

1 **Effects of cigarette butts on marine keystone species (*Ulva lactuca* L. and *Mytilus edulis* L.) and**
2 **sediment microphytobenthos**

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8

9 **Abstract**

10 Outdoor mesocosms with constantly flowing natural seawater were used to test the effects of
11 littered cigarette butts on the filter feeder *Mytilus edulis* (blue mussel), the macroalga, *Ulva*
12 *lactuca* (sea lettuce) and sediment microphytobenthos in a semi-natural marine setting. Either
13 conventional, cellulose acetate, or biodegradable, cellulose, smoked cigarette butts were added
14 at densities of 0.25 or 1 butt L⁻¹. The clearance rates of mussels exposed to 1 butt L⁻¹ of cellulose
15 acetate butts were three times less than the controls. The growth of *U. lactuca* was not
16 measurably affected by cigarette butts, however the sediment chlorophyll content was
17 significantly less in mesocosms exposed to 0.25 and 1 butt L⁻¹ of cellulose acetate butts. These
18 effects occurred despite constant replacement of seawater indicating how hazardous
19 conventional cigarette butts are to marine life. Biodegradable cellulose cigarette butts had
20 minimal effects on the measured variables but should still not be discarded as litter.

21

22 **Key words:** smoking, cellulose acetate, green butts, biodegradable, single use plastics,
23 hazardous.

24 **1. Introduction**

25 Global consumption of cigarettes has been rising steadily for years and tobacco consumption
26 is currently considered a global epidemic by the World Health Organization (WHO 2019). In
27 2016, ~5.7 trillion cigarettes were smoked worldwide and it is predicted that by 2025 at least 9
28 trillion cigarettes will be smoked annually worldwide (Araujo and Costa, 2019). With the
29 majority of smokers littering their used filters (i.e. cigarette butts) (~75%; Patel et al. 2013), it
30 is not surprising that they have maintained their position as the most abundant litter item found
31 in beach cleans for over 30 years (Ocean Conservancy 2019). They are difficult to collect as
32 litter, especially due to their small size, and many remain in the environment even after
33 organised litter picking events (Loizidou et al. 2018). Tourist holiday locations are particularly
34 prone to cigarette litter with densities up to 13.3 butts m⁻² on beaches in Thailand (Kungskulniti
35 et al. 2018) and a mean of 8 butts m⁻² on a beach in Uruguay (Rodríguez et al. 2020). Cigarette
36 butts are also one of the most common litter items caught by floating litter collection devices
37 used in marinas and harbours (Seabins™, The Seabin Project 2020). For example, they account
38 for ~29% of all litter collected by Seabins™ in France (Plastics Europe 2019). Once washed
39 or thrown into water, cigarette butts are only buoyant on the water surface for a short time
40 before they sink (Rech et al, 2014), potentially to be washed back onto shore or further out to
41 sea via waves or currents (Roman et al. 2020). Recently, a citizen-science program: “Dive
42 Against Debris®”, found that cigarette butts were the second most common single use plastic
43 item found on the Mediterranean seafloor (at <30 m depth); accounting for 5.14% and cigar
44 tips 3.4% of total debris (Consoli et al. 2020).

45 The majority (~90%; Pauly et al. 2002) of cigarette filters are still composed of cellulose
46 acetate and are not readily biodegradable (<15% weight loss per year in seawater; Gerritse et
47 al. 2020), but can fragment and persist as micro- or nano- sized plastic fibres (Chevalier et al.
48 2018). Even clean, unsmoked cellulose acetate cigarette filters can cause detrimental effects on

49 plants (Green et al. 2019), marine and freshwater fish (Slaughter et al. 2011) and amphibians
50 (Lawal and Ologundudu 2013). Once smoked, however, cigarette butts present a greater risk
51 to the environment than unsmoked filters due to thousands of chemicals including, for example,
52 nicotine, polycyclic aromatic hydrocarbons and heavy metals which are retained in the butt and
53 can leach into the water (Moerman and Potts 2011; Roder Green et al. 2014; Dobaradaran et
54 al. 2019; Dilip et al. 2021). Such leachate has been found to be lethal for marine fish (Slaughter
55 et al. 2011) and gastropods (Booth et al. 2015).

56 In response to concerns about plastic cigarette filters, alternative filters, composed of pure
57 cellulose have arrived on the market. Alternative cellulose filters have been described as
58 “green”, “biodegradable” and “environmentally friendly” implying they would be benign as
59 litter (Amos et al. 2017). A recent experiment, however, found that leachate derived from
60 cellulose cigarette butts had the same detrimental effects on freshwater invertebrates as
61 leachate derived from cellulose acetate cigarette butts (Green et al. 2020). The comparative
62 effects of cellulose acetate versus cellulose cigarette butts have not yet been tested in a marine
63 system. Indeed, despite their prevalence as litter, the effects of any type of cigarette butt on
64 benthic marine organisms has seldom been tested. Of the few studies carried out on benthic
65 marine organisms, lethal effects have been found on gastropods (*Austrocochlea porcata*, *Nerita*
66 *atramentosa* and *Bembicium nanum* exposed to 5 butts L⁻¹; Booth et al. 2015), sublethal
67 behavioural (when exposed to leachate from 4 – 8 butts L⁻¹) and genotoxic (when exposed to
68 leachate from 8 butts L⁻¹) effects on polychaetes (*Hediste diversicolor*; Wright et al. 2015) and
69 alterations to microbial assemblages (exposed to 25 butts L⁻¹; Quéméneur et al. 2020). These
70 experiments, however, were conducted using highly controlled, closed aquatic systems which
71 did not simulate the continuous flow and replacement of seawater that occurs in the marine
72 environment.

73 The aim of the current study was to assess the impacts of conventional versus alternative
74 smoked cigarette filters (butts) in a model benthic habitat with flowing seawater by examining
75 physiological responses of the benthic filter feeder *Mytilus edulis* Linnaeus (1753) (blue
76 mussel), the primary producer *Ulva lactuca* Linnaeus (1758) (sea lettuce) and sediment
77 microphytobenthos. It was hypothesised that butts made of cellulose and cellulose acetate
78 would have similar, but negative (i.e. reducing) effects on the clearance rate and attachment
79 strength of *M. edulis*, growth rate of *U. lactuca* and the concentration of chlorophyll-*a* and -*c*
80 as a proxy for the sediment microphytobenthos.

81

82 **2. Material and methods**

83 *2.1. Preparation of cigarette butts*

84 Cigarettes were rolled by hand using standard, bleached cigarette papers (Rizla, Bristol, UK)
85 filled with an average (\pm S.E.) of 0.543 ± 0.002 g per cigarette of a leading brand of tobacco.
86 Cigarettes contained either a cellulose acetate (slim size; 5 mm diameter x 14 mm length) or a
87 cellulose (unbleached) filter (slim size; 6 mm diameter x 15 mm length). All cigarettes were
88 smoked using a hand-operated vacuum pump in a fume cabinet with silicone tubing attached
89 to the filter of the cigarettes. After lighting, approximately 30 ± 1 mL of air was drawn into
90 each artificial “breath” and each cigarette was smoked for a total inhalation volume of ~ 600
91 mL per cigarette, thereby emulating a similar total inhalation volume smoked by humans (549
92 ± 166 to 585 ± 245 mL; McBride et al. 1984). Cigarette butts were added to mesocosms 24 -
93 28 hours after smoking.

94

95 *2.2. Experimental design and mesocosm set-up*

96 The experiment consisted of an asymmetric design with 2 fixed factors; Butts (2 levels;
97 cellulose versus cellulose acetate) and Concentration (2 levels; 0.25 and 1 butt L⁻¹ equivalents).
98 A single Control treatment was also included which consisted of no added butts. Each of the

99 five treatments was replicated using 6 separate mesocosms (n=6, N=30). The experiment was
100 carried out in an outdoor mesocosm system at the Queen's University Marine Laboratory
101 (QML), Portaferry, Northern Ireland, with natural light conditions (unenclosed system with no
102 roof) and continuously through-flowing, sand filtered seawater pumped from the adjacent
103 Strangford Lough. Mesocosms consisted of opaque polypropylene buckets with a 10 L capacity
104 (height = 25 cm, diameter = 25 cm), each filled up to 3 cm depth with clean coarse sand
105 (autoclaved, median grain size 500 – 1000 μm) and to a volume of 8 L with seawater and left
106 open at the top to ensure full natural light availability. Five individual *Mytilus edulis* (blue
107 mussel) with an average (\pm S.E.) length of 45.6 ± 0.2 mm and wet biomass of 14.17 ± 0.22 g
108 were added to each mesocosm onto a square, 25 cm^2 slate settlement plate. Mussels were
109 sourced from Strangford Lough and were acclimatised to the QML outdoor mesocosm system
110 for >3 months before being used in the experiment. In addition, one individual *Ulva lactuca*
111 (sea lettuce) was added to each mesocosm with an average wet biomass of 4.63 ± 0.04 g and
112 secured to a pebble using a piece of cotton string in order to simulate how they were found in
113 the field attached to the substratum. *Ulva lactuca* had been collected from the shore outside
114 QML and maintained within separate outdoor flow-through seawater tanks, for a period of 48
115 hours prior to commencement of the experiments. When in the mesocosm, *M. edulis* were fed
116 every 2 days throughout the experiment with 100 mL of $\sim 5 \times 10^5$ cells mL^{-1} of the microalga
117 *Nannochloris atomus*. The mesocosms were allowed to settle for 48 hours before introduction
118 of any cigarette butts, and on day 1 of the experiment, treatments were randomly assigned to
119 mesocosms and corresponding butts were added by dropping them onto the surface of the
120 water. Most ($\sim 90\%$) butts sank immediately to the sediment, but some remained floating at the
121 surface for up to 2 hours before sinking. Throughout the experiment, the water in the
122 mesocosms was $\sim 10^\circ\text{C}$ with a pH of ~ 8.2 and salinity of ~ 33 ppt and was continuously being
123 replaced via individual hoses at a rate of $\sim 500 \text{ mL min}^{-1}$ meaning that the water was completely

124 replaced >3 times per hour. Each mesocosm was a completely independent replicate and
125 wastewater discharged from mesocosms could not leak into any other mesocosm, with a mesh
126 on their outlet to prevent the butts from being inadvertently removed from the mesocosms. In
127 this way, butts were retained within the mesocosms and were added only once.

128

129 2.3. Measuring responses of *M. edulis* exposed to cigarette butts

130 After 5 days of exposure in the outdoor mesocosms, clearance rates were estimated using one
131 individual *M. edulis* from each mesocosm. *M. edulis* were held in separate 500 mL glass
132 beakers with an air bubbler and clean sand filtered seawater containing $\sim 5 \times 10^4$ cells mL⁻¹ of
133 the microalga *N. atomus*. *M. edulis* began filtering almost immediately and samples of 5 mL
134 were taken after 0, 20, and 40 min and algal cells were counted using a haemocytometer. This
135 time length was chosen because it is below the saturation reduction level for *M. edulis* whereby
136 clearance is reduced when feeding for > 2 hours at 3×10^4 or more cells ml⁻¹ (Pascoe et al.
137 2009). The dry biomass of each individual *M. edulis* used in the clearance rates was determined
138 by drying at 60°C for 24 hours and weighing to the nearest µg. Clearance rates were expressed
139 as litres of water cleared h⁻¹ g⁻¹ dry weight.

140 Tenacity (or attachment strength) of one mussel per mesocosm was measured after 5 days of
141 exposure using a portable dynamometer (Pesola, Sweden) scaled 0–10 N to measure the maximal
142 vertical force required for the individual to become dislodged (attachment strength, N). The
143 dynamometer had a small clamp attached to it that gripped individual mussels laterally without
144 displacing them. The maximum dislodgement force to the nearest 0.1 N was recorded for one
145 mussel from each mesocosm. The surface area of each mussel was approximated to an ellipse
146 using height and width (measured with Vernier callipers to 1 mm) as major and minor axes

147 (Bell and Gosline 1997). Tenacity is expressed as dislodgement force (N) per unit mussel area
148 (cm^{-2}).

149

150 *2.4. Measuring responses of primary producers to cigarette butts*

151 After 10 days, each individual *U. lactuca* was removed and spun dry with a handheld centrifuge
152 for 30 s before weighing fresh biomass to the nearest 0.01 g. Growth rates were calculated as
153 the increase in biomass between days 0 and 10.

154 The biomass of the microphytobenthos (MPB) was estimated after 10 days by chlorophyll
155 extraction. Approximately the top 1 cm of oxic sediment of was sampled and wrapped in tin
156 foil to protect from the sunlight. Chlorophyll was extracted immediately for 1 hour under
157 constant shaking at room temperature in the dark after adding 10 mL of 90% acetone to ~1 g
158 of wet, homogenised sand. Chlorophyll-a and chlorophyll-c concentrations were measured
159 from the supernatant using a spectrophotometer and calculated according to equations by
160 Jeffrey and Humphrey (1975). Concentrations are expressed as μg chlorophyll g^{-1} dry
161 sediment.

162

163 *2.5. Statistical analysis*

164 The design was asymmetrical (i.e. having a single control group for the two factors “Butt” and
165 “Concentration”), therefore the data were analysed by using the mean squares from two
166 independent ANOVAs (see Green et al. (2016) for an example of the calculations). Briefly,
167 this included partitioning of the variance by calculating (1) a one-way ANOVA with all
168 treatments as separate levels (five treatments \times six replicates each) and (2) a full-factorial two-
169 way ANOVA of “Butt” by “Concentration” without the Control (two factors \times two levels \times six
170 replicates each). The residuals of the 1st ANOVA were used to assess differences between the

171 levels within the 2nd ANOVA, allowing the variation associated with Control and that of the
172 other treatments to be distinguished (“Control vs. Others”), which is contrasted with one degree
173 of freedom (Underwood, 1997). When a significant effect in the “Control vs. Others” (C vs.
174 O) contrast was found, Dunnett’s test was used to contrast the Control versus each level of the
175 significant term. Post-hoc pairwise comparisons were also computed using Tukey HSD tests
176 when the main terms in the full-factorial ANOVA were significant. Statistical significance was
177 assumed at $\alpha = 0.05$. Data were screened for normality of distribution and homogeneity of
178 variance to check that they conformed to the assumptions of ANOVA. All statistical analyses
179 were done using R v3.6.2. (R Core Team, 2019).

180

181 **3. Results**

182 *3.1. Effects of cigarette butts on M. edulis*

183 No individuals of *M. edulis* died during the experiment. The dry biomass of *M. edulis* did not
184 significantly differ amongst treatments (Tables 1 & 2). Clearance rates of *M. edulis* were
185 significantly reduced by the addition of 1 cellulose acetate butt L⁻¹, causing a 2.6 times
186 reduction in clearance rates compared with *M. edulis* in the Control mesocosms or in those
187 dosed with cellulose butts (Table 1a). The tenacity of *M. edulis* was not significantly affected
188 by the addition of cigarette butts (Tables 1a and 2).

189

190 *3.2. Effects of cigarette butts on primary producers*

191 The growth rate of *U. lactuca* was positive in all mesocosms but was not significantly affected
192 by cigarette butts (Tables 1b and 2). Chlorophyll-*a* content of the sediment in mesocosms
193 exposed to 0.25 or 1 cellulose acetate butt L⁻¹ was 2.8 times less than that of the Control
194 mesocosms and 2.2 times less than of mesocosms with 0.25 cellulose butts L⁻¹ (Table 1b,
195 Figure 2). While mesocosms with 1 cellulose butt L⁻¹ had less chlorophyll-*a* than Control

196 mesocosms, this was not significantly different (Figure 2). Chlorophyll-*c* content was 3.5 times
197 less in sediment contaminated with cellulose acetate butts than in sediment with 0.25 cellulose
198 butts L⁻¹ (Table 1b and Figure 2).

199

200 **4. Discussion**

201 The current study found that even with constant replacement of seawater, simulating a
202 realistic marine environment, cellulose acetate cigarette butts significantly reduced the
203 clearance rates of *M. edulis* and the chlorophyll content of the sediment, whereas cellulose
204 cigarette butts had minimal impact.

205 Clearance rates of *M. edulis* are used in ecotoxicity testing because they are a sensitive and
206 ecologically relevant sub-lethal endpoint (Abel 1976). Reduced clearance rates have also
207 been found to occur in *M. edulis* in response to other contaminants including mercury
208 (Micallef and Tyler 1990), copper (Al-Subiai et al. 2011) microplastics (Woods et al. 2018)
209 and a range of hydrophobic organic chemicals (Donkin et al. 1989). A reduction in clearance
210 rates of these ecosystem engineers could lead to cascading effects on water quality, nutrient
211 cycling and primary productivity in sedimentary habitats due to their role in benthic-pelagic
212 coupling (van der Schatte et al. 2020; Barbier et al. 2011). A prolonged reduction in feeding
213 could lead to a reduction in health causing a decrease in reproductive output and/or growth
214 performance (Seed and Suchanek 1992). Longer term studies will help to elucidate
215 population level implications of the results of our short-term investigation.

216 Although there were no measurable effects on the growth rate of *U. lactuca*, the
217 concentration of chlorophyll-*a* and *c* of the sediment was reduced even when exposed to just
218 0.25 cellulose acetate butts L⁻¹. Effects on primary producers are important since they form
219 the base of food webs. The microphytobenthos, for example, deliver an array of vital

220 ecosystem services including nutrient cycling, primary productivity and sediment
221 stabilisation, and are an essential, but often overlooked, component of sedimentary habitats
222 (Hope et al. 2019). They are also a pivotal food source for heterotrophs in sandy subtidal
223 habitats (Evrard et al. 2012). In our study we quantified the effects on the early colonisation
224 of the sediment by using clean sand as a starting point. It is also likely, however, that
225 cigarette butts will affect established microphytobenthic communities as indicated by the
226 recent work of Quéméneur et al. (2020) who found that leachate from cigarette butts altered
227 established microbial communities in marine sand.

228 The effects on clearance rates of *M. edulis* and chlorophyll concentrations in the sediment
229 could be due to a combination of the chemicals accumulated in the butt after smoking
230 tobacco and the plastic itself in the cellulose acetate butts. Recently, Dilip et al. (2021)
231 characterised 98 chemicals from smoked cigarette butt leachate, a third of which are
232 classified as very toxic. In addition, leachate from unsmoked cellulose acetate cigarette filters
233 has been found to be toxic to marine and freshwater fish (Slaughter et al. 2011) and to
234 freshwater microalgae (Bonanomi et al. 2020) and unsmoked butts added as whole items
235 have been found to decrease the germination and growth of ryegrass and clover (Green et al.
236 2019) and to reduce the pH of seawater and alter microbial communities in marine sand
237 (Quéméneur et al. 2020). These effects could be due to plasticizers, such as diethyl phthalate,
238 which in isolation can be toxic to plants (Cheng, 2012) and animals (Liu et al.,2009). It is
239 possible that differences between the effects of cellulose acetate and cellulose cigarette butts
240 in the current study were due to (i) a greater concentration of chemicals retained in cellulose
241 acetate cigarettes after smoking, or (ii) leaching of plasticizers from cellulose acetate
242 cigarette butts. A complete characterisation of the chemical profiles of each type of cigarette
243 butt is needed in order to elucidate these mechanisms.

244 Although there was a marginal effect of 1 butt L⁻¹ of biodegradable cellulose cigarette butts on
245 sediment chlorophyll concentrations, there were no statistically detectable impacts on the
246 measured responses in the current study. In a closed system, however, such as a rockpool,
247 biodegradable cigarette butts would likely cause similar effects to non-biodegradable cigarette
248 butts due to the retention of leachate in the water (Booth et al. 2015). Indeed, a recent
249 experiment in a closed freshwater system showed that biodegradable cellulose butts had similar
250 detrimental effects as plastic cellulose acetate butts; causing mortality and a reduction of
251 movement of four invertebrate species (Green et al. 2020). Cigarette butts, regardless of their
252 biodegradability, pose a threat as litter in the environment and need to be disposed of
253 appropriately.

254

255 *Recommendations and conclusion*

256 It is likely that littering of cigarette butts occurs due to misconceptions that they are benign,
257 i.e. having no effect on the environment and that they are rapidly biodegradable. The majority
258 (43%) of smokers surveyed in Germany for example, were not aware that cigarette filters are
259 composed of synthetic material (Kotz and Kastaun 2020). Despite most cigarette butts being
260 composed of a type of plastic, cellulose acetate, they are still not widely classified as a single
261 use plastic. There is now evidence that cigarette butts can have detrimental effects on organisms
262 in terrestrial (Green et al. 2019), freshwater (Green et al. 2020) and marine habitats (Booth et
263 al. 2015, Wright et al. 2015 and the current study). To protect the environment, cellulose acetate
264 cigarette butts should be globally classified as single-use plastics as there is urgent need to
265 improve regulation relating to their use, collection and disposal. In addition, there needs to be
266 an increase in campaigns to raise awareness of the impacts of cigarette litter, an increase in
267 fines and smoking bans in areas of conservation importance (Axelsson and van Sebille, 2017)
268 and the introduction of extended producer responsibility for tobacco companies to hold

269 manufacturers responsible for collection, transport, processing and disposal of tobacco product
270 waste (Curtis et al. 2017).

271

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275

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Tables and figures

Table 1. Asymmetrical ANOVA results for (a) tenacity (Tenacity), dry weight (DW) and clearance rates of *M. edulis* (Clearance rates) and (b) growth of *U. lactuca* (*Ulva* growth), chlorophyll-*a* or -*c* content of the sediment (Chl-*a*, Chl-*c*). F ratios with P-values significant at $\alpha = 0.05$ are indicated in **bold**. MPB Chl-*a* and MPB Chl-*c* were square root and log (x+0.5) transformed respectively in order to meet the assumption of normality of distribution.

(a)		Tenacity			DW			Clearance rates		
Source of variation	d.f.	MS	F-ratio	P-value	MS	F-ratio	P-value	MS	F-ratio	P-value
One-way	4	0.02	0.57	0.686	0.01	0.62	0.651	23.94	4.75	0.005
Control vs others	1	0.03	0.82	0.374	0.00	0.42	0.521	21.80	4.32	0.048
Butt (B)	1	0.01	0.18	0.679	0.01	1.00	0.327	11.29	2.24	0.147
Concentration (C)	1	0.03	0.77	0.389	0.01	0.70	0.410	23.44	4.65	0.041
B x C	1	0.02	0.52	0.477	0.00	0.37	0.551	39.23	7.78	0.010

(b)		<i>Ulva</i> growth			MPB Chl- <i>a</i>			MPB Chl- <i>c</i>		
Source of variation	d.f.	MS	F-ratio	P-value	MS	F-ratio	P-value	MS	F-ratio	P-value
One-way	4	6.21	1.29	0.299	0.26	4.71	0.006	0.49	2.97	0.039
Control vs others	1	5.11	1.06	0.312	0.30	5.77	0.024	0.35	2.50	0.127
Butt (B)	1	6.66	1.39	0.250	0.43	7.86	0.010	0.94	6.45	0.018
Concentration (C)	1	9.55	1.99	0.171	0.18	3.89	0.060	0.35	1.66	0.210
B x C	1	3.51	0.73	0.400	0.10	1.30	0.264	0.30	1.27	0.271

Table 2. Tenacity (N cm⁻²) and dry weight of flesh (g) for *M. edulis* and growth (g) of *U. lactuca* after exposure to either no butts (Control) or to 0.25 or 1 butt L⁻¹ of smoked cellulose (C) or cellulose acetate (CA) cigarette butts. Data are mean ± S.E.M., n = 6.

Response / Treatment	Tenacity (N)	Dry weight flesh (g)	<i>U. lactuca</i> absolute growth (g)
Control	2.2 ± 0.6	0.32 ± 0.04	5.69 ± 0.95
C 0.25 butts L ⁻¹	1.6 ± 0.3	0.37 ± 0.05	4.38 ± 0.66
C 1 butt L ⁻¹	1.8 ± 0.3	0.37 ± 0.03	3.89 ± 0.56
CA 0.25 butts L ⁻¹	0.3 ± 0.1	0.30 ± 0.04	6.20 ± 0.92
CA 1 butt L ⁻¹	0.2 ± 0.1	0.36 ± 0.05	4.18 ± 1.23

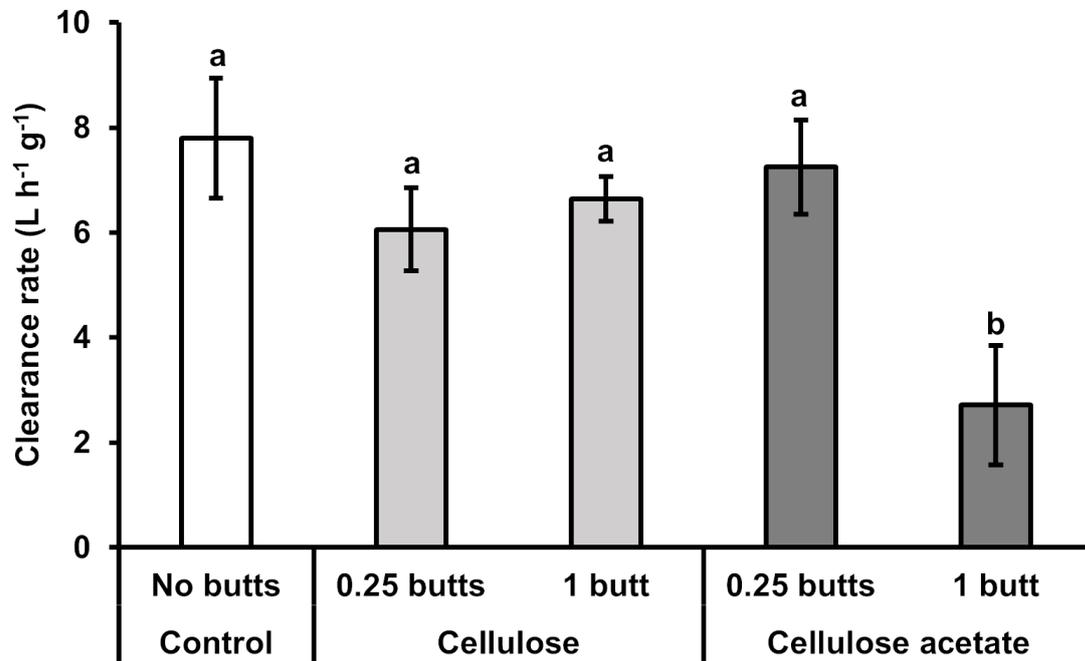


Figure 1. Clearance rates of *M. edulis* exposed to no butts or to smoked cellulose or smoked cellulose acetate cigarette butts at 0.25 or 1 butt L⁻¹. Data are mean ± S.E.M. based on dry weight, n = 6. Different superscript letters indicate a significant difference at $\alpha = 0.05$.

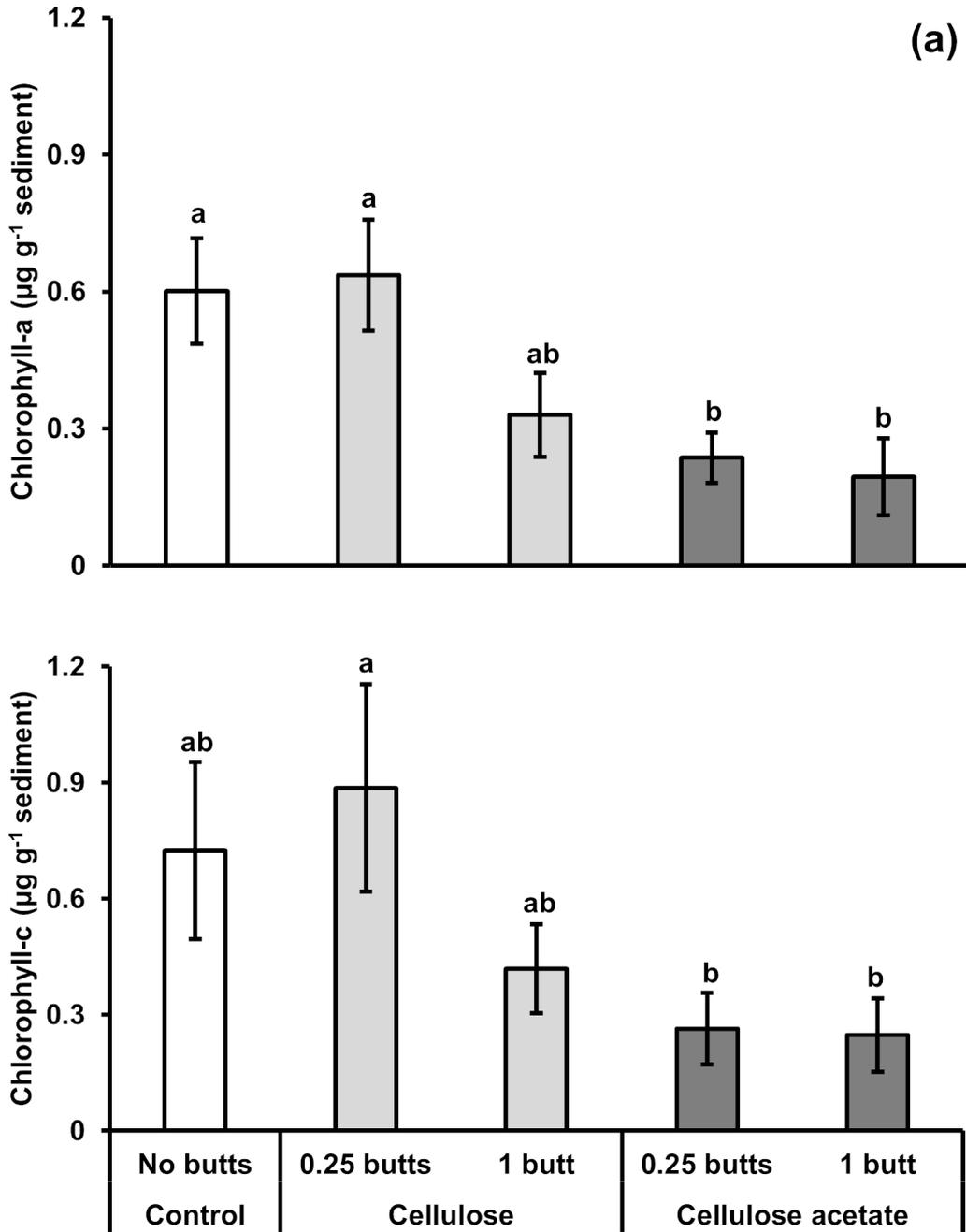


Figure 2. Chlorophyll-*a* (a) and chlorophyll-*c* (b) content extracted from sand exposed to either no butts (Control), smoked cellulose or smoked cellulose acetate butts at 0.25 or 1 butt L⁻¹. Data are mean ± S.E.M. based on dry sediment, n = 6. Different superscript letters indicate a significant difference at $\alpha = 0.05$.