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The effects of ocean acidification on calcification and incorporation of isotopes in Mediterranean pteropods and foraminifers

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The effects of ocean acidification on calcification and incorporation of isotopes and elements in Mediterranean pteropods and foraminifers

by

Ella Louise Howes

A thesis submitted in partial fulfilment

of the requirements for the degree of

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In Geosciences

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(Double Doctoral Agreement between Jacobs University and the University of Pierre et Marie Curie, Paris VI)

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Statutory Declaration

I, **Ella Louise Howes** , hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has been submitted to l'Université Pierre et Marie Curie and Jacobs University Bremen for conferral of a co-tutelle degree. This thesis has not been submitted to any other institutions for conferral of a degree elsewhere.

Bremen, March 18, 2014

Signature _____

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Les effets de l'acidification de l'océan sur la calcification et l'incorporation d'éléments et d'isotopes par des ptéropodes et foraminifères de Méditerranée.

Etant des producteurs pélagiques majeurs d'aragonite et de calcite, il est essentiel d'évaluer l'effet qu'aura le changement climatique global sur les ptéropodes et les foraminifères. Cette étude a utilisé une approche interdisciplinaire pour évaluer les effets de l'acidification de l'océan sur des espèces Méditerranéennes. Une comparaison d'échantillons conservés et modernes de deux espèces de ptéropodes a été réalisée pour étudier les effets du pH sur les propriétés de la coquille. Des populations de ptéropodes ont également été analysées à l'aide des données de séries temporelles. Pour permettre l'amélioration de futures expériences de perturbation, une revue collaborative des techniques de culture de ptéropodes a été produite. Enfin, le foraminifère *O. universa* a été cultivé dans des conditions de pH et de [CO_3^{2-}] découplés pour évaluer les effets des changements dans la chimie du carbonate sur la composition en bore et le fractionnement isotopique. Les coquilles des échantillons conservés de ptéropodes étaient plus épaisses que celles collectées en 2012 et les coquilles de *C. inflexa* recueillies en 1910 étaient nettement plus denses que celles de 2012, probablement en raison d'un effet de pH. Les abondances de ptéropodes ont montré une tendance croissante entre 1967-2003 et se sont révélées être influencées par les changements de température interannuelles, sans que des changements dans les propriétés des coquilles n'ai eu d'impacts négatifs. Lors de la dernière étude le pH était le seul paramètre du système de carbonate a affecter le δ11B de la calcite de *O. universa*. Le rapport B/Ca a diminué avec la diminution du [CO_3^{2-}] à pH constant mais n'a pas montré de tendance cohérente avec une [CO_3^{2-}] constant et un pH variable. Au lieu de cela, une étroite corrélation entre les rapports B / Ca et [HCO_3^-] a été observée suggérant que le bore est contrôlé par la [HCO_3^-], fournissant une proxy pour les reconstructions du système paléo-carbonate. Cette étude suggère que, dans la mer Méditerranée, les changements de taux de saturation en aragonite peuvent affecter la structure des coquilles de certaines espèces de ptéropodes, mais l'abondance est influencée par la température.

Mots-clés : L'acidification des océans, le changement global, thecosome ptéropode, foraminifères planctoniques.

The effects of ocean acidification on calcification and incorporation of isotopes and elements in Mediterranean pteropods and foraminifers

As the major pelagic producers of aragonite and calcite, it is essential to evaluate what effect climate change will have on pteropods and foraminifers. This study used an interdisciplinary approach to assess the effects of ocean acidification on Mediterranean species. A comparison of museum and modern samples of two pteropod species investigated the effects of pH on shell properties. Pteropod populations were analysed using time series data. To improve future perturbation experiments, a collaborative review of pteropod culture techniques was produced. The foraminifer *O. universa* was cultured under decoupled pH and [CO_3^{2-}] to assess the effects of changes in carbonate chemistry on boron incorporation and isotope fractionation. Museum pteropod samples were thicker than shells from 2012 and *C. inflexa* shells collected in 1910 were significantly denser than those from 2012, possibly due to a pH effect. Pteropod abundances displayed an increasing trend between 1967-2003 and are influenced by inter-annual temperature changes, with no sign of the observed changes in shell properties having had negative impacts. pH was the sole parameter of the carbonate system that affected the $\delta^{11}B$ of *O. universa* calcite. The B/Ca ratio decreased with decreasing [CO_3^{2-}] at constant pH but did not show consistent trends at constant [CO_3^{2-}] and varying pH. Instead, a close correlation of B/Ca ratios and [HCO_3^-] was observed suggesting that boron is controlled by the [HCO_3^-], providing a proxy for paleo-carbonate system reconstructions. This study suggests that, in the Mediterranean Sea, changes in Ω_a may affect shell structure of some pteropod species but abundance is influenced by temperature.

Key words: Ocean acidifcation, Global change, Thecosome pteropod, Planktonic Foraminifera

Les effets de l'acidification de l'océan sur la calcification et l'incorporation d'éléments et d'isotopes par des ptéropodes et foraminifères de Méditerranée.

Résumé :

L'acidification de l'océan est un processus mesurable en cours depuis le début de la révolution industrielle, cependant ces dernières années l'amélioration technologique et la croissance exponentielle de la population ont considérablement augmenté les émissions de carbone, altérant la chimie des carbonates, la spéciation d'éléments traces et des isotopes dans l'eau de mer. La mer Méditerranée a été identifiée comme une région particulièrement sensible au changement environnemental global et il a été suggéré que le taux d'acidification y a été plus élevé, surtout dans le bassin ouest, que dans les océans ouverts. Ces changements ont le potentiel d'affecter les capacités de calcification des organismes ainsi que la composition chimique et le fractionnement isotopique du carbonate de calcium qu'ils produisent. Etant les producteurs pélagiques majeurs respectivement de calcium et d'aragonite, les foraminifères et les ptéropodes ont une importance significative dans le cycle biogéochimique et les réseaux alimentaires. La connaissance de la réponse de ces organismes aux changements environnementaux actuels et lors d'évènements de perturbation du carbone comparables dans l'histoire géologique aidera à connaître les impacts potentiels des conditions futures prédites sur ces groupes.

La première étude utilise des échantillons collectés dans l'ouest de la Méditerranée durant les années 1910 à 1921 pour examiner si des changements environnementaux récents, causés par les émissions de CO_2, ont eu des effets mesurables sur les propriétés des coquilles de deux espèces communes de ptéropodes Méditerranéens. Les échantillons conservés et modernes (2012) de spécimens ont été comparés l'aide de la nouvelle approche de tomodensitométrie par rayon-X pour mesurer les biométriques des coquilles. Les échantillons conservés présentaient des coquilles plus épaisses que des coquilles de même longueur de 2012 et les coquilles collectées en 1910 de *C. inflexa* étaient significativement plus denses que celles de 2012. Ces changements impliquent que la protection de prédation et le control de la flottabilité offerts par les coquilles pourraient être compromis. Pour savoir si ces changements morphométriques des coquilles ont eu un effet délétère sur les populations de ptéropodes, lors d'une seconde étude, l'analyse d'une série temporelle de 37 ans des abondances de ptéropodes dans le nord-ouest de la Méditerranée a été réalisée. Le pH a été simulé

rétrospectivement pour la période totale d'étude et ses effets comparé avec d'autres variables incluant la température et salinité. Tous les groupes de ptéropodes ont montré une tendance à l'augmentation en abondance mais cependant n'ont pas montré d'effet délétère de la diminution de pH. Les populations de ptéropodes ont été influencées par des changements interannuels des températures d'été. Les analyses spectrales ont identifié une oscillation périodique de 14 ans dans toutes les espèces de ptéropodes, concomitant avec les oscillations de la température de surface de la mer, indiquant une influence possible du mode quasi décennal de l'Atlantique Nord sur les populations de ptéropodes. Ces résultats ne montrent pas de signes de changements dans les propriétés des coquilles vues au cours des 100 dernières années ayant eu des impacts négatifs sur l'abondance. Cependant, la sensibilité aux fluctuations de températures et aux changements d'épaisseur de coquille suggèrent que les ptéropodes Méditerranéens pourraient être en train d'approcher un « point seuil» comme les effets du changement global continuent d'accélérer.

Les études sur le long terme des effets de l'acidification de l'océan sur les ptéropodes a été rendu impossible par les difficultés de maintenir les cultures de laboratoires saines plus longtemps que 30 jours. Pendant le déroulement de cette thèse plusieurs techniques de culture de ptéropodes ont été testées sur une variété d'espèces. Le troisième chapitre présente une revue de différentes méthodologies pour maintenir des populations de ptéropodes en laboratoire, en s'appuyant sur les techniques développées pendant cette étude et en collaboration avec des expérimentateurs de premier plan dans le domaine.

Les proxys paléo-marins dérivés de la biogéochimie des coquilles fossiles implémente notre compréhension d'événements d'acidification passés et peut aider à valider les modèles des scénarios du futur. Pour comprendre comment la chimie des carbonates a été perturbée dans le passé (durée, taux de changement, couplage entre pH et taux de saturation, etc.), il est important de reconstruire complètement la chimie des carbonates. Ceci requiert la mesure de deux proxys indépendant, idéalement sur le même matériel. La composition isotopique du bore ($\delta^{11}B$) des coquilles de foraminifères planctoniques est établie comme un outil pour la reconstruction du pH de surface de l'océan. Le rapport B/Ca a été suggéré comme un proxy pour [CO_3^{2-}], qui si précis, permettrait aux paleocéanographes de contraindre pleinement le système des carbonates en utilisant ces deux proxys. Cependant les incertitudes demeurent sur lequel exerce un control majeur sur l'incorporation du bore dans la calcite des foraminifères car [CO_3^{2-}] et le pH varient de façon étroite dans les systèmes naturels. Le chapitre final

présente les résultats d'expériences avec le foraminifère planctonique *Orbulina universa*, cultivé dans des conditions qui découplaient les effets du pH et [CO_3^{2-}]. Aucun effet du [CO_3^{2-}] sur le $\delta^{11}B$ n'a été observe. Le rapport B/Ca a diminué avec la diminution du [CO_3^{2-}] à pH constant mais ne montre pas de tendance cohérente avec une [CO_3^{2-}] constant et un pH variable. Au lieu de cela, une étroite corrélation du rapport B/Ca et [HCO_3^-] a été observée suggérant que l'incorporation du bore dans la calcite des foraminifères est contrôlée par [HCO_3^-], fournissant un second proxy indépendant pour les reconstructions de systèmes paléo carbonatés.

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Chapter 1: Introduction

The global carbon cycle with all its abiotic and biotic feedbacks maintains a steady atmospheric CO_2 concentration that has typically varied between ca. 180 and 280 parts per million volume (ppmv) during the Pleistocene (Fig 1.1A) and has not exceeded its maximum value for the last ca. 25 million years (Myr) (Solomon et al. 2009, Gillett et al. 2011, Zeebe & Ridgwell 2011). Since the start of the industrial revolution, anthropogenic activities such as combustion of fossil fuels and cement production have released approximately 375 ± 30 Pg carbon into the atmosphere, this has been exacerbated by change in land usage which has added a further 180 ± 80 Pg (Ciais et al. 2013). By 2010 atmospheric CO_2 levels reached 390 ppmv, compared to a pre-industrial level of 280 ppmv (Fig 1.1B) (Orr 2011). Under the Intergovernmental Panel on Climate Change (IPCC) representative concentration pathway (RCP) 6.0 scenario, atmospheric CO_2 levels are predicted to reach around 670 ppmv by 2100 (Fig 1.1C), with a "worst case" scenario (RCP 8.5) of 931 ppmv (Ciais et al. 2013).

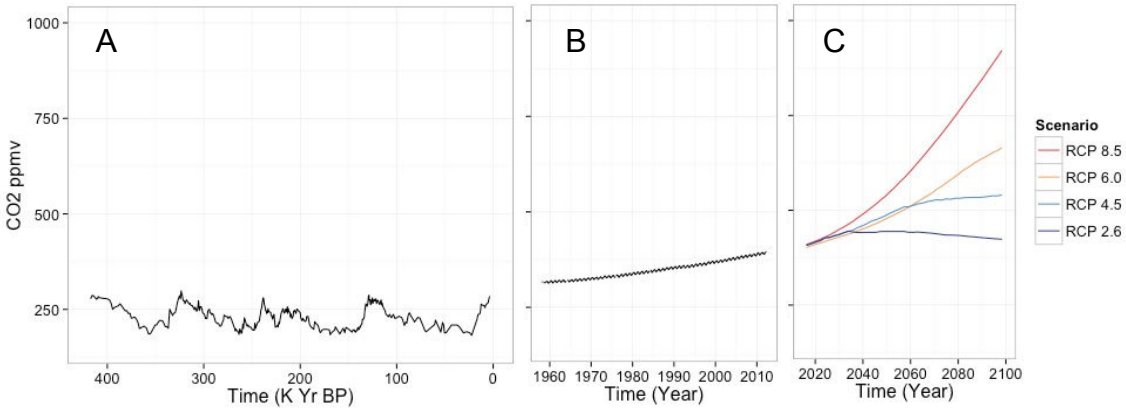

Figure 1.1: The fluctuation of atmospheric CO_2 (A) from 400,000 before present day to present (Vostok ice core, Barnola et al. 2003), (B) between 1958 and 2013 from the Mauna Loa time series (www.esrl.noaa.gov/gmd/ccgg/trends/). (C) IPCC, 2013 emission scenarios (Ciais et al. 2013).

Atmospheric CO_2 acts as a greenhouse gas and, alongside other effects such as surface albedo changes, increases global temperatures. Ocean heat content has risen by 0.40 x10^{22} J yr^{-1} (± 0.05) and accounts for 90% of total increase in heat content of the earth system (Rhein et al. 2013). Increasing sea surface temperatures (SSTs) decrease oxygen solubility and cause stronger stratification, resulting in areas of de-oxygenation and nutrient shoaling (Keeling et al. 2010). The thermal inertia of the oceans produces a time lag in greenhouse gas effects and

a further increase in SST of 1.8°C is predicted as a result of today's levels of atmospheric CO_2 (Hansen et al. 2008, Rhein et al. 2013). The effects of anthropogenic carbon release have been mediated by the buffering capacity of the oceans, which have absorbed approximately a quarter of CO_2 since the industrial revolution (Le Quéré et al. 2013). Despite this, the current level of carbon perturbation is unparalleled in the past 300 million years (Hönisch et al. 2012) and current atmospheric CO_2 levels are the highest they have been in the past 800,000 years (Lüthi et al. 2008). In this study, the focus is mainly on ocean acidification (ocean acidification) but note that this is only one of the three symptoms of a carbon perturbation (warming, ocean acidification and expansion of suboxic zones) and that the combination of stressors may aggravate each of the separate impacts (Bijma et al. 2013).

The major processes that control atmospheric CO_2 sequestration in the oceans are; instantaneous reactions with the carbonate system following gas exchange at the air-sea interface, the biological pump and export of carbon to the deep oceans (Fig 1.2), and finally ocean circulation (Zeebe & Wolf-Gladrow 2001). On a longer time scale, the weathering of rocks (supplying alkalinity to the ocean) buffers the alkalinity loss of biomineralised carbonates into the sediments (Aumont et al. 2001). Differences in the supply of alkalinity via rivers and the loss term into the sediment are visible by the shoaling or deepening of the lysocline. It is also important to note that when the CO_2 uptake by the oceans outpaces the resupply of total alkalinity (A_T) via rivers or sediment dissolution (shoaling of the lysocline), pH decrease is coupled to a lowering of the saturation state with respect to calcium carbonate and causing the buffer capacity of the oceans to decrease (Ridgwell & Schmidt 2010, Zeebe & Ridgwell 2011).

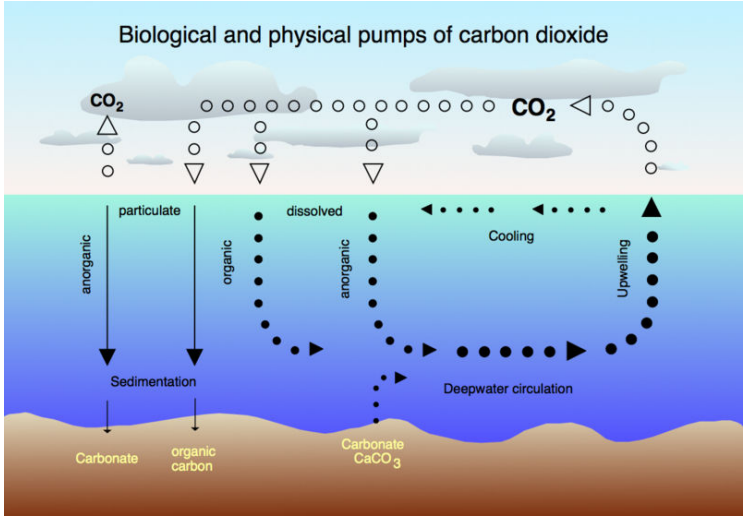

Fig 1.2: Biological and Physical carbon pumps. Phytoplankton fix carbon dioxide and form particulate organic carbon (POC). Most of the organic carbon is consumed and respired; some is exported below the thermocline to the ocean interior. Calcifiers produce skeletons from $CaCO_3$ particulate inorganic carbon (PIC). The flux of PIC and POC drawing C_T and A_T to the deep oceans (image courtesy Hannes Grobe, Alfred Wegener Institute).

Carbon dioxide absorbed by the oceans reacts with water and causes changes in the speciation of the total inorganic carbon (C_T). C_T in the oceans exists in a thermodynamic equilibrium of aqueous carbon dioxide (CO_2), carbonate ions (CO_3^{2-}), bicarbonate ions (HCO_3^-) and carbonic acid (H_2CO_3). The relative concentrations of seawater carbon species are pH dependent (Fig. 1.3).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad \text{Eq. 1}$$

As CO_2 is added to the system, it is quickly hydrated to form carbonic acid (H_2CO_3), a weak acid that is easily dissociated. The equilibrium (Eq. 1) is shifted towards the right, causing an increase in HCO_3^- and H^+ ions and a reduction of CO_3^{2-} ions. By the year 1994, average seawater pH had fallen from a preindustrial value of 8.18 to 8.10, with some models of future conditions predicting ocean pHs of 7.82 by the end of this century (Orr 2011).

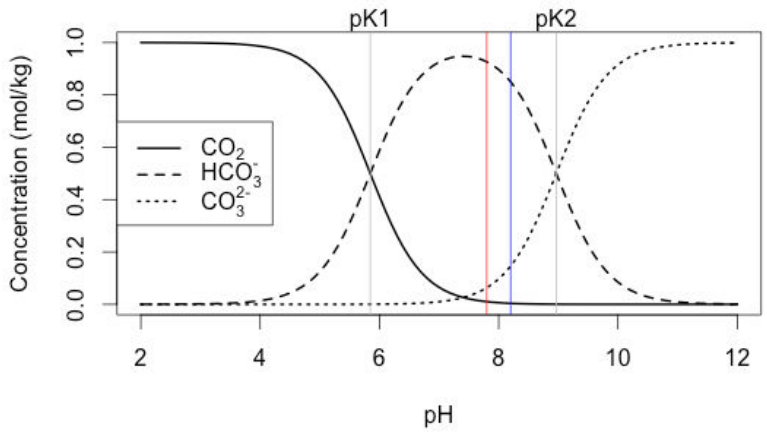

Fig. 1.3: The speciation and relative abundance of dissolved inorganic carbon present in seawater relative to pH. The blue line indicates conditions in the year 2000 and the red line the predicted changes by the year 2100 under a "business as usual" scenario (Rhein et al. 2013). Graph produced using seacarb (Lavigne & Gattuso 2013).

Some areas have been identified as being more vulnerable to the effects of climate change than others (Giorgi 2006). Due to its semi-enclosed nature and short residence time of water masses, the Mediterranean Sea is sensitive to external forcing, making it a "hotspot" for climate change effects (The Mermex Group 2011). This sensitivity means the region has the potential to provide an early warning for global ocean changes (Duarte et al. 1999). Evidence from time series and satellite data show a steady increase in SSTs since the year 1900 at a rate of 0.03 – 0.167 ˚C yr^{-1} (Nykjaer 2009, Vargas-Yáñez et al. 2010). The Mediterranean acts as a small to medium sink for CO_2, absorbing approximately 0.24 Gt C yr^{-1} (D'ortenzio et al. 2008). The rate of acidification is also relatively fast compared to the open oceans at a rate of -0.14 to -0.05 pH units yr^{-1}, with higher acidification in the western basin (Touratier & Goyet 2009, 2011).

The reduction in $[CO_3^{2-}]$ caused by ocean acidification has potentially negative effects for calcifying organisms as it will cause the calcium carbonate saturation state (Ω) to decrease. $[CO_3^{2-}]$ exerts the major control on saturation state as the residence time of $[Ca^{2+}]$ is ca. 1Myr (Broecker & Peng 1982) and hence remains constant over the short time scales considered here (Eq. 2). K_{sp} is the equilibrium constant for $CaCO_3$. If Ω is less than 1, the mineral phase is under saturated and dissolution will occur. Conversely, if Ω is greater than 1 it means that the mineral phase is supersaturated and precipitation will occur. Three different polymorphs of $CaCO_3$ are precipitated by marine calcifiers; high Mg calcite (Mg/Ca > 0.04), low Mg calcite (Mg/Ca < 0.04) and aragonite. The solubility of calcite is relative to the Mg/Ca ratio,

the more Mg it contains the higher its K_{sp} (Morse & Mackenzie 1990). Aragonite is approximately 50% more soluble than low Mg calcite (Mucci 1983)

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}} \qquad \text{Eq. 2}$$

Consequently, calcifying organisms have been observed to exhibit the strongest negative responses to ocean acidification, with meta-analysis revealing reductions in calcification, growth, abundance and survival rate with increasing pCO_2 (Kroeker et al. 2013b). Studies have reported a decrease in calcification in a variety of organisms, including; coccolithophores (Riebesell et al. 2000), Scleractinian corals (Gattuso et al. 1998), Foraminifera (Spero et al. 1997, Bijma et al. 1999) and pteropods (Comeau et al. 2009). There are also contradictory studies that have seen increased rates of calcification in certain organisms under increased pCO_2 (Iglesias-Rodriguez et al. 2008, Wood et al. 2008, Ries et al. 2009). The effects of decreased pH can also interact with other stressors, such as elevated temperatures, often increasing organisms' sensitivity (Kroeker et al. 2013b). As research progresses, it is emerging that some other processes, such as trophic interactions, are also affected by rising pCO_2 (Poore et al. 2013, Watson et al. 2014).

Chemical changes in the oceans will alter both the calcification potential of marine calcifiers as well as the incorporation of trace elements and isotope fractionation in the skeletal structures that they produce. Geochemical analysis of biogenic $CaCO_3$ exploits these changes in isotopic and elemental composition to make estimates of palaeoceanographic conditions by using them as proxy variables (proxies). The abundance of a proxy is correlated to a target environmental parameter, however biogenic calcification is a complex process and precipitation does not occur according to thermodynamic law (Wefer et al. 1999). These biological deviations, or so-called "vital effects", differ between species necessitating empirical calibrations to be undertaken for every species and proxy before they can be used as palaeo-climate estimates (Urey et al. 1951, Bemis et al. 1998, Bemis et al. 2000).

The entire carbonate system of seawater can be calculated if the temperature, depth and salinity are known, along with two parameters of the carbonate system (pH, $[CO_2]$, $[CO_3^{2-}]$, $[HCO_3^-]$, total alkalinity (A_T) and total dissolved carbon (C_T)) (Zeebe & Wolf-Gladrow 2001). The challenge for palaeoceanographers is to find robust proxies in marine calcium carbonate

that can be used to constrain two of the carbonate system parameters. Hershey et al. (1986) pioneered the use of boron isotope fractionation in carbonates as a potential proxy for pH. Boron exists in seawater in two forms; $B(OH)_3$ and $B(OH)_4^-$. The speciation of boron in seawater is pH dependent, (eq. 3) at high pH (> 10) almost all boron is in the form of $B(OH)_4^-$ and at low pH (< 7) it is almost completely $B(OH)_3$ (Fig. 1.4A).

$$B(OH)_3 + H_2O \leftrightarrow H^+ + B(OH)_4^- \qquad \text{Eq. 3}$$

$B(OH)_3$ has a greater abundance of the heavier ^{11}B isotope, relative to $B(OH)_4^-$. Hence, the isotope composition of the two species is also pH dependent, at a constant fractionation of ca. 27‰ (Fig. 1.4 B) (Zeebe & Wolf-Gladrow 2001, Klochko et al. 2006). Hemming and Hanson (1992) hypothesised that only $B(OH)_4^-$ is incorporated into marine carbonates (eq. 4) with the partition coefficient defined as per eq. 5, and thus the pH of the seawater the $CaCO_3$ was precipitated in can be can be derived from the $\delta^{11}B$ of $B(OH)_4^-$.

$$CaCO_3 + B(OH)_4^- \leftrightarrow Ca(HBO_3) + HCO_3^- + H_2O \qquad \text{Eq. 4}$$

$$K_D = \frac{[B/Ca]_{solid}}{[B(OH)_4^- / HCO_3^-]_{seawater}} \qquad \text{Eq. 5}$$

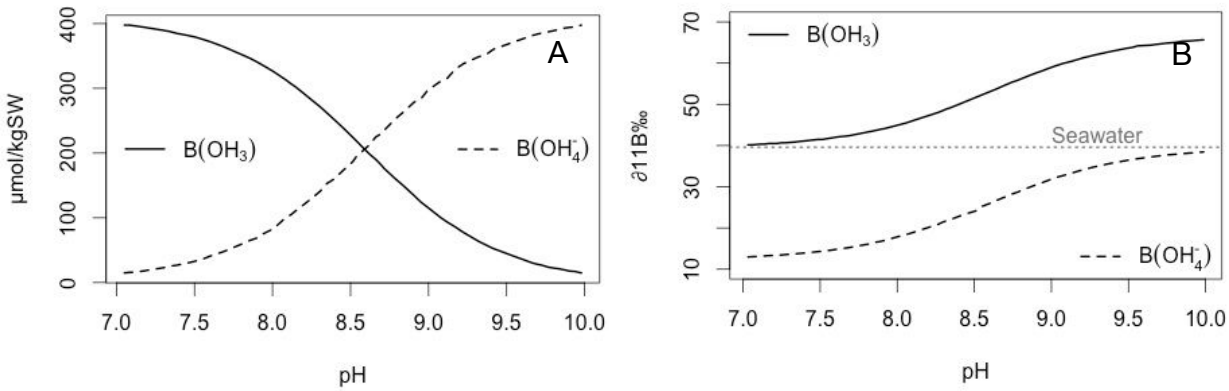

Fig. 1.4: (A) The effect of pH on concentration of dissolved boron speciation at T = 25°C, S = 35 and $[B]_{total}$ 416 µmol kg^{-1}. (B) Effect of pH on boron isotopic composition of $B(OH)_4^-$ and $B(OH)_3$ with thermodynamic fractionation factor (α_{4-3}) = 1.030 (Hemming & Hanson 1992).

As the incorporation of boron into $CaCO_3$ is a function of $[B(OH)_4^-]/[HCO_3^-]$, it has been suggested that the B/Ca ratio of carbonates could be used as a proxy for pH, temperature, $[HCO_3^-]$(Yu et al. 2007) or $[CO_3^{2-}]$ (Foster 2008). However there are still many uncertainties surrounding this relationship, which is complicated by the close co-variation of pH and carbonate species in natural systems. Early work showed a closer association of the B/Ca ratio with $[CO_3^{2-}]$ (Yu & Elderfield 2007, Foster 2008) and more recent work found a dependency between $[B(OH)_4^-]$ and inorganic carbonate species in seawater, but remained unable to distinguish between the effects of $[CO_3^{2-}]$ and $[HCO_3^-]$ (Allen et al. 2012).

Foraminifera are common proxy carriers, used widely in biogeochemical studies. Foraminifera are amoeboid protists, most of which secrete a calcite shell. They exist as both benthic and planktonic species (Sen Gupta 1999). In this work, only planktonic foraminifera were studied, the use of the term foraminifera herewith refers to planktonic species, unless otherwise stated. Shallow living species are often spinose and bear algal endosymbionts (Fig 1.5A) (Hemleben et al. 1989, Sen Gupta 1999). They adopt a dual feeding strategy depending on resource availability, they feed on zooplankton prey, mainly copepods, with nutrients from symbionts supplementing them when resources are limited (Fig. 1.5B) (Anderson & Be 1976). They are consumed by a wide range zooplankton, including polychaetes, holothurians, pteropods and tunicates and have also been found in the guts of shrimp and crabs (Culver & Lipps 2003). Foraminifera have been estimated to contribute 0.008–0.033 Gt yr^{-1} $CaCO_3$ to the global carbon budget (Schiebel & Movellan 2012).

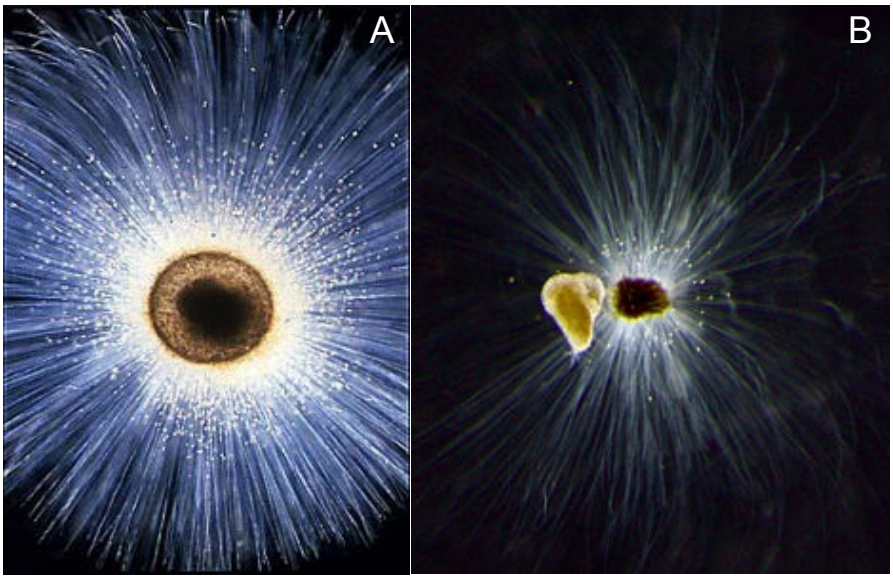

Fig 1.5: The planktonic foraminifer discussed in this thesis; *Orbulina universa*. (A) Adult specimen of *Orbulina. univsersa* with final, spherical chamber (photo courtesy of J. Bijma). (B) Pre-sphere juvenile with algal symbionts and *Artemia salina* prey captured in the sticky rhizopodial network in its spines.

Thecosome pteropods represent another major group of pelagic calcifiers. They are holoplanktonic opistobranchs that can be found in all the world's seas (Van Der Spoel 1967). The pteropod group consists of thecosome (shelled) and gymnosome (shell-less) species. Thecosome pteropods are represented by two families; *Limacinidae* and *Cavoliniidae*. *Limacinidae* have spiral shells and are less evolutionarily advanced than *Cavoliniidae*, which have lost their torsion to improve buoyancy (Lalli & Gilmer 1989). In this work, only shelled pteropods were studied, the term pteropod refers hereafter to thecosome pteropods (Fig. 1.6). Pteropods are omnivorous, using mucous webs to feed on a broad range of prey including phytoplankton, tintinnids, foraminifera, radiolaria, copepods and bivalve molluscs (Lalli & Gilmer 1989, Gilmer & Harbison 1991). They have high ingestion rates, studies have shown that in the Southern Ocean pteropods contribute up to 25% of phytoplankton grazing whilst constituting ~ 6% of total grazers (Hunt et al. 2008). Pteropods are a major link to higher trophic levels as important prey for a wide variety of organisms, including larger zooplankton, fish, birds and marine mammals (Lalli & Gilmer 1989, Armstrong et al. 2008). Pteropods are the only group of pelagic aragonite precipitators, and are estimated to constitute > 12% of global carbonate flux (Berner & Honjo 1981, Gangstø et al. 2008). They also contribute to the transport of organic matter to the deep oceans via faecal pellets, discarded mucous feeding

webs and the sinking shells of dead pteropods also speed the export of carbon to the deep oceans via the so-called ballast effect (Klaas & Archer 2002, Schiebel 2002).

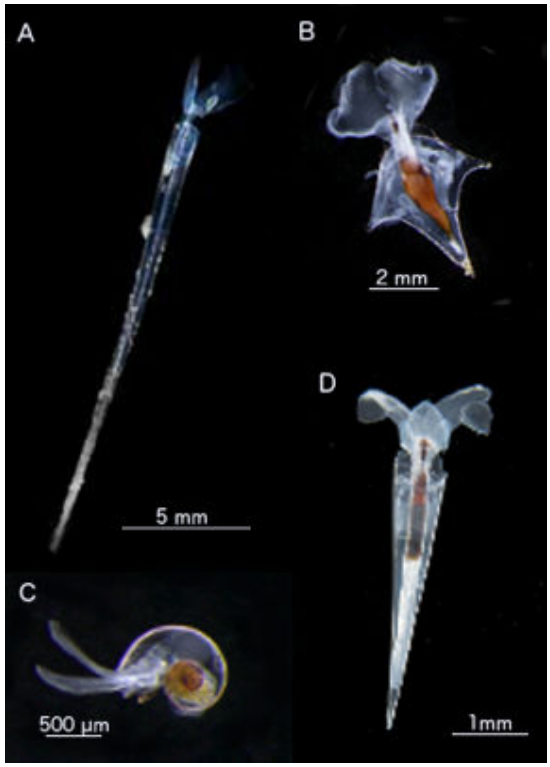

Figure 1.6: Some of the thecosome pteropod species included in the present study; *Creseis acicula* (A), *Cavolinia inflexa* (B), *Limacina inflata* (C) and *Styliola subula* (D).

Experimental work undertaken on pteropods and foraminifera has demonstrated a negative response to projected future conditions (summarised Table 1.1). Pteropods are predicted to be particularly vulnerable to the effects of lowered carbonate saturation

Table 1.1: Review of ocean acidification perturbation experiments undertaken on thecosome pteropods. Slopes denoting a negative, positive or neutral response to increasing levels of pCO_2. Table ordered by geographic region:, Arc = Arctic, Nor = Norwegian Sea, Atl = Atlantic, Med = Mediterranean, Ant = Antarctic, NA = not investigated.

Study	Species	Location	Methods	Calcification	Respiration	Mortality	Response details
Comeau et al. (2009)	*Limacina. helicina*	Arc	350 and 760 µatm pCO_2 with ^{45}Ca	↘	NA	NA	28% decrease calcification
Comeau et al. (2010b)	*L. helicina*	Arc	280, 380, 550, 760, 1020 µatm pCO_2, 0, 4˚C, ^{45}Ca	↘	↗	NA	• Decrease calcification at both temperatures • Interactive effect of temperature and pH, increased respiration rate
Lischka et al. (2011)	*L. helicina* (j)	Arc	130, 380, 750, 1150 µatm pCO_2 at 0, 5.5, 8˚C	↘	NA	↗	• Decrease shell increment & diameter • Dissolution at higher pH • Temperature and pH interactive effects on mortality
Lischka and Riebesell (2012)	*L. helicina* *Limacina retroversa*	Arc Arc	350, 650, 880 µatm pCO_2 at 2, 7 ˚C	↘ ↘	NA NA	— —	• Shell degradation and dissolution at higher pCO_2 • Synergistic effect of temperature on *L. helicina* • No synergistic effects of temperature on *L. retroversa*
Comeau et al. (2012a)	*L. helicina*	Arc	362, 530, 704 µatm pCO_2	↘	NA	—	Decrease in linear shell extension •
Manno et al. (2012b)	*L. retroversa*	Nor	280, 350, 750, 1000 at in situ salinity (33.8) 100%, 80%, 70%	↘	NA	↗	• Shell degradation and dissolution at higher pCO_2 Synergistic effect of freshening on mortality and shell dissolution
Orr et al. (2005)	*Clio pyramidata*	Atl	Short term incubation Ω_a <1	↘	NA	NA	Dissolution at shell edge with increased pCO_2
Fabry et al. (2008)	*C. pyramidata*	Atl	^{45}Ca, respiratory CO_2	↘	↘	NA	• Decreasing calcification rate 28% decrease respiration rate at 789 µatm pCO_2
(Comeau et al. 2010a)	*Cavolinia inflexa* (j)	Med	380, 857, 1713 µatm pCO_2	↘	NA	NA	• Reduced shell length at high pCO_2 • Shell-less in highest pCO_2
Comeau et al. (2012b)	*Creseis acicula*	Med	Decoupled pH and Ω_a	↘	↘	NA	• Ω_a driving factor • Decreased calcification • Decreased respiration rate
BednaršEk et al. (2012)	*L. helicina ant.*	Ant	375, 500, 750, 1200 µatm pCO_2	↘	NA	NA	• Increasing dissolution with increasing pCO_2
Seibel et al. (2012)	*L. helicina ant.*	Ant	180, 380, 790, 1000 µatm pCO_2	NA	↘	NA	• Respiration decreases as pCO_2 increases • Food depravation acts as synergistic stressor

states due to their thin shells and the 50% higher solubility of aragonite compared to low Mg calcite (Mucci 1983). Studies have confirmed that pteropod larvae and adults exhibit negative calcification responses to elevated pCO_2 with consistent evidence of decreased calcification (Comeau et al. 2010a, Comeau et al. 2010b, Lischka & Riebesell 2012) and increased shell dissolution (Lischka et al. 2011) (Table 1.1). Studies of metabolic rate have also revealed that pH acts in concert with other environmental stressors, such as temperature and salinity, influencing swimming speed and oxygen consumption in some species of pteropods (Comeau et al. 2010b, Maas et al. 2011, Bednaršek et al. 2012, Maas et al. 2012, Manno et al. 2012b). These studies were all short-term (< 30 days) due to constraints imposed by the difficulties of keeping pteropods alive in culture.

Despite their significant contribution to marine $CaCO_3$ production, there is a relative paucity of targeted investigations into the effects of ocean acidification on calcification in planktonic and symbiont bearing foraminifers (Hofmann et al. 2010). Several studies have highlighted sensitivity to ocean acidification in planktonic foraminifera. Size normalised shell weights (SNW) are often used as a response variable and the majority of studies using down cores and core tops have found decreases in SNW concurrent with periods of low pH and high pCO_2 (summarised in Keul et al. 2013). However it is unclear what parameter of the carbonate system is driving changes in SNW. Culture work has consistently found SNW to decrease under declining $[CO_3^{2-}]$ (Bijma et al. 1999, Russell et al. 2004, Lombard et al. 2010, Manno et al. 2012a) and Lombard et al. (2010) also observed decreases in calcification rate in two species under decreased $[CO_3^{2-}]$.

The tests of foraminifera have been shown to be accurate and reliable recorders of palaeo-conditions as evidenced by lab and field calibrations of isotopes and (trace) element concentration in their $CaCO_3$ (Spero & Williams 1988, Bemis et al. 1998, Bemis et al. 2000, Erez 2003) and are widely used in studies of palaeoceanographic proxies. $\partial^{11}B$ of planktonic foraminifera tests can provide reliable reconstructions of palaeo-pH (Sanyal et al. 1995, Hönisch & Hemming 2005), in agreement with data from ice cores (Zeebe et al. 2008). However, incorporation of elements into foraminiferal carbonate is often subject to controls imposed by a number of different environmental parameters that can confound the application of proxies, for instance the so-called "carbonate ion effect" on $\delta^{13}C$ and $\delta^{18}O$ (Spero & Lea 1996, Bijma et al. 1999). The accuracy of proxies can also be affected by a lack of knowledge surrounding the biology of the organism; calcification depth, effects of symbiont

photosynthesis and calcification mechanisms could all drastically alter the biogeochemistry of test calcite (Nürnberg et al. 1996).

In response to increased political interest and availability of funding, the number of publications reporting perturbation experiments assessing organism response to ocean acidification has rapidly increased over the last few years (Gattuso & Hansson 2011). However, as the field of knowledge expands, it becomes clearer that organism responses are highly variable. Effects differ interspecifically and even between different strains of the same species (Langer et al. 2006, Langer et al. 2009), complicating projection of future ecosystem effects (Gattuso et al. 2013). Evidence is also emerging that other stressors such as temperature, salinity, food/nutrient availability and local pollution can strongly modulate the effects of ocean acidification (Manno et al. 2012b, Seibel et al. 2012, Hoppe et al. 2013, Kroeker et al. 2013b, Götze et al. 2014). This highlights the need for targeted investigations on the effects of multiple stressors that consider not only global changes, but also specific local drivers. Many of the results on organism response to decreased pH are based on short-term investigations, with little known about the longer term effects on reproduction and population dynamics (Hendriks et al. 2010, Hofmann et al. 2010) or what effect ocean acidification has already had on key species (Turley & Boot 2011). There is also uncertainty regarding the capacity for adaptation to increasing acidification as experimental approaches represent the response to sudden, dramatic hypercapnia whereas the real process will occur relatively gradually over a period of decades (Widdicombe et al. 2010). The complexity and scale of these processes are difficult, if not impossible, to reproduce experimentally and necessitates the use of an interdisciplinary approach that uses a suite of methods such as analysis of sedimentary archives and time series to compliment and expand on experimental findings.

Geographically, research efforts have focused on the North Atlantic Ocean and the Pacific Ocean with studies in these areas constituting 73% of published articles in the year 2011 (Gattuso & Hansson 2011). The Mediterranean Sea has been identified as a region that is particularly sensitive to the global environmental change and it has been suggested that its rate of acidification has been higher, especially in the western basin, than in the open oceans. Despite its relatively small size, (0.82% of global ocean surface area) it supports between 4-18% of the planet's marine biodiversity. It is also a commercially important area, under huge anthropogenic pressure, with large coastal populations that rely on the marine environment

for fisheries, aquaculture and tourism (The Mermex Group 2011). In order to forecast future conditions, it is vital to gain a better understanding of how global change resulting from anthropogenic CO_2 perturbation is affecting marine ecosystems.

Ocean acidification research exhibits a strong taxonomic bias with the majority of work focussing on phytoplankton and corals and suffering from a relative paucity of studies on the responses of zooplankton to ocean acidification (Gattuso & Hansson 2011). It is vital that this imbalance is redressed because zooplankton are a vital component of marine ecosystems. They act as an important trophic link between the primary producers and larger, more complex organisms (Dam 2013), and are significant contributors to biogeochemical cycling (Longhurst 1991). Due to their short generation time, sensitivity to temperature, and lack of commercial exploitation, zooplankton populations have been suggested to be good indicators of climate variability (Perry et al. 2004) and their fossilised shells are frequently used for palaeo-climate reconstructions (Wefer et al. 1999). This study will investigate the effects of ocean acidification on two major groups of pelagic calcifiers; pteropods and foraminifera, focusing on Mediterranean species.

Knowledge of organisms' response to the current environmental changes and to comparable carbon perturbation events in geological history is necessary to project possible consequences for aragonitic organisms such as pteropods and calcitic ones such as foraminifers. The present study will focus on ocean acidification effects on Mediterranean species as, at present only two studies have investigated the impacts to Mediterranean pteropods (Comeau et al. 2010a, Comeau et al. 2012b). As little is known about the long-term effects on pteropod populations or their capacity for adaptation, recent environmental changes are examined using museum samples and time series analysis. The major barrier preventing further experimental progress on pteropods is the difficulty in maintaining laboratory populations. A wide variety of pteropod culture methods were tested and discussed in a detailed review of the state of the art, with the goal of making possible long-term experiments to investigate the effects of adaptation over several generations. Reliable culture methods for planktonic foraminifera are well established, however the use of boron proxies are still hampered by a lack of understanding about the controls on trace element incorporation in foraminiferal calcite. Culture experiments using *Orbulina universa* were undertaken with the aim of clarifying the issues surrounding the use of [B/Ca] ratio as a proxy for carbonate ion concentration. The results will be presented in the following four chapters:

Chapter 2: *Howes EL & Eagle R, Tripati A, Gattuso J-P, Bijma J, (in draft) Comparison of Mediterranean pteropod shell biometrics and ultrastructure from museum (1910 and 1921) and present day (2012) samples; effects of global change?*
To assess the effects of environmental change over the last 100 years, museum samples of *Cavolinia inflexa* and *Styliola subula* collected during the Mediterranean leg of the Thor (1910) and Dana (1921) cruises were compared with modern (2012) samples of the same species collected in the Ligurian Sea. Shell biometrics were measured applying the novel approach of micro CT scan and compared to manually measured parameters. Shell densities were calculated and scanning electron microscopy was used to compare the aragonite crystal morphology. pH for the collection years was hind-cast using temperature and salinity time series with atmospheric CO_2 concentrations from ice core data.

Chapter 3: *Howes EL, Stemmann L, Assailly C, Irisson J-O, Dima M, et al. (in review) Pteropod time-series from the North Western Mediterranean (1967-2003): impact of global change. Marine Ecology Progress Series.*
A time series of pteropod abundance covering the period 1967-2003 was extracted from the Point B (Northwestern Ligurian Sea) zooplankton time series. Inter- and intra-annual changes in the abundance of three groups (*Limacina* spp., *Cavolinia* spp. and "*Creseis*-like") were compared with a copepod time series to search for differential effects driven by ocean acidification and temperature. pH values were hind-cast from total alkalinity estimated from temperature and salinity, and atmospheric CO_2 taken from the Mauna Loa time series.

Chapter 4: *Howes EL, Bednaršek N, Büdenbender J, Comeau S, Doubleday A, Gallager SM, Hopcroft RR, Lischka S, Maas AE, Bijma J, Gattuso J-P, (2014) Sink and swim, a status review of thecosome pteropod culture techniques. Journal Plankton Research, 36 (2), 299-315.*
The widespread distribution of pteropods, their role in ocean food webs and their sensitivity to ocean acidification and warming has renewed scientific interest in this group of zooplankton. Unfortunately, their fragile shell, sensitivity to handling, unknowns surrounding buoyancy regulation and poorly described feeding mechanisms make thecosome pteropods notoriously difficult to maintain in the laboratory. The resultant high mortality rates and unnatural behaviours may confound experimental findings. The high mortality rate also discourages the use of periods of acclimation to experimental conditions and precludes vital

long-term studies. Here we summarize the current status of culture methodology to provide a comprehensive basis for future experimental work and culture system development.

Chapter 5: is a manuscript in draft form, *Howes EL, Kaczmarek K, Raitzsch M, Funcke A, Bijma N, Horn I, Gattuso J-P, Bijma J, (in draft) The major factor governing the incorporation of boron into Orbulina universa calcite: disentangling the carbonate system.* It is difficult to differentiate between the effects of pH and [CO_3^{2-}] on boron incorporation as they co-vary closely in natural systems. To disentangle the effects of pH and [CO_3^{2-}] on the B/Ca ratio and to test whether $\delta^{11}B$ remains constant at constant pH, but under changing [CO_3^{2-}] (pH 8.05 with 238, 285 and 532 µmol kg^{-1} CO_3^{2-}) and vice versa, we decoupled pH and [CO_3^{2-}] (276 ± 19.5 µmol kg^{-1} CO_3^{2-} with pH 7.7, 7.9 and 8.05) and cultured the planktonic foraminifer *Orbulina universa*. Measurements of the isotope composition of boron and trace elements were performed simultaneously using a femtosecond laser ablation system coupled to an MC ICP-MS and high resolution mass spectrometer. Results show that $\delta^{11}B$ is controlled by pH and does not respond to changes in [CO_3^{2-}]. On the other hand, the B/Ca ratio responds to [HCO_3^-] independently of pH. This suggests that B/Ca ratios in foraminiferal calcite may be used as a second, independent, proxy for paleo-carbonate system reconstructions.

Chapter 6 is a general discussion on the results presented in the previous chapters, followed by the general conclusions and perspectives.

Comparison of Mediterranean pteropod shell biometrics and ultrastructure from museum (1910 and 1921) and present day (2012) samples; effects of global change?

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Abstract

Anthropogenic carbon perturbation has caused decreases in seawater pH and increases in global temperatures since the start of the 20th century. The subsequent lowering of the saturation state of $CaCO_3$ makes the secretion of skeletons more problematic for marine calcifiers. Experimental work has indicated that thecosome pteropods are particularly sensitive to changes in the aragonite saturation state (Ω_a) however issues with maintaining populations in the laboratory has limited experiments to short term effects. To assess the effects of environmental change over the last 100 years, museum samples of *Cavolinia inflexa* and *Styliola subula* collected during the Mediterranean leg of the Thor (1910) and Dana (1921) cruises were compared with modern (2012) samples of the same species collected in the Ligurian Sea. Shell biometrics were measured, applying the novel approach of micro CT scan and compared to manually measured parameters. Shell densities were calculated and scanning electron microscopy was used to compare the aragonite crystal morphology. pH for the collection years was hind-cast using temperature and salinity time series with atmospheric CO_2 concentrations from ice core data. Museum samples were thicker than shells of the same length from 2012 (*S. subula*, 1921 = 40.6 µm, 2012 = 30.6 µm, *C. inflexa*, 1910 = 29 µm, 2012 = 28 µm) and *C. inflexa* shells collected in 1910 were significantly denser than those from 2012 (1910 = 1.1 mg mm^{-3}, 2012 = 1.3 mg mm^{-3}). These effects may have implications for predation pressure and buoyancy control, which could impact pteropod populations in the western Mediterranean Sea.

Introduction

From the start of the industrial era to the late 20th century, combustion of fossil fuels and cement production has released approximately 244 ± 20 Pg carbon into the atmosphere, this has been exacerbated by change in land usage which has added a further 140 ± 40 Pg (Sabine et al. 2004, Denman et al. 2007). By 2010, atmospheric CO_2 levels reached 390 ppmv, compared to a pre-industrial level of 280 ppmv (Orr 2011). The world's oceans have absorbed approximately a quarter of atmospheric CO_2 causing changes to the carbonate chemistry by decreasing pH; carbonate ion concentration, and thus, the saturation state (Ω) of calcite and aragonite. Lowered saturation state and pH can make the secretion of calcium carbonate skeletons more problematic and can increase dissolution of calcium carbonate structures (Gattuso et al. 1999). The Mediterranean Sea has been highlighted as a "hotspot" for climate change (The Mermex Group 2011) and is acidifying faster than the open oceans (Touratier & Goyet 2009, Touratier & Goyet 2011).

Thecosome pteropods are a cosmopolitan group of holoplanktonic opistobranchs, found globally and the only pelagic aragonite precipitators. They are important contributors to the biogeochemical cycling of carbon (Lalli & Gilmer 1989) and act as an important link in ocean food webs, exhibiting a top down control on smaller zooplankton and phytoplankton as well as being important prey for a wide variety of organisms including larger zooplankton, fish, marine birds and mammals (Lalli & Gilmer 1989, Doney et al. 2009). The shells of pteropods have adapted to suit their pelagic lifestyle, they are very small; < 1 mm to 15 mm in diameter and are extremely thin, ranging from about 6 µm to 100 µm in thickness. Despite their fragility, pteropod shells must serve as protection from predators and parasites and provide ballast and stability in the water column (Lalli & Gilmer 1989). The mechanical properties of the interlocked helical aragonite rods in the shells of Cavolindae provides a trade off between a light weight but strong and flexible shell (Zhang et al. 2011).

Experimental work has shown pteropods to be sensitive to the effects of global change, exhibiting decreased calcification rates and increased shell dissolution with declining pH and increasing temperatures (Comeau et al. 2010b, Lischka & Riebesell 2012). As polar and subpolar seas are predicted to be among the first to become under-saturated with respect to calcite and aragonite (Orr 2011), the focus of experimental work on pteropods has been on polar species, with only two studies undertaken in the Mediterranean (Comeau et al. 2010a,

Comeau et al. 2012). Due to difficulties maintaining laboratory cultures (Howes et al. 2014), empirical studies have only investigated acute effects and it remains unclear whether acclimation and adaption processes may mediate the effects of ocean acidification on shell formation. As development of culture methods are still underway (Howes et al. 2014), some of the best opportunities to assess long-term effects are to utilise time series data or museum collections to evaluate the effects of recent environmental change. Here we compare shell thickness and ultrastructure of two Mediterranean pteropod species (*Cavolinia inflexa* and *Styliola subula*) collected at the beginning of the twentieth century with samples collected in 2012.

Materials and Methods

Sample collection:

Modern specimens of *C. inflexa* and *S. subula* were collected from Point B, Villefranche-sur-Mer, 43.41˚N, 7.19˚E (Fig. 2.1) between August 2011 and August 2012. Sampling was undertaken with a 1 m mouth diameter, "Regent" plankton net, towed obliquely very slowly to avoid shell damage. Samples were immediately fixed and stored in in 90% ethanol. Museum samples of *C. inflexa* sampled by vertical net tow at station 125 (37.36˚ N, 2.55˚E) of the Thor Expedition in the year 1910 (Schmidt 1912). Museum samples of *S. subula* were sampled by vertical net tow at station 1124 (43.54˚ N, 7.19˚E) of the 1921, Dana expedition (Schmidt 1929). All museum samples were preserved in ethanol.

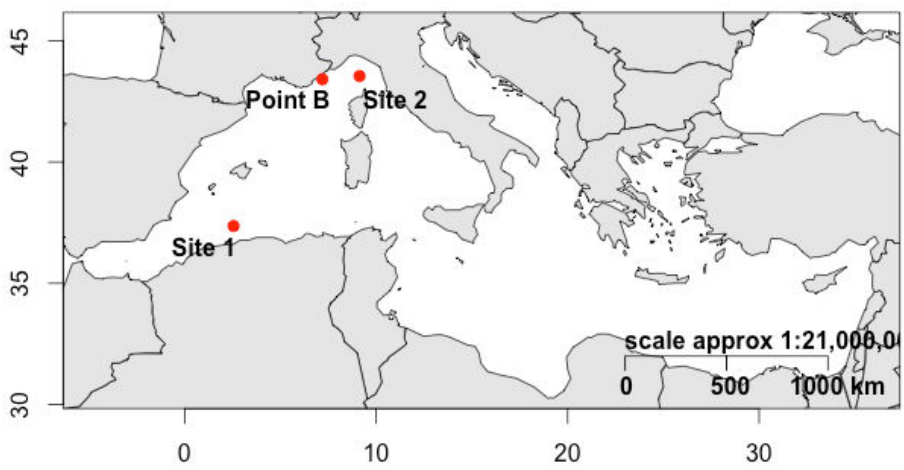

Fig. 2.1: Sampling locations for the museum samples of *Cavolinia inflexa* and *Styliola subula* and for the modern collections of both species (Point B). Map produced in R using maps and mapdata (Becker et al. 2013a, 2013b).

CT scanning:

X-ray computed tomograpahy (CT) imaging was carried out with a General Electric Company Phoenix Nanotom S Instrument. Samples were analysed in pairs using a custom fashioned balsa wood mount, alongside a calcite rhomb that acted as a reference material and was present in every scan. Sample mount, X-ray source and detector geometry was positioned to maximize resolution and was kept constant throughout all analysis of samples of a particular species. An X-ray tube voltage of 80 kV and current of 100 mA was used, without any physical x-ray filtering with metal strips. A voxel size of 5 μm^3 was typically achieved using this setup. Scan setup and assembly of 3D images was carried out with proprietary Datos/x 2.0 software. 1000 images of each mount were taken, and combined to build a 3D rendering of the image using Datos/x 2.0.

Image analysis was carried out with Volume Graphics VGStudio MAX version 2.2 software. Initial 3D renderings contained two pteropods and a calcite standard, which were extracted into separate renderings for analysis. We found that manual surface determination enabled the hard calcium carbonate shell to be separated from organic material comprising the soft-body of the pteropods and also separated the pteropod shell from the balsa wood mount without clipping the pteropod shell rendering. Object volume, average thickness, and surface area were determined using standard Volume Graphics analysis. In addition, dimension measurements were taken on 3D renderings of shells. For *C. inflexa*, three dimension measurements were taken, the width (at widest point), the width at half shell length and length (Fig. 2.2A). For *S. subula*, the dimension measurements that were taken were the total length, width (diameter at widest point) and the "diameter at half length" (Fig. 2.2B). Reproducibility was assessed by replica analysis of the carbonate rhomb standard and an individual *S. subula* specimen that was analysed multiple times. Accuracy of CT based measurements was assessed by comparison of the output of CT based morphometric analysis with measurements taken with a light microscope.

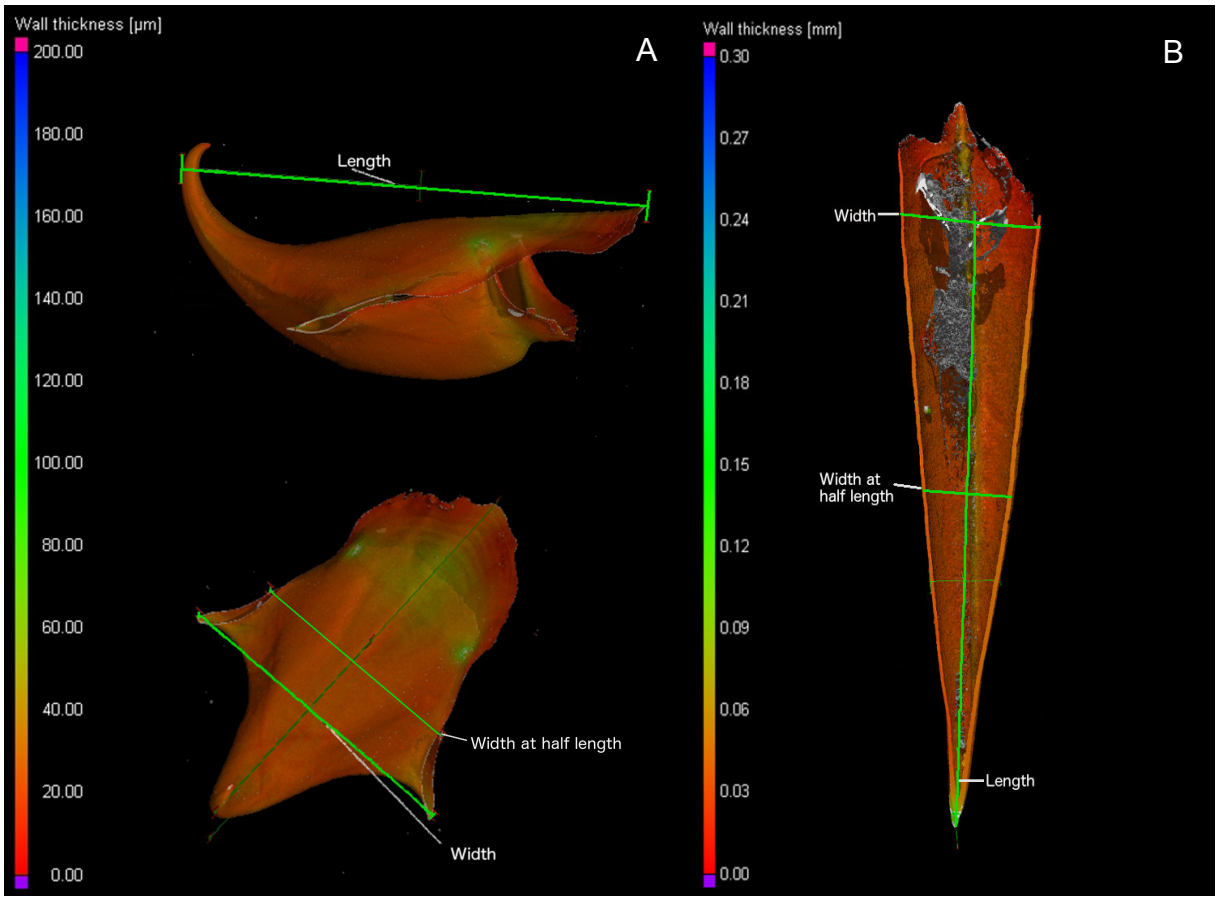

Fig 2.2. Parameters measured by CT scan for (A) *Cavolinia inflexa* and (B) *Styliola subula*

Manual shell measurements and weights:

Shell length and width (Fig. 2.2) were manually measured using a binocular microscope and camera with the imaging software AxioVision, version 6.1.7601. After scanning and manual measurement were complete, organic material was removed from the shell by soaking in 3:2 ratio of sodium hypochlorite (13%) and deionised water. Once the organics were completely dissolved, the empty shells were rinsed with deionised water and placed in the oven at 50˚C for 8 hours, followed by 24 hours in a dessicator. Each shell was individually weighed using a Mettler Toledo microbalance (precision = 0.1 µg). Shell density measurements were calculated by calculating volume from surface area and average thickness (Eq. 1) where V = volume, sa = surface area, t = thickness. Density was calculated from volume and mass using shown (Eq. 2) where ρ = density, V = volume, m = mass.

$$V = sa \cdot t \qquad \text{Eq. 1}$$

$$\rho = \frac{m}{V} \qquad \text{Eq. 2}$$

Crystal morphology:

Ten specimens from each sample group were embedded in resin (Araldite 2020) then successively ground with P1200/P2400/P4000 grinding paper (Buehler) and water to dorsal - ventral cross sections along the midline. The samples were then polished, first with 3 µm Buehler diamond polycrystalline suspension then 0.3 µm aluminium oxide suspension. The embedded samples were mounted on 1.8 mm (diameter) scanning electron microscopy (SEM) stubs. Specimens were coated with gold palladium for imaging using a Fei Quanta 220 F SEM with operating parameters of 10 kV, spot size 3 and 10 mm working distance.

pH hindcast:

The environmental times series run at Point B from Villefranche-sur-Mer *Service d'Observation en Milieu Littoral* (SOMLIT/CNRS-INSU) was used for pH estimation. Sampling has been undertaken on a weekly basis since 1957. The two parameters that have been continually measured throughout the study period are temperature and salinity. Since 2007, pH_T, dissolved inorganic carbon (C_T) and total alkalinity (A_T) have also been measured. Water for the determination of dissolved inorganic carbon (C_T) and total alkalinity (A_T) were collected from 10-L Niskin bottles, transferred to combusted glass bottles, overfilled, and poisoned with $HgCl_2$ as recommended by Dickson et al. (2007). C_T and A_T are determined colorimetrically and potentiometrically, respectively (Edmond, 1970; DOE, 1994). Further information on environmental sampling methods is provided at the SOMLIT web site (http://somlit.epoc.u-bordeaux1.fr/fr/).

From the SOMLIT time series, we were able to constrain a model for pH hindcasting. Total alkalinity (A_T) was calculated from monthly averages of temperature and salinity using a variation on the equations provided by Lee et al. (2006; Eq. 3), where: A_T, total alkalinity (µmol kg^{-1}); S, salinity, T, temperature and a, b, c, d, e are constants.

$$A_T = \mathrm{a} + \mathrm{b} \times \mathrm{S} + \mathrm{c} \times \mathrm{S}^2 + \mathrm{d} \times \mathrm{T} + \mathrm{e} \times \mathrm{T}^2 \qquad \text{Eq. 3}$$

The most parsimonious model was determined from eq. 3 using the Akaike information criterion measure (Akaike 1974) on sub-sampled (one per 10 weeks, to reduce the autocorrelation of the time series) alkalinity measurements ranging from 2007 to 2012. The temperature variables were dropped as they did not affect the goodness of fit of the model to the measured data. The constants a, b and c were then determined with all measures 2007-2012. The coeffcients a, b and c were derived from the weekly measurements of alkalinity taken from 2007-2012. Finally, the model was applied to annual averages of temperature and salinity from 1899-2012. Data for annual temperature values was taken from gridded sea surface temperatures (SST) are from the Hadley Data Centre (Hadley Centre for Climate Prediction and Research/Met Office/Ministry of Defence 2000); http://rda.ucar.edu/datasets/ds277.3.). Salinity data was unavailable for the full period so values were extrapolated from SOMLIT data as the change in surface salinity in the western Mediterranean has been shown to have been roughly linear since the year 1900 (Vargas-Yáñez et al. 2010).

The partial pressure of CO_2 ($pCO_{2\ sw}$) was estimated from temperature and concentration of CO_2 in the atmosphere ($CO_{2\ atm}$, ppm; Lefevre & Taylor 2002; Eq. 4).

$$pCO_{2_{sw}} = a \times T + b \times pCO_{2_{atm}} + c \qquad \text{Eq. 4}$$

There is no measurement of $CO_{2\ atm}$ in the Mediterranean region covering the entirety of the study period. The Mauna Loa (19˚53'N – 155˚57'W, Hawaii) atmospheric CO_2 time series was used, as this is the only station in the northern hemisphere that has data for the entire study period. To account for geographical differences, the data were compared to the 1979 - 1997 $CO_{2\ atm}$ time series from the Italian station, Monte Cimone (44˚18'N – 10˚7'E). Seasonal differences were minimised by lagging the Manuna Loa data by 2 months and adjusting seasonal maxima and minima of the values from the Mauna Loa time series to obtain the best fit with the data from Monte Cimone. The coefficients were determined using a linear model for 1 m depth based on $pCO_{2\ sw}$ computed from SOMLIT data from weekly measurements of dissolved inorganic carbon and total alkalinity ranging from January 2007 to March 2012. For values before the start of the Mauna Loa time series (pre 1958) values was taken from the Siple ice core dataset (Neftel et al. 1994).

Using the estimations of A_T and $pCO_{2\,sw}$ alongside the measured values of temperature, salinity and pressure, it was possible to calculate seawater pH_T using the R package seacarb (Lavigne & Gattuso 2013). The following constants were used: K_1 and K_2 from Lueker et al. (2000), K_f from Perez and Fraga (1987) and K_s from Dickson (1990). Weekly measurements of pH at Point B began in 2007. These measurements (2007-2012) are used to assess the goodness of fit for the calculated data.

Results:

The measured pH_T data for years 2007- 2012 display a seasonal oscillation with highest values in winter months (Feb/Mar) and lowest values in summer (Jul/Aug), the average seasonal variation over the 5 year time series is 0.07 ± 0.05 pH units (Fig. 2.3A). Comparison of calculated pH_T values to measured values (2007-2012) by linear regression produced an r^2 of 0.49 as the model failed to accurately predict the extreme low observations. However, the high values were well predicted and the overall trend and seasonal oscillations fit well with the real data (Fig. 2.3B). The hind cast shows a decreasing trend in both sites with a mean decrease of 0.1 and 0.08 pH_T units at site 1 and 2, respectively, since the start of the 20th century. Average annual temperature was 1.2˚C higher in 2012 than 1910 at site 1 and 0.41˚C higher in 2012 than 1921 at site 2. The calculated annual average pH at site 1 and 2 in the years of sample collection was 8.18 and 8.19 compared to 8 at Point B in 2012. Calculated annual average aragonite saturation state at site 1 and 2 during the years of sample collection was 3.97 and 3.88, respectively, in 2012, at Point B, average saturation state was 3.4 (Table 2.1, Fig. 2.4).

Table 2.1: Details of sampling time, location and hydrography

Species	Location	Date	Annual average temperature (˚C)	Annual average pH	Annual Ω_a
C. inflexa	Site 1: 37.36˚ N, 2.55˚E	09/07/1910	18.55	8.18	3.97
S. subula	Site 2: 3.54˚ N, 7.19˚E	27/09/1921	17.68	8.19	3.88
C. inflexa	Point B: 43.41˚N, 7.19˚E	01/01/2012 - 31/03/2012	18.85	8.09	3.4
S. subula	Point B: 43.41˚N, 7.19˚E	01/08/2012 - 31/08/2012	18.85	8.09	3.4

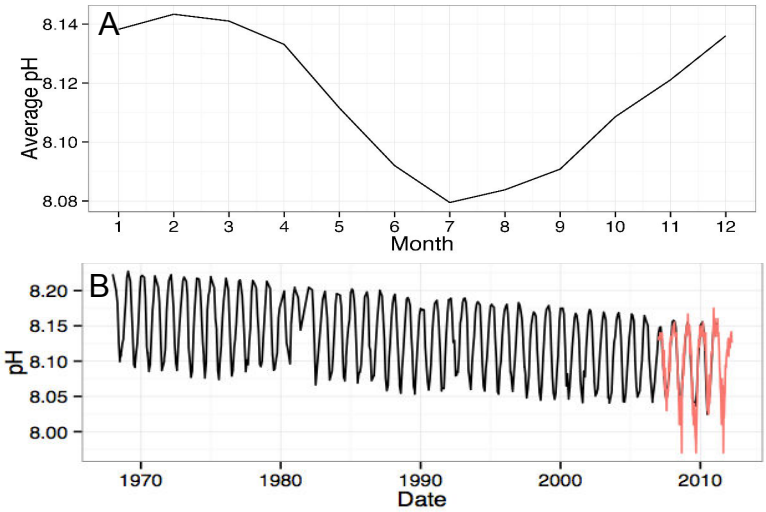

Fig. 2.3. (A) Average annual pH of measured data from SOMLIT at Point B from 2007-2012. (B) Comparison of measured pH values at Point B, Villefranche-sur-Mer, to calculated pH.

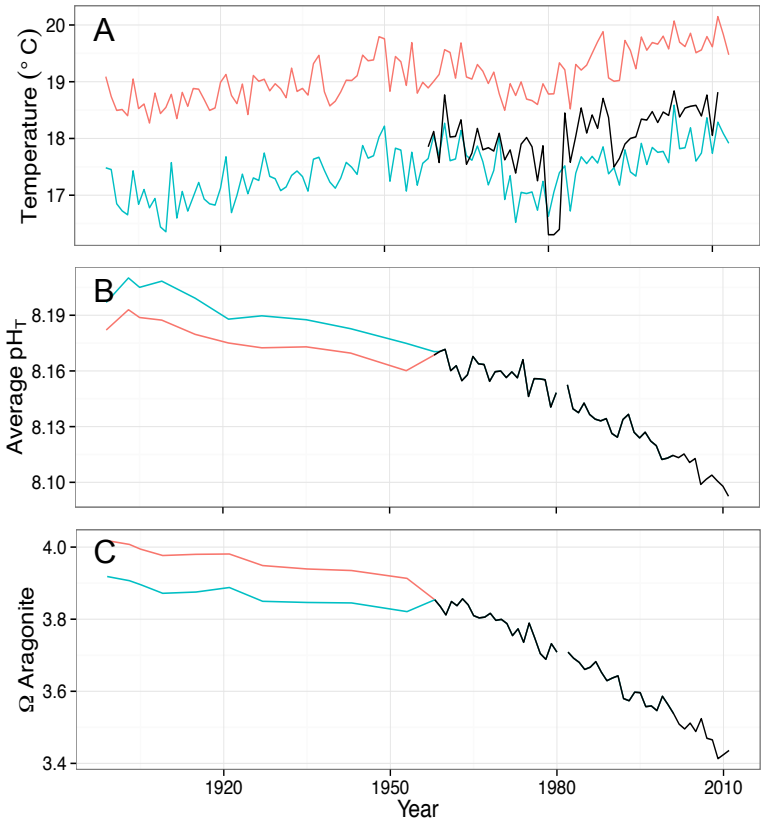

Fig. 2.4. Changes in temperature (A), pH (B) and Ω_a (C) since the 1900 at site 1 (red) and site 2 (blue) with data from Point B (black).

Shell properties:

Shell weight in museum samples was significantly higher than the 2012 for *Cavolinia inflexa* (W = 491, p = 0.004) and *Styliola subula* samples from 2012 (W = 598.5, p = 0.029). There was no significant difference between the average shell thickness of the old and new *C. inflexa* samples (W = 488, p = 0.188) although 1910 samples had a greater mean thickness than recent samples (mean 1910 samples = 29 µm ± 10.4 µm, mean 2012 samples = 28 µm ± 20.4 µm). The mean thickness of *S. subula* samples from 1921 (mean = 40.6 µm ± 12.7 µm) was significantly greater (W = 767, p = 0.002) than that of the 2012 samples (mean = 30.59 µm ± 27.5 µm; Fig. 2.5.). On average, museum samples from 1910 were thicker than samples of the same length from 2012 (Fig. 2.6). Museum samples of *C. inflexa* were significantly denser than 2012 samples (W = 568, p = < 0.001 mean 1910 samples = 1.3 ± 0.68, mean 2012 samples = 1.1 ± 0.78). No significant difference was present between densities of museum and modern *S. subula* samples (W = 380, p = 0.87; Fig. 2.5). Cross sections of shell showed the characteristic interlocked, helical, aragonite nanofibers in both species and no discernable differences in crystal structure or degradation between the old and new samples (Fig. 2.7).

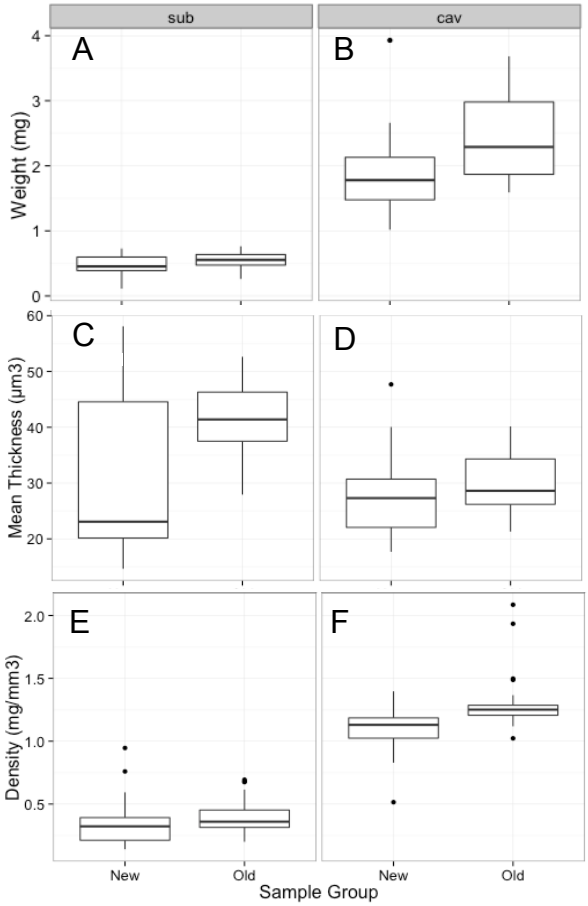

Fig. 2.5. Differences between average shell weight (A, B) thickness (C, D) and density (E, F) of new (2012) and old (1910/1921) *C. inflexa* (cav) and *S. subula* (sub).

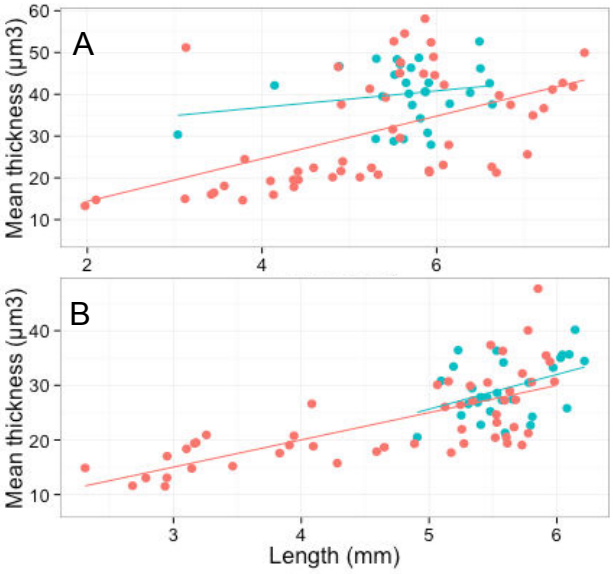

Fig. 2.6: Shell thickness (µm^3) plotted against shell length (mm) with linear regression. Museum samples shown in blue, 2012 samples shown in red. A. *Styliola subula.* B. *Cavolinia inflexa*.

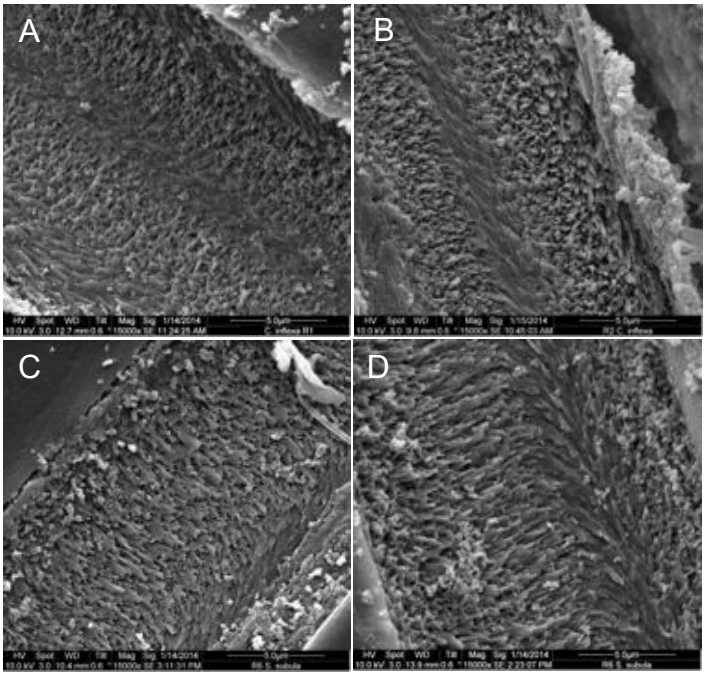

Fig. 2.7. SEM micrographs of cross sections of 1921 (A) and 2012 (B) *S. subula* shells and 1910 (C) and 2012 (D) *C. inflexa* shells.

Comparison of measured and scanned values:

To assess the accuracy of the CT scan, manually measured parameters were compared with the values obtained by the scan. In all parameters, the scanned values averaged lower than the measured ones (Fig. 2.8), for this reason, measured parameters were not compared with

scanned parameters. The average and standard deviation of the offset for measured and scanned length and width are displayed in table 2.2. During the relevant analytical period, the calcite standard yielded an average volume value of 4.124 +/- 0.062 mm^3. The standard pteropod that was analysed fifteen times over the same period yielded average shell thickness values of 40.8 +/- 3.6 µm, a volume of 0.943 +/- 0.176 mm^3, a surface area of 53.7 +/- 10.1 mm^2.

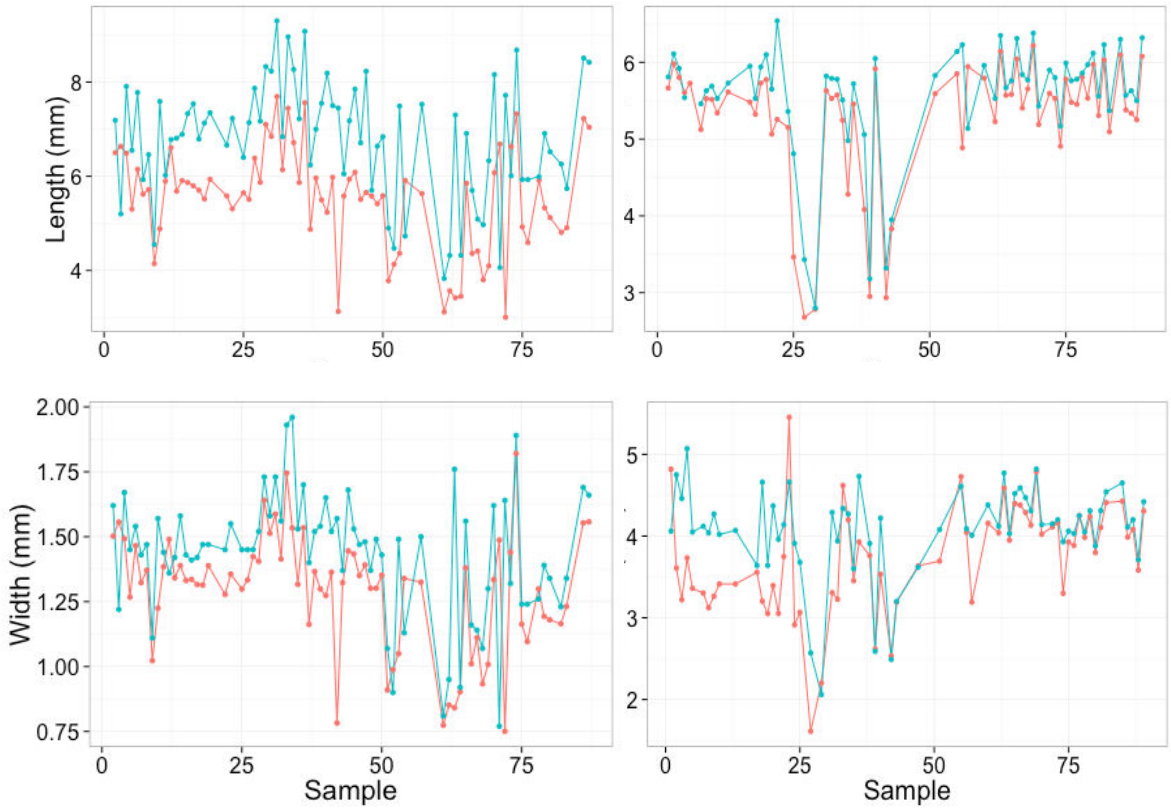

Fig. 2.8. Measured (blue) and scanned (red) shell parameters (length and width) of *C. inflexa* (A, C) and *S. subula* (B, D). Each point corresponds to a sample.

Table 2.2: Average offset and standard deviation between manually measured and scanned samples.

Species	Parameter	Average offset (mm)	Standard deviation (mm)
C. inflexa	Length	0.29	0.32
C. inflexa	Width	0.34	0.46
S. subula	Length	1.22	1.02
S. subula	Width	0.13	0.2

Discussion:

Despite a small offset between the manually measured and scanned samples, there is a broad agreement between the two datasets. The novel technique of using CT scans to assess shell properties offers the advantage of yielding many different measures per single scan. It also allows a holistic look at differences in the entire shell, not just small sections, giving an idea of the overall effects over the lifetime of the animal and taking into account areas of differing thickness due to shell structure morphology. Scanned samples showed a wide variation in wall thickness in different parts of the shell, particularly in *C. inflexa* (Fig. 2.9). Until now, it has been unclear that such variation existed and has implications for studies of shell thickness that use measurements at a single point on the shell. The different effect on density observed between the two species suggests that the density differences are not artefacts of calculations, measurement or storage and the greater thickness of the older samples also demonstrates that the differences are not due to their long term storage in ethanol producing dissolution.

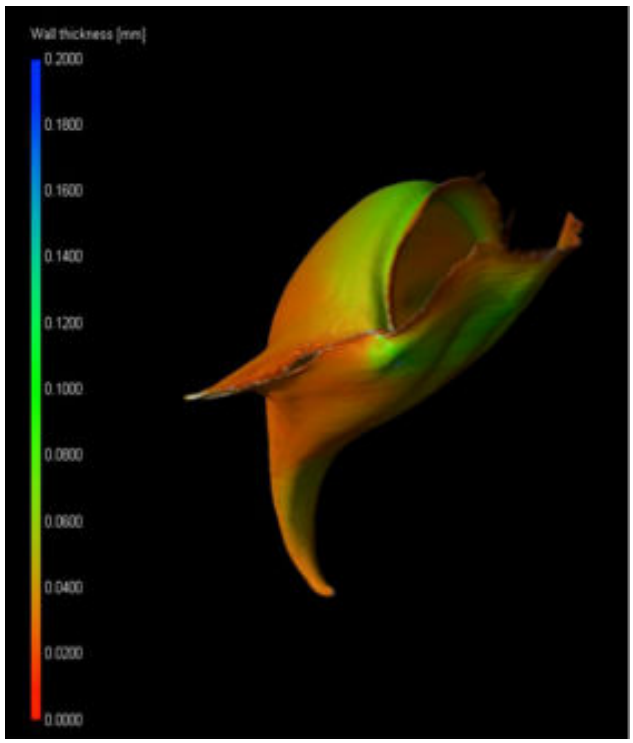

Fig. 2.9. Inhomogeneity in shell thickness of *C. inflexa.*

As ocean acidification is a relatively novel field of research, there is a paucity of long term pH and carbonate chemistry data, making it necessary to estimate historical conditions. Hind cast saturation state and pH show a steady decrease in the western Mediterranean since the turn of the 20th century at a rate of approximately 0.008 pH units per decade at site 1 and 0.007 units

per decade at site 2. This is comparable with rates reported by Touratier and Goyet (2011; pre-industrial - present day) of 0.01-0.003 pH units per decade. The effects of biological activity are not considered in the pH estimation, which may also have implications for predicting the exact pH. Records of atmospheric CO_2 are better documented, through the long-term time series at Mauna Loa, however values from ice core data must be used to attain atmospheric $pCO_{2\ atm}$ prior to the start of the series in 1958. Temperature and salinity records are more readily available, although with many gaps over the last hundred years. Both the estimated salinity values and the HadSST temperature values used for this study are in range of the measurements taken on during the 1910 and 1921 cruise sampling (salinity ± 0.5, temperature ± 0.3 ˚C; Schmidt 1912, 1929).

Mediterranean waters have remained supersaturated with respect to aragonite due to its temperate climate and high alkalinity. During the study period, the lowest saturations states of 3.18 occurred in September 2010, but even these "lows" can be considered favourable for calcification compared to projected values for 2100 and treatments in empirical studies. A recent time series study of pteropod abundances at Point B has shown no deleterious effect of the calculated decrease in pH on populations since 1967 (Howes et al. in review.). It has been demonstrated that migratory pteropod species, which are regularly exposed to a range of pH, are pre-acclimated and better able to cope with these conditions (Maas et al. 2012). As seasonal variation in pH at Point B is high, with a difference of approximately 0.07 pH units between summer and winter values, it suggests that the pteropods in the area have some degree of adaptability to cope with calcifying at a range of saturation states.

Despite the maintenance of super saturated conditions and unaffected pteropod abundances, differences were observed in shell thickness and density. Museum samples of *S. subula* were significantly thicker than modern but the same was not observed for *C. inflexa.* Modern specimens of *C. inflexa* were collected in winter (Jan-Mar) when pH is at its highest, whereas the 1910 collections were collected during July when pH is at its lowest. This suggests that seasonal variation may account for lack of significant difference in average thickness between the *C. inflexa* (*S. subula* modern and museum samples were collected at the same time of year).

This result is consistent with the findings of Roger et al. (2011) who used a different technique to compare thickness of *Diacavolinia longirostris* and *Creseis acicula* from samples ranging from 1960-2000, who found significant decreases in thickness in modern *D. longirostris* compared to older samples. Decreases in shell thickness in response to ocean acidification have been reasonably well documented in other mollusc species (Tunnicliffe et al. 2009, Gazeau et al. 2010, Gaylord et al. 2011). However, the comparison for pteropods is not as straight forward due to the shell growth phases. Studies of the shells of *Cuvierina columnella* indicate that Cavolinidae shell growth occurs in two phases; during phase 1 the shell grows to its maximum size and then, in phase 2, continues to thicken throughout the rest of the animal's life (Bé et al. 1972). To attempt to account for the continuous thickening, a wide range of modern samples were measured to compare the offset between thicknesses at the full range of shell lengths. Maximum shell length of adult *S. subula* is 13 mm and 8 mm in *C. inflexa* (Van Der Spoel 1967). The *S. subula* included in the study are far below maximum size and also collected during the same months as the 1921 samples (Table 2.1). Assuming no significant changes in breeding season, these factors minimise the likelihood of differences in shell thickness being related to ontogenetic effects. The *C. inflexa* shells are much closer to maximum length and it was not possible to collect samples from the same month of the year so the effects of life stage cannot be excluded.

In order to normalise for phase 2 shell growth, the density of the shells was calculated. *Cavolinia inflexa* shells from 1910 were significantly denser than modern shells while *S. subula* displayed no significant difference. Corals have also been observed to produce denser aragonite under higher pH conditions with more tightly packed and well-formed crystals compared to those accreted under lower pH conditions (Cohen et al. 2009, Holcomb et al. 2009). In the present study, it was it was not possible to observe any change in the packing or morphology of the aragonite rods, despite a significant change in density in one species. This could be due to the fact that the saturation states experienced by our specimens are much higher than those used in laboratory based perturbation experiments. It seems unlikely that pH conditions are the reason why density differences are seen in one species and not the other. The annual average pH and saturation states for the years of museum sample collection are very similar for the two sites, pH only differing by 0.01 units and Ω_a by 0.09 units (Table 2.1). As pteropods are thought to have life cycles of approximately 1 year (Lalli & Gilmer 1989), it is fair to assume that the annual averages are a good approximation of the conditions

experienced by individuals. Inter specific differences cannot be easily explained by depth distribution either, both have varying depth ranges in the Mediterranean, undergoing large diurnal vertical migrations. Adult *S. subula* are mesopelagic; 200-500 m with the juveniles inhabiting the upper 50 m, *C. inflexa* eurypelagic but in the Ligurian Sea a constant population can be found around 150-300 m (Rampal 1975, Andersen et al. 1998). The samples came from different locations so the effect of local environmental conditions, such as food availability, may be the cause.

Our results suggest that changes to pH and temperature have affected the shell thickness and density of two Mediterranean pteropods. However caution should be exercised as local scale changes in parameters such as dissolved inorganic carbon, salinity, and resource availability could also have affected shell properties. Geochemical comparisons of modern and museum samples could be used to clarify the hydrological conditions experienced during the lifetime of the animal but this will be the topic of a separate paper. Despite some uncertainties, the consistently greater thickness and shell weight in museum samples for two separate species, located at different sites is encouraging. The results are in agreement with the experimental work indicating that decreasing pH (or Ω_a) and increasing temperature reduce calcification (Comeau et al. 2010b, Lischka et al. 2011, Lischka & Riebesell 2012) and are also supported by the work on museum samples carried out by Roger et al. (2011). It is unclear whether the observed effects are due to solely pH or Ω_a change or an interactive effect with temperature as no studies on Mediterranean species have investigated the effects of temperature on calcification rate. The changes in density have not caused any change to the aragonite ultrastructure but work with corals has demonstrated structural changes to aragonite at saturation states as high as 2.28 (Cohen et al. 2009). At the projected levels of $CO_{2\ atm}$ for the year 2100 (Ciais et al. 2013) a reduction in Ω_a to around these values is predicted for the Mediterranean. The complex structures of pteropods shells give it flexibility and hardness whilst maintaining a light weight (Zhang et al. 2011) and any ultra-structural changes may compromise these properties. The suggestion that pteropod shells are getting thinner in synchrony with changes to temperature and saturation state indicates that there may be an increased risk of predation pressure on Mediterranean pteropods. Although long-term decreases have not yet been observed in pteropod populations in the north west Mediterranean (Howes et al. in review.), the observed changes to shell thickness and density could indicate that a tipping point may occur in the future.

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Chapter 3: Pteropod time-series from the North Western Mediterranean (1967-2003): impact of global change.

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Abstract:

Environmental changes resulting from anthropogenic CO_2 emissions occur at global and local levels and have potentially harmful effects, particularly for calcifying taxa. A time series of pteropod abundance covering the period 1967-2003 was extracted from the Point B (Northwestern Ligurian Sea) zooplankton time series. Inter and intra-annual changes in the abundance of three groups (*Limacina* spp., *Cavolinia* spp. and "*Creseis*-like") were compared with the copepods time series to search for differential effects driven by ocean acidification and temperature. pH values were hind-cast from total alkalinity estimated from temperature and salinity, and atmospheric CO_2 taken from the Mauna Loa time series. It is estimated that pH declined by 0.05 units over the study period. All pteropod groups display an increasing trend in abundance, therefore not showing any deleterious effect of declining pH. Pteropod populations are influenced by inter-annual changes in summer temperatures. Spectral analysis identified a 14-year periodic oscillation in SST and abundance of all pteropod species but not copepods, indicating a possible influence of the North Atlantic quasi-decadal mode on pteropod populations. Whilst laboratory studies have shown pteropods to be sensitive to changes in pH this analysis suggests that, in recent decades, local and regional scale drivers have a greater effect on pteropod populations in the north-western Mediterranean Sea. It should be noted that pH changes in laboratory studies clearly exceed 0.05 pH units and that the saturation state with respect to aragonite (Ω_a) is usually much lower than that for the Med.

Introduction

Anthropogenic activities such as the burning of fossil fuels, cement production and deforestation release CO_2 to the atmosphere at rates that are potentially unparalleled in the past 300 million years (Hönisch et al. 2012). Approximately one fourth of CO_2 is absorbed by the world's oceans resulting in decrease of 0.1 pH units since the industrial revolution (Le Quéré et al. 2013) with a further projected decrease of 0.3-0.4 pH units and a corresponding lowering of the saturation state of calcite (Ω_c) and aragonite (Ω_a) by the year 2100 in a business as usual scenario (Orr 2011). This can make production of shells and other calcareous structure more challenging. The effects of this carbon perturbation are also evident in increasing sea surface temperatures and expansion of anoxic areas (Gruber 2011, Bijma et al. 2013). Ocean heat content has risen by 0.40×10^{22} J yr^{-1} ($\pm$ 0.05) (Levitus et al. 2009) and accounts for 80% of total increase in heat content of the earth system (Levitus et al. 2005). The increasing sea surface temperatures (SSTs) lower the solubility of oxygen, cause stronger stratification and subsequent de-oxygenation (Keeling et al. 2010).

Due to their short generation time, sensitivity to temperature, and lack of commercial exploitation, zooplankton populations have been suggested as good indicators of climate variability (Perry et al. 2004). Experimental evidence showing decreased calcification rate, increased mortality and shell dissolution, and retarded larval development under elevated CO_2 suggests that pteropods will be particularly sensitive to ocean acidification and increasing SSTs (Comeau et al. 2009, Lischka et al. 2011, Lischka & Riebesell 2012). Studies of metabolic rate have also revealed that CO_2 acts in concert with other environmental stressors, influencing swimming speed and oxygen consumption of several pteropod species (Comeau et al. 2010, Maas et al. 2012, Manno et al. 2012). To date, experimental work on pteropods is limited to short-term (< 30 days) studies due to difficulties in maintaining laboratory cultures (Howes et al. in press). As pteropods play important roles in both the trophic system and biogeochemical cycling (Lalli & Gilmer 1989) it is important that the longer-term effects of climate change on pteropod populations are better understood.

As a result of high irradiance levels and extreme anthropogenic pressure, the Mediterranean has been suggested to be a "hotspot" for climate change effects (The Mermex Group 2011). Due to its semi enclosed nature and short residence time of waters, the Mediterranean Sea is

sensitive to external forcing, giving it the potential to act as an early warning for global ocean changes (Duarte et al. 1999). The long-term Mediterranean zooplankton time series at Point B, Villefranche-sur-Mer, have been analysed for the effects of climate change and other anthropogenic impacts (Molinero et al. 2005a, Molinero et al. 2005b, Garcia-Comas et al. 2011, Vandromme et al. 2011, Berline et al. 2012). However, the long-term changes in pteropod populations have never been investigated in the Mediterranean Sea.

Methods:

Sampling site:

Point B (43° 41,10 ' N - 7° 18.94 ' E) is located at the mouth of the Bay of Villefranche-sur-Mer (Fig. 3.1). The site has a depth of ~ 80 m and is situated on a narrow continental shelf, a few kilometres offshore the slope increases to ~ 2000 m depth, giving this coastal site the characteristics of an open ocean area. The North Atlantic Oscillation (NAO) has a significant influence on the area (Molinero et al. 2005a). The site experiences strong seasonality with chlorophyll-a maxima during the spring months. The conditions during winter have been shown to have a strong influence on the productivity of the following year (Buecher et al. 1997, Garcia-Comas et al. 2011).

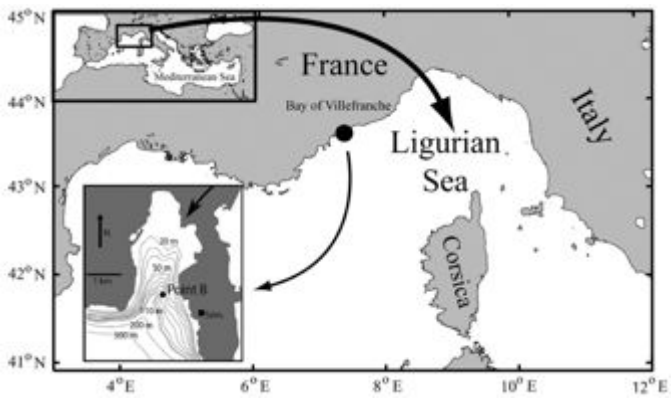

Fig. 3.1: Location of Point B.

Environmental variables:

Environmental variables were provided by the long term hydrological time-series run at Point B, now operated as part of the *Service d'Observation en Milieu Littoral* (SOMLIT/CNRS-INSU). Sampling is undertaken at depths of 0, 10, 20, 30, 50 and 75 m on a weekly basis. The two parameters that have been continually measured throughout the study period are temperature and salinity. Further information on environmental sampling methods is provided

at the SOMLIT web site (http://somlit.epoc.u-bordeaux1.fr/fr/). Temperature and salinity were averaged over the full depth profile for annual anomalies. Values for 20 m and 50 m depth were also used to assess differences above and below the thermocline. Wind speeds were provided by Météo France

Water for the determination of dissolved inorganic carbon (C_T) and total alkalinity (A_T) were collected from 10-L Niskin bottles, transferred to combusted glass bottles, overfilled, and poisoned with $HgCl_2$ as recommended by Dickson et al. (2007). C_T and A_T are determined colorimetrically and potentiometrically, respectively (Edmond, 1970; DOE, 1994).

Sea water density (kg m^{-3}) was calculated from temperature, salinity and pressure using the usual UNESCO formula implemented in the R package oce (Fofonoff & Millard Jr 1983, Kelley 2013). Total alkalinity (A_T) was calculated from monthly averages of temperature and salinity using a variation on the equations provided by Lee et al. (2006; Eq. 1), where: A_T, total alkalinity (µmol kg^{-1}); S, salinity, T, temperature and a, b, c, d, e are constants.

$$A_T = \mathrm{a} + \mathrm{b} \times \mathrm{S} + \mathrm{c} \times \mathrm{S}^2 + \mathrm{d} \times \mathrm{T} + \mathrm{e} \times \mathrm{T}^2 \qquad \text{(Eq. 1)}$$

The most parsimonious model was determined from eq. 1 using the Akaike information criterion measure on sub-sampled (one per 10 weeks, to reduce the autocorrelation of the time series) alkalinity measurements ranging from 2007 to 2012. The temperature variables were dropped as they did not affect the goodness of fit of the model to the measured data. The constants a, b and c were then determined with all measures 2007-2012. The coeffcients a, b and c were derived from the weekly measurements of alkalinity taken from 2007-2012. Finally, the calculation of A_T was extended for the years 1967 to 2006.

The partial pressure of CO_2 ($pCO_{2\,sw}$) was estimated from temperature and concentration of CO_2 in the atmosphere ($CO_{2\,atm}$, ppm; Lefevre & Taylor 2002; Eq. 2).

$$\mathrm{pCO_{2_{sw}}} = \mathrm{a} \times \mathrm{T} + \mathrm{b} \times \mathrm{pCO_{2_{atm}}} + \mathrm{c} \qquad \text{(Eq. 2)}$$

There is no measurement of $CO_{2\,atm}$ in the Mediterranean region covering the entirety of the study period. The Mauna Loa (19˚53'N – 155˚57'W, Hawaii) atmospheric CO_2 time series

was used, as this is the only station in the northern hemisphere that has data for the entire study period. To account for geographical differences, the data were compared to the 1979 - 1997, $CO_{2\ atm}$ time series from the Italian station, Monte Cimone (44˚18'N – 10˚7'E). Seasonal differences were minimised by lagging the Manuna Loa data by 2 months and adjusting seasonal maxima and minima of the values from the Mauna Loa time series to obtain the best fit with the data from Monte Cimone. The coefficients were determined using a linear model for 1 and 50 m based on $pCO_{2\ sw}$ computed from SOMLIT data from weekly measurements of dissolved inorganic carbon and total alkalinity ranging from January 2007 to March 2012.

Using the estimations of A_T and $pCO_{2\ sw}$, alongside the measured values of temperature, salinity and pressure, it was possible to calculate seawater pH using the R package seacarb (Lavigne & Gattuso 2013). The total boron concentration was calculated using the formulation of Lee et al. (2010). The following constants were used: K_1 and K_2 from Lueker et al. (2000), K_f from Perez and Fraga (1987) and K_s from Dickson (1990). Weekly measurements of pH_T at Point B began in 2007. These measurements (2007-2012) are used to assess the goodness of fit for the calculated data.

Zooplankton time series:

Daily zooplankton sampling (Monday to Friday) has been performed at Point B since 1968; the daily samples are pooled to form one, weekly, sample thereby overcoming issues with localised patchiness. Vertical hauls from 75 m depth to the surface were taken using a Juday-Bogorov net (330 µm mesh size and 50 cm opening diameter). The pooled weekly samples were preserved in formalin (2.5%) buffered with borax (sodium borate) until saturation. The preserved weekly samples were sorted using the Zooscan digital imaging system (http://www.zooscan.com, Gorsky et al. 2010). Each sample was split and analysed in two size fractions to avoid biased representation of large, rare organisms (Garcia-Comas et al. 2011). The pteropods were classified into three genus-based groups: Cavolinids (including *Clio* spp.), Limacinids and "*Creseis*-like" (including *Creseis* spp. and *Styliola subula*), the automated Zooscan classifications were manually verified. The copepod abundance was also analysed to provide a common zooplankton group as comparison for the pteropods.

Analyses:

Monthly averages of environmental and zooplankton data were used with missing data linearly interpolated. Zooplankton time series were log-transformed (log10(abundance +1)) in order to better describe periods outside of the bloom where abundances are very high. To assess the environmental forcing on zooplankton abundances, two Principal Component Analysis (PCA) of seasonal anomalies (normalised by standard deviation from the annual mean) were performed using the R package FactoMineR (Lê et al. 2008) on environmental data (with biological data added as supplementary variables) and on zooplankton abundances. Seasons were defined as per Vandromme et al. (2011); winter = Jan-Mar, spring = Apr-Jun, summer = Jul-Sept, autumn = Oct-Dec. Seawater densities were not included in the PCA because they were computed from temperature and salinity and therefore risk artificially altering PCA results as they are strongly correlated to these variables. Cumulative sum of differences to the mean (CUMSUM) analysis was used to highlight the turning points values above or below the mean of the series, for PC1 of biological and environmental variables (Ibanez et al. 1993).

Singular Spectrum Analysis (SSA, Allen & Smith 1996) was performed, using the R package Rssa (Korobeynikov 2010), on normalised monthly means, to search for statistically significant, periodic components in the environmental and zooplankton time series. SSA is well suited to use with short and/or noisy time series, identifying trend, oscillations and noise within the dataset. To minimise noise to signal ratio, as large a window as possible is used, whilst remaining under half the length of the time series (Allen & Smith 1996). Trends were analysed by SSA of non log transformed data as transformation can cause inaccurate estimation of trends. The window size used for the analysis presented herein was 200 months. As this is a relatively large window in comparison to the total length of the series, results were checked for robustness by comparison with analyses undertaken using a smaller window (150 months), which obtained similar results. All statistical analyses were undertaken using R 3.0.1 (R Core Team 2008).

Results:

The pH hind-cast shows a decreasing trend with a decrease in annual mean pH of 0.05 pH_T units over the study period (1967-2003) (Fig. 3.2A), or 0.014 units per decade. Standardised

anomalies of annual means of predicted pH_T values (Fig. 3.2B) reflect the steady downward trend with values mostly above average up to 1982 and mostly below average from 1983 to 2003. Comparison of calculated pH_T values to measured values (2007-2012) by linear regression produced an r^2 of 0.49 as the model failed to accurately predict the extreme low observations. However, the high values were well predicted and the overall trend and seasonal oscillations fit well with the real data (Fig. 3.2A).

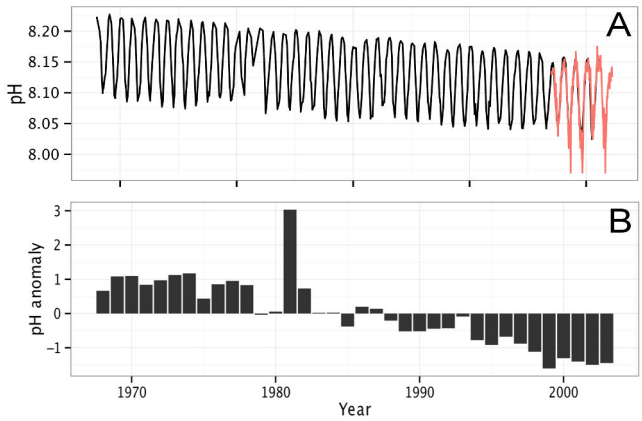

Fig. 3.2A: Estimation of pH values (black) and measured values from 2007 – 2012 (red). B: Standardised annual anomalies of the calculated pH at Point B from 1968-2003.

Sea temperature shows strong seasonal patterns (Fig. 3.3A) and ranged from winter minima of 12.4 to 14.7 ˚C and maxima in summer of 21.4 to 27.1 ˚C. At 50 m, the winter minima ranged from 12.5 to 14.4˚C, maxima were during autumn (14.0 to 22.30 ˚C). Variations in standardised anomalies of mean winter temperatures (Fig. 3.3D) display alternating periods of low and high temperatures. Temperatures were below the average for the study period until 1982, after which a warm period begins, lasting until 1990. Between 1990 and 1995 temperatures were again below average but increased after 1995 and remained above average for the rest of study period. Salinity is less seasonally influenced than temperature (Fig. 3.3B). It ranges between 36.46 and 38.52, with the lowest values observed in spring and winter. Inter-annual variations in winter salinity also alternate between periods of low and high salinity (Fig. 3.3E). Before 1981, values were below average. From 1981 to 1988 salinity was above average and displayed the highest values in the time series. Between 1989 and 1998 salinity is generally below average and from 1999 until the end of the series values are again above average. Winter density at Point B was slightly below average for the first three years of the series and tends to oscillate on short, 3-5 year, cycles, of high and low density (Fig. 3.3F). The variations from the mean are typically small except for two years in the 1980's (1981 and 1982). From 1996 onwards, values are all below average except for the years 2000

and 2003. Seasonal variations show lower densities in the summer during the early 1970's and the late 1990's to early 2000's (Fig. 3.3C). Higher winter densities are observed in the winter during the 1980's and early 1990's.

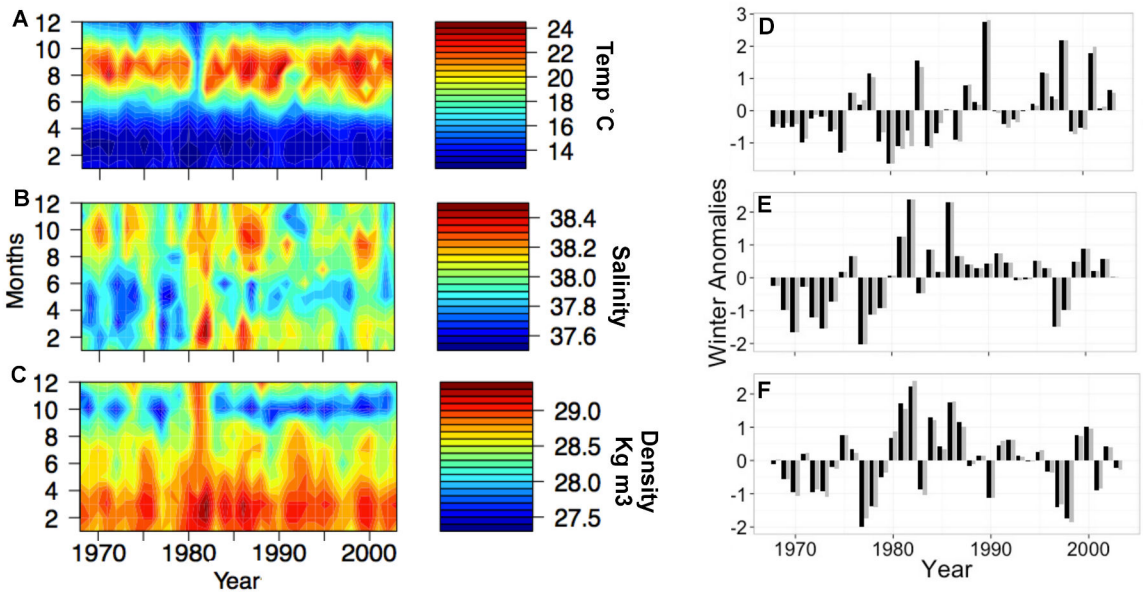

Fig. 3.3: Seasonal and inter-annual variability of (A) temperature, (B) salinity and (C) density at 20 m depth. (D) Standardised winter (Jan-Mar) anomalies of the temperature, (E) salinity and (F) density at 20 (grey bars) and 50 (black bars) m depth.

Creseis-like spp. constitute the majority of the pteropod species sampled at Point B, on average 15.7 ind m^{-3} (Fig 3.4). *Cavolinia* spp. are the next most abundant, with a mean of 13.8 ind m^{-3}. *Limacina* spp. were least abundant, on average 5.5 ind m^{-3} and maxima of 60.8 ind m^{-3}. Both *Cavolinia* and *Creseis* spp. have maxima (respectively 903.7 ind m^{-3} in 1999 and 677.8 ind m^{-3}, in 2001) significantly higher than average, which occurred within 2 years of each other. The maximum abundance of *Limacina* spp. occurred earlier in the series (60.8 ind m^{-3} in 1989; Fig. 3.4). The dominant species in each group is *Creseis acicula, Cavolinia inflexa* and *Limacina inflata*. Inter-annual abundances of all pteropod species alternate between low and high abundances on a quasi-decadal timescale (Fig. 3.4E-G). Abundances are below average from the start of the series until the early 1980's; this is followed by a period of higher than average abundances lasting until the early 1990's when numbers fall below average until approximately 1995. After 1995 abundances remain above average until the end of the series in 2003. Copepods display a similar trend at the start and end of the series with low abundances until the mid 1970's. From this point, numbers remain around the average for the series with slight oscillations of approximately 5 years in length (Fig. 3.4H). Abundances were very low from the early 1990's but recovered by 1997 and remained above

average until the end of the series. *Cavolinia* spp. exhibit earlier bloom times during the 1980's with high numbers appearing in late spring and summer months compared with the blooms of the 1970's and late 1960's which begun in late summer/early autumn and extended into the following spring (Fig. 3.4A). *Creseis*-like spp. bloom during summer and early winter, occurring earlier in the year as the series progresses (Fig. 3.4B). *Limacina* species tend to exhibit high abundances twice in the year with one bloom in spring and another in early autumn (Fig 3.4C).

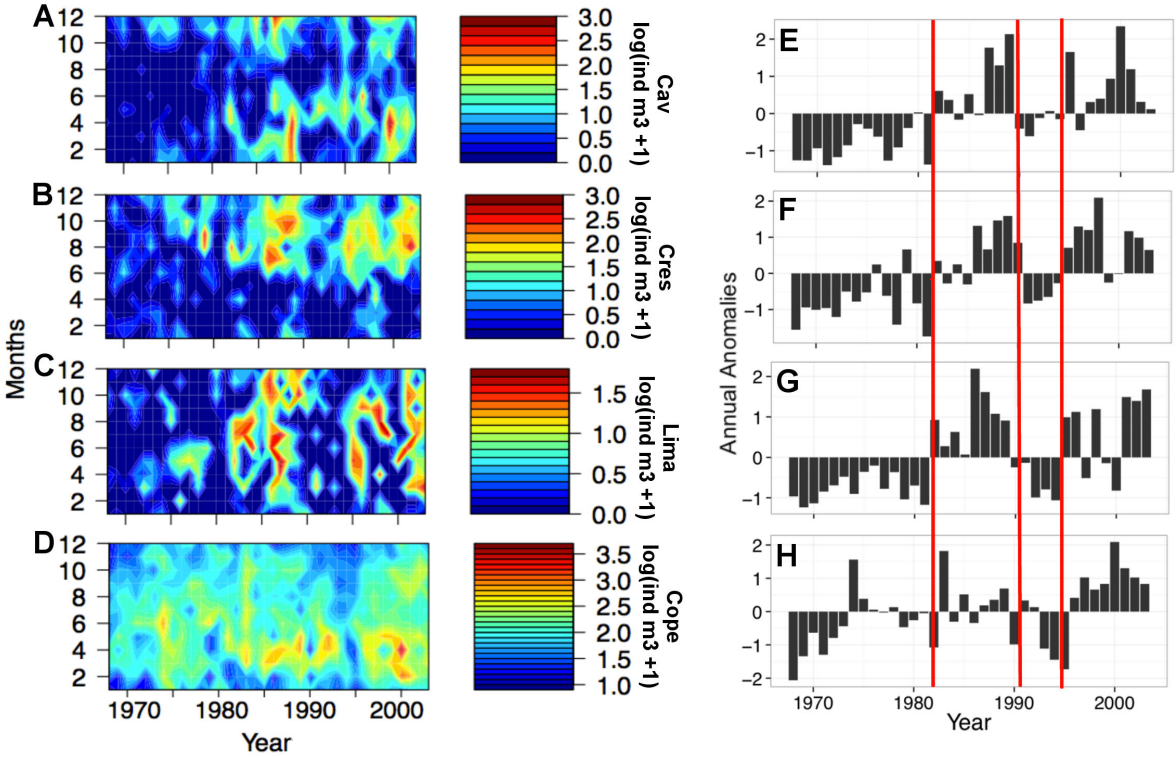

Fig. 3.4: :(A) Seasonal and inter-annual variability of *Cavolina*, (B) *Creseis*-like, (C) *Limacina* and (D) copepod abundances (log ind mm^{-3}). (E) Standardised annual anomalies of *Cavolina*, (F) *Creseis*-like, (G) *Limacina* and (H) copepod abundances zooplankton abundances at Point B, red lines separating alternating periods of high and low abundances.

The first and second principle components (PC) of environmental variables explained 46 and 32 % of the variance in the dataset. Temperature and salinity have strong positive loadings on PC1 while pH and wind speed have strong negative loadings (Fig 3.5A). Salinity is positively, and SST negatively, correlated with PC2. All pteropod groups are positively placed with respect to PC1, whilst copepods are negatively correlated with PC1. Both *Cavolina* and *Limacina* spp. are positively correlated to PC2, *Creseis*-like spp. are negatively placed with respect to PC2. *Creseis* abundances are strongly associated with surface temperatures and negatively with wind speed and pH whilst *Cavolinia* abundances correlate

to salinity below 50 m depth. *Limacina*. spp do not show a close association with any of the environmental variables. The cumulative sums of both PC1 for the environmental PCA and PC1 biological PCA show an oscillating pattern of 4 periods with turning points at 1981, 1989, and 1994 for biological variables and 1979, 1991 and 1998 for environmental variables (Fig. 3.5B, C). This related to the change in sign of the anomalies already highlighted above.

Table 3.1: Abbreviations used for the two PCA labels

1	surface	20	20 m	50	50 m	75	75 m
T	Sea temperature	S	Salinity	WS	Wind speed	pH	pH (calculated)
cope	Copepods	cres	*Creseis*-like spp.	cav	*Cavolina* spp.	lima	*Limacina* spp.

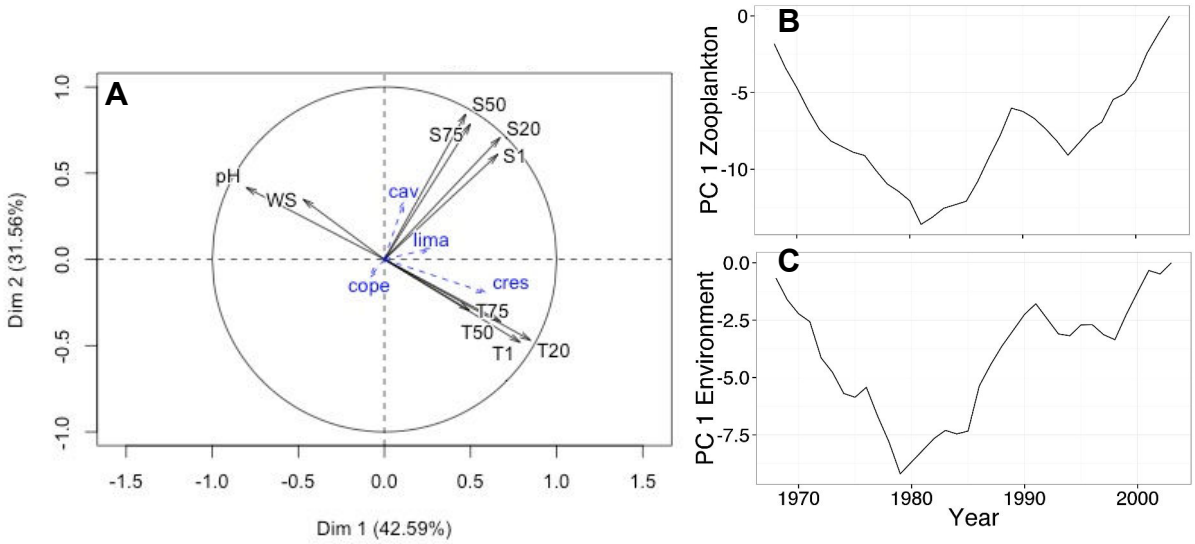

Fig. 3.5: (A) Principal component analysis of environmental variables at Point B, 1968-2003 with biological data as supplementary variables. CUMSUM of the first principle components of the PCA of (B) zooplankton abundances and (C) environmental variables.

SSA performed on non log-transformed abundances showed an increasing trend associated with the first eigenvalue for all pteropod species. The trend accounts for 10%, 13% and 19% of the total variance in *Cavolinia, Creseis* and *Limacina* spp., respectively. No significant trend was found for copepods. SSA analysis reveals a clear annual component in all zooplankton groups, which is expected in such a temperate, seasonal environment. All pteropod groups exhibit a quasi-decadal oscillation (Fig. 3.6A-C), above the noise level for all pteropod species groups and explaining 2%, 4%, 5% of the variation in *Creseis*-like spp., *Cavolinia* spp. and *Limacina* spp. respectively. The oscillations occur for *Cavolinia* every 11.2 years, for *Creseis* every 9.9 years and *Limacina* every 16.7 years. Sea temperature (averaged over depths 0, 20, 30, 50, 70 m) also displayed a quasi-decadal component of

approximately 15.3-year oscillations associated with the first and second eigenvalues (Fig. 7D), which accounted for 10% of the total variance. Copepods did not show a significant decadal component, however a 6.3-year oscillation (Fig. 3.7A) associated with the 9th and 10th eigenvalues was identified. This component was barely above the noise level and explained only 0.2% of the variance as the series is strongly dominated by the annual component. Density displays a similar pattern with a 6.9-year oscillation (Fig. 3.7B) on the 1st and 2nd eigenvalues, accounting for 17% of the variance in the series.

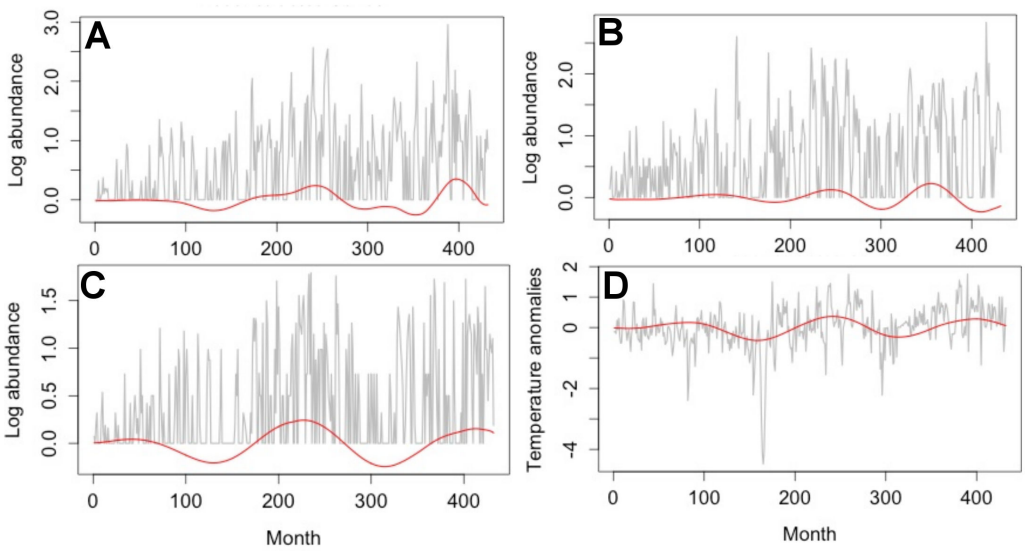

Fig. 3.6 Quasi decadal periodic component, indicated by red line, extracted from the time series: (A) *Cavolinia,* (B) *Creseis*-like, (C) *Limacina,* (D) normalised sea temperature, integrated 0-75 m depth.

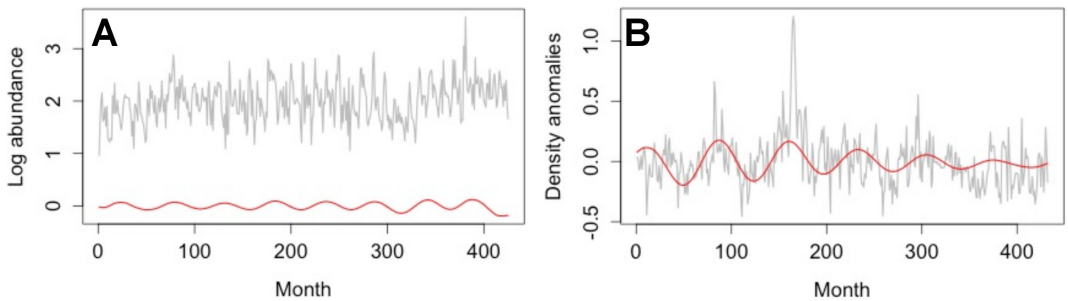

Fig. 3.7: Approximately 6.5-7 year periodic component, indicated by red line in (A) Copepod, (B) normalised density, integrated 0-75 m depth.

Discussion:

The main objective of the study was to investigate the inter-annual changes to pteropod populations at Point B and their response to anthropogenically driven changes in pH and temperature. The increasing trend in global and local temperatures is well documented (Bethoux & Gentili 1996, Vargas-Yanez et al. 2008, Levitus et al. 2009) but there is paucity of long-term pH and seawater carbonate system measurements. Records of atmospheric CO_2 are better documented, although the only northern hemisphere time series that covers the entire study period was the series at Mauna Loa. The effects of biological activity are not considered in the pH estimation, which may also have implications for predicting the exact pH. Despite inaccuracies in predicting extreme low values, pH hind-cast produced a decreasing trend that is towards the lower end of the range of –0.0014 and –0.0024 yr^{-1} reported in the 5th Assessment report of the IPCC (Rhein et al. 2013). This rate is comparable to the one reported by Touratier and Goyet (2011) for the Western Mediterranean from 1870 - present day. Attempts to calculate pH at 50 m depth were unsuccessful as the equations of Lefevre and Taylor (2002) refer specifically to SST so $pCO_{2\,sw}$ is not well constrained.

Despite a clear decreasing trend in estimated pH (Fig. 3.2) there is no evidence of any deleterious effect on pteropod populations, on the contrary, the abundance of all three species groups display positive trends. Due to the highly alkaline conditions in the evaporation basin of the Mediterranean, supersaturated conditions with respect to calcite and aragonite are maintained. At its highest during the study period, estimated Ω_a is 4.3 (1968), with a minimum of 3.1 in 2003. Studies that have shown pteropods to be sensitive to pH change have focused on future conditions and exposes individuals to saturation states that are well below those experienced during our study period. Evidence has shown that pteropods are capable of extending their shells in waters under-saturated with respect to aragonite (Comeau et al. 2012), suggesting that calcification of Mediterranean species will not have been adversely affected by a pH decrease that maintains super-saturation. Seasonal pH fluctuations are high, approximately 0.1 units, larger than the average decrease over the study period, suggesting that Mediterranean pteropods may also have a degree of short term adaptability to a range of saturation states. Despite some opportunity for adaptation, a continued downward trend in pH and saturation state will decrease annual minima, which may eventually produce the effects seen in experimental work.

These findings are in line with most other time series observations. Mackas and Galbraith (2011) found increased numbers of *Clio pyramidata* throughout their 30-year study period in the NE Pacific, despite a decreasing trend in pH. The abundance of *C. pyramidata* also increased over a 57-year times series (1951-2008) in the Central California Current System, alongside a decreasing pH trend, whilst *Limacina helicina* remained constant (Ohman et al. 2009). The same study also looked at the Southern California Current System where neither species displayed any changes in abundances. The analysis of a short time series (1994-2009) off the Antarctic Peninsula did not show any decreasing trend in pteropods populations (Loeb & Santora 2013). This is in contrast to recent work by Beare et al. (2013) who reported a decline in pteropods in the North Sea, over the last 50 years.

The effect of temperature on pteropod abundances appears to be more critical than pH, the above-mentioned studies generally found increases in cosmopolitan species concurrent with a rise in temperature and no change or a decrease in polar species with more limited tolerance. In the present study, the periods of high pteropod abundances are linked to higher summer temperatures. The majority of the pteropods sampled at Point B are cosmopolitan, warm-water species that are also abundant in tropical regions (Van Der Spoel 1967). The climate at Point B does not reach their maximum temperature ranges so it is feasible that warmer waters are conducive to increased fitness and higher abundances and that the increasing trend of all species groups over the course of the study period is linked to the effects of increased temperature. The sensitivity to temperature can also be observed in the extended bloom periods of *Cavolinia* spp. and the earlier timing of *Creseis*-like spp. blooms throughout the year. If this phenological shift continues to occur, it could have significant effects on populations as a result of trophic mismatches (Edwards & Richardson 2004).

The secondary aim of this study was to identify the factors controlling pteropod abundance. All pteropod series were characterised by long periods of low abundance with sudden extremely high abundances. A large portion of the variance (44%) in the zooplankton time series is explained by the oscillating trend shown in PC1 (Fig. 3.5B). This is similar to that identified for other zooplankton groups at Point B (Garcia-Comas et al. 2011, Berline et al. 2012) and bears a close resemblance to PC1 of the environmental data. This alternating trend is linked to inter-annual alternations between dry and wet years (Buecher et al. 1997) which,

as suggested by Garcia-Comas et al. (2011), drives a nutrient enrichment process, whereby favourable conditions in the previous winter increase phytoplankton growth in the following spring. The enhanced spring bloom leads to a stronger zooplankton community, exerting a "bottom-up" control on their abundances. During dry years, there is more intense convection (higher salinity, more heat loss from sea surface and colder winter temperature, despite higher annual temperature), loading more nutrients to the upper water layers. Prior to 1990, winter temperature and salinity interact as described to provide favourable conditions the following spring, the 1968-1970 low abundance period corresponds with a cool period with low salinity. The 1980's experienced warmer average years, due to warm summers but very cold winters and high salinities, leading to better mixing and the building up of zooplankton populations.

From the 1990's onwards, it seems that pteropod abundances at Point B co-vary more with summer temperature than winter salinities with higher abundances during warmer periods. The decrease in zooplankton abundance in the 90's is a common feature in all zooplankton groups analysed in the present study and by Garcia-Comas et al. (2011), although more pronounced for pteropods than for copepods. This turning point in the 1990s is correlated with a decrease in both winter and summer sea temperatures and above average winter salinity. The subsequent recovery of pteropod abundance coincides with a shift back to warmer summer temperatures (Fig. 3.3) and lower rainfall at the end of the 90's. It is well documented that zooplankton populations are highly sensitive to temperature often with complex and unpredictable responses (Richardson 2008). It is possible that higher temperatures in the late 1990s at the Point B station allowed faster growth and development for a larger breeding population as seen in the study by Ottersen and Stenseth (2001) on zooplankton of the Barents Sea. The singular spectrum analysis highlighted similar oscillating quasi-decadal periodicity between sea temperatures and all pteropod groups The periodicity identified in this study for temperature and pteropod groups is in the same range as the quasi-decadal oscillations of the NAO (Deser & Blackmon 1993, Dima et al. 2001). The cyclical population fluctuations caused by the NAO have the potential to be further impacted by climate change stressors, which could push organisms, already at the limits of the thermal tolerance (Beaugrand et al. 2008). However there is evidence to suggest that the centre of action of the NAO has shifted north-eastwards during the 1990's and early 2000's (Zhang et al. 2008) which was shown to diminish its influence on south western European temperatures (Beranova & Huth 2007). A correlation with inter annual temperature oscillations is not seen

in the copepod time series, which shows a closer link to seawater density, both series exhibiting approximately a 6-year cycle, supporting the strong link proposed by the "bottom-up" hypothesis.

Explanations for differences in pteropod and copepod abundances could be due to the differences in life history traits or trophic regime. Copepods generally feed by active prey detection and capture (Kiørboe 2011). Their peaks of abundance at Point B are correlated to optimum feeding conditions during spring phytoplankton blooms. The annual blooms of pteropods at Point B begin later in the year than the copepod blooms with most species appearing in summer or autumn. Blooms are related to seasonal vertical migrations linked to reproduction more than to food availability. Pteropods are passive feeders, utilising large mucous webs to collect food particles (Gilmer 1972) and so have a more omnivorous diet than many copepod species. Inter annual fluctuations of the pteropod time series co-vary more closely with those displayed by the jellyfish at Point B (Garcia-Comas et al. 2011). Like pteropods, the gelatinous predators at Point B (chaetognaths and jellyfish) bloom later in the year compared to copepods and suggesting that differences in the copepod and pteropod abundances can be related to trophic regime.

Pteropods were abundant in far lower numbers than copepods, with less consistent patterns of abundance. The vertical sampling depth of 0 to 70 m is not ideal for year round sampling of pteropods as the depth ranges of the main species present at the site reach far deeper, up to 1000 m (Rampal 1975). The site depth of 200 m is also a relatively shallow environment for pteropods. Despite these factors, the average abundances of *Cavolinia* spp. found in this study are comparable to those found across the wider Ligurian and Tyrrhenian Seas by Andersen et al. (1998). The *Limacina* group is particularly underrepresented with respect to the other two groups, likely due to sampling bias. *Limacina inflata* is by far the most abundant Limacinid at the site and its small body size (maximum shell diameter 1200 µm) may mean that the juvenile population is under sampled by the 330 µm mesh net compared to the larger bodied Cavolinid species. The low abundances may explain the lack of a strong association with any of the environmental PCA variables. The differences seen in the PCA between the *Cavolina* and *Creseis* groups could be related to differences in annual phenology. High *Creseis* abundances occur during summer months and are related to reproduction (Rampal 1975), it is likely that this process is governed by environmental cues such as temperature as is the case

for opisthobranch species (Thompson 1958), which could explain the strong associations with temperature. *Cavolinia* are abundant from late autumn to early spring, also migrating higher in the water column to reproduce (Rampal 1975). This timing means that they are concurrent with the spring bloom and so the success of the new generation population may be affected by the strength of the bloom, explaining the associations with salinity in its relation to the strength of winter convection of nutrients.

The mechanisms driving pteropod population fluctuations are complex but winter forcing and decadal temperature oscillations are the dominant factors controlling their abundance. Recent (1968-2012) trends in sea surface temperature and pH have not adversely affected populations which show an overall increase in abundance. This does not rule out future changes. The effects of climate change have accelerated over the last three decades (Romano 2003) and the Mediterranean is under significant pressure such as pollution, over fishing, and species invasions, which may eventually act in concert as a suit of stressors (The MerMex Group 2011). Time series studies have shown pteropods to be sensitive to temperature, and from empirical studies, it is clear that pteropods are highly affected by decreasing Ω_a. Recent environmental shifts are currently within the range of tolerance, this may not be the case for the conditions projected in the coming decades.

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Chapter 4: Sink and swim, a status review of thecosome pteropod culture techniques

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Abstract

The widespread distribution of pteropods, their role in ocean food webs, and their sensitivity to ocean acidification and warming has renewed scientific interest in this zooplanktonic group. Unfortunately, their fragile shell, sensitivity to handling, unknowns surrounding buoyancy regulation, and poorly described feeding mechanisms make thecosome pteropods notoriously difficult to maintain in the laboratory. The resultant high mortality rates and unnatural behaviours may confound experimental findings. High mortality rate also discourages the use of acclimation periods to experimental conditions, and precludes vital long-term studies. Here we summarise the current status of culture methodology to provide a comprehensive basis for future experimental work and culture system development.

Introduction:

Pteropods are a cosmopolitan group of holoplanktonic molluscs, which consist of thecosome (shelled) and gymnosome (shell-less) species, both of which play an important role in trophic and biogeochemical fluxes (Lalli & Gilmer 1989). For decades, pteropods have been particularly understudied, but recently thecosome species have become the focus of renewed scientific interest within the rapidly expanding field of ocean acidification research, and as such are the primary focus of this review. Since the industrial revolution the oceans have absorbed approximately one third of anthropogenic CO_2 (Sabine et al. 2004), altering seawater carbonate chemistry. Ocean pH has dropped by 0.1 pH units since the industrial revolution and is projected to decrease by up to 0.3-0.4 units by 2100 (Orr 2011).

Pteropods are predicted to be particularly vulnerable to the effects of lowered carbonate saturation states resulting from ocean acidification as their thin shells are made of aragonite, a form of calcium carbonate more soluble than calcite in seawater (Mucci 1983). Studies have confirmed that pteropod larvae and adults exhibit negative calcification responses to projected future conditions with decreased calcification (Comeau et al. 2010a, Comeau et al. 2010b, Lischka et al. 2011) and shell dissolution (Lischka et al. 2011). Studies of metabolic rate have also revealed that CO_2 acts in concert with other environmental stressors, such as temperature and salinity, influencing swim speed and oxygen consumption in some species of pteropods (Comeau et al. 2010b, Maas et al. 2011, Bednaršek et al. 2012a, Maas et al. 2012a, Manno et al. 2012). These studies have been primarily of short-term duration due to constraints imposed by the difficulties of keeping pteropods in culture.

In their natural environment, pteropods have periods of active swimming, sinking and neutral buoyancy (Lalli & Gilmer 1989). The weight of the shell causes the animals to sink relatively quickly and culture vessels do not provide a sufficiently high water column and/or adequate current conditions for the animals to attain neutral buoyancy. This often results in the animals repeatedly hitting the bottom of the culture vessel, causing shell breakage and the production of stress-induced mucous. The mucous can adhere their wings together, impairing swimming ability and can adhere them to the bottom of the tank.

Lack of buoyancy, in turn, impedes feeding behaviour. Thecosome pteropods are suspension feeders, producing mucous webs from their wings (Fig. 4.1), several times their body size, to entrap phytoplankton and small motile prey and other particles (Gilmer 1972). Production and retraction of the web can be relatively fast; as little as 5 seconds for deployment and < 20 seconds for retraction, although this is highly species specific (Gilmer & Harbison 1986). Whilst the web is deployed the animal hangs motionless with the web between the parapodia; eventually the animal consumes the entire mucous structure with its attached particles. In culture, there is insufficient time to extend the wings and deploy the feeding web. Food deprivation has been shown to function as a synergistic stressor with CO_2 (Seibel et al. 2012) and thus also acts as a confounding variable in long-term studies of captive animals.

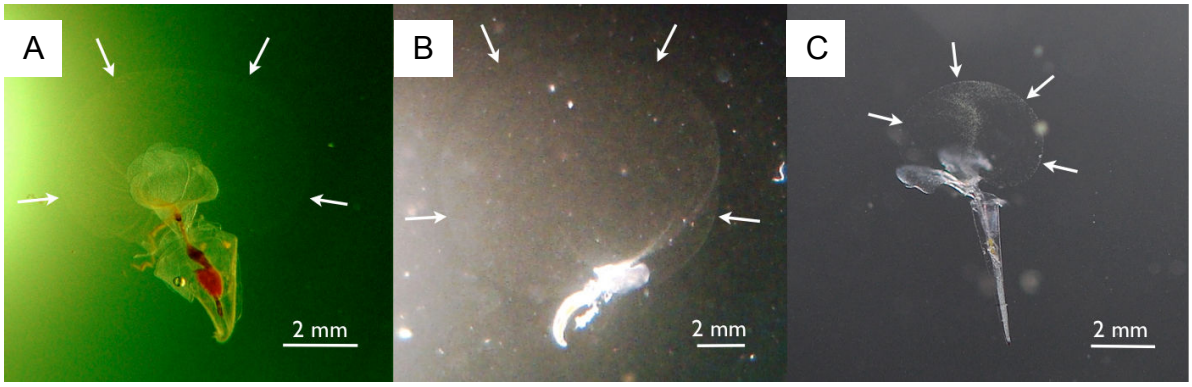

Fig. 4.1. Mucus web productions observed on pteropods maintained in temperature-gradient kreisel (Gorsky *et al.*, 1986). Panel (**A**) represents the mucus web being retracted and panel (**B**) shows the mucus web at its maximum extension in Mediterranean *C. inflexa*. Panel (**C**) shows the mucus production of the Mediterranean pteropod *C. virgula*. Photos: S. Comeau

In the course of experimental work with pteropods, various methods have been used to culture animals in the laboratory, with varying degrees of success. At the time of writing, only one laboratory has successfully reared a single species of pteropod, *Limacina retroversa*, through a full life cycle. Here we report, for the first time, the methods used. A common problem in developing culture systems is the patchy and seasonal abundance of pteropods, which limits opportunities for method testing and development. As negative results are often not published, these rare chances are often wasted by unknowingly repeating the unsuccessful tests of others, for this reason we also provide a summary of the varied and often-unpublished techniques tested in pteropod culture work. The aim of the present assessment is to summarise the different approaches used and discuss their efficacy as a starting point for the much-needed further development of pteropod culturing systems.

Methods:

Collection:

Pteropods are often classified as gelatinous animals in zooplankton collections, and are often in a poor state when retrieved using classical net tows (Hamner et al. 1975, Robison 2004). The shells of thecosome pteropods are fragile and many of the more highly evolved groups, including the Subfamily *Cavolinidae* and the Pseudothecosomes, have extremely delicate gelatinous pseudoconches and extensive external mantle structures. Damage by nets becomes particularly problematic when animals are collected for experimental or culturing purposes. Thus, collection of pteropods using specialized capture methods can greatly increase the proportion of undamaged animals. Although primarily open ocean animals, in some regions the topography and hydrography allow collection of pteropods from the shore or using small boats. In these locations, researchers have used "jelly dippers" (beakers strapped to long handles), to allow the collection of these delicate animals (Seibel et al. 2007, Maas et al. 2011).

Hand-collection can also be performed by sampling pteropods using SCUBA diving techniques (Gilmer 1974, Fabry 1990, Maas et al. 2012a) to collect larger organisms with minimal stress. Successful collection using diving is limited by animal density and is much more time and effort intensive than net sampling (Haddock & Heine 2005). It has, however, proven particularly useful in tropical regions (where Cavolinids and Pseudothecosomes are more abundant) and at night (when diel migratory individuals congregate in surface waters). Finally, a few species are exclusively meso- or bathy-pelagic, and gentle collection of these animals is particularly difficult. Such species have been sampled using remotely operated vehicles (Seibel et al. 2007) and using a net with a thermally-isolated cod end that closes at depth (i.e. Childress et al. 1978) maintaining the animals in water from the depth of collection until retrieval.

Plankton net tows remain the most widely used for collection since they are relatively easy to operate and offer a variety of mesh-sizes and designs (e.g. Bongo, WP2, MOCNESS). Many pteropod species can be captured using > 330 µm mesh net, although small species and juveniles may be better sampled using 153 µm mesh or less (Lischka et al. 2011, Bednaršek et al. 2012b). Plankton tows have the advantage that they enable the user to easily collect large

numbers of pteropods, including small individuals (< 500 µm), and/or individuals that occur at great depths (> 50 m). The disadvantage of damage to both the soft tissues and the shell by nets for work with live animals can be minimized if ship speeds of less than 1 knot are employed in conjunction with short duration, relatively vertical tows (Bednaršek et al. 2012a). The use of a specialized cod-end such as a home-made extra-large cod end (Bouquet et al. 2009) or thermally protected cod end (Childress et al. 1978) can further limit physical and thermal stress.

After collection, pteropods should be inspected to ensure that the shell and soft tissue were not damaged during collection. Damaged organisms must be discarded in order to avoid subsequent bacterial infection and the contamination/death of the whole population. Following inspection, it is recommended to immediately transfer the organisms into a large volume of filtered seawater at *in situ* temperature from the site of collection.

Water treatment

Pteropods produce mucous coverings, mucous webs and pseudofaeces, rejected particles, expelled in mucous strings (Gilmer & Harbison 1986), during feeding and buoyancy maintenance. These structures can attract bacterial populations that, in turn, can impact experiments and contaminate laboratory cultures. To deal with these issues researchers have used a combination of filtration, antibiotics and UV treatment of seawater (Comeau et al. 2009, Comeau et al. 2010a, Lischka et al. 2011, Maas et al. 2012a). Additionally, 0.5 mg L^{-1} disodium ethylenediaminetetraacetate (EDTA) can be used to chelate toxic metals from the seawater, even in areas where local water sources are relatively clean. Egg clutches/strings and juveniles are particularly susceptible to bacterial infections and successful rearing through to adult stages is only possible with rigorously clean techniques using antibiotics and careful handling procedures (see below). For adult cultures, water filtration through < 5 µm membrane filters is recommended as this reduces fouling and entanglement in aggregations of mucous and detritus; juvenile cultures require filtration using 1 µm filters.

To directly test whether antibiotics influenced the metabolism of pteropods, single adult *Limacina helicina* were held in glass syringe respiration chambers either treated or untreated with Streptomycin and Ampicillin (each 25 mg L^{-1}). During each experiment, a control syringe, without an animal, was simultaneously run as a control. After ~ 10-15 h, their oxygen

(O_2) consumption (µmol g^{-1} h^{-1}) was measured using the methods detailed by Maas et al. (2012a). There was a no significant effect of exposure to antibiotics for a period of ~ 12 h (ANCOVA $F_{1,15} = 0.098$, $p = 0.760$) on the metabolic rate of *L. helicina* (Fig. 4.2). These results indicate that antibiotics may be an effective way to control bacterial activity during short duration respiration runs without physiological side effects. This is also particularly useful when the oxygen consumption is being measured at warmer temperatures (> 10 °C) and longer duration experiments (> 8 h) where bacterial respiration can begin to be significant.

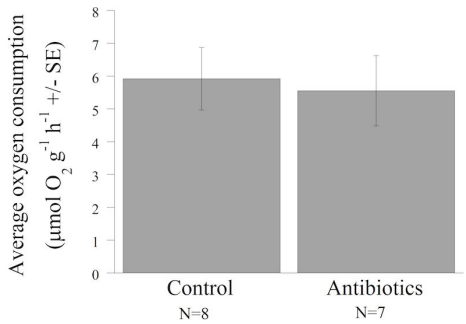

Fig 4.2. The effect of antibiotics (25 mg each of Streptomycin and Ampicillin L^{-1}) on the respiration rate of *L. helicina*.

Ultra violet and microwave sterilisation may also be used to sterilise culture water against fungi and viruses as well as bacteria. UV treatment is also more appropriate for use with large volumes than with antibiotics that need to be regularly replenished. A major drawback of UV sterilisation, however, is its inefficiency in closed, recirculating systems (Spotte & Adams 1981). In the Ny Ålesund Marine Lab, seawater is UV treated and 20 µm filtered, thus UV sterilisation has been used for a number of pteropod perturbation experiments (Comeau et al. 2009, Comeau et al. 2010b, Lischka et al. 2011). To our knowledge microwave sterilisation has never been used in conjunction with pteropod culturing.

Aeration and gas bubbling

Gas bubbling is often used for aeration or to control the carbonate chemistry in ocean acidification perturbation experiments, however, it can also cause shell damage; particularly in juvenile pteropods. Thus, in closed vessels the bubbling should be performed before addition of pteropods, while in open vessels, bubbling should be made in such a way that the animals are not in direct contact with the stream of bubbles. This can be achieved by bubbling within a vertical tube placed in the vessel, thereby offering excellent control of the carbonate

chemistry while also gently stirring the media. Veliger shells are hydrophobic (Gallager, pers. obs.), which can cause them to be trapped at the surface during bubbling or for gas bubbles to attach to the shell, causing buoyancy issues, in this instance the surface of the air-water interface can be covered with a few granules of polyvinal alcohol, which forms a non-toxic surface coating eliminating the hydrophobic/hydrophilic interface. If bubbling from a compressor, a series of filters decreasing in porosity to 0.45 µm should be used to avoid introducing airborne contaminates to the cultures. In humid conditions, air should be dehydrated using $CaCl_2$ or silica gel (Utting & Spencer 1991).

Diet:

To date, very little experimental work has been undertaken to establish the appropriate dietary regime for cultures. Previous studies utilising pteropod cultures have used enrichment of phytoplankton monocultures (Comeau et al. 2010b). Alternatively, cultures have not been enriched with food (Lischka et al. 2011) or in situ, unfiltered seawater is used. The use of motile species ensures that the entire water column remains evenly saturated with food with minimal agitation of culture vessels.

Successful cultures of *Limacina retroversa* have been maintained from juvenile stages through to adulthood with mixed algal diet. Based on feeding studies with artificial microspheres (Gallager, unpublished data), adults and juveniles were capable of feeding on particles 0.5 µm to 30 µm in diameter. Accordingly, the flagellate *Isochrysis galbana* (Clone T-ISO) and a Cryptophyte *Chroomonas salina* (Clone 3C) were used for larval and metamorphosing juvenile culture (Table 4.1). Concentrations of $1x10^4$ cells mL^{-1} are suitable for culture densities of about 1 veliger mL^{-1}. As parapodia developed, the dinoflagellate *Ceratium longipes* was added in addition to the two other species of microalgae. This dinoflagellate was used because of the frequent association of *Ceratium longipes* and *Limacina* in the field (Gallager et al. 1996). *Ceratium longipes, Chroomonas salina* and the flagellate *Isochrysis galbana* at a final concentration of $5x10^4$ cells mL^{-1} are an optimal combination of micro algae for juveniles and adults.

Food density was also found to be important during feeding trials using *L. helicina* juveniles to assess mixed algal diets at varying concentrations (Table 4.1). Two cultures were maintained with high food concentrations ($5x10^4$ cells mL^{-1}) and one with low food

concentration ($1x10^4$ cells mL^{-1}). Animals incubated in low food concentrations survived 21 days, whereas animals in the high food concentrations survived for 37 days and ciliary feeding was observed. These results suggest similar optimal algal concentrations for the two *Limacinid* species.

Table 4.1 Summary of dietary regimes used in thecosome pteropod culturing. For pteropod life-stages: (j) juveniles, with shell diameters where available. It should also be considered that many of the methods listed were employed as part of perturbation experiments, therefore other stressors outside of diet regime may have contributed to mortality.

Diet	Algal species	Conc.	Pteropod Species	Survival (days) & mortality (%)	Culture system	Reference
Unfed	-	-	*L. helicina* (j)	29 ~ 25% ~ 57% ~ 68% mortality	440 mL Jars, 3˚C 5˚C 8˚C	(Lischka et al. 2011)
Unfiltered seawater	-	-	*L. helicina* (j)	8 6% 8% 9% mortality	125 mL bottles, 1.6˚C pH 8.05 7.90 7.75	(Comeau et al. 2012)
Single algal species:	*Isochrisis galbana*	-	*C. inflexa* (j)	13 70% mortality	1 L bottle, plankton wheel, 13˚C	(Comeau et al. 2010a)
Mixed algal diet	*Tetraselmis* spp, *Skelotonema* spp., *Gymnodinium simplex*, *Amphidinium carterii*, *Rhodomonas salina* *Pavlova*.	1000 cells mL^{-1}	*L. helicina* (j) 100-2800 µm	5, 100% mortality	Stirred batch 2 µm filtered seawater, ~ 7°C	Doubleday, pers. obs.
Mixed algal diet	*Tetraselmis* spp, *Skelotonema* spp., *Gymnodinium simplex*, *Amphidinium carterii*, *Rhodomonas salina* *Pavlova*.	>50,000 cells mL^{-1}	*L. helicina* (j) ~ 500 µm	40, 50% mortality	Stirred batch 2 µm filtered seawater, ~ 7°C	Doubleday, pers. obs.
Mixed algal diet high conc.	*I. galbana, Chaetoceros calcitran Pavlova*	>50,000 cells mL^{-1}	*L. helicina* (j) 100-200 µm	37, 100% mortality	Stirred batch 2 µm filtered seawater, ~ 7°C	Doubleday, pers. obs.
Mixed algal diet low conc.	*I. galbana, Chaetoceros calcitran Pavlova* *I. galbana, Chroomonas salina* *I. galbana, Chroomonas salina, Ceratium longipes*	1000 cells mL^{-1} 1000 cells mL^{-1} 50,000 cells mL^{-1}	*L. helicina* (j) 100-200 µm Larvae *L. retroversa* *L. retroversa*, adult, and j	21 100% mortality to maturity	Stirred batch 2 µm filtered seawater, ~ 7°C 2 L flasks, 10°C 1000 L tubes, 10°C	Doubleday, pers. obs. Gallager, pers. obs. Gallager, pers. obs.

Culture methods

A wide range of approaches have been used to maintain pteropods for experimental purposes. They can be grouped into three broad categories: batch cultures (with various modifications; agitation, tethering) (Table 4.2), flow systems (circulating and flow through) (Table 4.3), and mesocosm systems.

Closed systems

Small individuals (< 1.5 mm) such as *Limacina inflata*, and juveniles of *Limacina helicina* (> 5 mm) or *L. retroversa* can survive reasonably well in closed batch cultures. These small individuals can be maintained in smaller closed flasks with no water changes for up to 29 d at 3, 5.5 and 8°C (L. helicina for percentage mortality see Table 4.2; Lischka et al. 2011), and 7 d at 14°C (*L. inflata*) (Howes pers. obs.). Adults and large species, however, appear to require much larger volumes for successful rearing. An appropriate size of culture vessels is difficult to define, although the bigger the water column depth the better chance of mucous web production.

Often if the culture vessels are not gently swirled or shaken periodically, food particles, mucous and/or pseudofaeces collected at the bottom of the vessel then become entangled around pteropods incubated in the batch cultures, considerably contributing to the mortality of organisms (Bednaršek et al. 2012a). As with most culturing systems, particulate waste should be gently siphoned out routinely, along with periodic water changes. To maintain the pteropods in suspension, tests have been undertaken to explore the effects of gently agitating cultures, either using external methods to move the whole vessel or by placing stirrers directly into the culture (Fig. 4.3; Table 4.2). While agitation methods work well for rearing egg clutches and juveniles (Table 4.2; Comeau et al. 2010a) due to their light weight and slow sinking speed which allows them to be maintained in constant suspension, these techniques were less successful for maintaining cultures of adults or juveniles > 3 mm in size. Several of the agitation methods tested did appear improve adult buoyancy; however, this was partially due to the pteropods swimming continually against the water currents precluding successful feeding behaviour. Alternatively, stirring can tumble pteropods either across the bottom of the culture vessels or into each other, and also cause an accumulation of mucous and detritus in the bottom of the culture vessel that can entangle individuals that fall into it. Vibrations produced by some of the systems also appeared to disturb the animals.

Table 4.2: Summary of variations on batch techniques attempted. For pteropod life-stages employed: (a) adults (j) juveniles, (l) veliger larvae (e) eggs. Note that the survival time represents the point at which the experiments were concluded and does not document the time at which all individuals died. It should also be considered that many of the methods listed were employed as part of perturbation experiments, therefore other stressors outside of the culture environment may have contributed to mortality.

Technique Description	Species	Survival (days) & mortality (%)	Reference	Comments
Batch: 440 mL jars, 10 individuals. Unfed. 3, 5, 8˚C	*L. helicina* (j)	29 ~ 25%, 57%, 68%	(Lischka et al. 2011)	-
Batch:1 l L, opaque, polyethylene jars. 1-6 individuals.	*C. pyramidata* *L. helicina*	≤ 1, 0%; short incubation	(Fabry 1989, 1990)	Feeding not observed
Batch: 1 litre Nalgene bottles, ≤15 individuals, -2˚C	*L. helicina*	13, 0%	(Maas *et al.*, 2012a)	Feeding not observed
Batch:2 L Schott bottles (opaque) 2 L Schott bottles 14˚C	*L. helicina ant.* *L. inflata*	14 3, 5%	(Bednaršek et al. 2012a) Moya *et al*. In prep	*L. inflata was* not observed feeding, but guts were full when inspected with a binocular microscope
20 L, 0˚C, 4˚C	*L. helicina*	2, 0% ; short incubation	(Comeau et al. 2010b)	Feeding not observed
1000 L tubes, 10°C	*L. retroversa*	To reproduction	Gallager, pers. obs.	Webs not observed, but guts full
50 000 L mesocosm (KOSMOS units)	*L. helicina* (a)	7, 95% 28, 99 %	(Riebesell et al. 2013)	Floating animals were observed
77 000 L mesocosm (KOSMOS units) (Fig. 4.7)	*L. retroversa* (a,j,l)	40, 50%	Büdenbender *et al*., in prep	All life stages present until the end of the experiment
Larvacean system; Fig. 4.3B) Plastic stirrers rotate (< 10 rpm) in round culture vessels (e.g. 20 L beakers). 5˚C 7˚C 15˚C	 *L. helicina* *C. inflexa* (e, j)	 5, 0% 40, 50% 10 100%	 (Comeau et al. 2009) Doubleday, pers. obs. Howes, pers. obs.	Technique effective for small individuals, egg clutches and larvae but failed to maintain pteropods > 2 mm; should be used preferentially with individuals of reduced sizes.
Rollers 6 rpm: (Fig 4.3A) Incubation 14˚C in 2 µm filtered seawater in 2 L glass bottles enriched with *I. galbana*. Bottles sealed with parafilm, ensuring no air bubbles. Every 2nd day water was changed by gently pouring through a 100-µm sieve. Inspection and removal of dead individuals.	*L. inflata*	7 , 60%	Howes pers. obs.	Slow speeds were more suitable for maintaining cultures,
Rotatory shakers (varying speeds) (Fig 4.3B): Preparation as described for rollers	*L. inflata*	7d , 60%	Howes pers. obs.	Slow speeds were suitable for maintaining cultures.
Plankton wheel (Fig. 4.3C) 1 L glass bottles prefilled with 0.2 µm-filtered seawater. Partial water change every 2nd day. 13˚C.	*C. inflexa* (e, j)	13, 70%	(Comeau et al. 2010a)	Successful for individual < 3 mm
Tethering: Rapid setting gel-based glue used to attach pteropods to needle. Needles and pteropods introduced to seawater (14˚C) immediately after attachment. (Fig 8). Disposable pipettes used to keep pteropods wet at all stages of procedure. Fed with *I. galbana*	*C. acicula* *C. inflexa* *C. pyramidata* *S. subula*	7, 100% 2, 100% 1, 100% 5, 100%	Howes pers. obs.	Stressful and time consuming attachment procedure Response species specific Feeding not observed

Table 4.3 Summary of variations on flow systems. For pteropod life-stages: (j) juveniles, (e) eggs. Note that the survival time represents the point at which the experiments were concluded and does not document the time at which all individuals died.

Technique Description	Species	Survival (days) & mortality (%)	Reference	Comments
Temperature gradient plankton Kreisel (Fig. 4.6)	*C. inflexa* *C. virgula*	7	(Gorsky et al. 1986)	Successful feeding observed at high algal concentrations.
Up flowing (Fig. 4.6A) Main body cylindrical water inflow at the base, positioned under 100 µm mesh, to disperse the flow evenly. Mesh at the top of the water column prevents animals being washed out.	*C. inflexa*	3, 100%	Howes pers. obs.	Animals sunk beneath the lowest current and struggled to swim back up through water flow. Use of meshes traps biofilm and pteropods. Requires exact flow rate control
Circular water current (Fig 4.6B): Circular current provided by water inflow at top of kreisel.	*C. acicula*	2, 100%	(Purcell et al. 2013)	System did not provide enough flow to maintain buoyancy and water column too small to allow production of mucous web.
Circulating upward flow (Fig. 4.6C) Main body is cylindrical. Three equally spaced inflows running vertically down one side. Each inflow fed by its own tube allowing the speed of the flows to be differentially controlled	*C. acicula*	3, 70%	(Graeve, unpubl., Howes, pers. obs.these)	System was more successful for the smaller individuals, < 1 cm, and one was observed with mucous feeding web. Once settled, they struggled to swim up the water column through water flows.
Bi-directional elliptical flow (Fig. 4.6D) Rectangular design. Two elliptical flows: one from the top of the tank flowing vertically and a second from one end, flowing horizontally	*C. acicula*	3, 80%	(Purcell et al. 2013)	The flows did not maintain the animals in suspension and forced them to swim continually or sink to the bottom.
110 L aquaria: 110 L V-shaped aquaria (40 x 40 x 70 cm) ca. 80 individuals	*L. helicina*	14, 90%	(Böer et al. 2006)	Feeding not observed Water column too short, sinking time ~15 sec
600 L system: Horizontal circular current, constant flow (10 L min^{-1}) 20 µm filtered water directly pumped from Kongsfjord. 100 individuals per tank	*L. helicina*	14, 50%	Büdenbender pers. obs.	Feeding not observed Animals were observed floating in water column Large volumes of water unsuited to perturbation experiments

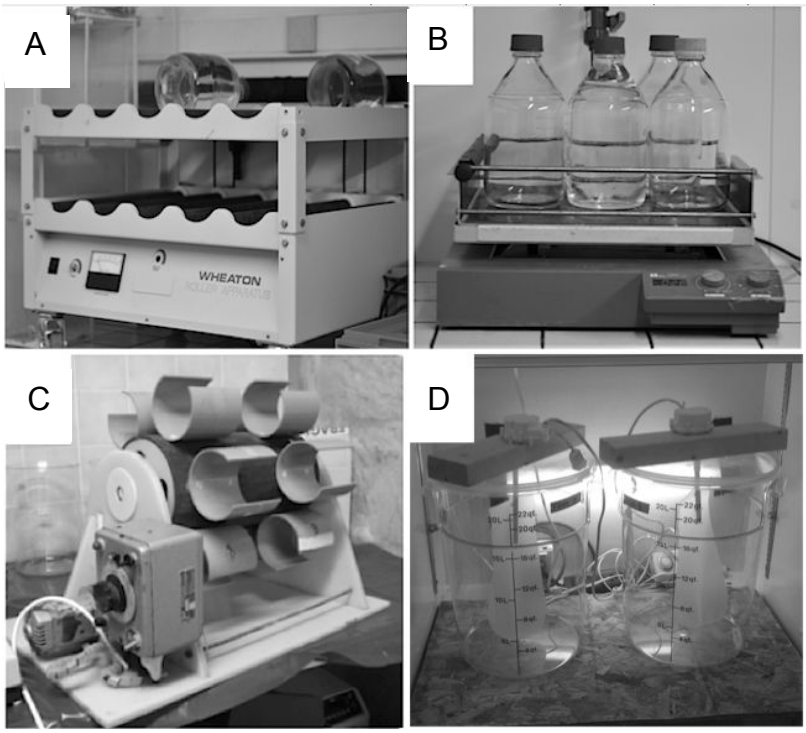

Fig. 4.3. Agitation methods: **A**: Rollers with sealed 2 L bottles containing *L. inflata* specimens. **B**: Gyratory shaker with 2 L bottles containing *L. inflata* cultures. **C**: Plankton wheel used for the culturing of *C. inflexa* egg clutches. **D**: The larvacean system, used for appendicularian culturing. In this system, the organisms are maintained in suspension by creating a gentle current using plastic paddles that rotate slowly (< 10 rpm) (Comeau et al. 2009).

To avoid some of the pitfalls of agitation, individual pteropods have been attached to mounted needles, pre-fixed in culture vessels, using gel-based superglue (Table 4.2, Fig. 4.4). This method allows the use of smaller volumes of water and less space requirement when culturing adults. Great care and dexterity are needed to avoid damaging the fragile pteropod shell, the process is time consuming and likely to be very stressful. The success of the method depends heavily on the shell shape: those species with simple, straight or conical shells responded better than those with more complex shell forms. This can probably be attributed to the animals being fixed at slightly the wrong orientation. Tethered animals were never observed feeding.

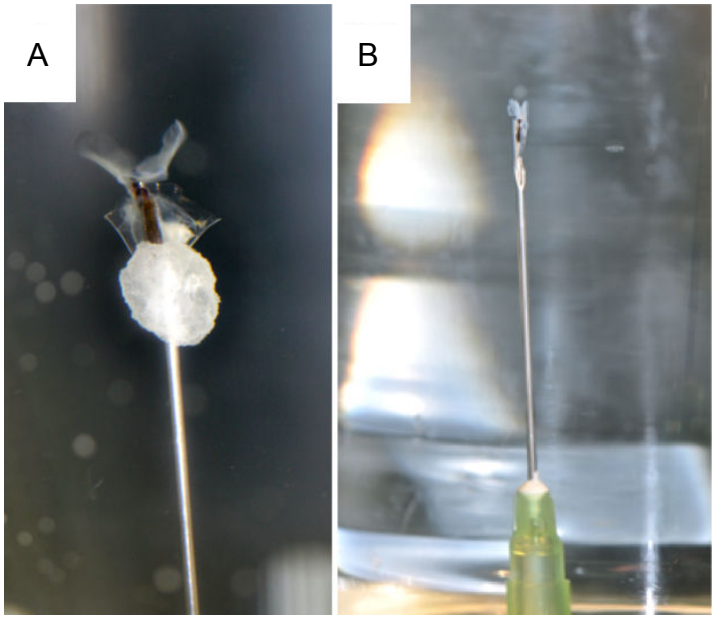

Fig. 4.4. Tethering method using needles and superglue. A : *C. pyramidata*. B : *S. styliola*.

To date, the most successful culture of pteropods has been accomplished using a closed system with regular water changes, developed over 8 separate attempts at culturing and detailed for the first time herein. In overview, egg strands of *Limacina retroversa* were collected from adults already in culture for several weeks. Larval development at 10°C in the egg capsules took ~ 10 days, followed by ~ 40 days for the veligers to reach metamorphic competency and begin to develop parapoda typical of the juvenile and adult. Metamorphosis took about 10 days to make the complete transition and loss of the velum. Development from metamorphosis to reproductive adult took approximately 40 to 50 days (Fig. 4.5). Egg strand production then began 90 days after hatching and continued until the F1 generation began to die out. After 110 days, high mortality occurred, and by 117 days, all of the adults had died. Complete generation time from reproductive adult to reproductive adult was around 90-110 days at 10°C. Several F2 generations were again raised to reproduction.

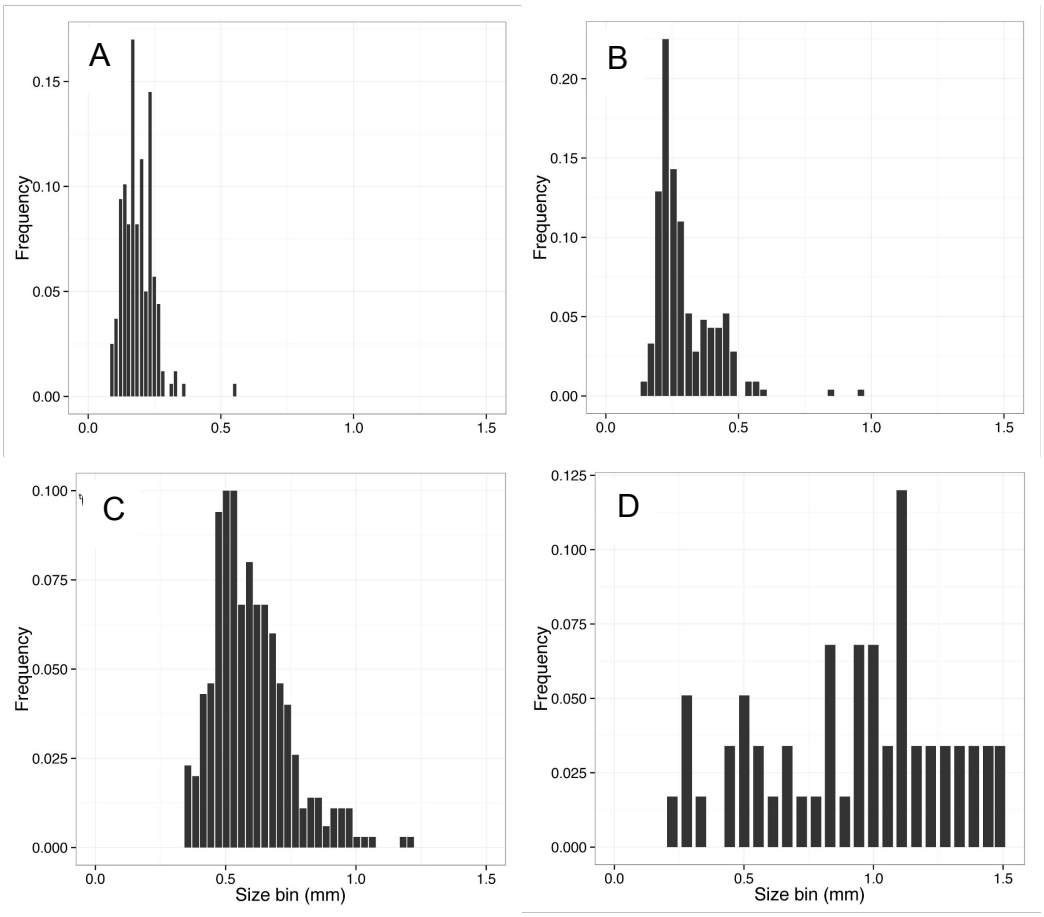

Fig. 4.5. Size distributions of juvenile and adult *Limacina retroversa* cultured at 10°C. **A.** Metamorphosis, 50 days post hatch, mean shell length 0.262 mm. **B.** 62 days post hatch, mean shell length 0.459 mm. **C.** 80 days post hatch, mean shell length 0.693 mm. **D.** 98 days post hatch, mean shell length 1.370 mm

To begin the overall process, adults were maintained for about 4 weeks in the laboratory in either 1000 L tubes or large tanks. Egg masses were separated by screening adult culture water through a 200 µm nitex screen, rinsed and re-suspended in 2000 mL Erlenmeyer flasks in 1 µm filtered seawater. Water in the ova cultures was aerated at ~ 100 mL min^{-1} with 1 µm filtered air and not changed during the ova incubation period. Before hatching, as the ova strands darkened, eggs were treated to reduce bacterial and fungal contamination for 30 min with 2 mg L^{-1} polyvinylpyrrolidone iodine complex (PVP-I, Sigma Chemical) seawater solution whose pH has been adjusted to ~ 8.3 with Tris-HCL buffer (Trizma, pH 8.3). The egg masses were then treated with 0.1 M sodium thiosulfate to reduce the iodine to iodide prior to re-suspension in culture flasks prepped with antibiotics, EDTA and algal mix. Gentle agitation of flasks might also be useful in keeping egg masses off the bottom, minimizing the risk of bacterial infection (Comeau et al. 2010a). To avoid culture entrapment at the surface, the flask was filled to near overflowing and covered with Parafilm to eliminate air bubbles.

Initially, about 1 veliger mL^{-1} of culture water was considered optimal. Algae were added to the cultures to achieve a final concentration of $1x10^4$ cells mL^{-1}. Flasks were inverted several times a day to mix algae and larvae. Cultures were screened using a 100 µm mesh and changed weekly at a minimum, and optimally every 3 days. At each culture water change, larvae were inspected and dead individuals removed using 2 drops of a 1:24 dilution of Surgical Scrub Surfactant (Purdue Frederick, Norwalk, CT) to re-suspend and facilitate handling the hydrophobic shells. Larvae were subsampled for measuring as necessary (typically 10-30 larvae were preserved per flask in 1% buffered formalin), and then concentrated into the centre of the dish by swirling. The larvae were then pipetted directly into a freshly prepared culture flask containing filtered seawater, algae, and antibiotics. This process minimized carryover of the surfactant from the glass dish. The flask was then sealed as before with parafilm or polyvinal alcohol. As larvae developed and approached metamorphic competency, densities were reduced to 1 individual 10 mL^{-1}, and once metamorphosed, each flask of juveniles was transferred to 50 L culture vessels.

Recirculating Systems:

Variants on the original design of the plankton kreisel (Greve 1968) have been used successfully to culture a variety different gelatinous zooplankton species (Dawson 2000, Purcell et al. 2013). A variety of kreisels with different flow patterns have been tested for pteropod culture including circular, up-flow and multidirectional flow patterns (Fig. 4.6; Table 4.3). Notably, one of the only observed instances of pteropods producing mucous webs under laboratory conditions (Fig. 4.1) was obtained using a variant of the "planktonkreisel" (Fig. 4.6E; Gorsky et al. 1986).

Kreisels systems typically use large volumes of water and the movement produced by the currents gives buoyancy aid. The flow rate appears to be critical and very fine control is required to keep the animals in suspension. If the flow is too strong, the pteropods will swim continuously, preventing feeding and resulting in rapid depletion of energy reserves. If too weak, it does not counteract the negative buoyancy of the shell and the animals come into contact with the sides and bottom of the vessel, causing shell damage and excess mucous production.

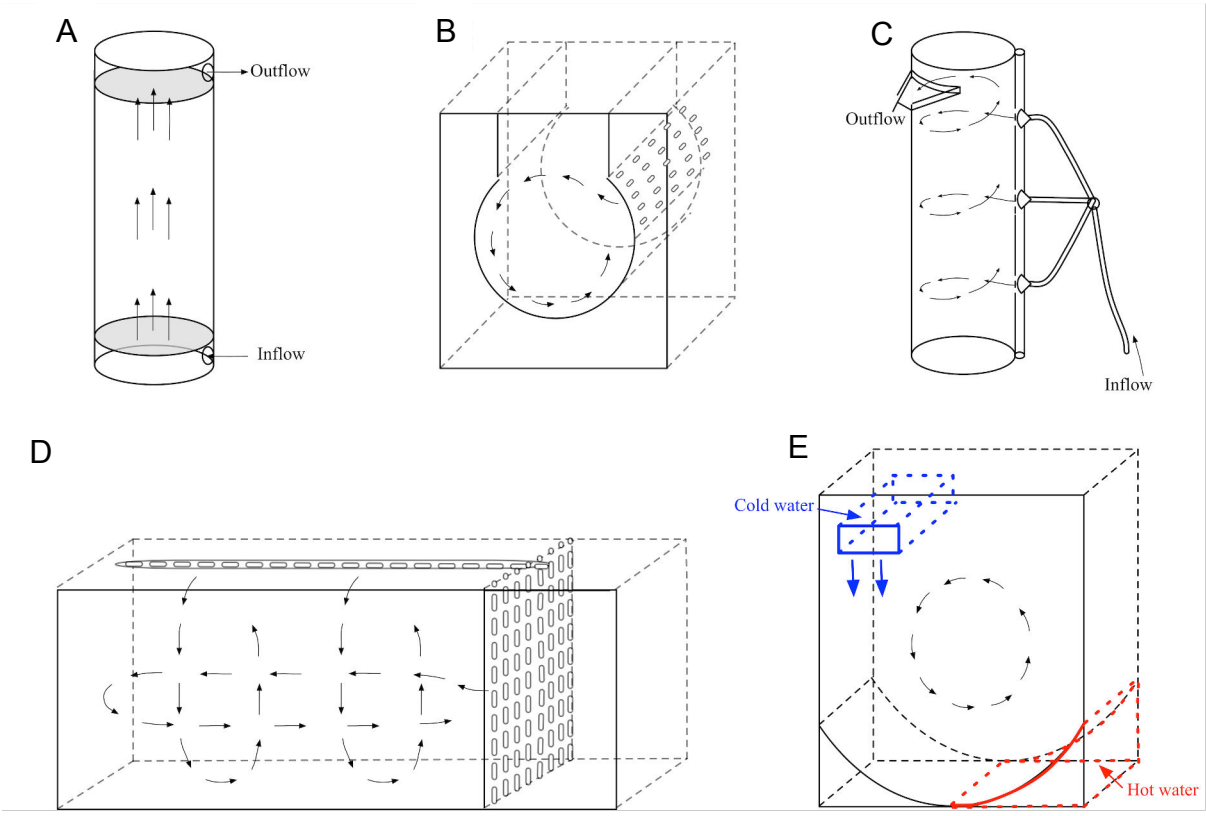

Fig. 4.6. Representations of the water flow kreisel systems, direction of water current represented by arrows. **A**: Upflow system designed in Villefranche-sur-Mer (Mahacek, Moya & Howes). **B**: Circular flow system used for ephyrae culture at Institut de Ciences del Mar, CSIC, Barcelona (Purcell et al. 2013). **C**: Circular upflow kreisel designed at Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research Dr Martin Graeve. **D**. Multidirectional jellyfish kreisel, Institut de Ciences del Mar, CSIC, Barcelona (Purcell *et al.*, 2013). **E:** the temperature gradient plankton kriesel (Gorsky et al. 1986). The water current (black arrows) is generated by the difference in water density between low density water on one side of the tank on the bottom (created by warming up the water) and high density water on the top of the tank on the opposite side (created by cooling down the water).

Flow through systems:

Flow through, or single pass systems, utilise water currents and flows in the same way as circulating systems, however, in these aquaria, when the overflow water leaves the aquaria it is discarded. Gymnosome pteropods have been successfully maintained in large numbers (ca. 80 individuals) for 365 days using a 110 L, V-shaped aquaria (40 x 40 x 70 cm; Lange & Kaiser 1995) with constant circulation, to keep the animals swimming (Böer et al. 2007). The same system was tested with *L. helicina*: the sinking rate from surface to the bottom of the aquaria was approximately 15 sec and after 14 days only 0-5 of 100 individuals survived.

Larger flow through systems have been more successful. In the Marine Laboratory in Ny-Ålesund, adult *L. helicina* individuals were reared in a 600 L flow through system (10 L min^{-1}; Table 4.3) successfully for 14 days at 100 individuals per tank; mortality was less than 50%. A horizontal circular current transported the pteropods, extending the time from surface to bottom by up to 1 minute during which no swimming activity or interface collision appeared. Mucous feeding structures were not observed.

Mesocosm systems:

The presence of a large water column, which can allow time for animals to deploy their mucous webs, makes mesocosm systems a candidate with high potential for successful pteropod culturing. To our knowledge, there have only been a few but promising attempts in this direction (Table. 4.2).

In 2010, nine KOSMOS mesocosms were deployed for 35 days in a high Arctic fjord. The mesocosms contained approximately 50 m^3 of natural fjord water, the enclosed water column was 15 m deep (Riebesell et al. 2013). Controlled quantities of pteropods collected using jelly dippers were added to each mesocosm (4 individuals per m^3). Most died during the first two weeks due to a design shortcoming that trapped pteropods in the dead volume below the built-in sediment trap or being collected by the sediment trap itself (Czerny et al. 2012, Riebesell et al. 2013). Only single individuals survived for the full period of the mesocosm deployment (30 days).

During the 2011 Bergen mesocosm campaign, nine KOSMOS units (Fig. 4.7) of approximately 77 m^3 volume and total bag length of 25 m were deployed in the Raunefjord, close to the Espegrend Biological station, Bergen Norway. *L. retroversa* individuals of all life stages were part of the natural plankton community and were entrapped in the mesocosms during the filling process, making the stressful collection process unnecessary. *L. retroversa*, as well as potential predators (e.g. *Clione limacina* and fish larvae), remained present for the full experiment (40 days; Büdenbender *et al*., in prep.). Mortality rates of veliger larvae at control conditions were approximately 50%.

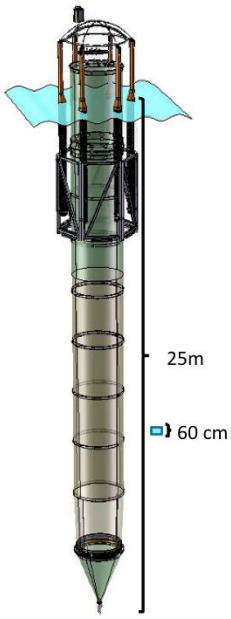

Fig. 4.7. KOSMOS system deployed for 40 days in Raunefjord Bergen Norway 2011 inhabiting a natural plankton community including *L. retroversa*. Mesocosm drawing by D. Hoffmann.

Discussion

Species selection:

In general, when culturing thecosomes it seems that the size of the animals and the size of the culture vessels are both important. Although small species/juveniles (< 1.5 mm) can be cultured in smaller vessels, larger species/adults require larger water volumes. Species of *Limacina* seem to be more robust than other body forma, with *L. retroversa* reared through larval stages to F3 generation under laboratory conditions. Healthy cultures of *L. inflata* have been maintained at least 7 days in low densities (fewer than 10 individuals L^{-1}) in simple closed containers. Also, the slower metabolic rate at colder temperatures for polar Limacinidae allows longer survival when individuals are unable to feed (Hopcroft, pers. obs.).

Cavoliniidae are more problematic, possibly due to the increased shell and mantle complexity making them more prone to damage. Tethering methods showed some early promise when applied to Cavoliniidae species with simple conical shells, while some success has been reported with *C. virgula* and *C. inflexa* in the temperature gradient plankton kreisel. Future attempts to improve culture systems should take into account that the different forma respond differently to the same culture vessels; one size does not fit all. With the goal of performing

successful perturbation experiments, effort should focus on developing techniques for several key and widespread cosmopolitan species.

Larval rearing:

Use of eggs laid in culture has been the only method that has successfully reared reproductive adults and refining this technique for polar species, e.g. *L. helicina*, and adapting it for Cavoliniidae, should be a priority for future work. Whilst the species, *L. retroversa* is relatively robust, there may also be advantages to working with F2 generations. The egg masses are not subjected to the rigours of sampling from the wild and can be kept in the most sterile conditions possible, limiting the risk of bacterial infestations. Experimentalists are able to work with many individuals of the same life stage that are already acclimatised to culture conditions. Successful work has already been undertaken using the egg clutches of *C. inflexa* (Comeau et al. 2010a), with clutches raised through veliger stages to metamorphosis with low mortality. However, once individuals reach sizes > 1 mm, culture methods must be adapted, as mortality rapidly decreases populations by up to 50% in 24 h. Large adults can be induced to mass spawning in the lab when held in crowded conditions or exposed to mild heat or light stress (Howes, pers. obs.) making them a good candidate for further work with careful application and tailoring of the methods presented here for *L. retroversa*.

Vessels:

Batch cultures are easy, non labour-intensive and cheap for pteropod cultivation, but are much more suitable in small volumes for juveniles and small species than for large adults as demonstrated for *L. retroversa*. With refinement, batch culturing should allow for easy experimental replication and could be implemented under field conditions. Nonetheless, fully closed batch culturing has its drawbacks: change in seawater chemistry generated by biological activities (i.e. changes in oxygen availability, carbonate chemistry and the accumulation of potentially lethal nitrogenous wastes), along with the accumulation of faecal wastes and bacteria. Small closed volumes are also handicapped by increased contact rates with the sides or the base of the culture vessel. General husbandry of culture vessels and water changes appear critical to rearing success. In this regard, recirculating systems such as plankton kreisels or flow through systems may offer some intermediate level of self-sufficiency albeit with higher cost and effort. With some specialisations, manipulations of

temperature and water chemistry are possible with kreisels and flow through systems, e.g. as implemented in the indoor mesocosm facility in the GEOMAR, Kiel.

Mesocosms such as those employed in the 2011 Bergen campaign show potential for new insights into pteropod ecology, particularly when tested with smaller Limacinid species. Mesocosms can provide conditions in which populations/species of pteropods are able to reach a state of neutral buoyancy and are able to feed with a mucus web on their natural food sources in a close to in-situ environment. There is a need for new instruments which allow *in-situ* observation and controlled capture of single individuals within these systems due their large body size/volume to water volume ratio (1:5.5 billion; e.g. volume based camera systems and remotely operated sampling devices like small ROV's). Mesocosms like the KOSMOS system, due to their size, provide the most realistic and favourable conditions for pteropod culturing. As demonstrated in the 2010 experiments, the size and design of mesocosms must be taken into careful consideration in relation to the size and sinking rates of the pteropod species. Mesocosms with built in sediment traps should be avoided as they act as a one-way gate, trapping animals at the bottom of the mesocosm (Büdenbender, pers. obs.). The labour, cost and logistics of setting up mesocosms are major drawbacks for experimental work where several treatments and replicates are required; the costs can however be greatly reduced by using pre-existing facilities such as the Espegrend biological station or by co-operation with existing projects. Further development of this work should focus on testing with non-polar or Cavoliniid species as it is not yet known how these less robust forma will respond to mesocosm conditions.

The common problem for all thecosome species is buoyancy and, as a direct result, feeding behaviour. As described above, many flow systems destabilise or trap the animals and even the use of much larger culture vessels does not allow adequate sinking space. In a range of culture vessels used by Gallager with *L. retroversa*, healthy adults and juveniles of various species tended to congregate at the surface. Surface aggregation may have been a response to gravity, illumination, tank currents, or food, with individuals observed vigorously swimming upwards and across the surface, followed by drifting downwards at various rates, usually quite slowly.

In the wild, pteropods undergo diurnal vertical migrations (DVM), for some species of > 300 m, which has been suggested to be linked to control of metabolic rate (Wormuth 1981, Lalli & Gilmer 1989, Maas et al. 2012b). The migratory behaviour might account for sinking and could exacerbate feeding difficulties experienced by culture specimens. The development of culture vessels, dark incubations, opaque tanks or graduated opaque tanks with darker bases and transparent tops, might exploit the natural upward migrations during dark hours and reduce sinking periods in laboratory animals. Variegated mesocosms have been used to culture jellyfish found in meromictic lakes (Dawson 2000) and such an introduction of a false halo, thermo or chemocline might counteract the downward migrations of the pteropods or satisfy metabolic needs related to DVM.

Diet and feeding:

As a result of abnormal feeding behaviour in many culture vessels, it is unclear whether inadequate diet may be a further stressor for lab animals; several taxa of gelatinous zooplankton require mixed diets when maintained in artificial environments (Raskoff et al. 2003). Sub-optimal food might account for the relatively slow growth rates encountered during culture of *L. retroversa*. Many other commonly cultured zooplankton genera can only achieve optimal fitness when provided with specific diets (Demott & Müller-Navarra 1997, Sommer 1998, Knuckey et al. 2005). Gut content analysis of wild-caught, adult *L. helicina* evidenced an omnivorous and, often, cannibalistic diet with juvenile life stages apparent in the gut contents (Gilmer & Harbison 1991). Small disturbances to the mucous web provoke rapid ingestion and it has been hypothesised that adults may, in fact, be mucous trappers, using their webs as a spider would (Gilmer & Harbison 1991). The guts of juveniles and smaller (< 2.6 mm shell diameter) individuals contained only phytoplankton (Hopkins 1987), suggesting a possible dietary shift in adult life stages. These *in situ* observations suggest that the mechanical feeding difficulties experienced by thecosomes in laboratory cultures of adults may be compounded by sub-optimal diets. The species selected for algal diets should be tailored to the size and developmental stage of the culture individuals. Algal species that occur concurrently with the culture species of pteropods should be used where possible.

Observations of cultured *L. retroversa* recorded periods of negligible and sometimes slightly buoyant drift usually associated with web production. However at no point during culture to the F3 generation were feeding webs observed, although phytoplankton was present in the

guts of all life stages. The same phenomenon was also observed in healthy cultures of *L. inflata* (Howes, pers. obs.) and *L. helicina* (Doubleday, pers. obs.). Close examination of the parapodia of tethered *L. retroversa* indicated that the edges of the parapodia were fringed with several sets of cilia (Fig. 4.8A). Long cilia (~30um) beat at a rate of about 15 Hz and together produced sufficient lift to keep the organisms floating with the parapodia extended but not moving. The longer cilia also provided flow to carry food particles past the parapodia edge where they were captured by the shorter cilia. These shorter cilia (~ 5 µm) surrounded the parapodia and formed a food groove (Fig. 4.8B) that transported particles to the mouth. Particles were observed moving at ~ 20 µm sec^{-1} in the food groove towards the mouth where some form of particle selection occurred. Feeding appeared to occur when the parapodia were extended and not moving as the organism slowly drifted about as if it were neutrally buoyant. Yonge (1926) proposed ciliary feeding in pteropods and it was later described in *L. retroversa* by Morton (1954). Since the SCUBA observations of Gilmer and Harbison (1986), the mucous web has become the accepted feeding mechanism in all pteropods, but these recent studies suggest that a mucous web is not essential for feeding under at least some conditions. If the mucous trapper theory is correct, production of a mucous net might only be triggered when larger, motile prey are detected: future studies should explore if mixed cultures of pteropods with smaller zooplankton prey induce web production. Alternatively, the high food concentrations in culture may only require ciliary feeding mechanisms and that webs, which are energetically expensive to produce, are only used to maximise capture when food is sparse.

Healthy *L. helicina* can survive starvation for 2-4 weeks (depending on condition at capture and body size) at 0-5˚C by living off lipid and body reserves (Lischka et al. 2011, Lischka & Riebesell 2012), as can *L. helicina antarctica* at -2˚C (Maas et al. 2011) and also at higher temperatures of up to 4˚C (Bednaršek et al. 2012a). Cultures of veliger stage *L. helicina* have been observed to survive starvation for up to seven days near 0°C (Hopcroft, pers. obs.). Ideally, successful culture methodology should confirm that feeding is taking place either by measuring metabolic rate (which decreases with food deprivation), or direct observation of feeding structures or full guts. Designs utilising camera systems may be required for feeding observations as production and retraction of feeding webs can be very rapid (Lalli & Gilmer 1989).

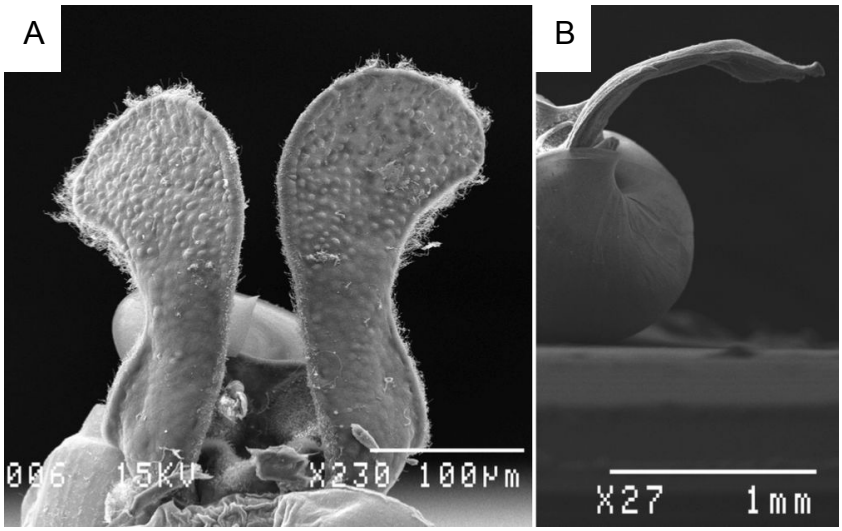

Fig. 4.8. **A**: Long cilia fringing the periphery of the parapodia of *Limacina retroversa* (arrow). **B**: Food groove travelling around the circumference of the parapodia of *Limacina retroversa* (arrow).

***Established protocols and aquaculture**:*

Culture of other taxa of gelatinous zooplankton has been markedly more successful than that of pteropods, although many of the challenges faced are very similar. The production of mucous feeding structures was a major issue for appendicularian culturing just as it is for thecosome pteropods (Paffenhöfer 1973). Delicate gelatinous bodies are prone to damage from collection and handling and must be kept in suspension, protected from impacts with the walls and base of culture vessels. The traditional plankton kreisel (Greve 1968) is suitable for many species and, in other cases, has provided an excellent starting point for more complex designs (Sommer 1992, 1993). Pteropod culture tests have been relatively successful using flow through and temperature gradient kreisels and these prototypes should now be expanded upon. Many attempts at culture development are side projects or a means to an end for a wider research project, such that the inevitable lack of time prevents the further development of promising systems. It is our belief that progress could be vastly improved with dedicated projects focused on improving systems and increased co-operation with aquaculturists. In this manner, it would be possible to capitalise on the potential economic value to fisheries as well as usefulness to the scientific community. The successful culture techniques used during rearing *L. retroversa* were developed in collaboration with mariculturists raising the opisthobranch *Aplysia californica* (Capo et al. 1997).

Current pteropod culture techniques are insufficient to address many questions about their future under changing climate conditions. There is a pressing need to develop improved cultivating methods, as thecosome pteropods are major exporters of carbon to the deep oceans in some regions (Berner & Honjo 1981) and important prey items for a number of animals, including commercially important species (Armstrong et al. 2008). Focus should first be on establishing successful culture methods before attempting to adapt these methods for suitability in undertaking perturbation experiments.

Recommendations:

1. Dedicated projects to develop systems and increased co-operation with aquaculturists. In this manner it would be possible to capitalise on the potential economic value to fisheries as well as usefulness to the scientific community. This would also allow the further development of reasonably successful methods reported here, several promising methods have been attempted once or twice and then abandoned due to lack of time or other priorities.
2. At present, perturbation experiments should focus on smaller (< 2 mm) species, larval or lipid rich life stages to ensure healthy cultures and minimise the effects of experimental stress on observed variables until solutions can be found for larger species and adults.
3. In the case that perturbation experiments with adults and larger species are attempted, large-scale facilities (> 500 L) should be considered (e.g. Bergen mesocosm facilities, Kiel KOSMOS system).
4. Tailoring of mixed algal diets depending on species size, life stage and algal species that are associated with pteropod blooms. Introduction of small zooplankton prey items for large species should be explored. Since web production has been observed with the temperature gradient kreisel, this apparatus should be used for investigation of potential links between food concentration and web production.
5. Improved communication between researchers: whilst compiling this paper it has become clear that the same, largely unsuccessful, methods have been attempted and re-attempted by several research groups. Thecosome pteropod abundance is patchy and difficulties in developing useful techniques are often compounded by limited

access to specimens. As such, it is vital that opportunities are not lost repeating unsuccessful methodology.

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Chapter 5: Decoupled carbonate chemistry controls on the incorporation of boron into *Orbulina universa*

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Abstract

In order to fully constrain paleo-carbonate systems, proxies for two out of seven parameters, plus temperature and salinity are required. The boron isotopic composition ($\delta^{11}B$) of planktonic foraminifera shells is a powerful tool to reconstruct changes in past surface ocean pH. As $B(OH)_4^-$ is substituted into the biogenic calcite lattice in place of CO_3^{2-}, it has been suggested that B/Ca ratios in biogenic calcite are a possible proxy for [CO_3^{2-}]. However, differentiating between the effects of pH and [CO_3^{2-}] is problematic, as they co-vary closely in natural systems, and so the major control on boron incorporation remains unclear. To disentangle the effects of pH and [CO_3^{2-}] on the B/Ca ratio and to test whether $\delta^{11}B$ remains constant at constant pH, but under changing [CO_3^{2-}] (pH 8.05 with 238, 285 and 532 µmol kg^{-1} CO_3^{2-}) and vice versa, we decoupled pH and [CO_3^{2-}] (276 ± 19.5 µmol kg^{-1} CO_3^{2-} with pH 7.7, 7.9 and 8.05) and cultured the planktonic foraminifer *Orbulina universa*. Measurements of the isotope composition of boron and trace elements were performed simultaneously using a femtosecond laser ablation system coupled to an MC ICP-MS and high resolution mass spectrometer. Results show that $\delta^{11}B$ is controlled by pH and does not respond to changes in [CO_3^{2-}]. On the other hand, the B/Ca ratio responds to [HCO_3^-] independently of pH. This suggests that B/Ca ratios in foraminiferal calcite may be used as a second, independent, proxy for paleo-carbonate system reconstructions.

Introduction

Before the Anthropocene, the atmospheric CO_2 concentration was governed by the surface ocean [CO_2], simply because the carbon content of the ocean is 65 times larger than that of the atmosphere (Siegenthaler & Sarmiento 1993). Hence, understanding the global carbon cycle and the evolution of atmospheric pCO_2 in Earth history requires knowledge of the dynamics of oceanic carbonate chemistry. The unprecedented magnitude and rate of carbon emissions since the industrial revolution (Ciais et al. 2013) has resulted in warming, and acidification of the oceans (Rhein et al. 2013). As a result, the interest in the reconstruction of seawater carbonate chemistry to identify ocean acidification in Earth history experienced another impetus (Hönisch et al. 2012). The reconstruction of the full oceanic carbonate chemistry requires proxies of at least two independent parameters of the carbonate system, in addition to temperature and salinity.

The most promising tool for reconstructing pH is the boron isotopic composition ($\delta^{11}B$) of biogenic carbonate producers such as foraminifera and corals (e.g., Sanyal et al. 1996, Sanyal et al. 2001, Hönisch et al. 2004, Rae et al. 2011). Boron exists in seawater primarily in the form of two species, boric acid ($B(OH)_3$) and borate ion ($B(OH)_4^-$). As for all weak acids, the relative abundance between these two species is controlled by pH. Because of the isotopic fractionation of 27.2‰ (Klochko et al. 2006, Klochko et al. 2009) between the two aqueous species ($\alpha_{4\text{-}3} = R_{B(OH)4-}/R_{B(OH)3}$), the boron isotopic composition of each species is also pH dependent (Palmer et al. 1987, Sanyal et al. 1996, Sanyal et al. 2000). At low pH (<7), nearly all boron is present in the form of boric acid, whereas at high *p*H (>10) boron primarily exists as borate. The stable isotope ^{11}B is enriched in $B(OH)_3$ compared to $B(OH)_4^-$, with a constant isotopic fractionation of 27.2 ‰ between the two boron species (Klochko et al., 2006). Consequently, as the relative concentration of the dissolved species changes with pH, so does their isotopic composition. Because it is assumed that only the charged species, borate, is incorporated into the calcite lattice (Hemming and Hanson, 1992) the boron isotopic composition of marine carbonates thus records the pH that prevailed when the calcium carbonate was precipitated.

Based on the boron systematics in seawater and the assumption that only the charged species is used in calcification, Vengosh et al. (1991) and Hemming and Hanson (1992) suggested that the boron isotopic composition of marine carbonates is controlled by seawater pH.

Consequently, the $\delta^{11}B$ of marine calcifiers such as corals, bivalves, foraminifera, pteropods, etc. can be used as a proxy for paleo pH and therefore provide clues on the evolution of an important parameter of the oceanic carbonate chemistry. For instance, Spivack et al. (1993) estimated that the pH of the surface ocean may have changed from 7.4 in the Miocene to 8.2 in the Holocene. Sanyal et al. (1995) estimated that, during the last glacial maximum, the deep ocean pH was about 0.3 pH unit higher and the surface ocean pH about 0.2 pH unit higher compared to that of the modern values. Inspired by such promising results, several groups took on the challenge to reconstruct ocean pH. In the subsequent years, many studies have dealt with reconstructing past oceanic pH across different time scales using the boron isotopic composition of mainly foraminifera but also corals (e.g. Spivack et al. 1993, Sanyal et al. 1995, Sanyal et al. 1997, Hemming et al. 1998, Palmer et al. 1998, Pearson & Palmer 1999, Sanyal & Bijma 1999, Pearson & Palmer 2000, Hönisch & Hemming 2005, Pagani et al. 2005, Foster et al. 2006, Hönisch et al. 2007, Hönisch et al. 2008, Hönisch et al. 2009, Honisch et al. 2011, Foster et al. 2012, Honisch et al. 2012).

$\delta^{11}B$ is proved to be a reliable recorder of pH but, in the aforementioned reconstructions, the second carbonate system parameter used to reconstruct pCO_2 was based on assumptions. In existing boron-based reconstructions, investigators attempted to estimate total alkalinity (A_T) or $[CO_3^{2-}]$ from modern ocean conditions or reconstructions of carbonate compensation depth (CCD). In the modern ocean A_T is linearly correlated with salinity, because total alkalinity is the charge balance between the major conservative ions in seawater, and this charge balance varies with salinity (Zeebe & Wolf-Gladrow 2001). Therefore, total alkalinity is a conservative parameter. It is assumed that the modern salinity–A_T relationship was constant over time so that A_T can be estimated from reconstructions of salinity using sea-level records (Foster 2008, Hönisch et al. 2009). However, salinity varies on a regional scale (e.g., it is higher at the equator than at higher latitudes, and lower in the vicinity of river mouths), but reliable proxies for regional salinity reconstructions are not available to date. Another approach is based on the assumption that seawater $[Ca^{2+}]$ has remained proportional to A_T over time so that A_T can be adjusted in a way that the water column is exactly saturated with respect to calcite at the lysocline (~500 m above the CCD) (Pearson & Palmer 2000). The CCD, however, is not uniform through space and time (van Andel, 1975), calling into question this approach for estimating past A_T. In other studies, $[CO_3^{2-}]$ was estimated through adjusting the surface ocean $[CO_3^{2-}]$ to match the CCD.

Considering the relatively large uncertainties afflicted with the constraints described above, it is of utmost importance to develop proxies for a carbonate system parameter other than pH. A potential one is the B/Ca ratio of planktonic foraminifera that has been used as a proxy for estimating past changes in [CO_3^{2-}] (Foster 2008), given that the concentration of borate $B(OH)_4^-$) increases with pH and pH co-varies with [CO_3^{2-}]. However, it is challenging, if not impossible, to identify the parameter controlling B/Ca based on samples that have grown in natural seawater because pH and carbonate chemistry parameters co-vary closely in natural systems. To disentangle their effects on a proxy, it is necessary to deconvolve the carbonate system.

A more recent study (Allen et al. 2012) has shown that the B/Ca ratio of planktonic foraminifera also decreases with increasing total inorganic carbon (C_T) or [HCO_3^-]) at constant pH (i.e. [$B(OH)_4^-$] was constant while [CO_3^{2-}] and [HCO_3^-] were increased), suggesting that borate and carbonate ions compete for the inclusion into the calcite lattice. Consequently, B/Ca would be influenced by the ratio between [$B(OH)_4^-$] and C_T (or [HCO_3^-]), rather than by pH or [CO_3^{2-}] alone. This new finding suggests that combined measurements of $\delta^{11}B_{calcite}$ and B/Ca of the same species might be used to estimate C_T from $\delta^{11}B_{calcite}$ (a proxy for pH and hence [$B(OH)_4^-$]) and B/Ca (a proxy for [$B(OH)_4^-$]-to- C_T ratio). However, in the study by Allen et al. (2012), pH was not decoupled from [CO_3^{2-}], leaving the question open whether the B/Ca ratio in planktonic foraminifera is influenced by the ratio between [$B(OH)_4^-$] and C_T or [HCO_3^-]. In our study we conducted experiments with decoupled pH and [CO_3^{2-}] in order to fill this gap in our knowledge of boron incorporation into biogenic carbonates.

Methods

Collection and culturing:

Living specimens of *Orbulina universa* were collected daily using a 57 cm diameter WP2 plankton net (200 µm mesh size), between July and September 2012 at Point B, Villefranche-sur-Mer, 43.41˚N, 7.19˚E and maintained until gametogenesis in laboratory cultures at the Laboratoire d'Oceanographie de Villefranche. Established procedures for maintaining planktonic foraminifera in laboratory culture were used (Spero & Lea 1993, Bemis et al. 1998, Bijma et al. 1998). Briefly, specimens were identified, measured with a light microscope, and transferred to 0.2 µm-filtered seawater, whose carbonate chemistry was

accurately determined and subsequently modified. Specimens were maintained individually in air-tight 100 ml acid-washed SCHOTT DURAN® bottles that were sealed without an air space and placed upside down into thermostated water baths maintained at a temperature of 23°C (±0.2°C). Light was provided by 4, 39 W fluorescent tubes (JBL Solar Ultra Marin Day), with reflectors, (at a distance of ca. 15 cm from the water surface), with a 12:12 h L: D photoperiod. The average irradiance, measured with a LI-193 sensor (LiCOR) in the culture jars was about 290 µmol photons m^{-2} s^{-1}.

The foraminifers were fed a one-day-old brine shrimp (*Artemia*) nauplius every second day until gametogenesis. The brine shrimps were hatched in modified seawater from the same batch as used for culturing the foraminifera. Just prior to feeding, hatched nauplii were transferred once again to fresh medium from the same batch. After feeding, culture jars were topped up with medium from the same batch to prevent the formation of a headspace. Empty shells were collected within 24 h after successful gametogenesis, rinsed in deionised water and archived in covered slides for later analysis. Approximately 35 tests were grown for each experimental treatment. Culture water samples were collected at the start and end of the experiments to verify the boron concentration, its isotopic composition and the carbonate system parameters.

Modified seawater chemistry

The objective of these experiments was to decouple seawater pH and [CO_3^{2-}] and create treatments with a constant pH and varying carbonate ion concentration and treatments with a constant carbonate ion concentration but varying pH. To decouple the effects of pH and [CO_3^{2-}], seawater carbonate chemistry was modified manipulating pH, using NaOH and HCl, and dissolved inorganic carbon (C_T) by adding gravimetrically carbonate and bicarbonate or bubbling with CO_2. Calculations were made using csys_vari.m (Zeebe & Wolf-Gladrow 2001) with carbonic acid dissociation constants of Mehrbach et al. (1973). Temperature (23°C) and salinity (38.0) were kept constant (Table 5.1).

To enable single shell analysis by LA-ICP-MS, the boron concentration was increased to about 10 times the concentration of natural seawater by adding boric acid to the culture water (see Sanyal et al. 1996). The pH_T and C_T were then modified via titration with boron free NaOH 1N and HCl 1N to bring the experimental pH to desired levels of 7.70 ± 0.05, 7.90 ±

0.05 and 8. 05 ± 0.05, respectively. Culture water samples collected at the start and at the end of each experiment showed that pH remained nearly constant throughout each experiment. The boron isotopic composition of each culture treatment is provided in Table 1. pH of the culture solutions was measured using a Metrohm, 826 pH mobile meter with glass electrode (Metrohm, electrode plus) calibrated to the total scale using TRIS and 2-aminopyridine buffer solutions (Dickson et al. 2007) adjusted to a salinity of 38.0 (Dickson et al. 2007). Total alkalinity (A_T) samples (150 mL) were filtered on GF/F and measured potentiometrically using a Metrohm Tritando 80 titrator and a Metrohm, electrode plus glass electrode (Dickson et al. 2007). 60 ml samples were also taken at the start and end of incubations and poisoned with 10 µL of saturated $HgCl_2$ pending determination of dissolved inorganic carbon (C_T). Samples were measured using an AIRICA (Marianda, Kiel) fitted with a Licor 6262 infra-red gas analyser. All parameters of the carbonate system were calculated from A_T (Hoppe et al. 2012) using the R package seacarb (Lavigne & Gattuso 2013).

Table 5.1: Average properties of the manipulated seawater culture medium from 4 samples (2 from the start of the incubation and two from the end of the incubation).

pH_T	s.d.	C_T (µmol kg^{-1})	s.d.	A_T (µmol kg^{-1})	s.d.	pCO_2 (µatm)	CO_3^{2-} (µmol kg^{-1})	HCO_3^- (µmol kg^{-1})	T (˚C)	S	$\delta^{11}B$ ‰	s.d.
8.05	0.05	2671.3	10	3466	27	515.8	285.6	2370.6	23 ± 0.7	38 ± 1.02	4.98	0.85
8.05	0.02	1269	15	2544.8	11	431.1	238.7	1981	23 ± 0.7	38 ± 0.6	5.35	0.53
8.05	0.03	4981.7	41	6398.3	38	962	532.6	4421	23 ± 0.7	38 ± 0.5	4.20	1.03
7.9	0.02	3806.4	26	4153.2	154	1069.1	296.6	3478.4	23 ± 0.7	38 ± 0.3	4.11	0.94
7.7	0.03	5117.8	54	5361.8	23	2334.1	257.8	4791.6	23 ± 0.7	38 ± 0.9	4.69	2.4

Boron analysis:

Prior to analysis, foraminiferal tests were soaked in NaOCl (10% chlorine) solution (1:3 deionized water) for ca. 6.5 h until all organic remains were removed (visual verification under the stereomicroscope), washed three times in deionized water and left overnight in a drying oven at 50˚C. For simultaneous determination of boron isotopes and B/Ca in single tests of foraminifers, a recently developed, novel method was applied. A detailed description of this methodology can be found in Kaczmarek et al. (in prep.). The isotopic composition of

foraminifers was determined using a Thermo Finnigan Neptune MC ICP-MS at the University of Hannover. Laser ablation of reference material (NIST 610) and samples was performed by an UV (193 nm)-femtosecond laser (Solstice Spectra Physics). All measurements were carried out in low mass resolution. Compact discrete dynode multipliers (CDD, Thermo) were attached to faraday cups at low site L4 and high site H4 with an applied voltage between 2100 and 2200 V. The low-resolution mode was sufficient for this study as H4 and L4 were fixed at a distance of 7 cm from each other. Possible interferences are $^{40}Ar^{4+}$ and $^{20}Ne^{2+}$, however, scanning the mass between 10.525 and 10.535 showed no interferences with these ions.

For analysis, a standard/sample bracketing procedure was adopted using a NIST 610 glass standard (351 ppm boron and 8.449 % calcium, $^{10}B/^{11}B$ of 4.049 (Le Roux et al. 2004) as reference material. The application of this external calibration technique allows correction of the instrumental mass discrimination and its temporal drift by using a reference material of known isotopic composition prior to, and after analysis of samples. The acquisition parameters in static mode for analysis of NIST 610 and samples were set to acquire 200 cycles of 1-second integrations each. Before starting sample analysis, measurements of NIST 610 were carried out until the drift of the instrument with respect to mass stability was less than 100 ppm over a period of twelve minutes (the time interval for a standard-sample-standard measuring sequence). In order to account for background signal, it was measured simultaneously for both isotopes for each measurement over the first 40 cycles by closing the laser shutter and reopening again for analysis of the reference material and samples respectively. Background counts were later subtracted from sample and standard measurements. Boron isotopic composition is reported using the delta notation (eq. 1):

$$\delta^{11}B_{sample}(‰) = \left[\frac{\left(^{11}B/^{10}B\right)_{sample}}{\left(^{11}B/^{10}B\right)_{NIST\ 610}} - 1\right] x\ 1000 \qquad \text{Eq. 1}$$

For the analysis of the foraminifers, we performed raster measurements with a spot size of 50 µm and 6-20 Hz (adjusted depending on sample). The size of the raster varied individually depending on the size of the focused surface. Reference material was measured in raster mode (100 µm x 100 µm) with a spot size of 50 µm and 5 Hz. Analysis of boron content and isotopic composition of seawater culture medium was undertaken at Cambridge University

using HR-ICP-MS as described by Misra et al. (2014). In short, trace elements were extracted from the seawater matrix via micro-sublimation and subsequently added to a mixture of nitric and hydrofluoric acid (0.1 M HNO_3 and 0.3 M HF) that acted as the sample matrix. Boron concentration and $\delta^{11}B$ was then measured via ICP-MS.

In order to determine the Ca^{2+} concentration, optical emission analysis was performed using a high-sensitivity spectrometer with a measuring range of 250 to 460 nm and a resolution of 0.11 nm. It was equipped with a back-thinned 2D FFT-CCD detector, and a grating with a groove density of 1200, which is most efficient between 200 and 400 nm. The detection limits are 500 and 60000 cps. Ca^{2+} ion lines were measured at a wavelength of 393.48 nm. At that wavelength, the spectra showed no interferences between the Ca^{2+} peak and other peaks. The acquisition parameters were set to 220 cycles per analysis with an integration time of 1 sec for each cycle. The intensity of the background signal was subtracted from the intensity of the reference material and sample for each analysis to obtain background corrected values. A sample/standard bracketing procedure was followed during measurement.

Statistics:

Lamtool was used for analysis and background correction of $\delta^{11}B$ data. All other statistics were carried out using R (R Core Team 2008). Error bars represent ± 2 standard deviations, correlations were calculated by linear regression.

Results:

B/Ca ratios:

The B/Ca ratio of *O. universa* shows a strong negative correlation ($R^2 = 0.93$) with C_T irrespective of the pH of the culture media (Fig 5.1A). It is also correlated to $[CO_2]$ but to a lesser extent ($R^2 = 0.63$) (Fig 5.1C). B/Ca also decreases with increasing $[CO_3^{2-}]$ in specimens grown under a pH of 8.05, (Fig 5.1E). However, the B/Ca ratio of specimens grown under lower pH_T values (7.9 and 7.7) is negatively offset from the relationship found at pH_T 8.05, while it increases with increasing $[B(OH)_4^-]/[CO_3^{2-}]$ ratio (Fig 5.1F). The overall correlation of B/Ca and $[CO_3^{2-}]$ is very low ($R^2 = 0.2$; Fig 1C). The B/Ca ratio exhibits a striking negative

relationship with increasing [HCO_3^-], irrespective of the pH of the culture medium (R^2 = 0.94; Fig 5.1G).

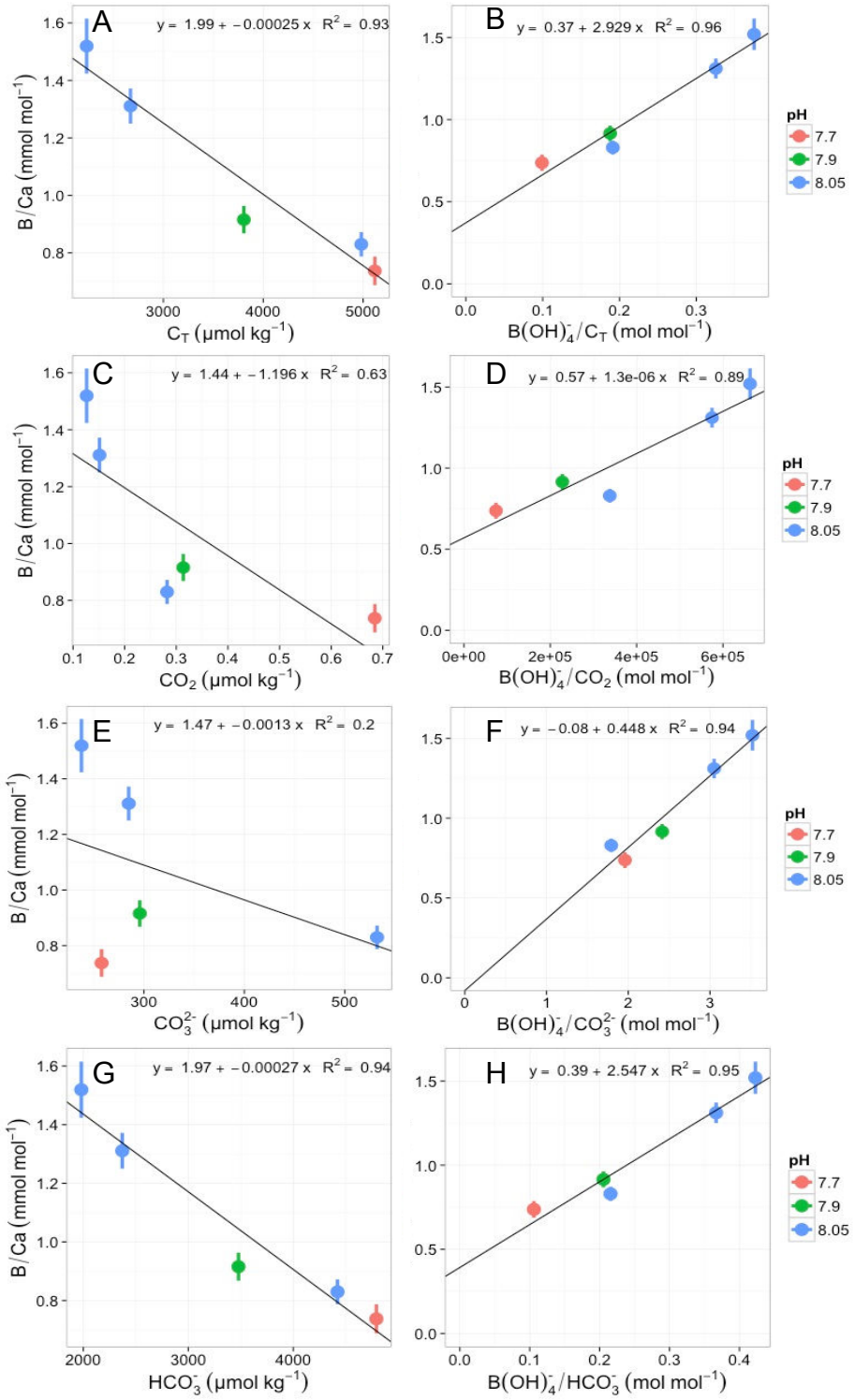

Fig. 5.1: B/Ca ratios plotted against (A) [C_T], (B) [$B(OH)_4^-$]/[C_T] ratio, (C) [CO_2], (D) [$B(OH)_4^-$]/[CO_2], (E) [CO_3^{2-}], (F) [$B(OH)_4^-$]/[CO_3^{2-}], (G) [HCO_3^-], (H) [$B(OH)_4^-$]/[HCO_3^-].

Boron isotopic fractionation ($\delta^{11}B$):

The fractionation of boron isotopes in the shells of *O. universa* is dependent on the pH of the culture medium, increasing with pH from 15‰ at pH_T 7.7 to 19.1‰ at pH_T 8.05. These values are close to the fractionation curve obtained for artificial seawater by Klochko et al. (2006) (Fig 5.2A). Changes in $[CO_3^{2-}]$ and $[HCO_3^-]$ at constant pH has no significant effect on $\delta^{11}B$ (Fig 5.2B).

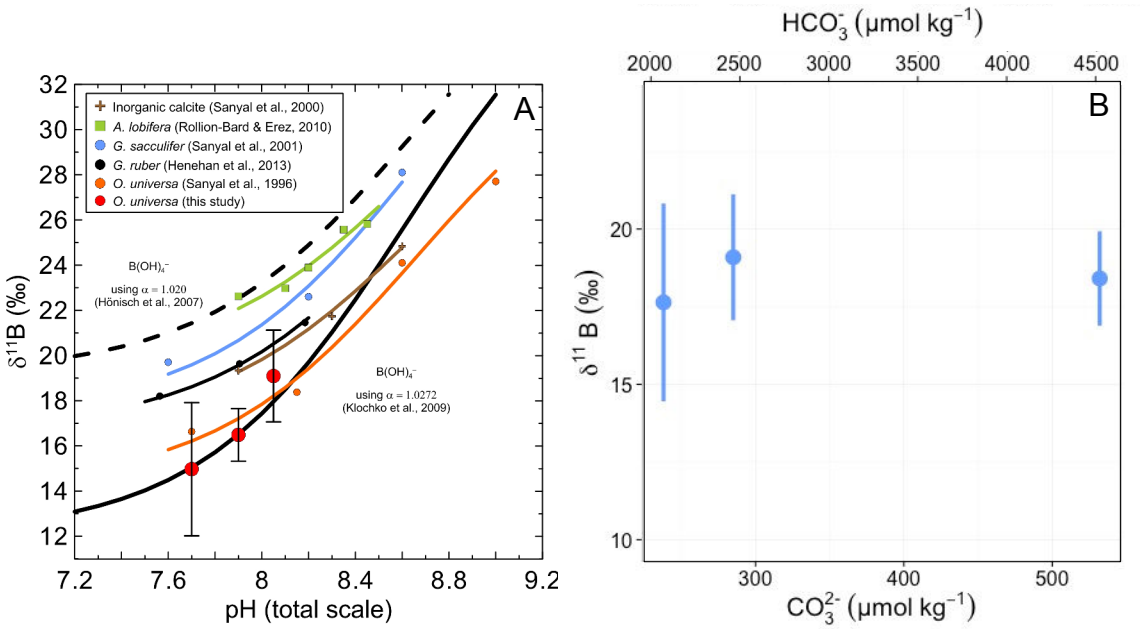

Fig 5.2: (A) Average $\delta^{11}B$ of $B(OH)_4^-$ in cultured *O. universa* calcite (red circles), solid black line shows empirical values for artificial seawater with a fractionation factor of ${}^{11\text{-}10}K_B$ = 1.0272 (Klochko et al. 2006) at T=23°C and S=38. Dashed black line shows empirical values for artificial seawater with a fractionation factor of ${}^{11\text{-}10}K_B$ = 1.020 (Klochko et al. 2006) at T=23°C and S=38. (B) Average $\delta^{11}B$ of $B(OH)_4^-$ in cultured *O. universa* calcite grown at the pH 8.05 and varying $[CO_3^{2-}]/[HCO_3^-]$. Error bars represent ± 2 sd across all single laser ablation analyses per treatment.

Discussion:

B/Ca:

Foster (2008) identified $[CO_3^{2-}]$ as having a major control on B/Ca in samples of foraminifera from down core and core tops. A similar conclusion was reached by Allen et al. (2011) for *O. universa*. These authors demonstrated a trend of decreasing B/Ca with increasing pH and $[CO_3^{2-}]$. However, due to the co-variations of the carbonate system in natural seawater, it is difficult to identify the differential effects of the individual parameters. Decoupling pH from other parameters of the carbonate system using artificial seawater enables us to disentangle the relationship and identify the controlling factors. Our results clearly show that the amount

of boron incorporated into *O. universa* calcite is a function of C_T (Fig. 5.1A). As C_T increases, B/Ca decreases, suggesting that $B(OH)_4^-$ competes with carbonate for inclusion in the calcite lattice. When B/Ca ratios are plotted against $[CO_2]$, the relationship is similar to that of C_T, however only <1% of C_T is in the form of CO_2 so this species is unlikely to have a major control on boron incorporation. The remaining >99% is ~10% CO_3^{2-} and ~ 90% HCO_3^- (Zeebe & Wolf-Gladrow 2001). Because of the strong correlation of the B/Ca ratio and $[B(OH)_4^-]/[C_T]$, one could argue that foraminifera utilize both HCO_3^- and CO_3^{2-} as substrate for calcification and therefore that C_T is the factor controlling the B/Ca ratios. However, because $[HCO_3^-]$ and $[CO_3^{2-}]$ respectively increase and decrease with decreasing pH in our treatments (Fig. 5.3), we can distinguish between bicarbonate and carbonate ion control over the B/Ca ratio.

At constant pH, the relationship between B/Ca and $[CO_3^{2-}]$ (Fig. 5.1C) supports the hypothesis of competition between CO_3^{2-} and $B(OH)_4^-$. However, when $[CO_3^{2-}]$ is held constant and pH is decreased, B/Ca significantly decreases despite the fact that $[CO_3^{2-}]$ remains more or less constant (Fig 1C and 1D, Table 1). If the same relationships are examined for B/Ca and $[HCO_3^-]$ a strong correlation between $[HCO_3^-]$ and B/Ca can be observed both for the absolute concentration of HCO_3^- (Fig. 5.1E) and also for the ratio of $[B(OH)_4^-]/[HCO_3^-]$ with no effect of changing pH (Fig. 5.1F). The close correlation between $[CO_3^{2-}]$ and B/Ca at constant pH can be explained by the corresponding increases in $[HCO_3^-]$ in these treatments (Table 5.1, Fig. 5.3).

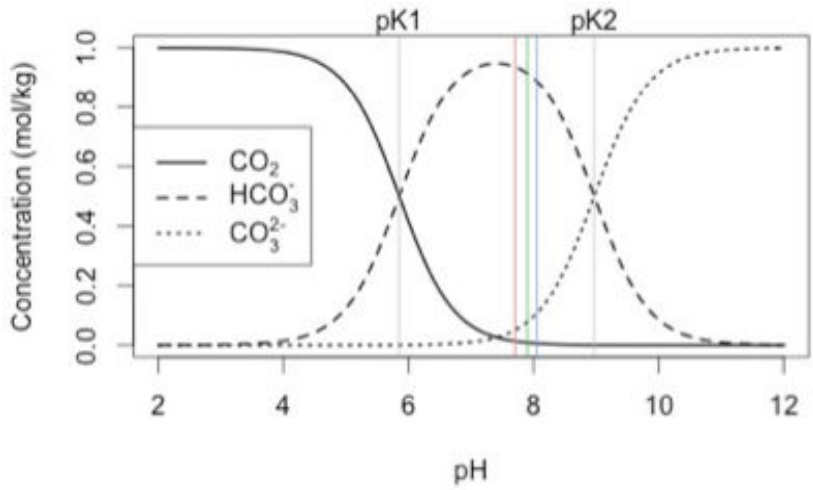

Fig 5.3: Bjerrum plot showing the relative speciation of carbonate species as a function of pH. Coloured lines correspond to the pH treatments used in this study, red = pH 7.7, green = 7.9, blue = 8.05. Plotted using seacarb (Lavigne & Gattuso 2013).

The finding that $[HCO_3^-]$ is the major control on boron incorporation in *O. universa* calcite is agreement with the hypotheses of Hemming and Hanson (1992) who suggested that only $B(OH)_4^-$ is incorporated into marine carbonates with the partition coefficient defined in eq. 2.

$$K_D = \frac{[B/Ca]_{solid}}{[B(OH)_4^- / HCO_3^-]_{seawater}} \qquad \text{Eq. 2}$$

Analysis of planktonic foraminifera in core tops also revealed correlation between the B/Ca ratio and $[HCO_3^-]$ (Yu et al. 2007). Another empirical study has investigated the effects of decoupling pH and the carbonate system on B/Ca and found the same effect of $[HCO_3^-]$ on the B/Ca ratio in *Globigerinoides sacculifer* (Allen et al. 2012) as for *O. universa* in the present study.

Boron isotopic fractionation ($\delta^{11}B$):

As the various species of inorganic carbon and pH are tightly linked, it is still not clear whether only pH and/or the concentration of one or several carbonate species might have an effect on $\delta^{11}B$. The results for treatments with varying pH and constant carbonate ion concentration displayed the same relationship as those from the calibration curve for *O. universa* produced by Sanyal et al. (1996) but the absolute values for a given pH are slightly lower by approximately 1 to 2‰ when compared to the values corrected to Klochko et al. (2006; in Zeebe et al. 2008). The effects of the unnaturally high C_T and A_T values in the treatments used cannot be discounted as the cause of this difference, although it is unlikely because the $\delta^{11}B$ values proved to be robust when a range of $[CO_3^{2-}]$ was used. The values produced for $\delta^{11}B$ in this study match closely with the values for artificial seawater given by Klochko et al. (2006) suggesting that the vital effects imposed by *O. universa* are not as strong as originally thought. There is no effect of varying $[CO_3^{2-}]$ on $\delta^{11}B$ of samples grown at the same pH but, most importantly, in light of the results obtained for the B/Ca ratio, there is also no effect of $[HCO_3^-]$ (Fig. 5.2B).

Proxy implications:

Based on this and other culture studies, it becomes clear that despite strong biological effects on the ambient carbonate chemistry (Rink et al. 1998, Wolf-Gladrow et al. 1999, Köhler-Rink & Kühl 2000, Köhler-Rink & Kuhl 2001, Zeebe et al. 2008), the boron isotopic composition

and the B/Ca are faithful predictors of pH and bicarbonate ion concentration, respectively. Our results provide strong evidence that [HCO_3^-] is recorded in the B/Ca ratio. A wide range of [HCO_3^-] was necessary to facilitate de-coupling the carbonate system from pH, however the high [HCO_3^-] in several of these treatments are unrealistic for natural seawater systems, particularly paleo-systems which exhibit lower [HCO_3^-] than present day oceans (calculated from values given in Zeebe 2012) and more environmentally-relevant values should be used for future calibration experiments. The proxy should therefore be ground-truthed using core top samples. The correlation of the B/Ca ratio to [HCO_3^-] rather than to [CO_3^{2-}] might have some implications for existing paleo-carbonate chemistry reconstructions based on this proxy such as the study by Foster (2008) and that of Yu et al. (2014), if the same relationship holds for benthic foraminifers as for planktonic ones.

A sound understanding of the effects of past carbon perturbations becomes increasingly urgent in an age where anthropogenic activities are producing such rapid changes in global climate (Knoll & Fischer 2011, Bijma et al. 2013). The usefulness of biogeochemical proxies to reconstruct paleoceanographic conditions is well established for environmental parameters such as temperature and salinity (Wefer et al. 1999) but uncertainties remain for proxies related to pH and the carbonate system (Pagani et al. 2005, Hönisch et al. 2007, Katz et al. 2010, Allen & Honisch 2012). This study confirms the robustness of $\delta^{11}B$ as an independent pH proxy and adds further weight to the growing body of evidence that B/Ca in planktonic foraminiferal calcite may be used as an independent proxy for [HCO_3^{2-}] (Yu et al. 2007, Allen et al. 2012), thereby allowing researchers to fully constrain the carbonate system.

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Chapter 6: Discussion

Anthropogenic activities such as the burning of fossil fuels, cement production and changes in land usage are causing perturbations to the global carbon cycle a rate and scale unparalleled in the last 300,000,000 years (Hönisch et al. 2012, Le Quéré et al. 2013). In the oceans the symptoms of global carbon perturbation manifest in three major processes: ocean acidification, warming and a decline in the global concentration of oxygen and an expansion of suboxic zones, which can act alone or in concert, to reduce the optimal performance of marine organisms (Gruber 2011, Bijma et al. 2013). In order to predict how these changes could impact ecosystems and biogeochemical cycles, it is vital for scientists to quantify the responses of key species. To do this effectively, an interdisciplinary approach is required as the effects of environmental changes will be experienced in many different, but interlinked, ways. Organism responses range in scale from molecular changes at the cellular level (Hofmann et al. 2008) to species composition shifts (Kroeker et al. 2013a, Kroeker et al. 2013c), population fluctuations (Parker et al. 2011) and extinction events (Thomas et al. 2004). Predictions can be greatly improved by also looking at the extent to which organisms have already reacted to climate change occurring in recent times and also to climate change events that took place in geological history.

Thecosome pteropods and planktonic foraminifers are key components of the pelagic ecosystem. They represent the largest contributors of pelagic aragonite and calcite, respectively (Berner & Honjo 1981, Schiebel & Movellan 2012) and are important links in the marine food web (Lalli & Gilmer 1989, Culver & Lipps 2003). The shells of foraminifera are also widely used in reconstructions of paleo-climate conditions. For these reasons it is vital to investigate the effects of climate change on these organisms.

Climate change in the Mediterranean:

Sea surface temperatures (SSTs) in the Mediterranean have steadily increased since the turn of the 20th century by approximately 0.039 to 0.167 °C yr^{-1} (Nykjaer 2009, Vargas-Yáñez et al. 2010). At the study site at Point B (north western Mediterranean), annual average SSTs have increased from approximately 17˚C to 17.9˚C (Fig 6.1). By the year 2100, mean temperatures are projected to rise by approximately 2-2.5˚C, pushing average summer maxima to > 28˚C (Lazzari et al. 2013). The rise in temperature will increase stratification, which can cause suboxia in deeper waters (Gruber 2011), although Mediterranean waters are relatively oxygen replete. Strong summer stratification can decrease oxygen transport through the thermocline but warming induced anoxia is not anticipated to be a significant risk (Zenetos et al. 2002). Increased stratification will cause further nutrient limitation in the already oligotrophic waters (The Mermex Group 2011).

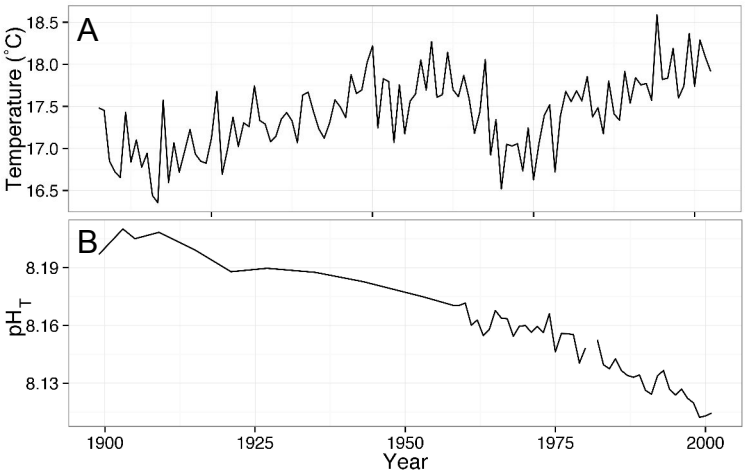

Fig 6.1: (A) Temperature time series for Point B location over the 20th century taken from Had SST dataset (Hadley Centre for Climate Prediction and Research/Met Office/Ministry of Defence 2000; http://rda.ucar.edu/datasets/ds277.3.). (B) pH hindcast for Point B over the 20th century, from 1899 – 1957, estimate produced from Siple ice core pCO_2 values (Neftel et al. 1994), from 1958 – present day, estimate produced from Mauna Loa CO_2 time series.

Due to high irradiance and summer temperatures as well as low riverine input, the Mediterranean acts as an evaporation basin with high salinity and, consequently, high total alkalinity (A_T). Seawater is therefore highly supersaturated with respect to calcite and aragonite, average annual Ω_a and Ω_c in 2012 (western basin) of 3.4 and 5.3, respectively (Touratier & Goyet 2009). At the study site investigated in this thesis there is a strong seasonal influence on pH_T with a difference of approximately 0.07 pH_T units between summer and winter values. Whilst historical records of SST are relatively numerous, there is a distinct paucity of pH measurements, making it necessary to estimate pH from other parameters. Hind-casting of pH_T values over the last century in the western Mediterranean suggest a decreasing trend in pH_T from approximately 8.19 to 8.09, which equates to a decrease in Ω_a of approximately 0.9 units, from 3.97 to 3.09 (Fig 6.1, Chap. 2). The hind-cast tends to be quite poor at estimating lower values and as a result is likely to give a conservative estimate of pH change. The estimate is based on measured values, each of which has an associated error, the multiple steps in calculation inevitably cause some propagation of these errors throughout the model (Ku 1966). Nonetheless, the methods used in this thesis have reproduced a trend with rates that agree with other published studies (Touratier & Goyet 2009, Rhein et al. 2013). Seawater pH is predicted to decrease further by an average of 0.3-0.4 units by 2100 under RCP8.5 (Rhein et al. 2013). Mediterranean waters will remain supersaturated, with Ω_a and Ω_c of about 2.1 and 3.2 at the end of this century.

An interdisciplinary approach:

There are several techniques available to measure the effects of environmental stressors, via field datasets, with perturbation experiments or by using the sedimentary archive to study analogous events in Earth history. This thesis employed an interdisciplinary approach to study the effects of climate change on Mediterranean pteropods and foraminifers. Time series and museum samples were used to assess the effects that ocean acidification and warming have already had on Mediterranean pteropods. Culture work was used to develop techniques for maintaining laboratory populations of pteropods with the aim of improving perturbation experiments. Planktonic foraminifers were used to define the major factor controlling $\delta^{11}B$ and B/Ca ratios for

developing tools to reconstruct carbonate chemistry with the aim of investigating ocean acidification analogues in Earth history. As with any scientific approach, there are considerations when using all of these methods. In the following section, the major advantages and disadvantages of each method are discussed.

By using the sedimentary archive to study climate change events in Earth history, it is possible to study the long-term (natural) perturbations of ocean chemistry and their impact on biogeochemical cycles (e.g. Hönisch et al. 2009). The advantage is that complexity is reduced as sediments integrate over space and time. It allows investigation of different time scales (historical/sub-recent, glacial-inter glacial, deep time) and is the only option to investigate the process of recovery after an ocean acidification event and to test different scenarios as case and sensitivity studies for models. The approach is limited by the accuracy of the proxies used for reconstructions (Wefer et al. 1999). Fossils used for reconstructions are often subject to dissolution or chemical changes in the sediments and biological information is limited to hard parts and biomarkers that remain well preserved in the sediment. The comparison of past climate change events to the current one is limited by the rate and scale of the carbon perturbation and the conditions directly preceding it (Zeebe & Ridgwell 2011, Hönisch et al. 2012). This reduces the number of potential analogues in the sedimentary archive.

Work with time series allows a relatively full assessment of the direct and indirect effects of recent climate change. The animals remain in their natural environment so information on trophic interactions, cascading effects and multiple stressors is fully captured. The use of long-term time series data also provides an indication of the adaptability of an organism, which cannot be easily inferred from laboratory-based experiments. However, many of the advantages of working with times series data have associated disadvantages. By analysing an animal in the natural environment, the researcher is faced with the full complexity of that environment, in many cases it can be difficult to ascertain which driver, or combination of drivers, produces the observed population responses. There is also temporal complexity; processes that are linked may be separated by lags or leads, which can make it difficult to identify dependencies. Work can also be limited by a lack of continuous, long-term data. Multi-decadal biological time series are relatively

rare and it can be problematic to find associated abiotic time series that cover the entire study period.

Comparing museum samples with modern ones offers a unique opportunity to compare the influence of changes in environmental conditions on physical structures and, if properly preserved, molecular genetics. They represent a "snapshot" of past conditions in specimens that are better preserved than if they were sampled from the sediments. As these samples are often taken during scientific expeditions, there are sometimes records of the abiotic conditions at the time of sampling. The major disadvantage of working with museum samples is that they are only representative of a very short timescale. As with time series data, the full complexity of the natural system can make it difficult to attribute environmental drivers to physical properties of collected specimens. Age effects, local scale environmental conditions and seasonal or inter-annual variability, may skew results. This variability is, to some extent, integrated over the lifetime of a long-lived animal. However, these factors can prove to be problematic when working with shorter-lived organisms. It can also be impossible to find records of environmental conditions at the time of collection; these data should be over longer time scales (> 1 yr) to provide an accurate description of the organism's environment and for comparison with modern conditions. How the samples have been stored must also be considered, many museum samples are rendered unsuitable by the use of inappropriate preservation procedures, which may have compromised structural integrity or altered chemical composition.

While proxies, time series data and museum samples are excellent tools for assessing changes that have already occurred, it is often desirable to analyse what effects future conditions may have. The most widely used method to do so is by perturbation experiments. They have the advantage that they allow the researcher to reduce the confounding variables present in natural systems by altering target parameters whilst maintaining all others unchanged. However, this reduction of complexity has the risk of masking interactive effects of other stressors, which may drastically alter the response of the organism. The logistics and time consuming nature of perturbation experiments often precludes long-term studies, making it difficult to assess an organism's capacity for acclimatisation or adaptation. There are also many animals that are

problematic to maintain in laboratory cultures due to size, dietary requirements or convoluted life cycles. In these cases, the stress imposed by unnatural conditions may compromise experimental results.

Effects of environmental change on Mediterranean pteropods:

Calcification

Museum samples of two common Mediterranean pteropods were compared to modern samples to assess whether the effects of environmental changes which took place over the last century could be observed in the shell biometrics (Chap. 2). Shell measurements were undertaken using a novel method of X-ray computed tomography (CT) imaging. Despite the fact that waters have remained supersaturated with respect to aragonite, some differences in pteropod shell structure can be detected between samples from the turn of the 20th century and the present day. *Styliola subula* shells from 1921 were significantly thicker than those from 2012 (Chap. 2; Fig. 2.5). *Cavolina inflexa* shells collected in 1910 also had a higher average thickness than shells from 2012 although the difference was not statistically significant (Chap. 2; Fig. 2.5). For both species, the average shell weight of museum samples was significantly higher than modern samples. Museum samples of *C. inflexa* were significantly denser than modern samples but no differences in aragonite ultrastructure were observed in either species (Chap. 2; Fig. 2.7). Similar results were observed by Roger et al. (2011) who reported thinner shells and increased $CaCO_3$ porosity with a decrease in Ω_a from 4 (± 0.15) to 3.5 (± 0.15) between the years 1980 and 2009.

The results suggest that changes in pH and temperature experienced over the last century have affected the shell thickness and density of two Mediterranean pteropod species. Experimental work has unanimously demonstrated that declining Ω_a negatively impacts pteropod calcification (Orr et al. 2005, Fabry et al. 2008, Comeau et al. 2009, Comeau et al. 2010a, Comeau et al. 2010b, Lischka et al. 2011, Bednaršek et al. 2012, Comeau et al. 2012a, Lischka & Riebesell 2012), although many of these studies were conducted at Ω_a well below those anticipated in future scenarios for the Mediterranean Sea. At present, there have been no experiments that have

investigated the effects of both warming and ocean acidification on Mediterranean species and it is therefore unclear whether the differences in shell thickness observed in this study were caused solely by ocean acidification or by the combined effect of multiple stressors. It has been demonstrated that the combined stress of increased temperature and lowered pH have synergistic effects on depressing calcification in *Limacina helicina* (Lischka & Riebesell 2012). However, no synergistic effect of temperature and pH on *L. helicina* calcification rate was observed by Comeau et al. (2010b), raising questions as to whether the results may have been affected by differences in methodology. As *L. helicina* is a polar species it is logical that increases in temperature are problematic, by contrast, many of the species present in the Mediterranean are warm water species also found in tropical waters so the effects might not be as severe. Local scale differences in parameters such as total dissolved carbon (C_T), salinity and resource availability could also have affected shell properties. Geochemical comparisons of modern and museum samples are planned to clarify the hydrological conditions experienced during the lifetime of the animal and quantify the influence of potentially confounding factors.

The differences in pteropod shell thickness observed alongside a decrease of 0.6 in Ω_a over the last 100 years suggests that the projected conditions for 2100 will further depress calcification. To test the response of Mediterranean pteropods to future conditions, perturbation experiments were undertaken on *Limacina inflata.* Two treatments were used, control pH, which represents the current conditions in the western Mediterranean and low pH, reflecting the projected conditions for 2100 (Table 6.1). Individuals of *L. inflata* (n =20) were incubated for 3 days with the radiotracer ^{45}Ca to measure calcification.

Table 6.1: Summary of the conditions in the two treatments

Treatment	**pH_T**	**pCO_2 (µatm)**	**HCO_3^- (µmol kg^{-1})**	**CO_3^{2-} (µmol kg^{-1})**	**DIC (µmol kg^{-1})**	**Alk (µmol kg^{-1})**	**Ω_a**
Control	8.08	404.3	2088.6	190.1	2294	2555.9	2.85
Low	7.92	619.1	2208.2	138.8	2370.4	2549.6	2.08

Individuals incubated in the low pH treatment exhibited a 50% decrease in gross calcification when exposed to waters of $\Omega_a = 2$ (Fig. 6.2), indicating that even this slight decrease in Ω_a could have repercussions for shell growth. There are very few measurements of calcification in Mediterranean pteropod species making comparisons with other studies somewhat difficult, as most previous work used polar species and were conducted at much lower Ω_a. Comeau et al. (2012b) provided the only other values for a Mediterranean species, *Creseis acicula*, and reported a 30% decrease in calcification with a decrease in Ω_a from 3.3 to 2.0. This decline of calcification is smaller than the one observed in the present study. A study with the polar limacinid *Limacina helicina*, found a decrease in calcification by a similar percentage as was observed in *L. inflata*. Saturation state was decreased by 0.77 (from 1.81 to 1.04) resulting in a 40% decrease in gross calcification. As calcification by *L. helicina* was demonstrated to decrease linearly with decreasing Ω, these results should be comparable, despite the absolute Ω being lower than those used in this study (Comeau et al. 2010b).

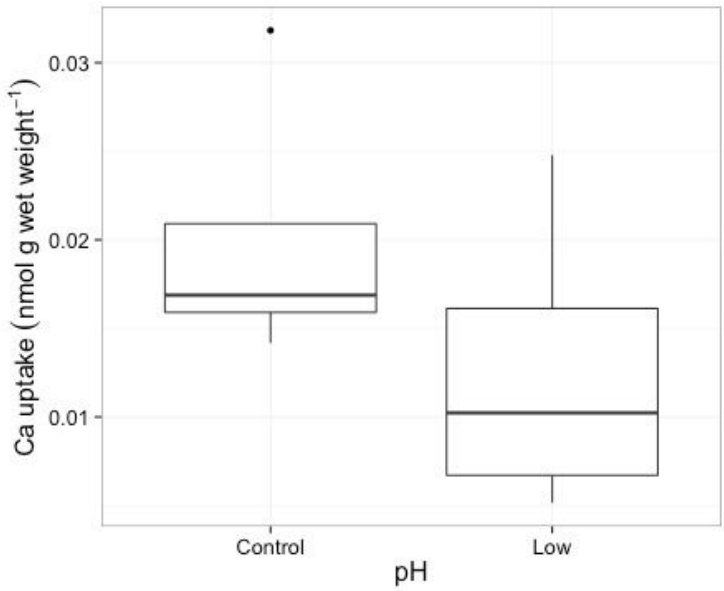

Fig 6.2 Incorporation of ^{45}Ca (nmol g wet weight^{-1}) into shells of *L. inflata* at two different pH levels. Specimens were incubated with radiotracer spike for 3 days at Ω_a 2.8 (Control) and Ω_a 2 (Low)

Abundance:

Analysis of the 37-year pteropod time series in the north western Mediterranean found no negative effect of potential thinning of the shells on pteropod abundances (Chap. 3). Populations appear to be more sensitive to temperature than pH and exhibit a trend of increasing abundance and an extension of annual periods of peak abundances, concurrent with higher annual temperatures. Synchronous oscillations of temperature and pteropod abundance can be observed on decadal timescales; periods of higher than average pteropod abundances coincide with higher temperatures (Fig 6.3; Chap. 3; Fig. 3.6). The North Atlantic Oscillation (NAO) is known to exert a strong effect on the north western Mediterranean environment and plankton communities (Molinero et al. 2005) and the observed oscillations in pteropod abundance occur at roughly the same intervals as the quasi-decadal oscillation of the NAO (Deser & Blackmon 1993). Similar effects of NAO influence can be seen in the regime shifts of zooplankton communities in a wider range of geographical locations (Conversi et al. 2010).

Analyses of other pteropod time series have produced similar results. No effect of decreasing pH was found on pteropod abundance in the NE Pacific Ocean (Mackas & Galbraith 2011), the Central and Southern California Current System (Ohman et al. 2009) and the Antarctic Peninsula (Loeb & Santora 2013). In theses studies there is also an indication of temperature sensitivities with cosmopolitan species generally increasing in numbers, concurrent with a rise in temperature and no change or a decrease in polar species with more limited tolerance. A recent analysis of continuous plankton recorder data from the North Sea is the only such study that has found a negative trend in pteropod abundances (Beare et al. 2013).

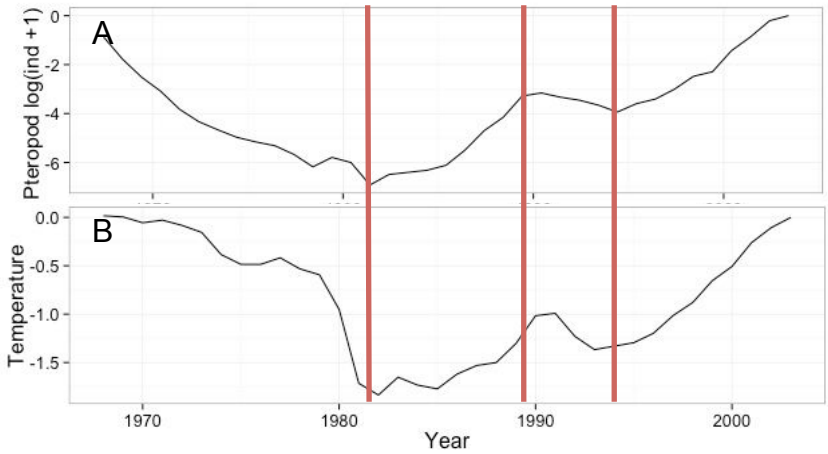

Fig 6.3. Cumulative sum of differences to the mean (CUMSUM) of annual temperature anomalies (˚C) and CUMSUM of annual anomalies of pteropod abundance (log10 (ind +1)). Red lines denote turning points in the pteropod times series.

Many of the dominant pteropod species found in the Mediterranean are cosmopolitan species that are also found in tropical waters and, at present, are living within the range of their recorded maxima of temperature tolerance. The level of temperature increase experienced in this area may not yet have reached pjeus (getting worse) thresholds for these species and, instead, may cause above average abundances by promoting faster growth and development through juvenile stages. This minimises the time spent in the vulnerable juvenile stages, enabling a higher number of individuals to become reproductive adults and could potentially explain the increased abundances in warm periods (Gillooly et al. 2002, Forster et al. 2011).

Perturbation experiments with pteropods

As well as quantifying what effects recent climate change has already had on pteropods, there is obvious interest from both the scientific community and society to predict accurately how they will respond to future conditions and what the consequences will be for higher trophic levels. In this section the methodological limitations of undertaking perturbation experiments with Mediterranean pteropod species are discussed.

Pteropod laboratory culture

One of the most common methods used to predict the response of an organism to future conditions is to undertake laboratory-based perturbation experiments. However, experimental work with Mediterranean pteropods represents a huge challenge for several reasons. In polar and sub-polar regions, pteropod abundance is typically very high, they can dominate the zooplankton biomass with abundances of 1000 -10,000 ind. m^{-3} (Fabry et al. 2008). In the Mediterranean, abundances are much lower, the highest recorded during the 37-year time series at Point B was 903 ind. m^{-3} but the onset of these short periods of high abundances are unpredictable and numbers very quickly return to background levels, averaging ~ 14 ind. m^{-3}, less than 5% of the zooplankton biomass. Populations are also highly seasonal and extremely patchy in distribution, making it difficult to obtain sufficient numbers of healthy individuals for experimental work.

Pteropods are also notoriously difficult to maintain in laboratory cultures, rendering long-term observations on multiple generations almost impossible. For some species, it is even difficult to allow an acclimatization period prior to commencing a perturbation experiment. This means it is also difficult to disentangle the effects of maintenance stress from treatment variables on pteropod mortality rates. The major issues are related to buoyancy and feeding. The weight of the shell causes the animals to sink relatively quickly and its fragile nature means that repeated impacts with the bottom or sides of culture vessels can cause damage. The lack of buoyancy impedes natural feeding behaviour. In the wild, pteropods feed by deploying a large mucous web. Once the web is deployed, it is held between the parapodia, which requires the animal to stop swimming (Gilmer 1972). The depth of the water column in many laboratory culture vessels does not provide sufficient time and space to deploy the feeding web. There is evidence that food deprivation can modulate the effects of pCO_2 in the polar pteropod, *Limacina helicina antarctica*, (Seibel et al. 2012). If this is the case for other species, it could have serious implications for future ocean acidification perturbation experiments.

In an attempt to improve future experimental work, many different culturing techniques were tested with several Mediterranean species (Chap. 4). From this work it is clear that certain species

are easier to maintain than others. *Limacina inflata* can be maintained in relatively small volumes of water (2 L) with low mortality rates (< 25 % wk^{-1}). After 7 days in culture, individuals with full guts were observed, indicating that they had fed during their incubation (Fig. 6.4). Larger species with more complex shell and mantle structures proved more problematic. *Cavolinia inflexa* could not be cultured past juvenile stages with mortality setting in after approximately 14 days. *Creseis acicula,* the other dominant Cavolinid species in the northwestern Mediterranean, experienced similar issues with 100% mortality after several days (Chap. 4; Table 4.2 and 4.3).

Fig 6.4: Cultured *L. inflata,* pictured after 7 days with phytoplankton present in guts (arrows).

The assessment of methods highlighted several critical factors for successful pteropod culturing. Perhaps the most important consideration is the collection of wild specimens; sampling must be carried out with great care to avoid damaging the fragile shell and mantle appendages. There is a high degree of mortality associated with even the most successful culture techniques so large and healthy starter cultures are preferable. Survival rates are greatly improved with increased water quality. Bacterial infestations can be minimised by using 2 µm-filtered seawater and antibiotics. In more polluted areas, survival rates can be improved by adding chelating agents to filtered seawater to bind trace metals. Vigorous water movements and bubbling must be avoided as this

can cause impacts with the culture vessel and forces the animals to swim continuously against the currents produced. Large water volumes are generally better for maintaining healthy cultures, although this can make it more difficult to attain the desired water chemistry for perturbation experiments.

Several promising methods have been identified. All life stages of *Limacina retroversa* could be maintained for 40 days in 77,000 L KOSMOS mesocosms (Riebesell et al. submitted). 1000 L tubes were also successfully used to culture *L. retroversa* through two complete life cycles (Howes et al. 2014). The temperature gradient kreisel has also proved to be suitable for maintaining cultures of *C. inflexa* and *Creseis virgula*, both of which were observed feeding with mucous webs (Howes et al. 2014). However, due to the temperature gradient that is used to produce water movement, this method is not suitable for use in ocean acidification perturbation experiments. It is the recommendation of this thesis that future ocean acidification investigations use the methods outlined in chapter 4 to minimise confounding results caused by culture stress (see also Howes et al. 2014).

Implications for the future of Pteropods in the Mediterranean

Despite the paucity of perturbation experiments with Mediterranean pteropods, it is possible to postulate some potential stressors that they may face in a high-CO_2 world, based on the results from this thesis and previous experimental work.

Reduced shell thickness

In this study it has been shown that, despite waters maintaining super-saturation with respect to aragonite, even slight changes in Ω_a have an impact on pteropod calcification rates, causing thinner shells and decreased density. A significant decrease in the rate of calcification was also observed for *L. inflata* when exposed to conditions projected for the end of the century. It is

unlikely that conditions in the Mediterranean will cause shell dissolution or impair growth completely but, based on the results of this thesis, it is likely that shells may become thinner and less dense under lowered Ω_a. In the present study it was not possible to observe any change in the packing or morphology of the aragonite rods (Chap. 2; Fig. 2.7), despite a significant change in density, probably because changes in ultrastructure are undetectable at elevated Ω_a. However, work with corals has demonstrated that small shifts in saturation state can have effects on aragonite ultrastructure (Cohen et al. 2009). The experimental values at which changes were observed are well within range of those predicted for 2100 in the Mediterranean suggesting that, under future conditions, the ultrastructure of pteropod shells may be compromised.

The complex ultrastructure of pteropod shells lends flexibility and hardness whilst maintaining a light weight (Zhang et al. 2011). Pteropod shells serve as protection from predators and parasites, they are important for buoyancy control and their weight is used to facilitate rapid sinking as an escape response when threatened (Lalli & Gilmer 1989). Laboratory reared individuals have been observed to survive without a shell (Comeau et al. 2010a). However, given the variety of its uses, it is unlikely that a reduction in shell function due to thinning or changes to ultrastructure and density would be without detrimental effects in the wild. Thinner and lighter shells may increase predation risks on two fronts: by reducing mechanical strength and slowing sinking capacity, which used as an escape response.

Thermal stress

The results from the analysis time series of pteropod abundance revealed sensitivity to temperature fluctuations. The analysis included species with tropical to sub-tropical distributions that are found in waters up to 28˚C (Van Der Spoel 1967). The highest SST observed in the time series was 27˚C (Chap. 3; Fig. 3.3) so an increase of 2 to 2.5˚C (Lazzari et al. 2013) produces maxima in SSTs that exceed the upper tolerance limit for these species (Van Der Spoel 1967). As these values represent surface maxima, for most of the year the entire water column will be within the optimum temperature window for these species. However highest temperatures will occur during late summer and early autumn, coinciding with the breeding seasons of *C. acicula*

and *L. inflata* and the presence of juveniles in surface waters (Rampal 1975). As juvenile stages are often more sensitive than adults (Kurihara 2008, Pineda et al. 2012), it could be that thermal stress has a disproportionately severe impact on recruitment. The decadal changes in temperatures in the Mediterranean are also strongly affected by the NAO (Molinero et al. 2005). If increased SSTs coincide with NAO-induced high temperatures, there is the potential to push already stressed populations over the limits of their tolerances (Beaugrand et al. 2008).

There are also temperate pteropod species found in the Mediterranean such as *Limacina retroversa*, *Styliola subula* and *Creseis virgula* (Van Der Spoel 1967, Rampal 1975) that may not be able to adapt to the higher temperatures. The enclosed nature of the Mediterranean leaves little possibility for migration to more favourable conditions and it seems likely that species that are unable to tolerate higher temperatures will be outcompeted by those that can (Coll et al. 2010). Temperature shifts have been observed to cause changes in the species compositions of pteropod communities (Herman 1971, Almogi-Labin et al. 1991, Bauerfeind et al. 2014) and it is probable that increasing temperatures will decrease pteropod diversity in the Mediterranean.

It is likely that thermal stress will have synergistic effects with pH on Mediterranean pteropods. This interaction can be explained by oxygen and capacity-limited thermal tolerance (OCCLT; Pörtner 2002, Pörtner 2010). Metabolic capacity is limited by insufficient oxygen supply at either extreme of an animal's temperature tolerance, creating an optimum thermal window (Pörtner 2002). Aerobic capacity and metabolic partitioning of an animal depends on environmental variables such as temperature and pH. Hypercapnia has negative effects on basic fitness related functions such as acid/base regulation, reproduction and calcification rates, which also limit aerobic scope (see Pörtner 2010). This increases the oxygen demand required to perform basic fitness processes and, as a result, multiple stressors act synergistically to reduce the thermal tolerance window. Conversely, tolerance to other stressors such as pH can diminish thermal stress (Metzger et al. 2007, Pörtner 2010). A study comparing synergistc effects of ocean acidification and temperature on *Limacina retroversa* and *L. helicina* by Lischka and Riebesell (2012) demonstrated this effect. Both species are found in polar waters but, of the two, *Limacina retroversa* has a broader geographic range and a higher thermal tolerance (Van Der Spoel 1967).

Statistically significant interactions between temperature and pH effect were only found for the less thermally tolerant *L. helicina.* Comeau et al. (2010b) also found synergistic effects of temperature and pCO_2 on *L. helicina*. At elevated temperatures, respiration increased as a function of increasing pCO_2. The projection that increases in SST may push many Mediterranean species to the edge of their thermal limits suggests that the effects could be exacerbated by decreasing pH and vice versa.

Trophic interactions

Gut content analysis has revealed pteropods to be omnivorous and generalist (Lalli & Gilmer 1989, Gilmer & Harbison 1991). Given their broad range of prey items, it seems unlikely that the changes in community composition induced by climate change will affect pteropod diets. It is not known whether their specialised mechanism of feeding may be impaired by ocean acidification or elevated temperatures. There have been no studies to investigate whether production of mucous webs, or the structure of the webs themselves, will be impaired under these conditions.

Climate change may affect the abundance of pteropod predators. Research on the effects of ocean acidification on finfish is relatively scarce but it has been observed that acidification has negative effects on prey detection (Cripps et al. 2011), predator avoidance (Dixson et al. 2010), juvenile survival (Franke & Clemmesen 2011), homing ability and olfactory discrimination (Munday et al. 2009). Warming has also been suggested to have negative consequences for fish (Pörtner et al. 2001, Pörtner & Knust 2007, Pörtner & Peck 2010). When this evidence is considered alongside the rates of over-fishing in the Mediterranean (Tudela 2004), it is likely that pteropods will not face increased fish predation as a result of climate change. Jellyfish are also predators of pteropods and it has been suggested that they are one of the groups that may thrive in future oceans (Richardson et al. 2009). Temperature increase has been shown to be favourable for growth and reproduction of some species (Purcell et al. 2007, Purcell et al. 2012). Increased stratification can produce more favourable feeding conditions (Cushing 1989) and over-fishing can remove predation pressure (Bakun & Weeks 2006). Studies on ocean acidification effects on

jellyfish are rare but current research indicates that jellyfish are tolerant to acidification (Winans & Purcell 2010, Klein et al. 2014). However, there is a lack of consensus in the scientific community as to whether jellyfish will thrive under future conditions (Condon et al. 2012). It is difficult to assess population dynamics, as data on historical population baselines is lacking (Condon et al. 2012). It has also been suggested that perceived increases in numbers may be highly localized and not representative of the wider area (Graham et al. 2001, Dawson & Hamner 2009).

There is potential for predation pressure on pteropods to increase in the future Mediterranean Sea, although it is impossible to make accurate predictions of complicated trophic interactions. There is also the possibility that ocean acidification could affect the escape response of pteropods. Several studies have shown mollusc escape responses to be impaired under lowered pH (Schalkhausser et al. 2013, Watson et al. 2014).

Proxy calibration in planktonic foraminifers

Mediterranean foraminifers are anticipated to be more robust to the effects of ocean acidification than pteropods due to the lower solubility of their calcite tests (Mucci 1983). The excellent preservation of foraminifera tests in highly saturated areas, such as the Mediterranean, makes them an excellent candidate for use as geochemical proxy carriers.

The oceanic carbonate chemistry has experienced considerable changes in Earth history (Zeebe 2012). Boron proxies can be used to analyse the evolution of the ocean carbon cycle and carbon perturbations over the last 14 Myr (the residency time of oceanic boron, Lemarchand et al. 2000). Although boron isotopic fractionation ($\delta^{11}B$) has been applied to make reconstructions of pH (Sanyal et al. 1996, Sanyal et al. 2001, Hönisch et al. 2004, Rae et al. 2011) it was previously unknown whether other, co-varying, changes in carbonate parameters have an additional impact and potentially affect the accuracy of the reconstruction. With a reliable pH proxy and an

independent proxy for one of the other carbonate system parameters (plus estimates for temperature and salinity), the full carbonate system can be completely constrained with a proxy for one other parameter, plus temperature and salinity values. The potential of the B/Ca ratio of foraminiferal calcite to provide this proxy has been widely recognized, although the application has been hampered by disagreements over the parameter of the carbonate system that exerts the major control on boron incorporation in foraminiferal calcite (Allen & Honisch 2012).

B/Ca ratios

Orbulina universa tests precipitated under decoupled carbonate chemistry revealed strong inverse correlation of the B/Ca ratio with the concentration of total inorganic carbon (C_T) suggesting that $B(OH)_4^-$ competes with carbonate for inclusion into the calcite lattice (Chap. 5; Fig. 5.1A). At constant pH, the B/Ca ratio is strongly correlated to carbonate ion (CO_3^{2-}) concentration, however at varying pH_T and constant [CO_3^{2-}] the B/Ca ratio does not remain constant (Chap. 5; Fig. 5.1E). When the B/Ca ratios are plotted against [CO_2], the relationship is similar to that of C_T (Chap. 5; Fig. 5.1C), however only <1% of C_T is in the form of CO_2 so this species is unlikely to have a major control on boron incorporation. The remaining >99% is ~10% CO_3^{2-} and ~ 90% HCO_3^- (Zeebe & Wolf-Gladrow 2001). The strongest correlations are observed between the B/Ca ratio and bicarbonate ion (HCO_3^-) concentration and the B/Ca ratio and the ratio of $B(OH)_4^-$ to [HCO_3^-] (Chap. 5; Fig. 5.1G, H). No pH effect was observed on these relationships. These data suggest that the B/Ca ratio can be used as a robust proxy for [HCO_3^-] but not, as has been previously suggested, for [CO_3^{2-}] (Foster 2008).

The work of this thesis provides experimental proof by properly decoupling the effects of pH, [CO_3^{2-}] and [HCO_3^-], that the B/Ca ratio of *O. universa* is controlled by [HCO_3^-], this is agreement with the hypothesis of Hemming and Hanson (1992), core top analyses (Yu et al. 2007) and culture work with another planktonic foraminifer, *Globigerinoides sacculifer* (Allen et al. 2012).

Boron isotopic fractionation

No variation in $\delta^{11}B$ was observed in the results of the experiments where pH was constant and $[CO_3^{2-}]$ and $[HCO_3^-]$ were varied (Chap. 5; Fig.5.2B). The results of experiments using varying pH and constant $[CO_3^{2-}]$ reveal an increasing trend when $\delta^{11}B$ is plotted against pH_T that is within range of the calibration curve produced for the same species by Sanyal et al. (1996). The slope of the trend is slightly steeper than the one produced by Sanyal et al. (1996) in their calibration study using the same species. It is unclear why but may be an artefact of the unnaturally high C_T and A_T used to decouple the carbonate system in our experiment. It has been observed that inconsistencies can exist between different measurement techniques (Tonarini et al. 2009), potentially affecting the steepness of the trend. Most studies of $\delta^{11}B$ in foraminifera (Fig 6.5) used thermal ionisation mass spectrometry (TIMS), except Rollion-Bard and Erez (2010), who used secondary ion mass spectrometry (SIMS). The measurements for this thesis were made using multi-collector laser ablation inductively coupled plasma mass spectrometry (MC-LA-ICPMS). Henehan et al. (2013) also used MC-LA-ICPMS but the trend is still shallower than the one produced by our study. However, for their measurements of $\delta^{11}B$ in seawater, they used column chemistry to remove the calcium matrix whereas the microsublimation technique was used in our study. Hence, differences might still be attributable to the different analytical methods used. This is an important topic recognized by the “boron community” and efforts are being undertaken to solve these problems.

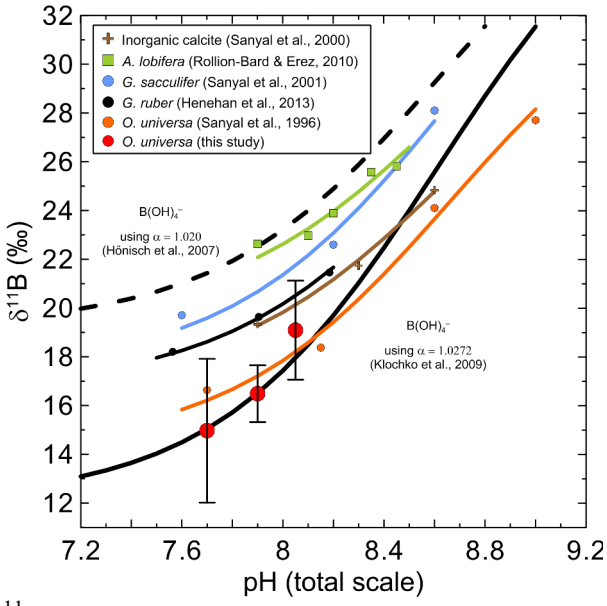

Fig 6.5: (A) Average $\delta^{11}B$ of $B(OH)_4^-$ in cultured *O. universa* calcite (red circles), solid black line shows empirical values for artificial seawater with a fractionation factor of ${}^{11\text{-}10}K_B = 1.0272$ (Klochko et al. 2006) at T=23°C and S=38. Dashed black line shows empirical values for artificial seawater with a fractionation factor of ${}^{11\text{-}10}K_B = 1.020$ (Klochko et al. 2006) at T=23°C and S=38.

The difference in slope could also be attributed to the boron fractionation constant (α). It is still unclear what the correct fractionation constant for boron in calcite is. Originally α = 1.0194, taken from the theoretical work of Kakihana et al. (1977), was used. The observation that the $\delta^{11}B$ of carbonates measured in calibration experiments produced curves that are depleted in ${}^{11}B$ compared to the "Kakihana curve" opened a debate on the possible reasons for these differences (Pagani et al. 2005, Hönisch et al. 2007, Pagani & Spivack 2007). Hönisch et al. (2007) argued that the slope of empirically produced curves matched that of the Kakihana curve and that, therefore, the offset was a species-specific vital effect. Experimental measurements of artificial seawater produced an α differing from the theoretical studies of 1.0272 (Klochko et al. 2006). The data points produced in our study fall almost exactly on the curve produced by Klochko et al. (2006) for fractionation of artificial seawater, α = 1.027 (Fig 6.5), suggesting that the vital effects imposed by foraminifera on the $\delta^{11}B$ could be less significant than originally thought.

Proxy application

This study confirms the robustness of $\delta^{11}B$ as an independent pH proxy and, for the first time, confirms the growing body of circumstantial evidence that B/Ca in planktonic foraminiferal calcite may be used as an independent proxy for [HCO_3^{2-}] (Yu et al. 2007, Allen et al. 2012), thereby allowing researchers to fully constrain the carbonate system. The application of these two proxies can provide vital information on natural carbon cycles and their variation within the last 14 Myr, thus providing context and scale for the current carbon perturbation.

Perspectives

Analysing past carbon perturbation events can provide valuable information on the potential consequences of the man-made one facing the planet today, although comparisons are limited by differences between environmental conditions and the rate of carbon release (Zeebe 2012). The PETM is considered to be the best analogue of the modern situation, given isotope proxies that infer warming of 5-8˚C (Zachos et al. 2003) and a 0.25 - 0.45 decrease in surface pH (Hönisch et al. 2012), causing the calcite compensation depth to shoal (CCD) by approximately 2 km and Ω_a to decrease between 1.5 - 2 units (Ridgwell & Schmidt 2010). This induced pole-ward migrations in warm water species and exerted high evolutionary pressure on calcifying plankton (Knoll & Fischer 2011, Hönisch et al. 2012). Benthic foraminifera suffered a 30-50% loss during this period (Thomas et al. 1990) but, conversely, planktonic foraminifera diversified (Petrizzo 2007). At this point in the fossil record it has been noted that pteropod fossils are very rare, probably reflecting the lowered Ω_a (Hart 2012). Despite reductions in calcifying taxa, there was no mass extinction event during the PETM (Knoll & Fischer 2011, Hart 2012, Hönisch et al. 2012). Five mass extinctions can be identified during the Phanerozoic Eon and at least one of the 3 main symptoms of carbon perturbation (warming, expansion of suboxic zones and ocean acidification) can be identified in all five of these events (Veron 2008, Bijma et al. 2013). All three of these symptoms can now be seen in modern oceans, indicating the potentially severe consequences of anthropogenic carbon perturbation.

With the potential for such severe and long lasting repercussions, the continued study of anthropogenic carbon perturbation is vital. Work on developing the B/Ca ratio/[HCO_3^-] proxy should continue with species specific, empirical calibrations undertaken at environmentally relevant [HCO_3^-]. The proxy should then be verified with core-top calibrations before being applied to paleo-reconstructions. In order to refine foraminiferal proxies further, it is essential to advance our understanding of the mechanisms involved in the construction of their tests, which contribute to the vital effects on element and isotope incorporation. It is clear from the complex shape and structure of the shells, as well as the fractionation effect on isotopes and trace metals, that biomineralization is tightly controlled (Erez 2003, Nehrke et al. 2013), however many of the processes involved remain unclear. There are conflicting theories on how the ions used for calcification enter the cell, either by vacuolization (Bentov et al. 2009) or trans-membrane transport (Nehrke et al. 2013). To investigate the issue further, culture work with foraminifera grown under differing trace metal concentrations and with fluorescent and isotopic tracers should be undertaken and analysed in conjunction with transmission electron microscopy and confocal microscopy.

Considering the ecological and biogeochemical significance of planktonic foraminifera, there has been relatively little investigation to examine the biological implications of climate change on these organisms and their observed response to ocean acidification is mixed (summarised by Keul et al. 2013). The continuation of perturbation experiments to confirm whether the inconsistencies in response are species-specific tolerances or methodological artefacts is required. There is also very little known about the effects of multiple stressors on foraminifera. This is an essential to consideration as the carbon perturbation will bring, not only the trio of warming, suboxia and ocean acidification but also freshening in polar areas, hyper-salination of warm seas and alterations in nutrient availability (Bijma et al. 2013). Emerging research suggests that, in some species of foraminifers, additional stressors such as elevated temperature can modulate the response to ocean acidification (Manno et al. 2012a) and some evidence also suggests other environmental factors such as local nutrient availability may have a stronger influence on foraminiferal shell weight than [CO_3^{2-}] (De Villiers 2004). In order to make reliable assessments

of future effects on foraminifera, it is essential to gather more information on their ecophysiological responses. Valuable information can also be extracted from paleoceanographic time series such as the study by Field et al. (2006) and the excellent preservation of foraminifera in Mediterranean sediments makes this area a good study site.

More is known about the response of pteropods to ocean acidification but there are still significant knowledge gaps. Only one study has assessed the impacts on larval stages (Comeau et al. 2010a), as juvenile stages are thought to be particularly sensitive to ocean acidification (Kurihara 2008), and further work should be undertaken. During this thesis, experimental work exposed pteropod eggs to increased temperatures and decreased pH, the results have not been included due to time restraints but will be analysed in due course. Work was also carried out to compare the ecophysiological and molecular responses of *L. inflata* to ocean acidification. RNA illumina sequencing was used to examine differential gene expression under acute exposure to lowered pH. Due to contamination of samples with non-pteropod RNA, presumably from food items, the bioinformatics analysis was much longer and more difficult than originally anticipated and the results were not ready in time to be included in this thesis. However, work to identify and extract pteropod sequences is on-going and, if successful, this will be the first dataset of its kind on thecosome pteropods.

Like foraminifera, more insight is needed on how pteropods will respond to multiple stressors. A few studies have addressed the effects of temperature and ocean acidification and found mixed responses (Comeau et al. 2010b, Lischka et al. 2011, Lischka & Riebesell 2012) and only one study has assessed the combined effects of ocean acidification and freshening (Manno et al. 2012b). The response of calcification rate in multi-stressor situations are consistent between studies but respiration and mortality are more confused and require further investigation and clarification. Several recent works have used mechanical techniques to measure the strength of pteropod shells (Zhang et al. 2011, Teniswood et al. 2013), this method has great potential for assessing the implications of shell thinning or dissolution as a result of ocean acidification. The exact site of calcification is also unknown for pteropods. It is assumed that it occurs at the mantle edge, as for other molluscs (Van Der Spoel 1967), but it is not clear if it is an intra or

extracellular process, if it occurs in a "privileged space" similar to foraminifera or if pH at the site of calcification can be altered as has been observed for some species of foraminifera (De Nooijer et al. 2009). Work on juveniles using florescent markers and confocal microscopy offers a good opportunity to resolve some of these questions.

There has been relatively little work undertaken on the geochemistry of pteropod shells (Fischer et al. 1999 and refs. therein, Juranek et al. 2003). This is most likely due to a combination of factors. Problems in culturing have prevented empirical proxy calibrations from being undertaken. Furthermore, their aragonitic shells are more susceptible to dissolution in sediments, limiting the number of potential study sites. However, in tropical and subtropical regions, pteropod ooze can constitute a high percentage of the sediment (Lalli & Gilmer 1989). In these areas, the use of pteropods for geochemical proxies offer several advantages; their shells are usually larger than those of foraminifera and they are longer lived (~ 1 year). This means that the conditions recorded in the entire shell reflect an annual average (Fischer et al. 1999) and, in some cases, it is possible to subsample individual shells to attain seasonal values (e.g. Kalberer et al. 1993). Several studies have also indicated that there is very little offset between pteropod aragonite and seawater isotopic values (Grossman et al. 1986, Grossman & Ku 1986, Almogi-Labin et al. 1991, Fabry & Deuser 1992). One of the original aims in this study was to measure the geochemistry of modern and museum samples for pteropod shells but, due to logistical problems with laboratory facilities, this has not yet been possible. However measurements will be carried out in the near future, as it would be a valuable contribution to the topic.

Conclusions

Considering the results of this work and others, it seems unlikely that ocean acidification alone will have significant effects on pteropods and planktonic foraminifers in the Mediterranean Sea. However, when considered amongst the numerous other stressors acting on this ecosystem, it will likely have an additive effect in reducing overall fitness. Planktonic foraminifera are likely to be less affected by the potential changes than pteropods as their low Mg calcite tests are 50% less

soluble than the aragonite shells of pteropods (Mucci 1983). Tropical and sub-tropical species of foraminifers, including those found in the Mediterranean, have been shown to have increased in abundance in parallel with global warming (Field et al. 2006). Neverless, this does not guarantee that thermal limits will not be reached in the near future. Pteropods are more sensitive to changes in Ω_a and increasing temperature may compound this. Still, by 2100, the Mediterranean is likely to be one of the more favourable habitats for pteropods especially considering the time scales projected for aragonite undersaturation to occur in other regions. The Arctic and Southern Oceans are projected to be undersaturated with respect to aragonite by as early as 2016 and 2050, respectively (Orr et al. 2005, Steinacher et al. 2009).

Nonetheless, conditions in the Mediterranean will certainly be less favourable for pteropods and foraminifera by 2100. It is also feasible that foraminifera will be producing lighter tests as a result of the changes to Ω (Bijma et al. 1999, Russell et al. 2004, Lombard et al. 2010, Manno et al. 2012a). This will have implications for biogeochemical cycling in the Mediterranean. Planktonic foraminifera have been estimated to contribute 0.008–0.033 Gt yr^{-1} $CaCO_3$ to the global carbon budget (Schiebel & Movellan 2012) and pteropods approximately 0.678 Gt yr^{-1} $CaCO_3$ (calculated from values given in Berner & Honjo 1981, Schiebel 2002). The sinking of pteropod faecal pellets and mucous structures also supplies carbon to the deep oceans and their shells can act as ballast, speeding the sinking of organic matter (Klaas & Archer 2002, Schiebel 2002). $CaCO_3$ precipitated in the surface oceans, increases atmospheric CO_2. Conversely, sinking of organic carbon decreases atmospheric CO_2. As pteropods are involved in both processes, it is difficult to predict the overall consequences of reductions in pteropod calcification on biogeochemical cycling. However, Zeebe (2012) calculates that the CO_2 produced by calcification is very little in comparison to the CO_2 taken up by primary producers that it helps to transfer to the ocean interior.

There may also be ecosystem alterations as a consequence of reduced pteropod abundances. They are important prey to many fish and reductions in pteropod populations may add to the stress already imposed by overfishing (Tudela 2004, The Mermex Group 2011). Pteropods are significant consumers of phytoplankton and, to a lesser extent, zooplankton (Gilmer & Harbison 1986, Hunt et al. 2008) reductions in pteropod grazing could cause increases in these groups

(Barry et al. 2011). The Mediterranean is under extreme anthropogenic pressure and it is likely that other stressors such as pollution, over-fishing and competition from invasive species will exacerbate the direct effects of climate change with the potential for drastic effects on biodiversity (The Mermex Group 2011). Compromised fitness of key Mediterranean species, such as pteropods and foraminifers, could have grave, cascading effects throughout the ecosystem (Barry et al. 2011). For an area with such high coastal populations and diverse groups of stakeholders, any loss of diversity could have severe socio-economic repercussions (Turley & Boot 2011, Rodrigues et al. 2013).

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Résumé

Etant des producteurs pélagiques majeurs d'aragonite et de calcite, il est essentiel d'évaluer l'effet qu'aura le changement climatique global sur les ptéropodes et les foraminifères. Cette étude a utilisé une approche interdisciplinaire pour évaluer les effets de l'acidification de l'océan sur des espèces Méditerranéennes. Une comparaison d'échantillons conservés et modernes de deux espèces de ptéropodes a été réalisée pour étudier les effets du pH sur les propriétés de la coquille. Des populations de ptéropodes ont également été analysées à l'aide des données de séries temporelles. Pour permettre l'amélioration de futures expériences de perturbation, une revue collaborative des techniques de culture de ptéropodes a été produite. Enfin, le foraminifère *O. universa* a été cultivé dans des conditions de pH et de $[CO_3^{2-}]$ découplés pour évaluer les effets des changements dans la chimie du carbonate sur la composition en bore et le fractionnement isotopique. Les coquilles des échantillons conservés de ptéropodes étaient plus épaisses que celles collectées en 2012 et les coquilles de *C. inflexa* recueillies en 1910 étaient nettement plus denses que celles de 2012, probablement en raison d'un effet de pH. Les abondances de ptéropodes ont montré une tendance croissante entre 1967-2003 et se sont révélées être influencées par les changements de température interannuelles, sans que des changements dans les propriétés des coquilles n'ai eu d'impacts négatifs. Lors de la dernière étude le pH était le seul paramètre du système de carbonate a affecter le δ11B de la calcite de *O. universa*. Le rapport B/Ca a diminué avec la diminution du $[CO_3^{2-}]$ à pH constant mais n'a pas montré de tendance cohérente avec une $[CO_3^{2-}]$ constant et un pH variable. Au lieu de cela, une étroite corrélation entre les rapports B / Ca et $[HCO_3^-]$ a été observée suggérant que le bore est contrôlé par la $[HCO_3^-]$, fournissant une proxy pour les reconstructions du système paléo-carbonate. Cette étude suggère que, dans la mer Méditerranée, les changements de taux de saturation en aragonite peuvent affecter la structure des coquilles de certaines espèces de ptéropodes, mais l'abondance est influencée par la température.

Abstract

As the major pelagic producers of aragonite and calcite, it is essential to evaluate what effect climate change will have on pteropods and foraminifers. This study used an interdisciplinary approach to assess the effects of ocean acidification on Mediterranean species. A comparison of museum and modern samples of two pteropod species investigated the effects of pH on shell properties. Pteropod populations were analysed using time series data. To improve future perturbation experiments, a collaborative review of pteropod culture techniques was produced. Finally, the foraminifer *O. universa* was cultured under decoupled pH and $[CO_3^{2-}]$ to assess the effects of changes in the carbonate chemistry on boron incorporation and isotope fractionation. Museum pteropod samples were thicker than shells from 2012 and *C. inflexa* shells collected in 1910 were significantly denser than those from 2012, possibly due to a pH effect. Pteropod abundances displayed an increasing trend between 1967-2003 and are influenced by inter-annual temperature changes, with no sign of the observed changes in shell properties having had negative impacts. pH was the sole parameter of the carbonate system that affected the $\delta^{11}B$ of *O. universa* calcite. The B/Ca ratio decreased with decreasing $[CO_3^{2-}]$ at constant pH but did not show consistent trends at constant $[CO_3^{2-}]$ and varying pH. Instead, a close correlation of B/Ca ratios and $[HCO_3^-]$ was observed suggesting that boron is controlled by the $[HCO_3^-]$, providing a proxy for paleo-carbonate system reconstructions. This study suggests that, in the Mediterranean Sea, changes in Ω_a may affect shell structure of some pteropod species but abundance is influenced by temperature.