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1 Investigation on the proteome response of transplanted Blue mussel (*Mytilus* sp.) during a long term
2 exposure experiment at differently impacted field stations in the German Bight (North Sea)

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20 Abstract

21 In a pilot field study the proteome response of *Mytilus* sp. was analyzed in relation to the concentration
22 of different trace metal contaminants. Over a period of eight month test organisms have been exposed
23 at a near-shore station in the anthropogenic impacted estuary of the river Elbe and at an off-shore
24 station in the vicinity of the Island of Helgoland in the German Bight (North Sea). The stations differ
25 in their hydrological as well as chemical characteristics. The physiological biomarkers, such as
26 condition index which have been continuously monitored during the experiment clearly indicate the
27 effects of the different environmental conditions. Multiple protein abundance changes were detected
28 utilizing the techniques of two dimensional gel electrophoresis (2dGE) and consequently proteins
29 arising as potential candidates for ecotoxicological monitoring have been identified by MALDI-ToF
30 and ToF/ToF mass spectrometry. Different cytoskeletal proteins, enzymes of energy metabolism,
31 stress proteins and one protein relevant for metal detoxification have been pointed out.

32

33 Keywords: *Mytilus*, proteomics, bioresponse, contaminants, North Sea, electrophoresis, mass
34 spectrometry

35

36 1. Introduction

37 Mussels are well established indicators for environmental pollution in marine and coastal habitats due
38 to their sessile and filter feeding existence and their ability to bioaccumulate a multitude of either trace
39 metal as well as organic contaminants. In consequence, their body burdens provide integrated
40 information on the pollution status of the near surrounding environment. Especially the utilization of
41 transplanted mussels compensates the biological diversity and scarcity related with the use of natural
42 mussel populations as indicators, which often complicates the final data interpretation as well as the
43 wide spread application of such approaches. The purposeful deployment of transplanted mussels in an
44 in situ experiment provides important information on the bioavailability of contaminants at one hand
45 and associated possible toxic effects on the other hand and it combines the advantages of realistic

46 environmental and semi-controlled experimental conditions (Salazar and Salazar, 1995). The
47 usefulness of applying caged mussels for biomonitoring purposes have been shown in several studies
48 (Bodin et al., 2004). Along the French Mediterranean coast differently polluted areas which were not
49 sampled before could be distinguished based on such approach. A good agreement with the
50 contamination level of parallelly investigated wild population was demonstrated (Andral et al., 2004).
51 A further successful example for a caged mussel experiment to distinguish polluted from less-polluted
52 sites at the Greek Mediterranean coastline is described by (Tsangaris et al., 2010). Bocchetti et al.,
53 (2008) applied caged mussels for an integrated biomonitoring study on the impact of dredging and
54 disposal operation in harbor areas, and they showed toxic effects related to elevated levels of inorganic
55 and organic contamination in the tissues of the caged mussels.
56 A long-term biomonitoring study with transplanted *Perna perna*, integrating data on bioaccumulation
57 of different classes of pollutants with data on biomarker related to defense mechanisms, pointed out
58 differences between sampling stations, seasons and critical areas in terms of contamination levels
59 (Pereira et al., 2012).
60 Even short-term exposure experiments with transplanted mussels as bioindicators were performed to
61 assess the water quality by separating impacted areas based on physicochemical and biochemical
62 parameters (Giarratano et al., 2010).
63 In the North Sea area the pilot study performed in the BECPELAG project is an excellent example for
64 the methodological performance as well as the integrative character of such caged mussel approach
65 (Hylland et al., 2006).
66 Classically selected physiological and biochemical parameters at the organismal and cellular level are
67 utilized as responsive elements (biomarker) to indicate chemical stress. Due to the multitude of
68 potential contaminants in the marine and coastal environment and due to the complexity of organism
69 responses the analysis of a selected set of biomarkers at different levels of biological organization have
70 been strongly recommended rather than focusing on a single marker of effects. Several suggestions for
71 a rational integrated assessment of biomarker responses have been made and applied for
72 environmental monitoring projects in order to achieve a comprehensive risk assessment and in

73 consequence to finally allow a description of the environmental health status (Beliaeff and Burgeot,
74 2002; Brooks et al., 2009; Gagne et al., 2008; Narbonne et al., 2001; Yeats et al., 2008).
75 Although biochemical and cellular events tend to be more sensitive than stress indices at the whole
76 organism level, it is often difficult to find correlations with ecological impacts (Amiard-Triquet and
77 Pavillon, 2004; Viarengo et al., 2007). However, it may provide an early warning of higher order
78 biological effects.
79 Since some years state-of-the-art proteomic techniques have been providing the opportunity to observe
80 a suite of responses in form of protein expression signatures (PES) at the molecular level. The pattern
81 of molecular biomarkers plays an important role in understanding the relationships between exposure
82 to pollutants and possible responses, in revealing modes of effects and in identifying key pathways in
83 the development of diseases. Major tools of proteomics are two dimensional gel-electrophoresis
84 (2dGE) or other high resolution multidimensional protein separation techniques which provide a
85 global expression pattern of the proteins present in a sample. These techniques are combined with
86 different mass spectrometric techniques which allow the identification of the individual regulated
87 proteins.
88 Environmental proteomics examines how multiple abundance changes are associated with a
89 contamination which is suspected to have a detrimental effect (Sanchez et al., 2011). Although the
90 utilization of such techniques represents a promising approach for a comprehensive assessment of
91 water quality, there are only few fundamental proteome analysis studies with mussels as bioindicators
92 available in literature and most of them are based on laboratory exposure experiments (Apraiz et al.,
93 2006; Campos et al., 2012; Dondero et al., 2010; Jonsson et al., 2006; Liu et al., 2012; Lopez et al.,
94 2002; Rodríguez-Ortega et al., 2003; Shepard et al., 2000).
95 The obtained data have to be carefully analyzed according to methods consistency, reproducibility,
96 statistical significance and accuracy to balance the biological variance, to filter out pronounced effects
97 and to process qualified biomarker. Facing the challenge related with the transfer from laboratory to
98 field samples, marine proteomics is an expanding and promising molecular research tool (Slattery et
99 al., 2012).

100 Within this background the present study describes the results of a long-term field exposure
101 experiment with transplanted *Mytilus* sp. at differently impacted coastal areas of the German Bight in
102 the North Sea using a combined approach which uses either chemical and biochemical analysis of
103 inorganic contamination as well as the analysis of a molecular response in mussel tissue. The main
104 objective was to demonstrate the suitability of using protein expression signatures (PES) to distinguish
105 different anthropogenic impacted areas and to identify major differently expressed proteins as
106 potential biomarker.
107
108 ## 2. Material and Methods
109
110 ### 2.1 Field exposure and sampling
111 Cohorts of mussels of the same origin (obtained from commercial fisheries at the Island of Sylt,
112 Germany) were deployed in cages at two different field stations; one located at the Island of
113 Helgoland, German Bight and the other at the estuary of the river Elbe in Cuxhaven, Germany from
114 May 2011 to January 2012. Oceanographic data such as Sea Surface Temperature (SST) and Salinity
115 were continuously recorded using the Coastal Observing System for Northern and Arctic Seas
116 COSYNA powered by the Helmholtz-Zentrum Geesthacht Centre of Materials and Coastal Research.
117 At the station in Cuxhaven the salinity ranges vary between 10 and 25. The mussels are continuously
118 submersed. The salinity at the off-shore station Helgoland was constantly above 30. The SST was
119 slightly higher at the station Cuxhaven at the beginning of the field exposure experiment with 17°C
120 compared to 14°C at Helgoland. A maximum of 20°C at Cuxhaven and 17°C at Helgoland was
121 reached in August and the temperature dropped to 5°C in Cuxhaven and 7°C at Helgoland in
122 December. Mussel sampling occurred every 6 weeks over an exposure period of eight month. The
123 sampling at Helgoland was done by the group of Scientific Diving, Alfred Wegener Institute for Polar-
124 and Marine Research Bremerhaven, Station Helgoland. After the recovery of the mussels from the
125 submerse station, the mussels were placed in filtered Helgoland seawater in a flow through tank over
126 night and shipped under cooled conditions to Cuxhaven . The cohort of mussels at Cuxhaven was

127 accessible via an elevator construction and both sample groups were transported to the laboratory
128 simultaneously wetted and cooled with respect to minimize stress related effects. Due to logistical
129 circumstances the mussels from Helgoland were transported about 4 h longer than the mussels from
130 Cuxhaven. The organisms of both groups were kept wetted and cooled until preparation.

131 2.2 Physiological parameter

132 Composite samples each composed of ten organisms were used for the measurement of the
133 physiological parameters Condition Index (CI tissue dry weight/shell dry weight) and Gonadosomatic
134 Index (GSI wet weight gonads / wet weight soft tissue x 100) (Pampanin et al., 2005). After the
135 sample preparation the tissue and shells were dried by lyophilization for five days. The protein
136 contents of gill extracts were measured after purification using Micro-Bio-Spin 6 columns (Biorad,
137 Munich, Germany) by Bradford Protein Assay and bicinchoninic acid (BCA) assay (Thermo Scientific
138 Pierce ™, Dreieich, Germany) using Bovine serum albumin (BSA ACS chemicals) as protein
139 standards (Bradford, 1976). Protein values were calculated as mean of one Bradford and two BCA
140 assay with 3 replicates each,

141 2.3 Trace element analysis of the whole mussel tissue

142 To avoid any contamination of the tissue samples every mussel was flushed with MilliQ water before
143 the opening of the shell. A cleaned ceramic knife was applied for the opening as well as for the tissue
144 removal from the shells in order to minimize trace element contamination. After the opening of the
145 shell the inside of the shell as well as the whole soft tissue were flushed with MilliQ water to remove
146 any remaining particles or sea water residues. Composite samples of the whole wet tissue were
147 homogenized using 50 mL in TubeDrive® devices (IKA, Staufen, Germany), freeze dried and
148 homogenized again in the same tubes. Three mussels were pooled per sample and three biological
149 replicate samples were produced. For trace element analysis an aliquot of approximately 200 mg dry
150 material was digested by microwave accelerated digestion (CEM, Kamp-Lintfort, Germany) using a
151 mixture of 5 mL HNO_3, 2 mL HCl and 1 mL of H_2O_2 and a temperature program according to the
152 EPA method 3052 (U.S. Environmental Protection Agency, 1996). All used acids and reagents were of
153 highest available purity to minimize blank levels (doubly subboiled or ultra pure quality). Indium and

154 rhodium were added as internal standard to the digestion solution. After digestion the samples were
155 quantitatively transferred into graduated 50 mL DigiTubes and filled up to 50 mL to reduce the
156 concentration of the acid. The diluted samples were measured using inductively coupled plasma
157 tandem mass spectrometry (Agilent 8800) (Pröfrock and Prange, 2012). An external calibration was
158 performed for quantification, which utilizes different multi element solutions to cover the targeted
159 analyte range.

160 2.4 Proteome analysis

161 2.4.1 Protein extraction

162 The gills of the fresh mussels were prepared and conserved individually at -80°C until further use. The
163 organs of three organisms were pooled and homogenized in a 25 mL Tube Drive ® device in 3 strokes
164 (1min at the highest rotation speed setting) followed by a cooling period on ice. A lysis buffer
165 containing 9 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS),
166 2% sodiumdesoxycholate, 1% dithiotreitol (DTT) and 2% ampholyte pH 3-10 was added in a wet
167 tissue – buffer ratio of 0.1 g /0.5 mL. The homogenates were further processed with the TubeDrive ®
168 homogenizer and a cooled ultra sound disintegrator (Branson Sonifier 450, G. Heinemann Ultraschall-
169 und Labortechnik, Schwäbisch Gmünd, Germany). The produced extract was centrifuged at 4°C for 30
170 min at 11000rpm in order to obtain a clear supernatant. Pre-concentration and protein purification was
171 achieved by precipitation with trichloracetic acid (TCA).

172 2.4.2 Two – dimensional gel electrophoresis

173 The dried TCA pellets were solubilized in rehydration buffer measuring 1 mg pellet in 200 µL buffer
174 at 4°C for 96h. The solubilized sample was finally applied on 11 cm IPG Strips pH 5-8 (BioRad,
175 Munich, Germany). Active rehydration was performed overnight. Isoelectric focusing was performed
176 on a Protean IEF Cell (BioRad) at 20 °C using the following program: rapid voltage slope at step 1:
177 200 V for 30 min; step 2: 500 V for 30 min; step 3: at 1000 V for 30 min; linear voltage slope at step
178 4: 8000V for 30 min, and step 5: at 8000V until it reached 35 000 Vh. Focused IPG Strips were kept
179 frozen at -80°C until further use. Frozen IPG strips were thawed, reduced (130 mM DTT) and then

180 alkylated (135 mM IAA) in equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 30% glycerol (v/v),
181 and 2% SDS (w/v) for 15 min, each. The second dimension was carried out on anykDa TGX Criterion
182 precast gels (BioRad,), at 120 V for 10 min and 200V for 40 min using a Criterion Dodeca Cell
183 (BioRad) thermostated at 15°C via an external cooling device. A protein marker covering the range of
184 6.5 kDa – 200 kDa was applied on each gel allowing the estimation of molecular weights of the
185 separated protein spots (AppliChem, Darmstadt, Germany).

186 2.4.3 Image acquisition and analysis

187 The protein spots were visualized by Coomassie Brilliant Blue CBB G250 staining according to (Kang
188 et al., 2002). The acquisition was done by utilizing a Proteineer SPII automated scanning and spot
189 picking system (Bruker Daltonics, Bremen, Germany). The software Delta2d 4.4 was applied for
190 image analysis and spot pattern comparison following the regular work flow (Decodon, Greifswald,
191 Germany). The spots were selected and sliced manually as well as automatically and further processed
192 for mass spectrometric analysis.

193 2.4.4 Protein identification by MALDI-ToF and -ToF/ToF mass spectrometry

194 The protocol for this process was modigied after Lassen et al., (2011). The destaining of protein gel
195 spots was performed by alternate washing with 40 mM ammonium bicarbonate and 40 mM
196 ammonium bicarbonate/acetonitrile (ACN) solution (1:1 v:v). The proteins were digested overnight at
197 37°C in a volume of 5 µL of 20 µg mL^{-1} trypsin solution (proteomic grade, Sigma-Aldrich, Munich,
198 Germany). The tryptic peptides were extracted by adding 10 µL 30% ACN/ 1% trifluoracetic acid
199 (TFA) for 30 min. After mixing the extracts with ACN (1:1 v:v), the MALDI matrix α-cyano-4-
200 hydroxy cinnamic acid (HCCA; 1 mg mL^{-1} ethanol:acetone 2:1 v:v) was added: Aliquots of 1 µL were
201 spotted on an 600 µm AnchorChip(TM) target (Bruker Daltonics, Bremen, Germany). Crystallisation
202 occurred at room temperature. Subsequently, an on–target washing step with 10 mM ammonium
203 phosphate (monobasic) in 0.1 % TFA and a recrystallization step with ethanol/acetone/0.1 % TFA
204 (6:3:1, v/v/v) were executed. The mass spectrometer was externally calibrated with the Peptide
205 Calibration Standard II (Bruker). Peptide mass fingerprints (PMF), and peptide fragmentation
206 fingerprints (PFF) were acquired using an Ultraflex II mass spectrometer (Bruker) controlled by the

207 Flex control software 3.1. The mass–to-charge ratios (*m/z*) in the resulting MS and MS/MS spectra
208 were annotated using the FlexAnalysis 3.0 software (Bruker) and further processed using BioTools 3.1
209 software (Bruker). For protein identification via the Mascot peptide mass fingerprint and MS/MS ion
210 search option (www. matrixscience.com) the peptide mass and the fragment mass tolerance were set to
211 50 ppm and 0.5 Da, respectively. At most, one missed cleavage for tryptic peptides was allowed.
212 Carbamidomethylation of cysteine, oxidation of methionine, and the formation of pyro-glutamic acid
213 from N-terminal glutamine (Gln→pyro-Glu (N-term Q)) were selected as possible modifications.
214 Databases used for the identification of homologous proteins were Swiss-Prot and NCBInr (National
215 Center for Biotechnology Information).

216

217 ## 2.5 Statistics

218 Physiological parameters were calculated from ten individuals and compared by using winSTAT ®
219 add on for Microsoft® Excel version 2012.1. For chemical analysis of inorganic contaminants, three
220 biological replicates, each consisting of three organisms, were measured in three technical replicates.
221 Mean and standard deviation were calculated from nine values. Significant differences were calculated
222 utilizing the non-paramaterical Mann – Whitney test.
223 The proteome analysis was performed with two biological replicates per sample group (primary
224 samples, Cuxhaven and Helgoland) each composed of pooled samples of three organisms. Overall six
225 technical replicates per sample group were analyzed in two independent 2d gelelectrophoresis
226 experiments. The statistical protein expression signature analysis as well as the Principal Components
227 Anaylsis (PCA) was performed with the Delta2d software 4.4 following the software work flow.
228 Significant differences in the protein abundance were calculation based on the normalized spot
229 volumes utilizing a students t-test provided by the Delta2d software.

230

231 # 3. Results

232 ## 3.1 Physiological Parameter

233 In order to obtain supplemental data on the physiology and growth of the transplanted mussels during
234 the exposure period, standard parameters were measured and summarized in table 1. The condition
235 index differed significantly in the bivalves transplanted for eight month at the stations in Helgoland
236 and Cuxhaven. In comparison to the primary samples, the organisms exposed in Cuxhaven indicated a
237 reduced fitness.
238 Due to the high biological variance, the GSI value showed no significant differences, but a tendency
239 for an improved reproduction rate of the Helgoland mussels can be assumed.
240 An increased protein content of the extracts and consequently of the gill tissue can be detected in the
241 mussels transplanted for eight month to the off-shore station at Helgoland. The protein value of the
242 mussels transplanted to Cuxhaven was slightly reduced compared to the primary samples. The
243 different extraction of the two pools of three individuals fitted and showed the same tendency.
244
245 3.2 Chemical contamination

246 The multi-element analysis of whole tissue preparations showed a broad spectrum of enriched
247 elements. A summary of the whole dried tissue concentration of selected priority and environmentally
248 relevant toxic elements is given in table 2. The elements arseniccadmium, chromium, iron, manganese,
249 molybdenum, nickel, lead, selenium and zinc were significantly enriched in the whole dry tissue
250 samples of mussels exposed at Cuxhavenin comparison to the primary and Helgoland samples. The
251 tissue of mussels exposed at the Helgoland off-shore station showed a lower element content. In
252 comparison to the primary samples only an increase of selenium and molybdenum concentrations over
253 the period of eight month was detected.
254
255 3.3 Protein expression signature analysis
256 The sample processing procedure of mussel gill tissue and the application of a pI range of 5-8 and
257 gradient anykDa SDS PAGE resulted in a reproducible and well distributed spot pattern for each
258 separated sample. In the fusion of all gel images including the two different extractions and gel
259 replicates of the primary, Cuxhaven and Helgoland samples, 215 spots were detected automatically,
260 refined manually and selected for the protein expression signature analysis of the different treated

261 mussel samples. An exemplary gel image of the primary group, used as "mastergel" in the delta2d
262 workflow is presented in figure 1A. Furthermore, exemplary gel images representing the proteome of
263 the gill tissue at the stations Helgoland and Cuxhaven after 8 month of field exposition are
264 documented in figure 1B and 1C.
265 The protein spot pattern of all samples was very similar and the majority of relative spots intensities
266 was consistent. When comparing the relative spot intensities, 37 spots (17.2%) differ significantly
267 between primary and Helgoland samples. Seventeen proteins were up- and 20 down-regulated. Twenty
268 spots (9.3%) were significantly differently expressed in the primary and Cuxhaven samples, which
269 showed 7 up- and 13 down-regulated.
270 The coefficient of variation of relative spot volumes was high. The number (percentage) of spots with
271 a relative variance below 25 was 117 (54.4%) for the primary, 95 (44.2%) for Cuxhaven and 115
272 (53.0%) for Helgoland samples.
273 Although the spot pattern looked similar and the gel to gel variance of spot volumes was high, the
274 samples can be grouped according to a PCA analysis performed as part of the delta2d software work
275 flow (figure 2) allowing the differentiation of the two field stations.
276
277 3.4 Protein identification by mass spectrometry
278 The spots for protein identification were selected according to their abundance, quality of separation
279 and significance of sample group specific expression. Overall, 22 spots have been selected and 15
280 proteins belonging to four major functional groups were identified, as summarized in table 3. These
281 proteins were actin, tubulin, tektin and paramyosin as components of the cytoskeleton, the enzymes
282 ATPsynthase subunits α and β, isocitrate dehydrogenase (IDH) and enolase, a protein able to bind
283 divalent ions called heavy metal binding protein and well known stress proteins. The mass
284 spectrometric analysis of the tryptic in-gel digestions using small volumes of trypsin-, peptide
285 extraction and washing solutions resulted in mass spectra (PMF and PFF) with sufficient intensities
286 suitable for database searches and final protein identification. A successful protein identification
287 included the PMF and/or PFF both with hits of significant scores ($p<0.05$) originating preferably from

288 bivalve species or at least from the phylum mollusks. The relevant proteins were confirmed at least in
289 two of the three different sample types (primary samples, Cuxhaven and Helgoland).
290 There were a few more interesting proteins arising as molecular biomarker candidates, e.g. peptidyl-
291 prolyl cis-trans isomerase (PPI), major vault protein (MVP) and guanine nucleotide-binding protein
292 subunit β (GbP β). However, they do not conform one of the restricted criteria, e.g. two sample
293 groups, score and class bivalvia. These criteria were selected in order to ensure a certain level of
294 reasonable and reliable information.
295 The protein abundance profiles of the identified spots were documented in the diagram in figure 3. Out
296 of these spots, G16, G34 and G37 were significantly down-regulated and G18 and G44 up-regulated in
297 Helgoland samples, whereas only a significant down-regulation can be observed for G13 and G34 in
298 gill tissue from Cuxhaven samples.
299
300 4. Discussion
301 4.1 Chemical stress
302 The two stations can be distinguished according to the hydrological, biological and physical data,
303 lower and oscillating salinity at Cuxhaven, as well as higher temperatures in spring and summer. Due
304 to the higher current and the concentration of suspended particulate matter, the turbidity in Cuxhaven
305 was much higher. Biofouling can be observed at both stations. In Cuxhaven it originated mostly from
306 barnacles whereas soft matter covered the cages at Helgoland. Consequently, different food
307 availability and therefore nutrition status have to be considered for the assessment of physiological and
308 biochemical parameters (González-Fernández et al., 2015).
309 The chemical contamination of the marine and coastal environment holds a continuous risk of toxic
310 effects on organisms, bioaccumulation of pollutants along the food web and impacting ecological
311 functions of habitats. According to the water framework directive (WFD), only cadmium, lead, nickel,
312 mercury, tin and their organic species belong to the list of priority substances. However, the spectrum
313 of inorganic substances which have to be observed due to their ecotoxicological potential is much
314 broader.

315 Environmental assessment criteria (EAC) (Ospar commission CEMP assessment criteria agreement
316 2009-2) for mussel tissue are available for cadmium (960 - 5000 µg kg^{-1} dry weight) and lead (1300 –
317 7500 µg kg^{-1} dry weight). The detected values at both stations (table 2) were below these limits. The
318 German Monitoring program (BLMP) status report for the North Sea 1999 – 2002 characterized the
319 Elbe estuary as a strong source for cadmium input into the North Sea which is reflected by the four
320 times increased concentration in whole mussel tissue from Cuxhaven compared to Helgoland
321 (Schmolke et al., 2005).
322 The picture is slightly different for lead. The BLMP report recorded a stagnation of lead reduction for
323 the last three decades and still an increased sediment concentration was observed in the North of
324 Helgoland. However the detected whole tissue concentrations were about the half compared to
325 Cuxhaven.
326 Especially in coastal areas the input of copper is on the rise caused by antifouling paintings and
327 coatings. The values for copper concentrations detected in mussels exposed at Helgoland and
328 Cuxhaven were similar and did not reflect the situation in the water phase with elevated concentration
329 in the Elbe estuary and natural mussels populations (BLMP report).
330 Likewise, zinc is a cofactor of enzymatic activities with low toxic potential in its inorganic
331 formulation. Opposite to the present data on transplanted mussels, the BLMP report documented
332 higher tissue concentrations in natural mussels obtained from Helgoland compared to samples from
333 the North Frisian Wadden Sea. The analysis of trace elements in mussels from German coastal waters
334 by instrumental neutron activation analysis (INAA) dated in 1978 provided an interesting opportunity
335 to compare recent data with that from 40 years ago (Schnier et al., 1978). A remarkable reduction was
336 detected for the elements Ag, Cd, Cr, and Zn at Helgoland. Almost the same level was observed for
337 Se, As, Ni, and Co tissue concentrations.
338 The Western Scheldt estuary at the Dutch North Sea coast can be seen as comparably highly impacted
339 estuary of anthropogenic and industrial use as the Elbe estuary. In order to show the recreation effect
340 of pollution reduction from the beginning of the 80ies in the last century, wild mussels were used as
341 bioindicators. In a long-term study until 2002, a reduction of the bioaccumulation of heavy metals

342 apart from Cr and Zn was observed (Mubiana et al., 2005). The determined values are generally in the
343 same order of magnitude as in the present study, although Cd and Cr were slightly reduced.
344 While analyzing internal and literature data it has to be taken into account that the metal
345 bioavailability decreases with increasing salinities, that there is a seasonal variation in metal uptake,
346 and that the principles of utilizing wild and transplanted mussels are different.
347 The contamination with persistent organic pollutants (POPs) was not an issue of this study. Data on
348 the water and sediment concentrations of priority substances are available from regular monitoring
349 programs combined e.g. in the BLMP status report for the North Sea 1999 – 2002 (Theobald and
350 Loewe, 2005). The rive Elbe is the major source for most of the analytes. Therefore the concentration
351 of non-polar lipophilic chlorinated hydrocarbons and polycyclic aromatic hydrocarbons is relatively
352 high in the Elbe estuary but is rapidly reduced from costal to off-shore regions. Therefore an impact of
353 organic compounds on the mussel physiology at the station in Cuxhaven can be assumed.
354

355 4.2 Proteome analysis and protein identification

356 The application of proteomic techniques for the identification of environmental stress indicators is a
357 rapidly expanding and powerful molecular research tool. Although *Mytilus* sp. is a sentinel organism
358 suited for ecotoxicological studies, only a fistful of proteins have been identified and verified as
359 responsive elements for environmental and chemical stress. Impact on mussel proteome often becomes
360 more manifest in form of protein modifications like ubiquitination or carbonylation and it is suggested
361 that the proteome is quite resistant to changes in the sense of absolute quantities (Dowling and
362 Sheehan, 2006). However, the PES in the present experiment showed a distinct pattern within the three
363 different sample groups. An improvement of the 2dGE technique towards a Difference Gel
364 Electrophoresis or a fluorescence prestaining of the samples could reduce the implication of staining
365 procedures and hence the number of necessary technical replicates. Thoroughly considerations should
366 be set on the consistency of sample preparation. The time-shifted treatment of the mussels of the
367 different sample groups might also influence the stress response on the protein level.
368 Comparing the PES is a useful instrument to get an overview but the challenge is to show their
369 feasibility for analyzing complex environments under multiple stressors (Bradley et al., 2002). PES

370 enable the selection and consequently the identification of proteins which is essential for
371 understanding response processes and functions.
372 Cytoskeletal proteins, playing a central role in essential physiological processes like intracellular
373 vesicle and organelle transport, cell motility and plasticity, were the most prominent functional group
374 of identified proteins. Among them were actin, tektin, β-tubulin and paramyosin. Actin is identified in
375 almost all bivalve proteome studies. This might be due to its high abundance but in particular the actin
376 cytoskeleton system is one of the first targets of oxidative stress in eukaryotic cells which results in
377 remarkable changes in their morphology and structure of the microfilament network (Dalle-Donne et
378 al., 2001; Fagotti et al., 1996). The expression profile of actin seems to be different in gills and
379 digestive glands as pointed out in a cadmium exposition study by (Chora et al., 2009). Actin is
380 impacted by exposition to copper nanoparticles (Gomes, 2014), copper salts as well as TBT, arsenic
381 and Aroclor (Rodríguez-Ortega et al., 2003) and North Sea oil (Manduzio et al., 2005).. The function
382 and aggregation of actin in the oyster *Crassostrea gigas* cultivated in urban sewage was influenced by
383 carbonylation resulting in a down-regulation of this protein (Flores-Nunes et al., 2014). In the present
384 study, all identified actin isoforms were decreased in the samples at Cuxhaven in comparison to the
385 samples obtained from Helgoland which might be an indication of the different chemical pollution
386 situation at both stations combined with oxidative stress.
387 The microtubules-forming cytoskeletal protein β-tubulin has been identified from two corresponding
388 spots G14 and G24 which were not completely resolved by isoelectric focusing at the edge pH 5. An
389 extension of the pH range using a micro range IPG strip e.g. 4.7 – 5.9 can be helpful to distinguish
390 these isoforms. In several contaminant-exposure experiments a down-regulation has been found for
391 this protein under chemical pressure (Apraiz, 2006; Chora, 2009). Combining the spot intensities of
392 G14 and G24, a reduced concentration of tubulin in Cuxhaven compared to the Helgoland *Mytilus* gill
393 samples has been detected which might correspond to the enlarged metal concentration.
394 Tektins are associated with tubulin in the microfilaments and they are present in the cilia of mussel
395 gills. The cilia are directly exposed to external medium and responsible for feeding and respiration.
396 These cytoskeletal proteins were not identified in *Mytilus* sp. up to now, however, there is a hint for a

397 down-regulation in oyster larvae due to mercury exposure and it seems that ocean acidification and
398 intensive air exposure also decrease the tektin expression (Dineshram et al., 2012; Fields et al., 2014)
399 Paramyosin is most abundant in tissue from the aductor muscle and have been found in gill tissue only
400 in traces. According to the relative high spot intensities reflecting the paramyosin tissue concentration,
401 an impurity during preparation can not be excluded. The intact paramyosin, isolated from the bivalve
402 *Mercenaria mercenaria* has a molecular weight of 220 kDa which corresponds to spot G47 consisting
403 of two interwinded identical α-helical polypeptide chains visible at spot G46 (Watabe et al., 2000). A
404 down-regulation of paramyosin extracted from digestive glands has been detected in a Cu-nanoparticle
405 exposition experiment performed by Gomes et al (2014). Due to the high gel-to-gel variance of spot
406 volumes, a significant impact can not be pointed out in the present study.
407 A further important functional group of proteins were stress proteins in form of Heat shock cognate 71
408 (HSC71), Heat shock protein 60 (HSP60), Heat shock protein 70 (HSP70) and the enzyme peptidyl-
409 prolyl cis trans isomerase (PPIase). The latter acts as chaperone, accelerating the folding of
410 oligopeptides N-terminal to proline.
411 PPIases are a conserved group of enzymes playing critical roles in regulatory mechanisms of cellular
412 function and pathophysiology of diseases. A study dedicated to the immune response of *Mytilus*
413 *galloprovincialis* to bacterial infection reveal the enzyme PPIase as sensitive element where a down-
414 regulation implies a disturbance in protein synthesis especially in gill tissue (Ji et al., 2013). In an
415 exposition experiment with the pharmaceutical substances diclofenac and gemfibrozil an increase of
416 this enzyme was detected (Schmidt et al., 2013). PPI was also recognized with higher abundancies
417 caused by benz(a)pyrene (BaP) and phenanthren exposition used as environmentally relevant model
418 contaminants in combination with tidal stress (Letendre et al., 2011). However, up to now there is no
419 further information available in the literature about a relationship of PPIase expression caused by
420 environmental pollution. In the present study a slightly increased content of PPIase in gill tissue
421 obtained from Helgoland samples can be observed and this protein could only be identified from spot
422 samples obtained from the Helgoland treatment group. Since this group of enzymes include important
423 targets for pharmaceutical therapeutics, further studies about the interaction with small bioactive
424 molecules and an organismal reaction in the marine environment would be interesting.

425 Heat shock proteins are well known elements indicating environmental stress (Monsinjon and Knigge,
426 2007). This family of proteins expresses a chaperone function by stabilizing new proteins to ensure
427 correct folding or by helping to refold proteins that were damaged by harsh living conditions. It is
428 known that in stressed cells the level of misfolded, aggregated or malfunctional proteins is increased
429 and therefor the necessity for helper molecules like chaperones also increases. Abiotic factors as
430 temperature and salinity as well as an intertidal habitat induce the HSP 70 synthesis (Lesser et al.,
431 2010; Lopez et al., 2001). Chapple et al., (1997) could show a tissue specific expression and especially
432 in gill tissue the HSP70 is increased within 48h after a temperature shift. A comprehensive study
433 comparing field and laboratory responses to metal contamination in relation to thermal stress pointed
434 out the potential of HSP70 isoforms as biomarker of marine pollution (Micovic et al., 2009). A
435 laboratory study focusing on HSP70 revealed the impact of BaP on the inducible HSP70 and the
436 constitutive HSC71 (Jurgen et al., 2011). An outcome of this study includes the hypothesis that HSC
437 levels reflect the capability of tissues for normal protein synthesis and that instead the amount of the
438 inducible isoform HSP represents a sensitive index of adaptation to stress. In addition to the study
439 especially dedicated to HSP, several laboratory exposition experiments can be found in recent
440 literature demonstrating the expression of HSP for example due to BaP and Cu^{2+} (Maria et al., 2013),
441 copper nanoparticles (Gomes et al., 2014), silver and silver nanoparticles (Gomes et al., 2013),
442 mercury (Zhang et al., 2013), TBT (Steinert and Pickwell, 1993), PCB and PAH in combination with
443 contaminated or suspended in sediments (Cruz-Rodríguez and Chu, 2002; Olsson et al., 2004) and
444 surfactants like SDS (Messina et al., 2014). In summary, HSP’s are one of the most potential
445 candidates as molecular biomarker of environmental stress in marine environments since antibodies
446 for immunological detection and specific quantification are available which enable a reliable
447 determination of this protein family (Hamer et al., 2005). In the present study all identified HSP
448 (HSC71, HSP70 and HSP60) were detected with higher abundances in the primary and Cuxhaven
449 samples compared to the gill tissues in mussels from the Helgoland station indicating an elevated level
450 of environmental stress.

451 Up to now the mitochondrial Enolase from *Mytilus* sp. has not been identified within the context of
452 environmental proteome studies. Toxicology studies with contaminated sediment showed a down-

453 regulation of enolase as metabolic enzyme in the flatfish *Solea senegalensis* (Costa et al., 2012). Also
454 methyl-parathion induces a down-regulation in *Sparus latus* liver and this enzyme is recognized as
455 target of oxidative stress (Chen and Huang, 2011). However, a significant impact can not be
456 documented in the present field study. Beyond the critical bioenergetic role of this enzyme, a second
457 important function as carrier for tRNA is supposed for *Saccharomyces* (Brandina et al., 2006). Due to
458 the association of this enzyme of the glycolytic pathway and the supporter function for protein
459 synthesis an up-regulation of this protein would be expected as protective responsive element.
460 Supplementary studies are necessary to reveal the role of enolase as potential contaminant-related
461 stress marker in bivalves.
462 Further identified enzymes with important functions within the context of the energy metabolism were
463 two subunits of ATPsynthase with spot G16 representing ATPsynthase subunit α and G44 identified
464 as ATPsynthase subunit β. Both subunits build the F1 unit located in the matrix of mitochondria
465 catalyzing the formation of the energy-storing molecule ATP. ATPsynthase has been identified as
466 potential indicator for chemical stress in few studies. Organotin compounds act as inhibitors of the
467 ATPsynthase in mussel digestive glands (Nesci et al., 2011). Apraiz et al. (2006) found a reduction of
468 this enzyme complex due to mussel exposition to BaP and brominated flame retardants. In contrast
469 Maria et al. (2013) found a significant up-regulation in *M. galloprovincialis* tissue after BaP
470 exposition that is explained by a higher energy consumption in gill tissue. Opposite to these findings,
471 in a field study with oysters deployed at metal-polluted sites a mitochondrial ATPase was down-
472 regulated (Liu and Wang, 2012). The ATPsynthase β chain was not influenced by North Sea oil
473 (Manduzio et al., 2005). A comprehensive exposition experiment utilizing Cu, Cd, Pb and Zn salts in
474 Sidney Rock Oysters showed the complex response of animals to the different metals (Thompson et
475 al., 2012). In the present field experiment a six to seven fold higher abundance of the ATPsynthase β
476 compared to the α-unit can be observed. The ATPsynthase subunit α seems to be balanced whereas the
477 subunit β is elevated in mussel gill tissue from both stations Cuxhaven and Helgoland. Because of all
478 these inconsistent findings, further investigations are necessary to reveal the impact on this important
479 molecule for energy metabolism and its feasibility as marker of environmental or even chemical stress.

480 Another identified protein belongs to the enzyme family of isocitratedehydrogenases (IDH). These
481 enzymes play a crucial role in the energy metabolism and catalyze the decarboxylation of isocitrate
482 under production of the energy equivalent NADPH as a major step in the citrate cycle. NADP-
483 dependent isocitratdehydrogenase was isolated from the hepatopancreas of *Mytilus* and a molecular
484 weight of 45-50 kDa was assigned (Head, 1980). It fits to spot G37 which was identified as IDH with
485 a reduced intensity in Helgoland samples compared to the primary and Cuxhaven samples. In an
486 exposition experiment with the pharmaceutical diclofenac an increased abundance of IDH was
487 measured by proteomic analysis (Jaafar et al., 2015). NADPH is also needed as cofactor for oxidative
488 biotransformation e.g. performed by glutathionreductase and therefore may be involved in
489 antioxidative processes. A higher IDH activity in digestive glands of caged mussels in Brest Harbor
490 (France) was measured by enzyme assays whereas the enzyme activity in gill tissue was not impacted
491 (Lacroix et al., 2015). Additionally in a field study analyzing wild mussels collected along the
492 Portuguese North West Coast impacted by petrochemical industries an elevated enzyme activity of
493 IDH was detected (Lima et al., 2007). These findings suggest a relationship of increased requirement
494 of redox equivalents under chemical stress conditions.
495 Only two of the identified proteins can be related to the biological function of detoxification. The
496 spots G45 and G34 showed homologies to a heavy metal binding protein (HIP) isolated from the
497 hemolymph of *Mytilus edulis*. Since it is supposed to be a glycoprotein, different isoforms might occur
498 resulting in corresponding spots at the 2d gel image (figure 1A). A specific pre-concentration utilizing
499 carbohydrate-selective lectin affinity separation and a narrow focusing pH range would be helpful to
500 separate and consequently characterize this interesting substance. The protein matches to a Ca-binding
501 protein from the extrapallial fluid (EP protein), playing a role in shell mineralization. However, it is
502 also able to bind other bivalent ions including Cu^{2+} and Cd^{2+} (Yin et al., 2005). This property
503 hypothesized a function as transport and detoxification protein although a significantly different
504 abundance can not be demonstrated in the present study due to the high spot intensity variances. For
505 this HIP-like protein extracted from gill tissue a molecular weight of about 33 kDa and pI of near 5
506 can be estimated (figure 1A) that fits very well to the characteristics of the EP protein (Yin et al.,
507 2005).

508 By investigating *Mytilus* gill tissue as target of oxidative stress the HIP was identified to be oxidized
509 and to build disulphide bridges as response to reactive oxygen species (McDonagh and Sheehan,
510 2007). A further protein also called heavy metal binding protein (MW ~35 kDa/ pI ~5) occurred in an
511 exposition experiment with crude oil and off-shore production water. The induction of the metal -
512 chelating protein is explained by the presence of vanadium, nickel and molybdenum in oil (Manduzio
513 et al., 2005).
514 Within the suite of proteins identified in the proteome of mussels exposed at different coastal stations
515 for 8 month the latter is of special interest as potential indicator for chemical stress induced by metal
516 contaminants since the tissue concentration of most of the analyzed elements is elevated at the near-
517 shore station in Cuxhaven. It may be an additional protein biomarker supplementing the group of
518 metal-binding proteins the metallothioneins that are discussed as biomarker candidate (Dallinger,
519 2007).
520 The second protein, which might be related to the detoxification and therefor is of special interest as
521 molecular biomarker candidate is the major vault protein (MVP). It is a large cytoplasmatic
522 ribonucleotideprotein corresponding to spot G11 with an estimated molecular weight of 100 kDa. The
523 exact function is still unclear but it is discussed as transport protein involved in the excretion of
524 conjugated metabolites and xenobiotics (Gomes et al., 2013; Luedeking and Koehler, 2004). It has
525 been found that this protein is overexpressed due to physical stress like anaerobiosis and chemical
526 stress caused by AgNP. In the present study it could only be identified in samples obtained from the
527 impacted near-shore station Cuxhaven. However the low abundance hindered the extensive molecular
528 analysis.
529 Analyzing the sum of identified proteins and detected proteome changes, a more stressful situation for
530 the caged mussels at the Cuxhaven station can be assumed due to higher intensities of stress proteins
531 and slightly lower levels of cytoskeletal proteins. Environmental studies often have shown the need to
532 integrate abiotic and biotic interactions in order to elucidate biomarkers of pollutant exposure (Bodin
533 et al., 2004). Abiotic conditions like salinity and temperature as well as the reproduction cycle as
534 intrinsic factor are especially important for the fitness and physiological adaptation processes of
535 mussels (Jarque et al., 2014; Tomanek et al., 2012). In comprehensive environmental monitoring

536 programs when a wide range of certain parameters is observed, a great variability in pollution
537 biomarker is determined and factors like food availability may mask the effect of contaminants on
538 biomarker regulation (González-Fernández et al., 2015). Since the two stations differ in salinity and
539 temperature as observed by the COSYNA monitoring program and the preparative sample treatment
540 was different, the proteome changes might also be partly related to these hydrological and
541 methodological parameters rather than to the determined chemical variances.
542

543 ## 5. Conclusion

544 Any animal response to variable stressors results in a complex reaction on different levels influenced
545 by many internal and external factors. Therefore, a direct correlation between chemical pollution of a
546 marine habitat to changes in the proteome of sentinel organism is difficult to establish even as a result
547 of long term field studies like in the present work. The localization of the stations and their technical
548 construct are suited to realize caged mussel experiments in order to indicate chemical stress due to
549 their different contamination level. These differences were reflected in the physiological condition
550 index as well as in the protein concentration and pattern of gill tissue that differs in samples from both
551 stations. A few protein biomarker candidates for environmental stress have been identified. The heavy
552 metal binding protein and the enzymes belonging to the energy metabolism are of special interest in
553 this context. However, a correlation to chemical stress and a verification of the impact of the natural
554 environmental conditions at the two routinely conducted near- and off-shore stations has to be
555 performed. In addition, controlled laboratory experiments under simulated environmental conditions in
556 terms of temperature, salinity, food availability etc., which cover different contamination situations
557 might be a suitable tool to better understand the role of the differentially expressed proteins as well as
558 their suitability as potential biomarkers.
559
560

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Figure 1

Exemplary gel images of gill tissue extract prepared from (A) primary samples (t=0), (B) Helgoland station and (C) Cuxhaven station (t = 36 weeks), identified spots were labelled in fig 1 this gel was used as master image

Figure 2:

PCA analysis of spot pattern according to the delta2d software workflow, each symbol represents a technical replicate

Figure 3:

Relative spot intensities of the identified proteins. Data represent the mean normalized spot volume calculated on the basis of the technical replicates T0, Cux n=6, Hel n=7, Mean (SD) * P>0,05 t-test

Table 1:

Physiological indices of *Mytilus* sp. exposed for eight month at a near- (Cuxhaven) and off-shore (Helgoland) field station and protein content of their gill extracts

Sample	CI[a]	GSI (%)[a]	Protein content of gills (mg/g_{ww})[b]
Primary samples (T0) t=0	0.207 (0.042)	20.505 (5.335)	70.50 76.56
Cuxhaven (Cux) t=36weeks	0.150 (0.018)*	21.536 (7.223)	65.19 66.44
Helgoland (Hel) t=36weeks	0,176 (0,016)**	25.107 (8.345)	83.62 87.19

a) mean (SD) T0 $n=10$; Cux/Hel $n=9$; Mann-Whitney (U) Test, $P> 0.05$; * significant reduction between primary and 8 month Cuxhaven samples; ** significant difference between 8 month Cuxhaven and Helgoland samples;
b) the two values represent the two extractions used as biological replicates each representing a pool of three mussels

Table 2:

Element concentrations measured in dry whole body tissue of *Mytilus* sp. exposed for eight month at a near- (Cuxhaven) and off-shore (Helgoland) field station

Element (mg/kg_{dw})	Primary samples t=0	Cuxhaven t=36weeks	Helgoland t=36weeks
Al	334.486 (45.915)[b]	308.683 (131.352)[c]	127.028 (13.277)[b, c]
As	11.261 (0.465)[a]	14.207 (1.000)[a, c]	11.435 (0.950)[c]
Ag	0.025 (0.002)[a]	0.048 (0.004)[a]	0.032 (0.014)
Cd	0.427 (0.036)[a]	1.635 (0.092)[a, c]	0.455 (0.024)[c]
Co	0.749 (0.048)[b]	0.789 (0.098)[c]	0.528 (0.030)[b, c]
Cr	1.055 (0.075)[b]	2.518 (1.878)[c]	0.811 (0.057)[b, c]
Cu	8.272 (0.282)[b]	7.465 (0.756)[c]	5.476 (0.303)[b, c]
Fe	460.265 (39.006)[b]	552.374 (160.893)[c]	192.625 (8.819)[b, c]
Mn	14.090 (1.289)[a, b]	23.041 (8.002)[a, c]	8.404 (0.531)[b, c]
Mo	0.819 (0.068)[a, b]	1.167 (0.077)[a, c]	1.012 (0.096)[b, c]
Ni	2.007 (0.151)[a, b]	3.055 (0.370)[a, c]	1.177 (0.051)[b, c]
Pb	1.201 (0.139)[a, b]	1.671 (0.184)[a, c]	0.960 (0.121)[b, c]
Se	3.031 (0.306)[a, b]	7.233 (0.281)[a, c]	4.621 (0.328)[b, c]
Zn	86.026 (19.139)[a]	128.959 (14.596)[a, c]	77.5437 (5.886)[c]

Mean (SD) $n=9$, Mann – Whitney (U) – Test $P>0.05$ [a] T0 vs C; [b] T0 vs H, [c] C vs H

Table 3: Identification of selected proteins by MALDI-ToF/ToF mass spectrometry in *Mytilus* sp. exposed for eight month at differently impacted field stations in the North Sea/German Bight

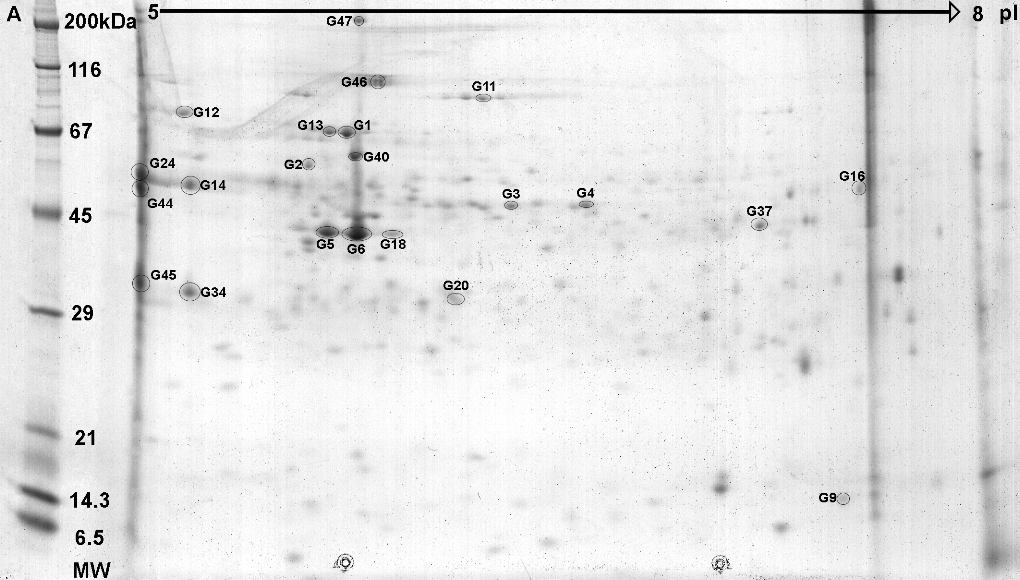
Spot no	Identified protein	Species	Accession no	MASCOT results						method
				Peptide mass fingerprint			MS/MS ion search			
				matched peptides	sequence coverage [%]	protein score[a]	peptide ions [*m/z*]	Sequence	Individual ions score[a]	
G3	Tektin	*Saccoglossus kowalevskii*					1065.4850	YYQSFSDR	44/42	MS/MS
G5	Actin	*M. galloprovincialis*	gi|5114428				1515.7505 1790.9040	IWHHTFYNELR SYELPDGQVITIGNER	61/51 110/51	MS/MS
G6	Actin	*M. galloprovincialis*	gi|5114428	10	31	89/74				MS
G18	Actin	*M. galloprovincialis*	gi|5114428				1790.9080	SYELPDGQVITIGNER	111/53	MS/MS
G40	Actin	*M. galloprovincialis*	gi|5114428				1516.724 1790.906	QEYDESGPSIVHR SYELPDGQVITIGNER	98/43 113/43	MS/MS
G14	Tubulin β-chain	*C. gigas*	gi|405965590	11	34	140/76	1130.6240 1959.0179	FPGQLNADLR GHYTEGAELVDSVLDVVR	82/46 137/46	MS + MS/MS
G24	Tubulin β-chain	*C. gigas*	gi|405965590	9	26	132/76	1130.6240	FPGQLNADLR	67/42	MS + MS/MS
G46	Paramyosin	*M. galloprovincialis*	gi|42559342	9	12	93/82	1239.7070 1315.6851	LAAAQAALNQLR ITIQQELEDAR	33/19 21/19	MS + MS/MS
G47	Paramyosin	*M. galloprovincialis*	gi|42559342	19	30	258/80	1239.7300 1330.6910	LAAAQAALNQLR ELELQLEEATR	71/49 51/49	MS + MS/MS
G34	HIP	*M. edulis*	gi|46395578	6	43	108/82				MS
G45	HIP	*M. edulis*	HIP_MYTED[c]	4	20	66/53	1052.5909	FIHHEIEK	36/13	MS + MS/MS

G4	Enolase	*Cerebratulus cf. lacteus*	gi|1839190				1804,9529	AAVPSGASTGIYEALELR	106/38	MS/MS
G16	ATP-synthase subunit α	*C. gigas*	gi|762100699	8	17	106/82				MS
G44	ATP-synthase subunit β, partial	*M. edulis*	gi|46909261	12	40	240/76	1860.9580	ILDPYVVGEEHYTVAR	74/53	MS + MS/MS
							1987.0280	GIAELGIYPAVDPLDSNSR	64/53	
G37	IDH	*M. trossulus*	gi|385268549	14	31	168/82	1033.5920	NILNGTVFR	48/43	MS + MS/MS
							1159.5830	YYDLGLPYR	56/43	
G1	HSC 71	*M. galloprovincialis*	gi|76780612	21	42	202/80	1253.6013	FEELNADLFR	71/50	MS + MS/MS
							1408.7772	AAVHEIVLVGGSTR	102/50	
							1487.6852	TTPSYVAFTDTER	97/50	
							2037.0288	LVNNSVITVPAYFNDSQR	138/50	
G13	HSC 71	*M. galloprovincialis*	gi|76780612	14	27	210/80	1253.6219	FEELNADLFR	61/45	MS + MS/MS
							1408.7980	AAVHEIVLVGGSTR	96/45	
							1487.7061	TTPSYVAFTDTER	63/45	
							2037.0330	LVNNSVITVPAYFNDSQR	89/45	
G2	60 kDa HSP	*C. gigas*	gi|405966599				1607.9161	AAVEEGIVPGGGVALLR	111/38	MS/MS
G12	70kDa HSP	*M. galloprovincialis*	gi|66766198				1183.6580	FDLTGIPPAPR	69/45	MS/MS
G9[b]	PPI	*Bombus impatiens*	gi|350420195	4	52	77/74				MS
G11[b]	MVP	*M. edulis*	gi|5714749				1874.0090	GIQNVYVLGEDEGVILR	69/40	MS/MS
G20[b]	GbP β	*Pinctada fucata*	GBB_PINFU[c]	4	12	66/53	1549.7070	ELPGHTGYLSCCR	22/14	MS +MS/MS

a – Protein score and individual ion score were significant

b – Identified in one of the three sample types (T0, Cux or Hel)

c – data base SwissProt, all other NCBInr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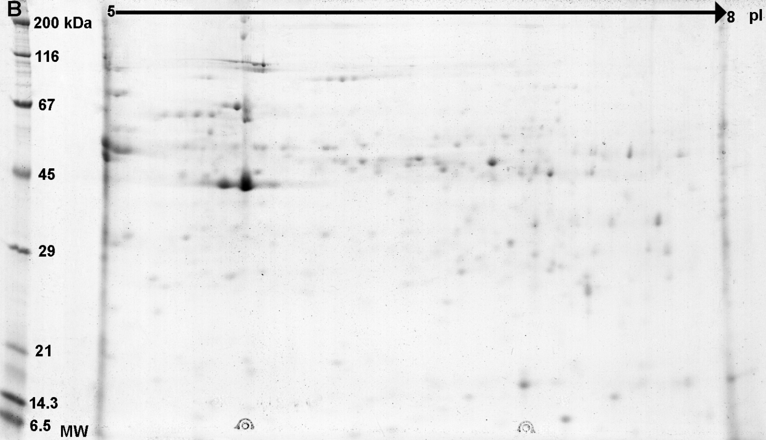
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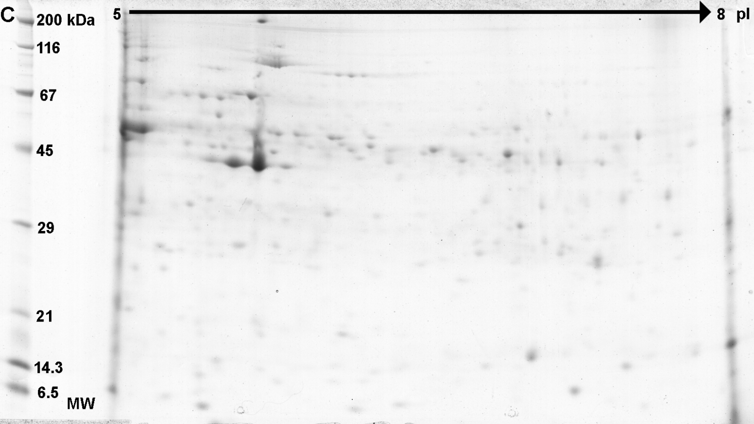
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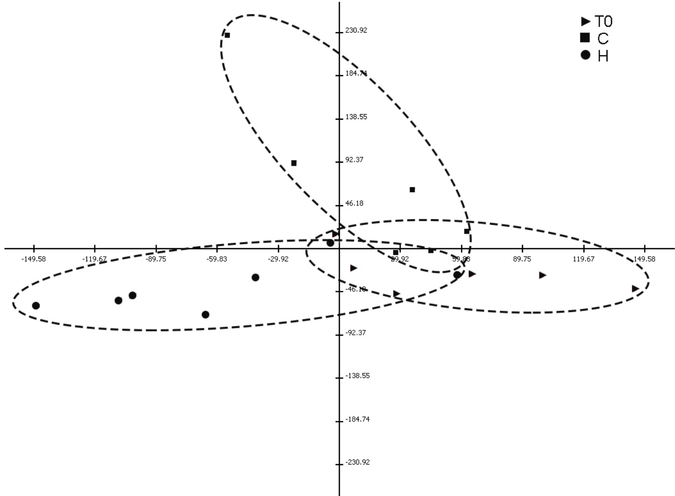

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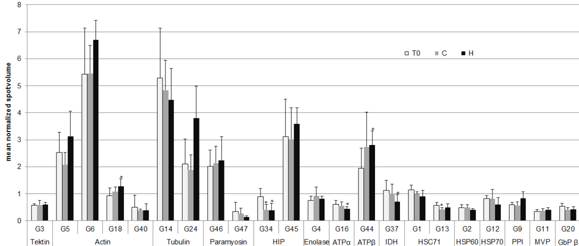

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G20
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