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RESEARCH

Evaluating the ability of macroalgae to create a chemical refuge for bivalves under ocean acidification conditions in closed-environment experiments

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Abstract

Ocean acidification (OA) can impact aquaculture because reduced pH may negatively affect the calcification in bivalve species. Photosynthetic activity can naturally generate an OA buffering effect, favouring the calcification process by increasing the surrounding seawater pH. Therefore, the incorporation of macroalgae into bivalve farms may be a strategy to mitigate the impacts of acidification on the industry. In this study, we evaluated the modification of seawater chemistry by the metabolic activity of the blue mussel *Mytilus chilensis* and three macroalgae (*Ulva* sp., *Chondracanthus chamissoi* and *Macrocystis pyrifera*), in monocultures and co-cultures under ambient and acidified initial conditions in three closed-environment experiments. In all three experiments, photosynthesis and respiration modulated seawater chemistry, resulting in higher values of pH, oxygen concentrations, and aragonite saturation state (Ω_{Ara}) in macroalgal monocultures compared to mussel monoculture. In co-cultures, pH, oxygen concentrations and Ω_{Ara} were higher than in mussel monoculture but lower than in macroalgal monoculture. In co-cultures, the OA buffering effect (pH > 7.7, $\Omega_{Ara} > 1$) was observed during daytime, but unfavourable conditions for calcification were observed during nighttime. These results are species-specific, with a greater capacity for pH increase for *Ulva* sp. and *Ch. chamissoi* and limited capacity for *M. pyrifera* in both initial pH treatments. Results of the enclosed environment experiments indicate that the presence of macroalgae in co-cultures did not guarantee favourable conditions for mussel calcification in acidified conditions.

Keywords Aquaculture · Photosynthesis · Reduced pH · Seaweeds · Shellfish

Introduction

Anthropogenic activities have increased global atmospheric CO_2, leading to the current climate change. Since the pre-industrial era, concentrations of atmospheric CO_2 have risen from 280 µatm to current levels of 422 µatm (IPCC 2021; NASA 2023), and projections indicate that atmospheric CO_2 could reach concentrations over 800 µatm by 2100 (IPCC 2021). The World´s oceans have absorbed more than 30% of that human-produced CO_2 which has led to physical and chemical changes in the ocean´ surface (Resplandy et al. 2018). One of the most significant drivers of climate change is the reduction in pH and chemical alterations in seawater, phenomena collectively known as ocean acidification, abbreviated as OA hereafter (IPCC 2021). OA can directly impact various marine organisms and the ecosystem services they provide to humans (Müller et al. 2009; Méléder et al. 2010; Kroeker et al. 2013; Krumhansl et al. 2016; Fernández et al.

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2019), including calcifying mollusks and macroalgae, which are important ecological engineers and crucial for economic activities such as aquaculture (FAO 2020).

OA can negatively affect aquaculture because reduced pH has been reported to have adverse effects on the physiology of bivalve species. These effects include increased vulnerability of early life stages, impairments in neurological functions, alterations in behavior, malformation, and dissolution of calcified shells (Kurihara et al. 2007; Kurihara 2008; Range et al. 2011; Barton et al. 2012; Barros et al. 2013; Vihtakari et al. 2013; Duarte et al. 2014; Frieder et al. 2014; Waldbusser et al. 2015; Guo et al. 2016; Benítez et al. 2018). Bivalve shells are composed partially or entirely of aragonite, a carbonate mineral, making them vulnerable to reductions in seawater pH that lead to a decline in the saturation state of aragonite (Ω_{Ara}) below 1 (Gazeau et al. 2013). Consequently, the energetic costs of calcification and the regulation of other physiological processes increase under OA conditions, affecting growth, development, and survival (Pörtner et al. 2004; Fabry et al. 2008; Gazeau et al. 2013), ultimately resulting in low biomass production.

The response of macroalgae to OA is influenced by their utilization of dissolved inorganic carbon (DIC) during photosynthesis. Approximately, 65% of marine macroalgae possess CO_2-concentrating mechanisms (CCMs) that enable them to actively take up HCO_3^- (Kübler and Dudgeon 2015; Stepien 2015). However, some species, mostly rhodophytes and chlorophytes, lack CCMs and are restricted to CO_2 uptake. CCM-equipped species are expected to react neutrally or positively to OA because their photosynthetic rates are already saturated for DIC, whereas non-CCM macroalgae are expected to respond positively to OA due to potential limitations in their photosynthetic rates caused by current $CO_{2(aq)}$ concentrations (Raven 1991; Giordano et al. 2005; Raven et al. 2005; Hepburn et al. 2011; Raven and Hurd 2012; Cornwall et al. 2015). Under progressively acidified conditions, the photosynthetic use of DIC is expected to reduce dissolved CO_2 concentrations, increase pH, and mitigate the negative of OA effects on bivalves in the surrounding environment.

The impacts of OA on bivalves can be especially problematic in areas where bivalves are the primary resource for aquaculture. In Chile, bivalve aquaculture is primarily focused on monocultures of the blue mussel *Mytilus chilensis*, producing 366,000 t in 2018, making it the second-largest global producer of mussels after China (FAO 2020). However, one of the industry's significant challenges is diversifying species and culture systems (Buschmann et al. 1996, 2008, 2013; Harvey et al. 2017; Fernández et al. 2019). To contribute to industry diversification, the incorporation of macroalgae has shown multiple environmental and productivity benefits in co-cultures with higher trophic level organisms such as finfish and bivalves. The cultivation of macroalgae may provide a chemical refuge against acidified conditions (Unsworth et al. 2012; Buapet et al. 2013; Hendriks et al. 2014; Krause-Jensen et al. 2016; Greiner et al. 2018; Groner et al. 2018; Fernández et al. 2019) potentially serving as a tool to buffer the negative impacts of OA on bivalve aquaculture.

Seawater pH may be reduced by bivalve respiration (and macroalgal photorespiration during nighttime) which releases CO_2 through the aerobic oxidation of organic carbon (Beer et al. 2014). Calcification processes in bivalves can also alter seawater carbonate equilibrium by generating one mole of dissolved CO_2 per one mole of $CaCO_3$ deposited (Ware et al. 1991). The increase in dissolved CO_2 concentrations resulting from metabolic activity is accompanied by a reduction in seawater pH, leading to levels expected under future OA conditions (Hofmann et al. 2011; Cornwall et al. 2013). These conditions are unfavourable for bivalve calcification and growth (i.e., $\Omega_{Ara} < 1$) in the local area. In contrast, macroalgae, during daytime, remove CO_2 through photosynthesis from the proximate seawater, increasing seawater pH to levels as high as 8.8 (Hofmann et al. 2011; Cornwall et al. 2013). This can create favourable conditions for shellfish calcification (i.e., $\Omega_{Ara} > 1$). This chemical refuge for calcifying organisms that counteract reduced pH effects on calcification has recently been termed "OA buffering effect" (Fernández et al. 2019). In addition, shellfish release CO_2, excrete NH_4^+ and urea that might enhance photosynthesis and growth of macroalgae (Harrison and Hurd 2001; Chung et al. 2013; Duarte et al. 2017; Roleda and Hurd 2019).

Metabolic interaction between bivalves and macroalgae has been observed in cultures. In OA experiments, the negative effects of exposure to elevated CO_2 (reduced pH) on valve and tissue growth rates of the clam *Mercenaria mercenaria* Linnaeus, the oyster *Crassostrea virginica* Gmelin, the scallop *Argopecten irradians* Lamarck and the blue mussel *Mytilus edulis* Linnaeus were mitigated by the photosynthetic activity of the kelp *Saccharina latissima* (Linnaeus) CE. Lane, C. Mayes, Druehl & GW. Saunders and the green macroalgae *Ulva* sp., creating a refuge for calcifying bivalves in an acidified environment (Young and Gobler 2018; Young et al. 2022). The co-culture of bivalves and macroalgae has also been reported to benefit macroalgal species. In tanks and field cultures, *S. latissima* displayed increased growth, pigments and carbon and nitrogen content related to the ammonium and phosphate released by *M. edulis* and the oyster *Magallana gigas* Thunberg (Hargrave et al. 2021, 2022). Furthermore, bivalves can feed on larvae and spores, reducing the amount of epibionts on macroalgal thallus. These studies suggest that macroalgae and bivalves can mutually benefit in co-culture conditions.

In summary, bivalve aquaculture may be negatively affected by climate change-related drivers because OA can affect calcification and growth of bivalves. Therefore,

mitigation strategies are urgently needed to reduce the effects of climate change on this economic sector (Alleway 2023; Cotas et al. 2023). One possible strategy is to incorporate macroalgae into bivalve aquaculture because photosynthetic activity can naturally generate an OA buffering effect (i.e., reduction of CO_2 excess and increases in seawater pH and Ω_{Ara}) which favors the calcification process (Fernández et al. 2019). However, is still necessary to better understand the metabolic interactions between bivalves and macroalgae under the current and future OA conditions. Therefore, the aim of this study was to assess how seawater chemistry is modified both in monocultures and co-cultures of the green alga *Ulva* sp., the red alga *Chondracanthus chamissoi* (C.Agardh) Kützing and the brown alga *Macrocystis pyrifera* (Linnaeus) C.Agardh, and the blue mussel *Mytilus chilensis* Hupé under acidified conditions in a closed environment for 24h. We hypothesized that, under OA, the seawater chemical conditions for calcification will be favourable in the co-culture and macroalgal monoculture treatments, but unfavourable in the bivalve monoculture treatment.

Materials and methods

Collection and acclimation

Adult individuals of *Mytilus chilensis* and the three macroalgae (*Ulva* sp., *Chondracanthus chamissoi* and *Macrocystis pyrifera*) were obtained from experimental farms located in Dalcahue, Chiloé Island, southern Chile. Those farms were subjected to variation in abiotic factors that included temperatures between 11 – 16ºC, salinity between 28 – 32 ‰ and PAR between 100 – 1400 µmol photon m^{-2} s^{-1} (Henríquez-Antipa et al. 2019; Instituto de Fomento Pesquero 2023) during the experimental dates (Table 1). Samples of each species were collected within one week before the start

Table 1 Initial experimental conditions (seawater pH and O_2) and actual cultured biomass (g FW) of the bivalve *Mytilus chilensis* and the three macroalgae (*Ulva* sp., *Chondracanthus chamissoi* and *Macrocystis pyrifera*) for co-culture combination in the three performed experiments. Values correspond to mean ± SD (n = 4)

Initial experimental conditions						
	Date	Nominal pH	Actual pH_T value	Oxygen (mg L^{-1})	Actual cultured biomass (g FW)	
A) *M. chilensis*:*Ulva* sp.	13-02-2020	Ambient	8.106 ± 0.057	9.21 ± 0.05	Bivalve mono-culture (1:0)	12.39 ± 0.92
					Macroalga mono-culture (0:1)	5.95 ± 0.45
					Co-culture (2:1)	12.69 ± 0.53:5.65 ± 0.13
		Extreme OA	7.095 ± 0.051	9.30 ± 0.11	Bivalve mono-culture (1:0)	12.72 ± 0.53
					Macroalga mono-culture (0:1)	5.95 ± 0.61
					Co-culture (2:1)	12.17 ± 0.64:5.75 ± 0.47
B) *M. chilensis*:*C. chamissoi*	17-12-2020	Ambient	7.870 ± 0.005	7.02 ± 0.20	Bivalve mono-culture (1:0)	11.09 ± 0.68
					Macroalga mono-culture (0:1)	3.66 ± 0.18
					Co-culture (3:1)	11.21 ± 0.75:3.39 ± 0.15
		Extreme OA	7.044 ± 0.029	7.02 ± 0.19	Bivalve mono-culture (1:0)	12.75 ± 0.68
					Macroalga mono-culture (0:1)	3.37 ± 0.20
					Co-culture (3:1)	10.29 ± 1.04:3.48 ± 0.10
C) *M. chilensis*:*M. pyrifera*	21-01-2021	Ambient	8.059 ± 0.031	9.27 ± 0.01	Bivalve mono-culture (1:0)	18.73 ± 0.56
					Macroalga mono-culture (0:1)	5.88 ± 0.21
					Co-culture (3:1)	18.74 ± 0.27:6.10 ± 0.18
		Extreme OA	7.045 ± 0.027	9.15 ± 0.41	Bivalve mono-culture (1:0)	18.36 ± 021
					Macroalga mono-culture (0:1)	5.82 ± 0.20
					Co-culture (3:1)	18.61 ± 0.28:5.87 ± 0.13

each experiment. These samples were separately packed and transported in a cool box to the ARM_{lab} facilities within 3 h of collection. In the laboratory, samples were lightly brushed and cleaned of visible epibionts using filtered seawater (1 µm, Whatman Polycap TC filter capsule, GE Healthcare Life Sciences, UK), and were kept in separated areas inside a common temperature-controlled room until the start of the experiment. The acclimation conditions in the area for bivalves consisted of low light (PAR of 12.30 ± 3.27 µmol photon m^{-2} s^{-1}; 12:12 h day:night) to avoid undesirable overgrowth of microalgae (mixture of *Nannochloris* sp. and *Chaetoceros* sp.) provided as *ad libitum* food, a temperature of 15.62 ± 0.28 °C and a salinity of 34 ± 0.7 ‰. For the macroalgae area, acclimation conditions consisted of a PAR of 152.11 ± 32.12 µmol photon m^{-2} s^{-1} (12:12 h day:night), a temperature of 16.86 °C ± 0.09 °C and a salinity of 34‰. Tissue samples used in each experiment were excised 24 hours before the experiment and were kept under acclimation conditions to allow for wound healing (Huovinen et al. 2010).

Co-culture experiments

Three separate experiments of bivalve-macroalga co-cultures were conducted: experiment A, *M. chilensis* and *Ulva* sp.; experiment B, *M. chilensis* and *C. chamissoi*; and experiment C, *M. chilensis* and *M. pyrifera* (Table 1). For each co-culture experiment, a given biomass of the two species were mixed (n = 4) at three different initial biomass proportions, as bivalve:macroalga (Fig. 1). The actual biomass for each proportion treatment is summarized in Table 1. The cultured organisms were exposed to two initial seawater pH treatments (ambient pH and extreme OA) for 24 h inside a sealed 500-mL glass flasks (Fig. 1) in a temperature-controlled growth room at 16°C. Culture flasks were continuously agitated at 100 rpm using two orbital shakers (SK-O330-PRO, DLAB Scientific, USA) under PAR conditions of 137.59 ± 17.1 µmol photons m^{-2} s^{-1} (LED tubes, T8 integrated light, 18 W, white colour, TEJiE Ltd., Chile) with a 14:10 h ligh:dark photoperiod. Light was measured with a quantum sensor (LP471 PAR, Delta OHM S.r.l., Italy) connected to a light meter (photo-radiometer HD2302.0, Delta OHM S.r.l.).

Seawater pH treatments

The seawater pH during the three experiments was measured in the total scale (pH_T) at 16°C using a spectrophotometric pH sensor (pHyter, Sunburst Sensors, USA) (Wang et al. 2019). In the laboratory, 30 L of seawater was filtered (0.22 µm, polyethersulfone membrane, MillextGP, Millipore Ireland Ltd., Ireland) for each experiment, and kept in previously sterilized 10 L-bottles at 16°C (experimental temperature) overnight before each experiment. The seawater pH for the extreme OA treatment was achieved by mixing CO_2 gas and air into the seawater inside an equilibration reservoir to reduce the ambient seawater pH to extreme OA (Riebesell et al. 2010). The gas flow to the equilibration reservoir was adjusted using a mass flow controlled connected to the gas

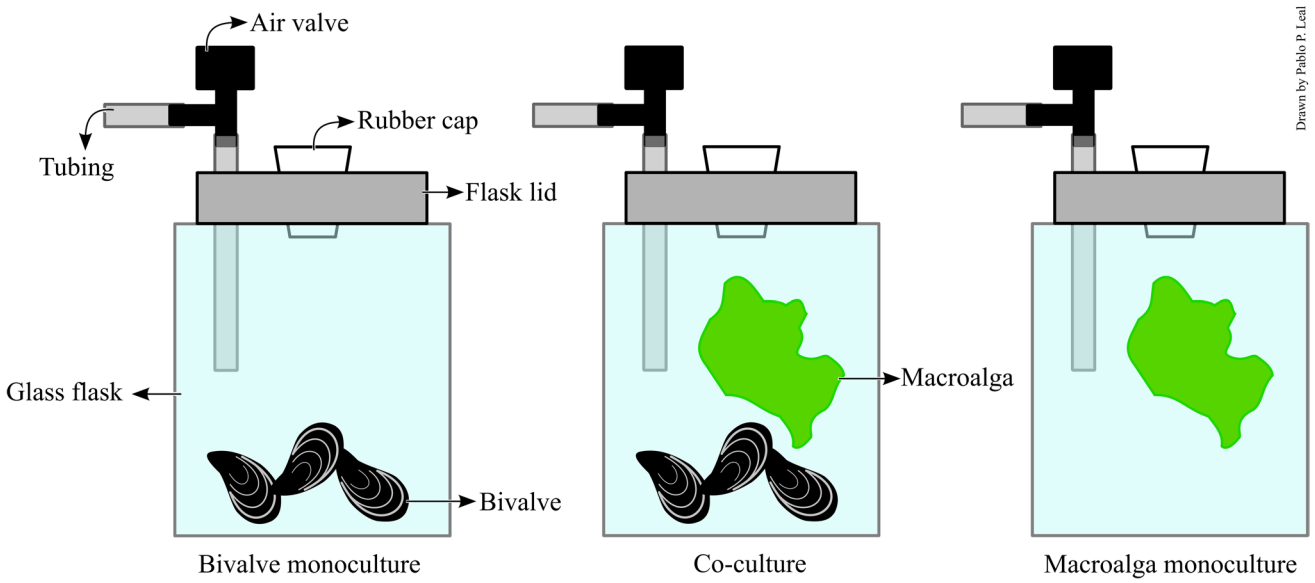

Fig. 1 Schematic representation of the experimental design showing the bivalve and macroalga mono-culture and co-culture conditions. Four replicates of each culture condition were prepared. Characteristics of the culture flasks are also detailed: glass flasks were totally filled with seawater and sealed with a flask lid; sealed conditions were kept using a rubber cap and a tubbed air valve to cover lid holes during oxygen measurements, and pH and nutrient sampling, respectively

tank (Fangue et al. 2010) while air was bubbled using an air pump. For the ambient pH treatment, air-bubbled seawater from the non-modified pH reservoir was obtained. The final values of seawater pH for the ambient and extreme OA treatments are detailed in Table 1.

Measurements of seawater parameters

Seawater pH and dissolved oxygen (DO) concentrations were measured every 3 h over the course of 24 h for each experiment, during day (1, 4, 7, 21 and 24 h) and night (10, 13, 16 and 19 h). From each replicate, 3 mL seawater samples were collected to measure pH through the tubbed air valve placed in the flask lid (Fig. 1). For O_2 measurements the oxygen electrode (HI 764080, HANNA instruments, USA), connected to a dissolved oxygen meter (HI-2004 Edge, HANNA instruments), was inserted into the experimental flask by removing a rubber cap from the flask lid (Fig. 1).

Seawater samples of 2 mL were taken after 1 h and 24 h after starting the experiment, and frozen at -80°C for nutrient analyses. Nitrate concentration analyses were performed following the USEPA procedure 40 CFR part 136 modified for the discrete analyzer for environmental testing AQ400 (SEAL Analytical, Inc., USA) according to Yarimizu et al. (2020). Nitrate was determined by reducing nitrate to nitrite by the addition of buffer that reacts with sulfanilamide producing a diazonium compound which reacts with N-(1-naphthyl)ethylenediamine dihydrochloride, forming a purple-red solution (520 nm).

Seawater samples (200 mL) representing both pH treatments were collected and fixed with 100 µL of saturated solution of mercuric chloride ($HgCl_2$, 7.4 g $(100\ mL)^{-1}$) for determining carbonate chemistry. Total alkalinity (AT) was measured using the closed-cell titration method and DIC was measured directly by acidifying the sample (Dickson et al. 2007). AT, DIC, pH, salinity and temperature were used to calculate seawater carbonate chemistry parameters (e.g, Ω_{Ara}; Table 1) using the program CO2SYS. After each sampling, experimental flasks were refilled using the seawater with the corresponding seawater pH treatment.

Statistical analyses

When Normality (Kolgomorov-Smirnow test) and homogeneity of variance (Levene's test) were not satisfied, data were rank-transformed (Conover and Iman 1981). The statistical significance of differences in variation in seawater pH, dissolved oxygen, nitrate concentration and aragonite saturations and their interaction were tested using the three-way ANOVA ($P < 0.05$). A post hoc Tukey test ($P < 0.05$) was applied when a significant effect (single, two and/or three-way interaction) of independent variables was observed. All the statistical analyses were run using the software SigmaPlot version 12.0 (Systat Software, Inc., USA).

Results

We observed that diurnal fluctuations in seawater pH_T and DO were driven by respiration and photosynthesis across the three experiments. As a general trend, seawater pH_T values were larger by 0.8 to 1.8 units in macroalgal monoculture compared to mussel monocultures, while seawater pH_T values in co-cultures were within that range of fluctuation under both pH_T treatments. DO concentrations were larger by 1.850 to 9.112 mg L^{-1}, with more pronounced intermediate concentrations observed in the experiment A compared to experiments B and C, under both pH_T treatments. In addition, Ω_{Ara} were consistently ≤ 1 in mussel monocultures, and consistently > 1 in macroalgal monocultures, under both pH_T treatments. However, in co-cultures under OA conditions, Ω_{Ara} exhibited diurnal fluctuations with values > 1 during the day and < 1 during the night, in contrast to Ω_{Ara} values in co-cultures under ambient pH, where Ω_{Ara} remained > 1 both during the day and night.

Variation in seawater pH

***M. chilensis – Ulva* sp. co-culture** Under the ambient treatment, pH averaged 7.486 during the day and 7.177 during the night in the *M. chilensis* monoculture. In the *Ulva* sp. monoculture, pH averaged 8.418 during the day and 8.616 during the night. In the *M. chilensis-Ulva* sp. co-culture, pH averaged 8.056 during the day and 7.862 during the night (Fig. 2). Under the extreme OA treatment, pH averaged 7.112 during the day and 7.002 during the night in the *M. chilensis* monoculture. In the *Ulva* sp. monoculture, pH averaged 8.156 during the day and 8.249 during the night. In the *M. chilensis-Ulva* sp. co-culture, pH averaged 7.495 during the day and 7.440 during the night (Fig. 2). There was a significant interactive effect of pH treatment × culture type × time on the seawater pH, with ambient pH treatment > OA treatment, *Ulva* sp. monoculture > *M. chilensis-Ulva* sp. co-culture > *M. chilensis* monoculture, with seawater pH greater during day than night in the *M. chilensis* monoculture and in the *M. chilensis-Ulva* sp. co-culture (Online Resource 1; Tukey, $P < 0.05$).

***M. chilensis – C. chamissoi* co-culture** Under the ambient treatment, pH averaged 7.492 during the day and 7.337 during the night in the *M. chilensis* monoculture. In the *C. chamissoi* monoculture, pH averaged 8.160 during the day and 8.181 during the night. In the *M. chilensis-C. chamissoi* co-culture, pH averaged 7.987 during the day and 7.830 during the night (Fig. 2). Under the extreme OA treatment,

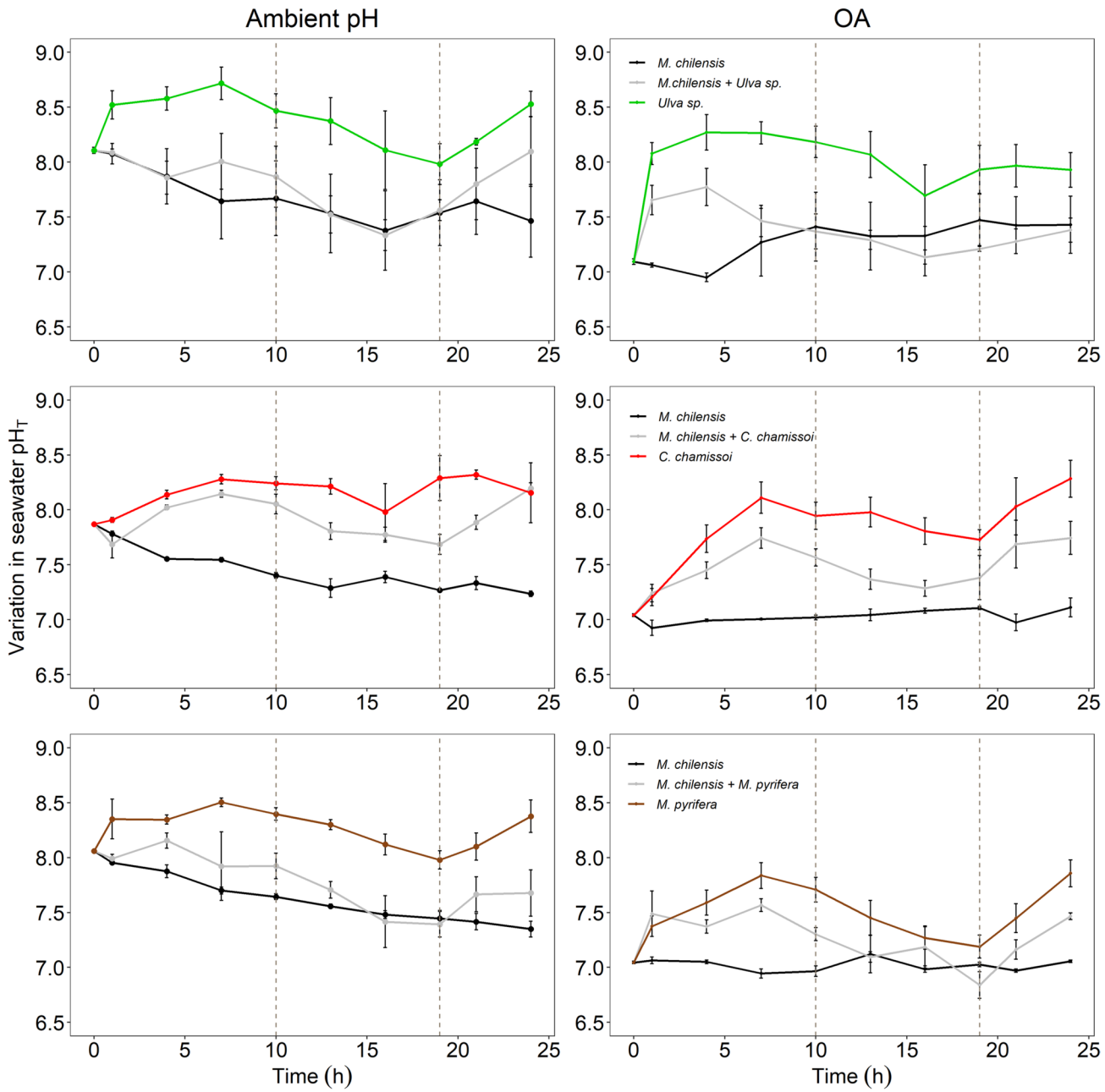

Fig. 2 Variation in pH_T in the seawater of monocultures and co-cultures of *M. chilensis*, *Ulva* sp., *C. chamissoi* and *M. pyrifera* under initial ambient pH and extreme OA after 24 h. Measurements were taken every 3 h. The time within vertical dashed lines indicate nighttime (10, 13, 16 and 19 h). Dots represent mean ± SD (n = 4)

pH averaged 7.002 during the day and 7.063 during the night in the *M. chilensis* monoculture. In the *C. chamissoi* monoculture, pH averaged 7.871 during the day and 7.865 during the night. In the *M. chilensis*-*C. chamissoi* co-culture, pH averaged 7.574 during the day and 7.401 during the night (Fig. 2). There was an interactive effect of pH treatment × time and culture type × time on the seawater pH, with ambient > extreme OA, *C. chamissoi* monoculture > *M. chilensis*- *C. chamissoi* co-culture > *M. chilensis* monoculture, with significantly greater values during day than at night (Online Resource 1; Tukey, $P < 0.05$).

***M. chilensis – M. pyrifera* co-culture** Under the ambient treatment, pH averaged 7.002 during the day and 7.532 during the night in the *M. chilensis* monoculture. In the *M. pyrifera* monoculture, pH averaged 7.871 during the day and 8.198 during the night. In the *M. chilensis*-*M. pyrifera* co-culture, pH averaged 7.574 during the day and 7.610 during the night

(Fig. 2). Under the extreme OA treatment, pH averaged 7.492 during the day and 7.025 during the night in the *M. chilensis* monoculture. In the *M. pyrifera* monoculture, pH averaged 8.160 during the day and 7.404 during the night. in the *M. chilensis-M. pyrifera* co-culture, pH averaged 7.987 during the day and 7.105 during the night (Fig. 2). There was an interactive effect of pH treatment × time and culture type × time on seawater pH being ambient treatment > extreme OA treatment, *M. pyrifera* monoculture = *M. chilensis-M. pyrifera* co-culture > *M. chilensis* monoculture, with significantly greater values during day than at night (Online Resource 1; Tukey, $P < 0.05$).

Variation in dissolved oxygen concentration

***M. chilensis – Ulva* sp. co-culture** Under the ambient treatment, DO averaged a concentration of 9.21 mg L^{-1} during the day and 2.05 mg L^{-1} during day the night in the *M. chilensis* monoculture. In the *Ulva* sp. monoculture, DO averaged 18.030 mg L^{-1} during the day and 8.487 mg L^{-1} during the night. In the *M. chilensis-Ulva* sp. co-culture, DO averaged mg L^{-1} during the day 13.12 and 3.88 mg L^{-1} during the night (Fig. 3). Under the extreme OA treatment, DO averaged a concentration of 9.29 mg L^{-1} during the day and 2.95 mg L^{-1} during the night. In the *Ulva* sp. monoculture, DO averaged 18.408 mg L^{-1} during the day and 8.343 mg L^{-1} during the night. In the *M. chilensis-Ulva* sp. co-culture, DO averaged 13.03 mg L^{-1} during the day and 3.51 mg L^{-1} during the night (Fig. 3). There was a significantly effect of pH treatment × culture type and culture type × time on DO being ambient treatment > extreme OA treatment, *Ulva* sp. monoculture > *M. chilensis-Ulva* sp. co-culture > *M. chilensis* monoculture, with DO greater during day than at night in the *Ulva* sp. monoculture and in the *M. chilensis-Ulva* sp. co-culture (Online Resource 2; Tukey, $P < 0.05$).

***M. chilensis – C. chamissoi* co-culture** Under the ambient treatment, DO averaged a concentration of 7.02 mg L^{-1} during the day and 4.41 mg L^{-1} during the night in the *M. chilensis* monoculture. In the *C. chamissoi* monoculture, DO averaged 10.53 mg L^{-1} during the day and 6.55 mg L^{-1} during the night. In the *M. chilensis-C. chamissoi* co-culture, DO averaged mg L^{-1} during the day 8.89 and 5.06 mg L^{-1} during the night (Fig. 3). Under the extreme OA treatment, DO averaged a concentration of 7.24 mg L^{-1} during the day and 4.71 mg L^{-1} during the night in the *M. chilensis* monoculture. In the *C. chamissoi* monoculture, DO averaged 10.93 mg L^{-1} during the day and 6.70 mg L^{-1} during the night. In the *M. chilensis-C. chamissoi* co-culture, DO averaged 10.44 mg L^{-1} during the day and 5.48 mg L^{-1} during the night (Fig. 3). There was an interactive effect of pH_T treatment × culture type × time on DO, with ambient treatment > extreme OA treatment, *C. chamissoi* monoculture > *M. chilensis-C. chamissoi* co-culture > *M. chilensis* monoculture, with DO greater during day than night in the *C. chamissoi* monoculture and in the *M. chilensis-C. chamissoi* co-culture (Online Resource 2; Tukey, $P < 0.05$).

***M. chilensis – M. pyrifera* co-culture** Under the ambient treatment, DO averaged a concentration of 9.27 mg L^{-1} during the day and 3.07 mg L^{-1} during the night in the *M. chilensis* monoculture. In the *M. pyrifera* monoculture, DO averaged 13.69 mg L^{-1} during the day and 5.56 mg L^{-1} during the night. In the *M. chilensis-M. pyrifera* co-culture, DO averaged 9.27 mg L^{-1} during the day and 3.92 mg L^{-1} during the night (Fig. 3). Under the extreme OA treatment, DO averaged a concentration of 9.15 mg L^{-1} during the day and 3.34 mg L^{-1} during the night in the *M. chilensis* monoculture. In the *M. pyrifera* monoculture, DO averaged 12.51 mg L^{-1} during the day and 5.19 mg L^{-1} during the night. In the *M. chilensis-M. pyrifera* co-culture, DO averaged 10.74 mg L^{-1} during the day and 3.29 mg L^{-1} during the night (Fig. 3). There was an interactive effect of culture type × time on DO being *M. pyrifera* monoculture > *M. chilensis-M. pyrifera* co-culture > *M. chilensis* monoculture with significantly greater values during day than at night (Online Resource 2; Tukey, $P < 0.05$).

Variation in the saturation state of aragonite (Ω_{Ara})

***M. chilensis – Ulva* sp. co-culture** Under the ambient treatment, Ω_{Ara} decreased to < 1 after 2 h of culture in the *M. chilensis* monoculture, increased to > 2 during 24 h of culture (> 7 and > 5 at day and night, respectively) in the *Ulva* sp. monoculture, and was > 2 during the day and > 1 during the night in the *M. chilensis-Ulva* sp. co-culture (Fig. 4). Under the extreme OA treatment, Ω_{Ara} remained < 1 during 24 h of culture in the *M. chilensis* monoculture, increased to > 2 during 24 h of culture (> 2 and > 3 at day and the night, respectively) in the *Ulva* sp. monoculture, and was > 1 during the day and < 1 during the night in the *M. chilensis-Ulva* sp. co-culture (Fig. 4). There was an interactive effect of pH treatment × time × culture type on Ω_{Ara}, with ambient treatment > extreme OA treatment, *Ulva* sp. monoculture > *M. chilensis-Ulva* sp. co-culture > *M. chilensis* monoculture, with significantly greater values during the day than at night (Online Resource 3; Tukey, $P < 0.05$).

***M. chilensis – C. chamissoi* co-culture** Under the ambient treatment, Ω_{Ara} decreased to < 1 after 2 h of culture in the *M. chilensis* monoculture, increased to > 2 during 24 h of culture (> 2 and > 3 at day and at night, respectively) in the *C. chamissoi* monoculture, and was > 2 during the day and > 1 during the night in the *M. chilensis-C. chamissoi* co-culture (Fig. 4). Under the extreme OA treatment, Ω_{Ara} decreased to < 1 after 2 h of culture in the *M. chilensis* monoculture,

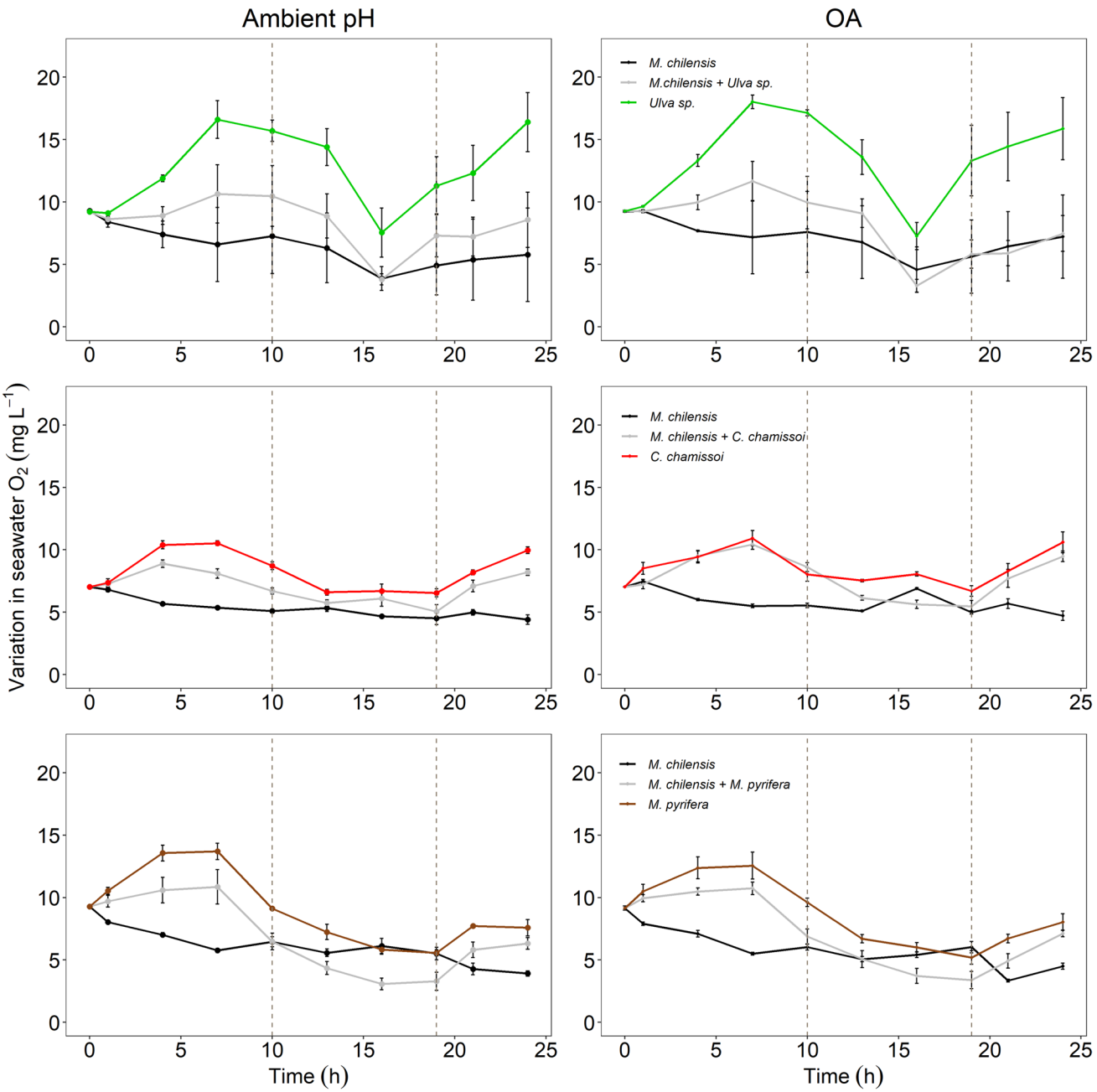

Fig. 3. Variation in O_2 concentration in the seawater of monocultures and co-cultures of *M. chilensis*, *Ulva* sp., *C. chamissoi* and *M. pyrifera* under initial ambient pH and extreme OA after 24 h. Measurements were taken every 3 h. The time within vertical dashed lines indicate nighttime (10, 13, 16 and 19 h). Dots represent mean ± SD (n = 4)

increased to > 1 during 24 h of culture (> 1 and > 2 at day and at night, respectively) in the *C. chamissoi* monoculture, and > 1 during the day and < 1 during the night in the *M. chilensis*-*C. chamissoi* co-culture (Fig. 4). There was an interactive effect of pH treatment × time and culture type × time on Ω_{Ara}, with ambient treatment > extreme OA treatment, *C. chamissoi* monoculture > *M. chilensis*-*C. chamissoi* co-culture > *M. chilensis* monoculture, with significantly greater values during the day than at night (Online Resource 3; Tukey, $P < 0.05$).

***M. chilensis – M. pyrifera* co-culture** Under the ambient treatment, Ω_{Ara} decreased to < 1 after 10 h of culture in the *M. chilensis* monoculture, increased to > 2 during 24 h of culture (> 3 and > 4 at day and at night, respectively) in the *M. pyrifera* monoculture, and > 2 during the day and > 1 during

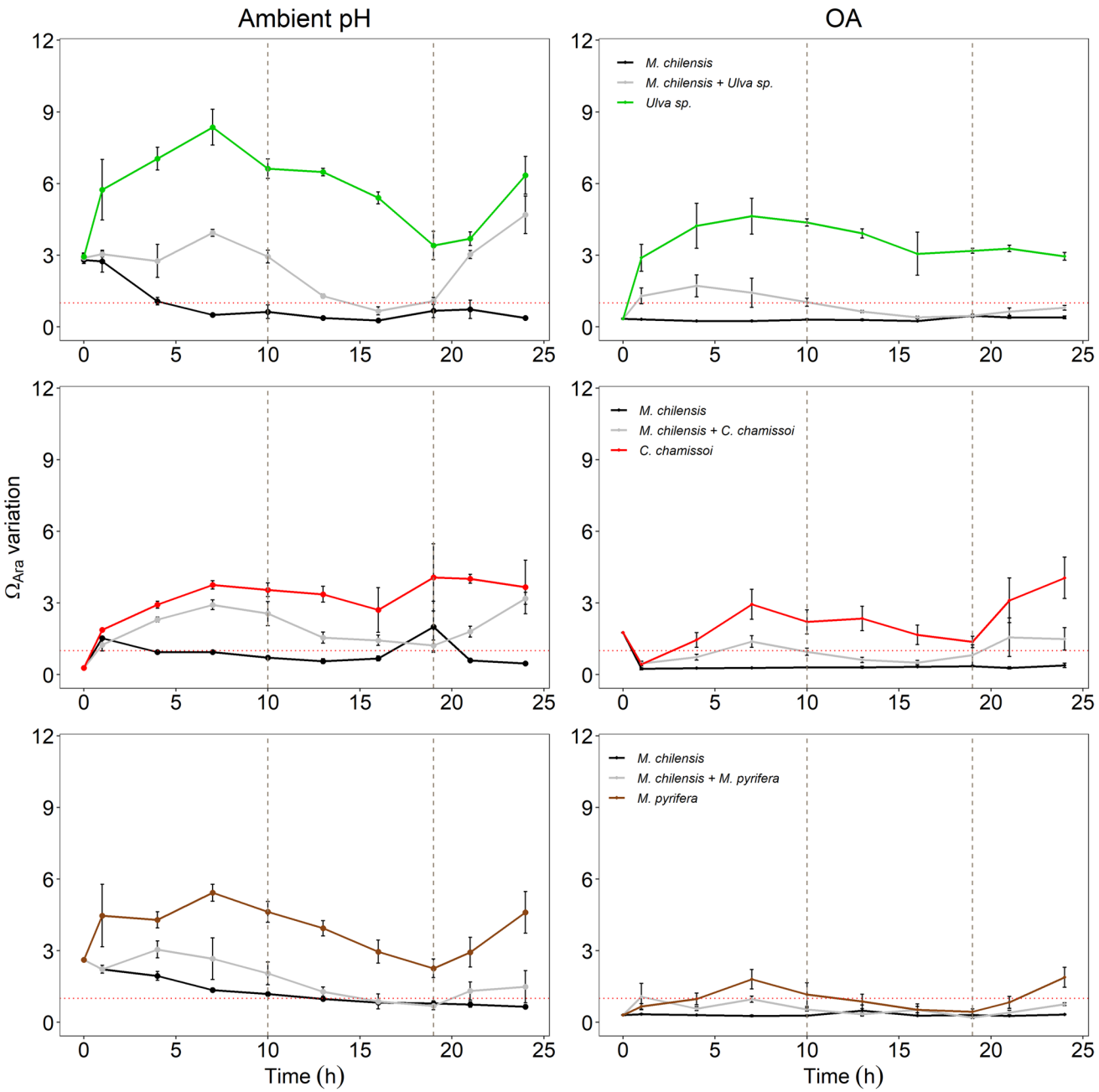

Fig. 4 Variation in the saturation state of aragonite (Ω_{Ara}) in the seawater of monocultures and co-cultures of *M. chilensis*, *Ulva* sp., *C. chamissoi* and *M. pyrifera* under initial ambient pH and extreme OA after 24 h. Measurements were taken every 3 h. The time within vertical dashed lines indicate nighttime (10, 13, 16 and 19 h). Dots represent mean ± SD (n = 4)

the night in the *M. chilensis-M. pyrifera* co-culture (Fig. 4). Under the extreme OA treatment, Ω_{Ara} remained < 1 during 24 h of culture in the *M. chilensis* monoculture, increased to > 1 during the day and < 1 during the night in the *M. pyrifera* monoculture, and < 1 in the *M. chilensis-M. pyrifera* co-culture (Fig. 4). There was an interactive effect of pH treatment × time and culture type × time on Ω_{Ara}, with ambient treatment > OA treatment, *M. pyrifera* monoculture > *M. chilensis-M. pyrifera* co-culture = *M. chilensis* monoculture with significantly greater values during the day than at night under OA conditions (Online Resource 3; Tukey, $P < 0.05$).

Variation in nitrate concentration

***M. chilensis – Ulva* sp. co-culture** Under the ambient treatment, NO_3^- concentration was reduced after 24 h of culture by 0.20 µM in the *M. chilensis* monoculture, by 4.97 µM in the *Ulva* sp. monoculture, and by 3.64 µM in the *M. chilensis-Ulva* sp.

co-culture (Fig. 5). Under the extreme OA treatment, NO_3^- was reduced after 24 h of culture by 1.79 in the *M. chilensis* monoculture, by 3.13 µM in the *Ulva* sp. monoculture, and by 0.20 µM in the *M. chilensis-Ulva* sp. co-culture (Fig. 5). There was a significantly interactive effect of time × culture type on NO_3^- concentration with significantly greater values after 24 h than 1 h in the *Ulva* sp. monoculture and in the *M. chilensis-Ulva* sp. co-culture (Online Resource 4; Tukey, $P < 0.05$).

***M. chilensis – C. chamissoi* co-culture** Under the ambient treatment, after 24 h of culture, NO_3^- concentration was increased by 2.06 µM in the *M. chilensis* monoculture, reduced by 3.45 µM in the *C. chamissoi* monoculture, and by 3.72 µM in the *M. chilensis-C. chamissoi* co-culture (Fig. 5). Under the extreme OA treatment, NO_3^- was increased by 0.70 µM in the *M. chilensis* monoculture, reduced by 3.36 µM in the *C. chamissoi* monoculture, and by 2.20 µM in the

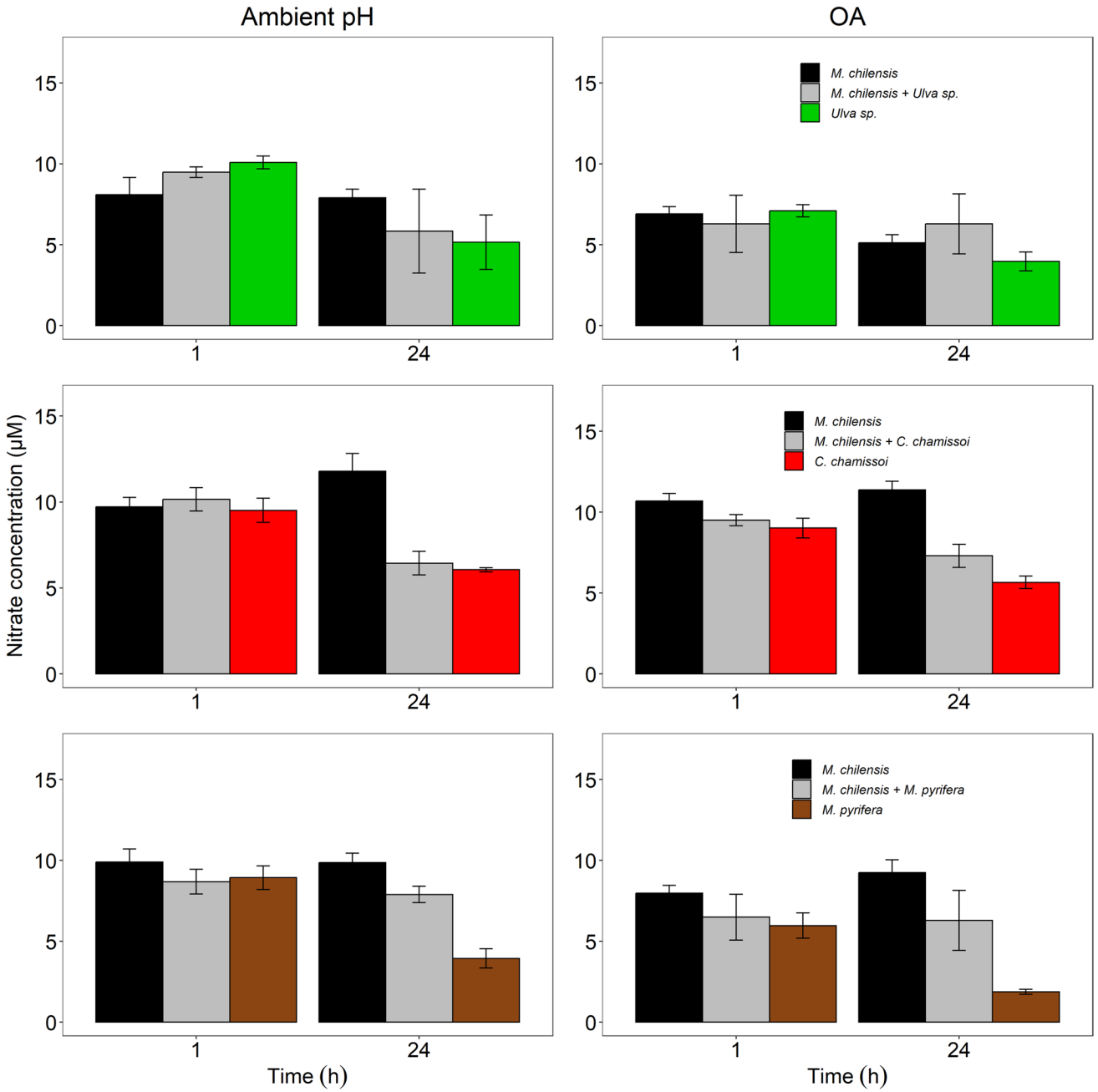

Fig. 5 Variation in nitrate concentration in the seawater of monocultures and co-cultures of *M. chilensis*, *Ulva* sp., *C. chamissoi* and *M. pyrifera* under initial ambient pH and extreme OA after 24 h. Measurements were taken every 3 h. The time within vertical dashed lines indicates nighttime (10, 13, 16 and 19 h). Dots represent mean ± SD (n = 4)

M. chilensis-C. chamissoi co-culture (Fig. 5). There was a significantly interactive effect of pH treatment × culture type × time on NO_3^- concentrations, with ambient treatment > extreme OA treatment, 1 h > 24 h, and significantly lower values after 24 h in the *C. chamissoi* monoculture and in the *M. chilensis-C. chamissoi* co-culture; and significantly greater after 24 h in the *M. chilensis* monoculture compared to those at 1 h (Online Resource 4; Tukey, $P < 0.05$).

***M. chilensis – M. pyrifera* co-culture** Under the ambient treatment, after 24 h of culture, NO_3^- concentration was reduced by 0.04 µM in the *M. chilensis* monoculture, by 4.99 µM in the *M. pyrifera* monoculture, and by 0.80 µM in the *M. chilensis-M. pyrifera* co-culture (Fig. 5). Under the extreme OA treatment, NO_3^- was increased by 1.29 µM in the *M. chilensis* monoculture, reduced by 4.09 µM in the *M. pyrifera* monoculture, and by 0.20 µM in the *M. chilensis-M. pyrifera* co-culture (Fig 5). There was a significantly interactive effect of time × culture type on NO_3^- concentrations being ambient treatment > extreme OA treatment, 1 h > 24 h, with significantly lower values after 24 h in in the *M. pyrifera* monoculture and in the *M. chilensis-M. pyrifera* co-culture under ambient pH_T treatment, and significantly greater after 24 h in the *M. chilensis* monoculture under OA. compared to those at 1 h (Online Resource 4; Tukey, $P < 0.05$).

Discussion

In this physiological study we observed that seawater chemical conditions in a closed environment are modulated by the metabolic activity of macroalgae and bivalves. In general, in monocultures of mussels, pH and Ω_{Ara} decreased, creating unfavourable conditions for calcification. In contrast, macroalgae in monocultures increased the values of these parameters, making the environment more favourable for calcification under both initial pH treatments. In co-cultures of mussels and macroalgae, pH and Ω_{Ara} increased compared to mussel monocultures, but remained below the levels observed in macroalgal monocultures. This suggests that the interaction between macroalgae and bivalves failed to create a beneficial environment for calcification, especially during the night (10 to 19 h). Diurnal fluctuations in O_2 concentrations driven by macroalgae and bivalve respiration and photosynthesis played a significant role in seawater chemistry. The balance of CO_2 production and consumption contributed to OA conditions in co-cultures. Thus, using macroalgae to ameliorate the negative effects of OA on bivalve calcification is more complex than thought.

Respiration releases CO_2 to the environment which can reduce surrounding seawater pH to levels below OA (Cornwall et al. 2013; Fernández et al. 2019). In the closed environment used here, mussel respiration resulted in significantly reduced pH relative to initial values, maintaining seawater pH around 7.0 and 7.5, and values of $\Omega_{Ara} < 1$ during day and night in all experiments. This is relevant because Ω_{Ara} values > 1.0 are considered necessary for optimal calcification in bivalves (Byrne and Fitzer 2019; Fernández et al. 2019). Below that saturation state, the availability of carbonate calcium is reduced, and the maintenance and production of the valves is outcompeted by their dissolution (Hendriks et al. 2015; Ramajo et al. 2016, 2019). Furthermore, CO_2 in excess due to OA and respiration can enters the extra- and intracellular compartments that causes acidosis (rise in H^+) of the internal body fluids. To restore the internal pH balance, excess of H^+ must be removed from intracellular compartments using active ion-exchange mechanisms (Gazeau et al. 2013; Hendriks et al. 2015). These physiological mechanisms to cope with OA conditions are energetically costly which negatively impact growth reproduction and survival in bivalves (Gazeau et al. 2013; Hendriks et al. 2015).

Macroalgae were able to modify the seawater chemistry conditions during both day and night in the closed environment. During the day, macroalgae removed CO_2 through photosynthesis, increasing pH. At night, CO_2 release from macroalgae led to a decrease in pH. (Fernández et al. 2019 and references therein). We observed an increase in seawater pH around 0.5 units in the ambient pH treatment and around 1.0 unit in the extreme OA treatment during daytime. At night, pH values remained > 7.7 ($\Omega_{Ara} > 2$) most of the time, indicating that daily photosynthetic modifications on seawater chemistry are carried over to nighttime. Similar results have been reported, indicating that the negative effects of OA conditions on growth rates of valve and/or tissue of different bivalve species were counteracted by the photosynthetic activity of *Ulva* sp. and *S. latissima* in laboratory and field experiments (Young and Gobler 2018; Young et al. 2022). In our study, the exception was the monoculture of *M. pyrifera* under extreme OA due to seawater pH varied between 7.3 and 7.9 with $\Omega_{Ara} < 1.0$ most of the experimental period. These pH values suggest that photosynthesis can ameliorate acidified conditions during daytime, but the results are species-specific.

To support photosynthesis, some macroalgae have CCMs that involve the take up of HCO_3^- and/or CO_2 by active transport (CCM-species) while other macroalgae rely on CO_2 uptake by passive diffusion, also known as non-CCM species (Roleda and Hurd 2012). The presence or absence of a CCM in macroalgae can be assessed using pH-drift experiments, which indicate that species that are not able to raise the pH above 9 rely on passive diffusion of CO_2, while species that can raise pH above 9 are HCO_3^- users because CO_2 is functionally absent in those pH conditions (Hepburn et al. 2011; Cornwall et al. 2015). However, we did not observe pH values > 9 in monocultures of *Ulva* sp. and *M. pyrifera* under

both initial pH treatments despite these two macroalgae are described as CCM-species (Fernández et al. 2014; Sun et al. 2023). The pH values observed were even lower in their respective co-culture with *M. chilensis*. A simple explanation may be that the diurnal experimental period was too short for the macroalgae to increase the pH to values greater than 9. A more complex explanation could be that CCM activity requires more energy than diffusive CO_2 uptake, thus high CO_2 concentrations (such as in OA conditions and in co-cultures) and/or declines in light activate a down-regulation of a CCM, switching to a less energetically expensive passive diffusion of CO_2 during photosynthesis (Hurd et al. 2009; Hepburn et al. 2011; Cornwall et al. 2015). For the case of *C. chamissoi*, that also failed to raise the pH over 9 during the experiments, it is possible that this species does not possess a CCM due to more than 80% of red macroalgae are non-CCM species (Cornwall et al. 2015), but this needs further empirical confirmation. These findings indicate that investigation of the mechanism that macroalgae employ to use inorganic carbon for photosynthesis is required to determine the ability of aquaculture relevant macroalgae to create a chemical refuge for bivalve calcification under OA conditions.

Here, the three species of macroalgae reduced the NO_3^- concentrations in mono- and co-cultures, with a greater reduction observed in the OA treatment compared to the ambient pH treatment. For macroalgae under enriched N conditions, the uptake and assimilation of NO_3^- are correlated with reduced seawater pH/elevated dissolved CO_2. In this condition, macroalgae can store N in intracellular pools, reducing the energy required for NO_3^- assimilation and allocating more energy for photosynthesis and growth compared to macroalgae in limiting N conditions (Fernández et al. 2017). This have been reported for some species, including the brown macroalgae *M. pyrifera* and *Hizikia fusiforme* (Harvey) Okamura, the red *Pyropia haitanensis* (T.J.Chang & B.F.Zheng) N.Kikuchi & M.Miyata, and *Ulva rigida* C.Agardh (Gordillo et al. 2001; Zou 2005; Liu and Zou 2015; Fernández et al. 2017). This information indicates that macroalgae in co-cultures can be used for the bioremediation of N in coastal areas with eutrophication problems (Gentry et al. 2019; Cotas et al. 2023; Walker et al. 2023). However, specie-specific studies on macroalgal N physiology are encouraged because it is affected by other abiotic factors such as light, temperature, and water movement (Roleda and Hurd 2019).

The OA threat to bivalve aquaculture can be ameliorated by the presence of macroalgae, but some aspects are to be considered before its implementation (Fernández et al. 2019). First, the understanding of the mechanisms for carbon acquisition of each species would help to design the co-culture farm to maximize the potential OA buffering effect. For instance, the optimal depth for cultivating CCM-species such as *M. pyrifera* may be close to the surface because CCMs require light to function, compared to non-CCM species, that do not require to spend energy to uptake CO_2 to support photosynthesis and growth (Fernández et al. 2019). Another aspect to consider is the proportion of biomass between the bivalve and macroalga co-cultured to maximize mutual benefits (Fernández et al. 2019). It has been suggested that a co-culturing proportion of 4:1 (for the oyster *Crassostrea angulata* Lamarck and the red macroalga *Gracilaria lemaneiformis* (Bory) Greville) and 3:1 (for the mussel *Mytilus galloprovincialis* Lamarck and *G. verrucosa* (Hudson) Papenfuss) for an efficient utilization of dissolved inorganic carbon, N and P uptake by the macroalgae, and a favoured calcification by the bivalve (Han et al. 2017; Ajjabi et al. 2018). In contrast, Young et al. (2022) showed that tissue and valve growth rates of *M. edulis* and *C. virginica* are faster with increasing biomass of co-cultured *S. latissima*, which suggest that a large amount of macroalgal biomass will be suitable to serve as a refuge for calcifying bivalves under OA conditions. Finally, evaluating local factors related to hydrodynamic regimes (e.g., current speeds and directions, water residency times and vertical mixing) are needed to understand how physiological responses of organisms (e.g., OA buffering effect, nutrient uptake, excretion rates, growth) will respond in co-culture farms (Fernández et al. 2019; Walker et al. 2023). Therefore, studies on these aspects are still needed to take advantage of any potential benefit of co-cultures as an OA mitigation tool.

Macroalgae have the potential to counteract the effects of OA on calcifying organisms by increasing the seawater pH in the surrounding environment (Fernández et al. 2019; Cotas et al. 2023; Edworthy et al. 2023). However, our hypothesis, suggesting that the seawater chemical conditions for calcification will be favourable in the co-culture and macroalgal monoculture treatments, but unfavourable in the bivalve monoculture treatment under OA conditions, was only partially supported by the results. The presence of macroalgae in closed-environment co-cultures created favourable conditions for mussel calcification only during daytime. Consequently, this study emphasizes the need for an understanding of the interactions between macroalgae and bivalves in co-culture systems and their effects on seawater chemistry under different environmental conditions (e.g., macroalga-bivalve culture proportions, multiple global driver interactions, and local hydrodynamic conditions), especially in the context of OA mitigation for aquaculture.

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Data availability The data obtained during the current study are available from the corresponding author on request.

Declarations

Competing interests The authors declare no competing interests.

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