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Flow cytometry and micro-Raman spectroscopy: Identification of hemocyte populations in the mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae) from Faro Lake and Tyrrhenian Sea (Sicily, Italy)

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ABSTRACT

Immunological and structural characteristics of hemocyte populations in the mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae), going from two different Sicilian habitats (Faro Lake and Tyrrhenian sea), was investigated by means of two different techniques (flow cytometric and micro-Raman spectroscopy analyses). For this purpose, three hundred and sixty mussels *Mytilus galloprovincialis* were analyzed during November 2017. They were divided into two equal groups (triplicate sample) on the basis of the site of collection (n = 60 caught in Faro Lake - group A, and n = 60 caught in Tyrrhenian Sea - group B). Some several differences between the species of Faro Lake and Tyrrhenian Sea are observed and ascribed to the disruption of immune parameters induced by the variations of some qualitative water parameters (temperature, salinity, dissolved oxygen, pH, ammonium 10, free chlorine, total chlorine, total phosphate, orthofhosphate) recorded in the two habitats. This study is relevant for monitoring the conditions of the sea and Faro Lake, which is strongly influenced by the currents of the Tyrrhenian Sea. Faro lake is well known for the cultivation of mussels and this is part of a coastal habitat of particular interest, consisted of a peculiar biocenotic complex. Further, for the first time, significant different arrangement in the mussels cell structural organization was evidenced by simply following their highly reproducible Raman biomolecular signatures.

1. Introduction

Hemocytes represent an important constituent of mussel hemolymph and play fundamental roles in the clearance of nitrogenous catabolites, the detoxification of toxic compounds, the storage of nutrients, and immunosurveillance, assuring the animal survival by coping with foreign, potentially pathogenic microbes entering the organism. In most cases, however, their high concentration may be induced by environmental contamination [1–3] to capture stressors such as handling [4], transportation [5] or, for aquatic animals, exposure to air [6]. Furthermore, intraindividual factors like injury and physical activity [7], starvation and individual metabolic condition [8,9] or spawning [10] may influence the hemocyte concentration. Thus, very different factors often give rise to a significant variation of hemocyte counts in individual molluscs of different classes [11,12] and in other invertebrates [12,13]. In fact, the number of hemocytes in circulation is not a characteristic feature of an invertebrate species; instead, it reflects the magnitude of a systemic response to endogenous or environmental stressors in individual animals, at any given time. Particularly, hemocytes play a critical role in the immune system of invertebrates.

In bivalves, hemocytes are essential for a number of processes, including wound healing, transport and digestion of nutrients, as well as immune defense [14,15]. Bivalves have two major types of hemocytes: agranular hemocytes, which lack granules, and granular hemocytes, which contain granules [14–16]. In mytilid mussels, hemocytes have been classified/characterized using different approaches such as the density gradient centrifugation method, carrying out transmission electron microscopy and lectin binding assay analyses [17–19]. To date

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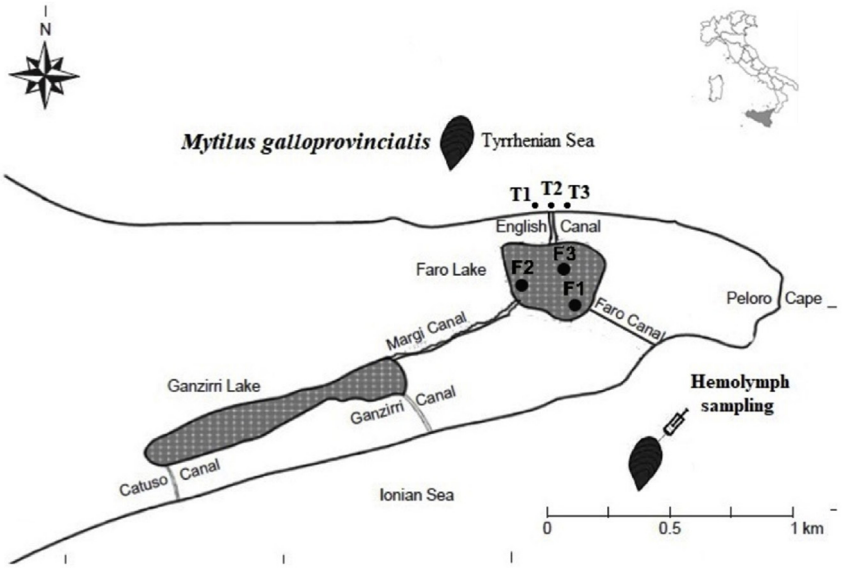


Fig. 1. Map depicting locations of the sampling sites, in the Tyrrhenian sea (T1, T2 and T3) and Faro Lake (F1, F2 and F3).

it is known that, while three or more types of hemocytes have been identified in bivalves, the nomenclature for these types varies between bivalve species [20]. Precisely, *Mytilus galloprovincialis* shows: 1) agranular hemocytes (called hyalinocytes), 2) three types of granular cells (called basophilic granulocytes), 3) acidophilic granulocytes, 4) intermediate cells which contain both acidophilic and basophilic granules [18]. However, the absence of available molecular databases means that classification of the cells remains based mainly on morphological, cytochemical, and functional characteristics, without the possibility to define separate cell lineages. Hence, actually key features defining the cells include the presence or absence of cytoplasmic granules, of acidic, basic or neutral staining of the granules and finally of phagocytic activity. This latter is the process by which certain immune cells recognize and eliminate non-self-components, such as invading and/or associating microorganisms, and it is one of the most important defensive function of hemocytes [21–24].

Faro lake is a typical example of a natural confined environment [25], where eutrophication can trigger stress with negative repercussions on the aquatic ecosystem. The lake is characterized by euryhaline waters, as there is an injection of fresh water from aquifers, which mixes with salt water through the channels communicating with the sea. It represents an example of a meromictic basin and, therefore, the sediments are not involved in a mixing process. The Tyrrhenian Sea, in general in the Strait of Messina, degrades slowly reaching 500 m of depth between the two shores of Sicily and Calabria. The Tyrrhenian waters are strongly influenced by tidal exchange regime, typical of the Strait of Messina [26–28]. In the area of sampling, the nature of the seabed is rocky.

In the last few years, flow cytometry, a well known routine tool used for vertebrate biomedical research, has been applied to bivalve immunology, allowing rapid, accurate, and quantitative analyses of hemocyte morphology and immune-related activities [29–32]. The combination of optical microscopy and flow cytometry allowed to study the alteration in DNA cell cycle characteristics of hemocytes of the *Mytilus galloprovincialis* mussel, collected at 17 different locations along the Adriatic coast, Croatia [33]. Brousseau et al. [34] have applied flow cytometry in the clam *Mya arenaria* hemocytes in which phagocytosis is well conserved and so too the sensitivity of this biological function to the environment in different habitat.

Microbial activities are strongly influenced by the natural habitats heterogeneity. Then, a full understanding of *in situ* profiling of microorganisms and their activities requires an ability to study these processes at the level of single cells [35], which cannot be performed with the methodology actually adopted. In this contest, micro-Raman spectroscopy could be efficiently used to detect the microorganisms activities in a rapidly and non-destructively way [36], adopting the same protocols used for the analysis of pharmaceutical formulations, also in microfluidic devices [37,38], providing simultaneously a molecular and a morphological signature of each cell, in turn, allowing an accurate discrimination of key immune cell subsets in a completely label-free manner [39]. In the present study, we analyzed the hemocytes of the *M. galloprovincialis* (Bivalvia: Mytilidae) collected from two different habitats, Lake Faro and Tyrrhenian Sea (Sicily, Italy). Samples's morphology and their immune-related activities were investigated by means of flow cytometry while cells structural organization was analyzed using the micro-Raman spectroscopy technique. The changes of specific Raman biomolecular signatures were followed to discriminate hemocytes going from Lake Faro and Tyrrhenian Sea. On the overall, we investigated about the effects of water temperature, salinity and pH on the immune response and also on the lipids and proteins conformational structures of mussels which live in two different habitat. The ultimate goal is to check if this species can be used as a biological indicator of environmental contaminants.

2. Material and methods

2.1. Animals and study area

Three hundred and sixty mussels *Mytilus galloprovincialis* were investigated during the study carried out in November 2017. They were divided into two equal groups on the basis of the site of collection. Sixty *Mytilus galloprovincialis* were caught in Faro Lake (group A), and sixty *Mytilus galloprovincialis* were caught in Tyrrhenian Sea (group B), and all mussels were sampled in triplicate for each site. For both sites of collection (Fig. 1), water physico-chemical parameters were measured. Water sampling was carried out in the same date of mussel sampling, in three stations of Faro Lake (F1, F2 and F3) and Tyrrhenian Sea (T1, T2 and T3), in triplicate. The three stations on each location were selected randomly and the distances among them were about 3 m (see Fig. 1). A multiparameter probe Multi 340i/SET – WTW was used to monitor the temperature (Table 1).

Table 1
Physico-chemical parameters of the Faro Lake and Tyrrhenian Sea during the autumn season.

Parameters	Faro Lake	Tyrrhenian Sea
Temperature (°C)	14.5 ± 0.1	17.8 ± 0.2*
Conducibility (mS/cm)	52.6 ± 0.1	56.5 ± 0.2*
Salinity (PSU)	37°/oo ± 0.1	36°/oo ± 0.2*
Dissolved Oxygen (mg/l)	7.6 ± 0.1	5.6 ± 0.2*
pH	8.13 ± 0.1	7.16 ± 0.3*
Ammonium 10 (mg/l)	0.2 ± 0.1	0.3 ± 0.2*
Free chlorine (mg/l)	0.07 ± 0.1	0.04 ± 0.2*
Total chlorine (mg/l)	0.18 ± 0.1	0.08 ± 0.3*
Total phosphate (mg/l)	1.6 ± 0.1	1.2 ± 0.2*
Orthophosphate (mg/l)	0.4 ± 0.1	0.3 ± 0.3*

Multi 340i/SET – WTW (*P < 0.05).

2.2. Water sampling and analysis

A portable Multi 340i/SET (WTW Wissenschaftlich, Weilheim, Germany) was used to estimate temperature, pH, conductivity and dissolved oxygen (DO), directly in the sampling sites. Hence, surface water samples were collected in polyethylene bottles and transferred, under refrigeration at temperature of 4 °C, to the laboratory. Here, the physico-chemical properties of the water samples were analyzed following standard methods [40]. Especially, nutrients (e.g. ammonia, phosphate, ortophosphate, nitrites) of samples, previously filtered through a 0.45-μm Millipore membrane filter paper, were estimated following the standard APHA (1995) colorimetric approach and using a Filterphotometer (PF-11 MN, Macherey-Nagel GmbH and Co. – D ren, Germany) spectrometer.

2.3. Collection of hemolymph and total hemocyte counts

Hemolymph was withdrawn from the posterior adductor muscle of each mussel using a 5-mL syringe and a 25-gauge needle. All sample were stored at temperature of 4 °C for 1 day before the analysis. The estimated number of circulating hemocytes or total hemocyte counts (THC) is (J: 18.5 ± 3.1°–106 cells mL−1), and then decreased drastically in sexually mature animals (M: 11.8 ± 1.1°–106 cells mL−1). After spawning, scallop THC returned to standard levels (S: 25.8 ± 2.8°–106 cells mL−1) (Table 2). No significant differences were observed between animals. Flow cytometric analyses were performed as previously described [9] within 5 h of drawing on whole hemocyte samples. Hemolymph (2–10 mL) was transferred to a plastic tube (whose volume is 2.0 or 15 mL) and kept on ice for less than 5 min until further use. So, the samples were collected in EDTA tubes and also by a pDC instrument to acquire cell membrane patches. All the so prepared samples were analyzed by a multispectral flow cytometer (ImageStreamx (Amnis, Seattle, WA), combining standard microscopy with flow cytometry. This technique allows to analyse up to 100 cells/s, acquiring simultaneously six images for each cell, including bright field, scatter, and multiple fluorescent images. To this scope, the integrated software INSPIRE was used to run the ImageStreamx. For each experiment, cells (pDCs, LCL, and mDCs) were stained with respective markers or PHK-26 and suspended in 50 μl buffer (cold PBS with 1% FCS and 0.05% sodium azide) in 0.6-ml microcentrifuge tubes. Samples were acquired in the following order: unlabelled, single-color fluorescence controls, and finally, the experimental samples. Samples were always left on ice. At least 10,000 cells/experimental sample and 2000 cells/single-color control were acquired for each sample. After each sample was injected into the flow cell and before the acquisition, we have waited for the formation of a single core stream. Then, the acquired mages were analyzed using the IDEAS image-analysis software (Amnis). Two hemocyte populations were found in the hemolymph of *Mytilus galloprovincialis*: hyaline hemocytes (HH) and granular hemocytes (GH). The GH are spherical to fusiform cells (6–12 μm), containing abundant cytoplasmic granules (≤1 μm) that appeared eosinophilic or basophilic on iemsa staining. Their nucleus (3–6 μm) was characteristically polymorphic, assuming a spherical or bi-to multilobulated shape (Figs. 4 and 5).

Table 2
Statistical results on hemocyte sub-populations from *Mytilus galloprovincialis* (Bivalvia: Mytilidae) captured in two different habitats, Faro Lake and Tyrrhenian Sea (Sicily, Italy).

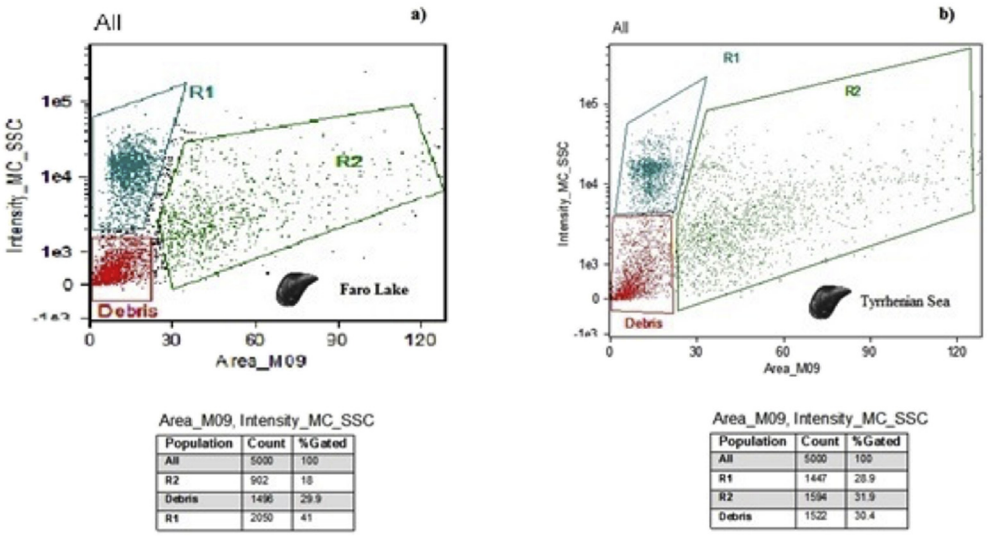
Mytilus galloprovincialis (n = 120)	Hemocyte	Mean ± SD	95% C.I.	25th-75th P
Faro Lake (n = 60)	Halinocytes (R1)	2055 ± 138[a]	1990–2119	2023–2090
	Granulocytes (R2)	909 ± 47[a]	887–931	890–930
	Total (R1 + R2)	2964		
Tyrrhenian Sea (n = 60)	Halinocytes (R1)	1453 ± 69[b]	1421–1485	1400–1500
	Granulocytes (R2)	1600 ± 125[b]	1542–1658	1500–1675
	Total (R1 + R2)	3053		

C.I. = Confidence interval of mean; P = percentile.
The mean values denoted with different letters within the same hemocyte subpopulation are statistically significant (P < 0.05).

2.4. Flow cytometry and micro-Raman spectroscopy

When a monochromatic laser light interacts with a sample, most of the photons are scattered: 1) without any change in energy (the so called elastic or Rayleigh scattering) which when the electrons in a molecule oscillate in resonance with the applied electric field of the incident light; 2) undergoing a change in energy (the so called inelastic scattering-i.e. Raman shift). The change of the incident photon energy indicates molecular polarizability (dipole moment induced by electric

Fig. 2. Raman setup instrument.



Area_M09, Intensity_MC_SSC

Population	Count	%Gated
All	5000	100
R2	902	18
Debris	1496	29.9
R1	2050	41

Area_M09, Intensity_MC_SSC

Population	Count	%Gated
All	5000	100
R1	1447	28.9
R2	1594	31.9
Debris	1522	30.4

Fig. 3. Flow cytometric analysis on hemocyte populations (R1: halinocytes and R2: granulocytes) with cells population count in mussel *Mytilus galloprovincialis* from Lake Faro (a) and Tyrrhenian Sea.

field) changes. A plot of the intensity of the inelastically scattered light as a function of the energy change is called Raman spectrum which is a distinct chemical fingerprint for each investigate molecule/material. For several years, The evolution in terms of Raman mapping has allowed to generate images based on the sample's Raman spectra, so showing the distribution of individual chemical components, polymorphs and phases, and variation in crystallinity [41]. Today they are available on the market either dispersive Raman spectrophotometer equipped with prisms or gratings and non-dispersive Raman spectrophotometer which uses a Michelson interferometer placed into a Fourier Transform Raman spectrophotometer [42]. In our case, to carry out micro-Raman measurements, the 20–25 μL hemolymph aliquot was deposited on a CaF_2 slide and left by air-drying in sterile conditions for one day. Then, each slide was directly observed and analyzed. All tests were performed in duplicate. In Fig. 2 is shown a photo of the micro-Raman setup adopted. Raman spectra were acquired using the 532 nm excitation wavelength coming from a solid state semiconductor laser. The optics of an Olympus BX40 confocal microscope allow to focalize the 1-mW laser beam onto an area of about 2 μm^2 on the sample surface. The elastically scattered radiation was rejected by an edge filter while the backscattered radiation was collected by the same optics of the microscope and dispersed by a monochromator. This latter is equipped with a 600 line/mm grating which allows investigation in the 200–2000 cm^{-1} spectral range, with a spectral resolution of about 2.0 cm^{-1}. Finally, a Peltier-cooled CCD sensor was used to record the spectra, usually averaged for a period of 10 s. We outline that, after the acquisition, no cell showed evidence of damage in the proximity of irradiated area. Further, the random collecting of Raman spectra in arbitrary cell location preserve data reproducibility of tested samples.

2.5. Statistical analysis

Data obtained for hemocyte populations were tested for normality using Kolmogorov-Smirnov test. Unpaired *t*-test was used to determine

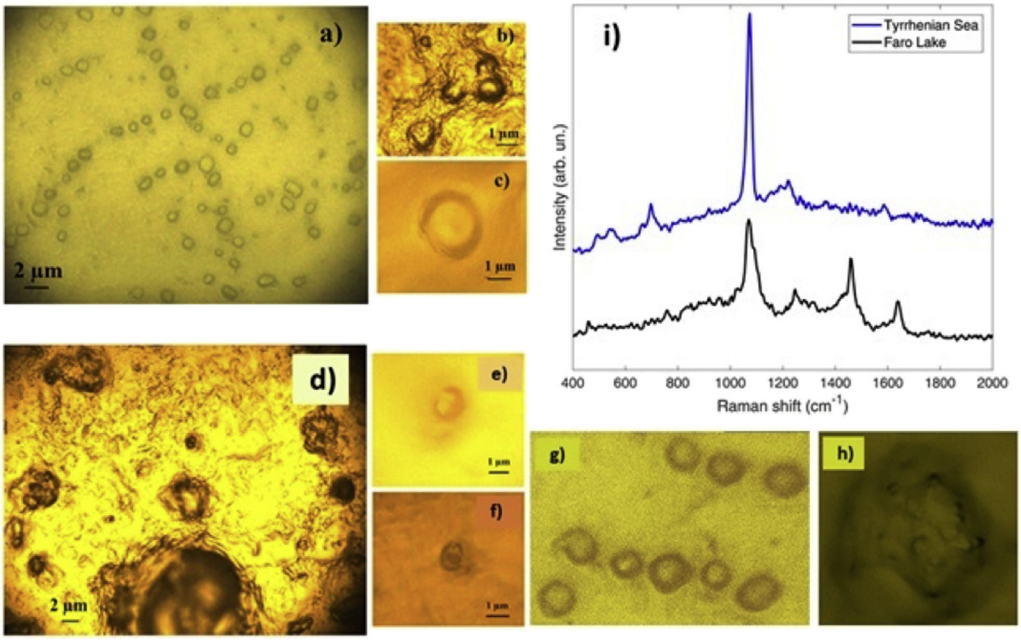


Fig. 4. Optical microscope images of the Tyrrhenian sea (a, b, c, g) and Faro Lake (d, e, f, h) samples and the corresponding representative Raman spectra (i).

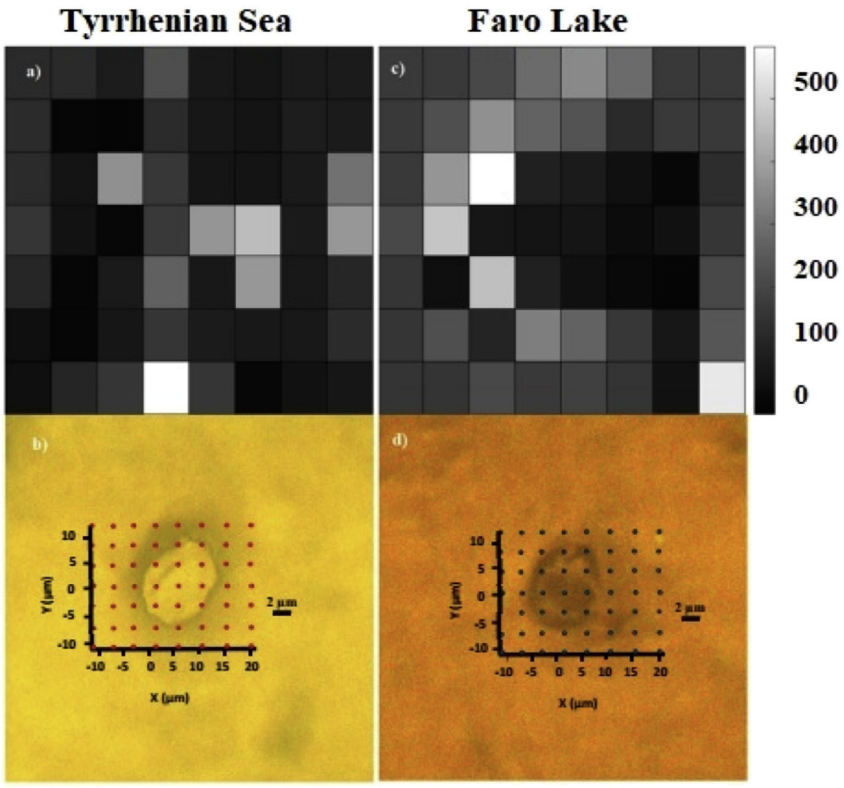


Fig. 5. Optical microscopy pictures and micro-Raman false color map (20 µm × 30 µm) obtained following the ratio of the (Amide II/Lipid) integrated area of the Tyrrhenian Sea (a,b) and Faro Lake (c,d) haemocytes samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

significant differences in chemical and physical parameters of two sampling sites and halinocytes (R1) and granulocytes (R2) measured in group A and group B. $P < 0.05$ was considered statistically significant for all analysis. Data were analyzed at 95% confidence level and all calculations were carried out. Data were analyzed using statistical software prism v. 5.00 (Graphpad Software Ldt., USA, 2003).

3. Results

The physico-chemical parameters and the statistical results of hemocyte sub-populations from *Mytilus galloprovincialis* (Bivalvia: Mytilidae) captured in two different habitats, Faro Lake and Tyrrhenian Sea (Sicily, Italy) are shown in Table 1.

As shown in Fig. 3, different hemocyte populations (R1: halinocytes and R2: granulocytes) with cells population count in mussel *Mytilus galloprovincialis* from Lake Faro (a) and Tyrrhenian Sea (b) - (Sicily, Italy) have been evidenced by flow cytometric analysis. Significant differences were found in halinocytes (R1) and granulocytes (R2) between the two groups of *Mytilus galloprovincialis* (Table 2). Furthermore, unpaired T-test showed that halinocytes (R1) values were significantly higher ($P < 0.001$) in group A with respect to group B. An opposite behaviour is shown by granulocytes (R2) [43,44].

In Fig. 4 are shown some optical microscopic pictures (a-f, g-h) of the Tyrrhenian Sea and Faro Lake haemocytes samples and the corresponding representative Raman spectra (Fig. 4i). Fig. 4b–c, g show that the spherical cells are very different in size (1–3 µm in size). In particular, the Faro Lake sample consists of few nanoparticles smaller than 5 µm in diameter. Some of them are isolated while others are agglomerated or overlapped (Fig. 4d–f, h). Moreover, as can be seen from Fig. 4i, the Raman spectrum of Faro Lake mussels look very different from that of Tyrrhenian Sea, reflecting different local structural arrangements. On the entire Tyrrhenian Sea cells, there is no remarkable evidence of the presence of the Amide II (α-helix) Raman scattering feature at 1458 cm^{-1} while the contribution at 1074 cm^{-1}, due to the lipid deformation, dominates. In details, Table 3 reports the main identified spectral bands, correlated to the presence of specific biomolecules, here used as markers for nucleic acids, lipids and proteins. Specifically, the contributions in the 650–1100 cm^{-1} region are attributed to DNA, triglycerides, glucose and nucleic acids, while those in the 1200–1800 cm^{-1} region are mainly ascribed to amides and lipids and partly to nucleic acids (i.e. adenine, guanine) [45–49].

Micro-Raman spectroscopy provided an excellent tool, in giving local structural information by selectively probing a microscopic scattering volume. Hence for a more in-depth analysis, we analyse the micro-Raman maps obtained by the integration of Amide II and lipid characteristic Raman bands. In Fig. 5 we report the maps obtained by the ratio between the Amide II and lipid integrated area of the Tyrrhenian Sea and Faro Lake haemocytes samples. The obtained values are represented on a 255 Gy scale (Fig. 5a,c). Brighter spots correspond to higher integrated area values. As can be seen from the images, in Tyrrhenian Sea mussel the contribution of the lipids dominates on the full cell while those of Amide II is nearly zero. On the contrary, the almost uniform intensity distribution of the Amide II/lipid ratio indicates that Amide II and lipid are homogeneously distributed inside the Faro lake cell even if, as expected, proteins features are localized mainly in the nucleus region while lipid ones are in the cytoplasmatic region [45,49]. Furthermore, we have observed that few Faro lake cells exhibiting an irregularly-shape, presumably associated to a spontaneous apoptotic process (Fig. 6). This modification of status is correlated with the projection of protrusions called “pseudopodia” (point #2 in Fig. 6a). We outline that these morphological features have been observed only in the Faro Lake sample.

4. Discussion

Mussels are considered as suitable sentinel organisms for the entire aquatic biocoenosis. Hemocytes represent the main mediators of immunity in invertebrates and their morpho-functional properties have been widely investigated as biomarkers in environmental monitoring [50]. The hemocytes count provides information on the physical and chemical parameters of water in which the mussel live, allowing to assess the relationship between these factors and the susceptibility of

Table 3
Main Raman marker bands and their assignments [45–49].

Raman band (cm^{-1})	Vibrational mode	Assignment
480–965	C-C twisting, δ (=CH) wagging	Protein, Lipid/Protein
990–995	C-O, C-C stretching	RNA (Ribose), benzene ring
1070	ν(C–C) or ν(C–O)	Lipids, Triglycerides, glucose
1074	ν(C–C) or ν(C–O)	Lipid (Phospholipids)
1090–1100	PO_2^- and O–P–O backbone stretching	DNA (Nucleic acids)
1140	ν(C–C)	Lipids
1180–1330	Amide III (β-sheet and random coil)	Protein (secondary structure)
1458	Amide II (α-helix)	Protein nucleic acid modes
1462	$\delta(CH_2)$ or CH_2/CH_3 wagging	Deformation of lipids, disaccharides
1490	C-N stretching, C-H bending	DNA (Guanine, Adenine), amino radical cations
1640	Amide I band	Protein band

organism changes in environmental conditions. An increase of total haemocytes can result from proliferation or movement of cells from tissues into hemolymph, whereas a haemocyte reduction can rely on cell lysis or recruitment from hemolymph to tissues [2]. These two compartments can be considered in a dynamic balance and various factors are involved in bilateral shifts of hemocytes, as well as presence of pathogens, body accumulation of contaminants, nutrients availability, genetic characteristics, or more generally stress-correlated situations [5,51–53].

In the present study, the recorded values of pH, ammonium 10, free chlorine, total chlorine, floride 2, total phosphate, potassium, although different between the two habitats, are within the permitted values (*European Commission, EEA, Primary Environmental Indicators, 2001*) and not visible between the two sites monitored. However, as shown in Table 2, an increase of total haemocytes (3053 cells) with a significant increase of granulocytes (R2) was observed in *Mytilus galloprovincialis* caught in Tyrrhenian sea with respect to Faro lake. The Tyrrhenian Sea water temperature of 17.8 °C was higher than those of 15.5 °C in Faro lake. Thus, it seems that total hemocytes value could be directly influenced by water temperature, as already observed in clams and mussels [10,54].

However, there are controversial data in the literature. Indeed, in marine bivalves it was observed that the number of circulating hemocytes increases upon reaching the temperature up to 28–32 °C [55] while other research reported that hyperthermia causes a significant decrease of total hemocytes in a temperature and time-dependent manner [56].

At this point, we analyzed the effects of dissolved oxygen (DO) and salinity on total hemocytes. As shown in Table 1, DO and salinity values are lower in Tyrrhenian Sea with respect to Faro Lake. Dissolved oxygen and salinity showed an inverse relationship with the total hemocytes count, probably depending on osmotic adjustment of the hemolymph compartment [57]. Moreover, the amount of circulating hemocytes increases under hypoxic conditions.

In this case, the low DO on the stimulation of heart rate acts as compensatory mechanism to maintain the oxygen tissue perfusion [58,59]. In any case, we have observed that total hemocytes count varies when exposed to specific environmental stressors [60,61] such as temperature and salinity.

Generally, hydrogen peroxide can produce an excess of hydroxyl radical via a Fenton mediated mechanism. This may induce acute oxidative injury if not scavenged or removed effectively by antioxidants. For example, acute oxidative stress induced changes in nicotinamide adenine dinucleotides in mouse skeletal muscles. There are several biochemical assay methods to estimate oxidative injury in cells. However, they do not provide information on the biochemical changes as the cells get damaged progressively under oxidative stress. Otherwise, micro- Raman spectroscopy combined to optical microscopy offers the possibility to monitor, point by point, the chemical composition of live cells undergoing oxidative stress [62,63].

In our case, we have observed that Faro Lake Raman peaks related to nucleic acids, lipids and proteins in the 700–1800 cm^{-1} showed several changes with respect to Tyrrhenian Sea (Table 3), indicating the breakdown of the phosphodiester backbone, a change of the protein conformational structure as well as of the nuclear bases [64]. The hemocyte degradation against the not-self (i.e. the real activation of the hemocyte) occurs through the production of oxygen free radicals (ROS) [65–67]. In some circumstances of ROS overproduction, the protection afforded by antioxidant defence mechanisms might be overcome, thereby leading to oxidative damage to tissue macromolecules including DNA, proteins and lipids. Xenobiotic-enhanced oxyradical generation can be a possible mechanism of pollution toxicity [68]. In our case, the observed spectral variations of the 1074 cm^{-1} and 1458 cm^{-1} peaks, ascribed to the lipid and Amide II (α-helix) contributions, support this hypothesis. In addition, the whole structure external to the hemocyte of the Faro Lake mussel was altered compared

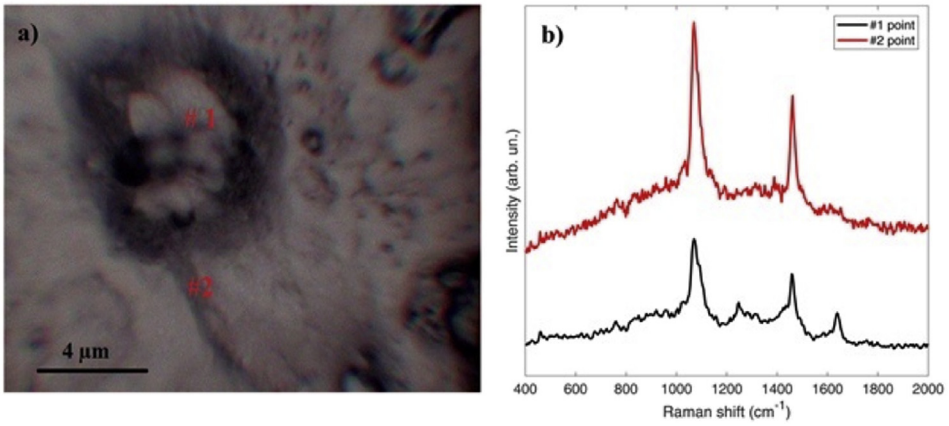


Fig. 6. Faro Lake optical microscope image characterized by a protrusion, called "pseudopodia" (a), and Raman spectra in two different cells points.

to those of Tyrrhenian Sea, not only for the extroflexion of the pseudopodia (point #2 in Fig. 6a) for which the disappearance of the Raman feature at about 1610 cm^{-1} was observed but also in terms of an increase in protein intake and specifically in actin, indicating a cytoskeleton reorganization (red spectrum in Fig. 6b).

On the overall, the observed biomolecular changes and the statistically significant increase in halinocytes (R1) in mussels sampled in Faro Lake with respect to those from the Tyrrhenian sea, are closely linked to the disruption of immune parameters, in turn, induced by the different values of some qualitative water parameters (temperature, salinity, dissolved oxygen, pH, ammonium 10, free chlorine, total chlorine, total phosphate, orthophosphate) in the two habitat. All these bio-chemical changes could be induced by the "glucose content" in the cells, probably used by lysosomes to activate enzymatic species involved in this process [64]. An intense metabolic cellular activity occurred in the hemolymph of Faro lake as indicated by the modifications of the C = C stretching vibrations of the Amide I α-helix structure and of the Amide III components linked to tubulin, heterodimeric globular protein containing subunits α and β and cytoskeleton element [69].

Summarizing, the combined effects of the environmental stressors have a great impact on bivalves, increasing the susceptibility to pathogens and also favouring natural mortality events. Finally, the aforementioned linkage between hemocyte counts and environmental parameters in *Mytilus galloprovincialis* can be considered as a useful tool to evaluate the effects of environmental stressors on the immune response, revealing in advance the development of potential critical situations for mussel survival. Nevertheless, further studies that compare the total hemocytes population of *Mytilus galloprovincialis*, collected in different sites, with several levels of pollution are necessary to clarify and fortify the knowledge of the linkage between hemocytes count population and habitat, to identify useful relationships on physiological adaptations to different environmental conditions.

5. Conclusion

Our result is innovative since, for the first time to our knowledge, detailed indications of cellular changes were obtained by using the spectroscopic data coming from the optical Raman scattering experiments. Ultimately, micro-Raman spectroscopy provides, in a short time, an accurate, non-invasive and low-cost method to monitor stress action on cells and a significant different arrangement in the cell structural organization by simply following their highly reproducible spectroscopic biomolecular signatures. In the future, the combined use of both techniques (flow cytometry and micro-Raman spectroscopy) will allow to perform more accurate investigations to better understand the variations of the hemocyte population in mussels in relation to anthropogenic loads, contributing to the identification of new "biomarkers".

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