Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (Mytilus edulis)

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Complete List of Authors:	Magara, Gabriele; Universita degli Studi di Perugia Khan, Farhan; Roskilde University, Department of Science and Environment Pinti, Marika; Universita degli Studi di Perugia Syberg, Kristian; Roskilde University, Department of Science and Environment Inzirillo, Angelo; Universita degli Studi di Perugia Elia, Antonia; Universita degli Studi di Perugia,
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1 **Effects of combined exposures of fluoranthene and polyethylene or**

2 **polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue**

3 **mussel (*Mytilus edulis*)**

4 *Gabriele Magara[a], Farhan R. Khan[b]*, Marika Pinti[a], Kristian Syberg[b], Angelo Inzirillo[a]*

5 *and Antonia Concetta Elia[a]**

6 *a Department of Chemistry, Biology and Biotechnology, via Elce di sotto 8, University of Perugia,*

7 *06123 Perugia, Italy*

8 *b Department of Science and Environment, Universitetsvej 1, P.O. Box 260, Roskilde University, DK-*

9 *4000 Roskilde, Denmark*

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12 ** Corresponding author: Dr. Antonia Concetta Elia*

13   *Department of Chemistry,*

14   *Biology and Biotechnology,*

15   *University of Perugia, Italy*

16   *Email: antonia.elia@unipg.it*

17   *Dr. Farhan R. Khan*

18   *Department of Science and Environment,*

19   *Roskilde University, Denmark*

20   *Email: frkhan@ruc.dk, farhan.khan@gmx.com*

21

22

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ABSTRACT

A growing interest in developing and commercialization of new ecofriendly plastic polymers is occurring attributed to the impact of marine plastics debris and microplastics that result from the degradation of oil-based polymers as these substances adversely affect ecosystem health. Recently, polyhydroxybutyrate (PHB) has become of interest due its biodegradability and physicochemical properties. However, biological consequences resulting from bioplastics exposure remains to be determined. Further, few data are apparently available regarding the potential for bioplastics to act as a vector for exogenous chemicals in the environment. The aim of the study was to compare the effects of polyethylene (PE MPs) and polyhydroxybutyrate (PHB MPs) microplastics administered alone or in combination with fluoranthene (Flu) on detoxifying enzymes in digestive glands and gills of *Mytilus edulis*. Blue mussels were exposed for 96hr to 8 experimental groups: control, Flu-only, PE MPs-only, PHB MPs-only, PE MPs-Flu co-exposure, PHB MPs-Flu co-exposure, Flu-incubated PE MPs and Flu-incubated PHB MPs. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), glutathione S-transferase (GST) and glutathione reductase (GR) were found to be significantly susceptible to Flu and plastics in both tissues. Interestingly, single exposure to PHB MPs led to decreased levels of CAT and GST in gills, SOD in digestive glands and SeGPx in both tissues. In co-exposure and incubation treatments, biochemical responses were generally comparable with those exerted by PE MPs or PHB MPs only, suggesting an apparent absence of combined effects with pollutant. Data demonstrate the ecotoxicological impact of bioplastics materials on digestive glands and gills of *Mytilus edulis*.

Keywords: biodegradable polymer, biomarker profile, bioplastics, blue mussels, microplastic

48 **Introduction**

49   Oil-based plastic debris and subsequently the microplastics (MPs) formed by their degradation are

50 one of the current major environmental concerns.   These plastics are defined chemically as organic or

51 semi-organic materials with long chains (macromolecules) and high molecular weight, generated by the

52 polymerization of monomers extracted from oil or gases (Cole et al., 2011). Once in seawater,

53 microplastics were shown to be ingested by aquatic species which resulted in   a number of deleterious

54 effects (Guzzetti et al., 2018; Alimba and Faggio, 2019) . The color, density, shape and size of MPs

55 often leads marine organisms to mistake MPs for food items and the consequences of this includes

56 blockage of the digestive system and false feeling of satiation (Wright et al., 2013). In addition, MPs

57 may act as a vehicle for transport of several contaminants dissolved in the aquatic environment

58 (Andrady, 2011). This 'vector effect' (Syberg et al., 2015), suggests that oil-based MPs may trap and

59 transport environmental pollutants including persistent organic pollutants (POPs) or metals (Oliveira et

60 al., 2013; Avio et al., 2015; Khan et al., 2015; Magara et al., 2018). The possibility that plastics might

61 then transport the adhered contaminants into aquatic biota is currently being debated (Koelmans et al.,

62 2016), and lab investigations into the vector effect remain ongoing.

63   There is a mounting interest in "green materials" and a focus on development and production of

64 biodegradable polymers (Mohanty et al., 2002). In contrast to oil-based polymers, these biopolymers

65 are derived from bioprocesses, using renewable resource in bio-refineries. Due to their mechanical and

66 physicochemical characteristics, these biodegradable polymers may be considered as an eco-friendly

67 substitutes to plastic (Anjum et al., 2016). In recent years, polyhydroxybutyrate (PHB) has received

68 growing interest for its characteristics similar to oil-based plastic (Dacosta et al., 2016).

69 Polyhydroxybutyrate is water insoluble, but highly biodegradable biopolymer, relatively resistant to

70 hydrolytic degradation which is extracted with chloroform from bacterial cultures grown on

71 carbohydrates. PHB (1) displays low oxygen permeability and reliable thermoplasticity with poor

72 mechanical properties, (2) high crystallinity degree and (3) is optically active with a mass about

73   approximately 0.5×10^6 Da (Anjum et al., 2016). However, although PHB is currently available

74   commercially, especially as main component of biodegradable market bags, to the best of our

75   knowledge little is known regarding the environmental impact or effects on aquatic organisms. By

76   sharing many characteristics with oil-based plastics, it is feasible that in the aquatic environment,

77   bioplastic undergoes modifications, resulting in generation of MPs as is the case for oil-based

78   polymers (Andrady, 2011). If due to environmental and societal pressures a move is made towards use

79   of bioplastics, then it is vital to gather information on the influence of these bioplastics on the

80   ecosystem health.

81   Oxidative stress results from an imbalance between pro-oxidants such as reactive oxygen species

82   (ROS), and the protective antioxidant system. Mechanisms that involve glutathione (GSH), and the

83   related antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases

84   (SeGPx and GPx) represent important protective metabolic pathways which are used as biomarkers

85   related to pollutant induced oxidative stress. Glutathione (GSH) in its reduced form is an important

86   non-enzymatic scavenger of oxyradicals, involved in the metabolism of toxic compounds and

87   endogenous substances (Meister and Anderson, 1983). Both SOD and CAT enzymes catalyze the

88   breakdown of ROS-generating O_2^- and H_2O_2, respectively and are key components within the primary

89   defense system against oxidative stress-induced damage. SeGPx enzyme reduces either hydrogen

90   peroxide or organic peroxides and GPx enzyme affects organic hydroperoxides. Polycyclic aromatic

91   hydrocarbons (PAHs) , of which fluoranthene (Flu) is often used as a model contaminant (and in

92   addition a priority aquatic pollutant (Directive 2008/105/EC)), are known to initiate oxidative stress

93   which was assessed by these aforementioned biomarkers (Cheung et al., 2001). The oxidative stress

94   biomarkers employed in the present study were utilized to determine the impact of a range of

95   xenobiotics on aquatic organisms (Al kaddissi et al., 2012; 2016; Cozzari et al., 2015; Elia et al.,

96   2017a; 2017 b; 2018; Magara et al., 2018). Previous studies utilizing these biomarkers to elucidate the

97 MP vector effect demonstrated a perturbation of intracellular antioxidant defenses (Oliveira et al.,

98 2013; Avio et al., 2015; Magara et al., 2018).

99   Previously Magara et al. (2018) investigated the influence of polyethylene microplastic beads

100 (PE MPs) on the accumulation and associated oxidative stress responses attributed to Flu in blue

101 mussels, *Mytilus edulis*. The mussels were exposed to 4 treatment groups: Flu-only, MP-only, Flu and

102 MP co-exposure, and Flu-incubated MP. Individual contaminant exposures to Flu or MP resulted in

103 varying responses, but co-exposures and incubated treatments (i.e. Flu was sorbed to plastic surfaces

104 prior to exposure) did not induce additive or synergistic responses. Further, MP-only exposure

105 appeared to be capable of eliciting direct effects on the oxidative stress system as evidenced by

106 enhanced activities of CAT and GPx (Magara et al., 2018).

107   Thus, the present study was designed to investigate the potential impact of PHB microplastics as a

108 single contaminant and in combination with Flu on the oxidative stress system of blue mussel (*Mytilus*

109 *edulis*), a well-regarded test species for MPs (Avio et al., 2015; Paul-Pont et al., 2016; Sahlmann et al.

110 2017; Von Moos, 2012). PE MPs, as an example of oil-based MPs, were employed as a comparison to

111 PHB MPs. The aim of the study was to examine the comparative changes on oxidative stress

112 biomarkers levels induced by oil based (PE) MPs and 'new' bioplastic PHB MPs as both single

113 contaminants and as potential vectors for PAHs.

114

Materials and methods

Mussels collection and maintenance

117   Blue mussel (*Mytilus edulis*) were collected in June, 2016, in Vellerup Vig – Isefjord, N 55°40.6'; E

118 11°48.7', in an non-polluted deep inlet of Baltic Sea near Roskilde (Denmark). The mussels were

119 maintained in a temperature-controlled room (10 ± 1°C). All specimens were placed in tanks (size 39 x

120 21 x 25 cm), filled with 10 L of field water. Field water was gradually replaced with lab water

121 maintaining similar salinity (20 salinity). Mussels were fed for the following three weeks twice weekly.

122 A 16:8 hr light/dark photoperiod was maintained throughout.

Chemicals and preparation

124 A primary stock solution of Flu (Fluka Chemika, Steinheim, Switzerland) was prepared at 1 mg/ml

125 in acetone. PE MPs were purchased from Cospheric LLC (Santa, Barbra, CA, USA) and PHB beads

126 provided by Prodotti Gianni S.R.L. (Milano, Italy). Both polymers were white in color and similar in

127 size (10–90 μm). Both MPs were treated with 0.1% Tween80 to enable dispersion in water column

128 (Khan et al., 2015).

129 To prepare each exposure treatment accurately a weight to MP number ratio was determined for

130 both PE and PHB particles. The weight associated to 1×10^6 MPs was weighed out for each treatment

131 group containing MPs. The MPs were dispersed in 5 ml of 0.1% Tween80 solution and shaken at 150

132 rpm overnight. The MPs were filtered through 1 μm nylon mesh and resuspended in 200 ml 20 salinity

133 water and kept shaking until the start of the experiment (150 rpm). MPs that required incubation with

134 Flu were resuspended into 20 ml acetone to which the appropriate amount of Flu stock was added to

135 yield the correct final concentration of 100 μg/L when made up to the final exposure solution. After an

136 initial mixing, dispersions were left under a fume hood for the acetone to evaporate and the Flu sorb to

137 the PE MPs. This method was adopted from use with other particulate contaminants (Al-Subiai et al.,

138 2012). When complete dryness was achieved overnight the Flu-incubated MPs were resuspended in

139 200 ml 20 salinity water and placed with the other dispersions on the shaking table overnight.

Mussels exposure

141 Mussels were exposed for 96 hr to 8 treatment groups as follows, (A) Control (no added

142 contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F)

143 PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs (n=4 per

144 treatment). The Flu and MP concentrations across all treatments were 100 μg/L and 1000 MPs/ml,

145 respectively. Although the concentrations exceeded environmental realistic conditions , the aim of this

146 study was to understand mechanistic responses that are more easily captured at higher exposure levels.

147 The concentrations used are consistent with previous studies (Al-Subiai et al.,2012; Cole et al., 2016;

148 Magara et al., 2018). Acetone and Tween80 were consistently added across treatments to prevent the

149 confounding influence of solvent and detergent. Ninety-six hr mussel exposures were conducted at

150 10±1°C and a 16/8 hr light/dark photoperiod. Mussels were individually exposed in aerated beakers

151 (250 ml) filled with 200 ml treatment solution. Four samples of two pooled mussels were exposed per

152 treatment group.

153 To start the experiment, each 200 ml dispersion was made up to 1 L with 20 salinity water and

154 vigorously stirred to ensure homogenous distribution of MPs in the water column. The 1 L dispersion

155 was then divided into 5 200 ml units again ensuring a homogenous distribution of MPs between

156 beakers. The appropriate amount of Flu stock was added to the Flu-only and co-exposures groups prior

157 to the start of the experiment. Post exposure (96 hr), mussels were removed and rinsed thoroughly

158 under 20 salinity water. Gill and digestive gland tissues were extracted from each individual and

159 placed in separate pre-weighed and pre-labelled vials. Tissues for biomarkers and Flu concentration

160 analysis were stored at -80°C and -20° C, respectively. No mortalities were recorded during the

161 experiments. .

Biochemical analysis

163 The analysis of biomarkers is described in detail elsewhere (Cozzari et al., 2015; Magara et al.,

164 2018). Briefly, total GSH levels as well as activities of SOD, CAT, GPx and SeGPx were measured in

165 cytosolic fraction of gills and digestive gland spectrophotometrically   (Varian Cary 50

166 spectrophotometer at 25°C). Protein concentrations in the cytosol were determined according to Lowry

167 et al. (1951) and used to normalize enzyme levels. All analyses were performed in triplicate along with

168 blank samples (buffer and reagents only). These absorbance values were subtracted from those of the

169   sample. SOD activity was determined according to an established method (McCord and Fridovich,

170   1969). Catalase levels were measured following the decrease in absorbance due to H_2O_2 consumption

171   at 240 nm (Greenwald, 1985). GPx activities (SeGPx and total GPx) were determined following the

172   oxidation of nicotinamide adenine dinucleotide phosphate reduced form (NADPH) at 340 nm

173   (Lawrence and Burk, 1976). GR activity was measured in 100 mM NaH2PO4 + Na2HPO4 buffer, pH

174   7, 1 mM oxidized glutathione (GSSG), and 60 μM NADPH; the decrease in absorbance due to the

175   oxidation of NADPH was measured at 340 nm (Chung et al. 1991). GST activity was determined in

176   100 mM NaH2PO4 + Na2HPO4 buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4 dinitrobenzene

177   (CDNB) as substrates and sample. Formation of the conjugate with GSH was read at 340 nm (Habig et

178   al. 1974). Total glutathione (GSH+2GSSG) was measured following homogenization in 5%

179   sulphosalicilic acid with 4mM EDTA by the GR recycling assay at 412 nm (Akerboom and Sies,

180   1981).

Statistical analysis

182   Data are reported as mean values ± the standard deviation (SD). Levene's test for normal distribution

183   was performed on datasets prior to statistical analysis. A one-way ANOVA with Bonferroni's multiple

184   comparison test was used to investigate differences between treatment groups (GraphPad Prism

185   software). Statistical significance was set at $P < 0.05$.

Results

187   In the gills, CAT activity levels significantly decreased in all treatment groups (up to 80%), except

188   for PE MPs only exposure compared to control (Figure 1C). The activity of SeGPx was markedly

189   lower (up to 90%) through the treatments (Figure 2C). GST levels in the treated groups were

190   significantly lower than control (up to 50%), except for Flu-incubated PHB MPs (Figure 3A). No

191   marked changes of SOD, GPx and GR activities were noted for all treatment compared to respective

192   controls (Figures 1A, 2A, 3C).

193   In the digestive glands, SOD activity was significantly reduced (up to 50%) in all treatments when

194   compared to control with the exception of the group incubated with to PE MPs only (Figure 1B). CAT

195   activity was markedly decreased similar to SOD (up to 60%)., However, no significant differences

196   were found in mussels exposed to PHB MPs only and Flu-incubated PHB MPs (Figure 1D). Total GPx

197   markedly fell (60%) following Flu exposure (Figure 2B). Levels of SeGPx activity were significantly

198   reduced compared to control (up to 50%), except for PE MPs only and Flu-incubated PHB MPs groups

199   (Figure 2D). GST activity peaked (1.5 fold higher) in Flu exposed group (Figure 3B), whereas GR

200   activity levels showed increasing trends in all treated groups. The GR activity was markedly higher

201   following PE MPs only exposure (1 fold), PE MPs-Flu co-exposure (1.2 fold) and Flu-incubated PHB

202   MPs treatment (1 fold, Figure 3D).

Discussion

204   The present study provides the first experimental evidence of stress-related effects initiated by

205   novel bio-microplastic exposure, either as a single contaminant or in combination with Flu, in the blue

206   mussel *Mytilus edulis.* The comparison was conducted with the more traditional oil-based plastic

207   polyethylene. Flu and both PE and PHB MPs modified the antioxidant responses in both gills and

208   digestive glands. The present results are largely in agreement with those previously reported for Flu

209   and PE MPs, both in single or combined exposures (Magara et al., 2018).

210   PE MPs altered the levels of several of the tested biomarkers. Although previous studies showed

211   that oil-based plastic led to enhanced antioxidant activities in copepods (Jeong et al., 2017) and fish

212   (Barboza et al., 2018), our results are in agreement with studies on mussels, showing a significant

213   inhibition of CAT, SeGPx and GST activity following oil-based MPs mussel exposure (Avio et al.,

214 2015; Paul-Pont et al., 2016). A decrease of CAT and GPx activities in digestive glands and of SeGPx

215 and GST in gills exposed to PE MPs may be related to the size and shape of microplastics, that play a

216 key role in initiation of biological changes (Browne et al., 2008; Von Moos, 2012; Avio et al., 2015).

217 Small polystyrene plastic particles may be translocated from the gut to the circulatory system of

218 *Mytilus edulis* and subsequently retained suggesting that these substances y might reach crucial organs

219 such as the heart or hepatic tissues to produce adverse effects (Browne et al., 2008). It is well known

220 that oil-based microplastics may accumulate within organisms and induce tissue abrasions, as

221 evidenced by histological changes in digestive cells and triggered inflammatory responses, formation

222 of granulocytomas and lysosomal destabilization (Avio et al., 2015; Von Moos, 2012). Therefore, in

223 this scenario, the imbalance of antioxidant defense markers might be expected and might be

224 attributed to physical damage induced by MPs enabling further accumulation of reactive products.

225 Further, in the present study increased GR activity in digestive glands might reflect the demand for

226 GSH as defense mechanism against oxidative stress induced by oil-based MPs (Jeong et al., 2016).

227 The present study also carried out a co-exposure and incubation scenario of both PE and PHB MPs

228 with Flu and reported information on oxidative stress related to the simultaneous exposure to

229 compounds. In this scenario, a mussel experimental group exposed to Flu-only was used as positive

230 control. Previously Pan et al (2005) found that at high benzo(k)fluoranthene, a PAH, concentrations the

231 activity of antioxidant enzymes was diminished in the scallop *Chlamys farreri* in a time-dependent

232 manner. Our findings that the marked decrease of SOD activity levels and both GPx in digestive

233 glands, as well as SeGPx in gills and CAT activity in both tissues indicate that this Flu may exert

234 detrimental effects when administered alone resulting in a severe oxidative stress in *Mytilus edulis*.

235 The impairment of antioxidant biomarkers levels may be attributed to a chemical-mediated damage.

236 Indeed, the enhanced GST activity measured in digestive glands might represent an important

237 outcome, since this indicate elevated of phase-II biotransformation metabolism. The significant

238 induction of GST activity in mussels exposed to Flu-only may be attributed to production of specific

239　substrates for GST by phase I enzymes, representing an effective defense line against Flu. Babson et al

240　(1986) showed that rat liver microsomes converted Flu to trans-2,3-dihydrodiol as the major

241　metabolite. Although data regarding Flu metabolites in blue mussels are lacking, it is suggested that

242　GST induction may be related to production of this intermediate.

243　　Biomarkers responses in digestive glands and gills of mussels exposed to both Flu and PE or PHB

244　MPs (co-exposure and incubated treatment) generally followed a trend similar to PAH alone or the

245　new plastic alone and no combined effects of these agents was apparent. The absence of combined

246　effects in *Mytilus edulis* exposed to PE MPs and Flu is in agreement with our previous results

247　(Magara et al., 2018), suggesting that MPs may act as a "sink" of environmental pollutants (Chua et al.

248　2014; Khan et al. 2015). Chua et al. (2014), showed that the concentration of polybrominated diphenyl

249　ethers (PBDE) was lower in *Allorchestes compressa* co-exposed to microplastics then specimens

250　treated with PBDE alone. Chua et al (2014) concluded that the presence of MPs may inhibit the

251　uptake of PBDE, perhaps because this contaminant is strongly absorbed onto microplastics surface

252　making it less bioavailable. It is already known that oil-based micropolymers have the propensity to

253　aggregate in water (Alimi et al., 2018) . Previously Khan et al (2015) found in zebrafish a decrease in

254　silver (Ag) uptake by fish attributed to diminished contact between Ag and tissues following oil-based

255　MP aggregation. Further, Magara et al. (2018) reported that Flu tissue concentrations were lower in

256　*Mytilus edulis* exposed to both co-exposure with polyethylene and PAH compared to specimens

257　treated with Flu alone. The absence of combined effects of PE MPs and Flu in *Mytilus edulis* and

258　previous results on Flu uptake in blue mussel (Magara et al., 2018), suggests that the interaction of

259　tissues with Flu might be delayed by this aggregation mechanism exerted by microplastics.

260

261　　Bioplastics are currently becoming the leading material for replacing oil-based polymers since it is

262　more desirable than traditional plastic due to a propensity to biodegrade in the environment (Anjum et

263　al., 2016). Recently Napper and Thompson (2019) demonstrated that biodegradable polymers may

not undergo any substantial deterioration over a 3 year period in marine environment, but may be reduced in small fragments similar to oil-based plastics. Our data suggest that PHB MPs results in altered levels of some oxidative stress biomarkers similar to oil-based MPs. The fact that bio-microplastics may exert stress effects and the potential low biodegradability rate indicate a novel threat for the marine environment. Therefore, it is crucial to understand degradation processes of bioplastics in the environment, but also gathering knowledge regarding   potential ecotoxicological impacts. Although the damage exerted by oil-based polymers is becoming increasingly understood, it is still necessary to investigate the possible consequences of bioplastics exposure both alone and in combination with other environmental pollutants present in the aquatic ecosystem.

Conclusions

   The results of the present study demonstrated that PHB MPs modify the baseline levels of biomarkers related to oxidative stress in *Mytilus edulis*. These alterations were similar to those exerted by PE MPs treatments for some of the antioxidant biomarkers . Further, the responses measured in both co-treatments were similar to those noted with MP-alone exposures. Data indicated the absence of any combined effects produced by oil-based MPs or bioplastics MPs and Flu.

Acknowledgements

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Legend of Figures

Figure 1. SOD (A and B) and CAT (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 2. GPx (A and B) and SeGPx (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 3. GST (A and B) and GR (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs.

 Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test).

1 Declarations of interest: none.

Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (*Mytilus edulis*)

Gabriele Magara[a], Farhan R. Khan[b]*, Marika Pinti[a], Kristian Syberg[b], Angelo Inzirillo[a] and Antonia Concetta Elia[a]*

a Department of Chemistry, Biology and Biotechnology, via Elce di sotto 8, University of Perugia, 06123 Perugia, Italy

b Department of Science and Environment, Universitetsvej 1, P.O. Box 260, Roskilde University, DK-4000 Roskilde, Denmark

Running title: Bioplastics as a vector for environmental pollutants

* Corresponding author: Dr. Antonia Concetta Elia

Department of Chemistry,

Biology and Biotechnology,

University of Perugia, Italy

Email: (antonia.elia@unipg.it

Dr. Farhan R. Khan

Department of Science and Environment,

Roskilde University, Denmark

Email: frkhan@ruc.dk, farhan.khan@gmx.com

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ABSTRACT

A growing interest in developing and commercialization of ~~ing~~ new ecofriendly plastic polymers is occurring attributed ~~owing~~ to the impact of marine plastics debris and microplastics that result from the degradation of oil-based polymers as these substances adversely affect ecosystem health. Recently, polyhydroxybutyrate (PHB) has become of interest due its biodegradability and physicoal ~~al~~-chemical properties. However, biological consequences~~impacts~~ resulting from bioplastics exposure remains to be determined.~~is yet unknown.~~ Moreover~~Further~~, few data are apparently available regarding ~~nothing is known about~~ the potential for bioplastics to act as a vector for exogenous chemicals in the environment. The aim of the study was to compare the effects of polyethylene (PE MPs) and polyhydroxybutyrate (PHB MPs) microplastics administered alone~~singularly~~ or in combination with fluoranthene (Flu) on detoxifying enzymes in digestive glands and gills of *Mytilus edulis*. Blue mussels were exposed for 96hr ~~r~~ to 8 ~~eight~~ experimental groups: control, Flu-only, PE MPs-only, PHB MPs-only, PE MPs-Flu co-exposure, PHB MPs-Flu co-exposure, Flu-incubated PE MPs and Flu-incubated PHB MPs. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), glutathione S-transferase (GST) and glutathione reductase (GR) were found~~have proved~~ to be significantly susceptible to Flu and plastics in both tissues. Interestingly, single exposure to PHB MPs ~~single exposure~~ led to decreased levels of CAT and GST in gills, SOD in digestive glands and SeGPx in both tissues ~~biomarkers biomarkers~~ STATE INCREASE OR DECREASE?~~in SOD, CAT, etc.impairments~~. In co-exposure and incubation treatments, biochemical responses were generally comparable with those exerted by PE MPs or PHB MPs only, suggesting an apparent absence of ~~no~~ combined effects with pollutant. ~~The present findings are amongst the first to describe~~ Data demonstrate the ecotoxicological ~~potential~~ impact of bioplastics materials on digestive glands and gills of *Mytilus edulis*.

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48 **Keywords**: biodegradable polymer, biomarker profile, bioplastics, blue mussels, microplastic

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Introduction

52   Oil-based plastic debris and subsequently the microplastics (MPs) formed by their degradation are

53 one of the current major biggest environmental concerns. issues of the day. These plastics are defined

54 chemically as organic or semi-organic materials with long chains (macromolecules) and high molecular

55 weight, generated by the polymerization of monomers extracted from oil or gases (Cole et al., 2011).

56 Once in seawater, microplastics were have been shown to be ingested by aquatic species which resulted

57 in can lead to a number of deleterious effects (Guzzetti et al., 2018; Alimba and Faggio, 2019) (NEED

58 REF??). The color, density, shape and size of MPs often leads marine organisms to mistake MPs for

59 food items and the consequences of this includes, blockage of the digestive system and false feeling of

60 satiation (Wright et al., 2013). In addition, , and also MPs may act as a vehicle for the transport of

61 several contaminants dissolved in the aquatic environment (Andrady, 2011). This 'vector effect'

62 (Syberg et al., 2015), suggests that oil-based MPs may trap and transport environmental pollutants

63 including, such as persistent organic pollutants (POPs) or metals (Oliveira et al., 2013; Avio et al.,

64 2015; Khan et al., 2015; Magara et al., 2018). The possibility that plastics might can then transport the

65 adhered contaminants into aquatic biota is currently being still debated (Koelmans et al., 2016), and but

66 laboratory investigations into the vector effect remain ongoing.

67   There is a mounting interest in "green materials" and a focus on the development and production of

68 biodegradable polymers (Mohanty et al., 2002). In contrast toUnlike oil-based polymers, these

69 biopolymers are derived from bioprocesses, using renewable resource in bio-refineries., and dDue to

70 their mechanical and physicochemical physical characteristics, these biodegradable polymers may be

71 considered as an eco-friendly substitutes to plastic (Anjum et al., 2016). In recent years,

72   polyhydroxybutyrate (PHB) has received ing growing interest for its characteristics similar to oil-based

73   plastic (NEED REF??)(Dacosta et al., 2016). It Polyhydroxybutyrate is a water insoluble, but highly

74   biodegradable biopolymer, relatively resistant to hydrolytic degradation which . It is extracted with

75   chloroform from bacterial cultures which is grown on carbohydrates. PHB (1) displayshas low oxygen

76   permeability and reliable good thermoplasticity with poor mechanical properties, (2) high crystallinity

77   degree and (3) is optically active, with a mass about approximately 0.5×10^6 Da (Anjum et al., 2016).

78   However, although PHB is currently available commercially, especially as main component of

79   biodegradable market bags, to the our best of our knowledge little is known regarding about it is the

80   environmental impact or effects on aquatic organisms. By sharing many characteristics with oil-based

81   plastics, it is feasible that in the aquatic environment, bioplastic undergoes modifications, resulting in

82   leading to generation of MPs as is the case for oil-based polymers (Andrady, 2011). If due to

83   environmental and societal pressures a move is made towards use of bioplastics, then it is vital to

84   gather information on the influence of these bioplastics on the ecosystem health.

85   Oxidative stress results from an imbalance between pro-oxidants, such as reactive oxygen species

86   (ROS), and the protective antioxidant system. Mechanisms that involve glutathione (GSH), and the

87   related antioxidant enzymes s superoxide dismutase (SOD), catalase (CAT), and glutathione

88   peroxidases (SeGPx and GPx) represent important protective metabolic pathways which and are used

89   as biomarkers related to pollutant induced oxidative stress. Glutathione (GSH) in its reduced form is an

90   important non-enzymatic scavenger of oxyradicals, and is involved in the metabolism of toxic

91   compounds and endogenous substances (Meister and Anderson, 1983). Both SOD and CAT enzymes

92   catalyze the breakdown of ROS-generatingcausing O_2^- and H_2O_2, respectively and are key components

93   within the primary defense system against oxidative stress-induced damage. SeGPx enzyme can

94   reduces either hydrogen peroxide or organic peroxides and GPx enzyme affects organic

95   hydroperoxides. Polycyclic aromatic hydrocarbons (PAHs) , of which fluoranthene (Flu) is often used

96   as a model contaminant (and in addition a priority aquatic pollutant (Directive 2008/105/EC)), are

97   known to initiategenerate oxidative stress which was has been assessed by these aforementioned

98   biomarkers (Cheung et al., 2001). The oxidative stress biomarkers employed used in the present study,

99   werehave been utilized to determine assess the impact of a range of xenobiotics on aquatic organisms

100 (Al kaddissi et al., 2012;, 2016; Cozzari et al., 2015; Elia et al., 2017a,; 2017 b,; 2018; Magara et al.,

101 2018). Previous studies utilizing these biomarkers to elucidate the MP vector effect have demonstrated

102 a perturbation of intracellular antioxidant defenses (Oliveira et al., 2013; Avio et al., 2015; Magara et

103 al., 2018).

104 Our p Previously study (Magara et al.,(2018) investigated the influence of polyethylene microplastic

105 beads (PE MPs) on the accumulation and associated oxidative stress responses attributed to

106 fluoranthene (Flu) in blue mussels, *Mytilus edulis*. The mussels were exposed to 4 four treatment

107 groups: Flu-only, MP-only, Flu and MP co-exposure, and Flu-incubated MP. Individual contaminant

108 exposures to Flu or MP resulted inled to varying responses, but co-exposures and

109 WHAT IS THISincubated ???DO YOU MEAN *IN VITRO*?treatments (i.e. Flu was sorbed to plastic

110 surfaces prior to exposure)exposures did not induce result in additive or synergistic responses.effects.

111 Moreover Further, MP-only exposure appeared to be capable of eliciting direct effects on the oxidative

112 stress system as evidenced demonstrated by the enhanced activities of CAT and GPx (Magara et al.,

113 2018).

114 If due to environmental and societal concerns a move is made towards bioplastics, then it is vital to

115 gather information on the impacts of the bioplastics. This includes the possibility of bioplastics as

116 vectors of other pollutants.

117 ___Thus, the present study was designed to investigate the potential impact of PHB microplastics as a

118 single contaminant and in combination with fluoranthene (Flu), a representative PAH, on the oxidative

119 stress system of blue mussel (*Mytilus edulis*), a well-regarded test species for MPs (Avio et al., 2015;

120 Paul-Pont et al., 2016; Sahlmann et al 2017; Von Moos, 2012). PE MPs, as an example of oil-based

121 MPs, were employed asused a comparison to the PHB MPs. The aim of the study was to examine the

comparative changes on oxidative stress biomarkers levels induced by oil based (PE) MPs and 'new'

bioplastic PHB MPs as both single contaminants and as potential vectors for PAHs.

~~SHIFT TO METHODSMussels were exposed for 96 h to seven treatment groups; Control (no added~~

~~contaminants), Flu only, PE MPs only, PHB MPs only, PE MPs-Flu co-exposure, PHB MPs-Flu co-~~

~~exposure, Flu-incubated PE MPs and Flu-incubated PHB MPs. !!~~

~~The aim of the study was to investigate the comparative changes on oxidative stress biomarkers levels~~

~~induced by oil based (PE) MPs and 'new' bioplastic PHB MPs as both single contaminants and as~~

~~potential vectors for PAHs.~~

Materials and methods

Mussels collection and maintenance

Blue mussel (*Mytilus edulis*) were collected in June, 2016, in Vellerup Vig – Isefjord, N 55°40.6'; E

11°48.7', in an ~~un~~ non-polluted deep inlet of Baltic Sea near Roskilde (Denmark). The mussels were

maintained ~~located~~ in a temperature-controlled room (10 ± 1°C). All specimens were placed in tanks

(size 39 x 21 x 25 cm), filled with 10 L of field water. Field water was gradually replaced with

lab~~oratory~~ water maintaining similar salinity (20 salinity). Mussels were fed for the following three

weeks twice weekly. A 16:8 h~~r~~ light/dark photoperiod was maintained throughout.

Chemicals and preparation

A primary stock solution of Flu (Fluka Chemika, Steinheim, Switzerland) was prepared at 1 mg/ml ~~L~~

in acetone. PE MPs were purchased from Cospheric LLC (Santa, Barbra, CA, USA) and PHB beads

~~were~~ provided by Prodotti Gianni S.R.L. (Milano, Italy). Both polymers were white in color and similar

in size (10–90 μm). Both MPs were treated with 0.1% Tween80 to enable ~~allow~~ dispersion in water

column (Khan et al., 2015).

146   To prepare each exposure treatment ~~correct~~ accurately a weight to MP number ratio was determined

147   for both PE and PHB particles. The weight associated to 1×10^6 MPs was weighed out for each

148   treatment group containing MPs. The MPs were dispersed in 5 ml of 0.1% Tween80 solution and

149   shaken at 150 rpm overnight. The MPs were filtered through 1 μm nylon mesh and resuspended in 200

150   ml ~~L of~~ 20 salinity water and kept shaking until the start of the experiment (150 rpm). MPs that

151   required incubation with Flu were resuspended into 20 ml ~~L of~~ acetone to which the appropriate

152   amount of Flu stock was added to yield~~give~~ the correct final concentration of 100 μg/-L~~⁻¹~~ when made

153   up to the final exposure solution. After an initial mixing, dispersions were left under a fume hood for

154   the acetone to evaporate and the Flu sorb to the PE MPs. This method was adopted from use with other

155   particulate contaminants (Al-Subiai et al., 2012). ~~Having achieved~~ When complete dryness was

156   achieved overnight the Flu-incubated MPs were resuspended in 200 ml~~L of~~ 20 salinity water and ~~were~~

157   placed with the other dispersions on the shaking table overnight.

Mussels exposure

159   Mussels were exposed for 96 hr to 8 ~~eight~~ treatment groups as follows, (A) Control (no added

160   contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F)

161   PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs (n=4 per

162   treatment).~~(1) Control (no added contaminants), (2) Flu only, (3) PE MPs only, (4) PHB MPs only, (5)~~

163   ~~PE MPs-Flu co-exposure, (6) PHB MPs-Flu co-exposure, (7) Flu-incubated PE MPs and (8) Flu-~~

164   ~~incubated PHB MPs.~~ The Flu and MP concentrations across all treatments were ~~as~~ 100 μg/-L~~⁻¹~~ and

165   1000 MPs/-ml~~L⁻¹~~, respectively. Although the concentrations exceeded environmental realistic

166   conditions ~~m~~, the aim of this study was to understand mechanistic responses that are more easily

167   captured at higher exposure ~~concentration~~ levels. The concentrations used are consistent with previous

168   studies (Al-Subiai et al.,2012; Cole et al., 2016; Magara et al., 2018). Acetone and tTween80 were

169   consistently added across treatments to prevent the confounding influence ~~mpact~~ of solvent and

170 detergent. Ninety-six hr our mussel exposures were conducted at 10±1°C and a 16/8 hr light/dark

171 photoperiod. Mussels were individually exposed in aerated beakers (250 mlL) filled with 200 mlL of

172 exposuretreatment solution. Five Four samples of two pooled mussels were exposed per treatment

173 group.

174   To start the experiment, each 200 ml L dispersion was made up to 1 L with 20 salinity water and

175 vigorously stirred to ensure the homogenous distribution of MPs in the water column. The 1 L

176 dispersion was then divided split into 5 five 200 ml units L exposures, again ensuring a homogenous

177 distribution of MPs between beakers. The appropriate amount of Flu stock was added to the Flu-only

178 and co-exposures groups prior to before the start of the experiment. Post exposure (96 hr), mussels

179 were removed and rinsed thoroughly under 20 salinity water. Gill and digestive gland tissues were

180 extracted dissected from each individual and placed in separate pre-weighed and pre-labelled vials.

181 Tissues for biomarkers and Flu concentration analysis were stored at -80°C and -20° C, respectively.

182 No mortalities were recorded during the experiments. osure.

Biochemical analysis

184   The analysis of biomarkers is described in detail elsewhere (Cozzari et al., 2015; Magara et al.,

185 2018). Briefly, total GSH levels glutathione, as well as activities of SOD, CAT, GPx and SeGPx were

186 measured in cytosolic fraction of gills and digestive gland by spectrophotometrically analysis (Varian

187 Cary 50 spectrophotometer at 25°C). Protein concentrations in the cytosol were determined according

188 to Lowry et al. (1951) and were used to normalize enzyme levels. All analyses were performed in

189 triplicate along with blank samples (buffer and reagents only). These absorbance values were

190 subtracted from those of the sample. SOD activity was determined according to an established method

191 (McCord and Fridovich, 1969). Catalase levels were measured following the decrease in absorbance

192 due to H_2O_2 consumption at 240 nm (Greenwald, 1985). GPx activities (SeGPx and total GPx) were

193 determined following the oxidation of nicotinamide adenine dinucleotide phosphate reduced form

194 (NADPH) at 340 nm (Lawrence and Burk, 1976). GR activity was measured in 100 mM NaH2PO4 +

195 Na2HPO4 buffer, pH 7, 1 mM oxidized glutathione (GSSG), and 60 μM NADPH; the decrease in

196 absorbance due to the oxidation of NADPH was measured at 340 nm (Chung et al. 1991). GST activity

197 was determined in 100 mM NaH2PO4 + Na2HPO4 buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4

198 dinitrobenzene (CDNB) as substrates and sample. Formation of the conjugate with GSH was read at

199 340 nm (Habig et al. 1974). Total glutathione (GSH+2GSSG) was measured following homogenization

200 in 5% sulphosalicilic acid with 4mM EDTA by the GR recycling assay at 412 nm (Akerboom and Sies,

201 1981).

Statistical analysis

203 ___Data are reported as mean values ± the standard deviation (SD). Levene's test for normal distribution

204 was performed on datasets prior to statistical analysis. A one-way ANOVA with Bonferroni's multiple

205 comparison test was used to investigate differences between treatment groups (GraphPad Prism

206 software). Statistical significance was set at $P < 0.05$.

Results

208 ___In the gills, CAT activity levels significantly decreased in all treatment groups (up to 80%), except

209 for PE MPs only exposure when compared to control (Figure 1C). The activity of SeGPx was markedly

210 consistently lower (up to 90%) through the treatments (Figure 2C). GST levels in the treated groups

211 were significantly lower than control (up to 50%), unless exposed toexcept for –Flu-incubated PHB

212 MPs (Figure 3A). No marked changes of SOD, GPx and GR activities y were notedrecorded for all the

213 treatment compared to respectivethe own controls (Figures 1A, 2A, 3C).

214 ___In the digestive glands, SOD activity concentrations were was significantly reduced consistently

215 lowered (up to 50%) in all treatments when compared to control with the exception of the group

216 incubated withexposed to PE MPs only (Figure 1B). CAT activity was markedly decreased lowered

217 similar~~ly~~ to SOD (up to 60%).~~,~~ ~~but no statistically~~ However, no significant differences were found
218 ~~recorded~~ in mussels exposed to PHB MPs only and Flu-incubated PHB MPs (Figure 1D). Total GPx
219 markedly fell ~~dropped~~ (60%) following Flu exposure (Figure 2B). Levels of SeGPx activity were
220 ~~reliably lower~~ significantly reduced compared to ~~than~~ control (up to 50%), except ~~unless~~ for PE MPs
221 only and Flu-incubated PHB MPs groups (Figure 2D). GST activity peaked (1.5 fold higher) in Flu
222 exposed group (Figure 3B), whereas GR activity levels showed increasing trends in all treated groups.~~;~~
223 ~~t~~The GR activity was markedly higher following PE MPs only exposure (1 fold), PE MPs-Flu co-
224 exposure (1.2 fold) and Flu-incubated PHB MPs treatment (1 fold, Figure 3D).

Discussion

226 　　　The present study provides the first experimental evidence~~s~~ of ~~the~~ stress-related effects
227 initiated~~caused~~ by novel bio-microplastic exposure, either as a single contaminant or in combination
228 with Flu, in ~~to~~ the blue mussel *Mytilus edulis*. The comparison was conducted~~made~~ with the more
229 traditional oil-based plastic polyethylene. Flu and both PE and PHB MPs modified the antioxidant
230 responses in both gills and digestive glands. The present results are largely in agreement ~~line~~ with those
231 previously reported for Flu and PE MPs, both in single or combined exposures (Magara et al., 2018).
232 　　　PE MPs altered the levels of several of the tested biomarkers. Although previous studies showed
233 that oil-based plastic led to enhanced antioxidant activities in copepods (Jeong et al., 2017) and fish
234 (Barboza et al., 2018), our results are in agreement with studies on mussels~~This outcome is in~~
235 ~~agreement line with previous studies~~, showing a significant inhibition of CAT, SeGPx and GST activity
236 following oil-based MPs mussel exposure (Avio et al., 2015; Paul-Pont et al., 2016). A ~~D~~ decrease of
237 CAT and GPx activities ~~-~~in digestive glands and of SeGPx and GST in gills exposed to PE MPs may be
238 related to the size and shape of microplastics, that ~~can~~ play a key role in initiation of ~~on promote~~
239 biological changes (Browne et al., 2008; Von Moos, 2012; Avio et al., 2015). Small polystyrene plastic
240 particles may ~~can~~ be translocated from the gut to the circulatory system of *Mytilus edulis* and

241 subsequently ~~be~~ retained~~,~~ suggesting that the~~se substances~~ y might ~~can~~ reach crucial organs such as the

242 heart or ~~the~~ hepatic tissues to produce adverse effects~~and then hurt~~ (Browne et al., 2008). It is well

243 known that oil-based microplastics may accumulate within organisms and induce ~~cause~~ tissue

244 abrasions, as evi~~de~~nced by histological changes in digestive cells and triggered inflammatory

245 responses, formation of granulocytomas and lysosomal destabilization (Avio et al., 2015; Von Moos,

246 2012). Therefore, in this scenario, the imbalance of antioxidant defense markers might be expected ~~is~~

247 ~~not surprising~~ and ~~can~~ might perhaps be attributed to ~~the~~ physical damage induced by MPs enabling

248 further accumulation of reactive products.   ~~leading to a slight accumulation of reactive products.~~

249 Further~~more~~, in the present study ~~the~~ increased GR activity in digestive glands might reflect the ~~can~~

250 ~~sustenance the rising~~ demand for ~~of~~ GSH as defense mechanism against oxidative stress induced

251 ~~caused~~ by oil-based MPs (Jeong et al., 2016).

252 ~~   THIS IS RESULTS! DELETEResults from this study described, for the first time, that exposure to~~

253 ~~bioplastic PHB MPs also showed impairment of antioxidant biomarkers. Activity of SOD decreased~~

254 ~~only in digestive glands, CAT in gills and Se-GPx in both tissues.~~

255 ~~NO EVIDENCE FOR INFLAMMATION DELETEAs for PE MPs, we may hypothesize a mechanic~~

256 ~~hurt, following inflammatory response. However, because of the biodegradability potential of PHB,~~

257 ~~chemical damages cannot be excluded. Indeed, it is feasible that potential toxic compounds have been~~

258 ~~generated during biopolymer degradation process. At our best knowledge, no studies have been carried~~

259 ~~out on the metabolism of PHB in marine mussels.METABOLISM NOT MEASURED Though, it is~~

260 ~~known that the PHB degradation rate is in the order of few months in anaerobic environment, and years~~

261 ~~in seawater (Madison and Huisman, 1999; Verlinden et al., 2007). Within the organisms, PHB has a~~

262 ~~degradation rate, albeit low, due to its physical-chemical characteristics, such as the high crystallinity~~

263 ~~(Zinn et al., 2001; Anjum et al., 2016). Therefore, we may suppose that during the 96h trial, *Mytilus*~~

264 ~~*edulis* has start to degraded PHB MPs and a higher hydroxybutyrate tissue concentration occurred,~~

265　leading to a triggered ROS production in combination with physical damage.YOU DID NOT

266　MEASURE THIS DELETE

267

268

269　　　The present study also carried out a co-exposure and incubation scenario of both PE and PHB MPs

270　with Flu and reportedthe results have provided information on oxidative stress related to the

271　simultaneous exposure to compounds. In this scenario, a mussel experimental group exposed to Flu-

272　only was used as positive control. Previously Pan et al (2005) found that at high benzo(k)fluoranthene,

273　a PAH, concentrations the activity of antioxidant enzymes WHICH STATE?? was diminished in the

274　scallop *Chlamys farreri* in a The time--dependent manner. course tendency of antioxidant enzymes to

275　restrain activities at high benzo(k)fluoranthene concentrations was already reported on scallop *Chlamys*

276　*farreri* (Pan et al., 2005). Accordingly, Our findings that the marked decrease of SOD activity levels

277　and both GPx's in digestive glands, as well as of SeGPx also in gills and CAT catalase activity in both

278　tissues indicate suggests that this Flu PAH may exert detrimental effects when singularly administered

279　alone resulting in, causing a severe oxidative stress oin *Mytilus edulis*. The impairment of antioxidant

280　biomarkers levels may be attributed to a chemical-mediated damage. Indeed, the enhanced triggered

281　GST activity measured in digestive glands might represent an important outcome, since this may

282　indicate elevateda strengthening of phase-II biotransformation metabolism. The significant induction of

283　GST activity in mussels exposed to Flu-only maycan be attributed explained due to the production of

284　specific substrates for GST by phase I enzymes, representing an effective defense line against

285　Flu.fluoranthene. Babson et al (1986) showed that rat liver microsomes converted Flu to trans-2,3-

286　dihydrodiol as the major metabolite. Metabolism study on rat liver microsomes showed that Flu is

287　converted to trans-2,3-dihydrodiol as the major metabolite (Babson et al., 1986). Although data

288　regarding about Flu metabolites in blue mussels are is lacking, we can it is suggested that GST

289　induction may be related to the production of this intermediate.

290 ~~However, b~~Biomarkers responses in digestive glands and gills of mussels exposed to both Flu and

291 PE or PHB MPs (co-exposure and incubated treatment) generally followed a trend similar to ~~the~~ PAH

292 alone ~~own~~ ~~traditional~~ ~~and~~ or the new plastic alone and no combined effects of these agents was

293 apparent. The absence of combined effects in *Mytilus edulis* exposed to PE MPs and Flu is in

294 agreement ~~consistent~~ with ~~the results of our previous study~~ our previous results (Magara et al., 2018)~~.~~

295 ~~This particular outcome may~~ could be related to a ~~MAKES NO SENSE retention mechanism?? of Flu~~

296 ~~exerted by PE MPs, due to MAKES NO SENSE specific physical properties~~, suggesting that MPs may

297 act as a "sink" of environmental pollutants (Chua et al. 2014; Khan et al. 2015). Chua et al. (2014),

298 showed that the concentration of ~~P~~polybrominated ~~D~~diphenyl ~~E~~ethers (PBDE) was lower in

299 *Allorchestes compressa* co-exposed to microplastics then specimens treated with ~~single~~ PBDE alone.

300 ~~The authors~~ Chua et al (2014) concluded that the presence of MPs may inhibit the uptake of PBDE,

301 perhaps because this contaminant is strongly absorbed onto microplastics surface~~,~~ making it less

302 bioavailable.~~ (Chua et al., 2014).~~ It is already known that oil-based micropolymers have the propensity

303 to aggregate in water (Alimi et al., 2018) ~~(NEED REF??)~~. ~~A p~~Previously ~~study~~ Khan et al (2015) found

304 ~~o~~in zebrafish ~~showed~~ a decrease in silver (Ag) uptake by fish attributed to ~~, probably due~~ to diminished

305 ~~a minimization of the~~ contacts~~s~~ between ~~the~~ Ag and tissues following oil-based MP aggregation.~~ (Khan~~

306 ~~et al., 2015).~~ Further~~more~~, Magara et al. (2018) reported that Flu tissue~~s~~ concentration~~s~~ w~~ere~~ ~~as~~ lower

307 in *Mytilus edulis* exposed to both co-exposure with ~~and incubated~~ polyethylene and PAH compared to

308 specimens treated with Flu alone. ~~fluoranthene only~~. The absence of combined effects of PE MPs and

309 Flu in *Mytilus edulis* ~~exposed to~~ ~~PE MPs, either in co-exposure or incubated treatment,~~ and previous

310 results on Flu uptake in ~~B~~blue mussel (Magara et al., 2018), suggests that the interaction of tissues with

311 Flu might ~~could~~ be delayed by this aggregation mechanism exerted by microplastics.

312 ~~THIS WAS NOT MEASURED, DELETE~~ ~~Concerning the biodegradable PHB polymer, biomarkers~~

313 ~~responses surprisingly allow to suppose characteristics similar to polyethylene, although knowledge on~~

314   PHB retention or aggregation properties are not available However, due to its ability to degrade in

315   tissues, further studies are needed to gather knowledge on the pro-oxidant role of bio-microplastic.

316

317   The results of the present research show for the first time a role of bioplastics in affecting antioxidant

318   and THIS WAS NOT DONEdetoxifying pathways.

319   ____Bioplastics are currently becoming the leading material for replacing oil-based polymers since it is

320   more desirable than traditional plastic due to a its propensity to biodegrade in the environment (Anjum

321   et al., 2016). A rRecenty Napper and Thompson (2019) study demonstrated that biodegradable

322   polymers may could not undergo any substantial deterioration over a 3 year period in marine

323   environment, but may be reduced in small fragments , as is similar to for oil-based plastics (Napper and

324   Thompson, 2019). Our data suggest that PHB MPs results in may lead to altered levels of some

325   oxidative stress biomarkers, again which is similar to oil-based MPs. The fact that bio-microplastics

326   may exert stress effects and the potential low biodegradability rate may indicate a novel threat for the

327   marine environment. Therefore, it is crucial to understand the degradation processes of bioplastics in

328   the environment, but whilst also gathering knowledge regarding about their potential ecotoxicological

329   impacts. Whilst Although the damage exerted by oil-based polymers is becoming increasingly

330   understood, it is still now necessary to investigate the possible consequences impacts of bioplastics

331   exposure, both alone and in combination with other environmental pollutants present in the aquatic

332   ecosystem.

Conclusions

333

334   __The results of the present study demonstrated suggest that PHB MPs may modify the baseline levels

335   of biomarkers related to oxidative stress in *Mytilus edulis*. These levels of alterations were similar to

336   those exerted by PE MPs treatments for somefew of the antioxidant detoxifying biomarkers.

337   Furthermore, the responses measured in both co-treatments were similar to those noted with of MP-

338   aloneonly exposures. Data indicated This suggests the absence of any no combined effects produced

caused by oil-based MPs or bioplastics MPs and Flu. ~~fluoranthene. Further studies are required to~~

~~gather information on the potential consequences of new and innovative bioplastic polymer in the~~

~~environment.~~

Acknowledgements

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Legend of Figures

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Figure 1. SOD (A and B) and CAT (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

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Figure 2. GPx (A and B) and SeGPx (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F)

PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show

mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-

way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 3. GST (A and B) and GR (C and D) activity in gills (A and C) and digestive gland (B and D)

of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added

contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F)

PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs.

 Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P <$

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Figure 2. GPx (A and B) and SeGPx (C and D) activity in gills (A and C) and digestive gland (B and

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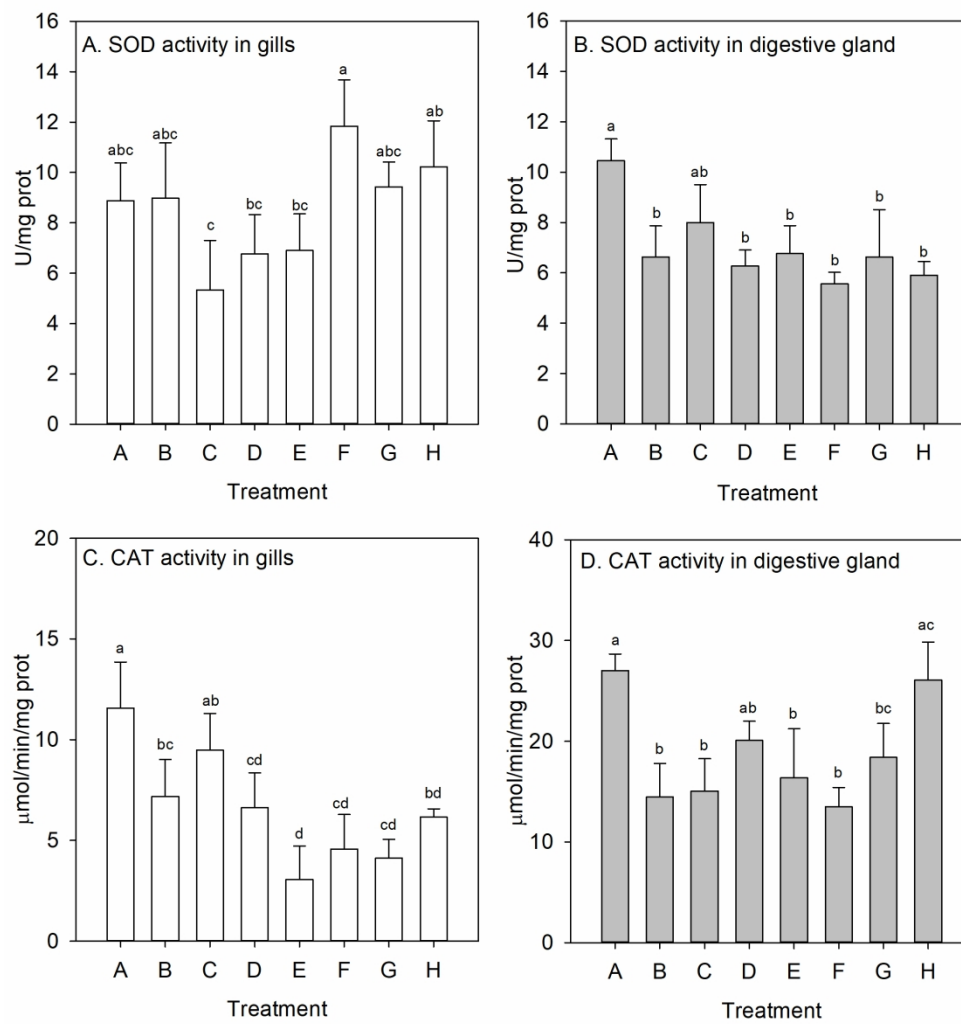48
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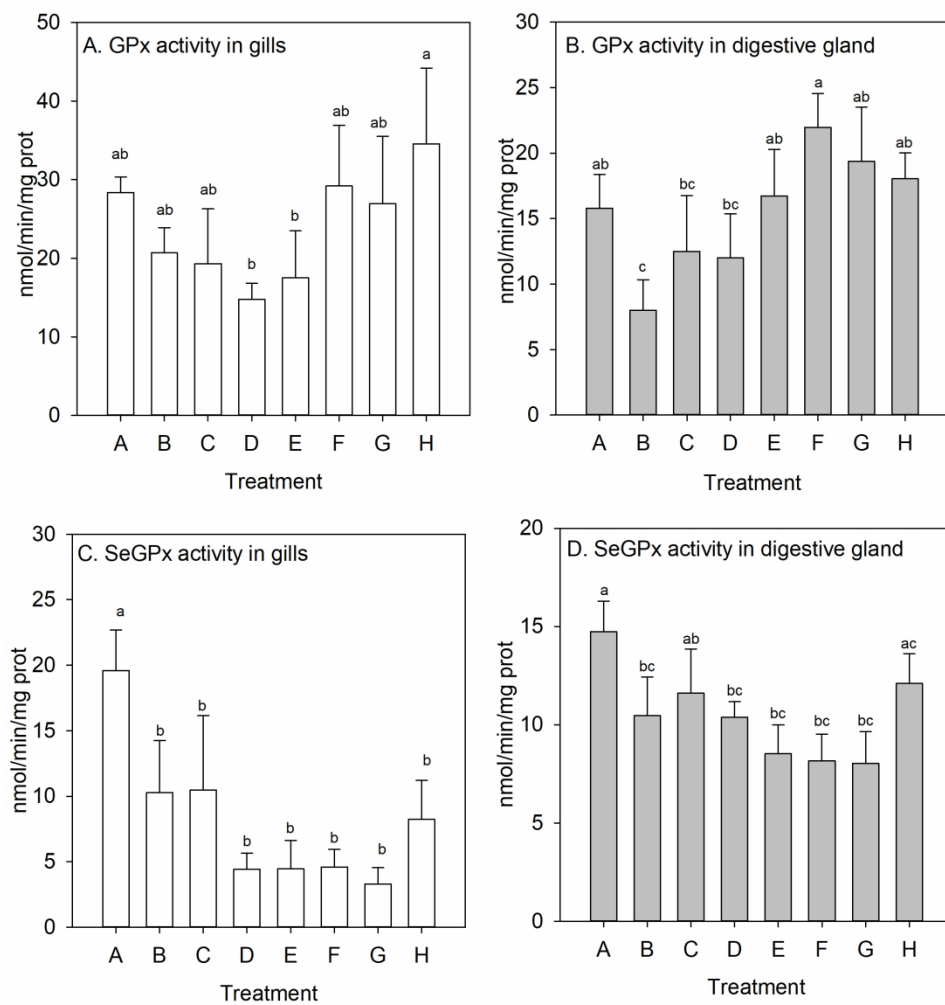58
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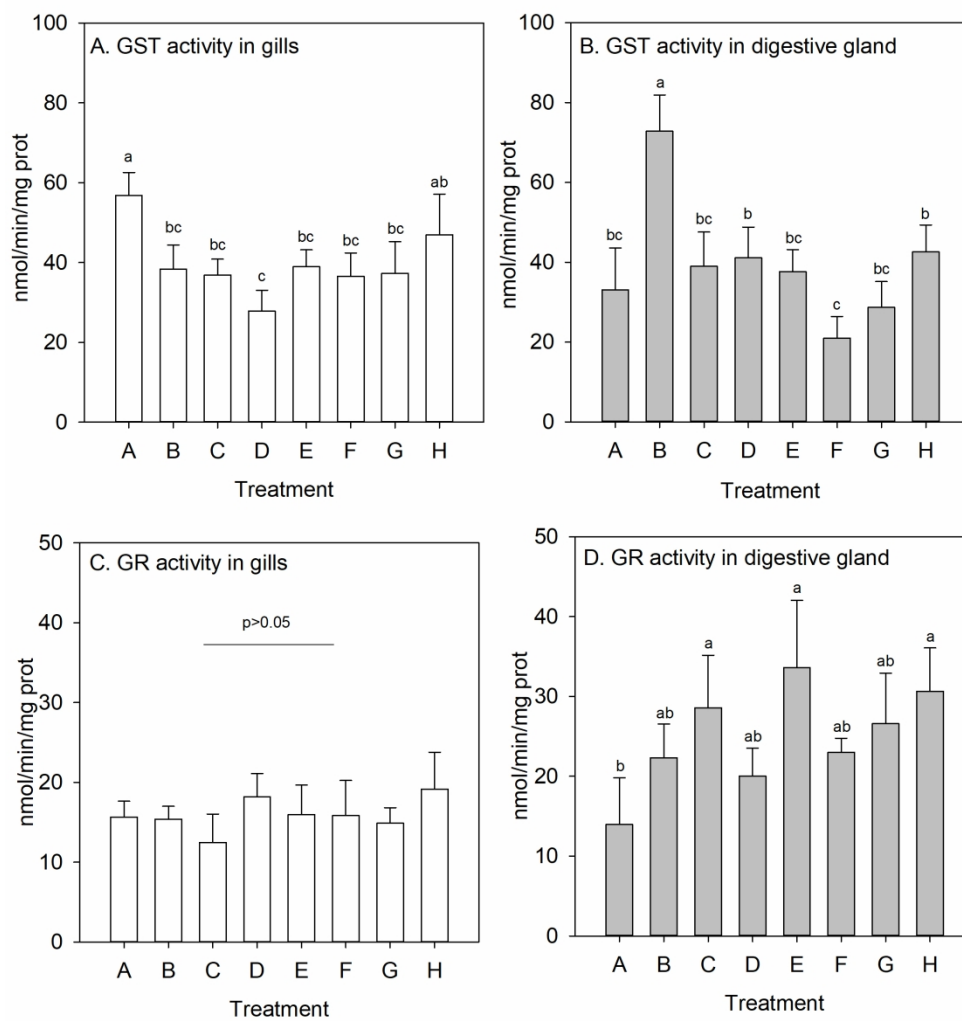

Figure 1

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Figure 2

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Figure 3

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