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RESEARCH ARTICLE

Expanding the upper-temperature boundary for the microalga *Picochlorum sp. (BPE23)* by adaptive laboratory evolution

Robin Barten[1] | **Teun Peeters**[1] | **Sofia Navalho**[1] | **Louis Fontowicz**[1] | **Rene H. Wijffels**[1,2] | **Maria Barbosa**[1]

[1] Bioprocess Engineering and AlgaePARC, Wageningen University and Research, Wageningen, The Netherlands

[2] Biosciences and Aquaculture, Nord University, Bodø, Norway

Correspondence
Robin Barten, Bioprocess Engineering and AlgaePARC, Wageningen University and Research, PO Box 16, 6700 AA Wageningen, The Netherlands.
Email: robin.barten@wur.nl

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Abstract

Closed photobioreactors reach temperatures that reduce microalgal production or even cause culture collapses. Cooling can maintain the temperature within tolerable boundaries, but cooling is energy-intensive and expensive. Thermotolerant microalgal strains can reduce dependence on such cooling. In this study, adaptive laboratory evolution was performed for 390 days to further increase the maximal tolerable temperature for the already thermotolerant microalgae *Picochlorum sp. (BPE23)*. The parental wild-type strain of *Picochlorum sp. (BPE23)* exhibited a maximum mid-day growth temperature of 47.5°C, whereas the isolated clones grew up to 49°C. At a lower temperature of 40°C, the growth rate and absorption cross-sectional area were similar for the wild-type strain and the evolved clones. Interestingly, the clones showed a 46% increase in cell volume compared to the wild-type strain. The evolved clones with an expanded upper-temperature boundary can be applied for broader temperature control of 1.5°C, without trade-off effects at lower temperatures.

KEYWORDS
adaptive laboratory evolution, diel temperature, microalgae, photobioreactor, temperature

1 | INTRODUCTION

Most industrial microalgal species have a maximal growth temperature between 25°C and 35°C. However, closed photobioreactors can reach peak temperatures up to 50°C due to climatological conditions such as high light levels.[1] Such temperatures have a disastrous effect on culture viability and productivity for most microalgal species. Therefore, the temperature is actively controlled in photobioreactor systems.[1] While cooling through shading, active cooling, or spraying of water is possible, these methods are expensive and not sustainable.[2] To reduce temperature control costs, species that are naturally more resistant to otherwise stressful temperatures, preferably in combination with tolerance to diel temperature fluctuations, should be employed.[1,3] Microalgal species that can maintain their maximal productivity rate under diel temperatures ranging from 30°C to 45°C would cause a cost reduction of 31%.[2] In addition, robust species with a high maximal growth temperature can prevent culture collapse due to photobioreactor overheating.[1,4]

Supra-optimal temperatures unbalance cellular metabolism in various ways, ultimately leading to reduced growth and productivity.[5,6] When exposed to supra-optimal temperatures, cells initially acclimate to rebalance cellular homeostasis. Cell membranes, photosystems, and protein composition are remodeled within hours to counteract the adverse effects of increased temperature.[3,7] Consecutively, due to the decreased metabolism and growth rate, cells allocate excess energy

Abbreviations: ALE, adaptive laboratory evolution; PAR, photosynthetically active radiation; DO, dissolved oxygen

into storage compounds such as fatty acids and lipids.[7,8] Acclimation will lead to a more stable cellular state, but growth will remain hindered when non-optimal growth conditions are maintained.

After prolonged exposure to supra-optimal temperature, mutants with improved phenotypes will periodically emerge due to de novo genetic mutations that cause fitness gains.[9] Natural genetic mutations can be exploited for strain optimization when combined with appropriate selective pressure. Adaptive laboratory evolution (ALE) has become a popular tool to select strains with improved phenotypes. Examples of traits suitable for improvement through ALE are: stress tolerance, substrate utilization, or growth rate and product yield.[9,10] It is especially applicable to improve complex traits as no prior genetic knowledge is required. Few studies have addressed the improvement of temperature robustness in microorganisms through ALE, in which a shift in optimal or maximal growth temperature of 2°C was observed on average.[11–14] However, ALE studies on microalgae concerning temperature, are scarce and the exact mechanisms for adaptation are unknown.[9,10] The green microalgae *Picochlorum costavermella* expresses a natural mutational rate of 3.23×10^{-10} to 10.12×10^{-10} nucleotide per generation, respectively.[15,16] Based on the 13.3 Mbp genome size of *Picochlorum costavermella*, this would lead to 0.013 genomic mutations per cell in one generation, which would require 74 generations for a single mutation.[17] However, the mutational rate is known to increase under stressful conditions to accelerate molecular evolution for rapid adaptation to new growth conditions.[18] In addition, the large number of cells in cell cultures significantly increase the chance of beneficial mutations.

Species of *Picochlorum* are often proposed as a platform for the production of various bulk and specialty products.[5,19,20] In prior research, *Picochlorum sp. (BPE23)* was isolated, characterized and selected for its high growth rate in combination with its robustness.[5] It shows optimal growth at temperatures between 35°C and 40°C, whereas it can survive mid-day peak temperatures of 47.5°C.[3] This study reports how *Picochlorum sp. (BPE23)* was improved through ALE to tolerate a 1.5°C higher maximum temperature.

2 | MATERIALS AND METHODS

2.1 | Cell cultivation

2.1.1 | Growth media and inoculum preparation

Picochlorum sp. (BPE23), isolated from a saltwater body of Bonaire, was pre-cultivated in shake flasks in an orbital shaker incubator (Multitron, Infors HT) under continuous light at an intensity of 100 μmol_{ph} m^{-2} s^{-1} (PAR).[5] The temperature was set at 40°C. The relative humidity of the air in the headspace was set to 60% and enriched with 2% CO_2. Cells were cultured in artificial seawater enriched with nutrients and trace elements. Elements were provided at the following concentrations (in g L^{-1}): NaCl, 24.5; $MgCl_2$ $6H_2O$, 9.80; Na_2SO_4, 3.20; $NaNO_3$ 2.12; K_2SO_4, 0.85; $CaCL_2$ $2H_2O$, 0.80; KH_2PO_4, 0.23; Na_2EDTA $2H_2O$, 0.105; Na_2EDTA, 0.06; $FeSO_4$ $7H_2O$, 0.0396; $MnCl_2$ $2H_2O$, 1.71 10^{-3}; $ZnSO_4$ $7H_2O$, 6.60 10^{-4}; Na_2Mo_4 $2H_2O$, 2.42 10^{-4}; $Co(NO_3)_2$ $6H_2O$, 7.00 10^{-5}; $NiSO_4$ $6H_2O$, 2.63 10^{-5}; $CuSO_4$ $5H_2O$, 2.40 10^{-5}; K_2CrO_4, 1.94 10^{-5}; Na_3VO_4, 1.84 10^{-5}; H_2SeO_3, 1.29 10^{-5}. HEPES (4.77 g L^{-1}) was added for Erlenmeyer cultures as a pH buffer. The medium pH was adjusted to 7.0, after which it was filter sterilized before use through filters with a 0.2 μm pore size. During photobioreactor cultivation, Antifoam B (J.T.Baker, Avantor, USA) was added at a concentration of 0.5 mL L^{-1} out of a 1% w/w stock. At the start of the cultivation, 0.168 g L^{-1} sodium bicarbonate ($NaHCO_3$) was added to provide sufficient CO_2. The photobioreactor was inoculated at a starting cell density of OD_{750} 0.2 (±0.07 g L^{-1}).

2.1.2 | ALE—photobioreactor operation

The ALE experiment was performed in a heat sterilized flat-panel photobioreactor with a 1.8 L working volume, a 20.7 mm light path and a 0.08 m^2 surface area for irradiation (Labfors 5 Lux, Infors HT, Switzerland).[3] Illumination was done from one side by 260 warm white LED lights with a spectrum of 450–620 nm. Day-night cycles (12/12 h/h) were applied in which light was given in a sinusoid pattern. The ingoing light reached 1500 μmol_{ph} m^{-2} s^{-1} (PAR) at its peak during mid-day. The outgoing light level was set at ~ 1% of the ingoing light, following a sinusoid pattern similar to the ingoing light. The outgoing light was controlled by automatic dilution of the cell culture (turbidostat mode) when below the set-point. The photobioreactor was aerated through sparging of compressed air at a rate of 980 mL min^{-1} (0.54 vesselvolume min^{-1}). CO_2 was provided on-demand through pH-controlled addition. The reactor pH was set at 7. The temperature was set at 25°C during nighttime and followed a sinusoid pattern during daytime. The peak temperature was increased stepwise over a period of 403 days at the following levels, 40°C, 45°C, 47°C, 48°C, 48.5°C, 49°C, and 49.5°C. The microalgae culture was monitored closely after a temperature increase.

2.1.3 | Culture tipping point—photobioreactor operation

The culture tipping point was measured for each temperature step throughout the evolution experiment by measuring the maximum temperature of oxygen production. When the growth of the cell culture was stable at each of the temperature steps, 400 mL of cell culture was transferred from the Laboratory evolution culture (2.1.2.) to a standalone Labfors photobioreactor system to reach a biomass concentration of 0.4 g L^{-1}. Air was supplied at a rate of 980 mL min^{-1}, and CO_2 was supplied at a fixed rate of 20 mL min^{-1}. Sulfuric acid and sodium hydroxide were used to control pH. The ingoing light was set at 200 μmol_{ph} m^{-2} s^{-1} (PAR), outgoing light was set at 35 μmo_{ph}l m^{-2} s^{-1} (PAR). The Dissolved oxygen concentration (DO) was measured online. After stabilization of the DO, the temperature was linearly increased at

a rate of 5°C per hour, from 30°C to 60°C. The measured DO value was normalized on a scale of 0 to 1.

2.1.4 | Isolation and characterization of ALE clones

Algal clones were isolated from the ALE culture at day 390. Clone isolation was done by plating on agar, followed by colony picking. Growth characterization of the parental wild-type strain and clones was done in algaemist photobioreactors (Technical Development studio, WUR, The Netherlands), under a repeated batch mode.[5] The microalgal culture was diluted daily at sunset to an OD_{750} value of 5 ($\pm$2 g L^{-1}).[5] The light was provided as a 12/12 h/h day/night cycle with light as a sinusoid with a mid-day value of 1500 μmol_{ph} m^{-2} s^{-1} (PAR). The temperature was set to follow a sinusoid trend. Four different midday peak temperatures were chosen: 40°C, 45°C, 47.5°C, and 49°C. Characterization experiments of the clones were performed as biological duplicates.

2.2 | Offline measurements

2.2.1 | Biomass concentration

The biomass concentration was determined by optical density measurements. Duplicate UV-VIS spectrophotometry measurements were performed at a wavelength of 750 nm (DR6000, Hach, USA). In addition, biomass concentration (in g L^{-1}) was measured in duplicate by dry weight determination. Empty Whatman glass microfiber filters (θ 55 mm, pore size 0.7 μm) were dried overnight at 95°C and placed in a desiccator for 2 h. Filters were then weighed and placed in the mild vacuum filtration setup. Cell culture containing 1 to 10 mg of microalgae biomass was diluted in 25 mL 0.5 M ammonium formate and filtered. The filter was washed twice with 25 mL 0.5 M ammonium formate to remove residual salts. The wet filter was dried overnight at 95°C, placed in a desiccator for 2 h, and weighed. Biomass concentration was calculated from the difference in filter weight before and after filtration and drying.

2.2.2 | Cell size and cell number

Cell size and cell number were determined in duplicate with the Multisizer III (Beckman Coulter Inc., USA, 50 μm aperture). Samples were diluted in two steps before analysis, initially by 5x dilution in fresh medium, followed by 100x dilution in Coulter Isoton II.

2.2.3 | Absorption cross-sectional area

The average dry-weight specific optical cross-section (m^2 kg^{-1}) was measured with a UV-VIS/double-beam spectrophotometer (Shimadzu UV-2600, Japan, light path: 2 mm), equipped with an integrating sphere module (ISR-2600). Absorbance was measured from 400 to 700 nm with a step size of 1 nm.

3 | RESULTS AND DISCUSSION

3.1 | The maximal diel peak temperature increased over time during ALE

During an ALE experiment of 403 days, 139 generations of microalgae were grown under mimicked commercial growth conditions. The culture temperature was increased stepwise to maintain a high selective pressure after cells adapted (Figure 1). The parental wild-type strain, *Picochlorum sp. (BPE23)*, was characterized in a prior study under comparable growth conditions and the same photobioreactor setup. It exhibited a maximal growth temperature of 47.5°C.[3]

The dilution rate of the photobioreactor, and therefore growth rate of the microalgae, was 0.77 d^{-1} at 40°C (Figure 1).[3] As the temperature increased to 45°C, growth initially reduced to 0.55 d^{-1} but recovered over time up to 0.65 d^{-1}, indicating acclimation or adaptation. A comparable trend for this gradual culture fitness recovery was observed after each increase in temperature. The consecutive steps to 47°C, 48°C, 48.5°C, and 49°C yielded dilution rates at the end of each steady-state of 0.44, 0.30, 0.35, and 0.25 d^{-1}, respectively. The temperature step to 45°C and 47.5°C little cellular stress and acclimation was rapid. However, the increase to 48°C, 48.5°C, and 49°C induced a significant stress response directly after the increase in temperature, resulting in a temporary decrease in dilution rate. Interestingly, the dilution rate at 48.5°C was higher than the dilution rate at 48°C. We hypothesized that the microalgae adapted to higher temperatures at this point. The cell culture collapsed after the temperature increase to 49.5°C. Hypothetically adaptation to 49.5°C should be possible when the ALE experiment was extended. However, while adaptation initially occurs rapidly, it slows after several hundred generations as most simple mutations have already occurred, and more complex multi-gene mutations are required for further improvement.[21]

3.2 | *Picochlorum sp. (BPE)* was able to produce oxygen at increased temperatures after ALE

The maximal temperature at which the ALE culture was capable of oxygen production was determined periodically to assess whether the microalgae were successfully adapting to increased mid-day peak temperatures. Measurements were done on days 30, 120, 170, 290, and 380 as the culture was in steady-state on these selected days (Figure 2). Cell culture was transferred to a stand-alone photobioreactor for which temperature was increased at a rate of 5°C per hour from 30°C to 60°C. The DO concentration was measured online throughout the experiment.

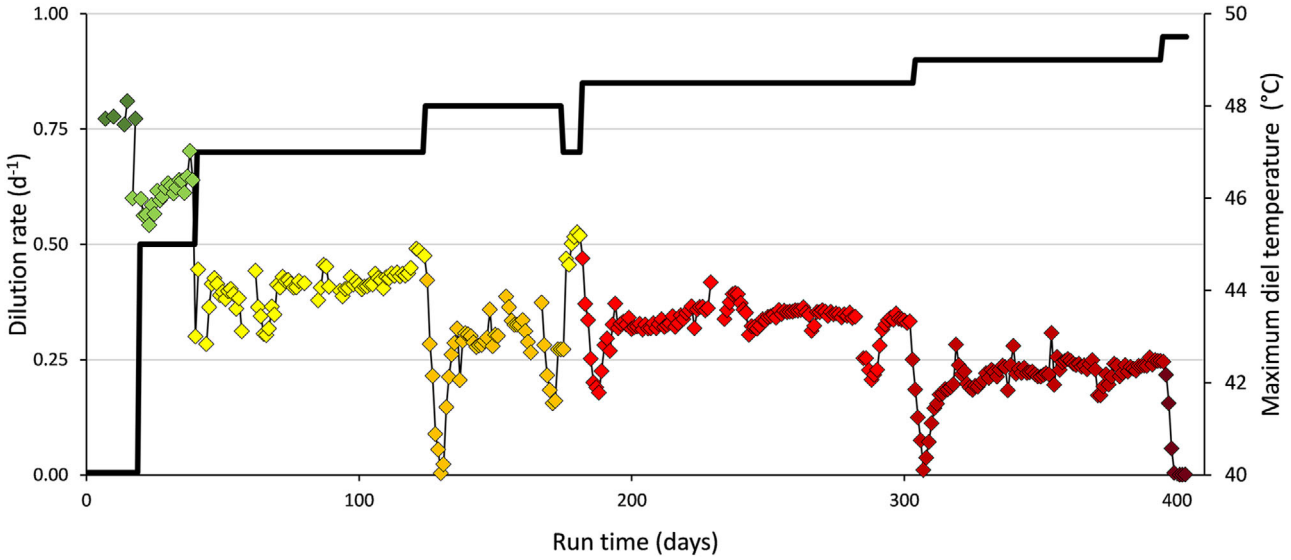

FIGURE 1 The measured dilution rate of the ALE photobioreactor per day. Diel cycles were applied to mimic growth conditions as found in outdoor photobioreactor systems. The dilution rate is displayed on the left y-axis while the mid-day peak temperature is displayed on the right y-axis

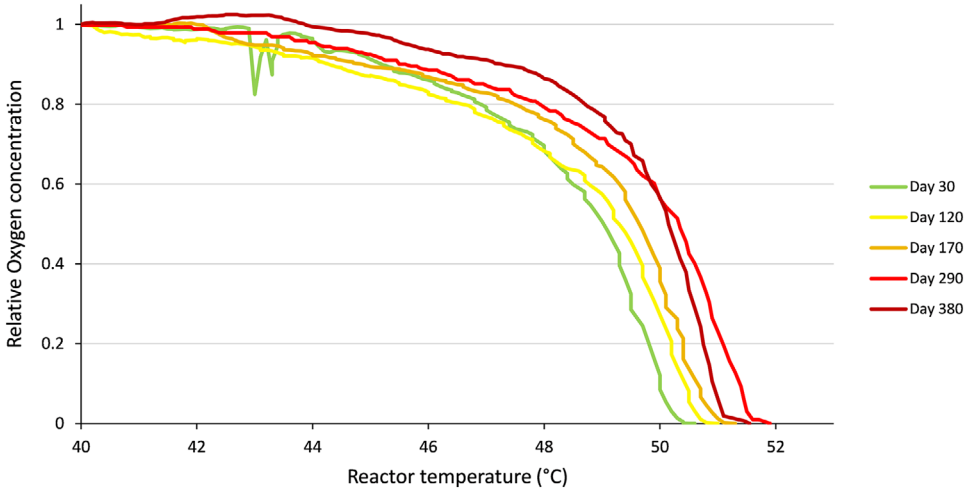

FIGURE 2 The capability of photosynthetic oxygen production during a temperature increase. The photosynthetic oxygen production was determined in stand-alone photobioreactors in which temperature was increased by 5°C per hour, from 30°C–60°C. Culture viability was monitored through the relative oxygen concentration which was normalized to the oxygen concentration at 40°C and displayed from 40°C–54°C. Cell culture was taken from the ALE photobioreactor for which the day number indicates the moment at which the experiments were done during the ALE experiment

A gradual decrease in oxygen concentration was observed due to increasing inhibition of photosynthesis at increasing temperatures. The maximal temperature at which oxygen production was observed shifted to higher temperatures as the ALE experiment proceeded. Each consecutive experiment showed a comparable curve with a shift of ±0.5°C. The shift of ±0.5°C is directly proportional to the increase in temperature in the ALE photobioreactor. The exception to the increasing trend was the last experiment at day 380, which compared to the experiment at day 290 had a higher relative DO concentration up to precisely 50°C, after which the DO decreased with a steeper trend than was observed in the previous experiments.

The rapid decrease in DO is most likely caused by the denaturation of enzymes involved in photosynthetic oxygen production. Photosynthesis is a thermo-sensitive process that severely impacts the metabolism and cell growth when disrupted.[7] While photosynthesis is not the only thermosensitive process, it is a good indicator of the point at which the cells die irreversibly. As it is amongst the most thermosensitive processes and simple to measure, it provides a way

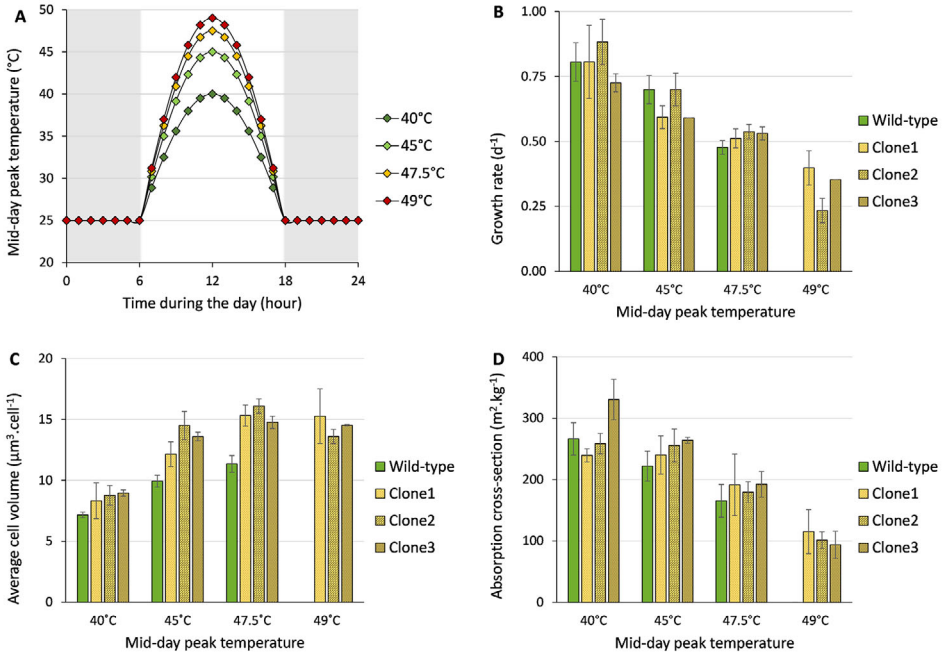

FIGURE 3 The (A) applied temperature regimes for characterization, (B) specific growth rate, (C) average cell volume, and (D) specific absorption cross-sectional area as measured for wild-type strain and three ALE clones of *Picochlorum sp. (BPE23)* during the characterization experiment. Data represents the average ± the propagated error of 3 days of steady-state growth for two biological duplicates photobioreactors operated in repeated batch mode

to assess and compare the state of the ALE culture at different times points.[7,22,23]

The initial increase in maximal temperature of oxygen production could be caused by short-term acclimation by exchange of photosynthetic pigment in the photosystems and by remodeling the cell membranes.[3,24] However, because the maximal temperature of photosynthetic oxygen production increased throughout the entire experiment, we expect that genetic adaptation took place in addition to short term acclimation. Genetic adaptation can cause structural differences for enzymes by which thermal tolerance can change.[25] Photosynthesis and carbon fixation are thermosensitive cellular processes that present a bottleneck at supra-optimal temperatures.[1] Especially Rubisco was reported to limit carbon assimilation under temperature-induced cell stress.[26] We hypothesize that enzymes in these pathways have adapted to tolerate higher temperatures. This hypothesis is strengthened by the fact that the maximal temperature for oxygen production increased throughout the ALE. In addition, a compositional adaptation of the cell and thylakoid membranes is often observed in response to increased temperature to counteract the impact of temperature-induced change in membrane fluidity.[3,11]

3.3 | Growth characterization of isolated clones

Three ALE clones were isolated at the end of the ALE experiment and subjected to growth characterization. Four diel temperature regimes were applied with different peak temperatures at mid-day (Figure 3). The wild-type and Clone1 show comparable growth at 40°C with a growth rate of 0.8 d^{-1}, whereas Clone2 grew faster and Clone3 grew slower at 45°C and 47.5°C. As expected, the clones were capable of growth at 49°C, while the growth rate of the wild-type strain decreased to 0 d^{-1} after 4 days at this temperature.

In previous research, a mid-day peak temperature of 40°C was reported to be optimal for wild-type *Picochlorum sp. (BPE23)*, whereas the maximum temperature tolerated was reported to be 47.5°C.[3] The current research shows a similar trend. The ability to grow at increased temperature makes the clones more robust, which is vital for maintaining a stable cell culture in commercial photobioreactors. In addition, the expanded upper-temperature boundary of 1.5°C can prevent culture crashes after periodic temperature increases. Both wild-type and clone strains could adapt to the new temperature regime within days, which indicates a rapid acclimation rate in response to changing growth conditions. Such rapid acclimation allows for less stringent process control, which can reduce operating costs.

ALE effectively shifts temperature optima and maximum in microalgae.[9,27] A trade-off in the lower temperature regions is commonly observed when evolving for higher temperature tolerance.[11,13] While growth at lower temperatures (20°C–40°C) was not characterized, the ALE clones obtained in this study did not display affected growth at the optimal growth temperature for the wild-type (40°C).

A shift in optimal temperature commonly accompanies a shift in maximal temperature due to the selective pressure in the supra-optimal temperature niche.[11,21] However, this was not observed for *Picochlorum sp. (BPE23)* in this study. In an ALE study with *Tisochrysis*

lutea, a shift of 3°C was observed for the optimal and maximal growth temperature.[14] *T. lutea* was able to tolerate a higher upper temperature and gained the ability to grow faster at temperatures above the optimal temperature for the wild-type.

3.4 | The cell size of *Picochlorum sp. (BPE23)* was impacted by ALE

On day 120 of the ALE experiment, a 0.5°C temperature increase to 48°C was made that led to an increase in the average cell volume from 9–13.3 μm^3 within 1 week time (Appendix A). A similar pattern was observed for the clone characterization as the isolated clones showed an increased cell size compared to the parental wild-type strain (Figure 3). While there was a slight difference in cell size between the wild-type and clones at 40°C, the difference in cell size became more pronounced at 45°C and higher temperatures. The adaptation is assumed to be genotypic due to the persistence of the cell size increase. Just as in a previous study, an increase in cell size was observed in *Picochlorum sp. (BPE23)* when exposed to increasing temperature.[3]

The consensus in the literature regarding whether cells increase or decrease in size during ALE under high temperatures is unclear. In theory, a decreased cell size allows for faster cell division which is beneficial for survival in continuously diluted cell culture.[1,28–30] In addition, a larger cell surface area to cell volume ratio is considered an evolutionary benefit of small cells in a high-temperature environment where gases are less soluble.[29] Indeed, a selection for small cells was applied during a 290 generation ALE study on *Dunaliella tertiolecta*, where the obtained smaller cells were found to be more tolerant to increased temperature.[28] We observed the opposite as cells became larger in response to increased temperature. In agreement with this, significant increases in cell size were observed during several ALE studies in *Escherichia coli*.[30,31] In these studies, comparable patterns were observed as in our study. It was hypothesized that the mutant strains accumulated larger metabolic reserves to be capable of faster acclimation under fluctuating growth conditions. This theory fits our case and is strengthened by the fact that *Picochlorum sp. (BPE23)* halts cell division at mid-day when stressful peak temperatures are applied, while at the same time, larger cells with more storage compounds were observed, as shown in a previously published study.[3] The capacity to accumulate storage compounds to store energy when energy is absorbed in excess and utilize this energy when energy is lacking would be an advantage. A second and often discussed hypothesis stated that mutant cells could simply grow slower in terms of cell division but grow equally fast in terms of biomass accumulation due to larger cells.[32] This hypothesis was found to be not valid when cells were forced to grow at an equal rate in chemostat operation, as the mutant cells remained larger than the parental cells, as was also observed in our study.[31] Unfortunately, different studies contradict each other, and the exact mechanisms behind cell size adaptation remain unknown.

3.5 | Changes in absorption cross-sectional area in response to increased temperatures

The biomass specific absorption cross-sectional area indicates the level of photosynthetic pigments in the cell since it reflects the level of light absorbed by the chromophores.[33] The absorption cross-sectional area decreased continuously throughout the ALE experiment, causing cells to become more transparent. A severe reduction event was observed around day 120 of the ALE, directly after the increase in temperature to 48°C (Appendix B). An average absorption cross-sectional area of 221.9 ± 18.9 was found between days 53 and 106 of the experiment, whereas an average absorption cross-sectional area of 143.4 ± 23.3 $m^2\ kg^{-1}$ was found between days 348 and 393 of the ALE experiment. As a result of the lower absorption cross-sectional area, the biomass concentration increased by 20–25% due to the turbidostat operation mode, which controls reactor dilution based on light absorption (Appendix C). Despite the decreased photobioreactor dilution rate, a larger biomass concentration caused the total productivity to remain at the same level.

Under non-optimal growth conditions, microalgae temporarily decrease their absorption cross-sectional area to decrease the energy flux from the photosystem.[3,34] This flexibility allows growth under fluctuating environmental conditions that affect the microalgal metabolism.[35] Both wild-type and clones of *Picochlorum sp. (BPE23)* showed their highest absorption cross-section value at 40°C, whereas the absorption cross-section decreased with increasing temperature.

A decreased absorption cross-sectional area is reported to lead to higher biomass yields on light as light is diluted and divided amongst a larger quantity of biomass.[33,36] As a result, less energy is dissipated into heat through photochemical quenching due to photosystem oversaturation.[35] Biomass specific absorption cross-sectional area was similar for the wild-type and the clones. Nonetheless, a difference in absorption cross-sectional area was observed at different cultivation temperatures, with lower values measured at higher temperatures (Figure 3). Based on the results, we conclude that the decrease in absorption cross-sectional area during ALE was a reversible acclimation effect.

4 | CONCLUSION

ALE was performed to improve the tolerance of *Picochlorum sp. (BPE23)* to high mid-day peak temperatures. The maximal temperature for growth was shifted from 47.5°C–49°C over 409 days (139 generations). Periodic assessment of the maximal temperature of oxygen production revealed that the tolerance to high temperatures increased throughout the ALE procedure. During clone characterization, no trade-off in productivity was observed compared to the wild-type strain at the lowest studied mid-day temperature of 40°C. The improved clones can be applied to reduce the risk of a culture crash on sunny days at which temperature can rise to lethal levels. The clones

can be applied without a trade-off at lower mid-day temperature, as low as 40°C.

The cell size and absorption cross-sectional area changed during ALE. The increase in cell size was persistent in the clones throughout the characterization experiments and became especially noticeable at supra-optimal temperatures. Due to its persistence, the cause for a cell size increase is assumed to be genotypic. Contrarily, the decreased absorption cross-sectional area was found to be reverted during later characterization experiments. This fast reversion highlights the flexibility of *Picochlorum sp. (BPE23)* to adapt its photosystems to new growth environments.

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. No conflicts on informed consent, or human or animal rights are applicable to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Robin Barten https://orcid.org/0000-0002-2695-9987

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