

## LETTER

# Fluctuating fortunes: Stressor synchronicity and fluctuating intensity influence biological impacts

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**Abstract**

Ecosystems remain under enormous pressure from multiple anthropogenic stressors. Manipulative experiments evaluating stressor interactions and impacts mostly apply stressors under static conditions without considering how variable stressor intensity (i.e. fluctuations) and synchronicity (i.e. timing of fluctuations) affect biological responses. We ask how variable stressor intensity and synchronicity, and interaction type, can influence how multiple stressors affect seagrass. At the highest intensities, fluctuating stressors applied asynchronously reduced seagrass biomass 36% more than for static stressors, yet no such difference occurred for photosynthetic capacity. Testing three separate hypotheses to predict underlying drivers of differences in biological responses highlighted alternative modes of action dependent on how stressors fluctuated over time. Given that environmental conditions are constantly changing, assessing static stressors may lead to inaccurate predictions of cumulative effects. Translating multiple stressor experiments to the real world, therefore, requires considering variability in stressor intensity and the synchronicity of fluctuations.

**KEYWORDS**

anthropogenic impact, asynchronous, ecological realism, experimental design, nonadditive effects, static stressors, synchronous

**INTRODUCTION**

Ecosystems around the globe are increasingly affected by human activities that introduce co-occurring stressors into the environment (Geldmann et al., 2014; Halpern et al., 2019). Multiple stressors can alter ecosystem structure and function, leading to habitat degradation and loss (Millennium Ecosystem Assessment, 2005). Recognition of unexpected impacts via synergistic or antagonistic stressor interactions ('ecological surprises'; Beauchesne et al., 2021; Crain et al., 2008; Jackson et al., 2016; Rillig et al., 2019; Stockbridge et al., 2020) has spurred research to better understand and predict multiple stressor effects (Orr et al., 2020). However, most experiments do

not capture the dynamic environmental conditions that influence stressors under natural conditions, such as variation in biological processes, temperature, precipitation patterns, freshwater flows and tidal cycles, which affect physicochemical properties and environmental conditions (Cloern & Jassby, 2012; Vase et al., 2018; Xu et al., 2020). Consequently, the intensity and synchronicity at which animals and plants are exposed to multiple stressors are rarely considered (Gunderson et al., 2016; Jackson et al., 2021).

The synchronicity and intensity of introduced stressors can influence biological responses, and thus should be accounted for when evaluating multiple stressor effects (Gunderson et al., 2016; Jackson et al., 2021). How close in

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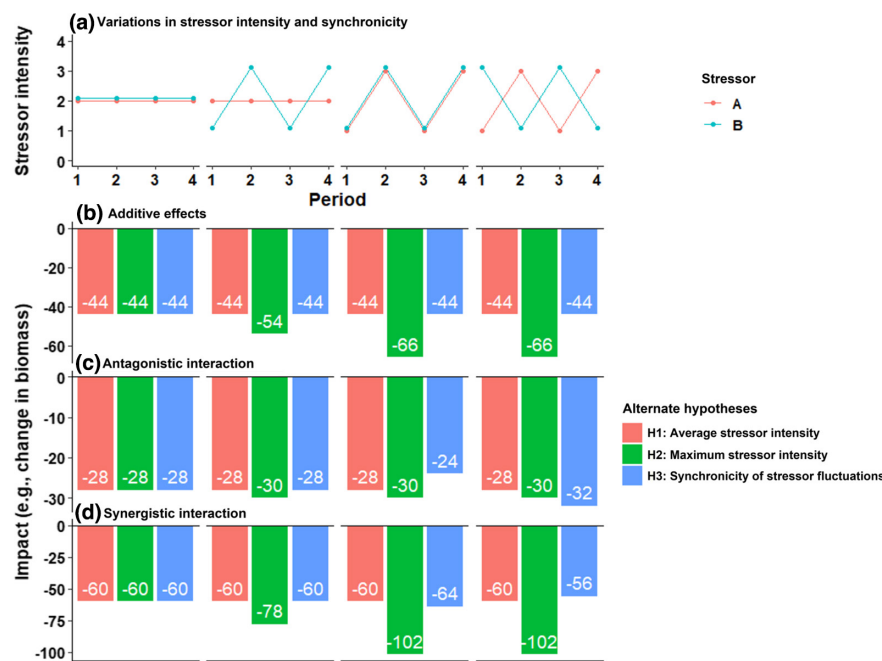
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time stressors occur can dictate whether exposure to one stressor increases (cross-tolerance) or decreases (cross-susceptibility) tolerance to subsequent stressors, which also depends on the capacity of organisms to physiologically compensate for the stress (Chen & Stillman, 2012; Gunderson et al., 2016; Todgham & Stillman, 2013). For example, a time lag between introduction of metal and biocide contamination resulted in amplified and longer-lasting effects on marine mussel assemblages compared to simultaneous introduction (Brooks & Crowe, 2019). How stressors fluctuate through time may also influence responses via relationships with the type of stressor interaction that exists (i.e. antagonistic, additive, synergistic; Gunderson et al., 2016). Fluctuations in stressor intensity can provide physiological refuge or make physiological compensation more challenging when exposed to additional stressors, depending on how they overlap in time (Clark & Gobler, 2016; Cross et al., 2019; Gunderson et al., 2016). Larval bivalves exposed to synchronous fluctuations of acidification and low dissolved oxygen, for example, had lower survival than larvae exposed to static (i.e. constant) stressors (Gobler et al., 2017).

Previous findings suggest that multiple stressor effects may vary in different ecological contexts, which has implications for how potential impacts to ecosystems are inferred. Therefore, consideration of variable stressor intensity (i.e. fluctuations) and synchronicity (i.e. timing

of fluctuations) within manipulative experiments should be common practice (Gunderson et al., 2016; Jackson et al., 2021; Przeslawski et al., 2015). However, few multiple stressor studies incorporate fluctuating stressor intensities or apply multiple stressors both synchronously and asynchronously (Ostrowski et al., 2021). Where responses to multiple stressors are shown to depend on variability in intensity and synchronicity, the ecological relevance and inferences drawn from some previous works using only static stressors may not provide a true understanding of multiple stressor effects in the environment.

Here, we determined whether variability in stressor intensity and the synchronicity of fluctuations influence responses to multiple stressors. We apply the stressors of reduced light and herbicide contamination to seagrass using five variations in stressor intensity and synchronicity (Figure 1A). Seagrasses support diverse wildlife (Sievers et al., 2019; Unsworth et al., 2019), sequester carbon (Fourqurean et al., 2012), improve water quality (de los Santos et al., 2020), and protect coastlines (Duarte et al., 2013), but are among the most threatened ecosystems (Halpern et al., 2019). Seagrasses are highly sensitive to disturbance from poor water quality (Turschwell et al., 2021) and are experiencing global declines in extent (Dunic et al., 2021), making them a model case study for this experiment.



**FIGURE 1** The variations in stressor intensity and synchronicity tested (A; from left to right): static-static, static-fluctuating or fluctuating-static, in-phase and out-of-phase. A conceptual framework for how variable stressor intensity and synchronicity can influence effects of multiple stressors, depending on whether the stressors have additive effects (B), or interact antagonistically (C) or synergistically (D). Impact of effects (e.g. change in biomass) is estimated for three alternate hypotheses (H1, H2, H3) based on fixed values for stressor A effects (-3), stressor B effects (-2.5) and the interactive effects (1, 0 and -1 for antagonistic, additive and synergistic, respectively). H1 and H2 assume variable stressor intensity and synchronicity do not matter, but rather the average or maximum stressor intensity, respectively, best predicts effects. H3 assumes variable stressor intensity and synchronicity do matter. This model also assumes stressors are always present (i.e. overlap). See Figure S1 for output when 'low' stressor intensities reach zero (i.e. periods without stressor overlap). Code used to model and plot impact is available at <https://github.com/ostro2al/fluctuating-multi-stressors>.

We first develop a conceptual framework of how variable stressor intensity and synchronicity might influence responses differently, as a function of interaction type. We then empirically test this conceptual model and use generalised additive models to test three alternative hypotheses regarding the mechanisms underpinning differences in biological responses to stressors. Finally, we use model predictions to interpret the type of stressor interaction (i.e. antagonistic, additive and synergistic) across treatments. Our results have implications for how multiple stressor experiments can be designed and conducted for more effective outcomes, and for how experimental conclusions are used to predict multiple stressor effects to manage ecosystems.

## MATERIALS AND METHODS

We developed a conceptual model to explain how variable stressor intensity and synchronicity can lead to differences in biological responses, depending on interaction type. We then tested our conceptual model experimentally by manipulating our applied stressors. We measured the effects of reduced light and herbicide contamination, and variable stressor intensity and synchronicity on seagrass growth and physiology. Finally, we developed a series of statistical models to test three hypotheses to explain how variable stressor intensity and synchronicity can influence biological responses to multiple stressors.

### Conceptual model of how stressor interactions can influence the effect of variable stressor intensity and synchronicity

We hypothesised that stressor interaction type and variable stressor intensity and synchronicity influence net biological responses to multiple stressors (Figure 1B–D, H3). Below we refer to the ‘net biological response’ as the outcome of the biological response at the end of an experimental treatment (i.e. individual or combined stressor treatment plus variable stressor intensity and synchronicity), the ‘effect’ as the effect size of a main effect or interactive effect, and the ‘impact’ as the biological response to a stressor combination (i.e. the effect times the stressor intensity relative to control outcome). When fluctuating stressors do not overlap in time, we predict there will be no stressor interactions. If there is an additive effect between two stressors, we predict the net biological response will be the same under static conditions or any variation in stressor intensity and synchronicity (Figure 1B). Where stressors interact antagonistically, synchronous fluctuations will result in greater mitigation of the main stressor effects, thereby reducing the total impact on the net biological response. Conversely, asynchronous fluctuations will

reduce the antagonistic effect, so the total impact will be greater than that of static stressors and synchronous fluctuations (Figure 1C). Finally, where stressors interact synergistically, we hypothesise that synchronous fluctuations will have greater effects compared to static application because the synergistic impact will be amplified at higher stressor intensities. Conversely, asynchronous fluctuations will mitigate the impacts of synergistic stressors (Figure 1D). We also hypothesise impacts across each stressor interaction type if variable stressor intensity and synchronicity do not matter, but rather the average or maximum stressor intensity affect net biological responses (Figure 1, H1 and H2, respectively).

### Variable stressor intensity and synchronicity

We evaluated the effects of variable stressor intensity and synchronicity on biological responses to multiple stressors. We tested reduced light and contamination with the photosystem II-inhibiting herbicide, diuron, across five variations in stressor intensity and synchronicity for a total of 29 treatments (Table S1). Application of stressors consisted of the ‘standard’ *static-static* where both stressors remained static throughout the experiment, *static-fluctuating* where light levels remained static while herbicide levels fluctuated every 48 h, *fluctuating-static* where light levels fluctuated every 48 h while herbicide levels remained static, *in-phase* where light and herbicide levels fluctuated every 48 h synchronously, and *out-of-phase* where light and herbicide levels fluctuated every 48 h asynchronously (Figure 1A).

### Experimental design

Seagrasses were exposed to reduced light and herbicide contamination in the laboratory. Seagrass plants (*Halophila ovalis*) collected from Moreton Bay, Queensland, Australia (−27.495590, 153.400453) were acclimated in the lab (24°C, 12 h light:12 h dark photoperiod) for 7 days. We then exposed replicate plants (5.22 ± 1.21 leaf pairs; mean ± SE) in individual 15 × 15 cm microcosms to environmentally relevant reduced light levels and herbicide contamination. We targeted light levels of 75% (‘medium light’; 90 μmol photons m<sup>−2</sup> s<sup>−1</sup>) and 25% (‘low light’; 30 μmol photons m<sup>−2</sup> s<sup>−1</sup>) of control light levels (measured mean ± SD; 84.3 ± 3.47 and 26.4 ± 3.91 μmol photons m<sup>−2</sup> s<sup>−1</sup>, respectively; LI-COR light meter; LI-250A), and 1 and 2 μg L<sup>−1</sup> of herbicide (mean ± SE; 0.89 ± 0.05 and 1.69 ± 0.09 μg L<sup>−1</sup>; ‘medium’ and ‘high’ herbicide, respectively). The selected concentrations of herbicide fall within the range of reported levels present in polluted estuaries in Queensland, Australia (e.g. Brodie &

Landos, 2019; Lewis et al., 2009). We achieved target light levels by placing individual microcosms at different distances from overhead LED lights. For detailed methodology on seagrass sample collection and herbicide stock solution preparation, refer to [Appendix A](#).

For static treatments, we drained and renewed 100% of the water in each microcosm every 48 h to maintain constant target herbicide concentrations while light conditions remained unchanged throughout the 7-day exposure. For fluctuating treatments, we applied herbicides as pulse inputs by renewing the water in each microcosm to target concentrations every 48 h. To achieve fluctuating  $1 \mu\text{g L}^{-1}$  herbicide, renewal of artificial seawater alternated from  $2 \mu\text{g L}^{-1}$  to  $0 \mu\text{g L}^{-1}$  herbicide treatments every 48 h. To achieve fluctuating  $2 \mu\text{g L}^{-1}$  herbicide, renewal of stock water alternated from  $3 \mu\text{g L}^{-1}$  to  $1 \mu\text{g L}^{-1}$  herbicide treatment every 48 h. All treatments, including the procedural control, were drained and renewed every 48 h. Additionally, to achieve fluctuating 75% light availability, light levels alternated from full light (100% control light) to 50% of control light conditions every 48 h. To achieve fluctuating 25% light availability, light levels alternated from 50% of control light to no light (0%; impermeable tarpaulin) every 48 h. Alternating between higher and lower than target stressor levels every 48 h ensured the mean herbicide concentration and mean light level per day across the experiment were the same between static and fluctuating treatments. Treatments where stressors fluctuate in-phase consisted of herbicide contamination (i.e. the 48 h following addition of highest herbicide concentration) paired with decreased light availability (i.e. the 48 h following greatest light reduction). Conversely, stressors fluctuating out-of-phase consisted of the less stressed condition of a single stressor (e.g. 100% control light) paired with the higher stressed condition of the second stressor (e.g.  $2 \mu\text{g L}^{-1}$  herbicide). Note 'fluctuating' control treatments are not possible (i.e. fluctuating  $0 \mu\text{g L}^{-1}$  herbicide or 100% light) and were not tested. All treatments were randomised and run in triplicate weekly. Trials were repeated across four periods for a total of 12 replicates per treatment (348 individual plants).

To demonstrate seagrass under no stress remained healthy, we included a procedural control exposed to full light (100% of control light = target  $120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ; measured mean  $\pm$  SD =  $119.4 \pm 3.26 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and no herbicide. These plants did not lose biomass over the 7-day experimental period ( $N = 12$ ). Photosynthetic capacity decreased during the first 48 h of the experiment, likely due to stress following transplant into microcosms, then remained constant for the duration of the experiment ( $\geq 0.6$ ; dimensionless). Given we were interested in comparing applications of stressors under conditions of variable intensity and synchronicity relative to the current standard (i.e. static–static), the procedural control was not included in the statistical analysis.

## Response variables

### Biomass

Initial biomass (g fresh weight) of each plant was recorded on day 0, prior to transplant into microcosms, and compared to final biomass measured on day 7 to quantify proportional change (i.e.  $[\text{final} - \text{initial}]/\text{initial}$ ).

### Photosynthetic capacity

Photosynthetic capacity was indicated by effective quantum yield, measured as chlorophyll *a* fluorescence, using a pulse amplitude-modulated fluorometer (Mini-PAM-II; Walz GmbH, Germany). We recorded measurements on light-adapted leaves on day 0 (prior to stressor exposure), during water changes on days 2, 4 and 6, and at the end of the experiment on day 7. For details on photosynthetic capacity measurements, refer to [Appendix A](#).

## Statistical analysis

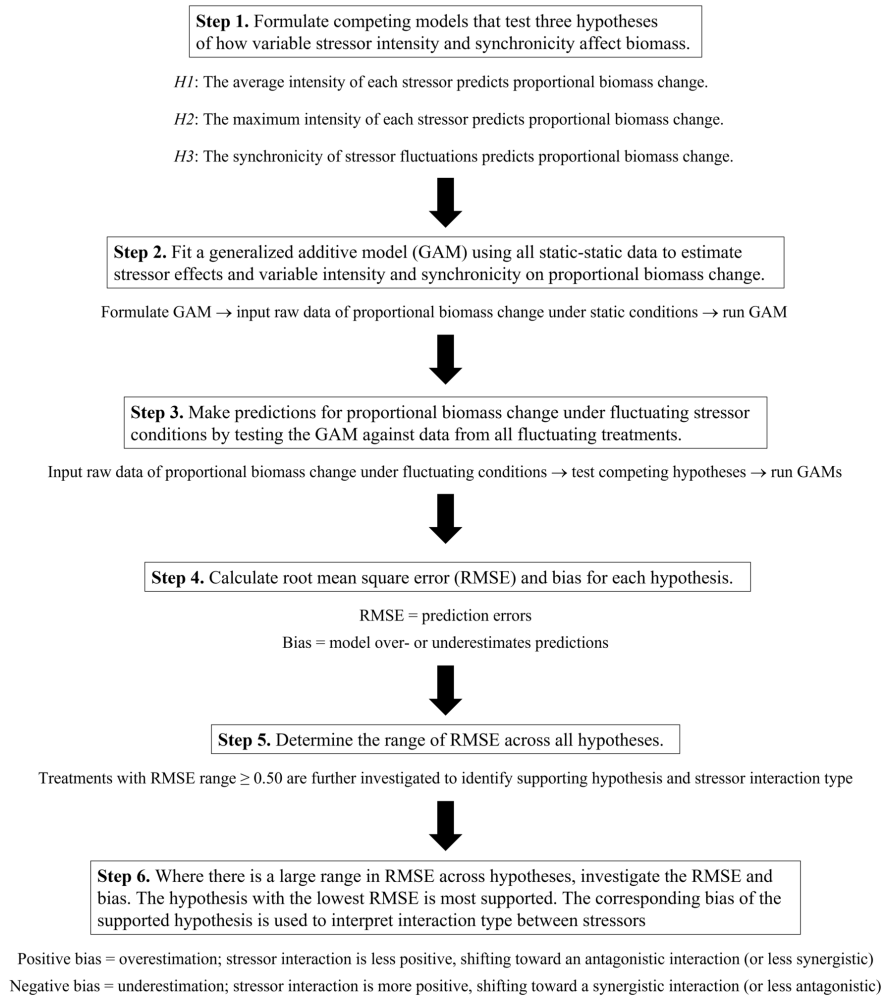
Our statistical analysis was conducted in two stages. We first analysed the effects of reduced light (medium, low), herbicide contamination (medium, high) and how stressor intensity and synchronicity vary over time (hereafter variable stressor intensity and synchronicity), on biomass and photosynthetic capacity using linear mixed-effects models. We then tested the conceptual model ([Figure 1B–D](#), H3), using only biomass data, by formalising it as a mathematical model and testing it against two competing models, which assumed stressor fluctuations do not matter ([Figure 1B–D](#), H1 and H2; [Figure 2](#)). We tested these hypotheses by parameterising the models with data from the static treatments, and then validating the predictions against data from the fluctuating treatments.

A fully crossed subset of the dataset that excluded treatment combinations where only one stressor was present (20 of the 29 combinations) was used to estimate the effects of variable stressor intensity and synchronicity on responses after confirming normality (Q–Q plot) and homogeneity of variance (residual plot). Because static stressor application is the standard method used in multiple stressor experiments, we made all comparisons to treatments in the static–static group. All statistical analyses were performed using R (version 4.1.2) and packages *lme4* (Bates, 2010) and *mgcv* (Wood, 2006).

### Biomass

We used a linear mixed-effects model to make predictions of the individual and interactive effects of variable





**FIGURE 2** Multi-step process to validate experimental results of proportional biomass change and interrogate how and why variable stressor intensity and synchronicity might influence net biological responses to multiple stressors.

stressor intensity and synchronicity (i.e. “stressor application” in model), reduced light and herbicide contamination, with a random effect of week, on proportional biomass change:

$$\text{Proportional biomass} \sim \text{Stressor application}^* \\ \text{Light level}^* \text{ Herbicide level} + (1|\text{Week}). \quad (1)$$

### Photosynthetic capacity

We analysed photosynthetic capacity (box-cox transformation;  $\lambda = 2$ ) as change over time relative to initial measurements taken prior to stressor exposure. We developed a series of models with different combinations of fixed (variable stressor intensity and synchronicity, reduced light, herbicide contamination and time) and random (plant ID) variables (and interaction terms) and used the Akaike information criterion (AIC) to select the best model for our data (Table S2). We used the following linear mixed-effects model to make predictions for changes in photosynthetic capacity:

$$\text{Photosynthetic capacity} \sim \text{Stressor application} + \text{Day} \\ + \text{Light level}^* \text{ Herbicide level} + (1|\text{ID}) + \text{offset}(\text{Day}0). \quad (2)$$

### Testing the conceptual model through validation of models against data

To test our conceptual model, we analysed proportional biomass change because we observed different effects of variable stressor intensity and synchronicity on this response.

We tested the following hypotheses (Figure 2, Step 1):

*H1:* The average intensity of each stressor during the experiment predicts proportional biomass change. This hypothesis equates to assuming stressor fluctuations do not matter, and only the average intensity is important (Figure 1B–D, H1):

$$y_T = f_1(\bar{H}) + f_2(\bar{L}) + f_3(\bar{H}, \bar{L}) \quad (3)$$

where  $y_T$  = proportional biomass change;  $f_1, f_2, f_3$  = effects of herbicide, light and their interaction on proportional biomass;  $\bar{H}$  = average herbicide level for a given treatment;  $\bar{L}$  = average light level for a given treatment.

**H2:** The maximum intensity of the stressor during the experiment predicts proportional biomass change. This hypothesis equates to assuming stressor fluctuations do not matter, but that the maximum intensity is important (Figure 1B–D, H2):

$$y_T = f_1(\max(H)) + f_2(\max(L)) + f_3(\max(H), \max(L)) \quad (4)$$

where  $y_T$  = proportional biomass change;  $f_1, f_2, f_3$  = effects of herbicide, light and their interaction on proportional biomass;  $\max()$  is the maximum function.

**H3:** The synchronicity of stressor fluctuations (i.e. in- and out-of-phase) predicts proportional biomass change. This hypothesis is a formalisation of our conceptual model (Figure 1B–D, H3):

$$y_T = \frac{f_1(H_a) + f_2(L_a) + f_3(H_a, L_a)}{2} + \frac{f_1(H_b) + f_2(L_b) + f_3(H_b, L_b)}{2} \quad (5)$$

where  $y_T$  = proportional biomass change;  $f_1, f_2, f_3$  = effects of herbicide, light and their interaction on proportional biomass;  $H_{a,b}$  and  $L_{a,b}$  = herbicide and light levels at each time interval when stressors were applied throughout the experiment. Note there are four intervals total, two where the highest stressor intensity was applied and two where the lowest stressor intensity was applied (i.e. fluctuating treatments alternated between high- and low-level intensities every 48 h for 7 days).

To test these models, we first fitted a generalised additive model for proportional biomass change, estimating the individual and interactive effects of light reduction and herbicide contamination under static intensities (Figure 2, Step 2):

$$\begin{aligned} \text{Model: Proportional biomass} & \sim \text{ti(Light level, k = 3, fx = TRUE)} \\ & + \text{ti(Herbicide level, k = 3, fx = TRUE)} \\ & + \text{ti(Light level, Herbicide level, k = 3, fx = TRUE)}. \end{aligned} \quad (6)$$

We then used this model to make predictions for biomass change under the three hypotheses for the fluctuating stressor treatments (Figure 2, Step 3). We calculated root mean square error (RMSE) and mean absolute bias statistics for each model to determine which hypothesis is best supported for each treatment, providing an explanation for underlying reasons variable stressor intensity and synchronicity affect responses differently (Figure 2, Step 4; data from all 29 treatments used in analysis).

## Interpreting stressor interactions

We used the results of the model validation to interpret stressor interactions for each treatment. We first identified the stressor interactions across treatments under static conditions using the coefficient estimate of the interaction terms from the static-static model (Table S3). The interaction type under static conditions was included in the model used to predict outcomes under the three hypotheses for fluctuating conditions. We interpreted these predictions with RMSE and bias values (Figure 2, Steps 5 and 6). The RMSE indicated the prediction errors and therefore, which model made the most accurate predictions (i.e. combined lowest bias and variance). The bias statistic indicated the tendency of the model to over- or underestimate experimental outcomes. A similar RMSE across all three hypotheses would occur when there was no stressor interaction in static treatments because all three models make the same prediction when there is an additive effect (Table 1). The hypothesis with the lowest RMSE was most supported, and the corresponding bias was used to interpret whether the stressor interaction in the fluctuating treatments was different to the interaction in static treatments. If the bias was negative, the model underestimated biomass change (i.e. effect was greater than expected), suggesting the interaction between herbicide and light becomes more synergistic (i.e. less antagonistic or stronger synergy). If bias was positive, the model overestimated biomass change (i.e. effect was less than expected), suggesting the interaction becomes more antagonistic (i.e. weaker synergy or stronger antagonism; Figure 2, Step 6).

## RESULTS

### How do variable stressor intensity and synchronicity influence biological responses?

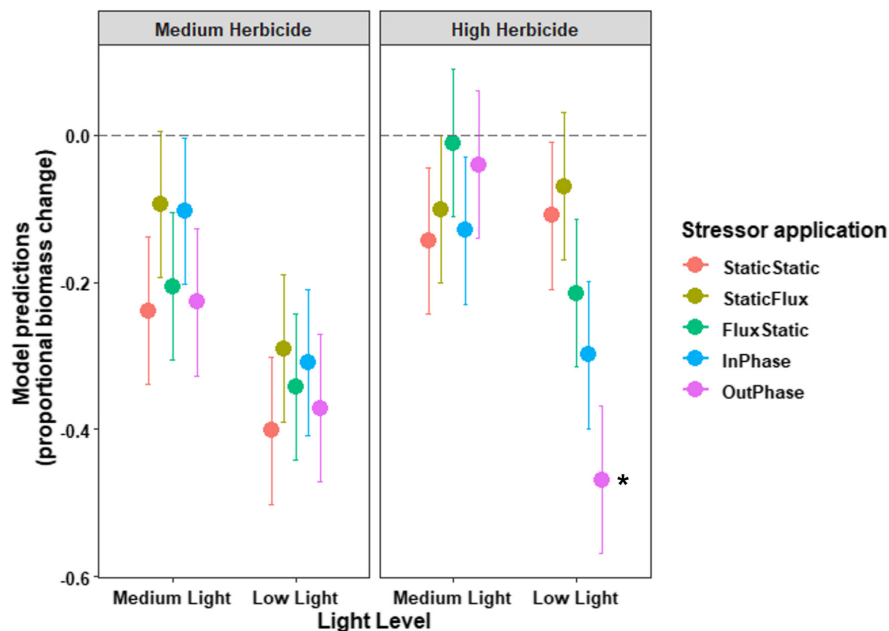
#### Proportional biomass

Seagrass biomass was reduced in all stressor treatments, and there were significant independent effects of light level ( $p < 0.001$ ) and herbicide level ( $p = 0.017$ ) on biomass. At the highest stressor intensities (i.e. low light paired with high herbicide), biomass change differed across the variations in stressor intensity and synchronicity. The out-of-phase application reduced biomass more than static-static application ( $p = 0.007$ ). In-phase application also caused greater biomass loss than the static-static method under the same treatment combination, although the difference was not statistically significant ( $p = 0.151$ ; Figure 3). Across other stressor intensities, variable intensity and synchronicity did not influence biomass.

**TABLE 1** Validation of models indicating root mean square error (RMSE) and bias for three alternate hypotheses explaining how variable stressor intensity and synchronicity might affect proportional biomass change

Stressor application	Light level	Herbicide level				RMSE range	H1 bias	H2 bias	H3 bias
			H1 RMSE	H2 RMSE	H3 RMSE				
Flux-Static	Medium	Control	0.31	0.31	0.33	0.02	0.06	0.01	0.11
	Low	Control	0.35	0.37	0.35	0.02	0.16	0.20	0.17
	Medium	Medium	0.37	0.38	0.37	0.01	-0.03	-0.09	-0.08
	Low	Medium	0.33	0.37	0.33	0.04	-0.06	-0.18	-0.06
	Medium	High	0.32	0.32	0.30	0.02	-0.13	-0.14	-0.10
	Low	High	0.28	0.30	0.28	0.02	0.11	0.15	0.11
Static-Flux	Control	Medium	0.34	0.39	0.41	0.07	-0.02	0.19	0.23
	Medium	Medium	0.38	0.36	0.36	0.02	-0.14	-0.05	-0.05
	Low	Medium	0.32	0.35	0.33	0.03	-0.11	0.18	0.15
	Control	High	0.58	0.72	0.60	0.14	0.11	0.43	0.17
	Medium	High	0.31	0.32	0.31	0.01	-0.04	0.10	-0.02
	Low	High	0.21	0.43	0.21	0.22	-0.04	0.38	0.03
In-Phase	Medium	Medium	0.68	0.67	0.67	0.01	-0.14	-0.05	0.03
	Low	Medium	0.32	0.39	0.35	0.07	-0.09	0.25	0.18
	Medium	High	0.20	0.27	0.20	0.07	-0.01	0.18	0.02
Out-Phase	Low	High	<b>0.24</b>	0.92	0.48	0.68	0.19	0.91	0.46
	Medium	Medium	0.18	0.19	0.20	0.02	-0.01	0.08	0.09
	Low	Medium	0.39	0.50	0.45	0.11	-0.03	0.31	0.23
	Medium	High	0.25	0.24	0.23	0.02	-0.10	0.09	0.02
	Low	High	0.48	1.10	<b>0.40</b>	0.70	0.36	1.10	0.23

Note: A similar RMSE across hypotheses indicates no stressor interaction (i.e. *additive*). For stressor combinations where the RMSE range was  $\geq 0.50$  (highlighted green), we further investigated the results. The hypothesis with the lowest RMSE was most supported (bold values), and the bias for that hypothesis was used for the interpretation of stressor interactions (highlighted grey). If bias was negative, the model underestimated biomass change, suggesting the interaction between stressors became more *synergistic*. If bias was positive, the model overestimated biomass change, suggesting the interaction became more *antagonistic*.

**FIGURE 3** Biomass change. Model predictions for mean ( $\pm$ SE) proportional biomass change for four stressor treatment combinations tested across the five variations in stressor intensity and synchronicity. The dashed line indicates no change from initial biomass prior to stressor exposure. Asterisk indicates a significant effect of variable stressor intensity and synchronicity relative to static-static ( $p < 0.05$ ). Each treatment combination was replicated 12 times.

## Photosynthetic capacity

There were significant independent effects of day ( $p < 0.001$ ), light level ( $p = 0.004$ ) and herbicide level ( $p = 0.011$ ) on photosynthetic capacity. However, there were no differences in photosynthetic capacity across the variations in stressor intensity and synchronicity. Photosynthetic capacity gradually decreased over time in all treatments, with the greatest reduction observed on the final day of the experiment. Although not statistically significant, in-phase application appeared to have a greater effect ( $p = 0.073$ ; Figure 4).

## Identifying how variable stressor intensity and synchronicity affect biomass

Validation of the models indicated which of the three competing hypotheses best-predicted the effect of each treatment on proportional biomass change. The treatment combination with highest stressor intensities showed the greatest range of RMSE values across the different hypotheses (Table 1), so we focus discussion on this treatment combination. The model for low light paired with high herbicide treatment under static conditions predicted no significant stressor interaction (Table S3). Recall that if there are no stressor interactions, there are no differences in the predictions of the different hypotheses (Figure 1B; additive). However, the coefficient estimate was positive ( $=0.098$ ), suggesting a weak antagonism. This antagonistic effect was

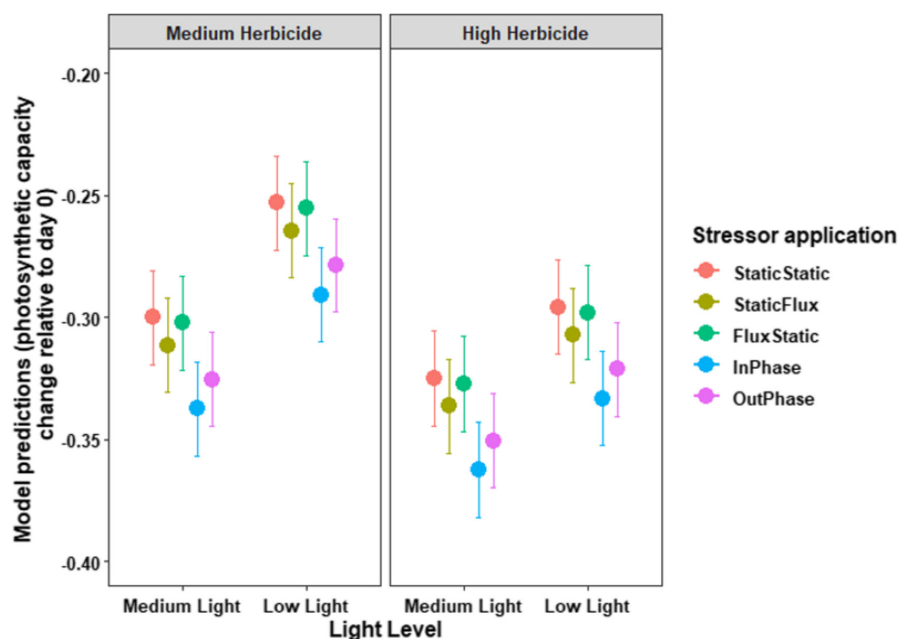
carried through to the predictions we made under fluctuating stressor conditions and could indicate changes in stressor interactions. We observed that the average stressor intensity (H1) best predicted the combined effect of high diuron and low light on biomass under the in-phase treatment (Table 1). For out-of-phase application, synchronicity of stressor introduction (H3) best predicted the biomass loss (Table 1). The corresponding bias statistic for each supported hypothesis for in- and out-of-phase treatments indicated that the model overestimated biomass change, suggesting that the interaction between low light and high herbicide became more antagonistic than in the static-static treatment, supporting the coefficient estimate (Table 1; grey highlighted cells). The bias statistic was high under these two treatments, indicating stronger stressor interactions than those observed under static conditions.

## DISCUSSION

### Effects of stressor intensity and synchronicity, and stressor interaction type on biological responses

#### Stressor intensity

We found that stressor interactions varied with mean stressor intensity, and variation in stressor intensities and synchronicities. The interaction between light and herbicide at the highest stressor intensities for biomass



**FIGURE 4** Photosynthetic capacity. Model predictions for mean ( $\pm$ SE) final change in photosynthetic capacity for four stressor treatment combinations tested across the five variations in stressor intensity and synchronicity. The results are relative change from initial measurement taken prior to stressor exposure. All comparisons are made relative to the static-static method. There is no significant effect of variable stressor intensity and synchronicity across treatments. Each treatment combination was replicated 12 times.



leans towards an antagonism when introduced in- and out-of-phase. High bias statistics for both in-and out-of-phase treatments also suggested the potential for stronger stressor interactions at higher intensities, a finding consistent with previous theoretical predictions and empirical experiments (King et al., 2022; Turschwell et al., 2022). For example, a stronger synergistic effect of reduced light and herbicide contamination on algal growth inhibition was observed at the highest stressor intensities (King et al., 2022). Therefore, we suggest that extreme synergisms and antagonisms can occur at higher stressor intensities, amplifying or reducing the impacts of in- and out-of-phase fluctuations than those hypothesised in our conceptual model.

### Out-of-phase effects

Our hypothesis that out-of-phase application will have amplified impacts compared to static conditions when there is an antagonistic interaction was supported, but only at high stressor intensities where a stronger interaction was observed. At lower intensities, there was no overlap of stressors (i.e. stressors not simultaneously present) and therefore no interactions. This resulted in no differences in biomass loss compared to static application, further supporting our initial hypothesis. At the highest intensities, the synchronicity of stressor fluctuations (H3) was important and best predicted biomass loss. Synchronicity is important because the antagonistic effect was dampened when introduced out-of-phase, resulting in a greater impact on the net biological response. Additionally, shorter duration between periods of stressor introduction under fluctuating treatments (e.g. intensities alternated every 48 h in this study) are likely to result in interactive effects, and at the highest stressor intensities there was a constant overlap of stressors when introduced out-of-phase, which could also explain the stronger interactions observed at higher intensities (Brooks & Crowe, 2019; Gunderson et al., 2016).

### In-phase effects

We observed that in-phase application could lead to a greater reduction of biomass than static application at the highest stressor intensities; the opposite of what we hypothesised for an antagonistic interaction if stressor synchronicity affects responses. The average concentration of stressors (H1) was instead the best predictor of biomass loss. Synchronicity might not matter here because of possible legacy effects, whereby initial exposure to stress continues to affect the seagrass irrespective of subsequent exposure frequencies or intensities (e.g. Ortiz et al., 2018; Pereda et al., 2019). Legacy effects are likely to occur when the recovery of biota takes longer relative to the timing between stressor introductions and

can influence future responses to additional stressors (Hughes et al., 2019; Ogle et al., 2015; Ryo et al., 2019). If legacy effects carry over into a period of no (or low) stress, we might see stronger stressor interactions and, thus, amplified or reduced impacts on net biological responses, which is consistent with the higher bias statistic for in-and out-of-phase applications at the highest intensities.

### Changes over time

Stressor interactions might change over time (Côté et al., 2016; Turschwell et al., 2022), which could lead to or mask differential responses to variable stressor intensity and synchronicity. As stressor intensity increases, physiological thresholds are often exceeded, resulting in increased energy required to compensate for stress, which can ultimately influence net biological impacts over time (Gunderson et al., 2016; Sokolova et al., 2012). Additionally, time lags between stressor presence may cause decoupled stressor effects if organisms can physiologically compensate for stress before additional exposure (e.g. Bible et al., 2017). Conversely, shorter lags may result in stressor interactions and latent effects (Cheng et al., 2015; Gunderson et al., 2016). Here, stressor fluctuations varied every 48 h, which might have prevented physiological refuge or impaired mechanisms of physiological compensation, thus amplifying stressor impacts under fluctuating conditions. If we applied fluctuating stressors further apart in time, however, we might expect to see additive effects. Ultimately, evaluating stressor interaction types across responses, time scales and variable stressor intensities and synchronicities can help us identify how stressor interactions and net biological responses vary across ecological contexts.

### Identifying underlying physiological mechanisms of multiple stressors and influences on responses

Fluctuating stressor intensity and synchronicity may affect compensatory physiological processes that mitigate stress events differently, depending on the intensity of fluctuations and how stressors overlap in time (Clark & Gobler, 2016; Cross et al., 2019). For example, stressors applied at high fluctuating intensities are more likely to overwhelm physiological responses and result in synergistic effects on net biological responses (Gunderson et al., 2016), which is supported by our findings for seagrass biomass loss. Under high-intensity fluctuating conditions, we exposed seagrass to periods where stressor intensity exceeded those under static conditions to achieve the same overall average intensity. Further, seagrass in this treatment was consistently exposed to stress (i.e. no periods where stressor levels reached zero).

These factors could further explain amplified impacts on biomass for in- and out-of-phase fluctuations.

Identifying the mechanisms by which an organism responds to stressors, and how similar the processes are, can help detect and predict novel stressor outcomes (Orr, Rillig, & Jackson, 2022). Multiple stressors may elicit a shared response meaning similar cellular pathways and physiological processes are triggered to mitigate effects, where overlap in responses may enhance or reduce tolerance to stressor exposure (Gunderson et al., 2016; Pandey et al., 2015; Todgham & Stillman, 2013). Light reduction and photosystem II-inhibiting herbicides affect photosynthetic capacity by impacting electron transport in the photosystem II (PSII) complex, however, the mode of action of each stressor is slightly different (Ahrens, 1994; Campbell et al., 2003; Oettmeier, 1992). Recent predictions suggest reduced light and PSII-inhibiting herbicides will have an antagonistic effect on seagrass photosynthetic capacity but an additive or synergistic effect on biomass (King et al., 2021). In our experiment, photosynthetic capacity was adversely affected by reduced light and herbicide contamination but did not differ across variable stressor intensities and synchronicities, which, based on our conceptual model, would suggest an additive effect (i.e. no stressor interaction). However, photosynthetic impacts increase at higher stressor intensities (e.g. Du et al., 2021; King et al., 2022), and in this study, critical thresholds might have been surpassed and mechanisms for physiological compensation overwhelmed, resulting in no differences across variable intensities and synchronicities. Additionally, differences in effects could be masked due to impacts on physiological endpoints not measured here that can affect photosynthetic capacity (e.g. reactive oxygen species and photosynthetic pigments), or because of different modes of action of each stressor (Gunderson et al., 2016; Sinclair et al., 2013). Overall, enhanced mechanistic understanding of stressor impacts at the physiological and individual levels can allow us to scale up to responses at higher levels of biological organisation and apply experimental conclusions across different ecological contexts for more effective monitoring and management strategies (Griffen et al., 2016; Turschwell et al., 2022).

## Management implications

Fluctuating environmental conditions, driven by both natural and anthropogenic factors, influence the presence of stressors and their impacts on ecosystems over time, and affects our ability to accurately predict stressor interaction types and net biological responses (Jackson et al., 2021; Ryo et al., 2019). Current experimental design for multiple stressor experiments rarely considers the complexity of dynamic physicochemical environments, potentially leading to inaccurate predictions of stressor effects and ineffective management (Sabater et al., 2021).

Identifying stressor interactions and how they change over time and across ecological contexts can influence management decisions aimed at controlling or modifying target stressors (Orr, Luijckx, et al., 2022). Given that stressor intensity and synchronicity can alter key metrics used in management decisions (e.g. habitat loss), greater consideration of spatial and temporal variability in stressor presence should be incorporated into ecosystem management. Manipulative studies conducted over longer timescales, as well as field experiments conducted *in situ* to evaluate effects of variable stressor intensity and synchronicity across higher levels of biological organisation (e.g. population, community and ecosystem processes), might prove useful in informing ecosystem management.

## Future directions

Although our experiment and model validation provide insight into how variable stressor intensity and synchronicity can influence biological responses, further research is needed to improve the models and test additional hypotheses. For example, continuous monitoring of biomass change and measuring additional physiological endpoints that can have flow-on effects at the individual response level (e.g. growth) may provide additional insight into how variable stressor intensity and synchronicity influence net biological responses. Additionally, incorporating more environmentally relevant stressor fluctuations, and testing various time lag durations between stressor introductions (e.g. Bible et al., 2017; Brooks & Crowe, 2019) and the order of consecutive stressor introductions (e.g. Ashauer et al., 2017; Pallarés et al., 2017) could help further elucidate how environmental variability influences stressor interactions and impacts. It is also possible that organisms are more resilient to, and readily initiate compensatory physiological responses, to predictable stressor fluctuations (e.g. seasonal changes) than to unexpected environmental changes. It would be useful to test whether a lack of predictability in fluctuations due to anthropogenic activities makes acclimation to changing environmental conditions more challenging. Measuring responses over longer durations can also increase ecological relevance of findings and provide insight on temporal variation of interactive effects if stressor interactions and compensatory physiological responses change over time (Turschwell et al., 2022).

## CONCLUSIONS

Variable stressor intensity and synchronicity due to dynamic physicochemical environments mean stressors rarely remain at static intensities for prolonged periods in aquatic ecosystems. However, this is how most multiple stressor experiments apply stressors, which

could lead to inaccurate conclusions and predictions of stressor effects. Here, variable stressor intensity and synchronicity led to differences in seagrass responses, with the strength of this effect dependent on the response variable measured, the intensity of stressors and the type of stressor interaction. Fluctuating, high-intensity stressors had greater effects on seagrass biomass loss than the equivalent intensity static stressor treatment, but no such differences were observed for photosynthetic capacity. Our study highlights the importance of considering environmental variability across ecologically relevant spatiotemporal scales, and how this might influence stressor intensity, synchronicity and interactions. Our results have implications for the design of multiple stressor experiments, and how past results are used to predict future responses. Ultimately, incorporating variability in stressor intensity and synchronicity in future experimental designs will aid in more accurately predicting stressor outcomes, and better informing the development of management strategies to effectively mitigate detrimental stressor impacts on ecosystems.

#### AUTHORS CONTRIBUTIONS

Andria Ostrowski, Rod M. Connolly and Michael Sievers participated in conceptualisation and developed the methodology for the experiment. Andria Ostrowski carried out laboratory work and data curation. Andria Ostrowski, Christopher J. Brown and Michael Sievers conducted the formal analysis. Andria Ostrowski wrote the first draft of the manuscript. All authors contributed to visualisation, review and editing of the manuscript.

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#### PEER REVIEW

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#### DATA AVAILABILITY STATEMENT

All data used in analyses are available in the manuscript. The data and code used in this article are available on Zenodo (<https://doi.org/10.5281/zenodo.7098575>).

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