

2019-03

Exposure to microplastics reduces attachment strength and alters the haemolymph proteome of blue mussels (*Mytilus edulis*)

Green, DS

<http://hdl.handle.net/10026.1/17624>

10.1016/j.envpol.2018.12.017

Environmental Pollution

Elsevier

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

1 Disclaimer

2 'This is a copy of the accepted paper as submitted for publication. Readers are advised to
3 refer to the final version of the paper which can be found at
4 *Environmental Pollution*, 246, 423-434. doi:[10.1016/j.envpol.2018.12.017](https://doi.org/10.1016/j.envpol.2018.12.017)

5

6

7 **Exposure to microplastics reduces attachment strength and alters the haemolymph**
8 **proteome of blue mussels (*Mytilus edulis*)**

9

10 **Authors:** Dannielle S. Green^{1*†}, Thomas J. Colgan^{2,3}, Richard C. Thompson⁴, James C.
11 Carolan⁵

12

13 **Affiliations:**

14 ¹*School of Life Sciences, Anglia Ruskin University, Cambridge, Cambridgeshire, CB11PT,*
15 *United Kingdom.*

16 ²*School of Biological, Earth and Environmental Sciences, University College Cork, Cork,*
17 *Ireland.*

18 ³*School of Biological and Chemical Sciences, Queen Mary University of London, London,*
19 *E14NS, United Kingdom.*

20 ⁴*School of Marine Science and Engineering, Plymouth University, Plymouth, Devon,*
21 *PL48AA, United Kingdom.*

22 ⁵*Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland.*

23

24 *Correspondence to: dannielle.green@anglia.ac.uk

25 †Lead contact

26 **Abstract**

27 The contamination of marine ecosystems with microplastics, such as the polymer
28 polyethylene, a commonly used component of single-use packaging, is of global concern.
29 Although it has been suggested that biodegradable polymers, such as polylactic acid, may be
30 used to replace some polyethylene packaging, little is known about their effects on marine
31 organisms. Blue mussels, *Mytilus edulis*, have become a “model organism” for investigating
32 the effects of microplastics in marine ecosystems. We show here that repeated exposure, over
33 a period of 52 days in an outdoor mesocosm setting, of *M. edulis* to polyethylene
34 microplastics reduced the number of byssal threads produced and the attachment strength
35 (tenacity) by ~50%. Exposure to either type of microplastic altered the haemolymph proteome
36 and, although a conserved response to microplastic exposure was observed, overall
37 polyethylene resulted in more changes to protein abundances than polylactic acid. Many of
38 the proteins affected are involved in vital biological processes, such as immune regulation,
39 detoxification, metabolism and structural development. Our study highlights the utility of
40 mass spectrometry-based proteomics to assess the health of key marine organisms and
41 identifies the potential mechanisms by which microplastics, both conventional and
42 biodegradable, could affect their ability to form and maintain reefs.

43

44 **Capsule**

45 Conventional microplastics alone reduced the attachment strength of blue mussels but both
46 conventional and biodegradable micoplastics altered the haemolymph proteome.

47

48 **Keywords**

49 Biodegradable microplastics, tenacity, proteins, polylactic acid, polyethylene, immunity.

50

51 **1. Introduction**

52 Microplastic particles (0.1 μm – 5 mm) are the most numerically abundant form of solid waste
53 on Earth (Eriksen et al. 2014) and are a potential threat to marine ecosystems globally
54 (Galloway et al. 2017). Global plastic production, which has risen from ~1.5 million metric
55 tonnes (MTT) in 1950 to ~335 MMT in 2016, is predicted to continue to rise (Plastics Europe,
56 2017). The majority (39.9%) of this production is designed for use in single-use packaging,
57 most of which is composed of polyethylene (PE) (Plastics Europe, 2017). Plastics that are
58 able to biodegrade in composting facilities (“biodegradable”), however, have been proposed
59 as suitable alternatives to conventional packagings, such as PE, and global production for
60 these polymers is also growing (Bioplastics Europe, 2016). Polylactic acid (PLA), derived
61 from e.g. starch, accounts for ~45% of the global market of biodegradable plastics and ~60%
62 of its production is used to make packaging (Markets and Markets, 2015). Most microplastics
63 arise from the fragmentation of larger plastic litter items rather than being directly littered as
64 micro-sized particles (for example, microbeads). Packaging items are also the most abundant
65 form of coastal litter (Galgani et al. 2015) and are, therefore, likely the greatest source of
66 microplastic litter.

67

68 Current estimates of environmental concentrations of microplastics in coastal waters are
69 biased towards larger particles because the mesh sizes used to sample are generally > 330
70 μm . When a smaller mesh is used, however, estimates of concentrations in seawater have
71 been found to be three orders of magnitude greater (e.g. from $\sim 0.005 \text{ L}^{-1}$ with a 335 μm net
72 to up to $\sim 6 \text{ L}^{-1}$ with grab samples filtered over a 0.45 μm filter; Barrows et al. 2017). Given
73 that global production of waste is not expected to peak before 2100 and that the amount of
74 plastic in the marine environment is expected to increase by an order of magnitude by 2025

75 (Jambeck et al. 2015), abundances of microplastics, and their potential impacts on marine
76 organisms, are likely to be even greater in the coming decades.

77

78 Understanding the impacts of microplastics is ongoing and has been the subject of several
79 recent reviews (such as Galloway et al. 2017, de Sá et al. 2018 and Anbumani and Kakkar
80 2018). Microplastics have been shown to negatively affect neural (neurotoxicity in
81 nematodes; Lei et al 2018), cellular (oxidative stress in leucocytes of gilthead seabream;
82 Espinosa et al. 2018) to population level (reduced reproductive output of oysters; Sussarellu
83 et al. 2016) biological processes and functions. One of the most well studied marine
84 organisms with respect to microplastics, is the blue mussel, *Mytilus edulis*. *M. edulis* is an
85 important ecosystem engineer, clinging together using byssal threads (extensible
86 proteinaceous fibres; Waite, 1991) to form reefs, which are important biogenic habitats (Seed,
87 1996). They are filter feeders and have been found to ingest microplastics in natural (Van
88 Cauwenberghe et al. 2015; De Witte et al. 2014) and experimental settings (Browne et al.
89 2008; von Moos et al. 2012). Despite this growing body of work, our understanding of how
90 microplastics affect the general health of *M. edulis*, ranging from its biological activity to its
91 underlying molecular phenotype, is currently lacking.

92

93 Haemolymph is the blood-plasma equivalent of the invertebrate circulatory system, involved
94 in the transportation of oxygen, nutrients, as well as proteins involved in important biological
95 processes, including the nervous system, reproduction and the immune system. Within
96 molluscs, the haemolymph has been a well-studied medium for understanding the immune
97 complement, as well as functionality, including aspects of both the cellular (Carballal et al.
98 1997) and humoral immune response (Campos et al. 2015). Directly assessing the cellular
99 and molecular composition of haemolymph, particularly proteins, provides an insight into the

100 general health of an invertebrate organism and can reveal the signatures of environmental
101 stress (Brown et al. 2006; Hannam et al. 2010). Invertebrate haemolymph protein abundances
102 may be assessed using enzyme assays, gel electrophoresis or directly using mass
103 spectrometry-based proteomics, which results in the unbiased identification and
104 quantification of multiple proteins in a given sample (e.g. Campos et al. 2015; McNamara et
105 al. 2017). The increasing incorporation of -omics techniques within the study of key
106 ecological species have improved our understanding of the immune complement (Campos et
107 al. 2015; Wu et al. 2016), structural composition (Mann et al. 2012; Mann and Edsinger,
108 2014), biomarkers of disease and pollution (Wu et al. 2016; Campos et al. 2016) as well as
109 responses of organisms to environmental change (Tomanek 2012; Tomanek 2014).

110

111 To date, the vast majority of experiments investigating detrimental effects of microplastics
112 exposure have used highly controlled laboratory conditions, with very high concentrations of
113 microplastics, for example 110 (Van Cauwenberghe et al. 2015) to >1 billion (Browne et al.
114 2008) particles ml⁻¹, which do not simulate natural conditions of the habitats in which the
115 organisms reside. Compared with indoor laboratories, outdoor mesocosm systems can
116 provide a better understanding of environmentally relevant effects of contaminants on
117 individuals, populations and communities (Miko et al. 2015). In order to estimate future risks,
118 an outdoor mesocosm experiment was used to expose *M. edulis* to a relatively low
119 concentration (~1 particle mL⁻¹ or 25 µg L⁻¹) of a range of particle sizes (~0.5 to ~330 µm)
120 of conventional (high-density polyethylene - HDPE) or biodegradable (polylactic acid - PLA)
121 microplastics in a natural outdoor setting for 52 days to assess impacts on (i) important
122 biological traits as ecosystem engineers including byssal thread production and attachment
123 strength and (ii) underlying molecular health through proteomic analysis of their
124 haemolymph.

125

126 **2. Methods**

127 *2.1. Experimental design and set-up*

128 Mesocosm experiments were set up at the outdoor flow-through mesocosm facility at Queen's
129 University Marine Laboratory, Portaferry, Northern Ireland. The experimental design
130 consisted of one fixed factor "Plastic", with three levels: no microplastics added (control),
131 polylactic acid (PLA) and high density polyethylene (HDPE).

132

133 Full details of the experimental set-up can be found in Green et al. (2017), but are described
134 here in brief. Mesocosms were 10 litre polypropylene buckets (height x diameter = 25 x 25
135 cm). The experiment had 5 replicates (n = 5, N = 15) and to simulate the habitat where the
136 mussels were collected from, each mesocosm contained a 5 cm layer of muddy sediment. On
137 the 26th of August 2014, after allowing 48 hours for the sediment to settle, each mesocosm
138 received 7 individuals of *M. edulis*. Mussels were measured, weighed and randomly allocated
139 to treatments in order to ensure that no biases due to size were introduced into the experiment.
140 The average (\pm S.E.M.) whole animal wet weight was 19.84 ± 0.51 g and there were no
141 differences among treatments (ANOVA: $F_{2,12} = 0.18$, $P = 0.831$). The average (\pm S.E.M.)
142 length of the mussels was 48.17 ± 0.83 mm and there were also no differences in length
143 among treatments (ANOVA: $F_{2,12} = 0.87$, $P = 0.469$). The experiment ran for 52 consecutive
144 days (until 14th October 2014). Sand-filtered seawater, sourced from Strangford Lough
145 ($54^{\circ}22'51.1''N$; $5^{\circ}33'04.0''W$) was continuously provided to each mesocosm at constant flow
146 rates (~ 500 mL minute^{-1}) via individual hoses resulting in an overlying water column of ~ 8
147 L.

148

149 *2.2. Microalgal culture and microplastic dosing*

150 Cultures of the microalgae *Isochrysis galbana* were prepared using seawater (35 psu), which
151 was filtered with 0.45 µm aperture membranes and sterilised with UV light. PLA or HDPE
152 microplastics were then added to 5 litre bottles with *I. galbana* and were continuously mixed
153 with air bubblers. Fresh batches of control and microplastic-dosed *I. galbana* cultures were
154 made weekly. Virgin (pre-production) white PLA and HDPE microplastic particles
155 (fragments not spheres) used in the experiments had a volume-weighted mean diameter of
156 65.6 µm (range = 0.6–363 µm) for PLA and 102.6 µm (range = 0.48–316 µm) for HDPE.
157 Every day, each mesocosm received 250 mL of $\sim 2 \times 10^6$ cells mL⁻¹ of microalgae containing
158 either 0 (control) or 800 µg L⁻¹ of PLA or HDPE microplastics, equating to a concentration
159 in the mesocosms of 25 µg L⁻¹. This corresponds to approximately 1296.3 (± 182.9) and 844.9
160 (± 138.7) particles L⁻¹ respectively (Green et al. 2017). Although this is high relative to current
161 environmental concentrations, due to the range of particle sizes included, these concentrations
162 are among the lowest used experimentally to date and, therefore, among the most
163 environmentally realistic (Lenz et al. 2016). In order to simulate a pulse of microplastics in
164 the environment (such as from wastewater effluent), mussels were exposed for two hours per
165 day. During dosing, the flow of water was paused and air bubblers were used to keep the
166 water moving and aerate the mesocosms. The water flow in the mesocosms was resumed after
167 2 hours and fresh seawater replaced the microplastic- contaminated water which was captured
168 for safe disposal.

169

170 2.3. Tenacity of mussels

171 The remaining individuals from each of the mesocosms (excluding those removed for
172 filtration and proteomics measurements) were placed onto glass petri dishes in clean buckets
173 with fresh flowing seawater. Feeding (without microplastics) was resumed for a further 3
174 days (in order to allow the mussels to attach their byssal threads) before tenacity was

175 measured for one randomly selected mussel from each replicate mesocosm. Tenacity was
176 measured following the method of Denny (1987). Briefly, a portable dynamometer (Pesola,
177 Sweden), scaled 0 to 10 N, was used to measure the maximal vertical force required for the
178 individual to become dislodged (attachment strength, N). The dynamometer had a small clamp
179 that gripped individual mussels. The maximum dislodgement force was marked by a ring on
180 the dynamometer, which remained in place after detachment indicating the maximum
181 extension of the spring (to the nearest 0.1 N). Shell planform area (A_{pl}) was approximated as
182 an ellipse with shell height and width as major and minor axes (measured with vernier
183 callipers to 1 mm), respectively (Bell and Gosline 1997). Tenacity was calculated as
184 dislodgement force per unit mussel planform area, in $N m^{-2}$. The number of byssal threads
185 deployed by each of the mussels used in tenacity measurements was also counted.

186

187 *2.4. Proteomic analysis of mussels*

188 After 50 days, haemolymph was extracted from one individual from each mesocosm. In order
189 to minimise disturbance, exposure to the air was not longer than 3 minutes before
190 haemolymph extraction. Prior to haemolymph extraction, the shells were temporarily opened
191 2-3 mm to allow for the removal of excess water from the interior. The shells were then
192 allowed to reclose and form a tight seal. An Omnican F syringe with an integrated 30 gauge
193 needle was inserted between the shells of the mussel into the posterior adductor muscle and
194 haemolymph slowly collected. The syringe was then removed from the needle and the
195 haemolymph was dispensed into a nuclease-free 1.5 ml microcentrifuge tube. The
196 haemolymph was stored temporarily at $-20^{\circ}C$ overnight before transfer to $-80^{\circ}C$ for long-
197 term storage. As haemocytes can be reactive to oxygen causing the production of melanin,
198 we took additional care to remove haemocytes prior to sample preparation for mass-
199 spectrometry.

200 200 µl crude haemolymph was removed and centrifuged at $8,000 \times g$ for 5 min to pellet
201 cellular debris. The supernatant was then removed, quantified and four independent biological
202 replicates were analysed from each treatment: control (n=4), PLA- (n=4) and HDPE-exposed
203 (n=4) mussels. 50 µg of each sample was precipitated using the 2D Clean-Up Kit (GE
204 HealthCare), following the manufacturer's instructions and the resulting protein pellet was
205 resuspended in 50 µl 6M urea, 2M thiourea, 0.1 M Tris-HCl, pH 8.0. 50mM ammonium
206 bicarbonate was added to each sample and proteins were reduced with 0.5M dithiothreitol
207 (DTT) at 56°C for 20 min and alkylated with 0.55M iodoacetamide (IAA) at room
208 temperature for 15 min, in the dark. 1 µl of a 1% w/v solution of Protease Max Surfactant
209 Trypsin Enhancer (Promega) and 0.5 µg of Sequence Grade Trypsin (Promega) was added to
210 give a protein:trypsin ratio of 100:1. The protein/trypsin mixture was incubated at 37°C for
211 18 h. Digestion was terminated by adding 1 µl of 100% trifluoroacetic acid (Sigma Aldrich)
212 and incubation at room temperature for 5 min. Samples were centrifuged for 10 min at $13,000$
213 $\times g$ and a volume equivalent to 40 µg of pre-digested protein was removed and purified for
214 mass spectrometry using C18 Spin Columns (Pierce), following the manufacturer's
215 instructions. The eluted peptides were dried using a SpeedyVac concentrator (Thermo
216 Scientific Savant DNA120) and resuspended in 2% v/v acetonitrile and 0.05% v/v
217 trifluoroacetic acid (TFA). Samples were sonicated for 5 min to aid peptide resuspension
218 followed by centrifugation for 5 min at $13,000 \times g$. The supernatant was removed and used
219 for mass spectrometry.

220

221 *2.5. Mass spectrometry*

222 1 µg of each digested sample was loaded onto a QExactive (ThermoFisher Scientific) high-
223 resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano)
224 chromatography system. The peptides were separated by a 4% to 35% gradient of acetonitrile

225 on a Biobasic C18 Picofrit™ column (100 mm length, 75 mm ID), using a 55 min reverse-
226 phase gradient at a flow rate of 250 nL min⁻¹. All data were acquired with the mass
227 spectrometer (MS) operating in automatic data dependent switching mode. A full MS scan at
228 140,000 resolution and a scan range of 400-2000 m/z were followed by an MS/MS scan,
229 resolution 17,500 and a range of 200-2000 m/z, selecting the 10 most intense ions prior to
230 MS/MS.

231

232 Protein identification and Label Free Quantification (LFQ) normalisation of MS/MS data
233 were performed using MaxQuant v1.5.6.5 (<http://www.maxquant.org>) following the general
234 procedures and settings outlined in Hubner et al. (2010). The Andromeda search algorithm
235 (Cox et al. 2011) incorporated in the MaxQuant software was used to correlate MS/MS data
236 against a predicted protein set generated from available RNA sequences for *Mytilus edulis*
237 (Bioproject: PRJEA75259), *Mytilus gallaprovincialis* (Bioproject: PRJNA167773) and
238 nucleotide/protein sequences available for *M. edulis* and *M. gallaprovincialis* from the
239 National Centre for Biotechnology and Information (NCBI) repository (8,305 entries,
240 downloaded September 2017) including the predicted protein set derived from the *M.*
241 *gallaprovincialis* genome (Murgarella) et al. 2016) (Bioproject: PRJNA262617). A 6-frame
242 translation was conducted on the RNA sequences for the transcriptome resources of *Mytilus*
243 species and an open reading frame extraction was performed. CD-Hit (Huang et al. 2010) was
244 utilized to remove redundant sequences that were similar by 98% and above. The final
245 predicted proteome comprised 12,453 sequences and was used in mass spectrometry searches
246 in addition to a contaminant sequence set provided by MaxQuant.

247 The following search parameters were used: first search peptide tolerance of 20 ppm, second
248 search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification
249 and N-acetylation of protein and oxidation of methionine as variable modifications and a

250 maximum of two missed cleavage sites allowed. False Discovery Rates (FDR) were set to
251 1% for both peptides and proteins and the FDR was estimated following searches against a
252 target-decoy database. LFQ intensities were calculated using the MaxLFQ algorithm (Cox et
253 al. 2014) from razor and unique peptides with a minimum ratio count of two peptides across
254 samples.

255

256 *2.6. Processing of proteomic data*

257 Perseus v.1.5.2.6 (www.maxquant.org/) was used for data processing and visualization. The
258 data matrix was first filtered for the removal of contaminants, decoy peptide matches and
259 peptides identified by site. To obtain annotations for all identified proteins a fasta file was
260 generated for the post-filtered proteins using BioEdit (Hall 1999) and BlastP searched using
261 Blast2Go (version: 4.1.9; Conesa et al. 2005) against the NCBI non redundant database with
262 the following search settings: number of blast hits: 20, high-scoring segment pair length
263 cutoff: 33, Blast expect value: $1.0e^{-5}$. These annotations were subsequently uploaded to
264 Perseus. Normalised LFQ intensity values (quantitative measurement of protein abundance)
265 were \log_2 transformed and each replicate was assigned to its corresponding sample (n=4).
266 Proteins not found in all 4 replicates of at least one treatment were removed from the analysis.
267 A data-imputation step was conducted to replace non-detected values with values that
268 simulate signals of lowest abundant proteins chosen randomly from a distribution specified
269 by a downshift of 1.8 times the mean standard deviation (SD) of all measured values and a
270 width of 0.3 times this SD. Volcano plots were generated in Perseus by plotting negative log
271 p-values against \log_2 fold-change values for each protein to visualize two-fold changes in
272 protein expression of the microplastic exposed mussels relative to the control mussels and
273 microplastic exposed mussels to each other. LFQ intensity values for proteins deemed
274 statistically significant (by ANOVA, see *Statistical data analyses*) were Z-score normalized
275 and hierarchical clustering was generated in order to summarise the overall differences among

276 treatments. Gene Ontology mapping and functional domain analysis were performed using
277 Blast2GO (v.4.1.9). The mass spectrometry proteomics data have been deposited to the
278 ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with
279 the dataset identifier PXD011567.

280

281 *2.7. Statistical data analyses*

282 Analysis of variance (ANOVA) was done using the R environment (R v3.1.3; R core team
283 2015) to test the null hypotheses that there would be no difference in the (i) tenacity (ii)
284 number of byssal threads (with $n = 5$) and (iii) relative (post imputation) abundances of
285 individual proteins (with $n = 4$). The data were screened for normality (q-q plots, and Shapiro-
286 Wilk tests) and homogeneity of variance (Levene's test, using the *car* package; Fox and
287 Weisberg 2011) to ascertain assumptions for ANOVA. Pairwise comparisons were computed
288 when the main test was significant. Statistical significance was assumed at $\alpha = 0.05$. In order
289 to explore the possible relationships between the effects of microplastics on protein
290 expression and effects on the biological functioning of blue mussels, Pearson's R correlation
291 coefficients were calculated.

292 PERMANOVA was performed on post imputation data to test the null hypothesis that the
293 haemolymph proteomes (composition and relative abundances of all 216 identified proteins)
294 of mussels exposed to PLA or HDPE microplastics or to no microplastics (controls) would
295 not differ. Patterns of difference in the haemolymph proteomes were visualized with
296 canonical analysis of principal coordinates based on Bray-Curtis resemblance matrices (using
297 PRIMER V6.1.12 with PERMANOVA+).

298

299 **3. Results**

300 *3.1. Effects of microplastics exposure on biological functions of M. edulis*

301 After 52 days of exposure to microplastics, the tenacity was lower ($F_{2,12} = 5.6$, $P = 0.019$) and
302 there were fewer byssal threads ($F_{2,12} = 10.3$, $P = 0.002$) produced by mussels exposed to
303 HDPE microplastics compared with mussels exposed to PLA or to no microplastics (Figure
304 1). Specifically, after exposure to HDPE, attachment strength of *M. edulis* was ~ 3.0 times less
305 compared to the controls, while it was ~ 2.2 times less compared with mussels exposed to
306 PLA (Figure 1a). Mirroring this, the number of byssal threads produced by mussels exposed
307 to HDPE was ~ 1.6 times less compared to the controls and ~ 1.8 times less compared to those
308 exposed to PLA (Figure 1b). Across all treatments, there was a significant positive
309 relationship between attachment strength and the number of byssal threads (Pearson's $r =$
310 0.78 , $t = 4.46$, $P < 0.001$).

311

312 3.2. Effects of microplastics on the haemolymph proteome of *M. edulis*

313 High resolution quantitative mass spectrometry identified 2,745 peptides, representing 216
314 high confidence proteins from *M. edulis* (Table S1). The haemolymph proteome was
315 significantly altered by microplastics ($pseudo-F_{2,9} = 2.89$, $P = 0.001$), with those exposed to
316 HDPE or to PLA having significantly different protein profiles compared to those not exposed
317 to microplastics (HDPE vs Control: $pseudo-t = 1.91$, $P = 0.023$ and PLA vs Control: $pseudo-$
318 $t = 1.48$, $P = 0.029$). The haemolymph proteome of mussels exposed to HDPE also differed
319 to those exposed to PLA (HDPE vs PLA: $pseudo-t = 1.67$, $P = 0.030$). This is illustrated by
320 ordination using canonical analysis of principal coordinates (Figure 2). Furthermore, $\sim 19\%$
321 of the haemolymph proteome (40 proteins) was significantly affected by exposure to
322 microplastics, with the majority being expressed at greater abundances (Table 1) when
323 microplastics were present, compared to the control.

324

325 Of the 40 statistically significant proteins, six were differentially expressed in mussels
326 exposed to both types of microplastic compared with controls. A further 11 were differentially
327 expressed only in those exposed to HDPE compared with controls and four were differentially
328 expressed only in those exposed to PLA compared with controls. Nine proteins differed
329 between HDPE and PLA while five proteins differed in expression between HDPE-exposed
330 and both PLA-exposed and control mussels (Table 1, Figure 3). Post-hoc tests were unable
331 to determine the differences in the remaining five proteins. Functional annotation through
332 homology searches and gene ontology mapping assigned functional information for
333 statistically significant differentially expressed proteins identifying proteins with putative
334 roles in the immune system, metabolism and detoxification, as well proteins of unknown
335 function (Table 1).

336

337 3.2.1. *Effects of microplastics exposure on the mussel immune complement*

338 Within the haemolymph of mussels exposed to microplastics, 11 putative immune-responsive
339 proteins were differentially expressed. Exposure to HDPE resulted in increased abundance of
340 four immune-responsive proteins, including a putative proinflammatory cytokine, macrophage
341 migration inhibitory factor (HE609105.1), as well as three complement C1q domain-
342 containing (C1qDC) proteins (FR715598.1; FR715581.1; HE609753.1) (Table 1, Figure 4).
343 One of these putative C1qDC proteins (FR715598.1) was also increased in mussels exposed
344 to PLA in comparison to the control. In addition, both microplastics treatments increased the
345 abundance of a fibrinogen-related protein (OPL33687.1). In contrast, a second fibrinogen-
346 related protein (OPL32613.1) was reduced within both treatments in comparison to control.
347 Six immune proteins were differentially expressed between mussels exposed to PLA compared
348 with those exposed to HDPE. In comparison to PLA, exposure to HDPE resulted in increased
349 abundance of a putative pore-forming apextrin-like protein (HQ709238.1), a galectin

350 (AJQ21509.1), as well as a putative antimicrobial peptide, mytimycin precursor
351 (AET85056.1). In contrast, PLA exposure increased the abundance of two C1qDC proteins
352 (FR715612.1; HE609604.1) in comparison to HDPE.

353

354 *3.2.2. Effects of microplastics exposure on the abundance of metabolic proteins*

355 Seven putative metabolic proteins were differentially expressed in response to one or both
356 microplastics treatments. HDPE exposure resulted in increased expression of a
357 glyceraldehyde-3-phosphate dehydrogenase (GAEN01008281.1), an aminopeptidase
358 (GAEN01005918.1) and a protein putatively involved in retinal metabolism, retinol
359 dehydrogenase 1 (OPL33362.1) (Table 1, Figure 4). Two putative metabolic enzymes, a
360 putative aspartate cytoplasmic protein (HE662841.1) and phosphoglycerate kinase
361 (GAEM01000061.1) were increased within mussels exposed to PLA in comparison to both
362 control and HDPE individuals. PLA exposure also resulted in a reduction in a
363 metalloproteinase inhibitor (GAEM01005782.1) in comparison to mussels exposed to HDPE.
364 Both HDPE and PLA also led to an elevated abundance of a putative peptidyl-prolyl cis-trans
365 isomerase protein (GAEN01009083.1) compared with control mussels.

366

367 *3.2.3. Potential detoxification proteins altered in response to microplastics exposure*

368 Four proteins previously identified to have putative roles as biomarkers of detoxification
369 within molluscs were differentially expressed in response to microplastics exposure.
370 Exposure to either microplastics treatment resulted in the increased expression of a putative
371 heavy metal binding protein (HE609570.1) and putative detoxification enzyme,
372 deferrochelatase peroxidase (GAEN01007747.1) in comparison to control mussels.
373 Furthermore, a ferritin heavy oocyte (GAEN01007405.1) was increased in response to HDPE
374 exposure in comparison to control mussels. A fourth protein, with a putative role in

375 detoxification (cathepsin D; GAEM01006053.1), was also identified to be significantly
376 affected by microplastics but post-hoc tests were not significant.

377

378 *3.2.4. Additional biomarkers associated with microplastics exposure*

379 Aside from variation in immune, metabolic and detoxification proteins, microplastics exposure
380 changed the abundance of an additional 19 proteins. Both HDPE and PLA reduced the
381 abundance of a protein of unknown function (OPL21291.1) compared with control mussels.
382 Exposure to HDPE increased the expression of proteins involved in a variety of biological
383 processes, including neurogenesis (GAEM01003123.1; OPL21044.1), structural integrity
384 (GAEM01002086.1; GAEM01005782.1; OPL21594.1), DNA binding (CAD37821.1;
385 CAC94907.1; GAEN01008605.1), and proteins of unknown function (OPL32817.1) in
386 comparison to control and/or PLA treatment. In contrast, HDPE reduced the abundance of two
387 proteins with roles in structural integrity (HE662833.1, GAEN01011200.1).

388

389 Exposure to PLA increased the expression of a putative growth factor protein
390 (GAEN01008261.1) and a protein of unknown function (HE609843.1) in comparison to
391 control and HDPE-exposed individuals, respectively. One putative titin-like protein
392 (GAEN01023435.1) was reduced within mussels exposed to PLA in comparison to control
393 mussels. Post-hoc tests were unable to determine the direction of differences in four additional
394 proteins found to be significantly affected, including proteins involved in structural integrity
395 (GAEN01011004.1; GAEN01007066.1), translation (GAEN01008711.1) and a protein of
396 unknown function (GAEN01005668.1).

397

398 **4. Discussion**

399 After long-term (52 days) exposure to ~ 1 particle mL^{-1} of HDPE microplastics, the number of
400 byssal threads produced and the tenacity of *M. edulis* were reduced by approximately 50%
401 when compared with mussels not exposed to microplastics. Tenacity is paramount to the
402 ability of mussels to form and maintain reefs without being dislodged by hydrodynamic forces
403 (Bell and Gosline, 1997). The ability to produce byssal threads and to form aggregations also
404 increases fertilization success, makes mussels more resistant to predation and, overall,
405 increases the probability of their survival (Christensen et al, 2015). Weakened attachment
406 strength in response to conventional microplastics could, therefore, result in cascading
407 ecological (by reducing the habitat availability for intertidal communities that depend on
408 mussel reefs) and economic (by reducing yields of suspension culturing of mussels in
409 aquaculture) consequences. For example, the mussel aquaculture industry is already worth ~ 3 -
410 4 billion USD globally per year (FAO 2015) and is expected to grow in the coming decades.
411 Reductions in the tenacity of two similar species of mussel, *Mytilus trossulus* (O'Donnell et
412 al. 2013) and *Mytilus coruscus* (Zhao et al. 2017) have also been found in response to ocean
413 acidification. Given that the concentrations of microplastics in the oceans (Jambeck et al.
414 2015) and the acidity of seawater (IPCC 2014) are both likely to increase in the coming
415 decades, future research should assess their combined effect on the health and tenacity of
416 mussels.

417

418 In order to complement the measures of tenacity and to provide a detailed assessment of mussel
419 health in response to microplastics exposure, we assessed changes in the proteome of the
420 mussel haemolymph. We chose the haemolymph because it plays an important role as a crucial
421 transporter of nutrients and oxygen, as well as being a primary site of immune activity and
422 xenobiotic detoxification (Malagoli et al. 2007). Information regarding the effects of
423 microplastic exposure on an organism's proteome are limited, but Sussarellu et al. (2016) found

424 that exposure to polystyrene microplastics altered proteins in the oocytes of oysters and that
425 this corresponded to a reduction in fertility. In the current study, exposure to HDPE or PLA
426 microplastics resulted in complex changes in a number of key biological processes, including
427 immunity, metabolism and detoxification.

428

429 Exposure of mussels to either PLA or HDPE microplastics resulted in changes in the
430 immunological profiles of their haemolymph. The immune system represents an important
431 obstacle to infection and disease, and has been extensively studied and characterised in mussels
432 (Campos et al. 2015; Wu et al. 2016) and while interactions between microplastics and aspects
433 of the mussel cellular immune response have been previously documented (von Moos et al.
434 2012; Avio et al. 2015; Paul-Pont et al. 2016), here we provide evidence of changes within the
435 humoral components of the mussel haemolymph in response to microplastics exposure.
436 Specifically, members of the C1qDC protein family were affected by exposure to
437 microplastics. C1qDC genes function in pathogen recognition (Gerdol et al. 2011) with certain
438 genes elevated in response to bacterial challenge (Gestal et al. 2010). Additionally, two
439 pathogen recognition molecules, galectin-2 and apextrin were upregulated in mussels exposed
440 to HDPE compared with PLA microplastics. Within molluscs, galectin-2 and apextrin have
441 been characterised to promote phagocytosis (Vasta et al. 2015) and function in membrane pore
442 formation (Estevez-Calvar et al. 2011) respectively. Other effector molecules altered by
443 microplastics exposure, included an increase in the antimicrobial peptide, myticin (Mitta et al.
444 1999), while fibrinogen-related proteins, functional in antigen recognition (Romero et al.
445 2011), were either up- or down- regulated in response to microplastics exposure. These
446 immunological changes may be due to physical abrasion from the microplastics after being
447 ingested by the mussels. Previous research by Avio et al. (2015) on a similar species of mussel
448 to those used in the current study, *Mytilus galloprovincialis*, found similar immunological

449 responses to virgin and contaminated microplastics (polyethylene and polystyrene) thus
450 suggesting physical abrasion as the cause of response. It is possible that microplastics can
451 translocate into tissues such as the gills and digestive tract (von Moos et al. 2012; Avio et al.
452 2015; Paul-Pont et al. 2016), as well as the haemolymph (Browne et al. 2008; Avio et al. 2015).
453 Although microplastics in the haemolymph were not quantified in the present study, it is
454 possible that physical abrasion of the tissue may have triggered the observed immunological
455 responses but further research is required.

456

457 Despite some differences in the effects of PLA and HDPE on the proteome, a number of
458 proteins responded similarly to both types of microplastic including complement C1q domain-
459 containing proteins (discussed above) and detoxification proteins, such as a peroxidase and a
460 heavy metal-binding protein. Aside from direct immunological activity, immune-responsive
461 proteins within the haemolymph may have roles in detoxification. Within *Mytilus* species,
462 Cq1DC protein expression has been identified to change in response to heavy metal exposure
463 suggesting a role in detoxification (Liu et al. 2014). Within the present study, a heavy metal
464 binding protein, which contained a complement-like domain, was increased in response to both
465 types of microplastics, indicating a potential conserved biomarker of microplastics exposure.
466 Heavy metal-binding proteins with strong reactivity to metal pollutants have been previously
467 characterised within the mussel haemolymph (Renwrantz and Werner 2007). While organisms
468 require metal ions in trace amounts, excessive quantities can be toxic (Mej re and B low 2001)
469 and require removal. Changes in immune proteins, as well as metal binding proteins, have been
470 characterized previously in mussels in response to other pollutants, highlighting the role of the
471 immune response as an indicator of a stressed phenotype (Coles et al. 1995). The ability of
472 mussels to detoxify microplastics and associated by-products has been investigated through the
473 measurement of detoxification enzyme activity (Paul-Pont et al. 2016), transcriptional

474 responses in antioxidant genes (Avio et al. 2015), as well as the assessment of oxidative damage
475 within exposed individuals (von Moos et al. 2012; Paul-Pont et al. 2016). We identified one
476 such detoxification enzyme, a peroxidase, to be increased within both microplastic treatments.
477 Peroxidases are vital enzymes involved in the degradation of by-products of respiration
478 (Brigelius-Flohé and Maiorino 2013) and have been identified to have increased enzyme
479 activity within mussels in response to exposure to other pollutants (Vidal-Liñán et al. 2015)
480 and within the marine copepod, *Paracyclops nana*, in response to microplastics (Jeong et al.
481 2017). While the exact role of this peroxidase within *Mytilus* is unknown (Tomanek 2015), it
482 has previously been found to change in abundance in response to fluctuations in temperature,
483 which suggests a role in oxidative stress (Fields et al. 2012). The generation of immune and
484 detoxification defenses can be metabolically costly, placing additional demands on a stressed
485 host. Within mussels, exposure to microplastics can affect metabolic enzymes, involved in
486 essential processes, such as energy metabolism and respiration. For example, metabolic
487 enzymes involved in glycolysis have been found to increase in response to microplastics
488 exposure, which has been suggested to be associated with mounting a detoxification response
489 (Paul-Pont et al. 2016). In the current experiment, the filtration rates of *M. edulis* were reduced
490 by exposure to either HDPE or PLA microplastics, compared with controls (results reported in
491 Green et al. 2017). Other contaminants can also cause similar responses, for example, in
492 response to anthracene, Mediterranean clams (*Ruditapes decussatus*) also had reduced
493 filtration rates and altered proteomes (Sellami et al. 2015). The reduction in the abundance of
494 metabolic proteins may, therefore, be associated with reduced feeding but further research is
495 needed to establish this causal link.

496

497 Myosin was reduced within mussels exposed to HDPE compared with controls. Myosin is
498 involved in generating muscle contraction in bivalves (Yamada et al. 2000) and has been found

499 to be altered by exposure to silver nanoparticles in a related mussel, *M. galloprovincialis*
500 (Gomes et al. 2013). The deficiency in myosin, coupled with the expression of immune and
501 detoxification proteins associated with mussels exposed to HDPE microplastics, may have
502 contributed to less byssal threads being secreted. The tenacity of mussels is primarily related
503 to byssal threads, either based on the number of threads or to their thickness (Carrington 2002).
504 The byssus proteome of another marine mussel of the same genus, *M. coruscus*, has been
505 previously characterised and a selection of other structural proteins (collagen-like) were
506 identified (Qin et al. 2016) and suggested to provide adjustable tension allowing for stable
507 attachment within dynamic rocky intertidal environments (Qin and Waite 1995).

508

509 Within the present study, exposure to conventional microplastics, HDPE, as well as a
510 biodegradable alternative, PLA, resulted in changes to the haemolymph proteome, including
511 proteins associated with stressed phenotypes. Certain proteins involved in immunity and
512 detoxification, affected by both microplastics, provide candidate biomarkers for further
513 research. Overall HDPE alone resulted in more proteomic changes in comparison to PLA.
514 Despite being less severe, the effects of PLA microplastics on the proteome of *M. edulis*
515 provides additional support to the growing body of literature on the potential issues of
516 biodegradable alternatives. For example, PLA microplastics have also been found to reduce
517 the biodiversity and abundance of organisms in marine invertebrate communities (Green 2016)
518 and to decrease the biomass of benthic primary producers (Green et al. 2017; Green et al. 2016).
519 Biodegradable plastics are set to become more dominant as packaging in the future, possibly
520 replacing some conventional plastics (Markets and Markets 2015). They are, therefore, also
521 more likely to become litter. Current testing methods, even those developed specifically for
522 marine habitats (ASTM D7991-15), are limited in their ability to predict the break-down and
523 ecological impacts of biodegradable plastics in the real world (Bioplastics Europe 2016).

524 Multidisciplinary research combining molecular, ecophysiological and traditional ecological
525 techniques is recommended in order to gain a more holistic understanding of the potential
526 impacts of conventional and biodegradable polymers.

527

528 **Acknowledgements**

529 Thank you to Dr Bas Boots for helpful comments on this manuscript and to the staff of
530 Portaferry Marine Laboratory for facilitating this research. This research was funded by the
531 Irish Research Council with a Postdoctoral Research Project Grant (GOIPD/2013/306)
532 awarded to DSG. The Maynooth University Q-Exactive Quantitative Mass Spectrometer was
533 funded under the SFI Research Infrastructure Call 2012; Grant Number: 12/RI/2346 (3).

534

535 **References**

536 Anbumani S, Kakkar P. 2018. Ecotoxicological effects of microplastics on biota: a review.
537 *Environmental Science and Pollution Research* 25: 14373.

538

539 Avio CG, Gorbi S, Milan M, Benedetti M, Fattorini D, d'Errico G, Pauletto M, Bargelloni L,
540 Regoli F. 2015. Pollutants bioavailability and toxicological risk from microplastics to marine
541 mussels. *Environmental pollution* 198: 211–222.

542

543 Barrows APW, Neumann CA, Berger ML, Shaw SD. 2017. Grab vs. neuston tow net: a
544 microplastic sampling performance comparison and possible advances in the field. *Analytical*
545 *Methods* 9(9):1446-1453.

546

547 Bell EC, Gosline JM. 1997. Strategies for life in flow: tenacity, morphometry, and probability
548 of dislodgment of two *Mytilus* species. *Marine Ecology Progress Series* 159:197-208.

549

550 Bioplastics Europe. 2016. Bioplastics - facts and figures. <http://www.european-bioplastics.org>.

551

552 Brigelius-Flohé R, Maiorino M. 2013. Glutathione peroxidases. *Biochimica et biophysica acta*,
553 1830(5): 3289–3303.

554

555 Brown RT, Galloway TS, Lowe D, Browne MA, Dissanayake A, Jones MB, Depledge MH.
556 2006. Differential sensitivity of three marine invertebrates to copper assessed using multiple
557 biomarkers. *Aquatic Toxicology* 66(3): 267-278.

558

559 Browne MA, Dissanayake A, Galloway TS, Lowe DM, Thompson RC. 2008. Ingested
560 microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.).
561 Environmental Science & Technology 42(13):5026-5031.
562

563 Campos A, Apraiz I, da Fonseca RR, Cristobal S. 2015. Shotgun analysis of the marine mussel
564 *Mytilus edulis* hemolymph proteome and mapping the innate immunity elements. Proteomics
565 15(23-24): 4021–4029.
566

567 Campos A, Danielsson G, Farinha AP, Kuruvilla J, Warholm P, Cristobal S. 2016. Shotgun
568 proteomics to unravel marine mussel (*Mytilus edulis*) response to long-term exposure to low
569 salinity and propranolol in a Baltic Sea microcosm. Journal of proteomics 137: 97–106.
570

571 Carballal MJ, Lopez C, Azevedo C, Villalba A. 1997. Enzymes Involved in Defense Functions
572 of Hemocytes of Mussel *Mytilus galloprovincialis*. Journal of invertebrate pathology 70(2):
573 96–105.
574

575 Carrington E. 2002. Seasonal variation in the attachment strength of blue mussels: Causes and
576 consequences. Limnology and Oceanography 47(6):1723-1733.
577

578 Christensen HT, Dolmer P, Hansen BW, Holmer M. 2015. Aggregation and attachment
579 responses of blue mussels, *Mytilus edulis*—impact of substrate composition, time scale and
580 source of mussel seed. Aquaculture 435: 245–251
581

582 Coles JA, Farley SR, Pipe RK. 1995. Alteration of the immune response of the common marine
583 mussel *Mytilus edulis* resulting from exposure to cadmium. Diseases of aquatic organisms 22:
584 59–65.
585

586 Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: a
587 universal tool for annotation, visualization and analysis in functional genomics research.
588 Bioinformatics 21(18):3674-3676.
589

590 Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. 2011. Andromeda: A
591 Peptide Search Engine Integrated into the MaxQuant Environment. Journal of Proteome
592 Research 10(4):1794-1805.
593

594 Cox J, Hein MY, Lubner CA, Paron I, Nagaraj N, Mann M. 2014. Accurate Proteome-wide
595 Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction,
596 Termed MaxLFQ. Molecular & Cellular Proteomics 13(9):2513-2526.
597

598 de Sá LC, Oliveira M, Ribeiro F, Rocha TL, Fütter MN. 2018. Studies of the effects of
599 microplastics on aquatic organisms: what do we know and where should we focus our efforts
600 in the future? Science of the Total Environment 645: 1029-1039.
601

602 Denny MW. 1987. Lift as a mechanism of patch initiation in mussel beds. *Journal of*
603 *Experimental Marine Biology and Ecology* 113(3):231-245.

604

605 De Witte B, Devriese L, Bekaert K, Hoffman S, Vandermeersch G, Cooreman K, Robbens J.
606 2014. Quality assessment of the blue mussel (*Mytilus edulis*): Comparison between commercial
607 and wild types. (Translated from English) *Marine pollution bulletin* 85(1):146-155.

608

609 Eriksen M, Lebreton LCM, Carson HS, Thiel M, Moore CJ, Borerro JC, Galgani F, Ryan PG,
610 Reisser J. 2014. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces
611 Weighing over 250,000 Tons Afloat at Sea. *PLoS ONE* 9(12).

612

613 Espinosa C, Beltran JMG, Esteban MA, Cuesta A. 2018. In vitro effects of virgin microplastics
614 on fish head-kidney leucocyte activities. *Environmental Pollution* 235:30–38.

615

616 Estevez-Calvar N, Romero A, Figueras A, Novoa B. 2011. Involvement of pore-forming
617 molecules in immune defense and development of the Mediterranean mussel (*Mytilus*
618 *galloprovincialis*). *Developmental and Comparative Immunology* 35(10):1015-1029.

619

620 FAO yearbook. Fishery and Aquaculture Statistics. 2015. Rome, Italy.

621

622 Fields PA, Zuzow MJ, Tomanek L. 2012. Proteomic Responses of Blue Mussel (*Mytilus*)
623 Congeners to Temperature Acclimation. *The Journal of Experimental Biology* 215: 1106–16.

624

625 Fox J, Weisberg S. 2011. An {R} Companion to Applied Regression, Second Edition (Sage) p
626 <http://socserv.socsci.mcmaster.ca/jfox/Books/Companion>.

627

628 Galgani F, Hanke G, Maes T. 2015. Global Distribution, Composition and Abundance of
629 Marine Litter. *Marine Anthropogenic Litter*, eds Bergmann M, Gutow L, & Klages M (Springer
630 International Publishing, Cham), pp 29-56.

631

632 Galloway TS, Cole M, Lewis C. 2017. Interactions of microplastic debris throughout the
633 marine ecosystem. *Nature Ecology and Evolution* 1: 0116

634

635 Gerdol M, Manfrin C, De Moro G, Figueras A, Novoa B, Venier P, Palavicini A. 2011. The
636 C1q domain containing proteins of the Mediterranean mussel *Mytilus galloprovincialis*: a
637 widespread and diverse family of immune-related molecules. *Developmental and comparative*
638 *immunology*, 35(6): 635–643.

639

640 Gestal C, Pallavicini A, Venier P, Novoa B, Figueras A. 2010. MgC1q, a novel C1q-domain-
641 containing protein involved in the immune response of *Mytilus galloprovincialis*.
642 *Developmental and Comparative Immunology* 34(9):926-934.

643

644 Gomes T, Pereira CG, Cardoso C, Bebianno MJ. 2013. Differential protein expression in
645 mussels *Mytilus galloprovincialis* exposed to nano and ionic Ag. *Aquatic Toxicology* 136:79-
646 90.

647

648 Green DS, 2016. Effects of microplastics on European flat oysters, *Ostrea edulis* and their
649 associated benthic communities. *Environmental pollution* 216: 95–103.

650

651 Green DS, Boots B, Sigwart J, Jiang S, Rocha C. 2016. Effects of conventional and
652 biodegradable microplastics on a marine ecosystem engineer (*Arenicola marina*) and sediment
653 nutrient cycling. *Environmental Pollution* 208: 426-434.

654

655 Green DS, Boots B, O'Connor NE, Thompson RC. 2017. Microplastics Affect the Ecological
656 Functioning of an Important Biogenic Habitat. *Environmental Science & Technology*
657 51(1):68-77.

658

659 Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
660 program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41:95-98.

661

662 Hannam ML, Bamber SD, Galloway TS, Moody AJ, Jones MB. 2010 Effects of the model
663 PAH phenanthrene on immune function and oxidative stress in the haemolymph of the
664 temperate scallop *Pecten maximus*. *Chemosphere* 78(7): 779-84.

665

666 Hubner NC, Bird AW, Cox J, Splettstoesser B, Bandilla P, Poser I. 2010. Quantitative
667 proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. *Journal*
668 *of Cell Biology* 189(4):739-754.

669

670 Huang Y, Niu B, Gao Y, Fu L, Li W. 2010. CD-HIT Suite: a web server for clustering and
671 comparing biological sequences. *Bioinformatics*. 26(5):680-682.

672

673 IPCC. 2014. *Climate change 2014: impacts, adaptation, and vulnerability. Part A: Global and*
674 *sectoral aspects. Contribution of Working Group II to the Fifth Assessment Report of the*
675 *Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.*

676

677 Jambeck JR, Geyer R, Wilcox C, Siegler TR, Perryman M, Andrady A, Narayan R, Lavender
678 Law K. 2015. Plastic waste inputs from land into the ocean. *Science* 347(6223):768-771.

679

680 Jeong CB, Kang HM, Lee MC, Kim DH, Han J, Hwang DS, Souissi S, Lee SJ, Shin KH, Heum
681 GP, Lee JS. 2017. Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2
682 pathway-mediated defense mechanisms in the marine copepod *Paracyclopsina nana*. *Scientific*
683 *reports* 7: p.41323.

684

685 Lenz R, Enders K, Nielsen TG. 2016. Microplastic exposure studies should be environmentally
686 realistic. *Proceedings of the National Academy of Sciences* 113(29):E4121-E4122.

687

688 Lei L, Liu M, Song Y, Lu S, Hu, J, Cao C, Xie B, Shic H, He D. 2018. Polystyrene
689 (nano)microplastics cause size-dependent neurotoxicity, oxidative damage and other adverse
690 effects in *Caenorhabditis elegans*. *Environmental Science: Nano* 5: 2009-2020.
691

692 Liu HH, Xiang LX, Shao JZ. 2014. A novel C1q-domain-containing (C1qDC) protein from
693 *Mytilus coruscus* with the transcriptional analysis against marine pathogens and heavy metals.
694 *Developmental and comparative immunology* 44(1): 70–75.
695

696 Malagoli D, Casarini L, Sacchi S, Ottaviani E. 2007. Stress and immune response in the mussel
697 *Mytilus galloprovincialis*. *Fish & shellfish immunology* 23(1): 171–177.
698

699 Mann K, Edsinger E. 2014. The *Lottia gigantea* shell matrix proteome: re-analysis including
700 MaxQuant iBAQ quantitation and phosphoproteome analysis. *Proteome science* 12(1): p.28.
701

702 Mann K, Edsinger-Gonzales E, Mann M. 2012. In-depth proteomic analysis of a mollusc shell:
703 acid-soluble and acid-insoluble matrix of the limpet *Lottia gigantea*. *Proteome science* 10(1):
704 p.28.
705

706 Markets and Markets. 2015. Biodegradable Plastics Market by Type (PLA, PHA, PBS, Starch-
707 Based Plastics, Regenerated Cellulose, PCL), by Application (Packaging, Fibers, Agriculture,
708 Injection Molding, and Others) - Global Trends & Forecasts to 2020.
709 <http://www.marketsandmarkets.com>. Accessed 12/05/18.
710

711 Mejáre M, Bülow L. 2001. Metal-binding proteins and peptides in bioremediation and
712 phytoremediation of heavy metals. *Trends in Biotechnology* 19(2): 67–73.
713

714 Miko Z, Ujszegi J, Gal Z, Imrei Z, Hettyey A. 2015. Choice of experimental venue matters in
715 ecotoxicology studies: Comparison of a laboratory-based and an outdoor mesocosm
716 experiment. *Aquatic Toxicology* 167:20-30.
717

718 Mitta G, Hubert F, Noël T, Roch P. 1999. Myticin, a novel cysteine-rich antimicrobial peptide
719 isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*. *European*
720 *journal of biochemistry* 265(1): 71–78.
721

722 Murgarella M, Puiu D, Novoa B, Figueras A, Posada D, Canchaya C. 2016. A First Insight into
723 the Genome of the Filter-Feeder Mussel *Mytilus Galloprovincialis*. *PloS One* 11 (3): e0151561.
724

725 McNamara LM, Carolan JC, Griffin CT, Fitzpatrick D, Kavanagh K. 2017. The effect of
726 entomopathogenic fungal culture filtrate on the immune response of the greater wax moth,
727 *Galleria mellonella* *Journal of Insect Physiology* 100:82-92.
728

729 O'Donnell MJ, George MN, Carrington E. 2013. Mussel byssus attachment weakened by ocean
730 acidification. *Nature Climate Change* 3(6):587-590.
731

732 Paul-Pont I, Lacroix C, González Fernández C, Hégaret H, Lambert C, Le Goïc N, Frère L,
733 Cassone AL, Sussarellu R, Fabioux C, Guyomarch J, Albentosa M, Huvet A, Soudant P. 2016.
734 Exposure of marine mussels *Mytilus spp.* to polystyrene microplastics: Toxicity and influence
735 on fluoranthene bioaccumulation. *Environmental pollution* 216:724–737.
736
737 Plastics Europe. 2017. *Plastics - the facts 2017. An analysis of European plastics production,*
738 *demand and waste data.* <http://www.plasticseurope.org>.
739
740 Sussarellu R, Suquet M, Thomas Y, Lambert C, Fabioux C, Pernet MEJ, Le Goïc N, Quillien
741 V, Mingant C, Epelboion Y, Corporeau C, Guyomarch J, Robbens J, PaulPont I, Soudant P,
742 Huvet A. 2016. Oyster reproduction is affected by exposure to polystyrene microplastics.
743 *Proceedings of the National Academy of Science* 113: 2430-2435
744
745 Qin CL, Pan QD, Qi Q, Fan M, Sun JJ, Li N, Liao Z. 2016. In-depth proteomic analysis of the
746 byssus from marine mussel *Mytilus coruscus*. *Journal of proteomics* 144:87–98.
747
748 Qin X, Waite JH. 1995. Exotic collagen gradients in the byssus of the mussel *Mytilus edulis*.
749 *The Journal of experimental biology* 198(3): 633–644.
750
751 Renwrantz L, Werner I. 2007. Origin of a metal-binding protein in serum of *Mytilus edulis*.
752 *The Journal of molluscan studies* 74(1): 11–17.
753
754 Romero A, Dios S, Poisa-Beiroa L, Costa MM, Posad D, Figueras A, Novoa B. 2011.
755 Individual sequence variability and functional activities of fibrinogen-related proteins (FREPs)
756 in the Mediterranean mussel (*Mytilus galloprovincialis*) suggest ancient and complex immune
757 recognition models in invertebrates. *Developmental and comparative immunology* 35(3):334–
758 344.
759
760 Seed R. 1996. Patterns of biodiversity in the macro-invertebrate fauna associated with mussel
761 patches on rocky shores. *Journal of the Marine Biological Association of the United Kingdom*
762 76(1): 203-210.
763
764 Sellami B, Khazri, Louati H, Della M, Ridha M, Driss, M, Aissa P, Mahmoudi E, Hamouda B,
765 Coelho AV, Sheehan D. 2015. Effects of anthracene on filtration rates, antioxidant defense
766 system, and redox proteomics in the Mediterranean clam *Ruditapes decussatus* (Mollusca:
767 Bivalvia). *Environmental Science and Pollution Research* 22(14):10956-10968.
768
769 Tomanek L. 2012. Environmental proteomics of the mussel *Mytilus*: implications for tolerance
770 to stress and change in limits of biogeographic ranges in response to climate change. *Integrative*
771 *and comparative biology* 52(5): 648–664.
772
773 Tomanek L. 2014. Proteomics to study adaptations in marine organisms to environmental
774 stress. *Journal of proteomics* 105:92–106.
775

776 Tomanek L. 2015. Proteomic Responses to Environmentally Induced Oxidative Stress. The
777 Journal of Experimental Biology 218(12): 1867–79.
778

779 Van Cauwenberghe L, Claessens M, Vandegehuchte MB, Janssen CR. 2015. Microplastics are
780 taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural
781 habitats. Environmental Pollution 199:10-17.
782

783 Vasta GR, Feng C, Bianchet MA, Bachvaroff TR, Tasumi S. 2015. Structural, functional, and
784 evolutionary aspects of galectins in aquatic mollusks: From a sweet tooth to the Trojan horse.
785 Fish & Shellfish Immunology 46(1):94-106.
786

787 Vidal-Liñán L, Bellas J, Salgueiro-González N, Muniategui S, Beiras R. 2015.
788 Bioaccumulation of 4-nonylphenol and effects on biomarkers, acetylcholinesterase,
789 glutathione-S-transferase and glutathione peroxidase, in *Mytilus galloprovincialis* mussel gills.
790 Environmental pollution 200: 133–139.
791

792 Vizcaíno JA, Csordas A, del-Toro N, Dianas JA, Griss J, Lavidas I, Mayer G, Perez-Riverol
793 Y, Reisinger F, Ternent T, Xu QW, Wang R, Hermjakob H. 2016. 2016 update of the PRIDE
794 database and related tools. Nucleic Acids Research 44(D1): D447-D456.
795

796 von Moos N, Burkhardt-Holm P, Koehler A. 2012. Uptake and Effects of Microplastics on
797 Cells and Tissue of the Blue Mussel *Mytilus edulis* L. after an Experimental Exposure.
798 Environmental Science & Technology 46(20): 11327-11335.
799

800 Waite JH. 1991. Mussel Beards - A coming of age. Chemistry and Industry (17):607-611.
801

802 Wu H, Xu L, Ji C, Yu D. 2016. Proteomic and metabolomic responses in D-shape larval
803 mussels *Mytilus galloprovincialis* exposed to cadmium and arsenic. Fish & shellfish
804 immunology 58: 514–520.
805

806 Yamada A, Yoshio M, Oiwa K, Nyitray L. 2000. Catchin, a novel protein in molluscan catch
807 muscles, is produced by alternative splicing from the myosin heavy chain gene. (Translated
808 from English) Journal of Molecular Biology 295(2):169-178.
809

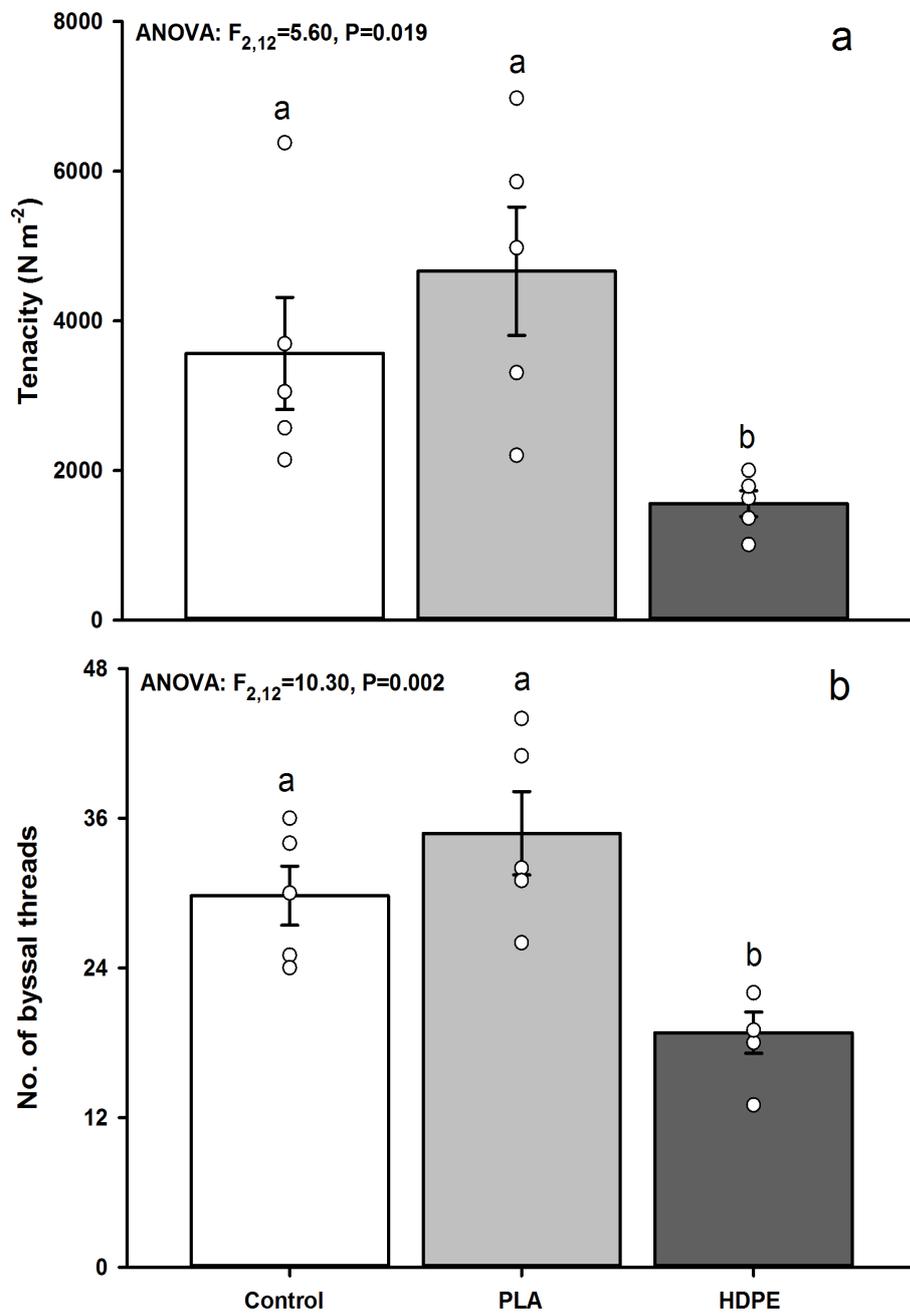
810 Zhao X, Guo C, Han Y, Che Z. 2017. Ocean acidification decreases mussel byssal attachment
811 strength and induces molecular byssal responses. Marine Ecology Progress Series 565: 67-77.

812 **Table 1.** Proteins in *M. edulis* haemolymph with significantly different relative abundances in individuals exposed to HDPE, PLA microplastics
813 or to no microplastics (CONT). Fold differences are indicated by “ δ fold”, and are in **bold** when significantly different according to post-hoc tests.
814 ANOVA (with F and P values) and pairwise post-hoc tests with *indicating significant differences at P<0.05). The source of the information on
815 protein annotation is detailed in Table S2.

	Protein ID	Protein Annotation	F	P	δ fold HDPE vs CONT	δ fold PLA vs CONT	δ fold HDPE vs PLA
Immune	FR715598.1	C1Q Domain Containing 1Q19	80.2	<0.001	7.0*	5.4*	1.5
	HE609753.1	Complement C1Q 4	58.0	<0.001	4.2*	-0.1	4.4*
	OPL33687.1	Fibrinogen-Related	8.0	0.009	3.7*	3.6*	0.1
	AJQ21509.1	Galectin 2	6.7	0.016	0.7	-0.5	1.2*
	HQ709238.1	Apextrin	5.6	0.026	1.9	-0.9	2.8*
	FR715612.1	C1Q Domain Containing 1Q33	5.5	0.027	2.3	-1.5	3.8*
	HE609604.1	Complement C1Q 2	5.1	0.032	-2.8	1.3	-4.1*
	FR715581.1	Complement C1Q Tumor Necrosis Factor	4.7	0.039	3.8*	2.6	1.1
	HE609105.1	Macrophage Migration Inhibitory Factor	4.5	0.045	0.8*	0.3	0.5
	AET85056.1	Mytimycin precursor	4.3	0.049	1.9	-3.3	5.1*
OPL32613.1	Microfibril-Associated Glyco 4	39.3	<0.001	-4.0*	-5.2*	1.3	
Metabolism	GAEN01005918.1	Aminopeptidase N	24.6	<0.001	3.3*	0.6	2.7*
	HE662841.1	Aspartate Cytoplasmic	9.0	0.007	0.0	0.7*	-0.7*
	GAEN01009083.1	Peptidyl-Prolyl Cis-Trans Isomerase	6.8	0.016	0.6*	0.5*	0.1
	GAEN01008281.1	Glyceraldehyde-3-Phosphate Dehydrogenase	6.3	0.020	0.5*	0.4	0.2
	GAEM01005782.1	Metallo Ase Inhibitor 3	5.4	0.029	1.5	-0.5	2.0*
	GAEM01000061.1	Phosphoglycerate Kinase	4.9	0.037	0.3	0.6*	-0.3
	GAEM01005782.1	Retinal Dehydrogenase 1	5.8	0.024	1.9*	1.4	0.5
Structural	GAEM01002086.1	Shell -5	12.5	0.002	2.8*	1.2	1.6
	HE662833.1	Calponin -1	6.4	0.019	-3.1*	-1.8	-1.3
	GAEN01011200.1	Myosin Essential Light Chain	6.1	0.021	-1.2*	-0.8	-0.4
	OPL21594.1	Singed Isoform X3	4.8	0.038	0.6*	0.5	0.2
	GAEN01011004.1	Myosin Heavy Non-Muscle-Like	4.6	0.043	2.1	2.0	0.1
	GAEN01023435.1	Titin-Like	4.5	0.045	-0.5	-0.7*	0.2
	GAEN01007066.1	Myosin Regulatory Light Chain Smooth Adductor Muscle-Like Isoform X3	4.4	0.047	-1.3	-1.1	-0.2
Detoxification	HE609570.1	Heavy Metal-Binding Protein	10.5	0.004	4.4*	2.9*	1.5
	GAEN01007747.1	Probable Deferrochelatae Peroxidase	7.2	0.013	2.3*	2.5*	-0.2
	GAEM01006053.1	Cathepsin D	4.6	0.042	2.2	2.2	0.0

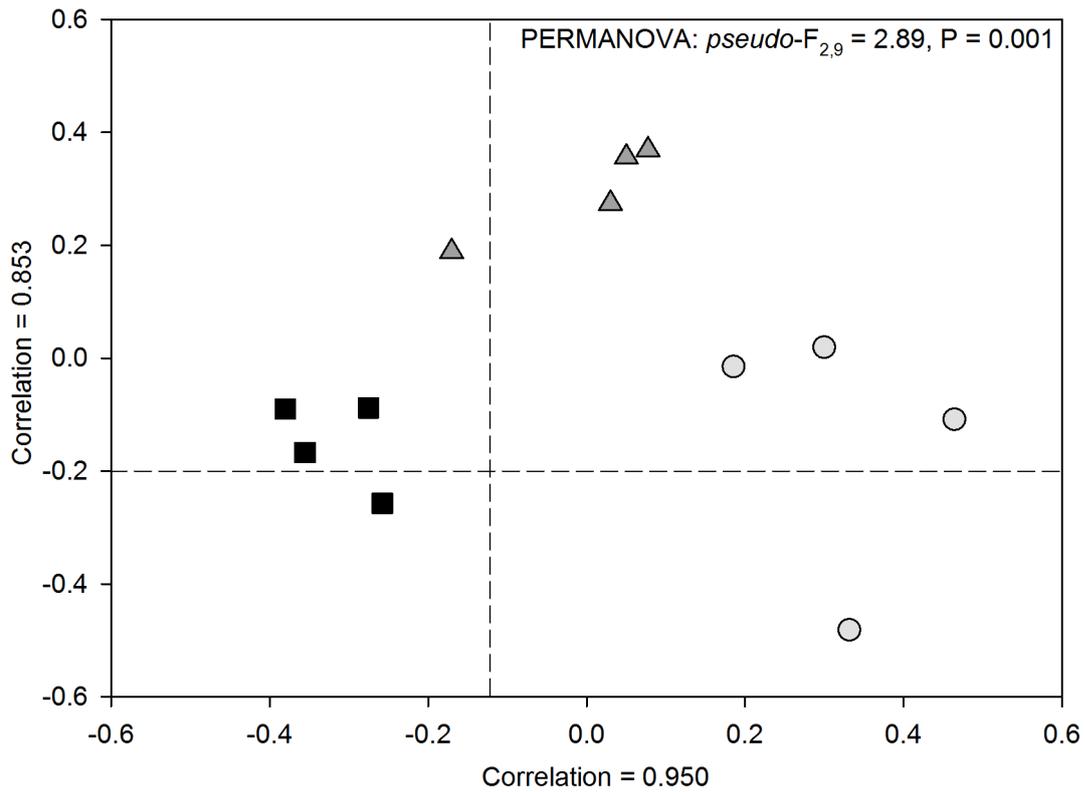
	GAEN01007405.1	Ferritin Heavy Oocyte Isoform	4.8	0.038	2.3*	1.7	0.6
DNA binding	CAD37821.1	Histone H2A	8.3	0.009	2.9*	-1.0	3.9*
	GAEN01008605.1	Cytoplasmic A3A	6.8	0.016	0.8*	0.7	0.2
	CAC94907.1	Histone H1	4.4	0.046	0.8*	0.2	0.5
Translation	GAEN01008711.1	60S Ribosomal L7	4.3	0.047	-1.5	-0.2	-1.4
Unknown	OPL21291.1	Hypothetical Protein Am593_06205	8.8	0.007	-0.9*	-0.8*	-0.2
	HE609843.1	Alpha-Crystallin A Chai	5.2	0.031	-0.9	0.3	-1.1*
	OPL32817.1	Hypothetical Protein Am593_04753	4.6	0.041	1.1	-2.1	3.1*
	GAEN01005668.1	Hypothetical Protein Brafldraft_84721	4.3	0.048	1.1	1.0	0.2
	GAEM01003123.1	Fatty Acid-Binding Brain	58.6	<0.001	4.7*	-0.1	4.8*
	GAEN01008261.1	Hypothetical Conserved 327	7.3	0.013	1.0	3.0*	-2.0
	OPL21044.1	Atrial Natriuretic Peptide Receptor A	12.7	0.002	4.9*	1.2	3.7*

816



818

819 **Figure 1.** Mean (\pm S.E.M.) tenacity (a) and number of byssal threads (b) of *M. edulis* after 52
 820 days of repeated exposure to $25 \mu\text{g L}^{-1}$ of PLA (polylactic acid) or HDPE (high-density
 821 polyethylene), or no microplastics (control). Letters denote significantly different treatments
 822 determined by posthoc tests (Tukey's).



823

824 **Figure 2.** Canonical analysis of principal coordinates (m=5, 75% of samples correctly
 825 allocated) of the composition and structure of haemolymph proteomes (using all 216
 826 identified proteins) from *M. edulis* after 52 days of repeated exposure to 25 $\mu\text{g L}^{-1}$ HDPE
 827 (high density polyethylene) microplastics (■), PLA (polylactic acid) microplastics (▲), or
 828 no microplastics (○).

829

830

831

832

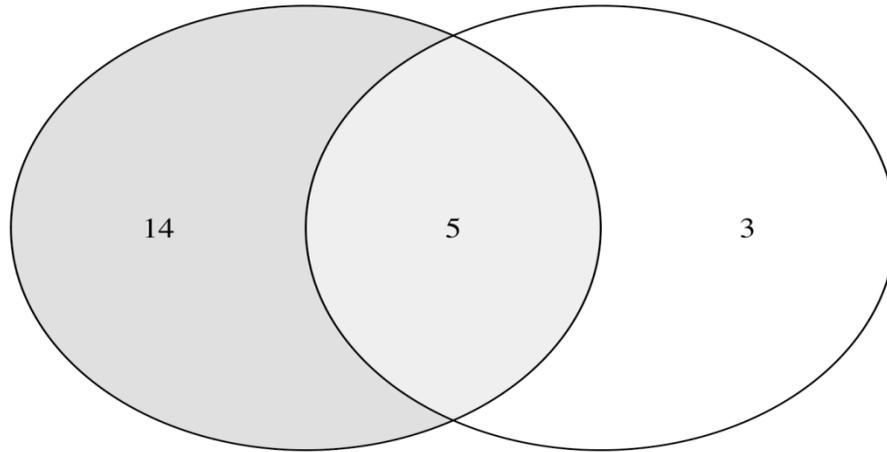
833

834

835

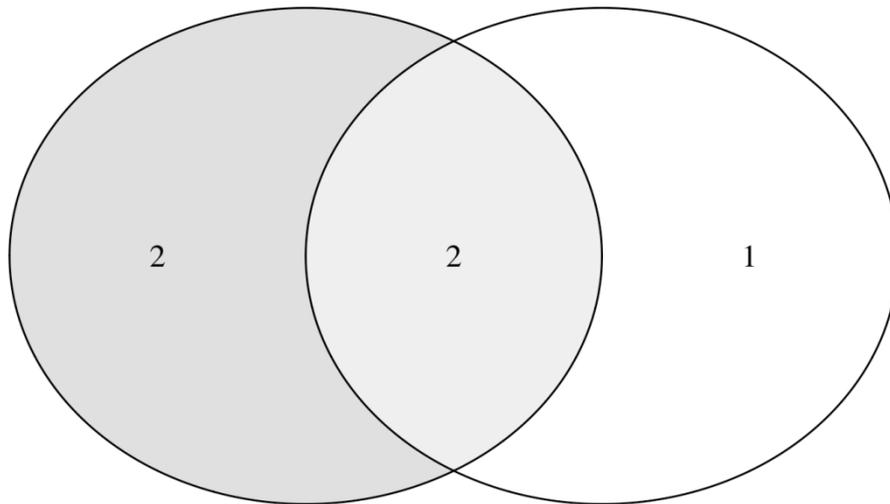
836

HDPE vs. Control PLA vs. Control



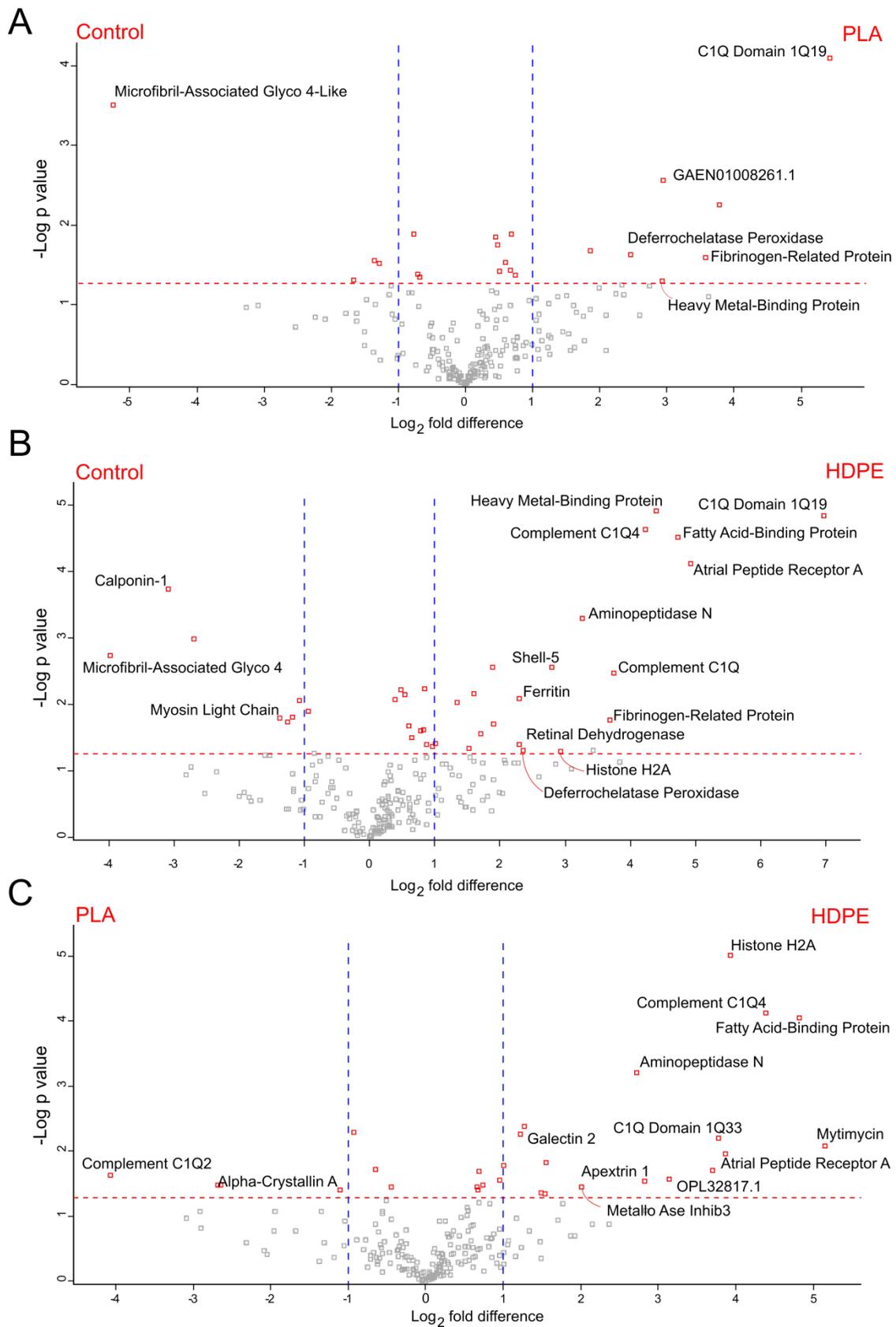
837 (A)

HDPE vs. Control PLA vs. Control



838 (B)

839 **Figure 3.** Venn Diagram depicting the number of proteins in mussel haemolymph altered in
840 abundance by exposure to either HDPE or PLA microplastics. For both treatments, the
841 number of proteins with A) increased expression, or B) decreased expression relative to
842 control treatment is shown.



843

844

Figure 4. Volcano plots based on post-imputation relative abundance of all identified proteins

845

comparing A) Control versus PLA, B) Control versus HDPE and C) PLA versus HDPE. Each

846 symbol represents a specific protein with those above the red line being considered
847 statistically significant (p -value < 0.05 based on post-hoc tests after ANOVA) and those to the
848 right and left of the vertical lines indicate relative fold changes of ≥ 2 .

849

850 **Supporting Information**

851 Table S1: 216 proteins identified with high confidence in the haemolymph of control and
852 HDPE- and PLA-exposed *M. edulis*. Normalised label free quantitative (LFQ) intensities
853 were determined for four biological replicates for each group and log₂ transformed. Missing
854 LFQ values were replaced through imputation. PEP, peptide error probability; MS/MS,
855 number of second mass spectra determined for each peptide. Additional protein annotations
856 were obtained by BLAST2Go analysis.