Université du Québec
à Rimouski

Impacts subcellulaires d'un déversement de bitumes dilués selon la méthode d'intervention sur deux espèces commerciales majeures du Québec, *Mytilus edulis* et *Homarus americanus*

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Aux 9 plus beaux cadeaux que mes frères m'aient offerts,

Lilian, Margaux, Léane, Julian, Sinia, Helena, Éline, Charline et Hannah

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AVANT-PROPOS

Cette thèse présente les résultats de recherches menées sur les conséquences et les mécanismes de dispersion d'un déversement de bitume dilué, selon la méthode d'intervention choisie, sur deux espèces d'importance commerciale au Québec, la moule bleue, *Mytilus edulis* et le homard américain, *Homarus americanus*.

Ce travail a été réalisé dans le cadre d'une subvention du Groupe National Consultatif sur les Contaminants, octroyée à R. Saint-Louis de l'Université du Québec à Rimouski, et dans le cadre d'une subvention de la Stratégie maritime du Québec. Cette étude s'inscrit donc dans un projet plus large visant à acquérir de nouvelles connaissances sur les pétroles non classiques extraits en Alberta, les bitumes dilués. L'importance de ce sujet nous a permis d'élargir nos collaborations avec S. Le Floch du Centre de Documentation, de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux (CEDRE) à Brest, France, ainsi qu'avec N. Toupoint et J. F. Laplante de Merinov aux Îles-de-la-Madeleine, Canada.

Les objectifs de cette thèse sont multiples et font appel à des expertises de différents domaines tels que l'écotoxicologie, l'écophysiologie et la chimie. Par une approche combinant l'étude du comportement de la nappe de pétrole et ses impacts, l'objectif est de déterminer le niveau des dommages physiologiques, subcellulaires et génotoxiques causé par les bitumes dilués sur deux organismes marins par rapport à un pétrole conventionnel, selon la technique d'intervention, ainsi que la saison. J'ai réalisé cette étude sous la direction de Richard Saint-Louis (directeur de recherche) et de Gaëlle Triffault-Bouchet (co-directrice).

RÉSUMÉ

L'extraction des pétroles non classiques de l'Alberta au Canada ne cesse de croître et entraîne une accélération de son exportation par oléoducs, train ou encore bateau. L'ajout de condensat pour le rendre transportable, en fait un pétrole particulier, le bitume dilué (dilbit). Les deux principaux bitumes dilués exportés d'Alberta sont l'Access Western Blend (AWB) et le Cold Lake Blend (CLB). Les risques de déversements ne peuvent être niés et doivent être étudiés afin de pouvoir sélectionner la meilleure méthode d'intervention. Le fleuve du Saint-Laurent, mis en danger par le rapprochement des réseaux d'oléoducs et le passage du transport maritime, regorge d'espèces de grandes importances commerciales pour le Québec, dont la moule bleue, *Mytilus edulis*, et le homard américain, *Homarus americanus*. Ainsi, connaître les impacts à court et long termes est fondamental pour la gestion des pêches commerciales.

La densité élevée de ce pétrole non classique soulève la question de l'efficacité de dispersants chimiques, méthode d'intervention autorisée au Canada, en plus du débat quant à l'augmentation possible de la toxicité de la nappe de pétrole dispersé par cette technique. Des expériences en laboratoires ont été réalisées sur les deux bitumes dilués les plus transportés, afin de vérifier l'efficacité de deux dispersants chimiques, Corexit® 9500A (CXT) et Finasol® OSR52 (FIN), selon une large gamme de températures et de salinités. On a constaté que le FIN est plus efficace sur les dilbits que le CXT dans les conditions de basses températures et salinités, mais que la taille des particules est plus grande lorsqu'elle est dispersée avec FIN que CXT.

Or, la taille des gouttelettes qui vont en découler détermine la vitesse de dilution de la nappe de pétrole, de sa dégradation naturelle, mais également de sa filtration par les organismes marins. De l'eau de mer a été contaminée, en laboratoire par des bitumes dilués, avec ajout ou non de dispersant chimique et utilisée pour exposer des moules. Afin d'analyser les impacts propres aux dilbits, des tests identiques ont été effectués avec un pétrole classique importé au Canada, l'Heidrun (HEI). Les impacts physiologiques, subcellulaires et génotoxiques, ainsi que les concentrations accumulées dans les tissus, ont été mesurés tout au long de trois saisons (printemps, automne et hiver). Notre étude démontre une bioaccumulation plus importante dans les moules exposées au HEI par rapport au dilbit, surtout lorsque le dispersant chimique est utilisé. Des déstabilisations de la membrane lysosomale, une diminution de la viabilité cellulaire, ainsi qu'une augmentation des dommages à l'ADN ont été mesurées. L'apport d'un indice intégré de réponses des biomarqueurs (IBR) permet de mettre en évidence des impacts globalement plus importants lors d'une dispersion chimique du bitume dilué CLB en été, entraînant même une importante mortalité.

Les dommages mesurés sur l'ADN soulèvent la question d'impact à long terme sur la moule bleue, augmentant le préjudice pour les mytiliculteurs en cas de contamination. Une exposition des moules bleues, suivie d'une période de dépuration, apporte de nouvelles données sur la capacité de ces organismes filtreurs à éliminer le contaminant et à réparer les dommages. La vitesse de dépuration varie en fonction des composés, mais semble assez rapide avec les HAP (< 48 h) après une exposition de 8 jours. Cependant, les concentrations d'autres composés mesurés dans les tissus des moules bleues sont toujours supérieures à celles des moules contrôles, même après 4 jours dans des eaux propres. Cependant, ce temps est suffisant afin de revenir aux valeurs de moules non exposées pour la dégradation de la membrane lysosomale, la viabilité cellulaire et, même, le niveau de dommages à l'ADN.

Toutes ces analyses biologiques ont été rendues possibles grâce à des analyses sur l'hémolymphe de la moule, permettant un suivi des mêmes individus. Les impacts d'un dilbit ont été comparés avec un produit pétrolier léger à risque pour les homards américains des Îles-de-la-Madeleine, le diesel marin. Une exposition au CLB sur le homard américain démontre que, comme chez la moule bleue, des impacts sur les membranes lysosomales et la viabilité cellulaire sont mesurés après une exposition au bitume dilué, ainsi qu'une induction de l'activité enzymatique EROD (ethoxyresorufin O-deethylase). Chez les homards, un retour au niveau basal des biomarqueurs a été mesuré après plusieurs jours, voire plusieurs semaines dans des eaux propres. De plus, des concentrations encore importantes d'hydrocarbures aromatiques polycycliques (HAP) ont été mesurées dans la chair des homards après 3 mois de maintien en eau propre, ainsi que dans les œufs, interrogeant sur un impact sur la génération suivante.

Mots clés : Bitume dilué, Dispersant chimique, *Mytilus edulis*, *Homarus americanus*, Bioaccumulation, Hydrocarbures, Effets subcellulaires, Dommage à l'ADN, Dépuration, Capacité de récupération.

ABSTRACT

The extraction of unconventional oils from Alberta, Canada, continues to grow, accelerating its export by pipeline, train, or boat. The risks of spills during oil transportation cannot be denied and must be studied to understand the best intervention strategy. The St. Lawrence River, endangered by the proximity of pipeline networks and the passage of maritime transport, abounds in species of great commercial importance for Quebec, including the blue mussel, *Mytilus edulis*, and the American lobster, *Homarus americanus*. Consequently, awareness of the short- and long-term impacts of a potential spill is fundamental to commercial fishery management. In case of a spill of unconventional Albertan oil, it is important to be able to rapidly employ effective clean-up strategies. However, condensate is added to these unconventional oils to facilitate their transportation; the result of the mixing of the two is known as diluted bitumen (dilbit) and possesses unique chemical properties.

The high density of diluted bitumen raises the question of the effectiveness of chemical dispersants (a method of intervention authorized in Canada), and the possible increase in the toxicity of oil dispersed by this technique is subject to debate. To fill these gaps in the knowledge, our laboratory experiments evaluated the most exported Albertan diluted bitumen: Access Western Blend (AWB) and Cold Lake Bland (CLB). Experiments allowed to verify the effectiveness of two chemical dispersants, Corexit® 9500A (CXT) and Finasol® OSR52 (FIN), over a wide range of temperatures and salinities. We found that FIN is more effective on dilbits than CXT at low temperatures and salinities, despite larger oil particle size after dispersion with FIN.

The size of the resulting droplets determines the speed of dilution of the oil slick, its natural degradation, and its filtration by marine organisms. In the laboratory, we contaminated seawater with diluted bitumen. We then exposed mussels to this contaminated water, with or without the addition of chemical dispersant. Identical tests carried out with a conventional oil imported into Canada (Heidrun, HEI) allowed to identify the specific impacts of dilbits. For three seasons (spring, autumn, and winter), we measured the diluted bitumen and chemical dispersant concentrations accumulated in the tissues, as well as the physiological, subcellular, and genotoxic impacts. Our study demonstrates greater bioaccumulation in mussels exposed to HEI than those exposed to dilbit, especially when using chemical dispersant. We measured destabilization of the lysosomal membrane, decreased cell viability, and increased DNA damage. The contribution of an integrated index of biomarker responses (IBR) makes it possible to highlight the consequences of chemical dispersion of CLB diluted bitumen in summer, which even results in significant mortality.

The measured DNA damage raises the question of long-term harm to the blue mussel, increasing the risk to mussel farm in the event of contamination. Blue mussels' exposure to dilbit, followed by a period of depuration, provides new data on the ability of these filter-feeding organisms to extract the contaminant and repair the damage. The depuration rate varies according to the compounds but seems rapid for polycyclic aromatic hydrocarbons (PAHs; < 48 h) after an exposure of eight days. However, the concentrations of other compounds measured in the tissues of exposed blue mussels are always higher than in our control mussels, even after four days in clean water. Moreover, this time period typically suffices to return to unexposed mussel values for lysosomal membrane damage, cell viability, and level of DNA damage.

We obtained this biological data by analysing mussels' hemolymph, which allowed to follow-up on individuals' health. American lobsters' exposure to CLB demonstrates that, as in blue mussels, impacts on lysosomal membranes and cell viability are noticeable after exposure to diluted bitumen. These results are validated by observing EROD (ethoxyresorufin-*o*-deethylase) activity in the exposed individuals. In lobsters, biomarkers recovery was measured after several days or several weeks in clean water. High concentrations of PAHs were measured in the flesh and eggs of lobsters three months after their exposure to CLB, raising questions about impacts on their reproductive capacities and the health of future generations.

Keywords: Diluted bitumen, Chemical dispersant, Mytilus edulis, Homarus americanus, Bioaccumulation, Hydrocarbons, Subcellular effects, DNA damage, Depuration, Recovery.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ABRÉVIATIONS, DES SIGLES ET DES ACRONYMES

AHR	Aryl hydrocarbon Receptor
ANOVA	Analyse de variance
API	American Petroleum Institute
APS	Average particle size
AWB	Access Western Blend
BTEX	Benzène, Toluène, Éthylbenzène, Xylènes
CCA	Canonical Correlation Analysis
CEWAF	Chemical Water Accommodated Fraction
CLB	Cold Lake Blend
cSt	Centistokes
CXT	Corexit® 9500A
CZDTs	Carbazoles and dibenzothiophenes
DE	Dispersant effectiveness
DEI	Dispersant effectiveness index
DILBIT	Bitume dilué (*diluted bitumen*)
EHL	Équilibre hydrophile lipophile
EMX	Enzymes du Métabolisme des Xénobiotiques

EROD	Ethoxyresorufin O-deethylase
FIN	Finasol® OSR52
HAP	Hydrocarbures aromatiques polycycliques
HEI	Heidrun
IBR	Indice intégré de réponses des biomarqueurs
IFP	Institut Français du Pétrole
Mb/j	Millions de barils par jour
PTSA	Tetrasodium 1,3,6,8-pyrenetetrasulfonate
SARA	Saturés, Aromatiques, Résines et Asphaltènes
SFG	Scope for Growth
TPH	Total Petroleum Hydrocarbons
VOC	Volatile organic compounds
WAF	Water Accommodated Fraction

INTRODUCTION GÉNÉRALE

Tout a commencé il y a plus d'un milliard d'années sur Terre, lorsque des végétaux et des organismes marins en décomposition suite à l'action de bactéries anaérobies se sont accumulés au fond des mers, dans des sédiments argileux très fins. Ils formèrent des couches riches en matière organique qui, sous l'effet de la tectonique des plaques, s'enfoncèrent lentement jusqu'à se décomposer en substances plus simples, les hydrocarbures, association d'hydrogène et de carbone. Ce mélange remonta ensuite vers la surface par des failles ou des roches poreuses, jusqu'à se retrouver piégé dans des « réservoirs » fermés hermétiquement par une couche imperméable, généralement constituée d'argile ou de sel. Ces réservoirs constituent les gisements pétrolifères classiques, mot issu du latin médiéval *petraoleum* « huile de roche ». Mais lorsque cette couche est absente, les hydrocarbures gagnent lentement la surface où les plus légers vont s'évaporer et les plus lourds se dégrader pour former des bitumes. Ce type de pétrole est le premier à être mentionné, bien avant d'être l'un des piliers de l'économie industrielle. Il y a plus de 3 000 ans en Mésopotamie, le bitume était utilisé comme mortier dans la construction des remparts, puis comme ingrédient essentiel au processus de momification chez les Égyptiens (Pigeaud, 2002). Il devient une arme redoutable au Moyen Âge sous le terme de « feux grégeois » capable d'enflammer la mer, ainsi que par les soldats incendiaires du califat de Bagdad, les *naffatun*, au IX^{e} siècle (Auzanneau, 2015). Il fut ensuite exploité au XVII^{e} siècle pour soigner des maladies, faire repousser les dents et les cheveux en Chine (Vogel, 1997) ou, par les Cris, pour calfater les canoës (Nikiforuk, 2010).

En 1858, James Miller Williams, qui dirigeait une carrière de bitume en Ontario (Canada), se mit à forer un puits à la recherche d'eau au cours d'une sécheresse. À la place, il en fit jaillir du pétrole (Kolbert, 2007). Mais c'est un an plus tard, en 1859, que l'industrie pétrolière voit officiellement le jour, lorsqu'Edwin Drake parvient à faire jaillir du pétrole brut d'un forage de 21 mètres de profondeur.

Il est alors utilisé comme nouveau combustible, remplaçant l'huile de baleine et de cachalot (Auzanneau, 2015 ; Figure 1).

Figure 1 : Dessin représentant un « grand bal donné par les baleines en l'honneur de la découverte des puits de pétrole de Pennsylvanie » (magazine américain *Vanity Fair* du 20 avril 1861)

En seulement un siècle et demi de développement industriel, l'humanité a pompé près de la moitié du pétrole brut exploitable sur Terre, que l'évolution géologique aura mis des dizaines de millions d'années à produire (Auzanneau, 2015). Des forages simples ont permis l'extraction du pétrole « classique », mais la demande de cette énergie fossile ne cessant de croître, la recherche vers de nouveaux puits s'est intensifiée jusqu'à développer de nouvelles techniques d'extraction plus complexes, permettant la production de pétrole « non classique », comme les sables bitumineux (Zou, 2017). Ces pétroles sont alors devenus un enjeu stratégique permettant de doubler les réserves accessibles et déplaçant cette richesse hors du Moyen-Orient, qui possédait la majorité des réserves de pétroles. Depuis 2010, la production de pétrole à partir des sables bitumineux a surpassé celle du pétrole classique et les réserves sont estimées comme étant plus importantes que les réserves de l'Iran et de l'Irak. Avec les sables bitumineux, les trois pays possédant désormais le plus de gisements de pétrole sont le Venezuela (18%), l'Arabie Saoudite (16%) et le Canada (10% ; RNCan, 2021).

DE L'OR NOIR AU CANADA

Le bitume provenant des sables bitumineux est un pétrole lourd que l'on trouve dans un mélange de sables et d'argiles originaires de la région du nord-est de l'Alberta. Les réserves de sables bitumineux au Canada furent décrites dès 1778 par l'explorateur Alexander Mackenzie comme des « fontaines de bitume ». Mais il faut attendre 1932 pour que des appels à la recherche pour « dégager les réserves de pétrole de ce tas de sable magique » soient lancés, puis 1960 pour qu'une mine à ciel ouvert et une usine de valorisation voient le jour en Alberta (Figure 2).

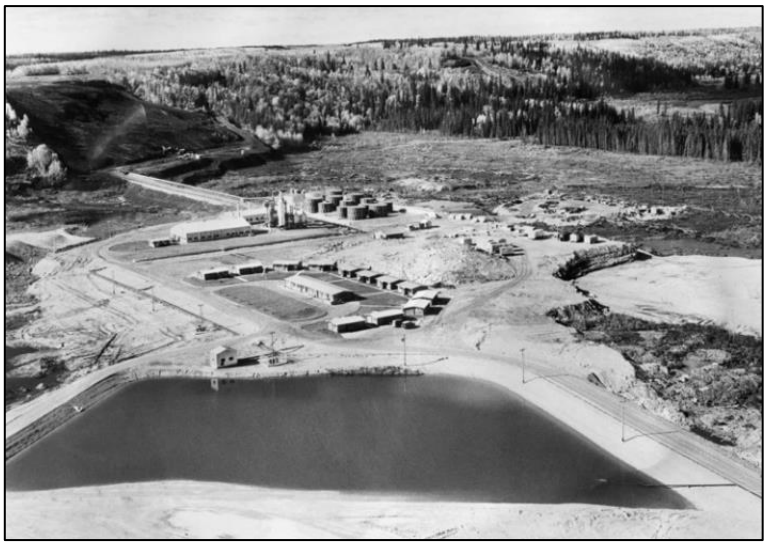

Figure 2 : Vue aérienne de l'usine pilote de sables bitumineux à Mildred Lake, Alberta, Canada, en 1960. Photo tirée du site du Gouvernement de l'Alberta (2021)

Désormais, les réserves prouvées de pétrole brut au Canada totalisent 166,7 milliards de barils dont 97% proviennent des sables bitumineux, permettant une production en 2020 de 2,8 Mb/j (RNCan, 2021).

Parmi les provinces canadiennes, la plus grande production de pétrole provient de l'Alberta (80,2 % en 2020 ; RNCan, 2021), dont les réserves de sables bitumineux sont localisées dans trois principaux gisements : Athabasca, Cold Lake et Peace River (Figure 3).

La Régie de l'énergie du Canada prédit une expansion d'environ 30% de la production de sables bitumineux d'ici 2050 (REC, 2021).

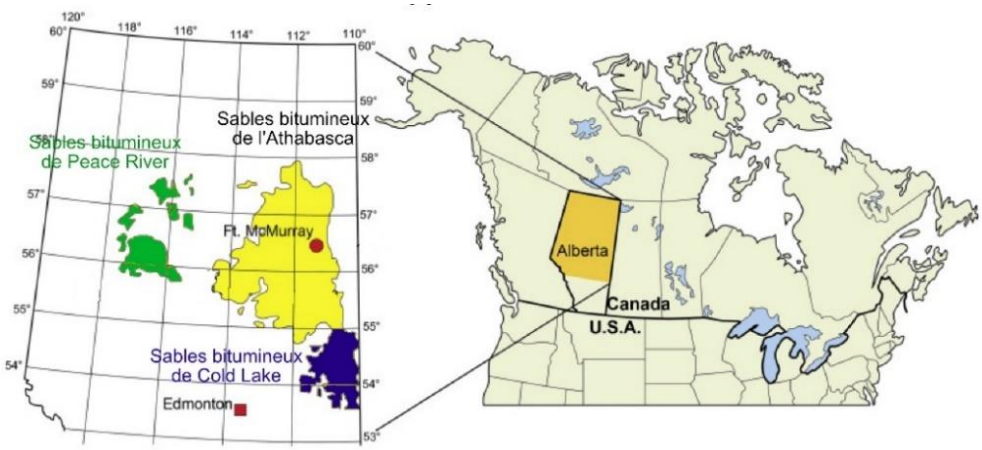

Figure 3 : Carte des gisements de sables bitumineux en Alberta, Canada. Figure modifiée d'après Martinius *et al.* (2017)

Un pétrole pas comme les autres

Le bitume est extrait des sables par extraction à ciel ouvert ou par extraction *in situ* en le chauffant avec de la vapeur, respectivement, si le gisement est proche de la surface, moins de 75 m, ou s'il est plus profond, environ 700 m (Appert *et al.*, 2005). Le bitume récupéré est une substance épaisse et visqueuse qui doit être diluée ou valorisée (transformée en pétrole synthétique) pour être transporté dans les oléoducs ou utilisé comme matière première dans les raffineries (Figure 4). La particularité de ce pétrole, en plus de son extraction, est l'ajout d'hydrocarbures plus légers appelés diluants afin de diminuer leur viscosité de 10 000 cSt à environ 350 cSt (Crosby *et al.*, 2013). On parle alors de bitume dilué ou dilbit (*diluted bitumen*). Les diluants légers comprennent les hydrocarbures dont la densité est inférieure à 760 kg/m^3, couramment appelés « condensats » (MPO, 2018a).

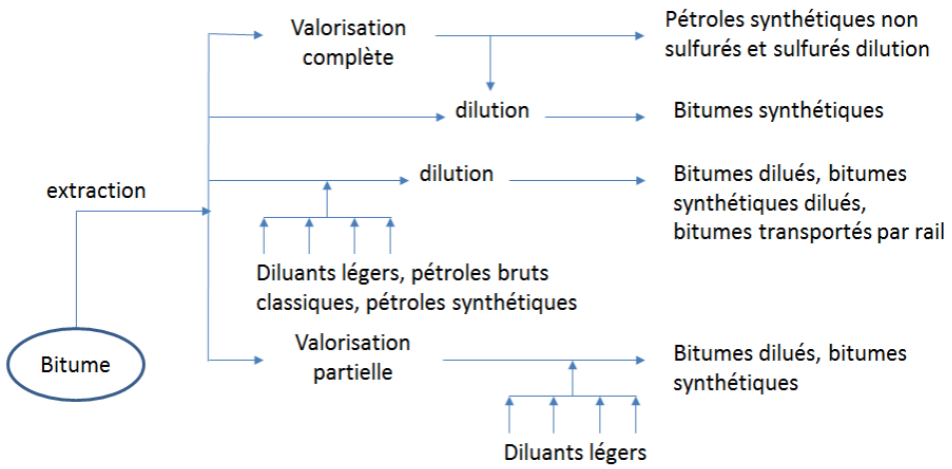

Figure 4 : Parcours généraux de la création du bitume dilué provenant de la région nordique de l'Alberta et de la Saskatchewan. Figure tirée de MPO, 2018a

Le devenir, le comportement et les impacts d'un bitume dilué lors d'un déversement dans l'environnement, sont dépendants de ses caractéristiques physico-chimiques, qui se différencient de celles des pétroles classiques, notamment du fait de leur formation et du type d'extraction. Les renseignements sur leur composition chimique et leurs propriétés physiques sont donc essentiels afin de prévenir et d'intervenir au mieux en cas d'accident. Parmi les bitumes dilués au Canada, les plus transportés et, donc, les plus étudiés sont l'Access Western Blend (AWB) et le Cold Lake Blend (CLB).

De façon générale, les bitumes se distinguent par une viscosité élevée et une densité faible. La densité, exprimée dans l'industrie pétrolière par un degré API (American Petroleum Institute), permet de distinguer les produits légers des lourds. Pour une densité API supérieure à 31,1°, le pétrole est dit léger ; entre 31,1° et 22,3°, moyen ; entre 22,3° et 10°, lourd et inférieur à 10°, le pétrole est classé comme extra-lourd. Les bitumes dilués AWB et CLB sont catégorisés dans les pétroles lourds, étant donné leur degré API (< 22,3°). Bien qu'extraits dans la même région, leurs caractéristiques physiques ne sont pas forcément identiques (Tableau 1), c'est pourquoi le mélange de condensats contient plus de fractions légères (C4 à C10) et de BTEX (benzène, toluène, éthylbenzène, xylène) avec le pétrole AWB que le CLB (MPO, 2013).

Tableau 1 : Propriétés physiques de deux bitumes dilués (*MPO, 2013), AWB (Crude Monitor, 2016a) et CLB (Crude Monitor, 2016b)

	Température (°C)	Access Western Blend (AWB)	Cold Lake Blend (CLB)
Degré API (degré API)	-	20,9*	21*
Densité (g/ml)	0	0,9399*	0,9376*
	15	0,9253*	0,9249
	20	0,9148*	0,9216*
Viscosité dynamique (cP)	0	1300*	803
	15	347*	285*
	40	59,8*	59*
Point d'éclair (°C)	-	< - 5*	< - 5*
Point d'écoulement (°C)	-	< - 25*	< - 25*
Tension superficielle (Air/pétrole, mN/m)	0	31,2	30,0
	15	30,2	28,8
	20	27,5	28,0
Tension interfaciale (pétrole/eau, mN/m)	0	24,8	30,6
	15	24,2	27,7
Tension interfaciale (pétrole /33 ‰ saumure, mN/m)	0	25,0	30,4
	15	23,8	26,3

En plus de leurs particularités physiques, les bitumes dilués se distinguent des pétroles classiques par leur composition chimique. De façon générale, le pétrole est un mélange complexe constitué principalement d'hydrocarbures, jusqu'à 97% dans certains produits, mais qui peut diminuer à 50% dans les pétroles lourds et les bitumes (Speight, 2006). Chaque pétrole a une composition chimique propre dont les composés sont regroupés en quatre classes (Corbett and Merz, 1975) : 1) composés Saturés ; 2) composés Aromatiques ; 3) Résines ; et 4) Asphaltènes (SARA ; Figure 5), schéma encore fréquemment utilisé dans la littérature. Les proportions de chacune de ces fractions apportent des informations sur la prédiction de propriétés comme la densité, la viscosité, la température d'ébullition et la stabilité des asphaltènes (Rudyk, 2018).

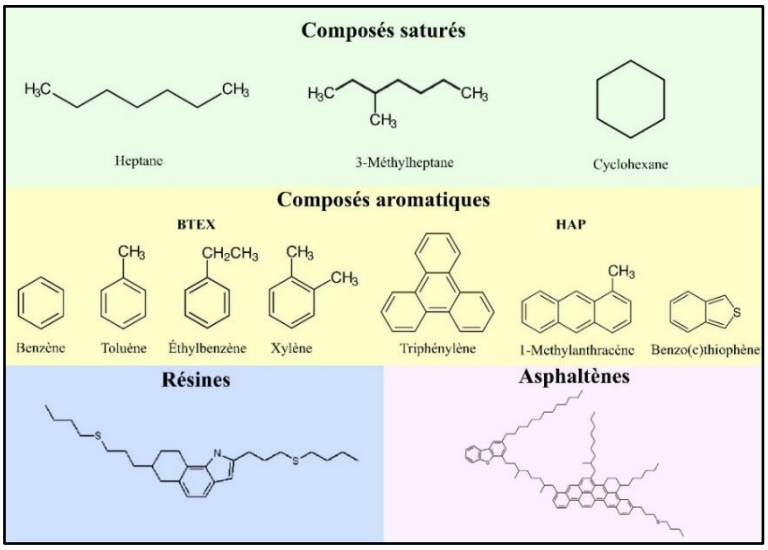

Figure 5 : Les 4 principales classes de composés chimiques des pétroles (SARA)

En comparaison avec un pétrole léger ou moyen (Figure 6), les bitumes dilués contiennent de plus fortes proportions de composés aromatiques, de résines et d'asphaltènes (Yang *et al.*, 2011 ; NASEM, 2016), et une plus faible proportion de composés saturés, et donc une composition moléculaire élevée.

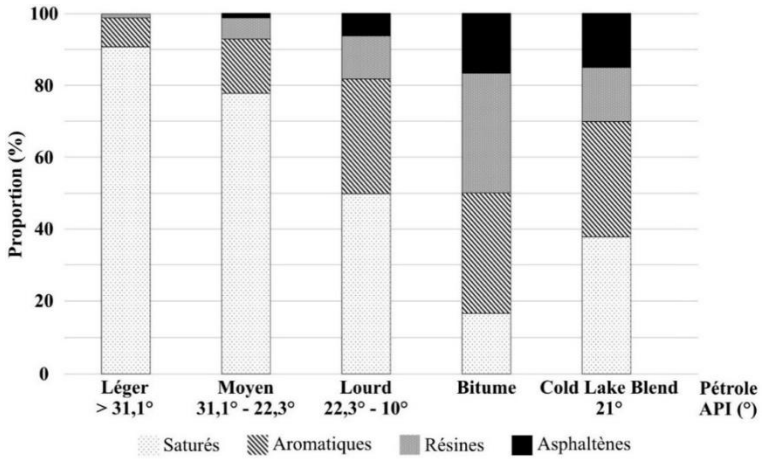

Figure 6 : Proportion de Saturés – Aromatiques – Résines - Asphaltènes (SARA) de différents types de pétroles. Figure modifiée d'après NASEM (2016)

Les bitumes dilués contiennent également des teneurs élevées en métaux traces comme l'aluminium (Al), le chrome (Cr), le nickel (Ni), et le vanadium (V) (Speight, 2014; Chauhan and de Klerk, 2020), ainsi que cinq à dix fois plus de soufre que les pétroles classiques (Swift *et al.*, 2011).

Leur composition chimique varie d'ailleurs par leur teneur en soufre, l'abondance totale des fractions légères et celle des BTEX (MPO, 2013), ce qui peut accentuer la variabilité de toxicité de ce type de pétrole. Les propriétés chimiques, comme physiques, ne sont pas identiques entre les deux bitumes dilués les plus transportés (Tableau 2), avec une plus forte teneur de composés aromatiques et de HAP alkylés chez le CLB comparé au AWB (King *et al.*, 2014).

Tableau 2 : Propriétés chimiques des deux produits pétroliers étudiés (MPO, 2013 ; Crude Monitor, 2016a; 2016b)

		Access Western Blend (AWB)	**Cold Lake Blend (CLB)**
Saturés (wt %)		≃ 37	≃ 45
Aromatiques (wt %)		≃ 31	≃ 30
Résines (wt %)		≃ 16	≃ 13
Asphaltènes (wt %)		≃ 15	≃ 13
Fractions légères	Butanes	0,93	1,40
	Pentanes	9,29	7,52
	Hexanes	6,00	5,22
	Heptanes	3,75	3,20
	Octanes	2,41	2,12
	Nonanes	1,35	1,35
	Décanes	0,67	0,69
	Total	24,4	21,5
BTEX (vol. en %)	Benzène	0,20	0,17
	Toluène	0,35	0,29
	Ethyl benzène	0,04	0,04
	Xylènes	0,32	0,27
	Total	0,91	0,77
Métaux (mg/kg)	Nickel	68	60,3
	Vanadium	177,5	155,5

Transport des bitumes

À des fins de transport, le condensat le plus couramment utilisé est un mélange d'hydrocarbures à base de naphta, qui peut comprendre des produits dérivés du gaz naturel (MPO, 2013). Le pourcentage ajouté dépend de la densité du bitume, de la saison (NRC, 2013) et du type de transport (CAPP, 2019), mais est en moyenne de 20 à 30% de condensat et 70 à 80% de bitume (Crosby *et al.*, 2013). L'ajout de ce condensat engendre des niveaux plus élevés de fractions légères et de composés BTEX (MPO, 2013).

En 2020, 3,5 millions de barils de pétrole brut sont exportés par jour vers les États-Unis, soit 97% de toutes les exportations canadiennes de pétrole brut et d'équivalent, ce qui fait du Canada le plus important fournisseur étranger de pétrole brut aux États-Unis (RNCan, 2021). Un réseau de 840 000 kilomètres d'oléoducs achemine le bitume dilué vers les États-Unis, mais également vers les différentes régions canadiennes (Figure 7).

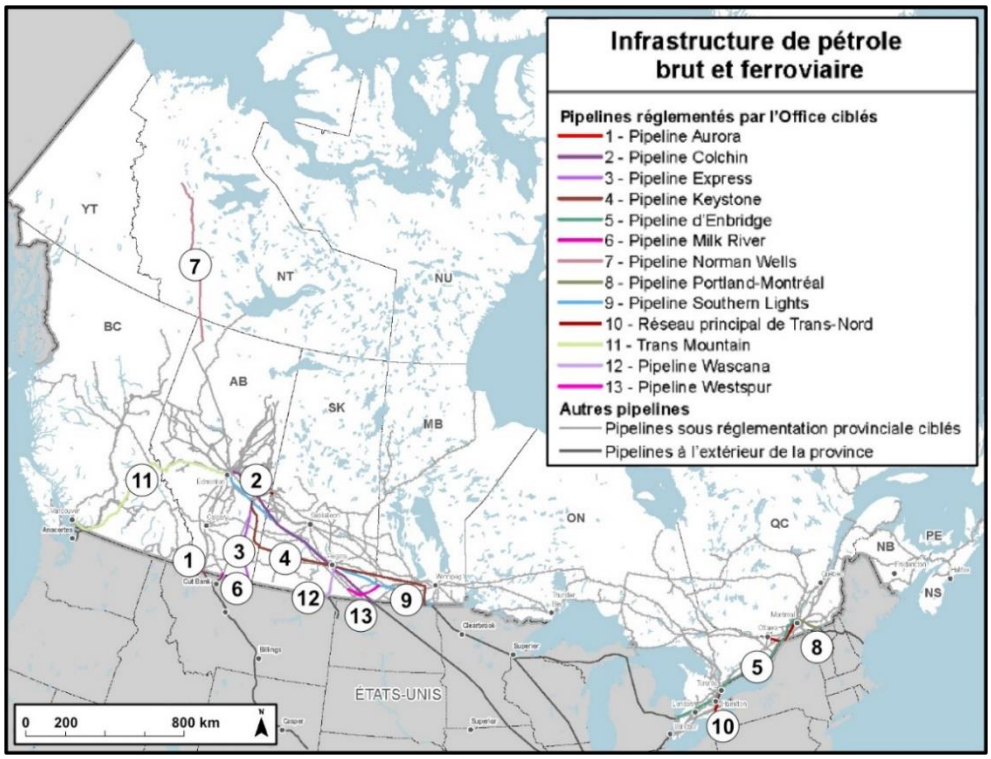

Figure 7 : Carte des principaux oléoducs de pétrole brut au Canada en 2022. Figure tirée de REC, 2022

Ce réseau continue d'être développé avec différents projets d'extension ou de renouvellement des oléoducs en place (CAPP, 2020), comme le projet d'oléoduc Trans Mountain approuvé en 2019, dans le but d'augmenter la capacité du réseau de 300 000 barils par jour à 890 000, de l'Alberta vers la côte Ouest du Canada (Colombie-Britannique ; Trans Mountain, 2019). Même si annulé en 2017, le projet d'oléoduc Énergie Est démontre l'intérêt d'acheminer le pétrole d'Alberta vers le Nouveau-Brunswick. Mais l'augmentation de l'exportation des bitumes vise aussi les trains et les navires, dont les solutions pour son transport sont en cours de développement (ONE, 2019), notamment pour permettre l'augmentation du nombre de pétroliers en transit par l'Arctique (ClearSeas, 2020).

De l'accident au déversement

Malgré le traitement des bitumes pour abaisser sa teneur en sable et en soufre dans le but de prévenir les risques de bris des oléoducs du fait d'une corrosion et érosion accélérée, les déversements des bitumes dilués sont à ce jour essentiellement dus à des bris d'oléoducs. Ces incidents sont potentiellement engendrés par leur effet corrosif, comme le soulignent certains auteurs, en raison de leur concentration plus élevée d'acides naphténiques, de composés soufrés et d'asphaltènes (Bakker, 2011 ; Swift *et al.*, 2011 ; Warmore, 2013). Mais d'autres affirment que les acides organiques présents ne semblent pas suffisamment corrosifs pour provoquer de la corrosion aux températures d'opération des oléoducs (Winter *et al.*, 2003 ; McIntyre *et al.*, 2014). La thèse de Liang (2020) précise que le bitume lui-même n'est pas corrosif, mais est due à la présence d'eau, principalement présente sous forme de gouttelettes émulsionnées (Wu, 2003) transportant des chlorures (CEPA, 2013), dont l'effet corrosif a été largement étudié (Evans, 1960; Li and Hihara, 2012; Schindelholz *et al.*, 2014). Ainsi, il apparaît que l'expansion rapide du transport des bitumes dilués et l'inquiétude de bris des oléoducs rendent le risque de leur déversement non négligeable.

Plusieurs déversements de bitume dilué ont déjà été répertoriés en Amérique du Nord, notamment causés par des bris d'oléoducs. En 2007, la rupture d'un oléoduc dû à des travaux de construction a entraîné le déversement de 232 000 litres d'un bitume dilué partiellement valorisé (Albian Heavy) dans la baie de Burnaby (Westridge, Colombie-Britannique). En 2010, le bris d'un oléoduc d'*Enbridge* dans le ruisseau Talmadge et la rivière Kalamazoo, près de Marshall (Michigan, États-Unis) a causé un déversement de 3 190 000 litres de bitume dilué (Cold Lake, Western Canada Select). Trois ans après le déversement, l'*Environmental Protection Agency* des États-Unis (US EPA) estimait que 680 000 ± 380 000 litres d'hydrocarbures restaient immergés dans la rivière (US-EPA, 2021a). En 2012, un joint d'étanchéité défectueux a causé la fuite de 230 000 litres de pétrole près d'Elk Point (Edmonton, Alberta, Canada). La même année, 461 000 litres ont été déversés dans la rivière Red Deer (Alberta, Canada) à la suite d'une fuite d'un oléoduc de la compagnie Plains Midstream Canada. Dans la même province, deux ans plus tard, une rupture d'oléoduc a entraîné le déversement de 70 000 litres de pétrole et d'eau traitée près de Slave Lake en avril, puis 60 000 litres de pétrole brut en novembre à Red Earth Creek. En 2015, 5 000 000 litres d'un mélange de bitume, de sable et d'eaux usées se sont déversés près de Fort McMurray en Alberta (Canada), en raison d'une fuite d'un oléoduc. Un an plus tard, c'est entre 200 000 et 250 000 litres de pétrole brut qui sont déversés dans la rivière Saskatchewan dans la province du même nom (Canada). Plus récemment, en août 2019, un oléoduc de la société *Bonterra Energy* a déversé 40 000 litres de pétrole dans le ruisseau Washout (Drayton Valley, Alberta, Canada). Même si les côtes maritimes du Québec n'ont pas encore été touchées par un déversement de bitume dilué, elles ont déjà été impactées par du mazout lourd dans la baie de Sept-Îles en 2013.

Une fois déversés dans l'environnement, des processus physiques, chimiques et biologiques altèrent la composition du pétrole (Fingas, 2013), ce qui influence son comportement au cours du temps (Cedre, 2009). Cette altération des hydrocarbures est souvent évoquée sous le terme de « vieillissement » (ITOPF, 2012), qui peut diminuer la sévérité d'un déversement et accélérer la restauration du milieu affecté (US-EPA, 2013), mais n'empêche pas l'atteinte à l'environnement (GENIVAR, 2013) (Figure 8).

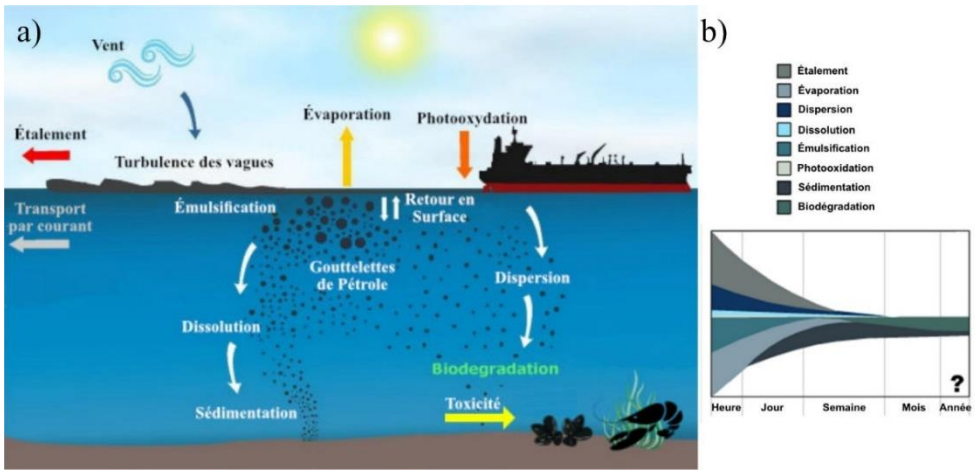

Figure 8 : a) Principaux processus de transport et d'altération affectant la nappe de pétrole. Figure modifiée à partir de Keramea *et al.*, 2021; b) Temps pour chaque processus. Figure tirée de CNOOC International (CNOOC, 2021)

Dans les premières heures, le bitume dilué s'étale à la surface du milieu marin pouvant former des nappes de quelques millimètres d'épaisseur (Fingas, 2013). Après évaporation des composants les plus volatils, comme les aliphatiques, les BTEX et les HAP de faible masse moléculaire (ITOPF, 2012), les propriétés physiques touchant la flottabilité du pétrole augmentent, comme la densité et la viscosité (Fingas and Punt, 2000; MPO, 2013) entraînant l'enfoncement d'hydrocarbures (Michel, 2010) que la mer soit agitée (Witt O'Briens, 2013) ou non (King *et al.*, 2014). Les hydrocarbures vont alors se disperser dans la colonne d'eau par le transfert de gouttelettes de pétrole de tailles variables, voir sédimenter par leur densité propre ou résultante, suite à l'adsorption sur la matière particulaire (Fingas and Punt, 2000). Au cours des semaines, mois, voire années, une biodégradation s'installe graduellement au fil de l'altération (Bocard, 2006 ; GENIVAR, 2013), constituant la principale voie d'élimination naturelle des hydrocarbures déversés dans l'environnement. Elle est due à des bactéries hydrocarbonoclastes qui ont la capacité de métaboliser les produits pétroliers (Head *et al.*, 2006), plus ou moins rapidement, selon la complexité des composés. De par leurs caractéristiques, le comportement des bitumes dilués se distingue par un temps de flottabilité plus long que pour des pétroles légers (King *et al.*, 2014).

Sans l'ajout de diluant, l'évaporation et l'étalement du bitume seraient très faible et son altération très lente (Barsauskas, 2014). Mais l'ajout du diluant augmente son étalement et sa submersion, et ainsi son interaction avec la matière organique et, éventuellement, sa sédimentation, mais les diluants s'évaporent rapidement. Ces processus varient d'un bitume dilué à l'autre selon sa composition chimique (Witt O'Briens, 2013 ; King *et al.*, 2014). Les effets du vieillissement sur les concentrations des groupes de composés saturés, aromatiques, résines et asphalthènes (SARA) vont varier selon le type de pétrole (Rial *et al.*, 2013). La photooxydation cause une diminution des composés aromatiques, tandis que l'évaporation entraîne leur augmentation. L'inverse fut observé par Rial *et al.* (2013) pour les résines. Les asphaltènes sont les plus résistantes au processus de vieillissement.

DU DILBIT EN FUITE

Du déversement à l'intervention

À la suite d'un déversement pétrolier, il est important d'intervenir au plus vite afin d'atténuer l'impact environnemental et d'accélérer la récupération de l'écosystème affecté. Le Canada dispose essentiellement d'équipements d'intervention mécaniques (barrages flottants, tampons absorbants, systèmes d'aspiration). Or, les particularités des pétroles non classiques rendent leur dégradation et dispersion naturelle plus longues et complexes que pour les pétroles classiques. Dans le but d'améliorer l'intervention, le gouvernement du Canada indique que « la planification de l'intervention ne devrait pas être axée exclusivement sur la capacité de récupération mécanique (…), mais être axée sur une stratégie qui tiendrait compte d'une plus vaste gamme de contre-mesures en cas de déversements, tels que (…) les agents dispersants » (Transports Canada, 2013). À l'heure actuelle, ces agents sont disponibles pour les activités pétrolières en mer dans l'est du Canada, et seul le Corexit® 9500A (CXT) est approuvé (MPO, 2021). L'efficacité de cette méthode d'intervention est dépendante, comme pour les autres, des conditions météorologiques, de la capacité des navires et du comportement de la nappe de pétrole (Figure 9).

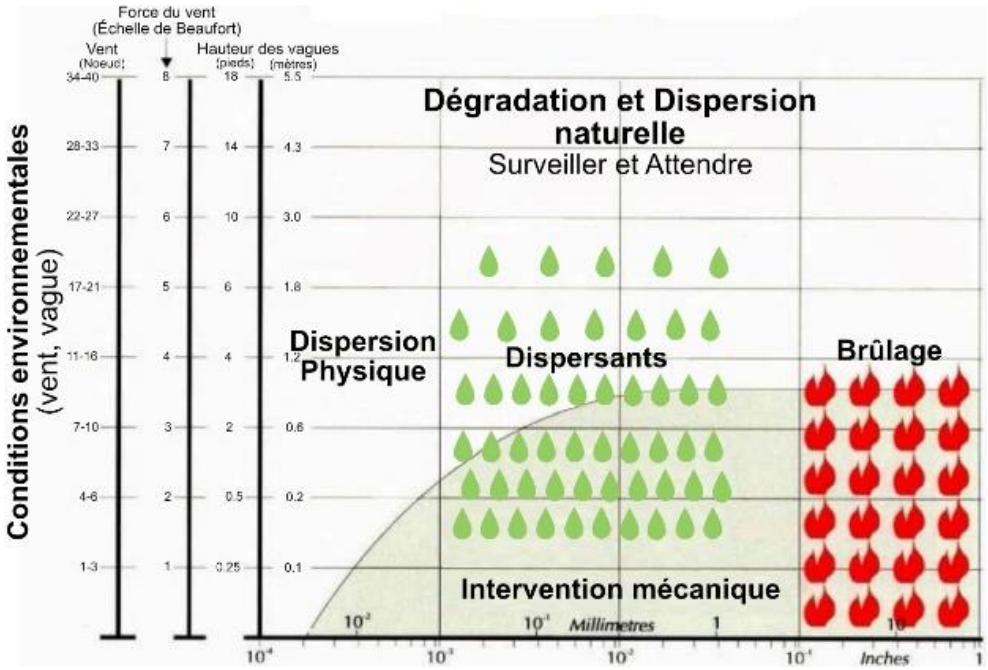

Figure 9 : Conditions de fonctionnement des options de lutte contre les déversements d'hydrocarbures. Figure tirée de CNOOC International (2021)

Les dispersants chimiques

Les dispersants chimiques ont pour objectif d'augmenter l'accessibilité du pétrole pour les microorganismes afin de faciliter la dégradation microbienne des hydrocarbures par le fractionnement de la nappe en une multitude de gouttelettes réparties dans la colonne d'eau, de quelques mètres à quelques dizaines de mètres de profondeur (Pelletier, 2015 ; Figure 10). Les principaux bénéfices pour l'environnement associé à cette méthode d'intervention sont la réduction/dispersion des nappes de pétrole afin de limiter l'impact direct sur les oiseaux, les mammifères et la frange littorale, et permet également de retarder la formation d'une émulsion persistante eau-dans-pétrole qui présente de nombreux défis en termes d'intervention.

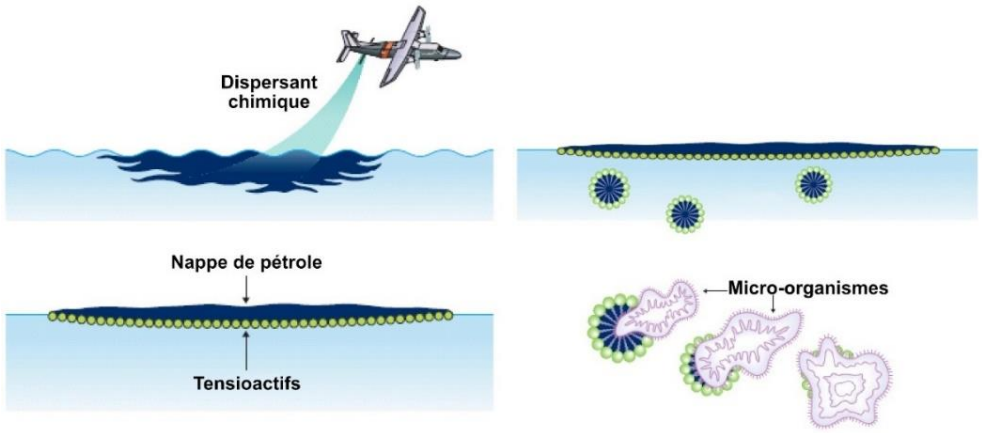

Figure 10 : Schéma de l'application et du fonctionnement des dispersants chimiques. Figure modifiée d'après le site de l'IPIECA-IOGP (2014b)

L'utilisation de dispersant chimique est dépendante de la localisation du déversement (distance des côtes), des conditions environnementales (température, salinité, énergie), des spécificités environnementales locales (sensibilité de l'habitat, particularités saisonnières : migrations des poissons, pêche ; Cedre, 2015) et de sa fenêtre de dispersibilité, c'est-à-dire le temps de vieillissement du pétrole (Cedre, 2015). Elle est privilégiée en eau profonde, où la circulation d'eau est importante (CEAEQ, 2015c).

Les dispersants chimiques sont composés d'un mélange de tensioactifs et de solvants qui diminue la tension de surface entre l'eau et la nappe de pétrole, favorisant ainsi la création de fines gouttelettes d'hydrocarbures qui se dispersent dans l'eau avec l'aide de l'agitation naturelle (Pelletier, 2015). Les principaux tensioactifs présents dans la majorité des dispersants sont ionique (Dioctyl sodium sulfosuccinate ; DOSS), et non ionique (Span et Tweens), possédant des affinités variables pour le pétrole et l'eau (Figure 11).

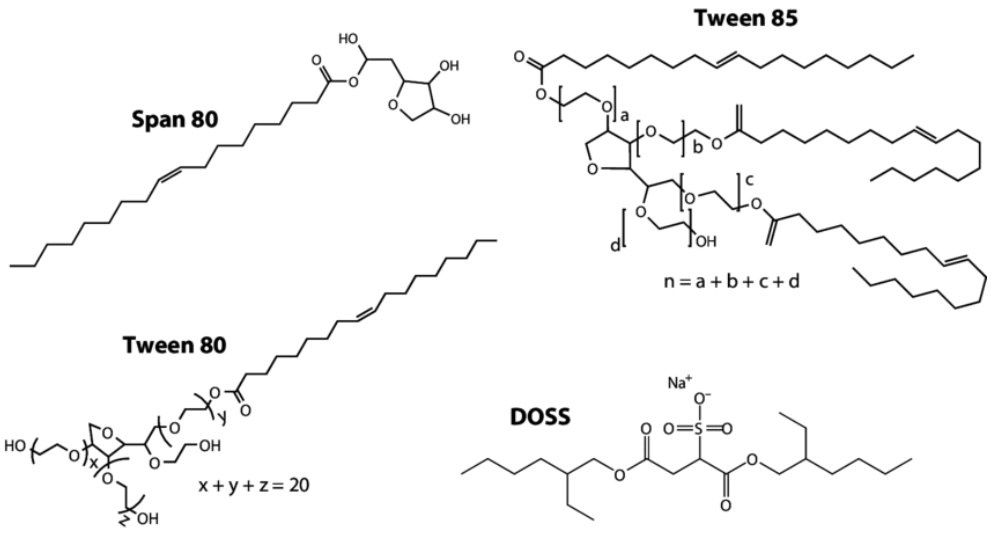

Figure 11 : Structure des tensioactifs utilisés dans les dispersants chimiques. Figure tirée de John *et al*. (2016)

Ces tensioactifs sont composés de deux parties : une partie oléophile et une partie hydrophile, qui vont attirer l'eau à une extrémité et le pétrole à l'autre extrémité (Figure 12). Leur affinité pour l'eau et le pétrole peut être classée par le nombre d'équilibres hydrophile lipophile (EHL). À un faible indice EHL (3-6), ils ont tendance à former des émulsions eau-dans-pétrole, tandis qu'à un indice plus élevé (8-18), ils ont tendance à former des émulsions pétrole-dans-eau, c.-à-d. des gouttelettes de pétrole dispersées dans l'eau (Ufford *et al.*, 2014), ce qui est recherché.

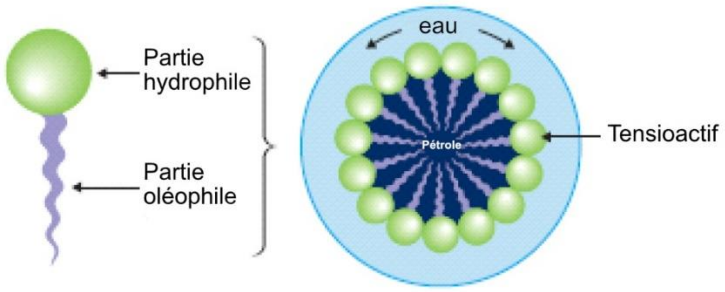

Figure 12 : Schéma de l'application et du fonctionnement des dispersants chimiques. Figure modifiée d'après le site de l'IPIECA-IOGP (2014b)

Le Span (EHL de 1,8 [Span 85] à 8,6 [Span 20] ; Kruglyakov, 2000) et les Tweens (EHL entre 10-17 ; Kruglyakov, 2000) sont des tensioactifs à base de saccharides non ioniques qui sont considérés comme facilement biodégradables en raison de leurs structures contenant des saccharides facilement métabolisées (Place *et al.*, 2016). Le DOSS est un surfactant anionique à double queue qui pourrait persister pendant des périodes beaucoup plus longues dans le milieu marin (Kujawinski *et al.*, 2011 ; Place *et al.*, 2016). Le tensioactif Span est insoluble dans l'eau tandis que le DOSS est peu soluble, mais les deux surfactants sont entièrement solubles dans les hydrocarbures. Le tensioactif Tween est totalement soluble dans l'eau.

Des additifs supplémentaires peuvent être présents pour améliorer la mobilité du tensioactif, augmenter la biodégradabilité du mélange pétrole/dispersant ou augmenter la stabilité à long terme de la dispersion (Clayton *et al.*, 1993). L'efficacité du dispersant dépend fortement de sa composition et de la quantité de tensioactif (Brandvik and Daling, 1998; Riehm and McCormick, 2014; Arnosti *et al.*, 2016).

Efficacité des dispersants chimiques

L'efficacité des dispersants chimique dépend de nombreux facteurs, comme la composition du dispersant et du pétrole, de la salinité, de la température et de l'énergie de la mer (Blondina *et al.*, 1999 ; George-Ares *et al.*, 2001 ; Chandrasekar *et al.*, 2006 ; Fingas, 2014 ; IPIECA-IOGP, 2014a ; Tansel *et al.*, 2014 ; King *et al.*, 2018). Effectivement, si l'agitation à la surface de l'eau est trop faible (< force 3), l'hydrocarbure reviendra presque inévitablement à la surface (CEAEQ, 2015c). Mais le principal facteur ayant un effet sur l'efficacité de la dispersion chimique des hydrocarbures pétroliers est la viscosité des hydrocarbures (Cedre, 2007), qui varie avec les conditions environnementales. En cas d'hydrocarbures très visqueux (entre 5 000 et 10 000 cSt), les dispersants sont inopérants puisqu'ils ont tendance à glisser sur les hydrocarbures et tomber dans la colonne d'eau avant la pénétration du solvant dans la nappe de pétrole (CEAEQ, 2015c).

Lorsque la viscosité est supérieure à 10 000 cSt ou qu'il y a une émulsion eau-dans-pétrole, les dispersants chimiques n'ont aucun effet (Lunel and Davies, 2001; CEAEQ, 2015c). Or, les bitumes dilués possèdent une plus forte proportion d'asphaltènes, qui contribue à l'augmentation de la viscosité et la densité des pétroles (Akmaz *et al.*, 2011), par rapport au pétrole classique. Ainsi, une teneur élevée en asphaltènes tend à réduire l'efficacité des agents dispersants (Chapman *et al.*, 2007). De plus, lorsque la température de l'eau est inférieure de 4 à 8 °C au point d'écoulement de l'hydrocarbure, le pétrole se solidifie très rapidement, ce qui va rendre la dispersion impossible (Fingas, 2011). Il a été également observé des efficacités maximales pour des salinités de 35 à 30 psu et minimales de 10 à 1 psu (Blondina *et al.*, 1999 ; Kulekeyev *et al.*, 2014). La salinité de l'eau est un facteur important lorsqu'elle est comprise entre 0 et 10 psu, et devient un facteur mineur au-delà de 10 psu (Chandrasekar *et al.*, 2006 ; Pelletier, 2015). Ces caractéristiques des bitumes dilués entraînent de nombreux questionnements quant à l'efficacité des dispersants sur ce type de pétrole et, notamment, dans des conditions de faible température et salinité (Lunel and Davies, 2001; MPO, 2013).

Malgré tout, il existe encore peu d'études sur l'efficacité des dispersants chimiques sur les bitumes dilués ou les pétroles lourds et donc peu de connaissances sur leur efficacité pour une large gamme de salinités et de températures (Srinivasan *et al.*, 2007 ; Li *et al.*, 2010 ; MPO, 2013 ; Witt O'Briens, 2013 ; King *et al.*, 2015, 2018 ; Pan *et al.*, 2017). De façon générale, leur efficacité est prouvée pour des salinités entre 25-30 psu et des températures au-dessus de 5 °C. Cependant, King *et al.* (2018) soulignent que les effets saisonniers sur l'efficacité des dispersants sur le bitume dilué sont plus importants que pour le pétrole brut synthétique (synbit), dû au fait que le diluant dans le synbit est moins volatil que le condensat dans le bitume dilué. Il manque donc encore des connaissances sur l'efficacité des dispersants dans différentes conditions environnementales. De plus, la concentration d'hydrocarbures pétroliers totaux dans la colonne d'eau étant plus élevée lors du traitement avec le dispersant (King *et al.*, 2015), la potentielle augmentation de la toxicité provoquée par l'utilisation de dispersant lors d'un déversement fait toujours l'objet d'études.

UNE MENACE POUR LES PÊCHES

L'estuaire et le golfe du Saint-Laurent soutiennent des secteurs importants de l'économie comme les pêcheries et l'écotourisme. Parmi les principales espèces marines consommées au Canada, deux sont d'une grande importance économique : la moule bleue, *Mytilus edulis*, et le homard américain, *Homarus americanus*. En 2020, la production de moules bleues du Canada s'élevait à 16 985 tonnes, pour une valeur d'environ 30,7 millions de dollars, représentant la deuxième production de mollusques la plus importante au Canada (MPO, 2020b). Le homard américain, *Homarus americanus*, est une espèce de première importance pour l'est du Canada. La capture de homards au Canada s'élevait à 68 070 tonnes en 2020, pour une valeur d'environ 761 millions de dollars (MPO, 2020a). Des impacts sur ces organismes auraient donc des incidences très significatives sur l'économie canadienne. En effet, lors d'un déversement, le pétrole se disperse sous forme de gouttelettes dans la masse d'eau, voire sédimenter sous la forme de fractions vieillies et ainsi atteindre les organismes pélagiques, comme les larves de homard, et benthiques, comme les homards et les moules bleues.

Cependant, l'emplacement des ports du transport de pétrole brut, des oléoducs et de leurs expansions, des mesures d'intervention pouvant être déployées en cas de déversement, ainsi que l'emplacement des sites de pêches et de cultures des moules bleues (Figure 13) montrent bien l'importance d'acquérir des connaissances sur l'impact d'un déversement sur ces espèces, ainsi que les dommages possibles pour les pêcheurs.

Les déversements de pétrole entraînent des préjudices considérables pour les pêcheurs rendant les espèces exploitées impropres à la consommation, impactant leur couleur, leur odeur (Michel and Abarnou, 1978) et/ou leur goût (Connell, 1974). On considère que l'altération se produit lorsque l'organisme accumule suffisamment d'hydrocarbures pour provoquer une saveur ou une odeur incommodante (Michel and Fingas, 2016). Ces organismes sont alors impropres à la consommation jusqu'à ce que cette altération disparaisse. Mais le retour à la normale peut prendre de quelques jours à un an après

l'exposition, selon les espèces (Spaulding *et al.*, 1996). À titre d'exemple, la catastrophe de la plateforme Deepwater Horizon (2010) dans le golfe du Mexique a fortement affecté les pêcheries de crustacés menant à leur fermeture pendant plus de cinq ans (Gallucci, 2015).

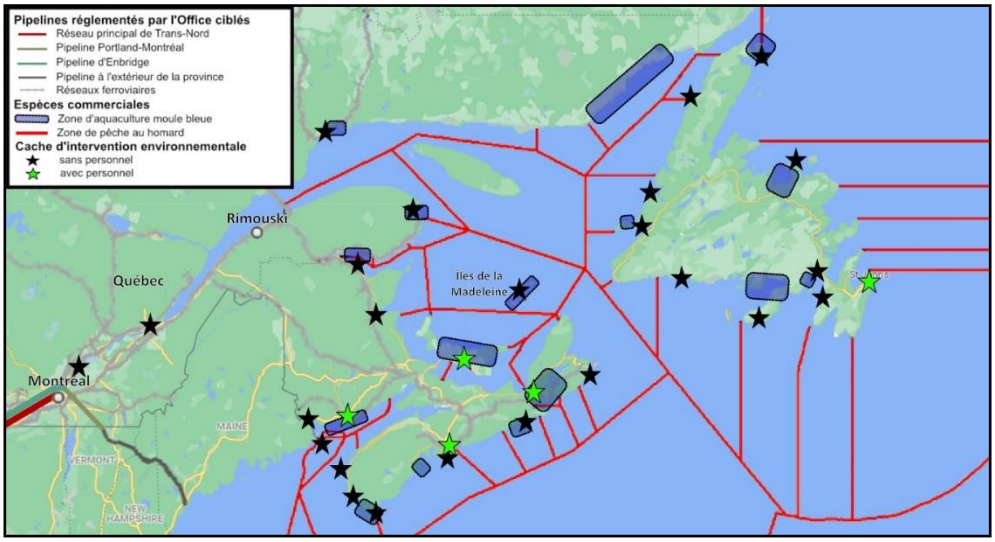

Figure 13 : Carte des transports pétroliers (oléoducs, réseaux ferroviaires), des zones de récolte des espèces commerciales (moule bleue et le homard américain) et des emplacements des sites de stockage des équipements d'intervention environnementale.
Répartition des zones de pêches du homard américain au Québec d'après MPO, 2015; zones d'élevage commercial de moules au Québec, d'après Cyr et al., 2015, et au Canada, d'après Nguyen and Williams, 2013; principaux oléoducs de pétrole brut au Québec, d'après Gouvernement du Canada, 2020; t emplacement d'équipements d'intervention spécialisés entreposés au Canada par la garde côtière canadienne, d'après Transports Canada, 2020.

Des molécules à risque

La toxicité d'un déversement de pétrole dépend de son vieillissement et ainsi que des composés qui vont atteindre la colonne d'eau. Lors d'un déversement les composés les plus volatils se perdent rapidement par évaporation (ITOPF, 2012), tandis que la dissolution rend les principaux composés solubles. Les hydrocarbures de faibles poids moléculaires, comme les monoaromatiques, les alcanes à chaîne courte ou les HAP de 2 à 3 cycles, peuvent atteindre rapidement après un déversement des concentrations importantes, mais diminuent avec la dilution et dispersion physique. Ils ne seront persistants que dans des conditions

météorologiques défavorables, comme une faible énergie, ou en cas de fuite de pétrole constante (Logan and Scott, 2015). Il n'est cependant pas impossible que ces composés rentrent en contact avec les organismes marins et soient dans ce cas responsable de la toxicité aiguë du déversement (Fingas and Punt, 2000; Dupuis and Ucan-Marin, 2015a; Philibert *et al.*, 2016), voire de mortalité.

La toxicité chronique est engendrée par les molécules les plus persistantes au processus de vieillissement, ainsi que leur capacité à se dissoudre dans la colonne d'eau. À titre d'exemple, le naphtalène (alcène à deux cycles) est considéré comme toxique pour les organismes aquatiques en raison de sa moindre sensibilité à la photooxydation et de son caractère persistant dans l'eau (Vijayavel *et al.*, 2004). Bien que peu de données soient disponibles à ce jour, la toxicité des résines est également soulevée étant solubles dans l'eau et résistantes à la biodégradation (Adams *et al.*, 2014 ; Cusson *et al.*, 2017). Cette toxicité est généralement associée à la présence des composés aromatiques, notamment les HAP alkylés, pour les espèces aquatiques (Fan *et al.*, 2002), car ils se séparent lentement du pétrole et deviennent plus biodisponibles pour une absorption passive par les branchies des organismes marins (Carls *et al.*, 2000).

Les composés aromatiques sont d'ailleurs considérés comme les hydrocarbures pétroliers les plus toxiques (Barron *et al.*, 1999), notamment par leur caractère cancérigène (Collins *et al.*, 1998 ; Kuo *et al.*, 1998 ; Rengarajan *et al.*, 2015 ; Devi *et al.*, 2016). Sur la base de cette toxicité, mais également de leur présence dans l'environnement, seize HAP sont classées comme polluants prioritaires par l'Agence de protection environnementale des États-Unis (US-EPA, 1982), dont 12 sont contrôlés par l'AFSCA dans l'alimentation animale (AFSCA, 2014) (Figure 14).

Les bitumes dilués ont également la particularité de contenir de plus fortes concentrations de métaux que les pétroles classiques. Cependant, leur toxicité résultant d'un déversement de dilbit n'est pas établie. Effectivement, de faibles concentrations en métaux ont été mesurées dans des eaux contaminées avec du bitume dilué, suggérant une faible bioaccumulation dans les tissus des moules (*Pyganodon grandis*) en raison d'une exposition

limitée (Séguin, 2021). De plus, la toxicité des dilbits CLB et AWB sur des poissons et invertébrés d'eau douce ne montre pas de corrélation avec les concentrations de métaux mesurés dans l'eau (Robidoux *et al.*, 2018).

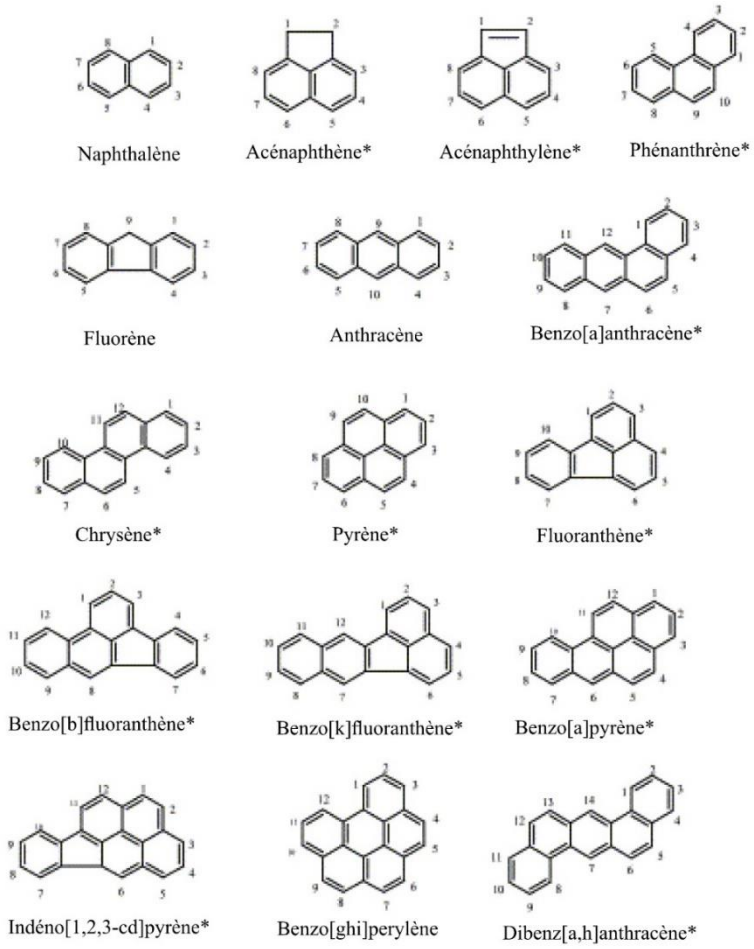

Figure 14 : Structures et nomenclatures des 16 HAP prioritaires pour l'US-EPA, dont les 12 HAP contrôlés par l'AFSCA (*; AFSCA, 2014)

Un risque accentué par le dispersant chimique ?

La toxicité et les effets à long terme des dispersants chimiques sont grandement débattus au sein de la communauté scientifique et font également l'objet de nombreuses préoccupations sociales (Bocard, 2006 ; Schrope, 2013 ; Prince, 2015). D'après certaines études, les dispersants sont reconnus comme étant peu toxiques, présentant des toxicités

semblables aux produits vaisselle courants (Parsons *et al.*, 1984 ; RUSSELL, 2003 ; Judson *et al.*, 2010 ; Word *et al.*, 2015). Les doses létales 50 % (DL50) mesurées par Fingas (2013) vont dans ce sens avec des valeurs comprises entre 200 à 400 mg/l, ce qui est dix fois moins toxique que la majorité des pétroles bruts. Cependant, la combinaison du pétrole avec le dispersant peut représenter une toxicité accrue, par rapport au pétrole seul. Tout d'abord parce que cette technique augmente la surface de contact entre l'eau et le pétrole et amplifie la diffusion des hydrocarbures solubles, notamment les HAP, mais aussi parce que les fines gouttelettes de pétrole sont plus facilement assimilables par les organismes via le système digestif, les branchies ou par contact dermique (CEAEQ, 2015c).

L'accident du pétrolier *Torrey Canyon*, en 1967, est le premier à attirer l'attention internationale sur les dangers liés à l'utilisation des dispersants. Lors de cet accident, environ 10 000 tonnes de dispersants sont utilisées sur les 121 000 tonnes de pétrole brut. Les résultats ont rapidement été reconnus comme désastreux sur le plan de la toxicité pour les organismes marins (Holme, 1969), ainsi qu'un temps de rétablissement bien plus long dans les zones où le dispersant fut utilisé par rapport aux zones sans (Hawkins *et al.*, 2017). Par la suite, lors de l'accident de la plateforme *Deepwater Horizon*, l'étude de Stefansson *et al.* (2016) a mis en évidence que l'impact du pétrole frais et vieilli n'était pas létal pour les larves d'échinodermes et de bivalves à moins d'être dispersées chimiquement. Plusieurs études ont également démontré que la toxicité du mélange pétrole/dispersant chimique est plus importante que celle du dispersant seul, mais aussi que celle du pétrole seul (Parsons *et al.*, 1984; Hemmer *et al.*, 2010; Hook and Osborn, 2012; Jung *et al.*, 2012; Rico-Martínez *et al.*, 2013; Almeda *et al.*, 2014; Anderson *et al.*, 2014; Fern *et al.*, 2015). Il a été démontré que l'addition de dispersant Corexit® 9500A après exposition à un pétrole léger (Louisiana sweet crude) induit une mortalité importante chez des crustacés (*Rhithropanopeus harrisii*) par rapport à une exposition au pétrole seul (Anderson *et al.*, 2014). Toutefois, les connaissances des impacts d'un déversement de bitume dilué traité aux dispersants chimiques sont encore faibles, en particulier pour les homards et les moules.

De l'eau vers l'organisme

Lors d'un déversement, les organismes benthiques sont exposés aux hydrocarbures présents sous forme dissoute, sous forme adsorbée et/ou complexée sur la matière organique dissoute et sous forme adsorbée sur les particules en suspension. Les moules bleues et les homards américains font partie des espèces les plus susceptibles d'accumuler les hydrocarbures de par leur mode de vie, même lorsque les concentrations sont faibles (Uthe and Musial, 1986 ; Stantec, 2012; Chalghmi, 2015). Une accumulation des composés pétroliers dans les chairs de mollusques et de crustacés (Stantec, 2012) a déjà été mesurée, même avec de faibles concentrations d'HAP dans les eaux, comme aux alentours de plates-formes gazières et pétrolières (Utvik, 1999 ; Durell *et al.*, 2006 ; Brooks *et al.*, 2011 ; Sundt *et al.*, 2011). Chez la moule, de fortes concentrations peuvent apparaître dans le tube digestif, en particulier dans la glande digestive ou le manteau, où les concentrations lipidiques ont tendance à être les plus élevées (Roberts, 1976 ; Moore *et al.*, 1984). D'ailleurs, son mode de vie sédentaire et son caractère sessile et filtreur en font de bons modèles reflétant la contamination des écosystèmes aquatiques (Meador *et al.*, 1995; Pêches et Océans Canada, 2003; Dupuis and Ucan-Marin, 2015a), à tel point que cette espèce est utilisée comme bioindicateur depuis le milieu des années 1970 dans le programme international *Mussel Watch Program*. Les moules bleues documentent par leur accumulation, le taux de contamination de la colonne d'eau et celui des sédiments (Smolders *et al.*, 2003). Elles sont même utilisées pour mesurer le taux de récupération après des accidents de marée noire (Loh *et al.*, 2017). Chez le homard, une bioaccumulation des contaminants est mesurable dans sa chaire, principalement dans son hépatopancréas (principal organe de détoxication), dont les concentrations varient selon la saison et le sexe (Canli and Furness, 1993a, 1993b; Barrento *et al.*, 2008). Les teneurs en métaux traces des pétroles peuvent être mesurées par ces deux espèces, dans la coquille de la moule bleue (Bellotto and Miekeley, 2007) ou dans la carapace des homards américains (Leblanc and Prince, 2012)..

La voie de pénétration la plus rapide des hydrocarbures pétroliers correspond à la simple absorption des polluants se trouvant dans l'eau de mer à travers la surface de contact

et de respiration (branchies, siphons respiratoires ; Fisher, 1995). En effet, une fois dissous, les hydrocarbures peuvent être directement absorbés au travers des branchies des moules ou des homards (Dupuis and Ucan-Marin, 2015a). Les branchies, ou cténidies, fonctionnent comme des tamis, permettant de concentrer des particules de tailles spécifiques. L'oxygène capté pénètre dans l'hémolymphe pour être distribué dans tout l'organisme. La taille moyenne des gouttelettes de dilbits formées lors de déversement en laboratoire, varie selon le dilbit et les conditions environnementales, pouvant être de 414 µm et 336 µm à 8 °C (28 ppt), respectivement pour le AWB et CLB (Zhao *et al.*, 2014) et de 30 et 70 µm pour une température plus élevée de 15 °C (Zhao *et al.*, 2014). Ceci suggère que la bioaccumulation est dépendante de la saison, non seulement pour la condition physiologique de l'organisme, mais également du comportement de la nappe de pétrole.

Chez le homard américain, l'absorption des contaminants peut également se produire lors de la mue du homard qui absorbe alors une grande quantité d'eau pour augmenter de volume de 15 à 20 % plus grands qu'avant et d'environ 40 à 50 % de son poids. Il peut muer 4 à 5 fois au début de sa vie benthique, puis une fois par année une fois adulte (Paille and Bourassa, 2020). De plus, la consommation d'organismes filtreurs, comme les moules bleues, est une source de contamination chez les homards. En effet, les composés adsorbés par la moule peuvent être incorporés par désorption-absorption à l'intérieur du système digestif des crustacés au cours de l'alimentation.

Des impacts sur nos espèces

Le principal mécanisme à l'origine de la toxicité des hydrocarbures aromatiques polycycliques est leur liaison directe aux sites hydrophobes des macromolécules, provoquant ainsi des perturbations de leur fonctionnement (Molven and Goksøyr, 1993). Les hydrocarbures possèdent un caractère lipophile et hydrophobe, qui leur confère la double capacité de franchir aisément l'ensemble des membranes cellulaires (épithélium intestinal, paroi vasculaire, membrane des cellules des organes cibles, épithélium des tubules rénaux…) et d'être éventuellement stockés dans les structures lipidiques (Chalghmi, 2015).

Une fois que les composés ont pénétré dans les cellules, ils induisent l'expression génique d'un groupe enzymatique du cytochrome P450 (CYP) (Jacob, 2008 ; Jorgensen *et al.*, 2008 ; Bekki *et al.*, 2013 ; Ikenaka *et al.*, 2013). Certains métabolites, produitspar les enzymes CYP, peuvent contribuer à la production d'espèces réactives d'oxygènes (ROS ; Di Giulio *et al.*, 1995 ; Regoli and Giuliani, 2014) et ainsi à un stress oxydant, ou bien interagir directement avec les composants cellulaires tels que l'ADN de par des caractéristiques électrophiles (Peters and Livingstone, 2001), pour former des adduits ou générer des cassures et provoquer des impacts à plus long terme (Akcha *et al.*, 2000a, 2000b). Les hydrocarbures s'accumulent au sein de l'organisme avant d'être excrétés, dépendamment de la capacité des organismes contaminés à métaboliser les composés.

Un déversement de pétrole peut avoir des effets indirects comme des changements comportementaux tels qu'une augmentation du délai entre la perception de la nourriture et sa poursuite (Atema and Stein, 1972; Gibson *et al.*, 1997; French, 1999; Felder *et al.*, 2014), un comportement désorienté ou des mouvements non coordonnés (Atema and Stein, 1974). Différentes études ont également mis en évidence l'apparition de pathologies, ainsi que des lésions des organes et des tissus (Aarab *et al.*, 2011 ; Ruiz *et al.*, 2011), comme l'apparition de tumeurs chez les moules (Cosson-Mannevy *et al.*, 1984), lors d'exposition aux HAP. Chez les crustacés, des dommages aux téguments peuvent apparaître, compromettant ainsi l'intégrité de leur épicuticule, qui sert de barrière physique entre l'environnement et les couches internes plus perméables de l'exosquelette (Spaulding *et al.*, 1996 ; Cobb *et al.*, 1999), ainsi qu'un ralentissement de la croissance et de la mue (Wang and Stickle, 1988; Giltz and Taylor, 2017; Barron *et al.*, 2018). Des impacts à plus long terme sont également mesurés, comme l'altération des processus bioénergétiques (Widdows *et al.*, 1997; McDowell *et al.*, 1999; Toro *et al.*, 2003; Peteiro *et al.*, 2007; Sureda *et al.*, 2011; Gonzalez-Fernandez *et al.*, 2016; Redmond *et al.*, 2016), l'induction de l'activité enzymatique (Mcdonald *et al.*, 1996), un stress oxydatif (Vijayavel *et al.*, 2004; Beyer *et al.*, 2017), de l'immunosuppression (Widdows *et al.*, 1982), de la génotoxicité et de la cytotoxicité (Pérez-Cadahía *et al.*, 2004) comme la perte de la stabilité lysosomale (Moore and Viarengo, 1987). Des effets néfastes sur la reproduction étaient relevés comme l'altération des gonades (Ortiz-

Zarragoitia and Cajaraville, 2006; Aarab *et al.*, 2011; Baussant *et al.*, 2011; Ortiz-Zarragoitia *et al.*, 2011; Ruiz *et al.*, 2011; Gonzalez-Fernandez *et al.*, 2016), une diminution du métabolisme, le développement ou la survie des larves (Blumer *et al.*, 1970; Toro *et al.*, 2003; Labarta *et al.*, 2005), ainsi qu'une perturbation endocrinienne (Vijayavel *et al.*, 2004; Ortiz-Zarragoitia and Cajaraville, 2006). Des impacts létaux ont été observés lors de déversement de pétrole chez les deux espèces étudiées. Après la marée noire de North Cape en 1996, une perte de 9 millions de homards a été répertoriée (Spaulding *et al.*, 1996).

Les données sur la toxicité spécifique des bitumes dilués sont limitées. Quelques études ont mis en évidence la toxicité des bitumes dilués, des extraits de bitume ou de l'eau de traitement de la région des sables bitumineux du Canada, mais essentiellement sur des espèces de poissons (Colavecchia *et al.*, 2004 ; Madison *et al.*, 2015, 2017, 2020 ; Alharbi *et al.*, 2016 ; Philibert *et al.*, 2016 ; Alderman *et al.*, 2017a, 2017b ; Bauer *et al.*, 2017 ; Barron *et al.*, 2018, 2021 ; Berube *et al.*, 2021).

Il existe encore très peu d'études publiées à ce jour de leur toxicité sur les crustacés ou les mollusques en milieu marin (Fingas, 2013 ; Barron *et al.*, 2018 ; Rhodenizer, 2019 ; Schmutz *et al.*, 2021). Pourtant, en plus de filtrer de grande quantité d'eau impactée, la bioaccumulation serait favorisée chez la moule et le homard, par un temps de demi-vie des HAP nettement plus long par rapport aux poissons (Stegeman and Lech, 1991; Meador *et al.*, 1995), ainsi qu'une vitesse de métabolisation plus lente (Seiser *et al.*, 2000).

DES ESPÈCES EN DÉFENSE

Lors d'une contamination importante, les homards peuvent fuir le lieu contaminé grâce à des petites antennes, organes sensoriels, qui permettent de percevoir différents signaux chimiques dans l'eau et donnent ainsi un signal de fuite. Ils peuvent également se protéger des xénobiotiques via leur excrétion dans la carapace, notamment via les métallothionéines (Chavez-Crooker *et al.*, 2003; Leblanc and Prince, 2012), permettant aux mues successives de jouer un rôle dans la détoxification (Ahearn *et al.*, 2004). Même si les moules bleues ne pourront pas fuir leur milieu environnant, elles vont pouvoir s'isoler de l'eau de mer ambiante en fermant leurs coquilles, au cours desquelles elles survivent en utilisant des processus métaboliques qui n'utilisent pas d'oxygène ou en réduisant leur taux de filtration. Mais chez ces deux espèces, un liquide circulatoire propulsé par le cœur vers les tissus afin d'assurer leur oxygénation et les échanges de nutriments, joue également un rôle de défense contre les xénobiotiques, l'hémolymphe.

L'hémolymphe

Dans sa fonction de défense, deux stratégies au niveau de la cellule hémocytaire ont été observées. La première consiste en une séquestration du contaminant, puis une élimination par voie rénale au niveau de la glande péricardique par un processus d'ultrafiltration. La seconde stratégie de défense vise à limiter l'accumulation des contaminants dans les membranes par des systèmes enzymatiques permettant l'élimination des xénobiotiques. Cette stratégie, appelée biotransformation, vise à limiter l'accumulation des contaminants dans les membranes grâce à des systèmes enzymatiques permettant l'élimination des xénobiotiques, mettant en jeu différentes enzymes de biotransformation et des transporteurs (Figure 15).

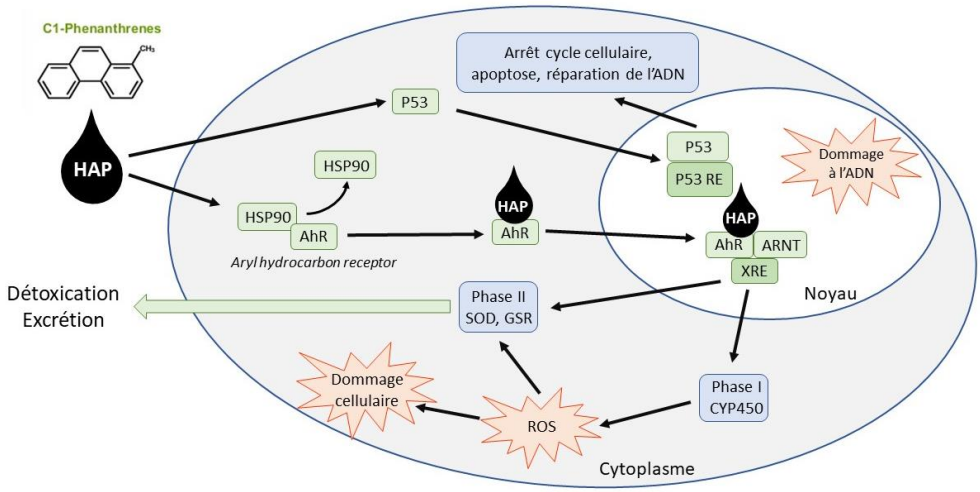

Figure 15 : Mécanisme de détoxication des HAP. Figure modifiée d'après McDonnell, 2017

Ces systèmes enzymatiques, regroupés sous le terme d'Enzymes du Métabolisme des Xénobiotiques (EMX), prennent en charge les xénobiotiques dans le but d'augmenter leur hydrosolubilité afin de faciliter leur excrétion dans les fluides biologiques. Ces processus de biotransformation se déroulent principalement au niveau des glandes antennaires, de l'hépatopancréas et de l'estomac chez les crustacés (Singer and Lee, 1977; Singer *et al.*, 1980) et de la glande digestive chez les mollusques (Livingstone *et al.*, 1989). La biotransformation des hydrocarbures s'effectue en trois phases que sont la fonctionnarisation (phase I), la conjugaison (phase II) et l'excrétion (phase III).

Lorsque les HAP atteignent la membrane cellulaire, ils se lient à des récepteurs d'aryle hydrocarbone (AhR), pour ensuite s'associer avec la protéine ARNt (*Aryl hydrocarbon receptor nuclear translocator*) une fois dans le noyau. Dans cette phase une enzyme monoxygénase à cytochrome P450, l'éthoxyrésorufine-O-dééthylase (EROD), permet la biotransformation des HAP en composés hydrosolubles (Milinkovitch, 2011), par ajout d'une nouvelle fonction chimique (-OH, -NH_2, -COOH).

Cette phase d'oxydation est la phase I (Zanette *et al.*, 2013). Ensuite (phase II), l'ajout d'un radical hydrophile sur la molécule issue de la fonctionnarisation en phase I, voire initiale, permet de diminuer leur toxicité et d'augmenter leur solubilité afin de faciliter leur transport. Les xénobiotiques sont alors véhiculés vers l'extérieur de la cellule (phase III) par

des protéines membranaires de transport composées de protéines membranaires de résistance aux xénobiotiques (« *Multixenobiotic resistance* » MXR). Dans le cas où la toxicité est trop importante et que les dommages de l'ADN sont irréparables, la cellule déclenche un mécanisme de mort cellulaire programmée (MCP), aussi appelé apoptose.

La dépuration selon les saisons

La vitesse de dépuration est fonction de multiples facteurs comme le type d'hydrocarbures, mais également la température de l'eau et le taux de filtration pour les bivalves (Michel and Fingas, 2016). Effectivement, les défenses varient selon les saisons, étant donné que les paramètres hémocytaires sont en lien avec le statut reproducteur. Chez la moule bleue, une baisse du phénomène de phagocytose est observée au moment de la ponte (Cartier *et al.*, 2004). Chez le homard, les mues sont plus fréquentes dans les eaux plus chaudes (Comeau and Savoie, 2001), ce qui influe sur la dépuration (Little *et al.*, 1985). De plus, en hiver le métabolisme de ces deux espèces est ralenti, avec des temps de fermeture plus longs chez la moule bleue (Hatcher *et al.*, 1997 ; Cusson *et al.*, 2005 ; Pernet *et al.*, 2007). À l'inverse, une « mortalité estivale » est observée pour les moules bleues (Myrand *et al.*, 2000), caractérisée par un budget énergétique négatif (Soletchnik *et al.*, 1997; Delaporte *et al.*, 2006; Samain and McCombie, 2008).

PROBLÉMATIQUE

C'est dans un contexte d'expansion de l'importation des bitumes dilués vers les marchés internationaux et du manque de connaissance des conséquences d'un déversement d'un tel pétrole que s'inscrit ce projet d'étude. L'impact des produits pétroliers a été démontré dans de précédentes études suivant des déversements de pétroles et/ou en recréant cette contamination en laboratoire. Le transfert de composés, notamment des hydrocarbures aromatiques polycycliques, du milieu environnant jusqu'aux cellules a été expliqué chez les organismes marins, et cause notamment des dommages à l'ADN.

Mais la particularité des pétroles non classiques extraient en Alberta, est le mélange de bitume avec un pourcentage de condensats, dont la composition exacte est inconnue. Or le niveau de toxicité d'un déversement dépend de la composition du pétrole, qui va influencer son vieillissement et donc les concentrations et composés retrouvés dans la colonne d'eau pouvant être bioaccumulés. Mais malgré une augmentation rapide de sa production, l'apport de connaissances pour intervenir au mieux en cas de déversement reste encore nécessaire.

De plus, la décision de la méthode d'intervention doit être prise le plus rapidement possible, avant la modification de la nappe de pétrole au cours du processus de vieillissement. Ces caractéristiques soulèvent notamment des questions sur l'utilisation de dispersant chimique en cas de déversement. Effectivement, sa densité diminuée par l'ajout de condensat devrait réaugmenter rapidement lors d'un déversement dû à l'évaporation rapide des composés les plus légers, dont ceux présents dans les condensats.

Le choix de l'intervention, en plus de son efficacité, dépend des impacts sur l'environnement qui en découleront. Les impacts d'un déversement de bitumes dilués, étudiés sur les organismes d'eau douce sont encore très méconnus sur les organismes marins. Or, ces données permettent d'évaluer les conséquences en fonction de la quantité déversée, ainsi que les effets à court, mais également à long terme. Si l'ajout de dispersant chimique est utilisé comme méthode d'intervention, ces mêmes questions se poseront.

Des études précédentes était d'ailleurs démontré des impacts accrus lors de l'utilisation de dispersant sur du pétrole classique, par rapport à une dispersion physique. Pourtant il existe encore trop peu de données sur les impacts d'un déversement de bitume dilué sur les moules bleues, et aucune sur le homard américain.

Des impacts trop importants et pouvant affecter les générations suivantes sur les moules bleues et les homards américains sont inquiétants pour l'économie du Québec. Cependant les organismes marins ont la capacité de se dépurer des xénobiotiques, mais qu'en est-il lors d'une contamination au bitume dilué ? Des connaissances sur leur capacité de se rétablir après un déversement de bitume dilué, autant de l'état cellulaire génétique que la vitesse, sont nécessaires pour la gestion des stocks.

OBJECTIFS

L'objectif général de cette étude consistait à déterminer le comportement et l'impact d'un déversement de deux bitumes dilués, Access Western Blend (AWB) et Cold Lake Blend (CLB) dans le fleuve du Saint-Laurent, sur deux espèces commerciales importantes : la moule bleue (*Mytilus edulis*) et le homard américain (*Homarus americanus*), ainsi que leur potentiel de rétablissement.

Le premier objectif de recherche visait à déterminer l'efficacité de deux dispersants à différentes températures et salinités sur les dilbits AWB et CLB. À la vue de la densité de ces pétroles, notre hypothèse était que l'efficacité du dispersant est fortement dépendante de la température et de la salinité de l'eau, voir devenir inefficace aux plus basses conditions. Afin de vérifier cette hypothèse, nous avons quantifié l'efficacité des dispersants par le test IFP (Institut Français du Pétrole) au centre de documentation, de recherche et d'expérimentations sur les pollutions accidentelles des eaux (CEDRE) à Brest, en France, ainsi que la taille moyenne des gouttelettes générées par le dispersant. Afin de visualiser l'efficacité globale, un indice d'efficacité du dispersant est proposé dans cette étude.

Le second objectif consistait à évaluer les impacts sous-létaux chez la moule bleue (*M. edulis*) lors d'un déversement de ces deux bitumes dilués dispersés physiquement et chimiquement à différentes saisons. Ces données ont été comparées aux impacts d'un déversement de pétrole classique, selon les mêmes modes de dispersion et des conditions saisonnières. Et enfin, ces mêmes conditions sont testées avec du dispersant seul, afin de comparer avec les impacts mesurés lors d'une dispersion chimique. Nos hypothèses étaient que l'utilisation de dispersant chimique augmente le niveau d'impact sur les moules bleues, que le bitume dilué est plus toxique que le pétrole classique, et que la capacité des moules à gérer cette contamination est dépendante de son état physiologique, et donc de la saison.

Le troisième objectif permettait de vérifier la capacité des moules bleues à se rétablir suite à un déversement d'un bitume dilué, c'est-à-dire à revenir à des dommages observés chez des moules contrôles (non exposées). Connaissant la génotoxicité des hydrocarbures

aromatiques polycycliques, la capacité de dépuration des moules bleues reste incertaine face à ce type de pollution. Notre hypothèse est que les dommages à l'ADN persistent même après une restitution des moules bleues dans des eaux propres.

Pour finir, un quatrième objectif était de vérifier l'utilisation des biomarqueurs validés sur l'hémolymphe de la moule bleue, sur l'hémolymphe du homard américain. Notre hypothèse est que les tests effectués sur l'hémolymphe de moules bleues sont transposables sur une autre espèce, comme le homard. De plus, le manque important de connaissance des impacts des produits pétroliers sur cette espèce d'une importance économique non négligeable est nécessaire. Il est possible que, comme la moule bleue, le homard américain puisse être utilisé comme bioindicateur de son milieu de vie via l'hémolymphe.

Ce projet apporte donc de nouvelles données originales sur l'impact des bitumes dilués sur deux espèces de forts intérêts commerciales, ainsi que sur leur capacité de rétablissement suite à une exposition de ce type de pétrole. De par l'utilisation de l'hémolymphe pour tous nos tests cellulaires, notre étude permet de vérifier l'intérêt de l'utilisation de ponction, permettant de garder l'individu vivant et ainsi faire un suivi plus précis avec les mêmes organismes. De plus, ce projet apporte également des éléments de réponses pour permettre au Gouvernement du Canada d'évaluer la possibilité de recourir aux dispersants chimiques dans les milieux aquatiques lors d'un déversement de pétroles non classiques.

CHAPITRE 1
UN INDICE D'EFFICACITE DE LA DISPERSION CHIMIQUE POUR COMPARER DEUX FORMULATIONS COMMERCIALES DE DISPERSANT SUR DU BITUME DILUE

1.1 RÉSUMÉ

Pour augmenter de manière responsable la production de bitume dilué (dilbit) et son transport, une solide connaissance des méthodes d'intervention en cas de déversement est requise, dont la dispersion chimique. En effet, il existe encore peu d'études sur l'efficacité des dispersants chimiques sur les dilbits pour une large gamme de températures et de salinités. Cette première étude a porté sur l'efficacité de deux dispersants, Corexit® 9500A (CXT) et Finasol® OSR52 (FIN), sur deux dilbits, Access Western Blend (AWB) et Cold Lake Blend (CLB). L'efficacité du dispersant (DE) à disperser les dilbits a été mesurée à l'aide du test IFP, la détermination de la taille moyenne des particules (APS) formées et leur distribution avec un granulomètre laser. Les essais, réalisés à trois températures et salinités, permettent de mesurer l'indice d'efficacité du dispersant (DEI). Les résultats montrent que l'efficacité de la dispersion dépend de la salinité et de la température, mais aussi des caractéristiques chimiques du pétrole et de celles du dispersant utilisé. Les tests d'efficacité IFP montrent que FIN est plus efficace sur les dilbits que CXT dans les conditions de basses températures et salinités, mais la taille des particules est plus grande lorsqu'elle est dispersée avec FIN que CXT. Contrairement à CXT, nous n'observons pas de corrélation entre DE et APS avec FIN. Effectivement, les résultats IFP montrent que le FIN a une meilleure efficacité sur les dilbits que le CXT, dans les conditions les plus basses, mais la taille des particules est plus grande lorsqu'elle est dispersée avec le FIN que le CXT. Ainsi, en tenant compte des différents paramètres, le DEI permet de déterminer l'efficacité globale du dispersant.

Cette étude fait l'objet d'un article, intitulé « A dispersant effectiveness index to compare two commercial formulations of dispersant on diluted bitumen ». Il a été soumis le 27 mars 2022 dans la revue Colloids and Surfaces A : Physicochemical and Engineering Aspects et est encore en révision. En tant que première auteure, ma contribution à ce travail fut la collection des données, la recherche sur l'état de l'art, le développement de la méthode, la réalisation des analyses, l'analyse et l'interprétation des résultats et la rédaction de l'article. Stéphane Le Floch, second auteur, et Richard Saint-Louis, directeur de recherche, ont fourni l'idée originale, aidé à la recherche sur l'état de la question, au développement de la méthode ainsi qu'à la révision de l'article. Gaëlle Triffault-Bouchet, troisième auteure et co-directrice de recherche, a également contribué à la révision de l'article.

Une version abrégée de l'article a été présentée avec une partie des résultats du chapitre 2 en mai 2018, lors du 14ème colloque international en écotoxicologie aquatique (EcoBIM), à Bordeaux (France) « *Cytotoxicité et génotoxicité du pétrole non classique (dilbit), dispersé physiquement et chimiquement en milieu marin, sur la moule bleue, Mytilus edulis* » C. Berthod, N. Lemaire, J. P. Gagné, C. Audet, R. Tremblay, S. Le Floch, G. Triffault-Bouchet et R. Saint-Louis, récompensé par le prix IFQM de la meilleure présentation orale.

1.1 A DISPERSANT EFFECTIVENESS INDEX TO COMPARE TWO COMMERCIAL FORMULATIONS OF CHEMICAL DISPERSANT ON DILUTED BITUMEN

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1.2 ABSTRACT

To responsibly increase the production of diluted bitumen (dilbit) and its transport, strong knowledge of spill response methods is required. However, there are still few studies on the effectiveness of different dispersants on dilbits over a wide range of temperatures and salinities. Our study proposed a dispersant effectiveness index (DEI) to improve knowledge of spill response methods on dilbit, using the IFP assay (dispersant effectiveness; DE) and a laser particle size analyzer (average particle size; APS). Two dispersants, Corexit® 9500A (CXT) and Finasol® OSR52 (FIN), were tested on two dilbits, Access Western Blend (AWB) and Cold Lake Blend (CLB). Our results show, additionally to new data on dispersant effectiveness, that DE and APS were not always correlated. So, for good efficiency measured with IFP, the APS was above the threshold at which droplets are considered dispersed. IFP shows that FIN has a better effectiveness on dilbits than CXT at the lowest conditions, but the particle size is larger when dispersed with FIN than CXT. The DEI was dependent on salinity and temperature, but also on the oil and dispersant composition, and so was important in order to determine the overall effectiveness of the dispersant, taking into account the different parameters.

Keywords : Dispersant, Bitumen-dilbit, Dispersant effectiveness index, IFP, Particle size

1.3 INTRODUCTION

The bitumen industry increases to response of the demand for petroleum hydrocarbons and the first bitumen reserves is in Canada (~ 46%; Hein, 2017), from where two dominant blends are transported by pipeline in high volumes across Canada: Access Western Blend (AWB) and Cold Lake Blend (CLB) (MPO, 2013; King *et al.*, 2014). Canada is a major exporter of crude oil, primarily to the USA., but the export of oils to Europe is increasing (RNCan, 2020a). For transportation purposes, 20 to 30% of lighter hydrocarbons called diluents are added to the bitumen to reduce its viscosity and density (Crosby *et al.*, 2013). In case of oil spill, the dispersant is one of several possible at-sea response techniques. It's a complex mixture of one or more surfactant with a solvent, often a hydrocarbon solvent (oil fraction), and other minor components for instance to improve its conservation and spraying (Merlin, 2015). The main purpose of a dispersant is to transfer the oil slick from the water surface into the water column by reducing the oil-water surface tension, and so create micro-droplets (< 70 µm) surrounded by dispersant, which will be partially degraded by marine micro-organisms (Clayton *et al.*, 1993). The most widely used and benchmarked chemical dispersant is the Corexit® class of dispersants, particularly EC9500A (John *et al.*, 2016), which was used during the Deepwater Horizon oil spill in the Gulf of Mexico in 2010. However certain chemical dispersant formulations, such Finasol® OSR52, have seen increased domestic interest in North America and are now included in products approved for oil spill response and mitigation in the U.S. Environmental Protection Agency's National Contingency Plan (EPA-NCP; US-EPA, 2021b). Corexit® 9500A and Finasol® OSR52 are used on many oils (George-Ares *et al.*, 2001) and represent, respectively, a large portion of the United States' and Europe's stockpiles (Steffek, 2015).

Several studies have demonstrated that chemical dispersant effectiveness depends on numerous factors, such as the water salinity, temperature, suspended mineral particles, sea energy, oil composition, and dispersant formulation (Blondina *et al.*, 1999; George-Ares *et al.*, 2001; Guyomarch *et al.*, 2002; Le Floch *et al.*, 2002; Chandrasekar *et al.*, 2006; Fingas, 2014; IPIECA-IOGP, 2014a; Tansel *et al.*, 2014; King *et al.*, 2018). The salinity is an important factor when it is between 0 and 10 psu, and becomes a minor factor beyond 10 psu

(Chandrasekar *et al.*, 2006; Pelletier, 2015). Indeed, the effectiveness of Corexit® 9500A and Finasol® OSR 52 dispersants demonstrate a maximum effectiveness at salinities of 35 to 30 psu and a minimum of 10 to 1 psu (Blondina *et al.*, 1999; Kulekeyev *et al.*, 2014). In addition to salinity, temperature also influences dispersion effectiveness, as it is related to oil viscosity (Fingas, 2014; IPIECA-IOGP, 2014a; Ufford *et al.*, 2014; Pelletier, 2015). In warmer water conditions (> 10°C), the dispersion efficacy of Corexit® 9500A is greater than in colder water (< 10°C; O'Laughlin *et al.*, 2020). A low temperature will significantly reduce chemical dispersant effectiveness due to an increase in oil viscosity (Stevens and Roberts, 2003; Lewis, 2004; Colcomb *et al.*, 2005; Holder, 2011), and lower salinities, due to seasonal melting or a river or estuary environment, will also decrease dispersion. Lewis (2004) reported that a 2000cP oil treated with Corexit® 9500A dispersed quickly and completely, but a more viscous 7000cP fuel oil did not.

The effectiveness of Corexit® 9500A on conventional crude oils has already been proven (Blondina *et al.*, 1999; George-Ares *et al.*, 2001; Moles *et al.*, 2002; Nedwed *et al.*, 2008; Belore *et al.*, 2009; Kulekeyev *et al.*, 2014; Tansel *et al.*, 2014; Steffek, 2015), and the weaker performance of the Finasol® OSR 52 dispersant (Kulekeyev *et al.*, 2014; Steffek, 2015). However, there are very few studies on the effectiveness of chemical dispersants on dilbits, despite their high viscosity (MPO, 2013; Fieldhouse *et al.*, 2014; King *et al.*, 2015, 2018; Pan *et al.*, 2017). These studies demonstrate the effectiveness of chemical dispersants on diluted bitumen depending on environmental conditions, such as temperature (between 3.7 and 19.7°C) or energy. King *et al*. (2018) underlined that the seasonal effects on chemical dispersant effectiveness (DE) for dilbit were greater than for synthetic crude oil (synbit). This is most likely due to the fact that the diluent portion in synbit is less volatile than the condensate in dilbit. Furthermore, the blending process for crude bitumen is at the discretion of the oil producer, so a wide variety of oil products of varying chemical composition is produced (King *et al.*, 2017). The properties of the spilled oil (such as viscosity, density, and chemistry) can significantly affect dispersant effectiveness and droplet-size distribution (Fingas *et al.*, 1991; Mukherjee *et al.*, 2011). The density of CLB increases at a slower rate compared to AWB, due to its high concentration of alkylated polycyclic aromatic hydrocarbons, which are more resistant to weathering (King *et al.*, 2014).

The concentration of the saturates, aromatics, resins, and asphaltenes (SARA) fractions, different according to the oil, have a particularly high impact (Mukherjee *et al.*, 2011; King *et al.*, 2018). Therefore, the dispersant effectiveness could be not the same between two diluted bitumen. A report by the Government of Canada (2013) and the studies by King *et al*. (2015; 2018) have observed different dispersion effectiveness and particles size between the two dilbits, AWB and CLB.

Dispersants are known to has low toxicity (Judson *et al.*, 2010; Word *et al.*, 2015), but it can increase with the combination with oil. First of all because this technique increases the contact surface between water and oil, and amplifies the diffusion of soluble hydrocarbons, in particular PAHs, but also because oil droplets are more easily assimilated by organisms (via the digestive system, the gills, by dermal contact; CEAEQ, 2015c). Small droplets will tend to remain in the water column or sink, while large droplets will resurface behind the slick. Oil droplets in a water column are usually sub-millimetre in size, typically ranging from about 10 µm to 200 µm, and a median size about 70 µm (Tan and Yao, 2001). Several studies have shown that the toxicity of the mixture is greater than the dispersant alone but also than the oil alone (Hemmer *et al.*, 2010; Almeda *et al.*, 2014; Anderson *et al.*, 2014; Fern *et al.*, 2015; Lara-Jacobo *et al.*, 2019), and appears to be influenced by particle size (Bobra *et al.*, 1989).

Decision making on the use of chemical dispersant is dependent on oil composition and environmental conditions, but also on the resulting impacts on the marine environment, influenced by the size of the droplets. However, previous works so far have focused on either droplet size or dispersion effectiveness. To our knowledge, there is no study proposing an index cumulating different measures to more precisely assess the effectiveness of a chemical dispersant. To fulfill this gap, our study proposes a dispersant effectiveness index to evaluate and compare the dispersion effectiveness of two chemical dispersants (Corexit® 9500A and Finasol® OSR 52) on two dilbits (Access Western Blend and Cold Lake Blend), over a range of salinities (5, 10, and 35 psu) and temperatures (0, 5, and 15°C), based on the French standardized IFP test and a laser particle size analyzer. These data will better inform decision makers in the event of a spill if the use of a chemical dispersant is considered, as well as the impacts associated with the size of dispersed oil's droplets.

1.4 MATERIALS AND METHODS

1.4.1 Sample Collection

The Access Western Blend (AWB) and Cold Lake Blend (CLB) dilbits were supplied by Thomas L. King's team, from Fisheries and Oceans Canada (winter blend containing ≃ 30% diluent). The AWB and CLB oils were classified as heavy crude oils by the American Petroleum Institute (API) value, respectively 21.7° and 20.9° (Table 3).

Table 3 : Physical properties and chemical composition of the two fresh dilbits (MPO, 2013)

	CLB	AWB
API	20.9	21
Density at 15°C (g/ml)	0.9249	0.9253
Density at 0°C (g/ml)	0.9376	0.9399
Viscosity at 15°C (cP)	285	347
Viscosity at 0°C (cP)	803	1,300
Saturate (%)	~45	~39
Aromatic (%)	~30	~30
Resin (%)	~12	~17
Asphaltene (%)	~13	~14

Corexit® 9500A (CXT) and Finasol® OSR 52 (FIN), supplied by CEDRE, are two third-generation concentrate marine dispersants, appear on the lists of dispersants approved by CEDRE and the US EPA (Cedre, 2018; US-EPA, 2021b) for oil spill response (Table 4).

Table 4 : Dispersant composition according to material safety data sheet of Corexit® 9500A (Nalco) and Finasol® OSR 52 (Total). * Data from Place et al. (2016)

Chemical composition	Corexit® 9500A (%)	Finasol® OSR 52 (%)
Docusate sodium	-	20–25
Hydrocarbons, C_{11}-C_{14}, n-alkanes, isoalkanes, cyclics, < 2 % aromatics	-	15–20
Carboxylic acids, di, C_6-C_{12} compounds with ethanolamine, boric acid compounds with ethanolamine		0–2

Chemical composition	Corexit® 9500A (%)	Finasol® OSR 52 (%)
Distillates (petroleum), hydrotreated light	10–30	-
(2-methoxymethylethoxy) propanol	-	15–20
2-aminoethanol	-	0–1
Nonionic surfactants	4,4 (Span80)* 18 (Tween80)* 4,6 (Tween 85)*	> 30
Anionic surfactants	18 (DOSS)*	15–30
Organic sulfonic acid salt	12	16

1.4.2 Experimental Conditions

The dispersant effectiveness was tested in a controlled temperature room at 0, 5, and 15°C. Instant Ocean Sea Salt (Aquarium Systems) and water were used to prepare the stock solutions at different salinity levels (5, 10, and 35 psu).

1.4.3 IFP Assay

The dispersibility of unweathered dilbit was measured with an approval laboratory test at low energy (2−5 m/s wind speed), the French IFP (Institut Français du Pétrole), agrees with the AFNOR NF T 90–345 standards (1990). Given that this study focuses on the dispersibility of two dilbits in cold waters and low energy conditions, i.e. a scenario involving a spill in Arctic ice-infested waters, the French IFP test was chosen. It appears to be well suited since it was designed to simulate offshore chemical dispersion as realistically as possible (Brandvik *et al.*, 2010; Ufford *et al.*, 2014). The IFP protocol used follow the methods of Chever *et al*. (2016). Briefly, each condition was tested in a 4.5 l vessel of synthetic sea water. Approximately 4 g of dilbit was deposited on the water surface inside a horizontal ring. Dispersant was evenly distributed on the oil with a syringe at a ratio of 1:20; the ratio recommended by the manufacturer. Energy was supplied by a flat ring agitator

periodically beating just under the water surface at a frequency of 15 cycles/min, used to transmit energy into the water column. The transmitted mixing energy was thought to be representative of low wave energies (2–5 m/s wind speed) for open sea conditions (Brandvik *et al.*, 2010). The ring was moved up and down by an electromagnet controlled by an electronic timer. For 1 hour, the outflow was recovered by supplying water at a surface inlet and an overflow pipe at the bottom of the vessel at 2.5 $l.h^{-1}$. The quantity of dispersed oil was determined by extraction from the sample, and measured by spectrophotometer at 580 nm. The concentration of emulsified oil follows the equation:

$$x = x_0 e^{-Dt},$$

where: x = oil concentration at time *t*; x_0 = initial oil concentration; D = dilution rate. The percentage of washed-out oil P at time *t* is:

$$P = 100\ (1 - x/\ x_0) = 100\ (1 - e^{-Dt}),$$

The experimental percentage of washed-out oil (Pa) by dispersant application follows the equation:

$$Pa = (100 * m_1)/m_0,$$

Where: m_0 = initial quantity of oil; m_1= quantity of oil determined by extraction from the sample. The dispersant effectiveness (DE) (%) is obtained by using this equation:

$$DE = 100\ Pa/P$$

The dispersant effectiveness was determined by calculating the average value of three tests conducted in identical conditions.

1.4.4 Oil Droplet Size

The average dispersed oil droplet size (APS) and their relative distributions, were measured with a laser particle size analyzer (Malvern Mastersizer 2000, United Kingdom).

The Malvern Mastersizer is a laser diffraction device that measures angular scattering distribution corresponding to droplet diameters ranging from 0.1 µm to 3000 µm. These measurements were taken at the end of each IFP assay, spaced out by 25 seconds and listed by the Mastersizer 2000 software.

1.4.5 Dispersant Effectiveness Index

The DEI is a quantitative index that allows comparisons of dispersant effectiveness among data sets (DE and APS), whose calculation is already used in ecotoxicology (Hagger *et al.*, 2008). First, each assay was ranked numerically to represent varying degrees of effectiveness (Table 3). The effectiveness was divided into three categories: good, uncertain, and poor. A dispersant is considered effective when the efficacy is greater than 60% (European Maritime Safety Agency, 2016). According to Chever *et al*. (2016), less than 20% of oil is considered as non-dispersible.

A droplet diameter of 70 µm was used as the cut-off point below which droplets were considered to be dispersed (Lunel, 1993; Li *et al.*, 2008, 2009). Lunel (1993) reported that 99% of the oil droplets contained within a good dispersion are < 70 µm. The second threshold, which was also determined by Lunel (1995), is 300 µm.

Table 5 : Assay rankings for the dispersant effectiveness index (DEI)

DE (%)	APS (µm)	Rank
> 60	< 70	1
20–60	70–300	2
< 20	> 300	3

The assays were also weighted according to the importance of the test. As the IFP assay measures the oil quantity collected after 1 hour of dispersion, it weighted as 1 and the average particle size as 2.

The DEI value was then calculated using the following equation to provide a DEI between 1 and 3 as illustrated below:

$$\text{DEI} = \frac{(\text{IFP rank} \times \text{IFP weighting}) + (\text{APS rank} \times \text{APS weighting})}{(\text{IFP weighting}) + (\text{APS weighting})}$$

The DEI was then assigned to one of four effectiveness categories, each associated with a colour: ≥ 1 (green) good; 1–1.5 (yellow) acceptable; 1.5–2 (orange) uncertain; > 2 (red) poor.

1.4.6 Statistical Analysis

All tests were regarded as statistically significant when $p < 0.05$. All data was statistically analyzed using R version 4.0.3. At first, the assumptions of normality and the homoscedasticity were verified respectively by Shapiro and Levene tests. IFP and APS data were analyzed by performing a two-way analysis of variance (ANOVA) to determine the significance of the effects of dispersant, temperature, and salinity. The post-hoc Tukey multiple comparison test was followed by the 'emmeans' function with Bonferroni corrections of the 'emmeans' package in R. Pearson's correlation coefficient (r) was performed between IFP and APS values for each dilbit.

1.5 RESULTS

1.5.1 Dispersant Effectiveness

The effectiveness of CXT on dilbits decreased significantly with decreasing salinity at temperatures tested (Figure 16).

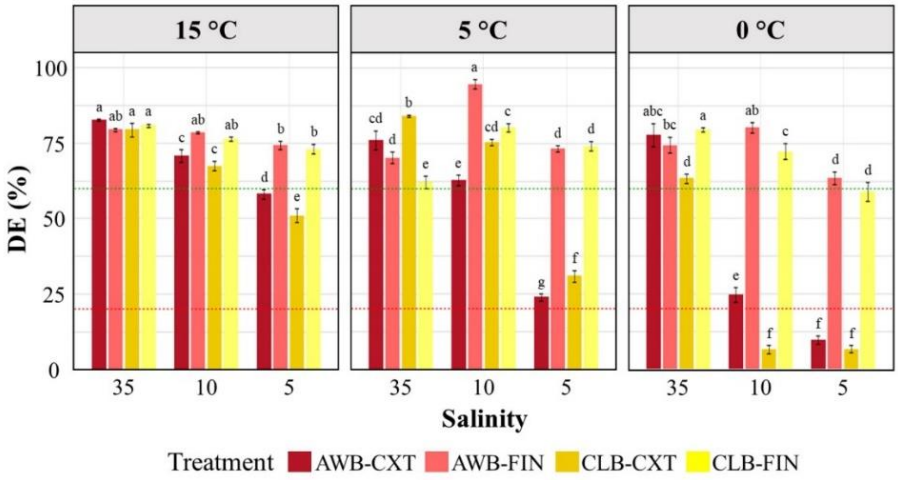

Figure 16 : Dispersant effectiveness (DE; %) for Corexit® 9500A (CXT) and Finasol® OSR52 (FIN) on Access Western Blend (AWB) and Cold Lake Blend (CLB) at three different temperatures (15, 5 and 0 °C) and three salinities (35, 10 and 5 psu) (mean ± SE)

At a salinity of 35 psu, the CXT effectiveness on AWB or CLB was considered effective (> 60%) at the temperatures tested. It was between 60 – 20% at 5 psu at two temperatures (15 – 5°C) on the two dilbits, and at 10 psu at 0°C, only on AWB. The CXT effectiveness was consider non-dispersible (< 20%) only at 0°C and 5 psu on the two dilbits, but also at 10 psu on CLB at the same temperature. With Finasol® OSR52 on AWB, the effectiveness was considered effective (> 60%) at all salinities and temperatures tested. On CLB, the effectiveness wasn't considered effective at 0°C and 5 psu, where the DE was between 60 – 20% (58.85 ± 2.44%).

On AWB, the two dispersants' effectiveness was similar at 35 psu for the three temperatures. At 10 and 5 psu, the effectiveness of FIN was significantly higher than CXT. On CLB at 35 psu, there is no significant difference between the two dispersants at 15°C, while CXT was significantly superior to FIN at 5°C, and the opposite was observed at 0°C. As with AWB, the effectiveness of FIN on CLB was significantly higher than the effectiveness of CXT for salinities of 10 and 5 psu, except in one condition: 5°C and 10 psu where the effectiveness was similar.

Between the two dilbits, the CXT effectiveness was significantly higher on AWB than CLB at 15°C and 5 psu, as well as at 0°C with salinities of 35 and 10 psu. The opposite was observed at 5°C. When the dilbits were dispersed with Finasol® OSR 52, no significant difference was measured between them at 15°C. However, the dispersion effectiveness was significantly higher for AWB at 10 psu for cold temperatures, 5 and 0°C, and at 35 psu but only at 5°C. At 35 psu and 0°C, the effectiveness was higher on CLB than AWB.

1.5.2 Dispersion Quality

Average Particle Size

The average particle size (APS) of AWB and CLB droplets dispersed by CXT or FIN was less than 300 µm (Figure 17). The largest droplet size was observed at the lowest temperature (0°C) for the two dilbits. The APS significantly increased when salinity decreased, except for one set of conditions: AWB dispersed with Finasol® OSR52 at 0°C and 5 psu.

At 15°C, the APS of the two dilbits dispersed by CXT or FIN was less than 70 µm for all the salinities tested, and so considered like a good dispersion. At 5°C, the APS increased and exceeds the 70 µm limit only at 5 psu with CXT for the two dilbits, and FIN but only with AWB (CXT-CLB: 91.24 ± 0.40 µm; CXT-AWB: 102.59 ± 1.58 µm; FIN-AWB: 105.68 ± 0.84 µm). At 0°C, the APS of the two dilbits was less than 70 µm for all the salinities tested, but only dispersed by FIN. Dispersed with CXT, the APS was higher than 70 µm at 10 and 5 psu, for the two dilbits.

At 35 psu, FIN induced a significantly higher APS than CXT with AWB and CLB. At 10 psu, it’s depended on temperature and dilbit: at 15°C, FIN induced a significantly higher APS than CXT for the two dilbits, and the opposite was measured at 0°C; at 5°C, FIN induced a significantly higher APS than CXT for AWB, and same APS for CLB. At 5 psu, the APS of AWB was similar between the two dispersants at 15 and 5°C, and higher with CXT dispersant than FIN at 0°C. The APS of CLB at 5 psu was higher with FIN than CXT at 15°C, and the opposite at 5 and 0°C. With CXT, the APS of the two dilbits was significantly

different under the three temperatures for a salinity of 5 psu. The AWB-APS was significantly larger than CLB–APS. This difference was also observed at 5°C – 35 psu. Under the other conditions, no significant difference was measured. With FIN, the AWB-APS was significantly larger than CLB-APS for all conditions, except two: 15°C – 10 psu and 0°C – 5 psu.

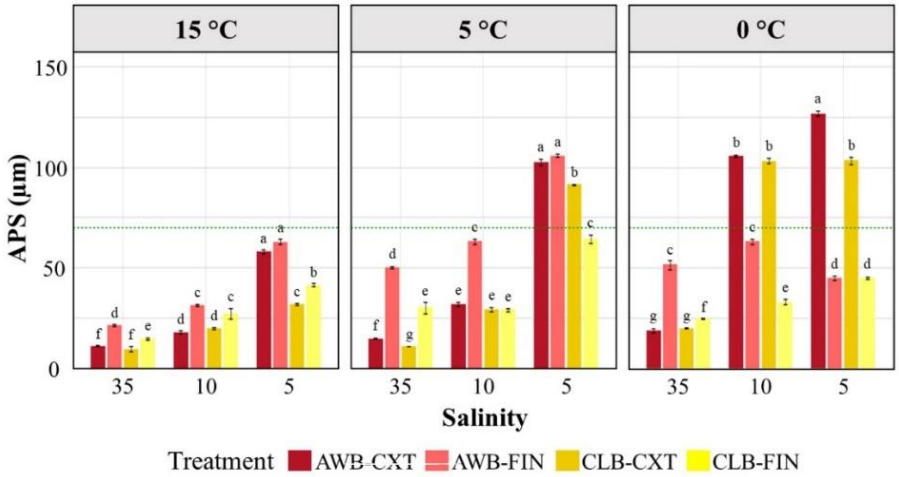

Figure 17: Average particle size (APS; µm) of Access Western Blend (AWB) and Cold Lake Blend (CLB) droplets dispersed with Corexit® 9500A or Finasol® OSR52 at three temperatures (15, 5, and 0°C) and three salinities (35, 10, and 5 psu) (mean ± SE)

Particle Size Distribution

With AWB, the curves are unimodal at 5 psu and bimodal at 35 psu and, sometimes, at 10 psu (Figure 18). The CLB particle size distribution (Figure 19) tends to be more bimodal than that of AWB at all three salinities, except at 0°C when CLB is dispersed with Corexit® 9500A. The AWB and CLB particle size distribution tends to be larger with decreasing salinity, except at 0°C, when dispersed by FIN or CXT. Overall, for both dilbits, the particle size distribution when dispersed by FIN is greater than that for CXT at a salinity of 35 psu.

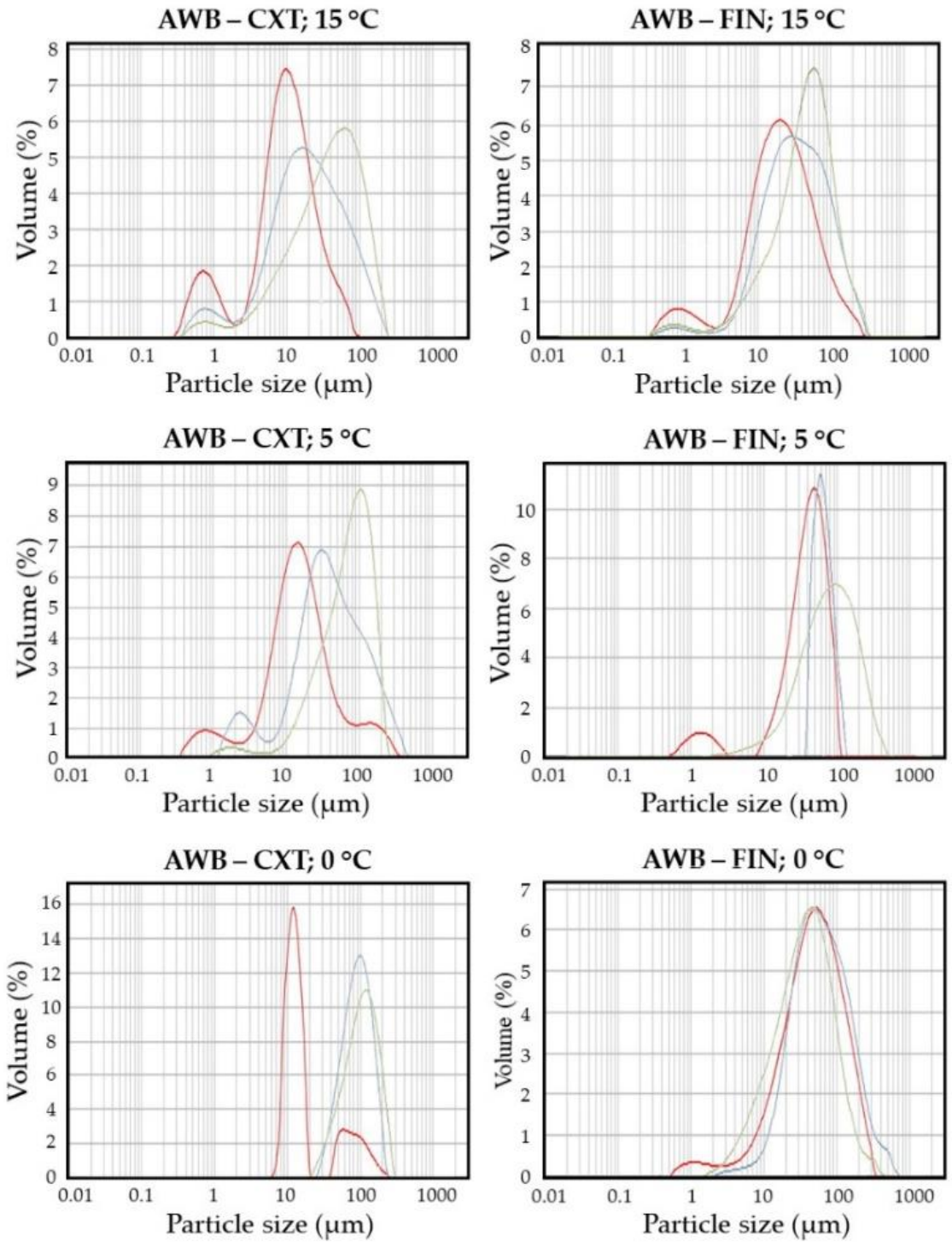

Figure 18 : Particle size distribution (µm) of Access Western Blend (AWB) dispersed with Corexit® 9500A (CXT) or Finasol® OSR52 (FIN) at three temperatures (15, 5, and 0°C) and three salinities: 35 psu (red), 10 psu (blue), and 5 psu (green)

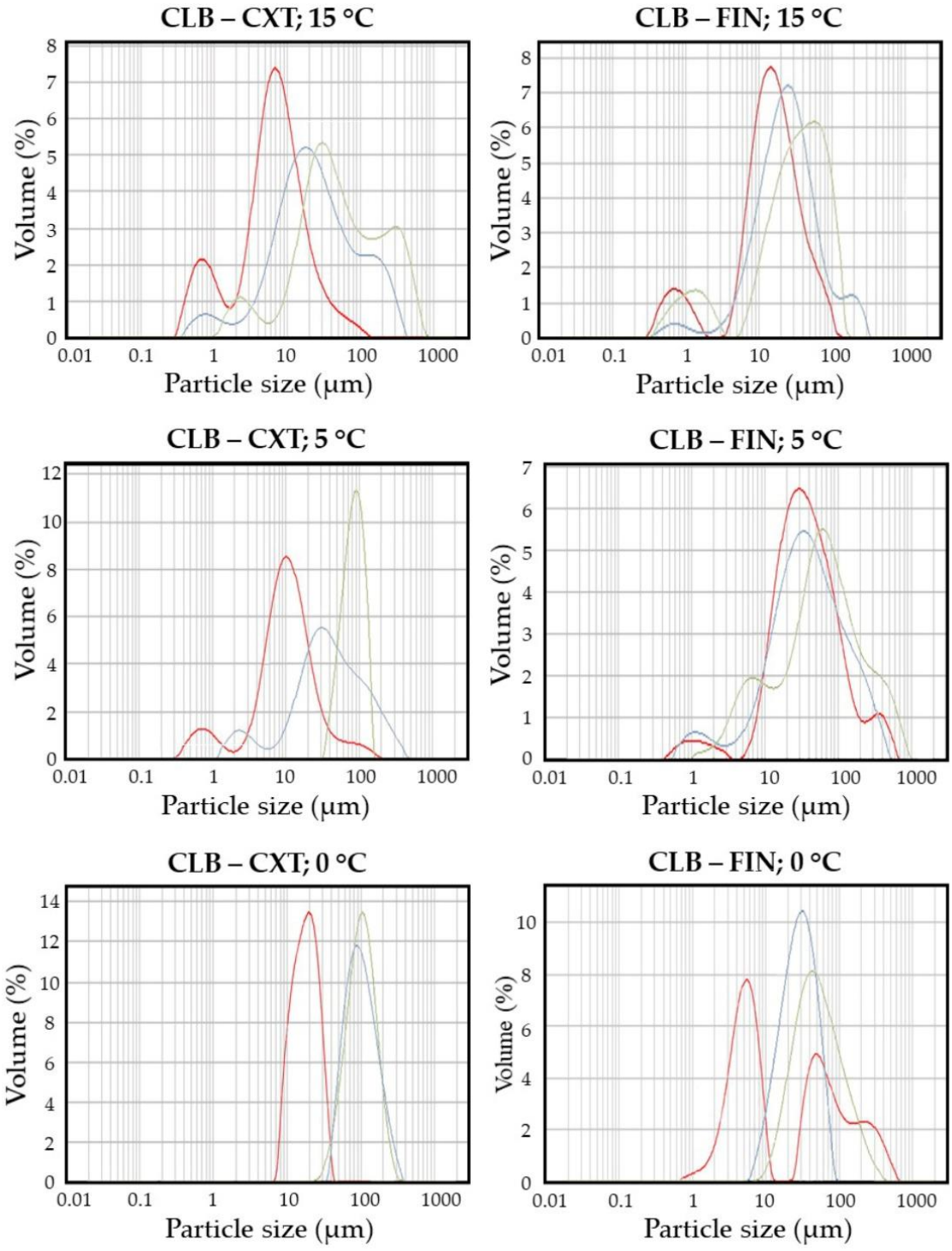

Figure 19 : Particle size distribution (µm) of Cold Lake Blend (CLB) dispersed with Corexit® 9500A (CXT) or Finasol® OSR52 (FIN) at three temperatures (15, 5, and 0°C) and three salinities: 35 psu (red), 10 psu (blue), and 5 psu (green)

1.5.3 Relationship between DE and APS

A decrease in dispersion effectiveness measured by the IFP test was significantly correlated with an increase in the average droplet size (Figure 20), but only with Corexit® 9500A (AWB: R^2 = - 0.9838; CLB: R^2 = - 0.9597). With Finasol® OSR 52, no correlation was measured between dispersion effectiveness and droplet size.

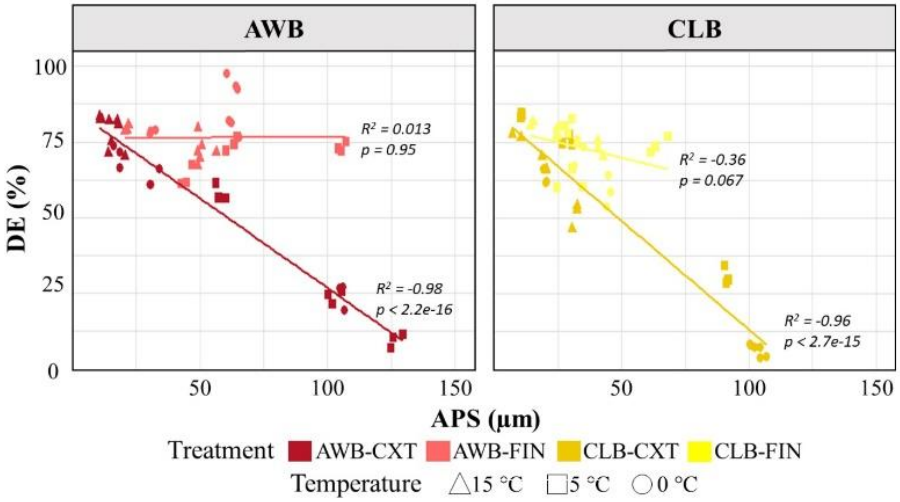

Figure 20 : Average particle size (APS; µm) according to IFP values (DE; %) for Access Western Blend (AWB) and Cold Lake Blend (CLB) dispersed by Corexit® 9500A (CXT) or Finasol® OSR 52 (FIN)

1.5.4 Dispersant Effectiveness Index

Correlation results highlight the importance to base the effectiveness of dispersant on one test (Figure 20). The dispersant effectiveness index allows comparisons of dispersant effectiveness among data sets (DE and APS). The lowest dispersant effectiveness index (DEI) was measured for the lowest temperature and salinity (Table 6). The DEI of Corexit® 9500A is higher at 0°C for dilbit AWB than CLB, and the DEI of Finasol® OSR 52 is higher for CLB than AWB at 5 psu for cold temperatures (5 and 0°C).

Table 6 : Assay rank determined in order to calculate the dispersant effectiveness index (DEI): Good (green), acceptable (yellow), uncertain (orange) and poor (red)

Dispersant	Dilbit	15°C			5°C			0°C		
		35	10	5	35	10	5	35	10	5
CXT	**AWB**									
CXT	**CLB**									
FIN	**AWB**									
FIN	**CLB**									

1.6 DISCUSSION

The aim of dispersant is to reduce the overall impact of an oil spill by reducing the exposure of birds and mammals at the surface of the ocean and improve the biodegradation of the hydrocarbons by allowing contacts between the molecules and microorganisms (Fingas, 2011). However, dispersion of oil in water can lead to increased chemical loading in benthic and coastal habitats (Ramachandran *et al.*, 2004), and more sensitive habitats can be impacted by the movement of currents and tides, transporting dispersed oil molecules, or even by their concentration in semi-enclosed coastal areas (DeLorenzo *et al.*, 2016). In addition, toxicity effects of dispersant are known to be influenced by its composition, and physical-chemical parameters, especially temperature and salinity (George-Ares and Clark, 2000; DeLorenzo *et al.*, 2016): the toxicity of dispersed oil increases with increasing temperature (National Research Council, 2005), and decreasing salinity (Kuhl *et al.*, 2013; DeLorenzo *et al.*, 2016). Our results show that marine dispersants, like Finasol® OSR52 and Corexit® 9500A, were very effective in marine conditions (salinity of 35 psu) at all the temperatures tested, but less effective in brackish waters (10 to 1 psu), as reported in previous studies (Blondina *et al.*, 1999; Kulekeyev *et al.*, 2014). However, the effectiveness of dispersion at low salinity was dependent on temperature. For example, the dispersant effectiveness index for Corexit® 9500A is classified as acceptable from 5 psu at 15°C, and as poor following a drop in temperature to 0°C (5 psu). Our results also show differences in dispersant effectiveness on the two dilbits, depending on the dispersant used, for the same environmental conditions that is the lowest temperatures (5 – 0°C) and the lowest salinities

(10 – 5 psu). At 15°C, there were no difference between the two dispersants effectiveness. This is consistent with King *et al*. (2015; 2018) who measured the effectiveness of the dispersant CXT at 25.5 to 30.4 psu on AWB to be 53.2% (17°C) and on CLB to be 59.4% (19°C) at temperatures around 15°C. The differences in the lowest temperatures and salinities can be explained by the dilbit difference in viscosity, related, in particular, to their contents of aromatics and asphaltenes. At 15°C, the viscosities of AWB and CLB are 347 cP and 285 cP, respectively, and at 0°C, 1,300 cP and 803 cP, respectively. As the difference in viscosity between the two dilbits is greater at 0°C than at 15°C, so is the dispersant effectiveness. Despite a higher viscosity for AWB, the dispersant effectiveness index shows better results with CXT, but not with FIN.

Our results indicate that, under the same conditions (temperature, salinity, type of oil), the FIN dispersant was effective on the two unweathered dilbit for a broader range of temperatures and salinities than CXT, which was more dependent on these parameters. In the case of the latter, the dispersant effectiveness index is uncertain at 15°C – 5 psu and from 10 psu at 0°C. The DE results show, in contrast to Resby *et al.*, 2007 and Steffek, 2015 for light and medium oils, that, the effectiveness of FIN was greater than that of CXT. So, as stated above, the DE is strongly dependent on many factors such as the type of oil (light, medium, heavy). Therefore, in addition to salinity, temperature, and oil composition, dispersant effectiveness is dependent on the dispersant formulation.

The APS is greater with FIN than CXT, except at the low salinities of 5 psu and 10 psu, and at the coldest temperature tested (0°C). This concurs with the study of Steffek, 2015, which showed that the droplet size is tends to be greater with FIN (95.28 μm) compared to CXT (83.85 μm), at 1°C and at salinities of 26 – 28 psu.

The particle size distribution is directly correlated with the oil viscosity, related to their chemical composition (Chever *et al.*, 2016). The APS measurement indicates that with both dispersants, AWB tends to have greater droplet sizes than CLB. This result confirms that the oil composition influences the multimodal distribution of the dispersed droplets. Mukherjee *et al.*, 2011 conclude that a high concentration of saturates increases the proportion of small

(< 70 μm) and medium-sized (70 – 200 μm) particles. Aromatics and asphaltenes reduce the proportion of small droplets and increase the number of larger ones (> 200 μm) (Mukherjee *et al.*, 2011). Indeed, the AWB composition is lower in saturates (38%) and higher in asphaltenes (14%) compared to CLB. Based on our results, CXT tends to produce smaller droplets with the two dilbits.

Smaller oil droplets could be more easily absorbed by filter organisms like mussel, but have larger surface areas and undergo higher rates of biodegradation due to enhanced oil availability (Gong *et al.*, 2014). However, the impact of chemical dispersants on biodegradation is still debated (Kleindienst *et al.*, 2015a, 2015b) because of the surfactants. For example, only Tween 20 assists the growth of hydrocarbon degrading bacterium, while the other ingredients slow it down (Bookstaver *et al.*, 2015). Smaller droplet size of CXT measured in our study can assume on higher toxicity for CXT than FIN.

Therefore, our data on dispersant effectiveness on dilbits will be useful to better intervene in the event of an oil spill in Canadian marine waters, taking into account the influence of the season on the dispersant effectiveness and related toxicity due to the particle size and composition of the dispersed oil. This will be helpful, in certain seasons, dependent on migration, when populations of birds and cetaceans are present in the Canadian waters in order to reduce oil spill and dispersant impact.

Based on our results, for AWB and CLB dilbits, one of the most important parameters for dispersant effectiveness seems to be the composition of the dispersant itself (IPIECA-IOGP, 2014a; Riehm and McCormick, 2014; John *et al.*, 2016). The optimal mixture of surfactants (Tween, Span, and DOSS) is needed for maximum effectiveness, defined as achieving the greatest reduction in oil-water interfacial tension (John *et al.*, 2016). According to a previous study, a DOSS-rich surfactant blend exhibited better dispersion effectiveness than a Span 80-rich surfactant blend (Riehm *et al.*, 2016). The low effectiveness of dispersants with low DOSS-to-Span 80 ratios was attributed in part to their slow rate of adsorption at the oil−water interface (Riehm *et al.*, 2016). According to Zhang *et al*. (2017), the proportion of DOSS anionic surfactant is 20% higher in the dispersant FIN compared to

CXT. The proportion of nonionic surfactants (Tween, Span) is also higher in FIN (> 30%) compared to CXT (< 30%). The proportions of these surfactants may explain the better dispersion effectiveness by FIN on our dilbit samples. Moreover, the key to dispersant effectiveness is the incorporation of solvents which can remain in the oil slick and resist extraction by seawater long enough to enable the surfactants to be effective (Lessard and Demarco, 2000). It can be assumed that FIN contains either higher concentrations of solvents or different solvents to CXT.

Finally, when measuring dispersion efficiency with the IFP test, Finasol® OSR52 was effective on dilbits under all our tested conditions (temperature and salinity). However, when using our dispersant effectiveness index, which combines IFP and particle size data, two conditions as acceptable (5°C, 5 psu), or even uncertain (0°C, 5 psu), as opposed to good with IFP. When measuring the APS of Corexit® 9500A was under the threshold of 70µm, so considered like good, the DEI was acceptable (5°C, 5 psu). Moreover, the dispersion efficiency measured with IFP at 0°C, 10 psu was similar between the two dilbits. But the DEI considered uncertain dispersion for AWB, and poor for CLB. Therefore, the use of the dispersant effectiveness index can help to improve these findings and therefore the choice of chemical dispersant taking into account more factors. Moreover, our index could be used to improve the decision table propose by Liu and Callies (2020), by specifying the dispersant efficacy, and with new data on unconventional oil.

1.7 CONCLUSION

The results of IFP tests show that Finasol® OSR 52 had a better effectiveness on dilbits at low temperatures (< 5°C) and low salinities (< 10 psu) compared to Corexit® 9500A. In addition, the average particle size was always less than 300 µm, even for dispersant effectiveness of less than 20%. Thus, there is a greater surface-to-volume ratio which leads to greater chemical reactivity of these particles and, consequently, to a potential of greater bacterial attack to mineralize them. The low correlation between dispersant effectiveness and the average particle size proves that our dispersant effectiveness index is a useful asset. It

allows a better visualization of the conditions leading to a good dispersion of the oil slick, taking into account various factors. It can be specified by compiling data already produced by the various studies, with the aim of specifying the best conditions of use like information on the diversity of some bacterial taxa known to be able to degrade oil, in order to take into account the possibility and time of natural oil degradation. The impacts on marine organisms as a function of particle sizes will further refine the choice of whether or not to use the chemical dispersant. Moreover, the viscosity of a floating oil increases over time, which decrease the dispersant effectiveness due to the reduced ability to penetrate through the oil-water interface. Given the high viscosity of dilbit, it is conceivable that dispersant stockpiles near pipelines' sensitive areas would enable a quick and effective response in the case of an accidental spill. Nevertheless, chemical dispersion should be considered a response option only to be implemented if recovery is not feasible.

1.8 FUNDINGS

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1.9 ACKNOWLEDGMENTS

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CHAPITRE 2
INDICE INTÉGRÉ DE RÉPONSE DES BIOMARQUEURS POUR ÉVALUER LES EFFETS DU PÉTROLE BRUT CLASSIQUE, DU BITUME DILUÉ ET DU DISPERSANT CHIMIQUE SUR LES MOULES BLEUES, *MYTILUS EDULIS* : INFLUENCE DES MÉTHODES DE DISPERSION ET DE LA SAISONNALITÉ

2.1 RÉSUMÉ

La croissance de la production de pétrole en Alberta a été principalement alimentée par le bitume dilué (dilbit). L'augmentation de la production et de l'exportation de dilbit nécessite des connaissances précises des risques générés par un déversement de dilbit, mais aussi sur les méthodes d'intervention en cas de déversement. Notre étude vise à comparer les impacts cellulaires et génotoxiques de deux dilbits (Access Western Blend, Cold Lake Blend) et d'un pétrole classique (Heidrun) sur la moule bleue, *Mytilus edulis*. Deux techniques de dispersion (physique et chimique) ont également été comparées sur trois saisons (été, automne et hiver), dans le but de favoriser une meilleure prise de décision après un déversement. Nos résultats ont montré une plus grande bioaccumulation dans les tissus des moules exposées au pétrole classique par rapport aux dilbits, en particulier avec la dispersion chimique, quelle que soit la saison. La déstabilisation des membranes lysosomale (25 à 60%), la diminution de la viabilité cellulaire (85 à 50%) et des dommages à l'ADN (10 à 40%) ont été mesurés après 48 h d'exposition sur les moules bleues. Même une mortalité (0 à 80%) a été observée en été pour l'exposition aux dilbits ou au dispersantseul. Le niveau des impacts dépendait du type de pétrole, de la méthode de dispersion et de la saison. L'indice de réponse des biomarqueurs intégrés montre des impacts globaux généralement plus élevés lors d'un déversement de dilbit dispersé chimiquement en été.

Ce deuxième article, intitulé « Integrated biomarker response index to assess the effects of conventional crude oil, diluted bitumen, and chemical dispersant on blue mussels, *Mytilus edulis*: a comparison of dispersion mechanisms and seasonal influence », sera soumis pour publication aux éditeurs de la revue *Science of the Total Environment*. En tant que première

auteure, ma contribution à ce travail fut la collecte des données, la recherche sur l'état de l'art, le développement de la méthode, la réalisation des expositions et des analyses, l'analyse et l'interprétation des résultats et la rédaction de l'article. Nicolas Lemaire, second auteur, a participé au développement de la méthode et la réalisation des expositions et des analyses. Réjean Tremblay, Jean-Pierre Gagné, et Céline Audet, ont fourni l'idée originale, aidé à la recherche sur l'état de la question, au développement de la méthode, et ils contribueront à la révision de l'article lors de la soumission. Richard Saint-Louis, directeur de recherche, et Gaëlle Triffault-Bouchet, co-directrice de recherche, ont fourni l'idée originale, aidé à la recherche sur l'état de la question, au développement de la méthode ainsi qu'à la révision de l'article.

Une version abrégée de l'article a été présentée à différentes conférences et réunions annuelles : en novembre 2016 par présentation d'un poster, lors de la 14ème réunion annuelle Québec Océan à Rimouski (Canada) « Effets biologiques sous-létaux sur la moule bleue des pétroles classique et non classiques dispersés chimiquement et physiquement en milieu marin » C. Berthod, N. Lemaire, G. Triffault-Bouchet, R. Tremblay, J. P. Gagné, C. Audet et R. Saint-Louis, puis en présentation orale lors du congrès Eau, Terre, Environnement (CETE), à Québec (Canada) « *Sublethal effects on the blue mussel of conventional and unconventional oils dispersed chemically and physically in the marine environment* » C. Berthod, N. Lemaire, G. Triffault-Bouchet, R. Tremblay, J. P. Gagné, C. Audet et R. Saint-Louis ; en octobre 2017 lors du 44ème congrès Canadian Ecotoxicity Workshop (CEW), à Guelph (Canada) *Sublethal effects of hydrocarbons by the blue mussel (Mytilus edulis) exposed to conventional and unconventional crude oils spilled* » C. Berthod, N. Lemaire, G. Triffault-Bouchet, R. Tremblay, J. P. Gagné, C. Audet et R. Saint-Louis ; en novembre 2017 lors de la 15ème réunion annuelle Québec Océan à Rivière-du-Loup (Canada) « Les moules bleues se questionnent : doit-on utiliser des dispersants chimiques comme moyen d'intervention lors d'un déversement de pétrole dans le Saint-Laurent ? » C. Berthod, N. Lemaire, G. Triffault-Bouchet, R. Tremblay, J. P. Gagné, C. Audet et R. Saint-Louis, puis lors du congrès Eau, Terre, Environnement (CETE), à Québec (Canada) « *Blue mussels wonder: should we use chemical dispersants as a response to an oil spill in the St. Lawrence?* » C. Berthod, N. Lemaire, G. Triffault-Bouchet, R. Tremblay, J. P. Gagné, C. Audet et R. Saint-Louis, récompensée par le premier prix de la meilleure présentation orale.

2.2 INTEGRATED BIOMARKER RESPONSE INDEX TO ASSESS THE EFFECTS OF CONVENTIONAL CRUDE OIL, DILUTED BITUMEN, AND CHEMICAL DISPERSANT ON BLUE MUSSELS, *MYTILUS EDULIS*: A COMPARISON OF DISPERSION MECHANISMS AND SEASONAL INFLUENCE

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2.3 ABSTRACT

Crude oil production in Alberta is rising to meet the demand for diluted bitumen (dilbit). The increase in dilbit production and exportation represents a greater need to understand the potential consequences of a dilbit spill and identify appropriate spill response methods. Our study aims to compare the cellular and genotoxic impacts of two dilbits (Access Western Blend and Cold Lake Blend) and a conventional oil (Heidrun) on the blue mussel, *Mytilus edulis*. Moreover, we compared two dispersion techniques (physical and chemical) over three seasons (summer, autumn, and winter) to understand the condition-specific effects of oil spill remediation. Our results showed greater contaminant bioaccumulation in mussel tissues exposed to conventional oil compared to dilbits, especially with chemical dispersion, regardless of the season. We measured lysosomal membrane destabilization (25–60%), decreased cell viability (85 - 50%), and DNA damage (10–40%) after a 48h exposure period. Mortality (0–80%) was observed in summer for dilbit exposure, or exposure to dispersant alone. We found that the severity of an oil spill's impact depends on the type of oil, dispersion technique, and season. The integrated biomarker response index shows higher global impacts after chemical dispersion, dilbit exposure, or at warmer temperatures.

Keywords: Diluted bitumen; Chemical dispersant; *Mytilus edulis*; Integrated biomarker response index; seasonal influence

2.4 INTRODUCTION

Total Canadian oil production is expected to rise to 5.86 million barrels per day (b/d) by 2035, including 4.25 million b/d from oil sands production (CAPP, 2019). Much of this is intended for export to European and Asian markets and involves using the country's sea lanes for its transportation. The unconventional oil from oil sands, known as diluted bitumen (dilbit), consists of bitumen mixed with petroleum distillate or condensate (up to 30%) to reduce its viscosity. Dilbit differs from conventional crude oils in its composition of monocyclic, polycyclic (PAH), and heterocyclic aromatic hydrocarbons (carbazoles and dibenzothiophenes). Dilbit also has greater density, viscosity, and adhesion properties, higher levels of asphaltenes, naphthenic acids, and sulfur, and contains metals such as nickel and vanadium (Monaghan *et al.*, 2021). Two dominant blends of dilbit, transported in high volumes across Canada, are Access Western Blend (AWB) and Cold Lake Blend (CLB). Despite significant dilbit production, Canada also imports and uses conventional oil, such as Heidrun (HEI) from the Norwegian Sea. Transport Canada must ensure that response measures can be applied to accidents up to 10,000 tonnes of oil in the marine environment. The Government of Canada intends to allow the use of chemical dispersants as an additional response measure, including Corexit® 9500A (Government of Canada, 2016). The Corexit® class of dispersants have been effective at dispersing diluted bitumen (Berthod *et al.*, 2022a) and are the most widely used, including in response to the Deepwater Horizon oil spill in the Gulf of Mexico in 2010. When dispersing agents are sprayed on an oil slick, the slick breaks down into small oil droplets (70 μm and smaller), facilitating dispersion in the water column (Cedre, 2005). The aim is to reduce the concentration of hydrocarbons in the affected environment as quickly as possible (Cedre, 2005). The use of dispersants is still debated, particularly due to potential correlation to increased toxicity (Dupuis and Ucan-Marin, 2015b; CEAEQ, 2015b; Murawski *et al.*, 2020).

Musselis recognized as a sentinel species to assess the health of marine ecosystems (Marigómez *et al.*, 2013; Martínez-Gómez *et al.*, 2017; Poulsen *et al.*, 2021). It has been used as a bioindicator of the health of marine ecosystems since the mid-1970s, when the first

international biomonitoring program was established: The Mussel Watch Program (Goldberg, 1986). Although all marine invertebrates are sensitive to an oil spill, benthic and sessile organisms are the most sensitive. In bivalves, the half-lives of pollutants such as polycyclic aromatic hydrocarbons (PAHs) are markedly longer than in fish (Stegeman and Lech, 1991; Meador *et al.*, 1995) and the rate of metabolism of PAHs is slower, which promotes bioaccumulation (Seiser *et al.*, 2000). Conventional oil spills or lab exposure experiments allow to observe impacts on mussels, such as alteration of growth (Peteiro *et al.*, 2006), biochemical components (Peteiro *et al.*, 2007), and bioenergetic processes (Peteiro *et al.*, 2006; Sureda *et al.*, 2011; Redmond *et al.*, 2016). Effects on reproduction, such as deterioration of the gonads (Baussant *et al.*, 2011; Ortiz-Zarragoitia *et al.*, 2011), decreased metabolism, or impaired development or survival of larvae (Labarta *et al.*, 2005; Schmutz *et al.*, 2021), as well as genotoxicity and cytotoxicity (Pérez-Cadahía *et al.*, 2004; Glad *et al.*, 2017; André *et al.*, 2022) have also been observed. Oil spills threaten the mussel aquaculture industry, *Mytilus edulis*, which is an important Canadian industry. The culture and export of mussels brought in $30.7 million in 2020 (MPO, 2020b). Moreover, this species is of great importance because of its role in the traditional diet of coastal populations.

The impacts of dilbit are still poorly studied and mainly focused on fish. Diluted bitumen induces oxidative stress (Madison *et al.*, 2015) and impacts the development (Madison *et al.*, 2015, 2017; Philibert *et al.*, 2016; Lin *et al.*, 2021) and behavior (Philibert *et al.*, 2016; Lin *et al.*, 2021) of fish. Barron *et al*. (2018), observed that estuarine species, compared to freshwater species, exhibit greater dilbit acute toxicity at lower concentrations. Other studies observe different biological effects between conventional and unconventional oil (Philibert *et al.*, 2016, 2021; Berube *et al.*, 2021, 2022). Depending on the chosen biomarker in fish, dilbit is more (development), less (mortality, pericardial edema, behaviour), or similarly (yolk sac edema, behaviour) toxic than conventional crude oil. Chemical composition plays a critical role in determining the sublethal toxicity of conventional and unconventional crude oils in freshwater ecosystems. But, to our knowledge, only one study has compared the impacts of dilbit to conventional oil on mussels (Schmutz *et al.*, 2021). In the study of Schmutz *et al*. (2021), significant negative effects were observed

at the cellular, physiological, and fitness levels, especially in offspring. The results suggest that dilbit is more toxic than conventional crude oil, despite lower rates of bioaccumulation of PAHs.

Besides oil type (proportion of aromatic hydrocarbons and viscosity), other factors will influence an oil spill's toxicity towards mussels. Water temperature, food availability, and reproductive stage may influence the flesh yield and biochemical composition of mussels (Fernández-Reiriz *et al.*, 1996; Okumus and Stirling, 1998), as well as the expression of various biomarkers (Aarab *et al.*, 2011). Studies show that some biological parameters used as biomarkers of mussel health can show seasonal fluctuations (Hagger *et al.*, 2010; Schmidt *et al.*, 2013; Dallas and Jha, 2015; Balbi *et al.*, 2017). The metabolism of organisms varies throughout the seasons, as does the behavior of oils. King *et al*. (2018), observed that the dispersion of dilbit is more sensitive to seasonal changes than conventional oil. According to Aarab *et al*. (2011), results should be interpreted according to the season and the reproductive stage of the organisms. The need for dilbit toxicity data has been highlighted in a number of comprehensive reviews (Dupuis and Ucan-Marin, 2015a; Lee *et al.*, 2015b; National Academies of Sciences, 2016; S.J. Wallace *et al.*, 2020), especially as it pertains to species such as the blue mussel. There is also a lack of information on toxicity when adding chemical dispersants, as well as seasonal influences.

Dispersing agents have low toxicity to many aquatic species, comparable to that of household cleaning agents (NOAA, 2012; Word *et al.*, 2015). However, the combination of oil with dispersant can increase toxicity. Increasing the contact area between water and oil amplifies the diffusion of soluble hydrocarbons (in particular PAHs) and smaller oil droplets are more easily assimilated by organisms (via the digestive system, gills, or skin contact) (Dupuis and Ucan-Marin, 2015a; CEAEQ, 2015a). Moreover, chemically dispersed contaminants are more likely to be ingested by organisms living in the water column (CEAEQ, 2015a). Marine species such as the common jellyfish (*Aurelia aurita*), which has a large surface area relative to its volume, are particularly sensitive to exposure to Corexit® 9500A (Echols *et al.*, 2016).

Several studies have shown that the toxicity of a dispersant-oil mixture is greater than that of either component alone (Hook and Osborn, 2012; Jung *et al.*, 2012; Rico-Martínez *et al.*, 2013; Anderson *et al.*, 2014). Anderson *et al.* (2014), observed no mortality in juvenile crabs (*Rhithropanopeus harrisii*) after exposure to Louisiana sweet crude oil, but significant mortality after exposure to oil dispersed with Corexit® 9500A. For AWB (Madison *et al.*, 2015) and CLB (Madison *et al.*, 2017) dilbit, water-accommodated fractions (WAF) have lesser toxicity to fish than chemically-enhanced WAF (CEWAF; Lara-Jacobo *et al.*, 2019).

The St. Lawrence seaway, which facilitates the marine transport of dilbit and conventional crude oil, is a complex ecosystem where the temperature varies greatly throughout the year: 1°C in winter, 5°C in autumn, and 15°C in summer (SLGO, 2020). In its maritime section, the seaway encompasses the Saguenay–St. Lawrence Marine Park, a marine protected area that covers 1,245 km² in the St. Lawrence Estuary and the Saguenay River. More than 2,200 species inhabit these waters, such as the beluga whale, the blue whale and the Barrow's goldeneye. Yet, there are only a few studies on the toxicity of oils in cold water (McFarlin *et al.*, 2011; Helle *et al.*, 2020) and particularly marked gaps in knowledge on the toxicity of dispersed dilbit in winter conditions (Lee *et al.*, 2015b).

The aim of this study is to provide new data on the sublethal impacts of an oil spill on blue mussels, *M. edulis*, depending on the type of oil (Heidrun, AWB, and CLB), the dispersion method (physical dispersion or Corexit® 9500A), and the season. The sublethal effects of a dispersing agent and dispersed crude oil were studied in winter, autumn, and summer. The exposure time was 48 hours, to mimic a real situation where the concentration of dispersed crude oil usually decreases rapidly (Le Floch *et al.*, 2014). Measured sublethal effects are scope for growth (respiration, assimilation, energy allocation), lysosomal stability, cell viability, and genetic alterations by the comet assay. The comet assay has demonstrated its relevance for measuring the genotoxic potential of chemically dispersed oil on bivalves (Martinovic *et al.*, 2015). Chemical analysis of water and mussel tissues allow a more precise comparison of our conditions. Biological measurements were chosen to be able to study toxicity at the organism level and to estimate potential effects at the community level. An

integrated biomarker response (IBR) index was used to summarize a series of biomarkers into one index.

The IBR method is an effective tool to visualize the biological effects of pollutants and simplify the interpretation of the relationships between multiple biomarkers and contamination levels (Devin *et al.*, 2014). It has become one of the most common integrative indexes in environmental biomonitoring programs and laboratory experiments (Zheng *et al.*, 2013; Lee *et al.*, 2015b; Yuan *et al.*, 2017). This study will therefore provide a better understanding of the toxic effects of potential oil spills on complex Canadian marine environments such as the St. Lawrence seaway.

2.5 MATERIALS AND METHODS

2.5.1 Reagents

All chemicals used were of the highest grade available. The following chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA): Phosphate Buffered Saline (PBS); Neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, CAS 553-24-2); dimethyl sulfoxide (DMSO, CAS 67-68-5); Trypan-blue, CAS 72-57-1); Methyl Methane Sulfonate (MMS, CAS 66-27-3); Sodium chloride (NaCl, CAS 7647-14-5); and Triton® X-100 (CAS 9002-93-1). Disodium ethylene diamine tetra acetic (Na_2EDTA, CAS 6381-92-6) was from Anachemia (Canada). Normal-melting-point agarose (NMA, CAS 9012-36-6) and low-melting-point agarose (LMA, CAS 9012-36-6) were from ThermoFisher Scientific Invitrogen (USA). Tris (hydroxymethyl aminomethane, CAS 77-86-1) and SYBR Green (CAS 163795-75-3) were from ThermoFisher Scientific (USA). TAE (2M Tris-Acetate, 0.05 EDTA, CAS 77-86-1,60-00-4,6850-28-8) was from Lonza (Switzerland).

2.5.2 Conditions of Oil Spill Stimulation

Oil Products

Three unweathered crude oils and one dispersant, Corexit® 9500A, were supplied by Thomas L. King's team from Fisheries and Oceans Canada. The chosen oils were one conventional crude oil from the North Sea, Heidrun (HEI), and two diluted bitumen, Access Western Blend (AWB) and Cold Lake Blend (CLB). The dilbits originated from the Athabasca oil sand deposit in Alberta (Canada). The physical properties and compositions of the oils and the dispersant are presented in tables 7 and 8.

Table 7 : Physical properties and composition (SARAs; percentage by weight) of the unweathered oils: Heidrun (Environment Canada, 1995), Access Western Blend, and Cold Lake Blend (MPO, 2013).

		Temperature (°C)	**Heidrun (HEI)**	**Access Western Blend (AWB)**	**Cold Lake Blend (CLB)**
Origin			Norway Sea	Alberta	Alberta
API	°		28.6	20.9	21
Density	*g/ml*	0	0.8942	0.9399	0.9376
		15	0.8833	0.9253	0.9249
Viscosity	*cP*	0	35	13,00	803
		15	18	347	285
Saturates	*% w/w*		55	~38	~45
Aromatics	*% w/w*		35	~30	~30
Resins	*% w/w*		9	~17	~12
Asphaltenes	*% w/w*		1	~14	~13

Table 8 : Chemical composition of the Corexit® 9500A dispersant (Technical sheet of Corexit® 9500A (Nalco); [1]Place *et al.*, 2016)

Chemical composition		CAS	Corexit® 9500A (%)
Distillates (petroleum), hydrotreated light		64742-47-8	10−30
Nonionic surfactants	Span 80	1338-43-8	4.4 [1]
	Tween 80	9005-65-6	18 [1]
	Tween 85	9005-70-3	4.6 [1]
Anionic surfactants	DOSS	577-11-7	18 [1]
Organic sulfonic acid salt		-	10−30
Propylene Glycol		57-55-6	1−5

Preparation of Crude Oil and Dispersant Exposure Solutions

Water-accommodated fraction (WAF) and chemically-enhanced WAF (CEWAF) were produced to simulate the physical and chemical dispersion of oil following a spill. WAF and CEWAF were prepared following the methods of Signer *et al.* (2000), with modifications to simulate subarctic environments (Barron and Ka'aihue, 2003) and to adapt to diluted bitumen (Madison *et al.*, 2015). WAF and CEWAF were prepared before each exposure in a fluorinated polyethylene carboy with 20% headspace, in darkness and at natural seasonal temperatures. The tested oil product (HEI, AWB, or CLB) and/or the dispersant (Corexit® 9500A) were added to filtered seawater pumped directly from the St. Lawrence Estuary (glass bed filtration; 50 µm). Oil product concentrations were 25 g/l. WAF was gently mixed for 18h then left to settle for 6h. CEWAF was prepared by the same procedure, but after 18h, the dispersant was injected on the surface layer of the residual oil (1:10, which was equivalent of 2.5 to 125 mg/l). CEWAF was then stirred for 1h and allowed to settle for another hour. Four sub-samples of WAF and CEWAF were diluted with filtered seawater for exposure tests. The concentrations of these dilutions were 20, 40, 60, and 80% and 0.1, 0.5, 1, and 5% for WAF and CEWAF, respectively.

Chemical dispersant exposure solutions were prepared by diluting Corexit® 9500A in filtered seawater to obtain four concentrations: 2, 20, 200, and 1000 mg/l, based on GESAMP rating scheme for acute aquatic toxicity (GESAMP, 2013). Stock solutions were made fresh

daily and diluted for static renewal exposures in a clean experimental microcosm. As a control, blue mussels were tested under the same conditions, but without petroleum products or dispersants.

Monitoring of Dissolved Aromatic Hydrocarbons by Fluorescence

At the start (T0) of each exposure, the concentration of the aromatic hydrocarbon dissolved fraction of crude oil was estimated in triplicate, using the Cyclops-7 submersible fluorometric sensor, equipped with the "O" sensor (Turner Designs, San José, CA, USA) for the detection of crude oil. It is one of five UV submersible fluorometers that are commercially available for *in situ* measurements of PAHs, and one of the most commonly used in delineating oil plumes in the field (Conmy *et al.*, 2014; Hwang *et al.*, 2020; Berthod *et al.*, 2021; Schmutz *et al.*, 2021). Its fluorescence optical specifications are excitation wavelengths at 325/120 nm and emission wavelengths at 410/600 nm. The Cyclops-7 sensor was controlled by a DataBank module (Turner Design, USA), which was connected to a computer by proprietary software (Turner Designs, San José, CA, USA). The sensor was calibrated with an aqueous solution of 100 µg/l of tetrasodium 1,3,6,8-pyrenetetrasulfonate (PTSA), a highly water-soluble pyrene derivative (CAS 6528-53-6; Sigma-Aldrich, Darmstadt, Germany). The fluorescence measurements were used as an indicator of hydrocarbon concentration and were consequently expressed in equivalent µg PTSA/l. The sensor response was linear within the PTSA concentration range used, from 5 to 550 µg/l.

2.5.3 Animals

The blue mussels, *M. edulis* (Linnaeus 1758), were collected from a mussel farm in St. Peter's Bay, Prince Edward Island (Canada; 42 °25'N, 62 °35'O). Upon their reception at UQAR's aquaculture facility in Rimouski (Quebec, Canada), they were acclimated in a large tank with running natural seawater pumped from the St. Lawrence Estuary and filtered through the glass bed filtration system of the station. This ensured their nutrition (particulate organic matter only). A total of 1,620 blue mussels, received in 4 batches (2 for winter, 1 for

autumn and, 1 for summer) were used for the experiment, with a mean length of 5.90 ± 0.56 cm and a mean mass of 19.92 ± 5.77 g.

2.5.4 Exposure

This study was realized during three seasons: summer (July-August), autumn (October-November), and winter (January-February). For each condition (season, treatment, dilution), three groups of six mussels were exposed to WAF, CEWAF, dispersant solution, or filtered seawater (control) for 48h in experimental microcosms (3.2L; Figure 21A), in a tank containing running natural seawater pumped directly from the St-Lawrence Estuary to obtain the natural temperature (Figure 21B). Of the six mussels, two were used for the condition index and tissues analysis, two for the scope for growth and the last two for cellular parameters.

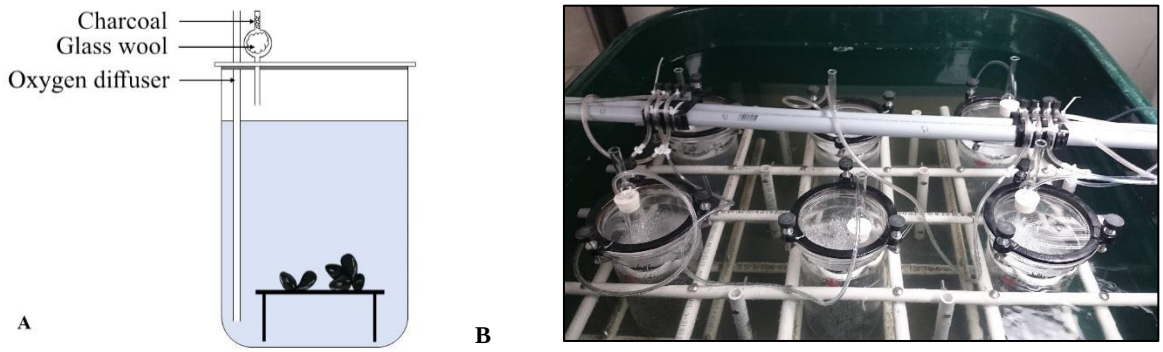

Figure 21 : **A.** Diagram of experimental microcosm and **B.** picture of the tank with six experimental microcosms

Experimental microcosms were cleaned with propanol to remove residual oil, rinsed with de-chlorinated water, and dried for re-use the following day. Before and after exposure, salinity, dissolved oxygen, and temperature were measured with the EXO2 multiparameter probe (YSI Inc., USA). Table 9 shows these parameters mean for each season.

Table 9 : Physico-chemical measurements mean in the experimental microcosms

	Temperature (°C)	Salinity (psu)	Oxygen saturation (%)
Winter	2.0 ± 0.4	27.0 ± 0.5	94.3 ± 3.6
Autumn	6.0 ± 0.6	26.7 ± 0.5	94.3 ± 3.6
Summer	16.2 ± 0.5	27.3 ± 1.2	89.4 ± 5.8

2.5.5 Tissues Analysis

Chemical analysis in whole mussel tissues was carried out according to the methods described by Schmutz *et al.* (2021; detail in Supplementary Methods). Analysis was performed on six organisms per condition (two mussels times three experimental microcosms), whose tissues were freeze dried (FreeZone, Labconco, USA) and reduced to a fine powder with a Virtis homogenizer. The concentrations of the following groups of molecules were determined in the mussel tissues: i) 16 PAHs classified as priority pollutants by the United States Environmental Protection Agency (US-EPA, 1984; Σ_{16}EPA-PAHs; C_{10} – C_{22}) due to their carcinogenicity (Honda and Suzuki, 2020); ii) 28 alkylated PAHs (Σ_{28}Alkylated-PAHs; C_{12} – C_{16}), given that alkyl-PAHs demonstrate different environmental behaviors and a significantly higher toxicity (Turcotte *et al.*, 2011; Mu *et al.*, 2014; Kang *et al.*, 2016; Cong *et al.*, 2021) than their non-alkyl forms due to their increased polarity and potentially different metabolic pathways and mechanisms experienced by organisms (Cong *et al.*, 2021); iii) 18 carbazoles and dibenzothiophenes (Σ_{18}CZDTs; C_{12} – C_{16}), which are representative of the nitrogen and sulfur heterocyclic PAHs known to be carcinogenic (Tsuda *et al.*, 1982; Jha and Bharti, 2002); and iv) 13 volatile organic compounds VOCs (Σ_{13}VOCs; C_9 – C_{10}) which seem to be a better predictor of mortality than PAHs (Philibert *et al.*, 2016; Bérubé *et al.*, 2021). Their sum is represented by Total Petroleum Hydrocarbons (TPH).

2.5.6 Physiological Parameters

Mortality

At the end of the exposure period, mussels' lethality was checked by mechanical stimulation of shell closure. Mussels were considered dead if they did not respond to closure stimulation.

Condition Index

The Condition Index (CI), a measure of the apparent health of bivalves, was calculated on four mussels per experimental microcosm, as the coefficient of dry flesh weight (DMW) and the shell length cubed (L^3/cm^3; Bodoy *et al.*, 1986; detail in Supplementary Methods):

$$\mathrm{CI} = \frac{DMW}{L^3}$$

Scope for Growth

Scope for growth (SFG) is used as a physiological energetic index for filter-feeding animals, determined by subtracting respired and excreted energy from energy assimilated through feeding (Widdows and Johnson, 1988). Scope for growth was measured according to Trembay *et al.* (1998c). Measurements were simultaneously realized on two mussels and one control (empty shell) per set of conditions in an experimental microcosm (Plexiglas cylinders of 1357 ml) filled with ultrafiltered (0.02 µm) and UV sterilized seawater. As the only significant difference was observed with the clearance rate in our study, detail of SFG method was described in Supplementary Methods. Clearance rate is defined as the volume of water cleared of suspended particles per unit time and biomass (Widdows and Johnson, 1988). Clearance rate was monitored by adding live microalgae to the experimental chambers and measuring their concentration over a 1h period. Food particles were counted from the outflow of all containers, including the control (an empty experimental chamber), every 15 min over 60 min (Gilek *et al.*, 1992) with a Zm Coulter Counter (Beckman Coulter Z series, ON, Canada) using a 200 µm orifice tube. Each measurement was done in triplicate. After mussels were fed during 24 – 48h with microalgae culture (60 cell $\mu l^{-1}.ind^{-1}$), faecal matter

and food samples were filtered onto GFC filters (47 mm for faeces and 25 mm for food) and rinsed with isotonic ammonium formate (3.2%). The samples were dried (65°C for 72h) and burned (450°C for 4h) to measure both dry and ash weights. This procedure provided estimates of the organic and inorganic fractions contained in the food and faeces.

2.5.7 Cellular Parameters

Hemolymph Extraction

Approximately 400 µl of hemolymph was collected from the adductor muscle of two specimens with a 23G needle and a 1 ml syringe pre-charged with 400 µl PBS-EDTA. The mix of hemolymph/PBS-EDTA was pooled in microtubes on ice to obtain 1.6 ml samples, which were then pelleted by centrifugation at 500 g for 5 min (Beckman Coulter, Microfuge 16). The cell suspension was made in 1 ml of residual supernatant. Prepared cell suspensions were used to measure lysosomal stability, cellular viability, and DNA damage.

Lysosomal Membrane Destabilization Index

The lysosomal membrane destabilisation index (LDI) was determined using the NRR assay according to the method described by Méthé *et al*. (2015; detail in Supplementary Methods). The lysosomal membrane destabilisation index (LDI) was calculated as the percentage of hemocytes with destabilized lysosomes (DH) in a total of 50 hemocytes, as follows:

$$LDI = \frac{DH}{50} X\ 100$$

Cell Viability

Hemocyte viability was assessed by a test of membrane integrity, the trypan blue exclusion method, according to McCarthy *et al.* (2014). This method has also been used in conjunction with the comet assay for a mussel exposure experiment (McCarthy *et al.*, 2014; Lacaze *et al.*, 2015). This assay is based on the principle that viable (live) cells possess intact membranes which can exclude this dye, while non-viable cells with damaged membranes

cannot. The cell viability (CV; detail in Supplementary Methods) was calculated as the percentage of viable cells in a total of 50 hemocytes, as follows:

$$CV = \frac{VC}{50} X\ 100$$

2.5.8 Genotoxicity

The comet assay (alkaline version) was performed according to the procedure described by Lacaze *et al.* (2015; detail in Supplementary Methods). Only hemocyte suspensions with a cellular viability rate higher than 70% were used (Lacaze *et al.*, 2015). At least 50 randomly selected nuclei per slide were counted for each sample, images were captured, and comets were analyzed with CASP 1.2.2 software (CASPLab, GNU public licence). Three measures are commonly used as a measure of DNA migration: % tail DNA, tail length, and tail moment. Percent tail DNA is increasingly the preferred assessment measure as it has the advantage of being expressed on a scale from 0 to 100%. Moreover, this factor is linearly related to dose in calibration studies, which further facilitates comparisons between studies (Collins *et al.*, 2008; Dhawan and Anderson, 2016).

2.5.9 Integrated Biomarker Response

Integrated Biomarker Response (IBR) was calculated with biomarker data (mortality, CR, LDI, CV, and DNA damage) obtained in the exposure experiment by the method proposed by Beliaeff and Burgeot (2002), with minor modifications by Yuan *et al*. (2017), adapted to use exposure group instead of site as a factor. Data was first standardized for a biomarker value (X), then the general mean (m) and standard deviation (s) of X were calculated for all exposure conditions and exposure seasons, depending on the comparisons to be made. X was standardized to obtain Y:

$$Y = \frac{(X - m)}{s}$$

Then, *Z* was calculated as *+Y* in the case of activation and *-Y* in the case of inhibition. *S* was calculated with the minimum value from all exposure groups across the seasons and for each biomarker, $S_i = Z_i + |Min|$. Finally, all *Si* values were shown on a radar diagram and the integrated biomarker response (IBR) was computed as the sum of the triangles defined by *k*-standardized biomarkers:

$$\text{IBR} = \sum_{i=1}^{k} A_i$$

$$A_i = S_i \times S_{i+1} \times \sin {}^{(2\pi/k)}\!/_{2}$$

To weaken the impact of biomarker sequence in the radar diagram, all possible circular arrangements of *k* biomarkers were calculated.

2.5.10 Statistical Analysis

All data was statistically analyzed using R software version 3.5.2. At first, the assumptions of normality and the homoscedasticity were verified using the Shapiro and Levene tests, respectively. When required, data were log-transformed to normalize their distribution. Differences between concentrations for each treatment group were determined by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences between treatments and season were determined by factorial ANOVA. Post hoc contrasts of means were made for significant effects with *P* values calculated using the Bonferroni procedure. All tests were regarded as statistically significant when $p < 0.05$.

Canonical correlation analysis (CCA), proposed by Hotelling (1936), is a multivariate exploratory statistical method used for exploring the relationships between two multivariate sets of quantitative variables observed on the same experimental units (Caron *et al.*, 2016). In this study, CCA was performed using XLSTAT (Addin-soft, USA) to explore the relationships between a first set of dependent variables (physiological, cellular parameters, and genotoxicity) and a second set of independent variables (tissue analysis).

2.6 RESULTS

2.6.1 Physical Dispersion

Water analysis

The concentration of aromatic hydrocarbons in HEI, AWB, and CLB microcosms was strongly correlated to nominal dilutions of WAF (%, v/v; Figure 22), with R^2 values > 0.9 ($p<0.05$; Pearson test).

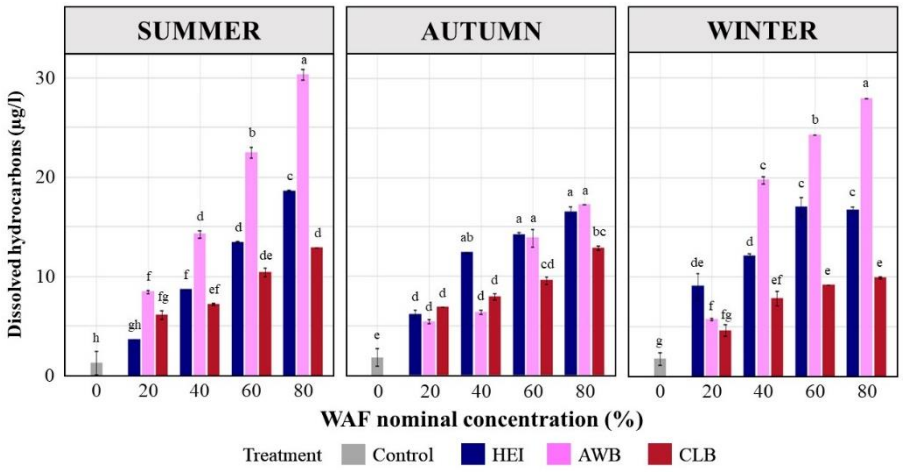

Figure 22. Fraction of dissolved hydrocarbons (µg/l) measured by fluorescence with the Cyclops-7 optical sensor in control (0%, v/v, WAF; n = 6) and WAF microcosms (HEI, AWB, and CLB; 20 to 80%, v/v; n = 3) for each season (mean ± SE; when error bars are not visible, SE was close to 0)

In summer and winter, the aromatic hydrocarbons concentration in microcosms was i) higher in AWB-WAF compared to HEI- and CLB-WAF from 40% (v/v) and above, and ii) lower in CLB-WAF for the same nominal concentrations in winter, and at 80% (v/v) in summer. In autumn, aromatic hydrocarbons concentrations were similar between AWB- and HEI-WAF microcosms, except at 40% (v/v) where HEI-WAF levels were greater than those of AWB-WAF. Concentrations were lowest in CLB-WAF microcosms from 60% (v/v) and above.

Tissue Analysis

The total petroleum hydrocarbons concentration (TPH; µg/g, dw) in whole tissues of mussels (Figure 23) was significantly correlated with dissolved hydrocarbons in microcosms contaminated with HEI at each season ($R^2 > 0.7$; $p < 0.05$; Pearson test), to AWB in summer and autumn ($R^2 > 0.6$; $p < 0.05$; Pearson test), and to CLB in autumn ($R^2 > 0.6$; $p < 0.05$; Pearson test). In winter, no correlation was found with dilbit exposure.

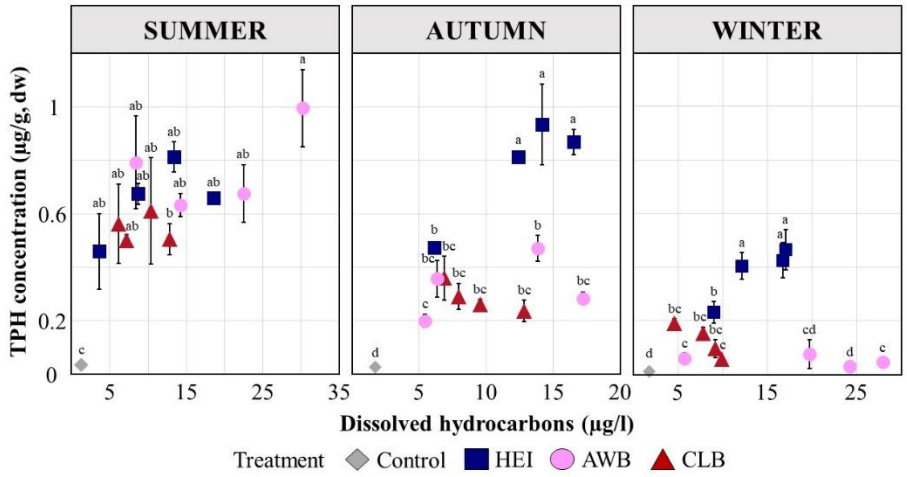

Figure 23 : Total petroleum hydrocarbons concentration (µg/g, dw) measured by GC-MS in whole tissues of *M. edulis* in control (0%, v/v, WAF; n = 6) and WAF microcosms (HEI, AWB, and CLB; n = 3) for each season, and compared to the dissolved hydrocarbons (µg/l) in microcosms (mean ± SE; when error bars are not visible, SE was close to 0)

TPH in whole tissues of mussels (µg/g, dw) was significantly different from controls in summer and autumn, regardless of the type of oil. In winter, only TPH measured in mussels exposed to HEI- and CLB-WAF was significantly different from controls.

In summer, TPH in mussels differed significantly between those held in CLB and AWB mesosocosms when dissolved hydrocarbons were ~13 and 30 µg/l respectively, which can be explained by lower concentrations in CLB-WAF microcosms. In autumn, TPH in mussel tissues increased with dissolved hydrocarbons, except for CLB-WAF contamination. In autumn, at higher concentration of dissolved hydrocarbons (> ~12 µg/l), TPH of mussels held in HEI mesoscosms differed significantly from those held in AWB and CLB ($p < 0.05$). TPH in mussels exposed to HEI-WAF was significantly higher than in those exposed to

dilbits, between 10 – 20 µg/l of dissolved hydrocarbons in microcosms. The same observation was made in winter, that is at higher concentration of dissolved hydrocarbons, TPH of mussels held in HEI mesoscosms differed significantly from those held in AWB and CLB. Dissolved hydrocarbons had an effect on TPH of mussels held in HEI at levels greater than 8 µg/l, compared to those held in AWB and CLB mesoscosms.

TPH in whole tissues of mussels were not significantly different between oil in summer, and higher for conventional oil than dilbits in autumn and winter. Significant differences were measured between seasons ($p<0.05$).

For the three oils, the proportion of Σ_{16}EPA-PAHs, Σ_{28}Alkylated-PAHs, Σ_{18}CZDTs, and Σ_{13}VOCs measured in mussel tissue was dependent on the season (Figure 24).

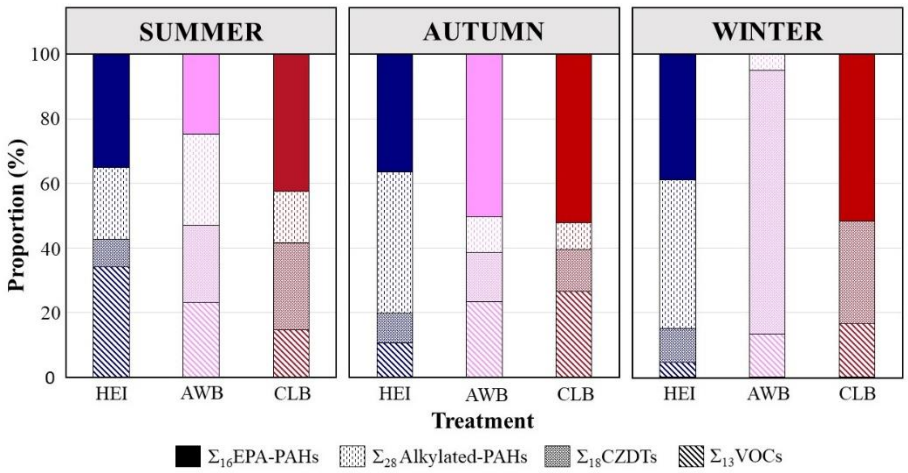

Figure 24 : Proportion of Σ_{28}Alkylated-PAHs, Σ_{16}EPA-PAHs, Σ_{18}CZDTs, and Σ_{13}VOCs (%) in whole tissues of *M. edulis* exposed to WAFs (HEI, AWB, and CLB) for each season

In mussel tissues exposed to HEI, the proportion of Σ_{16}EPA-PAHs and Σ_{18}CZDTs increased with decreasing temperature according to the season, and so between seasons, but the gap was small. The proportion of Σ_{28}Alkylated-PAHs increased too, especially between summer and autumn (22 to 44%), but with a small gap between autumn and winter (44 to 46%). Only the proportion of Σ_{13}VOCs decreased with the diminution of temperature. But for mussels exposed to dilbit, proportions of contaminants in their tissues were not the same. Contrary to HEI exposure, the proportion of Σ_{28}Alkylated-PAHs decreased with decreasing

temperature. The proportion of Σ_{18}CZDTs was higher in winter and lower in autumn. The two other compounds varied differently between the two dilbits. In mussel tissues exposed to AWB, the proportion of 1) Σ_{16}EPA-PAHs was higher in autumn and lower in winter, and 2) Σ_{13}VOCs represented the same proportion of between summer and autumn, and were lower in winter. In mussel tissues exposed to CLB, the proportion of 1) Σ_{16}EPA-PAHs was similar between autumn and winter, and lower in summer, and 2) Σ_{13}VOCs was higher in autumn and similar between summer and winter.

Physiological Parameters

A. Condition Index

The condition index (CI) varied between season for each treatment, but, at each season, no CI was under the control values (Figure 25).

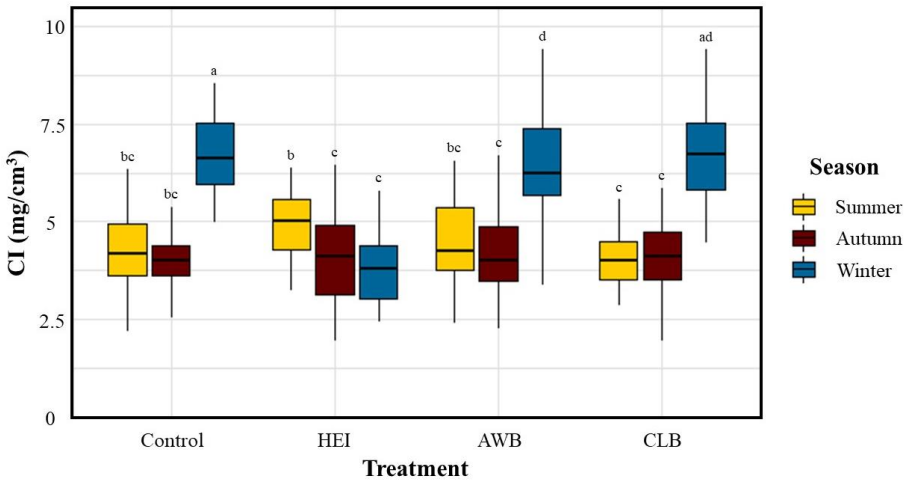

Figure 25 : Condition index (CI; mg/cm^3) of *M. edulis* in control (0%, v/v, WAF; n = 6) and WAF microcosms (HEI, AWB and CLB; n = 12) for each season (mean ± SE)

In summer, there was only a significant difference between HEI- and CLB-WAF. However, neither differed from control. In autumn, the CI was consistent between treatments. The CI was higher in winter except with HEI-WAF treatment, because a different batch of mussels was used for this experiment. As the overall health of the mussels appears to be higher in winter, and the concentrations in tissues were the lowest during this period, it was assumed that the impact is the lowest in this season.

B. Mortality

No mortality was observed in controls regardless of the season. For mussels exposed to WAF, no mortality was measured in winter and autumn. In summer, significant mortality was only measured for mussels exposed to 80% (v/v) of AWB-WAF (22%).

C. Clearance Rate

For the scope for growth (SFG) or its integrated metrics, the only significant difference was observed with the clearance rate (CR; Figure 26). In summer, no significant difference was measured, regardless of the type of oil.

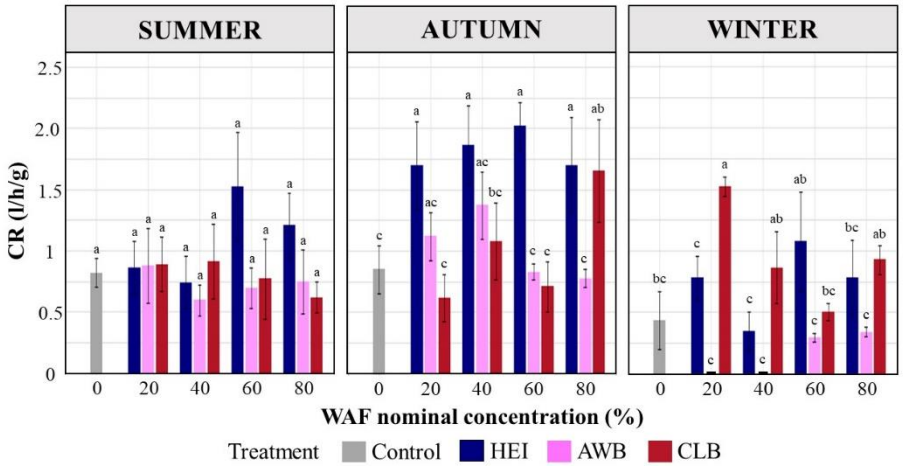

Figure 26 : Clearance rate (CR; l/h/g) in control (0%, v/v, WAF; n = 6) and WAF microcosms (HEI, AWB, and CLB; 20 to 80%, v/v; n = 3) for each season (mean ± SE)

In autumn, a significant increase in the CR compared to control was observed in mussels exposed to HEI-WAF for all nominal dilutions (20 to 80%, v/v), but not between them. A significant increase in the CR was also measured with CLB-WAF, but only at 80% (v/v). In autumn, the CR of mussels exposed to HEI-WAF was higher than AWB-WAF and CLB-WAF, from 60 to 80% (v/v) and from 20 to 60% (v/v), respectively. In winter, a significant increase in the CR compared to control was observed in mussels exposed to CLB-WAF at 20% (v/v), where it was significantly higher than for mussels exposed to HEI- or AWB-WAF.

Cellular Parameters

A. Lysosomal Membrane Destabilization

For each season, a significant difference was measured between control and mussels exposed to WAF, but for different nominal dilutions (Figure 27).

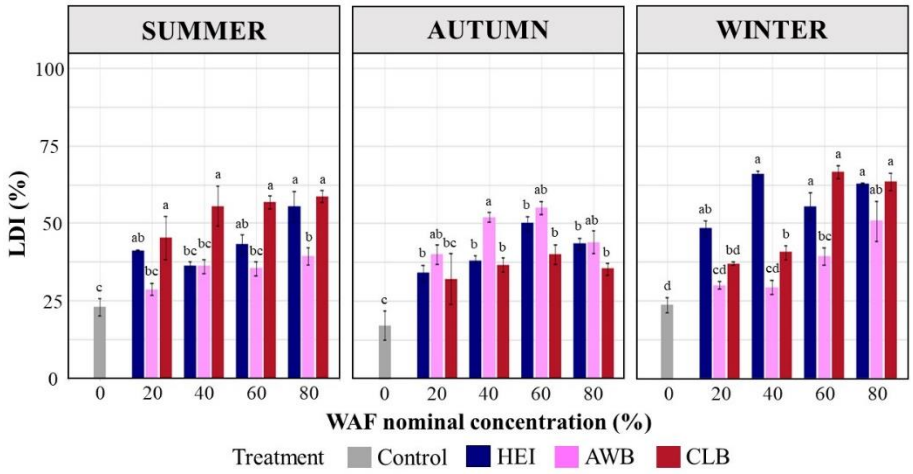

Figure 27 : Lysosomal destabilization index (LDI; %) in controls (0%, v/v WAF; n = 6) and WAF microcosms (HEI, AWB, and CLB; 20 to 80%, v/v; n = 3) for each season (mean ± SE)

For mussels exposed to HEI-WAF, the lysosomal membrane was destabilized from 20% (v/v) of WAF for each season, except summer at 40%, for TPH between 0.23 ± 0.04 and 0.47 ± 0.01 µg/g. With dilbit exposure, significant impacts significantly higher than control were measured at different WAF dilution according to the season. For AWB-WAF exposure, from 80% (v/v) in summer, 20% (v/v) in autumn and 60% (v/v) in winter, for TPH between 0.03 ± 0.01 and 1.00 ± 0.28 µg/g. For CLB-WAF, from 20% (v/v) in summer, and 40% (v/v) in autumn and winter, for TPH between 0.15 ± 0.02 and 0.56 ± 0.15 µg/g. There is no significant difference between WAF dilutions (20 to 80%), except for dilbit (AWB or CLB) exposure in winter.

In summer, the destabilization of the lysosomal membrane was higher for mussels exposed to CLB-WAF compared to AWB-WAF at all nominal dilutions, and to HEI-WAF at 40% (v/v). This value was higher for HEI- than AWB-WAF, only at 80% (v/v). In autumn,

a significant difference was measured between oil only at 40% of WAF, where mussels exposed to AWB-WAF showed a higher percentage of destabilization of the lysosomal membrane. All treatments were also significantly higher than controls, except CLB at 20%. In winter, the destabilization of the lysosomal membrane significantly increases from 60% (v/v) CLB-WAF and 60% (v/v) AWB-WAF. The LDI was higher for mussels exposed to HEI-WAF compared to dilbits at 40% (v/v). At 40 and 60% (v/v), the LDI was lower for mussels exposed to AWB-WAF, than for those exposed to HEI- or CLB-WAF.

B. Cell Viability

For each season, a significant difference was measured between control and mussels exposed to WAF, but for different nominal dilutions (Figure 28).

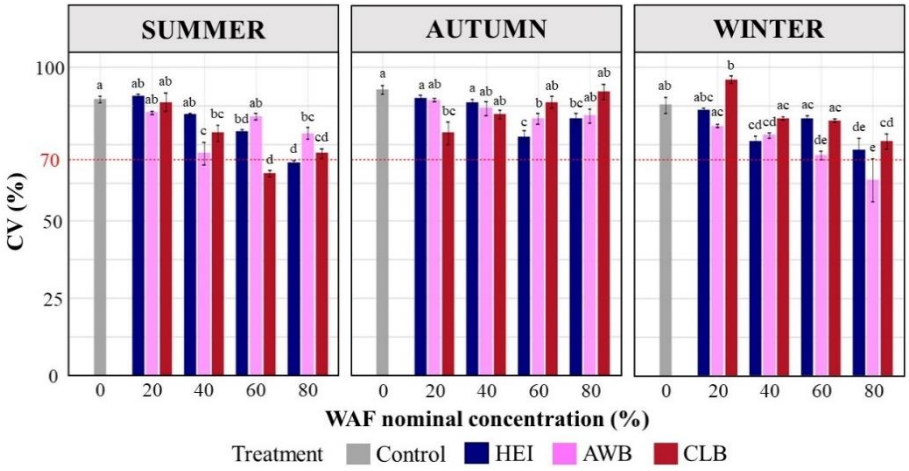

Figure 28 : Cell viability (CV; %) in control (0%, v/v WAF; n = 6) and WAF microcosms (HEI, AWB, and CLB; 20 to 80%, v/v; n = 3) for each season (mean ± SE; the red arrow (70%) shows the threshold value of cell viability recommended for comet assay)

In summer, CV decreased significantly, compared to control, from 40 and 60% (v/v) for CLB- (TPH: 0.50 ± 0.02 µg/g) and HEI-WAF (TPH: 0.81 ± 0.06 µg/g) respectively, and for two dilutions of AWB-WAF, 40 (TPH: 0.63 ± 0.04 µg/g) and 80%, v/v (TPH: 1.00 ± 0.28 µg/g). At 40% (v/v), only CV for mussels exposed to AWB-WAF was significantly lower than those exposed to HEI-WAF, and the opposite was measured at 80% (v/v). The lowest CV percentages were measured for CLB-WAF at 60% (v/v). In this season, CV decreased under the cellular viability rate for comet assay (70%), when mussels were exposed to HEI-

(80%, v/v, TPH: 0.66 ± 0.01 µg/g) or CLB- (60%, v/v, TPH: 0.61 ± 0.20 µg/g) WAF. In autumn, CV was significantly lower than controls for a few WAF nominal dilutions: 20% (v/v; TPH: 0.36 ± 0.08 µg/g) of CLB-WAF, 60% (v/v; TPH: 0.47 ± 0.05 µg/g) of AWB-WAF, and 60 - 80% (v/v; TPH: 0.87 ± 0.05 - 0.93 ± 0.15 µg/g) for HEI-WAF. Significant differences were measured between HEI-WAF and dilbits-WAF, but not between the two dilbits. Compared to HEI-WAF, CV was the lowest for dilbits-WAF at 20% (v/v) and the highest at 60% (v/v). In winter, CV decreased significantly, compared to control, with 40 and 80% (v/v; TPH: 0.40 ± 0.05 and 0.43 ± 0.07 µg/g) of HEI-WAF, from 40% (v/v; TPH: 0.08 ± 0.05 µg/g) of AWB-WAF, and only with 80% (v/v; TPH: 0.06 ± 0.01 µg/g) of CLB-WAF. Some significant differences were measured between exposure to different oils: at 20 and 80% (v/v), CV was higher for mussels exposed to CLB-WAF compared to AWB-WAF; and at 60% (v/v), CV was lower for AWB-WAF compared to the two other oils. In this season, CV decreased under the cellular viability rate for comet assay (70%), when mussels were exposed to AWB-WAF (80%, v/v; TPH: 0.05 ± 0.01 µg/g).

Genotoxicity

DNA impacts measured by tail DNA were observed on mussels exposed to WAF (Figure 29).

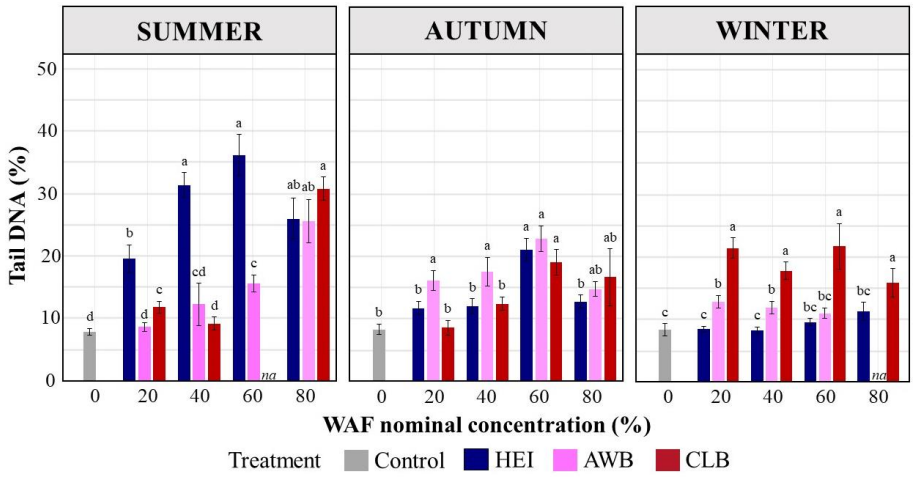

Figure 29 : Tail DNA (%) in control microcosm (0%, v/v, WAF; n = 6) and WAF microcosms (HEI, AWB, and CLB; 20 to 80%, v/v; n = 3) for each season (mean ± SE). When the cell viability is less than 70%, the results of the comet assay are not presented (noted *na*)

Differences were significant from controls for: HEI-WAF in summer (from 20%, v/v; TPH: 0.46 ± 0.14 µg/g) and autumn (at 60%, v/v; TPH: 0.93 ± 0.15 µg/g); AWB-WAF in summer (from 60%, v/v; TPH: 0.68 ± 0.11 µg/g), autumn (from 20 to 60%, v/v; TPH: 0.20 ± 0.03 to 0.47 ± 0.05 µg/g), and winter (from 20 and 40%, v/v; TPH: 0.06 ± 0.02 to 0.07 ± 0.05 µg/g); and CLB-WAF in summer (at 80%, v/v; TPH: 0.51 ± 0.06 µg/g), autumn (at 60%, v/v; TPH: 0.26 ± 0.02 µg/g), and winter (from 20%, v/v; TPH: 0.19 ± 0.02 µg/g).

In summer, tail DNA increased significantly for WAF dilutions from 40% (v/v) of HEI-WAF and from 80% (v/v) of AWB- or CLB-WAF. In summer, DNA degradation was greater in mussels exposed to HEI-WAF than for the two dilbits, except at 80% (v/v), at which the impacts were similar between the three oils. In autumn, tail DNA did not increase between the different dilutions for AWB-WAF. The same pattern was observed for HEI- and CLB-WAF, except at 60% (v/v). In winter, tail DNA did not increase between the different WAF dilutions for the three oils, however CLB was consistently higher at all dilutions than any other. In autumn and winter, the impacts on the DNA of mussels exposed to HEI-WAF were either similar or lower than those on mussels exposed to dilbit.

Proportion of tail DNA categories informed the impact level (Figure 30).

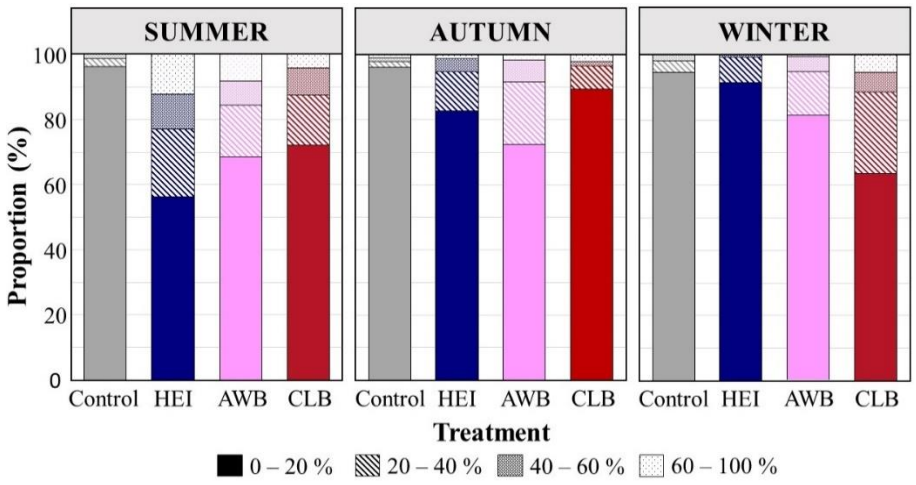

Figure 30 : Proportion (%) of the four categories of tail DNA (0-20, 20-40, 40-60 and, 60-100%) in hemocytes of *M. edulis* in control microcosms (n = 6) and WAF microcosms (HEI, AWB and CLB; n = 12) for each season

In autumn and winter, the proportion of different categories of tail DNA allows to highlight that the longest tails were measured after dilbit exposure, despite the same percentage of tail DNA. The opposite was observed in summer.

Canonical Correlation Analysis

The canonical correlation analysis (CCA) was performed to explore the relationships between a first set of variables considered as "dependent" variables (biometric measurements) and a second set considered as "independent" variables (tissue concentration of contaminants; Figure 31). Detail of statistical results is in Supplementary Data.

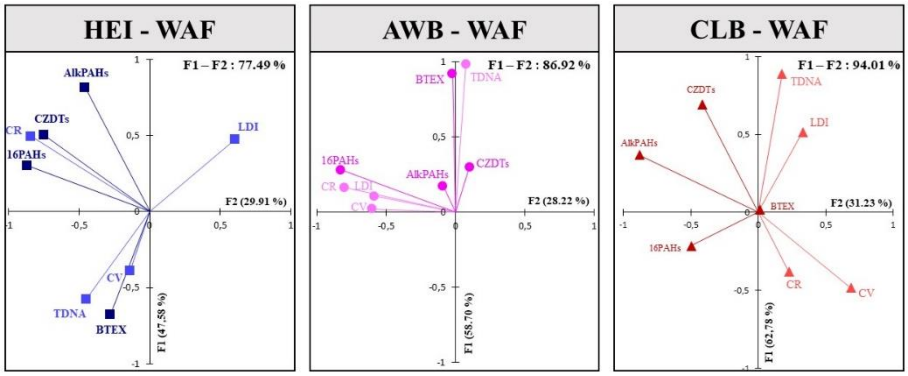

Figure 31 : Canonical correlation analysis (n = 36) showing the relationship between different biometric measurements and contaminant concentrations in *M. edulis* tissues exposed to WAF (HEI, AWB, or CLB)

CR = clearance rate; LDI = lysosomal destabilization index; CV = cell viability; TDNA: tail DNA; VOCs: Σ_{13}VOCs; 16PAHs: Σ_{16}EPA-PAHs; AlkPAHs: Σ_{28}Alkylated-PAHs; CZDTs: Σ_{18} carbazole/dibenzothiophene

In HEI- and AWB-WAF, a strong positive correlation (> 0.5) was measured between some biological parameters, and hydrocarbon concentrations in mussels: clearance rate and Σ_{16}EPA-PAHs, and tail DNA and Σ_{13}VOCs. Correlations were weak ($-0.5 < x < 0.5$) in mussels exposed to CLB-WAF. Cellular impacts tested in our study (lysosomal membrane destabilization and cell viability) seem to not be correlated with the hydrocarbon concentrations measured in mussel tissues.

2.6.2 Chemical Dispersion and Dispersant Alone

Water analysis

A. CEWAF

The concentrations of the aromatic hydrocarbons in HEI, AWB, and CLB microcosms were strongly correlated to CEWAF nominal dilutions (%, v/v; Figure 32), with R^2 values > 0.9 ($p<0.05$; Pearson test). Aromatic hydrocarbons concentrations in CEWAF microcosms were always significantly different from the controls regardless of the season, except in summer with 0.1% (v/v) of AWB-CEWAF (Figure 32).

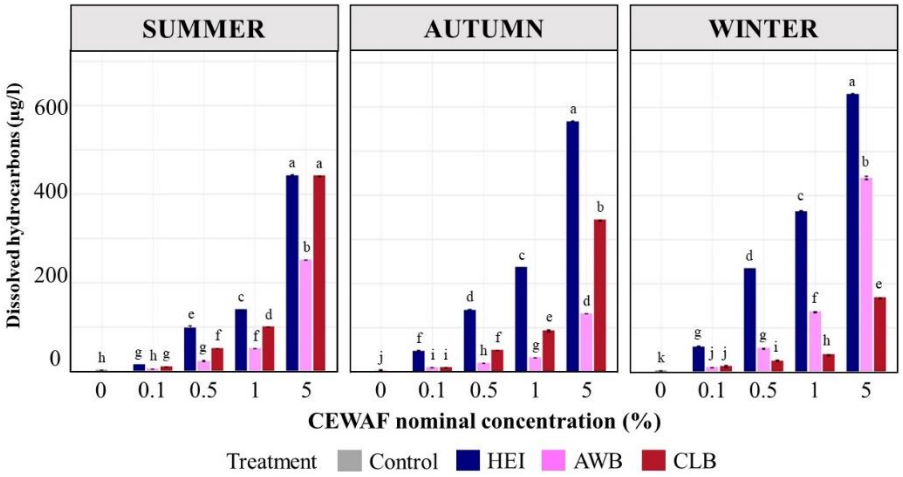

Figure 32 : Fraction of dissolved hydrocarbons measured by fluorescence with the Cyclops-7 optical sensor in CEWAF (HEI, AWB, and CLB; 0.1 to 5%, v/v; n = 3), and control microcosms (0%, v/v; n = 6) for each season (mean ± SE; when error bars are not visible, SE was close to 0)

The concentrations were higher in HEI-CEWAF microcosms compared to those with either dilbit for all CEWAF dilutions (0.1 to 5%, v/v) in autumn and winter, and 0.5 and 1% (v/v) in summer. For the other two dilbits (CLB, AWB), no significant differences were measured at 0.1% (v/v) of CEWAF, but from 0.5% (v/v) aromatic hydrocarbons concentrations in CLB-CEWAF microcosms were higher than the AWB-CEWAF ones in summer and autumn, and the opposite in winter.

B. Dispersant Solution

The concentrations of the aromatic hydrocarbons were strongly correlated to CXT nominal concentrations (Figure 33), with R^2 values > 0.9 ($p < 0.05$; Pearson test).

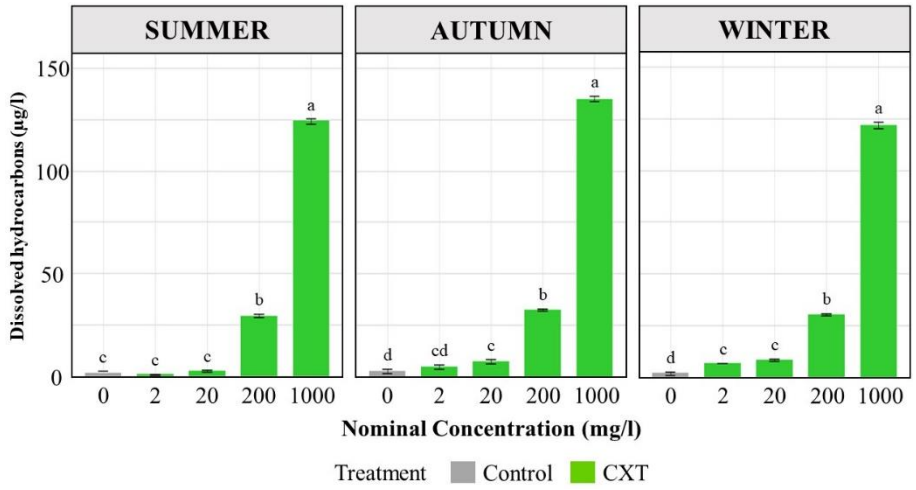

Figure 33 : Fraction of dissolved hydrocarbons measured by fluorescence with the Cyclops-7 optical sensor in CXT (2 to 1 000 mg/l; n = 3), and control microcosms (0 mg/l, v/v; n = 6) for each season (mean ± SE; when error bars are not visible, SE was close to 0)

In CXT microcosms, the aromatic hydrocarbon concentrations were significantly different from controls from different nominal concentration, according to the season: from 2 mg/l in winter, 20 mg/l in autumn, and 200 mg/l in summer.

Tissues Analysis

A. CEWAF

The total petroleum hydrocarbons concentration (TPH; µg/g, dw) in whole tissues of mussels (Figure 34) was significantly correlated with dissolved hydrocarbons in microcosms contaminated in summer and autumn by HEI ($R^2 > 0.7$; $p < 0.05$; Pearson test) or by CLB ($R^2 > 0.8$; $p < 0.05$; Pearson test). Lower correlation was measured with AWB-CEWAF exposure ($R^2 > 0.4$; $p < 0.05$; Pearson test) and only in autumn. In winter, no correlation was measured, regardless of the type of oil.

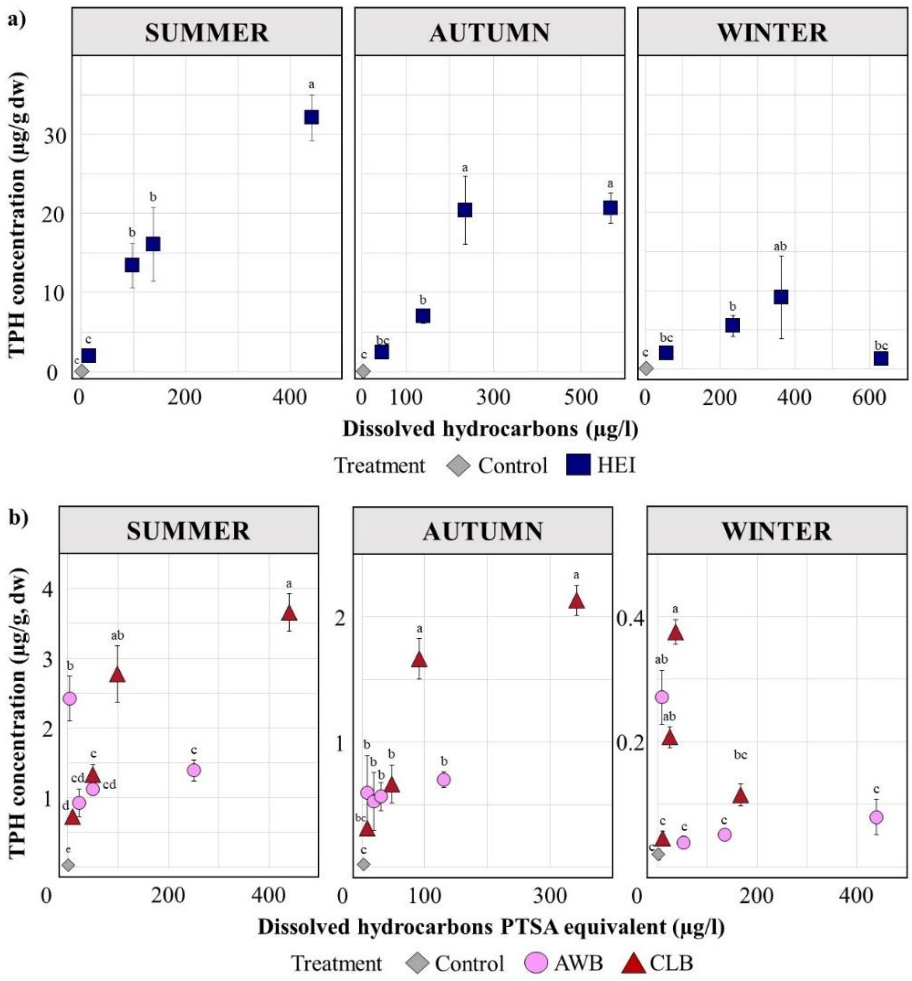

Figure 34 : Total petroleum hydrocarbons concentration (µg/g, dw) in whole tissues of *M. edulis*, measured by GC-MS, *vs*. the dissolved hydrocarbon concentrations (µg/l) in control microcosms (0%, v/v, CEWAF; n = 6) and CEWAF microcosms a) HEI (n = 3), b) AWB and CLB (n = 3) for each season (mean ± SE; when error bars are not visible, SE was close to 0)

TPH in whole tissues of mussels exposed to HEI-CEWAF was significantly different from controls from a dissolved hydrocarbons concentration of 100 µg/l in microcosms, regardless of season. For dilbit exposure (AWB or CLB), there was some significant difference from controls, but no with TPH concentration in microcosms < 100 µg/l. TPH increased with CEWAF nominal dilutions in mussel tissues exposed to HEI-CEWAF. In general, TPH concentrations in mussel tissues exposed to HEI-CEWAF were significantly higher than in mussel tissues exposed to the two dilbits from 100 µg/l of hydrocarbons in

microcosms. Between dilbits, TPH concentrations was higher for mussels exposed to CLB compared to AWB, at the higher dissolved hydrocarbon concentration in summer and autumn. Furthermore, the total hydrocarbons concentration measured in tissues of mussels exposed to HEI-WAF was under 1 µg/g (dw), while it was under 35 µg/g (dw) in tissues of mussels exposed to HEI-CEWAF. Such a difference was not observed between WAF and CEWAF prepared with dilbit (AWB or CLB).

B. Dispersant Solution

The TPH concentrations in whole tissues of mussels (Figure 35) were significantly correlated with dissolved hydrocarbons in microcosms contaminated to dispersant solution in summer and autumn ($R^2 > 0.7$; $p < 0.05$; Pearson test).

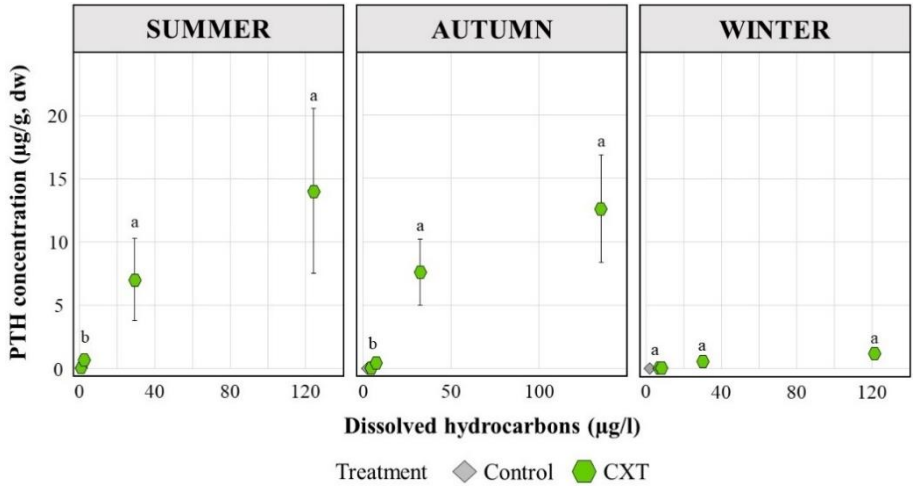

Figure 35 : Total petroleum hydrocarbons concentration (µg/g, dw) in whole tissues of *M. edulis*, measured by GC-MS, *vs*. the dissolved hydrocarbon concentrations (µg/l) in control microcosms (0%, v/v, CEWAF; n = 6) and CXT (2 to 1000 mg/l; n = 3) for each season (mean ± SE; when error bars are not visible, SE was close to 0)

In whole tissues of mussels exposed to dispersant alone (CXT), TPH concentration was significantly different from control, but only from a dissolved hydrocarbons concentration superior to 40 µg/l in microcosms in summer and autumn. No significant difference from control was measured in winter.

C. Total Hydrocarbon Proportion

Like for mussels exposed to WAF, the proportion of Alkylated-PAHs, EPA-PAHs, CZDTs, and VOCs measured in mussel tissue was dependent on the season (Figure 36).

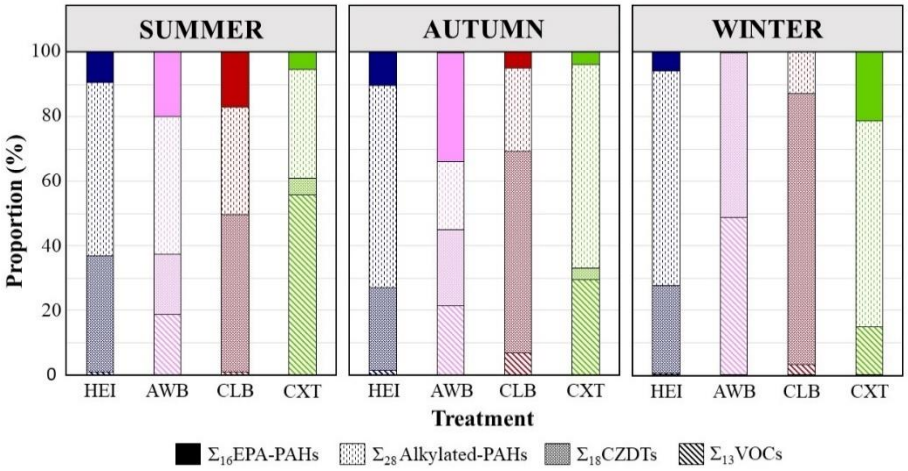

Figure 36 : Proportion of Σ_{28}Alkylated-PAHs, Σ_{16}EPA-PAHs, Σ_{18}CZDTs, and Σ_{13}VOCs (%) in tissue of *M. edulis* exposed to CEWAFs (HEI, AWB, and CLB) or dispersant solution (CXT) for each season

The proportion was different between the four treatments, regardless of season. In mussel tissue exposed to the HEI-CEWAF, the same pattern was observed between the three seasons: Σ_{28}Alkylated-PAHs > Σ_{18}CZDTs > Σ_{16}EPA-PAHs. The concentration of Σ_{13}VOCs was not under the limit of detection but was very low (< 1.5%). In mussel tissue exposed to AWB-CEWAF, the proportion pattern was different at each season. In summer, the highest proportion was Σ_{28}Alkylated-PAHs, Σ_{16}EPA-PAHs, and Σ_{18}CZDTs, and the lowest was Σ_{18}CZDTs, Σ_{28}Alkylated-PAHs, and Σ_{16}EPA-PAHs in summer, autumn and winter, respectively. In mussel tissue exposed to CLB-CEWAF, the highest proportion was Σ_{18}CZDTs and the lowest was Σ_{13}VOCs, regardless of the season. And finally, in mussel exposed to CXT, the lowest proportion was Σ_{18}CZDTs, regardless of the season. Values were equally low for Σ_{16}EPA-PAHs in summer and autumn. The highest proportion was Σ_{13}VOCs in summer and Σ_{28}Alkylated-PAHs in autumn and winter.

Physiological Parameters

A. Condition Index

In each season, no significant difference was measured for the condition index (CI) between treatments and nominal dilutions (CEWAF) or concentrations (CXT) (Figure 37).

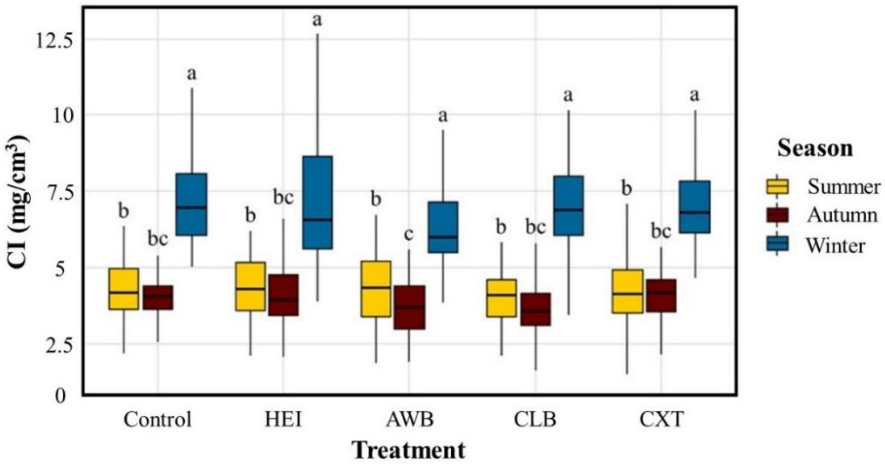

Figure 37 : Condition index (CI; mg/cm^3) of *M. edulis* in control microcosms (n = 6) CEWAF and dispersant solution microcosms (HEI, AWB, CLB, and CXT; n = 12) for each season (mean ± SE)

For all conditions, the quality of the mussels used was consistent with each season, but the CI varied seasonally: the CI in winter was significantly higher than in autumn and summer. For mussels exposed to AWB-CEWAF, CI was significantly different between the three seasons, which was not observed for those exposed to HEI-, CLB-CEWAF, or CXT.

B. Mortality

No mortality was observed in controls in each season. In summer, mortality was significantly higher than control with > 60% at the highest nominal concentrations (5%, v/v, or 200, 1000 mg/L) of AWB-CEWAF (67%), CLB-CEWAF (78%), and CXT (respectively 33 and 61%). In autumn, no mortality was measured.

C. Clearance Rate

Like in WAF exposure, the only significant difference was observed with the clearance rate (CR; Figure 38) for the scope for growth (SFG) or its integrated metrics, except in winter for mussels exposed to CEWAF, where the CR was not different from control, regardless of the type of oil.

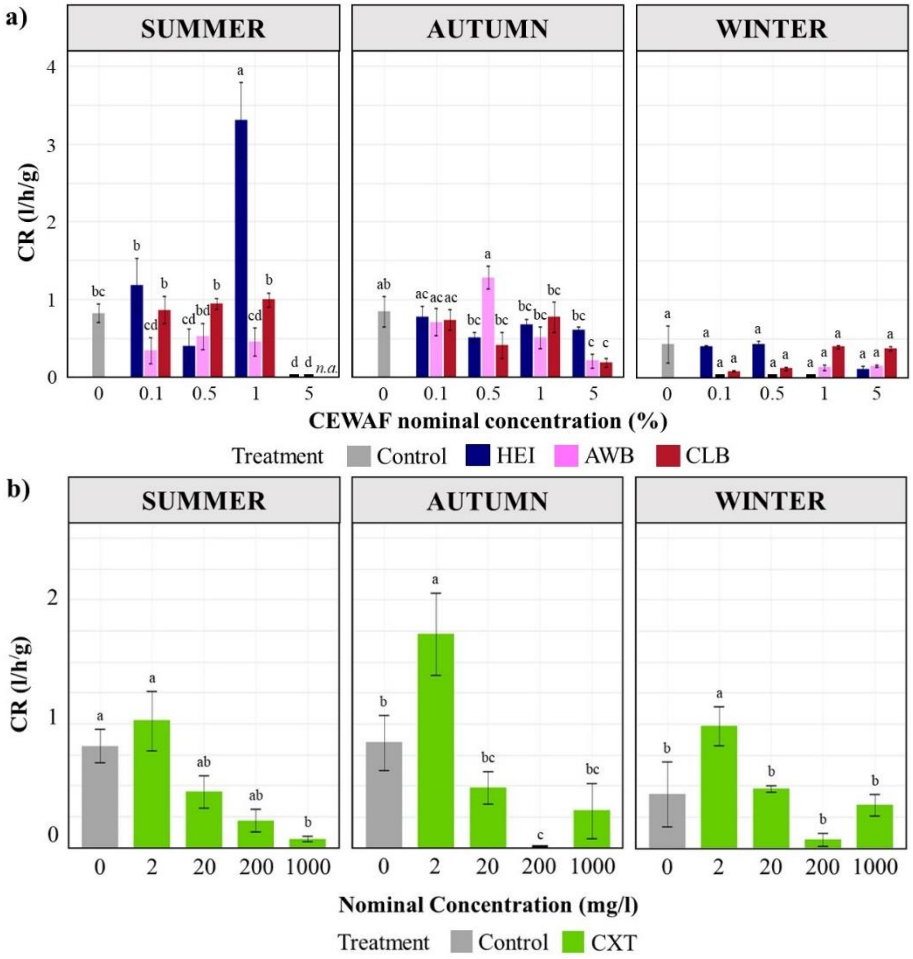

Figure 38 : Clearance rate (CR; l/h/g) in controls microcosms (0%, v/v, CEWAF or 0 mg/l; n = 6), and a) CEWAF (HEI, AWB, and CLB; 0.1 to 5%, v/v; n = 3), b) CXT (2 to 1 000 mg/l; n = 3) for each season (mean ± SE; *n.a.*: CR not measured because of high mortality)

In summer, a significant increase was measured at 1% of HEI-CEWAF and a decrease at 5% of HEI-, and AWB- CEWAF (Figure 38a). In autumn, a significant decrease in the CR was observed in mussels exposed at 5% (v/v) to AWB- and CLB-CEWAF. However, no significant difference was measured with HEI-CEWAF exposure. For mussels exposed to the dispersant solution (Figure 38b), the CR decreased significantly with 1000 or 200 mg/l in summer and autumn, respectively. A significant increase was measured at 2 mg/l in autumn and winter.

Cellular Parameters

B. Lysosomal Membrane Destabilization

The CEWAF contamination resulted in a destabilisation of lysosomal membranes in mussels (Figure 39). In each season, significant differences were measured between controls and mussels exposed to CEWAF, except for some dilutions: AWB-CEWAF in summer at 1 and 5% (v/v) and in winter at 0.1 and 0.5% (v/v). In mussels exposed to dispersant solution (CXT), significant differences from controls were measured in autumn and winter, from 200 mg/l, for TPH of 7.61 ± 2.60, and 0.59 ± 0.22 µg/g, respectively, but not in summer.

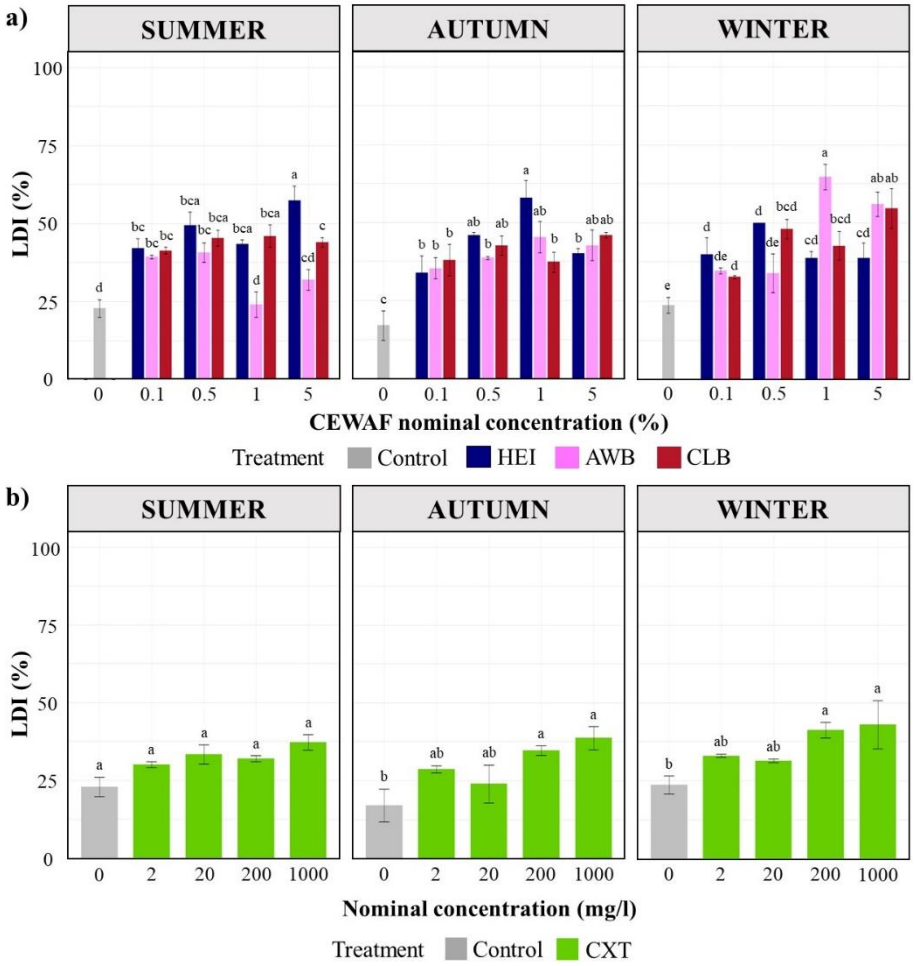

Figure 39 : Lysosomal destabilization index (LDI; %) in a) CEWAF (HEI, AWB, and CLB; 0.1 to 5%, v/v; n = 3), b) CXT (2 to 1 000 mg/l; n = 3), and control microcosms (0%, v/v, CEWAF or 0 mg/l CXT; n = 6) for each season (mean ± SE)

The lysosomal destabilization increased significantly when mussels were exposed to HEI-CEWAF in summer (5%, v/v; TPH: 32.11 ± 2.91 µg/g) and autumn (1%, v/v; TPH: 20.36 ± 4.32 µg/g), and to dilbits in winter, from 1% (v/v; TPH: 0.05 ± 0.004 µg/g) of AWB-CEWAF and 5% (v/v; TPH: 0.38 ± 0.10 µg/g) of CLB-CEWAF. In contrast to WAF exposure, a decrease was observed with increasing dilution of HEI-CEWAF in autumn and AWB-CEWAF in summer. Higher lysosomal destabilization was measured in mussels exposed to HEI-CEWAF in summer and autumn and to AWB-CEWAF in winter.

As with WAF exposure, significant differences between seasons were measured. AWB-CEWAF exposure (1-5%; v/v) in winter was globally more damaging to lysosomes than in summer. With 1% (v/v) of HEI-CEWAF, the lysosomal destabilization was higher in autumn than in winter and, with 5% (v/v), the lysosomal destabilization was higher in summer than in autumn or winter. For mussels exposed to dispersant solution, no significant differences between seasons were measured.

D. Cell Viability

In summer and winter, a significant difference in cell viability was measured between the control group and mussels exposed to CEWAF, but for different nominal dilutions (Figure 40 a). In summer and winter, a significant difference was measured between control and mussels exposed to CXT in summer (2 and from 200 mg/l; TPH: 0.07 ± 0.03, and from 7.02 ± 3.25 µg/g) and autumn (1000 mg/l; TPH: 12.61 ± 4.26 µg/g), but not in winter (Figure 40 b).

In summer, CV decreased significantly after exposure to 5% (v/v) of CEWAF regardless of the oil. At this dilution, the impact was significantly more important with dilbits than conventional oil. In this season, CV decreased under the cellular viability rate for comet assay (70%), when mussels were exposed to dilbit (AWB, CLB, 5%, v/v; TPH: 1.39 ± 0.15, and 3.66 ± 0.27 µg/g, respectively) or CXT (1000 mg/l, v/v; TPH: 14.04 ± 6.51 µg/g). In winter, CV decreased significantly from 1% (v/v) of AWB- (TPH: 0.05 ± 0.004 µg/g), and HEI-CEWAF (TPH: 9.10 ± 5.27 µg/g), and 5% (v/v) of CLB-CEWAF (TPH: 0.12 ± 0.08 µg/g). With AWB-CEWAF, a greater decrease was measured at 1 and 5% (v/v) compared to

CLB-, and at 5% (v/v) to HEI-CEWAF. In this season, CV decreased under the cellular viability rate for the comet assay (70%), when mussels were exposed to AWB- (1-5%, v/v) or CLB- (5%, v/v) CEWAF.

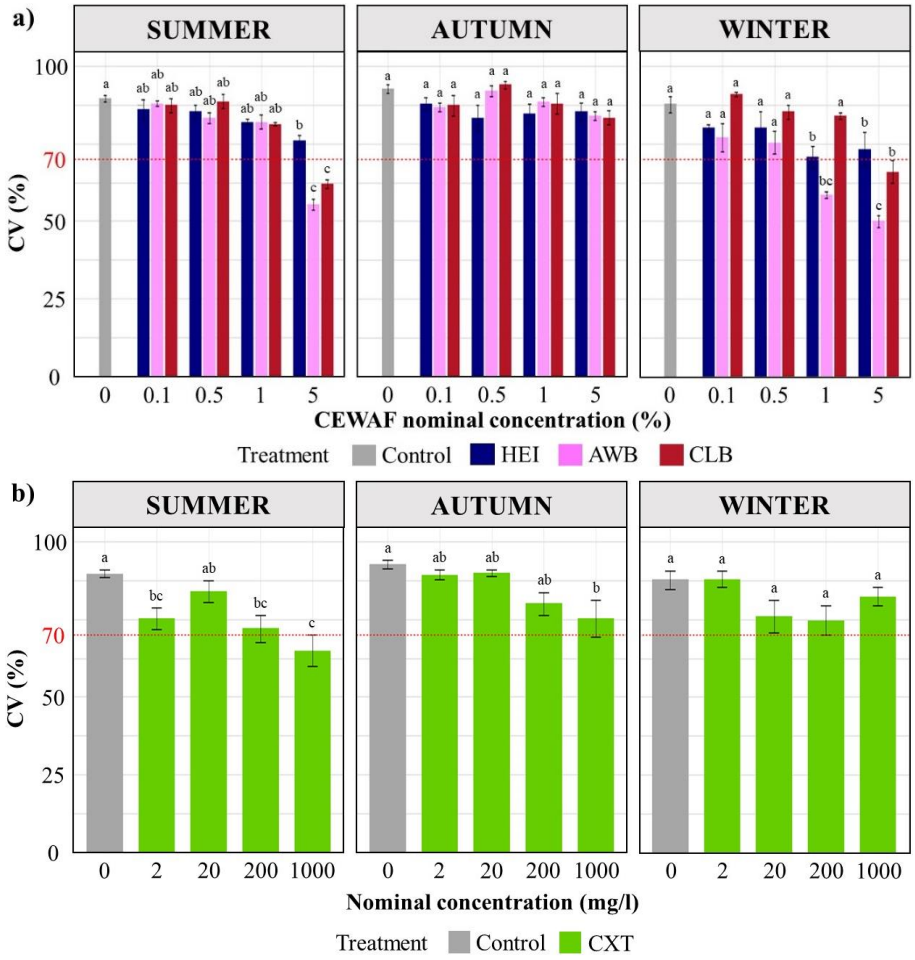

Figure 40 : Cell viability (CV; %) in a) CEWAF (HEI, AWB, and CLB; 0.1 to 5%, v/v; n = 3), b) CXT (2 to 1 000 mg/l; n = 3) and control microcosms (0%, v/v, CEWAF or 0 mg/l CXT; n = 6) for each season (mean ± SE)

As with WAF exposure, significant differences in cell viability were measured between the three seasons with different nominal dilutions. When the CV was significantly different, the lowest percentage was measured in winter and the highest in autumn for the three types of oils. With dispersant solution, a few significant differences of cell viability were measured between the three seasons depending on nominal concentrations. When the CV was

significantly different, the lowest percentage was measured in summer (1000 mg/l, v/v) and the highest in autumn (2 and 20 mg/l, v/v).

Genotoxicity

DNA impacts measured by tail DNA (%) were significantly different to controls for mussels exposed to CEWAF or CXT (Figure 41).

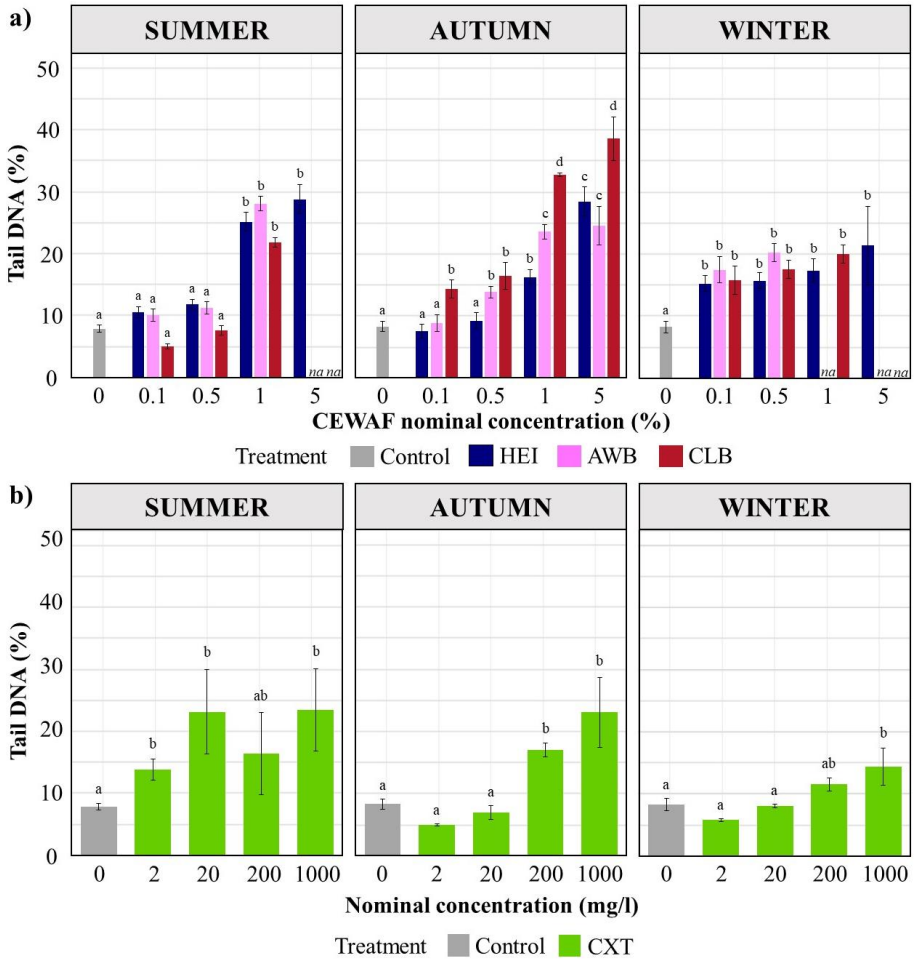

Figure 41 : Tail DNA (%) in a) CEWAF microcosms (HEI, AWB, and CLB; 0.1 to 5%, v/v; n = 3), b) CXT (2 to 1 000 mg/l; n = 3) and control microcosms (0%, v/v, CEWAF or 0 mg/l CXT; n = 6) for each season (mean ± SE)

DNA degradations were significantly different from controls from 1% (v/v) of CEWAF for the three oils in summer and in autumn, while in winter this difference was measured from 0.1% (v/v) of HEI- (TPH: 2.02 ± 0.83 µg/g), AWB-CEWAF (0.27 ± 0.24 µg/g), and of

CLB-CEWAF (TPH: 0.21 ± 0.05 µg/g). With CXT, DNA degradation increased compared to controls in the three seasons but for different nominal concentrations: from 2 mg/l in summer (0.07 ± 0.03 µg/g), 200 mg/l in autumn (TPH: 7.61 ± 2.60 µg/g), and 1000 mg/l in winter (TPH: 1.18 ± 0.16 µg/g).

In summer and winter, there are no significant differences to tail DNA between different CEWAF exposures. In autumn, higher DNA degradation was measured for mussels exposed to CLB-CEWAF (1-5%, v/v) than with HEI- or AWB-CEWAF, and higher DNA degradation to AWB- than HEI-CEWAF at 1% (v/v).

The proportion of tail DNA categories provided information on the DNA degradation level (Figure 42).

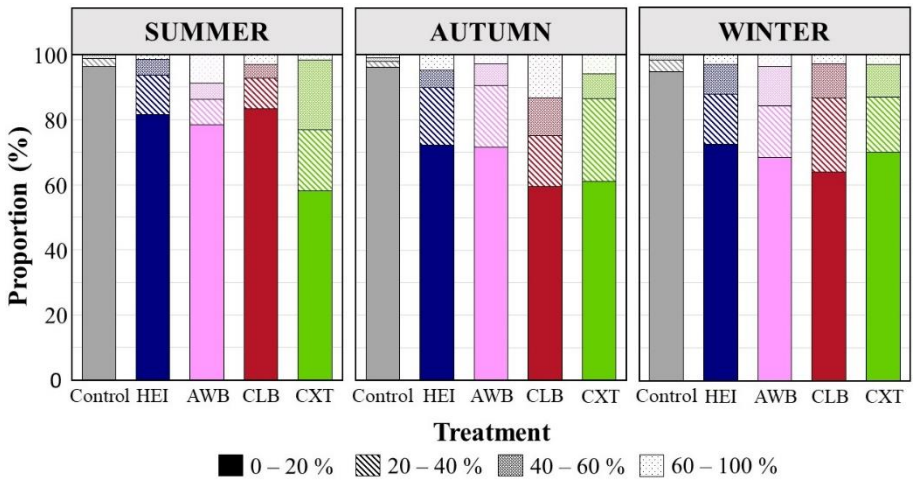

Figure 42 : Proportion (%) of the four categories of tail DNA (0-20, 20-40, 40-60 and, 60-100%) in hemocytes of *M. edulis* in control (n = 6) and CEWAF microcosms (HEI, AWB and CLB; n = 12) for each season

The proportion of DNA tail degradation above 20% was higher for CXT exposure in summer, and for CLB-CEWAF in autumn and winter. Percentages greater than 60% were predominantly measured with AWB-CEWAF in summer and winter, and CLB-CEWAF in autumn.

Canonical Correlation Analysis

The canonical correlation analysis (CCA) was performed to explore the relationships between a first set of variables considered as “dependent” variables (biometric measurements) and a second set considered as “independent” variables (tissue concentration of contaminants) (Figure 43). Detail of statistical results is in Supplementary Data.

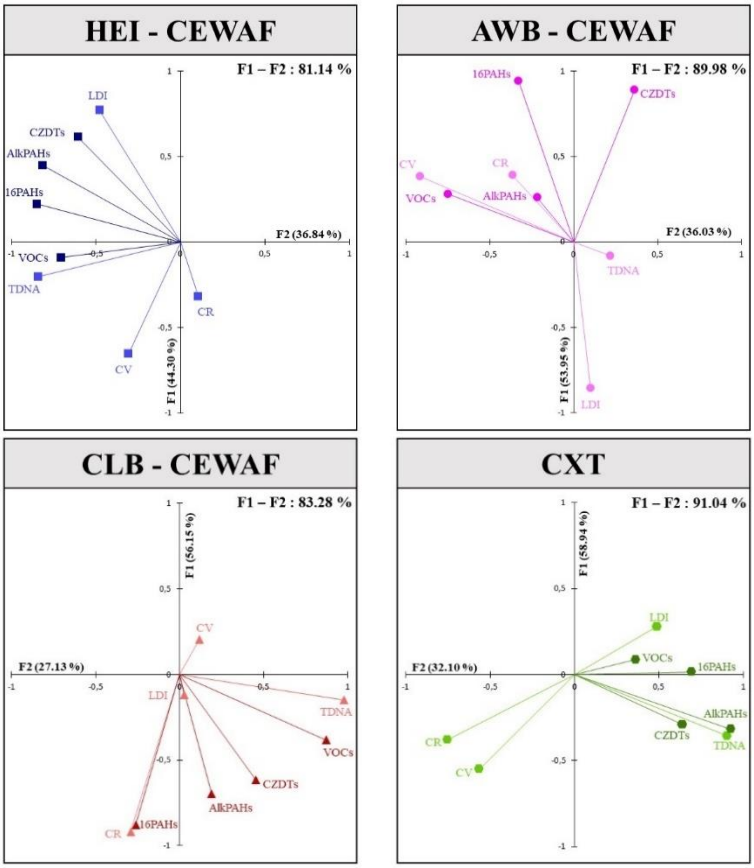

Figure 43 : Canonical correlation analysis (n = 36) showing the relationship between different biometric measurements and contaminant concentrations in *M. edulis* tissues exposed to CEWAF or dispersant solution

CR = clearance rate; LDI = lysosomal destabilization index; CV = cell viability; TDNA: tail DNA; VOCs: Σ_{13}VOCs; 16PAHs: Σ_{16}EPA-PAHs; AlkPAHs: Σ_{28}Alkylated-PAHs; CZDTs: Σ_{18} carbazole/dibenzothiophene dibenzothiophene

In HEI-CEWAF a strong positive correlation (> 0.5) was measured between lysosomal membrane destabilization (LDI) and hydrocarbon concentrations in mussels, Σ_{28}Alkylated-PAHs and Σ_{18}CZDTs. In CLB-CEWAF strong positive correlations (>0.5) were measured

between clearance rate (CR) and Σ_{16}EPA-PAHs, and DNA impact (TDNA) and Σ_{13}VOCs. For mussels exposed to AWB-CEWAF, correlations were weak between parameters. With the dispersant solution, a strong positive correlation was measured between tail DNA and Σ_{28}Alkylated-PAHs.

2.6.3 Integrated Biomarker Response

In order to better visualise the influence of the type of oil, the type of dispersion, and the season on the blue mussel, in case of a potential oil spill, integrated biomarker responses (IBRs) were established. Eight IBRs were calculated for each season by the averages of each respective condition: Control, WAF, CEWAF, and CXT for each oil and for each season, compared to the total hydrocarbon concentration measured in whole tissues of *M. edulis* (Figure 44).

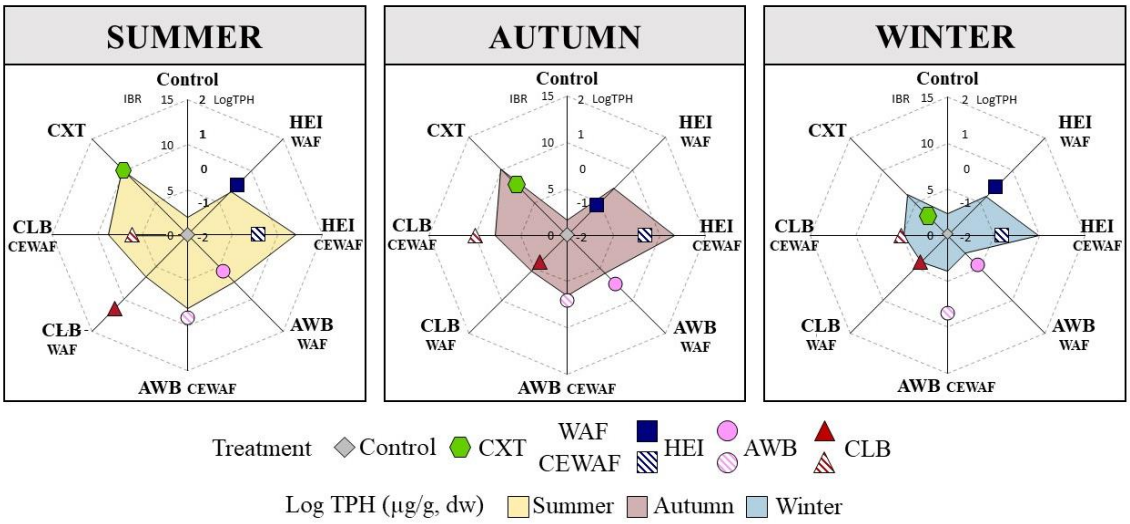

Figure 44 : Integrated biomarker response (IBR) measured with 5 biomarkers (mortality, clearance rate, lysosomal membrane destabilization, cell viability and DNA degradation) between exposure condition (Control, HEI, AWB, CLB, and CXT) for each season, compared to the mean of total hydrocarbon concentration (TPH; µg/g, dw) measured with all the nominal dilution or concentration.

Different patterns were observed between seasons. For each exposure, a higher IBR was observed compared to the control. In summer, the highest IBR was measured for mussels exposed to CLB-WAF despite the lowest TPH measured in tissues (except control), and the

lowest IBR was in the case of AWB-WAF (except control). In autumn, the highest IBR was measured for mussels exposed to CLB-CEWAF, and the lowest was for CLB-WAF (except control), which can be explained by the lowest TPH measured in mussels. In winter, the highest IBR measured for mussels exposed to AWB-CEWAF and the lowest (except control) was for CXT. At each season, despite higher TPH in mussels exposed to HEI-CEWAF, the IBR was not superior to all other conditions. The impact of dispersant solution alone was not negligeable, with an IBR superior to oil (WAF or CEWAF) exposure in summer and autumn. The only exceptions to this trend were CLB-WAF in summer, and HEI- and CLB-CEWAF in autumn. However, the IBR of CXT exposure was the lowest factor in winter. IBR was similar between WAF and CEWAF for exposure to conventional oil (HEI) in summer and winter, for AWB in autumn, and for CLB in winter. IBR of WAF conditions was superior to the CEWAF one for CLB in summer and AWB in winter. For the other conditions, the IBR was higher following an exposure to CEWAF than WAF.

The IBR value was dependent on the season for each treatment, but the lowest IBRs were always measured in autumn or winter, never in summer. To explain seasonal variation, IBRs were determined with control values (Figure 45).

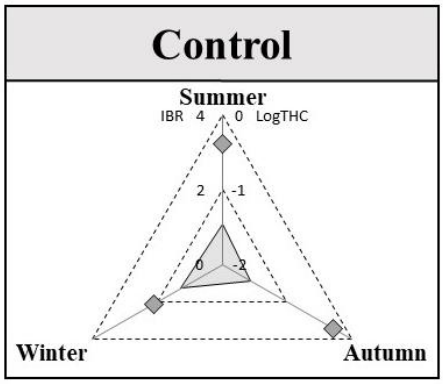

Figure 45 : Integrated biomarker response (IBR) of controls measured with 4 biomarkers (clearance rate, lysosomal membrane destabilization, cell viability and DNA degradation) on mussels *M. edulis* between season compared to the total hydrocarbon concentration (TPH; µg/g, dw).

The lowest IBR was measured in winter and the highest in autumn despite higher TPH in winter than in the two other seasons. The IBR in summer is only slightly lower than that in autumn.

2.7 DISCUSSION

WAF and CEWAF experiments reflected what could reasonably occur in the marine environment during a spill event, with dissolved our dispersed aromatic hydrocarbons concentrations between those measured after the Prestige (González *et al.*, 2006) and Deepwater Horizon (Sammarco *et al.*, 2013) incidents. Globally, in our study, aromatic hydrocarbon concentrations measured with the Cyclops-7 optical sensor were dependent on dispersion technique. Blue mussels, *M. edulis*, were exposed to aromatic hydrocarbon concentrations between 5 and 30 µg/l in WAF microcosms, while these concentrations were 5 times higher in CXT, and 10 to 20 times higher in CEWAF microcosms. The addition of chemical dispersant greatly increases the aromatic hydrocarbon concentrations as shown in many studies comparing WAF and CEWAF (Cohen and Nugegoda, 2000; Madison *et al.*, 2017; Robidoux *et al.*, 2018; McDonnell *et al.*, 2019). The high concentration of hydrocarbons can be attributed to the presence of oil droplets in CEWAF or increased dissolution of hydrocarbons following the increase in surface area (Ramachandran *et al.*, 2004; Lee *et al.*, 2013). Additionally, when facilitating oils' dispersion with Corexit® 9500A (CEWAF microcosms), the higher concentrations of dissolved hydrocarbons could be explained by the presence of hydrocarbons in dispersant itself, explaining a higher toxicity for mussels exposed to CEWAF than WAF. Indeed, hydrocarbons were already found in Corexit® 9500 because of its petroleum distillate fraction, but also due to the hydrocarbon side chains present in DOSS (Seidel *et al.*, 2016) and non-ionic surfactants (Word *et al.*, 2015). The petroleum distillate fraction of Corexit® 9500A consists of hydrocarbons (C_9 – C_{16}), including C_{10} cycloalkanes and iso- and n-paraffins (McFarlin *et al.*, 2018). Besides, differences between oils were observed for both dispersions. With physical dispersion, the hydrocarbon concentration was highest in microcosms contaminated with AWB, lower for HEI, then lowest for CLB. Bérubé *et al.* (2021) observed also differences in WAF according to the type of oil, with higher concentrations of PAHs in WAF prepared with a heavy conventional oil than dilbit, but lower VOCs and C_6 – C_{10} concentrations. Alsaadi *et al.* (2018 in supplementary data) also measured differences between dilbits, which can be explain by a

faster decrease of the hydrocarbon concentrations (PAHs, TPHs) for WAF prepared with CLB rather than AWB (Robidoux *et al.,* 2018). Furthermore, differences between WAFs may be explained by a fluorescence spectrum of crude oil dominated by the emission of resins and aromatics (Pantoja *et al.,* 2011). These occur in different concentrations in different oils, with higher levels of resins and aromatic compounds measured in dilbit and conventional oil, respectively. With chemical dispersion, hydrocarbon concentrations were generally higher in CEWAF prepared with HEI, AWB, then CLB, which indicates that the addition of chemical dispersant modified the dissolved hydrocarbons composition in microcosms compared to WAF. Contrary to the concentrations measured in the WAF microcosms, seawater contaminated with chemically dispersed CLB contained higher hydrocarbon concentrations than during contamination with AWB. This result is reversed in winter, following results of Berthod *et al.* (2022a), which shows a better efficiency of Corexit® 9500A on CLB compared to AWB at 5°C, and the opposite at 0°C. This result may explain the differences observed depending on oil type and seawater temperature. Belore *et al*. (2009) demonstrated a good efficiency of Corexit® 9500A on conventional oil even for cold temperatures, better than its efficiency on dilbits (Berthod *et al.*, 2022a).

Based on the aromatic hydrocarbon concentrations in water, one would expect to have different accumulations in the tissues depending on the type of oil and dispersion (physical or chemical). Aromatic hydrocarbon concentrations should be, in descending order, higher in mussel tissues exposed to CEWAF (HEI, then to diluted bitumen), to dispersant itself, then to WAF (AWB, HEI, then to CLB). The accumulation in mussel tissues followed this dispersion order, with generally more TPH in mussels exposed to CEWAF (< 35 µg/g), then to CXT (< 15 µg/g), and finally to WAF (< 1 µg/g). The difference between conventional oil and diluted bitumen was more significant with chemical (≃ 6 to 10 times higher) than physical (≃ 2 times higher) dispersion, explained by lower differences in water analysis in WAF than CEWAF. Furthermore, with CEWAF exposure, the accumulation in the tissues was effectively superior in mussels exposed to conventional oil than to diluted bitumen. But the TPH in mussel tissues exposed to WAF were higher after exposure to conventional oil

than to the two diluted bitumen, despite higher concentrations in AWB water microcosms. This result followed those of Schmutz *et al*. (2021) in winter.

The difference of accumulation in tissues between oil exposure could be explained by the hydrocarbon impact on clearance rate. Indeed, in some conditions (HEI-WAF and CEWAF, CLB-WAF, and CXT) an increase of CR was measured. In these conditions, higher hydrocarbon concentrations were measured in mussel tissues than in other treatments, or results were similar despite lower concentrations in contaminated seawater. This increase could be due to depuration (Kim *et al.*, 2007), but a decrease was also observed. With CXT or HEI-CEWAF exposure, an increase of CR was measured, followed by a decrease, related to the increase of the TH concentration in blue mussel tissues. This assumes that depuration was able to start from 1% of HEI-CEWAF (≃15 µg/g) or 2 mg/l of CXT (≃0.05 µg/g) in mussel tissues but seemed to stop from 5% (≃ 30 µg/g) or 200-1000 mg/l (≃8 µg/g), either by closing capacity or important impacts of PAHs. With chemical dispersion, mussels exposed to diluted bitumen even seem to decrease their CR, which can explain the important differences measured in tissues between conventional oil and diluted bitumen exposure. The mussels' capacity to remain closed in a contaminated environment is a defence mechanism (Durier *et al.*, 2021). It is possible that *M. edulis* could detect contaminants more quickly with diluted bitumen exposure than conventional oil. For mussels exposed to CXT, a decreased CR was measured at 200 or 1000 mg/l, following the study of Durier *et al.* (2021) who measured mussels' closure from 250 mg/l of Corexit® 9500A exposure. Furthermore, a decreasing of clearance rate could be due to a biological response to minimize absorption of PAHs (Jeong and Cho, 2007; Kim *et al.*, 2007), because of the ability of mussels to detect pollutant and decrease filtration in their presence (Wegner *et al.*, 2012) or to a decrease of ciliary activity due to narcotic effects of PAHs (Donkin *et al.*, 1989). Lower total hydrocarbons concentration measured in mussel tissues exposed to diluted bitumen could therefore be partly explained by earlier detection of pollutant or higher damage to ciliary activity.

The bioaccumulation in blue mussels, *Mytilus edulis*, after 48h of oil exposure resulted in cellular stress, destabilization of lysosomal membrane, and cell viability with varying impacts depending on the type of oil and the dispersion technique. Our study demonstrates that an exposure to a conventional oil or a diluted bitumen impacts lysosomal membrane in mussel hemocytes, from the lowest dilution of WAF (20%) or CEWAF (0.1%) and can cause up to more than 60% of cells to experience lysosomal membrane destabilization. For similar concentrations of oil, the impacts on lysosomal membranes appeared at higher dilutions for CEWAFs (from 0.1%) than WAFs (from 20% for HEI, and between 20 to 80% for diluted bitumen). In previous studies, lysosomal membrane destabilization was measured in mussels collected from conventional oil-impacted sites, with PAH concentrations in tissues varying from 0.1 to 160 $\mu g.g^{-1}$ (Fernley *et al.*, 2000; Sundt *et al.*, 2011; Hwang *et al.*, 2014). For diluted bitumen exposure, few data exists and is limited to laboratory assays (Schmutz *et al.*, 2021). Schmutz *et al.* (2021) compared the same three oils (HEI, CLB, AWB) in winter with physical dispersion, and observed, similarly to us, > 60% of lysosomal membrane destabilization with PAH concentrations between 0.6 to 5.0 µg/g, dw. In our study, destabilization of lysosomal membrane was measured from 0.2, 0.05, or 0.3 µg/g dw of TPH in mussel tissues, for conventional oil, diluted bitumen, or dispersant solution exposure, respectively. However, effect levels were similar between treatments, despite higher oil concentrations in the tissues of mussels exposed to CEWAFs and the presence of dispersant, which induced impacts on lysosomal membranes from 200 mg/l. The only correlation with Σ_{28}Alkylated-PAHs, which are known to have a high toxicity (Fan *et al.*, 2002), was measured with the chemically dispersed conventional oil. This could be explained by a higher proportion of alkylated PAHs in mussel tissues exposed to HEI-CEWAF than to dilbit-CEWAFs. As lysosomal activity is directly related to immune activity in bivalves, its destabilization can potentially lead to adverse effects, such as histopathological, developmental, or reproductive abnormalities, immunosuppression (Tapia-Morales *et al.*, 2019), higher mortality, or population decline (Martins *et al.*, 2005; Hwang *et al.*, 2008).

Cellular viability was also measured, but for higher percentages of dilution than those inducing lysosomal membrane impact. Indeed, significant differences from controls were

generally measured from 40% of WAF and 1% of CEWAF. Like for LDI, for similar concentrations of oil, damage was observed for lower concentrations of CEWAFs compared to WAFs. The dispersant itself caused damage to cell viability, especially in summer from 2 mg/l, which may explain the difference in impacts measured between dilutions of WAFs and CEWAFs. With physical dispersion, the decrease of the CV percentage was similar between the three oils. But with dispersant, lower percentages of CV were measured with diluted bitumen than conventional oil, which may be explained by a 10 times greater hydrocarbon concentration in the tissues of the mussels exposed to the CEWAFs. The CV decrease was measured from 0.4, 0.06, or 0.1 µg/g, dw of TPH in mussel tissues, with conventional oil, diluted bitumen, or dispersant solution, respectively, and could reach 50% of hemocytes viability compared to > 85% in mussel controls. Contrary to our laboratory experiments, previous studies in PAH pollution sites did not report an important decrease of cell viability in mussels, even with higher hydrocarbon concentrations in the tissues (Cajaraville *et al.*, 1996; Pérez-Cadahía *et al.*, 2004). This decrease was only observed with physically dispersed light crude oil. One study, Giannapas *et al.* (2012), measured a decrease of cell viability for mussels exposed to PAHs for a concentration of 0.2 mg/l. The authors highlight the fact that the reduction in hemocyte viability is a sign of an impaired immune efficiency in mussels, which is evidenced by the correlation between cell viability and levels of reactive oxygen species (ROS; $^{\bullet}O_2^-$; Giannapas *et al.*, 2012).

It is known that PAHs accumulated in mussels can induce the production of reactive oxygen species (ROS) via redox cycling, and binding to DNA via CYP transformation to arene oxides, forming DNA adducts, that are more effective sites for the production of stand breaks (Pérez-Cadahía *et al.*, 2004; Sureda *et al.*, 2011). Indeed, DNA damages, measured in our study with % of tail DNA, were observed after the exposure to WAFs and CEWAFs. The level of damage depends on the type of oil and the dispersion technique. An increase of DNA degradation compared to controls was generally measured with WAF from 20% and CEWAF from 0.1%. Like with the other biomarkers in our study, for similar concentrations of oil, DNA damages were observed for lower percentages of contaminated water during chemical dispersion compared to physical dispersion. Moreover, the dispersant itself damaged DNA,

especially in summer from 2 mg/l. However, despite the higher concentration of TH measured in the tissues of mussels exposed to CEWAF and the presence of CXT, chemically dispersed conventional oil did not result in a higher DNA damage than the other treatments. In our study, an increase of DNA degradation was measured from 0.5, 0.05, or 0.1 µg/g, dw of TPH in mussel tissues, after 48h of exposure to conventional oil, diluted bitumen, or dispersant solution. Previous studies measured DNA damage to mussels after a conventional oil spill (Pérez-Cadahía *et al.*, 2004), an exposure to a dispersed oil (Counihan, 2018), or PAH exposures (Hamoutene *et al.*, 2002; Michel *et al.*, 2013), but there is no data for diluted bitumen exposure. Our data generally shows a positive correlation between DNA damage and Σ_{13}VOCs for oil exposure, and with Σ_{28}Alkylated-PAHs for dispersant itself. Genetic and cellular damage can cause mortality resulting from malignancies that result from somatic cell DNA damage (Phillips and Arlt, 2009).

Indeed, important mortality was measured during our experiments, but only with dilbits chemically dispersed in summer and with the dispersant solution, despite the same or even lower TPH in mussel tissues than those exposed to conventional oil. The mussels undergo many biological processes that can make them more sensitive to external factors, like high temperature, food depletion, or reproduction (Myrand and Gaudreault, 1995; Tremblay *et al.*, 1998b; Tremblay *et al.*, 1998c). Like mussels in our study, mussel growers on Prince Edward Island have noted a possible link between major spawning and summer mortality (Myrand *et al.*, 2000). It seems that during summer, when mussels are more sensitive to contaminants, the risk of mortality is higher during a diluted bitumen spill, especially if a dispersant is used, as the dispersant itself causes some mortality.

All our biomarkers were summarized to an integrated biomarker response (IBR) index for each treatment, to visualize the global biological effects of an oil (WAF, CEWAF) or a dispersed (physically or chemically) oil exposure. With a chemically dispersed oil, despite higher concentrations in mussel tissues, the IBR was not always higher, depending rather on type of oil and season. Physical dispersion caused higher IBR in summer, autumn, and winter, for CLB, AWB, and HEI, respectively. The dispersant itself caused damage with high IBR

value (< 7.5) except in winter. At each season, a higher IBR was measured for dilbit exposure despite higher TPH in mussel tissues exposed to conventional oil.

The importance of the season was already evaluated by the aromatic hydrocarbon concentration in the microcosm water, which was higher in colder temperatures. This is consistent with the results of some natural environment studies, during which concentrations of PAHs decreased with increasing water temperature (Lohmann *et al.*, 2011; Vrana *et al.*, 2014). Freely dissolved PAH concentrations during the summer could decrease due to enhanced photolytic and OH-radical degradation of PAHs or by scavenging from higher particle concentrations (Lohmann *et al.*, 2011). Nevertheless, higher equilibrium concentrations in water are expected at lower temperatures as the Henry's law constant increases with temperature (Staudinger and Roberts, 2001). But with the dispersant solution, these concentrations were not different between seasons. The accumulation of contaminants was therefore dependent on the season, except with dispersant solution exposure, with lower total hydrocarbon concentration measured in mussels in winter than in the two other seasons. Furthermore, seasonal differences in mussel tissues could also be explained by natural variations of mussels' water filtration rate according to temperature (Comeau *et al.*, 2008). Indeed, the total hydrocarbon concentration in whole tissues depend on the type of oil, but also on the season. In summer, the impacts of an oil spill on mussels should be similar between the three oils. In autumn and winter, significantly higher impacts are assumed for mussels exposed to the conventional oil (HEI) compared to the diluted bitumen (AWB and CLB), suggesting potentially higher toxicity for conventional oil than for the two diluted bitumen.

Just as the clearance rate was seasonally dependent, bioaccumulation was also seasonally dependent. According to the CR results, depuration rates also varied seasonally, as the time required for depuration should be significantly higher at lower temperatures (Cusson *et al.*, 2005). This variation may explain the physiological, cellular, and genotoxic impacts on mussels. The final impacts measured will vary depending on the initial state of the blue mussels. However, seasonal changes in the physiological state of mussels have been

thoroughly reported in diverse aspects of the biology of mussels, including scope for growth, respiration and excretion, pollutant tissue burdens, and biomarker responses (Santarem *et al.*, 1994; Baumard *et al.*, 1999; Huang and Newell, 2002; Ringwood *et al.*, 2002; Wong and Cheung, 2003; Izagirre *et al.*, 2008). In nature, the condition index varies during the year, due to spawning (May to June), gamete development (September to December), then maturation (January to March) (Hagger *et al.*, 2010). CI decreased due to spawning, followed by a summer recovery phase, to then become higher in autumn. In our controls, a higher IBR was observed in winter, despite a higher condition index, then in summer, and finally, a lower IBR in autumn. According to our results, the response of the blue mussels (*M. edulis*) to the oil exposure was influenced by the season, which resulted in a natural variation of their physiological parameters. Lower CI were measured in summer (July - August) and autumn (October - November), which indicated a major biological effort, such as the production and release of gametes (Tremblay *et al.*, 1998a). In winter, the filtration rate was lower than during the other two seasons, both in the controls and in the impacted mussels. As in the study of Smaal *et al.* (1997) on *M. edulis* collected from Oosterschelde Estuary (Netherlands), the clearance rate was highest during the reproductive period (summer; July-August). However, during this season, Hagger *et al.* (2010) observed a decrease of the clearance rate of *M. edulis* collected from the mouth of the Exe Estuary (England).

Some biomarkers were not significantly different between seasons for controls but were for mussels exposed to the highest concentrations or nominal percentages of WAFs or CEWAFs. These variations can be explained by several factors: a difference in filtration, and therefore of the concentration of hydrocarbons in the tissues, as well as the physiological state of the mussel. Among these biomarkers, the results of the destabilization of the lysosomal membranes of the exposed mussels varied according to the seasons. In past studies, lysosomal membrane stability showed marked seasonal changes (Tremblay *et al.*, 1998a; Ringwood *et al.*, 2002; Izagirre *et al.*, 2008; Hagger *et al.*, 2010). In contrast to these results, our study generally measured higher lysosomal membrane destabilization in winter and not in summer. This can be explained by the fact that the mussels were exposed in late winter (February), which tends to increase this endpoint, more than in the other month, as observed

by Balbi *et al.* (2017). In addition, similar to the results of Padmini and Rani (2008), cell viability was dependant on season. With physical dispersion, the season with lowest value varied between the type of oil. The lowest CV was measured in winter with chemical dispersion and in summer for the dispersant itself. The degree of DNA damage was generally higher in summer in exposed mussels, during physical dispersion, while damage was not significantly different in our controls. For the three oils, as well as the chemical dispersant, the least DNA damage was measured in winter. Previously observed seasonal variations in genotoxic response in mussel hemocytes, with the greatest DNA damage observed during summer months (June – August; Akcha *et al.*, 2004; Kolarevic *et al.*, 2013) was echoed in our study. As reported by Michel *et al.* (Michel *et al.*, 2013), the level of DNA strand break measured in winter was low, only increasing in summer for conventional oil exposure, especially with physical dispersion.

2.8 CONCLUSION

Our results prove that the use of the chemical dispersant results in higher concentrations of total hydrocarbons in seawater compared to a physical dispersion for both conventional oil and diluted bitumen. Besides, the dispersant itself contributes to TPH concentration in seawater due to its chemical composition. However, the concentrations accumulated in mussel tissues were not always correlated with the TPH measured in seawater, whatever the season. With dilbit exposure, this correlation was low ($R^2 < 0.5$). In mussels exposed to conventional oil, TPH in mussel tissues increased with the dissolved hydrocarbons concentration in seawater, then leveled off or even decreased at the highest concentration. The clearance rate varied according to the season and on the level of pollution in the marine environment (TPH concentration). Indeed, the mussels can increase the clearance rate for depuration, or can keep their valves continuously closed, thus isolating themselves from the contaminant. They could detect some compounds more quickly and subsequently rapidly close their valves. Because of this response, our results call into question the use of the blue

mussel as a bioindicator of the health of marine ecosystems during an oil spill, particularly in the case of diluted bitumen.

The TPH accumulation in mussel tissues caused cellular damage to the immune system by degrading lysosomal membranes, but also induced an increase of unviable cells, DNA damage, and even high mortality rates. In the case of significant bioaccumulation of hydrocarbons (i.e., during the chemical dispersion of oils), the integrated biomarker response was generally higher during CEWAF than WAF exposure, but not consistently. The global impact on blue mussels will therefore depend on other parameters such as the initial mussel health, the season of the spill, and the hydrocarbons accumulated in mussels, which could determine its closing and limit its hydrocarbon exposure and bioaccumulation. Besides, despite significantly higher TPH in mussel tissues exposed to conventional oil than dilbit, the level of these impacts was similar, or even higher, with dilbit exposure. So, the integrated biomarker response was often higher for dilbit compared to conventional oil exposure, which suggests that the hydrocarbons accumulated in the mussels from dilbits are more toxic to this organism than those present in the conventional oil used in our study. A smaller spill of dilbit can cause the same impacts as a larger one of conventional oil. Our study also demonstrated that the dispersant itself can cause cellular and DNA damage, and even mortality in blue mussels.

Our study supports the importance of seasonal effects on blue mussels, both in terms of their clearance rate and the oil impact level. Spill countermeasures should account for the season due to the temperature-dependent effectiveness of chemical dispersants and for natural seasonal changes experienced by the blue mussel (its ability to depurate or stay closed, and its available energy). Indeed, in winter, it will be easier for the blue mussel to remain closed and avoid bioaccumulation. But it’s also possible that its depuration will be less effective, causing damage. In summer, higher clearance rates and a large part of mussels’ energy dedicated to reproduction leads to strong consequences, even significant mortality, especially during contamination with chemically dispersed dilbit.

Finally, DNA damage implies possible consequences to future generations and subsequent impacts on mussel farms several years after a spill. At lower TPH in mussel tissues after WAF or CEWAF dilbit exposure, DNA damage was similar or even greater than in mussels exposed to conventional oil WAF or CEWAF. It is therefore possible that a dilbit spill could be toxic over a longer period than a conventional oil spill.

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CHAPITRE 3
CAPACITE DE RECUPERATION DES MOULES BLEUES, *MYTILUS EDULIS*, APRES UNE EXPOSITION AU BITUME DILUE EN CONDITION ARCTIQUE

3.1 RÉSUMÉ

Avec les sables bitumineux, le Canada est le troisième pays possédant le plus de gisements de pétrole. La diminution des stocks de pétroles classiques accélère la production et l'exportation des non classiques, tels que les bitumes dilués (dilbit). De plus en plus d'études démontrent des impacts plus importants sur les moules bleues, *Mytilus edulis*, lors d'exposition au dilbit par rapport au pétrole classique. Les mytilicultures sont d'une grande importance économique au Canada, et sont à risque en cas de déversement, non seulement pour des impacts à court, mais également à long terme. Notre étude évalue la capacité des moules bleues à se rétablir à la suite d'une exposition au dlibit Cold Lake Blend (CLB) en condition subarctique. En plus d'un suivi des concentrations dans l'eau et dans les tissus, trois biomarqueurs sont utilisés pour évaluer cette capacité : la stabilité lysosomale, la viabilité cellulaire et la dégradation de l'ADN. Nos résultats montrent une bioaccumulation des hydrocarbures polycycliques aromatiques (HAP) dans les tissus des moules, et une dépuration rapide des HAP-alkylés. En sortie d'exposition, des impacts sur la stabilité lysosomale, la viabilité cellulaire et d'importants dommages sur l'ADN sont mesurés. Pourtant, ces biomarqueurs montrent une diminution jusqu'aux valeurs des moules contrôles, non impacté, au bout de quelques jours, voir au bout de 6 heures.

Ce troisième article, intitulé « Recovery capacity of blue mussels, *Mytilus edulis*, after an exposure to diluted bitumen in subarctic condition », sera soumis pour publication aux éditeurs de la revue *Aquatic Toxicology*. En tant que première auteure, ma contribution à ce travail fut l'apport de l'idée originale, la collection des données, la recherche sur l'état de l'art, le développement de la méthode, la réalisation des expositions et des analyses, l'analyse et l'interprétation des résultats et la rédaction de l'article. Anthony Schmutz, second auteur, a participé à la réalisation des expositions et des analyses. Richard Saint-Louis, directeur de recherche, et Gaëlle Triffault-Bouchet, co-directrice de recherche, ont aidé à la recherche sur l'état de la question et au développement de la méthode. Tous les auteurs contribueront à la révision de l'article lors de la soumission.

3.2 RECOVERY CAPACITY OF BLUE MUSSELS, *MYTILUS EDULIS*, AFTER AN EXPOSURE TO DILUTED BITUMEN IN ARCTIC CONDITION

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3.3 ABSTRACT

Canada has the is the third-largest oil deposits in the world, most of which is found in the western oil sands. The decrease in global conventional oil stocks is accelerating the production and export of non-conventional oils, such as diluted bitumen (dilbit) from these oil sands. A growing number of studies demonstrate the greater impacts of dilbit exposure on blue mussels, Mytilus edulis, when compared to conventional oil. Mussel farms are of great economic importance in Canada and are at risk in the event of a spill, for both short- and long-term impacts. Our study assesses the ability of blue mussels to recover following exposure to Cold Lake Blend (CLB) dilbit in subarctic conditions. In addition to monitoring concentrations in water and tissues, three biomarkers are used to assess this capacity: lysosomal stability, cell viability and DNA degradation. Our results show a bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in mussel tissues and a rapid depuration of PAH-alkylates. After exposure, impacts on lysosomal stability, cell viability and significant DNA damage are measured. However, these biomarkers are decreased compared to the values of control mussels a few days, or even six hours, following exposure.

Keywords: Diluted bitumen, Cold Lake Blend, Mytilus edulis, Depuration, Comet assay

3.4 INTRODUCTION

Petroleum production from Alberta's oil sands is expected to rise to 4.25 million b/d by 2035 (CAPP, 2019), accounting for 74% of total Alberta refinery demand (CER, 2022). Diluted bitumen (dilbit) is typically blended at ratios of 20:80 to 30:70 (diluent:bitumen), depending on the time of year, to decrease their high densities and viscosities, thus facilitating flow through pipelines (Crosby *et al.*, 2013). Dilbit exports depend on pipeline and rail transport across major Canadian watersheds to refineries or to marine ports. Much of this is intended for export to European and Asian markets and involves increasing use the country's sea lanes for its transportation, exposing marine ecosystems and species to spill risks (Green *et al.*, 2017). The two primary dilbit transported in large quantities across Canada are Access Western Blend (AWB) and Cold Lake Blend (CLB). The chemical makeup of dilbit may vary in relation to geographical source, extraction process, and additional propriety components within the diluent exclusive to the producer (Lee *et al.*, 2015b). Generally, bitumen have fewer saturates, more resins, more asphaltenes and same aromatic fraction than conventional crude oils (Woods *et al.*, 2008). Besides, the addition of diluent increases the chemical complexity of the bitumen and can affect how it behaves in aqueous systems (Lee *et al.*, 2015b). It composed mainly of lower molecular weight saturates and minor components of benzene, toluene, ethylbenzene, and xylenes (Lee *et al.*, 2015a; Speight, 2019).

While all marine invertebrates are sensitive to an oil spill, benthic and sessile organisms, like the blue mussel, are the most sensitive. The impacts of an oil spill have already been observed on different species, as well as the role of PAHs, but not so much for dilbit. After an oil spill, PAHs accumulate in the aquatic environment and are difficult to biodegrade. Due to their low solubility and high hydrophobicity, PAH contaminants in the aquatic ecosystem are adsorbed to suspended particles (Zhang *et al.*, 2016), which can be filtered by mussels and bioaccumulate through food absorption, transport through the respiratory surface, or inhalation (Zhou *et al.*, 2008). The low metabolic capacity of mussels and their high body burden allow them to accumulate wide ranges and high levels of

contaminants (Cossa, 1989). In addition, the bioaccumulation of PAHs in bivalves is favored by their significantly longer half-lives than in fish (Stegeman and Lech, 1991; Meador *et al.*, 1995) and a slower metabolic rate (Seiser *et al.*, 2000). When the level of pollution is high, bivalves can no longer purify contaminants at the same rate as accumulation, resulting in a higher level of contamination of their tissues. This concentration can then alter the defense mechanisms, which includes wound repair, coagulation, nodule formation, encapsulation, phagocytosis, and cytotoxicity (Coles *et al.*, 1994; Wootton *et al.*, 2003; McVeigh *et al.*, 2006; Bado-Nilles *et al.*, 2008).

Dilbit spills threaten the aquaculture of mussels, *M. edulis*, which is an important Canadian industry. In addition to loss of stocks, mussels' exposure to an oil spill could pose a risk to human health via the food chain, as reported by Lemiere et al. (2005) who described genotoxic effects in rats fed mussels contaminated by the Erika oil spill. Despite the importance of the culture and export of mussels, worth $30.7 million in 2020 (MPO, 2020b), and the risk to human health, there are only two previous studies of dilbit impact on the blue mussel, *M. edulis* (Schmutz *et al.*, 2021; Berthod *et al.*, 2022a). These two studies observed that AWB and CLB dilbits caused severe damage on blue mussels at physiological, cellular and DNA levels. The studies conclude that the bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in blue mussels after exposure to diluted bitumen was lower than exposure to conventional oil, but resulted in greater biological impacts and negative transgenerational effects on the unexposed F1 generation (Schmutz *et al.*, 2021). There could be an ecological risk that may lead to heritable mutations and loss in the total genetic diversity with significant implications for long-term survival of natural populations (Bickham *et al.*, 2000; Laffon *et al.*, 2006). Additionally, the consequences of a massive dilbit spill in a cold environment are still unknown. Marine cold-water ecosystems are generally considered sensitive despite low clearance rate (Berthod *et al.*, 2022b) and less resilient than temperate ones when faced with contaminant stress (Yang *et al.*, 2015). Besides, the *Mytilus edulis* depuration is lower during winter compared to summer conditions (Svensson, 2003).

The objective of this study is to observe the impacts of exposure to dilbit Cold Lake Blend on blue mussels, *Mytilus edulis*, in winter conditions, via cellular and genetic assay, and to monitor their depuration over several days. Monitoring lysosomal state provides us with information about their ability to defend themselves. In addition, genetic data is needed to better understand and consider the risks for future generations.

3.5 MATERIALS AND METHODS

3.5.1 Animals

Blue mussels, *Mytulis edulis* (Linnaeus 1758), were collected from the mussel farm of St. Peter's Bay in Prince Edward Island (Canada; 42 °25'N, 62 °35'O). Upon reception at UQAR's aquaculture facility in Rimouski (Quebec, Canada), they were placed in a large tank with running natural seawater pumped from the St. Lawrence Estuary and filtered through the glass beds filtration system of the station, ensuring the mussels' nutrition (particulate organic matter only). A total of 80 blue mussels were used for the experiment after an acclimatation of three weeks, with a mean shell length of 5.23 ± 0.44 cm and a mean mass of the organism of 14.31 ± 3.47 g. During the entire experimental period (exposure and post-exposure), mussels' lethality was checked by mechanical stimulation of shell closure. Mussels are considered dead when they do not respond to closure stimulation. No mortality was observed during exposure and depuration periods.

3.5.2 Oil Product

The unweathered crude oil, Cold Lake Blend (CLB), was supplied by Thomas L. King's team from Fisheries and Oceans Canada. This dilbit originates from the Athabasca oil sand deposit in Alberta (Canada). The physical properties and composition on oils and the dispersant are respectively presented in Table 10.

Table 10 : Physical properties and composition (SARAs; Percentage by Weight) for the CLB (MPO, 2013)

		Cold Lake Blend (CLB)
Origin		Alberta
API	°	21
Density at 0 °C	*g/ml*	0.9376
Density at 15 °C	*g/ml*	0.9249
Viscosity at 0 °C	*cP*	803
Viscosity at 15 °C	*cP*	285
Saturates	*% w/w*	~45
Aromatics	*% w/w*	~30
Resins	*% w/w*	~12
Asphaltenes	*% w/w*	~13

3.5.3 Exposure

Exposure took place over eight days in winter (November). Blue mussels were equally distributed in two experimental mesocosms (3.2 m height × 1.2 m diameter) containing 3500 L of filtered seawater (static) at UQAR's aquaculture facility (Figure 46). One of these was injected (at the surface) with 3 460 mL of dilbit Cold Lake Blend for the crude oil treatment, the other was used as a control. Mesocosm diameter and volume of oil used resulted in an estimated 2.5 mm thick oil slick which corresponded to a spill of 3000 m^3 over a 1 km^2 area. Bottom air bubbling was ensured to avoid the development of hypoxic conditions, with a good percent of oxygen (94.3% ± 3.6) and provide low energy mixing. Seawater temperature (2.85 ± 0.44°C) and salinity (30 ± 3 psu) were stable between treatments.

3.5.4 Post-Exposure Analysis

At the end of the exposure period, the exposed mussels were transferred to a dedicated indoor 3500 L mesocosm (open-system) for four days (Figure 46). Freshly filtered seawater

was continuously added at a flow rate of 5 L.min^{-1} in a top-bottom circulation for control and post-exposure treatments. Seawater temperature and salinity were stable during the post-exposure period (respectively 2.22 ± 0.81°C and 29 ± 1 psu). Before and after exposure, salinity, dissolved oxygen, and temperature were measured with the EXO2 multiparameter sensor (YSI Inc., USA).

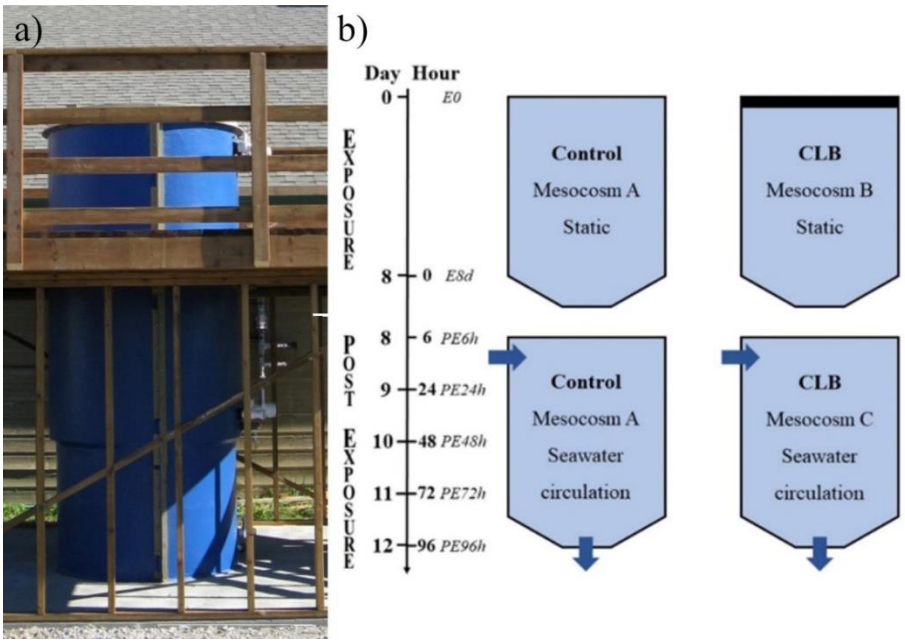

Figure 46 : a) Picture of mesocosm at UQAR's aquaculture facility in Rimouski (Quebec, Canada); b) Experimental set up of exposure and post-exposure (depuration)

3.5.5 Sampling

For each mesocosm (control and post-exposure), 10 blue mussels were evaluated before and after the exposure period, respectively named E0 and E8d. Then, the same 10 mussels were punctured during the depuration period, at 6, 24, 48, 72 and 96 hours after the exposure period (respectively named PE6h, PE24h, PE72h and PE96h).

From each specimen, approximately 500 µl of hemolymph was collected from the adductor muscle with a 23G needle and a 1 mL syringe pre-charged with 500 µl PBS-EDTA, to obtain a cell suspension. The hemolymph/PBS-EDTA mix was pelleted by centrifugation

at 500 g for 5 min (Beckman Coulter, Microfuge 16). The cell suspension was made in residual supernatant. Prepared cell suspension used to measure the lysosomal stability, the cellular viability, and DNA damage. Furthermore, five (5) blue mussels were recovered for condition index and tissues analysis at each experimental time period.

3.5.6 Chemical Analysis

Water Exposure Analysis

During each exposure, the concentration of the aromatic hydrocarbon dissolved fraction of crude oil was estimated using the Cyclops-7 submersible fluorometric sensor, equipped with the "O" sensor (Turner Designs, San José, CA, USA) for the detection of crude oil. It is one of five commercially available UV submersible fluorometers for *in situ* measurements of PAHs, and one of the most commonly used for delineating oil plumes in the field (Conmy *et al.*, 2014; Hwang *et al.*, 2020; Berthod *et al.*, 2021). Its fluorescence optical specifications are excitation wavelengths at 325 / 120 nm and emission wavelengths at 410–600 nm. The Cyclops-7 sensor was controlled by a DataBank module (Turner Design, USA), which was connected to a computer by proprietary software (Turner Designs, San José, CA, USA). The sensor was calibrated with an aqueous solution of 100 µg/l of tetrasodium 1,3,6,8-pyretetetrasulfonate (PTSA), a highly water-soluble pyrene derivative (CAS 6528-53-6; Sigma-Aldrich, Darmstadt, Germany). The fluorescence measurements were used as an indicator of hydrocarbon concentration and, thus, were expressed in equivalent µg PTSA/l. The sensor response was linear within the PTSA concentration range used, from 5 to 550 µg/l.

Tissues Analysis

Chemical analysis in whole mussel tissues followed the methods of Schmutz *et al.* (2021). It was performed on six organisms per condition (two mussels times three experimental microcosms), the tissues of which were freeze dried (FreeZone, Labconco, USA) and reduced to a fine powder with a Virtis homogenizer. The concentrations in the following groups were determined in the mussel tissues: i) 16 PAHs classified as priority

pollutants by the United States Environmental Protection Agency (US-EPA, 1984; Σ_{16}EPA-PAHs; C_{10} – C_{22}) due to their carcinogenicity (Honda and Suzuki, 2020); ii) 28 alkylated PAHs (Σ_{28}Alkylated-PAHs; C_{12} – C_{16}) given that alkyl-PAHs demonstrate different environmental behaviors and significantly higher toxicities (Turcotte *et al.*, 2011; Mu *et al.*, 2014; Kang *et al.*, 2016; Cong *et al.*, 2021) than their non-alkyl forms, due to the increasing polarity and potentially different metabolic pathways and mechanisms by organisms (Cong *et al.*, 2021); iii) 18 carbazole and dibenzothiophenes (Σ_{18}CzDBS; C_{12} – C_{16}) which are representative of the nitrogen and sulfur heterocyclic PAHs known to be carcinogenic (Tsuda *et al.*, 1982; Jha and Bharti, 2002); and iv) 13 volatile organic compounds VOCs (Σ_{13}VOCs; C_9 – C_{10}) which seemed to be a better predictor of mortality than PAHs (Philibert *et al.*, 2016; Bérubé *et al.*, 2021).

3.5.7 Biological Analysis

Condition Index

The condition index (CI) is a measure of the apparent health of bivalves. For condition index determination, five (5) blue mussels per experimental microcosm were cleaned of external byssus, washed, and measured with a digital caliper (CO 030150F1). Each mussel was opened with a stainless-steel scalpel and its ventral edge was placed on tissue paper to remove the internal water. Then, the total tissue of mussels was detached from the shell. The mussels' fleshwas dried (FreeZone, Labconco, USA), then measured with a digital scale. The condition index was calculated as the coefficient of dry meat weight (DMW) and the shell length cubed (L^3; mm^3) (Bodoy *et al.*, 1986):

$$\mathrm{CI} = \frac{DMW}{L^3}$$

Lysosomal membrane destabilization index

The lysosomal membrane destabilisation index was determined by using the NRR assay which was performed according to the method described by Méthé *et al.* (2015).

Briefly, 40 µl of hemolymph and filtered intervalvular fluid mix (1:1; 0.2 µm cellulose, acetate membrane, VWR, USA) were transferred to a positively charged microscope slide X-tra® (Product N°3800210; Leica Biosystems, France) and incubated in a dark humidity chamber at ambient temperature (20°C). After 15 min, 20 µl of working solution (Neutral red dye, DMSO, and filtered intervalvular fluid) was added to the slide, then, after another 15 min, a cover slip was installed. After 45 min, 50 cells were counted using a light microscope (Olympus BX 41, Olympus, Canada coupled to a camera Evolution VF Color and Image Pro Plus software, MediaCybernetics, USA; 400X magnification). The lysosomal membrane destabilisation index (LDI) was calculated as the percentage of hemocytes with destabilised lysosomes (DL) as follows:

$$LDI = \frac{\sum DL}{50} X\ 100$$

Cell Viability

Viability of hemocytes was assessed by a membrane integrity test: the trypan blue exclusion method, according to McCarthy *et al.* (2014). This method has also been used in conjunction with the comet assay for mussel exposure experiment (McCarthy *et al.*, 2014; Lacaze *et al.*, 2015). Equal quantities of cell suspension and trypan blue (20 µl: 20 µl) were mixed, and 20 µl of this solution was placed onto a slide (75x25mm; Fisher Scientific, Canada). A total of 50 cells per slide were counted and examined with the light microscope (Olympus BX 41, Olympus, Canada coupled to a camera Evolution VF Color and Image Pro Plus software, MediaCybernetics, USA; 400X magnification), and the viability percentage was recorded.

Comet assay

The comet assay (alkaline version) was performed according to the procedure described by Lacaze *et al.* (2015). Only hemocyte suspensions with a cellular viability rate higher than 70% were used (Lacaze *et al.*, 2015). Positive controls were carried out by exposing cells in vivo to 5 mM of MMS for 1 h at 4°C, to produce massive single-stranded DNA damage. Briefly, 20 µl of cell suspension was mixed with 20 µl of 0.65% LMA

prepared in phosphate buffered saine at 37°C. This was immediately deposited onto slides precoated with normal agarose (0.65%), then covered with a plastic coverslip (22x22 mm; Fisher Scientific, Canada). From this point, all steps were performed in the dark under red light to prevent additional DNA damage. Coverslips were withdrawn after agarose polymerization (4°C, 30 min), and slides were incubated in a freshly-made lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 mM Tris, 1.5 µl Triton X-100, 15 µl DMSO, pH 10, 4°C) for 1 h. Then, the slides were placed in an electrophoresis buffer (TAE 1X, pH > 13,4°C) for 20 min. Electrophoresis was conducted at 25 V and 310 mA for 24 min. The slides were then washed three times with freshly-made neutralization buffer (0.4 M Tris, pH 7.5, 4°C) for 5 min, then dried absolute ethanol for 30 min. Staining was performed with 20 µl of diluted Sybr Green solution (1 µl in 10 ml of DMSO). Slides were observed with a fluorescence microscope (Zeiss Axio Imager M2, Oberkochen, Germany). At least 100 randomly selected nuclei per slide were counted for each sample, images were captured, and comets were analyzed with CASP 1.2.2 software (CASPLab, GNU public licence). Three measures are now commonly used as a measure of DNA migration: % tail DNA, tail length, and tail moment, with an increasing tendency for the % tail DNA as the preferred measure for assessment. % Tail DNA has the advantage of being expressed on a scale from 0 to 100%, allowing for its linear relation to dose in calibration studies and simplifying comparisons between studies (Collins *et al.*, 2008; Dhawan and Anderson, 2016).

3.5.8 Statistical Analysis

All tests were regarded as statistically significant when $p < 0.05$. All data were statistically analyzed using R software version 3.5.2. At first, the assumptions of normality and the homoscedasticity were verified using the Shapiro and Levene tests, respectively. Differences between time and treatments were determined by factorial analysis of variance (ANOVA) followed by the Tukey's test. Post hoc contrasts of means were made for significant effects with *P* values calculated using the Bonferroni procedure. When required, data were log-transformed to normalize their distribution.

3.6 RESULTS

3.6.1 Chemical Analysis

Water Analysis

The concentration of aromatic hydrocarbons in CLB mesocosm, measured with the Cyclops-7 optical sensor, significantly increased over the eight days following the oil deposit (Figure 47).

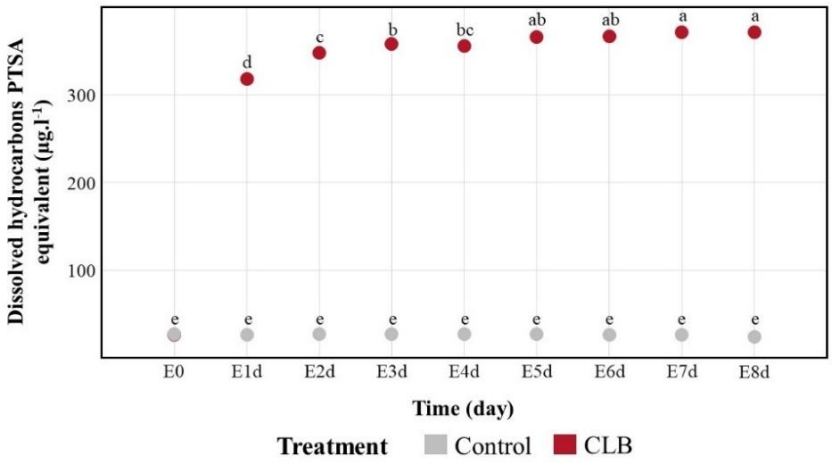

Figure 47 : Fraction of dissolved hydrocarbons measured by fluorescence with the Cyclops-7 optical sensor in Control and CLB-WAF mesocosms (n = 3) for each day during the exposure period (mean ± se; when error bars are not visible, se < 0.5)

The increase in the CLB-WAF mesocosm was exponential until it plateaued around 365 µg/l after three days. In mesocosm control, no significant differences were measured, and values were around 26.65 ± 0.36 µg/l. During the depuration period, the concentration of the dispersed aromatic hydrocarbons of crude oils measured with the Cyclops-7 optical sensor was similar between the control and CLB mesocosms, where the mean value was 23.82 ± 0.15 and 24.73 ± 1.52 µg/l, respectively.

Tissues Analysis

During exposure (E0 – E8d) and the depuration period (PE6h-PE96h), levels of polycyclic aromatic hydrocarbons (∑PAHs), carbazoles and dibenzothiophenes (∑CZDTs) in control mussels were low, sometimes under the limit of detection (Figure 48).

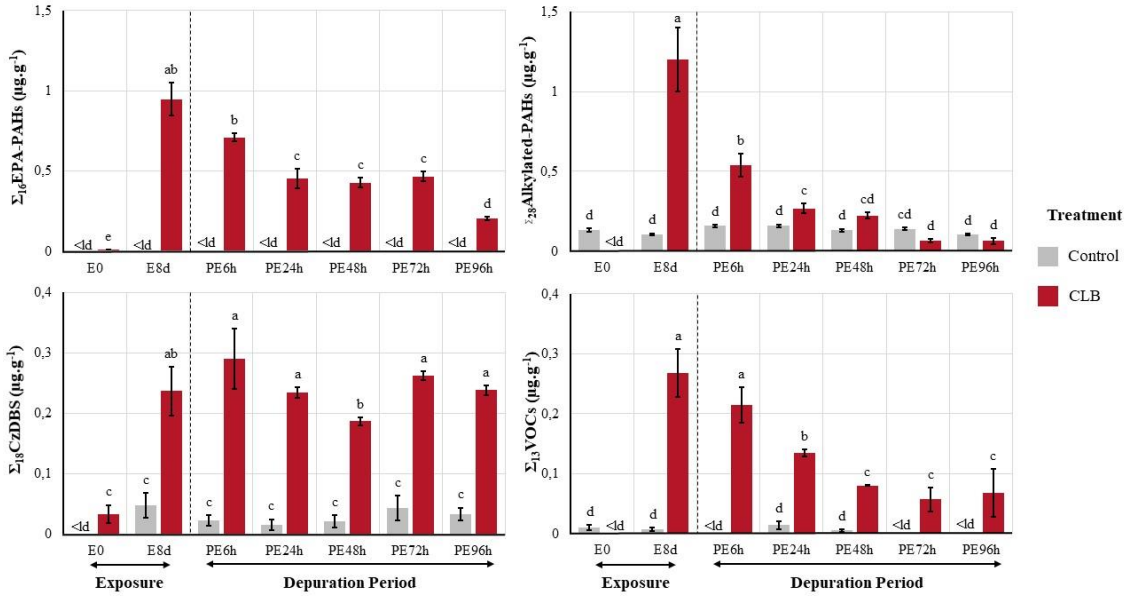

Figure 48 : Bioaccumulated Σ_{16}EPA-PAHs, Σ_{28}Alkylated-PAHs, Σ_{18}CZDTs, and Σ_{13}VOCs (µg/g, dw) in whole tissues of blue mussels during and after exposure for the control and diluted bitumen treatments (mean ± se; n = 5; ld = limit of detection)

After eight days in the CLB-contaminated mesocosm, PAHs were measured in mussel tissues, including the 16 PAHs listed by the EPA. A significant decrease was measured during the depuration period for both Σ_{16}EPA-PAHs and Σ_{28}Alkylated-PAHs but was quicker in the case of the latter. After 96 hours in clean seawater, the Σ_{16}EPA-PAHs concentrations in blue mussels were significantly higher for the CLB group than the control. The Σ_{28}Alkylated-PAHs concentration in mussel tissues was not significantly different to the control after 48 hours in clean seawater.

Concentrations of Σ_{18}CzDBS and Σ_{13}VOCs were lower than Σ_{16}EPA-PAHs and Σ_{28}Alkylated-PAHs. The concentration of Σ_{13}VOCs decreased during depuration period as for PAHs. After 48 hours in clean seawater, the concentration reached a plateau and was always higher than in control tissues after 96 hours. The concentration of Σ_{18}CzDBS was higher than in control tissues and did not decrease during the depuration period, 96 hours post exposure.

3.6.2 Biological Responses

Condition Index

Condition index values were similar between controls and mussels in the CLB-contaminated mesocosm. No significant difference was measured between them during the depuration period. The measured values were 6.5 ± 0.02 mg.cm^{-3} for the controls and 6.8 ± 0.01 mg.cm^{-3} for the mussels exposed to the CLB. So, the global health of mussels in both groups was similar.

Lysosomal Membrane Destabilization

Lysosomal membrane destabilization measured in blue mussels exposed to CLB-WAF was significantly higher than in control after eight days of exposure (Figure 49).

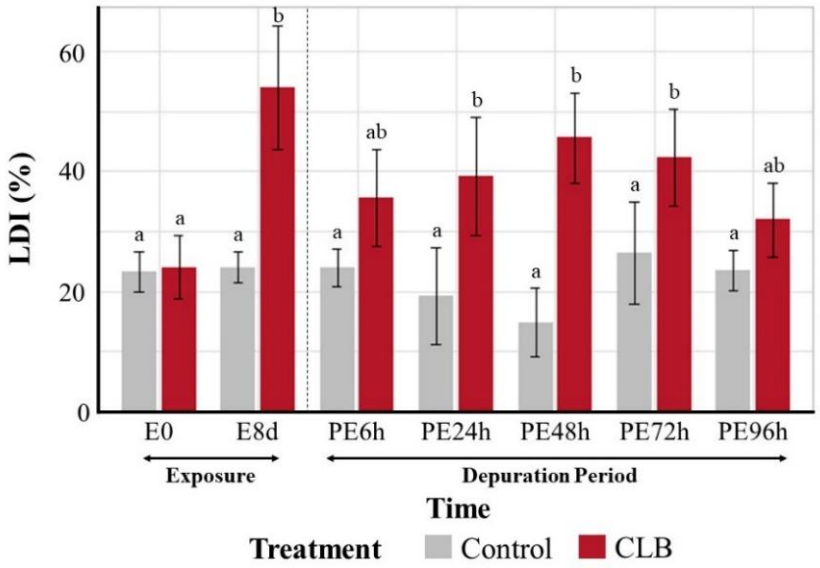

Figure 49 : Lysosomal destabilization index (LDI; %) in control (0%, v/v WAF; n = 5) and CLB-WAF mesocosms (n = 5) during exposure (E0; E8d) and depuration periods (PE6h; PE24h; PE48h; PE72h; PE96h) (mean ± SE)

A natural percentage of LDI (between 15 to 25%) was observed in control mussels during all experiments and was not significantly different between different times. The LDI seems to decrease after six hours in clean water, but was again significantly different from control mussels after 24 hours in clean water. Lysosomal damage was equal after 48 and 72

hours in clean water, then decreases to the control group's value after 96 hours in clean water. However, the decrease was not sufficient to be significantly different from the percentage of damage at the end of exposure. Lysosomal recovery seemed incomplete.

Cell Viability

Decrease in cell viability percentage was measured in blue mussels in the CLB-WAF mesocosm after eight days of exposure (Figure 50). This decrease was not measured in control mussels.

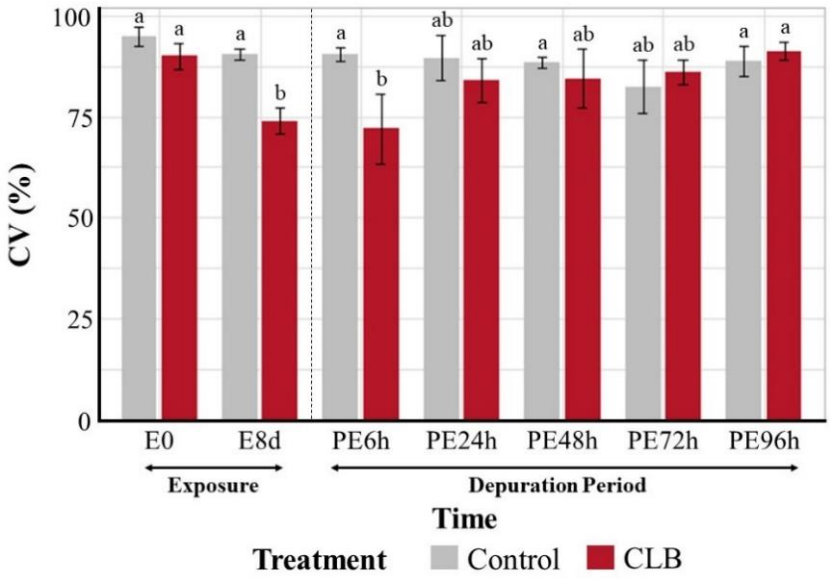

Figure 50 : Cell viability (CV; %) in control (0%, v/v WAF; n = 5) and CLB-WAF mesocosms (n = 5) during exposure (E0; E8d) and depuration periods (PE6h; PE24h; PE48h; PE72h; PE96h) (mean ± SE).

A natural percentage of unviable cells (between 10 to 20%) was observed in control mussels during all experiments and was not significantly different between different times. After six hours in clean seawater, mussels exposed to CLB-WAF always had a lower cell viability percentage than control mussels. But after 24 hours, no significant difference was measured between impacted mussels and controls, and this remained until the end of the depuration period (96 hours). Despite decreased CV, when mussels were exposed to CLB-WAF the percentage was always above the cellular viability rate for comet assay (70%).

Genotoxicity

Important DNA damage was measured in blue mussels in CLB-WAF mesocosm after eight days of exposure (E8d). This was significantly higher than in control mussels (Figure 51).

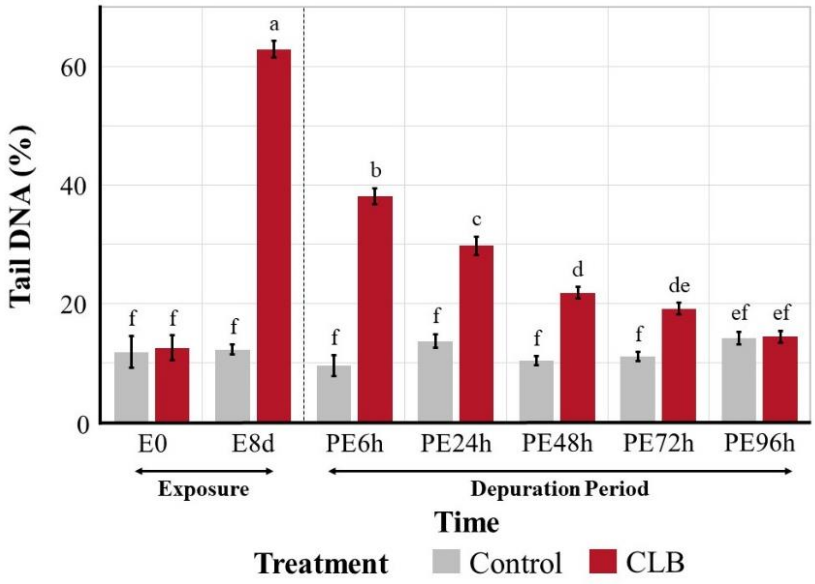

Figure 51 : DNA damage (Tail DNA; %) in control (0%, v/v WAF; n = 5) and CLB-WAF mesocosms (n = 5) during exposure (E0; E8d) and depuration periods (PE6h; PE24h; PE48h; PE72h; PE96h) (mean ± SE).

Natural DNA damage (about 20%) was observed in control mussels during all experiments and was not significantly different between different times. In the CLB group, DNA damage was significantly higher than controls at the end of the exposure period and during the depuration period. A significant decrease in DNA damage was measured in mussels exposed to CLB-WAF during the depuration period (from PE6h to PE96h). The values of DNA damage were not significantly different between the two groups (control and exposed) 96 hours after the end of the exposure period (PE96h).

Proportions of the tail DNA categories provided information on the impact level (Figure 52).

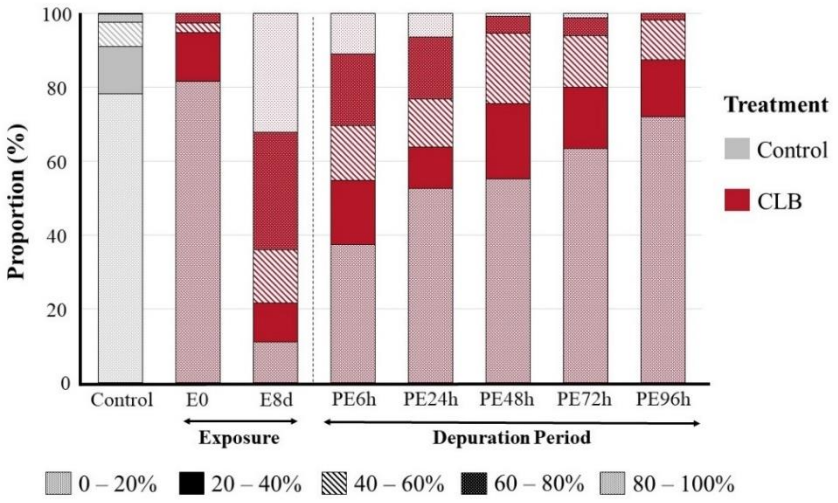

Figure 52 : Proportion (%) of five categories of tail DNA percentages (0-20, 20-40, 40-60, 60-80, and 80-100%) in hemocytes of *M. edulis* in control and CLB-WAF mesocosms.

After eight days of CLB-WAF exposure, DNA degradation was high (>60%; Figure 51), with an important proportion of tail DNA between 80-100%. During the depuration period, the proportion of tail DNA of 80-100% decreased after six hours in clean seawater. This continued over the first 48 hours of depuration. Between 48 and 96 hours, this proportion of this category was low, but the other categories between 20 to 80% decreased, and 0-20% increased. After 96 hours in clean water, the % of tail DNA was not significantly different between controls and exposed mussels (Figure 51). However, the percentage of the category 20 to 40% remained higher in exposed mussels than controls.

3.7 DISCUSSION

An increase of dissolved PAH concentrations was measured in the seawater of the CLB-containing mesocosm during the first three days. Then the concentration reached a plateau, which was due to the modification of oil spill after natural degradation (evaporation, photooxidation). The same observations were made by Stoyanovich *et al*. (2019) with Cold Lake Blend dilbit spilled in freshwater. In a natural environment, this value will vary more under the effect of waves and currents. The dissolved concentrations measured in our study

follow the values of Schmutz *et al.* (2021) in the same exposed condition (mesocosm). As in their study, our concentrations increase for the first 24 hours, then stabilize after 4-5 days. However, the concentrations of dissolved PAHs measured by the probe are a little higher in our study than those in Schmutz *et al.* (2021), which was performed in January with a seawater temperature of -1.85 ± 0.44 °C, with respective values of 371 and ≃ 325 µg/l after seven days. Moreover, the concentrations measured in our study increased about 50 µg/l in seven days, while Schmutz *et al.* (2021) observed an increase of approximately 100 µg/l. Although the lack of ice cover in our case represented greater evaporation, the concentration of dissolved PAHs seems to occur more quickly for Schmutz *et al.* (2021).

An accumulation of petroleum compounds was measured in mussel tissues after eight days, with higher concentrations of Σ_{28}Alkylated-PAHs and Σ_{16}EPA-PAHs compared to Σ_{18}CZDTs and Σ_{13}VOCs. This echoed the results of Schmutz *et al.* (2021), who measured concentrations higher in ΣPAHs in mussel tissues than in ΣCZDBs. But in our previous study (Berthod *et al.*, 2022b), where mussels were exposed to CLB-WAF in winter, their tissues contained Σ_{16}EPA-PAHs, Σ_{18}CZDTs, then Σ_{13}VOCs in decreasing order of concentration. Concentrations of Σ_{28}Alkylated-PAHs were below the limit of detection. In addition to the exposure technique, the exposure time was different between the two studies (48 hours versus 7 days).

During the post-exposure period, bioaccumulated Σ_{16}EPA-PAHs, Σ_{28}Alkylated-PAHs, and Σ_{13}VOCs gradually decreased during the first days, at a specific rate depending on the hydrocarbon group. As observed by Schmutz *et al.* (2021), accumulated ΣPAHs decreased more rapidly than the ΣCZDBs, and remained high four days post-exposure. Yet in their study, bioaccumulated ΣPAH were reduced by 50% after two weeks. Our results showed 50% of reduction after 24h for Σ_{16}EPA-PAHs and after 6h for Σ_{28}Alkylated-PAHs. The higher temperature could explain quicker depuration. It is known that mussels can exhibit a rapid depuration rate during the first days following exposure to hydrocarbons (Fossato and Canzonier, 1976). In our study, this was the case with the ΣPAHs and Σ_{13}VOCs, not Σ_{18}CZDTs.

Lysosomal stability has been classified as a useful and efficient parameter for distinguishing between polluted and clean sites, due to lysosomes' sensitivity to oil's lipophilic constituents (Hwang *et al.*, 2008), such as PAHs. They are likely to penetrate the lipid bilayers impacting membrane permeability and fluidity (Moore and Viarengo, 1987). In *Crassostera gigas* exposed to water accommodated fraction (WAF) of petroleum hydrocarbons, genes associated with the immune system were downregulated, showing possible immunosuppression (Tapia-Morales *et al.*, 2019). This can explain significant lysosomal destabilization observed directly following exposure. After four days in clean water, lysosomal stability was still not significantly different from directly after exposure. In the study of Schmutz *et al.* (2021), no significant effects to lysosomes were visible one month after exposure. Lysosomal integrity of green-lipped mussels (*Perna viridis*), exposed to benzo[a]pyrene returned to its background level after 20 days of depuration (Fang *et al.*, 2008). As lysosomal activity is directly related to immune activity in bivalves, its effectiveness is vital for protection against diseases, it seems that blue mussels need more time to depurate, as validated by the concentrations of Σ_{16}EPA-PAHs, Σ_{18}CZDTs, and Σ_{13}VOCs in mussel tissues.

Immune activity depends on viable cell-to-cell interactions, and therefore hemocyte cell viability is directly related to immune mechanisms (Liu and Gin, 2018). An increase in percentage of unviable cells was also measured after eight days of exposure. But, as opposed to lysosomal stability, significant differences were not observed four days after exposure, with the control treatment displaying the lowest cellular stress. The return to a percentage of viable cells similar to controls could suggest that the cells no longer need to undergo apoptosis to protect themselves, but instead continue with biotransformation. The same recovery rate was measured with comet assay for DNA degradation. Our results revealed the ability of *M. edulis* to repair DNA damage in accordance with previous studies on bivalves (Stambuk *et al.*, 2008; Kolarevic *et al.*, 2013). After exposure to cadmium (Pruski and Dixon, 2002), or environmental pollution like pentachlorophenol and copper sulphate (Villela *et al.*, 2006), levels of DNA damage decreased to healthy values, after 5h or even 2h of depuration, respectively. Although the average percentage of DNA damage decreases to meet control

values, after four days the proportion of 0-20% DNA damage remains lower in exposed mussels than in controls. So, it looks like the DNA damage has not fully returned to a healthy state. We measure a higher proportion of damage between 20 and 60% in exposed mussels. Although we showed that *M. edulis* have the capacity to recover DNA damage after few days, dilbit exposure caused negative transgenerational effects on the unexposed F1 generation (Schmutz *et al.*, 2021).

3.8 CONCLUSION

Quick depuration was observed during the post-exposure period (4 days) for Σ_{16}EPA-PAHs, Σ_{28}Alkylated-PAHs, and Σ_{13}VOCs, while the excretion of Σ_{18}CZDTs took longer. Despite significant bioaccumulation of petroleum hydrocarbons in their tissues, blue mussels showed good resilience, with negative effects on certain biomarkers disappearing within the first week. Even with high DNA damage, a significant decrease in negative effects was measured during the first day of the depuration period. It would therefore be interesting to link the level of DNA degradation in blue mussels to the genetic expression of genes involved in the biotransformation of contaminants and apoptosis.

3.9 FUNDINGS

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3.10 ACKNOWLEDGMENTS

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CHAPITRE 4
ÉTAT DE SANTE DES HEMOCYTES EN TANT QUE BIOMARQUEUR POUR EVALUER LA CAPACITE D'EPURATION DU HOMARD AMERICAIN (HOMARUS AMERICANUS) APRES EXPOSITION AU DIESEL MARIN ET AU BITUME DILUE (DILBIT)

4.1 RÉSUMÉ

Le transport croissant de produits pétroliers pose un risque important de déversement de bitume dilué (dilbit) ou de diesel marin en mer. Malgré l'importance économique du homard américain, il existe peu d'études d'impact d'un tel déversement. Pour surveiller l'état de santé de l'organisme et ainsi évaluer le niveau de contamination et d'épuration, des biomarqueurs sont souvent utilisés par l'industrie du homard, comme l'indice Brix. Notre recherche teste l'efficacité de quatre biomarqueurs (indice Brix, viabilité cellulaire, stabilité lysosomale et induction EROD) sur l'hémolymphe lors d'une contamination (96 h) par le dilbit (Cold Lake Blend) et le diesel marin, mais aussi une période de dépuration ultérieure selon deux cycles de température. À la fin de l'expérimentation, des tests chimiques et d'altération sont effectués. Nos résultats démontrent que parmi les 4 biomarqueurs testés, la stabilité lysosomale et l'induction EROD présentent une sensibilité plus élevée. L'augmentation de la température ne semble pas améliorer significativement la vitesse de dépuration. Les impacts cellulaires sont plus importants chez les homards exposés au dilbit, même si les concentrations d'HAP dans les tissus de homard exposés au CLB sont inférieures à celles exposées au diesel. De plus, un déversement de diesel marin semble être le plus dommageable pour la pêche au homard, car la chair de homard cuite sent encore les hydrocarbures même après dépuration. Enfin, la concentration élevée en HAP mesurée dans les œufs suggère fortement un effet néfaste transgénérationnel potentiel d'un tel déversement.

Les résultats du quatrième article ont été publiés sous forme d'un article intitulé « Hemocytes health status as a biomarker to assess depuration capacity in American lobster (*Homarus americanus*) after exposure to marine diesel and diluted bitumen (dilbit) » dans la revue Marine Science and Engineering (https://doi.org/10.3390/jmse9040370). En tant que premier auteur, ma contribution à ce travail fut l'essentiel de la collection des données, la recherche sur l'état de l'art, le développement de la méthode, la réalisation des analyses et des manipulations, l'analyse et l'interprétation des résultats et la rédaction de l'article. Richard Saint-Louis, directeur de recherche, et Nicolas Toupoint ont fourni l'idée originale, aidé à la recherche sur l'état de la question, au développement de la méthode, à la réalisation des analyses statistiques ainsi qu'à la révision de l'article. Gaëlle Triffault-Bouchet, co-directrice de recherche, et Madeleine Nadeau ont également contribué à la révision de l'article, et Nicolas Lemaire au développement de l'idée originale et à la recherche sur l'état de la question. Les auteurs Marie-Hélène Bénard-Déraspe et Jean-François Laplante ont aidé pour développement de la méthode et la réalisation des analyses et des manipulations.

Une version abrégée de l'article a été présentée à différentes conférences et réunions annuelles : en novembre 2018 par présentation d'un poster, lors de la 16^{e} réunion annuelle Québec Océan à Rivière-du-Loup (Canada) « *Évaluation de la capacité de dépuration chez le homard américain (Homarus americanus) après une contamination aux hydrocarbures pétroliers* » C. Berthod, N. Toupoint, J. F. Laplante, M. H. Bénard-Déraspe, N. Lemaire et R. Saint-Louis ; en mai 2019, lors du 15^{e} colloque international en écotoxicologie aquatique EcoBIM à Sousse (Tunisie) « *Influence of temperature rise on the depuration capacity in American lobster (Homarus americanus) after contamination with petroleum hydrocarbons* » C. Berthod, N. Toupoint, J. F. Laplante, M. H. Bénard-Déraspe, N. Lemaire et R. Saint-Louis, récompensés par le 2^{e} prix de la meilleure présentation ; et enfin, en juin 2019, lors du 23^{e} colloque annuel d'écotoxicologie Chapitre Saint-Laurent à Orford (Canada) « *Évaluation de la capacité de dépuration chez le homard américain (Homarus americanus) après une contamination aux hydrocarbures pétroliers* » C. Berthod, N. Toupoint, J. F. Laplante, M. H. Bénard-Déraspe, N. Lemaire et R. Saint-Louis, récompensés par le 1er prix de la meilleure présentation.

4.2 HEMOCYTES HEALTH STATUS AS A BIOMARKER TO ASSESS DEPURATION CAPACITY IN AMERICAN LOBSTER (*HOMARUS AMERICANUS*) AFTER EXPOSURE TO MARINE DIESEL AND DILUTED BITUMEN (DILBIT)

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4.3 ABSTRACT

The growing transportation of petroleum products pose a significant risk of marine diesel or diluted bitumen (dilbit) spills at sea. Despite the economic importance of the American lobster, there have been few studies assessing the impact study of such a spill on their population. In the lobster industry, lobster quality is monitored according to the Brix index of hemolymph. In our research, the effectiveness of three other biomarkers operative in the industry was assessed in hemolymph during contamination (over 96 h) by marine diesel and dilbit (Cold Lake Blend; CLB), as well as in the subsequent recovery period, according to two temperature cycles. At the end of the experiment, chemical and tainting assays were performed. Our results demonstrate that, among the four tested biomarkers, lysosomal stability and ethoxyresorufin O-deethylase (EROD) induction exhibit higher sensitivity. Increasing the temperature did not shorten the recovery period. Viability cellular impacts

were greater in lobsters exposed to dilbit than that in those exposed to marine diesel. Marine diesel exposure appears to be more problematic for the lobster fishery, as the cooked lobster meat still presented a hydrocarbon odor even after 3 months of live holding. Finally, the high PAH concentrations measured in lobster eggs suggest potential adverse transgenerational effects of marine diesel exposure.

4.4 INTRODUCTION

The American lobsters, Homarus americanus (Milne-Edwards, 1837), is found in the Northwest Atlantic Ocean, between Newfoundland (Canada) and North Carolina (USA). This species is one of the most important exploited crustaceans in the Northwest Atlantic and its fishery represents the major local economy for many North Atlantic coastal communities. The total catch reported for this species by the FAO (Food and Agriculture Organization) in 2016 was 162,547 t. In 2017, the countries with the highest catches were Canada (97,452 t) (DFO, 2017) and the United States (62,006 t) (NOAA, 2020). American lobster is the most valuable species for Canadian fisheries ($1.4 B), contributing to 45% of the whole commercial value of all fisheries in Atlantic Canada in 2018 (MPO, 2018b).

However, this economy has become threatened by another important sector in Canada. The oil industry. Indeed, Canada is one of the world's largest oil producers, benefiting from the third-largest oil reserves at the global scale, mostly in the form of unconventional oil (including diluted bitumen). In 2019, the proven crude oil reserves in Canada accumulated 168.5 billion barrels, of which 164.1 and 4.4 billion barrels were extracted from oil sands and conventional sources, respectively (NRC, 2020a). Most of the crude oil produced in Canada is exported to international markets for further refining. In 2018, 1.7 million barrels of oil (96 billion liters) were shipped daily to domestic refineries (NRC, 2020a). Domestic scales of petroleum products were 1.9 million barrels per day (110 billion liters), with 30% represented by diesel (NRC, 2020b). The exploitation and transport of this resource are still increasing, concomitantly with the demand for petroleum products (EIA-US, 2012). Although the frequency of large oil spills from tankers has been decreasing worldwide (Lee

et al., 2015b), inshore fisheries remain vulnerable to such a risk. Coastal areas are more likely to be exposed to small and medium petroleum product spills (< 1000 m^3), due to increased human activities (Marty and Potter, 2014), with small diesel spills (10–100 m^3) being the most common in Canada, following an annual frequency of 0.6 (Lee *et al.*, 2015b). Two small spill events occurring in the Gulf of St. Lawrence (eastern Canada) illustrate that for lobster fishery, the risk of exposure to hydrocarbons is real. In September 2013, 5 m^3 of heavy fuel were released into the marine waters of the Baie de Sept-Îles from an inland spill of 450 m^3, caused by human error at an industrial site. In September 2014, 100 m^3 of diesel leaked in Cap-aux-Meules Harbour (Îles-de-la-Madeleine), due to a failure in a pipeline that annually pipes 40 million liters of diesel from this harbor to a local power plant. Globally, there is an increased probability of small to medium spill events of various petroleum products, from diesel to diluted bitumen, due to the ongoing expansion of routine shipping operations and coastal activities.

Oil spills contaminate sediments (O'Sullivan, 1978) and the accumulated compounds degrade slowly (Liu *et al.*, 2012). Benthic invertebrates can take up hydrocarbons by feeding on contaminated material, or through direct absorption from sediments or water (Rumney *et al.*, 2011; Michel and Fingas, 2016). For lobsters, this results in high levels of PAHs, which can then persist over years: up to six and seven years after for the Braer (Kingston, 1999) and Amoco Cadiz (Law *et al.*, 2002) oil spills, respectively. Significantly elevated concentrations of total aromatics were found in the hepatopancreas of American lobster and their decrease was measured only after 11 days of depuration (Williams *et al.*, 1989). Few reviews have reported the impact of hydrocarbons on lobsters during oil spills or laboratory experiments, highlighting higher mortalities or lower abundances as major impacts (Spaulding *et al.*, 1996; Sanchez *et al.*, 2006). For example, a loss of 9 million lobsters was estimated after the North Cape oil spill in 1996 (23,000 barrels of home heating oil), based on observed abundances in the affected area compared to reference sites (Cobb and Clancy, 1998, 1999; French, 1999), in addition to the 2.9 million lobsters stranded on the beaches (Gibson *et al.*, 1997). In crustaceans, integument damage may appear following an oil spill, thus compromising the integrity of their epicuticle, which serves as a physical barrier between the environment and

the more permeable inner layers of the exoskeleton (Cobb *et al.*, 1999; Felder *et al.*, 2014). Behavioral effects have also been observed in lobsters, such as an increase in the delay between noticing food and pursuing it (Atema and Stein, 1972; 1974; Blumer *et al.*, 1973; Biedron and Evans, 2016), disorientated behavior, and uncoordinated movements (Richardson, 1984). Sublethal effects of heavy fuel oil have been observed in red rock lobster (*Jasus edwardsii*), such as a change in circulating immune cell populations, which recovered by the conclusion of a 96h exposure period (Webby and Ling, 2016). The depuration of PAHs appeared to be particularly slow in red rock lobster and significantly elevated PAH levels were still evident after 10 days of depuration (Webby and Ling, 2016). Furthermore, exposed lobsters may be unfit for human consumption due to gill-tissue damage (Payne *et al.*, 1983), as well as deterioration in meat color and smell (Michel and Abarnou, 1978). As a matter of fact, tainting occurs when the organism accumulates enough hydrocarbons to cause an "off" flavor or odor in the seafood (Michel and Fingas, 2016). These organisms are unsuitable for consumption until this tainting disappears, where associated delays can take from days to up to a year after exposure, depending on the species (Spaulding *et al.*, 1996). A study of Williams *et al*. (1989) observed that the odor of raw treated lobsters differed significantly from that of the control, even after 21 days of depuration; however, cooking appeared to remove the oily odor from the raw lobster. Despite the risk of diluted bitumen (dilbit) or diesel marine spills, to the best of our knowledge, there have been no studies dealing with the impacts of a dilbit spill on American lobsters and no studies on the sub-lethal impact of a marine diesel or dilbit spill. In addition, due to the bioavailability of soluble compounds and persistent polycyclic aromatic, organic sulfur, and heterocyclic compounds, it can be assumed that light petroleum products (e.g., marine diesel) are more toxic than heavy petroleum products (e.g., dilbit), despite their greater tendency to bind receiving organisms and environments.

Sub-lethal levels of contamination are usually quantified using biomarkers as exhibited observable or measurable changes at the molecular, biochemical, cellular, or physiological level. Biomarkers can reveal the exposure of a living organism to a present or past chemical substance and its associated effects (Lagadic, 1997). The monitoring of such markers can

facilitate the early detection of pathogens or anthropomorphic stressors. For example, McDonald *et al.* (1996) measured a significant induction of enzymatic activity in gray shrimp (*Penaeus aztecus*), for individuals sampled near (< 100m) an oil platform. Among the major physiological functions, the immune system is a significant mechanism that reflects the individual's health status. A deterioration in immune functions can quickly lead to morbidity or mortality (Fournier *et al.*, 2000). The efficiency of an individual's immune system can be compromised when specific tolerance thresholds of environmental conditions are exceeded, thus increasing their susceptibility to pathogens (Callaway *et al.*, 2012). In crustaceans, immunity is supported by hemocytes, circulating cells that are involved in multiple physiological functions and homeostasis processes. Therefore, the study of hemocyte functions is a prime target for use in the determination of health status.

In the lobster industry, the Brix index is routinely measured from the hemolymph, to evaluate the global lobster health. Simon *et al*. (2015) identified the Brix index as the most accurate and practical biomarker among eighteen hemolymph parameters, for reflecting the state of energetic reserves in spiny lobsters. Indeed, the Brix index value is highly correlated with the hemolymph total protein (Paterson *et al.*, 1999; Ozbay and Riley, 2002; Simon *et al.*, 2015; Mendo *et al.*, 2016), triglycerides, cholesterol, calcium, and phosphorus concentrations as well as with lipase activity (Simon *et al.*, 2015). To measure the lobster health status, various physiological changes can be assessed by assaying total blood proteins with the Brix index (Lorenzon *et al.*, 2007; Moisan *et al.*, 2008). As Battison (2018) pointed out, other applications could include the evaluation of lobsters subjected to different suspected exposure conditions to noxious or infectious agent(s). However, the effectiveness of the Brix index as an indicator of lobster quality following a petroleum product spill not yet known.

In this context, our study aims to test three biomarkers in addition to the Brix index, in punctured lobster hemolymph which is routinely monitored in industrial practices after contamination by two petroleum products: CLB or marine diesel. These are cell viability, lysosomal membrane stability, and activity of a phase I detoxification enzyme

(ethoxyresorufin O-deethylase; EROD). An initial sub-lethal effect is easily measured using Trypan blue (TB) dye exclusion to assess cell viability, which is one of the most common methods. It is based on the ability of the membrane of viable cells to exclude the dye. Destabilization of the lysosomal membrane is another recommended biomarker for monitoring the impact of petroleum hydrocarbon pollution (Martínez-Gómez *et al.*, 2015) as the excessive lysosome activity induced by dissolved hydrocarbons leads to an increase of enzymatic activity and can cause the destabilization of their membranes. The measurement of this destabilization by the neutral red retention (NRR) assay provides a sensitive index of the cell condition that can be related to the grade of contamination or recovery. Finally, the ethoxyresorufin-O-deethylase (EROD) biomarker is often used to assess exposure of marine organisms to PAHs. The EROD activity is measured to observe the induction of phase I P450 enzymes (Rewitz *et al.*, 2006; da Silva Rocha *et al.*, 2012a; CEAEQ, 2015c), which are responsible for xenobiotic biotransformation and are involved in reactive oxygen species production and accumulation. This enzyme family exhibits the highest levels in cell tissues of organs involved in food processing (Livingstone, 1998; Rewitz *et al.*, 2003, 2006), explaining why EROD activity is classically measured in the hepatopancreas for crustaceans. A few studies have measured this activity induction in hemolymph (Monari *et al.*, 2009), but not in lobsters.

These four biomarkers were tested at the end of exposure, as well as over 3 weeks following contamination. We also intended to explore the influence of water temperature on lobster recovery, as it may modulate the efficiency of this operation (Little *et al.*, 1985). In order to check the quality of lobsters in the context of industrial sales, the olfactory, visual, and taste quality were tested after the recovery phase. In addition, this study provides new data on the sublethal impacts of unconventional oil and marine diesel spills on American lobster and their recovery capacity. For exploratory purposes, chemical analyses of lobster tissue were carried out after several months in a fishpond.

4.5 MATERIALS AND METHODS

4.5.1 Conditions of oil spill simulation

Oil Products

Two types of petroleum products were tested: diluted bitumen (dilbit) from Alberta Cold Lake Blend (CLB) and a classical marine diesel from New Brunswick (Table 11).

Table 11 : Properties of Cold Lake Blend and marine diesel used in the assays. Data from (Crude Monitor, 2016b)

	Cold Lake Blend (CLB)	**Marine Diesel**
Origin	Alberta	New Brunswick
API (degree API)	20.9	35
Density at 15 °C (g/ml)	0.9249	0.84
Viscosity at 15 °C (cP)	285	2
Nickel ppm	65	*na*
Vanadium ppm	168	*na*
Saturated hydrocarbons (%)	45	65 - 95
Aromatics hydrocarbons (%)	30	5 - 25

Water Accommodated Fraction

In this study, lobsters were exposed to oil physically dispersed by the technique of water accommodated fraction (WAF). The WAF was prepared according to the standardized protocol of Singer *et al.* (2000), modified by Barron and Ka'aihue (2003) and Payne *et al.* (2014) for CLB and marine diesel, respectively. The WAF was prepared before each exposure by adding the tested oil products (1:10) to filtered natural seawater (0.2 µm), collected in Cap-aux-Meules, in a fluorinated polyethylene carboy with 20% headspace. The solution was gently mixed for 18 h then left to settle for 6 h after the cessation of stirring. The contaminated seawater was then recovered and diluted with filtered natural seawater for the exposure. The dilutions used were 0% and 60% of WAF for Control and CLB/marine diesel treatments, respectively.

Monitoring of dissolved aromatic hydrocarbons by fluorescence

During lobster exposure, the concentration of the aromatic hydrocarbon dissolved fraction of crude oil was estimated using the Cyclops-7 submersible fluorometric sensor, equipped with the "O" sensor (Turner Designs, San José, CA, USA) for the detection of crude oil. It is one of the five UV submersible fluorometers that are commercially available for in situ measurements of PAHs, and one of the most commonly used in delineating oil plumes in the field (Conmy *et al.*, 2014; Hwang *et al.*, 2020). Its fluorescence optical specifications are excitation wavelengths at 325/120 nm and emission wavelengths at 410–600 nm. The Cyclops-7 sensor was controlled by a DataBank module, which was connected to a computer by proprietary software (Turner Designs, San José, CA, USA). The sensor was calibrated with an aqueous solution of 100 µg/l of tetrasodium 1,3,6,8-pyretetetrasulfonate (PTSA), a highly water-soluble pyrene derivative (CAS 6528-53-6; Sigma-Aldrich, Darmstadt, Germany). The fluorescence measurements were used as an indicator of hydrocarbon concentration and, thus, were expressed in equivalent µg PTSA/l. The sensor response was linear within the PTSA concentration range used, from 5 to 550 µg/l. The measurements were made in situ in 15-liters exposure tanks.

4.5.2 Animals

One batch of lobsters (Homarus americanus; sex ratio 1:1), captured during the 2018 fishing season in Lobster Fishing Area 22 (LFA 22, Quebec City, Canada), was kept in a Merinov containment unit (without feeding) in Cap-aux-Meules, and maintained at 2°C to induce natural winter dormancy. A total of fifty-four (54) lobsters were used for the experiment, including 30 females and 24 males (88.3 ± 0.5 mm mean length and 559.5 ± 9.5 g).

4.5.3 Exposure and Recovery

Two series of assays were performed, with similar exposure conditions, but two different thermal regimes for recovery. For each series, twenty-seven (27) lobsters were

randomly assigned to nine (3 × 3) independent 15 L tanks (Figure 53) of oxygenated natural seawater, in order to carry out the following treatments in triplicate: Control (uncontaminated natural seawater), Cold Lake Blend (WAF-CLB) and marine diesel (WAF-Diesel). Three lobsters were used per replicate per treatment per series. Exposure was carried out for 96 h at 4°C with filtered natural seawater for Control and 60% diluted WAF for CLB and marine diesel (no feeding and water renewal). During the exposure, the physicochemical characteristics of the water were monitored daily (i.e., temperature, salinity, dissolved oxygen and ammonia).

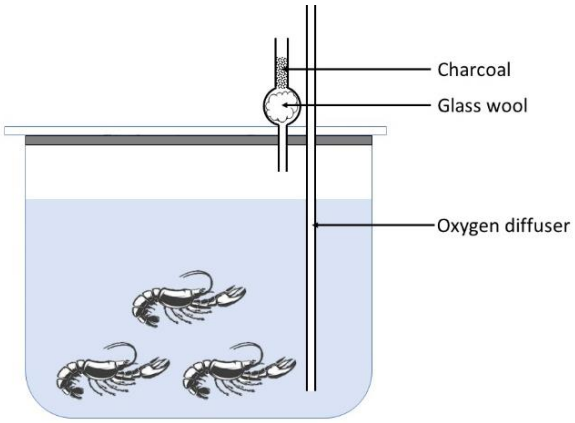

Figure 53 : Scheme of 15-liters exposure tank

At the end of the exposure, the lobsters were removed and placed in two series of new tanks filled with uncontaminated and unfiltered natural seawater, thus starting the depuration period. The recovery period is defined as the time required for an organism to return to its pre-exposure health state. This recovery time depends on the type of pollutant and the amount accumulated in tissues. At this stage, two thermal conditions were applied during the three weeks: series A, with a constant water temperature at 4°C, and series B, with a water temperature gradually increasing from 4 to 9°C. The tanks were cleaned, the water was renewed daily, and the physicochemical characteristics of the water were monitored daily (i.e., temperature, salinity, dissolved oxygen, and ammonia). The seawater physicochemical characteristics did not change during the assay, with a level of ammonia remaining lower than the toxic levels for lobster (5.2 ppm for adult lobsters at 5°C; Young-Lai *et al.*, 1991).

Finally, the dissolved aromatic compounds concentration of the crude oils was also measured daily, as described earlier (by fluorescence).

At the end of the three weeks of recovery, the lobsters were returned to open live holding tanks dedicated to long-term contention at a constant temperature of 2°C. Three months later, the organoleptic conditions (odor and color, before and after cooking) and PAH concentrations of tissues were checked.

Throughout the study (exposure and recovery) the lobsters were not fed in order to approximate industrial conditions, where no feed is provided to the lobster holding tanks. In addition, as benthic invertebrates can uptake hydrocarbons through the consumption of contaminated prey, the absence of food avoids the input of contamination from food as well as an additional variable in the analysis of results. The results presented for the exposure impacts combined the two series values for each condition. As the environmental conditions were the same for series A and B, the data of both were grouped together for each treatment ($n = 2 \times 3$). Then, the results of the recovery were presented by differentiating the two temperature templates.

4.5.4 Hemolymph Sampling

The Hemolymph puncture allows for rapid and non-lethal sampling. For each experimental condition, each lobster was punctured during the exposure period at the following times: 0 h (before exposure) and 96 h (end of exposure), and at: 24, 72, 168, 336 and 504 h in the recovery period (i.e., 1, 2, and 3 weeks of recovery). The 0 h time point during the exposure period corresponds to the puncturing of lobsters just before placing them in their tanks with associated samples, representing the initial health status.

The hemolymph was punctured under the abdomen (after the first pair of pleopods) with a 3 mL syringe and a 23 G needle. Two 0.5 mL hemolymph punctures were performed: the first for the Brix index test and a second one, using a preloaded syringe of a PBS_EDTA mixture (0.5 mL; 15 mM), for the three other biotests (TB, NRR, and EROD).

4.5.5 Biotests

Brix index

The Brix index (%) value was obtained by reading a 0.5 mL hemolymph volume in a refractometer (PAL-1; Atago), which had been calibrated beforehand using deionized water (0). The Brix index can be converted into a refractive index (RI), as follows (Leavitt and Bayer, 1977):

$$RI = 0.0015 \times Brix + 1.3325$$

The total protein (TP; mg/ml) in the hemolymph can then be determined, using the refractive index RI, as follows:

$$TP\ (mg/mL) = 5{,}449.417 \times RI - 7{,}295.321$$

Cellular viability

The viability of hemocytes was assessed by a membrane integrity test, the Trypan blue dye exclusion method. After the hemolymph puncture, as described above, 40 µl of cell suspension was added to 20 µl of Trypan blue dye solution (CAS 72-57-1). After 15 min, 20 µl of the mixture was deposited on a glass slide and observed using an optical microscope. Fifty cells were classified according to two categories: (1) Living cells, uncolored; and (2) dead cells, blue-colored.

Lysosomal membrane destabilization index

The lysosomal membrane destabilization index was determined using NRR assay, performed according to the method described by Song *et al.* (2007) modified by Small *et al.* (2007). A stock solution of neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, $C_{15}H_{17}ClN_4$, Sigma) was prepared by dissolving 2.22 mg in 1 ml of dimethyl sulfoxide (DMSO, C_2H_6OS, Sigma-Aldrich). The dye stock solution was kept at 4°C until required. A working solution was made daily by dissolving 8.5 µl of the dye stock solution in 500 µl of physiological saline solution (NaCl 19.31 g/l ; KCl 0.65 g/l ; $CaCl_2$ 1.38 g/l ; $MgSO_4$ 1.73 g/l ; Na_2SO_4 0.38 g/l ; HEPES 0.82 g/l adjusted to the pH of natural seawater).

The hemolymph (0.2 ml) was transferred to an Eppendorf tube with physiological saline solution (0.2 ml). An aliquot of 40 µl was transferred to a positively charged microscope slide and incubated in a dark humidity chamber at ambient temperature for 15 min to allow cells to attach to the slide. Then, the working solution (40 µl) was put on the slide. After 15 min in the same humidity chamber, a cover slip was placed on the slide. After 45 min, normal and destabilized cells were counted using a light microscope. In healthy cells, neutral red is retained in the lysosomes, turning them red, while the cytosol is colorless. For damaged cells, the efflux of red dye into the cytosol results in redness of the cytosol. Increased cellular retention of neutral red dye, therefore, corresponds to a healthier lobster. For each sample, at least 50 hemocytes were analyzed. The lysosomal membrane destabilization index (LDI; %) was calculated as the number of hemocytes with destabilized lysosomes (DH), as follows:

$$\text{LDI (\%)} = (\text{DH}/50) \times 100$$

EROD

The EROD activity test used for this study was based on the method described by Burgeot and Ménard (2004) and modified according to Monari et al. (2009) to adapt it to the hemolymph. The resorufin formation was followed by fluorimetry at 585 nm using a microplate reader (BioTek Epoch2), with an excitation wavelength of 530 nm. The fluorescence of the samples indicates the resorufin concentration by a standard curve. EROD activity is related to the protein concentration determined according to the Bradford assay (Bradford, 1976). The activities are expressed in picomoles per minute and per milligram of protein:

$$EROD = \frac{[resorufin]*\text{reaction } volume}{time\ (\text{min})*[protein]*volume},$$

4.5.6 Tainting Assay

An oil spill can affect the olfactory, visual, and taste quality of crustaceans, precluding their commercial value. Tainted seafood presents abnormal odor or flavor, atypical of the

seafood itself (ISO, 1992). This degradation may persist beyond the recovery period (Reilly and York, 2001). Three months after the experiments, one lobster per treatment and per series (for a total of 6) was sacrificed to perform olfactory and visual evaluations in fresh and cooked (after 15 min in boiling water) states. However, due to this low number of specimens, these qualitative results are only indicative.

4.5.7 Tissues Analysis for Polycyclic Aromatic Hydrocarbons (PAHs)

After 3 months of live holding, 2 lobsters per treatment and per series (for a total of 12) were sacrificed and tissues were analyzed for their PAH contents. Lobsters were boiled (15 min) and several tissues were recovered for chemical analysis: abdomen, claw, and hepatopancreas. In addition, three females of the sampled lobsters (1 in Control and 2 for marine diesel treatment) had spawned during this stage, thus permitting us to also assess PAHs in the eggs. Tissues were kept at −20°C until further analysis.

PAHs Extraction

Lobster tissues were freeze-dried (FreeZone, Labconco, USA) and homogenized into a fine powder using a Virtis homogenizer. A sub-sample of about 300 mg was suspended in 5 ml of tetramethylammonium hydroxide (TMAH, 25% water; Sigma Aldrich, Darmstadt, Germany) in a 12 mL glass tube with a Teflon-lined cap and vortexed for 1 min. The mixture was then heated (60°C) for one h with manual stirring every 15 min, in order to complete the alkaline digestion. After cooling the mixture to room temperature, 1.0 ml of deionized water, 1.0 g of NaCl, and 4.0 ml of hexane/toluene mix (1:1) were added before stirring for one h (Wrist Action Shaker; Burrell Scientific, Pittsburgh, PA, USA). The mixture was centrifuged (at 3000 g), and the upper organic layer was recovered. A second extraction step was performed with another 4 mL of hexane/toluene mix (1:1) and this recovered organic layer was added to the first one. The whole organic extract was then cleaned on a silica column topped with sodium sulfate. The volume of the cleaned extract was reduced under a gentle stream of nitrogen, at room temperature, to a final volume of 1000 µl. A volume of 150 µl of

the concentrated extract was pipetted and transferred to a GC vial equipped with a glass insert and a volume of 50 µl of a solution of deuterated PAHs was added as an internal standard.

PAHs Detection and Quantification

PAH analyses were performed using a gas chromatograph (GC, Agilent Technologies 6850 series II; Santa Clara, CA, USA) coupled to a mass spectrometer (MS, Agilent Technologies 5975B VL MSD). Injection (1µl) was performed using an Agilent Technologies Auto sampler 6850 series, at a temperature of 250°C with a splitless injection mode. The capillary column was an Rxi®-5ms (30 m × 0.25 mm ID × 0.25 µm FT, 5% diphenyl and 95% polysiloxane from RESTEK), with helium as the carrier gas at a flow rate of 1 ml/min. The oven temperature program was set as follows: 50°C for 2 min, 15°C/min until 275°C and 2 min hold, 15°C/min until 325°C and 15 min hold, and a post-run of 2 min at 300°C. The detection of PAHs was performed in scan mode between 50 to 500 amu, with positive ion detection, where quantification of each PAH was based on the ratio of the signal of their molecular ion relative to the signal of the appropriate internal standard. Sample blanks were processed and analyzed. Thirty-five PAHs (∑35Total-PAHs) were quantified. Among them, sixteen PAHs classified as priority pollutants by the United States Environmental Protection Agency (US-EPA, 1984) were quantified (∑16PAHs-EPA), along with 19 alkylated PAHs (∑19Alkylated-PAHs).

4.5.8 Statistical Treatment of Biomarker Responses

At first, the assumptions of normality and the homoscedasticity were verified using the Shapiro and Bartlett tests, respectively. Exposure data were analyzed by performing two-way repeated measures analysis of variance (ANOVA) to determine the significance of the effects of treatment and time. The post hoc Tukey's multiple comparison test was performed for comparison among the 2 times (0 and 96 h) and 3 treatments. Recovery data were first analyzed using one-way ANOVA followed by Tukey's test to compare the effects of petroleum treatments (CLB and marine diesel) to Control at each time. Secondly, each treatment was analyzed by performing a two-way repeated measures ANOVA, followed by

Tukey's test, to determine the significance of the effects of time and series. All tests were regarded as statistically significant when $p < 0.05$. All data were statistically analyzed using R software version 3.5.2 (R Core, 2018). The chemical analysis was not assessed statistically, due to the low number of replicates.

4.6 RESULTS

4.6.1 Exposure

Monitoring of dissolved aromatic hydrocarbons in experimental units

Figure 54 shows the dissolved aromatic hydrocarbons concentrations during exposure (0–96 h), expressed in equivalent PTSA and measured with the fluorescent probe during the experiments of series A and B combined.

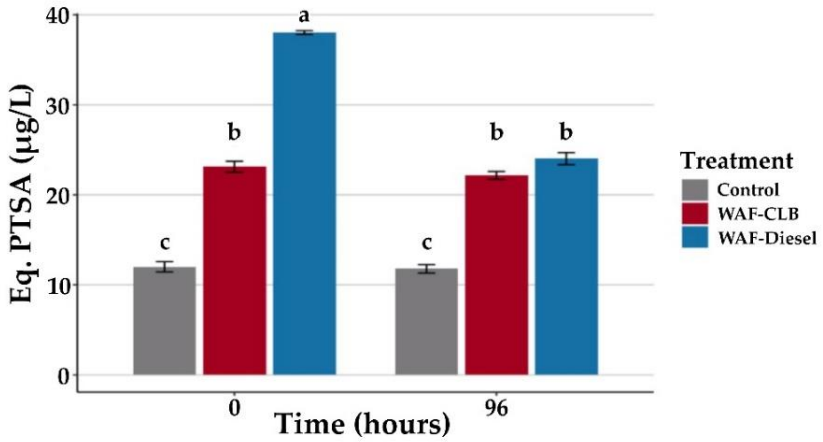

Figure 54**.** Hydrocarbon concentrations in each treatment (Control, WAF-CLB, and WAF-Diesel), measured by fluorescence (Cyclops-7) and expressed in PTSA equivalent (µg/L) before (0 h) and after 96 h of exposure. Series A and B are combined. Mean ± SE; n = 6. The letter symbols indicate a significant ($p < 0.05$) effect between time and treatment

At 0 h, the tanks contained either clean filtered sea-water or 60% WAF with CLB (WAF-CLB) or marine diesel (WAF-Diesel) mixed in clean sea-water, with no lobsters. The concentration of dissolved aromatic hydrocarbons was significantly higher in exposure tanks (WAF-CLB and WAF-Diesel) than in the Control. Moreover, this concentration was

significantly higher in WAF-Diesel tanks than in WAF-CLB. Hydrocarbon concentrations estimated by Cyclops-7 were in the range of LC50∞, which is 6–400 µg/l with the mean 50 µg/l of dissolved PAH concentration according to McCay (2003). At the end of the exposure (96h), the concentration of dissolved aromatic hydrocarbons in WAF-Diesel tanks was equal to that of WAF-CLB, thus decreasing by a 0.6 factor; both remained significantly greater than the Control. Despite an initial evaporation of the lightest compounds during the preparation of the WAFs (24h), evaporation seemed to continue during the exposure of the lobsters to the WAF-Diesel, unlike the WAF-CLB.

Sublethal Impacts after 96 h Exposure

Figure 55 shows the total protein concentrations, as inferred from measurements of the Brix index, before (0h) and after exposure (96h). This concentration did not differ significantly between the measuring points, nor between the three treatments (Control, WAF-CLB, and WAF-Diesel).

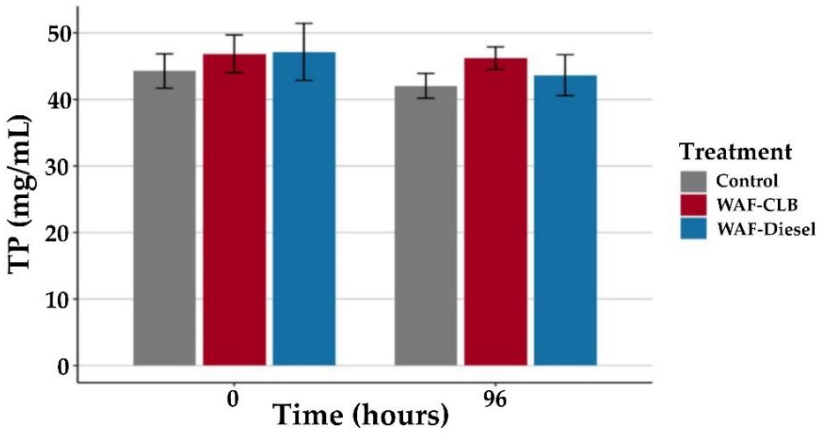

Figure 55 : Total protein content (TP; mg/ml) in hemolymph, as calculated from measurements of the Brix index, of lobsters for each treatment (Control, WAF-CLB, and WAF-Diesel) before (0h) and after 96h of exposure. Series A and B are combined. Mean ± SE; n = 6

Figure 56 and Figure 57 shows the cellular impacts, namely on cell viability and lysosomal stability, respectively, measured before (0h) and after 96h of exposure to hydrocarbons. Globally, the cell viability tended to drastically decrease in lobsters exposed to WAF-CLB (13.2 ± 2.5% nonviable at 0h vs. 19.9 ± 3.0% at 96h), whereas this trend was inverse for Control and stable for WAF-Diesel. An impact on cell viability after exposure was only observed in lobsters exposed to WAF-CLB, with a significant 2-fold higher fraction of nonviable cells when compared to both Control and WAF-Diesel treatments, which exhibited similar values.

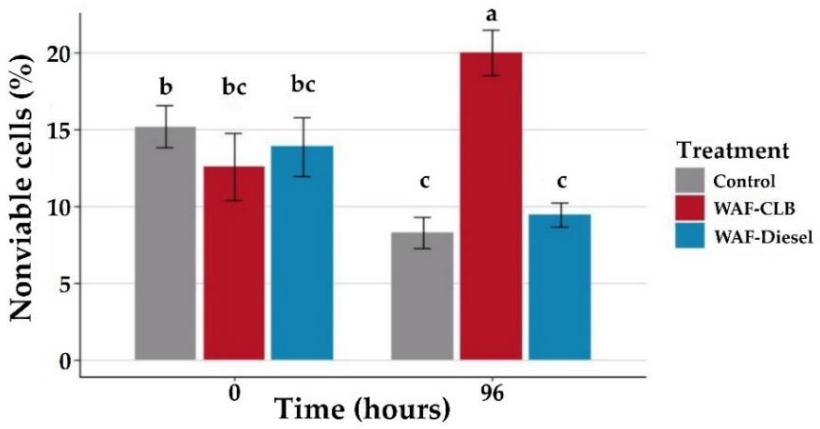

Figure 56 : Fractions of nonviable lobster' hemocyte cells for each treatment (Control, WAF-CLB, and WAF-Diesel) before (0 h) and after 96 h of exposure. Series A and B are combined; Mean ± SE; n = 6. The letter symbols indicate a significant ($p < 0.05$) effect between time and treatment

The lysosomal membranes (Figure 57) were significantly destabilized when exposed to both types of oil (CLB and marine diesel), affecting 45.4 ± 2.1% and 39.8 ± 2.5% of cells, respectively, vs. 16.0 ± 2% in the Control after 96 h of exposure. The two treatments affected the lysosomal membrane stability in the same way.

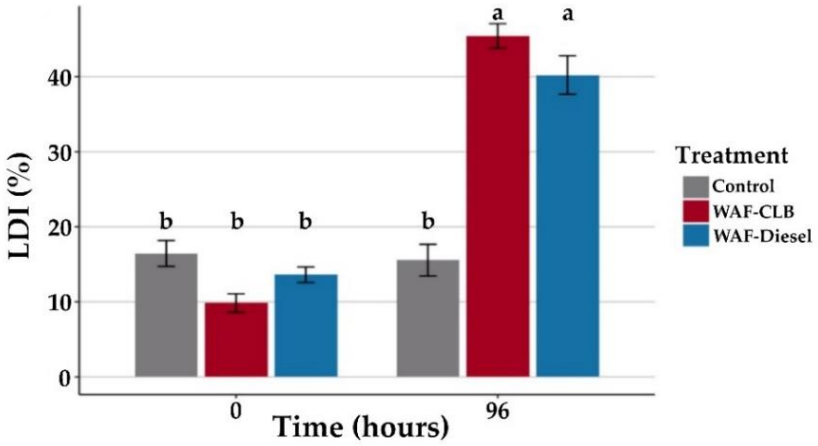

Figure 57 : Fraction of lysosomal membrane destabilization (LDI) in lobster' hemocyte cells for each for each treatment (Control, WAF-CLB, and WAF-Diesel) before (0 h) and after 96 h of exposure. Series A and B are combined; Mean ± SE; n = 6. The letter symbols indicate a significant ($p < 0.05$) effect between time and treatment

The EROD biomarker was used to measure the biotransformation activity of contamination in the hemolymph. EROD activities and associated induction rates between before (0h) and after exposure (96h) are presented in Table 12. Inductions of the hemolymph EROD activity were observed in lobsters exposed to both WAF-CLB and WAF-Diesel, with a significantly higher activity than the Control and an induction rate close to 2. As with the NRR biomarker, the two treatments had similar values for the EROD biomarker.

Table 12 : EROD activity in lobster hemolymph and associated induction rates for each treatment (Control, WAF-CLB, and WAF-Diesel) before (0 h) and after 96 h of exposure. Series A and B are combined. Mean ± SE; n = 6. The letter symbols indicate a significant ($p < 0.05$) effect between time and treatment

Treatment	**EROD activity**		**Induction rate**
	0h	**96h**	
Control	5.8 ± 0.3^{b}	5.5 ± 0.3^{b}	0
WAF-CLB	6.2 ± 0.6^{b}	12.3 ± 1.1^{a}	2
WAF-Diesel	5.9 ± 0.2^{b}	14.0 ± 0.9^{a}	2.4

4.6.2 Recovery Capacity

Brix index

Table 13 shows the results for the Brix index of series A and B obtained during the recovery period. No significant effect of oil product exposure was observed in terms of the total protein content in the hemolymph of lobsters during the recovery period, regardless the oil or thermal system.

Table 13 : Total protein content (mg/ml) in hemolymph, as calculated from measurements of the Brix index, of lobsters for each treatment (Control, WAF-CLB, and WAF-Diesel) during the recovery period in series A and B. Mean ± SE; n = 3

Treatment	End of Exposure	Recovery Period				
	96 h	24 h	72 h	168 h	336 h	504 h
	Series A					
Control	41.2 ± 2.5	39.4 ± 2.9	37.7 ± 2.2	35.7 ± 2.8	31.8 ± 3.3	29.7 ± 2.3
WAF-CLB	47.5 ± 3.3	47.4 ± 2.7	42.3 ± 2.1	39.1 ± 1.6	37.1 ± 2.6	37.2 ± 1.9
WAF-Diesel	42.4 ± 2.2	41.8 ± 2.0	37.1 ± 2.7	37.7 ± 1.7	32.3 ± 1.8	30.0 ± 1.4
	Series B					
Control	42.1 ± 4.7	44.3 ± 4.8	44.1 ± 4.6	43.8 ± 4.3	38.6 ± 4.1	37.1 ± 4.7
WAF-CLB	46.2 ± 6.2	43.5 ± 6.1	44.4 ± 5.3	45.3 ± 5.3	36.4 ± 5.0	38.9 ± 5.4
WAF-Diesel	45.7 ± 6.3	38.4 ± 5.0	41.0 ± 4.6	40.6 ± 4.3	32.1 ± 4.1	26.2 ± 3.1

Nevertheless, at the end of recovery (504 h), in lobsters exposed to hydrocarbons, the total protein content seems to be lower in lobsters exposed to WAF-Diesel than those exposed to WAF-CLB.

Figure 58 shows the time effect according to the oil treatments (WAF-CLB and WAF-Diesel). In Control lobsters, the Brix index did not change during the recovery in series B. In series A, a significant decrease was measured from 2 weeks of recovery (336h). For the oil treatments, the level of total protein tended to gradually reduce during the depuration phase in series A (Figure 58). In lobsters exposed to WAF-Diesel, the values significantly decreased during recovery in the two series, from 2 weeks (336h). In lobsters exposed to WAF-CLB in

series A, the difference was significant from 3 weeks of recovery (504h). Decreases in total protein may be due to the non-feeding condition applied during this study.

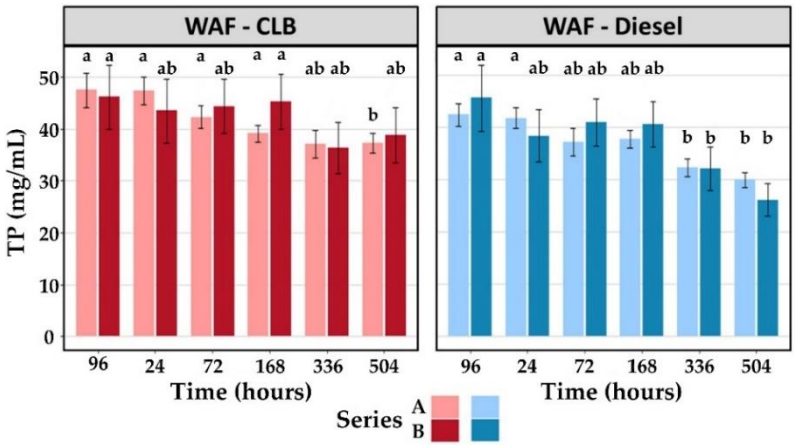

Figure 58 **:** Total protein content (TP; mg/ml) in hemolymph, as calculated from measurements of the Brix index, of lobsters exposed to WAF-CLB and WAF-Diesel during the recovery period in series A and B. Mean ± SE; n = 3. The letter symbols indicate a significant ($p < 0.05$) effect between time and series

Cellular viability

Globally, the percentage of dead cells was relatively low (< 25%), regardless the thermal conditions (series A and B; Table 14).

Table 14 : Fraction of nonviable lobster' hemocyte cells for each treatment (Control, WAF-CLB, and WAF-Diesel) during the recovery period in series A and B. Mean ± SE; n = 3. The * symbols indicate a significant ($p < 0.05$) effect of treatment for the considered time

Treatment	End of Exposure	Recovery Period				
	96 h	24 h	72 h	168 h	336 h	504 h
			Series A			
Control	9.8 ± 1.2	8.7 ± 1.8	8.3 ± 1.2	7.8 ± 1.4	8.8 ± 1.5	9.8 ± 1.2
WAF-CLB	18.3 ± 3.5*	9.6 ± 2.6	9.1 ± 0.8	8.0 ± 1.6	8.2 ± 1.0	10.0 ± 1.6
WAF-Diesel	10.4 ± 1.0	9.1 ± 0.7	9.7 ± 1.3	10.0 ± 2.8	9.0 ± 0.9	9.6 ± 1.3
			Series B			
Control	7.2 ± 1.1	12.2 ± 1.2	11.8 ± 2.1	12.4 ± 1.6	5.8 ± 0.7	5.6 ± 1.2
WAF-CLB	21.6 ± 5.2*	19.6 ± 3.2*	14.2 ± 1.6	7.3 ± 0.9	8.7 ± 2.1	6.7 ± 1.4
WAF-Diesel	8.4 ± 1.4	8.7 ± 1.7	15.6 ± 3.1	8.2 ± 1.3	5.9 ± 0.9	5.4 ± 1.2

In series A, the viability effect was significantly different in the WAF-CLB lobsters at the beginning of recovery (0 h), but reached the Control and WAF-Diesel values at 24 h of recovery. When the temperature increased in series B, the percentage of nonviable cells decreased and reached the control values from 72 h of recovery. In the lobsters exposed to WAF-Diesel, the cell viability was equivalent to the Control in both series (see Table 10).

During the recovery period (3 weeks), the percentage of nonviable cells did not vary significantly in Control lobsters and those exposed to WAF-Diesel, for both series. A significant decrease was measured in lobsters exposed to WAF-CLB in both series. Figure 59 shows the percentage of nonviable cells for these lobsters in both series.

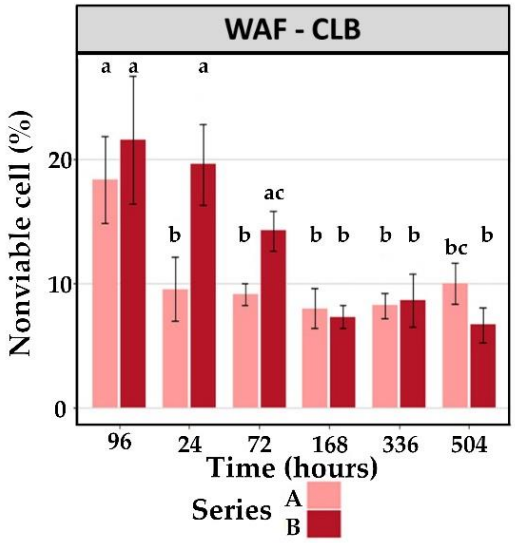

Figure 59. Fractions of nonviable lobster' hemocyte cells for WAF-CLB treatment during the recovery period in series A and B. Mean ± SE; n = 3. The letter symbols indicate a significant ($p < 0.05$) effect between time and series

In lobsters exposed to WAF-CLB in series A (constant 4°C), a significant decrease in the percentage of nonviable hemocytes was observed in the first 24h of recovery. In series B, this percentage significantly decreased from 1 week (168h) of depuration. The decrease under an increasing temperature (series B) was slower, than that under a constant temperature (series A).

Lysosomal membrane destabilization index

Table 15 shows the percentage of hemocytes with destabilization of the lysosomal membrane during the recovery period.

Table 15 : Fraction of lysosomal membrane destabilization (LDI) in lobster' hemocyte cells for each treatment (Control, WAF-CLB, and WAF-Diesel) during the recovery period in series A and B. Mean ± SE; n = 3. The * symbols indicate a significant ($p < 0.05$) effect of treatment for the considered time

Treatment	End of Exposure	Recovery Period				
	96 h	24 h	72 h	168 h	336 h	504 h
	Series A					
Control	12.8 ± 1.6	17.8 ± 0.7	17.6 ± 0.7	14.9 ± 1.0	13.1 ± 1.3	10.2 ± 1.8
WAF-CLB	46.0 ± 3.1 *	40.7 ± 3.8 *	39.3 ± 2.6 *	26.7 ± 2.7 *	16.9 ± 0.7	11.1 ± 1.5
WAF-Diesel	40.2 ± 2.6 *	38.6 ± 2.1 *	45.9 ± 1.8 *	32.8 ± 4.0 *	18.7 ± 1.8	11.8 ± 1.6
	Series B					
Control	18.4 ± 2.8	18.9 ± 1.2	19.6 ± 0.9	14.7 ± 2.2	11.8 ± 1.4	11.3 ± 1.7
WAF-CLB	44.9 ± 1.9 *	32.2 ± 6.3 *	25.3 ± 2.1	24.0 ± 1.7	11.8 ± 1.9	10.0 ± 1.9
WAF-Diesel	40.2 ± 3.5 *	40.9 ± 4.9 *	34.7 ± 4.2 *	18.7 ± 2.0	13.0 ± 1.5	13.7 ± 0.6

The percentage of destabilized lysosomal membrane of hemocytes decreased during recovery. In lobsters impacted by petroleum products (CLB or marine diesel), this percentage decreased and reached those of the controls from 2 weeks (336h) when the temperature remained constant (Table 15). However, when it increased, these values were reached more rapidly those of the Controls: from 72h for lobsters impacted by WAF-CLB and from 1 week (168h) for those impacted by WAF-Diesel.

During the 3 weeks of depuration (504h), the percentage of lysosomal membrane destabilization in lobsters exposed to WAF-CLB significantly decreased for both series (Figure 60).

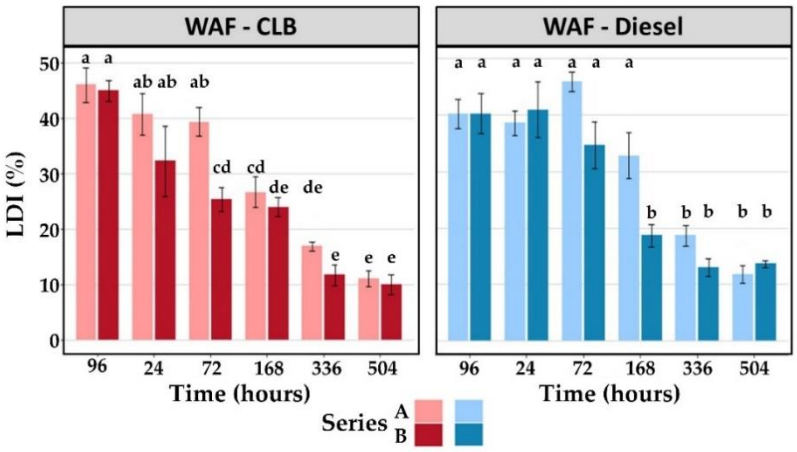

Figure 60. Fraction of lysosomal membrane destabilization (LDI) in lobster' hemocyte cells for WAF-CLB and WAF-Diesel treatments during the recovery period in series A and B. Mean ± SE; n = 3. The letter symbols indicate a significant ($p < 0.05$) effect between time and series.

This reduction was observed from 1 week (168h) of recovery and continue until 3 weeks (504h) in series A. When the temperature increased (series B), this reduction was observed from 24h and seemed to have stagnated from 2 weeks of recovery (336h) in lobsters exposed to WAF-CLB. For lobsters exposed to WAF-Diesel (Figure 60), the percentage of lysosomal membrane destabilization decreased significantly from 2 weeks in series A (336h) and 1 week in series B (168h). For both treatments (CLB and marine diesel), the decrease of percentage of destabilization of the lysosomal membrane was measured earlier when the temperature was increased (series B).

EROD

Table 16 shows the EROD activity during the recovery periods in both series (A and B). The EROD activity measured after 96 h of exposure decreased and reached the Control values during the recovery period. In series A, under all exposure conditions (WAF-CLB and WAF-Diesel), the induction of EROD enzyme activity was not significantly different from

the control from 72 h of recovery. The same pattern was observed when the temperature was increased (series B) for the lobsters exposed to WAF-Diesel. However, for those exposed to WAF-CLB, these values were not significantly different from the control after 24 h of recovery.

Table 16 : EROD activity in lobster hemolymph for each treatment (Control, WAF-CLB, and WAF-Diesel) during the recovery period in series A and B. Mean ± SE; n = 3. The * symbols indicate a significant ($p < 0.05$) effect of treatment for the considered time

Treatment	End of Exposure	Recovery Period				
	96 h	24 h	72 h	168 h	336 h	504 h
	Series A					
Control	5.8 ± 0.4	5.6 ± 0.4	6.0 ± 0.8	5.0 ± 0.4	5.7 ± 0.5	5.6 ± 0.8
WAF-CLB	10.6 ± 0.5 *	10.6 ± 0.8 *	8.9 ± 0.7	6.7 ± 0.6	5.7 ± 0.3	4.8 ± 0.2
WAF-Diesel	11.5 ± 0.7 *	10.8 ± 0.6 *	9.1 ± 0.7	6.3 ± 0.9	6.0 ± 0.5	5.3 ± 0.2
	Series B					
Control	5.5 ± 0.4	5.9 ± 0.5	6.2 ± 0.7	6.1 ± 0.4	5.7 ± 0.4	6.1 ± 0.3
WAF-CLB	13.6 ± 2.1 *	9.4 ± 0.6	8.3 ± 0.6	7.8 ± 0.3	7.0 ± 0.7	6.7 ± 0.4
WAF-Diesel	16.4 ± 1.2 *	14.2 ± 1.4 *	9.6 ± 0.6	7.4 ± 0.5	7.0 ± 0.5	5.8 ± 0.2

Figure 61 shows the significant decrease of EROD activity during recovery period in series A and B for lobsters exposed to petroleum product (CLB and marine diesel).

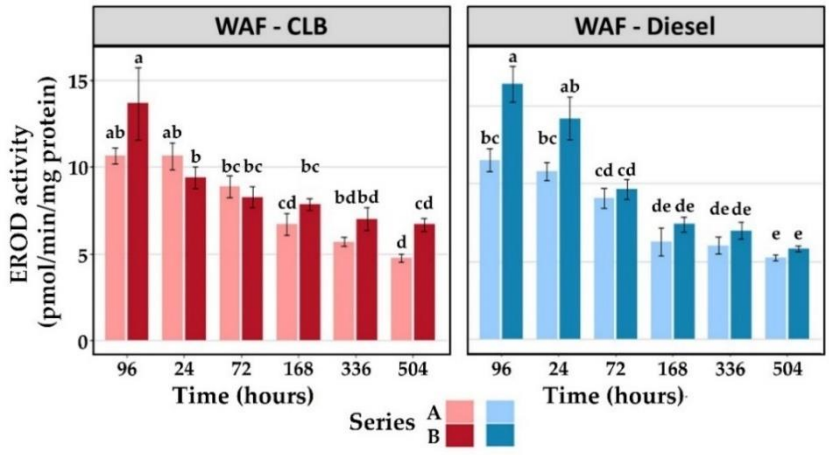

Figure 61**.** EROD activity in lobster hemolymph for WAF-CLB and WAF-Diesel treatments during the recovery period in series A and B. Mean ± SE; n = 3. The letter symbols indicate a significant ($p < 0.05$) effect between time and series

This decrease in lobsters exposed to WAF-CLB was significant after 168 h of recovery in series A and seemed to continue to decrease, although not significantly. In series B, two significant decreases were measured, after 24 and 504 h of recovery. This pattern corresponded to the observed percentage of degraded lysosomes. For those exposed to WAF-Diesel, this decrease was significant from 168 h of recovery in series A and 72 h in series B. In the two series, the value after 3 weeks of recovery (504 h) was significantly decreased compared with that after the first week of recovery (i.e., 168 h in series A and 72 h in series B).

A decrease in EROD activity was measured earlier when the temperature was increased (series B) for lobsters exposed to WAF-CLB and WAF-Diesel compared with those in series A. However, the initial values at the end of exposure were higher in series B than in series A, but not significantly for lobsters exposed to WAF-CLB. After 1 week of recovery (168 h), the EROD activity in lobsters exposed to WAF-CLB remained constant in series B while it continued to decrease in series A.

4.6.3 Tainting Assay

At the end of the exposure, the lobsters exposed to petroleum products all had a hydrocarbon smell; however, the odor was stronger in lobsters exposed to WAF-Diesel. After three weeks of recovery (504 h), only lobsters exposed to WAF-Diesel still had a marked hydrocarbon odor which persisted after three months of contention at 2°C. After cooking, this odor was even stronger. In the case of WAF-CLB lobsters, only one of the two lobsters (series B) had a slight hydrocarbon smell after cooking, especially in the hepatopancreas, even though this smell was not present before cooking.

In the fresh state, the dorsal view of the abdominal muscles showed no apparent difference in any of the treatments or series. Conversely, some variation in color was observed in the ventral view of the abdominal muscle depending on the treatment: (1) Control: dew and yellowish; (2) WAF-CLB: white; (3) WAF-Diesel: white or greenish. After cooking, only lobsters exposed to WAF-CLB in series A had a noticeable change of color of

the abdominal muscles (see Figure 62c). They were slightly duller white than the lobsters of other treatments.

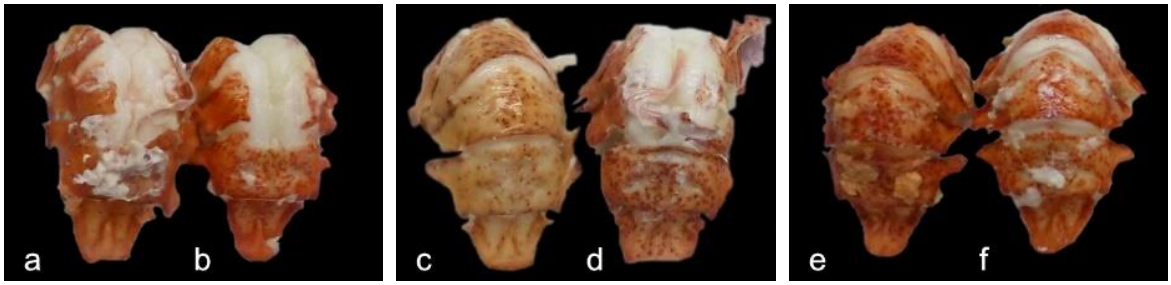

Figure 62. Dorsal view of abdomen muscle after cooking of (**a**) Control during series A; (**b**) Control during series B; (**c**) WAF-CLB during series A; (**d**) WAF-CLB during series B; (**e**) WAF-Diesel during series A; and (**f**) WAF-Diesel during series B

4.6.4 Chemical Analysis

Figure 63 shows the concentrations of 16 PAHs classified by the US_EPA as a priority pollutant, and 19 alkylated PAHs that were measured in different lobster tissues after 3 weeks of recovery and 3 months of live holding.

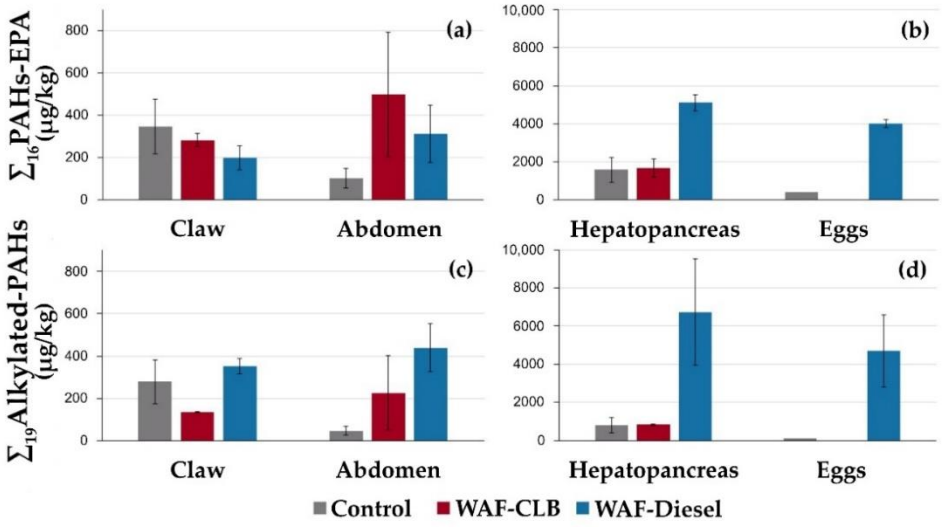

Figure 63**.** $\sum_{16}$PAHs-EPA and $\sum_{19}$Alkylated-PAHs measured in claw and abdomen (**a**,**c**, respectively) and hepatopancreas and eggs (**b**,**d**, respectively) for each treatment. Mean ± SE; n = 2, except for eggs in Control (n = 1) and WAF-CLB (n = 0)

The $\sum_{16}$PAHs-EPA was higher in the hepatopancreas of lobsters exposed to WAF-Diesel when compared with that in both Control and WAF-CLB treatments (Figure 63). Despite the few available sample eggs in Control lobsters, a difference in concentration was clearly demonstrated compared to eggs in lobsters exposed to WAF-Diesel. In claw and abdomen, $\sum_{16}$PAHs-EPA concentrations were globally lower than in the two other tissues (Figure 63a,b). It seems that there were no significant differences between treatments, due to the high standard errors (Figure 63a). Nevertheless, lobsters exposed to WAF-CLB seemed to have higher concentration of $\sum_{16}$PAHs-EPA in their abdomen, compared to those of the other treatments.

Similarly, as for $\sum_{16}$PAHs-EPA, $\sum_{19}$Alkylated-PAHs levels were also measured in tissues (Figure 63c,d). In claws, there did not seem to be any difference between the treatments. In the abdomen, $\sum_{19}$PAH-Alkyl appeared to be higher in lobsters exposed to WAF-CLB and WAF-Diesel compared to Control. However, the standard errors were too large to confirm any significant difference. The concentrations measured in hepatopancreas and eggs were clearly higher in lobsters exposed to WAF-Diesel compared with Control as well as WAF-CLB in the hepatopancreas.

Details of $\sum_{35}$Total-PAHs for each treatment are presented in Figure 64a,b, respectively in hepatopancreas and eggs.

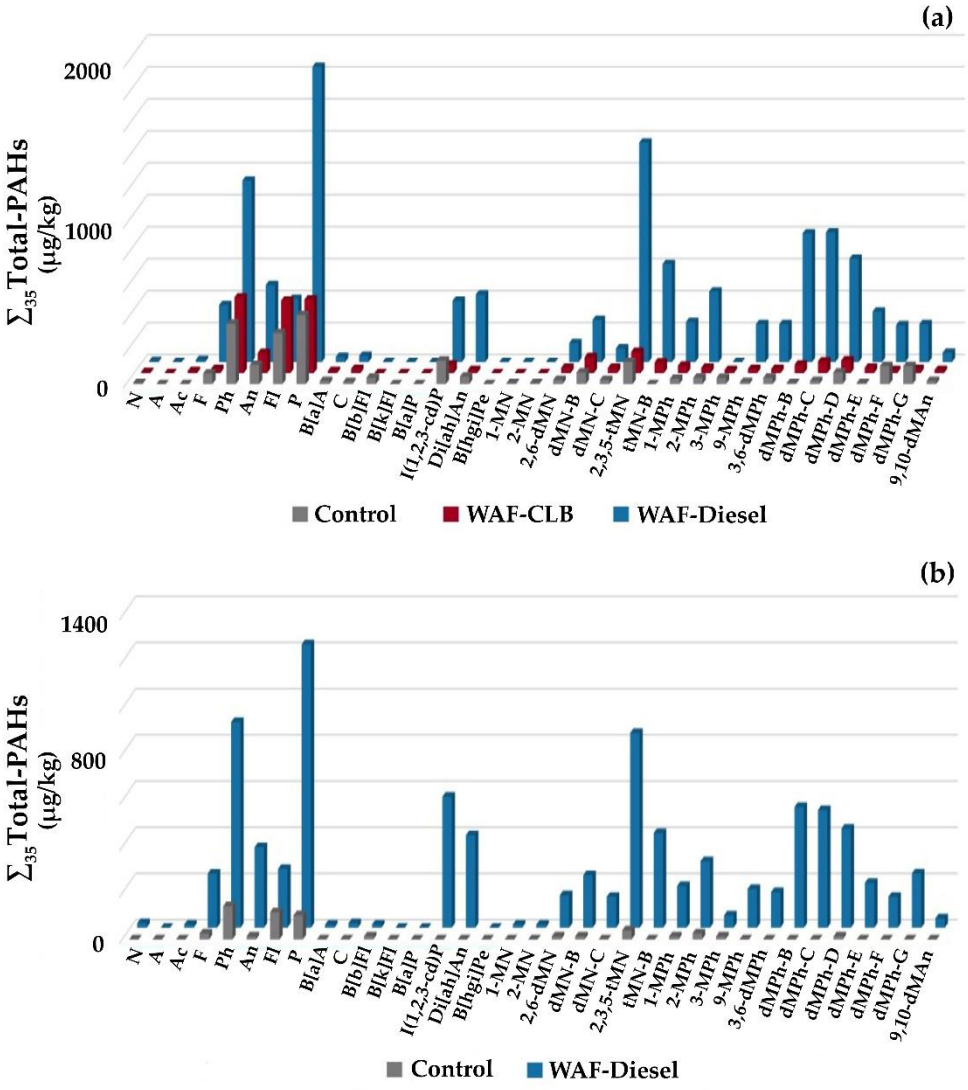

Figure 64. $\sum_{35}$Total-PAHs (µg/kg) measured in (**a**) hepatopancreas and (**b**) eggs of lobsters under each treatment (Control, WAF-CLB, and WAF-Diesel).

$\sum_{16}$**PAHs-EPA:** N: Naphthalene; A: Acenaphthylene; Ac: Acenaphthene; F: Fluorene; Ph: Phenanthrene; An: Anthracene; Fl: Fluoranthene; P: Pyrene; B[a]A: Benzo[a]anthracene; C: Chrysene; B[b]Fl: Benzo[b]fluoranthene; B[k]Fl: Benzo[k]fluoranthene; B[a]P: Benzo[a]pyrene; I[1,2,3-cd]P: Indeno[1,2,3-cd]pyrene; Di[ah]An: Dibenzo[ah]anthracene; B[hgi]Pe: Benzo[ghi]perylene.

$\sum_{19}$**Alkylated-PAHs:** 1-MN: 1-Methylnaphthalene; 2-MN: 2-Methylnaphthalene; 2,6-dMN: 2,6-Dimethylnaphthalene; dMN-B: Dimethylnaphthalene B; dMN-C: Dimethylnaphthalene C; 2,3,5-tMN: 2,3,5-Trimethylnaphthalene; tMN-B: Trimethylnaphthalene B; 1-MPh: 1-Methylphenanthrene; 2-MPh: 2-Methylphenanthrene; 3-MPh: 3-Methylphenanthrene; 9-MPh: 9-Methylphenanthrene; 3,6-dMPh: 3,6-Dimethylphenanthrene; dMPh-B: Dimethylphenanthrene B; dMPh-C: Dimethylphenanthrene C; dMPh-D: Dimethylphenanthrene D; dMPh-E: Dimethylphenanthrene E; dMPh-F: Dimethylphenanthrene F; dMPh-G: Dimethylphenanthrene G; 9,10-dMAn: 9,10-Dimethylanthracene.

4.7 DISCUSSION

4.7.1 Biomarkers and Industry

Our study demonstrated that the Brix index, as a biomarker of physiological condition status, is not sensitive enough to detect the adverse effects of exposure to diluted petroleum products. Besides, in applying this biomarker, it is important to pay attention to the sex ratio, as some studies have shown differences in the Brix index values according to the sex of lobsters. Moisan *et al.* (2008) observed lower Brix values in females than males under natural conditions without external contamination, while Battison (2018) observed higher Brix values in females than males. In our study, as observed by Moisan *et al.* (2008), the values were higher in males than females (approx. 10 mg/ml more in males). Although the Brix index was used during contamination and depuration monitoring experiments, we demonstrated that the implementation of other biomarkers is needed to support the industry in monitoring the health status of their catchments in case of dilbit or marine diesel spills. Our three selected biomarkers showed responses to contamination by petroleum products. However, only two had the appropriate sensitivity: Lysosomal stability (neutral red) and enzymatic activity (EROD). The results of our experiments showed that these can be used to characterize the impact of hydrocarbon exposure.

As hemolymph punctures are routinely performed in the industry to assess the health status and commercial value of lobsters via the Brix index, these biomarkers can be measured from the same samples. This does, however, require either sending frozen samples (−80°C) to a laboratory, or investing in a microscope, chemical reagents and training to analyze the results. The advantage of these biomarkers is that the results can be acquired quickly. However, despite a return to the baseline values of our biomarkers, the PAH concentrations in the edible parts after 3 months in clean water were still higher than those in control lobsters.

4.7.2 Impact of Hydrocarbon Exposure

This study allows us to observe some of the sub-lethal biological effects of 96h exposure to physically dispersed oils in lobsters under post-wintering conditions (i.e., 4°C average temperature in May in the Îles-de-la-Madeleine). Two petroleum products were tested, dilbit (originating from Cold Lake Blend; denoted CLB) and classical marine diesel using the water accommodated fraction (WAF) technique. Basically, no short-term impact was observed, in terms of both survival and hemolymph protein levels, at the tested concentrations (WAF 60%). Nevertheless, the membrane stability of hemocyte lysosomes (organelles necessary for defense against xenobiotics) was strongly impacted by oil exposure. The percentage of dead cells and cells with impacted lysosomes increased drastically after 96h of exposure to dilbit CLB and both CLB and marine diesel, respectively. Even though the Brix index indicated that lobsters were healthy before and after exposure, the complementary biomarkers clearly emphasized the detrimental impacts of CLB and marine diesel exposure at the cellular level. Despite WAF-CLB having lower concentrations of dissolved aromatic hydrocarbons compared with WAF-Diesel, the measured adverse impacts were slightly more pronounced in lobsters exposed to WAF-CLB than to WAF-Diesel, illustrating that, for identical spilled concentrations, a diluted bitumen spill would appear to be more damaging for lobsters than a marine diesel spill. It can be assumed that additional toxic compounds were present in WAF-CLB than in WAF-Diesel. However, the level of the EROD activity after 96h of exposure is encouraging in terms of indicating effective recovery in lobsters. Furthermore, the temperature increases for the B series showed a potential to accelerate recovery during the second period of the experiment.

The effects measured on lobsters were obtained in a controlled environment, without food or sediment, and the only contamination route was from intake water. Indeed, benthic invertebrates can uptake hydrocarbons from water or sediments (Rumney *et al.*, 2011; Michel and Fingas, 2016) or through the consumption of contaminated prey (Yender *et al.*, 2010; Turnbull *et al.*, 2020).

It is important to consider the long-term impacts of such a spill on American lobsters. Organisms contaminated by hydrocarbons produce reactive oxygen species (ROS), which results in DNA damage (Ross, 1991). In seabob shrimp, it has been shown that EROD activity and DNA damage are significantly correlated as well as with benzo-[a]-pyrene (B[a]P) tissue concentrations (da Silva Rocha *et al.*, 2012a). After 96h of exposure to 200 µg/l B[a]P, da Silva Rocha (2012a) measured an index of DNA damage of 350 in seabob shrimps and 4 pmol/min/mg protein for activity of hepatopancreas EROD for a B[a]P tissue content of 25.6 µg/g. In our study, even after three months of holding in clean water, lobsters exposed to petroleum products (CLB or marine diesel) still contained B[a]P in their hepatopancreas (respective concentrations of 230 and 231 µg/kg) at significantly higher rates than in the Control. We can, therefore, expect that the lobsters of our study may have suffered from significant DNA damage, which could cause long-term impacts.

Furthermore, the PAH concentrations measured in the WAF-Diesel lobster eggs could be of concern for the next generation, and therefore, for the long-term lobster stock. Indeed, several studies have already highlighted the genotoxicity of PAHs (da Silva Rocha *et al.*, 2012a; 2012b; Barron *et al.*, 2018; Yazdani, 2020), which can lead to egg malformations (Schmutz *et al.*, 2021) and impaired growth (Yazdani, 2020). At the larval stage, PAH contamination can lead to early mortality (Fulford *et al.*, 2014) or malformations (Schmutz *et al.*, 2021). Fulford *et al.* (2014) found that a concentration of 1 mg/l PAH was lethal for the first stage (zoea) of blue crab after 96 h exposure. Schmutz *et al.* (2021) observed a delayed effect of diluted bitumen exposure on blue mussels, with major malformations for larvae exposed to unconventional oils, including Cold Lake Blend.

4.7.3 Recovery Capacity in Case of Hydrocarbon Exposure

Most invertebrates can depurate hydrocarbons when the concentrations in water and sediment are restored to background level or if they are placed in a clean environment (Michel and Fingas, 2016). Globally, according to our biomarkers, the lobsters returned to a

healthy status when applying a recovery period between 1 and 2-weeks after a 96 h of exposure to petroleum products (diluted bitumen or marine diesel).

Based on our biomarker assessment, we cannot strictly conclude whether an increase in temperature improved lobsters' recovery. In fact, we note that the percentage of nonviable cells continued to increase in the beginning of the recovery period, when submitted to a growing temperature, such as in spring conditions (series B; from 4 to 9°C over 3 weeks). Additionally, the protein content decreased more strongly in lobsters exposed to WAF-Diesel when depurating in such conditions, thus indicating that a short exposure to marine diesel could affect the protein levels during spring. Conversely, the values for lysosomal membrane destabilization reached control values less rapidly when submitted to constant thermal conditions (4°C), similarly to the trend of the induction of the EROD enzyme. The latter suggests a longer need for lobsters to activate biotransformation. In addition, the activation of EROD activity in our study corroborated that organism exposed to hydrocarbons exhibit enzyme activation resulting from the production of reactive oxygen species (ROS; Ross, 1991). Phase 1 of detoxification (EROD activity) was therefore, active in all our tested conditions.

4.7.4 Consumption After an Oil Spill

After three months of live holding at 2°C following a 96h exposure to physically dispersed oil (CLB or marine diesel) and a 3 weeks recovery period, the lobsters remained inedible. The first element was related to the lingering odor, especially in lobsters exposed to marine diesel, thus corroborating the study by Reilly and York (2001), who demonstrated that even if crustaceans fished in a spill area satisfy PAH levels within the limits allowed by the human health risk assessment, the flavor or odor may still be affected. This inconvenience still persisted after cooking (boiling). Concerning lobsters exposed to dilbit CLB, no olfactory differences were noted, as in Williams *et al.* (1988). However, although boiling likely mitigated color variation already occurring in meat of unexposed individuals, this process seemed to reveal a distinctive smell of lobsters exposed to dilbit CLB. These results

are consistent with those found in the literature. As an example, following the Amoco Cadiz spill, Michel and Abarnou (1978) carried out experimental fisheries and showed that several spider crabs (*Maïa eguinadci*) and crabs (*Cancer pagurus*) had a distinct hydrocarbon taste.

During laboratory exposure to light oils (North Sea or Arabian crude oil), studies have reported minimum oil compound concentrations in fish tissues that were determined to be tainted by sensory testing as 5 to 100 ppm (Motohiro and Iseya, 1976; Howgate *et al.*, 1977; Heras *et al.*, 1993). After the Braer spill (spilling 85,000 t of Norwegian Gullfaks light crude oil), taint was readily perceived in caged salmon if the PAH concentration in the flesh was 1 ppm or greater (Motohiro and Iseya, 1976). In our study, the concentration of $\sum_{16}$PAHs-EPA in WAF-Diesel lobsters was 0.3 ± 0.1 ppm in the abdomen and 0.2 ± 0.06 ppm in the claws. Regarding WAF-CLB lobsters, despite having higher $\sum_{16}$PAHs-EPA concentrations in the abdomen and claws than WAF-Diesel lobsters, they did not exhibit an odor until being cooked.

The second aspect concerns the high levels of PAHs remaining in lobster tissues, including in the hepatopancreas, abdomen, and eggs, even after 3 months of live holding. Indeed, during consumption, PAHs can participate actively in metabolic activation in mammalian cells. Diol epoxies adhere to genetic material and tissues, causing mutations (Phillips, 1999). PAHs in foods are suggested to be one of the major contributors to skin and lung cancers (Kameda *et al.*, 2005; Lee and Shim, 2007; Yoon *et al.*, 2007; Zhang *et al.*, 2009). In Canada, there is no contamination threshold for seafood, including lobster [84]. However, in France, a guide value for $\sum_{16}$ PAHs-EPA is 100 µg/kg of dry weight in crustaceans as a guideline contamination value. The exclusion thresholds are 2 to 5 times greater than the guide value. During the Erika oil spill in 1999 and the Prestige spill at the end of 2002, the exclusion thresholds for crustaceans were 500 µg/kg for $\sum_{16}$ PAHsEPA (AFSSA, 2003). In the present study, even with low statistical power, our exploratory chemical results showed a difference between contaminated lobsters and controls. The abdomen of lobsters contaminated by dilbit CLB and marine diesel were, respectively 5 and 3 times greater than the control values, and superior than the lowest exclusion threshold

proposed by France (200 µg/kg), despite 3 months of holding in clean water. These data indicate the need for further research on the concentrations found in lobster tissue following a dilbit or diesel spill.

4.8 CONCLUSION

The objective of the study was to monitor four biomarkers on lobster hemolymph to assess impact and recovery capacity to diluted bitumen (CLB) and marine diesel. Our results show that the Brix index used by the lobster fishery is not relevant in the case of CLB or marine diesel exposure, so it is necessary to work with other biomarkers. Selected biomarkers tested in complement to the measurement of the Brix index allowed for monitoring the health status of hemocytes as a proxy to visualize the impacts of CLB and marine diesel exposure on the American lobster, as well as to follow the recovery efficiency. Despite a higher measured PTSA equivalent concentration in WAF-Diesel compared to WAF-CLB, the biomarkers did not show any difference between the two petroleum products, except in terms of cell viability, which was more impacted by CLB. However, the persistent odor and the PAH levels induced by a marine diesel exposure would be more damageable for the fisheries than lobsters exposed to CLB.

Lobster recovery following WAF exposure (to CLB and marine diesel) was observed over 3 weeks using 2 biomarkers—NRR and EROD—for assessment as well as one more for CLB exposure—cellular viability. According to these biomarkers, lobsters were able to recover in less than 3 weeks in clean water. However, even after 3 months of contention, lobsters still had high concentrations of PAHs, especially for those exposed to marine diesel. The effectiveness of an increase in temperature in improving recovery could not be confirmed by our study. Indeed, the NRR values reached the Control values more quickly with an increase in temperature, while the reverse was observed for the cellular viability.

In order to improve the monitoring of PAH concentrations in lobsters, it would be interesting to attempt to quantify these contaminants directly in the hemolymph, as proposed

in the study of Turnbull (2020) for paralytic shellfish toxins. Furthermore, monitoring of PAHs in lobsters in parallel with the biomarkers may represent a better strategy for monitoring recovery efficiency.

Further, to better assess the long-term impacts of a spill, it would be interesting to measure DNA damage; for example, by comet assay (Chao and Engelward, 2020). Finally, the PAH concentrations observed in lobster eggs suggest serious potential transgenerational effects which could severely impair the health of subsequent generations.

4.9 FUNDINGS

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DISCUSSION GÉNÉRALE

L'objectif principal de cette thèse était de générer de nouvelles connaissances pour améliorer la prise de décision en cas de déversement de bitume dilué, notamment dans le fleuve du Saint-Laurent. Les articles présentés dans cette thèse se déroulent selon les étapes du déversement, i) vérifier l'efficacité du dispersant chimique, ii) évaluer la toxicité du bitume dilué sur deux espèces marine d'importance commerciale au Québec, soit la moule bleue (*Mytilus edulis*) et le homard américain (*Homarus americanus*), et iii) vérifier leur capacité à se rétablir après une contamination aux produits pétroliers. Dans chaque chapitre, des objectifs secondaires spécifiques ont été déterminés afin d'analyser les effets des expositions aux pétroles sur ces deux espèces. Les prochaines sections présentent un retour sur le contexte de l'étude, les objectifs spécifiques de chaque article, les principaux résultats obtenus, en mettant en lien les quatre articles.

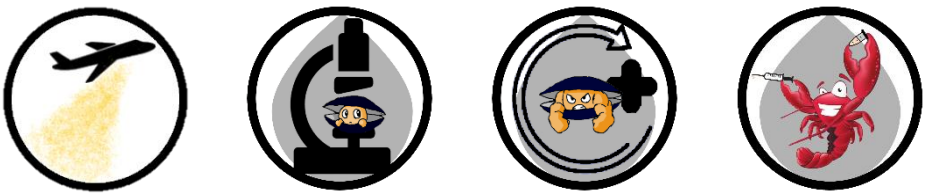
CONTEXTE DE L'ÉTUDE

Tandis que les réserves de pétroles classiques diminuent, l'intérêt des pétroles non classiques croît constamment. Depuis les années 1990, le Canada a développé le plus vaste gisement de pétrole brut sur la planète, les sables bitumineux. L'apport économique non négligeable pour le pays est un argument fort pour continuer à investir dans son développement. Cependant, de cette exploitation découle aussi 140 000 km^2 de forêt boréale détruite (Nikiforuk, 2010), plusieurs déversements de bitumes dilués dans les eaux douces et salées, entraînant des dommages sur les espèces aquatiques, et des répercussions économiques. Afin de pouvoir intervenir au plus vite et avec les meilleures solutions d'intervention, en cas de déversement, il est crucial de connaître les caractéristiques de ce pétrole si particulier. En plus de déterminer l'efficacité des méthodes d'intervention

existantes, il est important de connaître les effets toxiques que ce choix va entraîner sur le milieu, à court comme à long terme.

Parmi ces méthodes, le dispersant chimique prend une place non négligeable et est, depuis récemment, autorisé au Canada (MPO, 2021). Cependant, son efficacité est à questionner sur les bitumes dilués, dû à la viscosité importante de ces pétroles, mais également sur la période du déversement, étant dépendant de la température et de la salinité. Un déversement de bitume dilué en eau froide soulève donc des questions de l'utilité du dispersant dans ces conditions.

En plus de sa pertinence comme méthode d'intervention, le risque d'accroître la toxicité pour les organismes marins interpelle également la communauté scientifique. L'apport économique du Saint-Laurent est important, notamment pour les moules bleues, *Mytilus edulis*, et le homard américain, *Homarus americanus*. Ces deux espèces sont connues pour avoir une bioaccumulation des HAP plus importante que les poissons, dû à un temps de demi-vie de ces composés plus longs dans ces organismes (Stegeman and Lech, 1991; Meador *et al.*, 1995), ainsi qu'une vitesse de métabolisation plus lente (Seiser *et al.*, 2000). Or, un déversement au niveau du fleuve de Saint-Laurent entraînerait des pertes importantes pour les mytilicultures et les pêches. Il est donc important d'évaluer, en plus des niveaux de toxicité, la capacité de ces deux espèces à retrouver leur état de santé initial, avant l'exposition aux dilbits, ainsi que de se dépurer de façon efficace des composés toxiques accumulés lors du déversement. C'est dans ce contexte que s'inscrivait ce projet de thèse.



LE DISPERSANT CHIMIQUE COMME MÉTHODE POUR LES DILBITS

L'efficacité des dispersants chimiques sur les bitumes dilués étant toujours débattue, le premier chapitre de cette thèse a pour but de la déterminer à différentes températures (15, 5 et 0°C) et salinité (35, 10 et 5 psu). Une première collaboration entre l'UQAR (Canada) et le CEDRE (France) a permis de tester en laboratoire le pourcentage de bitume dilué récupéré après l'addition du dispersant, par le test français normalisé IFP, ainsi que la moyenne de la taille des gouttelettes générées. Deux dispersants, l'un utilisé en Amérique du Nord (Corexit® 9500A) l'autre en Europe (Finasol® OSR52), ont été comparés sur deux bitumes dilués (AWB, CLB). De plus, dans le but d'améliorer la prise de décision sur ce type de technique, un indice d'efficacité du dispersant (DEI) a été proposé. Il permet de cumuler les données obtenues sur le niveau de dispersion, mais également sur la taille des gouttelettes de pétrole, en fonction des conditions environnementales, du type de pétrole et du dispersant.

L'efficacité des dispersants a été mesurée au cours de ce premier chapitre, et était dépendante de la température, surtout à de faibles salinités (< 10 psu). De plus, l'efficacité varie entre les deux bitumes dilués, ce qui souligne l'importance de la composition du pétrole, notamment l'influence de leur viscosité. D'ailleurs, notre étude démontre une meilleure efficacité avec le Finasol® OSR52 que celle du Corexit® 9500A sur les bitumes dilués, considérés comme des pétroles lourds, tandis que l'inverse est observé par Resby *et al*. (2007) et Steffek (2015) pour des pétroles légers et moyens. Ces résultats appuient l'importance de générer des informations sur l'efficacité des dispersants selon différents paramètres, comme le type de pétrole, le dispersant et les conditions environnementales.

Cependant même si la dispersion est acceptable, il est important de vérifier la taille des gouttelettes qui n'est pas forcément corrélée avec les valeurs données par les tests d'efficacité, comme montré dans notre étude. L'indice d'efficacité proposé dans cette thèse permet d'obtenir une valeur plus globale, prenant en compte les résultats d'efficacité de dispersion et de taille des gouttelettes. Cet indice améliore donc le choix d'utilisation du dispersant chimique ou non.

La taille des gouttelettes donne une bonne idée d'un retour possible du pétrole en surface, mais elle peut être également importante pour évaluer le niveau de toxicité, notamment pour les organismes filtreurs. Des gouttelettes trop fines peuvent être filtrées en grandes quantités, mais de plus grandes vont être filtrées, et seront plus difficiles à excréter pour l'organisme. De plus, le fait qu'une partie des gouttelettes va s'agréger rapidement dans les sédiments est à prendre en compte pour les organismes tel que le homard étant donné son milieu de vie. Or, nos résultats montrent que le Finasol® OSR52 aura tendance à créer de plus grosses gouttelettes que le Corexit® 9500A, ce qui questionne leur toxicité. De plus, des différences de taille des gouttelettes sont observées entre les deux bitumes dilués, dues à leur composition différente en composés saturés, aromatiques et asphaltènes. Notre étude démontre de plus grosses gouttelettes avec AWB que CLB, ce qui sous-entend également une différence de toxicité entre les deux bitumes dilués, en raison d'une biodisponibilité différente.

Comme prévu par la définition du dispersant chimique, son utilisation augmente les concentrations d'hydrocarbures dissous dans la colonne d'eau, comme rapporté dans le chapitre 2. L'étude comparative de ce deuxième chapitre a permis de démontrer que la concentration obtenue dans la colonne d'eau, par l'ajout d'un dispersant, ne peut pas être considérée uniquement comme une augmentation de ce qu'on observe avec une dispersion physique. Le pétrole entraînant les plus fortes concentrations lors d'une dispersion physique n'est pas forcément le même lors d'une dispersion chimique. Effectivement, au cours de notre étude, on a mesuré des concentrations d'HAP plus importantes avec un bitume dilué lors d'une dispersion physique, comparativement avec le pétrole classique lors d'une dispersion chimique. Il faut considérer la composition du pétrole et, notamment, les concentrations de composés légers, ainsi que la composition du dispersant choisi et la saison ; des facteurs qui influent sur son efficacité et, donc, sur les concentrations retrouvées dans la colonne d'eau. La taille des gouttelettes et les variations observées de concentration dans la colonne d'eau selon différents facteurs questionnent sur la variation possible des toxicités selon ces mêmes facteurs.

BITUME DILUÉ ET PÉTROLE CLASSIQUE

Le deuxième chapitre de cette thèse présente les résultats de trois séries d'expériences en milieu contrôlé, ayant pour objectif de comparer les impacts d'un pétrole non classique (bitume dilué) par rapport à un pétrole classique sur la moule bleue, selon deux paramètres : la méthode d'intervention et la saison. Ces expériences, qui ont duré 15 mois, ont permis l'acquisition de données sur les moules bleues en été, automne et hiver, en utilisant essentiellement des biomarqueurs sur l'hémolymphe. Afin de visualiser l'ensemble des impacts, un indice, déjà utilisé en écologie, est appliquée à notre problématique.

Les variations d'efficacité du dispersant chimique et la concentration d'hydrocarbures, selon le pétrole et la saison, influent sur la concentration d'hydrocarbures bioaccumulés dans les tissus des moules. Comme attendu, l'ajout de dispersant chimique entraîne une bioaccumulation plus importante chez les moules bleues, due à une concentration plus importante dans la colonne d'eau. Toutefois, les résultats de notre étude démontrent également une bioaccumulation de composés pétroliers dans les tissus des moules exposées au dispersant seul. Donc, en plus de créer une concentration de produits pétroliers dans la colonne d'eau, le dispersant chimique augmente ces concentrations par sa composition. On mesure effectivement une bioaccumulation jusqu'à 30 fois plus importante pour un pétrole classique dispersé chimiquement, par rapport à une dispersion physique. Cependant, cet écart observé chez un pétrole classique est bien moins élevé pour un bitume dilué : on mesure seulement 2 fois plus de composés pétroliers dans les tissus des moules exposés à un bitume dilué, dispersé chimiquement, par rapport à une dispersion physique. Comme la concentration des composés dans la colonne d'eau n'explique pas cette différence, il est probable que ce soit lié : 1) à la présence de composés non mesurés par notre étude chez les bitumes dilués, ou 2) au déclenchement d'un système de protection (fermeture des valves) plus rapide lors d'une exposition au bitume dilué.

Cette bioaccumulation mesurée chez les moules bleues est responsable d'impacts cellulaires (stabilité lysosomale, viabilité cellulaire) et génotoxiques. Bien que les

concentrations en composés pétroliers dans les tissus des moules soient supérieures lors d'une contamination au pétrole classique, par rapport au bitume dilué, les valeurs d'IBR y sont généralement plus faibles ou équivalentes. Le bitume dilué est donc plus dommageable que le pétrole classique chez les moules bleues, causant des dommages similaires, voire plus élevés, pour des concentrations plus faibles dans les tissus. Cependant, aucune corrélation n'a pu être mesurée entre les groupes de composés et les impacts, de façon répétée entre les saisons, le type de pétrole et la dispersion. Cette information confirme notre hypothèse, qu'un ou plusieurs composés non quantifiés dans notre étude sur les bitumes dilués est/sont responsables de cette toxicité plus importante. On remarque néanmoins que la proportion de HAP alkylés est toujours la plus élevée dans les tissus des moules exposées aux eaux les plus concentrées (pétrole classique lors d'une dispersion chimique, ou dilbit lors d'une dispersion physique). De façon générale, les dommages mesurés sont plus importants lors d'un déversement de bitume dilué, dispersé chimiquement en été ou automne.

D'ailleurs, un paramètre, qui ne doit être négligé lors d'un déversement de pétrole, ressort également de notre étude : la saison. En plus d'impacter l'efficacité du dispersant chimique, la température de l'eau influence la bioaccumulation et donc le niveau de toxicité chez les moules bleues. Ces différences sont liées aux caractéristiques physiologiques de la moule bleue qui présentent des taux de filtration plus faibles en hiver et un risque accru en été dû à une dépense énergétique déjà portée sur la reproduction. Effectivement, c'est seulement en été que des mortalités ont été mesurées, mais uniquement lors d'une exposition au bitume dilué dispersé chimiquement. Ainsi, notre étude démontre bien une augmentation de la bioaccumulation dans les tissus des moules bleues avec la température (saison), ainsi qu'un IBR généralement plus élevé en été et plus faible en hiver.

RÉCUPÉRATION DES MOULES BLEUES

La toxicité démontrée des bitumes dilués plus importante qu'un pétrole classique soulève des questions sur la récupération des moules bleues après une exposition au bitume dilué, notamment en hiver au cours duquel la dépuration est plus lente (Svensson, 2003). Dans le troisième chapitre, la capacité de récupération des moules bleues, *Mytilus edulis*, après une exposition au bitume dilué CLB, est mesurée en hiver, en suivant les impacts cellulaires et génotoxiques observés au chapitre 2.

L'exposition au bitume dilué dispersé physiquement dans le mésocosme se différencie de la WAF utilisée pour les expositions précédentes (chapitre 2), par une concentration de HAP dissoute dans la colonne d'eau 10 fois plus importante. Dans les tissus, deux fois plus d'hydrocarbures sont mesurées après 8 jours d'exposition au bitume dilué CLB, contre 2 jours dans le précédent chapitre. Tout comme lors d'une exposition à une WAF, des dommages cellulaires (stabilité lysosomale et viabilité cellulaire) et génotoxiques sont mesurés. Cependant, malgré des dommages très importants, notamment sur le pourcentage de dégradation de l'ADN, notre étude montre une capacité de rétablissement de la moule bleue. Ainsi, après quelques heures dans une eau non contaminée, les biomarqueurs mesurés montrent déjà une diminution des effets, corrélée à celles des concentrations en produits pétroliers dans les tissus. Effectivement, la moule bleue se dépure rapidement des HAP et COVs mesurés dans notre étude. Même si les concentrations ne rejoignent pas celle des contrôles au bout de 4 jours, une forte diminution est observée. Néanmoins, les composés dibenzothiophene et carbazoles restent à des concentrations similaires à celles mesurées à la fin de l'exposition, soulignant leurs temps de demi-vie plus longs que pour les autres composés. Leurs concentrations encore élevées après 4 jours, contrairement aux autres composés mesurés, mettent en question leur responsabilité dans les impacts sur la moule bleue, notamment sur l'ADN.

LES BIOMARQUEURS DES MOULES VERS LES HOMARDS

Cette thèse démontre l'utilité de biomarqueurs pour le suivi de l'impact d'un déversement de pétrole sur la moule bleue, en utilisant son hémolymphe. Ce matériel biologique permet de conserver les individus et d'avoir accès rapidement à certains tests, comme la déstabilisation lysosomale ou la viabilité cellulaire. Or, le homard américain, une autre espèce d'importance économique au Québec, est également suivi via son hémolymphe. Déjà ponctionnée dans l'industrie, l'hémolymphe est utilisée pour vérifier l'état de santé global du homard par la concentration totale de protéines. Dans ce dernier chapitre, les biomarqueurs validés sur la moule bleue ont été testés chez le homard américain. Lors d'un déversement de bitume dilué, une contamination de cette espèce est possible via la colonne d'eau, mais également en lien direct avec les sédiments, qui se concentrent en HAP (Gong *et al.*, 2014 ; Beyer *et al.*, 2016). Cette espèce n'est donc pas à l'abri en cas de déversement, et pourtant très peu d'études apportent des données, et aucune concernant une contamination au dilbit. Ce chapitre en collaboration avec Merinov (Îles-de-la-Madeleine) est le premier à fournir des informations de l'impact d'une contamination au dilbit (CLB) dispersé physiquement, sur le homard américain. Les résultats démontrent, comme chez la moule bleue, des dommages sur la viabilité cellulaire et la stabilité lysosomale. Une augmentation de l'induction d'EROD en sortie d'exposition est également mesurée dans notre étude, bien que son induction chez les crustacés par les HAP est encore discutée, comme l'enzyme CYP responsable de l'activité EROD n'a pas encore été caractérisée (Zou, 2020).

En plus de ces nouvelles données, ce chapitre vérifie la capacité de récupération des homards. Avec la diminution des impacts pendant la période de dépuration, une diminution de l'activité d'EROD est également mesurée. L'augmentation de température ne semble pas améliorer le temps de dépuration.

Bien que la dépuration semble fonctionner rapidement chez le homard, on mesure encore des quantités de HAP significatives après 3 mois dans des eaux propres. Ces résultats préliminaires ne nous permettent pas de l'affirmer, mais nous pouvons supposer une

dépuration plus lente des homards par rapport aux moules bleues. De plus, ce projet permet de comparer un produit pétrolier léger, le diesel, avec un bitume dilué. On observe une plus forte concentration en HAP dans les tissus, lors d'une contamination au diesel par rapport au bitume dilué, même après 3 mois de dépuration. Il peut s'agir d'une bioaccumulation nettement supérieure lors de la phase d'exposition, ou une difficulté plus importante pour dépurer.

IMPORTANCE DE CE TYPE D'ÉTUDES POUR LA GESTION D'UN DÉVERSEMENT DE DILBIT

Le risque d'un déversement de bitume dilué ne peut pas être négligé et nécessite l'apport de connaissances afin de sélectionner les meilleures options de façon rapide pour intervenir au mieux, selon les conditions environnementales et les risques associés. La première prise de décision consiste à choisir la meilleure solution d'intervention. Pour ce faire, l'efficacité de la méthode doit être connue selon les conditions environnementales, puis le niveau de toxicité associé pour valider ou non ce choix.

Le choix des dispersants chimiques étant encore discuté pour les bitumes dilués, notre étude s'est focalisée sur cette méthode. Nos résultats mettent en évidence dans un premier temps, l'importance de prendre en compte, non seulement l'efficacité du dispersant chimique, mais également la taille des particules qui va en découler. Afin d'avoir une vue d'ensemble de ces deux effets, un indice d'efficacité est proposé et permet une prise de décision plus précise en fonction de la température et de la salinité du milieu. Cet indice est un outil utile pour la prise de décision. Dans un deuxième temps, pour valider la technique d'intervention sélectionnée, le niveau de toxicité doit être connu pour les espèces à risque du milieu. Dans le même esprit, un indice déjà utilisé en écologie a été utilisé dans notre étude pour visualiser l'impact global de toxicité d'un pétrole. Cet indice permet une comparaison rapide du niveau de toxicité d'un déversement selon plusieurs paramètres : le pétrole, la méthode d'intervention et la saison. Intégrer ces deux indices dans les directives pour la prise de décision dans le cadre d'une stratégie d'intervention, permettrait un choix plus précis et rapide.

Dans le but d'accumuler des données sur le niveau de toxicité des composés pétroliers chez une espèce, ainsi que de suivre les espèces présentes pour suivre leur dépuration et rétablissement, notre étude met en avant l'utilisation de l'hémolymphe chez deux espèces différentes, hémolymphe qui est déjà utilisée dans l'industrie pour vérifier l'état de santé global du homard américain. De plus, le choix de l'hémolymphe comme outil de travail permet d'éviter de sacrifier des individus, ainsi que de suivre un même groupe d'individus pour avoir un suivi plus précis. Nos travaux apportent de nouveaux biomarqueurs efficaces dans le suivi d'une contamination aux produits pétroliers. La viabilité cellulaire, la stabilité lysosomale, les dommages à l'ADN, ainsi que l'induction de l'activité enzymatique EROD sont des biomarqueurs validés dans cette thèse, non seulement pour évaluer le niveau de toxicité, mais également pour suivre le rétablissement de la moule bleue et du homard américain via leur hémolymphe.

LIMITES DE L'ÉTUDE ET PERSPECTIVES

Ce projet a apporté des connaissances essentielles afin d'améliorer les prises de décision lors d'un déversement de bitume dilué, mais également des pistes pour développer de nouveaux projets.

Tout d'abord, les tests effectués lors du premier chapitre traitant de l'efficacité du dispersant sur les bitumes dilués frais, pourraient être refaites sur du pétrole vieilli. L'utilisation de différents niveaux d'évaporation pourrait permettre de déterminer avec plus de précision la fenêtre de dispersibilité des bitumes dilués, selon le dispersant et les conditions environnementales. De plus, il serait intéressant de collecter davantage de données sur l'efficacité des dispersants chimiques selon l'énergie des vagues et les particules présentes. En plus de ces données sur l'efficacité de dispersion, la prise en compte de la quantité et de la diversité des bactéries hydrocarbonoclastes selon le lieu du déversement permettrait de considérer la dégradation naturelle du pétrole dans la prise de décision. Même si pour le moment seul le Corexit® 9500A est autorisé au Canada, notre étude démontre une efficacité plus importante d'un autre dispersant, le Finasol® OSR52. Une comparaison de

plusieurs dispersants selon leur composition apporterait des données très intéressantes pour définir le meilleur dispersant sur ce type de pétrole.

Une différence de taille de gouttelettes du pétrole dispersé est mesurée dans le premier chapitre, selon la composition du pétrole, du dispersant et les conditions environnementales. Il serait intéressant de faire un lien entre les tailles de gouttelettes formées par une dispersion chimique et le niveau de bioaccumulation observée chez les organismes marins. Ces informations pourraient aider, non seulement pour sélectionner le dispersant chimique le moins impactant lors d'un déversement, mais également pour le développement de ces produits. D'ailleurs, le développement d'études chimiques pour connaître avec plus de précision les composés retrouvés non seulement dans la colonne d'eau, mais également dans les sédiments, lors d'un déversement de bitumes dilués, serait pertinent pour comparer les composés accumulés par les espèces, par rapport à ceux retrouvées dans la colonne d'eau. Ces tests permettraient également d'évaluer le potentiel risque pour d'autres espèces et, en les comparant à différentes températures, apporterait une vision plus claire des risques d'un déversement tout au long de l'année. De plus, un équipement comme le générateur de flux du Cedre (France) permettrait d'effectuer des analyses des eaux en y ajoutant les impacts du rayonnement solaire, des vagues et du vent. Des analyses au GC-MS permettraient d'évaluer les composés retrouvés selon différentes températures et au cours du temps.

À la lumière des résultats obtenus dans cette thèse, les effets des pétroles classiques et non classiques sur l'environnement marin sont multiples et variables dus à leur composition hétérogène, aux effets de l'environnement sur le vieillissement des pétroles et à la saison à laquelle a lieu le déversement. Les observations très différentes entre le pétrole classique et non classique, ainsi qu'entre les deux bitumes dilués testés dans notre étude, confirment l'importance d'accroître les études sur ce type de pétrole. De plus, l'intérêt de comparer sur une même étude plusieurs types de pétroles comme dans notre chapitre 2, permet une meilleure vision des IBR obtenus. Ces différences soulignent l'importance de la composition chimique du pétrole sur le niveau de toxicité. Or, pour le moment, la plupart des études se focalisent sur l'effet des HAP au lieu de tenir compte de l'ensemble des composés pétroliers.

Ceci est d'autant plus important pour les bitumes dilués qui ont un pourcentage non négligeable de diluant, dont la composition est confidentielle. Or, nos résultats soulignent l'impact plus important des bitumes dilués par rapport au pétrole classique, en accord avec les récentes études sur le bitume dilué (Schmutz *et al.*, 2021 ; Berube *et al.*, 2021 ; Bérubé *et al.*, 2022).

Parmi les effets observés dans notre étude, des dommages à l'ADN ont été mesuré après plusieurs jours de contamination au bitume dilué, avec des pourcentages de queue de cpmet particulièrement important (> 60%) lors d'une dispersion physique de CLB. Lors de ce projet, des échantillons d'hémolymphe et de tissus ont été conservés au -80°C tout au long de l'expérimentation. Des analyses génétiques ont déjà démarré sur ces échantillons afin d'observer les gènes exprimés pendant la biotransformation lors d'une contamination au bitume dilué.

En terminant, les données de ces projets et les données déjà acquises pourraient être regroupées pour améliorer les résultats des indices proposés cette thèse. Et pour aller plus loin, un projet pour développer un indice plus complexe regroupant l'indice d'efficacité proposé au chapitre 1 avec l'indice IBR utilisé au chapitre 2 et 3. Les valeurs de L'IBR seraient un paramètre supplémentaire à ajouter aux deux paramètres déjà sélectionnés pour le DEI, l'efficacité de dispersant et la taille des gouttelettes. La combinaison de ces deux indices permettrait d'affiner le choix de l'utilisation du dispersant chimique, en prenant en compte son efficacité mais également le niveau de toxicité qui en découle. Effectivement, une variation saisonnière du niveau de toxicité, et donc des conditions environnementales, a été démontré au chapitre 2.

CONCLUSION GÉNÉRALE

Pour conclure, ce travail doctoral a grandement participé à enrichir les connaissances sur les bitumes dilués, en apportant des réponses pour améliorer l'intervention en cas de déversement. Dans un premier temps, un déversement de bitume dilué peut être traité avec un dispersant chimique, dont le choix final doit s'appuyer sur les résultats de l'indice de l'efficacité du dispersant. Effectivement, cette décision doit se prendre en fonction du dispersant, du type et composition du pétrole, mais également des conditions environnementales (température, salinité…). Cependant, ce choix d'intervention doit se prendre en ayant connaissance des toxicités à court et à long termes pouvant être engendrées. Un déversement de bitume dilué entraînera un niveau d'impact plus élevée qu'un pétrole classique. Ce niveau varie en fonction de la période de l'année du déversement, notamment pour les organismes filtreurs comme la moule bleue, *Mytilus edulis*. Dans le but de pouvoir suivre l'état de santé des espèces suivant un déversement, ce travail de thèse s'est basé sur les biomarqueurs via l'hémolymphe. En plus d'éviter de sacrifier l'organisme, ce prélèvement est rapide et donne accès à des tests pouvant s'appliquer directement sur l'hémolymphe, comme le test de la déstabilisation lysosomale ou la viabilité cellulaire. Cet aspect de rapidité et de conservation des populations est un atout pour la mytiliculture et les pêches. D'ailleurs, ces travaux de thèse démontrent la transposabilité des biomarqueurs utilisés sur l'hémolymphe de la moule bleue, vers l'hémolymphe d'homard américain, *Homarus americanus*. Un déversement de bitumes dilués, dispersé physiquement ou chimiquement, provoque des dommages cellulaires et génotoxiques. La capacité de ces espèces à se rétablir d'une contamination à ce type de pétrole est néanmoins possible, comme démontré dans cette thèse.

ANNEXE

CHAPITRE 2

Supplementary Methods

Tissues Analysis

A sub-sample of about 300 mg was suspended in 5 ml of tetramethylammonium hydroxide (TMAH, 25% water, Sigma Aldrich, Darm-stadt, Germany) in a 12 ml glass tube with a Teflon-lined cap and vortexed for 1 min. The mixture was then heated (60°C) for 1h with manual stirring every 15 min, in order to complete the alkaline digestion. After cooling the mixture to room temperature, 1.0 ml of deionized water, 1.0 g of NaCl, and 4.0 ml of hexane/toluene mix (1:1) were added before stirring for 1h (Wrist Action Shaker; Burrell Scientific, Pittsburgh, PA, USA). The mixture was centrifuged (at 3000 g), and the upper organic layer was recovered. A second extraction step was performed with another 4 ml of hexane/toluene mix (1:1) and this recovered organic layer was added to the first. The whole organic extract was then cleaned on a silica column topped with sodium sulfate. The volume of the cleaned extract was reduced under a gentle stream of nitrogen, at room temperature, to a final volume of 1000 µl. A volume of 150 µl of the concentrated extract was pipetted and transferred to a GC vial equipped with a glass insert and a volume of 50 µl of a solution of deuterated PAHs was added as an internal standard. PAH analyses were performed using a gas chromatograph (GC, Agilent Technolo-gies 6850 series II; Santa Clara, CA, USA) coupled to a mass spectrometer (MS, Agilent Technologies 5975B VL MSD). Injection (1µL) was performed using an Agilent Technologies Auto sampler 6850 series, at a temperature of 250°C with a splitless injection mode. The capillary column was an Rxi®-5ms (30 m × 0.25 mm ID × 0.25 µm FT, 5% diphenyl and 95% polysiloxane from RESTEK),

with helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature program was set as follows: 50°C for 2 min, 15°C / min until 275°C and 2 min hold, 15°C/min until 325°C and 15 min hold, and a post-run of 2 min at 300°C. The detection of PAHs was performed in scan mode between 50 to 500 amu, with positive ion detection, where quantification of each PAH was based on the ratio of the signal of their molecular ion relative to the signal of the appropriate internal standard. Sample blanks were processed and analyzed analysis.

Condition Index

Mussels were measured with a digital caliper (CO 030150F1). Each mussel was opened with a stainless-steel scalpel and its ventral edge was placed on tissue paper to remove internal water. Then, the total tissue of mussels was detached from the shell. The mussels' fleshes were dried (FreeZone, Labconco, USA) for 24 h, then weighed with a digital balance.

Scope for Growth

Scope for Growth is calculated ($J.g^{-1}.h^{-1}$) in exposed and control mussels as a function of energy excreted, catabolized (R), and absorbed from food (A). As the energy excreted is considered minimal (<5% of the energy budget; Pernet *et al.*, 2008), this parameter was not monitored. Oxygen uptake was measured for each mussel and control in a closed respirometer (580 mL) for a minimum of 1h and at O_2 partial pressures > 100 torr. The oxygen saturation level was measured by a polarographic electrode (YSI model 5775, USA) coupled to an oximeter (Cameron Instruments Company, model OM400) and a chart recorder (Linear, model 0585). When mussels spawned during the V_{O2} determinations, the data was eliminated.

Lysosomal Membrane Destabilization Index

Hemolymph (40 µl) and filtered intervalvular fluid mix (1:1; 0.2 µm cellulose, acetate membrane, VWR, USA) were transferred to a positively charged microscope slide X-tra® (Product N°3800210; Leica Biosystems, France) and incubated in a dark humidity chamber at ambient temperature (20°C). After 15 min, 20 µl of working solution (Neutral red dye, DMSO, and filtered intervalvular fluid) were added to the slide, then, after another 15 min, a cover slip was installed. After 45 min, 50 cells were counted using a light microscope

(Olympus BX 41, Olympus, Canada coupled to a camera Evolution VF Color and Image Pro Plus software, MediaCybernetics, USA; 400X magnification). Destabilized lysosomes (DH) were characterized by larger lysosomes or leakage of neutral red dye into the cytosol.

Cell Viability

Equal quantities of cell suspension and trypan blue (20 µl; 1:1) were mixed, 20 µl of this solution was placed onto a slide (75x25mm; Fisher Scientific, Canada), 50 cells were examined with a light microscope (Olympus BX 41, Olympus, Canada coupled to a camera Evolution VF Color and Image Pro Plus software, MediaCybernetics, USA; 400X magnification) and percentage viability was recorded.

Genotoxicity

Positive controls were carried out by exposing cells *in vivo* to 5 mM of MMS for 1h at 4°C, to produce massive single-stranded DNA damage. In short, 20 µl of cell suspension (prepared as described 1.2.7.1) were mixed with 20 µl of 0.65% LMA prepared in phosphate buffered saline at 37°C. This was immediately deposited onto the precoated slide with normal agarose (0.65%) and covered with a plastic coverslip (22x22 mm; Fisher Scientific, Canada). From this point, all steps were performed in the dark with a red light to prevent additional DNA damage. Coverslips were withdrawn after agarose polymerization (4°C, 30 min), and slides were incubated in a freshly-made lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 mM Tris, 1.5 µl Triton X-100, 15 µl DMSO, pH 10, 4°C) for 1h. Then, slides were placed in an electrophoresis buffer (TAE 1X, pH > 13, 4°C) for 20 min. Electrophoresis was conducted at 25 V and 310 mA for 24 min. The slides were then washed three times in freshly-made neutralization buffer (0.4 M Tris, pH 7.5, 4°C) for 5 min and dried with absolute ethanol for 30 min. Staining was performed with 20 µl of diluted Sybr Green solution (1 µl in 10 ml of DMSO). Slides were observed with a fluorescence microscope (Zeiss Axio Imager M2, Oberkochen, Germany).

Supplementary Data

CCA – WAF exposure

In the CCA of *M. edulis* tissues exposed to HEI-WAF, the first two factors explained 77.49% of the variability observed (Figure 31). F1 was mainly explained by CR, LDI, 16PAHs, and CZDTs, while the variables that most significantly correlated with F2 were TDNA, AlkPAHs, CZDTs, and VOCs. CV was the dependant variable that showed the weakest correlation with both F1 and F2, but a stronger association with F3 (21.07% of the variability). In the CCA of *M. edulis* tissues exposed to AWB-WAF, the first two factors explained 86.92% of the observed variability (Figure 31). F1 was mainly explained by CR, LDI, CV, and 16PAHs, while the variables that most correlated with F2 were TDNA and VOCs. AlkPAHs and CzDBS were contaminants that showed the weakest correlation with both F1 and F2, but a stronger correlation with F3 (12.89% of the variability), even if it was not a significant factor (eigenvalue < 1; data not shown). In the CCA of *M. edulis* tissues exposed to CLB-WAF, the first two factors explained 94.01% of the observed variability (Figure 31). F1 was mainly explained by CV and AlkPAHs. Even if F2 was not a significant factor (eigenvalue < 1), LDI, TDNA, and CZDTs were most significantly correlated with F2. CR and 16PAHs showed the weakest correlation with both F1 and F2, but stronger values regarding F3 (5.89% of the variability) and F4 (0.10% of the variability), even if these were not significant factors (eigenvalue < 1; data not shown).

CCA – CEWAF and CXT exposure

In the CCA of *M. edulis* tissues exposed to HEI-CEWAF, the first two factors explained 81.14% of the variability observed (Figure 43). F1 was mainly explained by TDNA and all contaminants, while the variables the most significantly correlated with F2 were LDI, CV, and CZDTs. On the other hand, CR was the dependant variable that showed the weakest correlation with both F1 and F2, but a stronger association with F3 (17.13% of the variability) even if it was not a significant factor (eigenvalue < 1; data not shown). In the CCA of *M. edulis* tissues exposed to AWB-CEWAF, the first two factors explained 89.98% of the observed variability (Figure 43). F1 was mainly explained by CV and VOCs, while the

variables the most significantly correlated with F2 were LDI, 16PAHs, and CZDTs. On the other hand, CR, TDNA, and AlkylPAHs were variables that showed the weakest correlation with both F1 and F2, but a stronger correlation with F3 (CR; 7.72% of the variability) or F4 (TDNA and AlkPAHs; 2.29% of the variability), even if it were not significant factors (eigenvalue < 1; data not shown). In the CCA of *M. edulis* tissues exposed to CLB-CEWAF, the first two factors explained 83.28% of the observed variability (Figure 43). F1 was mainly explained by TDNA and VOCs, while the variables the most correlated with F2 were CR, 16PAHs, AlkPAHs, and CZDTs. On the other hand, CV and LDI were variables that showed the weakest correlation with both F1 and F2, but stronger values regarding F3 (14.98% of the variability) and F4 (1.74% of the variability), even if these were not significant factors (eigenvalue < 1; data not shown).

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