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1 **Macroalgae as spatial and temporal bioindicators of coastal metal pollution following**
2 **remediation and diversion of acid mine drainage**

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21

22 **Abstract**

23 Acid mine drainage (AMD) is a significant contributor of metal pollution leading to ecosystem
24 damage. Bioindicator organisms such as intertidal brown macroalgae have an important role
25 in quantifying the risks of metal bioaccumulation in coastal locations exposed to AMD
26 contamination. Measurement of As, Cd, Cu, Fe, Pb, and Zn accumulation was performed in
27 *Fucus serratus*, *Fucus vesiculosus* and *Ascophyllum nodosum* sampled from two marine
28 locations near to an abandoned Cu mine in Anglesey, Wales, UK. Transect samples were
29 taken from a coastal location (Amlwch) that has seen a substantial increase in AMD
30 contamination over 15 years, in comparison to a nearby estuarine location (Dulas Estuary
31 leading to Dulas Bay) with a historic legacy of pollution. These were compared with samples
32 from the same sites taken 30 years earlier. Some of the Dulas macroalgae samples had Cd,
33 Cu and Zn concentrations that were above background but in general indicated a non-
34 polluted estuary in comparison to substantial pollution over previous decades. In contrast,
35 *Fucus* samples collected from directly below an AMD outflow at Amlwch showed extremely
36 elevated metal bioaccumulation ($> 250 \text{ mg Fe g}^{-1}$, $> 6 \text{ mg Cu g}^{-1}$, $> 2 \text{ mg Zn g}^{-1}$, $> 190 \text{ } \mu\text{g As}$
37 g^{-1}) and evidence of macroalgae toxicity, indicating severe pollution at this site. However, the
38 pollution dispersed within 200 m of the outflow source. This study has demonstrated the
39 efficiency of three brown macroalgae species as indicators for metal bioavailability at high
40 spatial resolution and over time.

41

42 **Keywords:** Acid mine drainage, Bioaccumulation, Bioindicator, Macroalgae, Marine
43 pollution, Trace metals

44 **1. Introduction**

45 Mining for minerals such as Cu is linked with the discharge of acid mine drainage
46 (AMD), an effluent produced when sulfide-containing rocks are exposed to water and
47 oxygen, leading to acid-induced mobilisation of toxic trace metals (Azapagic, 2004). The
48 highly acidic, metal polluted run-off enters nearby freshwaters and has substantial adverse
49 effects on aquatic biodiversity, reducing the value of the water for agricultural, recreational or
50 industrial uses, and rendering it unsafe for human consumption (Akcil and Koldas, 2006;
51 Tripole et al., 2006). For mine sites near to the sea, AMD may also be an important route for
52 coastal and marine pollution, the consequences of which are less well studied. Despite
53 adsorption reactions and geochemical precipitation that disperse and dissolve metals once
54 they enter the sea (Foster et al., 1978), the unique hydrodynamics of estuarine systems and
55 their action as sinks for pollutants makes them prone to a build-up of metal compounds in
56 sediments that increases the risks of bioaccumulation in biota and subsequent trophic
57 transfer (Simons et al., 2011). Use of bioindicators to detect metal pollution and determine
58 these risks of bioaccumulation are therefore important.

59 Macroalgae are ideal candidates as biological indicators for metal pollution
60 monitoring due to their wide distribution, and their ability to tolerate, accumulate and
61 concentrate metals with respect to the corresponding seawater concentration (Bryan and
62 Langston, 1992; Conti and Cecchetti, 2003; García-Seoane et al., 2018; Volterra and Conti,
63 2000). Furthermore, since macroalgae often cannot move away from the pollutant, their
64 sessile nature make them excellent indicators of the pollution source (García-Seoane et al.,
65 2018). Intertidal brown macroalgae such as *Fucus* spp. act as a good indicator of the
66 bioavailable forms of metals (Giusti, 2001; Ryan et al., 2012; Sánchez-Quiles et al., 2017).
67 For example, elevated metal concentrations in *Fucus vesiculosus* are indicative of trace
68 metal contamination (Barnett and Ashcroft, 1985; Forsberg et al., 1988; Stengel et al., 2005).
69 Likewise, *Fucus serratus* has been frequently used to detect trace metal effluent plumes in
70 estuaries caused by industrial activities (Fuge and James, 1973; Klumpp and Peterson,
71 1979). In addition, other brown macroalgae species including *Ascophyllum nodosum* have

72 been shown to be suitable bioindicators for metal contamination (Foster, 1976; Ryan et al.,
73 2012; Stengel et al., 2005). When using organisms as biological indicators potential
74 problems in interpreting the results of metal bioavailability can occur due to seasonal and
75 environmental variation (such as salinity and sea currents) during sampling. Factors such as
76 the age of an organism and variation in metal partitioning in different tissues are also
77 potential sources of error. To account for such variation more than one macroalgal species
78 over multiple sampling sites and tidal positions should be adopted (Conti and Cecchetti,
79 2003; García-Seoane et al., 2018).

80 This investigation makes use of a study site in Anglesey, Wales, UK (Fig. 1A), an
81 area long known for Cu mining. The Parys Mountain mine was the largest single producer of
82 Cu in the 18th century but mining at the site ceased in 1911 (Coupland and Johnson, 2004).
83 The continual discharge of this metal-rich water from the mine adits and spoil heaps enters
84 the Irish Sea via two rivers: the Northern and Southern Afon Goch, which allow significant
85 outflow of Fe, Cu, Cd and Zn into the sea (Mayes et al., 2010). Ochre precipitates are
86 formed when metal-rich and acidic water enters and mixes with neutral water of the sea.
87 These precipitates are periodically flushed out of the Afon Goch system under high
88 discharge, with metal precipitates from the Southern Afon Goch settling in the shallow Dulas
89 Estuary before flow into the sea at Dulas Bay at high tide (Parkman et al., 1996). Less is
90 known regarding the behaviour of the metals released via the Northern Afon Goch at
91 Amlwch although metal dispersion at this part of the coast is likely to be more rapid as the
92 discharge is directly into the sea.

93 In 2003 an underground dam at the Parys Mountain mine was dismantled as part of
94 drainage and flood risk management, which caused mine effluent to be redirected into the
95 Northern Afon Goch, whereas the Southern Afon Goch now only receives contamination
96 through spoil leachate runoff (Coupland and Johnson, 2004; Dean et al., 2013; Johnston et
97 al., 2008; Younger and Potter, 2012). The resulting shift in contamination allowed for down-
98 stream biotic recovery in the Southern Afon Goch that has seen a 3-fold improvement in
99 benthic invertebrate diversity (Dean et al., 2013). However, evidence of high metal

100 bioconcentration within tissues of the crustacean *Talitrus saltator* at Dulas Bay (Fialkowski et
101 al., 2009) suggests that the effluent plume discharged by the mine via the Southern Afon
102 Goch still enters the food chain. The ecological consequences of higher pollution load along
103 the Northern Afon Goch are unclear, but elevated trace metal/metalloid levels within marine
104 biota at Amlwch would be predicted.

105 Therefore this investigation assessed the degree of AMD pollution entering the Irish
106 Sea along the northeast coast of Anglesey, Wales adjacent to Dulas Bay and Amlwch,
107 resulting from the Parys Mountain Cu mine. The aim of this study was to assess the
108 efficiency of brown macroalgae to be used as indicators for metal contamination and
109 bioavailability due to coastal AMD pollution at high spatial resolution and over time. This was
110 achieved by measuring trace metal/metalloid concentrations in the macroalgae *F. serratus*,
111 *F. vesiculosus* and *A. nodosum* along intertidal and estuarine transects, and by comparing
112 present trace metal concentration in *F. serratus* and *F. vesiculosus* with past concentration
113 levels collected at the same sites. Through the use of such bioindicator data, an assessment
114 of risk of metal pollution transfer into the marine food chain at specific locations can be
115 gained, which would allow a determination as to whether remediation is needed.

116

117 **2. Materials and Methods**

118 *2.1. Study sites and macroalgae sampling regime*

119 To evaluate the marine pollution resulting from AMD from the Parys Mountain mine
120 on Anglesey, in north-west Wales, UK, two coastal areas that are currently or historically
121 associated with AMD from this mine were selected; Dulas and Amlwch on the north-east
122 coast of Anglesey (Fig. 1A). The Southern Afon Goch has allowed AMD to flow into Dulas
123 Estuary and then into the Irish Sea at Dulas Bay. A spit of land divides Dulas Estuary; this
124 prevents rapid dilution and dispersion of AMD beyond the estuary. At low tide seawater
125 retreats completely and only the narrow channel of the Southern Afon Goch is visible. Six
126 sites (DE1 – DE6) were chosen within Dulas Estuary for the collection of brown macroalgae
127 at rocky areas within the high tidal zone. One of the chosen sites (DE1) was on the land-side

128 of a spit of land and close to the river while sites DE2 – DE5 were along a transect on the
129 coastal side of the spit, and site DE6 was closest to Dulas Bay (Fig. 1B). *F. vesiculosus* was
130 the only *Fucus* sp. that was abundant throughout the estuary so for comparison samples of
131 *A. nodosum* were also collected from these sites. Twelve sites (DB1 – DB12) were chosen
132 along the coastline of Dulas Bay, split into two intertidal transects running either side of the
133 river mouth to identify a possible gradient in metal concentration. Two sites (DB6 and DB7)
134 were either side of the river mouth, sites DB1 – DB5 ranged between 100 m and 1600 m to
135 the north of the river outflow and sites DB8 – DB12 ranged between 100 m and 2000 m to
136 the south of the river outflow (Fig. 1E). All sites at Dulas Bay were chosen as having
137 sufficient abundance of both *F. serratus* and *F. vesiculosus*.

138 The Northern Afon Goch is a canalised stream that flows north where it enters the
139 Irish Sea at Amlwch via a dredge discharge pipe. Twelve sites (A1 – A12) were chosen for
140 the collection of brown macroalgae at rocky areas within the high tidal zone along the
141 coastline near Amlwch, again split into two intertidal transects running either side of the river
142 outflow to identify a possible gradient in metal concentration. One site (A6) was immediately
143 below the outflow, sites A1 – A5 ranged between 100 m and 1700 m to the west of the river
144 outflow and sites A7 – A12 ranged between 100 m and 1200 m to the south of the river
145 outflow (Fig. 1D). While *F. serratus* was abundant at all Amlwch coastal sites, *F. vesiculosus*
146 was only highly abundant at sites A1 and A2 but absent or only present as occasional
147 samples at all other sites.

148 Two locations on the west coast of Anglesey were chosen as control sites that were
149 not affected by AMD. The Afon Alaw Estuary was used in comparison to Dulas Estuary since
150 it is physically similar to Dulas Estuary. Three sites (AE1 – AE3) were chosen within the
151 Alaw Estuary for the collection of *F. vesiculosus* and *A. nodosum* at rocky areas within the
152 high tidal zone (Fig. 1C). A rocky shore site at Porth Cwyfan (PC1) (Fig. 1A) was used in
153 comparison to Amlwch and Dulas Bay for the collection of *F. serratus* and *F. vesiculosus*. All
154 sites were sampled in 2017 and a single site at Amlwch (A1), Dulas Bay (DB1), Dulas
155 Estuary (DE6) and Porth Cwyfan (PC1) was previously sampled in 1987 to allow for

156 temporal comparison of the AMD pollution impact. Location coordinates for each sample site
157 is shown in Table S1.

158 Three replicate samples of each macroalgal species were taken from each site
159 during the 2017 sampling and five replicate samples were taken during the 1987 sampling.
160 Standardised measures were employed to minimise problems of variability (García-Seoane
161 et al., 2018). To avoid age-related variation, macroalgae samples collected were of a similar
162 size at a fixed tidal height and were free of surface defects or evidence of predation. Care
163 was also taken to avoid wave-damaged and epiphyte-attached samples. Macroalgae in
164 direct contact with fine sediment was avoided as this hinders the ability of algae to act as an
165 indicator for metal (Rainbow et al., 2002). The samples were washed with seawater to rid
166 samples of surface contamination through adhesion. To avoid cross-contamination, samples
167 were placed in labelled polythene bags and transported to the lab in insulated cooler bags.

168

169 2.2. Macroalgae metal analysis

170 The concentrations of As, Cd, Cu, Fe, Pb and Zn associated with *F. serratus*, *F.*
171 *vesiculosus* and *A. nodosum* were measured. The macroalgae samples were first rinsed
172 with deionised water after being transported to the lab then placed in cool storage until
173 analysed. Samples were then well rinsed in deionised water three times and brushed to
174 remove any adhered particulates before being cut using stainless steel scissors. A 2 cm long
175 non-damaged, vesicle and epiphyte free area within the older part of the seaweed was
176 collected since metal concentrations in *Fucus* spp. are higher in the older part of the thallus
177 (Forsberg et al., 1988; Riget et al., 1997). Samples were dried at 80°C for 48 h and ground
178 using a pestle and mortar to facilitate digestion, then 0.1 g aliquots of dried material were
179 digested in 5 mL of 70% ultra-pure nitric acid for 2 h at 100°C refluxed on a hotplate. Digests
180 were then diluted to 2% nitric acid in deionized Milli-Q water (Millipore) and filtered (0.45 µm
181 filter) before analysis by inductively coupled plasma mass spectrometry (ICP-MS) using an
182 Agilent 7700x (Agilent, Stockport, UK), which was calibrated using a matrix matched serial
183 dilution of Specpure multi element plasma standard solution 4 (Alfa Aesar). A CRM of *Fucus*

184 *vesiculosus* (ERM-CD200; European Commission Joint Research Centre, Institute for
185 Reference Materials and Measurements, Geel, Belgium), certified for As, Cd, Cu, Hg, Pb, Se
186 and Zn, was used to test the accuracy of metal extraction and quantification from seaweed.
187 All of the values were >92.9% of the CRM concentration.

188

189 2.3. *Water sample data*

190 To assess water quality, specifically trace metal pollution (particulate and dissolved)
191 and pH from the point source discharges, historical water quality plus flow rate data for the
192 Northern Afon Goch at Amlwch and the Southern Afon Goch at Dulas Estuary between 1995
193 and 2017 were obtained from Natural Resources Wales.

194

195 2.4. *Data analysis*

196 Mean annual metal mass flux values were calculated as t yr^{-1} of total (dissolved and
197 particulate) Cu, Cd, Fe or Zn using discharge data and metal concentration data. A metal
198 pollution index (MPI) was used to aggregate total metal content in macroalgae to facilitate
199 spatial comparison, utilising the following equation (Usero et al., 1996):

$$200 \quad \text{MPI} = (\text{Cf}_1 \times \text{Cf}_2 \dots \times \text{Cf}_n)^{1/n}$$

201 where Cf_n = concentration of metal n in a given sample expressed as mg Kg^{-1} dry weight.

202 The critical index limit for MPI is 100, anything beyond this can be considered critically
203 polluted (Prasad and Sangita, 2008). A Kolmogorov-Smirnov test was performed to test for
204 normality within the data set. Statistical comparison of data was performed either using
205 Kruskal-Wallis test or one-way ANOVA ($P < 0.05$), as appropriate and Tukey's multiple
206 comparison post hoc test. Linear regression analyses were performed to compare metal
207 concentrations between two species. All statistical analyses were performed using
208 GraphPad Prism v6.04.

209

210

211 3. Results and Discussion

212

213 3.1. Current and historical status of metal pollution entering the Irish Sea via the Southern 214 and Northern Afon Goch

215 The analysis of long-term water quality data (1995 – 2017) indicates that alterations
216 in the drainage from the Parys Mountain mine in 2003 has re-routed and increased the AMD
217 outflow into the sea (Figs. 2 & 3; Fig. S1). Since 2003 total metal concentrations decreased
218 in the Southern Afon Goch as it enters Dulas Estuary while metal concentrations
219 dramatically increased in the Northern Afon Goch immediately prior to entering the Irish Sea
220 at Amlwch (Fig. 2). The change in the drainage regime also resulted in a marked reduction in
221 pH to ~ pH 3 in the Northern Afon Goch (Fig. 3C). Mean annual discharge data in both rivers
222 did not significantly vary over the years although the median discharge did show inter-annual
223 variation (Fig. 3A). The metal mass flux entering the Irish Sea from the Northern Afon Goch
224 increased markedly following the 2003 re-routing of mine drainage into the river (Fig. 3B).
225 For example, between 2004 – 2008 the mean annual mass flux of metals into the sea
226 increased by 30-fold for Fe, nearly 5-fold for Cu, 3-fold for Zn and nearly 10-fold for Cd
227 compared to the previous five years. Thus metal outflow via the Northern Afon Goch
228 accounts for the largest single marine release of Fe and Cu nationally within the UK, the
229 second largest release of Zn, and is also a major contributor of Cd (Mayes et al., 2010). No
230 other mine site in the country matches the size of Cu release from the Parys Mountain mine,
231 which makes up 71% of total Cu released nationally. In contrast, decreases in metal flux into
232 the Irish Sea via the Southern Afon Goch were observed, which were significantly lower for
233 Cu, Zn and Cd (Fig. 3B). However, the northern river is releasing greater amounts of metals
234 to the sea than at any time from the Southern Afon Goch, and as a consequence the total
235 metal mass flux from Parys Mountain to the Irish Sea is now higher than in the pre-2003
236 period, particularly for Fe and Cu. A natural wetland situated on the Southern Afon Goch is
237 acting as a sink for metals and has a substantial remedial effect on the AMD pollution
238 entering the system, so further reducing the metal loadings to the Irish Sea via Dulas

239 Estuary (Aguinaga et al., 2018). The wetland reduced concentrations of metals by around
240 50% before 2003 and now removes over 95% of the metals (Dean et al., 2013). In contrast,
241 the Northern Afon Goch is largely canalised and the effluent is not subject to passive
242 remediation by vegetation. Thus total metal loading to the Irish Sea from the Parys Mountain
243 mine has increased dramatically, and therefore this site remains a major source of metal
244 pollution to the Irish Sea.

245

246 3.2. Bioindicators for coastal metal bioavailability at Dulas Estuary and Dulas Bay

247 To determine whether reduced AMD outflow into Dulas Estuary and Dulas Bay was
248 associated with low metal bioconcentration at or near background levels within macroalgae
249 at these locations, samples were collected and analysed for metal concentration. It should
250 be noted that this study did not specifically differentiate between internalised and surface
251 bound metal such as by EDTA washing (García-Ríos et al., 2007), although all samples
252 were rigorously washed in deionised water prior to drying and tissue digestion. However,
253 other studies have indicated that for brown macroalgae species including *F. vesiculosus*
254 over 90% of the associated metals were intracellular bound (Ryan et al., 2012), suggesting
255 that these bioindicator species would be suitable to assess bioavailability of metal pollutants.

256 For the analysis of Dulas Estuary comparison was made with the Afon Alaw Estuary
257 as a control site since it is not exposed to AMD pollution. Furthermore, Dulas Estuary and
258 Afon Alaw Estuary have similar shore and substrate profiles suggesting comparable periods
259 of immersion and habitat types. *F. vesiculosus* was the only *Fucus* sp. that was abundant to
260 a similar extent across both estuaries; therefore comparisons were also made with the
261 equally ubiquitous brown algae *A. nodosum*. At Afon Alaw Estuary Cu, Zn, Cd and As
262 concentrations within *F. vesiculosus* from all sites, and Fe and Pb at two of the sites (AE2
263 and AE3) were within the typical background range for *Fucus* sp. in unpolluted environments
264 (Riget et al., 1997; Ryan et al., 2012; Tomlinson et al., 1980), and therefore considered as
265 baseline levels, whereas *F. vesiculosus* Fe and Pb concentrations from site AE1 were
266 unexpectedly high (Fig. 4A). Within the Dulas Estuary at all six sample sites, concentrations

267 of Fe, As and Pb in *F. vesiculosus* were also at baseline levels, as were concentrations of
268 Cu, Zn and Cd from sites DE3, DE4, DE5 and DE6. However, for these three metals, the
269 concentrations were significantly higher than baseline concentrations at sites DE1, furthest
270 from the sea (Fig. 1B), and at site DE2 (Fig. 4A). An equivalent metal association profile was
271 seen for *A. nodosum* (Fig. S2), and there was a significant positive correlation between *F.*
272 *vesiculosus* and *A. nodosum* for all metals, and individually for Zn and Cu (Fig. 4C).

273 Along the Dulas Bay shoreline transect, both *F. vesiculosus* and *F. serratus* were
274 abundant and so samples were taken from both species. Comparison was made with an
275 unpolluted control location at Porth Cwyfan where concentrations of all six measured metals
276 were at typical background (baseline) levels for both *F. vesiculosus* (Fig. 4B) and *F. serratus*
277 (Fig. S3). At Dulas Bay concentrations of Cd, As and Pb associated with both *Fucus* spp.
278 were also at or below baseline levels at all sites. However, concentrations of Cd, As and Pb
279 in *F. vesiculosus* sampled from the river mouth at sites DB6 and DB7 (Fig. 1E) were
280 significantly higher than in samples taken at many of the sites in both directions further along
281 the beach (Fig. 4B). A similar pattern was also seen for As in *F. serratus* (Fig. S3). For Fe,
282 Cu and Zn, only the *Fucus* samples collected from sites DB6 and/or DB7 had significantly
283 elevated concentrations of these metals, at 2 – 4-fold higher than the baseline levels (Fig.
284 4B, Fig. S3). There were also subtle but significantly higher concentrations of Fe and Cu in
285 *F. vesiculosus* at site DB4 (Fig. 4B) and in *F. serratus* at site DB8 (Fig. S3). There was also
286 a strong positive correlation between *F. vesiculosus* and *F. serratus*, which was significant
287 for all metals analysed together, and individually for Fe, Zn and Cu (Fig. 4D). While the metal
288 profiles were essentially equivalent for both *Fucus* spp., there was significantly higher Fe
289 associated with *F. serratus* from site DB7 ($3411 \mu\text{g g}^{-1}$) than with *F. vesiculosus* from this
290 site ($1345 \mu\text{g g}^{-1}$).

291 It is interesting that while there was significant elevated bioaccumulation of Fe at the
292 mouth of the Southern Afon Goch on the Dulas Bay shoreline at site DB7, there was no
293 evidence of bioaccumulation above baseline levels within the estuary. The bioavailability of
294 Fe into macroalgae will be reduced by the buffering effect of seawater causing Fe

295 precipitation (Foster, 1976). Nevertheless, it appears that sufficient quantities of Fe had
296 accumulated at the shoreline to mediate transfer into *Fucus* samples. Furthermore, Fe may
297 act as a vector for other metals via adsorption onto suspended iron oxyhydroxide colloidal
298 particulates (Stüben et al., 2003).

299

300

301 3.3. Bioindicators for coastal metal bioavailability at Amlwch

302 Since AMD outflow into the Irish Sea at Amlwch was found to generate a significantly
303 high metal pollution load over the last 15 years, macroalgae samples were collected and
304 analysed for metal concentration to examine whether there was evidence of high metal
305 bioconcentration close to the source of source and whether there was evidence of metal
306 dispersal. Comparison was again made with the unpolluted control location at Porth Cwyfan.
307 *F. vesiculosus* and *F. serratus* were both present along the shoreline adjacent to Amlwch but
308 *F. serratus* was more abundant and so replicate samples of *F. vesiculosus* could only be
309 taken at sites A1 and A2, which are over 950 m from the outflow (Fig. 1D).

310 The AMD from the Northern Afon Goch flows into the Irish Sea at site A6 via a
311 drainage pipe (Fig. 5A) then gives rise to a plume of metals that are clearly visible in the sea
312 (Fig. 5B), and which is composed of iron oxides and iron hydroxides, and other precipitated
313 metals and dissolved metals. The concentration of all measured metals (Fe, Cu, Zn, Cd, As,
314 Pb) associated with *F. serratus* biomass at site A6 was significantly higher than the baseline
315 concentrations within the macroalgae from site PC1 (Fig. 5E). However, the Cd
316 concentration within the *F. serratus* is perhaps lower than expected despite concentrations
317 within the Northern Afon Goch outflow typically reaching 30 – 40 $\mu\text{g L}^{-1}$ (Fig. 2). A likely
318 explanation, given the extremely high abundance of Zn is that Cd accumulation is being
319 suppressed as a result of competition with high Zn concentrations for limited binding sites
320 (Giusti, 2001). Though Zn may reduce toxicity of Cd at Amlwch for macroalgae, it is still of
321 concern. These free metal ions could be readily available for bioaccumulation as Cd quickly
322 attaches to sediment, where particle-bound and interstitial Cd may exert toxic effects on

323 benthic animals (Swartz et al., 1986). Another toxic metal that is of concern is Pb found in
324 the outflow at Amwlch at concentrations typically reaching $15 \mu\text{g L}^{-1}$ (Fig. S1), and
325 accumulating into *F. serratus* to nearly $70 \mu\text{g g}^{-1}$ here, substantially above baseline levels
326 (Riget et al., 1997; Ryan et al., 2012).

327 The effect of this AMD exposure was clearly evident by visual inspection of both
328 species of *Fucus*; in contrast to samples from all other sites, the macroalgae consistently
329 showed evidence of discolouration and reduced size of the fronds (Fig. 5C & D). The
330 concentrations of Fe (278.6 mg g^{-1}), Cu (6.3 mg g^{-1}), Zn (2.3 mg g^{-1}) and As ($196.0 \mu\text{g g}^{-1}$)
331 associated with *F. serratus* from site A6 were extremely high in comparison to the current
332 literature for *Fucus* spp. The Fe concentration was over 100 times higher than previous
333 measurements for *Fucus* spp. (Fuge and James, 1973) and over 10 times higher than the Fe
334 concentration value of 22.7 mg g^{-1} observed in the brown macroalga *Padina pavonica*
335 isolated from the Syrian coast (Al-Masri et al., 2003). Likewise this Cu concentration was
336 over 60 times higher than values seen in *F. vesiculosus* (Stenner and Nickless, 1974). The
337 highest Zn concentrations recorded in this study were substantially higher than the typical
338 concentrations for *Fucus* spp. even under polluted conditions, although equivalently high Zn
339 concentrations (3.6 mg g^{-1} for *F. serratus*; 4.2 mg g^{-1} for *F. vesiculosus*) have previously
340 been seen (Bryan and Gibbs, 1983; Stenner and Nickless, 1974). Finally, the As
341 concentration was slightly higher than previous uppermost recorded value of $147 \mu\text{g g}^{-1}$ for
342 *F. serratus* (Klumpp and Peterson, 1979) and $160 \mu\text{g g}^{-1}$ for *F. vesiculosus* (Langston, 2009).
343 This observation, alongside the high concentration of As in the Northern Afon Goch outflow
344 of between $75 - 100 \mu\text{g L}^{-1}$ (Fig. S1) indicates marine pollution at this site and marine
345 bioavailability of As. A large amount of the As in human diet comes from seafood and
346 various reports have investigated the relationship between As exposure from seafood and
347 carcinogenic diseases (Borak and Hosgood, 2007; Buchet et al., 1994). Arsenate is readily
348 taken up by plants and algae due to its chemical similarity to phosphate (Sanders et al.,
349 1989).

350 The concentrations of Fe, Cu, Zn, As and Pb in the macroalgae reduced dramatically
351 from sites within both directions of the outflow, indicating rapid dispersal of metals. Thus, the
352 *F. serratus* concentrations of Fe, As, Cd and Pb at all other sites along the coastline (sites
353 A1 – A5 and A7 – A12) were at baseline concentrations (Fig. 5E). However, for Zn there was
354 also significantly higher macroalgae concentration at site A5, which is situated 100 m to the
355 north-west from the outflow, and for Cu there were significantly higher macroalgae
356 concentrations at site A5 and at sites A7 and A8, which are 100 m and 200 m to the south-
357 east from the outflow (Fig. 1D). In *F. vesiculosus* concentrations of Fe, Cd, As and Pb at
358 sites A1 and A2 were not significantly different from the PC1 values, and while Cu and Zn
359 concentrations were significantly higher than the PC1 values (Fig. 5F), they were still close
360 to or within the metal concentration ranges seen in *F. vesiculosus* collected from other clean
361 sites (Giusti, 2001; Pedersen, 1984; Ryan et al., 2012).

362

363 3.4. Geographical distribution of present day metal pollution

364 A detailed spatial analysis is needed when determining the dispersal of a pollutant,
365 which should be therefore informing the sampling design. Many bioindicator studies do not
366 sample at high resolution and indeed many studies do not specify the distance between the
367 sample site and the point source of pollution (García-Seoane et al., 2018). In this study we
368 were able to use the *Fucus* spp. metal concentration values at the known distances to
369 calculate MPI values for each sample site associated with both outflow locations to generate
370 a spatial distribution of pollution risk at Amlwch and Dulas Bay (Fig. 6). MPI values for all
371 sites were < 35 (requiring no treatment) with the exception of site A6 at the mouth of the
372 Northern Afon Goch with a MPI value of 659, which is indicative of a critically polluted site
373 that requires immediate remediation efforts (Tomlinson et al., 1980). There was evidence of
374 a very steep pollution gradient from either direction of the Northern Afon Goch outflow at site
375 A6 (Fig. 6A) indicating rapid dispersal of the metal pollution within 100 m of outflow and
376 consequently a rapid reduction in bioaccumulation at these adjacent sites. At Dulas Bay,
377 which has a legacy of many decades of AMD pollution, there was no evidence for a metal

378 pollution gradient (Fig. 6B). No significant correlation was detected between distance and
379 MPI value.

380

381 3.5. Historical comparison of macroalgae bioindicators for coastal metal bioavailability

382 In addition to the *Fucus* samples collected in 2017, samples had been collected at
383 some of the same sites in 1987 on the Amlwch coast (site A1), Dulas Estuary (site DE6),
384 Dulas Bay (site DB1), and control site Porth Cwyfan (site PC1) and measured for Cu and Zn
385 concentration using the same methods as for the samples in 2017, allowing direct
386 comparison. A comparison of the samples between these 30 year periods would allow an
387 evaluation of the use of macroalgae as temporal bioindicators and determine whether the
388 alteration in direction of drainage outflow in 2003 could be seen on the basis of metal
389 bioaccumulation into the macroalgae. There was no significant difference in Cu and Zn
390 concentration within Porth Cwyfan *Fucus* samples between the two time periods and
391 likewise no significant difference between times within the Dulas Bay samples (Fig. 7). There
392 was a significantly increased Zn concentration in the macroalgae samples collected from site
393 A1 near the Northern Afon Goch outflow in 2017 compared to 1987, while there was a
394 significant, extremely large decrease in Cu concentration in the samples from site DE6 within
395 Dulas Estuary collected in 2017 in comparison to the samples collected at that site in 1987.
396 This observation is consistent with earlier studies that demonstrated that prior to the
397 alteration in drainage from the Parys Mountain mine in 2003 the Dulas Estuary and Dulas
398 Bay were heavily polluted sites that demonstrated high metal bioaccumulation into marine
399 organisms (Al-Thaqafi and White, 1991; Foster, 1976; Foster et al., 1978; Parkman et al.,
400 1996; Rainbow et al., 1999). For example, other past surveys of *F. vesiculosus* at the edge
401 of Dulas Estuary performed in 1997 have measured concentrations of Cu at $246 \pm 106 \mu\text{g g}^{-1}$
402 (Rainbow et al., 1999), while the Cu values are also substantially higher to the
403 concentrations measured in 2017 but not as high as those observed in 1987.

404

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406 3.6. Perspectives

407 While it is clear that the AMD discharge from the Parys Mountain mine site is still a
408 cause of significant levels of trace metal pollution at the Irish Sea coast (Fig. 3), the re-
409 routing of the discharge away from Dulas Estuary is likely to have longer-term benefits to the
410 ecology of this estuary. This reduced AMD flow along the Southern Afon Goch has been
411 further decreased and remediated by the action of the natural wetland, which is
412 approximately 7 km upstream of Dulas Estuary (Aguinaga et al., 2018; Dean et al., 2013).
413 Although there was an increase in metal bioavailability into macroalgae from specific sites at
414 the Southern Afon Goch as it passes through the estuary (sites DE1, DE2), and at the mouth
415 of the river as it enters the bay (sites DB6, DB7), the degree of pollution within Dulas Estuary
416 overall is no longer a concern. This is in stark contrast to the situation at the site in previous
417 decades (Al-Thaqafi and White, 1991; Parkman et al., 1996; Rainbow et al., 1999).
418 However, the upstream wetland has given rise to a build-up of significant amounts of Fe-rich
419 particulate metal sediment (Dean et al., 2013) that appears to be flushed into the estuary
420 and the bay during periodic high discharge events (Fig. 3A). This may explain some of the
421 above-baseline metal accumulation profiles seen in several of the macroalgae samples (Fig.
422 4). With regard to the AMD outflow at Amlwch, this is now responsible for an overall higher
423 metal flux into the Irish Sea, and this outflow gives rise to extremely high levels of metal
424 bioaccumulation into macroalgae (Fig. 5). However, the direct discharge into the Sea does
425 lead to rapid dispersal and dilution, and thus may be less damaging to the coastal
426 ecosystem. Future studies are needed to evaluate the biodiversity at both sites in more
427 detail in order to conclude whether the changes in AMD discharge from the Parys Mountain
428 mine have had significant beneficial and/or negative consequences.

429 Although macroalgae are very good bioindicators for dissolved metal bioavailability,
430 it is also possible that a macroalgal bioindicator is underestimating the total metal
431 bioavailability risks. Particulate metals will bind to the external surface of macroalgae, which
432 may overestimate bioaccumulation if samples are not sufficiently washed (García-Seoane et
433 al., 2018); however, macroalgae will not efficiently accumulate particulate metals unlike filter

434 feeders such as the barnacle (Rainbow et al., 1999). Therefore, it must be considered that
435 the risks of bioavailability by high accumulation of particulate metals within Dulas Estuary
436 could have been underestimated.

437

438 *3.7 Conclusions*

439 Overall, we can conclude that three species of brown macroalgae (*F. serratus*, *F.*
440 *vesiculosus* and *A. nodosum*) can be used as robust bioindicators to allow high resolution
441 mapping of trace metal pollution. Furthermore, the significant positive correlation between
442 the species, suggesting equivalent bioaccumulation mechanisms and characteristics,
443 indicates that they can be used interchangeably as bioindicators at sites with presence for
444 different species. We propose that by making use of macroalgae bioindicators at high spatial
445 resolution wherever possible, analysis can then be directed to further investigate the marine
446 food chain at specific high-risk locations in detail to quantify metal pollution transfer into
447 higher trophic levels and to determine ecological consequences of pollutants such as AMD.
448 The use of macroalgal bioindicators would then also be used to determine whether
449 remediation activities at heavily polluted sites are required. For example, the outcomes from
450 this present study indicate the need to reduce the substantial AMD outflow via the Northern
451 Afon Goch at Amlwch in order to reduce the metal pollution and risks of toxicity to marine
452 biota at this Irish Sea coastal site.

453

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458 Environmental Sciences) for ICP-AES analysis.

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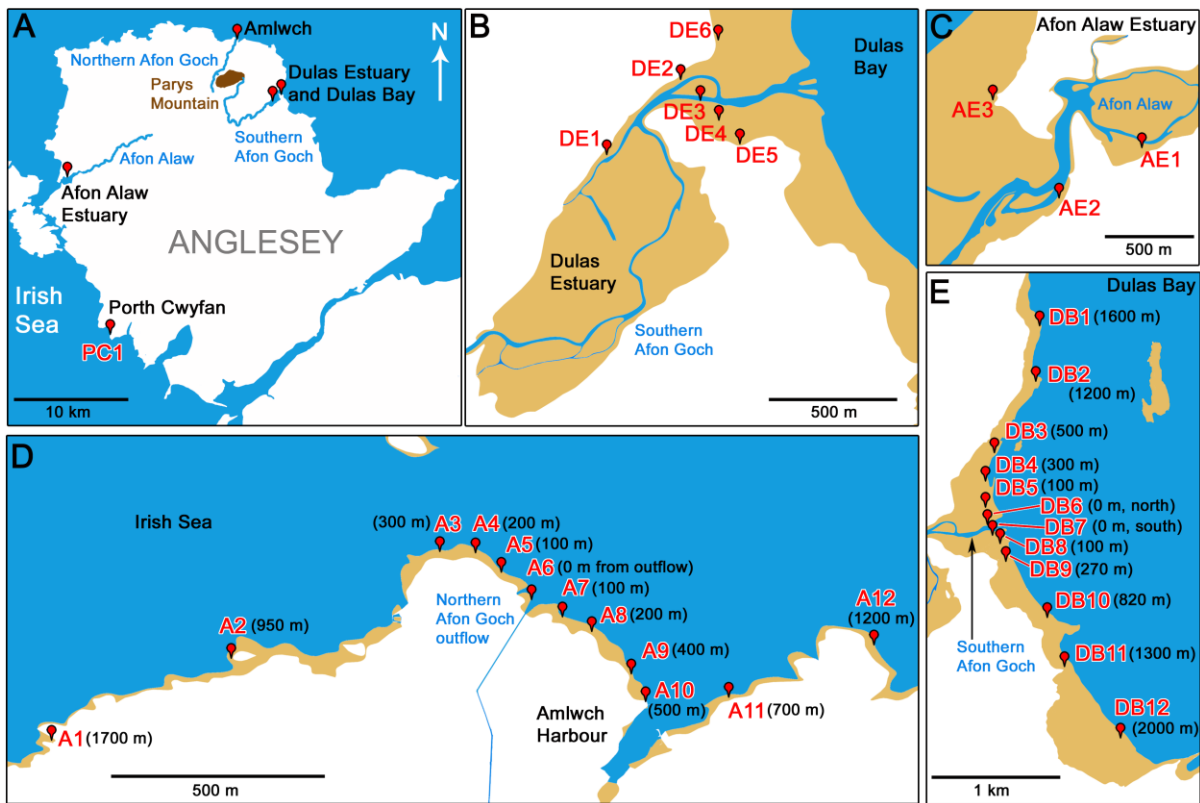
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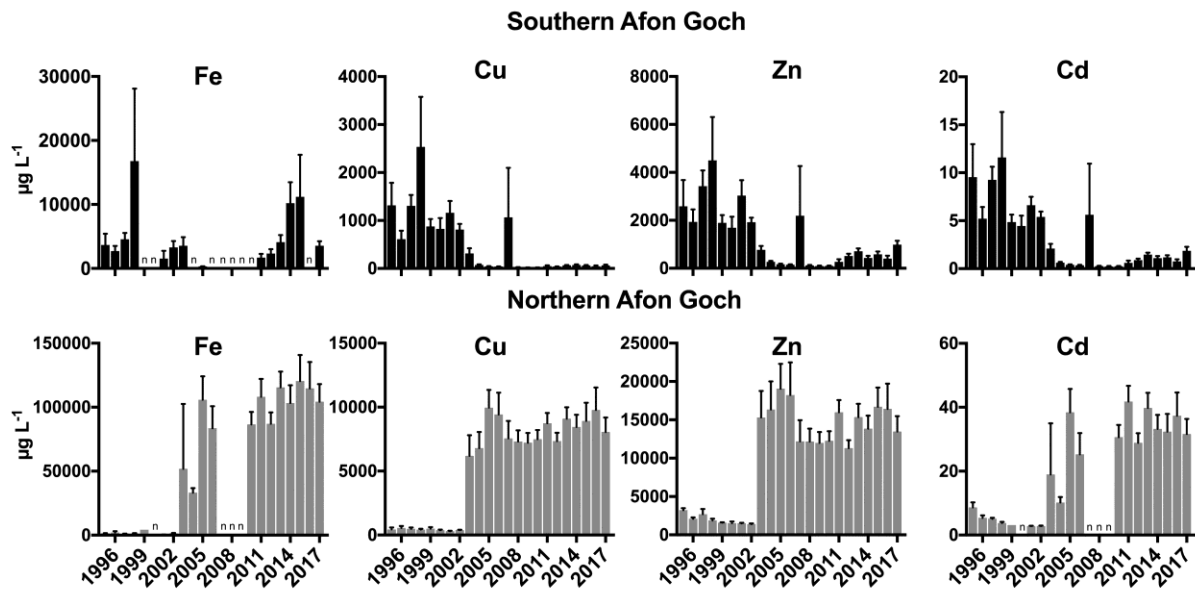
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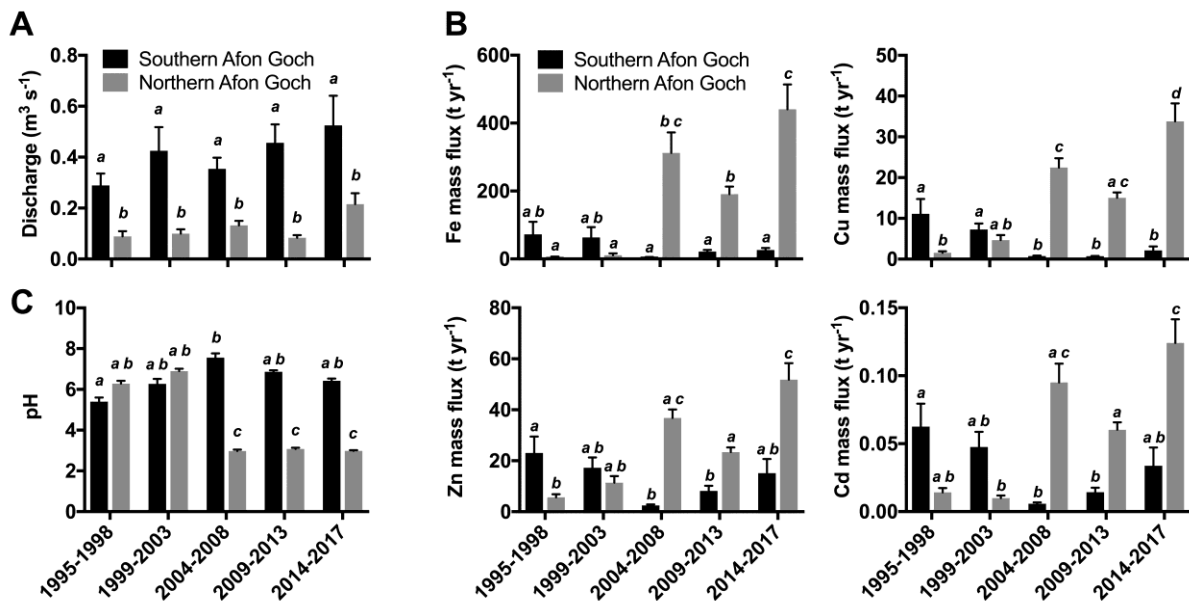


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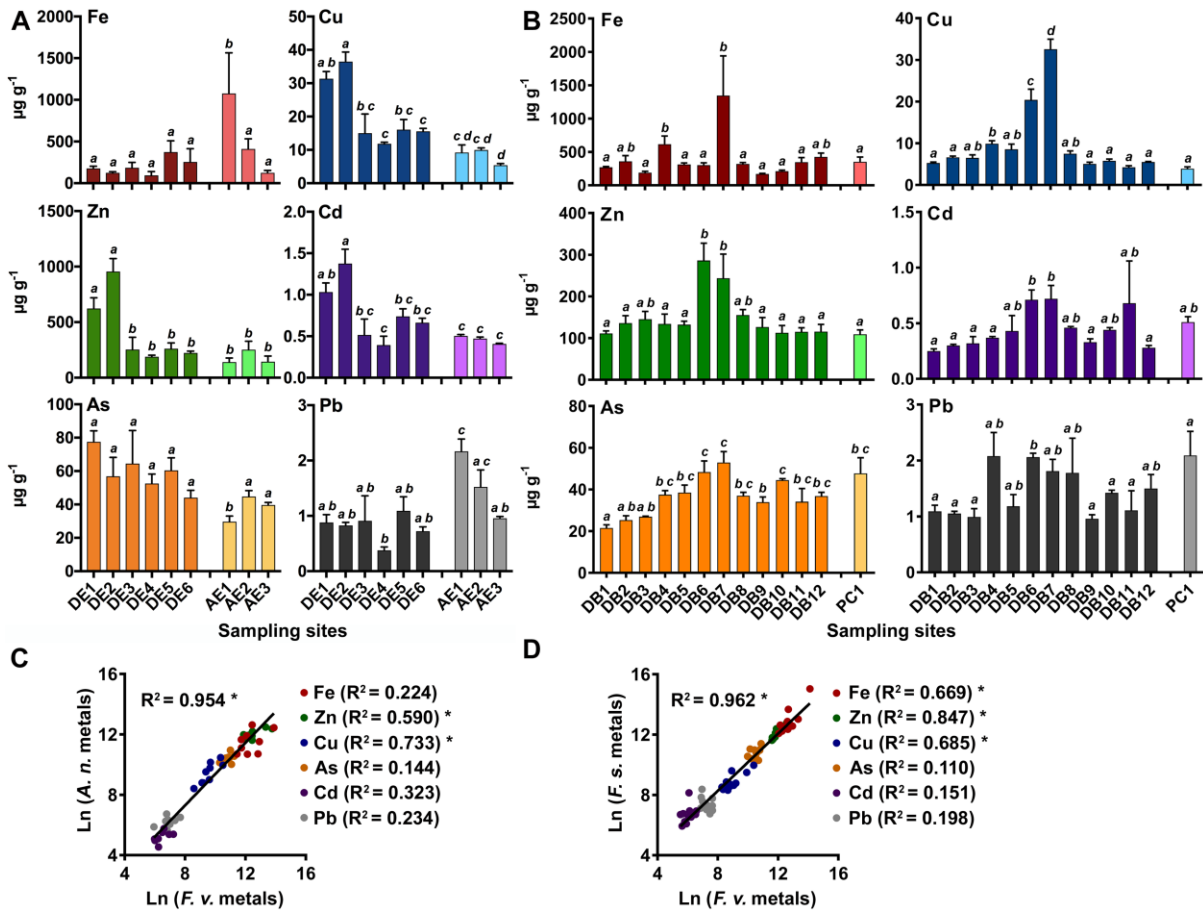
Figure 1. Location of the coastal sample sites and the AMD source at Parys Mountain mine. (A) Map of Anglesey, UK and location of sampling sites at Amlwch, Dulas Estuary, Dulas Bay, Afon Alaw Estuary (non-polluted control site) and Porth Cwyfan (non-polluted control site). The single sample site (PC1) at Porth Cwyfan is indicated. (B) Map of Dulas Estuary and sample sites DE1 – DE6. (C) Map of Afon Alaw Estuary and sample sites AE1 – AE3. (D) Map of Amlwch coast and sample sites A1 – A12. Site A6 is at the source of AMD outflow via the Northern Afon Goch. The distance of all other sample sites from the outflow is indicated. (E) Map of Dulas Bay and sample sites DB1 – DB12. Site DB6 and DB7 are on the north and south banks of the Southern Afon Goch river mouth, which is the source of AMD outflow. The distance of all other sample sites from the outflow is indicated.



595
 596 **Figure 2.** Historical metal concentration data (1995 – 2017) for water samples taken from the
 597 Southern Afon Goch before entering Dulas Estuary and the Northern Afon Goch before entering the
 598 Irish Sea at Amlwch. Data are mean (\pm SEM) values of total (dissolved and particulate) Fe, Cu, Zn
 599 and Cd concentrations for each year where data was available. Data was collected by Natural
 600 Resources Wales with 1 – 27 readings collected per year. For some years, individual metal datasets
 601 were not available (n: not determined).
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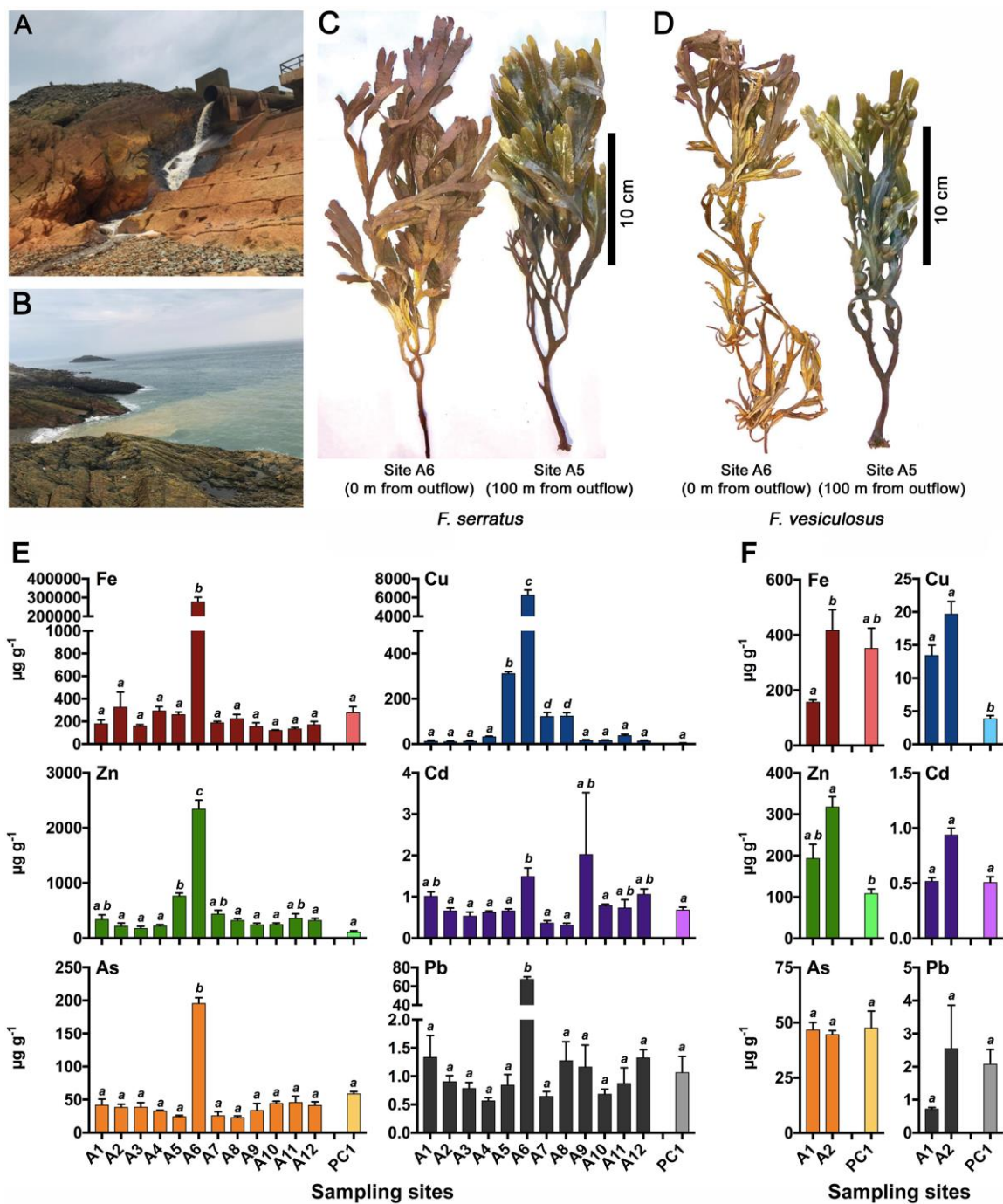


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 606 **Figure 3.** Historical (1995 – 2017) discharge (A), mass flux data for total (dissolved and particulate)
 607 Fe, Cu, Zn and Cd (B), and pH data (C) for water samples entering Dulas Estuary and Dulas Bay via
 608 the Southern Afon Goch and entering the Amlwch coastal water via the Northern Afon Goch. Data are
 609 mean (\pm SEM) values for the indicated time periods. Values that do not share lowercase letters are
 610 significantly different ($P < 0.05$). Data was collected by Natural Resources Wales with 1 – 27 readings
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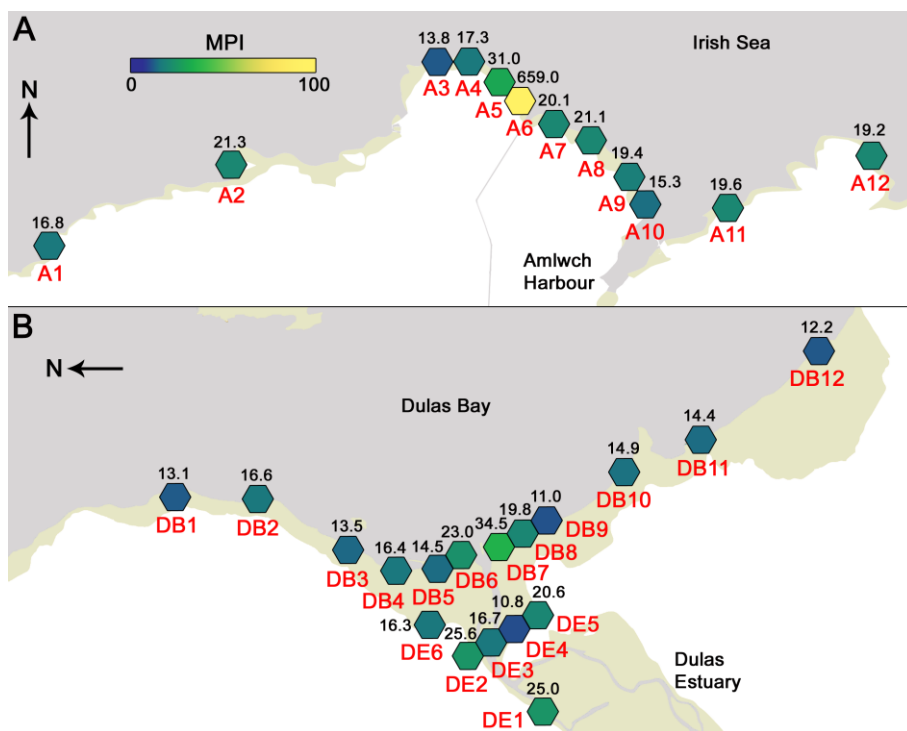
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Figure 4. (A and B) Metal concentrations associated with *Fucus vesiculosus* collected from sampling sites at Dulas Estuary (DE1 – DE6) and Afon Alaw Estuary (AE1 – AE3) (A), and from Dulas Bay (DB1 – DB12) and Porth Cwyfan (PC1) (B). All samples were collected in 2017. Data are mean (\pm SEM) of three *Fucus* samples from each site. Values that do not share lowercase letters are significantly different ($P < 0.05$). (C and D) Correlations between metal concentrations associated with *F. vesiculosus* (*F. v.*) and *Ascophyllum nodosum* (*A. n.*) collected from Dulas Estuary and Afon Alaw Estuary (C), and between metal concentrations associated with *F. vesiculosus* and *Fucus serratus* (*F. s.*) collected from Dulas Estuary and Afon Alaw Estuary (D). Correlations were performed for all metals grouped together and each metal individually. All metal concentration data (as μg per mg) was natural log transformed. An asterisk indicates a significant correlation ($P < 0.05$). Metal concentration data for *A. nodosum* and *F. serratus* are shown in Fig. S2 and Fig. S3, respectively.



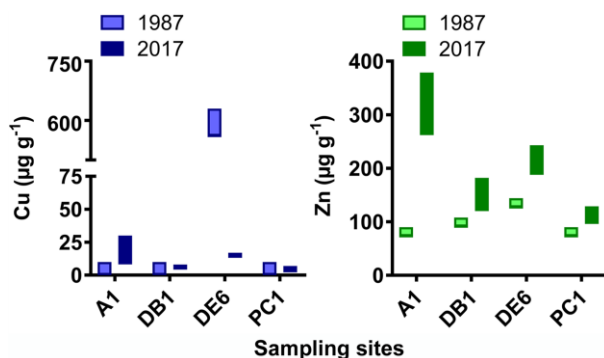
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Figure 5. AMD pollution at Amwlch from the Northern Afon Goch outflow. (A) Water from the Northern Afon Goch as it enters the Amwlch coast via a dredge pipe at site A6. (B) The effluent plume moving easterly after entering the Irish Sea at site A6. (C and D) Representative *Fucus serratus* (C) and *Fucus vesiculosus* (D) samples collected from site A6 at the source of AMD effluent outflow and from site A5, which is 100 m from the outflow. (E and F) Metal concentrations associated with *Fucus serratus* (E) and *Fucus vesiculosus* (F) collected from sampling sites at Amwlch (A1 – A12) and Porth Cwyfan (PC1) collected in 2017. *F. vesiculosus* was only present at high abundance along the Amwlch coast at sites A1 and A2. Data are mean (± SEM) of three *Fucus* samples from each site. Values that do not share lowercase letters are significantly different ($P < 0.05$).



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Figure 6. Geographical distribution of metal contamination using Metal Pollution Index (MPI) values in Anglesey coastal waters in 2017. MPI values were determined from cumulative Fe, Cu, Zn, Cd, As and Pb concentrations associated with *Fucus* sp. at each sample site.



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Figure 7. Historical comparison between *Fucus* sp. samples collected in 1987 and 2017 from Amlwch (site A1), Dulas Bay (site DB1), Dulas Estuary (site DE6) and Porth Cwyfan (site PC1) for Cu and Zn concentration. Boxes show minimum and maximum concentration ranges.