Acceptable Loss: Fitness Consequences of Salinity-Induced Cell Death in a Halotolerant Microalga

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Running title: Fitness of cell death

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Abstract:

Environmentally induced reductions in fitness components (survival, fecundity) are generally considered as passive, maladaptive responses to stress. However, there is also mounting evidence for active, programmed forms of environmentally induced cell death in unicellular organisms. While conceptual work has questioned how such programmed cell death (PCD) might be maintained by natural selection, few experimental studies have investigated how PCD influences genetic differences in longer-term fitness across environments. Here, we tracked the population dynamics of two closely related strains of the halotolerant microalga *Dunaliella salina*, following transfers across salinities.

We showed that after a salinity rise, only one of these strains displayed a massive population decline (-69% in one hour), largely attenuated by exposure to a PCD inhibitor. However, this decline was followed by a rapid demographic rebound, characterized by faster growth than the non-declining strain, such that sharper decline was correlated with faster subsequent growth across experiments and conditions. Strikingly, the decline was more pronounced in conditions more favourable to growth (more light, more nutrients, less competition), further suggesting that it was not simply passive. We explored several hypotheses that could explain this decline-rebound pattern, which suggests that successive stresses could select for higher environmentally induced death in this system.

Article type: major article.

Introduction

Living organisms are pervasively exposed to stressful environments that may reduce their demographic vital rates (survival, fecundity), in turn decreasing their population size, and making them vulnerable to extinction. A widespread mechanism by which organisms can cope with such environmental stresses is phenotypic plasticity, whereby a given genotype produces different phenotypes in response to the environment (Levins 1963; Bradshaw 1965). When the expressed phenotypes increase fitness across environments, then plasticity is said to be adaptive, while plasticity that reduces fitness across environments is described as maladaptive (Ghalambor et al. 2007). Adaptive plasticity and its evolution can reduce extinction risk in fluctuating environments that are sufficiently predictable (Reed et al. 2010; Ashander et al. 2016), and may also allow tolerance of higher rates of directional environmental change (Chevin et al. 2010).

However beyond these simple definitions, demonstrating whether or not a plastic response is adaptive, and why, may be challenging in practice. This is especially true for traits whose most immediate effect is to decrease a component of fitness. For instance, increased cell death rate in response to environmental stress would seem like the archetypical maladaptive, passive trait, which the organism can neither prevent nor control. Yet many unicellular organisms can undergo forms of cell death that are genetically controlled, and involve complex cellular cascades - from caspase-like activity to nuclear DNA degradation (Jiménez et al. 2009; Kroemer et al. 2009; Bidle 2015) - that share features with programmed cell death (hereafter PCD) in multicellular organisms (Ameisen 2002; Bidle and Falkowski 2004; Deponte 2008; Kasuba et al. 2015). Because such PCD appears as an active process, it is generally considered as adaptive, in effect representing a form of adaptive plasticity triggered by a variety of environmental stimuli. However, how and why PCD may be favored by natural selection in unicellular organisms (Franklin et al. 2006; Nedelcu et al. 2011; Durand and Ramsey 2019; Vostinar et al. 2019), where it amounts to cell suicide, remains difficult to understand in most cases, except for a few well-studied examples (Durand et al. 2011; Berngruber et

al. 2013). In general, the main argument for considering PCD as adaptive in unicells is that it is an active process, but this is not satisfying because (i) the line between PCD and less active forms of deaths is somewhat blurred, with a range of intermediate situations that are difficult to classify (Kroemer et al. 2009; Barreto Filho et al. 2021, 2022); and (ii) even plastic response that are not evidently active or programmed could still have been favoured by natural selection. Therefore, understanding the causes of selection on PCD – or any form of environmentally induced rapid death, regardless of where it stands along the PCD continuum - requires demonstrating that this trait varies genetically, and that it is associated with differential fitness (as highlighted by e.g. Reece et al. 2011). Even though PCD is unlikely to be favoured directly, since it causes an immediate demographic decline of the corresponding genotype*,* it may still be selected indirectly if it is associated with higher fitness in the long run, as any other trait that is selected through its covariance with fitness (Price 1970; Durand and Ramsey 2019). Therefore, understanding selection on PCD requires establishing its covariance with longer-term fitness across genotypes, as well as experimentally testing specific hypotheses for how its fitness costs and benefits arise.

Here, we undertake such an approach with the unicellular microalga *Dunaliella salina.* This halotolerant microalga is the main primary producer of hypersaline biomes such as continental saline lakes, coastal lagoons, and industrial salt ponds, where salinity fluctuates along the year (Ben-Amotz et al. 2009). *Dunaliella salina* can tolerate a broad range of salinities, from below seawater to saturated brine ([NaCl] ~6.2M), through a number of well-characterized, phenotypically plastic mechanisms (Oren 2005; Ben-Amotz et al. 2009; Chen and Jiang 2009). These include rapid morphological flexibility in response to sudden osmotic shock - allowed by absence of a cell wall -, followed by slower physiological adjustments, most notably the production of glycerol as osmoprotectant (Zidan et al. 1987). Overall, phenotypic plasticity is thus a key adaptive feature in this species, and was also recently shown to readily evolve in the laboratory (Leung et al. 2020).

Species of the genus *Dunaliella* can undergo PCD under various environmental triggers, such as darkness (Berges and Falkowski 1998; Segovia et al. 2003; Orellana et al. 2013), UV radiation

(Jiménez et al. 2009), or hyper-osmotic shock (Jiménez et al. 2009), the latter being directly relevant for adaptation to salinity. But here again, the potential benefits of PCD remains unclear. A study in *Dunaliella salina* has suggested an advantage of PCD via resource sharing, since dying cells can release large amounts of intracellular glycerol they produced for osmoregulation, which may then be remineralized by prokaryotes, or directly consumed via a heterotrophic pathway (Orellana et al. 2013). However, this study did not compare genotypes that differ in their rate of PCD, to investigate how these differences covary with fitness in the longer run. Recently, Leung et al. (2022) have identified two closely related strains of *Dunaliella salina* that vary in their rate of salinity-induced death. Interestingly, it has also been shown that strain-specific demographic response to salinity, including a rapid decline followed by rebound under hyperosmotic stress for one strain, is also related to gene expression response involved in chloroplast functions (Leung et al. 2022). This lends support to the hypothesis of chloroplast-mediated programmed cell death, as described in plants and different unicellular organisms (Zuppini et al. 2009; Thamatrakoln et al. 2013; Murik et al. 2014; Ambastha et al. 2015; Bidle 2016). We here use these strains to explore hypotheses about what drives the intensity of cell death, and more crucially, how this decline relates to later population growth. One of our most puzzling finding is that more cell death is associated to faster subsequent population growth across treatments.

Material & Methods

Strains

We focused on two closely related strains of *Dunaliella salina*, CCAP 19/12 (hereafter strain A) and CCAP 19/15 (strain C) (Emami et al. 2015), which were previously found to vary in their death rates in response to salinity (Leung et al. 2022). We received these strains in 2017 from the Culture Collection of Algae and Protozoa (UK) and maintained them at constant salinity ([NaCl] = 2.4M) for over 3 years prior to this experiment, transplanting them into fresh media twice a week for 5 months (as described in Rescan et al. 2020, constant treatment), and then about once per month. In all

experiments described below, we used 10 (nearly) isogenic lines (five for each strain), founded from a single haploid cell isolated using cells-sorting flow cytometry (BD FACSAria™ III ; Biosciences-US) in May 2019.

Common experimental design

Culture conditions

All lines were cultured in 50 ml flasks (CELLSTAR®; VWR 392-0016) with custom-made artificial saline water (Table S1) with modified NaCl concentration (hereafter [NaCl]), and enriched with 2% of Guillard's F/2 nutritive medium (Sigma, G0154-500 ML). The target culture salinity was obtained by mixing the required volumes of hyposaline ([NaCl] = 0M) and hypersaline ([NaCl] = 4.8M) solutions, taking into account the dilution of the inoculum for a final volume of 25 ml. Cultures were maintained in a growth chamber under 12h:12h light:dark cycles, with light intensity 100 $\mu mol.m^{-2}.s^{-1}$ (unless otherwise stated, Table 1), temperature 24°C, and position randomized with respect to treatment.

All assays followed the same general experimental design: we acclimated the lines at intermediate salinity ([NaCl] = 2.4M) for several days (between 4 to 13 days, see Table 1). Then we induced either hyper-osmotic shock or iso-osmotic transfer, by inoculating a volume of the acclimated cultures into fresh medium at the required expected initial density, and then followed demographic dynamics over 10 days. The initial densities varied between 5,000 and 50,000 cells/mL across experiments for practical reasons: we needed the minimal density (after putative population decline) to be large enough to be detectable with our flow cytometer, but still low enough for substantial exponential growth to occur following decline. More details on each experiment appear in Table 1 and the corresponding sections below.

Population density measures

Population density was assayed using a Guava® EasyCyte™ HT cytometer (Luminex Corporation, Texas, USA), with a laser emitting at 488 nm. We checked the cytometer performance before each

measure with the Guava® EasyCheck™ kit. The acquisition settings were 30 seconds, or until the number of counted events reached 200,000 (never reached). Band pass filters based on forward scatter (FSC), side scatter (SSC), and fluorescence emissions in natural red (695/50nm) and yellow (583/26nm), enabled the discrimination of live *Dunaliella* cells from other particles, owing to chlorophyll α natural fluorescence (Rescan et al. 2020). Note that some dead (or dying) cells had reduced red fluorescence relative to live cells, and were thus not taken into account for population density (Rescan et al. 2020), but these apparently dead cells do not account for the large population decline we focus on here (Fig S1). We also isolated single cells from doublets (i.e. when 2 cells were detected as a single event) based on the area-to-height ratio of the electronic pulse induced in the flow cytometer (Wersto et al. 2001). To determine population density, we sampled 200 µL of each population to count the number of live cells, at several time points. First, at the end of the acclimation step for the calculation of initial population density before each experiment; then, 1h after the osmotic stress (day = 0); and finally, once per day from day 1 to 10.

When the cytometer was unavailable (nutrient limitation experiment below, days 0-1), we measured whole-well fluorescence (excitation at 390/80 nm -wavelength/bandwidth-, and emission at 685/40 nm) of cultures in 96 well plates, using a BMG ClarioStar spectrometer. To convert from these fluorescence measurements into population density, we fitted a linear model (LM) between cytometer count and fluorescence for days 2 to 4 of the nutrient limitation experiment (see Fig. S2), for which both measurements were available and the population had not yet reached stationarity (Fig. S6).

Experiments and treatments

We have performed a series of experiments to test different hypotheses about the drivers and population consequences of rapid cell death, as summarized in Table 1.

Experiment 1: Salinity change effect

Since *Dunaliella salina* faces salinity variation along the year in its natural habitat, resulting from precipitation and climatic variables (sun and wind) affecting evaporation (Ben-Amotz et al. 2009), we investigated how salinity change affects its demographic dynamics. We compared iso-osmotic transfers (no change from the acclimation salinity) at intermediate ([NaCl] = 2.4M) or high ([NaCl] = 4M) salinity, to hyper-osmotic shock from intermediate to high salinity (from 2.4M to 4M of NaCl), to assess whether the response depends on salinity change or salinity value (3 replicates per condition).

Experiment 2: PCD inhibitor

A plausible explanation for the observed rapid population decline (Fig. 1) is programmed cell death, known to be common in *Dunaliella salina* (Orellana et al. 2013) and related species (Berges and Falkowski 1998; Segovia et al. 2003; Jiménez et al. 2009), notably in response to salt. To investigate this, we used Z-VAD(Ome)-FMK (CellSignaling), a cell permeable irreversible caspase inhibitor that prevents caspase-3-like from adopting its active form, and which has been used to inhibit cell death in a diversity of unicellulars (Bidle et al. 2007; Zuppini et al. 2007; Bidle and Bender 2008; Segovia and Berges 2009; Yordanova et al. 2013), including in *Dunaliella tertiolecta* (Segovia et al. 2003). Following a preliminary test (Fig. S3), we compared the decline rate of cells treated with four concentrations of this inhibitor: 0, 1.6, 3, and 4 µM. Treated cells were incubated in the dark for 30min, before performing iso-osmotic and hyper-osmotic transfers for both strains, with 3 replicates per treatment. Final volume in the flask was 10mL, with an initial total number of cells being 200 000. Population densities were then assessed through cytometer measurements 1 hour and 4 hours after the transfer on day 0, then on days 1, 2, 3. (see Fig. S4 for supplementary days 6, 7, 8 and for strain C dynamics).

Experiment 3: Initial density and population growth phase

The rapid cell death that we focus on strongly impacts population dynamics, which is indeed how it is detected (Fig. 1) (Berges and Falkowski 1998; Leung et al. 2022). It is thus likely to interact with

population density and competition occurring in the population (Ameisen et al. 1995; Christensen et al. 1995). In particular, the initial decline may influence post-decline growth rate, because fewer live individuals means reduced competition for resources (relaxed density dependence). These effects may also depend on the physiological state of the population prior to the transfer, which is itself influenced by competition and resource limitation in the recent past.

To test for these effects, we acclimated lines of strain A and C for 4 days (mid-exponential phase), 13 days (early-stationary phase) and 41 days (late-stationary phase), representing 3 different regimes influencing current physiological state, and then exposed them to hyper-osmotic stress, starting at 3 different initial densities (5,000; 20,000; 30,000 cells/mL). We used 4 different lines per strain as replicates, resulting in 72 flasks (2 strains x 3 Initial densities x 3 growth phases x 4 lines). For the control treatment (iso-osmotic transfer), populations were started at the acclimation salinity 2.4M, with only 1 line per strain (2 x 3 x 3 = 18 flasks). The 90 flasks were treated in 2 temporal blocs, one day apart.

Experiment 4: Nutrient limitation

It has been hypothesized that the population rebound observed following a population decline could result from a beneficial effect of nutrients released by the dying cells (Orellana et al. 2013), which may compensate for a lack of nutrients in the medium. To test this hypothesis, we compared a treatment with nutrient limitation (concentration of F/2 nutritive solution divided by 4 relative to our standard growth conditions) with a standard nutrient treatment, using 5 replicates per treatment. Population measures on day 0 and 1 were made with the spectrometer (fluorescence and optical density), while the following days were measured with the cytometer and spectrometer.

Experiment 5: Cultures on population filtrates

To further investigate whether the post-decline rebound is related to substances that dying cells might release in the culture medium (nutrients or informative molecules about environmental conditions), we used population filtrates as culture medium. Indeed, it was shown in another

microalgal species of the same order (*Chlamydomonas reinhardtii*) that supernatant of cells that have experienced PCD is more favourable to growth than filtrates of cells that have undergone other types of death (Durand et al. 2011), and that this effect depends on the recipient species (Durand et al. 2014). The latter is important, as it determines to what extent PCD may be favoured by kin selection (Durand et al. 2014), through so-called private goods that are preferentially used by relatives rather than public goods that may also be used by cheaters (Estrela et al. 2016, 2019). We thus tested for any strain-specific potential benefits of cell death in terms of demographic rebound in *D. salina*, more specifically by contrasting a declining and non-declining strain.

To prepare the filtrates, cultures of strains A and C (respectively 2 and 3 lines per strain) were grown in large volume (70 mL) for 4 days at intermediate salinity ([NaCl] = 2.4M), and then transferred to high salinity ([NaCl] = 4M) at an initial cell density of 100,000 cells/mL, in V=110 mL. On the next day, when the decline was expected to be maximal for strain A (according to Fig. 1), we verified that strain A actually showed a decline (*ca.* 50% in 24 hours), while strain C did not. We centrifuged and filtered these large volumes, so as to get our 2 filtrates as culture media (filtrate A and C respectively for strain A and C). We then inoculated 3 replicate lines per strain in 3 different culture media (filtrate A, filtrate C, and standard artificial saline water as a control), mixed in a ratio 1:1 with our artificial saline water to insure the presence of nutritive solution. We applied 3 salinity transfers: iso-osmotic at 2.4M and at 4M, and hyper-osmotic from 2.4M to 4M of NaCl, for a total of 54 flasks: 3 culture media x 3 salinity transfers x 2 strains x 3 replicates (minus 3 flasks because one acclimated population of strain A did not reach a sufficient density).

Experiment 6: Light intensity

Different studies have underlined the potential role of chloroplast activities in PCD phenomenon (Murik et al. 2014; Ambastha et al. 2015; Bidle 2016), and recent gene expression analysis on our focal strains revealed that strain-specific gene expression response to salinity changes involved mostly genes related to chloroplast structures and activities (Leung et al. 2022). In addition, light represents a resource that is not modified by putative elements released in their medium by dying

cells (as long as transparency is not affected). We thus tested whether light intensity influences the decline and rebound induced by a salinity rise, through an effect of photosynthetic activity on the physiological state of cells. To do so, we repeated experiment 1 but at 200 µmol.m^{-2}.s^{-1} light intensity instead of 100 µmol.m^{-2}.s^{-1}, during both acclimation and assay phases (Fig. S8). These two light levels are commonly considered in the literature as not stressful (Berges and Falkowski 1998; Sui and Harvey 2021), but 100 µmol.m^{-2}.s^{-1} is slightly limiting compared to 200.

Experiment 7: Successive osmotic shocks

The pattern of population decline and rebound that we observed in response to osmotic stress may be explained by the elimination of cells that were initially damaged or in low condition (e.g. old); a rapid population rebound would then be the result of faster multiplication of cells that were initially in better physiological state. This hypothesis implies a process akin to evolutionary rescue (Gomulkiewicz and Holt 1995), but where selection acts on a non-heritable phenotypic variation (as we used nearly isogenic lines). We would thus expect that the decline would be drastically reduced upon a second osmotic shock, because most cells in bad condition would already have been eliminated.

To test this hypothesis, we assessed the population decline for populations that were subjected to two successive hyper-osmotic shocks, separated by a return to intermediate salinity, which we compared to populations subjected to a single hyper-osmotic shock. We acclimated populations of strain A and C at intermediate salinity ([NaCl] = 2.4 M) for 14 days, transferred them a first time at high salinity ([NaCl] = 4 M) for 14 days. Then we transferred them back to [NaCl] = 2.4 M for 17 days (about 17 generations, assuming ~ 1 generation per day, Ben-Amotz et al. 2009), before inducing a second salinity rise at 4M, and (expected) initial density of 20,000 cells/mL. In the control condition, cultures were subjected to iso-osmotic transfer first (constant salinity at 2.4M), and exposed to hyper-osmotic shock only at the second transfer. We used 5 different lines per strain as replicates for each treatment (except 4 replicates for strain C in the successive shocks treatment, because of 1 broken flask).

Statistical analysis

To investigate effects of our treatments on population dynamics, we performed generalized linear models (GLM), using cells count data as response variable (except for the nutrient limitation experiment, based on whole-well fluorescence), with a log link function and a negative binomial error structure, as this has proved more accurate than Poisson in our setting (Rescan et al. 2020). First, to test for population decline following the transfer, we applied such a GLM to population size at day 1, using the logarithm of expected initial population density (based on population size at the end of the acclimation step) as offset. The linear predictor in this model thus estimated the logarithm of the relative change in population density from day 0 to 1. For the light experiment, we compared the experiment with brighter light against all other experiments under hyper-osmotic stress (except under nutrient limitation, where cytometer counts were not available for all days, and with the PCD inhibitor). For the nutrient limitation experiment, we applied a linear model to the logarithm of population density estimated by the whole-well fluorescence values (day 1) , because fluorescence is log-normally distributed (Rescan et al. 2020). The offset was the logarithm of the initial population density estimated by fluorescence of strain C (one hour after the transfer), as this strain does not display population decline (Fig. 1).

We then applied a second GLM for population growth in days corresponding to a phase of exponential growth (details in Table 1), visually detected as a linear trend on population dynamics on log scale (this phase is denoted as grey backgrounds on Fig. 1 and Figs. S4-S9). In this model, no offset was used for initial population size, but day was included as a continuous explanatory variable. The day effect in this model thus estimated the rate of exponential growth per day (linear trend on the log scale), and any interaction of day with other factors (treated as covariates in the regression) were effects of these factors on maximum, exponential growth. The fixed effects were experiment-dependent (Table 1). For population growth under nutrient limitation, we applied the same approach but using a LM on the logarithm of population densities based on whole-well fluorescence. For the

light experiment, we applied the GLM on the subset of days corresponding to the exponential phase in each experiment (determined as specified above).

Our results suggested that across experiments, larger initial decline was associated with faster maximal growth under hyper-osmotic stress. To formally test for this effect, we estimated the decline rate (on the log scale) predicted by the GLM as:

$$D = \ln\left(N_{0,exp}\right) - e_1 \quad \text{(Eq. 1)}$$

where e_1 is the linear predictor of the GLM (i.e. the estimator of population density on the log scale, no longer corrected by the offset) at day 1 after the shock, and $N_{0,exp}$ is the initial population density expected from the acclimation culture. A positive value of *D* means that the population has declined after the transfer. The standard error for the decline rate *D* is directly that of the linear predictor on the log scale. In the results, we also expressed the decline as proportional reduction in population size,

$$Population\ reduction\ (\%) = (1 - e^{-D}) \times 100 \quad \text{(Eq. 2)}$$

We also estimated the maximal daily growth rate *r* in the exponential phase as

$$r = \frac{e_{x_2} - e_{x_1}}{x_2 - x_1} \quad \text{(Eq. 3)}$$

where e_{x_1} and e_{x_2} are the linear predictors of the GLM (log population size) at days x_1 and x_2 after the shock, corresponding to the phase of exponential growth (grey backgrounds on Fig. 1 and Figs. S4-S9). We calculated the standard error of each estimated growth rate as:

$$\mathrm{SE}(r) = \frac{\sqrt{\mathrm{SE}(e_{x_2})^2 + \mathrm{SE}(e_{x_1})^2}}{x_2 - x_1} \quad \text{(Eq. 4)}$$

We then tested the significance of the relationship between the estimated decline rate and rebound growth rate using a bootstrap method. For each *D* and *r* pair (corresponding to a specific condition in a given experiment), we drew random values from a normal distribution with mean given by the estimate, and standard deviation given by the standard error of the estimate, to account for the uncertainty in each data point. A linear model was then fitted for the relationship between *D* and *r* across conditions in each of these simulations, and the corresponding regression slope and intercept

were drawn from normal distributions centred on their estimates and with SD given by their SE, to account for uncertainty in each regression. We repeated this process 10000 times, from which the proportion of positive slopes was used as a p-value.

Finally, the mean slope *b* and the intercept *a* over these 10000 simulations were used to compute the predicted population density at day *t* for non-declining population as

$$\ln(N_t) = \ln(N_0) + a \times t \quad \text{(Eq.5)}$$

or for a declining population starting to rebound at day *c* (corresponding to x_1 in eqs. 3-4) as

$$\ln(N_t) = \ln(N_0) - D + (a + bD) \times (t - c) \quad \text{(Eq.6)}$$

From this, we could then estimate the day at which a declining and a non-declining population would reach equal population density, assuming exponential rebound:

$$t_{eq} = c + \frac{1}{b} + \frac{ac}{bD} \quad \text{(Eq.7)}$$

All statistical analyses were performed on Rstudio (R version 3.6) using MASS version 7.3.53 package (Venables and Ripley 2002).

Results

We wished to understand the proximal determinants and fitness consequences of programmed cell death in a unicellular organism. Our aim was to find whether, and under which conditions, such apparently detrimental phenotype may be associated with genotype-dependent benefits for population growth, and thus potentially be favoured by natural selection.

Decline in response to osmotic shock is strain-specific

As salinity is a major component of *Dunaliella salina*'s niche, we first investigated its influence on demographic dynamics in this species. We observed that an iso-osmotic transfer into fresh medium without change in salinity (either at intermediate 2.4M or high 4M NaCl concentration) did not affect demography, since population densities measured 1-hour post-transfer matched the expected initial densities, accounting for the dilution into fresh medium (Fig. 1, Table S2). In contrast, transfer from

intermediate to high salinity induced a sharp demographic decline in strain A in the hours following the transfer, with population size reduced by 69% in one hour (Table S2), and reaching 77% decline at its minimum, 24 hours post-transfer. This decline was not observed for strain C (Fig. 1, Table S2), leading to a significant effect of strain identity on population density on day 1, and a significant interaction between strain and type of salinity transfer (Table 2). This indicates that the rate of rapid cell death varies among closely related strains of *D. salina* (as shown by Leung et al. 2022), and does so in a manner that depends on the type of salinity transfer, being trigged in strain A by a salinity rise rather than by high salinity *per se*. Note that the osmotic shock also led to an increase in the number of apparently dead cells (detected by their lower red fluorescence, Rescan et al 2020), but orders of magnitude lower than the observed decline of live cells, and of similar magnitude between both strains (Fig. S2).

Interestingly, the population decline of strain A was associated with a higher maximum growth rate in the following days, as compared to its growth rate without initial decline (Fig. 1, left and right panels; Table S3 P<3e-8 and P< 3e-15, respectively). In contrast, strain C, which did not decline following hyper-osmotic shock, had a weaker maximum growth rate compared to strain A in this salinity condition (Table S3, P<2e-16). As a result, even though strain A had a much lower population density than strain C after its initial decline under hyper-osmotic stress, it eventually compensated for its lag in population size, reaching a density similar to strain C after 6 days.

The decline is reduced by a programmed cell death inhibitor

To assess whether the rapid population decline may result from programmed cell death, as commonly documented in this species and genus (Berges and Falkowski 1998; Segovia et al. 2003; Jiménez et al. 2009; Orellana et al. 2013), we performed an experiment with a caspase-3-like PCD inhibitor, at four different concentrations. The PCD inhibitor had a clear quantitative effect on the intensity of the decline, with higher inhibitor concentration leading to less severe decline for strain A under hyper-osmotic shock (Fig. 2). Accordingly, the GLM treating inhibitor concentration as a

continuous variable had a slighter better AIC than the one treating it as a categorical factor (AIC = 220.78 vs 223.33). We found a significantly positive relationship (Table S4A, P<1e-3) between (log) population size at day 1 (post-decline) and inhibitor concentration, with a slope of 0.150 (SD = 0.045). The model thus predicts that the population size one day after hyper-osmotic shock is reduced by 70.8% without inhibitor (consistent with our previous experiment), but is proportionally increased by ~16% with each additional µM of inhibitor. In a preliminary experiment (with only 2 replicates), we even found that strain A already started to decline 15min after the osmotic shock (43% reduction in population size), while the 3 treatments with inhibitor did not (Fig. S3). In contrast, we found no effect of the inhibitor concentration on demographic dynamics in other conditions (strain A under iso-osmotic transfer, and strain C in any salinity transfer; Fig. S4). There was no significant difference effect of inhibitor concentration on maximum growth-rate between days 1 and 6, despite the observed differences in decline intensity (Fig. 5, Table S4B, P= 0.107). These results therefore indicate that the population decline observed for strain A under hyper-osmotic shock is at least partly explained by PCD.

Initial decline is greater under weaker competition

To understand what benefit such salinity-induced PCD may confer, we investigated hypotheses about reduced competition. A possible explanation for the higher growth rate of strain A following its initial decline under hyper-osmotic stress may be relaxed density dependence (competition release), whereby the resources unused by dead cells become available to those cells that survived. The influence of initial decline on later growth would thus be mediated by density-dependent processes. More specifically, we may expect the post-decline population to have a similar growth rate to a population that did not decline but started at similarly low density. In addition, the rate of death (and thus the population decline) could itself be modified by the initial population density, if PCD is a plastic trait that responds to cues of the density of conspecifics, or amount of resources. However, these influences of competition-mediated resource limitation on population growth are likely to be

modulated by the metabolic state of individual cells (Delong and Hanson 2009), which in turn depends not only on the immediate density of competitors, but also reflects the previous growth conditions during the acclimation phase (Rescan et al. 2020). This occurs because the influence of competition and resource limitation on the physiological states of individual cells (and hence population growth) depends on the rates of nutrient uptake and metabolic pathways involved, and may involve some delays (Droop 1973). We thus tested for an influence of not only the initial density of the inoculum, but also of the growth phase of the populations in the acclimation phase, as an indicator of the physiological state of the population. We report results about rates of initial decline and maximal growth rate in Figure 3, while the full population dynamics appear in Figure S5.

Overall, strain A showed greater decline in conditions where population growth is expected to be more rapid, that is, when cultures were transferred during their exponential or early stationary phase, and/or starting at low initial density (Fig. 3A). When the populations experienced osmotic shock in the mid-exponential or early-stationary phases (after 4 or 13 days of acclimation, respectively), the initial density did not impact the intensity of the observed decline in Strain A (Fig. 3A & Table S5A). In contrast, populations transferred in their late stationary phase (41 days) showed no decline when starting at high density (30,000 cells/mL), but a decline comparable to that in earlier phases when starting at low density (5,000 cells/mL) (Fig. 3A). Overall, across initial densities, populations transferred in their late stationary phase declined less than when transferred in earlier phases (Table. S5B). Contrary to strain A, strain C never declined in any demographic conditions (Fig. 3A).

Following the decline, strain A displayed higher maximum growth rate than strain C for the mid-exponential and early-stationary phase populations (Fig. 3B). Furthermore, the growth phase ("age" of the cultures before the transfer) influenced the maximal growth rate for strain A, but with an effect that also depended on density. The maximum growth rate was highest for populations transferred in their early-stationary phase, when starting at N_0 = 30,000 cells/mL (Table S6, P=0.037

for the comparison with exponential phase starting at N_0 = 5,000), while when starting at N_0 = 5,000 cells/mL they did not show a significantly different rebound that those transferred in their exponential phase (Table S6, P=0.839). In particular, the rebound growth rate of strain A inoculated at N_0 =30,000 cells/mL was higher than the growth rate of the non-declining strain C that started at a density similar to A post-decline (N_0 = 5000 cells/mL; see Fig.S5 and Fig. 3). This confirmed that density-dependent effects on population growth are largely mediated by persistent responses to past conditions of competition and resource limitation (as shown for salinity by Rescan et al. 2020). The weak and inconsistent effect of initial density on the rate of population rebound further suggests that this rebound is unlikely to be due to relaxed density dependence from many cells having died. In fact, our focus on the rate of exponential growth limits the possibility for density to have a large effect, since exponential growth is density-independent by definition.

Decline intensity depends on resources or cues from the culture medium

To more directly assess the role of resources competition on the observed pattern of decline and rebound, we manipulated nutrient abundance. When fewer nutrients were available, strain A declined significantly less than populations in control medium (Fig. 4A, Table S7 P=1e-4). (Note that the estimated decline and maximal growth rate were predicted based on spectrometer measures (Fig. S4A) instead of flow cytometry counts for this experiment.) The estimated maximal growth rate in exponential phase (from days 1 to 3 in this experiment) was clearly lower for strain A when nutrients were missing (Fig. 4B; Table S8 P=0.003). As in the previous experiment, in standard nutrient conditions strain A displayed a higher maximal growth rate compared to strain C (Table S8, P=3e-7).

Since the medium content affects the intensity of decline for strain A, we tested the hypothesis that the dying cells causing population decline release nutrients that may be used by the remaining cells to grow faster, or informative cues about the population demographic status. We also investigated whether the ability to use this resource or information was genotype-specific. To do

so, we grew populations of each strain in medium filtered from previous cultures that had undergone a salinity rise, either from strain A (filtrate A) or strain C (filtrate C) (Fig. 4C and 4D). Populations of strain A growing on filtrate A had a slightly but significantly lesser decline than control populations A growing in standard medium (Table S9: population reduction of 73% *vs* 77 %, P=0.026). Those growing on filtrate C instead showed a greater decline than the control (Table S9: population reduction of 81% *vs* 77 %, P=0.021). In contrast, we did not detect any effect of culture medium on the rebound growth rate for strain A (control: r=0.64, strain A in filtrate A: r=0.59, P=0.129; strain A in filtrate C: r=0.68, P=0.153 in Table S10). We also did not find any effect on the population growth of strain C, either in the first day (P=0.058 & P=0.219, respectively in filtrate A and in filtrate C compared to control culture, see Table S11A), or after day 1 (in all conditions r ∈[0.26;0.28]; P=0.092 & P=0.783 see Table S11B).

Decline and rebound depend on resources outside the culture medium

We also studied the effect of light intensity, an important resource for photosynthetic organisms, which unlike the medium should not be directly affected by the dying cells. We compared the control conditions from experiments above (plus one below with successive osmotic shocks), where populations faced a salinity rise at light intensity of 100 $\mu mol.m^{-2}.s^{-1}$ photons, to populations undergoing a salinity rise at higher light intensity 200 $\mu mol.m^{-2}.s^{-1}$ (Fig. 4E and 4F). The decline rate of strain A was significantly greater under brighter light (population reduced by 82% at 200 $\mu mol.m^{-2}.s^{-1}$ *vs* 57% at 100 $\mu mol.m^{-2}.s^{-1}$; P<0.001, Table S12A). Similarly, strain A grew significantly faster in the exponential phase (shaded area on demographic dynamics) under brighter light (r=0.87 vs 0.43 for 200 *vs* 100 $\mu mol.m^{-2}.s^{-1}$, P=0.007 in Table S12B). This confirmed that resource conditions more favourable to growth led to faster decline followed by greater growth rate.

A consequence of heterogeneity in cell condition?

Initial heterogeneity in the metabolic state or condition of cells could explain the decline-rebound pattern observed in response to hyper-osmotic shock (Fig. 1). Indeed, older or more damaged cells

within the population may be rapidly eliminated when facing hyper-osmotic shock, while those that were originally in good condition may survive and reproduce faster.

To investigate this hypothesis, we applied successive salinity rises to the two strains, separated by a short stay of 17 days at intermediate salinity (corresponding to ~17 generations). Our prediction under the hypothesis of an effect of cell condition was that a second osmotic shock imposed shortly after the first one should lead to a more moderate decline (if any), because damaged cells have already been eliminated. Contrary to this expectation, we found that the decline of strain A was greater upon a second salinity rise than after a single salinity rise (population reduction of 75% vs 62%, Fig. 4G, Table S13A P<2e-16), while the number of salinity rises did not impact the initial growth rate of strain C (no decline, Fig. 4G). For both strains A and C, the maximum growth-rate did not differ between the two treatments (Fig. 4H, Table S13B P=0.345).

Sharper decline is associated with faster rebound across all treatments

The results of these experiments suggest that faster initial decline was generally followed by a higher maximum growth rate (e.g. Fig. 4). In order to formally test for this pattern, we gathered all estimated pairs of decline intensity *D* (log reduction in population size between days 0 and 1, with larger positive values denoting sharper decline) and rebound rate *r* (daily rate of maximum exponential growth) for strain A facing a salinity rise, across experiments (Fig. 5). We found a highly significant positive relationship ($P = 8x10^{-4}$) between *D* and *r*. Decline intensity *D* ranged from 0.22 (19.8% reduction in population size for late stationary cultures transferred at high density) to 1.6 (79.8% reduction in population size under bright light). The slope of the regression of *r* against *D* is 0.268 (SD = 0.074). This means that relative to a putative population that would not decline, a population that initially declines by *D* but starts rebounding at day 1 would compensate for its initial demographic deficit by day 5 if *D* = 1.47 (77.0% initial reduction in population size), and by day 10 if *D* = 0.22, under exponential growth. In other words, a population that initially declines faster is predicted to match the size of a non-declining population more rapidly during exponential growth,

and/or may reach its carrying capacity first, thus potentially exerting competitive exclusion on the non-declining strain (Kot 2001).

Discussion

We investigated the factors influencing rapid, programmed cell death (PCD) in two closely related strains of the halotolerant microalga *Dunaliella salina*, and its demographic implications beyond the initial decline it produces. Our aim was to decipher how variation among strains in these demographic responses may impact selection on a trait that seems at first like an archetype of maladaptive plasticity.

Implications of the decline-rebound pattern

We established that the demographic response to high salinity characterized by a rapid decline followed by rebound (shown by Leung et al. 2022) is induced by salinity rise, rather than high salinity *per se* (Fig. 1). This fast response (population size reduced by 69% in one hour, Table S2, and even a reduction by 43%, in 15 min Fig. S3) matched the findings for the closely related species *D. viridis* (Jiménez et al. 2009), where about 70% of the population died one hour after a hyper-osmotic shock (from [NaCl] = 2M to [NaCl] = 5.5 M), as well as for another unicellular chlorophyte *Chlorella saccharophila* (Zuppini et al. 2010) (between 21.9% and 33.1% decline after 24 hours of hyper-osmotic stress). The decline-rebound pattern that we observed further matched the previous finding in *D. salina* that darkness (rather than salinity rise as here) causes the death of *ca.* 65% of cells, but later enhanced population growth (Orellana et al. 2013).

Interestingly, this phenotypic response varied drastically between two genetically close *Dunaliella* strains (Emami et al. 2015; Leung et al. 2022), one of which never displayed the characteristic decline-rebound pattern after a salinity rise, regardless of other experimental factors. While the specific genetic differences behind these contrasted responses to salinity are unknown, it is noteworthy that gene expression and DNA methylation responses to salinity vary between these

strains (Leung et al. 2022). Orellana et al. (2013) used yet another strain (CCAP-19/18, phylogenetically close to 19/12-strain A and 19/15-strain C used here, Assunção et al. 2013; Emami et al. 2015), which did decline, suggesting that there is genetic variation for this trait within *D. salina*, unlike in other species where such demographic responses have been investigated (Nedelcu et al. 2011; Durand 2020). The putative benefits of this decline also appeared to be strain-specific, as the growth of strain C was not influenced by filtrate of declining strain A (Fig. 4D, Table S11), mirroring the lack of significant effect of induced-death filtrate on 2 strains in *Chlamydomonas reinhardtii* (Durand et al. 2011). The markedly distinct demographic responses of these closely related strains imply that natural selection can act on rapid, environmentally induced cell death, favouring it in conditions where it is associated with higher accrued fitness during the demographic rebound.

Such a positive correlation between initial decline and later demographic rebound following osmotic shock was one of our most striking results (Fig. 5). This allowed the declining genotype to compensate for its initial lag, eventually reaching a density similar to - or even higher than - the non-declining genotype (Fig. 1, Fig. S6 and S7), before attaining the stationary phase where population growth stops (commonly described as carrying capacity). Assuming that the dynamics in monocultures predict what would happen in co-cultures, i.e. neglecting genotype-by-genotype demographic interactions causing frequency-dependent selection (Chevin 2011), these results suggest that the lethal, and at first glance disadvantageous, phenotype of rapid cell death is likely to be favoured in an environment with fluctuating salinity (including hyper-osmotic shock).

Causes of selection

Which mechanism could explain the positive correlation we found between decline and rebound rates for strain A? Our main hypothesis was that the dying cells release material that is beneficial to the remaining cells, thus representing a form of cooperation (Durand et al. 2011; Orellana et al. 2013). The importance of environmental dynamics for social evolution, especially in microbes, was recently emphasized (Estrela et al. 2016, 2019), and could involve different mechanisms (Kojic and

Milisavljevic 2020). For example, dying cells might provide resources by liberating nutrients (public goods) in the culture medium. This organic material may include intra-cellular glycerol (Zidan et al. 1987) that *D. salina* massively stores in its cytoplasm as osmo-protectant at high salinity, and could be used directly by the surviving cells (*D. Salina* being capable of heterotrophy (Chavoshi and Shariati 2019)), or metabolized by a halophilic archaea, as proposed by Orellana et al. 2013. However, we did not find a faster rebound for strains A growing on filtrate A, (and no "cheating" effect on the growth of strain C) and hence found no support for the altruistic nutrient-release hypothesis. Either this effect does not exist in this system, or it exists but could not be detected because death-induced molecules were too rare in the diluted filtrate, or because naturally co-occurring archaea that remineralize glycerol were absent. Another altruistic mechanisms could be the detoxification of the environment by dying cells (Estrela et al. 2019), but we are not aware of any mechanism through which *D. salina* might reduce salinity, which is the major stressful condition applied here. Signalling molecules could also be released, providing information about the environmental conditions, such as quorum-sensing molecules (Christensen et al. 1995; Durand 2020). This hypothesis cannot be discarded since strain A growing on filtrate A showed a more moderate decline (Fig. 4C), suggesting that live cells may perceive that a demographic decline already occurred, therefore modulating the proportion of cells that will die. To sum-up, our results did highlight that the declining strain is sensitive to the medium content, but we do not have clear evidence that this is an altruistic trait.

Another less adaptive hypothesis explaining the decline-rebound pattern could be a trade-off between reproduction and the rate of plastic change in a trait involved in salinity tolerance. Genotypes that invest more in salinity tolerance, by having more rapid plastic change in dedicated traits, should have higher survival probability when facing osmotic stress, but possibly at the expense of lower growth rate if salinity tolerance mechanisms are costly for reproduction. Such trade-off could involve the metabolism of glycerol, which is known to divert resources produced by photosynthesis or stored in starch (Ben-Amotz and Avron 1973), and is thus likely to impact population growth (Jones and Galloway 1979). A related concept in ecology is the trade-off between

resistance and recovery with respect to resilience, whereby a strain that is more susceptible to stress (less resistant) is able to recover faster from it (Hodgson et al. 2015). However, this hypothesis would not explain why for a given genotype (strain A), conditions that cause sharper decline also lead to faster growth during rebound.

A third hypothesis explaining the decline-rebound demography could be cell heterogeneity in the population, whereby damaged cells die when facing osmotic shock, while cells in good shape survive and have higher growth rate. However, we found that a second osmotic shock in fact induces more (rather than less) cell death than the first (Fig. 4G), so this explanation is unlikely to hold, unless a very high proportion of damaged cells is produced during the relatively short stay at intermediate salinity, which seems unlikely. These proposed hypotheses are not mutually exclusive and may partially explain the observed results depending on the ecological context.

Passive vs active plasticity and programmed cell death

A strong – albeit indirect – argument in favour of adaptive plasticity is when the plastic response can be established to involve an active mechanism (Pigliucci 1996). For the rapid decline we observed, a natural candidate of active mechanism would be programmed cell death (PCD), more commonly named cell suicide in unicellular organisms (Ameisen 2002; Durand 2020). PCD has been reported in unicellular chlorophytes under a range of environmental stresses (Bidle and Falkowski 2004; Zuppini et al. 2010; Durand et al. 2014). This includes *Dunaliella* species, such as *D. tertiolecta* (Berges and Falkowski 1998; Segovia et al. 2003), *D. viridis* (Jiménez et al. 2009), and our focal species *D. salina* (Orellana et al. 2013). Signatures of active cell suicide via PCD include the early externalisation of phosphatidylserine (PS) in the outer membrane, caspase-like activities in mitochondria, and DNA fragmentation (Barreto Filho et al. 2022). Our assays with caspase-like inhibition showed a clear, quantitative reduction of the population decline with increasing inhibitor concentration (Fig. 2). Although caspase-like activity is not specific to programmed cell death (Abraham and Shaham 2004; Barreto Filho et al. 2022), and PCD can occur without caspase activity (Leist and Jäättelä 2001;

Abraham and Shaham 2004), our finding that a caspase inhibitor routinely used to detect PCD distinctly reduces population decline is a clear indicator that the cell death causing this decline is not an entirely passive process. Furthermore the tested inhibitor was also used in the related species *D. tertiolecta*, where PCD has been firmly established using several assays and morphological criteria (Segovia et al. 2003).

In addition to this direct test for PCD, one consistent line of evidence towards an active process in our experiments was that the decline was more pronounced when the growing conditions were closer to optimal: brighter light (Fig. 4E, Table S12A), cells in better condition, and/or with less inter-individual competition (Fig. 3A, Table S5A), non-limiting nutrients (Fig. 4A, Table S7). All these effects point towards this death being an active and energy demanding process (Sathe et al. 2019).

In conclusion, our results indicate that the strain that does more PCD in response to hyper-osmotic stress eventually reaches higher population size in at least some conditions (Fig. S6 and Fig. S7). Future work should investigate whether these demographic dynamics in monoculture accurately predict what happens in competition between these strains, to better understand whether and how natural selection can favor a lethal phenotype as a form of adaptive plasticity in a stressful environment. The fact that salinity-induced death and its consequences for longer-term fitness varied drastically between closely related strains isolated in the same location suggests that PCD is a very evolvable trait, even on short evolutionary timescales.

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Tables

Table 1: Summary of experimental conditions.

Experiment	Acclimation [NaCl]	Osmotic conditions (n replicates)	Expected initial density N_0 (cells/mL)	Hypothesis about decline (D) and rebound (r)	Fixed effects for GLM
Salinity change	5d at 2.4M or 4M	Hyper (n=3) Iso (=3)	50,000	D induced by salinity change	Strain × salinity transfer Rebound on days 1-5
PCD inhibitor	6d at 2.4M	Hyper (n=3) Iso (n=3)	20,000	D reduced by PCD inhibitor	Inhibitor concentration
Initial density & Growth phase	4d, 13d or 41d at 2.4M	Hyper (n=4) Iso (n=1; control)	5,000 20,000 30,000	D and r modulated by metabolic state, r mediated by competition release	Strain × growth phase × initial density Rebound on days 4-9
Nutrient limitation	5d at 2.4M	Hyper (n=5)	50,000	D and r affected by nutrient limitation	Strain × nutrient treatment Rebound on days 1-3
Population filtrate	5d at 2.4M or 4M	Hyper (n=3) Iso (n=3)	50,000	Dying cells release molecules that affect D and r	Strain × culture media × salinity transfer Rebound on days 1-5
Light intensity	7d at 2.4M	Hyper (n=5) Iso (n=10)	Unknow (spectroscopy) 1/10 dilution	Light intensity as external (non-medium) resource influencing D and r	Strain × light Rebound on days 1-4
Successive osmotic shocks	14d at 2.4M, then 14d at 4M or 2.4M (control), then 17d at 2.4M	Hyper (n=5)	20,000	D caused by elimination of damaged cells, resulting in faster growth in r.	Strain × acclimation treatment Rebound on days 3-7

In the second column, d stands for days and M stands for molar of NaCl. All variables in the last column were treated as categorical, except the Inhibitor concentration treated as a continuous variable.

Table 2: Analysis of deviance table for the GLM of population density on day 1.

Factors	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)	R^2
NULL	16	964.93				
Strain	1	154.67	15	810.26	< 2.20E-16 ***	0.16
Transfer	2	444.95	13	365.31	< 2.20E-16 ***	0.46
Strain x Transfer	2	348.31	11	17	< 2.20E-16 ***	0.36

We used a GLM with log link function and a negative binomial error structure for population density at day 1, using the expected initial (log) density as offset. Tested effects are Strain, Transfer and Strain × Transfer interaction, and *** denote p-values <0.001. R^2 was calculated as a deviance ratio (Nakagawa and Schielzeth 2013).

Figures Legends

Figure 1. Demographic dynamics of *Dunaliella salina* following salinity transfers. Each panel represents a transfer condition from the acclimation salinity (S1: Salinity 1) to the assay salinity (S2: Salinity 2). Transfer occurs at day 0, and the first dot is measured 1 hour after the transfer, while the cross represents the expected initial density, predicted from dilution of the acclimated population. The number of live *D. salina* cells per mL in each day is represented on log scale, for strains A (dots) and C (triangles). Symbols are averages over 3 replicates and error bars indicate the standard error, while lighter points are the raw densities.

Figure 2. Influence of an inhibitor of programmed cell death. Demographic dynamics of strain A following a hyper-osmotic shock (2.4M to 4M NaCl) are shown under different concentrations (colours) of an inhibitor of caspase-3-like, a typical marker of programmed cell death. Transfers occurred at day 0, and the first measures were made 1 hour after the transfer, while the cross represents the expected initial density, predicted from dilution of the acclimated population. The number of live *D. salina* cells per mL in each day is represented on the log scale. Filled symbols are averages over 3 replicates, error bars indicate the standard error, and lighter points are the raw density measurements.

Figure 3. Impact of competitive conditions on initial decline and subsequent maximal growth following hyper-osmotic shock. Estimates and standard errors (across 4 replicates) from the negative binomial GLMs for (A) decline (days 0-1 after the osmotic shock), and (B) the maximal growth rate (corresponding to the shaded area in Fig. S5), are shown for different conditions of initial density (grey shades) and population growth phase (x-axis), for strains A (dots) and C (triangles). Population growth phase correspond to mid-exponential phase (Mid-exp: 4 days of acclimation), early-stationary phase (Early-stat: 13 days) and late-stationary phase (Late-stat: 41 days). In panel A, larger positive values indicate faster population decline, while negative values correspond to growing populations.

Figure 4. Influence of resources and previous stress on decline and rebound. Estimates and standard errors across 5 replicates (except for supernatant experiment: 3 replicates) from the negative binomial GLMs for decline on days 0-1 after the hyper-osmotic shock (left: A, C, E, G), and maximal growth rate on the exponential phase after the osmotic shock (right: B, D, F, H) are shown for different experimental conditions, for strains A (dots) and C (triangles). The factors investigated are the amount of nutrients in the culture medium (A-B); putative molecules released by cells that do or do not die in response to osmotic shock (C-D); light intensity, a resource type not directly modified by dying cells (E-F); and a previous salinity shock (G-H). All estimates are based on cytometer counts, except A-B on whole-well fluorescence.

Figure 5. Faster decline is associated to faster maximal growth across experiments. All estimates and standard errors for decline and rebound rates are shown for strain A following a salinity rise. Grey lines represent 10000 linear regressions of growth rate against decline rate, based on simulated samples where each pair of values (for decline and rebound) was randomly drawn from a normal distribution with mean set to the predicted value and standard deviation set to the standard error of estimators for each condition. Height of the 10000 replicates had a negative slope ($P = 8x10^{-4}$). Estimates from the nutrient experiment (blue empty circles) are not included in the linear regression, as they were based on whole-well fluorescence instead of cytometer counts.