometimes referred to as the nation's "Fertile Crescent" due to the high productivity, plentiful nutrients, and abundance of critical nursery habitats (Gunter, 1963; Moore et al. 1970).

A high diversity of marine life, including over 15,400 species, exists in the Gulf of Mexico, making it one of the most biodiverse oceanic water bodies on Earth (Felder and Camp, 2009). In addition to the 1000's of invertebrates, including over 2,400 seashells and over 2,500 crustaceans, there are over 1,500 species of fishes, 5 sea turtles, nearly 400 species of birds, and 30 species of marine mammals (Tunnell, 2009).

1.3 The Gulf Coast economy

The Gulf is a sea of contrasts, where a healthy environment and a healthy economy both coexist and contend with each other (McKinney, 2009). Economically, the five U.S. states that border the Gulf of Mexico have a gross domestic product of over $2.2 trillion, and the robust economy of the Gulf region provides jobs for more than 20 million people. Much of this economic activity is linked to Gulf of Mexico natural resources, such as tourism and recreation, commercial and recreational fishing, and petroleum production and exploration (NOS/NOAA 2008). Due to its geographical situation, the Gulf is highly productive and supports one of the U.S largest commercial fisheries, with estimated fish and shellfish landings of approximately 772 million kg per year (average 1997-2006), yielding a value of $662 million or about 25% of the total U.S. commercial fishing revenue (NMFS/NOAA, 2008). Recently (2006), 83% of the total U.S. shrimp landings, 56% of the oyster landings, and 14% of the commercial fishery landings came from the Gulf of Mexico. In addition to commercial fishing, recreational fishing activities on the gulf coast are a significant part of tourism (Tunnell, 2009). In 2009, 6 million anglers took 22.4 million recreational fishing trips in the Gulf of Mexico region, corresponding to more than $ 2 billion in revenue for fishing-related businesses (NMFS, 2010).

1.4 Oil and gas industry in the Gulf of Mexico

The Gulf of Mexico area, both onshore and offshore, is one of the major petroleum-producing areas of the United States. In 2007, Gulf of Mexico federal offshore oil production accounted for 25% of total U.S. crude oil production, and federal offshore natural gas production in the Gulf accounted for 14% of U.S natural gas production. In addition, over 40% of total U.S petroleum refining capacity is located along the Gulf coast, as well as 30% of total U.S natural gas processing plant capacity (US Energy Information Administration, 2012). Over 107,000 petroleum related workers are employed in the Gulf with over $12.7 billion annual wages earned (NOS/NOAA, 2008).

As technology has progressed over the years, oil companies have extended drilling and production farther and farther from shore, and into deeper and deeper waters. Production from water depths greater than 300 m began in 1979; currently, 72% of oil production in federal waters of the Gulf of Mexico comes from wells drilled in water depths of 300 m or greater. Sixty-five discoveries have been made in water depths greater than 1,500 m (Fig. 1). The deepest water depth in which a discovery has been made is 3,040 m (BOEM, 2012). In 2012, federal leases in the Gulf of Mexico produced 463 million barrels (73.6×10^6 m^3) of oil, which made up 19.5% of all US oil production that year (US Energy Information Administration, 2012) (Fig. 2). About 3,500 exploration and production platforms exist in the northwestern Gulf, down from nearly 7,000 totals, including over 25,000 miles of pipeline and approximately 50,000 total wells drilled (BOEM, 2010).

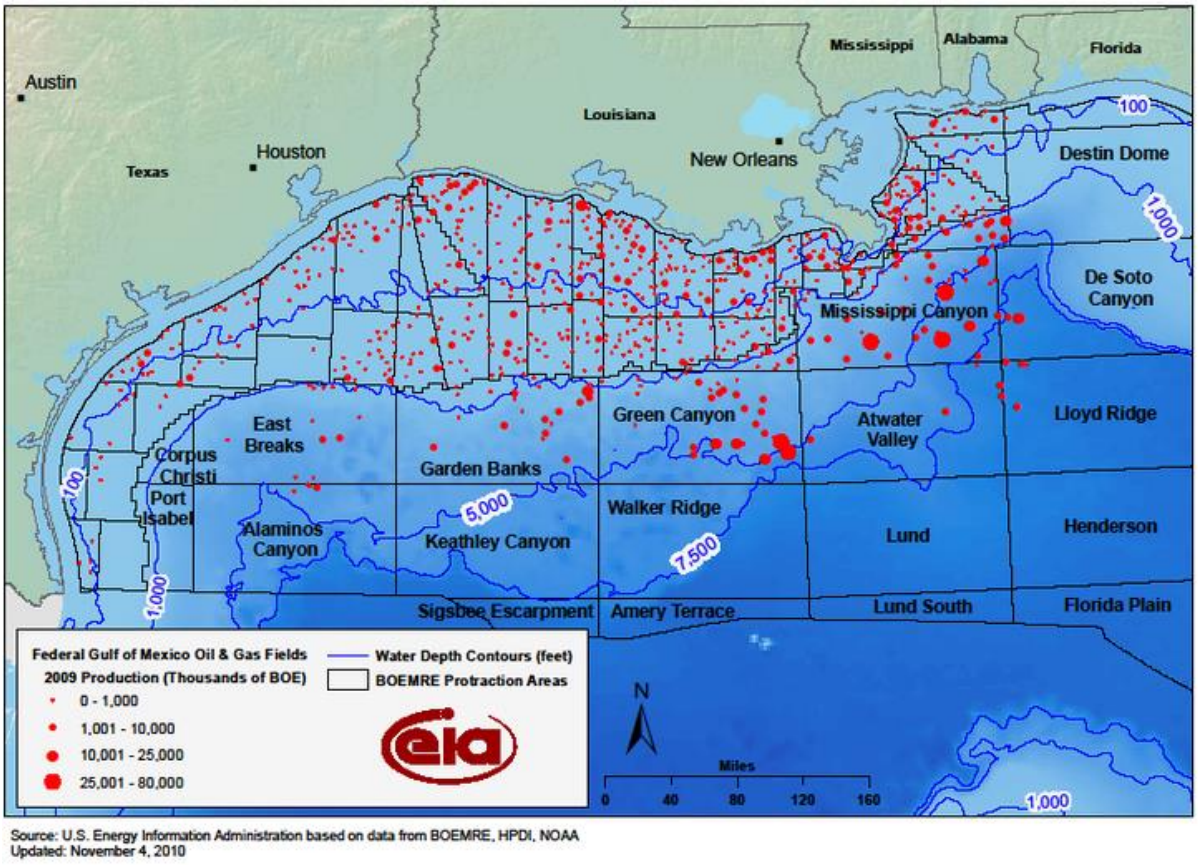


Figure 1: Producing oil and gas fields in the Gulf of Mexico in 2009. *From U.S. Energy Information Administration, based on data collected from BOEM (formerly BOEMRE), HPDI, NOAA.*

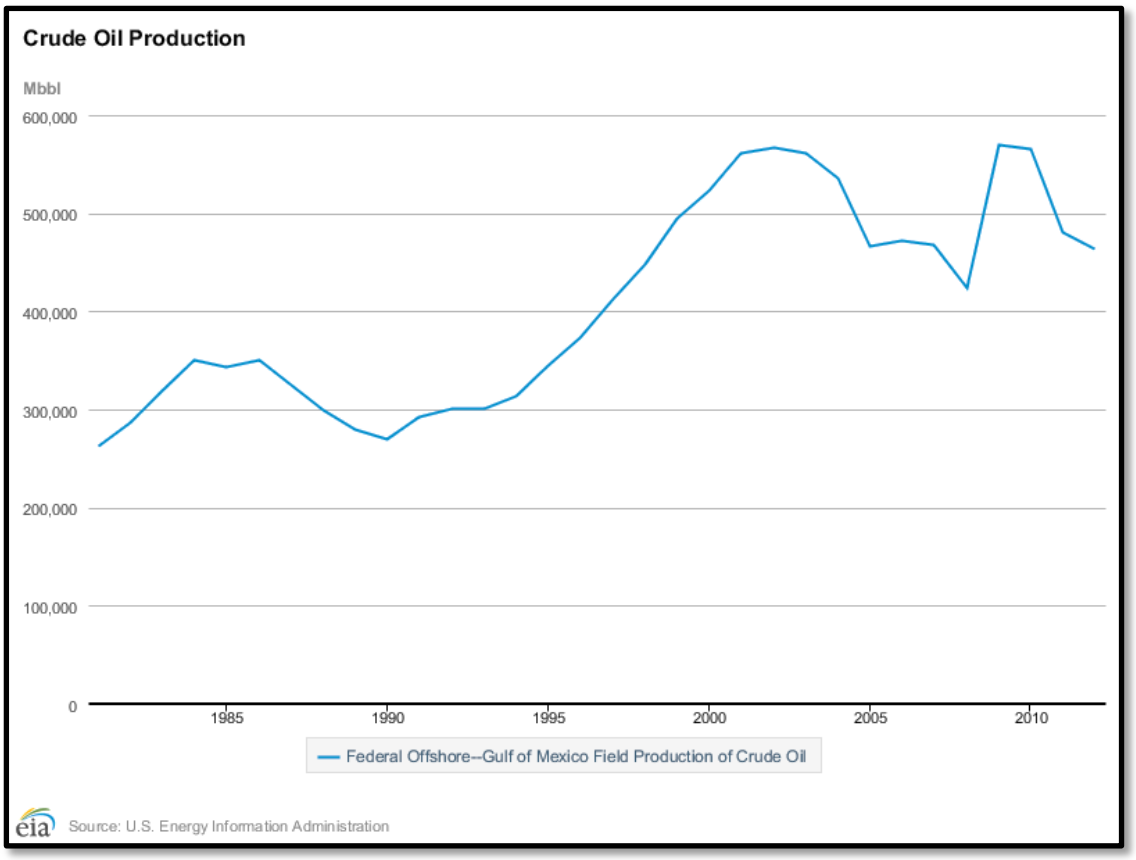


Figure 2: Evolution of Gulf of Mexico field production of crude oil. (*From U.S. Energy Information Administration*)

2. The *Deepwater Horizon* oil spill incident

2.1 Context

On April 22nd 2010, following an explosion that killed 11 workers 2 days earlier, the Deepwater Horizon (DWH) oil drilling platform sank and oil began to leak from the *Macondo-1* well (28°73'67''N, 88°38'69''W) into the Gulf of Mexico at a depth of approximately 5,000 ft. (1,500 m), creating a deep sea "cloud" or "plume" and a surface slick more than 50 miles (77 km) from the seashore (Camilli et al., 2010; JAG, 2010; OSAT, 2010). The blowout prevention device (BOP) at the wellhead and the entire emergency shut-off equipment failed. Upon sinking, the 21 in. (53 cm) riser pipe, from the wellhead to the drilling platform, collapsed onto the sea floor. Oil leaked from multiple locations along the riser pipe and the top of the BOP (Fig. 3) (Atlas and Hazen, 2011). Overall, it took 87 days to stop the flow of oil from the well (Crone and Tolstoy, 2010).

The DWH spill release was estimated by the National Incident Command's Flow Rate Technical Group (FRTG: Labson et al., 2010), McNutt et al. (2012) and Ryerson et al. (2012) at 4.9 million barrels (or about 779 million L) of light crude oil (Light Louisiana Oil, API gravity of 35.2), 0.8 million barrels of which were captured before release into the water column (Lehr et al., 2010). Previously the *IXTOC-1* well blowout in the bay of Campeche, estimated at 556 million L, was the largest oil spill in the Gulf of Mexico and the second largest in the world (the largest spill was in the Persian Gulf in 1991with nearly 2 million L as a result of intentional release of oil by Iraq: Tawfig and Olsen, 1993). The DWH oil spill was more than an order of magnitude greater in total volume of oil than the Exxon Valdez spill in 1989 (41.6 million L) (Moore, 1994; Atlas and Hazen, 2011).

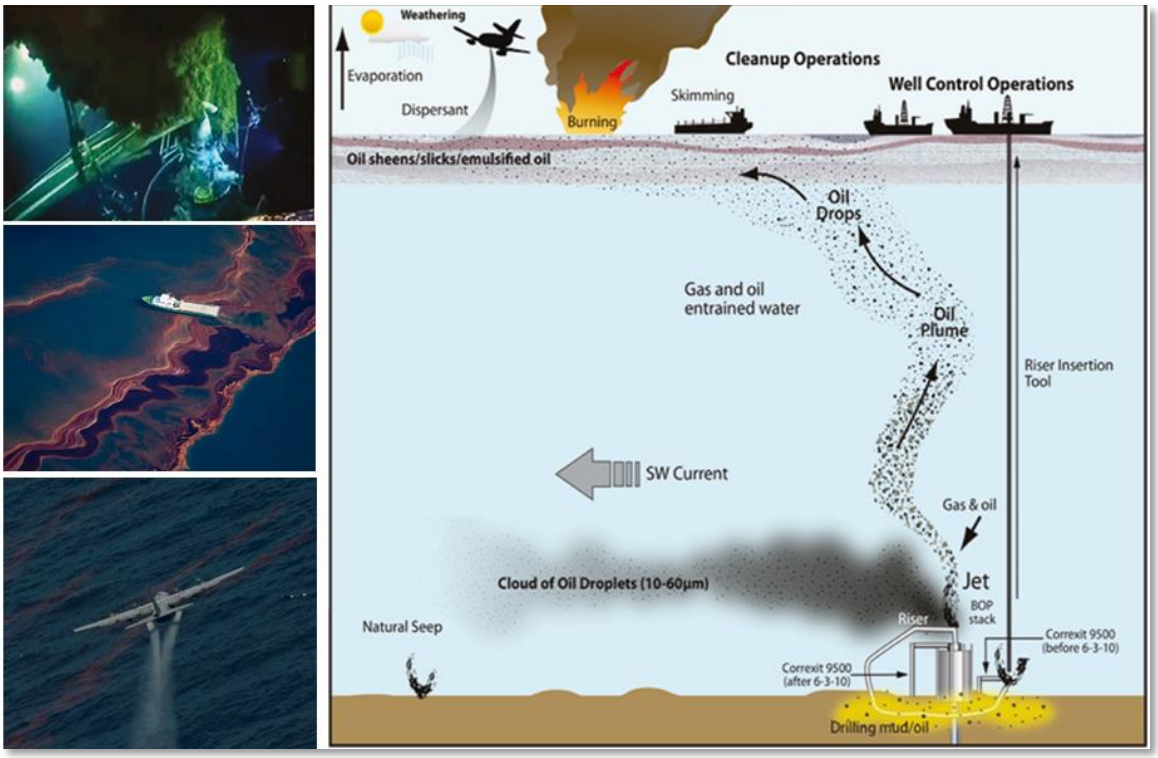


Figure 3: Graphic depiction of Deepwater Horizon spill and clean up (From Atlas and Hazen, 2011)

The oil budget calculator from the FRTG for the DWH well oil release estimated that 3% was skimmed, 5% was burned, 8% was chemically dispersed, 16% was naturally dispersed, 17% was captured directly at the wellhead, 25% was evaporated or dissolved, and 26% was remaining (Lehr et al., 2010; Barron, 2012) (Fig. 3). The BP spill also released considerable amounts of natural gas (mostly methane (CH_4)): Valentine et al (2010) estimated that 1.5 x 10^{10} moles of natural gas was potentially emitted in the deep water over the course of the spill in addition to oil.

According to estimates from aerial imaging by Labson et al (2010), on May 17th 2010, 23 days after the onset of the spill, total surface coverage of spilled oil was 17,700 km^2. Of this area, 2% was classified as "thick", 10% as "dull", and 88% as "sheen". On June 25th, satellite imagery provided estimates of surface coverage at over 63,000 km^2, primarily to the West, East and North of the DWH platform (Norse and Amos, 2010). Oil made landfall for the first time in Louisiana on May 15th, and eventually more than 1,600 km of shoreline was visibly oiled (Rosenbauer et al., 2010). Maximum oiling occurred along the shorelines and barrier islands of Louisiana, Mississippi, Alabama, and western Florida, as well as in the wetland areas of Louisiana (e.g., Barataria Bay). (Barron, 2012)

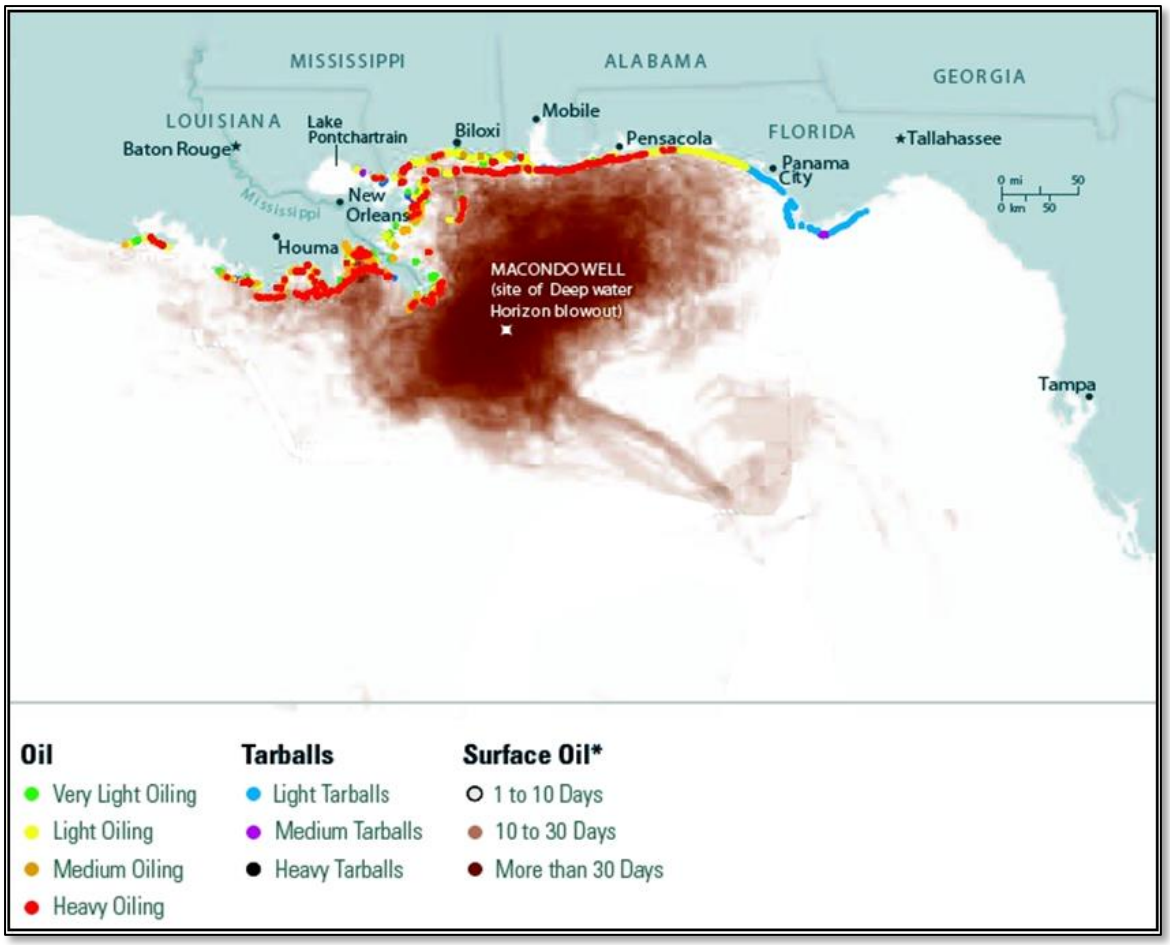


Figure 4: Cumulative Deepwater Horizon oil distribution in the Gulf of Mexico (days with oil present). Image downloaded August 2013 from www.GeoPlatform.gov/gulfresponse

It was estimated that 2 million gallons (about 8 million L) of dispersants (Corexit 9527 and Corexit 9500A®) was applied to floating oil offshore (≈ 1.2 million gal) and injected directly into the oil and gas plume at the wellhead (approx. 0.77 million gal) (Lehr et al., 2010; Kujawinski et al., 2011). The major focus was to protect the shorelines from oil contamination. This was also the first time dispersant had been applied to a deep water leaking well, primarily for safety reasons to prevent the highly flammable oil from reaching the surface immediately above the wellhead where many ships were involved in leak operations. In addition to dispersant, controlled burns, skimming, siphoning from the well-head, containment booms, shoreline scavenging, and beach sand mixing were used extensively to mitigate the spill's impact (On Scene Coordinator Report DWH spill, 2012). The BP DWH spill was the largest emergency response to a marine oil spill that the world has seen to date (Barron, 2012).

Table 1: Timeline of spill events and response actions (from Barron, 2012)

2010	Event and action
April 20	DWH rig explosion
April 25–30	Blowout preventer failure
	Leak estimates to 0.8 million L/day
	First oil reaches shorelines (Venice, LA)
	First in situ burning
May 2–7	Leak estimates to 4 million L/day
	First fishery closures
	First relief well started, 110 million L oil in gulf
	Containment dome failure/crystals
	Shoreline booming begins
May 14–18	Riser intubation 20% collection
	Fishery closures expanded
	Deep-water dispersion
	250 million L leaked
	Oil impacts mainland Louisiana
May 26–30	''Top kill'' attempted, abandoned
	380 million L leaked
	Declared largest U.S. environmental disaster
	Oiled wildlife reports increase
June 1–5	''Cut and cap'' attempted
	450 million L leaked
	Fishery closures in approximately 40% of gulf
June 9–15	Deep-water plumes detected
	Relief well drilling continues
	Leak rate estimates increased to 9.5 million L/day
June 23–30	BP response costs at $2.4 billion
	Hurricane Alex halts cleanup efforts
	Pensacola Beach oiled; 640 million L leaked
	Dispersant use continues (6.9 million L total)
July 11–16	New cap stops flow
	800 million L spilled oil
July 22–27	Evacuation from Tropical Storm Bonnie
August 2–8	''Static kill'' successful
	Relief wells continue
September 19	Well declared dead
	Cleanup continues
	Limited fishery closures

Besides, soon after the spill commenced, the State of Louisiana opened 2 diversions (with maximum discharge rates of 150-200 m^3 s^{-1}) from the Mississippi river to allow freshwater flow into Barataria Bay and Breton Sound. The main intended outcome of this operation was a countervailing force capable of preventing the flow of DWH oil into inland waters and coastal ecosystems. This diversion caused substantial drops in salinities in many normally brackish habitats (Bianchi et al., 2011).

2.2 Impacts of the DWH oil spill

2.2.1. Natural Resource Damage Assessment (NRDA)

The Oil Pollution Act of 1990, created following another spill disaster (*Exxon Valdez*), established liability for the discharge of oil to navigable waters and shorelines. The main goal of the Oil Pollution Act is to restore the natural resources and services that are lost as a result of oil spills. The responsibility for acting on behalf of the public lies with designated federal, state, tribal, and natural resource trustees (NOAA). The trustees for the DWH oil spill include the 5 affected states (Alabama, Florida, Louisiana, Mississippi and Texas) and the following federal trustees: U.S. Environmental Protection Agency (U.S. EPA), U.S. Department of Agriculture (USDA), the U.S. Department of Commerce (National Oceanic and Atmospheric Administration, NOAA) and the U.S Department of the Interior (Fish and Wildlife Service, National Park Service and Bureau of Land Management). The Natural Resource Damage Assessment (NRDA), aiming to assess injuries, damages, and restoration options for the DWH oil spill is still ongoing and has included over 90 offshore cruises, 30,000 samples of water, sediment, tissues, and oil from the deep-ocean, offshore and coastal areas (NOAA and US Department of Interior, 2011). Other activities consisted of complex modelling of oil distribution, fate and toxicity testing of multiple species. Over 7,000 km of shoreline have been surveyed for oil impacts (Fig. 4).

The present work was supported by funds provided as part of the NRDA for the DWH oil spill.

2.2.2. Oil spill impact assessment

The Deepwater Horizon oil spill was the largest environmental disaster and response effort in U.S. history (National Commission, 2011; Carriger and Barron, 2011). To assess and understand the true magnitude of the response to the spill, the Operational Science Advisory Team (OSAT, 2010), including representatives from BP and the federal agency NOAA, led several large-scale field efforts, including assessments of shoreline and wildlife oiling, as well as coastal waters and sediments (NOAA, 2011). The findings of OSAT (2010) included no deposits of liquid DWH oil beyond the shoreline, no exceedances of human health or dispersant toxicity benchmarks, and less than 1% incidence of water and sediment samples exceeding EPA aquatic toxicity benchmarks for Polycyclic Aromatic Hydrocarbons (PAHs) but none were consistent with DWH oil. OSAT (2010) concluded that the DWH crude oil was weathering, but oil degradation rates were variable. Finally,

deposits of oil found in drilling mud and PAHs concentrations exceeding aquatic toxicity benchmarks remained within 3 km of the well head (OSAT, 2010).

2.2.3. Post-spill economic assessment

In response to economic losses caused by the spill, the Gulf Coast Claims Facility (GCCF) opened in June 2010, as part of an agreement between the Obama Administration and BP to assist claimants in filing claims for costs and damages incurred as a result of the DWH oil spill. BP executives agreed to create a $ 20 billion spill response fund. The total number of people who have filed claims stands at 522,500 and as of June 2013, the total payments made from the fund amounted for $ 19.7 billion, and the remaining claims will be compensated from the company's future profits (GCCF, 2010).

One significant economic sector of the Gulf of Mexico (GoM), but difficult to assess, is the fishing industry as it requires the inclusion of both dockside losses and losses to seafood dealers, processors, and retailers. In addition, economic losses to the recreational fishing sector could prove equal to or greater than commercial fisheries losses (see § 1.3). NOAA Fisheries Service (NMFS) worked with the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), and the Gulf states to determine fishery closures in the northern GoM following the DWH spill. Closures started on 2 May 2010 in the area around the well blowout and continue to expand through the middle of July to reach 20 million ha or approximately 40% of the GoM exclusive economic zone (NMFS, 2012). Closures then decreased until 15 November when only the area around the *Macondo-1* (MC252) well was closed, equivalent to 260,000 ha (NMFS, 2010). Rather than direct effects on stocks, most of the economic losses to commercial and recreational fisheries were thus due to fishery closure. Nonetheless, the full extent of the impacts of the DWH oil itself and the responses used (e.g. dispersant, freshwater release) on fisheries will only become apparent after some years.

2.3 Chemical aspects

2.3.1 The Deepwater Horizon crude oil

2.3.1.1 Composition

Crude oils are complex mixtures containing thousands of different chemical compounds. Oils from different geological sources differ mainly in the ratios of large and interrelated families of compounds (Wang and Stout, 2007). The four classes of hydrocarbons in crude oil are saturates,

aromatics, asphaltenes, and resins: saturates and aromatics generally dominate (Leahy and Colwell, 1990). In addition, crude oils can include polar compounds containing nitrogen, sulfur or oxygen atoms (NSO compounds): the relative proportions of these compounds determine the general chemical characteristics of a given crude oil. Light Louisiana sweet crude oils such as the Deepwater Horizon (DWH) oil, is a low sulfur crude oil (Wang et al., 2003). As typical light Louisiana crude oil, the DWH oil also contains saturated n-alkanes, particularly resistant to biodegradation, (Leahy and Colwell, 1990) and polycyclic aromatic hydrocarbons (PAHs) and their alkylated homologues (alkylated PAHs), with over 50% as low-molecular-weight (LMW) hydrocarbons (methane and C_2-C_{11}) (Ryerson et al., 2011).

2.3.1.2 Weathering processes

Most petroleum hydrocarbons are highly insoluble in water. When oil enters the environment, it undergoes continuous compositional changes associated with weathering. The composition of light crude oil may result in rapid weathering in marine environments: weathering processes include evaporation, dissolution, emulsification, sedimentation, photo-oxidation, and biodegradation and generally favor LMW compounds (Landrum et al., 1987; Leahy and Colwell, 1990; Atlas, 1995; Wang and Stout, 2007, Daling et al., 2014). Fresh oil is usually more volatile, contains more water-soluble compounds, floats, is not very viscous, and easily disperses from the source (Mendelssohn, 2012), and for these reasons, is generally considered to be the most environmentally significant type of oil. On the contrary, due to the processes aforementioned, weathered oil is generally considered to have less potential for causing adverse toxic effects. The surface-collected DWH oil used during this research (Slick A) has all the characteristics of a naturally weathered crude oil, with the most volatile organic components (e.g. BTEX) mostly reduced or lost by evaporation and dissolution (Daling et al., 2014). Another weathering process which can change significantly the chemical composition of crude oil is biodegradation by hydrocarbon-eating bacteria (*Acinetobacter* and *Marinobacter*) (Atlas, 1995). Light crude oils such as DWH oil due to their higher proportion of simpler LMW hydrocarbons are more readily biodegraded than heavy crude oil. Hydrocarbon biodegradation takes place at the hydrocarbon-water interface. Thus the surface area to volume ratio of the oil can significantly impact the biodegradation rate (NRC, 2005).

2.3.2 The chemical dispersant Corexit 9500A®

2.3.2.1 Composition

Chemical dispersants such as Corexit 9500A® are complex mixtures, containing hazardous substances including petroleum distillates (solvent), propylene glycol (stabilizer), organic sulphonic acid salt or Dioctyl Sodium Sulfosuccinate or DOSS (surfactant), sorbitan and other ingredients (NALCO, 2012). These chemical compounds are used in many consumer products including food additive and personal care products, cosmetics, and household cleaning products (U.S National Library of Medicine). The primary anionic surfactant, DOSS, was selected by the trustees as the most appropriate indicator compound for Corexit 9500A® contamination in water, sediment and tissues, due to its bioactivity, extremely low volatility, and its potential to persist in the environment (OSAT/NOAA, 2010).

2.3.2.2 Mode of action

Surfactant components (DOSS) are amphipathic molecules possessing both hydrophobic and hydrophilic groups that act to decrease tension between the water and oil interface, stimulating the development of small oil-surfactants micelles less than 100 µm (NRC, 2005; Singer et al., 1996), and therefore facilitates the downward mixing of oil into the water (Canevari, 1973; Li and Garrett, 1998). Thus, by using dispersants, oil slicks can be dispersed to concentrations below toxicity thresholds limits (Lessard and DeMarco, 2000; Page et al., 2000) and become more accessible to hydrocarbon-degrading bacteria (Venosa and Zhu, 2003), enhancing the rates of biodegradation (Atlas, 1995).

2.3.2.3 Toxicity of Corexit 9500A®

Advances in dispersant formulations have improved their effectiveness over a broad range of conditions and drastically reduced earlier concern for their toxicity (Lunel et al., 1997). According to manufacturers, new generations of dispersant such as Corexit 9500A® are considered non-toxic and bio-degradable, when used on their own at recommended concentrations, at a minimum water depth of 20 m, with current speed higher than 1 $m.s^{-1}$, and at a minimum distance from the shore of 2 km (Lessard and DeMarco, 2000; Ramachandran et al., 2004; NALCO, 2012). According to MacKay (1995), maximum effectiveness of dispersion is reached at a ratio of 1 part of dispersant to 30 parts of oil, and up to 100 parts of oil if the crude is light and the sea has high energy. According to the U.S National Research Council, acute lethal toxicity of chemically dispersed oils resides not in the dispersant but primarily in the oil droplets (for some species) and the low molecular weight and dissolved, aromatic and aliphatic fractions of the oil (for most species) (NRC, 1989; Wolfe et al., 1998, 2000; Ramachandran et al., 2004). Toxicological considerations according to the MSDS

sheet indicate that none of the substances contained in the Corexit 9500A are listed as carcinogens by the International Agency for Research on Cancer (IARC), the National Toxicology Program (NTP), or the American Conference of Governmental Industrial Hygienists (ACGIH). In addition, based on their hazard characterization, the potential human hazard is low. Most of the toxicological testing of the Corexit 9500A has been conducted on rat, using acute oral, dermal and inhalation toxicity tests, or fish (e.g. inland silverside, common mummichog, trout, and turbot) using 96 h acute testing (for review, see George-Ares and Clark, 2000). Some model invertebrate species such as artemia (*Artemia salina*), mysid shrimp (*Americamysis bahia*) or copepods (*Acartia tonsa)* have also been investigated using acute toxicity testing: the lethal concentration obtained after 48 h were 20.7 mg L^{-1}, 32.2 mg L^{-1}, and 2 mg L^{-1} respectively (Hemmer et al., 2011; NALCO, 2012). In addition, toxicity tests using more conventional methods and adapted test species have been summarized by Singer et al. (1990; 1991; 1996). Four California marine species and lifestages tested included zoospores of giant kelp (*Macrocystis pyrifera*), embryos of red abalone (*Haliotis rufescens*), juveniles of the mysid (*Holmesimysis coastata*), and larvae of the topsmelt (*Atherinops affinis*). Abalone embryos revealed the greatest sensitivity to dispersant exposure (Corexit 9527) (Singer et al., 1990; 1991). Moreover, Corexit 9500 presented similar toxicity to the Corexit 9527 (Singer et al., 1996).

However, in view of the previous studies, very few data are available on the acute toxicity of the dispersant Corexit 9500A, alone or combined with crude oil, on other invertebrate species such as the American oyster *Crassostrea virginica.*

2.3.3 Polycyclic Aromatic Hydrocarbons (PAHs)

2.3.3.1 Characteristics and source in the environment

As previously stated, crude oils are complex mixtures of both hydrocarbons, such as alkanes, cycloalkanes and aromatic hydrocarbons, and non-hydrocarbon compounds. Polycyclic Aromatic Hydrocarbons (PAHs) generally refer to hydrocarbons containing at least one or more fused benzene rings, or aromatic rings (Fig. 5). PAHs are neutral, nonpolar, stable and lipophilic molecules (Varanasi, 1989). The larger compounds, with several aromatic rings, are less water-soluble and less volatile (Cerniglia, 1993). Because of these characteristics, PAHs are persistent in the environment and are found primarily in soil and sediment, but also in particulate matter suspended in air.

The main source of PAHs in the environment is in fossil fuels (oil and coal) and in tar deposits. They are natural products derived from aquatic algae laid down between 180 and 85 million years

ago, and generally produced when insufficient oxygen or other factors result in incomplete combustion of organic matter (Atlas, 1995). Their presence in the environment can result from natural processes such as wild fires, volcanic eruption, or natural oil seeping. They can also originate from human activities such as oil, coal, natural gas or wood combustion (pyrolytic origin) or from releases in the environment during extraction, transportation and accidental spills (petrogenic origin) (Neff, 1979; Readman et al., 1982; Soclo et al., 2000) (See § 3.1, Table 2). Toxicity of PAHs is structure-dependent: isomers can vary from being non-toxic to extremely toxic (e.g. benzo(a)pyrene and benzo(e)pyrene). In the present work, a sum of 50 PAHs, including parent and alkylated homologues, will be studied (see Table 3 in II.2.1). Figure 5 shows the list of the 16 PAHs compounds designated by the U.S. EPA as priority pollutants, often targeted for measurement in environmental samples.

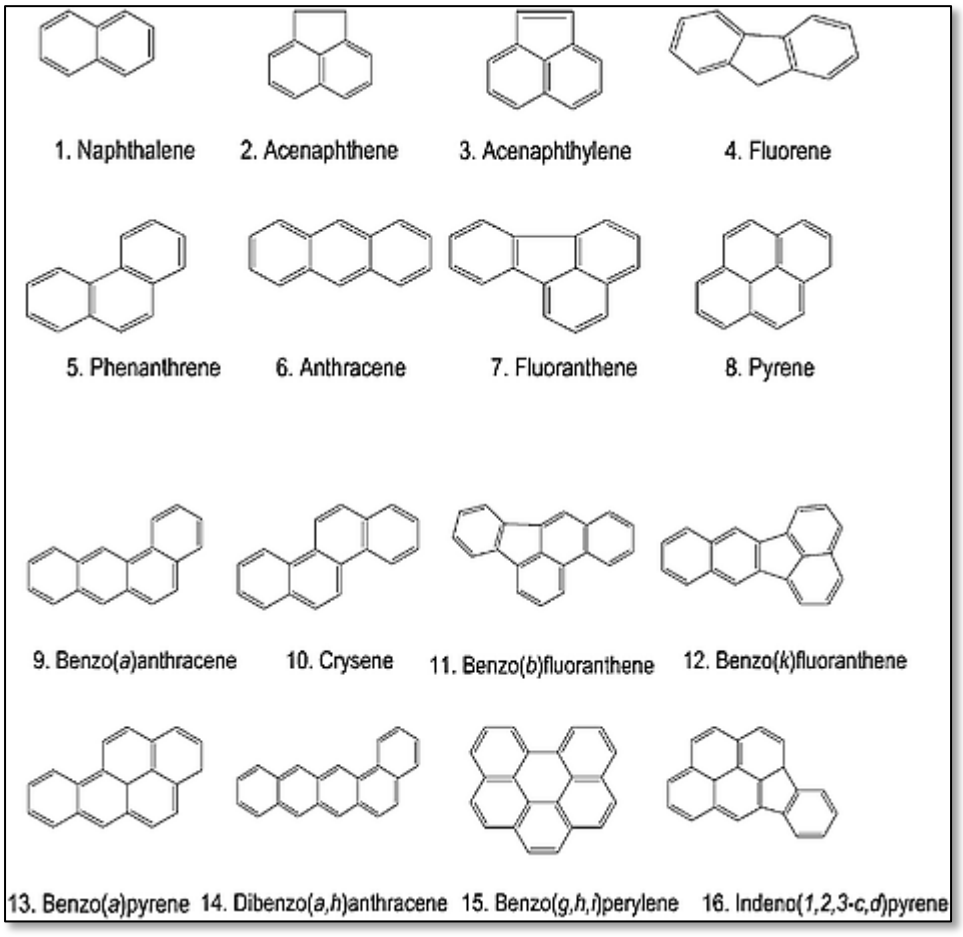


Figure 5: Chemical structure of EPA's 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs).

2.3.3.2 Bioavailability and uptake of PAHs

The fate of PAHs in aquatic ecosystems is mainly determined by (i) their molecular structure and solubility, and (ii) abiotic processes such as biodegradation, sedimentation, evaporation or

photochemical oxidation which will determine the actual concentrations of PAHs to which organisms will be exposed (Lee et al., 1978, Van Brummelen et al., 1998). Although PAHs as a group are considered to be hydrophobic, they possess a wide range of solubility (Varanasi, 1989). PAH solubility is characterized by their n-octanol/water partition coefficient (K_{ow}) (Lyman, 1990): solubility will decrease while the K_{ow} as well as the molecular weight increases (Porte & Albaigés, 1994; Djomo et al., 1996). The partitioning behavior of PAHs between sediments, overlying water (porewater), soil, particulate or colloidal matter, and dissolved organic matter is one of the major factors influencing the bioavailability of PAHs (Means et al., 1980; Readman et al., 1984; Landrum et al., 1990; Di Toro et al., 1991), and their binding affinity to these substrates is determined mainly by their hydrophobicity (Lee, 1977; Rubinstein et al., 1984). Because of this, lower molecular weight (LMW) compounds (1 or 2 benzene rings) will be preferentially dissolved while the heavier molecular weight (HMW) PAHs (3 or more benzene rings) will be adsorbed onto or associated with particles (Baumard et al., 1999).

Consequently, uptake of PAHs by organisms is governed by its bioavailability, and may take place from aqueous systems via passive diffusion through the gills or the skin, and from dietary sources via the gastro-intestinal tract (Kayal and Connell, 1990; Power and Chapman, 1992; Barron, 1995; Croxton et al., 2012). In general, elevated concentrations of PAHs in tissue can be correlated with the proximity of the organisms to the source of hydrocarbons discharge (Baumard et al., 1999). The majority of PAH body burden measurements have been made on bivalves. Given their biological characteristics (sessile, filter-feeder, benthic), bivalve can rapidly accumulate PAHs, and have little capacity for PAH metabolism (Stegeman and Teal, 1973; Neff et al., 1976; Viarengo et al., 1981; Meador et al., 1995) compared to fish which have generally low body burden of PAHs, due to their ability to metabolize PAHs (Neff, 1979; Farrington et al., 1983). Thus, contamination by high-molecular weight PAH, including those with carcinogenic potential and so of concern with regard to human consumers, is usually observed in shellfish, and particularly in bivalve mollusks. For this reason, PAHs are considered to be the best indicator of the potential toxicity of spilled crude oil to water-column and benthic organisms such as oysters (Anderson, 1977; Neff, 1979; Neff and Stubblefield, 1995).

2.3.3.3 Mechanisms of PAH toxicity: acute vs chronic

Toxicity of exposure to PAHs is generally defined as mechanisms which are responsible for negative effects on a variety of biological disruption such as growth, reproduction, and survival (Capuzzo et al., 1988). PAHs do not have one type of toxic action on aquatic organisms, but

several, which depend on the compound, the type of exposure (acute or chronic), the organism tested and the environmental conditions involved. Van Brummelen et al. (1998) postulated that PAH toxicity includes the following mechanisms:

a) Nonpolar narcosis or baseline toxicity, which is an aspecific mode of toxicity resulting from the accumulation of PAHs in biological membranes of an organism. This disturbance of the membrane structure can develop relatively rapidly during short-term exposure, and is a phenomenon typically observed in acute toxicity experiment (Van Brummelen et al., 1998).

b) Phototoxicity, where the presence of UV light increases the toxicity of certain PAHs by forming free radicals. This type of toxicity can also develop relatively quickly in short-term acute exposure. For example, anthracene, pyrene, benzo(a)pyrene, or fluoranthene are among the most phototoxic compounds whereas phenanthrene, fluorene or naphthalene do not elicit photo-induced toxicity (Landrum et al., 1987).

c) Biotransformation and subsequent adduct formation. High levels of PAHs in the tissue can cause an increase in the activity of the Mixed Function Oxygenase (MFO) system, containing the cytochrome P-450 enzymes, resulting in a rise in PAHs adducts formation. This induction will stimulate the elimination of PAHs from the tissue (detoxification) by excretion. During this enzymatic transformation, certain PAHs (usually large compounds) can be transformed into highly reactive compounds which may form covalent bonds with macromolecules such as protein and DNA, the adducts (Cavalieri and Rogan, 1998). DNA adducts may give rise to mutations, resulting in carcinogenic and teratogenic effects (Baird et al., 2005).

d) Disruption of hormone regulation. This mechanism of toxicity has been suggested by several authors and is thought to occur either by direct interaction of PAH metabolites with hormone receptors, or indirectly by interfering with hormone metabolism (Truscott et al., 1983; Payne et al., 1987). This phenomenon can only be elicited with chronic, long-term exposure to PAHs.

To conclude, a large array of studies is available in which adverse effects of PAHs on reproduction, survival or growth of bivalves have been described (see § 4.5 to 4.7). Under short-term toxicity studies (less than 96 h), nonpolar narcosis is the most likely mechanism of toxicity to be observed. Effects due to adduct formation or disturbance of hormonal regulation require long-term, chronic exposure to toxicant.

2.3.4 Use of bioassays in ecotoxicological studies

In aquatic ecotoxicology, results of ecotoxicity tests or bioassays are often used for risk assessment of substances, in order to estimate "safe" levels for the environment. Bioassays are carried out under well-defined laboratory conditions, using standardized water and organisms from laboratory stocks. After a defined time-interval (usually 48 to 96 h), the effects of the different concentrations tested are evaluated. Generally, endpoints such as survival, growth and reproduction are examined as these parameters are considered of significance for population growth. A study is deemed reliable if the design of the experiment is in agreement with standardized protocol and internationally accepted guidelines. For instance, exposure designs presented in the current study followed protocols described in "U.S. EPA. 1996. *Ecological Effects Test Guidelines: OPPTS 850.1055: Bivalve Acute Toxicity Test (embryo larval)".*

Data are usually presented as LCx/ECx for short-term tests (≤ 96-h), and LOEC for long-term tests (> 96h). The LC50 (also referred to as LD50) is the Lethal Concentration (or Lethal Dose) of a substance at which 50% of the organisms tested die. The EC50 (or ED50) is the 50% Effect Concentration, i.e. the concentration of a substance at which 50% effect occurs (e.g. instance of abnormalities, or reduction of larval growth). LCx and ECx values are often estimated applying a model to the data (e.g. sigmoid or log-logistic models) (Hamilton et al., 1977). The LOEC is the Lowest Observed Effect Concentration corresponding to the lowest dose in a series of test concentrations causing a significant adverse effect compared with the control. This threshold is often determined by comparing statistically the effect at the tested concentrations with the control (Gulley et al., 1989; His et al., 1996; Green et al., 2013). However, challenges on the use of LOEC in ecotoxicology have appeared in recent years, with a call for banning its use in favor of the x% effects concentration (ECx) (Landis and Chapman, 2011; Fox, 2012). Although the use of ECx value is appropriate in many situations, there are numerous real-world examples where it is not suitable. Some authors argue that estimation of ECx values using standard regression techniques are severely flawed, and favor the use of more biologically based models (Green et al., 2013) such as the hypothesis testing (e.g. ANOVA, t-test). In the present work, data will be presented using the 2 approaches, the regression (ECx/LCx) and the hypothesis testing analyses (LOEC).

2.3.5 Water Accommodated Fraction as an exposure solution

In order to assess the potential aquatic toxicity of the DWH crude oil, it is necessary to understand the types and concentrations of chemicals that are likely to dissolve into the water, because these dissolved compounds are what the organisms would be likely exposed to. As we mentioned

previously (§ 2.3.3.2), PAHs are characterized by low to very low solubility and therefore special attention must be paid to the way the exposure solutions are prepared. In the natural environment, prolonged turbulent mixing of crude oil by wind, currents and waves can result in the release of Water Accommodated Fraction (WAF) (Rossi et al., 1976; Barron et al., 1999). The "WAF approach" was chosen to provide the best estimate of what may solubilize from the oil into the overlying water (Singer et al., 1991; Barron et al., 1999; Singer et al., 2000). The methods reported in the literature to produce the WAFs are varied. Singer et al (2000) have noted a number of factors that influence the type and concentrations of constituents in WAF, such as initial amount of oil (loading rate), mixing and settling time, mixing energy, and the type of oil used in the WAF preparation. Consequently, different preparation methods can result in different toxicities of WAFs prepared from similar oils. To minimize the variability between research groups and make results more directly comparable, some researchers attempted to standardize testing conditions and created the CROSERF (*Chemical Response to Oil Spills: Ecological Effects Research Forum*). Utilizing the methods from Singer et al. (1991), CROSERF also sought to standardize a suite of marine test organisms (sensitive early life stages) and the preparation of both the water-accommodated fraction of crude oil (WAF) and the chemically-enhanced water accommodated fraction (CEWAF via Corexit 9500A) (Singer et al., 2000). In the same way, the method of preparation of WAF adopted in the current study followed procedure detailed in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (Version 4; February 4, 2014; Appendix A, pp A1-A9), NOAA. (see II.1: Material and Methods). WAF toxicity is commonly assessed through measurements of early larval growth, survival, and morphological abnormality in marine organisms such as fish (Couillard et al., 2005), starfish (Davis et al., 1981), urchins (Saco-Alvarez et al., 2008), crustacean (Cucci and Epifanio, 1979) or bivalves (Fucik, 1994; Saco-Alvarez, 2008).

3. Petroleum pollution: a review

3.1. Sources of oil in the marine environment

Following years of study and review of many sources of information, the National Research Council (2003) categorized all petroleum input into the sea into 4 categories: natural seeps, petroleum extraction, petroleum transportation, and petroleum consumption. Table 2 summarizes the average annual releases of oil into the environment by source categories, from 1990 to 1999. Natural seeps dominate all three of the geographic categories. In the Gulf of Mexico (GoM) during the 1990s decade, 82% of the input from coastal and offshore sources came from natural seeping.

However, these figures will change, particularly in the GoM, for the next decade due to the DWH oil spill event.

Table 2: Average, annual releases (1990-1999) of petroleum by source (in million of gallons) (NRC 2003)

Source	Gulf of Mexico	North America	Worldwide
Natural Seeps	43.1 (82%)	49.6 (63%)	184.7 (83%)
Extraction of Petroleum (platforms, atmospheric deposition, produced waters)	0.8 (2%)	0.9 (1%)	11.7 (5%)
Transportation of Petroleum (Pipeline spills, tanker spills, operational washings, coastal facility spills, atmospheric deposition)	1.3 (2%)	2.8 (4%)	6.3 (3%)
Consumption of Petroleum (Land based, recreational, operational discharges, atmospheric deposition, aircraft fuel)	7.1 (14%)	25.9 (33%)	20.2 (9%)
Total	**52.3**	**79.2**	**222.9**

3.2 Significant oil spills in the marine environment

3.2.1. Torrey Canyon and *Santa Barbara* spills

In 1967, the grounding of the Liberian oil tanker, the *Torrey Canyon*, off the coast of Cornwall, UK, made Europe aware of a risk which had been neglected, and gave birth to the first elements of European policies of prevention and response against oil spill disasters. About 120,000 t of Kuwait crude oil were spilled and some 1.6 million L of surfactants (alkylphenols) and solvents (aromatic hydrocarbons) were applied for 14 days following the release (NRC, 1989): this was the first time that such quantities of dispersant were used as a response to an oil spill (CEDRE, 2013). Unfortunately, impact studies indicated that some of the dispersants used were even more toxic than the oil itself (Southward and Southward, 1978) and led to substantial environmental damage (Lessard and DeMarco, 2000). In 1969, the blowout of a *Santa Barbara* platform in California

introduced in the modern era of oil spill concern and awareness, prompting planning for oil spill response, clean up, contingency plans, and studies of major oil spills. As a result, Earth Day was created in 1970 and the Clean Water Act was passed in 1972 in part because of the Santa Barbara spill (Anderson et al., 2014).

3.2.2. Amoco Cadiz

On March 1978, the oil tanker *Amoco Cadiz*, transporting 227,000 t of a mixture of Arabian and Iranian light crude oils, suffered a mechanical failure and ran aground rocks on the northern coast of Brittany, France, and contaminated about 350 km of shoreline (Berthou et al., 1987). This was the largest oil spill caused by a tanker grounding ever registered in the world. The consequences of this accident were significant and led to the creation of oil response plan by the French government and the imposition of traffic lanes in the Channel (CEDRE, 2013). After 14 years of complex proceedings and environmental impact assessment and monitoring, the French government finally obtained about 200 million euros in compensation, less than half of the claimed amount (CEDRE, 2013). However, this was the first time a long-term ecological survey was undertaken using many aquatic organisms, in particular oysters (Neff and Haensly, 1982; Balouet et al., 1986; Berthou et al., 1987).

3.2.3. Ixtoc 1

The most appropriate major spill for the Gulf of Mexico and probably the most similar to the DWH event is the *Ixtoc 1* spill. This platform blowout in the southern Gulf of Mexico, Bay of Campeche, 50 miles from the coast, was very similar to the DWH oil spill. The *Ixtoc* spill started in June 1979 and ended in March 1980, lasting almost 10 months and discharging about 550 million L of crude oil (CEDRE, 2013). The burning platform sank several days after the blowout, and similar clean up strategies (burning, dispersant, and skimmers), as well as containment strategies (top kill, relief wells) were used (Jernelov and Linden, 1981). Apart from lasting for a much longer period of time than the DWH oil spill, the main differences were the water depth, about 50 m for *Ixtoc* compared with 1500 m for the DWH, and the deepwater application of dispersant.

3.2.4. Exxon Valdez

When the *Exxon Valdez* tanker ran aground in 1989 and spilled almost 42 million L of Alaskan crude oil in the pristine waters of the Prince William Sound, Alaska, another era began in dealing with marine oil spills (Moore, 1994). In 1990, the US Oil Pollution Act was passed and the notion of responsible party and payment for recovery of damaged environments and economies, the Natural Resource Damage Assessment program (NRDA), were established in the U.S. Until the

DWH oil spill, the *Exxon Valdez* was the largest and the most significant spill in U.S history (NRC, 2005).

4. The Eastern oyster, *Crassostrea virginica*: model organism chosen for this study

4.1 Biological characteristics

4.1.1 Taxonomy and geographical distribution

4.1.1.1 Taxonomy

The Eastern oyster or American oyster, *Crassostrea virginica* (Gmelin, 1791), is classified in the following taxonomy:

Kingdom: Animalia

Phylum: Mollusca

Class: Bivalvia

Order: Lamellibranchia

Family: Ostreidae

Genus: *Crassostrea*

Species: *virginica*

4.1.1.2 Distribution

Crassostrea virginica is common in estuaries and coastal areas in the Western Atlantic, from the Gulf of St. Lawrence in Canada to the Gulf of Mexico, Carribean, and along the coasts of Brazil and Argentina (Galtsoff, 1964; Newball and Carriker, 1983; Garcia-Cubas et al., 1987; Andrews, 1991). It has been introduced to the west coast of North America, Hawaii, Australia, England, Japan and possibly other areas, but has not generally become established there (Quayle, 1988; Arakawa, 1990).

4.1.2 General anatomy

4.1.2.1 Eastern oyster adult

The Eastern oyster, given its wide range of geographical distribution and habitat, is a euryhaline species and can survive in waters with a broad range of salinity and temperature (Galtsoff, 1964).

a/ Physiology of feeding and digestion

Oysters are filter-feeders, drawing water in over their gills through the beating of cilia. They feed on suspended phytoplankton, organic matter, sediment, or bacteria (Newell and Jordan, 1983; Mann, 1988; Crosby et al., 1990). The large gills or ctenidia filter food particles from the water and direct it to the labial palps, surrounding the mouth (Fig. 6). Food particles are then sorted and transported to the mouth where they are ingested. A short esophagus leads from the mouth to the stomach, which is surrounded by the digestive diverticulum or digestive gland. The digestive diverticulum is the organ of absorption and intracellular digestion: it consists of a series of blind-ending tubules which communicate with the stomach by way of partially ciliated main ducts and non-ciliated secondary ducts (Owen, 1973). Food material are carried from the stomach to the ciliated digestive ducts, and then transferred to the lumina (or lumen) of the digestive tubules (Owen, 1973). From there, digested material is transported to the intestine and the rectum where it is excreted as feces (Fig. 6). Oysters feed generally most actively at temperature above 10 ˚C (Galtsoff, 1964).

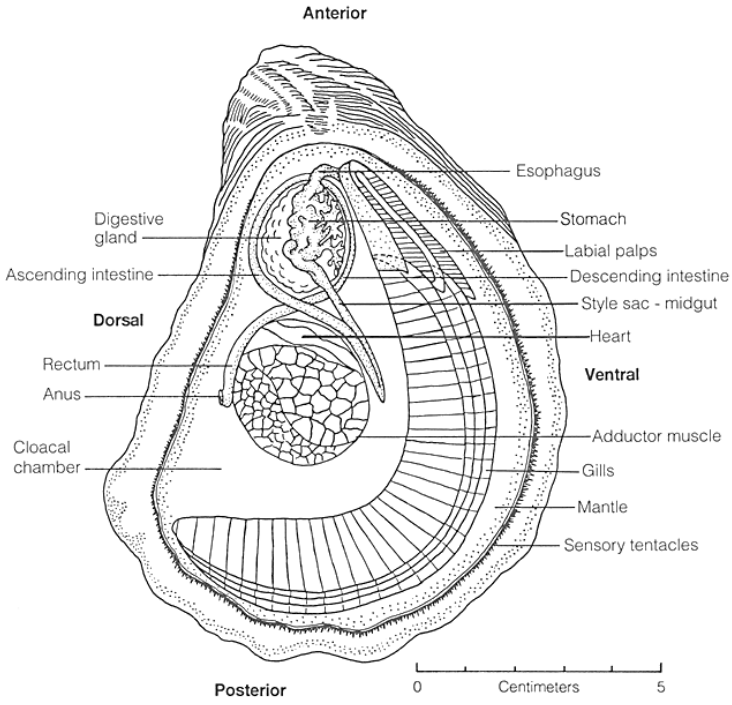


Figure 6: Diagrammatic representation of an adult *Crassostrea virginica*, in left valve, showing digestive system including labial palps and gills. Re-drawn from Galtsoff (1964).

b/ Immune system

A small, three-chambered heart, lying under the adductor muscle, pumps blood called hemolymph (Fig. 6). Oysters have an open circulatory system, in which hemocytes (blood cells) are not confined to the interior of the heart and vessels but also occur in sinuses and tissues (Cheng, 1996). Hemocytes are involved in many functions, including digestion and nutrient transport (Owen, 1966; Cheng and Garrabrant, 1977; Feng, 1977), wound healing (Pauley and Sparks, 1965), shell repair, excretion, and more importantly internal defense (Cheng, 1981; Fisher and Tamplin, 1988, Feng, 1988). In addition to the first line of defense, which consists of barriers such as the shell or mucous layer, phagocytosis, via circulating hemocytes, has been described as the main mechanism of cellular defense in oysters (Cheng et al., 1975; Galloway and Depledge, 2000). Many environmental chemicals such as oil/PAHs have been shown to affect the immune system of oysters and trigger phagocytosis process resulting in encapsulation and elimination of xenobiotics (Fisher and Tamplin, 1988; Sami et al., 1992; Luna-Acosta et al., 2011).

4.1.2.2 Eastern oyster gamete

a/ Spermatozoa

Sperm of *C. virginica* are about 40 µm long, with the head and middle piece measuring together about 2 µm. The head, middle piece, and tail are enclosed by a plasma membrane. The head consists of the nucleus, the axial body, the axial rod, and the acrosome (Fig. 7). The nucleus consists of thin DNA filaments and occupies most of the head, and is capped by the acrosome. The middle piece of each sperm consists of 4 laterally arranged mitochondria (Galtsoff, 1964). Mitochondria are organelles that generate most of the cell supply of energy, the ATP, necessary for the motility of sperm cells. Lastly, the tail of spermatozoa consists of a long flagellum, about 40 µm long (Fig. 7). Fertilization capacity of sperm cells or capacitation requires an activation of the motility of the flagellum and an induction of the acrosome reaction, which results in the binding and penetration of the spermatozoa into the membrane of the oocyte, initiating fertilization (Galtsoff, 1961; Hylander and Summers, 1977; Buffone et al., 2012).

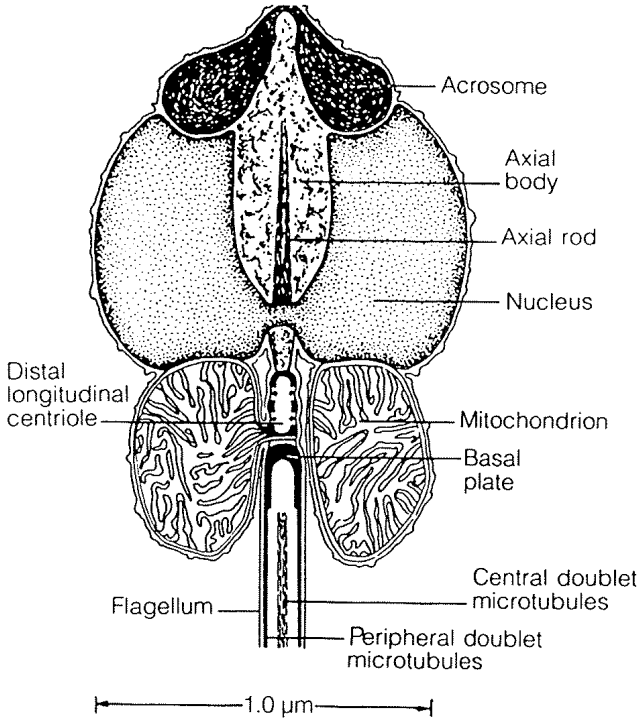


Figure 7: Fine structure of longitudinal section of sperm head, middle piece, and proximal portion of tail. From Galtsoff (1964).

b/ Oocytes

Eggs or oocytes of *C. virginica* are generally 50 to 60 µm in diameter. They have a typical "tear drop" shape and become round after hydration in seawater. Like many other aquatic organisms, oyster oocytes contain high levels of lipids: polar lipids (e.g. phospholipids) which function mainly as structural component of the membrane, and neutral lipids (e.g. triglycerides) which function as energy reserves (Trider and Castell, 1980; Thompson et al., 1996). Oocytes consist of a nucleus, a cytoplasm containing yolk granules and lipid globules, numerous mitochondria, and free ribosomes, and the plasma membrane, also called vitelline envelope (Daniels et al., 1973).

4.1.2.2 Eastern oyster larva

a/ Anatomy and physiology

The oyster larva contains specialized organs not present in the adult that are adapted to its great surface-to-volume ratio, motile lifestyle, and need to attach to a surface at metamorphosis. The length of the larval period varies depending on species and temperature but is typically in the range of 14 to 20 days for *Crassostrea virginica* larvae under culture conditions (Carriker and Palmer 1979; Bahr and Lanier, 1981).

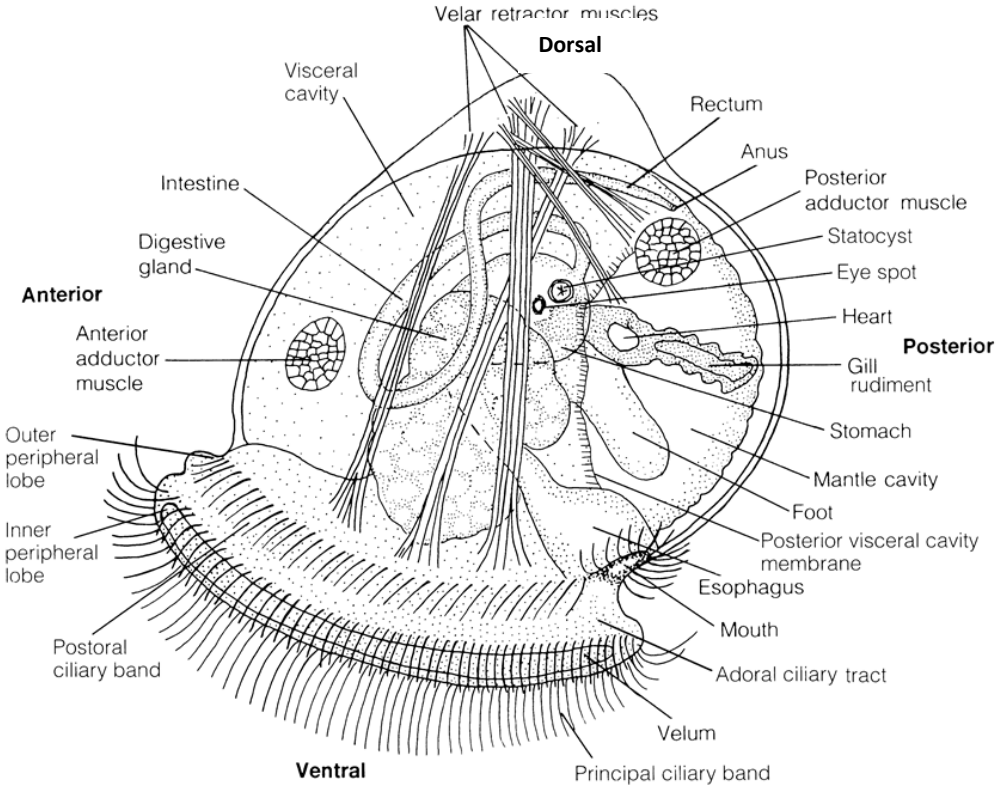


Figure 8: Diagrammatic illustration of prodissoconch II veliger larva viewed from the left (X 380). Based on drawings by Galtsoff (1964) and Elston (1980).

The shell of the larva consists of aragonite and has a shape similar to that of other bivalve species, which appears to diverge from this early similar morphology as they further develop. As in the adult oyster, beneath each valves of the shell lies the mantle, which is a thin sheet of tissue near the periphery but is fused to the visceral mass at the central and proximal aspects. In the larval stage, the visceral mass appears to have two prominent components: the velum, anchored to the visceral mass at its ventral aspect, and the fluid–filled visceral cavity containing the visceral organs (Elston, 1980) (Fig. 8). Velum consists of a primary ciliated ring composed of highly specialized columnar epithelium, which is the prominent feature of this organ (Erdmann, 1935). This organ has three functions: respiration, swimming and nutrition. The visceral cavity is essentially a fluid-filled bag containing the digestive organs (namely the mouth, esophagus, stomach, style sac, digestive gland, and intestine or midgut), hemocytes, the developing foot, and the adductor and retractor muscles (Elston, 1980) (Fig. 8).

b/ Larval nutrition

The egg yolk contains only sufficient protein and lipid reserves for embryos to develop essential tissues including feeding organs and a gut (endotrophic stage). Once this functional alimentary system develops, planktotrophic larvae must rely on exogenous food sources in order to grow

(exotrophic stage) (Newell and Langdon, 1996). Oyster larvae typically obtain food, such as phytoplankton, detritus or bacteria, by removing particles from the water column using their ciliated velum (Baldwin and Newell, 1991). As previously mentioned, the velum functions as an organ for both swimming and capturing food; it is also the principal site for absorption of dissolved organic material in seawater (Langdon and Newell, 1996). The mouth and alimentary system develop fully, allowing ingestion and assimilation of food particles. Generally, rates of particle clearance for molluscan larvae are proportional to the length of cirri on the principal band of the velum (Fig. 8) (Strathmann, 1987). The size range of food particles ingested by oyster larvae varies generally from 1 to 30 μm (Baldwin and Newell, 1991; Robert et al., 2004; Ponis et al., 2006), with microalgal species ranging in size from 3 to 10 μm supporting maximum larval growth (Webb and Chu, 1982).

4.1.3 Life cycle

4.1.3.1 Spawning

The early life history of the eastern oyster, which involves a number of developmental stages, can be summarized as follows. Adult eastern oysters are most common in estuaries, where they reproduce by releasing gametes into the seawater column at salinities above 5 to 10 PSU (Stanley and Sellers, 1986). Many adults will mature in their first year of life as males. As they age, an increasing percentage may switch sex and become female: this is known as protandric hermaphrodism. Gonads are located around the visceral mass, between the mantle and the digestive gland. Production of eggs and sperm is called gametogenesis, and is greatly influenced by the size of the adult, the temperature and the quantity and quality of the food. When the gonad is fully mature or ripe, gametes are released in the water where external fertilization occurs (Kennedy, 1996). In the northern part of the Gulf of Mexico, oyster spawning season typically occurs from mid-spring through late fall (Ingle, 1951; Hayes and Menzel, 1981), when water temperature is above 25 ˚C (Stanley and Sellers, 1986; Menzel, 1991).

4.1.3.2 Larval life: from fertilization to settlement

Eggs undergo meiotic division at fertilization and two polar bodies are released: cell division begins and within 30 min post fertilization, the egg divides into the two-celled stage. Within 12 h, the embryo passes through the multi-celled blastula and gastrula stages, and has developed into a motile and non-feeding trocophore. This form is followed by the prodissoconch I stage, commonly

referred to as the straight hinge stage, "D-larva" or veliger. Larvae continue to swim, feed and grow and within a few days, they develop into the prodissoconch II stage, often referred to as the late veliger or "umboned" larva (Fig. 9). These various larval stages are planktotrophic, grazing on phytoplankton, detritus, and bacteria. Near the end of the larval period, the oyster larva or pediveliger develops dark circular dots on each valve, the eyespot, a prominent foot containing the byssal gland, and gill rudiments. Between periods of swimming activity, "eyed" larvae sink to settle and use their foot to crawl on a substrate. When a suitable substrate is found, the larva is ready to metamorphose and begins its benthic existence as a spat or dissoconch (Kennedy, 1996) (Fig. 9). In the Gulf of Mexico, two peaks of settlement typically occur in early summer and late summer (Supan, 1983).

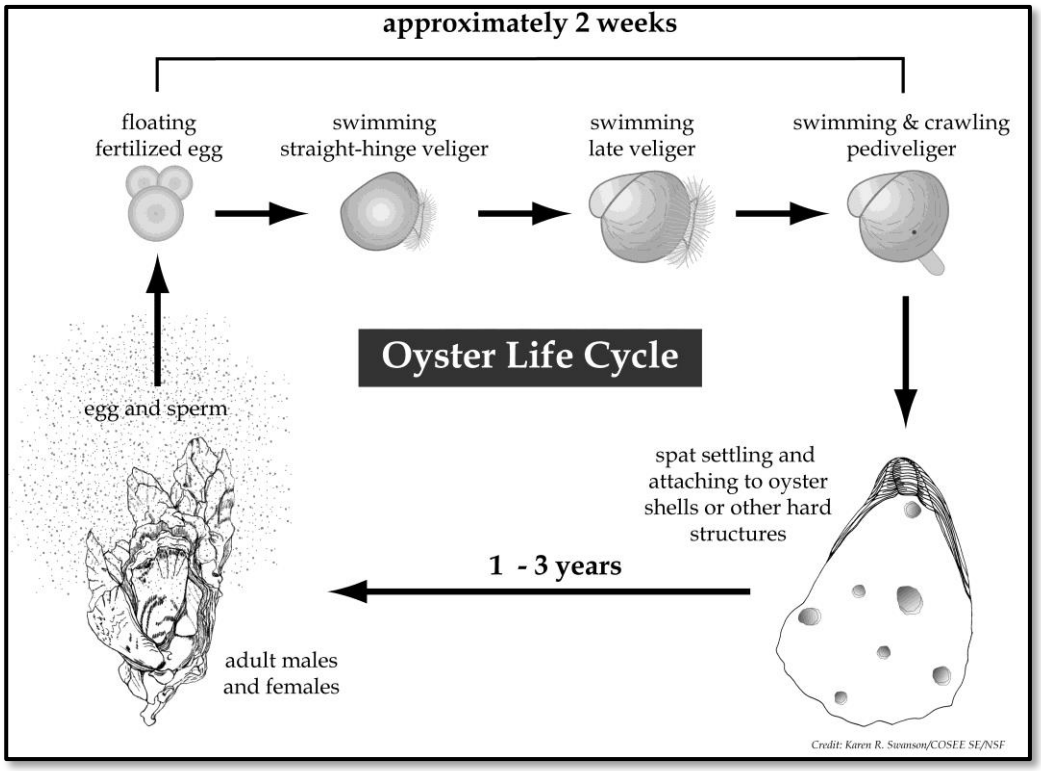


Figure 9: Eastern oyster life cycle (from COSEE SE/NSF)

4.2 Economic significance of oysters in the Gulf of Mexico

The Gulf of Mexico leads the U.S. in the production of *Crassostrea virginica*, producing in 2008 about 67% of the national total, including 9.4 million kg and $60.1 million (NMFS 2012). Louisiana leads the harvest with 5.8 million kg and $38.8 million. Following Louisiana in harvest is: Texas at 1.3 million kg and $8.83 million; Mississippi at 1.2 million kg and $6.87 million; West

Florida at 1.2 million kg and $5.47 million; and, Alabama at 33,000 kg and $243,414 (NMFS 2012). Harvest methods for oysters include hand picking in shallows, tonging from boats, and dragging or dredging from boats. Most Gulf landings are from publicly owned oyster beds but about 30% of the harvest is from privately leased beds (Mackenzie, 1989). Commercial fishery regulations for oysters vary from state to state, but all oysters harvested must be at least 3 inches (76 mm) in length according to the Gulf States Marine Fisheries Commission. Depending on location and local growth rate, oysters are harvested at about 1.5 - 2 years of age and live up to about 6 years (Galtsoff, 1964).

In 2012, overall harvests of *C. virginica* in the Gulf of Mexico were at similar levels to those before the DWH oil spill (≈ 9.4 million kg). However, in Louisiana for instance, total landings have decreased between 2008 and 2012, from 5.82 million kg to 4.9 million kg (NMFS, 2012).

4.3 Ecological significance of oysters in the Gulf of Mexico

Oyster reefs have been compared to coral reefs in terms of their structural heterogeneity (Harding and Mann, 1999). Their complex 3-dimensional structures create habitats for various macrofauna species, and serve as spawning substrate for many fish species. In addition, oyster reefs provide refuges from predation for many species (Tolley and Volety, 2005). Furthermore, shells that comprise oyster reefs form the primary settlement material for subsequent generations of eastern oysters in sediment-dominated estuaries enhancing directly oyster biomass (Coen et al., 2007; Volety et al., 2014).

Another ecological service provided by oyster reef concerns the improvement of water quality and the mitigation of eutrophication in estuaries: given their enormous filtration capacity, estimated to up to 50 gal per oyster per day, it is clear that oysters have the ability to alter water quality in eutrophic temperate estuaries found along the Gulf coast (Newell, 2004). Additionally, the consumption of particulate organic matter by oysters may significantly reduce re-mineralization on the ecosystem floor, hence controlling phytoplankton blooms and reducing hypoxia (Coen et al., 1999, 2007; Newell, 2004). Lastly, oyster reefs can stabilize benthic or intertidal habitats such as marshes, and influence water circulation patterns within estuaries (Wells, 1961; Newell, 2004; Volety et al., 2014).

4.4 Why this species was selected for this study?

Crassostrea virginica was selected as model organism for the following reasons:

• Native species with a commercial significance for the Gulf region (§ 4.2),

• Short-lived animals with an ecological significance for the Gulf (§ 4.3),

• Larvae are pelagic and filter-feeding (§ 4.1.2.2), hence likely vulnerable to oil pollution,

• After settlement, oysters are sessile and benthic,

• Have a reasonably high tolerance to abiotic factors (T˚C, S‰, pH…),

• Great filtering capacity combined with low capacity to metabolize toxic compounds potentially increase uptake and bioaccumulation in tissue (§ 2.3.3.2),

• As primary consumers, they are at the base of the food chain: risk of trophic transfer,

• Broadcast spawners with external fertilization (§ 4.1.3.2),

• Reproduction and larval rearing are reliable, standardized and well developed techniques,

• Extensive ecotoxicological data are available for this organism (§ 4.7).

From all these characteristics, *Crassostrea virginica* was chosen as the ideal candidate to evaluate the toxicity of the Deepwater Horizon oil spill in the present study.

4.5 Oyster and oil pollution: early works

Perhaps the first most complete study on the impact of crude oil on *Crassostrea virginica* was conducted by Galtsoff et al. (1935). Before that, most of the research was focused on the effects of oil and water-gas tar on aquatic life and particularly fish (Rushton and Jee, 1923; Lane et al., 1926; Roberts, 1926; American Petroleum Institute, 1933). Only a few studies were performed using oysters as a model organism (Leenhardt, 1925; Gowanloch, 1934). Massive mortality of oysters in Louisiana waters in 1932-1933, coincident with the development of oil and gas wells in the coastal region of the Gulf of Mexico, brought up great concerns about oil pollution and its effects on marine organisms. From their study in the laboratory, Galtsoff et al. (1935) demonstrated that crude oil did not induce significant mortality of oysters exposed for 6 to 8 weeks to a floating oil slick. Nonetheless, they showed that crude oil could reduce the feeding rates of oysters by narcosis of the ciliated epithelium of the gills (Galtsoff et al., 1935).

4.6 Oyster: a sentinel organism

The 1970s will see the beginning of a widespread research effort focusing on the effects of crude oil on bivalve species, and particularly on mussels and oysters. Oil components such as PAHs are highly resistant to degradation in the environment, and may accumulate in animal tissues and interfere with normal metabolic processes that affect growth, development, and reproduction (Capuzzo et al., 1988). Due to their biological characteristics, oysters are effective in taking up hydrocarbons via filtration and ingestion of particulate matter, and may accumulate them in their tissue to high concentrations, and thus be susceptible to the negative effects of these pollutants. For this reason, environmental research in the U.S. has suggested that mussels and oysters may be valuable as sentinel organisms for indicating levels of pollutants in estuarine and coastal marine waters on a worldwide scale: the U.S. Mussel Watch was born (Goldberg, 1975; Goldberg et al., 1978; Farrington et al., 1983). Meanwhile, off the coast of Brittany, the *Amoco Cadiz* ran aground and caused the largest oil spill ever registered in the world (Berthou et al., 1987). On account of the extensive damage caused by this disaster, many research and monitoring programs were immediately carried out to determine the long term effects of oil exposure on oysters (Balouet & Poder, 1981; Neff & Haensly, 1982; Balouet et al., 1986; Berthou et al, 1987). Berthou et al. (1987) found that 7 years after the spill, oysters from the field were still contaminated with oil. They also described a rapid accumulation of hydrocarbons which was followed by an initial rapid loss of alkanes and low molecular weight hydrocarbons, but heavier molecular weights hydrocarbons were persistent in the tissues. Their observations were consistent with other studies which related this two-phase depuration with the mobilization of lipid reserves (Neff, 1979; Farrington et al., 1982). Generally, biological effects of oil can be manifested at biochemical, cellular, and organismal levels before disturbances at the population level develop (Capuzzo, 1988). As described in § 2.3.3.3, adaptive responses such as resistance or metabolization of toxicants can exist in nature (Capuzzo, 1996). Consequently, all responses are not disruptive and do not necessarily result in deleterious impact at the next level of organization.

4.7 Use of oyster larvae in ecotoxicology

4.7.1 The bivalve embryo-toxicity assay

Early life stages are more sensitive than adults and represent a critical period in the life cycle of a marine organism such as oyster (Thorson, 1950; Connor, 1972; Chapman and Long, 1983; His and Robert, 1985; His and Beiras, 1999). For this reason, in order to assess rapidly water quality, Woelke (1972) proposed for the first time a standard method for conducting 48-h bioassays using *Crassostrea gigas* embryos. Consequently, early stages of oyster (embryos or larvae), due to their small size, high number produced in a spawning event and the extensive information on their culture in the lab, were selected as a reliable model organism in ecotoxicological studies to assess petroleum hydrocarbon pollution (Loosanoff and Davis, 1963; Renzoni, 1975; Chapman and Morgan, 1983; Chapman and Long, 1983; Chapman, 1989; Thain, 1992; Fucik, 1994; His et al., 1996; 1997; Geffard et al., 2001, 2002b, 2003).

The standard bivalve embryo toxicity assay consists of exposing bivalve embryos after fertilization to contaminants or effluents, typically for 48 h (Woelke, 1972; Chapman and Long, 1983; Chapman, 1989). The usual endpoint or response measured after 48 h of exposure is the percentage of abnormal (including deformed or absence of shells, or developmental failure) and dead larvae (Chapman, 1989; His et al., 1996). It is generally expressed as a median effective concentration resulting in 50% abnormal larvae (EC_{50}) or median lethal concentration (LC_{50}) (see § 2.3.4).

4.7.2 Oil/PAHs toxicity on oyster larvae

Although petroleum is one of the most studied pollutants of the aquatic environment and oyster species have been studied extensively in ecotoxicology, literature on the toxicity of crude oil and PAHs on early life stages of *Crassostrea virginica* is relatively scarce. Several authors have reported that acute exposure of early life stages of oysters to oil and/or PAH could result in reduced fertilization success, abnormal embryonic development, growth and ultimately increased mortality (Renzoni, 1975; Lyons et al., 2002; Geffard et al., 2001, 2002b, 2003; Jeong & Cho, 2005; Wessel et al., 2007). Early work from Renzoni (1975) found that, after exposing *C. virginica* sperm and eggs to different type of crude oils for 1 h before fertilization, fertilization was depressed in a dose-dependent way, with rates falling to 60-70% at 1,000 ppm. Similarly, Le Gore (1974) investigated the effects of Alaskan crude oil in developing embryos of *C. gigas* and concluded that oil induced severe abnormalities, with $EC50_{48h}$ value of 1,000 ppm of oil. Sigler and Leibovitz (1982) studied the effects of bilge cleaner alone and combined with No. 2 Fuel oil on *C. virginica* larvae and reported significant larval mortality within 24-h of exposure, with LC50 values ranging between 1 and 5 ppm. Jeong and Cho (2005) demonstrated that concentration of 10 individual PAHs from 50

to 200 µg L^{-1} could affect fertilization capabilities of *C. gigas* sperm and impact fertilization success and larval development. Another study by Wessel et al. (2007) indicated that oyster embryos, exposed to a mutagenic and teratogenic PAH, benzo(a)pyrene, showed abnormal development due to DNA strand breakage.

The use of PAH-contaminated sediment as a mode of exposure of oyster larvae has also been investigated by several authors (Chapman and Morgan, 1983; Phelps and Warner, 1990; Thain, 1992; His et al., 1997; Geffard et al., 2001; 2002b, 2003). Geffard et al. (2001) demonstrated that PAH released by contaminated sediment can have a deleterious effect on oyster embryos, and that embryotoxicity assays were more sensitive to PAHs than spermiotoxicity assays, resulting in less abnormal *C. gigas* larvae. They also reported that larval growth assay was more sensitive than the embryotoxicity assay (Geffard et al., 2002b). Besides, they found that some fraction of oil could be adsorbed on particle and become bioavailable to oyster larvae (Geffard et al., 2002b). Finally, they revealed that only the soluble fraction of PAHs was accumulated by oyster embryos, and the critical body burden of PAHs above which abnormalities were observed was 0.3 µg g^{-1} (Geffard et al., 2003). Despite the fact that oyster metamorphosis bioassay has been proved to be a rapid, sensitive and reliable method in toxicological assessment (Phelps and Warner, 1990), very few studies have used it as a biological response to study oil/PAH toxicity on oyster larvae. His et al. (1997) examined the toxicity of sediment-associated PAH on pediveliger stages of *C. gigas* and revealed a drastic reduction of metamorphosis, a critical process in the early life of oyster.

Finally, it has been often demonstrated that toxicity of oil and particularly certain PAHs is highly related to abiotic factors such as UV-radiation, temperature and salinity: for instance, Lyons et al. (2002) reported that photo-induced toxicity of specific PAHs (Pyr and BaP) could completely inhibit the normal development of *C. gigas* embryo and D-larvae at concentration of 5 µg PAH L^{-1}. In the same way, Jewell (1994) found that toxicity of individual PAHs (Benzene, Naphtalene) was highly dependent of temperature and salinity which affected directly its solubility. Concentrations of PAHs ranging from 10 to 100 ppm induced mild to severe abnormal development of oyster embryos.

4.7.3 Dispersant toxicity on oyster larvae

One of the first studies on dispersant toxicity was probably that of Hidu (1965): he investigated the effects of synthetic surfactants on the larvae of hard clams (*Mercenaria mercenaria*) and eastern oysters using embryotoxicity and larval growth assays. He found that larval growth was the most

sensitive endpoint compared to embryonic development, and that concentration of surfactant of 10 ppm could be deleterious. Very little information exists on the effects of Corexit dispersants on oyster larvae. In fact, most of the studies have been conducted on toxicological model of crustacean species such as mysid shrimps, amphipods, artemia, rotifers, or fish species such as inland silversides or trout (review from George-Ares and Clark, 2000; § 2.3.2). Fucik (1994) reported significant toxicity of Corexit 9527 on eastern oyster embryos with $LC50_{96h}$ value of 4.9 mg L^{-1}. Another study by Bragin and Clark (unpublished) found similar results with *C. gigas* embryos exposed for 48 h to Corexit 9527, with $LC50_{48h}$ ranging from 3.1 to 13.9 mg L^{-1}. In light of the limited knowledge on dispersant toxicity on *Crassostrea virginica* larvae, additional research looking at the acute effects of Corexit 9500A is crucial.

However, due to the variability of methodology used (e.g. different types of dispersant and crude oil tested, species and life stage exposed, duration of exposure, continuous or declining exposure, static *vs* flow-through system used, mode of preparation of WAF), the effective and lethal concentrations reported in the literature can be very variable, hence difficult to compare to a reference database. For example, Fucik (1994) demonstrated that eastern oyster embryos were adversely impacted by acute exposure to dispersed oil, with $LC50_{96h}$ values of 4 mg L^{-1} of total Hydrocarbons Petroleum (TPH). The difference of unit (TPH), crude oil (Gulf oil) and dispersant type (Corexit 9527) used in his study impedes comparability with other studies. As a result, it is essential that further effort should continue to support standardization of methods and procedures that would allow greater comparability and reproducibility of toxicological data.

Furthermore, as we previously described in § 2.3.4, the most common way of assessing the toxicity of a contaminant, is to determine a median lethal concentration (LC50). However, this method has shown limitations even though it can be a useful proxy for biological impacts of pollutant. A more ecologically sophisticated method, including an integrative approach using more sensitive indicators of population health (e.g. fertilization success, embryogenesis, growth, settlement success, feeding regime) would provide more appropriate input into post-spill impact assessments.

Objectives

The main goal of this study was to evaluate, in the laboratory, the toxicity of crude oil released during the *Deepwater Horizon* oil spill as well as dispersant (Corexit 9500A) on early life stages of the Eastern oyster, *Crassostrea virginica*.

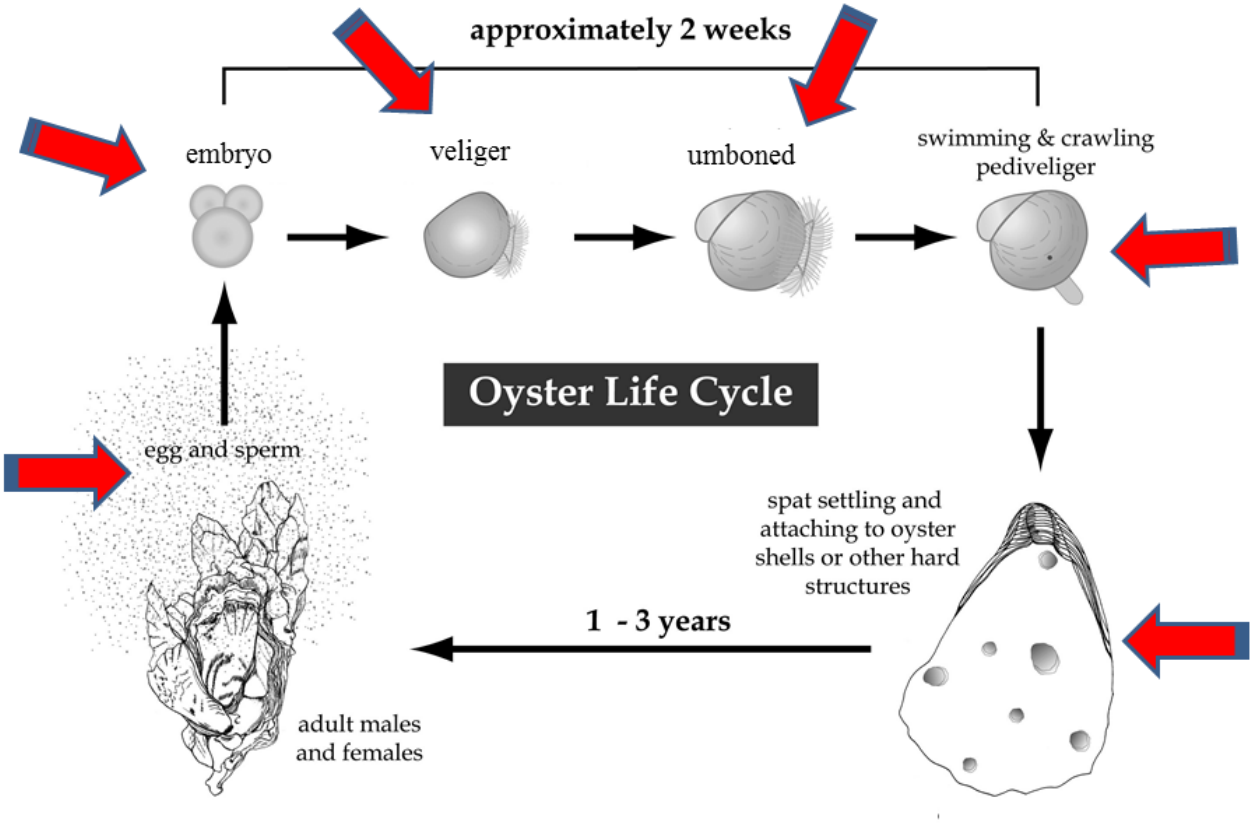


Figure 10: Summary of the different life stages of *Crassostrea virginica* investigated during this study: red arrows represent acute exposures to oil/dispersant.

In order to target this goal, four main objectives were investigated:

1/ Are the *Deepwater Horizon* oil and associated dispersant toxic to early life stages of *Crassostrea virginica*? If so, what are the concentration ranges where lethal and sub-lethal effects are observed?

2/ What are the most sensitive developmental stages and physiological processes of *Crassostrea virginica* to oil and/or dispersant?

3/ What are the mechanisms involved in the toxicity of oil and/or dispersant on these physiological processes?

4/ Are the results obtained in this study comparable with data collected from the field during the DWH oil spill?

This work will be divided in three main chapters, investigating the effects of acute but also dietary exposure of oyster to DWH oil (Slick A) and dispersant (Corexit 9500A®):

• **Impacts of DWH oil and dispersant on gametes and embryos of *Crassostrea virginica,***
(Chapter 1, Article 1 and 2)

• **Impacts of DWH oil and dispersant on larvae of *Crassostrea virginica,***
(Chapter 2, Article 3 and 4)

• **Impacts of DWH oil and dispersant on spat of *Crassostrea virginica*.**
(Chapter 3, Article 5)

From the present study, we will ultimately be able to establish whether there is a relationship between results gathered from laboratory toxicity testing and data collected from the field along the coast of the Gulf of Mexico. The implications of this research are multiple: data from this study will allow predictions of both acute and chronic impacts of crude oil on natural recruitment of oysters in the Gulf of Mexico. In addition, prior to the *Deepwater Horizon* incident, no deep water application of dispersant had been conducted. Consequently, this study will assess the impact of the use of dispersant on an economically and ecologically important species of the Gulf of Mexico and will enable informed decisions on the use of Corexit 9500A® in case of another oil spill. It will also provide valuable data on cost effective practices for fisheries/aquaculture as well as informed decisions on restoration and enhancement of wild stock to be made. The study is specific to *Crassostrea virginica*, but gathered information could be useful for another bivalve species widely distributed around the world, *Crassostrea gigas*, including many filter-feeding organisms.

II. GENERAL MATERIAL AND METHODS

II. General Material and Methods

1. Preparation of Water Accommodated Fractions (WAF)

WAF mode of preparation followed procedure detailed in the *Quality Assurance Project Plan: Deepwater Horizon Laboratory Toxicity Testing* (Version 4; February 4, 2014; Appendix A, pp A1-A9), NOAA.

The crude oil tested in the present study (sample CTC02404-02) was collected by NOAA personnel in cooperation with BP and transferred to Stratus Consulting Inc. under chain of custody. The DWH surface oil, or "Slick A", was collected near the source on 29 July 2010 from a barge holding mixed oil offloaded from different skimmer vessels. The dispersant Corexit 9500A®, manufactured by NALCO ®, was also provided by Stratus Consulting Inc.

1.1 CEWAF

The oil/dispersant mixtures or Chemically Enhanced Water Accommodated Fractions (CEWAF) were prepared under controlled temperature (25˚C) and under fluorescent lights to avoid photo-induced toxicity (Fig. 11). An aspirator bottle was filled with 2-L of UV-sterilized and 0.1 µm-filtered seawater (FSW), adjusted at a salinity of 20-25 PSU, and stirring was supplied using a stirring rod and a magnetic stirrer. Four grams of slick oil (Fig. 11.1) and 400 mg of dispersant (equivalent to an oil:dispersant ratio of 10:1) were then added to the aspirator bottle at the center of the vortex, using a gastight syringe (Fig. 11.2). The oil/dispersant mixture was then stirred at a vortex adjusted to 250 rpm (or 25 % of the solution height) for 18 h (Fig. 11.3). To allow separation of the solution from residual floating oil, the oil and dispersant mixture was left to stand for at least 3 h. The stock solution (final concentration of 2,000 ppm or 2 g oil L^{-1}) was obtained by carefully draining the bottom layer of the mixture from the aspirator bottle (Fig. 11.4). It was not filtered, and thus contained whole particulate oil in addition to dissolved PAHs. Exposure solutions were then obtained by adding FSW to the stock solution (Fig. 11.5).

Figure 11: Preparation of CEWAF exposure solutions

1.2 HEWAF

The oil only exposure solutions or High Energy Water Accommodated Fraction (HEWAF) were also prepared under similar controlled light and temperature as described above (Fig. 12). By dispersing the oil mechanically, the high energy method was used to artificially recreate the actions of waves, currents and stormy conditions. Two-liters of FSW were added to a stainless steel blender pitcher (Waring™CB15 commercial food blender) and then 4 g of slick oil (1:500 dilutions of oil) were added using a gastight syringe (Fig. 12.1 & 2). After 30 s at low speed blending (Fig. 12.3), the solution was transferred in a 2-L aspirator bottle and left to settle for at least one hour to allow separation of the solution from residual floating oil. The stock solution (final concentration of 2,000 ppm or 2 g oil L^{-1}) was obtained by carefully draining the bottom layer of the mixture from the aspirator bottle (Fig. 12.4). It was not filtered, and thus contained whole particulate oil in addition to dissolved PAHs. Dilutions were prepared by adding FSW to the stock solution (Fig. 12.5).

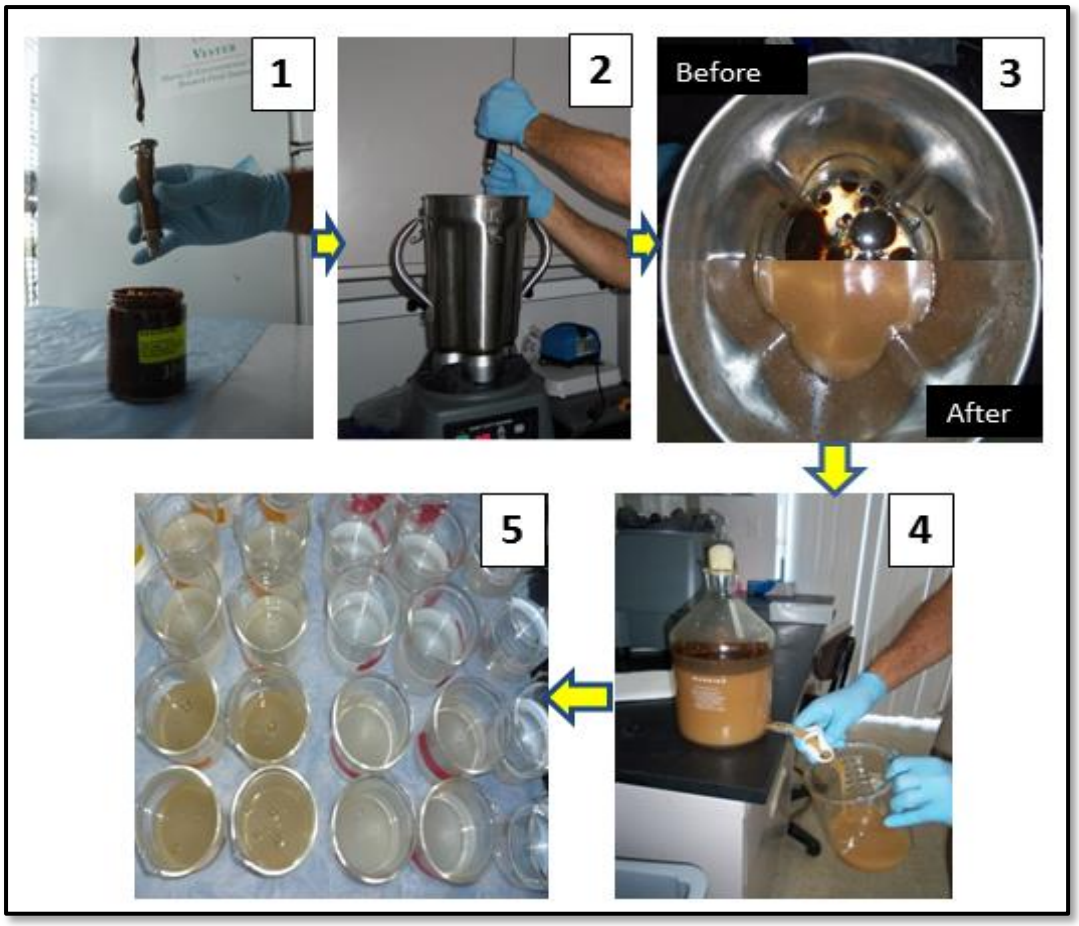


Figure 12: Preparation of HEWAF exposure solutions

1.3 Dispersant only and WAF (or LEWAF)

Stock solution of dispersant only was prepared by adding 400 mg of Corexit 9500A®, using a pipettor, to an aspirator bottle previously filled with 2-L of FSW (1: 50 dilution of dispersant) (Fig. 13). The solution was then stirred for 18 h, as described previously for CEWAF preparation. Different exposure concentrations were obtained by adding different amounts of FSW to the stock solution.

The WAF stock or LEWAF (Low Energy WAF) was prepared by adding 2-L of FSW to an aspirator bottle. Stirring was then provided with no vortex (≈ 180 rpm), and 4 g of oil were added to the seawater. In the same way as the other preparations, the top was covered with aluminum foil to avoid losses of volatile compounds, and the WAF solution was stirred for 18 to 24 h in the dark. No settling time was required, and exposure solutions were prepared by dilution of the stock.

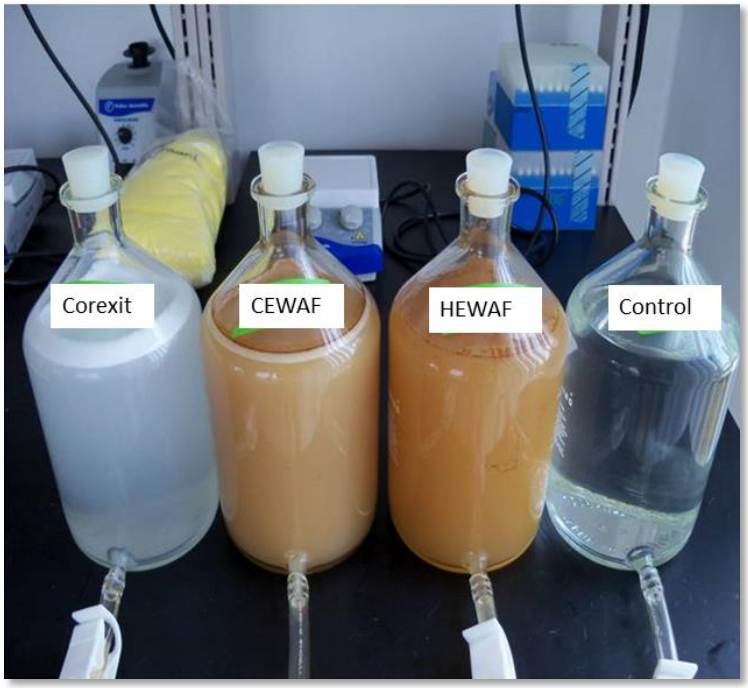


Figure 13: Stocks of different oil/dispersant preparations.

2. Analytical chemistry

2.1 Quantification of PAHs by GC/MS-SIM (from Tox Testing QAPP 2014-02-04 pdf, p 84)

Chemical analyses of water samples were conducted by ALS Environmental (Kelso, WA, USA) and validated by Ecochem Inc. (Seattle, WA, USA). For each stock solution of HEWAF and CEWAF, and the respective dilutions (including controls), PAHs including alkyl homologues were determined by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM) and a sum of 50 different PAHs, expressed as tPAHs, was quantified. The 250-mL unfiltered water samples were stabilized with hydrochloric acid, and stored in amber-bottles at 4˚C until shipment to the analytical laboratory by expedited courier. Samples were then extracted with dichloromethane and processed for GC/MS. The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the targeted analytes. Alkylated PAH homologues were quantified using a response factor assigned from the parent PAH compound. Analytes and target detection limits are listed in Table 3.

Table 3: List of 50 PAHs (parent and alkyl-homologues) quantified using GC/MS-SIM by ALS Environment (Kelso, WA, USA).

Naphthalene	(N0)	Fluoranthene	(FL0)
C1-Naphthalenes	(N1)	Pyrene	(PY0)
C2-Naphthalenes	(N2)	C1 - Fluoranthenes/Pyrenes	(FP1)
C3-Naphthalenes	(N3)	C2 - Fluoranthenes/Pyrenes	(FP2)
C4-Naphthalenes	(N4)	C3 - Fluoranthenes/Pyrenes	(FP3)
Biphenyl	(B)	C4-Fluoranthenes/Pyrenes	(FP4)
Dibenzofuran	(DF)	Naphthobenzothiophene	(NBT0)
Acenaphthylene	(AY)	C1-Naphthobenzothiophenes	(NBT1)
Acenaphthene	(AE)	C2-Naphthobenzothiophenes	(NBT2)
Fluorene	(F0)	C3-Naphthobenzothiophenes	(NBT3)
C1 - Fluorenes	(F1)	C4-Naphthobenzothiophenes	(NBT4)
C2 - Fluorenes	(F2)	Benz(a)anthracene	(BA0)
C3 - Fluorenes	(F3)	Chrysene+Triphenylene	(C0)
Anthracene	(A0)	C1 - Chrysenes	(C1)
Phenanthrene	(P0)	C2 - Chrysenes	(C2)
C1-Phenanthrenes/Anthracenes	(PA1)	C3 - Chrysenes	(C3)
C2-Phenanthrenes/Anthracenes	(PA2)	C4 - Chrysenes	(C4)
C3-Phenanthrenes/Anthracenes	(PA3)	Benzo(b)fluoranthene	(BBF)
C4-Phenanthrenes/Anthracenes	(PA4)	Benzo(j+k)fluoranthene	(BJKF)
Dibenzothiophene	(DBT0)	Benzo(a)fluoranthene	(BAF)
C1 - Dibenzothiophenes	(DBT1)	Benzo(e)pyrene	(BEP)
C2 - Dibenzothiophenes	(DBT2)	Benzo(a)pyrene	(BAP)
C3 - Dibenzothiophenes	(DBT3)	Indeno(1,2,3-cd)pyrene	(IND)
C4 - Dibenzothiophenes	(DBT4)	Dibenz(a,h)anthracene	(DA)
Benzo(b)fluorene	(BF)	Benzo(g,h,i)perylene	(GHI)

Target method detection limit range: 1 - 5 ng/L

2.2. Quantification of DOSS by LC/MS-MS

Water samples containing Corexit 9500A dispersant (Stock and dilutions) were analyzed using liquid chromatography tandem mass spectrometry (LC/MS-MS) for quantitative assessment of the following indicator compound, the surfactant dioctylsulfosuccinate sodium salt (DOSS). Method detection limits for DOSS were 20 μg L^{-1}.

3. Experimental oysters

3.1. Source, husbandry, conditioning and algal culture

Adult specimens of *Crassostrea virginica* (average weight of 75 g ± 20) were collected from natural populations in Estero Bay, Florida (Lat. 26˚19'50''N, Long. 81˚50'15''W). Estero Bay is State of Florida's first aquatic buffer preserve and has extensive, healthy oyster reefs, unexposed to the DWH oil spill (Fig. 14). Depending of the time of the year, adult oysters were brought in either ripe from the field or were further conditioned in the hatchery at 22˚C ± 1 following Loosanoff and Davis (1963), in a flow-through system supplied with coarsely filtered (30 µm sand filter) seawater, at ambient salinity (20-30 PSU). Oyster broodstock were maintained under natural light conditions, and fed a mixture of laboratory-cultured fresh microalgae (*Tetraselmis chui*, *Chaetoceros sp*. and *Tisochrysis lutea)* at a daily ration of 3% of oyster dry body weight for conditioning (Utting and Millican, 1997). Ten oysters were periodically sampled and examined for ripeness under a microscope.

Phytoplankton cultures were grown in f/2 culture medium (Guillard, 1975) prepared with FSW, and held in 10 L carboys and 90 L cylinders, at 22-23 ˚C and 30-32 PSU salinity on a 12:12 light:dark cycle and appropriate aeration.

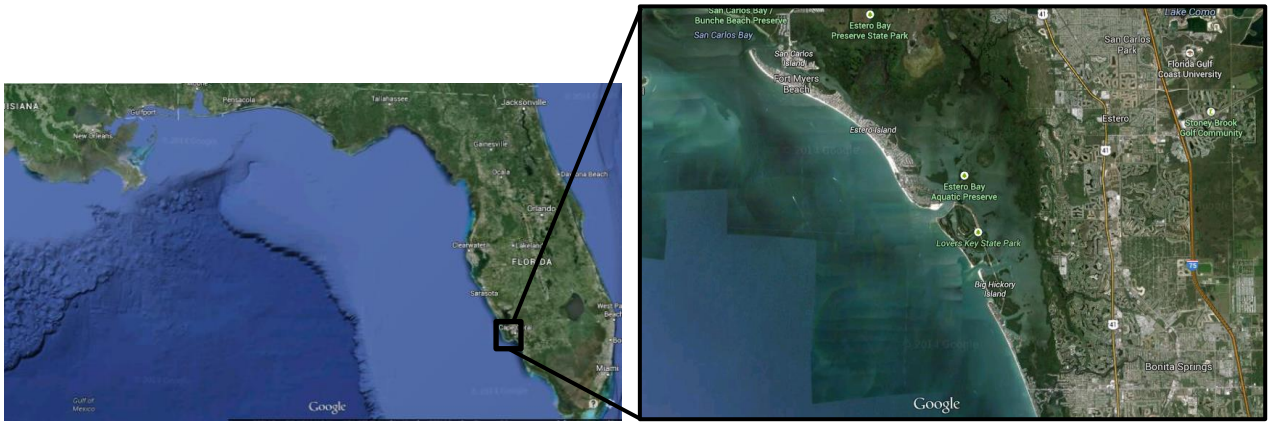


Figure 14: Estero Bay, on the west coast of Florida: zone of collection of oyster broodstock.
Source: https://www.google.com/maps/

3.2. Spawning and larval rearing

To obtain the best gamete quality, particularly for acute exposure of gamete, mature oysters were induced to spawn by thermal stimulation. When thermal shock was unsuccessful, gonad stripping was performed. To avoid cross-fertilization, spawning individuals were isolated after the first release of gamete. After verification under a microscope, gametes from at least 2-3 adults were

washed, counted and pooled for fertilization, at a ratio of 2.5 % (sperm : egg solution). Fertilized embryos were then stocked in the experimental hatchery in 50-L rearing tanks, at a density of 40 mL^{-1}, until hatching the next day. After evaluation of the hatching rate, newly hatched D-larvae were counted and stocked at a density of 10 mL^{-1} in the larval rearing tanks, filled up with UV-sterilized and 0.1 µm filtered seawater (FSW). Larval rearing was carried out for about 15 days at 28˚C, with a water renewal every other day, until larvae reached the pediveliger stage (see life cycle in § I.4.1.3). Settlement of competent larvae was conducted by providing microcultch on a suspended sieve. Through the whole larval cycle, oysters were fed a mixture of cultured microalgae (*Tisochrysis lutea, Chaetoceros muelleri*, and *Tetraselmis chui*) according to Helm and Bourne (2004).

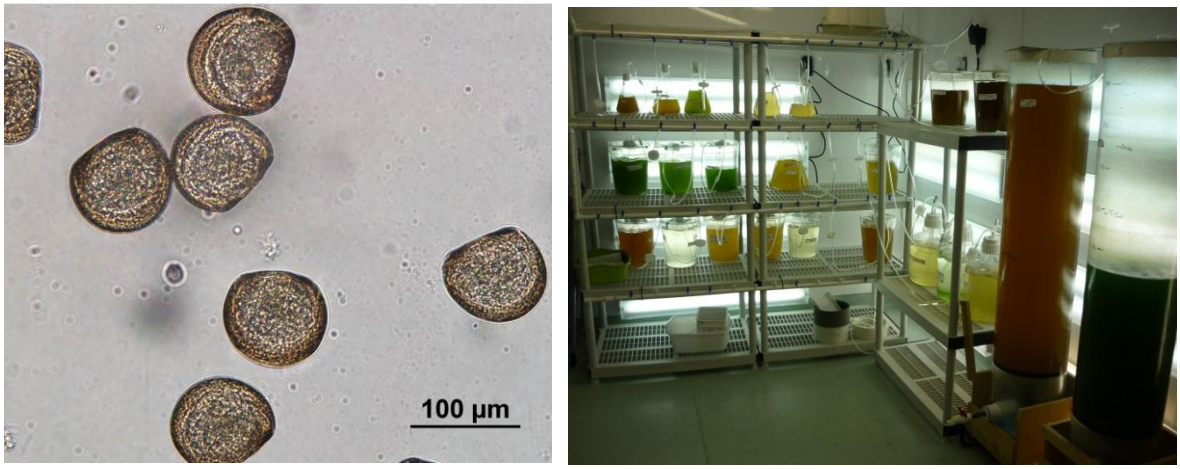


Figure 15: Four-day old veliger larvae (left). Microalgal culture in the experimental hatchery (right).

3.3. Origin of oyster spat

Two month-old oyster spat used in the acute exposure to DWH oil and dispersant (Chapter 3) were provided from the Auburn University Hatchery, Alabama (U.S), shipped in Styrofoam containers in next day delivery. After reception, spat were held at the experimental hatchery in a flow-through system for two weeks of acclimation, at ambient temperature (23˚C ± 2) and salinity (30 PSU ± 2). Spat were fed a mixture of Shellfish Diet (Reed Mariculture®) and naturally occurring phytoplankton from ambient water (filtration at 30 µm) for optimal growth.

III. CHEMICAL CHARACTERIZATION OF EXPOSURE SOLUTIONS

III. Chemical characterization of exposure solutions

1. Evolution of exposure solutions

Most of the toxicity tests carried out in the present study consisted of 96-h static assays, with no renewal of exposure solution. In addition, exposure containers were not covered, mimicking "real-world" exposure conditions.

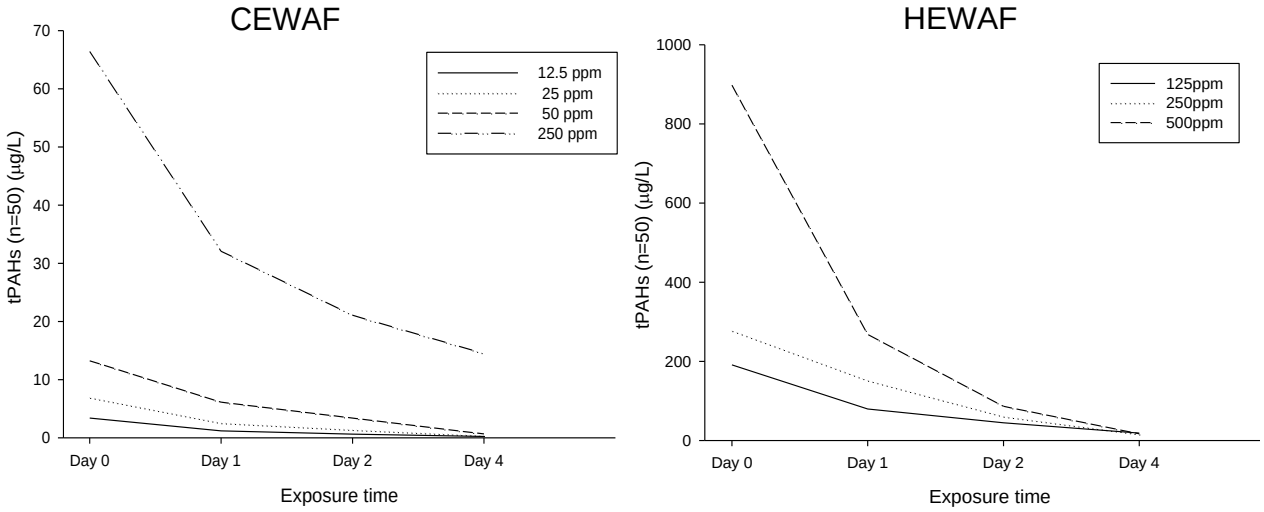


Figure 16: Evolution of tPAHs (n=50) measured in exposure solutions during 96 h static toxicity test, expressed in µg tPAHs L^{-1}. Nominal concentrations for CEWAF ranged from 12.5 to 250 ppm, and nominal concentrations for HEWAF ranged from 125 to 500 ppm.

Analytical chemistry revealed that concentrations of tPAHs contained in CEWAF or HEWAF solutions declined throughout exposure, with the most rapid decrease occurring during the first 24 h, particularly for the highest dose of HEWAF (500 ppm) which was reduced by 3 folds (Fig. 16). At the end of the 96 h exposure, all concentrations of HEWAF tested reached similar levels, around 17 µg tPAHs L^{-1} (± 1.5).

2. Dissolved *vs* particulate

First of all, comparison of filtered and non-filtered solutions of CEWAF and HEWAF (250 ppm) showed that the fraction of dissolved PAHs (blue) was variable between oil preparations. Dissolved fraction of both CEWAF and HEWAF consisted mostly of LMW PAHs with 1 or 2 rings (e.g. N, B, DF, AY, AE) but also 3 rings PAHs (Fig. 17). In addition, the particulate form of HEWAF and

to a lesser extent CEWAF consisted of a majority of HMW PAHs, with 3 rings and beyond. (Fig. 17)

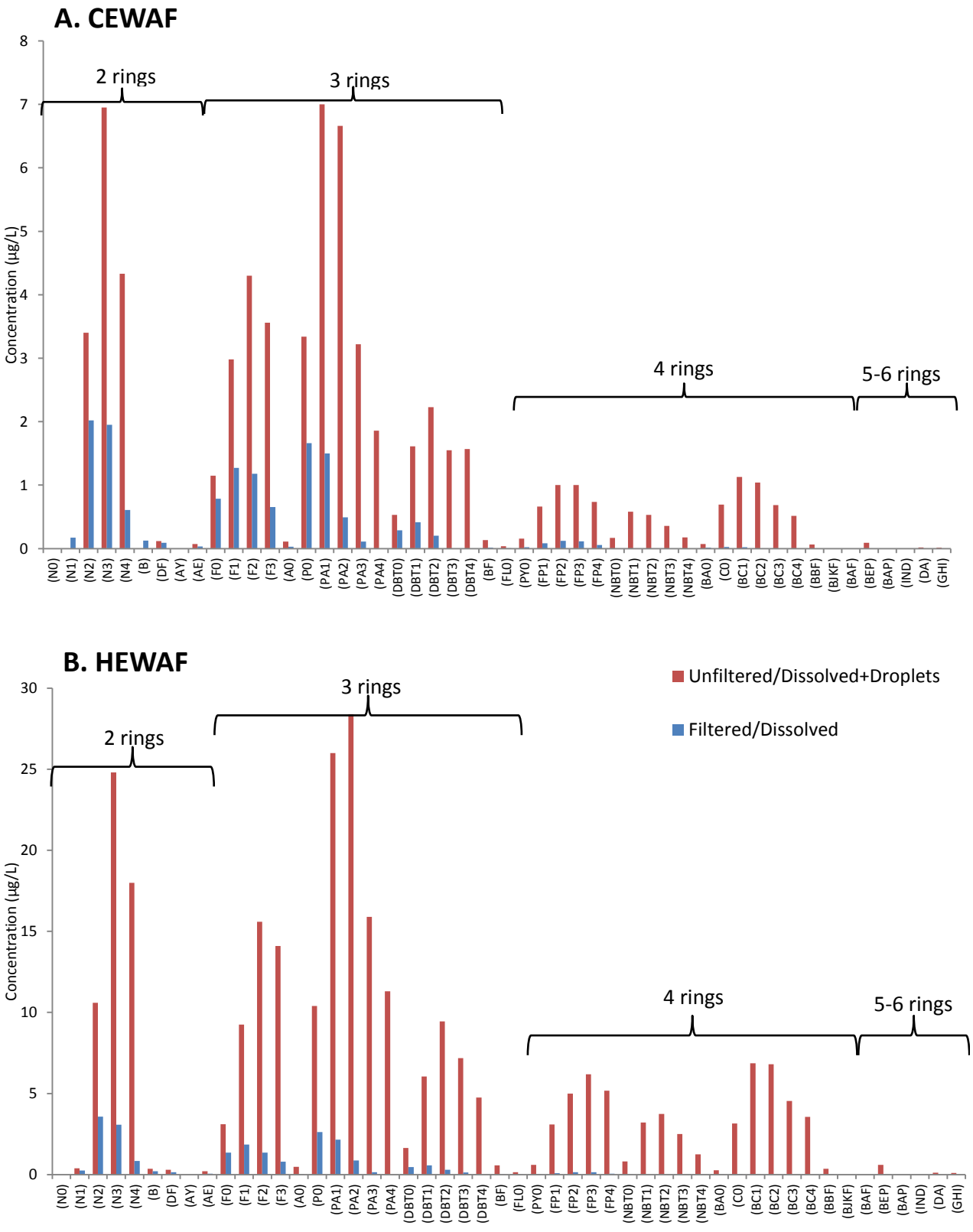


Figure 17: Contribution of dissolved PAH (blue) in total PAH profile (red) of CEWAF (A) and HEWAF (B) at nominal concentrations of 250 ppm, measured at test initiation (day 0). Data are expressed as measured PAH concentration in µg L^{-1}. TPAHs quantified by GC/MS-SIM. N0-4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene; PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0: Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4: Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF: Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA: Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0), while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc.

Chemical analysis of HEWAF revealed that, at equivalent nominal dose (i.e. 250 ppm) the proportion of particulate oil (droplets) was greater than for CEWAF (about 4 times), whereas the fraction of dissolved PAH was similar (Fig. 18).

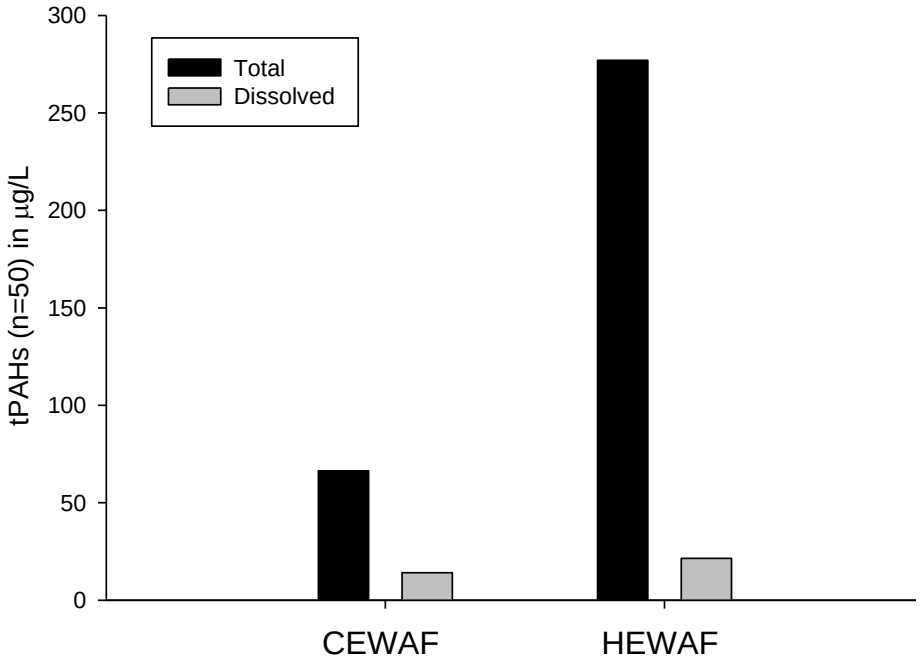


Figure 18: Fraction of dissolved PAH (grey) and total PAH (black) measured in 250 ppm doses of CEWAF and HEWAF at test initiation (day 0). Total PAH consist of unfiltered solution containing both dissolved and droplets. Data are expressed as concentration of the sum of 50 PAHs in µg L^{-1}. TPAHs quantified by GC/MS-SIM.

3. Droplet size distribution

Figure 19 shows an example of the size distribution (%) of oil droplets (particulate fraction) observed in a non-filtered stock solution of HEWAF (1:500 dilution of oil). Oil droplet diameters ranged from less than 1 μm to 22 μm, with the majority (≈ 90%) of droplets in the ≤ 1 to 5 μm range (Fig. 19). Mean diameter of these droplets equaled to 1.85 μm (± 0.6).

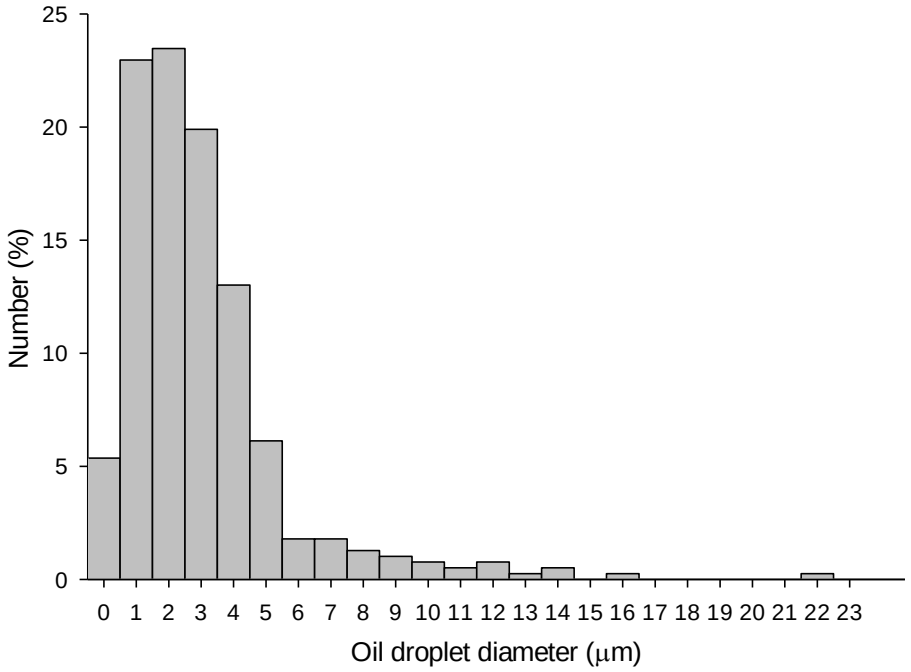
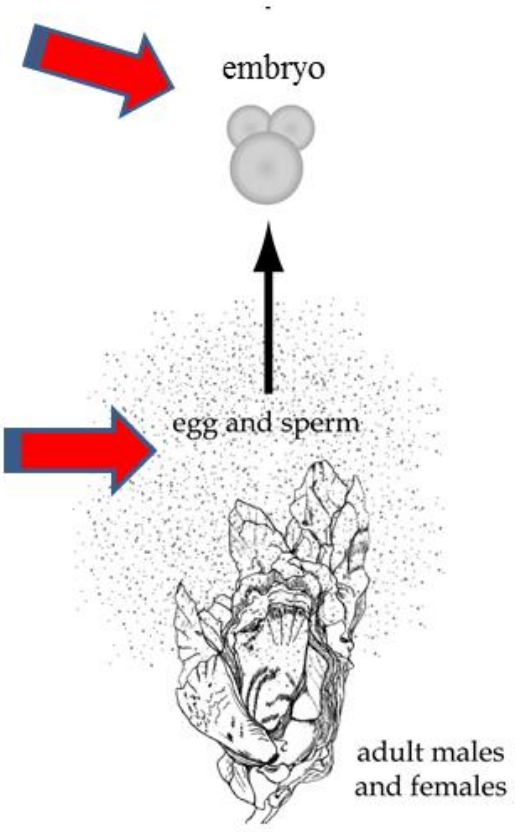


Figure 19: Example of size distribution (in %) of oil droplets in a sample of unfiltered HEWAF (2000 ppm), expressed as diameter (in μm).

Chapter 1: Impacts of DWH oil and dispersant on gametes and embryos of *Crassostrea virginica*



Introduction Chapter 1

The long-lasting nature of the DWH oil spill (85 days) coincided with the spawning season of the Eastern oyster in the Gulf of Mexico. Oysters are broadcast spawners, meaning that sperm and eggs are released in the surrounding environment where fertilization takes place. As a result, oyster gametes were likely exposed to oil/dispersant at the time of the spill, and fertilization and subsequent embryogenesis might be negatively affected by toxic compounds of oil (e.g. PAHs), dispersant, or a combination of both. This chapter attempted to determine the lethal and sublethal effects of an acute exposure to DWH oil, dispersed chemically (CEWAF) or mechanically (HEWAF), and dispersant alone on two sensitive early life stages (gametes and embryos) of *Crassostrea virginica*. Fertilization success, morphological development of the embryo, shell lengths and survival were assessed at different times of the acute exposure to contaminant.

In addition, further investigations looking at the cellular effects of CEWAF and HEWAF on gametes were undertaken. The mode of toxicity of oil/dispersant on sperm and oocyte functions were examined using *in vitro* and *in vivo* assays in order to relate them to successful fertilization and subsequent development, and the contribution of sperm and/or oocytes in those toxic effects was evaluated.

Article 1

Impacts of Deepwater Horizon oil and associated dispersant on early development of the American oyster *Crassostrea virginica*

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Abstract

In April 2010, the explosion of the Deepwater Horizon (DWH) offshore oil platform in the Gulf of Mexico spilled about 779 million L of crude oil 80 km off the coast of Louisiana, and was the largest oil spill in U.S. history. The period of the spill coincided with the reproductive season of oysters, one of the most ecologically and commercially important shellfish species along the Gulf coast of the U.S and Mexico. In addition, an estimated 7 million L of the chemical dispersant, Corexit 9500A®, were used at the wellhead and at the surface to disperse oil slicks. The present study evaluated in the laboratory the toxicity of surface-collected DWH oil, dispersant alone and dispersed oil on two sensitive early life stages (gametes and embryos) of the American oyster (*Crassostrea virginica*). Fertilization success, morphological development, growth and survival were assessed at different time points (1 h, 24 h and 96 h) as well as lethal and sub-lethal concentration ranges. Dispersed oil (CEWAF) and dispersant (Corexit) showed a similar toxicity. Gametes were more sensitive than embryos to dispersed oil and dispersant. Fertilization was significantly impacted by both CEWAF ($EC50_{1h}$ = 29.9 µg tPAHs L^{-1}) and dispersant ($EC50_{1h}$ = 1146 µg DOSS L^{-1}), and normal embryogenesis was reduced by CEWAF and dispersant. Similarly, veliger survival was affected by CEWAF ($LC50_{96h}$ = 8.5 µg tPAHs L^{-1}) and dispersant alone ($LC50_{96h}$ = 269 µg DOSS L^{-1}); shell length was also impaired in a dose-dependent way. HEWAF decreased fertilization and larval growth in a dose-dependent manner, significantly altered embryogenesis after 24 h of exposure and reduced subsequent survival after 96 h ($LC50_{96h}$ = 307 µg tPAHs L^{-1}). Similar trends were observed when embryos were exposed to CEWAF and Corexit after fertilization. These findings suggest that oil spills and the use of dispersant to disperse oil at the time of the spawning season of oysters can have deleterious effects on its sensitive early development and have drastic implications on oyster populations in the affected region.

Keywords*:* toxicity, Deepwater Horizon oil spill, fertilization, embryo-toxic, larval development, CEWAF, Corexit, PAH

Résumé

L'explosion de la plate-forme pétrolière off-shore "Deepwater Horizon" en Avril 2010 relâcha dans le Golfe du Mexique près de 779 million de litres de pétrole brut à 80 km au large des côtes de la Louisiane : ce fut la plus importante marée noire de l'histoire des Etats-Unis. La période de la marée noire a coïncidé avec la saison de reproduction de l'huitre, une des espèces de coquillage les plus importantes écologiquement et économiquement pour les régions côtières du Golfe du Mexique. Il a été estimé par ailleurs à 7 million de litres le volume de dispersant chimique, le Corexit 9500A®, utilisé directement à la fuite en profondeur et en surface pour disperser les nappes de brut. Cette étude a évalué en laboratoire la toxicité du pétrole brut récupéré en surface (Slick A), le dispersant, et le pétrole dispersé sur deux stades de vie sensibles (gamètes et embryons) de l'huitre Américaine, *Crassostrea virginica*. Le taux de fécondation, le développement morphologique, la croissance ainsi que la survie ainsi que des gammes de concentrations létales et sublétales ont été estimés à différents temps d'exposition (1 h, 24 h et 96 h). En général, le pétrole dispersé (CEWAF) et le dispersant seul (Corexit) ont induit des toxicités similaires. Les gamètes se sont montrés plus sensibles au CEWAF et au dispersant seul que les embryons. La fécondation a été affectée de façon significative par le CEWAF ($EC50_{1h}$=29.9 µg tPAHs L^{-1}) et le dispersant ($EC50_{1h}$=1146 µg DOSS L^{-1}), et l'embryogénèse normale a été réduite par le CEWAF et le dispersant. De la même façon, la survie des larves veligères a été impactée par le pétrole dispersée ($LC50_{96h}$ = 8.5 µg tPAHs L^{-1}) et par le dispersant ($LC50_{96h}$ = 269 µg DOSS L^{-1}), et les tailles de larves ont également été réduites. Le pétrole seul (HEWAF) a diminué la fécondation, l'embryogénèse après 24 h d'exposition, la croissance larvaire et la survie après 96 h ($LC50_{96h}$ = 307 µg tPAHs L^{-1}). Des tendances similaires ont été observées lorsque les embryons furent exposés au CEWAF et Corexit après la fécondation. Ces résultats suggèrent qu'une marée noire ainsi que l'utilisation de dispersant pour mitiger l'impact du pétrole au moment de la période de ponte des huitres peut avoir des effets néfastes sur les jeunes stades de vie très sensibles, et peuvent avoir des répercussions majeures sur les populations naturelles d'huitres dans la région affectée.

Mots-clés : toxicité, marée noire Deepwater Horizon, fécondation, embryo-toxique, développement larvaire, CEWAF, Corexit, HAP.

1. Introduction

The American oyster (*Crassostrea virginica)* is one of the most commercially and ecologically important shellfish species propagating along the east coasts of the U.S, from Maine to the Gulf of Mexico (Galtsoff, 1964, Volety et al. 2014). In 2012, total landings of *C. virginica* in the U.S represented a value of $104 million and about $74 million in coastal regions of the northern Gulf of Mexico (National Marine Fisheries Service, 2012). In addition to its economic value, it is also an ecologically vital species. Oyster reefs, which have been built through successive reproduction and settlement of larvae onto existing reef structure, provide food, shelter and habitat for many fish and shellfish species, improve water quality, stabilize bottom areas, and influence water circulation patterns within estuaries (Wells, 1961; Newell, 2004, Volety et al. 2014). In the northern part of the Gulf of Mexico, oyster spawning season typically occurs from mid-spring through late fall (Ingle, 1951). On April 20th, 2010, the explosion of the Deepwater Horizon (DWH) offshore oil platform, in the Gulf of Mexico, led to the release of an estimated 779,000 t (about 4.1 millions of barrels) of crude oil 80 km off the coast of Louisiana (OSAT/NOAA report, 2010; Camilli et al., 2012). The oil leak was discovered two days after the incident at a depth of 1544 m. After almost three months and several attempts to stop the leak, the well was finally cemented on July 15th, 2010, and about 8 million L of chemical dispersants, Corexit 9500A® and 9527A®, were used directly at the wellhead and at surface to disperse oil slicks (Kujawinski et al., 2011; On Scene Coordinator Report DWH, 2011). The DWH oil contaminated first the Louisiana coast and then, Mississippi, Alabama and Florida coasts (Rosenbauer et al., 2010).

Petroleum hydrocarbon contaminants pose a severe ecological risk. They can affect organisms by physical action [light reduction, asphyxia], by modification of habitat [change in pH (Neff, 1987), decrease of dissolved oxygen, decrease in food availability] and by toxic effects. Crude oil constituents are of particular concern due to their high chemical stability, low degradation and lipophilic nature. Most toxic effects of crude oil are typically attributed to the aromatic fraction, particularly Polycyclic Aromatic Hydrocarbons (PAHs), known to be persistent in the environment, and potentially mutagenic, genotoxic and carcinogenic to organisms (Roesijadi et al., 1978; Neff, 1985; Albers, 2003).

Chemical dispersants are complex mixtures, primarily containing surfactants (Dioctyl Sodium Sulfosuccinate i.e. DOSS) and solvents (Propylene Glycol), which reduce the interfacial tension at the oil-water interface, and therefore facilitates the downward mixing of

oil into the water (Canevari, 1973; Li and Garrett, 1998). Therefore, oil slicks can be dispersed to concentrations below toxicity thresholds for most marine and benthic species (Lessard and DeMarco, 2000; Page et al., 2000) and become more accessible to hydrocarbon-degrading bacteria (Venosa and Zhu, 2003). Because of higher exposure of aquatic organisms to petroleum compounds in coastal areas or estuaries, manufacturer recommends using chemical dispersants at a minimum water depth of 20 m, with current speed higher than 1 m s^{-1} and at a minimum distance from the shore or from off-shore islands of 2 km (Ramachandran et al., 2004). According to manufacturers, new dispersant generation such as Corexit 9500A® are considered non-toxic and bio-degradable, when used on their own and at recommended concentrations and depths. However, aquatic organisms are likely exposed to both dispersant and oil in combination, which may alter toxic effects (Getter and Baca, 1984; Gulec et al., 1997; Barron et al., 2003; Ramachandran et al., 2004; Rico-Martinez et al., 2013; Almeda et al., 2013). Moreover, little is known about the behavior and combined effects of oil and dispersants in the near shore environment (Allen, 1984).

Prolonged turbulent mixing of crude oil by wind, currents and waves results in the release of water accommodated fraction (WAF) (Rossi et al., 1976; Barron et al., 1999). WAF toxicity is commonly assessed through measurements of early larval growth, survival, and morphological abnormality in marine organisms such as fish (Couillard et al., 2005), starfish (Davis et al., 1981), crustaceans (Cucci and Epifanio, 1979) or bivalves (Fucik, 1994; Saco-Alvarez et al., 2008). Early life stages are typically more sensitive than adults and represent a critical period in the life cycle (Connor, 1972; Chapman and Long, 1983; Huffman Ringwood, 1991; His and Beiras, 1999).

In an aquatic ecosystem, physiological and ecological effects of environmental stress are numerous. Although the ultimate effect is mortality, sub-lethal effects include alteration of normal physiological activities (His and Robert, 1985; Beiras and His, 1994), increased expenditure of energy reserves affecting fecundity and subsequently reducing fertilization success, and larval viability and growth (Capuzzo, 1996; Thompson et al., 1996; McDowell et al., 1999). The Deepwater Horizon oil spill occurred at the beginning of *C. virginica* spawning season. Sperm and eggs were therefore likely exposed to toxicants as were oysters. Work on the effects of Alaskan crude oil reported severe abnormalities in developing embryos of Pacific oyster *(Crassostrea gigas)* (Le Gore, 1974). PAHs alone or associated to sediments, and other organic chemicals negatively impacted *C. gigas* sperm motility, embryonic development, larval growth and survival (Renzoni, 1975; His and Robert, 1983; Pelletier et al., 2000; Geffard et al., 2002b; 2003; Lyons et al., 2002; Akcha et al., 2012).

Moreover, exposure of Pacific oysters to PAHs significantly reduced fertilization success and larval development (Jeong and Cho, 2005). Lastly, *C. virginica* embryos were adversely impacted by acute exposure to dispersed oil with 96 h LC50 values of 4 mg L^{-1} of Total Hydrocarbons Petroleum (TPH), and by dispersant (Corexit 9527) with $LC50_{96h}$ values of 4.9 mg L^{-1} (Fucik et al., 1994). Therefore, fertilization success and subsequent embryogenesis and larval development of *C. virginica* might be negatively affected by exposure to oil, dispersed oil and Corexit 9500A® found in the Gulf of Mexico at that time. Although oysters have been used as a model organism in numerous ecotoxicological studies (Woelke, 1972; Chapman and Long, 1983; Chapman, 1989; Thain, 1991; 1992; His et al., 1996), little literature exists on the toxicity of dispersant and dispersed oil to *C. virginica* gametes and embryos; therefore effects of these toxicants on fertilization success and early development need further investigation.

The aim of this study was to examine the lethal and sub-lethal effects of acute exposure to surface-collected DWH oil (HEWAF), dispersant (Corexit 9500A®) or dispersed oil (CEWAF) on two sensitive early life stages (gametes and embryos) of *Crassostrea virginica*. Fertilization success, morphological development, shell lengths and survival were assessed at different time points (1 h, 24 h and 96 h). In addition, lethal and sub-lethal concentration ranges were also determined. Differences in gamete and embryo susceptibility to oil/dispersant may contribute to understand the mechanisms of toxicity on sensitive early life stages of bivalves.

2. Material and Methods

Range-finding tests were performed to establish the definitive test concentrations that cause lethal and sub lethal (developmental abnormalities, reduced growth) effects to oyster gametes and embryos. Exposure designs followed standardized protocols described in "U.S. EPA. 1996. *Ecological Effects Test Guidelines: OPPTS 850.1055: Bivalve Acute Toxicity Test (embryo larval)"*.

2.1. Water Accommodated Fractions

The crude oil tested in the present study (sample CTC02404-02) was collected by NOAA personnel in cooperation with BP and transferred to Stratus Consulting Inc. under chain of

custody. The DWH surface oil, or "Slick A", was collected near the source on 29 July 2010 from a barge holding mixed oil offloaded from different skimmer vessels. The dispersant, Corexit 9500A®, manufactured by NALCO ® was also provided by Stratus Consulting Inc.
For all exposure solutions, contaminants were added to UV-sterilized and 0.1 µm-filtered seawater (FSW), maintained at a salinity of 20-25 PSU.

2.1.1 HEWAF

Oil-only exposure solutions or High Energy Water Accommodated Fraction (HEWAF) were prepared at 25˚C under fluorescent lights to avoid photo-reactivity (Landrum et al., 1987). To artificially recreate wave and current action and stormy conditions, 2 L of filtered seawater (FSW) were added to a stainless steel blender pitcher (Waring™CB15, Waring Commercial, Torrington, CT) and 4 g of slick oil (1:500 dilution of oil) were added with a gastight syringe. After 30 s at the lowest speed blending, the solution was transferred to a 2-L aspirator bottle and left to settle for at least 1 h to separate residual floating oil. The bottom layer of the mixture was then carefully drained from the aspirator bottle and FSW was added to this stock to prepare dilutions for exposure testing. The stock and the different dilutions were not filtered, and thus contained whole particulate oil in addition to dissolved PAHs.

2.1.2 CEWAF

The oil/dispersant mixtures or Chemically Enhanced Water Accommodated Fractions (CEWAF) were also prepared at 25˚C under fluorescent lights. Two grams of slick oil and 200 mg dispersant (10:1 v:v) were added to an aspirator bottle filled with 2 L of FSW. Contaminants were added with a gastight syringe, and stirred at a vortex adjusted to 250 rpm using a stirring rod and a magnetic stirrer for 18 h. To allow separation of the solution from residual floating oil, the oil and dispersant mixture was left to stand for at least 3 h and the stock solution was carefully drained.

2.1.3 Corexit

Dispersant exposure solutions were prepared as described for the CEWAF above, except that no oil was added and the mixture was not settled. The dispersant stock was collected by draining the aspirator bottle and the stock solution was diluted with FSW to obtain different exposure concentrations.

In separate preliminary experiments, the median concentrations (LC50) of HEWAF, CEWAF and dispersant were determined in order to find the range of appropriate concentrations. For exposure experiments, nominal concentrations of HEWAF, CEWAF and dispersant used, and corresponding tPAHs and DOSS contents are listed in Table 1; PAH profiles of the stock solution of CEWAF and HEWAF are shown in Figure 1.

2.2. Oyster broodstock conditioning

Adult specimens of *Crassostrea virginica* (average weight of 75 g ±20) were collected between April and September 2011 and on October 2013, from natural populations in Estero Bay, Florida (Lat. 26˚19'50''N, Long. 81˚50'15''W). Adult oysters were held in the experimental hatchery at 23˚C ± 1, in a flow-through system supplied with coarsely filtered (30 µm sand filter) seawater, at ambient salinity (20-30 PSU), under natural light conditions, and fed a mixture of laboratory-cultured fresh microalgae (*Tetraselmis chui, Chaetoceros sp*. and *Tisochrysis lutea)* at a daily ration of 3% of oyster dry body weight for conditioning (Utting and Millican, 1997). Ten oysters were periodically sampled and examined for ripeness under a microscope.

2.3. Spawning and gamete recovery

Gamete and embryo exposures to CEWAF and dispersant were performed with offspring obtained from the same stock of adult oysters, at the end of August 2011. HEWAF exposures were conducted in November 2013, with an offspring obtained from different broodstock.

Mature oysters were thermally induced to spawn by alternating immersion in seawater at 18˚C and 30˚C for 30 min each time. To collect oocytes, spawning females were isolated in 1-L beakers filled with about 500 mL of FSW. To obtain a dense sperm solution, spawning males were isolated in approximately 200 mL of FSW in 1-L beakers. Oocytes and sperm were examined under a microscope for mobility (sperm), shape and absence of atresia (oocytes) for selection of the best gametes. After filtration through a 55 µm-mesh to remove debris, sperm from several males ($n \geq 3$) was pooled in a 1-L sterile beaker. Eggs from several females ($n \geq 3$), after successive sieving through 150-µm and 55-µm mesh to remove tissue and debris were rinsed on the 20-µm sieve and transferred into a sterile beaker filled with 2 L of FSW. Five subsamples of 50 µL of FSW/egg mixture were taken after continuous and gentle mixing. Subsamples of eggs were stained with 1% Lugol, and counted using a Sedgwick-Rafter® cell and a dissecting microscope.

2.4. Acute exposure of gametes (pre-fertilization)

2.4.1. Fertilization assay

Before fertilization, sperm and eggs were exposed separately to various concentrations of HEWAF, CEWAF or dispersant for 30 min (n=4 replicates for each concentration) (Table 1). Controls consisted of FSW only. Each 10-mL replicate of sperm (dense solution of 1.5 x 10^7 to 2.5 x 10^7 cells mL^{-1}) was incubated in test solution (40 mL HEWAF, CEWAF, or dispersant solution in 50-mL beakers). Oocytes (4,000-4,400) were also incubated in test solution (200 mL HEWAF, CEWAF, or dispersant in 400-mL beakers).

After the 30 min incubation, eggs from each exposure replicate were fertilized with 10 mL of sperm from corresponding sperm exposure replicates. Fertilization beakers were maintained in darkness at 26˚C ± 1 and at a salinity of 22 PSU ± 2 for 96 h (4 days). Each test chamber was subsampled 1 h post-fertilization and samples were preserved in 10% buffered formalin for later examination. To determine fertilization success (%), embryos presenting first cell cleavage/segmentation were counted under a microscope (minimum of 100 embryos per replicate).

2.4.2. Embryogenesis and larval development

Progress of embryogenesis and larval development was monitored after fertilization for 96 h and the exposure solutions were not renewed. Fresh cultured phytoplankton was added to each beaker at day 1 (5 x 10^4 cells mL^{-1}) and day 3 (1 x 10^5 cells mL^{-1}) post-fertilization. One day (24 h) and 4 days (96 h) after fertilization a subsample was removed from each beaker and fixed in 10 % formalin for later examination of abnormalities and larval shell lengths (see 2.6 for more details). Exposures were arrested after 96 h.

2.5. Acute exposure of embryos (post-fertilization)

Whilst gametes were incubated in exposure solutions, the remaining unexposed egg solution (around 5 million eggs in 2 L) was fertilized with 50 mL of the remaining unexposed sperm. Forty-five minutes later, fertilization success was verified by microscopic examination using the same method as previously described in 2.4.1. Fertilized eggs were then gently mixed with FSW, 50-µL subsamples (n=5) were taken and number of fertilized eggs counted as previously described. One hour after fertilization, when the 2-4 cell stage was reached, embryos were transferred volumetrically using a micropipette into 400 mL beakers filled with 200 mL of the different exposure concentrations of HEWAF, CEWAF or dispersant (Table 1)

(4 replicates per condition). Embryos were distributed at a targeted density of 20 mL^{-1} (≈ 4,000-4,400 individuals per beaker) and incubated for 96 h in the dark at 26 ˚C ± 1 and 22 PSU ± 2. Fresh cultured phytoplankton (*T. lutea*) was added to each exposure beaker at day 1 (5×10^4 cells mL^{-1}) and day 3 (1×10^5 cells mL^{-1}).

2.6 Subsampling, final survival assessment and measurements

Twenty four hours and 96 h after fertilization, a 5-10 mL subsample was collected from each exposure beaker and preserved with 10 % buffered formalin for later observations of abnormalities and shell measurements. After 96 h of exposure, the content of each beaker was filtered through a 20-µm nylon mesh and larvae were re-suspended in 20 mL FSW. Final survival was assessed by taking 200-µL subsamples (n=3) from the concentrate (20 mL) of each replicate and staining with 1% Lugol. Remaining samples were preserved in 10% buffered formalin for later observations of abnormalities and shell sizes at 96 h. At each sampling time (24 h and 96 h), a minimum of 50 randomly selected individuals for each replicate were examined under a microscope to determine the percentage of normal and abnormal larvae, and the percentage of dead larvae (translucent non-stained shells). Categories of abnormal larvae included (Fig. 4): (1) segmented eggs, normal embryos or malformed embryos that did not reach the D-larval stage; and (2) D-larvae with either convex hinge, indented shell margins, incomplete shells, protruded velum, or extrusion of mantle (from His et al., 1996).

In addition, at each sampling time, shell lengths of 25 randomly-selected larvae were measured in each beaker under a microscope equipped with an ocular micrometer.

2.7. Water quality and analytical chemistry

Temperature, dissolved oxygen, salinity, and pH were measured daily using a Pro ODO optic probe (YSI), a refractometer and a “Pinpoint” pH monitor (American Marine Inc.) respectively. Total ammonia was measured at the start and the end of each exposure experiment, using a Seal Analytical Auto Analyzer 3 and the G-171-96 method.

Chemical analyses of the stock solutions, the different HEWAF, CEWAF, dispersant concentrations and the FSW control were performed by ALS Environmental (Kelso, WA, USA). Polycyclic Aromatic Hydrocarbons (PAHs) including alkylated homologues were determined by gas chromatography with low resolution mass spectrometry using selected ion

monitoring (GC/MS-SIM) and a sum of 50 different PAHs or tPAHs were quantified (Fig. 1). The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the targeted analytes. Corexit 9500A was analyzed by liquid chromatography coupled with mass spectrometry (LC/MS-MS), and the marker Dioctylsulfosuccinate Sodium Salt (DOSS) was quantitatively assessed.

2.8. Statistical analyses

Measurement, percentage abnormality and mortality data were expressed as means ± standard deviation. As percentage data commonly form a binomial, rather than a normal distribution, all percentage data were arcsine-square root transformed (percentage of fertilized eggs, abnormally developed embryos or dead larvae) before statistical analysis. Data were statistically analyzed by analysis of variance (ANOVA), after checking normality (Shapiro-Wilk test) and homogeneity of variances between treatments (Levene's test) using SPSS® 19.0 statistical package. Whenever significant differences among groups were found ($p \leq 0.05$), a Tukey post-hoc test was performed. Kruskall-Wallis non-parametric test and Dunnett's T3 post-hoc test were performed whenever normality and homogeneity of variances requirements were not met after transformation of the data. Difference between life stages (Gamete vs Embryos) was tested using a 2-way ANOVA, treatments and stages being the fixed factors. Effective and lethal concentrations (ECx/LCx) were estimated using the *drc* and *bbmle* packages in R. For binomial response variables (mortality, fertilization), a 3-parameter log-logistic model was fitted.

Final survival was extrapolated from the number of larvae counted alive in the 96-h subsamples. To assess mortality, the extrapolated number alive was compared to the estimated number of larvae stocked at test initiation, while accounting for percent fertilization and the volume of test mixture removed at earlier sampling time points.

3. Results

3.1. Water quality and analytical chemistry

Temperature and salinity through the experiment ranged from 24.8 to 26.1˚C and from 22 to 25 PSU respectively. PH averaged 7.8 ± 0.5 and dissolved oxygen never decreased below 6 mg L^{-1} or 90% saturation. For each tested concentration of HEWAF, CEWAF, and dispersant, total ammonia concentrations remained at safe levels (NH_4^+ = 0.21 mg L^{-1} ± 0.095). FSW used for control contained very low levels of PAH at background levels (tPAHs = 0.008 µg.L^{-1} ± 0.01) (Table 1). PAH composition of HEWAF was similar to the CEWAF profile with similar peaks (Fig. 1).

Table 1: Range of nominal concentration (ppm or mg L^{-1}) used for HEWAF, CEWAF and dispersant exposures and corresponding PAH content (in µg L^{-1} = sum of 50 PAHs analyzed by GC/MS-SIM) and DOSS content (in µg L^{-1} analyzed by LC/MS-MS). CEWAF consisted of 1:10 oil to dispersant ratio.

HEWAF		CEWAF		Dispersant	
Nominal concentration (ppm)	Total Sum(PAH) (µg L^{-1})	Nominal concentration (ppm)	Total Sum(PAH) (µg L^{-1})	Nominal concentration (ppm)	Total DOSS (µg L^{-1})
0	0.017	0	0.06	0	0
62.5	108.3	6.3/0.63	1.3	0.62	62.5
125	198.1	12.5/1.25	3.3	1.25	125
250	417	25/2.5	6.4	2.5	250
500	839.4	50/5	14.2	5	500
1000	1634.5	100/10	26.2	10	1000

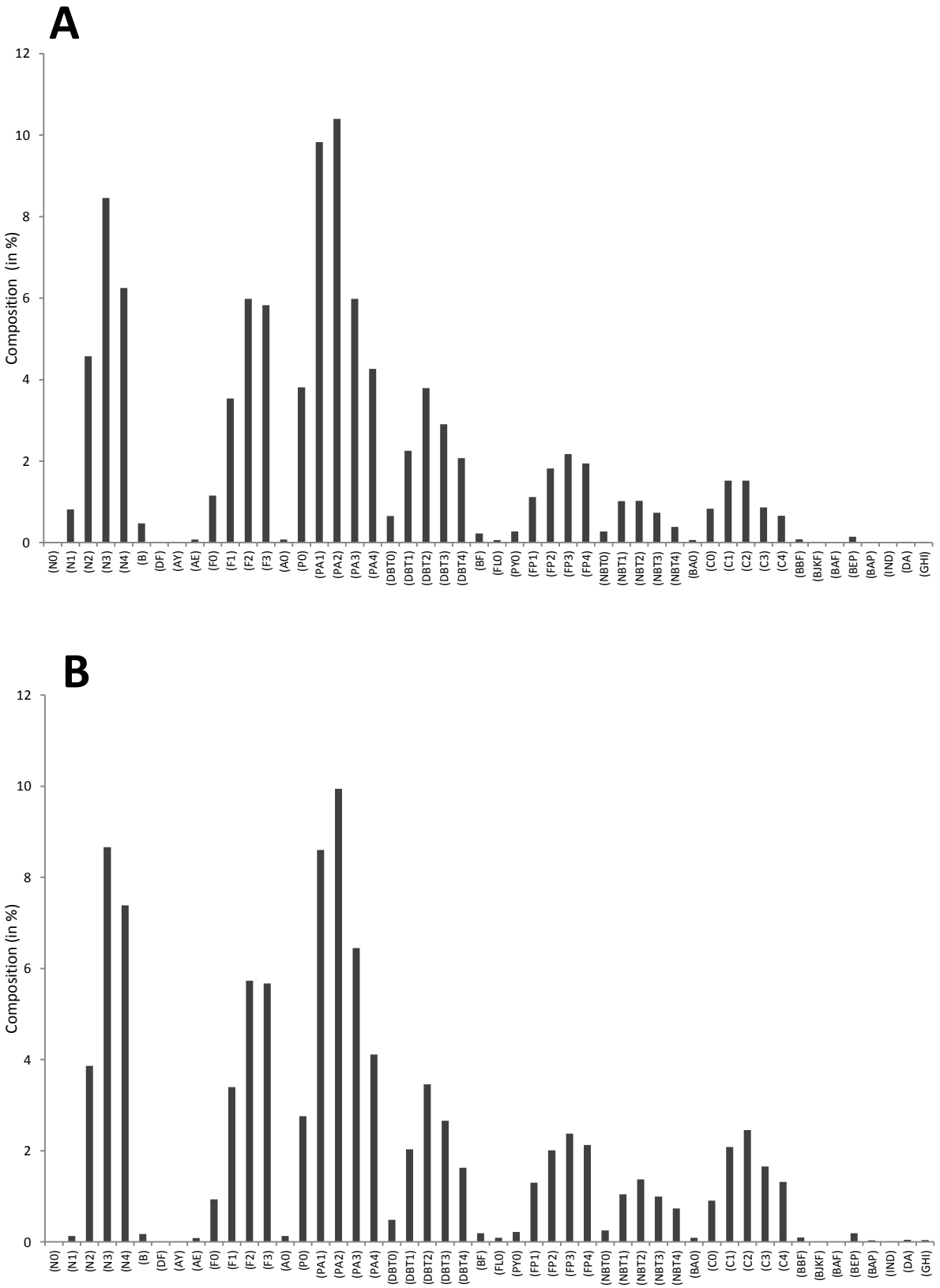

Figure 1: PAH composition of (A) CEWAF stock (1,000 mg oil L^{-1} corresponding to 262 µg Σ50 PAHs L^{-1}) and (B) HEWAF stock (2,000 mg oil L^{-1} corresponding to 3,209 µg Σ50 PAHs L^{-1}) used for gametes and embryos acute exposure, expressed in %. N0-4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene; PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0: Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4:

Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF: Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA: Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0), while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc

3.2. Effects on fertilization

Exposure to oil (HEWAF), dispersed oil (CEWAF) or dispersant for 30 min prior to fertilization, significantly affected the fertilization success in a dose-dependent manner (*p<0.001*) (Fig. 2). The lowest concentrations of HEWAF, CEWAF and dispersant alone having a significant effect on the fertilization success (LOEC) were respectively 1000 ppm (corresponding to 1,635 µg tPAHs L^{-1}), 50/5 ppm (corresponding to 14.2 µg tPAHs L^{-1}) and 5 ppm (corresponding to 500 µg DOSS L^{-1}) (Fig. 2). Fertilization success was inhibited by CEWAF and Corexit in a similar manner. Effect levels for fertilization inhibition ($EC20_{1h}$ and $EC50_{1h}$) for all solutions tested are listed in Table 2. For HEWAF, $EC50_{1h}$ value for fertilization inhibition was 1,394 ppm, corresponding to 2,250 µg tPAH L^{-1}. For CEWAF, nominal concentrations of 112/11.2 ppm (1:10 dispersant to oil ratio), corresponding to 29.9 µg tPAH L^{-1}, induced 50 % inhibition of the fertilization success. For Corexit 9500A®, the $EC50_{1h}$ for fertilization success reached 11.5 ppm (corresponding to 1,146 µg DOSS L^{-1}) (Table 2), indicating a similar toxicity between dispersant and CEWAF, at equivalent nominal concentration (1:10 ratio).

Table 2: Concentration causing 20 % and 50 % inhibition (EC20/EC50) of the fertilization success for gametes exposed for 1 h to HEWAF, CEWAF and dispersant, calculated using binomial response variables, fitted with a 3-parameter log-logistic model, using the *drc* and *bbmle* packages in R. Data are expressed as nominal concentrations (mg L^{-1} or ppm), measured concentrations of a sum of 50 PAHs (µg tPAH L^{-1}) for oil preparations (HEWAF and CEWAF) or µg DOSS L^{-1} for dispersant only (± 95% Confidence interval).

	EC20			**EC50**		
	HEWAF	**CEWAF**	**Dispersant**	**HEWAF**	**CEWAF**	**Dispersant**
Nominal (ppm)	**1020*** (896-1267)	**73/7.3** (64.3-80.5)	**6.9** (6.3-7.6)	**1394*** (1096-4000)	**112/11.2*** (102-133)	**11.5*** (10.2-12.7)
ΣPAH (µg L^{-1})	**1650*** (1450-2050)	**19.4** (17.1-21.4)	----------	**2250*** (1770-6460)	**29.9*** (27.2-35.6)	----------
DOSS (µg L^{-1})	----------	**#**	**695** (629-759)	----------	**#**	**1146*** (1023-1268)

*: ECx exceeded range tested. #: DOSS content not quantified for CEWAF.

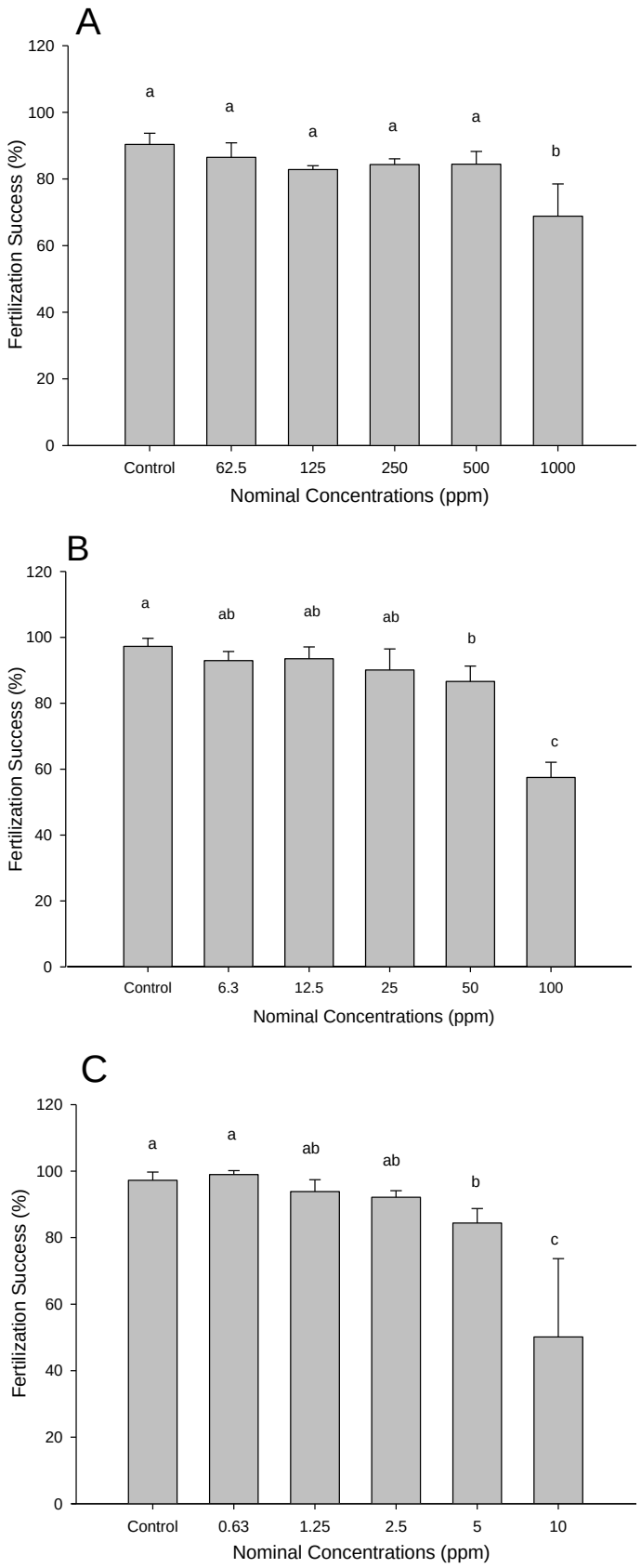

Figure 2: Fertilization success of *C. virginica* gametes (eggs and sperm) exposed to (A) HEWAF, (B) CEWAF and (C) Corexit 9500A, expressed as nominal concentrations of oil (ppm or mg L^{-1}) or dispersant (ppm; 1:10 ratio). (A): 62.5 ppm HEWAF corresponds to 108.3 µg tPAHs L^{-1} and 1,000 ppm HEWAF corresponds to 1,635 µg tPAHs L^{-1}. (B): 6.25 ppm CEWAF corresponds to 1.3 µg

tPAHs L^{-1} and 100 ppm CEWAF corresponds to 26.2 µg tPAHs L^{-1}. (C): 0.625 ppm Corexit corresponds to 62.5 µg DOSS L^{-1} and 10 ppm Corexit corresponds to 1000 µg DOSS L^{-1}. Data are presented as mean percentage ± standard deviation (SD). Different letters denote a significant difference at $p \leq 0.05$ (ANOVA, Tukey HSD post-hoc test).

3.3. Effects on embryogenesis

Percentages of abnormal larvae in the controls were relatively low and ranged from 0 to 10%. Continuous exposure of gametes from pre-fertilization until 24 h post-fertilization to HEWAF, CEWAF or Corexit 9500A® significantly and negatively impacted embryogenesis in a dose-dependent manner (*p<0.001*). Compared to controls, significantly higher number of morphological abnormalities were observed at nominal concentrations of 250 ppm (equivalent to 417 µg tPAHs L^{-1}) after exposure to HEWAF (*p<0.05*), and as low as 50/5 ppm (or 14.2 µg tPAHs L^{-1}) for CEWAF (*p<0.001*) and 5 ppm (or 500 µg DOSS L^{-1}) for dispersant (*p<0.001*) (Figs. 3 and 4). In a similar way, continuous exposure of newly segmented embryos (post-fertilization) for 24 h to all HEWAF, CEWAF and dispersant concentrations affected embryo development (*p<0.001*). Larval abnormalities were significantly higher in embryos exposed to HEWAF (125 ppm; *p<0.05*), CEWAF (50/5 ppm; *p<0.01*) and Corexit (10 ppm; *p<0.001*) compared to control treatments (Fig. 3). Although gametes exposed before fertilization to HEWAF showed a higher LOEC (250 ppm) than embryos exposed after fertilization (125 ppm, Fig. 3A and B), 2-way ANOVA showed that, overall, abnormalities in gametes were statistically higher than in embryos (*p<0.001*). From these results, sensitivity to CEWAF and Corexit appears to be stage-dependent, with LOEC values lower for the gamete exposure compared with the embryo exposure (Fig. 3C to F). In addition, when comparing CEWAF and Corexit, 50/5 ppm of CEWAF elicited higher abnormalities for both life stages tested than dispersant alone (5 ppm), at equivalent nominal doses tested (Fig. 3C to F).

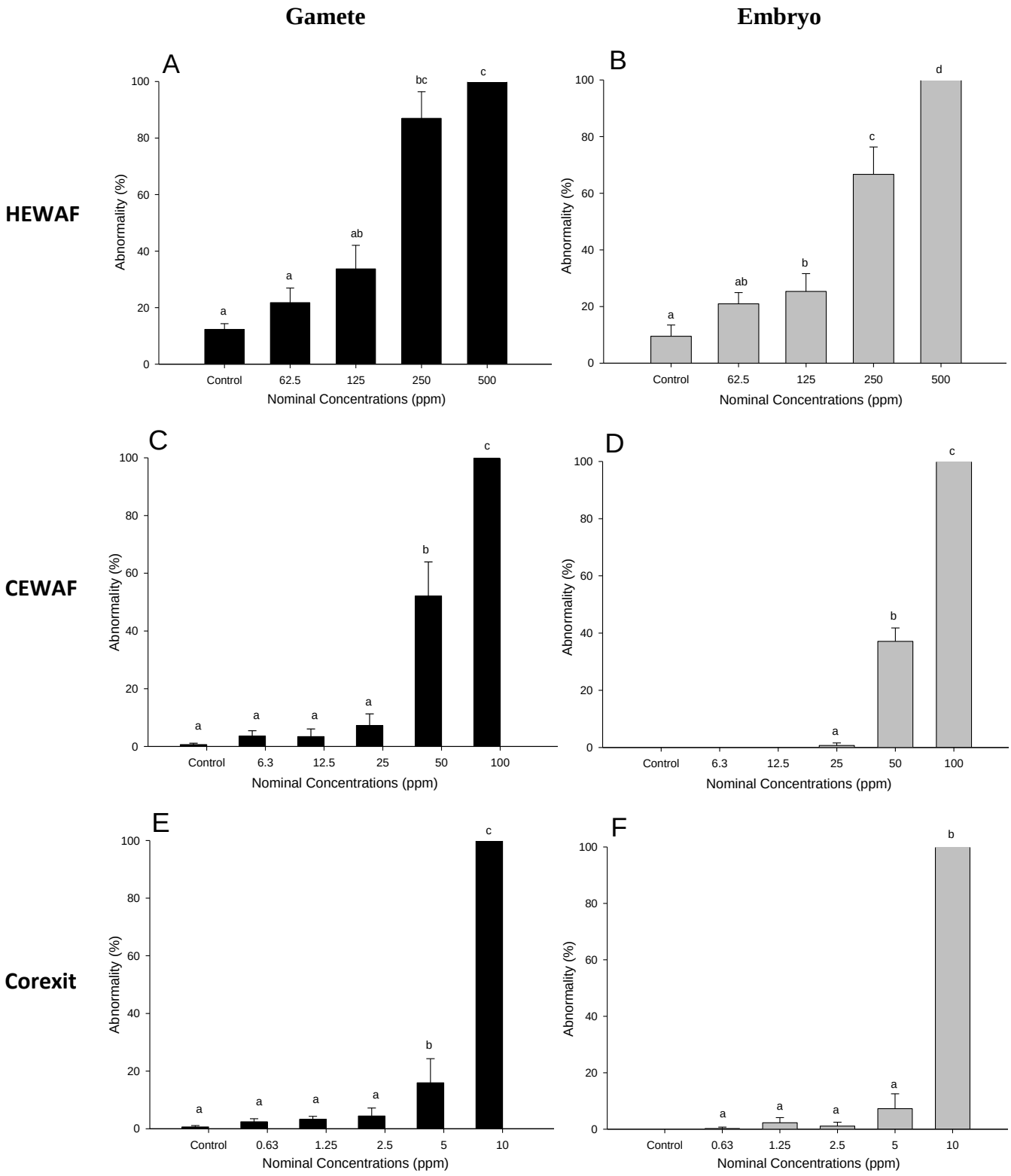

Figure 3: Mean percentages (±SD) of abnormal larvae derived from gametes (left) and embryos (right) exposed to HEWAF (A, B), CEWAF (C, D) and dispersant (E, F) expressed as nominal concentrations of oil (ppm or mg oil L^{-1}), or dispersant (ppm, 1:10 ratio) for 24 h post fertilization. Nominal concentrations of HEWAF (A, B) 62.5, 125, 250, 500 and 1000 ppm correspond to measured tPAHs (n=50) 108.3, 198.1, 417, 839.4 and 1,635 µg PAH L^{-1} respectively. Nominal concentrations of CEWAF (C, D) 6.25, 12.5, 25, 50 and 100 ppm correspond to measured tPAHs concentrations 1.3, 3.3, 6.4, 14.2 and 26.2 µg tPAHs L^{-1} respectively. Nominal concentrations for dispersant (E, F) 0.625, 1.25, 2.5, 5 and 10 ppm correspond to measured DOSS concentrations 62.5, 125, 250, 500 and 1000 µg DOSS L^{-1} respectively. Different letters denote a significant difference at $p \leq 0.05$ (ANOVA, Dunnett's post-hoc test).

After 24 h of incubation, visual observations revealed morphological abnormalities ranging from severely moribund to atrophied larvae (Fig. 4D), absence of shells, individuals with a convex-hinge, shell deformities (Fig. 4E), velum abnormally extended, incomplete shell and mantle extrusion (Fig. 4F) or indented shell margins.

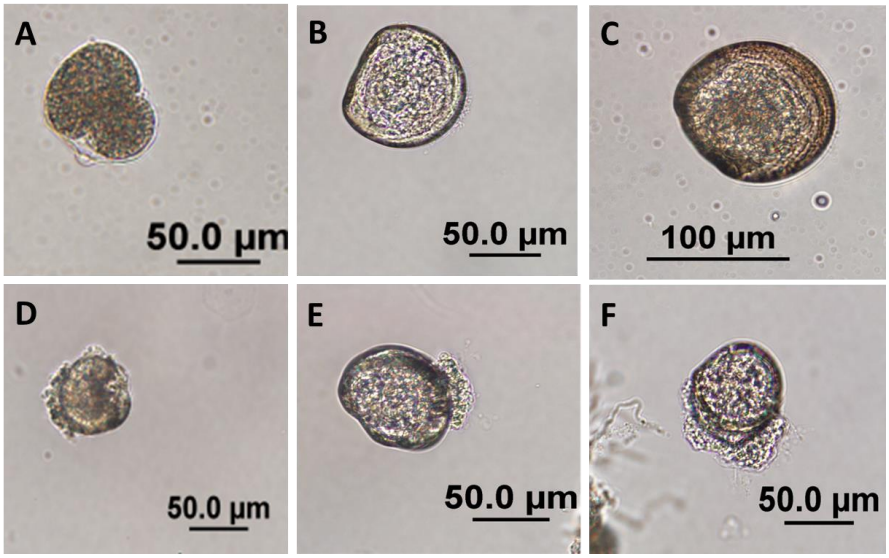

Figure 4: Normal embryo (A), normal 24 h-old (B) and 96 h-old larvae (C), and abnormal larvae (D, E, F) observed after exposure to HEWAF.

3.4. Effects on larval size

Control treatments showed an increase of mean shell lengths from 5 to 10 µm over 3 days. Mean shell lengths of larvae exposed as gametes or embryos to CEWAF, HEWAF, or dispersant for 24 and 96 h consistently declined as exposure concentrations increased. Regarding exposure to HEWAF, mean size of larvae derived from exposed gametes was significantly impaired at 125 ppm (equivalent to 198.1 µg tPAHs L^{-1}) from 24 h onward (Fig. 5A), whereas mean size of larvae derived from exposed embryos was inhibited significantly at 62.5 ppm (equivalent to 108.3 µg tPAHs L^{-1}) compared to control ($p<0.001$) (Fig. 5B). In addition, 2-way ANOVA showed that mean size after 96 h was significantly lower for larvae derived from embryo than gamete ($p=0.008$, data not shown).

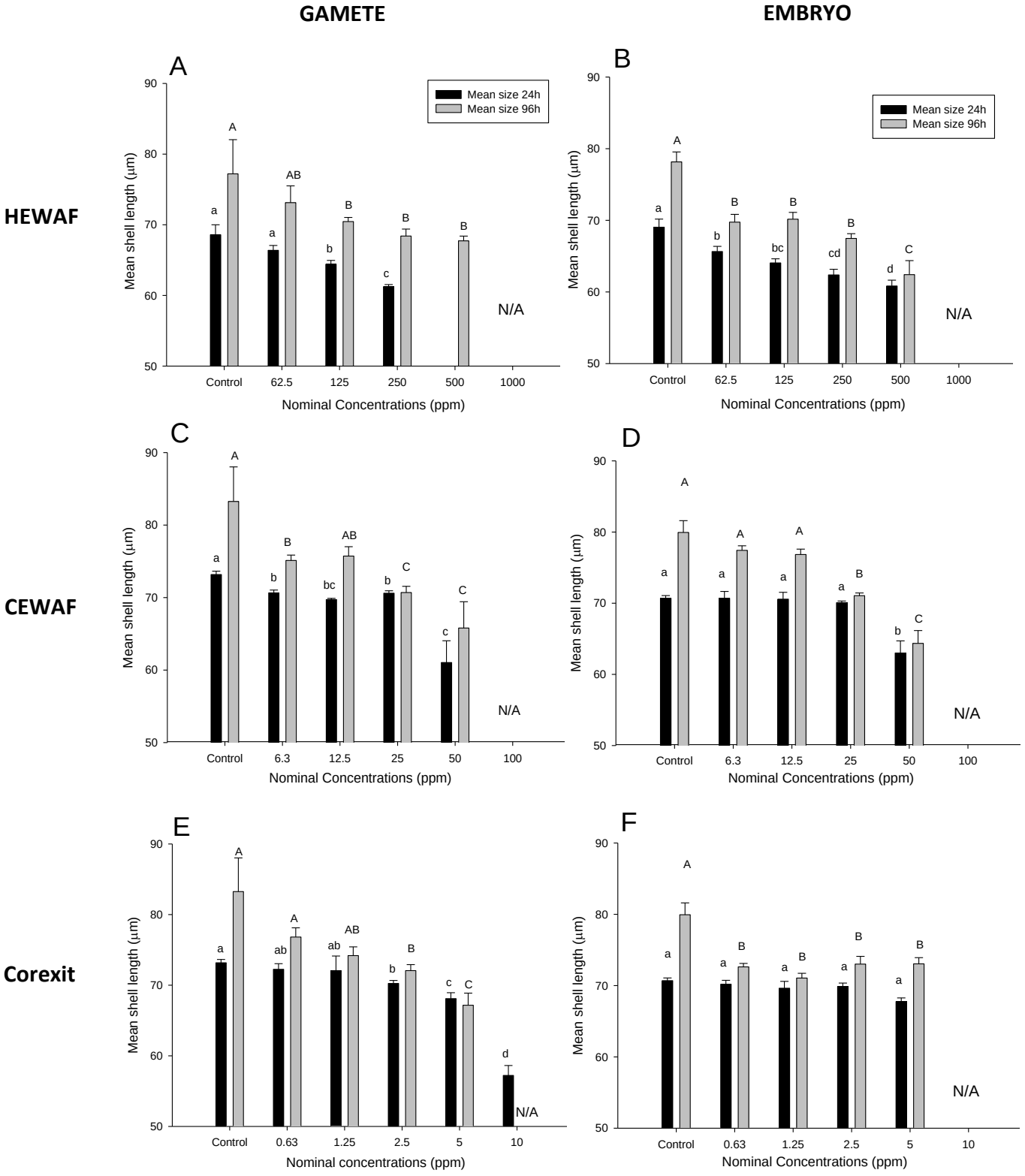

Figure 5: Mean shell lengths of larvae (n=100) derived from gametes (eggs and sperm) and embryos exposed to HEWAF (A, B), CEWAF (C, D) and dispersant (E, F), expressed as nominal concentrations of oil (ppm or mg oil L^{-1}), or dispersant (ppm, 1:10 ratio). Nominal concentrations for HEWAF (A, B) 62.5, 125, 250, 500 and 1000 ppm correspond to measured PAH concentrations (n = 50) 108.3, 198.1, 417, 839.4 and 1,635 µg tPAHs L^{-1} respectively. Nominal concentrations for CEWAF (C, D) 6.25, 12.5, 25, 50 and 100 ppm correspond to measured PAHs concentrations 1.3, 3.3, 6.4, 14.2 and 26.2 µg tPAHs L^{-1} respectively. Nominal concentrations for dispersant (E,F) 0.63, 1.25, 2.5, 5 and 10 ppm correspond to measured DOSS concentrations 62.5, 125, 250, 500 and 1000 µg DOSS L^{-1} respectively. Mean shell lengths are expressed in µm ± standard deviation (SD). N/A:

no larvae were observed. Different letters denote statistical difference at $p \leq 0.05$ (ANOVA, Tukey HSD or Dunnett's post-hoc test).

For the exposure to CEWAF, larvae derived from exposed gametes did not grow at concentration above 25/2.5 ppm (or 6.4 µg tPAHs L^{-1}) compared to control (*p<0.01*) (Fig. 5C). Moreover, all concentrations of CEWAF significantly impacted the mean shell length of larvae developed from exposed gametes after 24 h of exposure, compared to control (*p<0.001*) (Fig. 5C). Nominal concentrations of CEWAF as low as 6.3/0.63 ppm (corresponding to 1.3 µg L^{-1}) reduced significantly the mean shell length of larvae exposed for 24 h and 96 h to CEWAF from the gamete stage (*p<0.01,* Fig. 5C). On the contrary, even though CEWAF exposure reduced significantly the mean shell size of larvae exposed from embryos (*p<0.001*), only the 50/5 ppm and 25/2.5 ppm concentrations affected the mean shell lengths at 24 h and 96 h respectively (*p<0.001,* Fig. 5D). These results reflected an increase of sensitivity to CEWAF when exposure was done prior fertilization (*e.g.* embryos compared to gametes).

Corexit exposure induced a dose-response in shell length at 24 h and 96 h for larvae exposed from the gamete stage, with a LOEC value reaching 2.5 ppm (Fig. 5E). Contrastingly, no effect on mean size of larvae compared to control was observed after 24 h of exposure to dispersant from the embryo stage (*p=0.063*); whereas mean shell lengths were significantly inhibited after 96 h of exposure to concentration of 0.63 ppm (or 62.5 µg DOSS L^{-1}) and above (Fig. 5F). Absence of data at the highest concentrations was due to high mortalities.

3.5. Effects on survival after 96 h

Mortalities for every exposed group were dose-dependent. In the highest concentrations, dead larvae were noted with extruded and granulated tissues ("exploded" larvae). Compared to control, HEWAF, CEWAF and dispersant exposures significantly reduced larval survival at 96 h, regardless of the starting time of exposure (before or after fertilization) (data not shown). CEWAF was toxic to both life stages exposed, before or after fertilization, inducing 50 % of mortalities ($LC50_{96h}$) at nominal concentrations of 32 ppm (or 3.2 ppm of dispersant) corresponding to 8.5 µg tPAHs L^{-1} and 67 ppm (or 6.7 ppm of dispersant) corresponding to 17.7 µg tPAHs L^{-1} respectively (Table 3). In addition, $LC50_{96h}$ value of larvae derived from gametes exposed to Corexit only was 2.7 ppm (corresponding to 269 µg DOSS L^{-1}) (Table 3). Regarding HEWAF exposures, nominal concentrations of 184 ppm (or 307 µg tPAHs L^{-1})

and 130 ppm (corresponding to 220 µg tPAHs L^{-1}) significantly reduced (-50%) survival of larvae derived from exposed gamete and embryos respectively (Table 3).

According to the $LC50_{96h}$ values for CEWAF, larvae resulting from exposed gametes were about 2 times more sensitive than larvae resulting from exposed embryos (8.5 *vs* 17.7) and stage comparison using 2-way ANOVA confirmed this result (*p<0.001*). Contrastingly, according to the $LC50_{96h}$ values for HEWAF exposures, larvae resulting from exposed embryos were more sensitive than those exposed from gametes (307 *vs* 220) (Table 3) with 2-way ANOVA showing a significant difference between stage (*p<0.001*). $LC50_{96h}$ for Corexit exposure of embryos could not be calculated due to a lack of fit of the model. It is important to mention that for all exposures, initial fertilization success was accounted for in the $LC50_{96h}$ calculation.

Table 3: Concentrations causing 50 % of mortality (LC50) for gametes and embryos exposed for 96 h to HEWAF, CEWAF or dispersant, calculated using binomial response variables, fitted with a 3-parameter log-logistic model, using the *drc* and *bbmle* packages in R. Data are expressed as nominal concentrations (mg oil L^{-1} or ppm), measured concentrations of a sum of 50 PAHs (µg sum PAH L^{-1}) for oil preparations (HEWAF and CEWAF) or µg DOSS L^{-1} for dispersant (± 95 % CI). Non-fertilized eggs were accounted in total number (subtraction of unfertilized).

	Gamete			Embryo		
LC50	**HEWAF**	**CEWAF**	**Dispersant**	**HEWAF**	**CEWAF**	**Dispersant**
Nominal (ppm)	**184** (181-187)	**32/3.2** (32-33)	**2.7** (2.6-2.8)	**130** (127-132)	**67/6.7** (66-67.4)	**NC**
ΣPAH (µg L^{-1})	**307** (303-312)	**8.5** (8.4-8.7)	----------	**220** (216-224)	**17.7** (17.5-17.9)	----------
DOSS (µg L^{-1})	----------	**#**	**269** (262-276)	----------	**#**	**NC**

#: DOSS not quantified in CEWAF samples. NC: Not Calculable.

4. Discussion

The explosion of the Deepwater Horizon (DWH) oil platform on April 20th, 2010, resulted in the largest oil spill in U.S history (National Commission, 2011). This oil spill coincided with the spawning season of the Eastern oyster, *Crassostrea virginica*. Prior to the DWH oil spill incident, no deep-water application of dispersant such as the Corexit 9500A® had been conducted and there are no data concerning the environmental fate and the toxicity of dispersants, including Corexit 9500A®. Oysters, due to their ecological importance (e.g., filter feeding activity, sessile nature, ubiquity), have been employed as a model organism in eco-toxicological studies and for bio-monitoring the marine environment. But, the information concerning toxicity of crude oil, dispersed oil and dispersant itself on *C. virginica* gametes and embryos, and their effects on fertilization success and early development is limited. A detrimental effect of the oil and associated dispersant on the reproduction and early development of *C. virginica* could impact the recruitment and consequently decimate the oyster natural stocks in the affected areas, which has ecological and economic significance.

4.1. Effects on fertilization

Results of the present study showed that exposures of sperm and eggs of *Crassostrea virginica* to HEWAF, CEWAF or dispersant significantly reduced fertilization in a dose-dependent manner. Our study showed that mechanically mixed water accommodated fractions (HEWAF), reduced fertilization success with an $EC50_{1h}$ value reaching ≈ 1,400 mg DWH oil L^{-1} (equivalent to 2,250 µg tPAHs L^{-1}). In the same way, Renzoni (1975) found that, after exposing *C. virginica* sperm and eggs separately, for an hour before fertilization, to crude oils prepared in a similar way to the HEWAF, fertilization was depressed in a dose – dependent way, with fertilization success falling to 60-70 % at 1,000 mg oil L^{-1}.

After addition of Corexit 9500A® to DWH oil, concentrations of CEWAF inhibiting fertilization success were relatively low, reaching $EC20_{1h}$=19.4 µg tPAHs L^{-1} or 73 mg of crude oil L^{-1}. However, LOEC and ECx results indicated that fertilization success after CEWAF and dispersant exposure followed similar trends. Consequently, one can suggest that most of the toxic effect induced by the dispersed oil can be attributed to the dispersant fraction of the CEWAF (i.e. 1:10). Toxicity of Corexit dispersants on the fertilization success

of marine invertebrates has long been reported (Hagstrom and Lonning, 1977; Law, 1995). Negri and Heyward (2000) found that by dispersing oil chemically using Corexit 9527, fertilization capabilities were significantly reduced for coral species. However, it can become challenging to dissociate the contribution of dispersant from the toxic action of PAHs: as a consequence, an interactive/synergistic action of PAHs on the fertilization process following chemical dispersion must not be ruled out. According to Jeong and Cho (2005), movements of sperm cells from *Crassostrea gigas* were greatly affected upon exposure to 10 individual PAHs at concentrations as low as 50 ppb (or 50 μg L^{-1}), with viability and motility significantly decreased with increasing concentrations of PAHs (from 50 to 200 ppb). As a result, the inhibition of gamete fertilization observed in the present study could be explained by a potential direct effect of dispersant and/or oil on the swimming behavior of the sperm cells and a decrease in the rate of collision between eggs and spermatozoa, as Lewis et al. (2008) showed with a polychaete species, *Arenicola marina*, exposed to crude oil.

Our results are consistent with those of Donaghy et al. (2012), who found that exposure of *C. virginica* sperm to HEWAF, CEWAF or Corexit 9500A® had detrimental effects on cellular mechanisms that are potentially involved in fertilization processes. Donaghy et al. (2012) observed morphological modification of sperm cells (changing of size and increased internal complexity with increasing oil concentrations), alteration of acrosomal integrity, and inhibition of intracellular ROS production and mitochondrial metabolism. The physiological impairment of sperm cells could impact their motility and hence reduce their fertilization capabilities. In the same way, the spermiotoxicity of the herbicide diuron on *C. gigas* sperm induced an oxidative stress and impacted directly the motility of sperm cells (Akcha et al., 2012) but fertilization success was not measured concomitantly.

4.2. Effects on embryogenesis

Embryogenesis is a sensitive life stage where drastic physiological processes occur. For instance, shell formation begins early in embryogenesis by secretion of the very first shell material by shell field epithelial cells (Eyster & Morse, 1984). Our results indicated that continuous exposure of gametes from pre-fertilization until 24 h post-fertilization to HEWAF, CEWAF or Corexit 9500A® negatively impacted embryogenesis in a dose-dependent manner. Exposed gametes produced fewer embryos, and those embryos had higher

percentages of morphological abnormalities than control treatments. Similarly, continuous exposure of newly segmented embryos for 24 h to HEWAF, CEWAF and dispersant concentrations affected adversely embryonic development, suggesting a deleterious effect of both oil and dispersant on the mechanisms involved in shell secretion. Similar results were reported on *C. gigas* embryos contaminated with Alaskan crude oil which led to severe abnormalities, such as deformed and incomplete shells, with $EC50_{48h}$ value of 1,000 ppm or 1 ml L^{-1} (Le Gore, 1974). In a similar way, Le Pennec and Le Roux (1979) showed that exposure of early developing embryos to a different type of crude oil for only one hour induced high instances of shell abnormalities in *Mytilus edulis* larvae, which exhibited both great modifications of the hinge and crystallization of the shell. Since morphological effects of CEWAF, HEWAF and Corexit were only apparent after 24 h of exposure in the present work, it appears that calcification during oyster embryogenesis is one of the processes most sensitive to oil and/or dispersant. Hayakaze and Tanabe (1999) revealed that the organic matrix of the mussel *Mytilus galloprovincialis* shell began to be secreted by the shell gland during the late trocophore larval stage (≈ 12 h PF), and calcification was localized on the inner side of the organic matrix in the early veliger stage. Similarly, early veliger calcification follows the same process in the European oyster *Ostrea edulis* (Waller, 1981). Other aspects such as protein synthesis and metabolism require further investigation. Oil/PAHs or the dispersant itself could also interfere and inhibit certain enzymatic activities as it has been already reported with oyster embryos exposed to heavy metals, for instance carbonic anhydrase, involved in shell formation and calcium transport pathways (Hinkle et al., 1987). Finally, Wessel et al. (2007) described that exposure of *C. gigas* embryos to the PAH benzo[α]pyrene (BaP) could cause high rates of abnormal larvae and DNA strand breakage.

Overall, statistical analyses and percentages of abnormalities observed after 24 h of exposure to HEWAF, CEWAF and Corexit indicated that embryos were less sensitive than gametes in their toxicity response to PAH and/or dispersant. This finding is in contradiction with results reported by Geffard et al. (2001) which found that exposure of *C. gigas* embryos (post-fertilization) to PAH-polluted sediment induced more abnormalities than exposure of sperm (pre-fertilization). However, these authors exposed only sperm to PAH, while in the present study both eggs and sperm were exposed simultaneously, which could cause further damage to subsequent embryogenesis. From this, one may speculate that oocyte exposure to oil/PAHs and dispersant may be more likely to result in abnormal embryonic development than sperm exposure.

4.3. Effects on larval shell lengths

In addition to the significant impacts of oil and Corexit 9500A® on *Crassostrea virginica* developmental success, HEWAF, CEWAF or dispersant can subsequently inhibit shell growth. Our results showed a deleterious effect of all tested concentrations of oil, dispersed oil and dispersant on the mean size of veliger larvae after 24 h of exposure. Shell lengths were significantly smaller for larvae reared in exposure solutions compared to control. As mentioned above, oil and/or dispersant can disrupt normal embryogenesis and in particular shell formation, resulting in reduction or inhibition of shell growth. Our results also demonstrated that shell growth of exposed larvae consistently declined in a dose-dependent way, with level of tPAHs for CEWAF as low as 6.4 µg L^{-1} (or 25/2.5 ppm) inhibiting completely the growth of larvae after 96 h (Fig. 3C and D), concentration of PAHs lower than that which caused appreciable abnormality (14.2 µg tPAHs L^{-1}) in the 96-h experiment. This finding suggests that larval growth is a sensitive and valid indicator of toxicity of oil/dispersant. Hidu (1965), His and Robert (1985) and Geffard et al (2002b) all showed that larval growth assay with oyster was more sensitive than embryo assay using abnormality. A marked reduction in larval growth may increase chances of death by predation or other factors (Davis and Hidu, 1969; Calabrese et al., 1973). Moreover, Strathman and Hart (1987; 1995) have postulated that smaller larvae encounter and clear less food than larger ones, and are therefore more prone to starvation. Hence, oil and dispersant may reduce fitness of affected larvae by reducing feeding efficiency, even if the concentrations they are exposed to are not lethal. As previously mentioned, dispersant chemical compounds as well as PAH released from oil preparations could interfere with early larval development reducing intrinsically growth. Oil droplets resulting from dispersion could also have a negative physical effect such as clogging the gills or binding to the velum (Anderson et al., 1974; Renzoni, 1975; Sigler and Leibovitz, 1982) or inhibiting respiration by coating membranes, particularly with the HEWAF exposure. It is hypothesized that these droplets may also aggregate with algal cells (Rubinstein et al., 1984; Muschenheim and Lee, 2002) and may significantly reduce food availability (increase of size of particle, flocculation, and emulsification) and impact filtration rates of larvae, resulting in starvation and death. In order to address this issue, it would be interesting to monitor additional endpoints, such as swimming activity and feeding behavior, in order to get a more complete and sensitive approach.

4.4. Effects on larval survival

Preliminary range finding experiments showed that DWH oil prepared as low energy WAF (LEWAF or WAF) had little impact on the survival of early stages of oysters, with LOEC values ranging from 1,000 to 2,000 ppm (data not shown). Contrastingly, following exposure to mechanically dispersed DWH oil (HEWAF), larval survival was significantly reduced (-50%) at 130 to 184 ppm (equivalent to 220 to 307 µg tPAHs L^{-1}). In light of these results, high energy preparation (HEWAF) method seem to be a good surrogate for oil slicks submitted to the action of current, wind and waves, as demonstrated by Incardona et al (2013). Moreover, concentrations of CEWAF and Corexit inhibiting 50% of larval survival were relatively low, 32/3.2 to 67/6.7 ppm (equivalent to 8.5 to 17.7 µg tPAHs L^{-1}) and 2.7 ppm (equivalent to 269 µg DOSS L^{-1}) respectively. Various studies found that, compared to oil alone (WAF), addition of Corexit 9500A to oil (CEWAF) decreased survival during exposure of amphipods (Gulec et al., 1997), copepods (Lee et al., 2013), mussels and sea-urchins (Saco-Alvarez et al., 2008), and coral species (Negri and Heyward, 2000). Similarly, Rico-Martinez et al (2013) indicated that addition of Corexit 9500A® to Macondo oil (or DWH oil) at a ratio realistically encountered in the Gulf at the time of the spill (1:10) increased toxicity to rotifer *Brachionus plicatilis* by 52-fold compared to WAF. It cannot be excluded that increased toxicity observed in these studies could be mainly attributed to dispersant alone. However, at the highest doses tested (above 50 ppm of oil and 5 ppm of dispersant), CEWAF exposure resulted in a higher percentage of abnormality than Corexit alone (at 5 ppm) (Fig. 3).

According to the U.S National Research Council (1989), acute lethal toxicity of chemically dispersed oil resides not only in the dispersant but primarily in the bioavailability of oil droplets (for some species) and the low molecular weight and dissolved aromatic fractions of the oil (for most species). Apart from the results of abnormalities at the highest doses of CEWAF previously mentioned, the present study did not demonstrate an exacerbated effect of CEWAF on larval survival compared to Corexit alone. In fact, at equivalent nominal doses tested, our results demonstrated that in most cases, CEWAF was even less toxic than dispersant alone (Table 3). Fucik (1994) reported similar results with eastern oyster embryos that he exposed to the Corexit 9527 alone or mixed with Gulf of Mexico crude oil, with LC50 of 4.9 ppm and 11.2 ppm respectively. This finding suggests that most of the toxic effect of the dispersed oil is likely imputable to some of the chemical characteristics of the Corexit.

Due to the DOSS compound and its surfactant properties (NALCO, 2012), dispersant can break down oil into very small particles (NRC, 2005), which may make oil droplets more likely to affect filter-feeding organisms like Eastern oyster larvae through ingestion or by clogging of the gills. Additionally, acute toxicity of dispersant alone is usually attributed to its surface-active effect on bio membranes, including the disruption of respiratory cells resulting from electrolytic and/or osmotic imbalances (Abel, 1974; Singer et al., 1991; 1996). Accordingly, microscopic observations of organisms in 96 h samples of the present study exhibited severe damages such as membrane rupture and "explosion" of cells and extrusion of larval tissues which could be attributed to the dispersant. Overall, it can become challenging to dissociate the contribution of dispersant from the toxic action of PAHs: as a consequence, an interactive/synergistic action of PAHs on the embryogenesis process following chemical dispersion must not be ruled out. Thus, caution should be used when deciding to utilize chemical dispersion as a remedial for an oil spill, especially in a coastal marine environment.

$LC50_{96h}$ values determined for CEWAF and Corexit exposure were significantly lower for larvae derived from gametes exposed before fertilization than those of larvae exposed from embryos after fertilization, suggesting that gametes were more sensitive than embryos to chemically dispersed oil and dispersant. In contrast, our results showed that survival after 96 h of exposure to HEWAF was more affected for larvae derived from embryos compared to the survival of larvae derived from exposed gametes, suggesting that embryos were more sensitive than gametes to HEWAF.

Overall, our results demonstrated a stage-dependent response to CEWAF and Corexit, PAH and/or dispersant inducing more abnormal embryogenesis, retardation of growth and more mortality in larvae derived from exposed gametes than embryos. For HEWAF, the same trend was found for embryogenesis abnormalities. However, longer term exposure to HEWAF (96 h) seemed to impact further growth and survival of larvae derived from exposed embryos than gamete. This finding is in agreement with Geffard et al (2001)'s study which found that embryos were more sensitive to PAH exposure than sperm of *C. gigas*. This difference of stage sensitivity to HEWAF (embryo > gamete) may be due to high variability between treatments. Perhaps exposure to droplet-associated PAH contained in the HEWAF may be more toxic to embryos than sperm and oocytes. To answer this question, additional research on the toxicity related to the dissolved and particulate fraction of HEWAF, and particularly on the interaction of these droplets with various life stages of oyster is needed.

The ranges of concentration tested for this study were chosen to cause lethal as well as sub lethal effects on different life stages of oysters, in order to determine median lethal and effective concentrations. From these data, it is now possible to compare our findings with field-collected data, and predict acute effects likely to occur to eastern oysters as well as other filter-feeding species in field situations and improve the management of water quality of coastal zones worldwide. It is important to note that, in the natural environment, toxicity of crude oil not only depends on the concentration of oil and duration of exposure but also on environmental conditions. As a result, the impact of accidental oil spills on early life stages of oyster will vary depending on the specific circumstances of each catastrophe. For instance, a factor that we did not consider in this study is photo-induced toxicity. Ultraviolet radiation (UVR) may increase the toxicity of petroleum hydrocarbons (e.g. PAHs) on exposed marine organisms (Landrum et al., 1987). For example, it has been shown that exposure to UVR at environmentally typical levels could exacerbate PAH toxicity to bivalve embryos from 5 times to 100 times (Pelletier et al., 1997; Lyons et al., 2002). Although speculation and extrapolation of specific laboratory studies to the field needs to be taken cautiously, experimental studies are a reliable way of determining major toxic effects of hydrocarbons on sensitive aquatic organisms such as oyster larvae.

Harmful effects of PAHs on marine organisms (Barron et al., 1999; Ramachandran et al., 2004; Lee et al., 2013) and notably bivalves and oysters have long been documented (Pelletier et al., 2000; Geffard et al., 2002b; 2003; Lyons et al., 2002; Jeong and Cho, 2005; Wessel et al., 2007; Wang et al., 2012). The present study provided evidence that oil released during the DWH explosion, and particularly the application of dispersant can seriously impair the early development of the eastern oyster, even at very low concentrations. Oil spills could pose long term effects on aquatic animals and the ecosystem. Considering the persistence of PAHs in the environment, upward transfer of PAHs through the trophic hierarchy ultimately affecting human health is a realistic scenario. Oyster larvae may bio-accumulate various PAHs, especially those with high molecular weights by ingestion through filtration of oil micelles previously dispersed like other zooplankton species (Almeda et al., 2013; Lee et al., 2013). Thus, further research needs to be done on the potential for PAHs bio-accumulation in bivalve larvae.

5. Summary

The present study determined the potential impacts of the DWH oil spill and the application of dispersant to treat oil slicks on early life stages of the American oyster via short-term exposure experiments. Results revealed that exposure of oyster gametes and embryos to oil preparations and dispersant impaired fertilization, normal development of embryos to larval stage, and growth of resulting larvae. It also induced larval death, with dispersed oil and dispersant alone producing usually the highest impact. These results suggest that oil spills and employing dispersants to disperse oil at the time of oyster spawning season could affect oyster recruitment and ultimately oyster resources in affected regions.

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Article 2

Sensitivity of *Crassostrea virginica* gametes to dispersed oil: cellular responses of spermatozoa and oocytes and impacts on fertilization and embryogenesis

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Abstract

The recent Deepwater Horizon (DWH) oil spill incident in the Gulf of Mexico (April 2010) was unprecedented in total volume of crude oil (779 000 m^3) and dispersant (≈ 8 million L) released into a marine ecosystem. Due to the timing of the spill, coincident with the spawning season of *Crassostrea virginica*, unprotected eggs and sperm were likely exposed to the toxic effects of crude oil and dispersant. The aim of this study was to evaluate the potential acute cytotoxicity of DWH oil, dispersed mechanically (HEWAF) and chemically (CEWAF), on sperm and oocytes using flow-cytometry analyses (*in vitro*), and relate these cellular effects to fertilization success and embryogenesis using a 24-h *in vivo* assay. In addition, cross-fertilizations of oil-exposed gametes were performed to determine whether spermatozoa or oocytes were the most sensitive to CEWAF and HEWAF. *In vitro* results indicated that only morphology of sperm cells was altered by 30 min incubation with HEWAF solution, whereas viability of sperm was impacted by CEWAF exposure. Additionally, ROS production in exposed oocytes increased with increasing doses of DWH oil, suggesting an oxidative stress likely due to PAHs and/or dispersant. *In vivo* assays suggested dose-dependent decreases of fertilization (1 h) and survival (24 h) as well as an increase in abnormal embryos (6 h and 24 h) related to altered cellular characteristics. Finally, *in vivo* results alluded that oocytes were more susceptible to PAHs/dispersant toxicity than sperm and they may contribute to the deleterious effects of oil/dispersant on fertilization and embryogenesis.

Keywords: sperm, oocyte, Deepwater Horizon oil, cytotoxicity, fertilization, ROS, flow-cytometry

Résumé

La récente marée noire de DWH dans le Golfe du Mexique (Avril 2010) fut sans précédent en terme de volumes de pétrole brut (779,000 m^3) et dispersants (≈ 8 million L) relâchés dans un écosystème marin. Du fait de la période de la marée noire, coïncidant avec la saison de ponte de *Crassostrea virginica*, les œufs et sperme non-protégés furent potentiellement exposés aux effets toxiques du pétrole et du dispersant. Cette étude avait pour but d'évaluer l'éventuelle cytotoxicité aigüe du pétrole DWH, dispersé mécaniquement (HEWAF) et chimiquement (CEWAF), sur les spermatozoïdes et ovocytes en utilisant des analyses de cytométrie en flux (*in vitro*), et de rapporter ces effets aux taux de fécondations et développement embryonnaire observés durant les bioassays de 24-h (*in vivo*). Par ailleurs, des croisements de gamètes exposés au pétrole ont été effectués afin de déterminer si les spermatozoïdes ou les ovocytes étaient plus sensibles au CEWAF et HEWAF. Les résultats *in vitro* ont suggéré que seulement la morphologie des cellules de sperme fut altérée par une incubation de 30 min avec des solutions de HEWAF, et que la viabilité des cellules de sperme fut impactée par le CEWAF. Nous avons également établi que la production de ROS chez des ovocytes exposés ont augmenté avec des doses croissantes d'hydrocarbures, ce qui suggère un stress oxydatif probablement dû aux HAP/dispersant. Les assays *in vivo* ont montré des baisses dose-dépendantes de la fécondation (1 h) et de la survie (24 h) mais aussi une hausse du nombre d'embryons présentant des anomalies (6 h et 24 h), très certainement associées aux caractéristiques cellulaires modifiées. Enfin, il semblerait que les ovocytes soient plus susceptibles à la toxicité des HAP/dispersant que les spermatozoïdes, et qu'ils pourraient contribuer aux effets néfastes du pétrole/dispersant sur la fécondation et l'embryogénèse.

Mots-clés : sperme, ovocytes, pétrole brut DWH, cytotoxicité, fécondation, ROS, cytométrie en flux.

1. Introduction

The recent Deepwater Horizon (DWH) oil spill in the Gulf of Mexico, the world's largest accidental release of crude oil into the ocean in history (National Commission, 2011), resulted in the discharge of more than 4 million barrels of oil into the offshore waters between April 20th and July 15th, when the rig was finally capped (Crone and Tolstoy, 2010; Camilli et al., 2012). Although subsurface application of almost 3 million L of the dispersant Corexit 9500A near the wellhead (1500 m depth) enabled the retention of a considerable portion of oil in the water column and the creation of a "plume" (Camilli et al., 2010; JAG, 2010; Lehr et al., 2010; Kujawinski et al., 2011), oil also moved to the upper surface waters to form a slick. The surface coverage of that oil slick was estimated at ≈ 63,000 km^2 on June 25th, and reached land (mainland Louisiana) for the first time on May 15th (OSAT/NOAA, 2010).

Eastern oysters, *Crassostrea virginica*, are abundant in the northern region of the Gulf of Mexico. They are economically important as they are highly valued as food, but their ecological significance for the region is even more important (Coen et al., 1999) as oyster reefs provide valuable habitat for many estuarine species (Wells, 1961; Volety et al, 2014). In addition, oysters, like many other aquatic organisms, release their gametes in the surrounding waters where fertilization takes place. In the case of the DWH event, the spill overlapped with oyster spawning season, which occurs in the Gulf from late spring through late fall (Ingle, 1951). Consequently, unprotected eggs and sperm were likely susceptible to the toxic effects of petroleum hydrocarbons released during the DWH oil spill. Numerous studies have shown that acute exposure of sperm and eggs to organic pollutant could negatively impact fertilization success and embryogenesis of oysters (Renzoni, 1975; Geffard et al., 2001; Lyons et al., 2002; Jeong and Cho, 2005; Wessel et al., 2007; Akcha et al., 2012). Similarly, our previous work suggested that oyster gametes were sensitive to oil exposure and dispersant: CEWAF concentration of 110 ppm of oil (and 11 ppm of dispersant) and HEWAF concentration of 1,355 ppm reduced by 50% the fertilization success of gametes exposed for 30 min before fertilization (Vignier et al., in prep a/Article 1). After 24 h of continuous exposure, embryogenesis was also significantly affected (50% abnormal) at CEWAF concentration of 50 ppm oil and 5 ppm dispersant, and HEWAF concentration of 250 ppm (Vignier et al, in prep a/Article 1). In this previous study, for ecological relevance, eggs and sperm were exposed simultaneously to oil and dispersant. However, it is unclear from these results whether the reduced fertilization success

and subsequent altered embryogenesis were the consequences of impaired spermatozoa or oocyte function, or the result of an interaction of both.

Successful fertilization for any species depends on the production of high quality spermatozoa, resulting in appropriate motility of sperm and fertilizing capability (Lewis and Ford, 2012). Specifically, success of fertilization also depends on other various cellular characteristics of the spermatozoa and/or oocytes of which morphology, viability, DNA integrity, mitochondrial activity or production of reactive oxygen species (ROS) can be rapidly measured using flow-cytometry (Cordelli et al., 2005; Gillan et al., 2005; Martinez-Pastor et al., 2010; Le Goïc et al., 2014). Following their release during spawning, swimming sperm will reach oocytes and bind to the extracellular membrane of the eggs (Hylander and Summers, 1977). This binding may induce the acrosome reaction, allowing the penetration of the spermatozoa into the external membrane of the oocyte, initiating fertilization (Galtsoff, 1961; Buffone et al., 2012). Donaghy et al (2012) demonstrated that increasing concentrations of DWH oil and dispersant were inducing morphological modifications of sperm cells, alteration of acrosomal integrity and inhibition of ROS production and mitochondrial metabolism. Such physiological impairments of spermatozoa could impact their motility and reduce their fertilization capabilities as described by Jeong and Cho (2005), Nice (2005), Fitzpatrick et al. (2008) and Akcha et al. (2012).

Typically, spermiotoxicity is assessed by exposing sperm to the toxic agent for a short time period, then adding exposed sperm to eggs and following up to fertilization success and hatching (Pagano et al., 1982; Dinnel et al., 1989; Ringwood, 1992; Geffard et al., 2001; Bellas et al., 2004). Very few studies have examined precisely how oil/PAHs decreases fertilization success by assessing cellular characteristics of exposed gametes using flow-cytometry techniques, and particularly the fertilizing ability of oocyte that had been exposed to oil. Generally, appropriate morphology of oocyte, using simple visual observation, and subsequent fertilization success can be measured under light microscopy to assess egg quality following exposure to toxics (Fitzpatrick et al., 2008; Basti et al., 2013). In addition, oocyte viability can be determined using fluorescent dyes and epifluoresence microscopy (Valdez-Ramirez et al., 1999; Paniagua-Chavez et al., 2006). A recent study has demonstrated that the use of FCM was a reliable method to assess oocyte quality of *Crassostrea gigas* through viability and ROS production (Le Goïc et al., 2014).

The aim of the present study was (i) to evaluate the sublethal cellular effects of DWH oil, dispersed mechanically or chemically, to spermatozoa and oocytes stripped from *C. virginica* in order to relate them to the successful fertilization and embryogenesis after 24 h of continuous

exposure, and (ii) to determine whether sperm cells or oocytes were the most sensitive to oil and/or dispersant.

2. Material and methods

2.1 WAF preparation

Surface oil (Slick A) collected from skimmer vessels at the source during the *Deepwater Horizon* oil spill on July 29, 2010, and dispersant, Corexit 9500A®, were provided by Stratus Consulting Inc. The High Energy Water Accommodated Fractions (HEWAF) and the Chemically Enhanced Water Accommodated Fractions (CEWAF) exposure solutions were prepared following a standardized procedure extensively detailed in Vignier et al. (in prep.a/Article 1). CEWAF solutions contained 1:10 ratio of the dispersant Corexit 9500A. Two concentrations for CEWAF (50 ppm and 100 ppm) and HEWAF (250 ppm and 500 ppm) were tested; as demonstrated in Vignier et al. (in prep.a/Article 1) these doses were known to cause sub-lethal effects on fertilization success and embryogenesis of embryos exposed before fertilization.

2.2 Oyster sperm and oocytes

Ripe *Crassostrea virginica* broodstocks were collected in Estero Bay, Florida, in September 2013, and maintained at the experimental hatchery for acclimation for two weeks, at 22˚C ± 1, at a salinity of 20-25 PSU, and fed *ad libitum*. Fresh gametes, sperm and oocytes, were collected by stripping oyster gonads following Allen and Bushek (1992), and re-suspended in UV-treated and 0.1-µm filtered seawater (FSW). Oocytes and sperm were examined under a microscope for motility (sperm), and shape and absence of atresia (oocytes): individuals from which gametes did not fulfill these criteria were discarded. After filtration through a 55 µm mesh to remove debris, sperm from 3 males were kept separately in 50 mL sterile beaker. Eggs (mean diameter ≈ 50 µm) from 3 females, after successive sieving through 150 µm and 55 µm mesh to remove gonadal tissue and debris, were washed and collected on a 20 µm sieve. Gametes were pooled and re suspended with FSW in a sterile 1 L beaker for eggs and 100 mL beaker for sperm, and used for later fertilization assay. Three subsamples of 100 µL of FSW/egg mixture were taken after continuous and gentle mixing. Subsamples of eggs were stained with 1% Lugol, and counted using a Sedgwick-Rafter® cell and a dissecting microscope.

2.3 Cellular characteristics of exposed gametes

Sperm exposure

Sperm was exposed to two concentrations of HEWAF (final concentrations of 250 ppm and 500 ppm), and two concentrations of CEWAF (final concentrations of 50 ppm and 100 ppm) for 30 min at 25˚C, prior to flow cytometry analysis. Hundred sixty µL of prepared oil solution was then mixed with 40 µL of sperm working suspension to obtain a final concentration of 1 x 10^6 cell.mL-1. Suspension of exposed spermatozoa (200 µL) were then processed for flow cytometry assays including morphology, viability, mitochondrial membrane potential and oxidative activity using specific fluorescent probes, according to Le Goïc et al. (2013b). Individual males (n = 3) were exposed and analyzed separately. Sperm solution (i.e. pooled from 3 males) used for fertilization assay was exposed to the same treatments and analyzed subsequently using FCM.

Sperm morphology and viability

Sperm morphology was based upon relative flow-cytometric parameters, Forward SCatter (FSC: relative cell size) and Side SCatter (SSC: relative cell complexity).

Viability of spermatozoa was evaluated using a dual staining procedure with SYBR-14 and propidium iodide (PI) (Live/Dead® Sperm Viability kit, Molecular Probes). After ≈ 25 min of exposure to the different oil treatments, sperm was stained with both SYBR-14 (final concentration 1 µM) and PI (final concentration 10 µg mL^{-1}) for 10 min in the dark at room temperature. Proportion of live cells was estimated with SYBR-14, which only penetrates sperm cells with intact membrane, binds to double-stranded DNA and then emits in green fluorescence range (516 nm). Cell mortality was measured with PI, which penetrates only spermatozoa with damaged membrane, and then emits in the red fluorescence range (617 nm). Dying sperm cells were stained with both SYBR-14 and PI. Results were expressed as mean percentages of live, dying and dead spermatozoa.

Sperm mitochondrial membrane potential (MMP)

Mitochondrial membrane potential of spermatozoa was measured using the potential-dependent JC-1 (BD™ MitoScreen, BD Bioscience). This dye can enter selectively into mitochondria and reversibly change color from green to orange as the membrane potential increases. JC-1 exists as

two forms, monomeric or aggregate, depending upon membrane potential. The monomer form predominates in cells with low mitochondrial membrane potential and emits in the green wavelength (525-530 nm), whereas the aggregate form accumulates in mitochondria with high membrane potential and emits in the orange/yellow wavelength (590 nm). The JC-1 aggregate/monomer ratio is assumed to be proportional to mitochondrial membrane potential (Reers et al., 1991; Cossarizza et al., 1996; Le Goïc et al., 2013).

Aliquots of 200 µl of exposed spermatozoa (adjusted at a final concentration of ≈ 1 x 10^7 cell.mL^{-1}) were stained with JC-1 (final concentration 5 µM), incubated for 10 min in the dark at room temperature, then diluted at 1/10 to stop the reaction prior to FCM analysis.

Sperm ROS production

Determination of Reactive Oxygen Species (ROS) production was performed using 2'7'-dichlorofluorescein diacetate (DCFH-D: Molecular probes, Invitrogen), a membrane permeable, non-fluorescent dye. Inside cells, the -DA radical is first hydrolyzed by esterase enzymes. Intracellular hydrogen peroxide (H_2O_2), as well as superoxide ion (O_2^{-}•), then oxidizes DCFH to the fluorescent DCF molecule. DCF green fluorescence range (525 nm), detected on the flow cytometer, is proportional to ROS production of spermatozoa.

Briefly, the exposed spermatozoa suspension (200 µl) was incubated with DCFH-DA (final concentration 10 µM) for 10 min in the dark prior to flow cytometry analysis.

Oocytes exposure

Oocytes were exposed at a ratio of 4:1 (oil: oocyte suspension) to solutions of HEWAF (final concentrations of 250 and 500 ppm) and CEWAF (final concentrations of 50 and 100 ppm) for 30 min at 25˚C, prior to FCM analysis. Oocytes suspension was then left to settle for 5 min in the fridge and collected using a Pasteur-pipette (200 µl) and adjusted at 8,000 oocytes ml^{-1}, and then analyzed by flow cytometry for morphology, viability, and ROS production using specific fluorescent dyes according to Le Goïc et al. (2014) and briefly described hereafter. Oocytes from individual females (n = 3) were exposed and analyzed separately. Oocyte solution (i.e. pooled from 3 females) used for fertilization assay was exposed to the same concentrations and subsequently analyzed by FCM.

Oocytes morphology and viability

Values from Forward SCatter (FSC) and Side SCatter (SSC) detectors were used as descriptors of oocyte morphological characteristics. Viability of exposed oocytes was measured using propidium iodide (PI), which evaluates cell mortality by penetrating only oocytes with compromised membrane, and emits in the red fluorescence range (550-600 nm). Results were expressed as percentages of live cells. Aliquots of 200 µl exposed oocytes (final concentration of 8,000 mL^{-1}) were stained with PI (final concentration 10 µg mL^{-1}) for 10 min in the dark at room temperature.

Oocyte ROS production

Reactive oxygen species (ROS) were measured as previously described for sperm. Aliquots of 200 µl of exposed oocytes (at 8,000 mL^{-1}) were incubated with DCFH-DA (final concentration 10 µM) for 10 min in the dark at room temperature before FCM analyses.

Flow cytometry (FCM) analyses were performed on an EasyCyte 6HT cytometer (Guava Merck Millipore®) equipped with a 100 µm capillary opening, a 488 nm argon laser, and 3 fluorescence detectors: green (525 nm ± 15), yellow (583 nm ± 13), and red (680 nm ± 15). Samples were acquired during 30 s at a flow rate of 0.59 $\mu L.s^{-1}$. Collected data were analyzed with the software InCyte (Millipore®).

2.4 Fertilization and embryogenesis assay

Before fertilization, sperm and eggs obtained from 3 males and 3 females were exposed separately to two concentrations of HEWAF (250 and 500 ppm) and CEWAF (50 and 100 ppm) for 30 min (n = 4 replicates for each concentration). Controls consisted of FSW only.
Each replicate of sperm (10 mL at concentration of ≈ 2 x 10^7 cells ml^{-1}) was incubated in test solution (40 mL HEWAF or CEWAF). Simultaneously, about 4,000 oocytes were incubated in test solution (200 mL HEWAF or CEWAF). After the 30 min incubation, the 200-mL egg solutions from each exposure replicate were fertilized with 10 mL of sperm from corresponding sperm exposure replicates. In addition, oil-exposed oocytes were cross-fertilized with solution of control sperm (incubated in FSW).
Exposure beakers were maintained in darkness at 26 ˚C ± 1 and at a salinity of 23 PSU ± 2 for 24 h with no solution renewal. One hour, 6 h and 24 h post-fertilization, 10 mL subsamples were taken and preserved in 10% buffered formalin for later measurements

(fertilization success, abnormality, mortality). To determine fertilization success (%), appearance of a polar body and/or the first cell cleavage of embryos (a minimum of 100 embryos per replicate) were verified under light microscopy. Abnormality was evaluated according to the following criteria: (6 h) embryos that did not reach the blastula stage, with abnormally shaped cells, delayed and/or arrested development (polar body to 2-3 cells) (Thompson et al., 1996; Carriker, 2001); (24 h) D-larvae with indented shell margin, incomplete shell, protruded mantle, convex hinge, and arrested development at the embryo stage (Fig. 6) (His et al., 1996). Larval mortality was also assessed at the end of the 24-h exposure by observation of opened valves and/or translucent shells (no clear internal organization) as well as unfertilized eggs.

2.5 Statistical analyses

Data are presented as mean ± standard deviation (SD). All percentage data were arcsine-square root transformed prior to statistical analyses to ensure normality. The assumption of homogeneity of variance was verified using Levene's test, and normality was verified using Shapiro-Wilk test. To compare the effects of treatments (HEWAF and CEWAF) on the different cellular measurements and endpoints, one-way analyses of variance (ANOVA) were performed. Tukey HSD post-hoc test was used to specify significant differences between individual treatments. Kruskal-Wallis non-parametric test and Dunnett's post-hoc test were performed whenever normality and homogeneity of variances requirements were not met after transformation of the data. Differences were considered significant when p-values were ≤ 0.05. For data presented as % of control, statistical analysis was performed on raw data. Collected data from the FCM were analysed using Incyte software (Millipore). All statistical analyses were performed using SPSS 22.0 statistical software.

3. Results

3.1 Water quality

Through the exposure, temperature and salinity were 25˚C and 23 PSU respectively. Dissolved oxygen (D.O.) and pH averaged 6.9 mg L^{-1} ± 0.1 and 8.0 respectively. Filtered seawater (FSW) used for the control treatments showed levels of PAHs at background concentrations ($\sum_{42}$PAH=0.26 µg L^{-1} ± 0.25), considered negligible for the present study.

3.2 Cellular effects of oil/dispersant on sperm

3.2.1 Sperm morphology

The relative size of sperm cells (FSC) decreased significantly when exposed to both concentrations of HEWAF ($p<0.001$, Fig. 1A). Additionally, exposure of sperm to HEWAF induced a significant change of relative internal complexity ($p\leq0.05$, Fig. 1B). Sperm incubated for 30 min with CEWAF preparations (50 and 100 ppm) did not result in any modification of cell size (FSC) nor internal complexity (SSC) compared to control (Fig. 1A & B).

3.2.2 Sperm viability

Percentages of live and dying spermatozoa are presented in Figures 1D and E. When exposed to increasing concentrations of CEWAF, a decline in viable spermatozoa was observed, with 100 ppm inducing a significant reduction down to 59.5% ± 12.6 ($p<0.001$). In addition, the highest dose of CEWAF (100 ppm) elicited a significant negative effect on exposed sperm cells, with proportion of dying cells reaching 30% ± 12.6 ($p<0.001$, Fig. 1E). Exposure of sperm to HEWAF solutions for 30 min resulted in survival (≈ 90%) comparable to the one observed in the control group though (Fig. 1D). In the same way, percentages of dying sperm cells were not statistically different from the control with increasing doses of HEWAF as well as the 50 ppm dose of CEWAF (Fig. 1E).

3.2.3 Mitochondrial membrane potential

Our results showed that incubation of sperm with oil (HEWAF and CEWAF) did not have a significant effect on mitochondrial membrane potential (MMP) ratio in the range of concentrations tested ($p>0.05$, Fig. 1C). Nonetheless, a trend appeared, with an increase of active mitochondria in spermatozoa exposed to CEWAF and HEWAF in relation to oil concentration. Overall, the ratio of mitochondrial membrane potential was higher in oil-exposed sperm than in the control group, suggesting an increased activity of ATP-producing mitochondria related to exposure.

3.2.4 ROS production

Incubation of sperm with CEWAF had a significant effect on ROS production ($p<0.05$) and resulted in a dose-dependent decrease, with the highest dose of CEWAF (100 ppm) reducing it significantly compared to control (11.5 ± 0.4 and 21.1 ± 3 respectively) ($p<0.05$, Fig. 1F). For

the HEWAF-exposed sperm, ROS production could not be determined because of oil droplets fluorescence interfering with DCF fluorescence (see discussion).

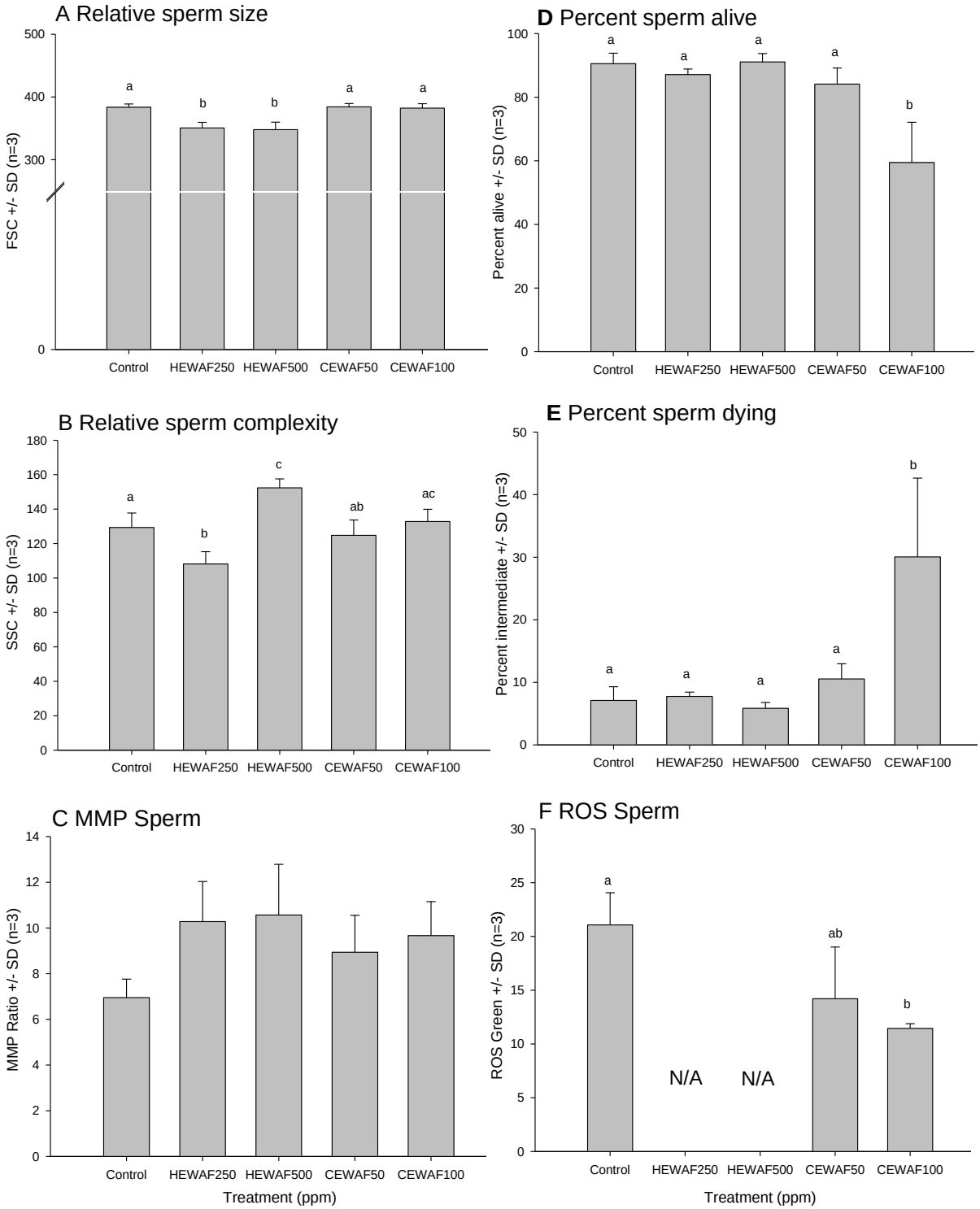

Figure 1: Relative size (A), internal complexity (B), MMP ratio (Yellow/Green) of active cells (C), percentage of viable (D) and dying (E), and ROS production (F) of *Crassostrea virginica* sperm cells after 30 min of exposure to 2 nominal concentrations of HEWAF (250 and 500) and CEWAF (50 and 100) expressed in ppm. Data are presented as means of 3 individual males ± SD, expressed in A.U or percentage. Different letters denote statistical difference (α=0.05) (ANOVA). N/A: not applicable.

3.3 Cellular effects of oil/dispersant on oocytes

Internal complexity (SSC) of oocytes was not affected by the 30 min exposure to oil (Fig. 2A). Incubation of oocytes with oil did not statistically affect the viability of oocytes, with no decrease in percentages of alive eggs compared to control (Fig. 2B). Exposure of eggs prior to fertilization to oil resulted in no significant difference in ROS production compared to control (Fig. 2C). However, a regular trend was observed, with ROS production regularly increasing with increasing concentrations. Between the control and the highest dose of CEWAF tested, mean ROS production more than doubled (3,947 to 9,070 A.U) after 30 min incubation, though not statistically different due to high individual variability (*p=0.782*, Fig. 2C).

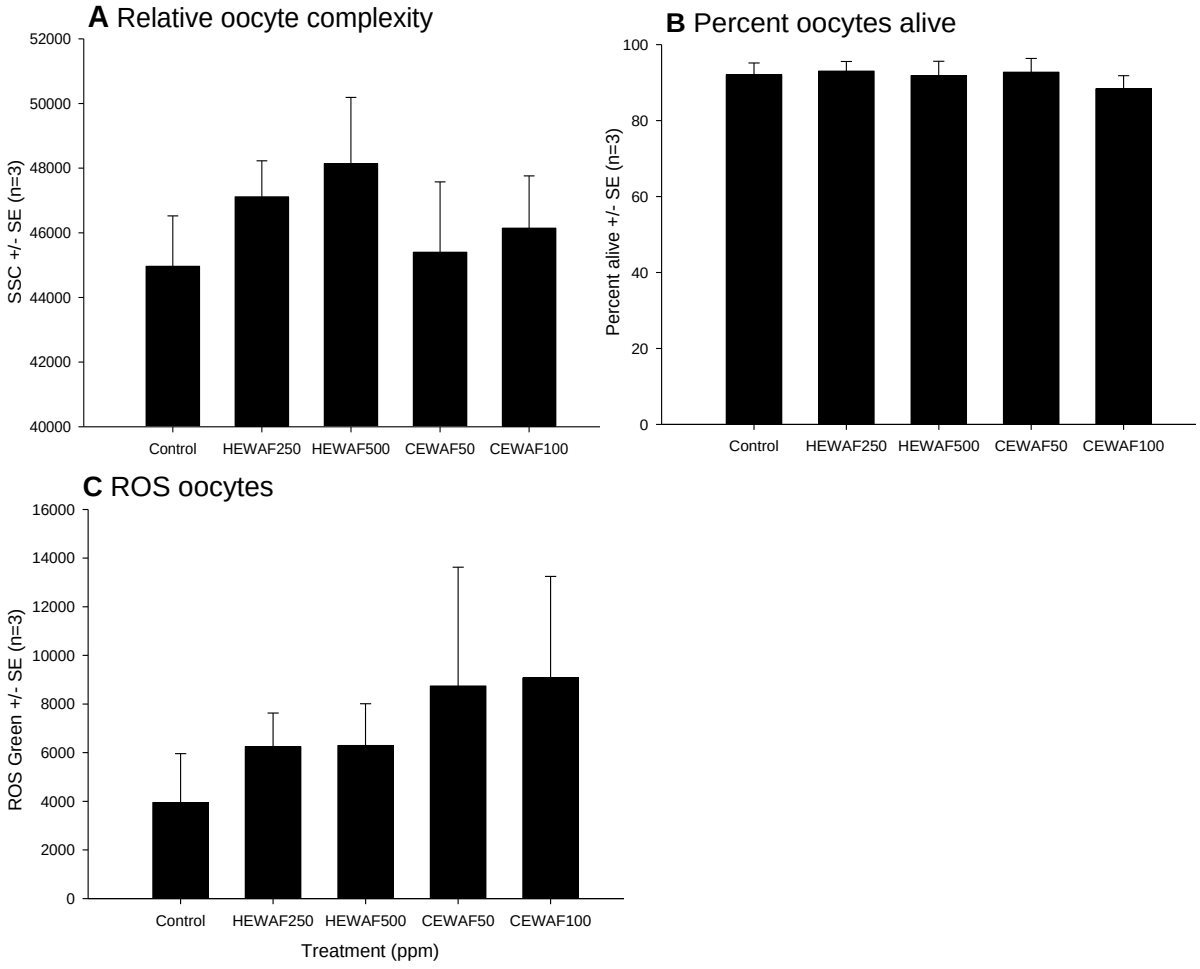

Figure 2: Relative internal complexity (A), percentage of live eggs (B), and ROS production (C) of *Crassostrea virginica* oocytes after 30 min of exposure to 2 nominal concentrations of HEWAF (250 and 500) and CEWAF (50 and 100) expressed in ppm. Data are presented as mean value, expressed in A.U or percentage, of 3 individual females ± SD.

3.4 Cross-fertilization: effects on fertilization success, embryogenesis and survival

Through the continuous 24-h exposure to HEWAF and CEWAF, different endpoints were measured at 1 h PF (fertilization), 6 and 24 h PF (abnormality) and at 24 h PF (mortality).

3.4.1 Effects on fertilization success

Fertilization success in the control group was 85.7% ± 3.9, an expected result for stripped gametes (data not shown). When male and female gametes were simultaneously incubated for 30 min prior to fertilization, fertilization success was significantly depressed in a dose-dependent manner by both HEWAF and CEWAF (Fig. 3A and B, $p<0.001$). HEWAF exposures (Fig. 3A) at nominal concentration of 500 ppm adversely impacted fertilization success (25.9% of the Control ± 10.6, $p<0.001$); whereas concentration of 250 ppm did not reduce fertilization significantly (88.5% of the Control ± 5.8). For the CEWAF exposure (Fig. 3B), nominal concentrations of 50 ppm and 100 ppm significantly impacted fertilization with rates falling to 10.1% of the control (± 4.1) and 0.1% of the control (± 0.2) respectively ($p<0.001$).

Fertilization of oocytes exposed to 500 ppm of HEWAF with control sperm (i.e. incubated in FSW only) showed statistical difference, i.e. 53.9% of the control (Fig. 3A, $p<0.001$). Similarly, oocytes incubated with both CEWAF treatments (50 and 100 ppm) and fertilized with non-exposed sperm induced significant reductions of the fertilization success, 20.1 % and 21.5% of control conditions respectively (Fig. 3B, $p<0.001$). However, no significant effect on fertilization success was observed with non-exposed sperm cells combined with eggs exposed to 250 ppm of HEWAF (Fig. 3A).

More specifically, compared to the control group, fertilization success obtained from eggs and sperm both exposed to 50 ppm of CEWAF was 10 times inhibited (≈ 10%), whereas fertilization was inhibited 5 times when only eggs were exposed (green circle, Fig. 3B). When exposing both gametes to 100 ppm of CEWAF, fertilization success was drastically reduced (≈ 0.1%); whereas, when only eggs were exposed to 100 ppm, fertilization was inhibited about 5 times (≈ 25%) compared to control (red/dash circle, Fig. 3B). For the 500 ppm exposure to HEWAF, the same trend as the 100 ppm CEWAF was observed (red/dash circle, Fig. 3A). Regarding HEWAF exposure, no significant difference was found for the 250 ppm treatment on both gametes and only on eggs (green circle, $p>0.05$).

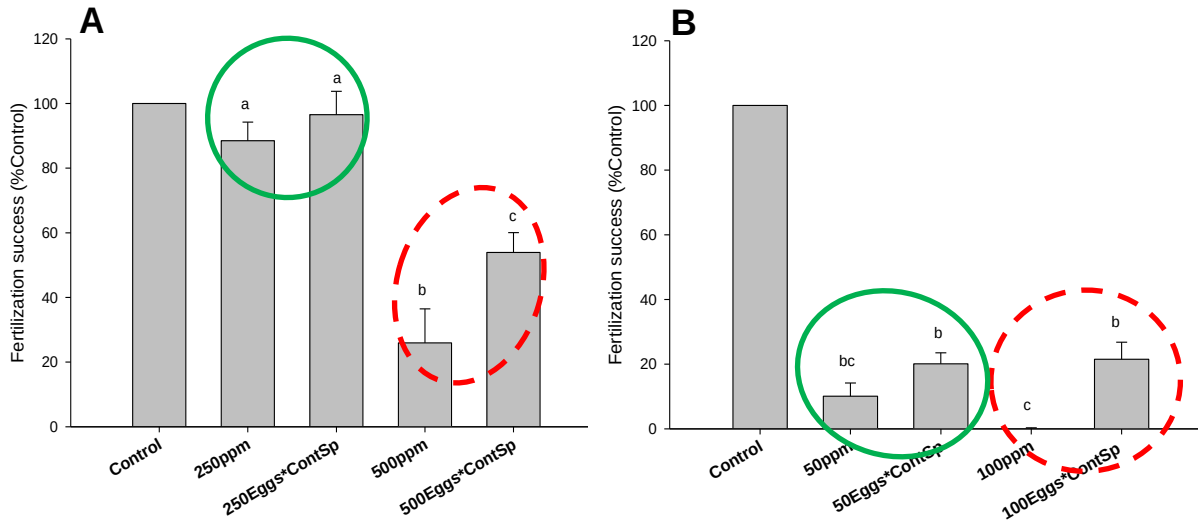

Figure 3: Fertilization success of *Crassostrea virginica* gametes (pooled from 3 individuals) exposed continuously to (A) HEWAF and (B) CEWAF, expressed as nominal concentrations (ppm). 250 ppm and 500 ppm treatments correspond to exposure of both gametes to HEWAF; "250Eggs*ContSp" treatment corresponds to crossing of exposed oocyte solution to 250 ppm of HEWAF with control sperm solution (FSW only); "500Eggs*ContSp" treatment corresponds to crossing of exposed egg solution to 500 ppm of HEWAF with control sperm solution. 50 and 100 ppm treatments correspond to exposure of both gametes to CEWAF. "50Eggs*ContSp" corresponds to egg solution exposed to 50 ppm of CEWAF and fertilized with control sperm solution; "100Eggs*ContSp" corresponds to cross-fertilization of eggs incubated with 100 ppm of CEWAF and control sperm. Data are presented as mean percentages ± SD of control (n = 4 replicate). Different letters and red/dash circles denote a significant difference at α=0.05 between conditions (ANOVA: Tukey HSD and Dunnett post-hoc test).

3.4.2 Effects on embryogenesis

Embryos observed in the control treatment 6 h after fertilization exhibited a normal development (Fig. 4a). After 6 h of incubation with oil solutions, embryos resulting from exposed gametes showed a dose-dependent increase in the number of abnormalities (Fig. 5, $p<0.001$). High instances of abnormally developed embryos with deformed cells (Fig. 4b), underdeveloped and arrested embryos (Fig. 4c), or swollen vitelline membrane were significantly observed in the 250 ppm HEWAF condition ($p<0.05$), and in the 50 ppm and 100 ppm doses of CEWAF (Fig. 5A and B, $p<0.01$).

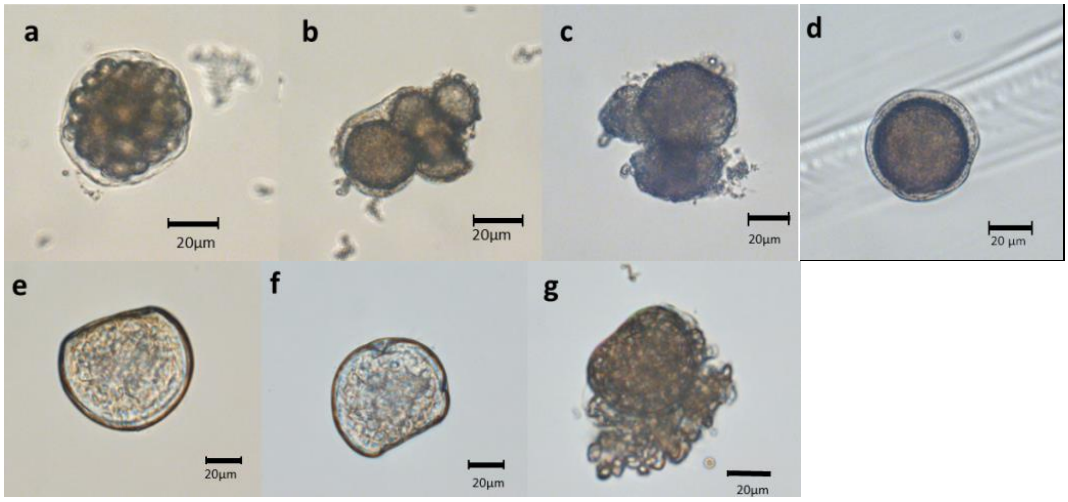

Figure 4: Normal 6 h-embryo (a); embryo with abnormal cell shape (b) or showing an arrested development, at 3-cell stage (c). (d) Abnormally fertilized embryo with swelling of vitelline envelope. Normal 24 h-old larva (e), and abnormal larvae (f, g) after 24 h of exposure to DWH oil.

After 24 h of exposure to oil solutions, abnormalities further increased in a dose-dependent manner (Fig. 5, *p<0.001*). Significantly higher occurrence of abnormalities such as D-larvae with indented shell margin (Fig. 4f), protruded mantle (Fig. 4g), convex hinge, or underdeveloped larvae at the embryo stage were observed in the 500 ppm HEWAF (Fig. 5A, *p<0.001*) and in the 50 ppm CEWAF treatment compared to control (Fig. 5B, *p<0.001*).

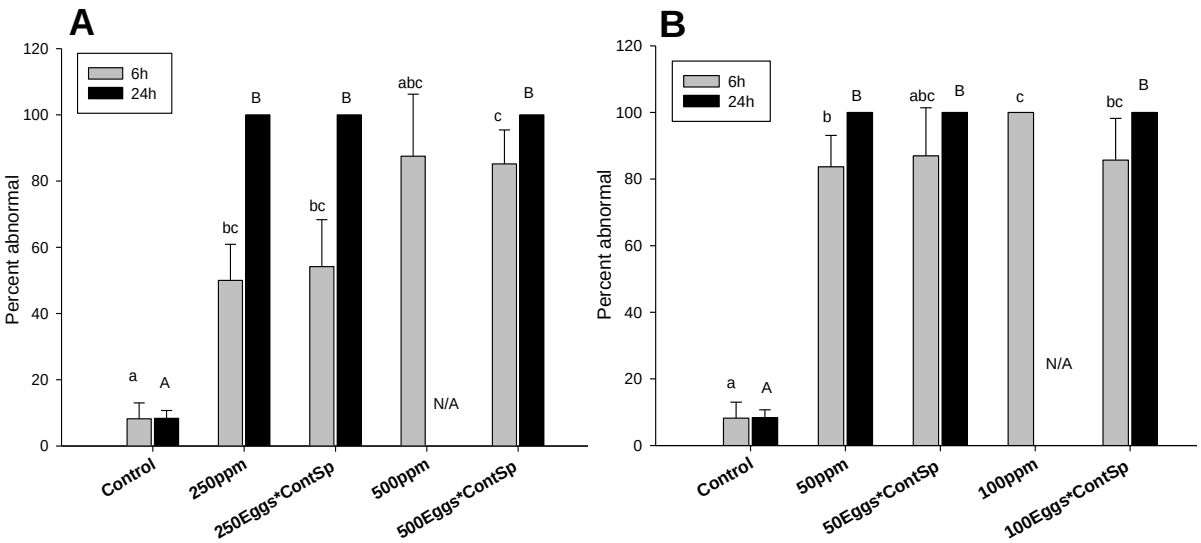

Figure 5: Percentages of abnormal embryos (grey) and D-larvae (black) resulting from cross-fertilized exposed gametes (sperm or oocytes) to HEWAF (A) and CEWAF (B), expressed as nominal concentrations (ppm). Data are presented as mean percentages ± SD (n=4). Different letters denote a statistical difference at α=0.05 (ANOVA, Tukey HSD or Dunnett post-hoc tests). N/A: no larvae were observed alive i.e. 100% mortality.

Embryos (6 h-old) and larvae (24 h-old) resulting from cross fertilization of oil-exposed oocytes and non-exposed sperm followed the same trends (Fig. 5) as the fertilization success results obtained 1 h post-fertilization (Fig. 3). Moreover, adverse effects on embryogenesis after 6 and

24 h of incubation with HEWAF (Fig. 5A) and CEWAF (Fig. 5B) were significantly more present compared to control.

3.4.3 Effects on larval survival

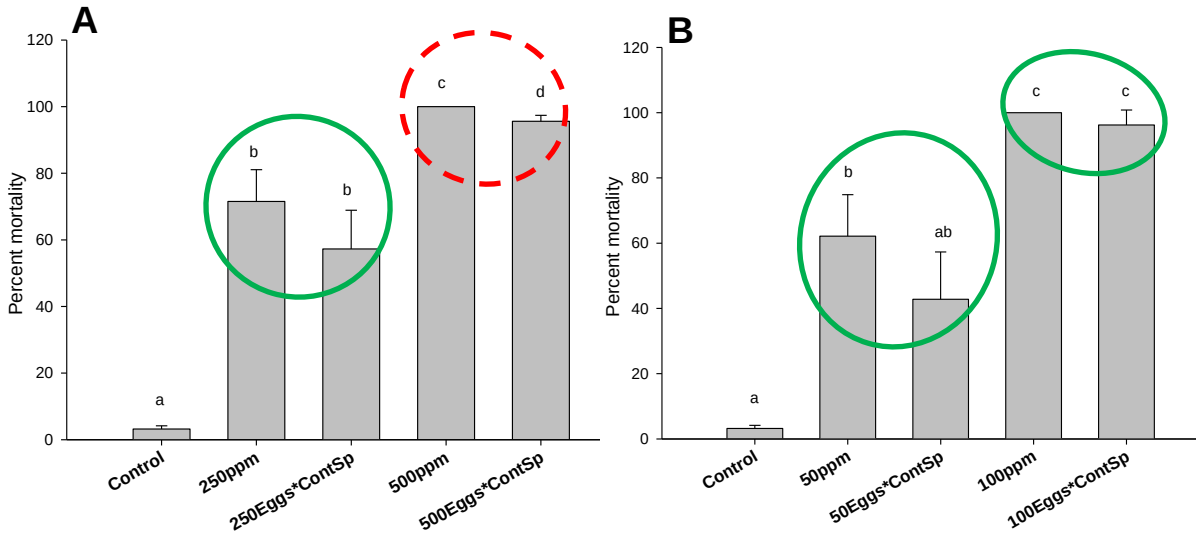

Figure 6: Final mortality of D-larvae resulting from cross-fertilized exposed gametes (sperm or oocytes) to HEWAF (A) and CEWAF (B) for 24 h, expressed as nominal concentrations (ppm). Data are presented as mean percentages ± SD (n=4). Different letters and red/dash circles denote a statistical difference at $\alpha=0.05$ (ANOVA, Tukey HSD or Dunnett post-hoc tests)

At the end of the exposure, survival was also impacted negatively with larval mortality increasing significantly for both HEWAF and CEWAF in relation to exposure doses (Fig. 6, $p<0.001$). Regarding the crossing of condition, deleterious effects on survival were observed for larvae resulting from oil-exposed oocytes fertilized with non-exposed sperm compared to control ($p<0.01$). In addition, final mortality results for both HEWAF and CEWAF exposures showed that no significant differences existed between oil-exposed eggs crossed with control sperm ($p>0.05$) (Fig. 6A and B: green circle) and both oil-exposed gametes, except for the highest dose of HEWAF (500 ppm) tested ($p<0.05$) (Fig. 6A: red/dash circle).

4. Discussion

The first objective of this study was to evaluate the cellular effects of DWH oil acute exposure, dispersed chemically (CEWAF) or mechanically (HEWAF), on spermatozoa and oocytes of *Crassostrea virginica*, and relate them to successful fertilization and embryogenesis. It is important to note here that *in vitro* analyses were performed on individual broodstock whereas *in vivo* assays were conducted using gametes pooled from several broodstocks. *In vivo* results showed that fertilization success, embryogenesis and survival were all impacted negatively by DWH oil in a dose-dependent manner. These findings are in agreement with our previous results (Vignier et al., in prep.a/Article 1).

Sperm cellular characteristics

Flow-cytometry (FCM) is a foremost technique to assess sperm quality and has already been used for sperm assessment in oysters in cryopreservation protocols (O'Connell et al., 2002; Gillan et al., 2005; Paniagua-Chavez et al., 2006; Martinez-Pastor et al., 2010). The use of FCM also showed a great potential in ecotoxicological studies using oyster sperm (Favret and Lynn, 2010; Akcha et al., 2012; Le Goïc et al., 2013). Morphology, viability, mitochondrial membrane potential and ROS production were selected as good indicators of sperm function in our *in vitro* study. We found that HEWAF exposure led to significant changes in both relative size and internal complexity of sperm cells compared to control. Donaghy et al. (2012) reported similar findings, with exposure to DWH oil prepared as HEWAF altering significantly sperm morphology and increasing internal complexity of spermatozoa. Sperm deformities are generally associated with functional deficiencies and can cause a reduction of sperm motility and thus fertilization capacity (Rurangwa et al., 2004). The change of size and complexity upon exposure to HEWAF may be explained by the presence of oil-droplets, more abundant in high energy dispersed oil (Li et al., 2008b; Carls et al., 2008; Carls and Thedinga, 2010), and the associated fluorescence of these oil-droplets which may interfere with light diffraction measured by FSC and SSC detectors. Further analysis showed that oil droplets could bind to sperm cells and form aggregates, changing therefore their morphology, which could explain the increase of complexity observed at the 500 ppm condition. More specifically, at the 250 ppm and 500 ppm doses of HEWAF, a reduction in the proportion of "free" sperm cells (not aggregated with oil) was observed, down to 24 % (± 3) and 7 % (± 3) respectively (data not shown). Even though quantitatively reduced, the remaining sperm cells were still viable (≈ 87%, Fig. 1D). However,

the reduced fertilization success observed at 500 ppm (25.9% of the control) was likely caused by the decrease of "free" sperm cells remaining to fertilize oocytes (< 40:1), a male to female gamete ratio limiting a successful fertilization, well below optimum ranges (from 100:1 to 5000:1) reported by Song and Suquet (2009) for *C. gigas*. Despite a mechanical action that may have induced morphological changes, oil-droplets derived from HEWAF preparation did not cause any adverse effect on the viability of spermatozoa. However, a reduction in the number of sperm cells due to aggregation with oil droplets may be responsible for the reduction in fertilization success observed at 500 ppm of HEWAF.

Contrastingly, results for CEWAF-exposed sperm did not show any significant morphological modification nor interference, probably due to the smaller fraction of oil-droplets contained in CEWAF (Li et al., 2009; Venosa et al., 2014). Nonetheless, viability of sperm cells exposed to 100/10 ppm of CEWAF (oil/dispersant) and subsequent fertilization decreased significantly. This could be directly associated with the toxic effect of dispersant as demonstrated in our previous study (Vignier et al., in prep.a/Article 1).

During mitochondrial respiration, most of the consumed oxygen is reduced to water but also to superoxide ion ($O_2^{\bullet-}$) which can be converted to hydrogen peroxide (H_2O_2) and the highly reactive $OH^{\bullet}$ (Poyton et al., 2009). Our results demonstrated that the production of these reactive oxygen species (ROS) was statistically reduced after acute exposure to oil. Authors like de Lamirande et al. (1997) showed that low concentrations of ROS could play an important role in sperm physiology, by their involvement in sperm capacitation, acrosome reaction and sperm-oocyte fusion. The decrease of ROS following exposure to CEWAF observed in the present study is in accordance with the study from Donaghy et al. (2012) who found a dose-related decrease of ROS after exposure to DWH oil and dispersant. It is plausible that the decline in intracellular ROS production observed after incubation with CEWAF, and the increased mortality might be directly related to the deleterious effect of the dispersant contained in the CEWAF (1:10), disrupting cellular respiration. Several studies showed evidence of acute toxicity of surfactant (one of the main compound of Corexit 9500A) by disruption of respiratory cells, which may lead to hypoxia and asphyxia (Abel, 1974; Nagell et al., 1974; Singer et al., 1995; 1996). Mitochondria are organelles that generate most of the cell supply of ATP during respiration. The roles of mitochondria and particularly ATP are primordial in fertilization process, as ATP is typically consumed in sperm flagella to provide energy for motility and is crucial for maintaining chemical gradients over membranes. In our study, oil exposure had no significant effect on the mitochondrial membrane potential (MMP), which is a key parameter governing fertilization. Nonetheless, a trend of increasing MMP was apparent after incubation

with oil and, over time, a decrease of cellular respiration may be detrimental to sperm cells as it could reduce the energy production of the sperm, hence impairing its motility. Regarding the absence of results for HEWAF, the relative fluorescence of droplet-associated oil, abundant within the HEWAF, have interfered and impeded the determination of sperm ROS production.

Oocytes cellular characteristics

Morphological characteristics of exposed oocytes were not statistically altered by DWH oil. A recent study by Le Goïc et al. (2014) also demonstrated that morphology of *Crassostrea gigas* oocytes following incubation with the toxic alga *Alexandrium minutum* was not affected. Nonetheless, similarly to the results obtained for sperm morphology, a non-significant trend of increasing complexity was noted after exposure to HEWAF: aggregation of oil droplets at the egg surface could be responsible for this morphological modification. In terms of oocyte viability, no significant impact of either HEWAF or CEWAF was detected in this study. This result is consistent with Fitzpatrick et al. (2008) who showed that exposure to copper did not influence egg viability of mussels. Similarly, Le Goïc et al. (2014) showed no change in the percentage of alive oocytes upon exposure to *A. minutum* toxin. The structure of the oocyte and the presence of a protecting membrane, the vitelline envelope, may explain the lack of effects of oil/PAHs exposure on eggs (Galstoff, 1961). However, as shown by Basti et al. (2013) with oocytes exposed to phytotoxin, we frequently observed in the CEWAF treatments abnormal embryos exhibiting swellings of the vitelline envelope (Fig. 4d). This oocyte membrane modification could be directly linked to the surfactant contained in the Corexit 9500A and its damaging effect on membrane (Singer et al., 1995, 1996). Besides, PAHs could also be responsible for the loss of membrane structure: Van Brummelen et al. (1998) described a non-specific mode of PAHs toxicity which can cause physical disturbance of bio-membrane structure.

Regarding ROS production by oocytes, although our results indicated no significant dose-response relationship between the exposed groups, there was evidence of an upward trend in ROS production, particularly for the CEWAF exposure. In fact, ROS production of oocytes more than doubled after 30 min incubation with CEWAF. Oxidative stress was shown to cause toxic effects on human oocytes and has been linked to reduced fertilization success (Tamura et al., 2008). Numerous authors have shown that stimulation of ROS production in bivalves can be induced by many environmental stresses, such as pollutants, pathogens, heat shock, UV exposure, or hypoxia (Roesijadi et al., 1997; Anderson et al., 1997; Lacoste et al., 2002; Hegaret et al., 2003b; Lambert et al., 2003). Like many other aquatic organisms, oyster oocytes contain

high levels of lipid: polar lipids (e.g. phospholipids) which function mainly as structural component of the membrane, and neutral lipids (e.g. triglycerides) which function as energy reserves (Trider and Castell, 1980; Thompson et al., 1996). The augmentation of ROS observed in the current study could therefore be associated with lipid peroxidation of the egg membranes (Di Giulio, 1989; Livingstone et al., 1994). Similarly, Livingstone et al. (1989) and Downs et al. (2002) described an oxidative stress and membrane impairments in bivalve following exposure to petroleum hydrocarbons. It can be speculated that excessive production of ROS observed herein may ultimately have severe implication for the subsequent fertilization as it can lead to DNA damage (Cavallo et al., 2003). In a similar way, Le Goïc et al. (2014) described an increase of ROS production in oocytes exposed for several days to *A. minutum* toxins.

Overall, cellular effects of DWH oil and associated dispersant on oyster oocytes were not as clear as those obtained with sperm following exposure. In fact, the method used in the present study had certain limitations, the main one relying on i) the relatively low number of individuals tested (n = 3) which resulted in high variability, and ii) the low numbers of oocytes analyzed, possibly increasing variability in the results. The collection of gametes by "stripping", i.e. bypassing the natural maturation process of oocytes (Valdez-Ramirez et al., 1999; Cannuel and Beninger, 2005), may be another cause of this intra-individual variability. High variability between individuals but also amongst oocyte population could therefore explain the non-significant trends obtained for most of the cellular parameters evaluated. Another issue encountered during FCM analyses of oocytes was the limited time allowed for incubation. In general hatchery procedure, fertilization of oocytes is usually achieved within 1-h of stripping or spawning of the females to avoid reduction of fertilization and subsequent alteration of embryonic and larval development as shown by Helm and Millican (1977). Consequently, in the present study, special care was given to incubate oocytes for no more than 30-40 min to allow the best results in term of fertilization. Despite these considerations, FCM analysis of oocytes was promising and has given more satisfactory results since the present study (Le Goïc et al., 2014).

The second objective of the present study was to determine *in vivo* the sensitivity of oil-exposed spermatozoa and/or oocytes and their respective contribution on successful fertilization and embryogenesis. We fertilized oil-exposed eggs with unexposed sperm and monitored subsequent fertilization success and embryogenesis until hatching of the D-larvae, 24 h after fertilization. However, due to problems sieving out sperm cells from the exposure solution after incubation with oil, we could not cross-fertilize oil-treated sperm with untreated oocytes. As comparatively

very few studies have dealt with pollutant-pretreated sperm, a protocol has not yet been developed which readily permits incubation of sperm without subsequent exposure of eggs during fertilization process. As a result, only fertilization success, abnormality and mortality of oil-exposed eggs crossed with control sperm were investigated and will be discussed.

Fertilization results as well as anomalies and mortalities obtained from the cross-fertilization assay followed the same trends. With the exception of the 500 ppm HEWAF condition, no significant differences in fertilization success or mortalities of embryos/larvae resulting from cross-fertilization of simultaneously exposed sperm and oocytes, or oil-exposed oocytes crossed with control sperm were found. The lack of statistical differences observed for fertilization success or mortalities results between crosses suggests that oocytes may be more sensitive than sperm to DWH oil/dispersant toxicity, and that they may contribute to the subsequent negative effects on fertilization, embryogenesis and survival. Likewise, Eyster and Morse (1984) demonstrated a marked sensitivity of *Spisula solidissima* eggs to silver compared to sperm. They showed that abnormal larvae were the consequence of toxicity of silver on oocyte, and suggested that microtubular disruption as well as changes in membrane permeability to ion could be the reason behind these anomalies. It has been well documented that surfactant, one of the main component of Corexit 9500A (Nalco Energy Services, 2012), but also PAHs can have adverse effects on membrane structures and can induce increased permeability of the membrane, a loss of barrier function or an osmotic imbalance (Benoit et al., 1987; Partearroyo et al., 1990, Singer et al., 1996; Van Brummelen et al., 1998). As previously mentioned, microscopic observations of embryos after 6 h of exposure to CEWAF indicated damages of the vitelline envelope, possibly imputable to either Corexit 9500A or PAHs. Furthermore, the increasing ROS measured in exposed oocytes, though not significant, is a typical sign of reduced fertility (Wang et al., 2003) and could explain our *in vivo* results. Consequently, given our previous results which showed minimal negative impact of oil/dispersant on sperm functions, we can suggest that detrimental effects of oil/dispersant on fertilization may be largely attributed to an impairment of oocyte functions. Oyster oocytes are typically richer in lipids than spermatozoa (Lee and Heffernan, 1991): as a result, lipophilic PAHs might be more bioavailable to diffuse across the egg membrane and elicit further damages (Connell, 1990) compared with sperm cells. In addition, after exposure of newly fertilized embryos, Wessel et al. (2007) showed that PAH induced high rates of abnormal embryos of *C. gigas* due to DNA strand breakage. The alkaline version of the single-cell electrophoresis technique, also known as the Comet assay, has been commonly used to assess DNA damage in cells, including bivalve sperm (Akcha et al., 2003; Lewis and Galloway, 2009; Kadar et al., 2011; Akcha et

al., 2012; Lewis and Ford, 2012), or oyster embryos (Wessel et al., 2007). It could be interesting to test this method with oocyte following oil and/or dispersant exposure as an alternative to FCM analysis.

On the other hand, several arguments implying a higher sensitivity of spermatozoa to DWH oil and/or dispersant, could be proposed. As opposed to oocytes, which contain a variety of proteins, anti-oxidants and certain enzymes that can repair the DNA and protect against environmentally induced damages, sperm cells are generally considered to have little or no capacity for DNA repair or anti-oxidant defense (Aitken et al., 2004). Additionally, sperm cells have a higher surface/volume ratio compared to oocytes (Seidel, 2006; Zhou and Li, 2009): this morphological characteristic could make sperm more prone to the toxicity of dissolved PAHs in solution compared to oocytes. Ultimately, even though sperm could be more sensitive to oil/dispersant cytotoxicity, we can hypothesize that reduced sperm function (e.g. viability) might be overcome by the great number of spermatozoa released. This is especially true in view of the results obtained with exposure to 250 ppm of HEWAF which showed that, even though reduced substantially in number because of oil droplets, sperm cells were viable and resulted in high fertilization rates. Consequently, and from the results obtained in the current study, it appears that successful fertilization and embryonic development may definitely be more related to oocyte quality rather than sperm quality.

Conclusion

This study demonstrated that chemically or mechanically dispersed DWH oil were cytotoxic to oyster gametes and could impact sensitive processes of the early life stage of *Crassostrea virginica*. Chemical characteristics of the dispersant Corexit 9500A as well as dissolved PAH seemed to induce the most severe effects, particularly on oocytes, resulting in impaired performance of embryo and larvae. To the best of our knowledge, this is the first time flow-cytometry was used to analyze cellular responses of oyster oocytes to petroleum hydrocarbons and dispersant exposures. Despite the need for further improvements, this method is encouraging. Furthermore, additional research is required to evaluate the cytotoxicity of oil and dispersant on the gamete of ecologically significant species, in order to inform effective responses to oil spill such as the use of chemical dispersant during the reproductive season of oysters.

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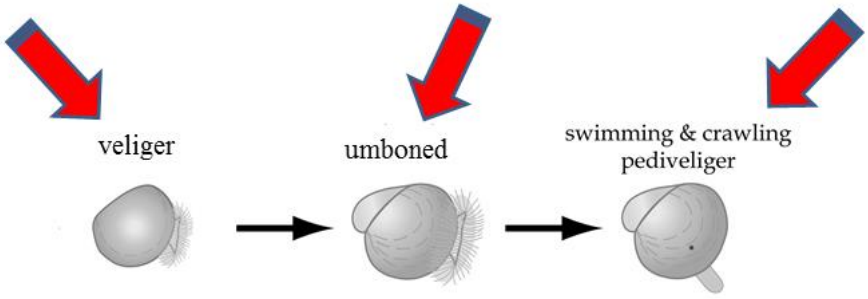
Synthesis Chapter 1

The aim of the previous chapter was to determine first of all the lethal and sublethal effects of an acute exposure to HEWAF, CEWAF and Corexit from before fertilization (gametes) and after fertilization (embryos), and try to understand the mechanisms of toxicity of oil/PAHs and dispersant on critical processes such as fertilization, embryogenesis (shell formation) and early larval development. Results revealed that exposure of oyster gametes and embryos to oil and dispersant impaired fertilization, normal development of embryos, growth of resulting larvae and ultimately survival, at concentration ranges of 1.3 to 11.5 ppm for Corexit (corresponding to 130 to 1,150 µg DOSS L^{-1}) and 1.3 to 30 µg tPAHs L^{-1} for CEWAF (corresponding to 6.3/0.63 ppm to 112/11.2 ppm). Dispersed oil and dispersant usually resulted in the highest impact compared to HEWAF, with most of the adverse effects of CEWAF due to its fraction of Corexit (1:10). Our results also showed that fertilization was a sensitive stage in the early development of oysters, with a stage-dependent response to CEWAF and dispersant, inducing further impairment when exposure started before fertilization (gamete more sensitive than embryos).

Additional investigations at the cellular level were conducted in order to evaluate the mode of toxicity of PAH and dispersant on spermatozoa and oocytes using flow-cytometry assays. Cellular analyses indicated that chemical characteristics of the dispersant (surfactant DOSS) as well as dissolved PAHs from CEWAF induced the most severe effects on gametes. Sperm viability was highly reduced at 100/10 ppm (oil/dispersant) of CEWAF and ROS production in oocytes was increased for both oil preparations, indicating a deleterious oxidative stress which may alter fertilization abilities and induce embryonic abnormalities. Incubation in HEWAF solution, due to a mechanical action of its particulate fraction, only altered sperm morphology and motility without affecting fertilization capabilities. *In vivo* assays using cross-fertilization treatments showed that oocytes were the most affected by PAHs/dispersant and confirmed their contribution to fertilization impairments and embryonic abnormalities.

Finally, fertilization success and larval growth were shown to be sensitive endpoints that must be included in toxicological assessment of DWH oil and dispersant on bivalve. Moreover, results on sperm and particularly oocyte cellular parameters using FCM demonstrated that the method was appropriate to evaluate gamete quality following exposure to oil and dispersant.

Chapter 2: Impacts of DWH oil and dispersant on larvae of *Crassostrea virginica*



Introduction Chapter 2

Typically, developing pelagic larvae of *Crassostrea virginica* spend 2 to 3 weeks in the water column, generally floating near the surface, until settlement. As shown in the previous chapter, early stages of *C. virginica* have the potential to be greatly affected by oil/PAHs/ dispersant and critical processes of oyster development such as larval growth, feeding, or metamorphosis could be impacted in the same manner. Alteration of feeding could result in retardation of larval growth and settlement, ultimately affecting survival and causing long term negative impacts on the whole population.

The aim of this chapter was to evaluate the lethal and sublethal effects of i) short-term acute exposures (96-h) to HEWAF, CEWAF and dispersant alone on various stages of oyster larvae (early veliger, umboned, and pediveliger), and ii) a long-term larval exposure (14-d) to oil/PAHs using a dietary mode of exposure. Sublethal responses such as growth, gut fullness, settlement success were monitored throughout exposures and special attention was given to the particulate form of oil and its interaction with larvae.

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Article 3

Lethal and sub-lethal effects of Deepwater Horizon oil and dispersant on *Crassostrea virginica* larvae

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Abstract

The eastern oyster, *Crassostrea virginica*, is one of the most commercially and ecologically important shellfish species in coastal region of the northern Gulf of Mexico. The long planktonic nature (14 to 21 days) of filter-feeding oyster larvae makes them vulnerable to acute exposure to contaminants. Following the explosion of the Deepwater Horizon (DWH) oil platform off the coast of Louisiana on the 20th April 2010, crude oil was spilled in the Gulf of Mexico for 85 days, coincident with the spawning season and recruitment of oysters. In the laboratory, newly hatched veliger (1 day-old), umbo (10 day-old) and pediveliger (15 day-old) larvae were acutely exposed for a duration of 3 to 4 days to increasing concentrations of unfiltered DWH oil (HEWAF), dispersed oil (CEWAF at 1:10 ratio of dispersant) and dispersant (Corexit 9500A®) in a static system. Feeding behavior, growth, mortality, and settlement success were assessed during the assays. Nominal concentrations of HEWAF (≥ 31.25 mg oil L^{-1}), CEWAF (≥ 54/5.4 mg L^{-1}) and dispersant (≥ 6.25 mg L^{-1}) were highly toxic to different stages of *C. virginica* larvae by affecting feeding efficiency, growth, settlement success and ultimately survival, in a dose-dependent manner. Acute exposure to increasing concentrations of CEWAF and dispersant reduced significantly survival of larvae, regardless of age (Day 1 or 10). The $LC50_{96h}$ for veliger, umbo and pediveliger for CEWAF exposure were 45, 72 and 80.4 µg tPAHs L^{-1} respectively. Our study also revealed that concentration of 18.4 µg tPAHs L^{-1} (corresponding to 58/5.8 mg oil L^{-1}) of CEWAF significantly reduced settlement success of larvae by 50%. Exposure to increasing doses of HEWAF for 96 h did not induce a dose-dependent response in mortality of any larval stages tested. Moreover, growth of larvae exposed to all contaminants was significantly affected in a dose-dependent manner, and older larvae were more resistant to toxicant than younger larvae. This work demonstrated that larval growth and metamorphosis/settlement of oyster larvae were valid indicators of toxicity. Consequently, the present study provided valuable data to assess the damage caused by the DWH oil spill as sublethal effects induced by oil and associated dispersant on these endpoints could have significant implications on population dynamics of oysters and the whole estuarine ecosystem.

Keywords: Deepwater Horizon oil, Corexit 9500A, oyster larvae, sub lethal, growth, settlement, PAH

Résumé

L'huître américaine, *Crassostrea virginica* est une des espèces les plus importantes sur le plan écologique et commercial pour les régions côtières du Golfe du Mexique. La phase planctonique relativement longue (14 à 21 j) de ses larves les rend vulnérables à une exposition aigüe à des contaminants en solution. A la suite de l'explosion de la plate-forme pétrolière Deepwater Horizon (DWH) au large des côtes de la Louisiane le 20 Avril 2010, du pétrole brut fut relâché dans le Golfe du Mexique durant 85 jours, période qui coïncide avec la saison de ponte des huîtres. En laboratoire, nous avons exposé de façon aigue de jeunes larves veligères (j1), des larves ombonés (j10), et des larves pediveligères (j15) durant 3 a 4 jours à des doses croissantes, non-filtrées, de pétrole seul (HEWAF), de pétrole dispersé (CEWAF) ou de dispersant seul (Corexit 9500A), dans un système statique. Alimentation larvaire, croissance, mortalité, et taux de fixation furent estimés durant les assays. Nos résultats ont démontré que des concentrations nominales de HEWAF ($\geq$ 31.25 mg L^{-1}), CEWAF ($\geq$ 54/5.4 mg L^{-1}) et dispersant ($\geq$ 6.25 mg L^{-1}) étaient hautement toxiques à différents stades larvaires de *C. virginica* en affectant la prise de nourriture, la croissance, le taux de fixation et finalement la survie, d'une façon dose-dépendante. Des expositions aigues à des doses croissantes de CEWAF et dispersant ont réduit de façon significative la survie des larves, indépendamment de l'âge (j1 ou j10). Les $LC50_{96h}$ pour les veligères, ombonés, et pediveligeres exposées au CEWAF furent 45, 72 et 80.4 µg tPAHs L^{-1} respectivement. Notre étude a également montré que des concentrations de CEWAF égales à 18.4 µg tPAHs L-1 (équivalent à 58/5.8 mg L^{-1}) pouvaient réduire les taux de fixation de moitié. Au contraire, l'exposition à des doses croissantes d'HEWAF pendant 96 h n'induisirent pas de réponse létale dose-dépendante pour les stades larvaires testés. En outre, nous avons mis en évidence que la croissance de larves exposées à tous les contaminants était affectée de façon dose-dépendante, et que les larves les plus âgées étaient les plus résistantes aux polluants. Cette étude a finalement démontré que la croissance et la métamorphose/fixation larvaire de l'huitre étaient des indices de toxicité valides. Par conséquent, ce travail a permis de fournir de précieuses données pour l'évaluation des dommages causés par la marée noire DWH, comme par exemple les effets sublétaux induits par le pétrole et le dispersant qui pourraient avoir des conséquences importantes sur les populations d'huitre et la dynamique générale des écosystèmes estuariens.

Mots-clés : pétrole Deepwater Horizon, Corexit 9500A, larves d'huitres, sublétal, croissance, fixation, HAP

1. Introduction

The Eastern oyster, *Crassostrea virginica* is one of the most commercially important shellfish species propagating along the east coasts of the U.S, from Maine to the Gulf of Mexico (Galtsoff, 1964) and an ecologically vital species for the Gulf of Mexico region. Oyster reefs, which have been built through successive reproduction and settlement of larvae onto existing reef structure, provide food, shelter and habitat for many fish and shellfish, improve water quality, stabilize bottom areas, and influence water circulation patterns within estuaries (Wells, 1961; Newell, 2004, Volety et al. 2014). In addition to its ecological significance, it is also an economically important species, with total landings of *C. virginica* in Louisiana representing about $42 million in value for 2012 (National Marine Fisheries Service, 2012). In the northern part of the Gulf of Mexico, oyster spawning season typically occurs from mid-spring through late fall (Ingle, 1951), when water temperature is above 25˚C (Stanley & Sellers, 1986; Menzel, 1991), with two peaks in settlement in early and late summer (Supan, 1983).

The accidental explosion of the Deepwater Horizon (DWH) oil drilling rig in April 2010 led to the largest marine oil spill in United States history, with an estimated 4.1 million barrels (779,000 t) of crude oil released into the Gulf of Mexico (Camilli et al., 2012; OSAT, 2010; McNutt et al., 2012). Spill response capabilities were tested to their limits by the continuous flow of crude oil spilling from the sea floor, and subsea dispersant application directly at the source of discharge was suggested as the best response option. As a result, an estimated 8 million L of dispersants (mostly Corexit 9500A®) were used, from which 2.9 million L were directly injected at the well head into the oil and gas plume at 1500 m depth (Lehr et al., 2010; Kujawinski et al., 2011). Prior to the DWH oil spill incident, no deep-water application of dispersant was ever conducted and behavior of such quantity of dispersant at depths, its environmental fate as well as its potential toxicity on pelagic and benthic organisms is poorly understood. From April 20th until the final capping of the leak on July 15th, DWH crude oil spilled from the *Macondo-1* well (Crone and Tolstoy, 2010), a period that coincides with the natural spawning and recruitment season of eastern oysters in the Gulf of Mexico. The developing pelagic larvae spend 2 to 3 weeks in the water column, generally floating near the surface, until they sink and settle on a suitable substrate (Bahr and Lanier, 1981). Among the biological components of marine ecosystems, planktonic organisms are particularly susceptible to crude oil pollution (Walsh, 1978; Graham et al., 2010; Almeda et al., 2013, 2014). Zooplankton such as oyster larvae cannot overcome the effects of currents, limiting

their capacity to avoid crude oil patches and, potentially forcing them to drift into highly polluted waters after oil spills.

Petroleum or crude oil is one of the most common pollutants released into the marine environment. Natural oil seepage, transportation, extraction, atmospheric deposition, surface run-offs and consumption are the main sources of crude oil into the sea (National Research Council NRC, 2003). Although catastrophic oil spills are not the most significant source of crude oil discharge in the marine environment, they have strong acute and long-term impacts on marine ecosystems, including effects from physical damages (asphyxia, physical contamination or coating of oil) to toxicity from their chemical compounds that constitute crude oil (NRC, 2003). Crude oil is a complex mixture of both hydrocarbons, such as alkanes, cycloalkanes and aromatic hydrocarbons, and non-hydrocarbon compounds. Polycyclic Aromatic Hydrocarbons (PAHs) are considered to be the most acutely toxic components of crude oil, exerting their toxicity by interfering with membrane fluidity (Neff, 1985; Van Brummelen et al., 1998; Barron et al., 1999). PAHs are also associated with potential carcinogenic, mutagenic and teratogenic effects in humans and aquatic animals (De Flora et al., 1990; 1991; Hylland, 2006). The water solubility and volatility of PAHs is highly dependent on their molecular weight, i.e. solubility decreases as their molecular weights increase (Porte and Albaiges, 1994).

Most bioassays have been traditionally focused on acute embryo-toxicity. It is recognized as one of the most sensitive tests (His et al., 1999). However, numerous studies reported that larval growth assays were even more sensitive than embryo toxicity tests (Hidu, 1965; His and Robert, 1985), with growth inhibition occurring at much lower concentrations than those required to induce embryos abnormality. Studies on the toxicity of crude oil and/or dispersant on larval growth of oyster are very limited though, making comparison very difficult. In a previous study, fertilization success and particularly early larval growth of oysters were shown to be negatively affected by exposure to DWH oil/dispersant and to be sensitive toxicological endpoints (Vignier et al., in prep.a/Article 1 and 2). Exposing gametes of *C. virginica* pre- and post-fertilization, showed that larvae resulting from exposed gametes were more affected than larvae resulting from embryos exposed to dispersed oil and dispersant (Vignier et al., in prep.a/Article 1), indicating a stage-dependent sensitivity to pollutant (Gamete > Embryo). Finally, toxicity responses of early life stages of oysters to CEWAF and dispersant alone followed similar trends, indicating that most of the toxicity was associated with the Corexit itself (Vignier et al., in prep.a/Article 1). It is also expected that sensitive processes such as metamorphosis and settlement of bivalve would likely be affected by acute

exposure of the larvae to pollutant, even for a short period of time and/or at relatively low levels of contaminants (Crisp & Austin, 1960). Despite the fact that the metamorphosis bioassay has been proved to be a rapid, sensitive, reliable and easy method (Phelps & Warner, 1990), only a few studies have used it as a biological response to study the impact of contaminants on oyster larvae (Beiras & His, 1994; His et al., 1997; Mottier et al., 2013).

The aim of the present study was the evaluation of the lethal and sublethal effects of DWH oil, dispersed oil and dispersant on different stages of the rapidly developing *C. virginica* larvae. Three separate experiments were carried out in the laboratory where the effects of increasing concentrations of HEWAF, CEWAF and dispersant using a 96 h static acute exposure were tested on (1) 24 h-old veliger, (2) late umbo larvae (10 day-old), and (3) pediveliger larvae (15 day-old). Finally, this study aimed to evaluate the validity of larval development and metamorphosis of oyster as toxicity endpoints in marine ecotoxicology, and to contribute to the DWH oil spill and dispersant assessment.

2. Material and Methods

2.1. Water Accommodated Fractions

Surface oil ("Slick A"), collected from skimmer vessels at the source during the Deepwater Horizon oil spill on 29 July 2010, and dispersant, Corexit 9500A®, were provided by Stratus Consulting Inc.

The oil only exposure solutions or High Energy Water Accommodated Fraction (HEWAF) were prepared under controlled temperature (25˚C) and under fluorescent lights to avoid photo-induced toxicity. The High Energy method was used to artificially recreate the action of waves, currents and stormy conditions, hence dispersing oil mechanically. Two-liters of UV-sterilized and 0.1 µm-filtered seawater (FSW) were added to a stainless steel blender pitcher (Waring™ CB15 commercial food blender) and then 4 g of "Slick A" (1:500 dilutions of oil) were added using a gastight syringe. After 30 s at low speed blending, the solution was transferred in a 2-L aspirator bottle and left to settle for at least one hour to allow separation of the solution from residual floating oil. The stock solution (2,000 ppm or 2 g oil L^{-1}) was obtained by carefully draining the bottom layer of the mixture from the aspirator bottle. It

was not filtered, and thus contained whole particulate oil in addition to dissolved PAHs. Dilutions were prepared by adding FSW to the stock solution.

The Oil/Dispersant mixtures or Chemically Enhanced Water Accommodated Fractions (CEWAF) were also prepared under similar controlled light and temperature as described above. Four grams of "Slick oil" and dispersant (400 mg) at a ratio of 10:1 (v:v) were added using a gastight syringe to an aspirator bottle, previously filled with 2-L of FSW at a salinity of 20-25 PSU, and stirred at a vortex adjusted to 250 rpm for 18 h on a magnetic stirrer. To allow separation of the solution from residual floating oil, the oil and dispersant mixture was left to stand for at least 3 h.

The dispersant exposure solution was prepared as described above, with no addition of oil. Different exposure concentrations were obtained by adding different amounts of FSW to the stock solution.

The median concentrations (LC50) of HEWAF, CEWAF and dispersant were determined prior to exposure experiments, in order to find the range of lethal and sub-lethal concentrations. For exposure experiments, nominal concentrations of HEWAF, CEWAF and dispersant used and corresponding PAH contents are listed in Table 1.

2.2. Experimental oysters and algae

Adult specimens of *Crassostrea virginica* (average weight of 75 g ± 20) were collected in June and September 2013, from natural populations in Estero Bay, Florida (Lat. 26˚19'50''N, Long. 81˚50'15''W). Adult oysters were conditioned in hatchery at 23˚C ± 1˚C, in a flow-through system supplied with coarsely filtered (30 µm sand filter) seawater, at ambient salinity (20-30 PSU), under natural light conditions, and fed a mixture of laboratory-cultured fresh microalgae (*Tetraselmis chui, Chaetoceros sp*. and *Tisochrysis lutea)* at a daily ration of 3% of oyster dry body weight (Utting & Millican, 1997). Phytoplankton cultures were grown in f/2 culture medium (Guillard, 1975) prepared with FSW, and held in 10 L carboys at 22-23 ˚C and 30-32 PSU salinity on a 12:12 light:dark cycle with cool-white fluorescent lights and appropriate aeration. Ten oysters were periodically sampled and examined for ripeness under a microscope.

2.3. Spawning and larval culture

Mature oysters were induced to spawn by thermal stimulation, by alternating immersion of oysters in seawater at 18˚C and 30˚C for 30 min each time. Spawning females were isolated in 1-L beaker filled with ≈ 500 mL of FSW for collection of oocytes; whereas spawning males were placed in ≈ 200 ml of FSW, to obtain a dense sperm solution. Gametes were examined under a microscope for mobility (sperm), shape and absence of atresia (oocytes) for selection of the best products. After filtration through a 55 µm mesh to remove debris, sperm from several males ($n \geq 3$) were pooled in a 1-L sterile beaker. Eggs from several females ($n \geq 3$), after successive sieving through 150 µm and 55 µm mesh to remove tissue and debris were rinsed on 20 µm sieve and transferred into a sterile beaker filled with 2 L of FSW. Oocytes were fertilized with 2.5 % of sperm solution (v:v), and gently mixed. After continuous and gentle mixing, five subsamples of 50 µL of newly fertilized embryos were stained with Lugol and counted using a Sedgwick-Rafter® cell and a dissecting microscope. After microscopic observation of the first cell cleavage, fertilization success was determined and embryos were thereafter transferred to hatching tank filled up with 50 L of FSW, at a density of 40 embryos mL^{-1}. About 24 h after fertilization at 28°C, embryos developed to swimming straight-hinge larvae or veliger and were retained on a 35 µm sieve. Veliger were then re-suspended in 2 L of FSW, counted as previously described, and used for the first acute exposure. The left-over veligers were placed in a tank at a density of 10 mL^{-1}, and were cultured in the hatchery to the late umboned (10 days) and pediveliger stages (15 days). Filtered seawater, maintained at 28°C, was changed every other day, and larvae were fed with live microalgae according to Helm and Bourne (2004).

2.4. Acute exposure of early veliger, late umboned, and pediveliger larvae

Acute exposures of veliger and umbo were performed with larvae originating from the same cohort in July 2013. Exposure of pediveliger was performed with a different cohort of larvae in October 2013.

2.4.1 Acute exposure of early veligers (day 1)

Early veligers (mean initial length = 70.8 ± 1.61 µm, n=25) were distributed at a density of 15 larvae mL^{-1} (approximately 3000 individuals per beaker) into 400 mL beakers filled with 200 mL of the different exposure concentrations of HEWAF, CEWAF or dispersant (Table 1). Control and treatment groups in quadruplicates, were maintained for 96 h in the dark at 26 ˚C ± 1 and 22 PSU ± 2, with no renewal of the exposure solutions. Gentle aeration was

provided for each beaker in order to maintain D.O levels above 4 mg L^{-1}. Fresh cultured phytoplankton (*T. lutea* and *C. muelleri*) was added to each exposure beaker at day 0 and day 2 (1 x 10^5 cells mL^{-1}). A 10-mL subsample was collected on the first day from the stock (T0), and after 48 h from each exposure beaker and preserved by addition of 300 µL of 10% buffered formalin. After 96 h of exposure, larvae from each beaker were concentrated to 30 mL by filtering larvae through a 35 µm mesh, and preserved with 10% buffered formalin for later observations of survival and shell measurements. Final survival was assessed by taking 300 µL subsamples (n=3) from the concentrate (30 mL) of each of the 4 replicates after homogenization, and observed under a microscope to evaluate live and dead larvae (translucent shell or opened valves). At each sampling time (0, 48 h and 96 h), shell lengths of 25 alive larvae were measured at random in each replicate (total of 100 per condition) using an inverted microscope (Olympus IX73) equipped with a camera Olympus DP73, and the CellSens Software. In addition, percentages of larvae with food in their stomach were assessed after 96 h of exposure in the same 25 individuals measured.

2.4.2 Acute exposure of late umboned larvae (day 10)

Ten day-old umboned larvae were retained on a 90-µm sieve, rinsed, re-suspended in FSW in a sterile 2-L beaker and counted as previously described. Acute exposure of late umboned (mean initial length = 139.4 ± 3.46 µm, n=200) were performed using the same protocol previously described, i.e. same exposure concentrations with 4 replicates per condition, excepted that larvae were loaded at a density of ≈ 2000 in 300 mL of FSW, and 50 larvae were randomly measured in each replicate. The same endpoints, i.e. shell lengths at 0, 48 and 96 h, gut fullness at 96 h, and final survival (considering the initial number of stocked larvae and the final number of survivors) were determined.

2.4.3 Acute exposure of pediveligers (day 15)

Fifteen days after fertilization, when oyster larvae reached the pediveliger stage or "eyed" stage, they were collected on a 200-µm sieve, rinsed, re-suspended in FSW in a 2-L beaker, and counted as previously described. Selected pediveligers (mean shell length ≥ 280 µm) were distributed at ≈ 1000 individuals into 600-mL beakers filled with 450 mL of the different exposure concentrations of HEWAF and CEWAF. Exposure consisted of 5 nominal concentrations and a FSW control, with 4 replicates per concentration (Table 1). Pediveligers were acutely exposed, for 72 h, in a static system at 26 °C ± 1 and 22 PSU ± 2 with no renewal of contaminant, in the dark to avoid photo-induced toxicity (Landrum et al., 1987).

Two settlement plates consisting of a cement board tile (120mm x 58mm), previously soaked/conditioned in sea water for a minimum of 2 weeks, were set-up vertically in the water column of each container. Gentle aeration was supplied to each beaker for 30 min every 2 h using a timer-controlled air pump, in order to maintain dissolved oxygen (D.O) levels > 4 mg L^{-1}. Fresh cultured phytoplankton (*T. lutea* and *C. muelleri*) was added to each exposure beaker at days 0 and 2 (1 x 10^5 cells mL^{-1}).

After 72 h of exposure, developmental success of pediveliger was determined by their progression to settlement as well as mortality. Settlement plates and container walls were observed under a dissecting microscope, and newly settled oysters counted. Remaining larvae were collected on a 150-µm sieve, rinsed with FSW and re-suspended in 30 mL in a 50-mL centrifuge tube. Samples were preserved with 10% buffered formalin for later estimation of survival and metamorphosed larvae. Final survival was assessed by taking three subsamples of 1000 µL from the concentrate (30 mL) of each replicate after homogenization, and observed under a microscope to discriminate between live, dead, and metamorphosed larvae (Fig. 1). Live larvae were distinguished by clarity of internal organs (Fig. 1A). Dead larvae were often grey and opaque, with opened valves, with no evidence of internal organization (Fig. 1B). Some dead larvae showed retraction or partial decomposition of tissues, and some with invasion of bacteria and protozoa (ciliates). Non-settled, metamorphosed larvae were identified by their larger size (>350-400 µm), and by the transition from rounded pediveliger to a flat shape with the new dissoconch (Fig. 1C). Settlement success was thus calculated by considering both metamorphosed and settled ones (Fig. 1D) on tiles versus number of non-metamorphosed (live pediveligers). In addition, as mortality of pediveligers is considered a more severe effect than a lack of metamorphosis, an overall median effective concentration ($EC50_{total}$) including both alive and dead individuals was also determined for HEWAF and CEWAF (Table 4).

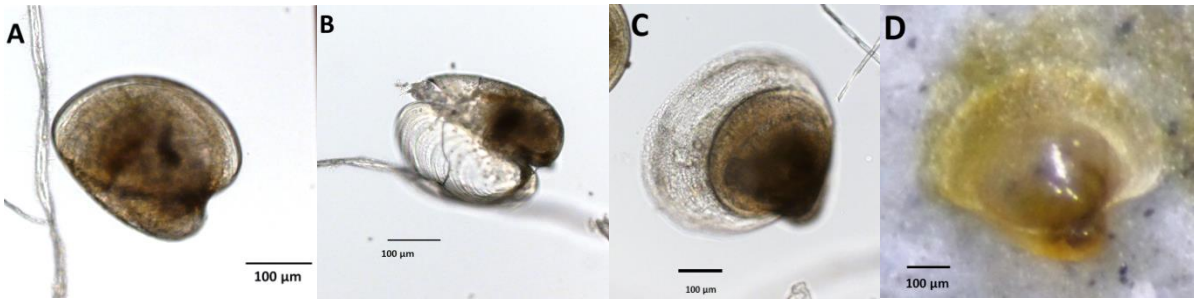

Figure 1: (A) Live *Crassostrea virginica* pediveliger larva, (B) dead larva, (C) metamorphosed larva, and (D) newly settled spat on tile.

2.4.4 Water quality and analytical chemistry

Temperature, dissolved oxygen, salinity, and pH were measured daily using Pro ODO optic probe (YSI), a refractometer and a "Pinpoint" pH monitor (American Marine Inc.). Total ammonia was measured at the start and the end of each exposure experiment, using a Seal Analytical Auto Analyzer 3 and the G-171-96 method.

Chemical analyses of hydrocarbon constituents and PAH composition of the different HEWAF, CEWAF, dispersant concentrations and the FSW control were performed by ALS Environment (Kelso, WA, USA). The 250-mL unfiltered water samples were stabilized with hydrochloric acid, and stored in amber-bottles at 4˚C until shipment to the analytical laboratory by expedited courier. Samples were then extracted with dichloromethane and processed for GC-MS. Polycyclic Aromatic Hydrocarbons including alkyl homologues were determined by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM). A sum of 50 different PAHs was quantified. The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the targeted analytes.

2.5. Statistical analyses

Shell length, settlement, gut fullness and mortality were expressed as means ± standard deviation. All percentage data (percentage of settled larvae or dead larvae) were arcsine-square root transformed prior to statistical analysis. Data were analyzed by analysis of variance (ANOVA), after checking normality (Shapiro-Wilk test) and homogeneity of variances between treatments (Levene's test) using SPSS® 22.0 statistical package. Whenever significant differences among groups were found (ANOVA: $p \leq 0.05$), a Tukey post-hoc test was performed. Kruskall-Wallis non-parametric test and Dunnett post-hoc test were performed whenever normality and homogeneity of variances requirements were not met after transformation of the data. Median-effects and lethal concentrations (EC50/LC50) were estimated using the *drc* and *bbmle* packages in R as well as the trimmed Spearman-Karber procedure (Hamilton et al., 1977). For binomial response variables (mortality, settlement and growth inhibition), data were fitted with a 3-parameter log-logistic model. Final survival was calculated using the number of alive larvae observed at the end of the exposure, divided by the total number initially stocked.

3. Results

3.1. Water quality and PAH analysis

Through the exposure, temperature and salinity were 25.4 ˚C ± 1.3 and 25.3 PSU ± 1.5 respectively. Dissolved oxygen (D.O.) and pH averaged 6.85 mg L^{-1} ± 0.28 and 8.04 ± 0.1 respectively. For each tested concentration of oil and/or dispersant, total ammonia concentration remained below levels causing deleterious effects to tested organisms (tNH_4^+ = 0.325 mg L^{-1} ± 0.360). Filtered seawater (FSW) used for the control treatments showed levels of PAHs at background concentrations (∑PAH=0.45µg L^{-1} ± 0.25), considered negligible for the present study. Sum of 50 PAHs measured for each concentration of HEWAF and CEWAF preparations are shown in Table 1 with corresponding nominal concentrations; whereas, PAH profiles of the stock solution of CEWAF and HEWAF are presented in Appendices B and C.

Table 1: Range of nominal concentrations (mg L^{-1}) of HEWAF and CEWAF used for exposures of 1 day-old veliger larvae, 10 day-old umbo larvae, and 15 day-old pediveliger larvae, and corresponding tPAHs concentrations in µg L^{-1} (sum of 50 PAHs quantified by GC/MS-SIM).

Larval stage exposed	**Veliger/Umbo**	**Pediveliger**
Oil Preparation	**Nominal (mg L^{-1}) => tPAHs (µg L^{-1})**	
HEWAF	0 => 0.5 62.5 => 95.3 125 => 202.0 250 => 389.9 500 => 761.7 1000 => 1605.5 2000 => 2985.2	0 => 0.1 31.25 => 48 62.5 => 113 125 => 191 250 => 399 500 => 719
CEWAF	0 => 0.4 62.5 => 14.0 125 => 25.3 250 => 44.9 500 => 91.2 1000 => 178.5	0 => 0.8 31.25 => 10.1 62.5 => 19.2 125 => 43.7 250 => 80.9 500 => 177.3

3.2. Lethal effects

For both veliger and umboned larvae, dose-dependent mortalities were observed after 96 h of exposure to both CEWAF and Corexit (Fig. 2B & C). At the highest concentrations tested, dead larvae were noted with extruded and vacuolated tissues as well as translucent shells and opened valves (Fig. 1B). Compared to control, CEWAF and Corexit exposures had

significant deleterious effects on larval survival regardless of the larval stage (veliger or umboned larvae) (*p<0.001*). Median lethal concentrations after 96 h ($LC50_{96h}$), expressed as measured PAH concentrations or nominal concentrations, are shown in Table 4 which summarized all experiments carried out in the present work. Nominal concentrations of CEWAF include 1:10 ratio of the dispersant. For exposure of veliger and umboned larvae to CEWAF, $LC50_{96h}$ reached 229/22.9 ppm (corresponding to 45 µg tPAHs L^{-1}) and 396/39.6 ppm (corresponding to 72 µg tPAHs L^{-1}) respectively (Table 4). Similar trends were observed with Corexit alone exposures, with $LC50_{96h}$ values for veliger and umbo larvae reaching 20.6 ppm (corresponding to 2.7 mg DOSS L^{-1}) and 58 ppm (corresponding to 7 mg DOSS L^{-1}) respectively. Median lethal concentrations and their 95% confidence intervals for HEWAF, CEWAF and Corexit were significantly lower for the exposed veliger compared to the exposed umboned larvae (Table 4).

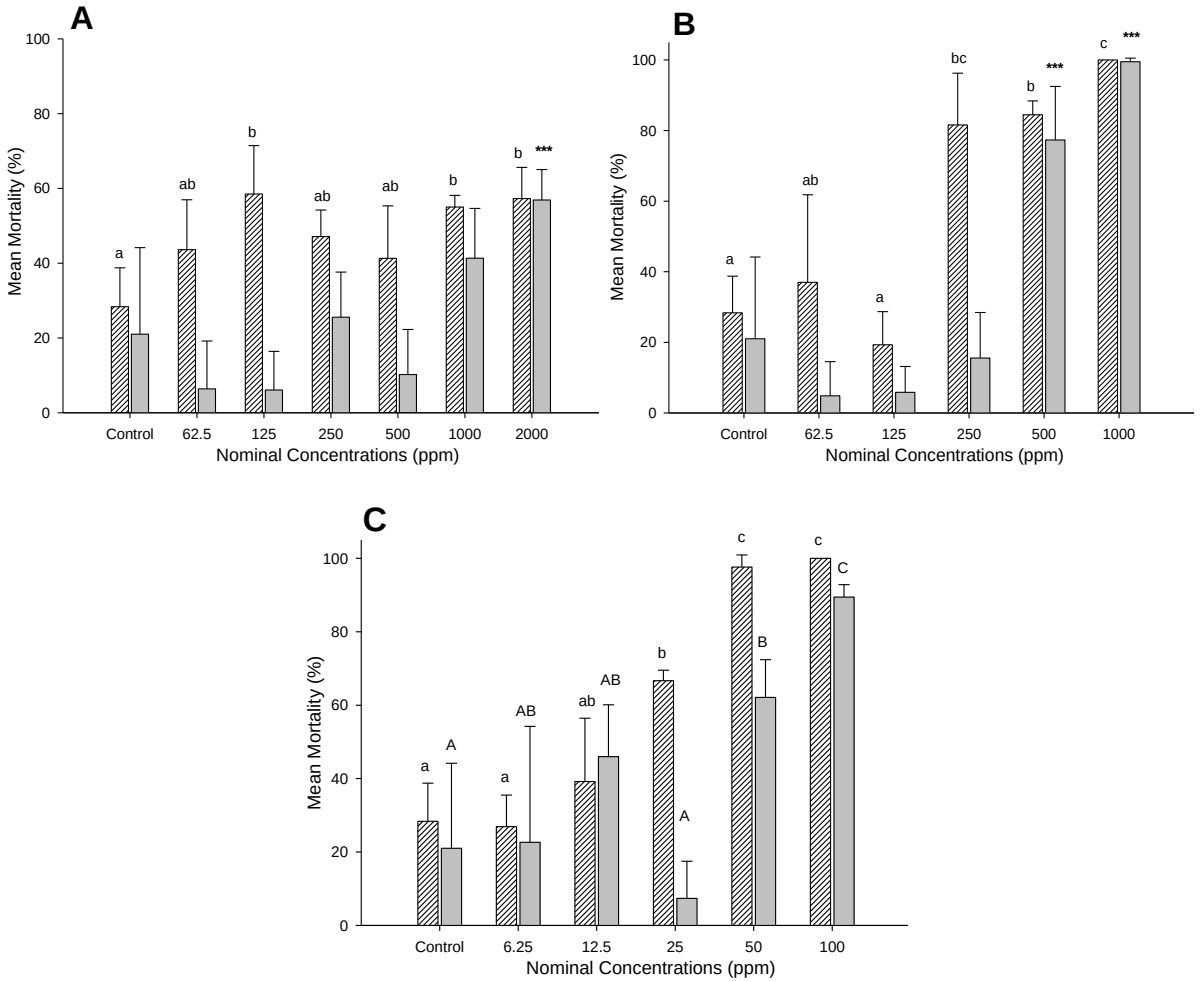

Figure 2: Mean percent mortalities (± SD) of veliger (striped) and umboned larvae (plain) exposed for 96 h to the same nominal concentrations of HEWAF (A), CEWAF (B), and Corexit (C), expressed in ppm. Nominal concentrations of HEWAF (A) 62.5, 125, 250, 500, 1000, and 2000 ppm correspond to measured sum PAH (n=50) 95.3, 202, 389.9, 761.7, 1605, and 2985 µg tPAHs L^{-1} respectively. Nominal concentrations of CEWAF (B) 62.5, 125, 250, 500, and 1000 ppm correspond to measured sum (PAH) 14, 25.3, 44.9, 91.2, and 178.5 µg

tPAHs L^{-1} respectively. Nominal concentrations of dispersant (C) 6.25, 12.5, 25, 50, and 100 ppm correspond to measured DOSS concentrations 0.77, 1.6, 3, 6.2, 12 mg L^{-1}. Different letters denote a statistical difference between treatments at $p \leq 0.05$ (ANOVA). ***: $p \leq 0.001$ (T-test: statistical difference from control). Mortalities were calculated from initial stocking numbers and implied number of dead larvae.

In contrast, acute exposure of both life stages to mechanically dispersed oil (HEWAF) did not induce a clear dose response in term of mortalities compared to CEWAF or Corexit (Fig. 2A). For the veliger exposure, significantly more larvae were dying at 125 ppm (*p<0.001*), 1000 ppm (*p=0.003*) and 2000 ppm (*p=0.001*) than in the control groups. Contrastingly, HEWAF exposure of older umboned larvae only induced significant mortalities at 2000 ppm compared to the control (*p=0.001*, Fig. 2A).

3.3. Sub-lethal effects on growth and feeding regime

Mean shell lengths of veliger and umboned larvae (n=100 per condition) exposed to HEWAF, CEWAF, and dispersant for 96 h are shown in Figure 3. Control treatments for the veliger exposure showed an increase of shell length of ≈ 13 µm in 96 h. In comparison, mean shell length of exposed larvae consistently declined with increasing tested concentrations. The shell increase of veliger larvae exposed to HEWAF was significantly reduced at 125 ppm (equivalent to 202 µg tPAHs L^{-1}) by about 50 %, and completely inhibited at 250 ppm (or 389.9 µg tPAHs L^{-1}) (Fig. 3A). The lowest concentration of HEWAF having a negative effect (LOEC) on shell length of veliger was 62.5 ppm, or 95.3 µg tPAHs L^{-1}. Effective concentrations causing 50 % of growth inhibition, or $EC50_{96h}$, were calculated and are shown in Table 4. The same trend was observed for umboned larvae exposure to HEWAF: mean shell lengths of larvae in the control treatment increased by 52 µm in 96 h; whereas, nominal concentrations of HEWAF equal to 250 ppm (corresponding to 389.9 µg PAHs L^{-1}) inhibited completely the growth of exposed larvae (Fig. 3B). In addition, the LOEC inhibiting shell lengths for umboned larvae was 62.5 ppm, equal to the one found for veliger. When comparing the $EC50_{96h}$ values between the veliger and umboned larvae experiments, a lower effective concentration was observed for umboned larvae exposure (81 ppm or 140 µg PAHs L^{-1}) compared to veliger exposure (141 ppm or 221 µg PAHs L^{-1}) (Table 4).

For CEWAF exposure, shell lengths of exposed veliger and umboned larvae were significantly and negatively affected at LOEC of 62.5/6.25 ppm (≈ 14 µg PAHs L^{-1}) and 125/12.5 ppm (equivalent to 25.3 µg PAHs L^{-1}) respectively (Fig. 3C & 3D). In addition, growth of veliger was completely inhibited at 62.5/6.25 ppm whereas growth of umboned larvae was inhibited at 250/25 ppm of CEWAF (corresponding to 44.9 µg tPAHs L^{-1}).

Effective concentrations of CEWAF inhibiting 50 % of the growth of veliger were lower (54/5.4 ppm) than EC50 values found for umboned larvae (140/14 ppm) (Table 4).

Exposure to dispersant alone showed very similar trends as CEWAF exposure, with shell increment of veliger larvae and umboned larvae completely inhibited at 6.3 ppm (or 770 µg DOSS L^{-1}) (Fig. 3E) and at 25 ppm (or 3000 µg DOSS L^{-1}) (Fig. 3F) respectively. The same trend for Corexit alone was observed for the EC50 values obtained for the 2 larval stages during the CEWAF exposure (Table 4). In addition, CEWAF induced more inhibition on shell lengths of umboned larvae than Corexit alone (Figs. 3D and F) and EC50 values were significantly lower for CEWAF (140/14 ppm) than Corexit (16.8 ppm) (Table 4).

Concerning feeding, table 2 presents the proportion of larvae containing algae in their stomach, characterized by a typical dark-brown color. Number of larvae with food in the stomach decreased with increasing concentrations of oil and/or dispersant ($p<0.001$). Dose-dependent responses were observed for both veliger and umboned larvae (Table 2). Nominal doses of HEWAF of 250 ppm inhibited significantly the feeding of veliger ($p<0.05$) whereas doses of 125 ppm of HEWAF significantly reduced the feeding of umbo larvae ($p<0.001$). However, for umboned larvae, feeding seemed to be less affected at highest concentrations.

Table 2: Mean percentages (± SD) of veliger and umboned larvae observed with food in the gut after 96 h of exposure to CEWAF, HEWAF, and dispersant. Larvae were fed with 100,000 cells/mL of *T. lutea*. Oil and dispersant treatments are expressed as nominal concentrations, in ppm. N/A: no larvae were observed alive, i.e. 100% mortality. Asterisks denote significant differences between exposed and controls (0 ppm): $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (ANOVA, Tukey or Dunnett post-hoc tests).

	Veliger	Umboned
HEWAF		
0 ppm	96% (±3)	100% (±0)
62.5ppm	92% (±2)	98% (±3)
125ppm	85% (±5)	80% (±5)***
250ppm	58% (±11)*	62% (±2)***
500ppm	35% (±10)**	46% (±6)***
1000ppm	1% (±3)***	44% (±5)***
2000ppm	N/A	41% (±6)***
CEWAF		
0 ppm	94% (±3)	89% (±8)
62.5ppm	39% (±14)*	83% (±5)
125ppm	6% (±6)***	86% (±2)
250ppm	3% (±3)***	78% (±2)
500ppm	N/A	70% (±7)***
1000ppm	N/A	N/A
Corexit		
0 ppm	98% (±3)	96% (±3)
6.25ppm	46% (±8)**	93% (±3)
12.5ppm	13% (±6)***	86% (±9)
25ppm	1% (±3)***	75% (±4)**
50ppm	N/A	55% (±9)**
100ppm	N/A	34% (±4)***

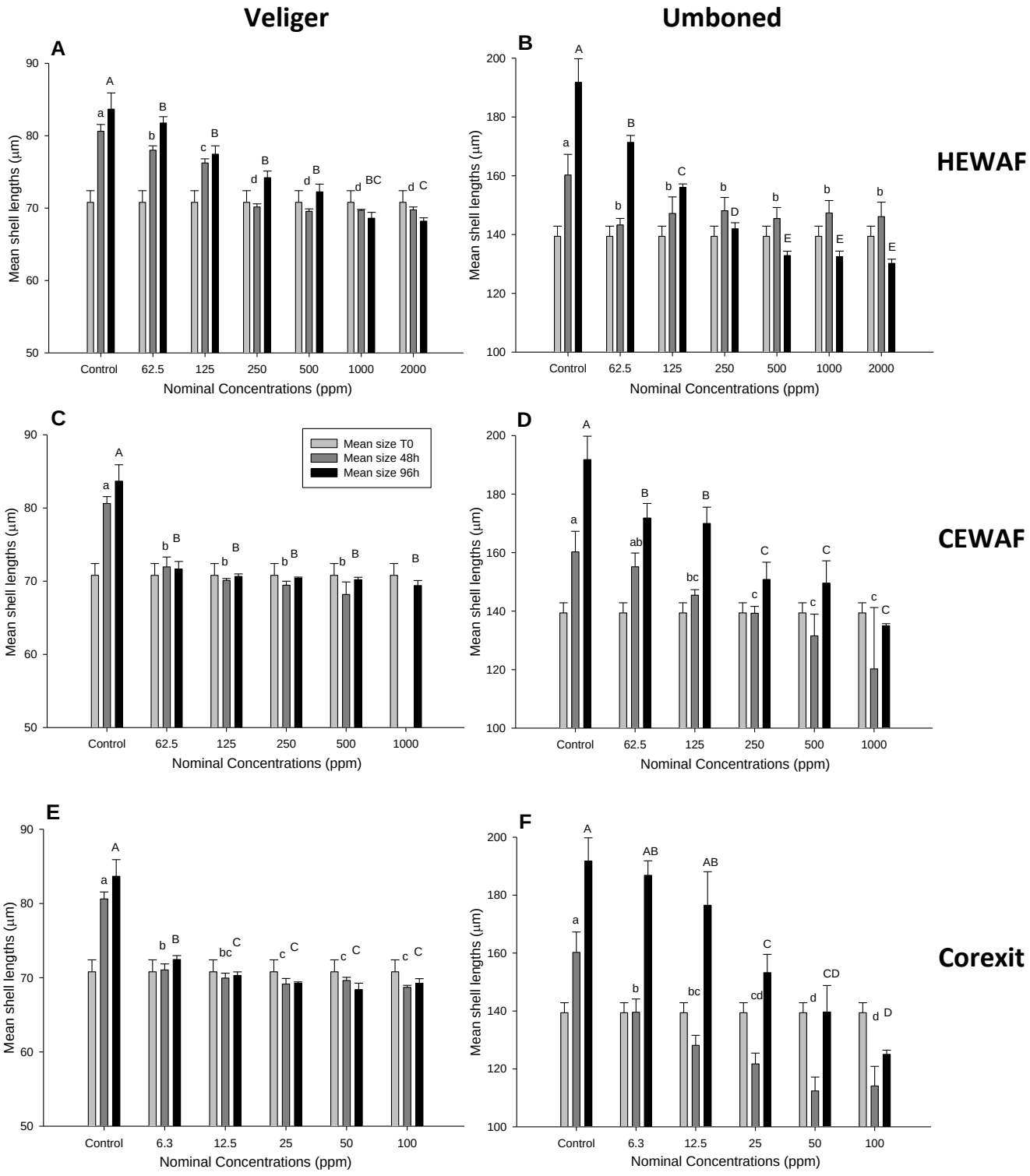

Figure 3: Mean shell lengths (µm ± SD; n=100) of veliger larvae (1 day-old) and umboned larvae (10 day-old) exposed to HEWAF (A & B), CEWAF (C & D) and the dispersant Corexit (E & F) for 96 h, expressed as nominal concentrations in ppm, with initial oil loading rate of 2 g L^{-1} and 0.2 g L^{-1} of dispersant. Nominal concentrations of HEWAF (A, B) 62.5, 125, 250, 500, 1000, and 2000 ppm correspond to measured total PAHs (n=50) 95.3, 202, 389.9, 761.7, 1605.5, and 2985.2 µg tPAHs L^{-1} respectively. Nominal concentrations of CEWAF (C, D) 62.5, 125, 250, 500, and 1000 ppm correspond to measured total PAHs (n=50) 14, 25.3, 44.9, 91.2, and 178.5 µg tPAHs L^{-1} respectively. Nominal concentrations of dispersant (E, F) 6.25, 12.5, 25, 50, and 100 ppm correspond to measured DOSS 0.77, 1.6, 3, 6.2, 12 mg L^{-1}. Different letters denote statistical difference at α=0.05 (ANOVA, Tukey or Dunnett post-hoc tests).

Moreover, there was a strong relationship between feeding regime and shell increment for each solution and life stage tested (*p=0.01,* Table 3). The presence of food in the gut was also strongly correlated (at 0.01 level) with the final survival of the experimental larvae (data not shown).

Table 3: Linear correlation between percent of food in gut *vs* mean growth. Statistical difference at p≤0.05

	Veliger		Umbo	
CEWAF	R=0.939	p=0.01	R=0.867	p=0.01
HEWAF	R=0.918	p=0.01	R=0.893	p=0.01
Corexit	R=0.936	p=0.01	R=0.863	p=0.01

3.4. Sub-lethal effects on settlement success

Mean settlement of pediveligers exposed to HEWAF (A) and CEWAF (B) are shown in Figure 4. Control group exhibited a mean settlement of 40.5 % (± 7.3) after 72 h. Pediveligers exposed acutely for 72 h to increasing concentrations of HEWAF showed a significant settlement inhibition compared to non-exposed larvae (Fig. 4A, *p<0.001*). The lowest concentration having an effect (LOEC) on settlement was 31.25 ppm (corresponding to 48 µg L^{-1} of tPAHs), reducing settlement success by more than 50 % (Fig. 4A, *p<0.001*). Concentration of HEWAF inhibiting settlement success (EC50) could not be calculated: however, EC50 was estimated to be below 31.25 ppm or 48 µg tPAHs L^{-1} (Table 4).

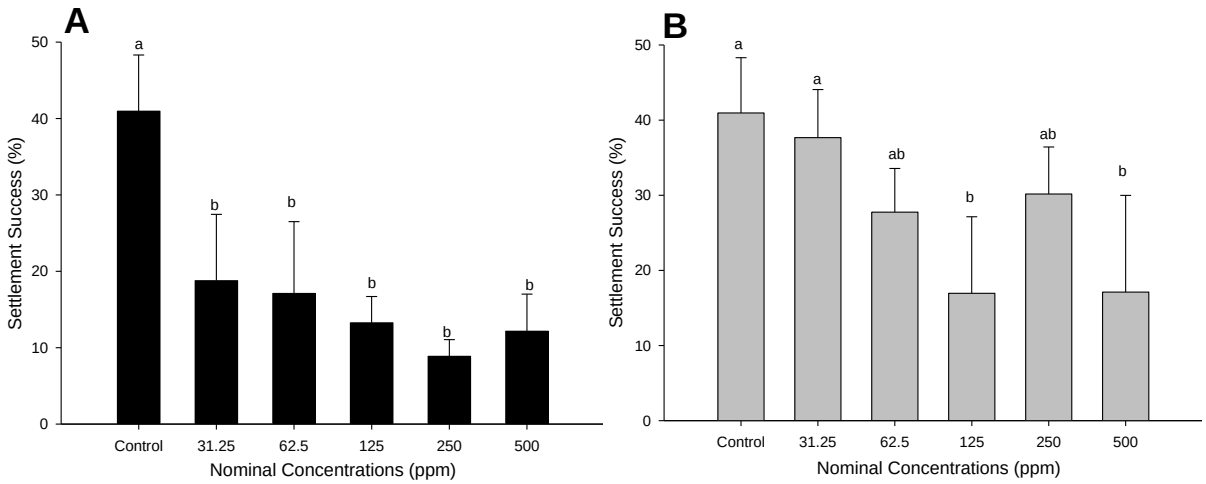

Figure 4: Mean settlement success ± SD, expressed in %, of pediveliger larvae after 72 h of acute exposure to HEWAF (A), and CEWAF (B), expressed as nominal concentrations, in ppm. Measured total PAHs (n=50) concentrations for HEWAF (A) 48, 113, 191, 399, and 719 µg L^{-1} correspond to nominal 31.25, 62.5, 125, 250, and 500 ppm respectively. Measured tPAHs concentrations for CEWAF (B) 10.1, 19.2, 43.7, 80.9, and 177.3 µg L^{-1} correspond to nominal 31.25, 62.5, 125, 250, and 500 ppm respectively. Sum of 50 PAHs quantified by GC/MS-SIM. Different letters denote significant difference between treatments (ANOVA: *p<0.05*). Percentages of settlement presented here were only considering live organisms.

Exposure of pediveliger larvae for 72 h to an increasing range of CEWAF also induced settlement inhibition (Fig. 4B, *p=0.001*). However, results for CEWAF exposure were not as consistent as those obtained with HEWAF. The LOEC reducing settlement success was 125 ppm or 43.7 µg tPAHs L^{-1} (*p=0.002*), with concentration of CEWAF of 500 ppm (or 177.3 µg tPAHs L^{-1}) reducing significantly settlement (Fig. 4B, *p=0.003*). The dose of CEWAF inhibiting 50 % of settlement success ($EC50_{72h}$) was 58.2 ppm (corresponding to 18.4 µg tPAHs L^{-1}, Table 4). Moreover, the overall median effective concentration ($EC50_{total}$), including both dead and non-settled organisms, reached 106 ppm (equivalent to 34.6 µg tPAHs L^{-1}) for CEWAF (Table 4).

Table 4: Median effective ($EC50_{96h}$) and lethal concentrations ($LC50_{96h}$) of veliger larvae (day 1 to 5), umboned larvae (day 10 to 14) and pediveliger (day 15 to 18) exposed to HEWAF, CEWAF and the Corexit dispersant (1:10 oil ratio) (± 95% CI). ECx and LCx values are expressed as nominal concentrations of oil and dispersant (in ppm or mg L^{-1}), and as measured tPAHs (n=50) in µg L^{-1} for oil preparations, and in µg DOSS L^{-1} for Corexit.

	Veliger		**Umboned**		**Pediveliger**		
	$EC50_{96h}$ (growth)	$LC50_{96h}$	$EC50_{96h}$ (growth)	$LC50_{96h}$	$EC50_{72h}$ (settlement)	$LC50_{72h}$	$EC50_{72h}$ (total)
HEWAF (ppm)	**141** (136-147)	**360** (NC)	**81** (78-84)	**1860** (1810-1900)	**< 31***	**> 500***	**NC**
HEWAF (µg $PAHL^{-1}$)	**221** (136-147)	**528** (NC)	**139[a]** (136-147)	**2814[a]** (2738-2875)	**< 48***	**> 719***	**NC**
CEWAF (ppm)	**54** (NC)	**229** (225-233)	**140** (130-150)	**396** (391-400)	**58.2** (54-62)	**242** (222-263)	**106** (100-112)
CEWAF (µg PAH L^{-1})	**10.2** (NC)	**45** (44-46)	**26.6[a]** (25-28)	**72[a]** (71-73)	**18.4** (17-20)	**80.4** (74-88)	**35** (33-37)
Corexit (ppm)	**≈ 5** (NC)	**20.6** (20-21)	**16.8** (16-17)	**58** (57-59)	**---**	**---**	**---**
Corexit (mg $DOSSL^{-1}$)	**0.63** (NC)	**2.7** (2.6-2.7)	**2.1[a]** (2-2.1)	**7[a]** (6.9-7.1)	**---**	**---**	**---**

[a]: extrapolated tPAH/DOSS measured values from veliger exposure solutions. *: Trim was too large or not reached: ECx/LCx could not be calculated. NC: Not calculable

4. Discussion

Despite the fact that oysters, due to their ecological significance, have been extensively studied as a model organism in ecotoxicological studies, information on the toxicity of crude oil and/or dispersant on *Crassostrea virginica* larvae remains limited. Results of the present work demonstrated that oil alone (HEWAF), dispersed oil (CEWAF) and dispersant (Corexit 9500A) were highly toxic to *C. virginica* larvae, regardless of the stage of development. DWH oil alone or in association with dispersant inhibited feeding ability, larval growth and settlement in a dose-dependent manner, and ultimately reduced survival.

Effects on survival

For both veliger and umbo, 96 h mortality figures were dose-dependent for CEWAF and Corexit exposures. At the highest concentrations of CEWAF and dispersant tested, high instances of larvae with translucent, opened shells and partial decomposition of tissue were consistently observed. Overall, toxicity of CEWAF and Corexit followed the same trends, which potentially suggests that most of the toxic effect of the dispersed oil may come from the Corexit, in particular for the veliger larvae tested.

Corexit 9500A® contains hazardous substances including petroleum distillates (solvent), propylene glycol (stabilizer), dioctyl sodium sulfosuccinate or DOSS (surfactant), sorbitan and other ingredients (Nalco Energy Services, 2012). Corexit 9500A® was the main dispersant type used to clean up the Deepwater Horizon oil spill in the Gulf of Mexico (National Commission, 2010) with nearly 7 million L released in the Gulf of Mexico (Kujawinski et al., 2011). Acute toxicity of dispersant alone is usually attributed to its surface active effects on bio-membranes, which include disruption of respiratory cells resulting from electrolytic and/or osmotic imbalances (Singer et al., 1991; 1996). Likewise, our previous work on *C. virginica* revealed severe damages on exposed oocytes and embryos and subsequent larvae (e.g. membrane disruption, extrusion of larval tissue), likely attributable to dispersant (Vignier et al., in prep.a/Article 1 and 2). In addition, gamete and embryo exposed to CEWAF and Corexit generally exhibited similar trends in toxicity.

In the present study, in the case of umbo exposure, CEWAF was shown to induce higher mortalities than Corexit alone (+ 150%) at equivalent nominal doses tested (Table 4). As observed by Almeda et al (2013) with marine microzooplankton exposed to DWH oil, prepared as WAF, and Corexit 9500A®, the increased lethality of chemically dispersed oil to oyster larvae may be simply associated with the additive effects of oil and dispersant. Our

preliminary studies on larvae exposed to WAF (Low Energy WAF) showed a very low toxicity (less than 15 % mortality was induced at nominal doses of 1,000 to 2,000 ppm) after 96 h of incubation (data not shown), which could suggest indeed an additional effect of both oil and dispersant. On the other hand, many authors have described a potential synergistic effect of chemically enhanced WAF compared to WAF, resulting in an exacerbated toxicity on aquatic organisms (Gulec et al., 1997; Negri and Heyward, 2000; Goodbody-Gringley et al., 2013; Rico-Martinez et al., 2013; Almeda et al., 2013). Even though none of these studies have been conducted on larval stages of bivalve such as oysters, we could speculate that an interaction of PAHs with the dispersant occurred, resulting in an increased sensitivity of umboned larvae to CEWAF compared to dispersant alone. Chemical dispersant in combination with crude oil may increase the dissolution of toxic soluble compounds of crude oil such as PAHs (Anderson et al., 1974; Couillard et al., 2005; Greer et al., 2012; Wu et al., 2012; Cohen et al., 2014) and increase their concentration in the water by up to 5 times as reported by Fucik (1994) and Cohen et al. (2001). Similarly, chemical analyses of the exposure solution used in the present study indicated that concentrations in the water of most PAHs were increased by 10 to 15 times for CEWAF compared to LEWAF, and that additional HMW compounds (3 and 4 rings) were dissolved in solution following dispersion with Corexit (see Appendix A and B).

Concerning the results obtained for the HEWAF exposure, interestingly, mortality results for veliger larvae as well as umboned followed the same trends, and were not concentration-related. Nevertheless, high levels of HEWAF and associated oil droplets may have adverse mechanical effects on the larvae such as coating of the gills, hence decreasing cell respiration and causing lethal effects (Anderson et al., 1974; Renzoni, 1975; Sigler and Leibovitz, 1982; Vignier et al., in prep.a/Article 1). Moreover, comparative PAH profiles of filtered *vs* non-filtered exposure solutions suggested that proportion of PAH derived from either the particulate form (as oil droplet) or the dissolved form were highly variable, and depended on the concentration tested and the exposure time (see § III.2). For instance, lower nominal concentrations of HEWAF contained high proportion of soluble PAH (up to 30-40 %) than higher concentrations of HEWAF, which contained mostly droplet-associated oil/PAH and a lower dissolved fraction (≈ 7-8 %) (Heather Forth, personal communications). From this, we could conclude that most of the toxic effects of HEWAF, particularly in respect to the high mortality observed at 125 ppm, may be directly associated with the dissolved fraction of oil/PAHs, as observed by Geffard et al., (2002b, 2003).

Overall, mortality was observed regardless of the larval stage exposed. However, umboned larvae as compared to veliger, showed an increased tolerance (2 to 5 times) to HEWAF, CEWAF or Corexit exposure. These results are in agreement with the previous study of Vignier et al., (in prep.a/Article 1), which showed that larvae resulting from exposed embryos were 2 to 3 times more tolerant to dispersed oil and dispersant alone than larvae resulting from exposed gametes (e.g. CEWAF: $LC50_{96h}$ = 67/6.7 ppm equivalent to 17.7 µg tPAHs L^{-1} *vs* 32/3.2 ppm equivalent to 8.5 µg tPAHs/L). This differential tolerance based on size is likely related to the higher surface area to volume ratio of small organisms which may increase the uptake of dissolved PAHs and/or toxic compounds of dispersant via passive diffusion. Similarly, results from other studies on coral larvae (Goodbody-Gringley et al., 2013) and copepods (Jiang et al., 2012) suggested that body size was inversely correlated with oil/PAH toxicity and that difference of sensitivity was related to variations in respiration rates. This size relationship could explain the difference in toxicity observed between the two larval stages tested.

Effects on feeding regime

Feeding regime is not a common endpoint reported in toxicological studies on bivalve larvae. A consistent decrease of the amount of food observed in the gut of larvae exposed to increasing concentrations of CEWAF, HEWAF and dispersant was shown. Valve closure is a well-known defense mechanism against environmental stress such as pollution in adult bivalve mollusks (Akberali and Trueman, 1985). Rapid shell closure and withdraw in to the shell in response to toxics were demonstrated as well on oyster larvae (Wisely and Blick, 1967). These authors also found that larvae exposed to the highest dose exhibited shells that were sometimes snapped together before the velum had completely retracted, leaving it protruding. Similarly, Renzoni (1975) revealed that after exposure to crude oil, oyster larvae exhibited an abnormal velum, which constantly protruded from the shell and bore inactive cilia. In the same way, protruded velums in larvae exposed to high concentrations of oil and dispersant were often observed in our study, indicating a sudden retraction of the larvae in their shell, which might consequently lead to starvation.

Narcotic effects manifested as sluggish behavior and/or a cessation of swimming is another sublethal effect resulting from oil exposure, and commonly observed in marine plankton species (Berdugo et al., 1977; Saiz et al., 2009; Almeda et al., 2013). Although narcosis is

reversible in most aquatic organisms after recovery in unpolluted waters (Berdugo et al., 1977), a prolonged exposure to narcosis may reduce feeding and growth, and consequently lead to death. Narcosis could therefore explain the reduced or absent feeding activity we observed at the highest treatments. Almeda et al. (2013) demonstrated that narcotic effects in copepods may be associated to both volatile components of hydrocarbons (BTEX) and low molecular weight PAHs such as naphtalene and acenaphtene. Analysis of exposure media showed that naphtalene was one of the most abundant PAH quantified (Appendix).

A significant correlation between feeding regime and growth for each stage and solution tested was also observed. This is in agreement with Strathmann (1987) and Hart & Strathmann (1995) who postulated that smaller larvae typically encounter and filter less food, and are therefore more prone to starvation than larger ones. Hence, oil, dispersed oil and dispersant may reduce the fitness of affected larvae by reducing feeding efficiency, even if the concentrations they are exposed to are not lethal. A stage-dependent feeding response to CEWAF and Corexit, with veliger larvae being more affected than umboned larvae, was revealed, whereas feeding abilities of umbo larvae were more impacted by HEWAF than those of veliger. The higher filtration capacity of umbo compared to veliger, as well as a mechanical action of the particulate oil on gills and velum may explain that difference (Webb and Chu, 1982; Rico-Villa et al., 2009).

Furthermore, oil and/or dispersant could have had a direct lethal effect on the algae used to feed the larvae. Also, due to their lipophilic properties, oil droplets might have aggregated or flocculated with algal cells (Rubinstein et al., 1984), hence reducing its availability to larvae. In the present study, oil droplets were observed in some of the larvae exposed to the highest doses of oil prepared as HEWAF, particularly in the body cavity and in the digestive system, as observed in Sigler and Leibovitz study (1982). This indicates that some larvae filtered directly oil droplets from the surrounding environment, demonstrating that the particulate form of oil and associated PAH could most definitely be a mode of toxicity, in addition to the passive absorption of dissolved PAHs and the direct toxicity of the dispersant.

Effects on growth

Another sublethal effect observed in the present work was a consistent decline of shell lengths in larvae exposed to HEWAF, CEWAF and Corexit solutions. Compared to control, shell lengths were significantly smaller for larvae reared in all exposure solutions after 48 or 96 h. This is in agreement with Renzoni (1975) who reported that *Mulinia lateralis* larvae

previously exposed to crude oil were significantly smaller than non-exposed ones. CEWAF exposure resulted in an absence of growth for veliger and umbo at concentrations of 62.5/6.25 ppm and 250/25 ppm (oil/dispersant) respectively. For Corexit exposure, the same trends were observed than with the CEWAF exposure (6.25 and 25 ppm), suggesting that most of the toxicity of the CEWAF could be attributed to the chemical properties of the dispersant itself. Larval growth in the current work was reduced at similar levels of CEWAF ($EC50_{96h}$ = 54/5.4 ppm equivalent to 10.2 µg tPAHs L^{-1}) than those affecting embryogenesis ($EC50_{24h}$= 50/5 ppm equivalent to 14.2 µg tPAHs L^{-1}) in the embryotoxicity test of Vignier et al. (in prep.a/Article 1), suggesting that larval growth is a valid endpoint as sensitive as embryogenesis. This is in accordance with other ecotoxicological studies exposing oyster larvae to heavy metals (Hidu, 1965; Brereton et al., 1973; Watling, 1982; His and Robert, 1985; Beiras and His, 1994). These results are of particular significance as a marked reduction in larval growth may lengthen the larval period and increase the risks of predation, disease or dispersion (Davis and Hidu, 1969; Calabrese et al, 1973; Beiras and His, 1994).

For oil only exposure (HEWAF), veliger or umboned larvae were both impacted in a dose-dependent way, shell lengths being significantly reduced compared to control. However, growth of umboned larvae was significantly more impaired by the same increasing doses of HEWAF than the growth of exposed veliger larvae. In fact, concentrations of HEWAF inhibiting 50% of growth for veliger was significantly higher ($EC50_{96h}$ = 141 ppm) than the one found for the umbo exposure ($EC50_{96h}$ = 81 ppm). This finding suggests that growth of umboned larvae is more sensitive to HEWAF than growth of veliger larvae, a result in contradiction with CEWAF and dispersant results, which showed that umboned larvae were more resistant than veliger. These contradictory results could be explained by the fact that veliger larvae are physiologically different from older larvae. Newly hatched larvae are mostly endotrophic and are able to survive and grow when deprived of food, using their own reserve up to 72 h PF as shown by Rico-Villa et al., (2009) with *C. gigas*. On the other hand, umboned larvae are exotrophic and rely essentially on food particles from their surroundings (Baldwin and Newell, 1991). When exposed to toxic solution, as previously described, veliger may close their shell as a defense mechanism and hence reduce their feeding activity, but would still grow; whereas, umboned larvae even though less sensitive to oil/PAHs will need to feed to sustain their growth (Ben Kheder et al., 2010b). In addition, larger umboned larvae are filtering more than newly hatched larvae, and therefore may be increasingly and simultaneously exposed to both dissolved and particulate oil found in HEWAF. Many studies

investigating oil toxicity on aquatic organisms highlighted the fact that most toxic effect of crude oil was related to the dissolved fraction of PAH (Barron et al., 1999, 2003; Ramachandran et al., 2004; Carls et al., 2008; Nordtug et al., 2011). However, it has been shown recently by several authors (Lee et al., 2012; Almeda et al., 2013, 2014) that crude oil toxicity could also be associated to its particulate fraction. It has been well documented that some filter-feeding plankton species could ingest these oil droplets which are in the same range as their food spectrum, and therefore be additionally exposed (Lee et al., 1978, 2012; Hansen et al, 2012; Almeda et al., 2014). Given the fact that oil droplets were observed in some of our exposed organisms, direct ingestion of particulate oil by oyster larvae is a potential route of exposure which may partly explain why umboned were more sensitive than veliger in light of growth inhibition and feeding data.

The physiological reason for the negative effect of oil/PAH/dispersant on the developing larvae is not fully understood. As it has been shown with metal exposure on *C. gigas* larvae, the effect of oil/PAHs/dispersant on growth and development is likely related to the inhibition of enzymes involved in the calcification of oyster shell such as alkaline phosphatase (Galtsoff, 1964), or carbonic anhydrase (Hinkle et al., 1987). Further research is needed to understand more precisely the modes of action of oil-related PAH and dispersant on larval development of bivalves.

Effects on settlement success

The present study reported sublethal effects of chemically dispersed oil (CEWAF) causing a 50% decrease in settlement success at concentrations of 18.4 µg tPAHs/L (equivalent to 58.2 mg oil L^{-1}). During oil spills, total PAHs concentration may frequently range from 1 to 150 µg L^{-1} (Neff and Stubblefield, 1995; Law et al., 1997). Reported concentrations of total PAHs in water samples collected during the DWH oil spill ranged from 100 µg L^{-1} near the wellhead to below detection limit in distant waters (Wade et al., 2011; Allan et al., 2012). The finding of the present study implies that relatively low concentrations of tPAHs (e.g. 18.4 µg L^{-1}), at levels realistically found in the environment at the time of the DWH oil spill, could have detrimental consequences on a critical process of the life cycle of *C. virginica* that is metamorphosis/settlement. For the exposure to mechanically dispersed oil (HEWAF), settlement was inhibited in a dose-dependent manner. In addition, concentrations inhibiting the settlement of pediveliger ($EC50_{72h}$) were significantly lower than the doses of HEWAF

inhibiting growth of veliger or umbo larvae (Table 4), indicating that settlement inhibition is a very sensitive endpoint. It would thus be interesting to include it in toxicological assessment of crude oil and dispersant. Several studies investigating the negative effects of heavy metals (Watling, 1983; Beiras & His, 1994), pesticides (Mottier et al., 2013), or contaminated sediments (Phelps & Warner, 1990, His et al., 1997) on the settlement of oyster larvae have shown that metamorphosis failure is a valid bio-indicator of general toxicity for exposure of *C. gigas* to contaminants. However, to our knowledge, this is the first time settlement success was studied as an endpoint using *C. virginica* pediveliger exposed acutely to oil and dispersant, particularly without the use of the chemical inducer epinephrine.

Furthermore, in a similar way to the growth inhibition of umboned larvae observed with HEWAF ($EC50_{96h}$ = 81ppm) and CEWAF ($EC50_{96h}$ = 140/14 ppm), it appears that sublethal effects of HEWAF on settlement were stronger compared to CEWAF (< 31 ppm *vs* 58.2 ppm). Again, chemical characteristics of HEWAF and the contribution of dissolved PAH found in higher proportion in low doses of HEWAF preparations, or the cumulative effects of dissolved and droplet-associated PAHs, may explain the increased impact of HEWAF on settlement compared to CEWAF, at equivalent nominal concentrations, as previously described with growth inhibition. In addition to the acute toxic effect of PAHs on pediveliger, we could also suspect that coating of settlement substrate by crude oil or oil-associated droplets may have been deleterious to the settlement of competent larvae. Nominal EC50 values found for HEWAF, lower than CEWAF, support that HEWAF-derived oil droplets could have definitely contributed to the lack of settlement observed. In a similar way, Smith & Hackney (1989) found that setting of larvae on oil-treated shells was delayed and spat recruitment on oiled-shells was significantly lower than control shells. Banks & Brown (2002) showed that clay tiles previously exposed to hydrocarbons in the laboratory depressed settlement success of *C. virginica* larvae. Further research is required to elucidate the mechanisms by which oil/PAHs and/or dispersant affect the processes of metamorphosis and settlement.

5. Conclusion

It can be concluded from the current study that larval growth, feeding regime and settlement success are sensitive physiological endpoints, which can be useful indicators for a realistic assessment of the impact of a major oil spill like the DWH event. Moreover, HEWAF

exposure of umbo larvae highlighted the necessity of considering the contribution of particulate oil as well as the dissolved fraction in toxicity study, as it can interact with the normal physiology of larvae (e.g. feeding rate) and impact negatively growth. Because it may be challenging to dissociate between the toxicity related to dissolve PAH *vs* droplet-associated PAHs, results should be reported using both nominal and measured concentrations. Additionally, due to its lipophilic properties, the particulate form of oil should be investigated in association with an algal species, using dietary pathway as a mode of exposure of oyster larvae.

Observed effects of oil/dispersant on larval development and metamorphosis led to wonder whether larvae could recover from a temporary acute exposure. It is evident that acute lethal effects of oil/dispersant are irreversible: nevertheless, more subtle effects on the feeding regime of oyster larvae may only be deleterious for a few days, and larvae could recover rapidly as shown by Ben Kheder et al. (2010b). Other processes such as settlement might be delayed substantially due to delays in growth, and potentially never occurs because of a temporary oil/dispersant exposure. Furthermore, adverse effects of crude oil and dispersant during the larval development may increase the larval predation risk, decrease the metamorphic success and impact the subsequent life history stages. In this light, more research should be done on the impacts of oil exposure to larvae and the subsequent capacity to recover in non-exposed seawater. This way, we could see whether deleterious effects on larval development, growth rates, metamorphosis success and survival are reversible or not.

Finally, it has to be denoted that in the natural environment, toxicity of crude oil depends not only on the concentration and duration of exposure, but also on environmental conditions. Consequently, the impact of accidental oil spills on early life stages of oyster will vary depending on the specific circumstances of each catastrophe. For instance, temperature, UV radiation or salinity may increase substantially the toxicity of crude oil to marine organisms (Jewell, 1994; Pelletier et al., 1997; Lyons et al., 2002; Ramachandran et al., 2006; Almeda et al., 2013). Alone or in combination with other environmental stressors, deleterious effects of DWH oil and dispersant on growth, feeding regime and metamorphosis, as observed in this study, could have important implications on the recruitment for the following year, and cause long term negative impacts on the population dynamics of oysters in the Gulf of Mexico. Although speculation and extrapolation of specific laboratory studies to the field needs to be taken cautiously, experimental studies are a reliable ways of determining major toxic effects of hydrocarbons on sensitive aquatic organisms such as oyster larvae. Therefore, our results could enable further understanding of the acute effects of chemically and mechanically

dispersed DWH oil and dispersant on oyster larvae, and help predict the potential impacts of catastrophic oil spills on an ecologically and economically important species of the Gulf of Mexico.

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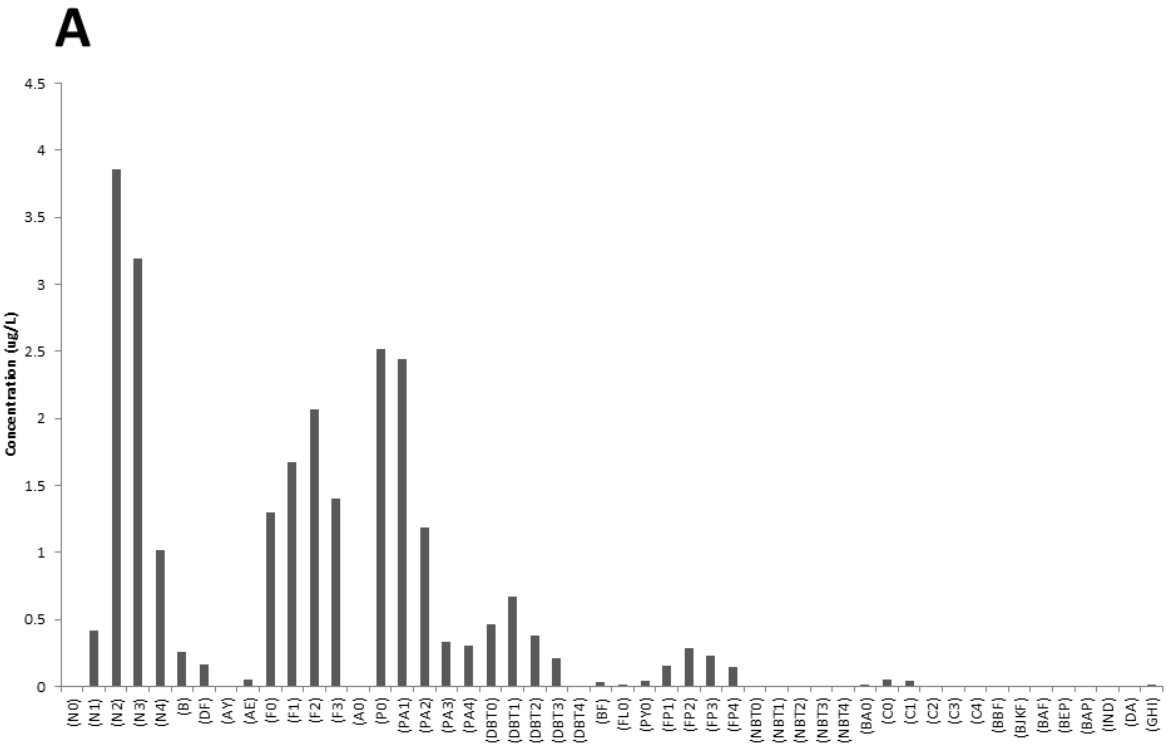
APPENDIX


A
Concentration (ug/L)
4.5
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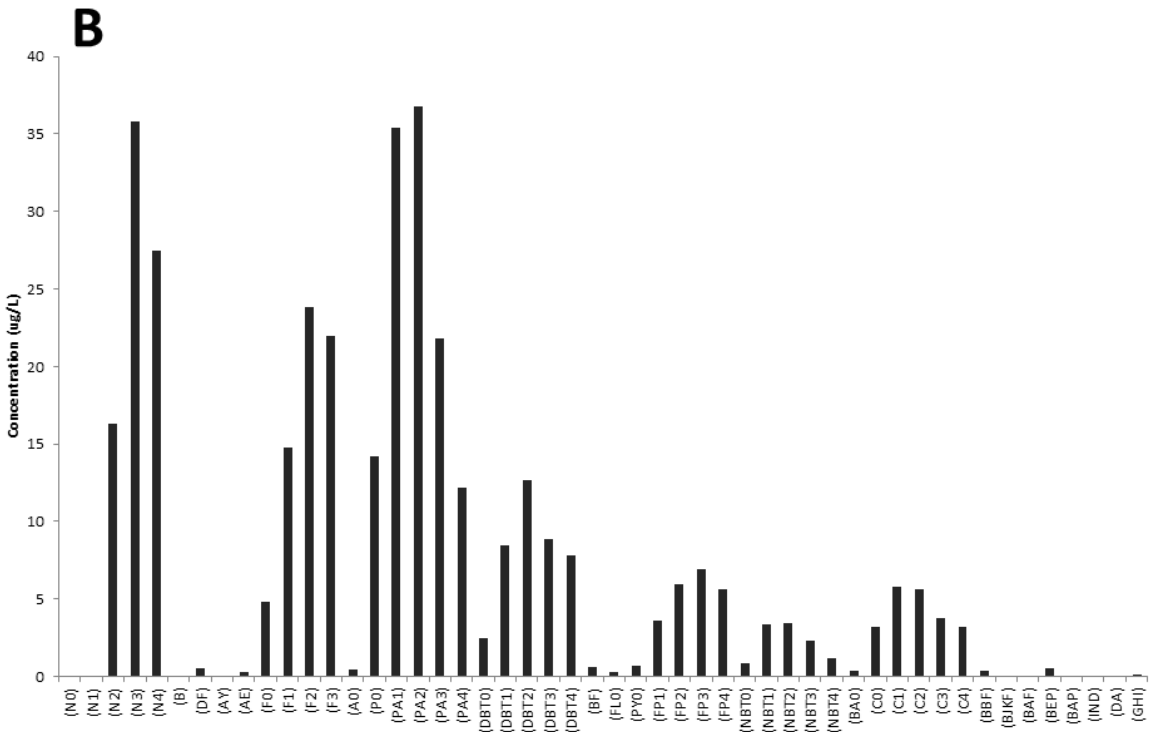
B
Concentration (ug/L)
40
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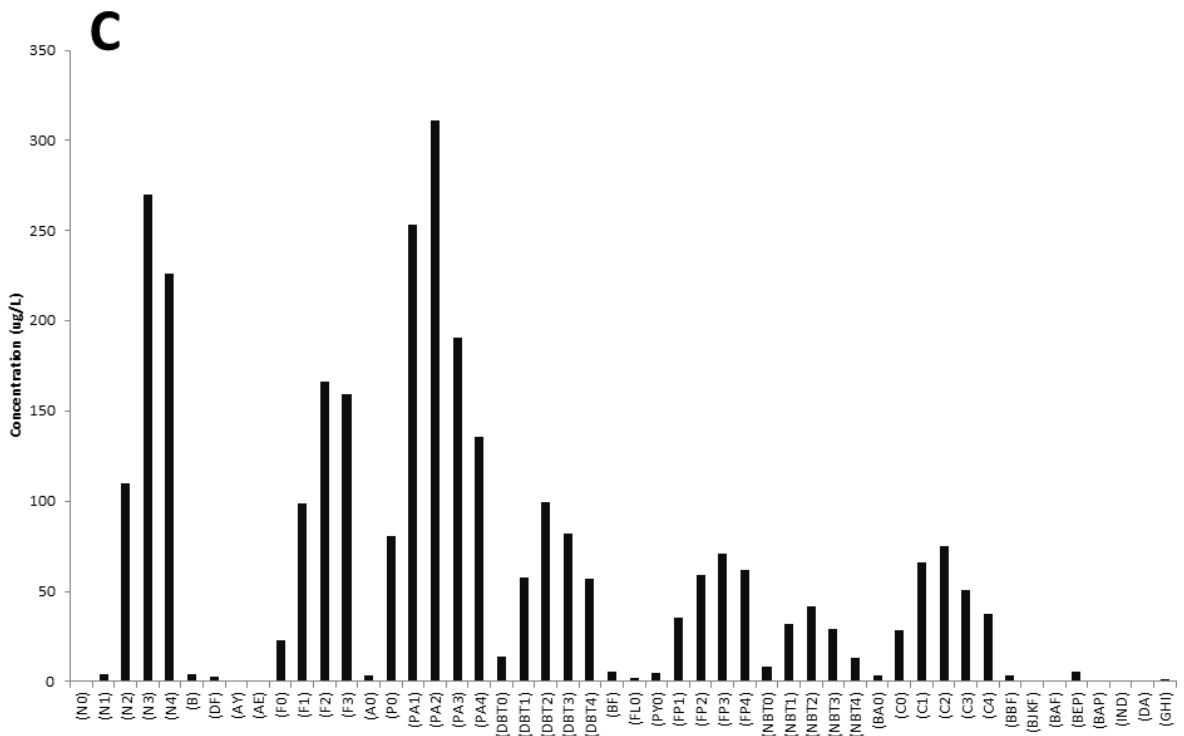

Appendix: PAHs content in stock solutions of (A) Low Energy WAF (LEWAF), (B) CEWAF, and (C) HEWAF, expressed in µg L^{-1} quantified by GC/MS-SIM. Stock solutions correspond to nominal oil load of 2000 ppm or 2 g oil L^{-1}. LEWAF (or WAF) was prepared by adding 2-L of FSW to an aspirator bottle. Stirring was then provided with no vortex (≈ 180 rpm), and 4 g of oil were added to the seawater. After covering the top with aluminum foil to avoid losses of volatile compounds, the WAF solution was stirred for 18 to 24 h in the dark. N0-4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene; PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0: Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4: Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF: Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA: Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0), while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc

Article 4

Interactions between *Crassostrea virginica* larvae and DWH oil: sublethal effects *via* dietary pathway

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Abstract

The *Deepwater Horizon* (DWH) disaster off the coast of Louisiana released crude oil in the Gulf of Mexico for 85 days, overlapping with the reproductive season of the oyster *Crassostrea virginica*. PAHs and oil droplets, based on their lipophilic properties, can adsorb onto phytoplankton. The long pelagic life of filter-feeding oyster larvae and their phytoplankton diet make them vulnerable to acute exposure to contaminants bound to suspended sediment, adsorbed onto algal and other particles, or in solution. In the laboratory, we simulated for 14 days an indirect mode of exposure by feeding 5 day-old larvae with algae (*Tisochrysis lutea*) mixed with four different concentrations of unfiltered DWH oil (HEWAF) known to cause sub-lethal effect in a static renewal system. Growth, feeding behavior, abnormality and mortality were monitored throughout the exposure. Total PAH (n=50) content of the water medium, in which larvae were grown, were quantified by GC/MS-SIM at test initiation, and before water change. Results revealed that oil droplets could bind to unicellular algae, resulting in particles which are in the size-range (1-30 µm) of food ingested by oyster larvae. When exposed for 14 days using a dietary pathway, larval growth and survival were negatively impacted at concentrations of tPAHs as low as 1.6 µg L^{-1}. GC/MS-MS analysis of the exposure medium confirmed that certain PAHs were also adsorbed by *T. lutea,* and potentially taken up by oyster larvae *via* ingestion of oil droplets and/or contaminated algae. The findings of the present study are of particular concern as they revealed that dietary exposure of oyster larvae is a realistic pathway of crude oil toxicity, which could have serious implication on the planktonic community and the food chain in general. Moreover, long-term exposure to levels of tPAHs realistically found during the DWH oil spill (1.6 to 78 µg L^{-1}) was shown to affect adversely larval survival.

Keywords: Deepwater Horizon oil, droplet, oyster larvae, sub-lethal, dietary exposure, PAHs

Résumé

La catastrophe *Deepwater Horizon* (DWH) au large des côtes de la Louisiane en 2010 libera du pétrole brut dans le Golfe du Mexique pendant 85 jours, à une période qui coïncida avec la saison de reproduction de l'huitre *Crassostrea virginica*. Les HAP et micelles de pétrole, en raison de leur propriétés lipophiles, peuvent s'adsorber sur du phytoplancton. La longue phase larvaire ainsi que le régime phytoplanctonique des huitres les rendent particulièrement vulnérables à une exposition aigue à des contaminants associés à des particules sédimentaires ou algales, ou simplement en solution. Nous avons simulé en laboratoire durant 14 jours un mode d'exposition indirecte en nourrissant des larves âgées de 5 jours avec une algue fourrage (*Tisochrysis lutea*) mélangée au préalable avec 4 différentes doses de pétrole DWH (HEWAF) non-filtrée, dans un système semi-statique avec renouvellement de solution tous les 2 jours. La croissance larvaire, l'alimentation, les anomalies et mortalités furent suivies tout au long des 14 j d'incubation. La somme de 50 HAP (exprimée en tPAHs) contenus dans le milieu d'exposition où les larves ont été cultivées, furent quantifiés par GC/MS-SIM au début et avant chaque changement d'eau. Nos résultats révélèrent que les gouttelettes de pétrole pouvaient adhérer aux cellules d'algue, créant des particules dont la gamme de taille (1-30 µm) coïncide avec la taille de nourriture ingérée par les larves de *C. virginica*. Au bout de 14 jours d'exposition indirecte, la croissance larvaire et la survie furent réduites à des concentrations d'HAP (n=50) aussi faibles que 1.6 µg L^{-1}. Les analyses GC/MS du milieu d'exposition confirmèrent que certains HAP ont été adsorbés par *T. lutea* et qu'ils pouvaient être ingérés par les larves *via* filtration des gouttes de pétrole et/ou d'algue contaminée. Les résultats de cette étude sont particulièrement important car ils suggèrent qu'une contamination par la voie trophique *via* une algue fourrage est un mode de toxicité possible par les hydrocarbures, qui pourrait avoir de sérieuses implications sur la communauté planctonique et la chaine alimentaire. En outre, des expositions à long-terme à des doses d'HAP vraisemblablement rencontrées dans l'environnement durant la marée noire DWH (1.6 à 78 µg L^{-1}) se sont montrées délétères pour la survie larvaire de *C. virginica*.

Mots-clés : pétrole *Deepwater Horizon*, gouttelettes, larves d'huitres, sublétales, HAP, exposition par voie trophique.

1. Introduction

In April 2010, the explosion of the *Deepwater Horizon* (DWH) oil drilling rig led to the largest marine oil spill in United States history, with an estimated 779 million L of crude oil and about 8 million L of dispersants (Corexit 9527 and 9500A®) released into the Gulf of Mexico (Camilli et al., 2012; OSAT/NOAA report, 2010; Kujawinski et al., 2011). DWH crude oil spilled from the *Macondo-1* well from April 20th until the final capping of the leak on July 15th (Crone and Tolstoy, 2010), a period that coincides with the natural spawning season of eastern oyster in the Gulf of Mexico (Ingle, 1951; Hayes and Menzel, 1981). The developing oyster pelagic larvae spend 2 to 3 weeks in the water column, generally floating near the surface, until they sink to settle on suitable substrate (Bahr and Lanier, 1981). Among the biological components of marine ecosystems, planktonic organisms are particularly susceptible to crude oil pollution (Walsh, 1978; Graham et al., 2010; Almeda et al., 2013; 2014). Zooplankton such as oyster larvae cannot overcome the effects of currents, limiting their capacity to avoid crude oil patches and, potentially forcing them to drift into highly polluted waters after oil spills.

After an oil spill, small crude oil droplets (1-100 µm in diameter) are generated naturally in the marine environment through the action of wind and breaking waves (natural dispersion). The wave energy causes the oil on the surface to break and effectively suspends the oil droplets in the water column (Delvigne and Sweeney, 1988; Mukherjee and Wrenn, 2009). In addition, the larger droplets will resurface and coalesce to form thin oil films, while plumes of the smaller dispersed oil droplets will have a much lower resurfacing velocity, and will therefore passively drift in subsurface waters (Lichtenthaler and Daling, 1985; Lewis and Daling, 2001). Chemical dispersants, like Corexit 9500A®, enhance this natural process of oil dispersion (Lessard and DeMarco, 2000; Li et al., 2008b). Moreover, these crude oil droplets are generally similar in size (1-50 µm) to the microalgae that are within the food spectra available to zooplankton (Hansen et al., 2012; Almeda et al., 2013) and filter-feeding organisms like oyster larvae (Baldwin and Newell, 1991; 1995; Robert et al., 2004; Ponis et al., 2006), with evidence of ingestion of oil droplets by some zooplankton species (Hansen et al., 2012; Lee et al., 2012; Almeda et al., 2014).

Polycyclic aromatic Hydrocarbons (PAHs) are considered to be the best indicator of the potential toxicity of spilled crude oil to water-column organisms (Anderson, 1977; Neff and Stubblefield, 1995), exerting its toxicity by interfering with membrane fluidity (Van

Brummelen et al., 1998). The water solubility and volatility of PAHs is highly dependent on their molecular weight, i.e. solubility decreases as their molecular weight increases (Porte and Albaigés, 1994; Djomo et al., 1996). As a result, lower molecular weight (LMW) compounds will be preferentially dissolved whereas heavier molecular weight (HMW) PAHs will be mainly found attached to particulate organic matter, sediment, detritus and microalgae (Lee, 1977; Rubinstein et al., 1984; Baumard et al., 1999). Due to their lipophilic properties, PAHs have a high affinity for biological compartments with high lipid content (Neff, 1979; Spacie et al., 1995). Microalgae are living photosynthetic particles rich in lipids to which lipophilic PAHs can adsorb. For instance, Widdows et al (1982) showed that PAHs could be associated with algal cells, in accordance with other studies (Herbes et al., 1977; Lee et al., 1978). The adsorption of organic pollutants to suspended particles in the water column has been described as an essential process influencing the bioavailability and toxicity of hydrophobic pollutants (Lyman, 1995; Chu et al., 2003). Pollutant uptake from water, sediments, and food sources are three routes by which filter-feeding animals, including oysters, can be exposed to PAHs. In the natural environment, these exposure routes occur simultaneously (Spacie et al., 1995). Consequently, PAHs can enter the food web and be potentially transferred to higher trophic level as these food particles are ingested and accumulated by filter-feeding organisms (Wolfe et al., 1998; 2000; Okay et al., 2000).

To sum up, oyster larvae may take up petroleum hydrocarbons directly, through passive uptake or absorption of the dissolved fraction of oil across biological membranes (Kayal and Connell, 1990), or ingestion of oil droplets, and/or through the ingestion of contaminated phytoplankton (Okay et al., 2000; Croxton et al., 2012). A previous study challenging various stages of oyster larvae with DWH oil and dispersant using 96-h acute assays (Vignier et al., in prep.b/Article 3) demonstrated that acute exposure to oil/dispersant altered survival, feeding regime and growth in a dose-dependent as well as stage-dependent manner. This study also showed that larvae could potentially filter directly oil droplets (presence of droplets in body cavity). This finding suggested that the ingestion of the particulate form of oil and associated PAHs was a realistic mode of toxicity, in addition to the passive absorption of soluble PAHs.

The overall goal of the present study was to i) evaluate the lethal and sublethal effects of DWH oil on *Crassostrea virginica* larvae using a dietary pathway and ii) determine whether larvae could ingest particulate oil because oil droplets are likely to be in their food size spectrum. To target these objectives, late veliger larvae (5 day-old) were fed throughout the

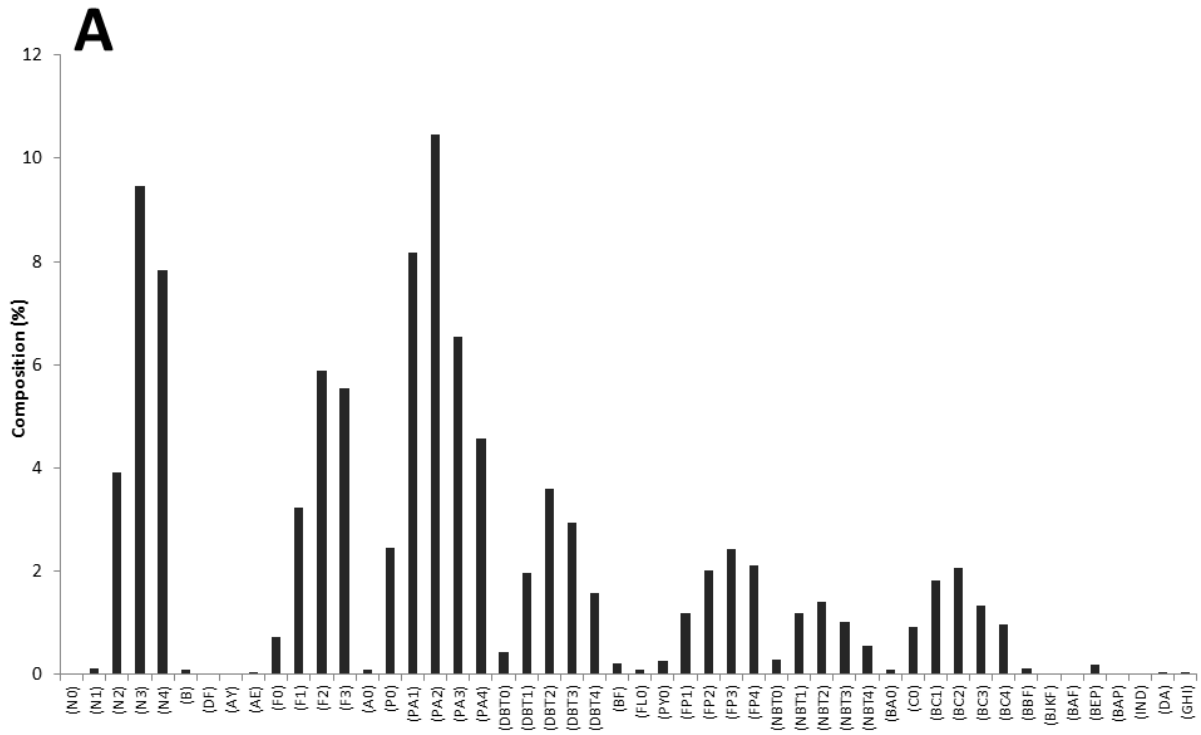
whole larval stage (14 days) with the microalgae *Tisochrysis lutea*, mixed with different concentrations of HEWAF.

2. Material and Methods

2.1. Water Accommodated Fractions

Surface oil (Slick A, sample CTC02404-02) was collected at the source during the Deepwater Horizon oil spill on 29 July 2010 from a barge holding mixed oil offloaded from a number of different skimmers, and was provided by Stratus Consulting Inc.

The High Energy Water Accommodated Fraction (HEWAF) exposure solutions were prepared following a standardized procedure detailed in Vignier et al. (in prep.a, b/ Article 1 and 3). The HEWAF stock solution (2,000 ppm or 2 g oil L^{-1}) was not filtered, and thus contained whole particulate oil in addition to dissolved PAHs. PAH profiles of DWH crude oil (Slick A) and HEWAF stock are shown in Figure 1A and B.



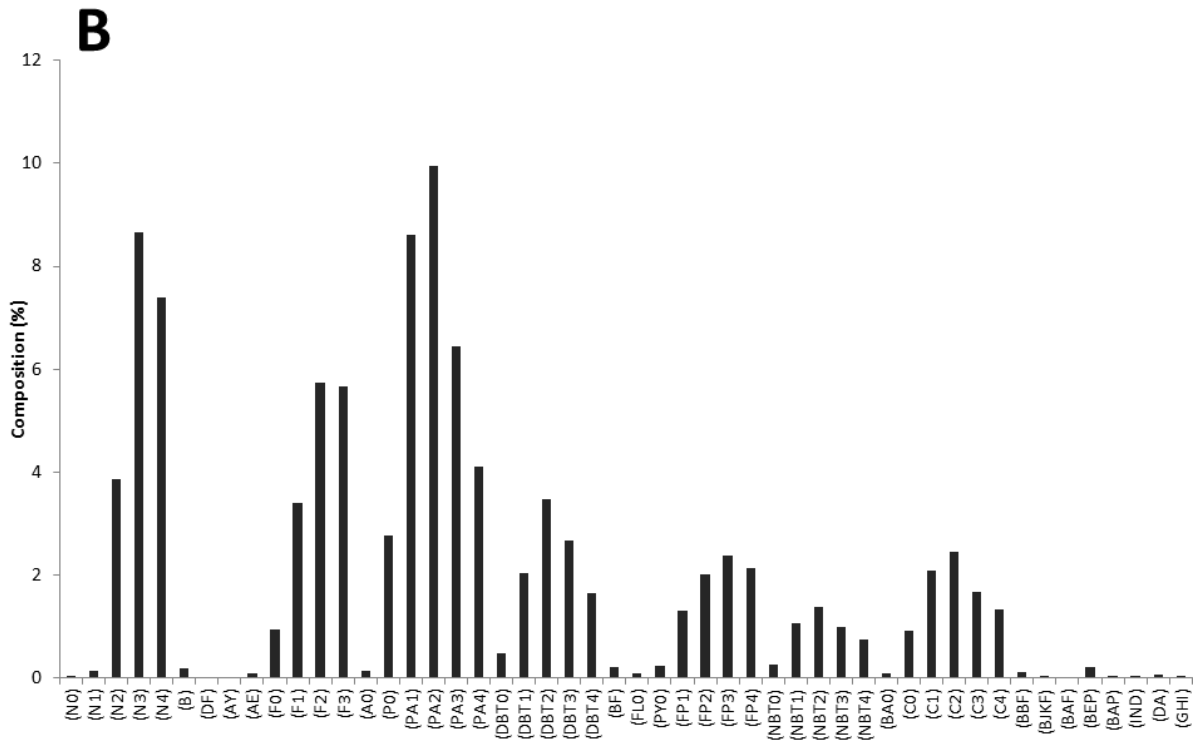


Figure 1: PAH profile (n=50) of crude oil "Slick A" (A) and HEWAF stock (B), expressed as % composition, quantified by GC/MS-SIM. Content of tPAHs (n=50) in crude oil corresponds to 0.31 % of the mass. HEWAF stock corresponds to nominal oil load of 2,000 ppm or 2 g oil L^{-1}.
N0-4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene; PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0: Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4: Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF: Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA: Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0), while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc.

2.2 Experimental oyster and microalgae

Adult specimens of *Crassostrea virginica* (average weight of 75 g ± 20) were collected in October 2012, from natural populations in Estero Bay, Florida. Adult oysters were acclimated and conditioned in the hatchery at 23˚C ± 1, in a flow-through system supplied with coarsely filtered (30-µm sand filter) seawater, at ambient salinity (20-30 PSU), and fed a mixture of fresh microalgae (*Tetraselmis chui, Chaetoceros muelleri* and *Tisochrysis lutea)* at a daily ration of 3% of oyster dry body weight (Utting and Millican, 1997). The microalgae, *T. chui, C. muelleri* and *T. lutea,* were grown in f/2 culture medium (Guillard, 1975) prepared with previously autoclaved FSW, and held in 10-L carboys at 22-23˚C and 30-32 PSU salinity on a 12:12 light:dark cycle with cool-white fluorescent lights and appropriate aeration.

Mature oysters were induced to spawn in the hatchery by thermal stimulation. Gamete collection, fertilization and larval rearing were carried out following methods previously detailed in Vignier et al. (in prep.b/Article 3).

2.3. Dietary exposure

2.3.1 Preparation of contaminated algae and feeding regime

Tisochrysis lutea (equivalent spherical diameter ESD ≈ 5 µm), formerly known as *Isochrysis* aff. *galbana* or T-*Iso* (Bendif et al., 2013), was used as the contaminant vector to feed oyster larvae for the duration of the exposure. Each exposure concentration was prepared by adding volumetrically stock solution of DWH oil prepared as HEWAF (2,000 ppm nominal oil load) to the algal suspension in a 400-mL glass beaker, to achieve a final algal concentration of 4.5 x 10^6 cells mL^{-1} and final oil nominal concentrations of 31.25, 62.5, 125 and 250 ppm. Experimental nominal concentrations chosen for this study were based upon results from a previous experiment, designed as "range finding" investigation, in which no significant effects on i) larval survival and ii) microalgae viability were observed. Each beaker, containing 300 mL of different nominal concentrations of algae-oil mixture, was gently stirred (250 rpm) using a stir bar for 6 h, allowing the oil to impregnate the algae, and left in the dark, covered with parafilm. Algae and oil mixtures were added to each exposure vessel at the beginning of the experiment and at each water change at a feeding regime of 150,000 cells mL^{-1} of *T. lutea* (total of 225 x 10^6 cells *T. lutea* per jar equivalent to about 50 mL of oil-contaminated algae, or about 3% of total volume of larval culture). Concentrations of PAHs (sum of 50) to which oyster larvae were exposed over the duration of the experiment ranged from 1.6 to 18.5 µg L^{-1}.

2.3.2 Oil droplet size distribution

Stock solution of HEWAF (2000 ppm) was examined on a glass slide using an inverted microscope Olympus IX73 equipped with a camera Olympus DP73. Oil droplet (n=392) diameters were randomly measured and counted using an image analysis software (CellSens), and a size distribution was determined.

2.3.3 Flow-cytometry analysis

To determine the different populations of particles (oil droplets, algae and algae/oil) as well as their sizes, each exposure oil and algae suspension was analyzed by flow cytometry using a Beckman-Coulter, Cytomics FC 500, and side scatter (SSC), forward scatter (FSC) and orange fluorescence (FL2) were used. Samples were acquired during 30 s at a fixed flow rate (0.59 $\mu L.s^{-1}$) and the percentages of algae and oil droplets, alone or combined, were determined by counting the number of events (particles) in 30 s.

2.3.4 Larval exposure protocol

Late veliger larvae (5 day-old, mean initial length = 97.7 µm ± 0.6, n=103), collected from the hatchery on a 55-µm sieve, were initially stocked at about 13 mL^{-1} (about 20,000 per jar) and exposed to a mixture of DWH oil and algae *(T. lutea*) for 14 days, in 1500-mL sterile glass jars, using a static-renewal system. To prevent the loss of some PAHs by evaporation or attachment to surfaces, which would reduce the potential toxicity of oil after several days, exposure media were changed every 2 days, during the 14 days. DWH oil stock was prepared as High Energy (HEWAF) method (see 2.1), mixed with the algae for 6 h (see 2.3.1 for more details), and then fed to larvae at the start of the exposure, and every other day after water renewal. Four concentrations of oil-contaminated algae (final concentrations of sum of 50 PAHs = 1.57 µg L^{-1}, 3.23 µg L^{-1}, 9.26 µg L^{-1} and 18.54 µg L^{-1}) were tested and control treatments were fed uncontaminated algae, with 4 replicates per treatment. Experiment was conducted for 14 days with gentle aeration to stimulate feeding and maintain sufficient dissolved oxygen (D.O). Water was renewed every other day by pouring the entire contents (water, larvae, remaining algae and oil) through a 55-µm sieve, separating the larvae from the exposure media (including algal and oil particles). Retained larvae were then gently washed and re-suspended into a cleaned exposure vessel containing fresh FSW. After filling exposure vessels, algae/oil mixtures were added.

2.3.5 Subsampling and measurements

On the first day of exposure and before each renewal, sub-samples of 10 mL were taken from all exposure vessels and fixed with 10% buffered formalin for later examination. These samples were examined using a microscope for abnormality, shell length and viability of the larvae. For example, abnormally extended or necrotic velum was noted as abnormalities. To

assess growth, shell length was measured every 2 days (minimum of 200 individuals per treatment) using an inverted microscope Olympus IX73 equipped with a camera Olympus DP73 and the CellSens Software. In addition, gut fullness or percentages of larvae with food in the stomach were assessed for each sample. At the end of the exposure (day 14), after taking a 10-mL sub-sample for examination of abnormality and shell length, the remaining larvae in each replicate of each treatment were collected on a 55-µm sieve, rinsed and re-suspended in 30 mL of FSW in 50-mL centrifuge tubes. Final survival was assessed by taking 300 µL subsamples (n=3) from the concentrate (30 mL) of each replicate after homogenization, and observed under a microscope to evaluate live and dead larvae (translucent shell or opened valves). In addition, effective (ECx) and lethal concentrations (LCx) were calculated using log-logistic and sigmoid models respectively (Table 1).

2.4 Analyses of water quality and PAHs

Temperature, salinity, pH and oxygen were measured daily by means of a YSI Pro ODO optic probe and a refractometer; whereas total ammonia was measured at the start, before each water change and at the end of the exposure experiment, using a Seal Analytical Auto Analyzer 3 and the G-171-96 method.

Analytical chemistry was performed by ALS Environment (Kelso, WA, USA). Polycyclic Aromatic Hydrocarbons, including alkyl homologues, were determined by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM) and a sum of 50 different PAHs were quantified. The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the targeted analytes. Two distinct sets of water samples were collected throughout the 14 days exposure. The first set was taken at test initiation (day 0), less than 1 h after feeding with algae, from one pseudo replicate (without larvae) from each treatment group including control. The second set consisted of effluent water samples (containing algae and oil) taken during the first water renewal from each treatment group, after sieving of the larvae.

2.5. Statistical analyses

Shell measurement, survival and PAH concentration data were expressed as means ± standard deviation. Percentage data were arcsine-square root transformed prior to statistical

analysis to normalize distribution. Data were analyzed by analysis of variance (ANOVA), after checking normality (Shapiro-Wilk test) and homogeneity of variances between treatments (Levene's test) using SPSS 22.0® statistical package. Whenever significant differences among groups were found (ANOVA: $\alpha = 0.05$), a Tukey post-hoc test was performed. Median-effects and lethal concentrations (ECx/LCx) were estimated using the *drc* and *bbmle* packages in R as well as the trimmed Spearman-Karber procedure (Hamilton et al., 1977). For binomial response variables (growth inhibition, abnormality), data were fitted to a 3-parameter log-logistic model.

3. Results

3.1 Water quality

Through the 2-weeks exposure, temperature and salinity were 25.5 ˚C ± 0.7 and 22.2 PSU ± 0.7 respectively. Dissolved oxygen (D.O.) and pH averaged 7.15 mg L^{-1} ± 0.13 and 7.74 ± 0.34 respectively. Moreover, total ammonia concentration remained below 0.5 mg L^{-1}, levels causing no deleterious effects to oyster larvae according to Losso et al. (2007: NOEC ≈ 4 mg L^{-1}).

3.2 Droplet size distribution and flow-cytometry analysis

Figure 2 shows the size distribution (%) of oil droplets observed in unfiltered HEWAF stock (1:500 dilutions of oil). Oil droplet diameters ranged from less than 1 µm to 22 µm, with the majority (≈ 90%) of droplets in the ≤ 1 to 5 µm range (Fig. 2). Diameter of these oil particles (mean diameter: 1.85 µm ± 0.62) was similar to small diatoms or flagellates, such as *Tisochrysis lutea* (ESD = 5 µm) (Robert et al., 2004) that oyster larvae feed on.

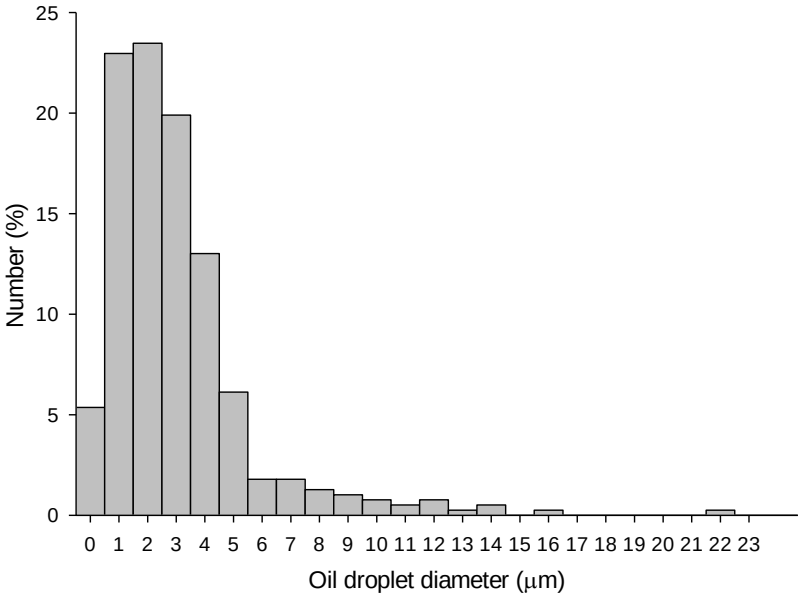


Figure 2: Size distribution (in %) of oil droplets expressed as diameter (in µm) of a sample of unfiltered HEWAF.

Flow-cytometer analysis of solutions of *T. lutea* mixed with increasing HEWAF allowed us to determine three distinct populations of particles with different fluorescence but with a similar range of relative size: *T. lutea*, oil droplets and *T. lutea*/oil droplets (Fig. 3A). Further analysis using flow-cytometry showed that the number of droplets associated with algal cells increased with increasing concentrations of HEWAF, up to about 15% for the highest HEWAF treatment, demonstrating that oil could adsorb to phytoplankton (Fig. 3B).

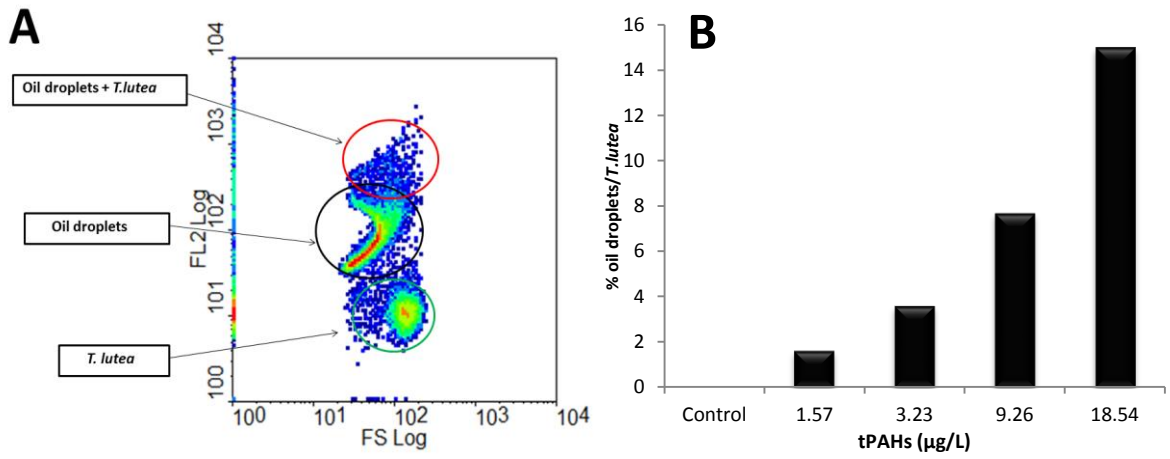


Figure 3: (A) Cytogram showing the different populations of particles distinguished according to relative size (FSC) and orange fluorescence (FL2). (B) Percentages of oil droplets associated with *T. lutea* cells after 6 h mixing with increasing concentrations of HEWAF, expressed as tPAHs (n=50) in µg L^{-1}.

3.3 Effects on growth, survival and feeding regime

Daily growth of larvae (n ≥ 100) continuously exposed to oil-contaminated algae, was negatively impacted in a dose-dependent manner (Fig. 4A). In the control treatment, larvae fed only *T. lutea* exhibited a daily growth of ≈ 8 µm (Fig. 4A), with mean shell length increasing from 97.7 µm (± 0.6) to 202.6 µm (± 3.7) after 14 days (Fig. 4B). Contrastingly, oil-contaminated algae, equivalent to 1.57 µg L^{-1} of tPAHs, affected growth significantly (*p≤0.01*, Fig. 4A). Compared to control group, growth was altered significantly even at intermediate concentrations of PAHs, with growth inhibition after 14 days reaching about 50% at levels of PAHs ranging from 3.23 to 9.25 µg L^{-1} (Fig. 4A). At the highest concentration tested, larvae were affected most (*p≤0.001*, Fig. 4A), with a cumulative growth of ≈ 30 µm in 14 days; the final mean size at the end of the experiment was 131.4 µm (± 6.8) (Fig. 4B).

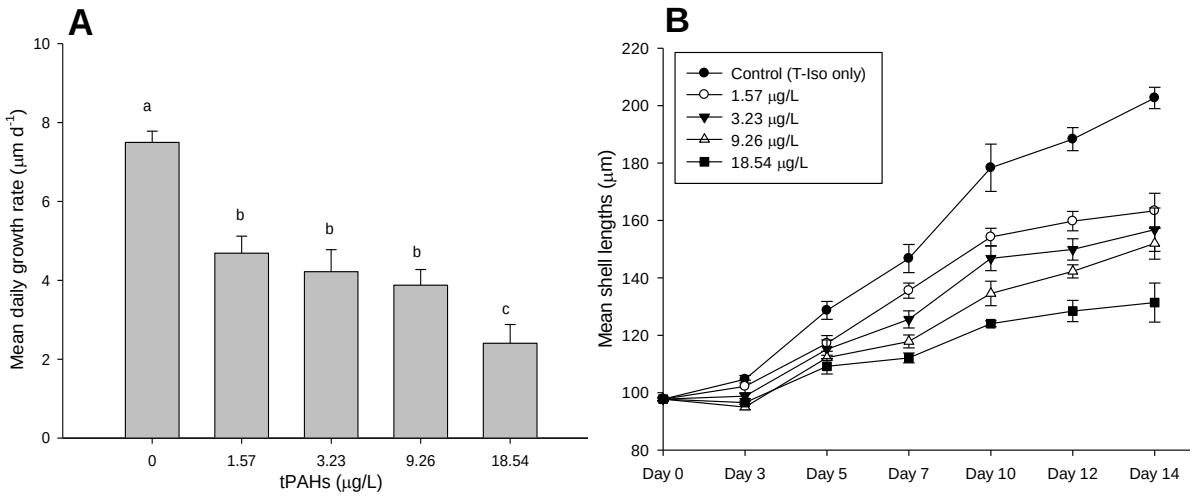


Figure 4: Growth of oyster larvae (n>100), expressed in daily growth rate (µm d^{-1}) (A) or evolution of shell length (µm) (B), fed *Tisochrysis lutea* mixed with increasing concentrations of oil/PAH (expressed in µg of tPAHs (n=50) L^{-1}) for 14 days. Data are presented as mean shell length ±SD. Different letters denote statistically significant differences between condition (ANOVA, α=0.05).

Survival of larvae at the end of the exposure (day 14) even though relatively high was negatively impacted in a dose-dependent way, with significant deleterious effects of oil-contaminated algae at concentrations as low as 3.23 µg tPAHs L^{-1} (ANOVA: *p=0.019*, Fig. 5). The highest concentration of oil-contaminated algae tested, corresponding to 18.54 µg tPAHs L^{-1}, reduced survival rates of larvae to about 67% on day 14 (Fig. 5).

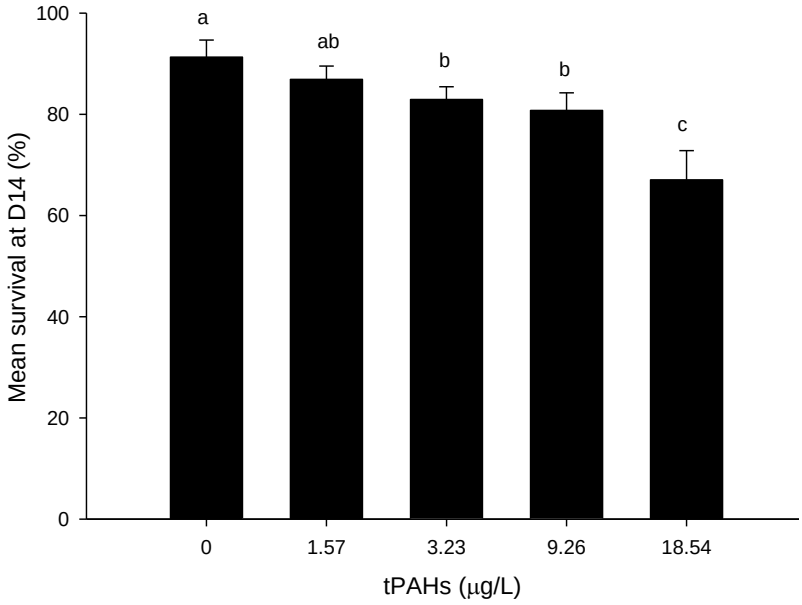


Figure 5: Survival of oyster larvae exposed for 14 days to *Tisochrysis lutea* mixed with increasing concentrations of oil, expressed in µg of tPAHs (n=50) L^{-1}. Data are presented as mean percentages ± SD. Different letters denote statistically significant differences between condition (ANOVA, α=0.05).

The concentration of PAHs causing 50 % of mortality after 14 days ($LC50_{336h}$) of exposure to oil-contaminated algae was estimated at 78 µg tPAHs L^{-1} (Table 1). Abnormalities were observed in exposed larvae, consisting of abnormally extended or necrotic velum. Concentrations of oil-contaminated *T. lutea* equivalent to 27.2 µg tPAHs L^{-1} induced 50% of abnormal larvae ($EC50_{336h}$) (Table 1).

Table 1: Effective (ECx) and lethal concentrations (LCx) of oil-contaminated *T. lutea* causing 50% abnormality, growth inhibition and mortality after 336 h of exposure, calculated using the *drc* and *bbmle* packages in R. Lowest concentration having an effect (LOEC) determined from Fig. 5 (ANOVA). ECx, LOEC and LCx values are expressed in µg tPAHs L^{-1} (± 95% C.I).

Abnormality	Growth Inhibition	Mortality	
$EC50_{336h}$	$EC50_{336h}$	$LOEC_{336h}$	$LC50_{336h}$
27.2 (3.1-51.3)	**6.8** (6.5-7.1)	**3.2**	**78** (52.8-132)

Gut content of the larvae (percentage of larvae with algae in stomach) was quantified at the start of the experiment and at various times during the exposure. No trend was observed between the control treatments and the exposed treatments (data not shown). Nonetheless, association of oil droplets in the body cavity of larvae was frequently observed.

3.4 PAHs content in exposure/culture media (oil-contaminated algae)

PAH profiles of the exposure medium (water + algae + oil) at test initiation and before water renewal are shown in Figs. 6 to 8.

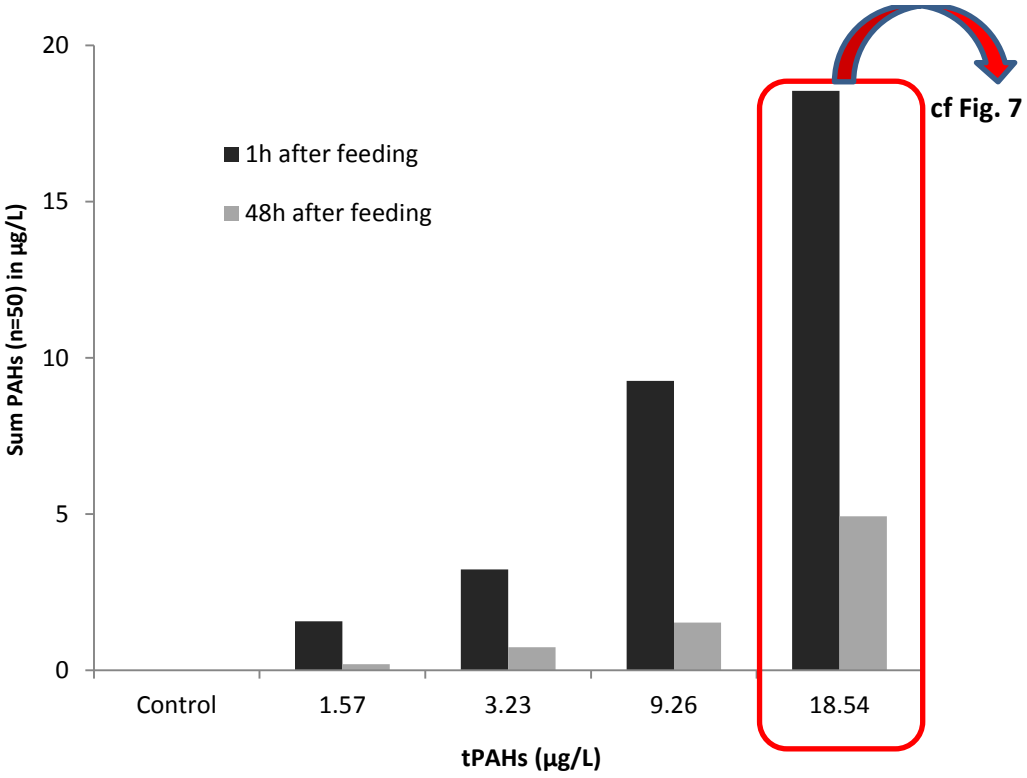


Figure 6: PAH content of the exposure medium, 1 h after feeding with oil-contaminated *T. lutea* (black), and after 48 h of exposure just before water renewal (grey). Sum of 50 PAHs, quantified using GC MS-SIM, is expressed in µg L^{-1}. Samples were not filtered (i.e. contain algae and oil).

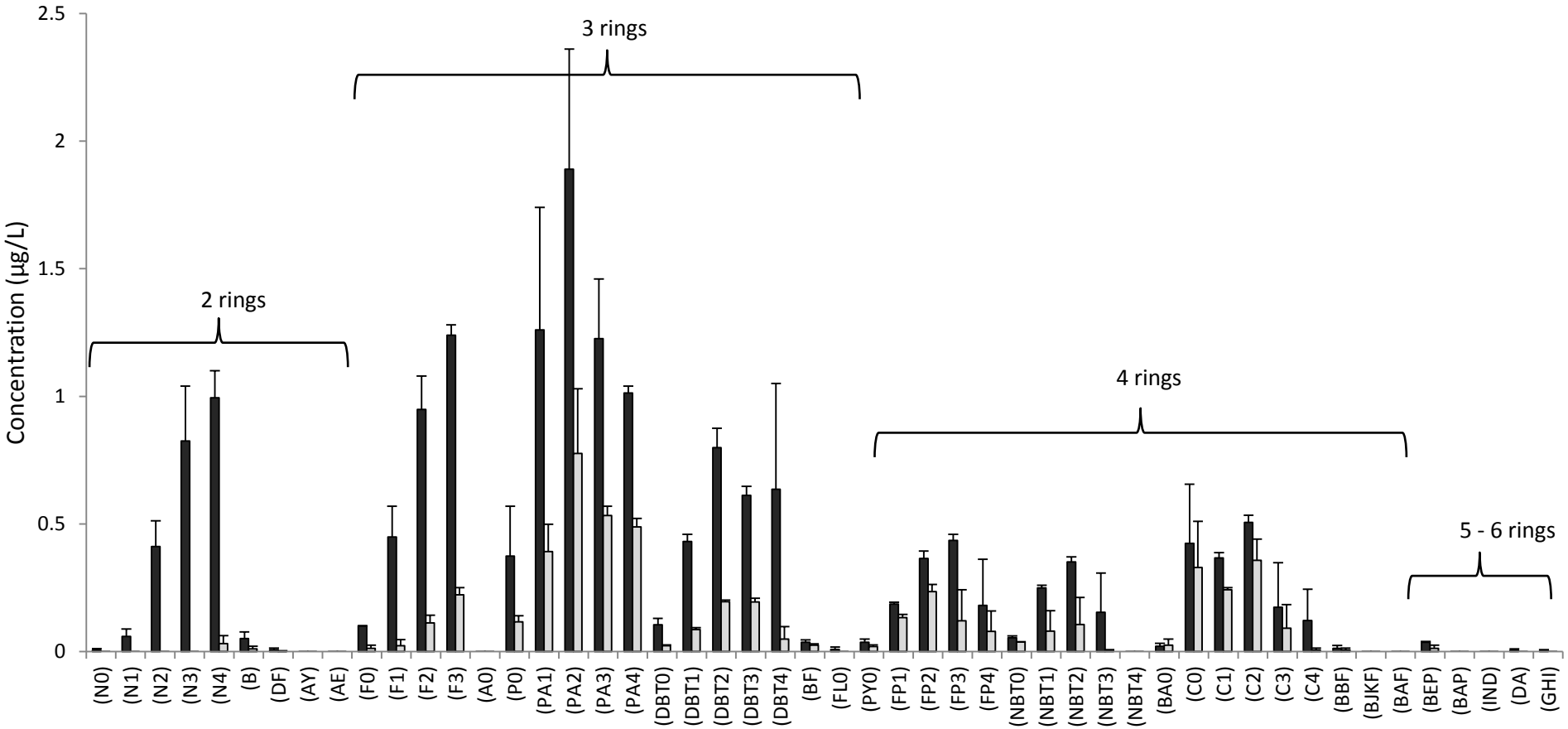


Figure 7: PAH profile of the culture medium, 1 h (black) and 48 h (grey) after feeding with the highest dose of oil-contaminated *T. lutea*. Concentration of individual PAH, quantified using GC MS-SIM, is expressed in µg L^{-1} (±SD). N0-4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene; PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0: Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4: Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF: Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA: Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0), while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc. Samples were not filtered.

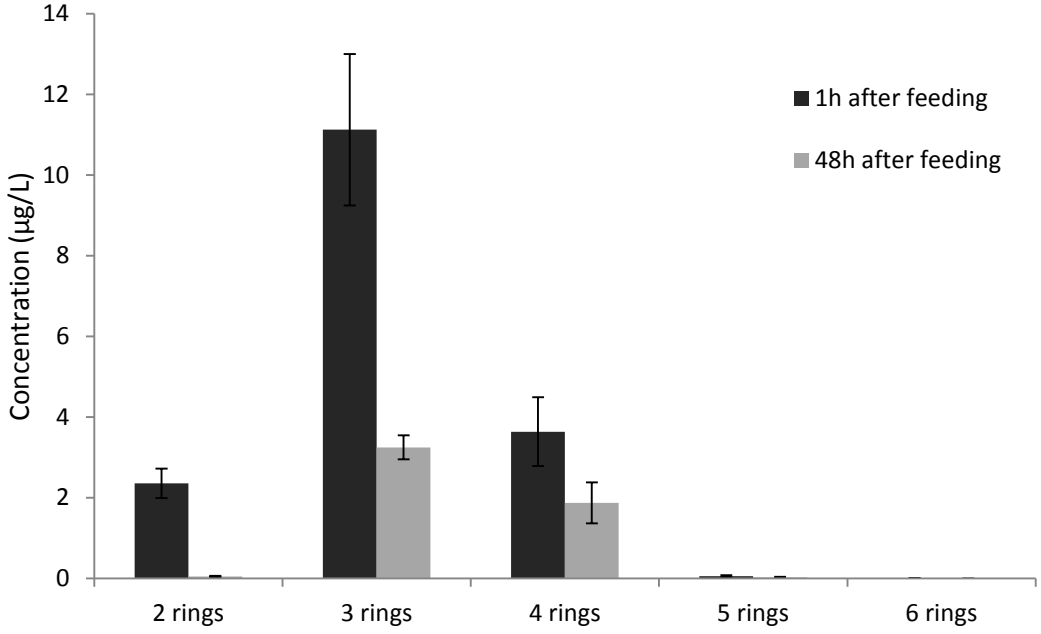


Figure 8: Same PAH profile of exposure medium at T_{1h} and T_{48h} (± SD) shown in Fig. 7, grouped as aromatic classes (2 to 6 rings).

Results indicated an increase of tPAHs (n=50) in the exposure medium with increasing doses of oil added to the algae (Fig. 6). In addition, GC/MS-SIM analysis showed a decline of tPAHs (about 4 times) measured in the exposure medium through time (Fig. 6). Figure 7 shows the profile of the exposure medium 1 h after oil-contaminated algae (highest dose) was fed to the larvae, and 48 h later before exposure medium was renewed. At the start of the exposure (1h), specific PAH peaks of the exposure solution (oil-contaminated algae) consisted of mostly bi-aromatic (Naphtalene), tri-aromatic (Fluorene, Anthracene/Phenanthrene, Dibenzothiophene) and to a lesser extent tetra-aromatic hydrocarbons (Pyrene, Naphtobenzothiophene, Chrysene) (Figs. 7 and 8). Before the water change and the renewing of exposure solution, the PAH profile of the medium in which larvae were exposed, consisted of tri- and tetra-aromatic compounds mainly, and penta-aromatic at very low levels (Figs. 7 and 8).

4. Discussion

Following acute exposures of larvae to DWH oil and dispersant using water accommodated fractions (Vignier et al., in prep.b/Article 3), the aim of the present study was to examine if a dietary exposure using oil-treated phytoplankton, at environmentally realistic levels of oil/PAH, could induce physiological impairments. The present study confirmed that chronic exposures of oyster during larval development to low doses of PAHs could have significant adverse effects. Results indicated that contaminated algae, equivalent to concentrations of tPAHs (n=50) as low as 1.6 µg L^{-1}, was sufficient to delay significantly growth of larvae, and that 6.8 µg tPAHs L^{-1} inhibited completely growth of 50% of the animal after 14 days of exposure. This finding is of great importance as it demonstrates that long-term exposure of oyster larvae to chronic levels of PAH can impact growth and ultimately metamorphosis and survival. These results are in agreement with Geffard et al. (2002b), who found that larval growth of *Crassostrea gigas* was impeded after being fed with *Isochrysis galbana* cultured in the presence of PAH-polluted sediment, at realistic environmental levels. As many authors reported, an increase in time spent as a pelagic form might increase the risk of larval death by predation or other factors (Davis and Hidu, 1969; Calabrese et al., 1973; Beiras and His, 1994). The total time delay to metamorphosis and settlement of oyster larvae may result in significant declines in spat. Drinnan and Stallworthy (1979) calculated that a 5 day delay to metamorphosis and settlement could cause a 63% decline in spat for *C. virginica*, resulting in a major impact on the population. Such reduced growth rates would compromise the ability of juvenile spat to compete for space and reduce their likelihood of successful recruitment to adult population.

Regarding final survival, median lethal concentration (LC50) after 14 days reached 78 µg tPAHs L^{-1}, with the lowest dose inducing significant mortality attaining 3.2 µg tPAHs L^{-1}.

Although total PAHs can reach extreme concentrations in seawater during oil spills, up to 600 µg L^{-1} according to Wade and Quinn (1975) or Oppenheimer et al. (1977), total PAHs concentrations frequently range from 1 to 150 µg L^{-1} following an oil spill (Neff and Stubblefield, 1995; Law et al., 1997). Similarly, reported concentrations of total PAHs (n=50) in water samples collected during the DWH oil spill ranged from below detection limit in distant waters to 100 µg L^{-1} near the well head (Wade et al., 2011; Allan et al., 2012). A study even reported level of tPAHs reaching 189 µg L^{-1} at depths between 1000 and 1400 m, 13 km from the well head (Diercks et al., 2010). In the present study, the minimum concentrations

of PAHs found to cause lethal impacts or growth impairments (e.g. 1.6 µg L^{-1}) overlap clearly with measured PAHs in the northern Gulf of Mexico surface waters following the DWH incident, with field detections in many cases at levels that were considerably higher. However, most of these field measurements were conducted offshore in the water column, and we can question whether coastal and estuary field measurements would still match our exposure concentrations. Kostka et al. (2011) reported levels of weathered DWH crude oil in sand from Florida beaches ranging from 3.1 to 4500 mg kg^{-1}, corresponding to 0.01 to 13.8 µg tPAHs L^{-1} (using a ratio of tPAHs (n=50) for crude oil equal to 0.31% of mass; see Fig. 1). In a field study monitoring waters around the UK, not after an oil spill event, Law et al. (1997) reported chronic concentrations of tPAHs (n=15) ranging from none detected to 10.7 µg L^{-1} in many coastal and estuarine samples. In light of these findings, the concentrations of PAHs tested in the present work were likely in the range of doses encountered in coastal and estuarine areas affected during the DWH oil spill.

The present work also revealed that, in addition to its dissolved fraction, toxicity of crude oil can also be attributed to its particulate fraction, and that dietary exposure is a possible and realistic way of contamination of oyster larvae. Exposing acutely umbo larvae for 96 h to mechanically dispersed oil (HEWAF) already demonstrated that droplet-associated oil and PAH could induce severe effects on growth, due in part to the mechanical action but also ingestion of these droplets (Vignier et al., in prep.b/Article 3). Measurements of the particulate form of DWH oil prepared as HEWAF method indicated that oil droplet size ranged from less than 1 µm to 22 µm with the majority between less than 1 µm and 5 µm, a result in agreement with other studies (Li and Garret, 1998; Hansen et al., 2012; Lee et al., 2012). Additionally, flow-cytometer analysis of solutions of oil-contaminated algae showed that a significant fraction (1/6) of particles present in the mixture were oil droplets aggregated with algae (Fig. 3B). This result showed evidence that highly lipophilic oil droplets can bind to the unicellular flagellate *T. lutea.* In addition, our flow-cytometry analysis indicated that droplet-associated algal cells had a similar size spectrum than algal cells alone (i.e. 5-15µm). This result suggests that droplet-associated algae as well as oil droplets were well within the size range of particles ingested by *C. virginica* larvae, i.e. between 0.2 and 30 µm (Baldwin and Newell, 1991; 1995; Robert et al., 2004; Ponis et al., 2006). Accordingly, higher instance of larvae with the presence of oil droplets in the body cavity and the stomach were observed at the highest dose of oil-contaminated algae, confirming that larvae were feeding directly on

oil droplets, associated or not to algae. Consequently, direct ingestion of particulate oil might have affected vital functions of the larvae such as swimming abilities or assimilation of food, as illustrated by the presence of abnormal and impaired velum, and ultimately impacted larval growth. Lee et al. (2012) observed the same phenomenon with *Dolioletta gegenbauri*, a species of pelagic tunicate abundant in the Gulf of Mexico, which ingested mechanically-dispersed oil droplets, ranging between 1 and 30 µm. In the same way, Hansen et al. (2012) demonstrated that the copepod, *Calanus finmarchicus,* could also feed on oil droplets (10-15 µm) which consequently decreased its filtration rates and survival.

Looking at the PAH profiles of the exposure media in which larvae were grown (oil , algae, water) between T_{1h} and T_{48h}, a reduction in total PAHs measured was observed. This decrease in PAH concentrations may be due to weathering processes such as evaporation, photo-chemical oxidation, microbial degradation (biodegradation), or to direct ingestion of oil droplets and/or algae by larvae. Because exposures were conducted using FSW treated with antibiotic and under artificial lights (no UV radiation), biodegradation and photo-oxidation could certainly be dismissed, suggesting that PAH depletion was due to evaporation or direct ingestion. For instance, PAH content of the exposure media (Fig. 7 and 8) revealed that bi-aromatic compounds (e.g. Naphtalene) were absent after 48 h, suggesting either a loss by evaporation (Atlas, 1995; Daling et al., 2014) and/or most likely an ingestion of contaminated algae by larvae.

Analytical chemistry of the exposure medium at T_{1h}, corresponding to oil droplets and the oil/algae mixture, showed that naphthalene (parent and homologs) and tri-aromatic compounds (Fluo, Phen, Anth, DBT) constituted the major classes of PAHs and to a lesser extent tetra-aromatic compounds (Pyr, NBT, Chry) (Fig. 7). Many studies have examined the partitioning of petroleum hydrocarbons between dissolved and particulate phases in waters and have found a significant adsorption of PAHs on particulate materials (Herbes, 1977; Lee, 1977; Fernandes et al., 1997; Geffard et al., 2003). Due to their hydrophobicity (K_{ow}), heavy molecular weight (HMW) hydrocarbons (3 to 6 rings) are preferentially adsorbed onto particles such as lipid-rich microalgae (Lyman, 1995; Baumard et al., 1999). Wolfe et al. (1998) reported an uptake of naphthalene and phenanthrene by *Isochrysis galbana (T-Iso)* following exposure to WAF crude oil. Similarly, Geffard et al. (2002b) demonstrated that *I. galbana,* previously grown with PAH-contaminated elutriate, accumulated soluble PAH (2 to 4 rings) as a function of their content in the exposure medium. In a similar way, in addition to

droplet-associated PAHs that can bind/adhere to algal cells, soluble PAHs (2-4 rings) may have also been adsorbed by *T. lutea.*

As a result, the relative decline over time of tri- and tetra-aromatic compounds (and possibly bi-aromatic) observed in the present study (Figs. 7 and 8) suggests a potential ingestion of these PAHs associated to algal particles by oyster larvae. Okay et al. (2000), Widdows et al. (1982) and Croxton et al. (2012) found that specific PAH could be ingested by adult bivalves using a dietary mode of exposure. More similarly, Geffard et al. (2002b) demonstrated that PAHs adsorbed on *I. galbana* were bio-available to *C. gigas* larvae, ingested and accumulated. As a result, we can assume that negative effects observed on growth and survival after 14 days of dietary exposure were likely related to the ingestion of these toxic PAHs (Napht, Fluo, Phen, DBT, Pyr, and NBT) by larvae.

Certainly, these findings provide crucial information concerning how particulate oil and its associates could be possibly assimilated by oyster larvae *via* a dietary pathway. Like other zooplankton species, oyster larvae may bio-accumulate various PAHs, especially those with high molecular weights by ingestion (Baumard et al., 1999; Geffard et al., 2002b; Lee et al., 2012). As a result, these toxic PAHs, including those with carcinogenic potential and so of concern with regard to human consumers (De Flora et al, 1991; Law et al., 1997; Hylland, 2006), could be potentially transferred upward through the food chain, and impact the whole ecosystem (Wolfe et al., 1998; Graham et al., 2010; Almeda et al., 2013).

Effects of a dietary exposure using chemically dispersed oil (CEWAF) were not investigated mainly because of the deleterious effect of dispersant on algal cells, and the surface-active agents shown to induce lysis of algal cells (Brown, 1973; Fedulova, 1976). However, it has been shown by several authors that dispersant combined with crude oil could increase significantly the uptake of some PAH (e.g. naphthalene, phenanthrene) by algal species (Wolfe et al, 1998; 2000) and be transferred *via* a primary consumer (e.g. rotifer) to higher trophic levels. This could be of great significance in view of the substantial volume of Corexit used at depth during the DWH oil spill and the presence of extensive plumes of oil that remained underwater (Camilli et al., 2010; JAG, 2010; Wade et al., 2011). Despite the great deal of research conducted on adult bivalves and their ability to bioaccumulate and biotransform organic contaminants, further research is required to understand the mechanisms of PAH uptake by larvae and their potential capacity to metabolize them.

Furthermore, it is important to note that the large majority of toxicity testing using bivalve larvae focuses solely on assessing the acute and short term effects of oil/PAH, without addressing chronic and long-term effects. This type of acute exposure may not reflect the true risk to aquatic organisms under field conditions. For instance, toxic levels of tPAHs determined in the present study were much lower (e.g. 7 to 35 times lower) than those found in our previous study in which veliger and umboned larvae were acutely exposed for 96 h (Vignier et al., in prep.b/Article 3). Additionally, compared to acute exposure in which larvae were exposed mainly to the dissolved fraction of oil but also to the particulate fraction, present findings showed that the combination of algae and oil may have increased PAH bioavailability and uptake effectiveness by the larvae *via* dietary pathway, hence increasing toxic effects.

5. Conclusion

To conclude, the present study clearly demonstrated that oil droplets could adhere and adsorb to phytoplankton, and that resulting particles (oil droplets and/or algae) and associated PAHs could become available to oyster larvae and be ingested during feeding. As a result, some persistent and toxic PAHs could be transferred to higher trophic levels, and impact the whole ecosystem, and ultimately affect human health, a phenomenon referred to as biomagnification. Moreover, long-term exposures to levels of tPAHs realistically encountered during the DWH oil spill (1.6 to 78 μg L^{-1}) were found to affect growth of larvae and ultimately survival. To our knowledge, the current study is the first of its kind to challenge *Crassostrea virginica* larvae to crude oil *via* a dietary pathway. Nonetheless, more research focusing on the bioaccumulation of PAHs and their potential biotransformation by bivalve larvae is needed. Examining if there is a relationship between PAHs bio-accumulation and larval survival and growth is also required to better evaluate the short and long term impacts of crude oil spills on oyster populations.

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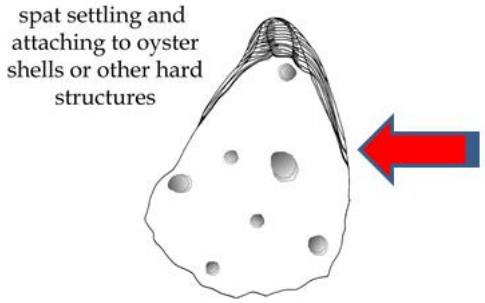
Synthesis Chapter 2

Despite the fact that oysters have been extensively studied as a model organism in ecotoxicology, information on the toxicity of crude oil on *Crassostrea virginica* larvae, in combination or not with dispersant, is very limited. The objective of the previous chapter was to evaluate the lethal and sublethal effects of i) a short-term acute exposure (96 h) to oil/PAHs and dispersant on various stages of the developing oyster larvae, and ii) a long-term larval exposure to oil/PAH (14 d) at environmentally realistic levels using a dietary mode of exposure (*T. lutea* + oil).

Firstly, our findings revealed a stage-dependent sensitivity to oil and dispersant in term of survival, with veliger larvae being logically the most sensitive. CEWAF usually elicited similar toxicity than Corexit, except for umbo exposure which showed enhanced adverse effects of CEWAF on growth and survival due probably to an additive/exacerbated toxic effect of PAHs. Secondly, settlement success of oil-exposed pediveliger demonstrated to be a sensitive endpoint (with $EC50_{72h}$ value for CEWAF reaching 18.4 µg tPAHs L^{-1}) which should be included, as well as larval growth or feeding inhibition, in toxicological assessment of oil pollution. Indeed, dietary exposure of larvae using oil-contaminated algae indicated that larval growth and subsequent survival were affected at levels of PAHs realistically encountered during the DWH oil spill (1.6 to 78 µg tPAHs L^{-1}).

Results collected during the HEWAF exposure of umbo also showed evidence that, in addition to its dissolved fraction, estimation of oil toxicity should consider the contribution of droplet-associated PAH in the induction of sublethal effects on feeding efficiency, growth, or settlement. In the same way, our second study confirmed that HEWAF-associated oil droplets and PAHs could interact and adhere/adsorb to lipid-rich phytoplankton, and be directly ingested by oyster larvae throughout larval development. As a result, some persistent toxic PAHs can become bioavailable to oyster larvae, and get ingested and potentially transferred upward through the food chain.

Chapter 3: Impacts of DWH oil and dispersant on spat of *Crassostrea virginica*



Introduction Chapter 3

Due to their biological characteristics (sessile, filter-feeding) and ecological significance in the Gulf regions, oysters have been employed as a model organism for bio-monitoring the environment. Following the DWH oil spill, an unprecedented amount of weathered crude oil reached the shore and contaminated estuaries where oyster reefs are essential. As opposed to larvae, oyster spat are more resistant and can withstand higher levels of oil contamination. However, from previous results using early larval stages, toxic effects from the dispersant and sublethal impacts of oil/PAHs may be expected with spat. The present chapter intended i) to determine the lethal effects of CEWAF, HEWAF and dispersant on 6 to 10 week-old spat, and ii) to examine the physiological (e.g. filtration rates), tissue (e.g. digestive system, inflammatory responses), and biochemical (lipid peroxidation) responses of spat exposed to crude oil prepared as HEWAF.

Article 5

Impacts of Deepwater Horizon oil and dispersant on spat of *Crassostrea virginica*

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Abstract

In April 2010, the explosion of the Deepwater Horizon (DWH) oil platform generated the largest oil spill in US history and released for nearly 3 months about 779,000 t of crude oil off the coast of Louisiana. As a response, about 8 million liters of dispersants were used to dissipate the slicks on the surface and directly at the well-head, at 1544 m depth. The Eastern oyster, *Crassostrea virginica* is an ecologically and economically important species in the northern coast of the Gulf of Mexico, especially in Louisiana where 60% of the US fishery for this species originate from. Due to its biological characteristics (sessile, filter-feeding) and the time of the oil spill, early life stages of oysters such as newly recruited spat might be potentially affected. The aim of this study was to investigate in the laboratory the effects of chemically dispersed DWH oil (CEWAF), oil alone prepared as a high energy method (HEWAF) and dispersant alone, on survival of 6 to 10 week-old oyster spat, and to evaluate the potential impacts of HEWAF on spat tissue. Results showed that exposure for 7 days to increasing concentrations of CEWAF and dispersant alone caused increased mortalities of early spat, $LC50_{7d}$ = 833.9 mg oil L^{-1} (or ≈ 148 µg tPAHs L^{-1}) and 84 mg L^{-1} (or 8.4 mg DOSS L^{-1}) respectively, suggesting that toxicity of CEWAF was only related to dispersant at equivalent concentrations (10:1 ratio). In contrast, exposure of spat for 10 to 14 days to HEWAF did not induce a dose-related response in mortality. When investigating the physiological impact of HEWAF on spat, clearance rates of algal food (*T. lutea*) were negatively affected by oil exposure. Consistently, histological observations showed that specific tissues in contact with HEWAF (gills, palp, CT, digestive gland) were adversely impacted, exhibiting pathological symptoms typical of an inflammatory response (e.g. hemocyte diapedesis and infiltration, ulcer, syncytia, epithelium sloughing). In addition, morphometric study of the digestive tubules (DGT) indicated a dose-dependent response to oil on lumen dilation and epithelium thinning of the DGT as well as a significant change in DGT synchrony. This finding suggests that structural changes occurred in the digestive gland of exposed oyster spat most likely due to an oil-related stress.

Keywords: oyster spat, Deepwater Horizon oil, HEWAF, inflammatory response, digestive tubule atrophy, histopathology.

Résumé

En Avril 2010, l'explosion de la plate-forme pétrolière DWH a entrainé la plus importante marée noire de l'histoire des Etats-Unis, libérant pendant près de 3 mois environ 779000 t de pétrole brut au large des côtes de la Louisiane. Environ 8 million de litres de dispersants furent utilisés comme moyen de réponse pour dissiper les nappes en surface mais aussi directement à la source de la fuite à 1544 m de profondeur. L'huitre américaine *Crassostrea virginica* est une espèce écologique et économique importante pour les régions côtières du nord du Golfe du Mexique, en particulier en Louisiane où elle représente 60 % des prises totales américaines. En raison de ses caractéristiques biologiques (sédentaire, filtreuse) et l'époque de la marée noire, les jeunes stades de vie de l'huitre tels que les jeunes naissains pourraient potentiellement être affectés. L'objectif de cette étude fut d'examiner en laboratoire les effets du pétrole DWH dispersé chimiquement (CEWAF), dispersé mécaniquement à l'aide d'une méthode « high energy » (HEWAF) et du dispersant seul (Corexit), sur la survie de naissain âgé de 6 à 10 semaines, ainsi que d'éventuels impacts du HEWAF au niveau des tissus. Nos résultats ont montré qu'une exposition aigue pendant 7 j à des doses croissantes de CEWAF et Corexit pouvait causer des mortalités de naissains, $LC50_{7d}$ = 834 mg L^{-1} (ou 148 µg tPAHs L^{-1}) et 84 mg L-1 (ou 8.4 mg DOSS L^{-1}) respectivement, suggérant que la plupart de la toxicité du CEWAF est due au dispersant, à concentration équivalente (ratio de 10:1). A l'inverse, l'exposition de naissain durant 10 à 14 j au HEWAF n'a pas induit de réponses létales dose-dépendantes. L'étude de l'impact physiologique du HEWAF sur le naissain a montré que les taux de filtration de phytoplancton (*T. lutea*) furent affectés négativement par le polluant. Les observations histologiques ont montré de façon consistante que les tissus en contact avec le pétrole (branchies, palpe labial, tissues conjonctifs, glande digestive) furent impactés défavorablement, exhibant des symptômes pathologiques caractéristiques d'une réponse inflammatoire (diapédèses et infiltrations d'hémocytes, ulcères, syncytia ou ruptures d'épithélium). De plus, les études morphométriques des tubules digestives (TD) indiquèrent une réponse au pétrole dose-dépendante se manifestant par une dilatation des lumens de TD, et une perte importante de la synchronisation de ces TD. Ces résultats suggèrent qu'un profond changement structurel de la glande digestive s'est produit chez les individus exposés, très certainement dû à un stress associé aux hydrocarbures.

Mots-clés : naissain, pétrole DWH, CEWAF, HEWAF, réponse inflammatoire, tubule digestive, histopathologie, atrophie

1. Introduction

On April 20th 2010, the lethal explosion at the *Deepwater Horizon* (DWH) oil drilling rig located 77 km southeast of the Louisiana Coast in Mississippi Canyon Block 252 (28˚55'12''N, 88˚23'14''W), led to the largest marine oil spill in United States history (National Commission, 2011; Carriger and Barron, 2011). Until the well was capped and the flow finally stopped, 85 days after the DWH oil platform sank, the National Incident Command's Flow Rate Technical Group (Labson et al., 2010; McNutt et al., 2012) estimated at 4.9 million barrels (or about 779 million liters) of light Louisiana crude oil that were released into the Gulf of Mexico, 0.8 million barrels of which were captured before their release into the water column (Federal Interagency Solutions Group, 2010). The escaping oil created a deep sea "cloud" or "plume", and a surface slick (JAG, 2010). Oil made landfall for the first time in mainland Louisiana on May 15th, and eventually the coastal extent of the spill was from Louisiana to Florida (Rosenbauer et al., 2010),with more than 1,600 km of shoreline visibly oiled (OSAT, 2010) (Fig. 1). Furthermore, an estimated 8 million L of dispersants (primarily Corexit 9500A) were applied at the ocean surface and directly at the wellhead, at 1544 m depth (Kujawinski et al., 2011). This was the first time oil dispersant was used in deep-sea environment, and the largest emergency response to a marine oil spill to date (National Commision, 2011).

Petroleum hydrocarbons or crude oil constituents are one of the most common pollutants released into the marine environment: they are of particular concern due to their high chemical stability, low solubility, low degradation, and lipophilic nature. Although oil spills represent a small fraction of the total crude oil discharge into the sea, they have strong acute and long term impacts on marine ecosystems, including effects from physical damages (physical contamination and smothering) and toxicity of their chemical compounds (NRC, 2003). Polycyclic aromatic hydrocarbons (PAHs) are considered to be the most acutely toxic components of crude oil (Roesijadi, et al., 1978; Neff, 1985; Neff and Stubblefield, 1995). Given their lipophilic nature, these contaminants can bind to particulate organic matter, sediment and microalgae, thus entering the food web (Herbes, 1977; Lee, 1977, Graham et al., 2010) and are potentially transferred to higher trophic level as these particles are ingested (Wolfe et al., 1998). Sedentary, benthic filter-feeding organisms such as oysters, given their enormous filtration capacity (Newell, 2004), are particularly effective in taking up hydrocarbons via filtration and ingestion of particulate matter, and may accumulate them in their tissue to high concentrations, and thus be susceptible to the negative effects of these pollutants. For this reason, bivalve mollusks, such as oysters and mussels, are employed on a world-wide scale as sentinel organisms to monitor levels of contaminants in estuarine and coastal environments as well as ecological impairment (Goldberg et al., 1978; Farrington et al., 1983; O'Connor and Lauenstein, 2006).

The American oyster (*Crassostrea virginica)* is one of the most commercially important shellfish species in the Gulf of Mexico, representing about $74 million in landings value in coastal regions of the northern Gulf of Mexico for 2012 (National Marine Fisheries Service, 2012). In addition to its economic value, it is also an ecologically vital species. Oyster reefs provide food, shelter and habitat for many fish and shellfish species and improve water quality in estuaries (Wells, 1961; Newell, 2004, Volety et al. 2014). Although the location of the spill was in deep water, currents carried the oil to shallow coastal waters and estuaries, which are spawning and nursery grounds for many marine species, including oysters (Fig. 1). Typically, spawning season of *C. virginica* is from mid-spring until late fall in the North of the Gulf of Mexico (Ingle, 1951; Hayes and Menzel, 1981), and two peaks in settlement occur in early and late summer (Supan, 1983). Given the time of the DWH spill, newly settled spat and juveniles of *C. virginica* are thus likely to have been exposed to DWH crude oil and associated dispersant and PAHs. The physiological and ecological effects of environmental stress are numerous. While the ultimate endpoint of stress is mortality, sub-lethal effects may interfere with normal metabolic processes of the animals, thus increasing expenditure of energy reserves reducing growth, survival and ultimately reproduction and recruitment (Capuzzo, 1996; Thompson et al., 1996; McDowell et al., 1999). Our previous studies demonstrated that DWH oil (CEWAF and HEWAF) and dispersant impacted in a dose-dependent manner survival, fertilization success and growth of *C. virginica* larvae exposed acutely for 96 h. Furthermore, our results indicated an increased tolerance to oil/PAHs with age (Vignier et al., in prep. a, b/Article 1 and 3).

Given their role of sentinel in coastal environment, bivalve species have the capacity to survive in highly polluted waters, despite the fact that pollutant levels often overload the normal physiological mechanism of biotransformation or detoxification present in the cell (Gilewicz et al., 1984; Moore, 1985; Viarengo et al., 1987). Axiak and George (1987) demonstrated that *Venus verrucosa* may assimilate oil by conducting oil droplets (4 to 10 μm) with the aid of cilia toward the mouth region in the same manner as with food particles. Extensive literature showed that exposure to some petroleum hydrocarbons can cause alterations in the condition of certain molluscs, by reducing the feeding efficiency of the animals (Galtsoff et al., 1935; Chipman and Galtsoff, 1949; Gilfillan, 1973; 1976; Barszcz et al., 1977; Axiak and George, 1987). As a result, variations in clearance rates of oil-exposed oysters may influence the degree of assimilation and exposure of specific tissue, and the subsequent damages/lesions. After ingestion, crude oil particles may be carried to the digestive diverticulum. The morphology and function of bivalve digestive diverticula have been well described for bivalve species (Owen, 1973; Langton, 1975; Morton, 1983). It consists of a series of blind-ending tubules which communicate with the stomach by way of ciliated main ducts and non-ciliated secondary ducts (Owen, 1970). Food material are carried from the stomach to the ciliated digestive

ducts, and then transferred to the lumina or lumen of the digestive tubules (Owen, 1973). After long-term and sub-lethal exposure to petroleum hydrocarbons, vacuolization and atrophy of the digestive cells leading to a thinning of the digestive epithelium and subsequently to an increase in tubule lumen size have been frequently reported for molluscs (Lowe et al., 1981; Widdows et al, 1982; Tripp et al., 1984; Couch, 1984; Lowe and Clark, 1989; Cajaraville et al., 1992). In addition, Lowe et al (1981) found that there was a loss of the normal synchrony of the digestive cells of mussels exposed long-term to oil, to a point where almost all the tubules exhibited a similar appearance (i.e. reconstituting stage). To avoid some of the subjectivity associated with descriptive histology, quantitative techniques, such as stereology and morphometric analysis, have been widely employed to measure alterations in cells following contaminant exposure (Lowe et al., 1981; Robinson, 1983; Lowe and Clark, 1989; Vega et al., 1989; Cajaraville et al., 1989a, Marigomez et al., 1990). Accordingly, in the present study, morphometric analyses were employed to quantify alterations on the digestive tubules of oil-exposed *C. virginica*.

Histopathological changes are powerful indicators of prior exposure to environmental stressors, especially xenobiotics, and histopathology is often seen as the easiest method for assessing both short- and long-term toxic effects in the field (Hinton and Lauren, 1990). Long-term effects of oil exposure in oysters and plaices collected from the field after the *Amoco Cadiz* spill reported necrosis, inflammation, lesions of the digestive tract epithelium, gonads, connective tissue, and gills (Neff and Haensly, 1982; Balouet et al., 1986; Berthou et al., 1987). After an exposure to petroleum hydrocarbons, Axiak et al. (1988) noted an inflammatory response in the gills, degeneration of epithelial cells in the foot and alimentary canal as well as an activation of mucus secretory cells in the gills of the clam *Venus verrucosa.* These observations suggest that after exposure to oil, an inflammatory response will likely occur, mainly located in the gills and the digestive system of exposed bivalves. Many authors have shown evidence that contaminant-stimulated "reactive oxygen species" (ROS) production and resulting oxidative damage may be a mechanism of toxicity in aquatic organisms exposed to pollution (Di Giulio et al., 1989; Ribera et al., 1991; Livingstone et al., 1994; 2001).

In the present study, the main objectives were i) to determine the lethal concentrations of oil/PAHs (CEWAF and HEWAF) and dispersant (Corexit) after acute exposure in a static renewal system, and ii) to determine the physiological (e.g. clearance rates), tissue (e.g. digestive tubules staging, tubule lumen sizes, inflammatory responses) and biochemical (e.g. lipid peroxidation) responses of *C. virginica* spat exposed to increasing concentrations of HEWAF (10 ppm to 2000 ppm). Because of the great dispersant dilution factor occurring at sea, and the fact that shore was heavily oiled by slick,

oyster spat were most likely exposed to oil alone: as a consequence, physiological and cellular responses of *C. virginica* spat were only investigated after exposure to HEWAF of DWH crude oil.

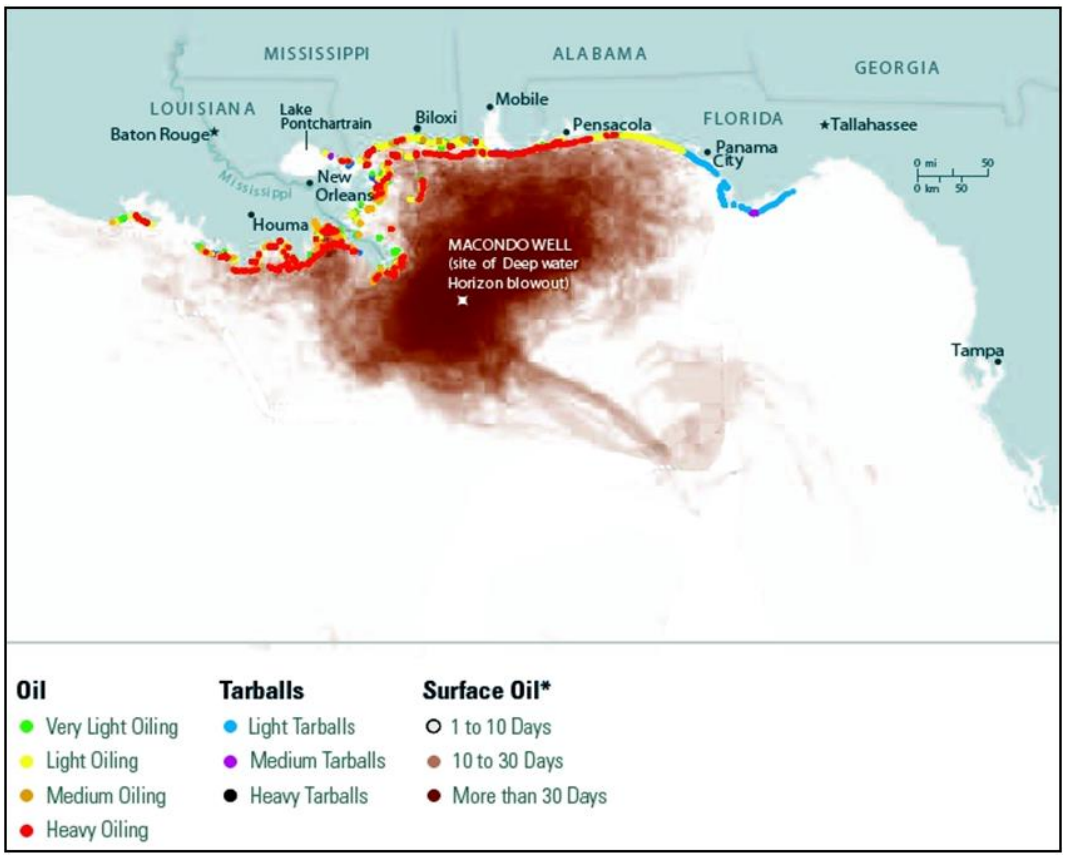


Figure 1: Cumulative Deepwater Horizon oil distribution in the Gulf of Mexico (number of days with oil present) and coastal impact of the spill. Image downloaded from **www.GeoPlatform.gov/gulfresponse**

2. Material and methods

2.1 Preparation of exposure solutions

Surface oil, also referred to as "Slick A", collected from skimmer vessels at the source during the *Deepwater Horizon* oil spill on July 29th, 2010, and dispersant, Corexit 9500A®, were provided by Stratus Consulting Inc. The "oil only" exposure solutions or High Energy Water Accommodated Fractions (HEWAF), the "oil/dispersant" mixtures (10:1) or Chemically Enhanced Water Accommodated Fractions (CEWAF) and the dispersant exposure solutions were prepared following a standardized procedure extensively detailed in Vignier et al. (in prep.a/Article 1).

2.2 Water quality and analytical chemistry

Water quality (T˚C, D.O and S‰) was monitored daily throughout the exposure period by means of a Pro ODO optic probe (YSI) and a refractometer, while pH was measured using a "Pinpoint" pH monitor (American Marine Inc.). Total ammonia was measured in one replicate per treatment at the start of the exposure and before each renewal of exposure solution, and analyzed using a Seal Analytical Auto Analyzer 3. Each test chamber was aerated to maintain oxygen levels above 5 mg L^{-1} and light photoperiod was maintained at 12h:12h using fluorescent lamps.

Chemical analyses of the stock solutions of HEWAF, CEWAF, dispersant as well as the FSW control, were conducted at the start and after each exposure solution renewal, and performed by ALS Environment (Kelso, WA, USA). Polycyclic Aromatic Hydrocarbons (PAHs), including alkyl homologues, were determined by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM) and a sum of 50 different PAHs were quantified. The analytical procedure was based on EPA Method 8270D with the GC and MS operating conditions optimized for separation and sensitivity of the targeted analytes.

2.3 Experimental design

Two month-old oyster spat (*C. virginica*) from the experimental hatchery of Auburn University, AL, were used in two separate experiments (March 2012 and May 2013). After reception, oyster spat were held for 2 weeks of acclimation at the experimental hatchery in a flow-through system, at ambient temperature (23 ˚C ± 1) and salinity (32 PSU ± 2), and fed *ad libitum* with Shellfish Diet (Reed Mariculture®) for optimal growth.

2.3.1 Experiment 1: acute exposure to HEWAF, CEWAF and Corexit

Experiment 1 was conducted in March 2012. Oyster spat (≈ 5 mm shell length) were counted and randomly distributed at a density of 25 individuals per beaker. Exposures were conducted in 600 mL sterile glass beakers filled up with 500 mL of solution, with four replicates per treatment, and lasted 7 days for CEWAF and Corexit, and 14 days for HEWAF. These exposure durations were chosen according to median lethal doses determined during preliminary range finding tests. For instance, no mortality was observed after 7 days of exposure to HEWAF, so the exposure was prolonged for an additional 7 days. Every other day, spat were collected on a 1 mm stainless steel sieve, and observed using a dissecting microscope for examination of dead spat, failing to close their valves. Mortalities were hence determined for each treatment through the experiment. Beakers were cleaned using deionized water and Kim-Wipes®, fresh exposure solutions (Table 1) were renewed, and oysters were daily fed 0.5 ml of Shellfish Diet.

2.3.2 Filtration rate measurements

In a separate experiment, filtration measurements were carried out during 24 h using *Tisochrysis lutea*. Spat (n = 10) were exposed to increasing concentrations of HEWAF (final concentrations ranged from 100 ppm to 2000 ppm), in triplicate, and fed *ad libitum* at the start of the trial with 100 mL of *T-lutea* per beaker. Residual algal cells were assessed through the exposure (T0 and T24) by taking from each replicate a 1.5 ml aliquot, and then fixed by addition of 100 µL of Glutaraldehyde. After thorough agitation, three 10 µl subsamples were taken from the fixed aliquots and observed under a microscope using a counting hemocytometer. Residual algal cells were then averaged for each sampling point and 24 h clearance rates were determined for each HEWAF condition. In order to compare among different treatment, clearance rates (CR) were normalized per gram of whole dry tissue weight of oyster, and determined using the following equation, adapted from Coughlan (1969):

$$CR = (V/(n \times t)) \times \ln (C_{ti}/C_{tf}))/\varpi$$

where "CR" is the Clearance Rate in L h^{-1} g^{-1}; "V" is the volume of each container in L; "n" is the number of spat; "t" is the time of experiment in h; "C_{ti}" and "C_{tf}" are the initial and final counts of *T. lutea* cells at t_i or t_f; "ϖ" is the mean individual dry weight of spat in g (ϖ=0.0005g).

Moreover, we assume that pumping rate of animal was constant, particle retention was 100 % efficient and the algal concentration was homogenous at all times.

2.3.3 Experiment 2: acute exposure to HEWAF

Following the inconclusive results obtained from the first exposure to HEWAF conducted in March 2012, we decided to re-run this test for 10 days, using a broader range of HEWAF concentration and add other endpoints such as histopathological analyses of specific tissues, morphometric analyses of the digestive diverticula, and lipid peroxidation assay.

2.3.3.1 Mortality measurement

Experiment 2 was conducted in May 2013. Oyster spat (10-15 mm shell length) were counted and randomly distributed at a density of 15 per beaker, in triplicates. Exposure was performed in 600 mL glass beakers filled up with 500 mL of solution, under static-renewal conditions, with the exposure media renewed every other day, and lasted 10 days. Beakers were cleaned using deionized water and Kim-Wipes®, and refilled with new exposure media. Effects of various treatments on early spat were determined by examining the survival at each water change, using a 2 mm sieve, and dead spat were

scored by their failure to close their valve. Spat from each beaker were daily fed 2 ml of Shellfish Diet®.

2.3.3.2 Histopathological study

At the end of the 10 day exposure to HEWAF (Experiment 2), 15 spat from each concentration and 25 from the control were randomly selected for histological examination. The number of individuals sampled represented a third of the population. Oyster spat were then fixed in a solution of Davidson's fixative for 48 h at 4˚C. After rinsing thoroughly with tap water, animals were placed in individual histological cassettes in a solution of 10 % EDTA (pH = 7.5) for decalcification (Howard et al., 2004). EDTA solution was changed every other day during the week of decalcification process. After decalcification and thorough rinsing, spat were transferred to cold 70% ethanol solution. Following dehydration through an ascending ethanol series, tissues were dealcoholized with xylene using a tissue processor (Shandon Citadel 1000), and embedded in paraffin wax. Sections (5 µm) were cut on a rotary microtome, Microm HM325, stained with Harris' hematoxylin and eosin (Winstead, 1995), and examined with light microscopy.

a) Morphometric analysis of the digestive tubules

At the end of the 10 day exposure to HEWAF (Experiment 2), digestive tubules were measured at random from histological sections of 6 animals per condition, using an inverted microscope Olympus IX73 equipped with a camera Olympus DP73 and the CellSens image analysis software. Morphometric analysis followed a protocol adapted and modified from Winstead (1995) and Cajaraville et al. (1989a; 1990). Histological cross sections of the digestive gland were divided into 4 fields of observation per animal. Seven to nine randomly selected tubules from each field (total of 30 tubules per spat) were measured and two sets of measurements, internal (luminal) surface and total tubule surface, for each tubule were determined using the image analysis CellSens software.

b) Grading of digestive tubules

In addition, the study of the phasic activity of the digestive gland was carried out by means of a subjective grading of the same tubules selected previously for morphometric measurements. Thus, at least 30 tubules per individual and a minimum of 180 per experimental group (6 oysters) were therefore categorized. Four tubule types were distinguished (Fig. 2), using a subjective method adapted from Langton (1975) and Robinson (1983), and modified following Cajaraville et al. (1989a): holding/absorpting (combined as stage I), disintegrating (stage II), and reconstituting (stage III). Besides, a fourth tubule type apparently not related to the digestion process and named

"necrotic" (stage IV), has been identified (Cajaraville et al., 1989a). The percentage of occurrence of the 4 different tubule types with respect to the total number measured was then calculated.

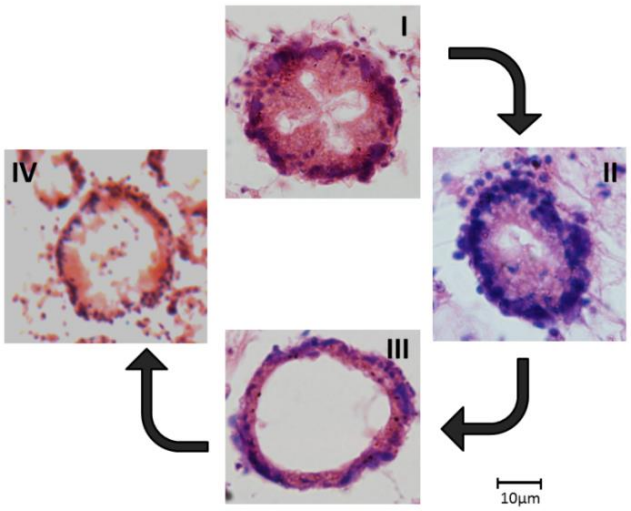


Figure 2: Subjective grading of digestive tubules into the 4 different stages observed. I: Absorbing / Holding; II: Disintegrating; III: Reconstituting; IV: Necrotic (sloughing) adapted from Langton (1975) & Robinson (1983) and modified from Cajaraville et al. (1989a).

2.3.3.3 Biochemical analysis (lipid peroxidation assay)

Surviving oysters from experiment 2 which were not processed for histological analyses were used for the lipid peroxidation assay. Lipid peroxides derived from polyunsaturated fatty acids are unstable and decompose to other complex compounds of which malondialdehyde (MDA) is the most abundant. The MDA was measured by a spectrophotometric assay using a commercial BIOXYTECH® MDA-586 kit (Oxis Research, Portland, OR, USA). The method is based on the reaction of a chromogenic agent, N methyl-2-phenylindole with MDA at 45˚C. A minimum of 100 mg wet weight of oyster powder was homogenized by sonication for 15 min in 990 µl of Phosphate Buffer Saline (PBS) pH = 7.4 and 10 µl of Butylated Hydrotoluene (BHT) to avoid intra-assay oxidation. The homogenate was then centrifuged at 10,000 g for 10 min, and the supernatant (200 µl) was added to a mixture of probucol in methanol, N-methyl-2-phenylindole, and hydrochloric acid as indicated in the MDA-586 kit. The reaction was conducted at 45˚C for 60 min. After centrifugation at 10,000 g for 10 min, the clear supernatant was transferred into a microtitration plate and the absorbance was measured at 586 nm. A standard curve was established with a solution of tetramethoxypropane (TMP) hydrolyzed during the acid incubation step generating MDA. Results were expressed in µmol of MDA per g of wet mass of oyster tissue.

2.4 Statistical analyses

All percentage data (mortality, tubule types) were normalized and arcsine-square root transformed prior to statistical analysis. Percent mortality, tubule staging, lumen ratio and lipid peroxidation data were analyzed by one-way analysis of variance (ANOVA), after checking for normality (Shapiro-Wilk test) and homogeneity of variances between treatments (Levene's test). Whenever significant differences among groups were found (ANOVA: α=0.05), treatments were compared using Tukey post-hoc test. Kruskall-Wallis non-parametric test and Dunnett post-hoc test were performed whenever normality and homogeneity of variance requirements were not met after transformation of the data. Median lethal concentrations (LC50) were estimated using Trimmed-Spearman Karber method (Hamilton et al., 1977).

Correlation analyses between morphometric parameters (lumen ratio) and (1) percentages of tubule types (to determine whether morphometric data can reflect the changes in the phasic activity of the digestive gland), and (2) percent mortality (to determine whether these modifications would correlate with mortalities) were conducted (Table 2). For histopathological results, a non-parametric Mann-Whitney/U-test (α=0.1) was used to assess the effect of HEWAF treatment on the prevalence of various pathological conditions compared to control (Table 3). Additionally, the total number of pathologies observed per individual (Fig. 10A) as well as gastro-intestinal pathologies, scored by intensity (Fig. 10B), were analyzed using Mann-Whitney/U-Test (α=0.1) and compared between treatments. All analyses were performed using SPSS® 22.0 statistical package.

3. Results

3.1 Water quality and analytical chemistry of oil solutions

Temperature and salinity throughout the different experiments were 24.4°C ± 0.9 and 22.7 PSU ± 1.9 respectively. Dissolved oxygen (D.O) and pH averaged 7.01 mg L^{-1} ± 0.5 and 8.27 ± 0.4 respectively whilst total ammonia concentrations remained at 3.18 mg L^{-1} ± 0.58. Seawater used for control treatments contained low levels of PAHs (n=50), at background levels (tPAHs = 0.24 µg L^{-1} ± 0.19); different nominal concentrations tested for each experiment with corresponding sum of PAHs (n=50) quantified by GC/MS-SIM are presented in Table 1.

Table 1: Range of nominal concentrations (ppm) of CEWAF and HEWAF used for acute exposures of oyster spat and corresponding tPAHs concentrations in µg/L (sum of 50 PAHs quantified by GC/MS-SIM)

CEWAF (Exp. 1)		HEWAF (Exp. 1)		HEWAF (Exp. 2)	
Nominal (ppm) <=> tPAHs (µg/L) (± SD)					
Control	0.4 ± 0.15	Control	0.3 ± 0.25	Control	0.03 ± 0.01
125	17 ± 10.2	62.5	41.9 ± 29.6	10	6.2 ± 7.1
250	33.8 ± 20	125	103.9 ± 19	50	74.5 ± 7.2
500	81.6 ± 17.8	250	191 ± 44.7	100	161.9 ± 9.7
1000	182.2 ± 21.6	500	414.5 ± 47.1	500	887.4 ± 28.5
		1000	826.8 ± 101.1	2000	3532.8 ± 145.8

3.2 Effects of CEWAF, Corexit and HEWAF (Experiment 1)

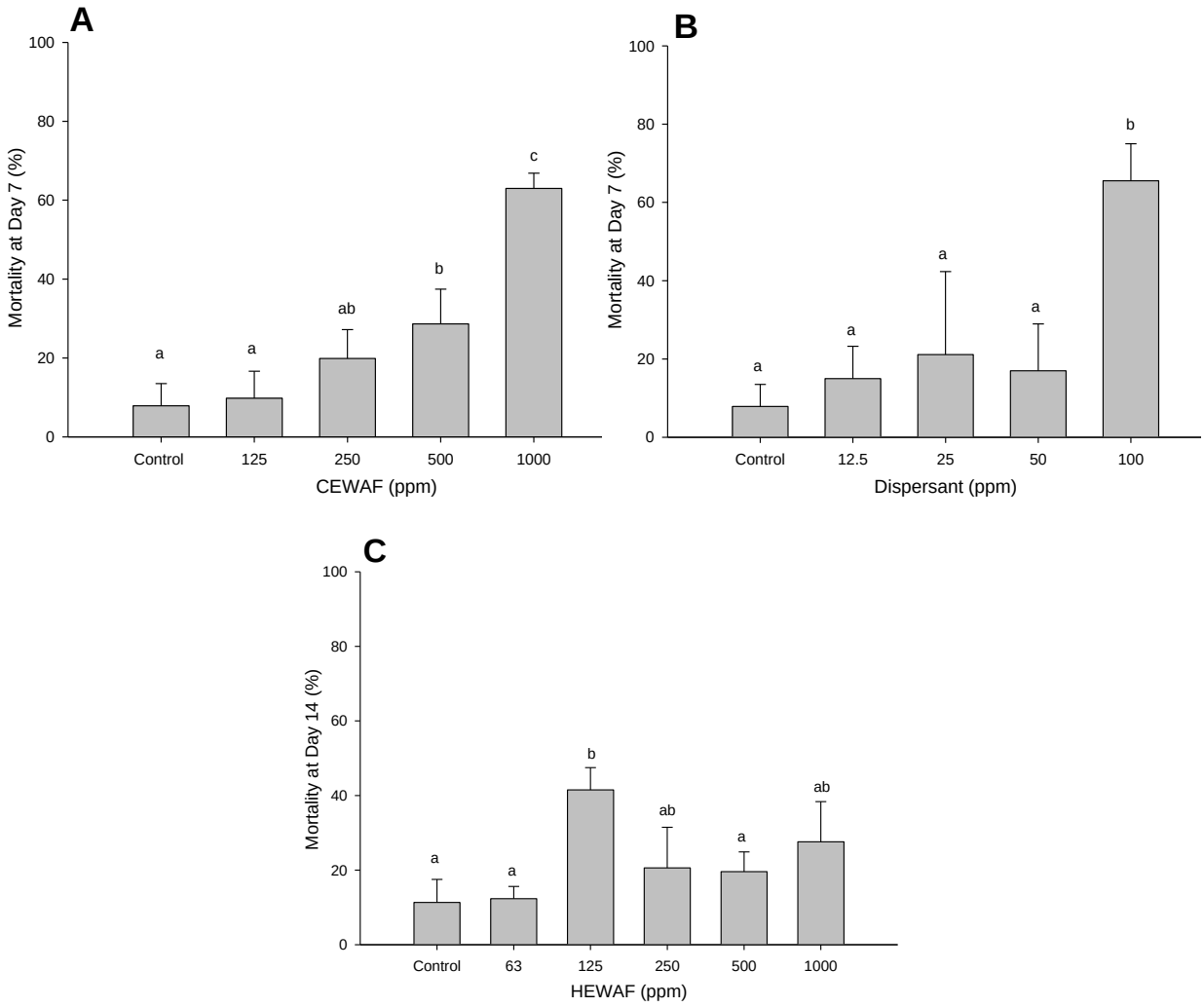


Figure 3: Mortality of spat after 7 days of exposure to CEWAF (A) and Corexit (B), and after 14 days of exposure to HEWAF (C), expressed in ppm. Nominal concentrations of CEWAF 125, 250, 500, and 1000 ppm correspond to 17, 33.8, 81.6, and 182.2 µg tPAHs (n=50) L^{-1} respectively. Nominal concentrations of dispersant 12.5, 25, 50 and 100 ppm correspond to 1.25, 2.5, 5 and 10 mg DOSS L^{-1}. Nominal concentrations of HEWAF 63, 125, 250, 500 and 1000 ppm correspond to 41.9, 103.9, 191, 414.5 and 826.8 µg tPAHs (n=50) L^{-1} respectively. Data are presented as mean percentage (n=4) ± standard deviation (SD). Treatments with the same letter were not significantly different (p>0.05) (ANOVA, *Tukey HSD* or *Dunnett's* post-hoc tests).

In experiment 1, results indicated that after 7 days of acute exposure to dispersed oil (CEWAF), spat mortality increased in a dose-dependent manner, with treatment having a significant effect on spat survival compared to control (*p<0.001;* Fig. 3A). At concentrations below 250 ppm or 33.8 µg tPAHs L^{-1} (mean tPAH of 4 samples), no significant effect on mortality was observed compared to control (*p=0.098*). Concentration of CEWAF of 500 ppm (LOEC) equivalent to 81.6 µg tPAHs L^{-1} however induced significant mortality compared to the non-exposed group (*p<0.01*). For the dispersant only exposure, results showed similar trends than CEWAF exposure, with spat mortality after 7 days being affected in a dose-dependent way compared to control *(p≤0.001;* Fig. 3B). No significant effect on mortality compared to control was observed below nominal concentrations of dispersant of 50 mg L^{-1}. Overall, trends in mortality for both CEWAF and Corexit were comparable at equivalent nominal concentrations (dispersant to oil ratio 1:10). Even though HEWAF treatments had a significant overall effect on mortality of spat after 14 days compared to control (*p≤0.001*), no dose-dependent effect was observed (Fig. 3C). Exposure to 125 ppm of HEWAF corresponding to 103.9 µg tPAHs L^{-1} was the only treatment with a significant effect on mortality of spat compared to control (*p<0.05*).

3.3 Effects of HEWAF on filtration rates (physiological impact)

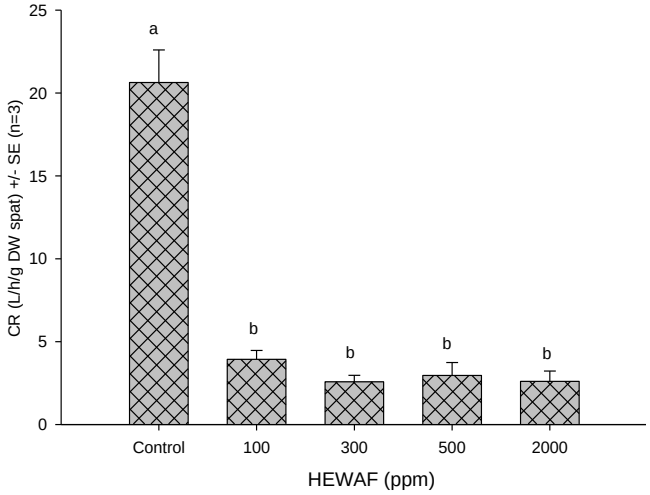


Figure 4: Clearance rates of *Tisochrysis lutea* by oyster spat exposed for 24 h to nominal concentrations of HEWAF (in ppm), expressed in L^{-1} h^{-1} g^{-1} DW oyster tissue. Data are presented as mean percentages (n=3) ± standard errors (SE). Different letters denote statistical differences compared to control (*ANOVA; α=0.05*)

In a separate experiment, results for 24 h-clearance rates of spat fed *Tisochrysis lutea* were significantly impacted at all concentrations of HEWAF tested (*p<0.001;* Fig. 4). Each treatment reduced oyster feeding activity by at least 5 folds compared to control (*p<0.05*). However, no difference was observed between treatments and all conditions had a comparable effect on clearance rates.

3.4 Effects of HEWAF (Experiment 2)

3.4.1 on mortality

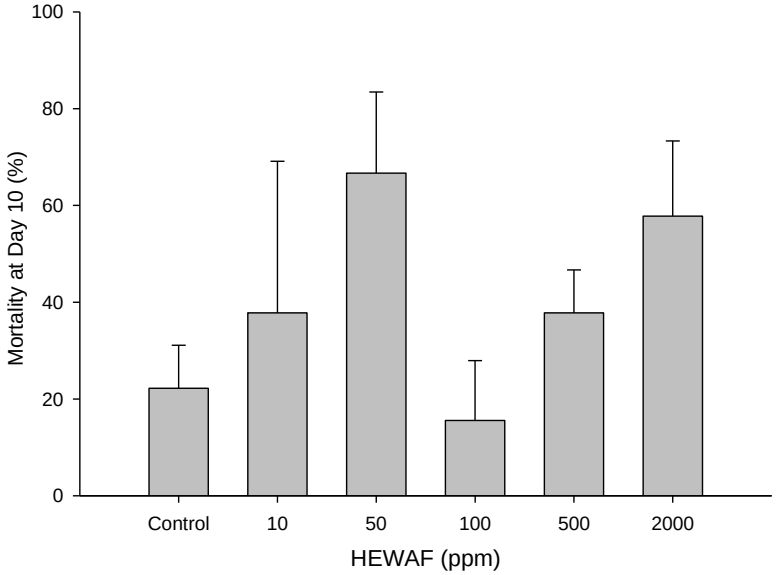


Figure 5: Mortality of spat after 10 days of exposure to increasing concentrations of HEWAF, expressed in nominal concentrations (ppm). Nominal concentrations 10, 50, 100, 500, and 2000 ppm correspond to 6.2, 74.5, 161.9, 887.4, and 3,533 µg tPAHs (n=50) L^{-1} respectively. Data are presented as mean percentages (n=3) ± standard errors (SE).

In experiment 2, larger juveniles (≈ 10-15 mm) were exposed to increasing concentrations of HEWAF (10 to 2000 ppm) for 10 days. Non-parametric statistical test showed no significant differences of mortality between control and exposed treatments (Kruskal-Wallis*: p=0.280*) (Fig. 5). Nevertheless, similar trends to the previous exposure to HEWAF were noted, with higher mortality observed at relatively low nominal concentrations (66.7% ± 16.8 at 50 ppm) compared to control (22.2% ± 8.9). In addition, the highest dose tested (2,000 ppm equivalent to 3,533 µg tPAHs L^{-1}) induced high percentage of mortality (57.8% ± 15.5) compared to control (Fig. 5).

3.4.2 on digestive processes (Morphometric analyses)

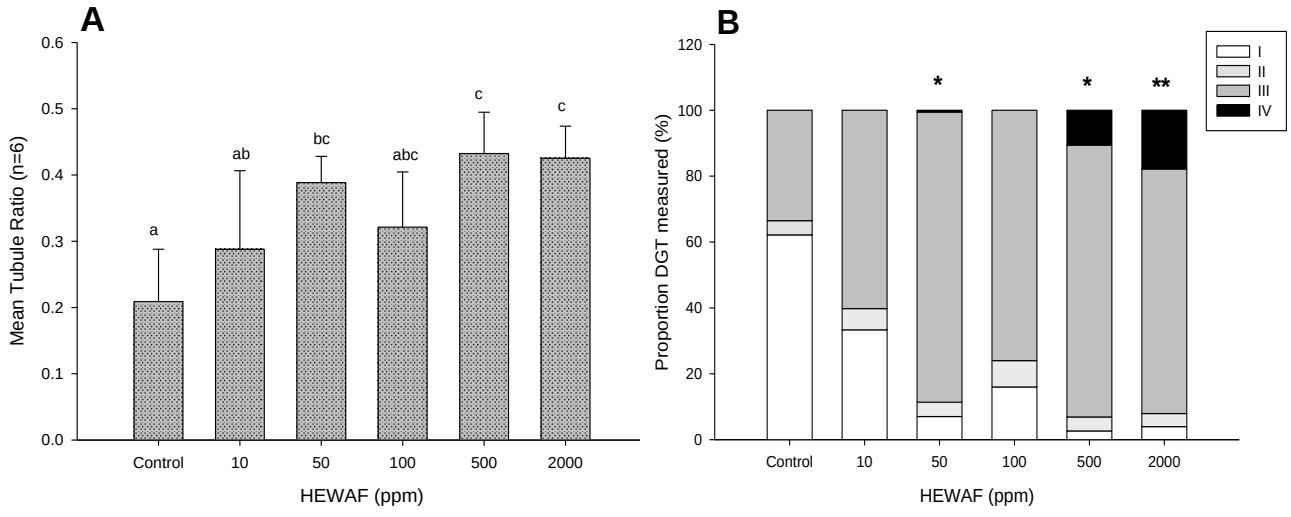


Figure 6: (A) Mean tubule ratio from 6 oysters exposed for 10 days to increasing nominal concentrations of HEWAF, expressed in ppm. Different letters denote a statistical difference (*ANOVA: α=0.05*). (B) Proportion (in percent) of the different digestive tubule (DGT) types observed in histological sections of spat (n=6), exposed for 10 days to HEWAF (ppm). Scoring method (2.3.3.2b) as follow: stage I: absorption; stage II: disintegration; stage III: reconstitution; stage IV: necrotic. Asterisks denote a statistical difference compared to control *(ANOVA: α=0.05*)

Measurements of the digestive tubule (DGT) lumen ratio in tissue sections of both control and experimental oysters indicated that exposure to increasing concentrations of HEWAF of DWH oil induced a significant increase of the lumen ratio compared to control ($p<0.001$, Fig. 6A). Dilation of the lumina of the DGT, as shown in Fig. 7B, increased in a dose-dependent manner, with nominal concentrations of HEWAF (e.g. 50, 500 or 2000 ppm) having a significant effect ($p\leq0.05$) (Fig. 6A). As a result of subjective tubule grading, mean proportions of each DGT stage were determined and are shown in Fig. 6B. Compared to control oysters, 10 days of exposure to HEWAF induced significant changes of synchrony between the different stages of tubules observed among treatments ($p<0.001$). In a similar way to the results obtained for the lumen ratio, the following nominal concentrations of HEWAF, 50 ppm, 500 ppm and 2000 ppm, impacted significantly the proportion of tubule types ($p\leq0.05$, Fig. 6B). Furthermore, analysis of variance showed that oil treatment had a significant effect on the occurrence of stage I, III and IV ($p<0.001$) DGT compared to non-exposed treatment. Distribution of tubule types was the most affected by exposure to oil, with a significant shift observed from stage I to III (Fig. 7A and B).

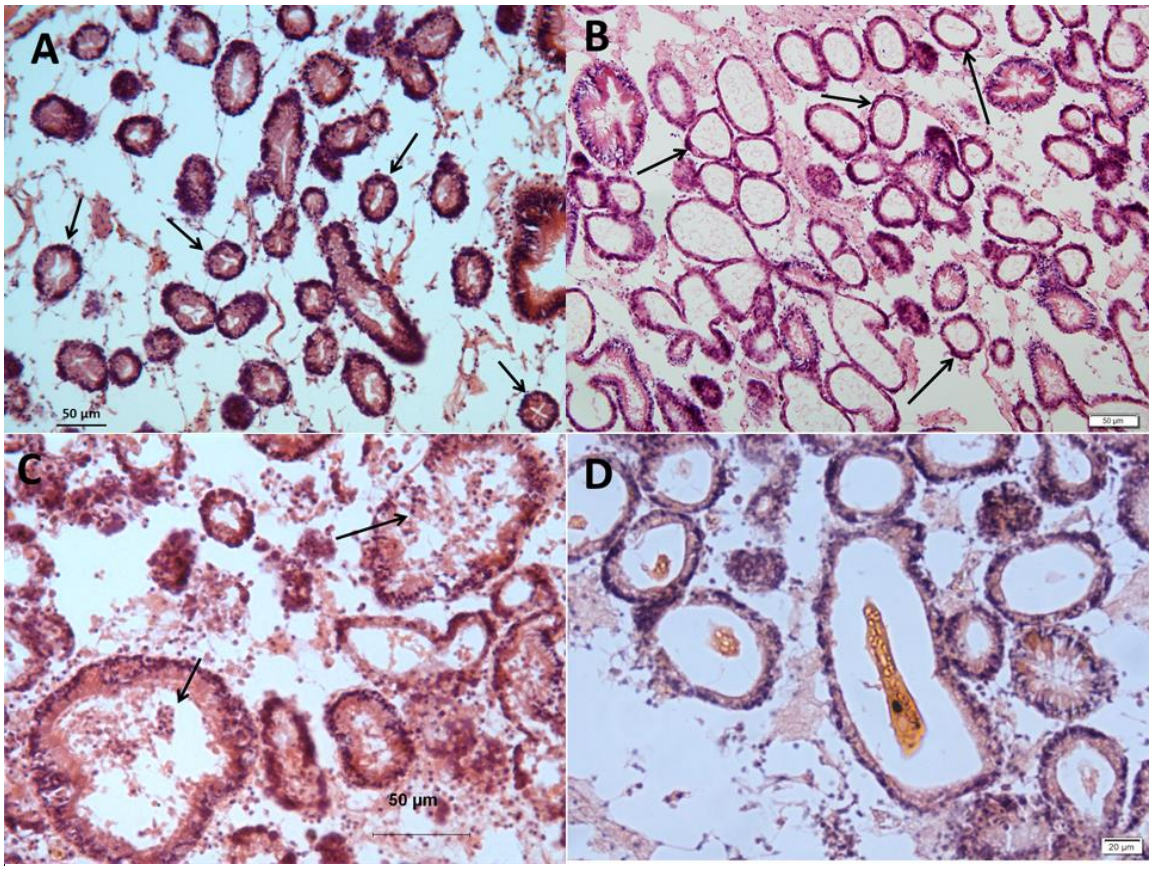


Figure 7: Histological preparations of *Crassostrea virginica* cross-sections (A) show predominantly normal (Type I) tubules (arrows); (B) arrows show severe metaplastic changes (Type III), with dilation of the lumina and loss of epithelial cell height; (C) shows necrotic stage of DGT with epithelial/digestive cells sloughing into the lumina (arrows); (D) example of tubules and ducts with presence of oil in lumen (2000 ppm).

In addition, statistical analysis showed that lumen ratio was significantly correlated with the proportion of stage I, stage III and stage IV tubules (Table 2), but not correlated with the proportion of stage II tubules. Disintegration of the DGT (stage II) was observed as either partial or complete and, as a result, it was often difficult to distinguish between classic degeneration, or poor preservation of tissue resulting from inadequate fixation. Generally, low lumen ratio corresponded with high proportion of stage I, and conversely, high lumen ratio corresponded with high proportion of stage III tubules. Lumen ratio was also positively correlated with mortality rates of corresponding spat at $p=0.05$ level (Table 2).

Table 2: Mean linear correlation between lumen ratio and various tubule stages and final mortalities. Significant correlation at $p \leq 0.05$.

	N	R	p
Lumen Ratio *vs* Stage I	***36***	***-0.652***	***0.010***
Lumen Ratio *vs* Stage II	*36*	*-0.189*	*0.271*
Lumen Ratio *vs* Stage III	***36***	***0.515***	***0.010***
Lumen Ratio *vs* Stage IV	***36***	***0.521***	***0.010***
Lumen Ratio *vs* Mortality	***18***	***0.497***	***0.050***

3.4.3 on histopathological conditions (tissue impact)

After exposure to mechanically dispersed oil (HEWAF) for 10 days, a variety of pathological conditions were observed using histology. Our observations focused on specific organs and tissues such as gills, labial palp, or mantle which were in direct contact with the oil solutions. In addition, connective tissue, and organs of the digestive system, such as stomach, intestine, digestive ducts and tubules were specifically studied. Conditions like erosion of gill filaments, hemocyte infiltration in the water tubules of the gills or in the labial palp (Fig. 8B), high instances of mucus secretory cells (mucus diapedesis) in the digestive tubules and ducts epithelia, or parasitic infestation (tylocephalum) were commonly observed in the oysters after 10 days, but distributed in all experimental treatments, exposed or not to oil. Nonetheless, oil-exposed spat differed from the control group in a number of respects. For instance, little food or digested material was found in the stomach, particularly in those exposed to 50 ppm and higher. One of the main histological features observed in exposed animals was the significant prevalence of syncytia in the connective tissue, in very high numbers compared to control (Fig. 8C and D) (U-test: $p<0.05$, Table 3). In some specific organs such as the gills, presence of syncytia was consistently observed in exposed groups, in significant proportions in some treatment (Table 3). In the labial palp epithelium, hemocytic diapedesis occurred in oil-exposed spat more often than in non-exposed spat, particularly at the highest doses (U-test: $p\leq0.1$, Table 3).

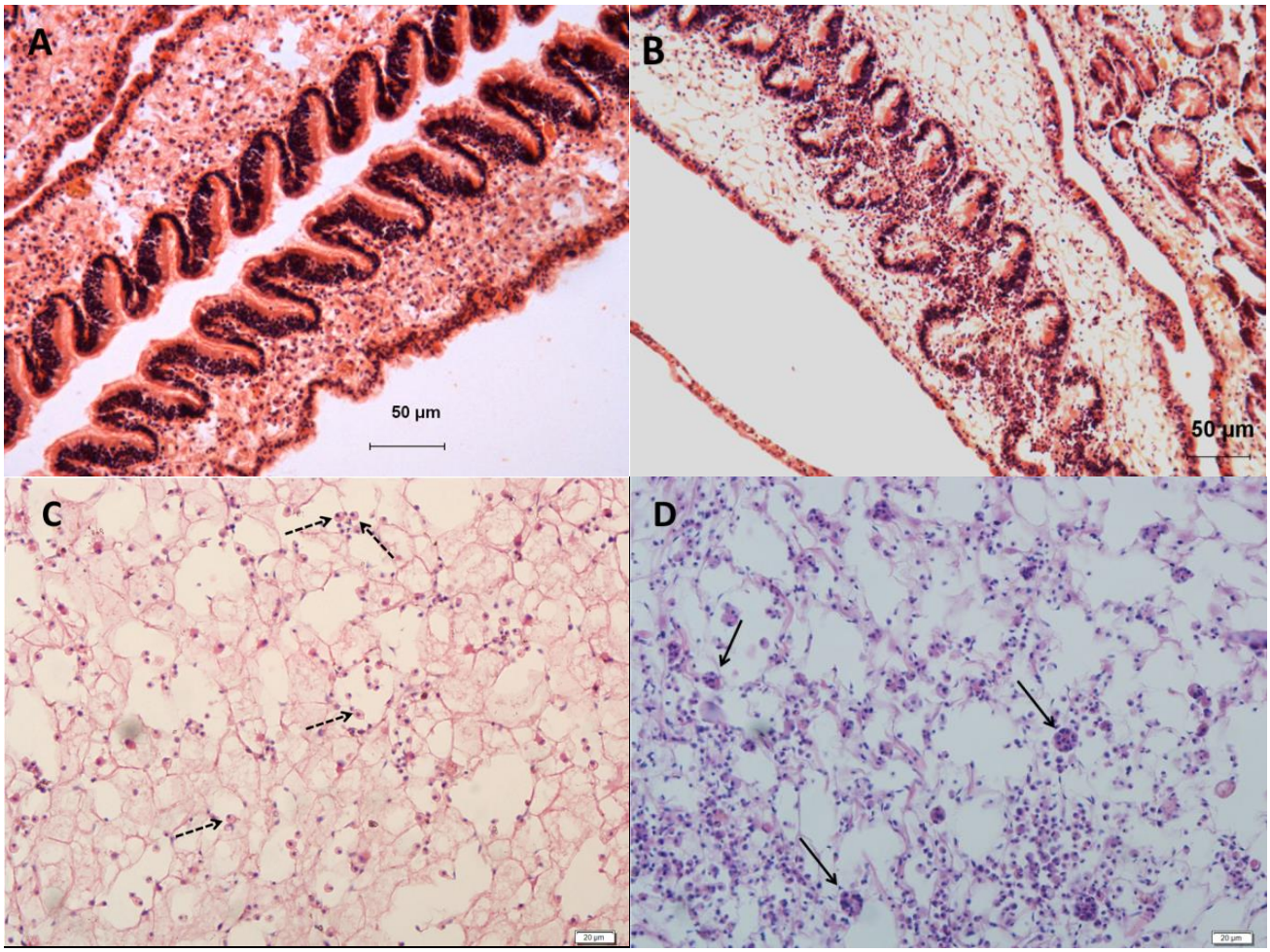


Figure 8: (A) Normal labial palp observed after 10 days in control oyster; (B) abnormal labial palp exhibiting major hemocyte infiltration (100 ppm); (C) healthy connective tissue with normal hemocytes and presence of phagocytosing hemocytes (broken arrows), observed in non-exposed group after 10 days; (D) connective tissue observed in oil-exposed spat (10 ppm) after 10 days showed high instance of syncytia (arrows).

Acute exposure to DWH oil prepared as HEWAF negatively affected the digestive system, including stomach, intestine and digestive diverticula, of oyster spat. After 10 days, an inflammatory response, characterized by severe hemocyte infiltration into the lumina of the stomach and the intestine and a prevalence of hypertrophic mucus secretory cells in the stomach epithelia, were observed (Table 3, Fig. 9D and E). In addition, exposure to oil caused severe hemocyte diapedesis into the stomach or the intestine after 10 days (Fig. 9C), occurring significantly more than in the non-exposed oysters (U-test, $p \leq 0.1$, Table 3). Besides, occurrence of severe pathological conditions in the stomach/intestine of exposed oysters such as ulcer (Fig. 9B) and epithelium sloughing (Fig. 9D) were found in a greater number of oysters at 50 and 100 ppm exposure concentrations (U-test, $p \leq 0.1$, Table 3).

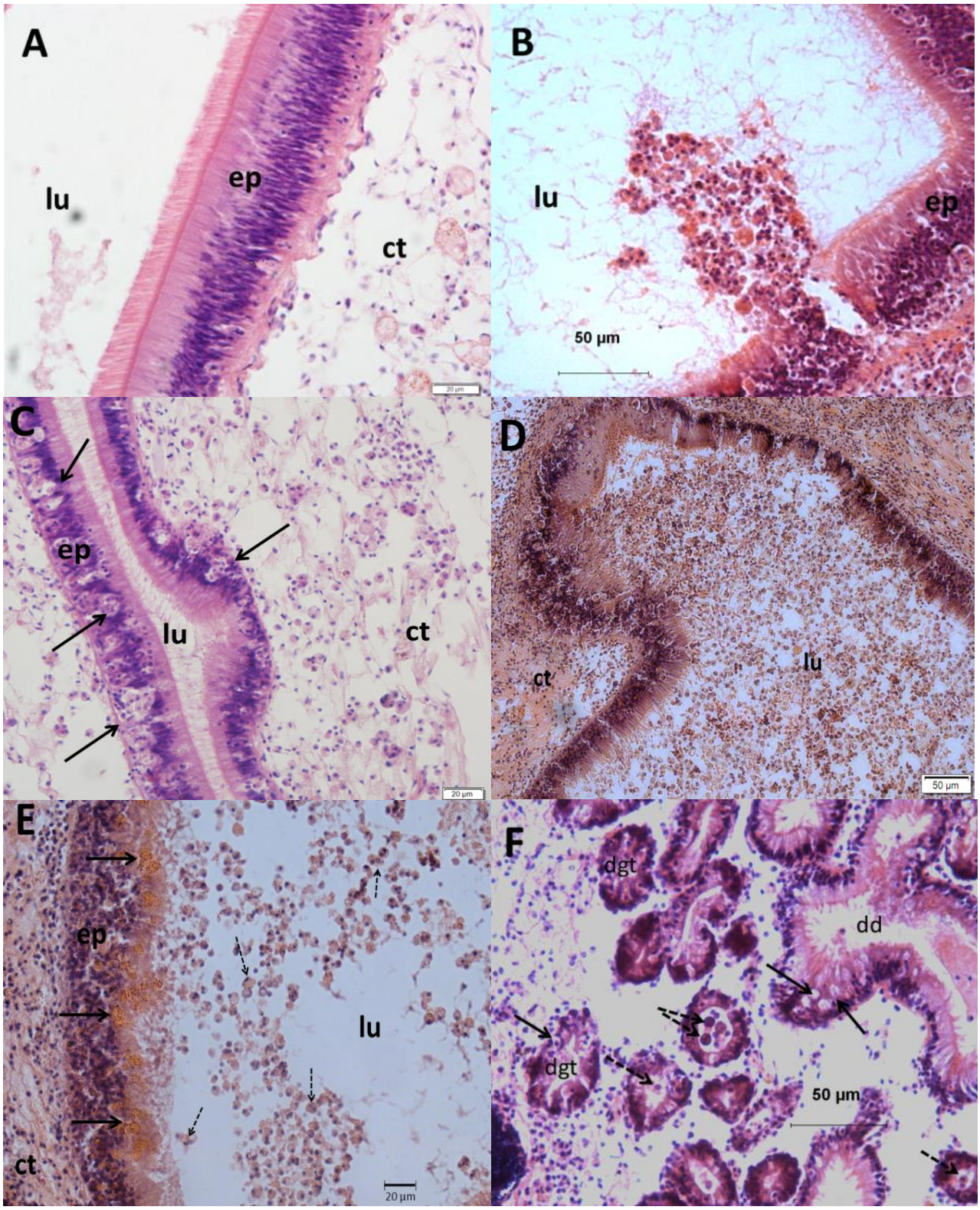


Figure 9: Section of the stomach of experimental oyster after 10 days of exposure to HEWF. (A) Normal epithelium in non-exposed oyster; (B) example of ulcer and consequent hemorrhaging of hemolymph in the stomach lumen observed in 50 ppm treatment; (C) heavy hemocyte diapedesis (arrows) from the connective tissue into the lumen of the stomach (10ppm). Note the presence of phagocytosing hemocytes as well as syncytia; (D) severe hemocyte infiltration in the stomach with massive epithelium sloughing observed in 100 ppm treatment. (E) Severe hemocyte infiltration coupled with epithelium sloughing and diapedesis (100 ppm); broken arrows show apoptotic hemocytes; arrows show oil accumulated in epithelia. (F) Section of digestive diverticulum (100 ppm) showing sloughing of digestive cells (broken arrow) in the lumen of digestive tubules as well as hemocyte diapedesis (arrows) in epithelia of tubules and digestive ducts. Ct: connective tissue; lu: lumen; ep: epithelium; dgt: digestive tubules; dd: digestive ducts.

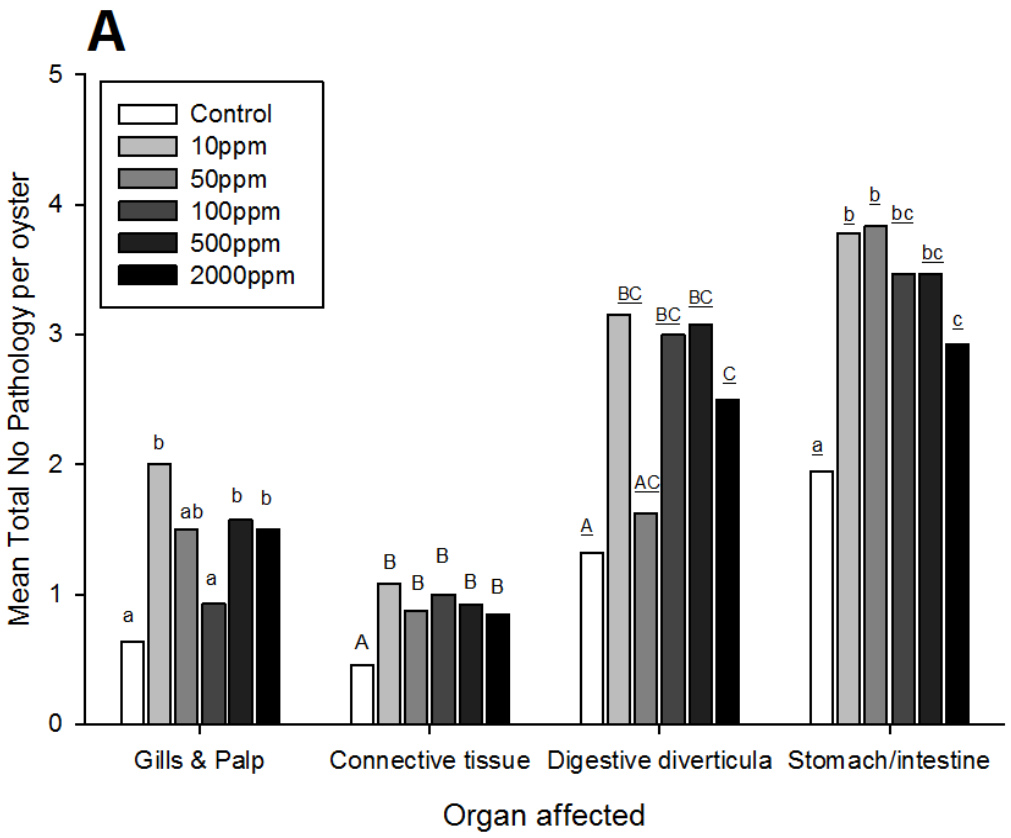
Table 3: Effects of DWH oil exposure (HEWAF) on *Crassostrea virginica* histological parameters after 10 days of exposure and compared to control. Data are presented in percent of occurrence per treatment. Non-parametric Mann-Whitney/U-test: statistical difference at α =0.1

Histological features	Control	10ppm	50ppm	100ppm	500ppm	2000ppm
GILLS						
Syncytia	5.3	46.2**	0	15.8	16.7	9.1
Hemocyte infiltration	0	15.4$^{\#}$	12.5	5.3	8.3	9.1
Erosion of filament	0	0	12.5	0	0	9.1
PALP						
Diapedesis	46.2	75	66.7	57.1	87.5$^{\#}$	100*
Hemocyte infiltration	7.7	50*	33.3	14.3	50*	28.6
CONNECTIVE TISSUE						
Syncytia	31.8	92.3***	75*	84.2***	92.3***	84.6**
STOMACH/INTESTINE						
Epithelium sloughing	5.3	11.1	50$^{\#}$	26.7$^{\#}$	15.4	0
Ulcer	0	0	33.3**	20*	7.7	0
Hemocyte diapedesis	79	100	100	100$^{\#}$	100$^{\#}$	84.6
Hyperplasia mucus secretory cells	21.1	77.8**	66.7*	66.7**	76.9**	69.2**
Hemocyte infiltration	57.9	100*	83.3	86.7$^{\#}$	92.3*	76.9
Mucus infiltration	31.6	88.9**	50	46.7	53.9	61.5$^{\#}$
DIGESTIVE DIVERTICULA						
Epithelium sloughing	31.8	100***	50	89.5***	76.9**	66.7*
Digestive cells in lumen	36.4	100***	50	94.7***	76.9*	66.7$^{\#}$
Hemocyte diapedesis	13.6	53.9**	37.5	47.4*	69.2***	25
Hyperplasia mucus secretory cells	9.1	30.8	25	21.1	38.5*	33.3$^{\#}$
Oil in lumen$^{@}$	0	0	0	5.3	7.7	8.3

Data were categorized as 0 or 1, according to the absence or presence of the observed feature; Mann-Whitney/U-tests were performed: $^{\#}$ $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, $^{@}$ Three oysters had this feature, all in the highest doses tested (100, 500, 2000 ppm) see Fig. 7D.

In addition, observation of the digestive diverticula showed a predominance of migration of hemocytes into the lumen of the digestive ducts by diapedesis in most of the exposure groups (U-test, *p<0.05*, Table 3) (Fig. 9F). At the end of the 10 day exposure, oyster spat exposed to oil showed evidence of sloughing of the epithelium of the digestive ducts and tubules (Fig. 7C), with the

presence of digestive cells in the lumina of the tubules and the ducts (U-test, $p<0.05$, Table 3) (Fig. 9F). Furthermore, presence of oil in the digestive ducts, as well as in the digestive tubules, was observed in several spat (n=3) exposed to the highest doses of HEWAF (Fig. 7D), but not in the control. Further, instead of partially digested food material, ready to be taken in by digestive cells for intracellular digestion, abnormal excretion product in the lumen of the tubule was observed. Finally, Figure 10A summarizes the mean number of pathological conditions observed per individual for each concentration of HEWAF tested. These results show an increase of the number of pathologies in every organ studied, and this for most concentrations of HEWAF tested compared to control oysters. Acute exposure to oil also induces most histopathological conditions in the digestive system, particularly in the gastro-intestinal system (Fig. 10A). A focus on the stomach and intestine revealed a significant increase in the intensity of pathologies compared to control (Fig. 10B). In addition, nominal concentration of 10 ppm seemed to induce the most severe pathologies compared to the highest dose (2,000ppm): for instance, hemocyte infiltration and diapedesis were significantly more intense at 10 ppm than 2,000ppm (U-test, $p\leq0.05$, Fig. 10B).



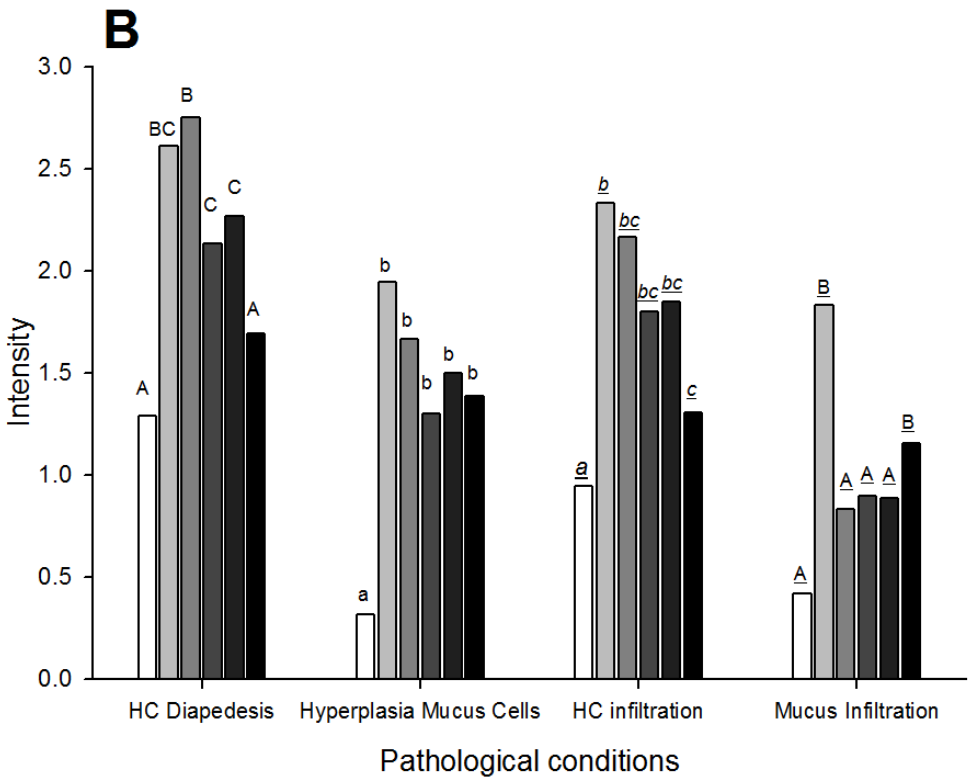


Figure 10: (A) Organ distribution of total pathological conditions observed per oyster (n ≥ 9). (B) Occurrence of various pathological conditions observed in the gastro-intestinal system (stomach/intestine) of oyster spat (n ≥ 9) after 10 days of exposure to HEWAF (ppm), categorized by intensity following a semi-quantitative scale; 0: absence, 1: light, 2: moderate, 3: severe. Different letters within each organ/pathological condition denote statistical difference at $p \leq 0.1$ (Mann-Whitney/U-test)

3.4.4 on lipid peroxidation

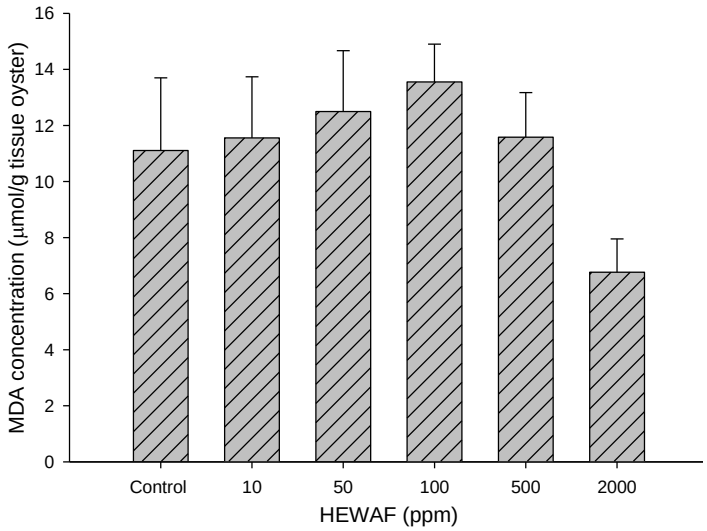


Figure 11: Mean malondialdehyde (MDA) content (±SE), expressed in µmol g^{-1} of oyster tissue (> 100mg), in spat exposed for 10 days to nominal doses of HEWAF (ppm).

For oysters exposed to increasing HEWAF concentrations, a trend to increased MDA levels was observed in the tissue (Fig. 11), but did not show any statistical significance compared to control ($p>0.05$). Concentrations of the lipid peroxidation byproduct were relatively high in the tissue sampled from the 100 ppm group (13.6 µmol g^{-1}) but not statistically different from the control group. Besides, the highest treatment (2,000 ppm) induced a non-significant decline in oxidative stress, with MDA levels lower than all the other treatments (6.8 µmol g^{-1}).

4. Discussion

At the end of the 7 day-exposure to CEWAF and Corexit alone, a concentration-related response was observed, with increasing doses of oil/dispersant causing an increased lethality. These results are in agreement with previous studies on early life stages of *Crassostrea virginica* (Vignier et al., in prep.a,b/Article 1 and 3). Overall, comparative results of median lethal concentrations for CEWAF ($LC50_{7d}$ = 833.9 ppm corresponding to 147.8 µg tPAHs L^{-1}) and Corexit ($LC50_{7d}$ = 84 ppm corresponding to 8.4 mg DOSS L^{-1}) suggest that dispersed oil has a similar toxicity to oyster spat than dispersant alone at equivalent concentrations (oil to dispersant ratio of 10:1). Most of CEWAF toxicity could be attributed to some chemical compounds of the dispersant as already shown by Vignier et al. (in prep.a, b/Article1 and 3). Surfactant, one of the main component of Corexit 9500A (Nalco Energy Services, 2012), have known effects on biomembranes such as increased permeability, loss of barrier function or osmotic imbalance (Benoit et al., 1987; Partearroyo et al., 1990, Singer et al., 1991; 1996). Additionally, the most obvious effect of dispersant in many aquatic species is gill damage resulting in death by asphyxiation (Abel, 1974; Nagell et al., 1974). Nuwayhid et al. (1980) observed other pathological impacts of dispersants in limpet gill tissue such as loss of cilia, and increased mucus production, lysosomal activity or vacuolation of mitochondria. Mortalities reported here indicate that spat are more tolerant to oil and/or dispersant compared to earlier developmental stages (Vignier et al., in prep.a,b/Article 1 and 3). Although bivalve species have been studied extensively in ecotoxicology, literature on the toxicity of DWH oil and/or dispersant Corexit 9500A on *C. virginica* is relatively scarce. Due to the variability of methodology used (e.g. different types of dispersant and crude oil tested, species and life stage exposed, duration of exposure, continuous or declining exposure, static *vs* flow-through system used, mode of preparation of WAF), the lethal concentrations recorded in the present study are therefore difficult to compare to a reference database. As a result, further effort should continue to support standardization of methods and procedures that would allow greater comparability and reproducibility of toxicological data.

Survival data obtained in the 2 separate HEWAF exposures showed similar trends, with increasing concentrations of DWH oil not inducing a dose-dependent mortality response. These results relate to

our previous study exposing oyster larvae to DWH oil in which survival followed the same trend (Vignier et al., in prep.b/Article 3). Several hypotheses have been speculated to try to explain higher mortalities at lower doses of HEWAF tested. Nonetheless, the most likely explanation seems to be more related to chemical processes than biological or physiological processes. Chemical analysis of DWH oil prepared as high energy method indicates that the proportion of soluble PAH and oil droplets is highly variable amongst concentrations and through time, with fraction of dissolved PAHs increasing with decreasing exposure concentrations (Vignier, unpublished data; Heather Forth, personal communication). It has been demonstrated by several authors that most of the toxicity to aquatic organisms can be imputed to the PAH derived from the dissolved fraction of oil rather than particulate oil (Barron et al, 1999; 2003; Carls et al., 2008; Nordtug et al., 2011; Hansen et al., 2012). If we consider solely the mortality data for HEWAF, it must be concluded that oyster spat are relatively tolerant to DWH oil. However, severe alterations in the normal physiology and metabolism of these animals can be expected, especially at sublethal levels, and the exact role of oil/PAHs in inducing these alterations is not clear. For this reason, the potential impacts of DWH oil prepared as high energy (HEWAF) on filtration, histopathological features and lipid peroxidation of exposed oysters were further investigated.

In response to an environmental stress such as oil pollution, filter-feeding oysters can reduce their clearance rates by closing their valves, as a defense mechanism (Akberali and Trueman, 1985). Concentrations ≥ 100 ppm of DWH oil caused a significant reduction in the clearance rate of oyster spat fed *Tisochrysis lutea* regardless of the concentration, suggesting valve closure as a potential cause. Numerous studies (Galtsoff et al., 1935; Chipman and Galtsoff 1949; Gilfillan, 1973; 1976; Barsczcz et al., 1977; Widdows et al., 1982) have also reported a decline in filtration rates of bivalves in response to hydrocarbons. Several reasons could explain this reduced feeding activity, the most obvious being a mechanical action of oil droplets by coating of the gills (Galtsoff et al., 1935), thus disabling the normal efficiency of the ctenidia to sort and absorb food particle. Gainey and Shumway (1991) found that exposure of gill tissue of hard clams to toxic algae exerted an irritation, causing a cessation of the ciliary activity. In the same way, oil/PAHs could have induced a cessation of the ciliary motion, inhibiting feeding rates. In the current study, instances of erosion of gill filaments were observed in some individuals, indicative of an apparent irritation and toxicity by contact. Our results could also be related to another study by Donkin and Widdows (1990) which suggested that bi- and tri-aromatic hydrocarbons could induce a narcotizing effect on the ciliary feeding mechanisms of *Mytilus edulis*, hence reducing clearance rates. In addition, occurrence of hemocyte infiltration and diapedesis in the water tubules of the gills or in the labial palp epithelia of

exposed oysters indicated a clear response of tissues in direct contact with HEWAF solution, confirming an exposure to dissolved PAHs and/or oil droplets by filtration. Similarly, Yamuma et al. (1996) observed significant hemocyte infiltration in the water tubules of the gills of freshwater prawns exposed to sublethal concentrations of PAHs. Lastly, abnormal production of mucus could also play an important role as a defense mechanism against oil exposure. Mucus contains lytic enzymes that act against a variety of stressors in bivalves such as phycotoxins (Shumway and Cucci, 1987; Galimany et al., 2008a). Enhanced mucus production in the gills can also incorporate and eliminate oil droplets as described by Axiak and George (1987) with the clam *Venus verrucosa*. In the present study, no substantial presence of mucus in the gills of oil-exposed oysters was observed. Perhaps mucus was present but may have been lost during preparation of spat and washed out through the decalcification process.

Following these results, further observations were carried out to determine the effects of oil/PAH exposure on the digestive system of oysters. Oil-exposed spat differed from the control group by the lack of food or digested material found in the stomach, particularly in those exposed to 50 ppm and higher. In a few cases, presence of oil in the lumen of the digestive tubules (DGT) or the digestive ducts (DD) was even reported, particularly at the highest doses. It is possible that oysters ingested directly oil as droplets, carried it to the stomach *via* the labial palp, and then transferred it to the lumina of DGT by the ciliated epithelia of the DD. Histological sections of oil-exposed oysters showed that digestive diverticula were similar in appearance to that seen in several species of mollusks typically starved (Langton, 1975; Morton, 1977; Robinson, 1983): for instance, severe atrophy of the DGT, with increased dilation of the lumina and a loss of epithelial cell height (stage III), beyond that demonstrated during a normal feeding/tidal cycle (Morton, 1977), were noted. This result suggests that crude oil/PAHs might disrupt the normal uptake of food by exposed oysters by contact, hence inducing a nutritional stress. This is in agreement with the works of Gilfillan (1973, 1976), Barszcz et al. (1977), Winstead (1995), or Wikfors and Smolowitz (1995) which showed that environmental stress can cause starvation. Ultimately, poor nutrition could have longer term deleterious implications, as it is known to play a role in reducing fecundity, gamete quality and subsequent larval performances, as observed with mussels (Holland and Spencer, 1973; Bayne et al., 1978) and oysters (Helm et al., 1973; Utting and Millican, 1997). Besides, similarly to Lowe et al. (1981), Robinson (1983) and Cajaraville et al. (1992), oyster mortalities were significantly correlated with DGT atrophy and the frequency of any tubule types. These two variables (lumen ratio and tubule staging) are simple indicators of DGT condition which could be used as a relevant index of general condition of bivalve in response to acute exposure to DWH oil.

Atrophy of the digestive diverticula after long-term and sub-lethal exposure to contaminants is a response that has been observed in numerous studies (Lowe et al., 1981; Rasmussen, 1982; Calabrese et al., 1984; Tripp et al., 1984; Auffret, 1988; Axiak et al, 1988; Lowe and Clark, 1989; Cajaraville et al., 1992). Like the current study, many authors reported that oil and PAHs exposures could lead to severe changes in the structure of the cells comprising the epithelium in the digestive gland of molluscs (Lowe et al., 1981; Widdows et al., 1982; Couch, 1984; Tripp et al., 1984; Axiak et al., 1988; Cajaraville et al., 1992; Bignell et al., 2011). Typically, the epithelium, which lines the DGT, is comprised of two cell types: digestive cells and basophilic cells (Owen, 1973). Digestive cells are responsible for the intracellular digestion of food material, while basophilic cells are involved in the synthesis and secretion of enzymes for extracellular digestion (Owen, 1973). As described by Lowe et al. (1981) and Moore (1985, 1990), PAHs could affect lysosomal activity, by altering their structure (size, number, membrane permeability) and function, leading to damages to digestive cells, thus impacting intracellular digestion. Calabrese et al (1984) revealed that modification of the normal structure of the DGT negatively altered digestive processes of mussels exposed to copper. The fact that oil was observed in the lumen of DGT clearly suggests a disruption of the mechanism of intracellular digestion, probably due to lysosome dysfunction.

In addition to tubule atrophy, histological examination of the digestive diverticula as well as the gastro-intestinal tract further confirmed a major inflammatory response in oil-exposed oysters. For instance, a strong occurrence of hemocyte diapedesis, ulcer or epithelium sloughing was observed in the stomach, intestine, digestive ducts or tubules. At the highest treatments (500 ppm and 2,000 ppm), a prevalence of necrotic tubules was noted, with cellular debris and digestive cells, most often in apoptosis, sloughed into the lumina of tubules and ducts (Fig. 9F). It is evident from these observations that a degeneration of the digestive cells lining the DGT epithelia and subsequent sloughing into the lumen was related to exposure to hydrocarbons. Likewise, Neff and Haensly (1982) described epithelium sloughing in oysters collected from the field following the *Amoco Cadiz* spill; whereas, Barszcz et al. (1977) noted that oysters exposed in the field to chronic concentration of oil exhibited digestive diverticula in poor condition, with high instances of tubule necrosis and sloughing of the epithelium. Finally, an excess of mucus in the alimentary canal and a high prevalence of hypertrophic mucus secretory cells in the epithelia of the stomach/intestine were some of the main histological features found in exposed spat. Although mucus secretory cells are a normal cell type in epithelia of the digestive gland, their increased number and abnormal size in oil-exposed oyster may indicate a hyperplasia, which could be a severe pathology representative of a precancerous condition, and may directly be imputable to contact with oil droplets/soluble PAHs.

An inflammatory response consisting of hemocyte proliferation in the connective tissue surrounding the digestive diverticula, as well as severe hemocyte diapedesis and infiltration into the lumen of the alimentary tract were consistently observed in oil-exposed individuals. Significant migration of hemocytes by diapedesis from the vascular system into the stomach and the intestine has also been described by Stauber (1950), Feng (1966) or Galimany et al. (2008a), as part of a defense mechanism in bivalves. The pathological changes we observed together suggest a potential non-specific response of hemocytes to oil/PAH exposure. Thus, Farley (1988) reported diapedesis of hemocytes into the alimentary canal in oysters exposed to heavy metal, whereas George and Goyer (1978) described migration of granular hemocytes in bivalve exposed to copper by diapedesis across the mantle surface. In fact, the response found in the present study is quite similar to the detoxification pathway described by George and Goyer (1978) and Thomson et al. (1985) for copper: divalent cations are accumulated in granular hemocytes and transferred *via* metal-binding proteins (e.g. metallothioneins) to tertiary lysosomes, resulting in the sequestration of these metals by the cells, which then migrate by diapedesis into the alimentary canal. In a similar way, we could hypothesize that after being assimilated in the gills or the digestive tract, either by passive absorption or direct ingestion, lipophilic PAH/oil droplets were transferred and accumulated in the lipid-rich connective tissue or epithelia of the gastrointestinal system, or remained in the alimentary canal. Hemocytes, originating from the surrounding tissue and the hemolymph, will then aggregate and/or sequestrate foreign PAHs by phagocytosis. Phagocytosis, *via* circulating hemocytes, has been described as an important process in mollusk immune response (Cheng et al., 1975; Galloway and Depledge, 2000). Pauley and Sparks (1965) showed that after injection of soluble toxic material into the connective tissue of oyster, necrotic cells were phagocytosed within a few hours, and phagocytes migrated to the exterior through epithelia. More specifically, in the presence of oil, Fisher and Tamplin (1988) and Sami et al. (1992) noted that oyster hemocytes can migrate to engulf and encapsulate foreign material. Moreover, Luna-Acosta et al. (2011) described that enzyme activities were activated in hemocytes of *C. gigas* exposed to crude oil, and may play an important role in protection against xenobiotics. Thus, as an attempt to eliminate oil, hemocytes may migrate from the connective tissue through the epithelia of the stomach/intestine by diapedesis, carrying the toxic compounds to the alimentary canal (intestine) for elimination by excretion in the feces. This process was further confirmed in our study by observation of actively phagocytosing hemocytes in the connective tissue and apoptotic hemocytes in the lumen of the stomach/intestine. Several studies on mussels have described this detoxification pathway with environmental stressors or toxic algae (Alves de Almeida et al., 2007; Galimany et al., 2008a): they also showed that this process was associated with an increase of lipid peroxidation due to an oxidative stress.

Indeed, chronic long-term exposure of bivalve to crude oil can induce an oxidative stress and impair the membrane integrity, associated with lipid peroxidation (Di Giulio et al., 1989; Livingstone et al., 1989; 1994; Downs et al., 2002). For oysters exposed to increasing HEWAF concentrations, a trend to increased MDA levels was observed in the tissue but did not show any statistical significance, mostly due to the high individual variability. Concentrations of MDA were relatively high in the tissue sampled from the 100 ppm group indicating an oxidative stress. It is noteworthy to mention that this concentration induced the lowest mortalities after 10 days of exposure whereas a high number of pathology per individual was reported, especially in term of hemocytic response in the connective tissue and digestive system. The same trend was observed with the 10 ppm treatment. Contrastingly, hemocytic response observed in the oysters exposed to 2000 ppm, particularly in the alimentary canal, was relatively low in comparison to the 10 ppm condition (Fig. 10B). As a result, low lipid peroxidation byproducts (MDA) were quantified in this treatment (2000 ppm), suggesting a direct correlation between hemocytic response and oxidative stress.

In addition to hemocytic proliferation, a strong occurrence of syncytia in the gills and connective tissue in exposed animals was also indicative of a severe inflammatory response. Syncytia are commonly defined as a multinucleate mass of protoplasm produced by the fusion of several cells (Dorland, 2011). They can be normal cells in animal biology (e.g. some type of fungi), but hemocytes are mononuclear cells and their merging together to form syncytia is a serious pathology. For instance, syncytia can form when cells are infected by certain types of viruses such as HIV, causing changes in immunology and surface antigens of the cells (Huerta et al., 2009) and ultimately apoptosis. Besides, formation of polykaryons cells (giant cells, syncytia) in response to inflammation has been frequently seen in vertebrates (Lewis and Lewis, 1926; Mariano and Spector, 1974) but more rarely reported in invertebrates (Anderson, 1987). This finding is of great significance as presence of syncytia may impair the immune response of affected organisms, and the relationship between syncytia formation and oil-exposure would require further research.

Lastly, histological analyses showed that oysters exposed to 10 ppm of HEWAF were significantly more affected (e.g. higher occurrence of hemocyte diapedesis and infiltration) than oysters exposed to 2,000 ppm of HEWAF. Calabrese et al. (1984) noticed the same trend with mussels exposed to 5 µg/L of copper which exhibited more histopathological alterations than those exposed to 10µg/L. Perhaps the chemical characteristics of low concentrations of HEWAF and particularly the proportion of dissolved/particulate hydrocarbons could have contributed to these augmented sub-lethal effects (Heather Forth, personal communication). Filtration results, which indicated no

difference of clearance between oil treatments, and the variable mortalities observed could support this hypothesis.

5. Conclusion

In summary, this study has revealed lethal effects of dispersed oil ($LC50_{7d}$ = 834 ppm equivalent to 148 µg tPAHs L^{-1}) and dispersant alone ($LC50_{7d}$ = 84 ppm equivalent to 8.4 mg DOSS L^{-1}) on an ecologically important organism of the Gulf of Mexico, *Crassostrea virginica*. Contrastingly, high concentrations of DWH oil (HEWAF) were necessary to elicit a lethal response (> 2000 ppm or 3,533 µg tPAHs L^{-1}) in oyster spat. Nevertheless, if we consider the mortality data in conjunction with the histopathological changes observed on surviving spat, it must be concluded that exposure to relatively low doses of HEWAF caused severe alterations in the normal physiology and metabolism of these animals. The present study also demonstrated that a non-specific inflammatory response occurred, with various degrees of severity depending of HEWAF doses, mostly located around the alimentary canal and the connective tissue. Our findings might contribute to a new approach on the effects of hydrocarbons and the role of hemocytes in detoxification pathways. It would be interesting to study more long term effects of oil exposure on oyster spat, and examine for instance, the consequences of a depletion of circulating hemocytes involved in immune response. Consequently, oysters would become immunocompromised, which could induce further severe pathological changes such as parasite infestation and disease.

Oysters are the principal benthic filter-feeders in the northern region of the Gulf of Mexico and appear to be, from the present study, sensitive to crude oil and dispersant alone. It is essential to mention that oysters may have also suffered from the synergistic effects of reduced salinity following the freshwater releases that were conducted in an attempt to prevent entry of oil into marshes (Bianchi et al., 2011). Gills are essential for ion regulation and osmotic balance in oysters: damages of hydrocarbons on the gills such as those observed in the present study may exacerbate impacts on physiology and general health. As a result, further research is required to understand the synergistic effects of multiple stressors exposure (e.g. salinity, temperature, hypoxia, UV-exposure) with DWH oil and/or dispersant, and their potential toxicity to oyster spat. Although these laboratory results showed significant effects in oysters exposed to DWH oil and/or dispersant, it cannot be extrapolated directly to the environment or to the natural oyster population. However, information collected in the present study can be used to assist in decision making such as the use of dispersant in response to oil spills, or the selection of meaningful endpoints other than lethality.

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Synthesis Chapter 3

Overall, acute exposure of oyster spat to dispersed oil or dispersant alone induced a similar toxic response, with concentrations of oil and dispersant of 834 mg L^{-1} (corresponding to 148 µg tPAHs L^{-1}) and 84 mg L^{-1} causing 50% of mortality after 7 days. Given their role of sentinel in coastal environment, oysters have the capacity to tolerate and survive highly polluted waters. This study has demonstrated indeed that high concentrations of HEWAF were necessary to elicit a lethal response. However, when considering the sublethal effects of oil/PAHs on physiological and cellular processes, our results demonstrated that much lower levels of oil/PAH could cause severe alterations of vital function, which will become deleterious to oyster population in the longer term. For instance, feeding rates were negatively affected at nominal doses of 100 mg oil L^{-1} resulting in starvation which will impact growth and ultimately reproduction and fecundity. Oyster spat may have been exposed to both dissolved PAHs, through passive diffusion through the gills or the digestive system epithelia, and droplet-associated PAH, *via* direct ingestion. As a consequence, specific tissue responses in gills, connective tissue or digestive system (e.g. hemocytic proliferation, ulcers, epithelium sloughing or digestive tubules necrosis) were observed at HEWAF concentrations as low as 10 mg L^{-1} (corresponding to 6.2 µg tPAHs L^{-1}). Exposure of the digestive system to PAHs also resulted in severe degeneration of the digestive diverticula which could be quantified by simple morphometric measurements. These results confirmed the essential role of hemocytes in inflammatory response and detoxification process following exposure to PAHs (soluble and droplet-associated). The present work highlighted the importance of including other endpoints than lethality in toxicological assessment in order to assess more accurately and realistically the impact of pollutant on organisms.

Chapter 4: GENERAL DISCUSSION AND CONCLUSIONS

Table of Content (General Discussion)

Chapter 4: General Discussion and Conclusions

1. Context of the study

The explosion of the *Deepwater Horizon* (DWH) oil platform on 20th April 2010 led to the tragic loss of 11 human lives, and resulted in the largest oil-related environmental disaster in U.S. history and the second largest worldwide in recorded history (Tawfig and Olsen, 1993; Carriger and Barron, 2011; National Commission, 2011). A failed blowout preventer below the DWH drilling rig caused an unprecedented amount of oil (779,000 t) and associated gas to discharge continuously in the Gulf of Mexico over a period of 3 months (OSAT/NOAA, 2010; Crone and Tolstoy, 2010; McNutt et al., 2012). The oil spill coincided with the spawning and recruitment season of many aquatic species in the Gulf of Mexico and particularly the Eastern oyster, *Crassostrea virginica*. Spill response capabilities were tested to their limits by the continuous flow of crude oil spilling from the sea floor, and subsea dispersant application directly at the source of discharge was suggested as the best response option. As a result, of the estimated 8 million L of dispersants used, about 2.9 million L were directly injected at the well head into the oil and gas plume at 1500 m depth (Lehr et al., 2010; Kujawinski et al., 2011). Prior to the DWH oil spill incident, no deep-water application of dispersant was ever conducted and behavior of such quantity of dispersant at depths, its environmental fate as well as its potential toxicity on pelagic and benthic organisms has been poorly understood.

Moreover, it has been documented that the treatment of oil with dispersant at the wellhead resulted in the formation of large, subsurface plumes made up of fine oil droplets (< 70 μm) suspended in the water column, which appeared to be degrading very slowly (Camilli et al., 2010; Diercks et al., 2010; JAG, 2010; Spier et al., 2013). In addition to the well-known toxicity of PAHs from the dissolved fraction of oil on aquatic organisms (NRC, 1989; Barron et al., 1999; 2003; Couillard et al., 2005; Carls et al., 2008), the potential toxicity of such droplets, through physical fouling or direct ingestion by filter-feeding organisms is likely to occur and not well understood.

Oysters, due to their biological characteristics (e.g. filter-feeding, sessile, ubiquity, broadcast spawners) and ecological importance in the Gulf region, have been employed as a model organism in eco-toxicological studies and for bio-monitoring the marine environment. However, information concerning the toxicity of crude oil, dispersed oil and dispersant alone

on various developmental stages of *C. virginica* and sensitive physiological processes are limited. The aim of this study was to investigate the effects (lethal and sublethal) of exposures of various life stages (gametes, embryos, veliger, pediveliger, or early spat) of oysters to surface-collected DWH oil, dispersant alone or dispersed oil, and to establish whether there is a relationship between the results gathered from this study and data collected from the field along the coast of the Gulf of Mexico. A detrimental effect of crude oil and associated dispersant on the reproduction and early development of the ecologically and economically important *C. virginica* could impact the recruitment and consequently decimate the oyster natural populations in the affected areas.

2. Main findings

2.1 Toxicity can be due to surface active compounds of Corexit 9500A

In most cases, the present study showed that CEWAF and Corexit 9500A exposures, at equivalent nominal concentrations, induced similar toxic responses, suggesting that most of CEWAF toxicity on oysters was likely imputable to surface-active agents contained in the Corexit. Surfactant compounds like DOSS have been reported to cause negative interaction with cell surfaces such as increased permeability, loss of barrier function or osmotic imbalance, or even cell lysis (Fedulova, 1976; Sigler and Leibovitz, 1982; Benoit et al., 1987; Partearroyo et al., 1990; Singer et al., 1990; 1996). Indeed, results of 24-h exposures of embryos to dispersant revealed severe abnormalities consisting of embryos with disrupted membranes or larvae with extruded tissue. Incubation of oocytes with CEWAF resulted in abnormal swelling of the vitelline envelope, indicating clearly a disruptive effect of surfactant (but also PAHs) which can interfere with membrane structures (Abel, 1974; Neff, 1985; Van Brummelen et al., 1998). Toxic compounds of dispersant could also induce damages on the gills of larvae or spat, resulting in death by asphyxiation as shown by Abel (1974) and Nagell et al. (1974) with other aquatic organisms.

2.2 Toxicity can be due to the dissolved form of oil

As described in the general introduction (§ 2.3.3.2 and 2.3.3.3), individual components of crude oil exhibit different levels of toxicity. Low-molecular weight (LMW) compounds, with one or two rings, are relatively soluble (low K_{ow}) in water and can reach high concentrations a few hours following an oil spill (Neff, 1979). Even though LMW PAHs are very

susceptible to weathering process (e.g. evaporation), they remain a concern as they can penetrate cell membrane *via* passive diffusion and cause toxicity through a non-specific mode of action, such as narcosis (Van Brummelen et al., 1998; Couillard et al., 2005). This mode of toxicity was definitely observed during acute exposure of early developmental stages, such as gametes, embryos or early larval stages. For instance, PAHs originating from the dissolved fraction of oil resulted in significant impairments of the fertilizing capacities of sperm (decline of viability) and oocytes (increase of ROS production) (Article 1 and 2), or an increase of abnormal development (inhibition of shell secretion) of the embryo (Article 1). In later stage of development such as early veliger, the reduction of feeding and lack of growth indicated clearly a narcotic effect due to contact with soluble PAHs (Article 1 and 3). Direct toxicity of these soluble PAHs, characterized by a disturbance of cell membranes, may have also played an important role in the mortalities observed in early, non-feeding developmental stages, but also in the inflammatory response observed in spat exposed to HEWAF, as a result of PAH diffusion through the gills (Article 5).

2.3 Toxicity can be due to combined effects of oil and Corexit 9500A

According to the U.S National Research Council (1989), acute lethal toxicity of chemically dispersed oil resides not only in the dispersant but primarily in the bioavailability of oil droplets (see next § 2.4) and the low molecular weight and dissolved aromatic fractions of the oil (for most species). In the present study, with the exception of the umboned larvae exposure (Article 3), CEWAF did not generally enhance toxic effects compared to Corexit alone. However, many authors have described a potential synergistic effect of chemically enhanced WAF (CEWAF) compared to WAF (LEWAF), resulting in an exacerbated toxicity on aquatic organisms (Gulec et al., 1997; Negri and Heyward, 2000; Ramachandran et al., 2004; Goodbody-Gringley et al., 2013; Rico-Martinez et al., 2013; Almeda et al., 2013). Chemical dispersant in combination with crude oil may increase the dissolution of LMW soluble compounds of crude oil (Anderson et al., 1974; Couillard et al., 2005; Greer et al., 2012; Wu et al., 2012; Cohen et al., 2014) and increase their concentration in the water by up to 5 times as reported by Fucik (1994) and Cohen et al. (2001). Similarly, chemical analyses of exposure solution used in the present study indicated that i) concentrations in the water of most PAHs were increased by 10 to 15 times for CEWAF compared to LEWAF (see Appendix 3 and 4), and ii) that additional HMW compounds (3 and 4 rings) were dissolved and found in solution following dispersion with Corexit (see Appendix 3, 4, 7 and 8).

2.4 Toxicity can be due to the particulate form of oil

Chemical dispersants generally serve to decrease the interfacial surface tension of oil, breaking down oil into very fine particles and thus facilitating its weathering (biodegradation and dissolution) (Canevari, 1973; Li and Garrett, 1998; Lessard and DeMarco, 2000). However, chemical dispersion also makes these oil droplets more bio-available to filter-feeding organisms like oysters and can cause adverse effects, either by direct ingestion of these droplets or by physical effects due to contact (e.g. surface coating) (Singer et al., 1998). Mechanical dispersion used to prepare HEWAF solutions enhanced further this process, with oil droplets in greater abundance than in chemically dispersed preparation of oil. This result is in agreement with Li et al. (2008b; 2009) who showed that distribution and characteristics of droplets were highly dependent of the mixing energy provided. In the present work, results clearly demonstrated that, by mechanical action, the particulate form of oil could drastically influence many physiological processes, such as fertilization by altering sperm motility and availability (Article 2), feeding behavior and growth of exotrophic umboned larvae (Article 3 and 4) or early spat (Article 5), or even survival by smothering of gills leading to asphyxiation. This finding is in agreement with several studies using filter-feeding species of zooplankton (Lee et al., 1978; 2012; Hansen et al., 2012; Almeda et al., 2013; 2014), but in contradiction with the works of Carls et al. (2008) and Nordtug et al. (2011) which showed that early life stages of fish (embryos, larvae) were only damaged by dissolved PAHs and not oil particles.

In addition, another concern associated with the particulate form of dispersed oil (CEWAF or HEWAF) is the bio-availability of Heavy Molecular Weight (HMW) compounds, more persistent in the environment and reported to be much more toxic than LMW hydrocarbons (Neff, 1979; 1985; Rice et al., 2001; Couillard et al., 2005). Analytical chemistry of solutions of HEWAF and CEWAF used in our toxicity testing indicated that the proportion of PAH derived from particulate/droplet-associated oil was greater than in chemically dispersed oil (CEWAF) (Fig.17A and B in III.2). Besides, ratio of dissolved PAHs to droplet-associated PAHs was equivalent to 1: 2 for CEWAF and 1: 10 for HEWAF (Fig. 18 in III.2). This is of particular concern as our results indicated that most of the persistent, toxic HMW compounds originated from the particulate fraction of oil (Fig. 17 in III.2). Toxicity results obtained after

acute exposure but also dietary exposure to HEWAF may confirm that most sublethal effects can be attributed to HMW PAHs contained in the particulate fraction of HEWAF.
Furthermore, as reported by Incardona et al. (2013), HEWAF preparation of oil might be a good proxy to assess the effects of slick oil submitted to the action of wind and waves. HEWAF method could also help to predict the toxicity of oil-droplets that form subsurface plumes on pelagic and benthic organisms, as they were shown by North et al. (2011) to be within a relevant size range (< 50 μm).

2.5 Lethal effects depend on exposure duration, concentrations, and life stage tested

First of all, the present study showed that short-term acute exposures to water accommodated fractions of oil and/or dispersant led to dose-dependent mortalities, with dispersed oil and dispersant resulting in the highest impact on survival of oysters, regardless of life stages tested. Mortality also tended to be more dependent upon exposure time than concentration of pollutant tested. Generally, mortalities observed following incubations with oil and dispersant were stage-dependent, i.e. sensitivity to oil/PAH and dispersant was greater for early developmental stages (Fig. 20-22). This stage-related sensitivity can be summarized as follow, from most sensitive to less sensitive:

Gametes > Embryos > Veliger > Umbo > Pediveliger > Spat

Many authors have reported that early life stages of aquatic organisms like oyster are more sensitive than adults, and represent a critical period in their life cycle (Thorson, 1950; Connor, 1972; Chapman and Long, 1983). This differential tolerance based mostly on size is likely related to the higher surface area to volume ratio of small organisms, which may increase their uptake of dissolved PAHs and toxic compounds of dispersant. As observed in other studies with copepods or coral larvae (Jiang et al., 2012; Goodbody-Gringley et al., 2013), body size was inversely correlated with oil/PAH toxicity and the difference of sensitivity was related to variations in respiration rates. Stage-dependent sensitivity could also be related to other factors such as the presence of a protective shell, which can act as a barrier and can be closed when conditions are too detrimental (Wisely and Blick, 1967; Akberali and Trueman, 1985). As previously discussed in § 2.4, ingestion of oil droplets by large enough larvae (e.g. late veliger or umboned) may additionally affect their survival.

2.6 Sublethal effects

In the present study, a multi-level, integrative approach was selected in order to assess more realistically the toxicity of oil and/or dispersant on oysters. As a result, effects at different levels of biological organization were determined: behavioral (feeding), physiological (growth, histopathology), ontogenetic (embryo or larval development), or population level (reproduction, mortality).

2.6.1 Oil and dispersant can affect fertilization success

Our results showed that fertilization was a sensitive stage in the early development of oysters. Acute exposure of gametes throughout the fertilization process (1h30) to HEWAF, CEWAF or Corexit alone resulted in a significant decline in fertilized embryos, with $LOEC_{1h}$ values reaching 1635 µg tPAHs L^{-1}, 14 µg tPAHs L^{-1}, and 500 µg DOSS L^{-1} respectively. Flow-cytometry analyses revealed that certain chemical properties of the Corexit in addition to the dissolved PAHs contained in CEWAF induced the most severe effects. A reduction of sperm viability and an alteration of ROS production for both spermatozoa and oocytes were revealed, which could certainly be related to unsuccessful fertilization. ROS production is essential in gamete physiology (de Lamirande et al., 1997; Aitken et al., 2004) and its modification can lead to DNA damage, lipid peroxidation and loss of viability (Livingstone et al., 1994; Cavallo et al., 2003; Koppers et al., 2010). Contrastingly, HEWAF did not affect sperm viability *per se* but the particulate fraction (oil droplets) interacted negatively and resulted, due to mechanical aggregation, in a reduction of "free" sperm cells available to fertilize eggs. Overall, in view of FCM results (ROS) and the cross-fertilization results, oocytes seemed to be more sensitive to PAH/dispersant toxicity and, as a consequence, contributed the most to fertilization impairments. Nevertheless, negative impact of CEWAF on sperm viability as well as an alteration of sperm function (morphology, motility) may have also contributed partly in the affected fertilization. Lastly, results of sperm and especially oocyte cellular parameters demonstrated that the use of FCM assays to evaluate gamete quality following exposure to oil and dispersant was a reliable and adapted tool.

2.6.2 Oil and dispersant can affect embryogenesis

Embryogenesis is a sensitive life stage where drastic physiological processes occur. Continuous acute 24-h exposure of oyster embryos throughout embryogenesis to HEWAF, CEWAF and Corexit resulted in high rates of morphological abnormalities, a finding in agreement with numerous authors (Le Gore, 1974; Le Pennec et Le Roux, 1979; Geffard et

al., 2001; Lyons et al., 2002; Wessel et al., 2007). CEWAF and Corexit induced significant abnormalities ($LOEC_{24h}$) at concentrations above 15 µg tPAHs L^{-1} and 500 µg DOSS L^{-1} respectively (Fig. 20 and 21), whereas HEWAF induced abnormality from doses of 400 µg tPAHs L^{-1} ($LOEC_{24h}$) (Fig. 22). Our results suggest that PAH/dispersant may interfere with the mechanisms involved in shell secretion, which begins early in embryogenesis (Eyster and Morse, 1984b). More specifically, they may disrupt and inhibit certain enzymatic activities such as carbonic anhydrase, involved in shell formation and calcium transport pathways (Galtsoff, 1969; Hinkle et al., 1987). Dissolved PAHs and/or toxic compounds of Corexit (surfactant) could also interfere with eggs and embryos membrane fluidity (Eyster and Morse, 1984a; Singer et al., 1990; 1996; Van Brummelen et al., 1998) and consequently damage DNA, resulting in abnormal development of embryos (Wessel et al., 2007). Further research is needed to understand more precisely the mode of action of PAHs and dispersant on embryogenesis of bivalves.

2.6.3 Oil and dispersant can affect feeding and larval growth

Of all the endpoints investigated in this study, feeding and larval growth were shown to be the most sensitive, with retardation of larval growth recorded at only 1 or 2 ppb of tPAHs (Figs. 21 and 22). As larval growth is strongly correlated and dependent on feeding ability (Strathmann, 1987), our results suggest that oil/dispersant could reduce the fitness of affected larvae by reducing their filtration rates. The lack of growth of early larval stages (e.g. veligers) was most likely a result of negative effects of dissolved PAHs/dispersant during embryogenesis, resulting in shell deformities which would consequently impair filtration. For the later stage (e.g. umbo) however, a reduction of feeding efficiency was mainly caused by the particulate form of oil (physical coating of the velum, aggregation with algae, and direct ingestion of droplets) which resulted in growth impairments as well. Narcotic effects from certain volatile PAHs (e.g. Naphtalene) could also explain reduction of feeding efficiency as it was shown with other invertebrate species (Berdugo et al., 1977; Almeda et al., 2013) (see § 2.2). Finally, larval digestive system is quite similar to that of juvenile or adult oysters (see § I.4.1.2.2), although simpler (Elston, 1980). Consequently, we can suspect that a similar mode of toxicity on the filtration ability of spat following acute exposure to oil/PAH (HEWAF) would occur with larvae (e.g. coating of the gills, irritation by contact of dissolved PAHs/dispersant, narcotizing effect of PAHs).

2.6.4 Oil and dispersant can affect settlement success

Metamorphosis and settlement success were both impacted by HEWAF and CEWAF, with $EC50_{72h}$ for CEWAF reaching 18.4 µg tPAHs L^{-1} for example (Fig. 21). From this, settlement inhibition was shown to be as sensitive as embryo-toxicity, highlighting the need to include this endpoint in toxicological assessment of oil or dispersant. The mechanisms involved in the toxicity of oil and/or dispersant on settlement are not clear. As previously mentioned, dissolved PAHs or surface-active compounds of the dispersant may have exerted a toxic effect on the metamorphosis process. More realistically, the physical action of particulate oil, especially abundant in the HEWAF, could result in the coating of settlement substrate and may have impeded settlement of competent larvae, as described by several authors (Smith and Hackney, 1989; Banks and Brown, 2002). It is essential to note that any impairment of feeding or substantial retardation of growth can result in an absence of settlement. During its larval life, oysters ingest sufficient food to supply all of the nutritive requirements associated with metamorphosis (Hickman and Gruffydd, 1971). As a result, any nutritional deficiency may result in a prolongation of the pelagic life (Holland and Spencer, 1973; Gallager and Mann, 1986). Additional research is required to elucidate the mechanisms by which oil, PAHs and/or dispersant affect the complex process of metamorphosis and settlement. Furthermore, the ongoing field monitoring program conducted under the NRDA (http://www.gulfspillrestoration.noaa.gov/oil-spill/gulf-spill-data/) on oyster recruitment will obtain precious data that will allow a better assessment of the impact of the spill along the coastal region of the GoM.

2.6.5 Oil/PAH can affect specific tissue and induce an inflammatory response

Following acute exposure of early spat to oil (HEWAF), results revealed that sublethal effects, caused by both the dissolved and the particulate fraction of oil, occurred from concentrations of 6.2 µg tPAHs L^{-1} and above (Fig. 22). Histological analyses indicated that severe alterations of tissue in direct contact with oil/PAHs (e.g. gills, connective tissue or digestive system) occurred, consisting of pathological conditions typical of an inflammatory response due to oil (Barszcz et al., 1977; Neff and Haensly, 1982; Berthou et al., 1987). For instance, hemocyte proliferation and infiltration, ulcer, lesions and necrosis of the digestive diverticula, or syncytia were consistently observed in exposed oysters. Our results confirmed the significant role of hemocytes in non-specific inflammatory response, and their

involvement in detoxification following contaminant exposures. Furthermore, exposure of the digestive system *via* ingested oil droplet or adsorbed PAH resulted in severe degeneration of the digestive diverticula (atrophy of tubules, loss of synchrony), which could be quantified by simple morphometric measurements, in agreement with other studies (Vega et al., 1989; Cajaraville et al., 1992; Wikfors and Smolowitz, 1995).

To sum up, a classification (excluding mortality) of the different biological functions (i.e. endpoints) impacted by oil/dispersant exposure can be suggested, from most sensitive to less sensitive:

Feeding ≈ Larval growth ≈ Spat tissue > Settlement > Embryogenesis $_{(24h)}$ **> Fertilization** $_{(1h)}$

2.7 The case of indirect exposure using dietary pathway

We saw previously that acute, short term exposure could lead to detrimental effects on critical physiological processes of larval development with dispersed oil and dispersant causing the most severe impacts compared to oil alone (HEWAF) (Fig. 22). Investigation of longer term, dietary exposure using oil-contaminated algae revealed that oyster larvae were able to ingest directly particulate oil, alone or in association with algal cells. This ingestion resulted in lethal and sublethal effects (growth inhibition) at levels of PAHs realistically encountered in the Gulf during the oil spill (e.g. from 1.6 to 78 µg tPAHs L^{-1}) (Fig. 23). Adhesion of HEWAF-associated oil droplets and/or adsorption of PAHs with lipid-rich phytoplankton are of particular concern as some persistent toxic PAHs (HMW) would become available to oyster larvae, and potentially be accumulated in a same way as described with adult oysters (Neff et al., 1976; Baumard et al., 1999; Geffard et al., 2002b). As a result, oil spills could pose long term effects on aquatic animals and the ecosystem, as an upward transfer of PAHs through the trophic hierarchy would be a realistic scenario that has been previously shown with other zooplankton species (Wolfe et al., 1998; Graham et al., 2010; Almeda et al., 2013). Thus, more research focusing on the bioaccumulation of PAHs and their potential biotransformation by bivalve larvae is needed. Examining if there is a relationship between bio-accumulation of PAHs and larval fitness is also required to better evaluate the short and long term impacts of crude oil spills on oyster populations.

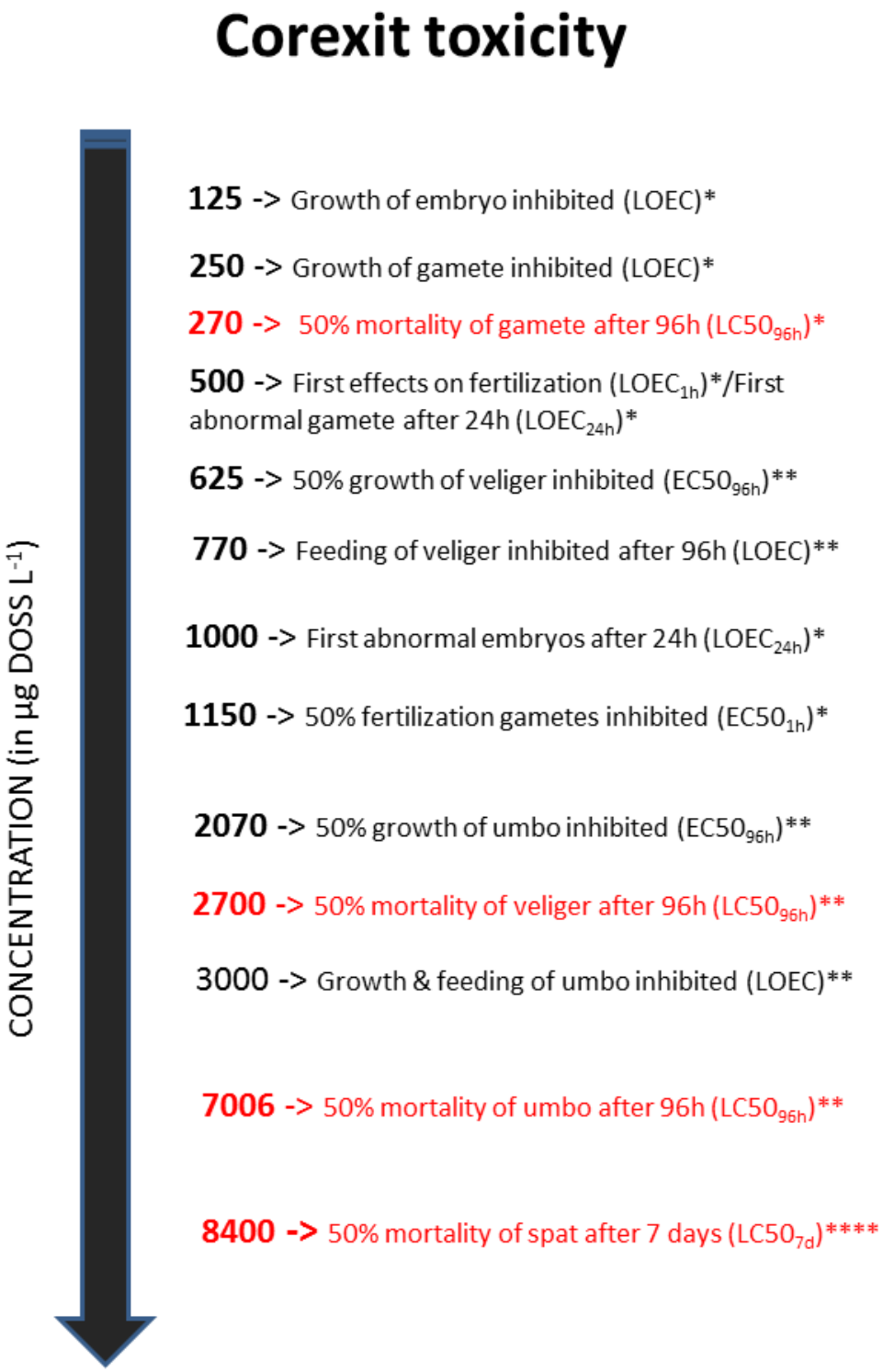

Figure 20: Toxicity levels and biological responses of different life stages of *Crassostrea virginica,* following acute exposure to Corexit 9500A, expressed as µg DOSS L^{-1}. Lowest effective concentration (LOEC), median effective concentration (EC50) and median lethal concentration (LC50) calculated in *: Article 1, **: Article 3, ***: Article 4, and ****: Article 5.

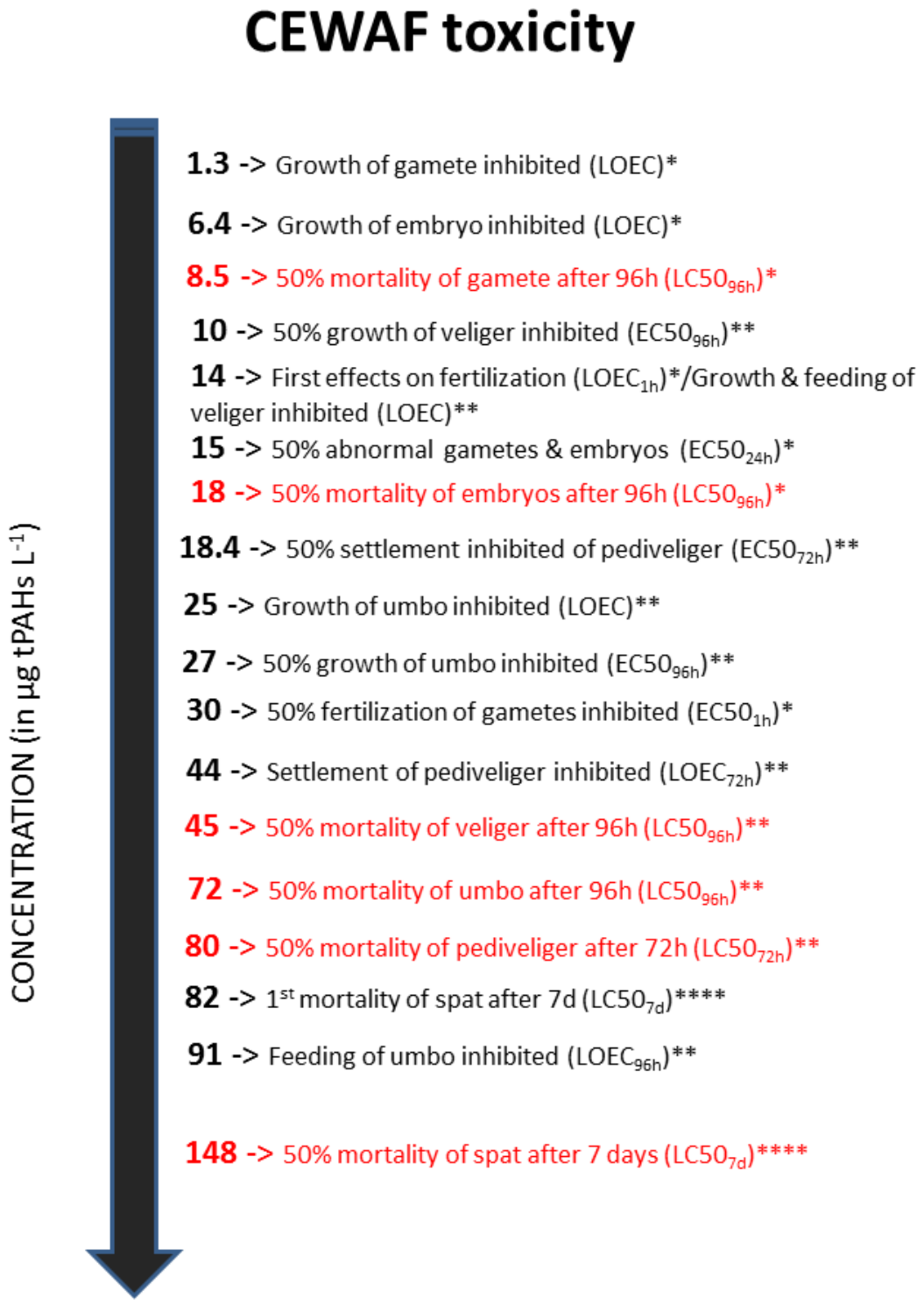

Figure 21: Toxicity levels and biological responses of different life stages of *Crassostrea virginica*, following acute exposure to CEWAF, expressed as µg tPAHs L^{-1}. Lowest effective concentration (LOEC), median effective concentration (EC50) and median lethal concentration (LC50) calculated in *: Article 1, **: Article 3, ***: Article 4, and ****: Article 5.

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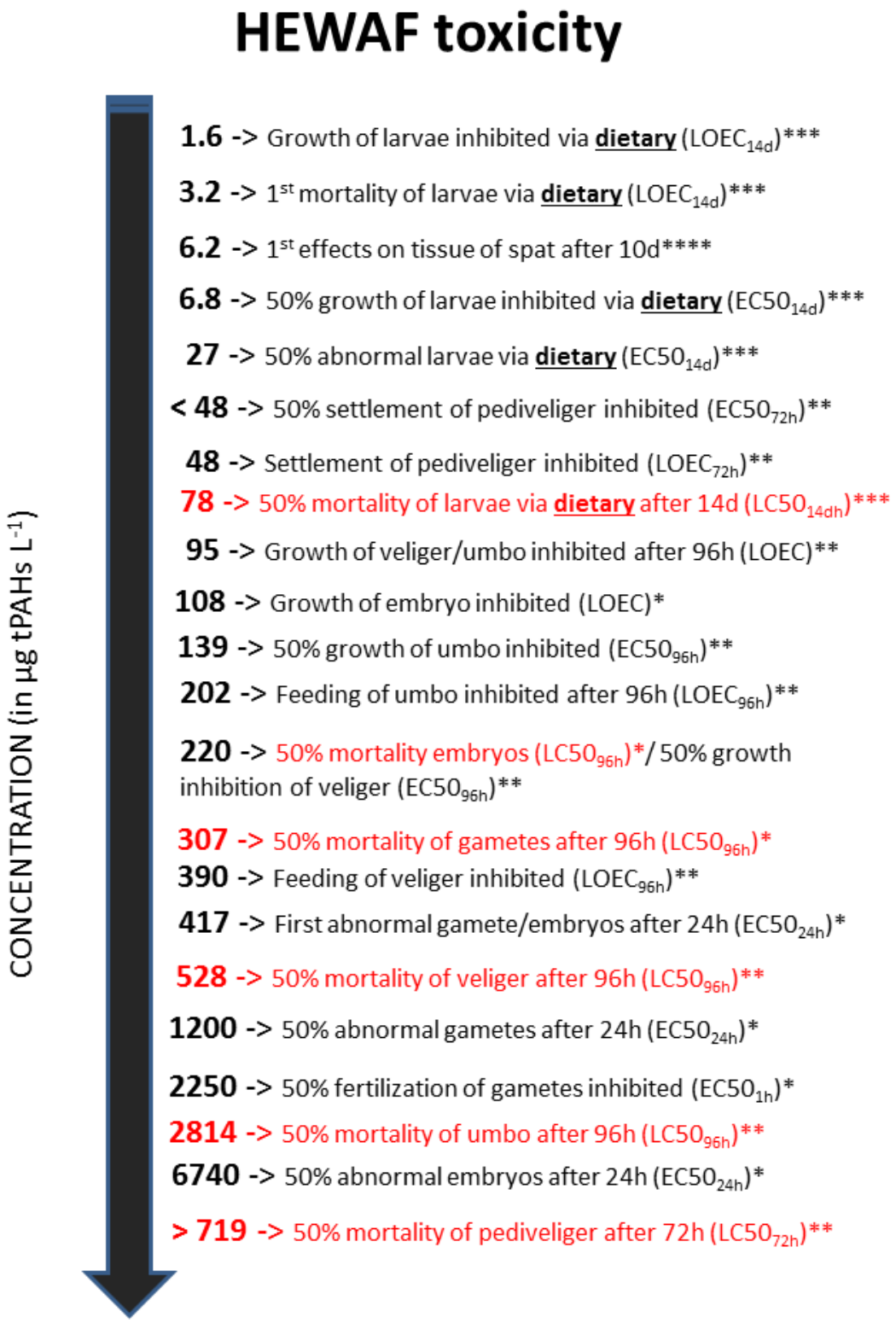

Figure 22: Toxicity levels and biological responses of different life stages of *Crassostrea virginica*, following acute and dietary exposure to HEWAF, expressed as µg tPAHs L^{-1}. Lowest effective concentration (LOEC), median effective concentration (EC50) and median lethal concentration (LC50) calculated in *: Article 1, **: Article 3, ***: Article 4, and ****: Article 5.

3. Comparison between toxic levels and field detections

As part of the Natural Resource Damage Assessment (NRDA) field sampling program led by NOAA, 90 offshore cruises were conducted and some 30,000 samples of water, sediment, tissue and oil from the deep-ocean, offshore and coastal areas were collected during the spill phase (NOAA and US Department of Interior, 2011). As a result, a large database of field sample data was generated. The NRDA sampling plans, protocols, and raw data are publicly available at the following link www.gulfspillrestoration.noaa.gov/oil-spill/gulf-spill-data. In order to compare with other ongoing NRDA studies and field data, a sum of 50 PAHs (parents and alkylated homologs) expressed as tPAHs, as well as DOSS concentrations (corresponding to the main marker of Corexit 9500A), were quantified in exposure solutions. Consequently, lethal and sublethal concentrations could be compared with field data. Figure 23 and 24 represent field measurements of total PAHs (n=50) and DOSS detected during the NRDA field sampling program: the source file for all Gulf water sample was obtained at http://54.243.205.138/gulfspillrestoration/qmmatrix/Water_chem_export.zip.

3.1 PAHs field detections

Acute toxicity testing conducted during the present study enabled us to determine lethal and sublethal effects of oil or dispersant on different stages of Eastern oyster. As indicated previously (§ 2.5), larval growth and feeding regime were usually the first parameters to be affected by oil/dispersant exposure, followed by settlement, and embryogenesis, all strongly dependent on the duration of exposure and the life stage challenged, particularly survival. For instance, chemically dispersed oil inhibited growth of larvae derived from exposed gametes, embryos, or veliger at concentrations below 10 µg tPAHs L^{-1}, whereas oysters exposed to CEWAF for 96 h prior fertilization resulted in LC50 of 8.5 µg tPAHs L^{-1} (Fig. 21). Concentrations equal to 15 and 18.4 µg tPAHs L^{-1} could also cause a 50% decrease in normal embryos and settlement success respectively (Fig. 21). When long-term, chronic exposure using dietary pathways were investigated, lower levels of tPAHs causing mortality were determined (e.g. LOEC=3.2 µg L^{-1}) (Fig. 22). Field detections of tPAHs measured between 0 and 20 m depths, ranged from 0 to 10 µg L^{-1} in most of the coastal regions of Louisiana, Alabama, Mississippi and part of Texas and North West Florida (Fig. 23). Even though field measurements in some areas near the DWH site reached levels up to 6000 µg L^{-1}, tPAHs were measured from 100 to 250 µg L^{-1} around the bay of Mobile, AL, or off the coast of

Texas (Fig. 23). In general, total PAHs concentrations range from 1 to 150 µg L^{-1} following an oil spill (Neff and Stubblefield, 1995; Law et al., 1997). In water samples collected during the DWH oil spill, some studies reported concentrations of total PAHs (n=50) that ranged from below detection limit in distant waters to 100 µg L^{-1} near the well head (Wade et al., 2011; Allan et al., 2012). Diercks et al. (2010) reported level of tPAHs reaching 189 µg L^{-1} at depths between 1000 and 1400 m, 13 km from the well head, consistent with the location of the subsurface plume. These field concentrations of PAHs measured in surface waters overlap clearly with the concentrations of PAHs found to cause lethal and sublethal impacts on oysters, with field detections in many cases at levels that were considerably higher.

However, it is important to note that it may be challenging to distinguish between chronic levels of PAH, resulting from routine oil discharge in the environment, and PAH originating from the DWH oil spill incident. For instance, much higher PAH levels were measured off the coast of Texas/Louisiana or in the bay of Mobile, AL, which could be the result of oil activity not related with the DWH event. In a field study monitoring waters around the UK, Law et al. (1997) reported that chronic concentrations of tPAHs (n=15) may range from none detected to 10.7 µg L^{-1} in many coastal and estuarine samples. Given the much higher levels of PAH detected in some bays and estuaries, chronic exposure can be excluded, and data collected during the NRDA sampling program were most likely related to the DWH oil spill. Furthermore, pre-spill field data were available as a reference baseline of contamination (EPA, 2008) and no confusion between chemical fingerprints of the DWH oil and natural seeping sources was demonstrated (OSAT, 2010).

3.2 DOSS field detections

The US EPA established analytical methods and screening levels for a selected dispersant-related chemical in water samples, the surfactant DOSS. From the 10,000 water samples collected for dispersant analysis during the NRDA field sampling program, most field measurements of DOSS did not exceed 1 µg L^{-1} (Fig. 24), levels which are well below the EPA aquatic toxicity benchmarks of 360 µg DOSS L^{-1} for acute and 40 µg DOSS L^{-1} for chronic exposure (http://www.epa.gov/bpspill/dispersant-methods.html). Nonetheless, field detections of DOSS ranging from 50 to 1000 µg L^{-1} were regularly measured, especially in coastal and estuarine areas where oyster populations are located (Fig. 24). In view of our toxicity thresholds (Fig. 20), it appears that early stages of oyster (e.g. gametes, embryos and

veligers) would be the most impacted by field levels of DOSS reported, with growth, feeding and embryogenesis being affected, but also survival ($LC50_{96h}$ = 270 µg DOSS L^{-1} for gamete) and fertilization success ($EC50_{1h}$=1,145 µg DOSS L^{-1}).

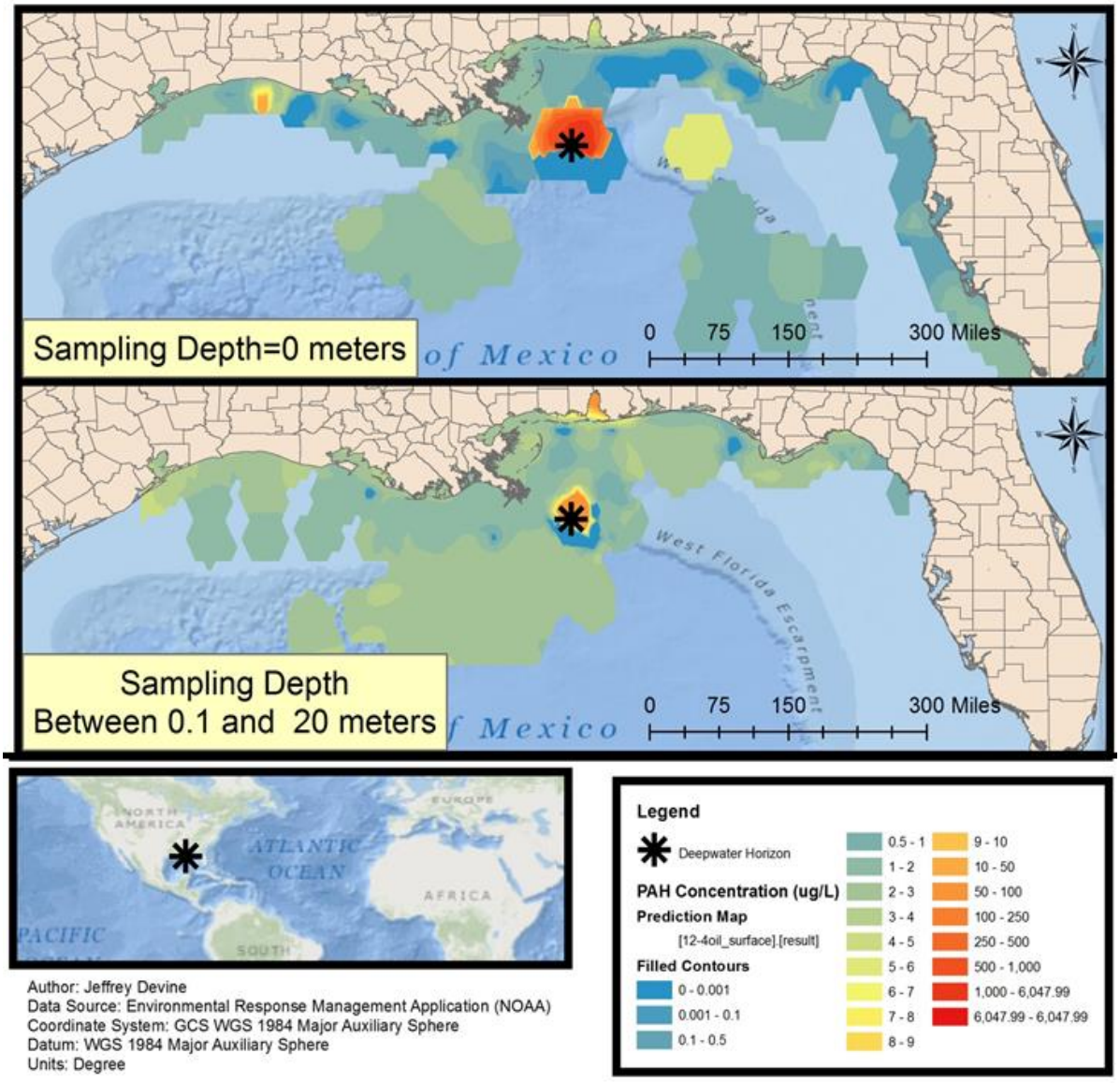

Figure 23: Field measurements of tPAHs (n=50) detected in the surface waters (0 to 20 m depths) of the Gulf of Mexico during the DWH oil spill, expressed in µg L^{-1}. Measured concentrations range from 0 to 6500 µg L^{-1}.
Data from Environmental Response Management Application (ERMA) and NOAA (available at http://gomex.erma.noaa.gov/) Author: Jeffrey Devine (FGCU).

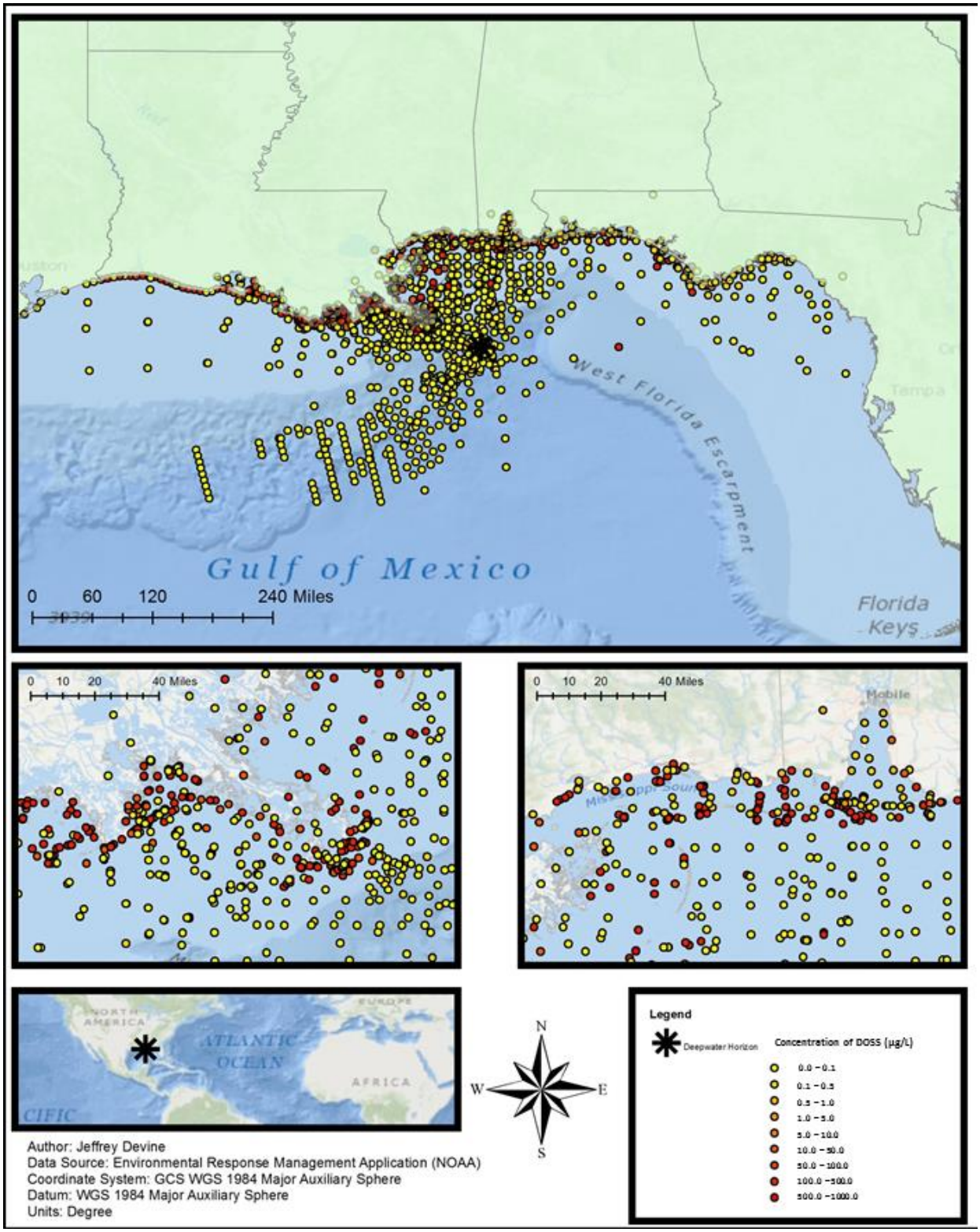

Figure 24: Field measurements of DOSS (main marker of Corexit 9500A) detected in the Gulf of Mexico during the DWH oil spill, expressed in µg L^{-1}. Measured concentrations range from 0 to 1,000 µg L^{-1}.
Data from Environmental Response Management Application (ERMA) and NOAA (available at http://gomex.erma.noaa.gov/) Author: Jeffrey Devine (FGCU).

These field measurements of DOSS are in contradiction with many studies which highlighted the fact that within a few hours (half-life of dispersant from 4 to 24 h), surfactant would dissolve and dissipate rapidly (Lessard and DeMarco, 2000; Fingas, 2002; Coelho et al., 2013), resulting in no threat for aquatic organisms. We could also wonder whether DOSS, which has been shown to have an extremely low volatility and a potential to persist in the environment (US FDA, 2014), could be accumulated by filter-feeding organisms like oysters. In order to assess the public health concern from consumption of GoM seafood, laboratory studies were conducted by NOAA, the FDA and EPA. They reported that little or no bio-concentration of DOSS in oyster tissue was evident (DOSS levels declined by > 99% within 72 h) after exposure to 100 mg dispersant L^{-1} (Benner et al., 2010).

These findings imply that concentrations of PAHs and DOSS tested in the present work were likely in the range of doses encountered in coastal and estuarine areas affected during the DWH oil spill, and could have detrimental consequences on reproduction and critical processes of the early life of *C. virginica*. However, even though helpful, it can be misleading to compare our results with only one marker of oil or dispersant, as toxicity of oil or dispersed oil are often related to synergistic effects of other toxic compounds contained in crude oil (e.g. normal alkanes, isoparrafins, diesel range organics) or Corexit (e.g. 2-Butoxyethanol or Di-Propylene Glycol Butyl Ether, DPnB) (Barron et al., 1999; Tjeerdema et al., 2013). In addition, despite the knowledge available on the increased bioavailability of certain PAHs after chemical dispersion of crude oil, little is known about the interaction of surfactant/DOSS with oil when mixed together, and how much would finally remain in solution.

Overall, valuable data were gathered during the present work, and thus could be compared with field data, enabling the prediction of acute and chronic effects likely to occur to Eastern oysters as well as other filter-feeding species in field situations.

4. Laboratory *vs* Field: underestimation or overestimation of toxicity?

4.1 Interactions of multiple factors in field situation

It is important to note that exposures in the laboratory are generally performed to establish a link between a cause (a specific stressor) and a particular effect observed in the field. Assessment of this toxic effect is usually conducted under optimal environmental and

controlled conditions. However, in a "real-world" situation, toxicity of crude oil not only depends on the concentration of oil and the duration of exposure, but also on combined effects of natural or anthropic stressors which can amplify the overall toxicity. As a result, our laboratory assessment of DWH oil toxicity and the severity of its effects may have been underestimated. Coastal estuaries are naturally dynamic habitats where fluctuations of temperature, salinity, nutrients, and hypoxia are common. Such environmental variability imposes to oysters and other resident species to compensate by investing on diverse physiological processes, and exposures to oil/dispersant, in addition to their toxic effects, may interfere with these compensative functions (Whitehead, 2013).

4.1.1 Salinity and temperature

Coastal estuaries can be subject to wide variations in salinity, due to discharge from rivers, marine flooding from storm surge or regular tidal and seasonal changes (Elliot and Quintino, 2007). By using their gills, oysters can compensate for these changes, regulating ions and maintaining osmotic balance. Damages of hydrocarbons on the gills such as those observed in the present study (Chapter 3) may exacerbate impacts on physiology and general health of oyster spat. Moreover, solubility of PAHs increases with decreasing salinity, hence increasing bioavailability and toxic risk for oysters in low salinity waters (Ramachandran et al., 2006). During the DWH spill, as a mitigation effort, massive releases of freshwater from the Mississippi river were carried out in order to limit slick oil from flowing into coastal ecosystems and estuaries (Bianchi et al., 2011). With regards to the previous comment, we can assume that such intervention may have unintended consequences on oyster populations.

In a similar way to salinity, water solubility of PAHs is directly related to temperature, i.e. it increases when the temperature increases (Lin and Tjeerdema, 2008). Despite the fact that weathering processes of oil will be accelerated, oyster population living in coastal waters of the Gulf of Mexico (GoM) may be at risk, as high summer temperatures in surface waters will increase the bioavailable fraction of oil, therefore enhancing its toxicity.

Besides, lifetime of droplets contained in the undersea plumes in the cold depths could be much greater than the one in warmer surface waters, where evaporation and photo-degradation can occur and bacterial degradation is much faster. For this reason, it is highly likely that there is still a significant amount of oil persisting below the surface of the GoM, posing a long-lasting threat to filter-feeding bivalves when some of these particles will eventually reach the coast.

4.1.2 UV radiation

Another factor that we did not consider in this study and that has been well documented is the synergistic action of ultraviolet (UV) radiation with oil, or photo-induced toxicity, occurring at the water surface. Indeed, UV radiation may increase the toxicity of petroleum hydrocarbons (e.g. PAHs) on exposed marine organisms (Landrum et al., 1987; Barron et al., 2003; Almeda et al., 2013). For instance, it has been shown that exposure to UV at environmentally typical levels could exacerbate PAH toxicity to bivalve embryos from 5 times to 100 times (Pelletier et al., 1997; Lyons et al., 2002). In the present study, all exposure solutions were prepared in the dark, and toxicity assays were conducted under artificial lights, hence minimizing photo-induced toxicity. Once again, a combined effect of this additional natural stress can greatly amplify the toxicity of oil exposure in a "real world" situation.

4.1.3 Dissolved oxygen and ammonia

During oil spills, another concern is the creation of potential 'anoxic dead zone" by the microbial degradation of petroleum compounds, since large amount of oxygen is consumed during the degrading process (Atlas, 1995; Camilli et al., 2010; Kostka et al., 2011). This phenomenon is particularly true in very large areas of the GoM where dissolved oxygen can be in short supply thanks to dramatically increased nutrient inputs from agriculture and municipalities throughout the Mississippi River watershed, which empties in the Gulf (Rabalais et al., 2002). The formation of oxygen-deficient dead zones could be dangerous for many marine organisms and impact the food chain (Kessler et al., 2011). As discussed above with salinity, oil-induced alterations on the gills may result in impaired functions involved in gas exchange and lead to mortality events. Throughout the present study however, dissolved oxygen (D.O) and total ammonia remained at safe levels during all exposures (Geffard et al., 2002a; Losso et al., 2007), suggesting that the lethal effects observed were most definitely due to chemical toxicants and not to interactive effects associated with low D.O or high ammonia.

4.1.4 Pathogens

Following exposure to oil/dispersant, the immune-system may be compromised, enabling outbreaks of pathogens that can further affect survival of oysters. Following the *Exxon Valdez* oil spill, disease was suspected as a contributing factor of the slow recovery of Pacific herring population (Carls et al., 1998; 2002). Increased susceptibility to infection from exposure to oil/dispersant could occur indirectly from epithelial damages, a condition frequently observed in exposed spat, causing a loss of the physical barrier to infection (Article 5). PAH and dispersant can also interact with hemocytes, the main component of immune system in oysters (Galloway and Depledge, 2000), and induce an inflammatory response using phagocytosis as a mechanism of detoxification (Article 5) (Cheng et al., 1975). However, prolonged exposure to hydrocarbons or dispersant could result in a depletion of circulating hemocytes and induce further pathological changes such as parasitic infestation or disease. Although oil and PAHs are known to affect the immune system of oysters (Sami et al., 1992; Auffret et al., 2004), immunotoxicity is not generally assessed during oil spills, mainly because of its long-term, sublethal effects, which may prove challenging to be linked with the initial cause.

4.1.5 Other stressors

Under field conditions, oyster populations may be subject to many other stressors such as heavy metals, pesticides and other organic contaminants from anthropogenic origin. These anthropogenic contaminants have been shown to induce severe developmental effects on bivalve larvae (Davis and Hidu, 1969; Calabrese et al., 1973; Brereton et al., 1973; Beiras and His, 1994; Akcha et al., 2012; Mottier et al., 2013), and exposure of organisms to multiple stressors at any given time could result in additive, synergistic or antagonistic responses (Jewell, 1994). Lastly, further exposure to toxic algal blooms (e.g. red tide *Karenia brevis*), present in the Gulf of Mexico and shown to affect early stages of oyster (Rolton et al., 2014), could additionally impact oyster population.

4.2 Other considerations

As opposed to adults, oyster larvae are pelagic and motile and can potentially avoid crude oil patches, mitigating therefore any negative impacts. It is evident that acute lethal effects of oil/dispersant are irreversible: however, more subtle effects on the feeding regime of oyster larvae may only be deleterious for a few days, and larvae could recover rapidly (Article 3;

Ben Kheder et al., 2010b). Nonetheless, when oiling is less severe, hydrocarbon concentrations in the environment may be too low to be detected and avoided. For instance, oyster can enter in contact and adsorb dissolved PAH, or directly ingest droplets or oil/PAH-contaminated particles as demonstrated in Article 4. As a result, toxic PAHs could enter the food chain (biomagnification) and impact the whole ecosystem many months after the incident (Graham et al., 2010; Whitehead et al., 2012). Another long-term consequence of exposure to oil and dispersant comes from the fact that some highly mutagenic PAHs contained in oils could only affect the next generations, as it has been demonstrated with herring populations years after the *Exxon Valdez* spill (Carls et al., 1999; Barron et al., 2003).

Oysters are euryhaline species and are able to withstand the highly variable conditions occurring in estuaries: for this reason, they are resistant to environmental changes and potentially able to rebound after a disaster such as the DWH incident. Their capacity to depurate and metabolize certain PAHs (Stegeman and Teal, 1973; Neff et al., 1976), although not as efficiently as other species such as fish (Neff, 1979; Farrington et al., 1983), proves that oysters are highly adaptable and resilient. Some studies argue that pre-existing acclimation to the presence of hydrocarbons in the Gulf of Mexico *via* natural seeping sources (Table 2, I.3.1) may have encouraged level of resilience (Abbriano et al., 2011; Silliman et al., 2012). We can also expect that oyster recruitment is likely to exhibit compensatory responses such as better growth and lower mortality of survivors to an episodic stress or mortality event from a spill. Therefore, a spill may not necessarily affect adult population and biomass if the exposure is not sustained or widespread (Tunnell, 2011). However, long lasting spill like the DWH event coincided with critical life stages of oysters (spawning, larval development, settlement), with certain coastal areas that were oiled for more than 30 consecutive days (see Fig. 4 in I.2.1). As we clearly demonstrated in this study, oil/dispersant could reduce recruitment of oysters and add a supplementary stress to population already burdened by reduced habitat quality, physiological stresses (e.g. spawning) or other environmental stressors.

We can also assume that most of the toxicological assessment conducted during the present study was based on conservative estimates of toxicity. For instance, the crude oil tested ("Slick A") was collected at the surface on the 29th July, several weeks after the final capping of the *Macondo-1* well (Crone and Tolstoy, 2010), and was as a result substantially weathered at sea. Because weathered oil has lost most of its volatile compounds which are also the most water-soluble components of oil (LMW), its potential for toxic impacts is

generally lessened compared to fresh oil (Leahy and Colwell, 1990). Moreover, all toxicity testing conducted by EPA during the response focused solely on assessing acute, short-term effects on commonly used test species (e.g. mysid shrimp or rotifers), using continuous exposure systems (Hemmer et al., 2011). These tests are considerably less sensitive than tests performed with early life stages of fish, bivalves or crustaceans. Moreover, these assays did not address the effects of long-term or declining exposures to oil/dispersant as it would happen in a dynamic environment where great dilution in the water column would be expected, resulting in rapidly declining exposure concentrations. By using declining exposure (Fig. 16 in III.1) and long-term (e.g. dietary) exposure on early stages of oyster, the present study proposed a more ecologically relevant approach to toxicity testing, using realistic indicators of population health.

Lastly, it is important to note that important underwater plumes were discovered after sub-water application of dispersant during the DWH spill, and a substantial amount of oil remained underwater and never reached the surface (Camilli et al., 2010; JAG, 2010; Wade et al., 2011). Undoubtedly, some of the oil has been oxidized completely or partly by bacteria, but no credible estimates of the rate of biodegradation exist. As oil droplets flocculate with suspended particles or are excreted with fecal pellets, they will settle onto the sediments and potentially affect benthic habitats. This could have further long term implications for many aquatic species when storm or hurricane may stir and reactivate toxic effects of oil/PAH from the sediment, or entrain lingering oil droplets to the shore as it was demonstrated following the tropical storm *Isaac* in August 2010 (Burns et al., 2014).

4.3 Future works and knowledge gaps

In regards to the point discussed above, future studies should focus on the toxicological effects resulting from the interaction of dispersed oil with sediments as well as the effects related to exposures to dissolved and particulate oil, especially when using filter-feeding species. As described during our HEWAF assays, complex routes of exposure can occur when considering the particulate oil as well as the dissolved oil. Chemical analysis of water samples that takes into account dissolved *vs* particulate fractions of oil are part of the NRDA evaluations, and data sets may become available in coming years (Tjeerdema et al., 2013). Other knowledge gaps include photo-induced toxicity of PAHs, toxicity tests that address delayed effects (recovery), dietary exposure of oyster larvae and particularly their potential to

bioaccumulate and metabolize PAHs, maternal transfer and effects on F2 generation, multiple stressors exposures or immunotoxicity tests.

Conclusions

The present study revealed that sub lethal concentrations of surface-collected DWH oil and associated dispersant could affect reproduction, early development and recruitment of the ecologically and commercially important *Crassostrea virginica*. These results suggest that application of the dispersant Corexit 9500A, at the surface or underwater, in response to an oil spill should be considered cautiously and all environmental tradeoffs should be evaluated. For instance, results gathered in the present work clearly indicated that the use of dispersant to dissipate oil slicks should be banned during the spawning season of oysters. The present study also demonstrated the significance of an integrative approach in ecotoxicology, in order to better assess toxic impacts of oil and dispersant, and predict long-term effects on populations. Field monitoring programs looking at post-spill oyster recruitment in regions affected by the DWH spill (http://www.gulfspillrestoration.noaa.gov/oil-spill/gulf-spill-data/) should certainly help to evaluate some of the impacts on the resource. However, because some responses are underlain by complex mechanisms, some sublethal effects can be difficult to detect and may take a generation or more to emerge. Thus, future research should focus on long-term effects of oil and dispersant using representative species, sensitive endpoints, and experimental conditions that mimic the natural environment.

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List of Abbreviations

ANOVA: Analysis of variance

Ant: Anthracene

ATP: Adenosine Triphosphate

BaP: Benzo(a)Pyrene

BeP: Benzo(e)Pyrene

BP: British Petroleum

CEWAF: Chemically Enhanced Water Accommodated Fraction

CR: Clearance Rate

CT: Connective Tissue

Chry: Chrysene

DNA: Deoxyribonucleic Acid

DBT: Dibenzothiophene

DGT: Digestive Tubule

DO: Dissolved Oxygen

DOSS: Dioctyl Sodium Sulfosuccinate

DW: Dry Weight

DWH: Deepwater Horizon

ECx: Effective Concentration causing x%

EDTA: Ethylenediaminetetraacetic acid

EPA: Environment Protection Agency

ESD: Equivalent Spherical Diameter

FCM: Flow-Cytometry

FDA: Food and Drugs Administration

FGCU: Florida Gulf Coast University

Fluo: Fluoranthene

FSC: Forward Side Scatter

FSW: Filtered Seawater

GoM: Gulf of Mexico

GC/MS-SIM: Gas Chromatography and Mass Spectrometry using Selected Ion Monitoring

HEWAF: High Energy Water Accommodated Fraction

HMW: High Molecular Weight

Kow: Octanol-Water Partition Coefficient

LC/MS-MS: Liquid Chromatography with coupled Mass Spectrometry

LCx: Concentration causing x% mortality

LEWAF: Low Energy Water Accommodated Fraction

LMW: Low Molecular Weight

LOEC: Lowest Observed Effective Concentration

MANOVA: Multiple Analyses of Variance

MDA: Malondialdehyde

MMP: Mitochondrial Membrane Potential

NA: Not Applicable

Naph: Naphtalene

NC: Not Calculable

NMFS: National Marine Fisheries Service

NOAA: National Oceanic and Atmospheric Administration

NOEC: No Observed Effective Concentration

NRDA: Natural Resource Damage Assessment

NBT: Naphtobenzothiophene

OSAT: On Scene Advisory Team

PAH: Polycyclic Aromatic Hydrocarbons

PF: Post Fertilization

Phe: Phenanthrene

Pyr: Pyrene

PPM: Part Per Million

PPB: Part Per Billion

ROS: Reactive Oxygen Species

S‰: Salinity

SD: Standard Deviation

SE: Standard Error

SSC: Side Scatter

T˚C: Temperature

TPH: Total Petroleum Hydrocarbons

USCG: United States Coast Guard

UV: Ultraviolet

WAF: Water Accommodated Fraction

APPENDICES

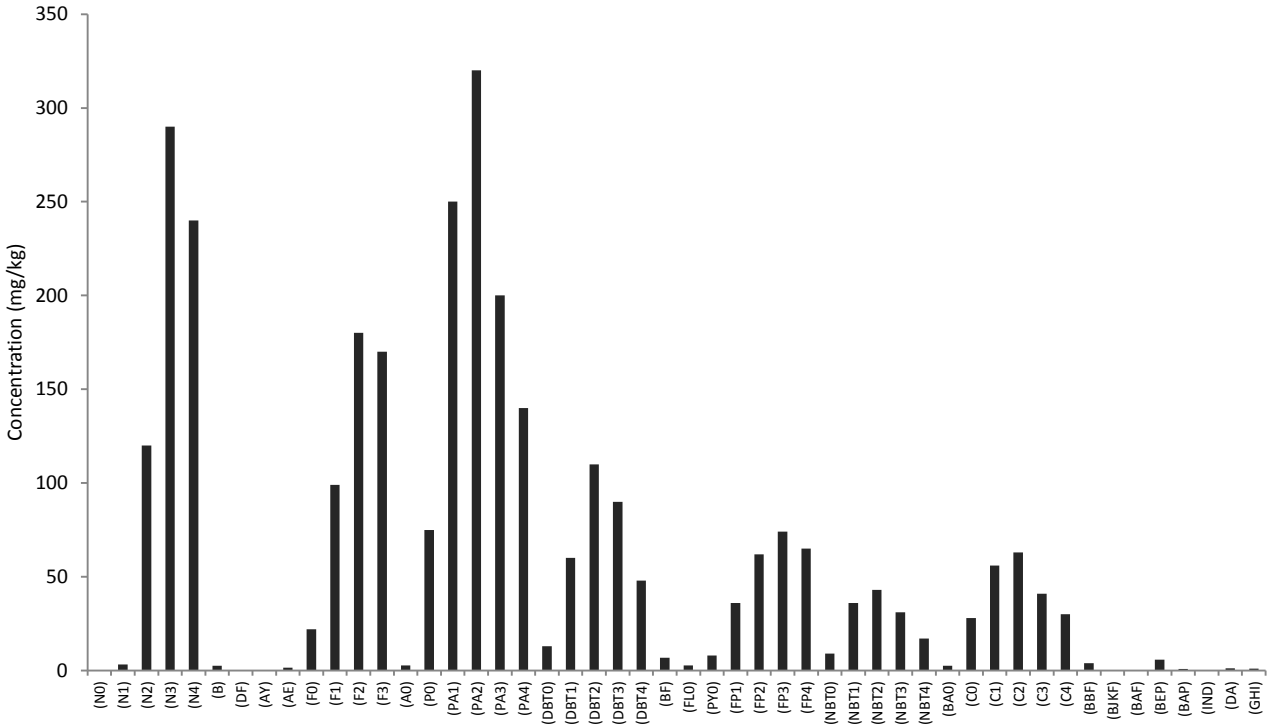
Appendix 1

PAH contents of (1) crude oil "Slick A" (sample CTC02404-02), (2) HEWAF, (3) CEWAF and (4) LEWAF, expressed in mg PAH/kg (Slick A) or µg PAHs/L. Sum of 50 PAHs quantified by GC/MS-SIM. Content of tPAHs (n=50) in crude oil corresponds to 0.31 % of the mass.

PAH profiles of (5) crude oil "Slick A", (6) HEWAF, (7) CEWAF and (8) LEWAF, expressed as percent composition (%) of total PAH (n=50), quantified using GC/MS-SIM.

N0-4: Napthalene; B: Biphenyl; AY: Acenaphtylene; AE: Acenaphtene; F0-3: Fluorene; A0: Anthracene; PA0-4: Phenanthrene; DBT0-4: Dibenzothiophene; BF:Benzo(b)fluorine; FLO: Fluoranthene; PY0: Pyrene; FP1-4: Fluoranthene/Pyrene; NBT0-4: Naphtobenzothiophene; BAO: Benz(a)anthracene; C0-4: Chrysene; BBF: Benzo(b)fluoranthene; BJKF: Benzo(j+k)fluoranthene; BAF: Benzo(a)fluoranthene; BEP: Benzo(e)pyrene; BAP: Benzo(a)pyrene; IND: Indeno(1,2,3)pyrene; DA: Dibenz(a,h)anthracene; GHI: Benzo(g,h,i)perylene. Parent compound is indicated by a 0 (e.g. N0), while numbers of additional carbons for alkylated homologs are indicated as N1, N2, etc.

1. Slick A



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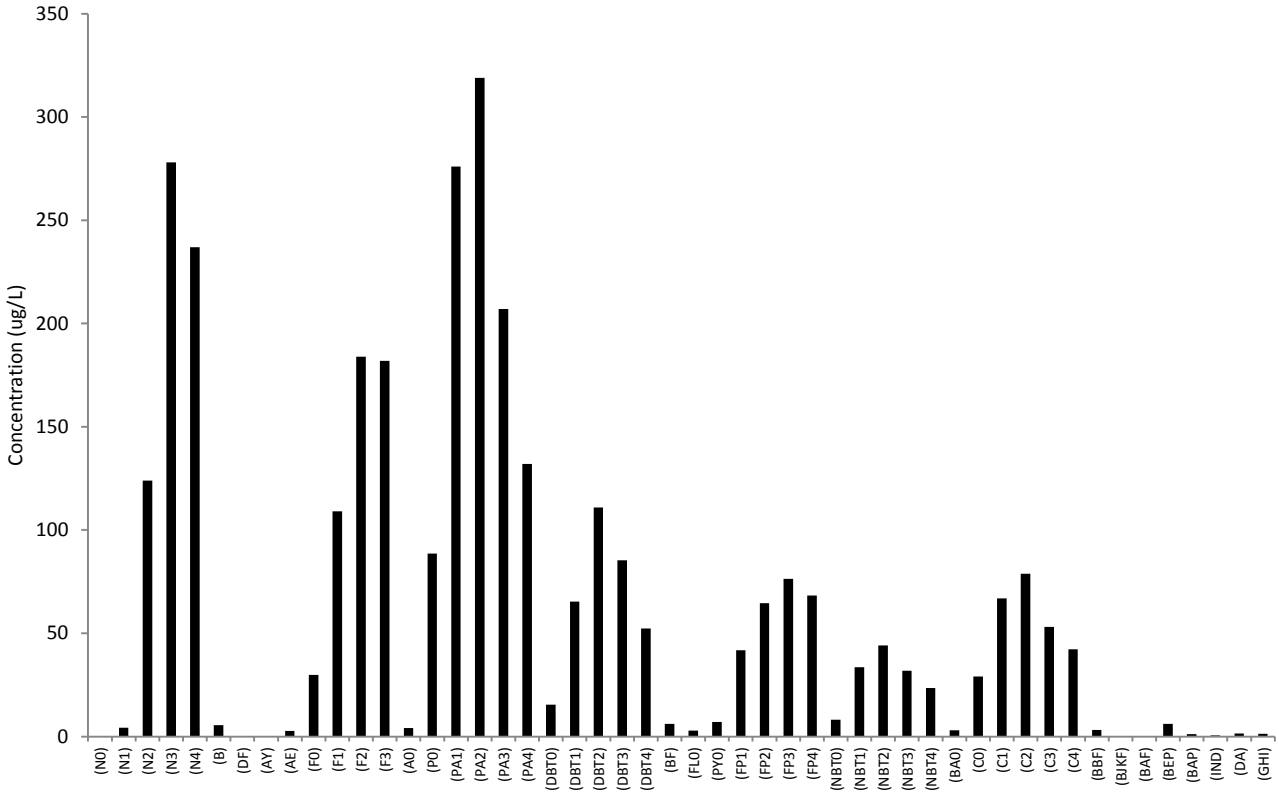

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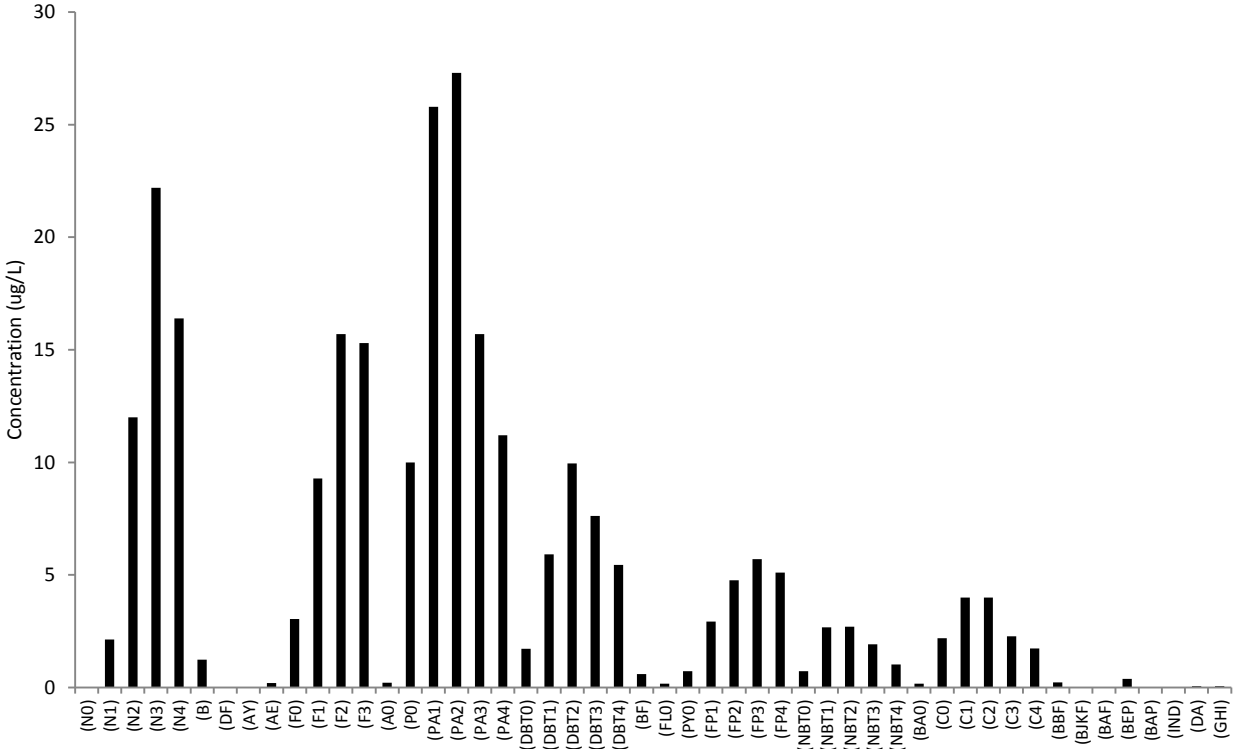

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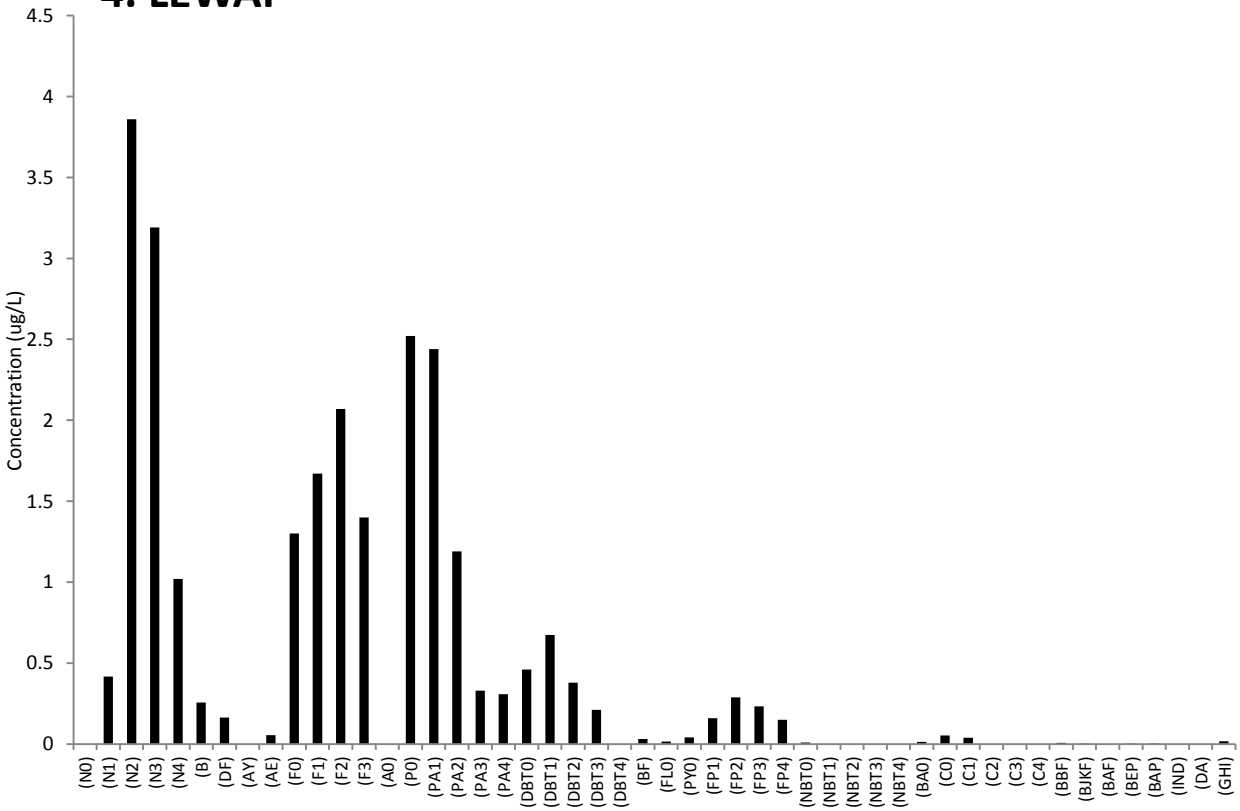

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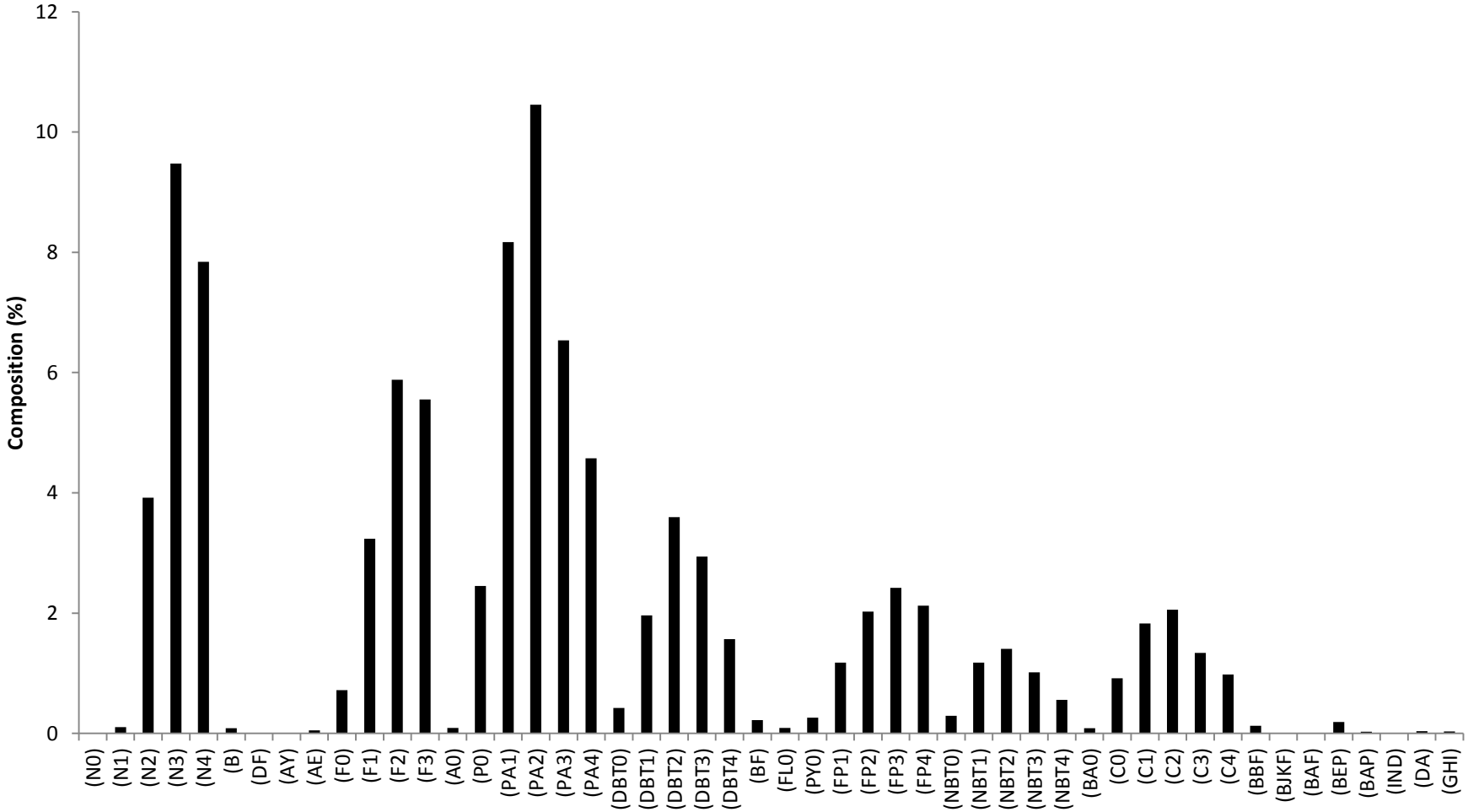
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(AE)
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(A0)
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(PA1)
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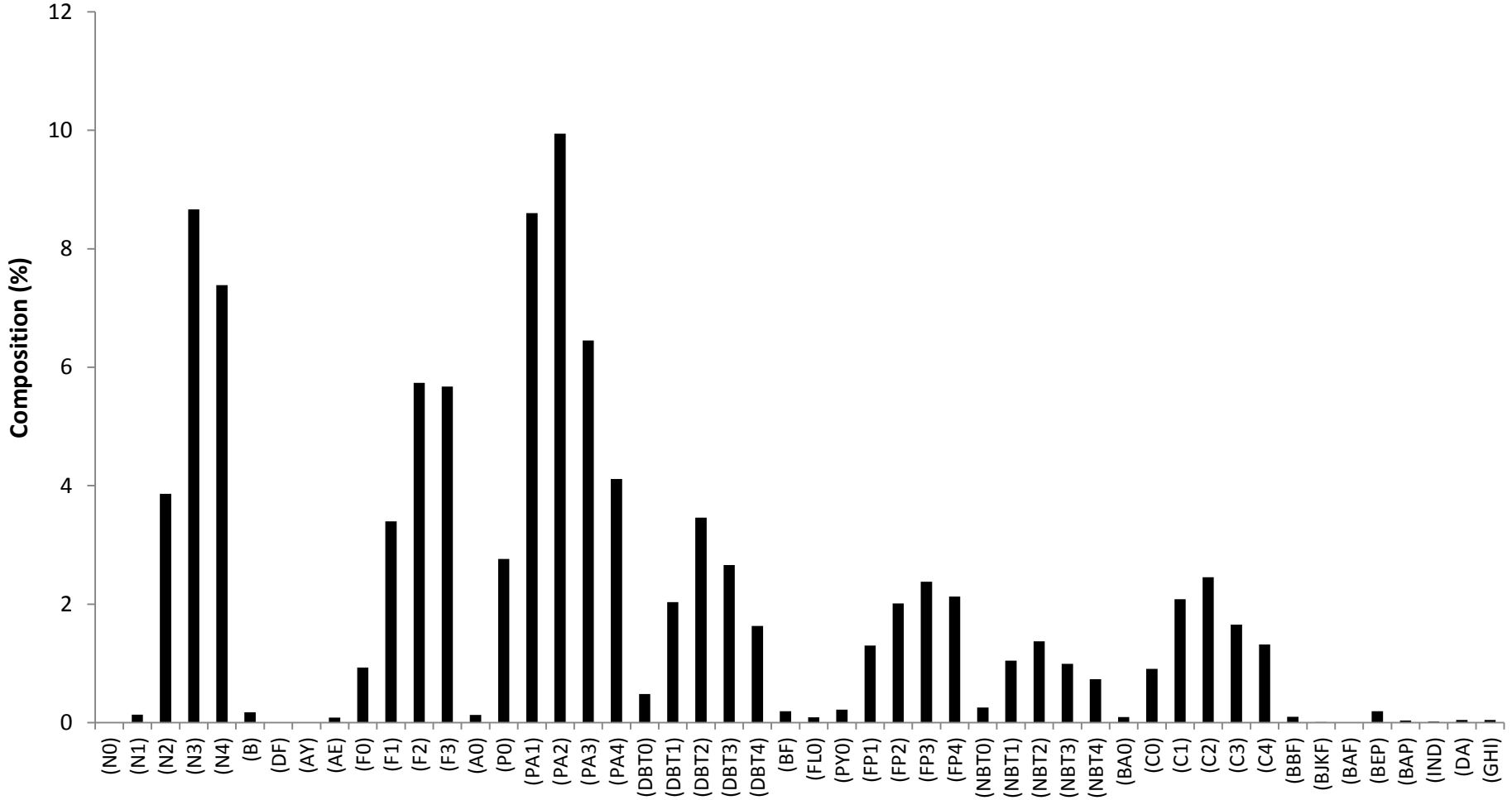
6. HEWAF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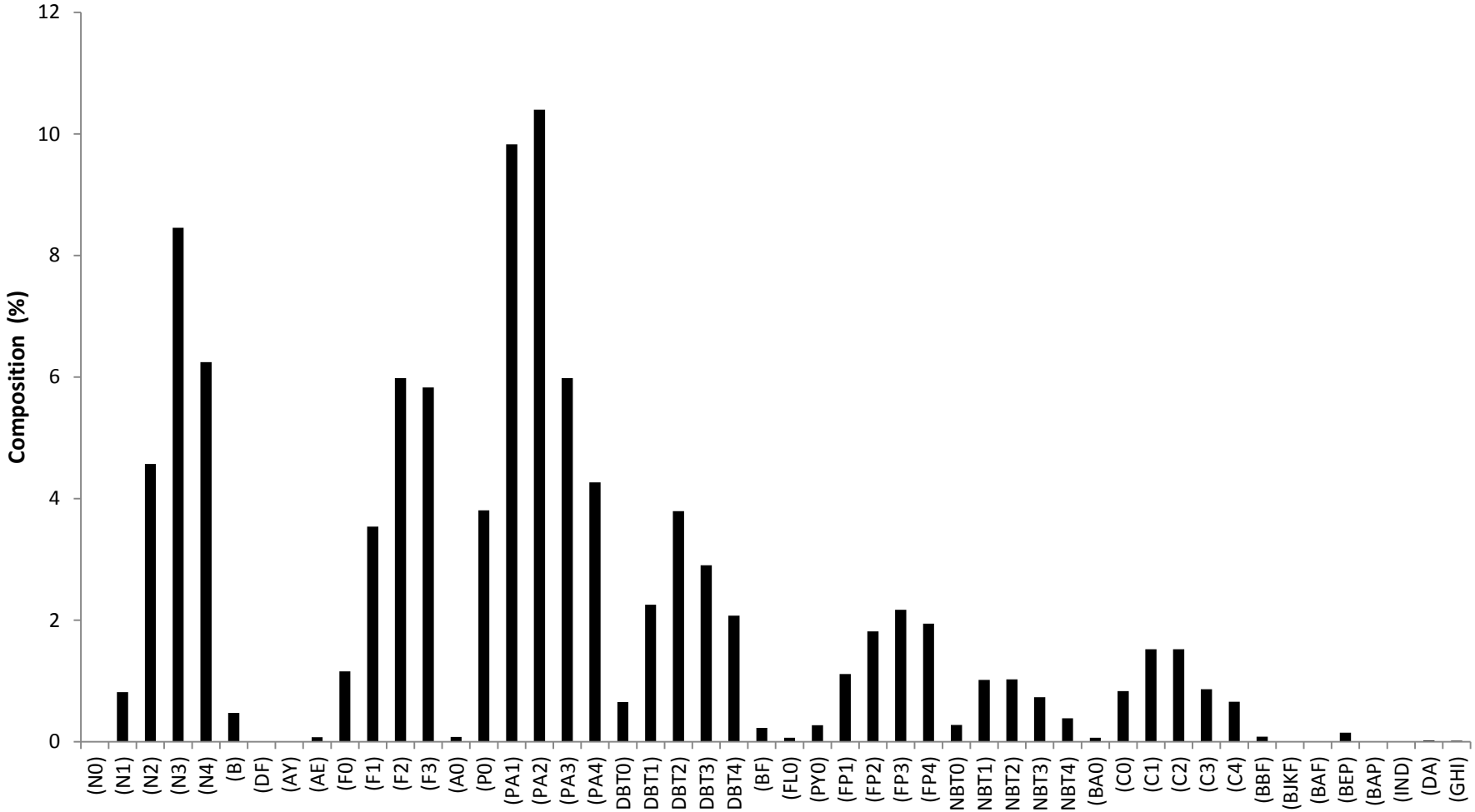
7. CEWAF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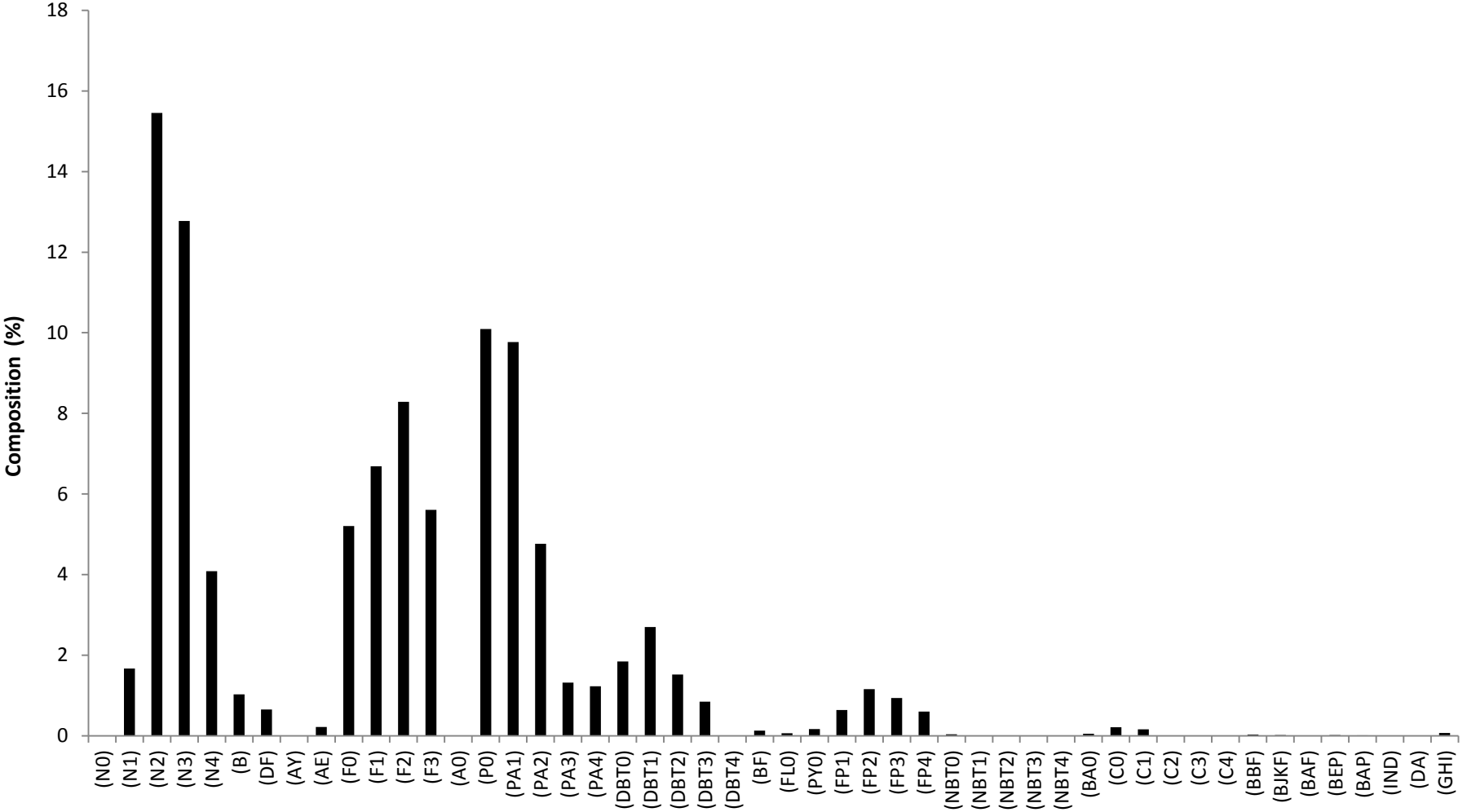
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Appendix 2

Peer reviewed articles

- **Vignier, J**., Donaghy, L., Denkert, B., Devine, J., Soudant, P., Chu, F.L.E, Morris, J.M., Carney, M.W., Lipton, J., Cacela, D., Lay, C., Krasnec, M., Robert, R., & Volety, A.K. Impacts of Deepwater Horizon oil and dispersant on early development of the American oyster, *Crassostrea virginica*. **In prep**.

- **Vignier, J.,** Le Goic, N., Soudant, P., Rolton, A., Robert, R., & Volety, A.K. Sensitivity of *Crassostrea virginica* gametes to Deepwater Horizon dispersed oil: cellular responses of spermatozoa and oocytes and impacts on fertilization and embryogenesis. **In prep**.

- **Vignier, J**., McEachern, K., Soudant, P., Chu, F.L.E, Morris, J.M., Carney, Lay, C., Krasnec, M., Robert, R., & Volety, A.K. Lethal and sublethal effects of Deepwater Horizon oil and dispersant on *Crassostrea virginica* larvae. **In prep**.

- **Vignier, J.,** Ketover, R., Rolton, A., Devine, J., Soudant, P., Chu, F.L.E., Morris, J, Carney, M., Lay, C., Krasnec, M., Robert, R., & Volety, A. K. Interactions between *Crassostrea virginica* larvae and DWH oil: toxic effects *via* dietary pathways. **In prep**.

- **Vignier, J.**, Rolton, A., Campbell, I., Nickols, E., Soudant, P., Chu, F.L.E., Morris, J., Carney, M., Lay, C., Krasnec, M., Lipton, J., Robert, R., & Volety, A.K. Impacts of Deepwater Horizon oil and associated dispersant on spat of *Crassostrea virginica*. **In prep**.

- Rolton, A.; **Vignier, J**; Soudant, P; Shumway, S; Bricelj, M; Volety, A (2014).Effects of the red tide dinoflagellate, *Karenia brevis*, on early development of the eastern oyster *Crassostrea virginica* and Northern Quahog *Mercenaria mercenaria*. *Aquatic Toxicology*, 155: 199-206.

- Carboni, S; **Vignier, J**; Chiantore, M; Tocher, D.R and Migaud, H (2012). Effects of dietary microalgae on growth, survival and fatty acid composition of sea urchin *Paracentrotus lividus* throughout larval development. *Aquaculture* 324-325 p250-258

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Résumé

L'explosion du forage et de la plate-forme pétrolière *Deepwater Horizon* (DWH) le 20 Avril 2010 a entrainé la plus importante catastrophe pétrolière de l'histoire des Etats-Unis, avec des quantités sans précédent de pétrole (779,000 T) et de gaz relâchées continuellement dans le Golfe du Mexique durant près de 3 mois. En retour et comme moyen de réponse, 8 million L de dispersant chimique (principalement Corexit 9500A®) furent utilisés pour disperser les nappes, dont 2.9 million L furent injectés directement à la tête du puit à 1500 m de profondeur. La marée noire coïncida avec la saison de ponte et de recrutement de l'huitre américaine *Crassostrea virginica*, une espèce à haute valeur écologique et commerciale dans le Golfe. En raison de ces caractéristiques biologiques (sédentaire, espèce filtreuse, répartition géographique, ponte et fécondation externe), les huitres ont été utilisées comme organisme modèle en écotoxicologie. Néanmoins, il existe très peu de données disponibles sur la toxicité du pétrole brute (HEWAF), du pétrole dispersé (CEWAF) ou du dispersant sur les jeunes stades de vie de *C. virginica*.

L'objectif de ce travail de thèse fut 1) de déterminer les effets létaux et sublétaux d'expositions aigues et chroniques à du pétrole DWH et/ou du Corexit 9500A® sur différents stades de développement des jeunes huitres, 2) d'examiner les mécanismes de toxicité des HAP (dissouts ou particulaires) issus du pétrole et du dispersant sur des processus physiologiques sensibles, et 3) d'établir si les résultats obtenus en laboratoire correspondent aux valeurs recueillies sur le terrain lors du programme NRDA.

Nos résultats ont démontré que le pétrole et/ou le dispersant pouvaient affecter la reproduction et le développement embryonnaire et larvaire de *C. virginica*, et que le pétrole dispersé et le dispersant induisaient en général le plus d'impact. En outre, des effets sublétaux tels que des inhibitions de croissance larvaire, de fixation ou de filtration furent observés à des niveaux d'HAP et de DOSS mesurés dans l'environnement. Ces résultats suggèrent que le pétrole et l'utilisation de dispersant, en particulier lors de la saison de ponte de l'huître, pourraient affecter son recrutement et impacter la ressource de façon délétère dans des régions touchées par une marée noire. Par ailleurs, des critères biologiques plus sensibles que la mortalité devraient être choisis afin d'estimer plus précisément l'impact environnemental des hydrocarbures et le devenir de ses constituants.

Mots-clés : pétrole Deepwater Horizon, dispersant, sublétaux, CEWAF, huitre, larves, toxicité.