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Departamento de Biologia

Andreia Cristina Ferreira de Carvalho

EFFECT OF DIFFERENT ENVIRONMENTAL CONDITIONS ON THE INCORPORATION OF TRACE ELEMENTS BY LARVAL SHELLS OF *Mytilus galloprovincialis*

EFEITO DE DIFERENTES CONDIÇÕES AMBIENTAIS NA INCORPORAÇÃO DE ELEMENTOS EM CONCHAS LARVARES DE *Mytilus galloprovincialis*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção de grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Henrique José de Barros Brito Queiroga, Professor do Departamento de Biologia da Universidade de Aveiro e da Doutora Laura Garcia Peteiro, Investigadora de Pós-doutoramento da Universidade de Aveiro.

O júri

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Palavras-chave

Assinaturas geoquímicas, concha larvar, mexilhão do mediterrâneo, dispersão, temperatura, salinidade

Resumo

A dispersão de organismos marinhos durante a sua fase larvar é determinante para a conectividade entre populações e o estabelecimento da complexa biogeografia das espécies marinhas. A determinação dos padrões de dispersão tem sido relevante para a conservação de espécies, a gestão de stocks de pesca e mesmo para a compreensão de processos ecológicos como a extinção e a recolonização de populações. As estruturas carbonatadas de organismos marinhos representam arquivos de informação ambiental. Através da análise geoquímica de estruturas como os otólitos de peixe e os estatólitos e conchas de invertebrados é possível descobrir as condições físico-químicas ambientais sob as quais se desenvolveram estes organismos e reconstruir padrões de dispersão e locais de origem de indivíduos. No entanto, a incorporação dos elementos químicos por estas estruturas é altamente regulado por um conjunto de fatores físico-químicos ambientais e biológicos que deverão ser ponderados para uma correta utilização de assinaturas geoquímicas como meio de identificação geográfica. Com o intuito de investigar como a composição química de conchas larvares do mexilhão *Mytilus galloprovincialis* é influenciada por algumas condições ambientais e temporais, indivíduos desta espécie foram submetidos a diferentes temperaturas, salinidades e concentrações de elementos. Pretendia-se verificar como difere a incorporação de elementos com os diferentes parâmetros de cada fator ambiental e se a mesma é influenciada pela idade do indivíduo. Com esse objetivo foram cultivadas larvas de *M. galloprovincialis* por um período de 6 e 14 dias e criados tratamentos combinados de temperatura (12, 17, 20 e 25°C) e de salinidade (26, 32 and 37). Foram quantificadas as concentrações dos elementos Mg, Mn, Ba, Sr, Cu e Zn pelo rácio de Ca através de análise de ICP-MS (inductively coupled plasma mass spectometry). Paralelamente um outro conjunto de indivíduos larvares foi submetido a concentrações de Cu e Zn durante aproximadamente 3 dias. Não foi encontrada variabilidade significativa nas assinaturas geoquímicas entre as larvas com 6 dias e 14 dias de idade, sugerindo uma composição química estável, para este período, que não foi afetada pelo desenvolvimento larvar.

Resumo (Cont.)

Numa outra abordagem, foi detetada a máxima incorporação destes elementos a uma temperatura de 17°C e a diferentes combinações de temperatura e salinidade, realçando uma interacção entre estes fatores. Estes resultados realçam a possível influência da temperatura sob a disponibilidade de cada elemento. Outro aspeto a ter em conta é o facto de a 17°C serem encontradas as condições ótimas para o desenvolvimento larvar, e por isso também da secreção da concha carbonatada do mexilhão, existindo uma maior incorporação dos elementos do ambiente. A incorporação de Cu, no entanto, não mostrou ser significativamente afetada pela temperatura nem pela salinidade. Relativamente ao Zn estes efeitos foram apenas significativos quando não combinados. Para além disso, e numa terceira abordagem, a disponibilidade destes últimos na água, não se mostrou determinante para a incorporação dos mesmos nas conchas, sugerindo-se um controlo da inclusão por parte de fatores fisiológicos do próprio organismo. Os resultados obtidos permitem compreender mais detalhadamente a relação que alguns fatores ambientais exercem sob a produção de assinaturas químicas em conchas larvares desta espécie de mexilhão e poderão contribuir para uma melhor interpretação de padrões de conectividade de populações no meio marinho.

Key words

Elemental fingerprinting, mussel larval shells, temperature, salinity, larval dispersal, Mediterranean mussel

Abstract

Larval dispersal is the main cause of connectivity between populations and the establishment of the complex biogeography of most marine species. The determination of dispersion patterns has been relevant to conservation studies of species, management of fisheries stocks and even for the understanding of ecological processes as the extinction and re-colonization of populations. Carbonate structures of marine organisms work as an archive of environmental information. Through geochemical analysis of fish otoliths, as well as statoliths and shells of invertebrates is possible to discover physical and chemical environmental conditions under which these organisms developed and trace dispersion patterns and natal habitats. However, the incorporation of trace elements in these structures is highly regulated by a range of physical and chemical environmental factors as well as physiological and biochemical. These factors should be considered for an accurate use of geochemical signatures as a means of geographical identification. In order to investigate how the geochemical composition of larval shells from the mussel species *Mytilus galloprovincialis* is influenced by environmental and temporal conditions, individuals were submitted to different temperatures, salinities and water elemental concentrations. The main objective was to understand how the elemental incorporation varies with different parameters of each environmental factor, and if the same is influenced by age. *M. galloprovincialis* larval were reared during 6 and 14 days under combined treatments of temperature (12, 17, 20 and 25° C) and salinity (26, 32, and 37). Concentrations of the elements Mg, Mn, Ba, Sr, Cu and Zn by the ratio of Ca were analyzed through ICP-MS (inductively wound couple plasma-mass spectrometry) analysis.

Abstract (Cont.)

Simultaneously another set of individuals were submitted to different concentrations of Cu and Zn for a period of approximately 3 days. No significant variability in chemical composition was found in larval shells between larval with 6 and 14 days of development, suggesting a stable geochemical signature, for this period, and no age effect on incorporation. On a second approach, was detected a maximum incorporation of these elements at 17° C and at different temperature-salinity combinations, suggesting an interaction between these factors. These results highlight the possible influence of temperature on each element's availability. Another aspect is the fact that at 17° C larval seem to find optimum conditions for its development and for carbonate shell secretion, having a higher incorporation of the elements from the water. Cu incorporation, however, was not significantly affected by temperature or salinity. With regard to the Zn these effects were significant only when not interacting. Finally, the availability of the lastly mentioned elements in the water was not significant for elemental concentrations in shells, suggesting that certain elements incorporation might be specifically controlled by physiological mechanisms of the organism. Results obtained allow understanding in more detail the influence some environmental factors have on geochemical signature production in mussel larval shells of this species and may contribute to a better use of elemental fingerprinting in the interpretation of connectivity patterns.

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1. Introduction

Carbonate structures, such as otoliths, statoliths, and protoconchs of marine organisms work as an archive of chemical and physical environmental variations experienced during growth (Vander Putten *et al.* 2000; Zacherl *et al.* 2003a; Zacherl, 2005; Becker *et al.* 2005). In association with specific environmental conditions non-calcium water elements are usually incorporated by the newly formed biogenic carbonate, leaving an elemental fingerprint (Thorrold *et al.* 2002; Becker *et* al. 2005). These signatures may be used to relate an isolated individual to its natal origin, working as a natural tag (Becker *et al.* 2005), and to investigate population connectivity mediated by larval dispersal by tracing a migration pathway (Fodrie *et al.* 2011). This can highly contribute to the understanding of marine population's dynamics and community structure, species evolution and ecology, which are of major importance in marine resources management (Becker *et al.* 2007).

Most marine organisms have a bi-phasic life cycle with a pre-settlement planktontrophic phase that can last from weeks to months (Becker *et al.* 2007), followed by a post larval phase, including a juvenile and adult stages. With very little to non-swimming capacity, larval are transported for several kilometers by ocean currents (Becker *et al.* 2005) contributing to a highly dispersed and connected demography among marine species (Becker *et al.* 2007; DiBacco&Levin, 2000). Attention has been given to this factor, and to the effect it has on marine populations since the beginning of the century (Hjort, 1914). Currently, it is theorized how marine species survival may even depend on this connectivity and how it can influence species persistence and local biodiversity maintenance (Thorrold *et al.* 2007 Frodie *et al.* 2011). Since most of population´s biogeography and abundance is determined during this early life stage, knowing how far larval can be transported and which trajectories they take might, for example, help managing commercial relevant fish and shellfish invertebrate stocks, as well as contributing to a more effective design of marine reserve networks (DiBacco&Levin, 2000; Thorrold *et al.* 2007), lead to the understanding of the spread of invasive species and the processes of extinction and recolonization (Becker, *et al.* 2005; Becker, *et al.* 2007). Therefore, understanding

larval dispersal mechanisms, rates and distances is, nowadays, crucial to determine MPAs optimal size, location and configuration (Levin, 2006), specially when the change of the environmental conditions may induce, in some cases, marine species redistribution (Sorte *et al.* 2013).

However, studying larval dispersal and therefore coastal population´s connectivity patterns has been a challenge (Levin *et al.*2006). Larvae reduced size and sparse concentration, relatively long planktonic periods of most larval and high mortality rates are some identified obstacles. Studying methods have included from traditional approaches like visual tracking (direct observation) and sampling routines (net tows sampling, settlement collectors) (Queiroga *et al.* 1994; Dibbaco and Levin, 2000; Levin and Largier, 2003; Thorrold *et al.*, 2007) to more complex such as hydrographic, behavior and energetic modeling, physical transport mechanisms and tagging techniques based on geochemical composition(Levin, 1990; Levin, 2006).

The geochemical composition of calcified structures of marine organisms based on geographical differences in water chemistry is increasingly being employed as a strategic tool to discriminate among source populations and understand connectivity patterns (Thorrold 2007; Lopez-Duarte *et al.* 2012; Carson *et al.* 2013). This natural tagging method follows the idea that physical and chemical conditions under which larval grow are recorded into their carbonate parts through its geochemical composition (Becker *et al.* 2005). At different water masses trace elements are incorporated in marine organism´s soft and hard tissues creating a chemical signature imprinted into mineral structures (Campana, 1999). This trace metal/calcium ratio signature can be related back to the location (or locations) where it was formed (Campana *et al.* 1994; Dibacco and Levin, 2000; Thorrold *et al.* 2002). However, an accurate use of elemental fingerprints as larval source identification depends on how different environmental conditions experienced at the several locations and on how stable are they over time (Thorrold *et al.* 2002; Becker *et al.* 2007) so that consistent spatial variations can be registered in the element composition (Zacherl *et al.* 2003). For instance, more geochemical tag studies have been undertaken using estuarine species than using open-coast species, most probably because in the ocean temperature, salinity and water chemistry variations

are less substantial (Becker et al 2007; Lopez-Duarte et al 2012; Carson *et al.* 2013). Therefore, the identification of geochemical differences in pelagic larval spawned in open coasts and consequently a rigorous determination of spatial and temporal scales, are problems still to overcome (Becker et al. 2007; Thorrold *et* al. 2007).

As it is referred, biogenic carbonate structures "use" chemical elements found in the water to build their own composition during development. However , this incorporation is intrinsically related and influenced by a range of environmental and biological factors including temperature, salinity, dissolved ion concentration in seawater, oxygen levels, nutrient loads, precipitation rate, ontogenetic characteristics and seasonal variations in animal´s physiology (Vander Puten *et al.* 2000; Zacherel *et al.*2003; Strasser, 2008). Comprehending how each factor can influence chemistry signature formation is fundamental to a correct study using elemental fingerprinting in larval dispersal studies (Strasser *et al.* 2008).

The first geochemical tagging studies ever made with hard tissues were applied to fish otoliths (ear-stones), though it was rapidly extended to statoliths and prodissoconchs, (larval aragonite shell segregated during embryonic development) (Thorrold *et al.* 2007). Several are the results successfully achieved in population studies using elemental fingerprinting, particularly in fish (Strasser *et al.* 2008). In mollusks, on the other hand, and especially in bivalves, studies developed are yet scarce (e.g. Becker *et al.* 2005, 2007; Carson, 2010a; 2010b). More recently, Fodrie *et al.* 2011 has investigated temporal patterns in two mussel species shells settling in one exposed region and one protected bay. Although mussels are commonly used as environmental quality indicators due to their wide geographic distribution and resistance to a broad range of environmental conditions (Vander Putten *et al.* 2000), very few studies have been carried out on their larval ecology (Strasser *et al.* 2008).

1.1Geochemical signatures formation

Trace elements in seawater have two major external sources: 1) atmospheric, anthropogenic or riverine input and 2) as a result of interactions between seawater and the newly formed oceanic basalt crust (Nriagu 1989; Donat & Bruland, 1995). Most of these elements are widely found in nature (Schroeder *et al.* 1966a; 1966b; 1967) hence its requirement for living organisms basic functioning (e.g. protein biological activity which is the case of enzyme cofactors Zn, Cu and Mn) (Singh *et al.* 2011).

Carbonate skeletons from hard corals and crustaceans (Finlay *et al.* 2011), mollusc shells and fish otoliths (Campana *et al.* 1994) are built through the deposition of calcium carbonate ($CaCO_3$), its main component (Campana *et al.* 1994; Campana *et al.* 1999). This compound can be found in aquatic systems as dissolved calcium ions are gained by the dissolution of minerals and rocks (e.g. silicates and carbonates) and carbonate ions are usually introduced by sources such as carbonate minerals, organic matter, atmospheric carbon dioxide and CO_2 with volcanic origin (Dietzel *et al.* 2004). Contrarly to other calcified structures, otoliths, statoliths and larval shells do not change the composition through growth (Campana *et al.* 1994). That's what makes these structures such success in paleoclimatic studies as recorders of environmental conditions (Carré *et al.*2006).

Concerning mussels, through water filtration, the carbonate element is deposited in the organism tissues whilst production of specific proteins will form the first aragonite-based shell (Gossling, 2004). At the same time, during this mineralization process, other elements available in the water are incorporated together with calcium that forms the skeletal structure of the animal (Richardson *et al.* 2001).

In general, bivalve shell segregation starts a few hours after fertilization, during the trochophore larval stage, the first of several planktonic stages (Figure 1) (Weiss *et al.* 1992). However, several authors (De Aguirre, 1975; Lutz and Kennish, 1992; Ruiz *et al.* 2008) have verified that in mussels this process can actually start under veliger

form, which is the second stage. During this process, a specialized group of ectodermal cells, the shell field, from the dorsal region of the embryo, secretes the shell gland. Subsequently, the outer mantle cells secrete a second and the outer most organic shell layer – the periostracum. In an inside-out process, the shell gland everts and becomes the larval mantle tissue while the periostracum spans the whole surface (Weiss *et al.* 1992; Gosling, 2004). This shell is called prodissoconch I and continues enlarging until the organism is able to close both valves. The production of prodissoconch II then follows and lasts until the last and third mobile stage, the pediveliger. Once the larval phase finishes the juvenile shell is called dissoconch but protoconchs I and II remain integrated into juvenile and adult shells as structurally distinguishable components (Weiss *et al.* 1992; Ruiz *et al.* 2008).

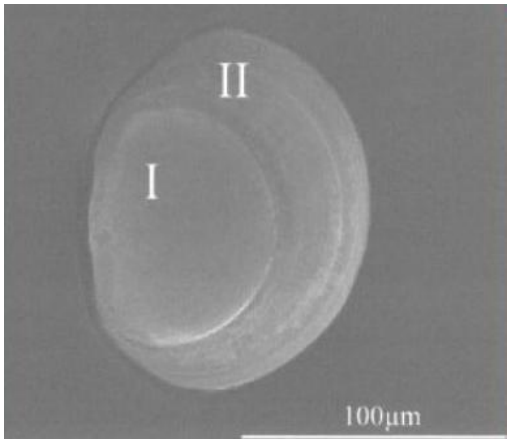

Figure 1 - Three day old larvae shells – I – protoconch I, II – protoconch II. Weiss et al. 2002

1.1.1 Factors influencing elements´ incorporation

Biogenic carbonate composition has been extensively used for paleo-environmental studies (Pilkey and Goodell, 1963). Although it is known that trace metal absorption is partially controlled by a wide range of environmental, biological and physiological/genetic factors, only recently have we been able to understand how each of these contribute to variability in microchemistry signatures(Strasser *et al*. 2008). Physical and chemical parameters such as temperature and salinity have been the most investigated factors over the years.

In fish otoliths and invertebrate statoliths, studies concerning temperature and salinity effect are numerous (Fowler *et al.* 1995; Elsdon and Gillanders, 2002; Martin *et al.* 2004; Martin and Thorrrold, 2005; Zacherl *et al.*, 2003b and Zacherl, 2005). Studies using bivalve shells have been growing over the years (e.g. Dodd, 1965; Dodd, 1967; Lerman, 1965; Lorens and Bender, 1980; Vander Putten, 2000; Lazareth *et al.*, 2003; Strasser *et al.* 2008). By studying elemental signatures in larval and juvenile

clam shells, Strasser (2008) was able to distinguish effects of temperature, salinity and age. It was found that the first two factors significantly influenced shell chemistry of the organisms. However, the authors highlighted that other physiological factors can create some confusion when interpreting elemental composition in carbonate structures.

1.2 Objectives

Geochemical tags are today undeniably important for larval tracking and general marine ecology studies. This dissertation focuses on the larval planktonic shells (prodissoconches) of the mussel *Mytilus galloprovincialis,* an ecological and economically relevant species. This study aims to contribute to a better comprehension of trace elements´ incorporation in relation to conditions of temperature, salinity, age and element´s concentration. The general objective is to understand the variability of elemental microchemistry composition of the larval shells in order to optimize its use as an elemental fingerprinting technique. For such purpose, a set of laboratory experiments were conducted where these organisms were submitted to constrained conditions of temperature, salinity, trace metals load and time. Effects of these parameters in concentrations of Ba, Cu, Mg, Mn, Sr and Zn relative to Ca concentration in D-veliger stage shells were analyzed using ICP-MS (Inductively Coupled Plasma Mass Spectrometry).

Three approaches were undertaken:

- Firstly, larval were reared for 14 days under the same temperature and salinity level.
- Secondly, mussel larvae were reared under different temperature and salinity treatments for 6 days.
- Thirdly, 26h reared larval were incubated into different concentrations of Cu and Zn at 17°C and salinity 32.

1.3 Research questions

Elements Intake

Being able to travel for several kilometers transported by ocean currents, individual larva experience different environmental conditions, such as the ones selected for this work. How do these different conditions affect the elemental signature creation in mussel prodissoconches is the major question of this thesis. Some of the guiding questions throughout this work should be:

- In which proportions are elements being incorporated?
- In which way are the different temperatures influencing incorporation?
- Is the increased availability of certain elements in higher salinity waters determining the deposition? Or are there other physiological factors determining the incorporation?
- Are there differences related to larval age?

Elements´ availability

Trace elements are found in aquatic systems abundantly, emerging from different known and unknown sources. A second major question is how different availabilities in the environment affect metal incorporation rate and consequently their contribution for shell-element signatures.

- What is the elements´ incorporation rate according to each added concentration?
- Whilst having more availability is also noticed higher incorporation?
- How is it found larval development at the different metal concentrations treatments?

2. Materials and Methods

2.1 Larvae rearing

Temperature and salinity

Adult *Mytilus galloprovincialis* were collected from Mira channel in the Ria de Aveiro and transported to the lab where they were cleaned and prepared for spawning induction. Mussel individuals were placed in plastic containers with artificial seawater. Thermal shock method was the most appropriate one for gamete release and several shifts of ~20 minutes of low temperature (3-4°C) and high temperature (22-25°C) took place. Both gametes were obtained in different glass containers and later passed through 40µm mesh filters and coupled. Fertilized eggs were filtered using the previous method and transferred to a new acid leached glass container where sub samples of 250µl were collected for counting and estimation of the stoking culture.

Approximately 16000 trochophore larvae with few hours were placed into each of the thirty-six 1 liter acid leached glasses. The larval containers were divided among 12 treatments that combine 4 temperatures (12, 17, 20 and 25°C) and 3 salinities (26, 32 and 37) in a factorial design with 3 replicates per treatment. Experiments room was kept at a 17°C temperature so it could be maintained the temperature of the third treatment. Water baths were created for three of the treatments. Aquarium water heaters were used for the 20 and 25°C treatments while for the 12°C treatment a cooler bath system was used. Temperature was continuously regulated by tank thermometers. Each larval container was covered with paraffin plastic and an air system was installed to provide oxygen to all rearing larvae.

Artificial seawater was obtained in laboratory by adding sea salt minerals to osmosis water (purified water by a reverse osmosis). Salinity was reduced for the low-salinity treatment by adding osmosis water and increased for the high-salinity treatment by adding artificial seawater. Water was filtered to exclude major contaminants and impurities and maintained in 5 liters water jugs for the whole time of the experiments.

Larvae were raised for 6 days under selected conditions except for one combination of temperature/salinity -17°C and 32 – where larvae were raised for 14

days. Water changes were executed every 3 days by filtering larval individuals with 40 µm filter meshes and depositing them in containers with new prepared and filtered artificial seawater. Larvae were fed *Isochrysis galbana* every 3 days, having all goblets received 5ml with a larval density of ≈20 000/L. Subsamples of 250µl of mussel larvae were collected for mortality and growth estimation from each container at each water change. Therefore organisms´ development was monitored and by the third day of experiment larvae were D-veliger stage and by the sixth, velum well developed veliger stage. At the 6^{th} and 14^{th} day (for the 17ºC 32 treatment) a sample of ≈ 1000 individuals per replicate were stored in acid leached Eppendorf tubes and frozen until sample processing.

Elemental availability

Two trace metals, Cu and Zn, were used to compare its incorporation, in larval shells, under different environmental concentrations during 60 hours. Approximately, 14000 larvae with 26h of development were distributed over 18 1l bakers of 3 treatments of Cu [0; 1; 5] µg/l and 3 treatments of Zn [0; 5; 10] µg/l with 3 replicates each. Stock solutions were prepared by adding a possible weighed concentration of each metal to 1 liter of filtered artificial seawater. Dilutions were made for each treatment. Room temperature was kept at 17°C.

2.2 Sample preparation

Water preparation

Water stock solutions for every salinity and element´s availability treatment were analyzed throughout the experiments for chemical composition. Sub samples of 900µl of each water salinity level (with three replicates each) were preserved with 100 µl of Ultrapure 60% nitric acid (HNO_3) and stored in acid leached 1,5ml ependorf tubes, at every water change, and stored at 4 °C until microchemistry analysis.

Larval shell preparation

Larva shell preparation followed Strasser *et al.* 2008 protocol. Each larva sample containing a "pool" of no more than 1000 larval individuals was defrosted and cleaned from organic matter. Using Petri plastic dishes, larvae "pools" were soaked in a solution of Suprapur hydrogen peroxyde (H_2O_2) 30% buffered with Suprapur

sodium hydroxide (NaOH) for 10 minutes to allow organic matter digestion and rinsed three times with Mili-Q water for larval shell decontamination before analysis. Using 40µm mesh filters larvae "pools" were filtered and transferred with a micropipette to acid leached 1, 5 ml ependorf tubes. Fluid excess was collected using a micropipette and 10µl of HNO_3 60% was added to each ependorfs to dissolve shells for ICP-MS analyses.

2.3 ICP-MS analysis

Both water and larval shell samples were analyzed for concentrations of barium (Ba), calcium (Ca), copper (Cu), magnesium (Mg), manganese (Mn), strontium (Sr) and zinc (Zn) by the Central Analytical Laboratory at the University of Aveiro. Selected elements for this study were already reported in previous studies, developed in laboratory, as having an interaction with temperature and salinity (Martin and Thorrold, 2005; Zacherl *et al.* 2003b; Strasser *et al.*2008) Ba, Mg, Mn and Sr and as successful proxies in tagging methods (Carson *et al.*2013).

Concentrations were determined with a Thermo X Series Inductively Coupled Plasma – Mass Spectrometer (ICP-MS), equipped with a Burgener Mira Mist nebulizer, set up with a Peltier conical cooled chamber, quartz torch and silver shielded nickel cones, coupled to a Cetax ASX-510 auto sampler. Indium (In) and Terbium (Tb) were used as internal standards and the CeO/Ce ratio was kept below <2%. The concentrations of ^{48}Ca, ^{24}Mg and ^{88}Sr were determined by a Jobin Yvon Activa M Inductively Coupled Plasma – Optical Omission Spectrometer (ICP-OES) equipped with a JY-AS500 auto sampler and a Burgener Mira Mist nebulizer.

2.4 Statistical analysis

Statistical routines were undertaken using software *Statistica*. Trace elemental concentrations of larval shells were standardized and ratios between each element and calcium (E: Ca) were calculated for data analysis. All ratio data was log normalized to correct for heteroscedasticity (heterogenity in error variance). Seawater chemical concentrations were interpreted through a linear regression as only the factor salinity was investigated for its effect in the different water treatments. Concentrations of trace elements in ratio to the amount of Ca were then

submitted to a factorial analysis of variance, factorial ANOVA, to explore the effects of temperature and salinity and the factors combined interaction on the incorporation of different elements. Tukey´s test was used as a post-hoc test. The effect of Cu and Zn concentration in the water under constant temperature (17°C) and salinity (32) was also tested with an ANOVA and pair-wise comparisons made using Tukey's test. Finally, the effect of age (6 and 14 day old) on the incorporation of different element: Ca at constant temperature (17°C) and salinity (32) was analyzed with a t-test.

3. Results

3.1 Temperature and Salinity effect

Water chemical composition

Seawater chemical composition for all elements varied among the tested salinity treatments, following an increasing trend as a function of salinity level (Fig 1). Increment was significant for all the seven elements as significant regressions confirm (Fig 1; p<0, 05). Zn concentrations in ultrapure Mili-Q water showed high concentrations of this metal (4 orders of magnitude higher than in salt water) suggesting contamination of deionized water sample. Therefore, those samples were not integrated in the analysis.

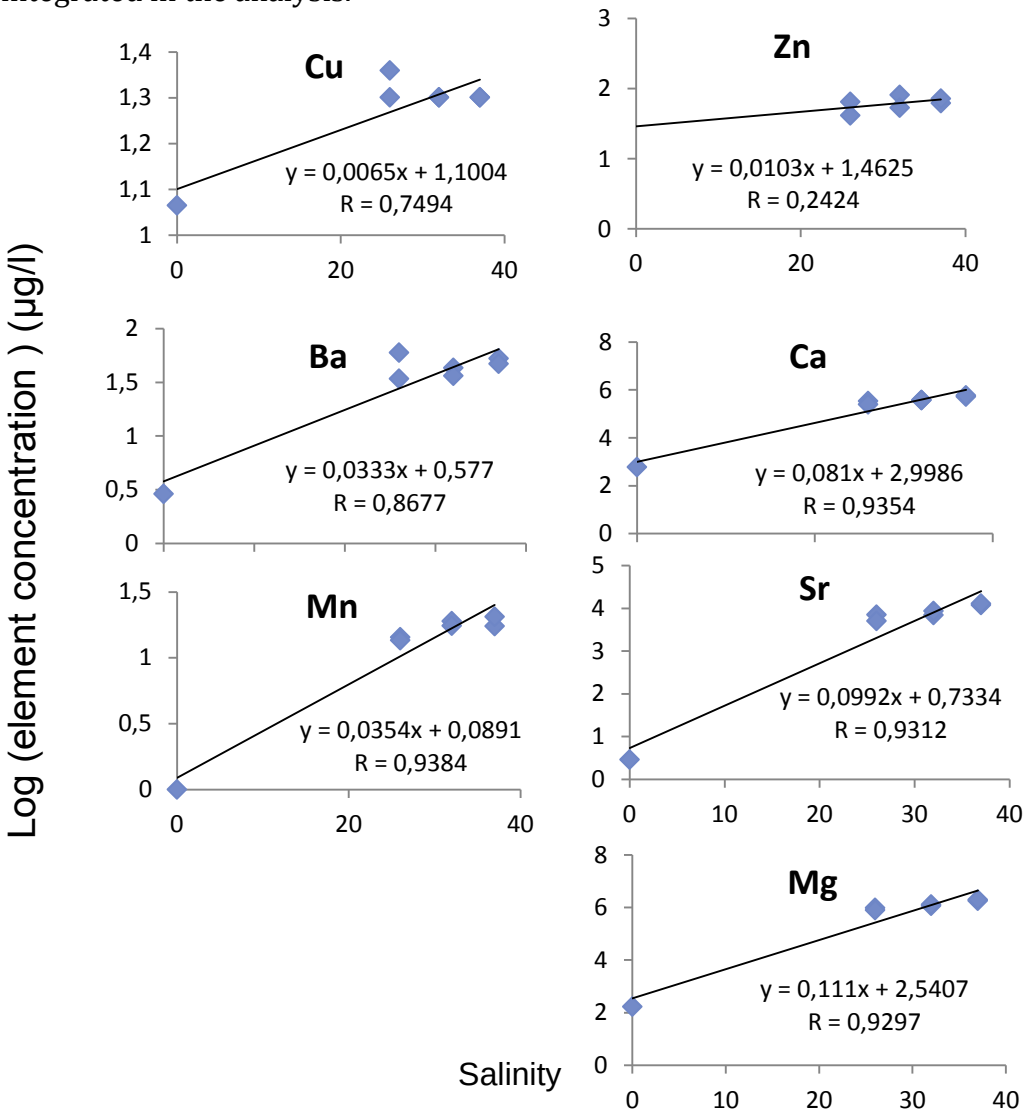

Figure 1- Mean seawater elemental concentrations of 26, 32 and 37 salinity treatments. Linear regression points represent log transformed concentrations of three replicates per salinity. Two samples of each salinity level were analyzed.

Larval shells

By the third day of the current experiment the 25°C treatment showed a larval mortality rate of nearly 100% which made this treatment no longer available for the study.

Temperature and salinity treatments showed to have a significant effect in all elements incorporation except for Cu (Table 1). Regarding temperature effect on concentrations of Mg/Ca, Mn/Ca, Zn/Ca, Sr/Ca and Ba/Ca, a common pattern was detected where the maximum incorporation is reached at 17C (Figure 2). While at 12C and 20C treatments incorporations were significantly lower. Although Cu/Ca also shows maximum incorporation at 17 C, the concentration was not significantly different from the other two temperature treatments.

Salinity also has a common effect on all the incorporation ratios according to the increasing trend observed on the elemental concentrations in the water (Figure 1). As salinity level increases element´s availability uniformly increases.

A significant interaction between temperature and salinity factors for all the elements, except for Cu and Zn, is visible in Table 1. It was also found a non-significant interaction effect on Mn/Ca ratios although very close (p=0, 07) from the statistic significance level. At each tested temperature it is seen that elemental concentration varies in all the salinity treatments.

Temperature and salinity interaction is distinguishable by a common decreasing tendency on shell incorporations at the highest temperature for every salinity level, although maximum incorporations occur at different salinity/temperature combinations for each element (Figure 4)

Table 1 – Results of ANOVA testing the effects of temperature (Temp), salinity (Sal) and interaction between temperature and salinity (Temp*Sal) on elemental ratios in larval shells from the different treatments. Significance for p< 0, 05.

	Mg:Ca				**Mn:Ca**				**Cu:Ca**	
	df	MS	*F*	p	MS	*F*	p	MS	*F*	p
Temp	2	2,5826	14,564	0,000108	5,5150	22,3011	0,000006	0,99997	0,91979	0,414072
Sal	2	2,5598	14,435	0,000114	4,6085	18,6352	0,000022	2,40793	2,21487	0,134033
Temp*Sal	4	0,6951	3,920	0,015720	0,6183	2,5000	0,073479	1,97594	1,81751	0,163155
Error	21	0,1773			0,2473			1,08716		
	Zn:Ca				**Sr:Ca**				**Ba:Ca**	
	df	MS	*F*	p	MS	*F*	p	MS	*F*	p
Temp	2	1,3168	15,600	0,000070	3,7461	15,825	0,000064	2,91086	8,77932	0,001694
Sal	2	1,0390	12,308	0,000290	3,1107	13,141	0,000199	3,63427	10,96115	0,000550
Temp*Sal	4	0,1816	2,152	0,109996	0,7114	3,005	0,041609	1,24095	3,74276	0,018870
Error	21	0,0844			0,2367			0,33156		

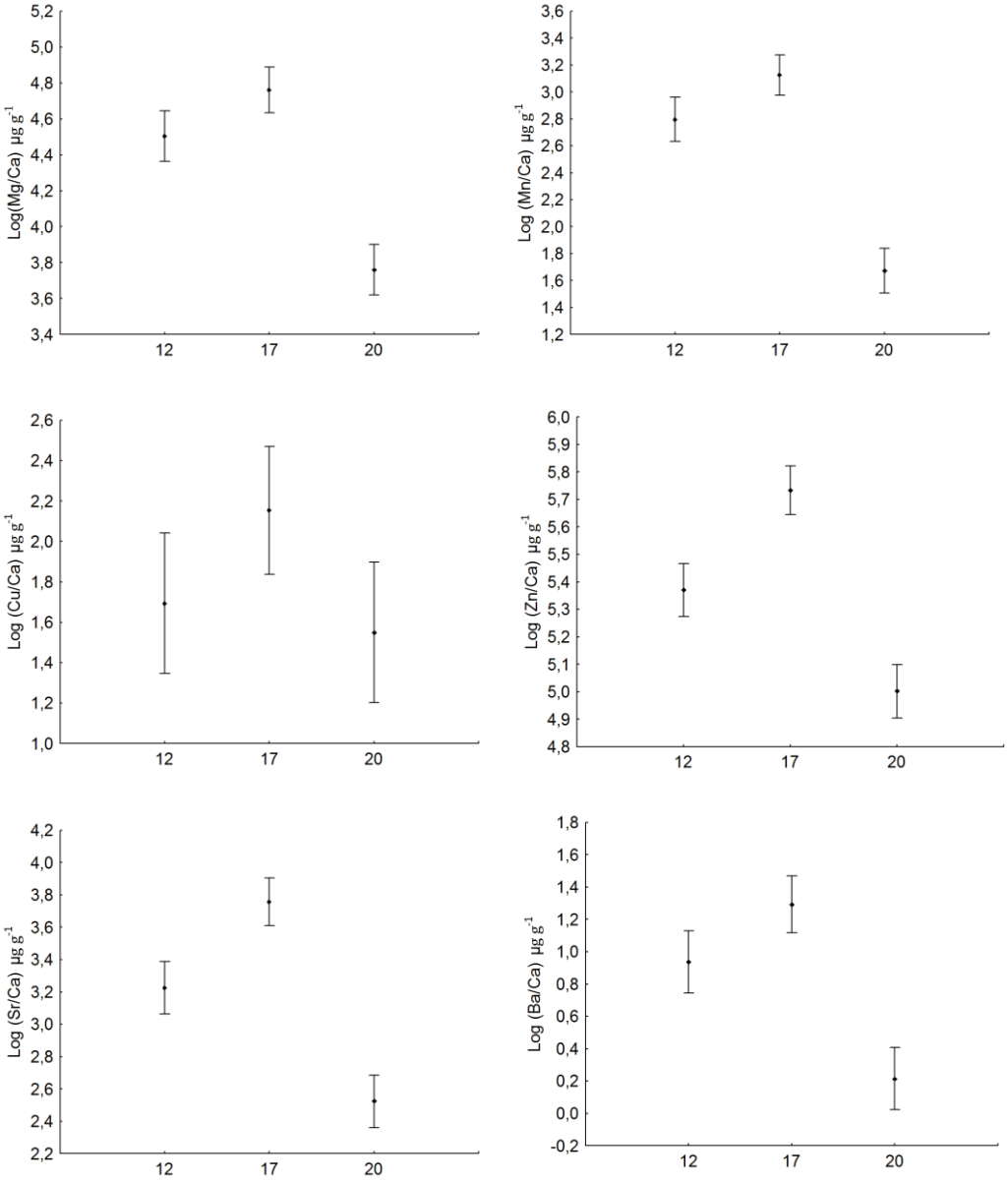

Figure 2 – Average ± s.e elemental ratios to Ca concentration on larval shells for the different temperature treatments. Points represent log transformed concentrations (µg g-1) and whiskers stand for the standard deviation.

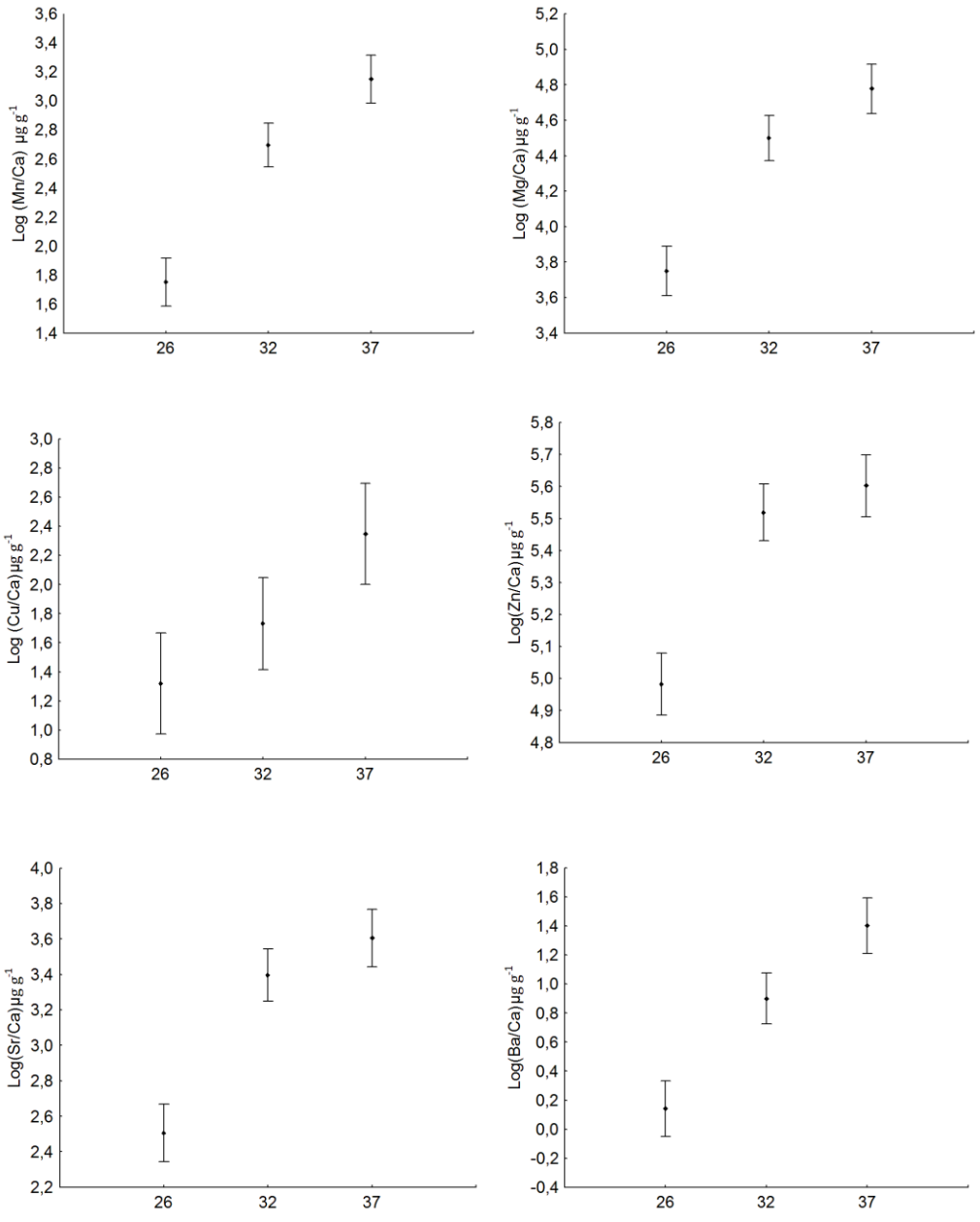

Figure 3 – Average ± s.e elemental ratios to Ca concentration on larval shells for the different salinity treatments. Points represent log transformed concentrations (µg g-1) and whiskers stand for the standard deviation.

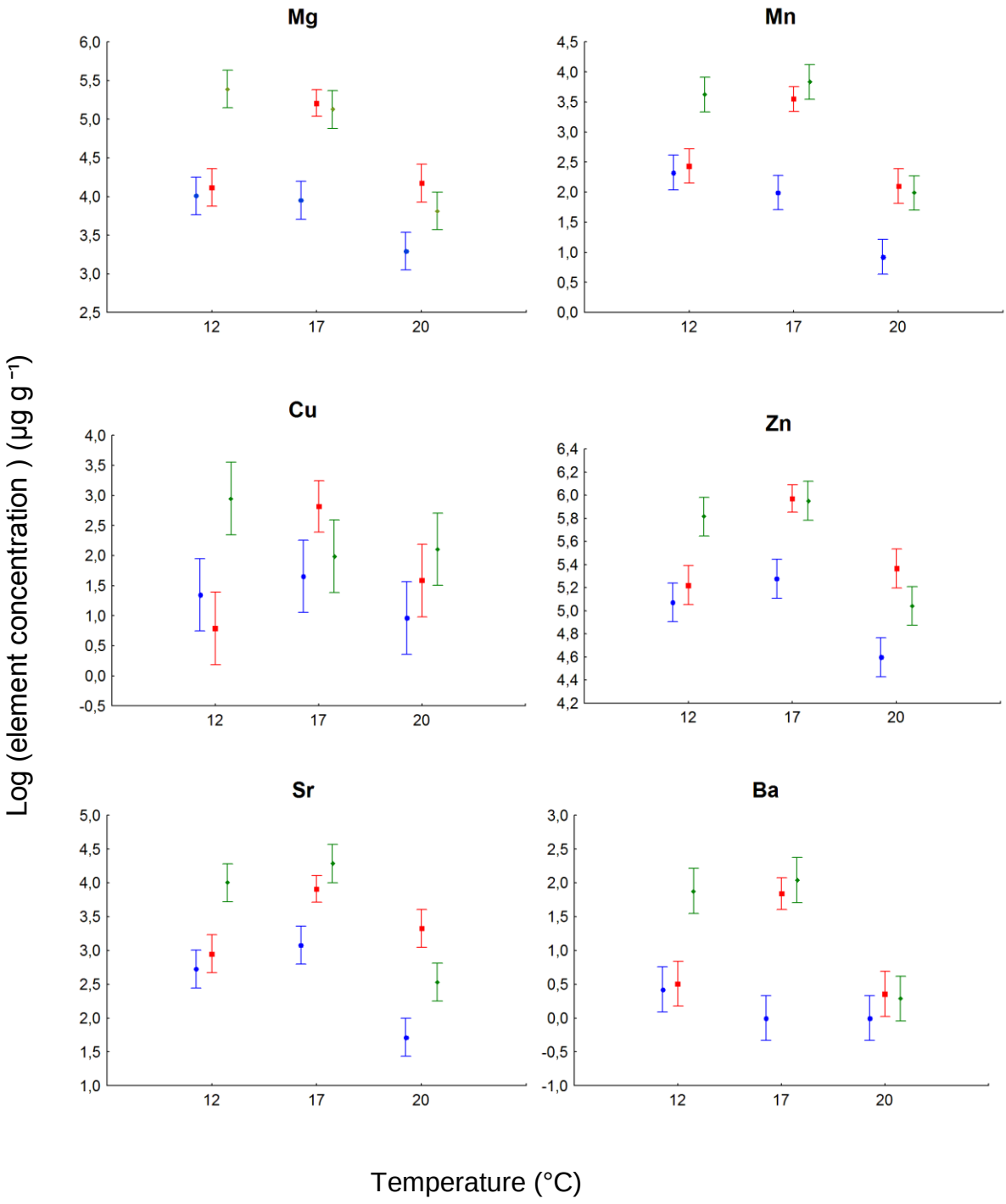

Figure 4 - Average ± s.e. elemental ratios to Ca concentration on larval shells for temperature and salinity interaction. Points represent log transformed concentrations (µg g-1) Mg (magnesium), Mn (manganese), Sr (strontium), Ba (barium), Cu (copper) and Zn (zinc) in larval shells and whiskers represent standard deviation. Blue, red and green correspondingly illustrate 26, 32 and 37 salinity treatments.

3.2 Age effect

Medium temperature and salinity treatment (17°C/32) was maintained for a total of 14 days in order to evaluate temporal variation of elements incorporation, to Ca ratio, into larval shells. T-test analysis was conducted to compare incorporations between the first and the second sampling events (6 and 14 days respectively). There were no significant differences on the incorporation ratio between sampling events of any of the analyzed elements (Mg/Ca: t=0, 5; p=0,6 ;Mn/Ca: t=0,7;p=0,5;Cu/Ca: t=1,03 ;p=0,3;Zn/Ca: t=2,23 ;p=0,08;Sr/Ca: t=0,49; p= 0,6; Ba/Ca: t=0,6;p=0,5), suggesting no ontogenetic variations on the incorporation ratio during this period.

3.3 Elemental availability effect

Water chemical concentration

Water analysis of Cu and Zn treatments illustrate a linear progression of their concentrations as a function of the tested concentration of each element.

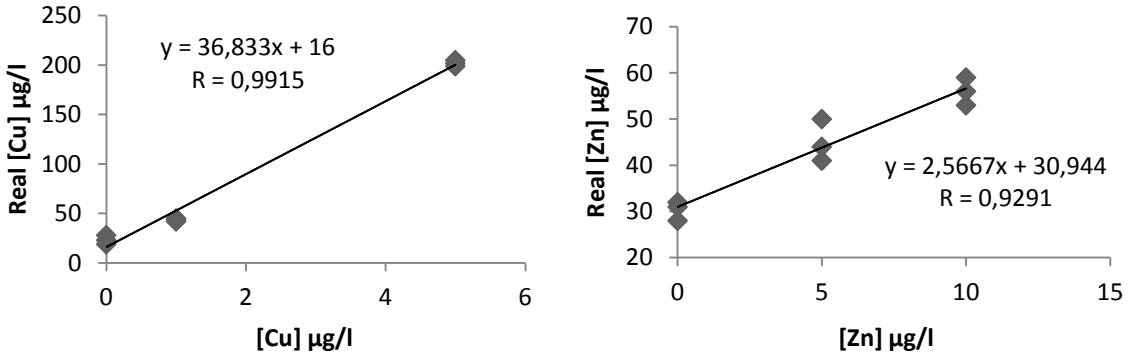

Figure 5 – Water chemical concentrations of Cu and Zn. Linear regression of theoretical water concentrations of Cu (0, 1, 5 µg/l) and Zn (0, 5, 10 µg/l) versus the real water concentrations.

Larval shells

Larvae reared in this experiment presented some individuals with morphological abnormalities by the third day of tests. Cu and Zn ratios in larval shells were quantified for different water concentrations. No statistically significant differences were found between the three levels of Cu (p>0, 05) (Table 2). On the other hand, Zn/Ca ratios showed to be significantly different according to Zn water concentration. Incorporations at the highest concentration decreases significantly (Fig. 6). Post-hoc comparisons using Tukey HSD (honestly significance difference) test indicated two significantly different groups in Zn treatment (Fig.7).

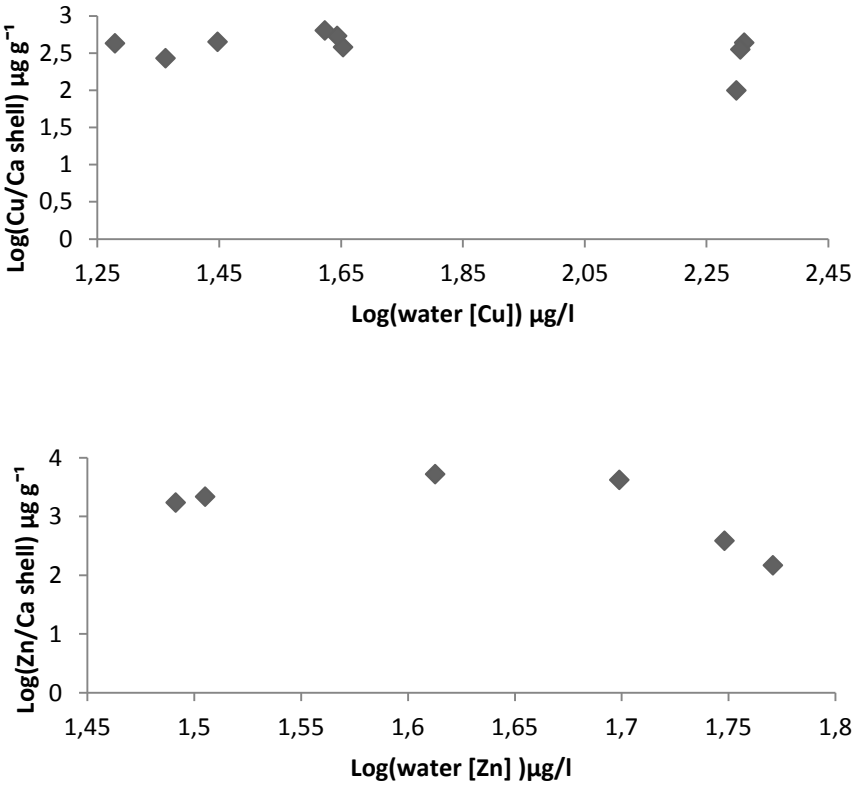

Figure 6 - Larval shells elemental ratios of Zn and Cu to calcium as a function of water concentrations (µg/l). Linear regression of logarithmic transformed data (points).

Table 2 - ANOVA results for Cu and Zn concentrations in larval shells

	Df	MS	F	P
Cu	2	0,07219	1,456	0,305091
Error	6	0,04957		
Zn	2	0,88176	27,296	0,011889
Error	3	0,03230		

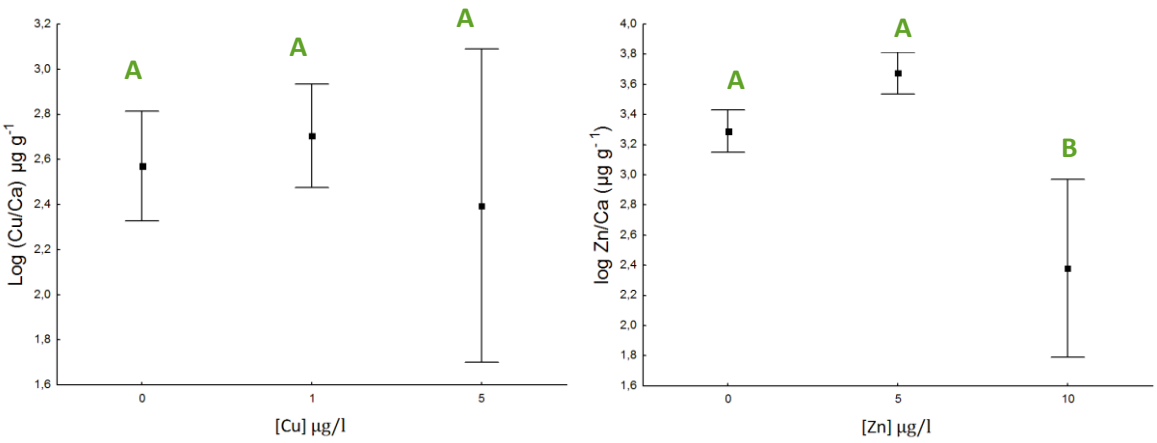

Figure 7 – Plot of mean concentrations of Cu and Zn ratios to calcium according to Cu and Zn theoretical concentration. Groups A and B represent dissimilar groups among treatments.

4. Discussion

4.1 Temperature and salinity effect

In this dissertation was investigated how temperature, salinity, elemental availability and age can influence variability in geochemical signatures in larval mussel shells. Firstly, the effect of temperature, salinity and the interaction of both factors were analyzed. It was found that the uptake of all the studied elements (barium, copper, magnesium, manganese, strontium and zinc) was affected by temperature and salinity and also found an interaction between both factors for most of the elements. The incorporation of Cu seemed to be the least affected by temperature, salinity and their interaction, while the uptake of Zn and Mn was not significant when considering factors´ interaction.

As expected, elemental incorporations increased with increasing salinity due to a higher availability of elements in the water (Figure 3). But when considering temperature, we see the highest elemental incorporation at 17°C (Figure 2) and the lowest incorporation at lower and higher temperature levels. These results may suggest the influence of temperature in element´s availability in the water allowing different concentrations of these at different temperature levels. This is also supported by the interaction of salinity and temperature where it is seen a maximum incorporation for each element at different temperature-salinity treatment pairs (Figure 4). On the other hand, although higher temperatures, in general, increase growth and reduce pelagic larval duration (Ruiz *et al.* 2008) also show detrimental effects on physiological status and survival over certain thresholds (Sánchez-Lazo and Martínez-Pita 2012). Although temperatures around 20C have been reported as optimum for mussel larval development (Ruiz *et al.* 2008; Sánchez-Lazo and Martínez-Pita, 2012), a decrease in survival was also observed for temperatures over 24°C (Sánchez-Lazo and Martínez-Pita 2012), in agreement with our results for the 25°C treatment where 100% mortality was observed.

The decrease of elemental incorporation in larval shells at higher temperatures may correspond to a physiological response to stress where elemental incorporation may occur at a slower rate as larvae metabolism is affected. On the other hand, the

decrease incorporations in larval shells at higher temperatures might not be directly related with survival or physiological stress but with other mechanisms directly related with shell segregation, with an optimum for element incorporation around 17°C.

Magnesium uptake

Results show that magnesium uptake in larval shells was significantly influenced by tested condition. Mg/Ca show an interaction between temperature and salinity, although incorporation increases with salinity there is a general lower incorporation at higher temperatures (Figure 4). Studies of bivalve aragonite and calcite larval shells haven't been consistent about the relation of Mg with temperature. Early studies with *M. edulis* (Dodd, 1965; Vander Putten, 2000) found that Mg concentrations in calcite shells increased with temperature. Lorens and Bender (1980) though, had already theorized that, regarding Mg in *Mytilus edulis,* the first should be regulated by mantle cells during calcite formation but not during aragonite formation. Although, more recent results from Strasser 2008 support a weak influence of temperature and salinity in Mg levels in both aragonite and calcite shells of *Mya arenaria,* the obtained results in this thesis can support temperature influence on Mg incorporation in mussel aragonite shells.

Manganese and Zinc uptake

Salinity and temperature seemed to contribute to Mn and Zn concentration variation in the larval shells but no interaction was observed between both factors (Figure2 and 3; Table 1) Elsdon and Gillanders (2003) that obtained no significant effects of salinity or temperature in Mn concentrations in laboratory reared fish otoliths justified these results by concluding that biological processes should be involved in Mn mediation in those structures. Still, Mn has been used successfully for other elemental tagging studies (e.g. Thorrold *et al.* 2001; Becker *et al.*2007).

Barium and Strontium uptake

Significant effect of temperature and salinity was found in both Sr/Ca and Ba/Ca concentrations in mussel larval shells. Incorporations followed a decreasing trend as a function of temperature and an increasing one with salinity level (Figure 2 and 3). Concerning Ba, these results agree with previous studies. Zacherl (2002) found a negative correlation between temperature and this element´s incorporation into aragonite statoliths of *Kalletia kalletii*, as well as Bath *et al.* 2000 in fish otoliths, Zacherl *et al.* 2003b in statoliths and Strasser *et al.* 2008 in mollusc shells. With exception of the last, the above referred studies have also confirmed a positive relation of Ba/Ca in calcified structures with Ba/Ca in the seawater (Zumholz, 2007), being also in agreement with obtained results in this work.

Regarding Sr, studies have defended that Sr incorporation in aragonite shells of molluscs are dependable on precipitation rate (Takesue and van Geen, 2004), while specifically in bivalve shells Sr/Ca ratios are mainly regulated by biological processes and not by thermodynamics (Gilikin *et al.* 2005).

Copper uptake

Copper was the element which showed to be the least affected by the tested conditions. No significant differences of Cu/Ca ratios were detected in larval shells between the different tested temperature and salinity levels. One interpretation for this case is that Cu incorporation might be highly regulated by the organisms during shell formation due to its high toxicity level (Beiras and Albentosa, 2004; Nadella *et al.*2009).

4.2 Age effect

Mussel larvae reared to evaluate age effect on elemental incorporations showed no significant differences in elemental ratios between the two development stages (6 and 14 days). This leads to the conclusion that during the first 14 days of larval development of *Mytilus galloprovincialis* there were no significant ontogenetic variations of water elemental incorporations and, therefore, geochemical signature remained stable during this time. This way, shell chemical signature obtained has a higher probability of being a good representative of water chemical composition.

These results exclude the possible influence of time in geochemical signature variation during the tested period, and confirm elemental fingerprinting as a reliable technique to represent original water masses as most of *in situ* studies incubate larvae for a period of 4-7 days.

On a bigger scale, considering connectivity between populations, elemental fingerprinting gains importance if this method can distinguish different water masses independently of the development stage of the organism.

4.3 Elemental availability effect

Copper and zinc concentrations in larval shells, according to current experimental results, proved to be the least affected by temperature and salinity. Aiming to understand if the incorporation of these elements in aragonite shells varies with another environmental factor, elemental availability was tested and mussel larvae were submitted to different levels of Cu and Zn.

Cu/Ca ratios showed no significant variance according to the level of Cu/Ca in the environmental water. Concerning Zn/Ca ratios, at higher water concentrations it is seen a significant decrease of incorporation in larval shells. Results may suggest that the uptake of these elements it is not controlled by environmental factors but intrinsically in the organism.

Metal toxicity in marine organisms has been reported in several studies and more recently in bivalves by Beiras & Albentosa (2004) and Nadella *et al.* (2009). According to the first study, among the tested metal range (including Zn, Co, Hg, Pb and Cd) Cu was the second most toxic element and the one with the highest risk factor for two species, including *Mytilus galloprovincialis*. Zn followed as the most toxic metal after Cu. In the second experiment, copper and Zn were considered the most toxic metals (compared with Zn, Ni e Cd) on *Mytilus trossolus* when analysed embryo development.

Biological factors regulating Cu and Zn uptake in aragonite shells may represent a physiological response to stress conditions.

5. Conclusions

Interest in elemental fingerprinting as a tool to detect larval dispersal and connectivity patterns among populations has been growing due to its successful use in the latter years. The present work originated results that contribute to the comprehension of how trace elements are incorporated in the larval shells of *Mytilus galloprovincialis* being influenced by temperature, salinity, elemental composition of the environment and age of the organisms.

A significant influence of temperature and salinity, for most of the tested elements, and a non significant influence from elemental availability and age are verified. Salinity significantly contributes to a higher availability of elements in the water and in the shells and its interaction with temperature is also significant for most of the elements. Moreover the effect temperature has in larval physiological processes regulation such as growth rate, planktonik periods, shell secretion and mortality is perhaps the main explanation for such incorporations according to temperature levels. Nonetheless, regarding age effect, results validate the importance of elemental fingerprinting in origin determination of larvae, in studies with a similar time range, as it is supported a consistency in chemical signature according to larval development stage. The different availability levels of Cu and Zn in water proved to be not significant in chemical signatures formation in larval shells. These were also the less variable elements found in shells with the effect of temperature and salinity which may reflect that its adherence was strongly regulated by other factors. Biological factors are here suggested as it is found in literature studies demonstrating these elements toxicity in mussel embryos.

More knowledge of the different factors conditioning elements uptake in carbonate structures is required for a more accurate interpretation and use of geochemical signatures in natal habitat identification.

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