Running title: Sodium sulfite induced hypoxia in anemones

Sodium sulfite can reliably induce chemical hypoxia without toxic effects in the model sea anemone species, *Exaiptasia diaphana*

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Abstract

Climate change is accelerating the deoxygenation of aquatic ecosystems, making understanding its effects on biological processes critical. An emerging method for inducing hypoxia during experimentation utilizes sodium sulfite to scavenge oxygen, however it remains unclear if hypoxia-inducing concentrations of sodium sulfite are toxic to aquatic species. We decoupled sodium sulfite's potential toxicity from its hypoxia-inducing effects by rapidly replacing the oxygen it removes via vigorous aeration. We examined the model species *Exaiptasia diaphana* (a sea anemone) and its endosymbiotic dinoflagellates by exposing them to a dosage that mimics the diel oxygen cycle for two weeks. We examined common stress indicators including, the anemone's metabolic rates and thermal tolerance, the algae's photosynthetic efficiency, cell density and reproduction in treated and untreated specimens, finding no effects of sodium sulfite exposure. A second trial assessed acute toxicity, finding that anemones lacked observable stress responses after 70x the concentration needed to induce anoxia (8.669 g/L). We therefore conclude that sodium sulfite is a viable, cost-effective and non-toxic way to induce hypoxia in laboratory settings.

Introduction

Hypoxia, the depletion of oxygen from an environment, is one of the main climate change-related stressors in aquatic environments, due to its links with global warming and acidification (Gobler and Baumann 2016; Tomasetti et al. 2021; Woods et al. 2022; Calvin et al. 2023). When temperatures increase, oxygen solubility in water declines (Dejours, 1981) because breaks in their weak molecular interactions become more common (Matear and Hirst 2003; Woods et al. 2022). However, oxygen's diffusivity also increases at higher temperatures leading to slightly increased bioavailability (Verberk and Atkinson, 2013; Boag et al. 2018). Hypoxia therefore, occurs not from decreased bioavailability but from an increase in metabolic demand that outpaces the additional bioavailability (Verberk and Atkinson, 2013; Audzijonyte et al. 2019; Rubalcaba et al. 2020). Hypoxia is also linked to the amount of dissolved carbon dioxide (CO_2) in aquatic environments due to the fluctuating dominance of photosynthesis and respiration during daily and seasonal cycles (Yates et al. 2007; Tomasetti et al. 2021).

The most common methodology used to induce hypoxic conditions in an experimental laboratory setting is the bubbling of nitrogen gas in the water (Flint et al. 2012). This causes the physical displacement of oxygen molecules by nitrogen ones, leading to hypoxia, however, there are some complications related to this methodology. Firstly, the nitrogen molecules also displace carbon dioxide molecules, resulting in hypoxic water conditions that are also less acidic (Flint et al. 2012; Klein et al. 2017). Furthermore, the addition of nitrogen gas can disrupt nitrogen dynamics in the study system due to the complicated interactions between the microbial communities that govern aquatic nitrogen cycling (i.e. diazotrophs, nitrifying and denitrifying bacteria/archaea). This may not directly affect animal study systems, but could certainly affect algal study systems which are often highly nitrogen

limited (Yodsuwan et al. 2017) as well as symbioses between animals and algae that contain microbes that mediate nitrogen dynamics (Rädecker et al. 2015).

The challenges involved with bubbling nitrogen to induce hypoxia have led to a few alternatives, such as custom gas mixes to aerate the water (Klein et al. 2017), however, this is a very costly option, making it difficult to adopt for long-term experiments and experiments that require high water volumes. Hypoxia chambers also provide an alternative but access to this equipment is very limited for most laboratories due to costs, and impractical for experiments that require opening the chamber often, for example, for animal husbandry purposes or recording physiological parameters (Muñoz-Sánchez and Chánez-Cárdenas 2019). Furthermore, this methodology is more suitable for experiments with low water volumes and small organisms, due to the limited dimensions of most commercially available chambers (Muñoz-Sánchez and Chánez-Cárdenas 2019).

Adding an oxygen scavenging chemical to the water surrounding an organism to induce chemical hypoxia is gaining traction as a promising method for inducing hypoxia, particularly in cell biology research. However, this method has not been widely tested or adopted in multicellular organisms, likely because potential toxicity is understudied and standardized methods have not been established. Two main chemicals have been identified to this end, cobalt chloride and sodium sulfite (SSI). Both scavenge oxygen efficiently, but a few studies have raised concerns over cobalt toxicity (Jiang et al. 2011; Muñoz-Sánchez and Chánez-Cárdenas 2019), so SSI is perhaps the most promising and less toxic option (Jiang et al. 2011). SSI is an antioxidant commonly used in commercial and industrial processes such as dried food preservation for human consumption (Marino et al. 2020). By scavenging oxygen, it induces deoxygenation and has been shown to cause hypoxia-related effects that are comparable to those caused when using nitrogen hypoxia (Gallegos-Saucedo et al. 2020; Marino et al. 2020). It is widely available, inexpensive, and its use is reproducible (Marino et

al. 2020). Its toxicity in multicellular organisms, however, has only been tested once to our knowledge, using the nematode, *Caenorhabditis elegans* (Jiang et al. 2011). This was done with the use of a sodium sulfate (SSA) solution as a control since SSA results from the oxidation of SSI and does not possess an oxygen scavenging capacity (Equation 1). The authors found that SSI hypoxia has the same effect on death rate, cellular morphology, and *C. elegans* hypoxia-inducible factor 1 (CeHIF-1) expression in comparison to nitrogen hypoxia, indicating that their effects are comparable (Jiang et al. 2011). Most importantly, the SSA control did not cause mortality, leading to the strong suggestion by the authors that SSI toxicity is negligible or non-existent, in comparison to its hypoxia-inducing effect (Jiang et al. 2011). However, the potential toxicity of SSA, and therefore SSI, has never been tested against an artificial seawater control.

$$2\ Na_2SO_{3\,(aq)} + O_{2\,(g)} = 2\ Na_2SO_{4\,(aq)}$$

Equation 1: Sodium sulfite (Na_2SO_3) scavenges O_2 by reacting with it to form sodium sulfate (Na_2SO_4).

To determine if chemical hypoxia using SSI is a reliable and non-toxic way to induce hypoxia for physiological studies on multicellular animals, we examined the effects of prolonged (Experiment 1) and acute (Experiment 2) SSI exposure on a sea anemone, *Exaiptasia diaphana* (Rapp 1829), a common marine model organism. To examine potential toxicity from the SSI itself, we decoupled the effects of exposure to the salt itself and the hypoxia it induces, by vigorously aerating all of the treatments, to ensure we overcompensated for any O_2 that was scavenged as quickly as possible. *Exaiptasia diaphana* is a popular and practical model used for stony corals, and therefore very relevant in climate change biology research

(Roberty et al. 2024). Furthermore, *E. diaphana* possesses a symbiotic relationship with photosynthetic dinoflagellates (Roberty et al. 2024), making it a representative species for those that could benefit the most from the experimental use of SSI instead of nitrogen hypoxia. This also allowed us to examine a variety of common performance indicators related to both animal and algal health, including the anemone's metabolic rates (Svoboda and Porrmann 1980) and critical thermal limits (Laetz and Verberk 2024) and the algae's photosynthetic efficiency (Schreiber 2004), cell density and reproductive rate (e.g. Suharsono and Brown 1992; Wilkerson et al. 1988). Each of these metrics has been previously established as an indicator of anemone and algae performance when in symbiosis.

Materials and procedures

Animal husbandry

All specimens were cultured in 20L tanks outfitted with air bubblers, temperature and oxygen probes (Neptune Systems, USA). Artificial seawater was made with Instant Ocean (IO) aquarium salt (Spectrum Brands, USA) and distilled water to maintain a consistent salinity of 35±1 PSU. Anemones were fed freshly hatched *Artemia* sp. shrimp (Superfish artemia) three times a week. Light was provided by full-spectrum LEDs on a 12:12 light-dark cycle, at a light intensity of 75 ± 5 μmol $m^{-2}s^{-1}$ which was measured with a PQ-500 quantum sensor (Apogee Instruments Inc, USA) enclosed in a waterproof flash housing (as detailed in Burgués Palau et al. 2024).

Experiment 1 - prolonged exposure to SSI and SSA

Experimental setup

Twelve 2 L aquaria were set up for each treatment (ASW, SSA + ASW and SSI + ASW) and covered by transparent plastic film to minimize evaporation. During the experiment, specimens were provided the same amount of light and brine shrimp as they had been before experimentation. The salinity was measured every other day with a refractometer (R12018, Red Sea, USA).

During experimentation, a stock solution of SSI (33.9 g/L; Cat: 1.06657, Merck KGaA, Germany) was added to six of the aquaria, making a final concentration of 0.125 g/L SSI diluted in ASW. This concentration successfully induced oxygen saturation decreases from 100 % saturation to 40 %, but this was minimized under excess air supply, decreasing only 3.79 ± 1.15 % (preliminary experiments conducted for this study). To compare the effect of additional sulfate ions without any oxygen scavenging capacity, a SSA stock solution (38 g/L concentration; Cat: 1.06649, Merck KGaA, Germany) was added to a second set of ASW-containing aquaria to make a final concentration of 0.133 g/L. These concentrations were chosen to standardize the molarity between our SSA and SSI treatments. The same volume of untreated, artificial seawater (ASW) was added to a set of aquaria containing anemones as a control. All additions (ASW, SSA + ASW, SSI + ASW) were performed once a day at 17:00 for two weeks.

Photosynthetic efficiency, algal cell density and mitotic index

To examine the health of the algae, we measured the maximum quantum yield of photosystem II (F_v/F_m), a metric that describes photosynthetic efficiency (Wägele and Johnsen, 2001; Hoogenboom et al. 2012). Healthy algae often display stable F_v/F_m values over time, while decreasing values often occur when algae are stressed (Bhagooli and Hidaka 2003). Specimens were provided 15 minutes of dark acclimation before they were measured using a Junior PAM fluorometer with a white measuring LED (Walz, GmBH Germany). We

then imaged chlorophyll fluorescence to examine algal cell density and assess if the anemone-algae symbiosis remains stable across treatments (i.e. there is no evidence of bleaching). We also determined the rate of algal cell division (Wilkerson et al., 1988), termed the mitotic index, in each specimen to determine if algal reproduction is affected by SSI or SSA exposure. To take these images, one tentacle was vivisected from each specimen, placed on a microscope slide and photographed using an epifluorescence microscope (Nikon Eclipse E800, Nikon USA, USA) containing a custom-built filter cube with a 400-440 nm band-pass excitation filter, 515 nm dichroic, and 610 nm long-pass emission filter (Chroma, USA). Images were analyzed in the FIJI/ImageJ (Schindelin et al. 2012), using the plugin 3D-AMP (Laetz et al. 2017) to determine the area that was filled with chlorophyll (percent coverage) in each tentacle. To determine the mitotic index, the number of symbionts in each sample was estimated by dividing the total area covered by algae divied the average area covered by a single algal cell. Subsequently, the number of symbionts undergoing cytokinesis was manually counted. The mitotic index was then calculated as the percentage of algae undergoing division relative to the total number of algae in each image. (Wilkerson et al. 1988).

Metabolic rate (O_2 consumption)

Measuring changes in oxygen consumption (by the animal) and production (due to photosynthesis) allowed us to detect changes in aerobic scope that can be associated with stress (e.g. metabolic depression). To calculate metabolic rates based on oxygen consumption, anemones were transferred to 2 and 22.2 mL respirometry chambers containing 100% O_2-saturated seawater and oxygen microsensors (PSt3 sensor type, PreSens GmBH, Germany). Oxygen saturation values were converted to concentration values (mgO_2/L) using an online calculator (https://water.usgs.gov/water-resources/software/DOTABLES/).

Specimens were given 30 minutes to recover from handling stress before the chambers were sealed. They were then placed in a thermostatic water tank under the same temperature and light intensity at the acclimation conditions. Then we measured the oxygen saturation every 10 minutes for an hour with an oxygen probe (Fibox Trace LCD 4, PreSens GmBH, Germany). During this time, temperature, salinity and air pressure were also recorded. The metabolic rate (MO_2) was calculated according to the protocol detailed in (Laetz and Verberk 2024). Negative O_2 values indicate that the anemone took up more O_2 from the seawater than the amount generated by its algae during photosynthesis. These values were then imported into R Studio (v.2024.04.2+764; RStudio Team, 2024) based on R (R version 4.4.1, 2024-06-14) for further analysis. Since metabolic rates scale non-linearly with body size, an allometric scaling exponent (0.61) was calculated for this species and used to account for the effects of body size (Laetz and Verberk 2024). These metabolic rates are therefore presented as the mgO_2 consumed/produced, per 0.61 grams, per hour ($mgO_2\ g^{-0.61}\ h^{-1}$). This ensured that any remaining variation could be explained by our treatments, rather than body size. Finally, each metabolic rate was multiplied by -1 so that specimens that took up O_2 from the seawater yielded negative metabolic rates, while specimens that produced so much O_2 that they oxygenated the surrounding seawater displayed positive metabolic rates, making interpretation more intuitive.

Critical thermal maxima

As a performance test, we also examined their Critical Thermal maxima (CTmax), the maximum temperature an individual can withstand before displaying a pre-defined stress behavior. In anemones, a few stress behaviors have been defined, a) complete tentacle retraction, b) detachment from the substrate, c) discharge of acontia, i.e. stinging threads of tissue filled with defensive stinging cells that some cnidarians release when threatened (Lam

et al. 2017; Laetz and Verberk 2024). Previous reports (Laetz and Verberk 2024) and preliminary experiments suggested that tentacle retraction and pedal disk detachment are reliable reactions to heat stress in *E. diaphana*, so they were used in this study.

To measure CTmax, each anemone was placed into a 2 L aquarium containing aerated seawater from their original aquaria (containing ASW, SSA+ASW or SSI+ASW). The aquaria were placed in a water bath at 25°C, under 75 ± 5 µmol $m^{-2}s^{-1}$ light. After acclimating for 30 minutes (Fig. 1A), the temperature was increased by 1 ℃ every ten minutes via tank heaters (Superfish, Pro Heater, 200W). CT1 was defined as the temperature at which an anemone retracted its tentacles (Fig. 1B) and CT2 was established as the temperature at which it detached from the wall of the respiratory chamber, Fig. 1C). Once CT2 was reached, the aquarium containing that specimen was immediately removed from the warm water bath and allowed to recover by cooling down ~1 °C every 5 minutes to 25 °C.

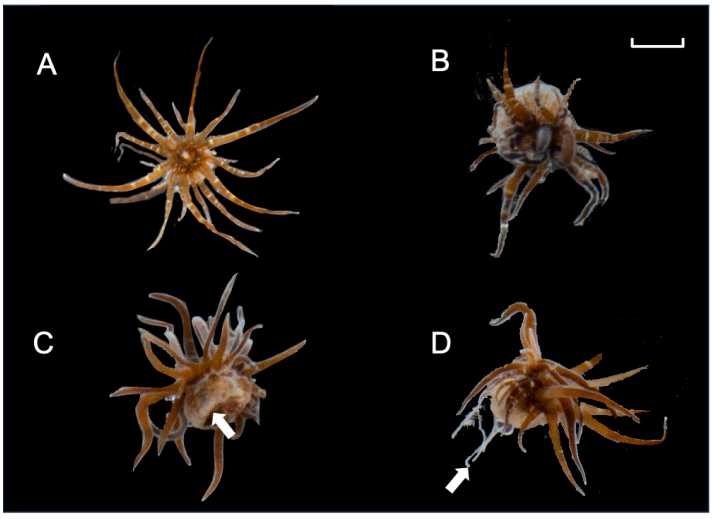

Figure 1. The behavior indicators of the *E. diaphana.* A) An unstressed specimen with tentacles outstretched, B) an anemone with retracted tentacles following acute stress, C) an anemone whose pedal disk is now detached from the surface of the aquarium. The white arrow shows the underside of the detached pedal disk, D) an anemone discharging acontia, indicated by the white arrow. The scale bar is 5 mm.

Experiment 2 - acute toxicity

To examine if SSI exposure (rather than the hypoxia it causes) can induce stress responses in *E. diaphana*, we performed the following acute toxicity test. Two rounds of 18 anemones were individually placed in 2 L aquaria containing 500 mL ASW and allowed to acclimate for 3 days. The temperature, light, and salinity were kept constant according to the parameters described above. Specimens were then randomly assigned to one of three treatments, ASW, ASW + SSA, and ASW + SSI and the order of these aquaria was randomized. The concentration of each salt was increased tenfold every 20 minutes until two of the following stress behaviors were observed: acontia release, complete tentacle retraction, pedal disk detachment. The starting concentrations of SSA and SSI were 0.133 g/L and 0.125 g/L respectively to provide exposure to equal molarities of each salt.

To decouple any effects of SSI from hypoxia, we vigorously aerated the aquaria to ensure that the O_2 that was scavenged by SSI was rapidly replaced. To examine if hypoxia was induced, O_2 saturation measurements were taken for each aquarium, every time the concentration was changed and the oxygen saturation was kept above 70% in the entire experiment (Fig. 2). To ensure the anemones were minimally disturbed by excess aeration, they were provided a glass vial turned on its side that functioned as a transparent semi-enclosed shelter, allowing water to mix but blocking fast water movement from direct contact with the anemone. Visual inspections ensured they were not agitated by fast-moving water.

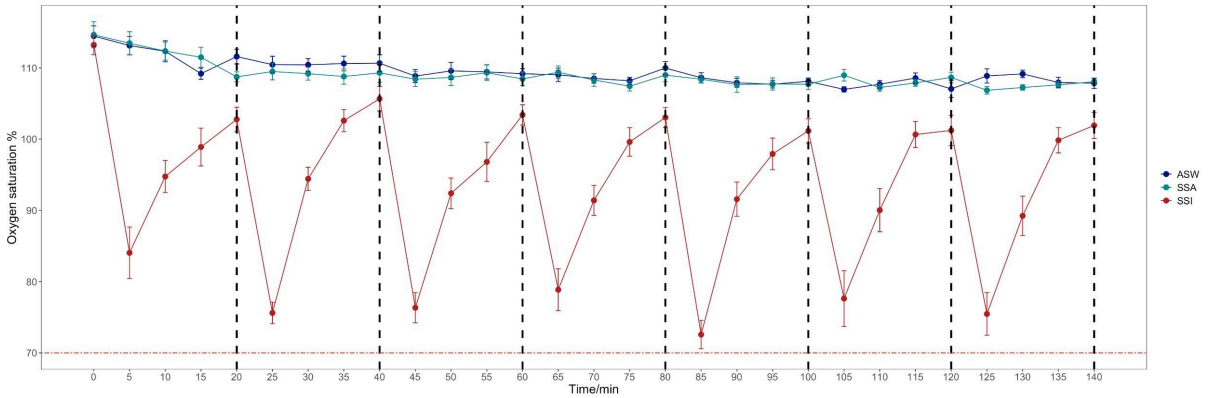

Figure 2. Oxygen saturation throughout Experiment 2 for each treatment. The concentration of each salt was increased every 20 minutes (seven times in total), as indicated by the vertical, black dashed lines. The oxygen saturation, expressed as a percentage, for each treatment, was measured every 5 minutes. The ASW-only control is depicted with a blue line, the SSA O_2 saturation is visualized with a turquoise line and the SSI O_2 saturation is shown with a solid red line. Note that the horizontal red dashed line indicates the 70% oxygen saturation threshold that is often used to denote hypoxia, and vigorous aeration prevented these specimens from experiencing hypoxia during this experiment.

Since adding SSA and SSI salts affects salinity, we developed a protocol to replace part of the IO salts with SSA and SSI at each step, thus keeping the salinity constant throughout the experiment (35 ± 1 PSU), while increasing the relative concentration of sulfite and sulfate ions. Each time the concentration was increased, the following steps were followed (detailed in Supplementary Tables 1-2). In Step 1 - the "removal phase", seawater was removed from each aquarium to prevent the overall volume from increasing exponentially. In Step 2, the "addition phase", a pre-determined volume (mL) of distilled water and amount of salt (ASW, SSA or SSI in g) were added to the respective treatment aquaria to increase the concentration of the target salt (ASW, SSA or SSI). In Step 3, the "compensation phase", additional salt and water were added to each tank to account for the fraction of salt that was removed in the first step, which caused a discrepancy between the actual concentration and the intended concentration of each target salt. The amount of salt and water that were added in Step 3 was calculated by determining the difference between the target concentration and actual concentration and adjusting the final volume to maintain the salinity at 35 PSU. Since the concentration of ions in the ASW control did not change during the experiment, we simply removed the same amount of ASW as we removed in each step for SSI and then replaced this volume with new ASW.

Statistical analyses

All data were analyzed with R studio (v.2024.04.2+764; Posit team, 2024) using functions included in the R base package (R Core Team, 2024) unless otherwise cited. In Experiment 1, the treatment (ASW, ASW + SSA, or ASW + SSI) was considered as the predictor variable. Each response variable (F_v/F_m, algal symbiont density, mitotic index, metabolic rate) was analyzed with the same statistical approach. First, the data was examined for outliers using the packages "outliers", "car" and "dplyr" (Fox and Weisberg 2019; Komsta, 2022; Wickham et al. 2023). Data was then tested for normality and homogeneity of variances using Shapiro-Wilk tests and Bartlett's tests. If all assumptions were met, a one-way ANOVA was performed, while a Kruskal Wallis rank sum test was performed when assumptions were not met. *P*-values of less than 0.05 were considered significant. Since a specimen with overall higher heat tolerance could be expected to have higher limits for both CTs, we first tested if CT1 and CT2 are correlated, however they were not (B =0.068, t_{34}=0.62, p =0.54, where B is the slope and t is the t-ratio), so we analyzed CT1 and CT2 separately according to the same steps described for the other responses above. Test results are displayed in Table 1. All plots were generated with "ggplot2" (Wickham 2016).

Response variable	Normality	Homogeneity of variances	Statistical test
F_v/F_m	Yes, p=0.09	Yes, p =0.68	One-way ANOVA
Algal cell density	Yes, p =0.09	Yes, p =0.13	One-way ANOVA
Mitotic index	Yes, p =0.82	Yes, p =0.81	One-way ANOVA
MO_2	Yes, p =0.08	Yes, p =0.69	One-way ANOVA

CT1	No, p =0.01	n.a.	Kruskal Wallis
CT2	Yes, p =0.07	Yes, p =0.27	One-way ANOVA

Table 1. Test results for normality, homogeneity of variances and the selected statistical tests

Experiment 2 investigated three behavioral responses (tentacle retraction, acontia discharge (Fig. 1D), and pedal disk detachment) that each anemone could have displayed following each increase in SSI, SSA or ASW concentration, so we first examined if any of the responses were correlated. After finding that detachment was positively correlated to both tentacle retraction and acontia discharge (Pearson, $r = 0.076$; $t = 14.98$, df = 286; $p = 2.0 \times 10^{-16}$ for retraction and $r = 0.048$, $t = 11.9$, df = 286; $p = 2 \times 10^{-16}$ for acontia discharge), we removed it from the analysis. A MANOVA model was then built to investigate the effect of treatment (ASW, ASW + SSA, and ASW + SSI) and concentration (0- 70x) on the behavioral changes: tentacle retraction and acontia discharge.

Assessment

These investigations illustrate that hypoxia-inducingconcentrations of sodium sulfate (0.125 g/L) andcan create a rapid chemical deoxygenation in laboratory experiments and that this deoxygenation can be overcome via vigorous aeration. Exposuring *Exaiptasia diaphana* and its endosymbiotic algae to SSA + ASW or SSI + ASW did not significantly affect any of the responses we measured in this study when compared to ASW alone in a long-time exposure. (detailed in Fig. 3, Table 2).

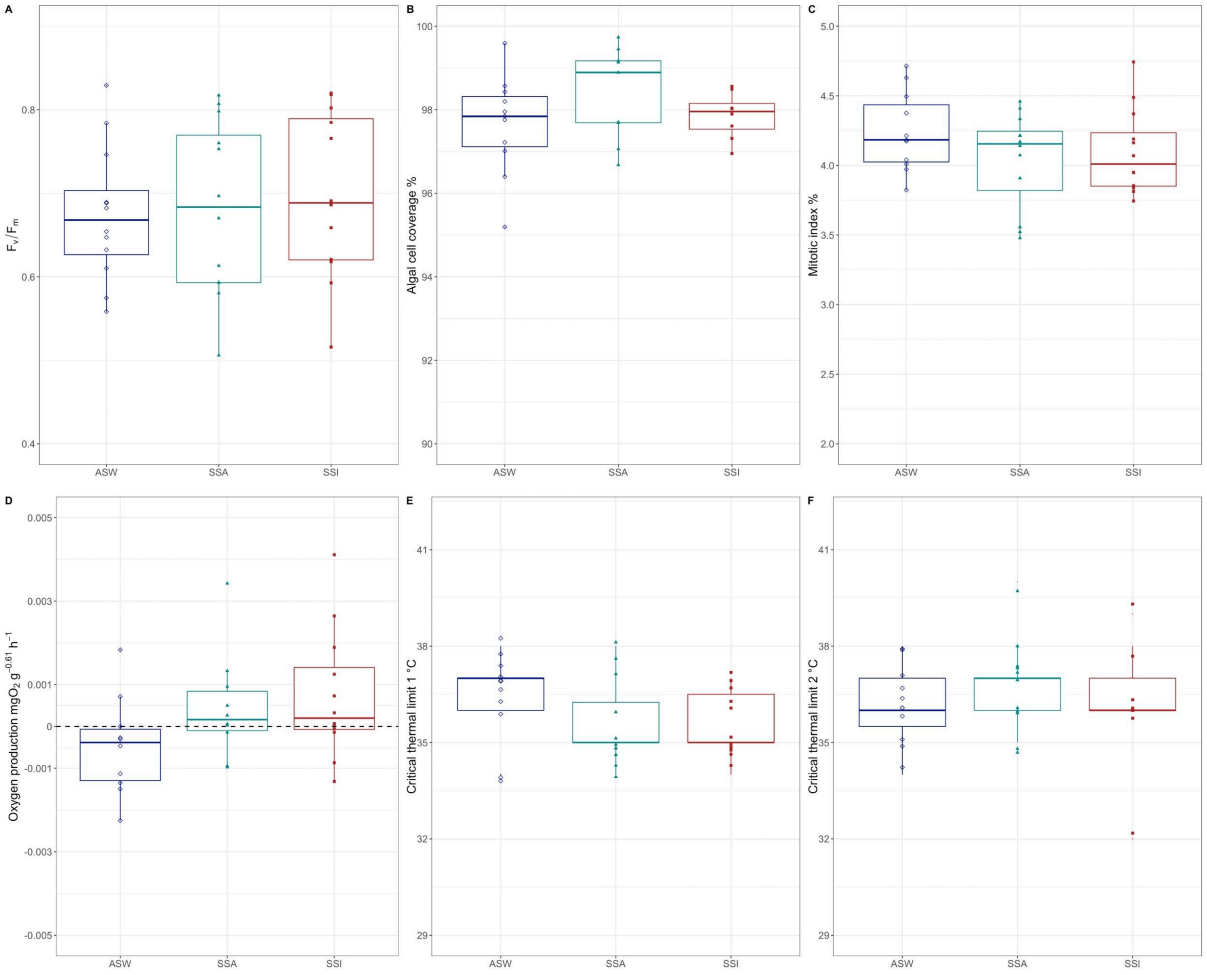

Figure 3. Each of the responses was examined in Experiment 1. In all panels, the artificial seawater (ASW) treatment is depicted in blue, sodium sulfate (SSA) +ASW-exposed specimens are in turquoise and sodium sulfate (SSI) +ASW-exposed specimens are in red. None of these treatments differed significantly from one another when the following responses were examined, A) maximum quantum yield of photosystem II (F_V/F_M), B) the percentage of endoderm tissue filled with symbiotic algae, C) the mitotic index (%), D) oxygen production in $mgO_2\ g^{-0.61}\ h^{-1}$, E) the temperature at which Critical Thermal limit 1 was reached (tentacle retraction), and F) the temperature at which Critical Thermal limit 2 was reached (pedal disk detachment).

Response variable	ASW	SSA + ASW	SSI + ASW	*P*-values
Fv/Fm	0.67 ± 0.08	0.68 ± 0.10	0.70 ± 0.10	not significant, $p = 0.83$
Algal cell density	97.7 ± 1.18%	96.8 ± 2.99%	94.5 ± 6.04 %	not significant, $p = 0.28$
Mitotic index	4.24 ± 0.28	4.04 ± 0.35	4.10 ± 0.31	not significant, $p = 0.31$
MO_2	-0.002 ± 0.007	-0.003 ± 0.007	-0.0005 ± 0.002	not significant, $p = 0.12$
CT1	35.9 ± 2.27℃	35.6 ± 1.38℃	35.2 ±1.90℃	not significant, $p = 0.20$
CT2	35.7 ± 2.15℃	36.2 ± 2.37℃	36.5 ± 3.97℃	not significant, $p = 0.65$

Table 2. The average (mean) values ± the standard deviation of each treatment (artificial seawater (ASW), sodium sulfate + artificial seawater (SSA + ASW) and sodium sulfite + artificial seawater (SSI + ASW)) for each response we measured and the results of either the one-way ANOVA or Kruskal-Wallis rank sum test that was performed. Since all *p*-values were > 0.05 and therefore considered non-significant, no post hoc testing was conducted.

Only three specimens displayed signs of stress when we examined stress responses to increasing concentrations of sulfate or sulfite ions (Experiment 2). One ejected acontia and

detached its pedal disk when exposed to 6.65 g/L (50x) of SSA + ASW, and the other detached its pedal disk and retracted its tentacles after exposure to 10x of ASW and 20x of SSI + ASW, respectively. The other specimens did not display stress behaviors at any step during this experiment. Furthermore, none of the predictors used in our MANOVA analysis had a significant effect on the stress behaviors: treatment ($F_{(4, 528)}$=0.99436, p =0.4101), concentration ($F_{(14, 528)}$=1.2351, p =0.2454), and their interaction ($F_{(28, 528)}$=0.8069, p =0.7497).

Discussion

Since none of the responses examined in Experiment 1 differed between treatments, we conclude that SSI+ASW and SSA+ASW do not have a demonstrable effect on photosynthetic efficiency, algal symbiont density, mitotic index, the anemone's metabolic rate, nor the anemone's critical thermal limits, as long as an O_2 saturation >70% is maintained during exposure (achieved here with vigorous aeration). Of these responses, metabolic rate (Fig. 3D) is the only response that, while statistically insignificant, could present a difference between SSI/SSA and ASW, which could indicate that exposure to higher sulfate concentrations either decrease metabolic rate or boost the rate of photosynthetic O_2 production (Zhang et al. 2020), although this will require further study. In a parallel study that examined how multiple stressors affected *E. diaphana* (Parodi et al. 2024), SSI induced hypoxia did lead to differences in metabolic rates, critical thermal limits and acclimation response ratios, a commonly used proxy for heat tolerance plasticity (Morley et al. 2019), especially when combined with other stressors. As these differences were not observed in this study, where hypoxia was prevented by vigorous aeration, we conclude that any negative effects of SSI exposure stem from hypoxia and are not triggered by increased sulfate availability.

The results of Experiment 2 further support our conclusion that exposure to SSI does not induce stress behaviors in *E. diaphana* as long as the O_2 saturation remains within the normoxic range. Only one (20x) of the SSI-exposed specimens demonstrated a stress behavior during the acute toxicity experiment, causing us to conclude that even a 70x dose (8.669 g/L) of SSI was not stressful, as long as normoxia and a constant salinity are maintained. Interestingly, acontia discharge, was only observed in one SSA-treated specimen in Experiment 2 of this study, and has not been observed in other stress trials in *E. diaphana* or other anemones (Laetz and Verberk 2024; Parodi et al. 2024). Therefore, we hypothesize that this behavior is unrelated to the experimentand is more likely caused by some other factor affecting that individual.

Comments and recommendations

Sodium sulfite has been used to induce hypoxia in studies examining single-celled algae or animal cells in culture for many years, but it has yet to be widely adopted for studies on multicellular organisms (Kramer and McClure 1982 ;Jiang et al., 2011). Our observations lead us to conclude that SSI is a reliable and practical way to induce hypoxia in experiments involving *E. diaphana*, and should be considered in future studies on the effects of hypoxia in other aquatic multicellular organisms. Furthermore, the ability to easily negate SSI's oxygen scavenging ability via vigorous aeration offers a promising way to include experimental controls that do not introduce other variables, unlike nitrogen immission, which changes both O_2 and CO_2 levels. This study corroborates findings by Jiang et al. 2011 in nematodes and further demonstrates that SSI is a promising, inexpensive, reliable and non-toxic method for inducing hypoxia in physiological experiments on multicellular organisms.

Author Contributions

ZQ: Data curation; formal analysis; methodology; validation; visualization; writing-original draft; writing - review and editing **LV:** Data curation; formal analysis; methodology; resources; writing - review and editing; **BAP:** Conceptualization; formal analysis; writing - original draft; writing- review and editing; **EMJL:** Conceptualization; data curation; formal analysis; methodology; resources; writing - original draft; writing - review and editing.

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Conflict of interest statement

All authors declare no conflict of interest.

Data availability statement

The data and R-scripts used in this study can be viewed using the following link and a permanent link will be generated if this manuscript is accepted: https://dataverse.nl/privateurl.xhtml?token=7e6b8016-93f7-4768-a240-883bd8d66701

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Supplementary Tables

Supplementary Table 1: The concentrations (g/L) of sodium sulfite (SSI) and Instant Ocean salt (IO) at each experimental step in Experiment 2, where the concentration of SSI was increased by 10x the amount used to induce hypoxic conditions (0.125 g/L). During the "addition phase", constant amounts of SSI (0.625 g) and distilled water (17.448 mL) were added to each SSI treatment aquarium, while the "compensation phase" replaced the SSI that was removed during Step 1 (the removal step needed to keep the volume from increasing exponentially and the salinity at 35 PSU). We also report the percentage of total salt derived from IO and SSI at each step, showing the progressive changes in ion concentrations during the experiment.

Step	Concentration of SSI (g/L)	SSI (g) added in Step 3 (compensation phase)	diH_2O (mL) addition in Step 3 (compensation phase)	Final percentage (out of 100 % total) contributed by IO to maintain 35 PSU	Final percentage (out of 100 % total) contributed by SSI to maintain 35 PSU
1 (0x SSI)	0.000	0	0	100	0
2 (10x SSI)	1.250	0.022	0.609	96.510	3.490
3 (20x SSI)	2.497	0.046	1.280	93.029	6.971
4 (30x SSI)	3.740	0.072	2.022	89.558	10.442
5 (40x SSI)	4.980	0.102	2.844	86.097	13.903
6 (50x SSI)	6.215	0.135	3.759	82.650	17.350
7 (60x SSI)	7.445	0.171	4.781	79.216	20.784
8 (70x SSI)	8.669	0.212	5.923	75.799	24.201

Supplementary Table 2 The concentrations (g/L) of sodium sulfate (SSA) and Instant Ocean salt (IO) at each experimental step in Experiment 2, where the concentration of SSA was increased by 10x. During the "addition phase", constant amounts of SSA (0.665 g) and distilled water (18.565 mL) were added to each SSA treatment aquarium, while the "compensation phase" replaced the SSA that was removed during Step 1 (the removal step needed to keep the volume from increasing exponentially and the salinity at 35 PSU). We also report the percentage of total salt derived from IO and SSA at each step, showing the progressive changes in ion concentrations during the experiment.

Step	Concentration of SSA (g/L)	SSA (g) added in Step 3 (compensation phase)	diH_2O (mL) addition in Step 3 (compensation phase)	Final percentage (out of 100 % total) contributed by IO to maintain 35 PSU	Final percentage (out of 100 % total) contributed by SSA to maintain 35 PSU
1 (0x SSA)	0.000	0	0	100	0
2 (10x SSA)	1.330	0.025	0.689	96.287	3.731
3 (20x SSA)	2.656	0.052	1.454	92.584	7.416
4 (30x SSA)	3.978	0.083	2.303	88.893	11.107
5 (40x SSA)	5.296	0.116	3.252	85.216	14.784
6 (50x SSA)	6.607	0.155	4.314	81.554	18.446
7 (60x SSA)	7.913	0.197	5.508	77.910	22.090
8 (70x SSA)	9.211	0.246	6.856	74.286	25.714