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ANALYSIS OF EPILITHIC COMMUNITIES ON SKELETAL REMAINS IN A PORT ENVIRONMENT FOR THE ESTIMATION OF THE POST-MORTEM SUBMERSION INTERVAL (PMSI)

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ABSTRACT

La tafonomia forense in ambienti marini costituisce un campo complesso in cui la degradazione dei resti organici è guidata dall'interazione tra fattori fisico-chimici e biologici. La presente tesi analizza la stima del Post-Mortem Submersion Interval (PMSI) mediante l'analisi delle comunità epilitiche che colonizzano i resti scheletrici in un contesto portuale.

Il femore di *Sus scrofa domesticus* è stato impiegato come modello sperimentale per le sue somiglianze strutturali e nanometriche con l'osso umano, rendendolo un proxy ampiamente riconosciuto nella ricerca forense. Lo studio è stato condotto nel porto di Genova, un ambiente costiero contraddistinto da dinamiche ecologiche peculiari rispetto ai contesti di mare profondo.

La ricerca si concentra sull'analisi della successione delle comunità colonizzatrici sulla superficie ossea. Le variazioni nella composizione, densità e tipologia delle popolazioni epilitiche sono risultate dipendenti dal tempo di esposizione, consentendo la ricostruzione di una cronologia biologica della permanenza in acqua. Parallelamente, la microscopia elettronica a scansione (SEM) è stata impiegata per valutare la progressione dell'erosione superficiale nei campioni posti a contatto con il fondale, fornendo una mappatura temporale della degradazione strutturale.

I risultati evidenziano che la colonizzazione segue schemi temporali coerenti e che l'erosione superficiale aumenta progressivamente, sostenendo il loro utilizzo come indicatori cronologici affidabili in ambienti portuali. Complessivamente, lo studio dimostra che l'integrazione tra dinamiche di colonizzazione e analisi dell'erosione permette di ottenere dati oggettivi per la stima del PMSI in contesti marini costieri. Questo approccio metodologico costituisce una base solida per future applicazioni in ambito forense, dove l'evidenza ecologica può essere tradotta in indicatori temporali robusti per la ricostruzione degli eventi.

La ricerca è stata condotta presso i laboratori del Dipartimento di Scienze della Terra, dell'Ambiente e della Vita (DISTAV).

Parole chiave: Tafonomia forense marina; Intervallo di sommersione post-mortem (PMSI); Comunità epilitiche; Degradazione ossea.

Forensic taphonomy in marine environments is an intricate discipline in which the degradation of organic remains is influenced by the interactions among physicochemical and biological factors. This thesis examines the estimation of the Post-Mortem Submersion Interval (PMSI) through the analysis of epilithic communities colonizing skeletal remains within a port setting.

The femur of *Sus scrofa domesticus* was used as an experimental model due to its structural and nanometric similarity to human bone, making it a widely accepted proxy in forensic research. The study was conducted in the port of Genoa, a coastal environment characterized by ecological dynamics distinct from those of deep-sea settings.

The research concentrates on the succession of colonizing communities on bone surfaces. Variations in the composition, density, and typology of epilithic populations were observed as a function of exposure time, thereby facilitating the reconstruction of a biological timeline of submersion. Concurrently, scanning electron microscopy (SEM) was utilized to evaluate the progression of surface erosion in samples in contact with the seabed, offering a temporal map of structural deterioration.

The findings indicate that colonization adheres to consistent temporal patterns and that surface erosion progressively intensifies, thereby supporting their application as dependable chronological indicators within port settings. Overall, this research illustrates that the integration of colonization dynamics with erosion analysis yields objective data for PMSI estimation in coastal marine environments. This methodological framework establishes a foundation for future applications in forensic science, wherein ecological evidence can be converted into reliable temporal indicators for event reconstruction.

The research was conducted at the laboratories of the Department of Earth, Environmental and Life Sciences (DISTAV).

Keywords: Marine forensic taphonomy; Post-mortem submersion interval (PMSI); Epilithic communities; Bone degradation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

1.1 Post-Mortem decomposition: a complex process and its forensic implications

Understanding post-mortem processes, specifically the biological, chemical, and physical changes that occur after death, is essential in forensic investigations. These processes are highly complex, and numerous variables can influence the nature and progression of post-mortem changes.

Among these processes, decomposition encompasses the physico-chemical and biological mechanisms responsible for the disintegration of a body or its components. Decomposition begins shortly after death, as the cessation of cardiac and respiratory functions interrupts blood circulation, thereby depriving cells of oxygen and nutrients, ultimately leading to cellular death. Although the decomposition process initiates promptly, visible signs at the macroscopic level generally become observable only several hours post-mortem.

During the initial stages, decomposition is principally propelled by two processes:

- Autolysis is the process by which molecules decompose cellular components as a result of lytic enzymes released when normal physiological processes are disrupted.
- Putrefaction, which involves the breakdown of tissue components by bacterial activity.
- These processes generate gases that add to the distinctive smell of decomposing bodies.
- Several factors influence the decomposition of a corpse:
- Environmental and atmospheric factors, including temperature, precipitation, humidity, and fire exposure.
- Biological activities, including microorganisms, insects, parasites, carnivores, and rodents;
- Burial conditions, including burial method and depth.
- Intrinsic characteristics of the body, such as age, body mass, body size, hydration status, and the proportion of lean and fat mass.
- The condition of the remains varies, as intact and dismembered bodies can decompose at different rates.

Decomposition is generally divided into a series of phases, commonly known as stages of decomposition.

- Fresh stage
- Chromatic stage
- Bloated stage

- Decay stage (including advanced decay)
- Dry stage (skeletonization)

A decomposing body undergoes a series of transformative processes that considerably alter its external appearance and internal structure. Each stage involves specific physical and biochemical changes. However, these stages should not be regarded as strictly distinct phases, but rather as a continuum of overlapping processes that progressively result in the complete decomposition of organic matter.

These general principles are applicable to both terrestrial and aquatic environments.

Decomposition in aquatic environments poses additional challenges due to interactions among various environmental factors. Variables such as water temperature, salinity, depth, dissolved oxygen levels, and the activity of necrophagous and colonizing organisms significantly influence both the rate and progression of decomposition.

Within this framework, marine forensic taphonomy examines the processes affecting organic remains from the moment of death until their recovery in aquatic environments. This discipline aims to interpret the intricate interactions that govern decomposition and to apply this understanding to forensic investigations. Research in this domain predominantly relies on experimental methodologies and observational studies, frequently utilizing animal models to enhance comprehension of decomposition processes pertinent to human forensic cases. As taphonomic processes are profoundly influenced by local environmental conditions, studies conducted across various ecological settings are indispensable, given that extrapolating results from one environment to another may result in considerable inaccuracies.

The post-mortem interval (PMI) signifies the duration between death and the discovery of the body. In forensic practice, however, investigators more frequently utilize the term minimum post-mortem interval (minPMI), which denotes the shortest conceivable duration elapsed between death and the recovery of remains.

An accurate estimation of the PMI is a critical element in reconstructing events and aiding in victim identification.

Therefore, studying decomposition in aquatic environments is crucial for enhancing the accuracy of PMI estimation, especially regarding the diagenetic and morphological changes that impact submerged skeletal remains. In this context, the degradation of organic remains is clearly illustrated by the phenomenon known as whale falls. These carcasses, extensively documented in scientific literature over the past twenty years, not only provide a significant source of organic carbon to dark marine environments but also act as natural in situ biogeochemical laboratories, enabling researchers to observe large-scale processes that influence the persistence and transformation of organic remains on the seafloor.

1.2 Review of existing literature on whale bone degradation in the deep sea

The degradation of a whale carcass that sinks to the ocean floor, a phenomenon commonly referred to as a whale fall**,** constitutes a complex process that leads to the development of distinctive benthic ecosystems (Smith, 2006). The ecological succession that occurs around the whale skeleton advances through multiple distinct stages (Smith & Baco, 2003).

The initial phase, referred to as the mobile scavenger stage, entails the swift removal of soft tissues by large scavengers such as sharks, hagfish, ratfish, and crabs (Amon et al., 2013; Smith et al., 2015). Depending on the size of the carcass, this phase may extend from several months to multiple years (Higgs et al., 2011).

This stage is succeeded by the enrichment opportunist phase, during which bones and adjacent sediments, enriched with organic material, are colonized by densely populated communities of opportunistic organisms such as polychaetes, crustaceans, and other invertebrates that feed on residual organic matter (Hilario et al., 2015). Despite the elevated organism density during this phase, species diversity typically remains comparatively low (Hilario et al., 2015).

The third phase, referred to as the sulphophilic stage, represents the most prolonged and intricate stage within the whale-fall succession. During this period, lipids contained in whale bones are subjected to anaerobic bacterial degradation, resulting in the production of sulphides (Higgs et al., 2011; Smith et al., 2015). These sulphides serve as an energy source for chemosynthetic organisms, thereby supporting a specialized ecosystem that can persist for several decades (Higgs et al., 2011; Smith et al., 2015).

Worms of the genus *Osedax* are instrumental during this stage, as they facilitate the breakdown of bone matrix via acid secretion and the metabolic activity of symbiotic bacteria, thereby allowing them to exploit the organic compounds within bone tissue (Höpner & Bertling, 2017).Finally, the reef stage occurs when the whale skeleton has predominantly decomposed into mineralized remains with minimal residual organic material (Smith & Baco, 2003; Higgs et al., 2011). At this stage, the skeletal framework may function as a substrate for filter-feeding organisms, although this stage is not invariably reached, depending on the rate and extent of decomposition (Smith & Baco, 2003; Higgs et al., 2011).

It is important to note that the majority of whale-fall studies have been conducted at depths exceeding 1000 meters, primarily within the bathyal or abyssal zones of the ocean (Lundsten et al., 2010).

The unique environmental conditions of the deep sea, marked by low temperatures and elevated hydrostatic pressure, considerably retard decomposition processes (Anderson & Bell, 2016). Moreover, deep-sea environments are generally nutrient-poor, making whale carcasses an immediate and concentrated nutritional resource for benthic communities (Smith & Baco, 2003).

For example, whale-fall studies have been conducted in Monterey Canyon at depths of approximately 3000 meters (Lundsten et al., 2010).

Although the clearly defined succession of organisms observed in deep-sea whale falls provides an important framework for understanding bone decomposition in marine environments, the composition of faunal communities and the rates of decay are likely to vary considerably in shallower coastal areas like the Port of Genoa (Watson & McClelland, 2018). Environmental conditions and the presence of different scavenger groups in port environments may influence taphonomic processes in ways that differ greatly from those in deep-sea ecosystems (Watson & McClelland, 2018).

Furthermore, the majority of extant research concentrates on cetacean bones, which are substantially larger and possess a markedly higher lipid content compared to porcine or human bones (Höpner & Bertling, 2017). This disparity suggests that the temporal dynamics and degradation processes observed in whale-fall systems may not be directly applicable to the decomposition of bones originating from smaller mammals (Watson & McClelland, 2018).

1.3 Comparative osteology: highlighting the differences between whale and human bones

Whale bones differ notably from human bones, and by extension, from those of pigs used as experimental models, in terms of composition, density, and structural organization (Höpner & Bertling, 2017).

A fundamental distinction exists in the composition of bones. Whale bones possess higher quantities of oil and lipids, serving as a long-term energy reserve for specialized decomposer organisms (Höpner & Bertling, 2017). This increased lipid content, often referred to as "biofuel reserves," is significantly less prominent in human and pig bones, which exhibit different ratios of organic to inorganic materials (Höpner & Bertling, 2017).

Regarding density and porosity, the skeletal structures of non-human animals, including cetaceans and porcine species, generally exhibit higher density and lower porosity than human bones (Watson & McClelland, 2018). For instance, in humans, the cortical thickness of the humerus and femur accounts for approximately one quarter of the total bone diameter, whereas in animals it approaches one half (Watson & McClelland, 2018).

Furthermore, trabecular bone is largely absent from the diaphysis of long bones in non-human animals, resulting in a more uniform medullary surface compared with the trabecular network commonly observed in human long bones (Watson & McClelland, 2018). Additionally, mineralization levels may vary between marine and terrestrial mammals (Höpner & Bertling, 2017). From a structural and morphological perspective, although all mammals—including whales, humans, and pigs—possess similar skeletal frameworks, whales exhibit notable adaptations to aquatic existence, such as the fusion of specific bones and the absence of hind limbs (Higgs et al., 2011).

Specifically focusing on the femur, the bone of interest in this study, relevant structural differences may exist between whales, pigs, and humans that could influence bone degradation processes (Watson & McClelland, 2018).

Differences in bone composition and size, as well as the unique environmental conditions—including temperature, pressure, oxygen levels, water flow, and salinity—present in deep-sea environments compared to port environments, elucidate why the degradation patterns observed in whale bones at extreme depths might not be directly applicable to human bones in shallower coastal areas (Watson & McClelland, 2018).

It is therefore probable that the microbial communities and necrophagous organisms in these two environments differ considerably (Höpner & Bertling, 2017).

Therefore, caution should be exercised when extrapolating taphonomic patterns observed in large marine vertebrates to human remains. The substantial differences in bone composition, lipid content, and structural organization, coupled with the disparate environmental conditions between deep-sea ecosystems and shallow coastal regions, can significantly influence degradation pathways. Consequently, controlled experimental models employing mammalian proxies, such as *Sus scrofa domesticus*, provide a valuable methodology for investigating bone alteration processes within marine contexts that more closely resemble forensic scenarios in coastal and port environments.

1.4 The experimental model: Sus scrofa domesticus

The domestic pig (*Sus scrofa domesticus*) is widely recognized as a preferred experimental model for the study of cortical bone, having been extensively utilized in both orthopedic and forensic research due to its notable similarities in size and structure to human bone (Taguchi et al., 2021). In the appendicular skeleton, the femur assumes a vital biomechanical function: as the principal long bone of the pelvic limb, it facilitates load transfer between the pelvis and the tibia and accommodates complex mechanical stresses such as compression, bending, and torsion (Enlow, 1990).

Although it serves as a valid proxy for humans, the porcine femur exhibits distinct histomorphological features, particularly in the organization of primary cortical bone and remodeling patterns (Enlow, 1990; Taguchi et al., 2021). These distinctions are essential for accurate interpretation of comparative data, especially when analyzing bone microstructure and the mechanical properties of skeletal tissue.

1.4.1 Macroscopic anatomy of the porcine femur

From a comprehensive perspective, the porcine femur may be segmented into three principal regions, each characterized by distinct morphological and functional features (Fig. 1):

- Proximal epiphysis**:** this region comprises the femoral head, a hemispherical joint surface covered with hyaline cartilage that articulates with the shaft through the femoral neck. It also includes the greater and lesser trochanters, bony prominences that serve as attachment points for major muscle groups. In pigs, the distinctive morphology of the femoral head and neck is engineered to optimize load distribution across the acetabulum, thereby augmenting joint stability during movement (Enlow, 1990).
- Diaphysis**:** this region features a thick, dense cortical layer surrounding the central medullary cavity and nutrient canal. Due to its organized structure and the predominance of compact bone tissue, the diaphysis is the preferred site for microstructural and ultrastructural investigations, particularly utilizing scanning electron microscopy (SEM) (Shah et al., 2019; Taguchi et al., 2021).
- Distal epiphysis encompasses the medial and lateral condyles, the intercondylar fossa, and the femoral trochlea, which articulate with the patella. In comparison to the diaphysis, this region contains a higher proportion of trabecular (spongy) bone, aiding in the absorption of mechanical stresses at the knee joint.

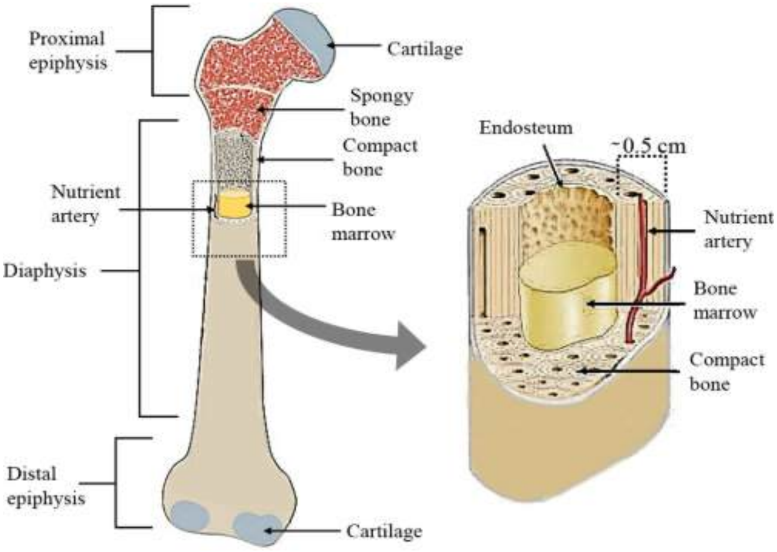


Figure 1 – Schematic representation of the macroscopic anatomy and internal structure of the femur. On the left: subdivision into epiphyses (proximal and distal) and diaphysis, highlighting the nutrient canal and tissue distribution. On the right: detail of a transverse section of the diaphysis showing the organization of the compact cortex, the endosteum, and the medullary cavity.

1.4.2 Ultrastructural examination with Scanning Electron Microscopy (SEM)

Ultrastructural analysis using SEM enables the examination of the hierarchical organization of bone tissue at multiple resolution levels, providing important insights into the functional morphology of the bone matrix (Shah et al., 2019):

- Micrometric Scale (μm): At this level, the investigation focuses on the organization of vascular canals and osteons. It is feasible to differentiate Haversian canals, which run longitudinally, from Volkmann's canals, which are oriented transversely, thereby permitting the passage of blood vessels and nerves through the compact cortical bone (Fig. 2) (Shah et al., 2019). An overview of the cortical structure (Fig. 3) indicates that in juvenile pigs, plexiform (or fibrolamellar) bone predominates; this bone exhibits a regular porosity with osteocyte lacunae uniformly dispersed throughout the matrix (Taguchi et al., 2021). This architecture, characteristic of swiftly growing species, features a highly organized arrangement of vascular canals within the primary bone matrix. Furthermore, the examination of the periosteal and endosteal surfaces facilitates the mapping of resorption lacunae and cortical porosity, parameters that show significant variation with age and bone remodeling rate.

- Nanometric scale (nm): at higher magnifications permit the observation of the intricate lacuno-canalicular network and the organization of mineralized type I collagen fibrils (Georgiadis et al., 2016). At this level, interspecies variations are minimal: both human and porcine specimens display the same D-periodicity of collagen fibrils, approximately 64–67 nm, thereby confirming the structural homogeneity of bone at the molecular level (Raguin et al., 2021). The employment of Scanning Electron Microscopy (SEM), often in conjunction with acid-etching techniques (Fig. 3), facilitates the high-precision visualization of canalicular interconnections, which are crucial for bone metabolism (Kubek et al., 2010).

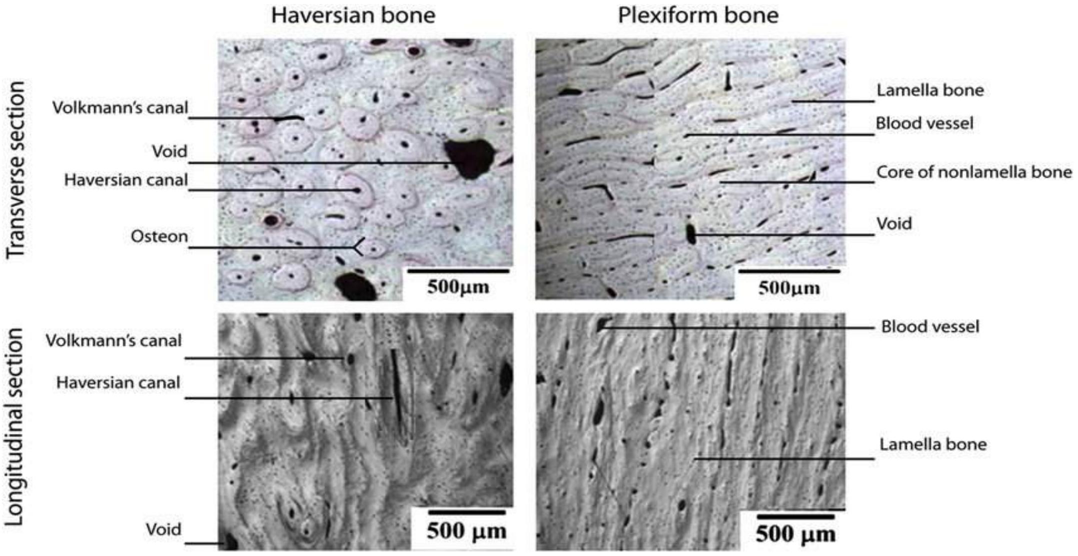


Figure 2 – Micrographs of transverse (top) and longitudinal (bottom) sections illustrating the organization of Haversian bone, including osteons, Haversian canals, and Volkmann's canals, as well as plexiform bone, characterized by parallel lamellae, blood vessels, and void spaces.

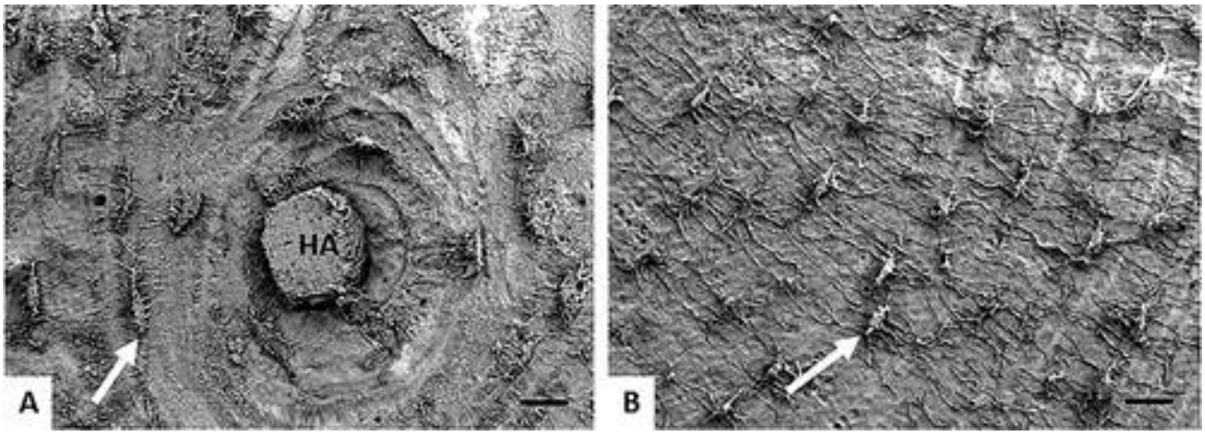


Figure 3 – SEM micrographs of cortical bone at different levels of observation. (A) Transverse section of an osteon showing the Haversian canal (HA) and surrounding concentric lamellae, representative of the microstructural organization of compact bone. (B) Detail of the bone surface highlighting the orientation of lamellar structures and collagen fibrils, enhanced by sample preparation, indicative of the hierarchical organization of bone tissue.

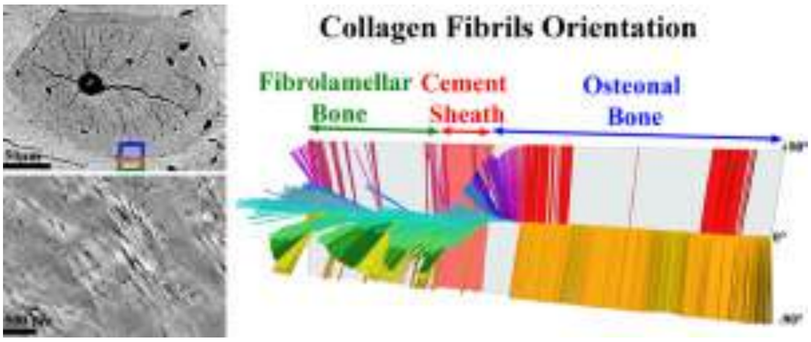


Figure 4 – Micrographs and representative schematic illustrate the arrangement, alignment, and three-dimensional reconstruction of collagen fibril orientation in different bone types, specifically fibrolamellar and osteonal bone, highlighting angular differences, hierarchical organization, and the nanoscale architecture of bone tissue.

1.4.3 Ultrastructural differences between porcine and human femur

Although the pig functions as an effective experimental substitute, comparative analysis indicates notable differences that should be taken into account during data interpretation. These disparities are observed at the histomorphological and microstructural levels, as well as in the distribution of mineralization and remodeling dynamics, and are especially prominent within the diaphyseal cortical bone (Fig. 5).

While adult human cortical bone exhibits a dense network of secondary osteons, juvenile pigs predominantly demonstrate plexiform (fibrolamellar) bone. This configuration enhances uniformity in the porcine femur and results in more isotropic mechanical behavior within plexiform regions, in contrast to the pronounced local anisotropy typically observed in human bone (Taguchi et al., 2021). Conversely, at the nanometric level, disparities between the two species are minimal: both exhibit a similar organization of collagen fibrils (Georgiadis et al., 2016).

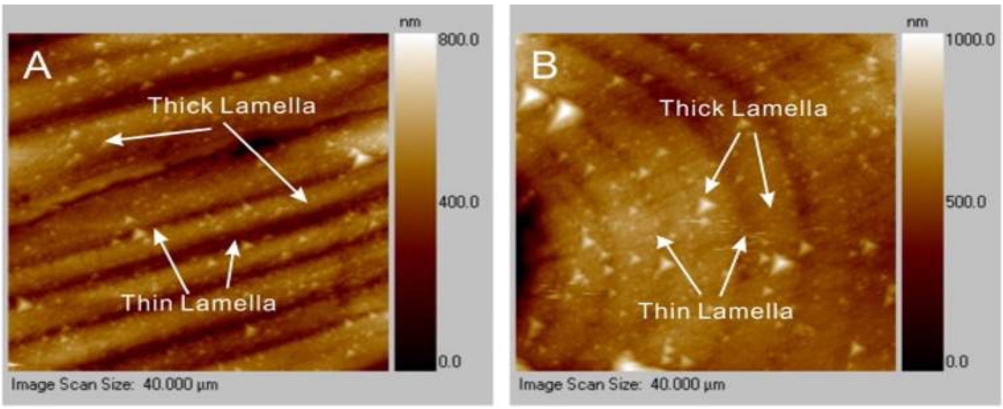


Figure 5 – AFM topography of plexiform bone (A) and secondary osteonal bone (B). The atomic force microscopy images highlight the organization of thick lamellae and thin lamellae in the two tissue types:
(A) Plexiform bone: characterized by a regular and parallel lamellar organization.
(B) Osteonal bone: exhibiting the concentric arrangement typical of bone remodeling.

The primary distinctions are summarized in **Table I**:

Table I - Comparative analysis of the morpho-structural and biomechanical characteristics of the swine (plexiform) and human (osteonal) cortical bone.

Category	**Model pig (Sus sofa)**	**Human model**
Predominant type	Marked presence of plexiform primary bone (especially in young/subadults).	Cortical dominated by secondary osteons (Havers systems).

Microstructure	Vascular canals in regular lattice; lower density of secondary osteones.	"Mosaic" structure due to high intracortical remodelling.
Osteons	Less numerous; prevalence of longitudinal primary channels.	Numerous, irregularly distributed; average diameter 150–250 mm.
Canals	Often more regular layout in primary areas.	More complex network due to secondary remodelling.
Growth and turnover	Rapid: extended primary deposition and accelerated skeletal growth.	Slow: gradual skeletal growth with high remodelling.
Subchondral bone	Generally thicker articular cartilage.	Progressive subchondral mineralisation.
Mineralisation	More homogeneous (in the young); lower heterogeneity.	Greater heterogeneity due to temporal remodelling differences.
Collagen (Type I)	More uniform fibrillar orientation in plexiform regions.	Greater directional variability due to remodelling effect.
Lacunar density	More orderly distribution; density related to rapid growth.	Relatively high density with less regular distribution.
Periostal surface	More regular, associated with primary deposition.	Greater irregularity related to mechanical adaptation and remodelling.
Biomechanics	More isotropic behaviour; greater structural homogeneity.	High local anisotropy; greater susceptibility to fractures (senescence).

1.4.4 Validity and limitations of the model

Sus scrofa domesticus is currently recognized as a gold standard model in forensic and biomedical research due to its notable morphological similarities to humans, thereby serving as a valuable experimental surrogate. However, the validity of this model requires assessment at both the microstructural and nanometric levels, highlighting its benefits as well as its intrinsic limitations.

The comparative analysis affirms that the porcine femur constitutes a highly reliable model for biomechanical and ultrastructural research, primarily owing to the stability of bone matrix organization at the nanometric scale. Scanning Electron Microscope (SEM) examinations demonstrate that the D-periodicity of collagen fibrils (approximately 64–67 nm) and the arrangement of the mineral matrix are fundamentally congruent across both species (Georgiadis et al., 2016; Shah et al., 2019). This ultrastructural uniformity provides a robust basis for high-resolution investigations, thereby establishing the pig as an exemplary reference for the study of diagenetic processes and tissue degradation within complex environments such as marine settings (Raguin et al., 2021).

Notwithstanding these similarities, the utilization of pigs presents certain limitations owing to significant histomorphological differences (Enlow, 1990). While adult human bone predominantly exhibits an osteonal pattern (secondary lamellar bone) resulting from continuous remodeling, the porcine femur—particularly in sub-adult specimens frequently employed in research—primarily consists of plexiform bone (Taguchi et al., 2021). This structure, which is associated with rapid growth, provides enhanced structural consistency and more uniform mechanical properties in comparison to the substantial local anisotropy observed in human bone.

In conclusion, although the porcine femur does not precisely replicate complex processes such as mature osteonal patterning, chronic intracortical remodeling, or the heterogeneous distribution of mineralization linked to aging, the significant similarity observed at the nanometric level justifies the utilization of *Sus scrofa* femurs as a reference standard for controlled biomechanical investigations and comparative ultrastructural analysis (Shah et al., 2019).

1.5 Degradation of terrestrial mammal bones in marine environments: a taphonomic overview

Understanding the fate of mammalian skeletal remains in marine environments is crucial for both forensic and archaeological taphonomy, as it provides the conceptual framework for interpreting degradation processes affecting pig femora (Anderson & Bell, 2016).

In contrast to the extensive decay models observed in large whale-fall ecosystems, which may require several decades for the complete breakdown of the skeletal remains, the post-mortem process of carcasses comparable in size to human bodies occurs at a substantially faster and more dynamic rate (Anderson & Bell, 2016). Experimental investigations utilizing pig carcasses—commonly regarded as a suitable proxy for human remains due to comparable body size and skin composition—have identified a sequence of taphonomic events that unfold through distinct stages.

1.5.1 Decomposition in marine waters: scavenging and quick skeletonization

The initial phase of marine decomposition in well-oxygenated deep waters is primarily facilitated by scavengers that swiftly remove soft tissues. Research conducted in the Salish Sea at an approximate depth of 300 meters demonstrated that pig carcasses could undergo complete skeletonization within three days during the autumn season and within four days during the spring (Anderson & Bell, 2016). This rapid tissue removal is primarily attributed to lysianassid amphipods, particularly species within the *Orchomene* complex. These scavengers are attracted to carcasses promptly upon their arrival on the seafloor and rapidly form dense aggregations that can reach a thickness of up to 4 cm (Anderson & Bell, 2016). Their feeding behavior generally begins internally within the carcass and progresses outward, frequently leaving the skin as the final tissue consumed.

In addition to the removal of soft tissue, the vigorous activity of these amphipods can induce significant biological reworking of the surrounding environment and displace skeletal elements from their original anatomical positions (Anderson & Bell, 2016).

Other organisms may also interact with the carcass; however, their role during the initial stages of decomposition appears to be minimal. Large predators, such as the bluntnose sixgill shark (*Hexanchus griseus*), might occasionally be attracted to carcasses but generally lose interest once amphipod swarms dominate the resource. Likewise, detritivorous species such as *Pandalus platyceros* and *Metacarcinus magister* typically consume the remaining tissues only after amphipods have completed skeletonizing, primarily ingesting residual cartilage (Anderson & Bell, 2016).

This sequence of colonization exemplifies a multi-stage taphonomic process, whereby rapid skeletonization driven by macro-scavengers is followed by extended microbial alteration of the remaining skeletal material.

1.5.2 The significant impact of environmental conditions on both macroscopic and microscopic bioerosion

The processes of skeletonization and subsequent bioerosion are interconnected and significantly influenced by environmental conditions on the seafloor (Anderson & Bell, 2016).

A principal influential factor is the availability of dissolved oxygen. During periods of increased amphipod activity, localized decreases in oxygen levels have been documented, potentially leading to hypoxic microenvironments that exclude other organisms unable to tolerate low oxygen conditions (Anderson & Bell, 2016).

Sedimentation is also a crucial environmental factor affecting skeletal preservation (Bell & Elkerton, 2008). Evidence from human remains recovered from the wreck of the *Mary Rose* (1545 CE) indicates that rapid burial in sediments can substantially inhibit the boring activity of endolithic organisms. Bones retrieved from the earliest sediment layer, deposited within a few months after the sinking, exhibited minimal to no alteration, whereas bones from subsequent layers, formed over decades in a more open marine environment, demonstrated extensive peripheral bioerosion (Bell & Elkerton, 2008).

Comparable patterns have been documented in porcine carcass experiments, wherein remains interred within sediment during the spring season were less appealing to scavengers than carcasses that were left exposed during autumn deployments (Anderson & Bell, 2016). These findings indicate that bioerosion should not be construed as an inevitable or linear process, but rather as a phenomenon profoundly affected by local taphonomic and environmental factors (Bell & Elkerton, 2008; Anderson & Bell, 2016).

1.5.3 Microbial decomposition and Post-Mortem micromodifications

Following the removal of soft tissues, skeletal remains become susceptible to microbial decomposition processes (Jans, 2008). At the microscopic level, degradation patterns exhibit considerable variation between aquatic and terrestrial environments (Jans, 2008; Pesquero et al., 2018).

In aquatic environments, microborings may be produced by fungi and cyanobacteria that can dissolve the mineralized bone matrix, creating branching tunnels known as *Wedl tunnels* (Jans, 2008). In contrast, bacterial degradation that is typically observed in terrestrial contexts results in Microscopical Focal Destructions (MFD), characterized by small pores and channels ranging from approximately 0.1 to 2.0 μm in diameter and closely associated with bone microvascular structures such as Haversian and Volkmann canals (Jans, 2008; Pesquero et al., 2018).

Marine micromodifications demonstrate distinct characteristics when compared to terrestrial bacterial alterations. Notably, they predominantly occur on the external surfaces of bones and teeth, whereas bacterial decomposition in terrestrial environments frequently initiates within internal blood vessel structures (Bell & Elkerton, 2008; Pesquero et al., 2018).

Marine endolithic micro-borings generally exhibit larger diameters, typically ranging from approximately 5 to 19 μm, and frequently display branching or bifurcating configurations (Bell & Elkerton, 2008; Pesquero et al., 2018). An essential diagnostic characteristic for distinguishing various types of microbial alteration is the presence or absence of a hypermineralized margin encircling the microtunnels (Jans, 2008; Bell & Elkerton, 2008; Pesquero et al., 2018).

Finally, the experimental work conducted by Ascenzi and Silvestrini (1984) furnished evidence indicating that certain organisms responsible for marine bone perforations may be amoeboid protozoa capable of actively phagocytosing apatite crystals from the bone matrix.

1.6 Ichnotaxonomy and interpretations of bone borings

The necessity for a structured classification system for evidence of biological activity on rigid substrates, such as bone, has prompted the advancement of ichnotaxonomy (Höpner & Bertling, 2017). This systematic approach surpasses mere morphological description, offering a foundation for ecological and behavioral interpretations of organisms responsible for trace production. In recent years, there has been a surge of interest in the classification of borings in bones, underscoring the increasing acknowledgment that this substrate demands specialized analytical consideration.

Ichnotaxonomy constitutes a specialized discipline within the field of paleontology that is dedicated to the systematic classification of biological traces produced by living organisms. Unlike traditional taxonomy, which primarily concerns itself with the classification of the organisms themselves, ichnotaxonomy focuses on ichnofossils—fossilized marks of biological activity, including burrows, borings, tracks, and trails. In the realm of forensic science, these traces include perforations and other modifications observed on bone surfaces. Therefore, within forensic taphonomy, ichnotaxonomy functions as a vital tool for identifying the agents responsible for bone boring and for reconstructing the post-mortem history of skeletal remains.

Recent studies have emphasized the importance of ichnotaxonomic analysis as a vital diagnostic instrument for differentiating modifications originating in terrestrial environments from those exclusively linked to marine contexts, thereby enhancing estimates of submersion duration (Guareschi et al., 2023). The identification of particular ichnotaxa can offer invaluable evidence concerning the environmental conditions encountered by a body—such as subaerial rather than submerged settings—as well as insights into the timing and progression of decomposition.

The integration of ichnotaxonomy into forensic investigations enhances the identification of agents responsible for bone modifications and facilitates the reconstruction of crime scenarios, thereby contributing significantly to the broader taphonomic analysis of skeletal remains.

1.6.1 Bone as an autonomous substrate in bioerosion

A fundamental principle in ichnotaxonomy asserts that the substrate in which a trace is produced may serve as a high-level classification criterion (ichnotaxobase) (Höpner & Bertling, 2017). Within this framework, bone should be regarded as an autonomous substrate, on par with lithic (rock) and xylitic (wood) substrates, in terms of taxonomic significance (Höpner & Bertling, 2017). This distinction is grounded in the concept of ichnotaxobases, which posits that the biomineralized nature of bone tissue distinctly influences the boring strategies employed by organisms, in contrast to those observed in rock or wood (Pirrone et al., 2014).

The fundamental rationale behind this proposal is predicated on the observation that most boring organisms tend to specialize in a singular type of substrate. Such specialization arises from the structural, mineralogical, and nutritional differences distinguishing bone, rock, and wood (Höpner & Bertling, 2017). Consequently, the identification of a specific ichnogenus within bone unequivocally indicates the substrate type involved and, accordingly, the ecological characteristics of the trace-producing organism (Höpner & Bertling, 2017). From this perspective, bone should not be regarded merely as an inert hard substrate; rather, it constitutes a biomineralized tissue whose distinctive organic and inorganic composition directly influences the boring strategies of colonizing organisms (Guareschi et al., 2023).

This principle applies exclusively when the bone maintains its original structure and composition, having not undergone significant modifications through diagenetic processes. Diagenesis pertains to the chemical and physical alterations experienced by sediments subsequent to their initial deposition and during or after their transformation into consolidated rock, excluding superficial modifications (Höpner & Bertling, 2017). In cases where the bone has experienced processes such as cementation or lithification, signs of bioerosion should alternatively be classified as if they were embedded within a lithic substrate (Höpner & Bertling, 2017).

1.6.2 Ichnogenera specific to bone and their classification

In order to address the inconsistency in nomenclature and to formalize the classification of bioerosion traces in bone, a new ichnofamily named Osteichnidae has been proposed, along with several ichnogenera specifically associated with bone borings (Höpner & Bertling, 2017). This framework conforms to the criteria established by Pirrone et al. (2014), who identified bone substrate as a pivotal taxonomic parameter capable of influencing trace morphology in ways that differ from those observed in rocks or wood.

The proposed nomenclature intends to surmount the constraints of previously established ichnogenera—such as *Gastrochaenolites* and *Trypanites*, which were originally defined for lithic substrates—and has subsequently been validated in global revisions of ichnotaxa (Wisshak et al., 2019).

Within the Osteichnidae, the principal ichnogenera have been delineated as follows (Höpner & Bertling, 2017):

- *Osteichnus* refers to uncomplicated U-shaped cavities excavated within bone substrates, generally oriented obliquely in relation to the surface.
- *Clavichnus* – unbranched, slender claviform tubes identified within bone substrates, typically oriented in a more or less perpendicular manner relative to the surface.
- *Cuniculichnus* – tunnels within bone oriented approximately parallel to the surface, characterised by a rounded cross-section. Early developmental stages appear as circular perforations or straight grooves with tapered ends. This ichnogenus encompasses a range of morphologies interpreted as successive growth stages of a single organism, suggesting a progressive pattern of excavation activity.

Within this context, the study conducted by Guareschi et al. (2023) emphasises the important role of poriferans in marine bioerosion, particularly boring sponges belonging to the genus *Cliona*. These organisms produce excavation chambers whose morphology reflects a specific adaptive response to the bone matrix. When correctly identified, such traces represent unequivocal indicators of prolonged marine exposure and can therefore provide objective evidence for reconstructing the post-mortem history of skeletal remains.

Ichnotaxonomy, therefore, transcends mere morphological description by providing an interpretative framework that associates trace characteristics with the behavioural ecology of the organisms that produce them. This approach significantly contributes to the reconstruction of taphonomic and post-mortem processes.

1.7 The unique environment of harbour contexts and its application to the Port of Genoa

The review of existing literature underscores that bioerosion and skeletal decomposition are closely linked to local environmental conditions. This principle is particularly relevant when evaluating the Port of Genoa, a coastal and harbor marine environment distinguished by dynamics that markedly differ from those typical of open marine contexts.

Harbour environments such as Genoa exemplify unique marine settings that are continually influenced by the interplay of natural processes and human activities. These features create environmental conditions that directly impact the preservation and degradation of skeletal remains. A primary factor is sedimentary dynamics. Ports regularly undergo dredging operations, construction projects, and seabed reworking, all of which can substantially modify sedimentation rates. Rapid burial of remains by sediments can inhibit the activity of endolithic organisms, thereby safeguarding bones from bioerosion (Bell & Elkerton, 2008). Conversely, seabed regions characterized by stronger currents or reduced sediment accumulation tend to offer prolonged exposure, enabling scavenging organisms to colonize and alter the remains.

PURPOSE OF THE STUDY

This thesis aims to investigate the possibility of estimating the Post-Mortem Submersion Interval (PMSI) from skeletal remains by analysing biological colonisation in a harbour environment. The PMSI refers to the time elapsed between the immersion of remains and their recovery in an underwater context. Its estimation represents a crucial component of forensic investigations, as it provides essential information for reconstructing the temporal sequence of post-mortem events.

This study explicitly emphasizes the succession and dynamics of organisms colonizing bone during submersion, with a particular focus on the interactions between environmental factors and degradation processes. The objective is to enhance understanding of post-mortem processes in harbor environments and to provide preliminary insights that could inform future advancements in the chronological estimation of skeletal remains recovered from aquatic settings.

MATERIALS AND METHODS

3.1 Immersion and recovery of samples

In the present study, a total of ten pig femurs (*Sus scrofa domesticus*) were utilized as analogues for human bone. The experimental procedures were conducted at the experimental floating platform of the National Research Council of Italy (CNR), located within the Port of Genoa. This site was selected to replicate forensic conditions in coastal and anthropogenically impacted waters.

The samples were divided into two experimental groups to evaluate the influence of their position within the water column on degradation processes. Five femurs were suspended at a depth of approximately 5 meters from the surface, while the remaining five were placed in direct contact with the seabed at a depth of approximately 14 meters.

Immersion commenced in May 2025 and persisted for a total of six months. Samples were collected monthly, excluding August, with the experimental cycle concluding in November 2025. During each sampling session, one bone was retrieved from each immersion condition (suspended and seabed), facilitating a systematic comparison of bone alteration and biological colonization over time.

Figure 6 - Experimental Marine Station located in the Port of Genoa, where the diving period of the samples was carried out. Images available on the website https://www.ias.cnr.it/infrastrutture/

3.2 Post-recovery treatment and storage

Immediately following recovery, the bones were properly stored in refrigerated containers and transported to the laboratories of the Department of Earth, Environment and Life Sciences (DISTAV) at the University of Genoa.

Upon entering the laboratory, the samples were meticulously labelled, securely packaged, and stored in a freezer to prevent post-recovery degradation processes. Following the completion of the entire recovery cycle, all bones underwent an air-drying process under controlled environmental conditions prior to subsequent morphological analyses.

3.3 Sampling for metagenomic analysis

To characterize the microbial communities and microbiome involved in bioerosion processes, a targeted sampling protocol was executed on the femoral diaphysis. For each specimen, biological material was collected employing both surface and destructive sampling methodologies.

Surface sampling was conducted employing sterile swabs to collect bacterial biofilm adhered to the external cortical surface. This method was complemented by destructive sampling, which involved the physical excision of aliquots of cortical bone tissue from the central shaft of the bone. This procedure was carried out manually employing a saw.

Both the bone fragments and the swab samples were placed in sterile tubes and stored in a freezer to preserve the integrity of the genetic material and to prevent contamination or thermal degradation. The material was subsequently sent to specialised laboratories in Trieste for DNA extraction, sequencing, and subsequent analyses, with the ultimate aim of identifying the microbial taxa responsible for bone degradation.

3.4 DNA extraction

The protocol commenced by adding a solution of tetrasodium pyrophosphate (final concentration of 5 mM) to each sample, followed by a 15-minute incubation in dark conditions and three cycles of sonication, each lasting 1 minute at 60 W (Branson Sonifier 2200). Component separation was achieved through centrifugation at 800×g for 10 minutes; the resulting sediments were resuspended in 4 mL of virus-free seawater and subjected to additional centrifugation cycles, a procedure repeated three times, with the respective supernatants collected for subsequent analysis. To eliminate extracellular DNA, the pooled supernatants were treated with DNase I (1 U/mL) for 15 minutes at room temperature in the absence of light. Removal of cellular organisms was conducted via pre-filtration using 0.2 µm membranes (Whatman Anopore), followed by concentration of viral particles onto 0.02 µm Al_2O_3 filters (Anodisc, diameter 47 mm). Genetic material was extracted from the filters using the DNeasy PowerSoil kit (Qiagen), in accordance with the manufacturer's instructions. The obtained viral DNA was divided into two aliquots: the first was used directly for library preparation and sequencing; the second was amplified utilizing Phi29 polymerase (GenomiPhi kit) and random hexameric primers, then purified using the DNeasy PowerClean kit (Qiagen). DNA quantification was performed fluorometrically using SYBR Gold on the NanoDrop ND-3000 platform, while fragment size distribution and quality were assessed via microfluidic electrophoresis on the Agilent 2100 system employing the DNA High-Sensitivity (HS) kit.

3.5 Ichnotaxonomic analysis and Scanning Electron Microscopy (SEM)

For the investigation of traces produced by colonizing organisms (ichnotaxonomy), quadrangular bone sections were extracted from the epiphyses of femurs located on the seabed, excluding those suspended within the water column (Fig. 7). Sectioning was conducted employing a rotary micromotor fitted with a diamond cutting disc, facilitating the extraction of standardized segments of both trabecular and cortical bone tissue.

Once removed, the samples were transferred to containers with hydrogen peroxide (H_2O_2) to meticulously eliminate organic residues and cleanse the bone surfaces. Subsequently, the specimens were prepared for examination using scanning electron microscopy (SEM), with the objective of documenting micro-borings and structural modifications within the bone matrix (Fig. 8).

Figure 7 - Instrumentation for cutting and sample preparation: micromotor with diamond disc, femoral epiphysis and test tubes intended for H_2O_2 treatment.

Figure 8 - Quadrangular bone sections mounted on stubs for scanning electron microscopy (SEM).

3.6 Identification of the colonising macrofauna

The taxonomic analysis of the epibiotic macrofauna was systematically conducted at the conclusion of the bone dehydration process. This method facilitated precise observation of sessile organisms and the remnants of vagile fauna that persisted attached to the bone matrix post-dehydration. Specimens were examined in the laboratories of the Department of Earth, Environment and Life Sciences (DISTAV) at the University of Genoa, utilizing stereomicroscopes. The primary systematic groups were classified to the genus level and, where feasible, to the species level.

For the samples suspended, the investigation additionally incorporated a quantitative analysis aimed at determining the extent of biological coverage. Specifically, individual counts were conducted for the primary colonizing groups, including Serpulidae, Bryozoa, and Balanidae (barnacles), on each bone surface. The resulting numerical data are presented in Table ?. The cataloging of taxa and their associated numerical data was employed to reconstruct ecological succession over months of immersion, thereby providing the foundational information necessary to interpret the biological impact on the degradation of the bone matrix.

3.7 Estimation of the percentage of surface coverage

To quantify the temporal progression of biological colonization, the percentage of total surface coverage for each sample was estimated utilizing digital image processing techniques. The analysis was performed on high-resolution photographs obtained subsequent to the drying phase for each facet of the specimen, thereby ensuring a comprehensive assessment of the anterior, posterior, and lateral surfaces.

The protocol entailed differentiating the original bone surface from regions inhabited by epibiotic biomass. Consequently, the coverage assessment incorporated both sessile macrofauna—such as cirripedes, serpulids, and bivalves—and colonial organisms as well as vegetal coverings, including encrusting bryozoans and algal films. For each temporal interval, the coverage value was calculated as the ratio of the area designated as colonised to the total area of the bone outline. This methodology facilitated the transformation of qualitative observations into quantitative data, which are instrumental in determining both the rate of colonisation and the extent to which the bone surface was protected from the surrounding environment.

3.8 Statistical analysis of variance

To standardize the quantification of settled organisms, each bone specimen was initially divided into three distinct anatomical sections: two epiphyseal portions (proximal and distal) and one diaphyseal portion (Fig. 9). This subdivision facilitated the assessment of potential spatial selectivity in biological colonisation.

Differences among communities established on the bone samples were examined using permutational analysis of variance (PERMANOVA), considering the following factors: time (five levels, fixed), bone portion (three levels, fixed), and species (three levels, fixed).

The original abundance dataset, encompassing the three identified taxonomic groups, was employed to compile the final data matrix. A square-root transformation was implemented to mitigate the influence of OTUs (Operational Taxonomic Units) that exhibited excessive prevalence. All statistical analyses were conducted using the Jamovi software.

Figure 9 - Division of the samples into three anatomical sections for the standardisation of the quantification of the settled organisms: two epiphyseal portions (proximal and distal) and one diaphyseal.

RESULTS

4.1. Biofouling and ecological succession analysis

The experimental exposure of suspended sog femurs in the Port of Genoa permitted the observation of dynamic biological colonization. Bone tissue offered a stable substrate for the attachment of a diverse biological community, facilitating the settlement and proliferation of numerous species.

Table II - Taxonomic list of the fouling community documented on the bone substrates during the immersion experiment.

Taxon
Bryozoa, Watersiporidae
Watersipora cucullata
Bryozoa, Schizoporellidae
Schizoporella errata
Bryozoa, Bugulidae
Bugula neritina
Bryozoa, Aeteidae
Aetea sp.
Arthropoda, Balanidae
Amphibalanus amphitrite
Arthropoda, Caprellidae
Caprella sp.
Arthropoda, Ischyroceridae
Jassa cfr. *falcata*
Mollusca, Mytilidae
Mytilus galloprovincialis
Mollusca, Anomiidae
Anomia ephippium
Anellida, Serpulidae
Hydroides elegans

4.1.1. Colonisation chronology

Since the initial month of immersion (June 2025), the development of a felt-like layer of algae, which cannot be taxonomically identified, has been observed on bones suspended at a depth of five meters. At this early stage, the bone surface exhibited no macroscopic signs of erosion. After one month, amphipod crustaceans were highly abundant (Fig. 11), likely attributable to the species *Jassa falcata*, which feeds on the periosteum; on clean bones, some individuals of the serpulid *Hydroides elegans* (Fig. 12) were identified. During this initial period, the total number of species recorded was two, covering approximately five percent of the area.

By the end of the second month, amphipods of the genus Jassa persisted, while *Hydroides elegans* exhibited greater abundance. Additionally, the settlement of the cirripede *Amphibalanus amphitrite* (Fig. 13, 14) was documented. The community was further supplemented by a complex assemblage of bryozoans (Fig. 15-19), comprising both encrusting species (*Watersipora cucullata* and *Schizoporella errata*) and erect species (*Bugula neritina* and species of the genus *Aetea*). Overall, there was an observable increase in biodiversity and biomass, with seven species identified and a coverage of 15%.

By the end of the fourth month, the community was supplemented with the bivalve *Mytilus galloprovincialis* (Fig. 20) and the amphipods of the genus *Caprella* (Fig. 21). While the number of species remained constant at seven, the proportion of covered area increased significantly, reaching approximately 80%.

Further additions to biodiversity were observed after the fifth month, with the appearance of the bivalve *Anomia ephippium* (Fig. 22, 23). At this stage, the number of species increased to eight, and the coverage reached approximately 85%.

Finally, in the sixth month of observation, a calcareous sponge (Fig. 24) was discovered; however, its taxonomic identification was not further investigated, and the succession reached its apex with nine species identified and approximately 95% surface coverage.

Figure 10 - Ecological succession on bone substrate over a six-month period. From left to right: samples S1 (1 month), S2 (2 months), S4 (4 months), S5 (5 months), and S6 (6 months).

Figure 11 – Detail of bone showing the presence of Amphipoda, genus *Jassa*.

Figure 12 – Portion of bone showing the presence of Serpulidae, *Hydroides elegans*.

Figure 13 – Portion of bone showing the presence of Balanidae, *A. amphitrite*.

Figure 14 – Detail of *A. amphitrite*.

Figure 15 – Portion of bone (epiphysis) showing the presence of Watersiporidae, *Watersipora cucullata*.

Figure 16 – Portion of bone showing the presence of *Schizoporella errata* (below) and *Bugula neritina* (above).

Figure 17 – Detail of Schizoporellidae, *S. errata*.

Figure 18 – Detail of Bugulidae, *B. neritina*.

Figure 19 – Detail of Serpulidae, *H. elegans* (left), and Aeteidae, genus *Aetea* (right).

Figure 20 – Detail of Mytilidae, *Mytilus galloprovincialis*.

Figure 21 – Detail of Caprellidae, genus *Caprella*.

Figure 22 – Detail of remains of Anomiidae, *Anomia ephippium* (left), and Serpulidae, *H. elegans* (right).

Figure 23 – Detail of a specimen of Anomiidae, *A. ephippium*.

Figure 24 – Detail of a calcareous sponge.

4.1.2 Abundance of colonising species and cover percentage

The examination of organism abundance revealed a trend shared by multiple species, characterized by a swift initial rise in numbers followed by a decline from the fourth month onward.

Table III – Quantitative count of the main groups of epibiont macro-fauna found on the samples in suspension (S) in the period May-November 2025.

Sample (Months)	Serpulidae	Bryozoa	Balanids
S1 (June)	2	-	-
S2 (July)	76	10	20
S4 (September)	50	5	69
S5 (October)	82	11	36
S6 (November)	69	15	30

Concerning the polychaete serpulid *Hydroides elegans*, initial observations during the first month documented eleven specimens across the entire bone sample. This number significantly increased to eighty individuals in the second month, then decreased to twenty-seven in the fourth month, and subsequently stabilized at approximately forty specimens during the fifth and sixth months.

A comparable pattern was observed for the cirripede *Amphibalanus amphitritis*: following its emergence in the second month with 20 individuals, the population reached a peak of approximately 60 individuals by the end of the fourth month, then declined to 30 over the following two months. Bryozoans, which had 6 colonies in total in the second month, also showed a marginal decline from the fourth month onward.

Overall, the organisms surveyed demonstrate a pattern of rapid colonization during the initial stages, followed by a steady decline in numbers beginning from the fourth month of immersion.

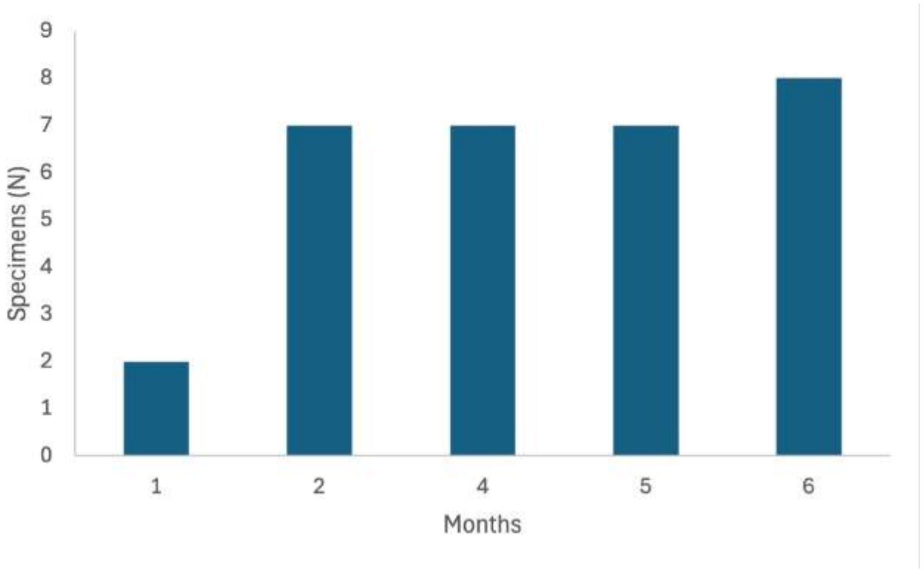


Figure 25 - Histogram representing the number of species found per sample.

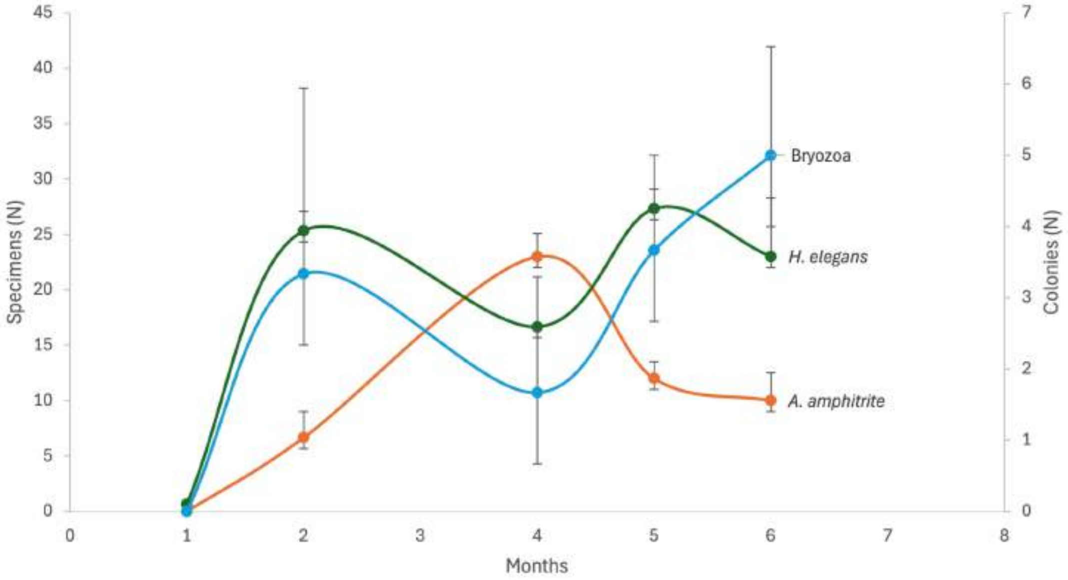


Figure 26 - Mean number of species and standard deviation identified on skeletal remains across the different sampling months. The graph highlights the temporal progression and the variability of the epilithic community colonization.

Concerning barnacles, the data demonstrate a temporally distinct colonization pattern: initial settlement predominantly occurs at the epiphyseal regions, subsequently progressing to the diaphysis (Fig. 27). Nevertheless, this discrepancy tends to decrease over the six-month period, after which the values are nearly homogeneous across various bone sections. Conversely, serpulids and bryozoans display a uniform settlement pattern (Fig. 28, 29), with no statistically significant differences in spatial distribution among the different portions of the specimen.

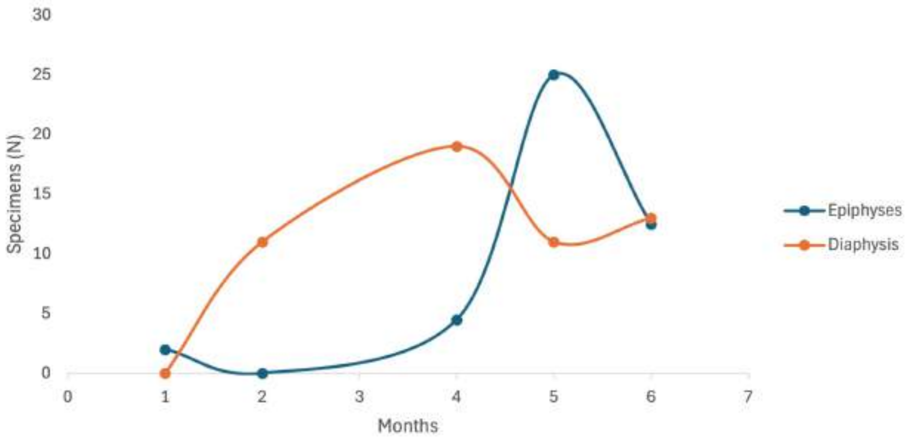


Figure 27 – Temporal dynamics of *Amphibalanus amphitrite* colonization across different bone regions (epiphyses and diaphysis).

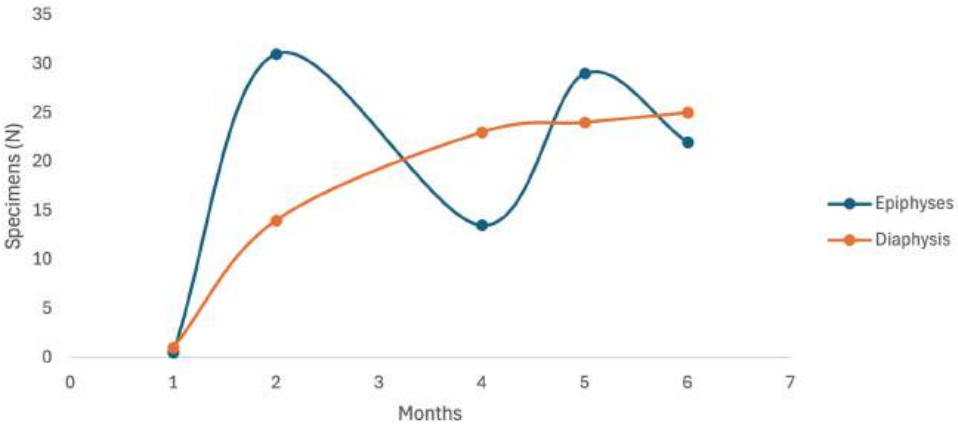


Figure 28 – Temporal dynamics of *Hydroides elegans* colonization across different bone regions (epiphyses and diaphysis).

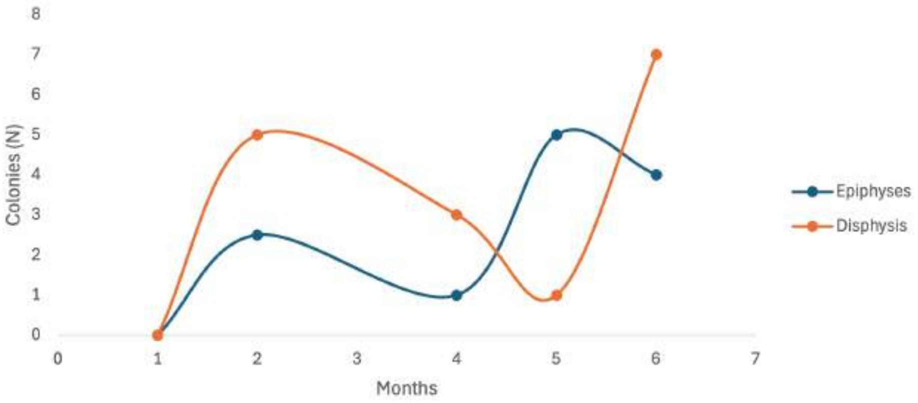


Figure 29 – Temporal dynamics of Bryozoa colonization across different bone regions (epiphyses and diaphysis).

Regarding surface coverage, the data showed a linear trend throughout the six-month immersion period, approaching nearly 100% by the end of the experimental period. This gradual increase reflects seasonal dynamics that facilitated the ongoing settlement and development of the benthic community commencing in June.

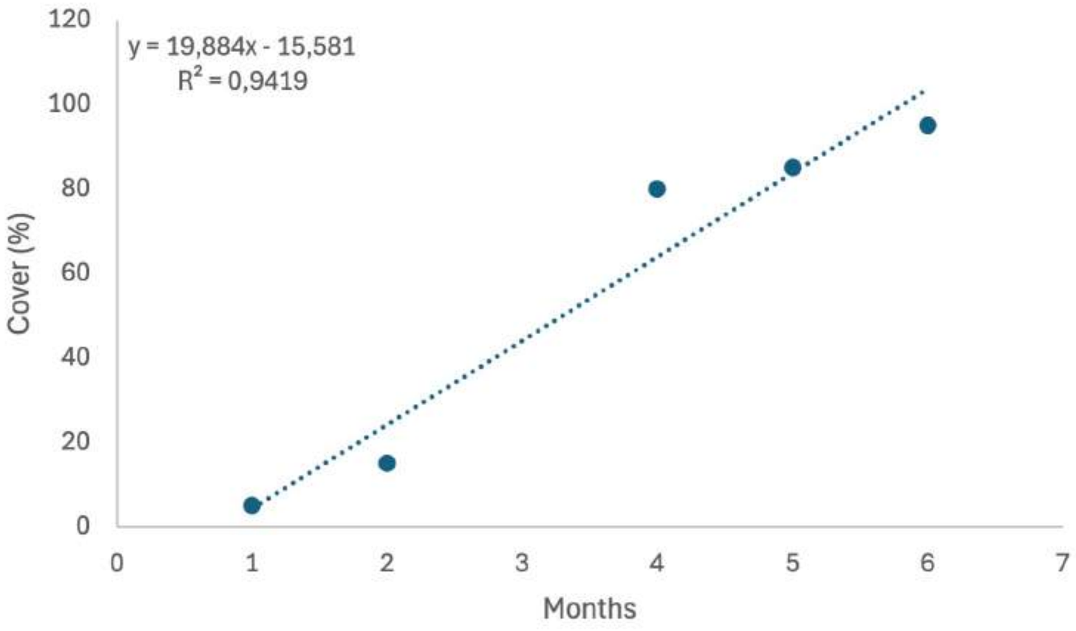


Figure 30 - Increase in the total surface coverage (%) of marine colonizers as a function of exposure time. The linear regression line is shown along with the corresponding coefficient of determination (R^2).

Table IV - Percentage of biotic coverage (Cover %) observed in analyzed samples relative to exposure duration.

Samples	**Cover (%)**
S1	5
S2	15
S4	80
S5	85
S6	95

4.2 Morphological and microstructural analysis of bone degradation via Scanning Electron Microscopy (SEM)

The micrographs obtained via scanning electron microscopy (SEM), included at the conclusion of this thesis, illustrate the progressive deterioration of the epiphyseal surface of bone specimens in direct contact with the seabed over a six-month immersion period.

Following the initial month of exposure (Fig. 31, row 1), the bone surface displays a finely spongy morphology, characterized by microcavities measuring 10-20 μm in diameter. At this preliminary stage, the vascular architecture remains distinctly visible: Volkmann's canals (diameters ranging from 400 to 600 μm) are clearly defined, originating from the periosteum and connecting to the underlying Haversian systems. In certain regions (Fig. 31, row 1), porosity increases, with microcavities reaching approximately 50 micrometers in diameter.

By the second month (Fig. 31, row 2), the widespread superficial sponginess starts to decrease, enabling more distinct visualization of the Haversian canals, which have diameters ranging from 20 to 50 μm. Under higher magnification, the trabecular surfaces begin to display fine, consistent erosion.

In the subsequent months (Fig. 31, rows 3–4), the surface gradually becomes increasingly irregular owing to ongoing erosive processes, resulting in a significant widening of the Haversian canals. By the sixth month, these canals attain diameters of at least 100 μm, signifying a considerable modification of the original compactness of the cortical surface.

This progressive alteration of cortical compactness and deterioration of the Haversian microstructure are consistent with taphonomic processes described by Bertoglio et al. (2021) in marine submersion contexts, although the present study provides micrometric quantification of canal widening in an animal model.

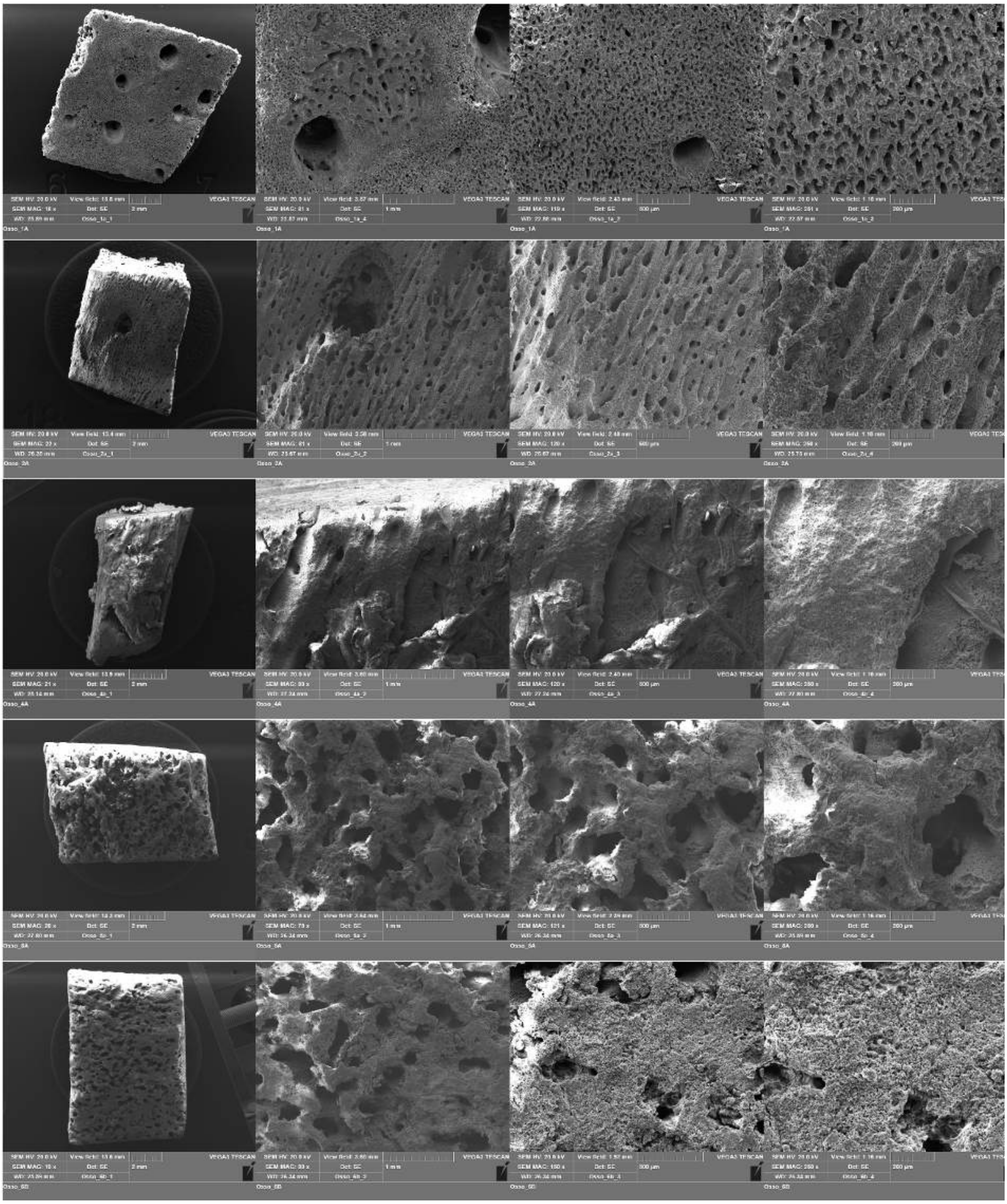
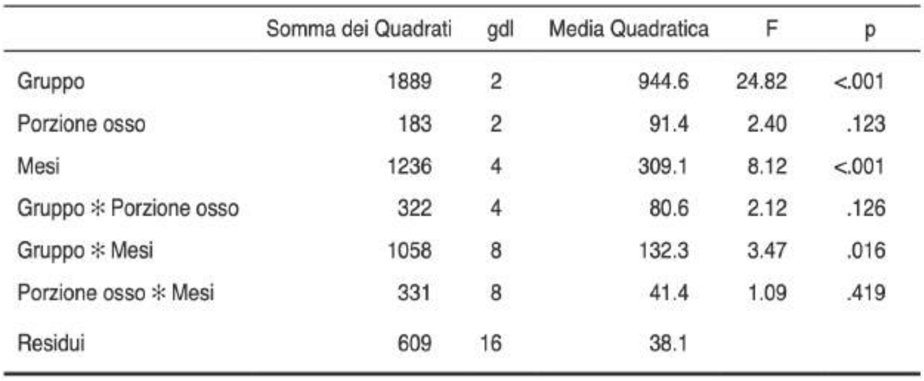


Figure 31 - Scanning electron microscopy (SEM) micrographs of bone sections at various magnifications, documenting bioerosion traces and structural alterations of the bone matrix.

4.3 Analysis of variance

The analysis of variance demonstrated significant differences at the taxonomic group and temporal levels (p < 0.001), while no significant differences were observed concerning the bone portion. Furthermore, the only statistically significant interaction among the factors was that between the taxonomic group and time (p = 0.016) (Table V).

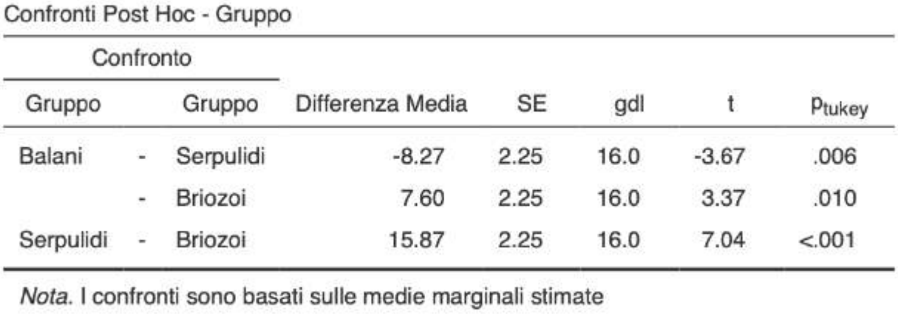
Table V - Results of the permutational multivariate analysis of variance (PERMANOVA) conducted on colonization data according to the factors Group, Bone Portion, and Months of Exposure.



	Somma dei Quadrati	gdl	Media Quadratica	F	p
Gruppo	1889	2	944.6	24.82	<.001
Porzione osso	183	2	91.4	2.40	.123
Mesi	1236	4	309.1	8.12	<.001
Gruppo ✻ Porzione osso	322	4	80.6	2.12	.126
Gruppo ✻ Mesi	1058	8	132.3	3.47	.016
Porzione osso ✻ Mesi	331	8	41.4	1.09	.419
Residui	609	16	38.1		

The post hoc comparison between groups revealed statistically significant differences among all three taxonomic groups.
Regarding the time factor, significant differences were observed only between the first month and all subsequent months (Table VI).

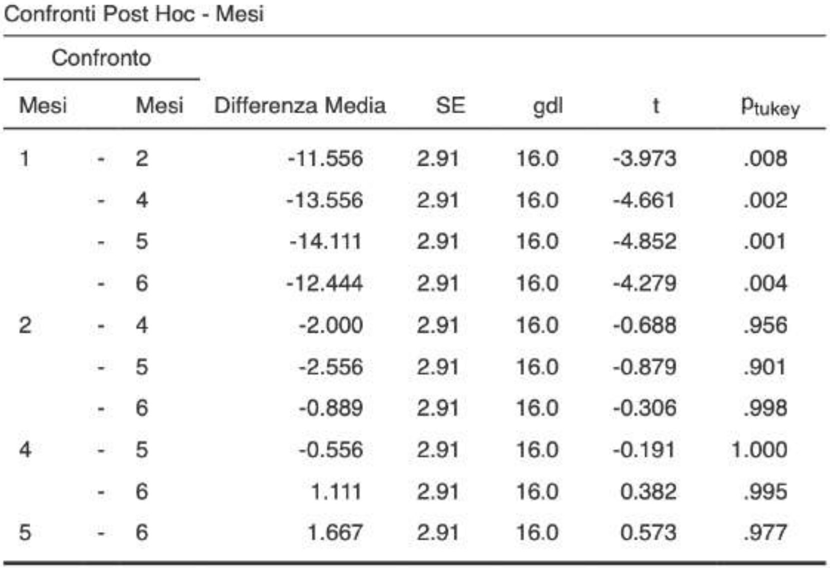
Table VI – Post hoc comparison analysis (Tukey's test) for the factors "Group" and "Months." The table reports the mean difference, standard error (SE), and significance level (p_{tukey}) for each pairwise comparison.



Confronti Post Hoc - Gruppo

Confronto							
Gruppo		Gruppo	Differenza Media	SE	gdl	t	p_{tukey}
Balani	-	Serpulidi	-8.27	2.25	16.0	-3.67	.006
	-	Briozoi	7.60	2.25	16.0	3.37	.010
Serpulidi	-	Briozoi	15.87	2.25	16.0	7.04	<.001

Nota. I confronti sono basati sulle medie marginali stimate



Confronti Post Hoc - Mesi

Confronto							
Mesi		Mesi	Differenza Media	SE	gdl	t	p_{tukey}
1	-	2	-11.556	2.91	16.0	-3.973	.008
	-	4	-13.556	2.91	16.0	-4.661	.002
	-	5	-14.111	2.91	16.0	-4.852	.001
	-	6	-12.444	2.91	16.0	-4.279	.004
2	-	4	-2.000	2.91	16.0	-0.688	.956
	-	5	-2.556	2.91	16.0	-0.879	.901
	-	6	-0.889	2.91	16.0	-0.306	.998
4	-	5	-0.556	2.91	16.0	-0.191	1.000
	-	6	1.111	2.91	16.0	0.382	.995
5	-	6	1.667	2.91	16.0	0.573	.977

DISCUSSION

5.1 Biology and ecology of the main colonizing species

The experimental phase conducted at the Port of Genoa demonstrates a diverse biological community composed of multiple taxa that colonize bone substrates at varying times and through different mechanisms. Consequently, interpreting submersion duration and the surface modifications observed on skeletal remains requires a biological and ecological framework focused on the primary colonizing species.

This section examines the biological characteristics, colonization techniques, and forensic significance of organisms within the phyla Bryozoa, Arthropoda, Mollusca, and Annelida. These colonizers range from pioneer species to those characteristic of mature fouling communities. Their analysis assists in reconstructing the ecological succession taking place at the interface between bone surfaces and the marine environment.

5.1.2 Bryozoa

The phylum Bryozoa primarily consists of marine coelomate organisms distinguished by complex, modular colony structures. Although historically considered a minor taxonomic group, recent studies emphasize their ecological significance in benthic ecosystems. Bryozoans serve as bioindicators and synthesize secondary metabolites—such as bryostatins—that hold notable potential in pharmacology and biotechnology (Gordon & Costello, 2016).

Colony development initiates with larval settlement. Following a brief planktonic phase, the larva undergoes metamorphosis, transforming into the ancestrula, which serves as the founding individual of the colony. Subsequently, modules known as zooids develop via asexual budding, a process referred to as astogeny. Although genetically identical, zooids may exhibit morphological and functional variations that enhance colony efficiency and survivability (Gordon & Costello, 2016).

All taxa discussed in this study belong to the order Cheilostomatida, the most diverse bryozoan group. Members of this group have a movable operculum that protects the zooidal opening. The operculum closes when the animal retracts the ciliated feeding structure known as the lophophore (Sokolover et al., 2018).

Genus *Aetea* Lamouroux, 1812

The genus *Aetea* comprises cheilostome bryozoans distinguished by simple zooids exhibiting a distinctive thread-like morphology. These zooids are composed of a creeping basal portion anchored to the substrate and an upright distal segment (Chae et al., 2017).

Each zooid consists of a prostrate region that attaches to the substrate and an upright tubular process commonly referred to as a “stem.” This process terminates in an expanded area comprising the membranous region and the operculum. The calcified external wall exhibits fine striations or punctuations, with their form and density serving as crucial diagnostic characteristics (Cook, 1977).

Colony development adheres to a conventional astogenetic pattern. The ancestrula gives rise to buds that extend linearly across the substrate, resulting in a network of stolons from which upright zooids ultimately emerge (Cook, 1977).

Species of this genus readily colonize flexible and ephemeral substrates. In dynamic habitats such as Mediterranean *Posidonia oceanica* seagrass meadows, some taxa—including *A. lepadiformis*—utilize specialized asexual reproduction strategies involving the production of brood sacs or resistant buds. These structures enable populations to persist in fragmented habitats or environments with strong hydrodynamic conditions, thereby facilitating rapid recolonization of seagrass leaves (Balduzzi et al., 1991).

***Watersipora cucullata* (Busk, 1854)**

Watersipora cucullata is an encrusting cheilostome bryozoan that forms dark colonies commonly observed within fouling communities. The species exhibits a distinctive arrangement of the orificial area and operculum, along with a dense distribution of pores on the calcified frontal wall (Reverter-Gil & Souto, 2019).

The genus *Watersipora* constitutes one of the most ecologically significant groups of bryozoans, yet it remains one of the most taxonomically challenging due to the paucity of conventional diagnostic features such as spines or avicularia (Ryland et al., 2009). Unlike several congeneric taxa that serve as pioneer organisms in global fouling assemblages, *W. cucullata* is regarded as native to the Mediterranean basin (Reverter-Gil & Souto, 2019).

Larvae demonstrate a sophisticated phototactic response. They initially navigate towards light; however, during settlement, they become photonegative and tend to colonize shaded surfaces, such as ship hulls (Wisely, 1958; cited in Ferrario et al., 2015).

This bryozoan also exhibits a high tolerance to copper-based antifouling coatings, a characteristic that facilitates its dissemination via maritime traffic (Weiss, 1947; cited in Ferrario et al., 2015).

Furthermore, colonies release inhibitory compounds that deter epibiosis, thereby maintaining the colony surface free of other organisms and enhancing gas exchange through the frontal pores.

Schizoporella errata **(Waters, 1878)**

Schizoporella errata is an encrusting cheilostome bryozoan that forms extensive, multilayered colonies varying in coloration from pink to dark red-orange (Micael et al., 2014). The species exhibits considerable phenotypic plasticity and possesses a high level of competitive capability. Colonies are capable of dominating substantial areas of substrate, attaining coverage levels approaching 99.25%, thereby actively inhibiting the growth of native organisms (Marasinghe & Ranatunga, 2018).

In the Mediterranean region, reproduction exhibits a seasonal pattern. Larval release reaches its peak when seawater temperatures range between 20°C and 25°C (Sokolover et al., 2018).

The resilience of this bryozoan in environments impacted by human activity is partly attributed to the synthesis of secondary metabolites, including alkaloids, sterols, and phthalate derivatives, which possess cytotoxic properties and provide chemical defenses against predators (Weerasinghe et al., 2018). Research conducted in harbor environments further indicates that the associated microbiota facilitates tolerance to hypoxic conditions.

Morphologically, the species exhibits clinal variation in the length of avicularia, specialized defensive zooids that respond to local environmental conditions (Schopf & Dutton, 1976). As a suspension-feeding filter feeder, *S. errata* also contributes to the removal of organic particulate matter and microplastics from the water column (Fortič et al., 2025).

Bugula neritina **(Linnaeus, 1758)**

Bugula neritina is an upright, tree-like cheilostome bryozoan that forms complex three-dimensional colonies. These structures serve as microhabitats for a wide variety of small invertebrates, including peracarid crustaceans, whose numbers fluctuate seasonally with colony growth (Conradi et al., 2000).

Although commonly characterized as dark reddish-purple, this bryozoan exhibits considerable variation in coloration, occasionally appearing in ochre or light brown hues. Such variation underscores the fact that *B. neritina* constitutes a complex of at least three cryptic species (Types S, N, and D). These lineages exhibit notable genetic differences and pigmentation patterns that vary across different regions and lineages (Fehlauer-Ale et al., 2014; McGovern & Hellberg, 2003).

A primary aspect of its biology pertains to larval energetic dynamics**.** Lecithotrophic larvae utilize approximately 20% of their lipid reserves per hour of free swimming**.** Extended planktonic phases diminish the energy accessible for metamorphosis and result in smaller ancestrulae with less efficient lophophores (Wendt, 1998).

The species sustains an obligate symbiotic relationship with the bacterium *Endobugula sertula*, which synthesizes bryostatins. These compounds function as chemical defenses, making the larvae unappealing to predators (McGovern & Hellberg, 2003).

Recent studies document notable transgenerational plasticity in response to copper pollution. Maternal colonies exposed to copper transmit heightened tolerance to their offspring through epigenetic mechanisms, thereby facilitating the colonization of environments affected by human activity (Neylan et al., 2022; Piola & Johnston, 2006).

Finally, the elevated filtration activity of this bryozoan significantly facilitates the removal of suspended contaminants from the water column (Fortič et al., 2025).

5.1.2 Arthropoda

The phylum Arthropoda constitutes one of the most extensive and diverse groups within the animal kingdom, characterized by a segmented body plan and an exoskeleton composed of chitin that offers both protection and mechanical support. Within this extensive taxonomic group, the class Crustacea demonstrates significant ecological plasticity, encompassing organisms that have successfully adapted to nearly all marine environments through highly diverse survival strategies. Notably, the orders (or infraclasses) Cirripedia and Amphipoda exemplify this adaptive divergence: the former are specialized for a sessile and gregarious lifestyle, sustained by calcified structures, while the latter have evolved towards a mobile existence among algal fronds or artificial substrates.

Amphibalanus amphitrite **(Darwin, 1854)**

Amphibalanus amphitrite is a balanomorph barnacle widely distributed in coastal environments and represents one of the most relevant species in the study of marine biofouling. The organism possesses a conical shell composed of a complex arrangement of calcareous plates whose growth is regulated by finely controlled biomineralization processes (Satheesh & Wesley, 2009). As highlighted in marine biological studies, the analysis of this organism relies on a rigorous scientific approach that includes observing biological traces left on the substrate to reconstruct its life history.

The development of the basal plate, composed of hierarchically organized calcite grains, facilitates the organism's firm adhesion to the substrate via a layer of cuticular tissue and specific adhesive proteins known as cement (De Gregorio et al., 2015; Burden et al., 2014). Basal growth proceeds radially through the expansion of internal channels, thereby ensuring robust attachment even during the volumetric growth of the lateral plates. From a biological perspective, the size and preservation state of these calcareous structures serve as key indicators for estimating the age of colonization and the duration of substrate exposure to the marine environment.

The reproductive biology of *A. amphitrite* exhibits considerable plasticity in response to environmental variations. In tropical regions or port environments characterized by elevated water temperatures, the species has the capacity to reproduce throughout the year, with peak activity levels positively correlated with increases in sea surface temperature and phytoplankton abundance (Satheesh & Wesley, 2009). The selectivity of cypris larvae during settlement is influenced by chemical and physical stimuli and exhibits varying sensitivity to antifouling extracts and biofilm characteristics compared to other balanid species (Maréchal & Hellio, 2011).

This species also functions as a genuine environmental sentinel by recording alterations in the surrounding ecosystem through its physiological responses. Populations of *A. amphitrite* demonstrate significant resilience to anthropogenic modifications, sustaining stable growth and egg production rates even under ocean acidification conditions down to pH 7.4, although the shell may exhibit diminished mechanical resistance in such scenarios (McDonald et al., 2009). The organism's non-selective filter-feeding behavior further exposes it to microplastics ingestion, thereby establishing it as a pertinent biological indicator for the surveillance of synthetic polymer dispersion within marine ecosystems (Wang et al., 2025).

From a forensic perspective, the cypris stage signifies a pivotal decision point for estimating immersion duration. Once the larva permanently affixes its basal plate to the osseous substrate, it serves as a significant temporal marker of colonization. As demonstrated by Pirtle and Magni (2019), the incremental growth of the lateral calcareous plates and the analysis of basal diameter enable accurate reconstruction of the number of weeks a specimen has been submerged, thus providing a quantitative parameter for determining the Minimum Post-Mortem Submersion Interval (PMSIm). Traces left by barnacle bases on cortical bone surfaces may also offer valuable insights into potential disarticulation or movement of remains along the seabed, contributing to the reconstruction of the taphonomic history of skeletal remains (Lopes et al., 2024).

Genus *Caprella* Lamarck, 1801

The genus *Caprella*, belonging to the family Caprellidae (order Amphipoda), comprises crustaceans characterized by an exceptionally slender and diminished body form, commonly known as “skeleton shrimps”. These organisms are predominantly epifaunal and tend to densely colonize beds of macroalgae or seagrass meadows, often displaying specific preferences related to the structural complexity of the algal substrate (Guerra-García, 2001). Within the genus, species such as *Caprella* *danilevskii* exhibit a cosmopolitan distribution (Guerra-García & Takeuchi, 2002) and have recently been documented in the Arabian Sea and the northern Indian Ocean (Gaikwad & Sautya, 2022).

In contrast to the planktonic life cycle characteristic of many other crustaceans, species of *Caprella* exhibit direct development, distinguished by an embryonic phase whose duration is highly dependent on water temperature (Takeuchi & Hirano, 1992). Upon completion of this phase, the juveniles—identified as instar I at the time of emergence from the maternal marsupium—are already morphologically akin to adults (Takeuchi & Hirano, 1991). Growth is marked by frequent molts, with intervals ranging from 2.5 to 6.5 days in *C. danilevskii* (Takeuchi & Hirano, 1991), and by significant sexual dimorphism, notably evident in the morphology of the gnathopods (Kim & Lee, 1975; Takeuchi & Hirano, 1991). Ecologically, several *Caprella* species often coexist sympatrically, with their abundance fluctuating seasonally in close relation to the biomass of vegetated substrates and recruitment dynamics (Paula et al., 2021).

***Jassa* cfr. *falcata* (Montagu, 1808)**

The genus *Jassa* comprises tube-building amphipods that frequently represent early and often dominant colonizers in marine fouling communities worldwide (Beermann, 2014; Beermann & Franke, 2012). These organisms function as opportunistic species capable of rapidly occupying newly available space through non-selective filtration, using their second antennae to capture suspended particles, while also supplementing their diet with detritus and small organisms (Nair & Anger, 1979; Chandrasekharan Nair & Anger, 1980). Their biology is strongly associated with the construction of residential tubes formed by cementing detrital material using secretions produced by pereopods 3 and 4 (Borowsky, 1983; Hill, 2000). These structures provide protection and serve as the basis for a complex social organization characterized by pronounced sexual dimorphism and a unique form of male polymorphism (Conlan, 1990; Conlan et al., 2021).

Within the same population, males develop according to two morphotypes: "major" males (or fighters), characterized by gnathopods bearing a conspicuous distal digitiform process used to defend females, and "minor" males (or sneakers), which maintain a morphology similar to females and adopt opportunistic reproductive tactics (Kurdziel & Knowles, 2002; Borowsky, 1985). The determination of these morphotypes is not purely genetic but strongly influenced by environmental factors and dietary quality, with high protein intake promoting the development of the "major" form (Kurdziel & Knowles, 2002). Reproductive behavior follows a mate-guarding strategy in which the male waits for the female's ovulatory molt before copulation (Borowsky, 1983). The digitiform process of terminal males has been observed to function as a key signal for female acceptance, as females are less tolerant of males lacking this structure (Borowsky, 1985).

Regarding *Jassa falcata*, a representative species of its genus, historical literature has documented its pronounced phenotypic plasticity owing to its substantial morphological variability during development, which has led to decades of taxonomic confusion (Sexton & Reid, 1951; Conlan, 1990). Individuals of this species exhibit average sizes that differ according to sex, with males reaching a maximum length of approximately 13 mm and females about 12 mm (Chandrasekharan Nair & Anger, 1980). Ecologically, *J. falcata* predominantly inhabits environments characterized by strong hydrodynamic forces and persistent currents, in contrast to congeners such as *J. marmorata*, which are typically found in more sheltered habitats (Karez & Ludynia, 2003). Despite this specific adaptation, *J. marmorata* can occasionally outcompete *J. falcata dur*ing the initial colonization of new substrates (Beerman & Franke, 2012).

The life cycle of *J. falcata* demonstrates a distinct thermal adaptation: lifespan extends as ambient temperature decreases, with females living up to 252 days at 10°C (Nair & Anger, 1979). The species reproduces year-round, with two primary reproductive peaks: one in winter and a more pronounced one in late spring (Chandrasekharan Nair & Anger, 1980). Biological investigations reveal seasonal fluctuations in the biochemical composition of populations, characterized by an inverse relationship between protein and lipid fractions that correlates with food availability (Chandrasekharan Nair & Anger, 1980). Fecundity exhibits a strong correlation with body size, with females reaching sexual maturity at 5–6 mm, and the species displays survival parameters that differ from those of sympatric species such as *J. herdmani* (Beermann & Purz, 2013). Finally, the capacity of this species to settle on a diverse array of substrates—from red algae to submerged bones—underscores its role as an opportunistic colonizer in marine ecological succession processes (Karez & Ludynia, 2003; Beermann, 2014).

5.1.3 Mollusca

The phylum Mollusca constitutes one of the most ancient and diverse taxonomic groups within the animal kingdom, distinguished by a fundamental body plan comprising a mantle, a muscular foot, and, in most classes, a protective calcareous shell. Within the class Bivalvia, the family Mytilidae encompasses species of significant ecological and economic importance, notably including *Mytilus galloprovincialis*.

Mytilus galloprovincialis **Lamarck, 1819**

The Mediterranean mussel is a sessile filter-feeding organism that colonizes intertidal and subtidal zones, demonstrating remarkable adaptability to heterogeneous environmental gradients and a pronounced invasive capacity (Hockey & van Erkom Schurink, 1992). The reproductive biology and recruitment dynamics of this species are strongly influenced by local hydrological conditions. In lagoon and coastal environments, the settlement of pediveliger larvae typically occurs with seasonal peaks between spring and early summer (Ceccherelli & Rossi, 1984). Growth follows mathematical models such as the von Bertalanffy growth function, with growth rates varying according to nutrient availability and temperature, allowing individuals to reach commercial size in approximately 12–18 months (Ceccherelli & Rossi, 1984; Batır et al., 2025).

From a physiological perspective, *M. galloprovincialis* is extensively recognized as a pivotal environmental sentinel species. Parameters such as lysosomal membrane stability and acetylcholinesterase (AChE) activity are frequently employed as early biomarkers of stress induced by contaminants (Taleb et al., 2007).

From a taphonomic and forensic perspective, a crucial element is represented by the mussel's anchoring apparatus: the byssus. This proteinaceous structure, composed of collagen-like filaments and adhesive proteins known as mussel foot proteins (Mfps), allows extremely strong physicochemical attachment even to porous surfaces such as cortical bone (Wiegemann, 2005). The persistence of these proteinaceous structures is an important indicator for reconstructing a specimen's immersion history, even in the absence of the living organism.

The species also demonstrates considerable resilience to stressors such as hypo-osmosis and ocean acidification. Nevertheless, the combined influence of rising temperatures and decreasing pH levels may modify the bioavailability of heavy metals, including cadmium, thereby eliciting tissue-specific metabolic responses (Hamer et al., 2008; Nardi et al., 2017). Owing to its high filtration efficiency, this species assumes a vital function within Integrated Multi-Trophic Aquaculture (IMTA) systems, serving as an extractor of organic nutrients (Batır et al., 2025).

***Anomia ephippium* Linnaeus, 1758**

Anomia ephippium exemplifies a distinctive instance of morphological adaptation among epibenthic bivalves. As a member of the family Anomiidae, this suspension-feeding mollusk is characterized by a peculiar attachment mechanism facilitated by a heavily calcified byssus passing through a specialized opening in the right valve, referred to as the byssal foramen or byssal notch (Studencka, 2018).

This anchoring system establishes a highly intimate adhesion to the substrate, leading to the phenomenon referred to as xenomorphism. During growth, the lower valve accurately replicates the irregularities, porosities, and micro-sculptures of the underlying bone surface, thereby molding its morphology in response (Studencka, 2018).

The recruitment patterns of *A. ephippium* in the Mediterranean region generally demonstrate seasonal peaks during the summer months, specifically between July and September. Following settlement, the species employs a notably rapid growth strategy, which is critical for effectively competing for space on hard substrates and minimizing post-larval mortality (Bramanti et al., 2003).

From an ecological perspective, the species is generally regarded as a characteristic component of advanced stages of benthic succession. Its presence is commonly correlated with mature, stabilized biofouling communities, in which the substrate has already undergone the requisite biochemical conditioning processes and colonization by pioneer species (Relini, 1980).

An additional element of particular biological interest lies in the shell microstructure known as "pseudo-nacre," whose elastic properties are directly related to organic pigmentation (Radhakrishnan et al., 2020). The presence of polyenic pigments, which produce shell coloration ranging from yellow to red, acts as a structural reinforcement, increasing mechanical rigidity and yielding elastic modulus values exceeding those of abalone nacre (Radhakrishnan et al., 2020). Although recent taxonomic revisions have reclassified some western Atlantic populations as *Anomia simplex*, *A. ephippium* remains the reference taxon for European populations (Radhakrishnan et al., 2021).

The preservation of this intricate biological architecture in Miocene fossil specimens exemplifies the evolutionary efficacy of an anchoring system that endures for millions of years within marine environments distinguished by high hydrodynamic energy (Studencka, 2018).

5.1.4 Annelida

The phylum Annelida encompasses a significant group of coelomate protostome invertebrates distinguished by a metameric body structure. Metamerism involves the serial repetition of comparable segments along the body's longitudinal axis, internally separated by transverse mesodermal septa. This compartmentalization of the coelom, serving as a hydrostatic skeleton, endows annelids with notable morphological adaptability and sophisticated hydrodynamic regulation.

Within the phylum, the class Polychaeta represents the phylogenetically oldest and most diverse group. Polychaetes are distinguished by the presence of parapodia bearing numerous chitinous chaetae. Based on their life strategies, they are traditionally divided into "errant" (mobile) and "sedentary" (tube-dwelling) forms. *Hydroides elegans* belongs to the order Canalipalpata, which includes sessile organisms that have evolved highly specialized filtration structures at the expense of active mobility.

The family Serpulidae (Rafinesque, 1815) comprises sedentary polychaetes specializing in secreting a protective calcareous tube that is permanently affixed to the substrate. These organisms feature a cephalic region modified into a crown of feather-like radioles, which serve functions in respiration and suspension feeding by capturing organic particles. A notable diagnostic characteristic is the operculum, a specialized structure that seals the tube opening when the animal retracts, thus providing protection against predation and environmental stressors.

Hydroides elegans **(Haswell, 1883)**

Hydroides elegans is a small serpulid polychaete measuring 15–20 mm, recognized as one of the pioneer species involved in calcareous biofouling within port environments. Its calcareous tube is the product of a complex biomineralization process facilitated by specialized calcifying glands. As demonstrated by Chan et al. (2015), the tube's microstructure undergoes considerable ontogenetic modifications, transitioning from an early stage predominantly composed of aragonite to an adult structure that is consolidated and optimized for mechanical resistance and sustained adhesion to substrates.

The successful colonization of *H. elegans* primarily results from its rapid life cycle. This species is dioecious and reproduces through external fertilization. Its eggs develop into planktotrophic trochophore larvae, which reach metamorphic competence in approximately five days at 25 °C. Research conducted by Seaver et al. (2005) has elucidated the growth patterns during segmentation: unlike many other annelids, the larval segments of *H. elegans* originate from a field of dividing cells distributed laterally rather than from a localized posterior growth zone. This developmental peculiarity facilitates the rapid transition to the post-metamorphic juvenile stage.

The essential transition to benthic existence is governed by environmental chemical signals. Recent studies (Nesbit & Shikuma, 2023; Farrell et al., 2025) affirm that larval settlement is exclusively initiated by the presence of a mature microbial biofilm. Certain marine bacteria, such as *Pseudoalteromonas luteoviolacea*, secrete protein complexes that activate signal transduction pathways within the larva, thereby inducing metamorphosis. Furthermore, Huggett et al. (2009) demonstrated that the initial wettability of a surface influences the formation of biofilm itself. In the context of the current thesis, the hydrophilic and porous characteristics of bone tissue may have contributed to an optimal biochemical interface, thereby facilitating the colonization processes.

The spatial distribution of individuals on the substrate is not stochastic. According to Walters et al. (1997), the aggregation of calcareous tubes results from the interaction between larval settlement behavior and local hydrodynamic conditions. Larvae tend to settle preferentially in areas characterized by reduced water flow or in proximity to conspecifics, explaining the tendency of *H. elegans* to form dense clustered encrustations.

H. elegans also exhibits significant resilience to anthropogenic stressors. While related species such as *Hydroides dirampha demon*strate greater tolerance to extreme temperatures and low salinity (Liu et al., 2020), *H. elegans* remains the predominant colonizer in Mediterranean ports. Its ability to dominate hard substrates, as documented by Schwan et al. (2015), in conjunction with its early appearance in immersion time series (S1), renders this polychaete a particularly valuable biological indicator for estimating the Minimum Post-Mortem Submersion Interval (PMSIm) in forensic investigations.

5.2 Estimation of submersion duration

The results obtained affirm the viability of estimating the duration of submersion (Post-Mortem Submersion Interval, PMSI) for both benthic and suspended samples. This temporal estimation can be accomplished by integrating two complementary parameters: the analysis of bone tissue degradation and the study of ecological succession within the colonizing communities. In this context, the present study adopts the approach of recent taphonomic investigations (e.g., Bertoglio et al., 2021), which, although not yet providing an absolute dating formula, have demonstrated that cortical modifications in marine environments follow predictable patterns that could potentially serve as chronological indicators.

It is essential to acknowledge that the observed colonization dynamics are significantly influenced by seasonal fluctuations. The experiment was conducted during the summer months—a period of peak reproductive activity for the local benthic organisms (Relini, 1980)—resulting in rapid and extensive colonization. Conversely, in winter, due to the reduced availability of planktonic larvae, ecological succession may experience considerable delays, thereby affecting the precision of temporal evaluations.

5.3 Biodiversity analysis and substrate specificity

From a biodiversity perspective, the communities established on bone matrices are significantly less diverse than those commonly observed on artificial or inert substrates. Systematic comparison with previous literature and recent monitoring in the study area highlights this difference: while Relini (1980) recorded 40 species at the experimental raft station, and Abate (2025) identified 14 species at the same site, the biological contingent detected on bones in the present study was markedly lower. This is further supported by the variance analysis (PERMANOVA), which revealed highly significant differences among the three identified taxonomic groups ($p < 0.001$), with post hoc testing indicating statistically relevant diversity across all pairwise comparisons. This statistical rigor confirms that the community is not a random assemblage but is structured into distinct groups defined by substrate characteristics.

This discrepancy indicates that bone tissue functions as an ecological filter, preferentially supporting a limited subset of generalist species exhibiting extensive ecological tolerance, which are already extensively distributed within the region. Significantly, no macro- or micro-boring organisms were observed, signifying that colonization during this temporal interval remains exclusively epibiotic, confined to the external surface of the bone.

5.4 Ecological succession and colonization chronology

The temporal analysis facilitated the identification of a definitive colonization sequence, commencing in the first month of submersion with the near-total loss of the periosteum. This process is attributed to the trophic activity of amphipod populations, presumably Jassa falcata, during which only isolated individuals of the genus *Hydroides* were observed.

From the second month onward, a stable settlement of barnacles and bryozoans was observed, persisting as the primary community components from their initial appearance until the sixth month. Ecological succession proceeded in the fourth month with the arrival of bivalve mollusks, *Mytilus galloprovincialis* and *Anomia ephippium*, indicating enhanced community maturity.

Statistically, significant differences over time emerged only when comparing the first month with all subsequent months ($p < 0.001$). This suggests that the transition from periosteal degradation to biofouling stabilization represents the most pronounced ecological discontinuity. Once this initial phase is overcome, the community appears to stabilize along a more consistent growth trajectory, as indicated by the significant Taxonomic Group × Time interaction ($p = 0.016$), reflecting differential colonization rates and development among the various taxa over the six-month period.

It is important to highlight that variations in the abundance of specific taxa may also indicate an intrinsic instability of the bone matrix, which is susceptible to ongoing dissolution. This degenerative process may be linked to water acidification observed in the study area (Abate, 2025), demonstrating a marked reduction in pH relative to previous decades.

5.5 Degradation dynamics in sediment-deposited samples

In the samples resting on the substrate, the benthic community was virtually absent, consistent with progressive burial of the remains by bottom sediments. In this context, bone degradation was primarily chemical, evolving linearly with exposure time. After the first month, the periosteum was nearly completely absent, accompanied by superficial dissolution, imparting a micro-spongy structure to the matrix. During this phase, the Volkmann canals became particularly prominent, their openings on the bone surface revealed by periosteal loss.

From the second month, the superficial plexiform bone was fully dissolved, permitting unobstructed observation of the Haversian systems, which became visible through the central canals. In subsequent months, the dissolution advanced, resulting in a highly irregular surface morphology, with Haversian canals expanding up to 100 µm. This significant degradation of sediment-covered bone is likely associated with the elevated concentrations of hydrogen sulfide (H_2S) commonly produced in anoxic sediments, which may influence early diagenetic processes and matrix biodeterioration (Eriksen et al., 2020).

5.6 Metagenomic Analysis

The integration of these data with metagenomic analysis is expected to be particularly insightful, enabling high-resolution characterization of both the macro-invertebrate component and microbial communities. Such analysis holds the potential to elucidate the role of microbial consortia in the degradation of bone matrix, thereby substantiating the hypothesis that marine bacterial succession may serve as a reliable indicator for estimating the post-mortem submersion interval (PMSI) (Dickson et al., 2011).

CONCLUSIONS

The current study investigated the processes of biological colonization and degradation of skeletal remains within a marine port environment, with the objective of evaluating the potential contribution of these processes to estimating the Post-Mortem Submersion Interval (PMSI). By experimentally immersing pig femora (*Sus scrofa domesticus*) in the Port of Genoa, the development of the associated communities was systematically documented, thereby elucidating the initial phases of ecological succession pertinent to this specific context.

The collected data indicate that bone tissue functions as an appropriate substrate for the settlement of various biological communities, following a well-defined temporal progression. Initially, the removal of the periosteum is observed, a phenomenon attributable to the trophic activity of amphipod crustaceans. These creatures, even after the first month of immersion, leave the bone surface exposed to colonization. This phase renders the cortical surface accessible for the settlement of pioneer epibionts, among which bryozoans are particularly prominent, notably *Schizoporella errata* and *Watersipora cucullata*. This is followed by the colonization of serpulid polychaetes such as *Hydroides elegans* and barnacles, including *Amphibalanus amphitrite*. As the duration of submersion extends to 90 days, the biological community becomes increasingly structurally complex, characterized by the introduction of larger sessile organisms, such as bivalve molluscs like *Mytilus galloprovincialis*, culminating in a progressively extensive and stratified biological coverage of the substrate.

The comparative analysis elucidates how the interval from the initial month to subsequent periods constitutes a discontinuity in the settlement process: during this interval, the transition from an exposed bone surface to a developed epibiont community indicates that the correlation between the condition of degradation and the biotic composition may serve as valuable temporal indicators for the chronological assessment of skeletal remains.

It was also observed that the community associated with bone demonstrates lower diversity relative to that found on other substrates within the same port basin. This evidence suggests a selective influence of the bone substrate, which appears to facilitate the settlement of opportunistic species. Throughout the entire observation period, no bioerosion phenomena or boring organisms were detected; during the initial months of immersion, taphonomic processes thus seem to be primarily characterized by epibiosis and superficial degradation.

While the identified succession patterns serve as definitive references, it is essential to emphasize that such processes are strictly constrained by environmental variables at the Port of Genoa. Seasonality played a crucial role; the experiment was conducted during the summer months, subjecting the samples to elevated water temperatures that likely accelerated growth rates and larval settlement. These physicochemical conditions, characteristic of coastal port environments during summer, distinctly differentiate these processes from those observed in deep-sea settings, where low average temperatures and high pressure significantly retard decomposition. Furthermore, in the port environment, the absence of specialized boring organisms such as *Osedax* indicates that, at least in the short to medium term, taphonomic processes remain limited to epibiosis and superficial degradation.

In light of these considerations, the present study constitutes an innovative contribution to the understanding of the degradation and colonization dynamics of skeletal remains in port environments. Despite its evident significance in forensic contexts, particularly for the recovery of bodies in urban coastal settings, this field of research remains inadequately documented in the literature compared to studies conducted in open sea or deep-sea environments. The experimental approach undertaken facilitated the identification of recurring patterns in the succession of epibiont communities, thereby providing an empirical basis for distinguishing the taphonomic processes characteristic of protected and anthropogenically influenced waters from those occurring in less disturbed natural systems. The documentation of this specific ecological succession on bone substrates thus offers preliminary yet essential insights for identifying novel temporal markers that may be employed in anthropological and forensic investigations within Mediterranean marine environments.

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