Supplementary Material

Decoupling salinity and carbonate chemistry: Low calcium ion concentration rather than salinity limits calcification in Baltic Sea mussels

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Datasets

Dataset #1
Product name
BALTIC SEA PHYSICS ANALYSIS AND FORECAST
Product identifier
BALTICSEA_ANALYSIS_FORECAST_PHY_003_006
Link
https://resources.marine.copernicus.eu/?option=com_csw&task=results?option=com_csw&view=details&product_id=BALTICSEA_ANALYSIS_FORECAST_PHY_003_006 last accessed 29/08/2017
Short description
This Baltic Sea physical model product provides forecasts for the physical conditions in the Baltic Sea. The Baltic forecast is updated twice daily providing a new six days forecast with hourly data for sea level variations, ice concentration and thickness at the surface, and temperature, salinity and horizontal velocities for the 3D field. The product is produced by the 3D ocean model code HBM developed within the Baltic ocean community. The product grid has a resolution of 1 nautical mile in the horizontal, and up to 25 vertical depth levels. The area covers the Baltic Sea including the transition area towards the North Sea (i.e. the Danish Belts, the Kattegat and Skagerrak).
Spatial resolution
2 km x 2 km
Vertical coverage
From -400 to 0 (25 levels)
Temporal resolution
Hourly – instantaneous, daily – mean, monthly - mean
Update frequency
2 x daily
Production unit
BAL-DMI-COPENHAGEN-DK; BAL-BSH-HAMBURG-GE

Table S1. Constituent chemicals added to solution to make calcium free artificial seawater (CFASW) after Kester, 1967. Mass of each chemical added was adjusted for the experimental salinities in this study (16, 11 and 6). All stock solutions also contained 5 % filtered seawater from the collections site to ensure presence of trace elements.

		g/L at respective salinity			
Salt	Molecular weight	35	16	11	6
NaCl	58.44	23.9260	10.9376	7.5196	4.1016
Na_2SO_4	142.04	4.0080	1.8322	1.2597	0.6871
$MgCL_2$	95.21	2.37	1.0834	0.7449	0.4063
KCl	74.56	0.6770	0.3095	0.2128	0.1161
$NaHCO_3$	84.00	0.1960	0.0896	0.0616	0.0336
KBr	119.01	0.0980	0.0448	0.0308	0.0168
H_3BO_3	61.83	0.0260	0.0119	0.0082	0.0045
$SrCl_2$	158.51	0.0085	0.0039	0.0027	0.0015
NaF	41.99	0.0030	0.0014	0.0009	0.0005

Table S2. A comparison of both laboratory experiments showing tank water volumes, number of animals per tank, mean mortality rates (no mortality was observed in the calcium experiment), ml of seawater per animal, body dry mass (BM) per animal, total body dry mass per tank, body dry mass per litre of seawater, number of feeds per day, final microalgae concentration in experimental vessels after feeding, total number of microalgae cells added per day and the number of cells per individual per day. The total microalgae cell no. ind^{-1} day^{-1} was also comparable between both laboratory experiments. For comparison, inter-treatment range values ae given below experimental means within the bicarbonate ion manipulation experiment, illustrating BM $litre^{-1}$ and microalgae cell no. ind^{-1} day^{-1} varied by a larger degree within the bicarbonate experiment than between experiments.

Experiment	volume (ml)	no. animals per tank	mortality rate end of exp.	ml per animal	BM per animal (mg)	total BM per tank (mg)	**BM per litre (mg l^{-1})**	no. of feeds per day	microalgae cell conc. in tanks (cells ml^{-1})	total no. of cells per day	**no. of cells per individual per day**
Calcium manipulation	50	2	N/A	25.0	0.329	0.658	**13.2**	1.0	10 000	5×10^5	**7.6×10^5**
Bicarbonate manipulation	2000	1600	53 % *(10 – 75 %)*	1.3 *(1.4 - 5)*	0.064 *(0.163 – 0.746)*	103.1 *(65.2 – 1074.2)*	**51.5** ***(32.6 – 537.1)***	2.5	10 000	5×10^7	**4.9×10^5** ***(4.7×10^4 – 7.7×10^5)***

Table S3. Model parameters for the power relationship between shell length and $CaCO_3$ mass with statistical results; standard error, *T* value and *p* value (Fig. S1). These relationships were used to calculate calcification rates in the field from shell length measurements.

Parameter	Site	value	Std. Error	*T*-value	*p*-value
intercept	Usedom	0.171	0.032	5.36	< **0.001**
	Ahrenshoop	0.050	0.012	4.29	< **0.001**
	Kiel	0.036	0.004	8.17	< **0.001**
power	Usedom	2.163	0.092	23.64	< **0.001**
	Ahrenshoop	2.716	0.107	25.43	< **0.001**
	Kiel	3.000	0.046	65.24	< **0.001**

Table S4. List of all R packages used for data analysis complete with versions, years, authors and intended use.

Package	*Complete name*	*Version*	*Year*	*Author*	*Use*
fitdistrplus	Fit distribution	1.0-14	2019	Marie Laure Delignette-Muller, Christophe Dutang (2015)	Fitting data distributions
ggplot2	Elegant graphics for data analysis	3.3.0	2016	Wickham H (2016)	Graphics
nlme	Linear and non-linar mixed effect models	3.1-147	2020	Pinheiro J, Bates D, Debroy S, Sarkar D, R Core Team (2020)	Fit and compare models
drc	Dose response analysis	3.0-1	2016	Ritz, C., Baty, F., Streibig, J. C., Gerhard, D. (2015)	Analysis of dose-response curves
ncdf4	Interface to Unidata netCDF files	1.17	2019	David Pierce (2010)	Read data from netCDF files
cmocean	cmocean	0.2	2019	Thyng, K., Richards, C. and Krylov, I.,	Colour maps for oceanography

Table S5. Model comparisons using AIC for selection of the most parsimonious negative exponential decay model fit to laboratory calcification rates across experimental [HCO_3^-] in the bicarbonate ion manipulation experiment. Model names and equations are shown with fixed parameters allowing direct comparison between salinity treatments. The lowest AIC value (in bold) represents the model which best explains the experimental data and was chosen for statistical analysis.

Model	*Equation*	*Fixed parameters*	*Estimated parameters*	*AIC*
Von Bertalanffy	$y \sim C_{max}*(1-e(-K*(x-s_0)))$	$s_0 = 380$	C_{max}, K	**115.58**
Gompertz	$y \sim C_{max}*(1+s_0*(e(-K*x)))^{-1}$	$s_0 = 380$	C_{max}, K	116.49
Logistic	$y \sim C_{max}*(1+s_0*(e(-K*x)))^{-1}$	$s_0 = 380$	C_{max}, K	115.79
Negative exponential	$y \sim C_{max}*(1-e(x/K))$		C_{max}, K	121.82
Michaelis-Menten	$y \sim C_{max}*x/(K+x)$		C_{max}, K	122.21

Table S6. Statistical test results of all analyses graphically depicted in figures 1-10. Pairwise comparisons (*post-hoc* tests) between treatments for statistically significant factors are listed on the right with significant *P*-values being shown in bold.

ANCOVA – laboratory calcification rates [Ca^{2+}] manipulation experiment (Fig. 2b)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*
Calcium	1	7160	7160	106.9	< **0.001**	11-16	0.988
Salinity	2	110.9	55.5	0.83	0.442	6-16	0.559
Calcium:Salinity	2	452.4	226.2	3.38	**0.041**	6-11	0.47
Residuals	54	3616	67				
Negative exponential decay model – Laboratory calcification and $\Omega_{aragonite}$ (Fig. 3a)							
parameter	*estimate*	*Std. Error*	*T-value*	*P-value*			
C_{max}	31.2315	5.244	5.956	< **0.001**			
K	0.374	0.1598	2.341	**0.027**			
Negative exponential decay model – Laboratory calcification and ESIR (Fig. 3b)							
parameter	*estimate*	*Std. Error*	*T-value*	*P-value*			
C_{max}	33.7696	5.9676	5.659	< **0.001**			
K	0.2752	0.1117	2.463	**0.020**			
ANCOVA – Field calcification rates (Fig. 4)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*
time	1	952304	952304	517.7	< **0.001**	Kie:Ahp	< **0.001**
population	2	2096837	1048419	570.0	< **0.001**	Use:Ahp	0.99
time:population	2	1769631	884815	491.0	< **0.001**	Use:Kie	< **0.001**
Residuals	12	30873	2573				
ANOVA – Mean salinity at field monitoring sites (Fig. 5)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*
site	2	1248	624.2	38518	< **0.001**	Ahp-Kie	< **0.001**
residuals	17253	279.6	0.02			Ahp-Use	< **0.001**
						Kie-Use	< **0.001**
ANOVA – Mean [HCO_3^-] at field monitoring sites (Fig. 6c)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*

site	2	414716	207358	38.80	< **0.001**	Ahp-Kie	< **0.001**
residuals	55	293930	5344			Ahp-Use	0.722
						Kie-Use	< **0.001**
ANOVA – Mean pH at field monitoring sites (Fig. 6a)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*		
site	2	0.092	0.046	1.217	0.304		
residuals	55	2.070	0.038				
ANOVA – Salinity-A_T relationship across all field monitoring sites (Fig. 7)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*		
salinity	1	571206	571206	86.6	< **0.001**		
residuals	56	369521	6599				
ANOVA – Mean $\Omega_{aragonite}$ at field monitoring sites (Fig. 6e)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*
site	2	1.89	0.94	7.22	**0.002**	Ahp-Kie	**0.044**
residuals	55	7.18	0.13			Ahp-Use	**0.002**
						Kie-Use	0.654
ANOVA – Mean ESIR at field monitoring sites (Fig. 6g)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*
site	2	3.98	1.99	10.88	< **0.001**	Ahp-Kie	**0.020**
residuals	55	10.07	0.18			Ahp-Use	0.363
						Kie-Use	< **0.001**
ANOVA - Mean Chl-*a* values at field monitoring sites (Fig. 6i)							
factor	*df*	*Sum Sq.*	*Mean Sq.*	*F-value*	*P-value*	*pair-wise comparisons*	*P-value*
site	2	11.41	5.7	13.8	< **0.001**	Ahp-Kie	< **0.001**
residuals	77	31.82	0.41			Ahp-Use	< **0.001**
						Kie-Use	0.35

Table S7. Parameters for the linear model fit to [Ca^{2+}] and calcification rates in the calcium ion manipulation experiment and the negative exponential decay model fit to [HCO_3^-] and calcification rates in the bicarbonate manipulation experiment. Significant *p*-values are shown in bold and graphical representations of these models are depicted in Fig. 3.

Linear model parameters – [Ca^{2+}]-calcification					
salinity	*treatment*	*value*	*Std. Error*	*T-value*	*P-value*
6	intercept	7.21	3.84	1.88	0.065
11		2.42	5.44	-0.88	0.382
16		-2.69	2.33	1.66	0.102
6	slope	1.67	1.67	3.9	< **0.001**
11		5.56	2.33	-1.78	0.081
16		2.37	2.37	2.57	**0.013**
Negative exponential decay model parameters - [HCO_3^-]-calcification					
salinity	*treatment*	*value*	*Std. Error*	*T-value*	*P-value*
6	C_{max}	4.68	2.03	2.31	< **0.001**
11		34.00	2.07	16.39	< **0.001**
16		35.25	1.78	19.79	< **0.001**
6	*K*	0.007	0.008	0.843	< **0.001**
11		0.007	0.001	5.966	< **0.001**
16		0.011	0.002	6.106	< **0.001**

Table S8. Parameters for negative exponential decay models fit to calcification rates (µg $CaCO_3$ d^{-1}) and [Ca^{2+}] and [HCO_3^-] predictors across both laboratory experiments. Parameters with significant estimates have *P*-values in bold.

Negative exponential model – [Ca^{2+}] – calcification (Fig. S4a)				
parameter	*value*	*Std. Error*	*T-value*	*P-value*
C_{max}	47.29	30.18	1.57	0.128
K	5.10	4.89	1.04	0.306
Negative exponential model – [HCO_3^-] – calcification (Fig. S4b)				
parameter	*value*	*Std. Error*	*T-value*	*P-value*
C_{max}	25.00	6.49	3.85	**< 0.001**
K	740.38	618.65	1.20	0.241

Table S9. Model parameters and R^2 values for the linear relationships: Salinity-A_T and $\Omega_{aragonite}$-ESIR. Also shown are the parameter estimates and residual sum of squares (RSS) for the negative exponential decay model fit to laboratory calcification rates in both experiments and substrate inhibitor ratio (SIR) not including $[Ca^{2+}]$.

Linear model – Salinity-A_T (Fig. 7)					
factor	*estimate*	*Std. Error*	*T-value*	*P-value*	R^2
intercept	1679.9	32.4	51.9	**<0.001**	0.6
slope	22.13	2.4	9.3	**<0.001**	
Linear model – Field $\Omega_{aragonite}$-ESIR relationship (Fig. S8)					
factor	*estimate*	*Std. Error*	*T-value*	*P-value*	R^2
intercept	0.337	0.049	6.89	**<0.001**	0.805
slope	0.781	0.051	15.39	**<0.001**	
Negative exponential decay model – Laboratory calcification and SIR (Fig. S5)					
parameter	*estimate*	*Std. Error*	*T-value*	*P-value*	*RSS*
C_{max}	22.66	2.75	8.23	**<0.001**	4631
K	0.02	0.01	1.45	0.157	

Table S10. Parameters and statistical results for the linear model fit to field calcification over time during the first 7 months. The linear slopes of these models express the calcification rates for each of the 3 populations (Table 3.)

Linear model - Field calcification rates over first 7 months.					
salinity	*parameter*	*value*	*Std. Error*	*T-value*	*P-value*
Usedom	intercept	-495.0	7657.9	-0.07	0.953
Ahrenshoop		-457.5	7657.9	-0.08	0.938
Kiel		-116675.0	5414.9	-15.24	< **0.001**
Usedom	slope	18.6	53.2	-0.33	0.761
Ahrenshoop		36.4	53.2	0.97	0.405
Kiel		2202.9	37.6	40.70	< **0.001**

Table S11. Statistical results for the Kruskal-Wallis test comparing temperatures (°C) at each site during the monitoring period.

Kruskal-Wallis test – Mean temperatures at field monitoring sites (Fig. S7)				
Kruskal-Wallis chi squared	*df*	*P-value*	*Dunn test*	*P-value*
122.73	2	<0.001	Ahp-Kie	**< 0.001**
			Ahp-Use	**0.042**
			Kie-Use	**< 0.001**

Supplementary figure legends

Figure S1: The relationship between juvenile shell length and $CaCO_3$ mass (mg) from the three study populations (data from Sanders, et al., 2018). This was used to calculate initial $CaCO_3$ mass in laboratory experiments and calculations of field calcification rates at the three monitoring sites in this study. Power model parameters are given in Table S3 with the insets listing the residual sum of squares (RSS) for each population.

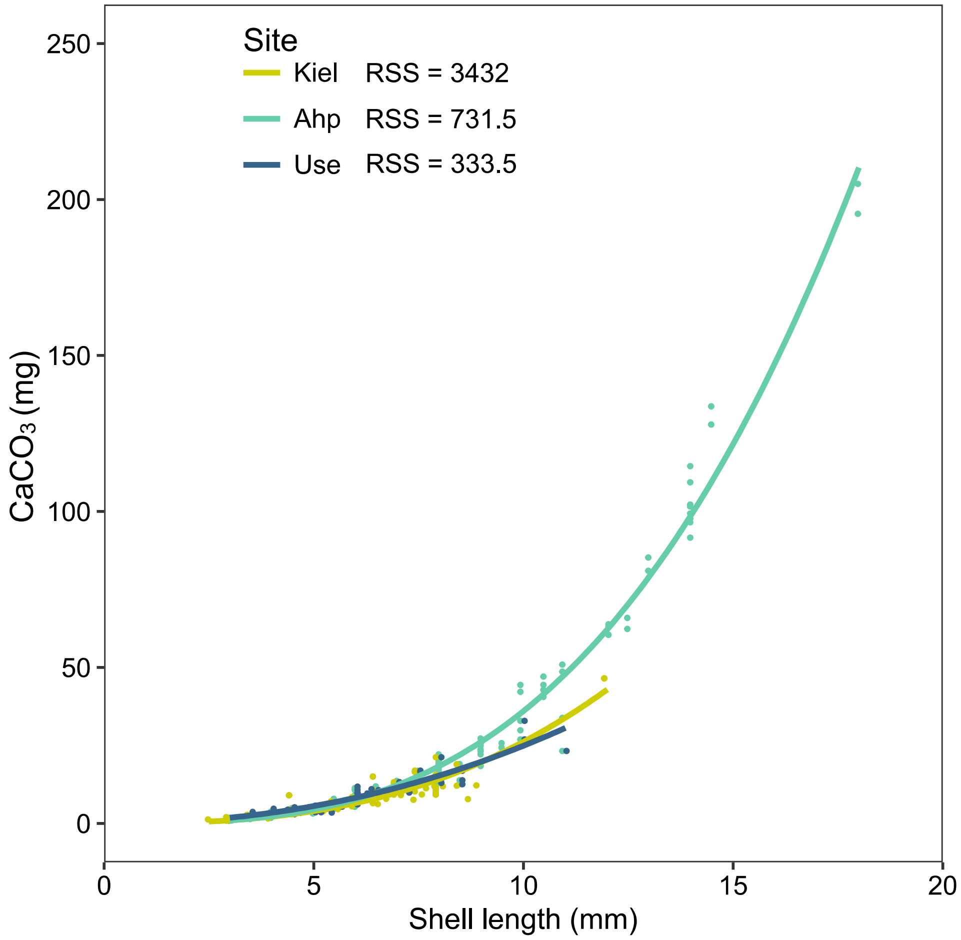


Figure S2: A comparison of the linear relationship between salinity and [Ca^{2+}] in the southwest Baltic Sea from calculated [Ca^{2+}] values (red dashed line) and measured [Ca^{2+}] values (blue dashed line). Methodologies for calculated and measured [Ca^{2+}] values are given in the text (section 2.5). Samples for the lowest salinity (3.15) were taken from the Achterwasser in Usedom (54° 0' 5" N, 14° 2' 47" E).

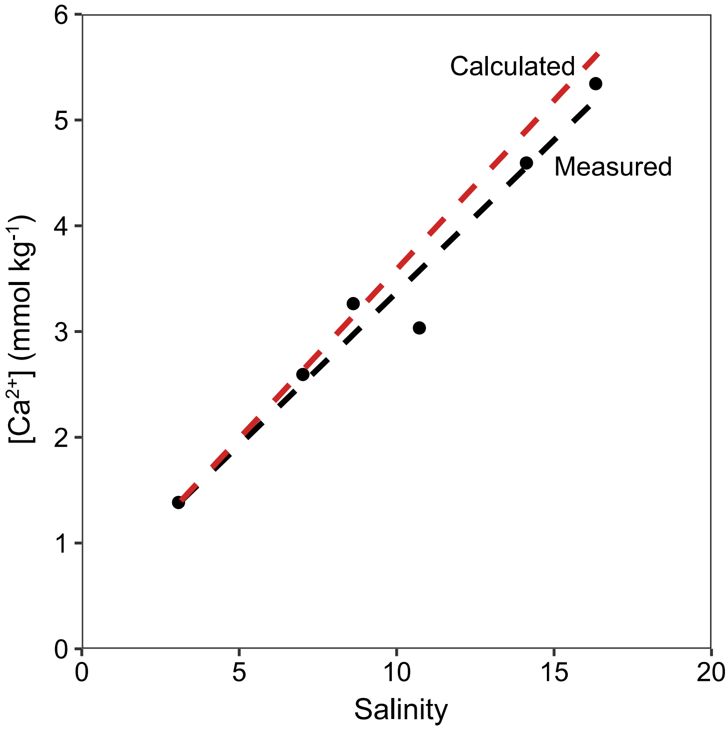


Figure S3: Settlement structures deployed at all three sites (Kiel, Ahrenshoop and Usedom) in March 2016 (a). A cross section of the settlement structures from 12 o'clock to 6 o'clock showing the orientation of the mesh net inside the cylinder (b). Cross section of the settlement structures showing the width of the mesh net inside the cylinder (c). A total of twenty 2 cm diameter holes were drilled into each cylinder and a 0.2 cm pore size cotton/nylon spat sock was diagonally positioned across the inside of the cylinders using cable ties. Numbers at the top of each figure represent the orientation of the cross sections in panels b and c.

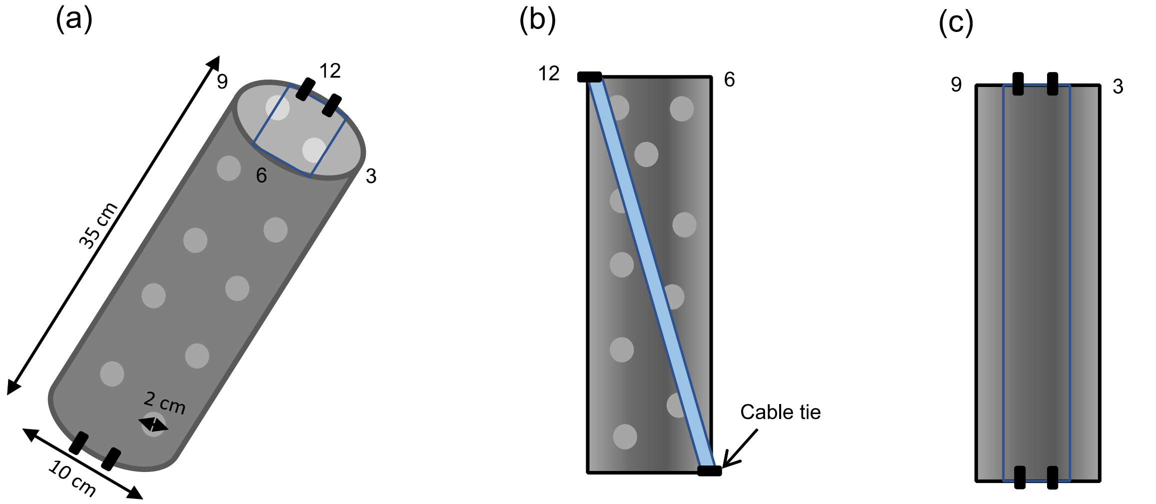


Figure S4: Calcification rates at all salinities from both laboratory experiments plotted across all [Ca^{2+}] (a) and all [HCO_3^-] (b). No significant relationship was found for either variable.

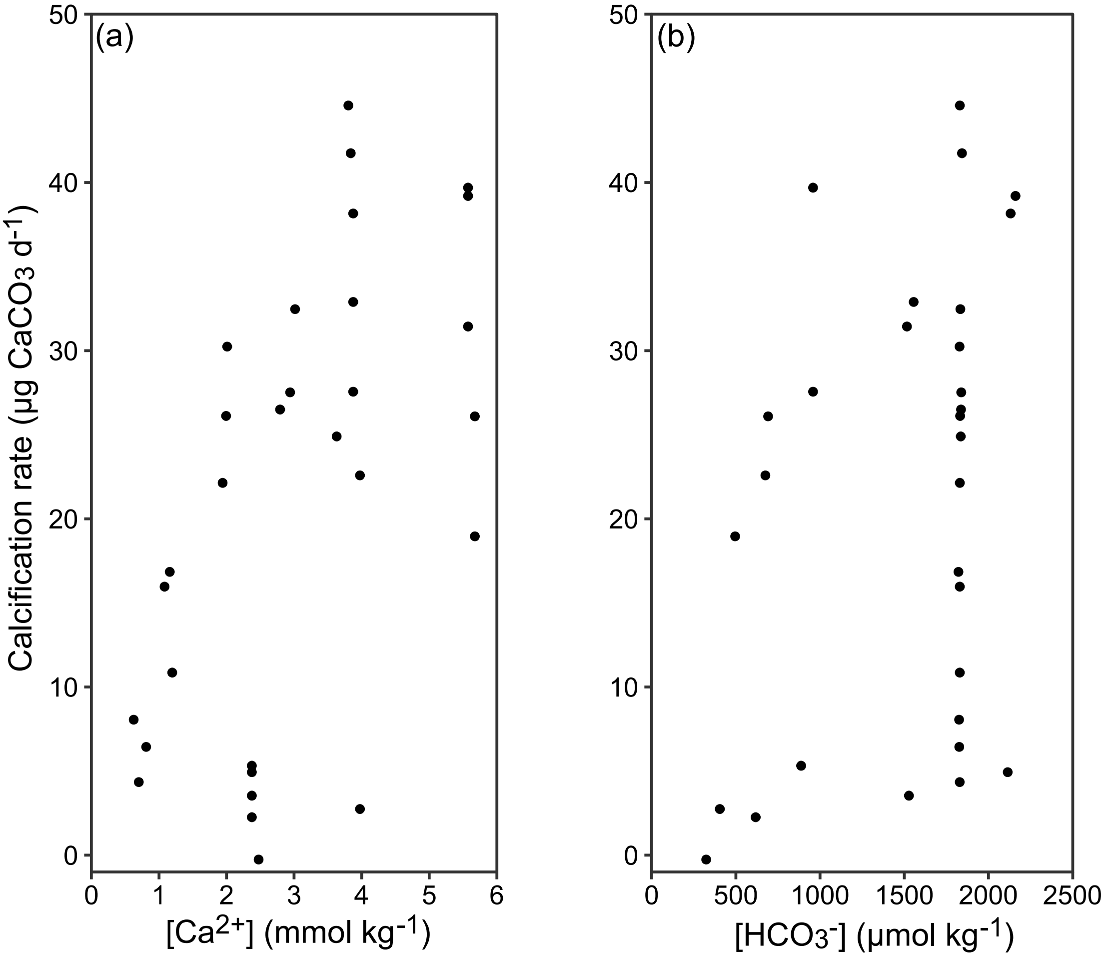


Figure S5. Calcification rates at all salinities from both laboratory experiments plotted across substrate inhibitor ratio (SIR). This measure excludes the effect of [Ca^{2+}] and no statistically significant model could be fit (Table S9).

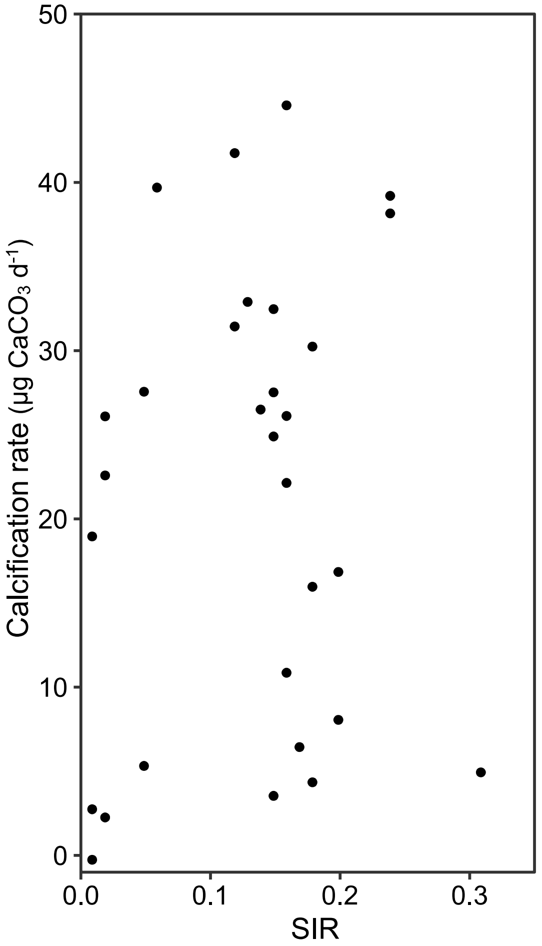


Figure S6: Graphical comparisons of parameters C_{max} (µg $CaCO_3$ d^{-1}) and K in the negative exponential decay model depicted in Fig. 3. Values are shown ± 95 % confidence intervals (CI). Overlaps in the CI indicates the two parameters in both models are not significantly different.

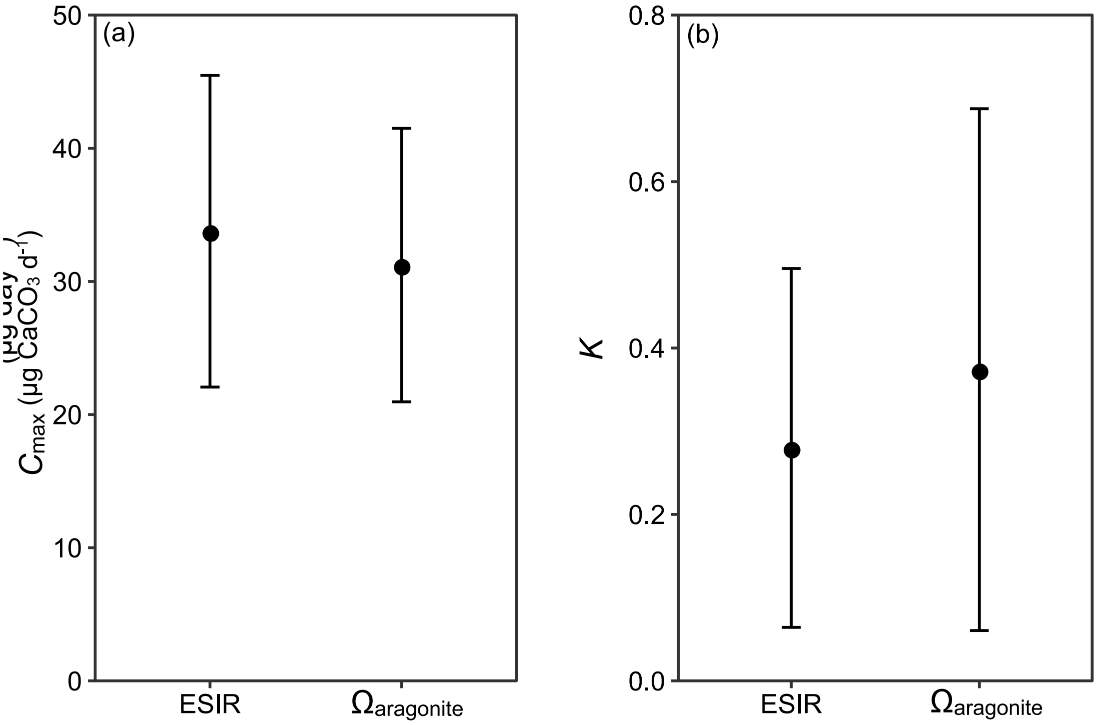


Figure S7: Temperature (°C) data from deployed CTD's at the three monitoring sites from Aug. 2015-Dec. 2017 (a). Box pots are shown on the right depicting median temperatures and interquartile ranges (b).

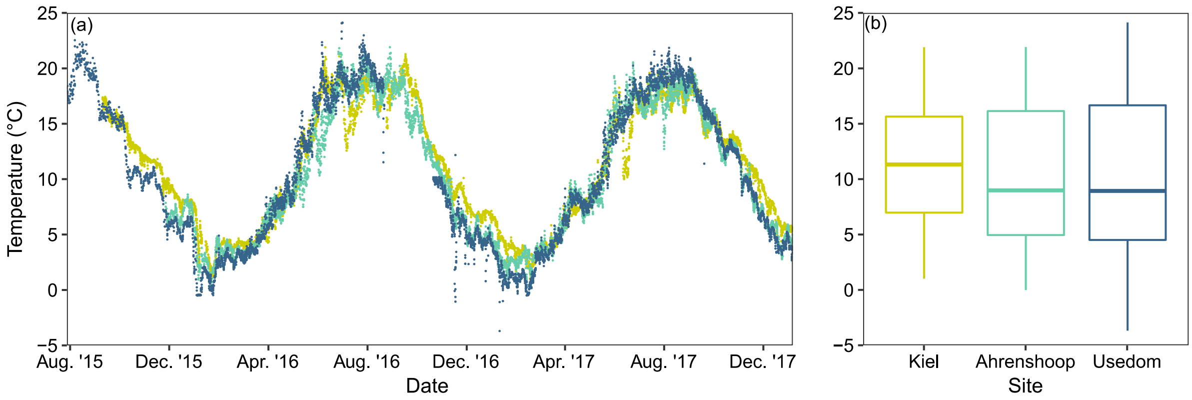


Figure S8: Field extended substrate inhibitor ratio (ESIR) plotted against aragonite saturation state ($\Omega_{aragonite}$) over the monitoring period at all three Baltic Sea sites. Parameters for the linear model are given in Table S9.

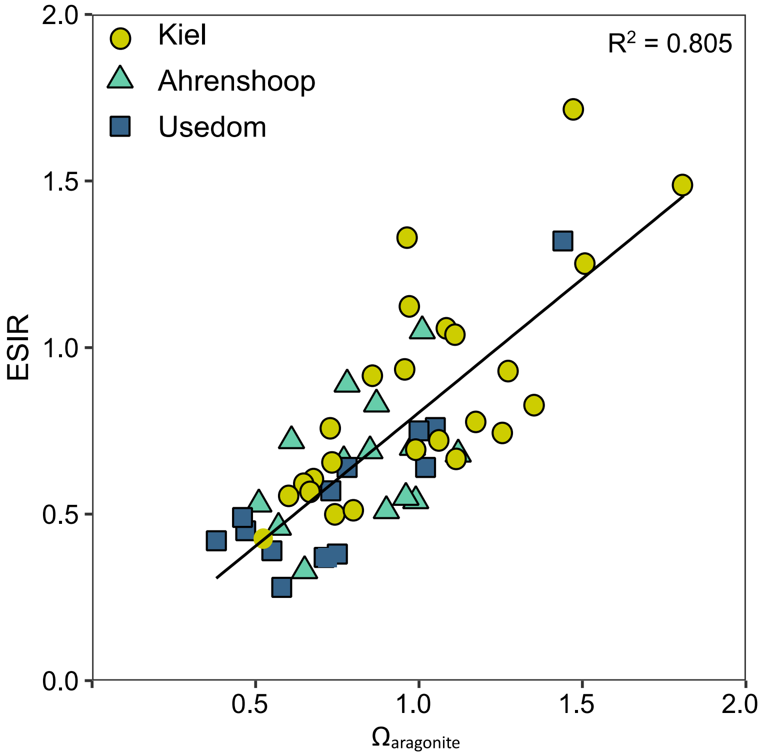
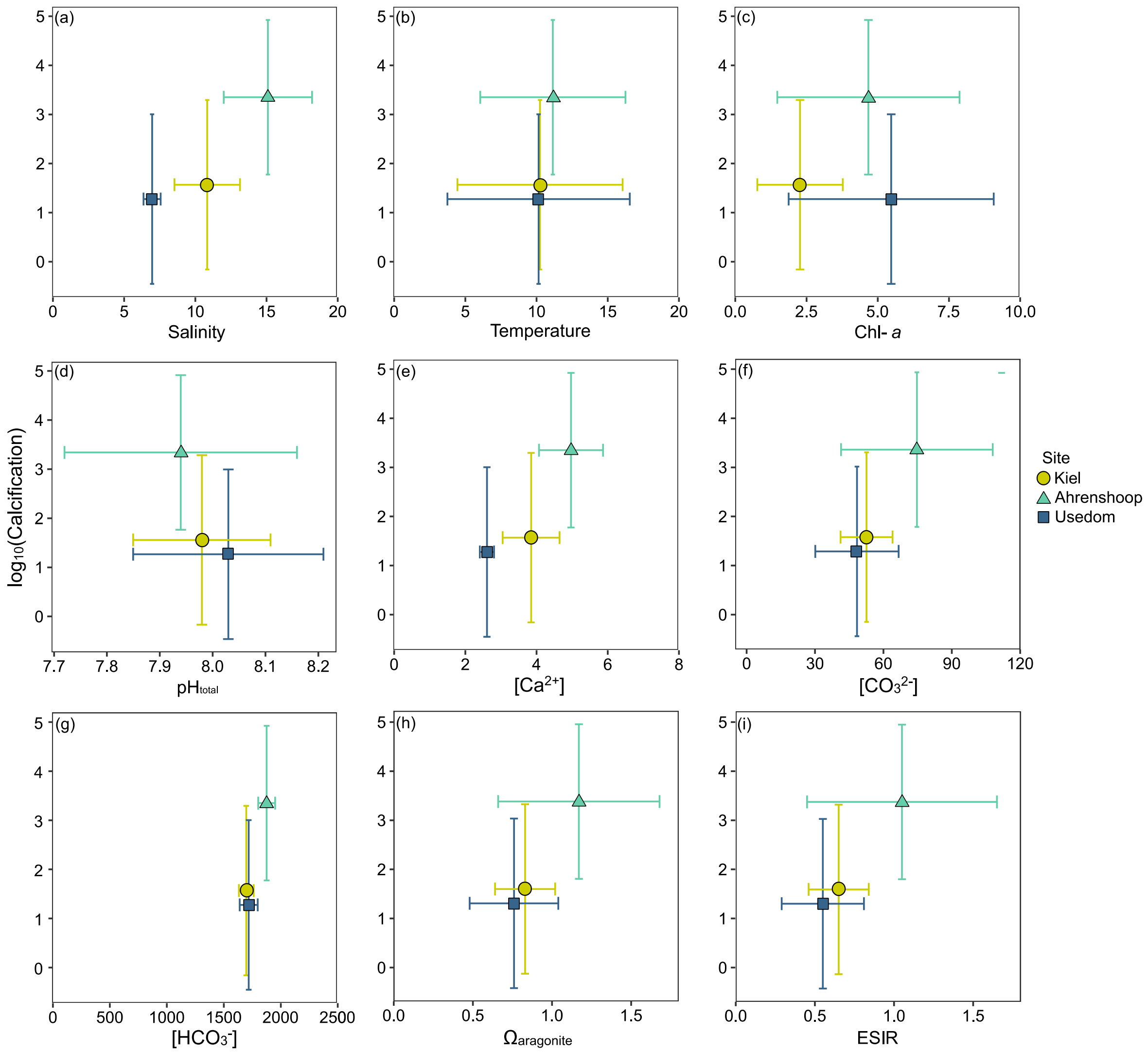


Figure S9: Log transformed field calcification rates (y-axes) plotted against multiple environmental parameters; Salinity (a), Temperature (°C) (b), chl-*a* (µg L^{-1}) (c), pH_{total} (d), $[Ca^{2+}]$ (mmol kg^{-1}) (e), $[CO_3^{2-}]$ (µmol kg^{-1}) (f), $[HCO_3^-]$ (µmol kg^{-1}) (g), $\Omega_{aragonite}$ (h), ESIR ($[Ca^{2+}][HCO_3^-]$ / $[H^+]$) (i). Values are shown ± standard deviation.



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