1   **Under ice spills of conventional crude oil and diluted bitumen:**

2   **Transgenerational effects and physiological resilience of blue mussel**

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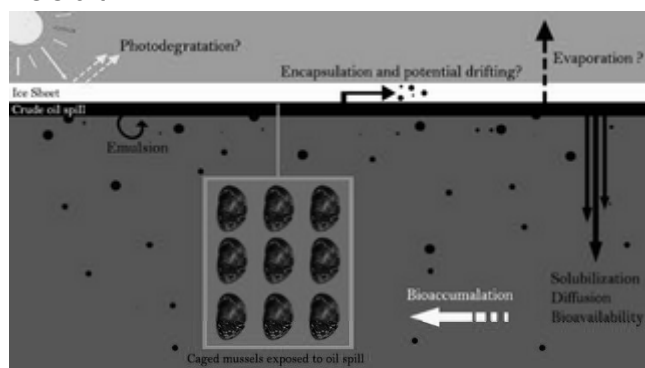
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14   ABSTRACT   3

15   Spillage at sea of diluted bitumen (dilbit) from oil sands has received little attention up to now. To our

16   knowledge, the impact of an acute exposure to dilbit has never been reported in blue mussels (*Mytilus*

17   *edulis*). Adult mussels were exposed to one conventional crude oil (Heidrun) and two dilbits (Cold Lake

18   Blend, Access Western Blend) for 7-days under icy conditions and then kept alive for three months until the

19   spawning season. Aromatic hydrocarbons bioaccumulation, physiological energetic budget, cellular stress,

20   byssus production and gametogenesis were monitored on exposed mussels. In spring time, spawning was

21   induced to characterize breeding success. The bioaccumulation of polycyclic aromatic hydrocarbons

22   (PAHs) in adults was detected after three days of exposure with higher PAHs tissues concentration related

23   to the conventional oil ($5.49\pm0.12\mu g.g^{-1}$ d.w.) compared to both dilbits ($0.91\pm0.02\mu g.g^{-1}$; $0.51\pm0.03\mu g.g^{-1}$

24   d.w.). Despite a rapid depuration combined with good resilience of exposed breeders, significant negative

25   effects were noted at cellular, physiological, and fitness levels especially in offspring. Our results suggest

26   a higher toxicity from the diluted bitumen compared to a conventional crude even with lower totalPAHs

27   bioaccumulation. Dilbits showed evident transgenerational negative effects on unexposed F1 generation.

28   Keywords: diluted bitumen, PAHs, blue mussel, winter oil spill, ice oil spill

29

30   INTRODUCTION

31

32   Worldwide oil demand is still on the rise due to increased needs from international markets, mainly in

33   developing countries. With present depletion of conventional oil reserves, unconventional oil sources

34   gradually gained attention to sustain future international petroleum demand [1]. Alternative unconventional

35   oil resources like bitumen and heavy-oil fields can mainly be found (~70%) in three countries: Venezuela,

36   Canada and U.S.A. In a smaller proportion, Middle-East, Asia, Africa and Russia also possess combined

37   bitumen heavy oil reserves [1]. The Athabasca geological formation (Western Canada) represents the biggest

38   know bitumen deposit with 45% of the world bitumen resources. Those tar sand deposits represent highly

39   biodegraded hydrocarbons from indigenous oil-degrading microorganisms. Low molecular weight

40   hydrocarbons being more sensitive to biodegradation, bitumen crudes contain a greater proportion of heavy

41   hydrocarbons, such as resins and asphaltenes [2,3]. Density and viscosity are therefore directly affected and

42   dilution of bitumen is mandatory prior to pipeline transit [3–5]. Diluents are mainly composed of light

43   hydrocarbons mixed with the bitumen resulting in diluted bitumen (dilbit), gas condensates are often used

44   to obtain dilbits in a proportion of 30% diluent and 70% bitumen [2–4]. As diluent composition can vary

45   depending on market availability and season (higher proportion of diluent in winter), the exact proportion of

46   light compounds is therefore unknown. This uncertainty is raising the question of the potential toxicity of

47   those highly complex crude mixtures. With international exportation expanding, seaborne oil trade will rise

48   [5,6] in upcoming years, exposing marine ecosystems and their species to spill risks [7]. Although the resilience

49   of cold marine communities has been thoroughly studied following the *Exxon Valdez* accident, the

50   consequences of a massive dilbit spill in a cold environment is still unpredictable. Those ecosystems are

51   generally considered more sensitive and less resilient than temperate ones, mainly due to harsher

52   environmental conditions induced by low water temperature, when facing contaminant stress [8].

53   Furthermore, low temperatures coupled with important ice coverage modify physical and chemical

54   properties of spilled oil such as viscosity, density, evaporation, dilution, and degradation potentially affecting

55   oil fate and weathering [8–14].

56

57   A limited number of studies have already focused on the chemical and physical fate of diluted bitumen

58   spilled at sea, reporting mainly on viscosity and density changes and weathering processes [5,8,14–18].

59   Dynamic changes in sea surface parameters such as wave action were included in studies, but none tested

60   the potential biological effects of an ice cover. In terms of ecotoxicological studies, only a few publications

61   covered physiological and ecological effects of toxic compounds associated with a dilbit spill. Most toxic

62 assessment studies used freshwater species [19–25], or early life stages of salmonid species [26–30], with

63 information lacking on reproductive process, larval supply and long-term exposure approach [3]. Potential

64 toxic effects of dilbit heavy crudes on subarctic bivalve populations have not yet been explored and

65 biological responses are expected to be different from those observed with temperate species [31].

66

67 In the present study, the bivalve *Mytilus edulis* was used as sentinel species exhibiting adequate traits for

68 ecotoxicological studies such as the daily filtration of a large volume of seawater providing efficient

69 bioaccumulation and proxy for bioavailable hydrocarbons and other toxicants. Their sessile nature, size

70 and robustness allow easy *in situ* monitoring while being also suitable for laboratory exposure studies [32,33].

71 During winter months, blue mussels reduce their overall activity although they keep filtering [34]. Mainly

72 depending on accumulated energetic reserve to survive through winter and start gametogenesis as soon

73 as environmental spring conditions becomes favorable [35]. Toxic effects of PAHs on bivalves have been

74 widely studied. Effects have been found on oxidative stress [32], endocrine system [36], immunosuppression

75 [37], bioenergetic status [38–40], genotoxicity and cytotoxicicty [41,42] pathology and disease susceptibility [43,44].

76 Also widely studied are negative impacts on reproductive endpoints like gonad alteration [36,39,43–45] and

77 development or survival of exposed larvae [46,47]. Among various papers covering effects of PAHs on various

78 mussel biomarkers, only one study addressed transgenerational effects of dispersed crude oil on blue

79 mussels [36].

80

81 To our best knowledge, no previous study has explored the toxic effects of spilled diluted bitumen on marine

82 species in ice-infested environment during pre-spawning season. Measuring dilbit effects on F0 breeders

83 and transgenerational impacts on F1 offspring is still unexplored. Our study was oriented in a global and

84 longer perspective to increase our understanding of short-term direct effects of dilbits on breeders coupled

85 with potential delayed indirect effects on the first generation following an oil spill. We therefore tested the

86 hypothesis that an under-ice crude oil spill (both conventional and dilbit) during gonad maturation period

87 will affect the integrity and resilience of adult mussels and the quality of their next breeding season, several

88 weeks after the exposure.

89

90 MATERIAL & METHODS

91 Details about experimental setting and analytical methods can be found in the Supporting Information

92 section.

93

94   Crude oil

95   Three different crude oils were tested without any alteration: one conventional oil from the North Sea,

96   Heidrun, and two unconventional oils, or dilbits, Cold Lake blend (CLB) and Access Western Blend (AWB).

97   CLB and AWB both originate from the Athabasca oil sand deposit in Alberta (Canada).

98

99   *Mytilus edulis*

100   Blue mussels (5-6cm, market size, 100% *M. edulis*) were obtained from a large mussel farm in St.Peter's

101   Bay, Prince Edward Island, Canada. Upon reception at UQAR's aquaculture facility in Rimouski (Quebec,

102   Canada), mussels were held in a large tank with running natural seawater pumped directly from the St.

103   Lawrence estuary and filtered through the glass beds filtration system of the station. No additional food,

104   other than natural particulate organic matter in the running water, was provided to mussels.

105

106   Exposure

107   Exposure experiments were conducted at UQAR's aquaculture facility, under a static regime, in a large

108   outdoor cylindrical mesocosm (3.2m height x 1.2m diameter) containing about 3500L of seawater delivered

109   by the station, and exposed to winter conditions prevailing from January to March 2016 (S1). Samples of

110   whole mussel tissues (n=7) were collected before exposure (t=0d exp.), after three days (t=3d exp.) and on

111   the last day (t=7d exp.) to monitor bioaccumulation of hydrocarbons during the exposure.

112

113   Post-exposure, depuration period

114   At the end of control and exposure treatments (corresponding to late March), each group of mussels were

115   transferred in clean mesh bags and suspended in an indoor mesocosm of the same design as the outdoor

116   mesocosm (3500L) with running clean seawater, until the beginning of the spawning season early May.

117   Mussel sampling started immediately after the 7-day exposure period and was repeated a month later. A

118   sampling event consisted of measurement of survival rate, followed by cellular (lysosomal membrane

119   stability; n=7), physiological (condition index, scope for growth, gonad histology, byssus thread; n=7) and

120   chemical (bioaccumulation of hydrocarbons; n=5) analyses on a total of 19 mussels.

121

122   Tissue analysis

123   Whole mussel tissues were freeze dried (FreeZone, Labconco, USA) and homogenized to a fine powder

124   with a Virtis homogenizer. A sub-sample of about 300mg was suspended in 5mL of tetramethylammonium

125   hydroxide (TMAH, 25% water, Sigma Aldrich) in a 12mL glass tube with a Teflon lined cap and vortexed

126   for one minute. The mixture was then heated (60°C) for one hour with manual stirring every 15min to

127   complete the alkaline digestion. After cooling the mixture to room temperature, 1.0mL of deionized water,

128   1.0g of NaCl and 4.0mL of hexane:toluene mixture (1:1) were added before stirring for one hour (Wrist

129   Action Shaker). The mixture was centrifuged (3000g) allowing the collection of the organic layer and

130   repetition of the extraction with another 4.0mL of hexane:toluene solution (1:1). The whole organic extract

131   was then cleaned on a silica column topped with sodium sulfate. The cleaned extract was evaporated under

132   nitrogen at room temperature to a final volume of 1000μL. A volume of 150μL of the concentrated extract

133   was pipetted and transferred to a GC vial equipped with a glass insert and a volume of 50μL of a solution

134   of deuterated PAHs and aliphatics was added as an internal standard. Organic extracts were analyzed by

135   gas chromatography (Trace GC, Thermo, USA) coupled with a mass spectrometer equipped with an ion

136   trap (Polaris Q, Thermo, USA). A capillary column (VB-5MS, 30mmx0.25mm i.d.; Valcobond™) and high

137   purity grade helium as carrier gas was used at a flow rate of 1mL.min^{-1}. Detection and quantification of

138   hydrocarbons (aliphatic, monoaromatic alkylated, polycyclic aromatic relatives and methyl derivatives,

139   heterocyclic aromatic carbazoles and dibenzothiophenes and methyl derivatives) were performed in scan

140   mode between 50-500amu with positive ions detection. Method blanks and analysis of a certified reference

141   material (SRM 2974a, Organics in freeze-dried mussel tissue, *Mytilus edulis*) were completed. Relative

142   standard error (n=5) for the quantitative analysis of thirteen PAHs in the SRM, varied between 5-20%

143   (median=11%). The median value for the recovery was 82% (41-118%). Results are presented in μg.g^{-1}.dry

144   weight and were not corrected for recovery yield. The LOD (mean of the blanks (n=6) plus 3 sigma), based

145   on 300mg size sample of dry tissue, varied from 0.7ng.g^{-1} to 24 ng.g^{-1} for 22 PAHs, with a median value of

146   2.4ng.g^{-1}.

147

148   Physiological energetic budget

149   Scope for growth is used as a physiological energetic index of filter-feeding animals and was obtained by

150   subtracting both energy respired and excreted from the energy assimilated from food, and illustrates the

151   mussels energy budget [48,49]. As the energy excreted being minimal (<5% of the energy budget) [50], this

152   parameter was not monitored. Measurements were realized simultaneously on seven mussels and one

153   control (empty shell) in individual experimental chamber (Plexiglas cylinders of 1357mL) filled with

154   ultrafiltered (0.02μm) and UV sterilized seawater maintained in a temperature-controlled water bath

155   (4.2ºC±0.82). The oxygen depletion was measured during one hour by a polarographic electrode (YSI

156   model 5775, USA). Clearance rate analysis, experimental chambers were opened, and air bubble injection

157 was added. The empty chamber control was used to determine the sedimentation level of microalgae. For

158 absorption efficiency, food and faecal samples (after continuous feeding during 24-48h; 60 cell.µL^{-1}.ind^{-1})

159 were filtered onto pre-combusted and pre-weighed GFC filters (47mm for faeces and 25mm for food), then

160 rinsed with isotonic ammonium formate (3.2%), dried at 65°C for 72h, cooled to room temperature in a

161 desiccator, and re-weighed. Afterwards, they were combusted overnight at 450°C for 4h, cooled to room

162 temperature in a desiccator, and finally weighed again. This procedure provided estimates of the organic

163 and inorganic fractions contained in the food and faeces.

164

165 Lysosomal stability or Neutral red retention assay

166 An adapted method from Wyatt and collaborators [51] was used to determine the lysosomal stability. Briefly,

167 the neutral red retention assay consisted in taking 700µL of hemolymph sampled from the posterior

168 adductor muscle of mussels with a 1000µL syringe (22.5-gauge needle). A subsample was then transferred

169 to a positively charged microscope slide and kept for 15min in a light-proof humidity chamber. Fresh neutral

170 red solution (8.5µl of stock solution containing 28.8mg of neutral red in 1000µL of dimethyl sulfoxide

171 (DMSO) mixed with 500µL of filtered intervalvular fluid) was then added. After 60min incubation period,

172 haemocytes were examined Stressed cells were characterized by larger lysosomes or leakage of neutral

173 red dye into the cytosol. Lysosomal stability or cellular stress is express as the relative abundance (%) of

174 stressed cells on total analyzed haemocytes.

175

176 Byssus thread

177 As byssus thread diameter can be related to energetic availability of mussels [52], this measure has been

178 added in this study. Two byssus threads were collected on five different mussels and kept in opened plastic

179 bags for analysis by digital microscope (VHX-2000, Keyence digital microscope system, Japan) mounted

180 with 250-2500X lenses (VH-Z250R). The threads were rehydrated, secured flat on microscope slides, and

181 their diameter was measured at five different locations on the distal section.

182

183 Gametogenesis

184 Gametogenesis development was determined in a portion of mussel mantle tissue preserved in 20mL

185 Davidson fixative solution [53] changed after 24h post-sampling. Prior to analysis, tissues were gradually

186 dehydrated in ethanol (75%, 70%, 90%, 95%) then infiltrated and embedded with methacrylate-based

187 embedding solution (kit JB-4, Sigma-Aldrich). Sections of 5µm thickness were stained with hematoxylin and

188 eosin (Ehrlich solution). Gamete maturation stage was determined based on the identification key by

189   Lemaire and collaborators [35]. Each sample was analyzed for gamete volume fraction (GVF), atresia volume

190   fraction (AVF) and oocytes diameter measurements.

191

192   Breeders conditioning and larvae monitoring

193   One month prior to the induction of spawning, 60 exposed mussels from each treatment were randomly

194   selected and conditioned in open system tanks of 100L, and fed *ad libitum* with microalgae (*Tisochrysis*

195   *lutea, Pavlova lutheri, Chaetoceros muelleri*, ratio of 1:1:1). Eggs from different females were pooled

196   together. A similar procedure was done to recover spermatozoids from males and fertilization was realized

197   at a ratio of 20 sperms per oocyte. Larvae were reared at 18°C with a final concentration of 10 larvae.mL^{-1}

198   in 1μm filtered and UV treated seawater renewed every two days. Egg size, shell growth and survival were

199   estimated every two days. Survival rates were expressed as the total number of individuals minus the

200   cumulative amount of empty shells and based on the first sampling timepoint.

201

202   Statistical analysis

203   The software R and his extension R-studio (R-Core Team 2012, V1.1.453) were used for the statistical

204   analysis of data produced. First, values were checked for uniformity of both data distribution and variance

205   with Shapiro-Wilk test (uniformity of distribution) and a visual observation of the distribution of variance.

206   When needed, data were log-transformed to normalize their distribution. Two-way ANOVAs, coupled with

207   Tuckey posteriori tests, were used to compare differences between treatments and sampling periods. The

208   ggplot2-package (V3.1.0) was used for data illustration purposes.

209

210

211   RESULTS & DISCUSSION

212   Bioaccumulation of hydrocarbons

213   The monitoring framework targeted 16PAHs selected by the United States Environmental Agency (USEPA)

214   as priority contaminants and their alkylated congeners for a total of 47 compounds. In addition, heterocyclic

215   hydrocarbons such as carbazoles and dibenzothiophene and their alkylated congeners including 10 and 8

216   compounds, respectively, were analyzed. This study monitored the presence of 65 petroleum hydrocarbons

217   to characterize their bioaccumulation and the potential toxicity of crude oils. Those petroleum hydrocarbon

218   congeners have demonstrated toxic effects on marine biota, and their occurrence and concentrations have

219   been used as proxies to predict or explain oil toxicity [3,26,54].

220

221   During winter exposure (days 0-7) and post-exposure period (days 8-35), levels of total polycyclic aromatic

222   hydrocarbons (TPAHs), carbazole (CBZ) and dibenzothiophene (DBT) in control mussels were very low

223   (<0.5µg.g^{-1}) compared to crude oil treatments (Figure 1). The highest bioaccumulation values were seen

224   after 3 and 7 days of exposure, followed by a gradual decrease after the end of exposure. Mussels exposed

225   to Heidrun treatment systematically showed the highest values for TPAHs, CBZ and DBT (up to 90% higher)

226   with maximal concentrations measured during the exposure. The two dilbit treatments showed a similar

227   bioaccumulation pattern resulting in lower concentrations than Heidrun crude for all analyzed hydrocarbons,

228   suggesting a potential higher toxicity from the conventional oil than the two diluted bitumen (Figure 1).

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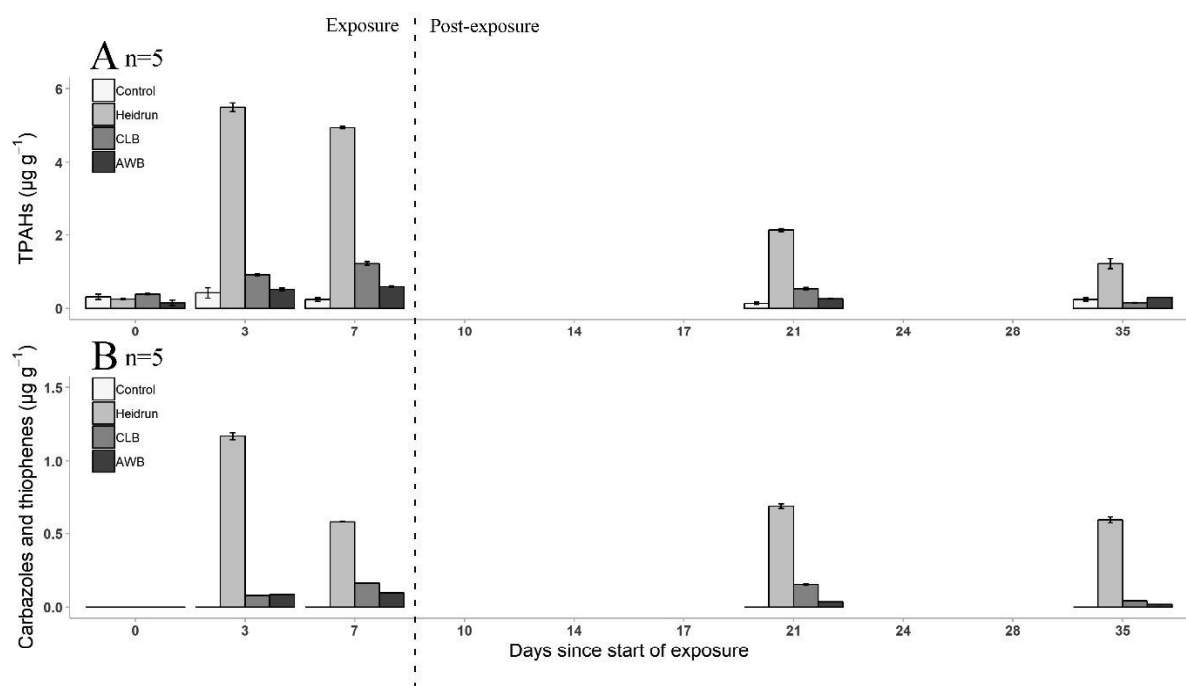
230
231   Figure 1. Bioaccumulated TPAHs (µg.g^{-1} dw) (A), CBZ and DBT (µg.g^{-1} dw) (B) in adult mussel whole tissue

232   during and after winter exposure for the control, the conventional oil and the diluted bitumen treatments.

233

234   Marine bivalves are filter feeding organisms removing suspended particles from a large volume of seawater

235   which can be exposed to pollutants through different pathways [55]: passive exposition via the passage of

236   water through the mantle cavity, food ingestion [38,56] or passive diffusion at the gill epithelium [38,57]. As

237   temperature influences the physiology of bivalves, winter causes a metabolic slowdown in blue mussels

238   [55,58] along with a lower filtration rate. Because of the reduced energy request coupled with a short exposure

239   time, exposed mussels could have passively avoided the dissolved hydrocarbon fraction by keeping their

240   valves firmly closed during the whole or a part of the exposure. Even with low seawater temperatures (-

241   1.44±0.58°C) and ice cover, we observed an accumulation of hydrocarbons from day 0 to day 3 in the

242   tissues of oil-exposed mussels following the different crude treatments. PAHs bioavailability being related

243   to their origin, and physical and chemical properties [59], different classes of crude oils could induce different

244   bioavailability of compounds in terms of congeners and concentrations. In this study, hydrocarbon

245   bioavailability was certainly different between treatments resulting in lower bioaccumulated values for both

246   dilbits, at least for the targeted hydrocarbons. The conventional oil led to bioaccumulation with values

247   sometimes ten times higher than for the dilbits. Bioaccumulation rate was also faster, with highest values

248   being recorded on the first sampling event, after three days of exposure. Compared to the conventional oil,

249   dilbits contained less compounds of low molecular weight (LMW) and a higher proportion of resins and

250   asphaltenes [3]. PAHs solubility being strongly influenced by their molecular weight, lower weight compounds

251   are mainly found in the dissolved fraction while higher molecular weight PAHs will tend to bind to particulate

252   and sediments [4]. Therefore, lower bioaccumulation observed with dilbit treatments seems in accordance

253   with their chemical characteristics.

254

255   During the depuration period, bioaccumulated TPAHs, CBZ and DBT gradually decreased during the first

256   month, with specific rate depending on the hydrocarbon group. Regardless of the oil treatment,

257   accumulated TPAHs decreased more rapidly than the CBZ and DBT. During the depuration period,

258   bioaccumulated TPAHs were reduced by 50% after two weeks, while CBZ and DBT had only slightly

259   decreased. After two additional weeks, the depuration rate of TPAHs was slightly reduced compared to the

260   first two weeks while CBZ and DBT were mostly depurated except for the conventional oil. It is known that

261   mussels can exhibit a rapid depuration rate for the first days following hydrocarbons exposure [55]. In this

262   study, this was only the case with the TPAHs concentration in contrast to CBZ and DBT (Table 1).

263

264   Table 1. Tissue bioaccumulation of carbazole (CBZ), dibenzothiophene (DBT) and TPAHs expressed in

265   concentration ($\mu g.g^{-1}$ d.w.) and relative importance (%) between measured petroleum hydrocarbons. Values

266   are classed between treatments and sampling time.

|	Tissue bioaccumulation	CBZs		DBTs		TPAHs	
		$\mu g\,g^{-1}\,dw$	%	$\mu g\,g^{-1}\,dw$	%	$\mu g\,g^{-1}\,dw$	%
Heidrun	7-day of exposure	0.26 ± 0.01	4.7	0.32 ± 0.01	5.8	4.95 ± 0.03	89.5
	2 weeks post-exposure	0.28 ± 0.01	10.0	0.39 ± 0.01	14.0	2.12 ± 0.04	76.0
	4 weeks post-exposure	*	< 1	0.59 ± 0.01	32.6	1.22 ± 0.04	67.4
CLB	7-day of exposure	*	< 1	0.16 ± 0.01	12.6	1.22 ± 0.05	88.4
	2 weeks post-exposure	*	< 1	0.15 ± 0.01	21.7	0.54 ± 0.03	78.3
	4 weeks post-exposure	*	< 1	0.04 ± 0.01	21.1	0.15 ± 0.01	78.9
AWB	7-day of exposure	0.03 ± 0.01	13.5	0.06 ± 0.01	11.1	0.59 ± 0.02	75.3
	2 weeks post-exposure	*	< 1	0.04 ± 0.01	13.3	0.26 ± 0.01	86.7
	4 weeks post-exposure	*	< 1	*	< 1	0.29 ± 0.01	100.0

267

268   * below detection limit

269

270   Physiological integrity of adults

271   Monitoring of the clearance rate (CR) and the scope for growth (SFG), which is used as an energetic index

272   of filter-feeding animals, were used to assess mussel physiological status [48,49]. For the SFG and his

273   integrated metrics, only the clearance rate showed a significant variation after the exposure period with

274   lower values for CLB and Heidrun treatments (Figure 2A). The recovery of CR after one month, as similar

275   values between oil treatments and control were observed (Figure 2A), suggests that physiological

276   performance impairment seems dependent of the targeted biomarker and hydrocarbon depuration [37].

277   Oxygen consumption (overall mean: 0.7205±0.13), assimilation (overall mean: 89.87±14.63) and SFG

278   (overall mean: -7.60±0.79) were not significantly impacted between treatments and time. These diverse

279   SFG metrics indicate that adult mussels showed a good overall physiological resistance to a short-term

280   petroleum hydrocarbon exposure. Time and concentration of hydrocarbon exposure are key characteristics

281   in the bivalves response. When a low level of PAHs exposure is extended, damage to metabolism could

282   be as severe as individuals exposed to high levels of PAHs [60]. Diminution of CR induced by contaminants

283   or PAHs exposure had already been reported [47,61]. Widdows and Johnson [49] also observed a relationship

284   between declining CR and increasing concentration of aromatic hydrocarbons in the body tissues of *Mytilus*

285   *edulis*, but their lower concentration induced no variation of CR. This dose dependence adjustment of the

286   CR was also recorded in the present study, as the bioaccumulation magnitude increased in the following

287   order: AWB<CLB<Heidrun. An increased CR could be due to depuration purpose [61], but it remains unclear

288   if the physiological reaction of slowing down the CR is induced by a variation of ciliary activity due to narcotic

289   effects of PAHs [62], or if it is a biological response to minimize intrusion of PAHs [60,61].

290
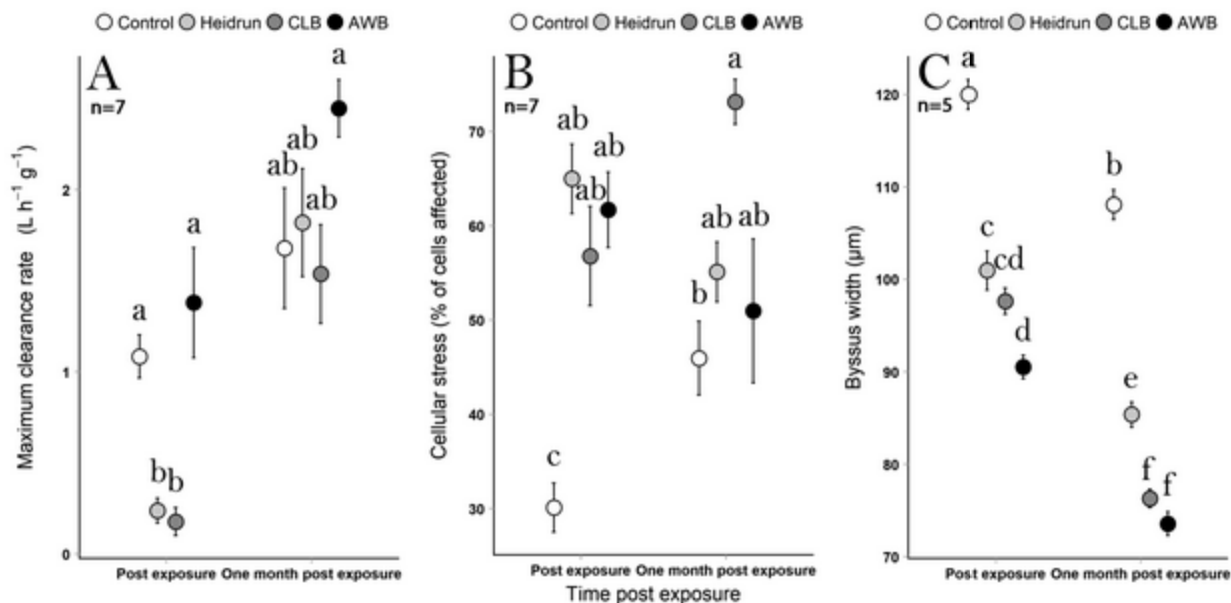
291
292   Figure 2. Maximum clearance rate (L.h^{-1}.g^{-1}) (A), cellular stress (% of affected cells) (B) and byssus

293   thickness (μm) (C) immediately after the exposure (within 12h) and one month later for the control, the

294   conventional oil and the diluted bitumen treatments. Mean ± SE. Different letters over each mean indicate

295   significant statistical differences between treatment and time (p<0.05).

296

297   Cellular stress

298   Immediately after the exposure, cellular stress was higher in the three oil treatments when compared to the

299   control (Figure 2B). Significant differences were not observed one month after the exposure while the

300   control kept the lowest cellular stress mean of all treatments (TreatmentxTime, DF=3 and 47, F=5.883,

301   p=0.0017). Lysosomal stability (cellular stress) has been classified as a useful and efficient screening

302   technique able to discriminate between polluted and clean sites, relying on its capacity to respond to

303   chemical challenges [63] and oil exposure [64]. Lysosomal activity being directly related to immune activity in

304   bivalves, its effectiveness is vital to protect the animals against diseases. Impaired lysosomal activities can

305   potentially induce adverse effects, such as histopathological, developmental, and reproductive

306   abnormalities or worst mortality and population decline [63,64]. Lipophilic compounds, as PAHs, will likely enter

307   the lipid bilayers impacting the membrane permeability and fluidity [65]. When *Mytilus edulis* were exposed

308   to phenanthrene, destabilizing effects on the lysosomal membrane seemed to be triggered by a critical

309   concentration accumulated by lysosomes [65]. In *Crassostera gigas* exposed to water accommodated fraction

310   (WAF) of petroleum hydrocarbons, genes related to immune system were down regulated, showing

311   potential immunosuppression [66]. This can explain the significant lysosomal destabilization observed right

312   after the exposure opposed to the absence of response one month later, when the mussels have already

313   eliminated more than half of their accumulated hydrocarbons. Like the clearance rate, no more significant

314   effects from oil exposure were visible one month after exposure (Figure 2B).

315

316   Byssus thickness

317   Byssus thread diameters showed significantly lower values for each oil treatment compared to control, with

318   diameters over 10% smaller with the AWB, the most impacted treatment (Figure 2C). After one-month,

319   negative impacts of oil treatments are still present except that the most impacted treatments were CLB and

320   AWB with byssus thread diameter over 25% lower than the control (TreatmentxTime, DF=6 and 1788,

321   F=11.08, p<0.001) (Figure 2B). Byssus secretion is a dynamic process influenced by both exogenous and

322   endogenous factors [67,68,62] and byssal thread production can represent over 10% of a mussel's monthly

323   energy expenditure [69]. A decrease in byssal thickness could indicate a potential individual energy trade-off

324   towards more important metabolic pathways potentially related to hydrocarbons depuration. As byssus

325   thickness morphology secreted under different endogenous conditions is the major parameter to explain

326   variability in attachment force [67] tinier byssal thread can increase mussel detachments. Considering that a

327   winter oil spill already causes higher energy demands in a harsh period of the year, production of new

328   byssus threads could worsen physiological integrity of mussels.

329

330   Overall, adult physiology biomarkers showed limited effects of oil immediately after the exposure with a

331   generally good resilience during the first month post-exposure, concomitantly with effective tissue

332   depuration. Even mortality (results not shown) wasn't strong with death toll never exceeding 15% in each

333   treatment. Despite significant negative effects of oil exposure immediately after the exposure for some

334   biomarkers, negative impacts generally disappeared at the end of the depuration month, except for the

335   diameter of byssus produced. Even with higher bioaccumulation for conventional oil compared to dilbit

336   treatments, no difference on physiological impacts were present between crude oils. Therefore, in

337   accordance with bioaccumulation values, diluted bitumen did not seem to induce strong toxic effects at the

338   physiological level.

339

340   Monitoring of breeding metrics

341   The gamete volume fraction (GVF) indicator (Figure 3A) showed the highest values in mussels from the

342   control treatment. Mussels exposed to oil treatments displayed lower GVF values, with means all under

343   50% without full recovery after one month (Figure 3A). Immediately after the exposure, mussels from the

344   CLB treatment showed the lowest one and mussels from the CLB and AWB treatments showed the lower

345   values one month later (TreatmentxTime, DF=3 and 340, F=4.062, p=0.00742). The atresia volume fraction

346   (AVF) (Figure 3B) indicator were very low in the control (<4%) and significantly higher in oil treatments

347   (Figure 3B) with dilbits showing means over 22% higher after the exposure and one month later

348   (TreatmentxTime, DF=3 and 340, F=14.41, p<0.001) (Figure 3B). Microscopy observation of gonad cross

349   sections gives a great insight of oil exposure effects on gametogenesis (Figure 3C). For control gonads,

350   follicles and individual oocytes displayed normal development with low occurrence of atretic oocytes. In

351   contrast, oil-exposed female mussels showed high gonad atresia level (▸) with hemocyte infiltration (*) in

352   the gametic tissue. AWB mussels displayed early stage atretic oocytes while CLB mussels showed late

353   stage atretic oocytes mainly already degraded, as observed after the 7-day exposure (Figure 3C).

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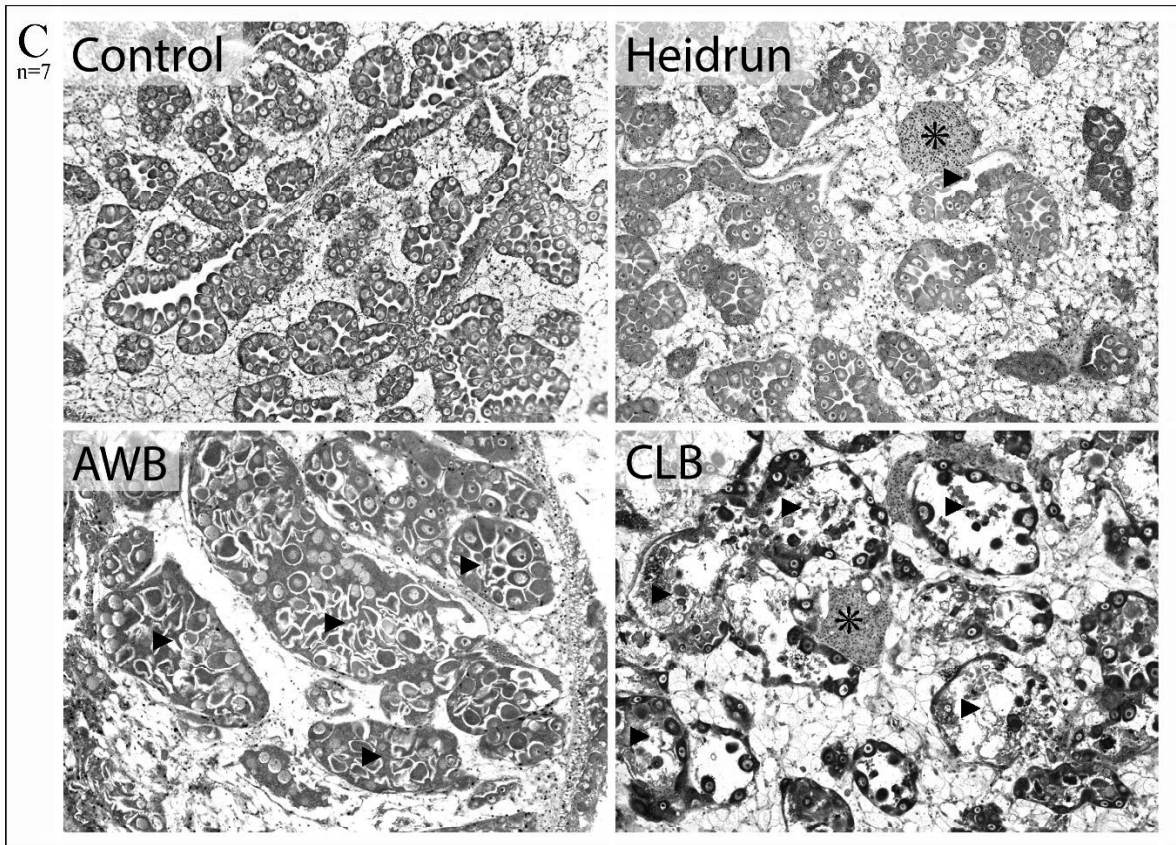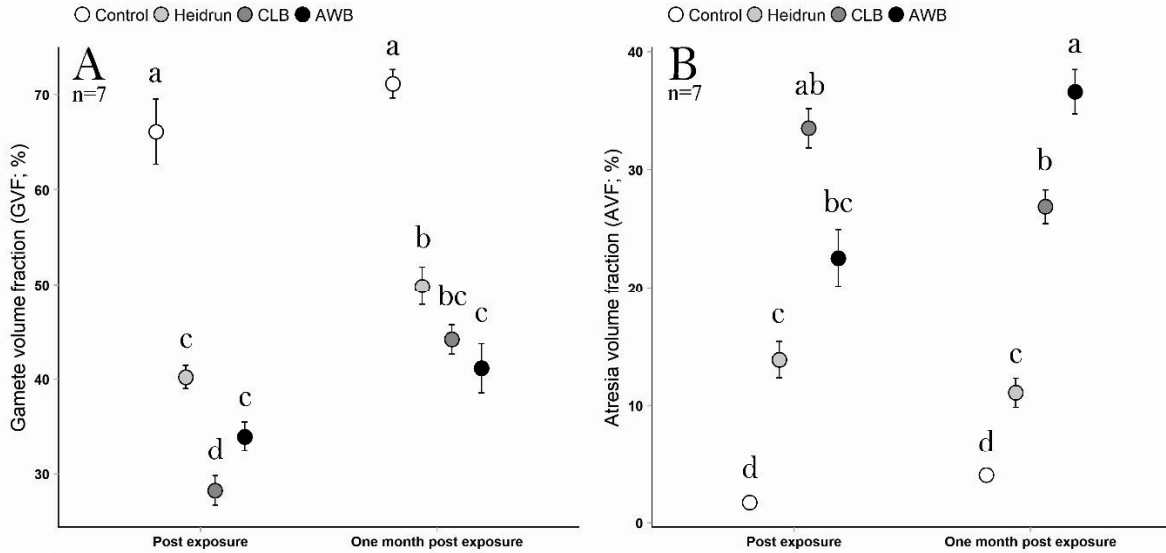
Figure 3. Gamete volume fraction (%) (A), atresia volume fraction (%) (B), and female gonads cross section (400X) (C). Female gonad cross sections are from samples obtained immediately after the exposure (within 12h). Hematoxylin and eosin (Ehrlich solution) staining, section of 5μm. Atresia is identified by an arrow

359   head " ▸ " and hemocyte infiltration by a star symbol " * ". Mean ± SE. Different letters over each mean

360   indicate significant statistical difference between treatment and time (p<0.05).

361

362

363   While adult mussels showed a good physiological resilience to a 7-day winter exposure, ecological

364   resilience seems strongly at risk. Omnipresence of oocyte atresia, sometimes over 40%, coupled with the

365   low proportion of viable oocytes showed important toxic effects of oil spill exposure on gametogenesis.

366   Bivalves histological analysis demonstrated to be a useful tool to assess ecotoxicity in bivalves exposed to

367   polluted environment [44,70]. Inflammation, necrosis and atrophy of gonadal cells were some of the effects

368   measured in *Ostrea edulis* and *Crassostrea gigas* exposed to a crude oil spill [71]. Multiple toxic effects in

369   *Mytilus edulis* have also been recorded as haemocytic infiltration of follicles, severe oocyte atresia or

370   neoplasia [44,45,70,72,73]. Atresia in female gonad is triggered when spawning copes with harsh environmental

371   conditions after gamete ripening occurs and is associated with over-maturation of gametes leading to the

372   end of gametogenic cycle, degeneration and resorption of gonadal tissue [70]. In *Crassostera gigas*, WAF of

373   petroleum hydrocarbons has been link to 22 sub-expressed genes linked to gonad maturation [66]. In our

374   study, the winter oil exposure rapidly impacted gonad integrity and maturation process. Even with the good

375   physiological resilience shown by exposed adult mussels, a significant increase in oocyte atresia was

376   present for the conventional oil and was even stronger for dilbits. Gonad tissue being used as energy

377   storage for mussels, oocyte atresia can be a useful way to relocate energy where most needed. An

378   unexpected oil exposure and the subsequent depuration activities could have induced higher energy

379   demands in a period of food scarcity, therefore inducing gonad atresia. In this case, the adult mussels

380   "choose" to maintain their own physiological integrity at the expense of reproduction. The gonad biomarker

381   is therefore the really first indicator of potential transgenerational effects, as genitors re-use energy stocked

382   under gonad form, the next mussel generation is put at risk.

383

384   Unexposed larvae (F1) produced by exposed genitors (F0)

385   The proportion of D-veliger larvae found two days post-fertilization was the highest in control mussels with

386   a mean of 60% compared to all oil treatments with means value below 25%. Mussels from the AWB

387   treatment produced the lowest proportion of D-veliger larvae with 4.9% (Treatment, DF=3 and 12, F=15.82,

388   p=0.00018) (Figure 4A). During their development until day 9 post-fertilization, control and Heidrun

389   treatments larvae showed higher final larval density opposed to dilbit treatments. After nine days of

390   development, no significant difference was found between the control and the Heidrun treatment (p=1) and

391   between the two diluted bitumen treatments (p=1) while both groups showed a significant difference

392   (TreatmentxTime, DF=6 and 36, F=18.55, p=<0.001) (Figure 4B). Considering the larval size progression

393   from fertilization until day 9 post-fertilization, controls showed the greatest size growth with a regular

394   increase over time. Larvae from oil treatments showed a step-like progression with no significant

395   differences, inside the same treatment, between sampling days 0-2, 2-4 and 7-9, except for the Heidrun

396   treatment between day 0-2. The only significant progression inside a same oil treatment was between day

397   4-7 (Treatment x Time, DF= 12 and 2198, F= 23.19, p<0.001) (Figure 4C).

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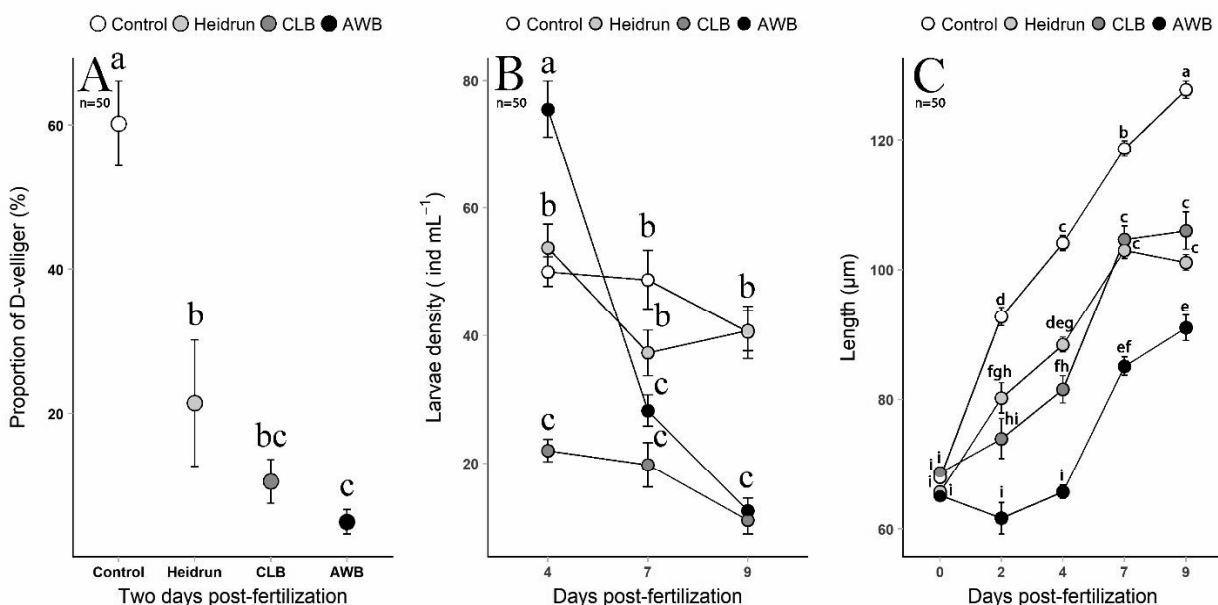
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401   Figure 4. Proportion (%) of larvae at D-veliger stage, two days post-fertilization (A), larvae density (larvae

402   mL^{-1}) at days 4, 7 and 9 post-fertilization (B) and larvae size growth (µm) from fertilization to nine days post-

403   fertilization (C) for the control, the conventional oil and the diluted bitumen treatments. Mean ± SE. Different

404   letters over each mean indicate significant statistical differences between treatment and time (p <0.05).

405

406   Embryogenesis, larval survival and growth were hardly impacted by oil treatment with larvae size lagging

407   five days behind the control treatment after nine days. Those strong effects of oil exposure, and particularly

408   of dilbits, on gonad maturation several weeks before spawning, support the hypothesis of strong

409   transgenerational effects of a winter oil exposure under ice cover. Studies have already shown negative

410   effects following PAHs exposure of gametes or growing larvae showing strong embryotoxicity [74–76].

411   However, in this study, gametes or larvae have never been directly exposed to any kind of petroleum

412   hydrocarbons. The only justification to the toxicity observed in the F1 larvae is through parental inheritance.

413   Observed impacts can therefore results from weakened energy reserves in eggs or maternal contaminant

414   transfer. Food-stressed mussels have been shown to produce lower quality larvae with increased

415   developmental abnormalities, diminished survival and growth rate potentially due to a significantly collapse

416   of energy inheritance [77,78]. Therefore, the energy trade-off observed in adult mussels after their exposure

417   to crude oil to assure their individual integrity seems to have reduced the energy passed on to the F1 larvae

418   generation. PAHs have been proved to accumulate in fish and copepod eggs, due to their high content in

419   lipids, and being transferred to offspring [79,80]. Specific hydrocarbon congener (phenanthrene) has also been

420   linked [81] to maternal transfer and photo-toxicity in larvae produced by exposed breeders of *Mulina lateralis* [81].

421   PAHs are also known for their genotoxicity potential on bivalve embryos exposed to dissolved fraction,

422   inducing strong DNA strand breakage even at low concentration [82].

423

424   Regardless the harsh abiotic conditions mussels can be exposed to during wintertime, they must

425   adequately balance their energy budget to allow gametogenesis to start as soon as possible to ensure

426   reproductive success. In this study, we highlight a cascade effect from the winter exposure and gonadal

427   impairments followed by lower gamete production and impaired larval metamorphosis, survival and growth

428   showing the potentially disastrous long-term consequences that a brief oil spill can induce on natural

429   populations. Essentially, a light (1:1000 crude oil:seawater) and short (7-day exposure) oil exposure during

430   winter under an ice cover induced higher bioaccumulation of measured hydrocarbons for conventional oil

431   compare to diluted bitumen. A fast depuration rate was obvious in the first month post exposure in the case

432   of TPAHs while CBZ and DBT were slower to be excreted. Even with important bioaccumulation of

433   petroleum hydrocarbons in the tissues of mussels, the physiological resilience of adults was strong with still

434   some negative effects on some biomarkers disappearing in the first month. At this point, if it was not for the

435   strong negative effects on gonad maturation, based on bioaccumulated values and mussel physiological

436   integrity, we had assumed that diluted bitumen were as toxic as conventional oils. Then, several weeks

437   after the winter oil spill, the breeding was induced, and the true toxic potential of diluted bitumen was

438   observed on every single reproductive biomarker measured.

439

440   However, based on the monitoring of targeted 16 PAHs, their alkylated congeners in addition to heterocyclic

441   hydrocarbons such as CBZ, DBT and their alkylated congeners were not enough to explain the observed

442   high toxicity. Novel unconventional oil products, such as diluted bitumen, possess differential chemical

443   composition and complexity, compared to conventional crude oil. In addition to significant amount of

444 unresolved complex mixture (UCMs), diluent is considered to enhance and complexify the LMW

445 hydrocarbon fractions [83] surely affecting both dissolved bioavailable compounds and, inevitably, their

446 toxicity. Also, we found essential to target a wider time frame to measure the strong toxic effects on

447 ecosystem integrity. Upcoming research on diluted bitumen should therefore include a wider range

448 monitoring of potential toxic compounds in a longer sampling time frame including genotoxicity, maternal

449 transfer and transgenerational effect biomarkers.

450

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