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1 TSI INCREASES FEEDING RATES ON *S. MUTICUM*

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3 **Time-since-invasion increases native mesoherbivore feeding rates on the invasive alga,**

4

***Sargassum muticum* (Yendo) Fensholt**

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17 *Abstract*

18

19 *Invasive algae can have substantial negative impacts in their invaded ranges. One widely-*
20 *cited mechanism that attempts to explain how invasive plants and algae are often able to*
21 *spread quickly, and even become dominant in their invaded ranges, is the Enemy Release*
22 *Hypothesis. This study assessed the feeding behaviours of two species of gastropod herbivore*
23 *from populations exposed to the invasive alga Sargassum muticum for different lengths of*
24 *time. Feeding-trials, consisting of both choice and no-choice, showed that the herbivores*
25 *from older stands (35–40 years established) of S. muticum were more likely to feed upon it*
26 *than those taken from younger (10–19 years established) stands. These findings provide*
27 *evidence in support of the ERH, by showing that herbivores consumed less S. muticum if they*
28 *were not experienced with it. These findings are in accordance with the results of other*
29 *feeding-trials with S. muticum, but in contrast to research that utilises observations of*
30 *herbivore abundance and diversity to assess top-down pressure. The former tend to validate*
31 *the ERH, and the latter typically reject it. The potential causes of this disparity are discussed,*
32 *as are the importance of palatability, herbivore species, and time-since-invasion when*
33 *considering research into the ERH. This study takes an important, yet neglected, approach to*
34 *the study of invasive ecology.*

35

36 *Key Words:* Behaviour, Enemy Release Hypothesis, Feeding trials, Herbivory, Invasive
37 species, Non-native species

38

39 1. INTRODUCTION

40

41 The introduction of invasive marine algae can have substantial negative impacts upon native
42 communities in their newly established range (Williams & Smith, 2007; Thomsen *et al.*,
43 2009). When an invasive plant or alga begins to proliferate in a new range, it presents a novel
44 food-source to native consumers. Although specialists may avoid the invasive organism,
45 generalist consumers can be attracted to such species and even prefer to consume them over
46 native species (Parker & Hay, 2005; Parker *et al.*, 2006). However, this is not always the case
47 and in many instances both plants (e.g. Jogesh *et al.*, 2008) and algae (e.g. Davis *et al.*, 2005)
48 have been shown to be avoided by generalist consumers. Herbivore preference may therefore
49 account for the invasibility of some species, a scenario described by the Enemy Release
50 Hypothesis (ERH) (Keane & Crawley, 2002).

51 *Sargassum muticum* Yendo (Fensholt) is a highly invasive marine alga which is not
52 controlled by large herbivores such as fish and urchins (Britton-Simmons, 2004; Thomsen *et*
53 *al.*, 2006; Engelen *et al.*, 2015). Although it attracts a range of mesoherbivores (Strong *et al.*,
54 2009), many of these still prefer to feed upon native algae or the epiphytes on *S. muticum*
55 (Norton & Benson, 1983; Critchley *et al.*, 1986; Viejo, 1999; Britton-Simmons, 2004;
56 Monteiro *et al.*, 2009; Cacabelos *et al.*, 2010a; Rossi *et al.*, 2010; Engelen *et al.*, 2011).

57 Because of their size, mesoherbivores are less mobile than large herbivores, and individuals
58 or localised populations can display strong host-plant specificity, even when the species as a
59 whole does not (Vesakoski *et al.*, 2009; Bell & Sotka, 2012; Mattila *et al.*, 2014). Specificity
60 is particularly evident in species that are slow-moving or brood their young (Sotka, 2005),
61 and may be because the host alga of a mesoherbivore is both its food and habitat, and some
62 algal species can provide better protection from predators (Watanabe, 1984; Jormalainen *et*
63 *al.*, 2001). As such it can be hypothesised that mesoherbivores, particularly species that are

64 less mobile, will establish a feeding preference for an invasive species such as *S. muticum*
65 when exposed to it, but the time scales involved in such development are unclear.

66 Optimally-foraging animals are expected to prefer readily available sources of food,
67 and in cases where host specificity is strong, an invasive population may therefore escape
68 local herbivores when it is first introduced to a new range (Maron & Vilá, 2001). However,
69 once an invasive species proliferates, encounter-rates with local consumers will increase and
70 these may then accept it as a food source. Therefore, it is likely that the longer such a species
71 has been present in an environment, the greater the propensity of local consumers to feed on
72 it will be (Schultheis *et al.* 2015). This has been shown to take as little as 20 years in beetles
73 that consume the leaves of invasive trees (Auerbach & Simberloff, 1988), and data on
74 introduced crops shows that species diversity can be as rich as that found in native
75 controphics after less than 200 years (Strong *et al.*, 1977). However, little is known about the
76 lengths of time it might take marine mesoherbivores to consume a species such as *S.*
77 *muticum*, and few studies that specifically incorporate time-since-invasion as a variable
78 utilise behavioural experiments (e.g. Trowbridge 2004). Most opt instead for observational
79 surveys of abundance or diversity to infer consumer choice (*sensu* Maron & Vilá, 2007).
80 Time-since-invasion is a vital and understudied element of invasive ecology, since both the
81 invader and the local community change the longer an invasive population has been
82 established (Strayer *et al.*, 2006).

83 This study aimed to enhance our understanding of the ERH by investigating the
84 feeding behaviours of slow-moving mesoherbivore grazers, taken from stands of *S. muticum*
85 that have been established for different lengths of time. The Space-for-Time Substitution
86 methodology is well established in invasive ecology and climate change studies, and although
87 it is not fool-proof it provides a convenient alternative to the time-for-time approach which is
88 often not viable (Pickett, 1989; Thomaz *et al.*, 2012; Blois *et al.*, 2013). *Sargassum muticum*

89 is an ideal species with which to investigate mesoherbivore responses with this approach,
90 because it is well known beyond the scientific community as a pest. It is large, conspicuous,
91 intertidal, and prefers sheltered habitats and so is quickly reported when it spreads to
92 harbours and inlets frequented by fishermen and sailors. As such, and in contrast to many
93 other marine invasions, a detailed and reliable chronology of its spread around western-
94 Europe and the British Isles exists (Davison, 2009). As a brown alga, *S. muticum* produces
95 quantifiable polyphenolic chemicals as a defence against herbivory (Van Alstyne & Paul,
96 1990; Pavia & Toth, 2000). Although not deterrent against all species, these phlorotannins
97 impede herbivory by a broad range of mesoherbivores, particularly slow-moving generalist
98 gastropod grazers (Pavia & Toth, 2008). Brown algae have been shown to increase
99 phlorotannin production in response to increased herbivory, making themselves less-palatable
100 (Van Alstyne & Paul, 1990; Pavia & Toth, 2008), although investigations into invasive plants
101 have shown variable responses in the production of chemical defences in their invaded ranges
102 (i.e. increases: Caño *et al.*, 2009; decreases: Willis *et al.*, 1999; no recorded change, but still
103 lowered palatability (i.e. the attractiveness of the food in the absence of others) in herbivore
104 performance trials: Hull-Sanders *et al.*, 2007). Using laboratory-based feeding experiments
105 with *S. muticum* and two species of common generalist gastropod grazers, this study tested
106 two hypotheses. Firstly, generalist gastropods are more likely to accept *S. muticum* as a food
107 source the longer it has been present in their local habitat. Secondly, the palatability of *S.*
108 *muticum* will be lower in long-established populations, compared to those recently
109 established. Testing these hypotheses furthers our knowledge of invasive ecology and time-
110 since-invasion effects, specifically with respects to the ERH.

111

112

113 2. MATERIALS AND METHODS

114

115 **2.1 Study organisms**

116 *Sargassum muticum* was used as a model invader because it bears all of the hallmarks of a
117 classic invasive marine species, being temperate, pseudo-annual, fast-growing and *r*-selected,
118 with broad physiological tolerances and a propensity for high dispersal rates and rapid growth
119 in areas of strong anthropogenic influences, such as harbours (Norton, 1977; Critchley *et al.*,
120 1986; Arenas *et al.*, 1995; Andrew & Viejo, 1998; Claridge & Franklin, 2002; Engelen &
121 Santos, 2009). *Sargassum muticum* was first sighted in the British Isles in 1973, on the
122 eastern coast of the Isle of Wight, and has subsequently spread as far east as Kent, and as far
123 north-west as the Firth of Clyde.

124 *Ascophyllum nodosum* (L.) Le Jolis and *Fucus serratus* (L.) were used as control
125 organisms to assess typical feeding rates of snails from the different sites. Both are common
126 native intertidal algal species, which are closely related to *S. muticum*. These three species are
127 all fucoids and as such, they bear a similar chemical composition (Davis *et al.* 2003), grow
128 well on sheltered shores, and are often found attached to hard substratum in the mid to lower
129 intertidal (Boaden *et al.*, 1975; Dudgeon & Petraitis, 2005). The two native control species
130 are consumed by the selected grazers, *Littorina obtusata* (L.) and *Littorina fabalis* (Turton),
131 which are common herbivores of seaweeds throughout Western Europe (Watson & Norton,
132 1987; Hayward & Ryland, 2006). Both grazers can be found on *Fucus spiralis*, *F.*
133 *vesiculosus*, *A. nodosum*, *F. serratus*, *S. muticum*, *Halidrys siliquosa*, *Ulva lactuca*, and *U.*
134 *intestinalis* in high abundances on sheltered shores, both in the intertidal and shallow subtidal
135 (Kurr, *unpublished data*). *Littorina obtusata* lives for 2-3 years and is therefore often slightly
136 larger than the annual *L. fabalis*, both of which spawn in late winter (Williams & Brailsford
137 1998). Although *L. obtusata* is slightly more common on the mid-shore and *L. fabalis* more

138 common on the low shore, both species share near-identical habitat preferences and
139 distributions (Hayward & Ryland, 2006). Adults of both species have similar preferences for
140 macroalgae, although epiphytic microalgae probably accounts for a larger component of the
141 diet of *L. fabalis* than *L. obtusata*, owing to the weaker buccal musculature and more ‘comb-
142 like’ radula of the former (Watson & Norton 1987). Both show an aversion towards algal
143 tissues containing high levels of phlorotannins, making them viable indicators for
144 ecologically relevant differences in algal defensive investment (Pavia & Toth, 2000; Pavia et
145 al., 2002).”

146 **2.2 Study sites**

147 Multiple potential sites were initially identified based on public records of first reported *S.*
148 *muticum* establishment. The suitability and comparability of these sites was estimated as far
149 as possible remotely, before being visited in July. Samples of *S. muticum*, the most abundant
150 native furoid in close proximity to the *S. muticum* stands, and a suitable number of whichever
151 littorinid was most common in the area were taken from each location. The experimental
152 designs and decisions about which grazer and native furoid were to be used were site-
153 specific, constrained predominantly by the necessity to find sites with comparable topography
154 and suitable time-since-invasion. Material from four *S. muticum* populations was used in
155 feeding trials (Figure 1). All stands of *S. muticum* grew on the upper sub-tidal of moderately
156 exposed sandy shores, in lagoons formed in the lee of a rock formations or sand-bars (Figure
157 2). Salinity and temperature differences at time-of-sampling were minimal (salinity within 1
158 unit, and temperature within 2 °C), and all populations were amongst or very near to common
159 native algae such as *F. serratus*, *A. nodosum* and/or *F. vesiculosus* (Figure 2). The
160 northernmost and southernmost sites differed by 3° of latitude, and whilst UV exposure
161 causes induction of phlorotannins (Pavia *et al.*, 1998), UV-R levels (which account for a
162 small percentage of total solar irradiance) differ by only 0.72% (estimated from Šúri *et al.*,

163 2007; Escobedo *et al.*, 2009). If *S. muticum* responds to UV in the same way as other fucooids,
164 this would equate to an approximate difference of 0.4% in phlorotannin abundance between
165 the northernmost and southernmost sites (Pavia *et al.*, 1998), a negligible amount,
166 considering that herbivory can induce phlorotannin production by 70% (Pavia & Toth, 2000).

167 For clarity, locations have been named by the length of time since the first
168 observation of *S. muticum* (Davison, 2009), as follows; ‘40YR’: Bembridge Ledge, on the
169 eastern coast of the Isle of Wight. ‘35YR’: Eastbourne on the south eastern coast of England.
170 ‘19YR’: West Angle Bay, near Milford Haven in south Wales. ‘10YR’: The northern shore of
171 the Foryd estuary near Caernarfon in North Wales (Figure 1). It can be assumed that the first
172 observation equates to time-since-invasion in *S. muticum* populations as it expresses limited
173 DNA polymorphism within UK and European populations, suggesting that it has spread from
174 a single point of invasion (Hallas, 2012; Le Cam, 2015). Communities impacted by an
175 invasive population with a time-since-invasion of 10 years or less, are expected to experience
176 considerably different effects to those exposed for 30 years or more, making the timescale
177 investigated here valid for detecting shifts in behaviour (see Strayer *et al.*, 2006).

178

179 **2.3 Feeding trials**

180 Four experiments were conducted: (1) *Sargassum muticum* from stands of different ages were
181 presented to *L. obtusata* collected from a site where *S. muticum* has not been recorded, to
182 assess the palatability of the algae (i.e. the attractiveness of the food in the absence of other
183 foods). (2) *Littorina obtusata* from *S. muticum* stands of different ages were presented with *S.*
184 *muticum* and *F. serratus* from one site, to assess the willingness-to-feed of the snails (i.e. how
185 eager the animals are to feed in the material in the absence of others). Finally, *L. fabalis* from
186 an ‘old’ and a ‘young’ *S. muticum* stand were presented with *S. muticum*, and the common
187 native alga *A. nodosum* from both sites in (3) no-choice trials to assess the willingness-to-

188 feed of grazers, and with *S. muticum* from the ‘young’ and ‘old’ stands in (4) choice trials to
189 determine feeding preferences of the snails (i.e. which material will be selected when given a
190 choice).

191 For each experiment, a standardised protocol was used. Firstly, all algae were
192 collected within one week of being used in trials, and maintained in ambient seawater (~19
193 °C, salinity 34) within the same outdoor aquaria used for feeding trials. *Sargassum muticum*
194 is notoriously fragile, and difficult to maintain in the laboratory. To minimise the degradation
195 of the fronds, whole individuals were collected by pulling the holdfast from the substrata.
196 Epiphytised fronds were removed, and all individuals were returned to the laboratory within
197 cool-boxes, inside of one day. Algae were inspected daily and gently washed with seawater.
198 Material showing signs of degradation was removed, and the whole individual was rotated in
199 the aquaria to limit self-shading. Only healthy material was used for feeding trials. From each
200 alga used, 500mg (+/- 50 mg) blotted-wet-weight clippings were taken from the apical region
201 (one clipping per apex) and autogenic changes in algal mass were corrected for by taking
202 control clippings and maintaining these in parallel to the experimental trials. For no-choice
203 trials, mean autogenic changes in mass were calculated and subtracted from the change in
204 mass in experimental trials to estimate change in mass due to consumption (Toth *et al.*,
205 2007). Additionally, ~5 clippings were taken for phlorotannin analysis in palatability trials.
206 Handling of algae was kept to a minimum, and great care was taken during blotting of fronds
207 to avoid dislodging vesicles or causing other damage. To further limit degradation, midway
208 through the trials aquaria were carefully upturned into a 1 mm sieve to remove snails and
209 algal material, the aquaria were cleaned using an abrasive pad, and all material was then
210 returned for the remainder of the experiment. Some, but not all, of the *S. muticum* clippings
211 used in autogenic trials lost mass, notably those used in Experiment 4 (Section 3.4, Figure 5).
212 This was probably due to loss of vesicles over the course of the trial because the clippings

213 appeared to in generally good condition. However some necrosis was evident near to the
214 clipping site at the base of some replicates in both experimental and control trials.

215 Secondly, all animals used in the trials were collected one week prior to experiments
216 and maintained as above, without food to ensure even levels of hunger. Because starved
217 herbivores can display compensatory feeding for the first two days (Cronin & Hay, 1996)
218 change in algal masses were only recorded after seven days. Change in algal mass was
219 corrected for snail ash-free dry mass and compared with autogenic changes in the controls
220 (identical treatments without grazers) (Monteiro *et al.*, 2009; Forslund *et al.*, 2010). All trials
221 were conducted in separate 250 ml aquaria with an individual water-line providing a
222 flushing-time of around 30 s.

223

224 **2.3.1 The palatability of *S. muticum* to naïve grazers**

225 To determine whether *S. muticum*'s palatability was different depending on time-since-
226 invasion, six algal individuals were collected at random from each of the 35YR, 19YR, and
227 10YR populations (n = 18). To capture individual *S. muticum* variation, three clippings from
228 each individual were provided to three *L. obtusata* (n = 54) collected from Bull Bay in North
229 Wales (53.422543°N, 4.368959°W) in a no-choice trial. *Sargassum muticum* does not grow
230 at Bull Bay, nor on the adjacent coastline, and so these individuals were extremely likely to
231 be naïve to this food-source. This was done to avoid biasing the study by using
232 mesoherbivores with a history of *S. muticum* consumption, thereby ensuring differences in
233 feeding responses were a product of the algal condition, and not that of the consumer.

234 Although the no-choice technique has been criticised for not producing 'true' feeding
235 responses (Roa, 1992), no-choice trials were used for two reasons. Firstly, *S. muticum*
236 fragments as it is fed upon, making it impossible to differentiate between algae from most
237 sites at the end of the trials. Secondly, the technique still holds value when determining the

238 ‘willingness-to-feed’ of a particular herbivore on a plant or alga (i.e. how much material are
239 herbivores willing to consume; sensu Jogesh *et al.*, 2008), or ‘palatability’ of a particular
240 plant or alga (i.e. how easy the material is to consume; sensu Toth *et al.*, 2007), as opposed to
241 purely ‘preference’ of consumers, which requires that a choice be offered. No-choice trials
242 can therefore be useful in predicting the results of direct interaction between grazers and
243 hosts (Pearse *et al.*, 2013). Data were analysed in a One-way ANOVA, with site as a fixed
244 factor, the response variable was the mean of the three clippings (minus mean autogenic
245 mass-change in controls) from each individual alga to provide a better estimate of the
246 palatability of an individual *S. muticum* and to avoid non-independence arising from using
247 clippings from individual alga in the analysis (n = 18).

248 Phlorotannin abundances in each of the six *S. muticum* individuals were determined.
249 Samples from the upper frond were washed in distilled water, frozen at -20 °C, and then
250 freeze-dried to constant weight. These were ground until homogenous, and 0.2 g subsampled
251 for chemical assay. 60 % aqueous acetone was used to extract phlorotannins over 1 hour
252 under constant agitation, in the dark. The algal pulp was separated by centrifugation (5300
253 rpm for 10 minutes) and the acetone removed using *in-vacuo* cold-distillation (80 kPa, 38
254 °C). Lipophilic compounds were filtered from this extract (Pavia & Toth, 2000) and 40%
255 Folin-Ciocalteu’s phenol reagent (Sigma-Aldrich F9252) was used in conjunction with 1M
256 aqueous sodium carbonate decahydrate solution (Sigma-Aldrich 71360) to act as a buffer.
257 The resultant solution was incubated in the dark for 30 minutes and analysed by
258 spectrophotometry at 760 nM, using phloroglucinol (1,3,5-trihydroxybenzene, Sigma-Aldrich
259 P3502) as a standard (Van Alstyne, 1995). Replicates were run in triplicate and phlorotannin
260 abundances compared with a one-way analysis of variance (ANOVA) with ‘site’ as a fixed
261 factor. Differences in means were compared using Tukey’s *post-hoc* tests.

262

263 **2.3.2 Willingness of grazers experienced with *S. muticum* to feed upon it**

264 To determine whether *L. obtusata* consumed more *S. muticum* when their population had
265 been exposed to it for longer, 30 individuals were collected at random from each of the
266 35YR, 19YR, and 10YR sites (n = 90). One *S. muticum* individual and one *F. serratus*
267 individual were collected from the 10YR site. Apex material from one large individual was
268 used for each algal species to keep phlorotannin abundances, algal condition, and any other
269 variables that may influence palatability, as constant as possible between treatments, given
270 that the focus in this experiment was on the grazers. Fifteen *L. obtusata* individuals were
271 provided with one clipping of the *S. muticum* individual each, and the other fifteen were
272 provided with one clipping of the *F. serratus* individual each to gauge for population-specific
273 differences in feeding-rates on a typical sympatric native alga. Mass change at the end of
274 trials were corrected for mean autogenic change in control clippings to estimate change in
275 mass due to consumption. Differences in ‘willingness-to-feed’ did not conform to the
276 assumption of homogeneity of variance because the variability in *F. serratus* consumption
277 was greater than that for *S. muticum* consumption. Therefore, means were compared using a
278 Kruskal-Wallis test, and paired Mann-Whitney U tests for *post-hoc* analysis.

279

280 **2.3.3 The willingness to consume *S. muticum* and a native alga by *L. fabalis***

281 To compare willingness-to-feed on *S. muticum* and a native sympatric alga by
282 mesoherbivores (*L. fabalis*) from a site invaded by *S. muticum* 40 years prior, to grazers from
283 a site invaded 10 years prior, three *S. muticum* and three *Ascophyllum nodosum* individuals
284 were collected at random from 40YR and 10YR sites (n = 6 of each species). *Littorina fabalis*
285 were collected at random from adjacent stands of fucoids, and from the substrata nearby to
286 the *S. muticum* and *A. nodosum* stands at these locations. One clipping of each alga was
287 provided to three randomly-assigned *L. fabalis* from each site, and all trials were run in

288 triplicate (n = 72). Changes in algal mass were analysed using a three-way nested-ANOVA
289 with ‘algal species’, ‘algal origin, and ‘grazer origin’ as fixed orthogonal factors, and
290 ‘individual’ (alga) nested in the interaction between ‘algal species’ and ‘algal origin’.
291 Differences in means were compared using Tukey’s *post-hoc* tests.

292

293 **2.3.4 *L. fabalis* preference for *S. muticum* from sites with different time-since-invasion**

294

295 Clippings from the same algal individuals used for the willingness to feed experiment
296 (Experiment 3) were used to compare the preference of *L. fabalis* collected from the 40YR
297 and 10YR sites when offered *S. muticum* from their site of origin, against algae collected
298 from the other site. Each treatment was allocated to six *L. fabalis* individuals in the following
299 randomly assigned treatments: (1) 40YR *L. fabalis* with 40YR *S. muticum* & 10YR *S.*
300 *muticum*, and (2) 10YR *L. fabalis* with 40YR *S. muticum* & 10 YR *S muticum*. All trials were
301 run in triplicate (n = 18). To capture autogenic changes in algal mass each experimental
302 aquaria (i.e. with grazers) was paired with a control aquaria containing the same algal
303 combination but no grazers. Following Forslund *et al.* (2010), a paired *t* test approach was
304 used to compare the differences in algal mass-change between clippings in both the
305 experimental and control aquaria, whereby, a significant result indicates that one clipping has
306 changed mass more than the other in the presence of grazers.

307 3. RESULTS

308

309 **3.1 The palatability of *S. muticum* to naïve grazers**

310 *Sargassum muticum* from 35YR contained the highest abundance of phlorotannins
311 (5.0% DW, S.E. = 0.15), and that from 19YR (3.6% DW, S.E. = 0.23) and 10YR (3.8%, S.E.
312 = 0.14) bore similar concentrations (ANOVA $F_{2,15} = 18.66$, $SS = 7.07$, $p < 0.001$, Tukey's
313 HSD; 35YR > 19YR = 10YR). However, the consumption of *S. muticum* tissue from
314 different sites was not significantly different (ANOVA $F_{2,15} = 0.0741$, $SS = 1902$, $p = 0.929$;
315 Figure 3a), suggesting that naïve *L. obtusata* taken from Bull Bay did not respond to
316 differences in phlorotannins and/or any differing palatability of *S. muticum* from sites with
317 different time-since-invasion.

318

319 **3.2 Willingness of grazers experienced with *S. muticum* to feed upon it**

320 There were significant differences between the willingness-to-feed on the *S. muticum*
321 individual by *L. obtusata* from all three sites (Kruskal-Wallis, $H = 28.69$, $p < 0.001$; Figure
322 3b). *Littorina obtusata* consumed broadly similar amounts of the *F. serratus* individual per
323 mg of animal dry mass regardless of site, although those from 19YR consumed more *F.*
324 *serratus* than those from 35YR. Consumption of the *S. muticum* individual relative to the *F.*
325 *serratus* individual showed an incremental increase with time-since-invasion. The 10YR
326 grazers consumed less of the *S. muticum* individual than those from the other populations,
327 and more of the *F. serratus* individual. The 19YR grazers consumed as much of the *S.*
328 *muticum* as those from 35YR, but consumed more of the *F. serratus* than the *S. muticum*. The
329 35YR grazers consumed as much of the *S. muticum* as they did the *F. serratus* individual,
330 indicating that they were equally willing to feed on the invasive and native algae.

331

332 **3.3 The willingness-to-feed on *S. muticum* and a native alga, by *L. fabalis***

333 Willingness to feed differed between species and grazer origin (Table 1, Figure 4). 40YR *S.*
334 *muticum* was the most readily consumed by both groups of grazers, however the 40YR
335 grazers consumed more of the invasive overall. Additionally, whilst the 10YR grazers were
336 willing to consume 10YR *A. nodosum* in similar quantities to 40YR *S. muticum*, 40YR
337 grazers consumed more of the invasive. No differences in consumption of the native *A.*
338 *nodosum* were detected between grazer populations, both consuming more of that from
339 the 10YR site.

340

341 **3.4 *L. fabalis* preference for *S. muticum* from sites with different time-since-invasion**

342 The experienced grazers demonstrated a clear preference for *S. muticum* from one location,
343 but these preferences were not evident in the more naïve group. 40YR *L. fabalis* preferred
344 40YR *S. muticum* to 10YR *S. muticum* ($t_9 = 2.44$, $p = 0.041$, Figure 5a), whereas no
345 preferences were observed for *S. muticum* from either site by 10YR grazers ($t_9 = 0.87$, $p =$
346 0.411, Figure 5b).

347

348 4. DISCUSSION

349 There were considerable differences in the acceptance and selection of *Sargassum muticum*
350 as a food source, by mesoherbivores from populations exposed to it for different lengths of
351 time. Overall, the greater the time-since-invasion, the greater the feeding rates on *S. muticum*
352 by grazers from those sites (Hypothesis 1, Experiments 2, 3 and 4). Chemical defences in *S.*
353 *muticum* were highest at the site with greatest time-since-invasion (Hypothesis 2, Experiment
354 1), although palatability, when tested by naïve grazers, did not differ (Hypothesis 2,
355 Experiment 1). Likewise, more experienced grazers demonstrated a clear preference for *S.*
356 *muticum* from one site when offered a choice, whereas naïve conspecifics consumed
357 indiscriminately (Hypothesis 1, Experiment 4). This suggests that some exposure to an alga
358 may be required to develop subjectivities based on chemical defence or condition
359 (Hypothesis 1, Experiment 1 and 4).

360 It is unclear whether the increased acceptance of *S. muticum* as a food source is the
361 result of exposure over decadal timescales, or differences in encounter rate because of greater
362 proliferation of the alga in older populations. However this point is probably moot, since the
363 abundance of a non-native species in an introduced range is also dependent on time (Bennett
364 *et al.* 2013; Byers *et al.*, 2015; Vicente *et al.*, 2014). Therefore the longer *S. muticum* has
365 been present, the more the local population of grazers will encounter it, and therefore the
366 more likely they will be to consume it. These results provide evidence in support of the
367 Enemy Release Hypothesis (ERH), which posits that non-native species experience lower
368 herbivore pressures in their introduced ranges because local consumers are unfamiliar with
369 them (Keane & Crawley, 2002). The ERH is a popular and widely cited explanation for the
370 invasibility of many photoautotrophs, but it is now apparent that the hypothesis typically fails
371 verification (Parker & Hay, 2005; Parker *et al.*, 2006). Only a few studies have specifically
372 tested the ERH with respect to time-since-invasion (Strayer *et al.* 2006, Heger and Jeschke

373 2014, Schultheis *et al.* 2015), and fewer still incorporate feeding-trials to directly assess
374 preferences or willingness-to-feed of consumers with TSI, particularly in the marine
375 environment (Trowbridge 2004).

376 Despite our findings, the question of whether or not the ERH applies to *S. muticum*
377 remains unclear since top-down control by specialist grazers in its native range has never
378 been demonstrated. Certainly the impacts of any increase in grazer preference have not been
379 sufficient to limit the spread of this species around the UK. Evidence for the ERH in *S.*
380 *muticum* can be divided into those studies that assessed grazer abundance and diversity (e.g.
381 Withers *et al.*, 1975; Norton & Benson, 1983; Viejo, 1999; Wernberg *et al.*, 2004; Strong *et*
382 *al.*, 2009; Cacabelos *et al.*, 2010a; Engelen *et al.*, 2013) and those like this the current study
383 that assessed feeding by grazers (e.g. Norton & Benson, 1983; Critchley *et al.*, 1986;
384 Pedersen *et al.*, 2005; Monteiro *et al.*, 2009; Strong *et al.*, 2009; Cacabelos *et al.*, 2010b;
385 Engelen *et al.*, 2011). Typically, observational studies on faunal abundance and diversity
386 demonstrated similar patterns between *S. muticum* and sympatric native algae, and therefore
387 show evidence against the ERH (e.g. Cacabelos *et al.*, 2010a; Engelen *et al.*, 2013) However,
388 the results of feeding-trials usually show an aversion towards *S. muticum* or a preference for
389 native species in feeding-trials, in support of the ERH (e.g. Monteiro *et al.* 2009, Cacabelos *et*
390 *al.* 2010b). Although different to previous feeding-trials because of the incorporation of time-
391 since-invasion, the present study also supports the ERH. These apparently contradictory
392 conclusions may be the result of animals moving into *S. muticum* stands during the day for
393 protection against visual predators, but then returning to native alga to feed during the night
394 (Buschmann, 1990; Machado *et al.*, 2015). Alternatively, grazers on *S. muticum* could be
395 feeding mainly on epiphytic material and detritus in the field (Viejo, 1999; Cacabelos *et al.*,
396 2010b), and may therefore avoid *S. muticum* under laboratory conditions due to experimental
397 removal of epiphytes or the selection of individuals that are relatively epiphyte free.

398 However, a small number of previous studies have found preferences for *S. muticum*
399 over native algae (e.g. Pedersen *et al.*, 2005), or a lack of preference for native algae when
400 paired with it (e.g. Engelen *et al.*, 2011). Strong *et al.* (2009) demonstrated that the amphipod
401 *Dexamine spinosa* from Strangford Lough in Northern Ireland, exhibited a clear preference
402 for *S. muticum* in feeding trials. The amphipod fed directly upon *S. muticum*'s fronds even
403 when they were epiphytised, showing neither the preference for, nor the aversion towards,
404 epiphytes seen in other species of crustacean (Karez *et al.*, 2000). *Sargassum muticum* was
405 present in Strangford Lough for eight years prior to these feeding-trials, although it was
406 probably abundant for less than five (Davison, 2009). These preferences therefore developed
407 quickly, far less than the 23 years Cacabelos *et al.* (2010a) show it took for grazers to adapt to
408 *S. muticum*, or the 19–35 years in this study. The findings by Strong *et al.* (2009) may be due
409 to the grazer studied, as swimming crustaceans are more mobile than benthic gastropods and
410 their feeding-modes differ, making it easier for them to feed on thinner algal fronds (Pavia &
411 Toth, 2000) such as those of *S. muticum*. In addition, the gastropods in Monteiro *et al.* (2009)
412 exhibited preference for native algae, whereas the crustaceans (both amphipods and isopods)
413 in Engelen *et al.* (2011) did not. These studies therefore arrive at different conclusions for the
414 ERH, possibly because of the capabilities of the grazers used.

415 Engelen *et al.* (2013) did not specifically test time-since-invasion in field
416 observations, but noted that the older *S. muticum* populations sampled had greater faunal
417 diversity with respect to sympatric native algae. Had the experiment been undertaken at the
418 older sites only, the ERH would also have appeared not to apply. Likewise, Monteiro *et al.*
419 (2009) discuss that the feeding-preferences for native algae over *S. muticum* in the grazers
420 they assayed, did not differ with time-since-invasions of 22, 12, and 7 years. However, the
421 results presented here suggest modifications of gastropod preferences do not begin until
422 somewhere between 19 and 35 years after invasion. Therefore, when drawing conclusions

423 from both field observations and feeding-trials with a view to testing the ERH, the nature of
424 the grazers included (their relative mobility, feeding-modes, and diet), and the time-since-
425 invasion at the site or sites being sampled (Strayer *et al.*, 2006; Schultheis *et al.*, 2015) must
426 be taken into consideration.

427 It is curious that despite differences in phlorotannin concentration in the *S. muticum*
428 sampled, palatability appeared unaffected in the first experiment. The younger two
429 populations bore similar abundances of phlorotannins, but the older population had around
430 1.5% more phlorotannin by dry mass. *Littorina obtusata* responds to differences in
431 phlorotannin of around 1% DM when feeding on *A. nodosum* (Pavia *et al.*, 2002). The lack of
432 differences in feeding response could have arisen because the animals used were entirely
433 naïve to *S. muticum*, since gastropods may display high rates of indiscriminate feeding on
434 novel foods (e.g. Whelan, 1982; Morrison & Hay, 2011). The naïve *L. obtusata* in the
435 palatability trials (experiment 1) consumed much more *S. muticum* material than the
436 experienced *L. obtusata* in the willingness-to-feed trials (experiment 2). These findings
437 suggest that biotic resistance in the very early stages of an invasion may be exceptionally
438 high (Parker & Hay, 2005), albeit temporary if the invasive species proves unpalatable (see
439 Whelan 1982).

440 These results represent a preliminary assessment of grazing preference with time-
441 since-invasion. Sampling a greater number of both sites and grazers would permit a more
442 concrete understanding of the potential behavioural shifts undertaken by these consumers, in
443 response to invasions. The logistical constraints of including time-since-invasion are notably
444 restrictive. Even equipped with the chronology of *S. muticum*'s spread, selecting sites similar
445 to one another proved to be difficult. Sites differed in terms of species composition and
446 compromises had to be made in the design of experiments. Caution must also be applied in
447 interpreting the broader ecological significance of these trials, since mesoherbivore feeding-

448 behaviours in the laboratory are not necessarily reflected in the field (Monteiro *et al.*, 2009),
449 and presence of grazers on algae in the field does not necessarily indicate direct herbivory on
450 the tissues of the macrophyte (Viejo, 1999; Pearse *et al.* 2013). It is worth noting that *S.*
451 *muticum* is vastly more abundant at both of the ‘older’ sites sampled (35YR and 40YR), and
452 although grazing marks are visible upon the fronds, they grew to similar thallus lengths as
453 observed from other sites elsewhere (Kurr, *unpublished data*). However, these findings do
454 suggest that native marine mesoherbivores have the potential to alter their behaviour in the
455 presence of non-native species, developing an ability to feed on novel foods over time
456 (Trowbridge 2004). This adds to the growing body of literature (e.g. Dostál *et al.*, 2013;
457 Harvey *et al.*, 2013; Byers *et al.*, 2015; Schultheis *et al.*, 2015, and references therein) which
458 shows that time-since-invasion is a fundamental component of invasive ecology.

459

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467

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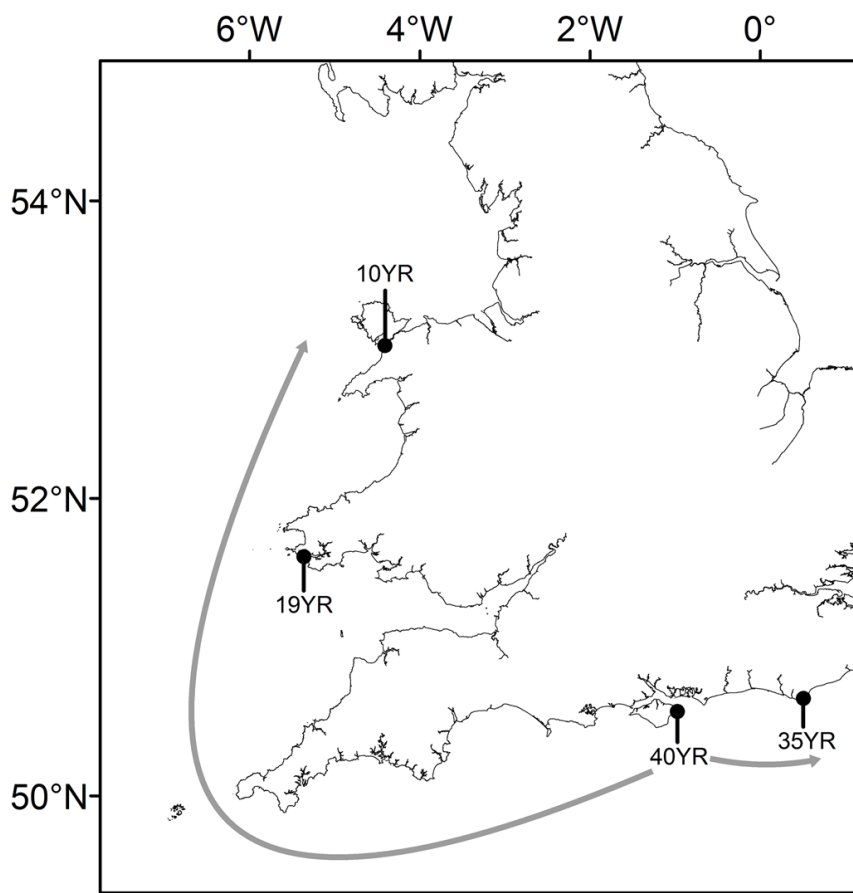
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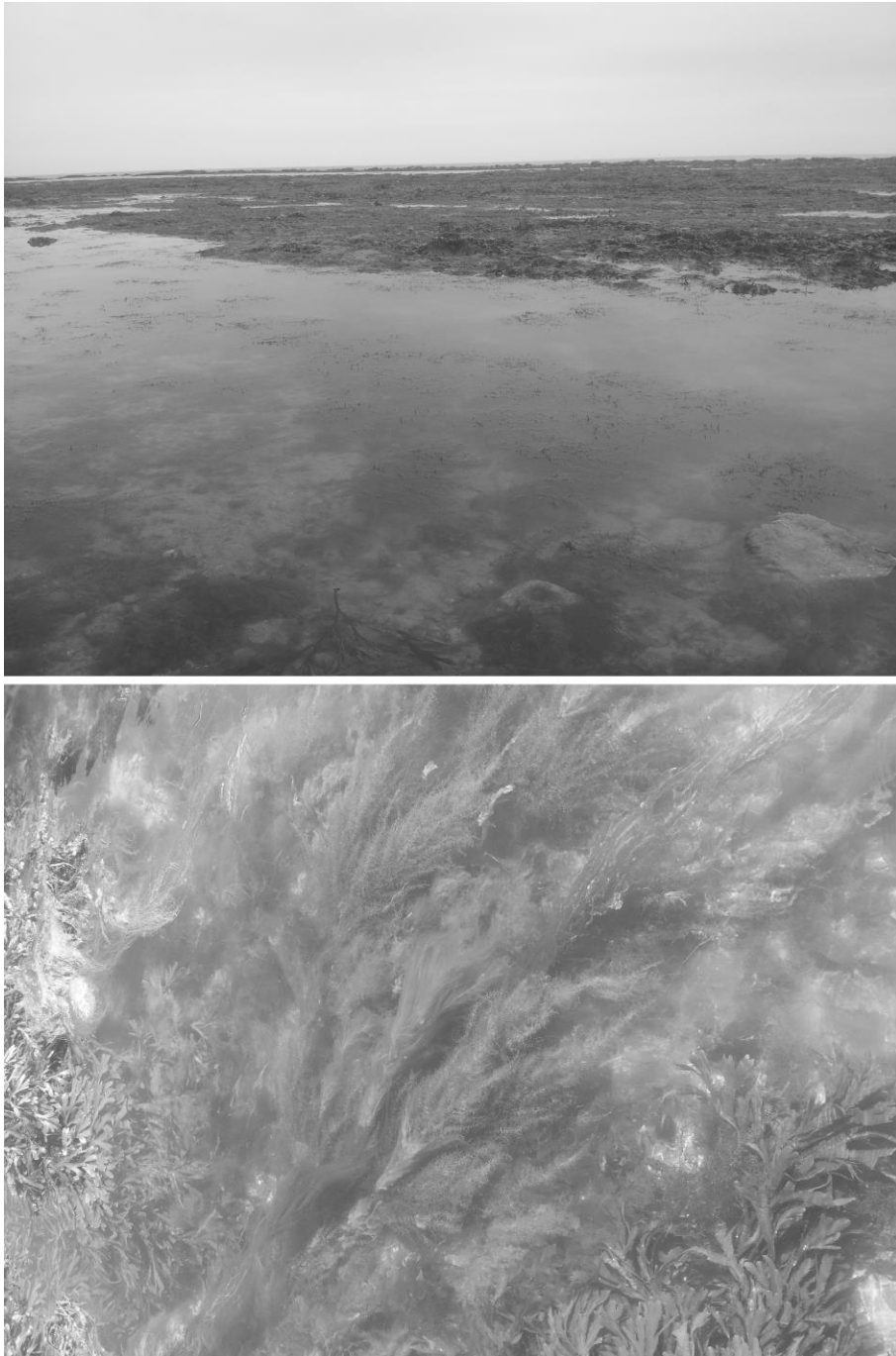
752 *Figures*

753 **Fig 1.** Locations of *Sargassum muticum* populations sampled for algal material and
 754 mesoherbivores. Sites are named by the time-since-invasion of *S. muticum*. Grey arrows
 755 represent general invasion path from first observed occurrence. Site 40YR: Bembridge Ledge
 756 (50.680466°N, 1.072554°W). Site 35YR: Eastbourne (50.750541°N, 0.270442°E). Site
 757 19YR: West Angle Bay, near Milford Haven (51.688676°N, 5.110854°W). Site 10YR: The
 758 northern shore of the Foryd estuary near Caernarfon (53.131581°N, 4.304016°W).



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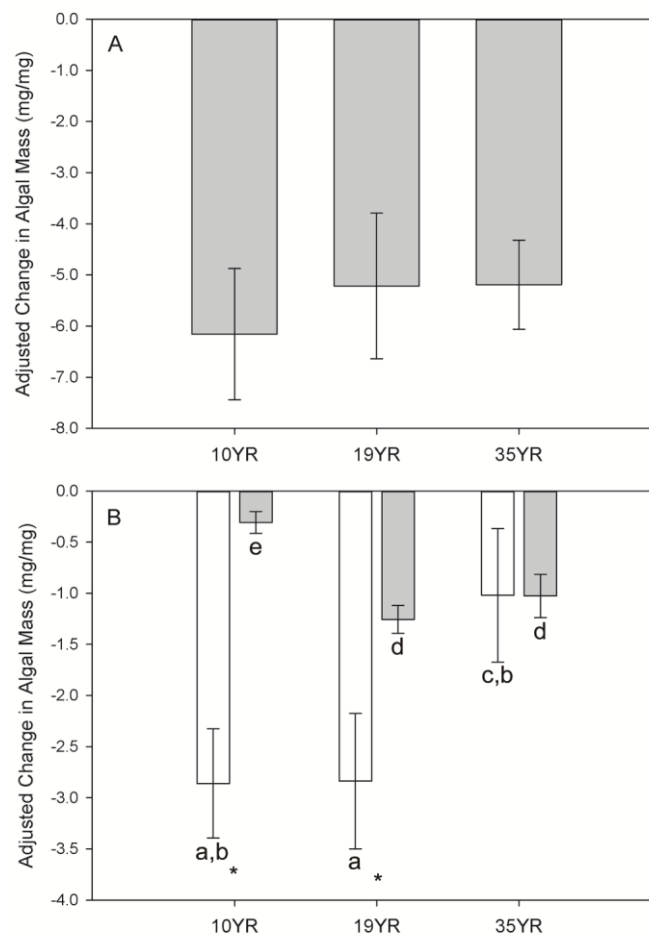
760 **Fig 2.** The lagoonal system at Site 35YR: Eastbourne showing the typical topography (upper
761 plate), and the typical position of *Sargassum muticum* individuals in relation to native algae
762 (lower plate), at the four sites sampled. *Sargassum muticum* fronds can be seen floating on
763 the surface waters close to *Fucus serratus*, *Ascophyllum nodosum*, *Ulva spp.*, and a number
764 of rhodophytes including *Heterosiphonia plumosa* and *Plumaria plumosa*.



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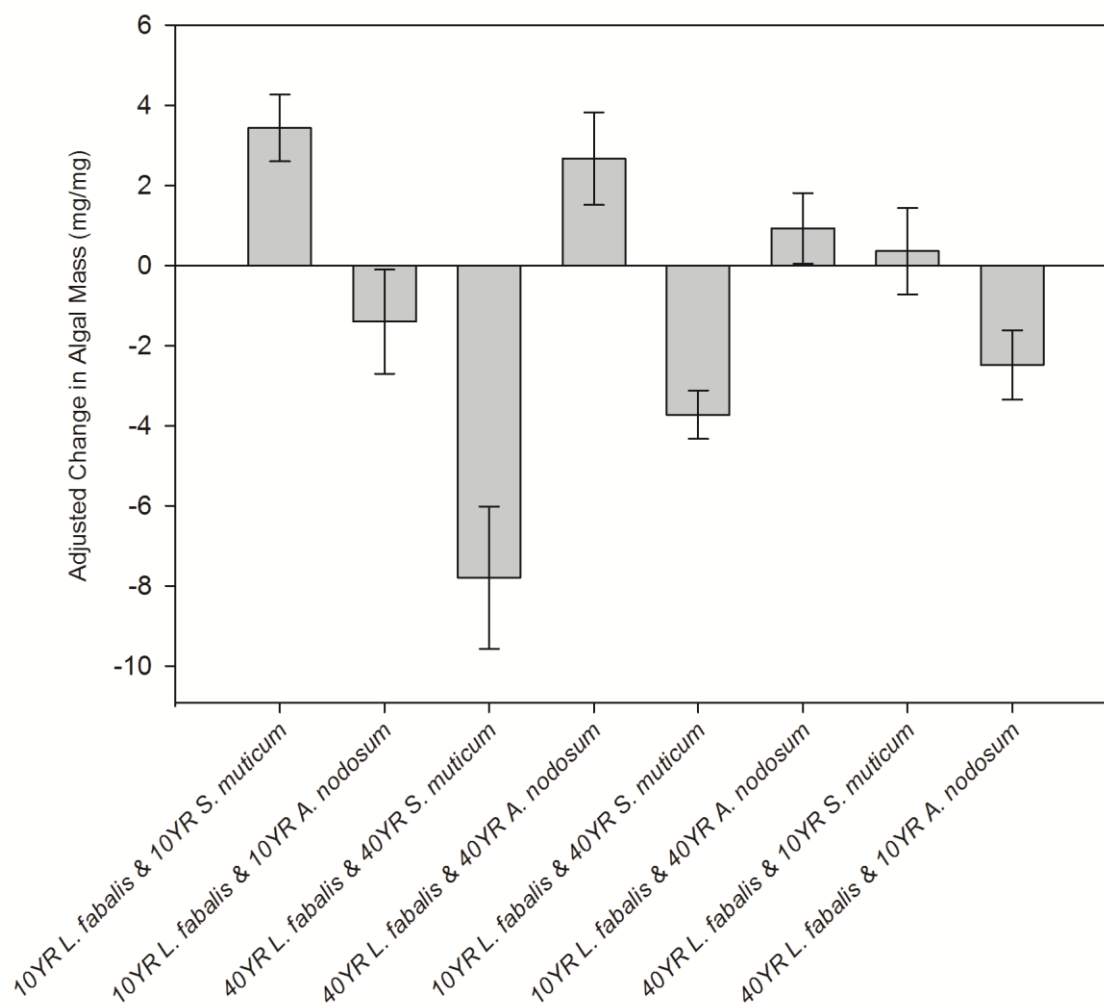
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767 **Fig 3.** Algal consumption by native herbivores: A) Change in algal mass per unit ash-free-
 768 dry-mass of herbivore (mg/mg) after seven days of *Sargassum muticum* fronds collected from
 769 populations established for different lengths of time (named by reported time-since-invasion),
 770 by *Littorina obtusata* naïve to *S. muticum*. B) Change in algal mass per unit ash-free-dry-
 771 mass of herbivore (mg/mg) of *Fucus serratus* (white bars) and *Sargassum muticum* (grey
 772 bars) after seven days in the presence of *Littorina obtusata* collected from *S. muticum*
 773 populations established for different lengths of time (named by reported time-since-invasion).
 774 Letters indicate groupings in consumption by each algal species and site, and * differences
 775 between algal species within a site based on Mann-Whitney U tests. All data are adjusted for
 776 autogenic changes in algal mass and error bars show +/- 1 S.E.



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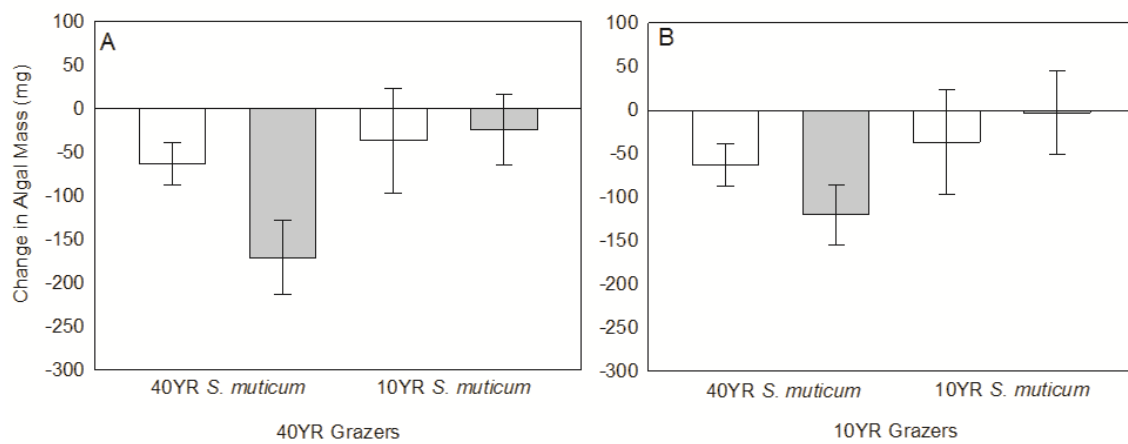
778 **Fig. 4.** Change in algal mass (m g/mg) of *Sargassum muticum* and *Ascophyllum nodosum*
 779 fronds collected from populations established for different lengths of time (named by
 780 reported time-since-invasion of *S. muticum*), after seven days of exposure to *Littorina fabalis*
 781 collected from those same sites in no-choice feeding trials. All data are adjusted for autogenic
 782 changes in algal mass. *Post hoc* analysis is presented in Table 1, and error bars show +/- 1
 783 S.E.



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785

786 **Fig. 5.** Change in algal mass of *Sargassum muticum* collected from sites bearing *S. muticum*
 787 populations of different ages (named by reported time-since-invasion of *S. muticum*), after
 788 seven days of exposure to *Littorina fabalis* collected from the same sites in choice feeding
 789 trials (grey bars), and autogenic controls that did not contain grazers (white bars). A) 40YR *L.*
 790 *fabalis* and B) 10YR *L. fabalis*, each treatment contained *S. muticum* from both sites. Error
 791 bars show +/- 1 S.E.



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794 **Table 1.** Three-way Nested ANOVA for the change in mass of algal individuals exposed to
 795 *Littorina fabalis* collected from two sites, one bearing *Sargassum muticum* for 40 years, and
 796 one for 10 years, in no-choice feeding trials on *Sargassum muticum* and
 797 *Ascophyllum nodosum*.

798

	DF	MS	F	P
Algal species	1	48724	8.01	0.007
Algal origin	1	54066	8.89	0.004
Grazer origin	1	37037	6.09	0.017
Algal species*Algal origin	1	458084	75.32	< 0.001
Algal species*Grazer origin	1	53737	8.84	0.005
Algal origin*Grazer origin	1	2952	0.49	0.489
Individual (Algal species*Algal origin)	1	23747	3.90	0.001
Algal species*Algal origin*Grazer origin	1	12827	2.11	0.153
Individual (Algal species*Algal origin)*Grazer origin	8	9899	1.63	0.142
Error	68	6082		

799

Tukey's *post-hoc* analysis:

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Algal Species*Algal Origin; 10YR *S. muticum* (A), 40YR *A. nodosum* (A), 10YR *A. nodosum* (B), 40YR *S. muticum* (C)

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Algal Species*Grazer Origin; *A. nodosum* and 40YR grazers (A), *S. muticum* and 10YR Grazers (A), *A. nodosum* and 10YR Grazers (A), *S. muticum* and 40YR Grazers (B)

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Algal Origin*Grazer Origin; 10YR Algae with 10YR Grazers (A), 10YR Algae with 40YR Grazers (AB), 40YR Algae with 10YR Grazers (AB), 40YR Algae with 40YR Grazers (B)

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