Preliminary investigation on the potential involvement of an ABC-like gene in *Halomicronema metazoicum* (Cyanobacteria) tolerance to low seawater pH in an ocean acidification scenario

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Abstract

Decreasing ocean surface pH, called ocean acidification (OA), is among the major risks for marine ecosystems due to human-driven atmospheric pCO_2 increase. Understanding the molecular mechanisms of adaptation enabling marine species to tolerate a lowered seawater pH could support predictions of consequences of future OA scenarios for marine life. This study examined whether the ATP-binding cassette (ABC)-like gene *slr2019* confers tolerance to the marine cyanobacterium *Halomicronema metazoicum* to low seawater pH conditions (7.7, 7.2, 6.5) in short- and long-term exposures (7 and 30 d). Photosynthetic pigment content indicated that the species can tolerate all three lowered-pH conditions. At day 7, *slr2019* was up-regulated at pH 7.7 while no changes were observed at lower pH. After 30-d exposure, a significant decrease in *slr2019* transcript levels was observed in all low-pH treatments. These first results indicate an effect of low pH on the examined transporter expression in *H. metazoicum*.

Keywords: *Halomicronema metazoicum*, Cyanobacteria, acid stress tolerance, ATP-binding cassette (ABC) membrane transporter proteins, ocean acidification (OA) scenarios, low pH

1. Introduction

Since the beginning of the industrial revolution, the mean surface pH of oceans dropped from approximately 8.2 to 8.1 due to past and current carbon dioxide (CO_2) emissions (Jiang et al., 2019), and the trend is expected to continue until the end of the century (estimated further decrease by up to 0.4 pH units by the year 2100) (Bindoff et al., 2019). The resulting ocean acidification (OA) represents a significant environmental threat to marine life (Alter et al., 2024; Doney et al., 2009). Relevant studies aimed at predicting the effects of future OA scenarios on coastal ecosystems have been carried out in naturally acidified areas (e.g. CO_2 vents), revealing several impacts from reductions in habitat complexity and species richness (Kroeker et al., 2013; Gambi et al., 2016; Muralisankar et al., 2021; Fanelli et al., 2022; Munari et al., 2022; Xie et al., 2023), to simplification of food webs and reduction of ecosystem functions (Vizzini et al., 2017; Teixidó et al., 2018). However, certain species exhibit remarkable abilities to tolerate natural low-pH/high-pCO_2 conditions (Gambi et al., 2016). Such adapted organisms can provide important insights into cellular and molecular mechanisms allowing marine species to cope with acidified environments.

Molecular responses affecting the cell integrity of marine species towards exposure to low pH have been examined but the cellular mechanisms driving tolerance remain to a large extent unclear (reviewed in Simonetti et al., 2022). Findings on prokaryotes dealing with highly acidified environments suggest the involvement of ATP-binding cassette (ABC) transport proteins in conferring resistance to acidic conditions by a relocation of endogenous substances, such as ATP, carbohydrates and lipids, that contribute to maintaining intracellular homeostasis (Zhu et al., 2019). This was found in cyanobacteria (*Synechocystis* sp. and *Anabaena* sp.; pH from 3.0 to 6.0) (Tahara et al., 2012; 2015; Matsuhashi et al., 2015; Uchiyama et al., 2019; Shvarev and Maldene, 2020), human gastric microbiota (*Helicobacter pylori*) (McGowan et al., 1998) and bacteria in fermented foods (*Acetobacter aceti*, *Acetobacter pasteurianus*, *Lactococcus lactis*) (Nakano et al., 2006; Gao et al., 2023, Zhu et al., 2019).

In the present study, the ABC transporter-like gene *slr2019* of a free-living Cyanobacterium strain of *Halomicronema metazoicum* was investigated for its potential role in conferring resistance to the impact of acidified conditions. Slr2019 was selected as a candidate protein and it has been recognized as essential to tolerate acid stress conditions in the cyanobacterium *Synechocystis* sp. PCC6803 (Matsuhashi et al., 2015). This work represents a first assessment of the involvement of ABC transporter proteins in *H. metazoicum*

resistance to low-pH/high-pCO_2 environments in order to clarify the general value of the hypothesis of the essential role of these proteins in bacterial survival to acid stress. The species was selected as an experimental model as it is known for its ability to cope with extreme environmental conditions, such as high temperature, high salinity, and high irradiance (Mutalipassi et al., 2019a), and it is found to inhabit naturally acidified marine areas (Ruocco et al., 2018). So far, there have been no investigations on an adaptive mechanism behind the tolerance of the low natural pH conditions in the habitat of *H. metazoicum*. We here present an experiment, in which cyanobacteria were exposed at pH conditions in the range from 0.4 to 1.6 units below the current natural seawater pH of 8.1, resembling potential future OA scenarios. Exposures were for 7 and for 30 d and *slr2019* transcript levels were determined in parallel to cellular viability. Since for *H. metazoicum* protocols for RNA extraction have not yet been established, different RNA extraction methods were evaluated in this study.

2. Materials and methods

2.1. *Halomicronema* culture conditions

Halomicronema metazoicum mattes were obtained from permanent cultures maintained at the Stazione Zoologica Anton Dohrn (Napoli, Italy), Ischia Marine Center at Villa Dohrn, Ischia and originating from leaves of *Posidonia oceanica*, in a meadow off Lacco Ameno d'Ischia (Bay of Naples, 40°44′56″ N,13°53′13″ E) (Ruocco et al., 2018). Pure strains were grown in Guillard's *f*/2 medium (Merck, Italy) and maintained under sterile conditions in a thermostatic chamber at a temperature of 18 °C with light irradiance of about 200 μE and a 12:12 light: dark photoperiod (see Mutalipassi et al., 2019b).

2.2. Development of an RNA extraction method for *H. metazoicum*

The extraction of high yield and high-quality RNA from polysaccharide-rich matrices, such as those of a filamentous cyanobacterium like *H. metazoicum*, poses specific challenges and no suitable RNA extraction protocol has been established for the here studied cyanobacteria. We therefore designed and tested eight different methods for RNA extraction from *H. metazoicum* cells (Figure 1A). Samples of approximately 100 mg fresh *H. metazoicum* mattes, stored in RNAlater (Sigma-Aldrich, USA) at -80 °C, were thawed, centrifuged

(4,500 rpm, 20 min, 4 °C) and the supernatant was removed. Prior to performing RNA isolation, cyanobacterium cells were homogenized by high-intensity (HI) ultra-sonication (26 W, BANDELIN electronic UW2070) as described by Kim Tiam et al. (2019), in order to successfully break up the cellular envelopes. Samples in the extraction buffer were briefly vortexed and sonicated on ice four times for 30 s. After each sonication, samples were vortexed. This homogenization step was used in all eight extraction methods.

Method 1. Manual RNA extraction. Samples were incubated in 1 ml TRIzol (Invitrogen, USA) extraction buffer on ice for 30 min and then homogenized. After centrifugation (12,000 *g*, 5 min, 4 °C), 50 µL 1-Bromo-3-Chloropropane (BCF) were added to the lower phase and re-centrifuged (12,000 *g*, 15 min, 4 °C). RNA was precipitated by adding 0.5 vol RNA precipitation solution (1.2 M sodium chloride, 0.8 M sodium citrate) and 0.5 vol isopropyl alcohol to the upper phase and centrifuged (12,000 *g*, 15 min, 4 °C). The resulting pellet was washed with 600 µL 75% ethanol, air dried and resuspended in 25 µL RNase-free water, followed by two incubation steps, one of 15 min at 60 °C and one of 5 min at room temperature.

Method 2. PureLink® RNA Mini Kit. RNA from samples was isolated using the PureLink® RNA Mini Kit (Ambion Life Technologies, USA) according to the manufacturer's protocol. Upon the procedure, RNA was diluted in 40 µL RNase-free water.

Method 3. TRIzol® reagent + PureLink® RNA Mini Kit. In order to improve the lysis effectiveness, the kit's lysis buffer was substituted with 1 mL TRIzol reagent. RNA isolation was performed following the 'Using TRIzol® Reagent with the PureLink® RNA Mini Kit' method from the user guide. Extracted RNA was re-suspended in a total volume of 40 µL RNase-free water.

Method 4. Sample clarification with NaOH + PureLink® RNA Mini Kit. An initial step with NaOH was performed to dissolve extracellular substances, which could lower the extraction yield (Defrancesco et al., 2002). Cyanobacterial mattes were incubated in 1 mL 1 M NaOH and centrifuged (4,500 rpm, 20 min, 4 °C). Then RNA was extracted following the 'Using TRIzol® Reagent with the PureLink® RNA Mini Kit' manufacturer's protocol.

Method 5. NucleoSpin® RNA II Kit. *H. metazoicum* mattes were incubated in 1 mL TRIzol reagent for 30 min on ice and homogenized. Then RNA was extracted by the NucleoSpin® RNA II kit (Macherey Nagel, Germany), following the manufacturer's protocol. RNA was eluted in 40 µL RNase-free water.

Method 6. NucleoSpin® miRNA Kit. 50 mg of *H. metazoicum* mattes were used to extract RNA following the NucleoSpin® miRNA Kit (Macherey Nagel, Germany) manufacturer's protocol. The purified RNA was eluted in 30 µL RNase-free water.

Method 7. TRIzol® reagent + NucleoSpin® miRNA Kit. The Lysis Buffer ML of the NucleoSpin® miRNA Kit was replaced with TRIzol reagent and then the manufacturer's protocol was followed. The isolated RNA was eluted in 30 µL RNase-free water.

Method 8. CTAB 2% buffer. The RNA isolation method developed by Wang and Stegemann (2010) was tested, with modification. Sixhundred µL pre-warmed (65 °C) 2% CTAB extraction buffer (200 mM Tris-HCl (pH 8.00), 20 mM ethylenediaminetetraacetic acid, 1.4 M di sodium chloride, 1% polyvinylpyrrolidone 40) were added to 100 mg of minced sample and vortexed for 5 min at room temperature. After the homogenization step, an equal volume of chloroform:isoamyl alcohol (24:1) was mixed with the sample and centrifuged (15,000 rcf, 5 min, room temperature). The clear upper phase was mixed again with an equal volume of chloroform:isoamyl alcohol (24:1) and the centrifuge was repeated. Sixhundred µL isopropanol were added to the upper phase and centrifuged (15,000 rcf, 15 min, room temperature). One mL 75% ethanol was added to the lower phase and centrifuged (15,000 rcf, 15 min, room temperature). The pellet was dissolved in 50 µL RNase-free water and purified following the manufacturer's protocol for the NucleoSpin® RNA II kit (Macherey Nagel, Germany). Isolated RNA was diluted in 60 µL RNA-free water.

The quality of RNA obtained with the above-mentioned methods was assessed based on $A_{260/A280}$ and $A_{260/A230}$ ratios measured on a BioPhotometer (Eppendorf 6131) and by electrophoresis on 1.5% agarose 'bleach gel' (Aranda et al., 2012 modified) (visual inspection of 18S and 28S ribosomal RNA bands and for the lack of visible genomic DNA contamination). The RNA concentration was quantified by absorbance at 260 nm on the BioPhotometer.

2.3. *In vivo* exposure of *H. metazoicum*

Halomicronema metazoicum mattes (approximately 1 g fresh weight) were isolated from the mother culture and cultivated in sterile Guillard's *f*/2 medium at pH 8.2, 7.7, 7.2, and 6.5, respectively, for 30 d in *ad hoc* devised photobioreactors (more details in Mutalipassi et al., 2019b; Figure S1), under controlled temperature and light conditions (18 °C, 12:12-h photoperiod). For each pH treatment, two photobioreactors were set up

(n = 2). Photobioreactors were composed of a Pyrex dish (2.4 L) covered with a heat-resistant glass plate provided with a narrow hole in the middle. There, a pH probe (InLab Micro pH, Mettler Toledo) was housed to constantly measure water pH. A pH controller (pH 201, Aqualight) was connected to the probe and to an electronic valve regulating a centrifuge pump and a CO_2 regulator (CO_2 Energy, Ferplast) linked to the photobioreactor through a glass pasteur pipette. The controller opened and closed the electronic valve, when necessary, supplying CO_2 in order to regulate the pH. The centrifuge pump (Askoll Pure pump 300) was placed inside the vessel and activated during CO_2 insufflation to avoid the formation of a pH gradient. Cyanobacterial mattes were put in a perforated box located inside the dish. The water pH was checked three times per day to ensure its stability (oscillations lower than 0.05 units). The chosen range of experimental pH values was representative of present (pH 8.2) and potential future ocean pH scenarios (pH 7.7; predicted by Jiang et al. (2019) for the end of the century in the scenario with CO_2 emissions remaining on the current high level). In addition, one treatment was at pH 6.5 representing naturally acidified conditions occurring in the high CO_2 venting area at the Castello Aragonese, Ischia Island (Kroeker et al., 2011; Foo et al., 2018).

Mattes from each group were collected after 7 and 30 d and stored in RNA later (Sigma-Aldrich, USA) at -80 °C until analysis.

2.4. Viability

Viability of mattes was assessed qualitatively by observations of morphology and color of filaments according to Ruocco et al. (2018) and by measuring chlorophyll *a* and carotenoid content according to the procedure of Zavřel et al. (2015). Briefly, samples of 20 mg were centrifuged (15,000 *g*, 7 min, room temperature) and 1 mL 100% methanol (precooled at 4 °C) was added. The suspension was homogenized by vortexing, covered with aluminum foil and incubated at 4 °C for 20 min to allow pigment extraction. After a second centrifugation step (15,000 *g*, 7 min, 4 °C) the absorbance of the supernatant was measured by a spectrophotometer (Shimadzu UV-VIS 160) at wavelengths 470, 665, and 720 nm against methanol as blank. The concentration of pigments was calculated as:

$$Chl_a\ [\mu g/mg] = 12.9447\ (A_{665} - A_{720}) / W$$

$$Carotenoids\ [\mu g/mg] = \{[1{,}000\ (A_{470} - A_{720}) - 2.86\ (Chl_a\ [\mu g/mg])] / 221\} / W$$

Where A_{470}, A_{665}, A_{720} were absorbance values measured at 470, 665 and 720 nm, respectively, and W was the sample weight.

Pigments concentration was expressed as μg of pigments/mg of mattes.

2.5. ABC gene expression analysis

Two samples of 100 mg *H. metazoicum* cells were collected from each photobioreactor (4 replicates for each pH treatments group) and RNA was extracted.

First-strand cDNA was synthesized from 175 ng of RNA (extracted with 'CTAB 2% buffer' method, method 8, see 3.1 below) in a final volume of 20 μL by using the qScript™ XLT cDNA SuperMix (Quantabio, USA) according to the manufacturer's instructions (10 μL RNA template, 4 μL qScript XLT cDNA SuperMix (5X), 6 μL Rnase/Dnase-free water).

qPCR was performed in triplicate on an iCycleriQ5 (Bio-Rad, USA) to amplify *slr2019* ABC-*like* transcript (identified by Matsuhashi et al., 2015) and *16S* (Ruocco et al., 2018) as a housekeeping gene. Each 20 μL reaction contained 1 μL cDNA (diluted 10 times), 10 μL PerfeCTa® SYBR® Green SuperMix, Low ROX (Quantabio, USA) and 1 μL of primer pairs. The thermal profile used was: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C, 15 s at specific melting temperature for each primer pair (59 °C for *16S* and 55 °C for *slr2019* gene), 10 s at 72 °C. The sequences of forward and reverse primers were 5′-ACGGGGCGGCTGATG-3′ and 5′-CAAAAATGCTCCACCAATCAC-3′ for *slr2019* and 5′-ATTGGGCGTAAAGCGTCCG-3′ and 5′- TTCACCGTACACTGGGAAT-3′ for *16S* (see Table S1 for PCR parameters). Relative quantification of gene expression levels was performed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

The reaction efficiency was determined by a five-point dilution series through the equation: $E = (10^{(-1/slope)} - 1) * 100$. The theoretical maximum of 100% indicates the doubling of the amount of product with each cycle.

2.6. Statistical analyses

The variance of pigment content and gene expression data was analyzed with the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Differences were considered significant if $p < 0.05$. Statistical analyses

were performed using the software GraphPad Prism version 9.5.1 (528) for macOS (GraphPad Software, USA).

3. Results and discussion

3.1. RNA extraction methods

The main obstacle to isolating pure, high-quality RNA from filamentous cyanobacteria is the matte's richness in polysaccharides, which can affect both the yield and the quality of the extracted RNA. We thus tested different protocols to isolate RNA from the filamentous cyanobacterium *H. metazoicum* to identify a method yielding RNA of reasonable quality.

Yield (as ng of extracted RNA per mg of initial sample) and purity (assessed through A_{260}/A_{280} ratio and A_{260}/A_{230} ratio) of RNA from each tested method are listed in Table 1; images of the bands of RNA isolates uponagarose gel electrophoresis are inFigure 1B. The average A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of the RNA extracted from *H. metazoicum* varied from 1.57 to 2.13 and from 0.10 to 2.05, respectively, while the yield was in the range of 1.13 ng_{RNA}/mg_{sample} and 14.88 ng_{RNA}/mg_{sample}. Only when using the 'CTAB 2% buffer' method (method 8), pure, high-quality total RNA was obtained, with A_{260}/A_{280} and A_{260}/A_{230} ratios > 2 (highest of all isolated) and the RNA yield among the highest (Table 1). The RNA isolate showed well-defined 28S and 18S ribosomal RNA upon gel electrophoresis (Figure 1B). In contrast, the RNA isolated with all other applied methods was less pure ($A_{260/230}$ ratios of 0.1 - 1.52), resulting in more indistinct electrophoresis bands (Figure 1B), and with a considerably lower yield in most cases (Table 1).

As suggested by Wang and Stegemann (2010), the 'CTAB 2% buffer' method seems to enable to obtain high-quality RNA isolates by efficiently breaking up the filamentous envelope of the bacterial cells. The RNA obtained with this method was suitable for qPCR analysis. The PCR efficiency resulted in 107% and 84% for *16S*- and *slr2019*-primer pairs, respectively (Table S1), indicating the robustness of the method.

Based on these results, the 'CTAB 2% buffer' method was here applied for RNA extraction from all experimental *H. metazoicum* samples.

3.2. Viability

Halomicronema metazoicum mattes of all four groups (three experimental groups and one control) were characterized by macroscopic aggregates of filaments with a vivid emerald-green color until the end of the experiment (Figure S2). According to our previous findings (Ruocco et al., 2018), the color of the *H. metazoicum* mattes represents a suitable marker for the health status in its exponential phase of growth. Thus, the color of the mattes indicates a good health status of the cyanobacteria incubated at different pH conditions. Across all pH treatments and exposure durations, the chlorophyll *a* contents of cyanobacteria cells varied between 0.13 (after 7 d exposure in mattes exposed to pH 7.7) and 0.31 μg/mg (after 7 d exposure in mattes exposed to pH 7.2). No significant differences were found in the chlorophyll *a* content of cells between control and low pH groups, neither after 7 d nor after 30 d of exposure (Figure 2A). The chlorophyll *a* concentration slightly increased between days 7 and 30 of exposure in control and pH-7.7 groups, while it was slightly lower in the pH-7.2 and pH-6.5 groups, but the differences were not significant. After 7 d of exposure, chlorophyll *a* slightly decreased in mattes exposed to pH 7.7 *vs.* controls while an increase in chlorophyll *a* was observed in the pH-7.2 treatment. Mattes exposed to the most severe low pH of 6.5 had a chlorophyll *a* content similar to the controls. After 30 d, no differences in chlorophyll *a* were found between the control and exposed groups (Figure 2A).

The carotenoid amount was lower than chlorophyll *a* being in the range of 0.05, after 7 d at pH 7.7 and 0.12 μg/mg, after 7 d at pH 7.2, as previously observed in *H. metazoicum* samples from the Gulf of Taranto (Northern Ionian Sea, Italy) (Caroppo et al., 2012). As for chlorophyll *a* content, the total carotenoids of cyanobacteria cells were not significantly different between controls and any of the experimental low-pH groups after 7 and 30 d of exposure (Figure 2B). The carotenoid concentrations in mattes derived from control groups and from pH 7.7 and 6.5 exposures were slightly but not significantly higher after 30 d than after 7 exposure days. After 7 and 30 d of exposure, the carotenoid contents were slightly lower in the pH-7.7 group and slightly higher in the pH-7.2 group than in the respective control groups ($p > 0.05$, no significance); in the pH-6.5 group, the carotenoid content was about the same as in the respective control group (Figure 2B).

Chlorophyll *a* and carotenoids are important light-absorbing pigments and are essential for the process of photosynthesis (Hirschberg and Chamovitz, 1994; Björn et al., 2009). Controversial findings have been limiting the use of chlorophyll *a* and carotenoids as suitable biomass indicators (Caroppo et al., 2012; Sinetova et al., 2012; Yu et al., 2023; Ramaraj et al., 2013; Prihantini et al., 2019). Therefore, chlorophyll *a* was here

used as a marker only for *H. metazoicum* viability and not for growth. The ability of *H. metazoicum* to face different environmental conditions such as high temperature, irradiance and salinity has been already reported (Mutalipassi et al., 2019a). Mattes of *H. metazoicum* that are in good conditions present a pigment profile with chlorophyll *a* being the major photosynthetic pigment and with carotenoids in lower abundance as shown in Figure 2A and 2B and in accordance with Caroppo et al. (2012), as seen in other cyanobacteria.

Here, results on the viability under acidified conditions suggest that *H. metazoicum* is able to tolerate low pH conditions down to severe ones as 6.5, thus confirming what was expected from field observations with mattes populating naturally acidified marine areas.

3.3. Gene expression

The ABC-like gene *slr2019* was found to be vital in *Synechocystis* sp. PCC6803 to tolerate acid stress conditions (Matsuashi et al., 2015). Through the use of a *slr2019*-defective mutant strain, Matsuashi et al. (2015) showed that *Synechocystis* sp. PCC6803 was not able to proliferate without this gene following exposure to pH 6.0 of 7 d. Moreover, after a short-term (4 h) exposure to pH 3.0, *slr2019* gene expression was found to be increased compared to a control, although the differences were not significant. Here, the *H. metazoicum slr2019*-like gene was upregulated by 2.8 fold in mattes exposed to pH 7.7 for 7 d; this change was not significant due to high variation across replicates, but upregulation was consistently seen in all replicates of this treatment (Figure 2C). In contrast, a slight but not significant downregulation in *slr2019* expression levels was observed in mattes from the pH-7.2 and pH-6.5 treatments (Figure 2C). On the opposite, after 30 d of exposure, significant down-regulation of the *slr2019* gene was observed at all experimental pH conditions compared to the control with fold changes in expression of 0.11 at pH 7.7, 0.03 at pH 7.2, and 0.04 at pH 6.5 (Figure 2C). Our results, at different pH and after 30-d exposure, provide additional information to data of Matsuashi et al. (2015) indicating the response of cyanobacteria to acidified conditions, showing the relationship between pH and its effects in gene regulation, and the importance of exposure of time. In order to explain our findings two possible hypotheses were formulated.

Our first hypothesis would be that exposure of *H. metazoicum* to low pH causes changes in the membrane's-lipopolysaccharide (LPS) structure as an acclimatation response to low-pH conditions. According to McGowan et al. (1998) and Martinić et al. (2011), changes in bacterial LPS structure occur upon exposure to

low pH (5.0 - 5.5). *Synechocystis* sp. PCC6803's Slr2019 is actually homologous to *Escherichia coli*'s inner membrane ABC transporter MsbA (Matsuashi et al., 2015) which is responsible for the synthesis and transportation of lipid A, one of the three major structures of LPS (Polissi and Georgopopulos, 1996; Zhou et al., 1998). LPS represents the main component of the outer membrane in Gram-negative bacteria, including cyanobacteria (Buttke and Ingram, 1975), and its synthesis and translocation is carried out by different proteins, including several ABC transporters (Polissi and Georgopopulos, 1996; Zhou et al., 1998; Sperandeo et al., 2007). LPS has a protective role for bacterial cells (Bertani and Ruiz, 2018) and previous findings have shown its importance for the tolerance of bacteria to acid conditions (Martinić et al., 2011; McGowan et al., 1998). In *Shigella flexneri* 2a, changes in the composition of two LPS regions, lipid A and O antigen, have been observed upon overnight exposure to pH 5.5 (Martinić et al., 2011). McGowan et al. (1998) identified the appearance of high-molecular-weight proteins and the down-regulated expression of other proteins in the LPS profile of *Helicobacter pylori* following a 72-96-h exposure to pH 5.0. Thus, the alteration in LPS structure seems to be due to an up-regulation of some LPS components and a down-regulation of other elements. Matsuashi et al. (2015) have demonstrated that *Slr2019* is involved in LPS synthesis in *Synechocystis* sp. PCC6803. Therefore, *Slr2019* of *H. metazoicum* may be involved in the synthesis and/or transportat of a LPS component which is more sensitive to moderate low-pH (7.7) in the short-term (7 d) and completely abolished upon prolonged exposure (30 d).

Our second hypothesis would be that changes in *slr2019* gene expression could be ascribed to the decrease of ATP caused by the increase of CO_2 intake which indeed has a strong effect on ATP pump transport functionality. A rise in external pCO_2 causes an increase in CO_2 uptake by bacterial cells with consequences for the intracellular ATP concentration. Physiological changes at higher CO_2 concentrations were reported by Klangpetch et al. (2013) as a decrease in intracellular ATP concentration in *E. coli* exposed to pCO_2 of 1–6 MPa for 16 min. The reduction of ATP may indeed result in lower availability of energy for other cellular pathways and may also impair ABC transporter activity. In order to compensate for lower ABC transporter activity and to maintain their functionality, cells might enhance the transcription of Slr2019. Therefore, the exposure to high-pCO_2 and the ATP depletion could be responsible for the observed up-regulation of the *slr2019* gene in the short-term (after 7 d) at pH 7.7. The response pattern shown by *H. metazoicum* at lower

pH conditions (7.2, 6.5) and in a longer time (30 d) suggests that this initial up-regulation of *slr2019* is too energetically too costly for cyanobacteria when exposed to more severe conditions.

4. Conclusions

This study represents a preliminary assessment of the modulation of ABC genes in *H. metazoicum* upon exposure to low pH resembling future OA scenarios in agreement with previous findings in cyanobacteria and other bacterial species, such as *Synechocystis* sp., through gene-deleted mutant strains and gene modulation analysis (Tahara et al., 2012; 2015; Uchiyama et al., 2019).

The evolution of different RNA extraction methods enabled to identify a protocol that is suitable to break up the cell walls of *H. metazoicum* so that RNA of high quality and quantity can be obtained. Such RNA could be used for transcript-level analyses of the ABC-like gene *slr2019*.

H. metazoicum has been shown to tolerate low pH conditions down to 6.5, far lower than environmental future predicted scenarios since pigment analyses indicated no adverse effects of acidified environments on cyanobacterial viability. Slr2019 function could thus compensate for adverse effects of low pH after short term exposure to environmental relevant value (7.7), this may be indicated by the up-regulation of *slr2019*. On the other hand, downregulation of *slr2019* after 30 d with decreasing pH suggests that this is not the case and further studies are needed to investigate whether this is associated with an adverse effect of low pH.

Since a different behaviour in *slr2019* gene expression was observed between 7 and 30 d, further investigations are necessary to unravel the mechanism of specific involvement. Such knowledge will allow us to further investigate the ability of marine species to deal with OA scenarios and predict future impacts on biodiversity.

CRediT authorship contribution statement

Romano Patrizia: Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing. **Simonetti Silvia**: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision. **Maria Cristina Gambi**: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project

administration, Funding acquisition. **Till Luckenbach**: Writing - Review & Editing. **Valerio Zupo**: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Ilaria Corsi**: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

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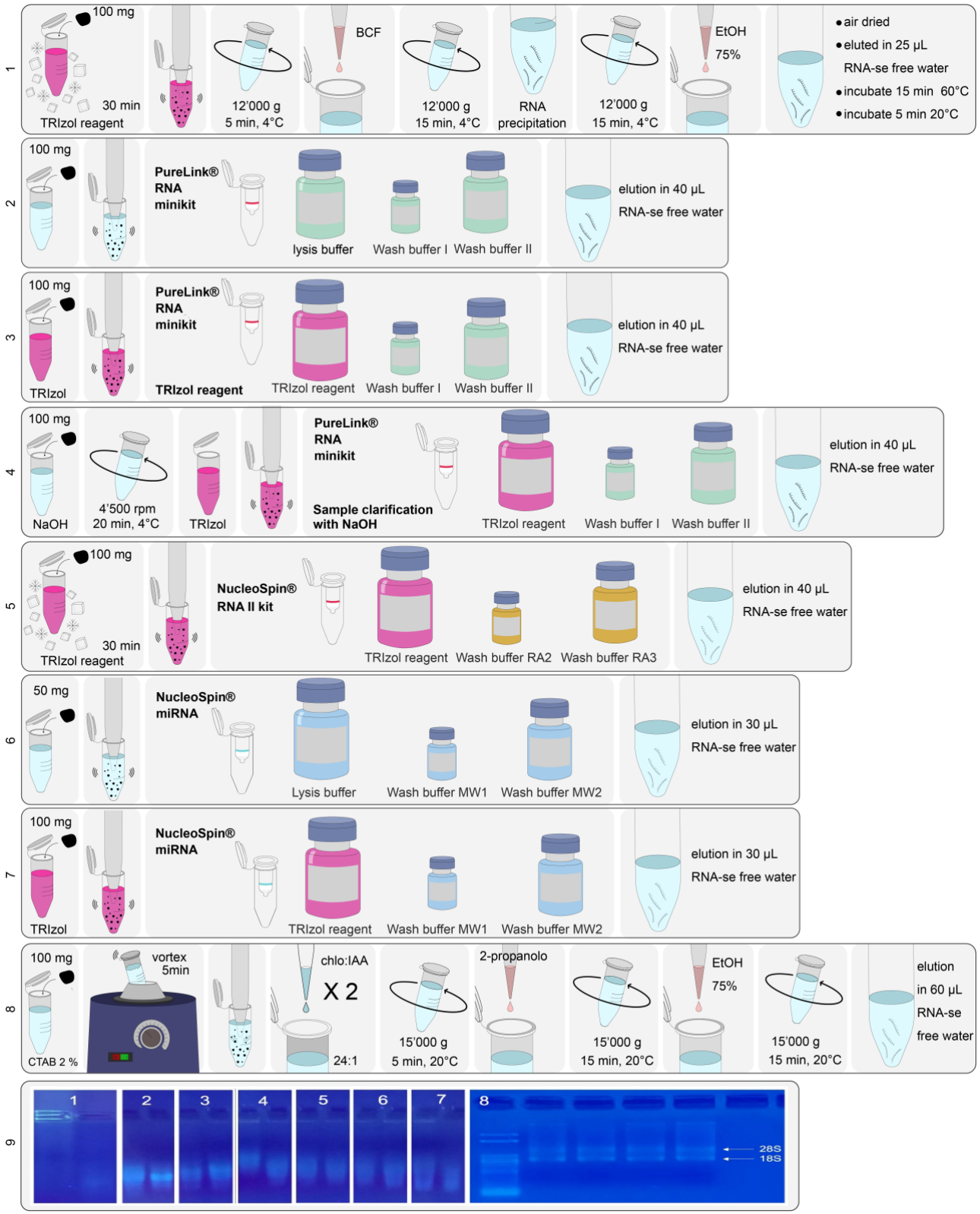
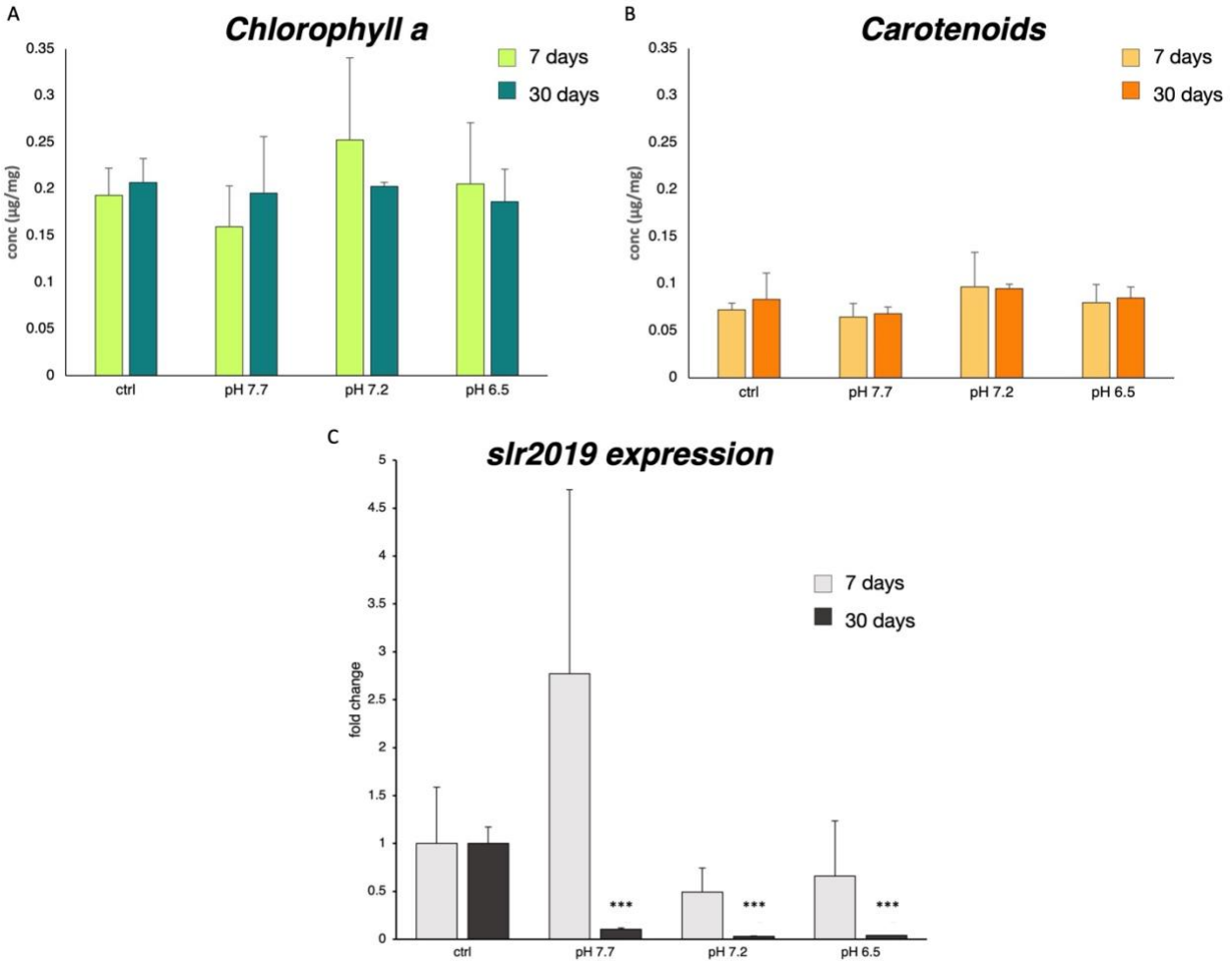


Figure 1. Schematic description of the extraction methods (1-8) and 1.5% agarose 'bleach gel' showing the quality of the extracted RNA from each method (9). White arrows indicate 18S and 28S ribosomal RNA bands

2-column fitting image, color online only



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493 Figure 2. Physiological and transcriptomic results assessed in *H. metazoicum* mattes exposed to four different pH (8.2,
494 7.7, 7.2, 6.5). The upper charts represent chlorophyll *a* (A) and carotenoid (B) contents (µg/mg) measured in control and
495 treated groups after 7 and 30 d of exposure. The chart below (C) represents the expression of *slr2019* gene in control and
496 treated groups after 7 and 30 d of exposure. Results are shown as mean ± standard deviation (SD). *** indicates significant
497 differences ($p < 0.001$) with controls group

498 2-column fitting image, color online only

Table 1. Table of purity, concentrations and yield (mean ± standard deviation) of total RNA extracted through the eight different methods

	$A_{260/280}$	$A_{260/230}$	Conc ($ng_{RNA}/\mu L$)	Yield (ng_{RNA}/mg_{sample})
Manual extraction	1.86 ± 0.14	0.10 ± 0.00	105.97 ± 29.77	12.89 ± 3.78
PureLink® RNA Mini Kit	1.80 ± 0.03	0.61 ± 0.07	74.70 ± 18.24	14.88 ± 3.73
TRIzol + PureLink	1.99 ± 0.07	1.23 ± 0.44	23.67 ± 29.57	4.63 ± 5.68
NaOH + PureLink	1.94 ± 0.07	0.63 ± 0.31	8.05 ± 3.05	1.13 ± 0.14
NucleoSpin® RNA II Kit	1.57 ± 0.38	0.42 ± 0.48	33.65 ± 5.59	6.72 ± 1.47
NucleoSpin® miRNA Kit	2.10 ± 0.02	1.52 ± 0.03	9.60 ± 0.42	5.97 ± 0.26
TRIzol + NucleoSpin® miRNA Kit	2.01 ± 0.08	1.24 ± 0.33	7.15 ± 3.08	2.91 ± 1.30
CTAB 2% buffer	2.13 ± 0.02	2.05 ± 0.15	24.82 ± 4.06	14.88 ± 2.43