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Invited review

Shelled pteropods in peril: Assessing vulnerability in a high CO_2 ocean

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ABSTRACT

The impact of anthropogenic ocean acidification (OA) on marine ecosystems is a vital concern facing marine scientists and managers of ocean resources. Euthecosomatous pteropods (holoplanktonic gastropods) represent an excellent sentinel for indicating exposure to anthropogenic OA because of the sensitivity of their aragonite shells to the OA conditions less favorable for calcification. However, an integration of observations, experiments and modelling efforts is needed to make accurate predictions of how these organisms will respond to future changes to their environment. Our understanding of the underlying organismal biology and life history is far from complete and must be improved if we are to comprehend fully the responses of these organisms to the multitude of stressors in their environment beyond OA. This review considers the present state of research and understanding of euthecosomatous pteropod biology and ecology of these organisms and considers promising new laboratory methods, advances in instrumentation (such as molecular, trace elements, stable isotopes,

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palaeobiology alongside autonomous sampling platforms, CT scanning and high-quality video recording) and novel field-based approaches (i.e. studies of upwelling and CO_2 vent regions) that may allow us to improve our predictive capacity of their vulnerability and/or resilience. In addition to playing a critical ecological and biogeochemical role, pteropods can offer a significant value as an early-indicator of anthropogenic OA. This role as a sentinel species should be developed further to consolidate their potential use within marine environmental management policy making.

1. Introduction

Anthropogenic ocean acidification (OA) poses a serious, global threat to marine ecosystems and the services they provide. Its potential influence on marine fisheries has led to anthropogenic OA being increasingly appreciated as a pressing societal concern, and a top research priority (Rudd, 2014). Many international programmes and projects are now investigating the impacts of anthropogenic OA on marine biodiversity and its wider implications. The international advisory groups IOC-UNESCO and OSPAR-ICES recommended that future studies into marine ecosystems should focus on species that are particularly sensitive to anthropogenic OA since these species can indicate when and where the first impacts will occur.

Continuous uptake of about a third of anthropogenically produced carbon dioxide (CO_2) since 1750 (Le Quéré et al., 2015), combined with ocean warming and increased freshwater run-off in high latitude regions, are predicted to cause major changes in seawater chemistry. Anthropogenic carbon dioxide emissions induce OA, where pH and the concentration of carbonate ions decrease, resulting in shoaling of the Calcium Carbonate ($CaCO_3$) saturation horizon (Feely et al., 2009). Ocean pH has already declined by 0.1 unit since pre-industrial times and is predicted to fall another 0.3 unit by 2100 (Caldeira and Wickett, 2003). These changes are likely to affect marine calcifiers, in turn altering biodiversity, trophic interactions and large-scale marine biogeochemical processes (Fabry et al., 2008).

Euthecosomatous pteropods (holoplanktonic shelled marine gastropods) have been identified as candidates for indicating the onset of anthropogenic OA, since the shells of species in the vulnerable polar and sub-polar regions have been demonstrated to be particularly prone to dissolution (Comeau et al., 2010a; Lischka and Riebesell 2012). Euthecosomatous pteropods are a cosmopolitan group widely distributed in the world's oceans. They are otherwise known as “sea butterflies” because they flap their wing-like parapodia to “fly” through the water. Euthecosomatous shells are relatively small, ranging from less than a millimetre to a few centimetres in length (Lalli and Gilmer, 1989) and are made from aragonite, a metastable and relatively soluble form of biogenic $CaCO_3$ (Mucci, 1983). Pteropods are an important ecological as well as biogeochemical component of the marine ecosystem. They are a major food source of carnivorous zooplankton, commercially exploited fishes, such as cod, salmon, herring, mackerel, and a number of higher predators, such as seabirds, polar cod and whales (Lalli and Gilmer, 1989; Hunt et al., 2008; Falk-Petersen et al., 2001). From a biogeochemical point of view, pteropods play an important role in the direct export of organic carbon and carbonate to the deep ocean through the sinking of dead individuals (Manno et al., 2007; Tsurumi et al., 2005). The shells of dead individuals also act as ballast for sinking matter while the discarded mucus webs of these organisms facilitate the aggregation of particles, and both factors in combination facilitate carbon export and sequestration (Noji et al., 1997; Klaas and Archer, 2002)).

In order to use Euthecosomatous pteropods as biological indicators for anthropogenic OA monitoring and to inform policy makers about the impacts of OA on marine ecosystems, it is crucial to parameterize the relationship between expected seawater carbonate chemistry changes and the biological responses of pteropods, both at individual and population levels. A full understanding of the vulnerability of pteropods to anthropogenic OA requires the integration of observations, experiments and modelling. This demands a coordinated effort from a wide range of disciplines throughout the international scientific community.

In June 2015, an international workshop was held at the British Antarctic Survey in Cambridge to consider the “Response of shelled pteropods to OA”. It was attended by scientists from biomechanical engineering, ocean carbonate chemistry, geology, palaeontology, biogeochemistry, taxonomy, physiology and ecology. The aim of the workshop was to consolidate progress made so far in pteropod research and to identify knowledge gaps and future research needs.

Here, we present the outcomes from the workshop which consider

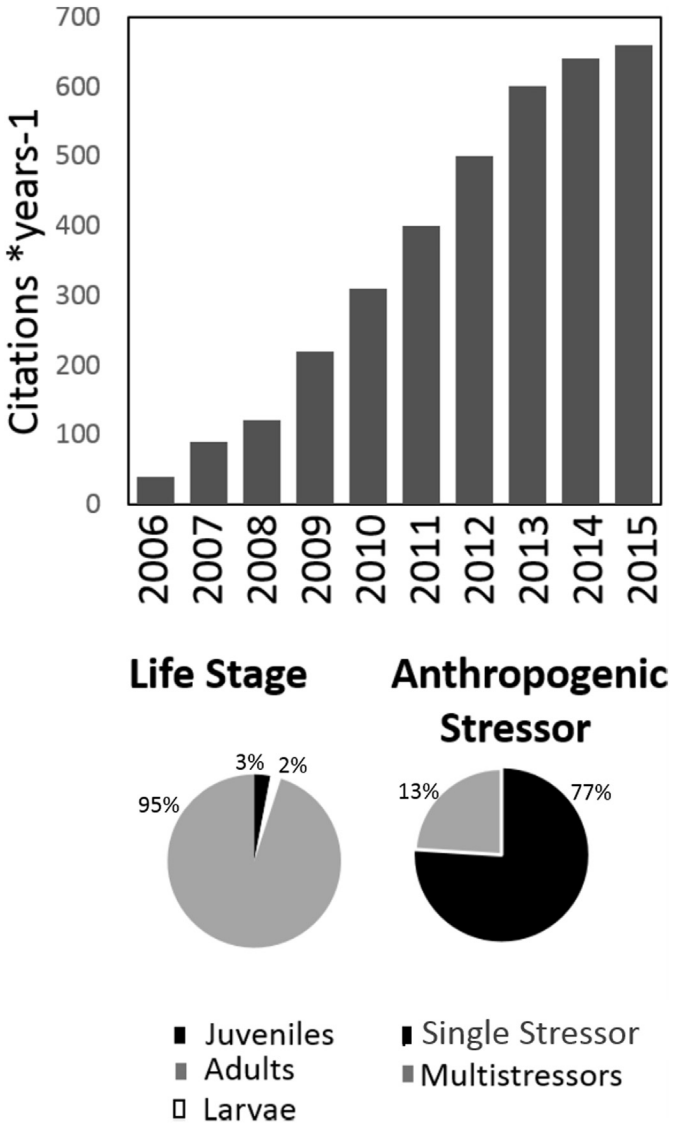

Fig. 1. On the top: histograms show the increase in citations each year of peer-review publications focusing on pteropods and ocean acidification; on the bottom: pie charts highlights the focus of publications (as %) on life stage and on ocean acidification as a single stressor, while the citation records demonstrates larvae/juvenile stages and multistressor scenarios are not adequate addressed.

the present state of pteropod knowledge and gaps (Fig. 1) and provide recommendations for future research and monitoring. In the first section (*Present state of knowledge and gaps*), we consider our present understanding of biomass distribution, species boundaries, synergistic impacts of multistressors, life cycles, energy budgets and early-stage development. In the second section (*Technological advancements*), we identify promising new methods, such as molecular, trace elemental and stable isotopic measurements, computed tomography scanning, high-speed video imaging and active multifrequency acoustics, which will provide new perspectives on the biology of pteropods, as well as fill knowledge gaps that currently exist. In the final section, we identify major needs in pteropod research and how they can be addressed to improve our understanding of pteropod vulnerability to anthropogenic OA. Finally we discuss how to provide stakeholder with info they can potentially use as tools for policy-making and management.

2. Present state of knowledge and gaps in the pteropod research

2.1. The carbonate chemistry environment

Assessing the impact of anthropogenic OA on pteropod biology and ecology relies on accurate characterization and assessment of trends in marine carbonate chemistry. Given the long-term mean trend in surface ocean pH (− 0.002 pH units/year, Feely et al., 2009) and the accuracy of state-of-the-art pH measurements in seawater (± 0.005 pH units, Dickson, 2011), only the most accurate time-series measurements are suitable for trend analysis. The ongoing development of pH and pCO_2 sensors and instrumentation for use on moorings, drifters and gliders (e.g. SeaFet, Contros, Sunburst) offers new opportunities such as observations with high-temporal resolution (Sutton et al., 2014), autonomous deployment in remote oceans (Monteiro et al., 2015) and vertical profiling (Williams et al., 2017). However, detection of long-term trends in ocean pH or pCO_2 requires accuracy and stability of sensors, as well as frequent in situ calibration, something which few novel sensors can (yet) achieve (Bakker et al., 2016). Synthesis products of ocean carbon, such as the Surface Ocean CO_2 Atlas (SOCAT) (Bakker et al., 2014a) for the surface ocean and the Global Data Analysis Project version 2 (GLODAPv2) (Olsen et al., 2016) for the interior ocean, are important tools for studying marine carbonate chemistry and how it is changing.

Biological activity, temperature change, upwelling and fresh water inputs from rivers, groundwater, sea ice and glacial melting, are all parameters that impact marine carbonate chemistry in addition to OA (Chierici et al., 2011; Fransson et al., 2016). As a result, pH and Ω_{ar} (aragonite saturation state) display large temporal (diurnal, seasonal, year-to-year, decadal) and spatial variation, particularly in coastal waters (e.g. Legge et al., 2016). Surface ocean pH varies seasonally by about 0.2 to 0.6 pH units at open ocean sites such as HOT (22°45′N, 158°W) and ESTOC (European Station for Time-series in the Ocean, 29°N 15°W). These time series also reveal considerable year-to-year variation in surface ocean pH (Feely et al., 2009; Bakker et al., 2014b).

Temporal and spatial variations in carbonate chemistry are much larger in near-shore areas, coastal seas and near major river outflows than in the open ocean (Bakker et al., 2016).

The key question from the biological point of view is how to carry out carbonate chemistry measurements to provide an adequate context for the interpretation of pteropod responses to the natural environment (i.e. in situ). For instance, both Ωar values and the duration of exposure should be combined within metrics such as “undersaturation days” or “exposure severity” (Hauri et al., 2013), to provide a comprehensive understanding of the exposure history of the individuals. Nevertheless, to evaluate the exposure history of individuals fully across time and space in situ, one also needs to combine the carbonate chemistry field with the modelled advective trajectory that individuals have taken through it, which requires coupling observations with particle tracking models.

Sites of high pCO_2 or “natural OA hotspots” have been the focus of a number of studies considering in situ biological responses to critical carbonate chemistry environmental conditions. Long term monitoring of the in situ population dynamics across natural small scale gradients could be relevant to understand the plasticity and adaptive-response of pteropods to anthropogenic OA. Studies of the response of pteropods to carbonate chemistry gradients in upwelling regions, such as along the US west coast and in the Scotia Sea (Bednaršek et al., 2014a, 2014b, 2012c, respectively), have been insightful in this regard. Such areas contain a range of aragonite saturation state values, from levels ($\Omega_{ar} \geq 1$) where we expect little change in shell dissolution, to gradually more severe undersaturation ($0.8 \leq \Omega_{ar} \leq 1$) where an effect of OA is more significant. Maas et al. (2016) used a similar comparative approach to explore respiration rate responses, testing individuals from the US west coast, where CO_2 values are already high, in contrast to those from the US east coast which never experiences undersaturation in the upper water column. These studies rely on the tight integration between carbonate chemistry and biological-response studies, which must be an essential element of all future studies investigating the sensitivity of pteropods to anthropogenic OA.

Currently, there is little overlap between expeditions which focus on biological sampling and processes and those which measure water column carbonate chemistry. We encourage better coordination between these two research areas even to the extent that one group “piggybacks” onto the expeditions of the other. This will have a number of mutual benefits to scientists in both fields since pteropods may influence water chemistry as much as in situ chemical conditions affect the biology of pteropods. A major requirement of experimentalists is to have a fundamental knowledge of current regional carbonate chemistry conditions and their variability to design experiments that are grounded in environmentally relevant conditions as well as to be able to interpret in situ biological responses. This encompasses understanding the magnitude, duration and frequency of baseline conditions, diel and seasonal variability, and identification of ‘natural hot spots’ of OA which coincide with regionally high pteropod biomass.

2.2. Biomass and databases

Although the ecological and biogeochemical importance of pteropods is now well recognised, their global biomass distribution remains poorly resolved. Such information is crucial for assessing the present size of pteropod populations and quantifying their contribution to the carbon export within ocean biogeochemical models. There is a wide body of pteropod net-catch data available within on-line databases (e.g. OBIS, PANGAEA, NMFS-COPEPOD, ZooDB, GBIF) with the most comprehensive being in the North Pacific, Southern Ocean and North Atlantic. Further pteropod databases are also in their early stages of construction for the Mediterranean and Arctic. A more thorough assessment of the coverage and uses of these databases are presented in the External Supplementary material “ESM_data source”. Most of the data is open access provided there is appropriate acknowledgement of its use. Those databases have the potential to be a valuable tool to improve the assessment of the effects of anthropogenic OA, particularly in terms of the vulnerability of calcifying species, since it provides a benchmark against which modelling projections and future sampling efforts can be compared.

Bednaršek et al. (2012a) have provided a comprehensive global data coverage of pteropod distribution. The study was part of the MARine Ecosystem DATa project (MAREDAT), which aimed to construct a database of field measurements to determine the biomass of plankton functional types (PFTs, Le Quere et al., 2005). The pteropod database (doi.pangaea.de/http://dx.doi.org/10.1594/PANGAEA.777387) was one of 10 PFT databases set up as part of this initiative, which are now all publicly available (https://www.uea.ac.uk/green-ocean/data#biomass). The Bednaršek et al. (2012a) study amassed data from net catches to a maximum depth of 2000 m between the years 1950 and

2010 from PANGEA, ZooDB, NMFS COPEPOD as well as 41 scientific articles. This amounted to 25,939 data points from 15,134 net-sampling stations. Details on pteropod abundance, biomass, species composition and life stages were documented within the database, alongside sampling information (i.e. maximum net depth, net mesh size as well as time of the year/day, collection methods etc.).

Although pteropod observations were available for all ocean basins (Fig. 2), there was a clear bias of the data towards observations in the Northern Hemisphere (NH) (85% of entries), with the remaining 15% in the Southern Hemisphere (SH). Based on these results, it was estimated that shelled pteropods constituted a mean global carbonate biomass of 23.17 mg $CaCO_3$ m^{-3} and they are especially prevalent in the coastal to shelf areas (Fig. 2). By extrapolating regional biomass estimates to a global scale, Bednaršek et al. (2012a) estimated global pteropod biomass to amount to 500 Tg C, contributing between 20 and 40% to global carbonate production. Berner and Honjo (1981), by comparison, considered that pteropods may constitute at least 12% of the total carbonate flux worldwide. This is a significant number since it corresponds to five times the global estimated planktonic foraminifera biomass production (Schiebel and Movellan, 2012) and around one fifth of the global diatom production (Leblanc et al., 2012).

Unfortunately, most databases contain information on pteropod distribution and abundance but not on biogeochemical parameters and processes, such as aragonite production, shell dissolution, or inorganic to organic carbon ratios. Depth discrete information to characterise vertical distribution patterns is also generally lacking. However, with the rapidly growing interest in the ecological response of pteropods to anthropogenic OA and ocean warming, it is crucial that a common metadata platform is put in place to aid data management and facilitate further global as well as regional analyses on pteropod biomass and distribution, particularly to determine evidence of recent change.

2.3. Biogeography, species boundaries and adaptive potential

Most research to date has focused on ecological responses of pteropods to ocean changes over very short time scales (< 1 month), and we know little about the evolutionary framework or the adaptive potential of pteropods. It is important to have a solid phylogenetic framework to be able to identify species accurately. It is also important to quantify variability in natural populations, to examine partitioning of populations across space and time and to estimate the amount of gene flow between them, if we are to predict the potential of pteropods to adapt to changing ocean conditions (Peijnenburg and Goetze, 2013).

In contrast to the ecological relevance of pteropods, and the increasing amount of experimental work done on their physiology and resilience to environmental stress, there has been little modern advance regarding studies of their morphology (Kubilius et al., 2014). In the past, several pteropod taxa have been identified using the traditional approach of univariate measurements of shell dimensions (e.g. Janssen, 2005). The shells of Euthecosomatous pteropods exhibit complex and highly diverse shapes, which enable detailed geometric morphometric analyses to identify species and geographic variation within species in a more powerful way (e.g. Aiello et al., 2007). Geometric morphometric analyses were successfully applied to distinguish between morphotypes of the tropical and subtropical *Cuvierina* pteropods (Burridge et al., 2015). 2D geometric morphometrics is a suitable method for processing well-preserved specimens in large numbers and comparing between populations and species. Regarding the external and internal anatomy and organ function, most of what is known originates from early monographic works (e.g. Meisenheimer, 1905). A recent microanatomical study of the species *Creseis clava* by Kubilius et al. (2014) showed that 3D-reconstruction methods are a suitable tool to verify and complement the sparse and partly outdated morphological data of pteropods.

The phylogenetic relationships between major groups of pteropods, and also in relation to other gastropod molluscs, are still largely unresolved. So far, data of several genetic markers from 11 pteropod genera have been included in phylogenetic analyses of higher taxonomic levels (16S in Thollesson, 1999; 18S, 28S and Cytochrome Oxidase 1 (CO1) in Klussmann-Kolb and Dinapoli, 2006). Combined analyses of morphological and molecular data (28S and COI) were used to assess phylogenetic relationships within most shelled pteropod species (Corse et al., 2013). However, the phylogenetic resolution, especially above genus level, has been limited due to large heterogeneity in evolutionary rates. DNA barcoding studies based on partial CO1 sequences have also been conducted in pteropods: 1) to distinguish Arctic and Antarctic populations (Hunt et al., 2010; Sromek et al., 2015), 2) to assess species diversity in different ocean basins (Bucklin et al., 2010), and 3) to delimit species boundaries (Maas et al., 2013; Burridge et al., 2015).

An accurate assessment of species distribution patterns is an essential step to predicting species-specific ecological and evolutionary responses to ocean change. An important tool for assessing species distributions is Ecological Niche Modelling (ENM), which has been applied in the marine environment in the past ~10 years (e.g. Robinson et al., 2011). This method aims to estimate the ecological tolerances of different taxa based on presence-data and spatial data layers of remotely sensed or measured and interpolated marine environmental data. An essential step in the use of environmental data is to test for collinearity and to remove correlated variables. ENM was used by Burridge et al. (2015) to estimate the ecological tolerances of six *Cuvierina* morphotypes. Studies of pteropod biogeography have traditionally applied presence data only to estimate species distribution patterns in a qualitative manner (e.g. Bé and Gilmer, 1977; van der Spoel and Heyman, 1983), however, a recent study reported distribution patterns of pteropods based on quantitative sampling in the Atlantic Ocean (Burridge et al., 2016).

Revisions of most pteropod taxa are urgently needed to be able to accurately identify species and monitor any changes in species distributions. This should be based on an integrative approach, combining

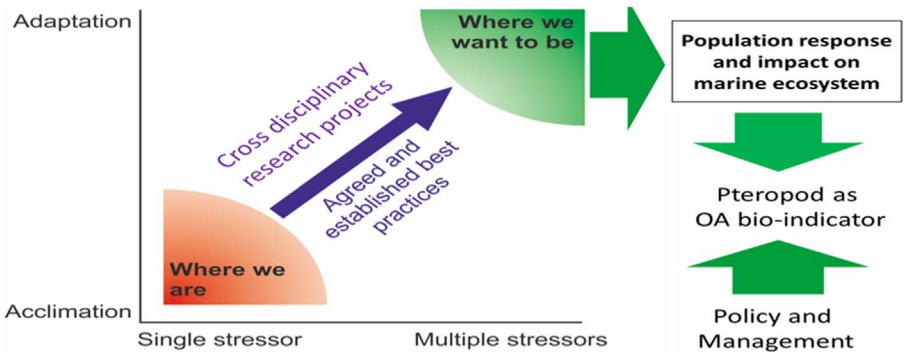

Fig. 2. Global distribution of quality controlled data of pteropod biomass (μg C L^{-1}; based on Bednaršek et al., 2012a) averaged over the top 62.5 m. White areas indicate the areas with presence of data below 62.5 while grey areas indicate data absence. The areas of high biomass correspond with high biomass hotspots.

morphological, morphometric, molecular, fossil, and/or geographic analyses. It is often still unknown whether the described pteropod species are made up of cryptic species assemblages, whether genetically distinct forms can be morphologically separated, or whether regional differences within pteropod species could be responsible for distinct physiological responses to OA or add to their resilience to ocean changes. Reduced-representation genomic methods using Next Generation Sequencing (NGS) techniques (e.g. Davey et al., 2011) hold great promise for assessing species boundaries as well as resolving barriers to gene flow between populations of pteropods.

2.4. Life history

A key requirement for understanding organisms' responses to anthropogenic OA change is knowledge of its life history because different life history stages may have different tolerance to OA. However, fundamental details in the life history of euthecosome pteropods, such as life cycle and growth rates, remain poorly parameterised (Hunt et al., 2008). A hindrance to defining their life cycle patterns is that study of shell-size distribution to determine cohort structure is not a straightforward means of determining life-span. Cohorts do not necessarily represent different generations, since multiple spawning events have repeatedly been observed within one year (e.g. Gannefors et al., 2005; Wang, 2014). However, cohort analysis of monthly sampling over three years in a north Pacific fjord indicates *L. helicina* has a longevity of 10 months (Wang et al., 2017). Recent successes in culturing more temperate species *L. retroversa* has provided some insight to this species' life cycle under laboratory conditions (Howes et al., 2014; Thabet et al., 2015) revealing a complete generation time from reproductive adult to reproductive adult for this species to be around 3 months at 8 °C. Generation times are likely to be species- and temperature-specific. Species that span a wide geographical range are exposed to a wide range of temperatures, seasonal cycles and levels of food availability which will affect growth rates and spawning times (Bednaršek et al., 2012a). For instance, *L. helicina* specimens found in the sub-arctic regions are 2–3 times larger than the same species found north of the Polar Front (Kobayashi, 1974; Gilmer and Harbison, 1991; Gannefors et al., 2005). Compared to polar species, the life span of temperate and tropical species is less well known but is likely to be shorter and less influenced by seasonal factors.

Another outstanding question is whether the rate of shell growth is continuous throughout the life of the pteropod or influenced by ontogenic and/or seasonal factors. Understanding controls on growth rate will enable more accurate observations of response to future OA. While Bednaršek et al., 2012a assumed shell growth to continue over the autumn and winter months at rates almost equivalent to those in summer in *Limacina helicina antarctica* collected in the Scotia Sea, seasonal differences in growth rates have been observed by Van der Spoel and Dadon (1999) in Antarctic and sub-Antarctic pteropods. Lischka and Riebesell (2012) show that *Limacina helicina* growth ceases during winter in Kongsfjord (Svalbard) when food is scarce. Similarly, Wang et al. (2017) observe that *Limacina helicina* is highly responsive to temperature and food availability in a fjordal setting in the north Pacific, with rapid spring time growth following minimal growth over winter. The level of tolerance of pteropods to a habitat with natural high seasonal variability in pH (e.g. fjords, sea-ice and coastal upwelling) is important indicators of how future OA change will impact pteropod populations. Understanding the baseline life cycle of pteropod species from a range of geographical settings is critical for monitoring and predicting future change due to OA, since responses may be region-specific. Bednaršek et al. (2016) used a projection matrix to consider the importance of fecundity, survival rates, stage duration, stage structure stability in populations (i.e. which stages contribute the most towards the population), and life stage weighting (i.e. which of the population stages are most vulnerable to stressors). Juvenile and the subsequent early stage of sub-adult stages were identified as being the most critical to population dynamics by this modelling approach. Understanding which life-stages may be most sensitive to the change is a key factor in assessing potential impacts of future OA conditions. In addition, consideration of generation times and therefore the opportunity to adapt genetically to the changing conditions over the coming decades should be factored in.

2.5. Sensitivity of the early stages

Juvenile stages of marine calcifiers are particularly sensitive to anthropogenic OA (Kroeker et al., 2013). Comeau et al. (2010a, 2010b) carried out one of the earliest studies, finding that larvae of the Mediterranean pteropod *Cavolinia inflexa* exhibited malformations and lower shell growth under elevated pCO_2 (870 ppm, Ωar = 0.92). At extremely high pCO_2 (1700 ppm, Ωar = 0.67), the larvae did not build shells but were still viable, although their functionality was questioned.

Thabet et al. (2015) found that survival during the early development of *L. retroversa* was sensitive to elevated levels of CO_2, with larval mortality at the gastrula stage significantly increasing when incubated at both 800 ppm (Ω_{ar} = 0.96) and 1200 ppm (Ω_{ar} = 0.73) and development being delayed at 1200 ppm. Manno et al. (2016) found that pteropod eggs of *Limacina helicina antarctica* spawned under high pCO_2 (Ω_{ar} = 0.76) lacked resilience to anthropogenic OA. Maternal OA stress (i.e. OA exposure only during the egg-brood phase) resulted in eggs that were smaller and had a lower C content in comparison to those that were not exposed, while embryonic OA stress (OA exposure during only the embryonic development phase) delayed development rate. The combination of maternal and embryonic OA stress reduced the percentage of eggs successfully reaching the organogenesis stage by 80%.

Cutting-edge microscopic and molecular techniques (see Sections 3.1 and 3.2) pioneered with adult pteropods should be adapted for use also with juveniles and larvae. Ideally microscopy techniques such as micro Computed Tomography scans of developing shells and Raman spectroscopy to discern the type of $CaCO_3$ in the early shell/protoconch could be used to assess the sensitivity of the early calcification process to acidification (Waldbusser et al., 2013; Waldbusser et al., 2016). Molecular techniques could further assess stress responses, transgenerational effects and the heritability of fitness. Finally, little is known about the metabolic rate and swimming behaviours of pelagic larvae under OA. The small size of the early life stages of thecosomes, will require the miniaturization of techniques for exploring these factors.

One of the main limitations with current research techniques is the difficulty in maintaining pteropods in culture for extended periods (Harbison and Gilmer 1992; Howes et al., 2014). Likely factors preventing successful culture are the inability of adults to feed, combined with rapid sinking when not actively swimming. Some species appear to respond more favourably to laboratory conditions, notably *L. retroversa*, which has been cultured through three generations, and *Heliconoides inflatus* and *Limacina helicina*, which have been observed to feed in small closed systems (Howes et al., 2014; Thabet et al., 2015). Different techniques are required to incubate eggs and early larvae successfully compared to adults. For instance, gas bubbling may damage the shells of early life stages even though the technique has no adverse effect on adult or juveniles. Exposure via post-bubbled sea water should therefore be undertaken, rather than direct bubbling. Nevertheless, incubation remains the most feasible way to harvest pteropod eggs although it potentially adds artefacts related to captivity. In the future, incubation experiments should be designed to differentiate between the effects of the various components of the carbonate chemistry (pH, CO_3^{2-}, HCO_3^-, CO_2, Ω), as well as their relative relevance. This has already been demonstrated for corals (i.e. Comeau et al. 2013; Jury et al., 2010). As example a recent experiments on calcifies coral demonstrated as the negative effect of declining CO_3^{2-} on the calcification can be partly mitigated by the use of the increasing HCO_3^- (Comeau et al. 2013).

2.6. Energy budget

The development of energy budgets is critical to understanding how pteropods acquire resources from their environment and use nutrients and energy for growth, calcification, maintenance, and reproduction under OA scenarios. The only available data on minimal energy requirements and assimilation efficiency of carbon in Euthecosome pteropods are summarized in Lalli and Gilmer (1989) for *Cavolinia longirostris*. Other physiological measurements conducted on pteropods include oxygen consumption rates, ammonium and phosphate excretion rates, atomic ratios (O:N, N:P, O:P), and C, N, P daily loss rates (e.g. Ikeda and Skoldal, 1989, and references therein).

With regards to calcification, it has been shown that pteropods can continue calcification in undersaturated conditions and potentially repair their shell (e.g. Comeau et al., 2010a, 2010b; Lischka et al., 2011). This may place an extra energy burden on these animals and cause increased oxygen consumption rates (Comeau et al., 2010a, 2010b). In terms of food availability, after 4 days starvation, Maas et al. (2011) found metabolic suppression took place with ~20% lower oxygen consumption rates in *L. helicina* antarctica although ammonia excretion rates were not significantly affected. During low phytoplankton abundance years, the effect of pCO_2 may be masked by reduced metabolic rates, while CO_2-induced effects may be measurable during years of high phytoplankton abundances (Seibel et al., 2012).

Conducting reliable physiological measurements to calculate energy budgets is still a largely underutilized approach. It is nevertheless a major undertaking in that rates of ingestion, food composition, growth and excretion rates, faecal pellet production, and protein and lipid content must all be measured.

2.7. Synergic effect and multiple stressors

Anthropogenic OA occurs concomitantly with other global environmental changes. These “multiple stressors” include warming, deoxygenation, freshening and increased stratification (Pörtner et al., 2014; Bednaršek et al., 2016). Owing to the interactive effects of two or more drivers, which can be additive, synergistic or antagonistic, it is generally not possible to extrapolate from single- to multiple-driver responses. Thus, investigation of single drivers can produce misleading inferences about individual responses to multiple stressors (Bopp et al., 2013) which may also be specific with regards region, habitat, species, and life-history stage (Hobday and Lough, 2011). Bednaršek et al. (2016) synthesized all the available studies on pteropod effects due to multiple stressors. Here, we provide a short overview of experimental studies that have measured physiological responses in pteropods and can be used to develop functional relationships for predictive models.

Combined effect of Warming and OA

Comeau et al. (2010b) investigated calcification and respiration of adult *L. helicina* in response to elevated temperature and pCO_2 in the Arctic, demonstrating reduced calcification as a function of pCO_2 and elevated temperature. Respiration was unaffected at the control temperature but increased significantly as a function of pCO_2 at higher temperatures. In the same region, Lischka et al. (2011) examined the interactive effects of low pH and higher temperatures on the mortality, shell degradation, and shell growth of pre-wintering early juvenile *L. helicina*. Results revealed a significant temperature effect on mortality and a pCO_2 effect on shell growth and degradation, but no synergistic effects were revealed. Lischka and Riebesell (2012) investigated the impact of acidification and warming on shell degradation and mortality of two pteropods species during the Arctic winter: the polar *L. helicina* and the boreal *L. retroversa*. For overwintering *L. helicina*, a negative synergistic effect between temperature and pCO_2 on shell degradation was found while no synergistic effect on shell degradation was detected on *L. retroversa*. It was suggested that the different thermal windows of the two species resulted in *L. helicina* enduring higher levels of stress compared with *L. retroversa*.

Combined effect of acidification and freshening

The combined effect of anthropogenic OA and freshening was investigated by Manno et al. (2012) on *L. retroversa*, in the Northern Norwegian Sea (Sub-Arctic costal water). Mortality of *L. retroversa* strongly increased when both pH and salinity were reduced simultaneously. The synergy of freshening and acidification also had a negative effect on pteropod locomotory speed and their ability to swim upwards. It was suggested that the energy required to avoid sinking (in a low salinity scenario), combined with the extra energetic cost necessary to counteract shell dissolution (in the high pCO_2 scenario), exceeded the available energy budget, so affecting the behaviour and decreasing the survivorship of these pteropods (Manno et al., 2012).

Combined effect of hypoxia and acidification

Maas et al. (2011) measured the oxygen consumption and ammonia excretion in seven species of North Pacific Ocean pteropods, some of which migrate vertically each day into cold (10–15 °C) hypoxic water (< 20 μmol O_2 kg^{-1}) and others remain continuously within oxygenated surface waters. It was found that the combination of low temperature and hypoxia suppresses metabolic rate by 80–90%. The pteropods species were noted to have different vertical distributions, metabolic rates and physiological responses to temperature and hypoxia. In the same region, Maas et al. (2012) examined the oxygen consumption and ammonia excretion of five pteropod species to elevated levels of CO_2. Metabolic rates of pteropods that naturally migrate into the hypoxic water were not influenced by short-term exposure to high CO_2 conditions. Similarly, Maas et al. (2016) compared the response of multiple species from the North Atlantic and the North Pacific to short term high CO_2 and additionally exposed three species in each region to low oxygen as a co-stressor (10%; ~130 μmol O_2 kg^{-1}). They found that only one species from the Atlantic (*Limacina retroversa)* showed a significant change in respiration rate in relation to the combined treatment and suggested that natural environmental exposure to these co-variables influences metabolic sensitivity. Bednaršek and Ohman (2015) observed altered vertical migration behaviour as well as changes in species community composition as a result of co-variation of high CO_2 and low dissolved oxygen across a frontal system in the Southern California Current System.

To expand from single to multiple stressors is one of the major challenges of OA research on pteropods and relatively few multiple stressors studies have been conducted with these organisms. Multifactorial experimental designs rapidly become increasingly challenging when testing for two or more environmental stressors. There is also a risk that comparability between data sets is lost and evidence becomes more conflicting (Riebesell and Gattuso, 2015). Dealing with this level of complexity requires interdisciplinary efforts that integrate empirical, experimental, and modelling approaches. For pteropods, the combined effects of increased temperature and decreased aragonite saturation state could have major controlling influences on their future physiological responses in the open ocean. In coastal regions, the additional stressors of decreased oxygen and increased pollution may also play significant roles (Reum et al., 2014). The challenges of the complex experimental designs can be partially overcome by carrying out studies in the natural environment where such stressors naturally co-vary. However, it is often difficult to derive a mechanistic understanding of the main drivers of the stress response in such settings which limits our ability to formulate predictive models. Another step will be to translate the results obtained from short-term observations and experiments conducted at the physiological and individual level into the long-term population-level assessments. So far, such population level studies have been region specific and have just considered OA as the sole stressor (Loeb and Santora, 2013).

3. Technological advancements

This section will consider the recent technological advances in the pteropods research and suggest how this technology can be used as powerful tools to help fill identified knowledge gaps.

3.1. "Omics" technologies

An increasing number of studies have employed molecular methods to disentangle the complex picture associated with CO_2 responses of marine invertebrates. These reveal changes in expression of genes, proteins and metabolites with a known or predicted role in acid-base balance, apoptosis (cell death), biomineralization/cytoskeleton, development, protein synthesis, energetic metabolism, and stress responses (Tomanek et al., 2011; Kurihara et al., 2012; Moya et al., 2012; Hüning et al., 2013; Pespeni et al., 2013; Thompson et al., 2015; Wei et al., 2015; Moya et al., 2016). Application of these technologies to the study of pteropods is only just beginning but shows promise for further understanding the effects of acidification.

Gene expressions studies (transcriptomics), based on quantification of mRNA, are a fruitful avenue for examining physiological questions, particularly when manipulations or natural experiments can be used to tease out differential patterns of expression. Historically, these were done as single gene quantitative PCR (qPCR) analyses, or with microarrays. These technologies require a priori knowledge of gene sequences and provide information about a limited set of genes (tens to hundreds) typically of well-known model organisms. However, the recent development of RNA "Next Generation" high-throughput sequencing technology (RNAseq) allows all the genes being expressed at a given time to be identified without any previous knowledge of gene sequences. With the advent of sufficiently inexpensive high-throughput sequencing and the development of robust analysis pipelines to enable assembly, annotation, and quantification of these data, transcriptional profiling is no longer restricted to model organisms. Transcriptomics is the only "omics" technology that has so far been used with pteropods. Recently Johnson and Hofmann (2016) developed a transcriptomic resource for *Limacina helicina* where the RNA sequencing libraries were prepared from *Limacina* that had been exposed to a range of pH levels and an elevated temperature to maximize the diversity of expressed genes. Previous studies suggest that some biomineralization associated sequences, specifically c-type lectins and collagens, may be sensitive to acidified conditions (Koh et al., 2015; Maas et al., 2015). Interestingly, the expression patterns of these groups of genes were opposite in the two pteropod species studied. In response to raised level of dissolved CO_2, *Limacina helicina* showed downregulation of biomineralization associated sequences, while *Clio pyramidata* showed upregulation. A newer study by Moya et al. (2016) on *Heliconoides inflatus* showed an increase in biomineralization associated transcripts, including collagen, while in addition they also documented an increase in neural signalling, and a downregulation of protein synthesis as response to low pH conditions. Further studies exploring whether these differences in the biomineralization response are species-specific, or are due to other factors such as duration or severity of exposure, are warranted. Proteomics, a technique which uses spectral analyses of fragmented peptides to infer protein sequence, and metabolomics, which identifies the low molecular weight metabolites in a sample using nuclear magnetic resonance (NMR) and mass spectroscopy, are technologies only just becoming available for non-model organisms. No studies to date have been done with pteropods, but ideally all three "omic" technologies will eventually be interrogated simultaneously to provide metrics that detail physiological responses to environmental change at the whole-organism level in pteropods. However, studies of this sort are hampered by the level of technology required, its cost, and the development of bioinformatic techniques capable of interpreting complex data outputs.

3.2. Computed tomography (CT) scanning

Pteropod shell condition and morphology has historically been studied using 2D–techniques such as light and scanning electron microscopy. Advances in computed tomography (CT) scanning (Cormack and Hounsfield, 1979) enable pteropod shells and internal structures to be reconstructed at micrometer resolution (micro-CT) in 3D. Micro-CT enables pteropod shell properties such as thickness, volume, density, and shape, to be quantified. As a non-destructive technique, it can be combined with other methods, such as genetics and geochemistry, to obtain a more complete understanding of the organism responses to anthropogenic OA.

Micro-CT has been established as a useful method to assess the effects of anthropogenic OA on calcareous structures of corals (Foster et al., 2014) and brachiopods (Schreiber, et al. 2014). The development of highly accurate industrial micro-CT scanners now enable this technology to be used on shelled pteropods. Micro-CT scanners generate a series of X-ray images at different angles around the shell. The 2D X-ray images are reconstructed to make a 3D model, which is used for measurements and visualization of features.

Some metrics of shell state that can be derived through CT scanning are:

Shell volume and thickness

Shell volume can be calculated by multiplying the number of voxels (3D pixels) with 'shell' greyscale values by the voxel size. Shell thickness can be assessed by determining the wall thickness of the pteropod at every voxel point around the shell, a tool which could be used to assess aragonite production or dissolution during an incubation experiment. The advantage of these micro-CT methods is that quantitative values of shell properties can be calculated objectively, making this an impartial way to compare results between different scientific teams.

Shell density

Sub-micron scale micro-CT has been used to investigate the density of carbonate shells (e.g. Iwasaki et al., 2015). The shell density of an individual specimen can be qualitatively represented by the greyscale values.

3D morphometrics

3D geometric morphometrics provides a quantitative means to distinguish between pteropod species and population to quantify shell growth. An exportable surface is generated from reconstructed micro-CT scan data. Each of the growth lines of the specimen are outlined to create a 3D mesh model of the shell, which contains coordinates unique for each shell shape (Liew et al., 2014). These coordinates can be used for quantitative measurements of shell ontogeny, growth, and morphometric variation in pteropods. This tool will help to quantify morphological variability in pteropod species, for instance, across natural gradients of OA.

CT scanning has certain limitations. In particular, X-ray intensity and the sensitivity of the detector are not always constant because of electric fluctuations, so raw greyscale numbers are not consistent between scans. To calculate actual shell density, however, calibration between standard materials with a known density and greyscale values is needed. At present, such reference materials for calcium carbonate do not exist, so it is a priority to identify a standard for global comparisons. Finally, international consensus on methodology, quality control, sample-storage, and sharing of CT data is a requirement to ensure the potential of CT scanning is optimised in resolving responses of pteropods to anthropogenic OA across the globe.

3.3. Palaeo applications

Pteropod shells often make up a large proportion of the microfossils

found in sea floor sediments (Lalli and Gilmer, 1989), and the pteropod fossil record extends 72 million years, from the Late Cretaceous to the present (Janssen and Goedert, 2016). Fossil pteropods, however, have rarely been used in OA research, largely due to the susceptibility of the delicate aragonitic shells to post-mortem dissolution in the water column, on the sea floor and within the sediment. Diagenesis, during lithification of the sediments, also affects the preservation of pteropod shells on all time-scales and limits the usable fossil record for OA research to soft, unaltered, marine sediments. In older sediments in particular, separating in-life dissolution from post-mortem is challenging. In marine sediments that have not been affected by post-depositional (post-mortem) dissolution, the fossil record of pteropods can be used as an indicator of OA. A number of studies have identified fluctuations in the shell condition of pteropods through time within sediment cores (Wall-Palmer, 2013a and references therein) but such results have usually reflected variations in post-mortem dissolution that fluctuate with global environmental conditions.

Thus far, only two studies have identified variations in pteropod shell condition in the fossil record that reflect the in-life dissolution due to OA (Wall-Palmer et al., 2012; Wall-Palmer et al., 2013b). These studies were carried out on the abundant cosmopolitan species *Heliconoides inflatus* in sediment cores from the Caribbean Sea and the Indian Ocean. A semi-quantitative dissolution index (*Limacina* Dissolution Index, LDX) based on shell transparency and luster (Gerhardt and Henrich, 2001) was applied using light microscopy and scanning electron microscopy. This method was originally developed to detect post-depositional dissolution, but has subsequently been adapted and used in studies of living pteropods (Manno et al., 2010; Roberts et al., 2011; Lischka et al., 2011) to detect changes in the shell condition that reflect in-life dissolution due to low carbonate saturation in the surface waters.

Wall-Palmer et al. (2012, 2013b) found that the LDX of *H. inflatus* in sediment showed a significant correlation to the Vostok atmospheric CO_2 concentrations over the entire time period recorded in the sediment cores (up to 450 ka). Shells were badly corroded and smaller during periods of high atmospheric CO_2 concentration compared to when atmospheric CO_2 concentrations were low. This suggests that the living pteropods experienced increased shell dissolution and found it significantly more difficult to calcify their shells when pCO_2 was high, resulting in corroded and smaller shells. This is in agreement with calcification experiments upon modern *H. inflatus*, which exhibited a 37% decrease in calcification at pH 7.9 compared to control conditions of pH 8.1 (Moya et al., 2016).

Although only LDX and shell size have been used to identify OA in the fossil record thus far, theoretically, any technique that is used on the shells of living pteropods may be applied to shells in the fossil record. This includes nanoindentation (Teniswood et al., 2013, 2016), stable isotope analysis (see Section 3.4), porosity measurements (Roger et al., 2012) and thickness and density measurements from X-ray tomography.

There are many advantages and disadvantages of using fossil pteropod shells in OA research. Arguably the principal advantage is that microfossils in seafloor sediments act as a natural laboratory, recording actual changes in diversity, abundance, geochemistry and shell characteristics. The complexity of oceanic systems, our lack of understanding about the ecology and biology of living pteropods, and the difficulty of keeping them under laboratory conditions, limits our appreciation of how pteropods will react to a changing ocean. The fossil record can provide us with this information. Investigation of this natural laboratory, however, also comes with caveats, because many of the factors affecting pteropods in the natural environment cannot be separated. This makes it extremely difficult to determine whether factors such as food availability and temperature have had synergistic effects upon shell growth and maintenance.

The main limitation of the pteropod fossil record is the susceptibility of the aragonitic shells to post-depositional dissolution, particularly below the aragonite lysocline. It is estimated that pteropods are preserved in as little as 2% of the ocean floor (Lalli and Gilmer, 1989). However, this still amounts to around 7 million km^2 of potential pteropod fossil records. There are also hundreds of kilometers of suitable sediment cores already available upon request from core stores around the world. Despite the current spatial and temporal limitations of the pteropod fossil record, it is still a vast resource and should be more utilized in future OA research.

3.4. Trace elements and stable isotopes

The possibility to assess the future consequences of anthropogenic OA on pteropods using field sampling and live culturing is limited (see above). The geological record, however, offers long-term evidence for several global environmental perturbations, where the effect of these threats can be studied directly.

Environmental conditions (e.g. temperature, salinity, carbonate chemistry) during calcification are recorded in the geochemical composition as well as the “quality” of the calcium carbonate shells produced. These measureable, compositional parameters have a known relationship to environmental conditions and are called “proxy” variables (Fischer and Wefer, 1999). Calibrations of these proxies have been inferred from inorganic precipitation experiments and field samples (e.g. Devilliers et al., 1994). While the analysis of the trace elemental and stable isotopic composition of foraminifera and corals is widely used in paleoceanography, few studies have assessed these in pteropod shells (e.g. Grossman et al., 1986).

Establishing trace elemental and stable isotopic composition of pteropod shells as proxies of environmental water condition should start with the analyses of field samples and/or cultured specimens. Analysis of field specimens, e.g. from sediment trap studies, allows natural variability among specimens as well as seasonality to be assessed. Alternatively, culturing pteropods under controlled laboratory conditions offers the opportunity to calibrate proxies accurately under a range of conditions, including those that are currently not found in nature (Howes et al., 2014). Additionally, culturing experiments allow varying a single (target) parameter while all others remain constant. Hence, it allows assessment of the effect of a single target parameter (e.g. carbonate ion concentration) on pteropod shell composition. Using this approach, important advancements in foraminiferal and coral studies have been made (e.g. Langdon et al., 2000).

Both trace elements and stable isotopes are measured using mass spectrometry. This instrumentation is widely available but on older machines requires relatively large numbers of individuals. Recent technical advancements allow analyses to be carried out on very small samples and/or in situ. SIMS (Secondary Ion Mass Spectrometry) and LA-ICP-MS (Laser Ablation Inductively Coupled Mass Spectrometry) are such examples where the stable isotopic and trace elemental composition is measured on very small regions (10–50 μm typically) within individual shells (e.g. Hathorne et al., 2003). Analysing the geochemical composition of pteropod shells offers exciting new possibilities, not only in a paleoceanographic, but also in an ecological context. For instance, the incorporation of Uranium (U) into biogenic calcium carbonate depends on the carbonate chemistry of seawater during calcification such that the U to calcium ratio of the shell can be used to back-calculate pH and or carbonate ion concentration (see for instance foraminifera: Keul et al., 2013; mollusks: Frieder et al., 2014). Seawater pH can be estimated through analysing boron isotopic composition within shells (e.g. Hönisch and Hemming, 2005), but the importance of the so-called “vital effect” has to be determined for pteropods. $\delta^{18}O$, a widely used temperature proxy, and ^{13}C, a proxy for feeding conditions, have also been determined in thecosome pteropods (Juranek et al., 2003).

Compared to the commonly studied foraminifera and corals, pteropods have a number of advantages as taxa on which to determine environmental proxies. Their relatively large shell size and long

lifespans of some species enables seasonal patterns to be resolved through analysing different parts of the shell. Conversely, whole-shell analyses integrate these seasonal patterns, reducing levels of seasonal bias. Finally, pteropods are symbiont free and have no specific gametogenic calcification at depth, which can otherwise obscure the proxy-signals derived from foraminifera.

3.5. Swimming behaviour

Pteropods exhibit strategic movements including diel vertical migrations and escape responses from predators. Pteropods achieve mobility with negatively buoyant shells and flapping parapodia. Some of their observed movements include swimming in a saw tooth-like pattern, and sinking with extended or withdrawn parapodia. Thus, changes to the condition of their shells by anthropogenic OA will likely affect their locomotion, with consequences to individual energy budgets and fitness. Childress and Dudley (2004) studied the swimming characteristics of a shell-less pteropod *Clione limacina*, which transitions from ciliary to flapping locomotion through ontogeny, depending on their Reynolds number (a non-dimensional quantity that is defined by the ratio of the inertial and viscous forces on the organism, hence, indicating the type of flow field surrounding it). Murphy et al. (2016) found that, in a remarkable example of convergent evolution, *Limacina helicina* 'flies' underwater in the same way that very small insects fly in the air. In the only study to date of the potential consequences of anthropogenic OA to pteropod locomotion, Manno et al. (2012), reported that swimming behaviour of the pteropod *Limacina retroversa* was affected by the synergistic stress of enhanced dissolved CO_2 and decreased salinity.

Understanding the underlying principles of a swimming pteropod requires investigation of the body kinematics and fluid velocity field generated during propulsion. This involves measurement of metrics such as velocity, acceleration, parapodia flapping frequency, pitching angular frequency, and tortuosity of the swimming trajectory. These can be resolved through traditional techniques such as high-speed video imaging for swimming behaviour and dye visualization to analyse qualitatively the fluid flow. Further quantitative information on fluid momentum transferred by flapping wings to the surrounding fluid can be achieved via tomographic particle image velocimetry (Adhikari et al., 2016) where the fluid is seeded with small inert particles and multiple cameras (typically four) are used to view the swimming pteropod. Additionally, quantitative measurement of the body kinematics can be acquired through orthogonal projections analysis where two orthogonal views of the same pteropod are obtained, either via a mirrored tank or multiple cameras. By calibrating the distances within the images, specific points on the pteropod can be resolved in three dimensions. These emerging technologies are already providing new insight into the fundamental nature of pteropod locomotion, an important component of addressing current knowledge gaps surrounding pteropod energetics and the likely impacts of anthropogenic OA on pteropod fitness.

3.6. Advancements to traditional sampling technologies

Studies of the distribution and behaviour of thecosome pteropods in the wild, monitoring of pteropod abundance and shell condition in relation to anthropogenic OA, as well as laboratory studies of the response of live individuals to various stressors, all share a common need for effective sampling methodologies. Pteropods are fragile animals and are difficult to recover whole and even more so to capture in a live state that in any way replicates their behaviour in the open ocean. They also have a patchy distribution across vertical gradients, making it challenging to collect individuals and appropriate life stages.

Pteropods have traditionally been sampled using plankton nets (simple vs. closing or multiple opening/closing), which enable collecting large numbers of organisms with little effort. In order to sample effectively smaller pteropod species and early life stages, a relatively small mesh size is often employed (200 μm or less). However, analyses of long-term variability in pteropod abundance and species composition in relation to anthropogenic OA (Mackas and Galbraith, 2012) have typically relied on zooplankton monitoring surveys where net design was chosen to optimize the sampling of a variety of zooplankton types other than pteropods. Future monitoring efforts more targeted at pteropods might re-assess the exact choice of nets. High-speed samplers, such as the Continuous Plankton Recorder, allowing more continuous sampling, have also been in use for long time periods in many parts of the world's oceans, providing key information on long-term trends in pteropod abundance (Beaugrand et al., 2013).

Careful sampling is needed to prevent potential mechanical damage both to the soft tissues and to the shell of the collected organisms. This necessitates additional modifications to typical net sampling methods when live, healthy animals are required. Rather than the small volume cod-ends typical of most plankton nets (e.g. ca. 1.5 L), large and weighted cod-ends (30 − 110 L), such as those specifically designed for gelatinous plankton (Howes et al., 2014), in combination with slow haul rates (e.g. 5–10 m/min at 2knots per hour) and limited tow duration, can be used to maintain specimens in healthy condition during capture and recovery of the net. Motion compensated devices may also decrease levels of shell damage during capture (Bednaršek et al., 2012a, 2012b). Thermally protected cod-ends can minimize the degree of acclimation required by experimental animals to captive conditions when animals are sampled at depth in cold temperatures (Childress et al., 1978). In regions where thecosomes are sufficiently large and abundant, direct collection in jars or beakers by scuba diving (Maas et al., 2012) or from the surface (Comeau et al., 2009) can also be an effective means of sampling the animals, being one of the least stressful techniques for organisms. Sediment traps provide valuable information on pteropod population dynamics, life cycles, seasonal succession and inter-annual variations in species composition (Bauerfeind et al., 2014) since they collect pteropods year-round, covering periods when traditional sampling by nets cannot be performed. The thecosome shell is a highly refractive structure, lending itself to effective detection via a variety of current and emerging optical techniques. As blue water scuba diving first gained popularity in marine research, visual observations along with imagery collected via still and video cameras by divers provided key insights into pteropod biology (Gilmer, 1974). More recently, towed or profiled high magnification underwater image-forming optical systems such as the Video Plankton Recorder have been used to quantify the vertical distribution and associations with hydrography of pteropods at very fine spatial resolution (Gallager et al., 1996). The small sampling volumes of such devices, however, require that the target species be present at sufficiently high densities to allow statistically reliable detection. Hence careful consideration of the likely densities and scales of variability is warranted in employing small-volume optical methods (Benfield et al., 1996).

High frequency active acoustic methods likewise can offer an attractive means of sampling thecosome pteropods remotely since the hard shell makes them efficient scatterers of sound relative to other similarly sized zooplankton (ca. two orders of magnitude stronger returns than for a crustacean of similar size, (Stanton et al., 1996). The fast sampling rates afforded by acoustic methods offer the potential for rapidly sampling pteropods throughout the water column at fine resolution, but only few studies have capitalized on acoustic methods for ecological investigations. Tarling et al., 2001, for instance, characterized the speed and timing of diel vertical migration of scattering layers associated with *Cavolina inflexa* in the Ligurian Sea using a ship-mounted acoustic Doppler current profiler, while ongoing analyses of multi-frequency acoustic data collected in the northwest Atlantic Ocean allow the characterization of *Limacina retroversa* swarm structure and distribution (Gareth Lawson, 2017, personal communication). Based on the few studies seeking to quantify the contribution of pteropods to

regional patterns of acoustic backscattering, it seems that pteropods are less often the dominant scatterer in comparison to taxa like euphausiids and copepods (Lawson et al., 2004; Lavery et al. 2007) but, nonetheless, acoustics offer a profitable sampling approach, in those times and places where pteropods can be shown to dominate acoustic returns. With the current proliferation of ocean observatories and autonomous sampling platforms, (such as vehicles, moorings, and floats, methods such as acoustics and optics) there is great potential for remote, autonomous observations of pteropods which provides particularly attractive sampling options as the international community continues to develop global anthropogenic OA monitoring observing networks (Newton et al., 2014).

4. Conclusions

4.1. Recommendations

Understanding and predicting the response of pteropods to anthropogenic OA, in concert with other environmental stressors, is a top research priority, both to determine their vulnerability and population resilience and for their use as an indicator of global environmental change. Ultimately, robust predictions of the likely response of these organisms to future environmental change should be addressed across at both the individual and population levels (Fig.3). This synthesis has provided an overview of our present understanding of pteropod responses and vulnerabilities. Overall, it is clear that current research has mainly been based on short-term observations and experiments conducted at the physiological and individual level, mostly to single stressors. There is now a need to expand these findings to population levels while also considering multiple stressors, including temperature, salinity and dissolved oxygen, alongside OA. Recent advancements in the use of molecular, stable isotopes and palaeobiology techniques in pteropod research, alongside the incorporation of advanced instrumentations, such as autonomous sampling platforms, CT scanning and high-quality video recording, should be used in multidisciplinary research efforts. Future progress and understanding will be contingent on our ability to build cross-disciplinary research projects in order to tackle these research aims.

Below we summarize our recommendations as to where efforts in both research and marine environmental management should be focused:

4.1.1. Improvements to base-line knowledge of pteropod biology

Culturing techniques are still a critical tool in learning more about the basic physiology of pteropods. At present, life cycle characteristics, including life-span, and rates of growth, fecundity, calcification mechanisms and mortality rates of many pteropod species are not sufficiently well known. These parameters are essential to assess ecosystem impacts that result from population processes and to scale up from laboratory studies of individuals to natural populations. Studies on predator-prey interactions combined with food web models is necessary to constrain the potential impacts of reductions in pteropod availability. Open-access species databases are rapidly synthesising existing datasets, allowing access to little-used datasets and identifying geographic areas where few data exist and further fieldwork is required.

4.1.2. Improvements to base-line knowledge of pteropod biogeography and biogeochemical impact

Measurement of particulate aragonite and calcite as an Essential Ocean Variable (EOV) would highlight the relative importance of pteropods, the main pelagic aragonite producers, compared to coccolithophores and foraminifers, the main producers of calcite. This has several applications of global significance, including determining the impacts of anthropogenic OA on these organisms, and further resolving the changing buffering capacity of the ocean to increased CO_2.

4.1.3. Identification of critical life stages

The long-term success of a species depends on successful recruitment, which in turn depends on the most vulnerable stages in the life cycle being successfully negotiated. Identification and parameterisation of these stages are important to the improvement of models that otherwise assume an invariant mortality rate. Further experiments and development of demographic models are required to identify vulnerable life stages and threshold levels of environmental stress. These thresholds can be identified through sub-lethal responses in physiological rates and genomic, proteomic or even metabolomic expression levels.

4.1.4. Greater use of natural environmental gradients

Identifying natural responses to stressors is difficult in laboratory conditions since the behaviour of organisms is constrained and the feeding environment is poorly simulated. Meso- and small-scale gradients can be found across natural features such as regions of upwelling

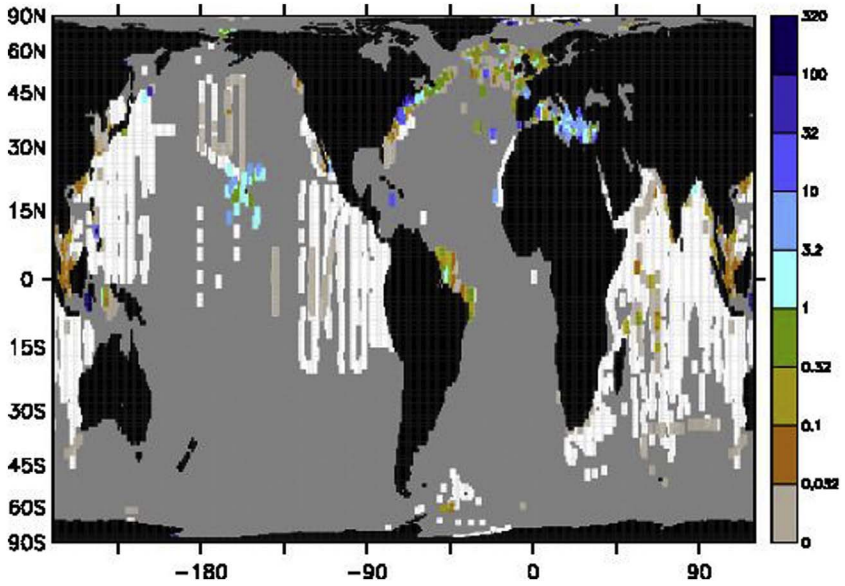

Fig. 3. Schematic diagram representing the future direction in pteropod research in multistressor driven environment. Black axes indicate the direction of research needs to assess pteropod population response and marine ecosystems with high pteropod biomass to future ocean conditions. Pteropods as early stage indicators should be related to the needs to policy makers and marine resource managers. Adaptation from the original figure in Riebesell and Gattuso, 2015.

eddies and CO_2 vents, which provide settings for 'natural' experiments. For instance, pH and carbonate chemistry may vary across these gradients (while other variables remain relatively constant). Furthermore, carbonate chemistry varies seasonally in many shelf systems, providing a temporal natural experiment. Long term monitoring of the in-situ populations across these gradients to determine physiological- and shell-state can identify the capacity of pteropod species to respond to these stressors over short (< 10 year) to medium (10–100 year) temporal scales. The difficulty with this approach is to determine the residence-times of populations within these gradients, so as to parameterise the duration of their exposure to the stressor. This necessitates integration with Lagrangian modelling studies to be able to track pteropods across temporal and spatial scales.

4.1.5. Assessing the potential of pteropods as a bio-indicator of anthropogenic OA

Currently, only one parameter (i.e. shell dissolution) has been demonstrated as a potential useful proxy for anthropogenic OA exposure, and this has only clearly been demonstrated in the genera *Limacina* and *Heliconoides*. Future studies should establish the use of simple metrics such as shell thickness that can be measured as a response to anthropogenic OA which then can be used by the regulatory-management community. It is essential that all such metrics are functionally related to prevailing carbonate chemistry conditions. Simultaneous measurement of chemical and biological parameters can and should be part of the global network of monitoring stations. This review documents the many different ways in which pteropods can be studied, with the ultimate aim to enable comparisons between different regions.

4.1.6. Using pteropods to communicate the issue of anthropogenic OA

Pteropods have an emblematic role as a sentinel of anthropogenic OA since their shell-state response illustrates the anthropogenic OA problem in a way that the public and policy-makers find tangible. As such, pteropods can be a very effective outreach component in communicating the impact of OA on ecosystems and identifying the most vulnerable regions.

4.2. Pteropods as sentinels for anthropogenic OA

The scientific and management communities need to work closely together to identify the challenges, benefits and uncertainties in defining species vulnerability within broader assessment frameworks. In order to act, management bodies use vulnerability assessments based on a set of objective metrics with clearly identified uncertainty and errors around the end-points. These include the identification how cumulative exposure history, including the magnitude, frequency, and duration of exposure, impacts vulnerability. To meet these requirements, the scientific community must provide information on natural variability in exposure, as well as species sensitivity thresholds and end-points to define how different stressors are impacting biology over time. Scientific consensus with quantified uncertainty can be used for interpretation of observational and modelling outputs and identification of anthropogenic OA-vulnerable areas, in order to address one of the pressing issues currently facing the management community involved with coastal resources and water quality.

After the first studies suggesting the sensitivity of pteropods to anthropogenic OA more than a decade ago (Orr et al., 2005) there was a rapid development in the application of novel methods to detect early OA-responses in the field. The rapid growth in pteropod research that resulted has, among other things, sought to qualify whether pteropods could be used as a sensitive indicator of OA for environmental monitoring and management (Weisberg et al., 2016). One of the most important features of a sentinel species is a strong correlation between the stressor and the species response, which for pteropods is the direct link between their shell state and the level of aragonite saturation. As well as shell dissolution, there may also be other approaches that could be used to link biological responses and anthropogenic OA, such as the measurement of shell thickness by micro CT scanning. To fully assess the use of pteropds as sentinel species, it will be crucial in the next future to fill the identified knowledge gaps (i.e. generation of a common metadata platform, scaling up to population levels) with the help of the technological advancements highlighted in this overview. However, the outcome of this workshop unequivocally shows that the pteropod research community has reached a stage where there is agreement on the utility of pteropods as a sentinel species. This knowledge must now be translated to make it a usable tool for policy making and environmental management.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.earscirev.2017.04.005.

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