SI Appendix for

Evolutionary consequences of multidriver environmental change in an aquatic primary producer

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Supplementary Methods & Materials

Divers used in multidriver environments

Details of how individual drivers were manipulated and our reasoning behind specific manipulations are below. Driver intensities were kept in line with future climate change scenarios where possible (1–3), however, some adjustments were made so that a) the drivers could be easily manipulated in the lab; b) each driver had an effect on growth rate so that changes in fitness could be quantified; and c) single drivers environments did not cause extinctions during the initial response. With the exception of CO_2/pH (Tris HCl was added to prevent pH of the media fluctuating with changing CO_2), we did not attempt to control chemical interactions between drivers as these interactions may contribute to organismal responses and to subsequent patterns of how response scales with the number of drivers.

Temperature: A conductive heat-mat (Exo Terra heat wave substrate heat mat) was placed under experimental plates to increase the temperature of the culture media to 26^{o}C. This did not affect the control temperature set within the incubator and was controlled using a thermostat (Rootit heat mat thermostat). Our reasoning is that a 1 ^{o}C rise in temperature a) could be produced without affecting the overall temperature of the incubator or causing condensation on the culture vessel lid, b) falls within the range of predicted temperature rises for aquatic ecosystems (2) and c) produces a change in growth rate in *C. reinhardtii* and can thus act as a driver, but does not cause mortality (we wanted to avoid large numbers of extinctions during the experiment).

CO_2: Sterile breathable films (AeraSeal breathable sealing film) were used instead of the of the 96-well plate lids that came with the plates. This allows increased CO_2 diffusion into the media. While we did not quantify the precise level of CO_2 in the media, growth in the high-CO_2 conditions was stimulated, indicating that it was acting as a driver, which is all that was needed for the purpose of this study. CO_2 levels in the test environments were chosen based on projected CO_2 levels, and are in line with other experiments investigating microalgal responses to CO_2 enrichment.

pH: The pH of the culture media was altered by adding 2% HCl. This required one to two drops per litre of HSMT, so the concentration of nutrients was not altered by

changes in volume. The pH was measured with a pH meter (Thermo Orion Star A121 pH Portable Meter) and buffered by adding Tris-HCL. Even though this drop in pH (0.7 units) is large relative to changes expected in marine ecosystems (2) it is well within those experienced in freshwater systems (4). Based on pilot work, this drop reliably affects growth in the *C. reinhardtii* in our laboratory cultures.

UV: A UV lamp (UVM-57) was used in order to provide a dose of UV radiation twice weekly (Table S4). The breathable films were removed from the culture plates under sterile conditions during UV radiation. The lamp was mounted 5.1 cm from the surface of the culture plates providing an irradiative force of 33.75 $W.cm^{-2}$. Populations were irradiated for 4 mins and this corresponds to a UV dose of 8.1 $KJ.m^{-2}$.

Light intensity: Overall light intensity was reduced by approximately 40% using a neutral density light filter (0.15 Neutral Density filter), designed to reduce the light intensity across all wavelengths equally and attenuate light by absorption with minimal reflection. The filter was secured to the top of the experimental plates allowing sufficient room for CO_2 to circulate. Our rationale for decreasing light was pragmatic; it is possible to put a filter on some of the culture vessels, but difficult to selectively increase light levels reliably for only a few populations during an experiment of this size.

Herbicide: Atrazine was used at a concentration of 0.5μM in HSMT. Atrazine was then added to the culture media used for this treatment freshly whenever populations were transferred into fresh media. Based on pilot work, this concentration of atrazine reliably affects growth in the *C. reinhardtii* genotype used.

Nutrients: All nutrients within Hunter's trace elements (HTE) were reduced equally to 25% relative to the control concentration (see Table S2 for concentration of each nutrient within HTE). Since laboratory strains are used to growing in rich media such as HSMT, increasing trace nutrients has no measurable effect on growth. The reduction in nutrients needed to act as a driver in this experiment was determined empirically during pilot studies.

Phosphate: Phosphate was reduced to 1.69 mM, a concentration factor of 0.125 relative to the control concentration (13.56mM) (4). Salts lost by the removal of dipotassium phosphate (K_2HPO_4) and monopotassium phosphate (KH_2PO_4) were replaced with potassium chloride (KCl). The level of phosphate needed to act as a driver was based on pilot work and previous studies by (5).

Batch culture transfers

All populations were grown in 96-well plates and propagated by batch culture (50 µL of growing cells transferred every 3-4 days into 200 µL of fresh media), for 95 transfers (~450 asexual generations). Sterile breathable films (AeraSeal) were used to allow equal air diffusion across plates. Some populations went extinct during the selection experiment; all populations went extinct in the single driver herbicide and the eight driver environments. After the extinction of all populations in the eight driver environment at transfer four, populations were rescued by increasing the

transfer volume by 100%. Populations from the 8-driver environment were excluded from statistical analyses but are included in figures for comparison.

Acclimation periods

So long as the genetic composition of the population does not have time to change during the acclimation period, evolution will not contribute substantially to the measure of acclimation. Initially the starting populations are essentially lacking any genetic variation, a dozen or so generations is not enough time for a mutation to rise in frequency to a point where it affects average population trait values, if the mutation starts from an initial frequency of 1/(population size) in a population with ~10^5 individuals. Evolved populations may be more variable, though genetic variation in adapting populations under strong selection (as is the case here), should be very low. In practice, we cannot completely exclude the possibility that changes in genotype frequencies had a small effect on our acclimation measurements in the evolved populations, or that some small amount of backselection occurred when evolved populations were transferred back to the control selection environment. In both cases, this would result in our slightly underestimating the magnitude of evolutionary responses.

Growth rate and response to selection measurement details

For calculations of the rate of cell division (d^{-1}), the initial drop in growth rate and the direct response to selection, populations were acclimated to the assay environment and then transferred to fresh medium at equal cell density (~ 41,000 cells/ml). Cell counts were performed after 0 and 72 hours of growth using a BD FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer calibrated with CS&T beads. The data were acquired with the BD FACSDiva v6 software. Due to the size of the assays, cell counts were performed in batches which are included in the statistical analysis.

For calculations of the direct response to selection, the initial drop in population growth rate was measured by comparing the growth rate of the evolved control populations in each regime with the growth rate the control population in the control environment. The initial drop in growth rate is analogous to the plastic response, and is good indicator of the strength of selection. Using the evolved control populations (Figure 1) accounts for the effects of adaptation to general culturing and laboratory conditions.

Flow cytometric analysis of physiological parameters

A FACS CANTO, calibrated with CS&T beads, was used to measure trait values. Relative chlorophyll autofluorescence intensity was detected in the PerCP-Cy5.5 channel (Ex-Max 488 nm/Em-Max long pass (LP) 670 – 725 nm). Samples were run from 96 well plates, at flow rates of 1µl/second. Contaminants do not affect cell counts (see supplementary methods for details of fungal contamination). In addition, outside the range of normal cell size (6, 7) we classified as dead and excluded from analyses. Forward scatter (FSC) is correlated with cell size (6, 8, 9). FSC was calibrated with size calibration beads (Bang Laboratories, Inc.; (10) (Fig. S8). Chlorophyll autofluorescence per cell volume ($1/\mu m^3$) was calculated assuming spherical cells (11).

Fungal contamination

Populations were initially sterile but became contaminated with a fungus by transfer number 95. Since the culture media had no carbon source, fungal growth was limited and we found no significant effect of contamination on the number of divisions per day between populations with and without a fungal contaminant (Fig. S7A; t = -1.703, df = 1.432, P = 0.277). In addition, there is no difference between the final cell density of populations with and without a fungal contaminant (Fig. S7B; t = 2.4995, df = 1.891, P = 0.137) (fungal contaminant = $3.5x10^6$ cells per ml ± $4.0x10^5$ cells per ml; Mean ± SD and no contaminant = $4.4x10^6$ cells per ml ± $3.1x10^5$ cells per ml; Mean ± SD).

Fungal spores were identified using a light microscope at 40x magnification and the number of cells per ml of *C. reinhardtii* cultures were determined by counting cells using a haemocytometer (Fig. S7). To quantify the effect of fungal contaminants on the growth rate of the *C. reinhardtii* populations, our measure of population fitness, we measured the number of cell divisions per day (equation 2). A two sample t-test (R base package) was used to detect any differences in the number of divisions per day and final cells densities between populations growing with and without fungal spores in the culture media. In addition, growth curves showing the average number of cells per ml of two populations with and two populations without fungal contamination, over 72 h, are shown in Fig. S7B.

Mixed model analysis of direct response to selection

The effect of the identity of the environmental drivers, the plastic response and number of drivers on the direct response to selection was analysed using a mixed model in R (12), using the packages lme4 and lmerTest. The plastic response, number of drivers and the identity of the environmental drivers are fixed effects and batch and identity of evolved populations are random effects in the mixed model. However, the model cannot run when all eight environmental drivers and number of drivers are used to explain variation in the direct response, and this is because the model is overparametrised. We found that no additional variation in the direct response was explained by the inclusion of number of drivers and the environmental driver UV, and the majority of the variation in the direct response was explained by the plastic response (Table S4). For this reason, number of drivers was removed from the model and we have included all eight environmental drivers so that we can measure how much of the variation in the direct response is explained by each environmental driver and the strength of selection (Fig. 2) (see methods and materials in the main text).

Mixed model analysis of growth rates of C. reinhardtii under multidriver environments

The effect of the number of drivers and the identity of the drivers on absolute growth rate after evolution was analysed using a mixed model. Number of drivers (0-7) and the identity of the environmental drivers are fixed factors, however this model is overparameterised when all eight environmental drivers are used, and since UV explains none of the variation in the growth rate, UV was removed from the mixed

model. The identity of each regime, batch number and evolved populations within each regime were taken as random factors the mixed model analysis.

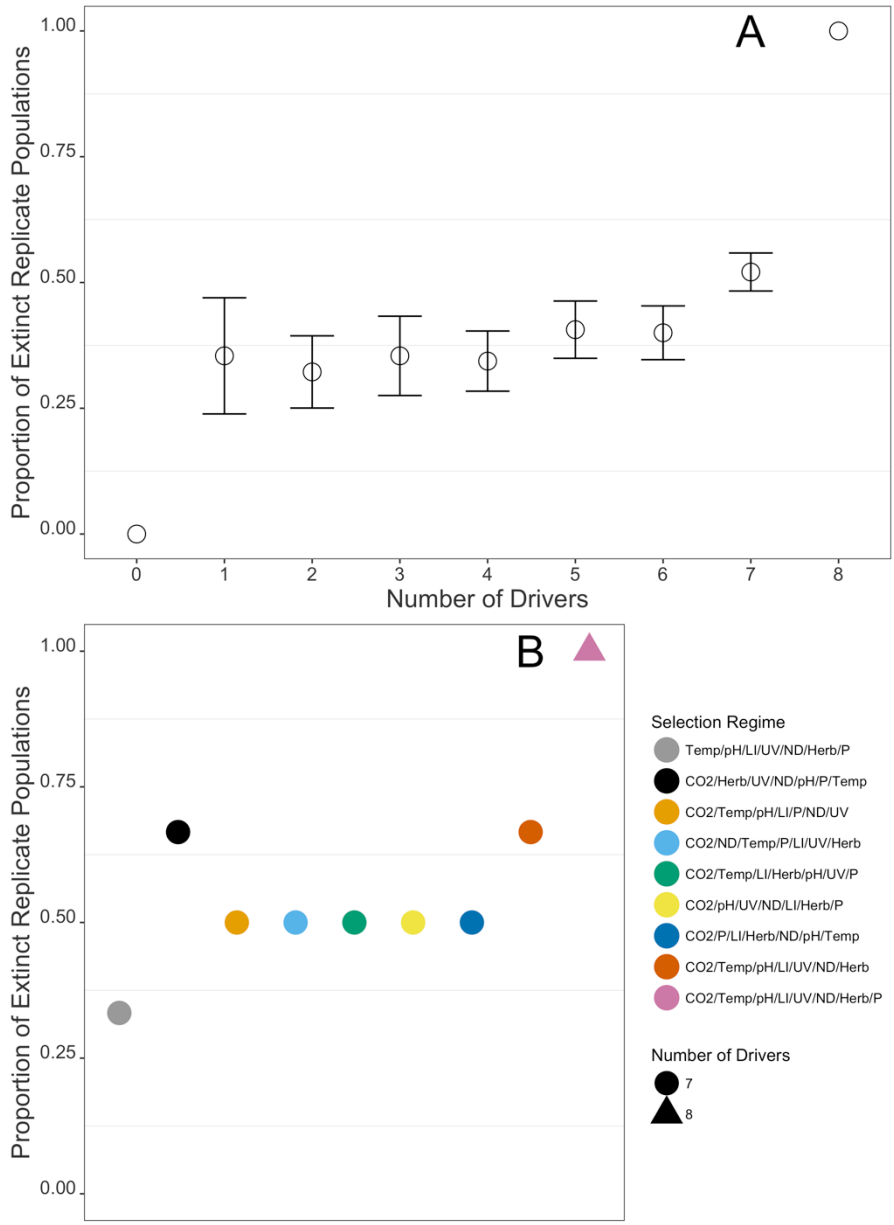

Fig. S1. The proportion of populations of *C. reinhardtii* that went extinct under increasing number of drivers. (A) Six replicate populations were evolved in one of 96 regimes (see Table S1). Open circles show the average (±SE) proportion of extinct populations over all regimes for each driver number category. As the number of drivers increases to seven and eight, the proportion of extinct populations within each regime increases significantly ($F_{1,94} = 5.91$, $P = 0.017$). (B) All populations within evolved with eight drivers went extinct at transfer number four, approximately 12 generations. The majority of populations evolved under regimes with seven drivers went extinct between transfer number 24 and transfer number 46 (approximately 50 – 100 generations), however two populations, one in regime CO2/P/LI/Herb/ND/pH/Temp and a second in regime CO2/Herb/UV/ND/pH/P/Temp, went extinct at transfer number 67 (approximately 150 generations).

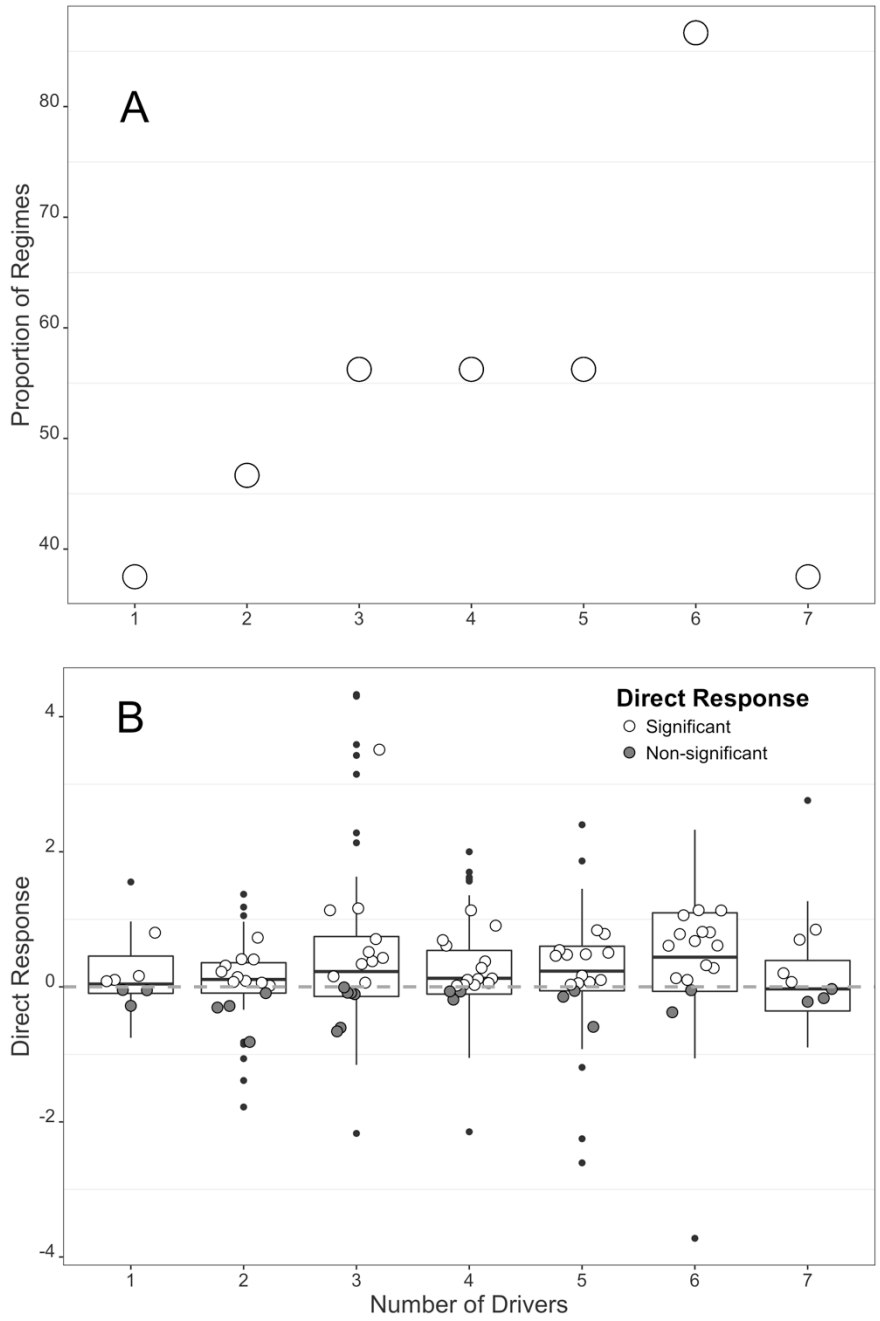

Fig. S2. **The proportion significant positive direct responses to selection in multidriver environments.** (A) As the number of drivers increases the proportion of regimes where the multidriver-evolved populations have a significant direct response to selection initially increases with the number of drivers when few drivers are present (1-3 drivers). There is no effect of the number of drivers for intermediate numbers of drivers (3-5 drivers). The proportion of environments with direct responses to selection is highest in the 6 driver environment, and then falls off sharply in the 7 driver environments. (B) The direct response of *C. reinhardtii* populations within each regime under increasing number of drivers (see Fig. 3); open circles indicate regimes that have a significant direct response to selection which is greater than the median direct response of regimes with a single driver. Filled circles indicate regimes that fall below the median direct response of regimes with a single driver. Note all populations growth in regimes with eight drivers went extinct.

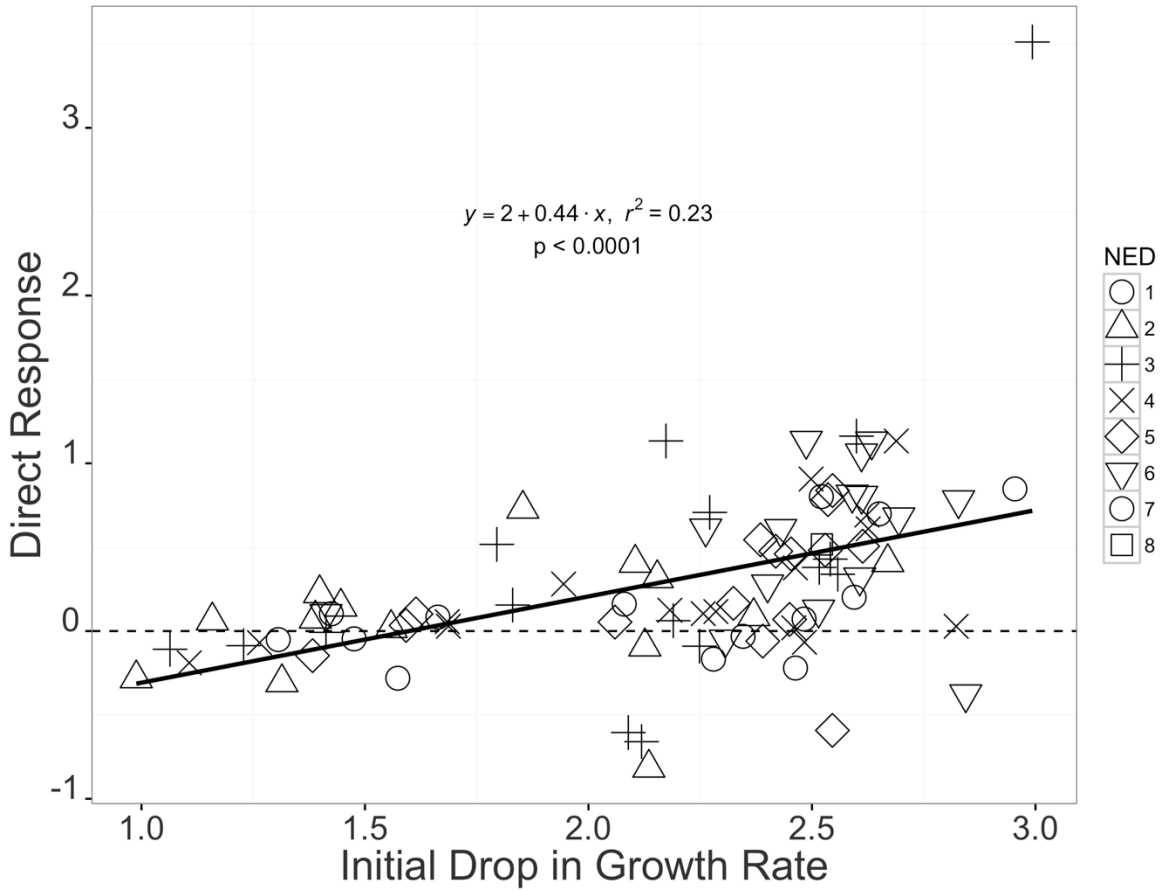

Fig. S3. There is a positive relationship between the initial drop in growth rate and the direct response to selection under increasing number of drivers for populations that persist. The initial drop in growth rate is measured as the difference between the average rate of cell division between the evolved control in the control environment and the average rate of cell division of the evolved control in the multidriver environments. Data points show the average response of populations within each regime and the number of drivers (1 to 8) is indicated by the shape of the data points. Solid line shows the results of the linear regression, and the dashed line indicates that there is no difference between the growth rate of the evolved control and the multidriver-evolved populations, in the same selection environment.

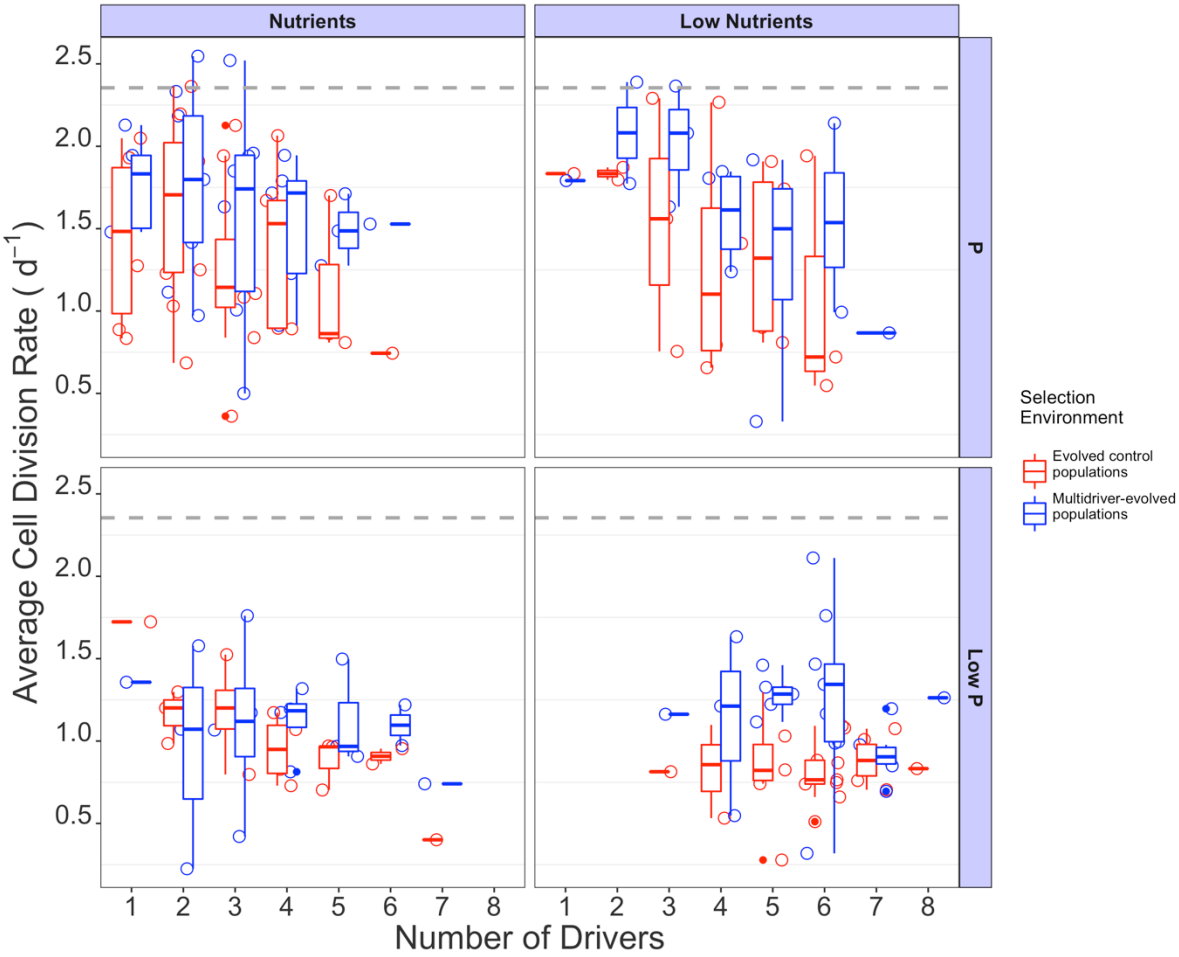

Fig. S4. Nutritional availability determines the average rate of cell division (d^{-1}) of multidriver-evolved populations. Each panel label indicates the nutritional quality of the environment; top left, general nutrient replete and phosphate (P) replete; top right, low nutrients and P replete; bottom left, general nutrients replete and low P; bottom left, low nutrients and low P. Dashed line indicates the growth rate of the evolved control population in the control environment.

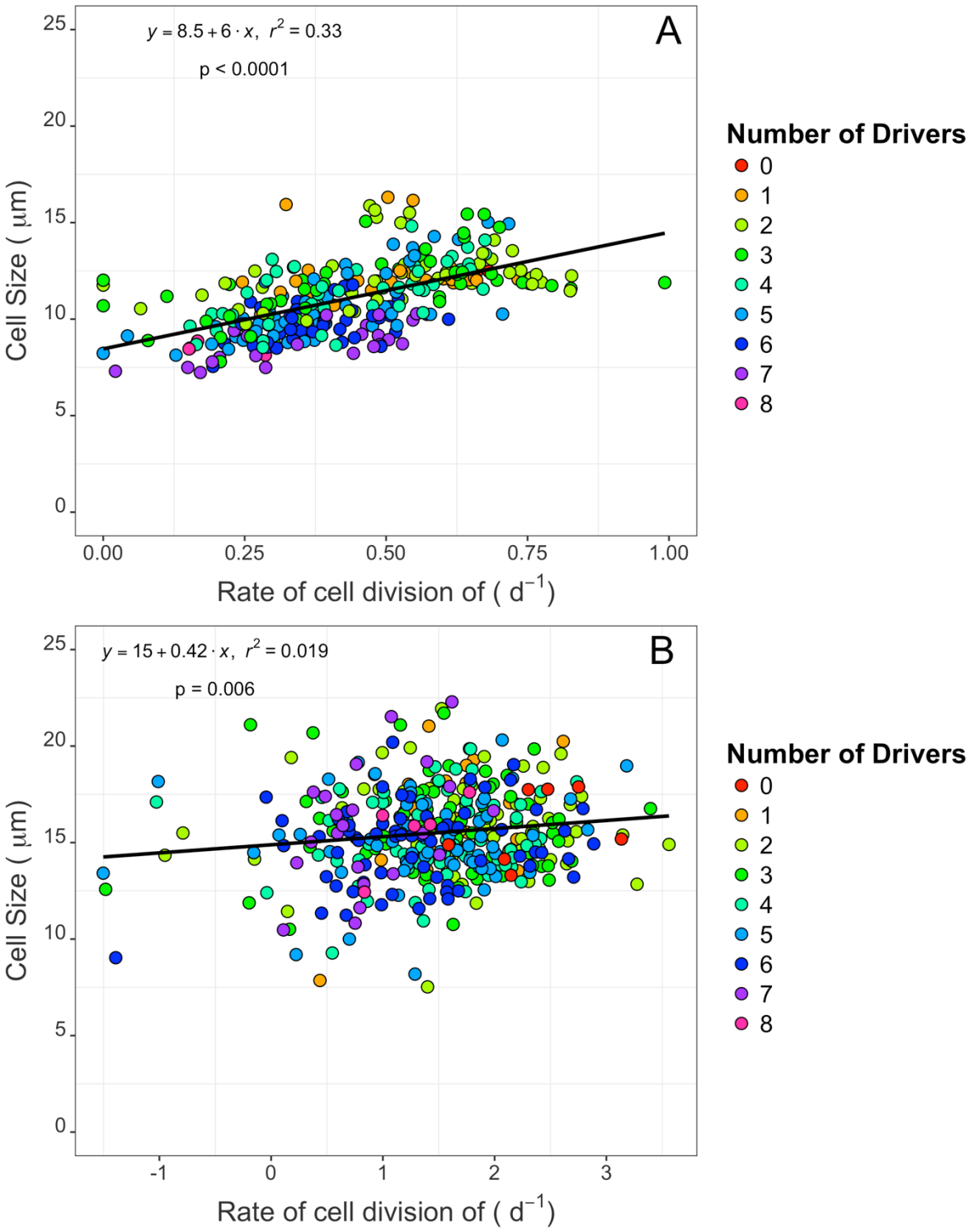

Fig. S5 Correlation between cell size and the rate of cell division (d^{-1}) before and after evolution. Filled circles show the rate of cell division (d^{-1}) and cell size (µm) of replicate populations within each regime (A) before and (B) after evolution. The number of drivers of each regime is indicated by the colour of filled circles. Solid line shows the results of the linear regression.

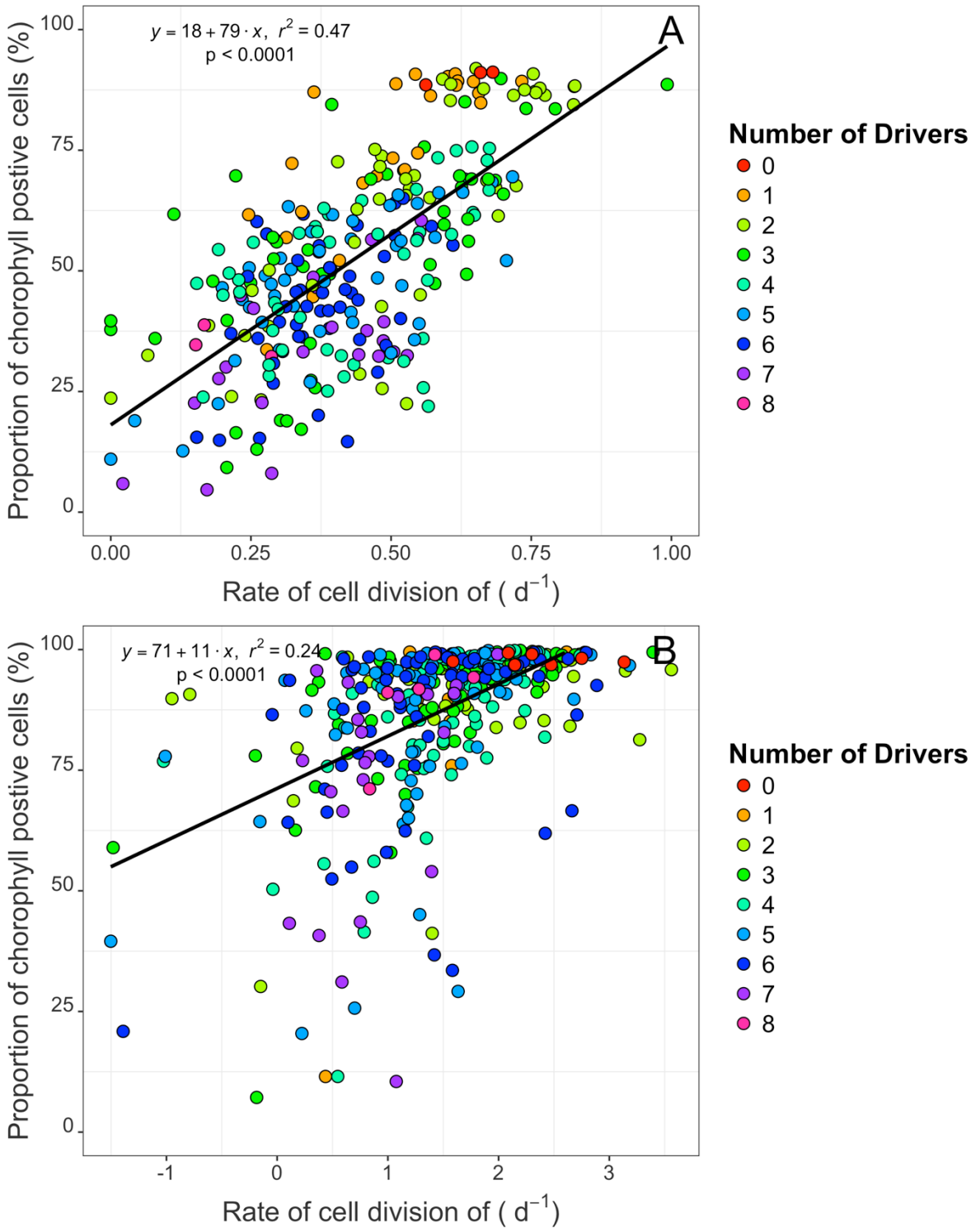

Fig. S6. Correlation between proportion of chlorophyll positive cells and the rate of cell division (d^{-1}) before and after evolution. Filled circles show the rate of cell division (d^{-1}) and cell size (μm) of replicate populations within each regime (A) before and (B) after evolution. The number of drivers of each regime is indicated by the colour of filled circles. Solid line shows the results of the linear regression.

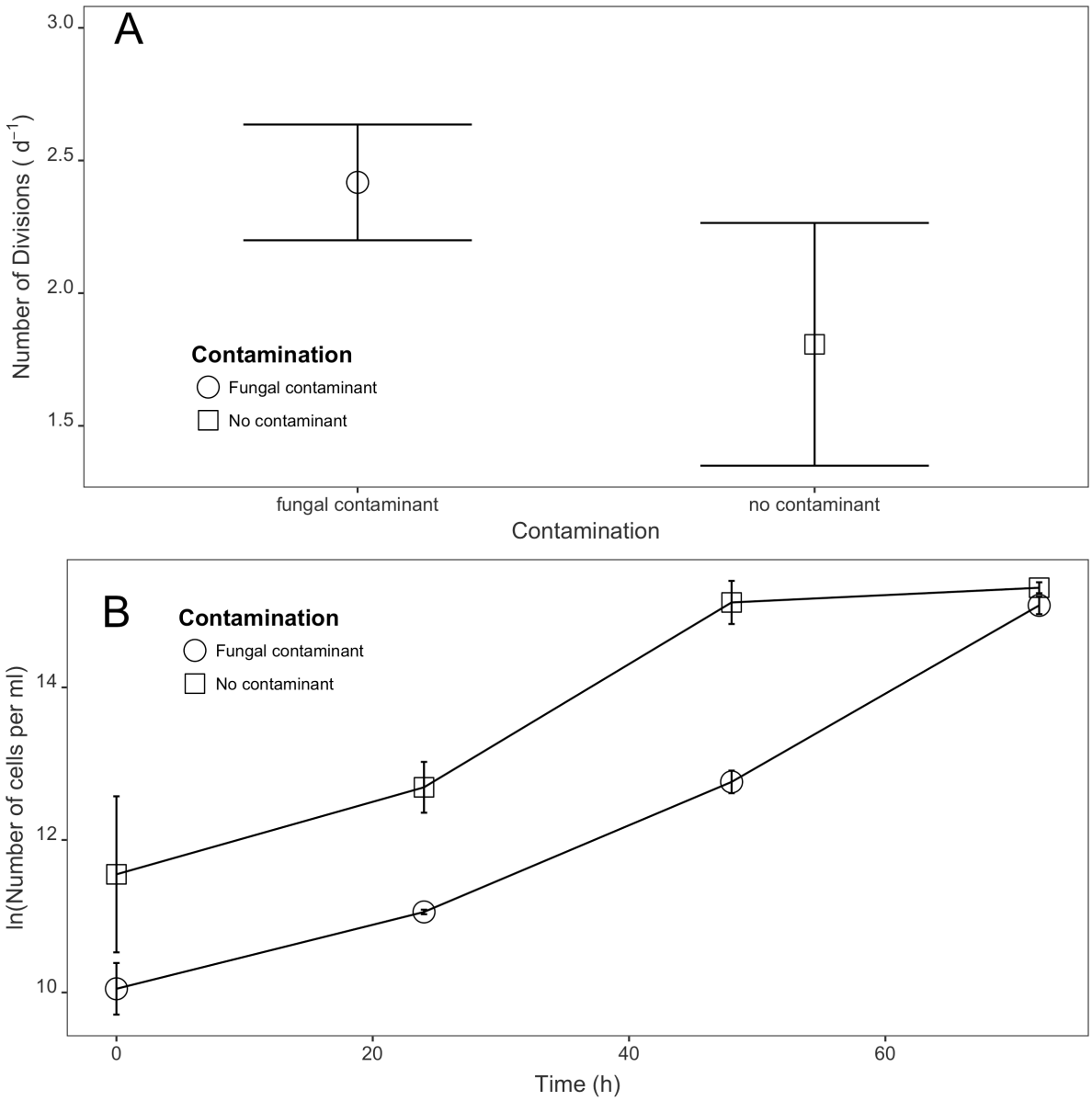

Fig. S7. Fungal contamination has no effect on the growth rate of *C. reinhardtii* populations. (A) Data points show the average number of divisions (d^{-1}) of *C. reinhardtii* (±SD), with and without a fungal contaminant. (B) Growth curves show the average number of cells per ml (±SD) of *C. reinhardtii*, measured every 24 h between 0 h to 72 h. Open circles show the average number of cells per ml (±SD) of *C. reinhardtii* growing with a fungal contaminant and open squares show the average number of cells per ml (±SD) of *C. reinhardtii* growing without a fungal contaminant.

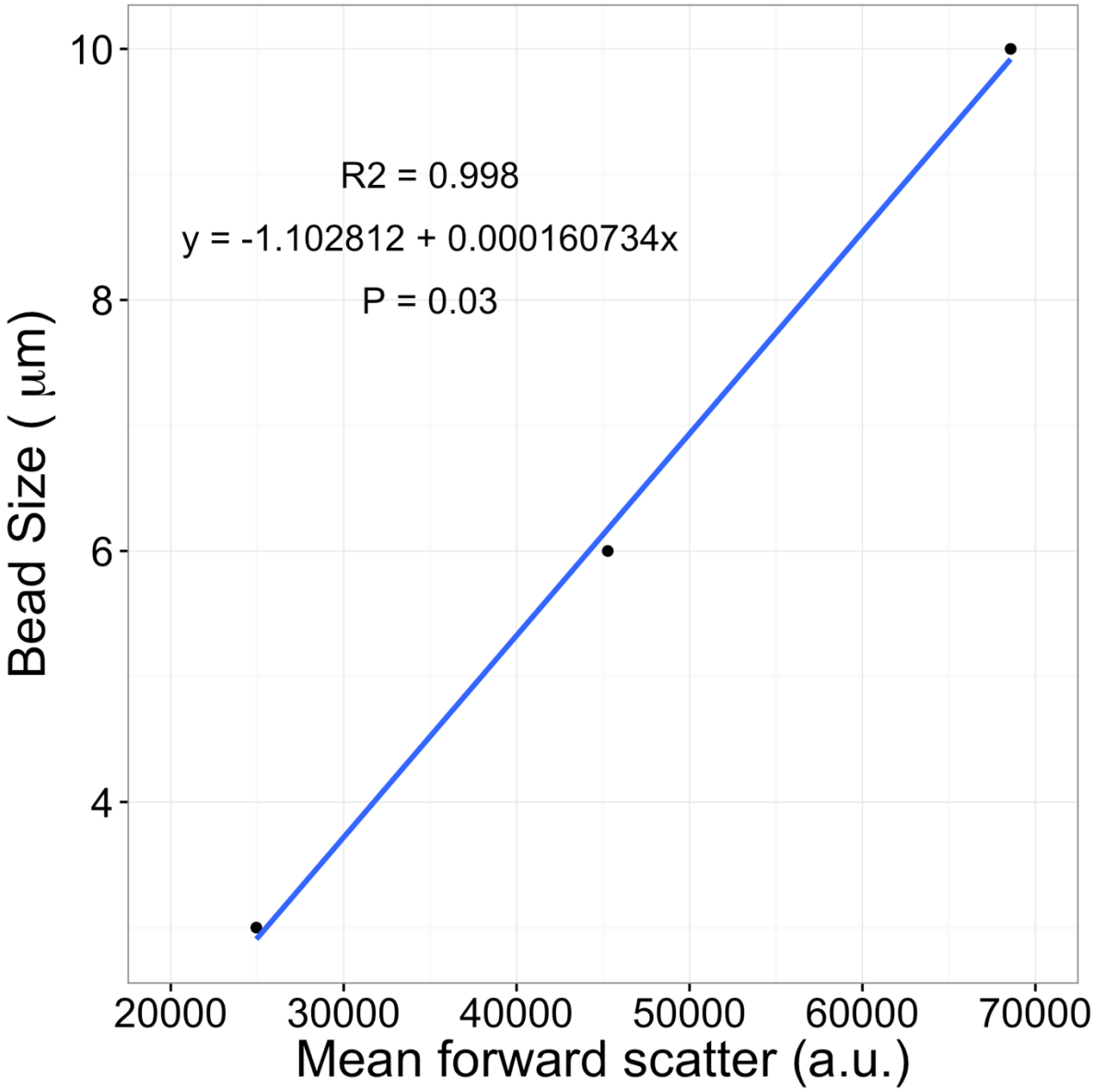

Fig. S8. Calibration of mean forward satter (a.u.) to cell size (µm). Standard curve of mean forward scatter (a.u.) of calibration beads and size (µm) of calibration beads, measured using a FACS Canto. Solid line shows the results of the linear regression.

Table S1 Environmental drivers and their combinations in each unique regime environment.

Number of Environmental Drivers	**Regimes**								**No. of Regimes**
0	Control								**1**
1	CO_2	Temp	LI	pH	P	Herb	ND	UV	**8**
2	CO_2/Temp	CO_2/ LI	CO_2/ pH	CO_2/ P	CO_2/ Herb	CO_2/ ND	CO_2/ UV		**15**
	Temp/ pH	Temp/ LI	pH/ UV	P/ LI	Herb/ UV	UV/ ND	UV/ LI	pH/ P	
3	CO_2/ Temp/ pH	CO_2/ Temp/ LI	CO_2/ pH/ UV	CO2/ P/ LI	CO_2/ Herb/ UV	CO_2/ UV/ ND	CO_2/ UV/ LI	CO_2/ pH/ P	**16**
	Temp/ pH/ LI	Temp/ LI/ Herb	pH/ UV/ ND	P/ LI/ Herb	Herb/ UV/ ND	UV/ ND/ P	UV/ LI/ pH	pH/ P/ UV	
4	CO_2/ Temp/ pH/ LI	CO_2/ Temp/ LI/ Herb	CO_2/ pH/ UV/ ND	CO_2/ LI/ Herb	CO_2/ Herb/ UV/ ND	CO_2/ UV/ ND/ P	CO_2/ UV/ LI/ pH	CO_2/ pH/ P/ UV	**16**
	Temp/ pH/ LI/ UV	Temp/ LI/ Herb/ pH	pH/ UV/ ND/ LI	P/ LI/ Herb/ ND	Herb/ UV/ ND/ pH	UV/ ND/ P/ LI	UV/ LI/ pH/ P	pH/ P/ UV/ Herb	
5	CO_2/ Temp/ pH/ LI/ UV	CO_2/ Temp/ LI/ Herb/ pH	CO_2/ pH/ UV/ ND/ LI	CO_2/ P/ LI/ Herb/ ND	CO_2/ Herb/ UV/ ND/ pH	CO_2/ UV/ ND/ P/ LI	CO_2/ UV/ LI/ pH/ P	CO_2/ pH/ P/ UV/ Herb	**16**
	Temp/ pH/ LI/ UV/ ND	Temp/ LI/ Herb/ pH/ UV	pH/ UV/ ND/ LI/ Herb	P/ LI/ Herb/ ND/ pH	Herb/ UV/ ND/ pH/ P	UV/ ND/ P/ LI/ Herb	UV/ LI/ pH/ P/ ND	pH/ P/ UV/ Herb/ LI	
6	CO_2/ Temp/ pH/ LI/ UV/ ND	CO_2/ Temp/ LI/ Herb/ pH/ UV	CO_2/ pH/ UV/ ND/ LI/ Herb	CO_2/ P/ LI/ Herb/ ND/ pH	CO_2/ Herb/ UV/ ND/ pH/ P	CO_2/ UV/ ND/ P/ LI/ Herb	CO_2/ UV/ LI/ pH/ P/ ND	CO_2/ pH/ P/ UV/ Herb/ LI	**15**
	Temp/ pH/ LI/ UV/ ND/ Herb	Temp/ LI/ Herb/ pH/ UV/ P	pH/ UV/ ND/ LI/ Herb/ P	P/ LI/ Herb/ ND/ pH/ Temp	Herb/ UV/ ND/ pH/ P/ Temp	ND/ Temp/ P/ LI/ UV/ Herb	Temp/ pH/ LI/ P/ ND/ UV		
7	CO_2/ Temp/ pH/ LI/ UV/ ND/ Herb	CO_2/ Temp/ LI/ Herb/ pH/ UV/ P	CO_2/ pH/ UV/ ND/ LI/ Herb/ P	CO_2/ P/ LI/ Herb/ ND/ pH/ Temp	CO_2/ Herb/ UV/ ND/ pH/ P/ Temp	CO_2/ ND/ Temp/ P/ LI/ UV/ Herb	CO_2/ Temp/ pH/ LI/ P/ ND/ UV	Temp/ pH/ LI/ UV/ ND/ Herb/ P	**8**
8	CO_2/ Temp/ pH/ LI/ UV/ ND/ Herb/ P								**1**

Eight single environmental drivers were used in combinations of 1-8 drivers. Drivers were: CO_2, CO_2 enrichment; Temp, elevated temperature; LI, reduced light intensity; pH, reduced pH; P, phosphate starvation; Herb, herbicide; ND, general nutrient depletion; UV, UV radiation.

Table S2 A comparison of the control environment and the environmental drivers used in the test environments.

Environmental drivers	Control	Treatment	pH of Culture Media
CO_2 (ppm)	420	2000	7.2
pH	7.2	6.5	6.5
Temperature (^oC)	25	26	7.2
Phosphorus (mM)	13.56	1.69	7.2
Nutrients (%)*	100	25	7.2
Herbicide (µM)**	0	0.5	7.2
UVB dose ($KJ.m^{-2}$)	0	8.1	7.2
Light intensity ($\mu mol\ m^{-2}\ s^{-1}$)	32	18	7.2

* see Table S2 for concentration of all nutrients.

** Atrazine was stored as stock solutions of 10mM in ethanol. Further dilutions were made in HSM media to achieve a working solution of 50µM.

Table S3 A comparison of the concentration of Hutner's Trace Elements in 1 liter of HSMT culture media in control and treatment (nutrient depletion) environments.

Hutner's Trace Elements	Control (mM)	Treatment (mM)
$Na_2EDTA \cdot 2H_2O$	0.134	0.034
$ZnSO_4 \cdot 7H_2O$	0.077	0.019
H_3BO_3	0.184	0.046
$MnCl_2 \cdot 4H_2O$	0.026	0.006
$FeSO_4 \cdot 7H_2O$	0.013	0.003
$CoCl_2 \cdot 6H_2O$	0.007	0.002
$CuSO_4 \cdot 5H_2O$	0.006	0.002
$(NH_4)6Mo_7O_{24} \cdot 4H_2O$	0.890	0.222

Table S4 Specifications of UVB lamp.

Model UVM-57 Lamp specification: 15W.cm^{-2} @ 7.32cm	Treatment
Irradiative force (W.cm^{-2})	33.75
Distance from plate (cm)	5.08
Time (min)	4
UVB dose (KJ.m^{-2})	8.1

Table S5 percentage variation that each part of the mixed model explains on the **direct response** to selection. Including, all environmental drivers apart from UV and number of drivers.

Source	Groups	% variation
Number of drivers	fixed	0.29
Strength of selection	fixed	25.81
P	fixed	6.24
Herb	fixed	3.23
Temp	fixed	0.26
CO_2	fixed	1.95
pH	fixed	0.01
LI	fixed	0.33
ND	fixed	0.43
Evolved population	random	38.99
Batch	random	7.62
Error within evolved populations		14.85

Seven single environmental drivers were used in combinations of 1-8 drivers; CO_2, CO_2 enrichment; Temp, elevated temperature; LI, reduced light intensity; pH, reduced pH; P, phosphate starvation; Herb, herbicide; ND, general nutrient depletion.

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