

A common contaminant shifts impacts of climate change on a plant-microbe mutualism: effects of temperature, CO₂ and leachate from tire wear particles

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Running title: Multi-stressors & plant-microbe interactions

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Abstract

1 Anthropogenic stressors, such as climate change or chemical pollution, affect indi-
2 vidual species and alter species interactions. Moreover, species interactions can modify
3 effects of anthropogenic stressors on interacting species - a process which may vary
4 amongst stressors or stressor combinations. Most ecotoxicological work focuses on
5 single stressors on single species. Here, we test hypotheses about multiple stressors
6 (climate change and tire wear particles) and interacting species, and whether species
7 interactions modify responses. We use duckweed and its microbiome to model responses
8 of plant-microbe interactions. Climate change is occurring globally, and with increasing
9 urbanization, tire wear particles increasingly contaminate road runoff. Their leachate is
10 associated with zinc, PAHs, plastic additives, and other toxic compounds. We crossed
11 perpendicular gradients of temperature and CO₂ in a well plate with factorial manip-
12 ulation of leachate from tire wear particles and presence of duckweed microbiomes.
13 We measured duckweed and microbial growth, duckweed greenness, and plant-microbe
14 growth correlations. We found that tire leachate and warmer temperatures enhanced
15 duckweed and microbial growth, but microbes diminished positive responses in duck-
16 weed, meaning microbiomes became costly for duckweed. These costs of microbiomes
17 were less-than-additive with warming and leachate, and might be caused by leachate-
18 disrupted endocrine signaling in duckweed. We observed reduced greenness at higher
19 CO₂ without tire leachate, suggesting a relative increase in plant nutrient demand, and
20 possibly underlying positive plant-microbe growth correlations in these conditions, as
21 microbes presumably increase nutrient availability. However, with tire leachate, growth
22 correlations were never positive, and shifted negative at lower CO₂, further suggesting
23 leachate favors mutualism disruption. In summary, while individual stressors of global
24 change can affect individual species, in ecology we know species interact; and in ecotox-
25 icology, we know stressors interact. Our results demonstrate this complexity: multiple
26 stressors can affect species interactions, and species interactions can alter effects of
27 multiple stressors.
28

29 Key Words: duckweed, Lemnaceae, *Lemna minor*, microbiome, species interactions, mul-

30 tiple stressors, urban pollution, climate change, tire wear particles, rhizosphere

31 Introduction

32 Global change can disrupt species interactions. Famously, rising temperatures cause corals
33 to expel mutualistic symbionts (Hoegh-Guldberg, 1999), and eutrophic conditions cause cas-
34 cading effects on lake food chains (Carpenter et al., 2001) and decouple fitnesses in nutrient
35 exchange mutualisms (Shantz et al., 2016). With the increase of human influence, global
36 change extends beyond CO₂, temperature, and nutrients, as these factors are now matched
37 or exceeded in rates of increase by synthetic contaminants (Bernhardt et al., 2017). Despite
38 proportionally less attention in the ecological literature (Bernhardt et al., 2017), synthetic
39 contaminants have similarly far-reaching impacts on species interactions and food webs.
40 Upon exposure to ozone, the anti-herbivory benefits of hosting a fungus disappeared for
41 plants (Ueno et al., 2016), and certain groups of synthetic contaminants shift rates and
42 diversity of whole clades of parasites (Blanar et al., 2009). Contaminants can also have
43 pervasive indirect effects via trophic cascades in aquatic ecosystems (Fleeger et al., 2003).
44 A synthetic oestrogen in a lake caused a prey fish species to crash, indirectly reducing the
45 top predator and increasing zooplankton biomass (Kidd et al., 2014). Importantly, synthetic
46 contaminants should be considered in the suite of global change stressors.

47 Species interactions may also alter the individual species-level effect of stressors - includ-
48 ing chemical contaminants. In addition to affecting a physiological response, they can alter
49 the dosage and/or mechanism of exposure that individuals receive. From DDT to microplas-
50 tics, pollutants can move to predators from prey via consumptive interactions (Hickey and
51 Anderson, 1968; Nelms et al., 2018). Likewise, mutualistic rhizosphere microbes can cause
52 higher concentrations of heavy metals in plant tissues than the plant would accumulate alone
53 (Braud et al., 2009). Direct effects of contaminants on one species can also combine with the
54 effects on species interactions, i.e. to increase or decrease the rates at which species interact,
55 and therefore the rates of trophic transfer. For example, neonicotinoid pesticides reach and
56 harm non-target insect predators through consumption of contaminated prey, which reduces
57 predation pressure and leads to a population increases of tolerant prey (Douglas et al., 2015).

58 Contaminants may also change physiology and behaviors, producing trait-mediated shifts in
59 interactions (Saaristo et al., 2018), such as psychoactive pharmaceuticals that change preda-
60 tor avoidance behavior and therefore predation rates on fish (Martiny et al., 2013).

61 We have a vested interest in the outcomes of certain species interactions. Plant-microbiomes
62 are broadly tied to human well-being through influences on ecosystem productivity, crop
63 health, and even on our own microbiomes via vegetable consumption (Berg et al., 2014).
64 Further, a subset of plant-microbe interactions supply the majority of terrestrial plant ni-
65 trogen and phosphorus (Smith et al., 2011; Fowler et al., 2013; Coskun et al., 2017). Plant-
66 microbiome interactions are largely mutually beneficial (Avis et al., 2008; Dijkstra et al.,
67 2013), and often ameliorate stressors (Porter et al., 2019), including temperature and drought
68 (Compant et al., 2010; Kivlin et al., 2013), yet can alternatively exacerbate negative effects
69 (David et al., 2018). While microbes often dilute contaminant effects on plants because they
70 either promote growth (Rajkumar et al., 2012), or reduce uptake by metabolizing or bioad-
71 sorbing compounds (Chaudhry et al., 2005; Madhaiyan et al., 2007), some microbes instead
72 enhance effects by increasing contaminant bioavailability (Braud et al., 2009). Effects of
73 synthetic contaminants on plant-microbe mutualisms are poorly understood, and here, we
74 use interactions between duckweed *Lemna minor* and its microbiome as a model. Duckweed
75 has an extensive history in ecotoxicology, owing to its ability to adsorb or transform a wide
76 variety of anthropogenic contaminants, from heavy metals (Mo et al., 1989), to nutrients
77 (Zhao et al., 2014) and organic compounds (Gatidou et al., 2017; O'Brien et al., 2019).
78 Duckweed has also proven to be a highly tractable experimental system due to its clonal
79 reproduction, small size (a few mm, Landolt, 1975), short generation time (as few as 3 days,
80 Liu et al., 2017), and host-microbiome interactions similar to those of other plants, in which
81 microbiomes promote duckweed growth in benign and stressful conditions (O'Brien et al.,
82 2019; O'Brien et al., 2020*b,a*).

83 We aim to quantify the effects of a single chemical stressor, tire wear particles, on
84 duckweed-microbiome interactions. Recent estimates suggest a massive 1 million t/a of tire

85 wear particles enter the environment in the USA, with increasing inputs in recent decades
86 due to mounting vehicle traffic (Wagner et al., 2018). Tire wear particles are the main source
87 of total suspended solids (Göbel et al., 2007), zinc (Councell et al., 2004), and polycyclic
88 aromatic hydrocarbons (PAHs, Boonyatumanond et al., 2007) in urban runoff, and leachate
89 from tires has been linked to acute lethality in coho salmon (Peter et al., 2018) and devel-
90 opmental abnormalities in fathead minnow (Kolomijeca et al., 2020), but has shown milder
91 effects on other organisms (Marwood et al., 2011; Panko et al., 2013; Redondo-Hasselerharm
92 et al., 2018). Since multiple stressors very often underlie “ecological surprises” (non-additive
93 effects e.g. Darling and Côté, 2008; Crain et al., 2008; Jackson et al., 2016), and since the
94 multiple facets of global change do not occur in isolation, we consider effects of tire wear par-
95 ticles across gradients of climate change. We evaluate these global change factors for effects
96 at multiple levels, from single-stressor on single-species, to multi-stressor on interaction out-
97 comes, and we specifically consider how shifts in variation within interaction outcomes could
98 alter long-term responses. In mutualisms, fitness feedbacks (correlations between fitnesses of
99 interacting species, i.e. Sachs et al., 2004), might shift with environmental conditions (Shantz
100 et al., 2016), with positive fitness feedbacks enhancing mutualisms, and weak or negative
101 feedbacks potentially leading to evolutionary disruptions (Weese et al., 2015).

102 The well-documented positive effect of CO₂ on both microbial and plant growth (Treseder,
103 2004; Norby and Zak, 2011), is linked to increases in root exudates (Phillips et al., 2006),
104 which appear to drive microbial growth responses that in turn enhance nitrogen turnover
105 and feed back to plant growth (Phillips et al., 2011). Thus, we predict that elevated CO₂ will
106 enhance the main benefits of microbes to duckweed and investment by duckweed in microbes,
107 as well as enhance positive plant-microbe fitness correlations. Likewise, the impacts of
108 microbes on plant growth in response to increases in temperature are most often positive,
109 even if the main effects of temperature are sometimes not (Compant et al., 2010; Kivlin
110 et al., 2013), therefore we expect temperature could also enhance both positive effects of
111 interactions and fitness correlations. Conversely, predicting effects of leachate from tire wear

112 particles is less straightforward, as the two chemicals often implicated in leachate effects, zinc
113 and PAHs, would be expected to cause contrasting responses. Elevated zinc levels negatively
114 affect duckweed, and while its microbiome can reduce negative impacts in the short term
115 (O'Brien et al., 2020b), long-term benefits of microbiomes may erode, as zinc may cause
116 negative fitness feedbacks between duckweed and microbes (O'Brien et al., 2020a). In other
117 systems, microbes often enhance uptake of metal contaminants under warming and CO₂
118 (Rajkumar et al., 2013), suggesting that climate change may exacerbate both short- and long-
119 term effects of zinc. Yet for PAHs, microbes may mitigate negative effects: PAHs generally
120 have negative effects on plants, including duckweed (Becker et al., 2002; Zezulka et al.,
121 2013), but may be rapidly degraded by microbes (Heitkamp and Cerniglia, 1987; Haritash
122 and Kaushik, 2009). While zinc and PAHs are most often expected to drive biological effects,
123 leachate from tires contains a complex mixture of compounds (Peter et al., 2018; Kolomijeca
124 et al., 2020; Capolupo et al., 2020) and main effects on responses of organisms are highly
125 varied (Panko et al., 2013; Peter et al., 2018), precluding clear predictions. However, several
126 studies have found greater effects of leachate at higher temperatures (Marwood et al., 2011;
127 Kolomijeca et al., 2020), so we might predict that warming would exacerbate leachate effects
128 on duckweed, microbes, and their fitness correlations.

129 **Methods**

130 **Biological materials**

131 We collected *Lemna minor* and associated microbes from the University of Toronto's field
132 station, the Koffler Scientific Reserve (King City, Ontario, Canada), in the summer of 2017.
133 We used one single frond (bleached to remove all source-site microbes) to start an isogenic
134 line (or nearly so), which grew to high numbers (>1,000) in just a few months. *Lemna*
135 *minor* is known to reproduce primarily via vegetative clonal budding of daughter fronds
136 but it can also very occasionally undergo sexual reproduction (Ho, 2017). Although we

137 never observed sexual reproduction in the lab, flowers are cryptic (Landolt, 1975). Even if
138 sexual reproduction occurred in our cultivated line, given little segregating variation within
139 duckweed from our source site (Ho, 2017), we expect our line is still essentially isogenic, and
140 assume so from here forward. Plants in our isogenic line were cultured in growth chambers
141 (ENCONAIR AC80, Winnipeg, Canada) under 16-hour 23 °C day and 8-hour 18 °C night
142 cycles, in Krazčič's media (Krazčič et al., 1995), in vented 500 mL mason jars. We refreshed
143 media approximately twice per month, as the isogenic line was maintained at high density.

144 When duckweeds were collected, we also isolated the microbes associated with them by
145 pulverizing one clonal unit of duckweed (e.g. a mother and daughter frond), plating on
146 yeast-mannitol agar media, and culturing at 29 °C for 5 days before storing at 4 °C until
147 the experiment. This microbial culture represents the fraction of culturable microbes in
148 both the external and internal microbiome (e.g. epiphytic and endophytic), and includes
149 a subset of bacterial taxa that are representative of the field sampled bacterial microbiome
150 (O'Brien et al., 2019; O'Brien et al., 2020b), but also may include other taxa, as fungi and
151 diatoms are known to associate with duckweed (Rejmankova et al., 1986; Goldsborough,
152 1993), and may have persisted in lab cultures. Previous sequencing of the bacterial fraction
153 of the microbial culture identified 17 unique members, largely from Gammaproteobacteria
154 (but also Alphaproteobacteria, Bacilli, Flavobacteriia, Firmicutes, and Sphingobacteriia),
155 with Aeromonadaceae (including *Aeromonas* spp.) and *Pseudomonas* spp. in relatively
156 high abundance (O'Brien et al., 2019).

157 Three to four days before adding duckweeds to the experiment, we sterilized the external
158 surfaces, as our cultures are vented to lab air and not gnotobiotic. We shook for 5 minutes in
159 reverse osmosis water, submerged them in 1% NaOCl (diluted from Lavo ProTM, Montréal)
160 for one minute in a biosafety cabinet, then rinsed with autoclaved water four times: the
161 first rinse short and vigorous to remove most bleach, then three 10 minute submerged soaks.
162 While this procedure does not remove all endophytic microbes inside tissues, we have found
163 that it is successful at greatly reducing microbes (Figure S1, O'Brien et al., 2019), and that

164 duckweed generally does not recover from longer or more concentrated bleach treatment
165 (O'Brien, pers obs).

166 **Experimental Device**

167 We used an experimental device for our multi-stressor and fully factorial experiment that was
168 based on previously reported designs applying CO₂ gradients over multi-well plates (Nguyen
169 et al., 2018; Yang et al., 2020). Here, we included orthogonal temperature manipulation,
170 added gas delivery tubes to allow internal lighting, improved the CO₂ concentration control
171 system, and humidified the air (see Figure S2, for a graphical representation).

172 In brief, CO₂ from a gas tank (Praxair) was manually adjusted via regulator and needle
173 valves in a hand-assembled gas mixing board. The control system maintained a constant
174 concentration of CO₂ in ppm, as described by Yang et al. (2020), and was comprised of an
175 Arduino microprocessor connected to a solenoid valve, a 12V/5V relay module, and a CO₂
176 sensor. The valve and relay were controlled by a PID algorithm in response to sensor output
177 within the CO₂-air mixer, maintaining a steady concentration of CO₂ into the experiment by
178 turning the flow on and off. The CO₂ concentration in the CO₂ rich stream was monitored
179 continuously using the Arduino user interface. We humidified CO₂-rich air and ambient air
180 in separate hand-assembled bubble humidifiers, which forced gas into water-submerged air-
181 stones. Finally, we pumped (Pawfly Adjustable Air Pump 4-LPM) humidified CO₂-rich air
182 and ambient air into opposite sides of each aerogel bar, developing a spatially linear gradient
183 of CO₂ concentration (Nguyen et al., 2018; Yang et al., 2020) from 1,000 to 400 ppm (across
184 ranges from RCP8.5, USGCRP et al., 2017).

185 Since we aimed to quantify both independent and combined effects of climate change
186 variables, we applied a thermal linear gradient orthogonal to the CO₂ gradient. We used
187 aluminum plates cut to fit 96-well plates, with aluminum tubes attached below the aluminum
188 plate at both sides with thermal adhesive tape. We pumped (Esky EAP-03 2500L/H Sub-
189 mersible Water Pump) hot water through the tube under the plate on one side, and cold

190 water on the other using flexible tubing from hot and cold tanks, heated (Anova Precision
191 Cooker 4.0) to 35 °C and cooled (Active Aqua Chiller Refrigeration Unit, 1/10HP) to 7.2 °C.
192 We monitored temperature daily throughout the experiment with thermocouples (Omega
193 HHP806), and achieved a realistic global change temperature gradient based on the range of
194 July stream water temperatures from rural to urban sites in the Greater Toronto Area (13-27
195 °C, Toronto Regional Conservation Authority, 2016). Temperature periodically fluctuated
196 due to cycles in lab temperature that made chilling more and less effective, and also due to
197 periodic leaks and tube-blockages in the system, which we corrected as they appeared.

198 We supplied light to the experiment by connecting experimental well plates to the aerogel
199 gas gradient via delivery tubes with interlaced lighting. Delivery tubes consisted of two PCR
200 96-well plates, each with the tube ends removed with a hot wire foam cutter, with the second
201 plate inverted and the open tube ends of the second pressed inside the open tube ends of
202 the first. LED striplights (LEDMO 6000K 2835 SMD LED) were placed between each row
203 of PCR plate wells (twice per row and including outside edges, so that each experimental
204 row received light from both sides). The “top” side of one PCR was placed on top of the
205 experimental 96-well plate opening, and the “top” side of the other against the aerogel.
206 LED lights were set to their lowest brightness setting via a LED controller (ER CHEN)
207 and emitted light parallel to the experimental plate liquid surface (indirect). All layers were
208 pressed together with 3-1/2’ steel screws and nuts, and possible leakage of gas exiting directly
209 from the well plate (rather than exiting out the aerogel as intended) was slowed by wrapping
210 the device with parafilm.

211 **Tire wear particle leachate**

212 We used a Michelin energy saver tire (a/s, all season, sidewall markings 205/60R16 91Vtire),
213 and sliced strips from the tread portion, which we hand cut to small cubes ($\approx 0.5 \text{ cm}^2$). We
214 ground cubes in a Cuisinart Supreme Grind Automatic Burr Mill (DBM-8C) with plate
215 grinders, freezing the tire sample in liquid nitrogen before each grinding, and passing all tire

216 particles through each setting (from most coarse to most fine). We then passed resulting
217 particles through a burr mill with cone grinders (10903-913US, BODUM) three times, on
218 the finest setting and at room temperature, which ripped tire pieces to provide a surface
219 texture similar to road wear.

220 We characterized the size distribution of our lab-created tire wear particles by placing a
221 50 mg subsample in surfactant (10% w/v Alcojet, Alconox, Inc) on a glass slide. Particles
222 were largely aggregated without surfactant, and some even with surfactant (Figure S3). We
223 imaged all portions of this slide with a Leica (M205 A) microscope with camera (DFC425
224 C), at gain 1, gamma 0.57, whites blown out (“cut” to 25 or 26), and an automatic exposure
225 time (ranged from 38.5 to 46.8 ms), with an added scale bar, using Leica Application Suite
226 (version 3.8.0) software. We used ImageJ to analyze the number and size distribution of
227 particles, with brightness thresholding (set to 195) and “Analyze Particles” to measure
228 maximum caliper and top (facing camera) surface area. Our particle size distribution is
229 coarsely similar to many measured size distributions for tire particles generated by road
230 wear (Kreider et al., 2010; Wagner et al., 2018). We estimated 11.7 particles per milligram.
231 The maximum caliper of particles ranged from 1.7 μm to 1.7 mm, and facing surface area
232 from 0.002 μm^2 to 0.9 mm^2 (Figure S4), with particles generally in highly complex shapes
233 (Figure S3).

234 Tire particles do enter waterways (e.g. Grbić et al., 2020), yet highly concentrated leach-
235 ing near the roadside with dilution in recipient streams is expected to be the primary source
236 of tire leachate contaminants, as the bulk of tire particles seem to remain near the road-
237 side (Wagner et al., 2018). We sought to mimic this process. We leached tire particles
238 immediately prior to use at a concentration of 20 g/L. Leaching took place in amber bottles
239 wrapped in foil in autoclaved reverse osmosis water, with paired bottles for with (leachate)
240 and without (negative control) tire particles. We set bottles on a shaker at 20 rpm for 10
241 days at ambient temperature. Leachate and negative controls were filter-sterilized (auto-
242 claving would alter chemistry) with water-wettable polytetrafluoroethylene filters of 0.2 μm

243 maximum pore width (Acrodisc® syringe filters, Pall Corporation, NY, USA) which in turn
244 were sterilized by passing 1% NaOCl in sterile reverse osmosis water through the filter and
245 letting it sit for 5 minutes, followed by triple rinsing with sterile reverse osmosis water. We
246 expect that filtering removed the majority of tire particulate, limiting any observed effects
247 to the leached chemicals. Still, while we did not detect tire particles smaller than $0.2\mu\text{m}$ in
248 diameter in our image analysis (minimum $1.7\mu\text{m}$, Figure S4), any that existed would have
249 passed through this filter. We then split leachate into two solutions, one undiluted and one
250 diluted by 50% with the negative control. To each experimental well, we added $100\mu\text{L}$ of a
251 leachate treatment (negative control, 50% diluted, or full strength) depending on the design
252 (see below), and we also added $100\mu\text{L}$ of double strength Krazčič's growth media. Each well
253 then holds $200\mu\text{L}$ of $1\times$ strength Krazčič's media with a concentration of leachate that is
254 0 , $0.25\times$, or $0.5\times$ the original leachate concentration. Our $0.5\times$ leachate treatment is meant
255 to replicate the max reasonable dosage that a pond near a highway might receive, 10 g/L
256 of leaching tire wear particles, and our $0.25\times$ treatment a dose that a less-travelled road or
257 further pond might receive, 5 g/L of leaching tire wear particles (see ranges in Wagner et al.,
258 2018). Leaching rates from tire wear particles may differ across leaching concentrations, but
259 we expect these effects to be small (Rhodes et al., 2012), and so refer to our treatments as
260 5 and 10 g/L leachate throughout.

261 **Experimental set up**

262 We experimentally exposed duckweed in well plates to climate-change gradients, crossed
263 with tire leachate and microbial treatments. We used our device to generate perpendicular
264 temperature and CO_2 gradients over plates, so that each well in a plate is a unique combina-
265 tion of temperature and CO_2 ppm. Within plates, each well received one sterilized duckweed
266 clonal unit (e.g. one mother-daughter frond pair), and we alternated microbial re-inoculation
267 across columns of wells, so that both re-inoculated and uninoculated treatments spanned the
268 temperature gradient.

269 To generate inocula, we placed a swab from the cultured agar plate (see above) into
270 2 mL of autoclaved liquid yeast mannitol media in a glass vial (previously cleaned and
271 sterilized in a muffle furnace) and cultured at 30°C for approximately 24 hours at 200 rpm
272 in a shaking incubator (VWR, Radnor, PA, USA), together with an identical vial of yeast-
273 mannitol media with no swab added as sham inoculum. We then diluted so that 10 μ L
274 of inocula would bring a well to approximately 5,000 cells per μ L based on an estimate of
275 cell density from optical density (as described in O'Brien et al., 2020b). We diluted sham
276 inocula by the same amount, added 10 μ L of inocula or sham to each well, and sealed plates
277 with BreatheEasier (Millipore-Sigma, Diversified Biotech, Dedham, MA, USA) membranes.
278 We crossed this design with three levels of tire leachate (96 unique temperature and CO₂
279 conditions \times 3 = 288 treatments) and treated an entire plate with a particular level (0 g/L =
280 none, 5 g/L, or 10 g/L, see above) to prevent cross-contamination between treatments (Birch
281 et al., 2019). We expect that many components of tire leachate could be volatile (U.S. EPA
282 CDC/ATSDR, 2019), yet we require gas exchange for CO₂ treatments and living organisms.
283 Plates in devices were connected to CO₂, air, hot water, and cold water in parallel for 7
284 days. This three-plate setup constituted one replicate of the experiment, and we repeated
285 the setup three times (i.e. 3 blocks, for 9 total plates and 864 total experimental units),
286 where plates with different tire particle leachate treatments were randomly assigned to the
287 three parallel devices within each replicate (see Figure S2).

288 All experiment setup including hand sterilization, plate filling, and microbial manipula-
289 tion was conducted in a biosafety cabinet (ESCO Micro Pte. Ltd., Labculture®[®], Singapore)
290 and glassware was used where possible, with standard cleaning followed by a muffle furnace
291 treatment at 450°C for 7 hours (ThermoFisher Scientific, F30428C-80). We used glass-coated
292 96-well plates (Thermo Scientific, 60180-P304) that were bleach sterilized (5 minutes in 1%
293 NaOCl, followed by three rinses of autoclaved reverse-osmosis water) and cleaned of con-
294 taminants (three rinses of acetone followed by one rinse of hexane left to evaporate (both
295 Fisher Chemical HPLC grade, \geq 99.5% and \geq 98.5%, respectively). When glassware was not

296 possible, plastic labware was autoclaved or purchased pre-sterilized.

297 **Data collection**

298 At the end of the experiment, we disconnected plates from the device and recorded duckweed
299 growth and traits with image analysis, and microbial growth with optical density.

300 We photographed plates using a custom camera rig for a Nikon D3200 (with AF-S DX
301 NIKKOR 18-55mm f/3.5-5.6G VR lens, Minato, Tokyo, Japan) and a standard backlit light-
302 ing regime (created with Yongnuo YN-300 light, Shenzhen, China). We analyzed images in
303 ImageJ, using color threshold settings to select only “live” duckweed fronds. Thresholds
304 were set subjectively by the image scorer (blind to plate conditions) to include duckweeds
305 having any green hue, but exclude algae. The same thresholds for minimum pixels and hue
306 were applied across all plates in a round, but individual images differed slightly, so small
307 adjustments to brightness and saturation cutoffs were necessary. We then measured pixel
308 area and greenness (ratio of green brightness to total brightness in pixels across RGB chan-
309 nels) of all duckweed fronds in a well (with “Analyze Particles,” see examples in Figure S5).
310 Greenness is associated with leaf nitrogen (Thind et al., 2012), including greenness in digital
311 images (Rorie et al., 2011). This is likely due to the relationship between chlorophyll and
312 nitrogen content (Schepers et al., 1992; Ma et al., 1996), which may be reduced by increasing
313 CO₂ (Ellsworth et al., 2004), as plants become nitrogen limited. We used a custom R script
314 to sum (area) or average (greenness) measures for duckweed fronds in the same well.

315 Optical density was measured on a 70 μ L sample of liquid from each well at the end
316 of the experiment after imaging. We recorded optical density at 600 nm (BioTek Synergy
317 HT plate reader, Gen5 1.10 software, Winooski, VT, USA). Plates could not be measured
318 simultaneously, and were incubated at 4°C 0.5-2 hours between imaging and optical density
319 measures. Each reading had the optical density of the background (plate and reverse osmosis
320 water) subtracted. The minimum optical density that was greater than 0 was taken to be
321 the threshold reading, and all values lower than 0 were set to this threshold. We expect

322 optical density to be correlated with the live colony forming units of microbes in solution at
323 the end of the experiment, as verified in O'Brien et al. (2020*b*).

324 **Data analysis**

325 We analyzed data in R (R Core Team, 2019) using linear models and with package MCM-
326 Cglmm (Hadfield, 2010). We fit models from more complex (all linear interactions of all
327 treatment parameters) to less complex in reverse stepwise regression. At each step, we re-
328 moved the most complex (highest order interaction) parameter, unless more than one most
329 complex parameter was non-significant, in which case we removed the term with the highest
330 pMCMC (the Bayesian equivalent of the p-value, Hadfield, 2010). We repeated the process
331 until no terms were non-significant (unless they were components of significant higher-order
332 interactions, in which case they were retained), or the simpler model fit worse, and we call
333 the resulting model the “best” model. We evaluated significance with pMCMC and model fit
334 with the Bayesian equivalent of AIC, or deviance information criteria (Spiegelhalter et al.,
335 2002). We report highest posterior density intervals (HPDIs) as the Bayesian equivalent
336 of confidence intervals and use 95% for pMCMC <0.05 and 90% for effects with marginal
337 pMCMC values (<0.1). All models included the random effect of round (1, 2, or 3), ran for
338 100,000 iterations thinned by 50 and with 500 iterations of burnin. We refit best models
339 with increased iterations (1,000,000), for reporting estimated parameter values. We applied
340 our best-model procedure to the response variables of duckweed growth in pixel area, the log
341 of optical density (data from inoculated wells only; we fit log of optical density as a function
342 of inoculation as a separate model, see Figure S1), and greenness.

343 For better behavior of model fitting functions, we modified CO₂ concentrations to the
344 same order of magnitude as temperature by dividing by 100 (ppm/100 or, parts per 10,000).
345 We interpolated average measured temperatures over the course of the experiment for each
346 well (due to slight variations, see above), and these differed somewhat in range across treat-
347 ments and tire particle leachate treatments (wider in control, 13.8-29.3°C vs 19.0-26.5°C all

348 others), but means were similar (21.7, 22.2°C for 0 g/L and all others, respectively). We do not
349 anticipate that this affected the estimated main effect of leachate, given similar mean tem-
350 peratures, nor do we expect that the wider range of temperatures in plates without leachate
351 increased power to detect temperature effects in this vs other tire treatments, given that
352 temperature effects are not strongest at 0 g/L (see Results). Finally, some wells partially
353 dried due to loose seals with gas supply in the device. In these wells, duckweed generally
354 died due to sticking to well walls as liquid levels dropped, rather than due to treatment
355 exposure, and optical density-based estimates of microbial growth will be inaccurate. These
356 wells were difficult to identify with certainty in some cases, so we excluded all wells in which
357 duckweed had fully died (104 of 864 wells).

358 If microbes and duckweed independently respond to the same treatments, this could cause
359 positive fitness correlations between the two without fitness feedbacks. However, we can use
360 the residual fitness variation and covariation after accounting for the effects of treatments on
361 duckweed and total microbial growth, to ask how selection might act on duckweed or microbe
362 genotypes that increase the fitness of their partner. We further explored the residuals of
363 duckweed and microbial growth from a fully fitted model with all terms, including non-
364 significant treatment terms (in case weak trends were missed in best models), and using the
365 data from only inoculated wells in which duckweed survived (n=372). We modeled residuals
366 of duckweed pixel area increase as the response, and explanatory variables were residual log
367 optical density and the interaction between residual log optical density and each treatment
368 or combination of treatments. As above, we performed reverse stepwise regression to select
369 the best model.

370 **Results**

371 Duckweed was affected by all anthropogenic stressors in either growth, traits or both. We
372 also observed effects from interaction with the microbiome, and altered interactions in the

373 presence of anthropogenic stressors, i.e., non-additive effects between the microbiome and
374 anthropogenic stressors.

375 For duckweed growth, the best model found that microbiome effects were conditional
376 (main effect $pMCMC > 0.1$) on both tire leachate and temperature (interaction terms pM -
377 $CMC < 0.05$, Table 1, Figure 1), such that increasing tire leachate and temperature sepa-
378 rately increased costs of microbiomes to duckweed. At both lower temperature and without
379 any tire leachate, inoculated and uninoculated plants grew equally little (Figure 1b, see
380 overlapping HPDI at lowest temperatures). Without any tire leachate, uninoculated plants
381 responded positively to warming temperature ($pMCMC < 0.05$), but plants inoculated with
382 microbiomes did not ($pMCMC < 0.05$), producing negative effects of microbiomes at warmer
383 temperatures (Figure 1b). With 10 g/L tire particle leachate, uninoculated duckweed grew
384 about three times as much as inoculated plants, on average across other treatments (means
385 2694 and 876 pixels, $SE \pm 225$ and 143 pixels, both respectively, Figure 1a, Tire×Microbe
386 negative with $pMCMC < 0.05$, Table 1), also producing costs of microbiomes. However,
387 costs of microbiomes at higher temperatures and with tire leachate were less than additive,
388 with marginal significance (Temperature×Tire×Microbes $pMCMC < 0.1$), meaning that
389 plants without microbiomes did not grow much more than plants with microbiomes when
390 temperatures were warmest and the tire leachate treatment was 10 g/L of particles (Figure
391 1c). The best fit model did not include an effect of CO₂ on duckweed growth.

392 Total microbial growth (putative average “fitness” across the microbiome community),
393 was also affected by temperature ($pMCMC < 0.05$) and tire leachate ($pMCMC < 0.05$), but
394 not CO₂ treatments (not included in the best model). Like duckweed, microbes grew more at
395 higher temperatures (mean predicted optical densities 0.003 and 0.025 at coldest and highest
396 applied temperatures, respectively) and at higher tire particle leachate concentrations (mean
397 predicted optical densities of 0.005 at 0 g/L and 0.015 at 10 g/L, non-overlapping 95% HPDI,
398 Figure 2). There was some contaminant microbial growth in uninoculated wells, but there
399 was less microbial growth, on average, in uninoculated wells (optical density means 0.0065

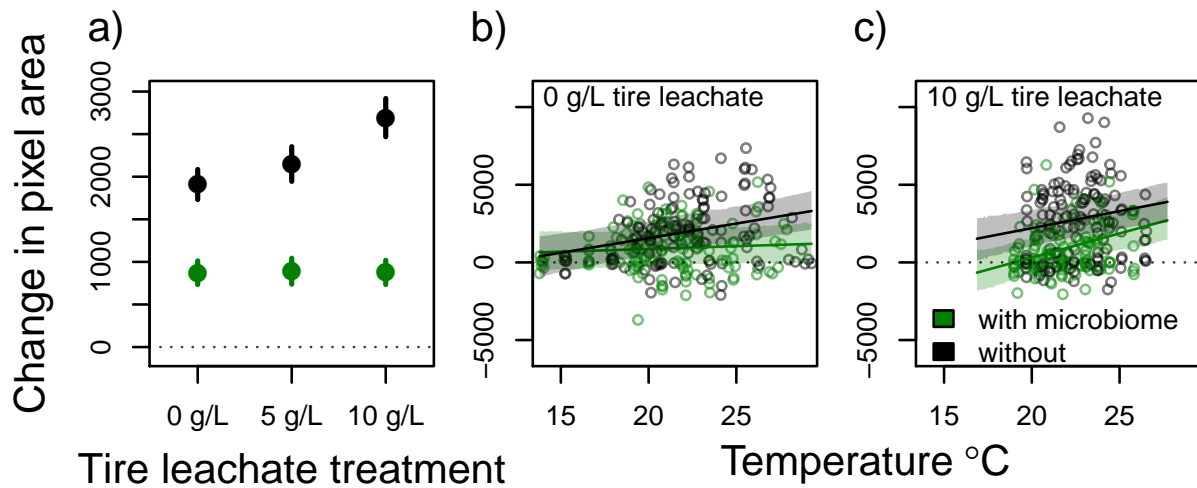


Figure 1: Duckweed growth when re-inoculated (green) and when not re-inoculated (black) with their microbiome across experimental treatments with significant effects. a) Growth means (points) across different levels of tire particle leachate treatments (x-axis), with one standard error of the mean (bars). b) & c) Duckweed growth across temperature ($^{\circ}\text{C}$, x-axis), at 0 g/L (b) and 10 g/L (c) tire particle leachate treatments. Points are observed growth data with interpolated average temperature values during the experiment. Shaded backgrounds indicate 90% HPDIs for the predicted mean (lines) from the best-fit model. Data and fitted model predictions for concentrations of 5 g/L leachate treatments are not shown, but are intermediate. The temperature range extends further in (b) due to temperature anomalies in one round of the experiment.

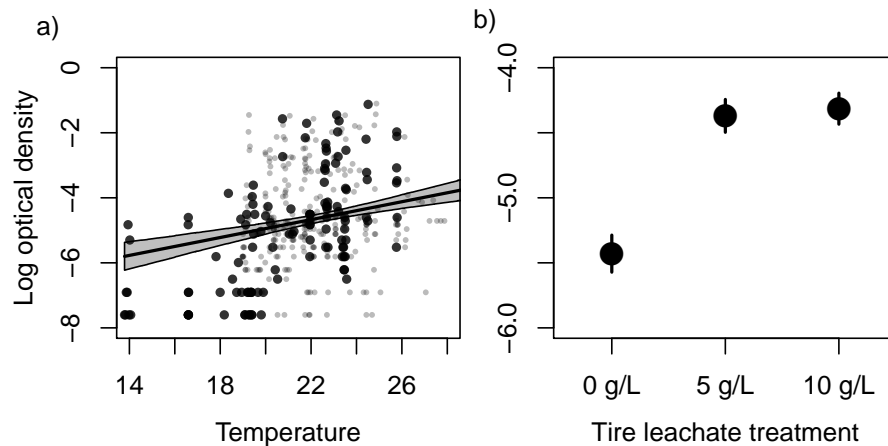


Figure 2: Response of total microbial growth to treatments in the best model. Total microbial growth (log of the optical density) is putative average “fitness” across microbiome component species. a) Points are observed optical densities and interpolated average temperatures (grey and smaller = 10 g/L or 0 g/L, black and larger = 5 g/L tire particle leachate treatments) for each well. Grey background indicates 95% HPDI for the predicted mean (line) from the best-fit model at 5 g/L leachate treatments. b) Means of logged observed optical densities (bars are standard errors) across 0 g/L, 5 g/L, and 10 g/L tire particle leachate treatments.

400 and 0.0090, with SE range 0.0061-0.0070 and 0.0083-0.0097 in uninoculated and inoculated
401 wells, respectively, pMCMC < 0.05, Figure S1).

402 The best model for duckweed frond greenness found that CO₂, temperature and leachate
403 from tires all affected outcomes. Temperature increased greenness in wells by about 0.04
404 from coldest to warmest (from model-predicted mean of 0.408 to 0.440, Figure 3a, Table
405 1), similar to growth effects. In contrast, increasing CO₂ from the lowest to highest applied
406 level decreased greenness proportion by about 0.02 (from predicted mean of Figure 3b).
407 Interestingly, with tire particle leachate, greenness was less reduced for higher CO₂ levels,
408 with only half as much change across the CO₂ range, 0.01 (for 10 g/L, though still non-
409 overlapping 95% HPDIs between lowest and highest CO₂).

410 Raw fitness correlation was essentially neutral ($\rho=-0.016$), despite similar responses of
411 duckweed and microbes to treatments, which might have inflated correlation. Indeed, resid-
412 ual fitness correlations accounting for main treatment responses were largely negative, and
413 the best fit model suggested significant influence of experimental treatments on the sign

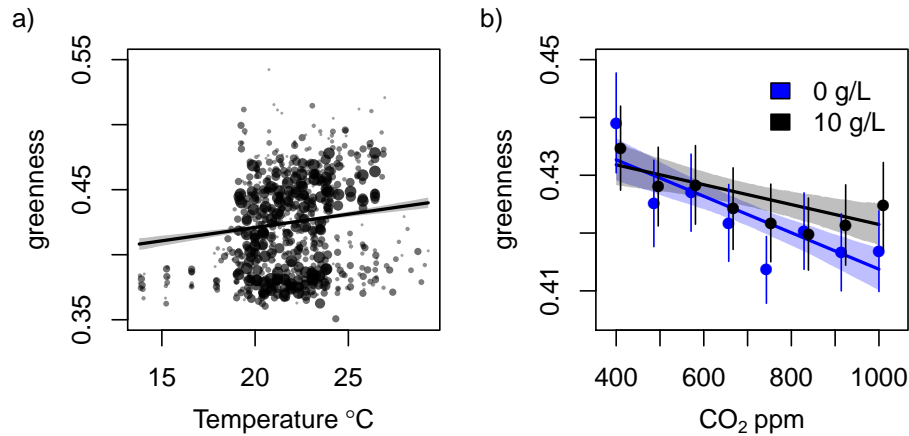


Figure 3: Response of greenness of duckweed fronds to treatments. a) Points are observed greenness across interpolated average temperatures. Background shading indicates 95% HPDI for the predicted mean (line) from the best-fit model for the parameters tested, at 700 ppm CO₂ and 5 g/L tire particle leachate treatments. Points from treatments further from these values are smaller and fainter. b) Greenness in CO₂ and tire particle leachate treatments (blue, 0 g/L and black, 10 g/L) averaged across temperature (bars are one standard error), offset slightly along the x-axis for visibility. Model predictions (lines) and 95 % HPDI (shading) are for average temperature. Microbiome treatment did not affect greenness, so observations in both panels are included as points (a) or averages (b) without regard to microbe treatment.

parameter	duckweed growth	log optical density	greenness
Intercept	-2280	-8.38 *	0.400
Microbes	2410 .	NA	–
Temperature	187 *	0.151 *	0.002 *
CO ₂	–	–	-0.003 *
Tire	0.821	0.100*	-0.0007
CO ₂ × Tire	–	–	0.0001 .
Microbes × Tire	-608 *	NA	–
Temperature × Tire	3.16	–	–
Temperature × Microbes	-152 *	NA	–
Temperature × Tire × Microbes	24.4 .	NA	–

Table 1: Best fit models between treatments and response variables: duckweed growth, microbial growth (optical density), and duckweed frond greenness (proportion). CO₂ was fit with ppm / 100 (or, parts per 10,000, ranging from 4-10), see Methods. “–” indicates this term is not in the best model for the response variable, “*” indicates pMCMC < 0.05 and “.” indicates pMCMC < 0.1

parameter	fitness covariation
Intercept	-23.4
ODres	-1020 .
ODres×Temp	33.8
ODres×CO ₂	44.8 .
ODres×Tire	-22.9 .

Table 2: Best fit models for the slope between fitness residuals across treatments. “ODres” is short for the residuals of the log of optical density, and “.” indicates pMCMC < 0.1.

414 and strength of fitness correlations. There was a marginally significant negative correla-
415 tion between residual duckweed and total microbial growth. This was shifted positive by
416 CO₂ (marginally, pMCMC < 0.1) and temperature (n.s.), but further decreased in higher
417 tire particle leachate concentration treatments (marginally, pMCMC < 0.1). While effects
418 were all marginally or not significant, all simpler models (factorial combination of remain-
419 ing terms, and intercept only model) fit worse when evaluated by DIC. We visualized these
420 marginally significant effects by splitting the data into two scenarios for weaker and stronger
421 climate change (temperature and CO₂ above or below the average level we applied, 21.7°C
422 and 700 ppm, respectively) crossed with no, medium, and higher prevalence of tire wear
423 particles. With weaker climate change conditions (13.7-21.7°C and ambient-700 ppm CO₂),
424 residual fitness correlations were marginally negative without leachate from tire particles,
425 but strongly negative (significant, HPDIs for duckweed growth do not overlap for extremes
426 of residual microbial growth) with 10 g/L leachate (Figure 4a-c). Under stronger climate
427 change conditions (21.7-29.3°C and 700-1000 ppm CO₂), fitness correlations were marginally
428 positive without leachate, but shifted to neutral with 10 g/L leachate treatments (Figure
429 4d-f).

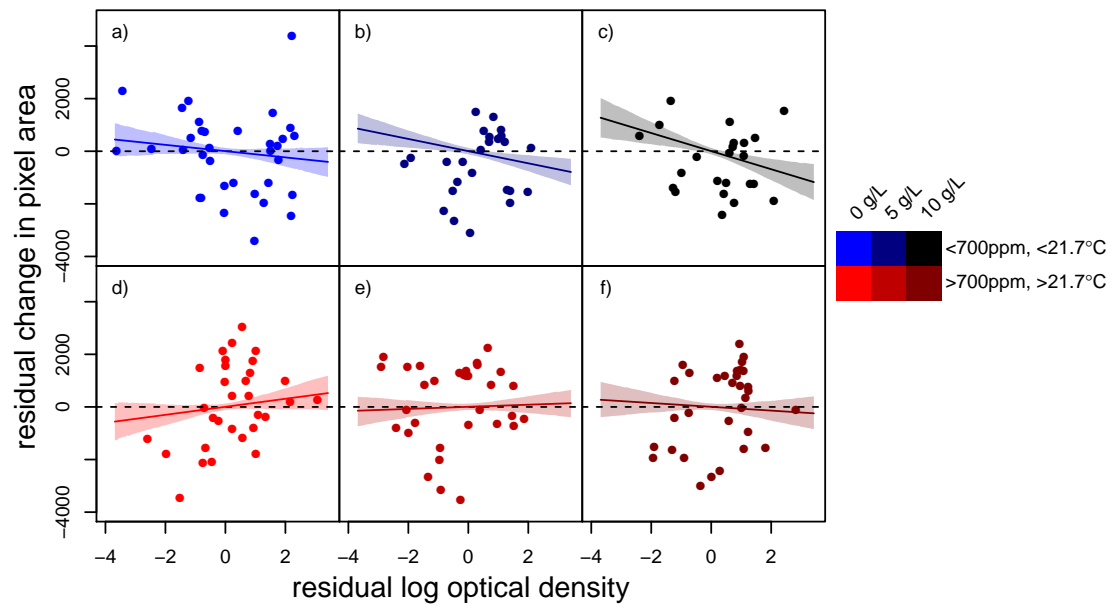


Figure 4: Residual growth (fitness proxy) for duckweeds and microbes for subsets of data falling into scenarios of weaker (a - c) or stronger global change (d - f) with 10 g/L (c and f), 5 g/L (b and e) and without (a and d, 0 g/L) contamination with leachate from tire wear particles. Stronger global change is defined as CO₂ and temperature both above average treatment means, 21.7°C and 700 ppm, and shown in reds. Weaker global change is defined as CO₂ and temperature below these means, and shown in blues. Tire particle leachate treatment is indicated by color darkness, with brightest colors indicating no leachate, and darkest indicating 10 g/L. Points are data observations from these treatment categories, with shaded background indicating the 90% HPDI for the predicted mean response (lines) of the best fit model at the mean of the treatment ranges within the category. Strong warming with low CO₂ and vice versa represent less likely global change scenarios and data and model predictions are not depicted here.

430 Discussion

431 Climate change and tire wear particle leachate have non-additive 432 effects on duckweed-microbiome interactions

433 Our observations show the importance of considering synthetic contaminants in global change
434 science. Synthetic contaminant concentrations in nature, including pesticides, plastic addi-
435 tives, and trace metals, are prevalent and increasing - and in step with other global change
436 parameters (Bernhardt et al., 2017). As shown here, synthetic contaminants can have effects
437 that can both percolate through species interactions (Fleeger et al., 2003) and shift across cli-
438 mate change backdrops (Yang et al., 2020). We aimed to characterize the simultaneous and
439 individual effects of climate change and a model synthetic contaminant (leachate from tire
440 wear particles) on duckweed and its microbiome. Leachate from tire wear particles altered
441 duckweed growth, microbial growth, and duckweed-microbiome interactions, but effects var-
442 ied with different climate change parameters. Both duckweed and microbes grew better
443 under conditions increasingly resembling urban and future scenarios, i.e. warmer and more
444 concentrated leachate from tire particles, but effects were not linearly additive for duckweed
445 when microbes were present. We have previously found that duckweed microbiomes gener-
446 ally increase duckweed growth in both benign and a variety of stressful conditions (O'Brien
447 et al., 2019; O'Brien et al., 2020*b,a*). Despite our strong prior that the relationship between
448 duckweed and its microbiome would be mutualistic and would increase the benefits of CO₂,
449 microbes largely reduced the positive effects of temperature and leachate (but less so for
450 combined warm temperatures and concentrated leachate, Table 1 and Figure 1), and there
451 was no main effect of CO₂ on growth of either duckweed or microbes. In other words, global
452 change scenarios increased the costs of microbes as a whole relative to the benefits, shifting
453 the interaction from essentially neutral at current, non-urban conditions (no leachate, low
454 temperature, Figure 1b), to costly in future scenarios.

455 **Growth correlations, greenness and longer-term impacts on duckweed-** 456 **microbiome interactions**

457 The microbiome-free state may be irrelevant in nature: as no genotype of duckweed is
458 microbe-free in the field, it cannot be favored by selection. Instead, variation in the quantity
459 of microbes supported by hosts is a more meaningful metric (Partida-Martinez and Heil,
460 2011). In sections of the experiment representing only weak global change (Figure 4a),
461 duckweed and microbe residual growth (variation after accounting for responses to temper-
462 ature and tire particle leachate) were not positively correlated (Figure 4a). Stronger climate
463 change manipulations (temperature and CO₂ increases only) increased positive growth corre-
464 lations (Figure 4d), but leachate from tire wear particles shifted correlations negative (Figure
465 4c,f). Growth correlations may indicate fitness conflicts and alignment in mutualisms (neg-
466 ative and positive correlations, respectively), and phenotypic correlations can be a good
467 proxy for genetic correlations (Waitt and Levin, 1998; Stepan et al., 2002). However, this
468 proxy is not always reliable (Stinchcombe et al., 2002) and may somewhat depend on ge-
469 netic variation contributing substantially to phenotypic variation, and here we have only one
470 genotype. While even clonal duckweed can accumulate mutations on which selection could
471 act (Ho, 2017), our experimental duckweed are an unknown and relatively small number of
472 clonal generations apart. Still, the relationship between growth of plants and the growth of
473 microbes may be mechanistically the same regardless of whether variation is generated by
474 stochastic or genetic effects. If so, over longer time, climate change could select for enhanced
475 duckweed-microbiome mutualisms in uncontaminated sites, but for disrupted mutualisms in
476 sites that receive road runoff.

477 The effects of treatments on plant greenness may help explain patterns, and provide a
478 common mechanism that could link stochastic and genetic fitness correlations. Greenness is
479 positively linked to leaf nitrogen and chlorophyll content (Rorie et al., 2011; Thind et al.,
480 2012), and is often decreased when plants become nitrogen limited at elevated CO₂ (Ellsworth
481 et al., 2004). Therefore, reduced greenness may be a signal that plants are more nitrogen

482 limited than carbon limited, and that microbial provisioning of nitrogen would have more
483 fitness benefits and microbial use of carbon fewer fitness costs. Here, duckweed greenness
484 was low with high CO₂ when there was also no tire leachate pollution, and indeed, these
485 same conditions (high CO₂ and no tire leachate) were the only conditions in which microbe
486 and duckweed growth were positively linked (Figures 3b & 4c, Table 2), suggesting microbes
487 alleviate the nitrogen limitation of duckweed at elevated CO₂. Indeed, relative strengths of
488 carbon and nitrogen limitation appear to drive results across many different experimental
489 manipulations of CO₂ (Treseder, 2004; Phillips et al., 2011).

490 **Possible mechanisms of tire wear particle leachate effects**

491 Another important question to consider is what components of the tire leachate might drive
492 the effects we observed. Leachate of our exact tire is known to contain both zinc and PAHs
493 (Kolomijeca et al., 2020), as does the leachate of many tires (Wagner et al., 2018). Zinc
494 reduces duckweed growth at ambient climate (O'Brien et al., 2020*a,b*), so increased growth
495 at higher leachate concentrations indicates that other components of tire leachate contribute
496 to effects, masking or altering negative effects of zinc. One PAH is known to affect duckweed
497 growth (phenanthrene, Becker et al., 2002), and a number of others inhibit growth and
498 induce chlorosis in a close relative (anthracene, phenanthrene, benzo[a]pyrene, fluoranthene,
499 pyrene and naphthalene, Huang et al., 1993; Ren et al., 1994). The tire we used contains
500 all the above PAHs, and all but fluoranthene have been detected in its leachate (Kolomijeca
501 et al., 2020). Surprisingly, we observed only positive effects on duckweed: increased growth
502 and greenness at higher leachate concentrations (Figures 1a & 3b). Effects of PAHs may
503 also depend on photodegradation pathways (Huang et al., 1993; Ren et al., 1994), and,
504 interestingly, some effects of leachate from tires may likewise depend on light (Wik and Dave,
505 2006). Alternatively, very low doses of phenanthrene actually stimulated growth in duckweed
506 (Becker et al., 2002), and increased growth at low doses due to compensatory mechanisms, a
507 common biological response to toxins (Calabrese, 2008). Our growth responses could suggest

508 low doses of PAHs in leachate, but the growth continues increasing from 5 to 10 g/L. More
509 likely, PAHs could be disrupting hormone signaling in duckweed. Phenanthrene inhibits
510 ethylene responses in *Arabidopsis thaliana* (Weisman et al., 2010), and as ethylene may
511 promote or inhibit growth depending on the dose (Pierik et al., 2006), this could explain
512 why effects vary across dose and plant species, beyond results here. In fact, plant endocrine
513 disruption appears to be common in a number of classes of ubiquitous contaminants (Couée
514 et al., 2013), and may be the mode of action here even if PAHs are not the component of
515 leachate causing the observed effects.

516 Indeed, tires contain a great many other biologically active compounds (U.S. EPA CDC/ATSDR,
517 2019), many of which are known to leach into water (Zahn et al., 2019; Capolupo et al., 2020).
518 Of those that are known to leach, mixtures of 1,3-diphenylguanidine and hexa(methoxymethyl)melamine
519 are associated with toxic effects on coho salmon (Peter et al., 2018), and mixtures of benzoth-
520 iazole, 2(3H)-benzothiazolone, phthalimide, phthalide, bisphenol-A, and n-cyclohexylformamide
521 may underlie toxicity in algae and mussels (Capolupo et al., 2020). Much less is known about
522 likely effects of these on duckweed. General toxicity of the transformation products of 1,3-
523 diphenylguanidine is expected across organisms (Sieira et al., 2020), and the same is true
524 for benzothiazole, with documented toxicity across a number of other species, and poten-
525 tial similar mode of action as PAHs, due to activation of aryl hydrocarbon receptors (Liao
526 et al., 2018). Bisphenol-A toxicity to duckweed and other *Lemna* is known (Mihaich et al.,
527 2009; Fekete-Kertész et al., 2015), and like PAHs may increase growth at low concentrations
528 (Mihaich et al., 2009). Lastly, while hexa(methoxymethyl)melamine is expected to have
529 low toxicity to aquatic organisms, (U.S. EPA, 2004) it has been associated with negative
530 effects on *Daphnia* (de Hoogh et al., 2006). While the effects of these other compounds are
531 largely expected to be negative, not enough is known to rule them out as sources of positive
532 effects here. Future work should undertake non-targeted chemical analysis to ask whether
533 there are unexpected chemicals in the leachate. Moreover, fractionating leachate could be
534 included to determine which chemicals or suites of chemicals drive leachate effects on plants

535 and microbes.

536 Regardless of the source of the growth promotion, microbes remove it, providing a clear
537 example of a species interaction altering a contaminant effect. If duckweed responses to PAHs
538 in tire leachate caused the increased growth, microbes may have removed effects by rapid
539 mineralization. While we do not expect that the source of our biological materials has much
540 history of contaminant exposure (field site in a natural area), even freshwater microbes from
541 pristine sites may rapidly mineralize PAHs at appreciable rates (Heitkamp and Cerniglia,
542 1987; Haritash and Kaushik, 2009). PAH degraders include many *Pseudomonas* strains
543 (Haritash and Kaushik, 2009), and several *Pseudomonas* strains have also been previously
544 identified in this microbiome (O'Brien et al., 2019). This mechanism could also account for
545 negative growth correlations induced by tire leachate: as microbes mineralize PAHs, they
546 may increase in abundance, duckweeds may then be less hormonally disrupted and grow less.

547 One pervasive theme across studies of leachate from tire wear particles, is that not all
548 tires, organisms, and methods are equivalent. Diverse methods find diverse biological effects
549 ranging from acute lethality in coho salmon (Peter et al., 2018) to no effects at even high
550 doses for some invertebrates (Redondo-Hasselerharm et al., 2018). Studies on tire particles
551 vary in a number of factors that may influence toxicity, not exhaustively including: the
552 brand of tire (Wik and Dave, 2006), size of tire particles used (though perhaps not always,
553 Rhodes et al., 2012; Khanal et al., 2014), the age of the tire (Day et al., 1993; Sharma et al.,
554 2010), whether or not road wear is simultaneously considered (Redondo-Hasselerharm et al.,
555 2018), whether or not the particles or leachate or both are tested (Khan et al., 2019), leach-
556 ing time (Wik and Dave, 2006; Rhodes et al., 2012), leaching conditions (Marwood et al.,
557 2011) and leachate storage (Khanal et al., 2019). It is hard to predict how differences in
558 methods, tires, or study species might have affected our results, but we note that the studies
559 on similar organisms above do not find opposing signs of effects across factors, but rather
560 stronger and weaker effects (for example, Panko et al., 2013; Kolomijeca et al., 2020). Fi-
561 nally, we note commonalities in the effects of temperature. Kolomijeca et al. (2020) similarly

562 observed stronger effects of leachate from tire wear particles at high temperature on fathead
563 minnow, and Marwood et al. (2011) observed that leachate produced at higher temperatures
564 had greater effects, though these studies both observed stronger negative effects, while we
565 observed stronger positive effects of temperature and tire when duckweed were inoculated
566 with microbiomes. Broadening our view, it appears that temperature exacerbation of con-
567 taminant effects may be a fairly common outcome across contaminants in aquatic systems
568 (Crain et al., 2008; Jackson et al., 2016).

569 **Conclusions**

570 Changing environments can disrupt species interactions, and duckweed-microbiome interac-
571 tions are no exception: microbiomes were more costly to duckweed with tire particle leachate
572 or warming, and leachate may shift selection pressures towards microbes that reduce duck-
573 weed growth (or duckweeds that reduce microbial growth). Further, with our finding that
574 some effects of leachate from tire particles depended on temperature or CO₂, our study joins
575 a vast array of cases where multiple global change factors have non-additive effects (Darling
576 and Côté, 2008; Crain et al., 2008; Jackson et al., 2016). With an ever increasing suite of
577 anthropogenic stressors comes more potential for such “ecological surprises,” including for
578 disrupted species interactions. Effects of anthropogenic contaminants on species interactions
579 therefore continues to be a critical area of research: shifted strengths or flipped signs of in-
580 teraction outcomes could echo through food webs and alter basic ecosystem functions from
581 productivity to stability.

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595 **Data Accessibility:** Data will be accessible upon publication via figshare and Github.
596 Scripts will also be accessible on Github.

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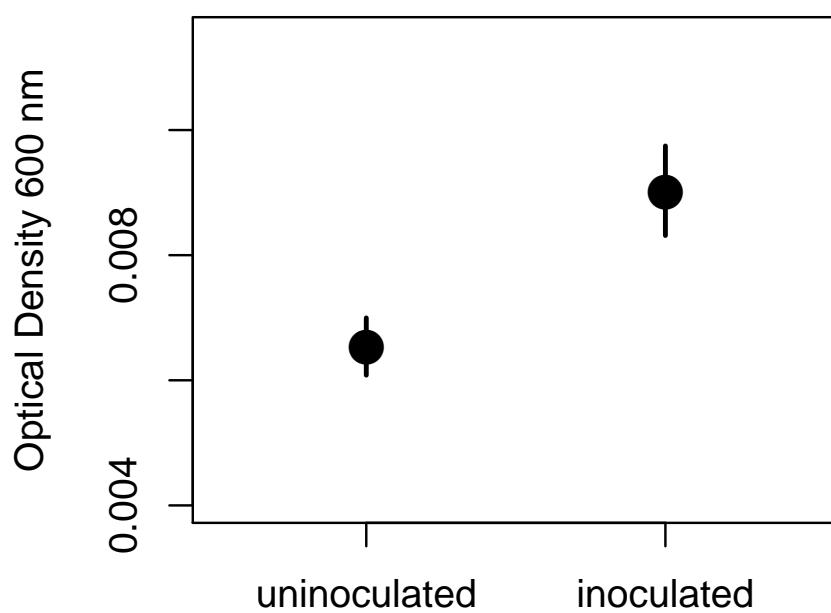


Figure S1: Effect of inoculation with microbiome on total microbial growth (optical density). Means (points) and standard errors (bars) are shown.

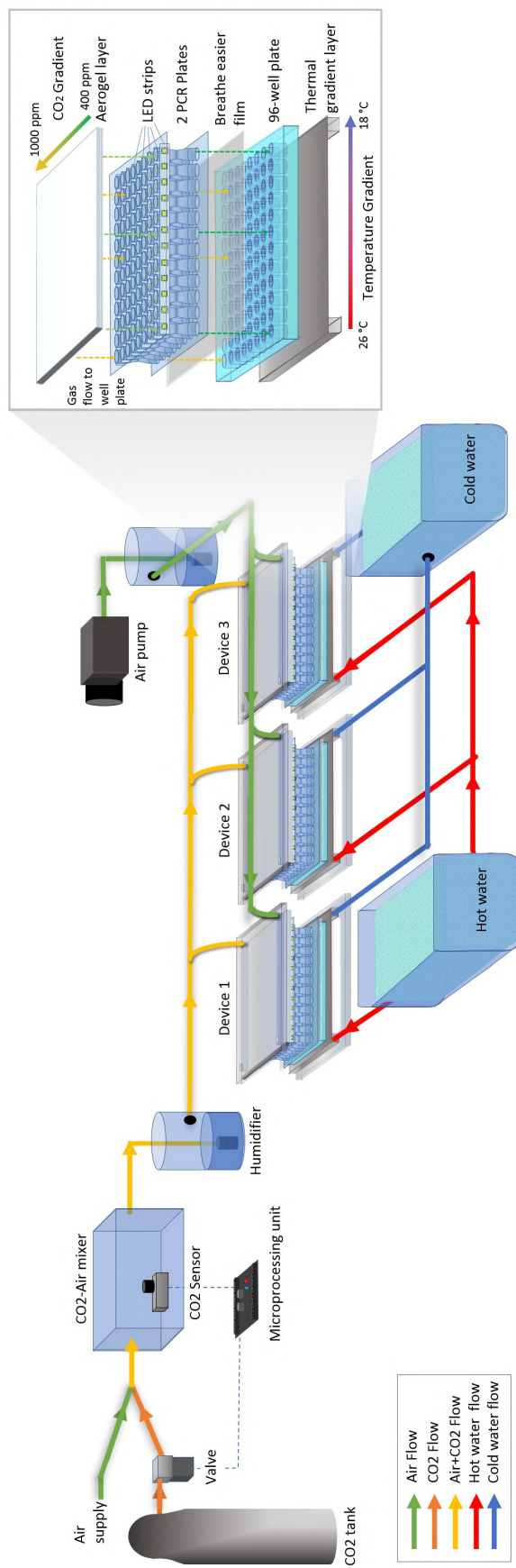


Figure S2: Graphical representation of device setup. Briefly, a targeted elevated CO₂ concentration for the highest treatment (yellow) was achieved by mixing pure CO₂ (orange) and air (green) via the controlling action of a microprocessing unit, valve and sensor (see text and Nguyen et al., 2018; Yang et al., 2020). Then, both this mix and air (green) were humidified and delivered to the plate on opposite sides, and an even gradient was generated with an aerogel. Below this aerogel layer, we placed tubes that connect the aerogel gas gradient to experimental wells of a 96-well plate. LED light strips were interlaced in the spacing between these tubes. Next, we placed a breathable, cell-barrier membrane over the wells. Finally, below the plate, we applied a temperature gradient using an aluminum plate with hot and cold water piped across either end (orthogonal to the gas gradient, see inset, Methods text). The three plates in parallel depicted here show one replicate, which was repeated three times. The schematics of one of the three plates is shown in inset on the right, with the different layers shown. The three leachate treatments would be randomly assigned across plates within a replicate and are therefore not shown.

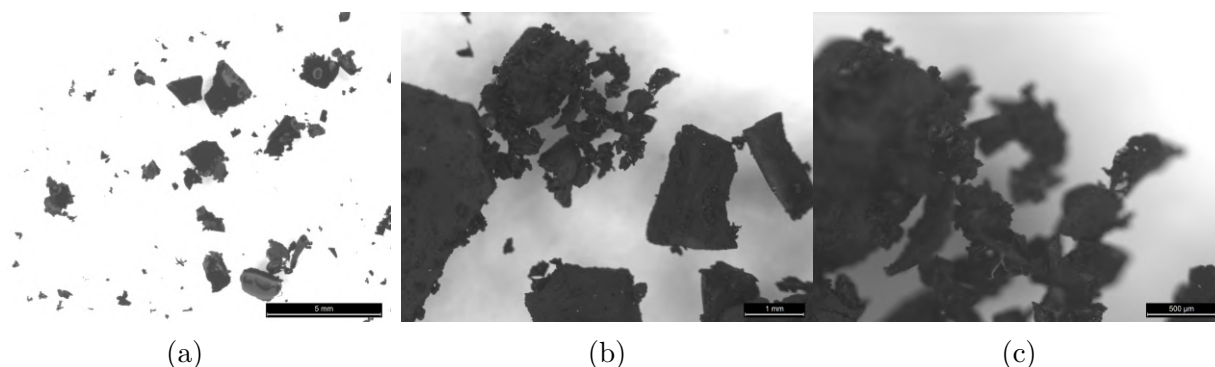


Figure S3: Characteristic images of tire particles generated and used in the experiment. a) tire particles in surfactant, removing most static attractions and images blown out as described in Methods. b) tire particles without surfactant. c) a region of the same particles at higher magnification.

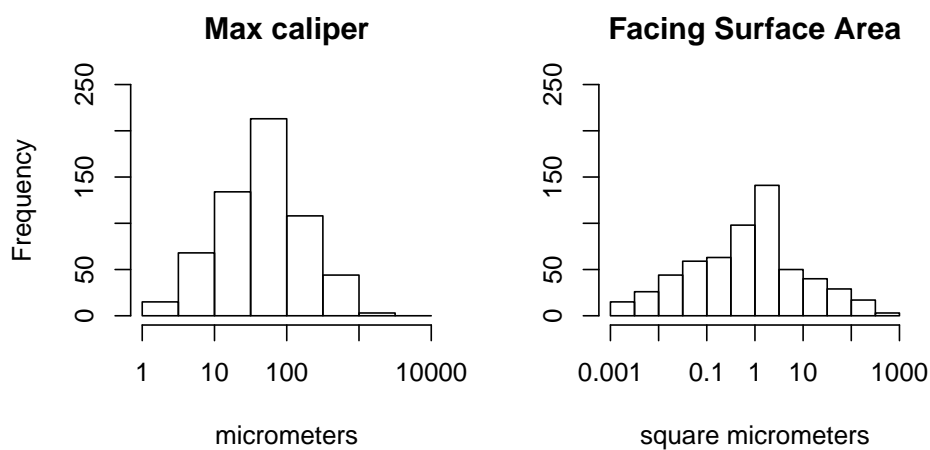


Figure S4: Distribution of tire particle size measurements. The maximum caliper (Feret's Diameter, left) and the top surface area (visible in the in the image, right).

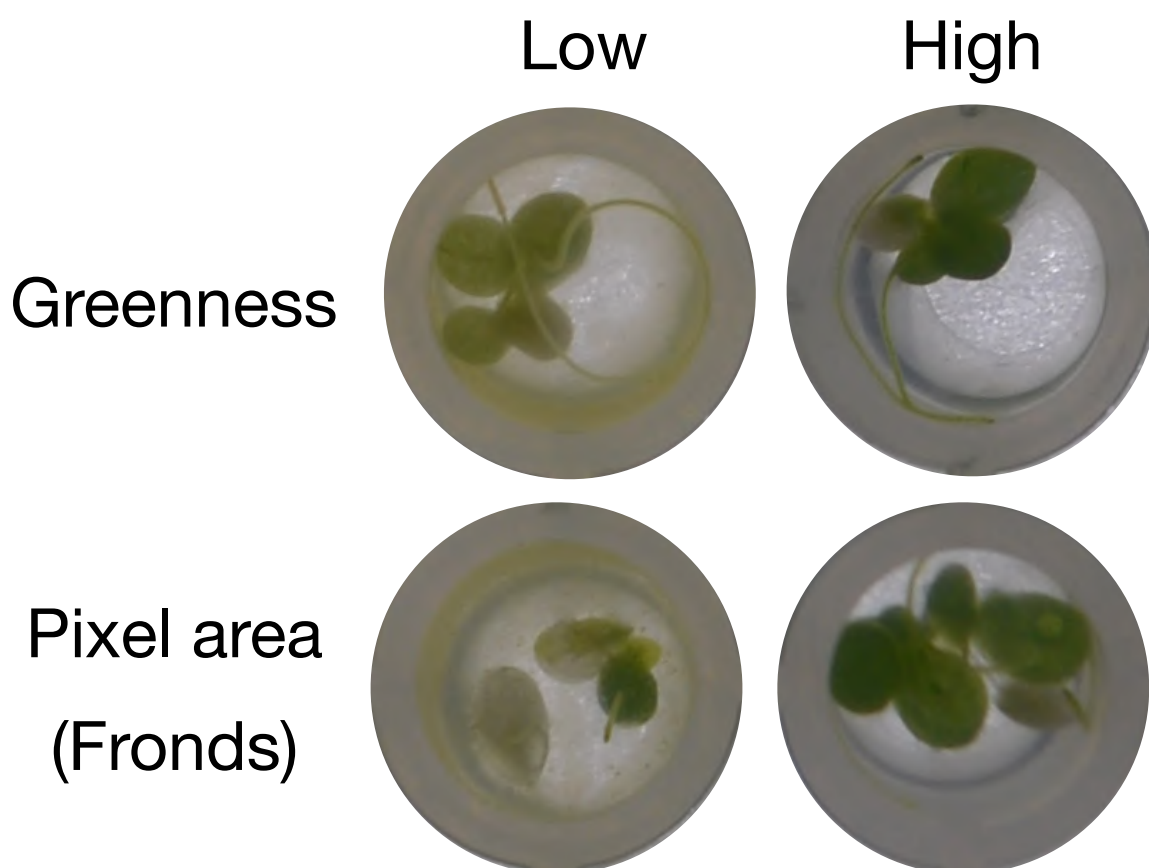


Figure S5: Example images of duckweed from the experiment, for duckweeds scored in ImageJ to have lower and higher greenness and pixel area (related to frond number). These are from the third round, and are of the plate from the tire particle leachate treatment of 10 g/L concentration.