Marine fish carbonates – contribution to sediment production in temperate environments

Submitted by Christine Elizabeth Stephens, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, September 2016.

I certify that all material in this thesis/dissertation* which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

In the past, oceans have strongly influenced atmospheric CO_2 levels through organic and inorganic carbon cycling. The inorganic carbon pump relies on the formation of calcium carbonate which releases CO_2 into the surface ocean and traps alkalinity in solid form which sinks to deeper ocean layers and sediments. After sinking, calcium carbonate can either then become trapped in the sediments or dissolve increasing the alkalinity of deeper ocean layers. The net effect is of acidifying surface oceans and encouraging release of CO_2 to the atmosphere. The present thesis focuses on marine teleost (bony) fish in temperate areas as previously poorly understood but potentially major producers of calcium carbonate in the ocean. Fish in temperate areas may be contributing to carbonate sediment production and as such the inorganic carbon pump. Prior to this thesis only tropical fish have been investigated as major piscine sediment producers. The present thesis describes the composition and morphology of carbonates produced by many different species of temperate fish providing a basis for the understanding the fate of these carbonates in the environments and their potential contribution to sediment production and the inorganic carbon cycle. Characteristics of carbonates produced by fish in the wild were fairly consistent within a species upon examination of carbonates produced by poor cod (*Trisopterus minutus*) over the course of a year. However, despite the likely consistent and distinct characteristics of fish carbonates, little evidence of them was found in temperate shallow sediments beneath pens of farmed Atlantic salmon (*Salmo salar*) where there theoretically should be very high production rates. Reduced salinity, often a feature of temperate areas compared to tropical areas, was found to reduce production rates of carbonate from fish compared to higher salinities. However, salinity reductions below the ocean average of 35 psu (practical salinity units) had less impact on production rates than increases above 35psu. As such it is argued that production rates in temperate environments should still be relatively high considering high fish biomasses in some temperate regions and could still mean fish in temperate areas are an important source of carbonate production and potential sediment production.

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1 Introduction

The world's oceans cover approximately 71% of the surface of the Earth (Tait and Dipper, 1998a) and contain approximately 1.36 billion cubic kilometres of seawater (Ocean, 2008). Oceans contain approximately 50 times the amount of dissolved inorganic carbon to that in the atmosphere and strongly influence atmospheric CO_2 levels over millennial timescales (Falkowski et al., 2000; Zeebe, 2012). In pre-industrial times, the ocean acted a key controller of atmospheric CO_2 concentrations (Lea, 2014), and considering these have been rapidly rising since then there is considerable interest in understanding the fluxes of CO_2 between the ocean and atmosphere and how these relate to the ocean carbon cycles (Millero, 2007). This thesis intends to add to the body of knowledge surrounding ocean carbon cycles by addressing gaps in our current understanding which are outlined in this introduction below.

1.1 The ocean carbon cycles

The surface of the ocean acts as an interface with the atmosphere where CO_2 can be exchanged. Dissolved CO_2 in the seawater can be released to the atmosphere or atmospheric CO_2 can be dissolved in the seawater. CO_2 dissolves into seawater and reacts with water as shown in Equation 1.

Equation 1

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

An increase in the concentration of CO_2 in seawater leads to the equilibria presented in Equation 1 being pushed to the right creating acidic hydrogen ions and decreasing the pH of the seawater. Concentrations of dissolved CO_2 in seawater can be affected by either changes in atmospheric CO_2 concentrations or by processes within the oceans that result in the release or sequestration of CO_2. Such oceanic processes can subsequently impact the equilibrium of CO_2 between the ocean and the atmosphere, and vice versa. The main processes within the ocean which act to control atmospheric CO_2 are the solubility pump, the organic carbon pump and the counter carbonate pump.

The solubility pump acts to transports dissolved CO_2 in surface waters into the ocean interior. CO_2 is more soluble at cooler temperatures, and tends to favourably dissolve into the dense cool water masses present at high latitudes,

especially in the North Atlantic and the Southern Ocean (Falkowski et al., 2000). The high density of these cool water masses causes them to sink to deeper ocean layers. Due to thermal and salinity stratification, and the pathway of the ocean currents, deeper layers of the ocean may not be remixed with surface waters for 500 to 1000 years (depending on the ocean basin) (Devries et al., 2012). This effectively sequesters CO_2 from the atmosphere in the deep ocean until it can remix with surface layers.

The organic carbon pump and the counter carbonate pump (or the inorganic carbon pump) are slightly different in that they rely on biological processes in the ocean. As such predicting the role of these systems in ocean-atmosphere CO_2 exchanges across changing climates can be complex.

The processes involved in both the organic carbon pump and carbonate counter pump are summarized in Figure 1. The organic carbon pump relies on uptake of CO_2 by photosynthesising organisms from surface ocean layers (specifically within the photic zone) and sequestration of the carbon as organic matter in their tissues. This reduction in the dissolved CO_2 effectively favours pushing the equilibria presented in Equation 1 to the left, favouring an increased pH locally. Some of this organic carbon is re-cycled in the surface waters immediately by respiration. This includes respiration of the photosynthesising organisms themselves and organisms which subsequently feed on them (either through predation of living organisms or degradation of dead organic matter). However, a portion of this organic carbon remains in the tissues and excreted matter of organisms. When organisms die or faecal pellets are released, they tend to sink. A portion of this sinking matter reaches deeper ocean layers. As mentioned above in reference to the solubility pump, these deeper ocean layers may not be remixed with surface waters for 500 to 1000 years (depending on the ocean basin) (Devries et al., 2012). As such CO_2 from the atmosphere is effectively sequestered in deeper layers until they become remixed with surface layers. On top of this, some of the organic matter that sinks to depth avoids being broken down and mineralised (dissolved) into the water and sinks further to the sediments. If organic carbon becomes buried sediment it may not be recycled through emersion and subsequent weathering of sedimentary rocks for approximately 300 million years (Lea, 2014), offering the longest term storage option for carbon from the atmosphere and ocean.

The counter carbonate pump (or the inorganic carbon pump) works on similar principles to yield almost opposite net results. Calcium carbonate ($CaCO_3$) is formed by many calcifying marine organisms such as marine plankton, corals and shellfish across many different environments. Calcium carbonate can be formed by organisms from calcium (Ca^{2+}), carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions as follows in Equation 2 and Equation 3:

Equation 2

$$Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3 + H_2O + CO_2$$

Equation 3

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3$$

As shown in Equation 2, calcium carbonate formation can release CO_2, and can trap alkaline carbonate and bicarbonate ion as solid calcium carbonate. Removal of either of these ions will result in a decrease in the pH of the surrounding seawater. The solid carbonate precipitates are generally used to form skeletons and protective shells for various organisms such as coccolithophores which use them to form plates to cover their surface. When these organisms die these dense skeletal carbonate minerals sink transporting the solid alkalinity deeper into the ocean and removing it from the surface layers. This leaves the surface layers more acidic unlike the organic carbon pump which removes acidic dissolved CO_2 as organic matter from surface layers, leaving surface layers more alkaline. Similar to the organic carbon pump the solid trapped ions can be recycled into seawater, however unlike the organic carbon pump this occurs not through respiration but through direct dissolution of the solid calcium carbonate. Calcium carbonate is more likely to dissolve under the cooler temperatures and higher pressures in the deeper ocean. As such, the alkaline carbonate and bicarbonate ions tend to be released in deeper ocean layers where it remains until ocean circulation mixes it with the surfaces layers. Alternatively, if any survives to be buried in the sediment, it can effectively remove it from the ocean carbon cycle. The net result is that the carbonate pump tends to remove alkalinity from the surface

waters decreasing the pH. A decrease in the pH of surface waters means CO_2 is less able to dissolve into the water and react as shown in Equation 1 which is in direct contrast to the organic carbon pump which acts to facilitate the uptake of CO_2 from the atmosphere. The relative efficiencies of the organic carbon pump and the inorganic carbonate pump can therefore control whether there is net efflux or uptake of CO_2 by the ocean due to biological processes. Calculating the relative efficiency of these processes and understanding how these might change will therefore be important in predicting the role our oceans might play in mitigating the exponentially rising atmospheric CO_2 concentrations we are currently experiencing.

The efficiency of both the organic carbon pump and the inorganic carbon will in part be dependent on how much carbon dioxide or alkalinity is trapped in solid forms at the surface oceans and what proportion of this makes it to deeper ocean layers or sediments, and by the difference, what proportion is re-cycled into the seawater by respiration (for organic carbon) and dissolution (for carbonates). As the amounts of carbon or alkalinity stored in sediments and recycled should total the amount produced these are often referred to as carbon or carbonate budgets. Figure 2 shows a visual representation of the carbonate budget and its relation to ocean and atmospheric chemistry.

In order to understand rates of carbon sequestration by the carbon pump in the ocean, extensive efforts have been put into estimating organic carbon budgets: the amounts of carbon trapped through photosynthesis, sinking rates of organic carbon in the ocean and the amounts that are subsequently sequestered in sediments versus recycled via respiration (Houghton, 2007).

Many efforts have also been made to understand carbonate budgets. Components of the carbonate budget include the rate of carbonate production in the ocean, the rate of carbonate accumulation in the sediments and (by difference) how much of the produced carbonate undergoes dissolution. However, our understanding of the carbonate budget is likely still incomplete. Analysis by Smith and Mackenzie (2016) shows that previous budgets are likely to underestimate the amount of carbonate produced across the world’s oceans. Traditionally carbonate budgets have focused predominantly on estimating carbonate production from planktonic sources and tropical regions which have been relatively well studied. For example, production rates for coral reefs have

been available for a long time and are relatively well established (Milliman, 1993; Smith and Mackenzie, 2016). By combining reef production rates with measurements of near surface coral reef area which can be obtained through satellite imagery (Spalding et al., 2002) and estimates of submerged reef area it is possible to provide relatively good estimates of coral reef carbonate production rates (Smith and Mackenzie, 2016). However, Smith and Mackenzie (2016) note that extratropical areas have been comparatively less well studied which may have led to an underestimation of total oceanic carbonate production. Providing and using a preliminary summary of “typical rates” of carbonate production for some extratropical areas they argue that the contributions of extratropical areas to carbonate production have previously been underestimated and that estimates of the global ocean carbonate production should be revised upwards.

For an accurate global production budget it is important to quantify carbonate production rates across different areas of the world. This is also important as carbonate minerals produced in different regions can have very different rates of dissolution. The proportion of carbonate production that accumulates in the sediments is what removes alkalinity from the seawater over long time scales. Sedimented carbonate that then dissolves at depth also removes alkalinity from the surface ocean as deeper layers can take between 500 and 1000 years to remix with surface layers (Devries et al., 2012). As such is not only useful to estimate carbonate budgets in terms of production, dissolution and accumulation, there is also value in estimating the dissolution taking place in different ocean layers. Environmental conditions such as lower temperature and seawater alkalinity, that vary geospatially, have the potential to alter this balance by favouring the dissolution of carbonates (Morse et al., 2007). As such even if cooler areas have high carbonate production rates they may not contribute as much to the carbonate pump as warmer areas. However, temperature and alkalinity are not the only important factors. The time which deeper layers of ocean remain unmixed with surface layers varies depending on the ocean basin due to differences in ocean currents. For example, in the temperate and polar latitudes of the North Atlantic, relatively saline surface waters from the tropics are carried by the Gulf Stream and become cooler and denser resulting in a major area of surface water movement to the deeper ocean. The deeper waters here are effectively at the beginning of the ocean

conveyor so any carbon sequestered into the deeper layers has the potential to remain sequestered for a long time (hundreds of years) before remixing with surface waters leading to the these waters being some of the most efficient at carbon sequestration (Devries et al., 2012). Presumably these areas will also be some of the most efficient at trapping alkalinity from carbonates for the same reason. As there are regional differences in the efficiency of oceans at contributing to the carbon and carbonate pumps it would be useful to accurately estimate carbonate budgets regionally.

Considering there currently seems to be a relative lack of information of carbonate production rates in extratropical areas, their importance in relation to global carbon budget and the high efficiency with which temperate oceans can store carbonates short term, there is a pressing need for more information on carbonate production, dissolution and accumulation rates in temperate seas.

1.2 Fish as carbonate producers in extratropical environments

Smith and Mackenzie (2016) summarize carbonate production rates for extratropical areas for sites where carbonate production is predominantly attributed to organisms such as calcifying algae (such as rhodoliths) and shell producing organisms (such as clams and limpets). One potential source of carbonate production in extratropical regions that has not been considered previously is teleost (bony) fish.

Marine teleosts produce calcium carbonate in their intestines as a result of a physiological strategy to deal with drinking seawater (Wilson et al., 2002). Once precipitated in the intestine they subsequently excrete calcium carbonate into the environment, either as mucus coated pellets in the absence of food, or mixed into faecal pellets. Consequently individual fish release calcium carbonate into the environment almost continuously throughout their life, as opposed to organisms which use calcium carbonate skeletally, which will only release their calcium carbonate skeletons upon death.

Marine teleost fish have recently been recognised as potential major contributors to oceanic calcium carbonate production with estimates that fish could be responsible for between 3 and 15 % of total global oceanic production (or potentially even between 9 and 45 % using less conservative parameters) (Wilson et al., 2009). Additionally marine fish are relatively abundant in

temperate latitudes compared to tropical latitudes (Jennings et al., 2008). As such it seems likely that fish would be major contributors to carbonate production in extratropical regions and it may be useful to include fish produced carbonates in future carbonate budgets for extratropical areas.

In addition to fish being likely contributors to carbonate production there is also some evidence that the carbonates they make can accumulate in sediments in tropical areas (Perry et al., 2011; Salter et al., 2014). If fish carbonates can contribute to sediments in tropical areas it suggests that fish can contribute to accumulation of carbonates in global budgets. However, there is little information on what proportion of the carbonates produced by fish can contribute to carbonate accumulation. It is unlikely that all of the carbonates produced by fish remain solid for long enough in the ocean to contribute to accumulation of sediments rather than dissolving. Previously, fish carbonates have generally been described as being composed predominantly of high magnesium calcite (Perry et al., 2011; Walsh et al., 1991) and have been found to be relatively soluble compared to other forms of carbonate such as aragonite and calcite (Woosley et al., 2012). However further studies in tropical species have found that fish can produce varying types of calcium carbonate including lower magnesium calcite varieties (Salter et al., 2012). As magnesium contents of calcite has been found to alter solubility (Morse et al., 2007), it may be that previously measured solubility is not reflective of fish carbonates in general. There are therefore questions regarding how soluble fish carbonates generally are in relation to carbonates produced by other marine calcifying organisms.

However, currently little information is available that would allow inclusion of fish carbonates in regional budgets for carbonate production, dissolution and accumulation. Considering fish are particularly abundant in temperate regions, further investigation into the role of temperate fish may be useful in tackling the current deficit of knowledge of carbonate budgets in extratropical areas. As such, the studies presented in the current thesis take the first steps and make progress in investigating the solubility, sedimentary accumulation and the production rates of carbonates produced by temperate fish species.

1.3 The characteristics and solubility of fish produced carbonates

All previous information that has been collected on the characteristics of fish produced carbonates and their potential contribution to sediments has been

collected from tropical fish species. Currently no information is available for fish from extratropical and temperate regions. In tropical species, however, it has been observed that fish can produce carbonate crystals in a variety of different morphologies with a wide range in magnesium content (between 0.5 and 40 mol%) (Salter et al., 2012). Both the morphology and magnesium content have the ability to affect solubility of carbonates, with higher magnesium content increasing solubility (Morse et al., 2007) and smaller particles having higher surface area to volume ratio which will also increase solubility. It may be that temperate fish also produce carbonates with a range of different solubilities. Therefore, identifying the typical morphology and composition of carbonates produced by temperate fish (together with potential causes of their variation) would provide a starting point for investigation of this issue. Clearly this information should better inform any future estimates of the role of fish in the carbonate pump.

Furthermore, without this information, it will not be possible to successfully identify carbonates produced by fish in temperate sediments. We cannot assume that previous studies of carbonates produced by tropical fish can be applied to temperate fish. By definition, temperate oceans are much cooler than tropical ones, which favours dissolution of calcium carbonates, and they are generally much less saturated in calcite and aragonites (Millero, 2007). Chapter 2 in the present thesis aimed to characterise carbonates produced by temperate marine fish species. Previous studies have noted that often different species of fish tend to produce different types of carbonates (Salter et al., 2012) although do not identify a reason for this. The study presented in chapter 2 therefore examined the morphology and composition of carbonates produced by a 21 different temperate fish species. The study in chapter 2 also repeatedly examined carbonates produced by one species across the course of a year, to identify the biotic and abiotic factors which can modify these carbonate characteristics. In chapter 2 it is hypothesised that various factors such as diet and temperature and therefore season might impact the characteristics of carbonates produced by fish. Chapter 2 therefore provides a platform for further study to understand the solubility of carbonates produced by fish in temperate environments.

1.4 Accumulation of fish carbonates in sediments

As noted above, the environmental conditions present in temperate oceans are more favourable toward the dissolution of carbonates than in tropical oceans. Chapter 3 aimed to address whether carbonates from temperate fish can actually contribute to sediment accumulation in carbonate budgets. Even if fish do contribute to sediment production it may be challenging to identify particles of fish origin if there are very few present in sediments. A first step to investigate this topic would be to examine sediments from areas which should have very high carbonate production rates from fish. Additionally, it would be better to start by examining relatively shallow sediments which will limit the potential for carbonate dissolution whilst sinking. Fish farms that are moored in the sea and open to the environment provide such an opportunity with a discreet area in which large numbers of fish are held for prolonged periods. If fish carbonates can be easily found in shallow areas where production rates are high then it might be appropriate to start looking at sediments at different depths in order to ascertain how deep fish carbonates can sink in temperate oceans and contribute to sediments. It might then also be possible to gain information about what types of carbonates produced by fish are likely to survive at different depths by observing the abundance of different morphologies found in sediments at different depths. In order to utilise the opportunity that fish farms present chapter 3 examines sediments obtained from below pens of farmed Atlantic salmon in Scotland. Sediments were examined directly using scanning electron microscopy for evidence of particles that might be of fish origin based on characterisation of particles obtained directly from salmon. In addition, carbonate contents of sediments from beneath the cages were compared with sediments elsewhere in the areas in order to identify whether carbonate production from the salmon might accumulate in sediments and raise average sediment carbonate content. Chapter 3 therefore makes a first step towards investigating accumulation of carbonates from fish in carbonate budgets for temperate areas.

1.5 Fish carbonate production rates

The final study presented in this thesis (chapter 4) aimed to refine our understanding of fish carbonates production rates in temperate areas. Current estimates of fish carbonate production rates in the ocean were obtained through

combining ecological models of fish biomass distribution with information on ocean temperatures and an understanding of the effect of temperature on the production rates of individuals (Wilson et al., 2009). There are, however, other environmental factors that are likely to have an effect on carbonate production rates in fish.

In order to understand what environmental factors are likely to have an impact on the carbonate production rates of fish, an understanding of the underlying mechanism of fish carbonate production is required. Marine teleost fish are hypo-osmotic compared to the seawater around them meaning they have a relatively low salt and ionic content compared to the seawater. They therefore tend to lose water from their tissues to the seawater environment through osmosis. One strategy they use to counter act this, is to drink large quantities of seawater. This seawater, however, contains high concentrations of various ions, the absorption of which needs to be minimised in order to maintain their hypo-osmotic state. In order to do this while still being able to absorb water in the intestine they exchange chloride ions from the seawater with bicarbonate ions produced from respiratory carbon dioxide (Wilson et al., 2002; Wilson and Grosell, 2003). The uptake of chloride ions aids intestinal fluid absorption, while excess blood chloride can be excreted through specialised gill ion-transporting cells back into the seawater. Similarly the creation of bicarbonate from respiratory carbon dioxide results in an excess of acidic hydrogen ions which are also excreted back into the seawater via the gills (Genz et al., 2008). The bicarbonate secreted into the intestine creates a high pH environment, sometime in excess of pH 9 (Grosell, 2006), and precipitates calcium ion (and to a lesser extent, magnesium ions) into crystals of calcium carbonate as shown in Equation 2. By precipitating the calcium ions it both prevents them from being absorbed and lowers the osmolality of the intestinal fluids which makes it easier to absorb water (Whittamore et al., 2010). Precipitated crystals are then excreted into the environment.

Increased temperature is expected to affect the rate of carbonate precipitation by marine fish by speeding up the processes outlined above. Metabolic rates are the sum of all energy consuming processes in an organism. This includes the osmoregulatory process that lead to carbonate precipitation in marine fish. Therefore well established relationships between metabolic rate and

temperature were used in previous models to predict fish carbonate production across the globe in different temperature areas (Wilson et al., 2009).

Temperature is an important factor to consider when estimating the carbonate production rate of fish in extratropical regions as, by definition, temperatures in tropical areas are higher than those in temperate areas. Additionally it affects not only the rate of calcium carbonate production but also the solubility. Calcium carbonates are more soluble at lower temperatures than higher ones. Lower temperatures increase the solubility of CO_2 in seawater, as such more CO_2 can dissolve in cooler higher pressure seawater and CO_2 reacts with calcium carbonate to make it dissolve (Equation 2 followed to the left). Understanding the effects of factors that impact both fish carbonate production rates and potential solubility of carbonates will be particularly useful in calculating carbonate budgets and in understanding whether fish contribute more to areas where the carbonate sink contributing to transport of alkalinity or areas in which carbonates are more likely to dissolve.

Salinity is another factor that may play a role both fish carbonate production rates and solubility of carbonates. Salinity is a factor that is often higher in warmer tropical areas due to increased evaporation, which also leads to an increase in alkalinity of the more concentrated seawater in these areas. Conversely, in cooler temperate areas with increased precipitation or area with more freshwater input, such as from seasonal ice melts and riverine inputs, have lower salinities and alkalinities (Lee et al., 2006; Millero et al., 1998). Warmer tropical areas are where fish carbonates are more likely to be preserved rather dissolve due to the higher temperatures and increased alkalinity. Being more saline, warmer tropical areas are also likely to be areas in which carbonate production rates for individual fish are high. This is because an increased salinity will lead to an increased osmotic gradient between the fish and the seawater which will exacerbate water loss to the environment. To compensate for this, fish would have to drink seawater and extract water from it at a faster rate. Greater drinking will lead to an increase in the rate of supply of calcium ions to the intestine, and presumably also a need to increase bicarbonate ion secretion rates and therefore an increase in the calcium carbonate precipitation rates. Over large changes in salinities (9, 35 and 50 practical salinity units - psu) carbonate production rates do increase at higher

salinities (Genz et al., 2008), however these salinity differences are much larger than is generally observed in the oceans. Large scale ocean salinity ranges are more typically between 30 and 40 psu (Millero et al., 1998; Vine et al., 2015). So far, the effect of salinity on fish carbonate production rates across this narrower range of salinities has not been tested. It could be that rates of production are higher in tropical areas, compared to temperate ones, due to salinity in addition to temperature.

In order to provide information that will help refine our understanding of how fish carbonate production rates can change and better estimate production rates across different ocean regions, chapter 4 examines carbonate production rates from fish held at 30, 35 and 40 psu as salinities that are found across the natural ocean range. To assess whether any changes in production rates observed are likely to be applicable across fish species in general, production rates were measured in two different species. Additionally, other physiological parameters (such as oxygen consumption and drinking rates) which could be related to osmoregulation and carbonate production rates in attempt to identify the cause of any difference between the two species examined. Therefore, chapter 4 provides information that might be used to better estimate fish carbonate production rates across various ocean regions in the future.

1.6 Summary of key questions

Accurate ocean carbonate budgets of production, dissolution and accumulation will ultimately aid our understanding of atmosphere and ocean CO_2 fluxes. The discussion above highlights the current deficit in our knowledge of the inorganic carbon or carbonate pump, especially concerning the estimation of accurate carbonate budgets for extratropical areas. This is particularly concerning as some oceans in extratropical and temperate areas are likely to be some of the most efficient at sequestering carbon and carbonate on our planet. As such this thesis aims to enhance our understanding of carbonate budgets in temperate areas by considering a previously unrecognised, but potentially large, source of carbonate production in temperate areas: teleost fish.

The studies presented in this thesis systematically address the solubility, sedimentary accumulation and production rate of carbonates produced by fish with particular attention to temperate areas and species.

1.7 Figures

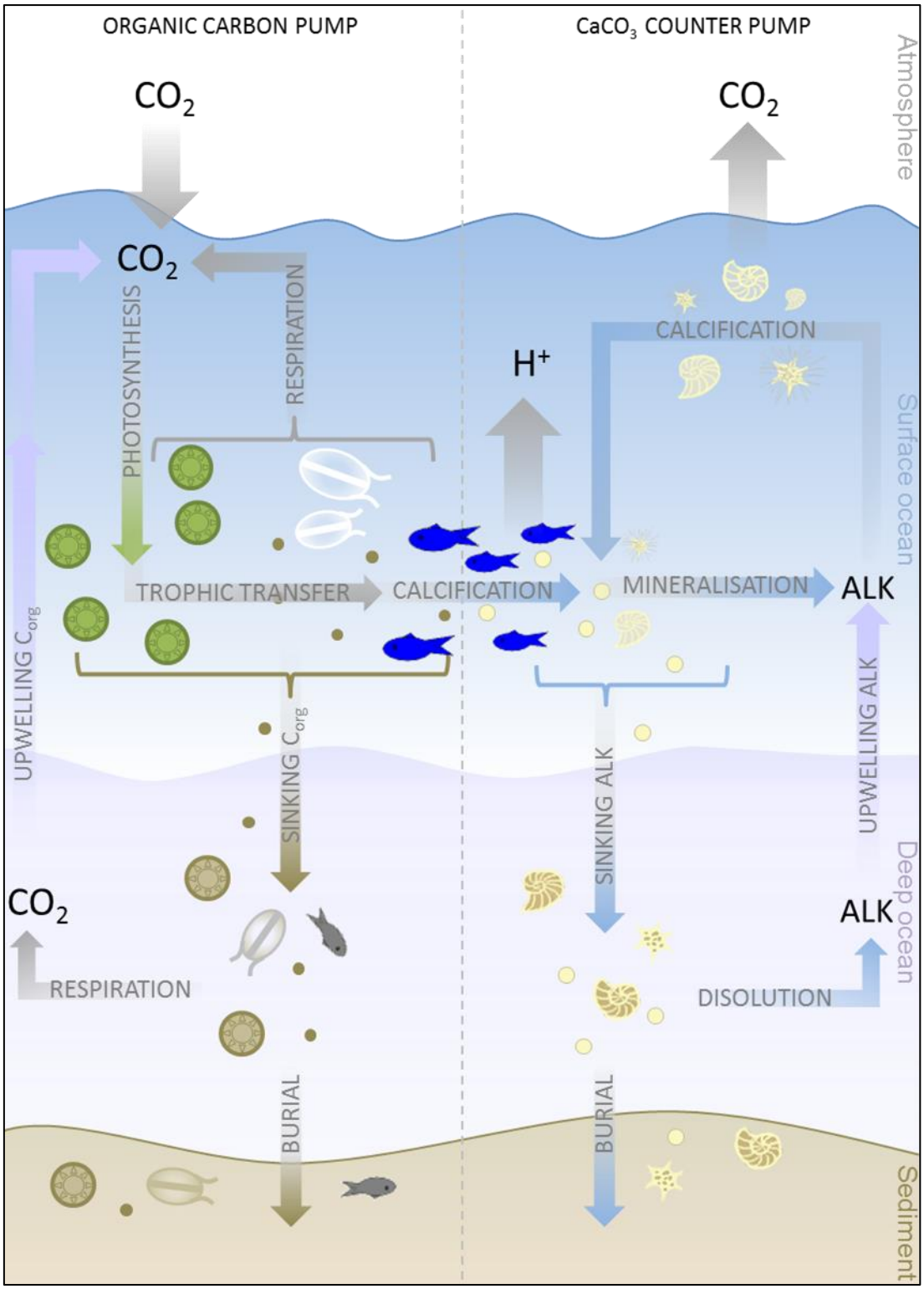

Figure 1 Ocean carbon pumps

A schematic of the oceans “organic carbon pump” (left) and “carbonate counter pump” (right). The organic carbon pump acts to sequester CO_2 from the atmosphere through incorporation into organic matter (C_{org}) by photosynthesis. C_{org} is then transported to the deeper ocean layers and sediments through

sinking. Conversely the creation of calcium carbonate through calcification in marine organisms releases CO_2 in ocean surface layers and subsequently the atmosphere, while trapping surface ocean alkalinity and transporting it to deeper ocean layers and sediment. Fish are distinct from many other calcifying organisms in that they use respiratory CO_2 (which would otherwise be excreted directly into ocean surfaces waters) to create calcium carbonate rather than relying on pre-dissolved seawater alkalinity. As fish are heterotrophic they ultimately gain this carbon from photosynthesis of primary producers which removes CO_2 from the seawater. This figure is adapted from diagrams presented by Lea (2014).

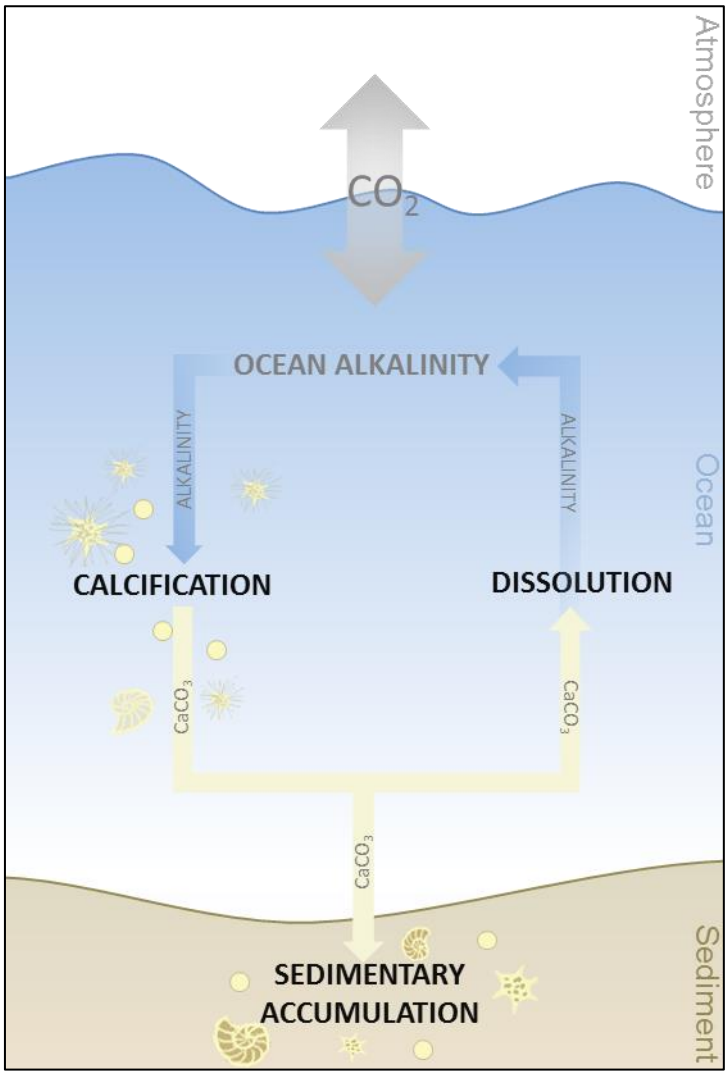

Figure 2 Carbonate budgets

A diagram showing the major components of carbonate budgets (bold black text) and the effect on ocean alkalinity and subsequently atmosphere and ocean CO_2 exchange. Calcification (production of calcium carbonate) removes alkalinity from the ocean forming solid calcium carbonate which either accumulates in sediments, removing it the alkalinity from the ocean, or dissolves, returning alkalinity to the seawater. The subsequent effect on ocean alkalinity impacts the ability of CO_2 to dissolve in seawater. The sum of the dissolution and accumulation rates of carbonates should be equal to the total production rate of carbonate in the ocean and as such is often referred to as a "budget". This diagram is a visual representation of some of the concepts presented by Smith and Mackenzie (2016).

2 Factors affecting intestinal carbonate crystal characteristics of wild temperate fish species

2.1 Summary

Marine teleost fish are volumetrically important producers of calcium carbonate in the global ocean and of carbonate sediments in tropical areas in particular. As such marine fish therefore make a significant contribution to ocean inorganic carbon cycle. It is however currently unclear how much of the carbonate produced by marine fish contributes to sediment production rather than rapid dissolution. Calcium carbonates produced by fish can have varying morphologies and magnesium contents, both of which can alter the solubility of carbonates. However, there is currently no knowledge of the characteristics of carbonates that could be produced by temperate fish. Marine teleost fish are relatively abundant in temperate climates and therefore are likely to be volumetrically important producers of calcium carbonate. Although different species tend to consistently produced carbonates of similar characteristics, previous studies have not yet identified factors that relate to variability in the characteristics of carbonates produced. As such the current study collected and examined the morphology, size and elemental composition of carbonates from 21 different species of temperate fish caught in the wild. Additionally carbonates were repeatedly collected from a single species, poor cod (*Trisopterus minutus*) in ten different months across an entire year to examine variation relating to seasons. Data on carbonate characteristics were examined in relation to collection month, fish length, fish condition and sex in an attempt. It was found that the largest variation in elemental composition and size of crystals occurred between distinctly different morphology types of carbonates identified. By contrast, generally there was low variation in crystal characteristics within these morphology types across different collection months, species, fish lengths, condition and sexes. As such it was concluded that in future research determination of the morphology types produced by fish may be sufficient to predict the elemental composition of fish carbonate crystals. As elemental composition is likely to be related to solubility, knowledge of the proportion of different morphology types produced by fish will be useful in predicting the solubility of fish carbonates in the ocean.

2.2 Introduction

Marine teleost fish have been recognised as major contributors to the production of oceanic calcium carbonate (Wilson et al., 2009). They precipitate crystals of calcium carbonate within their intestines as part of an osmoregulatory strategy (Whittamore et al., 2010). These calcium carbonate crystals are then excreted into the environment. Oceanic calcium carbonate production is a part of biogeochemical processes which control ocean chemistry. The production of calcium carbonate decreases ocean pH locally, however it's dissolution raises pH locally. If calcium carbonate formed at the surface sinks to the ocean floor it contributes to carbonate sediment production and a decrease in surface ocean pH. Alternatively, rapid dissolution would regenerates alkalinity in the surface ocean as proposed by Wilson et al. (2009).

Previous studies have shown evidence that fish carbonates have the potential to contribute to sediment production in the Bahamas. Perry et al. (2011) showed that fine-grained high magnesium calcite particles produced by tropical fish are morphologically and mineralogically distinct from other common forms of carbonate mud in Bahamian surface sediments. In addition, it was shown that carbonates that appear to be of fish origin (based on their morphology and elemental composition) were present in Bahamian surface sediments. By integrating site specific fish biomass and fish carbonate excretion rate data they estimated that approximately 14 % of overall mud production in shallow water habitats of the Bahamas could be derived from fish. Subsequent work by Salter et al. (2014) examined the preservation potential of these particles in seawater and shows that clusters of fish-derived carbonate particles that form pellets can stay more intact than previously thought and as such fish produced carbonates may contribute to the fine sand fraction (>63 µm diameter particles) of sediments in addition to the mud fraction.

Although fish produced carbonates have the potential to contribute to sediment production, it is currently unclear how much of the carbonate produced by fish contributes to carbonate sediments without first dissolving. The depth at which calcium carbonate particles dissolve in the ocean is dependent on the solubility of the crystals. The solubility of the crystals will be dependent on size and composition of particles. Smaller crystal particles have a higher surface to volume ratio allowing for more surface contact with the surrounding seawater

and faster dissolution. Crystals which include different elements in their matrix will also have different solubility; for example, increased magnesium content can enhance solubility of calcium carbonates (Morse et al., 2007; Walter, 1984). In 1989, energy dispersive X-ray spectroscopy (EDS) revealed that crystals of calcium carbonate precipitated in the intestine of European eels (*Anguilla anguilla*) contained other elements such as magnesium as part of their structure (Humbert et al., 1989). In subsequent research it was confirmed that magnesium content is generally high in fish carbonates, contributing up to 40 mol% magnesium (Salter et al., 2012). This is higher than typically found in calcium carbonates produced by other marine organisms (up to about 18 mol%) (Cros et al., 2013; Pickett and Andersson, 2015). As such, high Mg calcite produced by fish may well be less stable than calcium carbonate produced by other organism in the ocean (Woosley et al., 2012). Additionally, it has been shown that various morphologies and elemental compositions of calcium carbonate crystals are produced by a range of tropical marine fish (Salter et al., 2014, 2012). Distinct crystal morphologies, particle sizes and magnesium contents, will undoubtedly be associated with distinctly different solubilities as well. Thus the solubility of fish carbonates is likely to be quite variable, in addition to the prior conclusion that they will collectively have a higher solubility than other more studied forms of carbonates in the ocean (e.g. calcite and aragonite). With this in mind, current understanding of calcium carbonate transport, solubility and fate in the ocean based on more conventionally studied calcium carbonate producers (such as coccolithophores) may inadequately predict the impact fish have on ocean chemistry and sediments.

In order to understand the impact of fish carbonate production on sediments and ocean chemistry, we need to know how much the solubility of fish produced carbonates varies naturally, which relates to crystal size, shape and composition. As such, determining how much these aspects vary across different species in different environments will provide a starting point for identifying which factors will be key for making predictions.

In nature variation in crystal characteristics of carbonates produced by fish is likely to occur both between species and within species potentially as a result of both endogenous and exogenous factors. For example, diet is likely to impact carbonate crystallisation in fish and vary naturally. Dietary intake and the

subsequent absorption of selected nutrients will clearly alter the intestinal chemical environment for crystallisation, and therefore the composition of the resulting crystals. Crystal shape and size may also be influenced by the molecules available as a result of feeding and digestion. *In vitro* studies which have yielded precipitates similar to those seen in fish, have shown that changing the ratio of calcium and magnesium ions or calcium to carbonate ions in solution affects the magnesium content and morphology of precipitated calcium carbonates produced (Blue and Dove, 2015; Yang et al., 2015). Even dietary intake of substance not directly incorporated into the crystal matrices have the potential to impact crystallisation of carbonates. For example, various organic compounds can affect crystallisation and stabilisation of calcium magnesium carbonates *in vitro* (Karakostis et al., 2016; Lauth et al., 2016; Qi et al., 2014; Wang et al., 2015; Wolf et al., 2015).

Diet clearly varies in the wild between different species of fish that fill different ecological niches. Also, even when dietary intake is similar different species may have different physiological needs and uptake of specific nutrients resulting in different intestinal chemistries and therefore crystals with different characteristics.

Additionally dietary intake and nutrient absorption can vary within fish species across different seasons as well as with life stage and developmental needs. Younger fish will have different prey to older, larger fish. Sexually developing males and females have different nutritional requirements. In aquaculture it is known that broodstock fish have special dietary requirements related to quality of gamete production (Izquierdo et al., 2001). Egg production for female fish is especially resource intensive; it has previously been shown that oestrogen (a hormone related to the development of female gonads in fish) has the potential to reduce calcium carbonate precipitation rates in fish, attributed to increased calcium uptake by the intestine required for egg production (Al-Jandal et al., 2011). If oestrogen in fish has the ability to alter calcium uptake to the extent it reduces precipitation rates it might also alter other characteristics of the carbonates. One effect of removing calcium from the intestine could be that carbonate precipitates contain more magnesium instead. It might be expected that within a species of fish, intestinal supply and demand may vary across fish of different size and sex and these are both factors which will relate to the state

of sexual development. In certain fish such as poor cod (*Trisopterus minutus*) spawning state can also be related to condition index. Condition index in fish is a measure of how heavy fish are compared to their length (i.e. equivalent to the body mass index in humans). Fish which have large amounts of fat and muscle reserves, or have well developed gonads will have a higher condition index. It has been shown previously that condition index is related to spawning for poor cod, increasing prior to spawning and decreasing rapidly after (Šantić et al., 2010). It might be expected that fish in poor condition, such as after spawning (Šantić et al., 2010), require a larger uptake of nutrients such as calcium in order to regain condition, whereas fish in good condition require less nutrients. Fish condition may therefore be another factor that relates to intestinal supply and demand within a fish species that could potentially alter the precipitation of carbonate.

In addition to changes in intestinal supply and demand (across fish length, sex and condition), temperature may be another factor that causes natural variation in the characteristics of carbonates within a species. Temperature has been shown to alter the magnesium and calcium ratios in other biogenic calcium carbonates with warmer temperatures favouring higher amounts of magnesium inclusion into carbonates (Butler et al., 2015; Lea et al., 1999; Xiao et al., 2014). It may be that this is also the case in fish-produced carbonates. Temperature naturally varies with season in the marine environment; thus fish of the same species may precipitate carbonates with different characteristics depending on the season.

So far it has been shown that tropical fish can produce a range of different carbonate types from which it might be possible to start to infer solubility (Salter et al., 2012). Previous studies have only ever linked variability of carbonates produced to differences in fish species (Perry et al., 2011; Salter et al., 2012) and have never explored the potential for variation in carbonate characteristics within species. Additionally these studies only examine carbonates produced by tropical species. There is currently no information available on the types of carbonates produced by fish living in other climate regions. Given the high abundance of marine teleost fish at temperate latitudes (Jennings et al., 2008) information on the characteristics of carbonates they produce would therefore be useful to understand the global impact of fish on the inorganic carbon cycle.

Another aspect not explored in these previous studies is the impact of food intake on the characteristics of carbonates produced by fish. Previous studies have focused on documenting the carbonates produced by unfed fish (Perry et al., 2011; Salter et al., 2014, 2012). Examining precipitates from unfed fish ensures there is no contamination of endogenously produced material with exogenous material from feeding. However, most wild fish will be feeding almost continuously as a necessary part of survival. It is possible that the presences of food in the intestine may alter the characteristics of carbonates produced by fish. One previous study shows an image of endogenously produced carbonate alongside exogenous material collected from fish soon after capture from the wild (Salter et al., 2012), however further work is required to investigate potential effects feeding might have on the characteristics of fish derived carbonates.

The present study aims to address some of the gaps in the knowledge outlined above. The present study aimed to assess what natural variation might occur in the characteristics of carbonates produced by fish both across different species and within a species across a year. Characteristics of calcium carbonates produced by 21 different species of temperate marine fish were observed. Carbonates were collected directly from the intestine of fish captured off the coast of the UK ensuring carbonates examined were precipitated in the intestine of fish which had consumed a natural diet and experienced natural environmental conditions such as temperature. Furthermore, fish were captured at different times of the year, with poor cod (*Tripsopterus mintus*) being sampled across 11 months of a year in an attempt to capture natural temporal variation in carbonate characteristics within a single species. Characteristics of carbonates (including crystal dimensions and elemental composition) were measured and compared to other variables related to their collection such as fish species, length, condition index, sex, and month in which they were collected.

2.3 Methods

2.3.1 Study area and sampling

Fish from a total of 21 different species of marine teleosts were collected via otter trawls in from research vessels in the Western English Channel and the

North Sea. Fish were captured in the North Sea from a variety of locations as part of the International Bottom Trawl Survey (ICES, 2012) in the months of August and September between 2011 and 2013. Fish were captured in the Western English Channel 15 km south west of Plymouth, England (50° 15' N, 4° 13' W) as part of the Marine Biological Association of the United Kingdom Standard Haul Time Series Otter Trawl Survey (MEDIN, 2017). The site is designated as "L4" as part of the Western Channel Observatory long term observations (WCO, 2017) and trawls were carried out of at a depth of approximately 55 m on 20 different days in 11 different months between April 2014 and May 2015. For ease of analysis data has been grouped by month, only rather than day. Due to vessel maintenance it was not possible to collect fish in August. A non-random subsample of fish was taken from hauls for dissection and collection of intestinal contents, biased to be large enough in size for easy dissection. See Table 1 for details of which species were obtained from which sites, the taxonomic order they belong to and the numbers of individuals sampled. See Table 2 for details of which species were collected in each month, and see Table 5 for details of the sizes categories. From L4, a second random subsample of poor cod was taken throughout the year to assess monthly average fish sizes.

Following collection of fish from both subsamples, body lengths were measured to the nearest mm from the tip of the snout to the longest extent of the tail and fish wet mass was measured to the nearest gram using a spring balance.

Once fish mass and length were obtained, fish subsampled for dissection were opened through an incision into the body cavity and the sex was noted if discernible. The intestines were then dissected as soon as possible following collection, and contents squeezed into clip top 1.5 ml microcentrifuge tubes which were stored in cool bags for approximately 3 to 6 hours until they could be transferred to -20ºC for longer term storage. Samples were then thawed, 5% sodium hypochlorite (bleach) was added and tubes placed on a shaker until all contents appeared white as a method of removing organic matter without affecting the carbonate crystals (Pingitore Jr et al., 1993). Samples were then centrifuged at 5000 rpm for 3 minutes (Eppendorf 5804R centrifuge) and the liquid supernatant was removed leaving solid particles (including intestinal precipitates) in the bottom of the tube. To rinse off the bleach, ultrapure water

was then added to the remaining solid particles and the tubes shaken to mix. Samples were then centrifuged, and supernatant liquid removed. This rinsing process was repeated 3 times or until no noticeable odour of bleach remained. After as much liquid as possible was removed the samples were then dried at 40 °C. Once dry samples were then stored at room temperature in sealed microcentrifuge tubes until analysis with scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS).

2.3.2 Morphological analysis

Analysis of intestinal precipitate morphology was carried out by SEM using a Hitachi S3200N SEM-EDS microscope using a working distance of 15 mm and an operating voltage of 20 KeV. Clean dry intestinal precipitates from an individual fish were sprinkled onto self-adhesive carbon spectrotabs mounted on stubs. Samples were subsequently sputter coated with a 20 nm gold palladium layer to aid conductance. At 3 distinct locations on each stub secondary electron images were taken at 500, 2000 and 5000 x magnification. Locations were chosen in an attempt to incorporate and show the maximum number of different endogenous morphologies present on the entire stub. Material on the stub was considered to be endogenously precipitated if it displayed a regular structure at some level and was morphologically dissimilar from more obviously exogenous particles such as shell fragment or coccolithophores. This was further confirmed by EDS analysis; endogenous particles were assumed to be those emitting a strong calcium or magnesium signal. Particles with strong sodium and chloride readings (indicating they were present in greater quantity than calcium and magnesium) were disregarded as salt crystal contamination. Typical examples of all types of particles considered to be of endogenous origin can be seen in the figures presented in section 2.4.1. Crystal length and width measurements were taken from 10 different crystals on each image at 5000 x magnification using Image J if possible; if there were less than ten individual crystals present in an image then all crystals were measured. Crystal aspect ratios were calculated as crystal length divided by the width.

2.3.3 Energy dispersive X-ray spectroscopy (EDS)

At each of the 3 locations where secondary electron microscopy images were taken on each stub (as described in section 2.3.2), at least 3 EDS spot measurements were taken. If more than one distinct crystal morphology was present at a location, 3 EDS spot measurements were take on each of the crystal types present. The magnesium content as Mg mol% was obtained by calculating the magnesium content as a percent of the sum of both magnesium and calcium atomic % values. Magnesium content was expressed as a percentage of the calcium and magnesium content because the EDS also picked up varying percentages of carbon and oxygen, the measurements of which are not likely to accurately reflect the carbonate content of the crystals due to potential contamination from the carbon spectrotabs used to mount the samples and the atmosphere during storage and handling. Contamination from other sources of carbon and oxygen will lead to variations in the percentage of calcium and magnesium being detected. Fish intestinal carbonate precipitates should contain calcium and magnesium as the major cations, as such, one mole of precipitate should contain 1 mole of a combination of calcium and magnesium ions. By calculating the magnesium as a percent of the sum of the calcium and magnesium atomic % values obtained from the EDS the resulting number should therefore represent the percentage of a mole of precipitates that magnesium contributes to being the cation for.

Phosphorus was an element that was also consistently detected in high amounts relative to other elements in certain crystal morphologies. It was assumed that this elemental phosphorus was likely to be in the form of phosphate. If phosphorus is in the form of phosphate, for every calcium or magnesium ion in calcium carbonates it would be expected that there is also a carbonate or phosphate ion. As such, the phosphorous content as P mol% values were calculated as a percentage of the combined Mg and Ca atomic %. This assumes that the remaining anionic content is made up by carbonate, which is not directly measureable by EDS due to the high levels of background carbon and oxygen. Calculated Mg mol% and P mol% values for each EDS spot were then averaged per image and then the mean of all images per individual fish were calculated for each morphology type before calculating any

further means for morphologies or taxonomic order. This was to avoid pseudo replication as variation was greatest between individual fish.

2.3.4 Statistical analysis

Values for crystal phosphorus and magnesium content, and crystal dimensions (length, width and aspect ratio) were averaged per image (see sections 2.3.2 and 2.3.3 for details) and then the mean of all images per individual fish were calculated for each morphology type before calculating any further means for capture months, fish size, sex, or before testing for correlations. This was to avoid pseudo replication as variation was greatest between individual fish. The only exception to this was for monthly averages of crystal dimensions taken on nanospheres obtained only from poor cod due to nanospheres of a measurable size only being present in one individual each month. In this case, on Figure 19, length, width and aspect ratio of nanospheres represent the mean and standard error from all images taken within the same individual fish. Means are presented as mean ±standard error.

Fish condition index was calculated from randomly subsampled fish as described in Equation 4, where M is the mass of fish in grams and L is the total length of fish in cm.

Equation 4

$$Condition\ index = M/L^3 \times 100$$

To test whether crystal characteristics (magnesium content, phosphorus content, crystal length, width and aspect ratios) could vary significantly over time, ANOVAs were carried out for characteristics of ellipsoids over months on data obtained from all species combined and for data obtained from just poor cod. Nanospheres were observed in too few individual fish to allow statistical testing over months in data obtained from just poor cod. Differences in crystal characteristics between male, female and individuals whose sex could not be identified were tested through ANOVAs on data obtained just from poor cod. Additionally, correlation tests were carried out between poor cod condition and carbonate characteristics (crystal size and composition). Correlation tests were also carried out between average crystal characteristics for each fish (length,

width, aspect ratio, magnesium content and phosphorus content) and fish length utilising data from all species collected and also from all poor cod separately. To investigate whether particular carbonate types might correlate with the presence of other carbonate types, their frequencies were correlated against each other (for all species and for poor cod separately). All correlation tests were carried out using Pearson's correlation in IBM SPSS V23 statistics package. ANOVAs were carried out using the same statistical package.

2.4 Results

SEM examination of fish intestinal contents revealed plenty of carbonate precipitates that were very similar to the endogenously produced precipitates described by Salter et al. (2012), alongside large amounts of apparently exogenous matter. Material that was identified as endogenous (as determined by the methods described in section 2.3.2) was classified into categories described below based on their distinctly different elemental compositions and crystal morphologies.

2.4.1 Types of precipitate

Four main morphological categories of endogenous precipitates were identified: monocrystalline ellipsoids, nanospheres (and variants, see section 2.4.1.2), granular particles and polycrystalline (composed of multiple inter-grown crystals) structures. The most common crystal types across all species examined were monocrystalline ellipsoids and nanospheres (Table 1). Both these types were less than 5 µm in length or diameter (Figure 3). Polycrystalline structures contributed the largest particles, some of which were in excess of 180 µm in length. Average magnesium contents varied between morphologies (Figure 2). Phosphorus was found to be a major component of granular and nanosphere crystals, but was absent or present in very low levels in polycrystalline structures and monocrystalline ellipsoids (Figure 2). Below are detailed descriptions of morphologies and compositions for each category of crystal morphology observed. Fish species are referred to with their common English name, but for a full list of species see Table 1.

2.4.1.1 Monocrystalline ellipsoids

The monocrystalline ellipsoids observed in the temperate wild fish in this study showed a high degree of consistency to those reported from unfed tropical

species by Salter et al. (2012), however magnesium content was generally lower (in the range of 8 to 13 mol% compared to 20 to 30 mol% observed in tropical species (Salter et al., 2012). Monocrystalline ellipsoids were present across all taxonomic orders of fish sampled (although not from all species within each taxonomic order) and were the second most predominant crystal type when considering all species (Table 1). Across all taxonomic orders sampled, magnesium content of monocrystalline ellipsoids was fairly similar, averaging 10.3 mol % and phosphorus content was low, averaging 1.7 mol% (Figure 5). Examples of typical monocrystalline ellipsoids from a variety of species are shown in Figure 6.

2.4.1.2 Nanospheres and derivatives

Nanosphere type particles were the most ubiquitous morphology type, being present in 19 out of 21 fish species studied and present in most individuals (Table 1). These were similar to the nanospheres described from tropical species by Salter et al. (2012). Nanospheres in the present study tended to extend to a slightly larger size (up to 3.07 µm) than those described previously (Figure 3), examples of which are shown in Figure 7 E and F. The ability to measure fine scale nanospheres was limited due to working distances required for EDS analysis limiting resolution of images obtained at high magnification. As such it was difficult to measure nanosphere type particles less than 0.2 µm and these very fine particles may be underrepresented in Figure 8. Examples of this very fine nanosphere material can be seen in Figure 7 (G, H and I). In some cases nanospheres were extended, to varying degrees, along one axis, becoming “pill” or “jelly bean” shaped (Figure 7, A, B and C). These differ from the shape of monocrystalline ellipsoids as they are rounded hemispherically at the ends rather than tapering and are generally found alongside the monocrystalline spheres they appear to be related to. Across all types of nanosphere variations described above, magnesium content averaged at 23.2 mol% and phosphorus content at 27.2 mol%, greater than any of the other particle types examined in this study (Figure 4).

2.4.1.3 Polycrystalline structures

Polycrystalline particles were not as common as the monocrystalline particles described above only being observed in 8 out of the 21 species studied and never in more than 67 % of the individuals examined in those species (Table 1).

These structures ranged greatly in size with some particles attaining sizes greater than 180 µm in length (sizes larger than this were observed in 4 out of the 25 examples of polycrystalline structures examined but were not possible to measure due to the methods used in this study) (Figure 3). A number of morphologies were observed for the polycrystalline structures present in this study and both the individual constituent crystals and the overall superstructures they formed varied. Superstructures included dumbbell, wheatsheaf, polycrystalline ellipsoidal and interlocking spherical structures similar to those describe by Salter et al. (2012) (Figure 9, D, E, F and C respectively). In addition to previously described crystalline superstructures, crystalline “chunks” with no apparent regular superstructure (Figure 9, A and B), and polycrystalline structures adhered to previously ingested material were observed (Figure 9, G and H). Average magnesium and phosphorus contents of polycrystalline structures were relatively low (4.96 mol% and 0.44 mol%, respectively) across all taxonomic orders sampled (Figure 5).

2.4.1.4 Granules

In addition to the precipitates described above an additional category of particle was observed which appeared to be endogenously precipitated (as determined by the methods described in section 2.3.2), although were difficult to allocate to any of the above described categories due to inconsistent particle sizes and shapes. This was possibly due to contamination with exogenous material or the presence of a mixture of the above described particles in close proximity. In these cases material was classified as “granules”. Examples of the typical variety of particles classed as granules are shown in Figure 10. These particles were all less than 4.06 µm in length. Granules were only present in 7 of the 21 species examined and only in cod they appeared to be present in more than 50 % of the individuals within those species because only one individual of cod was sampled (Table 1). Average magnesium and phosphorus content of particles classed as granules had high variation compared to other morphology types; standard errors for average granule magnesium and phosphorus content were 32 and 34 % of their respective means (Figure 4 and Figure 11).

2.4.2 Variation between species and taxonomic orders

2.4.2.1 Morphological types produced

The frequency with which different morphology types were observed in different species sampled is shown in Table 1. Species within the same taxonomic order do not necessarily produce similar morphology types.

2.4.2.2 Crystal sizes

Fish from different taxonomic orders produce monocrystalline ellipsoids with slightly different size distributions. Pleuronectiforme and perciforme species only produced monocrystalline ellipsoids up to a maximum of 1.57 µm and 1.50 µm in length respectively, whereas Gadiformes and Scorpaeniformes produced ellipsoids over a wider range of sizes, up to 2.41 µm in length (Figure 8). Size distributions of nanosphere type crystals were less distinct between fish from different taxonomic orders (Figure 8). All taxonomic orders produced nanospheres up to 2 µm in length except for Scorpaeniformes which only produced nanospheres up to 1.3 µm.

2.4.2.3 Crystal composition

For all morphology types observed, magnesium and phosphorus content was fairly consistent across different taxonomic orders sampled (Figure 5 and Figure 11), yet composition was distinct between morphology types (Figure 4).

2.4.3 Variation across sample months

2.4.3.1 Morphological types produced

The frequency with which different morphology types of carbonates were observed across all species sampled in the Western English Channel varied each month with no obvious pattern (Figure 12 and Table 2). Generally ellipsoids and nanospheres were the most common morphology type across the year, being present in 46 and 49 % of individuals from the Western English Channel, respectively (Table 2). It was not possible to statistically test whether the frequencies were significantly different between months due to the low number of fish observed each month.

The frequency varied across months for poor cod as well (Figure 13 and Table 3). Ellipsoids were present in 100 % of individuals sampled in May, November, December and January, whereas they were only present in 33 % of individuals in September. Nanospheres were most common in October, when they were

observed in 67 % of all the fish sampled, whereas they were not observed at all in fish sampled in May, November and December. Polycrystalline structures were most common in May when they were observed in 50 % of the individuals sampled, yet they were not present in any individuals sampled September through to January. Granules were most common in April and January where they were present in 50 % of the sampled individuals but were not present in any individuals sampled between May and December, except in 1 of the three fish sampled in September. It was not possible to statistically test whether the frequencies were significantly different between months due to the low number of fish observed each month.

2.4.3.2 Crystal sizes

Summaries of the highest and lowest monthly average crystal sizes in relation to the average crystal sizes across the whole year are shown in Table 4.

Across all species sampled from the Western English Channel monthly averages for ellipsoid crystal sizes are shown in Figure 14. For details on the numbers of each species sampled each month see Table 2. Monthly average sizes for ellipsoids sampled in poor cod are shown in Figure 15. When tested by ANOVA, there was no significant difference in crystal length, width and aspect ratio between months for ellipsoids measured from all species and for ellipsoids measured from poor cod only.

In general the monthly average size of nanospheres across all species sampled from the Western English Channel was more variable than for ellipsoids (Figure 14 and Figure 16) with no obvious pattern. For details on the numbers of each species sampled each month see Table 2. There was no significant different between months (ANOVA) for any of the measured crystal dimensions from all species.

Not all observed nanospheres were large enough to measure from images taken due to a limitation in resolution. As such, low numbers of possible measurements in some months meant it was not possible to calculate averages across different individuals sampled when examining nanosphere dimensions from poor cod. Instead the mean dimensions of nanospheres examined from poor cod only are as an average of image averages per month or year, rather than a mean of individual fish. Crystal sizes measured in this way are marked with an “*” on Table 4. Monthly averages are shown in Figure 17. Due to the low

numbers of observations possible (because of image resolution limitations) of nanospheres it was not possible to statically test differences between months.

2.4.3.3 Crystal composition

Summaries of the highest and lowest monthly average elemental compositions in relation to the average elemental composition across the whole year are shown in Table 4.

Across all species sampled from the Western English Channel generally magnesium content of ellipsoids seemed fairly consistent across months until January when they appeared to decline until sampling finished in March (Figure 18). Average phosphorus content was more variable. Monthly average nanosphere magnesium and phosphorus content was highly variable (Figure 18). There were no significant differences in magnesium or phosphorus content of ellipsoids and nanospheres between months when tested by ANOVA.

Patterns in monthly average magnesium and phosphorus content of ellipsoids and nanospheres were similar when examined for just poor cod (Figure 19). Phosphorus measured in ellipsoids was generally low and variable, averaging at a maximum of 2.9 ±2.6 mol% in October and non-existent in April, September and December. However, there was no significant difference in magnesium or phosphorus content of ellipsoids observed in poor cod between months. Due to the low numbers of observations of nanospheres it was not possible to statically test differences between months in just poor cod.

2.4.3.4 Average monthly poor cod lengths

Poor cod subsampled for intestinal contents were generally larger on average than fish randomly sampled for length but appeared to be fairly representative of monthly changes present in fish randomly sampled from the haul (Figure 20).

2.4.4 Variation across fish of different lengths

2.4.4.1 Morphological types produced

The frequency with which different morphology types were observed varied with fish length for all fish species sampled (Figure 21). Ellipsoids were only absent in the 230-249 mm size category, and the prevalence of ellipsoids remained low in larger size categories (e.g. present in ≤20 % of fish that were >270 mm in length; Figure 21 and Table 5). Apart from a decrease in the prevalence of

ellipsoids in larger fish there were no other obvious patterns for frequency of crystal morphology versus fish size.

By contrast, within poor cod the fish larger than 190 mm was the only size category where none of the morphology categories were absent (**Table 6** and Figure 22). Apart from this there was no obvious pattern to the frequency versus size in poor cod. It was not possible to test the effect of fish length on the frequency of morphology observations due to low numbers of fish sampled.

2.4.4.2 Crystal sizes

There was no significant correlation between any aspects of ellipsoid or nanosphere size (crystal length, width and aspect ratio) with fish lengths for either all species collected from the Western English Channel or just poor cod (Table 6).

2.4.4.3 Crystal composition

Across all species there were no significant relationships between fish length and elemental composition of ellipsoids and nanospheres (Table 8). However, when poor cod were examined separately, there was a significant positive relationship between magnesium content of ellipsoids and fish length. For a doubling in body length from 100 to 200 mm, the magnesium content of ellipsoids nearly doubled. Additionally there was a significant negative relationship between phosphorus content of nanospheres and length of fish (Figure 23). For a doubling in in body length the phosphorus content decreased by approximately 4 fold.

2.4.5 Variation across fish of different sexes

2.4.5.1 Morphological types produced

Overall the different morphology types of carbonate were equally common in females, males and poor cod whose sex could not be identified. The only potential exception to this was that polycrystalline structures could have been more common in male poor cod; however, only 5 fish were successfully identified as males out of the 40 fish sampled, which increases the risk that this increase in frequency compared to other sexes was due to chance (Figure 24). Unfortunately due to the low number of males observed in the study it was not possible to statistically test whether the frequency of observations of morphology types was different between sexes.

2.4.5.2 Crystal size and composition

There were no significant differences between the dimensions or elemental composition of ellipsoids or nanospheres collected from male, female and unidentified poor cod (Figure 25 and Figure 26) as tested by ANOVA.

2.4.6 Variation across condition index

2.4.6.1 Morphological types produced, crystal size and composition

Across poor cod there were no significant correlations between fish condition index and frequencies of different crystal morphology types (Table 9), dimensions of ellipsoids or nanosphere crystals (Table 9), or their elemental composition (Table 10). Correlations with fish condition index were not tested for all species combined as condition index is so variable between species.

2.4.7 Relationships between the occurrence frequencies of different crystal morphology types

There was an inverse relationship between the occurrence of ellipsoids and nanospheres in each fish species (Table 12 and Figure 27). This was also true for all species sampled in each month (Table 13 and Figure 27). However, this negative correlation was not significant for poor cod alone across different months (Table 14) or different size categories (Table 15 and Figure 28). Additionally, this correlation was not significant across different size categories of all species combined (Table 16). The only other significant correlations between frequencies of other morphology types was a significant positive correlation between the frequency of ellipsoids and polycrystalline structures in poor cod across different size categories.

2.5 Discussion

The biggest variation in the carbonate characteristics found in wild-caught fish was between the different types of crystal morphology. The four distinct morphological types (ellipsoids, nanospheres, polycrystalline structures and granules) are described in section 2.4.1 and encompass those previously described in tropical species (Salter et al., 2012). The size, shape and composition of these different morphology types did not vary greatly between species that are members of different taxonomic orders (Figure 5, Figure 7 and Figure 11), or at different times of the year, or by fish of different length,

condition or sexes (Figure 14, Figure 16, Figure 18, Figure 25 and Figure 26). As such, the morphological type of carbonate produced is the greatest determining factor for the characteristics which relate to solubility of the carbonates produced by a fish. In order to determine the proportions of fish carbonates that contribute to sediment production versus rapid dissolution, it would therefore be useful to be able to predict the types of carbonates fish tend to produce based endogenous and exogenous factors. These are discussed below.

2.5.1 Differences between temperate and tropical species

There were several differences between the characteristics and morphologies of carbonates produced by the temperate species examined in the current study compared to precipitates observed previously from tropical species. Many of the morphologies described previously in tropical species (Salter et al., 2012) were also observed in the present study, although in the latter they were observed inter-mixed with exogenous ingested materials. In prior studies it was noted that generally the types of precipitates produced were fairly consistent within species and as such using morphological and compositional data, Salter et al. (2012) categorised tropical species as follows: Category 1 species produce predominantly monocrystalline ellipsoids. Category 2 species produced no monocrystalline ellipsoids and predominantly low magnesium (0.8-7 mol%) polycrystalline structures such as spheres and ellipsoids. Category 3 species have a dominant Mg carbonate and Mg-calcite phase in the form of rhombohedral structures. Category 4 have polycrystalline structures such as spheres and ellipsoids which are high in magnesium (19-23 mol%). In the present study, for some species, no morphology type was observed consistently across all members sampled limiting the usefulness of the categorisation system proposed by Salter et al. (2012). This could, however, in some species, be partly due to the difficulty of distinguishing and identifying endogenous material from other exogenous materials, thus leading to morphology types being missed in some samples. There were some species that appeared to produce primarily monocrystalline ellipsoids and as such could be classed as category 1 producers, but assigning the other species to the remaining categories is difficult. Polycrystalline material appeared in only 15% of all sampled fish and never consistently throughout all members of a sampled

species (Table 1). Also among those that were observed there were additional types of polycrystalline structures to those described by Salter et al. (2012). Some structures seemed to show no clear overall structure (Figure 9, A and B) and some were found adhered to apparently exogenous material which strongly influenced their overall shape (Figure 9, G and H). This type of precipitation, which uses existing structures as a base, has never before been documented in fish and could have important implications for modification of sinking and transport rates of particles to the deeper ocean. It additionally could mean fish are contributing carbonate mass to skeletal grains from other organisms when they ingest them, which has not been recognised in previous studies.

There were several differences between the frequency of different morphology types in the temperate species in this study and the tropical species observed previously. These differences may have either been caused by the differences the temperature, the diet of the fish and the collection methods between the studies. In the current study, species which produced predominantly polycrystalline structures were rare (Table 1) which contrasts with the tropical species examined previously (Salter et al., 2012). Tropical species which predominantly produced monocrystalline ellipsoids or a mixture of polycrystalline structures and nanospheres were comparatively rare. Nanospheres were only found in two tropical species (*Sphyraena barracuda* and *Albula vulpes*) out of the 21 different tropical species sampled by Salter et al. (2012), whereas in the present study nanospheres were observed in 18 of the 21 temperate species sampled.

Another difference between the precipitates from temperate species in the current study and from tropical species observed previously is the large amounts of phosphorus observed in some morphology types. The nanospheres observed in the present study contained a fairly high amount of phosphorus (average of ~27 mol%) (Figure 4). Other morphologies examined in the present study had typically low phosphorus contents (averaging <2 mol%) except granules which were hard to categorise and as such could contain a mixture of the other morphology types and phosphorus contents. Phosphorus content was only ever detected at levels of > 1 wt. % in the precipitates observed in tropical species by Salter et al. (2012).

The greater abundance of phosphorus containing nanospheres observed in the temperate fish here compared to previous studies on tropical species (Perry et al., 2011; Salter et al., 2014, 2012) may be more to do with feeding than the temperature. Previous studies on tropical species studied wild-captured fish that were held without food for at least two days prior to collection of precipitates thus avoiding contamination from exogenous material previously eaten by the fish. However, this would not be representative of most fish in the wild which will normally be eating continuously. By contrast, the current study examined carbonates from fish which had been feeding naturally in the wild and the presence of dietary material in the intestine could clearly affect the carbonate precipitation process as discussed in section 2.2. In particular, the presence of ingested material could explain the high proportion of phosphorus observed in nanospheres in the current study. Phosphorus is an element that is extremely low in seawater, less than 0.8 µM in the Western English Channel (Smyth et al., 2010) and all fish require phosphorus which is abundant in natural diets in order to maintain healthy growth (Robinson et al., 1987). The only potential source of phosphorus observed in the intestinal nanospheres is therefore via the diet. To further support this concept, unpublished lab-based studies from Rod Wilson's research group (Reardon et al., 2016), have shown that feeding fish (on various natural and commercial aquaculture diets) results in high levels of phosphorus in the intestinal precipitates compared to starved fish which is incorporated into the matrix of nanospheres.

However, there have been cases where nanosphere like material has been observed in fish species that were not fed. Nanospheres were found in two tropical species (*Sphyraena barracuda* and *Albula vulpes*) out of the 21 different tropical species sampled by Salter et al. (2012). Additionally, nanospheres were also observed in starved gilt-head seabream (*Sparus aurata*) and was identified as amorphous calcium carbonate (ACC) by FTIR spectroscopy (Foran et al., 2013). This would indicate that a source of phosphorus from the diet is not required for the formation of nanospheres. However, it may be that phosphate from the diet helps favour nanosphere formation over other morphology types. ACC can be stabilised by a number of factors including the addition of various organic macromolecules or phosphate to the surrounding solution (Qi et al., 2014). It may be that certain fish species are able to produce stabilising compounds in their intestine that allow ACC to form, but the presence of

phosphate from the diet may allow even more fish species to precipitate ACC. ACC can also adsorb phosphate (Xu et al., 2014) which may explain the apparent inclusion of phosphorus in the nanospheres in the present study and those in examined by Reardon et al (2016).

The previous studies on tropical species (Perry et al., 2011; Salter et al., 2014, 2012) also collected carbonates after excretion by fish into the seawater, whereas in the current study, material was collected directly from the intestine of fish following capture. It is possible that biological molecules are able to stabilise ACC within the intestine environment, but once excreted into seawater rapid recrystallization occurs into more complex polycrystalline species. Previous instances where material has been collected directly from the intestine of fish have shown predominantly amorphous carbonates even under starved conditions (Foran et al., 2013), however interpretation should be cautious as this was only in one species. Incubation of ACC in water can lead to crystallisation into more complex structures such as ellipsoids in approximately 12 to 24 hours (Wang et al., 2015). This could be the reason why more complex precipitates were observed in the tropical species compared to the temperate species in this study. Additionally it has been observed that ACC from starved fish dissolves rapidly in seawater (Foran et al., 2013); it could be that in the previous tropical studies that ACC was excreted by the fish but dissolved in the seawater before collection.

It still remains that there were high levels of phosphorus included in the nanospheres observed in the present study which is not the case in nanospheres produced by starved animals (Reardon et al., 2016). Higher levels of phosphorus are thus likely to be representative of fish in the wild due to feeding. Further work is needed to establish the effect of pre- and post-excretion sampling and feeding on carbonates produced by fish. Experiments which examine the morphology of carbonates from fed and starved fish both collected after excretion into the seawater and directly from the intestine should help to determine whether feeding or the method/timing of collection has an impact on the presence of nanospheres produced and excreted by marine fish.

The current study suggests that calcium carbonate excreted by wild fish is dominated by slightly smaller crystals and with a distinctively high phosphorous content than those seen previously in tropical species (Salter et al., 2014). This

may have an implication for the solubility of the carbonates precipitated from fish as smaller particles will have a higher surface area to volume ratio leading to a higher dissolution potential. In addition to being small the high phosphate content can also alter the solubility of calcium carbonates (Bentov et al., 2010; Greenwald, 1945; Morse et al., 2007; Raz et al., 2002; Sawada, 1997). Additionally, nanospheres in the present study tended to have higher magnesium content compared to the other morphologies examined (Figure 4) and magnesium content generally leads to less stable precipitates of carbonates (Walter, 1984). As such it will be important to establish the relative abundance of high phosphorus and magnesium containing nanospheres compared to large polycrystalline structures which are likely less soluble.

Monocrystalline ellipsoidal crystals in the current study generally had lower magnesium content (8 to 13 mol%) than in the ellipsoids from tropical species (20 to 30 mol%) (Salter et al., 2012). This might be due to the difference in temperature between these two climates, as cooler temperatures have been associated with reduced magnesium content of carbonates from other biogenic carbonate sources (Butler et al., 2015; Lea et al., 1999; Xiao et al., 2014). Previous unpublished lab-based studies from Rod Wilson's group have also suggested that experimentally reduced temperatures lower the magnesium content of precipitates produced by European flounder (Cobb et al., 2016). This will be important for understanding the global role of fish in the inorganic carbon cycle as solubility increases with magnesium content in calcium carbonates (Bertram and Mackenzie, 1991) (Morse et al., 2007).

2.5.2 Differences between temperate species

In the current study there was some variation in types of carbonates observed in different species (Table 1). In some species ellipsoids, which are lower in phosphorus, dominated and high phosphorus containing nanospheres were absent. If ACC nanospheres are the precursors to more complex structures (Wang et al., 2015), and phosphate stabilises calcium carbonate in the amorphous form and inhibits crystallization (Walter and Burton, 1986), it is interesting to consider that some species have a mechanism of excluding phosphorus and allowing the formation of more complex structures such as ellipsoids. If large amounts of phosphorus can be adsorbed by ACC (Xu et al., 2014) then this may reduce the bioavailability of this essential nutrient for

uptake, and ultimately growth and maintenance (Andrews et al., 1973; Chávez-Sánchez et al., 2000; Robinson et al., 1987). Indeed, for marine fish at least this could contribute to the notoriously inefficient uptake of dietary P in fish (only about half is typically absorbed). This is an issue of environmental importance as it can lead to significant eutrophication caused by high-P effluents from aquaculture (Sugiura et al., 2006). The mechanisms behind the precipitation of lower and higher phosphorus carbonates is unclear. However as phosphate can alter the solubility of calcium carbonates (Bentov et al., 2010; Greenwald, 1945; Morse et al., 2007; Raz et al., 2002; Sawada, 1997) it may therefore be important to identify species that produce different phosphorus content of precipitates to consider that they might have separate post excretory pathways.

2.5.3 Differences across sample months

Despite variability in the frequency of different morphology types over time, there was no discernible pattern to this monthly variation (Figure 12). This would indicate that seasonally variable factors do not have the same effect across multiple species with respect to the type of carbonate morphology produced by fish. However, the tendency for poor cod to produce predominantly ellipsoids was maintained across the whole year (Table 3). Ellipsoids were the predominant morphology found in poor cod in all months. Although the consistent production of the same morphologies has been previously suggested (Perry et al., 2011; Salter et al., 2012) this is the first substantial evidence of a single species producing predominantly one type of morphology over the entire annual cycle and the changing environmental and biotic factors this entails.

In addition to ellipsoids always being the most predominant form of carbonate produced by poor cod, they were also relatively consistent in their average dimensions and elemental composition throughout the year. However, some minor temporal patterns were apparent: the fall in Mg content from December to March (Figure 19), which was potentially be due to the lower winter temperatures as discussed above (Butler et al., 2015; Lea et al., 1999; Xiao et al., 2014). Temperature at depth (50 m) at L4 has previously been shown to be highest in September and lowest in March (Smyth et al., 2010). However, it seems strange that magnesium contents of ellipsoids from the preceding April is much higher, despite temperatures not rising much compared to March (Smyth et al., 2010). It could be that particular year that the samples were taken in had

an unusually warm April. Alternatively, it could be related to the length of the fish sampled. It was found that magnesium content of ellipsoids was correlated significantly with the length of fish sampled (discussed in section 2.5.5). The average length of the fish sampled also appeared to have the pattern where it decreased from December to March (Figure 19). Without details of the exact temperature of across the sampling time, or further lab based studies which control different factors, it may not be possible to detangle the effects of fish length and temperate on magnesium content of ellipsoids. Similar trends in the ellipsoid characteristics was seen across all species sampled, however this may be because poor cod make up large fraction of all the fish sampled throughout the year.

In general the dimensions and elemental composition were more variable across months than those of ellipsoids (Figure 19). However, due to the lower number of nanospheres observed in poor cod it was not possible to test whether the differences between months were statistically significant. When it was possible to statistically test for significant differences in nanosphere characteristics between months due to inclusion of data from all species, no significant differences were found. Characteristics of nanospheres were general very variable within each month.

Dietary phosphorus input may explain the variations in the frequency with which nanospheres were observed across the year in poor cod in the current study. Feeding changes seasonally with previous studies showing that more fish have empty stomachs in the winter and spring (Šantić et al., 2009). This may explain why in many of the cooler months nanospheres were not observed at all in poor cod, whereas in other warmer months they were observed in up to 67 % of the sampled fish (Figure 13). However, only two fish were sampled in January, and one of these had nanospheres. It seems likely that this apparent “peak” in a winter month is simply an artefact of the very low sample numbers. Interestingly, this trend across time was not present when data from all different species were included. This could be because different species feed on different prey types which are more abundant at different times of the year or it could be because carbonate production across different species responds differently to the presence of food in the intestine.

Overall there appears to be little change in the characteristics of carbonates produced by a species over the annual cycle.

2.5.4 Differences between fish of different sexes and condition

Poor cod was the only species collected in sufficient number to provide males and females collected consistently across different times of the year. The present study found no clear differences in the frequency of different types of morphology due to sex by comparing females, males and fish with unidentified sex (Figure 24). Similarly, there was no significant effect of sex on precipitate dimensions or elemental composition for ellipsoid and nanosphere carbonates (Figure 25 and Figure 26). Thus it would appear that sex of the fish did not influence the carbonates produced by fish in this study. However, this is based on comparing the sexes across a whole year, whereas poor cod typically only have one main spawning period per year (Unluoglu, 2015) which is between February and May in the Plymouth area (Western English Channel) (Menon, 1950). It could be that any difference between males and females would only be apparent in the build up to spawning, in particular while females are developing eggs. It was not, however, possible to examine differences between males and females on a monthly basis due to the low numbers of fish sampled, 40 in total, of which only 5 were successfully identified as males. It was however possible to examine the effect of body condition index on carbonate characteristics of poor cod, as hypothesised in Section 2.2. However, no significant correlation was observed between monthly average fish condition and monthly observation frequencies of different carbonate types (Table 9) or monthly average carbonate crystal dimensions or elemental composition (Table 10 and Table 11). The present study shows little evidence that the spawning and sexual development have an impact on the characteristics of carbonates produced by fish.

2.5.5 Differences across fish length

A factor which was found to have a strong relation with carbonate crystal characteristics was fish length of poor cod. Body length was found to positively correlate with the magnesium content of ellipsoids and negatively correlate with the phosphorus content of nanospheres (Table 8 and Figure 23). One potential explanation why body size may influence this relates to the diet of poor cod which generally alters with their length (Mattson, 1990; Šantić et al., 2009)

which in turn may alter the environment of intestine sufficiently to alter the elemental composition of the carbonates precipitated.

When all species were examined, no significant correlations were observed between fish length and any crystal characteristics. However, it might be that differences between species obscure any relationships between fish length and carbonate characteristics when data from all species are combined together.

The frequency with which different types of carbonates were observed changed across length of fish when all fish and just poor cod were grouped into length categories (Figure 21 and Figure 22), although there was no obvious overall pattern especially across all species examined together. Unfortunately it was not possible to statistically test the significance of fish length on the types of carbonate produced due to the low numbers of fish sampled.

Overall, fish length could be a factor that relates to the characteristics of carbonate produced by fish within a species but it would appear that this is not the case across multiple species.

2.5.6 Conclusions and future steps

This study shows that different carbonate morphology types provide the greatest variation in particle sizes and elemental compositions that are likely to lead to the most important differences in solubility after excretion by fish. In turn, this is likely to have the greatest influence on the fate of fish carbonates in nature, i.e. how much fish carbonates contribute to sediment production versus rapid dissolution. Furthermore, this study shows that the characteristics of the carbonates produced within a species tend to be fairly consistent in nature. Therefore, the next biggest challenge to predicting the solubility of fish carbonates in general across the oceans is to predict what morphology types different species are likely to be produced in different climate zones. Unfortunately limited sample numbers limit the conclusions that can be drawn regarding the influence of time of year and taxonomic orders on the frequency with which different morphology types are produced by fish. Sampling a larger population of fish within each month and taxonomic group would be required to make the analysis more statistically robust.

It was, however, noticeable that across different months and species sampled there was a significant negative correlation between the frequency of

nanospheres and ellipsoids (Figure 27). Further, in poor cod in isolation, there was a significant positive correlation in the frequency of occurrence of ellipsoids and polycrystalline structures (Figure 28). This is quite surprising as some categories had low numbers of fish sampled in total which limits the number of possible percentage frequencies that can occur. For example a category with only 3 fish sampled in total in (such as for poor cod less than 110 mm long), it is only possible to observe a morphology in 0, 33, 66 or 100 % of the fish sampled. This may disguise potential correlations between the frequency observations. Considering that, despite the low quality data, the frequency of ellipsoids correlated significantly with the frequency of polycrystalline structure carbonates, it seems likely there is a factor (or factors) that is (are) conducive to the formation of polycrystalline structures and ellipsoids but unfavourable to the formation of nanospheres. Sections 2.5.1 and 2.5.2 discuss some reasons why ellipsoids might form over nanospheres in some species. Identifying the mechanism by which some species precipitate different types of ellipsoids may allow more generalised predictions of what morphology types a broader range of different species are likely to produce in different climate zones.

This study provides valuable information based on wild-caught fish on the variation that can occur naturally in the characteristics of carbonates produced by wild fish. However, in future it may be necessary to perform more controlled experiments under laboratory conditions in order to determine the specific influence of various endogenous and exogenous factors. It is clear that diet and temperature may be important factors to investigate further although these may not completely explain the variability in carbonates produced by different species.

Additionally this study has raised important questions about the method of collection for gut carbonates, i.e. directly from the intestine or post-excretion into the seawater and how this might affect the resulting carbonates to be examined. It is unclear whether the abundance of nanospheres observed in the current study compared to previous studies is due to direct intestinal sampling or the fish feeding prior to sampling. Carbonates that are collected following excretion into the seawater may be more representative of the carbonates that contribute to sediment production. However, if there is rapid dissolution of some phases of gut carbonates in the initial minutes and hours after excretion, it is also vital to

understand their morphology and chemistry if we are to interpret and predict the impact of fish on surface ocean chemistry. However, currently little is known about the recrystallisation processes that might occur in seawater that may alter the characteristics of carbonates produced by fish. Unless carbonates can be collected from the seawater either immediately after excretion or after a set period of time, carbonates may undergo varying amounts of recrystallisation which might complicate the interpretation of any results collected. This is something that should be taken into account, and ideally standardised, in future studies relating to the environmental significance of gut carbonate production by fish.

Finally this study has highlighted that variation in carbonate characteristics within morphology types was low relative to differences between different morphology types. As such, future work should perhaps focus on measuring the frequency with which different morphology types occur under different conditions, rather than collecting large amounts of detail on the characteristics of each morphology type present.

Overall this study raises important questions and provides a platform for further work to investigate the contribution of fish across different climates to sediment production and the ocean inorganic carbon cycle as a whole.

2.6 Tables

Table 1 List of species sampled and which crystal types present

A list of species sampled, and the percentage presence of each crystal type among the individuals sampled in each species. Species obtained only from North Sea sites are marked *. Species obtained in both the North Sea and Western English Channel are marked †. All other species were obtained only from the Western English Channel. N indicates the number of individuals from which measurements were taken within each species.

Order	Species	Common Name	Percent presence of morphologies in sampled individuals				N
			Polycrystalline	Ellipsoids	Granules	Nanospheres	
Clupeiformes	† *Clupea harengus*	Herring				100	4
	Sprattus sprattus	Sprat	33			67	3
Gadiformes	*Trisopterus luscus*	Bib		100			2
	Gadus morhua	Cod		100		100	1
	† *Melanogrammus aeglefinus*	Haddock		33		67	3
	Trisopterus minutus	Poor cod	14	90	17	21	29
	* *Pollachius virens*	Saithe			100	100	1
	† *Merlangius merlangus*	Whiting	40	40	40	20	5
Lophiiformes	*Lophius piscatorius*	Monkfish	50	50	50	50	2
Perciformes	*Callionymus lyra*	Dragonet	50			50	2
	† *Scomber scombrus*	Mackerel	25		25	100	4
	Cepola macrophthalma	Red bandfish			50	50	2
	Trachurus trachurus	Scad		33		100	3
	Mullus surmuletus	Striped red mullet		33	33	100	3
Pleuronectiformes	† *Limanda limanda*	Dab				100	2
	Microstomus kitt	Lemon sole	67			67	3
	† *Pleuronectes platessa*	Plaice				100	3
	Arnoglossus laterna	Scaldfish		100			1
	Microchirus variegatus	Thickback sole	14	14		71	7
Scorpaeniformes	† *Chelidonichthys cuculus*	Red gurnard		100		25	4
	* *Trigloporus lastoviza*	Streaked gurnard		100			1
All species			15	49	14	51	85

Table 2 Table of species sampled each month and crystal morphology types observed

Table shows the monthly frequency with which different morphology types were observed as a percentage of all species sampled from the Western English Channel each month. Additionally, the table shows the numbers of each different species sampled each month.

	Month	April	May	June	July	September	October	November	December	January	February	March	Entire Year
Occurrence (% of fish)	Polycrystalline	9	25	14	14	14			20		11	50	16
	Ellipsoids	36	50	50	71	29	50	100	20	100	33	25	46
	Granules	27		7		14	13		20	33	11	13	12
	Nanospheres	64	25	43	29	43	63	33	80	33	56	63	49
	Total number of fish	11	8	14	7	7	8	3	5	3	9	8	83
Number of each species sampled	Herring	1	2										3
	Sprat										2	1	3
	Bib	1								1			2
	Cod	1											1
	Haddock	1	1										2
	Poor cod	3	4	5	4	2	2	2		2	3	2	29
	Saithe												0
	Whiting	1		1		1			1				4
	Monkfish			2									2
	Dragonet					2					1	1	4
	Mackerel								3				3
	Red Bandfish			3									3
	Scad	1				1			1				3
	Striped Red Mullet						3						3
	Dab	1	1	1									3
	Lemon Sole	1										2	3
	Plaice					1					1	1	3
	Scaldfish						2						2
	Thickback Sole			1	2		1				2	1	7
	Red Gurnard			1	1			1					3
	Streaked Gurnard												0

Table 3 Occurrence frequency of different types of carbonates across poor cod captured in different months

Percentage of individuals in which a morphology type of carbonate was observed in poor cod collected in a given month. Column labelled “N” shows total number of individuals sampled within a month.

	Presence of morphologies as a percent of sampled individuals				
Month	Polycrystalline	Ellipsoids	Granules	Nanospheres	N
April	25	50	50	25	4
May	50	100	0	0	4
June	14	71	0	29	7
July	17	67	0	33	6
September	0	33	33	33	3
October	0	67	0	67	3
November	0	100	0	0	2
December	0	100	0	0	1
January	0	100	50	50	2
February	20	60	20	20	5
March	33	67	33	33	3
Entire year	18	70	15	28	40

Table 4 Summary of monthly ellipsoids and nanosphere characteristics

Table shows a summary of the highest and lowest monthly averages and the annual average for fish carbonates with ellipsoidal and nanosphere morphologies. Averages were calculated as average of all individuals sampled in the time period excluding characteristics labels with “*”. Characteristics labelled as “*” are presented as an average of all images taken in from fish in the time period due to the low numbers of individuals where the characteristic could be examined.

				Annual Average			Highest month average				Lowest month average			
Morphology	Species	Characteristic	Fig	Mean	SE	n	Month	Mean	SE	n	Month	Mean	SE	n
Ellipsoids	All	Crystal Length	12	1.09	0.03	37	Jul	1.21	0.10	5	Nov	0.97	0.01	3
		Crystal Width	12	0.52	0.01	37	Jan	0.58	0.02	3	Sep	0.44	0.06	2
		Crystal Aspect	12	2.12	0.05	37	April	2.41	0.08	4	Nov	1.80	0.03	3
	Poor Cod	Crystal Length	13	1.07	0.03	27	Apr	1.26	0.16	2	Nov	0.97	0.03	2
		Crystal Width	13	0.53	0.01	27	Jan	0.58	0.04	2	Dec	0.48	na	1
		Crystal Aspect	13	2.01	0.04	27	Apr	2.36	0.17	2	Nov	1.79	0.04	2
Nanospheres	All	Crystal Length	14	0.97	0.08	27	Jul	1.90	na	1	Feb	0.57	0.18	4
		Crystal Width	14	0.77	0.05	27	Jul	1.24	na	1	Feb	0.52	0.20	4
		Crystal Aspect	14	1.26	0.04	27	Jan	1.61	na	1	Dec	1.00	na	1
	Poor Cod	Crystal Length*	15	1.05	0.11	17	Jul	1.80	0.31	3	Sep	0.46	0.05	4
		Crystal Width*	15	0.75	0.08	17	Jul	1.21	0.16	3	Sep	0.33	0.04	4
		Crystal Aspect*	15	1.40	0.04	17	Jan	1.61	na	1	Feb	1.17	0.18	2
Ellipsoids	All	Mg Content	16	10.42	1.04	41	Oct	14.58	2.32	4	Mar	4.37	0.21	2
		P Content	16	1.98	0.54	41	Apr	3.61	2.11	4	Dec	0.00	na	1
	Poor Cod	Mg Content	17	8.13	0.62	28	Oct	10.81	1.96	2	Mar	4.37	0.21	2
		P Content	17	1.11	0.42	28	Oct	2.87	2.59	2	Aprl	0.00	0.00	2
Nanospheres	All	Mg Content	16	21.24	1.78	45	Dec	35.19	6.25	4	Nov	6.85	na	1
		P Content	16	24.88	1.78	45	May	34.58	5.50	3	Nov	0.00	na	1
	Poor Cod	Mg Content	17	15.07	2.94	11	Feb	32.29	na	1	Apr	5.96	na	1
		P Content	17	23.19	3.57	11	June	32.18	9.63	2	Jan	7.62	na	1

Table 5 Table of species sampled size categories and crystal morphology types observed

Table shows the monthly frequency with which different morphology types were observed as a percentage of all species sampled from the Western English Channel in a particular size category based on the length of the fish. Additionally, the table shows the numbers of each different species sampled each size category.

	Size category (mm)	<129	130-149	150-169	170-189	190-209	210-229	230-249	250-269	270+
Occurrence (% of fish)	Polycrystalline	22	10		30	10	18		30	7
	Ellipsoids	56	60	46	80	60	27		10	20
	Granules	22		15		10	27	17		7
	Nanospheres	22	50	54	20	70	36	50	50	40
	Total number of fish	9	10	13	10	10	11	6	10	15
Number of each species per size category	Herring							1	2	1
	Sprat	1	2							
	Bib					1				1
	Cod									1
	Haddock					1		1		1
	Poor cod	4	4	8	5	5	3			
	Saithe									1
	Whiting				1		2		1	1
	Monkfish									2
	Dragonet					1	1	1	1	
	Mackerel							2	3	
	Red Bandfish									3
	Scad			1	1					1
	Striped Red Mullet	1	1	1						
	Dab				1		2	1		
	Lemon Sole					1	1		1	
	Plaice						1		2	1
	Scaldfish	2								
	Thickback Sole	1	3	2	1					
	Red Gurnard			1	1	1				1
	Streaked Gurnard						1			

Table 6 Occurrence frequency of different types of carbonate produced by different size categories of poor cod

Percentage of individuals in which a morphology type of carbonate was observed in a given size category of length in mm. Column labelled “N” shows total number of individuals sampled within a size category.

	Presence of morphologies as a percent of sampled individuals				
Fish length	Crystalline	Ellipsoid	Granules	Nanospheres	N
<110	33	100	33	0	3
110-130	0	67	0	33	3
130-150	17	67	0	33	6
150-170	0	50	10	40	10
170-190	38	88	0	0	8
190-210	20	80	40	60	5
>210	25	75	50	25	5
All sizes	18	70	15	28	40

Table 7 Correlation analysis between fish lengths and crystal sizes

Table shows Pearson's product-moment correlation between average crystal sizes and fish length for all species collected from the Western English Channel and for just poor cod collected from the Western English Channel. There were no significant relationships.

			Fish lengths	
			All species	Poor cod
Ellipsoids	Crystal Lengths	Pearson Correlation	.311	.215
		P (2-tailed)	.069	.283
		N	35	27
	Crystal Widths	Pearson Correlation	.165	-.067
		P (2-tailed)	.344	.738
		N	35	27
	Aspect Ratio	Pearson Correlation	.120	.315
		P (2-tailed)	.491	.110
		N	35	27
Nanospheres	Crystal Lengths	Pearson Correlation	-.237	-.206
		P (2-tailed)	.233	.624
		N	27	8
	Crystal Widths	Pearson Correlation	-.216	-.415
		P (2-tailed)	.280	.306
		N	27	8
	Aspect Ratio	Pearson Correlation	.022	.644
		P (2-tailed)	.911	.085
		N	29	8

Table 8 Correlation between fish lengths and elemental composition of ellipsoids and nanospheres

Table shows Pearson's product-moment correlation between average crystal elemental composition (magnesium and phosphorus content) and fish length for all species collected from the Western English Channel and poor cod analysed separately. Significant relationships are denoted by **.

			Fish length	
			All species	Poor cod only
Ellipsoids	Mg mol%	Pearson Correlation	.129	.513**
		P (2-tailed)	.421	.005
		N	41	28
	P mol%	Pearson Correlation	.170	.133
		P (2-tailed)	.287	.500
		N	41	28
Nanospheres	Mg mol%	Pearson Correlation	.162	-.275
		P (2-tailed)	.288	.413
		N	45	11
	P mol%	Pearson Correlation	.122	-.803**
		P (2-tailed)	.423	.003
		N	45	11

Table 9 Correlation tests between monthly average fish condition and occurrence of carbonate morphology types

Table shows the result of Pearson's correlation test between average condition of poor cod each month and the frequency with which different morphology types of carbonates were observed each individual fish each month. No significant correlations were observed.

		Average fish condition
Polycrystalline frequency	Pearson Correlation	-.027
	P (2-tailed)	.937
	N	11
Ellipsoid frequency	Pearson Correlation	.216
	P (2-tailed)	.524
	N	11
Granule frequency	Pearson Correlation	-.032
	P (2-tailed)	.924
	N	11
Nanosphere frequency	Pearson Correlation	-.119
	P (2-tailed)	.728
	N	11

Table 10 Correlation analysis between crystal sizes of ellipsoids and nanospheres and fish condition index

Table shows Pearson's product-moment correlation between average crystal sizes (crystal length, width and aspect ratio) and fish condition index for poor cod collected from the Western English Channel. There were no significant relationships.

			Fish Condition
Ellipsoids	Crystal Length	Pearson Correlation	.040
		P (2-tailed)	.908
		N	11
	Crystal Width	Pearson Correlation	.170
		P (2-tailed)	.618
		N	11
	Aspect ratio	Pearson Correlation	-.045
		P (2-tailed)	.895
		N	11
Nanospheres	Crystal Length	Pearson Correlation	-.399
		P (2-tailed)	.328
		N	8
	Crystal Width	Pearson Correlation	-.480
		P (2-tailed)	.229
		N	8
	Aspect ratio	Pearson Correlation	.118
		P (2-tailed)	.780
		N	8

Table 11 Correlation analysis between fish condition and elemental composition of ellipsoids and nanospheres

Table shows Pearson's product-moment correlation between average crystal elemental composition (magnesium and phosphorus content) and fish condition index for poor cod collected from the Western English Channel. There were no significant relationships.

			Fish Condition
Ellipsoids	Mg mol%	Pearson Correlation	.218
		P (2-tailed)	.520
		N	11
	P mol%	Pearson Correlation	.059
		P (2-tailed)	.864
		N	11
Nanospheres	Mg mol%	Pearson Correlation	-.574
		P (2-tailed)	.136
		N	8
	P mol%	Pearson Correlation	.119
		P (2-tailed)	.779
		N	8

Table 12 Correlations between observance frequencies of morphology types in different species

Result of Pearson's correlation analysis between the frequency with which ellipsoids, granules, nanospheres and polycrystalline structured carbonates were observed across different species of temperate fish. Significant values are highlighted with **.

		Polycrystalline	Ellipsoids	Granules	Nanospheres
Polycrystalline	Pearson Correlation	1	-.308	.050	-.105
	P (2-tailed)		.175	.831	.650
	N	21	21	21	21
Ellipsoids	Pearson Correlation	-.308	1	-.232	-.662**
	P (2-tailed)	.175		.311	.001
	N	21	21	21	21
Granules	Pearson Correlation	.050	-.232	1	.133
	P (2-tailed)	.831	.311		.565
	N	21	21	21	21
Nanospheres	Pearson Correlation	-.105	-.662**	.133	1
	P (2-tailed)	.650	.001	.565	
	N	21	21	21	21

Table 13 Correlations between observance frequencies of morphology types across all species sampled in different months

Result of Pearson's correlation analysis between the frequency with which ellipsoids, granules, nanospheres and polycrystalline structured carbonates were observed across all species captured from the Western English Channel observed in each month. Significant values are highlighted with *.

		Polycrystalline	Ellipsoids	Granules	Nanospheres
Polycrystalline	Pearson Correlation	1	-.586	-.178	.220
	P (2-tailed)		.058	.600	.516
	N	11	11	11	11
Ellipsoids	Pearson Correlation	-.586	1	-.095	-.691*
	P (2-tailed)	.058		.781	.018
	N	11	11	11	11
Granules	Pearson Correlation	-.178	-.095	1	.455
	P (2-tailed)	.600	.781		.160
	N	11	11	11	11
Nanospheres	Pearson Correlation	.220	-.691*	.455	1
	P (2-tailed)	.516	.018	.160	
	N	11	11	11	11

Table 14 Correlations between observance frequencies of morphology types across poor cod sampled in different months

Result of Pearson's correlation analysis between the frequency with which ellipsoids, granules, nanospheres and polycrystalline structured carbonates were observed across poor cod captured from the Western English Channel observed in each month. There were no significant correlations.

		Polycrystalline	Ellipsoids	Granules	Nanospheres
Polycrystalline	Pearson Correlation	1	0.004	0.014	-0.314
	P (2-tailed)		0.992	0.967	0.347
	N	11	11	11	11
Ellipsoids	Pearson Correlation	0.004	1	-0.367	-0.405
	P (2-tailed)	0.992		0.267	0.216
	N	11	11	11	11
Granules	Pearson Correlation	0.014	-0.367	1	0.32
	P (2-tailed)	0.967	0.267		0.338
	N	11	11	11	11
Nanospheres	Pearson Correlation	-0.314	-0.405	0.32	1
	P (2-tailed)	0.347	0.216	0.338	
	N	11	11	11	11

Table 15 Correlations between observance frequencies of morphology types across poor cod sampled in different size categories

Result of Pearson's correlation analysis between the frequency with which ellipsoids, granules, nanospheres and polycrystalline structured carbonates were observed across poor cod captured from the Western English Channel observed in each size category. Significant values are highlighted with *.

		Polycrystalline	Ellipsoids	Granules	Nanospheres
Polycrystalline	Pearson Correlation	1	.864*	0.321	-0.648
	P (2-tailed)		0.012	0.483	0.116
	N	7	7	7	7
Ellipsoids	Pearson Correlation	.864*	1	0.354	-0.612
	P (2-tailed)	0.012		0.436	0.144
	N	7	7	7	7
Granules	Pearson Correlation	0.321	0.354	1	0.152
	P (2-tailed)	0.483	0.436		0.746
	N	7	7	7	7
Nanospheres	Pearson Correlation	-0.648	-0.612	0.152	1
	P (2-tailed)	0.116	0.144	0.746	
	N	7	7	7	7

Table 16 Correlations between observance frequencies of morphology types across all species sampled in different size categories

Result of Pearson's correlation analysis between the frequency with which ellipsoids, granules, nanospheres and polycrystalline structured carbonates were observed across all species captured from the Western English Channel observed in each size category. There were no significant correlations.

		Polycrystalline	Ellipsoids	Granules	Nanospheres
Polycrystalline	Pearson Correlation	1	.280	-.268	-.558
	P (2-tailed)		.466	.485	.118
	N	9	9	9	9
Ellipsoids	Pearson Correlation	.280	1	-.228	-.248
	P (2-tailed)	.466		.555	.520
	N	9	9	9	9
Granules	Pearson Correlation	-.268	-.228	1	-.131
	P (2-tailed)	.485	.555		.737
	N	9	9	9	9
Nanospheres	Pearson Correlation	-.558	-.248	-.131	1
	P (2-tailed)	.118	.520	.737	
	N	9	9	9	9

2.7 Figures

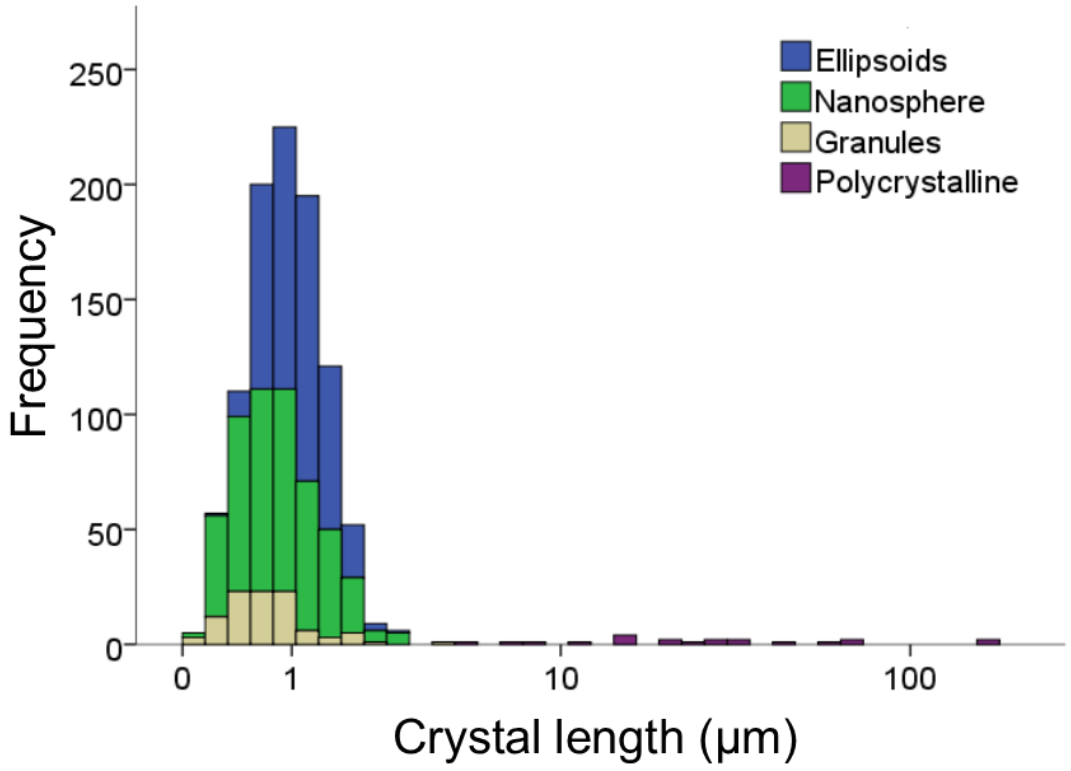

Figure 3 Size distribution of common morphologies

Size distribution of all the common morphology types of endogenously produced intestinal precipitates in temperate fish species caught in the wild. Frequency represents numbers of individual crystals measured.

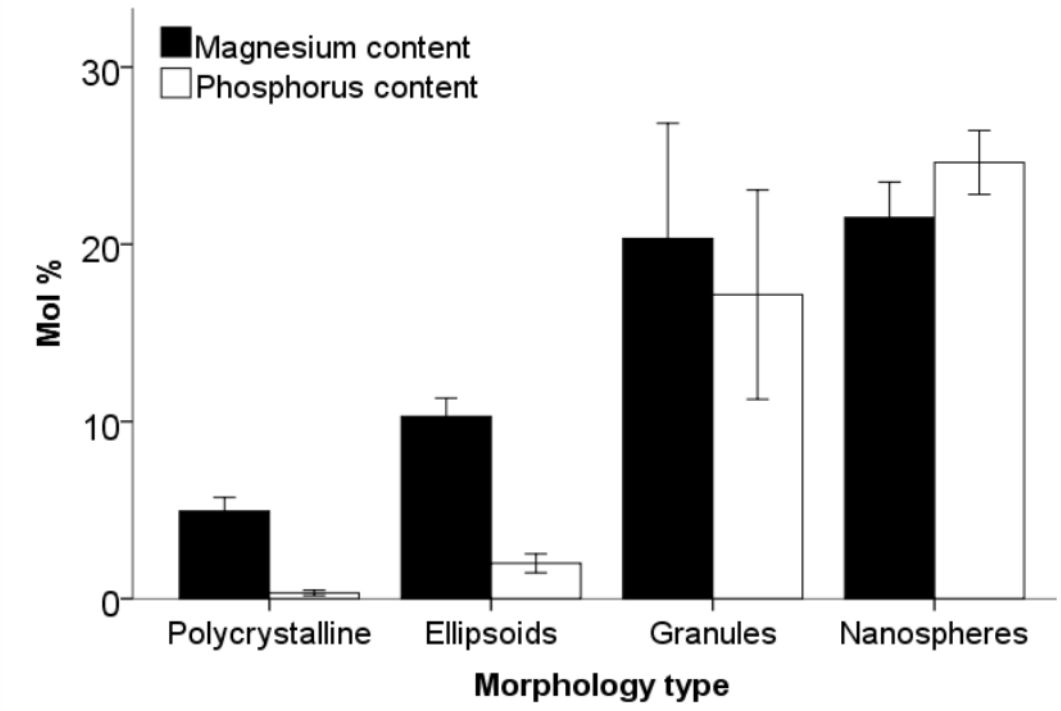

Figure 4 Mean magnesium and phosphorus contents of different morphologies

Means magnesium and phosphorus contents (as mol%) of common crystal morphologies found in the intestine of wild-caught temperate marine fish. Error bars represent the standard error of the mean.

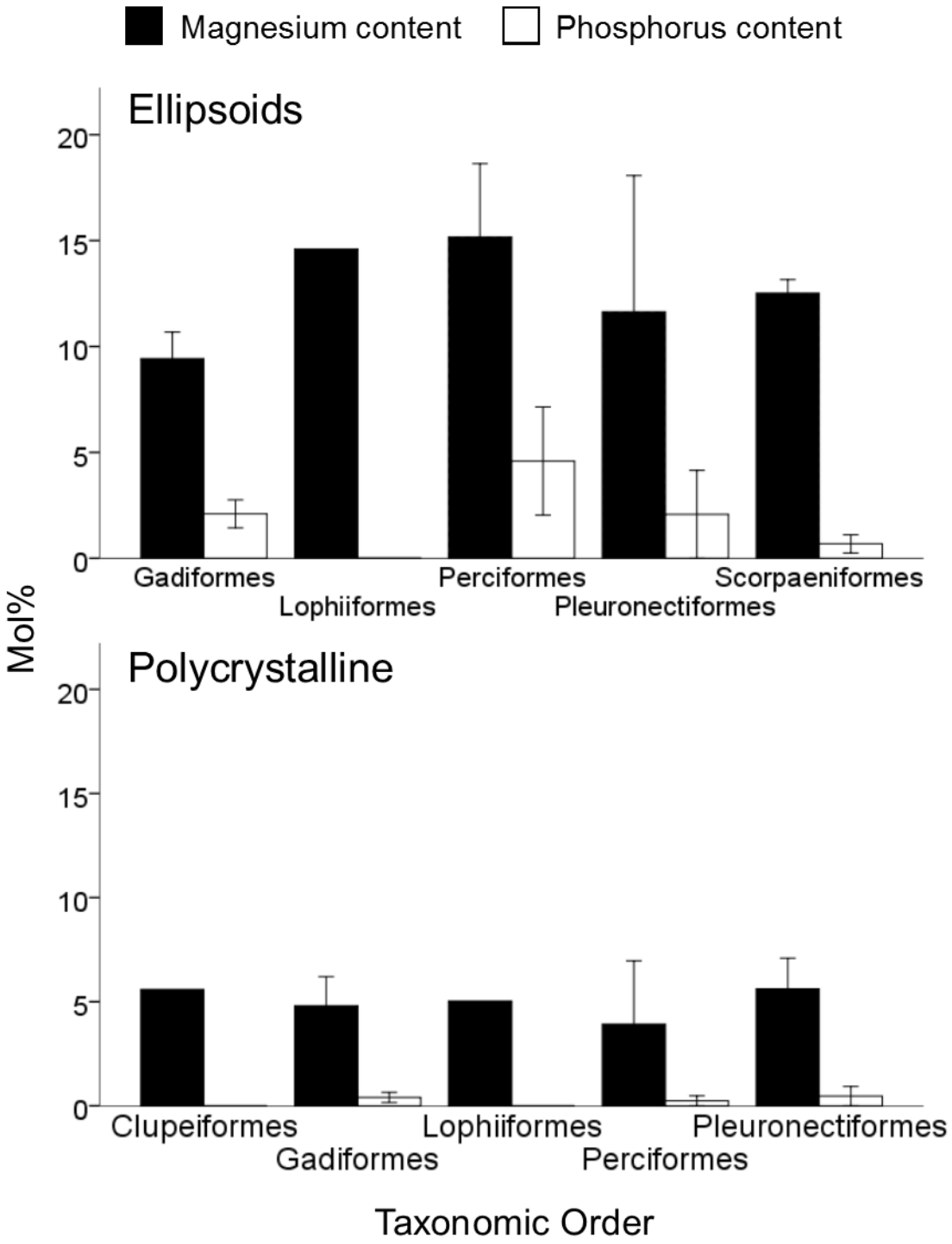

Figure 5 Magnesium and phosphorus content of ellipsoidal and polycrystalline carbonates across different taxonomic orders of temperate marine fish.

Mean magnesium (black bars) and phosphorus (white bars) content as mol% monocrystalline ellipsoids (top) and polycrystalline structures (bottom) of carbonates produced by different taxonomic orders of temperate marine fish. Error bars represent the standard error of the mean.

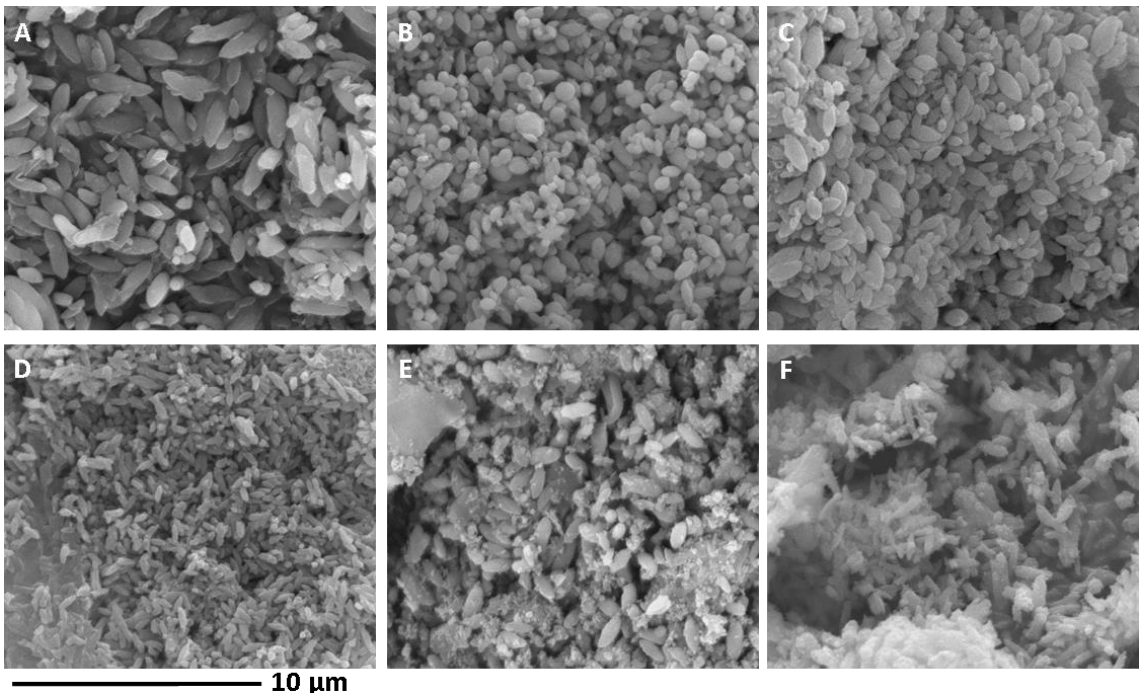

Figure 6 Examples of typical monocrystalline ellipsoids

Secondary electron microscope images of typical monocrystalline ellipsoids produced by A) red gurnard, B) thickback sole, C) poor cod, D) scaldfish, E) striped red mullet, F) monkfish. All images are to the same scale, shown by the 10 µm bar

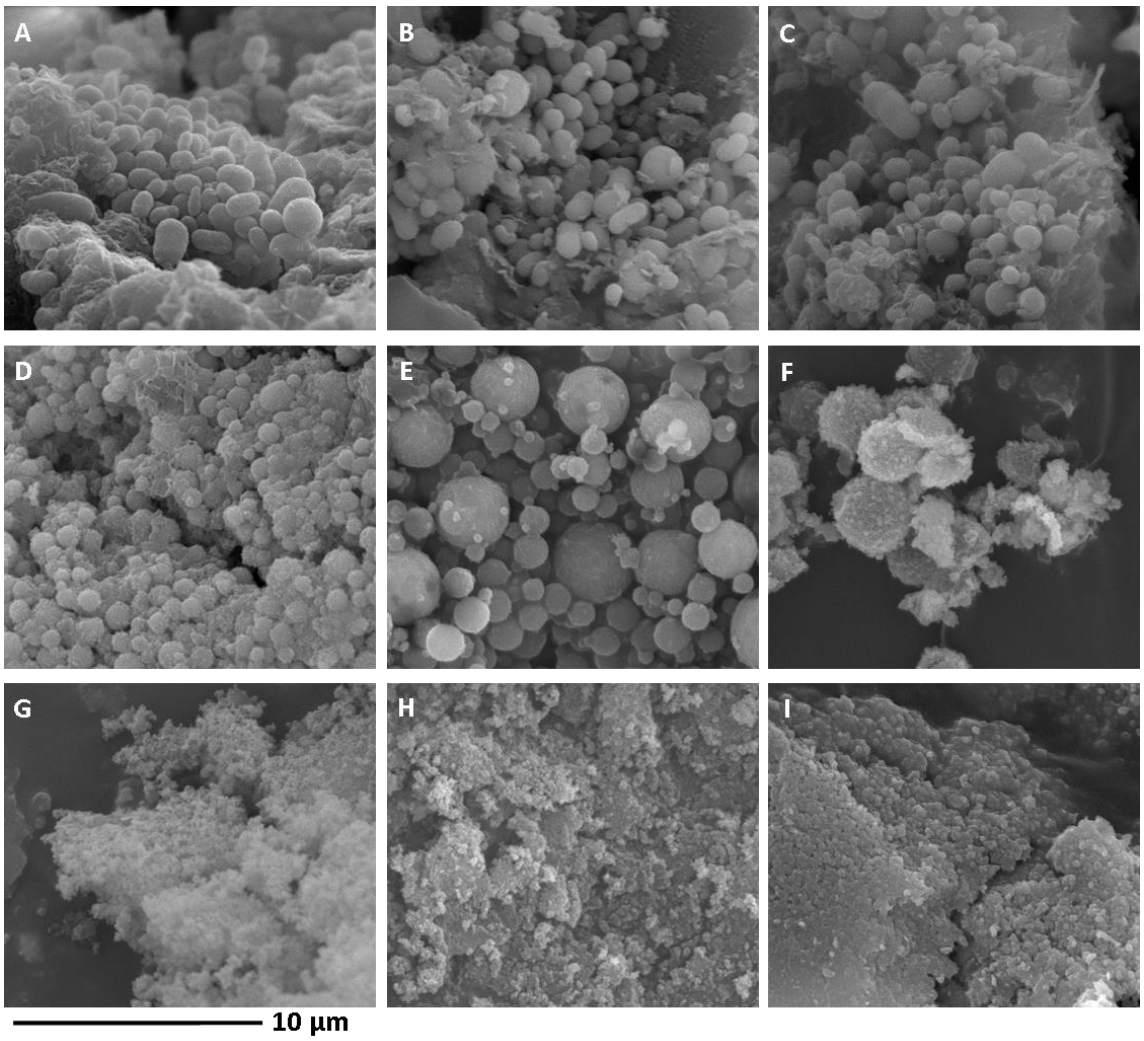

Figure 7 Examples of nanosphere type precipitates

Secondary electron microscopy images of nanosphere type precipitates produced in the intestine of a variety of temperate marine fish species. Variations include: “jelly bean” shaped extended spheres produced by A) dab, B) poor cod, C) plaice; nanospheres spheres produced by D) herring, E) lemon sole, F) monkfish; and very fine particulate nanosphere type material produced by G) haddock, H) scad, I) lemon sole. All images to the same scale.

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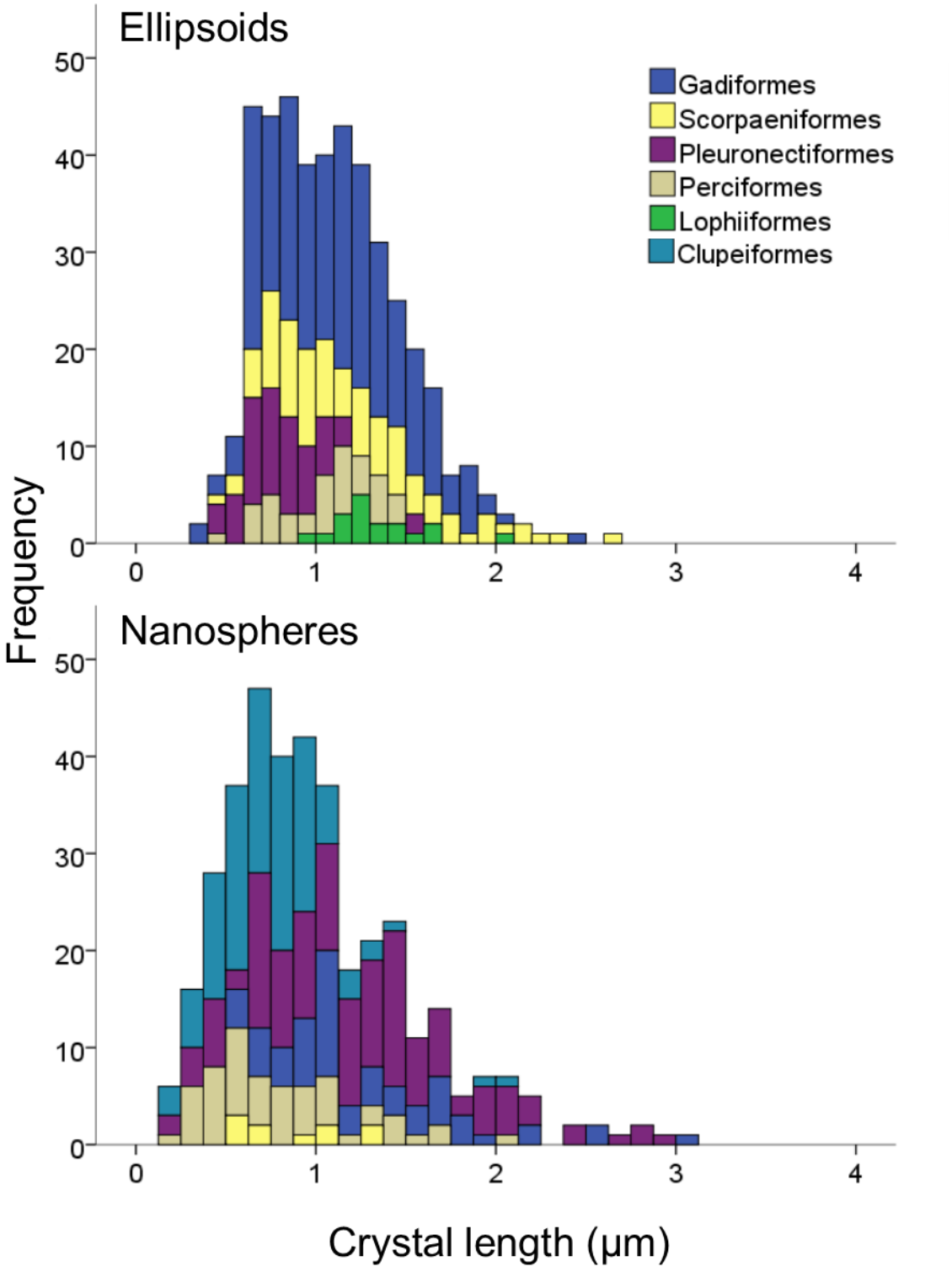

Figure 8 Size distribution of monocrystalline ellipsoids and nanospheres

Histogram showing size distribution of monocrystalline ellipsoid and nanosphere types of precipitate produced by temperate marine fish species within different taxonomic orders. Frequency represents number of individual crystals measured.

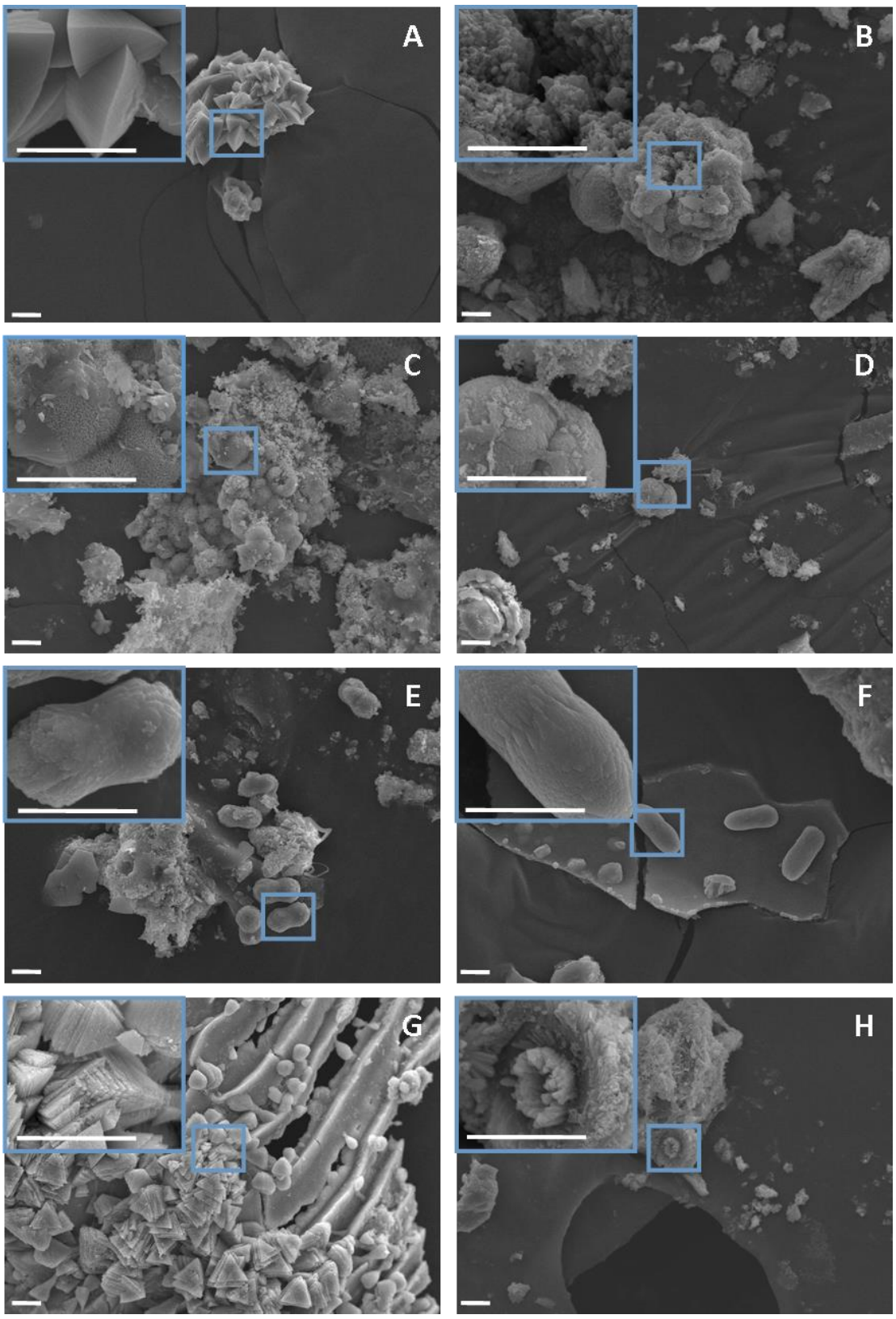

Figure 9 Examples of polycrystalline structures

Secondary electron microscopy images of polycrystalline structured intestinal precipitates produced by A) common dragonet, B) mackerel showing no distinct superstructure. Precipitates showing intergrown spherical superstructures from C) poor cod and D) lemon sole. Precipitates showing “dumbell” type

superstructures produced by E) whiting and F) thickback sole. Precipitates which have adhered to exogenous material produced by G) whiting and H) mackerel. White bars on images indicate 10 µm.

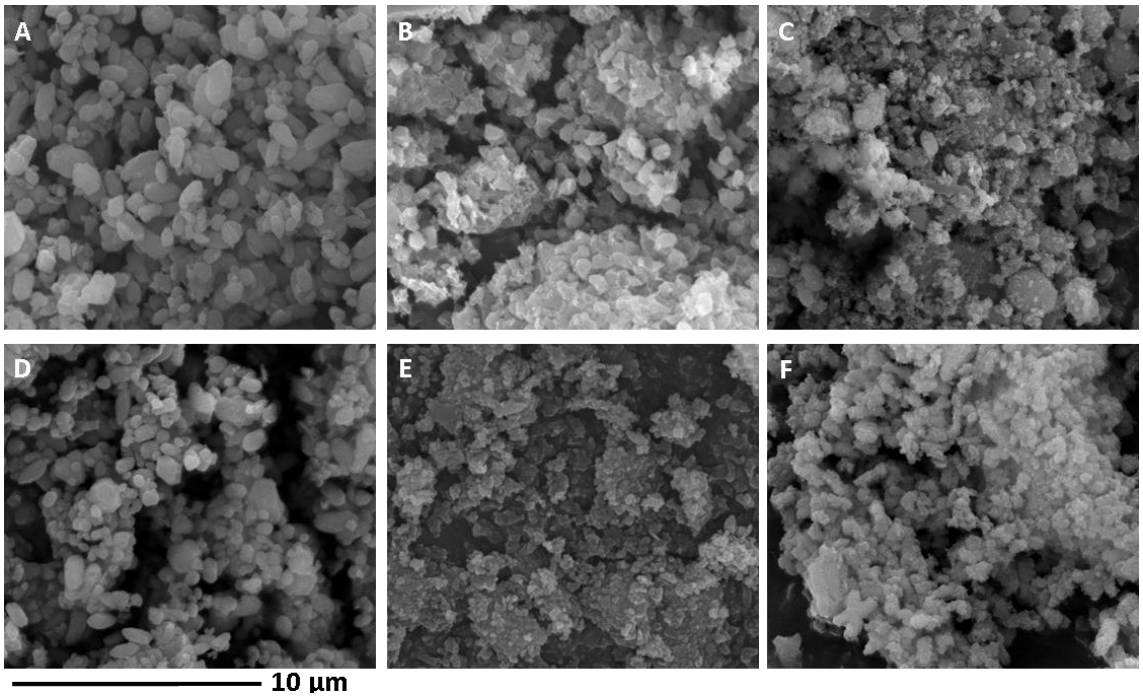

Figure 10 Examples of granular precipitates not described by other categories

Secondary electron microscopy images of granular precipitates produced A), B) and C) poor cod; D) whiting; E) striped red mullet; and F) monkfish. All images are to the same scale.

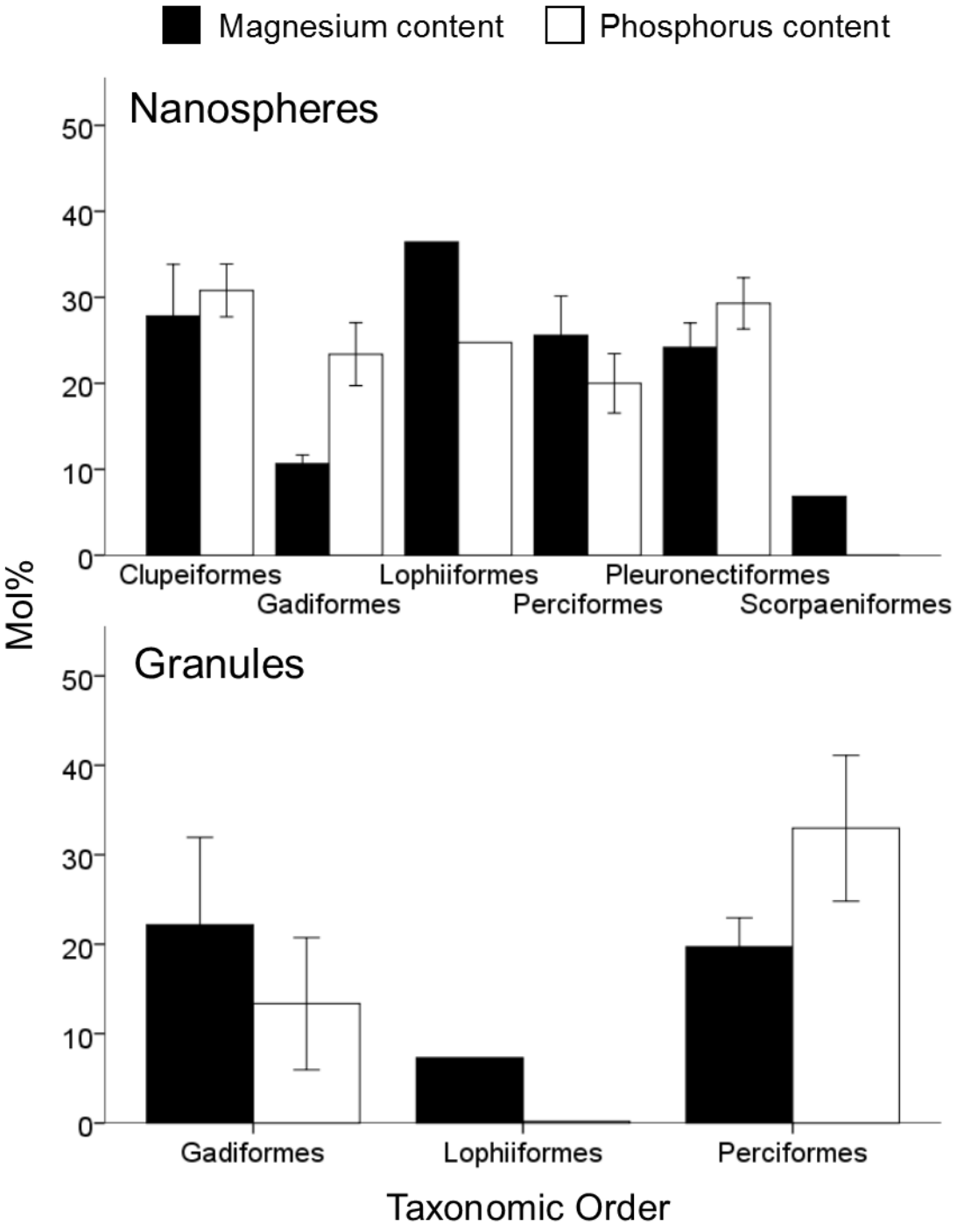

Figure 11 Magnesium and phosphorus content of nanosphere and granular carbonates across different taxonomic orders of temperate marine fish.

Mean magnesium (black bars) and phosphorus (white bars) content as mol% monocrystalline ellipsoids (top) and polycrystalline structures (bottom) of carbonates produced by different taxonomic orders of temperate marine fish. Error bars represent the standard error of the mean.

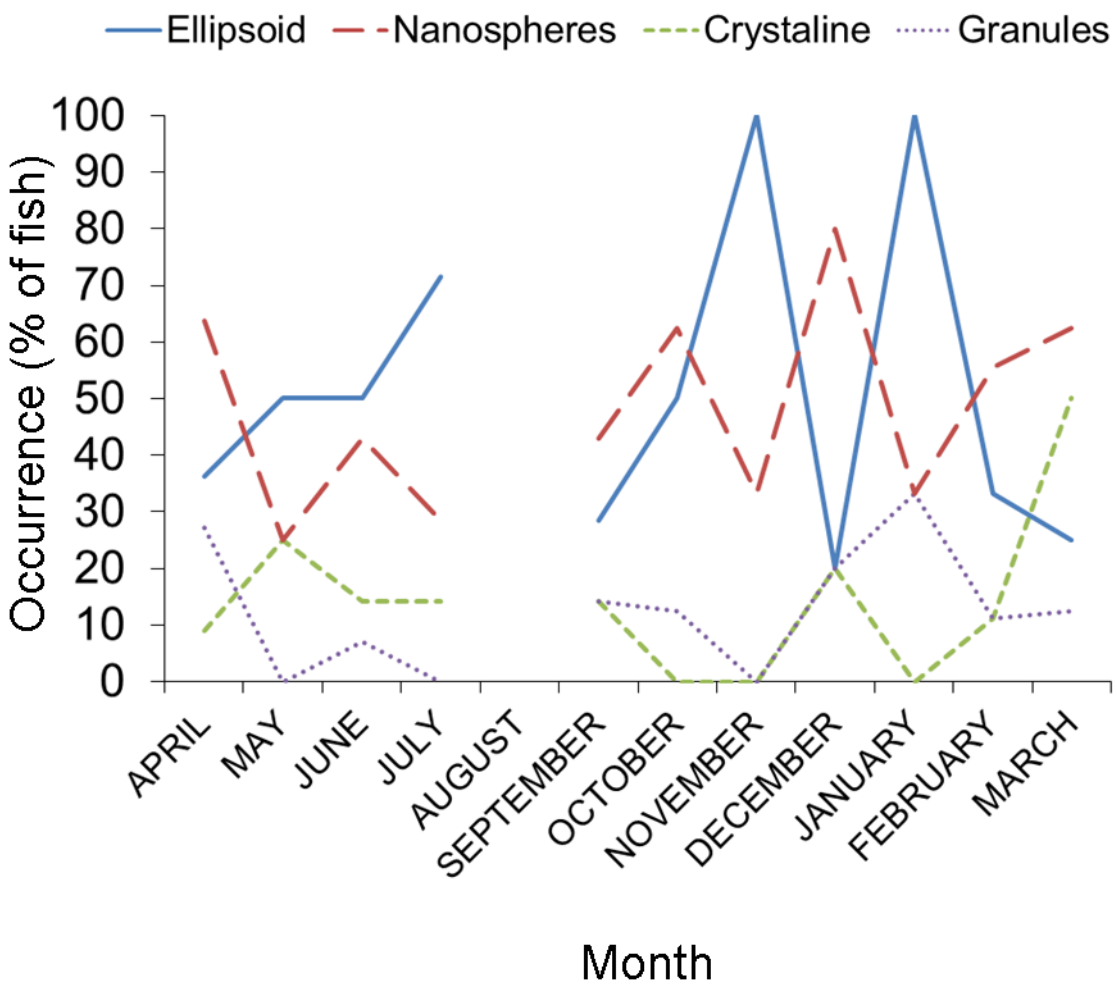

Figure 12 Occurrence frequency of different morphology types across all sampled Western English Channel species each month

The occurrence frequency of different carbonate morphology types in all species sampled from the Western English Channel. Occurrence rate is presented as the number of fish in which morphology types were observed as a percentage of total number of fish sampled in that month. No data is available for August.

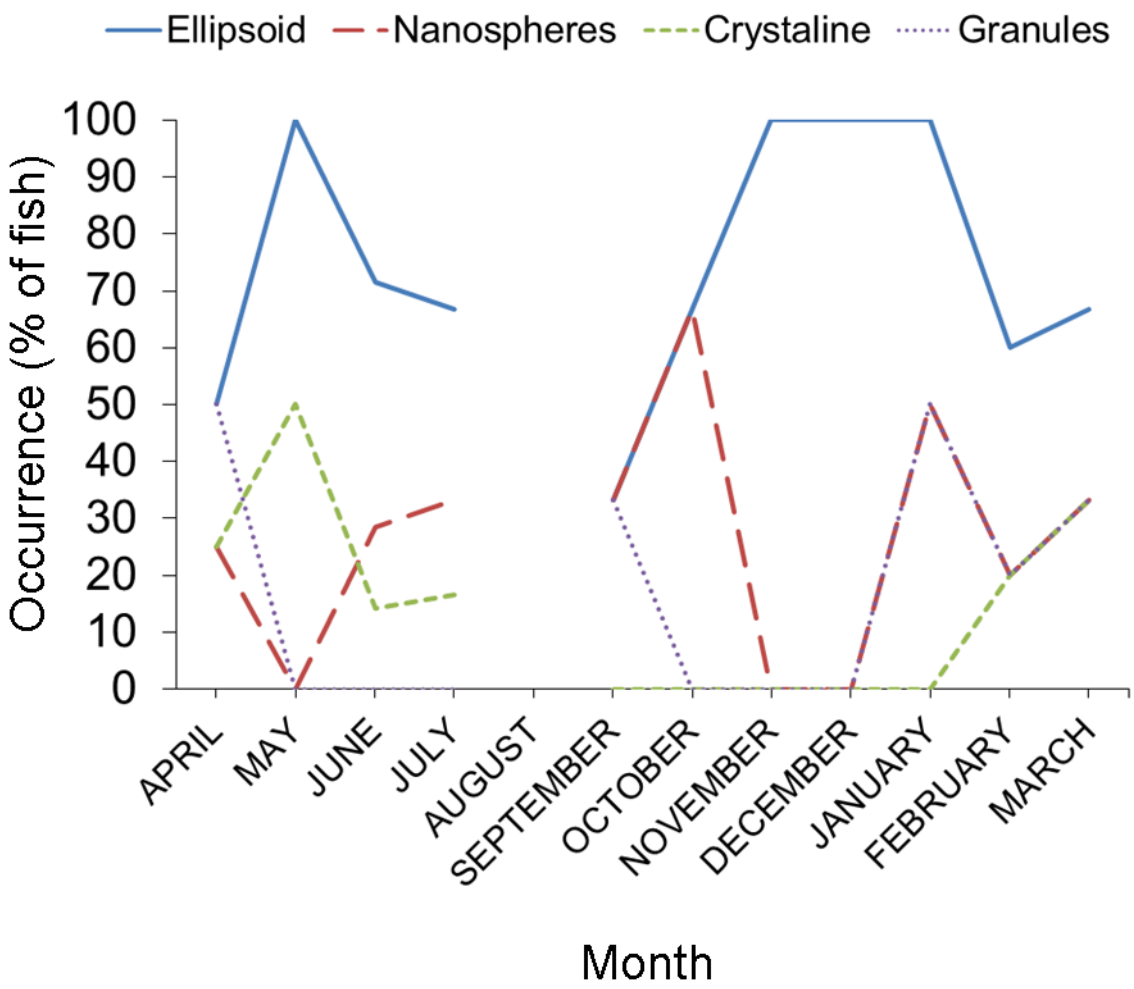

Figure 13 Occurrence frequency of different morphology types in poor cod each month

The occurrence frequency of different carbonate morphology types in poor cod sampled from the Western English Channel. Occurrence rate is presented as the number of fish in which morphology types were observed as a percentage of total number of fish sampled in that month. No data is available for August.

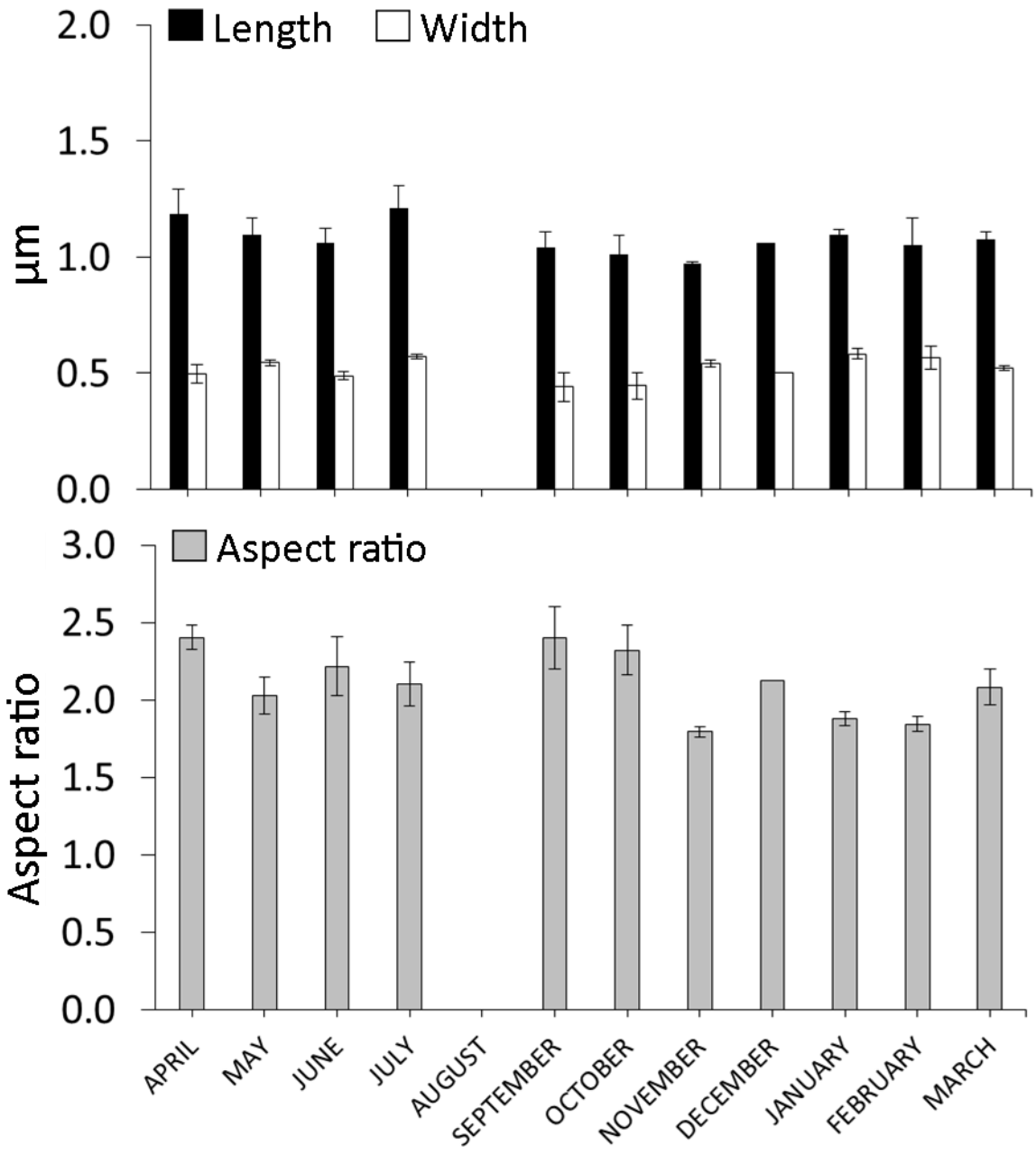

Figure 14 Monthly average ellipsoid sizes across all Western English Chanel species

Monthly mean average crystal sizes of ellipsoids observed across all species sampled from the Western English Chanel. Top panel shows average crystal length (black bars) and width (white bars). Bottom panel shows average aspect ratio (grey bars) of crystals. Error bars represent standard error of the mean. No data is available for August.

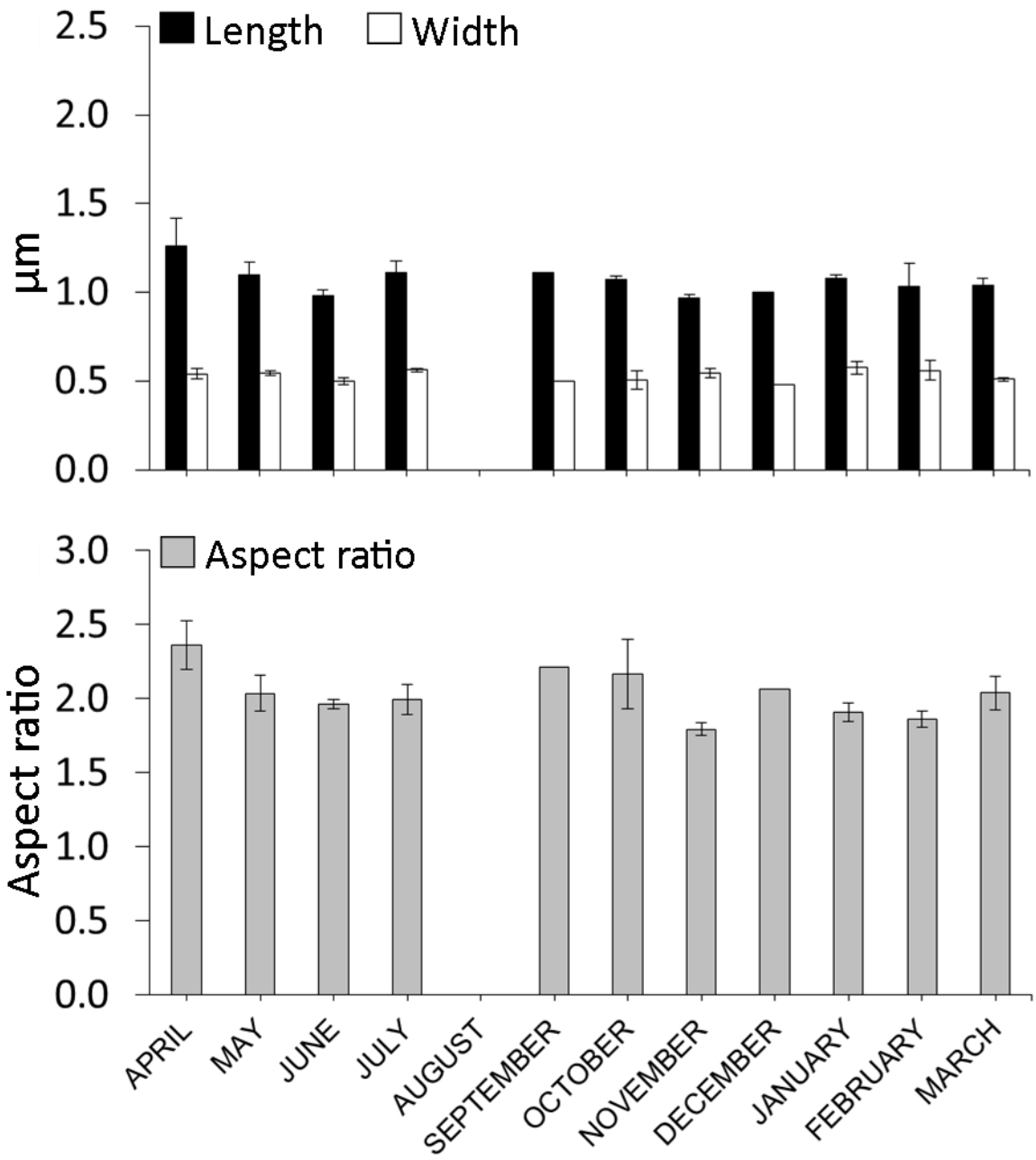

Figure 15 Monthly average ellipsoid sizes from poor cod sampled from the Western English Channel

Monthly mean average crystal sizes of ellipsoids observed in poor cod sampled from the Western English Chanel. Top panel shows average crystal length (black bars) and width (white bars). Bottom panel shows average aspect ratio (grey bars) of crystals. Error bars represent standard error of the mean. No data is available for August.

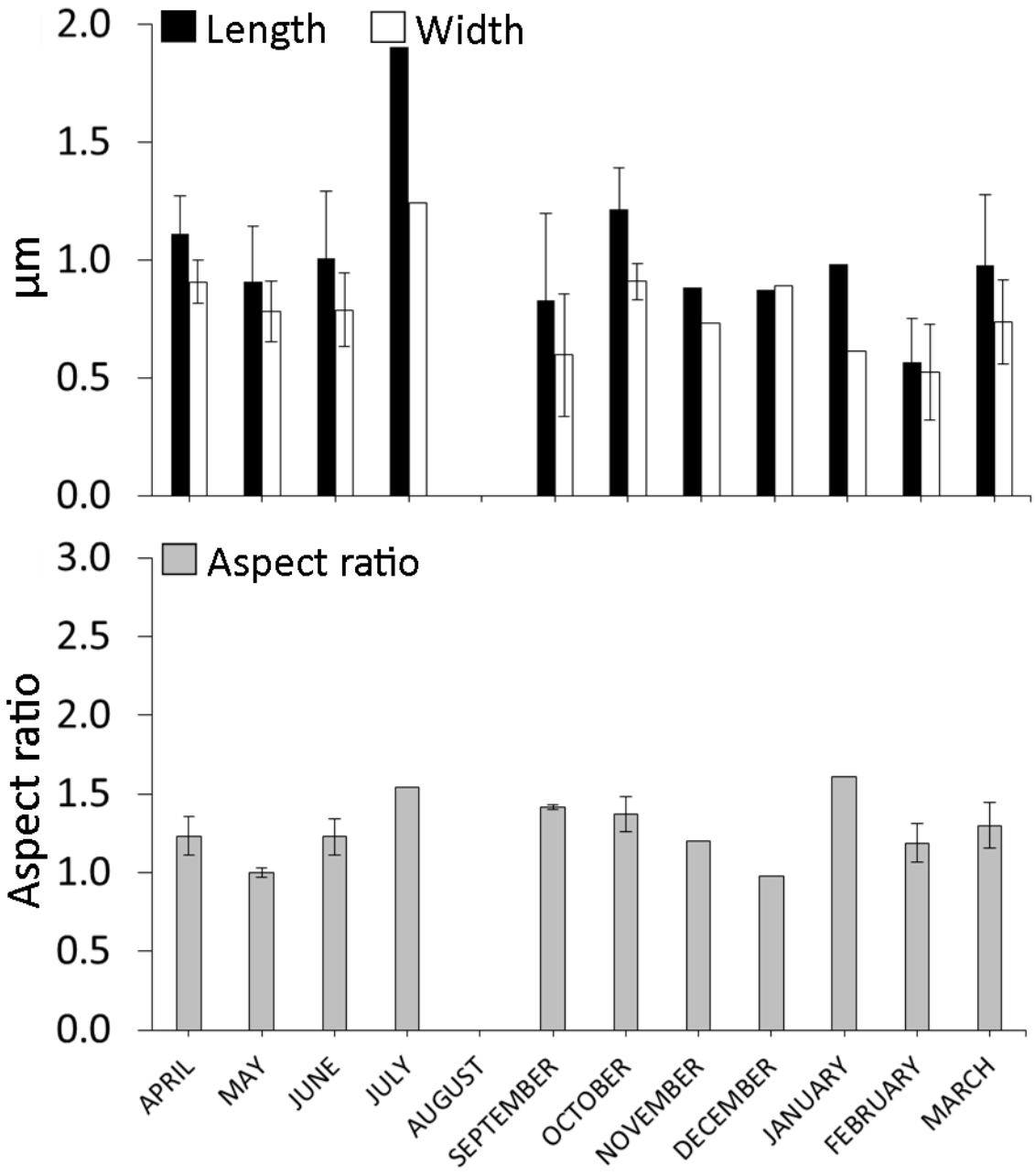

Figure 16 Monthly average nanosphere sizes across all Western English Chanel species

Monthly mean average crystal sizes of nanospheres observed across all species sampled from the Western English Chanel. Top panel shows average crystal length (black bars) and width (white bars). Bottom panel shows average aspect ratio (grey bars) of crystals. Error bars represent standard error of the mean. No data is available for August.

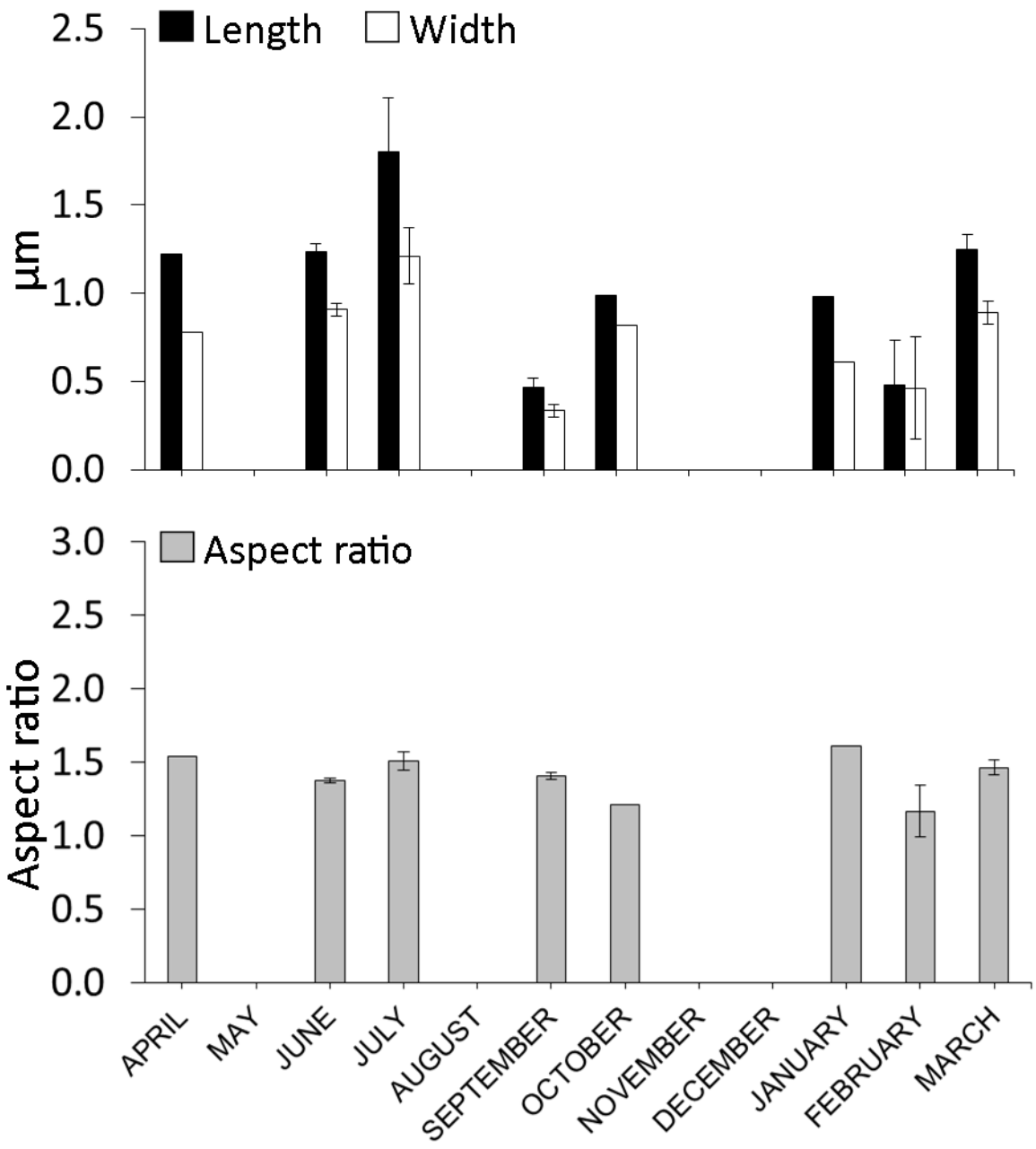

Figure 17 Monthly average nanosphere sizes from poor cod sampled from the Western English Channel

Monthly mean average crystal sizes of nanospheres observed across poor cod sampled from the Western English Chanel. Top panel shows average crystal length (black bars) and width (white bars). Bottom panel shows average aspect ratio (grey bars) of crystals. Error bars represent standard error of the mean. No data is available for August.

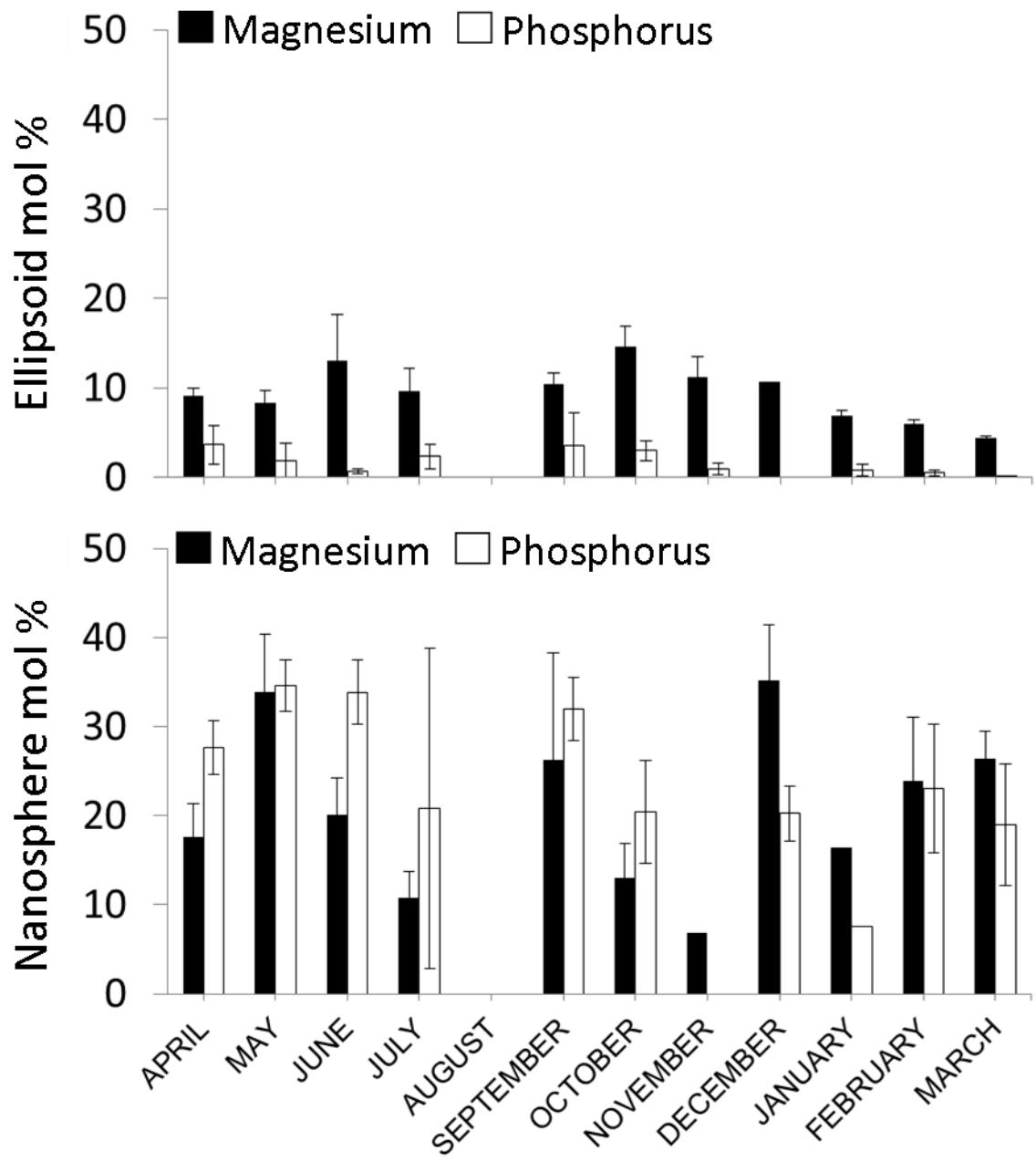

Figure 18 Monthly average ellipsoid and nanosphere elemental compositions from all fish

Monthly average magnesium content (black bars) and phosphorus content (white bars) of ellipsoids (top panel) and nanospheres (bottom panel) collected from all species sampled from the Western English Channel. Error bars represent standard error of the mean.

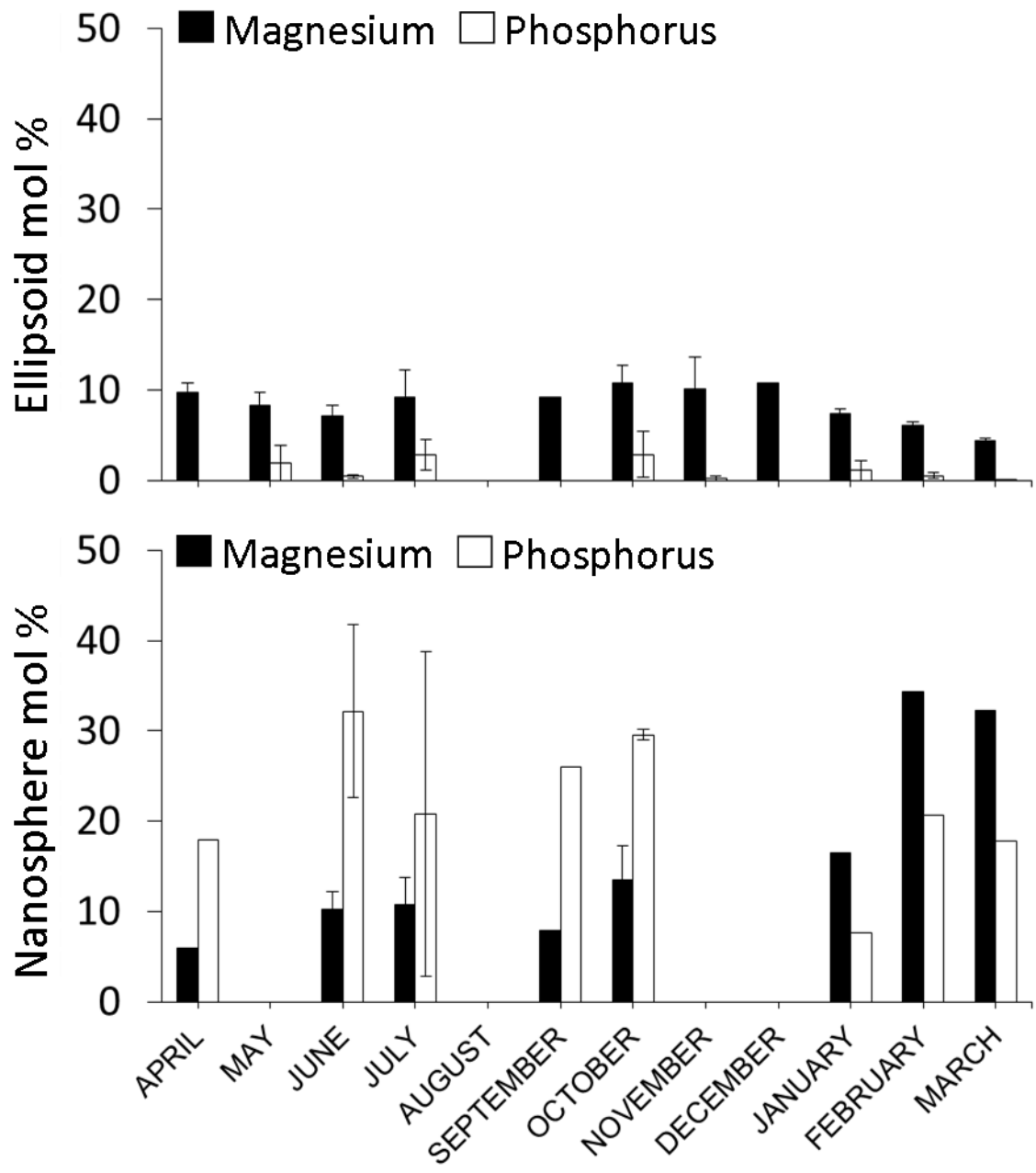

Figure 19 Monthly average ellipsoid and nanosphere elemental compositions from poor cod

Monthly average magnesium content (black bars) and phosphorus content (white bars) of ellipsoids (top panel) and nanospheres (bottom panel) collected from poor cod sampled from the Western English Channel. Error bars represent standard error of the mean.

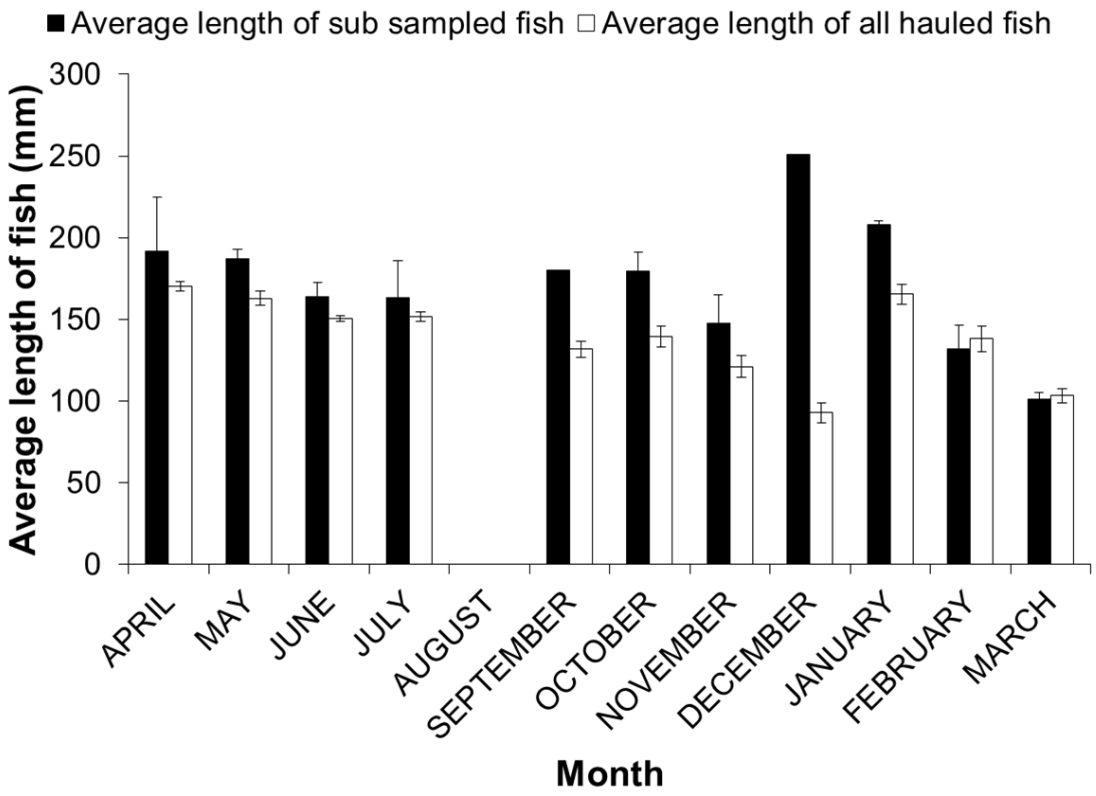

Figure 20 Monthly average length of poor cod

Monthly average lengths of fish sub sampled from haul for dissection and collection of intestinal precipitates (black bars), and average lengths of fish randomly subsampled from haul for length (white bars). Error bars show standard error of the mean.

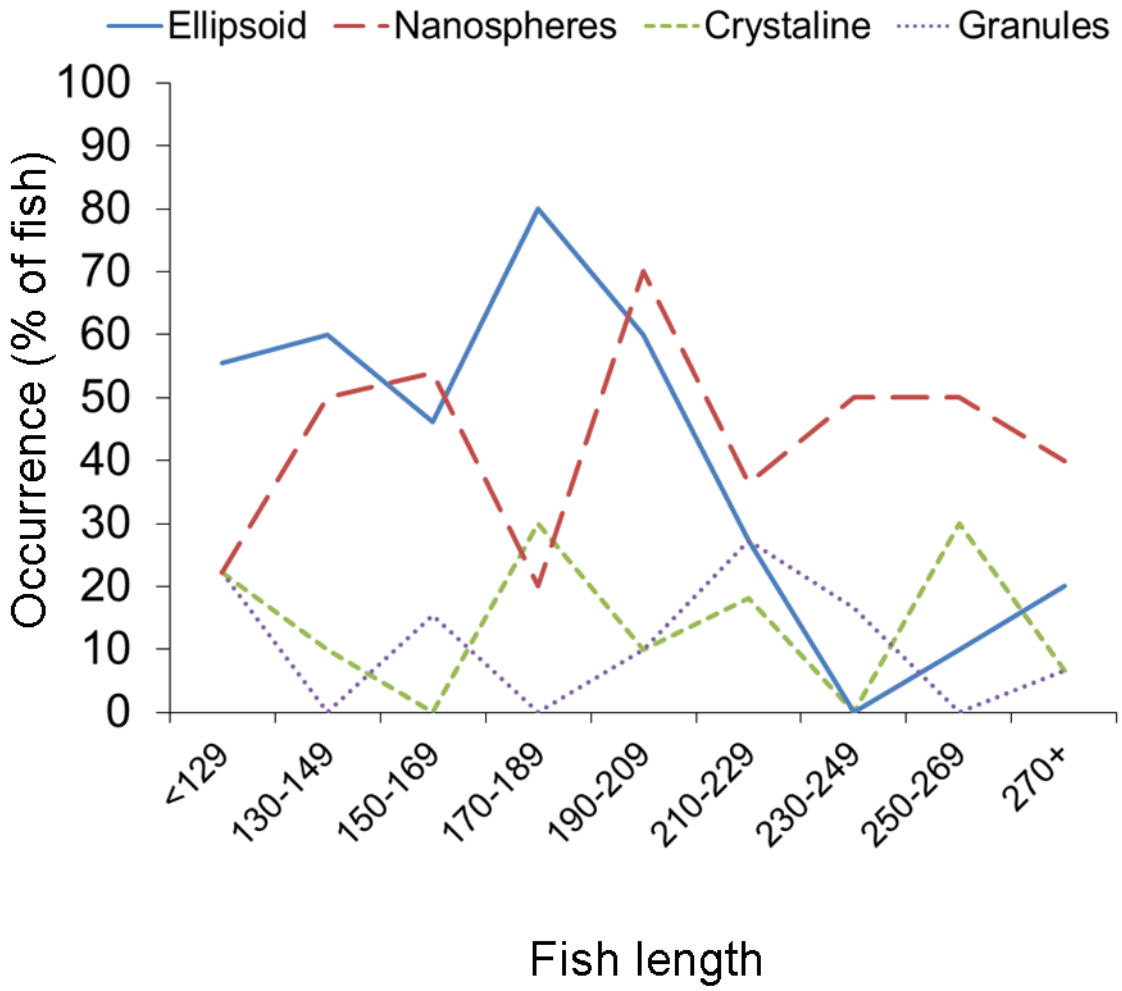

Figure 21 Occurrence frequency of different morphology types in all species across different fish sizes

The occurrence frequency of different carbonate morphology types in all species sampled from the Western English Channel across different size categories based on fish length. Occurrence rate is presented as the number of fish in which morphology types were observed as a percentage of total number of fish sampled in that size category.

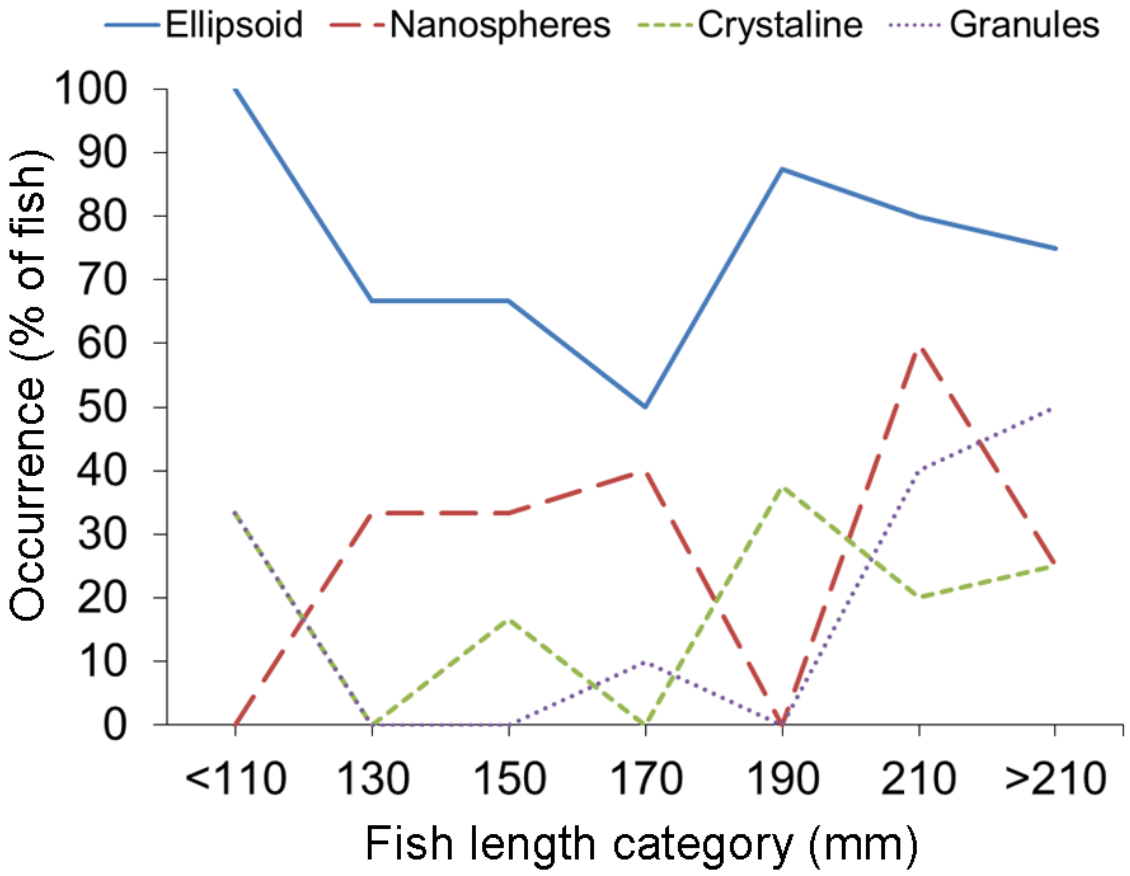

Figure 22 Occurrences of different morphology types across poor cod of different lengths

The occurrence rate of different fish carbonate morphology types are shown as the number of poor cod in which a morphology type were observed as a percentage of total number of poor cod sampled in that size categories. Length category number indicates the top end of the category unless otherwise stated.

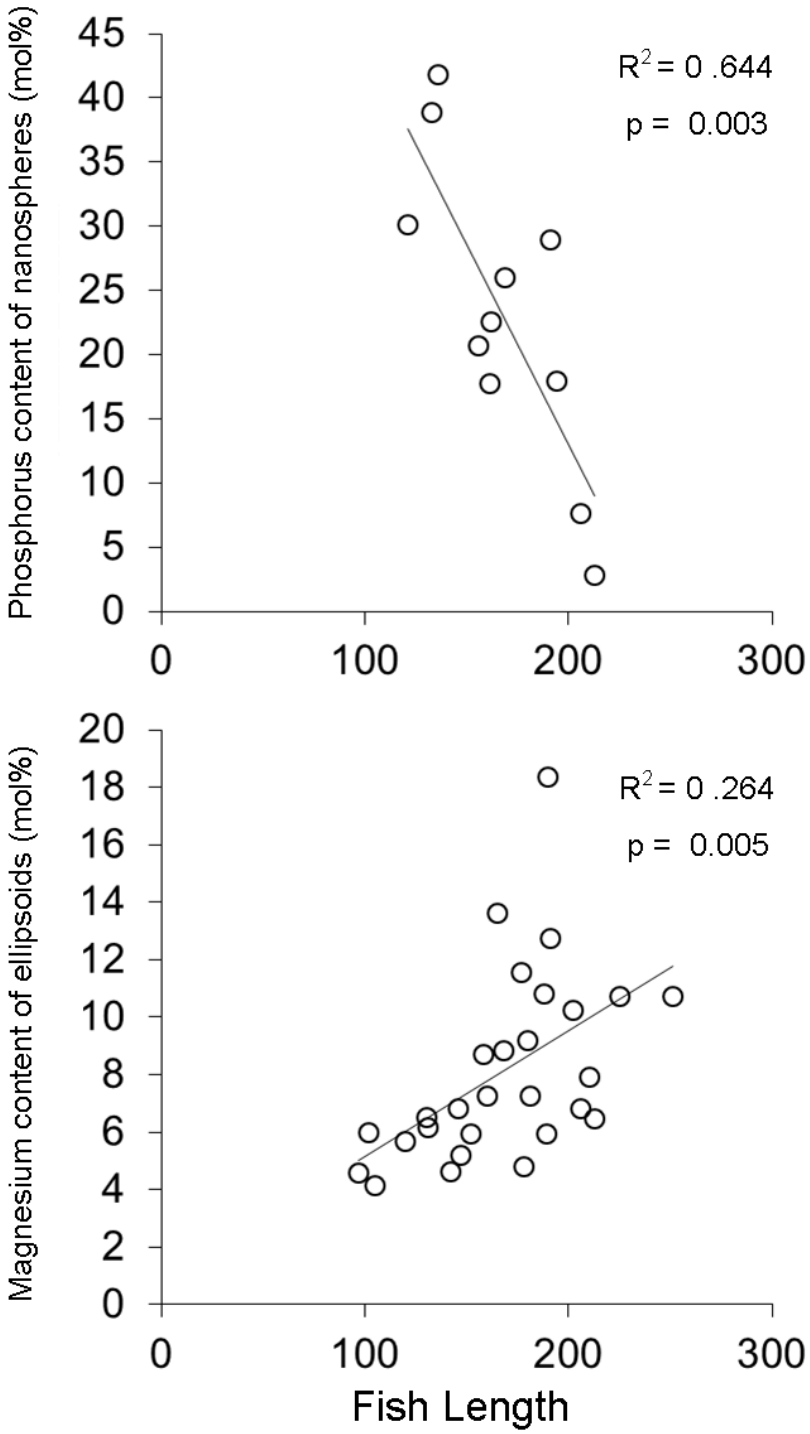

Figure 23 Scatter graphs of fish length and average phosphorus content of nanospheres (top) and average magnesium content of ellipsoids (bottom).

Scatter graphs of fish length and average phosphorus content of nanospheres (top) and average magnesium content of ellipsoids (bottom). P values are the result of Pearson's product-moment correlation test.

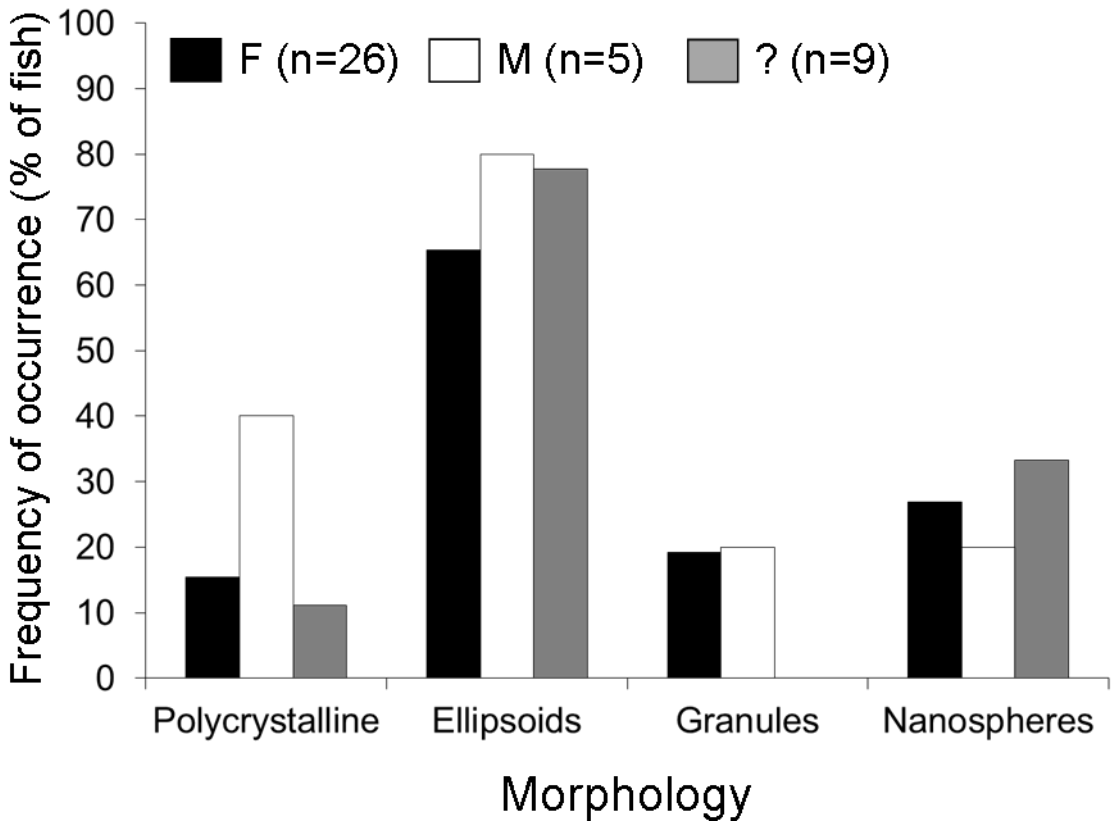

Figure 24 Occurrences of different morphology types in poor cod of different sexes

The occurrence rate of different crystal morphology types shown as the number of poor cod in which a morphology type was observed as a percentage of total number of poor cod sampled classed as females (F, black bars), males (M, white bars) and those where sex could not be identified (?, grey bars).

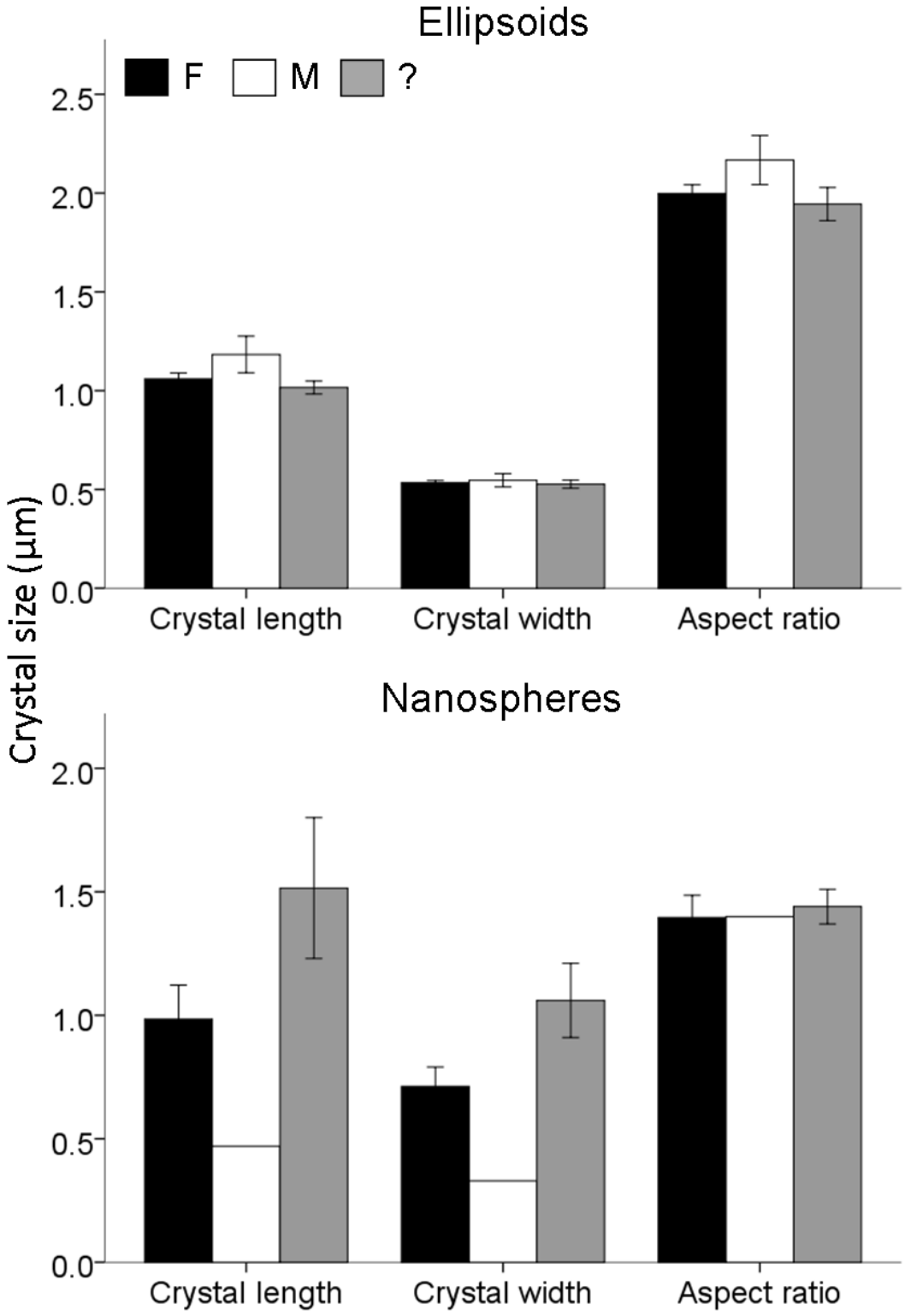

Figure 25 Crystal size and aspect ratio of carbonates sampled from fish of different sex

Average crystal size (length and width) and aspect ratio (length/width) of ellipsoidal carbonates (top) and nanospheres (bottom) sampled in female (black bars), male (white bars) and unidentified poor cod. Error bars represent standard error of the mean.

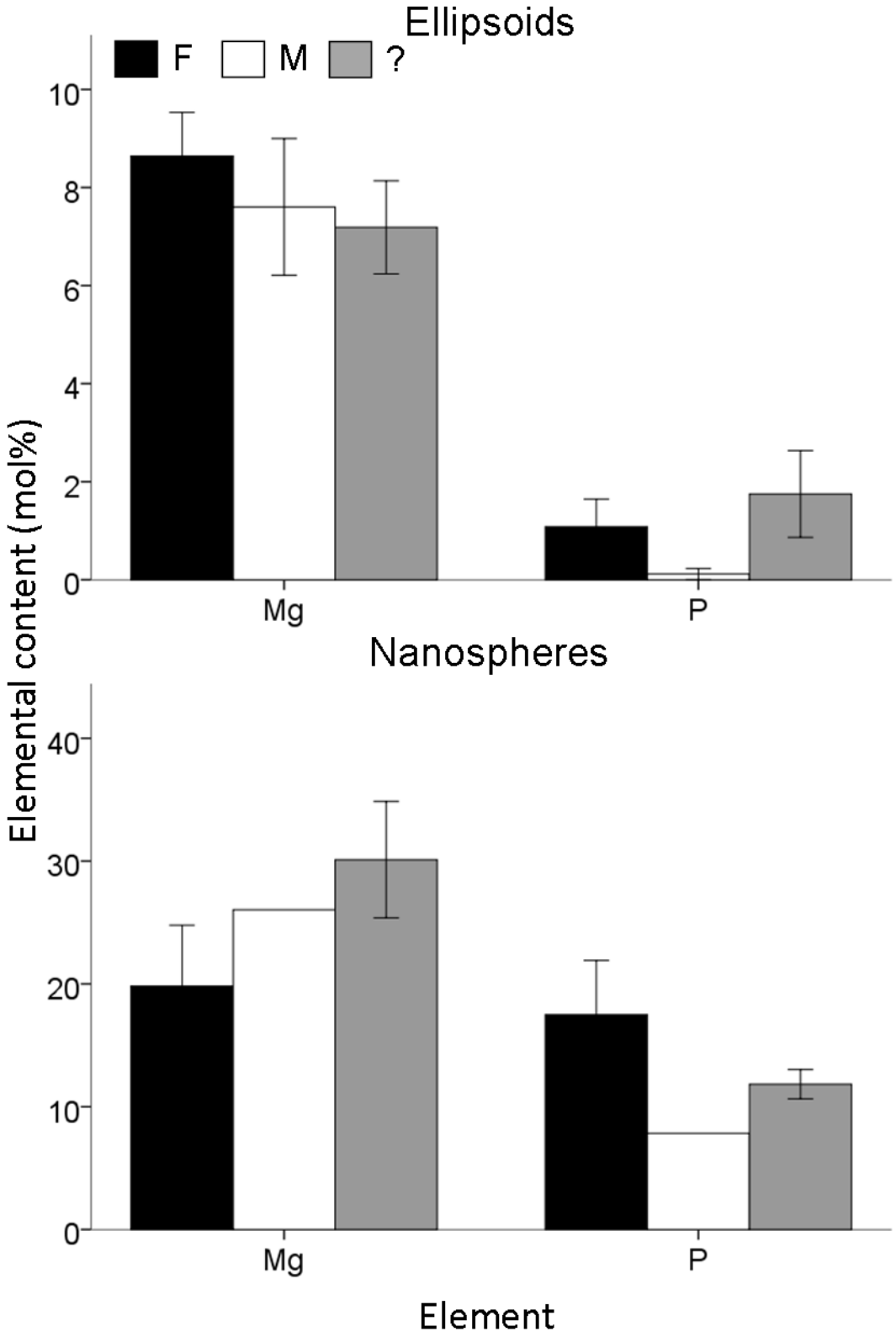

Figure 26 Elemental composition of carbonates from different sex fish

Average magnesium and phosphorus content of ellipsoidal carbonates (top) and nanospheres (bottom) sampled in female (black bars), male (white bars) and unidentified poor cod. Error bars represent standard error of the mean.

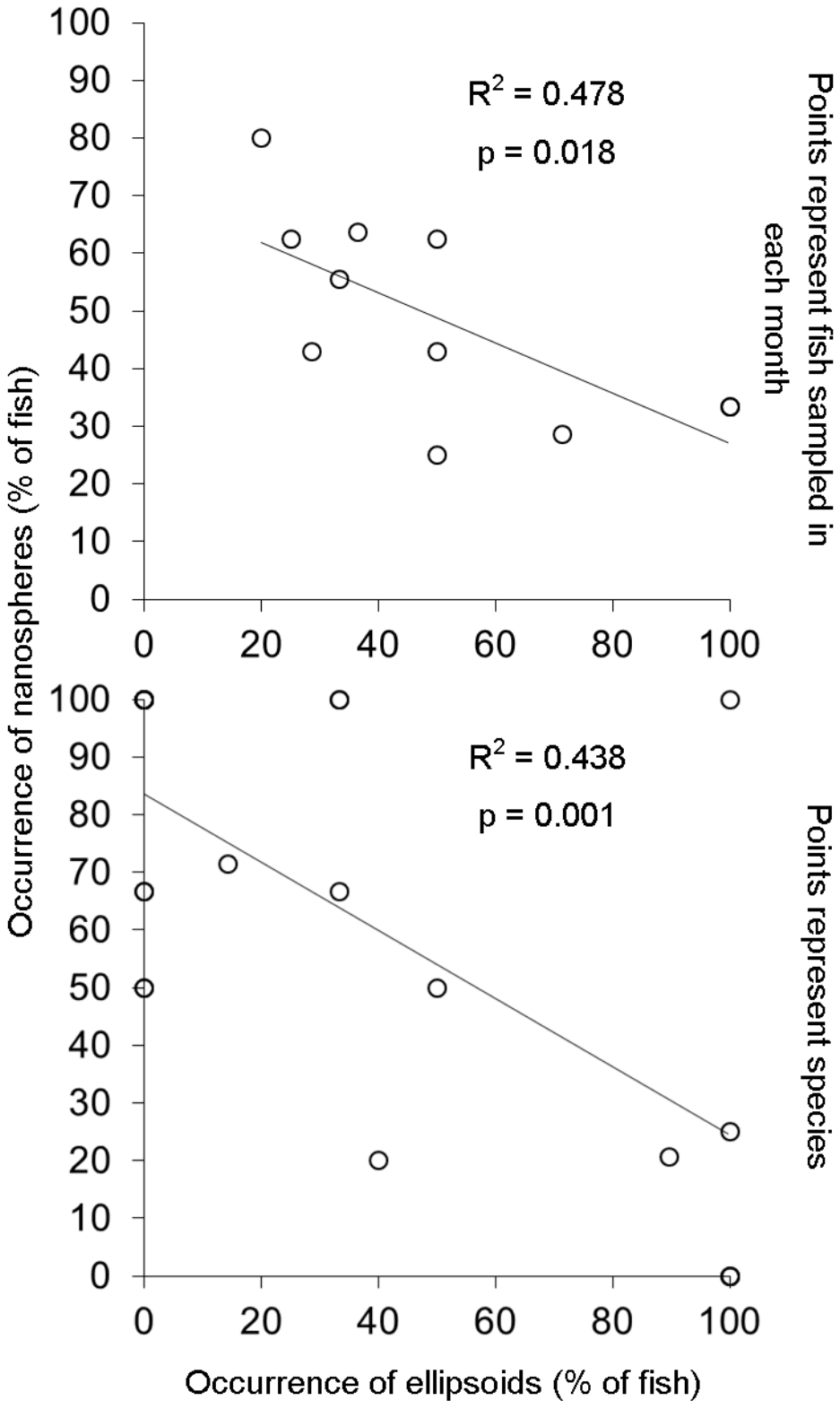

Figure 27 Scatter plots of the occurrence frequency of nanospheres compared to the frequency of ellipsoidal crystals

Scatter plots shows the frequency of ellipsoid occurrence compared to the frequency of nanosphere occurrence as percentages of the total number of fish sampled in each month (top panel) and each species (bottom panel).

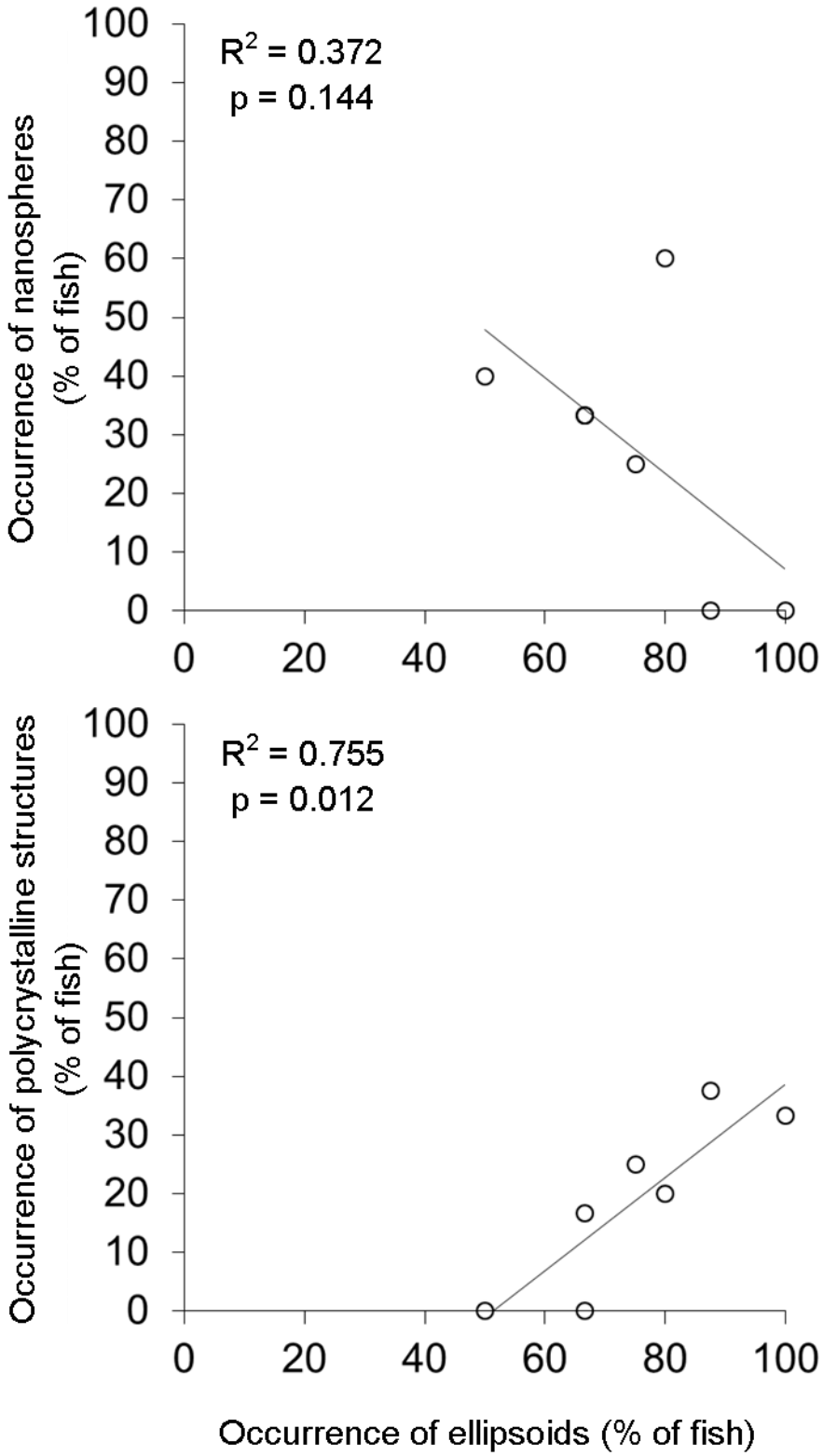

Figure 28 Scatter graphs of the frequency of ellipsoids against the frequency of nanospheres (top) and polycrystalline structures (bottom) in poor cod of different size categories

Scatter graphs of frequency with which ellipsoids were observed in various length categories of poor cod against the frequency with which nanospheres

were observed (top) and polycrystalline structures were observed as a percentage of the poor cod sampled in that size category.

3 Sedimentary preservation of temperate fish carbonates

3.1 Summary

Marine fish have previously been estimated to produce calcium carbonate in the ocean at a rate which could be significant to the ocean inorganic carbon pump. Understanding where fish carbonates end up in the ocean will enhance our understanding of how they might impact the global carbon cycle. So far there has been evidence that fish produced carbonates can contribute to shallow water sediment production in tropical areas. However, the potential for fish in temperate environments to contribute to sediment production has not been investigated. The current study examines and characterises the precipitates produced by farmed Atlantic salmon. Information on the precipitates produced by farmed salmon was subsequently used to inform searches for fish produced carbonates in sediment beneath salmon pens. Sediments beneath salmon pens were examined using scanning electron microscopy (SEM) for particles similar to those characterised as being produced by the Atlantic salmon. Additionally carbonate content of sediments beneath salmon pens was measured and compared to sediments in reference areas outside the impact of effluent from the salmon pens. No particles were observed through SEM of sediments from beneath cages that looked similar to fish produced carbonates. Additionally there was also little evidence that salmon influenced the carbonate content of sediments beneath salmon pens. It was unclear whether this was due to environmental factors unique to areas beneath fish farms (that may promote rapid dissolution) such as high organic loading or low temperatures associated with temperate seas. Either way, this study emphasises that fish carbonates have the potential to dissolve even in shallow water settings given the right environmental conditions.

3.2 Introduction

Marine teleost fish precipitate and excrete calcium carbonates in their intestines as a result of osmoregulatory mechanisms (Wilson et al., 2002; Wilson and Grosell, 2003) and the crystals are subsequently excreted into the environment. In the environment one of two processes happens; the carbonates either dissolve, raising ocean alkalinity, or persist, sinking to the seabed and contributing to carbon transport and storage as part of the sediments. The scale of this excretion is such that fish are potentially major contributors to ocean

sediment production and carbon cycling (Perry et al., 2011; Wilson et al., 2009). Understanding more about these two fates therefore may enhance our appreciation of the interactions that occur between the atmosphere and oceans in the future. Previous studies have shown evidence that excreted fish carbonates may end up in the sediments in tropical environments (Perry et al., 2011). Additionally they have the potential to be preserved over reasonably long periods; in a previous study, fish carbonate pellets agitated very gently in seawater showed little change after 200 days (Salter et al., 2014). However, it remains to be seen whether precipitates produced by fish in temperate latitudes have the potential to persist in sediments, or whether they dissolve soon after excretion.

Chapters 2 identify that fish carbonates produced by temperate species share many characteristics with those studied in tropical species. However temperate oceans have environmental characteristics that may make fish carbonates more likely to dissolve than in tropical settings. For example, temperate environments have by definition lower temperatures, between 5 and 18°C mean annual surface seawater temperatures (Tait and Dipper, 1998b). Additionally temperate areas often have lower seawater alkalinity (Millero et al., 1998). Both lower temperatures and seawater alkalinity increase the solubility of calcium carbonates (Morse et al., 2007). As such it may be that fish carbonates in temperate oceans dissolve at shallower depths compared to those in tropical areas. This may prevent fish from being major contributors to sediments in temperate areas. However, currently there is no empirical data to quantify the fate of fish carbonates in temperate environments, such as at what depth they can remain solid at while sinking or, how long once on the seabed they can remain there. Fish carbonates are most likely to be present in sediments at shallow depths and in areas where production rates are high. If no evidence of fish carbonates can be found in sediments in shallow water areas with high production rates it seems unlikely that fish carbonate production in temperate areas contribute greatly to deeper oceans and more widely through temperate environments. As it has never previously been investigated, this study aims to investigate whether fish carbonates are present in sediments of a temperate environmental setting that has high carbonate production rates from fish. Fish farms in temperate seas provide an ideal opportunity to investigate this. Farmed Atlantic salmon (*Salmo salar*) smolts are typically transferred to sea pens for a

period of 14 to 24 months before harvest (Marine Harvest 2015). Compared to wild fish, salmon farms provide a relatively high density of fish confined to an area for a prolonged period; the excrement and uneaten food from these fish is sufficient to create an area enriched in organic matter in the benthic sediments and pelagic water (Brown et al., 1987; Navarro et al., 2008). As Atlantic salmon are marine teleosts, they are likely to precipitate inorganic calcium carbonate in their intestines which they will excrete in addition to organic faecal matter (i.e. undigested food). Areas beneath salmon pens are therefore likely to contain a high abundance of both intestinally precipitated calcium carbonate and organic waste. Additionally sea pens for salmon can be moored close to shore in relatively shallow water settings. This maximises the potential of retrieving fish-produced carbonates from sediments, if they do persist there.

In order to identify fish carbonates in the sediments below salmon farms, this study utilised several techniques. Firstly the intestinal contents of farmed, Atlantic salmon were examined to characterise the morphology, size and elemental composition of endogenously produced precipitates of calcium carbonate using scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS). Intestinal precipitates from some fish that were not fed for several days prior to sampling were examined. This allowed precipitates uncontaminated by exogenous material to be characterised. Intestinal precipitates were also identified and characterised from fish that were feeding prior to sampling, which should be more typical of the products from farmed salmon that are fed continuously to encourage high growth rates. Subsequently, sediments from beneath salmon pens were sieved to concentrate potential fish carbonate by size separation of sediments based on the sizes of particles observed in the fish intestinal contents. This size-sorted fraction of sediments was then examined using SEM and EDS to compare with particles characterised from the fish intestinal contents.

Additionally, organic matter contents of sediments beneath salmon pens were compared to “reference” sediments in areas that should be unaffected by salmon pens to confirm that excreted matter was reaching the sediments examined for precipitates. Carbonate content of sediments beneath salmon pens were also measured and compared to these “reference” sites to assess

whether the excreted carbonate precipitates from salmon were sufficient to raise the carbonate content of sediments beneath them.

3.3 Methods

3.3.1 Study area and sampling

Samples were taken from three different salmon farm sites (Gorsten, Linnhe and Leven) run by Marine Harvest Scotland that were located in sea lochs on the west coast of Scotland (Figure 29). As part of routine environmental monitoring, sediment samples were hand collected at each site in 1 litre tubs by divers from both below salmon pens and at “reference” areas from each site. Reference areas were a distance away from the pens predicted to be far enough to avoid impact from the salmon effluent based on previous environmental monitoring at each site. Reference samples were taken at distances of more than 80 m, 90 m and 234 m from salmon pens at Leven, Linnhe and Gorsten respectively. Tubs of sediment samples were then frozen at -20ºC for storage. Tubs of sediment from under pens and at reference sites were later thawed to subsample for sieving (n=3 at each site) (see section 3.3.3) and analysis of organic matter and carbonate content on whole sediments (n=3 at each site) (see section 3.3.4). Intestinal contents were sampled from fish obtained from the same sites. Sampled fish at Gorsten (n=3) were withheld food for 2 days prior to sampling. Fish from Linnhe and Leven (n= 5) were fed a commercial pellet diet to satiation prior to sampling. Fish were sampled similarly to the methods described in the previous chapter. Intestinal contents were then frozen at -20°C for storage and transport before being thawed to prepare them for scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS).

3.3.2 Salmon intestinal content SEM and EDS analysis

Morphological analysis (by SEM) and compositional analysis (by EDS) of fish intestinal contents were carried out using the methods described in chapter 2 In brief, precipitates were treated to remove organic matter with bleach, rinsed, dried and mounted onto SEM stubs using self-adhesive carbon spectrotabs. Each sample was examined using a Hitachi S3200N SEM-EDS microscope with an attached Oxford instruments EDS system and secondary electron images were taken at 500, 2000 and 5000 x magnification of particles that

appeared to be endogenously produced (see chapter 2). At 5000 x magnification, particles were analysed by EDS readings taken at least 3 different points to assess elemental composition of particles. Particle sizes were measured at a later date from images using Image J.

3.3.3 Preparation of sediments for analysis

Particle sizes of intestinal precipitates produced by salmon were generally found to be less than 50 µm. Therefore sediments from each sampled site were separated into coarse (>63 µm) and fine (<63 µm) size fractions to concentrate the majority of potential fish carbonates into part of the sediment making them easier to find evidence of. To aid separation of size classes, and disaggregate any faecal matter, sediment samples were first cleaned of organic matter. A 5% sodium hypochlorite (bleach) solution was added to thawed subsamples of sediment at approximately a 1:1 ratio of hypochlorite solution to sediment volume. Sediment samples with bleach were then placed on a shaker for approximately 1 day, until the bleach appeared to no longer alter in colour. If a large colour change in the bleach occurred it was removed and replaced with fresh bleach. This was to remove organic matter without altering the carbonate crystals (Pingitore Jr et al., 1993). Samples were then centrifuged at 3000 rpm for 5 minutes (Eppendorf 5804R centrifuge with A-4-44 swing bucket rotor) and the bleach supernatant removed leaving any inorganic precipitates at the bottom of each tube. Following the removal of the bleach supernatant, ultrapure water was added and shaken with the sample to rinse off any leftover bleach. The sample was then passed through a 63 µm test sieve. Each fraction was rinsed into a vacuum filtration unit containing pre-weighed 0.45 µm filter papers to collect the sediment fractions. Sediment fractions on the filter paper were then rinsed with ultrapure water to remove any final traces of bleach. Once any liquid had drained filter papers were carefully transferred to pre-weighed petri dishes and dried at 40ºC to a constant weight which was recorded and used to calculate the percentage contribution of fine and course fractions to total sediment weight. Sub samples were then taken from fine fractions for SEM and EDS analysis using methods similar to those described in section 3.3.2, paying particular attention to particles which appeared similar to the endogenous carbonates observed in the salmon intestinal contents. Secondary electron images were taken of particles which looked the most like endogenous particles

from salmon intestinal contents and their elemental composition was analysed using EDS. Remaining fine fractions and course fractions were then subject to the “loss on ignition” method described below to quantify carbonate content.

3.3.4 Loss on ignition and carbonate analysis

Both whole sediment samples and samples which were sub-divided into coarse and fine fractions were subjected to loss on ignition and carbonate content analysis. Whole sediment samples were not cleaned of organic matter but were prepared as follows: Thawed subsamples of whole sediments from each fish farm site were centrifuged at 3000 rpm for 5 minutes (Eppendorf 5804R centrifuge with A-4-44 swing bucket rotor) to allow removal of as much seawater as possible. Following this ultrapure water was added to and shaken with samples. The samples were then centrifuged at 3000 rpm for 10 minutes using the same equipment described above to allow the removal of remaining salts and as much water as possible. Samples were then dried at 40ºC and ground in a pestle and mortar. Approximately 5 g of whole dried sediments subsamples were then transferred to pre-weighed crucibles. Additionally, approximately 1 g of dried samples of sediment separated into course and fine fractions (as prepared in section 3.3.3) were carefully scraped from the filters and ground in a pestle and mortar before being transferred to other pre-weighed crucibles. All samples in crucibles were then dried overnight at 105 ºC to remove any moisture from sediments. Crucibles containing sediments were then left to cool in desiccators then weighed again to obtain exact dry sample weight in each crucible. Once weighed, samples were then placed in a muffle furnace at 550 ºC for 4 hours. Samples were then cooled in desiccators and reweighed. Weight loss was used to calculate dry weight lost on ignition which is a measure of organic matter in whole sediment samples but not for samples separated into different size fractions. This was not a true measure of organic matter for samples separated into different size fractions as the cleaning process using bleaching to aid sieving would have removed some organic content prior to removal through loss on ignition. The weighed ash samples were then returned to the furnace at 925 ºC for 1 hour to convert carbonates to oxides lost as CO_2. Samples were then removed, allowed to cool in desiccators and reweighed. Weight loss at this stage was multiplied by 1.36 to give the weight of carbonate lost (Heiri et al., 2001) and then calculated as a percentage

of the dry weight (after heating to 105 °C) to show percentage carbonate content of sediments.

3.3.5 Statistical analysis

A two-way ANOVA with whether or not samples were from beneath pens or reference sediments used as fixed factor, and site as a random factor, revealed a significant interaction between the two factors for the variables: weight percent of fine size class sediments to whole sediments, organic content and carbonate content of sediments. As such, these variables at each site were analysed separately for differences between sediments from beneath pens and reference sediments using one-way ANOVAs. A two-way ANOVA was used to test for differences in magnesium and phosphorus content between carbonates of different morphology types in fed and unfed fish. Variance tests were carried out using IBM SPSS Statistics V.23 software.

3.4 Results

3.4.1 Characteristics of intestinal precipitates produced by salmon

SEM examination of intestinal contents from the farmed salmon revealed that salmon appear to predominantly produce intestinal precipitates as polycrystalline structures when both fed and unfed (Table 17 and Figure 30). The mean size for polycrystalline structures in fed and unfed fish were 44 and 17 µm respectively. Polycrystalline structures from fed fish were produced over a larger size range than unfed fish; the majority from fed fish (70 %) were less than 50 µm, whereas nearly all from unfed fish (98 %) of polycrystalline structures were shorter than 50 µm (Figure 31). Polycrystalline structures observed in unfed fish had more regular structures than those in fed fish (Figure 30). The only other morphology of endogenously produced precipitates observed was nanospheres (as describe in chapter 2) which were present in 80 % of fed fish (Table 17). Nanospheres were smooth appearing spheres no bigger than 8 µm but on average were 2.4 µm (Figure 31). Typical examples of all morphology types observed in salmon are shown in Figure 30. Nanospheres were contained a similar amount of magnesium (18.7 mol% on average) compared to the polycrystalline structures observed in salmon (mean 16.7 mol% for unfed fish and 9.6 mol% for fed fish). Nanospheres contained much more phosphorus than polycrystalline structures; nanospheres contained an

average of 42.4 mol% phosphorus, whereas phosphorus was only observed in polycrystalline structures from fed fish and only on average at 0.5 mol% (Figure 32). Morphology had a significant effect on the phosphorus content of the precipitates, whereas whether the fish had been fed or not, had no effect as tested by a two way ANOVA. There was no significant effect of either morphology or feeding on the magnesium content of precipitates.

3.4.2 Sediment characteristics

Upon examination by SEM of sediment fine fractions (<63 µm) collected from under salmon pens, no particles were observed that had morphologies or chemical compositions similar to those observed in the salmon intestinal contents. Individual sediment particles in general contained very little calcium or magnesium. The particle that was found to have the most calcium contained 1.6% calcium (atomic %) relative to 18.3 % silicon (Figure 33, A). The particle with the most magnesium contained 7.8 % magnesium (atomic %) relative to 6.5 % aluminium and 20.2 % silicon (Figure 33, C). Both Leven and Linnhe had significantly higher organic content in sediment under salmon cages (8.8% and 12.4 % respectively) than compared to reference sediments (3.6 % and 2.8 % respectively), whereas Gorsten did not (Figure 34). There was no significant different in carbonate content of whole sediments between samples taken beneath salmon cages and reference sites at Gorsten and Leven (Figure 35). At Linnhe, the mean carbonate content was significantly higher under salmon cages (3.2 %) compared to reference sediments (0.8 %) (Figure 35). The fine size (<63 µm) fraction made just over half of the total sediment weight at Gorsten and there was no significant difference between sediment from beneath salmon cages and reference sediments. The fine fractions at Leven and Linnhe contributed to significantly more of the sediment total weight under salmon pens (48 % and 28 % at Leven and Linnhe respectively) than it did to reference sediments (22 % and 20 % at Leven and Linnhe respectively) (Figure 36). There was no significant difference between the carbonate content of the fine fraction of sediments from under salmon pens and reference sediments at Gorsten; both contained 1.2% carbonate. At Leven there was significantly less carbonate under salmon pens (2.0 %) compared to the reference sediment (3.0 %) in the fine fraction of sediment. In the fine fraction of sediment at Linnhe there was significantly more carbonate in sediments collected under pens (2.4

%) than reference sediments (0.8 %) (Figure 37). Carbonate content was similar in samples taken from beneath pens and their reference sites at Gorsten and Leven, but was higher in samples from beneath pens at Linnhe (Figure 35).

3.5 Discussion

This is the first study to search for and attempt to identify the presence of fish produced carbonates in temperate sediments of any kind. As such, the present study investigated an area that theoretically would have a large chance of fish carbonates being present in the sediments, under salmon farm pens. As a component of searching for fish produced carbonates in the sediments, this study also describes the morphology, size and composition of calcium carbonates produced by Atlantic salmon. However, despite choosing to examine sediments that theoretically should maximise the chance of locating fish carbonates, little evidence was found of fish-derived carbonates in the sediment below salmon pens. Evidence of fish carbonates in sediments has, however, been found previously in tropical sediments (Perry et al., 2011; Salter et al., 2014). Below discusses the lack of evidence observed in the present study for fish carbonates in the sediments and some of the reasons why this may be the case.

3.5.1 Evidence of precipitates under salmon pens

The precipitates from Atlantic salmon were mostly smaller than 50 µm in length. As such, sediment was sieved in to separate out the <63 µm size class of particles which should have in theory concentrated most fish produced carbonates into a smaller sediment volume making it easier to find them. In spite of this, no particles that appeared to be similar to the morphology and composition of the carbonates examined in intestinal precipitates were observed. This, however, is not conclusive evidence that they were absent. A direct observation of particles in the sediment with a similar morphology and chemical composition to intestinal precipitates would provide strong evidence for the contribution of fish carbonates to the sediment. However it is only possible to examine very small amounts of sediments through SEM and as such it could be that there happened to be no fish carbonates in the small subsamples of sediment taken for SEM observations. Additionally it relies on human observation to visually spot particles that potentially look like fish

carbonates among many other particles in the sediment. This can be especially difficult due to the size range of particles; when viewing sediments at a suitable magnification to see larger, for example 20 – 50 µm size particles, it difficult to properly see smaller (1 – 10 µm) particles. This combined with human inefficiency at spotting particles may have led to potential fish carbonates being missed in sediment observed by SEM. As such in the present study, bulk carbonate content of sediments was also measured both under the salmon pens and at reference sites which should be outside the area impacted by salmon effluent from pens. In theory, if salmon in pens are producing carbonates which contribute to the sediments, carbonate contents of sediments from beneath cages should be higher than the carbonate content of the reference sediments. However, only one of the three sites studied, Linnhe, had significantly more carbonate in the sediment beneath salmon pens (3.2 %) compared to reference sediment (0.8 %) (Figure 35). However, this appeared to be mostly due to the course fraction (>63 µm) having increased carbonate content. The course fraction of sediments makes up the majority of the sediment weight at Linnhe both under salmon cages and in the reference sediments (fine fraction representing 28 % and 20 % of total sediment respectively) (Figure 36). Additionally the difference between the carbonate content of sediments beneath pens and reference sediments was bigger in the course fraction of sediments at Linnhe, there was 1.6 % more carbonate under pens in the course fraction compared to only 0.7 % more under pens in the fine fraction (Figure 37). The majority (70%) of the carbonates examined in intestinal contents from salmon were smaller than 50 µm, it might be expected that the fine fraction (<63 µm) would be responsible for any increases in carbonate of whole sediments rather than the course fraction (>63 µm) if the carbonate content increase was being caused by the salmon.

For the other two of the three sites examined, Gorsten and Leven, carbonate content was extremely similar between whole sediments taken from beneath pens and reference sediments taken from outside the plume (Figure 35). Overall there is no convincing evidence that salmon are contributing much to carbonate sediment production. The reasons for this need to be considered as previously there has been evidence in tropical studies that fish can contribute to carbonate sediment production (Perry et al., 2011; Salter et al., 2014).

3.5.2 Salmon precipitate characteristics

It could have been that a characteristic of the precipitates themselves predisposes carbonates produced by Atlantic salmon to dissolve quickly. However, the morphology of Atlantic salmon precipitates was not unlike precipitates observed in tropical fish previously in the tropics. This study found that salmon produce predominantly polycrystalline intestinal precipitates. Polycrystalline structured precipitates were observed in 100 % of the sampled salmon regardless of whether they were fed prior to sampling or not (Table 17). Previous studies in tropical species also reported polycrystalline structures being produced from a variety of species (Salter et al., 2012). Bluehead wrasse (*Thalassoma bifasciatum*), checkered puffer (*Sphoeroides testudineus*) and bonefish (*Albula vulpes*) produced polycrystalline carbonate structures between 4 and 125 µm in length in a previous study (Salter et al., 2014), similar to Atlantic salmon observed in the present study (Figure 31). Atlantic salmon polycrystalline structured carbonates contained an average of 16.7 and 9.6 mol % magnesium (for unfed and fed fish respectively) which is within the range of magnesium contents reported for tropical fish carbonates (between 0.5 and 40 mol %) (Salter et al., 2012), all be it at the lower end of this range. Polycrystalline structured carbonate produced by Atlantic salmon therefore seem fairly similar to those produced by tropical species in both size and magnesium content.

A major difference observed between the Atlantic salmon carbonates and the carbonate precipitates described previously in tropical fish was the presence of phosphorus containing nanospheres in fed fish. Phosphorus containing nanospheres were observed in 80% of fed Atlantic salmon but not in salmon that were withheld food prior to sampling. As such it appears that the presence of food in the intestine favours the production of phosphorus containing nanospheres. This may explain why none were reported to be produced from tropical species in previous studies, as these were all withheld food prior to sampling (Salter et al., 2014, 2012).

Both the size of particles and the magnesium content have the potential to alter how quickly carbonates dissolve. Smaller particles will have a higher surface area to volume ratio resulting in a large amount of the carbonate being exposed to the seawater aiding dissolution. Magnesium content can also decrease the

stability of calcium carbonate precipitates (Walter, 1984). As polycrystalline structures were similar to those described as being produced by tropical species in both size and magnesium content it is likely that they have similar stabilities in seawater. It is currently unknown how phosphorus will affect the solubility of nanospheres, but these were always produced alongside polycrystalline structures by fed Atlantic salmon. As such even if nanospheres were to dissolve rapidly on excretion, it might be expected that the polycrystalline structures still have the potential to remain solid in seawater and contribute to sediments as has been seen for tropical fish species (Perry et al., 2011; Salter et al., 2014). Therefore there must be other reasons why little evidence of carbonates from salmon was observed in the sediments beneath salmon pens in the current study.

3.5.3 Solubility of carbonates in temperate environments

It seems unlikely that any unique characteristic of the precipitates themselves produced by salmon is leading to their absence in the sediment below fish pens as fish carbonates from tropical species had similar characteristics. In tropical environments, evidence has been found that fish contribute carbonates to the sediments (Perry et al., 2011; Salter et al., 2014). It could be that environmental factors that differ between the tropical reef environments and the temperate sea lochs examined in the present study caused salmon precipitates to dissolve quickly in the present study. The major environmental differences that might affect solubility of carbonates are the seawater temperature and chemistry, including total alkalinity and calcite saturation state.

Higher temperatures cause lower solubility in magnesium calcites (Bertram and Mackenzie, 1991). Seawater temperatures were reported to be between 25 and 30 °C in the previous tropical study examining fish precipitate preservation in sediments (Salter et al., 2014), i.e. considerably higher than those experienced in the Scottish sea lochs studied here. Temperate seas generally tend to reach a maximum annual average of 18 °C (Tait and Dipper, 1998b) and the study area used here is typically much lower. For example, temperatures typically between 5 and 10 °C over the month of May have been reported previously in the Loch Linnhe area (Price et al., 2015). Lower temperature could therefore be a major factor behind lower preservation potential for fish carbonates in current study.

Additionally, total alkalinity of the seawater will affect the solubility of solid calcium carbonates. The majority of total alkalinity in seawater is from dissolved carbonate and bicarbonate ions. Higher alkalinity will push the equilibrium toward favouring less dissolution and thus greater preservation of fish carbonates in the sediment (Butler, 1991). The main controllers of total alkalinity in oceans are inputs and removal of freshwater (through processes such as precipitation and evaporation); and as such salinity is correlated with total alkalinity in seawater (Lee et al., 2006; Millero et al., 1998). Generally oceans in tropical latitudes have higher alkalinity seawater than those in temperate latitudes due to less precipitation and freshwater input and more evaporation (Millero et al., 1998). Additionally the salinity of the seawater examined in the previous tropical study in The Bahamas was reported to be between 36 and 37 practical salinity units (psu), whereas the area in the current temperate study in Scotland generally had a salinity of around 30 psu, when measured over about a month using a data buoy (Price et al., 2015). It is therefore likely that the study conducted in the tropics, where fish carbonates were preserved in the sediment, was in much higher alkalinity seawater than the Scottish sea lochs examined in the present study. Overall, seawater conditions in the present study were likely not conducive to preservation of fish precipitated calcium carbonates in the sediment which could be why the high density of salmon did not appear to contribute much carbonate material to the sediments beneath them. However, there are other reasons why there may have been few fish carbonates evident in the sediments examined from under salmon pens in the present study.

3.5.4 The depositional environment below farmed fish

It could be that a feature unique to the environment below the farmed fish in the present study caused the dissolution of the carbonates produced by the penned salmon rather than the temperate climate. It is possible that the reference sediments were from subtly different depositional environments compared to the sediments below the pens. This could mask any increase caused by the fish under the salmon pens. At two of the three sites examined, the sediment beneath the salmon pens had a significantly different proportion of fine (<63 μm) sediment compared to reference sites, with sediments from under cages containing 1.4 and 2.2 fold more fine particles (as a weight % of total sediments) than reference sediments at Linnhe and Leven respectively (Figure

36). The difference in the contribution of the fine fraction to total sediment composition could be caused by the presence of the salmon themselves. Pellet foods for fish often contain fish meal, among other substances, which contain small particles such as bone. Undigested food from the salmon could cause an increase in various size categories of particles in the sediment. If these non-carbonate particles are excreted in a greater quantity than the carbonates it could mean that an increase in the % carbonate content of sediments may not be observed under salmon pens. Either way, sediments from other sources may have obscured the impact that the penned salmon have on sediment carbonate.

Another possibility could be that the carbonates produced by the penned salmon are exported over large horizontal distances and therefore contribute to carbonate content of sediments in areas further from the salmon cages than previously thought. Alternatively it could have been that the reference sediments were taken too close to the pens and as such were still affected by excreted matter from the salmon leading to no observable difference. However, both of these explanations seem unlikely as at two of the three sites studied, organic content of sediments beneath salmon cages was significantly higher than the reference sediments (Figure 34). This would indicate at these sites at least, excrement from the fish was reaching the sediment in higher amounts under the pens compared to at the location of reference sediments rather than being transported away.

The presence of organic matter from the fish could have another major effect on carbonates in the sediment, however. The organic matter present in fish faeces can provide nutrients for various organisms to feed upon. Sediments close to fish farms have been shown to take up oxygen from the water (Brown et al., 1987; Cathalot et al., 2012). This is presumably due to increased respiration of microorganisms and so it would be associated with a reciprocal increase in carbon dioxide input to the seawater. The salmon themselves will also excrete their own respiratory carbon dioxide into the seawater. Inside intensive aquaculture systems, levels of carbon dioxide reach much higher levels than occur naturally due to the high stocking density (Ellis et al., 2016), and this is likely to be the case for water around and inside sea pens. Higher levels of carbon dioxide in the water would decrease the pH, carbonate ion concentration and saturation state, favouring dissolution of carbonates from the salmon that

settle in the area of enriched organic matter around salmon pens. Unfortunately data on the pH of water near the sediments and in the salmon pens were not obtained in this study. As such, the extent to which increased organic matter and respiration in the environment of the salmon pens affected the dissolution of any carbonates is unknown.

3.5.5 Conclusions and future work

The current study was the first to attempt to identify evidence of fish produced carbonates in temperate sediments. Previous studies have shown evidence of fish carbonate contribution to tropical sediment production (Perry et al., 2011; Salter et al., 2014). The current study has shown that Atlantic salmon appear to produce carbonates similar in morphology and composition to tropical species. However, despite this, little evidence of fish produced carbonates in sediments was found beneath penned farmed Atlantic salmon. It is therefore likely that the carbonates produced by the salmon dissolved fairly rapidly preventing an accumulation in the sediments below pens.

It is unclear, however, whether this will extend to carbonates produced by fish across all temperate areas. The density of fish carbonate production provided by farmed salmon may be similar to fish species that form large schools, but it is unlikely to be representative of all fish carbonate production in the natural environment. Section 3.5.4 discusses how the high concentration of organic matter from the salmon pens could have facilitated the dissolution of the carbonates produced by the fish.

It was chosen to examine the sediments beneath fish farms in the current study as they present an opportunity to examine sediments from an area with relatively high production rates of fish carbonates. If it were possible to identify and measure fish carbonates in sediments below fish farms it would have further presented opportunities to investigate factors that might impact the persistence of fish carbonates in sediments, for example, by measuring how long fish carbonates remain in sediments after a fish farm lies fallow. However, the current study failed to identify any carbonates produced by fish in the sediments below salmon pens. As such, utilising areas of high fish density may not be a practical way of investigating whether fish can contribute to carbonate

sediment production. Alternative methods of investigating fish contribution to carbonate sediment production may prove more useful in the future.

It may be impractical to try and simply identify fish produced carbonates in sediments from different locations using SEM. Section 3.5.1 discusses reason why observing fish produced carbonates in sediments can be difficult. Additionally, although identifying fish carbonates in temperate sediments would confirm whether fish can contribute to sediment production, it will not provide information on the extent to which fish contribute to carbonate sediment accumulation. It may be that only a fraction of the carbonates produced by fish actually reach the sediment before dissolving depending on the environmental parameters (such as temperature and water alkalinity) and seabed depth. The current study highlights the potential for fish produced carbonates to be able to dissolve in the environment. In the future it may be more important to understand how much of the carbonates produced by fish have the potential to contribute to sediment production across different environments rather than simply whether they do or not.

In order to investigate how much of the carbonates produced by fish contribute to sediment production it may be more effective to directly examine the solubility of the carbonates themselves rather than examining sediments. If the solubility of different types of carbonate produced by fish is known, it might be possible to predict whether particles are capable of reaching the seabed across different areas of the ocean according to the depth and temperature.

Additionally it would be useful to know how long carbonates produced by fish can remain intact once reaching the ocean floor. To contribute to sediments fish carbonates will need to remain solid long enough to allow burial to take place and limit contact with seawater. Experiments that look at long term dissolution of fish carbonates may be required to yield such information. Salter et al. (2014) examined the longer term effects of seawater exposure and motion on carbonates produced by tropical fish in laboratory based experiments using containers of seawater and shaker plates. However such experiments in sealed containers are unlikely to be completely representative of conditions on the seabed due to the closed nature of the system.

One experimental approach, which may be more representative of processes occurring in the environment, could be to anchor fish carbonates to the seafloor

in a container permeable to seawater. The carbonates could then be found and examined over time for evidence of dissolution. Although it is unlikely that the environment directly around the carbonates would be completely representative of the environment found in sediments it would be more so than those found in a closed system. Additionally it avoids the difficulty of finding fish produced carbonates among sediments which occurred in the current study.

Overall, although the current study did not identify evidence that precipitates from farmed salmon were contributing to carbonate sediment production, it is not possible to discount that fish produced carbonates may contribute to sediment production in other temperate climate areas. Future work may need to utilise alternative methods in order to successfully determine the extent to which fish in temperate areas may contribute to carbonate sediment production. Either way, the present study highlights that fish produced carbonates can be susceptible to dissolution in the ocean. As such, it highlights the importance in understanding fish carbonate solubility in being able to predict what fate befalls fish carbonates in the ocean and how they are contributing to the ocean carbon cycle.

3.6 Tables

Table 17 Frequency with which different morphologies of intestinal precipitates are produced by salmon

Frequency with which different morphologies of intestinal precipitates are produced by fed and unfed salmon expressed as a percentage of individuals in which the morphology was observed. The number of individuals observed in each category is shown the column labelled “Total”

	Polycrystalline	Nanospheres	Total
Unfed	100 %	0 %	3
Fed	100 %	80 %	5

3.7 Figures

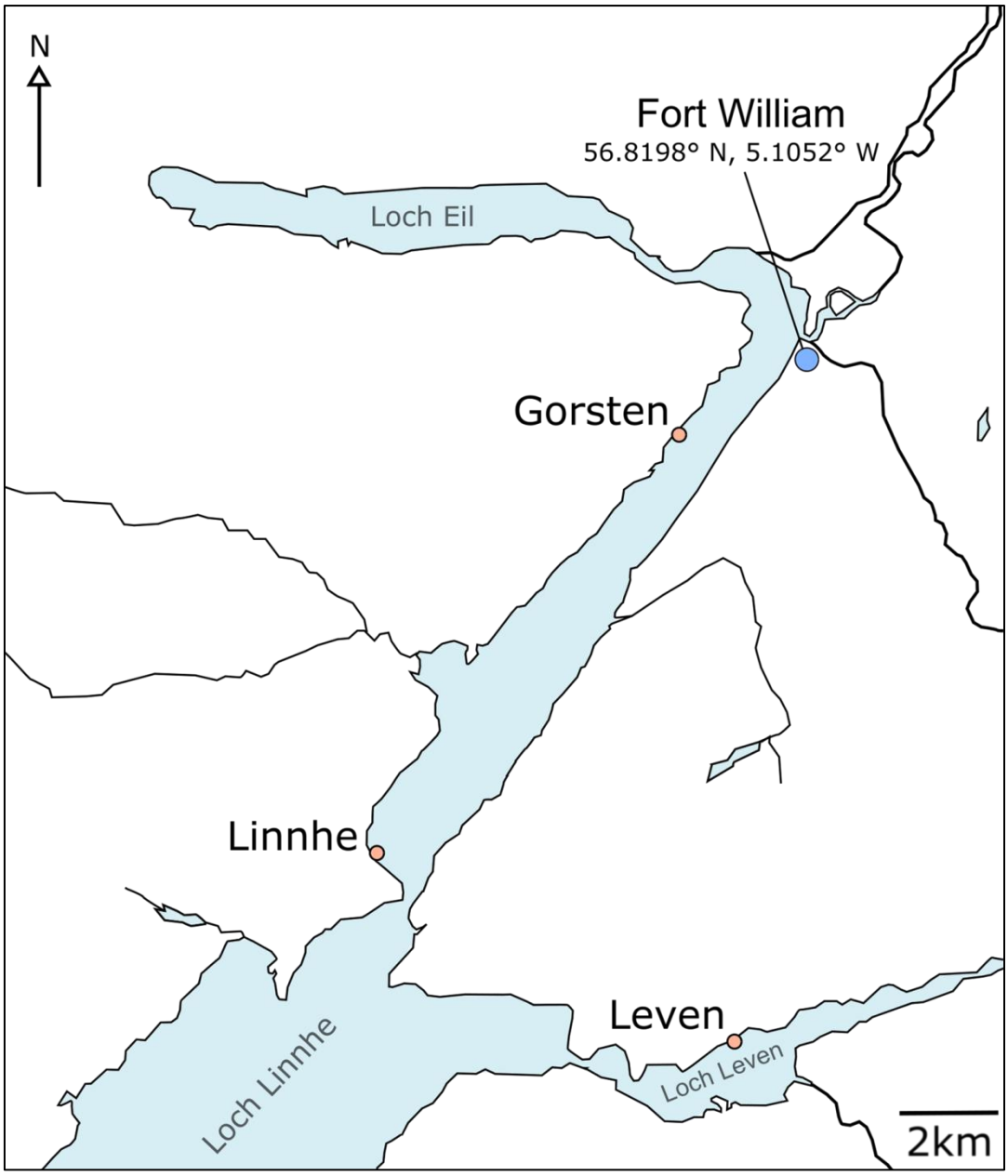

Figure 29 Map showing salmon pen study sites

Map showing the location of salmon pens studied: Gorsten, Linnhe and Leven.

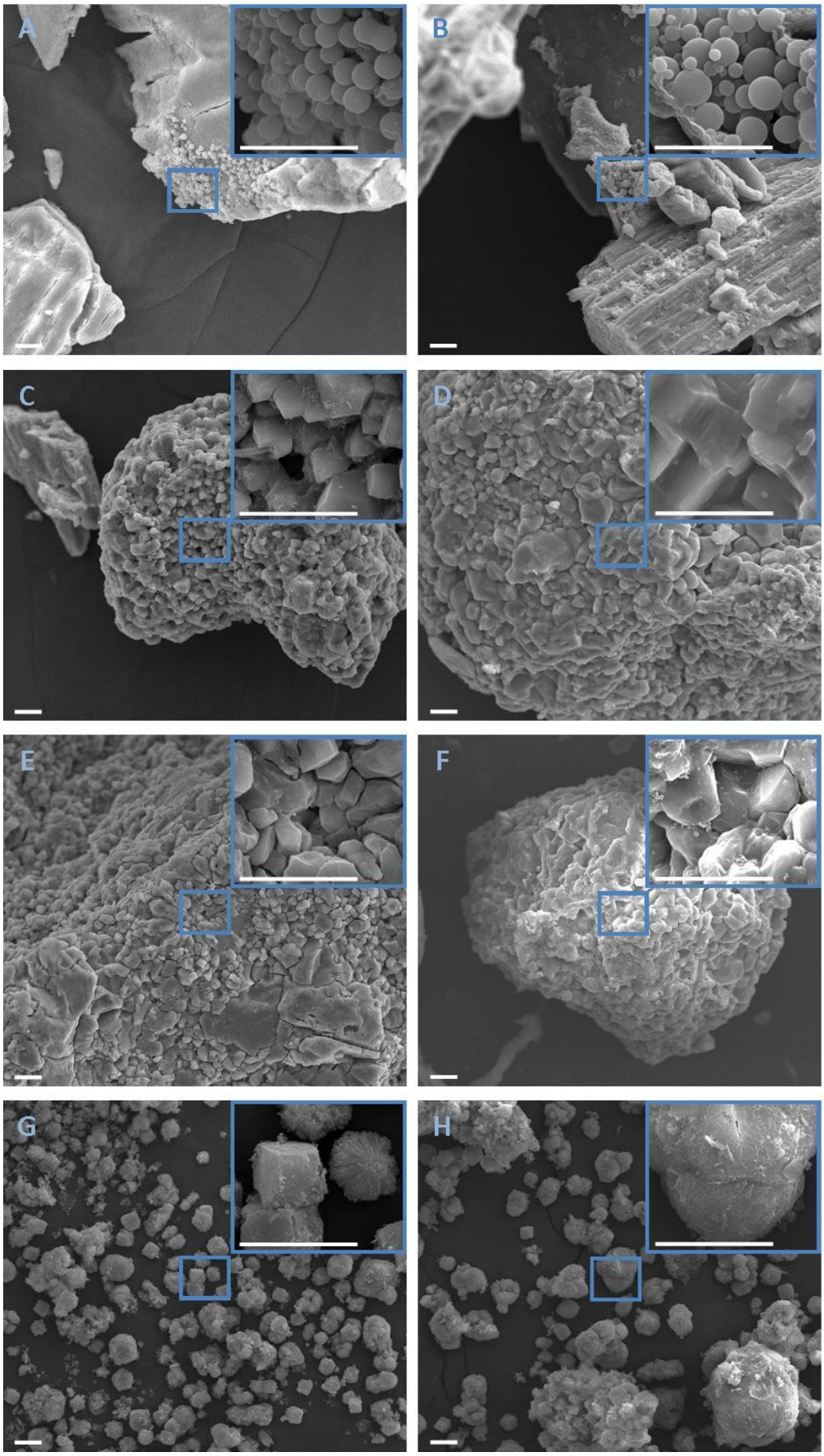

Figure 30 Examples of typical intestinal precipitate morphologies

observed in salmon.

SEM images of typical intestinal precipitate morphologies observed in salmon. A) and B) show typical nanospheres from fed salmon; C), D), E) and F) show polycrystalline structures observed in fed salmon, whereas G) and H) show polycrystalline structures observed in salmon withheld food for 2 days. White bars indicate 10 µm.

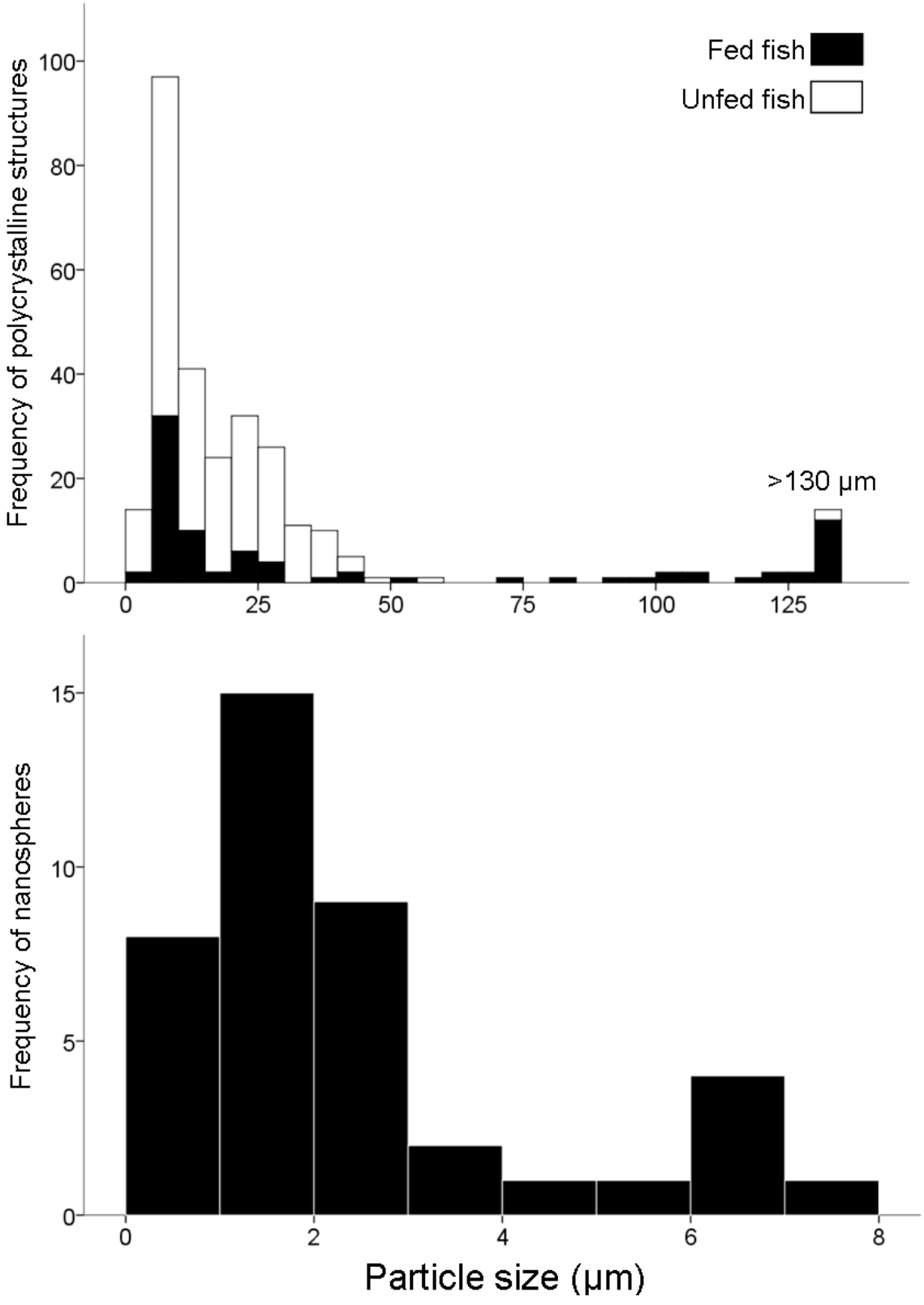

Figure 31 Size distribution of intestinal precipitates produced by salmon

Histogram of sizes of polycrystalline structured carbonates (top) and nanospheres (bottom) produced by Atlantic salmon. White bars represent precipitated carbonates obtained from the intestine of fish withheld food for 2 days prior to sampling whereas black bars represent precipitates obtained from

fish feeding until the time of sampling.

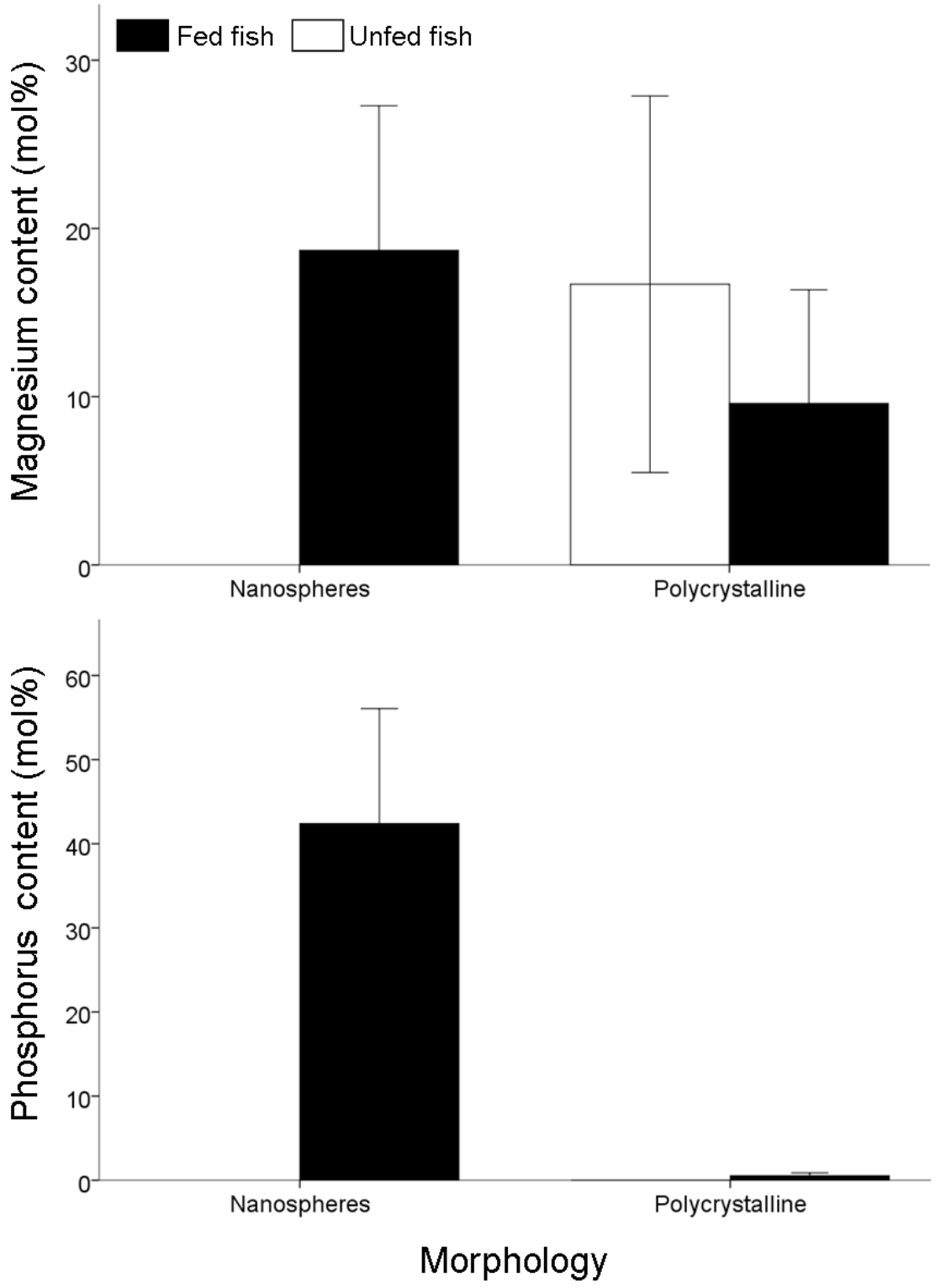

Figure 32 Magnesium and phosphorus content of intestinal precipitates produced by Atlantic salmon.

Average magnesium (top) and phosphorus (bottom) content of different morphologies of intestinal precipitates produced by farmed salmon. Bars represent means for precipitates obtained from fish withheld food for two days prior to sampling (white) or fish feeding prior to sampling (black). Error bars are

equivalent to the standard error of the mean.

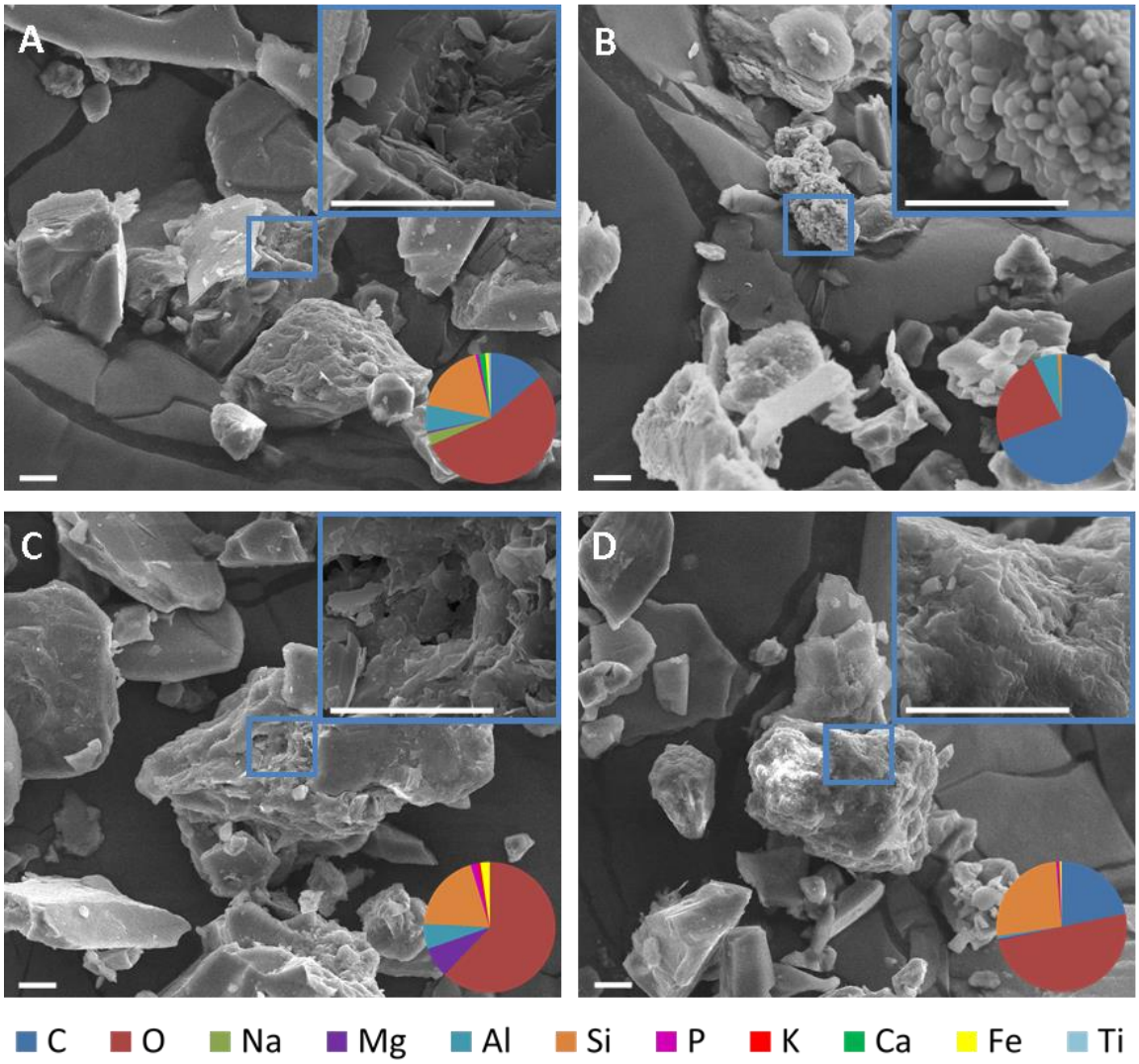

Figure 33 SEM images and chemical compositions of fine sediment fraction from beneath salmon pens.

SEM images of <63 µm fraction of sediments collected beneath salmon pens. Pie charts show the average % elemental composition (by atomic %, not weight) of expanded areas as measured by EDS on three points. White bars represent 10 µm.

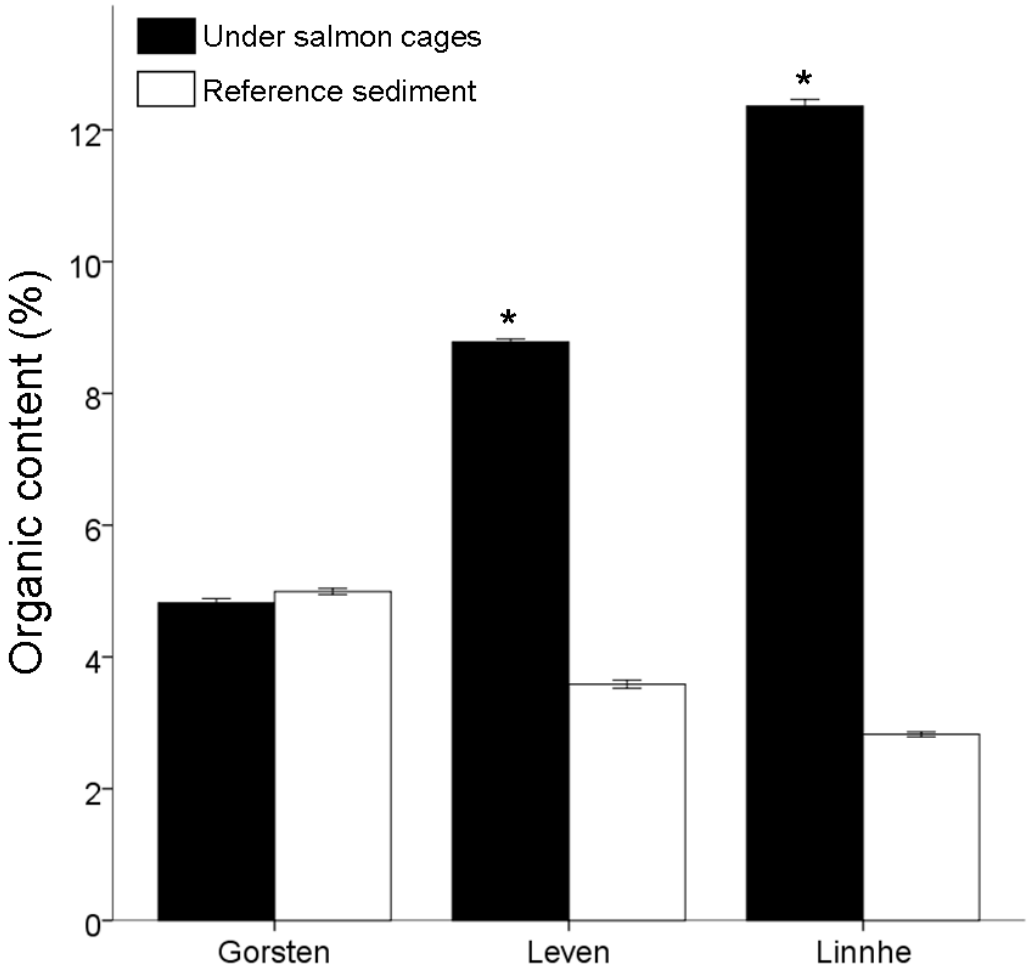

Figure 34 Organic content of sediments sampled beneath salmon pens and reference samples.

Average organic content (measured by loss on ignition) of sediments sampled beneath salmon pens and reference samples taken from outside the plume of impact from penned salmon across all three study sites, Gorsten, Linnhe and Leven. Error bars show standard error of the mean. Asterisk show significant differences between samples from beneath cages and reference sites.

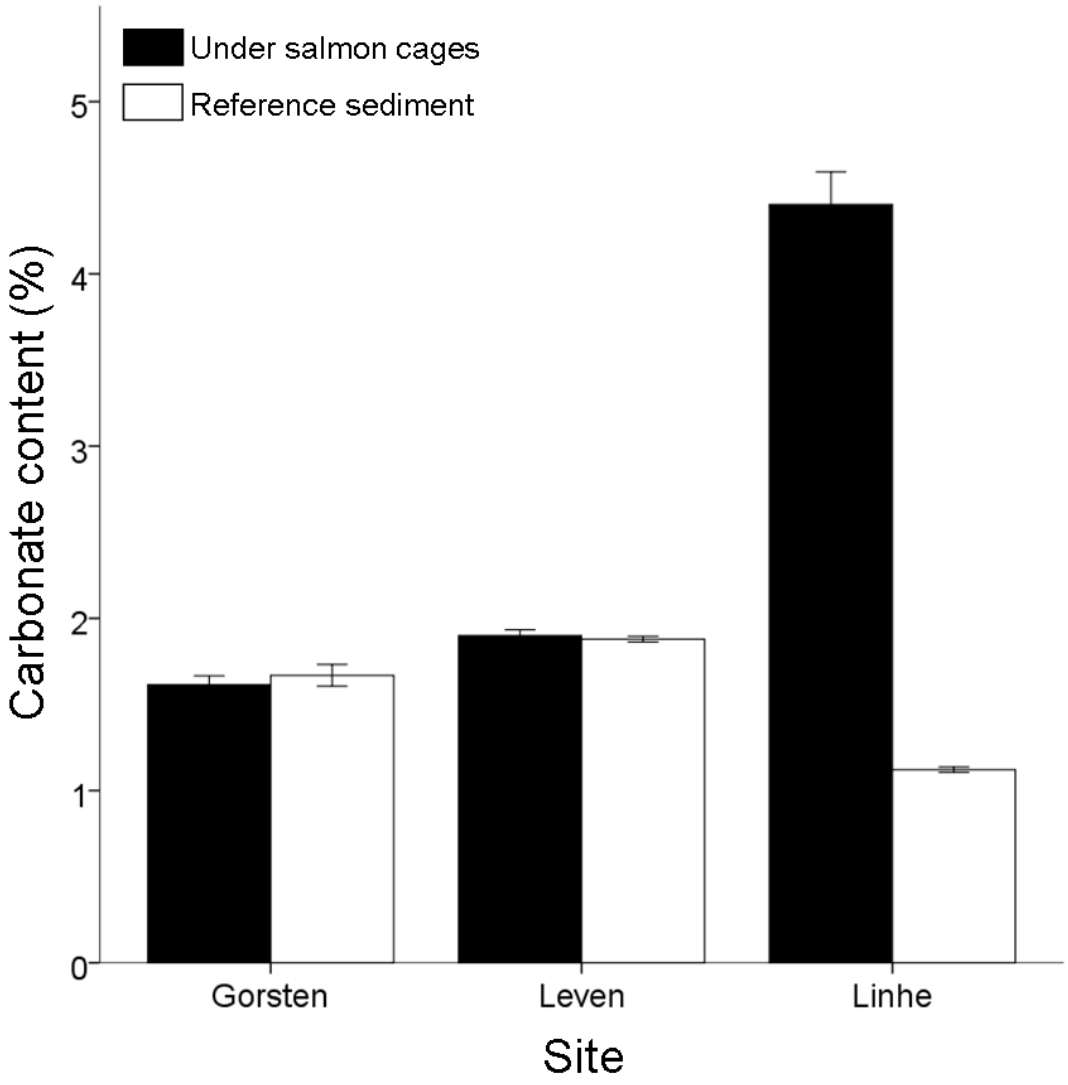

Figure 35 Carbonate content of sediments sampled from beneath salmon pens and reference sediments outside the impact of salmon effluent.

Mean carbonate content of sediments collected from directly beneath salmon pens (black bars) and reference sediments which were not influenced by effluent material from salmon pens (white bars) at Gorsten, Leven and Linnhe. Error bars show standard error of the mean. Asterisk (*) indicates significant differences between samples from under salmon cages and reference samples.

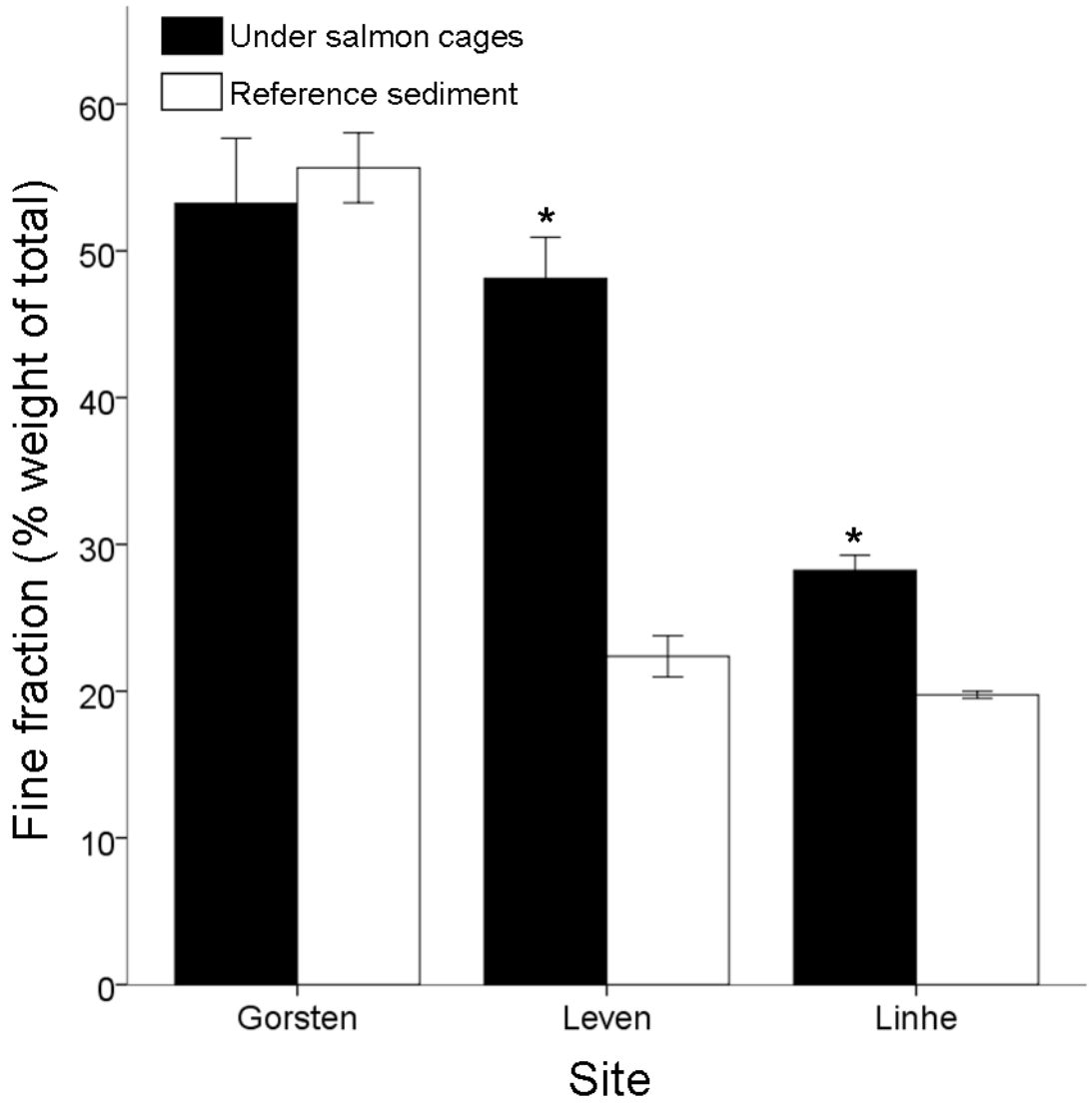

Figure 36 Fraction of sediment <63 µm in size at each site

Mean percentage contribution of sediment fine fraction (<63 µm) to total sediment weight for sediments from beneath salmon pens (black bars) and reference sediments (white bars) which are outside the area of impact from salmon pen effluent. Error bars represent standard error of the mean. Asterisks (*) show significant differences between sediments beneath pens and reference sediments.

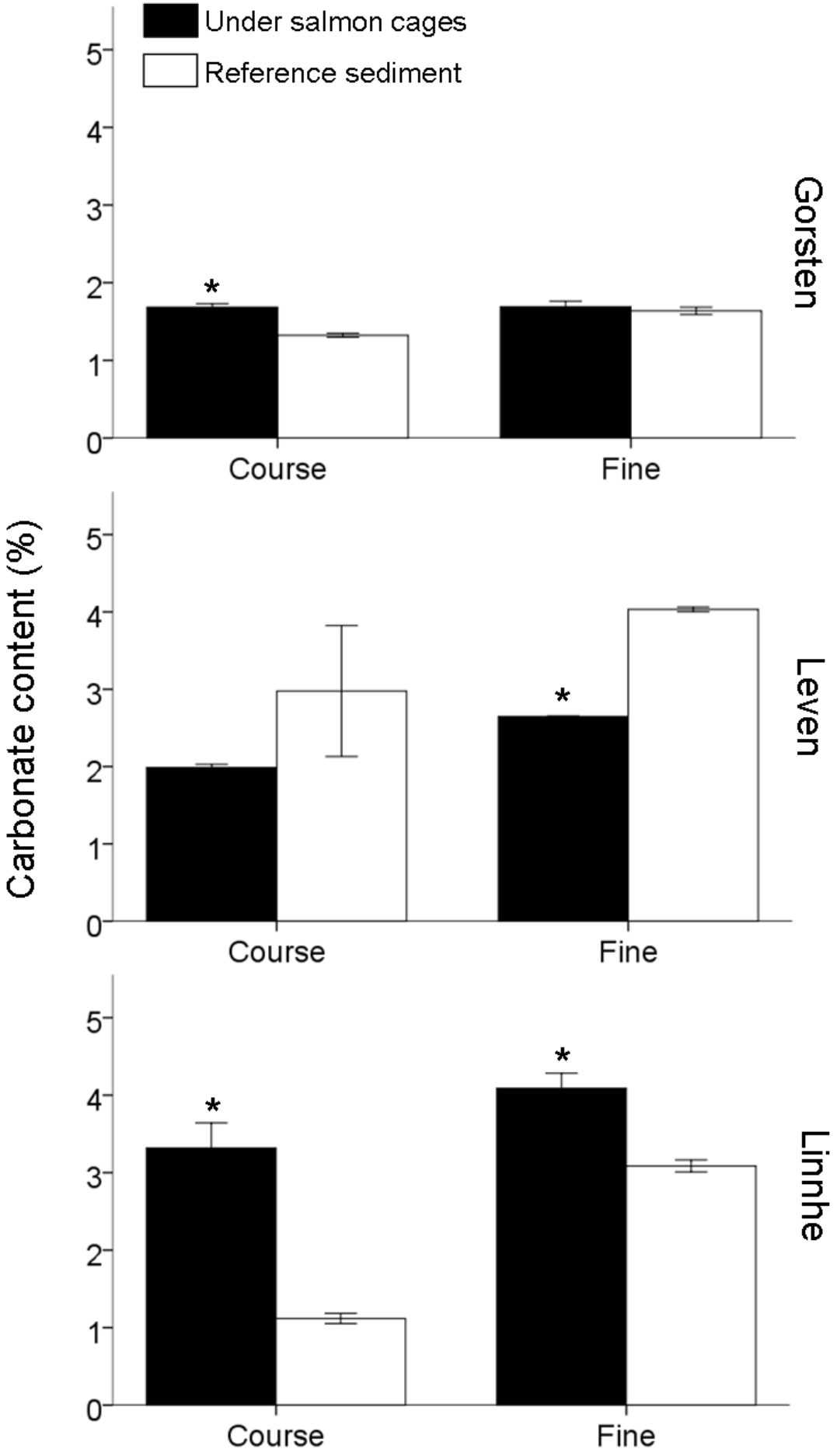
Under salmon cages
Reference sediment
Carbonate content (%)
Gorsten
Leven
Linnhe
Course
Fine
0
1
2
3
4
5
*

Figure 37 Carbonate % content for course and fine fractions of sediment at each site

Average carbonate content (%) for course (>65 µm) and fine (<63 µm) size fractions of sediment from both under salmon pens (black bars) and reference sediments away from the influence of salmon pen effluent (white bars) at sites Gorsten (top), Leven (middle) and Linnhe (bottom). Error bars indicate standard error of the mean. Asterisks (*) show significant differences between sampled from under salmon pens and reference sediments.

4 The effects of salinity on gut carbonate excretion in marine fish

4.1 Summary

Oceans across the globe vary spatially in various parameters including temperature, salinity and alkalinity. Additionally fish biomass is distributed unevenly throughout different ocean environments. These environmental parameters can potentially alter both the rate at which individual fish produce carbonates and the solubility of them in the environment. Understanding how fish carbonate production rates vary through such parameters will be essential in estimating how much fish are contributing to carbonate production in different areas. Although the effects of temperature have already been considered and included in previous models of ocean fish carbonate production, salinity has not. This chapter examines effects that changes in salinity, within an environmentally relevant range, can have on carbonate production from two temperate marine fish species. Goldsinny wrasse (*Ctenolabrus rupestris*) and shanny (*Lipophrys pholis*) were held at 30, 35 or 40 practical salinity units (psu). Precipitates were collected and quantified to obtain carbonate production rates. Drinking rates were also measured in order to investigate the mechanism behind the relationship between salinity and carbonate production rates. Each species displayed different responses in drinking rate increases between salinities increases. Additionally there were differences between species in the rate that calcium ions were precipitated relative to the rate of supply to the intestine through drinking. Despite differing responses between species to increased salinity, both species exhibited increase carbonate production rates at higher salinities. Carbonate production rates increased with greater magnitude between 35 and 40 psu than between 30 and 35 psu for both species. If this is the case for fish more widely across the oceans, current estimates for global fish carbonate production rates could be lower than in reality.

4.2 Introduction

In 2009 marine fish became recognised as a potential major contributor to global marine calcium carbonate production. Based on the known geo-spatial variability in fish biomass and seawater temperature globally, it was estimated

conservatively that marine fish are responsible for between 3 and 15 % of total oceanic carbonate production (Wilson et al., 2009). Furthermore, less conservative (though physiologically realistic) estimates suggest the contribution of fish could be as high as 45 %. Calcium carbonate in the ocean contributes to the inorganic carbon pump; its formation sequesters ocean alkalinity into a solid form and releases acidic carbon dioxide into surface waters. The sequestered alkalinity can then be transported to deeper parts of the ocean and sediments removing it from surface waters and the ocean-atmosphere interface. This carbonate “pump” works antagonistically to the organic carbon pump which favours carbon dioxide sequestration into deep oceans through photosynthesis and sinking of organic matter. The role of fish in these processes is currently poorly understood despite the significant amount of carbonate they are estimated to produce in the ocean.

To understand how fish-derived carbonates impact ocean chemistry we also need information regarding its solubility; less soluble carbonates are more likely to reach the deeper ocean and sediments and therefore be more efficient at sequestering ocean alkalinity. Some factors are likely to affect both the production rate and the solubility once these carbonates are excreted into the environment. Temperature is one such factor, the effects of which have already been considered and applied to models of fish global carbonate production (Wilson et al., 2009). Salinity is another factor with the potential to affect both the production rate and solubility of carbonates, and across the world’s oceans salinity varies naturally between approximately 30 and 40 practical salinity units (psu) (Vine et al., 2015), with an average value of approximately 35 psu. The same factors that impact salinity also impact alkalinity of the seawater, so these variables often correlate (Lee et al., 2006; Millero et al., 1998). Alkalinity can affect the solubility of excreted calcium carbonates (Morse et al., 2007) while the salinity directly impacts the production rate of calcium carbonates from fish. Understanding of the mechanism behind intestinal carbonate production helps to explain this influence of salinity.

Marine teleost fish precipitate carbonate in their intestines as part of their osmoregulatory strategy (Grosell, 2011; Wilson et al., 2002; Wilson and Grosell, 2003). They are hypo-osmotic to their seawater environment and so employ various strategies to counteract passive water loss and ion uptake. One

component of this is to drink large quantities of seawater. Calcium in this ingested seawater is precipitated as calcium carbonate in the intestine by the secretion of large amounts of endogenous HCO_3^- ions by the intestinal epithelium (Wilson et al. 2002; Grosell 2011; Guffey et al. 2011). This avoids the uptake of excess calcium and reduces the osmolality of intestinal fluid to aid water uptake (Wilson et al., 2002; Wilson and Grosell, 2003). The calcium carbonate precipitates are then excreted as mucus coated aggregates of crystals into the environment.

Marine fish tend to regulate their internal osmolality between about 310 and 352 mOsm kg^{-1} (Whittamore, 2011) which is equivalent to a salinity of 10 to 12 psu. Therefore at a higher environmental salinity, there is a larger osmotic gradient between the internal body fluids of a fish and the surrounding seawater, and hence a greater passive loss of water and gain of ions. To counter this, drinking rate must therefore increase along with salinity. Imbibed seawater is the principle source of calcium ions for the production of calcium carbonate in fish intestines. Thus an increased supply of calcium ions to the intestine from increased drinking should result in an increase in the production rate of calcium carbonate.

In theory, if all the ingested calcium ions are precipitated as carbonate in the intestine, then the production rate of calcium carbonate (µmol/kg/h) can be estimated as the product of the seawater drinking rate (ml/kg/h) and the calcium ion concentration in the ingested seawater (µmol/ml) as shown in Equation 5.

Equation 5

$$CaCO_3 \text{ precipitation rate} = \text{Drinking rate} \times [Ca^{2+}]$$

However, both drinking rate and the concentration of the calcium ions in seawater increase with rising salinity, which means the carbonate precipitation rate should increase exponentially, rather than linearly, with a rise in salinity. It is possible to make a simple estimate of the change in drinking rate with salinity. Assuming the efficiencies of passive water loss and regulated water absorption in the intestine are the same at different salinities, then the relative change in drinking rate should be proportional to the relative change in osmotic gradient (environmental osmolality minus the fish body fluid osmolality). The relative change in carbonate precipitation rate can then be estimated as the product of

the relative change in drinking rate and the relative change in calcium ion concentration at two different salinities.

However, this simple estimate of change in carbonate production with salinity is likely inadequate for accurate predictions as two assumptions are probably not always correct. The first was that the efficiency of passive water loss and regulated (intestinal) water uptake does not vary with osmotic gradient. However, previous studies have found that when salinity is increased, water absorption by the gut increased proportionally less than the increase in osmotic gradient between the fish and the seawater (Table 18). So, for example in a study conducted on gulf toadfish (*Opsanus beta*) a 1.58 fold increase in the osmotic gradient between the fish and seawater lead to only a 1.24 fold increase in the rate of water absorption (Genz et al., 2008). Similarly, other studies found that when salinity is increased, drinking rates also increased proportionally less than the increase in osmotic gradient (Table 18). For example, in European seabass (*Dicentrarchus labrax*) a 2.2 fold increase in the osmotic gradient resulted in drinking rate increasing by only 1.2 fold (Varsamos et al., 2004). As such, it would seem that fish are able to minimise the impact of osmotic gradient on water loss and subsequent drinking requirements at increased salinities. Depending on the mechanism by which this happens this may lead to carbonate production rates not increasing as much as predicted at higher salinities as calcium ions may not be supplied to the intestine at rates as high as predicted.

The second assumption is that the same fraction of ingested calcium ions will be precipitated as calcium carbonate across all salinities. In reality if this were to happen, it would mean that fish would effectively end up with increasing amounts of calcium left in solution in their intestinal fluid as salinity increases. For example, if fish precipitate 90% of the ingested calcium at all salinities, then the amount of calcium remaining in solution will be higher at 40 psu than 35 psu as the ingested fluid in the former starts with a higher concentration of calcium ions. This extra calcium would either be absorbed by the intestine or it would contribute more to the osmotic potential of the intestinal fluid making water absorption into the blood harder. In order to maintain fluid absorption efficiency it would make more sense to precipitate a higher proportion of the ingestion calcium at higher salinities. In support of this, fractional calcium precipitation

values in a previous study were 26.8 and 61.2 % of the ingested calcium at 35 and 50 psu, respectively (Genz et al., 2008).

Calcium carbonate production rates from fish have been shown to increase over large increases in salinity (between 9, 35 and 50 psu) (Genz et al., 2008), however oceanic salinities tend to vary only between approximately 30 and 40 psu on large spatial scales (Antonov et al., 2010; Vine et al., 2015). So far it is not known how changes to salinity within these ranges affect carbonate production rates from fish. Without such information, it seems unlikely that we can accurately predict how carbonate production rate will change with different salinities that exist in the marine environment today.

Considering the importance of accurately estimating global fish calcium carbonate production rates, especially regarding factors that also relate to solubility, the current study investigated the effect of environmentally relevant salinity differences on fish carbonate production rates. Carbonate production rates, drinking rates and other physiological parameters were measured in two fish species, goldsinny wrasse (*Ctenolabrus rupestris*) and shanny (*Lipophrys pholis*), held at 30, 35 and 40 psu. Shanny are native to temperate rocky shore tidal pools so experience large changes on a regular basis while goldsinny wrasse are generally found on temperate rocky reefs in deeper water, so presumably do not regularly experience great fluctuations in salinity. By comparing changes in carbonate production rates, metabolic rates and drinking rates caused by salinity in these two species, this study aimed to produce data that can be used to more accurately predict how carbonate production rates change across different salinities throughout many species of fish in the ocean.

4.3 Materials and methods

4.3.1 Experimental animals and design

Shanny (*Lipophrys pholis;* range: 8.3 - 38.7 g) and goldsinny wrasse (*Ctenolabrus rupestris;* range: 10.9 - 34.4 g) were obtained from Native Marine Centre (Weymouth, UK). Prior to experiments fish were maintained in 35 psu artificial seawater (Tropic Marin® salts dissolved in reverse osmosis (RO) water) recirculating through a biofiltration system at 15 °C. Fish were fed on a diet of chopped prawn, squid and mussel daily to satiation. Upon initiation of the experiment, shanny (n=10) and goldsinny wrasse (n=10) were transferred to

individual 2.9 litre plastic tanks connected to each one of three closed recirculating systems which were maintained nominally at 30, 35 or 40 psu. Upon transfer, each fish was blotted dry with paper towel to remove excess water and weighed. Individual plastic tanks were aerated and equipped with a plastic mesh basket that allow excreted faeces and precipitates to fall through while preventing access of the fish to minimise subsequent re-ingestion. Mesh baskets were removable and thus also provided a way to rapidly and easily transfer fish to a separate volume of seawater with minimum stress to allow collection of excreted intestinal precipitates from their original tank.

Each closed recirculating system had a total volume of 250 litres and a total fish biomass of 344, 355 and 407 grams for 30, 35 and 40 psu systems, respectively. Water was changed completely every 5 days and water samples were taken to monitor ammonia levels, which reached a maximum of 107 µM. Salinity was maintained through daily monitoring (YSI 90, Yellow Springs, Colorado, USA) and addition of RO water to compensate for evaporation. Salinity and pH (Hanna HI 8314 pH meter with Radiometer Analytical Red Rod pHC2401 electrode) were recorded every day.

Fish were acclimated to the individual tanks within the treatment salinities for 6 days without food. At the end of the acclimation period tanks were cleaned to remove faeces. Precipitates were then collected for analysis from each tank using a wide mouth pipette every 24h after initial cleaning for 5 days (further analysis of collected precipitates is described in section 4.3.2).

Oxygen consumption rates were measured 1 day after the initial acclimation period (described in section 4.3.3).

At 8 days past the acclimation period fish were transferred to new individual 2.9 l plastic aerated tanks for drinking rate measurements. Tanks contained clean aerated seawater of the same salinity with no mesh baskets. Fish were acclimated to these new tanks overnight before drinking rate was measured and blood samples were taken (described in section 4.3.4).

4.3.2 Carbonate precipitate analysis

Collected precipitates were briefly rinsed with deionised water to remove any superficial gut fluid and seawater before adding 5% (w/v) sodium hypochlorite (Fischer Scientific) to digest organic components. Precipitates were stored in

this until analysis could be carried out. Collected precipitates stored in sodium hypochlorite were centrifuged at 5000 rpm for 3 minutes and rinsed in deionised water, three times to remove traces of hypochlorite before being dried at 40 °C. Samples from each fish were then combined in another vessel for analysis through the addition of ultrapure water (20 ml total) and sonication (Vibra-Cell, Sonics and Material Inc.) to release any adhered precipitates and minimize particle size. Bicarbonate equivalent (HCO_3^- + $2CO_3^{2-}$) content of precipitate and seawater samples were determined by double titration using the method of Hills (1973) as described by Wilson et al. (2002) with an autotritration set up (Metrohm 870 Dosino dosing units, 815 Robotic USB sample processor (XL), 970 Titrando controlled by Tiamo titration software (v2.3)). Samples were then re-acidified by addition of HCl in equal quantity to the amount of base added in titration to prevent re precipitation of any ions. Samples were then diluted at an appropriate ratio with ultrapure water and frozen for later analysis of calcium content by ion chromatography (Dionex ICS-1000, Sunnyvale, CA, USA).

The rate of carbonate excretion ($C_{excretion\ rate}$, in µequivalents/kg/h) was calculated from the amount of carbonate and bicarbonate equivalents in each precipitate sample (C_{amount}, in µequivalents), the mass of the fish (M, in kg) and the collection time period (T, in hours) as described in Equation 6.

Equation 6

$$C_{excretion\ rate} = C_{amount} / (M \times T)$$

The rate of calcium excretion (as precipitated carbonate) ($Ca_{excretion\ rate}$, in µmol/kg/h) was calculated from the amount of calcium in precipitates (Ca_{amount}, in µmol), the fish mass (M, in kg) and the collection period time (T, in hours) as described in Equation 7.

Equation 7

$$Ca_{excretion\ rate} = Ca_{amount} / (M \times T)$$

4.3.3 Oxygen consumption rates

Oxygen consumption rate was measured in each experimental tank using static respirometry. Individual tanks were disconnected from the closed recirculation systems and aeration was stopped. To measure the initial partial pressure of oxygen, samples were taken by using a 2 ml syringe to mix the water within the tank before then withdrawing a sample. The PO_2 was measured using a Strathkelvin Instruments 1302 oxygen electrode housed within a thermostatted water-jacket maintained at 15 °C using a Grant LT D6G water bath and pump. The oxygen electrode was connected to a Strathkelvin meter (model 781). Each fish was left undisturbed for between 1.5 and 4.2 hours depending on the size of the fish. A second sample was then taken in the same manner (to ensure complete mixing of water in the tank prior to sampling) and the PO_2 measured. The time period was sufficient time to deplete the oxygen in the tank to at least 90 % and no less than 78 % of air-saturation. Tanks were then re connected to the recirculating system and aeration returned.

Metabolic rate (MO_2, in mmol/kg/h) was calculated from change in the partial pressure of oxygen in the water (ΔPO_2 in kPa), the respiration time period (Δt in hours), the solubility of oxygen in the water ($O_{2\ solubility}$ in mmol of O_2/litre/kPa), the respiration volume (V_{resp} in litres) and the mass of the fish (M in kg) measurements using Equation 8. Values for oxygen solubility at the relevant salinities and temperature were obtained from Boutilier et al. (1984).

Equation 8

$$MO_2 = ((\Delta PO_2/\Delta t) \times O_{2\ solubility} \times V_{resp}) / M$$

The change in partial pressure of oxygen for the respiration period ($\Delta PO_2/\Delta t$) was calculated from the initial and final partial pressure ($PO_{2\ initial}$ and $PO_{2\ final}$ respectively, both in kPa) and the initial and final times ($t_{initial}$ and t_{final} both in hours) as described in Equation 9.

Equation 9

$$(\Delta PO_2/\Delta t) = (PO_{2\ initial} - PO_{2\ final}) / (t_{\ initial} - t_{\ final})$$

It was assumed that the density of the fish is roughly equal to that of the water as such respiration volume (V_{resp} in litres) was calculated from the tank volume (V_{tank} in litres) and the fish mass (M in kg) as described in Equation 10.

Equation 10

$$V_{resp} = V_{tank} - M$$

4.3.4 Drinking rate, calcium ingestion rates and blood sampling

The drinking rate of each fish was measured by using a modification of methods described previously (Fuentes and Eddy, 1997; Usher et al., 1988). Water was drained from each tank via a siphon to leave approximately 0.5 l. To each tank 1 ml of a concentrated (0.1 mCi/ml) ^{51}Cr-EDTA stock was added and allowed to mix via the effect of aeration. Tanks were then darkened and left for 2 h. Water samples were taken either side of this time period and a gamma counter (2480 Wizard^2 Automatic Gamma Counter, Perkin Elmer) was used to measure activity of the radiolabelled marker (^{51}Cr-EDTA) in precise volumes of triplicate water samples from each tank. Following the measurement time period, each fish was terminated via the introduction of buffered tricaine methane sulfonate (MS222) to the tanks to overdose. Fish were then rinsed in label free water. Blood samples were collected by caudal puncture into heparinised syringes and transferred to tubes. The blood tubes were spun at 5000 rpm for 5 minutes to separate the plasma from the red blood cells. An aliquot of plasma was diluted with ultrapure water and frozen for later analysis of Na^+ and Cl^- ions via ion chromatography (Dionex ICS-1000, Sunnyvale, CA, USA). The remaining plasma was also frozen (due to time constraints) for later osmolality analysis on a vapour pressure osmometer (Wescor Vapro 5520). Then the gastrointestinal tract was ligated using silk sutures at each end and removed and transferred into individual tubes for gamma counting to determine radioactivity.

Drinking rates (*DR* in ml/kg/hour) were calculated from the activity of the gut and the activity for 1 ml of seawater (Ac_{gut} and Ac_{water} both in the same units), fish mass (*M* in kg) and incubation time of the fish in the radio labelled water (*t* in hours) as described in Equation 11.

Equation 11

$$DR = (Ac_{gut} / Ac_{water}) / (M \times t)$$

The calcium ingestion rate of fish ($Ca_{ingestion\ rate}$ in µmol/kg/h) was calculated by from drinking rate (*DR* in litres/kg/h) and the concentration of calcium in the seawater ([Ca] in µM) as described in Equation 12. Seawater calcium concentration for was obtained from Atkinson and Bingham (1997) for Tropic Marin salts.

Equation 12

$$Ca_{ingestion\ rate} = DR \times [Ca]$$

Calcium precipitate rates ($Ca_{excretion\ rate}$) were then calculated as a percentage of the calcium ingestion rate ($Ca_{ingestion\ rate}$) to give the fractional calcium precipitation rate.

4.3.5 Statistical analysis

To examine the effect of salinity and species on drinking rate, metabolic rate, plasma osmolality and carbonate precipitation each species one-way ANOVAs were preformed and Gabriel's pairwise comparison test used post hoc to identify which between-group differences were significant due to varying sample sizes. To examine the effect of different salinity exposures on carbonate production rates and fractional calcium precipitation rates, Welch's ANOVA was used due to violation of the assumption of equal variances. Games-Howell post hoc test was used to identify any significant differences as it does not assume equal variances between groups. To test for a significant difference in overall average drinking rates between species and independent samples T-test was used. All statistical analyses were carried out using IBM SPSS Statistics 23.

4.4 Results

4.4.1 Carbonate excretion rates

Carbonate excretion rates increased with rising salinity for both goldsinny wrasse and shanny (Figure 38). Production rates for goldsinny wrasse were significantly higher at 40 psu than at 30 psu (Figure 38) and were 2.3 times the average production rates measured at 35 psu (Table 19 and Table 20). Production rates for shanny at 40 psu were 1.6 times the average measured production rates at 35 psu which was also a significant increase (Table 19 and Table 20). Increases in carbonate production rate between 30 and 35 psu were of lower magnitude than the increase between 35 and 40 psu for both species. Average carbonate production rates by goldsinny wrasse increased by 7 µequivalents/kg/h between 30 and 35 psu, and 12 µequivalents/kg/h between 35 and 40 psu; average carbonate production rates for shanny increased by 21 µequivalents/kg/h between 30 and 35 psu, and 52 µequivalents/kg/h between 35 and 40 psu (Table 19 and Figure 38).

4.4.2 Drinking rates

Average drinking rates for both goldsinny wrasse and shanny increased with higher salinity (Figure 39). For shanny fold change in drinking rate closely corresponded to the fold change in the osmotic gradient between the fish and the environment at each salinity increase (Table 20). However, for goldsinny wrasse the fold change in drinking rate was much higher between 35 and 40 psu than the fold change in osmotic gradient. The average drinking rate at 40 psu was also significantly higher than the average drinking rates measured at 30 and 35 psu (Table 19 and Figure 39). There was no significant difference between the species in the average drinking rate across all salinities ($p = 0.248$).

4.4.3 Fractional calcium precipitation

Goldsinny wrasse precipitated a significantly lower percentage of the ingested calcium ions (9.6 %) at 30 psu compared to 35 and 40 psu (33 and 30 % respectively) (Figure 41 and Table 19). Shanny seemed to be able to precipitate calcium (as calcium carbonate) at a faster rate than could have been supplied by the measured drinking rates at all salinities. Shanny seemed to precipitate calcium at about double the rate than should have been supplied by drinking

and there was no significant difference in the averages between salinities (Figure 41 and Table 19).

4.4.4 Oxygen consumption rate

Oxygen consumption rates were similar across salinities for both species however it peaked significantly at 35 psu for goldsinny and shanny (significantly compared to 40 psu but not 30 psu) (Figure 40).

4.4.5 Blood plasma osmolality and ion concentrations

There was no significant difference in average plasma osmolality or Na^+, Cl^- or Ca^{2+} plasma concentration between fish held at different salinities for goldsinny wrasse or shanny (Table 19).

4.5 Discussion

This study found that calcium carbonate production rates from fish can be altered by changes in salinity within an environmentally relevant range. For both goldsinny wrasse and shanny, increasing salinity increased the average carbonate production rate. Average carbonate production rates for goldsinny held at 40 psu were 2.3 times the average production rates measured at 35 psu for goldsinny and 1.6 times for shanny (Table 20). This may impact how much carbonate fish are estimated to produce across the oceans depending on how much ocean salinity varies and whether areas of variation from average salinity overlap with areas where fish are living. Addition it is important to consider how representative these results are of fish more widely across different environments.

4.5.1 Ocean salinities and areas of fish production

Average ocean salinities are generally considered to be 35 psu, however rates of evaporation and fresh water input from precipitation can affect salinity of the oceans. As such salinity varies between about 30 and 40 psu both spatially and temporally across the world's oceans (Vine et al., 2015). Large areas of higher than average salinity include the Mediterranean Sea, Red Sea and large open ocean areas of the North and South Atlantic which can typically see surface salinities close to 40 psu (Antonov et al., 2010). These areas have the potential to overlap with areas of large fish biomass especially considering a recent study that surveyed mesopelagic fishes (Irigoien et al., 2014), which suggests that

global fish biomass may have been greatly underestimated in areas of open ocean. Additionally in one of the previous models of global carbonate production, the Arabian Sea, stood out as an area of very high production due large numbers of smaller fish and high temperatures (Wilson et al., 2009). This is also an area of ocean which has generally high salinity. The salinity in the Arabian Sea has been measured as being above average (>35 psu) for up to depths deeper than 200 m and reaching salinities of up to 37.2 psu closer to the surface (Joseph and Freeland, 2005). With this in mind it could be that fish carbonate production in this area is higher than expected.

Areas of salinity generally lower than average tend to be at high northern latitudes, areas of the North Pacific and areas around large terrestrial fresh water inputs such as the Bay of Bengal (Antonov et al., 2010). Although some of these areas may overlap with areas of high fish biomass, the difference between average rates at 30 and 35 psu in this study were lower than the difference between 35 and 40 psu for both species. As such it may be that the decrease in carbonate production for fish in lower salinities will not be as large as the increase seen from areas that higher in production rates sue to increased salinity. If this is the case it would mean that the overall estimates of fish carbonate production may increase globally. Ultimately, however, it depends on how much areas of high fish biomass and low fish sizes overlap with areas of lower or higher salinity.

4.5.2 Relevance to other species

It is also important to consider whether the results seen in the two temperate species examined in this study are representative of fish more widely living in different environmental salinities. Understanding the osmoregulatory mechanisms by which salinity had an impact on the carbonate production rates in the species examined in this study will allow comparison with previous studies in the literature which study various osmoregulatory mechanisms on other species. This may help us understand whether the results observed here are applicable and representative of species more widely across different environments.

Prior to this study it was hypothesised an increase in osmotic gradient caused by an increase in salinity would lead to an increase in water loss. And that this would need to be replaced by water absorbed from drinking in order to maintain

tissue volume. As such drinking rate (which is also affectively the rate of supply of calcium ions to the intestine which are precipitated are calcium carbonate) would need to increase proportionally to osmotic gradient. However it was discussed that this generally is not the case for many studies which have examined changes in drinking rate and water absorption over different salinities.

Studies which have examined water absorption showed that generally water absorption increased relatively less than the osmotic gradient faced by fish with increasing salinity (Table 18). As such it would seem that fish are able to minimise the rate at which water is lost (and there for must be absorbed) with increasing salinities. Laverty and Skadhauge (2012) in a review discuss studies that seem to provide evidence that branchial integument permeability can alter in response to hyper salinity and hypothesise that this may be due to altered expression of aquaporins.

In addition to minimising water loss, fish in previous studies increased drinking rates similar to or less than the change in osmotic gradient caused by increased salinity (Table 18). For species which increased drinking rate less than the osmotic gradient increase this may partly be the result of minimising water loss. If water loss is reduced then the rate at which water needs to be absorbed is also reduced. The supply of water for absorption comes from drinking rate, as such it may be that the drinking rate does not need to increase as much as the osmotic gradient. In order for this to be possible, the efficiency with which water is absorbed from ingested seawater needs to be maintained or increased despite the increased osmotic gradient. Increase osmotic gradient should in theory mean that water absorption becomes less efficient at higher salinities unless other mechanisms are involved to mitigate this. Many factors individually have been identified to have the potential to alter water absorption efficiency in the intestine by either altering Na^+ and Cl^- uptake rates or HCO_3^- excretion rates that stimulate calcium precipitation, both of which will raise local blood osmolality or reduce luminal osmolality to increase water uptake by the intestine. Various regulatory hormones can have these effects such as the guanylin family of peptides (Ruhr et al., 2015) and the parathyroid hormone-related peptide (PTHrP) (Carvalho et al., 2015), and environmental stimuli such as the concentration of calcium ions in the imbibed seawater (Cooper et al., 2010).

In studies which have measured both water absorption and drinking rate at different salinities, water absorption increase at higher salinities by a relatively the same factor as drinking rate (Table 18). For two of the three species examined the water absorption increased slightly more than drinking rate with a salinity change. So, for example a 1.42 fold increase in water absorption in rainbow trout was supplied by only a 1.36 fold increase in drinking rate (Shehadeh and Gordon, 1969). In herring a 2.57 fold increase in water absorption was supplied by only a 2.40 fold increase in drinking rate (Tytler and Blaxter, 1988). For both of these species the efficiency with which water was absorbed from imbibed seawater must have increased slightly, potentially through some of the mechanisms briefly discussed above. For the third (and final) species where water absorption and drinking rate was observed at different salinities, the gulf toadfish, a 1.46 fold change in drinking rate only yielded a 1.24 fold changed in water absorption indicating water absorption was slightly less efficient at the higher salinity (Genz et al., 2008). The study in gulf toadfish, however, examines a change in salinity from 35 to 50 psu, as opposed to studies which examined rainbow trout and herring which observed salinity changes below 35 psu. The reduced efficiency of water absorption in gulf toadfish may have been because 50 psu is a relative high salinity. Either way, for all of the studies, increases in water absorption were not that different from the increases in drinking rates indicating fish have the potential to used mechanisms that maintain water absorption efficiencies at higher salinities. Over all it seems likely that fish do not have to increase drinking rates by as much as the increase in osmotic gradient as they can somehow reduce the rate at water is lost (and therefore water absorption rate), and simultaneously maintain the efficiency with water is absorbed from higher salinity seawater.

In the present study, unfortunately water absorption was not measured, but drinking rates were. Between 30 and 35 psu drinking rates increased similarly to the change in osmotic gradient for both goldsinny wrasse and shanny (Table 20). This is similar to some of the species in previous studies although most increase drinking rates were less than the osmotic gradient increases (Table 18). Between 35 and 40 psu in the present study, shanny continue to increase drinking rate similar to the increase in osmotic gradient however goldsinny wrasse increase drinking rates a lot more than the osmotic gradient increases. Between 35 and 40 psu represents approximately an osmotic gradient change

of 1.2 fold for both species. For this change in osmotic gradient, shanny changed their drinking rates by 1.3 fold whereas goldsinny wrasse exhibited a 2.4 fold change in drinking rate. No other species examined previously in the literature increase drinking rates more than the increase in osmotic gradient at higher salinities (Table 18). As such it may be that carbonate production rate results seen in the present study for goldsinny wrasse are unusual. It could be caused by either a decrease in the efficiency of water absorption at higher salinities or an increase in water loss. A possible reason for fish to have increased water loss may be because it could be exacerbated by metabolic activity. The gills are a major site of water loss in fish and as such the rate at which water passes over the gills has the potential to impact water loss (Nilsson, 1989), by altering diffusion boundary layers and hence osmotic movement of water. Higher metabolic costs would result in an increase in oxygen demand which would require faster gill ventilation rates, increasing water loss. It has been hypothesised previously that there should be a higher metabolic cost to living at higher salinity (although evidence for this is conflicting) as the process of osmoregulation requires active transport of ions (Ern et al., 2014). In the present study, oxygen consumption rates did not change much with salinity and the only significant differences observed were small and did not follow the pattern that oxygen consumption rates were higher at higher salinities (Figure 40). It is therefore unlikely that this had an impact on water loss for goldsinny wrasse. Instead it could be that goldsinny wrasse are unable to maintain the efficiency of water from drinking salinities, meaning they need to drinking more in order to absorb the water they need.

The large increase in goldsinny drinking rate could be the main reason why goldsinny wrasse showed a large increases (2.3 fold) in carbonate production rates between 35 and 40 psu. The relationship between drinking rates and carbonate production rates will depend on what fractions of the ingested calcium ions are precipitated as calcium carbonate. This may alter across different salinities. The only previous study (to my knowledge) that has reported fractional calcium precipitation rates, reports them as being 26.8 % and 61.2 % at 35 and 50 psu respectively for gulf toadfish(*Opsanus beta*) (Genz et al., 2008). This indicates that larger fractions of the ingested calcium ions may be precipitated by fish at higher salinities. As precipitation of calcium ions removes them from solution it lowers the osmolality of the intestinal fluid which aids water

absorption. It would be beneficial to precipitate larger fractions of the calcium ions at higher salinities in order to maintain the efficiency of water absorption from drinking at higher salinities. If larger fractions of the ingested calcium ions are precipitated, combined with higher concentrations of calcium ions in seawater at higher salinities and faster rates of supply from increase drinking rates, it could go towards increasing calcium carbonate production rates of fish at higher salinities.

In the present study, fractional calcium precipitation increased for goldsinny wrasse between 30 and 35 psu from 8% to 30% (Figure 41). However, between 35 and 40 psu the fraction of calcium that was precipitated did not change significantly. If fractional calcium precipitation did not change above 35 psu it may have meant more calcium was present in solution in intestinal fluids, which may have hindered water absorption. A decrease in the efficiency of water absorption from high amounts of calcium left in solution fits with the large increases in drinking rates observed in goldsinny between 35 and 40 psu – increased drinking would potentially be required in order to supply more seawater to the intestine so that sufficient can absorbed. As such it seems likely that the large increase seen in carbonate production rates between 35 and 40 psu in goldsinny wrasse was due predominantly to the large increase in drinking rate they exhibited between these salinities.

Shanny, however, did not seem to increase their fractional calcium precipitation with increased salinity. Even more interestingly, they appeared to be able to precipitate calcium ions in their intestine into calcium carbonates at a faster rate than calcium ions were ingested through drinking (Figure 41). Shanny appeared to consistently precipitate calcium ions at approximately double the rate that should have been supplied to the intestine from the measured drinking rates. Drinking rate was measured over the period of 2 hours compared to the calcium precipitation rates which were a 5-day average. It could be that the measured drinking rate was not representative of average drinking rates over the 5 days which calcium precipitation rates were measured. It has been shown that fish can exhibit diurnal changes in drinking rate (Cooper et al., 2016) and it could be that in this instance drinking rate was measured at a period of relatively low drinking. The measured drinking rates, however, were in the range of those previously reported for marine fish generally (Marshall and Grosell, 2006), and

when examined, drinking rates of shanny and goldsinny were not significantly different from each other in the present study. This would suggest that the measured drinking rates for shanny were not particularly low. The alternative is that the shanny were actively excreting extra calcium ions into the intestinal lumen. Fuentes et al. (2010) show *in vitro* in the sea bream intestine PTHrP and stanniocalcin 1 (STC 1) can act antagonistically to each other to control intestinal calcium absorption to the point where STC 1 can actually create a net efflux of calcium into lumen. However, it would make little sense osmotically to add calcium ions to the intestinal lumen which would increase the osmotic potential in the lumen and inhibit water uptake, unless it were a mechanism to deal with extra bodily calcium ions taken up elsewhere, such as through the gills. STC 1 is produced in response to hypercalcaemia in fish in order to initiate mechanisms to reduce excess calcium levels (Radman et al., 2002). It is therefore possible that the intestine act as an excretory organ for excess calcium potentially taken in through the gills or other surfaces in contact with the seawater, which would explain why shanny in this study appeared to precipitate calcium ions at a greater rate than could have been provided by drinking. This could be partly responsible for the generally high carbonate production rates seen in shanny in this study. Shanny consistently produced carbonates at a much faster rate than goldsinny at all salinities (Figure 38) which could be due to the supply of extra calcium ions from sources other than drinking. Either way, it appears that it may not be wise to assume that a fold change in the rate of supply of calcium ions to the intestine through drinking seawater will always result in the same fold change in precipitation rates.

Overall it appears that in all the species studied so far, the relationship between salinity, drinking rate and calcium precipitation as calcium carbonate has been different for each species. Goldsinny wrasse exhibited increases in drinking rate in excess to the increase in the osmotic gradient caused by the seawater. This increase in drinking rate seemed to be the cause of the large increase in the carbonate production rate observed between 35 and 40 psu. However, no other species from studies examined from the literature or in the present study increased drinking rates more than a change in osmotic gradient. Shanny in the present study show increases in drinking rates with salinity increases which were more representative of fish previously studied in the literature. However, unlike the goldsinny wrasse examined in the present study and gulf toadfish

examined in a previous study (Genz et al., 2008), shanny did not show changes in fractional calcium precipitation rates across different salinities and additionally appeared to be able to precipitate calcium at a faster rate than was supplied by drinking. The gulf toadfish, examined in a previous study, neither exhibited changes in drinking rate larger than changes in the osmotic gradient like the goldsinny wrasse, nor were they similar to the shanny in that they changed the fraction of ingested calcium ions they precipitated at different salinities and it they did not appear to ever precipitate calcium at a faster rate than it was ingested. Additionally, gulf toadfish in the previous study examined changes over a large differences in salinity (9, 35 and 50 psu) which are not representative of large scale oceanic salinity differences. Until calcium carbonate production rates and drinking rates are measured in more species across environmentally relevant salinities it is hard to suggest how relevant the results measured here are to the relationship between salinity and fish carbonate production rates in general. However, despite the differences in drinking rate changes and fractional calcium precipitation observed all the species, all species exhibited increases in carbonate production rates at higher salinity.

4.5.3 Conclusions

Overall fish seem to increase carbonate production rates with increased salinity, although further research is required to tease apart species differences in the exact nature of the relationship between salinity and fish carbonate production rates. Despite differences between species in the present study, both species examined exhibited major increases in carbonate production rates above 35 psu, which is generally considered the ocean average, that were larger than the decreases observed below 35 psu. If 35 psu is a true ocean average and fish are evenly distributed through areas of lower and higher salinity, based on the results observed here we could predict estimates of global fish carbonate production rates to increase.

4.6 Tables

Table 18 Relative drinking rates and intestinal water absorption rates at different salinities

Literature data from studies which have measured drinking rates and intestinal water absorption rates at different salinities. Columns show the relative increases in osmotic gradient between the fish and seawater, drinking rate and intestinal water absorption when measured at the "Compared salinity" compared to the "Relative salinity". Source studies are as follows: 1 (Shehadeh and Gordon, 1969), 2 (Tytler and Blaxter, 1988), 3 (Brown and Tytler, 1993), 4 (Genz et al., 2008), 5 (Maetz and Skadhauge, 1968), 6 (Gonzalez et al., 2005), 7 (Sardella et al., 2004), 8 (Guerreiro et al., 2004), 9 (Sardella et al., 2004), 10 (Varsamos et al., 2004), 11 (Webb et al., 2001), 12 (Zhang and Wang, 2007), 13 (Tytler and Blaxter, 1988), 14 (Tytler and Blaxter, 1988). Data was not available for cells containing "na".

Species	Relative salinity (psu)	Compared salinty (psu)	Fold increase in osmotic gradient	Fold increase in drinking rate	Fold increase in water absorption	Reference
Rainbow trout (*Oncorhynchus mykiss*)	17.5	35	4.18	1.36	1.42	1
Herring (*Clupea harengus L.*)	16	32	5.00	2.40	2.57	2
Turbot (*Scophthalmus maximus*)	17.5	35	4.18	na	2.17	3
Gulf Toadfish (*Opsanus beta*)	35	50	1.58	1.46	1.24	4
Eel (*Anguilla anguilla*)	35	70	2.5	2.5	na	5
Sailfin Molly (*Poecilia latipinna*)	35	60	2.1	1.9	na	6
Mozambique tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*)	35	75	2.7	2.4	na	7
Sea bream (*Sparus auratus L.)*	35.5	55	1.8	1.2	na	8
Mozambique tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*)	35	55	1.9	1.2	na	9
European seabass (*Dicentrarchus labrax*)	25	40	2.2	1.2	na	10
Tidepool sculpins (*Oligocottus maculosus*)	18	30	3.0	1.2	na	11
Black sea bream (*Acanthopagrus schlegeli*)	20	35	2.9	1.0	na	12
Cod (*Gadus Morhua L.*)	16	32	5.0	2.7	na	13
Plaice (*Pleuronectes platessa*)	16	32	5.0	1.4	na	14

Table 19 Various physiological parameters of fish at different salinities

Summary of data and statistical tests from parameters measured in fish held at different salinities. Columns show mean, number of individuals (N), the standard error of the mean (SE) and results significance values obtained from ANOVAs, Welch's robust test and Levene's test for homogeneity of various. Significant values are highlighted in bold.

		30psu			35psu			40psu			ANOVA	Welch's	Levene's
Species	Variable	Mean	N	SE	Mean	N	SE	Mean	N	SE	P	P	P
Goldsinny	Fish mass (g)	18.00	10	1.11	19.28	10	1.27	20.17	10	2.17	0.629	0.610	0.219
	Carbonate production rate (µequiv/kg/h)	1.77	10	0.42	9.10	10	3.09	21.34	10	5.60	**0.003**	**0.005**	**0.001**
	Drinking Rate (ml/kg/h)	1.12	10	0.20	1.30	10	0.18	2.76	10	0.36	**0.000**	**0.004**	0.190
	Metabolic Rate (mmol/kg/h)	1.71	10	0.10	2.27	10	0.10	1.91	10	0.06	**0.000**	**0.004**	0.375
	Fractional calcium precipitation (%)	9.6	8	2.1	33.0	8	8.4	30.0	10	9.5	0.106	**0.024**	**0.028**
	Plasma osmolality (mmol/kg)	322	8	3	324	10	4	333	9	3	0.083	0.051	0.611
	Plasma chloride concentration (mM)	142.79	7	2.08	141.37	10	3.37	139.29	9	4.03	0.787	0.746	0.236
	Plasma sodium concentration (mM)	158.84	7	2.19	155.14	10	2.39	152.91	9	3.59	0.393	0.335	0.425
	Plasma calcium concentration (mM)	2.59	7	0.19	2.15	10	0.13	3.01	9	0.60	0.269	0.130	**0.000**
	Precipitate magnesium content (%)	39.25	9	8.75	47.45	10	11.07	15.90	10	2.54	**0.029**	**0.012**	**0.006**
Shanny	Fish mass (g)	18.178	9	3.01	16.24	10	2.13	20.55	10	3.16	0.547	0.546	0.463
	Carbonate production rate (µequiv/kg/h)	59.572	9	6.44	80.70	10	6.86	132.74	9	9.78	**0.000**	**0.000**	0.354
	Drinking Rate (ml/kg/h)	1.565	9	0.21	2.06	10	0.27	2.43	10	0.34	0.120	0.110	0.361
	Metabolic Rate (mmol/kg/h)	2.2282	9	0.26	2.66	10	0.15	1.95	10	0.15	**0.040**	**0.017**	0.639
	Fractional calcium precipitation (%)	203.6	9	39.5	177.6	10	59.1	222.8	10	48.3	0.815	0.846	0.442
	Plasma osmolality (mmol/kg)	317	8	8	336	10	5	329	9	6	0.140	0.207	0.653
	Plasma chloride concentration (mM)	148.26	7	2.63	150.35	10	2.09	146.45	9	2.84	0.527	0.557	0.717
	Plasma sodium concentration (mM)	164.06	7	3.13	168.40	10	2.39	167.65	9	1.63	0.435	0.547	0.204
	Plasma calcium concentration (mM)	2.8128	7	0.11	2.59	10	0.13	3.05	9	0.56	0.625	0.395	**0.000**
	Precipitate magnesium content (%)	25.76	9	3.75	25.65	10	4.38	23.12	9	3.21	0.865	0.841	0.545

Table 20 Relative changes in precipitate rates and other parameters

Table shows fold changes for various observed parameters across differences in salinities for species observed in the present study (Goldsinny: *Ctenolabrus rupestris*; and Shanny: *Lipophrys pholis*) and one species (Gulf toadfish: *Opsanus beta*) observed in a previous study (Genz et al., 2008). All fold change data is calculated by dividing the value observed at the "Compared salinity" by the value observed at the "Relative salinity". Calcium excretion rate refers to the rate of calcium excretion as a solid precipitate of calcium carbonate.

Species	Relative Salinity (psu)	Compared salinity (psu)	Fold change in osmotic gradient	Fold change in drinking rate	Fold change in calcium ingestion rate	Fold change in calcium excretion rate	Fold change in carbonate production rate
Goldsinny	30	35	1.3	1.2	1.3	6.3	5.1
Shanny	30	35	1.3	1.3	1.1	1.1	1.4
Goldsinny	35	40	1.2	2.4	2.8	1.9	2.3
Shanny	35	40	1.2	1.3	1.8	1.8	1.6
Gulf Toadfish	35	50	1.6	1.5	2.4	4.8	9.0

4.7 Figures

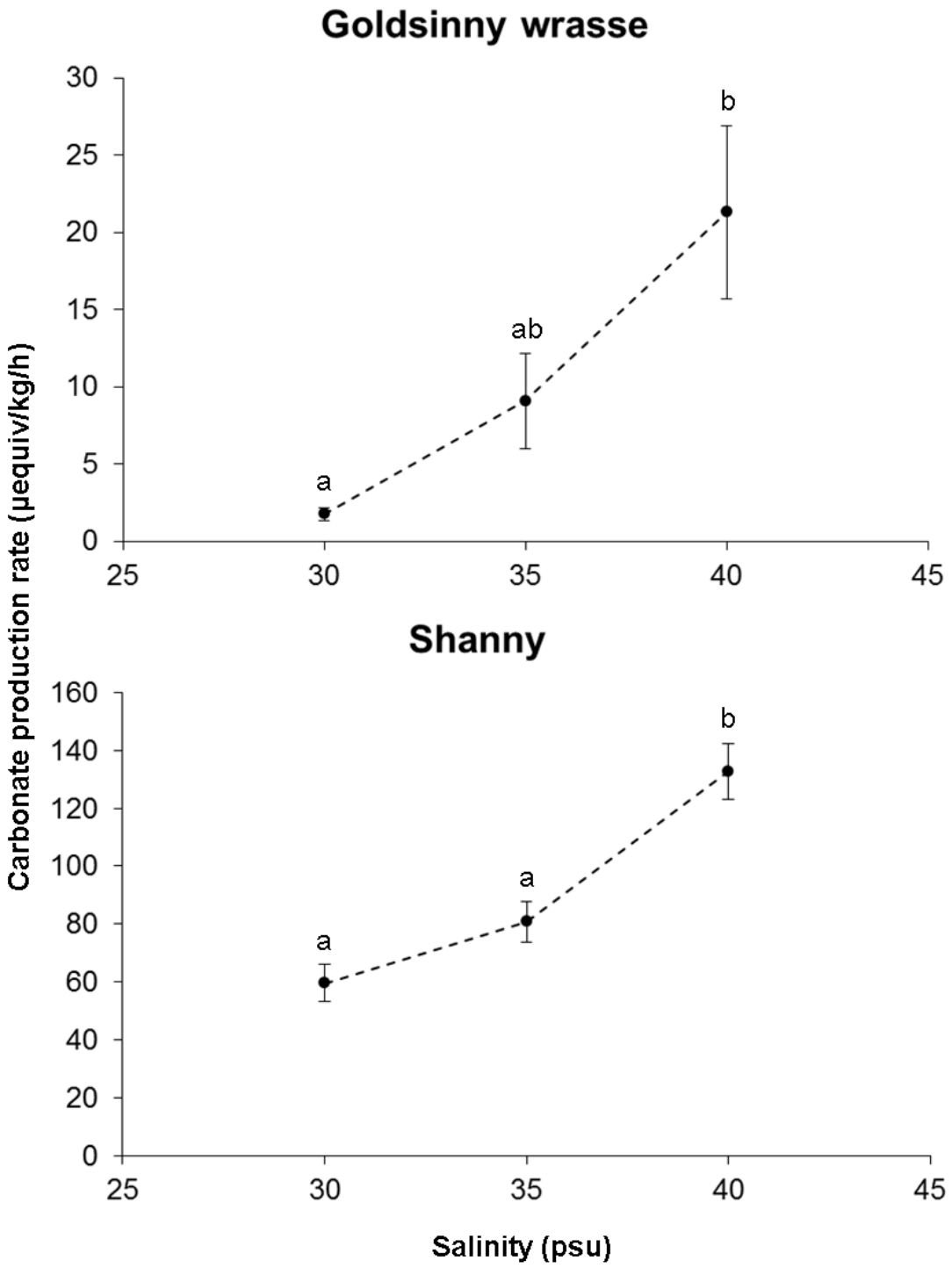

Figure 38 Fish carbonate precipitation rate across different salinities

Plots of mean measured carbonate production rates at different salinities for goldsinny wrasse (top) and shanny (bottom). Error bars represent standard error of the mean. Different letters above points shows groups which are statically significantly different from each other.

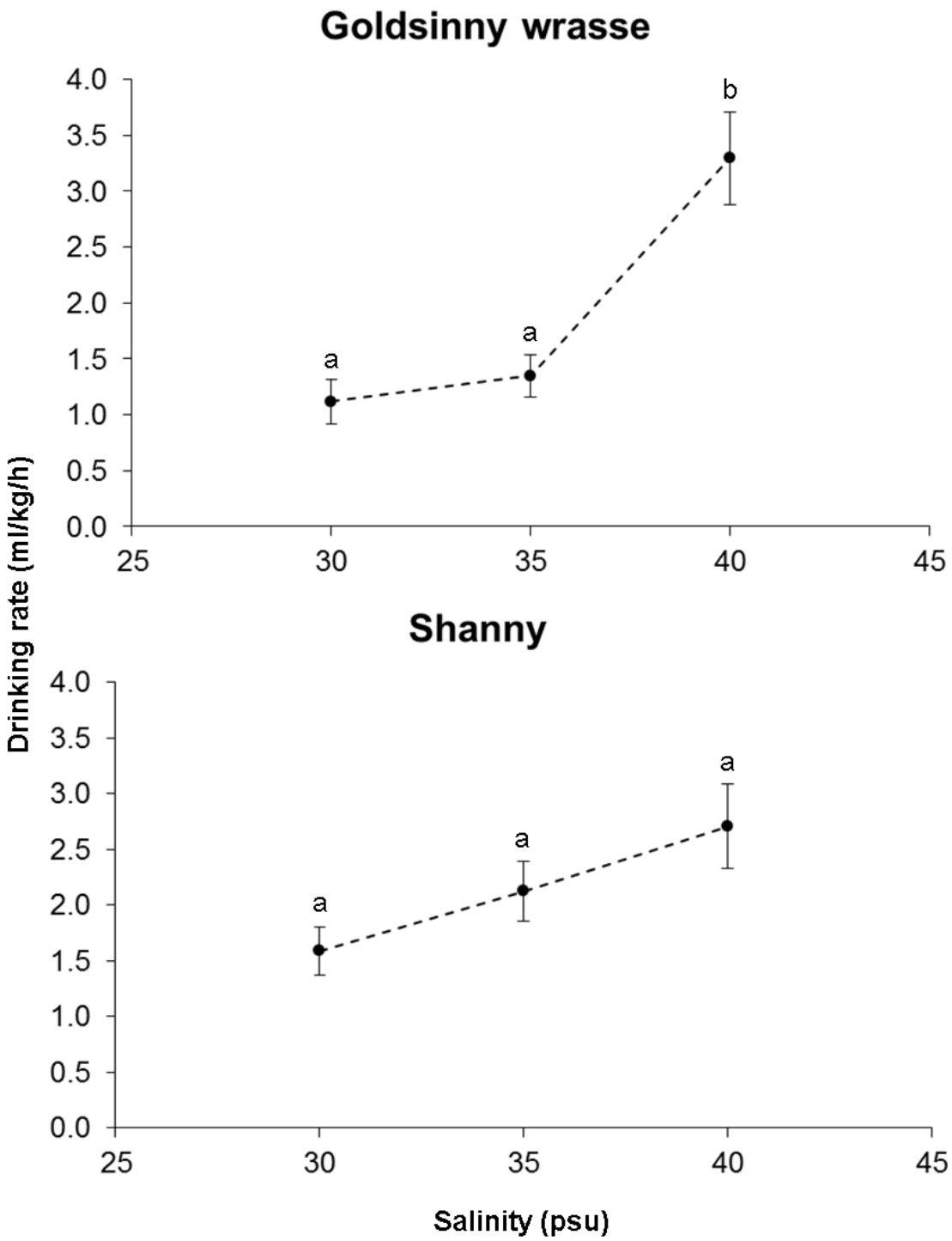

Figure 39 Drinking rates of fish across different salinities

Plots of mean measured drinking rates at different salinities for goldsinny (top) wrasse and shanny (bottom). Error bars represent standard error of the mean. Different letters above points shows groups which are statically significantly different from each other.

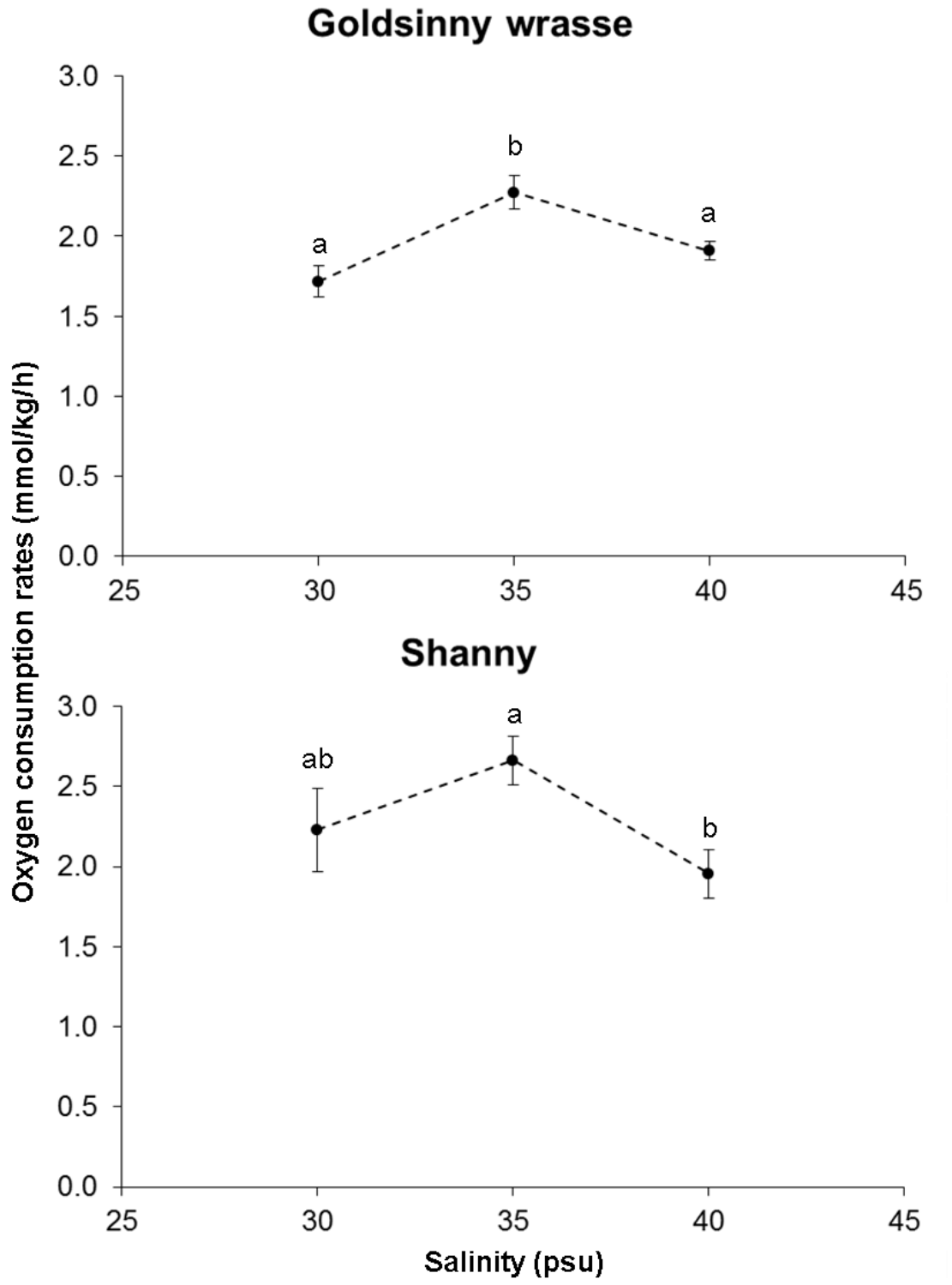

Figure 40 Oxygen consumption rates of fish across different salinities

Graph of fish oxygen consumptions rates at different salinities. Points show mean measured values for goldsinny (top) wrasse and shanny (bottom). Error bars represent standard error of the means.

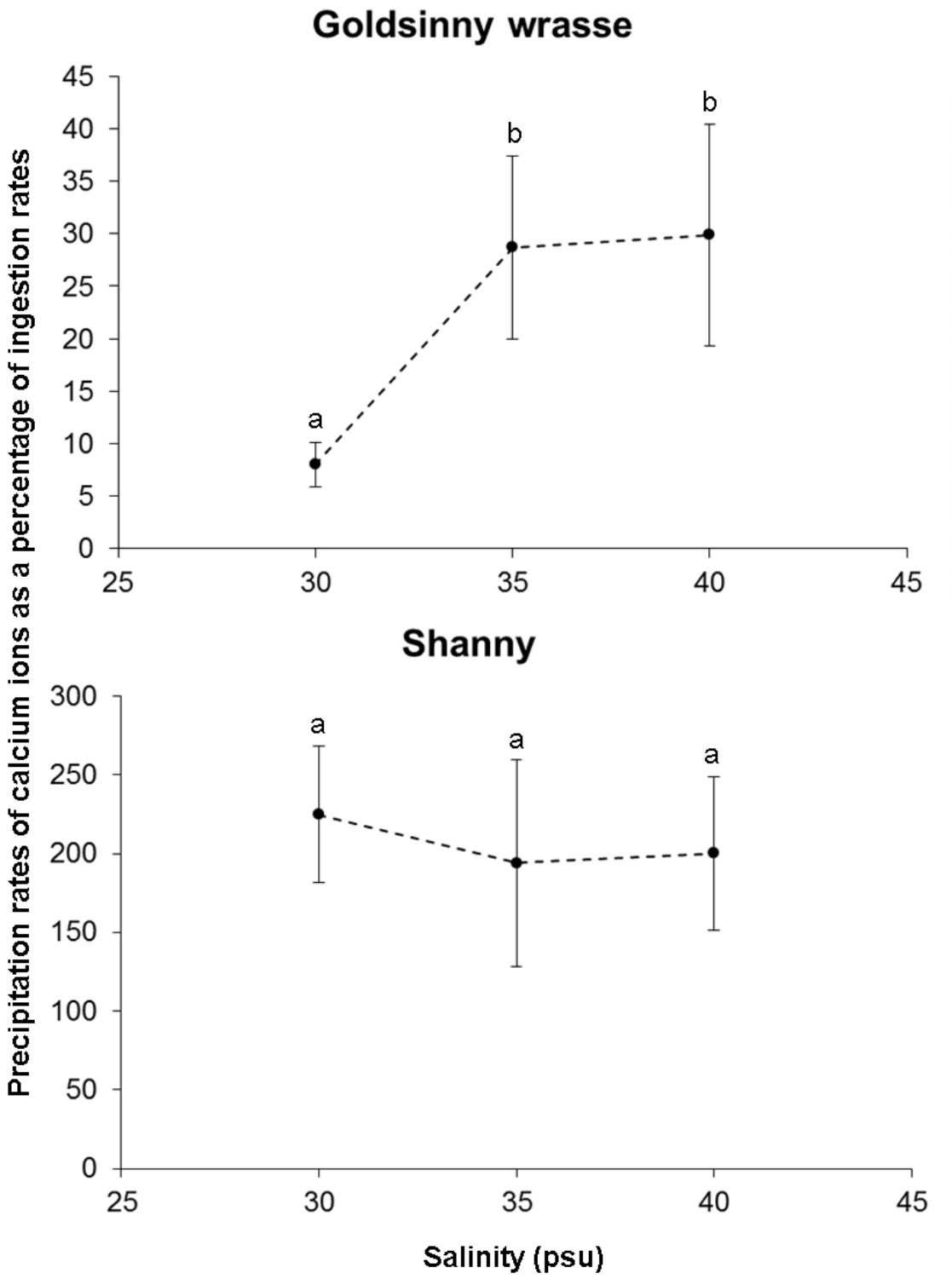

Figure 41 Fractional calcium precipitation from seawater ingested by fish

Graph shows mean calcium precipitation rates by goldsinny wrasse (top) and shanny (bottom) at different salinities expressed as a percentage of the rate of calcium ion ingestion. Error bars represent standard error of the means. Shanny appear to be capable of precipitating more calcium than is supplied by drinking.

5 General discussion

The aim of the studies presented in this thesis was to further our knowledge of the processes involved in the inorganic carbon, or carbonate, pump in the ocean in order to ultimately enhance understanding of ocean and atmosphere CO_2 fluxes. To predict ocean-atmosphere CO_2 fluxes, accurate estimates of carbonate budgets in terms of production, dissolution and accumulation rates of carbonates across different ocean regions are required which is explained in Chapter 1. Chapter 1 also highlighted the importance of estimating carbonate budgets in extratropical areas (which are likely to be important on a global scale) and the lack of information currently available to do so. Teleost fish are a major source of carbonate production across the world's oceans (Wilson et al., 2009) and are likely to be relatively important in the production of carbonate in temperate areas. In order to provide information that might be useful in determining carbonate budgets in temperate areas, this thesis examines the roles that temperate fish species play in carbonate production, dissolution and accumulation in sediments. Below I discuss the progress that the studies presented here have made towards understanding the contribution of fish to carbonate production, the solubility of fish carbonates and their potential for accumulation in sediments. Future steps towards further understanding the role of fish in carbonate budgets are also discussed along with further implications towards understanding our oceans present, past and future.

5.1 Solubility of temperate fish carbonates

Chapter 2 presents the first available information on the typical morphology and elemental composition of carbonates produced by temperate marine fish species. This consequently forms a foundation for further investigating the solubility of these carbonates. Carbonates produced by marine fish have been shown to have varying morphological and compositional (magnesium and phosphorus content) traits in both the current study and previous ones (Salter et al., 2012). Although it is possible to define the elemental composition of carbonates using the continuous variables of magnesium and phosphorus content, shape is not so easily defined by one or two continuous variables. Chapter 2 addressed this issue by classifying morphologies into three morphologically and compositionally distinct categories: polycrystalline structures (low magnesium and phosphorus); monocrystalline ellipsoids (higher

magnesium content, but low phosphorus), and nanospheres (higher magnesium and phosphorus contents). There was little variation observed in the elemental composition of these categories, even across different species and environmental parameters. In future studies it may therefore be possible to infer the elemental composition of fish calcium carbonate particles based on their morphological category.

These categories could also be used to describe the carbonates seen in previous studies in tropical species, although these noted that there was considerable variation within the abundant polycrystalline structures (Salter et al., 2012). For this reason, Salter et al (2012) used further categories, in addition to those in the present thesis, to describe polycrystalline structures which had different magnesium contents. However, as discussed in chapter 2 the variety of different polycrystalline structures observed in tropical species may be the result of collecting samples from precipitates excreted into seawater by unfed fish as opposed to collecting them directly from the intestine of feeding wild fish as was done for temperate species in chapter 2. Fish obviously feed most of the time in the wild, so it could be that samples collected from unfed fish under laboratory conditions are not an ideal representation of carbonates produced by wild fish. Samples could be examined from the intestine of fed, wild-caught, tropical species to assess whether additional categories would be useful to describe different kinds of polycrystalline carbonates produced by fish across other environments.

Additionally, chapter 2 raises the question of whether magnesium content can alter within morphology types due to natural temperature variation over an annual cycle in wild-caught fish. Although there was no significant difference in magnesium content of ellipsoidal shaped carbonates from all species sampled across months when tested statistically, there was a steady decrease from October through to March from an average of 14.58 mol% to 4.37 mol%. March is generally time of the year with the coolest sea temperatures (Smyth et al., 2010) which have been associated with reduced magnesium content of carbonates from other biogenic carbonate sources (Butler et al., 2015; Lea et al., 1999; Xiao et al., 2014). Previous unpublished lab based studies have also suggested that this might be the case in carbonates produced by flounder (Cobb et al., 2016). Additionally, in chapter 2 of the present thesis, the

magnesium content of ellipsoids produced by a range of temperate species was generally lower (in the range of 8 to 13 mol%) compared to ellipsoids from tropical species (20 to 30 mol%) (Salter et al., 2012). As such, it may be that the three distinct morphological categories identified in chapter 2 do not reliably predict magnesium content of fish carbonates produced across different temperatures. Further investigation is required to determine the effects of temperature and feeding on the elemental composition of different carbonate morphology types.

However, the three distinct categories identified in chapter 2 would appear sufficient for describing carbonates produced fish in the limited temperature range of temperate regions and feeding on natural diets. With the identification of this classification system of carbonates in temperate fish, the next steps towards estimating the amount of fish-derived carbonate that dissolves in temperate oceans would be to assess the solubility of each of these carbonate types.

Chapter 2 contains speculative discussion upon the fates of different types of carbonate produced by fish based on the distinct chemical compositions of the different types of carbonates. Previous data on the solubility of fish carbonates has only been collected from precipitates produced by one species, the Gulf toadfish (*Opsanus beta*), under starved conditions (Woosley et al., 2012) Considering different fish species make various different types of carbonate precipitates it is unlikely that solubility data collected on a single, unfed species is representative of the solubility of fish carbonates across the ocean. The electron microscopy images of the carbonates examined by Woosley et al. (2012) show that they are likely to fit into the monocrystalline ellipsoid category of carbonates. However the magnesium content of the monocrystalline ellipsoid carbonates produced by gulf toadfish were measured to be 47.9 ± 0.7 mol% (Woosley et al., 2012). This is much higher than the magnesium content measured in the ellipsoids produced by temperate fish in chapter 2 (in the range of 8 to 13 mol%) which could be to do with the higher temperatures experienced by the gulf toadfish or being withheld food as discussed above. As increased magnesium content is associated with increased solubility in calcium carbonates (Bertram and Mackenzie, 1991), then the solubility measured by

Woosley et al. (2012) is unlikely to be applicable to the ellipsoids produced by temperate fish.

Solubility data on the different categories of carbonates produced by temperate fish would therefore be useful. If overall carbonate production from fish in a certain area consists of different categories of carbonates, and the proportions of those different categories known, then by knowing the solubility potential of each category it would be possible to estimate how much might dissolve or persist in the sediment. This approach would also require an accurate estimate of the proportions with which different categories of carbonate are produced in temperate oceans. This might be achievable if the factors responsible for causing different types of carbonate to be produced can be determined.

Studies on tropical species (Salter et al., 2012) noted that fish species tended to be fairly consistent in the carbonate morphology types they produce. Chapter 2 confirmed a species would persist in predominantly producing the same type of carbonate over a whole annual cycle. No link was observed between morphology types observed and the sex or condition of fish within the species of poor cod. Negative correlations were observed, however, between the observations of ellipsoids and the observations of nanospheres in some circumstances. Additionally, in some circumstance there were positive correlations between the observations of ellipsoids and the observation of polycrystalline structures. Perhaps conditions which are conducive to the production of nanospheres are not conducive to the production of ellipsoids and vice versa. However, the exact factors which control these conditions have yet to be identified.

Chapter 2 identifies dietary input, dietary requirement and fish length as potential factors which might influence the type of carbonates produced within a species. As diet (and possibly average length) varies between species it is also possible that these factors may play a part in a species consistency in the types of carbonates it produces. Examining precipitates from species that have a range of different diets and species over a range of different lengths might help clarify the role these factors play. Many species of fish change greatly in size throughout their lives, as such, it would be possible to examine different size individuals from many species in future studies. Future studies to investigate the effects of diet could investigate differences between species with herbivorous

diets and species with predominantly carnivorous diets; alternatively studies could investigate differences in diet and carbonate characteristics of benthic and pelagic species. This approach might be useful for providing information on carbonate characteristics of fish that could potentially be applied to ecosystem models to better understand what types of carbonates are produced across different environments. Examining carbonate collected from wild captured fish would provide the most representative information carbonate produced by fish in the ocean, however, many factors, such as complex diet variations may make it difficult to understand how dietary components affect carbonate characteristics in wild fish. Lab based studies would provide the opportunity to control and manipulate diet which may provide better information on the effects of specific dietary components. A combination of both lab and field based studies may prove useful in the future to determine the role of diet on the determination on the types of carbonates produced by fish.

Diet may not be the only factor to influence the characteristics of carbonate produced by fish. The unfed tropical species in previous studies also seemed to be consistent in producing different types of carbonates between species, without the influence of diet (Salter et al., 2014, 2012), it seems likely that diet is only part of the story, and rather some other trait related to species is also a contributing factor. If this is the case, genetically related species might be expected to share such traits. Examining precipitates from species across different taxonomic groups may help clarify this. The previously published data on a variety of tropical fish species predominantly features species of the order perciformes (Salter et al., 2012) making it difficult to compared between difference taxonomic orders. Chapter 2 does examine species from a few different taxonomic orders, although it there is not always a clear pattern between species from the same order producing similar types of precipitates (Table 1). It may be traits that relate to which precipitate types are produced differentiate at taxonomic levels above that of order.

As the exact factors which control the category of carbonate produced by fish and their solubilties are still unclear, it is also not possible to accurately estimate the proportion of carbonate that is produced in each category or their rates of dissolution in the ocean. However, the present thesis has identified categories of carbonate for which solubility should be investigated and provided insights

into some potential factors that could be related to the determination of which categories are produced in temperate fish.

5.2 Sedimentary accumulation of temperate fish carbonates

Chapter 3 is the first study to ever attempt to identify fish carbonates in temperate sediments. As such the sediments beneath fish farms were examined as an area where accumulation of fish carbonates is likely due to the high density of fish above. However, both methods employed, direct visual examination of sediments using SEM and measurement of the carbonate content failed to detect any evidence of fish carbonate accumulation in sediments. However, it was hypothesised that this is most likely due to the high organic content of sediments beneath fish pens resulting in high rates of microbial respiration and therefore lower pH sediments and water which would favour carbonate dissolution rather than accumulation. It was concluded fish farms therefore do not provide a good opportunity to observe accumulation of fish carbonates in sediments.

It was also noted that direct examination of sediments using SEM may not be an effective method of detecting and measuring fish carbonate accumulation in sediments. The absence of carbonates from fish in the specific sediments examined in chapter 3, does not mean that fish carbonates are absent in any temperate sediments. A future alternative to identify fish carbonates in sediments could be to use stable isotope analysis. It may be that fish carbonates have a distinct isotopic signature compared to carbonates produced from other sources. Fish utilise respiratory CO_2 to make bicarbonate which reacts with ingested calcium ions to form calcium carbonate in their intestines (Wilson et al., 2002). Unless fractionation of different isotopes occurs in this process, it is expected that the carbonates produced by fish will have similar carbon isotope ratios to that of their diet (i.e. the source of organic carbon that ultimately yields their respiratory CO_2); this should be distinct compared to organisms which utilise seawater bicarbonate for construction of calcium carbonate. This difference is because photosynthesis preferentially selects lighter carbon isotopes from the medium they reside in for synthesis of organic matter. Therefore, photosynthesis gives carbon in nearly all organic matter a distinctly different ratio of isotopes compared to the ratio present in the environment (atmosphere or seawater). As such it could be that the carbon

present in fish carbonate (ultimately derived from photosynthesis at the first stage of the food chain) has a distinct isotopic ratio compared to carbon in carbonates derived directly from seawater bicarbonate. This may be useful for identification of fish carbonates if they are present in high enough concentration within sediments. Measurement of carbonate stable isotopes in fish produced carbonates and comparisons to other carbonate sources would be required in order to confirm whether isotope ratios are distinct in fish produced carbonates.

If the presence of fish carbonates can be confirmed within sediments, it would then be worth attempting to measure fish carbonate sedimentary accumulation rates across different ocean regions in order to estimate the contribution of fish to global carbonate accumulation.

5.3 The production rate of fish carbonates

Previous estimations of fish carbonate production rates across the world utilised the relationship between fish individual body mass, temperature and carbonate production rates applied to two different global ecosystem models (Wilson et al., 2009). In that model, individual fish tend to produce more carbonate in warm ocean areas due to an increase in metabolic rate with temperature. However, salinity was not included as a factor which might affect the production rates of individual fish. Chapter 4 has shown that carbonate production rates increase with salinity. With this in consideration it would seem that warm, high salinity areas of oceans will be the areas in which the most carbonate is produced by individual fish although this has yet to be incorporated in a model. As such it would seem that tropical areas are likely to be conducive to high carbonate production rates compared to temperate areas. However, although individual production rates may be greater in tropical areas, it is estimated that there is a greater abundance of fish across temperate latitudes compared tropical ones (Jennings et al., 2008). Consequently, in previous models temperate oceans are still relatively important in terms of fish carbonate production rates (Wilson et al., 2009) but the inclusion of salinity in future models of fish calcium carbonate production would help clarify this.

Even though the estimates of production rates of fish carbonates in temperate areas may be revised downward due to the impact of salinity, discussion in chapter 4 notes that estimates of fish carbonate production globally may increase. Some high salinity and high temperature areas of the ocean overlap

with some areas in which fish are relatively abundant, especially considering recent studies that suggest we have greatly underestimated the abundance if fish in open ocean (Irigoien et al., 2014). There are some areas of high fish abundance that also overlap with low salinity but the results in chapter 4 would indicate that the relative decrease in production rate seen below the ocean average of 35 psu is smaller than the relative increase in production rates seen above 35 psu. This would suggest that global estimates of carbonate production might increase if salinity is taken into account. An estimate of the increase can be roughly calculated and demonstrates this likely increase if some assumptions are made. Assume that 35 psu is a true ocean average and fish are equally distributed in areas above and below and at 35 psu, and suppose that current carbonate production rates (which were presumably calculated at the ocean average of 35 psu) are equal to 1. In current models no change occurs in the fish above and below 35 psu, i.e. these are both also equal to 1. The sum of the current production rates with fish at, above and below 35 would then be 3 using current models. Crudely utilising the data obtained in shanny examined in chapter 4, fish at 35 psu would again be considered to produce carbonate at a rate of 1, fish at 30 psu would produce carbonate at a rate of 0.7 and fish at 40 psu would produce carbonate at a rate of 1.6. The sum of the production rates at 30, 35 and 40 psu is then 3.3. The sum of the production rates when salinity is accounted for (based on the data for shanny) is therefore 10 % higher than the production rates estimated when salinity is not accounted for. When the data from goldsinny wrasse is used instead in the same manner, the production rate is 17 % higher. Although this is obviously over-simplified it does demonstrate that the estimates of global carbonate production need to be revised based on salinity, and the most likely impact will be an increase.

5.4 The overall carbonate budget and the carbonate pump

The efficiency of the action of the carbonate pump is determined by the amount of produced carbonate that remains solid long enough to accumulate in sediments or reach deeper ocean layers. Therefore, differences between ocean regions in rates of production, dissolution and accumulation rates of carbonates will result in differences in the efficiency with which the carbonate pump acts in those regions.

Temperate areas by definition are cooler than tropical areas (Tait and Dipper, 1998b) and are also generally associated with lower salinities and alkalinity due to reduced evaporation and increased precipitation or freshwater input (Millero et al., 1998). Lower temperature, salinity and alkalinity are conducive to both lower fish carbonate production rates and increased dissolution. This may mean that fish in temperate areas individually produce less carbonate and what they do produce is able to sink less far and contribute less to the carbonate pump. However, there are several other factors which may work to increase the impact of temperate fish to the carbonate pump.

Fish have been estimated to be relatively abundant in temperate latitudes compared to tropical latitudes (Jennings et al., 2008) and as such, even though individual production rates from fish may be lower, the overall rate of carbonate production from fish in temperate areas may still be large. The data in chapter 4 may now allow us to further refine models which estimate production rates of fish carbonates across the globe to assess the relative importance of temperate areas in fish production.

Cooler temperatures and lower salinities in temperate areas which favour dissolution of carbonates may also be counteracted somewhat by the reduced magnesium content of the carbonates from temperate fish. Magnesium contents measured in ellipsoidal morphology carbonates in chapter 2 were less than half the values of magnesium measure in ellipsoids from tropical species previously (Salter et al., 2012). It was speculated that the lower magnesium contents could be related to the lower temperatures as is seen in other calcifying organisms. Confirmation of the potential effect of temperature on magnesium contents of fish carbonates would be useful. Additionally information on the solubility of different fish carbonate types with different magnesium contents is required before conclusions can be made about the relative solubility of fish carbonates in temperate areas compared to tropical ones.

It is currently difficult to make a direct comparison between the potential solubility of fish carbonates in temperate and tropical areas due to methodological differences in the information available about the characteristics of temperate and tropical fish carbonates. The information provided in chapter 2 is based on carbonates collected directly from fish captured from the wild with intestines full of food whereas the information available on tropical species has

been collected from holding tanks after a period of starvation. The effects that this may have had are discussed in chapter 2, and until comparable data collected on feeding tropical fish it is difficult to assess the relative solubility of carbonates from fish produced in different areas.

Even if fewer fish carbonates are produced in temperate areas and these dissolve at a faster rate, temperate areas may still have an important role in the carbonate pump. As discussed previously in section 1.1, some temperate oceans are likely to be relatively efficient at contributing to the carbonate pump due the long length of time with which it takes for the deeper ocean to mix with the surface ocean. This means even if fewer fish carbonates reach the deeper ocean, in these temperate areas alkalinity from dissolved carbonates remains sequestered from the surface for a longer period of time.

Overall, further research is required to determine how much fish contribute to different parts of the carbonate budget and the relative importance of fish in temperate oceans on the efficiency of the global carbonate pump. However, it still seems likely that carbonate produced in temperate oceans is likely to play an important part.

5.5 Using fish carbonates to investigate past oceans

Further investigation is required in order to establish the exact cause of variation in carbonate characteristics produced by marine fish. However, if fish carbonate characteristics do prove to be controlled by species, diet, or temperature, it could be that fish carbonates in preserved in sediments could provide information about the geographical area on past ecosystem assemblages of fish or environmental temperatures. Magnesium-calcium ratios have already been proposed as a proxy for past seawater temperatures based on other biogenic carbonates, although it has been noted that the change in magnesium-calcium ratios with temperature can be different across different species (Borremans et al., 2009; Butler et al., 2015; Cros et al., 2013; Xiao et al., 2014). It is currently unknown whether different fish species will have differences in how calcium and magnesium composition changes across temperatures. This technique would also rely on being able to successfully identify fish carbonates in sediments and sedimentary rock. Identifying fish carbonates in sediments presents a challenge due to their relatively small size, and as discussed in chapter 2, can prove difficult. A potential approach not

attempted in the studies of the current thesis, but discussed in section 4.9, might be to use stable isotopes of the carbon present in carbonates to identify fish carbonates within sediments.

Many of the potential techniques discussed above regarding fish carbonates in sediments and sedimentary rocks are likely only useful if fish carbonates are present in high enough concentration in sediments; chapter 3 showed little evidence that this is the case in modern temperate sediments. However, there are periods in the geological past where sediments (now converted to sedimentary rocks) are likely to contain much more fish produced carbonate than present sediments. For, example the Cretaceous period had a climate far more conducive to the production of fish produced carbonates than modern oceans. The Cretaceous period is famous for having much higher average temperatures, higher atmospheric CO_2, and higher ocean salinities compared to modern oceans (Friedrich et al., 2012; Rohling, 2007; Wagner et al., 2008; Zeebe, 2012). Increased temperature is known to increase the metabolic rate of fishes and as such estimates of carbonate production for warmer areas of the modern ocean are higher than those of cooler areas (Wilson et al., 2009). Higher average temperatures in the Cretaceous should be expected to increase carbonate production rate of fishes also. Higher atmospheric and ocean CO_2 levels in the Cretaceous period should also lead to increases calcium carbonate production from fish. Fish calcium carbonate production is dependent on secretion of bicarbonate ions from the fish blood to the intestine where it precipitates with calcium to form calcium carbonate. At higher seawater CO_2 concentrations fish exhibit increased blood CO_2 concentrations (Heisler, 1984) causing acidosis which is compensated by accumulating bicarbonate ions in the blood to restore pH (Melzner et al., 2009). The increased blood CO_2 concentration and bicarbonate ion concentrations causes increased bicarbonate intestinal excretion (Esbaugh et al., 2012) which precipitates in the intestine to form calcium carbonate. As such increased CO_2 concentrations in the Cretaceous period should act to increase carbonate production rates from fish. Additionally, chapter 4 in the current study showed that higher salinities lead to higher carbonate production rates in fish. Overall the increased temperatures, CO_2 concentrations and salinities of the cretaceous period should lead to greatly increased production rates of calcium carbonates from fish. Additionally the Cretaceous period is when fish species underwent massive diversification to

become the dominant taxa of vertebrates in the ocean (Finn and Kristoffersen, 2007). If the abundance of fish in Cretaceous oceans was high it could be that fish are major contributors to the large amounts of chalk deposited in the era considering the environmental conditions. Therefore, if further investigation reveals clear links between environmental or ecological parameters and fish carbonate characteristics, it may be that more can be revealed from Cretaceous carbonate rocks about the environmental and ecological conditions present in that period of Earth's history.

5.6 The role of fish carbonate in future oceans

In pre-industrial times over the timescale of millennia, the ocean was the major controller of atmospheric CO_2 (Falkowski et al., 2000; Zeebe, 2012). However, we have now entered an age where anthropogenic CO_2 emissions have cause atmospheric CO_2 concentrations to rise. During the past 800,000 years until 1750 (before the start of the industrial revolution) atmospheric CO_2 concentrations varied between 180 and 300 ppm, but in 2011 the average were measured at 390.5 ppm (Ciais et al., 2013) and in 2012 measurements exceeded 400ppm (McGee, 2015). Anthropogenic CO_2 emissions are predicted to increase unless stringent mitigation strategies are put in place (IPCC, 2014). This increase in atmospheric CO_2 has driven climate change; global temperature has risen, levels of ice and snow have decreased and mean global sea levels have risen; these effects are predicted to continue as atmospheric CO_2 concentrations go on rising (IPCC, 2014).

So far the oceans have mitigated anthropogenic CO_2 emissions by absorbing approximately 30% of anthropogenic emissions since 1750 (IPCC, 2014). There has therefore been great interest in predicting the capacity of the ocean to take up further CO_2 emissions in the future. It has been suggested that for the biological pumps in the oceans (such as the organic carbon pump and the inorganic carbon or carbonate pump) to take up further CO_2 in the coming century, their efficiency must increase (Falkowski et al., 2000). One way in which biological pumps could become more efficient in facilitating ocean uptake of CO_2 is by increasing the ratio of organic matter that sinks as part of the carbon pump compared to the amount of calcium carbonate that sinks as part of the carbonate pump (Falkowski et al., 2000). Changes in the inorganic carbon (or carbonate) pump therefore have the ability to effect ocean uptake of CO_2 in

the future. It has been hypothesised that a decrease in the amount of carbonate produced would allow the oceans to absorb more CO_2 (Doney et al., 2009; Millero, 2007). However it has also been noted that the carbonate pump and organic carbon pump may be intrinsically linked. Relatively dense calcium carbonate particles can combine with organic matter and act as ballast promoting sinking rates (Armstrong et al., 2002). As such decreasing the carbonate pump efficiencies may result in decreasing the organic carbon pumps efficiency at trapping CO_2.

It is predicted calcification rates for many organisms may decrease in response to the increased levels of ocean and atmospheric CO_2 levels predicted for the future due to the acidifying effects of CO_2 in seawater (Doney et al., 2009; Fabry et al., 2008; Gazeau et al., 2007; Jokiel et al., 2008; Kroeker et al., 2013). However this is unlikely to be the case for fish. Section 4.12 explains why fish are likely to increases production rates of carbonate under elevated CO_2 and temperature conditions. Additionally the effects of salinity on carbonate production rates of fish may cause an increase in fish carbonate production in future oceans. One predicted impact of increased atmospheric CO_2 is an increase in the hydrological cycle across the globe due to warming. This increase in the hydrological cycle is predicted to impact ocean salinities by making them more extreme. Increased precipitation in areas of low salinity will lead to salinities becoming even lower in these areas; increases evaporation will lead to the salinity of already relatively saline areas to increase (Durack et al., 2012; Durack and Wijffels, 2010). Considering that chapter 4 revealed that increases in carbonate production rates from fish increase more above 35 psu compared to the decrease below 35 psu, it could mean that more extreme areas of high and low salinity in the ocean actually acts to increase the amount of carbonate produced by fish globally.

Overall it seems likely that fish calcium carbonate production rates will increase in the future whereas production rates from other organism are likely to decrease. If fish carbonate does not act as effective ballast for organic carbon, then the increased carbonate production from fish may result in a decrease in the ability of the ocean to uptake CO_2 in the future despite falling carbonate production rates from other carbonate sources. However, if the carbonate pump is linked to the organic carbon pump through the ballast effect, then it could be

that the relative importance of fish in carbon transport and sequestration in the ocean increases in the future.

It is additionally worth considering that increase in CO_2 predicted for the future will result in the oceans having a lower pH. This is favourable to the dissolution of calcium carbonates. Quicker dissolution of carbonates in the ocean may counteract the effect of any increases in carbonate production and reduce the impact of the carbonate pump.

Ultimately, understanding how the impact of the carbonate pump on future ocean-atmosphere CO_2 fluxes is a complex problem. As of yet there seems to be insufficient information available to predict how the action of the carbonate pump will change in the future. It is even more complex to determine the overall response of the ocean to increasing CO_2. Net CO_2 fluxes are determined by the action of not only the carbonate pump, but the organic carbon pump and the solubility pump as well. Knowledge of not only how each of these individual systems will react in the future but knowledge of how they interact with each other will be required to successfully understand the role of the ocean on future atmospheric CO_2 concentrations.

5.7 Concluding remarks

Overall, the chapters in the present thesis have started to investigate the role of fish in the ocean carbonate pump. They have provided information that can be used to fill a gap in our understanding of carbonate budgets in temperate areas, and refine our understanding of carbonate budgets across other oceans. The chapters have contributed to understanding the role of fish in all three major parts of carbonate budgets: production, dissolution and accumulation in sediments. Production rates are probably the component of the budget currently best understood. Models had been developed previously to estimate production rates of fish carbonate across the world's oceans and the new information about the effect of salinity on fish carbonate production rates presented in this thesis can be used to improve on these models further. However understanding carbonate production rates alone are of scant use in determining the impact of the carbonate pump on ocean atmosphere CO_2 fluxes. It is critical that we also accurately understand the amount which accumulates in sediments or the amount which dissolves in the ocean. In theory, if accurate estimations of two parts of the budget are known, then the third can be calculated. However, in

practice having some knowledge of the third is useful for checking estimates of the other two components. Currently we have a poor understanding of both the dissolution and accumulation rates of carbonate produced by fish. However, the chapters above have started to investigate fish carbonate dissolution and accumulation rates. The information provided here provides a platform for future investigation on fish carbonate dissolution and accumulation rates and will hopefully encourage further research on the role of fish in the activity of the carbonate pump on ocean atmosphere CO_2 fluxes.

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