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ORIGINAL ARTICLE

Ocean Acidification Influences Strain Selection and Metabolism of the Benthic Diatom *Cocconeis neothumensis* var. *marina*

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Abstract

The uptake of carbon dioxide (CO_2) by oceans is dramatically altering the chemistry of seawater, leading to a continuous decrease of pH over the last century. This phenomenon, called ocean acidification (OA), has raised concerns due to its negative effects on marine biodiversity, including plankton communities and seagrass meadows. The most relevant seagrass in the Mediterranean is *Posidonia oceanica*, producing complex and stable benthic ecosystems. OA markedly affects the colonization and settlement patterns of epibionts within the leaf communities of *P. oceanic*a. Epiphytic diatoms associated with *P. oceanica* are influenced by complex chemical and trophic interactions and play a fundamental role in the ecological successions characterizing the leaf stratum. In this study, we isolated two strains of *Cocconeis neothumensis* var. *marina*, one of the main epiphyte diatoms associated with *P. oceanica,* from two sites off the Island of Ischia (Italy) characterized by different pH conditions, i.e., a naturally low pH site (pH 7.6) influenced by volcanic CO_2 emissions, and an adjacent location with ambient pH conditions (pH 8.1). We further cultured both strains of *C. neothumensis* under both pH conditions, resulting in four treatment conditions. Four significantly different growth curves were obtained, and metabolomic studies confirmed that the physiology of the strains differed according to pH conditions. Overall, this

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study demonstrated that OA is likely to trigger the selection of specific diatom strains, with possible consequences for trophic and chemical relationships among the associated consumers.

Key words: benthos; CO_2 vents; epiphyte; metabolomics; ocean acidification; *Posidonia oceanica*; seagrass.

Introduction

Diatoms are widespread and important autotrophs within the phylum Heterokontophyta (class Bacillariophyceae). Globally, they are considered to be the most diverse group of eukaryotic algae (Bork and others 2015) and are a major component of both benthic and planktonic primary production. Diatoms inhabit terrestrial (humid), freshwater, and marine environments (Mann and Droop 1996; Zupo and others 2007). They exhibit a unique cellular morphology, being enclosed in a microscopic shell, the frustule, which is a siliceous double-capsuled structure. Frustules are among the most complex examples of natural micro- and nanostructured materials (Rogato and De Tommasi 2020). This characteristic enhances the industrial and biotechnological importance of diatoms, leading to their use in producing bioactive compounds, functional foods, feeds, and biofuels (Rabiee and others 2021). The biotechnological significance of diatoms is primarily due to the variety of bioactive compounds they synthesize, which are valuable for biomedical and industrial applications (Riccio and others 2020; Esposito and others 2022).

From an ecological point of view, diatoms play an important trophic role, forming the foundation of most marine food webs and contributing to the maintenance of oceanic biogeochemical cycles (Bowler and others 2009, 2010). Collectively, diatoms account for 40% of marine primary production (Clement and others 2016; Li and others 2017) and contribute up to 20% of global carbon dioxide (CO_2) fixation, thereby playing a key role in regulating our climate (Falkowski and others 2004; Tréguer and others 2018). Therefore, it is useful to unravel the effects of ocean acidification (OA) on the physiology and ecology of diatoms, to better predict how marine ecosystems will change in the near future.

While the direct effects of OA are extensively described in several studies (for example, Wingert and Cochlan 2021; Wu and others 2014), its indirect effects on the ecology of resilient ecosystems, such as *Posidonia oceanica* (L.) Delile meadows (Zunino and others 2019), still require comprehensive understanding. The resilience of seagrass environments is maintained by complex chemical and trophic relationships with associated animal communities. Rising CO_2 levels impact plant–animal chemical relationships in aquatic environments (Zupo and others 2015), potentially leading to detrimental ecological impairments. However, physiological responses to increasing CO_2 concentrations are species-specific (Gao and Campbell 2014). Studies on diatoms dominating the epiphytic communities of *P. oceanica* leaves indicate that high CO_2 levels alter the responses of invertebrates to wound-activated infochemicals produced by epiphytes (Mutalipassi and others 2020). Specifically, OA influences the metabolism of such diatoms as *Cocconeis scutellum* var. *parva*, affecting their production of volatile organic compounds (Mutalipassi and others 2019, 2022). These compounds, which play the role of infochemicals, are involved in plant–animal communications and influence the food webs of crucial environments, thereby impacting their proper functioning. Additionally, diatoms of the genus *Cocconeis* synthesize programmed cell death (PCD) metabolites (Zupo and others 2023) that dramatically influence the physiology of their consumers.

Consequently, *Cocconeis neothumensis* var. *marina* De Stefano and others (2000), is considered a well-established model diatom (Zupo 2000), belonging to a genus that seasonally dominates the leaves of *P. oceanica* (Zupo and others 2007) and substantially influences the food webs of this Mediterranean seagrass. The physiological effects of *Cocconeis* spp. have also driven biotechnological interest (Raniello and others 2007), as they produce programmed cell death (PCD) triggers, potentially valuable for biotechnological applications (Nappo and others 2012).

We collected two strains of *C. neothumensis* var. *marina* from *P. oceanica* meadows at two sites characterized by different pH conditions: ambient pH (8.1) and low pH (7.6). These sites included: (i) a meadow near shallow volcanic vents off the Island of Ischia (Gulf of Naples, Italy), characterized by naturally low pH conditions, and (ii) an adjacent coastal site hosting another meadow thriving under ambient pH conditions. The diatom strains were isolated and cultured axenically. Differences in the growth dynamics of the two strains, both collected and cultured under ambient and low pH conditions, were recorded. Similarly, variations in the metabolic profiles of the two strains, according to culture conditions, were assessed using metabo-

lomic tools. These investigations aimed at assessing whether OA may locally influence the selection of these diatoms, which are involved in important plant–animal chemical relationships.

Material and Methods

Diatom Isolation

We used sampling matrices which were specifically designed for epiphytic diatoms to collect species that strongly adhere to the substrate (for example, *Cocconeis* spp.). Each collecting matrix consisted of a plastic frame holding eight replicate glass disks (each with a surface area of 23.75 cm^2) painted with a low-adhesion silicone coating (Figure S1). They were anchored to the sand bottom using a mooring latch and maintained in a vertical position with a small buoy on the upper side. This setup ensured that the sampling devices remained vertical while freely floating, emulating the position of *P. oceanica* leaves, where epiphytes continuously settle. The matrices were deployed by scuba divers at the margin of *P. oceanica* meadows, at a depth of about 6 m, at two sites off the island of Ischia (Gulf of Naples, Italy; Figure 1) and kept in situ for 30 days, during spring. The first collecting matrix was located in Cartaromana bay, near Sant'Anna rocks (40°43′34.68″ N, 13°57′40.92″ E), and served as a control site under ambient pH conditions (8.1). The second device was deployed off the Castello Aragonese (40°43′50.62″ N, 13°57′47.99″ E) to exploit shallow CO_2 volcanic vents (low pH site) to collect diatoms under low pH conditions. CO_2 bubbles emerging from the seabed in this location acidify the water to a pH of 7.6 (Hall-Spencer and others 2008), mirroring the pH changes of the future world's oceans under SSP5-8.5 scenario by 2100 (Kwiatkowski and others 2020). After one month, the devices were transferred to the laboratory. The low-adhesion slides were removed, rinsed with filtered seawater (0.22 µm TPP ''rapid'' Filtermax), and gently scraped with a sterile glass slide to collect the organisms that colonized the smooth surface. Epiphytes scraped from each of 8 replicate low-adhesion slides were then divided into six subsamples, transferred into individual wells of a multiwell plate, and analyzed under Leica DM IL inverted microscopy (Leica microsystems DM IL, Germany) to isolate diatoms presumably belonging to the genus *Cocconeis*, recognized using optical microscopy (OM) based on their ovoidal shape. Diatoms of interest were isolated by sequentially transferring single cells with a micromanipulator (Leica Microsystems, Leitz Micromanipulator, Germany) equipped with a microinjector syringe (Narishige IM-5B, Japan) and a sterilized glass capillary .

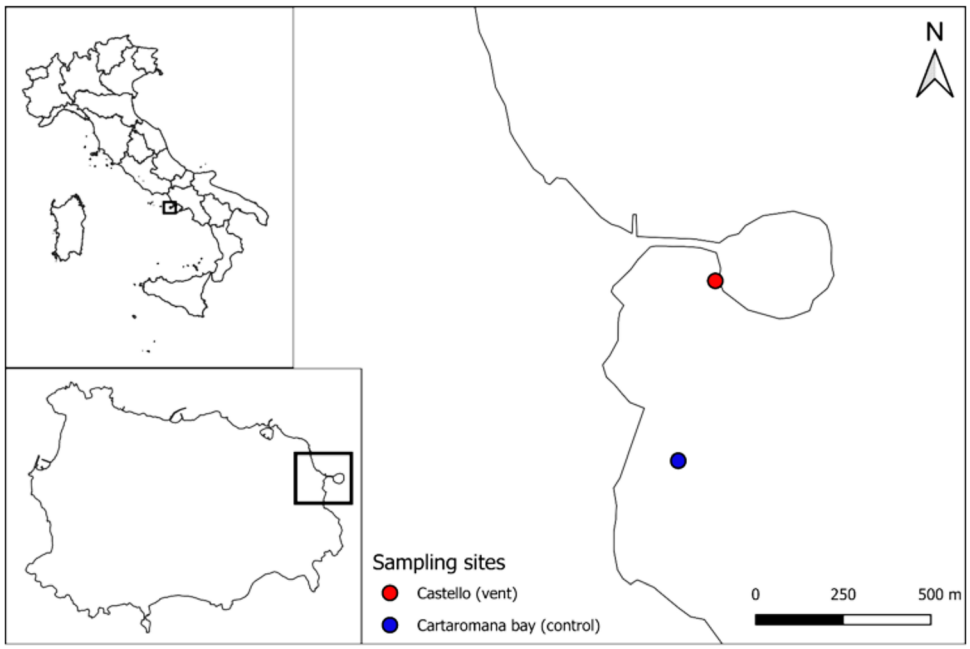

Figure 1. Map of the sampling sites in Italy (upper left square) off the Island of Ischia. A red dot indicates the site Castello Aragonese (40°43′50.62″ N, 13°57′47.99″ E; pH 7.6) and a blue dot indicates the site Cartaromana Bay (40°43′34.68″ N, 13°57′40.92″ E; pH 8.1).

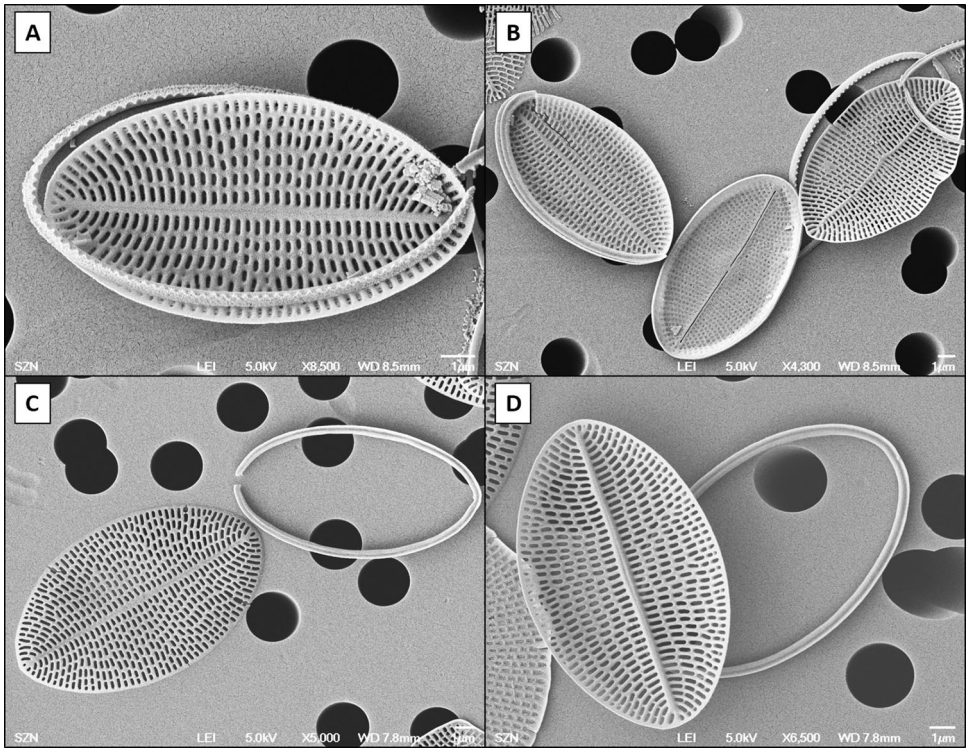

Figure 2. SEM images of the two strains of *C. neothumensis* isolated in this study. **A**, **B** CnA collected at pH 7.6 at Castello Aragonese. C and D) CnN collected at pH 8.1 at Cartaromana Bay

Following presumptive identification of *Cocconeis* spp., diatom cells were individually placed in wells containing 6 mL of sterilized seawater to obtain axenic monoclonal cultures, which were then identified under scanning electron microscopy (SEM) (Figure 2). The wells were checked daily under inverted OM to confirm the absence of contaminants. The purification of the strains required several further transfers of single cells. Once parent cultures of monoclonal strains were obtained, they were kept under controlled conditions in a thermostatic chamber at 18 °C with a 12:12 light/dark photoperiod (provided by Sylvania Gro-Lux neon lamps; Osram Sylvania Inc., USA) set at an irradiance of 140 μE m^{-2} s^{-2}. All strains were transferred approximately every 15–20 days to new sterile dishes. Transfers and renewals of cultures were performed under a laminar flow hood using sterile glassware. The isolation procedures yielded two strains of *C. neothumensis* var. *marina*, one from each sampling site, namely, a strain isolated from the ambient pH condition site (hereafter CnN) and a strain from the low pH condition site (hereafter CnA).

Morphological Identification

Morphological identification of diatoms was based on the ultrastructure of their frustules analyzed on SEM images. A portion of biofilm was collected from the mother cultures, centrifuged, cleaned with acids, and transferred to two replicate stubs. Diatom samples underwent a cleaning protocol with HNO_3 [65%] and H_2SO_4 [98%] to eliminate the organic material from the siliceous frustules. After the cleaning process with strong acids (according to von Stosch, 1974), samples were rinsed with distilled water, dehydrated, and transferred in a drop of pure EtOH onto SEM stubs. The stubs were dried overnight under a hood, sputtered with gold, and observed under SEM (Microscope JEOL 6700 F).

Molecular Identification

Lyses & Bacteria/Fungi PCR-GO Kit (DNA-TECH Spin-off) was used to perform PCR from single colonies of CnN and CnA, according to manufacturer's instructions (Somma and others 2025). PCR was performed with specific primers rbcL-F, 5′-3′ ATGTCTCAATCTGTAWCAGAACGGACTC and rbcL-R, 5′-3′ TAARAAWCKYTCTCTCCAACGCA, to amplify a rbcL fragment of 660 bp (Evans and

others 2007; Guo and others 2015). The purified PCR products (using the QIAquick Gel Extraction kit; Qiagen, Milan, Italy) were sequenced on an Applied Biosystems 3730 DNA Analyzer (48 capillaries; Life Technologies) using BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies). The sequences obtained from CnN and CnA were submitted to GenBank using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the species and then aligned to highly similar sequences using MultiAlin (http://multalin.toulouse.inra.fr/multalin/).

Cultures at Controlled pH Conditions

Photobioreactors for benthic diatoms were developed ad hoc to obtain cultures under ambient (pH = 8.1 ± 0.1) and low pH (pH = 7.6 ± 0.1) conditions (Mutalipassi and others 2019). Each photobioreactor was assembled in a Pyrex dish (300 × 200 × 40 mm, with a total volume of 2.4 L) covered with a glass lid and equipped with a pH probe (InLab®Micro pH, Mettler Toledo) designed to work in a reduced volume. A pH controller (pH 201, Aqualight) was connected to an InLab Micro-Probe (using a BNC cable) via an electronic valve connected to a CO_2 regulator (CO_2 Energy, Ferplast). A sterile glass tube reached the bottom, directing CO_2 bubbles to a position close to a water pump (Askoll Pure pump 300), to avoid water stratification. The CO_2 regulator and the centrifuge pump were operated simultaneously, when necessary, to adjust the pH to the pre-set values. Three replicates of the two diatom strains, CnN and CnA, were inoculated and cultured in individual photobioreactors, each at one of the two pH conditions, with controllers set at 7.6 (low pH) and 8.1 (ambient pH). This experimental plan allowed for the cultivation of both strains at both pH levels (7.6 ± 0.1 and 8.1 ± 0.1) in a fully randomized experiment consisting of three replicates for each of four treatments.

Six sterile square cover slides (2 × 2 cm in surface area) were deployed in each photobioreactor to evaluate the growth under inverted OM. Starting five days after inoculation, a cover slide was collected from each plate every other day, and the diatoms were photographed and counted in three random quadrats (surface area 0.2 × 0.2 mm) using a gridded eyepiece. The data collected were used to compute a growth curve for each strain (CnN and CnA) at the two culture conditions (pH 7.6 ± 0.1 and 8.1 ± 0.1). The medium was removed after 16 days of culture, and the plates were quickly rinsed with distilled water to remove residual salts, frozen at − 20 °C, and dried in a freeze dryer (Lio 5P, Italy) to collect the diatoms by scraping them with a sterile iron blade. The collected diatoms were then weighed and stored in glass vessels at − 20 °C.

^{1}H-NMR Metabolomic Analysis

For each strain (CnA and CnN), three replicates were obtained at each pH condition (pH 7.6 ± 0.1 and 8.1 ± 0.1). Diatoms were re-suspended in 170 µl of H_2O and 700 µl of methanol and then sonicated for 30 s. Subsequently, 350 µl of chloroform was added, and samples were mixed in an orbital shaker for 10 min on ice. Afterward, 350 µl of H_2O/chloroform (1:1, v/v) was added to each cell suspension and centrifuged at 4000 rpm for 10 min at 4 °C. The aqueous (polar) and lipophilic (apolar) phases were then collected separately, transferred to a glass vial, and dried under a nitrogen flow. Samples were analyzed using nuclear magnetic resonance (NMR) with a 600-MHz Bruker Avance DRX spectrometer equipped with a TCI probe. The polar fractions were dissolved in 630 µl of PBS-D_2O, with the pH adjusted to 7.20. As an internal standard, 70 µl of the sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid (1% in D_2O) was used. Concurrently, the lipophilic fractions were dissolved in 700 µl of deuterated chloroform. All ^{1}H-NMR spectra of the polar phases were acquired at 300 K using the excitation sculpting pulse sequence to suppress water resonance. A double-pulsed field gradient echo was used, with a soft square pulse of 4 ms at the water resonance frequency and gradient pulses of 1 ms duration. This sequence added 128 transients of 64 k complex points, with an acquisition time of 4 s per transient. Time domain data were zero-filled to 256 k complex points, and an exponential amplification of 0.6 Hz was applied prior to Fourier transformation.

In ^{1}H-NMR, each metabolite can produce multiple proton signals. The number of signals and their positions (chemical shifts) are determined by the unique chemical environments surrounding each hydrogen (proton) within the molecule.

Statistical and Pathway Analysis

The 0.50–8.60 ppm spectral region of the ^{1}H-NMR spectra was integrated into buckets of 0.04 ppm using the AMIX package (Bruker, Biospin GmbH, Rheinstetten, Germany). The water resonance region (4.5–5.2 ppm) was excluded during the analysis, and the bucketed region was normalized to the total spectrum area using Pareto scaling. Partial

least squares discriminant analysis (PLS-DA, multivariate analysis) were used to compare the spectra obtained from the polar and apolar phases of diatoms. A score plot is a visualization technique used in principal component analysis (PCA) to represent the relationships between data points based on their scores on principal components. It essentially shows how each data point is positioned in the reduced dimensional space created by PCA, allowing for the identification of clusters, outliers, and trends. Variable importance in projection (VIP) plot displays the most important metabolite features identified by projections to latent structures discriminant analysis (PLS-DA, multivariate analysis, PLS-DA). The scores in VIP plot represent the importance of each variable in a partial least squares (PLS) or orthogonal partial least squares discriminant analysis (OPLS-DA) model. A higher VIP score indicates that a variable has a greater influence on explaining the variation in the response variable (Y). Variables with VIP scores greater than 1 are often considered influential.

We used NLS methods with the Gompertz curve to model the growth curves of the strains cultivated under both pH conditions. The growth increments (number of cells per surface unit) of the two strains fit the sigmoid Gompertz curve (Tjørve and Tjørve, 2017), described by the following equation:

$$y(t) = \alpha^{-\beta^{-k_t}} \quad (1)$$

The growth curves (Figure 3) exhibited high correlation indices (R^2) ranging from 0.77 to 0.85 (Table 1). A Kruskal–Wallis test was applied to test the significance of differences among the growth curves of the strains and between pH levels of cultures. Graphs and statistical analyses were performed using the software R (version 4.3.1, R Core Team 2023).

Results

Identification and Growth Curves

The isolation of diatoms from benthic collectors resulted in fifteen strains of highly adhesive species,

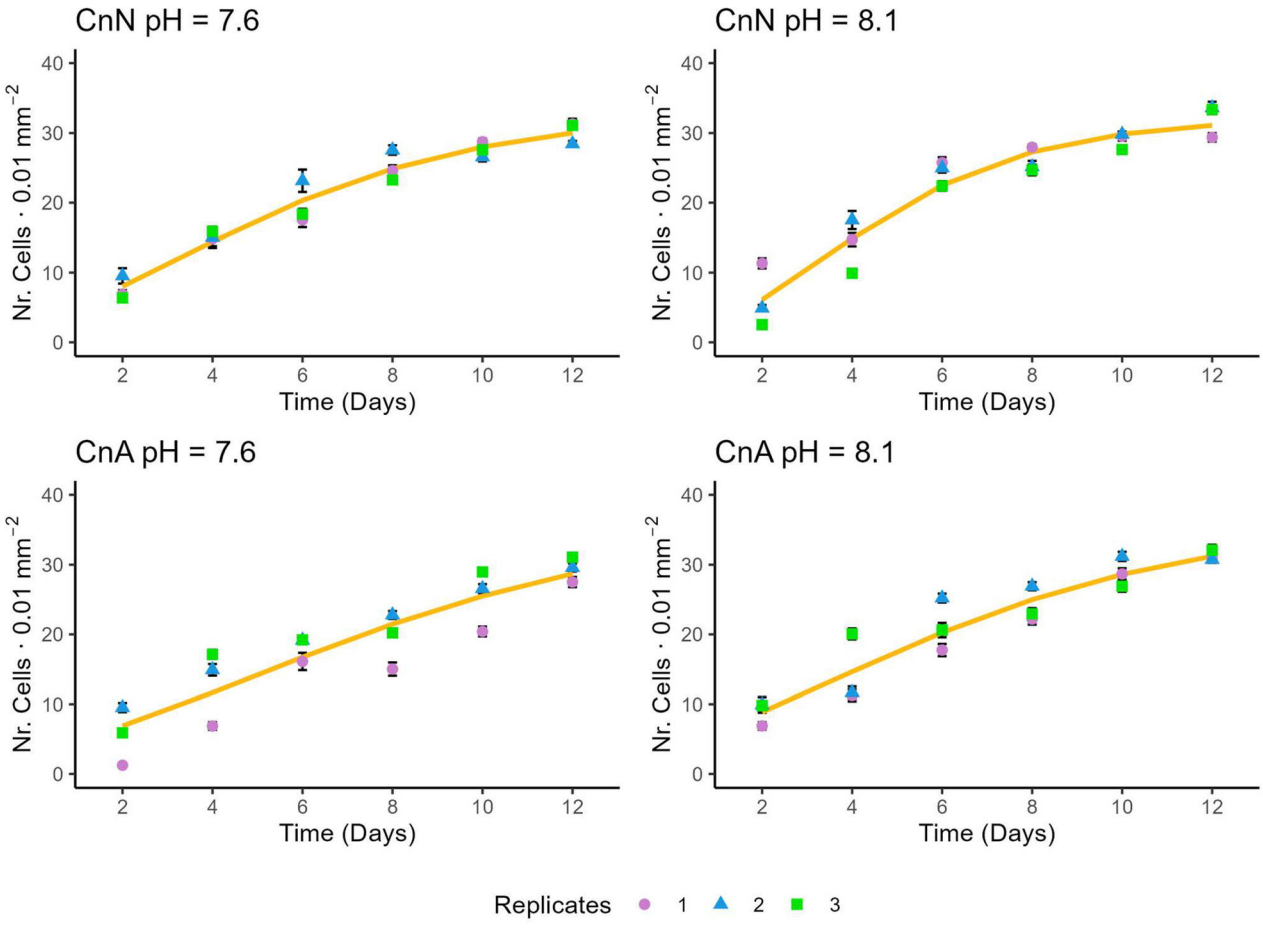

Figure 3. Growth curves for the two strains CnN and CnA, cultured at two pH conditions (7.6 and 8.1).

Table 1. Growth Parameters of *Cocconeis neothumensis* at Two pH Regimes, with R^2

Strain (and pH at collection sites)	Culture pH	α	β	k	R^2
CnN (8.1)	7.6	33.12718	2.417448	0.2666841	0.8268
CnN (8.1)	8.1	32.25347	3.578462	0.3829807	0.8529
CnA (7.6)	7.6	37.33126	2.451702	0.186097	0.7707
CnA (7.6)	8.1	36.64513	2.195439	0.2183115	0.8066

primarily belonging to the genera *Mastogloia* and *Cocconeis*, which are common epiphytes of *P. oceanica* leaves. This research focused on two conspecific strains collected from two sites with different pH (control, 8.1 vs. low, 7.6). Morphological identification of the diatom frustule ultrastructure indicated that both strains were *Cocconeis neothumensis* var. *marina* (Figure 2). This identification was confirmed through molecular techniques. Preliminary observations of the two cultured strains highlighted macroscopic differences in their growth patterns. The strain collected at the control site (CnN) exhibited a spatially homogeneous growth, due to continuous biofilm formation in the dishes. The strain collected at low pH (CnA) exhibited a ''patchy'' growth, with diatoms aggregated in small denser patches and empty spaces, was observed among them. These macroscopic differences in the spatial distribution patterns of the two strains were consistently maintained during several generations of cultured diatoms.

While replicates revealed variability due to patchiness in the growth patterns, the growth curves showed significant differences (Table 1). Specifically, the CnN strain cultured under both pH conditions grew faster and exhibited a steeper slope than the CnA strain. The CnN strain reached a plateau earlier, as supported by the highest growth rate parameter (k) in the computed equation (Table 1). Furthermore, the growth of the two strains under both culture pH exhibited statistically significant differences according to the Kruskal–Wallis test (Figures S2, S3). The average cell size in the two cultures also differed, with the CnN strain exhibiting an average maximum diameter of 13 µm (± 3.2), while CnA exhibited an average maximum diameter of 9 µm (± 2.1). Cell size varied over time in the asynchronous culture, depending on the frequency of sexual reproduction events.

^{1}H-NMR Analysis of Metabolites and Lipids

^{1}H-NMR spectra were obtained from aqueous extracts of the strains CnN and CnA cultivated at both pH conditions. Considering polar metabolites from CnN at pH 7.6 and 8.1, the score plot (Figure 4A) showed that both clustered into separate classes, suggesting the presence of different levels of metabolites (Figure 4B). In particular, (i) the levels of lactate, 2-hydroxybutyrate, formate, glutathione, and of some amino acids, such as valine, glutamate, leucine, and isoleucine, as well as glutathione, were higher in CnN cultivated at pH 7.6 compared to CnN cultivated at pH 8.1. In contrast, asparagine, phosphocholine, ornithine, glycine, choline, glycerol-phosphocholine, and glucose were higher in CnN cultivated at pH 8.1. Polar metabolites from CnA cultivated at both pH 7.6 and 8.1 (see the score plot in Figure 5A) showed two groups, although the pH 7.6 group exhibited higher variability. In particular, glucose, glycine, glycerol-phosphocholine, phosphocholine, lactate, and choline were higher in CnA cultivated at pH 7.6, whereas the aminoacids proline, arginine, leucine, valine, and hydroxyproline were higher in CnA cultivated at pH 8.1, as well as acetate and ATP (Figure 5B).

^{1}H-NMR spectra were also obtained from lipophilic extracts of CnN cultivated at both pH 7.6 and 8.1 and from CnA cultivated at pH 7.6 and 8.1. The score plot (Figs. 6A, 7A) showed that they both clustered, indicating the presence of statistically different levels of lipidic signals. In the case of CnN cultivated at pH 8.1, higher levels of proton signals of cholesterol, omega-3, docosahexaenoic acid and arachidonic acid were detected, whereas some proton signals of fatty acids and triglycerides were higher in CnN cultivated at pH 7.6 (Figure 6B). Statistically different levels of proton signals of phospholipids, linoleic acid, and fatty acids (Figure 7B) were observed in CnA cultivated at pH 8.1. Conversely, CnA cultivated at pH 7.6 showed increased proton signals of cholesterol, arachidonic acid and other fatty acids.

Discussion

Main Effects of OA on Diatoms

Ocean acidification (OA) elicits several physiological effects on marine organisms (Shi and Li 2024),

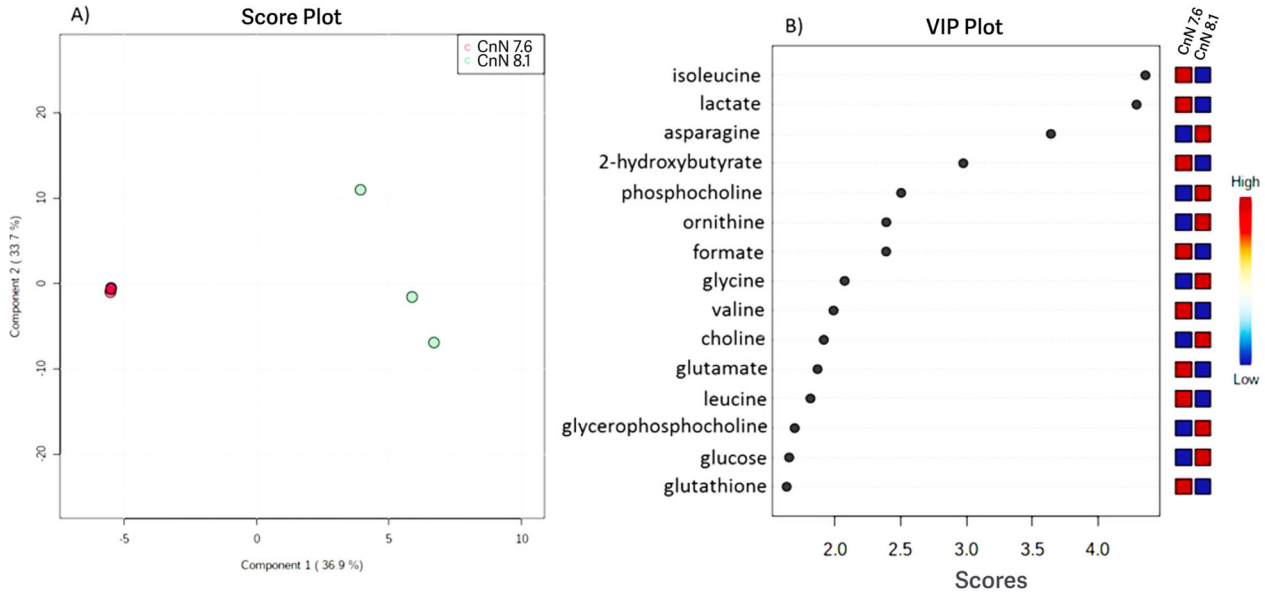

Figure 4. **A** Score plot and **B** variable importance in projection (VIP) plot of the metabolites. Colored boxes on right indicate relative concentration of metabolites that increase (red squares) and decrease (blue squares) in the aqueous extracts from CnN grown at pH 7.6 and pH 8.1. The scores in the VIP plot represent the importance of each variable in a partial least squares (PLS) or orthogonal partial least squares discriminant analysis (OPLS-DA) model. For details, see Materials and Methods section.

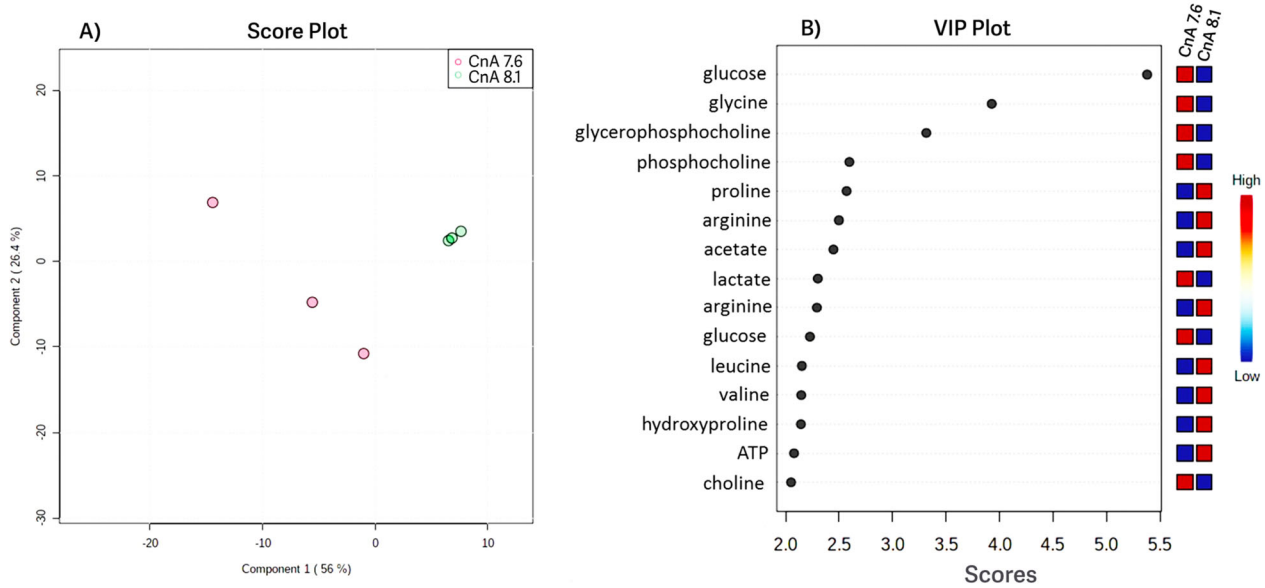

Figure 5. **A** Score plot **B** and variable importance in projection (VIP) plot of the metabolites. Colored boxes on right indicate relative concentration of corresponding metabolites that increase (red squares) and decrease (blue squares) in the aqueous extracts from CnA grown at pH 7.6 and pH 8.1. Glucose and arginine repeated twice correspond to two proton signals for each metabolite, which were found to be significant. For details, see Materials and Methods section and legend to Figure 4.

including oxidative stress, metabolic impairments, retarded growth, abnormal development, and various genetic influences (Ivanina and Sokolova 2015). These effects impact animal behavior (Zupo and others 2015) and affect the structure of invertebrate populations (Tai and others 2021). Our results revealed strong physiological differences between two strains collected from sites with

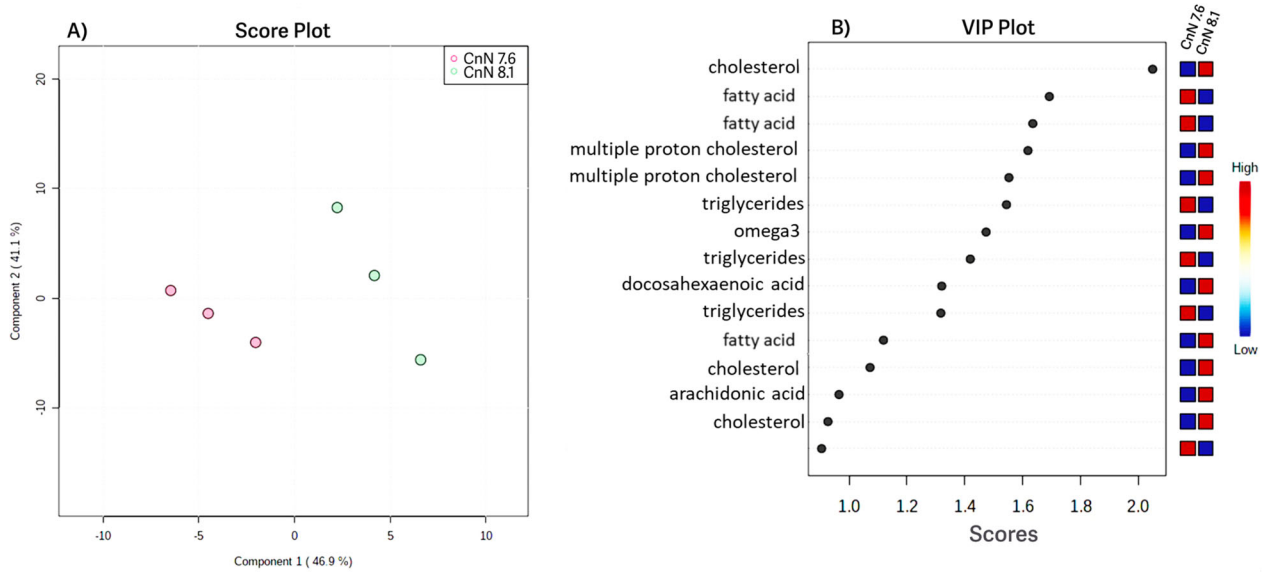

Figure 6. **A** Score plot and **B** variable importance in projection (VIP) plot of the metabolites. Colored boxes on right indicate relative concentration of corresponding metabolite for samples that increase (red squares) and decrease (blue squares) in the lipophilic extracts from CnN grown at pH 7.6 and pH 8.1. Cholesterol repeated five times, fatty acids, and triglycerides repeated three times correspond to proton signals for each metabolite, which were found to be significant. For details, see Materials and Methods section and legend to Figure 4.

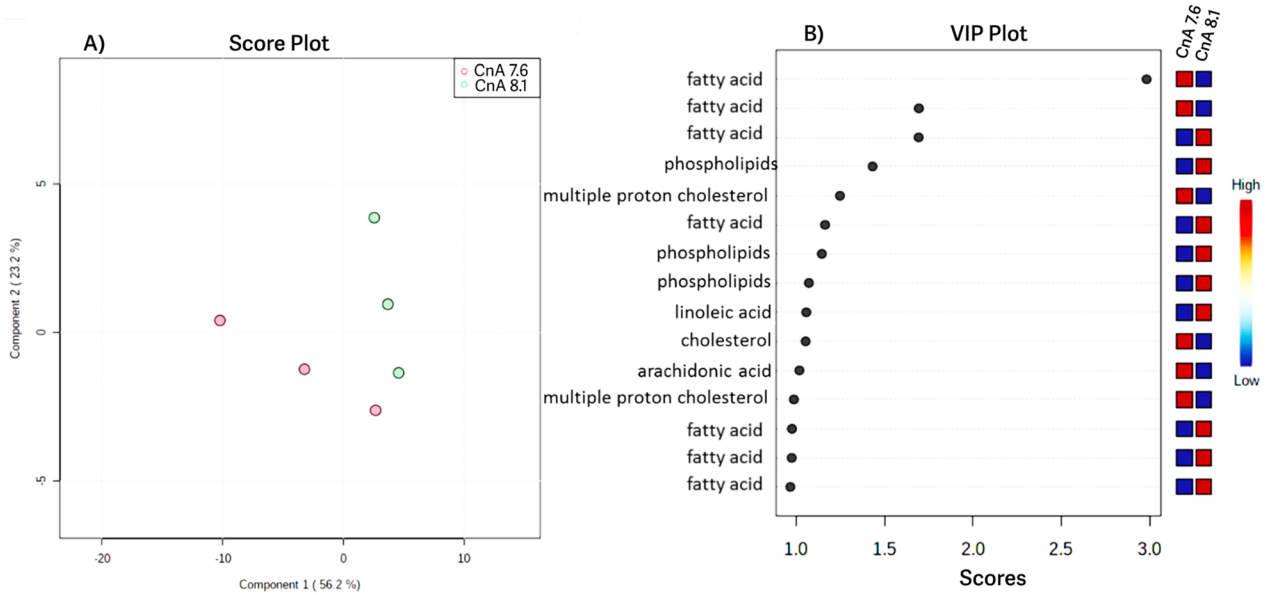

Figure 7. **A** Score plot and **B** variable importance in projection (VIP) plot of the metabolites. Colored boxes on right indicate relative concentration of corresponding metabolites for samples that increase (red squares) and decrease (blue squares) in the lipophilic extracts from CnA grown at pH 7.6 and pH 8.1. Fatty acids repeated seven times, cholesterol and phospholipids repeated three times correspond to proton signals for each metabolite, which were found to be significant. For details, see Materials and Methods section and legend to Figure 4.

different pH conditions, influencing their growth patterns, the average size of their frustules, and their replication speed. The growth curves of strains isolated from ambient pH sites and low pH sites were significantly different. Both strains grew faster at pH 8.1, exhibiting distinct exponential phases. Although the influence of OA on diatom proliferation is known (Wu and others 2014; Li and others

2023), this study highlights that, under both pH conditions, strains collected under ambient pH conditions (which consistently showed larger cells) grew faster than strains collected under low pH conditions (which regularly showed smaller cells in cultures). Contrasting influences of pH on the growth of several planktonic diatoms have been demonstrated (Mus and others 2013; Bermúdez and others 2015). For instance, a low pH declining to 7.77 did not affect the growth rates of the toxic diatom *Pseudonitzschia australis* (Wingert and Cochlan, 2021). Similarly, the growth rates of the non-toxic diatom *P. fraudulenta* were unaffected by pH levels ranging from 7.40 to 8.07, and rising pCO_2 did not significantly impact the growth of *Phaeodactylum tricornutum* (Cai and others 2022). However, OA promoted the growth of *Thalassiosira* sp., particularly at lower temperatures (15 °C), while negative effects of OA were demonstrated by Torstensson and others (2012) on planktonic diatoms. Evidently, the sensitivity of diatoms to increasing CO_2 levels varies according to the species, and relates to specific genetic traits, such as those influencing the cell size (Wu and others 2014) and the carbon fixation pathways (Lone and Malik 2021). However, interactions with environmental factors like nutrients, temperature, and light irradiance (Pérez-Rojas and others 2022) may also influence these patterns.

Laboratory analyses showed that exposure to higher CO_2 levels had negligible effects on some diatoms (Tortell and others 2000), but recent investigations demonstrated that increased CO_2 may positively influence the growth of some diatoms (Mutalipassi and others 2019; Brodie and others 2014; Harvey and others 2021) by reducing the energetic costs of their highly efficient concentrating mechanisms (CCMs). The CCMs, which enable microalgae to proliferate when CO_2 concentration limits photosynthesis, result from an adaptive response to gradual CO_2 increases (Matsuda and others 2017). Lower surface area-to-volume ratios lead to a lower diffusive flux of CO_2 relative to their carbon demand for growth. In addition, under low pH conditions, a higher availability of dissolved CO_2 reduces the energetic cost of active bicarbonate (HCO_2^-) uptake, leading to a potential downregulation of CCMs. This permits cells to redirect metabolic energy toward biosynthetic pathways, increasing the production of specific metabolites (Moore and others 2015; Lone and Malik 2021). Conversely, under ambient pH conditions diatoms could rely on active bicarbonate transporters, requiring additional ATP investment. This may lead to increased synthesis of phospholipids and cholesterol, crucial for maintaining membrane integrity and cellular signaling (Cai and others 2022; Sunshine and Iruela-Arispe 2017). Tortell and others (2008) showed increased multiplication rates and a shift of diatom communities in response to OA in situ.

These results demonstrate that OA may interact with other environmental factors to amplify its effects (Hatje and others 2022) and that medium acidification produces species-specific, often contrasting, effects. Studies on diatom communities revealed that centric diatoms, primarily planktonic, successfully resist seawater acidification and are favored by lower pH compared to pennate species (Tortell and others 2002; Wu and others 2014; Feng and others 2021; Cai and others 2022). This is mainly due to cell size and morphology. Considering that diatoms contribute about 40% of the primary production in seas and play key roles in aquatic food webs, their fate in acidified oceans is likely to have dramatic implications for the stability and resilience of several ecosystems. Indeed, research on centric diatoms demonstrated a frequent size dependence of CO_2 which stimulated growth (Wu and others 2014).

The photosynthetic efficiency of planktonic diatoms is less affected by elevated CO_2 than other microalgal groups (Cai and others 2022), but several studies demonstrated how cell size influences photosynthetic capacity in response to OA (Key and others 2010; Li and Gao 2013). Larger planktonic diatoms (diameter $>$ 30 µm) showed significant enhancement of growth rates, while smaller diatoms (cell diameter within 4–30 µm) exhibited reduced effects. The strains of *C. neothumensis* var. *marina* collected in benthic environments and investigated in this study exhibited a maximum total length of the apical axis of about 13 µm for CnN, while CnA exhibited a smaller size. These relationships indicate that OA positively influenced the growth of species with larger size (such as observed for CnN) in the size range characterizing the two isolated strains. Other investigations (Moore and others 2017) showed that nutrient concentration induces key sexual morphologies, such as oogonia, auxospores, and spermatogonia, in strains of *Thalassiosira pseudonana*, *T. weissflogii*, and *Cyclotella cryptica*. In this case, higher nutrient concentration was a trigger for sexual reproduction, producing larger cells, while in our study, CO_2-acidified waters selected strains with a smaller size. These differences add to the plethora of information already available but not yet completely understood, regarding the mechanisms determining the reproduction and size of diatoms.

Our results highlight that, beyond the influence of OA on the size and growth patterns of diatoms, two strains of *C. neothumensis* exhibiting different metabolism (even when cultivated under the same conditions) were isolated at different pH conditions. Although they corresponded to the same species, according to morphological and molecular identification, the differences were unlikely to result from random variation. Previous investigations (Somma and others 2023) demonstrated that global changes alter the successions of early colonizers of benthic surfaces, including diatoms. Consequently, we cannot exclude that future OA scenarios will determine the selection of specific diatom strains, which exhibit remarkable differences in their physiology, size, growth patterns, and biosynthesis of essential metabolites. In such cases, noteworthy effects on the physiology of consumers might be anticipated.

Selection and Metabolic Shifts under OA

The physiological divergence observed between the two strains of *C. neothumensis* reinforces the view that OA acts as a selective pressure. Our results align with predictions that non-calcified primary producers, particularly diatoms, might expand under higher CO_2 concentrations, while calcified epiphytes decline (Brodie and others 2014). Such shifts, inferred from natural CO_2 vents, suggest that acidification-tolerant taxa might dominate future ecosystems. Diatoms have been shown to characterize marine food webs under elevated CO_2, often outcompeting macroalgae and reshaping trophic dynamics (Harvey and others 2019). Moreover, benthic assemblages exposed to long-term acidification may become locked in early successional stages, with reduced diversity and complexity. The distinct physiological profiles of *C. neothumensis* strains isolated from low pH sites are consistent with this selection, indicating that OA may stabilize epiphytic communities in simplified, fast-growing stages. While primary productivity may be conserved, community development could be constrained. Moreover, additional stressors (*for example,* nutrient enrichment and sedimentation) can amplify the effects of acidification, further favoring stress-tolerant taxa (Johnson and others 2015). At a microbial scale, OA alters both taxonomic and functional composition of biofilms (Kerfahi and others 2023), a pattern mirrored in our metabolomic data. Our results provide a mechanistic foundation for predicting community shifts across benthic systems, by revealing intraspecific physiological plasticity of diatoms.

In addition, while several effects of OA on the growth and expansion of diatoms have been previously demonstrated, it is worth noting that most research focused on planktonic diatoms. Here, for the first time, the metabolic profiles of a benthic diatom were analyzed in response to OA. Metabolomics is a challenging technique because, unlike genomic and proteomic methods, it allows for the analysis of molecules with a range of physical properties, from water-soluble organic acids to highly lipophilic compounds (Clish 2015). To date, metabolomic approaches to the effects of OA on microalgae were only provided by a few studies (Tan and others 2019; Sanchez-Arcos 2024).

We showed that strong differences in the metabolic profiles may be demonstrated not only within the two strains cultivated at each of the two pH conditions, but also between them. In fact, polar metabolites produced by the two strains significantly differed. A higher proportion of glycine, involved in the photorespiratory glycolate pathways (Kroth and others 2008), was produced by the CnN strain cultured at pH 8.1 and, conversely, by the CnA strain cultured at pH 7.6. These differences could be relevant for the metabolism of crustacean consumers (Li and others 2019) and also important for biotechnological purposes, as glycine may become conditionally essential in mammals and may be limiting for the normal development of the fetus (Jackson and others 2002). Consequently, our results demonstrated that correct culture conditions for this diatom, in an OSMAC (One Strain Many Compounds; Romano and others 2018) perspective, may lead to the maximization of the glycine production.

In contrast, both strains showed similar trends in the production of choline, phosphocholine, and glycerol-phosphocholine, at ambient pH (8.1). The production of valine and leucine was higher in CnN cultured at low pH (7.6), but increased in CnA cultured at pH 8.1. Diatoms produce these amino acids starting from cellular carbon (C) and nitrogen (N), adopting glycolysis, gluconeogenesis, respiration, and the citric acid cycle (Bromke 2013). Experimental evidence indicated the species specificity of essential and non-essential amino acid composition of diatoms within individual taxonomic groups (Kolmakova & Kolmakov 2020; Khatuoon and others 2009; Consequently, the medium pH was demonstrated to be a strong determinant for the biosynthesis of specific amino acids, and this evidence might be exploited during the massive production of diatoms for biotechnological purposes (Fouillaud and Dufossé, 2022). Notably, a lower pH reduced the production of

lactate, glucose, and formate, which are involved in the glycidic metabolism of consumers, along with isoleucine, valine, and leucine (branched-chain amino acids, BCAAs, and essential amino acids), as well as glutathione, which is involved in oxidative stress responses.

In some cases, diatoms can lower the intracellular pH, for example to maintain the cross-membrane electrochemical gradient for H^+ efflux (Shi and others 2019). Several key enzymes are pH dependent although the sensitivities to pH might be complex and vary by organism, potentially disrupting, for example, the protein synthesis machinery (ribosomes, tRNAs also pH-sensitive). This is the case of phosphofructokinase-1 (PFK-1; Wang and others 2024) and glutathione synthetase (Matsuda and others 1996). If low pH leads to cellular stress and damage, the demand for glutathione might increase, even if the production capacity is hampered. In addition, pH gradients are crucial for transport processes. In fact, low pH might disrupt membrane potential or the activity of membrane transporters needed for substrate uptake, or even product exports related to these pathways (Leuthold and others 2009; Flynn and others 2012). Extreme pH can sometimes directly affect the stability of substrates and products, although this is less than a primary driver for reduced production, as compared to the effects of enzymes. Significant differences were also observed in the metabolic profiles of lipophilic compounds, along with varying levels of lipidic signals, showcasing the crucial role played by several lipidic compounds produced by diatoms in the physiology of their consumers (Gerecht 2010; Russo 2019; Sanchez-Arcos and others 2024). Cholesterol and arachidonic acid were present at higher proportions in CnN cultured at pH 8.1 and CnA cultured at pH 7.6 (that is, in their native conditions of isolation). These differences have an ecological significance, as cholesterol is an essential compound for copepods feeding on planktonic diatoms (Hassett and others 2004). In fact, diatoms exposed to ambient pH appear to likely provide a higher-quality food source for their consumers. Cholesterol positively affects egg production rates in several species of copepods, including *Acartia hudsonica*, *Acartia tonsa*, and *Calanus finmarchicus*. Several studies (Wichard and others 2008; Gerecht 2010) indicated that diatoms under stressful conditions (triggered by the presence of grazers) produce polyunsaturated aldehydes (PUAs) and other deleterious compounds, probably as part of a defensive mechanism against oxidative damage (Bermúdez and others 2015), reducing the fertility of consumers. The metabolic differences revealed here confirm that stressed strains (in this case, due to the effect of abnormal pH) tend to be a worse food for animal consumers (Russo 2019). This is specifically due to misregulation of PUAs and PUFAs production within the biosynthetic pathways of fatty acids, affecting their nutritional parameters. Such alteration of PUAs production should be considered in the context of chemical communications, because PUAs can act as signals for consumers and have a defensive role, triggering detrimental effects on grazers (for example, impairing reproductive success of copepods; Ianora and others 2004, 2015.)Furthermore, OA modified the metabolism of cholesterol, triglycerides, and fatty acids in both strains, with direct implications for membrane fluidity (Kaddah and others 2018) and structural stability (Hąc-Wydro and Wydro 2007). Consequently, it impacts nutrients uptake, waste removal and several cellular functions under changing environmental conditions (Yoon and others, 2021; Chadova 2024). Investigations also demonstrated that OA negatively influences the production of specific fatty acids involved in the reproductive physiology of benthic shrimps feeding on *Cocconeis* spp. (Mutalipassi and others 2019). Consistent with these findings, other studies (Sanchez-Arcos and others 2024) performed on a strain of *Coconeis scutellum parva* collected under ambient pH, based on metabolomics and bioactivity-based fractionation, identified a fatty acid ester and a phospholipid, LPG (lysophosphatidylglycerol, 16:1), as potential apoptogenic metabolites responsible for dramatic physiological consequences on diatom consumers. Their production was largely prevented by cultivating diatoms under lower pH regimes. Therefore, the results of this study will be useful to modulate the culture conditions of diatoms and maximize the biosynthesis of bioactive molecules for medical, nutraceutical, and other biotechnological purposes.

Overall, our findings demonstrate that OA alters the metabolic profiles and growth dynamics of diatom strains, by reducing the synthesis pf phospholipids. The differences observed between strains isolated at naturally low pH and ambient pH environments may drive the selection of specific diatoms, with far-reaching consequences for marine food webs, in future OA scenarios.

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AUTHOR CONTRIBUTIONS

MC and VZ conceptualized and designed the study; VZ, MC, and AT supervised the whole experimental research; ES, MC, NR, MM, and FI performed the field and laboratory activities; ES,MC, and JC analyzed the data; MC and ES led the writing of the manuscript, and AT and VZ revised the first draft. All authors critically contributed to the drafts and gave final approval for publication.

DATA AVAILABILITY

Data are available at https://www.szn.it/index.php/it/.

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