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

Zhen Qin[1], Lorenzo Vassura[1], Bianca A. Parodi[1], Elise M. J. Laetz[1°]

[1]Groningen Institute for Evolutionary Life Sciences, University of Groningen, Netherlands
°Corresponding author: e.m.j.laetz@rug.nl

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Abstract

Climate change is accelerating deoxygenation in aquatic ecosystems worldwide, causing consequences for aerobic organisms. Empirically studying the effects of deoxygenation on biological processes is therefore critical. Multiple methods for inducing hypoxia in physiological studies have been developed, each with pros and cons. Using oxygen scavenger chemicals, such as sodium sulfite, to reliably and inexpensively induce hypoxia in aquatic systems is gaining popularity, however its potential toxicity remains unknown and studies decoupling exposure to the salt from its hypoxic effects are lacking. To address this, we investigated the effects of sodium sulfite and the product of its oxidation, sodium sulfate, on the model sea anemone, *Exaiptasia diaphana* and its endosymbiotic dinoflagellates. To separate the effects of the salt itself and hypoxia, we provided vigorous aeration which quickly replaced sodium sulfite scavenged oxygen. In the first experiment, we exposed anemones to a hypoxia-inducing dosage of sodium sulfite (0.125 g/L), the equivalent concentration of sodium sulfate or a seawater control for two weeks under vigorous aeration to prevent actual hypoxia. Then, we measured the anemone's metabolic rates and their thermal tolerance. We also measured the algae's photosynthetic efficiency, cell density and reproduction. Neither sodium sulfite nor sodium sulfate exposure affected any of these parameters, allowing us to conclude that exposure to these salts at these concentrations is not toxic for *E. diaphana*. We then conducted a second experiment to determine how much sodium sulfite and sodium sulfate anemones could withstand before displaying behavioral signs of stress. After exposure to 70x (8.75 g/L), well beyond the concentration needed to induce anoxia when vigorous aeration is not provided, anemones showed no sign of stress, indicating that exposure to these salts at these concentrations is not acutely stressful. We therefore conclude that sodium sulfite is a viable and non-toxic way to scavenge oxygen and induce hypoxia in laboratory settings.

1. 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 (Calvin et al., 2023; Gobler & Baumann, 2016; Tomasetti et al., 2021; Woods et al., 2022). When temperatures increase, oxygen solubility in water declines (Dejours, 1981) because breaks in their weak molecular interactions become more common (Matear & Hirst, 2003; Woods et al., 2022). However, oxygen's diffusivity also increases at higher temperatures leading to slightly increased bioavailability (Boag et al., 2018; Verberk & Atkinson, 2013). Hypoxia therefore occurs, not from decreased bioavailability, but from an increase in metabolic demand that outpaces the additional bioavailability (Audzijonyte et al., 2019; Rubalcaba et al., 2020; Verberk & Atkinson, 2013). 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 (Tomasetti et al., 2021; Yates et al., 2007). Since warming, acidification (from increased CO_2) and deoxygenation are physically linked, all three - nicknamed the deadly trio - must be considered if we are to predict organismal and ecosystem responses to climate change in aquatic habitats. While a wealth of data exists to examine organismal responses to temperature and acidification increases, deoxygenation is chronically understudied, a gap that must be filled if we are to predict how species and ecosystems will respond to climate change (Allan et al., 2006; Glazier, 2015; Killen et al., 2010; Woods et al., 2022).

The most common methodology used to simulate hypoxic conditions in an experimental laboratory setting is the immission 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, however again, 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 & 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 & Chánez-Cárdenas, 2019).

Adding an oxygen scavenging chemical to the water surrounding an organism to induce chemical hypoxia is gaining traction as a promising (but not new) 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 & 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). It emulates physical hypoxia processes and has been shown to cause hypoxia-related effects that are comparable to the ones 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, was never 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 the ones 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 and critical thermal limits and the algae's photosynthetic efficiency, cell density and reproductive rate.

2. Methodology

2.1 Animal husbandry

All specimens were cultured in 20 L 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).

2.2 Experiment 1 - prolonged exposure to SSI and SSA

2.2.1 Experimental Setup

Six aquaria (3.3 L) 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 during the acclimation phase. The salinity was measured every other day with a refractometer (R12018, Red Sea, USA).

After acclimation, SSI stock solution (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 had only a minor effect under excess air supply decreasing by 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 seawater was added to a set of aquaria containing untreated (ASW only) anemones as a control. All additions (ASW, SSA + ASW, SSI + ASW) were performed once a day at 17:00 for two weeks.

2.2.2 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 (Hoogenboom et al., 2012; Wägele & Johnsen, 2001). Healthy algae often display stable F_v/F_m values over time, while decreasing values often occur when algae are stressed (Bhagooli & 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, 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 by 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)).

2.2.3 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 (*USGS DO TABLES*, 2018). 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 & Verberk, 2024). Negative O_2 values indicate that the anemone took up O_2 from the seawater, therefore requiring more 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 & Verberk, 2024). The final 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.

2.2.4 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 (Laetz & Verberk, 2024; Lam et al., 2017). Previous reports (Laetz & 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.

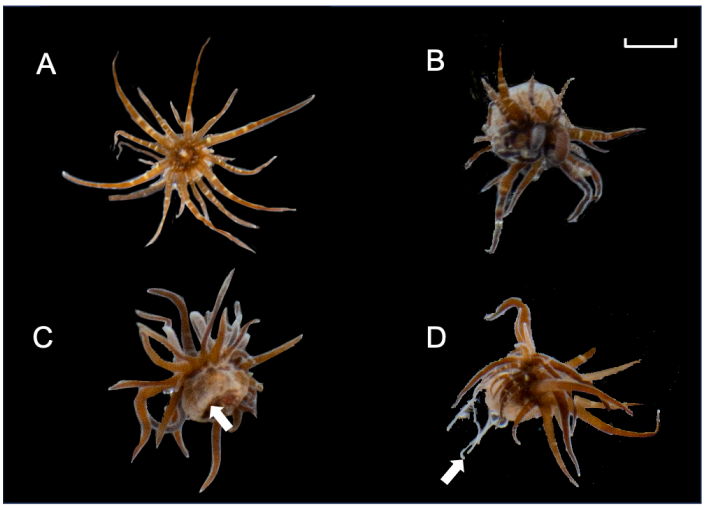

Figure 1. The behavior indicators of the *E. diaphana.* A) An unstressed specimen with tentacles outstretched, B) an anemone with retracted tentacles following acute stress, C) an anemone whose pedal disk is now detached from the surface of the aquarium. The white arrow shows the underside of the detached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

2.3 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. Eighteen 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 ten-fold 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.

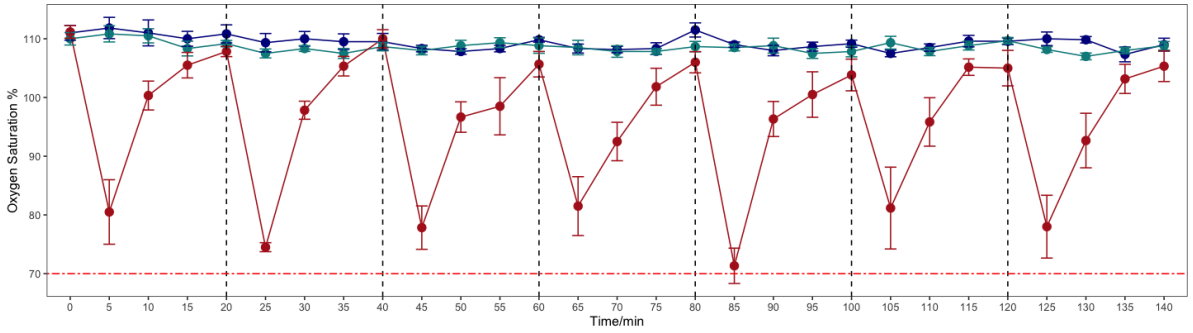

Figure 2. Oxygen saturation throughout Experiment 2 for each treatment. The concentration of each salt was increased every 20 minutes (seven times total), as indicated by the vertical, black dashed lines. The oxygen saturation, expressed as a percentage, for each treatment, was measured every 5 minutes. 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. The horizontal red dashed line indicates the 70 % oxygen saturation threshold that is often used to denote hypoxia.

Since adding SSA and SSI salts to examine their potential toxicity 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 (IO, SSA or SSI in g) were added to the respective treatment aquaria to increase the concentration of the target salt (IO, 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 were 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.

2.4 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 rates) was analyzed with the same statistical approach. First, the data was examined for outliers using the packages “outliers”, “car” and “dplyr” (Fox & 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 ($B=0.095$; $t_{1,15}=0.57$; $p=0.58$, 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).

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, $r = 0.705$; $t=11.83$, $df = 142$; $P < 2e^{-16}$ for both), 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.

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

Response variable	Normality?	Homogeneity of variances?	Statistical test
F_v/F_m	No, p=0.04	n.a.	Kruskal Wallis
Algal cell density	No, p=0.09	n.a.	Kruskal Wallis
Mitotic index	Yes, p=0.41	Yes, p=0.22	One-way ANOVA
MO_2	Yes, p=0.49	Yes, p=0.94	One-way ANOVA
CT1	No, p=0.01	n.a.	Kruskal Wallis
CT2	Yes, p=0.38	Yes, p=0.21	One-way ANOVA

3. Results

3.1 Experiment 1 - prolonged exposure

Exposure to SSA + ASW or SSI + ASW did not significantly affect any of the responses we measured in this study when compared to ASW alone (Table 2, Fig. 3).

Table 2. The average (mean) values ± the standard deviation of each treatment (ASW, SSA + ASW and 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.

Response variable	ASW	SSA	SSI	Significant difference?
Fv/Fm	0.75 ± 0.06	0.77 ± 0.05	0.78 ± 0.05	no, p = 0.48
Algal cell density	95.4 ± 1.78%	95.7 ± 3.3%	96.9 ± 2.0 %	no, p = 0.49
Mitotic index	0.69 ± 0.15	0.47 ± 0.39	0.38 ± 0.28	no, p = 0.23
MO_2	-0.01 ± 0.02	-0.0001 ± 0.003	-0.002 ± 0.005	no, p = 0.43
CT1	34.3 ± 3.09℃	35.2 ± 1.17℃	34.9 ± 2.64℃	no, p = 0.67
CT2	36.6 ± 1.67℃	35.5 ± 3.27℃	36.7 ± 4.37℃	no, p = 0.65

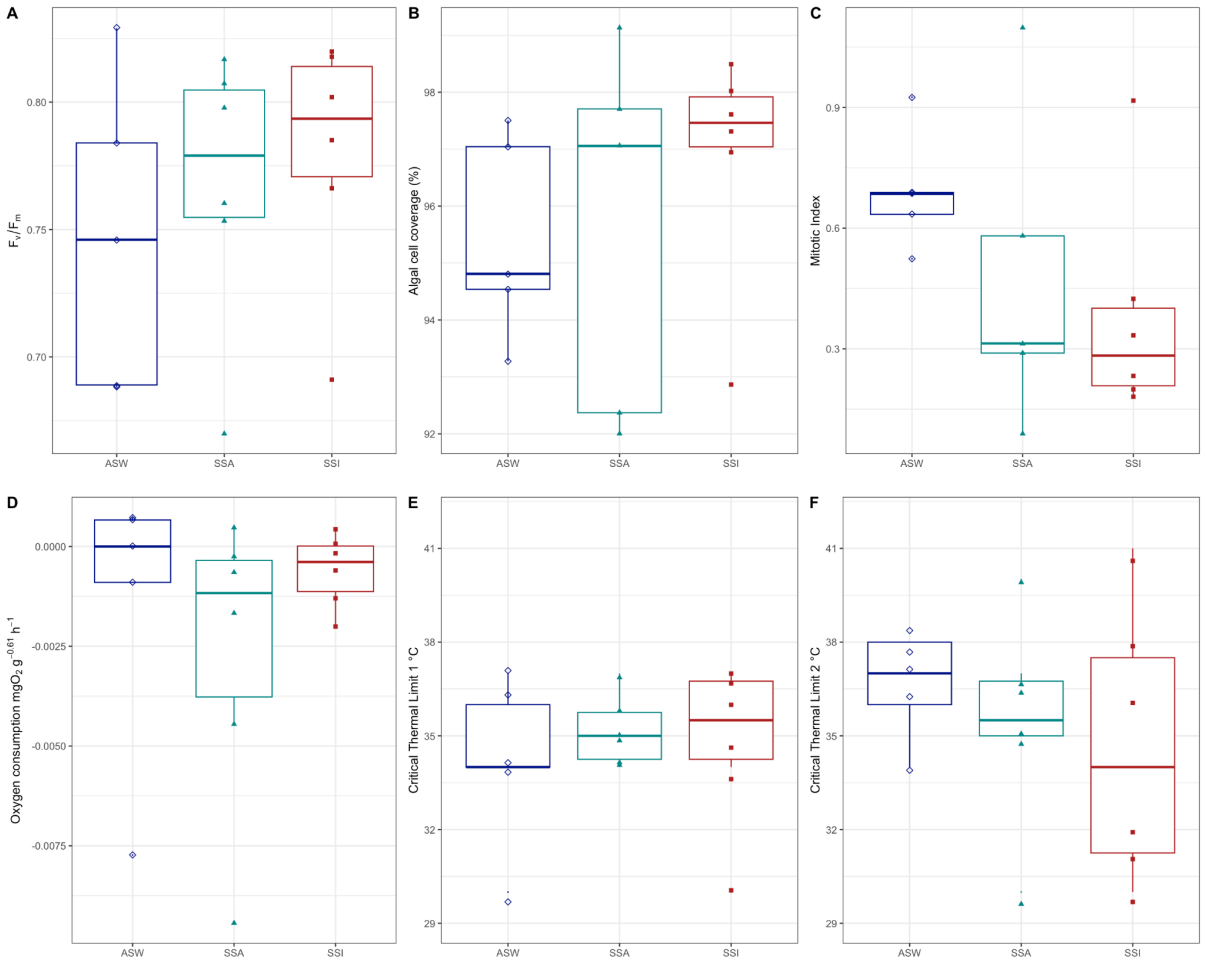

Figure 3. Each of the responses examined in Experiment 1. In all panels, the ASW treatment is depicted in blue, SSA+ASW-exposed specimens are in turquoise and SSI+ASW-exposed specimens are in red. None of these treatments differed significantly from one another when the following responses were examined, A) maximum photochemical quantum yield (F_V/F_M), B) the percentage of endoderm tissue filled with symbiotic algae, C) the mitotic index (%), D) oxygen consumption 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).

3.2 Experiment 2 - Acute toxicity of SSI and SSA

Only two specimens displayed signs of stress when we examined stress responses to increasing concentrations of sulfate or sulfite ions. One ejected acontia and detached its pedal disk when exposed to 6.65g/L (50x) of SSA, and the other detached its pedal disk and retracted its tentacles after exposure to 10x of ASW. The other specimens did not display stress behaviors at any step during this experiment. Furthermore, none of our predictors had a significant effect on the stress behaviors: treatment ($F(4,240)=0.996$, $p=0.4104$), concentration ($F(14,240)=0.999$, $p=0.4551$), and their interaction ($F(28,240)=0.999$, $p=0.4710$) .

4. 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, mitotic index (Fig. 3C) is the only response that, while statistically insignificant, appears to have a difference between SSI/SSA and ASW, which could indicate that exposure to higher concentrations of sulfur lowers the rate of symbiotic algae division, although this will require further study. In a parallel study that examined how multiple stressors affected *E. diaphana* (BAP, ZQ, EMJL unpublished results), 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 rather than exposure to the salt itself.

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. None of the SSI-exposed specimens demonstrated a stress behavior during the acute toxicity experiment, causing us to conclude that even a 70x dose (8.75 g/L) of SSI was not stressful, as long as normoxia and a constant salinity were maintained. Interestingly, the behavioral stress response, 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 & Verberk, 2024, BAP, ZQ, EMJL unpublished results). Therefore, we hypothesize that this behavior is unrelated to the experiments we conducted here, and more likely related to some other attribute of that individual.

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 & McClure, 1982). Combined, our observations lead us to conclude that SSI is a reliable and practical way to induce hypoxia in *E. diaphana*, that 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 an inexpensive, reliable and controllable 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 will be made available upon reasonable request.

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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. 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)	8.755	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