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Gradu Amaierako Lana / Trabajo Fin de Grado/ Bachelor´s Thesis
Biologiako Gradua / Grado en Biologia/ Degree in Biology

Biological responses of two mytilid species to WAF of crude oil and dispersed crude oil

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Leioa, 2019ko ekainaren 21a /Leioa, 21 de junio de 2019/ Leioa, 21st of June of 2019

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1. ABSTRACT

Mytilus species are important organisms in marine systems being highly abundant and widely distributed along the coasts of Europe and worldwide. They are widely used in monitoring programmes to assess the health status of coastal and estuarine ecosystems, and to assess the effects produced after exposure to contaminants (single and mixtures) under laboratory exposure conditions. In order to ascertain the effects of oil spills and their responses an experiment was carried out in the facilities of Plentzia Marine Station (PiE-UPV/EHU). After a period of acclimatization to laboratory conditions two mussel species from the Mytilus complex (*Mytilus trossulus, M. edulis*) were exposed to produced Water Accommodated Fraction of crude oil (WAF) at a high concentration (WAF25%) and WAF of dispersed crude oil (Finasol OSR52) (WAF5%D) at 15 ºC and 20 psu (Practical Salinity Unit). Mussels were dissected at days 0 and 7 and different endpoints were measured, such as tissue-level biomarkers, including cell type composition (volume density of basophilic cells, V_{vBAS}) in digestive gland epithelium, structural changes of digestive alveoli (MLR/MET, MET/MDR) and connective to diverticula ratio (CTD). Moreover, gonadal development of mussels (as a supporting parameter) was also determined.

1. LABURPENA

Mytilus espezieak izaki garrantzitsuak dira sistema itsastarretan oso ugariak direlako eta beraien sakabanakuntza Europako kostaldean zehar eta munduan zehar ematen delako. Monitorizazio programetan asko erabiltzen dira estuarioetako eta kostaldeko ekosistemen osasun egoera ebaluatzeko, baita laborategiko esposizio egoeretan kutsatzaileek (bakarrik edo nahastuta) eratzen dituzten efektuak ikertzeko erabiltzen dira. Fuel isurkiek eta hauen erantzunak dituzten efektuak determinatzeko esperimentu bat burutu zen Plentziako Itsas Estazioko (PiE, EHU/UPV) instalazioetan. Aklimatazio periodo baten ondoren laborategiko egoeretara, Mytilus konplexuko bi muskuilu espezie (*Mytilus trossulus, M. edulis*) ekoiztutako fuelaren Uretan Moldatutako Frakzioa (WAF) kontzentrazio altuan (WAF25%) eta sakabanatutako fuelaren (Finasol OSR 52) Uretan Moldatutako Frakzioaren (WAF5%D) eraginpean jarri ziren 15 ºC-tan eta 20 psu-tan (Practical Salinity Unit). Muskuiluak 0 eta 7 egunetan disekzionatu ziren parametro ezberdinak aztertzeko: ehun-mailako biomarkatzaileak, hala nola, zelula-mota osaera (zelula basofilikoen dentsitate bolumetrikoaren aldaketa, V_{vBAS}) liseri-guruineko epitelioetan, liseri-guruinaren aldaketa egituralak (MLR/MET,MET/MDR), ehun konektibo

eta dibertikuluen arteko ratioa (CTD). Gainera, muskuiluen gonaden garapena determinatu zen (parametro laguntzaile moduan).

2. INTRODUCTION

Oceans and seas provide 99% of the available living space on the planet covering 71% of the Earth's surface and contain 90% of the biosphere and consequently harbour more biological diversity than terrestrial and freshwater ecosystems together. The Oceanic environment is a complex ecosystem with countless biotic and abiotic components that has been directly and indirectly influenced by both natural and anthropogenic activities that can affect the health status of marine biota (fauna and flora) and can pose an effect on human health (Jha, 2004; Di., 2012; Depledge et al., 2013). The major source of pollution affecting oceans and seas comes from the land through runoff, industrial and agricultural waste releases (pesticide, herbicide, sewage etc) (Dallas, 2013). In addition, ocean mining (i.e. silver, gold, copper, cobalt and zinc), toxic liquids, plastic debris and large-scale oil spills have a negative influence on the environment (Blumer, et al., 1972, Clark et al., 1989).

Petroleum has been recognized as a critical environmental contaminant since the beginning of the Twentieth Century. Reports of the biological consequences of shipwrecks involving cargos of crude and fuel oil began to appear in the mass media and scientific press. The accumulated literature on this well-studied contaminant is vast, covering topics as diverse as analytical chemistry, chemical fate, oil spill prevention and response, mitigation and restoration, economic and social analysis, and biological effects on all forms of plant and animal life in saltwater, freshwater, and terrestrial environments. (see review by Albers, 1998)

The industrial use of fuel is spread worldwide and its production is one of the most concerning processes regarding the environmental impact. The world´s energy consumption is increasing very quickly, so new sources have to be found. Fuel is the most demanded energy source, especially the crude oil.

Consequently, searching for new fields for oil production and oils transportation is the main objective of many countries, being the Arctic Ocean one of the most interesting targets. The richness of the Arctic Ocean in hydrocarbon resources and its divided governance between 5 different countries (Canada, United States, Russia, Norway and Denmark/Greenland) have ended in an over exploitation of these area (Gulas, et al. 2016). Hence, the Arctic, through the years, has become an extremely fragile

area due to its extreme seasonality, low temperatures, the persistence of hydrocarbons at those temperatures and the slow habitat recovery (Knoll, 2014; Gulas, et al. 2016).

A recently started research project funded by the EU through the Horizon 2020 programme called "Integrated oil spill response actions and environmental effects-GRACE project" is focused on developing, comparing and evaluating the environmental effects of oil spill response methods in a cold climate and ice infested waters. The present work has been conducted in the framework of abovementioned project.

The oil contains a significant amount of polycyclic aromatic hydrocarbons (PAHs), known to be damaging for marine organisms. A typical crude oil may contain from 0.2% to more than 7% total PAHs (Ma, et al. 2017). The chemical properties of the oil determine its release impact in the marine ecosystem (evaporation, spreading…). The viscosity and the thickness of the oil are dependent on the natural processes interacting while an oil spill is occurring. These natural processes happen in several steps and are influenced by cold temperatures. The thinner the oil, the more the solubility and the number of volatile compounds increase. The mixing of the water with oil compounds due to the wind, waves and currents may end up in the release of Water-Accommodated Fraction (WAF). WAF is known to be the part of the petroleum that persist in the aqueous phase after the separation period as a consequence of the mixing forces. Petroleum is mainly composed by light hydrocarbons (Rufli, et al. 1998; Neff, et al. 2000), which are the remaining components after the evaporation period (from few hours to a day after the spill).

Petroleum seems to be highly toxic for the organisms living in the water column and for the reproduction of marine invertebrates (Lewis, et al. 2008), and even displays carcinogenic effects among others. PAHs have the tendency to bind/adsorb with particulate matter due to their hydrophobic nature and their low aqueous solubility. The consequent particles' deposition allows filter-feeders organisms (like mussels) to uptake the molecules and accumulate them in their tissues. The pollutant mixtures that are created from the deposited particles in the sediments, induce toxic effects at different levels of biological organization (cellular, molecular, physiological…) (Neff, 2002). In order to minimize those effects and increase the speed of natural biodegradation after an oil spill, different oil spill responses have been developed including the use of chemical dispersants.

In the Arctic waters, the spreading and the volatility rate of oil are reduced by cold conditions. Hence, the use of dispersant products is highly applied in order to accelerate the biodegradability process (Word, 2014). The dispersants are chemical mixtures composed by bio-surfactants that enhance the solubility of the oil, reducing the surface tension at the oil water interface, converting it into small droplets and facilitating its dilution in the water column and further accelerating the natural biodegradation process made by microbes (NRC, 2014). However, the increasing aqueous concentrations of oil available in the environment have been demonstrated to produce a toxic effect in the marine biota observed at cellular and molecular level (Major, et al. 2016). Moreover, the dispersant itself provoked deleterious effects in the biota, sometimes more intense than oil spills themselves (Almeda, et al. 2014). These effects can be monitored using sentinel species inhabiting the areas where the oil was spilled and where dispersant was used.

The most widely used invertebrate and marine sentinel species are mussels from the genus *Mytilus.* They have been frequently used in biological effects studies in both laboratory exposure tests and biomonitoring programmes worldwide. This is mostly due to their wide world geographic distribution and the ability to bioaccumulate contaminants from the water column as well as having a large number of validated biological effect endpoints (biomarkers) that can be measured and quantified. In consequence, they have a higher probability of accumulating toxicants present in the water in their tissues, taking into account the high tolerance they have against pollutants. For instance, PAHs are accumulated in fat tissues of mussels being an ideal bio-indicator of contamination (Fasulo, et al. 2012; Capello, et al. 2013). Another important feature of mussels is that they have very low metabolic activity; therefore, contaminant concentrations in their tissues accurately reflect the magnitude of environmental contamination (Zorita et al., 2007; Vassilenko, 2012). Moreover, mussel populations are sessile, relatively stable and large, and also tolerant to a wide range of environmental conditions so they can provide statistically significant data, and are easy to collect and maintain in laboratory, making possible their very extensive use in experimentation (Vassilenko, 2012). Furthermore, mussels are economically relevant. For all these reasons, filter feeders in general, and mussels in particular are a recommended biomonitoring organism of the International Corporation for the Exploration of the Seas (ICES) integrated biomonitoring scheme (ICES, 2011). In this experiment the species used were *Mytilus edulis* and *Mytilus trossulus.*

The *Mytilus edulis* complex consists of three basic taxa (*M. edulis, M. galloprovincialis,* and *M. trossulus*) (Fig. 2). *Mytilus trossulus* known as foolish mussel is one of them distributed on the temperate to subarctic coasts in Northern Hemisphere especially in the intertidal and subtidal territory of North Atlantic West coast and Baltic Sea (Fig. 1) (Innes and Bates, 1999; Väinölä and Strelkov, 2011). This mussel species is used as bioindicator organism in Baltic Sea because in Europe it's only found in Baltic Sea (Varvio et al., 1988; Turja et al., 2014).

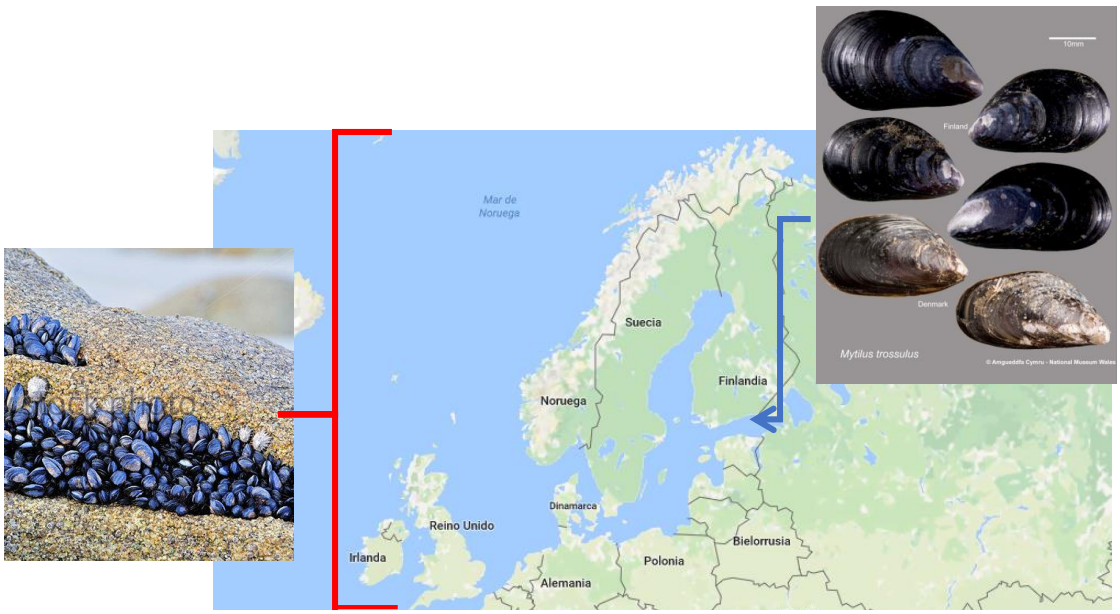


Fig. 1. Approximate range of both mussel species (left for *Mytilus edulis*, right for *M. trossulus*). In the Northern Hemisphere where they overlap. Variable amounts of hybridisation occur between species.

Fig. 2. The Taxa of Mytilus complex (from left to right *Mytilus trossulus, M. edulis, M. galloprovincialis* (Credit: Endika Gil Uriarte)

The blue mussel, also known as common mussel (*Mytilus edulis*) is native to the North East Atlantic. As mentioned before, it is widely used for experimentation due to its availability and characteristics that make it an ideal sentinel organism. When compared to *M. trossulus* within the same size category the later would tend to be older and experience a longer exposure history and opportunity to bioaccumulate. The suggested slower growth rates exhibited by *M. trossulus* compared to *M. edulis* (Lobel et al., 1990) can be inferred having in mind that the Baltic *M. trossulus* is a variety which carries *M. edulis* mtDNA (Riisgård et al., 2014). This particular mussel is able to inhabit the particular haline gradient of the Baltic, from Danish Strait, where salinity resembles that of the Atlantic (around 35 psu), and tolerate the extremely low salinity conditions towards the Gulf of Bothnia (<3 psu). The price to pay is that shell growth rates of this dwarf mussel are controlled by salinity, which has been proved the limiting factor for shell growth, but not for somatic growth. Thus, even if small, Baltic mussels are speculated to be extremely long-lived animals, and therefore ecologically relevant in the Baltic Sea (Riisgård et al., 2014).

The use of biomarkers as predictors for long-term responses is a highly used approach when studying the possible toxic effects of pollutants. Biomarkers are measurements done at different response levels (cellular, molecular, biochemical and tissue level) that indicate the good health status of the studied organisms. In order to know the extent of the responses under pollutants, biomarkers of effect are used (Sanni, et al. 2017). Changes in the epithelium thickness and cell type composition (digestive and basophilic cells) along with histopathological alterations are commonly used biomarkers in ecotoxicological studies (Garmendia, et al. 2011; Costa, et al. 2013; Marigómez, et al. 2013). In a normal condition, the number of digestive cells in comparison to basophilic cells is much higher, because they are the ones in charge of the intracellular digestion. Nevertheless, under a stress situation, as in the case of hydrocarbons exposure, the quantity of basophilic cells, which are secretory cells, increase (Zaldibar, et al. 2007).

Histopathological alterations are the result of changes at cellular, molecular and tissue level of the organism, which can end up in alterations at higher biological levels having serious consequences at the individual or population level. Therefore, histopathological studies are used to provide information for the determination of the environmental health status.

3. HYPOTHESIS

Histopathological alterations as well as tissue-level biomarkers are suitable indicators to assess the adverse effects produced by the exposure to WAF of crude oil and WAF of dispersed crude oil in two species of mytilids that do not show species-specific responses.

4. OBJECTIVES

The overall objective of the study is to determine the biological responses in mussels produced after exposure to WAF of crude oil and WAF of dispersed crude oil (WAF-D) mixtures at set experimental timescales. More specifically to assess species-specific histological response to oil pollution in both mussel species and to assess mussel biological response under sub-optimal physiological conditions (i.e. osmotic and thermal stress).

5. MATERIAL AND METHODS

5.1 Collection of mussels and acclimation

Two mussel species from the *Mytilus* genus, *M. edulis, M. trossulus* were studied in this experiment. Mussels were collected from two separate locations and then brought to the installations of the PiE-UPV/EHU in Plentzia (Basque Country). Mussels *Mytilus edulis* with a size of 3,5-4,5 cm were purchased from the Snadder & Snaskum farm in Rissa (Norway) the 25th of October 2016. At the time they were collected, water temperature was 9°C with a salinity of 33 PSU. Exactly one year later in 2017, mussels *Mytilus trossulus* with a size between 2 and 3 cm were purchased from the Kalmarsund farm in Vassmolösa (Sweden). At the time they were collected, water was at 10°C and 7 PSU. Each species was kept in an aquarium for 9 days with constantly running seawater to acclimate to a water temperature of 15°C and a salinity of 20 PSU before starting the experiment.

5.2 Exposure treatments

Mussels were exposed to 25% WAF (WAF25) and 5% WAF oil+dispersant (DWAF5) for seven days within a flow-through seawater system with a water temperature of 15°C and a salinity of 20 PSU. The WAF25 mixture was made by mixing Naphthenic North Atlantic crude oil from Equinor with water (ratio 1:200 oil/water). For the DWAF5 mixture, the same oil was used but first mixed with Finasol OSR52 dispersant (Total Co.) at a ratio 1:10 dispersant/oil, and then added to the water. In both cases, the WAF was produced at low energy (stirred, with no vortex 40 hours) at 10°C and

20 PSU water. This allowed larger oil droplets to rise to the surface. The produced WAF could then be harvested from the bottom of the mixing in the Mariotte bottles.

To set up the experiment, 12 glass aquaria with a volume of 30 litres were placed in two industrial refrigerators (as shown in Fig. 3to control the exposure temperature at 15 ºC). There were two aquariums per exposure group for every mussel species. Measurements of ammonia, salinity, oxygen, and temperature were taken in the treated and control aquariums. Every two days the water was changed and new WAF was added in the aquariums. After refilling the tanks, microalgae food (Microalgae Composed Diet®, Acuinuga, *Isochrysis* spp., *Tetraselmis* spp., *Pavlova* spp., *Nannochloropsis* spp. and *Spirulina* spp.) was given to the mussels.

Fig. 3 The tanks set up in the refrigerators with the maintenance system

5.3 Mussel sampling

The sampling times were at the start of the experiment (t0) and after seven days (t7). 20 mussels were collected from the control groups, 20 from the WAF25 and 20 from the DWAF5 exposed mussels. All the individuals were sampled before being fed.

5.4 Tissue-level biomarkers

The mussels were opened and a cross-section of the soft body (containing digestive gland, mantle, gill and foot) was dissected and fixed in 4% formaldehyde in seawater for histological analyses.

The fixed samples were dehydrated in graded ethanol series, and embedded in paraffin. Sections (5 µm thick) were cut in a rotary microtome (Leica RM2125 RTS), and stained with hematoxylin-eosin. Slides were viewed at 40× magnification using a drawing tube attached to a light microscope (Nikon eclipse Ni-U). A Weibel graticule (multipurpose system M-168) was used, and hits of basophilic and digestive cells, luminal area and connective tissue were recorded.

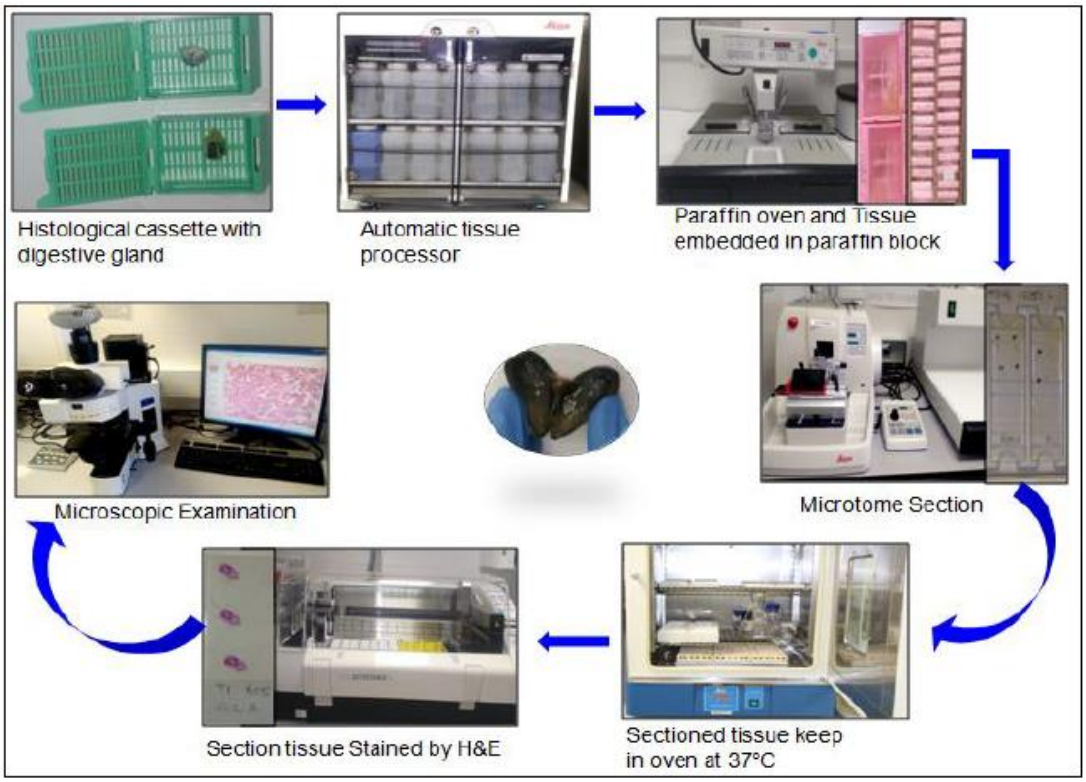


Fig. 4 Flowchart to illustrate the process followed in the histopathological analysis of tissues and organs. Credit to Joyanta Bir

The volume density of basophilic cells (Vv_{BAS}; $\mu m^3/\mu m^3$) in digestive gland of mussels was determined according to Soto et al. (2002). The mean epithelial thickness of the digestive alveoli (MET; µm) was determined according to Garmendia, et al. (2011) together with other estimates of changes in alveolus morphology such as the mean luminal radius (MLR; µm) and the mean diverticular radius (MDR; µm); then the MLR-to-MET ratio (MLR/MET; µm/µm) and MET-to-MDR ratio (MET/MDR; µm/µm) (Vega et al., 1989; Cajaraville et al., 2006) (Fig. 5). Likewise, the integrity of the digestive gland tissue was simultaneously determined as the extent of the interstitial connective tissue relative to the space occupied by digestive diverticula (connective-to-

diverticula (CTD) ratio) (Brooks et al., 2011; Garmendia et al., 2011) on the basis of the same stereological data set.

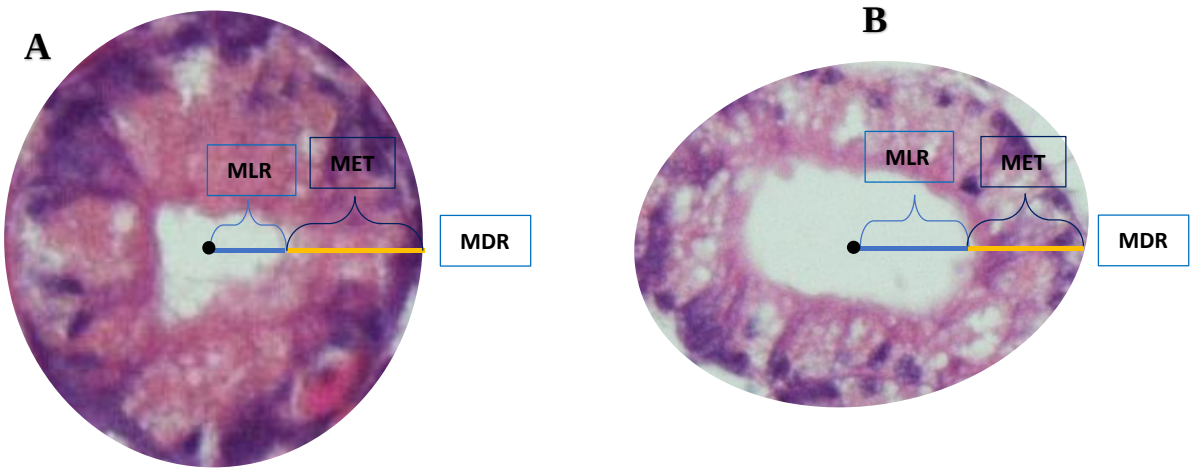


Fig. 5. Schematic diagrams of the planimetric parameters over digestive alveoli. **MLR**: Mean Luminal Radius, **MET**: Mean Epithelial Thickness, **MDR**: Mean diverticular Radius. **A** *M. trossulus* **B** *M. edulis*

5.5 Sex ratio, gamete developmental stages and gonad index

Using the gonad from the mantle in the cross-sections a determination of developmental stages of gametes and gonad index (GI) at the light microscope was carried out, according to Ortiz-Zarragoitia et al., (2011). Briefly, gamete developmental stages were distinguished (Fig. 6 and Fig. 7) and a GI value was assigned to each mussel depending on its gamete developmental stage which varies from zero, when no sexual activity is noted, to five, when all individuals are mature (adapted after Kim et al., 2006), and the average GI of 20 mussels was calculated per experimental group.

5.6 Statistical analysis

The statistical analyses were made using IBM® SPSS® Statistics ver. 24.0.0.0 (IBM Corp., Armonk, NY, USA). Biological parameters were tested for normality (Kolmogorov-Smirnov's test) and homogeneity (Levene's test). V_{vBAS}, MLR/MET, MET/MDR and CTD ratio were analysed by means of one-way analysis of variance (ANOVA) and statistical differences were established using Duncan's post-hoc test. Sex ratio bias was studied using Chi-square test, comparing total number of female and male mussels and normalizing for theoretical gender bias (1:1) A 95% significance level ($p<0.05$) was established for all statistical analyses carried out.

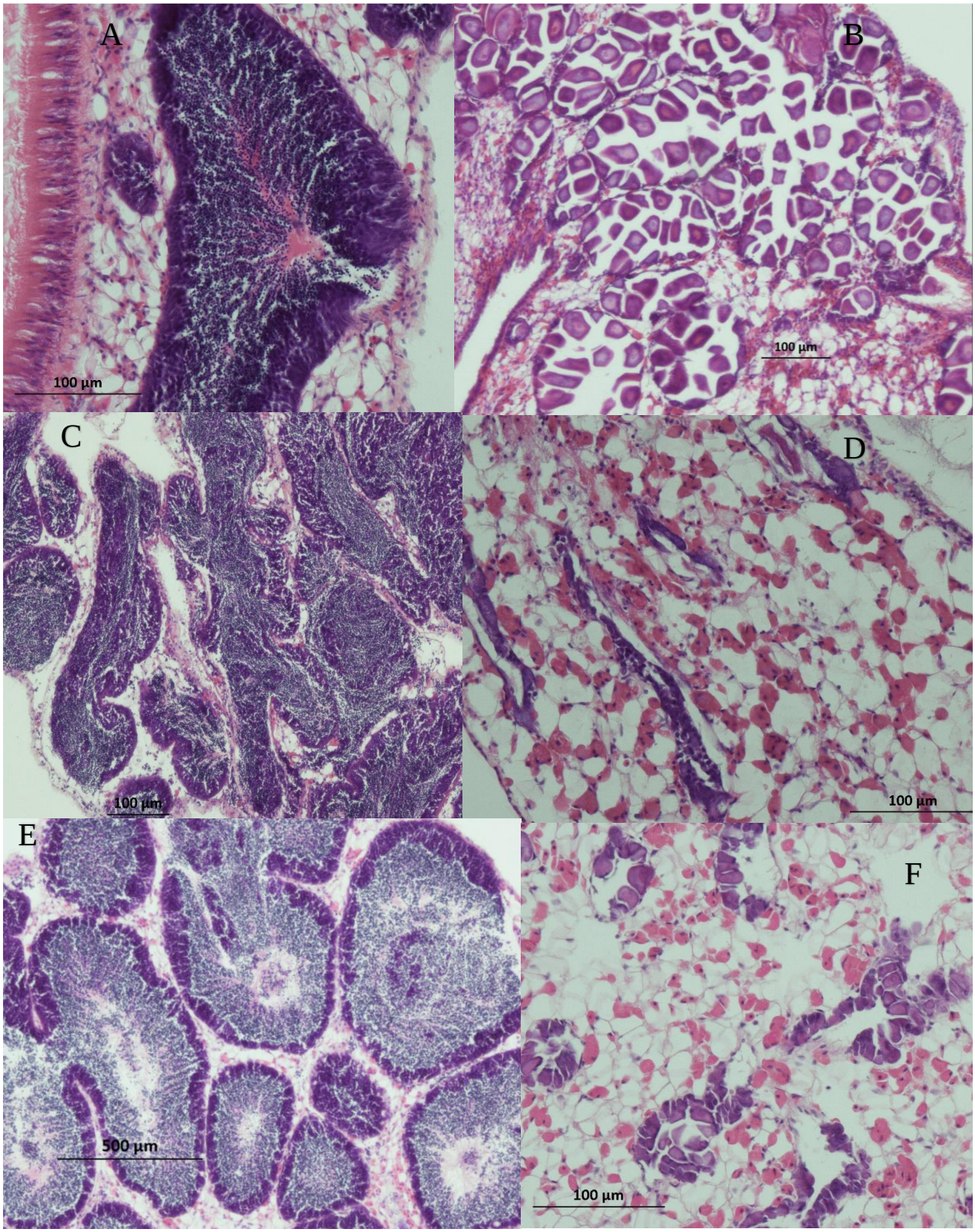


Fig. 6. Micrograph of mussel *M. trossulus* gonad stained with hematoxylin-eosin. A) Spawning male gonad, stage 2 B) Spawning female gonad, stage 4 C) Developing male gonad, stage 4 D) Developing female gonad, stage 1 E) Spawning male gonad, stage 4 F) Developing female gonad, stage 2.

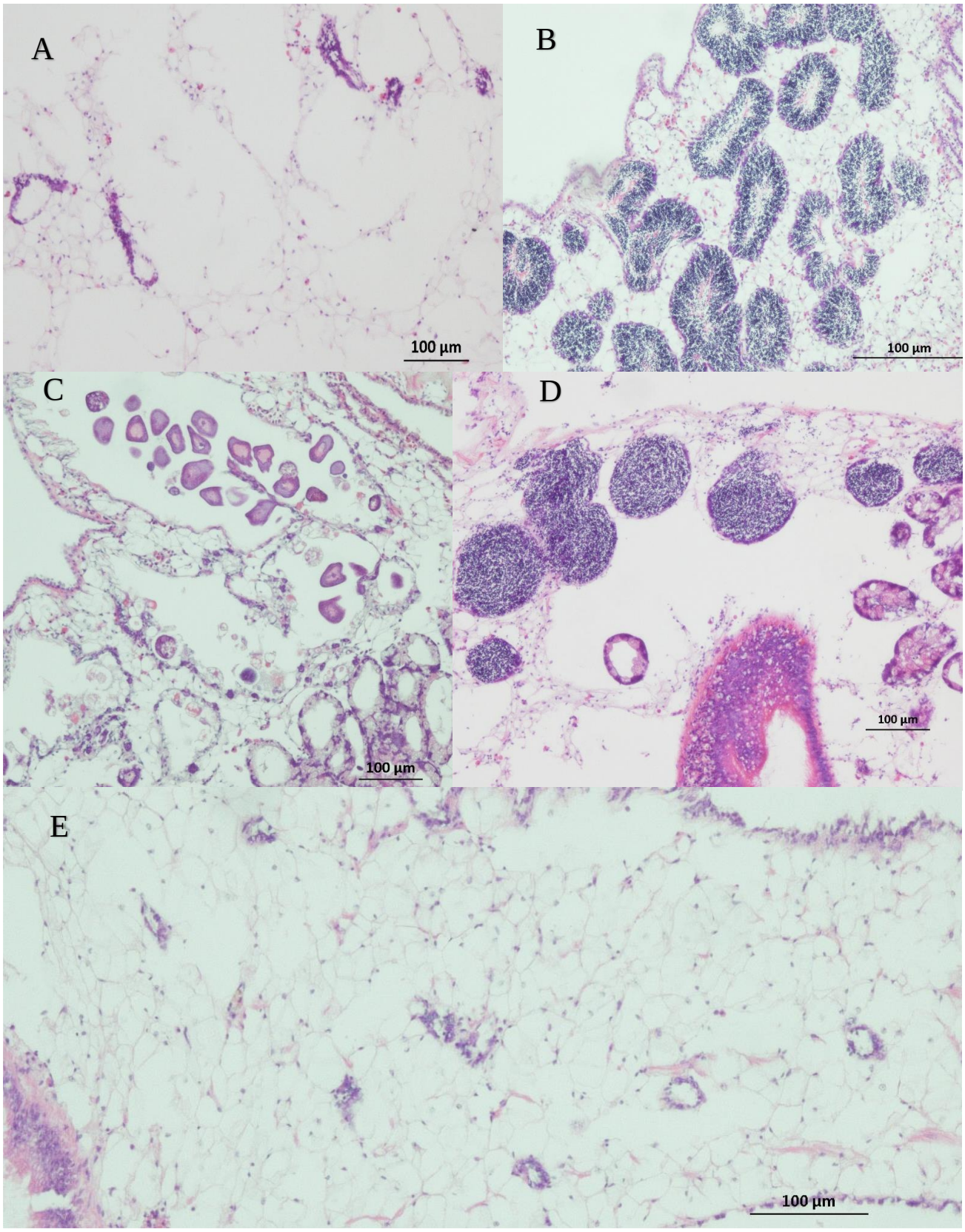


Fig. 7. Micrograph of mussel *M. edulis* stained with hematoxylin-eosin. A) Developing female gonad pre-stage 1 B) Spawning male gonad, stage 3 C) Spawning female gonad, stage 3 D) Developing male gonad, stage 3 E) Resting/spent gonad, stage 0

6. RESULTS

6.1 Tissue-level biomarkers

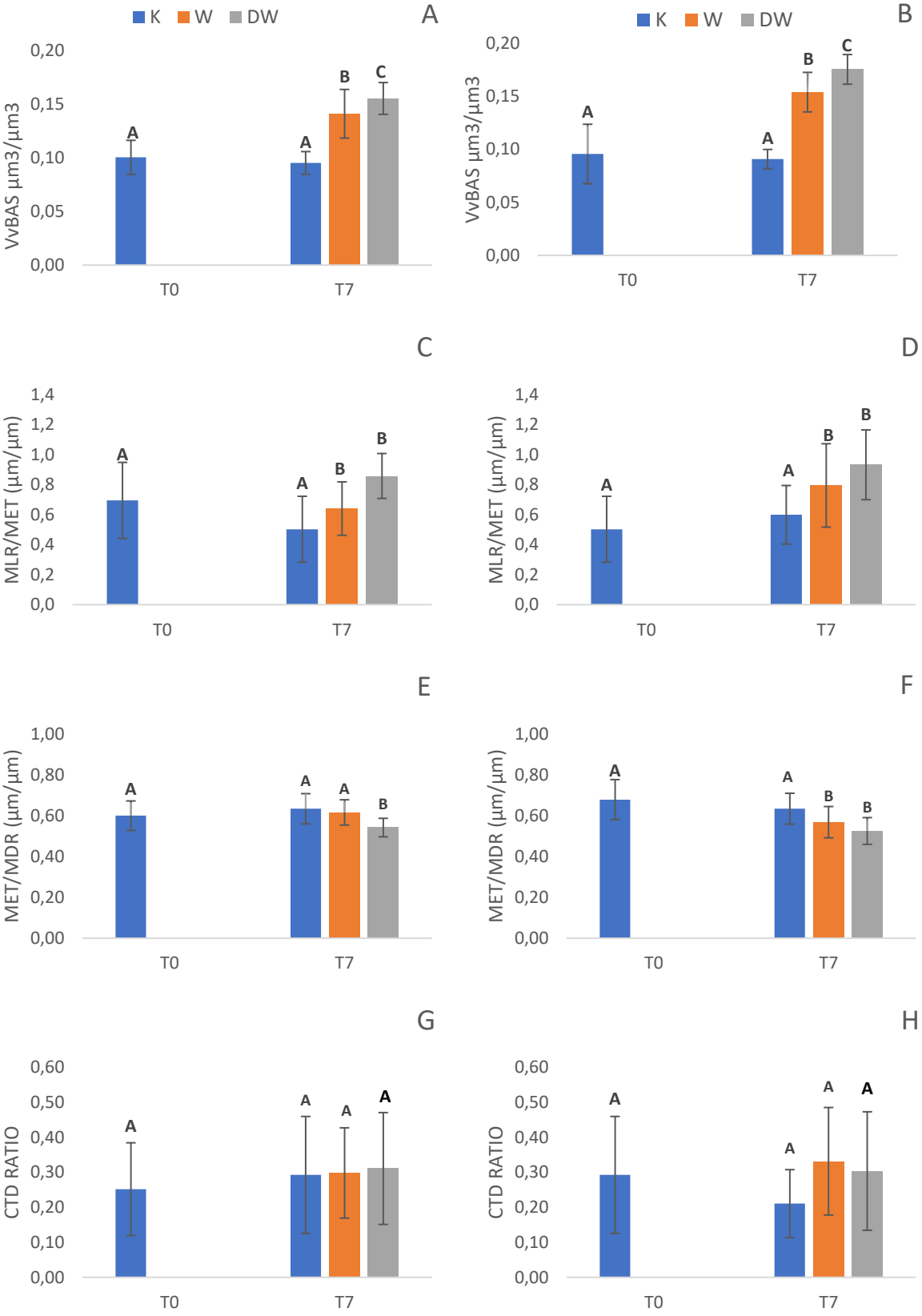
The volume density of basophilic cells (Vv_{BAS}), the MLR-to-MET ratio (MLR/MET), MET-to-MDR ratio (MET/MDR) and the connective-to-diverticula (CTD) ratio) for all exposure conditions are illustrated in Figure 8. For all the analysed biomarkers the controls did not show significant differences between T0 and T7.

Vv_{BAS} showed similar values in both controls of both species (below 0.1 µm3/µm3). A significant increase in the Vv_{BAS} in both Mytilid was observed for WAF and WAF-D in comparison with the control ones, being the higher values, the ones obtained after the exposure to WAF-D (*M. trossulus*; Fig.8A and *M. edulis*; Fig.8B).

MLR/MET ratio significantly increased with exposure in both species in comparison with control groups. The highest values occurred for the WAF-D groups in *M. trossulus* (Fig.8C) and *M. edulis* (Fig.8D), although the differences between both were not significant.

In the case of MET/MDR ratio the exposed groups of the two mussel species presented a decrease of the ratio when compared with the control ones that was significant only for the WAF-D group for *M. trossulus* (Figs. 8E and F).

CTD ratio did not show significant differences among exposed and control groups for both species (Figs.8G and 8H).


A
K W DW
VvBAS µm3/µm3
0,20
0,15
0,10
0,05
0,00
A A B C
T0 T7
B
K W DW
VvBAS µm3/µm3
0,20
0,15
0,10
0,05
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A A B C
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MET/MDR (µm/µm)
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CTD RATIO
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CTD RATIO
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Fig 8. (previous page) Volume density of basophilic cells (Vv_{BAS}) (A. *M. trossulus*, B. *M. edulis*); MLR/MET: Mean luminal radius to mean epithelial thickness ratio (C *M. trossulus*, D. *M. edulis*); MET/MDR: Mean epithelial thickness to Mean Diverticular Radius ratio (E. *M. trossulus*, F. *M. edulis*). CTD: Connective to diverticula (G *M. trossulus*, H. *M. edulis*). Intervals indicate standard deviation. Letters indicate significant differences among exposures according to the Duncan's test performed after a one-way ANOVA ($p<0.05$). Each colour (Blue, Orange & Grey) corresponds to Control, WAF and WAF-D respectively.

6.2 Sex ratio, gamete developmental stages and gonad index

Sex ratio values for each sampling group at each sampling time are shown in Table 1. The sex ratio in *Mytilus trossulus* showed a slight difference in T0 depicting a 1:1 male to female proportion, whereas it did present a small difference in T7 0,82:1 which is not considered statistically different from the theorical 1:1 ratio. There was no mussel in a resting state. This was different when compared to *Mytilus edulis* where the majority of the mussels were found in a resting state (75% in T0 and 66,67% in T7). This means that there was no possible way to clearly identify the sex of the mussel. At T7, a statistically different sex ratio compared to the theorical one with a 0,15:1 ratio respectively was obtained. No hermaphrodites were identified in any of the groups.

Table 1. Sex of mussels *Mytilus trossulus* and *Mytilus edulis* in both different times and exposures. Sex ratio value with asterisk indicate that is different in comparison with the theoretical 1:1 between female and males, according to χ^2 test ($p< 0.05$).

		Mytilus trossulus		*Mytilus edulis*	
		T0	T7	T0	T7
Control	Female	10	12	4	3
	Male	10	8	1	0
	Resting	0	0	15	17
WAF	Female		10		10
	Male		10		1
	Resting		0		9
WAFD	Female		11		7
	Male		9		2
	Resting		0		11
Sex ratio		1	0,82	0,25	0,15*
(*p*) χ^2		1	0,43858	0,17971	0,00039
% Resting		-	-	75	61,67

Microscopical observations of the gonad showed differences in gamete development between both species and exposures (Fig. 6-7). In *M. trossulus* (Fig. 9A) the gonad index displayed values around 2,5-3 while in *M. edulis* (Fig. 9B) a significant decrease in the gonad index values was observed, near the 1 value.

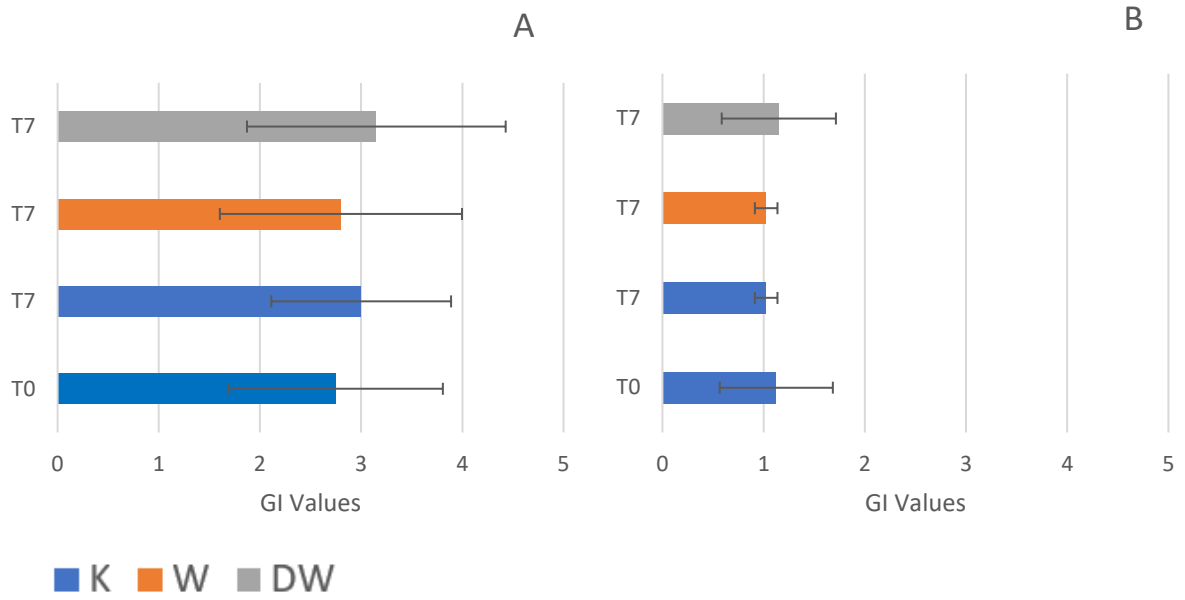

Fig. 9. Gamete development in mussels (**A** *M. trossulus* **B** *M. edulis*) represented by the Gonad index (GI) values. Intervals indicate standard deviation. K, control; W, WAF; DW, WAF-D.

7. DISCUSSION

In this work a battery of tissue-level biomarkers such as Vv_{BAS}, MLR/MET, MET/MDR and CTD ratio has been applied to determine the biological response of two species of mussels, *Mytilus trossulus* (collected from the Baltic Sea) and mussels *Mytilus edulis* (collected from the North Atlantic) elicited after exposure to the accommodated Fraction of oil (WAF) and to oil-dispersed (Finasol OSR52) mixture (WAF5D 15 ºC, and a salinity of 20 psu.

Furthermore, sex ratio, gamete developmental stages and gonad index (GI) were recorded as supporting parameters for both species. It has been proposed that a shift of the sex ratio from the theorical one (1:1, male: female) can be due to varying factors which can include pollutants (Marigómez et al., 2004). Presently, in the case of *M. trossulus* the sex ratio was close to 1:1 with slight variations that cannot be attributed to the exposure to WAF or WAF-D as proposed by other authors that conducted exposure studies in the laboratory (Orbea et al., 2006; Garmendia et al., 2010; Cuevas et al., 2015). On the other hand, the sex ratio in *M. edulis* was far away from the theorical one, with a ratio of 0,15:1 after 7 d of exposure to WAF and WAF-D although this skewed ratio could be related more than to the exposure to pollutants, to the high proportion of mussels in a resting state which made impossible the sexual identification of the specimens of this group. Once the histological identification under the microscope is feasible the ratio might be close to 1:1 as

found for M. trossulus. The gonad index supported these results since reflected the developmental stage of the mussels: spawning in *M. trossulus* and resting in *M. edulis.*

For the tissue-level biomarkers, the values of Vv_{BAS} for the control groups depict a good health status since values below 0,1 $\mu m^3/\mu m^3$ are typical from reference locations, whilst upon exposure to pollutants values can overcome 0,12 $\mu m^3/\mu m^3$ (Cajaraville et al., 1990; Marigómez et al., 2006; Garmendia et al., 2011). The response given by the mussels in the exposure groups were higher than the 0,12 threshold value that might indicate the existence of health disturbance evidences due to the exposure to WAF, and mainly to the WAF mixed with the dispersant. It seems that a 7-day exposure to the dispersant (mimicking a possible remediation strategy after a crude oil spill in the field) is long enough to produce distress in both mussel species.

Similar results were obtained for MET/MDR. This ratio compares the thickness of the epithelium to the radius of the alveola and decreases as an indication of the thinning of the epithelium (Vega et al., 1989) resembling a stress situation. In *M. trossulus* the value decreased enough after exposure to WAF-D and showed significant differences when compared to the other two groups. This confirmed a typical stress response, while the response after exposure to WAF alone was not able to discriminate from controls. However, the responses observed in M. edulis were slightly different since the exposure groups are grouped together and apart from the control.

Changes in the digestive morphology such as thinning of the digestive epithelium, alteration in the cell type composition of the digestive gland, and an increase in CTD ratio are characteristic of stressed mussels (Brooks et al., 2011; Garmendia et al., 2011). However, CTD ratio was the only biomarker that showed no statistical difference between any of the groups, neither in *M. trossulus* nor in *M. edulis.*

In summary, the results of the tissue-level biomarkers showed similar responses in both species to the same type of exposure conditions and exposure treatments. It is clear that the habitats in which these mussel species live are very different, for instance, in terms of salinity, brackish waters (*M. trossulus*) where the salinity barely reaches 10 psu, and open sea (*M. edulis*) where the salinity is around 30 psu. Therefore, large differences could be expected a priori. However, most of the biomarkers only reflected slight differences for the same exposure conditions to WAF and WAF-D. It is more feasible than these differences might be related to the acclimation to laboratory conditions (17ºC, 33 PSU; Plentzia), dissimilar to the natural ones for Mytilus edulis (9ºC, 33 PSU, North Atlantic) that produced a certain degree of vacuolization throughout the whole digestive gland (Fig. 10). This degree of vacuolization could be a signal of stress in mussels. In addition, the

high resting percentage of individuals might add an "extra stress" to already stressed Mytilus edulis mussels. Accordingly, vacuolization did not appear in the digestive gland of *M. trossulus* (10ºC, 7 PSU; Baltic Sea) even in the exposed groups, which means that they were not caused by the exposure to the pollutant. However, despite of all this, the overall differences were not significant enough to create discrepancies between the response of both species to the exposure.

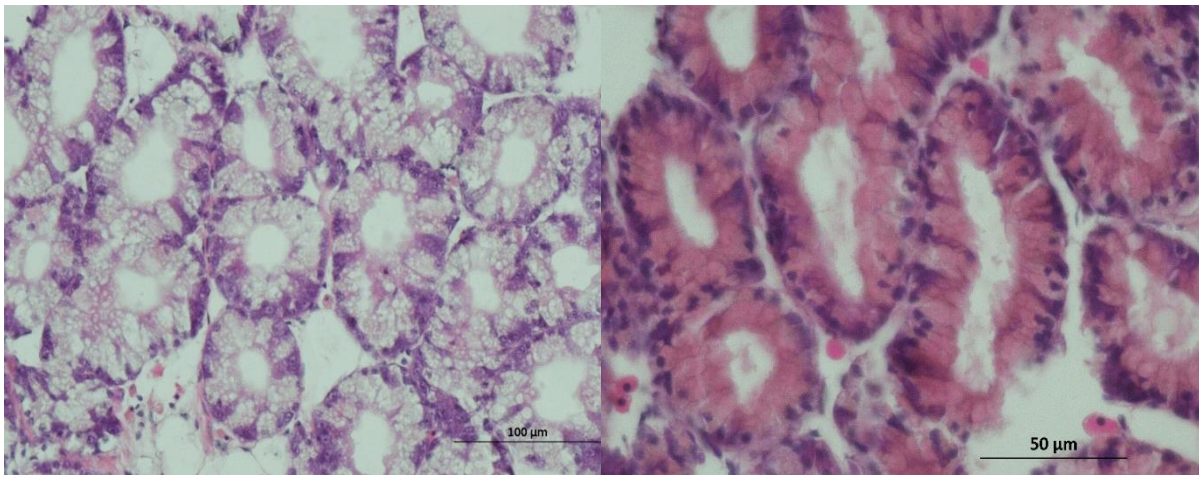

Fig. 10. High degree of vacuolization present in the digestive gland of *Mytilus edulis* (left), *Mytilus trossulus* digestive gland with no vacuolization for comparison (right).

The strength of the tissue-level biomarkers applied herein is proved even further when our results are compared with the results of another experiment which dealt with cell-level responses (changes in digestive cell lysosomes; De Koninck, 2019). According to this author *M. trossulus* was the only species that did not respond to either lipofuscin or neutral lipid accumulation after exposure since the endo-lysosomal system was different to that of the other species. In general, this species possesses larger sized lysosomes compared to the others. In general terms, lysosomes become enlarged under stress conditions, which are reflected as an increase in volume density of lysosomes and low surface-to-volume ratio. However, the exposure to WAF-D caused a size reduction deviated from these general responses. These results proved that the lysosomes show a clear distinction in response to the exposure between *M. trossulus* and *M. edulis.*

8. CONCLUSION

- Tissue-level biomarkers and histopathology of both Baltic mussels and North Atlantic mussels is a valuable tool to assess the effect produced by crude oil spills and subsequent remediation strategies based on the use of dispersants.
- WAF-D produced more marked stress in mussels compared to WAF, which means that in a short time frame (1-7 D) the dispersant generates higher stress than the crude oil itself.
- The similarity of the responses recorded by both mytilid species after exposure to WAF and WAF-D regarding tissue-level biomarkers were not species-specific.
- Further research is recommended to confirm the results obtained in this work at longer exposure times and at different conditions (for example decreased or increased salinity/temperature and different crude oil WAF and dispersed crude oil WAF concentrations)

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