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ORGANIC MATRICES OF CALCIUM CARBONATE BIOMINERALS IMPROVE OSTEOBLASTIC MINERALIZATION

Sarah Nahle¹, Camille Lutet-Toti^{2,3}, Yuto Namikawa⁴, Marie-Hélène Piet⁵, Alice Brion⁶, Sylvie Peyroche¹, Michio Suzuki⁴, Frédéric Marin², Marthe Rousseau^{1,7}

1 Jean Monnet University Saint-Étienne, INSERM, Mines Saint Etienne, SAINBIOSE U1059, Saint-Étienne, France ;

2 Biogeosciences Laboratory, UMR CNRS-EPHE 6282, University of Burgundy, Dijon, France;

3 Dipartimento di Chimica "Giacomo Ciamician", Alma Mater Studiorum Università di Bologna.

4 Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan;

5 UMR 7365 CNRS-University of Lorraine, Molecular Engineering & Articular Pathophysiology (IMoPA), Vandœuvre-lès-Nancy, France;

6 Laboratory of Genome Structure and Instability, National Museum of Natural History (MNHN), INSERM, U1154, CNRS UMR7196, Paris, France;

7 UMR5510 MATEIS, CNRS, University of Lyon, INSA-Lyon, Lyon, France.

Corresponding author: Dr Marthe ROUSSEAU, marthe.rousseau@univ-st-etienne.fr

Abstract: Many organisms incorporate inorganic solids into their tissues to improve functional and mechanical properties. The resulting mineralized tissues are called biominerals. Several studies have shown that nacreous biominerals induce osteoblastic extracellular mineralization. Among them, *Pinctada margaritifera* is well known for the ability of its organic matrix to stimulate bone cells. In this context, we aimed to study the effects of shell extracts from three other *Pinctada* species (*Pinctada radiata*, *Pinctada maxima*, and *Pinctada fucata*) on osteoblastic extracellular matrix mineralization, by using an *in vitro* model of mouse osteoblastic precursor cells (MC3T3-E1). For a better understanding of the *Pinctada*-bone mineralization relationship, we evaluated the effects of 4 other nacreous mollusks that are phylogenetically distant and distinct from the *Pinctada* genus. In addition, we tested 12 non-nacreous mollusks and one extra-group. Biomineral shell powders were prepared, and their organic matrix was partially extracted using ethanol. Firstly, the effect of these powders and extracts was assessed on the viability of MC3T3-E1. Our results indicated that neither the powder nor the ethanol-soluble matrix (ESM) affected cell viability at low concentrations. Then, we evaluated osteoblastic mineralization using Alizarin Red staining and we found a prominent MC3T3-E1 mineralization mainly induced by nacreous biominerals, especially those belonging to the *Pinctada* genus. However, few non-nacreous biominerals were also able to stimulate the extracellular mineralization. Overall, our findings validate the remarkable ability of CaCO₃ biomineral extracts to promote bone mineralization. Nevertheless, further *in vitro* and *in vivo* studies are needed to uncover the mechanisms of action of biominerals in bone.

Keywords: biomineral, nacre, skeletal matrix, mineralization, bone

Introduction:

Osteoporosis (OP) is a serious global health concern characterized by an alarming incidence of osteoporosis-related fractures occurring at a frequency of one every 3 seconds worldwide (Ganesan et al. 2023; Johnell and Kanis 2006). Such a disease is marked by a disruption in the dynamic equilibrium of bone remodeling with reduced bone formation by osteoblasts and increased bone resorption by osteoclasts resulting in weaker bones that are more prone to fracture (Munoz, Robinson, and Shibli-Rahhal 2020; Riggs, Khosla, and Melton 1998).

The existing treatments face major constraints related to their effectiveness as well as their long-term use. One of the most commonly used antiresorptive medications for OP is bisphosphonates, which are effective in reducing fracture risk. However, these treatments, while beneficial, come with limitations, including modest gains in bone density and the potential for rare but severe side effects (Adler et al. 2016). In contrast to antiresorptive treatments, anabolic agents work by stimulating bone formation and increasing bone density. Such treatments are exemplified by teriparatide, a synthetic form of parathyroid hormone (PTH), which is the one and only clinically available anabolic reagent approved for the treatment of osteoporosis (Canalis, Giustina, and Bilezikian 2007). Teriparatide, when daily administered, has demonstrated high efficacy in improving bone density. Ongoing treatment is required to maintain such benefits which makes the treatment costly (Canalis et al. 2007; Carson and Clarke 2018). Biominerals, which have garnered attention in the field of bone research, can be defined as minerals produced by living organisms (Carson and Clarke 2018). Mollusks, the second largest phylum of metazoans, have played and continue to play a crucial role in the comprehension of the mechanisms of biomineralization (Evans 2019). Mollusk shells are primarily composed of calcium carbonate in the form of aragonite or calcite (**Table 1**) or a mix of both polymorphs. Some variations in the arrangement of the crystallites are observed between different species (Marin, Le Roy, and Marie 2012), in particular in the bivalve class, characterized by an extraordinary diversity of shell microstructures (Taylor, Kennedy, and Hall, 1969, 1973).

Many bivalve shells – in particular within the pteriomorphids clade that contains edible mussels and pearl oysters – are of the 'nacro-prismatic' type and exhibit a three-layered structure. The outer layer called the periostracum, is mostly organic and results from a

quinone-tanning process. Ontogenetically speaking, it represents the first shell layer formed by bivalve larvae and its role is to support the early underlying mineralization and to protect the shell against dissolution. The middle layer is prismatic and made of long needles of calcite, developed perpendicularly or obliquely to the outer shell surface. At last, the inner layer is aragonitic and presents a lustrous aspect; this is nacre also known as mother-of-pearl and the focus of the present study (Hahn et al. 2012). In bivalves, nacre consists of a stack of tiny flat tablets arranged in a brick-wall manner, while, in gastropods and cephalopods, nacre tablets are arranged in columns. Like bone, nacre contains both organic and inorganic components (Marie et al. 2009). Its organic matrix is a mixture of proteins, peptides, glycoproteins, lipids, chitin, and pigments (De Muizon et al. 2022). In their paper, Gonzalez and Vallejo reviewed the historical therapeutic applications of marine mollusk shells in Spanish ethnomedicine (González J. and Vallejo J. 2023). They uncovered intricate practices, such as using nacre from seashells for various dermatological purposes, including treating acne, eliminating facial spots through maceration in lemon juice, and dissolving mother-of-pearl buttons in lemon juice or vinegar for addressing skin conditions like chloasma (also called 'pregnancy mask', *i.e.*, pigmentation disorder) and freckles. Additionally, the authors highlighted the traditional remedy of placing small mother-of-pearl buttons under the eyelid for extracting foreign bodies and discussed the potential pharmacological activities of these marine-derived products (González J. and Vallejo J. 2023). In addition, nacre demonstrated the capacity of increasing the cell osteogenic activity without exhibiting toxicity (Lopez et al. 1992). This remarkable property led to the hypothesis that nacre might contain elements capable of inducing mineralization and promoting the proper functioning of human bone (Lopez et al. 2004; De Muizon et al. 2022; Westbroek and Marin 1998).

Within nacreous molluscs, the Polynesian pearl oyster *Pinctada margaritifera* has proven having the capacity to induce mineralization in mouse pre-osteoblastic cell line MC3T3-E1 (Brion et al. 2015; Rousseau et al. 2003, 2008). On the other hand, the water-soluble-matrix (WSM) of *Pinctada fucata*, the Japanese 'Akoya' pearl oyster, confirmed its efficacy in enhancing osteoblast differentiation (Chaturvedi, Singha, and Dey 2013). Taken together, these findings emphasize the effects of the nacre matrix on bone.

In their review, (Zhang et al. 2017) provided a comprehensive overview of the nacre species studied for their effects on bone. However, these studies primarily focused on a

few nacreous mollusks, specifically on different species of the *Pinctada* genus; to our knowledge, no studies have investigated the effects of non-nacreous mollusk biominerals on bone regeneration.

Therefore, in the present study, we have broadened the scope by investigating the osteogenic capacity of other nacreous mollusks different from *Pinctada* genus. We cautiously selected species that are taxonomically and geographically distant from *Pinctada* (**fig.1**). To gain a more comprehensive understanding of nacre-bone mineralization relationship, we also investigated the osteogenic capacity of non-nacreous mollusks, including one cephalopod (the cuttlefish) and two gastropods, among which, one nacreous. At last, we also added in the study an extra-group representative, the purple sea urchin. We assessed the effects of all these biominerals on osteoblasts using the pre-osteoblastic MC3T3-E1 cell line.

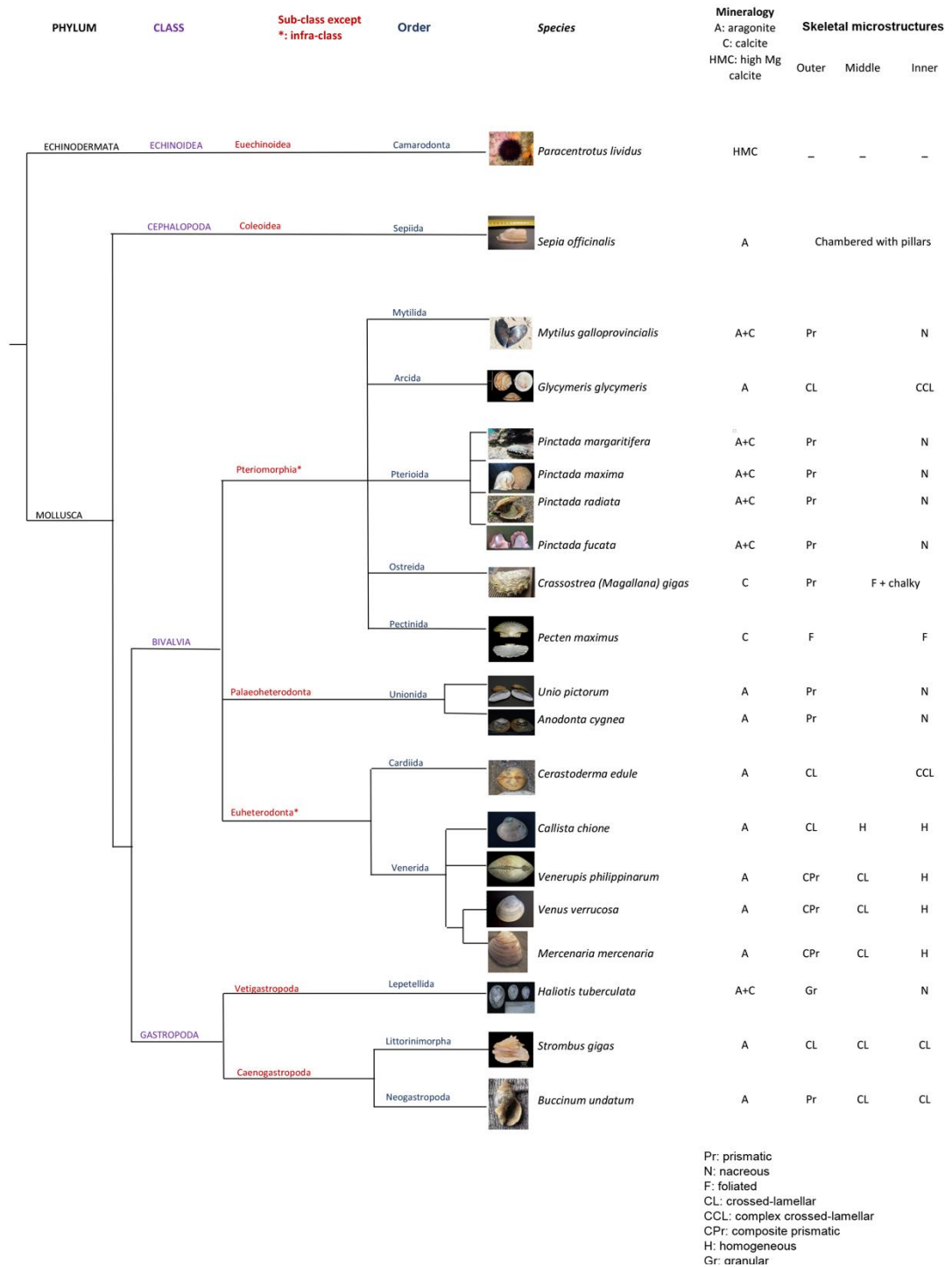


Fig. 1 Evolutionary relationships among selected species. Latin names written in blue correspond to seawater species while freshwater species names are written in black.

Material and methods

Shell powder preparation

The source and location of the 20 different skeletons used in this study are indicated in Table 1. Biomineral shell powders were prepared at UMR CNRS 6282 Biogeosciences, Dijon, France, or at the University of Tokyo, Japan. In brief, shells were scrupulously brushed and, when necessary, their outer surfaces were manually sanded or gently (to avoid heating) abraded with a mini-drill (Dremel-type) equipped either with a 20 mm diameter diamond saw or with a dentist drill. In particular, as indicated in Table 1, for all nacreous mollusks (*Pinctada*, *Mytilus*, *Unio*, *Anodonta*, *Haliotis*), we took care to obtain a pure nacreous layer devoid of outer prismatic layer. Shells of large size (like *Strombus gigas*) were sliced with a Dremel diamond saw. Shells and shell slices were extensively bleached with sodium hypochlorite NaClO (0.26% v/v active chlorine) for days with several changes of bleaching solution. They were then thoroughly rinsed with deionized water and dried at 37°C, before being crushed in a jaw crusher (Retsch, model BB 200). The obtained millimetric fragments were subsequently ground in a fine powder in a Pulverisette 2 grinder (Fritsch) equipped with an agate bowl and mortar. The powder was sieved and the fraction below 200 µm was collected in 50 mL sample pots while the fraction above was ground again until passing through the 200 µm mesh. In total, between 89 g (*Sepia officinalis*) and 469 g (*Strombus gigas*) of clean, size-calibrated powders were obtained and stored at room temperature until use.

Organic matrices extraction

Skeletal organic matrices were extracted without a demineralizing process using ethanol following the methodology described by (Brion et al. 2015; De Muizon et al. 2022) . 20 g of powders were suspended in absolute ethanol (100%, w/v) containing (0.1%, v/v) hydrochloric acid with continuous stirring for 24h at 37°C. Suspensions were centrifuged for 20 min at 1500 g, sterile-filtered through a 0.22 µm filter, and then evaporated for 48h at 37°C in an incubator. The resulting ethanol-soluble matrix (ESM) extracts were quantified by weighing on precision balance then dissolved in culture medium to obtain a stock solution of 10 mg/mL concentration. As described by (Zhang et

al. 2016), these ESM contain calcium cations as well as carbonate/bicarbonate anions, in addition to soluble organic macromolecules.

Cell culture

Mice osteoblastic precursor cells (MC3T3-E1) were provided from the European Collection of Cell Cultures (ECACC-99072810). MC3T3-E1 cells were cultured in Minimum Essential Medium Eagle (Sigma Aldrich M4526) supplemented with (10%, v/v) foetal bovine serum (MP-S00H8), 200 mM L-Glutamine (Sigma Aldrich G7513), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich P4458) at 37°C in a humidified atmosphere of 5% CO₂. To stimulate the differentiation and the mineralization of the extracellular matrix, cells were cultured with 10mM β-Glycerophosphate and 50µg/mL ascorbic acid. This medium is identified as an osteogenic differentiation medium.

Cell viability

Cell viability was determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay (MTT; Sigma-Aldrich M5655-1G). Briefly, cells were seeded at a density of 8,2x10³ cells/well in a 96-well plate and allowed to adhere overnight. Cells were then treated for 24h with varying concentrations of powder or ESM (100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, and 5000 µg/mL). Then, 20µL of MTT solution (5 mg/mL) was added per well in a fresh medium (100µL) and cells were incubated at 37°C. After 4h, the medium was removed, formazan crystals were dissolved by 200µL of dimethyl sulfoxide (DMSO, Sigma Aldrich D5879) and the absorbance was measured at 570 nm using a microplate reader TECAN (INFINITE M-PLEX).

Alizarin-red staining

Matrix mineralization was assessed by Alizarin-red staining. Cells were treated with 100 µg/mL of ESM. ESM-treated and non-treated cells were cultured in osteogenic differentiation medium for 7, 14, and 21 days at a density of 2,5x10⁴ cells/well in a 48-well plate. Cells were then fixed with 4% paraformaldehyde for 30 minutes at 4°C,

washed with deionized water, and incubated with 1% (w/v) Alizarin-red solution (Sigma Aldrich, A5533) for 5 minutes. Staining was observed under a light microscope (ZEISS axiovert 4pcfl). To quantify Alizarin-red stained nodules, 10% acetic acid was added to the cells and incubated for 30 minutes at room temperature. Cells were gently scrapped. Lysates were heated at 85°C for 10 minutes and centrifuged for 15 minutes at 4°C. The supernatant was recovered and treated with 10% of ammonium hydroxide. Subsequently, 150µL were transferred to 96 well-plate and the absorbance representative of mineralized nodule formation was measured at 570 nm.

Phylums	Classes	Species Name	Common name	Origin	Shell Composition	Environment
<i>Mollusca</i>	<i>Bivalve</i>	<i>Pinctada margaritifera</i>	Pearl Oyster	Pacific and Indian Oceans	Nacre	Seawater
		<i>Pinctada maxima</i>	South Sea Pearl Oyster	Western Pacific, Australia	Nacre	Seawater
		<i>Pinctada radiata</i>	Gulf Pearl Oyster	Red Sea, Indian Ocean	Nacre	Seawater
		<i>Pinctada fucata</i>	Akoya Pearl Oyster	Indo-Pacific, Japan	Nacre	Seawater
		<i>Mytilus galloprovincialis</i>	Mediterranean Mussel	Mediterranean, Atlantic	Nacre	Seawater
		<i>Unio pictorum</i>	Painter's Mussel	Europe, Asia	Nacre	Freshwater
		<i>Anodonta cygnea</i>	Swan Mussel	Europe, North America	Nacre	Freshwater
		<i>Pecten maximus</i>	Great Scallop	European coasts	Calcite	Seawater
		<i>Crassostrea gigas</i>	Pacific Oyster	Indo-West Pacific	Calcite	Seawater
		<i>Glycymeris glycymeris</i>	Bittersweet Clam	European coasts	Aragonite	Seawater
		<i>Cerastoderma edule</i>	Common Cockle	European coasts	Aragonite	Seawater
		<i>Venus verrucosa</i>	Warty Venus	European coasts	Aragonite	Seawater
		<i>Callista chione</i>	Smooth Clam	Mediterranean, Atlantic	Aragonite	Seawater
		<i>Venerupis philippinarum</i>	Manila Clam	Global coastlines	Aragonite	Seawater
	<i>Mercenaria mercenaria</i>	Northern Quahog	North America	Aragonite	Seawater	
	<i>Gastropod</i>	<i>Strombus gigas</i>	Queen Conch	Caribbean, Gulf of Mexico	Aragonite	Seawater
<i>Buccinum undatum</i>		Common Whelk	North Atlantic	Aragonite	Seawater	
<i>Haliotis tuberculata</i>		Abalone	Mediterranean, Atlantic	Nacre	Seawater	
<i>Cephalopod</i>	<i>Sepia officinalis</i>	Common Cuttlefish	Global oceans	Aragonite	Seawater	
<i>Echinodermata</i>	<i>Echinoidea</i>	<i>Paracentrotus lividus</i>	Purple Sea Urchin	Mediterranean, Atlantic	Calcite	Seawater

Table 1: Biomineral Characteristics of Selected Species (Information sourced from the Ocean Biodiversity Information System (obis.org))

Results

Shell powder viability assessment on MC3T3-E1 cells

Prior to any shell powder extraction, we first conducted a comprehensive assessment of their impact on MC3T3-E1 cell viability. This evaluation aimed to determine their potential toxicity. Shell powders were mixed in α -MEM culture medium and subsequently tested on cells at various concentrations. As shown in **fig.2**, five nacre powders (the 4 *Pinctada* and *U. pictorum*) induce a decrease of cell viability from low to high concentrations: this decrease is particularly drastic for *Pinctada radiata* and *Pinctada maxima*, which reaches 58.4% at 2500 $\mu\text{g/mL}$ and 59.2% at 3500 $\mu\text{g/mL}$, respectively. Intriguingly, at low concentrations, three nacre powders - that of *Pinctada margaritifera* (between 500 and 1000 $\mu\text{g/mL}$), of *Unio pictorum* (between 500 and 2000 $\mu\text{g/mL}$) or of *Haliotis tuberculata* (between 1000 and 2000 $\mu\text{g/mL}$) - exhibited an enhancement of cell proliferation, with viability reaching approximately 118%. The cell viability induced by *M. galloprovincialis* and *A. cygnea* nacres was stable whatever concentration used.

As shown by Fig.3, the 12 non-nacreous powders- including that of the sea urchin *P. lividus* - did not exert any toxic effect on MC3T3-E1 cells. Most of them induced a high cell viability (above 80%) at high concentration. Single exception: the powder of the cuttlefish *Sepia officinalis*, which induced a relatively important decrease from low to high concentration.

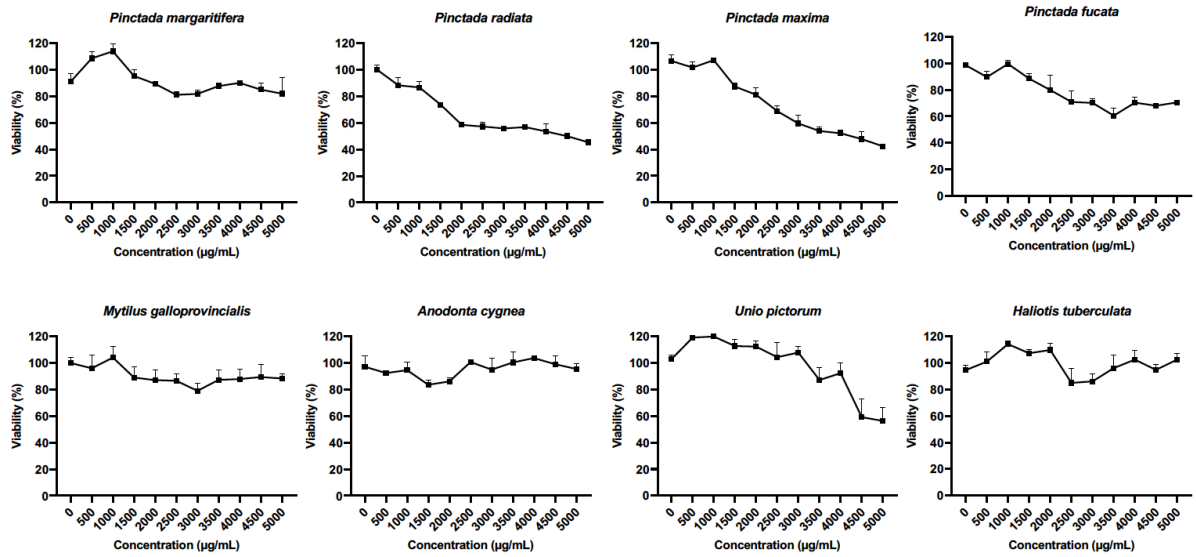


Fig. 2 Effect of shell powder from nacreous species on the viability of MC3T3-E1 cells. Cells were treated with varying concentrations of biomineral shell powder mixed in culture medium α -MEM for 24h. Results are standardized to the control group that did not receive any treatment and are represented as the mean \pm SEM of an experiment performed in four replicates.

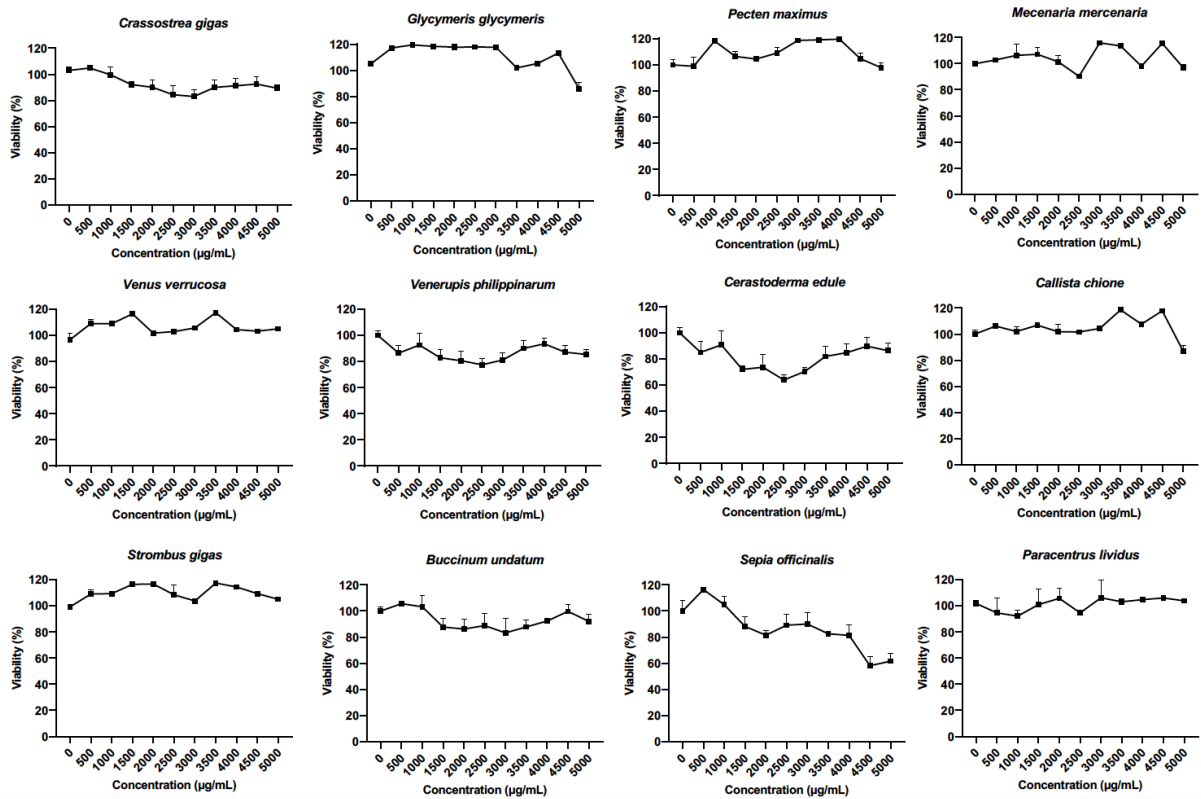


Fig. 3 Effect of shell powder from non-nacreous species on the viability of MC3T3-E1 cells. Cells were treated with varying concentrations of biomineral shell powder dissolved in culture medium α -MEM for 24h. Results are standardized to the control non-treated group and are represented as the mean \pm SEM of an experiment performed in four replicates.

ESM viability assessment on MC3T3-E1 cells

To study the ability of the different biomineral ESM on mineralizing osteoblastic cells, we assessed its effect on the viability of MC3T3-E1 cells after 24h of treatment. At low concentrations, the ESM from *P. margaritifera*, *P. fucata*, *P. maxima*, *P. radiata*, *A. cygnea*, *U. pictorum*, *H.tuberculata*, and *M. galloprovincialis* showed no noticeable toxic effects on cell viability, as depicted in **fig.4**. However, at high concentrations of ESM from *A. cygnea*, and *U. pictorum*, a slight reduction in cell viability was observed, reaching approximately 80% at 2500 $\mu\text{g/mL}$. The reduction of cell viability was more pronounced for *M. galloprovincialis* (around 60% at 4000 $\mu\text{g/mL}$).

For non-nacreous biominerals, including *P. maximus*, *S. officinalis*, *G. glycymeris*, *M. mercenaria*, *V. verrucosa*, *S. gigas*, *C. chione*, and *P. lividus*, no inhibition of cell proliferation was detected (**fig. 5**). However, the ESM extracted from *B. undatum*, *V. philippinarum*, *C. edule*, and *C. gigas* exhibited toxicity at 2500 $\mu\text{g/mL}$, resulting in a reduction of cell viability to less than 50%. These findings highlight the varying effects of ESM from different biominerals on cell viability and underscore the importance of considering both concentration and biomineral type in cell-based assays.

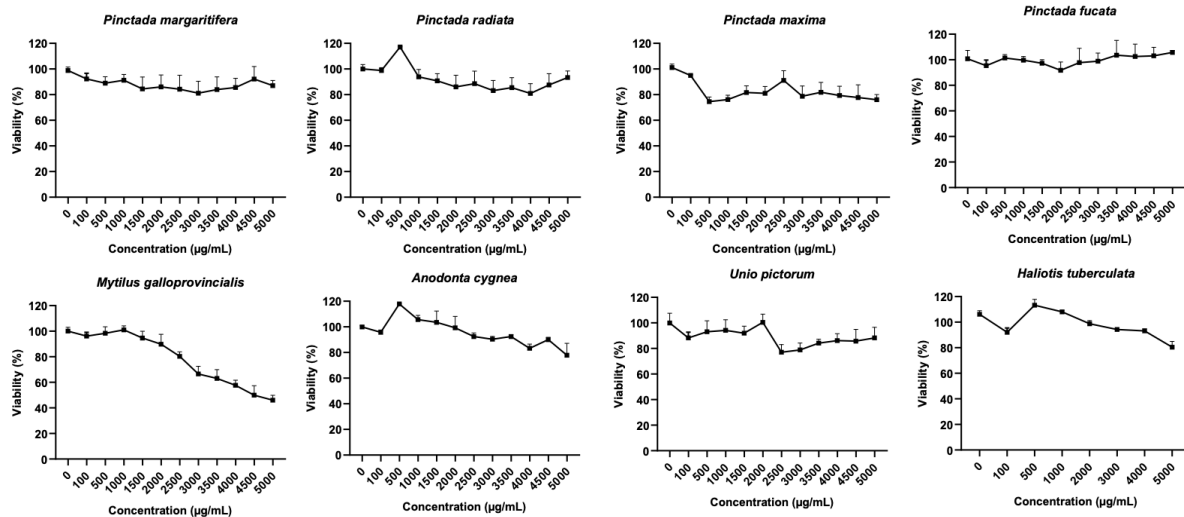


Fig. 4 Effect of nacreous ESM on the viability of MC3T3-E1 cells. Cells were treated with varying concentrations of ESM for 24h. Results are standardized to the control group that did not receive any treatment and are represented as the mean \pm SEM of an experiment performed in four replicates.

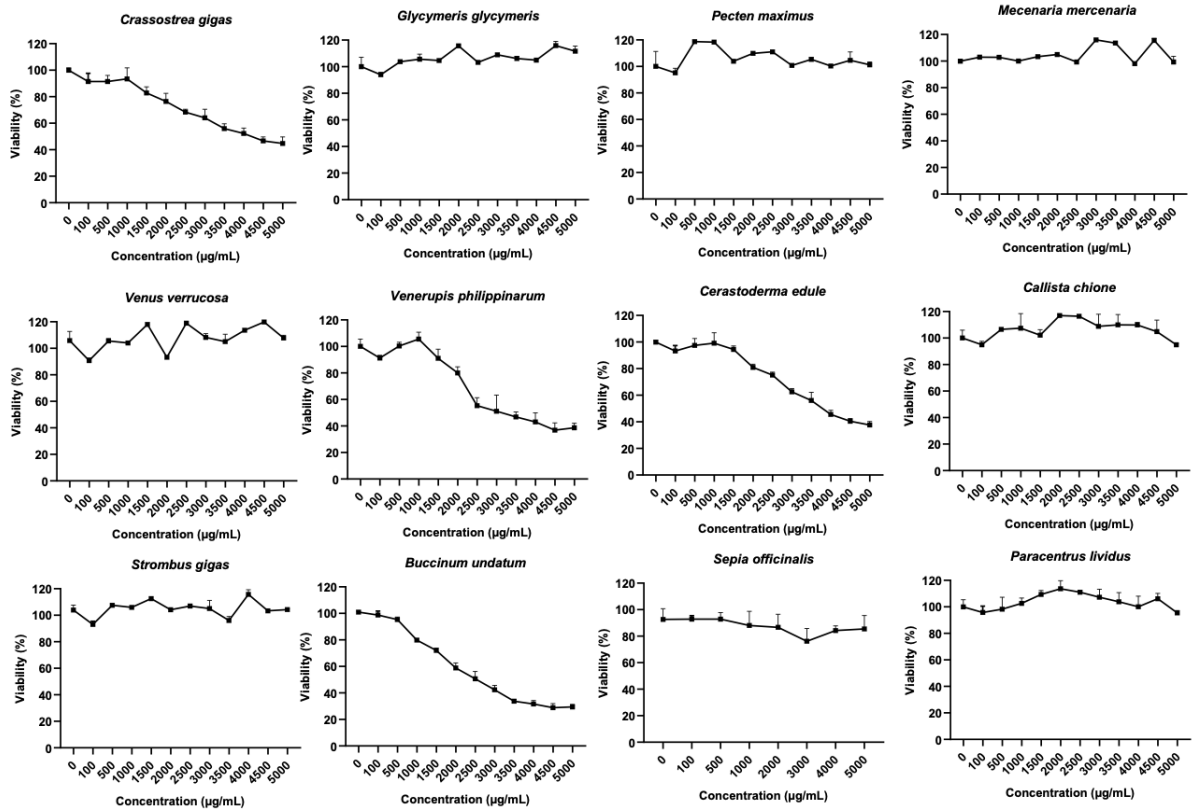


Fig. 5 Effect of non-nacreous ESM on the viability of MC3T3-E1 cells. Cells were treated with varying concentrations of ESM for 24h. Results are standardized to the control group that did not receive any treatment and are represented as the mean \pm SEM of an experiment performed in four replicates.

Mineralization Responses Across Biominerals

We investigated the effects of ESM on the mineralizing process in cultured MC3T3-E1 cells. ESM was added throughout the culture period (21 days) and the concentration of 100 $\mu\text{g}/\text{mL}$ was considered since its non-toxicity was confirmed across all species by the MTT viability test. The control consisted of cells treated with 10mM β -Glycerophosphate and 50 $\mu\text{g}/\text{mL}$ ascorbic acid. Mineralized nodules were seen as red and dark brown to black spots. Distinct mineralization responses were observed in MC3T3-E1 cells treated with ESM, as shown by **fig.6** (nacreous) , **fig.7** (non-nacreous) and **fig.8**.

Among the nacreous species, red crystals indicative of mineralization were intensely expressed in cells exposed to ESM from all species within the *Pinctada* genus, highlighting their significant role in promoting mineralization (**fig.6**). Moreover, *A. cygnea* exhibited the capacity to induce mineralization in MC3T3-E1 cells, while *U. pictorum*, which is closely related to *Anodonta* (unionid bivalves), had a much more reduced effect, similar to that of the nacreous gastropod, *H. tuberculata*. The nacre of the

mussel *M. galloprovincialis* was even less effective in stimulating the mineralization of the MC3T3-E1 cells, since few red spots were observed.

On the other hand, among the non-nacreous biominerals, *S. officinalis* and *G. glycymeris* demonstrated no discernible capacity to induce mineralization in the cultured cells **(fig.7)**. The bivalves *M. mercenaria*, *V. verrucosa*, *V. philippinarum*, *C. gigas*, the two gastropods *S. gigas* and *B. undatum* and finally, the sea urchin *P. lividus* exhibited modest effects on the mineralization of the MC3T3-E1 cells when compared to the nacreous species. The great scallop *P. maximus* induced a higher effect. However, the most astonishing insight was the substantial mineralization potential demonstrated by *C. edule* and *C. chione*.

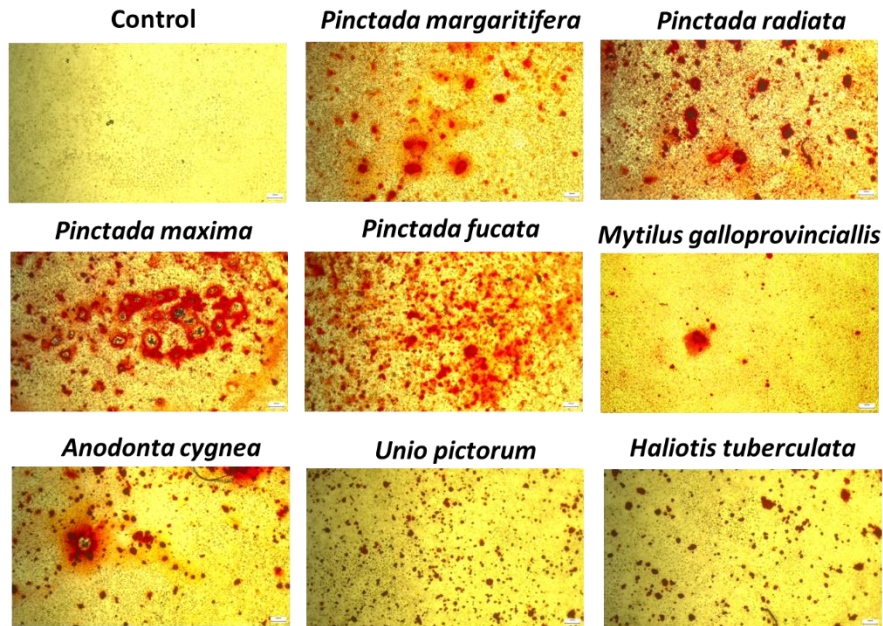


Fig. 6 Evaluation of MC3T3-E1 mineralization following 21 days of treatment with nacreous ESM using Alizarin Red staining. Representative pictures of alizarin red stained MC3T3-E1. The images were obtained using phase contrast microscopy; scale bar = 1000 μ m. Cells were cultured in osteogenic differentiation medium w/o ESM for 21 days at a density of $2,5 \times 10^4$ cells/well in a 48-well plate. Results are representative of three experiments.

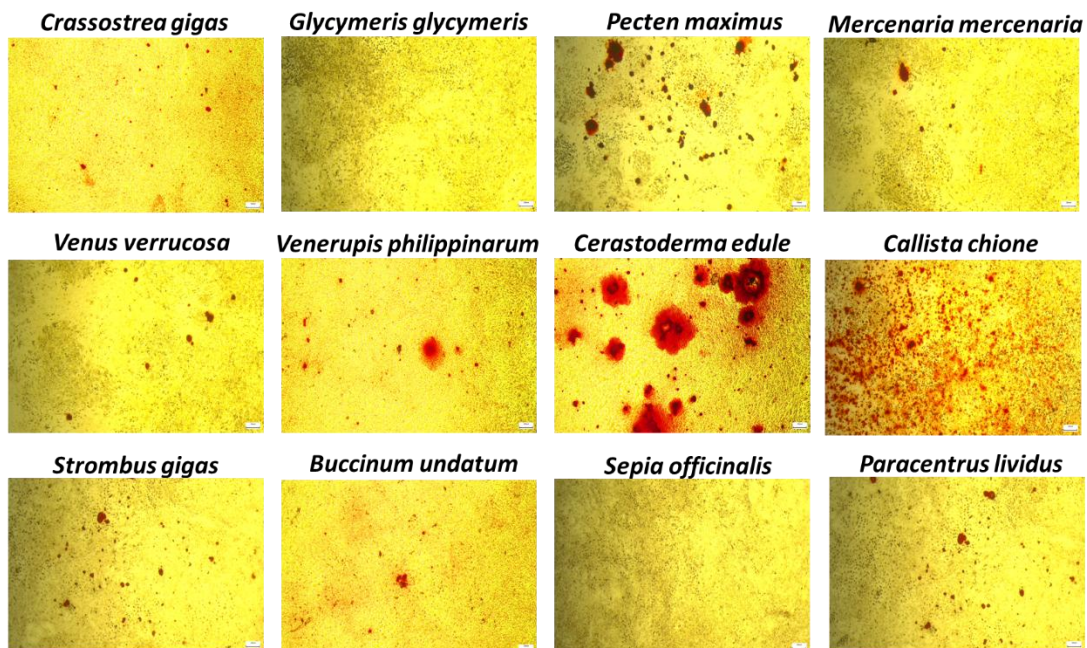


Fig. 7 Evaluation of MC3T3-E1 matrix mineralization following 21 days of treatment with non-nacreous ESM using Alizarin Red staining. Representative pictures of alizarin red stained MC3T3-E1. The images were obtained using phase contrast microscopy; scale bar = 1000 μ m. Cells were cultured in osteogenic differentiation medium w/o ESM for 21 days at a density of $2,5 \times 10^4$ cells/well in a 48-well plate. Results are representative of three experiments.

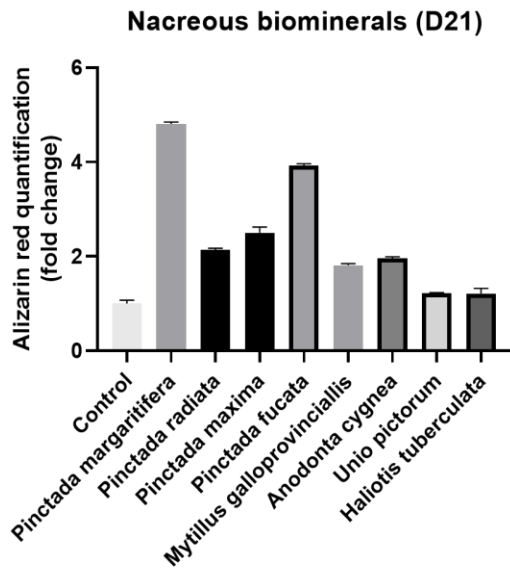
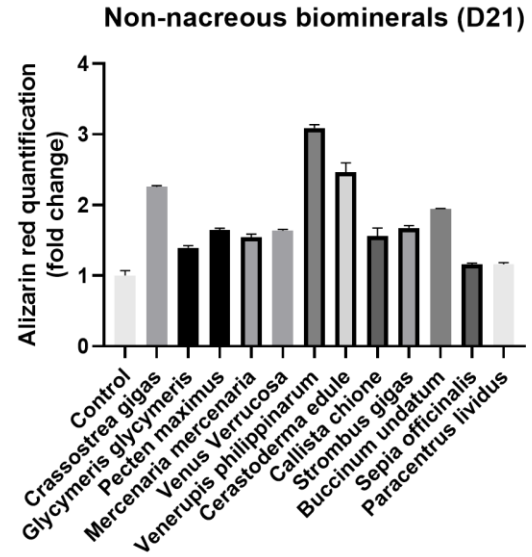
A**B**

Fig. 8: Alizarin red quantification. A- nacreous species and B- non-nacreous species. Red nodules were quantified using 10% acetic acid and 10% ammonium hydroxide. Cells were cultured in osteogenic differentiation medium w/o ESM for 14 days at a density of 2.5×10^4 cells/well in a 48-well plate. Results are representative of three experiments.

Discussion

The osteogenic potential of nacre is now well-documented (Zhang et al. 2017). However, most of the data accumulated on this shell microstructure focus on the water-soluble matrix (WSM) from a few species (Chaturvedi et al. 2013; Pereira Mouriès et al. 2002; Rousseau et al. 2003; Sud et al. 2001). Eight years ago, an ethanol extraction method was used to extract the organic matrix from the pearl oyster *Pinctada margaritifera* (Brion et al. 2015). The resulting ethanol-soluble matrix (referred to as ESM) proved having the capacity to induce the mineralization of MC3T3-E1 as well as the human subchondral osteoarthritic osteoblasts (Brion et al. 2015). So far, studies using ESM extracts have only investigated the osteogenic potential of *Pinctada margaritifera* mother-of-pearl (Brion et al. 2015; Willemin et al. 2019; Zhang et al. 2016). There are no reports available on other species, neither on other microstructures.

Herein, we initiated a pilot study on the effects exerted by both nacreous and non-nacreous species on MC3T3-E1 matrix mineralization. This study presented challenges as acquiring shell powder from these special species was particularly difficult, making it hard to obtain an adequate quantity for the long experimentation (21 days). Prior to any experiments, we rigorously assessed the toxicity profiles of the shell powders. As illustrated in **Table 1**, these shells primarily consist of calcite or aragonite. Nacre samples belong to the second category. All studied samples have natural solubility in acidic solutions. Knowing the toxic nature of acids towards cells, we developed a methodology involving the mixing of shell powders with culture medium and leaving them undissolved when exposed to the cells. To ensure that this approach did not compromise the accuracy of absorbance measurements in the MTT assays, we adjusted the protocol and incorporated centrifugation techniques to meticulously remove the powder residues from the cultures before making any absorbance readings. Our findings revealed that at low concentrations, these powders presented no visible toxicity concerns. However, at higher concentrations, some molluscan species did exhibit a modest impact on cell viability (**fig.4; fig.5**).

We subsequently proceeded to extract the ESM following the methodology outlined by (Brion et al. 2015). Our investigation extended to assessing the potential toxicity of these ESM extracts on MC3T3-E1 cells. Remarkably, at lower concentrations, we

observed no toxicity, aligning with our earlier findings regarding shell powders. However, it became apparent that at higher concentrations, some of the tested species, such as *B. undatum*, *M. galloprovincialis*, *V. philipinarum*, *C. edule*, and *C. gigas* exhibit some degree of cytotoxicity. These observations underscore the importance of carefully evaluating the concentration-dependent effects of ESM components.

As a consequence of these findings, we made an informed decision to select a non-toxic concentration of 100 µg/mL for the rest of the study. This concentration, established through rigorous viability assessments, allows us to effectively investigate the various effects of ESM components on MC3T3-E1 cells without compromising cell viability.

The osteogenesis starts with the differentiation of mesenchymal cells into pre-osteoblasts. These pre-osteoblasts subsequently progress into mature osteoblasts which finally induce the deposition of bone matrix proteins and the mineralization of the matrix, leading to bone formation. To assess the calcium deposition within the matrix, we employed Red Alizarin staining. The murine pre-osteoblastic cells MC3T3-E1 were treated with 100 µg/mL of both nacreous and non-nacreous ESM for 7, 14 (**Supp. Fig 1; Supp. Fig 2**) and 21 days (**fig.6; fig.7; fig.8**). Our findings revealed an important difference in mineralization effects between nacreous and non-nacreous species. With the exception of *M. galloprovincialis* – explainable by its relative cytotoxicity to MC3T3-E1 cells - all nacreous species exhibited a more obvious impact on mineralization. Among them, all *Pinctada* species played a prominent role in inducing mineralization, emphasizing their potential importance in bone formation. Conversely, within the non-nacreous category, *Cerastoderma edule* and *Callista chione* (both belonging to Venerida order and both predominantly crossed-lamellar) yielded to our surprise strongly positive effects, deserving further investigations to elucidate their organic matrix composition responsible for this phenomenon. In a lesser extent, *Pecten maximus* also recorded interesting effects. To our knowledge, our study represents the first report of the positive effects of non-nacreous mollusk extracts on bone-forming cells.

Indeed, our findings underscore the efficacy of the ethanol-soluble matrix (ESM) from biomineral shells as a worthy matrix for bone formation. These insights not only improve our knowledge of the biological roles of these matrix constituents but also illuminate promising avenues for future research and therapeutic discovery. The potential applications of this research could have consequences in the field of bone health, offering new opportunities for the development of innovative treatments.

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Statements and Declarations

Availability of data and material

The datasets are included in this article and available from the corresponding author on reasonable request.

Competing Interests

MR provides scientific consultation for Megabiopharma.

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Author's contribution

Study design: Frédéric Marin, Marthe Rousseau. Study conduct: Sarah Nahle, Camille Lutet-Toti, Yuto Namikawa, Marie-Hélène Piet, Alice Brion, Sylvie Peyroche. Data analysis and visualization: Sarah Nahle, Frédéric Marin. Data interpretation: Sarah Nahle, Michio Suzuki, Frédéric Marin, Marthe Rousseau. Writing original draft: Sarah Nahle. Revising manuscript content : Sarah Nahle, Sylvie Peyroche, Michio Suzuki, Frédéric Marin, Marthe Rousseau. Approving final version of manuscript: all authors.

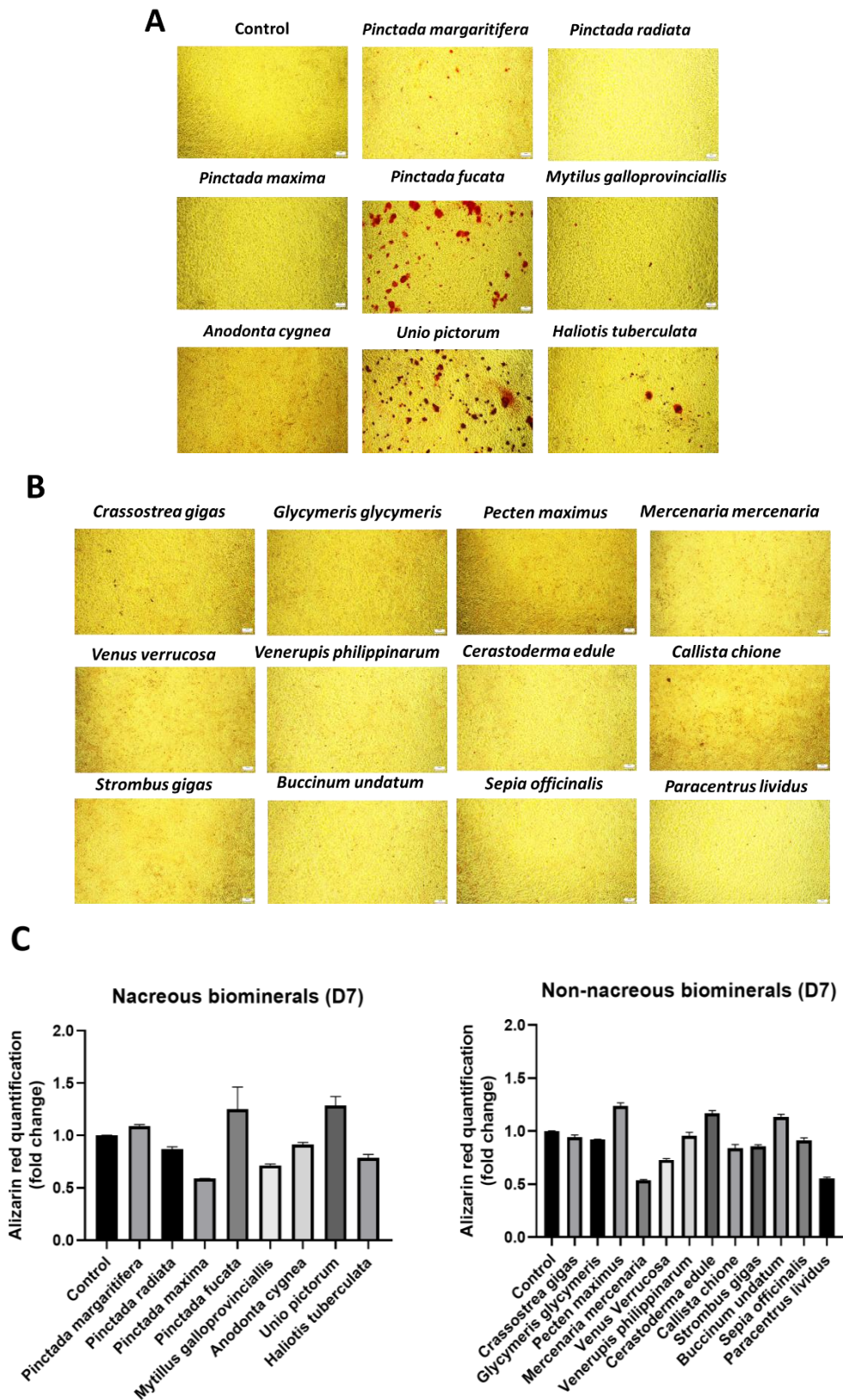
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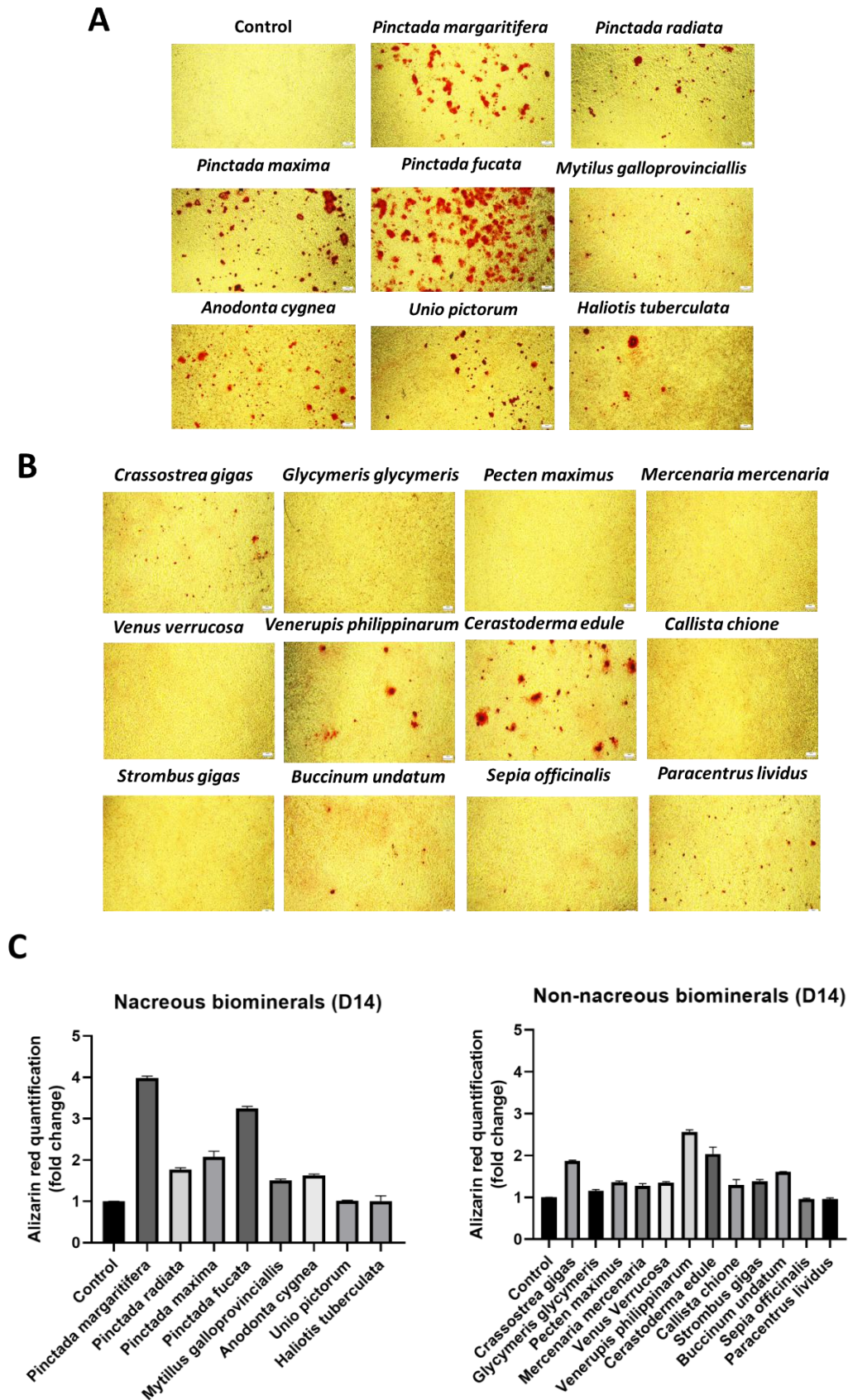
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Supplementary information



Supplementary figure 1: Evaluation of MC3T3-E1 mineralization following 7 days of treatment A- with nacreous ESM B- with non-nacreous species; using Alizarin Red staining. C- Alizarin red nodules were quantified using 10% acetic acid and 10% ammonium hydroxide. Cells were cultured in osteogenic differentiation medium w/o ESM for 7 days at a density of 2.5×10^4 cells/well in a 48-well plate. The images were obtained using phase contrast microscopy; scale bar = 1000 μ m. Results are representative of three experiments.



Supplementary figure 2: Evaluation of MC3T3-E1 mineralization following 14 days of treatment **A-** with nacreous ESM **B-** with non-nacreous species; using Alizarin Red staining. **C-**Alizarin red nodules were quantified using 10% acetic acid and 10% ammonium hydroxide. Cells were cultured in osteogenic differentiation medium w/o ESM for 14 days at a density of 2.5×10^4 cells/well in a 48-well plate. The images were obtained using phase contrast microscopy; scale bar = 1000 μ m. Results are representative of three experiments.

