Developing techniques to better assess the impacts of ocean acidification on net community production and calcification rates.

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Abstract

The research included in this manuscript will allow scientists to better assess the impacts of ocean acidification (OA) on net community production and calcification rates. First, I collaborated with the European Free Ocean Carbon Dioxide Enrichment (eFOCE) project. eFOCE aims to better understand and project the impacts of OA by precisely controlling pH in the field and follow the responses of natural communities. I describe here an incubation-based methodology to study marine benthic community metabolism that takes into account different ecosystem components. I incubate the above ground and below ground aspects of the seagrass community and the below ground separately. This methodology is being developed to investigate the effects of decreasing pH on a seagrass meadow community. Thus one can identify the impact of pH on a whole community and its separate parts. Two partially enclosed chambers were placed in the bay of Villefranche-sur-Mer, in a seagrass community at 12 m depth. In one chamber the pH will be lowered by 0.3 units and maintained at this offset for the long term while the other chamber will serve as a control. For my part of the study, sediment and seagrass incubations were performed separately, in daylight and darkness for 4-24 hours, inside each of the eFOCE chambers. Repeated incubations were conducted in July and September 2013 prior to pH perturbation. Water samples were collected from incubations and changes in dissolved oxygen, dissolved inorganic carbon, and total alkalinity were analyzed to determine net primary production (NPP), community respiration (CR) and, gross primary production (GPP) as well as light and dark net calcification rates. My goal was to assess the status of the meadow and treatment chambers prior to incubation and assess the success of this methodology. The studied seagrass meadow was autotrophic (NCP of ~238 ±123 mmol O_2 m^{-2} d^{-1}, and ~158 ±61 mmol O_2 m^{-2} d^{-1} in July and September 2013, respectively) and the sediment varied between autotrophic and heterotrophic conditions in July and was clearly heterotrophic in September. The NCP_{O2}:NCP_{DIC} ratio was on average 1.8 for seagrass and 1.9 for sediment. Calcification rates ranged from -107 to -150 mmol $CaCO_3/m^2$/day in July. In a separate study, I describe a method to test the validity of the alkalinity anomaly technique often used to determine net calcification rates. Mediterranean mussel, *Mytilus galloprovincialis*, specimen were incubated for 24 h in a beaker with constant air bubbling. The G_{Ca}:G_{AT} ratio based on these measurements was 0.77 ±0.06, uncorrected for alkalinity, and 0.82 ±0.06 when corrected for alkalinity (G^*_{AT}). Both ratios are significantly different from the adjusted calcification A_T:Ca^{2+} ratio of 1. The release of dissolved organic carbon could explain the over/underestimation of calcification based on alkalinity. However, most of the produced DOC (Urea) seems to be uncharged and not affect the corrected alkalinity rates. There must be an additional sink of alkalinity that remains unknown. Recently indicated measurements suggested that the ammonium release, which is implemented within the corrected alkalinity rates, was too low. A recalculated G^*_{AT} with estimated ammonium release rates gave a G_{Ca}:G^*_{AT} of 1.09 ±0.08. Within this study the alkalinity anomaly method appears to be not valid for bivalve species; however with the estimated ammonium release taken into account the method seems valid.

Layman summary / Leken samenvatting

De laatste eeuwen is het klimaat aan het veranderen door de constante uitstoot van broeikasgassen, waaronder koolstofdioxide (CO_2). Deze veranderingen betreffen o.a. de opwarming en verzuring van oceanen en zeeën. De verzuring van oceanen komt vooral door de opname van koolstofdioxide door het zeewater. Deze chemische verandering van een hoger koolstofdioxide gehalte van zeewater heeft zowel negatieve als positieve gevolgen.

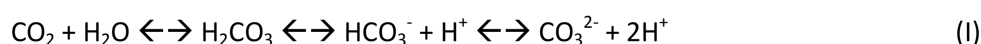
Voor het onderzoeksproject (genaamd eFOCE) is er onderzoek gedaan naar effecten van hogere CO_2 concentraties van zeewater op zeegras gemeenschappen. Eerdere onderzoeken hebben aangetoond dat het effect van een hogere CO_2 concentratie van zeewater verschillende effecten hebben op zeegras. Tijdens dit project waren twee plexiglas boxen op de bodem van de Middellandse Zee geplaats. Deze boxen zouden uiteindelijk kunstmatig verzuurd worden door de toevoeging van zeewater met verhoogde CO_2 waardes. In dit onderzoeksproject heb ik gezocht naar een techniek om de effecten van verzuring en hoger CO_2 gehalte op zeegras gemeenschappen te meten. Ik gebruikte hiervoor dag en nacht incubaties van zeegras planten of het sediment van de zeegras gemeenschap. Van deze incubaties werden water samples afgenomen bij het begin en het einde van de inzet. De zuurstofgehaltes, alkaliniteit (zuurbufferende capaciteit) en opgeloste inorganische koolstof (DIC) van deze samples werden bepaald. Hiermee kon ik de netto primaire productie (NPP) en calcificatie van de planten en het sediment bepalen voor de maanden juli en september. Het zeegrass was autotroof ("zelfvoedend") met een NPP van 238 ±123 mmol O_2 m^{-2} d^{-1} in juli 2013 en ~158 ±61 mmol O_2 m^{-2} d^{-1} in september 2013, respectivelijk. Het zeegras sediment varieerde tussen autotroof en heterotroof ("celmateriaal afkomstig van andere organismen") in juli en was heterotroof in september. Calcificatie waardes varieerde tussen -107 en -150 mmol $CaCO_3$ m^{-2} d^{-1} in juli.

Daarnaast wilde ik de deugdelijkheid van een techniek testen waarmee normaal calcificatie waardes worden bepaald. Deze methode werd uitgevoerd op *Mytilus galloprovincialis* (diepwater mosselen) die voor 24 uur in een vat werden geïncubeerd. Normaal worden calcificatie waardes bepaald door de alkaliniteit van het water (G_{AT}) te meten. Met de nieuwere, nauwkeurigere beschouwde techniek worden de calcium waardes (G_{Ca}) van het omliggende water waarin de organismes leven gemeten. In deze studie zijn beide factoren gemeten en wordt aan de hand van de ratio bepaald of de techniek deugd. De G_{Ca}:G_{AT} ratio was 0.82 met een standaardafwijking van 0.06 en deze ratio verschilde significant van de verwachte ratio van 1. Alkaliniteit waardes worden beinvloed door verschillende factoren waaronder de uitstoot of opname van ammonium, opgeloste inorganisch koolstof, opgeloste organische koolstof, nitriet, nitraat en fosfaat door de organismes. Vervolgens is onderzocht of de alkaliniteit wordt beïnvloed door de extra hoeveelheid ammonium die wordt uitgestoot. Door G_{AT} met de aangepaste ammonium waarde te herberekenen, bleek dit inderdaad het geval te zijn (ratio van 1.09 t.o.v. een verachte ratio van 1). Hiermee lijkt de deugdelijkheid van deze methode voor Diepwater mossels te kloppen.

Chapter 1: Measures of production and calcification rates in a seagrass meadow provides the baseline necessary to assess the impact of ocean acidification.

1. Introduction

Since the early 1800s increasing fossil fuel combustion, deforestation, and construction have contributed rapidly to rising atmospheric carbon dioxide levels in the atmosphere and oceanic reservoirs (Sabine et al. 2004; Meehl et al. 2007). The value in 2012 was 393.82 ± 0.12 ppm (mean value for 2012 at Mauna Loa, Conway and Tans, 2011); and the rate of increase was ~0.6 % year $^{-1}$ from 2000 to 2010 (Peter et al. 2012). This increase in CO_2 is causing global warming and ocean acidification (Caldeira and Wicket, 2003; Caldeira and Wicket, 2005). The global oceans serve as a significant sink of anthropogenic CO_2 (Sabine et al. 2004) which has important consequences for the carbonate chemistry of the surface waters (Feely et al. 2004).
An increase in the surface seawater dissolved inorganic carbon (DIC) content and partial pressure of CO_2 (pCO_2), and a decrease in *p*H of 0.1 unit have been noticed since pre-industrial times (Caldeira and Wicket, 2003; Caldeira and Wicket, 2005; Bates, 2007). The pH of the surface seawater (0-10 m) is expected to decline 0.36 units by the end of the 21st century relative to recent values (Orr, 2011). Ocean acidification arises from the formation and dissociation of carbonic acid (H_2CO_3), which releases hydrogen ions (H^+). This shifts the equilibrium of seawater carbonate and increases CO_2 and HCO_3^- concentrations at the expense of CO_3^{2-} concentrations (Eqs. I; Zeebe and Wolf-Gladrow, 2003):



$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad (I)$$

Because of these changing chemical conditions a large number of studies focus on the consequences for marine ecosystems (Guinotte and Fabry, 2008). Changes in ocean chemistry could have significant impacts on the functioning of aquatic and marine plant communities (Guinotte and Fabry, 2008; Hall-Spencer and Rodolfo-Metalpa, 2009; Kleypas et al. 2009; Semesi et al. 2009). A decrease in pH may have dramatic effects on the diversity within seagrass habitats (Martin et al. 2008). The percentage cover of epiphytic coralline algae is reduced with increasing acidification of seawater both in short-term aquaria and near natural CO_2 vents (Martin et al. 2008). Beneficial effects are suggested for seagrass taxa exposed to increasing CO_2 levels (Palacios and Zimmerman, 2007; Hall-Spencer and Rodolfo-Metalpa, 2009; Jiang et al. 2010; Alexandre et al. 2012). Seagrasses are carbon-limited because of the inefficient use of HCO_3^- and low concentration of $CO_2(aq)$ in seawater (Beer and Koch, 1996; Invers et al. 2001). Increase in photosynthetic performances has been noticed in both short-term- (ca. 45 days) (Zimmerman et al. 1997) and long-term (1 year) CO_2-enriched mesocosm experiments (Palacios and Zimmerman, 2007).
Most experiments focusing on the effects of increasing CO_2 on benthic aquatic plants have been performed in mesocosms (Zimmerman et al. 1997; Palacios and Zimmerman, 2007). Laboratory mesocosm experiments generally provide ideal conditions which could over- or underestimate the responses of the natural communities. Indeed, the differences between *in situ* and *ex situ* responses of terrestrial plant community

dynamics to CO_2 enrichment have been documented in a review of several studies (Ainsworth and Long, 2005). *In situ* investigations on the responses of seagrass meadows to CO_2 enrichment are scarce and mostly based on observational studies near volcanic vents (Martin et al. 2008; Hall-Spencer and Rodolfo-Metalpa, 2009). Also *in situ* seagrass metabolism studies generally estimate the whole community metabolism instead of focusing on the separate inputs of the parts, e.g. plants and sediment (Barrón et al, 2006; Champenois and Borges, 2011).

I describe here an incubation-based methodology to study marine benthic community metabolism that takes into account different ecosystem components. I incubate the above ground and below ground aspects of the seagrass community separately. This methodology is being developed to investigate the effects of decreasing pH on a seagrass meadow community. Thus one can identify the impact of pH on a whole community and its separate parts. If proven successful, it will be used within a technological sophisticated experimental system that allows the precise control of pH *in situ*. The system consists of two clear Perspex enclosures and an open plot located in a seagrass meadow of *Posidonia oceanica* in the bay of Villefranche. Sediment and seagrass incubations were performed separately and repeatedly, inside each and outside of the enclosures prior to pH perturbation. Water samples were collected from incubations and changes in dissolved oxygen, dissolved inorganic carbon (DIC), and total alkalinity (A_T) were analyzed to determine net community production (NCP), community respiration (CR) and gross primary production (GPP), as well as light and dark net calcification rates. With these methods and analyses I describe the overall metabolism of seagrass meadows in the bay of Villefranche.

The aims of this study is to assess metabolic variation both spatially and temporally, for both the above ground and below ground parts, of seagrass community prior to pH manipulation and to assess the applicability of this methodology. Specifically, this study addresses the following questions for seagrass blade and the below-ground community: are there differences in metabolism among the different enclosures and study plots and will metabolism differ between the months of July and September? Because there are large seasonal differences in the bay and because the study area is similar in habitat and encompasses a relatively small area, I hypothesize that the metabolisms of the different components of the seagrass community in the different plots will be similar and that these metabolic rates will differ between months.

2. Material and Methods

2.1 Site and system description

The eFOCE project will be using a carbon dioxide enrichment system which is connected to the water inflow of the mesocosms to precisely control pH *in situ*. The eFOCE system is located in a seagrass meadow of *Posidonia oceanica* in the bay of Villefranche at ~12 m depth (Fig. 1). The bay of Villefranche is subject to minimal tidal changes (~20 cm) and is mostly protected from wave action.

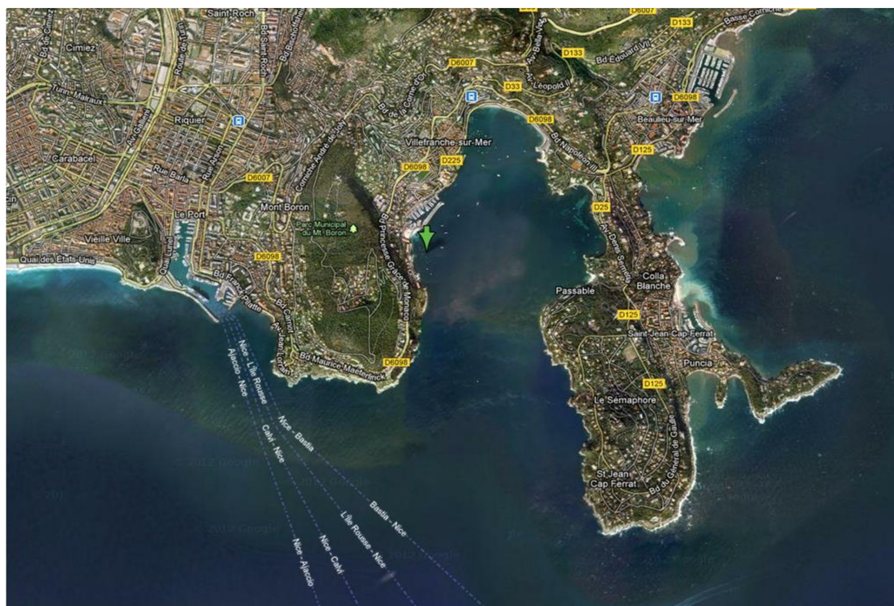


Figure 1. The site location (green arrow) in the bay of Villefranche (43°42'00"N, 7°19'00"E).

The system consists of two clear, open-top, flow-through Perspex chambers (2 x 1 x 1 m^2) with the possibility for long time continuous CO_2 enrichment (Fig. 2). The open-top, clear Perspex chambers allows light to reach the target organisms. Additionally the open-top chamber allows easy access to the benthic community for sampling.

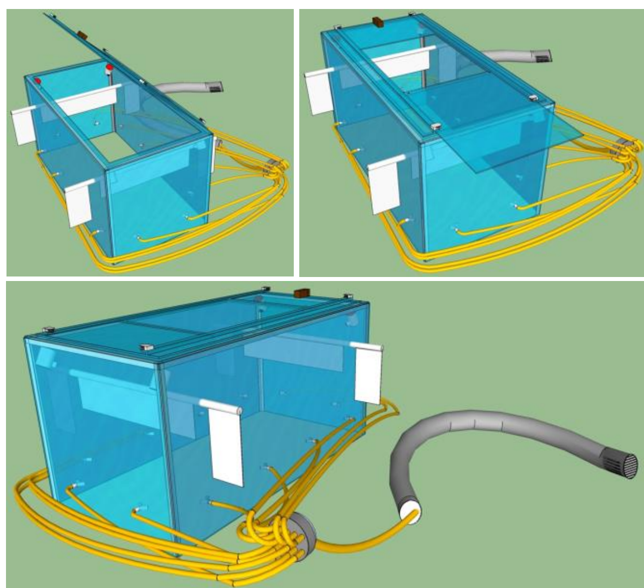


Figure 2. The eFOCE system with two clear Perspex chambers.

The incubations were conducted in the bay of Villefranche within two enclosures and in a nearby area of similar size and in similar habitat. One of the chambers, referred to as Experiment (E), eventually will be enriched with CO_2. The other chamber will serve as a control; referred to as the Control (C) chamber. The nearby area exposed to ambient conditions, or Reference (R) plot, will be used to account for any structural effect that may result from the chamber. The chambers and the reference exclude large fishes with a net (mesh size of 2 x 2 cm) that covers the open top of the chamber or encloses the plot.

2.2 Incubations

The seagrass incubations were conducted on 3 haphazardly chosen seagrass shoots inside the partially closed chambers. Each shoot and surrounding water was enclosed with a vinyl bag. Then a dense piece of foam and elastic were used to seal the bag at the base of the blades. At the end of the incubations seagrass leaves were sampled in order to estimate their dry weight and the dry weight of epibionts.

Incubations of sediment were performed separately on different patches during each incubation. The incubations were conducted on a bare sediment patch located in the chamber and within the reference plot. The sediment patch was clear of above-ground biomass. However, rhizomes and other organisms could still be present. A polyvinyl chloride rigid cylinder (PVC) (radius = 7.25 cm) was hammered into the sediment (down to 6 cm) and the open top enclosed with a gas-tight vinyl bag sealed to the base with elastic cords.

Seagrass incubations were conducted in light (incubation time ~ 4 h) and dark (incubation time ~ 7 h) conditions in July (and one during the beginning of Augustus) and September, 2013. Dark conditions were created by covering the vinyl bags with a light impermeable bag. Sediment incubations were performed within 14 days from the incubation of seagrass. Sediment incubations were conducted in July (n=2), in both light and dark (incubation time ~ 8 h), and in September 2013 (n=3) for a whole day (incubation time ~24 h). During the 8 h sediment incubations, dark conditions were created by covering the incubation design with a light impermeable bag.

For all incubations, seawater samples were collected from incubation bags immediately after setup (T_0) and at the completion of the incubation (T_F; Fig. 3). Seawater was drawn into 7x 100 ml syringes that were able to attach to the two-way valve on incubation bags. These water samples were analyzed for dissolved oxygen and DIC concentrations to determine production and analyzed for alkalinity to determine calcification rates.

Figure 3. An example of the collection of the sample from a sediment incubation.

2.3 Sampling and measuring of parameters

Oxygen

For each incubation, 60 mL of collected seawater was transferred immediately into biological oxygen demand (BOD) bottles and dissolved oxygen concentrations were determined according to the Winkler method using a potentiometric end-point determination with an estimated accuracy of ±2 μmol kg^{-1}. Analyses were performed with a Metrohm Titrando 888 and with a Metrohm ion electrode. Reagents and standardizations were similar to those described by Knap et al. (1996).

Dissolved Inorganic Carbon

Water samples used for DIC analyses were stored in 60 mL brown borosilicate bottles and poisoned with 10 μL of mercury chloride ($HgCl_2$). DIC was determined immediately after opening the bottle, in triplicate (1.2 mL subsamples), using an inorganic carbon analyser (AIRICA, Marianda, Kiel, Germany) coupled to an infrared gas analyzer (LI-COR 6262). This system was calibrated prior to sample analysis against a certified standard provided by A. Dickson (batch 129).

Total alkalinity

For total alkalinity (A_T) measurements, 200 mL of collected seawater was filtered through 47 mm GF/F filter, poisoned with 40 μL of $HgCl_2$ and preserved at room temperature pending analysis. A_T was determined on triplicate 50 mL subsamples by potentiometric titration on a Metrohm Titrando 80 titrator coupled to a glass electrode (Metrohm, electrode plus) and a thermometer. The pH electrode was calibrated on the total scale using TRIS buffers of salinity 35 provided by A. Dickson. Measurements were carried out at 25 °C and A_T was calculated as described by Dickson et al. (2007). A standard provided by A. Dickson (batch 129) was used to verify the accuracy of the measurements.

Volume of incubation bag

The volume of incubation bags was estimated by injecting a known volume of a concentrated brine solution into the incubators and comparing the determined salinity of the resulting solution to a laboratory dilution.

Specifically, a water sample of 60 mL was taken at the beginning of each incubation setup. Then at the end of the incubation 10 mL of a concentrated brine solution was injected into the bag and allowed to mix for 5 minutes and a final water sample of 60 mL collected. In the laboratory 10 ml of the brine solution was added to range of volumes (0.5, 1, 2, 5 and 10 L) and the salinity of the resulting solutions and water samples measured with a GuildLine 8400B Autosal Salinometer. The volume of the bags was determined by fitting a regression line with the salinity of the dilutions.

Seagrass treatment

Seagrass shoots that were used for the incubation were collected, epibionts were scraped off and both the shoots and the epibionts were dried in a dry-oven (~ 70 °C). The length and weight of the shoots were then determinate.

2.4 Computations and statistical analysis

For seagrass, each estimated rate was estimated as:

Metabolism rate = ΔX * V / area

For sediment:

Metabolism rate = ΔX * V / area

Hereby, X is the rate of the parameter calculated (expressed per day) and V is the volume of the incubation (in L). The rate is expressed as mmol (O_2 or C or $CaCO_3$) per m^2 per day.

NCP was integrated over 24 h by first determining the hourly rate in daylight and then multiplying this rate by the length of the photoperiod (H_d) ($H_n = 24 – H_d$). CR was also integrated over 24 hours by multiplying the hourly rate in the dark by 24. CR is expressed as a positive value. Daily integrated GPP was computed as the difference of daily integrated NCP and daily integrated CR. In September a 24 h sediment incubation was conducted which was only integrated over 24 h. The measurement of changes in A_T of seawater is considered as the most convenient method to estimate calcification/dissolution processes (Smith and Key, 1975; Chisholm and Gattuso, 1991).

DIC* was calculated from DIC and total alkalinity (A_T) values according to Broecker and Peng [1982] (Eqs. II):

$$DIC^* = DIC - 0.5\ A_T \quad \text{(II)}$$

The hourly change of DIC during the incubations was calculated in the same manner as the oxygen units.

A single factor ANOVA is used to determine the statistical significance of the differences between the different months and the different plots. A Deming regression (Model II) was used to compare the relationship between NCP(O_2) and NCP(DIC) and the relationship between CR(O_2) and CR(DIC) for both seagrass and sediment incubations. The Deming regression technique considers that both X and Y variable measurements may contain errors.

3. Results

3.1 Incubation metabolism

The seagrass shoot and epibiont communities were autotrophic with mean NCP values ranging from 25 to 419 mmol O_2 m² d^{-1} and 146 to 765 mmol C m² d^{-1} (Fig. 4). Overall, NCP rates were higher in July (mean: 238 ± 123 mmol O_2 m^{-2} d^{-1} and 483 ± 222 mmol C m² d^{-1}) when compared to values in September (mean: 158 ± 61 mmol O_2 m^{-2} d^{-1}).

Net community production (NCP) varied significantly among the C, E, and R in July, while September measures were more consistent among treatments (Fig. 4). For example, mean NCP rates of the seagrass shoots in July based on oxygen were 94, 342 ± 75 and 229 ± 74 mmol O_2 m² d^{-1} for the E, C and R, respectively while the mean NCP in September for the E, C and R shoots were 200 ± 49, 153 ± 87, 122 ± 13 mmol O_2 m² d^{-1} respectively (Fig. 4). There was only significant difference between the two months in the control plot. The mean NCP rates of seagrass in July based on DIC showed a similar trend, however no significant difference (Fig. 4).

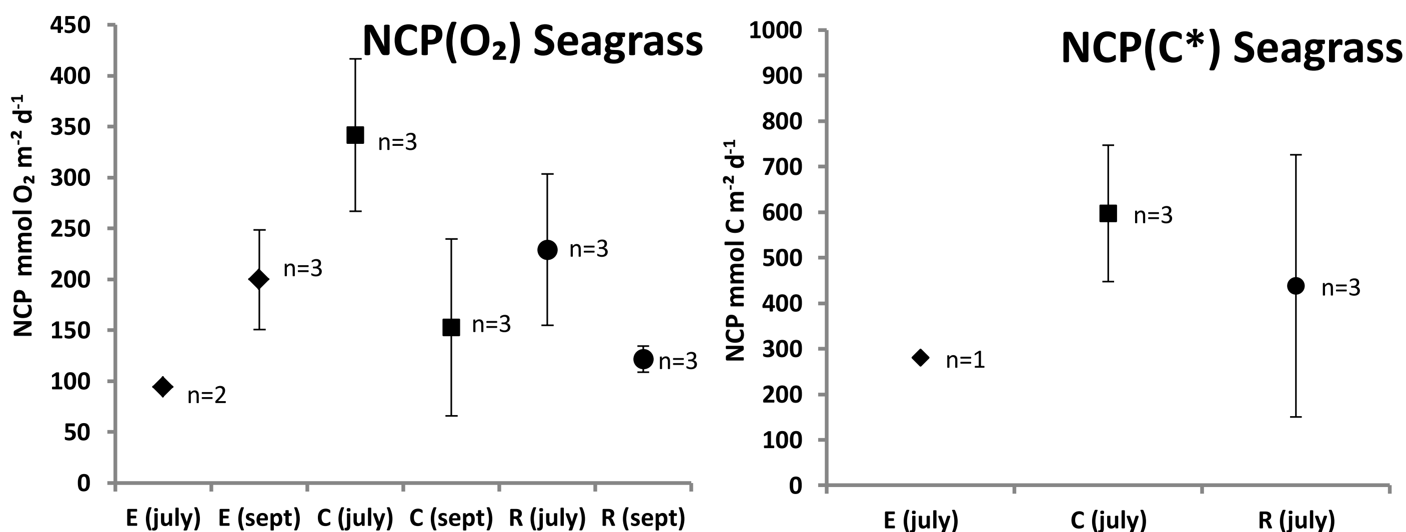


Figure 4. The mean NCP ± SD of seagrass shoots in July and September in the three chambers (Experiment = E, Control = C, and Reference = R) in both oxygen (left) and carbon (right) units.

The seagrass shoot and epibiont community respiration (CR) rates were similar between the E and R treatments and significantly different from the shoots in the C treatment, for both months. Mean CR in July based on oxygen were 36, 59 ± 28, and 34 ± 11 mmol O_2 m² d^{-1} for E, C and R shoots, respectively. The mean CR of seagrass in July based on DIC were 199, 132 ± 74, and 92 mmol C m² d^{-1} for E, C and R, respectively. The mean CR in September for the E, C and R shoots were significantly different with rates of respectively, 19 ± 9, 47 ± 10, 18 ± 10 mmol O_2 m^{-2} d^{-1} (Fig. 5). There was no significant difference between the two months.

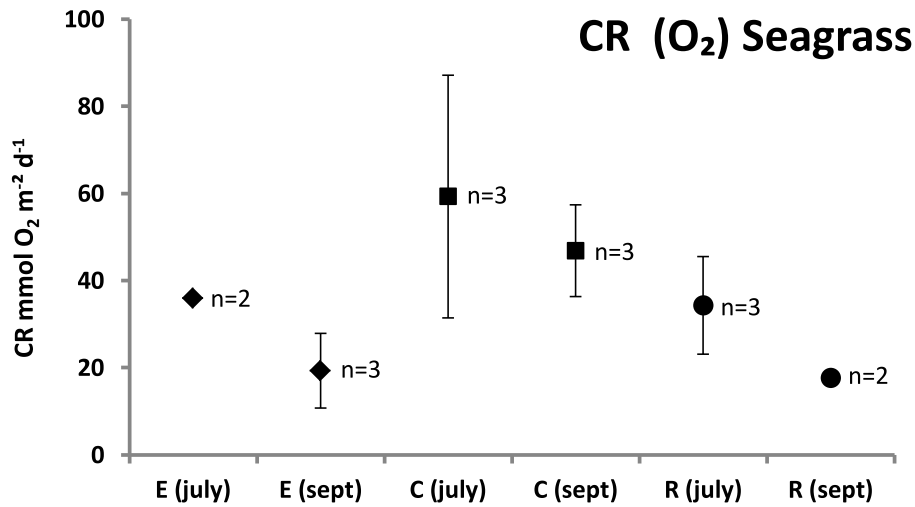


Figure 5. The mean CR of seagrass shoots in July and September in all three chambers. Standard deviation error bars are shown, except for Experiment (July) (range 31.7 and 40.3) and Reference (September) (range 14.6 and 20.8) were only two samples were measured and therefore the mean is shown.

Gross primary production (GPP) rates for the shoots showed similar trends as the NCP. Values varied among treatments (E, C and R). In July GPP rates were significantly different between the E, C, and R treatments while the rates in September were more consistent (Fig. 6). Mean rates for July 2013 were 265 ± 136 mmol O_2 m^{-2} d^{-1} and 575 ± 232 mmol C m^2 d^{-1}, while the mean rate in September was 185 ± 44 mmol O_2 m^{-2} d^{-1}. There was only significant difference between the two months in the control plot.

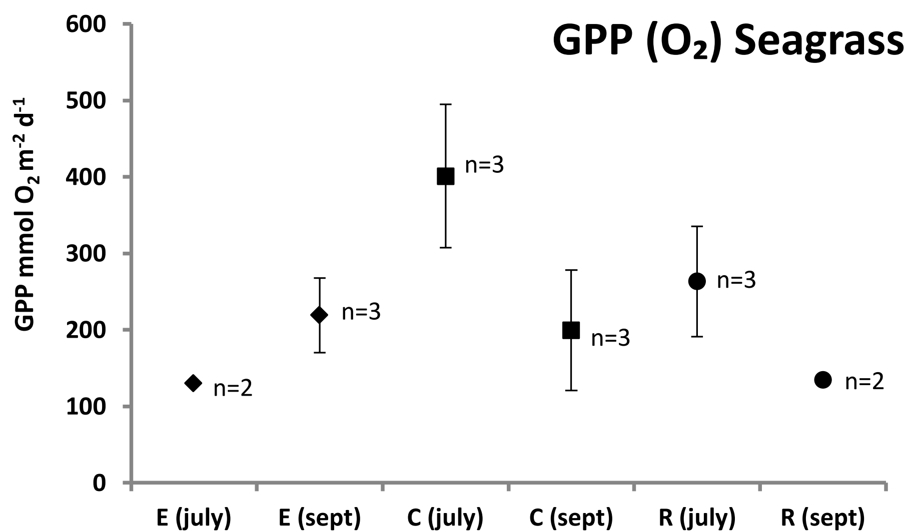


Figure 6. The mean GPP (O_2) of seagrass plants in July and September in all three boxes in mmol O_2/m^2/day. Standard deviation error bars are shown, except for Experiment (July; range: [65, 195]) and Reference (September; range: [128, 142]) where only two samples were measured and therefore the mean is shown.

Calcification rates varied widely among the treatments and months in the light period (between -66 and -200 mmol $CaCO_3$ $m^{-2}d^{-1}$ in July and between -21.5 and -272.1 mmol $CaCO_3$ $m^{-2}d^{-1}$ in September); however rates were not significantly different. The concentrations in the dark were more consistent, except for one experiment September value, with rates varying between -37.9 and 12.8 mmol $CaCO_3$ m^{-2} d^{-1} in July and between -10.6 and -150.5 mmol $CaCO_3$ m^{-2} d^{-1} in September. The calcification in daylight for seagrass shoots

was similar in both months.

The sediment community varied between being heterotrophic and autotrophic status and varied among the E, C, and R treatments (Fig. 7). In July, the sediment community was heterotrophic for the experiment and control chambers and autotrophic for the reference plot. The NCP rates varied from -9.2 to 9.7 mmol O_2 m^{-2} d^{-1} and -21.9 to 10.6 mmol C m^2 d^{-1}. Similarly the CR and GPP rates vary within and among the different treatments, however the production and respiration rates are consistent for sediment community within the control chamber (Fig. 7). CR varied from 0.8 to 19.6 mmol O_2 m^{-2} d^{-1} and -6.8 to 11.7 mmol C m^2 d^{-1}, while GPP varied from -0.3 to 19.4 mmol O_2 m^{-2} d^{-1} and -22.3 to 11.0 mmol C m^2 d^{-1}. There was no significant difference within the production or respiration rates.

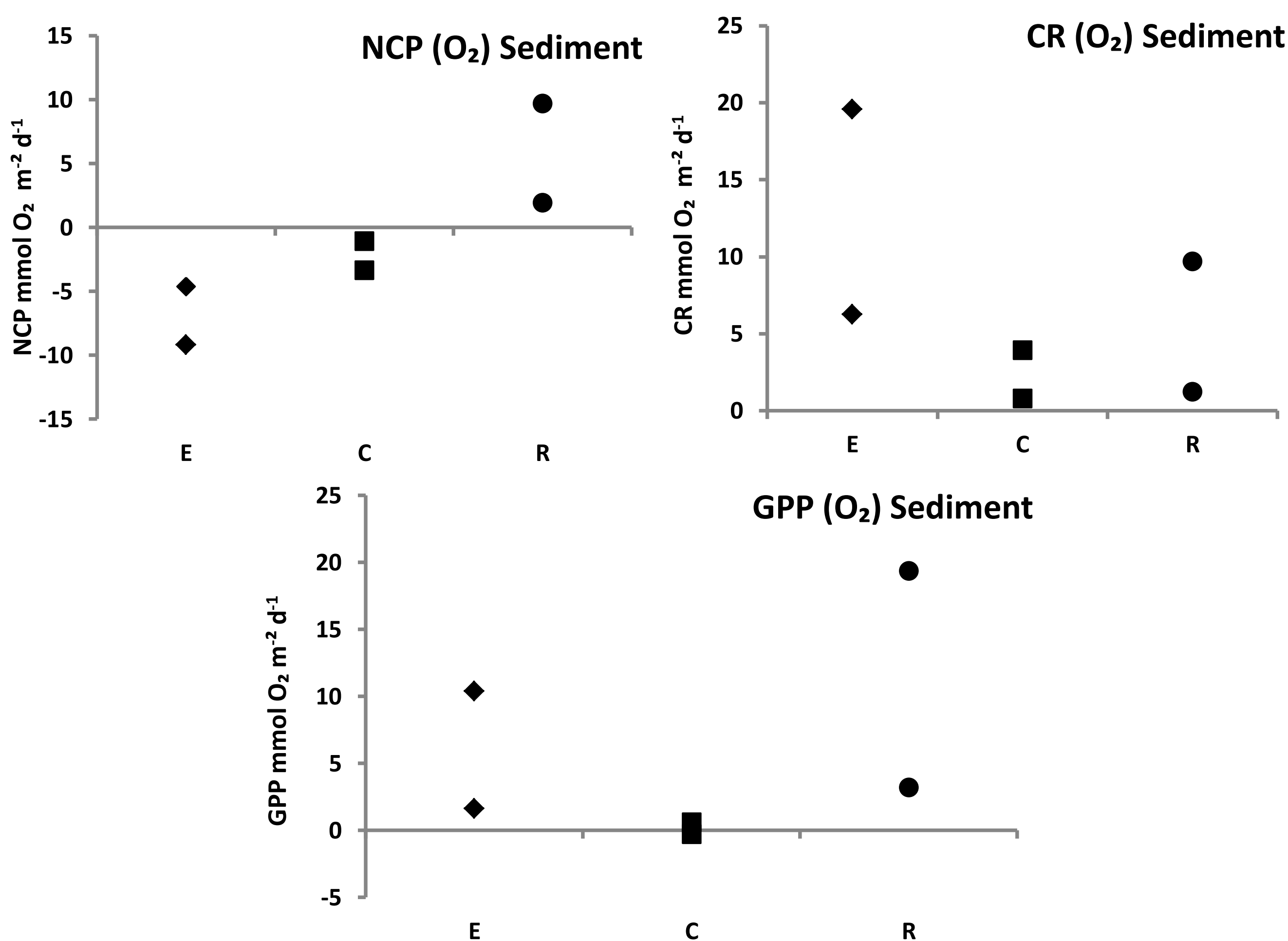


Figure 7. The NCP (top-left), CR (top-right) and GPP (bottom) rates for two sediment incubations in July in E, C and R.

Rates based on oxygen and DIC for the sediment incubation over 24 hours are all negative. The experiment plot gave the highest value, however variation among E, C, and R was consistent between oxygen and DIC based measurements. The mean rates for the 24h incubation in September for the sediment were -13 ± 6 mmol O_2 m^{-2} d^{-1} and -18 ± 7 mmol C m^{-2} d^{-1}.

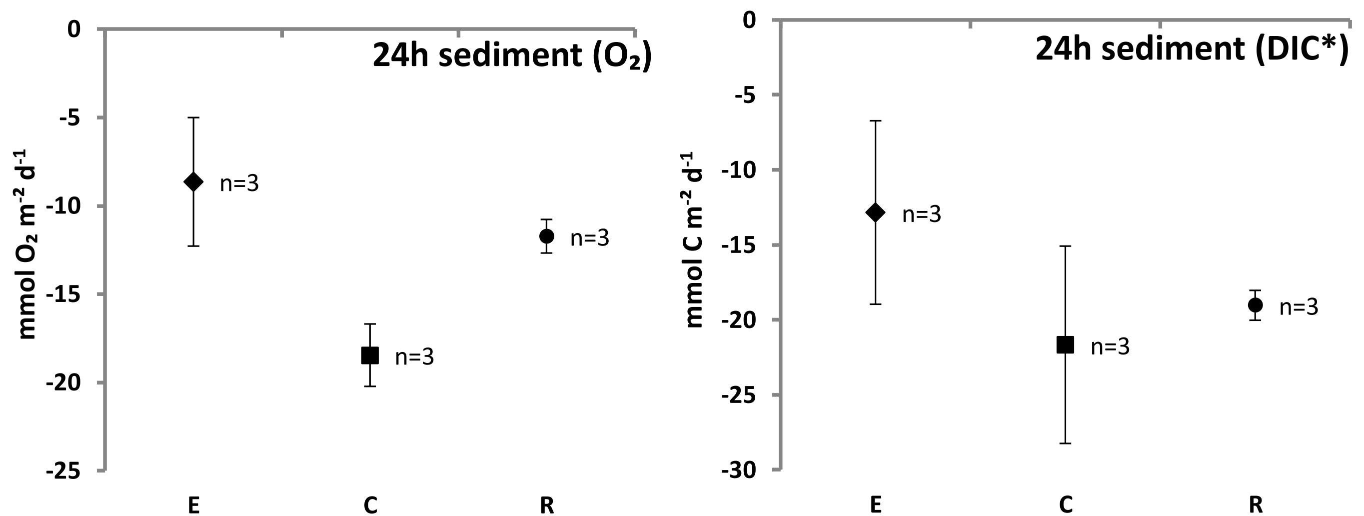


Figure 8. Rates for the 24h sediment incubations in mmol O_2 m^{-2} d^{-1}in September, 2013. Standard deviations are shown by error bars (left). Rates for the 24h sediment incubations in mmol C m^{-2} d^{-1} are shown for two sediment incubations in September, 2013 (right).

The calcification rates estimated for the sediment incubation in July are varying between -1.2 and -13.6 mmol $CaCO_3$ m^{-2} d^{-1} during light conditions and between -11.2 and 6.8 mmol $CaCO_3$ m^{-2} d^{-1} during the dark incubation. The mean daily calcification rates in July are -12 ± 13, -12 ± 7, and -11 ± 10 mmol $CaCO_3$ $m^{-2}d^{-1}$ for E, C and R, respectively. The daily mean calcification rates estimated for the sediment incubation in September are -4 ± 2, -2 ± 1 and 3 ± 1 mmol $CaCO_3$ $m^{-2}d^{-1}$ for E, C and R, respectively.

3.2 Other parameters

Production and respiration were related to leaf biomass ($r^2 = 0.36$ and 0.21, $p < 0.001$ respectively). The regression lines between the oxygen rates and leaf biomasses are significantly different from zero (Fig. 9).

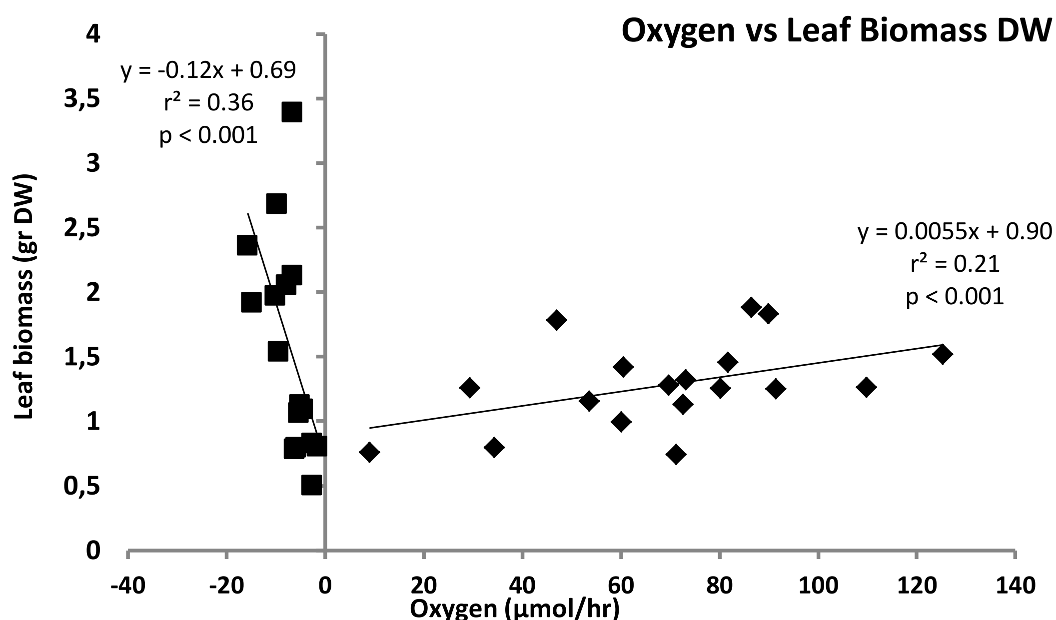


Figure 9. The leaf biomass (gr DW) is related to production and respiration rates (μmol O_2/hr) for both light incubations (diamonds) and dark incubations (squares).

Calcification and the epiphyte biomass are significantly related (r^2 = 0.14, p-value < 0.001). The regression line between the calcification rates and epiphyte biomass is significantly different from zero (Fig. 10).

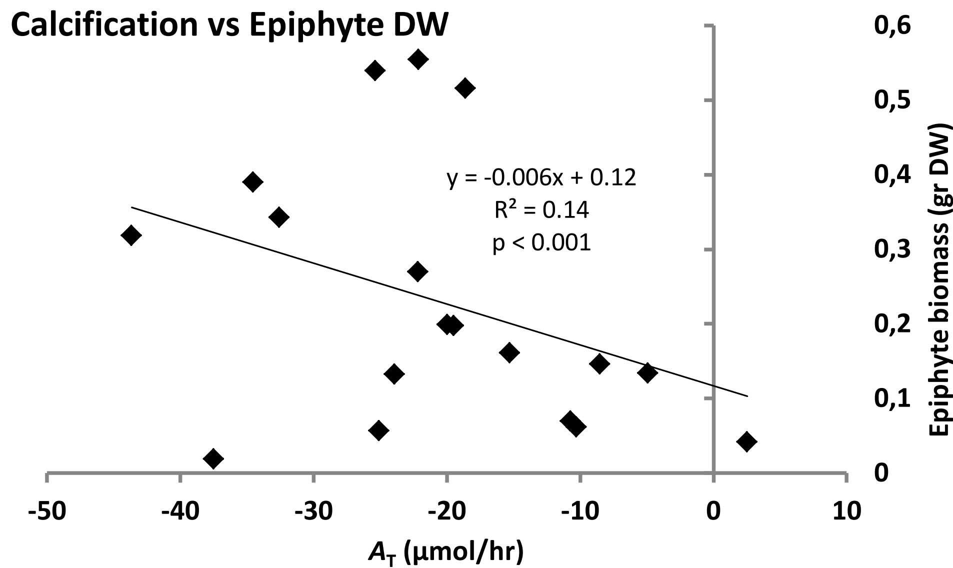
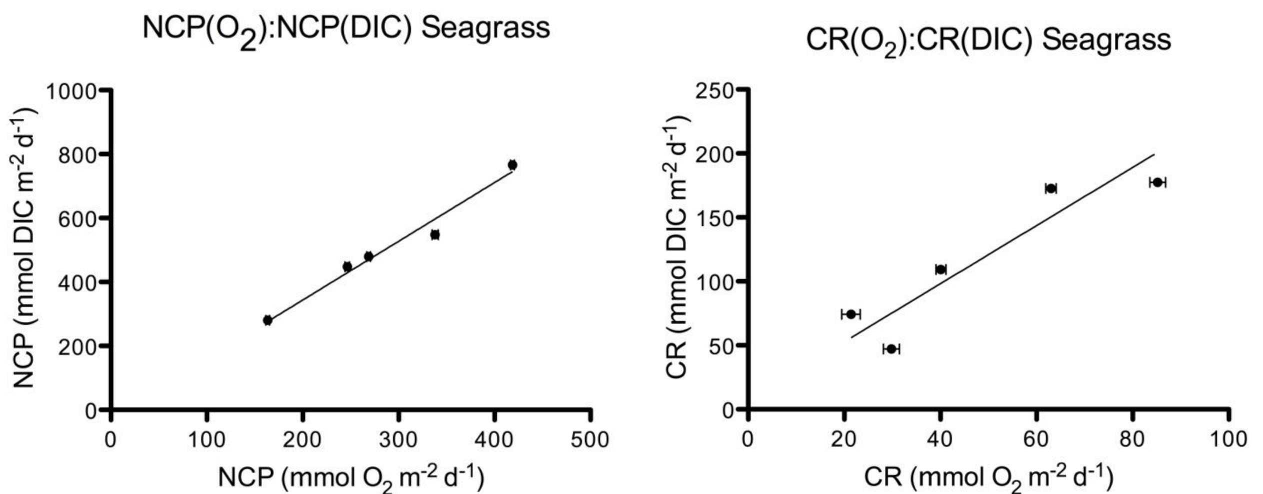


Figure 10. The total alkalinity (μmol/hr) plotted against the epiphyte biomass (gr DW) for seagrass light incubations.

3.3 Metabolism ratios

A Deming Regression gave a NCP_{O2}:NCP_C ratio that is 1.8 ± 0.2 for seagrass shoots and 1.9 ± 1.5 for sediment incubations (Fig. 11). The CR_{O2}:CR_C ratio is 2.2 ± 0.6 for the seagrass and 1.0 ± 0.4 for sediment (Fig. 11).



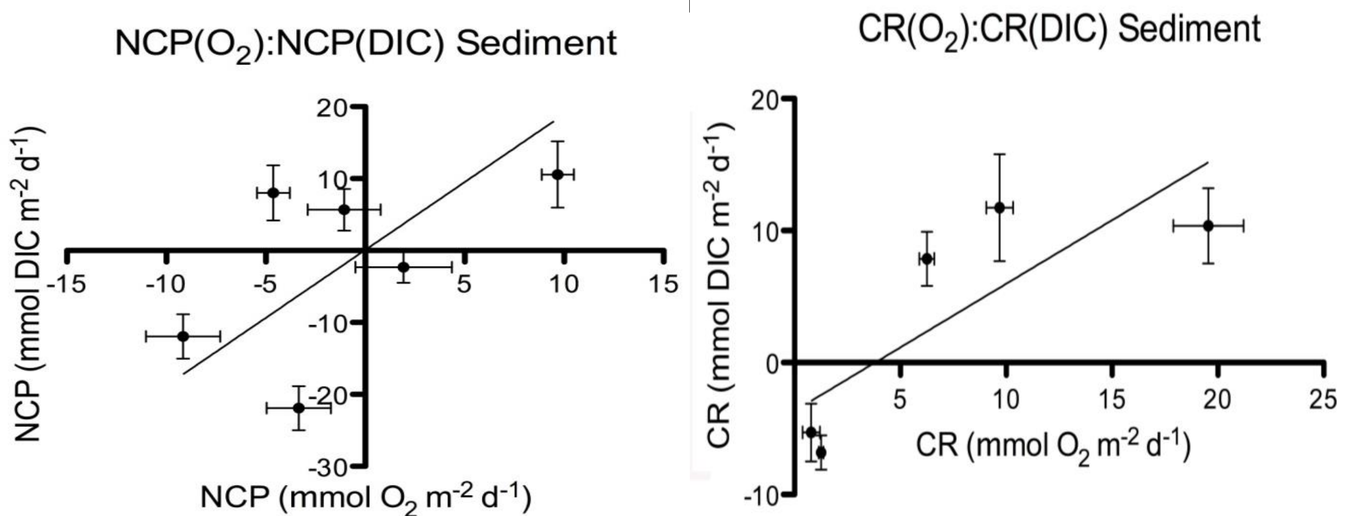


Figure 11. The NCP_{O2}:NCP_{DIC} ratio for seagrass (~1.8) (top-left); the CR_{O2}:CR_{DIC} ratio for seagrass (~1.9) (top-right); the NCP_{O2}:NCP_{DIC} ratio for sediment incubations in July 2013 (~2.2) (bottom-left) and; the CR_{O2}:CR_{DIC} ratio for sediment in July 2013 (~1.0) (bottom-right). A Deming regression technique is used.

A Deming regression gave a ratio between O_2 and C NCP rates of 1.5 ±0.3 with a 95% confidence interval of 0.55 to 2.42 for the sediment incubation in September 2013 in the bay of Villefranche (Fig. 12).

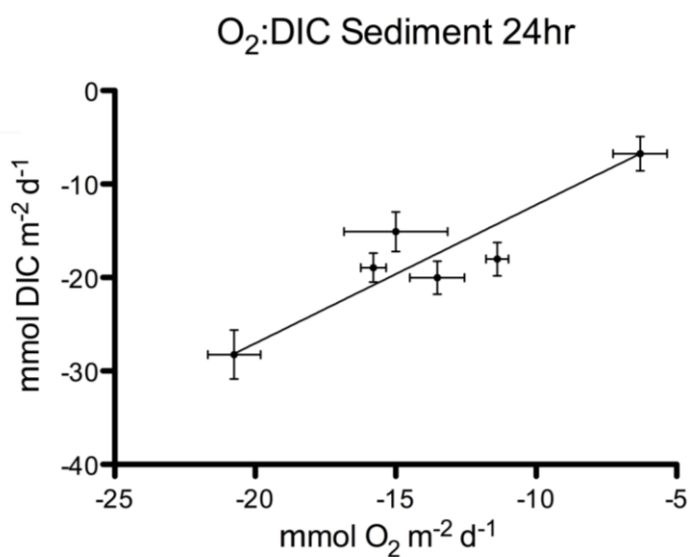


Figure 12. The NCP_{O2}:NCP_{DIC} ratio for sediment (~1.5) in September, 2013, in the bay of Villefranche. A Deming regression technique is used.

4. Discussion

I described the production and calcification rates in above and below-ground parts of a seagrass meadow. Overall results show seagrass shoots to be net autotrophic and the sediment to be heterotrophic in July. There was some variability in production and calcification rates among treatments and between months, however the difference was only significant in a few cases. This variability in measurements with the current sample size might prevent accurate assessments of OA impact. There was a significant difference between the months July and September in the NCP and GPP rates of the control plot. A significant difference was also observed between the different plots in July for NCP and GPP and in September for CR.

Autotrophic seagrass communities have been observed in studies on seagrass meadows in the Mediterranean Sea. Values measured in this study are much higher compared to values observed in e.g. Mallorca Island, Spain (Barrón et al. 2006); Palma Bay, Mallorca (Gazeau et al. 2005); and Sounion, Greece (Apostolaki et al. 2010) (Table 1). However, the incubation setup in our study only encloses seagrass blades and part of the stem, and the other parts of the plant and other organisms are not enclosed.

Table 1. The metabolism values of the different studies conducted in the Bay of Villefranche, Magalluf Bay, Palma Bay and the Aegean Sea. The bay of Villefranche incubation setup only encloses seagrass blades, and not encloses encounters like the other three studies.

		NCP		CR		GPP	
		mmol O_2 m^{-2} d^{-1}	mmol C m^{-2} d^{-1}	mmol O_2 m^{-2} d^{-1}	mmol C m^{-2} d^{-1}	mmol O_2 m^{-2} d^{-1}	mmol C m^{-2} d^{-1}
Bay of Villefranche, France	July	238	483	44		282	
	Sept.	158		29		191	
Magalluf Bay, Mallorca (Barrón et al. 2006)	July		40		100		135
	Sept.		-10		40		20
Palma Bay, Mallorca (Gazeau et al. 2005)	July	40					
	Sept.	-10					
Aegean Sea, Greece (Apostolaki et al. 2010)	July		55		120		175
	Sept.		20		50		75

4.1 Seagrass metabolism

The variability in the parts of the community measured and/or measurement methods might explain a large part of the differences between the studies mentioned above. All studies show lower metabolism values compared to the values in the bay of Villefranche. Differences between the different studies might be explainable by difference in local conditions, e.g. light penetration, temperature, current velocity, form of inorganic carbon, and nutrient availability (Hemminga and Duarte, 2000). Barrón *et al* [2006] and Apostolaki *et al* [2010] present their values in carbon units instead of oxygen units, which gives an overestimation of 1.1 to 1.2 for the NCP and CR, respectively (Apostolaki et al. 2010). All studies used an in situ benthic chamber

consisting of a PVC cylinder and a polyethylene plastic bag fitted to this cylinder (Hansen et al. 2000; Gazeau et al. 2005; Barrón et al. 2006; Apostolaki et al. 2010). This design comprises the whole seagrass plant, including the below-ground roots and the sediment (Gazeau et al. 2005; Barrón et al. 2006; Apostolaki et al. 2010). Roots and rhizomes contribute about 15-57% to the respiration of the whole seagrass plant (Hemminga and Duarte, 2000). NCP is corrected for the respiration during the night. Therefore, the difference between our experiment and the other studies might be explained by the separation of the leaves from the shoot, roots and rhizomes of the plant. The heterotrophic values in September of seagrass of both studies on Mallorca Island might be explained by the difference of incubation device and thereby inclusion of different plant parts in the incubation. The metabolism values of seagrass shoots in July were on average 1.5 fold higher compared to those in September. The study in the Aegean Sea also found a ratio of 1.5 for July and September (June and August for the Aegean Sea study) for seagrass communities (Apostolaki et al, 2010). The temperature in July was with 23.5°C higher than the 20.3°C in September, while *in situ* PAR was ~140 μmol m^{-2} sec^{-1} in July and ~83 μmol m^{-2} sec^{-1} in September. Both a higher temperature and irradiance in July (Appendix A) might explain the difference in NCP partially (Marbà et al. 1996).

4.2 Sediment metabolism

The sediment gave different outcomes for the different plots varying between autotrophic and heterotrophic in July. The NCP (O_2) rates of the bare sediment vary between 10 and -10 mmol O_2 m^{-2} d^{-1} in July. Other studies observed mostly autotrophic sediment in July; however some of the metabolism rates of the sediment were around zero. Apostolaki *et al* [2010] observed NCP of ~5 mmol C m^{-2} d^{-1} in June 2006 in the Aegean Sea. In Magalluf Bay, Mallorca, the NCP was ~5 mmol C m^{-2} d^{-1} in July (Barrón et al. 2006). A NCP of 0 was observed in Palma Bay, Mallorca, in July, 2001 and 2002, for the bare sediment (Gazeau et al. 2005). The NCP, CR and GPP of the studies by Barrón *et al* [2006] and Apostolaki *et al* [2010] are displayed in carbon units instead of oxygen units, which gives an overestimation of 1.1 to 1.2 (Apostolaki et al. 2010). Sediment incubations in September gave a mean NCP of -13 ±6 mmol O_2 m^{-2} d^{-1}, while the other studies had sediment which was autotrophic. NCP in Magalluf Bay in September 2001 was ~2 mmol C m^{-2} d^{-1} (Barrón et al. 2006). Apostolaki *et al* [2010] observed in September 2006 a NCP of ~35 mmol C m^{-2} d^{-1} for sediment in the Aegean Sea.

CR rates measured during our sediment incubations were quiet comparable to studies in the Aegean Sea and Magalluf Bay. Our rates were fluctuating between 1 and 20 mmol O_2 m^{-2} d^{-1} in July, while to the mean rate in June in the Aegean Sea was ~20 mmol C m^{-2} d^{-1} (Apostolaki et al. 2010) and was ~5 mmol C m^{-2} d^{-1} in July in Magalluf Bay (Barrón et al. 2006).

The same counts for GPP rates measured in our study compared to the studies conducted in the Aegean Sea and Magalluf Bay, Mallorca. GPP was 6 ±8 mmol O_2 m^{-2} d^{-1} in July 2013 for the sediment in the bay of Villefranche-sur-Mer. Barrón *et al* [2006] observed a GPP of ~5 mmol C m^{-2} d^{-1} in July in Magalluf Bay. The GPP in the study on a seagrass meadow in the Aegean Sea in June 2006 was ~15 mmol C m^{-2} d^{-1} (Apostolaki et al. 2010).

4.3 NCP_{O2}:NCP_{DIC} ratios

The mean NCP value in July was 483.8 ±222.4 mmol C m^{-2} d^{-1} for the seagrass which is high compared to the oxygen NCP value observed in the bay of Villefranche. The NCP_{O2}:NCP_{DIC} ratio seen in the seagrass incubations in the bay of Villefranche was 1.8. Gazeau et al [2005] estimates a NEP(DIC):NEP(O_2) ratio of 2:1 over a *Posidonia oceanica* meadow based on surface water DIC and O_2. However, after correction of the DIC budget for the $CaCO_3$ production (net DIC consumption) the ratio comes close to 1:1. Barrón *et al* [2006] observed NCP_{O2}:NCP_{DIC} ratios ranging from ~0.5 to 3.0 for *Posidonia oceanica* benthos (mean 0.7). A study in Laguna Madre (Texas, US) observed GPP ratios which ranged from 0.15 to 1.11 (Ziegler and Benner, 1998). The CR ratio for seagrass was 2.2 ±0.6. An earlier study observed large standard deviations in the CR ratio of a *Thalassia testudium* meadow in Laguna Madre as well (Ziegler and Benner, 1998).

The NCP and CR ratios for sediment incubations in July were 1.9 ±1.4 and 1.0 ±0.4, respectively. The NCP_{O2}:NCP_{DIC} ratio for 24 h sediment incubations in September was 1.5 ±0.3. These values are lower than the range of ratios observed in a study at Magalluf Bay which acquired NCP_{O2}:NCP_{DIC} ratios fluctuating between 0.2 and 1.2 for unvegetated sediments (Barrón et al. 2006). The variability in the NCP/CR rates of sediment estimated from oxygen and dissolved inorganic carbon has been observed before in two different studies (Ziegler and Benner, 1998; Barrón et al. 2006).

4.4 Calcification

Precipitation of 1 mole of $CaCO_3$ leads to a decrease of 2 moles in total alkalinity (Wolf-Gladrow et al. 2007). Total alkalinity measurements in our incubations are not corrected for the electro neutrality; nitrification; denitrification; nitrogen, phosphorus, silicon and sulfur assimilation and remineralization; methane oxidation by sulphate reduction; and the effect of production by marine plankton (Wolf-Gladrow et al. 2007). The measurement of changes in A_T of seawater is considered as the most convenient method to estimate calcification/dissolution processes (Smith and Key, 1975; Chisholm and Gattuso, 1991). Barrón *et al* [2006] observed calcification rates in Magalluf Bay values of ~-13 and ~5 mmol $CaCO_3$ m^{-2} d^{-1} for July and September, 2001, respectively. The mean rates of ~-107 and ~-150 mmol $CaCO_3$ m^{-2} d^{-1} we observed in the bay of Villefranche on seagrass plants in July and September 2013, respectively, are very high compared to these rates. Similar to the variations observed in the oxygen and DIC rates, part of the variation might be explained by the difference in experimental design. In our experimental design the sediment, roots, rhizomes and shoots are excluded which gives a difference in the calcification rates. The relation observed between the calcification rates and the epiphyte biomass is significantly from zero. This might be explained by the fact that dead epiphytes are included in the biomass as well.

The sediment in the bay of Villefranche gave mean rates of -12.0 ±18.2 and -1.0 ±2.6 mmol $CaCO_3$ m^{-2} d^{-1} for July and September 2013, respectively. A correction for the experimental design by including the sediment may not explain the high variations between the two studies. However, previous studies show that the effect of sediment on alkalinity depends on the many factors including: grain size and composition of the sediment,

nutrient and organic input, temperature and hydrodynamics (Alongi et al. 1996). The reactions affecting alkalinity can be calculated by using TA_{EC} as described by Wolf-Gladrow *et al* [2007]. A difference in sediment composition and metabolic and geochemical reactions of this sediment might explain the variations between the study in Magalluf Bay and the bay of Villefranche.

5. Conclusion

The analysis of oxygen and DIC fluxes depicted the studied seagrass meadow as a net autotrophic community at the daily time scale. The analysis of the seagrass metabolism gave variable results. However, they were only significantly different within the plots in July for NCP and GPP and in September for the CR, and significantly different between the two months (July and September) in the control patch for NCP and GPP. The analysis of the sediment patches gave variable results, however not significantly different. The NCP_{O2}:NCP_{DIC} ratio was similar for seagrass shoots and sediment patches and was within the range of ratios observed before in Magalluf Bay, Mallorca. The metabolism findings in the bay of Villefranche usually gave rates much higher compared to other analyses of daily seagrass community metabolism. While our experiment incubates the plant from the top of the leaf until the start of the shoot, these other analyses use incubation designs which includes the sediment, roots, rhizomes and shoots in their seagrass incubation. This variability in incubation design might explain the differences partly. The mean calcification rates observed on the seagrass meadow in the bay of Villefranche differ from rates observed in Magalluf Bay, Mallorca, in the same months in 2006. This variation could be due to a difference in experimental design, including the possible death calcifying epiphytes in the calculation, and local factors varying between time and place.

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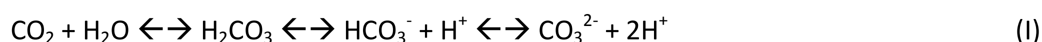
Chapter 2: Validation of alkalinity anomaly technique to quantify calcification rates in *Mytilus galloprovincialis*.

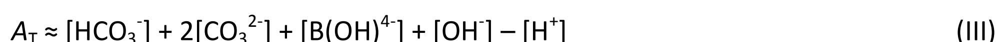
1. Introduction

The absorption of anthropogenic carbon dioxide (CO_2) by the ocean is causing changes in seawater chemistry (Sabine et al. 2004; Solomon et al. 2007). The absorbed CO_2 causes an increasing concentration of H^+ ions and therefore a decrease in the pH of seawater commonly known as ocean acidification (OA) (Caldeira and Wicket, 2003; Caldeira and Wicket, 2005). This shifts the equilibrium of seawater carbonate towards more CO_2 and HCO_3^- and less CO_3^{2-} (Eqs. I; Zeebe and Wolf-Gladrow, 2003):



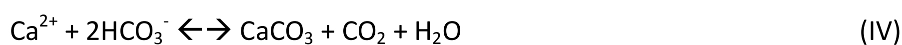
$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad \text{(I)}$$

The average pH and carbonate ions (CO_3^{2-}) of surface seawater could decrease by 0.2 - 0.4 unit and by 30-50% by the end of the 21st century, respectively (Caldeira and Wicket, 2003; Caldeira and Wicket, 2005).
Ocean acidification affects marine organisms with a calcareous skeleton because of their requirement of carbonate ions (Kleypas et al. 2006). Calcification and decalcification, known as the formation and dissolution of calcium carbonate ($CaCO_3$), respectively, are important components of the oceanic carbon cycle (Kleypas et al, 2009). Studies have shown the negative impact of ocean acidification on calcifying benthic organisms, such as corals (Kleypas et al. 2006), coralline algae (Semesi et al. 2009) or mollusks (Gazeau et al. 2007).
Calcification is generally estimated using total alkalinity (A_T) and dissolved inorganic carbon (DIC) rates (Smith and Key. 1975, Gattuso et al. 1993). A_T is known as the excess of bases (proton acceptors) over acids (proton donors). In seawater, A_T is mostly determined by the proton acceptors bicarbonate, carbonate, borate and hydroxide, and the hydrogen ion as a proton donor (Eqs. III; Zeebe and Wolf-Gladrow, 2003):



$$A_T \approx [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)^{4-}] + [OH^-] - [H^+] \qquad \text{(III)}$$

However, natural water samples contain various acid-base systems that can accept and donate protons (Wolf-Gladrow et al. 2007).These acid-base systems are included in the expression based on the choice of zero protons level which classifies chemical species into proton acceptors and proton donors for each acid-base system (Wolf-Gladrow et al. 2007). Based on the expression of inorganic carbon metabolism (Eqs. IV):



$$Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3 + CO_2 + H_2O \qquad \text{(IV)}$$

the production or dissolution of 1 mole of $CaCO_3$ involves a variation of 2 moles in A_T and 1 mole in dissolved calcium ions (Ca^{2+}) (Wolf-Gladrow et al. 2007). However, A_T can vary as well depending on other processes such as nitrification, denitrification, nitrogen- and phosphorus assimilation and remineralization (Wolf-Gladrow et al. 2007). Calcium does not suffer from such potential issues and the excess Ca^{2+} can only have originated from $CaCO_3$ dissolution, especially in shallow regions (de Villiers and Nelson, 1999). By measuring A_T and Ca^{2+} before and after incubation of calcifying organisms, we can estimate net calcification rates. The

alkalinity anomaly technique has been used to quantify calcification rates of coral reef communities (Smith and Kinsey, 1978; Gattuso et al. 1998), mollusks (Gazeau et al. 2007) and calcareous algae (Martin et al. 2009). However, this technique has only been validated for corals (Chisholm and Gattuso, 1991) and has not been tested for most calcifying organisms.

In the present study, the alkalinity anomaly technique has been used to quantify calcification rates of the Mediterranean Mussel, *Mytilus galloprovincialis* (Fig. 13) and these results have been compared to rates derived from calcium measurements. Mussels were incubated for 24 hours in a beaker with constant bubbling of air and a continuously water movement. Ca^{2+} and A_T measurements from water samples taken at 0, 2, 4, 6, 8 and 24 hours were used to estimate calcification rates based on both techniques. The A_T:Ca^{2+} ratio can be calculated based on these measurements and was compared with the adjusted A_T:Ca^{2+} ratio of 1.

Figure 13. Mediterranean mussels (*Mytilus galloprovincialis*) (http://www.marinespecies.org/photogallery.php?album=700&pic=49986).

2. Material and methods

2.1 Biological material

Mytilus galloprovincialis is considered native to the Mediterranean-, Black- and Adriatic Sea, however can be found worldwide in marine and estuarine habitats mainly due to human activities (Seed, 1976). *Mytilus galloprovincialis* is a dark colored mussel which can grow up to 15 cm, although usually is within the range of 5 to 8 cm (Seed, 1976).

2.2 Experiment description

A similar biomass of mussels was placed in three 5 L beakers with 4 L of filtered seawater. The mussels were incubated for 24 h with a constant bubbling of air and the water was stirred continuously with a magnetic bar (Fig. 14). Water samples were taken at 0, 2, 4, 6, 8 and 24 h. Alkalinity and calcium values were measured on each of these samples. During a previous study the values for ammonium, nitrite, nitrate and phosphorus were acquired.

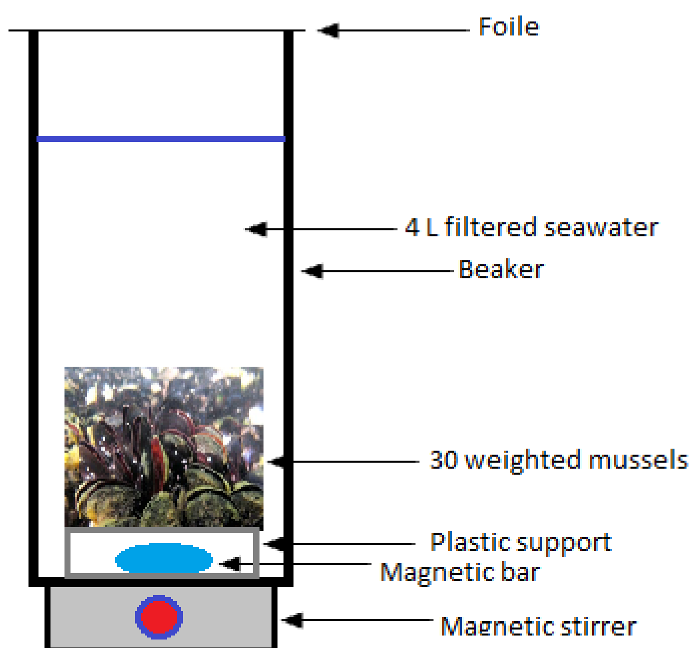


Figure 14. The setup of the 5 L beaker with the Mediterranean Mussel (*Mytilus galloprovincialis*) and a constant air-flow and water movement.

2.3 Analyses of parameters

A_T was determined based on the methods described in the book "Guide to Best Practices for Ocean CO_2 Measurements" Dickson (2007). A sample of 200 mL was filtered through GF/F 47mm diameter (Whatman®) filters and kept refrigerated until analysis (we added HgCl2). The measurements were performed in triplicate, from sub-samples of 50 mL by potentiometric titration using 0.1 N hydrochloric acid and a Metrohm Titrando® (at 22°C). The pH glass electrode was calibrated daily with (or against) a TRIS buffer provided by A. Dickson. The accuracy of the method was estimated by dosing 3 to 5 samples of standard seawater with known

alkalinity (commercial standards supplied by A. Dickson; http://andrew.ucsd.edu/co2qc/index.html) and this resulted in a coefficient of variation smaller than 0.11 %.
Calcium was determined using the ethylene glycol tetra acetic acid (EGTA) potentiometric titration modified from *Cao and Dai* [2011]. A sample of 60 mL was filtered through GF/F 47mm diameter (Whatman®) filters and kept refrigerated until analysis. 4 g of seawater (salinity of 38) and 4 g of $HgCl_2$ solution (~1 mmol L^{-1}) were accurately weighed. Then 5 g of a concentrated EGTA solution (~10 mmol L^{-1}) were added to complex Hg^{2+} completely and nearby 95% of Ca^{2+}. After adding 4 mL of borate buffer, the remaining Ca^{2+} is titrated by ~2 mmol L^{-1} EGTA up to the end point potential. This end point potential was given by the Metrohm Titrando potentiometer with an amalgamated silver combined electrode (Metrohm Ag Titrode). The software Metrohm TiAMO 2.3 gave the volume of the diluted solution of EGTA necessary for the remaining ~5% of Ca^{2+}. In the statistical software package R we then used a polynomial fit of the titration curve after which the equivalence point and corresponding volume were given. The titer of the EGTA solution was estimated daily based on the titration of IAPSO standard seawater (Osil). The coefficient of variation was smaller than 0.15 %.
Ammonium, nitrite, nitrate and phosphate values were measured in a previous study and used in this study to correct calcification rates estimated from A_T variations. Nitrite, nitrate and phosphate measurements had an average coefficient of variation of 3-4%. Ammonium measurements had an average coefficient of variation of 1 %.

2.4 Computations and statistical analysis

Calcification estimated from alkalinity are based on the formula:

$$G_{AT} = -1/2\ \Delta A_T \qquad (V)$$

Calcification estimated from calcium measurements are based on the formula:

$$G_{Ca} = -\ \Delta Ca^{2+} \qquad (VI)$$

The ratio of A_T:Ca^{2+} was estimated in previous studies at a value of 2 (Smith and Key, 1975). Because we measure calcification rates and corrected G_{AT} (=-1/2 ΔA_T) we use a A_T:Ca^{2+} calcification ratio of 1.
A_T corrected for ammonium excretion ($\Delta A_T = E_N = \Delta NH_4 + \Delta NO_2^- + \Delta NO_3^-$), phosphate excretion ($\Delta A_T = -E_P = -\Delta PO_4$) and nitrification ($\Delta A_T = -2N = -2 (\Delta NO_2^- + \Delta NO_3^-)$).

$$\Delta A_T = -2G_{AT} + E_N - E_P - 2N = -2G + \Delta NH_4 + \Delta NO_2^- + \Delta NO_3^- - \Delta PO_4 - 2 (\Delta NO_2^- + \Delta NO_3^-) \qquad (VII)$$

This gives the formula:

$$G^*_{AT} = -1/2\ \Delta A_T + \Delta NH_4 - \Delta NO_2^- - \Delta NO_3^- - \Delta PO_4 \qquad (VIII)$$

For total alkalinity, calcium, ammonium, nitrate, nitrite, and phosphate a linear regression is utilized to determine the variation in concentrations. A Deming regression is used to determine the ratio of G_{Ca} and G^*_{AT}.

3. Results

3.1 Chemistry conditions

A_T concentrations decreased linearly with a rate of -28.2 ± 0.78 μmol kg^{-1} hr^{-1} ($p < 0.0001$, N = 3, Fig. 15). The calcium concentrations decreased linearly with a rate of -17.5 ± 0.78 μmol kg^{-1} hr^{-1} ($p < 0.0001$, N = 3, Fig. 15).

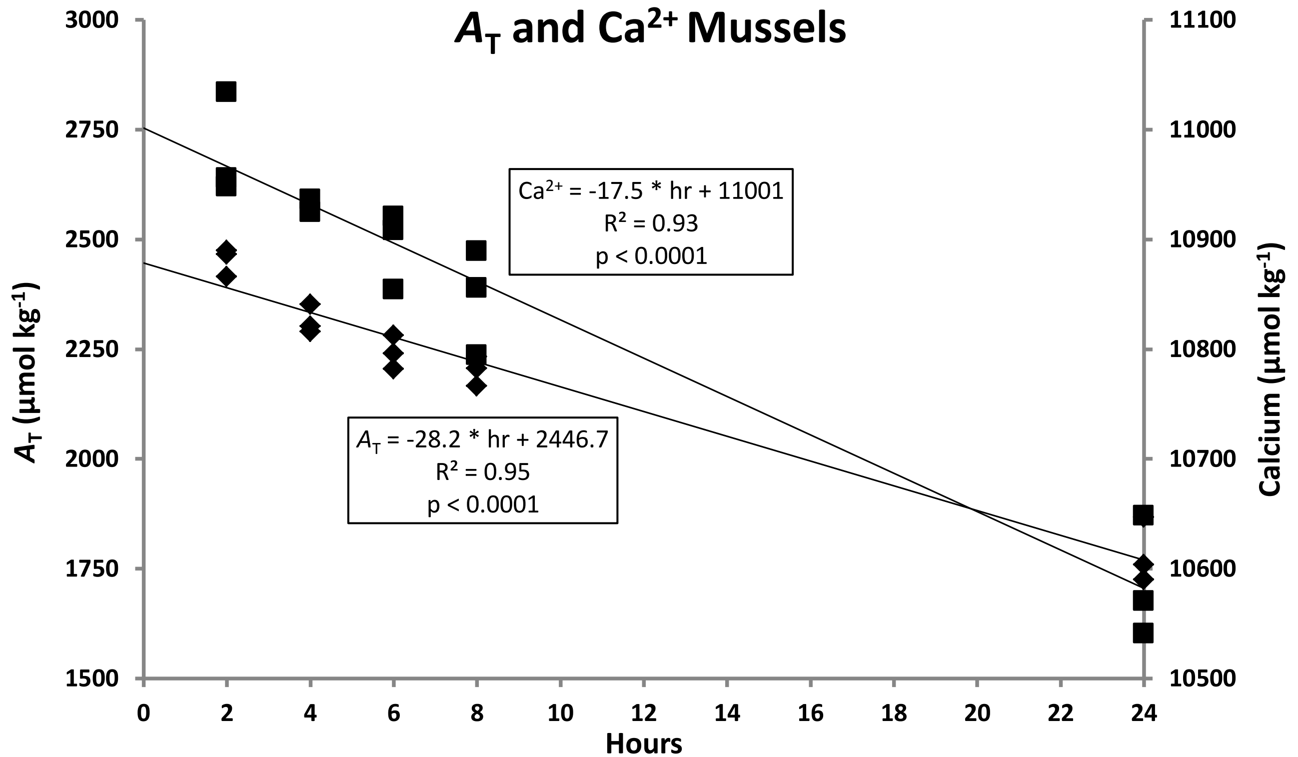


Figure 15. Variation in total alkalinity (diamonds) and calcium (squares) (both in μmol kg^{-1}) during the 24 h incubation of Mediterranean mussels (*Mytilus galloprovincialis*).

Ammonium concentrations increased linearly with a rate of 2.2 ±0.1 μmol kg^{-1} hr^{-1} ($p < 0.0001$, N = 3, Fig. 16). Phosphate concentrations increased linearly with a rate of 0.78 ±0.02 μmol kg^{-1} hr^{-1} ($p < 0.0001$, N = 3, Fig. 16).

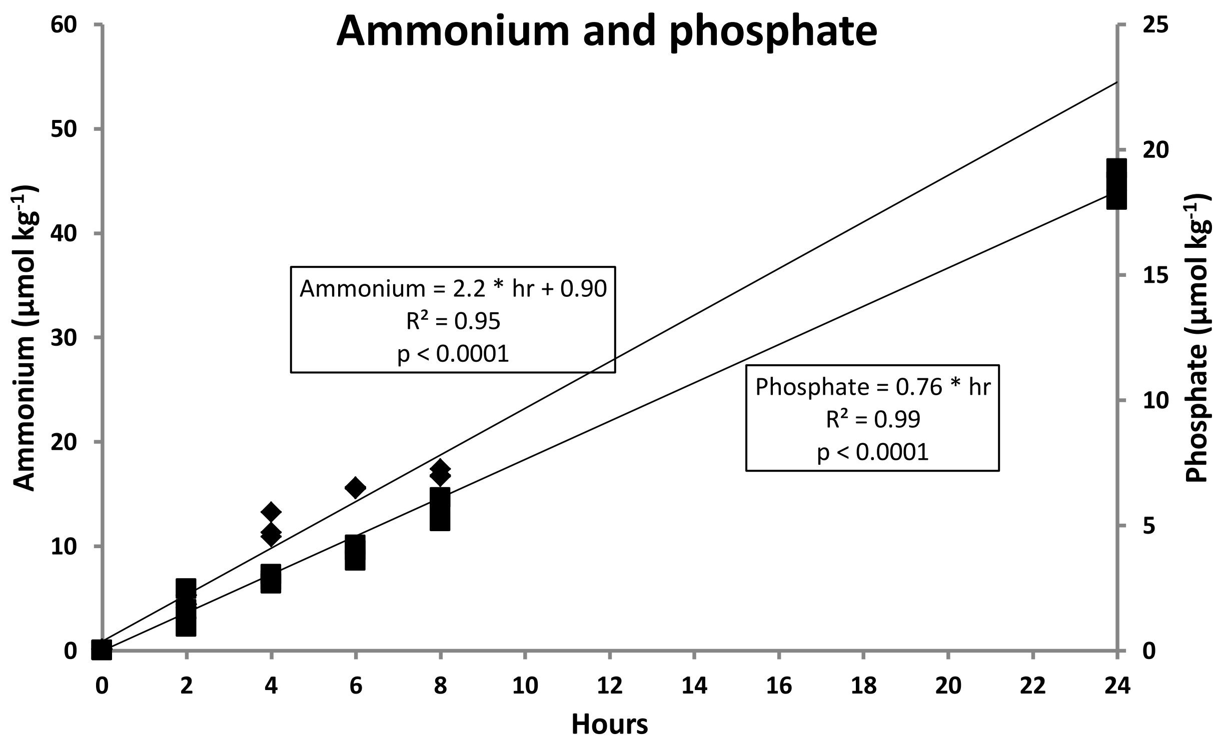


Figure 16. Variation in ammonium (diamonds) and phosphate (squares) concentrations (μmol kg^{-1}) during the 24 h incubation of Mediterranean mussels (*Mytilus galloprovincialis*).

Nitrate concentrations increased linearly with a rate of 0.08 ±6.4 E-03 μmol kg^{-1} hr^{-1} ($p < 0.0001$, N = 3, Fig. 17) and nitrate concentrations increased linearly with a rate of 0.03 ± 8.8 E-04 μmol kg^{-1} hr^{-1} ($p < 0.0001$, N = 3, Fig. 17).

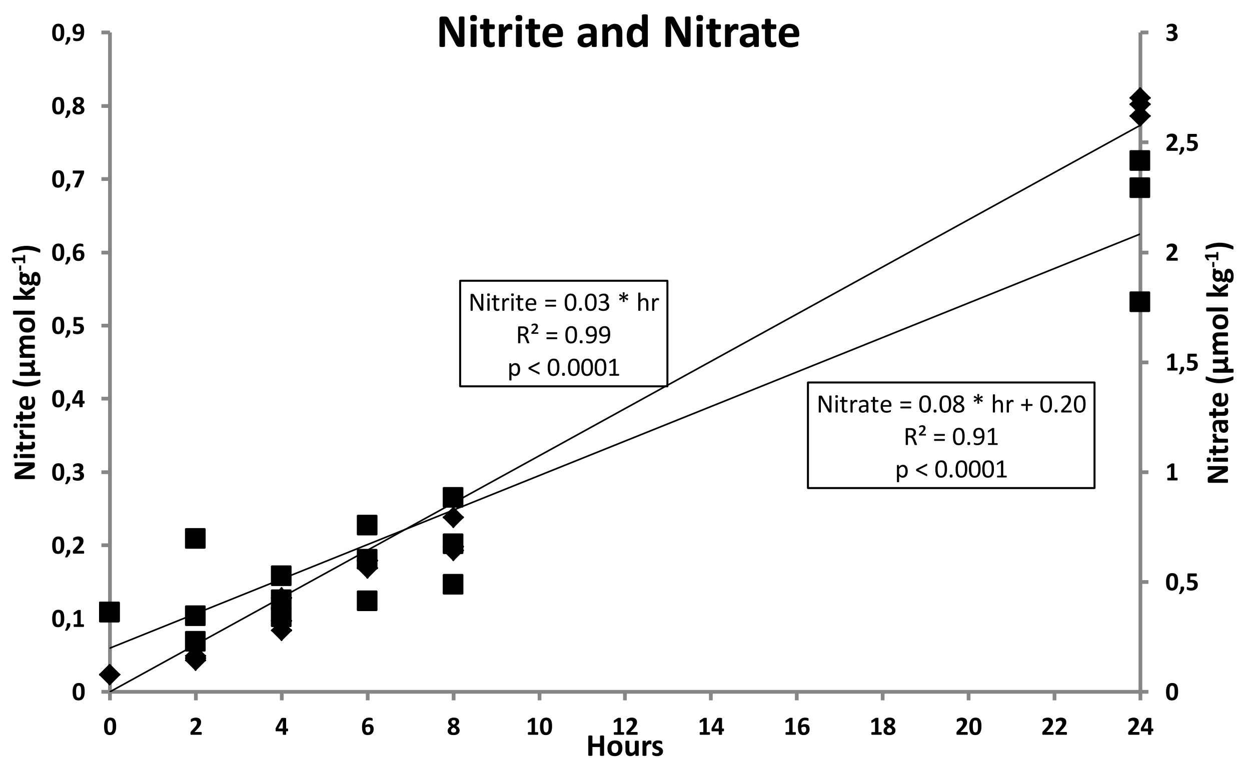


Figure 17. Variations in nitrate (squares) and nitrite (diamonds) concentrations (μmol kg^{-1}) during the 24 h incubation of Mediterranean mussels (*Mytilus galloprovincialis*).

Releases of Nitrogen (= NH^4 - NO^{2-} - NO^{3-}) and Phosphorus (P) were significantly correlated with each other ($r = 0.94$). The ΔN:ΔP ratio for *Mytilus galloprovincialis* was 3.62 ±0.6.

3.2 Calcification

The calcification estimated from calcium variations (G_{Ca}) and calcification estimated from alkalinity variations considering that only calcification/dissolution are important (G_{AT}) give a ratio of 0.77 ±0.06 ($p < 0.0001$; 95% Confidence interval: 0.6 to 0.9) (Fig. 18).

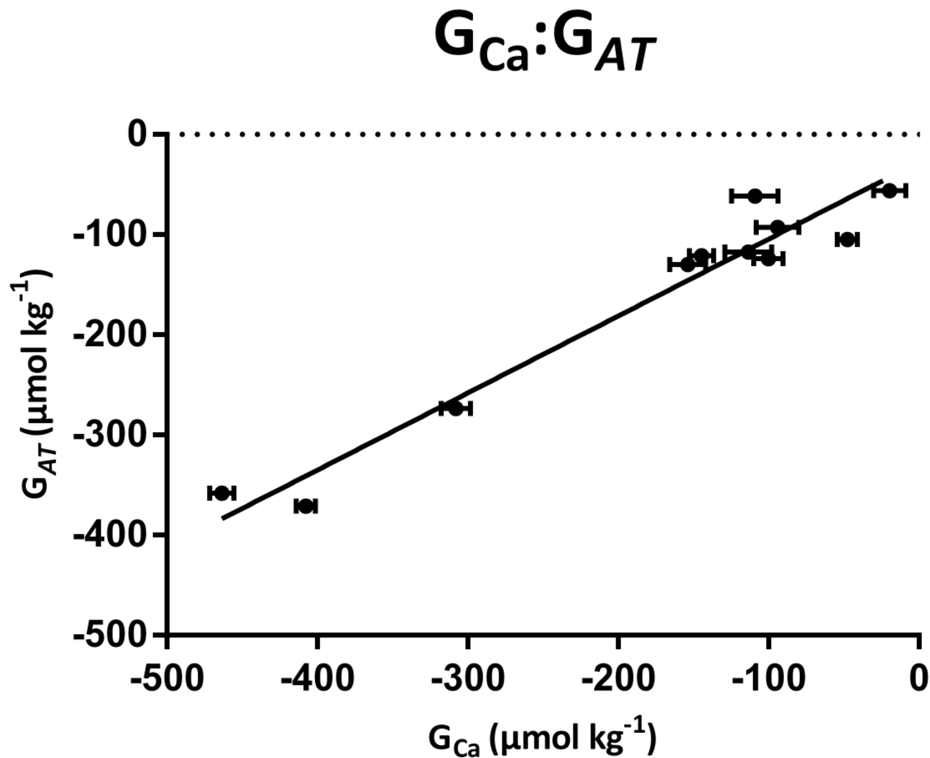


Figure 18. The G_{AT} plotted against the G_{Ca} (µmol kg^{-1}).

After correction of the total alkalinity, the ratio of G_{Ca} and G^*_{AT} was 0.82 ± 0.06 ($p < 0.0001$; 95% Confidence interval: 0.7 to 1.0) (Fig. 19).

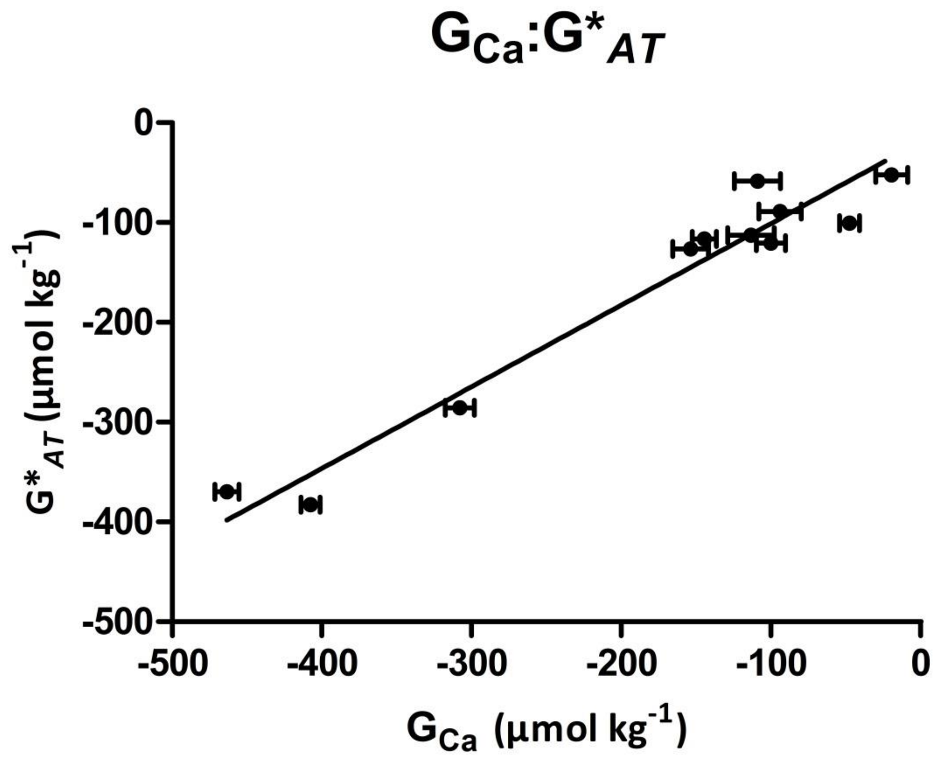


Figure 19. The G^*_{AT} plotted against the G_{Ca} (μmol kg^{-1}).

4. Discussion

4.1 Chemistry conditions

Total alkalinity (A_T) is affected by both inorganic and organic carbon production/consumption (Wolf-Gladrow et al. 2007). The A_T approach usually includes the introduction of an A_T* term, which includes the influence of organic carbon production/consumption, electro neutrality and uptake of nutrients, nutrient fluxes and more (Wolf-Gladrow et al. 2007). This study includes the effect of nitrification, remineralization of nitrogen and remineralization of phosphorus on total alkalinity. Recent measurements indicated that ammonium concentrations were (2.5 x too) low, because the ratio of ammonium and phosphate in these measurements was ~8. After estimating the new ammonium release values, a new calcification rate could be calculated. The effects of organic carbon release are not included which might give an over/underestimation of the calcification based on A_T (Kim and Lee, 2009). However, most of DOC, excreted as Urea, is uncharged and thereby not affects A_T (Seed et al, 1976).

4.2 Calcification

Net calcification estimated from calcium variations (G_{Ca}) and net calcification estimated from alkalinity variations considering that only calcification/dissolution are important (G_{AT}) give a ratio of 0.77 ±0.06, which is significantly different from the adjusted theoretically estimated ratio of 1 (Smith and Key, 1975). After correcting the variations in alkalinity (G^*_{AT}) for the effect of excretion of N (nitrification and remineralization of nitrogen) and excretion of P (remineralization of phosphorus) the ratio becomes 0.82 ±0.06. The fact that the ratio, with corrected alkalinity, is different from 1 must be explained by an additional source/sink of alkalinity that remains unknown. Most of the produced DOC (Urea) seems to be uncharged and seems to not affect the corrected alkalinity rates. However, the ratio with estimated ammonium release, based on the ratio of ~8 with phosphate uptake, was 1.09 ±0.08. The main reason that the ratio of G_{Ca} and G_{AT} is different from 1 seems to be the incorrect measurements for ammonium release. Further research with the correct ammonium measurements might confirm this presumption. Within this study the alkalinity anomaly method appears to be not valid for bivalve species, however with the estimated ammonium release taken into account the method seems valid.

5. Conclusion

The Mediterranean mussel (*Mytilus galloprovincialis*) incubation measurements of chemistry conditions gave ratios for uncorrected alkalinity (G_{Ca}:G_{AT}) and corrected alkalinity ($G_{Ca:}G^*_{AT}$) which were both significantly different from the adjusted theoretically estimated ratio of 1. The fact that the corrected alkalinity and calcium ratio is different from 1 must be explained by an additional source/sink of alkalinity that remains unknown. A suggestion is that the ammonium release was measurement incorrectly which is indicated by a recalculation based on estimated ammonium release. The alkalinity anomaly method appears to be not valid for bivalve species; however with the estimated ammonium release taken into account the method seems valid. Further research has to be done to investigate the (suggested) factor for this difference.

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Appendix A

Table 2. The temperature (°C) and PAR (μmol/sec/m^2) data for the different incubations conducted from July till September.

Date	Incubation	Temperature (°C)	PAR (μmol/sec/m^2)
17-07-2013	Sediment Light Experiment	20.94 (±0.2)	136.5 (±78)
17-07-2013	Sediment Light Control	21.33 (±0.4)	128.9 (±80)
17-07-2013	Sediment Light Reference	20.61 (±0.5)	128.2 (±56)
25-07-2013	Seagrass Light Experiment	23.39 (±0.25)	146.0 (±49)
25-07-2013	Seagrass Light Control	23.60 (±0.23)	114.5 (±58)
25-07-2013	Seagrass Light Reference	23.34 (±0.25)	157.3 (±44)
31-07-2013/05-08-2013	Seagrass Dark Experiment	19.12 (±0.24)	ND
31-07-2013	Seagrass Dark Control	19.37 (±0.30)	ND
31-07-2013/05-08-2013	Seagrass Dark Reference	19.21 (±0.24)	ND
28-08-2013	Sediment 24hr Experiment	27.72 (±0.62)	43.7 (±81)
28-08-2013	Sediment 24hr Control	24.07 (±0.09)	44.2 (±81)
28-08-2013	Sediment 24hr Reference	24.05 (±0.13)	30.9 (±58)
04-09-2013	Sediment 24hr Experiment	23.07 (±0.64)	45.5 (±72)
04-09-2013	Sediment 24hr Control	23.15 (±0.65)	49.5 (±71)
04-09-2013	Sediment 24hr Reference	23.07 (±0.71)	54.5 (±84)
05-09-2013	Sediment 24hr Experiment	23.71 (±0.32)	61.3 (±88)
05-09-2013	Sediment 24hr Control	23.79 (±0.31)	58.4 (±84)
05-09-2013	Sediment 24hr Reference	23.82 (±0.34)	57.2 (±81)
10-09-2013	Seagrass Light Experiment	20.28 (±0.44)	80.0 (±66)
10-09-2013	Seagrass Light Control	20.42 (±0.54)	72.3 (±63)
10-09-2013	Seagrass Light Reference	ND	98.0 (±70)
12-09-2013	Seagrass Dark Experiment	22.62 (±0.07)	ND
12-09-2013	Seagrass Dark Control	22.65 (±0.07)	ND
12-09-2013	Seagrass Dark Reference	ND	ND