Ecotoxicity effects of oil Water Accommodated Fractions and oil Water Accommodated Fractions + Dispersant on cold environments: *Acarita tonsa* based bioassays and microbial community dynamics as monitoring tools

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General Abstract

Oil pollution is an emerging problem in the world seas, especially in cold environments where remediation procedures are difficult. After an oil spill, wave dynamics and other weathering factors introduce soluble oil hydrocarbons, especially polycyclic aromatic hydrocarbons (PAHs) in the water column, creating Water Accommodated Fractions (WAF). As a form of remediation, dispersant are added to assist in biodegradation. However, studies have shown that dispersant have adverse effect on marine organisms. The objective of this thesis is to estimate the effects of the weathered oils (WAF) in cold environment, focusing on marine fauna using in vivo bioassays and microbial communities. In addition, it is important to evaluate the toxicological effects of added chemical dispersant to WAF and its efficiency in hydrocarbon degradation. The in vivo toxicological assays, conducted using the model organism *Acartia tonsa*, showed that WAF prepared from distilled oils (IFO 180 and Marine diesel) exhibited high toxicity toward the copepod considering the lethality, reproduction and molecular endpoints such as gene transcription patterns. This suggests that the chemical composition of WAF from the distilled oils contributed to toxicity higher than that of WAF from the crude oil. However, the addition of dispersant to crude oil WAF also increased the toxicity toward *Acartia tonsa*. In addition, *Acartia tonsa* exposed to WAF with dispersant has also shown low fecundity, high mortality and affected gene transcription patterns, further highlighting the toxicity effects of dispersants. Upon examining the effects of WAF and WAF with dispersant on microbial communities using microcosm setups, dispersant added to WAF did not improve biodegradation rates. However, WAF with dispersant contributed to changes in the microbial community dynamics in both water and in sediment by selecting certain taxa. Results have shown that microbial communities with initial hydrocarbon degrading bacteria due to history of chronic hydrocarbon exposure

(*legacy effects*) were the main driver of the degradation rate of PAHs in water and in sediment. In conclusion, integrating both in vivo toxicity assays and studying microbial communities dynamics has paved the way to understand the fate of oil spilled in the cold environment after weathering and whether the traditional use of dispersant mitigated the oil spill effects.

La pollution par les hydrocarbures est un problème émergent dans les mers du monde, en particulier dans les environnements froids où les procédures d'assainissement sont difficiles. Après un déversement de pétrole, la dynamique des vagues et d'autres facteurs d'altération introduisent la solubilisation des hydrocarbures, en particulier des hydrocarbures aromatiques polycycliques (HAP) dans la colonne d'eau, créant des fractions d'hydrocarbures solubilisés dans l'eau (WAF). Après une marée noire, des stratégies de lutte impliquent l'addition de dispersant afin de favoriser la biodégradation. Cependant, des études ont montré que les dispersants ont des effets néfastes sur les organismes marins. L'objectif de cette thèse est d'étudier les effets des hydrocarbures altérés par le temps (WAF) dans les environnements froid, en focalisant sur la faune marine à l'aide de bio-essais in vivo et sur les communautés microbiennes. De plus, il est important d'étudier les effets toxicologiques de l'ajout de dispersant chimique au WAF et son efficacité dans la dégradation des hydrocarbures. Les essais toxicologiques *in vivo*, réalisés en utilisant *Acartia tonsa* comme organisme modèle, ont montré que les WAF préparées à partir de pétroles distillés (IFO 180 et Marine diesel) présentaient une toxicité élevée envers le copépode considérant les paramètres de létalité, de reproduction et indicateurs moléculaires Tels que les modèles de transcription des gènes. Ces résultats suggèrent que la composition chimique des WAF obtenues à partir de pétroles distillés ont contribué à une toxicité supérieure à celle des WAF obtenues à partir de pétrole brut.

Cependant, l'ajout de dispersant dans les WAF obtenues à partir de pétrole brut a augmenté la toxicité envers *Acartia tonsa*. En outre, *Acartia tonsa* exposé au WAF contenant un dispersant a également montré une faible fécondité, une mortalité élevée et des profils d'expression génique affectés, soulignant davantage les effets toxique des dispersants. Après avoir examiné les effets du WAF et du WAF avec dispersant sur les communautés microbiennes dans des expérimentations en microcosmes, le dispersant ajouté au WAF n'a pas amélioré les taux de biodégradation. Cependant, le WAF avec dispersant a contribué à des changements dans la structure des communautés microbiennes et leur dynamique, à la fois dans l'eau et dans les sédiments, résultant en la sélection de certains taxons. Les résultats ont montré que les communautés microbiennes possédant des microorganismes dégradant les hydrocarbures, en raison d'une exposition chronique aux hydrocarbures (*effet héritage*), étaient responsables des taux de dégradation élevés des HAP dans l'eau et dans les sédiments. En conclusion, l'intégration à la fois des essais de toxicité *in vivo* et de l'étude de la dynamique des communautés microbiennes a ouvert la voie pour comprendre le devenir du pétrole, déversé dans l'environnement froid, après leur altération. Nous avons également évalué si l'utilisation traditionnelle de dispersant atténuait les effets du déversement de pétrole.

La contaminación por hidrocarburos es un problema emergente en los mares del mundo especialmente en ambientes fríos, donde los procesos de remediación son más complejos. Cuando el petróleo es derramado en el mar diversos factores meteorológicos y el movimiento de las olas favorecen la integración de los hidrocarburos hidrófilos procedentes del petróleo en la columna de agua, al producto resultante se le conoce como fracción acomodada del petróleo en el agua (WAF) y suele estar principalmente compuesta por hidrocarburos aromáticos policíclicos (HAPs). Históricamente como

forma de remediación ante los vertidos de petróleo se ha utilizado la incorporación de dispersantes químicos que favorecen la degradación del petróleo. Sin embargo, diversos estudios han demostrado que los dispersantes pueden tener efectos adversos sobre los organismos marinos. El objetivo de esta tesis es estimar el posible impacto de los aceites envejecidos (WAF) en los organismos marinos procedentes de un ambiente frio mediante bioensayos *in vivo* y el estudio de comunidades microbianas. Además, se evalua el posible impacto toxicológico derivado de añadir un dispersante químico al WAF y su efectividad a la hora de favorecer la degradación de hidrocarburos. Los ensayos toxicológicos realizados *in vivo* con el organismo modelo *Acartia tonsa* demostraron que el WAF obtenido a partir de aceites destilados (IFO 180 y *Marine diesel*) tiene una alta toxicidad hacia el copépodo a diferentes niveles de organización biológica (transcripción genética, reproducción y mortalidad). Siendo la composición química del WAF procedente de aceites destilados lo que contribuyó a una mayor toxicidad en comparación al WAF procedente de un petróleo crudo. Al añadir dispersante al petróleo crudo el WAF obtenido tuvo una mayor toxicidad hacia *Acartia tonsa* en términos de alteración de la transcripción genética, reproducción y mortalidad. En el caso de las comunidades microbianas, al examinar los efectos del WAF en solitario y WAF con dispersante utilizando microcosmos se pudo observar que el dispersante no favoreció la biodegradación de los compuestos procedentes del WAF. Sin embargo, el WAF con dispersante sí alteró las dinámicas de la comunidad microbiana, favoreciendo ciertos taxones tanto en el agua como en el sedimento. Observando que las comunidades microbianas que poseían inicialmente bacterias degradantes de hidrocarburos, debido a un historial de exposición crónico a estos compuestos (*legacy effects)*, fueron el principal impulsor de la degradación de los HAPs en el agua y en los sedimentos. En conclusión, la integración de los ensayos de toxicidad *in vivo* y el estudio de la dinámica de las comunidades

microbianas ha facilitado la comprensión del posible impacto de los vertidos de petróleo envejecido y si el uso de los dispersantes tradicionales reduce o no su posible impacto en entornos marinos de clima frio.

General Introduction

1. The Arctic and Subarctic marine ecosystem

The Arctic and subarctic environment are regions that lie beyond 50^{o} North (Figure 1). These environments are characterized seasonally by periods of midnight sun and the warmest months do not exceed 10^{o}C (Hoberg et al., 2012). The Arctic and subarctic ecosystems are unique in the extreme conditions it presents for living organisms. Although its surface is mostly covered with ice sheets throughout the year, subarctic and Arctic environments are teeming with life. This ecosystem consists of two thousand algal species, tens of thousands of microbes and approximately five thousand species of animals (Meltofte et al., 2013). Like any other ecosystem, this ecosystem consists of complex food webs with interactions between microbes, phytoplankton, zooplankton, macro-invertebrates, fish, seabirds and mammals. The primary production of phytoplankton and algae in the Arctic Sea usually peaks during the spring season, when sunlight penetrates the water column. This plant life provides energy for primary consumers such as zooplankton communities, which are food sources for several species of fish and marine mammals (Soreide et al., 2010).

In the Arctic and subarctic environment, zooplankton communities are dominated by the *Calanus* genus (Ashjian et al., 2003). Examples of native *Calanus* species in the Arctic are *Calanus glacialis* and *Calanus hyperboreus* (Daase, 2013). Examples of newly introduced Arctic *Calanus* species are *Calanus finmarchicus* and *Calanus marshallae* (Scott, 2000). Members of the *Calanus* genus have larger body masses than other groups of copepods. Arctic copepods have the ability to store huge lipid reserves, mostly as wax esters, which consist of up to 60% of their total body weight at the end of the feeding season (Falk Peterson et al., 2009). The wax esters in *Calanus* contain omega-3 polyunsaturated fatty acids, which are obtained from dinoflagellates. These fatty acids are important components of the lipid membranes in all animals (Falk Peterson et al.,

2009, Soreide, 2010). Arctic copepods are a vital food source for many different organisms such as the Arctic cod *Boreogadus saida*, the Pacific herring *Clupea pallasii*, the bowhead whale *Balaena mysticetus*, different species of seabirds, the amphipods *Themisto libellula* and gelatinous zooplankton (Ctenophores) (Darnis et al., 2012).

Threats to the Arctic and subarctic marine environment

Over the last two decades, the global average temperature has increased by 0.6°C in the Arctic (Bockris, 2010), provoking seasonal retreats of ice sheets in the western Arctic (Stroeve et al., 2007). Models have predicted that by 2050, the Arctic will be ice-free in the summer (Arzel et al., 2006). A warmer seawater temperature in the Arctic as well as the shrinking coverage of ice sheets has significantly affected the whole Arctic ecosystem and its biota (Karcher, 2013). During the spring, phytoplankton blooms in the Arctic sea, boosting the primary productivity in the region. However, a second phytoplankton bloom has started to appear in the fall, which is a common feature in warmer oceans (Ardyna, 2014). This extra bloom disturbs the carbon cycle in the ocean and the food web. These conditions are shifting Arctic ecosystems to become more like temperate ecosystems. As a result, temperate species shift more northward toward the Arctic and subarctic environments. Recently, the presence of the Pacific native diatom *Neodenticula seminae* has been reported in the Nordic sea (Miettinen et al., 2013). In the North Sea, species such as the Atlantic cod *Gadus morhua*, the common sole *Solea solea* and the scaldfish *Arnoglossus laterna* have shifted their habitats northward to higher latitudes (Perry et al.; 2005).

In addition, the Arctic environment is vulnerable to acidification that is caused by the high solubility of atmospheric carbon dioxide in cold waters (Steinacher et al., 2009). This acidification promotes changes in calcium deposits, negatively affecting Arctic

invertebrates such as zooplankton pteropods (Orr et al., 2005), sea urchins (Fabry et al., 2008) and cold-water corals (Langdon et al., 2005).

Oil pollution in the Arctic or subarctic environment

With the increasing demand for fossil oils, oil extraction and transport in the oceans have significantly increased during the last decade. Consequently, the risks of oil spill incidents are escalating rapidly. Between 2010 and 2018, 59 major oil spills occurred, resulting in the release of approximately 163,000 tons of oil into the marine environment (ITOPF 2019). Currently, it has been estimated that the Arctic and subarctic seas contain up to 13% of the undiscovered oils in the world (Camus et al 2017).

From 1996 to 1999, more than 409 oil spill accidents have occurred in Alaska (Poland et al 2003). The biggest oil spill incident in history was during the 1991 Gulf war, where 6 million barrels were deliberately spilled in the Gulf of Kuwait (Saadoun 2015). Another major oil spill incident was the explosion of the deep water horizon oil rig in 2010, which released at least 4 million barrels in the gulf of Mexico (Redmond et al 2011). The best-known example of an oil spill in cold environments was the *Exxon Valdez* oil spill near Alaska, which occurred in 1989. A volume of 41.6 million L of North Slope heavy oil was spilled near island shorelines (Atlas, 2011). Storms that occurred after the spill incident greatly dispersed the oil to cover 15% of the shoreline along Prince William Sound and the Gulf of Alaska shoreline (Short et al., 2003). This incident had a great impact on the fish and invertebrate species in the area. For example, there were greater abnormality rates among Pacific herring, *Clupea pallasi*, larvae that hatched in oiled areas compared with non-impacted areas (Fakseness et al 2020). The amphipod populations impacted by this oil spill did not recover in terms of population size until 1995 (Falk Peterson, 2009).

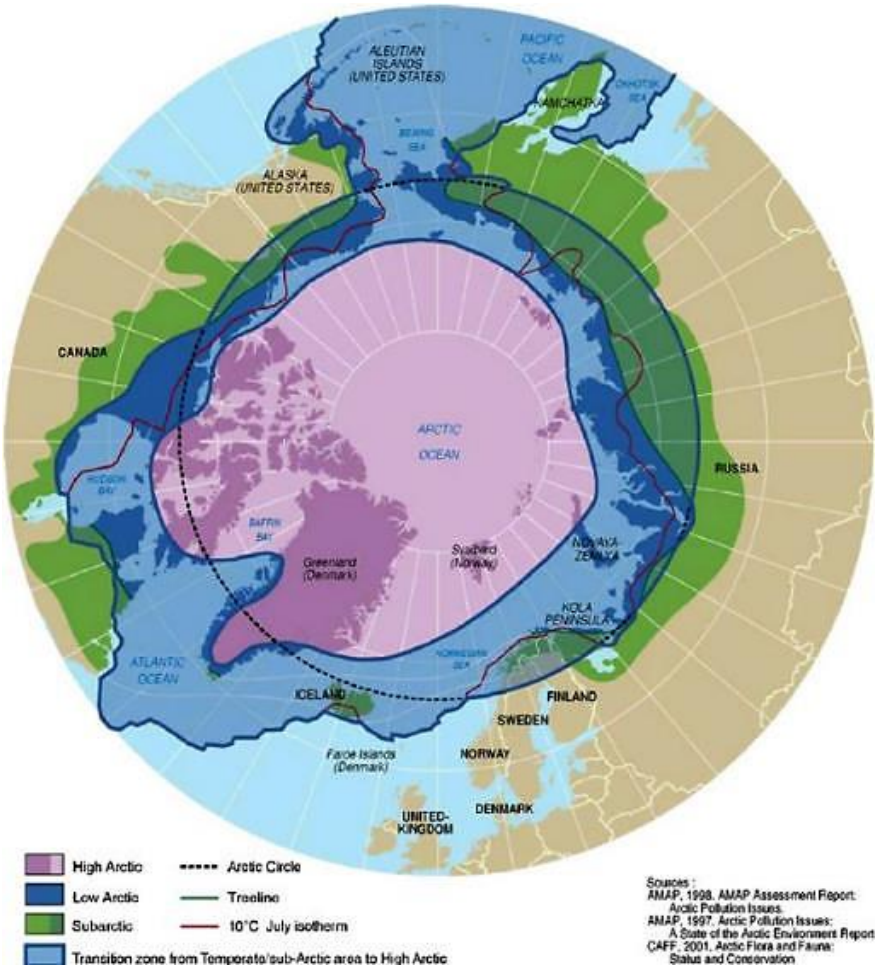

Figure (1) The borders of Arctic and subarctic environments (Hoberg et al 2012)

Microbial ecology of oil degradation in Arctic and subarctic ecosystems

In cold environments, bioavailability of hydrocarbon decreases due to the solidification of the substrates (Giudice et al 2015). Little is known about ecological succession of microbial communities at low temperature after the introduction of pollutants such as oil. However, using next generation sequencing, thoroughly studying aftermath of deep water Horizon helped in understanding how oil contamination can result in shifts in the resident microbial communities (King et al 2014). Specialization disturbance hypothesis can explain microbial succession patterns shortly after oil spill, where after disturbances,

generalists are favored while specialists are selected against (Robbins et al 2021). Once oil spill occurred, early responders are often consisted of generalist communities, which are rapidly stimulated due to their tolerance to severe disturbances (Rodrigues et al 2015). However, during the depletion of resources, resident microbial communities are shifted toward more specialist's communities, which are adapted to carbon limited conditions or are capable to utilize alternative energy sources.

However, several bacterial taxa has capabilities for hydrocarbon biodegradation at low temperature. For example, *Gamma protobacteria*, especially from the genera of *Colwellia* and *Oleispera* showed significant increase in abundance after the enrichment of crude oil and ammonium phosphate to Antarctic seawater under pack of ice (Yakimov et al 2004). Genera of the psychrotolerant *Rhodococcus* isolates showed ability to degrade alkanes and PCB at temperatures ranging from 4 to 35 º C (De Domenico et al 2004). In addition, *Rhodococcus* bacteria have shown to be able to produce biosurfactants (Yakimov et al 1999). In fact, biosurfactants play important roles in bacterial biodegradation capabilities by allowing bacteria to interact with the droplets of hydrocarbons (Giudice et al 2015). Biosurfactants are considered as a valuable alternative to commercial designed dispersants in the solubilization and dispersion of the hydrocarbons, because biosurfactants are easily biodegradable and do not accumulate in the environment (Desai Banat, 1997).

Microbial biodegradation rates in Arctic and subarctic environments

Cold adapted bacteria, which are known to thrive in temperatures as low as 0º C, are widely distributed at temperatures below 5º C (Margesin and schinner 1999). However, temperature is a major contributor to the biodegradation rates of hydrocarbons. Atlas et al (1998) demonstrated that biodegradation rates were several folds faster in 25º C than

in 5º C. Therefore, in Arctic and subarctic environments, biodegradation rates in winter are significantly slower than in summer season (Siron et al 1995). However, at subzero temperatures, slow biodegradation still take place until -6º C (Rike et al., 2005). At such low temperatures, microbial communities need to have specific adaptations for survival and metabolism. For example, when temperature falls, cytoplasmic membrane fluidity decreases and membrane associated metabolic process are slower (Rike et al., 2005). Consequently, cold adapted microorganisms (*Psychrophili*c) have modifications of membrane lipids in which increase the fluidity of the cytoplasmic membrane (Gounot and Russel, 1999). Cryo protective compounds, such as trehalose, can be used as source of energy to maintain viability at freezing temperatures (Kandror et al 2002).

Aside from temperature, other factors also contribute to the biodegradation rates of oil in the environment. For example, availability of nutrients plays an important role in the microbial biodegradation rates of hydrocarbons in the environment. Nutrients, such as phosphorus and nitrogen, are crucial elements needed for the oil degraders (Xu et al 2013). More than 150 g of nitrogen and 30 g of phosphorus are consumed to convert 1 Kg of hydrocarbons in bacterial cells (Ron and Rosenberg 2014). The initial bacterial communities with hydrocarbon degradation capabilities highlights the biodegradation rates of the communities. In an unpolluted site, hydrocarbon-degrading communities comprise 0.1% of the total microbial communities, while sites with history of oil pollution have up to 100% of hydrocarbon degraders (Atlas, 1981).

2. Calanoids, the most abundant order of copepods

The order Calanoida has 40 families of both freshwater and saltwater copepods. Calanoids are the most abundant copepods in the planktonic communities, making from 55% to 95 % of the total planktonic populations in a given environment (Mauchline, 1998).

Calanoids main distinguishable feature are the two tall antennae protruding from the opposite sides of the copepod cephalosome. Such antennae are usually as long as the whole body. In addition, Calanoids have five pairs of legs used for swimming. Calanoids are considered food for many important commercial fish, making them of commercial importance for culturing. Most calanoids are considered to be herbivores feeding on small phytoplankton (Naganuma, 1996). Calanoid life cycle has four major stages: egg stage, nauplius stage, copepodite stage and adult stage (Figure 2). Generally, calanoids have six naupliar stages. In the first naupliar stage, the newly hatched nauplius are around 0.1 mm long, they develop maxillopodian eyes, which are simple centered eyes with several photoreceptors. Naupliar stages undergo six stages of molts until the copepodite stages. There are five copepodite stages, in which the organisms lose the naupliar maxillopodian eye and develop until it closely resembles the adult copepod shape. Finally, copepods will reach the final adult stage and become sexually mature, capable to reproduce (Trujillo Ortiz, 1986). Calanoid copepods have both of their sexes reproductively active throughout the year. During mating, a male will grasp the females by their antennae and males will deposit spermatophores onto female urosomes to fertilize the eggs. Upon fertilization, eggs are released from the urosomes. Additionally, males can mate consequently with several females (Halsband, 2001).

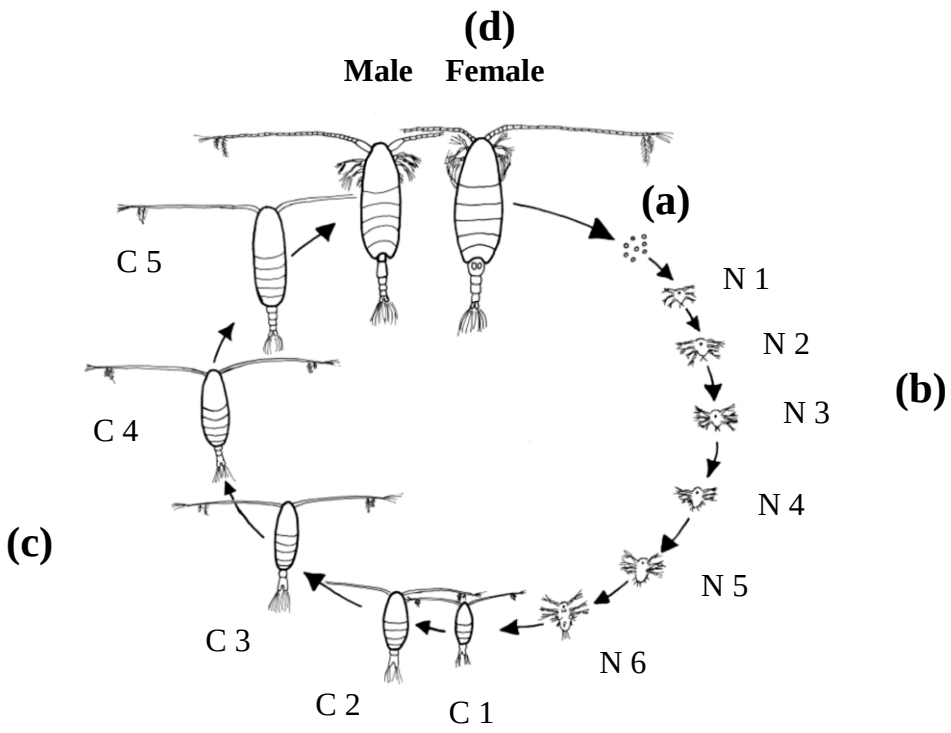

Figure (2) Calanoid life cycle: (a) egg hatching stage, (b) 6 naupliar stages, (c) 5 copepodite stages, (d) adult stage
http://www.sahfos.ac.uk/TaxonManual/figures/image/ARTHROPODA/callifecycl.JPG

Acartia tonsa, an invasive species in the cold environments

Acartia tonsa is a species from the order Calanoida, family Acartiidae. *A. tonsa* is a coastal and estuarine species. Its adult sizes range from 1.1 to 1.3 mm, and it is sexually dimorphic (Pastorinho et al, 2003). *A. tonsa* has a wide geographical distribution, covering several regions of the North Atlantic, Indian and Pacific oceans, the North Sea, Mediterranean Sea and recently the Arctic Sea (Figure 4) (Holste, 2006). *A. tonsa* is a great example of temperate species that are shifting towards Arctic and subarctic environments. It tolerate a wide range of salinity (from 1 to 32 psu) and temperature (1 to 32°C), making it a suitable invasive species that is capable of living in many environments (Holste, 2006). Recently, *Acartia tonsa* has been introduced to the North Sea through aquaculture or through shipping activity (Streftaris et al., 2005). In addition, *Acartia tonsa* has become one of the most abundant copepod species in the boreal coastal

waters and estuaries (Jorgensen et al., 2019). *A. tonsa* produces highly resistant diapause eggs as well as eggs that can be induced into quiescence, and this trait has probably played an important role in its wide distribution (Jensen and Carroll 2010).

Acartia tonsa females are larger and live longer than males. Females are distinguished from males by having broader urosome and thicker antennae (Figure 3) (Sabatini, 1990). *Acartia tonsa* females are broadcast spawners, meaning they do not carry their own eggs as other species and spawn them as soon as the eggs are mature (Marcus et al., 2007). Females release around 18 to 50 eggs per brood and can produce up to 718 eggs during their lifetime (Mauchline, 1998). In *Acartia*, the ovary is situated at the dorsal side of the females. Oocytes of all developmental stages are present in females, indicating that oocyte maturation is a continuous process (Niehoff 2007). The level of maturation of the oocytes increases with the distance from the ovary, with mature oocytes close to the genital segment ready for spawning (Niehoff 2007). The constant egg spawning abilities make *Acartia tonsa* females great candidate for toxicological studies in respect of reproduction capabilities.

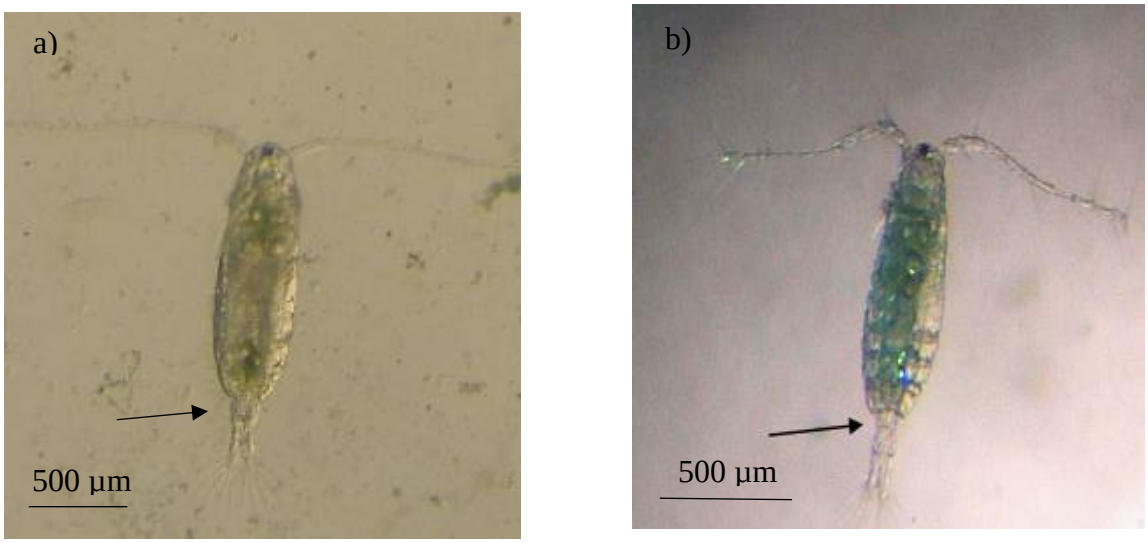

Figure (3) Sexual dimorphisms in *Acartia tonsa*. (a) Female *Acartia tonsa*, black arrow shows round urosome fused to the main body. (b) Male *Acartia tonsa* , the urosome is more straight

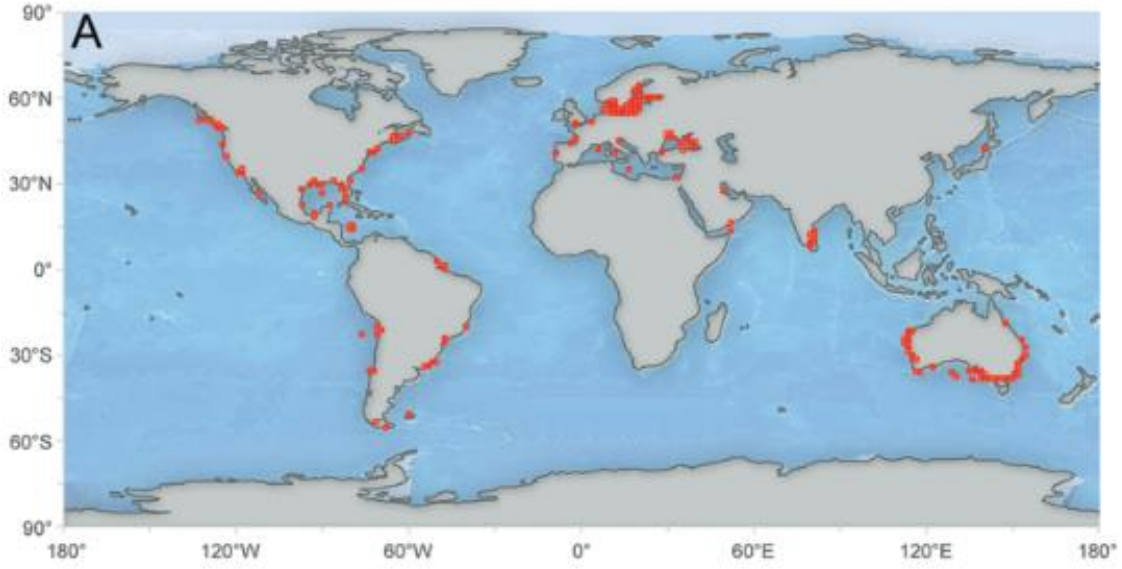

Figure (4) Geographic distribution of *Acartia tonsa (*World register of marine species*)*

Lipids in Acartia tonsa

In copepods, lipids, such as polyunsaturated fatty acids, are important for the metabolism of chemicals responsible for regulating cell differentiation, egg production and hatching (Acheampong et al., 2011). Consumers, such as copepods, cannot produce enough essential fatty acids (EFAs) to cover their basic biochemical needs. Therefore, the quality of EFA source (i.e phytoplankton) plays an important role in copepods growth, development and egg production (Bi et al., 2020).

Compared to other genus of copepods, *Acartia* sp. are known to have low amount of storage lipids (Lee et al., 2006). *Acartia* copepods lack the wax esters in comparison to higher latitude calanoids. Consequently relying on its small lipid reserve consisting of triacylglycerol (TAG) for their temporary energy need (Lee et al., 2006, Werbrouck et al., 2016). However, when a lipid rich food source is available for *Acartia tonsa*, the

original lipid storage levels can rapidly be magnified up to 60 factors (Werbrouck et al., 2016), which will be highly invested in egg production (Bi et al., 2020). Hazzard and Kleppel (2003) have demonstrated that adults of *Acartia tonsa* contained fatty acids up to 40% of the total lipids, high proportions of these fatty acids are of omega-3. Therefore, *Acartia tonsa* is considered as a valuable live feed for several species of fish as its omega -3 profile exceeds that of enriched rotifers and enriched *Artemia* (Marcus et al., 2007).

Acartia tonsa, a model organism for toxicological assays

Acartia tonsa has been considered for toxicity assays of marine pollution since 1977 (Gorbi et al 2012). The International Organization for Standardization (ISO) recommended *Acartia tonsa* as a model organism for acute, chronic and semi chronic bioassays to test for the toxicity of several contaminants (Gorbi et al., 2012). Recently, *Acartia tonsa* has been included by the Italian Ministry of Environment and by the Government of Canada in the list of species used for the toxicological evaluation of sediment pollution (Carotenuto et al., 2020). The Organization for Economic Co operation and Development (OECD) has one standardized test (OECD, 2004 number 79) for studying full life cycle with sexually reproducing *Acartia tonsa*. *Acartia tonsa*'s wide distribution, short life cycle, continuous ability to produce eggs and the ability to be easily cultured in lab conditions make it a proper candidate for toxicological studies (Bellas et al., 2007). *Acartia tonsa* females can produce dormant eggs which can be stored at 3º C for up to four months (Vitielo et al., 2016). Additionally, *Acartia tonsa* has shown sensitivity under a range of different contaminants and toxins (Andersen et al., 1999, Bielmyer et al., 2006, Pinho et al., 2007; Sahlmann et al., 2019). Several endpoints have been studied in *Acartia tonsa* (Table 1) ranging from mortality to reproductive assays.

Table1: An overview of some toxicological studies done using *Acartia tonsa* as a model organism

Endpoints studied	**Contaminant**	**Concentration range**	**Exposure Period**	**References**
Egg production rate, Lethality , recruitment rate	Flouranthene, phenanthrene, pyrene	Flu: 200-800 nM Phe:200- 1800 nM Py: 40-640 nM	48 hours	Bellas et al 2007
Lethality	Water Accommodated Fraction (WAF) from crude oils	0- 100% WAF	48 hours	Faksness et al 2015
Egg hatching, nauplar mortality, Egg production rate	Nickel and Nickel nanoparticles	5- 50 mg/L 1-10 mg/L	7 days, 4 days, 48 hours	Zhou et al 2016
GST gene expression levels	Dimethylnapht-halene	0.54-57.358 µg/L	24, 48,72, 96 hours	Zhou et al 2020
Mortality, concentration of magnesium ,chloride, sodium / Dry weight, ATPase activity	AgNO3	5-178 µg/L	48 hours	Pedroso et al 2007
Mortality	Polyethylene Microbeads	0.5- 2.5 µg/L	48 hours	Syberg et al 2017
Egg production rate, Egg Hatching rate,	Copper	0-160 µg/L	6 days	Lauer et al 2010
Mortality, Larval development	Nitro and polycyclic synthetic Musks	0.6- 5.0 mg/L	5 days	Wollenberger et al 2003
Reproduction, Mortality	ZnO nanoparticles	50- 300 µg/L	7 days	Jarvis et al 2013
Reproduction, Mortality	Ag, Zn, Cu, Ni	5.4- 15.4 µg/gm	7 days	Bielmyer et al 2006
Feeding, reproduction	Scrubber discharge water	0-100%	24 hours	Koski et al 2017

3. Crude oil and Water accommodated fractions (WAF)

Crude oil contains a mixture between different compounds such as hydrocarbons, heterocyclic compounds and some heavy metals (Saadoun, 2015). Crude oils are mainly classified into three categories: Paraffinic, Naphthenic and Aromatic (Simanzhenkov 2003). In addition, the types of crude oils are usually named after their properties. For example, a sweet crude oil contains low amount of sulfur while sour crude oil contains more sulfur (Petro Online, 2014). Light and heavy crude oils depend on the oil density. Light crude oil has high percentage of light hydrocarbons, which can be inexpensively recovered during distillation. Heavy crude oil contains high concentrations of bitumen, nickel and sulfur. Compared to light crude oil, heavy crude oil requires more refining for good quality oil products (Petro Online, 2014).

After an oil spill, weathering processes and wave dynamics in the water column can introduce the oil soluble hydrocarbons into the water column and thus be more bioavailable to marine organisms (Barron, 2004). These Water-soluble fractions, called water accommodated fractions (WAF) contain harmful aromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene and xylenes) and polycyclic aromatic hydrocarbons (PAHs) (Jiang et al., 2010). However, the chemical composition of WAF is mainly dependent on the crude oil and the weathering conditions (Katsumiti et al., 2019). In WAF, PAHs are mostly two to five rings hydrocarbons, higher rings PAHs are difficult to dissolve in water for their high lipophilic ability (Jiang et al., 2010).

The fate of oil Spill Pollution in the cold environments

Upon the introduction of oil to the water column, it faces several changes in its physical and chemical properties. Oil faces weathering processes such as spread, evaporation, dissolution, emulsification, dispersion, absorption, sedimentation, photo oxidation and

biological degradation (Figure 5) (Sun et al., 2019). However, oil spills behave differently in the cold environment compared to warmer environments. For example, during high production periods, polynyas or opening in ice packs are very susceptible to oil pollution because oil slicks can be concentrated in the small shallow areas. Low temperature leads to increase in viscosity of oil, reduced evaporation of volatiles and increased water solubility (Yang et al., 2009). In addition, the different hydrocarbon constituents in oil behave differently after an oil spill. For example, aromatic hydrocarbons are more soluble than the aliphatic hydrocarbons with the same molecular weight and can be washed out preferentially (Prince et al., 2002). Biodegradation of linear alkanes is more preferential than branched alkanes (Prince et al., 2002). Consequently, the natural process of oil biodegradation is very slow, and thus, untreated oil slicks remain in the environment for more than 50 years. The dynamic movement of ice packs combined with the high residence time of the oil particles in the water would have a significant effect on the *albedo* effect on ice sheets (Van angelen, 2012). In addition, several weathering processes of oil are highly influenced by low temperatures (Brandvik et al., 2008).

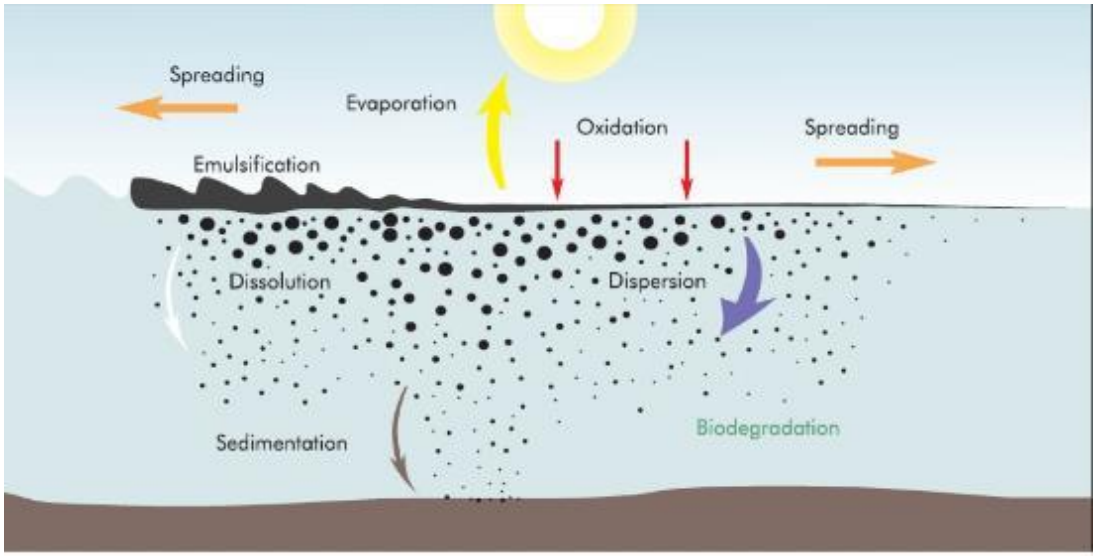

Figure (5): Different weathering effects facing oil after the oil spill (ITOPF, 2019)

Oil cleanup techniques

Oil slick cleanups can be conducted using different techniques. For example, sorbent-like materials such as polypropylene are used to absorb the oil from the water (Bayat, 2005). Booms are buoyant barriers, which limit the spread of oil without changing the physical and chemical properties of it (Dave et al., 2011). Another technique involves the controlled burning of the spilled oil to decrease the amount of oil in the water (Mullin, 2003). One well-known technique is "bio-stimulation", which was used after the *Exxon Valdes* oil spill. This process involves adding nitrogen-containing fertilizers to the water, which will stimulate hydrocarbon biodegradation bacteria and speed up the biodegradation process (Atlas, 2011).

One of the preferred methods in several countries as a form of remediation after oil spills is the addition of dispersants in order to break down the oil particles and thus increase their surface area for more efficient bacterial biodegradation (George-Ares, 2000). Further details on dispersants will be discussed in the next section.

The use of dispersants and their effects on bacterial biodegradation rates

Dispersants are surfactants that are made of anionic and nonionic molecules, which orient the oil water interface and lower interfacial tension (Ramachandran et al., 2004). Consequently, dispersants facilitate the formation of small mixed oil surfactant micelles (Ramachandran et al 2004), increasing the bioavailability of oil for bacterial biodegradation (Sun et al., 201). Dispersion of oil in the water surface reduces the potential impact on shorelines and coastal ecosystems (Personna et al., 2016). However, several studies have shown that dispersants have harmful effects on the environment because they facilitate the introduction of PAHs to the water column, making them more bioavailable to marine organisms (Yamada et al., 2003). In addition, several studies have

shown that dispersants have toxic effects to several species of crustaceans, fish and mollusks (Wise et al., 2011)

An example of a widespread use of dispersants was during the Deepwater Horizon oil spill accident, where 2.1 million gallons of the dispersant, Corexit 9500, were applied at the water surface and seafloor (Techtman et al., 2017). Although the use of dispersants is widely used as a form of emergency response, the efficiency of dispersants to enhance biodegradation rates is still debated (Sun et al., 2019). For example, Techtmann et al (2017) saw slight increase in biodegradation rates of oil dispersed with the dispersant Corexit 9500 at both 5 and 25º C. In addition, Venosa et al (2007) also observed slight increase in biodegradation at 5º C and rapid increase of biodegradation rates at 20º C of oil dispersed with Corexit 9500. In contrast, Kleindienst et al (2015) illustrated that microorganisms select the hydrocarbons in dispersant as a carbon substrate and consequently attenuate biodegradation. Additionally, experiments with chemically enhanced WAF (CEWAF) showed that dispersant stimulates and selects the dispersant degrading bacteria against the more effective hydrocarbon degraders (Kleindienst et al., 2015, 2016). Therefore, further research is definitely needed for understanding the effects of dispersant on biodegradation rates.

4. Polycyclic Aromatic Hydrocarbons (PAHs) and their effects on zooplankton communities

PAHs are aromatic hydrocarbons form by the fusion of two or more benzene rings (Haritash et al., 2009). The common forms of PAHs are shown in Figure (6). PAHs are usually formed from the incomplete combustion of organic matter such as burning of fossil fuels or coal and during the subjection of several organic processes (EFSA, 2008).

Natural sources of PAHs can be as well forest fires, oil seeps and volcanic eruptions (Haritash et al., 2009). Several forms of PAHs are considered carcinogenic, mutagenic, genotoxic and can bio accumulate in marine organisms (Pane et al., 2005). A number of PAHs are currently listed in the Water Framework Directive (WFD) among the 33 top priority hazardous substances (Boonen et al., 2020). In the marine environment, two to four rings PAHs are persistent in the water column and are highly soluble (Bellas et al., 2007). Therefore, naphthalene, fluorene, phenanthrene and anthracene have significant toxicity towards organisms (Arias et al., 2016). High molecular weight PAHs, such as those with more than four rings, are known carcinogens, but do not have long lasting threat to the marine environment due to their extreme low solubility in water (Albers et al., 1996). PAHs are hydrophobic in nature; therefore, PAHs accumulate in the lipid of aquatic biota (Hsieh et al., 2019; Lee et al., 2006). Therefore, PAHs accumulating in marine plankton have a big risk to the whole marine biota due to the transfer of PAHs to higher trophic levels in the food chain (Hsieh et al., 2019).

In some copepods, PAHs such as naphthalene are excreted through feces (Berrojalbiz et al., 2009). PAHs with more rings such as fluoranthene and pyrene may accumulate in zooplankton for long duration (Mitra et al., 2012). Copepod eggs are high in lipid content, they are therefore in great risk to accumulate PAHs. Some PAHs, such as phenantherene, fluoranthene, and chrysene, were reported to be bio-accumulated in eggs of *Acartia tonsa*, suggesting the transfer of PAHs to the next generation (Almeda et al., 2013). In addition to PAHs lipophilicity, PAHs have shown to be interfering with the fluidity of the cell membrane (Van Brummelen et al., 1996). On a cellular level, PAHs damage the DNA by forming PAH-DNA adducts (Xue et al., 2005) and enhance the generation of reactive oxygen species (ROS) during the metabolism of PAHs (Patri et al., 2009)

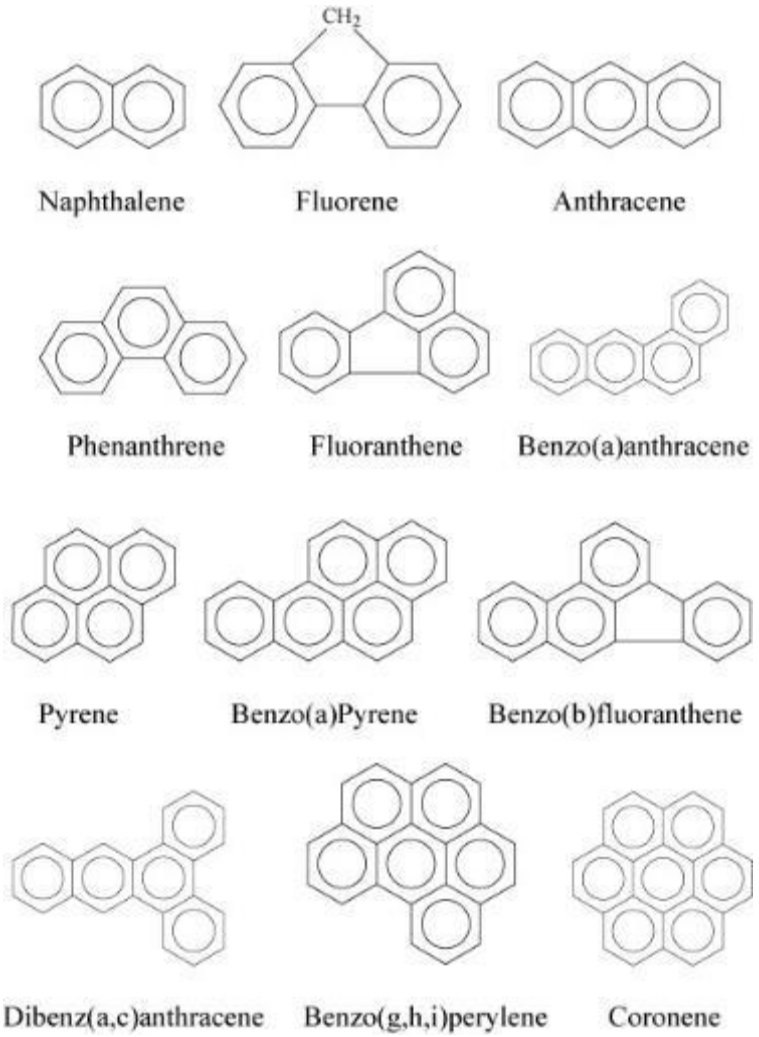

Figure (6): commonly known PAHs with different numbers of aromatic rings (Haritash et al 2009)

Cellular metabolism of PAHs

In aquatic organisms, the cellular metabolism of xenobiotic compounds is sub-divided into three phases (Ikenaka et al., 2013). At phase I, xenobiotics undergo bio-transformations through oxidation or reduction processes. At phase II, conjugation reactions produce polar derivatives, which are more easily to be excreted than the parent compound. At phase III, metabolites are excreted from the cell through membrane proteins, such as the ATP binding cassette transporters (ABC) (Ikenaka et al., 2013). In vertebrates, CYP1A1 and CYP1B1 coding proteins are some of the responsible for the metabolism of PAHs under phase I (Shimada, 2003; Han et al.; 2017). Glutathione S-

transferases (GSTs) are important isoenzymes, which are involved in the phase II detoxification system, limit oxidative damage and are involved in several conjugation reactions (Zhou et al. 2020). Several studies have demonstrated that the phase II conjugation metabolites in invertebrates have not yet been confirmed in vertebrates (Ikenaka et al., 2007).

In vertebrates, xenobiotic compounds such as PAHs binds to the Aryl hydrocarbon receptor (AHR) which activates the expression of CYP1A1 gene. However, AHR CYP metabolism pathway is a source of reactive intermediate metabolites, which is carcinogenic (Nerbert and Dalton, 2006). For example, oxidative metabolites such as diol epoxide covalently bind to DNA and cause several adverse effects such as cancer (Boonen et al., 2020). PAHs toxicity effects and CYP1A1 metabolic pathways on marine vertebrates has been well studied (Kim et al., 2015; Ryu et al., 2009). However, AHR CYP1A pathways is still poorly understood in marine invertebrates as CYP1 gene family has not been found in invertebrate genomes yet (Han et al., 2017). Some CYP genes have shown potentials to be involved in signaling pathways similar to AHR CYP1A in vertebrates (Kim et al., 2015).

A PAH-AHR-CYP signaling pathway for marine copepods was proposed by Han et al (2017) (Figure 7). Inside the cytosol, the PAHs in WAF cause oxidative stress by generating (ROS) and damage DNA. Inactive (AHR) usually forms complex molecules with the molecular chaperone HSP90 and other proteins. Activated AHR bind with PAHs from WAF and translocate to the nucleus. In the nucleus, the PAH-AhR dimerizes with the transcriptionally active Aryl hydrocarbon receptor nuclear translator (AhRnt) in the promoter region for the transcription of AhR regulated genes. As CYP1A family genes are yet to be found in marine invertebrates, other genes from the CYP family such as *CYP*

3024, *CYP 3027* found in the copepod *T.japonicus* and *P. nana* could have similar function (Han et al., 2017).

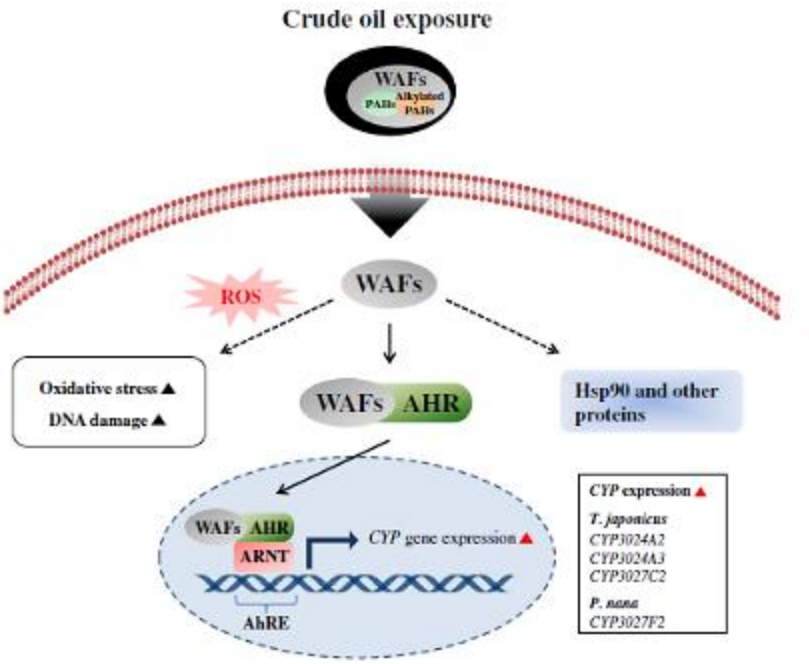

Figure (7): Proposed PAH-AhR-CYP signaling pathway in marine copepods (Han et al., 2017).

Microbial degradation pathways of PAH:

In general, microbes have developed strategies to degrade polycyclic aromatic hydrocarbons under aerobic and anaerobic conditions (Figure 8). Under aerobic conditions, ring hydroxylating dioxygenases enzymes (RHD) directly incorporates two oxygen molecules into an aromatic ring, forming cis- dihydrodiol (Duran and Cravo Laureau, 2016). Using dehydrogenase enzymes, cis dihydrodiol is converted to catechol which then undergo an aromatic ring fission into aliphatic products. At this stage, aliphatic products can enter metabolic pathways via the tricarboxylic acid cycle (Cernigilia, 1992).

However, under anaerobic conditions, PAH molecules are activated through the carboxylation of the aromatic rings using a carboxylase enzyme (Rabus et al., 2016).

Additionally, another activation mechanism is via methyl transferase which methylates the aromatic ring. The methylation process is then followed by naphthyl-2-methyl-succinate synthase, which catalyzes the addition in fumarate (Meckenstock and Moutakki, 2011). However, in both mechanisms mentioned, the degradation pathways proceeds through beta oxidation after the oxidation with coenzyme A (Meckenstock and Moutakki, 2011).

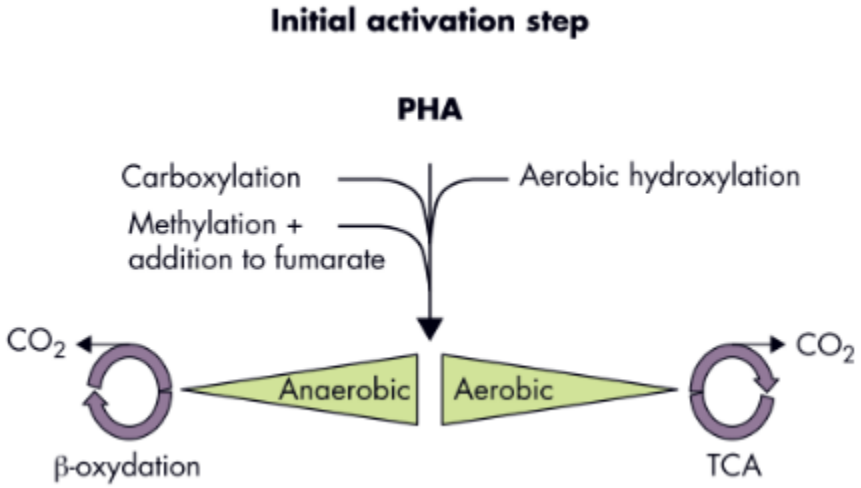

Figure (6): Main activation mechanisms of the PAH biodegradation pathways (Duran and Cravo Laureau, 2016)

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State of the art, Hypothesis and Objectives

State of the art

Oil spill pollution has become an emerging problem in the world seas. This issue has bigger impact on cold environments due to the harsh conditions these environments possess. Weathered oil from oil spills, in the form of Water Accommodated Fractions, introduce PAHs in the water column and have detrimental effects on the marine fauna inhabiting these environments. As a form of bioremediation, chemical dispersants are added to the oil in order to break down the oil slick into smaller droplets and therefore facilitate microbial biodegradation of oil. However, the use of dispersant as a form of remediation have shown to have detrimental effects on marine fauna.

Due to its sensitivity to toxins and being a crucial link in the marine food chain, the copepod *Acartia tonsa* has proved to be a suitable organism to toxicologically investigate the effects of several toxins. To date, little is known about the toxicological effects of WAF and WAF+Dispersant on the copepod *Acartia tonsa*. Studying different types reproductive capabilities endpoints, such as of egg production rate and egg hatching successes will help in understanding how WAF or WAF+Dispersant affect the populations dynamics of *Acartia tonsa* after exposure. Molecular endpoints, such biomarker genes of oxidative stress and metabolism of xenobiotic compounds, illustrate the cellular stresses facing *Acartia tonsa* after exposure and be early markers for exposure stress. Therefore, integrating endpoints of different biological levels, will provide a comprehensive insight on the toxicological effects of WAF and WAF+Dispersant on marine fauna.

In addition, microbial microcosms are suitable in mimicking environments to understand the fate of WAF and WAF+Dispersant and their effects on bacterial communities. Analyzing the resident microbial communities through next generation sequencing will assist in understanding how bacterial communities respond to the introduction of WAF at

low temperature. The addition of dispersant to WAF in microcosms further helps in understanding the role of dispersant in stimulating the microbial communities to biodegrade oil. In conclusion, both in vivo assays and microcosm experiments pave the way for fully understand the fate of weathered oil in the marine environment.

Hypothesis

A wide range of *in vivo* bioassays and a battery of biological multi-level responses using the model organism *Acartia tonsa* could help to determine how WAF from different oils have different toxicities. The addition of bioremediation chemicals such as dispersants to WAF has more severe toxic effects on marine copepods than WAF alone. Finally, at low temperature conditions, WAF with and without the addition of chemical dispersants affect the dynamics of microbial communities inhabiting the water column and the sediments.

Objectives

1) To assess the toxicity profiles of WAF prepared from two different types of distilled oils and a naphthenic North Sea crude oil using copepods *Acartia tonsa*, as experimental organism.

2) To investigate the toxic effects of WAF prepared from naphthenic North Sea oil single or in combination with a commercial chemical dispersant using the model organism *Acartia tonsa.*

3) To describe the changes produced by the exposure to naphthenic North Sea oil WAF with and without dispersant on native microbial communities in water and in sediment samples from subarctic areas.

4) To determine whether the addition of a commercial chemical dispersant to a naphthenic North Sea oil WAF improves the biodegradation rates of PAHs in subarctic water or sediment experimental samples and changes its toxicity to *Acartia tonsa* copepods

Results and Discussions

Chapter 1: Application of a biological multilevel response approach in the copepod *Acartia tonsa* for toxicity testing of three oil Water Accommodated Fractions

This chapter has been presented in the 39th international symposium on Halogenated Persistent Organic Pollutants (DIOXIN 2019). Kyoto Japan

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Abstract:

Copepods play a critical role in the marine food webs, being a food source for marine organisms. In this study, we investigated the toxic effects of Water Accommodated Fractions (WAF) from three types of oil: Naphthenic North Sea crude oil (NNS), Intermediate Fuel Oil (IFO 180) and a commercial Marine Gas Oil (MGO). The WAF were prepared at 10°C and 30 *PSU* (practical salinity unit), and tested on the marine copepod *Acartia tonsa* at different endpoints and at different levels of biological organizations. We determined the median lethal concentrations after 96 hours (LC50) and reproduction capabilities were calculated in adult females following seven days of exposure to sublethal WAF doses. The total lipid content was measured in reproductive females using Nile red lipophilic dye after 96 hours of WAF exposure. We also measured the transcription levels of genes involved in antioxidant response and xenobiotic biotransformation after short exposure for 48 hours. High doses (7% WAF) of MGO affected survival, percentage of fecund females, egg hatching success, and total lipid content. The IFO 180 WAF affected, at medium (20%) and high (40%) doses, the number of fecund females, mortality and produced significant effects on gene expression levels. In conclusion, toxicity assays showed that the WAF prepared from refined oils were more toxic than crude oil WAF to *Acartia tonsa*.

Keywords: *Acartia tonsa*, oil Water Acommodated Fractions, Fecundity, LC50, total lipids, Gene expression

Introduction

Due to the increasing oil demand and the melting of the polar ice caps, oil extraction and transport in the oceans have significantly increased during the last decade. Consequently,

the risks of oil spill incidents are escalating rapidly. Between 2010 and 2018, 59 major oil spills occurred, resulting in the release of approximately 163,000 tons of oil into the marine environment (ITOPF, 2020). Water Accommodated Fractions (WAF) are solutions of *low molecular weight hydrocarbons*, which with appropriate wave dynamics and weathering processes, are naturally released from *petroleum hydrocarbon* mixtures or spilled oils. WAF contain harmful polycyclic aromatic hydrocarbons (PAHs), aliphatic compounds and alkylated chemicals known to be bioavailable and very toxic to several aquatic species (Abbriano et al., 2011; Barron et al., 2003). Several environmental factors, such as temperature and light, influence the degree of bioavailability and toxicity of PAHs in WAF (Lyons et al., 2011; Lee, 2003). At low temperatures, PAHs show low solubility and reduced bioavailability, but at high temperatures, both low and high molecular weight hydrocarbons can be introduced in the water column (Lyons et al., 2011). Photo-oxidation of PAHs results in the production of free radicals that react with oxygen to generate toxic reactive oxygen species (ROS) (Lee, 2003). Several members of the superfamily of cytochrome P450 genes participate in the metabolism and biotransformation of organic chemicals, including PAHs, and are considered biomarkers for WAF and oil exposure in aquatic animals (Snyder et al., 1998; Rhee et al., 2013). The role of the aryl-hydrocarbon receptor (AhR) dependent pathway and the activity of cytochrome P450 enzymes (CYPs) in the detoxification of PAHs is well characterized in vertebrates, but poorly described in invertebrates (Han et al., 2017; Nelson, 2018).

PAHs are known to have a deleterious impact on cellular metabolism and functions (Allan et al., 2012; Han et al., 2014). Inside the cell, the metabolism of PAHs generates ROS (Hannam et al., 2009) and an imbalance between generation of ROS and ROS neutralization via antioxidants or oxyradical scavenging systems. An imbalance of these

processes in the cell triggers oxidative stress ((Valavanidis et al., 2006) that can result in DNA damage,oxidation of biomolecules and cell death (Martins et al., 2013; Wilk et al., 2013).

Consequently, the expression of several genes involved in antioxidant defenses represents a valuable tool for understanding the toxicity response mechanisms against oxidative stress. Antioxidant enzymes (e.g Catalase and glutathione transferase (GST)) (Kim et al, 2011), molecular chaperones (e.g. heat shock proteins) and iron storage proteins (i.e. ferritin) have been used as biomarkers of ROS induced responses in aquatic organisms (Tarrant et al. 2019).

The calanoid copepod, *Acartia tonsa*, is a cosmopolitan species inhabiting temperate regions and is usually abundant in coastal and estuarine areas (Cervetto et al. 1995). Copepods are considered ecologically crucial to the marine food web, serving as a primary food source for secondary consumers, and exerting trophic effects on primary producers such as phytoplankton (Gorbi et al. 2012). The International Organization for Standardization (ISO) recommended the use of *Acartia tonsa* for the evaluation of lethality and toxicity of several contaminants (Gorbi et al., 2012), since it is sensitive to different types of contaminants and toxins (Andersen et al., 1999, Bielmyer et al., 2006, Pinho et al., 2007; Sahlmann et al., 2019). Thus, *Acartia tonsa* is considered a valuable model organism for understanding pollutants' effects on the marine zooplankton communities.

Few studies have investigated the effects of oil WAF on *Acartia tonsa* (Avila et al., 2010, Faksness et al., 2014), mainly focusing on lethality without concurrently considering different sublethal endpoints. The toxic effects of WAF on several other copepod species

with respect to mortality, development, egg production, and motility have been established (Cowles and Remillard, 1983; Bejarano, 2006; Jiang et al., 2012; Hansen, 2013 and 2018; Lee et al., 2013). Exposure to dispersed oil alters the lipid sac volume in the cold-water copepod *Calanus finmarchicus* (Hansen et al., 2016), leading the authors to conclude that changes in lipid content due to contaminant exposition might have adverse effects on the reproduction capabilities in copepods. It has been shown that fatty acid composition in *Acartia tonsa* influenced egg hatching success and egg production rates (Broglio et al., 2003; Cavallo and Peck, 2020), but to date, the effects of oil WAF on the lipid composition in *Acartia tonsa* are poorly understood.

A potential role in PAH metabolism for genes belonging to the CYP family has been suggested in copepods. Several genes of CYP3 clan showed differential transcription profiles after exposure to individual PAHs and WAF (Hansen et al., 2007 and 2008; Han et al., 2014, 2015 and 2017). A role of genes in the CYP 3 clan in PAH and oil metabolism has been suggested in marine copepods, such as *Tigriopus japonicus* and *Paracyclopina nana* (Han et al., 2014 and 2015).

In order to better understand the effects of oil WAF on *Acartia tonsa* copepods, we studed the effects of three oil WAF (crude and refined) with different water solubility and bioaccumulative characteristics. We selected a naphtenic crude oil from the North Sea (NNS) and two refined oils: Intermediate Fuel Oil 180 (IFO 180) and a commercial Marine gas oil (MGO). NNS and IFO 180 have low dispersion potential and a low concentration of water-soluble components (Hansen et al., 2013). In contrast, MGO, commonly used as fuel for different types of ships, has low viscosity and high volatility, which facilitate its dispersion in the marine environment (Hansen et al. 2013). We applied

a biological multilevel response approach to integrate effects on lethality, fecundity (egg-laying), total lipid content and transcription levels of genes related to stress responses and PAH metabolism. In addition, potential transgenerational effects on egg hatching success were assessed.

Materials and Methods

Selected Oils and preparation of Water Accommodated Fractions (WAF)

The three oils used for WAF preparation were NNS, IFO 180, and commercial MGO. NNS oil is a naphthenic-based light crude oil characterized by a low aromatic content, a low wax content, a low viscosity index (299 cP at 2° C), and a density of 0.900 g/ml . It is also rich in branched and cyclic saturated hydrocarbons (Katsumiti et al., 2019). IFO 180 is a blend of heavy fuel oil and gas oil composing approximately of 88% residual oil and 12% distillate oil. It is characterized by a very high viscosity (180 cSt at 50° C), high density >0.967 g/ml , and has a maximum sulfur content of up to 3.5% (Johann et al., 2020). IFO 180 is mainly used in the machinery and engines of large shipping vessels (Alexander et al., 2016). MGO is used in the engines of small ships and fast boats. It is a blend between gas oil and heavy fuel oils and is highly volatile, with low viscosity (2-4 cSt at 40° C), low density 0.856 g/ml containing up to 60% aromatic hydrocarbons (Hansen et al., 2013). Oils were provided by the Norwegian University of Science and Technology (NTNU; Trondheim, Norway), within the framework of the EU-funded project GRACE (Integrated oil spill response actions and environmental effects) (Jørgensen et al., 2020).

The WAF were prepared according to the protocol by Singer et al. (2000) for low-energy WAF with slight modifications. Briefly, oil was added to 0.2 µm filtered seawater in 4 L glass bottles with bottom tap, at a final loading concentration of 5 g oil per 1 L of seawater. The water-oil mixture was continuously stirred at 800 rpm (low-energy) using a magnetic stirrer without forming any vortex for 40 h. The stirring was conducted at 10°C under dark conditions to avoid photo oxidation and degradation of oil elements. Finally, 3.5 L of WAF were carefully extracted from the bottles using the tap, avoiding the presence of non diluted oil, and serial dilutions of each WAF were immediately prepared using clean seawater.

Table 1: Selected doses of water accommodated fractions (WAF) of studied oils in the different analyzed biological endpoints.

	NNS WAF			**IFO 180 WAF**			**MGO WAF**		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
Reproductive assays	10%	30%	50%	10%	25%	40%	3%	5%	7%
Lipid levels	-	25%	50%	-	20%	40%	-	5%	7%
Gene transcription levels	-	25%	50%	-	20%	40%	-	5%	7%

NNS: Naphthenic crude oil from North Sea; IFO 180: Intermediate fuel oil 180; MGO: Marine gas oil.
(-): not tested

Copepod culture

Copepods, *Acartia tonsa* (Plentzia Marine Station, PiE-UPV/EHU), were maintained in a 10 L tank filled with 0.2 µm filtered seawater at 18°C, a salinity of 30 practical salinity unit (PSU) and a constant photoperiod of 16/8 h light/dark cycle. Copepods were fed every two days using a 1:1 mixture of the brown microalgae *Isochrysis galbana* and green microalgae *Tetraselmis chuii.* To prevent potential cannibalism,

recently hatched nauplii were transferred from the main tank into a smaller 5 L tank until adulthood.

Chemical analysis of the PAH content

The PAH composition and concentrations of the different types of WAF were determined by GC/MS analysis. A mixed standard solution of 16 PAHs was used [Norwegian Standard (NS 9815: S-4008-100-T): phenanthrene (Phe), anthracene (An), fluoranthene (Fluo), pyrene (Pyr), 11 H-benzo[a]fluorene (11H-B[a]F), 11 H-benzo[b]fluorene (11H-B[b]F), benz[a]anthracene (B[a]A), chrysene (Chry), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), indeno[1,2,3-cd]pyrene (Ind), dibenz[a,h]anthracene (D[a,h]A), benzo[g,h,i]perylene (B[g,h,i]P)] and dibenzo[a,e]pyrene (D[a,e]P). Passive samplers of poly dimethylsiloxane polymer coat (Gerstel GmbH, Germany), with 10 mm in length and 0.5 mm film thick, were introduced in aqueous samples (35 mL) and stirred during 315 minutes. Once the extraction step was over, samplers were cleaned with Milli-Q water in order to eliminate seawater and dried with a paper tissue. Passive samplers were desorbed using a commercial thermal desorption TDS-2 unit (Gerstel GmbH) connected to a CIS-4 injector (Gerstel GmbH) with the following conditions: desorption time (10 min), desorption temperature (300º C), desorption flow (23 mL min^{-1}), cryo-focusing temperature (−50ºC) and vent pressure (7 psi). This desorption unit was coupled in an Agilent 6890 gas chromatograph also coupled to an Agilent 5975 mass spectrometer system (Agilent Technologies, USA). The Mass spectrometer (MS) was operated in selected ion monitoring (SIM) to quantify target compounds. An Agilent DB-5MS+DG column with the following dimensions 30 m x 0.25 mm and 0.25 μm was used. Analytes were separated using the following conditions: helium as carrier gas (1.3 mL/min); ion source transfer line and quadrupole analyzer temperatures maintained at 300, 230, and 150ºC,

respectively. The following temperature program was used for target PAHs and lineal hydrocarbons: 170 ºC for 5 min; ramp at 30ºC min^{-1} to 260 ºC; ramp at 8 ºC/min to 300 º C and hold 15 min. Three replicates per WAF were analyzed and concentrations calculated at the ng/L level.

Lethal toxicity assays 96 h

Lethality tests were conducted according to the international standard for water quality determination of acute lethal toxicity to marine copepods (ISO14669). Thirty adult copepods were evenly distributed among six replicates, 5 copepods in each container. During the assay duration, the copepods were not fed, and no sex preference was considered when selecting adult copepods. Copepods in each replicate were exposed to the contaminant for 96 h in 250 mL glass beakers containing 50 mL of 0% (control), 10%, 30%, 50%, 70% and 100% WAF diluted in seawater. Fifty percent WAF+seawater volume was replaced every 48 h. Each day of exposure, replicates were checked for mortality, and dead copepods were counted and removed. A copepod was considered dead if it did not move shortly after a gentle stimulation using a pipette. The LC50 values obtained from the lethal toxicity assays determined the low, medium and high concentrations to be further used in the sub-lethal endpoints assays (Table 1).

Fecundity and egg hatching assays

Twenty adult gravid females were selected under a 10X magnification dissecting microscope. Additionally, five adult males were included in each treatment to maintain female fertility throughout the experiment (Holste et al., 2006). The fecundity assay was performed similarly to the exposure methods described by Olsen et al. (2013), with some modifications in exposure time and methods. The fecundity assay was divided into three consecutive stages: exposure, recovery and individual egg collection.

In the exposure stage, copepods were placed into 300 mL glass beakers filled with 150 mL of the corresponding WAF dilution. Copepods were exposed in a semi-static condition for a duration of seven days. Every two days, 50% of the WAF+seawater volume was renewed. Containers were cleaned every day to remove fecal pellets, dead algae and any dead copepods . Throughout the experiment, copepods were fed daily with the algae *Tetraselmis chuii* at a concentration of 3000 cells per mL. In the recovery stage, the surviving females were transferred to clean, WAF-free seawater, for four days. Water was changed every two days, and copepods were fed daily with *Tetraselmis chuii* as in the exposure stage. This experimental procedure allows the identification of delayed effects on exposed female reproductive capacity to mimic environmental realistic scenarios of point oil spills or discharges.

In the fecundity egg collection stage, each surviving female was individually transferred to an incubation chamber with clean seawater for three days. Incubation chambers were divided into two compartments, spawning chamber and brooding chamber, similar to the device proposed by Kleppel et al (1998). The upper spawning chamber consisted of a plastic tube (50 mL, 10 cm in diameter), which housed an individual female for egg laying. A mesh of 200 µm was attached at the tube's base to prevent the females from entering the bottom compartment (brooding chamber) where eggs were deposited. The copepods were fed daily as in the previous stages. Each day, eggs were counted, carefully collected by pipetting, and transferred to individual vials containing seawater to allow them to hatch. Only females producing two or more eggs during the three days of the fecundity test stage were considered reproductive females. After 48 h, the unhatched eggs were considered unviable.

Total lipid quantification using Nile red lipophilic dye

We used a slightly modified protocol from Tingaud et al. (2011) and Jordao et al. (2015) to quantify fecund females' total lipid content utilizing Nile red lipophilic dye. The working solution of Nile red dye (Sigma Aldrich) was prepared by dissolving the dye in acetone at a concentration of 334 µg/L. Briefly, adult females (*n*=10) were maintained and exposed to WAF for 96 h as described for the fecundity assay. Two doses for each WAF (medium and high, Table 1) were analyzed. After the exposure, copepods were rinsed with seawater for 5 minutes and then transferred to 20 mL of seawater mixed with 29 µL of Nile red dye working solution. The adult females were stained for one hour at room temperature in darkness. For lipid level quantification in adult females, the stained copepods were washed with seawater for 5 minutes. Then, each copepod was placed into a vial with 200 mL isopropanol and homogenized using a tissue homogenizer (Precellys 24 homogenizer, Bertin Instruments, France) at 2500 vibrations per second for 45 seconds. The homogenate was centrifuged at 12000 rpm for 2 minutes. Then, 150 mL of the extract was pipetted in a black 96 well microplate for fluorescence determination, and the plate was read at a wavelength of 530/590 nm excitation/emission using a fluorimeter (Biotek Cytation 5 imaging reader, USA).

A histological and histochemical study of lipid distribution in copepod tissues was carried out in parallel to detect which tissue/organ showed the highest accumulation pattern for lipids. The procedure and results are presented in the Supplementary Materials section.

Gene transcription analysis

Exposure experiments

A total of 175 adult copepods were exposed to 150 mL of WAF for 48 h. Exposure concentrations were the same as described in section 2.6. Adult copepods were divided

into five replicates per treatment (35 copepods in each replicate). The copepods were fed daily with *Tetraselmis chuii*, and no gender preferences were taken during copepod collection for exposure. After 48 h of exposure, the copepods were transferred to cryovials filled with RNA later solution (AMBION, USA) and stored at -80ºC until extraction.

RNA extraction and cDNA synthesis

RNA was extracted using RNeasy Minikit (Qiagen,Germany) following the manufacturer protocol. Total RNA was treated with DNase-1 using the DNA-free kit (Invitrogen, USA) to remove genomic DNA. RNA integrity and concentration were measured using the Bioanalyzer 2100 (Agilent Technologies, USA). All RNA samples obtained a RIN score higher than 7, suitable for downstream qPCR analysis (Fleige et al., 2006). RNA purity was determined by measuring sample absorbance ratios of 260/280 nm using a UV-spectrophotometer (Epoch, Biotek, USA). All samples had acceptable ratios between 1.8–2.3. cDNA was synthesized from purified RNA using the AffinityScript Multiple Temperature cDNA synthesis kit (Agilent Technologies, USA) with random primers and following the manufacturer's protocol.

Primer design

Catalase, *ferritin* and members of *gst, hsp90, hsp70* families genes were targeted as biomarkers for the ROS response. A CYP gene from clan 3 (*cyp3026b*) was targeted as a biomarker for PAH metabolism. Primers for catalase (*cat*), *cyp3026b* and *gst* were designed *de novo* from conserved regions in closely related crustacean species and scaffolds from the *Acartia tonsa* genome project (Jorgensen et al. 2019) (Accession number PRJEB20069). Clustal Omega (Madiera et al 2019) was used for multiple sequence alignment. For *cyp3026b*, *Acartia tonsa* genome sequence LS054218.1 was

aligned with the gene sequences from *Tigriopus japonicus* (KF639998.1) and *Eurytemora affinis* (XM 023493699.1). For catalase, the genome sequence LS054460.1 was aligned with sequences from *Calanus finmarchicus* (EL965956.1) and *Eurytemora affinis* (XM023472406.1). For *gst*, the genome sequence LS085988.1 was aligned with *Tortanus forcipatus* (KT755427.1) and *Acartia pacifica* (KT754520.1). Specific primers to amplify fragments of *hsp70*, *hsp90* and *ferritin* genes were obtained from Nilsson et al (2014). *β-actin* gene was used for transcription normalization as previously proposed (Zhou et al. 2020), as it showed low coefficient of variability (*CV* < 5%). The identity of targeted genes was confirmed by sequencing (General Genomics Service Sequencing and Genotyping Unit, SGIker, UPV/EHU). Primer sequences and the primers' amplicon regions' multi-sequence alignments are presented in Table 2 and (Supplementary Materials, chapter 1, figure S2) respectively. Amplification efficiency for each primer pairs was calculated by conducting a 1:2 serial dilution curve generated from pooled experimental samples. All primer pairs showed amplification efficiency between 90% and 100%.

RT-qPCR

Real-time quantitative PCR was conducted using FastStart SYBR Green fluorescence dye (ROCHE Diagnostics, Switzerland) with ROX passive dye included in the mix. The reaction medium (25 µL) consisted of 12.5 µL FastStart SYBR Green master mix, 0.3 µL forward primer (800 nM), 0.3 µL reverse primer (800 nM) and 3 µL diluted cDNA template (1:5 v/v). qPCR was performed in 384 optical well plates (Applied Biosystems, USA) using ViiA 7 Real-Time PCR system (Applied Biosystems, USA). The amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and then 1 minute of annealing at the corresponding Tm

for each gene (Table 2). A dissociation curve was performed after the PCR cycles to determine the product specificity and check the absence of primer dimers. All qPCR assays were conducted in triplicates, and a non-template control (NTC) was included for each plate.

Table 2: Primer sequences and melting temperature (Tm) for all the genes analyzed in this study.

Gene	Primer sequences 5' to 3'	Tm (°C)	References
hsp70	*Fw*: TTCAATGATTCACAGAGACAAGC *Rv* :TCCTTGTGATGTTAAGACCAGCTAT	57.1 59.7	Nilsson *et al.*, 2014
hsp90	*Fw*:GTCACATCCCAGTATGGTTGG *Rv* :CCATGGTGGAGGTGTCACGG	59.8 63.5	Nilsson *et al.*, 2014
ferritin	*Fw*:ACGCTTGCACTGATAATCCA *Rv* :AGTTCTACCGTGACGCATCC	55.3 59.4	Nilsson *et al.*, 2014
β-actin	*Fw*:TTGGGTATGGAGTCCTGTGG *Rv* :CCTGGATACATAGTGGTGCC	59.4 59.4	Zhou *et al*., 2020
cyp3026b	*Fw*:TGCCATTGGCATCCACCATGAC *Rv*:AGCCTCCAGCAAAGCAAACCTC	62.1 62.1	Current study
gst	*Fw*:TGCTTGATTCACTTCTACAAGAGA *Rv*:GTCACCATCAACAACAGTTGGA	57.6 58.4	Current study
cat	*Fw*:GGAGAGAATCCCAGAAAGAGTG *Rv*:ACAGTCCAATTTCCCCTCCC	60.3 59.4	Current study

Integrated Biological Response (IBR)/n index

The selected endpoints reflect a hierarchy of biological organization from molecular to an individual level. The percentage of reproductive females, egg hatching success, total lipid levels, and *cyp3026b* and *hsp70* transcription levels were used to develop an integrated biomarker response index (IBR). The IBR constitutes a practical and robust tool to assess the responsiveness using multiple levels of biological responses (Broeg and Lehtonen, 2006). Standardized values of each biological response were calculated and plotted using star plots according to Beliaeff and Burgeot (2002). IBR index was

calculated by summing up the triangular surfaces of the star plots (IBR= $\sum A_i$) according to the procedure discussed by Beliaeff and Burgeot (2002) with the modifications suggested by Devin et al (2014), which avoids the biomarkers order bias in the IBR calculation. Since the IBR index is directly dependent on the number of biomarkers in the data set, the obtained IBR index value was divided by the number of biomarkers used (Broeg and Lehtonen, 2006, Marigomez et al., 2013). To facilitate data representation of the different concentrations of the selected endpoints for IBR/n index, only medium and high doses of each WAF type were only used in the star plot visualization.

Statistical data analysis

Copepod mortality at different times (48, 72, 96 h) from lethal toxicity assays were analyzed using the PROBIT regression model (Vincent et al., 2008) to calculate the LC50 of the different WAF types. Significant differences among LC50 values were determined based on the absence of 95% confidence interval limits overlap. Data from egg production rate, egg-hatching success, Nile red fluorescence and gene expression ratios was initially checked for normal distribution using Kolmogorov-Smirnov test, while homogeneity of variances were tested using Levene´s test . One-way ANOVA analyzed data showing normal distribution. Significant differences were further analyzed using Dunnett´s post-hoc test to identify differences among treated and control groups. Data showing non-homogenous variance but normal distribution was analyzed using Welch one-way ANOVA, followed by the Games-Howell post hoc test. Data showing non-normal distribution were analyzed using the non-parametric Kruskal-Wallis test, followed by Mann-Whitney post hoc test. All these analyses were performed with the aid of the statistical package IBM-SPSS v26. The relationship between the count data of the number of reproductive females and WAF doses was analyzed by a modified Fisher exact test (Freeman Hamilton test), due to the small sample size and the contingency table larger

than 2x2 (VassarStats, Lowry 1998-2019). The association between both variables was determined by a linear regression coefficient (IBM SPSS v26). Egg hatching success during individual fecundity tests was calculated as follows: (number of hatched egg per female/total number of eggs produced per female) x100. Finally, relative gene transcription levels were determined using the Pfaffl method (Pfaffl et al., 2001). The transcript levels were log2 transformed and statistical differences between control and treatments were determined using one-way ANOVA. The Dunnet post-hoc test was further used to determine the statistical difference compared to control (IBM SPSS v26). *Z*-test was used to determine significant differences in the IBR/n indices among the experimental groups

Results

WAF chemical composition

The three studied types of WAF contained mostly (99%) low-molecular-weight PAHs (< 3 rings), with naphthalene being predominant (Table 3). Among the 16 quantified PAHs, high-molecular-weight PAHs (> 4 rings) were below the detection limit for all three WAF. NNS WAF showed the highest content of total PAHs, particularly naphthalene (Table 3). IFO 180 WAF had the lowest total PAH concentration.

Table 3: Concentrations of the most prevalent PAHs in the three different types of 100% WAF prepared at 10°C. All concentrations are presented at ng/L (mean values ± SD; *n*=3). PAHs below detection limit for the three tested oils were: 11 H-benzo[a]fluorene (11H-B[a]F), 11 H-benzo[b]fluorene (11H-B[b]F), chrysene (Chry), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), indeno[1,2,3-cd]pyrene (Ind), dibenz[a,h]anthracene (D[a,h]A), benzo[g,h,i]perylene (B[g,h,i]P)] and dibenzo[a,e]pyrene (D[a,e]P).

PAH	# of rings	NNS	IFO 180	MGO
Naphthalene	2	19295 ± 759	8441±660	11213± 1166
Acenaphthene	2	347±25	965±3	362±37
Fluorene	2	1008±3	909±115	1161±95
Phenanthrene	3	1115±15	1968±63	1953±71
Anthracene	3	LDL	132±18	LDL
Fluoranthene	3	LQL	23±2.3	25±3
Pyrene	4	141±33	56±2	37±3
Benzo[a]anthracene	4	LQL	29±3	LQL
∑ PAHs		21934±7141	12524±2861	14759±4080

LDL: Low detection limit.
LQL: Lower than quantification limit.

WAF Lethal toxicity

The lethal toxicity assays showed that WAF toxicity to adult *Acartia tonsa* was different according to the fuel oil. The MGO WAF showed to be the most toxic, while the NNS was the least toxic to copepods (Table 4). For MGO WAF, LC50 were as low as 34%, 10% and 8 % after 48, 72 and 96 h, respectively. The IFO 180 WAF had the second most lethal effect to adult *Acartia tonsa* with LC50 of 74%, 52%, and 40% after 48, 72 and 96 h, respectively. The NNS WAF showed the least lethal effect with LC50 of 74% after 72 hours and 52% after 96 h, and no lethal effects on the first 48 h. Noteworthy, the three types of WAF showed LC50 at 72 h and 96 h significantly lower than their respective LC50 at 48 h.

Table 4: Lethal concentrations (LC50) for the adult *Acartia tonsa* exposed to the different WAF after 48, 72, 96 hours expressed as percentage of WAF diluted in seawater. The upper and lower limits of 95% confidence interval are indicated in parenthesis. Different superscript letters in each row denote significant differences among times, based on the absence of confidence interval overlap. Different superscript numbers in each column denote significant differences between oils, based on the absence of confidence interval overlap.

	48 h	72 h	96 h
NNS	>100%[a 1] (95-176)	74%[b 1] (61-94)	52%[b 1] (40-65)
IFO180	74%[a 2] (67-81)	52%[b 1] (45-61)	40%[b 1] (32-49)
MGO	34% [a 3] (29-42)	10%[b 2] (0.3-16)	8%[b 2] (0-13)

Effect of WAF on fecundity

Exposure of female copepods to IFO 180 WAF and MGO WAF produced a significant reduced the proportion of individuals laying eggs (Figure 1). The proportion of reproductive females was negatively associated with the doses of IFO 180 WAF (Fisher exact test $p < 0.0005$, $a = -23$) and MGO WAF (Fisher exact test $p < 0.05$, $a = -17.4$). The effect was very pronounced with the high IFO 180 WAF dose, with only two females laying eggs. Therefore, the high dose of IFO 180 WAF was removed from further statistical analysis. No significant difference was observed between the egg production rates of control fecund females and the fecund females exposed to NNS WAF, IFO 180 WAF or MGO WAF (Figure 2). However, MGO WAF at high dose negatively affected the viability of produced eggs (Figure 3) compared to controls, suggesting a disruption in the egg production process.

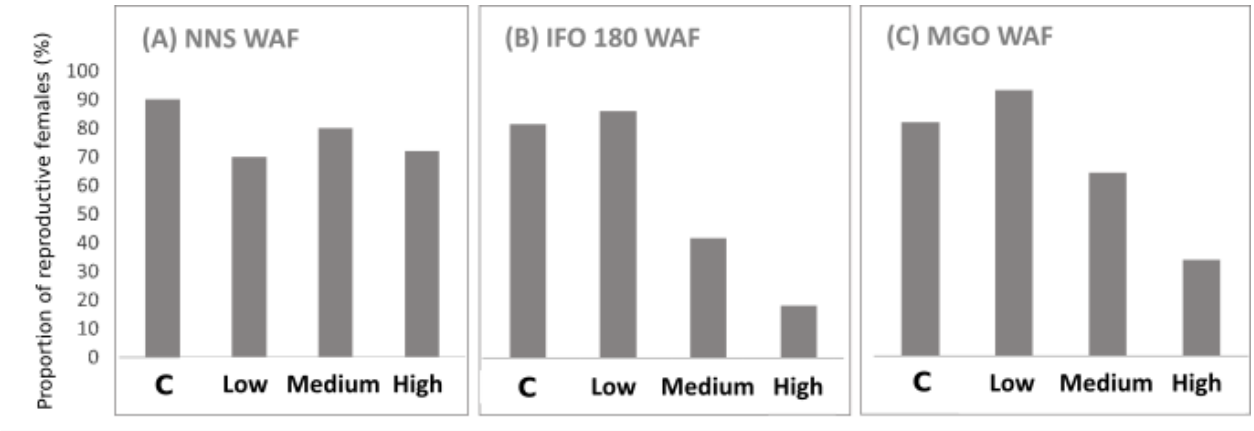


Figure 1: Proportion of reproductive (egg producing) females after exposure to different doses of a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. X-axis represents the selected sub-lethal WAF doses. A negative association between number of reproductive females and doses in IFO 180 WAF (Fisher exact test $p < 0.0005$, $a = -23$) and MGO WAF (Fisher exact test $p < 0.05$, $a = -17.4$) was detected. C: control. Only two females produced eggs in high IFO WAF treatment, therefore, high IFO 180 WAF dose was not included in the statistical analysis

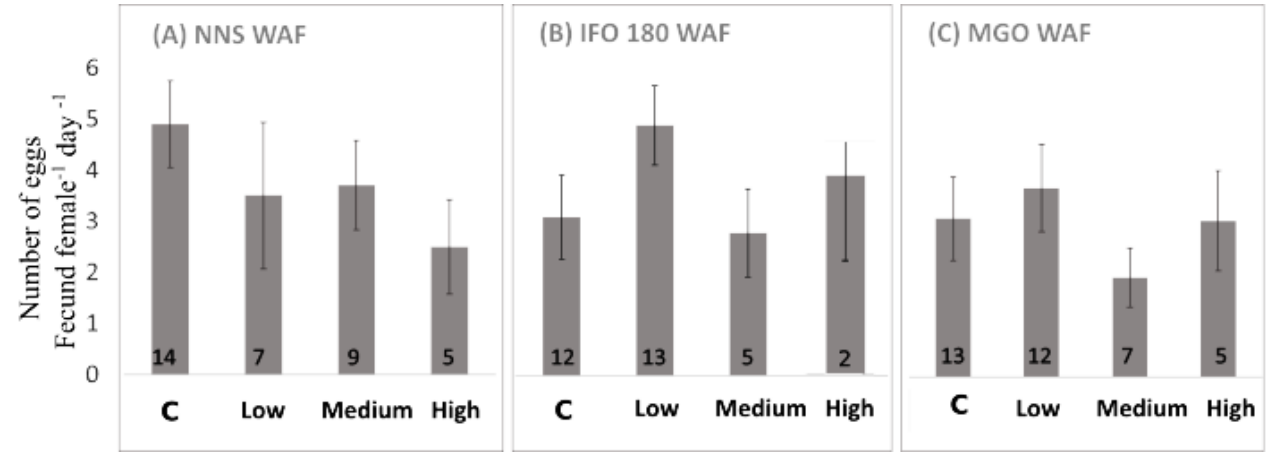


Figure 2: Average egg production rate (number of eggs per fecund female^{-1} day $^{-1}$) for the three days individual experimental period of a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. Numbers in each bar indicate the total number of reproducing females in the experimental group (n). No significant differences were detected between controls and treatments (one way ANOVA). Only two females produced eggs in high IFO WAF treatment, therefore, high IFO 180 WAF dose was not included in the statistical analysis. X-axis represents the selected sub-lethal WAF doses. C: control. Graphs show mean ± standard error.

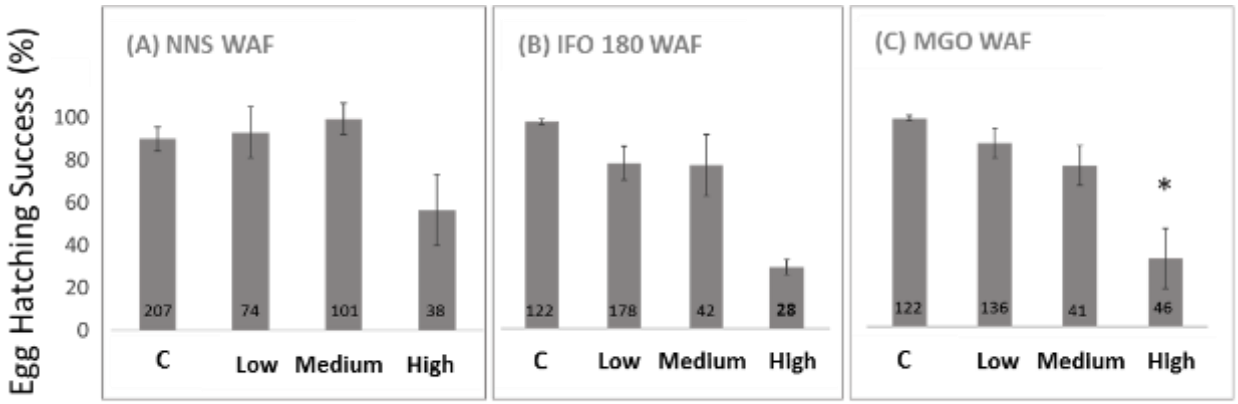


Figure 3: Average percentage of egg hatching success of a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. X-axis represents the selected sub-lethal WAF doses. Numbers in each bar indicate the total number of eggs analyzed in each treatment. Asterisks denote significant difference from control according to Kruskal-Wallis test ($p<0.05$), followed by Mann-Whitney post hoc test . C: control. Graphs show mean ± standard error.

Effect of WAF on total lipid

Ovaries were demonstrated as a vital accumulation tissue for lipids in female *Acartia tonsa* copepods (see Supplementary Material, chapter 1 ,Figure S1). We quantified the lipid content using the Nile red lipophilic dye to give further insights into how exposure to the three types of WAF affected the amount of accumulated lipids in females. Overall, the collected data was highly variable in all treatments and control (Figure 4). Nevertheless, Nile red fluorescence values of the copepods exposed to the high MGO WAF dose were significantly lower than those observed in the control copepods (Figure 4c), indicating a loss in total lipid levels.

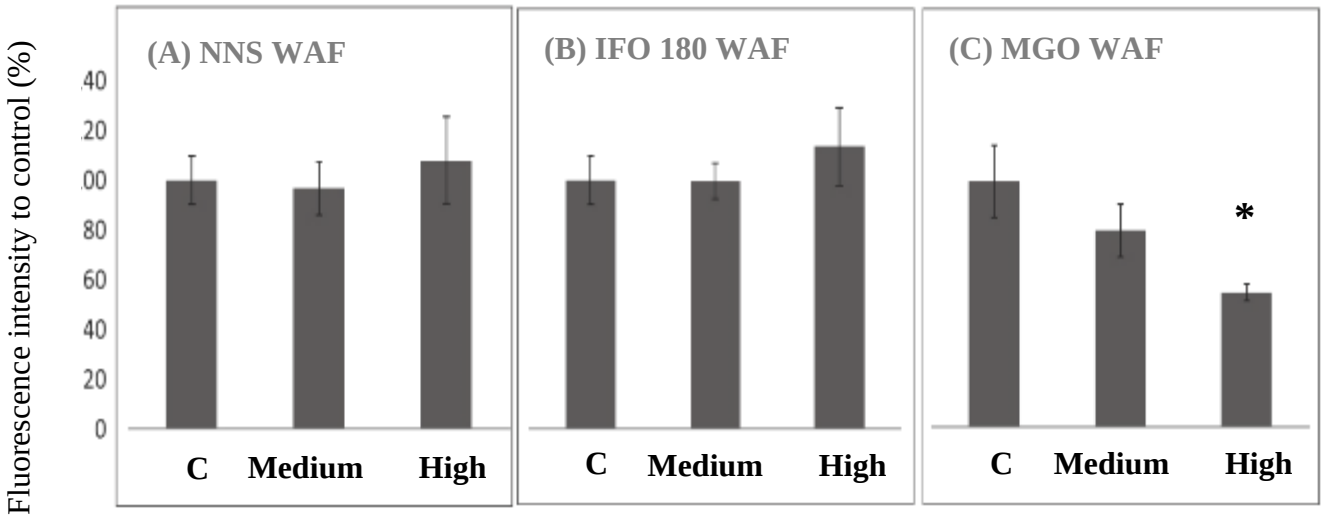


Figure 4: Total lipid content corresponding to the percentage of Nile red fluorescence intensity compared to control (100%) after 96 hours of exposure in a) NNS WAF, b) IFO 180 WAF and c) MGO WAF. X-axis represent the selected sub-lethal WAF doses. Asterisk denotes significant difference from control after one-way Welsh ANOVA, followed by Games-Howell post hoc test ($p<0.05$). C: control. Graphs show mean ± standard error.

Effect of WAF on gene expression

Changes in transcription levels of the studied genes indicated toxic effects of tested WAF in *Acartia tonsa* (Figure 5). Down-regulation was observed for *hsp70* transcription levels in copepods exposed to WAF, being statistically significant for copepods exposed to the medium dose of IFO 180 WAF. In contrast, *cyp3026b* up-regulation was observed in copepods exposed to the high dose of IFO 180 WAF condition. Statistical significant differences were absent for *cat*, *gst*, *hsp 90* and *ferritin*.

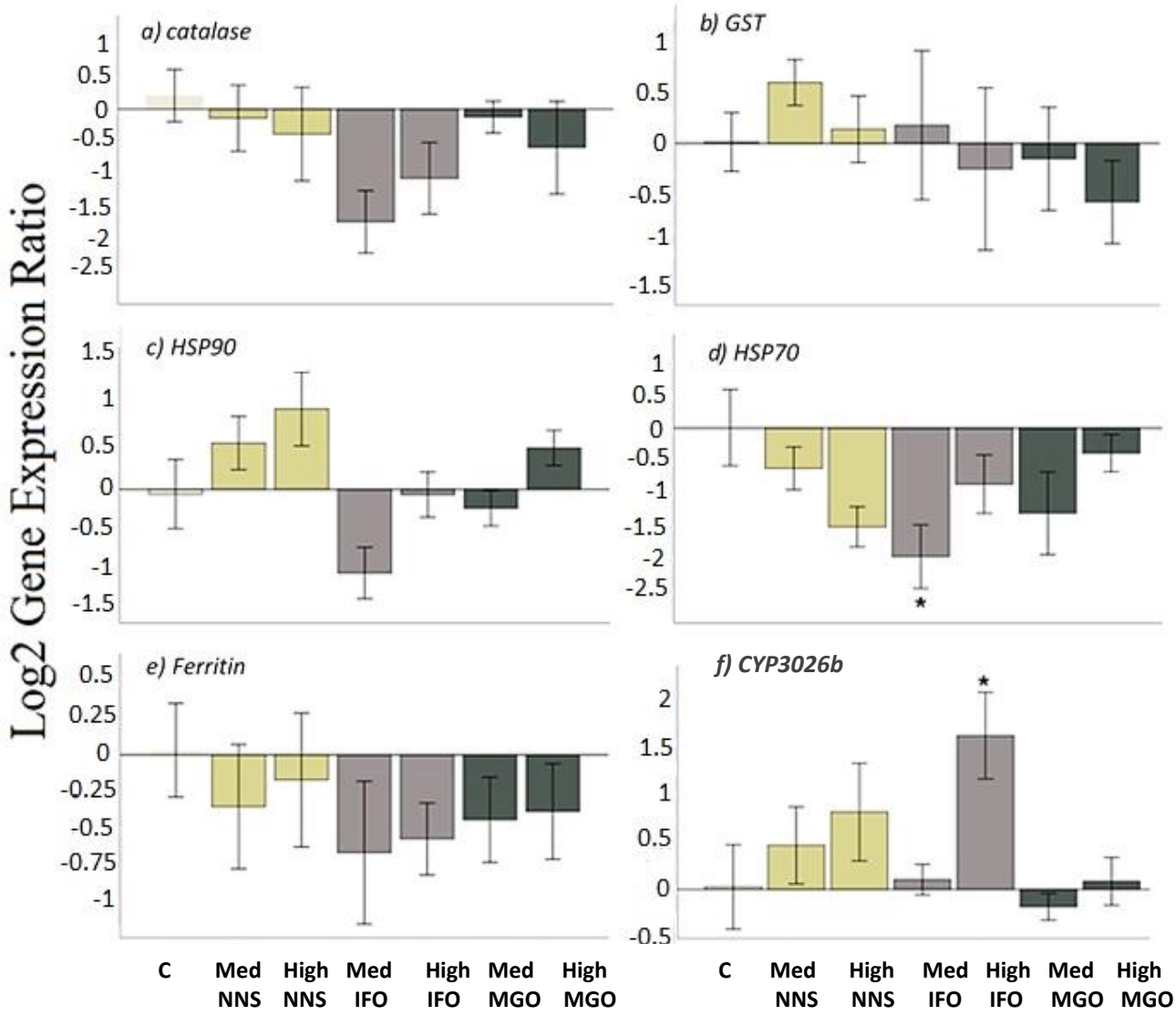
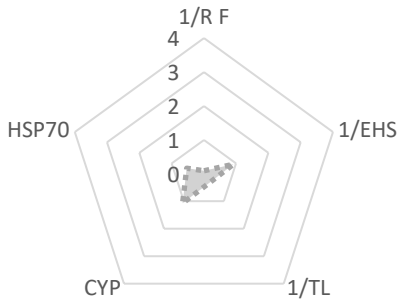


Figure 5: Relative gene transcription levels (RQ) for a) Catalase (*cat*), b) Glutathione S-transferase (*gst*), c) Heat shock protein 90 (*hsp90*), d) Heat shock protein 70 (*hsp70*), e) Ferritin and f) Cytochrome P450 3026b (*cyp3026b*). Transcription levels are represented in log2 scale (mean ± standard error). Asterisks denote statistically significant differences from control according to one-way ANOVA followed by Dunnett´s post hoc-test ($p<0.05$).

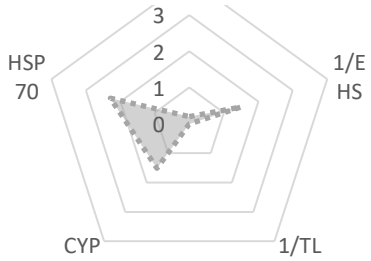
IBR/n index

Figure 6 (a-f) shows star plots for each experimental group. The surface value inside the dotted areas was calculated in order to obtain the IBR/n index value (Figure 6g). For high IFO 180 WAF and MGO WAF doses, IBR/n indices were significantly higher compared to their corresponding control, indicating toxic effects of both WAF for copepods. For the IFO 180 WAF, the low number of fecund females and up-regulation of *cyp3026b*

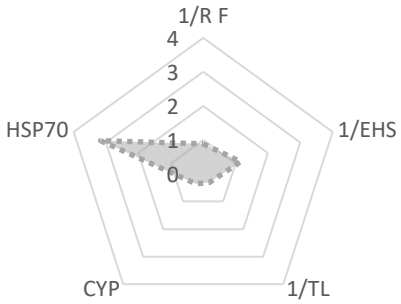
a) Med NNS WAF



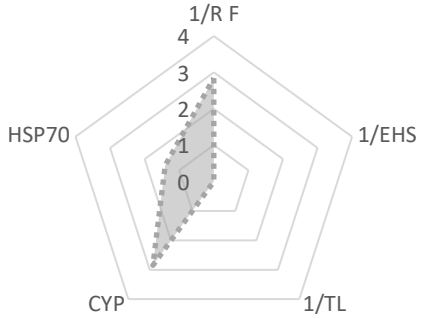
b) High NNS WAF



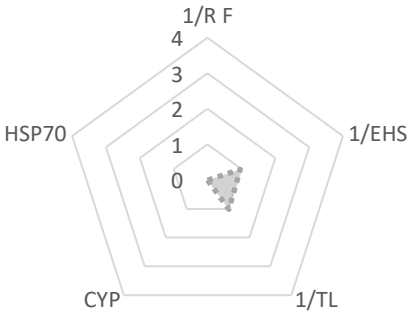
c) Med IFO 180 WAF



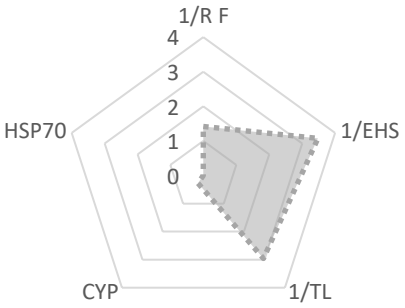
d) High IFO 180 WAF



e) Med MGO WAF



f) High MGO WAF



g)

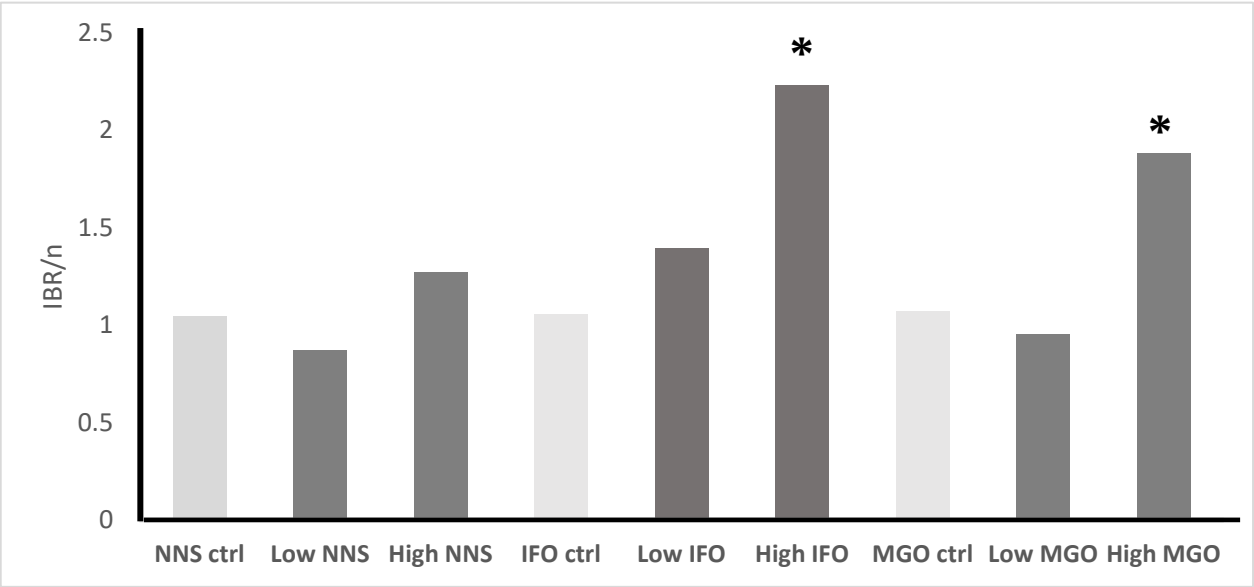


Figure 6: (a-f) Star plots constructed using five endpoints according to their hierarchical biological level representativeness for each experimental group of *A. tonsa*. The biological parameters were percentage of reproductive females (RF), egg hatching success (EHS), total lipid content (TL), *cyp 3026b* transcription levels (*cyp*) and *hsp70* transcript levels (*hsp70*). g) IBR/n index calculated based on the surface values within the area delimited by dotted lines in star-plots. Asterisks denote significant differences between control and treated groups according to *Z*-test ($p<0.05$).

contributed significantly to obtain a high IBR/n index value. Reduced egg hatchability and low total lipid amount explained the high IBR/n index value for the MGO WAF condition.

Discussion

In this study, a multilevel biological response approach was applied to assess the toxicity of three different oil WAF in the copepod *Acartia tonsa*. Adult mortality, female fecundity, egg hatchability and individual total lipid levels were analyzed in copepods exposed to a light crude oil (NNS) and two refined oils (IFO 180 and MGO). Besides,

transcriptional responses of members of gene families involved in ROS metabolism (*cat* and *gst*), stress and iron homeostasis (*hsp70*, *hsp90*, and *ferritin*), and xenobiotic metabolism (*cyp3026b*) were investigated. As expected, throughout the different endpoints studied, MGO WAF caused the most detrimental effects on the copepods considering mortality, percentage of fecund females, egg hatching success, and total lipid content. Conversely, MGO WAF produced relatively mild effects on gene expression levels. IFO 180 WAF was also toxic to copepods, resulting in increased mortality, a decreased percentage of fecund females, and significant effects on gene expression compared to control. In contrast, NNS WAF was the least toxic considering mortality, reproductive, lipid endpoints and the gene expression changes. The high doses of both IFO 180 and MGO WAF had a significantly higher IBR/n index than the other WAF conditions, demonstrating the toxicity of IFO180 and MGO to *Acartia tonsa*.

It was not surprising to observe MGO toxicity, as previous studies have highlighted the toxicity of MGO WAF to marine organisms. The larvae of the marine Pejerrey *Odontesthes argentinensis*, exhibited LC50 of 5.86% after 96 h exposure to MGO WAF (Rodrigues et al., 2010). Similarly, several studies showed that the observed toxicity of MGO on marine fish and crustaceans is due to the highly volatile BTEX compounds (benzene, toluene, ethylbenzene, and xylene) in the WAF (Gonzalez-Doncel et al. 2008; Neff et al. 2000). Indeed, WAF from crude oil derivatives (i.e., MGO) has higher BTEX content and additives (e.g., phenols and heterocyclic compounds containing toxic nitrogen and sulfur) than WAF from crude oils (Saeed and Al-Mutairi, 1999; Rodrigues et al., 2010). It has been reported that MGO WAF prepared by mixing at 1:40 (oil : water) ratio, contained BTEX compounds up to 1008 µg/L. Therefore, aside from PAHs, further chemical analysis of other types of hydrocarbons is necessary to better understand the WAF toxicity profiles

Several studies in copepods highlighted a decrease in the egg production rates due to toxic effects of oil WAF compounds (Hansen et al., 2015 and 2017; Olsen 2013). For example, copepods of the genus *Calanus* exposed up to 5.5 mg/L naphthenic crude oil, that is similar to the NNS crude oil tested in the present study, with mechanically and chemically dispersed conditions, showed reduced egg and nauplii production (Hansen et al., 2015). The copepod *Paracartia grani* showed decreased egg production under exposure to high doses of naphthalene (Calbet et al., 2007). Accordingly, the results of the present study demonstrated that both IFO 180 WAF and MGO WAF showed lower percentage of egg-laying females than the control. Indeed, only two females laid eggs in the high dose of IFO 180.

The egg production rate of fecund females did not vary among the studied WAF conditions and controls, suggesting a possible compensatory mechanism in exposed fecund female reproductive capacity. A compensatory effect in egg-laying was also described for the harpacticoid *Nitocra spinipes* exposed to musk fragrances (Breitholtz et al., 2003). In *Calanus glacialis*, a reduction in the number of reproductive females was described after exposure to WAF prepared with 10 µg/L crude oil without affecting the cumulative produced egg number, suggesting that egg-laying was at a normal rate, similar to controls (Jensen and Carroll, 2010), as shown in the present study for *Acartia tonsa*. Similarly, *C. finmarchicus* females exposed to 16.5 mg/L arctic crude oil WAF showed a reduction in the number of egg-laying females, but the number of produced eggs in the exposed females was not different from that of control females (Olsen et al., 2013). Furthermore, as shown in the present study, the four-day recovery period was not enough for the *Acartia tonsa* females to recover from the observed toxicity effects, suggesting a long-term effect of the exposure conditions. This observation is consistent with effects

detected in *Calanus* copepods, which showed signs of recovery after exposure to oil in fecundity after 20 days in clean water (Hansen et al., 2015 and 2017).

The females exposed to the high dose of MGO WAF showed lower lipid content levels than the non-exposed control females. Linking both lipid and egg viability endpoints suggested that the short exposure to MGO WAF affected egg development, lipid metabolism and lipid accumulation in females. In copepods, lipids sacs, when present, are often close to developing oocytes (Lee et al., 2006; Hansen et al., 2017). We have shown that in *Acartia tonsa*, the ovary is the main organ for lipid accumulation. Thus, it is likely that the disruption of lipid levels caused by exposure to MGO WAF resulted in decreasing the percentage of egg-laying females and egg-hatching success. Such observation is consistent with previous works on *Calanus finmarchicus* females showing that exposure to naphthenic crude oil reduced lipid sac size and egg-laying rate. In addition, egg hatching was negatively affected by maternal transfer of PAHs in lipid sacs (Hansen et al., 2017). In the marine copepod *Centropages hamatus*, long-lasting effects on egg viability were detected after oil WAF exposure (Cowles and Remillard, 1983), suggesting that the biosynthetic pathway involved in oogenesis was affected.

Although the expression patterns of *catalase, gst* and *hsp90* genes in oil WAF exposed copepods were not significantly different from those of non-exposed control copepods, the medium dose of IFO 180 showed a significant downregulation of the selected form of heat shock protein 70 gene (*hsp70*). Several studies showed up-regulation of some members in the heat shock proteins family in response to environmental pollutants (Yoshimi et al 2002; Rhee et al., 2009). However, fluoranthene, cadmium, nonylphenol, and octylphenol have shown to inhibit the expression of heat shock proteins (Werner et al., 1997; Rhee et al., 2009), as shown in the present work. Noteworthy, the sublethal concentrations of IFO 180 WAF contained fluoranthene, suggesting that fluoranthene

might be involved in the observed *hsp70* downregulation. We observed that the *cyp3026b* gene was up-regulated in *Acartia tonsa* after exposure to IFO 180 WAF. Such response was consistent with previous studies reporting that genes from the cytochrome P450 (*cyp*) clan 3 showed up-regulation in the copepods *T. japonicus* and *P. nana* after exposure to crude oil WAF (Han et al., 2014, 2015 and 2017). Although little is known about the regulation of the *cyp* genes of the PAHs detoxification process in invertebrates, there is some evidence of their involvement in the PAHs detoxification pathway (Han et al., 2014, 2015 and 2017). Indeed, several core sequence motifs (*e.g.*, xenobiotic response element, aryl-hydrocarbon response element, and estrogen response element) were described in the promoter regions of *cyp* clan 3 members of *T. japonicus* and *P. nana* (Han et al., 2014; 2015 and 2017). We advocate that the forms of *hsp70* and *cyp3026b* genes used in this study can be suitable biomarker genes for estimating the toxic effect of WAF exposure because they showed early responses after oil WAF exposure conditions. The other assessed genes involved in ROS and stress dependent responses (*hsp90*, *ferritin*, *gst*, and *catalase*) showed variability in their transcription profiles, but did not differ to levels determined in controls. Future transcriptomic studies such as, RNA-seq, can provide a further comprehensive view of changes in gene expression.

Conclusions

In conclusion, the results of the present work demonstrated that exposure to IFO 180 and MGO WAF had harmful effects on *Acartia tonsa*. The NNS showed less toxicity in comparison to refined oil WAF. The biological multi level approach applied in the present study, highlights the importance of integrating the effects elicited by environmental pollutants at different biological levels. The combination of multilevel biological endpoints provided a comprehensive picture of how an oils (crude and refined) affect

Acartia tonsa individuals. Suppression in egg spawning was observed in treated females, but the egg-producing females still produced eggs at rates similar to controls, probably as a results of unknown compensatory mechanisms. However, in the case of high doses of MGO WAF, the females produced non-viable eggs which could be explained as a consequence of altered lipid levels in exposed females. In addition, transcriptional levels of the forms of *Cyp3026b*and *hsp 70* used in this study could be potential biomarkers of oil WAF exposure in *Acartia tonsa*. Metabolic, reproductive and transgenerational toxic effects detected in copepods exposed to refined oil WAF raise concerns about the risks of copepod communities in regions with high maritime activities such as harbors and high maritime traffic routes. Such areas can have concentations of PAHs at similar levels to those used in this study. Disruption in the copepod population can strongly impact the whole food web by transferring the effects to the polluted ecosystem's higher trophic levels.

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Chapter 2: Toxicity effects Of Naphthenic Crude Oil Water Accommodated Fraction with and without Dispersant on *Acartia tonsa*

This chapter has been presented in the 19th international symposium on Pollutant Responses in Marine Organisms (PRIMO). Matsuyama, Japan

Abstract

Zooplankton, such as copepods, plays a key role in the marine food web, being the primary consumers of phytoplankton and a rich energy source for other invertebrates and vertebrates. Alterations in copepod populations after oil spills have been reported at several places in temperate waters. In this study, we investigated the effects on mortality, fecundity and gene expression of a naphthenic crude oil Water Accommodated Fraction (WAF) prepared alone or in combination with a commercial dispersant (Finasol 52) on the model species *Acartia tonsa*. Toxicity assays (96 h) were carried out on adult copepods and LC50 values were calculated. Gravid females were exposed to WAF or NNS WAF+ Dispersant for 7 days, then transferred to clean water for 3 days (to mimic an exposure and recovery scenario) to assess effects on fecundity. The reproductive endpoints investigated were: egg laying, egg production rate, egg hatching success and the number of productive females. In addition, relative transcript levels of key genes involved in oxidative stress and xenobiotic metabolism were determined. According to the lethality test, the WAF + Dispersant mixture was the most lethal toward copepods. In terms of egg production rate, female copepods showed the highest disrupted fecundity after exposure to NNS WAF+ Dispersant conditions. However, neither the percentage of fecund females nor the egg hatching rate after maternal exposure were affected by WAF or NNS WAF+Dispersant exposure. In addition, 10% WAF + Dispersant treatment showed significant transcription level of hsp70 gene. Principal component analysis (PCA) showed that WAF treatment and 10% NNS WAF+Dispersant had different transcription pattern than that of control. In conclusion, results show that NNS WAF+Dispersant was more toxic toward *Acartia tonsa* than WAF alone.

Keywords: *Acartia tonsa* Water Acommodated Fractions, Toxicity, Fecundity, Gene transcription, Dispersant.

Introduction

Overexploitation of oil resources resulted in the boost of the maritime traffic, expansion of oil extraction platforms and the construction of new oil transportation pipelines. Consequently, there is an increasing risk of oil spill accidents towards the marine environment.

During an oil spill, the oil slick can be mitigated with several cleanup techniques such as the *in- situ* burning of oil (Mullins et al., 2003), introduction of nitrogen containing fertilizers to stimulate the biodegradation bacteria *on site* (Atlas, 2011) and using sorbent like materials such as polypropylene to absorb oil slick (Bayat, 2005). Another technique to emulsify the oil slick during spill accidents is the introduction of chemical dispersant agents. Dispersants split the oil into smaller droplets, increasing the surface area of the oil vesicles and making the oil more bioavailable (Kliendienst et al., 2015). The increased surface area and bioavailability facilitate bacterial biodegradation of the oil droplets (Sun et al., 2019). For example, more than 7 million liters of chemical dispersants have been used during the Deepwater Horizon well blowout in order to stimulate the bacterial biodegradation of oil (Kleindienst et al., 2015). However, several studies have demonstrated that dispersant is toxic for several marine organisms (Mitchell and Holdway, 2000; Lee et al., 2013; Penagos et al., 2020; Kastumiti et al,. 2019).

Water Accommodated Fractions (WAF) are hydrocarbon fractions with low molecular weight that with adequate wave dynamics and weathering processes will be soluble in water. Hydrocarbons in WAF are more bioavailable for marine organisms (Barron, 2003). WAF contains harmful aromatic hydrocarbons such as benzene, toluene, xylenes and

polycyclic aromatic hydrocarbons (PAHs). PAHs have deleterious effects on cellular metabolism and function (Han et al., 2014). For example, PAHs form DNA damage by forming DNA adducts which cause cancer (Xue et al., 2005). In addition, PAHs increase the generation of reactive oxygen species (ROS) in organisms, which result in oxidative damage on lipid, nucleic acids and proteins (Dvorak et al., 2021). Consequently, cells respond to this oxidative stress through the activation of antioxidant enzymes, which interact with the ROS and protecting the cells from oxidative stress (Kim et al., 2011).

Copepod dominated zooplankton play a vital role in the food chain of marine communities by transferring energy from primary producers (phytoplankton) to secondary and tertiary consumers (i.e., planktivorous fish) (Ustun, 2014). Because of the copepod's ecological importance, any disruption in their populations have adverse effects on the whole food chain cycle and therefore disrupt the entire web fauna (Darnis et al., 2012). Similar to any other organisms, copepods are vulnerable to contaminants that are released in oil spills as water-soluble fractions. The effect of water-soluble oil fractions on selected species of copepods in terms of mortality, development, egg production and motility is well established (Bejarano, 2006; Avila et al., 2010; Lee et al., 2013). In copepods, several genes, such as glutathione S transferases (GST) catalase, and Heat shock proteins have been considered as valuable biomarkers for cellular stress due to ROS (Zhou et al.,2020; Hansen et al., 2008). Additionally, members of the superfamily of cytochrome P450 genes have been considered as valuable biomarkers for WAF and oil exposure due to their roles in the biotransformation of a wide range of compounds (Rhee et al., 2013). Until now, little is known about cytochrome P450 detoxification functions in copepods. However, some *cyp* genes, members of the clan 3, have been shown to be WAF sensitive in *Tigriopus japonicus* and *Paracyclopina nana* (Han et al., 2014).

This work aims to investigate the toxicity profile of WAF and WAF with dispersant (NNS WAF+ Dispersant) using the model organism *Acartia tonsa*. *Acartia tonsa* short life cycle, easy maintenance and the high continuous ability to produce eggs, make it a suitable candidate for toxicological assays (Gorbi et al,. 2012). Cosequently, *Acartia tonsa* has been studied after exposure to different contaminants and showed sensitivity to several toxins (Bellas et al,. 2007, Pedroso et al., 2007; Lauer et al., 2010). Freshly prepared Water accommodated fraction from a Naphthenic North Atlantic sea oil was tested mimicking an oil spill scenario. In addition, the commercial dispersant Finasol 52 was added to the WAF was also tested as a measure of remediation procedure after an oil spill.

The effects of the WAF and NNS WAF+Dispersant to copepods will be studied using different biological endpoints. Lethality will be primarily investigated by calculating the lethal concentration (LC50). Afterward, reproductive capabilities after exposure to sub lethal concentrations will be studied through investigating different endpoints such as: the number of reproductive females, the egg reproduction rate and the percentage of successfully hatched eggs. Finally, studying different transcriptome levels of genes related to PAH exposure and oxidative stress will highlight the exposure effects at molecular level.

Materials and methods

Preparation of the Water Accommodated Fractions (WAF)

Two conditions of WAF were prepared for the exposure experiments. 1) Fresh prepared WAF and Dispersant added to WAF (NNS WAF+ Dispersant). The soluble fractions were made from a naphthenic North Sea crude oil provided by the Norwegian University

of Science and Technology (NTNU) (Trodheim Norway) within the framework of the GRACE project. Naphthenic North Sea oil (NNS) is a naphthenic based light crude oil. It is characterized by a low aromatic content, a low wax content, a low viscosity index (299 cP at 2°) and a density of 0.900 g/ml. In addition, it is rich in branched and cyclic aromatic hydrocarbons (Katsumiti et al., 2019). The dispersant selected for this study was Finasol 52 (Total oils, France). Finasol 52 is a second generation dispersant composed of a complex mixture of surfactants and petroleum distillates (Katsumiti et al., 2019). Detailed information regarding the composition of Finasol 52 dispersant is presented in Table 1 (Total oils, France).

Table 1: Chemical composition of dispersant Finasol 52 obtained from Total oils, France

Chemical name	**Percentage weight %**
docusate sodium	20-25%
Hydrocarbons, C11-C14,n-alkanes, isoalkanes, cyclics,<2% aromatics	15-20%
(2-methoxymethylethoxy) propanol	15-20%
Carboxylic acids, di, C6-12 cmpds, with ethanolamine, boric acid cmpd with ethanolamine	0-2%
Ethanolamine	0-1%

Preparation of WAF was similar to the protocol described by Singer et al (2000) but with slight modifications. Briefly, crude oil was added to filtered seawater at a ratio of 5 g of

oil to every 1 liter of water. The oil, water mixture was stirred for 42 hour at 10° C and under dark conditions to avoid photo oxidation of oil elements using a magnetic stirrer without creating vortex. Serial dilutions were prepared for the WAF. For NNS WAF+Dispersant, the commercial dispersant Finasol 52 was added first to the oil at a ratio of 1 g of dispersant to every 10 g of crude oil, gently mixed, then WAF was produced as mentioned before.

Copepod culture

Different generations of *Acartia tonsa* were obtained from a continuous culture established for six months in the aquaria facility of PiE (UPV/EHU). Copepods were cultured in a 10 L tank with 0.1 µm filtered seawater at a temperature of 18°C, salinity of 30 Practical salinity unit and constant photoperiod of 16:8 light/dark cycle. Copepods were daily fed with a mixture of the brown micro algae *Isochrysis galbana* and the green microalgae *Tetraselmis chuii* at a ratio of 1:1

Lethal toxicity assays 96 hours:

Lethality tests were conducted according to the international standard for water quality, for the determination of acute lethal toxicity to marine copepods, (ISO14669). For each condition of WAF, 30 adult copepods were exposed in 250 ml glass concentrations containing 50 ml of filtered seawater and WAF conditions for up to 96 hours. Lethal assays were done in triplicates and total of five dilutions were assessed in each WAF condition. The tested WAF doses were: 10%, 30%, 50%, 70% and 100%. NNS WAF+DISPERSANT doses were initially considered to be the same for the NNS WAF. However, the first experiment showed 100% lethality of the WAF +D at 30% dose. As a result, the selected doses for NNS WAF+Dispersant assay were 5%, 10%, 15% ,20% and 25%

Each day of exposure, replicates were checked for mortality and dead copepods were removed. A copepod was considered dead if it didn't move shortly after a gentle stimulation using a pipette. Similar to the works done by Jiang et al. (2012) copepods were not fed during the exposure period and no gender preference was considered when selecting adult copepods for the experiment. The LC50 results from the toxicity assays determined the sub lethal concentrations to be used in further assays.

Fecundity and egg hatching assays:

Exposure doses which were selected ranged from low to very high sub lethal concentrations. For NNS WAF, the selected exposure doses were 10%, 30% and 50% NNS WAF+Dispersant exposure were 7.5%, 12.5% and 20% . In each treatment, 20 adult females were selected under a dissecting microscope. In addition, five adult males were included in each treatment to assist in maintaining female fertility throughout the whole experiment (Holste and Peck, 2006). The fecundity assay was divided into three consecutive stages: exposure stage, recovery stage and individual female fecundity test stage. This allows to test for the delayed effects caused by exposure scenarios.

In the exposure stage, females were transferred to 300 ml glass tanks filled with the different WAF conditions and left for seven days. Each day, eggs and dead copepods were removed. Every two days, 150 ml of the experimental tanks was replaced and tanks cleaned from fecal pellets and dead algae. Copepods were fed daily with the algae *Tetraselmis chuii* throughout the whole experiment at a concentration of 3000 cells per ml. During the recovery stage, the surviving copepods were gently transferred to filtered clean seawater for four days. Similar to the procedure during the exposure stage, the eggs were daily removed and water was replaced every two days.

In the individual fecundity experimental stage, the surviving females were transferred to incubating chambers containing filtered clean seawater for three days. Incubation chambers were constructed similar to the device proposed by Kleppel et al (1998). The system was divided into two chambers (spawning chamber and brooding chamber). The upper spawning chamber was a 10 cm in diameter plastic tube, which housed one female for egg production. A 200 µm mesh was attached at the base of the tube to prevent female individuals from entering the bottom compartment where eggs are stored. Each day, eggs were counted and copepods were fed at the same algal concentration mentioned before. Recently laid eggs from each treatment were pipetted to individual petri dishes containing seawater to allow to hatch. Hatched eggs were counted and unhatched eggs after 48 hours were considered non viable.

Gene transcription analysis

Exposure experiments

Adult copepods were exposed in five replicates, with each replicate containing 35 adult copepods exposed to 150 ml of NNS WAF or NNS WAF+Dispersant solutions for 48 h. Two exposure doses were chosen per WAF condition: NNS WAF (25% and 50%), NNS WAF+Dispersant (10% and 20%) . The copepods were fed daily with *Tetraselmis chuii* at a concentration of 3000 cells per ml, and no gender preferences were considered during copepod collection for exposure. After 48 h of exposure, the copepods were transferred to cryovials filled with RNA later solution (AMBION, USA) and stored at -80ºC until RNA extraction.

RNA extraction and cDNA synthesis

RNA was extracted using RNeasy Minikit (Qiagen, Germany) following the manufacturer protocol. Total RNA was treated with DNase-1 using the DNA-free kit

(Invitrogen, USA) to remove traces of genomic DNA. RNA integrity and concentration were measured using the Bioanalyzer 2100 (Agilent Technologies, USA). All RNA samples obtained a RIN score higher than 7, suitable for downstream qPCR analysis (Fleige et al., 2006). RNA purity was determined by measuring sample absorbance ratios of 260/280 nm using a UV-spectrophotometer (Epoch, Biotek, USA). All samples had ratios between 1.8–2.3. cDNA was synthesized from purified RNA using the AffinityScript Multiple Temperature cDNA synthesis kit (Agilent Technologies, USA) with random primers and following the manufacturer's protocol.

Primer design

Catalase (cat), glutathione S transferase (*gst),* heat shock protein 90 (*hsp90*) *, heat shock protein 70 (hsp70),* and *ferritin* genes were targeted as biomarkers of cellular oxidative stress and PAH metabolism. Cytochrome p 450 gene (*cyp*) from clan 3 (*cyp3026b*), a biomarker for PAH metabolism, was analyzed. Primers for *cat*, *cyp3026b* and *gst* were designed *de novo* from conserved regions in closely related crustacean species and scaffolds from the *Acartia tonsa* genome project (Jorgensen et al., 2019) (Accession number PRJEB20069). Clustal Omega (Madiera et al., 2019) was used for multiple sequence alignment. For *cyp3026b, Acartia tonsa* genome sequence LS054218.1 was aligned with the gene sequences from *Tigriopus japonicus* (KF639998.1) and *Eurytemora affinis* (XM 023493699.1). For catalase, the genome sequence LS054460.1 was aligned with sequences from *Calanus finmarchicus* (EL965956.1) and *Eurytemora affinis* (XM023472406.1). For *gst,* the genome sequence LS085988.1 was aligned with *Tortanus forcipatus* (KT755427.1) and *Acartia pacifica* (KT754520.1). Specific primers to amplify fragments of *hsp70*, *hsp90* and *ferritin* genes were obtained from Nilsson et al (2014). *β-actin* gene was used for transcription normalization as previously proposed (Zhou et al. 2020), showing in the current experiment a low coefficient of variability (*CV* < 5%). The

identity of all targeted genes was confirmed by sequencing (General Genomics Service Sequencing and Genotyping Unit, SGIker, UPV/EHU). Primer sequences and the primers' amplicon regions' multi-sequence alignments are presented in Table 2 and (Supplementary Material chapter 1, figure S1) respectively. Amplification efficiency for each primer pairs was calculated by conducting a 1:2 serial dilution curve generated from pooled experimental samples. All primer pairs showed amplification efficiency between 90% and 100%.

Table 2: Primer sequences and melting temperature (Tm) for all the genes analyzed in this study.

Gene	Primer sequences 5' to 3'	Tm (°C)	References
hsp70	*Fw*: TTCAATGATTCACAGAGACAAGC	57.1	Nilsson *et al.*, 2014
	Rv :TCCTTGTGATGTTAAGACCAGCTAT	59.7	
hsp90	*Fw*:GTCACATCCCAGTATGGTTGG	59.8	Nilsson *et al.*, 2014
	Rv :CCATGGTGGAGGTGTCACGG	63.5	
ferritin	*Fw*:ACGCTTGCACTGATAATCCA	55.3	Nilsson *et al.*, 2014
	Rv :AGTTCTACCGTGACGCATCC	59.4	
β-actin	*Fw*:TTGGGTATGGAGTCCTGTGG	59.4	Zhou *et al*., 2020
	Rv :CCTGGATACATAGTGGTGCC	59.4	
cyp3026b	*Fw*:TGCCATTGGCATCCACCATGAC	62.1	Current study
	Rv:AGCCTCCAGCAAAGCAAACCTC	62.1	
gst	*Fw*:TGCTTGATTCACTTCTACAAGAGA	57.6	Current study
	Rv:GTCACCATCAACAACAGTTGGA	58.4	
cat	*Fw*:GGAGAGAATCCCAGAAAGAGTG	60.3	Current study
	Rv:ACAGTCCAATTTCCCCTCCC	59.4	

RT-qPCR

Real-time quantitative PCR was conducted using FastStart SYBR Green fluorescence dye (ROCHE Diagnostics, Switzerland) with ROX passive dye included in the mix. The reaction medium (25 µL) consisted of 12.5 µL FastStart SYBR Green master mix, 0.3 µL forward primer (800 nM), 0.3 µL reverse primer (800 nM) and 3 µL diluted cDNA template (1:5 v/v). qPCR was performed in 384 optical well plates (Applied Biosystems, USA) using ViiA 7 Real-Time PCR system (Applied Biosystems, USA). The amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 second, and then 1 minute of annealing at the corresponding Tm for each gene (Table 2). A dissociation curve was performed after the PCR cycles to determine the product specificity and check the absence of primer dimers. All qPCR assays were conducted in triplicates, and a non-template control (NTC) was included for each plate.

Data analysis

Mortality of copepods at different timeframes (48 ,72, 96 hours) from the lethal toxicity assay were analyzed using PROBIT analysis (Vincent, 2008) to calculate the LC 50 of different WAF conditions. Data from egg production rate and egg hatching success were initially checked for normality using Kolmogrov-Smirnov test. Numbers of eggs produced during the exposure phase and the egg production rate of females (eggs $Females^{-1}$ day^{-1}) during the individual female fecundity stage were analyzed by one way ANOVA. Significant differences were further analyzed using Dunnett's and Tukey Kramer post-hoc tests (IBM SPSS v26). Percentage of reproductive females was analyzed by Chi-square test. Egg hatching success during individual fecundity test was calculated as follows: (number of hatched eggs per female /Total number produced per female) x100; average results per treatment were then analyzed using one -way ANOVA.

Finally, relative gene transcription levels were determined using the Pfaffl method (Pfaffl et al., 2001). The transcript levels were log2 transformed and statistical differences among experimental groups and their respective controls were determined using one-way ANOVA. The Dunnet post-hoc test was further used to determine the statistical difference compared to control (IBM SPSS v26). A principal component analysis (PCA) for transcription results integration was performed using PAST statistical software version 4.03 (Hammer et al 2020)

Results

Table 3: Lethal concentration values (LC50) determined at 48, 72, 96 hours with adult *Acartia tonsa* exposed to the different WAF conditions (*n*=30). Number in parenthesis denote 95% confidence intervals. Letter superscript denote significant difference based on the absence of confidence interval overlap

OIL WAF	**48hr LC50**	**72hr LC50**	**96hr LC50**
NNS WAF	>100% (95-176)[a]	74% (61- 94)[b]	52% (40- 65)[b]
NNS WAF +Dispersant	34% (24 - 100)[a]	31% (24 – 55)[a]	21% (11.7 – 63.5)[a]

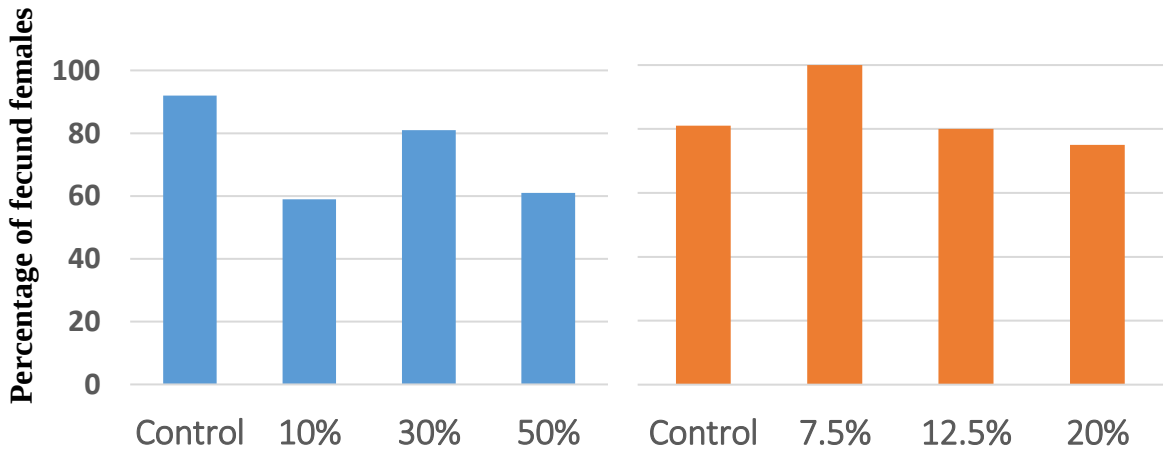

Figure (1): Percentage of egg producing females after the 7 days exposure to NNS WAF (blue) and NNS WAF+Dispersant (orange). Females having more than 2 eggs were considered as a reproductive female. Fisher exact test showed no significant association between the reproductive females and the NNS WAF & NNS WAF+Dispersant

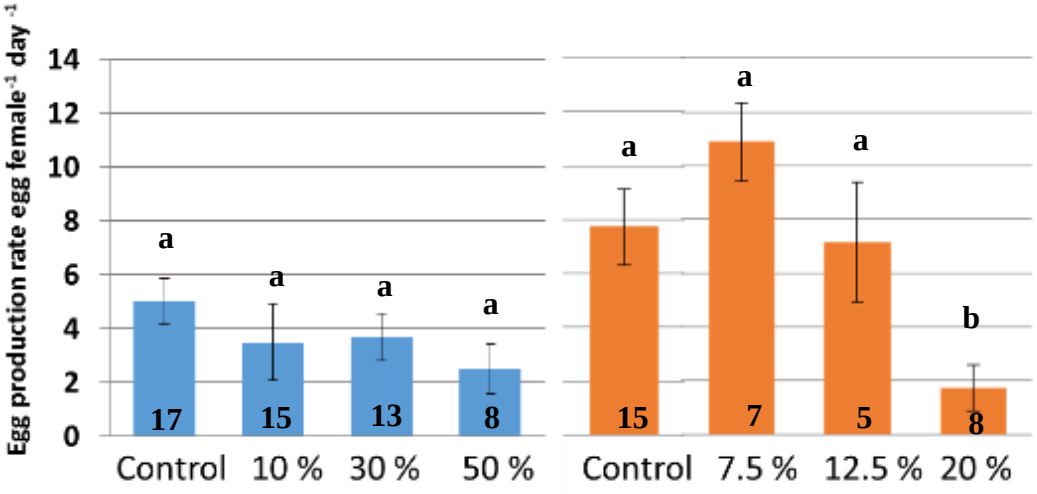

Figure (2): Average egg production rates of reproductive females (number of eggs per fecund female^{-1} day $^{-1}$) during the three individual experimentation days of NNS WAF (blue), NNS WAF+Dispersant (orange). Numbers within each bar indicate the total number of reproducing females in the experimental group. Different letters denote statistically significant differences among groups according to one-way ANOVA followed by Tukey Kramer post hoc-test ($p<0.05$). Graphs show mean ± standard error.

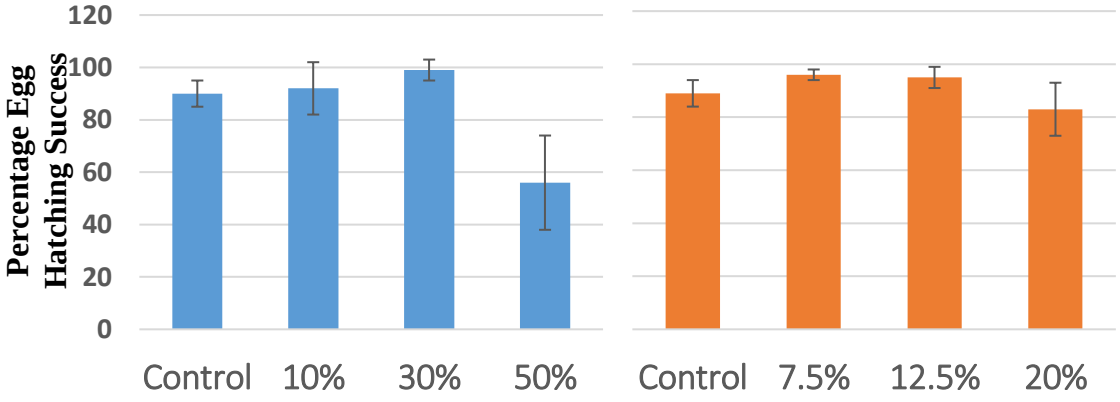

Figure (3): Egg hatching success during the three experimental days after exposure to WAF (blue) and NNS WAF+Dispersant (orange). Graphs show mean ± standard error.

The PROBIT analysis (Table 1) showed that the mixture of NNS WAF+Dispersant had the highest lethality on adults *Acartia tonsa*. LC50 values were as low as 34%, 31% and 21 % of NNS WAF+Dispersant dose during the 48, 72, 96 hours, respectively. Compared to the NNS WAF+Dispersant mixture, NNS WAF showed to have lower lethality on *Acartia tonsa* adults. LC50 values for NNS WAF were above 100% at 48 hours, and 74 % at 72 hours and 52% at 96 hours.

After the end of the exposure and recovery periods, adult females were individually placed in incubation chambers for three days to check the female reproductive capabilities. Females producing more than 2 eggs during the three days of the experimentation period were the only ones considered reproductive. Chi-square analysis showed that there was no significant association between the percentage of reproductive females and the doses of NNS WAF or NNS WAF+Dispersant (figure 1), suggesting that exposure did not affect the ability of laying eggs in adult females.

The number of surviving and reproductive females after exposure and recovery stage are presented in Figure 2. The egg production rate of females exposed to NNS WAF (Figure 3a) showed a slight dose dependent reducing trend, although not significant. In contrast, females exposed to 20% NNS WAF+Dispersant had a significant reduction in egg production rate compared to other experimental groups ($p<0.05$). None of NNS WAF and NNS WAF+Dispersant did significantly affect egg hatching (Figure 4).

High variability in relative gene transcription levels was observed. According to the expression patterns of the biomarkers, NNS WAF treatments and the 10% treatment of NNS WAF+Dispersant showed to have transcription levels different from the control. Antioxidant enzyme coding genes, *cat* and *gst*, showed noticeable down regulation in the

10% NNS WAF+Dispersant treatment (Figure 4 a & b). In addition, *hsp70*, showed significant transcription downregulation, although non-significant, in the 10% NNS WAF + Dispersant treatment (Figure 4,d). *Hsp*70 also showed downregulation in expression levels at 25% NNS WAF treatment. Ferritin, *hsp 90* and *cyp 3026b* did not show any significant changes expression patterns compared to controls (figure 4). The principal component analysis explained the variability of combined transcription levels with two components. Both axes explained 91% of the total variance, with the first axis explaining 73% of the total variance, while the second axis explained 18% of the total variance. *Hsp 70, ferritin*, *catalase* and *gst* showed to be the highest positive correlation with the first component. Whereas *hsp 90* and *cyp3026b* were negatively correlated with the first component. *Ferritin* and *hsp70* were weakly negatively correlated with the second component, while *gst*, *hsp90, cyp3026b* and *catalase* were positively correlated with the second component (Table 3). Upon looking at the expression patterns of the different genes, the two WAF treatment groups were clustered together, suggesting similar expression patterns and different from control. However, 10% NNS WAF +Dispersant treatment showed to be different from control, WAF treatments and 20% NNS WAF+Dispersant. Interestingly, treatment of 20% NNS WAF +Dispersant was closely clustered with control, suggesting similar transcription patterns.

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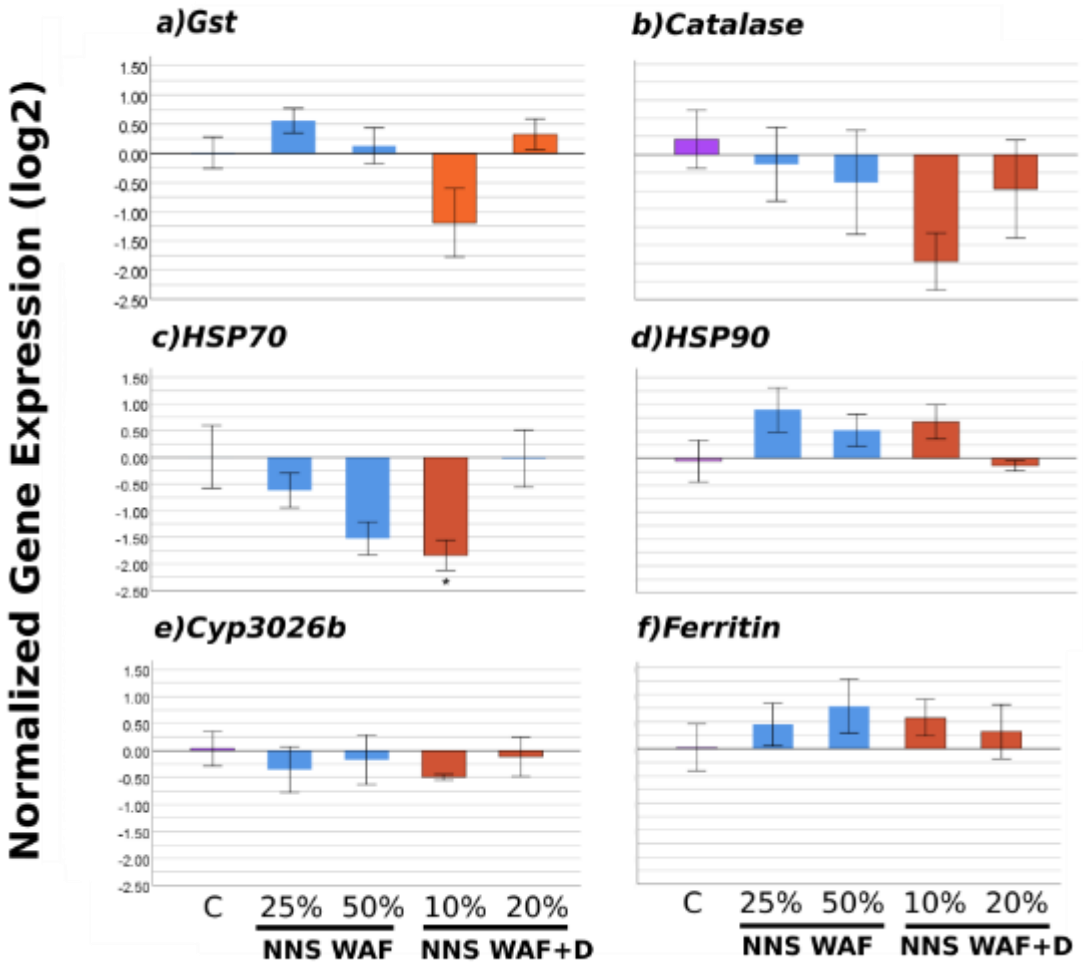

Figure(4): Relative gene transcription levels (RQ) for a) Glutathione S-transferase (*gst*), b) Catalase (*cat*), c) Ferritin, d) Heat shock protein 70 (*hsp70*), e) Cytochrome P450 3026b (*cyp3026b*) and f) Heat shock protein 90 (*hsp90*). Purple bar is control, blue is NNS WAF doses and orange NNS WAF+Dispersant doses. Transcription levels are represented in log2 scale (mean ± standard error). Asterisks denote statistically significant differences from the corresponding control according to one-way ANOVA followed by Dunnett´s post hoc-test ($p<0.05$)

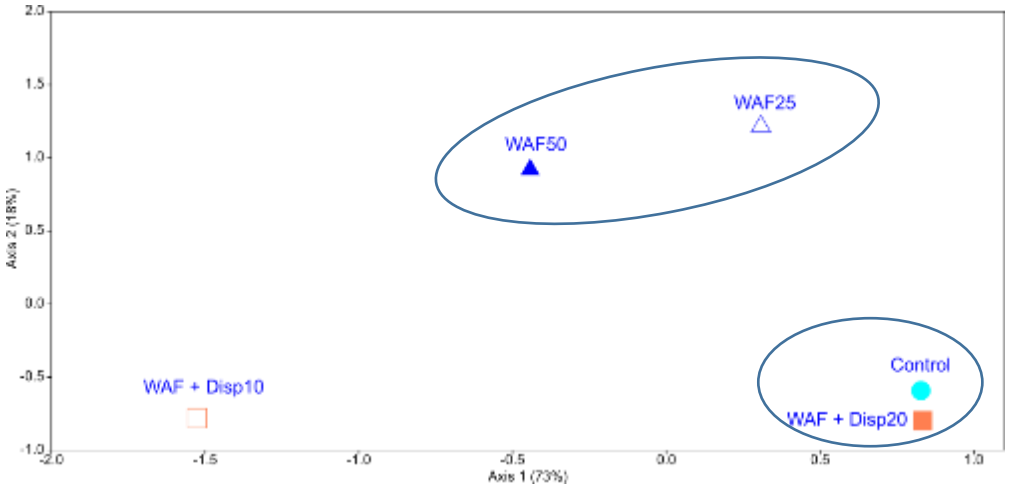

Figure (5): Principal Component Analysis (PCA) of the combined transcriptional patterns of the different biomarker genes used in this study.

Discussion:

In this study, we investigated the toxicity of NNS WAF with and without the addition of a commercial dispersant (Finasol 52). NNS WAF+Dispersant had the most negative effect on the copepod *Acartia tonsa*. Such toxicity was highlighted by the low LC50, the low egg production rate at high doses and the changes in gene expression patterns such as the significant downregulation of *hsp* 70 gene. However, NNS WAF showed to have a slight, non-statistically significant, response regarding some of the reproductive and molecular endpoints.

During the last decades, the use of dispersants showed its efficiency in dispersing oil slicks into small droplets and minimize the oil slick area in several oil spill incidents, thus facilitating natural oil degradation (Noirungsee et al., 2020). However, it has been shown that the toxicity of dispersants to organisms can be due to the effects of surface active components on the biological membrane of organisms. In addition, exposure to dispersants has been described to cause the disruption of respiratory cells resulting from electrolytic and/ or osmotic imbalance (Wolfe et al., 2001). Oil dispersed with chemicals

can be more toxic than oil or dispersant alone as dispersants increase the concentration the dissolved PAH in the water column (Lyons et al., 2011). Lee et al (2013) observed a similar pattern of lethality when studying the effects of crude oil WAF with and without the addition of Corexit 9500 on the rock pool copepod *Trigriopus japonicus*. WAF+Dispersant had 96 hours LC50 values as low as 45% whereas the 96 hours LC50 of WAF was higher than 100% dose (Lee et al.,2013). Almeida et al. (2014) showed that *Acartia tonsa* exposed to dispersant treated emulsions had higher mortality than adults exposed for 48 hours to crude oil emulsion alone. Additionally, dispersant added to crude oil emulsions decreased *Acartia tonsa's* egg production rates and affected naupilar development (Almeida et al., 2014). Aside from direct toxicity, oil + dispersant cause narcosis and affect the feeding behaviors of the adult female copepods, causing decrease in egg production rates as a consequence

WAF with and without dispersant did not have significant effect on the number of fecund females, as most of the females in all NNS WAF treatments produced eggs. In addition, considering the production of viable eggs, the eggs obtained from almost all exposed adult females showed high percentage of hatching success. Olsen et al. (2013) also observed high egg hatching success from C*alanus finmarchicus* exposed to dispersed crude oil droplets. Our results suggest that the WAF conditions did not have a significant effect on the female capability to produce viable eggs. However, further transgenerational studies are needed to investigate whether offsprings develop normally. Bejarano et al (2006) demonstrated with a full life cycle experiment on the meiobenthic copepod *Amphiascus tenuiremis* that up to 50% WAF from light crude oil had detrimental effects on the development time and fecundity of the exposed females. Our results also highlight that the WAF conditions mainly affected the eggs production rates. In addition, the significant reduction of egg production rate in NNS WAF+Dispersant exposure in the individual

fecundity stage even after the four days recovery period, suggest that the toxic damage from the exposure stage can be long-termed and persistent. Such toxic effects after exposure period highlights that the toxicity is still persistent even after the WAF remediation from the environment or after the migration of the copepods from the WAF polluted area during for example vertical migration (Calbet et al., 2007).

Studying the co-expression of different genes involved in oxidative stress and xenobiotic metabolism provides a valuable insight on gene co-regulation patterns in *Acartia tonsa* in response to the exposure to NNS WAF or NNS WAF+Dispersant. PCA showed that both NNS WAF and NNS WAF+Dispersant have distinct expression profiles from each other (Figure 5), suggesting thus different cellular toxicity effects. The co-downregulation patterns exhibited in 10% WAF +Dispersant might suggest a decrease in the cellular antioxidant machinery, which probably result in the over accumulation of ROS in cells (Dvorak at al., 2021). Similar to our findings, the intertidal copepod, *Tigripus japonicus*, showed significant downregulation in the expression levels of *gst* after exposure to 6.25 μM of PCB (Lee et al., 2006).

Interestingly, the transcription patterns of NNS WAF+Dispersant exposed adults were showing a non monotonic response, in which the effects of the high dose cannot predict the effects of low doses (Bicho et al., 2016). We hypothesize that the exposure to 20% NNS WAF+Dispersant dose had narcotic effects on *Acartia tonsa*, resulting to low metabolism and therefore affected reproduction capabilities and gene transcription levels. Similar to the results we observed, PAHs in WAF was shown to narcotize the copepod *O. davisae* that result in severely impaired behavioral and reproductive responses (Barata et al., 2005). Several contaminants, such as fluoranthene, cadmium, nonylphenol, and octylphenol, inhibit the expression of heat shock proteins, which consequently reduce the cellular cyto-protective mechanisms (Werner et al., 1997; Rhee et al., 2009). Compared

to WAF alone, dispersant increase the bioavailability of petroleum hydrocarbons in water (Yamada et al., 2003). Therefore, we speculate that the addition of dispersant increased the bioavailable concentrations of PAHs, such as fluoranthene, possibly resulting to *hsp70* downregulation. However, to fully understand the toxicity effects of dispersants chemical analysis of WAF +Dispersant is needed .

Conclusions

In conclusion, WAF+Dispersant had marked effects on the adult *Acartia tonsa* at different biological levels. As shown, WAF+Dispersant had detrimental effects on *Acartia tonsa* in respect of mortality, reproduction capabilities and caused cellular stress. Therefore, we speculate that exposure to WAF+Dispersant disrupt the population of *Acarita tonsa* by inhibiting reproduction, which might disrupt the whole food chain. Transgenerational studies will provide further information on the effects of contamination on the different generation of *Acartia tonsa* after exposure.

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Chapter 3: Microbial community dynamics in Crude oil Water Accommodated Fraction and Water Accommodated Fraction + Dispersant contaminated cold seawater

This chapter has been presented in the *30th SETAC Europe online annual meeting*

Abstract

We investigated the bacterial community dynamics in cold water contaminated by polycyclic aromatic hydrocarbons (PAH) in presence or absence of Dispersant (Finasol 52). Water-accommodated fractions (WAF) were prepared with Naphthenic North Sea (NNS) crude oil in order to contaminate seawater from Norwegian cold sites, one oil-polluted and the other pristine. The WAF-contaminated waters were maintained in microcosms with and without Dispersant at 4° C for 21 days. PAH contents and bacterial compositions (16S rRNA gene sequencing) were determined at days 0, 7, 14 and 21. In addition, the 96 hours toxicity assay with adult *Acartia tonsa* was performed at days 0 and 21. The presence of Dispersant increased the toxicity against *Acartia tonsa* but facilitated the reduction of the toxicity at day 21 although the PAH removal was not enhanced by the presence of Dispersant. The highest PAH removal was obtained with the water from the oil-polluted site revealing the legacy effect, the presence of adapted microorganisms to the presence of PAH. The PAH removal was accompanied by bacterial community changes during the incubation, revealing: i) the plasticity of the bacterial community to adapt to the presence of PAH and Dispersant, ii) the presence of specific bacterial taxa probably involved in PAH degradation, and iii) the effects of Dispersant in shaping the bacterial communities dynamics through the rapid stimulation of certain potentially degrading Dispersant taxa, such as *Fusibacter*. Thus, our results provide valuable insights on the role of bacterial community in determining the fate of water solubilized hydrocarbon in cold environments questioning the role of Dispersant used for fighting oil spill.

Keywords: Water Accommodated Fractions, Dispersant, Cold water, Microbial communities, Bioremediation, Crude oil.

Introduction

Cold areas, such as the sub arctic area, are increasingly threatened by human activities due to climate change effects opening novel sea lines (Farrington, 2014). Particularly, the Arctic seas are exposed to oil-related activities presenting a high risk of oil spill accidents (Vergeynest et al., 2019). Among hydrocarbon compounds, polycyclic aromatic hydrocarbons (PAH) are the most toxic, their fate in marine ecosystems being dependent largely on microbial activities and abiotic weathering processes (for review see, Duran and Cravo-Laureau, 2016). The abiotic weathering processes, including mechanical actions (e.g. wave movements) and chemical reactions (e.g. photo-oxidation), favor PAH solubility, especially low molecular weight PAH (LMW-PAH), increase the hydrocarbon bioavailability and thus toxicity (Duran and Cravo-Laureau, 2016; Neff et al., 2005). In order to simulate weathering processes, scientists are using water-accommodated fractions (WAF), which contains harmful aromatic hydrocarbons, aliphatic compounds, and PAHs (Jiang et al., 2012). WAF is considered as useful model for studying oil spills dynamics, determining the fate of solubilized hydrocarbons, their toxicity and degradation by microorganisms (Faksness et al., 2020). Because the oil recovery is difficult in ice-covered waters, biodegradation by microorganisms remains the most promising alternative in oil spill response (Vergeynest et al., 2019). In cold environments, the PAH biodegradation by microorganisms is limited by the low temperature and the seasonal variation of nutrient availability (Vergeynest et al., 2019; Brakstad et al., 2008). Therefore, further knowledge on microbial hydrocarbon degradation in cold conditions is required in order to develop adapted microbial management strategies for fighting oil spills. The use of Dispersants is a strategy implemented to stimulate the microbial degradation by increasing hydrocarbon solubility and thus bioavailability (George Ares et al., 2000; Yamada et al., 2003), but unfortunately increasing also toxicity (Baca et al.,

2006). Indeed, several studies have shown that Dispersant have adverse toxic effects on marine organisms (Lee et al., 2013; Cohen and Nugegoda, 2000; Mitchell and Holdway, 2000), including benthic organisms (Cuny et al., 2015) and microbial communities (Barkstad et al., 2015; McFarlin et al., 2018; Garneau et al., 2016; Kleindienst et al., 2015). However, only few studies have investigated the effect of Dispersant on PAH microbial degradation at low temperature (Pan et al., 2017). We hypothesize that microbial communities have their own PAH degradation capacity at low temperature, which probably depend whether or not they have been adapted to the presence of PAH and to the presence of Dispersant. In order to verify the hypothesis, we investigated the effect of WAF, prepared in presence of Dispersant or not, on water from two cold environments, one oil-polluted and the other pristine, in microcosm experiment during 21 days. The study focused on three main objectives: i) determine the PAH degradation capacities of the bacterial communities assessed by GC-MS analysis, ii) the resulting toxic effect by toxicity assays on the copepod *Acartia tonsa*, and iii) characterize microbial community changes in the water column identifying microbial taxa linked with PAH degradation by 16S rRNA gene Illumina sequencing. The comparison of microbial communities behavior from oil-polluted site to that from pristine site allowed the characterization of the microbial legacy effect, providing useful information for the development of strategies for the management of microbial communities in oil-spill response.

Materials and methods

Sampling sites

Water samples were collected from two sites, a pristine and a contaminated site. The polluted site was a harbor in the middle of Tromso Norway (69°39'08.7"N 18°57'50.2"E)

characterized with busy boat activity. Whereas the pristine site is a sound in Tisnes Norway (69°36'24.4"N 18°49'56.3"E) with little anthropogenic influences nearby. Water was collected from the coast at a shallow depth, stored in polypropylene containers at 4 degrees. The water containers were then shipped to Pau, France at constant temperature of 4 degrees.

Water accommodated fractions preparation:

Two types of water accommodated fractions were prepared for the microcosm experiments: 1) crude oil WAF, and 2) Dispersant added to WAF (WAF+ Dispersant). The soluble fractions were made from an arctic crude oil provided by Norwegian university of science and technology (NTNU). Naphthenic North Sea (NNS) is a naphthenic based light crude oil with a low aromatic hydrocarbons content, low wax content, low viscosity index (299 cP at 2°) and density of (0.900 g/ml). The crude oil is characterized by high amount of Polycyclic Aromatic Hydrocarbons (Katsumiti et al., 2019). Preparation of WAF was similar to the protocol described by Singer et al., (2000) but with slight modifications. Briefly, in 8 liters glass bottles, crude oil was added to artificial seawater of salinity 30 ppt at a ratio of 5 gram of oil per 1 liters water. The oil water mixture was stirred for 42 hour at 10 degrees and under dark conditions using a magnetic stirrer without creating vortex. For WAF+ Dispersant, the Dispersant finasol 52 (Total oil company, France) was added first to the oil at a ratio of 1 gram of Dispersant per 10 grams of crude oil, gently mixed, then the WAF was produced similar to the procedures mentioned before.

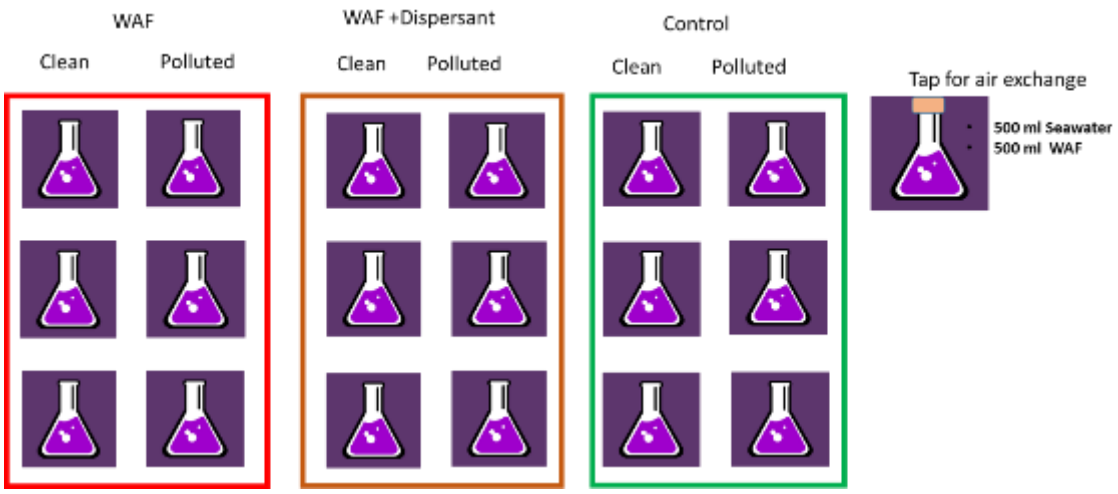


Figure (1): Schematic representation of the microcosm experiment at 4 degrees for 21 days

Microcosm set up:

For each microcosm (Figure 1), 500 ml of collected water and 500 ml of WAF were added to a 2 liter sterile glass flasks covered with filter rubber mesh tap to allow for gas exchange. Each microcosm condition was done in triplicates for a total number of 18 microcosms: 3 controls from clean conditions, 3 controls from polluted conditions, 3 WAF treatments with clean conditions, 3 WAF treatments with polluted conditions, 3 WAF+ Dispersant treatments with clean conditions and 3 WAF + Dispersant treatments with polluted conditions. All microcosms were incubated at 4 ° C for 3 days before the addition of WAF to the treatments to allow for acclimatization. Microcosms were set up for 21 days at 4 ° C in the dark to prevent photosynthesis and photoreactions. Every day, each microcosm was gently mixed to allow for resuspension of particles. Water samples were collected after 0, 7,14, 21 days. At each time collection, samples for microbial analysis, toxicity assays, PAHs analysis were collected. Microbial analysis samples from water were collected through vacuum filtering 100 ml of microcosm water through 0.46 microns membrane filter.

Chemical analysis of the PAH content

The PAH composition and concentrations of WAF and WAF+ Dispersant were determined by GC/MS analysis. A mix standard solution of 16 PAHs was used [Norwegian Standard (NS 9815: S-4008-100-T): phenanthrene (Phe), anthracene (An), fluoranthene (Fluo), pyrene (Pyr), 11 H-benzo[a]fluorene (11H-B[a]F), 11 H-benzo[b]fluorene (11H-B[b]F), benz[a]anthracene (B[a]A), chrysene (Chry), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), indeno[1,2,3-cd]pyrene (Ind), dibenz[a,h]anthracene (D[a,h]A), benzo[g,h,i]perylene (B[g,h,i]P)] and dibenzo[a,e]pyrene (D[a,e]P). The employed stir bars (poly dimethylsiloxane polymer coated twisters, supplied by Gerstel GmbH, Germany) were 10 mm length and 0.5 mm film thick. The twisters were introduced in aqueous samples (35 mL) and stirred during 315 minutes. Once the extraction step was over, twisters were cleaned with Milli-Q water, in order to eliminate seawater, and dried with paper tissue. Twisters were desorbed using a commercial thermal desorption TDS-2 unit (Gerstel GmbH) connected to a CIS-4 injector (Gerstel GmbH) with the following conditions: desorption time (10 min), desorption temperature (300º C), desorption flow (23 mL min^{-1}), cryo-focusing temperature (−50ºC) and vent pressure (7 psi). This desorption unit was coupled in an Agilent 6890 gas chromatograph also coupled to an Agilent 5975 mass spectrometer system (Agilent Technologies, USA). The Mass spectrometer (MS) was operated in selected ion monitoring (SIM) for quantification of target compounds. An Agilent DB-5MS+DG column with the following dimensions 30 m x 0.25 mm and 0.25 μm was used. Analytes were separated using the following conditions: carrier gas, helium (1.3 mL/min); transfer line, ion source and quadrupole analyser temperatures were maintained at 300, 230 and 150ºC, respectively. The following temperature program was used for target PAHs and lineal hydrocarbons: 170ºC for 5 min; ramp at 30ºC min^{-1} to 260ºC; ramp at 8ºC/min to 300ºC and hold 15 min.

DNA extraction and 16S rRNA gene PCR amplification

The membrane filters were cut into small pieces then homogenized using metal beads. The total DNA was extracted using power soil total DNA isolation kit (Qiagen, Germany) following the extraction protocol recommended by manufacturer. DNA quantity was determined using Quant it dsDNA HS kit (Theremofisher scientific, USA). DNA was amplified using the bacterial universal primers 515f and 928r (Wang et al., 2009), which amplifies the V4 region of the 16S rRNA gene. The reaction medium (25 µl) consisted of 12.5 Taq polymerase (Thermofisher scientific, USA), 1 µl of forward and reverse primer at concentration of (20µM), 5.5 µl water and 5 µl undiluted DNA template. The polymerase chain reaction was performed using thermocycler (Applied biosystems, USA) with the following cycling conditions: 10 minutes at 95 ° C followed by 30 cycles of: 30 seconds at 95 ° C, 30 seconds at 60 ° C and 40 second at 72 ° C, followed by a final step of 10 minutes at 72 ° C. Successful amplification of the PCR was checked on 2% agarose gel electrophoresis, stained with ethidium bromide. PCRs were performed in triplicates and then pooled for further sequencing.

Amplicon sequencing and bioinformatics analysis

Genetic barcoding was performed using NGS illumina Miseq by sequencing the V4 region 16S rRNA gene. Demultiplexed sequences obtained were treated with Qiime 2 software (Quantitative insights into microbial ecology; Caporaso et al., 2010) according to Quince et al. (2009). Briefly, sequences were trimmed and then denoised using DADA2 pipeline integrated in the Qiime2 software to remove chimeric sequences. The total number of sequences for each sample obtained after denoising is presented in (Supplementary Materials, chapter 3,Table S1). Denoised sequences were assigned with taxonomic affiliation using RNA gene reference database SILVA version 132 (Quast et al., 2013). In addition, singletons were filtered. Finally, sequences were rarefied

according to the lowest number of total sequences observed in samples. Rarefaction curves are presented in (Supplementary Materials, chapter 3, Figure S1). An abundance table with the total ASV observed was generated and rare taxa were removed. The top 300 taxa based on abundances were used for further analysis.

Copepod mortality assays and gene transcription assays:

Total of 30 adults of the copepod *Acartia tonsa* were selected under a dissection microscope. Adult copepods were placed in 75 ml water collected from the microcosm for 96 hours. Copepods were not fed during exposure and no gender preference was considered when collecting the copepods for the experiments. Everyday, copepod mortality was checked and dead copepods were removed. A copepod was considered dead when is not moving after a gentle stimulation with a pipette. After 96 hours, the surviving copepods were collected and placed in cryotubes filled with RNA later (Invitrogen, USA). The pooled copepods were homogenized using beads tissue homogenizer (Precellys 24 tissue homogenizer, France) with the following program: 45 secs at 6500 rpm, then 2 minutes in ice, then another 6500 rpm. RNA was extracted using RNA easy minikit (Qiagen, Germany) following the manufacture protocol. The extracted RNA quantity and quality were observed using 2100 bioanalyzer (Agilent technologies, USA). RNA from the extracted samples showed to have minimum Rin score of 7, indicating intact extracted RNA. In addition, RNA purity was determined by measuring samples' absorbance ratios of 260/280 nm using a spectrophotometer (Epoch, Biotek, USA). All samples had acceptable ratios between 1.8–2.3. cDNA was synthesized from purified RNA using the AffinityScript Multiple Temperature cDNA synthesis kit (Agilent Technologies, USA) with random primers and following the manufacturer´s protocol. In addition, cDNA concentration was quantified using Quant –it Oil green stain (Invitrogen, USA)

Primer design and Real time quantitative PCR

Gst primers were designed from conserved regions observed by aligning *Acartia tonsa* genome (PRJEB20069) with *Tortanus forcipatus* (KT755427.1) and *Acartia pacifica* (KT754520.1) using Clustal omega (Madiera et al., 2019). Hybridization Taqman Probes were designed according to the sequenced amplicons obtained from the *gst* primers. For the quantitative PCR, the reaction medium (25 µl) consisted of 12.5 µl Taqman gene expression master mix (Applied Biosystems, USA), 0.3 µl (800 nM) forward and reverse primer, 0.25 µl hybridization probe, 8.2 µl water and 3 µl cDNA template, undiluted. Quantitiative PCR was performed in 384 optical well plates (Applied Biosystems, USA) using ViiA 7 Real-Time PCR system (Applied Biosystems, USA). The amplification conditions were 50° C for 2 min., 95° C for 10 min., followed by 40 cycles of 95° C for 15 s and then 1 min. of annealing at 60° C.

Statistical analysis

Analysis of the bacterial community dynamics such as alpha diversity indices, bacterial abundances at the phylum level, were conducted on R software using RAM package (Wen Chen et al., 2018). CA, CCA, PERMANOVA and SIMPER analyses were performed using PAST statistical software version 4.03 (Hammer et al., 2020).

Linear discriminant analysis effect size (LEfse, Segata et al., 2011) was performed with galaxy (http://huttenhower.sph.harvard.edu/lefse/). Briefly, non parametric Kluskal Wallis was used to detect taxa with significant abundances ($p < 0.05$). Biological consistency was conducted using pair wise Wilcoxon test. Finally, LDA with threshold score of 4.0 and 1000 bootstraps was applied.

Network analyses of bacterial communities overtime were constructed using Molecular Ecological Network Analysis (MENA) pipeline (http://129.15.40.240/mena/; Deng et al.,

2012). Briefly, a pair wise similarity matrix was constructed from log transformed ASV abundance table using spearman correlation. A similarity threshold cut off value was automatically generated for the similarity matrix. Network topological features, such as global network properties, individual nodes' centrality, module separation and modularity, were performed. Mantel test was performed using Pearson correlation to investigate correlation between Bacterial taxa connectivity and the concentrations of total PAHs. Finally, the network modules were generated with "greedy modularity optimization mode" and the contaminated and pristine networks were visualized using *cytoscape (*Shannon et al., 2003). Finally, *Acartia tonsa* gene transcript levels were normalized with the amount of input cDNA using formula adapted from the ΔΔCT normalization method (Bartolome et al., 2016).

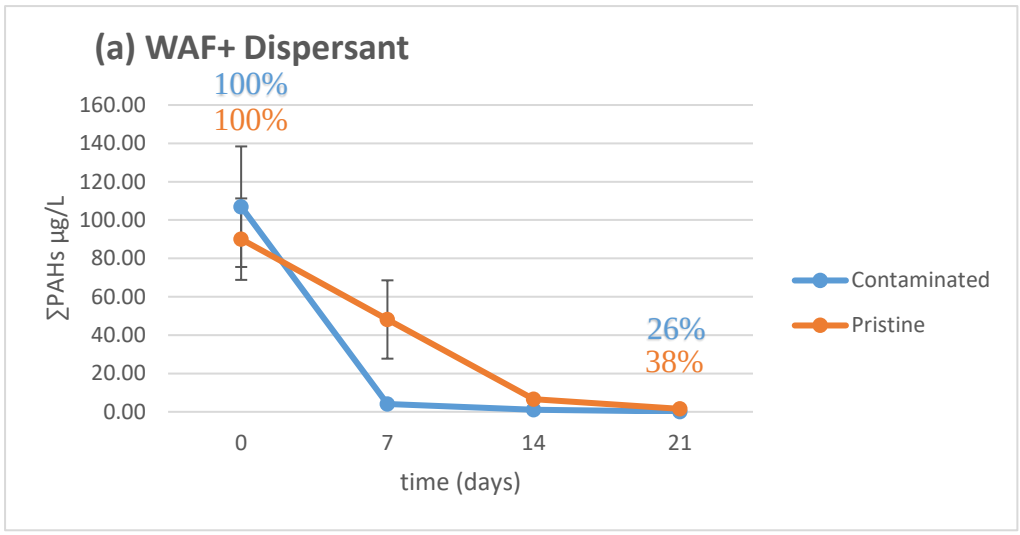
Results and Discussion

PAH degradation and toxicity

The initial water accommodated fractions (WAF) contained similar total PAH concentration whether they were prepared with Dispersant or not, and irrespective of the water origin. They exhibited similar PAH composition (unpaired t test $p>0.05$) containing mainly naphthalene and derivatives (acinaphtalene and acenaphtlene, >50 µg/L), while the content of high molecular weight PAH (> 3 rings) was below 1 µg/L. However, the WAF prepared with Dispersant showed higher toxicity with 100% copepod (*Acartia tonsa*) mortality whereas the WAF without Dispersant exhibited 20% copepod mortality (Figure 2), showing the toxic effect of the Dispersant. During microcosm incubation, the PAH content decreased drastically, faster with water coming from contaminated sites with removal of almost all total PAH (96%) within 7 days while with pristine water the

complete PAH removal (98%) was obtained after 14 days (Figure 2). Noteworthy, the presence of Dispersant did not increase the PAH degradation rates (Figure 2). At low temperature, the volatility of low molecular weight hydrocarbons decreases while viscosity increases, but WAF degradation has been reported in 14 days at 10 ° C (Huang et al., 2019). Irwin et al., (1997) demonstrated that in soil, volatilization only accounted for 30% and 20% of the total loss of naphthalene and methyl naphthalene. In our study, although some amount of PAHs might have lost by volatilization, the rapid decrease of PAHs after seven days in water from the contaminated site suggest that biodegradation participate to PAH removal.

At the end of the experiment (21 days) the toxicity, assessed by *Acartia tonsa* mortality, was similar in all conditions (unpaired t test $P>0.05$), close to that observed at the beginning of the experiment in WAF without Dispersant (around 30 %). Mortality in the control microcosms at time 0 and time 21 days, and in water from both sites, was below 15 %. It has been shown that WAF+ Dispersant has high toxicity toward marine organisms compared to WAF without Dispersant (Lee et al., 2013), especially toward *Acartia tonsa* (Rattes Almeida et al., 2014). In addition, dispersed oil droplets have been shown more toxic than WAF due to the bioavailability of low soluble and high molecular weight PAHs (Berrojalbiz et al., 2009)



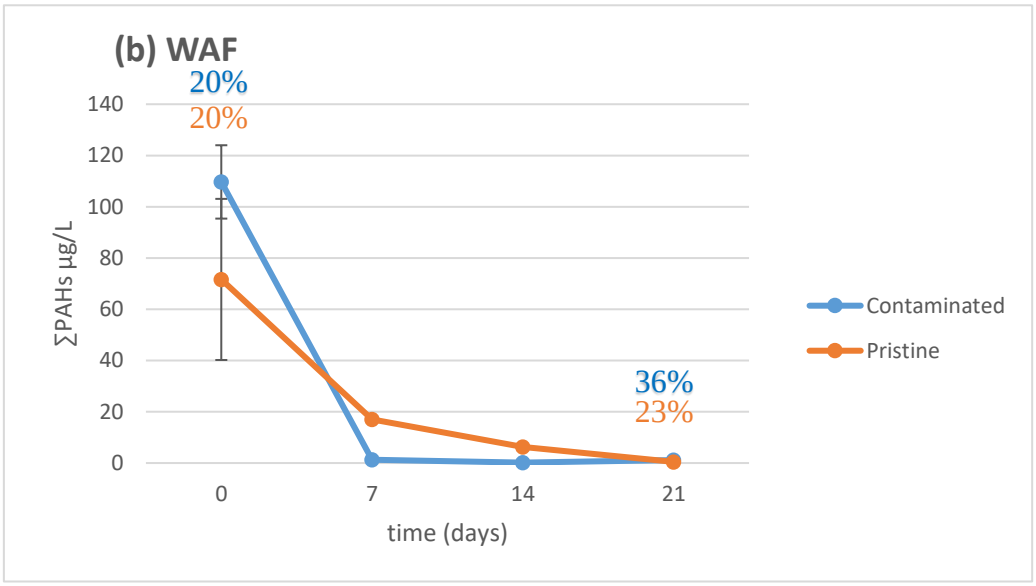


Figure (2): Concentrations of PAHs (∑PAHs µg/L) in microcosms of a) WAF + Dispersant and b) WAF. Blue line denote microcosm prepared with water from contaminated site, orange line denote microcosm prepared with water from pristine site. Numbers above points denote percentage of mortality of *Acartia tonsa* after 96 hours exposure at microcosm water samples obtained at day 0 and day 21. Colors of numbers represent which site the exposure water was taken from. N=3 Error bars = ±1 Standard error

Microbial communities characterization and composition

After trimming, the retained 296,386 sequences were rarefied to 3224 sequences per samples, corresponding to the lowest sequences number in a sample. The retained sequences were then dispatched within 4024 ASVs (Supplementary Materials, chapter 3, Table S1). The rarefaction curves for the different conditions indicated that the sequencing depth was sufficient to reveal the microbial diversity (Supplementary Materials, chapter 3, Figure S1). Nevertheless, despite several attempts, it was not possible to characterize the microbial communities from contaminated water at day 14 under the WAF with Dispersant condition, and at days 14 and 21 under the WAF condition, probably because the microbial abundance was low. In fact, we hypothesize that the depletion of hydrocarbon (Figure 2 a & b) in water caused the collapse of microbial communities inhabiting the contaminated water at day 14 in WAF and WAF+ Dispersant. Hydrocarbons available in WAF and in WAF+ Dispersant probably represent the main carbon source for the growth of oil degraders (Xu et al., 2018). However, in the case of WAF+ Dispersant, the added hydrocarbons and Dispersant represent an easily degradable carbon source for bacteria rapidly consumed (after 7 days, Figure 2) corresponding to a priming effect usually described after addition of fresh bioavailable carbon source (Pascault et al., 2013). Then after adaptation (around 21 days, Figure 2), microbial community use less bioavailable carbon source present on the water.

The Shannon index was between 2.95 and 4.26, increasing in pristine water during the experiment while decreasing in polluted water (Figure 3, and Supplementary Materials, chapter 3, Table S1). However, according to one way ANOVA, no significant differences were observed among Shannon indices during the experiment in all conditions.

The microbial communities were dominated by *Proteobacteria* (65%- 25%), *Bacteroidetes* (60%- 10%), *Firmicutes* (40%-5%) and *Nanoarchaeaota* (20%-1%) phyla,

while *Cyanobacteria, Actinobacteria, Fusobacteria, Thaumarchaeota,* phyla were present at lower abundance, less than 10% (Figure 3). Noteworthy, changes in microbial community composition were observable during the incubation period characterized by increasing abundance of *Bacteroidetes* with concomitant decreasing of *Proteobacteria* and *Firmicutes* abundances irrespective of the condition and the origin of water (Figure 3). However, it can be noticed that at the beginning of the experiment, the *Bacteroidetes* abundance was lower in the pristine water but reached similar abundance to that observed in contaminated water at the end of the experiment. Such observation suggested that members of the phyla *Proteobacteria, Bacteroidetes* and *Firmicutes* play crucial roles in PAH degradation within the microbial community. These phyla have been described to be dominant in hydrocarbon-enriched environments (Kwon et al., 2019). For example, during the Deepwater horizon oil spill accident, members of *Bacteriodetes* became the major phyla in the water column (Redmond and Valentine, 2012), contributing to 3% of the total hydrocarbon degradation activity (Kwon et al., 2019). Crisafi et al., (2016) demonstrated that at 4 C° for 90 days, oil enriched samples favored several hydrocarbon degrader strains belonging to *Gammaproteobacteria* and *Alphaproteobacteria*, being capable to degrade up to 80% of the available hydrocarbons. In addition, *Firmicutes* showed to be important components in the bacterial communities composition from samples from western Siberia oil fields (Frank et al., 2016). In fact, several members of the phyla *Firmicutes* and *Proteobacteria* are considered to be opportunistic heterotroph (Neguyen Lopez Lozano et al., 2013) that probably explains the increase abundance of such phyla after the addition of WAF and WAF+ Dispersant.

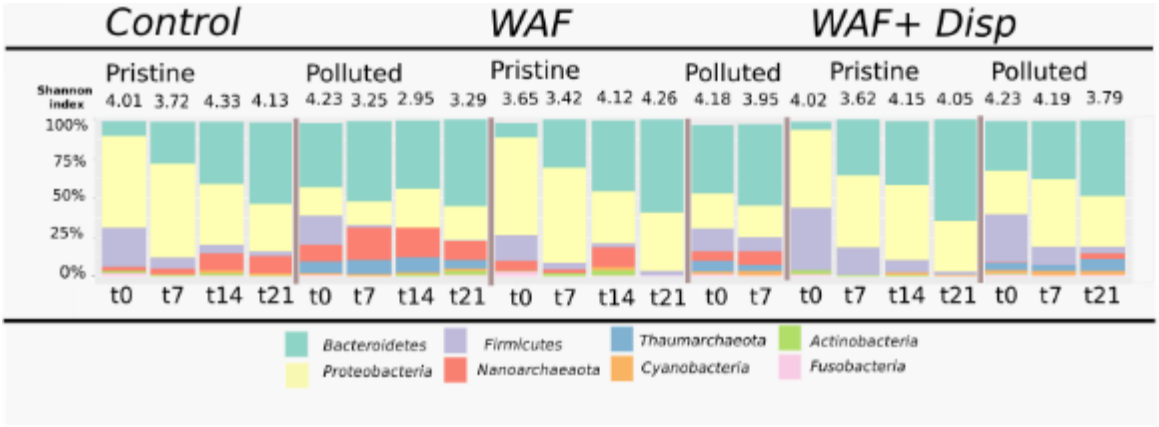


Figure (3): Bacterial composition at the Phylum level in pristine and oil-polluted waters for the three treatments (controls, WAF, WAF+ Dispersant) during the incubation. Number above each bar indicates Shannon index (alpha diversity).

Comparison of microbial communities

The correspondence analysis (CA) separated pristine water apart from contaminated water into two main clusters along the axis 1 that explained 19% of the data distribution (Figure 4a), indicating that the microbial community structures were different according to the site. Noteworthy, the microbial communities of both sites have shown separation according to time within each main cluster along the axis 2 that explained 13% of the data distribution despite the missing samples of polluted water at days 14 and 21 (Figure 4a). Such observation further confirmed the modifications of microbial communities during the experiment, which corresponds to the ecological succession relating the microbial plasticity for the adaptation to the environmental conditions. Additionally, similarity percentage analysis (SIMPER) revealed differences between the microbial community composition according to the conditions (control, WAF, and WAF + Dispersant) with dissimilarity between 38% and 54% at the beginning of the experiment, and above 55% for the rest of the experiment. The dissimilarity was higher between control and WAF with Dispersant (60% < SIMPER > 80%) than between control and WAF (32% < SIMPER > 62%), revealing the effect of both WAF and WAF + Dispersant on the

microbial communities. These observations were supported by permutation analysis of variance (PERMANOVA) indicating that the origin of water was the main factor explaining data distribution followed by time and then the conditions (Supplementary Materials chapter 3, Table S2). The initial composition of the bacterial communities plays important roles in shaping the responses toward oil contamination as observed (Ortmann et al., 2015; Al Jawasim et al., 2015).

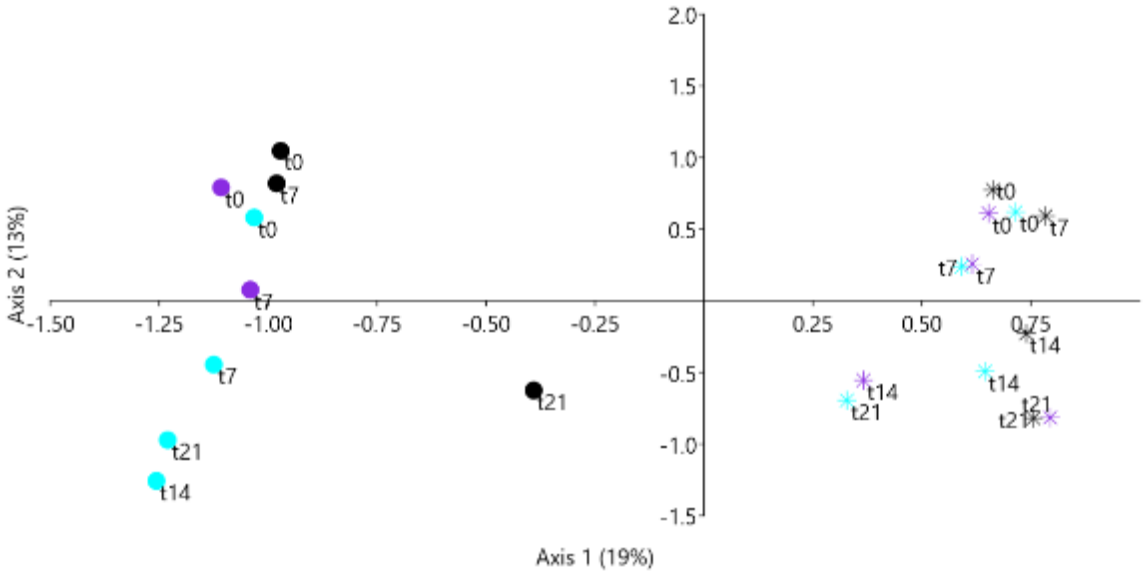


Figure (4): Correspondence analysis (CA) comparing microbial communities from pristine and oil-polluted waters in the three treatments (controls, WAF, WAF+ Dispersant), at different incubation time. Dots, microbial community of water from oil-polluted site. Stars, microbial community of water from pristine site. Light blue, Control; Purple, WAF; Black, WAF + Dispersant. Numbers indicate the incubation time.

Linear discriminant analysis (LDA) effect size (LEfse) revealed taxa significantly more abundant according to the condition for both pristine and contaminated waters (Figure 5, Supplementary Material, chapter 3, figure S2) explaining the observed differences between the microbial communities. Taxa found significantly more abundant in a condition have been proposed as bioindicator (Segata et al., 2011). More bioindicators

for contamination were observed for contaminated water than for pristine water while, in contrast, more bioindicators were detected for the control in the pristine water. Such observation indicates that due to legacy effects, the contaminated water microbial community was adapted to the presence of hydrocarbon. Such legacy effect has been described in temperate aquatic environments (Ben Salem et al., 2019; Duran et al., 2015; Misson et al., 2016) but not yet in cold environments.

The abundance distribution of the identified bioindicators by LEfSe according to the treatment (control, WAF, and WAF+ Dispersant) further revealed the specificity of taxa to the treatment (Figure 5a). For the pristine water, seven bioindicators were exclusive for the WAF+ Dispersant (Figure 5a) while no exclusive ASVs were found for the other conditions. It is likely that most of taxa associated to the bioindicators for pristine water were able to cope with the presence of contaminant as they are equally distributed within control and contaminated treatments, but only the WAF+ Dispersant treatment provides favorable conditions for the development of specific microorganisms able to use hydrocarbon and/or Dispersant as carbon source. The seven bioindicators exclusive for the WAF+Dispersant include ASVs belonging to *Fusibacter* (ASV 75, ASV 2, ASV 154), *Flavobacterium* (ASV 49), *Proteobacteria Psychrobacter* (ASV 71), *Psychromonas* (ASV 106), and *Shewanella* (ASV 161).

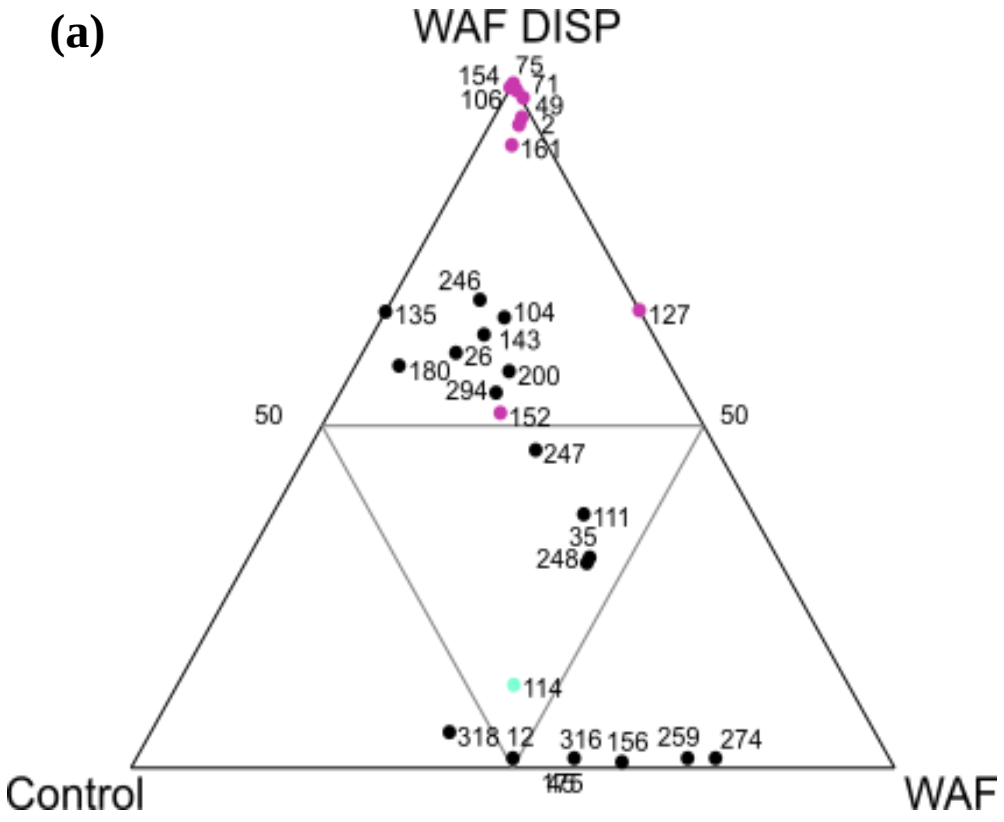
For the contaminated water, the bioindicator ASVs were specifically distributed according to the conditions (Figure 5b), showing abundance above 50% in their respective condition. The ASVs which are specifically identified for the WAF + Dispersant treatment were members of the *Proteobacteria* phyla, belonging to *Colwellia* (ASV 14, ASV 232, ASV 7), *Marinomonas* (ASV 86, ASV 126, ASV 73), *Moritella* (ASV 95), *Sulfitobacter* (ASV 139), *Paraperlucidibaca* (ASV 118, ASV 179, ASV 292) genera. The exclusive bioindicators for the WAF treatment included ASVs belonging to

Bacteroidales (ASV 189) *Cyanobacteria* (ASV 243), *Arcobacter* (ASV 63), *Sulfurovum* (ASV 244), *Moritella* (ASV 36), *Marinifilium* (ASV 145), and *Fusibacter* (ASV 181) genera. Interestingly, some bioindicator ASVs were equally distributed between WAF and WAF+DISPERSANTispersant treatments suggesting that they respond to the presence of hydrocarbons. These bioindicator ASVs belong to *Caedibacteraceae* (ASV 21) family, *Moritella* (ASV 109), *Olispera*, (ASV 89), *Fusibacter* (ASV 182, ASV 131, ASV 291), and *Marinifilium* (ASV 201) genera.

The most striking observation was that among the bioindicators taxa, *Colwellia* was exclusively found in WAF+Dispersant treatment for contaminated water. Such observation was in agreement with previous studies showing that *Colwellia* was detected in oil containing cold seawater (Garneau et al., 2016), dominating bacterial communities after the Deepwater Horizon oil spill, and presenting the capacity to biodegrade aromatic hydrocarbons (Mason et al., 2014). Additionally, *Colwellia* has been shown to benefit from the presence of Dispersant (Kliendiest et al., 2015).

Noteworthy, several ASVs belonging to *Fusibacter*, genera able to degrade low molecular weight PAHs (Folwell et al., 2016), were identified as contamination bioindicator (WAF and WAF+Dispersant) in contaminated and pristine waters (Figure 5b). Interestingly, it was demonstrated that *Fusibacter* dominated the bacterial community after Dispersant addition in oiled sediments, probably by using it as carbon source (Calderon et al., 2018). Most of the ASVs revealed as contamination bioindicators were related to taxa known as hydrocarbon degraders such as *Moritella* (Bagi et al., 2014), *Paraperlucidibaca* (Brakstad et al., 2008). Similarly, the psychrophilic *Shewanella* have shown pyrene degradation potential (Rathour et al., 2018), some strains have even been isolated from Arctic Sea PAH-rich sediment (Dong et al., 2015). In addition, it is important to note that two ASVs belonging to the *Woesarchaeia* archaeal class (ASV 314

and ASV 318) were identified as exclusive bioindicators for control contaminated water, suggesting that they were sensitive to the addition of WAF and WAF + Dispersant.



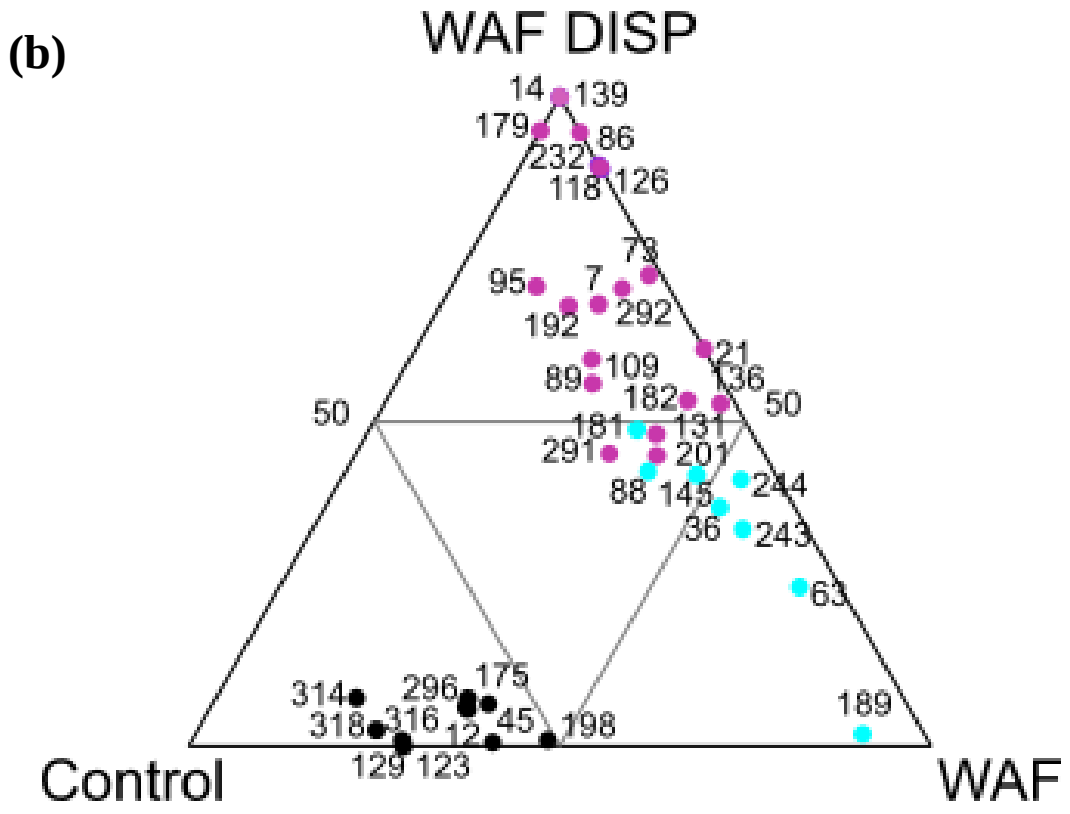


Figure (5): Ternary plots showing the abundance of bio indicator taxa identified by LEFse in pristine water (a) and contaminated water (b). Numbers refer to ASV taxa. Bio indicator taxa identified for WAF+ Dispersant treatments are purple dots, for WAF are light blue dots, and for Control are black dots.

The relation between bacterial taxa and PAHs toxicity

Canonical correspondence analysis (CCA) assisted in visualizing how microbial taxa interacted with the PAHs toxicity. For pristine water, the first to axis of the CCA explained 99% of the observed variance (axis 1: 72% and axis 2: 27%; Figure 6a). The axis 1 was positively explained by PAH concentration and *Acartia tonsa* mortality but negatively explained by the down-regulation levels of *gst* gene. Such observation indicated the correlation between PAHs concentrations and toxicity. Bacterial

communities at t0 and those with Dispersant (WAF + Dispersant at t0) were strongly correlated with the mortality and the concentration of PAHs (Figure 6a). These microbial communities were dominated by ASVs belonging mainly to the *Firmicutes, Fusibacter* (11 ASVs), the *Proteobacteria Burkholderiaceae* family (3 ASVs), and to the *Actinobacteria Microbacteriaceae* family. Stress levels in copepods, measured by the transcription of *gst* gene, were positively correlated with WAF + Dispersant samples collected after 21 days, in which ASVs affiliated to *Flavobacterium, Salinirepens* (2 ASVs), and *Psychrobacter* were positively correlated with the *gst* gene expression levels (Figure 6a).

For contaminated water (Figure 6 b), the CCA explained 99% of the variance (axis 1: 87%, axis 2: 11%). The PAHs concentrations and *Acartia tonsa* mortality were positively explained by the first axis while *gst* gene downregulation levels was negatively explained by the second axis (Figure 6b). In general, the communities of WAF and WAF+ Dispersant microcosms at time 0 were correlated with PAH concentrations and with copepod mortality. However, the communities at microcosm treatments at time 21 correlated with the *gst* gene down transcription patterns.

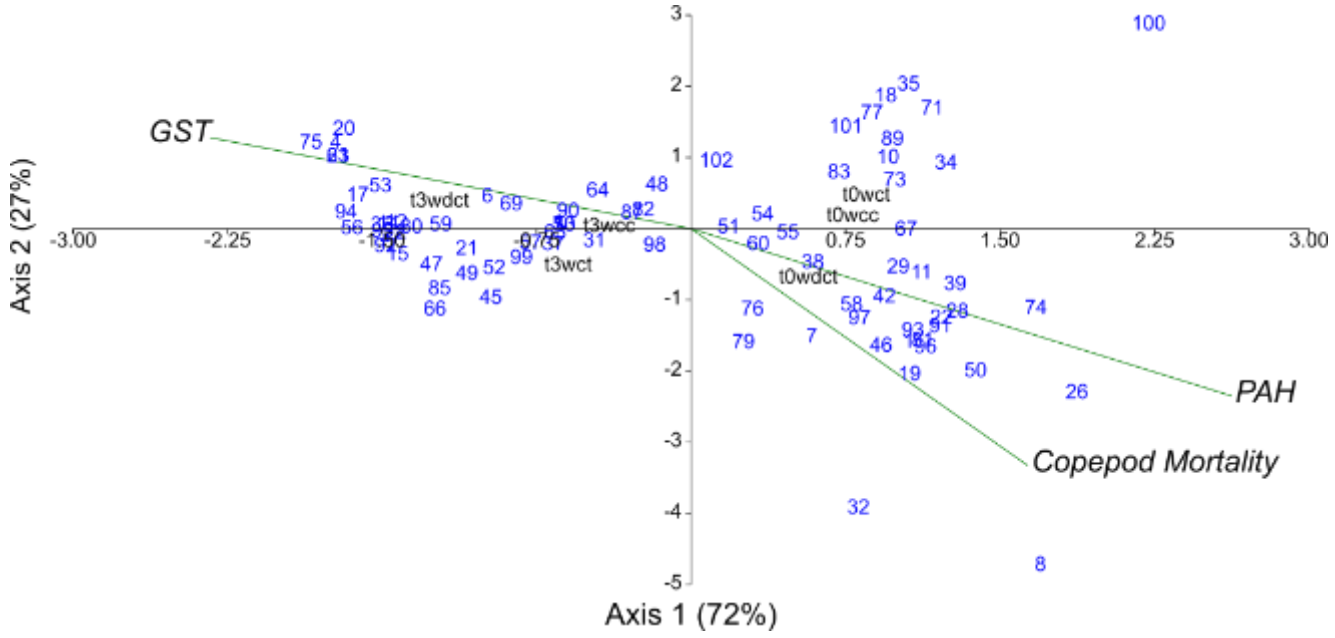
The abundance of several ASVs showed strong positive correlation with copepod mortality such as *Fusibacter* (8 ASV), *Moritella,* and an ASV from the family *Rhodobacteraceae*. In addition, the abundance of some ASVs was correlated with the concentration of PAHs. These ASVs were *Marinifilum* (7 ASVs), ASV from family *Rhodobacteraceae,* and an ASV of the genus *Colwellia*. In both pristine and contaminated microbial communities, the *Firmicutes, Fusibacter*, showed to be an important taxa capable to survive the toxicity of Dispersant. Currently, *Fusibacter* is yet to be directly linked to PAHs biodegradation (Kappel et al., 2014). However, we hypothesize that *Fusibacter,* among the other correlated ASVs identified, played an important role in

reducing the toxicity of Dispersant. The mineralization of Dispersant (e.g. Corexit EC9500A) by a consortium of marine microbes has been shown to be faster than that of crude oil at low temperature (Lindstrom et al., 2002).

Several ASVs correlated with *gst* gene expression belonged to *Marinifilum*, *Lutibacter*, *Salinirepens, Polaromonas*, *Colwellia.* These correlations probably result from the reduction of Dispersant toxicity, suggesting the sensitivity of these taxa toward the toxicity of Dispersant. It is likely that such taxa, stimulated after 21 days of microcosm experiment, were initially out competed by the opportunistic taxa, which were rapidly stimulated by the Dispersant at the beginning. Then, later on the experiment, such stimulated taxa were replaced upon the depletion of the carbon source provided from Dispersant. Dispersant have shown to be able to alter bacterial communities by favoring Dispersant stimulated taxa while inhibiting others (Yoshida et al., 2006).

As shown by *Acartia tonsa* toxicity assays (figure 2), the toxicity of the Dispersant decreased from the beginning to the end of the microcosm experiment, the WAF+ Dispersant from day 21 microcosms still had deleterious sub-lethal effects towards *Acartia tonsa* through the down expression of the *gst* gene. The *gst* genes are known as antioxidant genes capable to detoxify several xenobiotics and protect the cell from oxidative stress (Lee et al., 2006). The low expression levels of *gst* gene observed in exposed *Acartia tonsa* suggests a decrease in the cellular antioxidant machinery resulting probably in the over accumulation of ROS in cells (Dvorak at al., 2021). However, further study is needed to understand the significance of transcript downregulation of *gst* gene in *Acartia tonsa*.

(a) Pristine site



(b) Contaminated site

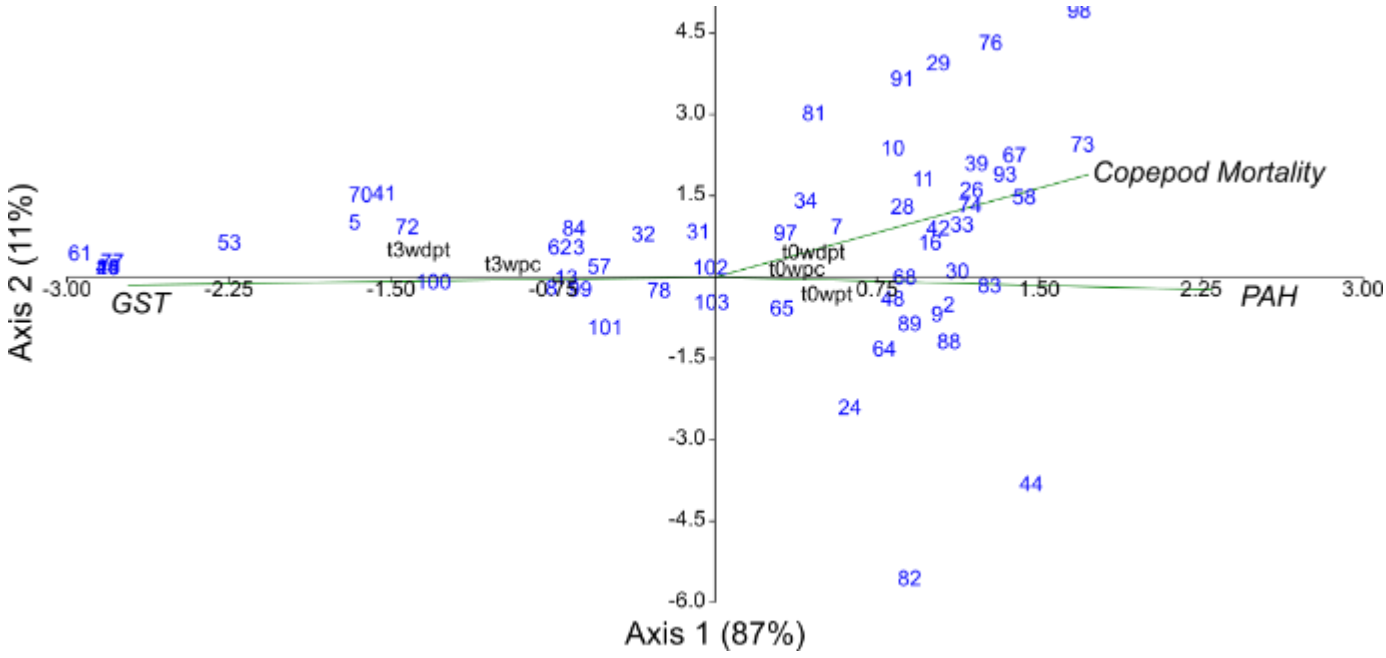


Figure (6): Canonical correspondence analysis (CCA) showing correlation between the top 100 abundant ASV and the sum of concentration of PAH (PAH), expression levels of *gst* gene in *Acartia tonsa* (GST) and the mortality exhibited by *Acartia tonsa* in the microcosm water (Copepod mortality). Blue numbers denote the number of ASV, black names denote the microcosm communities

Ecological association network MENA

In order to obtain information on the microbial interactions occurring in response to WAF or WAF+Dispersant exposures, a network analysis was performed. Networks describing strong correlations (spearman correlation threshold at 0.910 and 0.950; p value < 0.05) were obtained (Supplementary Materials, chapter 3, Table S4). Pristine communities' network showed more density by having 171 total nodes and 468 links, while contaminated communities network showed 112 total nodes and 225 links. However, R^2 of power law fitted better the pristine site network (0.68) compared to contaminated site network (0.48), indicating that pristine site fits better as a scale free network, where fewer taxa accounts for the majority of interactions. However, both networks exhibited average path distance of 3.925 for contaminated and 4.296 for pristine and similar average clustering coefficient (0.307 contaminated, 0.306 pristine). Network indices from both network were higher than the random network indices, suggesting that both networks exhibited small world features, meaning that networks are highly clustered and nodes are related (Watts and Strogatz, 1998). However, no association was found between ASV and PAHs concentrations (Mantel test, for pristine (r =0.007318, P= 0.375) and contaminated (r= -0.02143, P= 0.625) networks). Nevertheless, the networks, for both pristine and contaminated waters, were modular. Contaminated site network yielded 17 modules with modularity value of 0.65 while pristine site network yielded 14 modules with modularity value of 0.60. A plot was constructed from the ASVs connectivity within the modules (Zi) and ASVs connectivity among other modules (Pi). According to Zi and

Pi values, the roles of ASVs in modules were classified as: 1) Peripheral, 2) Connectors, 3) Module hubs, 4) Network Hubs (Liang et al., 2016). The threshold for these classifications were selected at Zi = 2.5 and Pi = 0.62 as previously described by Guimera and Amaral (2005).

In general, most of the ASVs in both networks were peripherals, and therefore considered as specialists (Figure 7a). In contaminated network, there are distinct modules with little interconnection, evidenced by the ASVs with low Pi value. In contrast, pristine network showed with more interconnected modules, evidenced by the several ASVs with high Pi value (Figure 7a). In addition, several connector ASVs were identified in pristine network, suggesting the complexity of the ecological interactions within the network. Connectors are generalist species, which are capable to connect different modules (Liang et al., 2016). The connector taxa identified belonged to *Flavobacterium* (ASV 49) genus, 3 ASVs of uncultured bacteria from the order *Rickettsiales* (ASV 259, ASV 224, ASV 174), *Paraperlucidibacta* (ASV 227), ASV from class *Nitrososphaeria* (ASV 321), an ASV from family *Cyanobiaceae* (ASV 153), and 2 ASV from *Fusibacter* (ASV 37 and ASV 290) genus.

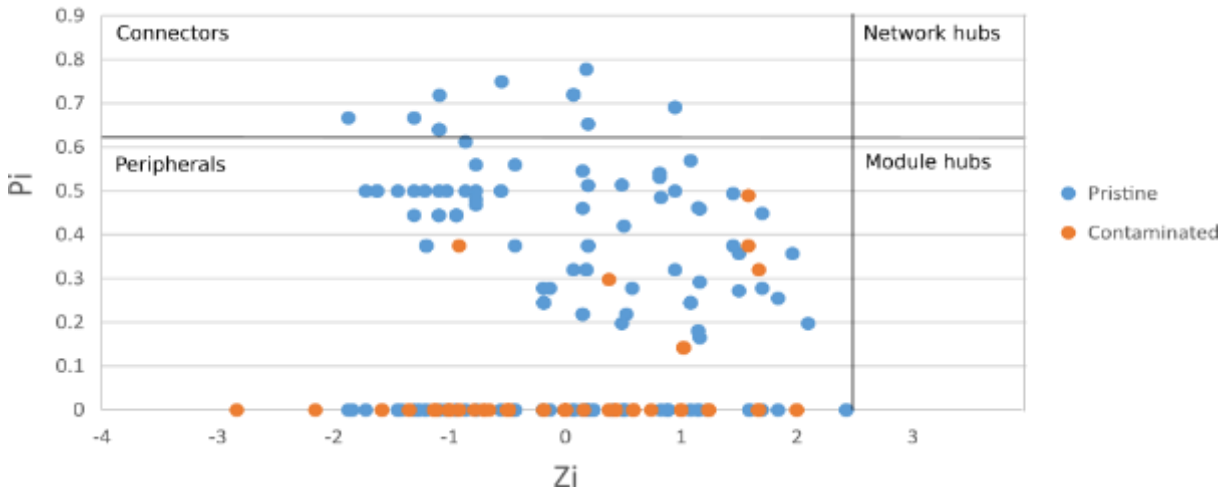
The networks, both pristine and contaminated water, showed a modularization according to time (Figure 7b & c) as evidenced by the fact that most interactions were observed at time 14 and time 21 for the pristine network (Figure 7a), and at time 0 for the contaminated site network (Figure 7b). Such observation further supports the ecological succession observed by CCA. Interestingly, one module in the pristine network clustered ASVs showing high bacterial abundances in WAF+ Dispersant treatments, indicating that microbial community composition changes in response to the presence of Dispersant. Among the ASVs in this module, 7 ASVs belonged to the genus *Fusibacter*, further

showing the importance of such genus in the microbial community dynamics, and four ASVs were connector nodes (ASV 321, ASV 277, ASV 153, ASV 290).

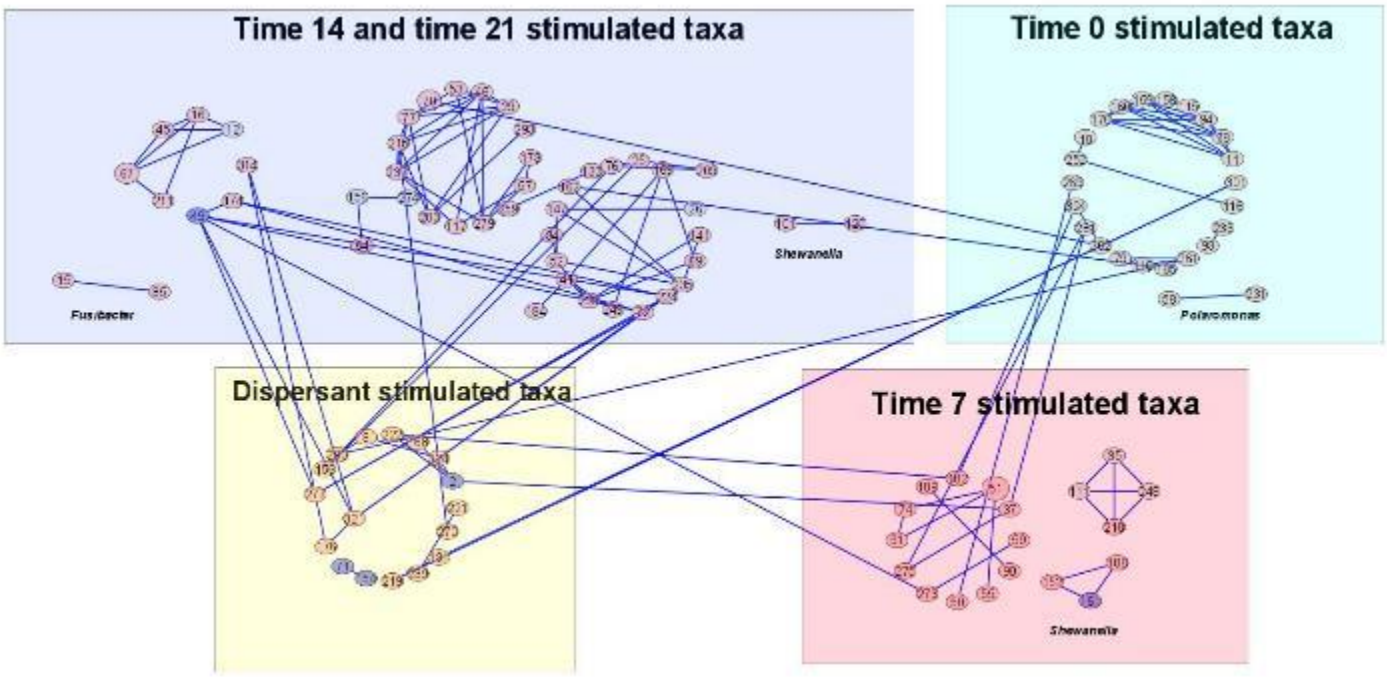
Interestingly, some taxa showed noticeable patterns in the networks, indicating their ecological role on the bacterial communities upon WAF or WAF+ dispersant enrichment. For example, in pristine network, four small modules exclusively belonged to three taxa: *Fusibacter*, *Shewanella* and *Polarimonas* and in contaminated water network (Figure 7 c), there were several small modules of *Fusibacter* at t0. These small modules, which are lacking co occurring interactions, suggest that such taxa could be exhibiting other type of interactions with the other communities, such as competition. At t0, ASVs showing high node degree in contaminated water network, belonged to the genus *Marinifilium*, suggesting that *Marinifilium* is an important key species.

The ASVs identified as bioindicator by LEfse were found in specific modules within the contaminated water network at time 7, further suggesting that these ASVs are important for the organization of microbial assemblage. Such observation was in agreement with a previous study showing that soil with a previous history of contamination has fewer interactions than pristine soil (Sheng et al., 2021). It is likely that the selection pressure exerted by the presence of contaminant reduces diversity, and thus the interconnectivity among taxa

(a)



(b)


Time 14 and time 21 stimulated taxa
Time 0 stimulated taxa
Dispersant stimulated taxa
Time 7 stimulated taxa
Fusibacter
Shewanella
Polaromonas
Shewanella

(c)

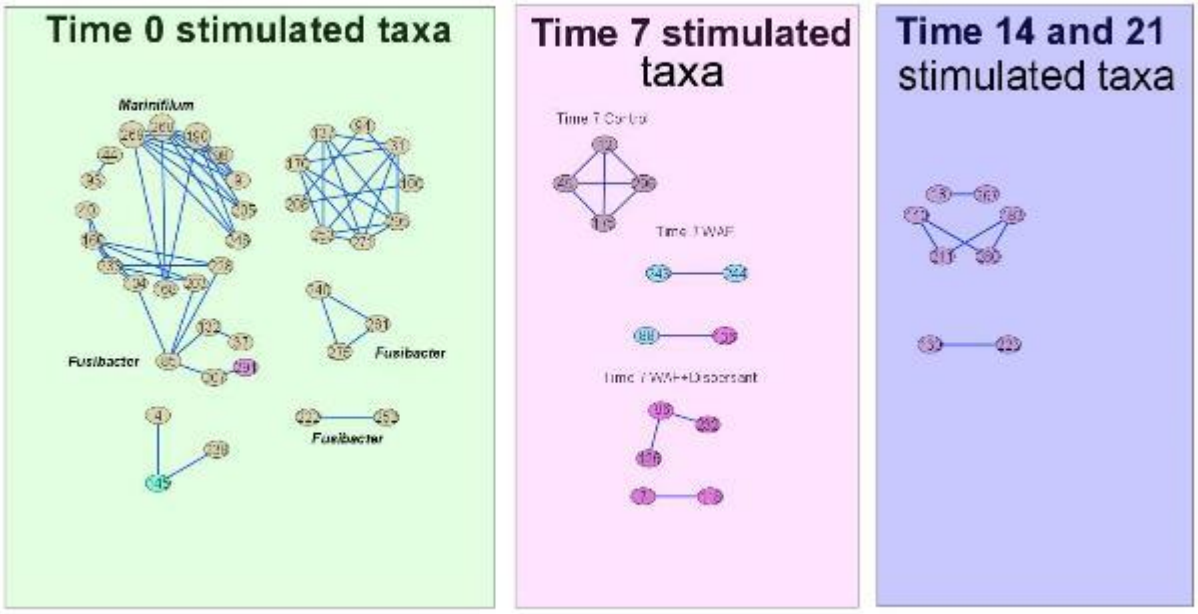

Time 0 stimulated taxa
Marinifilum
Fusibacter
Fusibacter
Fusibacter
Time 7 stimulated taxa
Time 7 WAF
Time 7 WAF+Dispersant
Time 14 and 21 stimulated taxa

Figure (8): MENA networks of microbial communities interactions over the 21 days of the microcosm from pristine and contaminated site. (a) Zi, Pi plot of ASV distribution based on their topological features: within module connectivity (Zi) and among module connectivity (Pi). **Network from the pristine site** (b) is the total microbial interactions in pristine site over the course of the microcosm duration, blue lines are positive interactions (Co-occurrence). Purple nodes are biomarker taxa identified by LEfse for WAF+ Dispersant treatment, White nodes are taxa identified for Control. **Network from the contaminated site** (c) is the total microbial interactions in contaminated site respectively over the course of the microcosm duration, blue lines are positive interaction (Co-occurrence). Purple nodes are biomarker taxa identified by LEfse for WAF+ Dispersant treatment, Light blue nodes are taxa identified for WAF treatment .White nodes are taxa identified for Control. Bigger nodes indicate ASVs with the highest node degree.

Conclusions

In cold environments, microbes are not dormant, active bacterial communities were observed in response to the addition of WAF and WAF+ Dispersant. Our results showed that the legacy effect plays a vital role in shaping the microbial response toward WAF and WAF+ Dispersant. Adapted microbial communities presented higher removal rates allowing the identification of several WAF stimulated taxa, but the contribution of Dispersant to the PAHs removal is still questionable. Network analysis demonstrated that the microbial interactions patterns were time dependent, but the presence of Dispersant also changed the bacterial community structure, probably by stimulating taxa with potential biodegradation capabilities. We demonstrated that WAF+ Dispersant rapidly stimulated the *Firmucutes Fusibacter*, which consequently affected the microbial communities' dynamics. The PAHs removal decreased the toxicity against marine organism (copepod). We assume that the toxicity decrease was due to PAHs and Dispersant removal by taxa, such as *Fusibacter* and *Colwellia*. In conclusion, this study has helped to further understand the behavior of water microbial communities at 4 C°

upon the introduction of WAF during oil spill scenarios and the introduction of WAF+ Dispersant during bioremediation efforts. Consequently, our results illustrate how responsive microbial communities are to oil contamination at low temperature.

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Chapter 4: Cold sediment microbial community shifts in response to Crude oil Water Accommodated Fraction and Water Accommodated Fraction + Dispersant

Abstract:

In cold environment, the low temperature slows down microbial metabolisms, especially the biodegradation processes of hydrocarbons, which are often stimulated by the addition of dispersants in oil spill disasters. In this study, we investigated the effects of hydrocarbon Water Accommodated Fraction (WAF) prepared with and without dispersant on benthic microbial community in a microcosm experiment in which hydrocarbon removal was observed. The prepared WAF contained similar poly-aromatic hydrocarbons (PAH) content. The microcosm experiment, setup with either pristine or contaminated sediments, was conducted during 21 days at 4 ° C under WAF and WAF+ Dispersant conditions. The behavior of bacterial communities in response to WAF was examined at both DNA and RNA levels, revealing the effect of WAF on the resident and active communities respectively. The contaminated sediment showed less taxa responsive to the addition of both WAF than the pristine sediment, indicating the legacy effect by the presence of hydrocarbon degrading and dispersant resistant taxa inhabiting the contaminated sediment.

Keywords: Sediment microbial communities, Water Accommodated Fractions, Dispersant, Microcosms

Introduction:

The constant polar ice cap melting has open novel marine areas where oil extraction and transportation has dramatically increased threatening such cold environments (e.g. arctic) with oil spill accidents (Farrington, 2014). Hydrocarbon degradation via microbial communities is often considered as the most cost effective remediation technique (Chen et al., 2015). However, because low temperatures slow down the microbial activities,

especially the hydrocarbon degradation functions, the bioremediation is hindered in cold environments (Yang et al., 2009). In addition, low temperatures change the physical and chemical properties of oil by increasing its viscosity favoring the sorption of harmful compounds, such as polycyclic aromatic hydrocarbons (PAHs), to organic matter (Crisafi et al., 2016). It is thus crucial to develop remediation techniques for mitigating the adverse oil spill effects adapted to cold environments

After an oil spill, the fate of the oil in the environment depends on several weathering processes such as evaporation, dispersion, dissolution, emulsification, biodegradation, photo oxidation and sedimentation (Sun et al., 2019). For example, low molecular weight hydrocarbons (LMWH) from crude oil solubilize in the water column with sufficient wave dynamics. Such process result in the formation of water accommodated fractions (WAF), which contain harmful aromatic hydrocarbons, polycyclic aromatic hydrocarbon (PAHs), and aliphatic compounds (Jiang et al., 2012). Because of their bioavailability, the compounds in WAF are very toxic for several aquatic species (Abbriano et al., 2011).

In case of an oil spill, the introduction of dispersants is often used to stimulate the bioremediation processes. Dispersants are introduced to the oil slick to reduce the surface tension of the oil, facilitating the microbial degradation processes (Suja et al., 2017). Dispersants have been applied as early as the Exxon Valdez oil spill accident in Alaska (Etkin et al., 2003), and extensively used during the Deepwater Horizon oil spill accident, where 2.1 millions gallons of the dispersant Corexit 9500 were spread at the water surface and seafloor (Techtman et al., 2017). The efficiency of dispersants to improve hydrocarbon degradation by microbial communities has been under debate (Techtman et al., 2017, Kleindiest et al., 2015, Rahsepar et al., 2016), while their harmful effect has been shown on various marine organisms (Almeda et al., 2013, George Ares et al.,. 2000), including benthic micro-and macro-fauna (Cuny et al.,., 2015; Duran et al., 2015).

Although the effects of WAF, with or without dispersant addition, has been studied on the water microbial community (Brakstad et al., 2000; 2018; Personna et al., 2016), little is known about the effect of WAF hydrocarbons on the benthic microbial community after their sedimentation (Cuny et al., 2015; Duran et al., 2015). It is thus crucial to understand the effects of WAF, with or without dispersant addition, on benthic microbial communities and activity, in order to propose adapted mitigation procedures, especially in cold environment. Most studies analyzing the effect of hydrocarbons on microbial community were performed at the DNA level providing information on the genetic potential but hide the real microorganisms at work, i.e. the active microbial community (Stauffert et al., 2013, 2014), because the DNA extracts contain dormant taxa and non growth active cells (Argudo et al., 2020). As RNA, involved in metabolic processes, is stable in active cells (Argudo et al., 2020), several studies have performed the analysis at the RNA level providing information on the active microbial communities modifications in response to the presence of oil in microbial mats, (Bordenave et al.,2008, 2004) and marine sediments (Stauffert et al., 2013, 2014, Terrisse et al., 2017). The analysis of active microbial communities (RNA level) is particularly well adapted to follow the microbial dynamic in short course microcosm experiment (Cravo-Laureau and Duran, 2014).

The objective of this study was to investigate the effects of WAF, prepared with or without dispersant addition, on total (DNA level) and active (RNA level) microbial communities inhabiting either pristine or contaminated cold marine sediments in microcosm experiment maintained at 4° C. We specifically investigated 1) the dynamics of both total and active microbial communities, 2) the legacy effect comparing pristine and contaminated sediments, and 3) the PAHs biodegradation potential by predicting functional pathways.

Materials and methods

Sampling site

Water and sediment samples were collected from pristine and contaminated sites. The contaminated site was located in a harbor in the middle of Tromso, Norway (69°39'08.7"N 18°57'50.2"E) characterized with busy boat activity. The pristine site was located in a sound in Tisnes, Norway (69°36'24.4"N 18°49'56.3"E) with little anthropogenic influences. Sediment were collected by hands with a sterile spatula, and then stored in polypropylene containers at 4° C. Water and sediment containers were shipped to Pau, France at constant temperature of 4° C.

Chemical analysis:

Extraction of the organic compounds:

The extraction was carried out in a closed MARX 6 microwave (CEM, USA) with temperature controller. About 1.0 grams of each of freeze dried samples were loaded into the Teflon lined extraction vessel and 15 mL of acetone was added. In addition, 25 μL(5 μg L-1) of 6 deuterated PAHs solution in acetone that acted as surrogate was added. Three blank samples were used in the analysis. The microwave extraction program consisted of 5 minutes reaching a temperature of 110° C for 15 minutes. Once samples were extracted, the extracts were filtered (Millipore PTFE, 0.45μm), evaporated and reconstituted in 1 mL of hexane.

Extract cleaning up stage:

Standard solution containing all 16 PAHs were prepared in n-hexane. The extracts were loaded on top of 1 g of Florisil ® cartridges previously conditioned with 8mL of n-hexane . Elution was performed with 12 mL of a mixture of hexane: toluene (80:20). These 12 mL fractions were evaporated using a stream of nitrogen and at a constant temperature

(40° C) until dryness. About 1 mL of hexane was then added, filtered again and stored in chromatographic vials until GC MS analysis.

Extract chemical analysis:

The 16 PAHs were quantified using GC-MS / MS according to (Navarro et al.,2006). Briefly, the extracted compounds were analyzed on a 6890 N Agilent gas chromatograph coupled to a 5973 N Agilent mass spectrometer (Agilent Technologies, USA) with a 7683 Agilent autosampler. 2 µL of the sample were injected in the splitless mode at 270 ° C into a 30 m × 0.25 mm × 0.25 µm HP-5 capillary column. The temperature programme used for the chromatographic separation was as follows: 60 ° C for 2 min, temperature increase at 10 ° C min^{-1} to 290 ° C where it was finally held for 10 min. The carrier gas was helium (C-50) and was kept at a constant flux of 1.5 mL min^{-1}. The mass spectrometer was operated in the electron impact ionisation mode and the energy of the electrons was kept at 70 eV. The interface was kept at 300 ° C and the ionisation source and the quadrupole at 230 and 150 ° C, respectively.

Water accommodated fractions preparation

Two types of water accommodated fractions were prepared for the microcosm experiments: 1) WAF from crude oil, and 2) WAF+ Dispersant from crude oil added with dispersant. The soluble fractions were made from a Naphthenic North Sea (NNS) crude oil provided by the Norwegian University of science and technology (NTNU). This arctic crude oil, naphthenic based light crude oil, is characterized by a low aromatic and wax contents, a low viscosity index (299 cP at 2° C) and a density of 0.900 g/ml. It is rich in branched and cyclic aromatic hydrocarbons (Katsumiti et al., 2019). Preparation of WAF was similar to the protocol described by singer et al., (2000) but with slight modifications. Briefly, in 8 L glass bottles, crude oil was added to artificial seawater of salinity 30 ppt

at a ratio of 5 gram of oil per 1 L water. The oil water mixture was stirred for 42 hour at 10 ° C and under dark conditions using a magnetic stirrer without creating vortex. For WAF+ Dispersant, the dispersant Finasol 52 (total oil company) was added first to the oil at a ratio of 1 gram of dispersant per 10 grams of crude oil, gently mixed, then the WAF was produced following the same procedure.

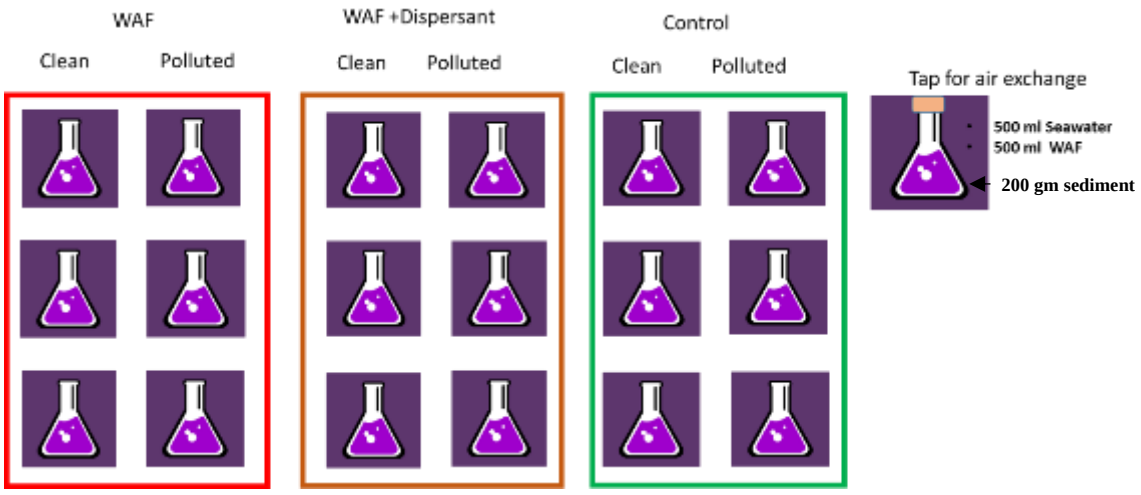


Figure (1) Microcosm experiment set up scheme at 4 ° C for 21 days

Microcosm set up

For each microcosm 500 ml of water, 500 ml of WAF, and 200 g of sediment were added to a 2 L sterile glass flasks covered with filter rubber mesh tap to allow gas exchange. Each microcosm condition was done in triplicates for a total number of 18 microcosms: 3 controls from clean conditions, 3 controls from polluted conditions, 3 WAF treatments with clean conditions, 3 WAF treatments with polluted conditions, 3 WAF dispersant treatments with clean conditions and 3 WAF dispersant treatments with polluted conditions (Figure 1). The sediment were homogenized and dispatched in the 2 L bottles (9 containing pristine sediments and 9 containing contaminated sediments). The different

conditions were applied after 3 days acclimatization at 4 ° C by adding the different type of WAF. Microcosms were maintained for 21 days at 4 ° C in the dark. Every day, the microcosms were gently shaken to allow the suspension of particles and for homogenization of sediments. Sub-samples of sediments were collected after 0, 7,14, 21 days of incubation for microbial analysis and PAHs analysis. Sub-samples were stored at -80° C until analysis.

Total DNA and RNA Co-extraction

From each sub-sample, genomic DNA and RNA transcripts were co-extracted. First, the total RNA was extracted using power sediment total RNA isolation kit (Qiagen, Germany) following the extraction protocol. Then, microbial DNA was further eluted from the RNA spinning column kit using the RNeasy Power-sediment DNA elution kit (Qiagen, Germany). RNA was quantified using Quant it RNA BR kit (Theremofisher scientific, USA), and DNA was quantified using Quant it dsDNA HS kit (Theremofisher scientific, USA).

CDNA RT and DNA amplification

Extracted RNA were initially treated with DNase (Invitrogen,USA) to remove genomic DNA carried over from the extraction procedure. DNase treated RNA were reverse transcribed using M-MLV reverse transcriptase (Invitrogen, USA) using random hexamers and following the manufacturer protocols.

The V4 region of the 16S rRNA gene was amplified using the bacterial universal primers 515f and 928r (Wang et al., 2009). The reaction medium (25 µl) consisted of 12.5 µl Taq polymerase (Invitrogen, USA) , 1 µl of forward and reverse primers at concentration of (20 µM), 5.5 µl water and 5 µl undiluted DNA template. The polymerase chain reaction was performed using with the following cycling conditions: 10 minutes at 95 ° C,

followed by 30 cycles of: 30 seconds at 95 ° C, 30 seconds at 60 ° C and 40 second at 72 ° C, followed by a final step of 10 minutes at 72 degree. Successful amplification of the PCR was checked on 2% agarose gel electrophoresis, stained with ethidium bromide. PCRs were performed in triplicates and are eventually pooled for further sequencing.

Amplicon sequencing and bioinformatics analysis

The 16S rRNA (gene and transcript) barcoding was performed using NGS Illumina MiSeq platform Toulouse (France). The obtained demultiplexed sequences were treated with Qiime 2 software (Caporaso et al., 2010) according to Quince et al., (2009). Briefly, DNA and cDNA sequences were trimmed and then denoised using DADA2 pipeline integrated in the Qiime2 software to remove chimeric sequences. The total number of sequences for each sample obtained after denoising is presented in Table S1. Denoised sequences were assigned with taxonomic affiliation using RNA gene reference database SILVA version 132 (Quast et al 2013) and singletons were then removed. Finally, sequences were rarefied according to the lowest number of total sequences observed in samples. Rarefaction curves are presented in (Supplementary Materials , chapter 4, Figure S1). An abundance table with the total available amplicon sequence variant (ASV) was produced. Rare taxa were removed and the top 350 taxa based on abundances were used for analysis.

Statistical analysis

Statistical analysis on the bacterial community dynamics such as alpha diversity indices, total bacterial abundances at the phylum level, were conducted on R software using RAM package (Chen et al., 2018). Procrustes analysis on DNA and RNA abundances was conducted using vegan package (Okansen et al., 2020). RNA: DNA ratio was calculated by dividing RNA abundances by DNA abundance of the same taxa.

Linear discriminant analysis effect size (LEfse, Segata et al., 2011) was performed with galaxy ((http://huttenhower.sph.harvard.edu/galaxy/)). Briefly, non parametric kluskal wallis was used to detect taxa with significant abundances (*p-value* < 0.05). Biological consistency was conducted using pair wise Wilcoxon test (*p-value* < 0.05). Finally, LDA with threshold score of 3.0 and 1000 bootstraps was applied. The RNA/DNA ratio of Taxa with the highest LDA scores were plotted overtime.

Non metric NMDS, Canonical correspondence analysis, and PERMANOVA were performed by PAST statistical software v4.03 (Hammer et al., 2020). The analysis were based on Bray Curtis dissimilarity.

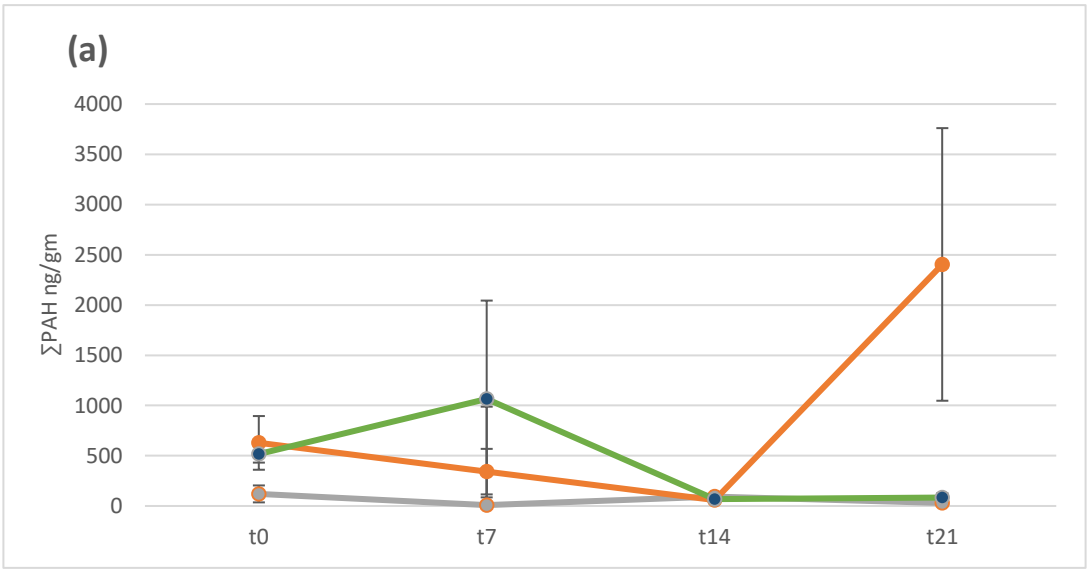
Tax4fun2 package (Asshauer 2014) was used to predict the gene function of all the ASV classified from the RNA abundance table. Predicted gene involved in PAHs biodegradation pathways were selected. Finally, heatmaps and clustered correlations were generated from functional genes using CIMminer (http://discover.nci.nih.gov/cimminer/). Correlations made were based on Pearson correlation. Only significant correlations (Pearson correlation > XXX, and p-value < 0.05) were considered.

Results and Discussion

PAH removal

The concentration of total PAHs was below 3 µg per gram of dry sediment in the majority of samples, the highest PAH concentration being measured in contaminated sediment containing WAF+ Dispersant (Figure 2). PAH concentration increased in control from contaminated site during the experiment, suggesting that the PAHs present in the sediment become more available under the experimental conditions. In the WAF + dispersant treatments, the total PAH concentration significantly decreased during the

experiment with Pristine sediment ($p < 0.01$), being close to 0 at day 21 (Figure2). In presence of dispersant (WAF + Dispersant), the PAH concentration decrease was notable from day 7. Additionally, the proportion of low molecular weight PAHs (LMW-PAHs, 2 - 3 rings such as naphthalene, acenaphthene, acenaphthylene , fluorene , phenanthrene, anthracene) decreased during the experiment, whereas that of high molecular weight PAHs (HMW-PAHs, > 3 rings such as fluoranthene, pyrene, chrysene) increased (Figure 3). This observation was made in both types of sediments irrespective of the treatment (WAF and WAF + Dispersant), but the proportion of HMW-PAHs was higher in sediments from the contaminated site, confirming the chronic pollution (Figure 3 b). The ratio LMW-PAHs/ HMW-PAHs decreasing during the experiment substantiated the removal of PAHs, as LMW-PAHs are more susceptible to microbial degradation and volatilization (Shilla et al., 2018; Olayinka et al., 2019; Lukic et al., 2016). It is likely that PAHs were removed by biotic processes considering the conditions (at 4° C in the dark limiting abiotic processes) under which the experiment was conducted, although abiotic processes could not be excluded



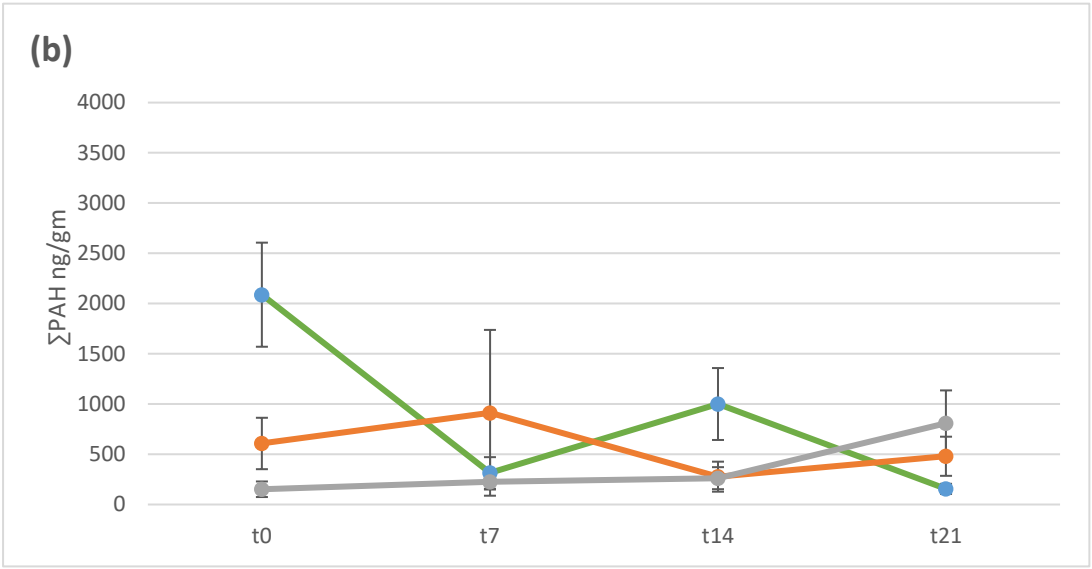
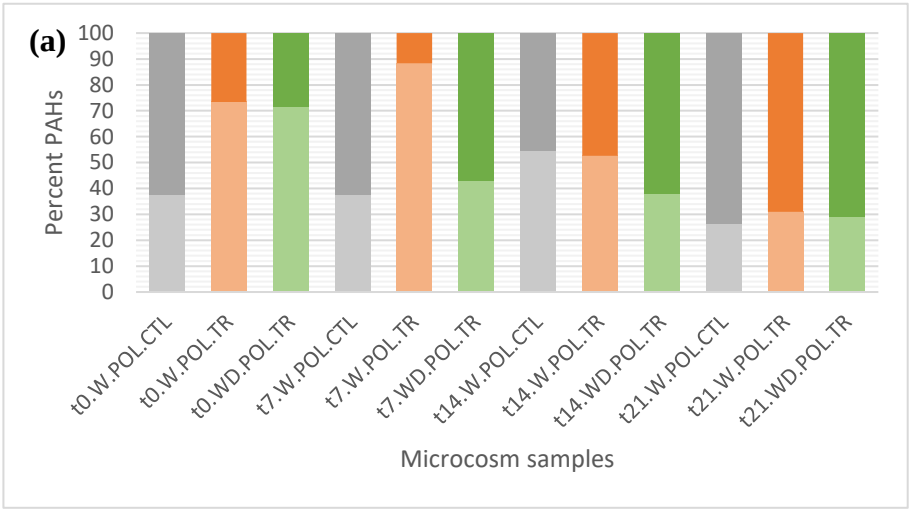


Figure (2): concentration of total PAHs in ng/gm in pristine sediment (a) and contaminated sediment (b) microcosms over the course of 21 days. Grey= control, Orange = WAF, Blue= WAF+ dispersant , (n=3) Error bars ±1 Standard error



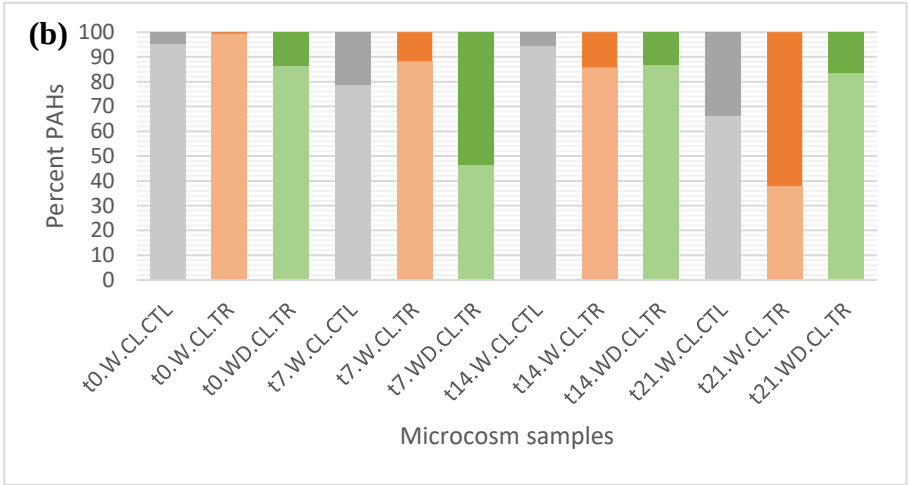


Figure (3): Percentage of low molecular weight PAHs (LMW) and high molecular weight PAHs (HMW) in pristine (a) and contaminated sediment (b). Grey= Control, Orange= WAF, Green =WAF + Dispersant. Darker color denote (HMW) PAHs while lighter color denote (LMW) PAHs. (N=3)

Bacterial community structures

After trimming a total of 631207 reads was obtained for sediment samples (Supplementary Material chapter 4, Table S1). The rarefaction depth was selected at 4396 for sediment samples according to the sample with the lowest sequence count. Rarefaction curves indicated that sufficient sequences were obtained to estimate the microbial diversity (Supplementary Material, chapter 4, Figure S1). Interestingly, the total community (DNA level analysis) showed more observed ASV than the active community (RNA level analysis), suggesting that just a portion of the total bacterial communities was active during the experiment.

The total (DNA) microbial communities were dominated by Proteobacteria (60%-50%) and Bacteroidetes (35%- 25%) phyla (Figure 4). Cyanobacteria, Actinobacteria, Planctomycetes, Nanoarchaeaeota phyla were observed at low abundance. Several bacterial genera from the phylum Proteobacteria, such as *Marinobacter*, *Oleispira*, *Rhodobacter*, have shown PAHs degradation potentials at 4 ° C (Crisafi et al., 2016). In general, the abundance of the different phyla did not showed noticeable changes during the experiment, except members of the Cyanobacteria phylum decreasing during the experiment in all treatments, probably because of the incubation in the dark. In addition, one way ANOVA showed no significant differences among the samples in respect of species richness (Shannon diversity index).

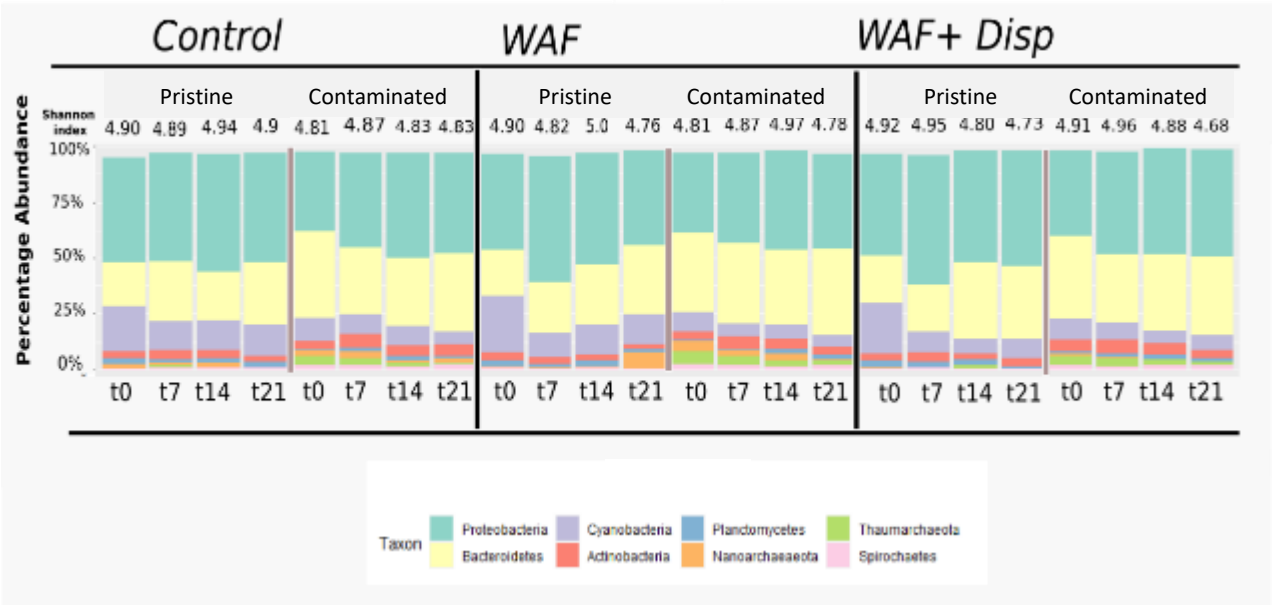


Figure (4): Relative abundances of total bacterial communities at the Phylum level in the three treatments (controls, WAF, WAF+ Dispersant) overtime. Number above each bar denotes Shannon diversity index (n=3)

Microbial community comparisons

The Procrustes analysis showed significant differences between total (DNA) and active (RNA) microbial communities with pristine sediment (P=0.4, M^2= 0.94, permutations 999) while with sediments total and active communities were similar (P = 0.04, M^2 = 0.8, permutations 999); (Supplementary Material, chapter 4, Figure S2). It is likely that the microbial communities from contaminated sediments were well adapted to the presence of PAHs explaining that total and active microbial communities were similar. In contrast, for the microbial community from pristine sediment the presence of WAF and WAF+ dispersant probably activated hydrocarbon-degrading bacteria, representing only a proportion of the total community. Thereafter, the ratio RNA to DNA was used to compare the active microbial communities. Additionally, the comparison of the behavior of microbial community from contaminated sediment to that of pristine sediment highlighted the legacy effect of the PAHs-adapted microbial community inhabiting the

contaminated sediment. The legacy effect, initially reported for microbial communities adapted to the presence of hydrocarbon (Paissé et al., 2008) and other contaminants (Ben Salem et al., 2019) in temperate marine sediments, is thus also observable for microbial communities inhabiting cold environments.

The comparison of total microbial communities (DNA) by nNMDs, based on Bray Curtis dissimilarity index revealed the modification of the microbial communities during the experiment for both pristine and contaminated sediments (Figure 5). Noteworthy, the microbial communities of the control showed less modification during the experiment than those of the WAF and WAF+Dispersant treatments (Figure 5a &b)

At time 0 in pristine site, total microbial communities clustered together, suggesting similar communities at the beginning of the experiment (Figure 5 b). Then, changes were observed within the microbial communities according to the treatments during the experiment. Particularly, for microbial communities from pristine sediment, the presence of dispersant resulted in changes more important than WAF alone. This suggest that upon the introduction of dispersant to pristine sediment, continuous microbial succession in the total communities was occurring overtime.

In general, according to RNA: DNA ratios (Figure 5 a & b), microcosms' active communities are not showing distinctive clustering. The NMDS shows that in pristine and contaminated sediment, the samples of the same treatment are distant from each other, therefore changes in active microbial community is observed overtime in the three treatments. However, in contaminated sediment, microbial communities (both total and active) showed an important shift in the WAF+Dispersant treatment at time 14. In addition, PERMANOVA based on bray Curtis dissimilarity only showed significant difference between contaminated and pristine sediments in respect of total (DNA) and active (RNA:DNA) communities (PERMANOVA $p<0.0001$), demonstrating the

significant diversity between the pristine and contaminated bacterial communities. Chemically enhanced WAF (CEWAF) has been demonstrated that can trigger bacterial communities' shifts in sediment after 30 days of incubation (Al Jawasim et al., 2015). Doyle et al., (2018) observed rapid changes within the microbial composition in surface water upon exposure to chemically enhanced WAF with Corexit dispersant. The authors explained that such rapid shifts in communities is due to the availability hydrocarbon degraders in the environment chronically exposed to hydrocarbons. On the other hand, Louvado et al., (2019) observed that after the addition of dispersant, no significant changes in the structure of sediment microbial communities from port facing chronic pollution due to the preconditioning of the sediment to contamination.

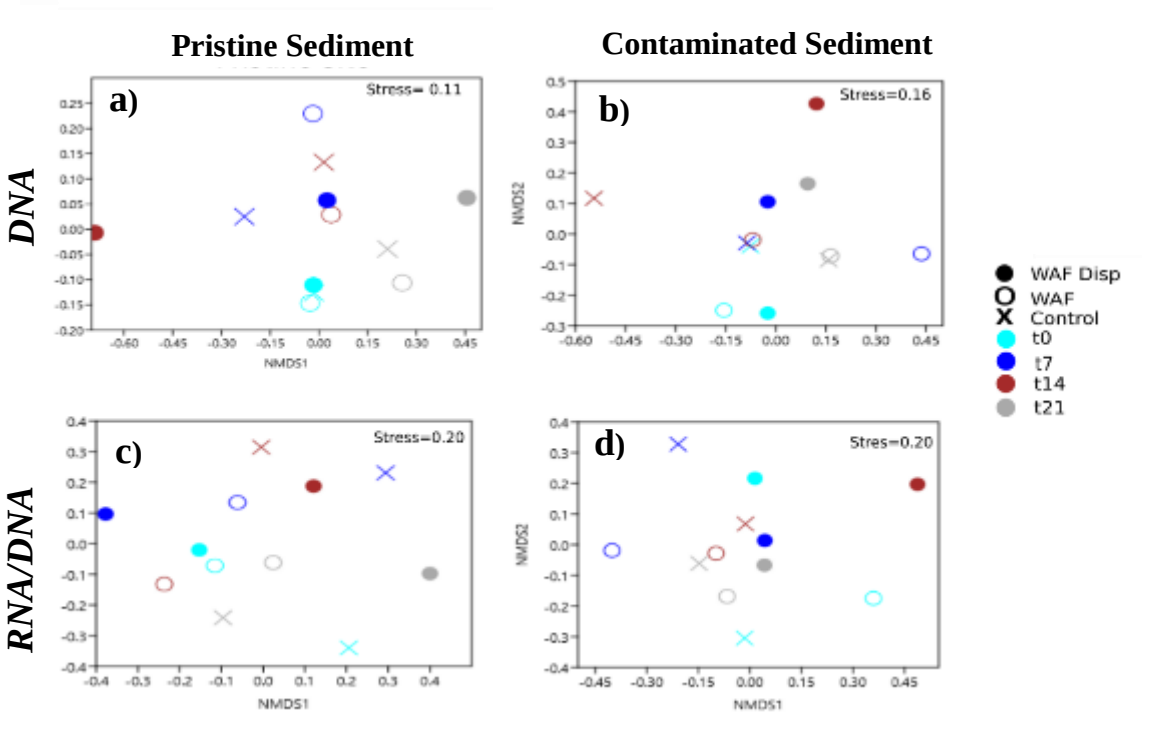


Figure (5): Non metric Multi Dimensional Scaling (NMDS) on samples diversity based on Bray Curtis Dissimilarity. (a,b) NMDS based on abundances of the total bacterial communities using DNA. (c,d) NMDS based on abundances of the active bacterial communities using the ratio between RNA to DNA.

Linear discriminant analysis (LDA) effect size (LEfse), performed on the active microbial community (ratio RNA:DNA), identified several taxa showing significant different abundances according to the treatment that represent potential bio-indicators of the effect of WAF or WAF + dispersant for both contaminated and pristine sediments (Figure 6). For contaminated sediment 13 bioindicator taxa were identified with WAF and 11 with the WAF+Dispersant (Figure 6b). The bioindicator taxa with the highest LDA score belonged to the *Gamma proteobacteria* and *Bacteriodetes* orders (Figure 6b). Interestingly, 5 of the taxa identified as bioindicator for WAF+Dispersant belonged to the genus *Colwellia*, highlighting the high activity of this genus in the WAF+ Dispersant treatment. Such observation was in agreement with previous reports showing that the presence of dispersant favor the *Colwellia* growth as it is able to use dispersant as carbon source (Kliendienst et al., 2015; Pena Montenegro 2020; Noirungsee et al., 2020). In contrast, ASV related to the gram negative *Thermoanaerobacteraceae* and to *Bacteroidales* identified as bioindicator in WAF treatments, have been described to be able to degrade PAHs (Marozava et al., 2018; Miller et al., 2019). It is also important to note that an ASV (ASV 1178) belonging to the *Desulfobulbaceae* family was identified as bioindicator for both WAF and WAF+ Dispersant treatments in comparison to the control (Figure 6b), suggesting that this ASV was active in both conditions that reveal its capacity to thrive in different environmental conditions. This result was consistent with the fact that members of the *Desulfobulbaceae* have been found abundant in oil-contaminated marine sediment with the capacity to degrade hydrocarbon anaerobically under sulfate reducing conditions (Matturo et al., 2017; Beazley et al., 2012). The presence of active ASVs related to *Bacteroidales* and *Desulfobulbaceae* significantly abundant on both WAF treatments suggested that anaerobic processes are involved in hydrocarbon degradation.

For Pristine sediment, only one ASV belonging to the *Gammaproteobacteria* class was identified as bioindicator for WAF+ Dispersant treatment (Figure 6A). Regarding the WAF treatment, LEfse identified 8 bioindicator taxa, an ASV related to the cyanobacteria *Microcystacae* family showing the highest LDA score. The other ASVs identified as WAF bioindicators belonged to *Gamma Proteobacteria* and *Alpha Proteobacteria*, genera detected in coastal salt march after the Deepwater Horizon oil spill (Beazley et al., 2012). ASVs related to *Desulfobulbaceae* were also revealed as bioindicator of WAF treatment (Figure 6a) indicating that anaerobic processes participate to PAH degradation.

The fact that large number of bioindicator taxa were identified in contaminated sediment than in pristine sediment further supported hydrocarbon-adapted microorganism inhabit the contaminated sediment. It is likely that the constant exposure to anthropogenic pressures such as oil spillage from boats select microbial communities with taxa able to degrade PAHs (Storey et al., 2018).

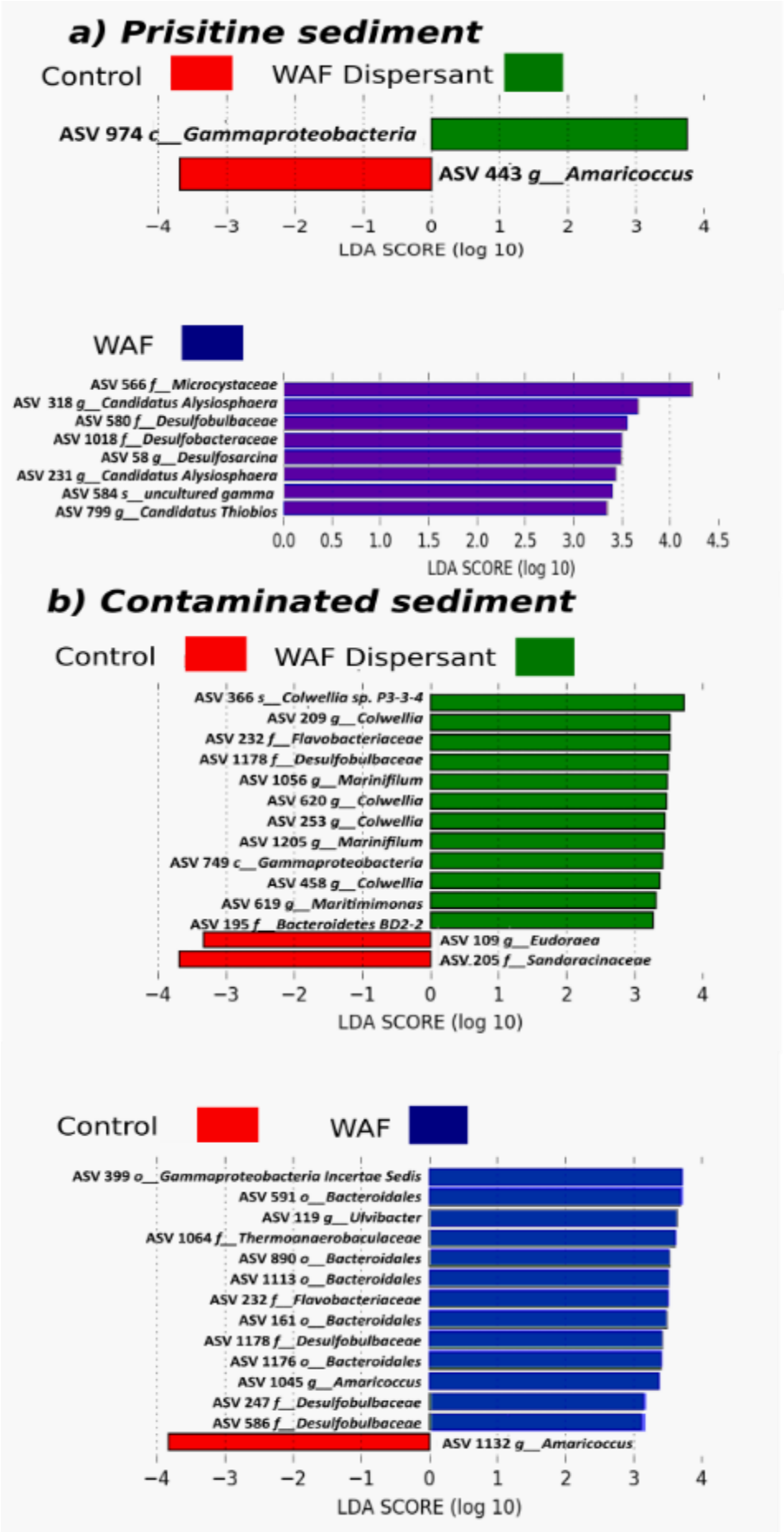

a) Prisitine sediment
Control
WAF Dispersant
ASV 974 c__Gammaproteobacteria
ASV 443 g__Amaricoccus
−4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)
WAF
ASV 566 f__Microcystaceae
ASV 318 g__Candidatus Alysiosphaera
ASV 580 f__Desulfobulbaceae
ASV 1018 f__Desulfobacteraceae
ASV 58 g__Desulfosarcina
ASV 231 g__Candidatus Alysiosphaera
ASV 584 s__uncultured gamma
ASV 799 g__Candidatus Thiobios
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
LDA SCORE (log 10)
b) Contaminated sediment
Control
WAF Dispersant
ASV 366 s__Colwellia sp. P3-3-4
ASV 209 g__Colwellia
ASV 232 f__Flavobacteriaceae
ASV 1178 f__Desulfobulbaceae
ASV 1056 g__Marinifilum
ASV 620 g__Colwellia
ASV 253 g__Colwellia
ASV 1205 g__Marinifilum
ASV 749 c__Gammaproteobacteria
ASV 458 g__Colwellia
ASV 619 g__Maritimimonas
ASV 195 f__Bacteroidetes BD2-2
ASV 109 g__Eudoraea
ASV 205 f__Sandaracinaceae
−4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)
Control
WAF
ASV 399 o__Gammaproteobacteria Incertae Sedis
ASV 591 o__Bacteroidales
ASV 119 g__Ulvibacter
ASV 1064 f__Thermoanaerobaculaceae
ASV 890 o__Bacteroidales
ASV 1113 o__Bacteroidales
ASV 232 f__Flavobacteriaceae
ASV 161 o__Bacteroidales
ASV 1178 f__Desulfobulbaceae
ASV 1176 o__Bacteroidales
ASV 1045 g__Amaricoccus
ASV 247 f__Desulfobulbaceae
ASV 586 f__Desulfobulbaceae
ASV 1132 g__Amaricoccus
−4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)

Figure (6): LefSE based on Linear Discriminant analysis (LDA) of taxa with RNA: DNA ratio significantly different from control. A) Treatments from Pristine sediment. B) Treatments from Contaminated sediment.

Dynamic of the active bacterial communities in response to WAF and WAF+ Dispersant

The ASVs with the highest LDA score for each treatment were selected to follow the dynamic change of their abundance pattern during the experiment (Figure 7). The highest change of the RNA:DNA ratio was observed for the cyanobacteria *Microcystaceae,* decreasing overtime. This can be explained by the lack of light during the experiment, inhibiting photosynthesis and therefore inhibiting the growth of photosynthetic bacteria. Interestingly, the ASV 974 *Gamma Proteobacteria*, a bioindicator for WAF+ dispersant in pristine sediment, showed more than five folds RNA:DNA increase at the seventh day followed by decrease in RNA:DNA for the rest of the experiment. In addition, *Colwellia* (ASV 366), also showed rapid increase in activity until day 7. However, unlike *Gamma Proteobacteria*, *Colwellia* was detected in the active community after day 7, suggesting its resilience. However, other selected ASVs, *(Candidatus Alysiosphaera, Gamma Proteobacteria Incertae Sedis, Bacteroidales, Flavobacteriaceae)* showed a delayed increase in the RNA:DNA ratios after 14 days of the microcosm experiment.

Gamma Proteobacteria and *Colwellia* ASVs showed active patterns similar to those observed for opportunistic taxa with r strategy (Storey et al., 2018). The r strategists have high and rapid growth rates, capable to colonize environment with high resource availability. However, r strategists have low competitive capability and lack stability (Vadstein et al., 2018). In the case of *Gamma Proteobacteria,* the initial growth is probably stimulated by the hydrocarbon enrichment (WAF or the WAF + dispersant), but then they collapse probably because of the depletion of carbon source. In contrast, the

delayed response in RNA:DNA ratio exhibited by *Candidatus Alysiosphaera, Gamma Proteobacteria Incertae Sedis, Bacteroidales, Flavobacteriaceae* follows the growth K strategist taxa. The K strategist taxa exhibit slow growth, evidenced in peak active ommunities after 14 days or more (Figures 7 a & c & d). They eventually succeed the collapse of r strategist because they are better competitor for resources (Vadstein et al., 2018).

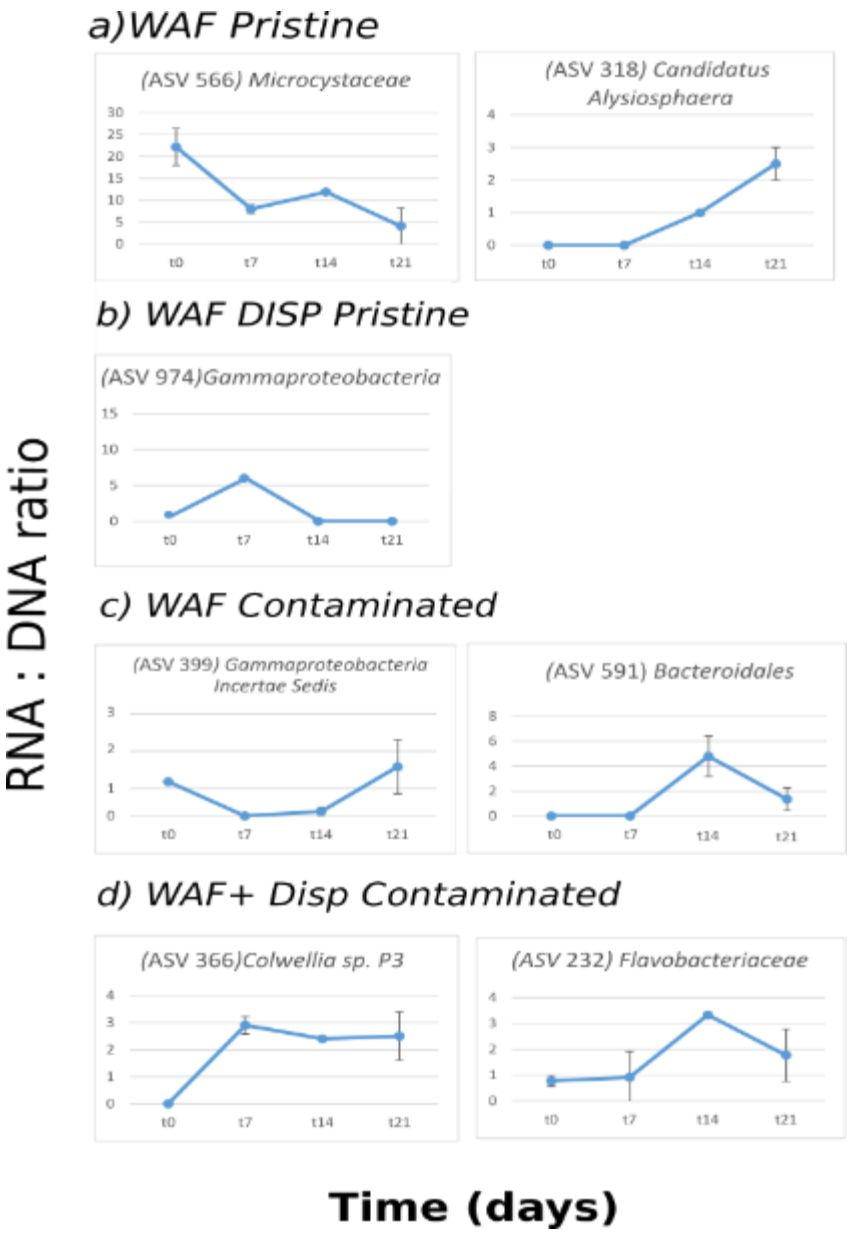


Figure 7: Dynamics of RNA: DNA ratios overtime of bioindicator microbial communities identified by lefSE

Relationship between the predicted functional genes and the concentrations of PAHs

The study of Tax4fun2 inferred functional genes in the active microbial communities allowed to understand the degradation patterns observed in response of WAF or WAF+ Dispersant enrichment. Among the 8426 functional genes predicted using tax4fun2, we selected 18 genes linked to PAH degradation pathways (Supplementary Materials chapter 4, Table 2). Most of the selected predicted genes were mainly involved in naphthalene degradation pathways, only six were involved in degradation pathways of PAHs with more than 2 rings. Canonical Correspondence Analysis (CCA) demonstrated the relationship between the predicted PAH degradation functional genes and the concentrations of the different types of PAHs.

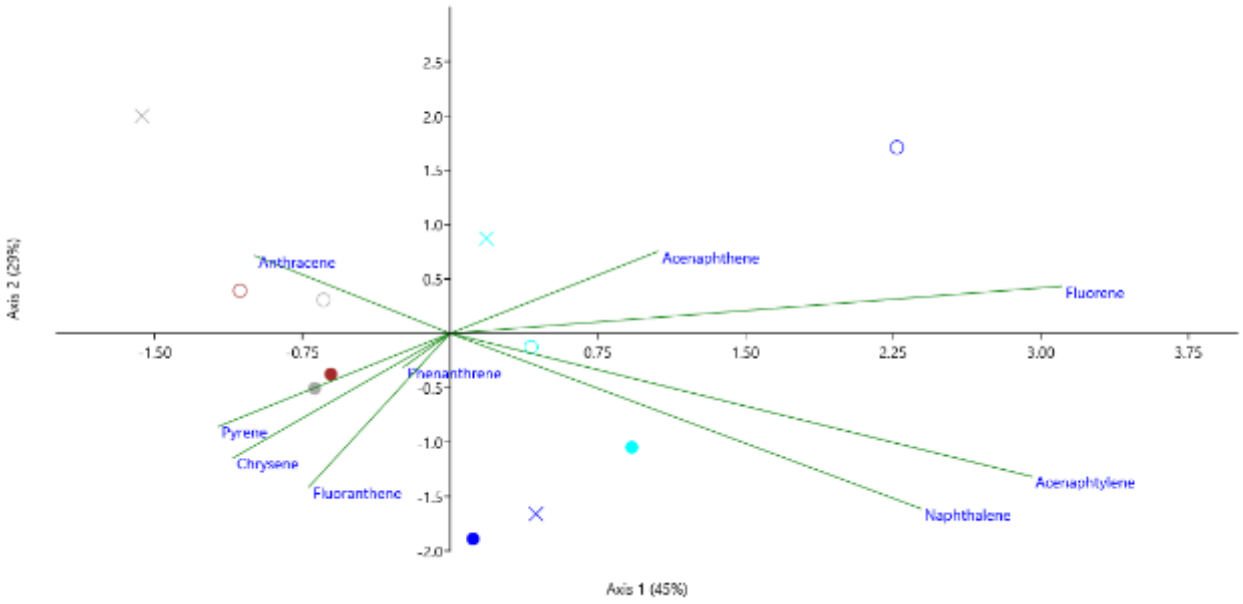
For pristine sediment (Figure 8 a), both CCA axes explained 74% of the total variance observed (axis 1: 45% and axis 2: 29%). The CCA demonstrated that the first axis correlated with 2-3 ring PAHs (fluorene , naphtalene , acenaphthene and acenaphtylene), whereas PAHs with more rings (anthracene, flouranthene, phenanterene and pyrene) were negatively correlated. The functional microbial community in WAF and WAF+ dispersant microcosms at time zero were associated with naphthalene and acenaphtylene, while at time 14 and time 21 they were associated with pyrene and chrysene, as the microbial community from WAF treatment at time 14. The functional communities of time 21 WAF and control were associated with anthracene. Control sample at time 14 was highly variable from the rest of the samples and therefore it was removed from the CCA analysis.

For contaminated sediment (Figure 8 b), both CCA axes explained 72% of the total variance observed (axis 1: 41% and axis 2: 31%). The first axis was positively correlated with naphthalene and its derivatives (acenaphthene and acenaphtylene), while the second axis was negatively correlated with PAHs with more rings (fluorene, pyrene, anthracene,

fluoranthene, phenantherene). Functional communities of control and WAF at time 7 and time 14 respectively were associated with low molecular PAHs of two rings (naphthalene, acenaphthene, acenaphtylene). Additionally, WAF+ Dispersant functional communities of time 0,7 and 14 were also associated with low molecular PAHs of two rings. Whereas functional communities of WAF + Dispersant and WAF at time 21 and control at time 7 were associated with PAHs with more than two rings (fluorene, pyrene, anthracene, fluoranthene and phenanthrene).

As shown by the CCA of pristine and contaminated sediments communities, the correlation between the WAF and WAF+Dispersant exposed functional communities at time 14 ,21 and the high molecular weight PAHs ,such as pyrene chrysene , could have several explanations. HMW PAHs biodegradation rates slower than LMW due to the slow microbial degradation of four aromatic rings (Garneau et al., 2016). In addition, degraders of HMW PAH, are not commonly in high abundance in the environment (Rathour et al., 2018), therefore it would take some time to stimulate such taxa to abundances adequate for noticeable biodegradation processes.

(a) Pristine Sediment



(b) Contaminated Sediment

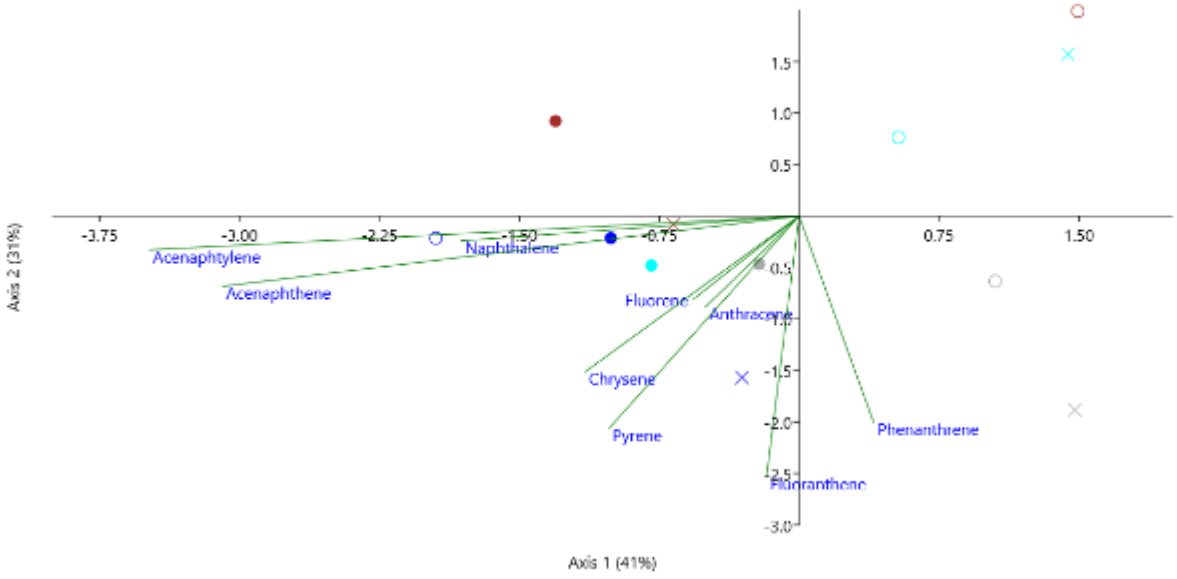


Figure (8): Canonical Correspondence analysis (CCA) between the selected functional gene in active bacterial communities and the Concentrations of different PAHs. a) Microcosms samples prepared by contaminated sediment, b) Microcosms samples prepared by pristine sediment.

Relationship between each predicted functional gene and the different types of PAHs

In order to understand the dynamics of PAH metabolisms within communities, the predicted functional genes of the PAH metabolic pathways were analyzed. The PAH degradation pathways are complex and involve several metabolic steps. Generally, PAH degradation pathways are initialized through the hydroxylation or deoxygenation of PAHs through the catalysis of oxygenases. PAHs are then transformed into several key intermediate metabolites such as protocatechuate, salicylate, gentisate and catechol, which are then metabolized through central pathways (Cao et al., 2015).

The heat map (Figure 9 a) showed that in the pristine site functional communities in WAF+ Dispersant microcosms, 15 functional genes showed strong positive correlations (0.80< Pearson correlation <0.99) with 2/ 3 rings PAHs (naphthalene, acenaphthene, flourene, acenaphtylene), while in WAF treatment, 6 functional genes showed strong positive correlation (0.80< Pearson correlation <0.99) with naphthalene and acenaphtylene.

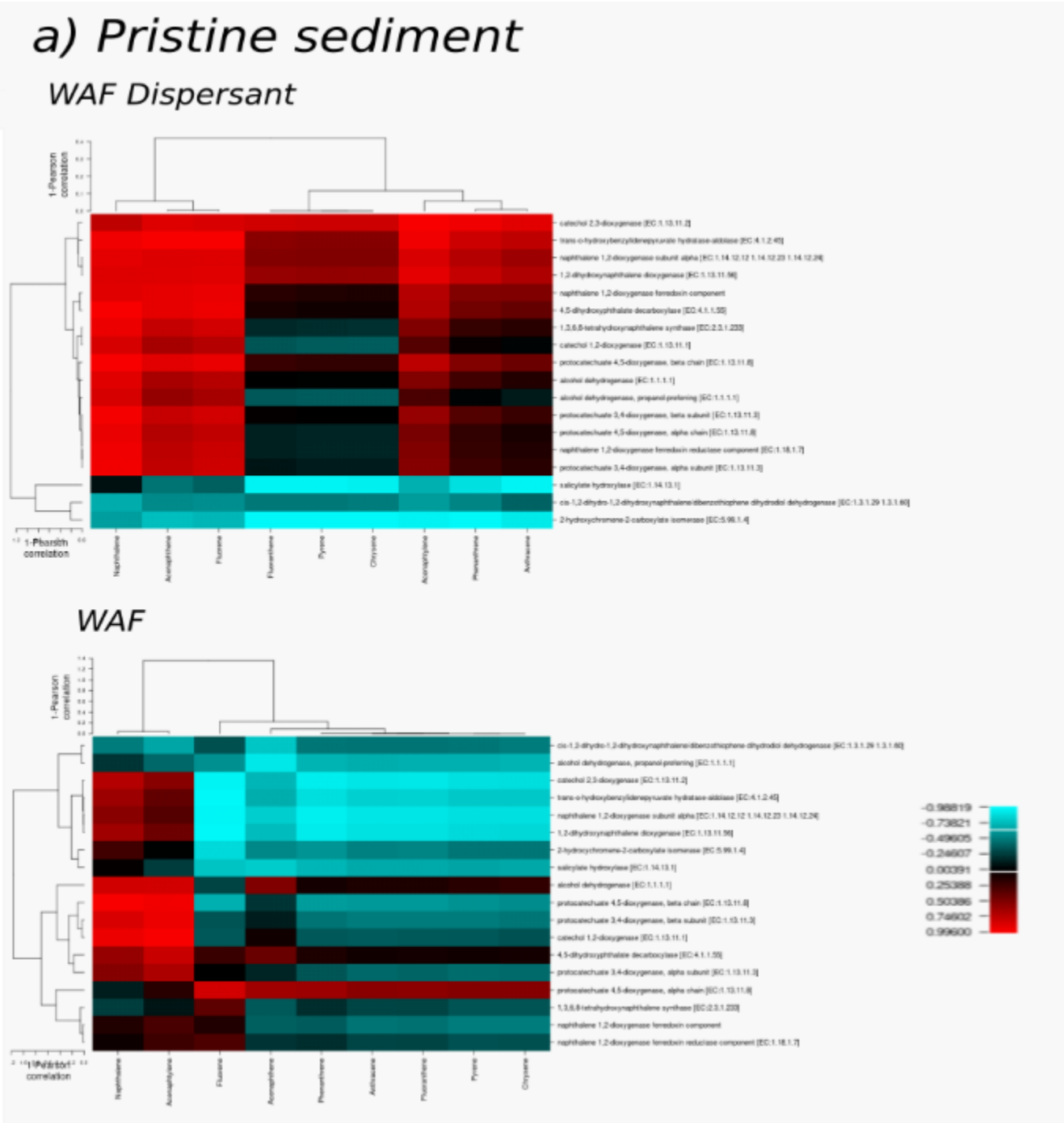
In the heat map for the functional microbial community from contaminated sediment (Figure 9 b), several functional genes showed strong correlation (0.74< Pearson correlation <0.99) with the concentrations of PAHs. One functional gene, 4,5 dihydroxylphalate decarboxylase showed high correlation (0.74< Pearson correlation <0.99) with all PAHs studied except chrysene in WAF+ Dispersant treatments (Figure 9 b). However, in the WAF treatments, 4,5 dihydroxylphalate decarboxylase was strongly correlated (0.74< Pearson correlation <0.99) with all PAHs except naphthalene and its

derivatives. The 4,5 dihydroxylphalate decarboxylase is known as a major intermediate metabolite in the microbial degradation of PAHs such as fluoranthene, fluorene and phenanthrene (Kasai et al., 2019).

Additionally, seven functional genes in WAF+Dispersant treatments were strongly correlated with chrysene, suggesting the availability of active taxa involved in the degradation pathways of chrysene. Four more functional genes were positively correlated with PAHs with more than 2 rings (pyrene, fluoranthene, Anthracene, phenanthrene) (0.70< Pearson correlation <0.85). Interestingly, in WAF treatments, (acenaphtylene and acenaphthene) showed strong correlation (0.80< Pearson correlation <0.99) with 4 genes. Whereas, PAHs with more rings were positively correlated with 3 different genes classified as intermediate metabolites, (Dihydroxylphalate, Protochatecuate, Catechol) (0.70< Pearson correlation <0.85). Consequently, our result demonstrates that the active functional communities from contaminated sediment are efficient to degrade PAH with more than 2 rings.

We can conclude that according to the correlation patterns observed in the heat maps, the functional communities from pristine sediment were enriched with active populations involved with LMW biodegradation pathways. The addition of WAF+Dispersant stimulated taxa with functional genes involved in LMW PAHs degradation pathways. The observed strong correlation patterns of functional communities in pristine sediment could be because in pristine environments, contamination could be a strong driver that can rapidly stimulate and shift the active bacteria communities (Coehlo et al., 2015). On the other hand, the contaminated sediment functional communities are already adapted to the chronic presence of the hydrocarbon and consequently, are not highly stimulated as observed in the pristine sediment. Although our results showed that active microbial community exhibited promising predicted functional patterns, it is important to highlight

that such functional genes are predicted and not directly measured. Therefore, more research is needed to measure metabolites of PAH degradation pathways to fully understand these pathways.



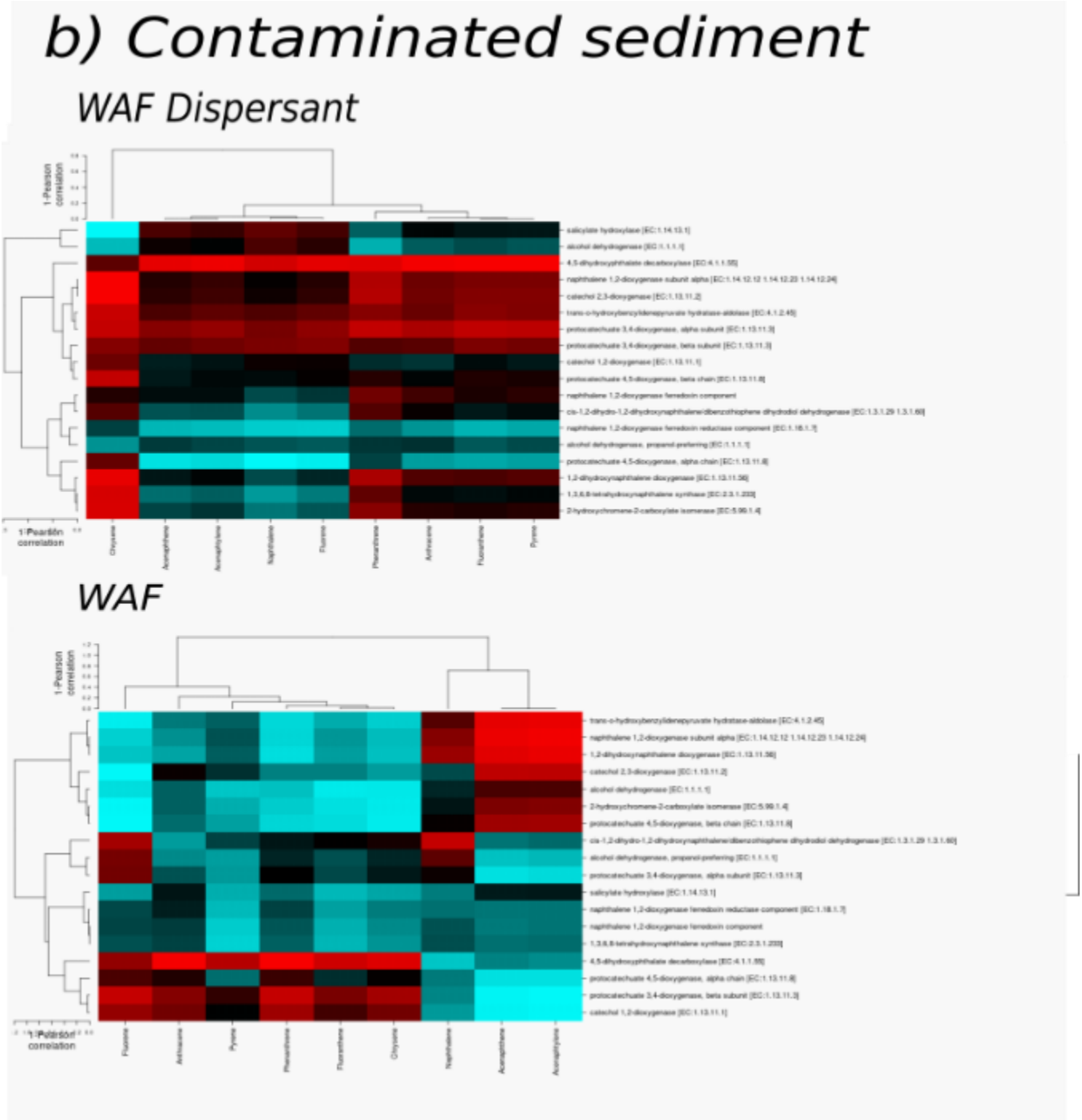


Figure (9): Heatmap with cluster analysis showing the correlation between each predicted functional gene and the concentration of the different PAHs in a) Pristine sediment communities and b) Contaminated sediment communities . Correlation and cluster analysis are based on Pearson correlation

Conclusions:

This study highlighted the behavior of microbial communities in sediments upon the introduction of WAF and WAF + Dispersant at 4° C. The initial microbial communities showed to be an important player on the fate of PAHs in sediment. Sediment with a chronic history of contamination owned taxa responsive to the PAHs in the WAF. In addition, dispersant showed to have impact on the total and active microbial community dynamics, especially for those inhabiting pristine sediment. Throughout the course of the experiment, active taxa followed an ecological succession degrading first LMW PAHs and then HMW PAHs, possibly because the degradation of HMW PAHs is challenge. However, the difference in microbial communities between the two types of sediment also highlighted the different biodegradation capabilities in the sediments. Dispersant added to pristine sediment showed to stimulate PAH biodegradation pathways of LMW PAHs such as naphthalene and its derivatives. Whereas in contaminated sediment, both WAF with and without dispersant stimulated PAH biodegradation pathways. We conclude that although dispersant did not stimulate several specific taxa, it stimulated the biodegradation rates in the sediment. In contaminated sediment, there was no changes in the biodegradation rates between WAF and WAF+ Dispersant.

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General Discussion

Through the different bioassays performed and endpoints studied, the copepod *Acartia tonsa* has shown to be a valuable toxicological tool in understanding the effects of contaminants on the marine environments. The different levels of endpoints used in this thesis has further demonstrated how cheap and valuable can toxicity assays be in order to rapidly diagnose lethal and sub lethal toxicity effects of environmental contaminants. In addition, *Acartia tonsa* geographic distribution and its tolerance to a wide range of temperature and salinities represent this species as a valuable candidate for different environments such as tropical, temperate and cold environments.

Water Accommodated Fractions prepared from a crude oil and two types of distilled oils (IFO 180 and marine diesel) showed to have different chemical compositions and consequently different toxicity effects on the copepod *Acartia tonsa*. NNS WAF had the highest concentration of total PAH, mainly naphthalene. Whereas, distilled oils WAF have high concentrations of phenanthrene. Although PAHs compositions contribute to the observed toxicity profiles, it has been shown that additives added to oil derivatives (e.g. phenol and sulfur) play a major role in the toxicity toward *Acartia tonsa* (Rodrigues et al., 2010). WAF prepared from distilled oils (marine diesel and IFO180) was the most toxic in respect of mortality and reproductive capabilities. In the case of marine diesel, both histology and Nile red imaging have shown that lipid deposits in *Acartia tonsa* are majorly accumulated in eggs and ovaries. At the molecular level exposure to IFO 180 WAF showed to initiate cellular damage, evidenced by the early significant expression patterns of CYP 3026b and heat shock protein 70 genes. In addition, our results have demonstrated that these genes act as valuable biomarkers for assessment of early cellular damage due to exposure. Although of the high naphthalene content, WAF from the NNS crude oil showed very little toxicity effects on *Acartia tonsa,* further suggesting that distilled oils have more toxic effects than the crude oil. Results have shown that the

toxicity effects after exposure to WAF from distilled oils are highly focused on offspring production, therefore affecting the copepod population through the inability of producing viable offspring. This decrease in reproduction capabilities has detrimental effects on the different trophic levels of the food web as *Acartia tonsa* are dominant components in their ecosystems (Mauchline, 1998). However, further research on transgenerational effects of distilled oils of WAF will provide wider picture on the effects on the population of *Acartia tonsa*.

Dispersant added to Water Accommodated Fraction (WAF) has shown to have toxic effects on *Acartia tonsa.* WAF with dispersant had caused high mortality rates, affected the rates of egg production and affected the transcription levels. It is likely that the addition of the dispersant had more detrimental effects on marine fauna than the WAF alone. As illustrated from the in vivo and microcosm experiment, we have a good understanding on the toxicity effects of WAF+ Dispersant. Although we observed that the initial concentrations of PAHs in WAF+ Dispersant in the microcosms were similar to PAHs concentrations in WAF, WAF +Dispersant has been shown to increase the bioavailability of PAHs in the water column (Milinkovitch et al., 2011). However, as demonstrated in chapter 3, the initial high toxicity exhibited by the WAF+ dispersant is not long lasting. The microcosm study has shown that after 21 days, the toxicity of dispersant has noticeably decreased due to the possible microbial degradation of the dispersant and PAHs. However, at day 21, potential sub-lethal toxicity was still present as shown by the downregulation of the antioxidant genes in the exposed *Acartia tonsa*. The biodegradation of the dispersant was due to the rapid stimulation of several opportunistic taxa, such as *Colwellia* and *Fusibacter*, which have shown to be able to use dispersant as carbon source for growth (Kleindiest et at., 2015; Calderon et al., 2018).

The efficiency of dispersant in improving biodegradation rates has been widely debated in literature. Our results participate in better understanding the role of dispersant on the biodegradation rates after introducing oil under low temperature. In water, our microcosms results did not provide clear evidence that the dispersant improved the PAH biodegradation rates as the concentration of PAHs in WAF and WAF+ Dispersant were similar. In fact, at low temperature, the addition of dispersant to oil has shown to initially inhibit biodegradation rates and lower the degradation rates of aromatic hydrocarbon (Foght et al., 1982; Rahespar et al., 2016).

Our results support the fact that the primary factor influencing the biodegradation rates is the initial composition of bacterial communities that own PAH-degrading taxa (Storey et al., 2018). Compared to microbial communities inhabiting pristine water communities, those inhabiting contaminated water contained more hydrocarbon degrading taxa. This corresponds to the legacy effect explaining the presence of adapted microbial populations to the presence of PAH, indicating that the whole microbial community is "PAH-immunized" with the ability to respond rapidly after WAF addition. However, WAF+ Dispersant has clearly shown to play a key role in shaping the dynamics of bacterial communities by stimulating several opportunistic taxa, which exerted ecological pressure on other bacterial populations. However, to fully understand the biodegradation rates in water in response to the addition of WAF of WAF+ Dispersant further work is still needed by studying the bacterial communities at the functional level through the analysis of RNA and DNA sequences. Unfortunately, we were not able to obtain RNA from water samples probably because of low microbial biomass in cold temperature, constituting a limitation in this thesis.

In sediments, the concentrations of PAHs were significantly lower than in the water column. Several reasons could explain these differences in concentrations. For example,

more time was needed for further sedimentation of PAHs from the water column. In addition, PAHs could have been lost due to evaporation during the freeze dry pre treatment (Law et al., 1994), a problem with wet sediments extractions. In aquatic environments, dispersants can speed up the sedimentation of suspended particles and can assist in the formation of oil mineral aggregates in the water column , therefore promoting the transfer of PAHs into marine sediment (Cai et al., 2017). In addition, the strong adhesion force between oil and the sediment decrease the bioavailability of PAHs, thus reducing the bacterial biodegradation rates (Ansari et al., 2018; Louvado et al., 2019). Our study has demonstrated that the integrative study of both total and active bacterial communities through both DNA and RNA sequencing assisted in understanding the functional microbial communities, which are involved in PAHs degradation in response to the addition of WAF and WAF+ Dispersant.

Interestingly, in pristine sediment, the abundance of active communities corresponding to different functional genes of PAH degradation pathways demonstrated that biodegradation mechanisms occurred in sediment in response to the introduction of dispersant. In pristine and contaminated sediments, functional communities demonstrated that low molecular weight PAHs, such as naphthalene, are degraded faster than heavier PAHs with more than 3 rings. In fact, we hypothesize that legacy effect is an important contributor to the responses of microbial communities toward WAF. Low response of functional taxa to WAF and WAF+ Dispersant is due to the legacy effects resulting in the high abundance of hydrocarbon degrading and dispersant resistant taxa in the contaminated sediment (Louvado et al., 2019; Paisse et al., 2010).

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Conclusions and Thesis

Conclusions:

1) Different types of oil have showed different toxicological effects in exposed *Acartia tonsa* copepods. WAF prepared from marine diesel showed to have detrimental effects on mortality, fecundity, total lipid content and egg hatching success to copepods. The combination of such endpoints demonstrates that the sub lethal concentrations of marine diesel affect the egg production capabilities of female copepods. IFO 180 shown detrimental effects on mortality, fecundity and transcription levels of selected genes involved in ROS and xenobiotic metabolism. Therefore, WAF prepared with distilled oils showed more toxic effects than NNS crude oil.

2) Exposure of copepods to WAF+ Dispersant had detrimental effects on survival and egg production rates in females and downregulation of key genes in the ROS and xenobiotic metabolism, in comparison to NNS WAF alone. This indicates that the use of chemical dispersants of the FINASOL52 type as a form of remediation after an oil spill could have detrimental effect on the marine fauna.

3) Studying endpoints at different biological levels, ranging from molecular to reproductive endpoints, demonstrated that the copepod *Acartia tonsa* is a valuable model organism suitable for studying the effects of contaminants.

4) In the water column at 4°C, bacterial communities structures are not static, they are actively changing overtime due to the addition of WAF with and without the dispersant. WAF+Dispersant had great impact on the structure of the bacterial communities. The presence of dispersant stimulates certain taxa, which play a role in driving the community structure. However, there is no enough evidence that dispersant improve the biodegradation rates of PAHs in the water column. However, the toxicity of WAF+ dispersant is acute but not long lasting. The dispersant stimulated taxa are able to degrade the dispersant, and consequently decreased the dispersant toxicity.

5) The initial structure of the bacterial communities plays a key role in PAHs removal in both sediment and water column. Microbial communities of subarctic areas with historical expososure to chronic contamination contain bacterial taxa capable of PAHs-biodegradation. Whereas bacterial communities from pristine site, not exposed to PAH-contamination, exhibited slower removal rates, indicating that pristine sites are sensitive to oil spills.

6) Analysis of RNA of bacterial communities from subarctic areas paved the way to understand the functional communities in sediment upon the introduction of WAF and WAF+ Dispersant. Dispersant showed to play a role stimulating active communities in pristine sediment, suggesting the possible involvement of several taxa in biodegradation due to dispersant. On the other hand, functional communities in contaminated sediment showed that legacy effects contributed in shaping the microbial response towards WAF and WAF+ Dispersant

Thesis:

Bioassays using the marine copepod *Acartia tonsa* in combination with a biological multi-level approach demonstrated that refined oils are more toxic to copepods than naphthenic crude oil. The addition of chemical dispersant Finasol 52 provokes more prominent toxic effects in copepods. This raise the environmental concern on the application of these compounds in oil spill scenarios. Moreover, at 4°C, microbial biodegradation rates are more influenced by the initial composition of bacterial communities than by the addition of dispersant. However, dispersant has shown to affect the microbial community dynamics by favoring certain microbial taxa than others.

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Supplementary Materials

Chapter 1:

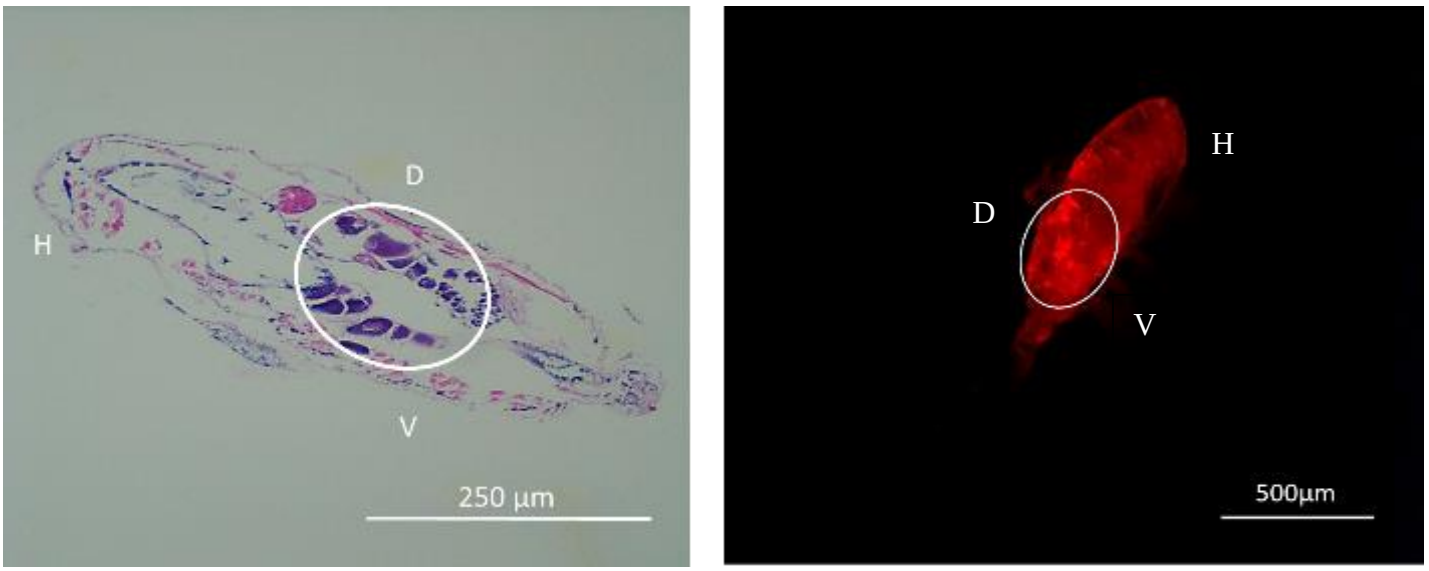

Figure S1: (a) Longitudinal section of a fecund female stained with hematoxylin and eosin. Circle represents ovary with eggs at different developmental stages. Scale bar: 250 µm. D: dorsal side, V: ventral side, H: head. (b) Fluorescence microscope image of a fecund female stained with Nile Red. Bright red spots (in circle) represent high lipid deposit areas around the ovaries. Scale bar: 500 µm. D: dorsal side, V: ventral side, H: head.

A) Cyp3026b

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LS054218.1          CTGACTTCCAGCTTCCAGGTACAGACTATTGGGTGAGAAAGAACGACCTGCTCTCACTGT        6497
KF639998.1          GTGATTACAAGATCCCTGGTCACGATGTCATCATACCAAAAGGTACCGATGTGTATATGA        1318
XM_023493699.1      AAGACTACAAGTTCCCTAACACCCATGTTGAAGTAAAAAAAAATGATGTTGTTTTGATAC        1344
                      ** * * ** * **        *        *   ***          * *   *

LS054218.1          CTGCCATTGGCATCCACCATGACGAGCGCTACTACCCCAACCCTAGCCAGTTCAATCCTG        6557
KF639998.1          TGGCTTACAGCATCATGCGAGATGAGAGGTATTTCGATCAACCCACTGAATTCAATCCAG        1378
XM_023493699.1      CAGCGTCCGGAATTCACCGAGATGGAAAATACTATCCAAACCCAAACCAGTTCAATCCAG        1404
                      **     * **    *  ** *     ** *      * ** *   * ******** *

LS054218.1          ATAATTTCTCCAAAGAGGCCCGCCAATCACGCAGCCCCTACACTTTCCTGTCATTTGGAC        6617
KF639998.1          ATAACTTCTCCAAAGAGGCCAAGGAGAAGAGAAGTCCCTACGCTTATCTCCCATTTGGTC        1438
XM_023493699.1      AAAACTTTTCTAAGGAAGCCAAGCAGTCTAGAAGTCCTTATGCATTCTTGGGATTTGGTC        1464
                    * ** ** ** ** ** ***    *     * ** ** **  * *   *   ****** *

LS054218.1          AAGGTCCCCGAGCTTGCATAGGCATGAGGTTTGCTTTGCTGGAGGCTAAAGTCGCCATCG        6677
KF639998.1          AAGGTCCTAGAGCTTGCATTGGCATGAGATTTGCTCTTCTGGAGGCTAAAATTGGATTGG        1498
XM_023493699.1      AAGGTCCTCGAGCTTGCATAGGAATGAGGTTCGCACTTCTGGAAGCCAAGGTGGCAATGA        1524
                    *******  ********** ** ***** ** **  * ***** ** **  * *   *

LS054218.1          CAATGGTGCTGCGCAAGTTTAATTTTCTGCCCAGCAACAAGAATCCGGAGCAGCTGGAAT        6737
KF639998.1          TGTCCGTTCTGACCAAATATCGGATCAAAACTTGCGAGGAAACTCCTCAGCATGCCATTC        1558
XM_023493699.1      TGATGGTTCTCTCAAATTACACATTTGTCTACAGTCAAAAGAATCCAGACAAACATGTTA        1584
                         ** **    ** *      *        *  *  * * ***  *  *
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B) Catalase

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LS054460.1          CATTTTCTTGTTCAAGGAGAACCATATCTGTTCAGTGGTTAGCTAAATTTTTCAGTACAG        1380
XM_023472406.1      CAAGGATTACGAGAAAAAAAATAATGG-AACTGATGTCCTGACTACAG--GTCATGGTGC        147
EL965956.1          AGAAAGCTTTAGAAGAAAGAATGGGAT-GGTGGAGACCCTGACAACAG--GGCACGGAGC        135
                           *     *   * **          *     *  * * *     **

LS054460.1          ACATAATGACACAAACAAACATCCTGTTACTTTTATATAAGAATTTAAAATTCTTTTTTA        1440
XM_023472406.1      TCCAGTCGCGGATAAAATAAATTCTATAACTGCAGGACCTAGGGGACCAATCCTTCTCCA        207
EL965956.1          TCCAATTGCTGACAAGCTTAACTCTATGTCAGTAGGTCCCAGGGGACCTCTTCTAATCCA        195
                     *     *     **     *  ** *  *                  * **  *  *

LS054460.1          GGATCATGATCTTATTGATGAGATGGCTCATTTTGACAGGGAGAGAATCCCAGAAAGAGT        1500
XM_023472406.1      GGATCATGTTCTAATTGAAGAGCTGGCCCACTTTGACCGTGAGAGGATTCCTGAAAGAGT        267
EL965956.1          GGACCATGTGCTCTTGGAGGAAATGGCACATTTTGATCGTGAGAGAATCCCAGAAAGGGT        255
                    *** ****  **  * ** **  **** ** *****  * ***** ** ** ***** **

LS054460.1          GGTACATGCTAAAGGAGCCGGGGCCTTTGGATACTTTGAGGTAATTATCAGGGATAATTT        1560
XM_023472406.1      TGTTCATGCAAAG-------------------------------------------------        280
EL965956.1          TGTACATGCAAAG-------------------------------------------------        268
                     ** ***** **
```

C) GST

Figure S2: Multi sequence alignment (a) cyp3026b (b) catalase (c) gst with homologs of other species. Grey areas denote amplicon regions.

Chapter 3:

a)

b)

c)

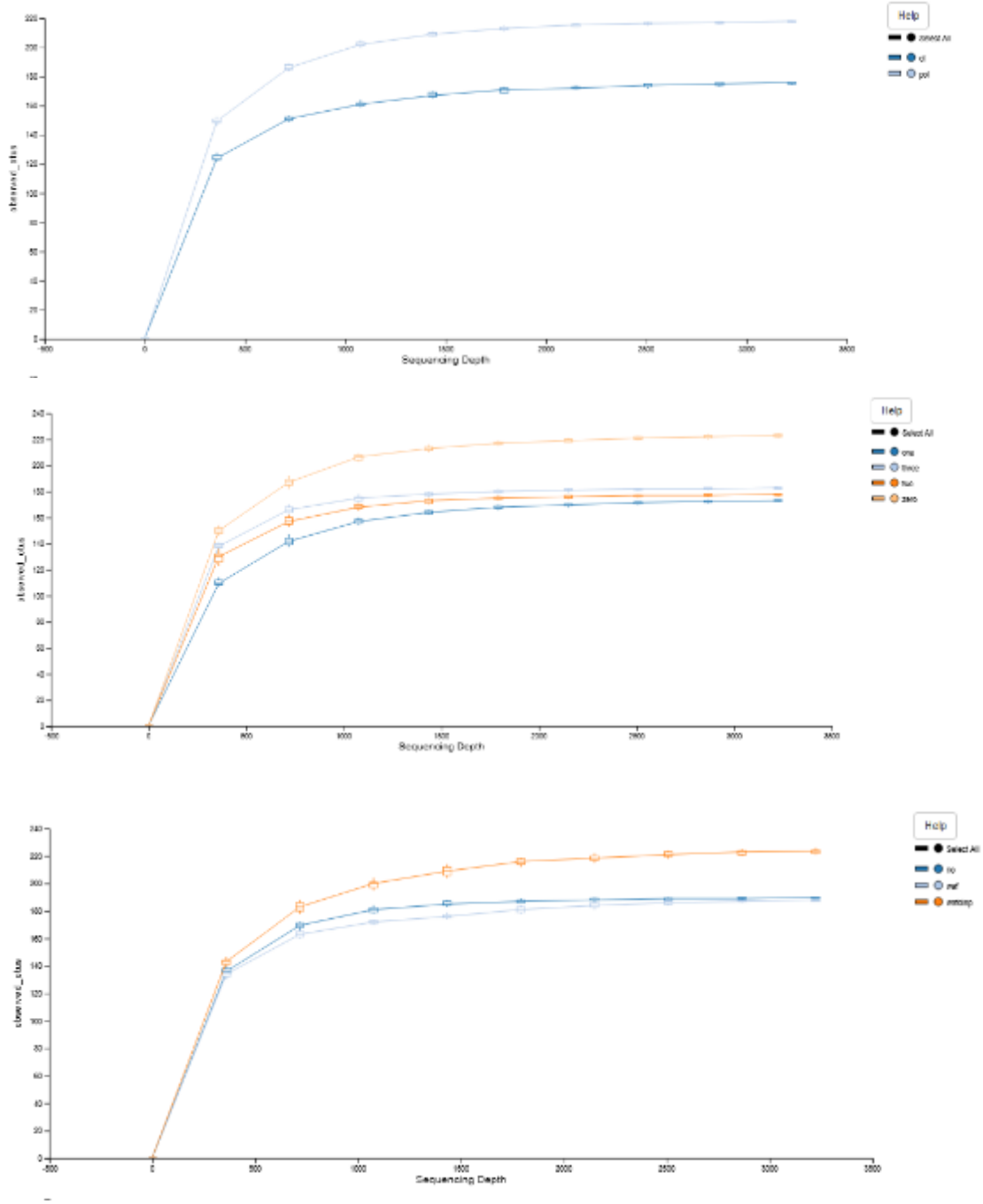


Figure S1: Rarefaction curves in respect of a)site, b)treatment , c) time

Table S2: denoising Features and alpha diversites for all samples used in this study

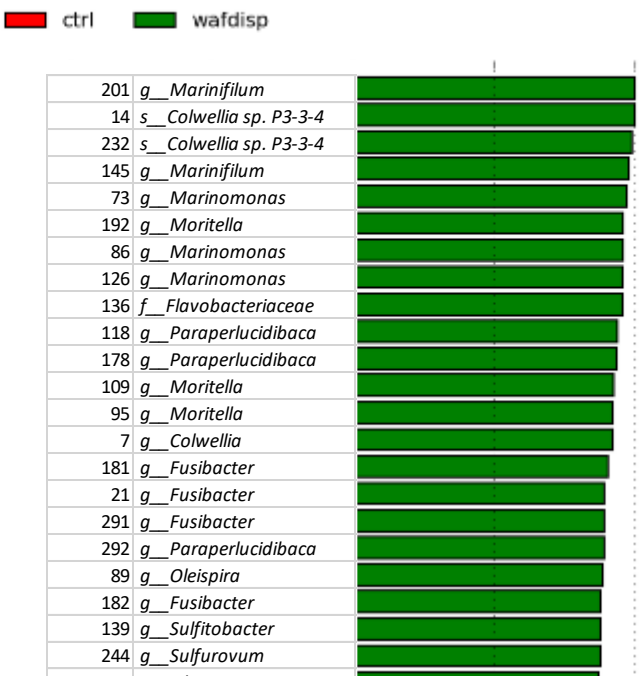
Sample ID	Input	Filtered	Denoised	Merged	Non Chimeric	Total Features Count	Shannnon	Chao
t0wcc1	17714	14094	12471	9082	5967	5956	4.05009	80
t0wcc3	18416	13877	12082	9060	5620	5523	4.050962	81
t0wct1	20436	11995	10851	8780	5524	5397	3.829928	72
t0wct2	36900	27361	25076	19857	11079	10778	3.761543	96
t0wdct1	24753	14305	12402	8126	4461	4460	4.04409	77
t0wdct2	17589	13453	12878	11286	6986	6935	4.037667	74
t0wdct3	31555	24670	22391	16670	9057	9027	4.047305	88
t0wdpt2	25244	20001	17482	12490	8041	7955	4.144615	88
t0wdpt3	24279	19057	15889	10061	7043	6941	4.349169	88
t0wpc1	17527	13194	10374	6793	4952	4573	4.138095	83
t0wpc2	22876	18466	15949	11713	7682	7397	4.122577	86
t0wpc3	26151	20763	17002	11104	8059	7612	4.299992	112
t0wpt1	21163	16089	11964	6761	5439	5086	4.236977	93
t0wpt2	21614	17412	13322	7579	5247	5096	4.070794	81
t0wpt3	20568	16118	13155	8861	6245	5974	4.147163	84
t1wcc2	20594	16784	15303	12250	7481	7420	3.658691	80
t1wcc3	25805	18280	16429	11733	7086	6960	3.745895	90
t1wct1	30942	24789	22315	16756	10293	10168	3.592754	85
t1wct2	21146	16877	15376	11208	5359	5351	3.322226	62
t1wct3	22530	17841	16168	12638	6764	6708	3.354788	55
t1wdct1	25786	20768	19506	15097	5243	5243	3.446853	56
t1wdct3	27089	19959	19075	15496	6549	6549	3.789411	82
t1wdpt1	25918	20697	18686	13458	7060	6940	4.151746	90
t1wdpt2	33188	25020	23053	17400	8633	8556	4.146219	90
t1wdpt3	27619	22403	20701	16481	8630	8571	4.229703	95
t1wpc1	24915	19015	15774	10972	6997	6281	3.3736	62
t1wpc3	24872	19845	16569	11479	7821	6922	3.400236	66
t1wpt2	22609	17939	14488	9008	6066	5748	4.030255	84
t1wpt3	11992	8670	6755	4649	3496	3224	3.885882	64
t2wcc1	21155	16929	14420	6667	3476	3476	3.947329	62
t2wcc2	23882	17701	14897	9618	6941	6298	4.024696	106
t2wcc3	24886	18238	15060	6246	4310	4259	4.364533	92
t2wct1	22016	14721	12215	8380	6244	5863	3.853566	83
t2wct2	28448	22641	19521	10289	6019	5841	4.389007	104
t2wct3	18021	14385	12048	7845	5982	5553	4.110718	94
t2wdct1	21660	17674	15545	9190	5231	5227	4.234618	93
t2wdct3	18685	15058	13881	10040	5038	5026	4.106162	88
t2wpc1	19291	15614	11702	6263	4025	3474	3.087738	30
t3wcc1	24454	17483	15638	9261	4640	4631	4.087585	74
t3wcc2	21790	17660	14979	8376	5789	5338	4.074292	91
t3wcc3	19824	13147	11095	7290	5456	5145	3.813043	76
t3wct2	20372	16360	14102	6340	3707	3707	4.29972	83

t3wdct1	33068	25989	24988	19488	10292	10270	4.178824	105
t3wdct2	21274	16110	14399	7949	3798	3783	3.957842	72
t3wdpt2	27205	20520	18267	11346	6361	6237	3.804102	64
t3wpc1	22133	18379	15265	9681	6511	6178	3.082392	40
t3wpc2	20989	16575	13526	5799	3707	3556	3.501361	40

Table S3: Permutational multivariate analysis of variance (PERMANOVA) between Pristine and Contaminated microbial communities (water community). The sample collection time (collection time). Control, WAF, WAF+ dispersant (condition)

	***PERMANOVA P* value**
Water community	0.0001
Collection time	0.0179
Condition	0.1

a)



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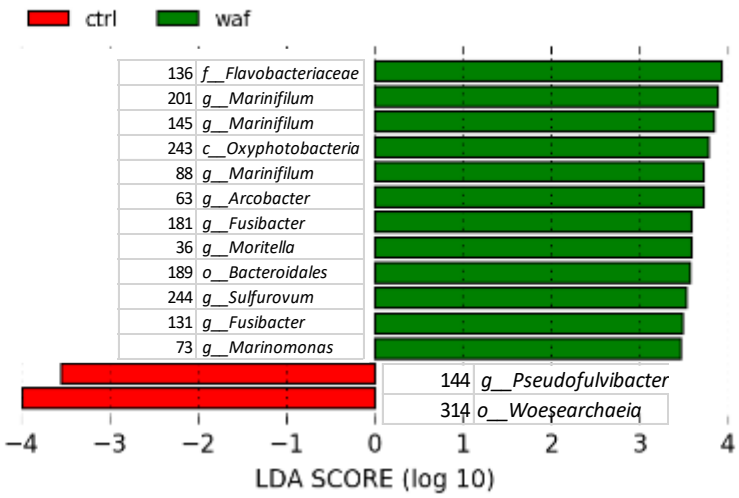

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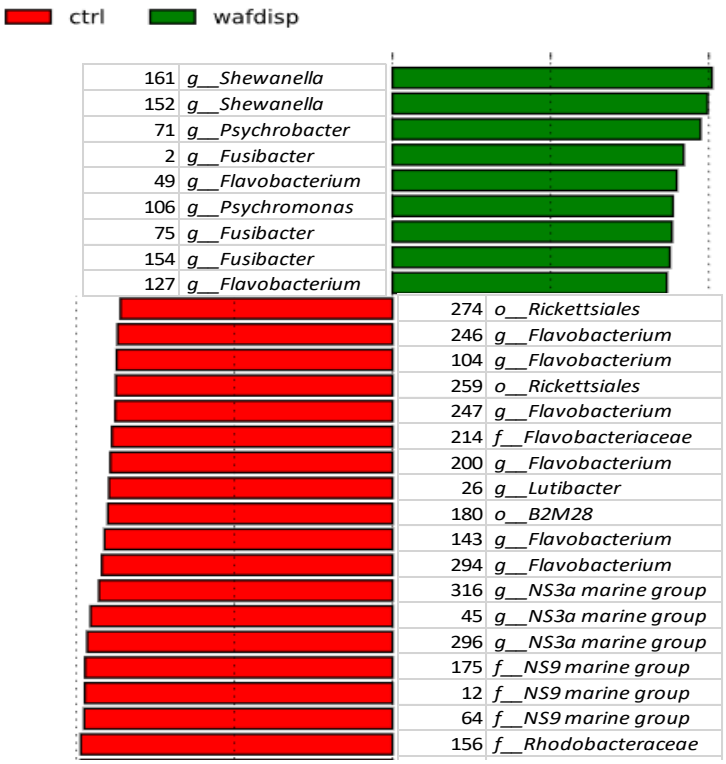

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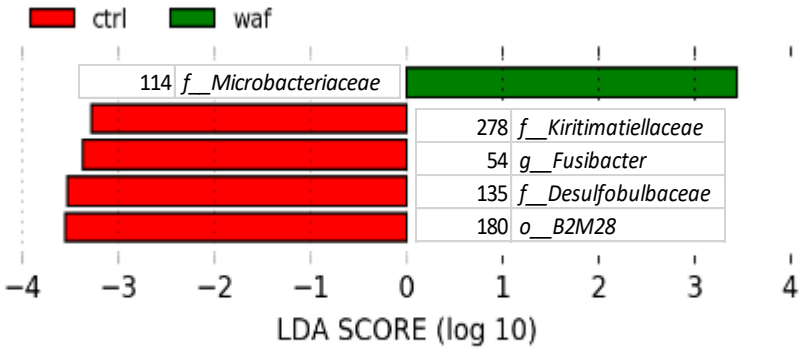


Figure S2: LefSE based on Linear Discriminant analysis (LDA) of taxa with abundances ratio significantly different from control. A) WAF+ Dispersant contaminated water. B) WAF Contaminated sediment. C) WAF+ Dispersant pristine water. D) WAF pristine water.

a)

Community	RMT threshold	Total nodes	Total links	R^2 power law	Average Degree (avgKK)	Average Path distance (GD)	Average Clustering Coefficient (avg CC)	Modularity And number of nodes
Contaminated	0.950	112	225	0.448	4.018	3.925	0.307	0.65 (17)
Pristine	0.910	171	468	0.663	5.474	4.296	0.308	0.60 (14)

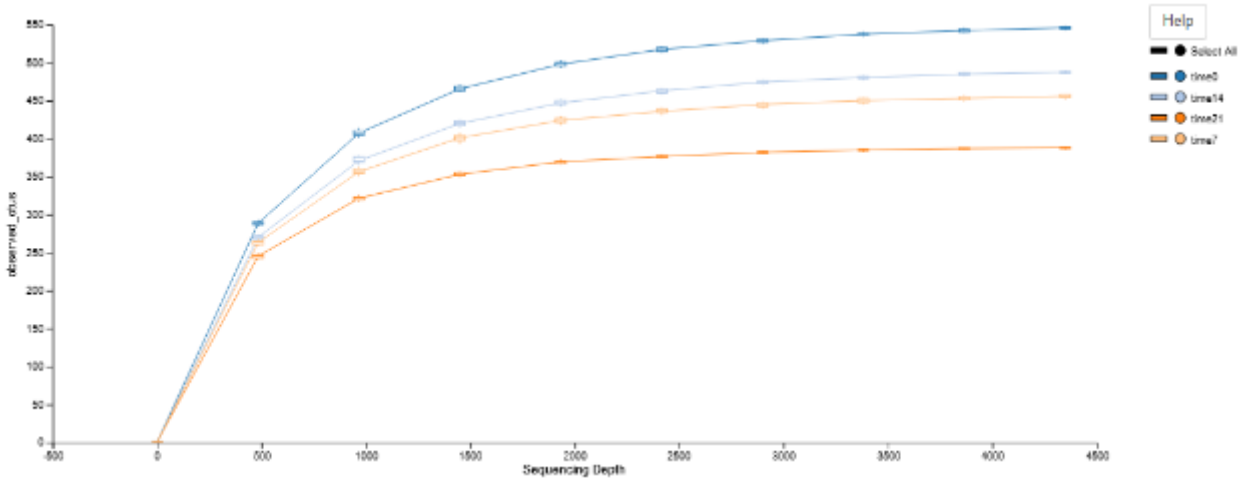
b)

Table S3: Major topological characteristics of (a) empirical networks generated for pristine and contaminated site. (b) Random networks generated for pristine and contaminated site.

Community	Average Path distance (GD)	Average Clustering Coefficient (avg CC)	Modularity and number of nodes
Contaminated	3.356 +/- 0.098	0.074 +/- 0.016	0.439 +/- 0.011
Pristine	3.197 +/- 0.032	0.043 +/- 0.008	0.385 +/- 0.009

Chapter 4:

a)



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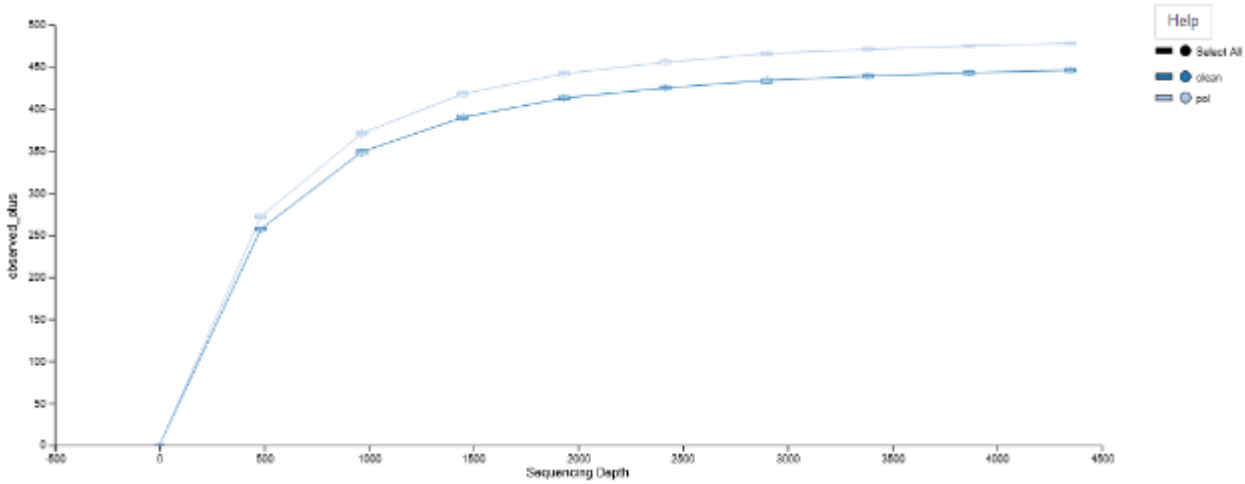

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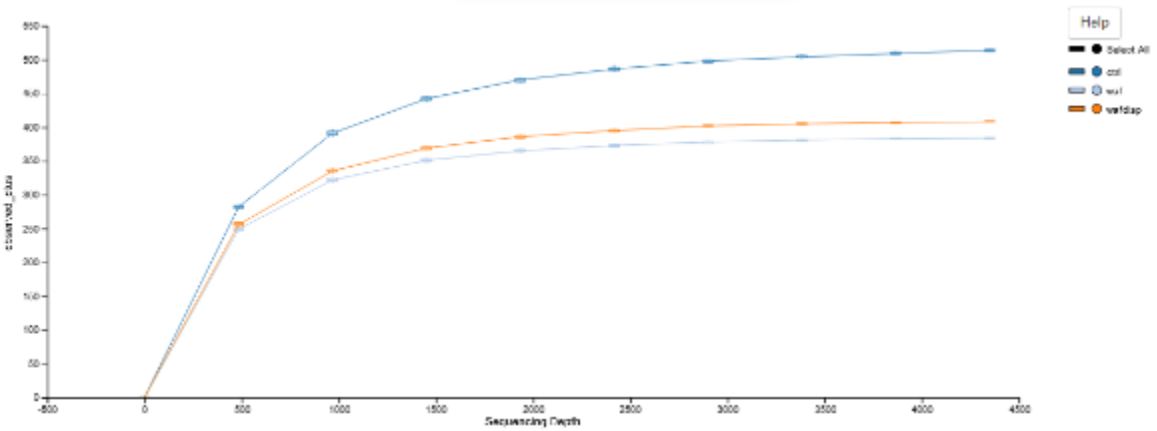

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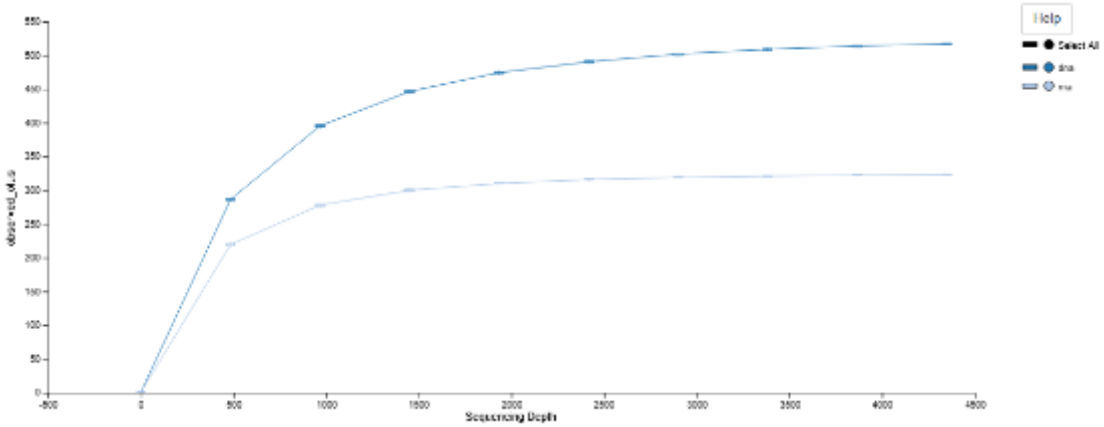


Figure S1: Rarefaction curves in respect of a) time, b) sediment type, c) treatment, d) Active and total communities.

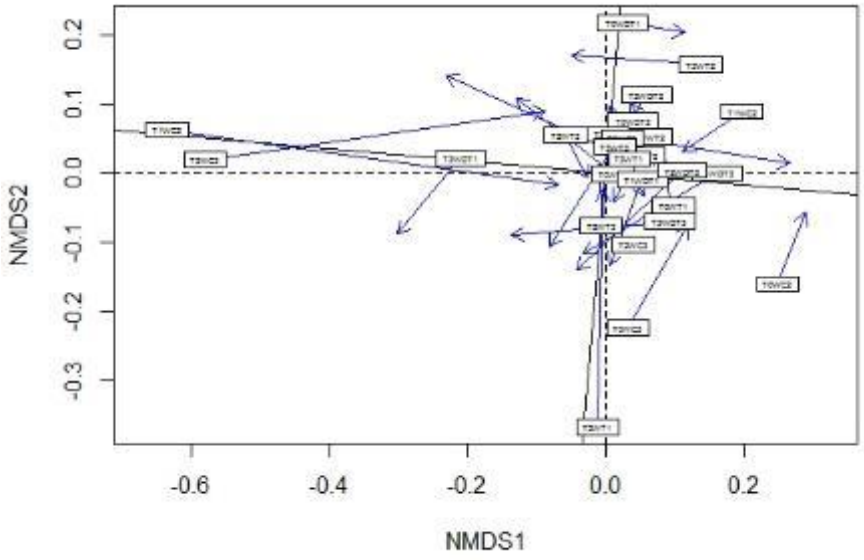
Table S1: denoising Features and alpha diversites for all samples used in this study

	input	**filtered**	**denoised**	**merged**	**non-chimeric**	**Shannon**	**Chao**
DNA SEQUENCES							
RD-t0wcc1	33507	26431	23303	11720	9583	461.4643	5.886716
RD-t0wcc2	45066	35024	31876	22859	19137	845.2542	6.465483
RD-t0wcc3	46926	36470	33151	23763	19306	843.9008	6.429745
RD-t0wct1	39645	30587	27000	14916	12103	611.9048	6.116946
RD-t0wct3	38319	29318	25963	16397	13650	605.3382	6.106314
RD-t0wdct2	38802	30181	27302	21391	17649	492.3714	5.938021
RD-t0wdct3	42265	32774	29389	16873	13876	652.6296	6.205546
RD-t0wdpt1	30210	23731	19850	9457	7991	414.125	5.830066
RD-t0wdpt2	49620	37137	32863	17878	14749	757.6515	6.357009
RD-t0wpc1-C2	31034	24778	21829	15217	13256	979.1007	6.567135
RD-t0wpc3	39355	31219	26760	12677	10634	535.5581	6.056632
RD-t0wpt1	49100	36128	31884	20321	17429	812.4211	6.435952
RD-t1wcc2bis	35612	27781	23836	12836	11089	530.6667	6.030321
RD-t1wct3-E3	33413	26311	23240	12746	10261	395.9286	5.778493
RD-t1wdct1	46194	32828	29896	20355	16571	717.8	6.251663
RD-t1wdct3	39668	30272	26810	12631	9981	526.1818	5.986085
RD-t1wdpt1	55720	38051	33499	17338	14428	699.8298	6.263242
RD-t1wdpt2	45237	33762	29890	16043	13154	664.6793	6.25482
RD-t1wdpt3	40155	30264	26426	13148	10847	531.8947	6.00363
RD-t1wpc1	28648	21887	20591	15442	13733	526.7568	6.013139
RD-t1wpc2	40500	29255	25327	12061	9862	643.1875	6.203608

RD-t1wpt1	38240	29148	25019	11828	10098	469.4444	5.935639
RD-t2wcc1	37032	28925	25751	13124	9524	451.7576	5.862341
RD-t2wcc2	39067	30527	28202	21861	18182	788.1852	6.349056
RD-t2wcc3	37741	30263	26842	14457	11517	536.8781	6.024408
RD-t2wct1	41502	30104	26502	12994	10248	520.0625	5.977933
RD-t2wdct1	37101	29127	26079	13955	11008	526.6	5.968498
RD-t2wdct3	35938	28546	24422	10661	9167	487.871	5.972768
RD-t2wdpt3	40839	29063	25171	12414	10506	512	5.97558
RD-t2wpc1	38993	30513	27871	14966	11414	520	5.930838
RD-t2wpc2	38091	29380	25730	13178	10710	561.2222	6.009221
RD-t2wpc3	35481	27666	23790	10864	9297	487.5357	5.926593
RD-t2wpt1	35213	26894	23293	12816	11011	531.15	6.029325
RD-t2wpt2	47634	33392	28967	14863	12813	654.5313	6.210578
RD-t2wpt3	43331	33821	29358	13836	11607	626.65	6.204462
RD-t3wcc3	41908	33579	30032	15060	11591	590.0784	6.108076
RD-t3wct1	36610	29669	26837	15373	10994	447.6897	5.809806
RD-t3wct1-H8	35090	27568	24429	12512	9948	485.6	5.932296
RD-t3wdct1-C8	38765	30995	28151	13897	9748	407.4444	5.735535
RD-t3wdct2	34974	26521	24098	12946	9589	567.8781	6.090369
RD-t3wdct3	39647	28425	25885	13381	8766	389	5.624712
RD-t3wdpt1	28639	22723	19321	7801	6500	347.1667	5.639325
RD-t3wdpt2	43681	34177	30445	14254	11221	567.8781	6.090369
RD-t3wdpt3	33346	24986	21246	9678	8110	399.8125	5.776157
RD-t3wpc2	44188	30157	26126	11957	9989	506.8864	5.990301
RD-t3wpc3	30025	23834	20310	10251	8793	436.2353	5.871304
RD-t3wpt1	26388	19583	16273	6785	5861	287.0909	5.431072
RD-t3wpt2	41088	31154	27034	12016	9998	492.7241	5.945963
RD-t3wpt3	41755	31112	26751	11176	9398	482.871	5.974083
RNA SEQUENCES							
RD-T0-W-CL-CTL-1	22144	17042	14802	10486	8225	245.5	4.910319
RD-T0-W-CL-CTL-2	50583	34568	34074	33359	23049	89.11111	2.566646
RD-T0-W-CL-CTL-3	19066	14065	11613	6770	5470	214.8571	5.040803
RD-T0-W-CL-TR-1	33908	24723	21449	13556	11293	387.55	5.491083
RD-T0-W-CL-TR-3	23294	18191	15725	10310	8447	324	5.438406
RD-T0-W-POL-CTL-1	32701	25259	22757	14703	12916	634.56	6.195111
RD-T0-W-POL-CTL-2	15246	12086	11614	9543	8397	318.1429	5.285694
RD-T0-W-POL-CTL-3	28700	21537	20014	16145	14271	606.5185	6.111494
RD-T0-W-POL-TR-1	25737	20660	18137	11901	9179	360.0667	5.598031
RD-T0-WD-CL-TR-1	31788	24802	23553	18795	13995	574.386	5.972961
RD-T0-WD-CL-TR-3	25442	19700	17398	11516	9233	342.75	5.572118
RD-T0-WD-POL-TR-1	18730	14961	11888	5899	5129	218.6	5.111547
RD-T0-WD-POL-TR-2	31247	23917	19605	10543	9100	413.5652	5.77581
RD-T1-W-CL-CTL-2a	41920	29314	26157	16759	13734	557.4	6.038159
RD-T1-W-CL-CTL-2b	38239	28607	24909	14794	12176	459.0385	5.8254
RD-T1-W-CL-TR-3a	36361	25174	21652	11457	9496	296.5	5.148989

RD-T1-W-CL-TR-3b	23451	18392	17595	14957	12549	439.3	5.716358
RD-T1-WD-CL-TR1	31242	24674	22279	15240	12194	356.25	5.661721
RD-T1-WD-CL-TR-1	30543	23033	18643	8569	7390	479.4643	5.860585
RD-T1-WD-CL-TR-3	37692	30172	26743	14878	11462	398.0455	5.53652
RD-T1-WD-POL-TR-1	27124	21293	18331	10436	9257	410.6471	5.790889
RD-T1-WD-POL-TR-2	43366	33788	28477	13174	11080	497.6207	5.991764
RD-T1-WD-POL-TR-3	23717	17946	14504	6880	5927	237.6	5.243632
RD-T1-W-POL-CTL-2	20440	15400	12431	6239	5488	228	5.242531
RD-T1-W-POL-CTL-3	23822	17997	15752	9066	6961	278.6667	5.375562
RD-T2-W-CL-CTL-1	32731	20800	18157	9818	6096	200	4.939236
RD-T2-W-CL-CTL-2	34262	26269	25648	23743	17627	491.8	5.556216
RD-T2-W-CL-CTL-3	37122	26944	26787	25620	11802	124	4.06818
RD-T2-W-CL-TR-1	40013	30833	27077	14795	11961	389.4762	5.305829
RD-T2-WD-CL-TR-2	18437	14571	12480	6814	5360	307.75	5.363798
RD-T2-WD-CL-TR-3	28682	21487	19125	10778	8306	307.3333	5.421371
RD-T2-WD-POL-TR-3a	30690	23580	19647	9126	7892	317	5.542073
RD-T2-W-POL-CTL-1	29533	21375	17380	8007	6998	297.5	5.473954
RD-T2-W-POL-CTL-2	30031	23833	20002	10231	8462	362.7143	5.654444
RD-T2-W-POL-CTL-3	25722	20251	16718	9268	7675	330.9091	5.576549
RD-T2-W-POL-TR-1	46781	36943	36047	33206	23703	710.7802	5.953491
RD-T2-W-POL-TR-2	28184	22142	18450	9159	8014	322.1539	5.553523
RD-T2-W-POL-TR-3	28421	20656	17524	9716	8065	367.875	5.692873
RD-T3-W-CL-CTL-3	26774	20188	17001	8379	6692	201.6	4.680973
RD-T3-W-CL-TR-1a	27582	21204	18328	10290	8412	256.2308	4.930345
RD-T3-W-CL-TR-2a	24947	18104	16138	11459	7680	267.6667	5.294825
RD-T3-WD-CL-TR-2	27106	21627	18909	10702	8439	291	5.222959
RD-T3-WD-CL-TR-3	27074	21134	17110	7834	6620	265.4286	5.368881
RD-T3-WD-POL-TR-1	41042	32378	28037	15790	12882	541.42	6.047255
RD-T3-WD-POL-TR-2	25424	20451	17396	7857	6582	272.2	5.378478
RD-T3-WD-POL-TR-3	30028	21492	17601	7992	6711	280.75	5.426634
RD-T3-W-POL-CTL-1	32368	23716	18698	7720	6807	389.6667	5.742673
RD-T3-W-POL-CTL-2	32601	24522	20484	10278	8847	336.5	5.418379
RD-T3-W-POL-TR1	25938	21000	17860	10408	9006	370.1	5.704215
RD-T3-W-POL-TR-2	18199	14515	11667	6054	5345	215	5.123931
RD-T3-W-POL-TR-3	36120	25974	21733	10968	9217	423.8125	5.858397

a)



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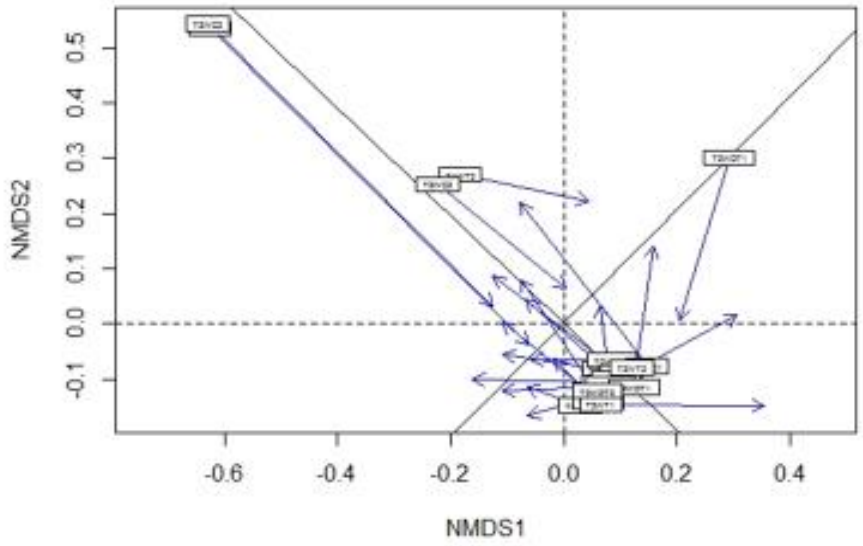


Figure 2S: Procrustes analysis of total communities (DNA) and Active communities (RNA) using Non metric MDS. A) NMDS for Contaminated sediment, b) NMDS for pristine sediment.

Table S2: Predicted functional genes selected for this study and their function in the PAH degradation pathways.

Predicted gene	function	References
naphthalene 1,2-dioxygenase ferredoxin component	Naphthalene/ Phenanthrene Degradation Pathway, initial breakdown	Parales 2003
naphthalene 1,2-dioxygenase subunit alpha	Naphthalene degradation pathway, initial breakdown	Parales 2003
naphthalene 1,2-dioxygenase ferredoxin reductase component	Naphthalene degradation pathway, initial breakdown	Parales 2003

cis-1,2-dihydro-1,2-dihydroxynaphthalene /dibenzothiophene dihydrodiol dehydrogenase	Naphthalene degradation pathway	Denome 1994
1,2-dihydroxynaphthalene dioxygenase	Naphthalene degradation pathway, initial breakdown	Patel 1980
1,3,6,8-tetrahydroxynaphthalene synthase	Naphthalene degradation pathway	Ghimire et al 2008
2-hydroxychromene-2-carboxylate isomerase	Naphthalene degradation pathway	Denome 1994
trans-o-hydroxybenzylidenepyruvate hydratase-aldolase	Naphthalene degradation pathway	Eaton 1994
salicylate hydroxylase	Naphthalene and other HMW PAHs	Jouanneau 2007
alcohol dehydrogenase	Naphthalene and other PAHs	Haritash 2009
alcohol dehydrogenase, propanol-preferring	Naphthalene and other PAHs	Haritash 2009
4,5-dihydroxyphthalate decarboxylase	Metabolite for flouranthene, flourene phenanthrene biodegradation pathways	Kasai et al 2019
catechol 1,2-dioxygenase	Pyrene degradation pathway, intermediate in PAH pathway	Singh et al 2013
catechol 2,3-dioxygenase	Pyrene degradation pathway, intermediate in PAH pathway	Singh et al 2013
protocatechuate 4,5-dioxygenase, alpha chain	Pyrene, Phenanthrene degradation pathways, intermediate in PAH pathway	Thomas et al 2016
protocatechuate 4,5-dioxygenase, beta chain	Pyrene, Phenanthrene degradation pathways, intermediate in PAH pathway	Thomas et al 2016
protocatechuate 3,4-dioxygenase, beta subunit	Pyrene, Phenanthrene degradation pathways, intermediate in PAH pathway	Thomas et al 2016
protocatechuate 3,4-dioxygenase, alpha subunit	Pyrene, Phenanthrene degradation pathways, intermediate in PAH pathway	Thomas et al 2016

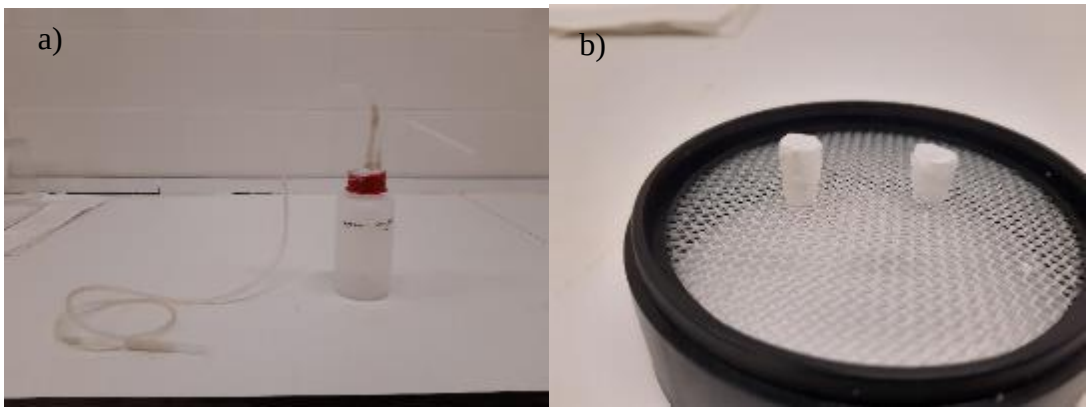
Appendix

Acartia tonsa intensive culturing:

Good practices of culturing and maintenance are crucial for good supply of *Acartia tonsa* for experimentation. In literature, several methods of intensive culturing of *Acartia tonsa* has been suggested (Marcus et al 2007, Medina et al 2004). In this section, we discuss the culturing practices followed for *Acartia tonsa* production. *Acartia tonsa* were initially purchased from an aquaculture distributer, either in the form of dormant eggs or adults. Adults purchased were first acclimatized to temperature and salinity of the laboratory. Copepods were cultured in 20 liters plastic tanks with gentle aeriation. Copepods were fed every 2 days with 1:1 algae of *Tetraselmis chuii* and *Isochrysis galbana*. at a concentration of 3000 cells per ml.

To avoid of adults consuming the newly hatched nauplii, nauplii were collected from the main tank to smaller culturing tank every weeks. The early stage naupli were feed only with *Isochrysis galbana* for the first week. Once copepods reach late copepodite stages, they were returned to the main stock tank.

A simple, small siphon was made to be used for copepod collection with gentle force and water removal (figure a). Depending on the function, small tips made with 200 µm or 45 µm mesh (figure b) to be attach to the siphon. Siphon with 45 um mesh tip was used to remove water from the culturing tanks for cleaning without removing adult and naupli. To collect naupli, the 200 um tip was used.



Nile red in vivo staining of Adult Acartia tonsa:

Based on Tingaud-Sequeira *et al*. (2011)

Nile Red Solution

- For the stock of Nile Red, use acetone (334 ppm aprox). Keep at -20 °C;

- Prepare freshly working solution at 0.5 ppm (each time should be prepared), dilute stock solution in ASTM (or water which you use for your animals),

Staining *in vivo*

- Place 10 juveniles in a vial with 100 ml of ASTM to clean all the medium and algae after exposure
- Prepare a 20ml of working solution at 0.5 ppm for each treatment and put 10animals of the correspondent treatment in a vial of 20 ml;
- Incubate for 1 hour protected from light exposure;
- After 1 hour …immediately:
- Remove animals and clean them in 50 ml of ASTM;
- Place one copepod per microtube of 1.5 ml (sonicator extraction) or 2 ml (tissuelyser extraction);
- Remove all water using fine tip glass Pasteur pipettes (note: there could be water droplets on the walls of the tube that must also be removed);
- Add 0.3 ml of isopropanol and put on ice;

Extraction and quantification

- Extraction can be made immediately or samples can be conserved on ice in the dark for a few hours… but the quantification must be done just after extraction;
- Start the spectrophotometer 5 minutes before use. Open the "protocol" for quantification (wavelength: 530-590nm by fluorescence);
- Homogenize the samples by sonication at 2 volts for 5 second (verify that all daphnia are well homogenized), in the tissuelyser use 50 vibrations/40s with 2 beads (5 mm)
- Always work on ice, under laminar flow closet;
- Centrifuge sample at 12.000 rpm 2 min;
- Place 0.200 ml of extract (supernatant) per well in a black 96 well microplate and read fluorescence at 530(ext) – 590 (em) nm (be careful: do not resuspend the pellet after centrifugation and while removing supernatant.

Histochemical visualization of lipid droplets using Nile Red lipophilic dye

- In order to identify lipid-accumulation body regions stained females were observed under fluorescent microscope at 40X magnification. Immediately after

staining, the animals were mounted on glass slides without a coverslip and photographed under a fluorescence microscope (Nikon eclipse E400, Japan) using a green filter.

Histological visualization of adult females

- About 30 Female copepods were introduced in 1.5 mL Eppendorf tubes filled with warm liquid 2.5% agarose and placed in a warm water bath at 40ºC.
- Copepods were left to suspend through the liquid agarose for a few minutes and then the tubes were placed on ice to solidify the gel.
- After solidification, the gel containing copepods was pushed out of the tube and cut into 1 cm thick gel cores.
- The gel cores were placed in histology cassettes and fixed with Bouin solution for 8 hours at 4ºC.
- After fixation, the samples were rinsed with 70% ethanol and processed for paraffin embedding using the ASP300 Automatic tissue processor (Nussloch, Germany).
- Samples were formed into histology blocks and sectioned into 5 µm thick sections using an automated microtome (Leica RM225, Germany).
- Sections were mounted on glass slides and stained using hematoxylin and eosin (Leica Autostainer ST5010, Germany).

RNA Extraction in Acartia tonsa using RNA easy mini kit:

- Upon exposure, copepods were filtered through a 200 µm mesh for collection and placed in cryotubes filled with RNA later (Invitrogen,USA) and stored in -80 c
- Using a small needle with syringe, RNA later was carefully removed without removing copepods in the process.
- The cryto tubes containing the copepods were filled with RLT lysis buffer with beads for tissue homogenization
- Copepod were homogenized using a Precelis tissue disruptor at the following settings: 45 seconds disruptions twice, then place samples on ice for a minute then tissue disruption for 45 seconds twice again.
- Samples were centrifuged for 3 minutes at maximum speed and supernatant was collected in new tubes
- 1 volume of 70% ethanol was added to the supernatant and mixed well by pipetting.
- 700 µL of samples were pipetted in RNeasy spin column placed in 2ml collection tube and centrifuged at 8000 G for 15 seconds. To get maximum yield, this step was repeated with the remainder of the supernatant from the previous step using the same spin column.
- 700 µL of RW1 buffer was added to the spin column and centrifuged at 8000 G for 15 seconds. Then the flow through was discarded
- 500 µL of RPE buffer was added to the spin column and centrifuged at 8000 G for 15 seconds. Then the flow through was discarded
- 500 µL of RPE buffer was added to the spin column and centrifuged at 8000 G for 2 minutes seconds. Then the flow through was discarded
- An optional step was performed in which the spin column was placed into a new tube and centrifuged for one minute in order to dry the membrane
- The spin column was placed into a 1.5 collection tube, 30 µL of RNAse free water was placed in the spin column, then centrifuged at 8000G for one minute to elute the RNA collected.

DNase treatment of the Extracted RNA

- Add 0.1 volume of 10X DNase 1 Buffer and 1 µL rDNase1 to the RNA and mix gently
- Incubate at 37C for 20- 30 minutes
- Add resuspended DNase Inactivation Reagent and mix well
- Incubate at 2 minutes at room temperature while mixing
- Centrifuge at 10 000 G for 1.5 minutes and transfer the RNA in a new tube

First Strand cDNA synthesis

- Total RNA, RNAse free water and 3 µL of random primers are mixed at a total volume of 15.7 µL
- Incubate the reaction at 65 C for 5 minutes
- The reaction was cooled for 10 minutes at room temperature.
- Each of the following component were added to the reaction for a final volume of 20 µL:
    - 2 µL 10X Affinity Script RT buffer
    - 0.8 µL dNTP mix
    - 0.5 µL RNase block ribonuclease inhibitor
    - 1 µL Affinity Script Multiple Temperature RT
- The reaction was incubated at 25 C for 10 minutes, then incubated at 55 C for 1 hour. Finally, the reaction was terminated by incubating at 70C for 15 minutes.