Strain-specific metabarcoding reveals rapid evolution of copper tolerance in populations of the coastal diatom Skeletonema marinoi

Björn Andersson[1], Olof Berglund[2], Helena Filipsson[2], Olga Kourtchenko[1], Anna Godhe[3], Kerstin Johannesson[4], Mats Töpel[5], Matthew Pinder[5], Lara Hoepfner[6], and Karin Rengefors[2]

[1]University of Gothenburg
[2]Lund University
[3]Goteborgs Universitet
[4]Affiliation not available
[5]University of Gothenburg Faculty of Science
[6]University of Münster

April 5, 2023

Abstract

Phytoplankton have short generation times, flexible reproduction strategies, large population sizes, and high standing genetic diversity, traits that should facilitate rapid evolution under directional selection. We quantified local adaptation of copper tolerance in a population of the diatom Skeletonema marinoi from a mining exposed inlet in the Baltic Sea and in a non-exposed population 100 km away. We hypothesized that mining pollution has driven evolution of elevated copper tolerance in the impacted population of S. marinoi. Assays of 58 strains originating from sediment resting stages revealed no difference in the average tolerance to copper between the two populations. However, variation within populations was greater at the mining site, with three strains displaying hyper-tolerant phenotypes. In an artificial evolution experiment, we used a novel intraspecific metabarcoding locus to track selection and quantify fitness of all 58 strains during co-cultivation in one control and one toxic copper treatment. As expected, the hyper-tolerant strains enabled rapid evolution of copper tolerance in the mining exposed population through selection on available strain diversity. Within 42 days, in each experimental replicate a single strain dominated (30-99% abundance) but different strains dominated the different treatments. The reference population developed tolerance beyond expectations primarily due to slowly developing plastic response in one strain, suggesting that different modes of copper tolerance are present in the two populations. Our findings provide novel empirical evidence that standing genetic diversity of phytoplankton resting stage allows populations to evolve rapidly (20-50 generations) and flexibly on timescales relevant for seasonal bloom progressions.

Introduction

All organisms require trace amounts of metals to function and produce new biomass. However, elevated metal concentrations quickly becomes toxic (Lemire et al., 2013; Waldron & Robinson, 2009), with structural effects on polluted ecosystems (Blanck, 2002; Blanck & Dahl, 1996; Pesce et al., 2010; Schmitt et al., 2005). Field studies on plant communities growing on polluted mine tailings have shown that dominant species rely primarily on migration of tolerant individuals from adjacent populations, rather than *de-novo* mutations (Macnair, 1987). More recently, the critical role of standing genetic variability in evolution of metal tolerance has been shown also for invertebrates (Janssens et al., 2009), yeast (Grangeteau et al., 2017), mycorrhizal

fungi (Bazzicalupo et al., 2020), and bacteria (Carlson et al., 2019). With their rapid generation turn over and huge population sizes, phytoplankton might evolve metal tolerance from either standing genetic variation or new mutations. Metal tolerant species of phytoplankton have been reported to dominate polluted aquatic environments (Foster, 1982; Kalinowska & Pawlik-Skowrońska, 2008) but, it is not know to what extent these tolerant species relies on adaptation through selection from standing genetic variation, or novel mutations (Xu et al., 2018; Zhao et al., 2017).

It has been argued that selection from standing genetic variation could enable phytoplankton populations to rapidly adapt to changing environmental conditions (Godhe & Rynearson, 2017; Rengefors et al., 2017). These selection arguments are based on indirect observations such as large population sizes (Sassenhagen et al., 2021), high dispersal rates (Hutchinson, 1961), and high genetic diversity in phytoplankton (Flowers et al., 2015; Kashtan et al., 2014; Osuna-Cruz et al., 2020). However, empirical evidence of phytoplankton phenotypic variability and capacity of adaptation is limited, with knowledge limited to a few key climate change traits and model species (Lohbeck et al., 2012; Schaum et al., 2017; Schaum et al., 2018; Wolf et al., 2019). The evolutionary potential of phytoplankton has mostly been assayed through artificial evolution experiments using a low-diverse genetic background. In such artificial evolution experiments, a single strain or, in some cases, a mixture of a few strains – often a random assembly of strains from different culture collections and geographical origins – is subjected to strong directional selection pressure, and the change in growth rate is recorded as a proxy for evolutionary potential (Collins & Bell, 2004; Lohbeck et al., 2012; Reusch & Boyd, 2013; Schaum et al., 2018). Such experiments reveal how unexposed populations may respond to novel selection pressures, like metal stress, through *de-novo* mutations and they can also explore how plastic responses contribute to phenotypic changes over time (Schaum & Collins, 2014; Xu et al., 2018). However, they contain insufficient diversity to account for selection from the standing genetic diversity already present in natural populations composed of thousands of unique clones (Sassenhagen et al., 2021).

We have identified the local populations of the diatom *Skeletonema marinoi* Sarno & Zingone, in and around the Baltic Sea, as a system to study intraspecific diversity and adaptation in phytoplankton. The genetic structure of *S. marinoi* in the Baltic Sea is primarily linked to the strong salinity gradient (Godhe et al., 2016; Pinseel et al., 2022; Sjöqvist et al., 2015). However, like many other coastal phytoplankton (McQuoid et al., 2002; Montresor et al., 2013),*S. marinoi* produces benthic resting stages that can anchor it to geographical locations (Sundqvist et al., 2018). Consequently, population structure can develop over distances as short as a few kilometers (Härnström et al., 2011; Sefbom et al., 2018), although the drivers of such differentiations have not been identified. The Baltic Sea also has an extensive and well-documented history of human pollution (HELCOM, 2010; Lehtonen et al., 2017; Reusch et al., 2018), which could be one driving force of local adaptation, as has been shown for *Daphnia* populations (Kerfoot et al., 1999). At a small (ca. 5 km^2) copper mining polluted inlet in the Baltic Sea, we have previously observed that strains of *S. marinoi* appear overly tolerant to several metals present in the mining ore (Andersson et al., 2020) and that toxic metal stress can affect interspecific competition between diatoms (Andersson et al., 2022). The mine is located on the shoreline and was active from the 17^{th}until the early 20^{th} century (Söderhielm & Sundblad, 1996). The sediment in the inlet is polluted with several metals whose concentrations across depth layers correlate negatively with the abundance of *S. marinoi* micro-fossils (Ning et al., 2018), suggesting that metal pollution has had an adverse effect on this species.

The present study aimed to test if copper tolerance has evolved in the mining-exposed *S. marinoi* population and to what extent intraspecific trait variation enables the population to adapt rapidly to a toxic environment. Our primary hypotheses were that centuries of mining exposure have caused copper tolerant strains to evolve and that during renewed exposure to toxic stress, the copper-exposed population has an evolutionary advantage over an unexposed reference population. To test these hypotheses, we isolated a large number *S. marinoi*strains from the mining-exposed inlet (30 strains) and an unexposed reference inlet (28 strains) and quantified their copper tolerance in mono-clonal experiments. We then assembled the populations back together and performed a 42-day long artificial evolution experiment (Fig. S1) where we tracked the strain selection process using amplicon sequencing of a recently developed nuclear locus with exceptional

intraspecific diversity (Pinder et al., 2023). This metabarcoding approach allowed us to observe the selection process with unprecedented resolution, and to quantify how selection from intraspecific diversity allow phytoplankton populations to adapt to adverse environmental conditions.

Method

Sampling procedure and sites description

Västervik Gåsfjärden (57°34.35'N, 16°34.98'E) is a semi-enclosed inlet on the Swedish coast of the Baltic Sea. It has been exposed to varying mining activity from the Solstad copper mine from the 17th to the early 20th century (Söderhielm & Sundblad, 1996). We have previously documented detailed sedimentological features of Gåsfjärden and inferred past environmental changes from this data (Ning, Ghosh, et al., 2016; Ning et al., 2018; Ning, Tang, et al., 2016). In this study, we use the unpolluted Gropviken (58°19.92N 16°42.35'E) inlet as a reference site since it also has hypoxic and laminated sediments (Karlsson et al., 2010), but to our knowledge it has not been exposed to mining activity. The sites are ˜100 km apart and have similar bathymetry and a cross-section of one to three km. Salinity was between 6 and 7 at the time of sampling, with a thermocline around six meters depth and a 10-20% decrease in oxygen concentration towards the bottom. The sediment is generally not bioturbated, and lamination patterns suggest frequent hypoxia periods (Ning, Ghosh, et al., 2016). Throughout this manuscript, we refer to Västervik Gåsfjärden [VG] as the 'mining inlet' and Gropviken [GP] as the 'reference inlet', while strains are named using the respective acronym.

Strain isolation

Sediment samples were collected using a Gemax corer and processed as described in the Supplemental Material and Methods. We have previously shown that *S. marinoi* is the most abundant resting stage species in the sediments, with up to a million viable cells per gram sediment (Andersson, Rengefors, et al., 2023). In this study, individual strains of *S. marinoi* were germinated and isolated from resting stages in the sediment one to one-and-a-half years after sampling using the sediment immediately below the surface (1-2 cm depth for the mining and 2-4 cm depth for the reference inlet). Resting stages were germinated and cultured in standard f/2 media (Guillard 1975) amended with 106 µM SiO_2. All media was mixed from the same batch of sterile-filtered (Sarstedt's [Helsingborg, Sweden] 0.2 mm polyethersulfone membrane filter) water sourced from Askö Marine Laboratory (station B1: www.smhi.se) with a salinity of 7. Sediment (0.1 mg) was diluted in 1 mL medium in replicates on 24-well plates (Polystyrene, Falcon^{TM}), incubated at 16°C and 10 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) at a 12:12h light:dark cycle. After 3-6 days, the light intensity was increased to 180 µmol photons m^{-2} s^{-1}, and only one individual chain of *S. marinoi* was isolated from each well using standard micro-pipetting techniques (Härnström et al. 2011).

Sixty-nine and 55 strains were isolated from the mining and reference inlet with 88% and 94% survival, respectively. We did not attempt to make cultures axenic as this stresses cells and could impose unwanted selection. Strains were re-inoculated from one to 100 mL of medium, divided and processed in parallel for DNA samples, phenotyping, and the artificial evolution experiments. To minimize laboratory adaptation, all strains were processed within one month after germination, corresponding to <37 generations. During each sub-culture step, the culture was screened for contamination of other phytoplankton species and auxospore formation or bimodal cell sizes indicating sexual inbreeding in *S. marinoi* (Ferrante et al. 2019), and such cultures were discarded (ca. 30%). This left 30 mining and 28 reference strains available for experiments.

Phenotyping of growth rate and copper tolerance

Culture density was routinely measured using *in vivo* chlorophyll*a* (chl *a*) fluorescence (referred to as RFU [relative fluorescence units] from here on) using a Varioscan^{TM} Flash Multimode Reader (ThermoScientific). During re-inoculation, individual culture densities were normalized and reduced to low enough densities to maintain exponential growth until the subsequent dilution, as described in (Andersson et al., 2020). In tandem with seeding the artificial evolution experiment (described below), growth curves (N=4) were collected on individual strains in one mL media on 24-well plates (Polystyrene, Falcon^{TM}) for four days with daily RFU measurements midway into the diel light cycle. Microscopic cell counts and dimension measurements of all

strains (N=58) were collected to convert strain-specific RFU measurements to cell density. Three hundred cells of each strain were counted in Sedgewick-Rafter cell counting chamber (Wildlife Supply CompanyVR, U.S.A.) using an inverted microscope (Axiovert 135, Zeiss), at ×200 magnification. To compute the strain-specific cellular surface-to-volume of strains (Hillebrand et al., 1999), the dimensions of 20 cells per strain were measured at ×400 magnification.

In conjunction with the start of assays, signs of disruption of photosynthetic activity was monitored in all strains using the maximum quantum yield of photosynthesis (F_V/F_M), measured using pulse amplitude modulated chl *a* fluorescence (PAM) on a Phyto-PAM (Schreiber, 1998). Cultures were dark-adapted for 10 min to relax any quenching of chl *a* fluorescence and establish minimal fluorescence level (F_0). After 10 min in darkness, a saturating pulse (200 ms of 10,000 µmol photons m^{-2} s^{-1}) was used to elicit F_M. The maximum quantum yield of photosynthesis (F_V/F_M) was calculated as $(F_M - F_0)/F_M$ using the 630 nm excitation channel.

Acute toxic 72-hrs dose-response curves were used to measure individual strains tolerance to copper. The method was based on the OECD standard (OECD, 2006), and adapted for *S. marinoi* as described in Andersson et al. (2020), with modification to the range of copper concentrations to improve the resolution. Specifically, this study used twelve 1.08-fold dilution steps, covering the range between 5.1 and 13 µM $CuSO_4$, with triplicate replicates around the 5% (7.6 µM), 50% (8.9 µM), and 95% (10 µM) growth inhibitory concentrations established for *S. marinoi* strain RO5AC in pilot experiments. Throughout the experiments we employ strain RO5AC as a mono-clonal reference control to control for experimental artifacts, development of chronic toxic stress and phenotypic plasticity. The copper-amended medium was mixed from 100 µM $CuSO_4$ stocks ˜3 hrs before adding cells. Each strain was treated on a single 24-well plate with the 18 copper treatments, five controls with standard f/2 media (containing f/2 amended levels of 0.04 µM Cu), and an internal plate control containing strain RO5AC in standard f/2. Each replicate was inoculated at 0.003 RFU (˜900 cells mL^{-1}). After 72-hrs exposure, the RFU was measured, and the specific growth rate (day^{-1}) was calculated as follows:

Eq. 1: $$\mu = \frac{\mathrm{LN}(\mathrm{RFU}_{72h}) - LN(\mathrm{RFU}_{0h})}{3}$$

For the dose-response curves, inhibition of growth rate was computed as the growth rate in a copper treatment divided by the average growth rate across the five control replicates. Some strains exhibited strong inhibition of growth in the controls relative to the low copper exposure, which generated normalization artifacts. To mitigate this,t -tests were performed on all dose-response curves, and whenever the control replicates had a significantly (p <0.05) lower growth rate than the three lowest copper treatment concentrations, normalization was done using the average of these values instead of the control. From the dose-response curves, inhibitory concentrations (EC) of 5, 50, and 95 percent were calculated using the drc-package version 3.0-1 (Ritz et al., 2015) in R (R Core Team 2018) with the Weibull type II function [W2.2]. The copper response range was computed as the EC95/EC05. The absolute concentration of copper in the treatment was measured (ICP-OES) several times during the experiments, including before and after cells had grown in the media for three days. Across all samples (N=8), the nominal concentration (Cu_n) correlated well (R^2=0.992) with the observed concentration (Cu_a), and all concentrations presented have been corrected to the absolute µM values using the relationship $Cu_a = Cu_n \times 0.703 - 0.142$.

Artificial evolution experiments

We set up a 42-day-long artificial evolution experiment to assess how strain-selection was affected by copper stress. We aimed for a period that would be long enough to exhaust the strain selection but short enough not to allow for novel mutations to play a role. *De-novo*mutations are unlikely to affect the same strains across multiple replicates (separate experimental bottles), which is how we later validated the assumption of a low impact from new mutations. The two original populations were re-assembled by pooling the 28-30 strains at equal densities based on RFU and distributed at 0.0015 RFU density into ten 100 mL cultures in tissue culture flasks (Polystyrene, Sarstedt). This corresponded to ˜10,000 *S. marinoi* chains per replicate bottle (assuming five cells per chain), which are sufficiently large populations to minimize genetic drift while

allowing for substantial effects of selection (Schlüter et al., 2014). Five replicates were subjected to 8.65 µM copper exposure (strain RO5AC's EC50) and five to non-toxic control conditions. Exponential growth was maintained through semi-continuous cultivation (Wood et al., 2005), with dilutions every third day to maintain the density below 0.5 RFU (Andersson et al., 2020). The two populations were processed six months apart, with one replicate bottle in each treatment containing only strain RO5AC as an internal reference to control for experimental artifacts.

pH, Fv/Fm, and RFU were measured in connection with 3-day re-inoculations and with daily resolution around days 0-3, 15-18, and 30-33. Measurements of pH were used as a control for inorganic carbon limitation, which in our experimental setup occurs above pH˜8.2 (Andersson et al., 2020), and the Fv/Fm as a control for development of chronic stress (Andersson et al., 2022). Aliquot samples were preserved in Lugol's solution and later screened for signs of contamination, sexual reproduction, or spore formation, but there were no such observations throughout the experiment. RFU was used to calculate the growth rate across the three-day re-inoculation periods.

Changes in copper tolerance of the populations was quantified using two approaches. First, 72-h dose-response curves were collected as described for individual strains after zero (N=1), 30 (N=3), and 42 (N=5) days. For the copper selection treatment, copper toxicity was also relaxed for three days at the end of the experimental selection phase, and additional dose-response curves were determined (N=5) to assess if increases in copper tolerance were reversible (plastic) or permanent (evolved). Secondly, a rapid assay of copper tolerance was developed that restricts the cells' capacity to induce plastic responses during the test period. For this test, cells were collected via gentle centrifugation (10 min at 1500 g) and inoculated in lethal concentrations of copper (15 µM), and loss of photosynthetic capacity was quantified using a Phyto-PAM. Specifically, the yield of Photosystem II was monitored under 166 µmol photons $m^{-2}s^{-1}$ actinic light throughout a three hour period. This PAM assay was implemented in the artifical evolution experiments on days three, 24 and 39.

The strain selection process was tracked using a recently developed*S. marinoi* barcoding locus (Pinder et al., 2023), as outlined in the metabarcoding section below. Cell samples for this analysis were collected from the artificial evolution experiment on day zero (N=4, with two DNA extraction replicates × two PCR replicates), nine (N=5 bottle replicates), and 42 (N=5 bottle replicates), via centrifugation (3000 g for 10 min), flash-freezing in liquid N_2, and storage at -80°C until subsequential DNA extraction.

DNA extraction and metabarcoding

DNA was extracted using a CTAB-phenol-chloroform protocol as described in Godhe et al. (2001), with an added RNA digestion step during cell lysis (65ºC for 60 min using one mg RNaseA [mL CTAB buffer]$^{-1}$). Genomic DNA yield was quantified using Qubit (Thermo Fisher Scientific). The evolution experiment used a single barcoding locus to track strain selection. This 523 bp locus,*Sm_C12W1* , is located on contig 12 inside a pentatricopeptide (PPR) repeat region of gene Sm_t00009768-RA, encoding an RNA-binding protein. The locus has 38 SNP positions amongst the 58 strains used in this study, and 110 unique alleles with 100% heterozygosity, including two triploid/aneuploid strains (Pinder et al., 2023). *Sm_C12W1*was amplified from 100 ng DNA from the evolution experiments using the Phusion® High-Fidelity PCR Kit (Thermo Scientific). The primer concentration used was 0.1 µM each of Sm_C12W1-F (5'AGGYTTCGCCTCCTCAAAC3') and Sm_C12W1-R (5'GGCACGATGCACACGCAAAG3'). A One-step PCR reaction with Illumina-adapter extended primers was run for 30 cycles, with five s denaturation (98ºC), five s annealing (62.5ºC), 30 s extension (72ºC), and a final five min extension. The PCR products were quantified using PicoGreen dsDNA kit (Invitrogen) and normalized to 100 ng per sample. Magnet bead-based size selection (>300 bp), Nextera Dual-indexing, pooling, and further library preparation, as well as amplicon sequencing (1/2 of an Illumina MiSeq lane with 300 bp pair-end read with v3 chemistry), was carried out by the National Genomics Infrastructure (Uppsala, Sweden).

Amplicon sequence data were quality controlled and processed as described in (Pinder et al., 2023). Briefly, adapter sequences were removed from reads using Cutadapt version 3.2 (Martin, 2011), trimmed using a

PHRED score >28 and a minimum read length of 180 bp, and merged into amplicons using BBmerge version 38.86 (Bushnell et al., 2017), with default settings. Although DADA2's denoising algorithms (Callahan et al., 2016) could correct a substantial part of amplicons with sequencing errors, it also removed the majority of low frequency allelic observations, potentially generating false negatives (Pinder et al., 2023). We therefore opted to not use DADA2. Instead, we chose to rely on exact sequence matches to known alleles, and utilized strains heterozygosity to quality control and filter data. First, alleles shared between two to three strains were divided according to the abundance of each strain's second unique allele using a set of differential equations (Table S1). In general, the proportion of shared allelei_1 belonging to a specific strain x, y, and z was computed individually for each sample based on the strain's second unique allele (x_2, y_2, andz_2):

Eq. 2:Reads of $i1$ belonging to strain $x = i1 \times \frac{\text{strain } x \text{ allele } x2}{(strain\ x \text{ allele } x2 + strain\ y \text{ allele } y2 + strain\ z \text{ allele } z2)}$

The two heterozygous allele counts were then summed up to represent the abundance of each strain. During this step any strain without observations of both alleles was disregarded as a false positive and counted as absent (through the first part of Eq. 3 below). The third allele of triploid strains was removed. Amplicon counts per strain were normalized to relative abundance (RA) as:

Eq. 3:RA strain $x = \frac{\text{counts allele } x1}{\text{counts allele } x1 \times counts \text{ allele x2}} \times \frac{\text{counts allele } x1 + counts \text{ allele x2}}{\sum \text{counts allels}_{a-z}}$

The changes in the relative abundance of each strain's alleles, combined with the population's overall growth rate over the first nine days ($\mu_{9\text{days}}$) of the evolution experiment, were used to compute each strain's specific growth rate during co-cultivation in the evolution experiment.

Eq. 4:u strain $x = u_{9days} + \frac{\text{LN(RA strain } x \text{ day 9)} - LN(\text{RA strain } x \text{ day 0})}{9}$

Strains not seen in any of the five experimental replicates at day nine were presumed to be extinct since dilution bottelnecks in population size were generally smaller than the sequensing depth. The false discovery rate (FDR) of this approch was quantified by taking advantage of the fact that the two populations were processed independently. FDR was computed by comparing the total number of strain observations (TO) to the False Positive (FP) observations of mining strain seen in a reference sample, or vice versa, accounting for the fact that only half of all FP can be detected this way:

Eq. 5: FDR $= \frac{(FP \times 2)}{\text{TO}}$

All data, scripts and metadata will be made publicaly available opun acceptance (Andersson, Berglund, et al., 2023), or can be accessed at: https://github.com/Bearstar85/Cu_evolution**.**

Results

Strain variability in growth and copper tolerance based on mono-clonal observations

Copper, nickel and lead concentrations were elevated in the sediment deposited at the mining inlet during the past century (see Supplemental information, and Table S2). Across both populations, the copper tolerance (EC50) of mono-clonal strains was close to eight µM copper, and the means did not differ significantly between the two populations (Fig. 1A, Welch t -test, $p = 0.4$. However, the variation was larger in the mining inlet population (F -test, $p = 0.04$). This was driven by three very tolerant strains (VG1-2_81, VG1-2_89, and VG1-2_105) with EC50 above nine µM copper, and VG1-2_67, which was a particularly sensitive strain (Fig. 1A). The comparatively low variability in copper tolerance in the reference inlet population made it difficult to distinguish between the strains' acute 72-hrs dose-responses to copper (Fig. 1B), while amongst the mining inlet strains, more copper tolerance differences could be clearly resolved (Fig. 1C).

Each strain's maximum growth rate was used as a proxy for fitness in a non-toxic environment (Fig S2A). Several strains in both populations experienced sudden cessation, or even periods of negative growth, during the initial growth cycle (Fig. S2B and C). Overall strains from the mining inlet grew faster than the reference (average 1.5±0.28 versus 1.3±0.24 day^{-1}, Welch t -test, $p = 0.02$). Unlike the EC50, which followed a Gaussian distribution (Fig. 2A), the growth rates leveled at an apparent maximum value for each population (Fig. S2A). In the mining site population, three strains stood out with rapid and statistically indistinguishable

growth rates around 1.9 day^{-1} (VG1-2_74, VG1-2_78, and VG1-2_103), while the growth rate of more than half (15) of the fastest growing strains from the reference inlet ranged between 1.4-1.6 day^{-1}(Fig. S2A).

Evolution of copper tolerance

The artificial evolution experiment was performed to test if selection on a population's standing variation of strains allows rapid evolution of copper tolerance (Fig. S1). A detailed description of the experimental approach and the outcome (changes in density, growth rate, pH, and Fv/Fm) is included in the Supplement (Fig. S3). Briefly, chronic toxic effects developed over time, and the copper concentration had to be modified to allow stable growth and maintain inhibition levels at around 50%. Strain RO5AC served as a reference point between the two experiments and was used to control for these treatment differences. During the experiment, the mining population's growth rate increased to about 0.2 day^{-1} higher than RO5AC, while the reference population grew slower than RO5AC throughout the experiment (Fig. S3). The absolute growth rate increases in the mining population, from the start to the end of the selection phase, was 3-fold higher than the growth rate of the reference population (0.50 versus 0.15 day^{-1}). In both populations, and the RO5AC mono-clonal control, the EC50 values increased from around eight to ten µM copper (Fig. 2), which was similar to the mining inlet strain VG1-2_81's initial EC50, but significantly higher than any strain in the reference population (Fig. 1A). The response ranges to copper also expanded relative to the control (Table 1), resulting in adapted populations being tolerant to a wider range of copper concentrations beyond what was lethal to all strains during acute 72-hrs exposure.

In the reference population and in the RO5AC strain, a large part of the increase in copper tolerance (50-100%) was plastic and rapidly lost once the toxic stress was relaxed (Fig. 2A and B). The PAM assay showed that this tolerance was induced in RO5AC after only three days of exposure to copper, whereas it took up to 21 days before it was attained in the reference population (Fig. 3). In the reference population, four of the copper-exposed experimental replicates grew poorly in ambient media without excessive copper (Fig. S4), indicating that copper tolerance carries a cost in terms of limited ability to reach high cell densities in low copper environments. Collectively, these observations show that at least some strains of the reference population acclimated to toxic conditions via a plastic response to copper stress, and that this effect was not accurately captured by the acute 72-h dose-response experiment.

Strain selection during artificial evolution

The strain selection process was tracked using strain-specific metabarcoding of a locus with high allelic richness of 110 unique alleles in the 59 strains. At the start of the experiment, individual strains contributed between 1.9 to 6.3% cells of the start population based on microscopic counts. Both alleles of all strains were also detected initially (Fig. 4), although some were observed at up to three-fold higher or lower relative abundance compared with microscopic cell counts. Out of 669 strain observations across all samples, only 22 were of a strain in the wrong population, likely because of sequencing errors in key SNP positions or PCR chimeras, suggesting a FDR of 0.065. These false positive observations amounted to a negligible amount of total strain observations (on average 0.02% [SD: 0.05] per sample) but could affect absent/present scoring and underestimate the number of extinct strains. After 42 days, a single strain generally dominated 30-99% of the total strain abundance and replicates typically delivered the same dominant strain, but different strains were selected for in the copper and control treatment (Fig. 4).

The metabarcoding revealed that, as expected for the mining population, the tolerant VG1-2_81 and VG1-2_74 strains became the most abundant ones in all copper selection replicates, with a joint final abundance of 37 to 99% (Fig S5). VG1-2_81 was initially highly competitive in the control conditions but VG1-2_103 eventually outcompeted it and other strains in this treatment, making up 22-90% of all amplicons in all five replicates on day 42 (Table 1). VG1-2_103 was also the second fastest-growing strain and statistically indistinguishable from the other two when grown as a mono-clonal culture (Fig. S2).

The selection outcome of the reference population deviated more from expectations based on mono-clonal traits (Fig. 5). In the control, GP2-4_40, a strain with an observed average growth rate of only 1.32 ± 0.11 day^{-1}, dominated all five replicates after 42 days of selection (37-90%, Fig. 4), with several more strains

retained at 1-25% abundance (Table 1, Fig S4). In the copper treatment, GP2-4_27, which had a below-average EC50 (7.8 µM Cu; Fig. S6), outcompeted everyone else in four out of five replicates (93-97%, Fig. 3) and was thus identified as the strain responsible for the plastic tolerance developing in this population (Fig. 2 and 3). In the last replicate, GP2-4_57, a strain that was extinct in the other four, as well as in all the controls, became dominant (78%, Fig. S5). Importantly, like all strains at the beginning of the experiment, GP2-4_57 was heterozygous for the barcoding locus *Sm_C12W1* , but all 9,099 amplicons observed at day 42 were from only one of its two alleles (Fig. S6), indicating a loss of heterozygosity, which could be explained through inbreeding. Furthermore, this bottle replicate had its own evolutionary trajectory and developed higher copper tolerance (EC50 11.2 µM Cu, versus 9.45-10.8) but slower growth rate (46 generations versus 54-59) than the GP2-4_27 dominated replicates (Table 1).

The metabarcoded relative abundances of strains were used to disentangle individual growth rates during co-cultivation. The barcoded copper tolerance traits were not correlated with other mono-clonal strain traits like cellular surface-to-volume ratios, Fv/Fm, or growth rate (Fig. S7 and S8). Several strains had already gone extinct after nine days, especially in the Mining population and copper treatment, where nine out of 30 were lost (Fig. 5). The remaining strains' growth rates in the reference population correlated poorly against what was predicted from mono-clonal observations, with R^2 of 0.003,p =0.8 (copper), and 0.07, p =0.2 (control, without the outlier-strain GP2-4_42). Correlations were higher in the Mining experiments with R^2 of 0.39, p =0.002 (copper) and 0.24, p =0.006 (control, without outlier-strain VG1-2_63), but it was still difficult to distinguish growth rates between many strains with confidence (Fig. 1, S1, and 5). Importantly the precision of the metabarcode-derived growth rates was, on average, three times higher for the barcoded growth rates (95% conf. +/- 0.038 day^{-1}) compared with the mono-clonally estimated rates (+/- 0.11 day^{-1}), showing that this approach has much higher chance of detecting subtle strain differences in fitness. Furthermore, the evolutionary trajectories observed via metabarcoding on day nine generally persisted for the final 33 days of the evolution experiment (Fig. 4, 5, S4, and S5). Consequently, a short selection experiment of pooled populations of strains, combined with observations of strain abundance using intraspecific metabarcoding, appears to be a robust approach to estimate both fitness of individual strains and the evolutionary potential of phytoplankton populations.

Discussion

Phytoplankton communities respond quickly to environmental changes across seasons largely by a dynamic relationship among species, however, the evolutionary potential and adaptability of individual species are still not well understood. Past experimental evolution studies have shown that strong directional selection pressures can lead to adaptation through *de novo* mutations, often within a few hundred generations (Collins & Bell, 2004; Collins et al., 2014; Malerba et al., 2020; Schaum et al., 2018). However, the role of selection on standing genetic variation has to date not been directly assessed. In this study, we utilized a newly developed strain-specific metabarcoding approach to track selection among strains in two populations of a common pelagic diatom, and we were able to assess how rapidly selection acts on standing genetic variation. Our first hypothesis was that the diatom*S. marinoi* had evolved elevated tolerance to copper at a mining-exposed inlet. We found some support for this hypothesis from the observation that a small subset (10-15%) of the strains were much more tolerant to copper stress than any of the strains from a reference inlet. Secondly, we hypothesized that genetic variation would allow the population exposed to copper close to a mining site to evolve copper tolerance more rapidly, and with a larger amplitude, than from a single strain genotype, or from an unexposed population. This hypothesis was also supported by the outcome of the artificial evolution experiment where the mining population rapidly selected for the most tolerant strain in less than 50 generations and recovered more than three times more fitness (growth rate) than the mono-clonal RO5AC strain and the reference population.

Metabarcoding revealed that strain selection drove the evolutionary responses of our artificial populations, providing empirical support to the hypothesis that standing genetic variation within phytoplankton populations can support rapid adaptation (Godhe & Rynearson, 2017). In contrast, our results did not suggest that *de-novo* mutations were involved. Mutations are, of course, the ultimate source of genetic variation,

and that we observed no tolerant strains at the reference site suggests that the trait had evolved locally during centuries of mining exposure, presumably through mutations and/or recombination. Acquiring *de-novo* mutations is generally a slow process because the vast majority of mutations are near neutral or deleterious (Kimura, 1983; Ohta, 1992) and restricted to affecting one locus at a time (Karve & Wagner, 2022; Tupin et al., 2010), which is why such processes could not rival selection from standing variation over relatively short experimental periods, such as in our experiment (50-100 generations).

In one isolated replicate, sexual recombination and loss of heterozygosity caused a strain to develop a hyper copper tolerant phenotype. This observation suggests that much of the fitness variability that *de-novo* mutations could rapidly generate may already reside in natural populations and be available for selection to act upon, especially if recombined into diverse combinations. This is a reasonable expectation as phytoplankton populations harbor up to three percent single nucleotide polymorphic diversity across the genome (Flowers et al., 2015; Mock et al., 2017), contain large-scale re-arrangements across species pan genomes (Blanc-Mathieu et al., 2017; Kashtan et al., 2014; Osuna-Cruz et al., 2020; Read et al., 2013), and can facilitate substantial rates of horizontal gene transfer (Vancaester et al., 2020). Such high standing genetic variation, combined with recombination during meiosis or horizontal gene transfer, should enable phytoplankton populations to rapidly combine favorable alleles from distant loci, strains, or even separate taxa. It is therefore reasonable to expect that similar to the situation in macroorganisms (Barrett & Schluter, 2008), outcrossing and selection from standing genetic variation should provide the primary potential for evolutionary change in phytoplankton populations, and provide short-term adaptations to both seasonal changes, spatial heterogeneity, and anthropogenic stressors such as metal pollution.

Strong toxic selection pressures are expected to purge sensitive genotypes from a population (Blanck, 2002). In contrast, our results showed that sensitive strains persisted in the mining exposed population but that a small subset of strains (three out of 30) had evolved, and retained, permanently high tolerance to copper. This suggests that the mining site population is currently not experiencing a constant strong selection pressure from copper, or that a trade-off between toxic copper tolerance and other components of fitness exists, such as nutritional copper uptake (Sunda, 2012). The lack of tolerant strains at the reference site, and inhibition of adapted cultures by growth media with low/regular copper concentrations, support the notion of a fitness cost associated with high copper tolerance. There is unfortunately no available timeseries of monitoring data of water concentrations of metals from the mining area around Västervik Gåsfjärden, and we can only speculate on the selective processes that shaped copper tolerance of the two *S. marinoi* populations.

Since the mining activity ceased ca. 1920, and because metal concentrations in the sediment have declined since the 1980s (Ning et al., 2018), toxic exposure could be a historical event dating back to the active mining period. Alternatively, the mining inlet population may still experience fluctuating selection pressures between toxic and non-toxic copper conditions, as the mining tailings are still exposed to varying degrees of weathering. The fact that we chose to assay copper tolerance in the resting stage population rather than the actively growing planktonic population may also have influenced the copper tolerance trait distribution. Resting stages can remain viable for at least a decade (Lewis et al., 1999), potentially even centuries (Härnström et al., 2011), providing an evolutionary buffer against loss of diversity during periods of strong directional selection on the planktonic population (Sundqvist et al., 2018). Therefore, our populations likely contain resting stages from multiple bloom seasons, and tolerant strains may have been deposited during phases of high copper concentration, and sensitive ones during ambient conditions. However, we also cannot exclude the possibility that the larger variation in the mining population stems from spurious effect from having only two sampling locations, or other sampling artifacts. Because of the 10-fold slower sedimentation rate at the mining inlet (Supplemental Method and Results), we germinated resting stages from sediment deposited between 1995-2010, compared with 2012-2015 at the reference inlet. The potentially older and more extended deposition range at the mining inlet may have captured a more diverse set of strains than at the reference inlet. Irresectable of the driver of the larger variation in copper tolerance in the mining exposed population, our finding highlights the important of incorporate large amounts of strain diversity in adaptive studies of phytoplankton.

The variation in copper tolerance among distinct experimental strains raises the question of how much variation there is in a natural population. With a few notable exceptions (Ajani et al., 2020; Gross et al., 2017; Schaum et al., 2016), artificial evolution experiments and monoculture phenotyping studies incorporate less than ten strains to represent an entire population or species (Lohbeck et al., 2012; Ribeiro et al., 2011; Sassenhagen et al., 2015; Wolf et al., 2019). This is likely insufficient to represent the actual diversity of most phytoplankton populations, not least during blooms when they are predicted to contain thousands to millions of unique clones (Sassenhagen et al., 2021). Furthermore, pan-genome studies show that even if hundreds of strains are sequenced, they generally do not saturate (Kashtan et al., 2014). Mesocosm experiments using natural communities can incorporate sufficiently large population sizes to represent clonal population diversity, yet such experiments are challenging to analyze and maintain, and still rare [but see (Schaum et al., 2017; Scheinin et al., 2015)].

Using a strain-specific metabarcoding approach (Pinder et al., 2023) improve our experimental analysis in several ways. First, strain-specific metabarcoding estimates of fitness (growth rate and copper tolerance) provided, on average, three times higher precision than mono-clonal experiments. Second, the reduced number of experimental bottle replicates of mixed-culture experiments enabled longer and more complex experiments to be performed (Gresham & Dunham, 2014). Running selection experiments for a long time added the benefit that it also resolved if plastic responses develop over time, and if the plastic potential differed between strains. In our experiments, metabarcoding revealed that certain strains could develop a high degree of plasticity relatively slowly (over 2-10 generations), which the mono-clonal dose-response curves failed to capture. This was somewhat surprising since the 72-hrs dose-response assay proceeded over multiple generations (3-7 at <EC50), which is often sufficient to capture the complete acclimation response in phytoplankton (Falkowski & LaRoche, 1991).

Third, with the strain-specific metabarcoding we could incorporate more strains without added experimental effort. Since we have developed three additional barcode loci for *S. marinoi* (Pinder et al., 2023), multiplexing of several barcodes should enable separation between even more strains, or strains that are not clones, but homologous in the locus *Sm_C12W1* . However, such approaches will only be possible if the allelic genotypes of all strains in the selection experiment are known, something that in diploid taxa requires strain isolation and genotyping with molecule resolution (through molecular resolution sequencing) to parse out the alleles. Yet with this added sequencing effort, strain-specific metabarcoding experiments should be able to incorporate much higher amounts of diversity than the 58 strains used in our study.

Finally, a strain-specific metabarcoding approach is arguably more accurate in determining the relative fitness of strains, compared with mono-clonal fitness estimations. In part, this is because growth in mixed population removes bottle effects and other experimental artifacts associated with mono-clonal phenotyping (Robinson et al., 2014). More importantly, mixed culture experiments can incorporate interactions between strains and their shared aqueous environment. Strain-specific metabarcoding should therefore be compatible with mesocosm experiments on natural plankton communities (Scheinin et al., 2015; Tatters et al., 2013), or experiments investigating the evolutionary effects of predation (Sjöqvist et al., 2014), nutrient competition within (Collins, 2011) and between species (Descamps-Julien & Gonzalez, 2005), or other fitness traits that are challenging to determine using mono-clonal assays.

Conclusions

We have shown that copper tolerance has evolved in a small subset of strains in a local mining-exposed population of *S. marinoi* , and that the resting stage population have retained these strains even though the mining activity and pollution level has declined. Our artificial evolution experiment highlights that selection from such standing genetic variation in phytoplankton enables populations to respond to environmental stress much faster than through *de-novo*mutations. It also suggests that extensive species dynamics might be due to evolutionarily important strains that sometimes occur in low frequency but can be swept to high frequencies by directional selection under specific conditions. Importantly, our experimental approach exemplifies how strain-specific metabarcoding can be employed to track selection and quantify strain fitness during co-cultivation, enabling incorporation of higher amounts of strains than other approaches permit.

With careful experimental design, future strain-specific metabarcoding experiments should be able to track selection processes in diverse populations inhabiting dynamic and complex environments, more similar to the challenges that phytoplankton face in their natural habitat. Although further development and evaluations of the analytical approaches in mixed DNA samples of unknown allele composition are warranted, it should in theory be possible to track selection processes in natural populations based *in situ* sampling of environmental DNA from monitoring programs or targeted sampling efforts. Collectively such approaches will yield important new insight into how the intraspecific diversity enables phytoplankton to adapt to life in a dynamic environment.

Data Accessibility and Benefit-Sharing

Data has been deposited to Swedish National Data Service (https://snd.gu.se/en) and will be publicly available with DOI upon acceptance. Analyses and R-scripts [together with relevant data and metadata] can be accessed at https://github.com/Bearstar85/Cu_evolution, except the metabarcoding sequence mapping pipeline, which is available at https://github.com/topel-research-group/Live2Tell. Data has been deposited to Swedish National Data Service (*https://snd.gu.se/en*) and will be publicly available with DOI upon acceptance. Raw Illumina MiSeq sequences have been deposited at NCBI under BioProject PRJNA939970.

Author Contributions

BA, AG, OB, HLF, and KR identified the research question and designed the experiments. BA, OK, and LH conducted the experiments and laboratory work. MT, MIMP, and BA performed the bioinformatic work and analyzed the data. BA drafted the first version of the manuscript and all authors contributed to the final version.

Acknowledgments

The Captain and the crew of r/v Electra af Askö and Johan Burman, and Kotaro Hirose for sediment sampling assistance. Jakob Walve for facilitating water collection at Askö B1 station. The project was funded by the Swedish Research Council Formas grant number 2016-00594 and the Oscar and Lili Lamm Foundation grant number FO2018-0042. The authors acknowledge support from the National Genomics Infrastructure in Stockholm funded by Science for Life Laboratory, the Knut and Alice Wallenberg Foundation and the Swedish Research Council, and SNIC/Uppsala Multidisciplinary Center for Advanced Computational Science for assistance with massively parallel sequencing and access to the UPPMAX computational infrastructure.

References

Ajani, P. A., Petrou, K., Larsson, M. E., Nielsen, D. A., Burke, J., & Murray, S. A. (2020). Phenotypic trait variability as an indication of adaptive capacity in a cosmopolitan marine diatom. *Environmental Microbiology, 23* , 207–223. doi:10.1111/1462-2920.15294

Andersson, B., Berglund, O., Filipsson, H. L., Kourtchenko, O., Godhe, A., Johannesson, K., . . . Rengefors, K. (2023). Dataset: Evolution of copper tolerance in the coastal diatom Skeletonema marinoi.*https://snd.gu.se/en/, 2023-47* . doi:Included upon acceptance

Andersson, B., Godhe, A., Filipsson, H. L., Rengefors, K., & Berglund, O. (2020). Differences in metal tolerance among strains, populations, and species of marine diatoms-importance of exponential growth for quantification. *Aquatic Toxicology, 226* , 105551. doi:10.1016/j.aquatox.2020.105551.

Andersson, B., Godhe, A., Filipsson, H. L., Zetterholm, L., Edler, L., Berglund, O., & Rengefors, K. (2022). Intraspecific variation in metal tolerance modulate competition between two marine diatoms. *The ISME journal, 16* , 511–520. doi:10.1038/s41396-021-01092-9

Andersson, B., Rengefors, K., Kourtchenko, O., Johannesson, K., Berglund, O., & Filipsson, H. L. (2023). Cross-contamination risks in sediment-based resurrection studies of phytoplankton. *Limnology and Oceanography Letters* . doi:10.1002/lol2.10291

Barrett, R. D., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in Ecology & Evolution, 23* , 38-44.

Bazzicalupo, A. L., Ruytinx, J., Ke, Y.-H., Coninx, L., Colpaert, J. V., Nguyen, N. H., . . . Branco, S. (2020). Fungal heavy metal adaptation through single nucleotide polymorphisms and copy-number variation.*Molecular Ecology, 29* , 4157-4169. doi:10.1111/mec.15618

Blanc-Mathieu, R., Krasovec, M., Hebrard, M., Yau, S., Desgranges, E., Martin, J., . . . Donnadieu, C. (2017). Population genomics of picophytoplankton unveils novel chromosome hypervariability.*Science advances, 3* , e1700239. doi:10.1126/sciadv.1700239

Blanck, H. (2002). A critical review of procedures and approaches used for assessing pollution-induced community tolerance (PICT) in biotic communities. *Human and Ecological Risk Assessment, 8* , 1003-1034. doi:10.1080/1080-700291905792

Blanck, H., & Dahl, B. (1996). Pollution-induced community tolerance (PICT) in marine periphyton in a gradient of tri-n-butyltin (TBT) contamination. *Aquatic Toxicology, 35* , 59-77. doi:10.1016/0166-445X(96)00007-0

Bushnell, B., Rood, J., & Singer, E. (2017). BBMerge–accurate paired shotgun read merging via overlap. *PloS one, 12* , e0185056. doi:10.1371/journal.pone.0185056

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13* , 581-583. doi:10.1038/nmeth.3869

Carlson, H. K., Price, M. N., Callaghan, M., Aaring, A., Chakraborty, R., Liu, H., . . . Deutschbauer, A. M. (2019). The selective pressures on the microbial community in a metal-contaminated aquifer. *The ISME journal, 13* , 937-949.

Collins, S. (2011). Competition limits adaptation and productivity in a photosynthetic alga at elevated CO2. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 278* , 247-255. doi:10.1098/rspb.2010.1173

Collins, S., & Bell, G. (2004). Phenotypic consequences of 1,000 generations of selection at elevated CO2 in a green alga. *Nature, 431* , 566-569. doi:10.1038/nature02945

Collins, S., Rost, B., & Rynearson, T. A. (2014). Evolutionary potential of marine phytoplankton under ocean acidification. *Evol Appl, 7* , 140-155. doi:10.1111/eva.12120

Descamps-Julien, B., & Gonzalez, A. (2005). Stable coexistence in a fluctuating environment: an experimental demonstration. *Ecology, 86* , 2815-2824. doi:10.1890/04-1700

Falkowski, P. G., & LaRoche, J. (1991). Acclimation to spectral irradiance in algae. *Journal of Phycology, 27* , 8-14. doi:10.1111/j.0022-3646.1991.00008.x

Flowers, J. M., Hazzouri, K. M., Pham, G. M., Rosas, U., Bahmani, T., Khraiwesh, B., . . . Harris, E. H. (2015). Whole-genome resequencing reveals extensive natural variation in the model green alga*Chlamydomonas reinhardtii* . *The Plant Cell, 27* , 2353-2369. doi:10.1105/tpc.15.00492

Foster, P. L. (1982). Metal resistances of Chlorophyta from rivers polluted by heavy metals. *Freshwater Biology, 12* , 41-61. doi:10.1111/j.1365-2427.1982.tb00602.x

Godhe, A., Otta, S. K., Rehnstam-Holm, A. S., Karunasagar, I., & Karunasagar, I. (2001). Polymerase chain reaction in detection of*Gymnodinium mikimotoi* and *Alexandrium minutum* in field samples from southwest India. *Marine Biotechnology, 3* , 152-162. doi:10.1007/s101260000052

Godhe, A., & Rynearson, T. (2017). The role of intraspecific variation in the ecological and evolutionary success of diatoms in changing environments. *Philosophical Transactions of the Royal Society B: Biological*

Sciences, 372 , 20160399. doi:10.1098/rstb.2016.0399

Godhe, A., Sjöqvist, C., Sildever, S., Sefbom, J., Harardóttir, S., Bertos-Fortis, M., . . . Jonsson, P. R. (2016). Physical barriers and environmental gradients cause spatial and temporal genetic differentiation of an extensive algal bloom. *Journal of Biogeography, 43* , 1130-1142. doi:10.1111/jbi.12722

Grangeteau, C., David, V., Herve, A., Guilloux-Benatier, M., & Rousseaux, S. (2017). The sensitivity of yeasts and yeasts-like fungi to copper and sulfur could explain lower yeast biodiversity in organic vineyards. *FEMS Yeast Research, 17* , fox092.

Gresham, D., & Dunham, M. J. (2014). The enduring utility of continuous culturing in experimental evolution. *Genomics, 104* , 399-405.

Gross, S., Kourtchenko, O., Rajala, T., Andersson, B., Fernandez, L., Blomberg, A., & Godhe, A. (2017). Optimization of a high-throughput phenotyping method for chain-forming phytoplankton species.*Limnology and Oceanography: Methods, 16* , 57-67 doi:10.1002/lom3.10226

HELCOM. (2010). Hazardous substances in the Baltic Sea. *Balt. Sea Environ. Proc. No. 120B.* , Helsinki Commission,116. Retrieved from*http://www.helcom.fi/Lists/Publications/BSEP120B.pdf*

Hillebrand, H., Durselen, C. D., Kirschtel, D., Pollingher, U., & Zohary, T. (1999). Biovolume calculation for pelagic and benthic microalgae. *Journal of Phycology, 35* , 403-424. doi:10.1046/j.1529-8817.1999.3520403.x

Hutchinson, G. E. (1961). The paradox of the plankton. *American Naturalist, 95* , 137-145.

Harnstrom, K., Ellegaard, M., Andersen, T. J., & Godhe, A. (2011). Hundred years of genetic structure in a sediment revived diatom population. *Proceedings of the National Academy of Sciences, USA, 108* , 4252–4257. doi:10.1073/pnas.1013528108

Janssens, T. K., Roelofs, D., & Van Straalen, N. M. (2009). Molecular mechanisms of heavy metal tolerance and evolution in invertebrates.*Insect Science, 16* , 3-18. doi:10.1111/j.1744-7917.2009.00249.x

Kalinowska, R., & Pawlik-Skowrońska, B. (2008). Metal resistance of soil algae (Chlorophyta) occurring in post-flotation Zn/Pb-and Cu-tailing ponds. *Polish Journal of Ecology, 56* , 415-430.

Karlsson, M., Malmaeus, M., Rydin, E., & Jonsson, P. (2010). Bottenundersökningar i Upplands, Stockholms, Södermanlands och Östergötlands skärgårdar 2008-2009. *Rapport B1928, IVL Svenska Miljöinstitutet, Stockholm* . Retrieved from*www.ivl.se*

Karve, S., & Wagner, A. (2022). Environmental complexity is more important than mutation in driving the evolution of latent novel traits in *E. coli* . *Nature Communications, 13* , 5904. doi:10.1038/s41467-022-33634-w

Kashtan, N., Roggensack, S. E., Rodrigue, S., Thompson, J. W., Biller, S. J., Coe, A., . . . Chisholm, S. W. (2014). Single-cell genomics reveals hundreds of coexisting subpopulations in wild*Prochlorococcus* . *Science, 344* , 416-420. doi:10.1126/science.1248575

Kerfoot, W. C., Robbins, J. A., & Weider, L. J. (1999). A new approach to historical reconstruction: combining descriptive and experimental paleolimnology. *Limnology and Oceanography, 44* , 1232–1247. doi:10.4319/lo.1999.44.5.1232

Kimura, M. (1983). *The neutral theory of molecular evolution* : Cambridge University Press.

Lehtonen, K. K., Bignert, A., Bradshaw, C., Broeg, K., & Schiedek, D. (2017). Chemical pollution and ecotoxicology. In P. S. Snoeijs-Leijonmalm, H.: & T. Radziejewska (Eds.), *Biological Oceanography of the Baltic Sea* (pp. 547-589). Dordrecht, The Netherlands: Springer Nature.

Lemire, J. A., Harrison, J. J., & Turner, R. J. (2013). Antimicrobial activity of metals: mechanisms, molecular targets and applications.*Nature Reviews Microbiology, 11* , 371-384. doi:10.1038/nrmicro3028

Lewis, J., Harris, A. S. D., Jones, K. J., & Edmonds, R. L. (1999). Long-term survival of marine planktonic diatoms and dinoflagellates in stored sediment samples. *Journal of Plankton Research, 21* , 343–354. doi:10.1080/00071618000650361

Lohbeck, K. T., Riebesell, U., & Reusch, T. B. (2012). Adaptive evolution of a key phytoplankton species to ocean acidification.*Nature Geoscience, 5* , 346. doi:10.1038/ngeo1441

Macnair, M. R. (1987). Heavy metal tolerance in plants: A model evolutionary system. *Trends in Ecology & Evolution, 2* , 354-359. doi:10.1016/0169-5347(87)90135-2

Malerba, M. E., Marshall, D. J., Palacios, M. M., Raven, J. A., & Beardall, J. (2020). Cell size influences inorganic carbon acquisition in artificially selected phytoplankton. *New Phytologist, 229* , 2647-2659. doi:10.1111/nph.17068

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal, 17* , 10-12. doi:10.14806/ej.17.1.200

McQuoid, M. R., Godhe, A., & Nordberg, K. (2002). Viability of phytoplankton resting stages in the sediments of a coastal Swedish fjord. *European Journal of Phycology, 37* , 191–201. doi:10.1017/S0967026202003670

Mock, T., Otillar, R. P., Strauss, J., McMullan, M., Paajanen, P., Schmutz, J., . . . Grigoriev, I. V. (2017). Evolutionary genomics of the cold-adapted diatom *Fragilariopsis cylindrus* . *Nature, 541* , 536-540. doi:10.1038/nature20803

Montresor, M., Di Prisco, C., Sarno, D., Margiotta, F., & Zingone, A. (2013). Diversity and germination patterns of diatom resting stages at a coastal Mediterranean site. *Marine Ecology Progress Series, 484* , 79–95. doi:10.3354/meps10236

Ning, W., Ghosh, A., Jilbert, T., Slomp, C. P., Khan, M., Nyberg, J., . . . Filipsson, H. L. (2016). Evolving coastal character of a Baltic Sea inlet during the Holocene shoreline regression: impact on coastal zone hypoxia. *Journal of Paleolimnology, 55* , 319–338. doi:10.1007/s10933-016-9882-6

Ning, W., Nielsen, A., Ivarsson, L. N., Jilbert, T., Åkesson, C., Slomp, C., . . . Filipsson, H. (2018). Anthropogenic and climatic impacts on a coastal environment in the Baltic Sea over the last 1000 years.*Anthropocene, 21* , 66–79. doi:10.1016/j.ancene.2018.02.003

Ning, W., Tang, J., & Filipsson, H. L. (2016). Long-term coastal openness variation and its impact on sediment grain-size distribution: a case study from the Baltic Sea. *Earth Surface Dynamics, 4* , 773–780. doi:10.5194/esurf-4-773-2016

OECD. (2006). *Test No. 201: Alga, Growth Inhibition Test. Retrived from: https://www.oecd-ilibrary.org/content/publication/9789264069923-en.*.

Ohta, T. (1992). The Nearly Neutral Theory of Molecular Evolution.*Annual Review of Ecology and Systematics, 23* , 263-286. doi:10.1146/annurev.es.23.110192.001403

Osuna-Cruz, C. M., Bilcke, G., Vancaester, E., De Decker, S., Bones, A. M., Winge, P., . . . Audoor, S. (2020). The *Seminavis robusta*genome provides insights into the evolutionary adaptations of benthic diatoms. *Nature Communications, 11* , 3320. doi:10.1038/s41467-020-17191-8

Pesce, S., Lissalde, S., Lavieille, D., Margoum, C., Mazzella, N., Roubeix, V., & Montuelle, B. (2010). Evaluation of single and joint toxic effects of diuron and its main metabolites on natural phototrophic biofilms using a pollution-induced community tolerance (PICT) approach.*Aquatic Toxicology, 99* , 492-499. doi:10.1016/j.aquatox.2010.06.006

Pinder, M. I. M., Andersson, B., Rengefors, K., Blossom, H. B., Svensson, M., & Töpel, M. (2023). Bamboozle: A bioinformatic tool for identification and quantification of intraspecific barcodes.*bioRxiv* . doi:10.1101/2023.03.16.532925

Pinseel, E., Nakov, T., Van den Berge, K., Downey, K. M., Judy, K. J., Kourtchenko, O., . . . Töpel, M. (2022). Strain-specific transcriptional responses overshadow salinity effects in a marine diatom sampled along the Baltic Sea salinity cline. *The ISME journal, 16* , 1776–1787. doi:10.1038/s41396-022-01230-x

Read, B. A., Kegel, J., Klute, M. J., Kuo, A., Lefebvre, S. C., Maumus, F., . . . Salamov, A. (2013). Pan genome of the phytoplankton*Emiliania* underpins its global distribution. *Nature, 499* , 209-213. doi:10.1038/nature12221

Rengefors, K., Kremp, A., Reusch, T. B., & Wood, A. M. (2017). Genetic diversity and evolution in eukaryotic phytoplankton: revelations from population genetic studies. *Journal of Plankton Research, 39* , 165-179. doi:10.1093/plankt/fbw098

Reusch, T. B., & Boyd, P. W. (2013). Experimental evolution meets marine phytoplankton. *Evolution, 67* , 1849-1859. doi:10.1111/evo.12035

Reusch, T. B., Dierking, J., Andersson, H. C., Bonsdorff, E., Carstensen, J., Casini, M., . . . Hyytiäinen, K. (2018). The Baltic Sea as a time machine for the future coastal ocean. *Science advances, 4* , eaar8195. doi:10.1126/sciadv.aar8195

Ribeiro, S., Berge, T., Lundholm, N., Andersen, T. J., Abrantes, F., & Ellegaard, M. (2011). Phytoplankton growth after a century of dormancy illuminates past resilience to catastrophic darkness. *Nature Communications, 2* , 311. doi:10.1038/ncomms1314

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-response analysis using R. *PloS one, 10* , e0146021. doi:10.1371/journal.pone.0146021

Robinson, D. G., Chen, W., Storey, J. D., & Gresham, D. (2014). Design and analysis of Bar-seq experiments. *G3: Genes, Genomes, Genetics, 4* , 11-18. doi:10.1534/g3.113.008565

Sassenhagen, I., Erdner, D., Lougheed, B., Richlen, M., & Sjöqvist, C. (2021). Estimating proportion of clones and genotype richness in aquatic microalgae. *Authorea Preprints* . doi:10.22541/au.161876383.32271114/v1

Sassenhagen, I., Wilken, S., Godhe, A., & Rengefors, K. (2015). Phenotypic plasticity and differentiation in an invasive freshwater microalga. *Harmful Algae, 41* , 38-45. doi:10.1016/j.hal.2014.11.001

Schaum, C.-E., Barton, S., Bestion, E., Buckling, A., Garcia-Carreras, B., Lopez, P., . . . Trimmer, M. (2017). Adaptation of phytoplankton to a decade of experimental warming linked to increased photosynthesis.*Nature Ecology & Evolution, 1* , 1-7. doi:10.1038/s41559-017-0094

Schaum, C.-E., Buckling, A., Smirnoff, N., Studholme, D., & Yvon-Durocher, G. (2018). Environmental fluctuations accelerate molecular evolution of thermal tolerance in a marine diatom.*Nature Communications, 9* , 1719. doi:10.1038/s41467-018-03906-5

Schaum, C.-E., Rost, B., & Collins, S. (2016). Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. *The ISME journal, 10* , 75-84. doi:10.1038/ismej.2015.102

Schaum, C. E., & Collins, S. (2014). Plasticity predicts evolution in a marine alga. *Proceedings of the Royal Society B: Biological Sciences, 281* , 20141486. doi:doi:10.1098/rspb.2014.1486

Scheinin, M., Riebesell, U., Rynearson, T. A., Lohbeck, K. T., & Collins, S. (2015). Experimental evolution gone wild. *Journal of the Royal Society Interface, 12* , 20150056. doi:10.1098/rsif.2015.0056

Schlüter, L., Lohbeck, K. T., Gutowska, M. A., Gröger, J. P., Riebesell, U., & Reusch, T. B. (2014). Adaptation of a globally important coccolithophore to ocean warming and acidification. *Nature Climate Change, 4* , 1024.

Schmitt, H., Haapakangas, H., & van Beelen, P. (2005). Effects of antibiotics on soil microorganisms: time and nutrients influence pollution-induced community tolerance. *Soil Biology and Biochemistry, 37* , 1882-1892.

Schreiber, U. (1998). Chlorophyll fluorescence: New Instruments for Special Applications. In G. Garab (Ed.), *Photosynthesis: mechanisms and effects* (pp. 4253-4258). Springer, Dordrecht: Springer.

Sefbom, J., Kremp, A., Rengefors, K., Jonsson, P. R., Sjoqvist, C., & Godhe, A. (2018). A planktonic diatom displays genetic structure over small spatial scales. *Environmental Microbiology, 20* , 2783-2795. doi:10.1111/1462-2920.14117

Sjöqvist, C., Godhe, A., Jonsson, P. R., Sundqvist, L., & Kremp, A. (2015). Local adaptation and oceanographic connectivity patterns explain genetic differentiation of a marine diatom across the North Sea-Baltic Sea salinity gradient. *Molecular Ecology, 24* , 2871-2885. doi:10.1111/mec.13208

Sjöqvist, C., Kremp, A., Lindehoff, E., Båmstedt, U., Egardt, J., Gross, S., . . . Richter, H. (2014). Effects of grazer presence on genetic structure of a phenotypically diverse diatom population. *Microbial Ecology, 67* , 83-95. doi:10.1007/s00248-013-0327-8

Sunda, W. (2012). Feedback Interactions between Trace Metal Nutrients and Phytoplankton in the Ocean. *Frontiers in Microbiology, 3* , 204. doi:10.3389/fmicb.2012.00204

Sundqvist, L., Godhe, A., Jonsson, P. R., & Sefbom, J. (2018). The anchoring effect-long-term dormancy and genetic population structure. *The ISME journal, 12* , 2929-2941. doi:10.1038/s41396-018-0216-8

Söderhielm, J., & Sundblad, K. (1996). The Solstad Cu-Co-Au mineralization and its relation to post-Svecofennian regional shear zones in southeastern Sweden. *GFF, 118* , 47-47. doi:10.1080/11035899609546323

Tatters, A. O., Roleda, M. Y., Schnetzer, A., Fu, F., Hurd, C. L., Boyd, P. W., . . . Hutchins, D. A. (2013). Short-and long-term conditioning of a temperate marine diatom community to acidification and warming. *Philosophical Transactions of the Royal Society B: Biological Sciences, 368* , 20120437. doi:10.1098/rstb.2012.0437

Tupin, A., Gualtieri, M., Roquet-Baneres, F., Morichaud, Z., Brodolin, K., & Leonetti, J.-P. (2010). Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. *International Journal of Antimicrobial Agents, 35* , 519-523. doi:10.1016/j.ijantimicag.2009.12.017

Vancaester, E., Depuydt, T., Osuna-Cruz, C. M., & Vandepoele, K. (2020). Comprehensive and Functional Analysis of Horizontal Gene Transfer Events in Diatoms. *Molecular Biology and Evolution, 37* , 3243-3257. doi:10.1093/molbev/msaa182

Waldron, K. J., & Robinson, N. J. (2009). How do bacterial cells ensure that metalloproteins get the correct metal? *Nature Reviews: Microbiology, 7* , 25. doi:10.1038/nrmicro2057

Wolf, K. K., Romanelli, E., Rost, B., John, U., Collins, S., Weigand, H., & Hoppe, C. J. (2019). Company matters: The presence of other genotypes alters traits and intraspecific selection in an Arctic diatom under climate change. *Global Change Biology, 25* , 2869-2884. doi:10.1111/gcb.14675

Wood, A. M., Everroad, R., & Wingard, L. (2005). Measuring growth rates in microalgal cultures, p. 269-288. *Andersen, R. A. (Ed.): Algal culturing techniques. Elsevier.*

Xu, C., Sun, T., Li, S., Chen, L., & Zhang, W. (2018). Adaptive laboratory evolution of cadmium tolerance in Synechocystis sp. PCC 6803. *Biotechnology for biofuels, 11* , 1-15.

Zhao, Y., Dominska, M., Petrova, A., Bagshaw, H., Kokoska, R. J., & Petes, T. D. (2017). Properties of mitotic and meiotic recombination in the tandemly-repeated CUP1 gene cluster in the yeast *Saccharomyces cerevisiae* . *Genetics, 206* , 785-800. doi:10.1534/genetics.117.201285

Tables

Table 1: Summary of key observations during the artificial evolution experiment on a replicate-by-replicate basis. Several barcode samples at day 42 were cross-contaminated during the library preparation and have

been omitted from further quantitative analysis (see Fig S4 for more details). RO5AC is a mono-clonal strain run as a reference control between the two populations experiments. Abbreviations: C; control treatment, Cu; copper treatment, GP; Gropviken [reference inlet], VG; Gasfjarden [mining inlet]

Bottle replicate	Growth rate first nine days (day^{-1})	Growth rate first nine days (day^{-1})	Total number of generations during the experiment	The most abundant strain on day 42 (%)	Other strains (Number of strains/strain and their abundance)	Cross-contaminated barcode sample? (day 42)	EC50 (day 42)	Respons range (EC05/
Reference inlet	Reference inlet	Reference inlet	Reference inlet	Reference inlet	Reference inlet	Reference inlet	Reference inlet	
GP_C1	GP_C1	1.62	108	GP2-4_-40(58%)	Six(4-17%)	No	7.50	1.40
GP_C2	GP_C2	1.64	107	GP2-4_-40(65%)	Three(4-13%)	No	7.69	1.50
GP_C3	GP_C3	1.73	113	GP2-4_-40(90%)	Three(˜4%)	No	7.34	1.59
GP_C4	GP_C4	1.68	111	GP2-4_-40(78%)	Three(˜6%)	No	7.16	1.71
GP_C5	GP_C5	1.71	106	GP2-4_-40(37%)	Five(6-25%)	No	8.03	1.77
GP_Cu1	GP_Cu1	0.587	55	GP2-4_-27(99%)	Three(<1%)	No	10.8	1.40
GP_Cu2	GP_Cu2	0.498	54	GP2-4_-27(99%)	GP2-4_-72(<1%)	No	9.84	1.83
GP_Cu3	GP_Cu3	0.458	46	GP2-4_-57(78%)*	GP2-4_-27(10%)	No	11.2	1.78
GP_Cu4	GP_Cu4	0.634	59	GP2-4_-27(100%)	None	No	10.6	2.06
GP_Cu5	GP_Cu5	0.472	57	GP2-4_-27(100%)	None	No	9.45	1.78
RO5AC_-GP_C	RO5AC_-GP_C	NA	NA	GP2-4_-40(80%)	Three(˜4%)	No**	NA	NA
RO5AC_-GP_Cu	RO5AC_-GP_Cu	0.799	66	RO5AC(100%)	None	No	9.88	1.78
Mining inlet	Mining inlet	Mining inlet	Mining inlet	Mining inlet	Mining inlet	Mining inlet	Mining inlet	
VG_C1	VG_C1	1.70	105	VG1-2_-103(90%)	VG1-2_-74(6%)	No	8.20	1.53
VG_C2	VG_C2	1.71	106	VG1-2_-103(89%)	VG1-2_-74(7%)	No	8.06	1.65
VG_C3	VG_C3	1.76	108	VG1-2_-103(91%)	VG1-2_-74(8%)	No	8.38	1.64
VG_C4	VG_C4	1.76	109	VG1-2_-103(22%)	VG1-2_-81(17%)	Yes (˜70%)	8.14	1.55
VG_C5	VG_C5	1.80	110	VG1-2_-103(48%)	VG1-2_-81(10%)	Yes (˜40%)	7.94	1.48
VG_Cu1	VG_Cu1	0.662	52	VG1-2_-74(72%)	VG1-2_-81(28%)	No	9.96	1.65

Bottle replicate	Growth rate first nine days (day^{-1})	Growth rate first nine days (day^{-1})	Total number of generations during the experiment	The most abundant strain on day 42 (%)	Other strains (Number of strains/strain and their abundance)	Cross-contaminated barcode sample? (day 42)	EC50 (day 42)	Respons range (EC05/
VG_Cu2	VG_Cu2	0.558	44	VG1-2_-81(20%)	VG1-2_-95(17%)	Yes (˜60%)	9.94	1.69
VG_Cu3	VG_Cu3	0.657	54	VG1-2_-81(75%)	VG1-2_-74(10%)	Yes (˜15%)	9.93	1.93
VG_Cu4	VG_Cu4	0.555	53	VG1-2_-81(95%)	VG1-2_-74(4%)	No	9.86	2.12
VG_Cu5	VG_Cu5	0.487	40	VG1-2_-81(60%)	VG1-2_-74(20%)	Yes (˜20%)	9.93	2.00
RO5AC_-VG_C	RO5AC_-VG_C	1.63	98	RO5AC(100%)	None	No	9.10	1.38
RO5AC_-VG_Cu	RO5AC_-VG_Cu	0.327	25	RO5AC(100%)	None	No	10.3	1.41

**Loss of heterozygosity with only one out of the two Sm_C12W1 alleles observed in the prevailing genotype*

***Barcoding indicated that the RO5AC_GP_C bottle was physically cross-contaminated at the start of the experiment and rapidly outcompeted.*

Figure legends

Figure 1 : Population-wide characterization of copper tolerance in the two populations (mining and reference inlet), based on mono-clonal acute 72-h dose-response curve exposure. A) mining and the reference inlets population distribution of copper tolerance estimated based on EC50, the effective concentration inhibiting growth rate by 50% (N=30 and 28 strains, respectively). B) and C) are examples of full dose-response curves for the most sensitive, tolerant, and median strain (see symbol legend for strain names) per population based on ranked EC50. N=5 for control and 18 copper treatments for dose-response curves. Strain RO5AC is included as an internal control between populations. Error bars/shaded areas show 95% confidence intervals, and the median as well as RO5AC's EC50 is indicated with a vertical line.

Figure 2: Changes in copper tolerance during the artificial evolution experiment in a copper-toxic (Copper) and non-toxic environment (Control). At the end of the selection phase, copper toxicity was relaxed for three days to test if the developed tolerance was reversible (plastic) or permanent (evolved). Panel B shows a mono-clonal culture of RO5AC which was run in parallel with the reference (Rep. 1) and the mining population (Rep. 2). Metabarcoding showed that RO5AC was cross-contaminated with the GP-Control treatment from day nine and onwards (Table 1). These samples are omitted in panel B. Error bars show 95% confidence intervals based on dose-response curve data gathered as shown in Fig. 1.

Figure. 3. Photosynthetic activity was inhibited at a slower rate in evolved or pre-exposed (Mining population) cultures, compared with naïve strains (Reference population). The yield of Photosystem II (PSII) was monitored during a short exposure (3h) to lethal copper concentrations. Shown are means with 95% confidence intervals with N=3 replicates per time-point (N=1-2 for single strain RO5AC). Note that both populations require more than three days of selection to develop tolerance in contrast to the single strain RO5AC, which reaches its minimum inhibition rate within three days.

Figure. 4. Changes in strain abundances based on metabarcoding during the artificial evolution experiment. Shown are time-point averages based on quantification of strain abundances recorded at days 0, 9, and 42 (N range between 1 and 5). Cross-contaminated samples (Fig S4) have been excluded from the Mining

population at day 42. Strain GP2-4_45 and 46 are clones and cannot be distinguished. Strain VG1-2_65 and VG1-2_99 lack strain-specific DNA sequencing data but correspond to two pairs of unknown amplicons tightly linked at a 1:1 ratio across the metabarcoding data (Pinder et al. in prep). They have been annotated as belonging to either of these strains. Abundant strains are highlighted in plots with the strain names according to the legend, and the sequential order of strains follows the legend top-down and left-to-right.

Figure 5. Comparison of metabarcoded fitness estimates during the selection experiment (Observed growth rate) against mono-clonally predicted ones (Modeled). Growth rate was modeled for the copper treatment using mono-clonal 72-h dose-response curves (N=23 total replicates) and the applied 8.65 μM copper exposure, or the specific growth rate in the control environment. The observed growth rate corresponds to mean growth rates (N=1 to 5 depending on strain and treatment) during the first nine days of the selection experiment. Extinct strains (missing in all selection experiment replicates) for each panel are highlighted in the legend and not included in plots. Colors of the strains are the same as in the legend of Fig. 4, with key strains from Fig. 1 and 4 shown with names. Error bars show 95% confidence intervals when N>1. Bolded strain names highlight cases when modeled selection outcome agrees with the selection experiment at day 42.