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Unusual Crystal Formation in Organisms - Exceptions that Confirm Biomineralization Rules

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The universe is full of things that care of themselves.
Atoms spontanously assemble into molecules.
Mountains steadily rise out of the sea.
Stars continously cluster into galaxies.
Yet nowhere is self-organization more impressive than in crystals.
(Fagan, J.F. & Ward, M.D. 1992)

1. Introduction

Biomineralization is a complex combination of biochemical and physiological processes, depending on endogenous activity of an organism and the exogenous environmental influence. In marine organisms (bacteria, calcareous algae, molluscs, crustaceans, fish and mammals) biomineralization starts at early stages of development and continues during the entire life. These processes take place in different tissues, shells and skeletons, and are manifested in calcification, decalcification and in polymorph transitions of calcium carbonate in particular shell or skeleton layers (Lowenstam, 1981; McConnaughey, 1989). Biomineralization processes depend on a number of conditions of which the main ones, that influence the type and structure of the biomineral, are the following: supersaturation of the medium with calcium and other ions, the nature of the critical nucleus, characteristics of the organic matrix, promotors and inhibitors of phase transitions, environmental temperature, salinity concentration and pH ranges (Wilbur & Saleuddin, 1983; Addadi & Weiner, 1992; Mann, 1991, 2001; Bauerlein, 2004; Veis, 2008). The research related to the biomineralization processes is interdisciplinary and the main aim of the present review is to express and interpret results obtained by different methods and approaches. In this review we address three major topics.

First, we turn back to the period before the modern biomineralization theory, which presents several innovative hypotheses and proposes mechanisms fundamental in understanding crystal formation in the living organisms. These advanced ideas were randomly or deliberately neglected and minimized in recent biomineralization literature, and they deserve attention.

Next, we take up the role of different research methodologies that directly promote and address some of the pressing profiles in explanation or acceptance of unusual-usual biomineralization processes.

Third, we turn to areas that require a closer attention and a more intense effort in presenting the recent synthesis and generalization of the mineralization of the organisms. One of these is well confirmed and accepted knowledge and techniques underlying the environmentally induced biomineralization and the influence of different extrinsic conditions on crystal formation in adult invertebrates. The area is apparently much intricate and faces an increasing difficulty in well-trained understanding of the biomineralization related to starting-initial biomineralization processes, the first crystals formation and growth during early embryogenesis up to the juvenile and adult organisms. Major achievements and examples of fundamental results in recent years will be presented. It depends of the personal scientific judgement whether the distinction between the basic mechanisms of the initial crystal formation and evolutionary role of these processes on the general aspects of the biomineralization represents a real difference.

2. Usual-unusual biomineralization

Biomineralization is recognized like a separate scientific discipline comprising and interacting within natural and technical scientific fields in the late sixties - early seventies of the previous century. According to Bauerlein (2000), the modern biomineralization era began by Lowenstam's synthesized review entitled "Minerals formed by Organisms" (Lowenstam, 1981), and continued in numerous books, book serials and conference volumes as well as extraordinary reviews up to recent. The biomineralization research could be presented in few graduated steps as:
-    structural and morphological recognition –differentiation - at the cell, tissue or organism level,
-    physiological function determination - propositions on the tissue, organism, species or family level,
-    possible mechanisms used in appropriate synthesis of different species,
-    application/correcting deviations and production of new materials with unexpected properties.

The level of reliable recognition and evaluation of contribution of biomineralization to the general knowledge in life sciences is dependent on the methodology applied and exploited in the research as well as on spatial and temporal approach in explanation of unusual to usual biomineralization.

Definition of UNUSUAL: not usual: uncommon, rare (Webster Dictionary). Related to UNUSUAL - Synonyms: curious, extraordinary, funny, odd, offbeat, out-of-the-way, peculiar, queer, rare, singular, strange, unaccustomed, uncommon, uncustomary, unique, weird

Related Words: bizarre, eccentric, far-out, kooky (also kookie), oddball, outlandish, outré, way-out; aberrant, abnormal, atypical, exceptional, irregular; newsworthy, notable, noteworthy, noticeable, particular, remarkable, special

Increasing interests of diverse groups of scientists for inclusion in the biomineralization research and application of new instrumental methods resulted in exponential growth of the general knowledge and in numerous crucial studies and research that enormously broaden understanding in the field. Remarkable literature data could explain the difference and

gradation in our understanding from *unusual* to *usual* biomineralization. Furthermore, only several basic studies and experimental data that improve step by step growing importance in the biomineralization field could be enumerated. Odum (1951) described strontium content of sea water celestite radiolarian and strontianite snail shells. Vinogradov (1953) presented chemical series of skeletal formation in marine organisms. Beside expected carbonate polymorphs calcite, aragonite and vaterite he emphasized importance of $CaCO_3$ crystallohydrates (amorphous $CaCO_3$ in further literature - ACC), and its role in biomineral formation. Amorphous $CaCO_3$ is found frequently in rather dispersed state in young Mollusca and Crustacea and later it changes into calcite or aragonite. He also observed that formation of aragonite in marine organisms is stimulated by temperature, pH and "the presence of a crystalline seed of isomorphic matter," especially concentration of magnesium, phosphate and carbonate salts. Both inspired findings of Vinogradov (1953) were confirmed in further basic biomineralization research and interpreted as crucial for starting crystals formation in the organisms, ACC as a template and nucleation centre as promotors of the biomineralization. Lowenstam (1954) compared environmental conditions on the modification of carbonate secretion in several marine invertebrates. Stenzel finding of aragonite in the resilium of oysters (1962), and different calcite and aragonite regions in the adult oysters *Crassostrea virginica* (1963), deserves attention and it was "unusual" only during a short period. In the next years, using X-ray diffraction, Stenzel (1964) found that the larval shell of the common North American oyster, *Crassostrea virginica*, in a strait-hinge stage to the latest umbo stage (before metamorphosis), was composed of mineral aragonite and that no calcite was indicated. He also stated that it was most likely that all, or nearly all, Bivalvia had aragonitic larval shells because most of them had aragonitic adult shells. According to Stenzel one might assume that the larvae of oysters conform to the general pattern in the Bivalvia. Larval oyster shells are aragonitic because their ancestors had aragonitic shells, and there was, and is, no adaptive need for the free-swimming larvae to have shells of a composition other than aragonitic. Many further morphological studies and research related to bivalve larval shells are based on and support the Stenzel findings and hypothesis (Taylor et al., 1969; Carriker & Palmer, 1979; Carriker et al., 1980; Waller, 1981; Watabe, 1988). Hare (1963) analyzed amino acids in the proteins from aragonite and calcite layers in the shells of the mussels *Mytilus californianus* and expressed importance of the organic matrix in the biomineralization at organisms' level and contributed to general interpretation of the invertebrate biomineralization. The molluscs produce a shell composed mostly of biogenic calcium carbonate, $CaCO_3$. The most common precipitating biominerals are either calcite or aragonite. Vaterite, the third polymorph of calcium carbonate, was registered in the freshwater gastropod *Viviparus intertextus* and in newly formed layers of bivalve shells (Wilbur, 1964; Taylor et al., 1969; Watabe, 1988). Also, some authors showed that, beside calcium carbonates, other minerals could be incorporated in the structure of mollusc shells. In the larval shell of the pearl oyster *Pinctada martensi*, Watabe (1988) observed mineral dahlite-carbonate hydroxylapatite, $Ca_5(PO_4,CO_3)_3(OH)$. Barite, $BaSO_4$, which is usually mineralised only by two protozoan organisms, was recorded in the shell of field-collected freshwater Asian bivalve *Corbicula fluminea* (Fritz et al., 1990). In laboratory experiments the same species co-precipitated barite crystals with aragonite in the inner shell surface (Fritz et al., 1992). Carriker et al., (1980 and 1991) demonstrated a remarkable capacity of oysters to incorporate other elements in their shells. Fritz et al., (1990) suggested that minerals of elemental composition similar to that of calcium carbonate could also be mineralised by molluscs.

3. Environmentaly induced biomineralization

Many recent reports and ecological studies have correlated harmful environmental factors (lack of oxygen, toxic algae, heavy metals, organic pollutants, antifouling paints) with disease, deformation, mutagenicity or genotoxicity of biomineralization, calcification processes and mineral composition of marine organisms. The calcareous skeletons of marine organisms act as concentrators of dissolved minor polluting elements in the seawater, permitting easier analysis and more reliable measurements than those carried out directly on water or on soft tissues. The minor elements, including heavy metals, nanoparticles and organic molecules could be incorporated into the crystal structure through isomorphism, where remain stabilized in the shell and skeleton structures and thus can be used as bioindicators of the different contamination in the sea.

Marine organisms have been commonly used as bio-indicators of changes in marine environment. Bioaccumulation of metals and lipophilic organic compounds through extraction from sea water is typically confirmed by chemical analysis of soft tissue. However, the analysis of the hard structures consisting of biominerals is rarely applied, despite the possibility that it could provide additional information on toxic elements concentration, understanding of biomineralization process and environmental evaluation.

In this review unusual biominerals influenced by unexpected environmental events, and caused by anthropogenic activity in several groups of marine organisms, freshwater and subterranean snails will be presented. In order to explain how complex and sentinel mechanisms regulate environmental and physiological functions in the mollusks, a concise report of outstanding study related to the shell microgrowth patterns of bivalve *Ruditapes philippinarum* performed by Kanazawa and Sato (2008) will be presented. They found that each microgrowth increment in the shell corresponds to one lunar day and the local tidal periodicity could be easily determined from it (Figure 1). However, spawning season and spawning breaks of some specimens could be also recognized and distinguished from the shell microgrowth patterns. Also, it may be possible to estimate the age of sexual maturity and the number of spawning events that occurred per year in fossil bivalves.

3.1 Shell crystals of marine bivalve as a site of pollutant deposition
3.1.1 Pink coloured nacreous layer of the Mediterranean mussels *Mytilus galloprovincialis*

This chapter reports on the unusual crystal morphology and color found in parts of the inner nacreous layer of the Mediterranean mussels *Mytilus galloprovincialis* during intensive anoxic conditions caused by intensive phytoplancton bloom in the Northern Adriatic in the years 1989-1991.

Molluscs in general are believed to exert only a minimal vital effect over their isotopic composition (Jones, 1985), and thus their isotopic values are representative of environmental water conditions. However, calcium carbonate may not always be precipitated in equilibrium with the environment, and in such cases stable isotope analysis of the shell carbonate may be an unreliable technique for environmental reconstruction. Isotopic disequilibrium may be in part due to metabolic effects or kinetic effects that are inherent in fast-growing shells, or areas of the shell (Mitchel et al., 1994). Many molluscs use aragonite to build their shells but calcitic and those with mixed mineralogy also are abudant. In shells with mixed aragonite-calcite composition, low temperatures are favourable for deposition of calcite, while warm waters favour formation of aragonite (Schifano & Censi, 1986).
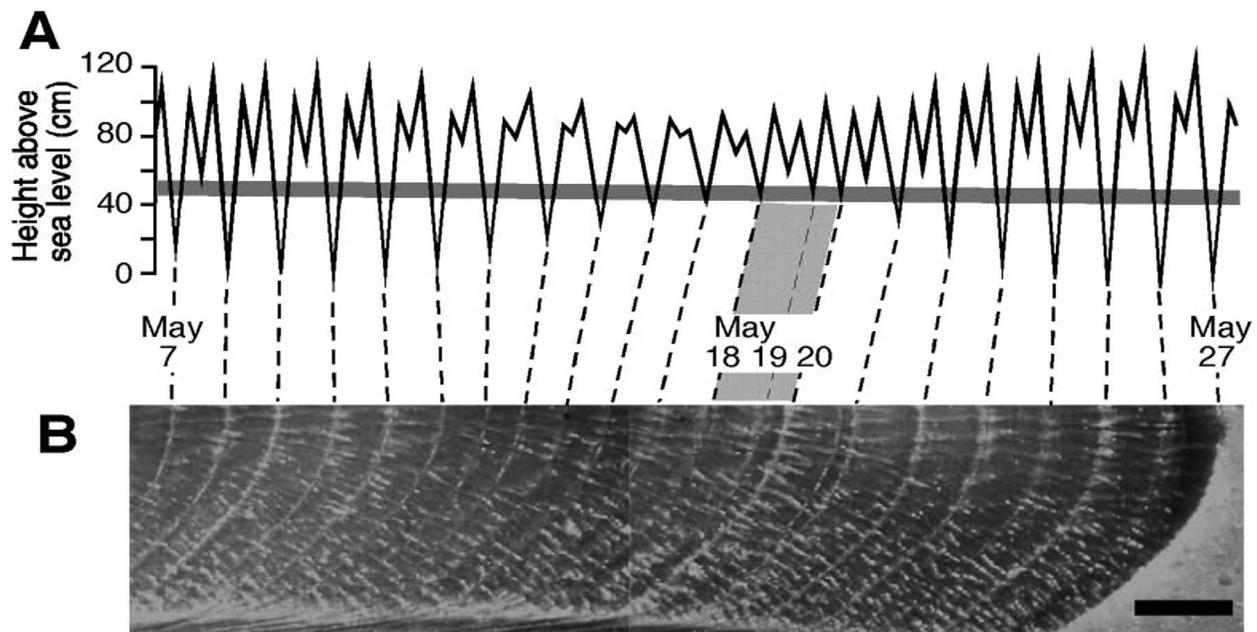
A. Local tidal periodicity, as determined using TIDE for WIN. The bold grey line indicates the hight above sea level of the sampling site. B. Shell microgrowth pattern formed from 7 to 27 May 2005 for the individual (shell length, 29 mm; age, 3) of *Ruditapes philippinarum* collected from Matsukawa-ura on 27 May 2005. Each microgrowth line is respective to immersion event in each day. The grey area indicates shell microgrowth increments in 1.5 lunar day during a neap tide, from 18 to 20 May 2005. Scale bar = 100 μm.

Fig. 1. Copy from: Kanazawa, T., & Sato, S. (2008). *Journal of Molluscan Studies*, 74, 89-95; doi:10.1093/mollus/eym049.

The impact of environmental factors such as marine anoxia in the coastal zones of the Northern Adriatic on the growth of Mediterranean mussels *Mytilus galloprovincialis* shell is indicated by changes in the growth structure of the outer part of the nacreous aragonitic layer, which was pink in colour and out of isotopic equilibrium with oxygen and carbon from the ambient seawater (Figure 2a). The inner nacreous shell layer was roughly divided into two sublayers, and according to scanning electrone microscope (SEM) analysis, the white uncoloured part of the nacreous layer consisted of flattened aragonite prisms (Figure 2b). The outer pink coloured sublayer was up to 900 μm thick and composed of irregular aragonite grains showing a massive crystal structure. These aragonite crystals are precipitated during a prolonged stress condition and deposited more rapidly than ucoloured ones. The comparison of results obtained by energy dispersive spectroscopy (EDS) and stable isotope analysis showed that the pink coloured aragonite contains more carbon than oxygen relative to the uncoloured aragonite, thus pink aragonite layer indicates anoxic conditions during its precipitation. Isotopic disequilibrium most probably resulted due to a faster precipitation of aragonite during the summer period, as well as due to a lack of oxygen and higher concentrations of isotopically light organic derived CO_2 in the anoxic environment. The influence of higher concentrations of ambiental CO_2 is also reflected in a higher C/O ratio of pink coloured aragonite as compared to the uncoloured one (Dolenec et al., 2000).
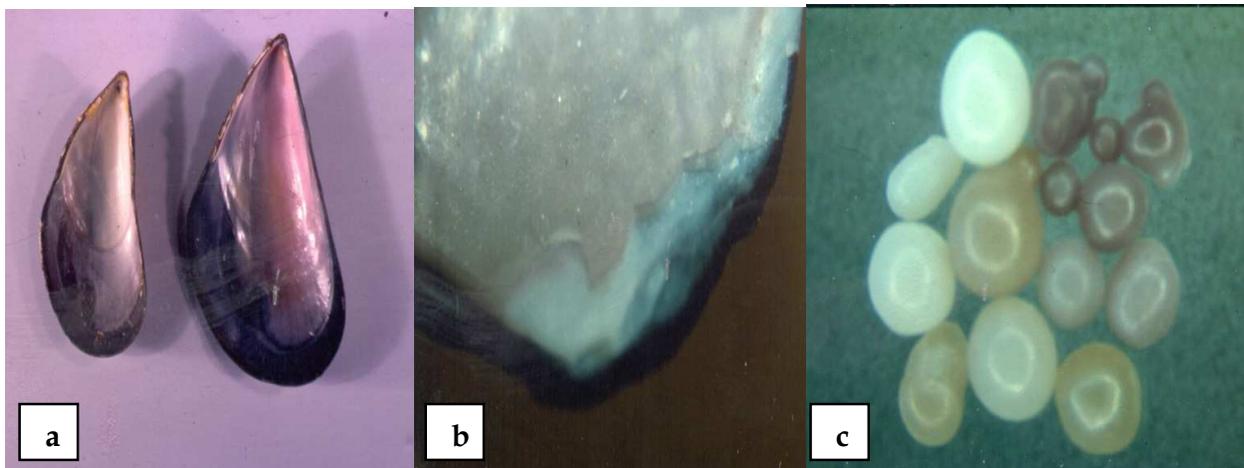
Fig. 2. a) Right valves of mussel *Mytilus galloprovincialis* - uncoloured and Pink colored sampled during anoxic conditions; b) transsection of the shell valve – upper part of pink colored nacreous layer; middle white part of the "normal" nacreous layer; down black colored outside prismatic calcite layer; c) white mussel pearls collected in "normal" environmental condition and colored pearls separated from the shells during anoxic period.

The inside pink layer of the shell was carefully extracted and powdered and analysed by X-ray diffraction. The results were compared by those for the white inside layer from the shell collected in the year that preceded the apearance of the pink shells. Diffraction patterns showed that both samples consisted of dominant aragonite fraction and some calcite crystals. As it was confirmed by SEM analysis the pink layer contained approximately 10 % of calcite. The diffraction lines of calcite in the sample of the pink layer were broadened. Further, the electron spin resonance (ESR) spectroscopy of Mn^{2+} ions that are paramagnetic substitutes for Ca^{2+} in the host calcite was also performed in order to obtain more details of the structural properties of calcium carbonate in the shell (Brečević at al., 1996). The ESR analysis confirmed that the pink layer contained a smaller fraction of calcite than the white nacreous part, and showed that the symmetry of the nearest environment of Mn^{2+} (Ca^{2+}) was lower in the pink layer than in the white. Also, the ESR spectrum of the pink layer indicated the presence of other paramagnetic ions, besides Mn^{2+}, that appeared in an unevenly dispersed clusters. On the other hand, the standard chemical analysis and X-ray spectroscopy could not find any significant difference in the composition of the examined pink and white layers from the mussel shells.

A long term monitoring of the pink mussels showed that in 1990, in locally more polluted zones the number of pink mussels ranged between 40 and 100%, while in the Mirna River estuary and some other areas under the influence of freshwater inflow, the number was much lower and ranged from 8 to 14%. The analyzed mussel samples indicated a high number of pink mussels among specimens longer than 40 mm. The smallest pink colored mussel was 22 mm in length. It is important that pink colored nacreous shell layers were ocasionally noted in some other bivalve species (e.g. *Calista chione*, *Venus verucosa, Pecten jascobaeus*), and in several mussels pink or brown pearls were found (Figure 2c). The named other bivalve species were found only at few different localities in very low number, and this result was not included in a systematic study. Southward along the shallow eastern Adriatic coast pink colored mussels have never been observed. In the research period of 1991 and 1992 a decrease in percentage of pink mussels and in their shell coloring intensity

was observed, and in 1993 pink colored mussels were not registered (Medaković et al., 1995a). The present results show a difference between the samples collected during short/long unexpected natural events – like anoxia, or by different human activities manipulating near harbors, city waste or sewage and chemical industry centers, indicating different levels of contamination or pollution (Degobbis, 1989; Hrs-Brenko et al., 1990).

Consequently, hard structure of marine organisms, i.e. biominerals should be found to provide useful data on determination of environmental status and also on estimation of pollution caused by anthropogenic influences as it was confirmed in several recent studies during whole east Adriatic coast (Hamer et al., 2008, 2010; Rončević et al., 2009, Kanduč et al., 2011).

Effects of environmental factors have to be considered in sampling strategies for monitoring programmes to prevent possible false interpretation of the results. In their research Hamer et al., (2008), examined salinity and temperature variations at investigated sites from northern to southern Adriatic coast (from Limski kanal to Dubrovnik). The results showed that mussel *Mytilus galloprovincialis* sensitively responded to subtle changes in the environmental conditions. Results of the isotopic analysis presented in this study support the hypothesis that the δ13C in mussel's shell might be used as an indicator of environmental salinity stress and the freshwater influx. The values of δ13C mussel was also reflected in lower *M.galloprovincialis* shell carbonates. Furthermore, the calculated temperatures for *M.galloprovincialis* shell growth in the investigated area range from 13.4 to 20.9 ℃ for calcite and from 16.6 to 23.1 ℃ for aragonite. According to the δ18O and δ13C values of shell layers, the investigated area could be separated into three locations: those with a bigger influence of freshwater, those with a smaller influence of freshwater and those with marine environments. Therefore such investigations might represent an additional tool for waste water management and environmental protection (Hamer et al., 2010). The results are confirmed by recent study of Kanduč et al., (2011). Oxygen and carbon isotopes were analysed for calcite and aragonite in separate shell layers, while major, minor and trace elements in the bulk shell were analysed to evaluate environmental conditions such as the temperature of carbonate deposition, freshwater influence and locations of anthropogenic pollution. The highest concentrations of manganese, barium, boron, arsenic, nickel and chromium were observed in shells from Omiš, Bačvice and Zablaće (Central Adriatic) and Sv. Ivan (South Adriatic), where chemical and heavy industries are located and where sewage is known to be discharged into coastal areas. The highest concentrations of zinc, lead and copper were measured in samples from Pula, Rijeka and Gruž, where there are also ports in addition to industry.

As a conclusion of this chapter and confirmation of Carriker et al., (1980 and 1991) findings that bivalve posses a remarkable capacity to incorporate other elements in their shells, the results of the metal content in shell samples of *Mytilus galloprovincialis* mussel will be presented. Using inductively coupled plasma atomic emission spectrometry (ICP-AES), Rončević et al., (2010) analysed bioaccumulation of metals in the shell layers of mussels sampled along eastern Adriatic coast. The efficiency of conversion of crude samples into solution by acid digestion in an open plate and in a microwave oven was examined by use of certified reference material of marine sediment and laboratory made standards of calcite and aragonite. Influence of high Ca content matrix on emission intensities of Al, Ba, Cd, Cu, Fe, Mg, Mn, Na, Ni, Pb, Sr and Zn was observed as depression of emission signal for most of the measured elements, ranging from 0.8% to 8%. Greater values were noted for Ba and Ni emission lines. Enhancement of signals was observed for Na and Mg lines. The

determination of As, Sb, Se and Sn was performed by HG/ICP-AES. A greater abundance of Sn was found in samples collected near the Al-processing industry centre. No detectable concentrations of As, Sb, and Se were found in the shell samples. Results of ICP-AES metal analysis showed that samples collected near harbours, city waste or sewage outlets, and chemical industry centres indicate a certain level of contamination. It is important that different mineralogical structures of the shell layers imply incorporation of metals with different ionic radii into a calcitic or aragonitic layer. These studies show that bivalve shell analysis provides useful data on determination of marine environment status.

3.1.2 Influence of antropogenic pollution on the crystal growth in *Ostrea edulis* oyster shell

Coastal resources are used and exploited for economic and social objectives: urbanization, industry, tourism, fishery, aquaculture, energy production and transportation. Such activities produce combined environmental impacts resulting in marine and freshwater contamination and/or pollution, loss of marine resources, endangered biodiversity, loss of public access to the coast, etc. The number of new chemicals that appear on the market is increasing each year. Most of these substances are insoluble and non-biodegradable and their effect on the environment is rarely or not understood completely. The effects and impact of compound tributyltin (TBT) released from antifouling paints on marine environment was taken as a case study.

Organotin pollution started in the mid-1960s, when tributyltin (TBT) was used in antifouling ship paints intensively. TBT is organo-metallic complex that leaches from the paint and enters the marine environment. TBT accumulates in the sediments, in particular, in areas with many ships like harbors and ports. This compound was responsible for the disruption of the endocrine system of marine mollusks, leading to the development of male sex characteristics in female marine snails and causing harmful effects on other marine organisms. The number of harmfull effects of TBT were recognized like: killing spat, causing growth deformities, causing shell thickening and layering and other malformations in oyster shells due to alterations in the calcium metabolism (Alzieu, 1991; Dyrinda, 1992). Letal and subletal effects have been found in other bivalves, mussels and cockles. One of the most singular effect of TBT is the masculinisation induced in female gastropods, termed imposex, occurs worldwide in over 150 species following exposure to low levels of TBT. Females exposed to TBT grow Accessory Sex Organs (ASO), including sperm duct, seminal vesicle, external sperm groove and penis, because TBT disrupts their immune and endocrine systems (Fent, 1996). TBT is widely distributed in the world. It has been found in phytoplankton, which lies at a base of most marine food webs, thus transfering contaminants to higher organisms, fish and also salmon held in net pens, dolphins, seals and sperm whales of the deep sea, and so enter the human food chain (Fisher & Reinfelder, 1995). It is toxic to humans.

An extremely low level of TBT in the seawater significantly affects shellfish larval growth, impairs the immune system of organisms and causes malformations in the shells of adult shellfish (Alzieu et al., 1982; Thain et al., 1989). Shell calcification anomalies caused by TBT, which consists of stacked 'chambers' filled with a gelatinous substance that gives the shell a ball-like shape, were observed in the oyster *Crassostrea gigas* sampled in the French Atlantic coast and in California (Alzieu et al., 1989; Stephenson, 1991). Similar deformation effects were seen in the shells of adult oysters *C. gigas* and European flat oyster *Ostrea edulis*

sampled at several localities along the East Adriatic coast (Medaković, 1995). In our previous research, X-ray powder diffraction (XRD) was used to study calcification and changes in the biomineralization process of the bivalvia and other invertebrates influenced by environmental changes (Medaković et al., 1997, 2003). Although mollusk shells are considered to be a powerful tool for monitoring environmental conditions, alterations in the biomineralization processes influenced by TBT have been insufficiently investigated so far. In this chapter we follow the accepted idea that the elemental composition of each polymorph can vary within the organisms, although the calcified material is produced in similar environments.

In the work of Medaković et al., (2006), X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM) equipped with X-ray energy dispersive microprobe (EDX) was used because these techniques provide qualitative and quantitative information about environmentally induced elements in oyster shell layers in a fast, sensitive and simple way. In the recent literature, numerous papers describe morphological changes in mollusk shells caused by TBT. No data are available on mineral components and minor elements incorporated in shell layers thus deformed, and this is the first result related to tin in the chambers of oyster *O. edulis.*

The shells of European flat oyster *Ostrea edulis* are composed mainly of calcite. Aragonite is present only in the small area of the inner shell layers on the imprints of the muscle attachment (Taylor et al., 1969). XRD patterns of the analysed samples gave the average mineral composition in the milled shell parts. Calcite is the dominant component in the outerprismatic shell layers of both samples (Table 1). Also, both samples indicated a small presence of halite (NaCl), which could be eliminated by additional washing in distilled water. Sample OE-RV1 contained traces of aragonite in this layer. Furthermore, SEM and XPS analyses confirmed that this shell part sample, OE-RV1, contained 0.1 to 0.3 at.% of silica, in contrast to OE-MS1, in which Si was present in amounts of 1.2 to 1.5 at.% (Table 1). It was shown that a longtime exposure of organisms to environmental conditions might result in the presence of foreign particles in the shell. Clays or other suspended particles could be 'captured' in the structure of the shells during periods of withdrawal of the mantle and fast growth (Carriker et al., 1991). In the outer prismatic layers of the oyster *O. edulis,* a small amount of silica mineral (quartz 1 to 2 mol%) was registered, and detailed studies showed that it was characteristic only for one locality in the northern Adriatic (Medaković, 1995).

The above inferences and our results indicate that the promoting mechanism of incorporation of silica in oyster shells can not only be related to the structural characteristic of the *Ostrea edulis* shell but probably even more to specific environmental factors. Factors for precipitation of carbonate polymers in the shells include a complex interaction between the organisms and environmental conditions, inorganic ions, dissolved organic materials, enzymatic activity, macromolecules, etc. Although the importance of organic tissue in the formation of shell polymorph is indispensable, only the presence of TBT in the environment induces tin in the oyster soft tissue. Moreover, accumulated tin in the digestive gland and in the gills of the oyster causes morphological changes in their shells, unusual chamber formation and suggested mechanisms of TBT action (Alzieu et al., 1986). TBT is known to inhibit oxidative phosphorylation and it has been suggested that this forms the basis of its action on the shell. Thain & Waldock (1986) confirmed in laboratory experiments that only TBT influences growth and causes shell abnormalities in oysters.

The results of the present research showed, for the first time, that the only malformed chambers of *Ostrea edulis* contained tin. The XPS spectra confirm that tin is present in sample OE-RV2 in amounts of about 0.1 at.%, and in the OE-MS2 in amounts ranging from ~ 0.1 to 0.2 at.% (Table 1). The Sn 3d XPS spectrum of the OE-MS2 sample clearly shows two characteristic Sn peaks that could be associated with the presence of tin oxide compounds. The quantitative SEM analysis confirms the results given by XPS, and shows a negligible amount of tin in these samples.

			Mineral components (M %)				Minor elements (at. %)	
Locality	Sample code	Shell layer	A	C	H	MC	Si	Sn
Stn 1	OE-MS1	Outer	-	dom	tr	-	1.2 – 1.5	-
	OE-MS2	Inner	-	min	-	dom	0.05 – 0.2	0.1 – 0.2
Stn 2	OE-RV1	Outer	tr	dom	tr	-	0.1 – 0.3	-
	OE-RV2	Inner	-	dom	-	-	0.0 – 0.1	0.1

Table 1. Mineral components and minor elements in different shell layers of the oyster *Ostrea edulis* expressed in molar percentages (M %), and in atomic percentages (at. %). A, aragonite; C, calcite; H, halite; MC, magnesium calcite; Si (silica); Sn (tin); dom, dominant component (mostly 100 %); min, minor component (about 2-3%); tr, traces (much less than 1%); - no component is present; Stn 1- shellfish farm, Stn 2 – marina, both in East Adriatic Sea, Croatia.

3.2 Benthic marine organisms and fouling communities
3.2.1 Calcareous algae
The corralinacean algae make an important contribution to sediment production and reef limestone formation (Lewin, 1962; Cabioch & Giraud 1986; James et al., 1988), producing over 50% of the calcareous material in coral reefs (Aharon, 1991). In shallow tropical waters, carbonate deposition of calcareous green and red algae can reach levels of 61% of bulk sediments (Wefer, 1980). Because of their habits and abundance, corralinacean algae play very important role in some assemblages (Peres & Picard, 1964).

Many papers have been published on diverse genera of calcareous algae, which attempt to explain still unknown mechanisms of biomineralization. Crystallization processes depend on a number of conditions, but the main ones which influence the type and structure of the biomineral are the following: supersaturation of medium with calcium ions, nature of critical nucleus, organic matrix, inhibitors of phase transitions, environmental temperature, salinity concentrations, pH range. McConnaughey (1989) stated that, regardless of the type of new forming minerals, the main conditions included: transport mechanisms, existence of semipermeable membranes for precipitation, divalent cations for removal of protons, presence of Ca^{2+} enzyme ATP-ase and passive electronuclear anion exchange. Furthermore,

some authors have pointed to the significance of environmental stress and other endogenous factors.

In this chapter we present mineral composition of nine species of red calcareous algae (Corallinaceae, Rhodophyta) collected in the Adriatic Sea in 1987 and 1988, examined by X-ray powder diffraction (counter diffractometer, monochromatized Cu$K\alpha$ radiation). In addition, a comparison between the calcareous algae from the north Adriatic (Rovinj area) and the central Adriatic (Kornati Islands) with regard to genus, species and environmental factors was undertaken (Medaković et al., 1995).

The results of this study show that all analyzed samples contained magnesium calcite, which was dominant in all but two cases, where aragonite was the main phase (Table 2). The fact that magnesium calcite is the dominant component is in agreement with statements of numerous literature data (Linck, 1930; Babička, 1936; Vinogradov, 1953; Chave, 1954; Chave & Wheeler, 1965; Walker & Moss, 1984; James at al., 1988; Borowitzka, 1989; Aharon, 1991; Pueschel et al., 1992). Most of the authors found that the magnesium content in Corallinacea ranged from 2 to 30 molar % (M %). Diffraction lines of magnesium calcite in our study were broadened and shifted toward higher Bragg angles in relation to pure calcite. Supposing that in the calcite crystal lattice only magnesium replaces calcium, it follows that the fraction of magnesium in magnesium calcite, found from diffraction line shifts, would be 18 molar % (M%), for all analyzed species at all stations and sampled seasons. One may conclude that content of magnesium in magnesium calcite is rather constant in all species analysed, although the molar fraction of magnesium calcite varies from one sample to the other (Table 2). The broadening of the diffraction lines could be explained due to small crystallite sizes. Similar line broadening was also observed in X-ray diffraction patterns of the first larval shell, prodissoconch I, of oyster, *Ostrea edulis*, containing crystalline aragonite (Medaković et al., 1989). The broadening of aragonite diffraction lines decreased with further development of larval stages, through prodissoconch II to the juvenile oyster veliconcha, before its settlement and metamorphosis to the adult oyster. In the present study the similar diffraction broadening observed in nine different algal species indicated similar crystallite size. This could indicate a similar age of algal plants sampled, but this is not possible because the sampled species of Corallinacea did not reproduce in the same seasons. In the present study all parts of a given alga were used, i.e. basal and middle parts and younger tips or "apices". King & Schramm, (1982), showed that in *Phymatolithon calcareum*, different parts of the tallus exhibit different calcification rates. The same authors observed that calcification of crustose corralines was slower than that in articulated species. Taking into consideration all these facts we may accept the hypothesis that the mineral composition of corralinacean algae varies with algal age (Vinogradov, 1953) and growth rate (Chave & Wheeler, 1965), but we cannot explain why algal species show similar crystallite sizes.

Unusual and unexplained results described in this study relate on high amount (75 to 80 M%) of mineral aragonite in the algae *Pseudolithophyllum expansum* collected at Kornati Islands in two independent sampling during 1987 and 1988. These two samples also contained magnesium calcite and a small fraction of calcite (5 to 10 M%). In other studied samples aragonite was detected in small fractions, up to 10 M%. The elemental analysis of corralinacean algae obtained by X-ray spectroscopy showed that the fraction of the metals Sr, Fe, Mn, Zn, Pb, Br, Cu and Rb was very small (15 to 2000 ppm). In most samples other expected minerals were detected in small fractions, such as sylvite (KCl, up to 2 M%), quartz (α-SiO2, up to 2 M%) and magnesite ($MgCO_3$, only in one sample, 1 M%). The results show that calcareous algae are able to deposit a mixture of magnesium calcite, calcite and aragonite,

and even dolomite but such a large molar fraction of aragonite in the alga *P. expansum*, or in any other corallinacean algae, has not been noted in recent literature (Table 2).

Algae species	Sample station	Calcareous components				Other minerlas [a]		
		Magn. calcite $(Ca,Mg)CO_3$	Aragonite $CaCO_3$	Calcite $CaCO_3$	Dolomite $CaMg(CO_3)_2$	Sylvite KCl	Quartz α-SiO_2	Magnesite $MgCO_3$
Amphiroa cryptarthrodia	1	100	-	-	-	1	-	-
	7	100	tr	-	-	tr	-	-
	8	100	-	-	-	-	-	-
Amphiroa rigida	1	100	tr	-	-	-	-	-
	7,8	99	1	-	-	-	-	-
Jania longifurca	1	98	2	-	-	-	1	-
	7	100	tr	-	-	1	-	-
	8	99	-	-	-	1	-	-
	7,8	100	tr	-	-	-	-	-
Corallina officinalis	1	99	1	-	-	1	1	-
	1	99	tr	-	1	1	1	-
	7,8	100	-	-	-	-	-	-
Lithophyllum lichenoides	5	98	2	-	-	-	-	-
	7,8	98	2	-	-	-	-	-
Lithophyllum racemus	2	90	5	-	5	-	2	-
	4	100	-	-	-	-	-	-
	8	100	tr	-	-	-	-	-
Phymatolithon calcareum	5	97	3	-	-	-	-	-
Tenarea undulosa	7,8	100	-	-	-	-	-	-
	8	100	tr	-	-	-	-	-
Pseudolitho-phyllum expansum	1	97	3	-	-	2	-	-
	1	90	10	-	-	1	1	1
	3	97	3	-	-	-	-	-
	6	100	tr	-	-	-	-	-
	7,8	15	75	10	-	-	1	-
	7,8	15	80	5	tr	-	1	-

[a] In relation to: magnesium calcite + aragonite + calcite + dolomite = 100 %

Table 2. Phase composition (molar fraction, %) of calcareous algae from the Adriatic Sea. (*tr* trace component; - no data).

The other unusual biomineral, dolomite, was registered as a minor component (from traces up to 5 molar %) in calcareous algae *Coralina*, *Lithophyllum* and *Pseudolithophyllum sp*. (Table 2). The appearance of mineral dolomite in different living organisms in recent literature is well presented; however, all authors could not agree with the theory of the dolomite formation (Deelman, 2008). Detailed explanation of possible dolomite formation in the living organisms will be presented in the last chapter on unusual biomineralization in the freshwater and subterranean snails.

The hypotheses that algal anatomy and various organic constituents of the cell modify and regulate the effects of CO_2 (or HCO_3^-) on calcium carbonate nucleation and crystal growth (Borowitzka, 1989), and that under certain conditions the same organism can form different minerals from the same tissue (McConnaughey, 1989), confirm our statement that complexity of microclimatic and oceanographic characteristics may influence the diversity of algal mineral composition and that environmental effects were imprinted in the skeletal composition of the Corallinacea (Medaković et al., 1995). Due to numerous factors which could potentially influence biomineralization in the calcareous algae, more algal species should be monitored on a longer time scale with a higher sampling frequency in order to asses the influence of the environment on the mineral composition and phase fractions in the calcareous algae.

3.2.2 Long term influence on crystals growth of fouling communities or how marine calcareous organisms protect and preserve antique bronze Sculpture of Apoxyomenos during 25 centuries

Benthic organisms that colonize and attach themselves to the natural or artificial substrates in the sea are often called fouling organisms. The attachments differ in style and range in permanence from temporarily simple or multiple muscular attachments (e.g., limpets, octopus), or holdfasts by releasable byssus threads (mussels), by organic glues (some barnacle species), up to permanent whole life cementation (oysters), or boring by chemical or mechanical means (bivalve *Lithophaga sp.*). The adhesion between the organisms and surfaces is characterized by the extreme complexity and depends on numerous physical and biological features. One of the most important processes includes the interaction between the attached surface and biologically formed minerals. The factors that control these properties are, however, poorly understood and represent a crucial problem in understanding the biomineralization mechanisms of marine organisms. All types of fouling organisms are able to attach themselves to various surfaces, to erode and degrade the surfaces, and in the same time to incorporate some unusual ions from the attached surface and environment in their calcareous structure.

Chemical and physical properties of the sea water cause a severe corrosion of any submerged material. A complex combination of living creature activities named fouling organisms (communities) may erode, degrade and deteriorate natural or artificial substrates. The interaction of specific artificial surface with mineral composition of several groups of marine fouling organisms attached during long term period to the bronze antic sculpture of Apoxyomenos were studied by X-ray diffraction (XRD) and elemental analysis (ICP-AES). Whole empty bivalve shells, tubes of the serpulid polyhaete worms, calcareous algae and encrusted calcareous structures contained small parts of several groups of different marine organisms, were collected from the inside and outside surfaces of the antique bronze sculpture of Apoxyomenos found at the depth of 45 m on the Adriatic sea bottom close to Lošinj Island (Croatia) in the 1999.

Two kinds of effects and active interaction between fouling calcareous organisms and the artificial surface of the bronze structure were observed. The parts of the statue directly immersed in the sediment showed much more serious damages and decomposition of the material surfaces than external parts covered by mixture of fouling organisms, communities and hard calcareous structures. The results of X-ray diffraction showed that calcite and/or aragonite were dominant components in all analyzed samples. In some shell parts and calcareous structures both calcite and magnesium calcite were found. Small amount of

mineral feldspar was registered in the shells of boring bivalve *Rocellaria dubia* and in the tube of serpulid polychaete worm *Hydroides elegans*. However, beside other components, mineral quartz in traces was found in several parts of the undetermined bivalve shells, in calcareous structure and calcareous algae *Pseudolithophyllum expansum* (Table 3, Figure 3).

O	Sample code	Sample description	Mineral components in molar fraction			
			Calcite	Aragonite	Feldspar	Quartz
1	1As1	Shells from the sediment	traces	dom	-	-
2	1As2		-	dom	minor	-
3	1As3		traces	dom	traces	-
4	1As4	*Hydroides elegans*	dom 0.6 +Mg calcite	0.4	minor	traces
5	1As5	*Rocellaria dubia* +Mixture	dom 0.6	0.4	-	-
6	1As6	Shell parts	0.3 + Mg calcite	0.7	-	traces
7	1As7	Calcareous structure	dom +Mg calcite	minor	-	traces
8	1As9	Calcareous algae	dom 0.7	0.3	-	traces

Table 3. Mineral components in the analyzed samples. Calcite ($CaCO_3$); Magnesium calcite ($Mg_xCa_{1-x}CO_3$); Aragonite ($CaCO_3$); Feldspars (e.g. albite $NaAlSi_3O_8$ and microcline $KAlSi_3O_8$); Quartz (α-SiO_2); dom = dominant component; minor = minor component; tr = component in traces.

X-ray diffraction patterns showed that aragonite was the dominant component in the analyzed bivalve shells (samples 1As1, 1As2, and 1As3). Beside aragonite, the shells of *Abra alba* and *Rocellaria dubia* contained traces of calcite (less than 1 molar %, 1M %). Mineral feldspar (e.g. albite, $NaAlSi_3O_8$, and microcline, $KAlSi_3O_8$), as a minor component (~ 3 M%), was found in the shell of *Divaricella divaricata* and in *R. dubia* was present only in traces. The second group of samples (1As4 to 1As9) was characterized as mixtures of calcite, magnesium calcite and aragonite, with traces of quartz except for the sample 1As5 (Table 3). Moreover, mineral composition and phase fraction of thus attached bivalve shells, polyhaete tubes and calcareous algae have been changed and differ from the same organisms living on the natural surfaces (Table 4). Inductively coupled plasma atomic emission spectroscopy analysis showed all samples to have a high metal content, with the specific metals and their

concentrations being consistent with the sculpture as the source rather than normal seawater. No metal-based crystalline phases were found by powder X-ray diffraction apart from the presence of iron and copper hydroxides in one sample that showed a distinctly green coloration. Accumulation of non-native metals in the calcareous structures of these organisms and the unusual phase compositions of the latter suggest metal-induced changes to the biological pathways controlling the transport of calcium and magnesium and incorporation of heavy metals in growth sites of inorganic matrix (Lyons et al., 2009).
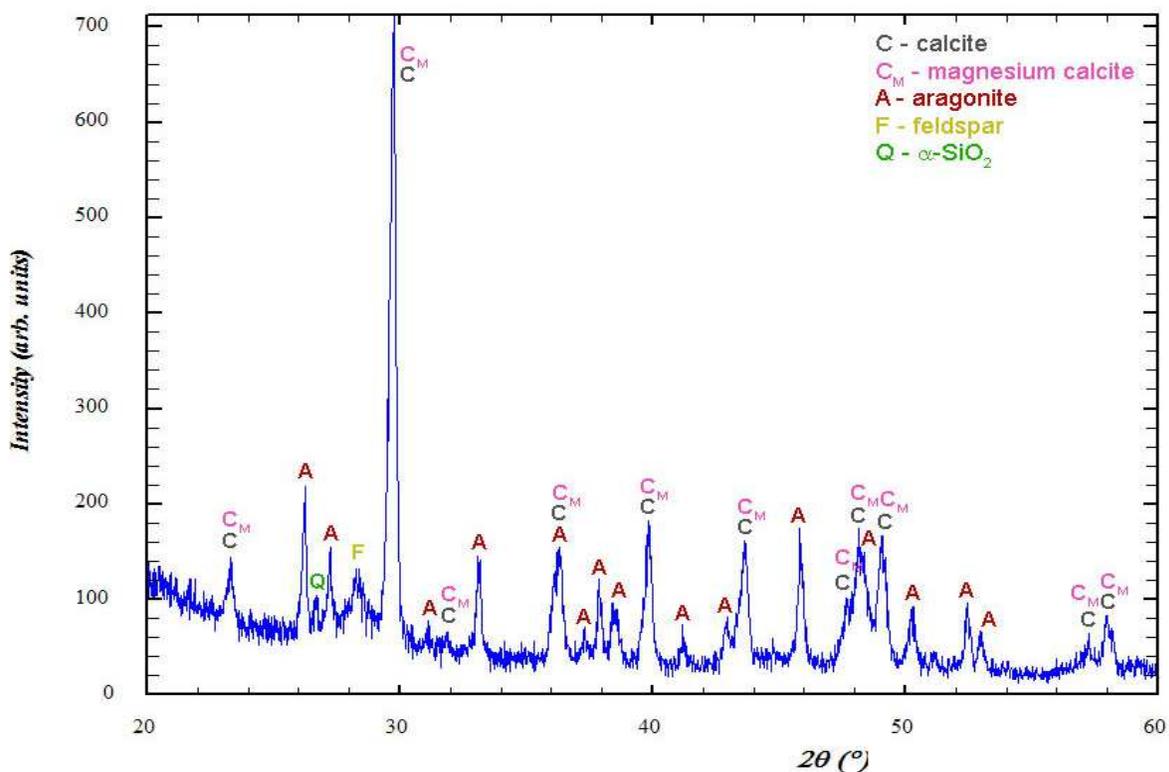
Fig. 3. Characteristic part of X-ray diffraction pattern of the tubes of the serpulid polyhaete worms *Hydroides elegans* (sample 1As4). Calcite (C) and magnesium calcite (C_M) are dominant (~ 60 M %), aragonite (A) is present in an amount of ~ 40 M %, feldspar is a minor phase (several M %), while quartz α-SiO_2 (Q) is present in traces.

The biomineral coating on the exposed part of the statue had accumulated over the centuries, reaching a thickness of 30 to 50 mm. The investigated samples removed from the outermost layer, indicated the gradual transport of metals from the statue surface to the most recently formed biomineral layer. The specific metal concentrations found in the minerals were higher than could be accounted for the bioaccumulation from seawater and were consistent with the composition of the ancient bronze, which was based on copper-tin alloys with lead and zinc added to improve the casting characteristics and durability of the sculptures. Evaluation of the mineral layers and fossilized organisms on the statue enable some presumptions and consideration that underwater fouling organisms and communities interacted with the statue as well as the effect of certain mineral deposits on the bronze sculpture slowing down its deterioration (Medaković, 2009).

	Attachment surface	Calcite (molar fraction)	Aragonite (molar fraction)
Hydroides elegans	natural	0.99	0.01
	bronze (sample 1As4)	0.60 (C+C_M)	0.40
Rocellaria dubia	natural	0.01	0.99
	bronze (sample 1As5)	0.60 (C+C_M)	0.40
Calcareous algae	natural-Istria	0.99 (C+C_M)	0.01
	natural-Kornati	0.10 (C) + 0.15 (C_M)	0.75
	bronze (sample 1As9)	0.70 (C+C_M)	0.3

Table 4. Comparison of phase analysis results of samples taken from different attachment surfaces. The tubes of the serpulid polychaete worm *H. elegans* attached to the outer sculpture surface (sample 1As4) contain 60 M% of C+C_M and 40 M% of aragonite, while those found on the natural surface have 99 M% of calcite and 1 M% of aragonite. The shells of boring bivalves *Rocellaria dubia* sampled from natural surface contain 99 M% of A and 1 M% of calcite. That sample also contains traces of feldspar. Empty siphons of *R.dubia*, mixed with other calcareous structures contain calcite as the dominant phase, while the amount of A is 40 M% (sample 1As5). Similarly, the amounts of calcite and A in calcareous algae attached on the natural surfaces in the Adriatic Sea, Istrian region: 99 M% of C+C_M, and 1 M% of A; Kornati National park region: 10 M% of C, 15 M% of C_M, 75 M% of A; bronze sculpture surface: 70 M% of C+C_M, 30 M% of A (Medaković et al., 1995).

Studies of long-term biofouled manmade structure are limited; the finding an ancient sculpture immersed for two millennia in the sea provided a unique opportunity to test the long-term impact of a specific artificial substrate on biomineralizing organisms and the effects of biocorrosion (Medaković, 2009). The results showed that there was some metal sequestration by the marine organisms that were attached to the statue although this amount of metal, being significant to alter metabolic processes in the organisms, was relatively insignificant in view of the materials perspective. This give us a hint that we may be able to mitigate the negative effects of corrosion by using biogenic materials as passivating layers on metal surfaces.

While a lot more work has to be done on this topic, it has become an exciting area of research for the future to direct and controlled growth of these passivating layers directly on the metal surfaces, particulary by mimicking similar biological pathways that mineralizing organisms use. The advantage of growing these biolayers based on anchored enzymes, for example, is that the mechanical properties may be superior in many cases to those of the layers that can presently be deposited by physical or chemical deposition. Such efforts at reducing corrosion are also applicable to other surroundings such as land or subterranean areas, and not just marine environments. This study underlines the importance of long-term investigations when attempting to understand benthic community succession. Long-term studies allow detection of the effects of rare events and slow acting processes, revealing subtle but consistent trends and environmental changes.

3.3 Freshwater and subterranean snails

The present research is based on mineralogical and crystallographic characteristics of the gastropod shells that sometimes beside other taxonomical character could help in differentiating similar or overlapping species, or reflect specific environmental conditions of the locality. X-ray powder diffraction (XRD) was used to study the mineral composition and phase fractions of the shells of freshwater snails *Belgrandiella fontinalis* (F. Schmidt 1847) and *B. kuesteri* (Boeters 1970) collected from three springs in north-eastern Slovenia and subterranean snails *Zospeum alpestre* (Freyer 1855) and *Z. isselianum* (Pollonera 1886) sampled in four caves in Kamnik - Savinja Alps, Slovenia (Medaković et al., 1999, 2003; Slapnik & Medaković, 2007).

The gastropod shells contain an outer organic periostracum layer and an inner carbonate layer. Carbonates in the form of calcite, aragonite or vaterite could be distributed in two or more shell layers that differ in size, orientation and the way of crystals packing. The fractions of minerals depend on many factors, and are characteristics of a given family, genus, or even species. The recent research shows that crystal components in different organisms are influenced by biological conditions, and that crystal forms in specified tissues are adapted to their function. The mollusc shell morphology and mineralogy do not change gradually after organism's death, thus shells are equally important for basic biological research of the organisms and for monitoring environmental contamination (Addadi & Weiner, 1992; Albeck et al., 1993; Berman et al., 1993; Davidson et al., 1995).

Two independent measurements of as-found shells of both *Belgrandiella* species showed aragonite to be dominant in an outer inorganic layer (Table 5). Results of powdered snail shells showed the mineralogy of all shell layers. In the inner layers, aragonite was accompanied with calcite, quartz and dolomite as minor phases. The mineralogy differs according to locality and species indicating the influence of the environmental conditions on biomineralization processes in the inner inorganic layer (Table 5, Figure 4). The factors for precipitation of carbonate polymorphs in the shells include a complex interaction between organisms and environmental conditions, inorganic ions, dissolved organic materials, enzymatic activity, macromolecules, etc. Whereas the importance of organic tissue in formation of shell polymorphs is indispensable, several environmental factors that could influence different mixtures of minerals and the presence of dolomite in *Belgrandiella* shells will be described.

Species	Locality	Aragonite $CaCO_3$	Calcite $CaCO_3$	Quartz α-SiO_2	Dolomite $CaMg(CO_3)_2$	$SiCa_2$
Belgrandiella fontinalis	Stn 1	98	2	-	-	-
	Stn 2	78	2	-	20	-
Belgrandiella kuesteri	Stn 1	90	3	7	-	-
	Stn 2	80	-	-	20	-
	Stn 3	93	2	5	-	-
Zospeum alpestre	Stn 4	92	5	3	-	-
	Stn 5	60	37	3	-	-
Zospeum isselianum	Stn 6	85	10	-	-	5
	Stn 7	80	10	-	-	10

Table 5. The results of X-ray diffraction phase analysis of powdered snail shells (in molar %) for *Belgrandiella fontinalis*, *Belgrandiella kuesteri*, *Zospeum alpestre* and *Zospeum isselianum* inhabiting Kropa (Stn 1), Vidmar (Stn 2) and Markelc springs (Stn 3) and caves Kamniška jama (Stn 4), Jerohi 1 (Stn 5), Jama pod Mokrico (Stn 6) and Konečka zijalka (Stn 7). Both *Belgrandiella* species collected from Stn 2 contained 20 % of dolomite in the shells. *Zospeum isselianum* sampled at Stn 6 and Stn 7 have 5 to 10 % of unusual $SiCa_2$ phase.

Because of its crystallographic properties calcite in the shell layers provides a better temperature protection of the organism from the environment than aragonite, and for this reason most mollusks form their shells by both calcite and aragonite (Lowenstam, 1954). For example, subterranean gastropod *Zospeum alpestre* from the locality with a higher and more stable annual temperature contained in the inner shell layer 5 molar% of calcite, but the same species from a locality with a lower and oscillating annual temperature contained up to 37 molar% of calcite (Medaković et al., 1999) (Table 5). The measured hydrographic parameters at Stn 1 and Stn 3 were similar, resulting in similar calcite fractions (2–3 molar%) in the *Belgrandiella* shells (Table 5). Calcite in the *Belgrandiella* shells could be induced by the annual temperature variation, but it seems that quartz in *B. questeri* at Stn 1 and 3, and dolomite formations of both snails at Stn2, were promoted by other complex environmental activities.

The presence of mineral quartz in the adult mollusks could be environmentally induced by "capturing" small particles in the structure of the shell during periods of fast growth and intensive biomineralization (Carriker, 1992; Medaković, 1995; Medaković et al., 1999; Mutvei et al., 1996). During the sample preparation for XRD snail shells were occasionally washed with distilled water and by that procedure all possible impurities from inside and outside of the shells were removed (Medaković et al., 1997, 1999, 2003a). The fact that all analyzed species are characterized by a very slow annual growth (Slapnik, 1998), and therefore by slow biomineralization processes indicates that quartz in *B. questeri* and *Z. alpestre* shells is rather a structural characteristic of these species and not environmentally induced by specific conditions of the localities. A possible promoting mechanism of incorporation of quartz could be related to structural characteristic of the inner shell layer of *B. questeri* (it being a characteristic of this species) and/or specific hydrographic conditions at the given localities.
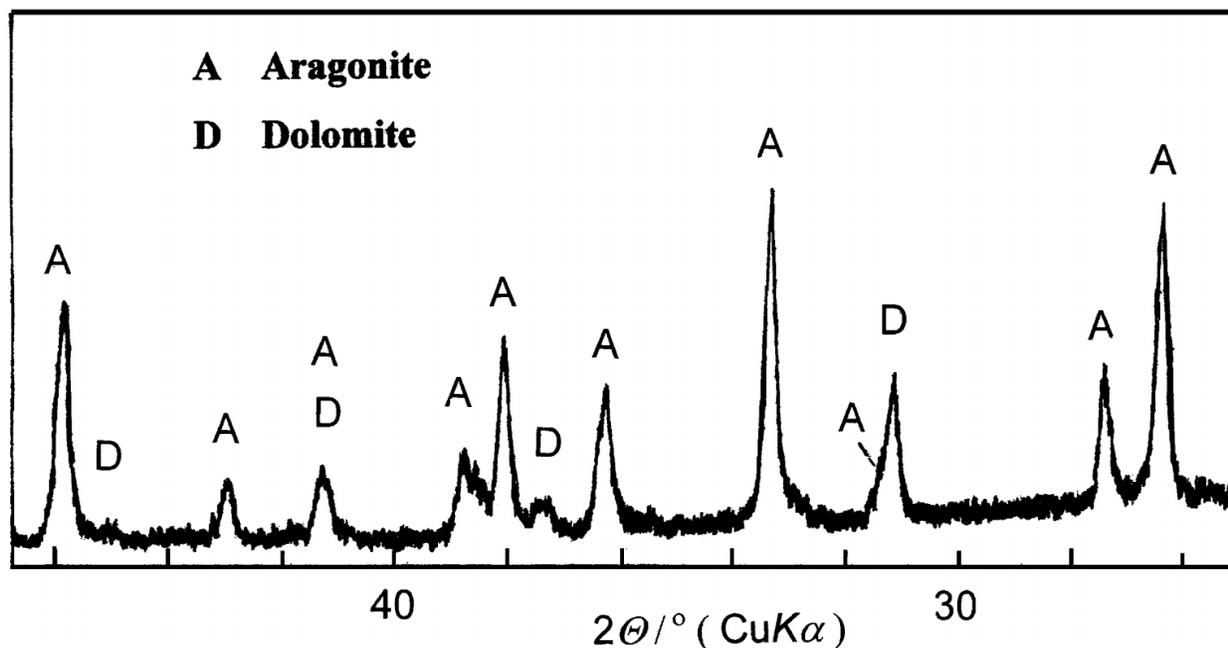
Fig. 4. *Belgrandiella kuesteri*. Characteristic part of X-ray diffraction patterns of freshwater snail shells sampled at Stn 2, Vidmar spring near Podkraj and Hrastnik, Slovenia. Diffraction pattern presents the mineral composition of powdered shells. The amounts of dominant aragonite (A) is 80 molar%, and dolomite (D) 20 molar%. *Belgrandiella fontinalis* sampled at the same station contained approximately 78 molar% of aragonite, 20 molar% of dolomite and 2 molar% of calcite. Both *B. kuesteri* and *B. fontinalis* were the first freshwater organisms found to precipitate dolomite. Mineral dolomite has been never before registered in the literature as a constituent of adult gastropod and even mollusks shells.

We postulate that higher concentrations of Mg ions in all springs result in formation of aragonite as a dominant phase in both *Belgrandiella* species. Obviously, the concentration of Mg ions in water could be a critical factor determining the shell mineralization, and maybe one of important factors that could lead to the dolomite formation in *Belgrandiella* shells from Stn 2. Unfortunately, relevant literature data about dolomite formation in the adult molluscs does not exist. Here we will discuss two possible factors for dolomite formation in *Belgrandiella*.

The first factor is related to magnesium ions concentration in the shell environment. Fritz et al., (1990) found that bivalves decrease high concentrations of certain elements in the tissue by secreting them into the extrapallial fluid as divalent cations. Falini et al., (1996) confirmed that double charged ions, particularly Mg^{2+}, present in $CaCO_3$ solution favours the formation of aragonite. The increase in the Mg concentration in the external solution is directly proportional to the increase of Mg concentration in aragonite of the shell, and at the same time high Mg concentrations in seawater inhibit the growth of calcite in the shells of *M. edulis* (Lorens & Bender, 1980). Lorens & Bender (1980) found that *M. edulis* may produce aragonite in the calcitic layer when Mg concentrations in the extrapallial fluid become very high, and that the mantle could mediate the transfer of Mg ions, thus, fractioning ions and producing carbonates that are not 'characteristic'. Contents of magnesium ions in the operculum and the shell layers of the snails of the genera *Neritina*, *Puperita* and *Astraea* can be directly correlated with differences in shell mineralogy (Adegoke, 1973).

An unusual mineral in the gastropods and even mollusks shell, dolomite $CaMg(CO_3)_2$, in amounts of 20 M% was present in both *Belgrandiella* species sampled from Stn 2. Another unusual and unexpected mineral phase, $SiCa_2$, was registered only in *Zospeum isselianum* shells. It is well established that mineralogy and composition of the calcified structure reflect endogenous and exogenous condition in the environment (Dodd, 1967; Kennedy et al., 1969; Carriker et al., 1991). Fritz et al., (1990) indicated that minerals of elemental composition similar to calcium carbonates could be mineralized by mollusks. Findings of a small amount of dolomite, beside other minerals in the embryos and larvae of the mussels *Mytilus edulis* (Medaković, 1995) in the embryos, early echinopluteus, and in spines, teeth and skeleton of adult Antarctic Sea urchins *Sterechinus neumayeri* (Medaković & Popović, 2000; Medaković et al., 2003) confirm the statement of the above authors, and indicate two possible formation mechanisms of dolomite in both *Belgrandiella* species, as well as $SiCa_2$ in *Zospeum isselianum*. We believe that dolomite in both *Belgrandiella* species is influenced by specific environmental characteristics of the locality at Stn 2. Fallini et al., (1996), and Fritz et al., (1990) showed that the presence of double charged ions (Mg^{2+} or Ba^{2+}) in the environment changes the "normal" biomineralization process and causes formation of unusual biominerals (e.g. barite in the freshwater bivalvia). The hardness of the water, ° dH measured at Stn 2, amounted 17.6 that was almost two times higher than at other two stations, Stn 1 and Stn 3. On the contrary, Stn 2 showed a much lower level of dissolved oxygen, 17.1 mg l^{-1} O_2, in comparison to 24.6 mg l^{-1} O_2, at Stn1 and 23.4 mg l^{-1} O_2 at Stn 3 (Medaković et al., 2003a; Slapnik, 1994). We believe that these hydrologic characteristics at Stn 2 and a complex interaction of other environmental conditions of the Stn 2 caused the formation of dolomite in both *Belgrandiella species*. Contrary to that, $SiCa_2$ phase in *Zospeunm isselianum* indicates possible selectivity in the mineral deposition that belongs to the individual shell structure (Dodd, 1967).

The appearance of mineral dolomite in different living organisms in recent literature is well presented; however, all authors could not agree with the theory of the dolomite formation (Deelman, 2008). In his book, in the chapter entitled *Organic or inorganic?*, Deelman (2008) refers in detail on mechanisms of natural low-temperature formation of dolomite by activity of sulphate or methane reducing and urea decomposing bacteria, like the components in two species of tape worms, in freshwater snails, in pearls, in bladder stones of the Dalmatian dog, as well as in human granulomas, tooth enamel, kidney and urinary stones. Our former research confirmed the formation of dolomite as a minor component (from traces up to 5 molar %) in calcareous algae *Coralina*, *Lithophyllum* and *Pseudolithophyllum sp.* from the Adriatic sea (Medaković et al., 1995). However, dolomite is registered as a starting biomineral between other unusual biominerals only in the embryos of the bivalve *Mytilus edulis*, and in the embryos, larvae and adults of the Antarctic sea urchins *Sterechinus neumayeri* (Medaković, 1995; Medaković et al., 2003).

A different approach could be used to explain the presence of dolomite in the organisms. It is well known that carbonate minerals are seldom pure but tend to incorporate other cations within their crystal lattice. The rhombohedral calcite tends to incorporate cations with smaller ionic radius, and orthorhombic aragonite large cations. Mg, as a small cation, substitutes Ca in calcite and this process can result equally in low Mg-calcite (Mg content < 1 molar %) or in high Mg-calcite (4-20 molar % of Mg). As the concentration of Mg ions in the sea water is almost 5 times higher than that of Ca ions, a number of marine organisms form their hard structures and skeletons of high Mg-calcite. Dolomite is formed when the

amount of Ca ions is similar to that of Mg ions in the rhombohedral crystal lattice, and once formed dolomite is relatively a stable mineral. Mg ion is smaller than Ca ion; so the lattice spacings in pure dolomite are considerably smaller than those in calcite. The organisms are capable to selectively precipitate different minerals; their specific regulation can overcome the strict physical parameters of ambient ion concentrations. Because of thermodynamical *reasons,* organisms favour less energetically consuming processes. According to the Ostwald step rule related to the nucleation and growth of crystalline phases, the organisms form a sequence of metastable phases before the formation of the final stable phase (Navrotsky, 2004; Weiner, 2006). This could be an explanation of the dolomite deposition in *Belgrandiella* snails.

Another approach comprises influence of Mg/Ca seawater chemistry oscillation during geological periods on skeletal secretion of different marine organisms (algae, sponge, corals, bryozoans, molluscs) that are reflected on initial evolution on phosphatic to calcitic invertebrate biominerals (Kempe & Kazimiercak, 1994). Mg/Ca ratio of seawater appears to control also evolution of biomineralization and favoured evolution of calcitic skeleton in one geoperiod or high Mg-calcitic or aragonitic during another period (Stanly & Hardie, 1998). Yongding et al., (1977) observed, on the basis of marine sediments, that the mineral components in fossils show an evolution sequence: silica and dolomite—phosphate—organic matter—calcite—magnesium calcite and aragonite. Thomas et al. (2000) showed that evolution and strategy for constructing hard skeleton are imprinted in the organism RNA sequences and patterns of early embryonic development. Recurring features of parallel evolution of complex organic and inorganic skeletons are controlled by genes that were already established in ancestral organisms. An important aspect of the evolutionary history of biomineralization is the finding that from the first up to presently known mineralized organism *Cloudina* contained in its shell walls organic materials with only microdolomite crystals (Grant, 1990; Knoll, 2003).

Belgrandiella fontinalis and *B. kuesteri* are the first freshwater gastropods found to precipitate an unusual mineral, dolomite, in their shells. The mineral composition and fractions of the minerals in shells of both *Belgrandiella* species show that the analyzed species respond to stress, and that ecological factors of the locality influence the shell biomineralization. The findings have a taxonomic and ecological significance.

4. Conclusion

The final aim of the study will be also to describe ecological and morphological importance of the crystal formation in several „model" organisms, to express importance in understanding, and to distinguish terminology between unusual and usual biomineralization. Generally accepted statements have comprised that crystal components in different organisms are under biological control and that crystal forms and morphology in specified tissues are adapted to their function being a characteristic for family, genus and species.

As a support to this review our unpublished experimental results, related to the findings of "unusual" initial biomineral struvite, $(NH_4)MgPO_4 \cdot 6(H_2O)$, in the embryos of the Mediterranean mussels *Mytilus galloprovincialis*, brushite, $CaHPO_4 \cdot 2(H_2O)$, and small amount of feldspars in tropic bivalve species *Codakia orbicularis* and *Tivela mactroides*, and finally dolomite in embryos of the Nudibranch gastropod *Aplysia punctata*, lead us to the conclusion that in specific conditions contemporary marine invertebrates are capable of following ancient feature and models of the biominerals formation in the early ancestors.

Moreover, the presented experimental results clarify and explain well known biomineralization hypotheses:

- calcium carbonate and phosphate ions are abundant in the oceans and thus would prevail in biomineralization over much rarer materials (Lowenstam & Margulis, 1980; Lowenstam, 1981);
- the evolutionary pattern described as "exaptation" (Gould & Vrba, 1982; Kirschvink & Hagadorn, 2000) and some essential immunological similarities between macromolecules involved in the invertebrate and vertebrate biominerals arguing for such common ancestor;
- the powerful idea that molecular inhibitors evolved early as anti-calcification protectors in highly oversaturated Proterozoic oceans were later recruited for the physiological control of crystal formation (Marin et al., 1996, 2000);
- hypothesis of Westbroek & Marin (1998), and Marin et al., (2008) that origins of calcareous skeletons reflect multiple independent co-optations of molecular and physiological processes, which are widely shared by eukaryotic organisms.

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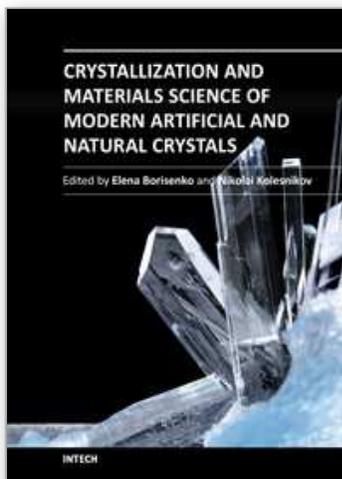
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Crystal growth is an important process, which forms the basis for a wide variety of natural phenomena and engineering developments. This book provides a unique opportunity for a reader to gain knowledge about various aspects of crystal growth from advanced inorganic materials to inorganic/organic composites, it unravels some problems of molecular crystallizations and shows advances in growth of pharmaceutical crystals, it tells about biomineralization of mollusks and cryoprotection of living cells, it gives a chance to learn about statistics of chiral asymmetry in crystal structure.

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