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Ecological and Molecular Analyses of Macrophyte Ecosystems for the Assessment of the Environmental Health of Coastal and Transitional Areas

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1. INTRODUCTION

Background, scope, and aims

Aquatic ecosystems are among the largest and most productive systems in the earth. Marine ecosystems and especially coastal and transitional ecosystems can adjust the dynamic balance of the global sources of food and services, which is of great significance to human survival and development (Tang et al., 2018). However, they are also the ultimate sink for many global pollutants.

Coastal and transitional water bodies exhibit a high level of heterogeneity at biological and environmental level, particularly in areas of intensive anthropogenic activities (Guallar and Flos, 2017). This consequently leads to physicochemical alterations, habitat destruction and changes in biodiversity (Borja et al., 2012).

Recent worldwide concerns in ecosystem protection and monitoring programs have focused on the degradation of marine water quality caused by environmental pressures, including habitat loss, decreased biodiversity, harmful algal blooms, anoxia, and contamination by sewage, pesticides, polycyclic aromatic hydrocarbons, heavy metals and other organic and inorganic pollutants (Diaz and Rosenberg, 2008). Marine environmental researches conducted during the last two decades indicted that coastal and transitional ecosystems are mainly in a sub-healthy or an unhealthy state (Tang et al., 2018). Direct or indirect human influences have significantly modified coastal ecosystems, leading to alteration of their functions (Franzo et al., 2015). The ecological integrity of marine environments is threatened by increasing urbanization, intensive agricultural and industrial activities (Torres et al., 2008). Heavy metals, petroleum hydrocarbons, pesticides and herbicides are the most prevalent and persistent toxic chemical

substances that ultimately are discharged into marine coastal and transitional water bodies. These chemicals, which are associated with anthropogenic stressors, are able to affect the aquatic ecosystem health (Hook et al., 2014). The adverse effects of these toxics to the marine organisms (Bohmer et al., 2001; Brack, 2005; Burton et al., 2002; Olivares et al., 2016) and human health (EPA 2000; Sany et al., 2014) are very dangerous. In particular, organic pollutants are hydrophobic and bioaccumulates in fatty tissues of plants and animals whereas heavy metals bind to proteins (Gautam et al. 2016; Vidal-Liñan and Bellas, 2013; Wahsha et al., 2012). The ecosystem degradation, therefore, has increased the attention to evaluate the aquatic health to improve the ecosystem conservation and monitoring programs (Martinez-Haro et al., 2015; Prat et al., 2013).

The Ecosystem Approach is a reflection of Europe's increasing efforts to improve, protect and conserve aquatic ecosystems in line with the aim of European Directives (i.e. Habitats and Species Directive 92/43/EEC, Water Framework Directive - 2000/60/EC, Marine Strategy Directive - 2008/56/EC, Environmental Impact Assessment Directive - 2011/92/EU). The European Water Framework Directive (WFD) (EC, 2000) represents a transformation of the guidelines for water quality assessment and monitoring across all the EU Member States in terms of protection and management of inland surface, transitional, coastal and ground waters. The inherent aim of the WFD is to protect and prevent deterioration of European waters on the basis of their ecological community structure. Under WFD, the monitoring of both ecological and chemical status using the Biological Quality Elements (BQEs) is seen as an extremely important tool to achieve the main aim of "Good" water status for all EU water bodies.

Marine benthic macrophytes (macroalgae and angiosperms) are considered fundamental BQEs for the monitoring and assessment of the ecological status of coastal and transitional waters (Buosi and Sfriso, 2017). They are often used as indicators of water quality, as they integrate the effects of long-term exposure to nutrients or other pollutants, and alteration of the bottom or hydrodynamic conditions (Ballesteros et al., 2007; Nikolic et al., 2013; Orfanidis et al., 2011; Sfriso et al., 2014). Macrophyte communities are sensitive to anthropogenic stressors, modifying their structure and function accordingly (Sfriso et al., 2009; Sfriso and Curiel, 2007). Macrophytes respond with a decrease or even the disappearance of the most sensitive species and their replacement by highly resistant or opportunistic species (Orfanidis et al., 2001; Orfanidis et al., 2011; Sfriso et al., 2009, 2014; Sfriso and Curiel, 2007). Thus, macrophytes provide information on the health of the environment and consequently are used as early warning signals for general or punctual stressors (Ferrat et al., 2003).

The use of macrophyte community changes to evaluate and diagnose water quality status necessitates an understanding of the underlying resulted ecological processes (Rindi and Guiry, 2004). This is related to their sessile nature and rapid response to anthropogenic stressors in aquatic environments (Sfriso et al., 2017). Macrophyte biotic indices, based on community analysis at the species level have been developed to evaluate the health status of coastal and transitional waters (Mouillot et al., 2005; Orfanidis et al., 2001, 2011). During the last two decades, different macrophyte biotic indices have been developed (Giaccone and Catra, 2004; Orfanidis et al., 2001, 2011; Sfriso et al., 2007, 2009; Viaroli et al., 2008). For instance, Sfriso et al., (2007, 2009) proposed a rapid (R-MaQI) and an expert version (E-MaQI) of MaQI (Macrophyte Quality Index), specifically for Mediterranean transitional environments, which have been validated on several

Italian lagoons. The final version of MaQI, which was successfully inter-calibrated in the European inter-calibration exercise, integrated the two previous versions of the index (Sfriso et al., 2014) and was adopted by the Italian Ministry of Environment to assess the ecological status of coastal waters according to the WFD (2000/60/EC) requirements. For coastal waters a valuable tool for monitoring ecosystem health and water quality using macrophyte communities was the index CARLIT (Cartography of littoral rocky-shore community index (CARLIT) (Ballesteros et al., 2007; Buosi and Sfriso, 2017; Nikoli´c et al., 2013).

Within the scope of WFD, increasing interest nowadays takes the ecotoxicological line to better diagnose the impairment of the biological quality elements within the ecological system at the early stage of exposure, and to establish cause-effect relationship in ecological quality assessment (EC - 2009, 2010). Furthermore, the ecotoxicological studies may provide more realistic assessment of the impact and exposure of aquatic organisms to anthropogenic pollutants. Indeed, biomarkers can offer a good connection between both classical (physico–chemical) and biological approaches, thus providing early warning signals to anticipate potential impacts at higher levels of biological organization (Allan et al., 2006).

In transitional and coastal environments, macrophytes can be exposed to several anthropogenic pollutants resulting in intermittent intracellular oxidative stress because of the accumulation of Reactive Oxygen Species (ROS) (Mitler, 2002; Pinto et al., 2003). There, different types of pollutants can stimulate the production of ROS causing toxic effects in aquatic organisms (Torres et al., 2002) like cellular oxidative damage to lipids, proteins or DNA. The determination of the oxidative stress can be quick and cost effective tool to provide an early warning signal of ecosystem disturbance (Wahsha et al., 2017).

Oxidative stress deteriorates the structure and functionality of the cells. Fatty acid rich structures, such as the cell membranes may be oxidized by overproduction of ROS, causing a loss of rigidity and permeability resulting ultimately in cell death (Sharma et al., 2012; Wahsha et al., 2010). In the oxidation process, known as lipid peroxidation (LPO), the determination of lipid peroxide malondialdehyde (MDA) content is a widely-used method to estimate the oxidative stress level in biological material (Wahsha et al., 2012). The estimation of LPO is considered a highly predictive biomarker of pollution effects.

Beside the previous approaches, the shifts of microbial communities caused by anthropogenic stressors have been increasingly studied in the marine environment, particularly when they are associated with eukaryotes, such as corals (Ainsworth et al., 2010; Rosenberg et al., 2007), macroalgae (Egan et al., 2013; Marzinelli et al., 2015; Singh and Reddy, 2014; Wahl et al., 2012) and sponges (Hentschel et al., 2012; Webster and Bourne, 2012). Due to their sensitive and rapid response to environmental stressors, marine microbial communities act as sentinels of environmental impacts (Sun et al., 2012). Environmental stressors affects significantly the microbial community's structure of the water column (Meziti et al., 2016; Staley et al., 2013), marine eukaryotes and several critical functions of the ecosystem (Thompson et al., 2015). Studies on marine macrophytes suggest that bacterial assemblages which are hosted on cortical tissues can be disrupted by environmental stressors (e.g., anthropogenic pollution; Marzinelli et al., 2015; Staley et al., 2013).

The shift of microbial communities on macrophyte walls can be a reliable indicator for detecting or diagnosing changes in marine ecosystems due to their sensitivity and rapid response to hydrologic and water quality changes (Won et al.,

2017). For instance, some bacterial strains associated to invasive macroalgae and absent in the native species, are suggested to play a key role in stress tolerance (Aires et al., 2013; 2015). Furthermore, these microbial communities can also rapidly respond to seasonal fluctuations, including phytoplankton abundance, grazing pressure and the excess of nutrient concentrations (Gilbert et al., 2009; 2012; Meziti et al., 2015).

The recent development of Next-Generation Sequencing (NGS) technologies provides opportunities to explore these complex microbial communities and their response mechanisms to anthropogenic stressors (Kysela et al., 2005; Sogin et al., 2006). Particularly, NGS technologies are powerful tools to study the diversity, composition, and structure of microbial communities (Zhang et al., 2014). For instance, the sequencing of 16S rRNA genes has been used to explore the shifts of macrophyte-microbial communities inhabiting coastal and transitional water bodies characterized by intense anthropogenic activities. Nevertheless, few studies of macrophyte microbial communities using NSG technologies were conducted.

Examples of marine areas affected by high anthropogenic pressures are the lagoon of Venice (Italy) and the Gulf of Aqaba (Jordan). They are unique and highly variable ecosystems. The semi-enclosed nature of both areas exposes them to various forms of anthropogenic disturbances. Both areas are heavily impacted by multiple pressures that generate large amounts of pollutants from urban, agricultural and industrial activities (Al-Rousan et al., 2016; Micheletti et al., 2011; Sfriso et al., 2014) and the ecological status and benthic macrophyte communities are highly correlated with the anthropogenic contamination.

The expected results of the present study will provide new insights into the aquatic ecosystem functions and describe the response to anthropogenic stress in

the macrophytes, by applying holistic and multidisciplinary ecological approaches. In this context, the present study aims to use macrophytes to assess the ecological status for marine coastal and transitional environments employing integrated approaches based on ecological indices and oxidative stress biomarkers as well as macrophyte associated microbial communities. The results will provide a more complete assessment of the anthropogenic stressors affecting the health of the marine environments, with potential implications for monitoring and improving marine ecosystems.

Objectives

The significances and purposes of this study are to:

1. Assess the ecological status of coastal and transitional marine environments using ecological indices (Macrophyte quality index).
2. Assess the ecological status of coastal and transitional marine environments using oxidative stress biomarker (lipid peroxidation).
3. Assess the concentration and accumulation of heavy metals in sediments and selected macrophyte tissues.
4. Investigate the shift of the microbial communities associated to macrophytes under different source of anthropogenic pressures.
5. Investigate the correlation between anthropogenic pressures, environmental parameters and the ecological status using statistical analyses.
6. Evaluate the suitability of the adopted integrated approach for monitoring water quality in costal and transitional ecosystems.

LITERATURE REVIEW

1. Anthropogenic pressures in aquatic environment

During the most recent decade the evaluation of the impacts of anthropogenic stressors on the health of aquatic ecosystems is expanding around the world. Aquatic ecosystems can be stressed by multiple environmental factors which originate from a variety of anthropogenic sources. Stressors challenge the integrity of ecosystems and the quality of the environment (Hook et al., 2014). For instance, the Gulf of Aqaba and the lagoon of Venice are aquatic environments which suffer of significant anthropogenic pressures.

1.1 The Lagoon of Venice

The lagoon of Venice (LV) is a shallow transitional water system with an average depth of 1.2 m and a total surface area of 549 km^2 (**Fig. 1**). It is divided into three main sections, named the southern, the northern and the central basins. The city of Venice is located in the central basin. The daily tidal action enable the renewal of about 60% of the lagoon water every 12 hrs (Masiol et al., 2014). The LV is connected to the northern Adriatic Sea by three wide inlets, with an averaged water exchange of ca. 8000 m^3/s (Gačić et al., 2005). Ecosystem seasonality of LV is highly variable. In late winter-spring the increase of the autotrophic community activity results from an increased level of available inorganic nutrients and favorable light and temperature conditions. In summer, the ecosystem productivity reaches its maximum level when concentrations of dissolved inorganic nutrients are low and production is mainly sustained by nutrient recycling (Bandelj et al., 2008). The productivity during autumn is limited by unfavorable light and temperature conditions and waters rich in inorganic nutrients (Solidoro et al.,

2004). The annual variability of the ecosystem productivity is mostly related to the climate conditions (Cossarini et al., 2008; Facca et al., 2002). Water circulation in the LV is mainly driven by tidal action (Gačić et al., 2004). Residence time varies over the lagoon and with meteorological conditions, with average values ranging from few days in the areas close to the inlets to 40 days in the inner, more confined areas (Cucco et al., 2009). Water nutrients (dissolved inorganic nitrogen, phosphate and silicate), chlorophyll-*a* and plankton abundance and diversity are spatially different among the LV (Bandelj et al., 2008; Sfriso et al., 2016). These variations are related with the uneven distribution of nutrient loads from rivers and other discharge points along the marginal areas (Solidoro et al., 2004) and the complex hydrodynamics.

Biologically, the LV is a highly productive ecosystem, characterized by high diversity of macrophytes. During the last years, the macrophyte abundance and diversity have experienced continuous changes related to stresses by anthropogenic pressures (Sfriso and Facca, 2007). Macroalgal blooms have been reported since 1960s, 1980s and until 1990s and consisted of abnormal growth of Ulvaceae, which replaced seagrass populations (Sfriso et al., 2003). The high densities of these blooms covered large areas affecting nutrient cycles both in surface sediments and the water column (Sfriso et al., 2003; Solidoro et al., 1997a, 1997b). In the following years, a combination of factors (Sfriso and Marcomini, 1996) decreased macroalgal blooms until 2005, particularly in the central basin (Miotti et al., 2007). However, the high biomasses recorded in the past have not been observed anymore (Sfriso et al., 2007).

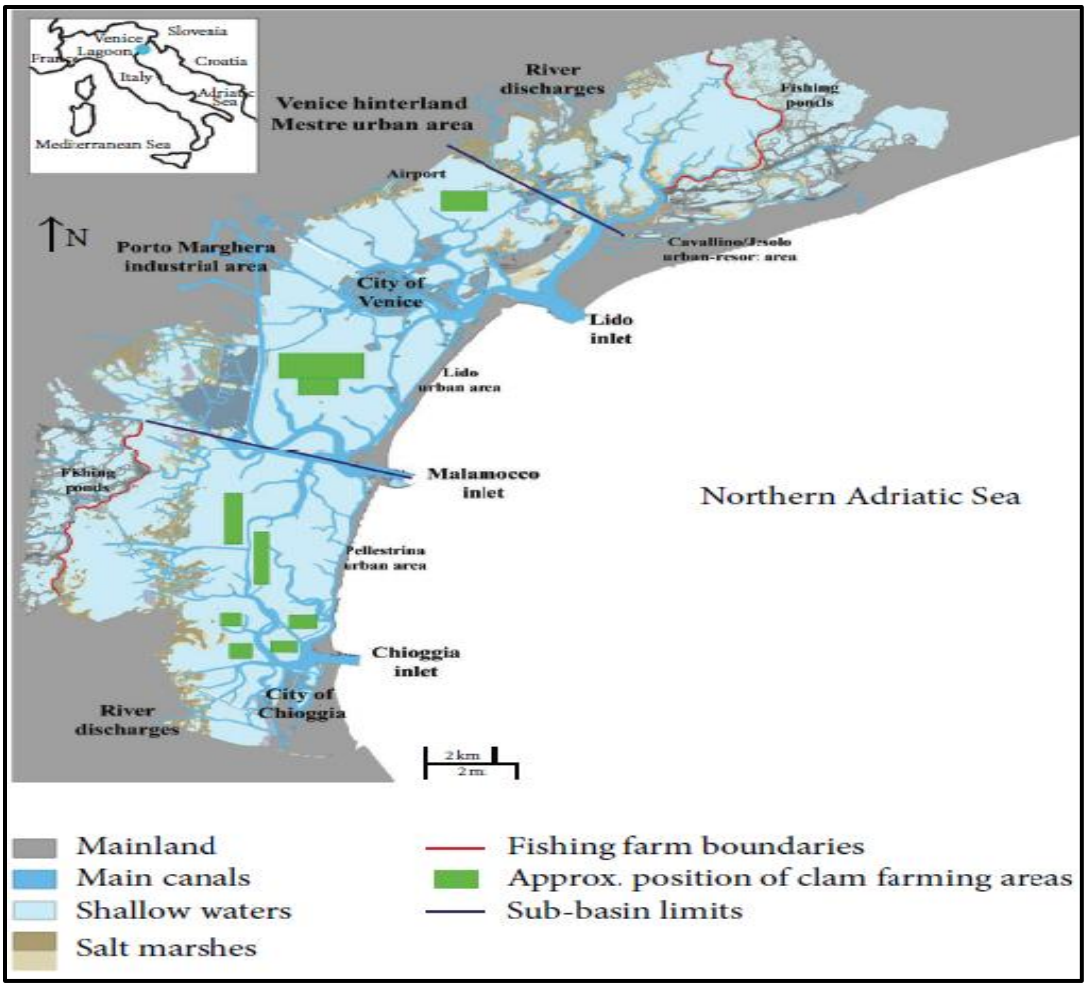

Fig. 1: map of the lagoon of Venice with major sources of anthropogenic activities (Facca et al., 2014).

Approximately 300 species of macroalgae have been recorded in the LV (Sfriso and Cavolo, 1983; Sfriso and Curiel, 2007) and the number of species is increasing, although 87 taxa (54 Rhodophyceae, 25 Phaeophyceae, and 8 Chlorophyceae) recorded in the past have not been found in the lagoon in recent years. Interestingly, some newly recorded species, such as *Sargassum muticum* (Yendo) Fensholt and *Undaria pinnatifida* (Harvey) Suringar have largely colonized the lagoon. They are large species of extra-Mediterranean origin introduced into the lagoon in the early 1990s which colonized hard substrata replacing the native species (Curiel and Marzocchi, 2010).

At present, the main primary producers of the LV are aquatic angiosperms. The first studies of seagrasses have been carried out in 1990 by Caniglia et al., (1990). These authors mapped the distribution of the three main species that colonize the LV (i.e., *Cymodocea nodosa* (Ucria) Ascherson, *Zostera marina* Linnaeus and *Z. noltei* Hornemann). Totally, in 2002 these species covered ca. 5431 ha (Rismondo et al., 2003). However, during the years the areal distribution of the seagrass species had changed. The increase of marine conditions in the lagoon favoured the distribution of *C. nodosa*, the most adaptive species (Sfriso et al., 2007), whereas the restoring of good conditions in the inner areas, recently, triggered the spread of *Z. marina* and *Z. noltei*. Since 2014, *Z. noltei* and *Z. marina* were successfully transplanted in the northern part of the LV, where they disappeared, in order to recovery good ecological conditions also in this confined area. This activity was funded by the European Community through a LIFE nature project (LIFE12 NAT/IT/000331-SERESTO) "Habitat 1150* (Coastal lagoon) recovery by SEagrass RESTOration, a new strategic approach to meet HD & WFD objectives". The Department of Environmental Sciences of the Ca`Foscari University of Venice was the partner which coordinated the transplanting activities and the monitoring of the ecological status of the project area.

1.1.1 Potential Sources of anthropogenic stress

The Venice lagoon is a transitional water system that experienced many natural and anthropogenic pressures. Since the mid 20th century, the ecosystem was exposed to several anthropogenic pressures causing sediment (Bellucci et al., 2000; Guerzoni et al., 2007; Secco et al., 2005; Zonta et al., 2007) and water (Micheletti et al., 2011) contamination. Eutrophication (Sfriso et al., 2003), overexploitation of biological resources (Pranovi et al., 2004), degradation of biota due to

bioaccumulation of pollutants through the food chain (Raccanelli et al., 2004; Sfriso et al., 2008; Turetta et al., 2005), sediment erosion (Sfriso et al., 2005; Sarretta et al., 2010; Rapaglia et al., 2011) and salt marsh losses (Molinaroli et al., 2009) are considered of the major pressures on the lagoon ecosystem.

Different stressors act on the lagoon ecosystem, causing multiple environmental impacts. Increased urbanization and land reclamation for agriculture, aquaculture and industry reduced the total surface of the lagoon by ~3280 ha between 1924 and 1960 (Ravera, 2000). A major industrial area, including chemical and oil industries, was established, leading to the dredging of deep navigation canals.

The industrial and agricultural activities grew significantly from the early 1940s to 1960s, seriously affecting the lagoon ecosystem. Pollutants discharged into the lagoon are still present in the sediments and have been bioaccumulate in the food chain (Dalla Valle et al., 2005; Frignani et al., 2005; Pavoni et al., 1992). Pollutant contamination is particularly high in the sediment of the industrial canals (Guerzoni et al., 2007; Guerzoni and Raccanelli, 2003). Studies of contaminants in the system (Micheletti et al., 2008; Raccanelli et al., 2004) suggest that bioaccumulation of persistent organic pollutants (POPs) in lagoon organisms may be significant. These conclusions have important implications both in terms of risk for human health (Frangipane, 1999; Raccanelli et al., 2008) and of increase in natural mortality and/or energy expenditure for metabolic detoxification of species (Carrer et al., 2005; Guerzoni et al., 2007; Micheletti et al., 2008). A study by Guédron et al., (2012), shows that some canals of the lagoon are contaminated by Hg coming from two chloralkali plants of the industrial area of Porto Marghera. Until a few years ago, attention has been mainly focused on heavy metals (Hg, Pb, As, Cd, Zn, and Ni), whereas recently studies addressed toward all the classes of

POPs, such as dioxins, polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB) and polycyclic aromatic hydrocarbons (PAHs). Industries are the major sources of these pollutants, which are delivered to the lagoon through point and nonpoint pollution pathways, as well as atmospheric fallout (Guerzoni et al., 2007). Frignani et al., (2005) reported that the highest values of micro-pollutants (Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and hexaclorobenzene (HCB)) have been found in the San Giuliano Canal that is likely the major conveyor of pollutants to the area of Campalto. Moreover, Sfriso et al., (2014) reported that the lagoon was contaminated by organochlorine compounds and clam bioaccumulation was significantly higher in the polluted sites.

Recent studies on the LV showed that some toxics, such as Hg and As, are widespread and exhibit high concentrations in surface sediments (Bellucci et al., 2002; Bernardello et al., 2006). The clam *Tapes philippinarum* Adams & Reeve growing in the contaminated sediment of San Giuliano and Fusina exhibited high tissue bioaccumulation of As, Cd and Hg because the high level of pollutants settled in these areas (Sfriso et al. 2008). Another recent study by Sfriso et al., (2018) investigated the relationship between metal concentration in surface sediments and their bioaccumulation on three bivalve species: razor clam (*Solen marginatus* Pulteney, 1799), Manila clam (*Ruditapes philippinarum* Adams and Reeve, 1850) and lagoon cockle (*Cerastoderma glaucum* Poiret, 1789). The results showed that bivalve contamination, especially for razor clams, was significantly influenced by environmental conditions and sediment granulometric composition.

Industrial activities also caused a number of indirect impacts on the lagoon like the dredging of large navigation canals that altered the morphology of salt marshes and modified permanently a large part of the central basin. In addition, the

diversion of rivers outside the lagoon, together with the building of jetties and digging of new canals, increased the intrusion of seawater, leading to habitat and species changes with reduction of oligohaline habitats. These morphological changes clearly impacted the spatial distribution, structure, and composition of the macrobenthic communities and brackish species have been replaced with marine species (Giordani Soika and Perin, 1974).

An exponential increase of fertilizers and pollutants from the industrial area, contributed to increase nutrient loads flowing into the lagoon. The eutrophication was the main effect that between 1960s and 1980s affected the lagoon when annual dystrophic crises occurred (Pavoni et al., 1990) and the release of hydrogen sulphide adversely affected human activities (Ravera, 2000). Dystrophic crises also had significant impacts on the lagoon ecosystem, influencing biogeochemical cycles, promoting the release of large amounts of nutrients, enrichment of organic matter in surface sediments and affecting benthic communities (Solidoro et al., 1997a, 1997b). The decrease of fish, shrimps and crabs was one of the main consequences of these changes (Sfriso et al., 1992; Sfriso and Marcomini, 1996). *Ulva* mats acted as traps for nutrients entering the lagoon, storing them in surface sediments and algae tissues. In summer, phosphorus and nitrogen entering the lagoon from the Adriatic Sea during tidal exchanges contributed to support macroalgal growth (Sfriso and Marcomini, 1996).

The lacking of sewage systems in the city of Venice (Scearce, 2007) is another source of contamination because the waste coming from the historical centre of Venice is discharged directly into canals. Furthermore, clam harvesting and aquaculture activities increase nutrients and pollutants in the lagoon water (Cloern, 2001).

1.2 Gulf of Aqaba

The Gulf of Aqaba (GA) is one of two northward extensions of the Red Sea. It is a semi-enclosed basin separated from the Red Sea by the Straits of Tiran. The gulf is surrounded by Egypt in the west, Saudi Arabia to the east, and Jordan and Israel to the north **(Fig. 2**). The length of the Jordanian coast in GA is extremely short: ca. 26.5 km and the semi-enclosed nature of the GA provide a unique ecosystem with unique biological communities. In fact, the Red Sea supports species diversity higher than any other eastern Indian Ocean region (Crosby et al., 2000).

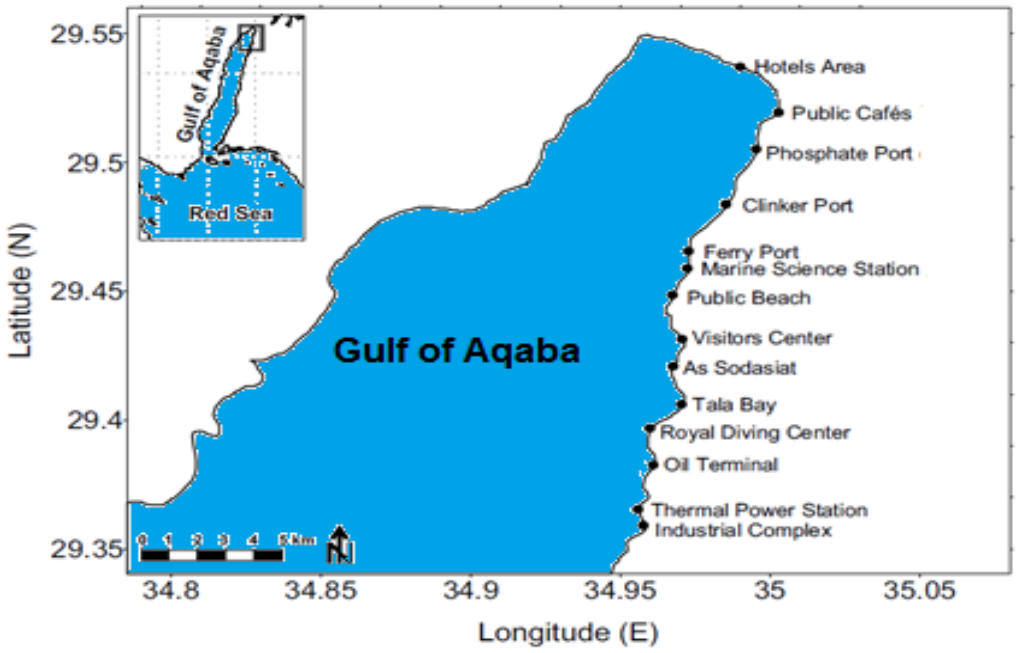

Fig. 2: The Jordanian coasts of the GA. Map represent the major activities along the coast of GA

The GA is characterized by exceptional high water transparency due to the absence of major rivers and streams flowing into the sea (Hulings, 1989) as well as by low levels of suspended matter, low phytoplankton biomass (Wahbah and Zughul, 2001). The exposure to dry and hot climate, with high evaporation, increase temperature and salinity, these values are higher than the average of other

seas around the world (Manasrah et al., 2006). Indeed, salinity is relatively high and ranges between 40 and 41 psu. During summer, the surface water temperature reaches 28 ºC, whereas it falls to above 20 ºC in winter (Manasrah et al., 2004). The seasonal variation is about 6-7°C (Manasrah et al., 2004). The upper 200 m of the water column during summer display characteristics quite different from winter (Manasrah et al., 2004). In summer, a strong thermocline is established with a temperature of 21-27 °C, whereas in winter water column mixes when temperature is 20.5-21.0°C (Manasrah et al., 2006).

Nutrient concentrations, particularly nitrates, phosphates and silicates are quickly depleted in the surface layers during summer. In contrast, in winter the thermocline weakens and deep convective mixing persists for several months, bringing up relatively nutrient-rich water to the surface (Al-Rousan et al., 2004). Furthermore, inorganic nutrient concentrations are within the typical range reported for oligotrophic tropical oceans (Al-Rousan et al., 2004; Badran, 2001). Concurrently, the extremely oligotrophic conditions of GA are related to the arid climate of the area and the nutrient depleted water received from the Red Sea through the Straits of Tiran (Al-Rousan et al., 2016). The low chlorophyll-*a* concentration is related to the low phytoplankton biomass, which partly explains the high water transparency.

The semi-enclosed nature of GA contributes to the limited water exchange with the Red Sea and the Indian Ocean. The mean water residence time in the GA ranges from one to three years (World Bank, 1996). This, along with the relatively small volume of the Gulf, contributes to the accumulation of pollutants, which is one of the most important sources of environment disturbances in the Gulf area.

On the other hand, the extensive sunlight, clear visibility, deep light penetration, and the warm waters are the driving factors of the luxuriant development of corals (Al-Horani et al., 2006; Schuhmacher et al., 1995). GA exhibits a high diversified environment of hard and soft corals (Al-Horani et al., 2006) and about 512 fish species have been recorded (Khalaf and Kochzius, 2002) in addition with thousands of other reef associated organisms. Unfortunately, little literature related to the diversity of macrophytes in the GA area is available. Natour et al., (1979) recorded 25 species of green and brown macroalgae and 28 species of red macroalgae in the Jordanian coastal waters. High diversity of macroalgae is observed in localities having minimal exposure to wave action, substrate stability and slope at different sites along the coast. According to Littler and Littler (1984) six functional groups of macroalgae were identified in the coastal region of the GA: filamentous algae, calcareous algae, sheet-like algae, thick lathery algae, coarsely-branched algae and crusts algae.

Another study in the GA carried out by Al-Zibdah and Damhoureyeh (2006), reports 28 genera of benthic macroalgae, including 7 Chlorophyceae, 10 Phaeophyceae and 11 Rhodophyceae. The result clearly demonstrated that brown algae (Phaeophyceae) had the highest cover and biomass. The highest abundance of algae was during the spring between March and May.

In the Red Sea, the seagrass meadows colonize the mid-tidal areas (Hulings, 1979; Edwards and Head, 1987; Lipkin, 1979; Lipkin et al., 2003). In the GA *Halophila stipulacea* (Forsskål) Ascherson, *H. ovalis* (R. Brown) J.D. Hooker and *Halodule uninervis* (Forsskål) Ascherson are only present (Wahbeh, 1980; 1982). Seagrass meadows are important nursery for many commercially important fishes and crustaceans. In the GA, Wahbeh (1980) found more than 49 species of

invertebrates (mostly molluscs) living in seagrass meadows, either attached to the plant (gastropods) or buried in the sediment (bivalves).

The city of Aqaba, which lies on the Jordanian side of the GA, is the only access of Jordan on the sea. The development of the city during the last two decades for commerce, tourism and technology increased the total population, the number of tourists, industrial and commercial activities and wastewater discharge, which became the main source of anthropogenic disturbances for the marine ecosystem (Al-Halasah and Ammary, 2007).

1.2.1 Potential Sources of anthropogenic stressors in Gulf of Aqaba

The major source of anthropogenic pressures for the aquatic ecosystems in the GA includes urbanization and tourism, oil transport, industrial pollution and sewage discharges (Wahsha et al., 2017). These pressures have consequently affected the health status of the aquatic biological communities.

Urban development and industrial pollution contributed to high environmental pressure on the GA (Abu-Hilal and Badran, 1990; Mancy, 1993; Wahsha et al., 2016). The rapid development of industrial and port activities during the last 30 years caused degradation and death of coral reefs, and loss of the biodiversity (Khalaf and Kochzius, 2002). Furthermore, the industrial discharges into the Gulf are the major source of suspended particles and heavy metals. Sources of metals include land-based operations such as clinker production and fertilizer manufacture in Aqaba and seawater desalination plants in Eilat (Abu-Hilal and Badran, 1990). The dust settles during phosphate exportation and contributes to increasing the waterborne phosphate concentration, reducing water transparency and increasing sediment load. In fact, phosphate dust deposition was

suggested as a factor contributing to reduce coral growth rates (Al-Rousan et al., 2012). The heated cooling water discharged by power plants and fertilizer production increases water temperature by several degrees, affecting the aquatic life (Wahbeh, 1993).

The concentration of phosphate and heavy metals were found to be relatively higher in areas of dense industrial activities (Wahsha et al., 2016). Moreover, the study of Al-Absi et al., (2016) found that uranium concentrations were significant in sediment and seagrass samples collected from the area near phosphate port site in the north part of the reef area. They found that uranium levels were also higher compared to those from other regions of the world. In addition, Khalaf and Kochzius (2002) noticed a reduction in fish abundance and changes in the relative abundance of different fish communities in contaminated areas in GA.

Numerous shipping and industrial activities are the major source of oil spill. Accidental or intentional releases of oil-contaminated bilge or ballast water from oil tankers contribute to damage the reproductive system of corals, interfere with the production of larvae, and inhibit normal settling (Al-Horani et al., 2006). Oil spills reduce the light penetration in water reducing photosynthesis and affecting macrophyte biodiversity (Al-Halasah and Ammary, 2007).

Recreational activities in GA are the major source of solid waste and litter. This poses a constant, high visible environmental problem. Seagrass meadows, fish and the benthic macro fauna are heavily impacted by discarded plastic and other synthetic materials (Abu-Hilal and Al-Najjar, 2004).

1.3 Impacts of anthropogenic stressors on Macrophyte communities

Ecosystem health can be defined as the ability of a system to maintain its organizational structure, self-regulate and recover after stress (Rapport et al., 1985). Aquatic macrophytes undergo change and are subject to a number of natural or anthropogenic stresses. Hence, it is essential to assess the consequences of the anthropogenic stress impact on species and the overall aquatic ecosystem (Martinez-Haro et al., 2015). Macrophytes can be effectively used as long-term indicators of the conditions to which they are subjected (Orfanidis et al., 2001) and are therefore considered as a fundamental biological quality element in the WFD. Their temporal and spatial distribution is affected by several environmental factors and anthropogenic stressors. **Table 1** summarizes some examples of the impact of anthropogenic stresses on macrophytes.

The environment degradation by anthropogenic stresses, such as high nutrients load, introduction of toxic chemicals and sediment re-suspension, contribute to the shrinking of seagrass meadows or the disappearance of sensitive macroalgae that are replaced by thionitrophilous taxa, especially Ulvaceae, Cladophoraceae, Glacilariacae and Solieriaceae (Sfriso et al., 2017). In the worst cases, the bottom can be completely deprived of vegetation (Sfriso et al., 2014, 2009) and phytoplankton and/or cyanobacteria become dominant (Viaroli et al., 2008). Under these conditions the coastal and transitional water bodies are affected by sudden changes in the production and decay of macrophytes, which can trigger anoxic crises, leading to the death of the benthic macro fauna and fish. These disturbances in the aquatic environment are negatively affecting the ecological status and degrade the ecosystem integrity.

Table (1): Macrophyte communities' response to anthropogenic stress

Anthropogenic stress	Macrophytes	Impact	References
Heavy metals	Macroalgae	Inhibition of reproduction and development	Caliceti et al. (2002); Crowe et al. (2000); Lobban and Harrison (1994)
	Seagrasses	Modify morphology of seageass cells	Wahsha et al. (2016)
Petroleum hydrocarbons	Macroalgae	Short term growth reduction of intertidal species	Lobban and Harrison (1994)
	Seagrasses	Decline of meadows by reducing light penetration	Al-Halasah and Ammary. (2007)
Organic matter	Macroalgae	Change community structure resulting from reduced light penetration	Lobban and Harrison (1994)
	Seagrasses	Decline of meadows by reduced light penetration and organic matter accumulation in sediment	Hemminga and Duarte (2002)
Eutrophication	Macroalgae	Dominance of opportunistic species, algal blooms	Lotze and Schramm (2000); Sfriso et al., (1992)
	Seagrasses	Meadows divergent, dominance of fleshy macroalgae	Larkum et al. (1989); Sfriso and Facca (2007)

Over the last decade, efforts have been made by researchers to develop methodologies for assessing the ecological status of aquatic environments under anthropogenic interferences using the biological quality elements. These methodologies provide reliable tools for evaluating the effects of anthropogenic pressures on the quality of the ecological systems in marine life.

1.4 Approaches for the assessment of the ecological health status

1.4.1 Community based approach to assess the ecological status: Ecological Indices

In the last decade, several ecological indices have been adopted to assess the quality of marine ecosystems based upon the community levels. Ecological indices provide numerical a dimensional values to express the general status of ecosystems through the description of different aspects of the structure and the sensitivity of communities (diversity, abundance, tolerance and/or its combination) (Connon et al., 2012). This type of indices employs the "biological quality elements" (BQE) to classify water bodies into "ecological status classes" (ESC). According to WFD, the ecological status is expressed as an "ecological quality ratio" or EQR (ranging from 0 – low quality to 1 – high quality), which is calculated as a quotient between the value of an indicator in a site and a reference value which is defined by incorporating the natural variation of the considered BQE (EC, 2003).

The European Union WFD (EC, 2000) sets a legal framework for monitoring the ecological quality of European waters to protect and improve coastal and transitional waters (Borja, 2005). After the WFD entered into force, several studies on coastal and transitional environments in Spain, Portugal and Greece started (i.e. Ballesteros et al., 2007; Borja et al., 2000, 2004, 2006; Orfanidis et al., 2001, 2003, 2011; Prat et al., 2013; Simboura and Zenetos, 2002; Simboura, 2004; Simboura et al., 2005). Some researchers from those countries proposed to assess the ecological status of coastal and transitional environments by studying macrobenthic communities (AZTI Marine Biotic Index -AMBI- Borja et al., 2000, 2004; Biotic index -BENTIX- Simboura, 2004) or macroalgal associations (Borja et al., 2004; Ecological Evaluation Index -EEI- Orfanidis et al.,

2001, 2003, 2011; Panayotidis et al., 2004) due to their sessile nature and the relatively long life span.

The first attempt to assess the ecological status of transitional environments by ecological quality elements in Italy was the results obtained by some macroalgal taxonomic studies (Sfriso et al., 2002, 2006) in the LV. These studies gave evidence of a high correlation between the Rhodophyceae/Chlorophyceae (R/C) ratio and the ecological status of the environment. They are based on the fact that, in general, Chlorophyceae taxa prevail in eutrophic and polluted areas whereas the number of Rhodophyceae is more abundant in less polluted areas. After that, different studies by national programs in Italy were conducted to assess the ecological status of transitional waters in different Italian lagoons (i.e. Lesina, Goro, Venice, Orbetello, etc.). The later investigations in LV were employed to set up a new Quality Index, based mainly on macrophytes (MaQI = Macrophyte Quality Index). Initially, two versions were proposed: an expert (E-MaQI, Sfriso et al., 2006a) and a rapid assessment (R-MaQI, Sfriso et al., 2007) index. E-MaQI is based on the taxonomic determinations of all the macroalgal species present in the sampling sites. In addition, it is difficult to apply because it requires a good taxonomic knowledge. In fact, macroalgae are sorted into three groups (score: 0 =opportunistic taxa, 1=indifferent taxa, 2=sensitive taxa) on the basis of their relationship with the environmental conditions (nutrients, pollutants, and other stressors) related to pressures recorded in many Italian lagoons. Instead, R-MaQI was based on the determination of the main taxa and some environmental parameters. Because the WFD excluded the determination of physico-chemical parameters, successively the two indices were combined in one index, named MaQI, based both on macroalgae and aquatic angiosperms. This new index, contrarily to the indices R/C and E-MaQI, does not require a minimum of 20

macroalgal taxa per each station, which is a rare condition to be found in the soft substrate of the Italian transitional waters (Sfriso et al., 2009), but it can be applied also in the presence of one species. This modified version of MaQI was adopted by Italian Law to be accepted for all EU transitional water bodies. The calculation of the ecological status by this modified version is based on different metrics: number and percentage of sensitive macroalgal taxa; relative abundance (wet weight) of Chlorophyta and Rhodophyta; total percent macroalgal cover and percent cover of aquatic angiosperms. The last revision for MaQI, obtained after the European inter-calibration exercise, was successfully completed by the Mediterranean Geographic Inter-calibration Group (Med-GIG) (EC, 2010; Orfanidis et al., 2011).

Recently, an assessment of the ecological status of the transitional environment in the Mediterranean eco-regions was conducted by Falace et al., (2009). Phytobenthic indices (EEI-Ecological Evaluation Index and MaQI) were used to analyze macrophyte assemblages in Marano-Grado Lagoon in the north Adriatic Sea.

In the coastal areas of the northern Adriatic Sea, other ecological indices were proposed to qualify the ecological status. In fact, the sea conditions are different from that of transitional environments and indices as MaQI are not applicable whereas the R/C ratio or the index EEI can be used. However, the R/C ratios recorded in the marine stations are higher than those recorded in the lagoon and can be only compared with those obtained from other marine areas. Another index used to assess the ecological status of coastal area is CARLIT (Cartography of littoral rocky-shore community index) that can be applied only in rocky coasts (Ballesteros et al., 2007). Therefore, Sfriso and Facca (2011) proposed an

integration of CARLIT to consider also the sandy shores of the north-western Adriatic Sea. The suggested integration focused on three points: (a) the application of the index also to the artificial substrata; (b) its application to a transect (linear surface) of ca. 0.05 km (length of marine breakwaters is ca 50–70 m); (c) the introduction of new categories taking into account the macroalgal assemblages recorded in the north-western Adriatic Sea. The application of the modified CARLIT to the dikes of the lagoon inlets and the breakwaters parallel to the coast allowed assessing the EQR of marine areas of the Venice coastline in the "Moderate" status. The EQR values of that study were similar to the values recorded by Buosi and Sfriso (2017) for the coastal area of Lido Island in the study to assess the ecological status of the coasts of the northern Adriatic Sea. In this study both the modified CARLIT and EEI were applied.

Regarding the Red Sea (mainly GA), little data is available from the literature dealing with the diversity and the ecological aspects of the benthic macrophyte communities. Green and Short (2003) published an atlas of the seagrasses in the world also including the seagrass distribution in the coasts of the Red Sea, instead El Shaffai (2011) prepared a field guide of the seagrasses that colonize the Red Sea Area. The distribution and abundance of seagrass communities were investigated along the Jordanian coast of the GA by Al-Rousan et al., (2011). The seasonal and spatial distribution of some dominant macroalgal species was carried out by Al-Zibdah and Damhoureyeh (2006), whereas Benayahu and Loya (1979) focused their attention on the coral epilithic and endolithic algae. No researches were carried out during the past years using macrophytes in ecological indices to assess the ecological status of the coastal area in the Red Sea. However, some researchers investigated the effects of

anthropogenic stressors on the coral reefs in the area of GA (Egypt side) using Shannon species diversity index (Shokry et al., 2013).

1.4.2 Ecotoxicological approach to assess the ecological status: Biomarkers

Aquatic ecosystems are often impacted from anthropogenic pollutants originating from point and diffuse sources which are toxic for aquatic inhabitants (Backhaus and Faust, 2012). The ecotoxicological approach studies the effects of anthropogenic stresses on ecosystems at different levels of biological organization, from the molecular and cellular level to entire ecosystems (Connon et al., 2012). Consequently, ecotoxicology contributes to understand the mechanisms by which contaminants disorganize the normal biological performance, in order to develop appropriate measures to prevent their adverse outcomes.

The increased level of toxic chemicals that have been introduced into aquatic systems highlight the need for techniques that detect not only the damage to organisms exposed to pollutants, but also the less obvious biochemical and physiological impairment that might ultimately result in ecological damages (Depledge, 2005). Therefore, the combination of ecological relevant indicators of anthropogenic disturbance with ecotoxicological tools (i.e. Biotests) provides us with early-warning signals that help to maintain the ecological health of the ecosystem before the ecological damage occurs (Marin-Guirao et al., 2005). Biotests can perform different levels of biological organization, from the level of molecules and cells to tissues and organs, individuals, populations, and communities, under standardized conditions in laboratories or in the field, by means of *in situ* exposures (**Fig. 3**).

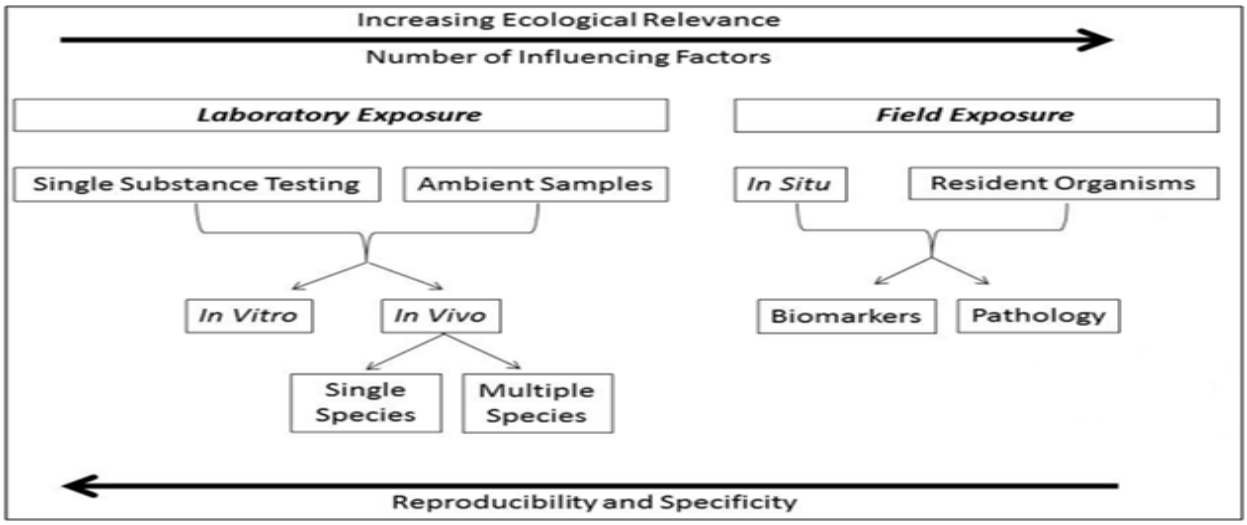

Fig. 3: Biological approaches for measuring toxicity of chemicals and their effects in the aquatic environment (modified after Connon et al., (2012))

Biomarkers are important tools used to assess the effects of anthropogenic toxics in marine ecosystems, directly in cells and tissues of exposed organisms. Within the concept of pollution monitoring, biomarkers can be defined as a quantitative measurements of changes occurring at cellular, biochemical, molecular, or physiological levels that can be measured in cells, body fluids, tissues or organs within an organism as indicative of alteration caused by external stressors (Huggett et al., 1992; Vidal-Li˜nan and Bellas, 2013). Biomarkers are considered as sensitive (early-warning), rapid and cost-effective tools to detect the potential risk of damage to the ecosystem by contaminants present in a given ecosystem. Furthermore, to assess the ecological status of aquatic environments it is of great importance to discover effects related to toxic chemicals before significant effects at the population level can occur (Hook et al., 2014). Oxidative stress biomarkers have recently employed as a reliable tool to study the adverse effects of anthropogenic contaminants in marine ecosystems, providing insight to cause-effect relationship.

1.4.2.1 Oxidative stress biomarkers

Exposure to environmental contaminants can affect the survival of aquatic communities via numerous mechanisms, including direct toxicity (Connon et al., 2012). Heavy metals, polycyclic aromatic hydrocarbons, organochlorine and organophosphate pesticides, polychlorinated biphenyls are among the harmful pollutants that are competent of inducing toxicity in living organisms due to their capability of interacting with the nuclear proteins and nucleic acids causing oxidative deterioration of biomolecules (Leonard et al., 2004). The releasing of these pollutants into the environment stimulate the generation of ROS resulting in oxidative damage to membrane lipids, DNA, and proteins (Wahsha et al., 2012).

Oxidative stress is defined as the potential deterioration of tissues and cellular components caused by increased level of oxygen free radicals and other ROS. Oxidative stress is induced as a result of three factors: (a) an increase in oxidant generation, (b) a decrease in antioxidant protection, and (c) failure to repair oxidative damage (Valavanidis et al., 2006). Wahsha and Al-Jassabi (2009) reported that during times of environmental stress, ROS levels can increase dramatically and this may damage significantly the cell.

The deleterious effect of ROS excess for cell membranes is the oxidative degradation of lipids, especially polyunsaturated fatty acids (PUFA) known as lipid peroxidation, which can directly result in the loss of membrane integrity (Gobert et al., 2010; Timbrell, 2009). Several studies reported that excess of ROS can initiate lipid peroxidation through the action of highly reactive hydroxyl radicals even at very low concentration (Katoch and Begum, 2003; Wahsha et al., 2010). The lipid peroxidation is a chain reaction driven by free radicals that induce the oxidation of

PUFA (Abuja and Albertini, 2001). The steps and end-products of chain reaction are illustrated in **Fig. 4**.

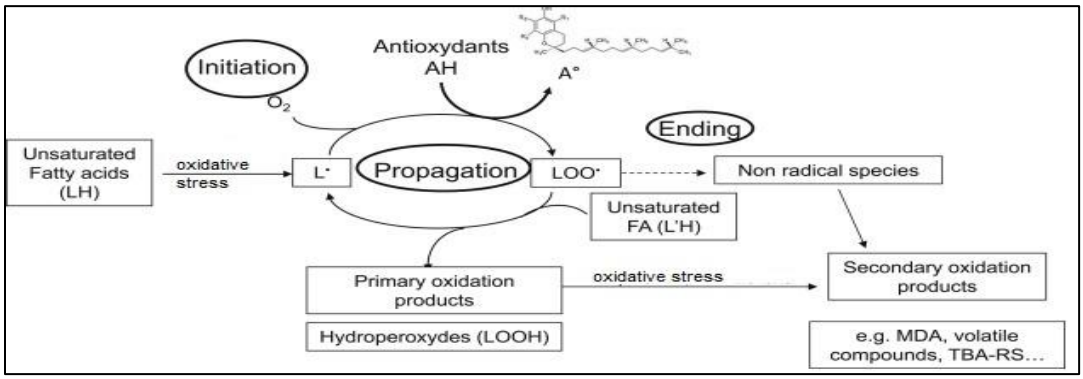

Fig. 4: steps of Lipid Peroxidation (edited after Guyon et al., 2016)

The lipid peroxide Malondialdehyde (MDA) is one of the major secondary oxidation end-product of lipid peroxidation process (Wahsha et al., 2010; Yadav, 2010). In this case, membrane destabilization and fusion are directly correlated with MDA production (Wahsha and Al-Jassabi, 2009; Wahsha et al., 2010).

The determination of MDA content is widely used as a reliable tool to detect the level of oxidative damage by estimating the formation of lipid peroxides (Taulavuori et al., 2001; Wahsha et al., 2017). Furthermore, various studies on different type of aquatic organisms indicated that the formation of ROS and an increased MDA production were observed as a result of exposure to different heavy metals under laboratory condition (Avci et al., 2005; Bocchetti et al., 2004; Company et al., 2004; Geret et al., 2002)

In a related context, it's well known that the determination of the MDA level in biological material is used successfully as a highly predictive biomarker for evaluating the oxidative damage in response to variety of toxic chemicals in marine environments (Valavanidis et al., 2006), especially for fish (Hamoutene et al., 2000), marine invertebrates (Livingstone and Pipe, 1992; Viarengo and Canesi,

1991), mussels and oysters (Belcheva et al., 2015; Cherian and Chan, 1993). Recently, marine macrophytes have been employed in oxidative stress biomarker assays under chronic and acute exposure to anthropogenic stressors (Ferrat et al., 2003; Wahsha et al., 2017) at early stages of stress (Wahsha et al., 2017).

Pereira et al., (2014) used the macroalgae *Ulva* spp. for in field transplantation experiment at short term exposure of 24 hr. in an area stressed by anthropogenic contaminants. They implied that the induction of LPO was recorded in *Ulva* spp. as a response to the higher incorporation of Mn, Fe, and Pb in combination with the lack of dissolved oxygen in the water. Similar findings were proposed by Olivares et al., (2016) that found the concentration of metals in waters and macroalgae (*Scytosiphon lomentaria* (Lyngbye) Link and *Ulva rigida* C. Agardh) were related with oxidative stress biomarkers (MDA) in areas with high mining activities.

Lin et al., (2016) investigated the potential influences of anthropogenic pollutants by evaluating the responses of the intertidal seagrass *Zostera japonica* Ascherson & Graebner to heavy metals: Cu, Pb, and Cd. Using the MDA biomarkers, the results revealed that heavy metal concentration increased in seagrass exposed to high levels of metals. Similarly, Vavilin et al., (1998) showed a significant increase of MDA after exposure to trace metals in marine macrophyte species. MDA increased in the foliar sheaths of seagrass *Posidonia oceanica* (Linnaeus) Delile after 48h exposure to mercury chloride ($HgCl_2$) (Ferrat et al., 2002).

1.4.3 Macrophyte associated microbial community approach

The evaluation of the structure and diversity of macrophyte associated microbial communities in coastal and transitional ecosystems was recently

suggested to be critical to better understand their environmental status, particularly for those that were affected by anthropogenic stressors (Jeffries et al., 2016; Meziti et al., 2016).

Marine microbial communities are one of the most complex and highly diverse ecosystems. The surfaces of macrophytes establish a suitable substratum for the settlement of microorganisms and excrete a variety of organic substances that provide nutrient for the multiplication of the bacteria and formation of bacterial biofilms (Steinberg et al., 2002; Singh and Reddy, 2014). Microbial communities on the surface of the macrophytes are highly complex, dynamic and consists of a consortium of microorganisms including bacteria, fungi, diatoms, protozoa, spores and larvae of marine invertebrates (Goecke et al., 2010; Lachnit et al., 2009, 2011). In particular, the bacterial communities associated with the distinct marine eukaryotes include groups of organisms involved in important metabolic processes such as nitrification, nitrogen fixation (Chisholm et al., 1996), sulphate reduction (Crump and Koch, 2008), photosynthesis (Barott et al., 2011), plant growth enhancement (Orole and Adejumo, 2011), morphogenesis induction (Nakanishi and Nishijima, 1996), or chemical defence (Burke et al., 2011; Lee et al., 2009; Wahl et al., 2012). Correspondingly, macroalgae associated to microbial communities contribute in the production of plant-growth promoting substances, quorum sensing signalling molecules (Joint et al., 2002), bioactive and other effective molecules that are responsible for the morphology, development, and growth of macroalgae (Singh and Reddy, 2014).

1.4.3.1 Key functions of macroalgae-associated bacterial communities

The symbiotic interaction of macroalgae and the associated bacterial communities have been reported as essential for normal morphological

development and growth of the macroalgal host (**Fig. 5**). Some nitrogen fixing associated bacterial strains can significantly influence the growth of green and red macroalgae (Chisholm et al., 1996; Singh et al., 2011). Additionally, associated bacterial communities are also known to induce settlement of zoospores of *Ulva* species on appropriate surfaces (Joint et al., 2007; Weinberger et al., 2007). Bacteria present within the macroalgae biofilm express a unique set of genes, including those involved in adhesion, auto-aggregation and anoxic growth (Schembri et al., 2003). The sensing signalling molecules produced by the established association is important for maintaining the attachment of bacteria to surfaces and the biofilm mode of growth (Joint et al., 2007). Moreover, the macroalgal biofilms can alter the host's interaction with various physico-chemical conditions and may control further fouler, consumers or pathogens (Wahl et al., 2012; Singh and Reddy, 2014).

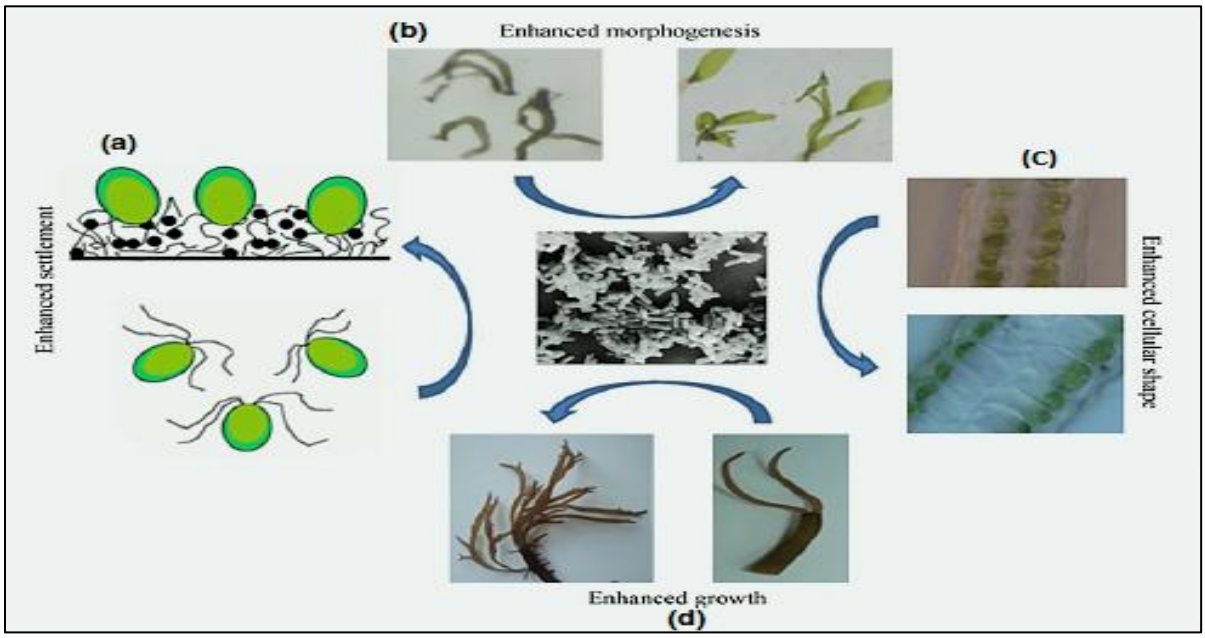

Fig. 5: bacterial role in macroalgae development: (a) promoting *Ulva* zoospores settlement on bacterial polysaccharide; (b) reverting normal morphogenesis in axenic culture of *Ulva* upon putative morphology that induce bacterial strains; (c) reverting wild type cell structure of *Ulva* in the presence of appropriate bacteria; and (d) regeneration of new buds and growth from individual fronds of *Gracilaria dura* with plant hormone producing and nitrogen-fixing bacterial strains (Source: Singh and Reddy, 2014).

1.4.3.2 Factors influencing the assembly and maintenance of bacterial communities on macroalgal hosts

The qualitative and quantitative structure of the microbial community associated to macroalgae and its metabolic activity are influenced by a wide range of biological, physical, and chemical properties on the macroalgal surface (Egan et al., 2013). The parameters that affect the macroalgal surface environment include algal metabolites, the existing resident microbial community with its pool of microbial derived secondary metabolites, and physico-chemical conditions on the thallus surface such as oxygen, carbon dioxide, temperature, oxidative stress and osmotic stress (**Fig. 6**). Many of these parameters are subject to daily (Spilling et al., 2010) or seasonal modulations (Hellio et al., 2004). Bacteria entering into a stable association with a macroalgal host exhibit adaptive traits that reflect these niche conditions.

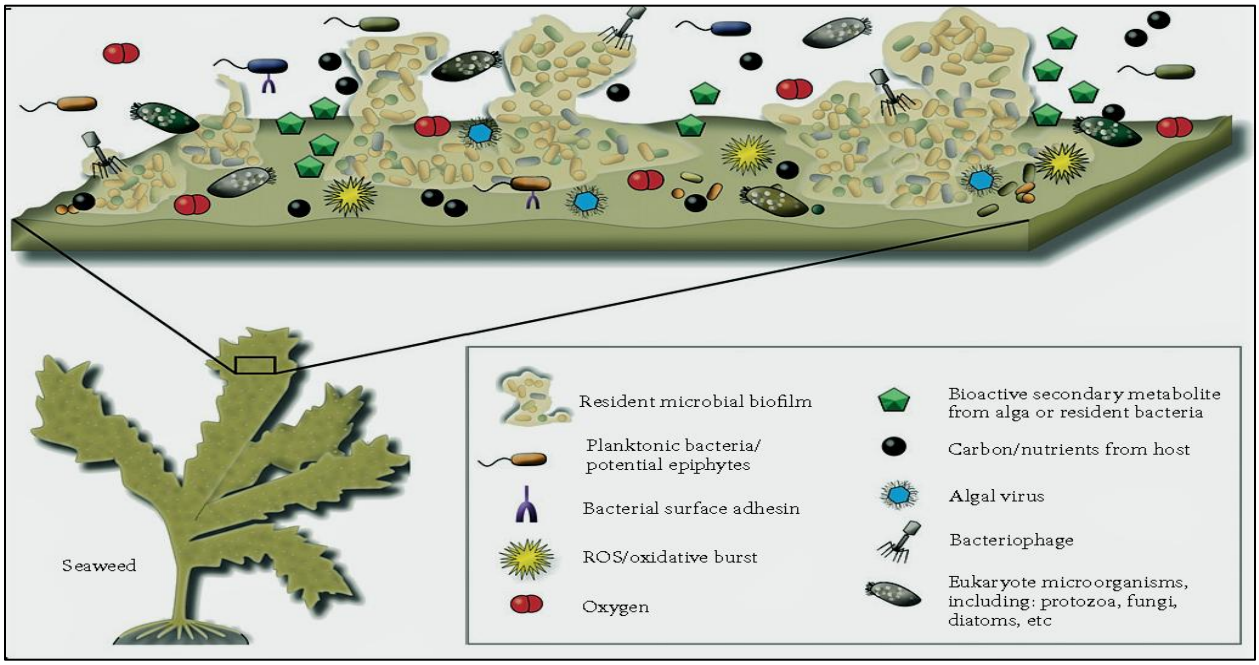

Fig. 6: parameters affecting the macrophyte-bacterial communities association (Modified after Egan et al., (2013)).

1.4.3.3 Microbial community shift and anthropogenic pressures

Various categories of potentially toxic compounds originating from industrial and agricultural activities are ultimately settled in the water bodies (Schlesinger et al., 2011). These compounds comprise the toxic chemicals released from the input of organic and inorganic nutrients from sewage effluents (Beck and Birch, 2012) and agricultural run-off, loads of inorganic nutrients from fertilizers or toxins from pesticides (Vieira et al., 2008), industrial waste enriched in heavy metals and other toxins (McCready et al., 2006) or thermal pollution associated with the cooling waters from power plants and other large industry (Shiah et al., 2006).

Microbial communities associated to macrophyte are extremely sensitive to rapid changes in the environment and can be used as indicators of stress (Hollants et al., 2012; Sun et al., 2012) as changes in the relative abundance of specific taxa or functional genes are related to shifts in the physicochemical dynamics within transitional and coastal ecosystems (Fortunato et al., 2012; Smith et al., 2010). Similarly, bacterial response to stress, mainly related to DNA damage, have general stress response like expression of many stress response genes that enhance bacteria sporulation, long-term survival especially in the stationary phase (Marles-Wright and Lewis, 2007). Bacterial responses to these stresses can lead to altered and often unbalanced nutrient cycles, anoxic conditions, blooms of harmful algae (Anderson et al., 2002; Paerl, 2006) and increase in enteric and endemic pathogen concentrations (Hsieh et al., 2007). Conversely, bacterial populations may also remediating marine water conditions by rapidly degrading contaminants, including hydrocarbons and fertilizers (Head et al., 2006).

Despite the potentially fundamental role played by marine macrophyte associated microorganisms on the lives of their hosts, there is little or no

information (particularly for key marine habitat-forming macrophyte hosts) on how these communities vary at large spatial scales, and what are the main drivers of this variation (Egan et al., 2008; Goecke et al., 2013; Wahl et al., 2012;). The characterization of the macrophyte-associated microbial communities through new innovative next-generation sequencing (NGS) provides an assessment of bacterial community structure and diversity and has recently emerged as a powerful tool to examine bacterial communities in marine eukaryotes.

NGS techniques have greatly increased sequencing by implementing massively parallel sequencing (Sogin et al., 2006). The amplification of small but highly variable regions of the 16S rRNA gene (e.g. the V3, V5 or V6 hypervariable regions) has resulted in extremely deep sequencing of bacterial communities. This facilitated the identification of rare populations in bacterial communities that may account for functional diversity and ecosystem stability (Kysela et al., 2005; Sogin et al., 2006). These NSG techniques have recently been utilized to characterize bacterial communities in marine waters (Brown et al., 2012) and other marine eukaryotes (Egan et al., 2008; Goecke et al., 2013; Morrow et al., 2012; Sunagawa et al., 2015), in order to assess the impacts of anthropogenic activities on the structure and biodiversity of microbial communities in the marine ecosystems (Edlund and Jansson, 2006; Meziti et al., 2016). For instance, Illumina HiSeq® sequencing is one of the recently used platforms within NGS in marine host-associated microorganisms. Previous studies of environmental monitoring demonstrated that this platform can provide new insights to study the diversity of microbial communities in the environment (Yang et al., 2016; Zhang et al., 2015).

NGS techniques during the last decade have evolved in the characterization of microbial communities associated with marine eukaryotes, including

macrophytes, to address the effects of anthropogenic stressors. Among these studies an assessment was conducted by Michelou et al., (2013) in Marine Station at Monterey, USA, to study the diversity of bacteria communities associated with a kelp forest of *Macrocystis pyrifera* (Linnaeus) C. Agardh which is characterized by high biodiversity and productivity. The results obtained via the 16S RNA gene pyrosequencing technique highlight the temporal variation of the kelp associated microbial communities. They indicated that the distribution of some phyla of bacteria differed between the surface of the kelp and the surrounding water, and bacterial community structure varied over time.

Staley et al., (2013) performed a study that was among the first to characterize a large river (Mississippi river) bacterial community using the Illumina-based sequencing approach to evaluate shifts in the community potentially resulting from upstream inputs and land use changes. From the bacteria phyla collected in the sites of the study area, they concluded that shifts in community structure was related to changes in the relative abundance of bacterial phyla present throughout the Upper Mississippi River, which was influenced by potential anthropogenic impacts.

Using 16S-rRNA gene sequencing, Marzinelli et al., (2015) conducted a research study to characterize the bacterial and archaeal communities associated to the kelp *Ecklonia radiata* (C. Agardh) J. Agardh across three geographical areas along the Australian continent. These phylogenetically and taxonomically diverse communities were more strongly and consistently associated to host conditions than geographical location or environmental variables. In addition they found that the ‘core’ microbial community characteristic of healthy kelps appeared to be lost when hosts become stressed. They also found that microbial communities on stressed individuals were more similar to each other among locations than those on

healthy hosts, proving that the variation of bacterial communities associated to macrophyte host is a function of host conditions (including environmental stresses) rather than geography.

Habitats change, due to global and local pressures, population resilience and adaptive processes, depend not only on the habitats gene pools but also on their associated bacteria communities. For this purpose Aires et al., (2016) focused on the associated bacteria of the two most invasive macroalgae in southwest Iberia (coastal mainland) and nearby offshore Atlantic islands, *Asparagopsis taxiformis* (Delile) Trevisan and *Asparagopsis armata* Harvey. Bacterial communities were characterized using 16S rRNA barcoding through 454 next generation sequencing and shotgun metagenomics. The bacterial community composition was clearly different between the species *A. taxiformis* and *A. armata* and between continental and island habitats. The metabolic assignment for the higher abundance bacterial orders (*Acidimicrobiales*, *Sphingomonadales*, *Xanthomonadales*, *Myxococcales*, and *Alteromonadales*) contained a high number of reads with functions related to oxidative stress and resistance to toxic compounds, more precisely heavy metals. They also found that *A. taxiformis* from islands contained more bacteria related to oligotrophic environments which might putatively play a role in mineralization of dissolved organic matter related to the nutrient limitation. These results support the hypotheses that the bacterial communities associated to macrophytes have host-specificity and are modulated by environmental conditions.

The small tropical seagrass *Halophila stipulacea* is native from the Red Sea; this species has invaded the southern Mediterranean Sea and recently also has colonized the Caribbean Sea. Due to its invasive nature, there is growing interest to understand this species' capacity to adapt to new conditions, which might be attributed to its ability to thrive in a broad range of ecological niches. The study of

Rotini et al., (2017) used a multidisciplinary approach to depict variations in epiphytic bacterial communities along a depth gradient in the GA. *H. stipulacea* displayed a well conserved core bacteriome, as assessed by 454-pyrosequencing of 16S rRNA gene reads amplified from metagenomic DNA. The dominant bacterial classes were belonging to *Alphaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* across all depths. The richness of associated communities was showed higher within depth variability. The overall results demonstrated the pivotal role of epiphytic bacterial communities in helping plants to cope with environmental and ecological variations. The plant/holobiont capability to persist and adapt to environmental changes probably has an important role in its ecological resilience and invasiveness.

In marine systems, coastal urbanization via the addition of artificial structures is a major source of stress to habitat formers that can negatively affect the host performance and survival. To elucidate this effect, Marzinelli et al., (2018) performed a study to characterize the kelp-associated microbial communities in two of the most common and abundant artificial structures in Sydney Harbour - pier-pilings and seawalls - and neighbouring natural rocky reefs. The kelp *Ecklonia radiata* is the dominant habitat-forming species along 8000 km of the temperate Australian coast. Kelp-associated microbial communities on pilings differed significantly from those on seawalls and natural rocky reefs, possibly due to differences in abiotic (e.g., shade) and biotic (e.g., grazing) factors between habitats. Surprisingly, they found many bacteria that were more abundant on kelp on pilings belonged to taxa often associated with macroalgal diseases, including tissue bleaching in *Ecklonia*. The study demonstrated that urbanization can alter the microbiota of key habitat-forming species with potential ecological consequences.

2. MATERIALS and METHODS

2.1 Description of study area

2.1.1 Lagoon of Venice (LV)

The LV is a complex, heterogeneous, and continuously evolving dynamic system, sensitive to an array of external drivers and pressures. Both natural and anthropogenic stressors significantly affect the lagoon ecosystems (Solidoro et al., 2010). The lagoon is characterized by high number of sub-basins and habitats, with diverse biological communities, nutrient loadings, trophic status, salinity and hydro-morphological conditions (Tagliapietra and Volpi Ghirardini, 2006) According to literature (Acri et al., 2004; Facca et al., 2002; Facca and Sfriso, 2009; Sfriso et al., 2009), three sampling sites were selected, with particular reference to the central basin (**Fig. 7**). Sampling sites were chosen according to the variation in the source of anthropogenic pressures that affects the macrophyte communities (**Fig. 8**).

- **Santa Maria del Mare (SMM)** (45° 19.30.081 N; 12° 18.30.65 E)

SMM is located close to the Malamocco port-entrance which connects the lagoon with the northern Adriatic Sea and, therefore, experiences very effective tidal water exchanges. It is characterized by a high ecological status (high hydrodynamics, low nutrient concentrations, and the absence of algal blooms). This area is poorly impacted by pollutants indicating anthropogenic pressures. SMM features a high dense seagrass beds and a variety of macroalgal taxa, especially Rhodophyceae with high ecological values (Sfriso et al., 2013). Water are clear (>3 m) for most of the year. Environmental parameters, such as oxygen

and salinity, showed low seasonal changes. Sediments are mostly coarse or sandy and well oxidized (Sfriso et al., 1992).

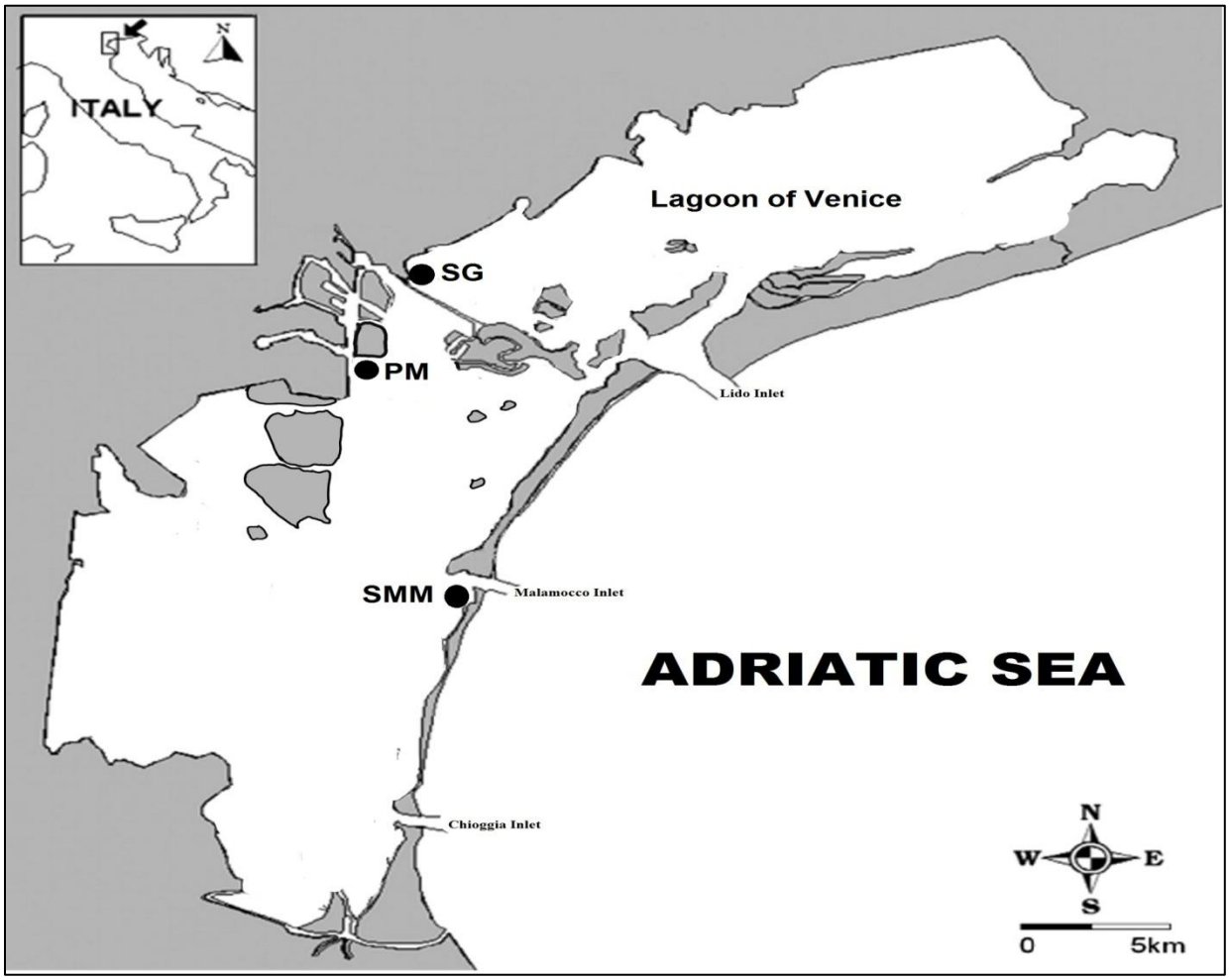

Fig. 7: Map of Lagoon of Venice (LV) sampling sites (SMM, PM & SG).

- **Porto Marghera (PM)** (45° 25.55.29 N; 12° 15.39.16 E)

PM is located close to the inner border of the lagoon, at the center of the SIN area (National Interest Site) which is identified as an area of high environmental risk. PM is influenced by the pollutants released from the industrial activities of the petrochemical industrial pole and by urban and agricultural water inputs that

discharges pollutants into the lagoon (Masiol et al., 2014; Raccanelli et al., 2008). Several previous studies reported that the sediment in area of PM was heavily polluted by heavy metals and organic micro-pollutants (Masiol et al., 2014; Zonta et al., 2007). A marked gradient of sediment contamination from the industrial area to the sea was evidenced by a chemical monitoring programme (MAV-CNV, 1999). Macroalgae were quite absent and phytoplankton blooms occurred frequently. The water is turbid, seasonally changeable. Oxygen saturation seasonally reaches 200–300%, followed by anoxic events.

- **San Giuliano (SG)** (45° 28.15.34 N; 12° 17.57.94 E)

SG is close to the inner border of the lagoon and, therefore, it has very poor water exchange and is characterized by hypertrophic conditions and marked industrial and urban contamination (Guerzoni and Raccanelli, 2003; Sfriso et al., 2005). SG is influenced by the freshwater inputs of the Osellino River which conveys in the lagoon nutrients and pollutants (Sfriso et al., 2008). Macrophytes are abundant and mainly represented by Gracilariaceae, Solieriaceae and Ulvaceae. Waters are very turbid and seasonally changeable, however, on average, Secchi disk is <0.5–0.8 m, due both to phytoplankton blooms and sediment re-suspension phenomena. It is also characterized by the presence of anoxic sediments and persistent water anoxia in spring-summer, and high variability of environmental parameters such as water transparency and salinity.

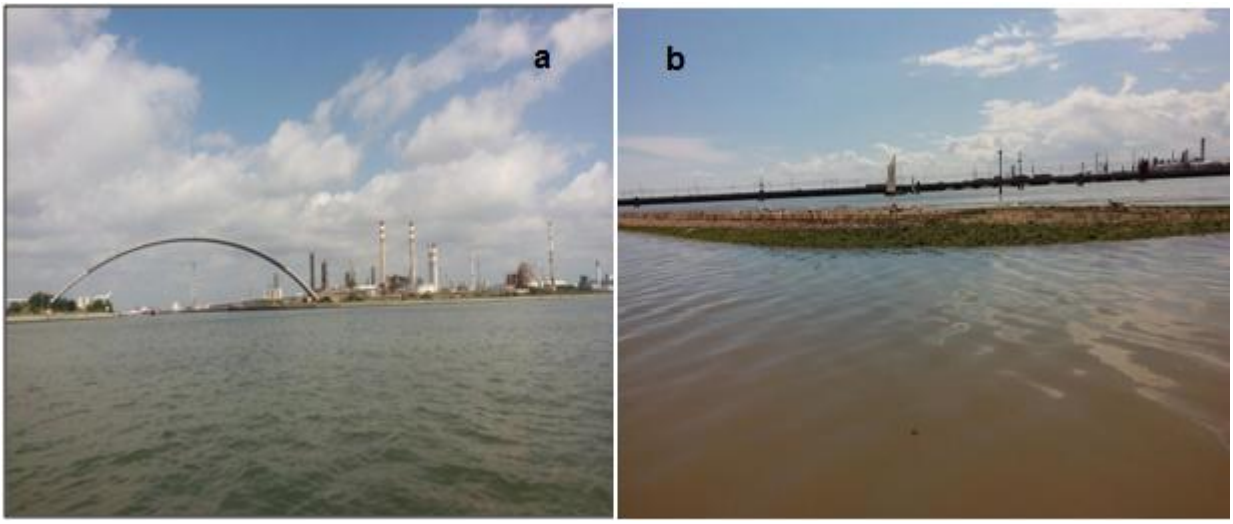

Fig. 8: Examples of sources of anthropogenic pressures at LV. (**a**: chemical manufacturing; **b**: touristic activities)

2.1.2 Gulf of Aqaba (GA)

The Jordanian coastline of the GA is the only marine access of Jordan. Several features make it unique compared with any other ecosystems. GA consists of a series of embayments, each one including similar communities typical of rocky shore, flat reef, face reef, fore reef, sandy shore, and sandy bottom and seagrass ecosystems (**Fig. 9**). GA is biologically extremely diverse and contains rare and endemic species of algae. The coral reefs support a higher marine biodiversity than that found at similar latitudes elsewhere, (Hulings, 1989). Several activities developed on the coastal area include tourism, industrial constructions of new ports and resorts accompanied by intensive shipping and terrestrial transportation, which increased the pressures on the marine (Al-Rousan et al., 2005). Anthropogenic stressors, including fishing activities, shipping processes and industrial activities (**Fig. 10**), have been reported to affect the ecosystem health in the coastal area (Wahsha et al., 2017). Based on the variation of the anthropogenic

stressors, three sites were selected along the coast of GA representing the major anthropogenic stressors threaten the area.

- **Marine Science Station (MSS)** (29º 27.513 N; 34º 58.561 E)

The MSS is a well-developed coral reef site declared since the mid-1970s a protected Marine Reserve area. It was selected as an uncontaminated site with low levels of pollutants (Al-Rousan et al., 2016; Wahsha et al., 2017). The flat reef is quite wide with a definite reef front and reef slope. In this area, the sediment is mainly carbonatic, composed of coral reef fragments, remains of foraminifera, calcareous red algae and sea urchins (Rasheed et al., 2005). Water is clear with low turbidity and low nutrients content during summer season (Manasrah et al., 2004).

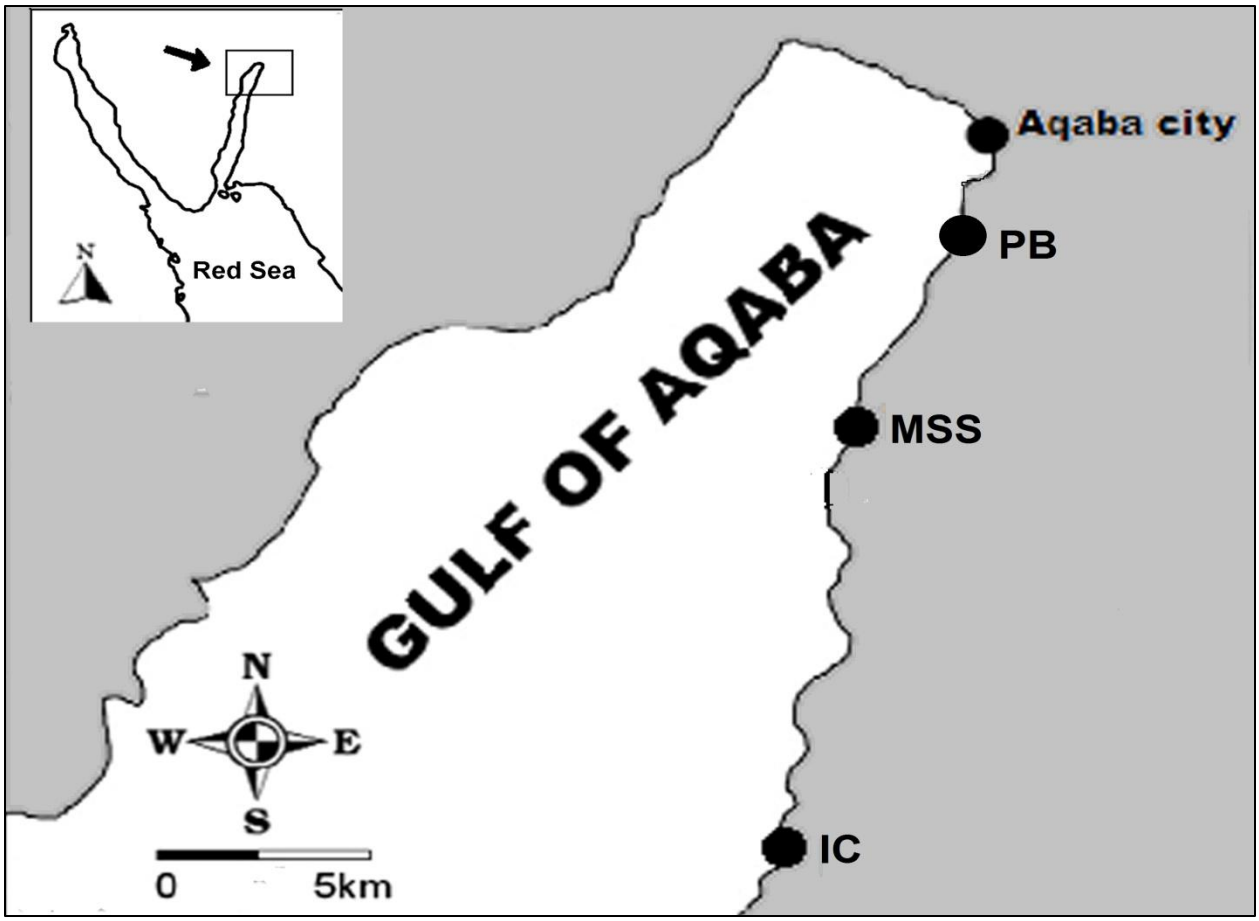

Fig. 9: Map of Gulf of Aqaba (GA) sampling sites (MSS, PB & IC).

- **Public Beach (PB)** (29° 52.611 N; 35° 00.080 E)

The PB is located in the northern tip of the GA coast. This site is mainly characterized by sandy bottoms and scattered seagrass beds. The coastal water of this site is heavily utilized by visitors for sea sports and boat navigation. This area is influenced by solid waste discharge and engines exhaust gas emissions from boat activities (Al-Absi et al., 2016). Water is relatively turbid and high organic matter content released from touristic activities (Al-Zibdah and Damhoureyeh, 2006). High diversity of macroalgal species characterizes this area.

- **Industrial Complex (IC)** (29° 45.845 N; 34° 97.631 E)

The IC is located in the southern part of the Jordanian coast dominated by coral reef. The IC area hosts a Timber Plant, a Thermal Power Station, a Phosphate Fertilizer Complex, a Potash Export Terminal and a Mixed Fertilizers plant. It also comprises the main platform in which a mega investment project of moving the ports from the northern part of the coast was being implemented. These industrial activities pose a potential threat to the adjacent coastal ecosystem (Al-Zibdah and Damhoureyeh, 2006). Water is less turbid with increased level of nutrients released from industrial activities. The macroalgal coverage is characterized by the dominance of tolerant species (Al-Zibdah and Damhoureyeh, 2006).

Fig. 10: Examples of sources of anthropogenic pressures at GA. (**a**: oil transportation; **b**: industrial effluents)

2.2 Sample collection and preparation

2.2.1 Water sampling and monitoring of environmental parameters

The monitoring of environmental parameters in water and sediment was performed in both study areas. In LV the samples were collected in the four seasons (winter 2016, spring, summer and autumn 2017), whereas for GA the samples were collected in summer 2016 and winter 2017, due to the slight seasonal changes in this areas as stated earlier (Manasrah et al., 2004).

Sampling and field measurement of water physico-chemical parameters were performed within the sampling area of the macrophyte sampling (ca. 100 linear meters) at a depth of 50 cm. Temperature, pH (accuracy ±0.015 units) and redox potential (Eh) (accuracy ±0.15%) were measured with a portable pH-meter (PH25 + CRISON). The concentration of dissolved oxygen (DO) was obtained with a portable oximeter (OXI45 + CRISON). It depends on physico-chemical factors (temperature and salinity) that determine its solubility and biological activities (photosynthesis and respiration). The percentage of DO saturation was calculated by the formula of Weiss (1970).

Furthermore, water samples were collected at a depth of 50 cm by means of 3-liter polyethylene bottles, subsequently sorted into different aliquots for bio-chemical analyzes. An aliquot of 50 ml was stored for the determination of salinity, while 1-liter aliquot was filtered with Whatman GF/F glass fiber filters (porosity: 0.7 μm) for the determination of nutrients (reactive phosphorus, ammonium, nitrates, nitrites and silicates). Filters were stored at -18 °C for chlorophyll-*a* (Chl-*a*) concentrations. In addition, two 1-liter water aliquots (two replicas) were filtered on cellulose acetate filters (previously dried in oven and weighed) to determine the amount of suspended particulate in the water column (Strickland and

Parsons, 1972). The amount of suspended particulate expressed in mg/L allows information on the turbidity of water from the various stations.

2.2.2 Sediment sampling and environmental parameter monitoring

Samples of the 5 cm surface top layer were collected through Plexiglas corer (i.d. 10 cm). Three "pools" from three different points in the same area were carefully homogenized for the determination of the grain-size distribution, sediment moisture and the concentration of carbon, phosphorus, nitrogen and total metals. Sediment pH (accuracy ±0.015 units) and redox potential (Eh) (accuracy ±0.15%) were measured on site with a portable pH-meter (pH meter PH25 + CRISON). The samples obtained were placed in 200 ml PVC (polyvinyl chloride) containers and frozen at -20 ° C, to avoid bio-chemical alterations. In LV, due to the high sedimentation rates that reduce water transparency affecting macrophyte communities, sedimentation traps to collect the Settled Particulate Matter (SPM) were also used. Each SPM sample was sorted after homogenisation in two subsamples: one for the determination of grain-size distribution and the other for the analysis of total metals and other for carbon, phosphorus, nitrogen determination. Sampled were stored frozen until laboratory analyses.

2.2.3. Macroalgae sampling

The macroalgae of the genus *Ulva* have been proposed as good pollution biosentinels (e.g. Pereira et al., 2009; Villares et al., 2002). These macroalgae are widely distributed in coastal and transitional environments, are sedentary, widely studied, easy to sample and able to survive under laboratory conditions. In addition, they are highly sensitive to environmental changes (Rainbow and Phillips, 1993). Their high surface area/volume ratio (provided by a laminar

structure) and a structurally uniform and physiologically active thallus (Villares et al., 2001) are other favourable characteristics. *Ulva leatevirens* Areschoug was selected for biomarker assays, metals determination and the analysis of the associated microbial community.

2.2.3.1 Ulva leatevirens Areschoug

U. leatevirens is widely distributed in temperate warm areas. These algae have a limited development during winter whereas it grows a lot in late spring and summer. The greater presence of nutrients in sea water, also deriving from mild eutrophic phenomena, favours the increase of these algae which tend to consume the excess of nutrients. *Ulva* is common in the most populated areas, especially in the area affected by tides and in tide pools, while it meet less frequently in the most pristine areas (**Fig. 11**).

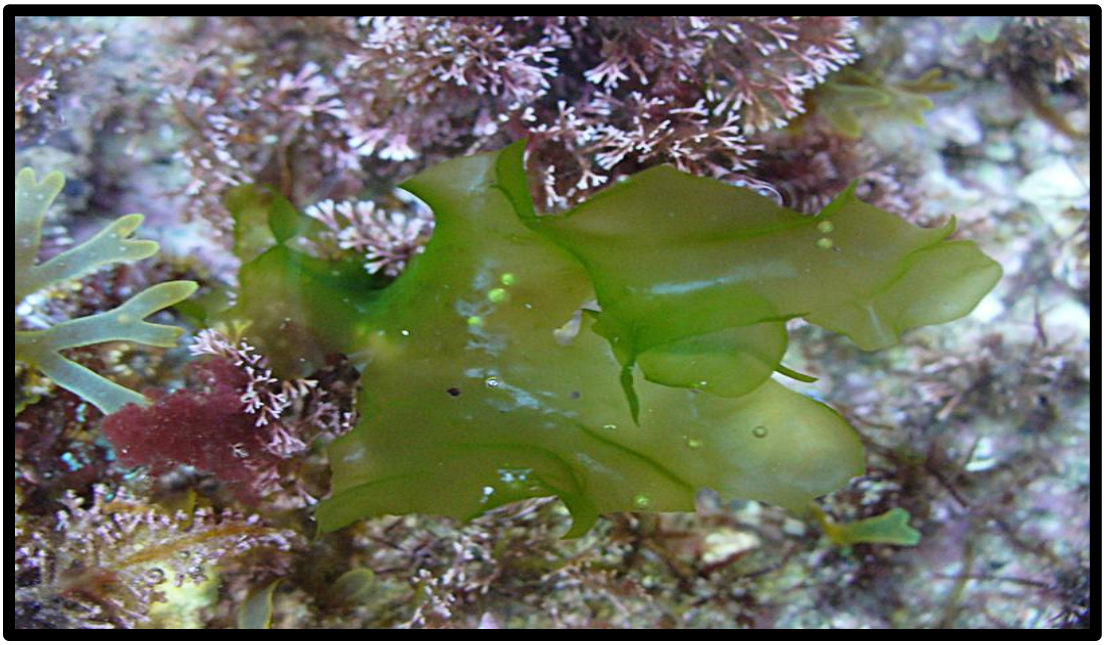

Fig. 11: The macroalgae *U. leatevirens (*source*:* www.algaebase.org)

2.3 Laboratory analyses

2.3.1 Water parameters

Dissolved nutrients

The concentration of nutrients in the water was determined following the filtration of water by Whatman GF/F glass fiber filters (porosity: 0.7 μm) according to the spectrophotometric analysis procedures reported in Strickland and Parsons (1972). The quantification at different wavelengths of phosphates, silicates and inorganic nitrogen compounds occurs by comparison with calibration curves made with sea water. All colorimetric analyses were performed in triplicate.

Reactive Phosphorus

Sea orthophosphates may be present in water with different fractions of ions PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$. The reactive phosphorus (RP) was determined using the spectrophotometric method of Murphy and Riley (1962) based on the reaction of the orthophosphate ion (PO_4^{3-}) with ammonium heptamolibdate ($(NH_4)\ 6Mo_7O_{24}$). The reaction, catalyzed by the antimonyl ion (SbO +) in acidic environment with sulphuric acid leads to the formation of a yellow phosphomolybdic compound reduced from ascorbic acid to a blue coloured compound with maximum absorbance at 885 nm.

Ammonium

The determination of ammonium was performed by the spectrophotometric method of the phenol-hypochlorite (Riley, 1953) modified by Solarzano, (1969) by the formation in alkaline environment of indophenol blue by reaction between ammonia, hypochlorite and phenol catalyzed by sodium nitroprusside. The addition of trisodium citrate prevents the interference due to the precipitation of magnesium hydroxide, leading to the development of the blue color with the

maximum absorbance at 640 nm. The colorimetric reaction has a duration that depends on temperature and concentration. Therefore, the spectrophotometric reading is made after at least 3 hours of reaction to avoid photochemical interferences (Gravitz and Gleye, 1975) where the sample is kept in the dark.

Determination of Nitrites and Nitrates

The simultaneous measurement of nitrites and nitrite is determined by the formation of the diazonium salt for the reaction between nitrite and sulfonyl-amide (SULF) in an acid medium with HCl. The formed salt reacts with N- (1-Naphthyl) ethylenediamine (NED) forming a nitrogen-compound with the intense pink color (Benschneider and Robinson, 1952). The reaction takes place in 10 minutes and the absorbance reading is performed at 543 nm. The photometric analysis of nitrites occurs 10 minutes after the addition of the reagents to the water samples. Nitrates are determined by the passage of water sample in a copper-plated cadmium column where they are transformed in nitrites. Therefore, the filtered sample contains both the original nitrites and the nitrites resulting from nitrate reduction. Nitrate concentration is obtained multiplying the factor of the column obtained before each set of analyses by the absorbance of the filtered sample after subtracting the absorbance of the nitrites.

Determination of Silicates

The reactive silicate was quantified using the spectrophotometric method according to Mullin and Riley (1965) modified by Strickland and Parsons (1972). The silicate reacts with the ammonium heptamolibdate, with the methyl-sulphite and the oxalic acid, in acidic environment of sulphuric acid, leading to the formation of a blue colored compound with maximum absorbance at 830 nm.

Determination of Chl-*a*

Chlorophyll-*a* is a photosynthetic pigment present in plant cell chloroplasts that is generally used for a gross estimation of the phytoplankton concentration in marine-coastal environments. Chl-*a* was quantified by spectrophotometric analysis using the method proposed by Lorenzen (1967). After the filtration of water by Whatman GF/F glass fiber filters (porosity: 0.7 μm) the filters are immersed in 90% acetone solution (Lorenzen, 1967). Chl-*a* is extracted by breaking the cell walls in an ultrasonic bath for about 30 min, followed by overnight complete extraction of the pigment. The pigment concentration is quantified by a spectrophotometer at 665 nm. A further measurement of absorbance at 750 nm is a corrective value for turbidity. Absorbance values, filtered volumes of water (ca. 500ml) (Brito et al., 2009) and the volume of solvent for extraction (in ml) are integrated into the equations of Lorenzen (1967) obtaining values in μg/L. Extractions and analyses were performed in duplicate.

Determination of Salinity

Salinity is a measure of the content of salts dissolved in sea water. The relative composition of the salts (Na^+, K^+, Cl^-...) is constant without inference of freshwater discharged. Salinity is obtained from the chlorinity (Cl^-) (Oxner, 1962), using the formula: Salinity = Cl^-*1.805 + 0.03. A known volume of sea water is titrated with a known solution of silver nitrate, using K_2CrO_4 as indicator, monitoring the reaction term with the change from yellow to red. A correction factor is obtained by titration of the Copenhagen water by a known title. The analysis was made in duplicate.

Determination of filtered Suspended Particulate Matter

The suspended particulate, consisting of resuspended sediments, organic matter, bacteria and phytoplankton, indicates the turbidity of water. The growth of primary producers directly influences the amount of light that receives the water column or the bottom.

The suspended particulate was obtained by filtration with cellulose acetate filters (porosity: 0.45 μm). The filters were dried in an oven at 50-60 °C and weighed to determine the particulate matter as a difference in weight, expressed in mg/L (Strickland and Parsons, 1972). The analysis was carried out in duplicate.

2.3.2 Analyses of sediment parameters

Grain-size analysis and density

The grain-size is perhaps the most representative parameter of a sediment matrix. The sediment texture influences the physico-chemical properties, the oxidation state, and often is linked to the degree of contamination (Zonta et al., 1994). As well, grain-size influences the quantity of nutrients and pollutants trapped in the sediments and the benthic communities that inhabit the sediment. Density, on the other hand, is a parameter that indicates the sediment content per unit volume, thus describing its degree of dilution, which accompanies any parameter linked to the sediment.

The grain-size was determined by wet separation method using a 63 μm sieve (Sfriso et al., 2005) for the subdivision of the silt and sandy fraction. The quantification is expressed as dry weight value on total dry weight. The sediment density was determined by weight difference of the sediment on crucibles of known weight and volume after drying in oven at 110 °C, thus quantifying by the dry weight per unit of volume (Sfriso et al., 2005).

Determination of Total Organic Matter (TOM)

Organic matter in sediment consists of compound containing carbon, nitrogen and phosphorus. Organic matter influence the partitioning of contaminates in sediments. To determinate the total organic matter (%TOM) in sediment or settled particulate matter (SPM), the sediment was distributed on tree crucibles and dried in an oven lab at 60ºC for 48h to determinate the dry weight. Then, the samples were burned at 450 °C for 3h in a muffle furnace. This last process allows obtaining the ash weight as described by Byers et al., (1978). The TOM was determined with the following equation:

%TOM = (dry weight – ash weight) / dry weight) x 100

Total and inorganic phosphorus in sediment and Settled Particulate Matter (SPM)

The sediment is a reservoir for the phosphorus that can be released to the water column both when the redox conditions change or as a result of anthropogenic pressures as seabed scraping (Sfriso et al., 2005). The particulate matter usually presents a nutrient concentration 2-3 times higher than surface sediments. The presence of phosphorus in the water is closely linked to the availability of phosphorus in the sediment, especially where the phosphorus inputs are strictly limited and regulated, becoming a parameter of significant importance for the understanding of the trophic state of the study areas, especially in LV.

For the analysis of the concentration of organic and inorganic phosphorus in the sediment and/or SPM the procedure proposed by Aspila et al., (1976) was applied. It allows the phosphorus to be extracted from the solid component of the sediment in an aqueous matrix. The frozen samples first are lyophilized and then pulverized using pestle and mortar. Subsequently, 0.3 - 0.4 g of sediment are

weighed using an analytical balance. For the analysis of inorganic phosphorus, the weighed sample is placed in polyethylene bottles; 50 ml of 1N hydrochloric acid are added and, to obtain extraction, the sediment is sonicated for 30 minutes. To allow the solid part to settle on the bottom and separate it from the limpid aqueous part, the sample is left to rest for at least an hour. Then 0.5 ml are taken from the supernatant with an analytical syringe and diluted with milli-Q to 10 ml before the spectrophotometric determination according to Strickland and Parsons, (1972). Finally, the concentration was calculated taking into account the quantity of weighted sediment. The concentration of organic phosphorus was obtained by difference between the extraction with and without combustion at 550 °C for 2 hrs. The analyses were done in duplicate and repeated on different days, retaining the values only when their difference was less than 5%, otherwise they were repeated until the desired precision was obtained.

Determination of carbon and nitrogen in sediment, SPM and macroalgae

The analysis of the concentrations of organic and inorganic carbon (C) and nitrogen (N) in sediments and SPM is fundamental to understand the relationships between the substrate and the water column in the biogeochemical cycles of marine-coastal environments. Nitrogen and phosphorus are important nutrients for the primary producers as macrophytes while the carbon, both into inorganic and organic forms, mainly allow obtaining information on the concentration of carbonates and on the degradation of organic matter in the marine environment.

The frozen samples are lyophilized to eliminate the water present and subsequently are milled using pestle and mortar. The concentration of carbon and nitrogen was obtained by an elementary analyzer (Vario Micro Cube, CHNS, Fisher Elementar). The instrument, which uses a continuous flow of helium and an

instantaneous flow of oxygen for the combustion reaction, allows to obtain the area of the peak concentration of C and N by passing the sample through a combustion column at 1200 °C (combustion reaction) and a reduction column at 800 °C (reduction reaction).In the final phase there is a column (trap) that divides the gases obtained from the previous reactions and an analyzer that detects the area of the peaks of the various elemental components. About 0.5 mg of sediments or SPM was injected in the instrument. Sulphanilamide (N: 16.25%, C: 41.81%, S: 18.62%, H: 4.65%) was used as standard in relation to the substrate background. The per cent concentration of C and N was calculated after the creation of a calibration curve. Analyses were performed in triplicate.

2.3.3 **Metal analyses**

In order to verify the environmental contamination and bioaccumulation and the potential source of anthropogenic stress for *U. leatevirens*, the quantification of total metals and of arsenic (metalloid) was carried out in surface sediment and macroalgal tissues. The metals analysed in the surface sediments and *U. leatevirens* tissues were: As, Hg, Cd, Fe, Pb, Cu, Cr, Mn, Ni, V and Zn.

Matrix digestions, dilutions and solutions were prepared with analytical grade reagents and Milli-Q water (resistivity 18.2Ω), while the containers and the glassware were treated with 25% nitric acid bath (HNO_3 69%) for 48 hours and rinsed with Milli-Q water to remove all traces of residual metals that could interfere with the results.

Macroalgal collection and preparation

Macroalgal samples were collected at low tide from the intertidal zone. Approximately 15 to 25 equal size thalli of the same species from different

locations on the studied site were collected and pooled. *U. leatevirens* thalli were washed on site by seawater to remove all trace of sediment and epiphytes. Samples were kept in seawater until arrive in the laboratory. In the laboratory the samples were washed by Milli-Q water and blot dried. Macroalgal samples were attached to the substrate to verify site source of tissues. The collected thalli were pooled together and divided into three replicates. Samples were freeze dried, pulverized and sieved to a particle size of <500 µm. Samples were stored, at room temperature, in airtight polyethylene bottles until required for analysis (Murphy et al., 2007).

Sediment collection and preparation

Superficial sediment was collected from the three selected sites of the study areas. The upper 5 cm sediments from each site were removed by a polyethene scoop and immediately mixed to form homogenized samples which were stored in polyethene bottles. In the laboratory, the samples were freeze-dried and ground using pestle and mortar and sieved. The 63 µm fractions were stored until analyses.

Macroalgae metal extraction

The extraction of metals in macroalgal tissues was performed by digestion using Teflon bombs. 100 mg of dried sample was digested by mixture of 3 ml of nitric acid, 4 ml of Milli-Q water and 3 ml of perchloric acid at 130 °C for 2 hrs (Rigollet et al., 2004). Each digest was brought to 50 ml with Milli-Q water. The extractions were made in triplicate on different days to insure the reliability and reproducibility of the extraction method.

Sediment metal extraction

Samples of 0.2 g were digested in 3 ml of concentrated nitric acid and 3 ml of concentrated perchloric acid and 4 ml Milli-Q water using Teflon bombs for 2

hrs at 130 °C (Rigollet et al., 2004). Following digestion, the vessel contents were filtered and diluted to a final volume of 50 ml with ultrapure water. The extractions were made in triplicate on different days to insure the reliability and reproducibility of the extraction method.

Metals analysis

The analyses of metals of both macroalgae and sediment were performed by atomic absorption with the Spectra 250 Plus Varian double-beam instrument with a continuous source background corrector (deuterium lamp), equipped with a rotating turret with 4 hollow cathode lamps. The instrument was used in the air /acetylene flame configuration for the elements at higher concentrations (mg /L). For lower concentrations (µg/L) the instrument was configured with the graphite furnace (GTA-96 Varian) combined with rotating auto-sampler. The metal concentrations in the macroalgal tissues and sediment were expressed as µg/g in dry weight (DW) and were compared to the sediment quality values specifically designed for the respected areas (GA or LV). In fact, a specific regulation is applied to Venice Lagoon sediments because of the peculiarity of this ecosystem. According to Italian Law and especially the so called "Venice Special Law" (Ministero per l'Ambiente, 1993), three Sediment Quality Classes had to be implemented for the management of dredged materials, depending on the future use of the sediments (Classes L, M, and H). To validate the analytical methodology and verify the accuracy of the analysis, certified reference materials were used: MESS-3 (Marine Sediment Reference Materials for Trace Metals and other Constituents – National Research Council Canada) and CRM N° 0974 (*Olea Europaea)* that have undergone the same extractions of the samples. The recoveries were higher than 94% whilst the mean coefficient of variation was within 8%. All the quantifications were done with blank and sample blank (HNO3

1%) procedure run in parallel. For each sample, the sample blank was subtracted and the mean values and respective standard deviations were calculated (Figueira et al., 2011).

2.3.4 Estimation of lipid peroxidation (Oxidative stress biomarker)

The induction of biomarkers such as lipid peroxidation (LPO) is a potential indicator for the stress of macroalgae (*U. leatevirens*) exposed to anthropogenic pressures. In the lipid peroxidation assay, malonaldehyde (MDA) is the major secondary oxidation end-product of lipid peroxidation process. MDA reacts with thiobarbituric acid (TBA) as a TBA reactive substance (MDA-TBA) to produce a red colored complex which has peak absorbance at 535nm (Wahsha et al., 2012), as in the following reaction (**Fig. 12**).

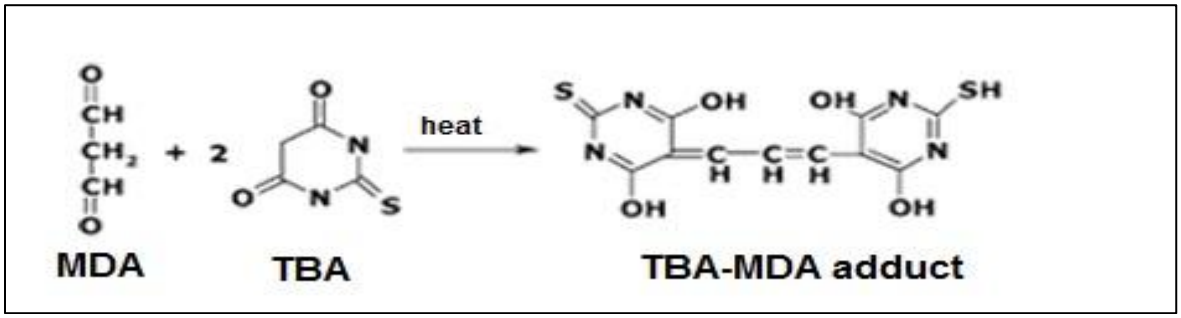

Fig. 12: an illustration of the process of lipid peroxidation. (Edited after: Grotto et al., 2009)

Macroalgal sampling

Macroalgal samples were collected during the period of low tide from the upper layer of the intertidal zone according to Benton (2001) with some minor modifications. At least ten equally sized specimens of selected macroalgal species (at the early vegetative phase and normal morphological appearance) have been sampled at each site and pooled together with their corresponding surrounding water. Samples were packed in plastic bags not completely closed with a non-

metallic closure, to minimize the stress during transportation and to allow gas exchange, and transported to the laboratory. Macroalgae (*U. leatevirens* Areschoug) were classified according to Sfriso and Curiel, (2007). All macroalgal samples were gently washed and rinsed with standardized seawater for 10 seconds to avoid any appreciable stress decomposition.

Estimation of lipid peroxidation (LPO)

The LPO level (as MDA) was determined by the thiobarbituric acid reactive substances (TBARS) reaction as described by Wahsha et al., (2012). Homogenate of 0.10 g fresh weight of the macroalgal tissues in 5 mL solution of 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) was incubated at 95 °C for 30 min, followed by quick cooling, and centrifuged at 10,000 g for 10 min. The absorbance of the clear supernatant was measured by spectrophotometer at 532 nm, and correction for unspecific turbidity was done by subtracting the absorbance of the sample at 600 nm. A 20 ml of 0.25%TBA in 10% TCA was used as blank. The concentrations of MDA were quantified and expressed using Beer's law with an extinction coefficient of 155 mM^{-1} cm^{-1}. For quality control of the evaluation of the lipid peroxidation process, ten macroalgal specimens were collected from uncontaminated areas both in the Lagoon of Venice (SMM) and the Gulf of Aqaba (MSS).

2.3.5 Macrophyte analyses (Ecological Index)

Macrophyte collection (qualitative approach)

For the study of macrophyte biodiversity of each marine area, it is necessary to obtain a check-list of the species present. The taxonomic study of macrophytes requires detailed observations of the morphology of the thallus and of the tissues of

each species sampled, using the optical microscope and the stereoscope. For this reason, a sampling was also performed by direct collection of samples for the taxonomic study in the laboratory. All the macrophyte species were collected within an area of 100 linear meters, along different transects, with a pattern from the shore towards deeper depths.

The direct collection from the coastal areas (GA) was carried out by snorkeling. During the sampling, attention was paid to the integrity of the thallus structure, in particular in the presence of the basal part, since this region is often decisive for the recognition of the alga or the angiosperms. The collected material was stored in sea water and 4% formaldehyde and stored in cold rooms in the dark at 4 °C, for the subsequent determination in the laboratory. The biomass at each station was estimated in duplicate by a square of 50cm x 50cm. All the vegetation inside the square was collected and placed in a separate plastic bag. About 4% formaldehyde/seawater solution was added to each bag. In the laboratory, the algal samples were rinsed in water, and sorted by species. Biomass (expressed as dry weight in grams per m^2) was calculated according to protocol of English et al., (1997).

Morphological identification

The taxonomic identification of macrophytes requires for the majority of species an extensive observation of morphological characters (anatomy/cytology), even at the cellular level. The indicative characters for the identification of a species depend on the type of tested macrophyte. There are some species that are simply recognized by examining the morphological characteristics of the thallus without the use of microscopes. For example, the determination of the different species of marine seagrasses, in general, can be performed through the observation of the characters, such as the size of the leaves, the number of ribs on the leaves

and the morphological characteristics of the rhizome. Although in the macroalgae this method is less realistic, there are numerous species in which a macroscopic observation of the thallus allows recognition at the species level. However, this method is often inappropriate in most macroalgae, so it is necessary to observe the microscopic characters, which require the use of a stereoscope and an optical microscope, and sometimes even an eco-physiological study. In addition, the morphology and colour of the thallus are not always useful for the determination of the species. The peculiarities that are often significant for the species determination are: ramification and size of the arms of the thallus, cross section of a branch (cell size and structure), disposition and abundance of the number of cortical cells and mostly reproductive organs (gametophyte, sporophyte, carposporophyte). The taxonomic study of the species sampled was carried out through the use of a stereoscope and an optical microscope with related software that allowed measuring and taking images of the species and the most significant characters.

2.3.5.1 Applied index (MaQI)

Macrophyte Sampling

For application of MaQI in LV, samples were collected from the three sampling sites during two seasons (spring and autumn 2017). In all the sites the main environmental parameters related to water, surface sediment, macroalgal and angiosperm composition and cover were determined.

Macrophyte cover

The cover of macrophyte was determined according to the monitoring protocols by ISPRA (2011). The presence/absence of macrophyte in each site was assessed by touching the bottom of an area about 20-30m by a rake for at least 20 times in order to obtain an accuracy ≥95% as required by MaQI (Sfriso, 2010). The relative abundance of the main taxa was obtained by sorting and wet weighting the

macroalgae collected in 6 random samples obtained from the bottom with a rake for approx. 1m. Macrophytes were preserved in 4% formaldehyde seawater for the determination at specific and intraspecific level by means of a stereo-zoom microscope and a light microscope. Some samples of uncertain identification were also kept in silica gel for further future molecular analyses. Total angiosperm cover, in the sites where they were present, was recorded by means of the Visual Census Technique only to apply MaQI: two researchers in the field determined the cover of angiosperm separately and the final value was the mean of the two determinations.

Ecological status assessment: MaQI structure

The ecological status (ES) of the studied areas was assessed by applying the Macrophyte Quality Index (MaQI) modified version of Sfriso et al., (2014) using MaQI scheme intercalibrated by the European Commission. The application of MaQI for the ecological assessment is based on several metrics: (1) number and percentage of sensitive macroalgal taxa (score=2; Sfriso et al., 2009); (2) relative abundance (wet weight) of Chlorophyta and Rhodophyta; (3) total percent macroalgal cover; (4) percent cover of aquatic angiosperms. For the calculation of EQR two entries are necessary, one for macroalgae and the other for angiosperms (**Fig. 13**). The total (spring + autumn) taxonomic list, the relative abundance of the dominant taxa, the percent number of sensitive taxa, the maximum macroalgal and angiosperm cover were taken into consideration for the MaQI implementation.

MaQI is deliberately categorical in order to be also applied in the presence of a very low macrophyte cover or number of taxa, especially if the assessment is supported by historical data which indicate that the studied water body in the past

was populated by macroalgae and/or covered by aquatic angiosperms which disappeared during the second half of 20th century.

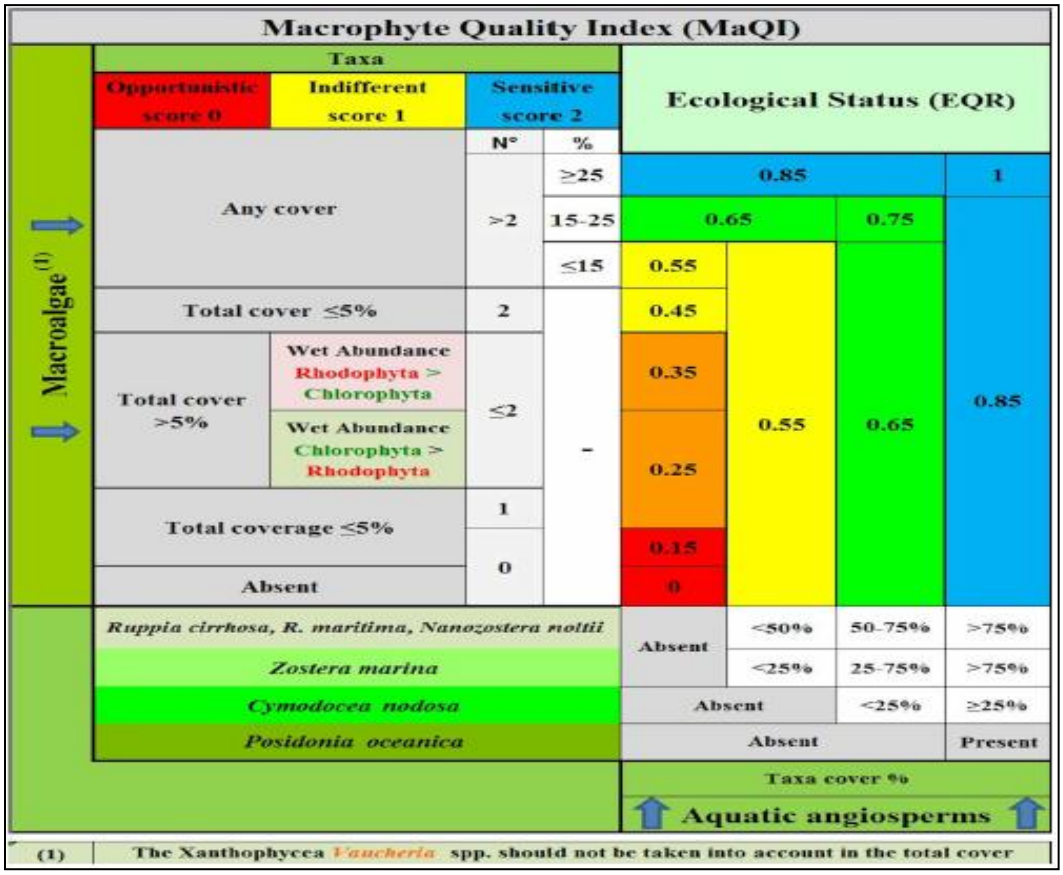

Fig. 13: The MaQI Scheme for the determination of the EQR (Source: Sfriso et al., 2014)

2.3.6 Microbial community analyses

Macroalgal sampling

To study the microbial communities associated with the macrophytes in both LV and GA sampling areas, the thalli of *U. leatevirens* (fixed on the substratum) were sampled from the intertidal zone. The selected thalli were directly preserved in pre-sterilized bottles of Artificial Sea Water (ASW) to avoid inappropriate contamination. The thalli were washed with seawater to eliminate or reduce the epiphytes and/or invertebrates and to remove settled sediment and unattached

microorganisms. The bottles were preserved in ice until the laboratory analysis. Two replicates per station were used for microbial community analysis. Each replicate was composed of at least 10 equally size thalli of *U. leatevirens*.

Bacterial pellets and total DNA extraction

In the laboratory, samples were immediately subjected to smooth sonication (3*30s) to ensure detachment of bacterial cells on *U. leatevirens* surface. The macroalgal parts were removed with sterile tweezers under sterilized conditions followed by filtration of the remaining ASW (including bacterial cells) with 0.20 μm nitrocellose filters. The filtration was necessary for trap bacterial cells. The filters for each replicate were divided by sterile blade and resuspended in sterile tube containing 10 ml of washing buffer solution (200 mM Tris–HCl pH 8, 10 mM EDTA, and 0.24% Triton X-100; Kadivar and Stapleton, 2003). The filters were gently rubbed against the collection tubes walls and vortexed (3*30 s) to detach and collect the bacteria and filter fragments were removed with sterile tweezers. The tubes containing the washing buffer solution were centrifuged at 5000 rpm for 20 min to obtain the bacterial pellets. Metagenomic bacterial DNA was extracted from the pellets using the DNeasy® PowerSoil® Kit (Qiagen, USA) following the protocol provided by the manufacturer. The purity and quantity of DNA extracts were determined by agarose gel electrophoresis and Qubit Fluorometer 2.0 (Invitrogen, CA). DNA was stored at −20 °C for further use. For comparison of microbial communities, total DNA was extracted from seawater samples (i.e near the macroalgae) collected during summer and autumn seasons following the same metagenomic DNA extraction protocol.

PCR amplicon libraries for Illumina NextSeq 500 platform sequencing were constructed, using bacterial primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′)

and 806R (5′-GGACTACNVGGGTWTCTAAT-3) targeting V4 hyper-variable region of bacterial 16S rRNA genes (Apprill et al., 2015). The conditions for amplification were as follows: 98°C for 30 s; 25 cycles of 98°C for 20 s, 57°C for 30 s, followed by 72°C for 30 s, with a final extension 72°C for 5 min. Phusion® High-fidelity DNA polymerase was used in the amplification reaction. The PCR products were purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) and quantified using a Qubit system (Invitrogen). The amplicons were tagged using Nextera XT DNA index kit (Illumina, San Diego, CA, USA) following the manufacturer instruction. Equimolar amounts of purified tagged amplicons were pooled and stored at −20°C until sequenced. Tagged amplicons from 16S rRNA amplification were analyzed through Illumina® NextSeq 500 platform (University of Padua, Italy) using the Nextseq® 500/550 High-output KT v2 (Illumina, Inc., San Diego, CA, United States), according to manufacturer's instructions.

The 16S rRNA gene raw sequences were processed using the R-package high-resolution sample inference from amplicon sequencing data (DADA2) (v. 1.4) (Callahan et al., 2016) and included primer removal and truncating the forward reads at 125 bp. The SILVA SSU database v. 128 (Quast et al., 2012) with a 50% confidence threshold were used to assign taxonomy to each inferred 16S rRNA gene sequence; defined here as an exact Amplicon Sequence Variants (ASVs). ASV method infers the biological sequences in the sample prior to the introduction of amplification and sequencing errors, and distinguishes sequence variants differing by as little as one nucleotide (Callahan et al., 2017). Data were normalized by relative abundance of ASVs using the selected threshold; the threshold was set to be at $3*10^{-5}$, and used for explorative characterization of the bacterial communities.

2.4 Statistical analyses

2.4.1 Metal content statistical analyses

One-way ANOVA was used to compare the metal concentration in sampling sites. The Spearman rank order correlation test allowed checking the significant correlations of metals in macroalgae and sediment. The different statistical methods were applied with a 95% confidence interval (significance, $p < 0.05$). Data were processed using STATISTICA 10.1 (Stat. Soft. Inc., 1998, Tulsa) and reported as mean values ± standard deviation for all macroalgae and sediment samples. The standard deviations of pooled samples of algae refer to the variability within different replicates. The overall metal content in sediment or macroalgae was calculated for different sites to compare the degree of metal pollution with the Metals Pollution Index (MPI) using the formula reported by Usero et al., (1997).

$$\mathbf{MPI} = (Cf1 \times Cf2 \times \ldots\ldots Cfn)^{1/n}$$

Where $Cf1$, $Cf2$…up to Cfn are the concentrations of the metal 'n' in the sample.

2.4.2 Oxidative stress biomarker statistical analyses

Mann–Whitney and Kruskal–Wallis tests were used to evaluate significant differences in oxidative stress biomarker (LPO) among sites. Pearson's correlation coefficients were used to evaluate the relationships between metal concentration and LPO levels. Data were processed using STATISTICA 10.1 (Stat. Soft. Inc., 1998, Tulsa).

2.4.3 MaQI and environmental parameters statistical analyses

The correlations between the environmental parameters (natural parameters and parameters related to anthropogenic pressures) and the macrophyte variables were obtained by calculating the Spearman coefficients ($p < 0.05$). Furthermore, the

same matrix was used for the principal component analysis (PCA) which processes data in order to reduce the number of variables for an easier understanding of the total variance of the system. PCA allowed determining the main factors that affect the system variance and also the affinity of the different macrophyte taxa with the environmental parameters obtained by plotting the results of the first two components in a plane surface. The factor loadings of the vectors were considered significant with values >0.7. All the analyses were performed by using Statistica version 10.1 (Statsoft, Inc. Tulsa, USA).

2.4.4 Microbial community statistical analyses

Community's diversity (*α*- Diversity)

The normalized ASVs table to the minimum number of sequences (after removal of chimeras, eukaryotic sequences, unassigned sequences, chloroplast and mitochondrial ASVs) was used for statistical analyses. The Chao 1 richness (Chao, 1984), Shannon and Simpson indices and number of distinct ASVs were calculated to assess diversity values within samples (*α*-Diversity) using estimate richness on phyloseq analysis (Callahan et al., 2017). Analyses of variance (one-way ANOVA) were performed to access significant differences in *α*- diversities among sites using STATISTICA 10.1 (Stat. Soft. Inc., 1998, Tulsa).

Community Structure and Characterization (*ß* -Diversity)

Using the normalized (rarefied) data set, diversity between the different samples (*ß*-Diversity) was estimated using Bray-Curtis dissimilarity measure to build the distance matrix (Bray and Curtis, 1957) after square root transformation of the data. Permutational multivariate analysis of variance (PERMANOVA+) analyses were done by PRIMER v6 (PRIMER-E, UK) to test for differences between samples with the factors: Type of Sample (macroalgae vs. water), season,

and site. The samples were considered statistically different when p-values were <0.05 and r-values were close to one (Clarke and Warwick, 1994). Analyses were done using 9999 permutations of residuals under a reduced model. PERMDISP was used to test for homogeneity of multivariate dispersion within groups. Non-metric multidimensional scaling (nMDS) was generated as an ordination method to visualize the variation of microbial community structure and composition in different sample groups (Kenkel and Orloci 1986). To identify ASVs specific and shared among defined sample groups a Venn-Diagram was constructed using Venny 2.0 (Oliveros, 2007).

Distance-based Redundancy analysis (db-RDA) allowed investigating the effect of environmental parameters and the total associated microbial community by using CANOCO (version 5.0) software. The normalized relative abundance of ASVs and the measured environmental parameters (natural parameters and parameters related to anthropogenic pressures) were used as species input and environmental input, respectively.

3. RESULTS

Part I: Environmental parameters

3.1 Environmental parameters for water (Lagoon of Venice)

The Mean ± Standard Deviation (SD) values of the environmental parameters measured in the water column (pH, redox potential (Eh), temperature (Temp.), dissolved oxygen saturation (DO), Salinity and Filtered particulate matter (FPM)) in the stations in LV are shown in **Table 2**. The highest values of pH were recorded at SMM. Redox potential (Eh) exhibited high variability among seasons (high SD) where the lowest values were recorded in spring for all sites. Water column temperatures increased from the sea inlet (SMM) to the mainland (PM and SG); the highest seasonal variation was recorded for SG (14.0 – 29.8 °C). The seasonal measurements of water environmental parameters are listed in appendix (A).

Table 2: Values of pH, redox potential (Eh), temperature (Temp), dissolved oxygen saturation (DO), salinity and Filtered particulate matter (FPM) for water column samples of LV sampling sites.

Parameter	SMM		PM		SG	
	Mean	± SD	Mean	± SD	Mean	± SD
pH	8.30	0.14	8.15	0.13	8.22	0.36
Eh (mv)	283	25.0	281	41.0	358	150
Temp. (°C)	19.7	4.50	23.0	6.50	22.3	7.00
DO (%)	155	8.00	137	31.0	157	68.0
Salinity (PSU)	26.6	4.40	24.7	2.10	19.3	9.60
FPM (mg/L)	29.0	11.8	33.8	4.30	56.2	45.0

The highest %DO fluctuations were recorded at SG (**Fig. 14**) with a peak concentration in spring (258%). Moreover, salinity was less fluctuating among

sites and seasons; hence, lower value (6.40 psu) was measured at SG during winter. The highest value of salinity (32.5 psu) was recorded in summer at SMM. The concentration of FPM in water column varied among sites with average values of 29.0 and 33.8 mg/L at SMM and PM, respectively. At SG, FPM was higher (56.2 ± 45.0 mg/L) and more variable among seasons. High value of FPM (121.5 mg/L) during spring resulted from the increased turbidity (i.e at SG).

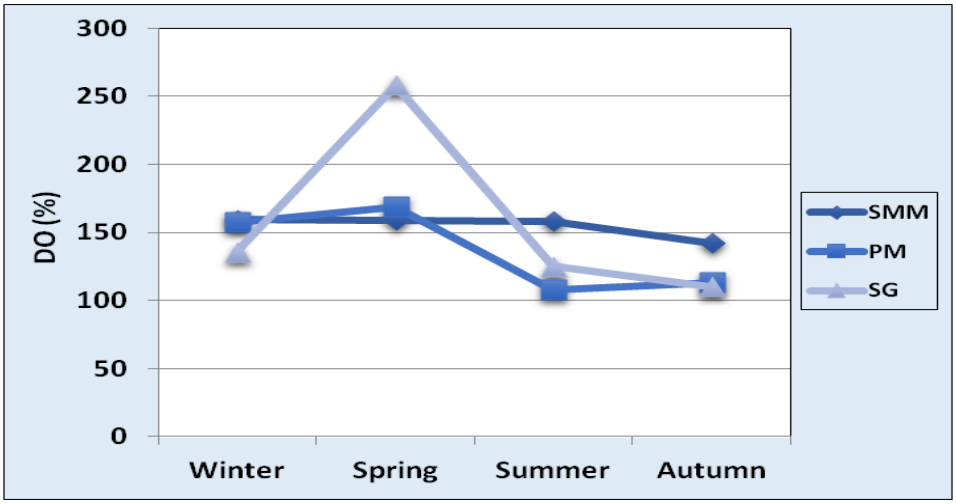

Fig. 14: Dissolved oxygen saturation (DO) during seasons at LV sampling sites.

Reactive silicates (SiO_4) in the water column varied between 5 and 15 µM, with slight seasonal variations at SMM and PM (**Fig. 15**). The concentration was slightly higher in winter. No specific variation in the concentration of SiO_4 was showed among seasons. SiO_4 concentrations at SG were significantly higher in comparison with the other sites with a maximum in winter (43.7 µM).

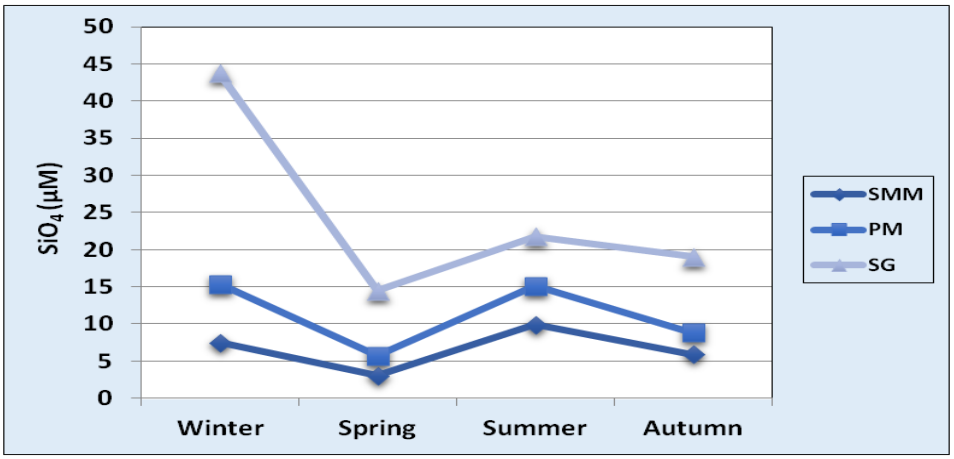

Fig. 15: Reactive silicate (SiO_4) concentration (µM) during seasons at LV sampling sites.

The Dissolved Inorganic Nitrogen (DIN = sum of Ammonium, Nitrite and Nitrate) concentrations were generally high at SG. The lowest DIN concentrations were recorded at SMM in all the seasons. The highest concentration was found at SG in winter where a high concentration of ammonium was also noticed, contributing to about 70% of DIN concentration. Conversely, the highest nitrate contribution to the DIN concentration (63%) was recorded in winter and autumn. Higher concentrations of ammonium were measured at SG in comparison to the other sites for all seasons (**Fig. 16**).

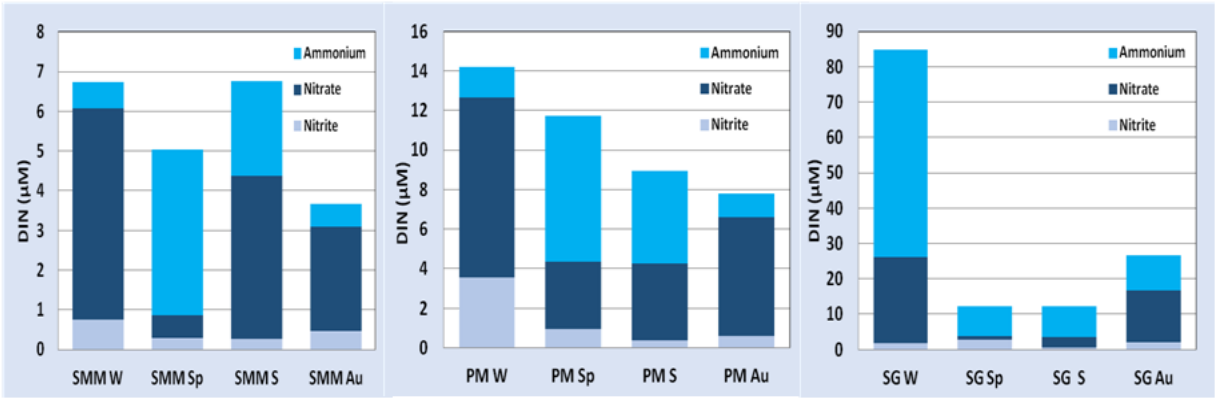

Fig. 16: Dissolved Inorganic Nitrogen (DIN) concentration (µM) during seasons at LV sampling sites.

Reactive Phosphorus (RP) (**Fig. 17**) showed very low concentrations, at the limit of detection, with small fluctuations among seasons (mean values: 0.15 and 0.52 µM at SMM and PM, respectively). At SG, however, an extremely high concentration (3.15 µM) was measured in spring. RP at SG exhibited the highest concentrations in comparison to the other sites (0.65 – 3.15 µM).

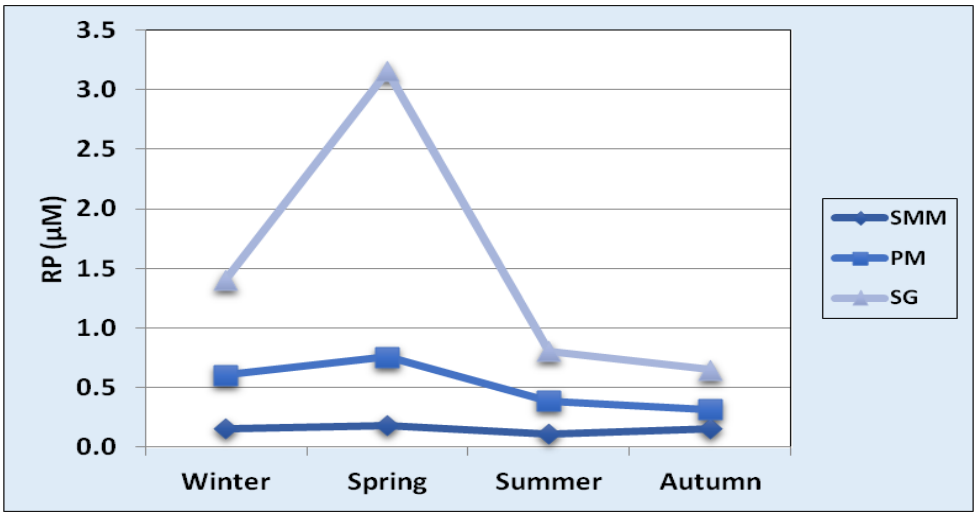

Fig. 17: Reactive phosphorus (RP) concentration (µM) during seasons at LV sampling sites.

Chlorophyll-*a* (Chl-*a*) showed the highest values at SG in spring (**Fig. 18**) reaching 29.9 µg/L. For both SMM and PM the average Chl-*a* concentration was quite similar: 2.10 and 2.20 µg/L, respectively. Chl-*a* didn't exhibit a specific seasonal trend among sites. The highest concentration of Chl-*a* at PM were recorded during summer season (3.29 µg/L).

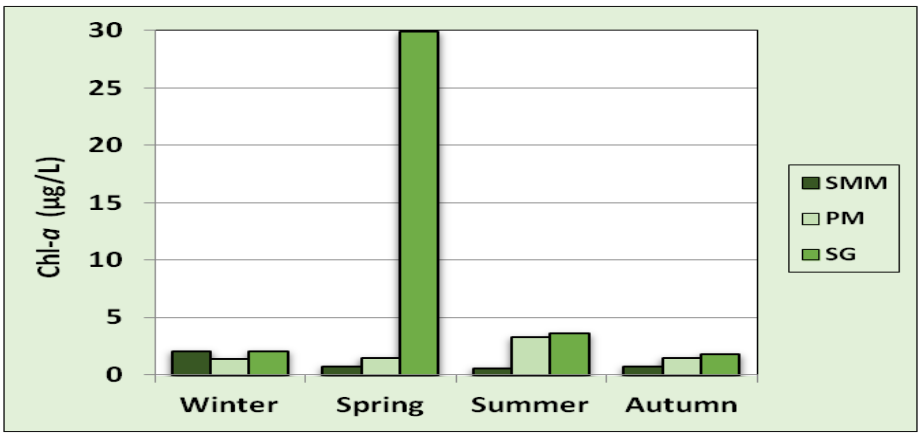

Fig. 18: Chlorophyll-*a* (Chl-*a*) concentration (µg/L) during seasons at LV sampling sites.

3.2 Environmental parameters for surface sediments (Lagoon of Venice)

The in-field and laboratory analyses of sediment collected from LV sampling sites are shown in **Table 3**. The local variability of carbon, nitrogen, phosphorus and grain-size in sediment and settled particulate matter (SPM) samples was investigated by combining 3 pools for each sampling sites.

Table 3: Values of pH, Eh, humidity, wet density, total organic matter (TOM), total carbon (TC) and total nitrogen (TN) for sediment of LV sampling sites.

Site	pH	Eh	Humidity	Wet density	TOM	TC	TN	Fines
		(mv)	(%)	(g/cm^3)	%	mg/g	mg/g	(%)
SMM	8.00	167	36.0	1.73	1.54	78.6	0.10	21.4
PM	7.43	-192	21.0	1.81	4.57	67.0	0.80	74.8
SG	7.85	-80	48.0	1.49	6.57	68.3	1.40	93.0

The values of pH, Eh, moisture and density of sediments in LV sampling sites are reported in **Table 3**. PM and SG sediment samples were characterized by lower pH and Eh in comparison to SMM. The grain-size distribution varied among

sites. At SMM the sediment was prevalently sandy (ca. 79%), whereas at SG and PM it was more pelitic (ca. 93 and 75% of clay, respectively). Furthermore, PM and SG exhibited a high TOM content (ca. 4.6 and 6.6 %, respectively). Total carbon showed similar concentrations in all the sites ranging from 67.0 mg/g at PM to 78.6 mg/g at SMM. In contrast, TN concentration changed remarkably from 0.1 mg/g at SMM to 1.4 mg/g at SG.

The total concentration of phosphorus (TP) in sediment at PM and SG was quite similar. Higher variations were recorded in the organic and inorganic fractions of phosphorus. P org. was the highest at SG (89.5 µg/g; 19.0% of the TP) whereas it reduced markedly at PM and SMM **(Fig. 19)**. P org. at PM was extremely low (2.4% of TP).

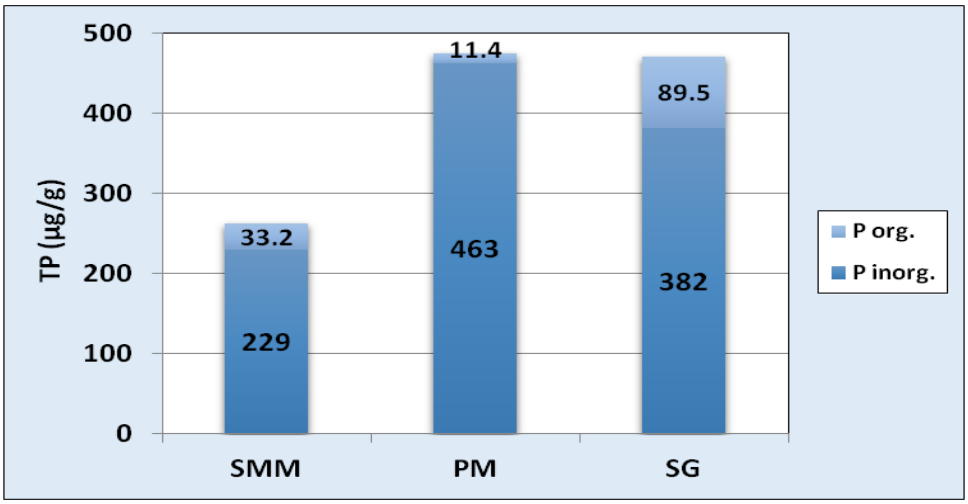

Fig. 19: Concentration of total phosphorus (TP) fractions: organic phosphorus (P org.) and inorganic phosphorus (P inorg.) for sediment in LV sampling sites.

3.2.1 Environmental parameters for settled particulate matter (Lagoon of Venice)

The settled particulate matter (SPM) in the traps at SG was almost completely pelitic (99.4%) with high concentration of TN, TP (P org. and P inorg.)

and TOM in comparison to all the other sites **(Table 4)**. The prevalent sandy nature of SPM at SMM was linked to the low concentration of organic (P org.) and inorganic (P inorg) phosphorus fractions and TN which in turn were consistent with the lowest %TOM content (3.52 %). The concentration of both P org. and P inorg. was higher at SG (ca. 217 and 606 µg/g, respectively) in comparison to the other sites. Additionally, the lowest percentage of P org. among sites was recorded at PM site (15.5% of the TP). The low variability among sites was noticed for the content of TC, although a slight increase was found at PM (78.9 mg/g).

Table 4: Values of total carbon (TC), total nitrogen (TN), organic phosphorus (P org.), inorganic phosphorus (P inorg.), total organic matter (TOM), and fine sediment (<63 µm) percent (Fines) for SPM from LV sampling sites

Site	TC	TN	P org.	P inorg.	TOM	Fines
	mg/g	mg/g	µg/g	µg/g	(%)	(%)
SMM	71.7	1.00	81.0	281	3.52	35.4
PM	78.9	1.60	91.0	493	6.10	70.0
SG	71.3	2.30	217	606	6.92	99.4

3.3 Environmental parameters for water (Gulf of Aqaba)

The in-field measurements in water column of the GA during the two sampling periods are shown in the **Table 5**. The pH values were slightly higher in winter in comparison to summer in all sites. This was also noticed for salinity that showed very few variations among sites and seasons. Temperatures showed slight seasonal changes during winter and summer. The same trend was also recorded for the %DO saturation which was super-saturated in all the water samples during the two seasons. The highest % DO saturation (ca. 149%) was recorded at IC.

Table 5: Values of pH, temperature (Temp), salinity and dissolved oxygen saturation (DO) of water column samples at GA sampling sites

Parameter	Winter			Summer		
	MSS	**PB**	**IC**	**MSS**	**PB**	**IC**
pH	8.38	8.32	8.43	8.12	7.89	7.91
Temp (°C)	21.5	21.6	21.6	23.9	26.6	26.8
Salinity (PSU)	40.8	40.9	40.7	40.7	40.7	40.8
DO (%)	146	146	147	140	147	149

The low concentrations of nutrients (DIN, RP and SiO_4) were noticed for all the sampling sites of GA. Silicates were always below 2.50 µM with a bit higher variability at MSS, for instance, SiO_4 reduced from 2.31 µM in winter to 1.24 µM, in summer (**Fig. 20**).

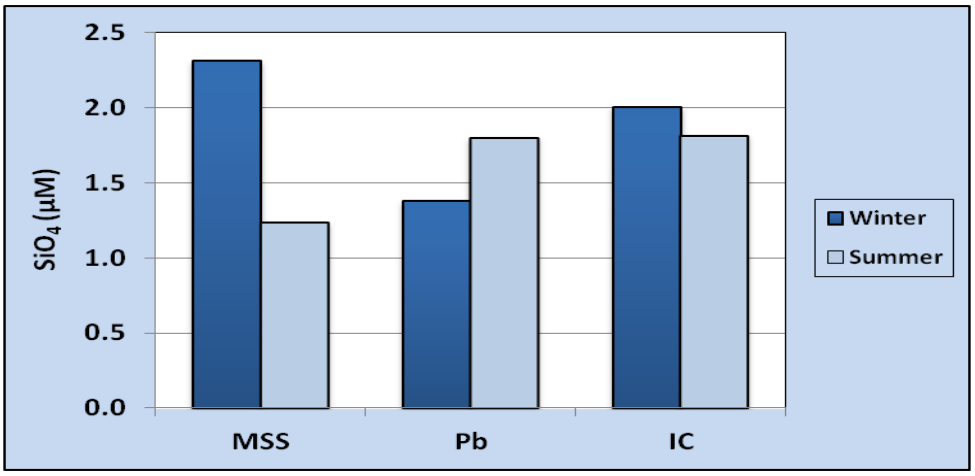

Fig. 20: Reactive silicate (SiO4) concentration (µM) during seasons at GA sampling sites.

The concentration of DIN was higher in winter than in summer. However, DIN concentration was very low in all the three sampling sites. The highest concentration of DIN (1.74 µM) was measured at PB. In summer the highest contribution to DIN concentration (ca. 70%) was due to ammonium, especially at

MSS and PB. Furthermore, during winter ammonium concentration contribution decreased to 42% in all sites (**Fig. 21**).

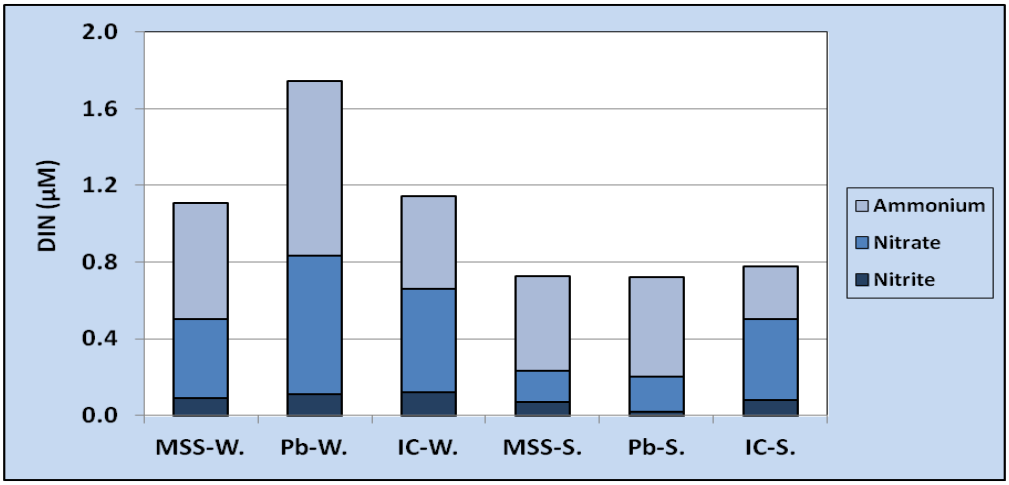

Fig. 21: Dissolved inorganic nitrogen (DIN) concentration (µM) in water column at GA sampling sites. (W: Winter, S: Summer)

The RP concentration was generally higher in summer among all sampling sites. No specific variations were noticed in the RP concentration among sites during summer (**Fig. 22**). The lowest RP concentration was measured at MSS in winter (0.05 µM), then it increased by 68% in summer.

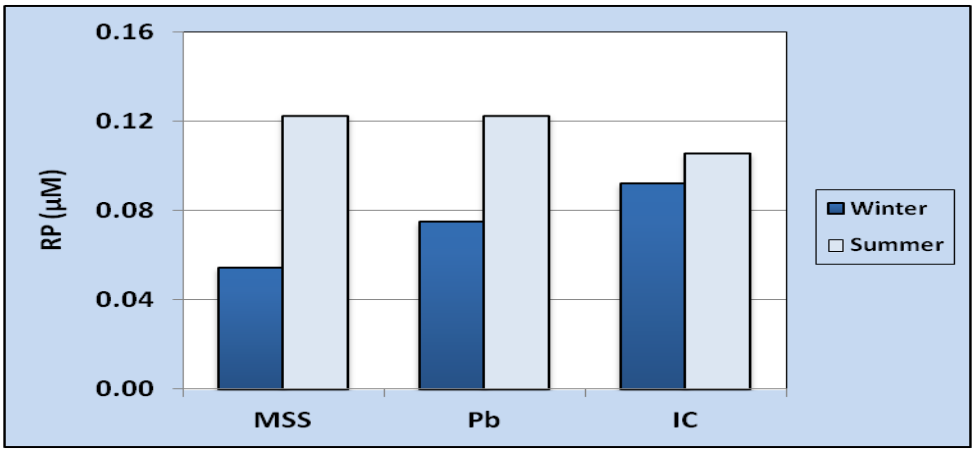

Fig. 22: Reactive phosphorus (RP) concentration (µM) during seasons at GA sampling sites.

GA sampling sites showed also low Chl-*a* concentrations (**Fig. 23**), but contrarily to the lagoon of Venice, the highest concentrations were measured in winter with 0.32 and 0.31 µg/L at PB and IC, respectively. The concentration of Chl-*a* in summer reduced averagely by 38% from the winter concentrations.

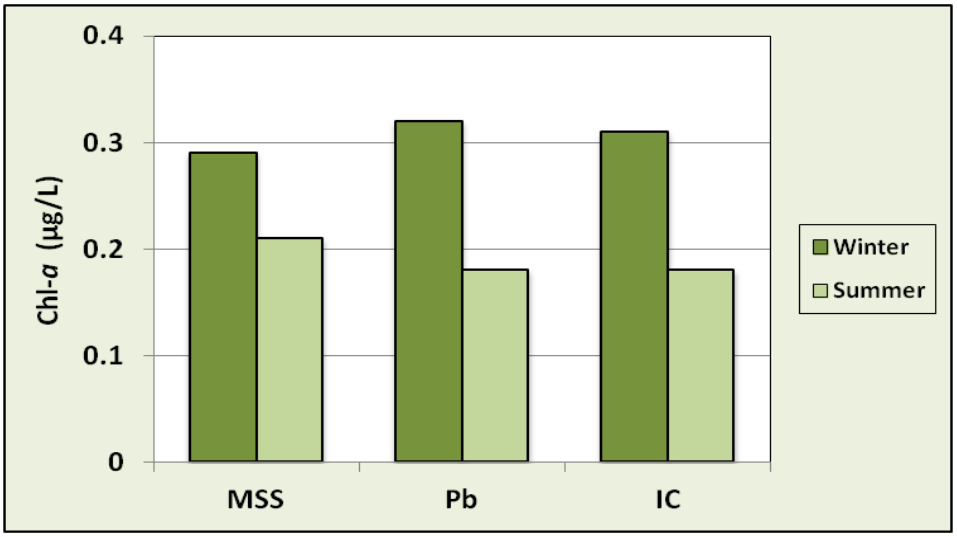

Fig. 23: Chlorophyll-*a* (Chl-*a*) concentration (µg/L) during seasons at GA sampling sites.

3.4 Environmental parameters for surface sediments (Gulf of Aqaba)

The sediment of GA was characterized by a sandy grain-size (**Fig. 24**) which was high at PB (5.6 % of sediment were <63µm) and a bit lower in the other stations. High TOM content was measured at MSS and IC, whilst the lowest was measured at PB (0.32 %).

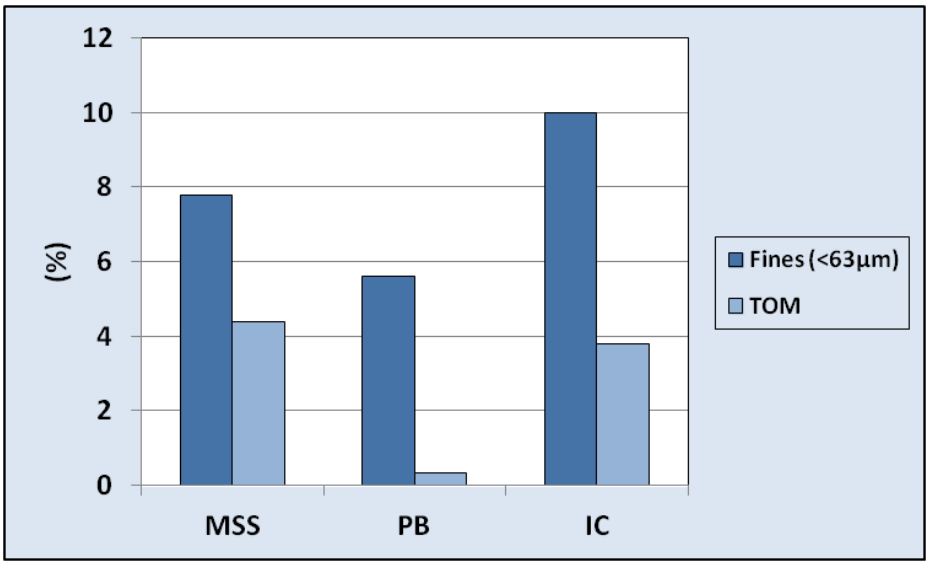

Fig. 24: Sediment total organic matter content (TOM) and percent of fine sediment (<63µm) for GA sampling sites.

In agreement with the TOM content, higher concentrations of OC and TN were found at MSS. TP was higher in the sediment at IC (0.83 mg/g). Low concentration of TN in sediment was detected at both PB and IC (0.08 mg/g) (**Fig. 25**). Lower OC and TN concentrations were noticed at PB (0.95, 0.08 mg/g, respectively).

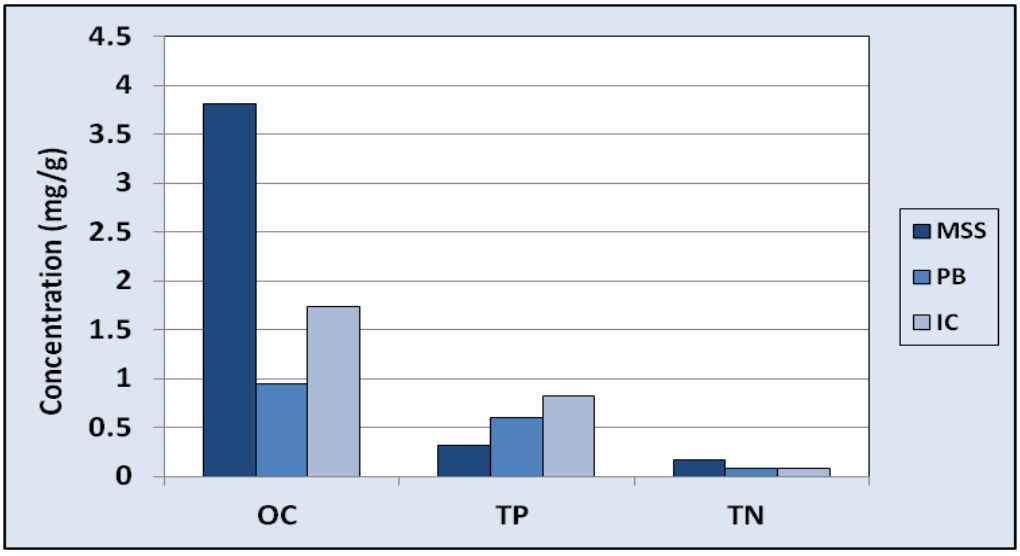

Fig. 25: Concentration of organic carbon (OC), total phosphorus (TP) and total nitrogen (TN) in sediment of GA sampling sites.

Part II: Macrophyte diversity and quality index

3.5 Macrophyte diversity (Lagoon of Venice)

The diversity of macrophyte in the Lagoon of Venice was investigated during two sampling seasons (spring and autumn) in the three sampling sites. Fifty three taxa (16 Chlorophyta, 5 Ochrophyta and 32 Rhodophyta) and 2 aquatic angiosperms (*Zostera noltei* Hornemann and C*ymodocea nodosa* (Ucria) Ascherson) were found (**Fig. 26)** among the sampling sites. The highest number of taxa (i.e. highest diversity) was recorded at SMM with 46 macroalgae and 2 angiosperms. Whereas, the lowest number of taxa was found at SG with 9 taxa (4 Rhodophyta and 5 Chlorophyta). No significant differences between the sampling periods and seasonal species change were observed among sites. Additionally, aquatic angiosperms were exclusively present at SMM.

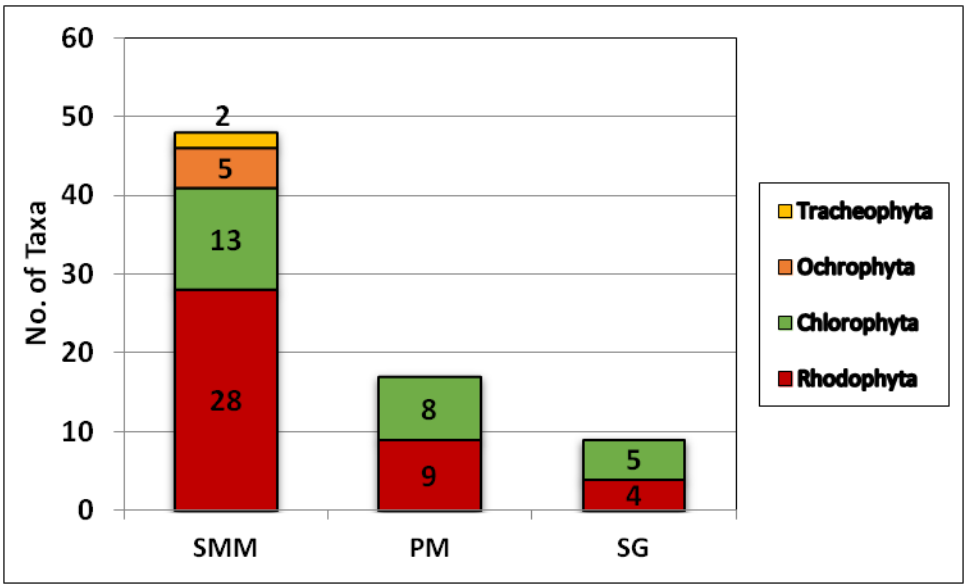

Fig. 26: Number of macrophyte taxa (at phylum level) at LV sampling sites.

The percentage of Rhodophyta was found to be the highest at SMM (61%). This site was also characterized by the presence of Ochrophyta (11%) which in the other stations were missing (**Fig. 27**). In general, the dominant taxa recorded at SG and PM was belonged to the families: Gracilariaceae, Solieriaceae and Ulvaceae. The list of identified macroalgal species is included in Appendix (B).

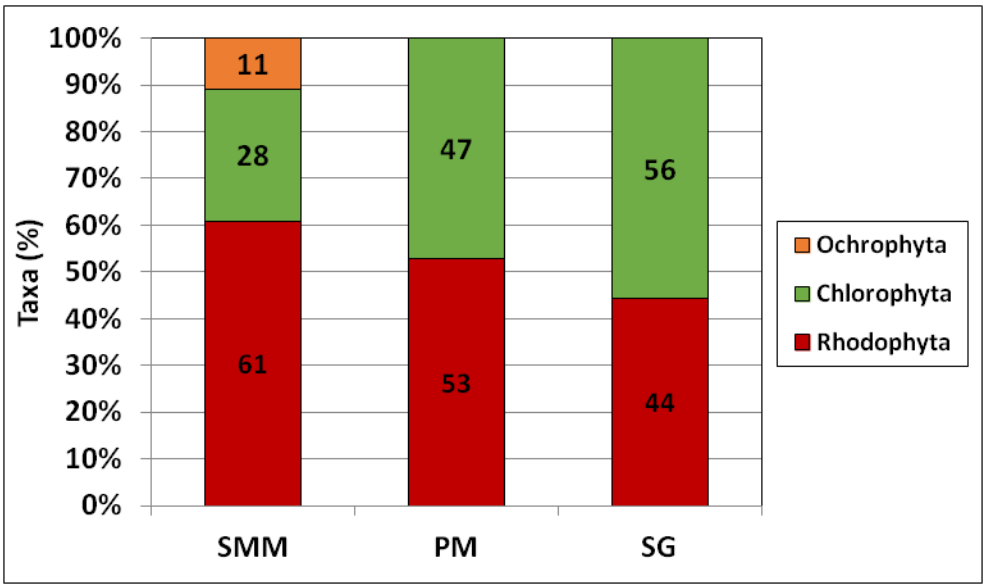

Fig. 27: Percentage of macroalgal taxa (at phylum level) distributed along LV sampling sites.

3.5.1 Macrophyte Quality Index (Lagoon of Venice)

The values of metrics required for the application of MaQI were also derived from spring and autumn surveys of the three sampling sites selected in this study. The implementation of the MaQI index requires several metrics: number and percentage of sensitive macroalgal taxa (score = 2); relative abundance (wet weight) of Chlorophyta and Rhodophyta; total percentage of macroalgal cover; per cent cover of aquatic angiosperms. The total (spring + autumn) number of macroalgal taxa, the relative abundance of the dominant taxa, the per cent number

of sensitive taxa, the maximum macroalgal and angiosperm cover for the three sampling sites (SMM, PM and SG) were included in the implementation of MaQI to calculate the EQR value in comparison to the reference site (**Table 6**).

Table 6: Macrophyte metrics and MaQI determination in LV sites

		SMM	PM	SG
No. of total Macroalgal taxa		46	17	9
Sensitive taxa	No. of sensitive taxa	14	0	0
	Sensitive taxa (%)	32	0	0
Relative Abundance (%)	Rhodophyta (%)	64	53	44
	Chlorophyta (%)	30	47	56
Macroalgal cover (%)	Max. cover (spring + autumn)	100	60	50
Angiosperm cover (%)	*Cymodosa nodosa*	65	0	0
	Zostera noltei	35	0	0
	Zostera marina	0	0	0
MaQI	**EQR**	1.00	0.35	0.25
	Ecological status	**H (High)**	**P (Poor)**	**P (Poor)**

Table 6 shows that SMM was the only site which presented sensitive macroalgae (32% of the total recorded taxa) and angiosperm cover of *Cymodosa nodosa* and *Zostera noltei*. By the application of these metrics included in the MaQI scheme, SMM was assessed in the HIGH ecological status. This was in opposition to the worst site (SG) which was assessed in the POOR ecological status supported by the absence of sensitive macroalgal and angiosperm species, as well as the higher relative abundance of Chlorophyta (score 0.25). A similar situation was observed at PM, except for the higher Rhodophyta abundance with respect to Chlorophyta, (Score 0.35).

Spearman rank order correlations were applied for both samples of spring and autumn. A high number of correlations were observed between the water column environmental parameters and the macrophyte variables. Nutrient concentrations in the water column were found correlated with the percentage of Chlorophyta and inversely with that of Rhodophyta. Similarly, clear inverse correlations were recorded between nutrients, angiosperms cover and EQR values (**Table 7**). Chl-*a* concentrations were found positively correlated with the percentage of Chlorophyta and negatively with the other macrophyte variables.

Table 7: Spearman correlations between water parameters (LV) and macrophyte variables (Bold correlations were significant at $p < 0.05$, $|r| \geq 0.61$)

Parameter	No. of taxa	% sensitive species	% Rhodophyta	% Chlorophyta	% cover (macroalgae)	% cover Angiosperm	EQR
RP	**-0.82**	**-0.85**	**-0.79**	**0.81**	**-0.86**	**-0.79**	**-0.89**
Nitrite	**-0.69**	**-0.76**	-0.48	0.53	-0.36	-0.53	-0.42
Nitrate	-0.43	-0.45	-0.30	0.29	-0.04	-0.35	-0.16
ammonium	**-0.70**	**-0.71**	**-0.74**	**0.78**	**-0.78**	**-0.66**	**-0.90**
DIN	**-0.85**	**-0.95**	**-0.77**	**0.79**	**-0.74**	**-0.83**	**-0.82**
SiO_4	**-0.73**	**-0.79**	**-0.76**	**0.71**	**-0.70**	**-0.62**	**-0.74**
Chl-*a*	**-0.62**	**-0.61**	**-0.77**	**0.75**	**-0.76**	-0.54	**-0.74**
pH	0.42	0.14	0.36	-0.31	0.16	0.29	0.21
Eh	-0.33	-0.20	-0.23	0.15	-0.21	-0.08	-0.16
DO (%)	0.39	0.17	0.39	-0.39	0.26	0.35	0.32
Salinity	0.34	0.32	0.16	-0.29	0.13	0.22	0.22
Temp	0.06	-0.03	-0.28	0.23	-0.50	-0.33	-0.39
FPM	-0.43	-0.27	-0.49	0.40	-0.40	-0.30	-0.36

The measurements of sediment parameters supported these significant correlations with macrophyte variables. High percentages of fine sediment and SPM were negatively correlated with macrophyte variables except the percent of Chlorophyta.

Table 8: Spearman correlations between sediment parameters (LV) and macrophyte variables (Bold correlations were significant at $p < 0.05$, $|r| \geq 0.81$)

Parameter	No. of taxa	% sensitive species	% Rhodophyta	% Chlorophyta	% cover (macroalgae)	% cover Angiosperm	EQR
pH-sed	0.43	0.56	0.52	-0.54	0.58	0.78	0.44
Eh-sed	0.43	0.68	0.46	-0.43	0.55	**0.85**	0.50
TOM-sed	**-0.89**	-0.80	**-0.99**	**1.00**	**-0.93**	-0.78	**-0.91**
TP-sed	-0.49	-0.56	-0.46	0.49	-0.49	-0.78	-0.44
P org.-sed	-0.54	-0.31	-0.41	0.43	-0.23	0.03	-0.44
Fines -sed	**-0.96**	**-0.90**	**-0.97**	**0.96**	**-0.91**	**-0.85**	**-0.98**
Humidity-sed	-0.37	-0.19	-0.52	0.49	-0.46	-0.03	-0.50
Density -sed	0.49	0.31	0.41	-0.37	0.29	0.03	0.50
TN - sed	**-0.83**	-0.80	**-0.99**	**0.94**	**-0.99**	**-0.85**	**-0.97**
TC -sed	0.43	0.68	0.46	-0.43	0.55	**0.85**	0.50
TOM-SPM	**-0.94**	-0.80	**-0.93**	**0.94**	**-0.84**	-0.78	**-0.91**
TP -SPM	**-0.89**	-0.80	**-0.99**	**1.00**	**-0.93**	-0.78	**-0.91**
P org. -SPM	**-0.89**	-0.80	**-0.93**	**0.89**	**-0.90**	**-0.85**	**-0.97**
Fines-SPM	**-0.94**	**-0.93**	**-0.87**	**0.83**	**-0.81**	**-0.85**	**-0.97**
TN -SPM	**-0.89**	**-0.93**	**-0.93**	**0.89**	**-0.90**	**-0.85**	**-0.97**
TC -SPM	0.43	0.19	0.46	-0.43	0.38	0.03	0.50

Significant negative correlations were found between macrophyte variables and TOM in both sediment and SPM. The cover of macroalgae was negatively correlated with nutrients (TOM, TN, TP) in sediment and SPM, whereas these parameters were positively correlated with the percentage of Chlorophyta (**Table 8**).

3.6 Macrophyte diversity (Gulf of Aqaba)

On the whole, 35 species of macroalgae and only a single angiosperm were collected from the three sites in the GA. Macroalgae were sorted in 7 Chlorophyta, 18 Rhodophyta and 10 Ochrophyta, whereas, the angiosperm species was *Halophila stipulacea* (Forsskål) Ascherson. Macroalgae were possibly identified at specific and intraspecific level. Some taxa were identified at genus level due to the lack of distinct references for the macroalgae of the Red Sea.

MSS and PB were characterized by high abundance of Rhodophyta (47, 65 %, respectively) (**Fig. 28**) while IC was mainly dominated by Ochrophyta (56%). The highest percentage of Chlorophyta was recorded at IC (33%), while the lowest one was found at PB (11%). The list of macroalgal taxa is included in appendix (C).

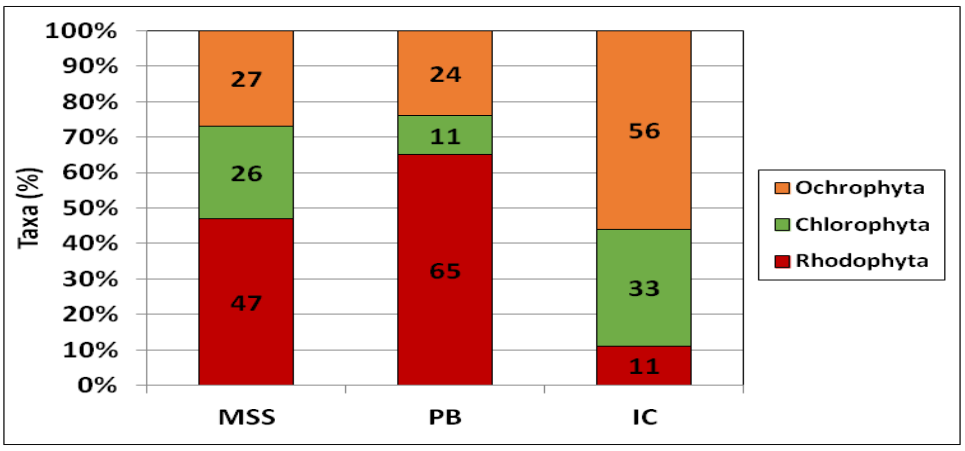

Fig. 28: Percentage of macroalgal taxa (at phylum level) distributed along GA sampling sites.

The average cover (%) and biomass (g/m^2) of macroalgae for the three sampling sites is reported in **Fig. 29**. Ochrophyta represented the highest cover and biomass among the macroalgal taxa, whereas Rhodophyta exhibited the lowest cover and biomass (1.5% and 1.4 g/m^2). This was clearly noticed during sampling collection especially for Rhodophyta which were characterized by small size species with low cover and biomass.

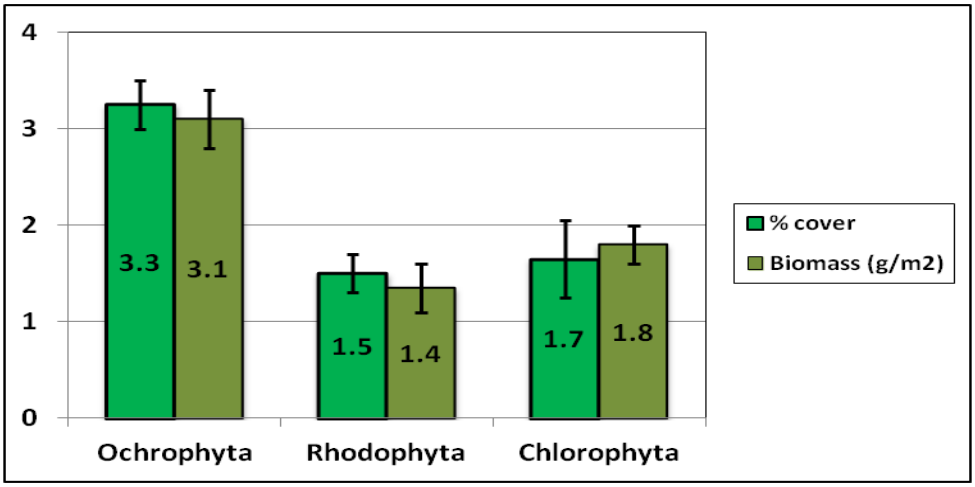

Fig. 29: The averages cover percentage and biomass for macroalgal taxa (at phylum level) at GA sampling sites.

The comparison of macroalgal taxa collected from the GA sampling sites is showed in **Fig. 30**. By comparing the sampling sites, PB showed a cover (%) and macroalgal biomass (g/m^2) a bit higher than the other stations. No significant variations between the cover and biomass of macroalgal taxa were noticed across the sampling sites. Conversely, the angiosperm cover (%) was higher with comparison to macroalgae. The highest percent cover in the selected transects was measured at MSS (ca.80 %); whereas in the other stations (IC and PB) the cover was slightly lower (ca. 70 % and 65%, respectively).

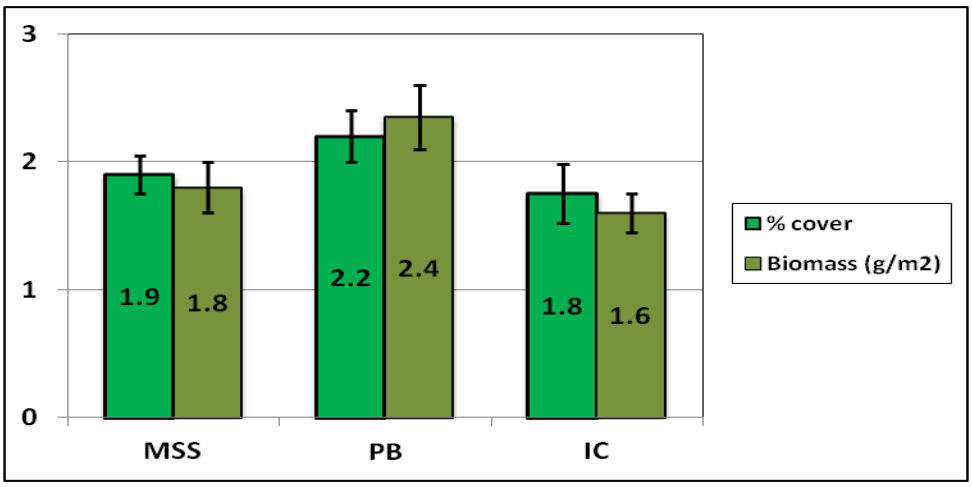

Fig. 30: Cover percentage and biomass of macroalgae for GA sampling sites.

The Venn diagram (**Fig. 31**) described the distribution of macroalgal species among the three sampling sites in the GA. Twelve macroalgal species were specifically found only at PB, while only 4 species were exclusively found at IC. Two species were present in all sites (i.e. *Sphacelaria tribuloides* Meneghini, *Ulva laetevirens* Areschoug). It's worthy to state that the old name of *Ulva lactuca* (which was used in the taxonomical studies in the Red Sea area) was not currently accepted in taxonomy. In fact, the morphological and interspecific characteristics of this species were similar to *Ulva laetevirens* Areschoug).

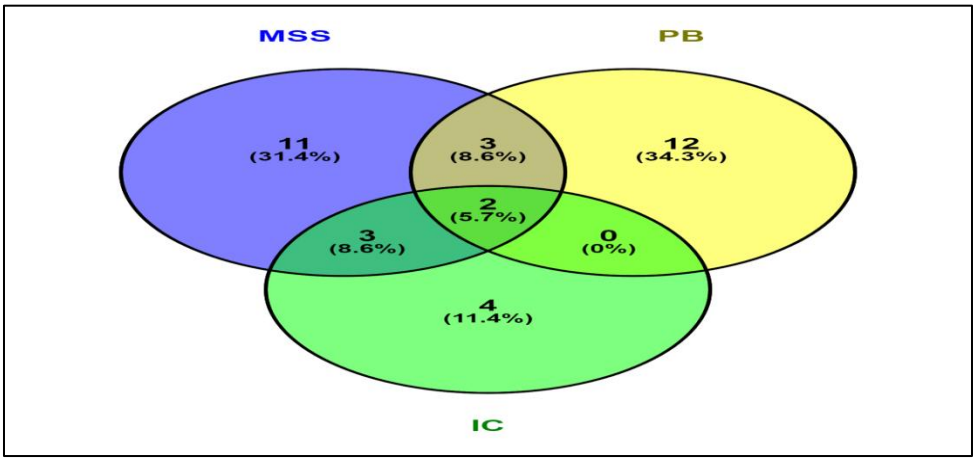

Fig. 31: Venn diagram of macroalgal species distribution in GA sampling sites.

The water column parameters and macrophyte variables in GA showed significant correlations between the total number of taxa and nutrients, pH and Chl-*a*. Instead angiosperm cover was significantly correlated with ammonium and salinity. No significant correlations were recorded between Chlorophyta and Rhodophyta and the environmental parameters, whereas temperature and pH were significantly correlated with Ochrophyta (r = 0.64, - 0.77, respectively) (**Table 9**).

Table 9: Spearman correlation between water parameters (GA) and macrophyte variables (in **Bold** correlations were significant at $p < 0.05$, $|r| \geq 0.64$)

Parameter	No. taxa	% Ochrophyta	% Rhodophyta	% Chlorophyta	Macroalgae Biomass	% Angiosperm
Nitrite	**0.66**	-0.49	0.43	-0.43	0.31	0.09
Nitrate	0.49	0.03	0.03	-0.03	-0.09	0.37
Ammonium	**0.77**	-0.03	-0.03	0.03	0.14	**0.66**
DIN	**0.77**	-0.31	0.37	-0.37	0.26	0.37
RP	-0.75	0.17	-0.06	0.06	0.03	-0.58
Silicate	0.09	-0.09	-0.31	0.31	-0.37	0.03
Chl-*a*	**0.93**	-0.58	0.58	-0.58	0.58	0.23
pH	**0.71**	**-0.77**	0.60	-0.60	0.54	-0.09
Temp.	**-0.90**	**0.64**	-0.41	0.41	-0.46	-0.23
Salinity	0.60	0.26	-0.20	0.20	-0.31	**0.83**
DO	-0.49	0.43	-0.49	0.49	-0.60	-0.14
FPM	-0.32	0.00	0.12	-0.12	0.06	-0.06

As shown in **Table 10**, the TOM and OC content in sediments were significantly correlated with the angiosperm cover, whereas no significant correlations were found with the macroalgal biomass. Fine sediments exhibited

significant negative correlation with the macroalgal biomass and cover. Moreover, no significant correlations were found between macroalgal taxa and nutrients in sediment (OC, TN, TP). Finally, the number of taxa showed opposite correlations with TP and TN in surface sediments (**Table 10**).

Table 10: Spearman correlation between sediment parameters (GA) and macrophyte variables (**Bold** correlations were significant at $p < 0.05$, $|r| \geq 0.89$)

Parameter	No. taxa	% Ochrophyta	% Rhodophyta	% Chlorophyta	Macroalgae Biomass	% Angiosperm
OC -sed	0.37	0.49	-0.43	0.43	-0.49	**0.94**
TP -sed	**-0.94**	0.54	-0.37	0.37	-0.43	-0.37
TN -sed	**0.94**	-0.43	0.49	-0.49	0.43	0.49
TOM -sed	0.43	0.54	-0.37	0.37	-0.43	**1.00**
Fines	-0.48	**0.96**	**-0.96**	**0.96**	**-0.96**	0.48

Part III: Metal analyses and Oxidative Stress Biomarker

3.7 Metal Analyses (Lagoon of Venice)

3.7.1 Metal concentration in surface sediments and SPM

Metal and metalloid (As) concentrations in the surface sediment and SPM are shown in **Table 11**, specifically for As, Cu, Hg, Mn, Ni, Pb, V, Zn. Data are expressed as µg/g (dw). For both sediment and SPM, the concentrations of many metals (Ni, Pb, V and Zn) were significantly higher (Tukey`s test, $p<0.05$) than those of the uncontaminated control site (SMM). Sediments from SG showed the highest concentrations of metals (Cu, Mn, Zn) and metalloid (As), whilst the

sediments at PM showed the highest concentrations of the other measured metals (V, Ni and Pb).

Most of the measured metals and metalloid from **sediment** showed concentration within the Low Contamination Class (LCC) limits of the "Venice Special Law". Zn exceeded the limits of the LLC and reached values above the medium class contamination (MCC). No metals exceeded the MCC levels entering in the limits established for High Class Contamination (HCC).

The concentrations of Cu, Ni and As from **SPM** exhibited significant variations at SG and PM, whereas the highest Pb, V and Zn concentrations were recorded at PM. The majority of metals showed higher concentrations in SPM in comparison to sediment. Moreover, As and Cu concentrations from SPM contaminated sites exceeded the LLC limits, whilst Pb and Zn exceeds the MCC limits.

Table (11): Metals and metalloids (As) concentrations (Mean ± SD) (µg/g dw) in surface sediments and SPM collected at SMM, PM, SG in the LV. LCC (Low Class Contamination) and MCC (Medium Class Contamination) for each metal, according to safe criteria to excavate, transport and reuse sediments from the Venice canals (Ministero per l'Ambiente, 1993) are indicated in brackets. "L" represents values above LCC and "M" values above MCC. Statistically significant differences from SMM area (for both sediment and SPM) are represented by "a". (n.a): not available. d.l: detection limit, MPI: metal pollution index.

Site	Metals and metalloid (µg/g)								
Sediment	**As (15,25)**	**Cu (40,50)**	**Hg (0.5,2)**	**Mn (n.a)**	**Ni (45,50)**	**Pb (45,100)**	**V (n.a)**	**Zn (200,400)**	**MPI**
SMM	1.00 ± 0.14	6.40 ±0.85	< d.l	259 ±1.77	8.65 ±1.63	4.75 ±0.07	13.9 ±0.21	17.3 ±1.13	**4.50**
PM	4.89 ± 0.21	58.4 ±1.06^{L,a}	1.65 ±0.05^{L}	243 ±1.34	22.0 ±0.85^{a}	68.1 ±1.84^{L,a}	32.5 ±1.27^{a}	327 ±3.39^{M}	**31.1**
SG	16.17 ±0.89^{a}	45.4 ±2.76^{L,a}	0.86 ±0.02^{L}	469 ±29.0^{a}	15.8 ±1.27	56.1 ±0.99^{L,a}	22.8 ±0.64	429 ±12.5^{M,a}	**32.9**
SPM									
SMM	13.6 ± 3.39	9.2 ±0.22	0.29 ± 0.03	366 ±0.71	14.6 ±0.85	13.4 ±0.78	19.1 ±0.47	46.8 ±0.07	**14.8**
PM	16.9 ±0.49^{L}	49.1 ±0.85^{L}	1.09 ±0.04^{L}	486 ±3.54	22.2 ±0.35^{a}	72.9 ±0.21^{M,a}	53.6 ±1.41^{a}	659 ±2.12^{M,a}	**47.3**
SG	17.9 ±3.89^{L}	49.2 ±0.01^{L}	0.88 ±0.05^{L}	606 ±2.83	23.6 ±0.21^{a}	45.5 ±0.78^{M}	39.5 ±0.85^{a}	325 ±2.83^{L,a}	**39.9**

Pearson correlations among metals from sediment and SPM are shown in **Table 12**. Linear positive correlations were showed for many metals except Mn from sediment and As from SPM. Furthermore, no significant correlations were found between As in SPM and the metals in sediment **(Table 12)**.

Table (12): Pearson correlation calculated on the concentrations of metals and metalloid from sediment and SPM. (In Bold correlations are significant at $p < 0.05$, $|r| \geq 0.86$)

	As -SPM	**Cu -SPM**	**Hg -SPM**	**Mn -SPM**	**Ni -SPM**	**Pb -SPM**	**V -SPM**	**Zn -SPM**
As-sed	0.63	0.79	0.60	**0.99**	**0.86**	0.42	0.47	0.33
Cu-sed	0.69	**0.90**	0.75	**0.99**	**0.95**	0.60	0.64	0.52
Hg-sed	0.49	**0.88**	**0.97**	0.52	0.80	**1.00**	**1.00**	**1.00**
Mn-sed	0.49	0.39	0.13	0.79	0.51	-0.07	-0.02	-0.16
Ni-sed	0.38	**0.87**	**0.96**	0.52	0.78	**0.99**	**0.98**	**0.98**
Pb -sed	0.59	**0.98**	**0.99**	0.76	**0.94**	**0.95**	**0.97**	**0.92**
V-sed	0.49	**0.85**	**0.96**	0.48	0.76	**1.00**	**0.98**	**1.00**
Zn-sed	0.67	**0.97**	**0.87**	**0.96**	**0.99**	0.75	0.79	0.69

(sed: sediment, SPM: settled particulate matter)

The Metal Pollution Index (MPI) was used to compare total metals and metalloids from sediment and SPM. The highest values of MPI are measured in contaminated sites (from both sediment and SPM). SMM exhibited the lowest index value for sediment and SPM (4.5, 14.8, respectively). The higher concentration of metals in SPM in comparison with sediment resulted in the increase of the MPI values for PM and SG sites (47.3, 39.9, respectively).

3.7.2 Metals concentration in macroalgae (*Ulva laetevirens*)

Data on macroalgal (*U. laetevirens*) metal contamination are reported in Appendix (C). The concentrations of metals/metalloids recorded during the four sampling seasons in the sites of the lagoon of Venice are shown in the **Figures 32-39**. The highest bioaccumulation values for *U. laetevirens* were observed at SG as calculated by the MPI for the overall determined metals (data included in Appendix D).

Table 13: Pearson correlations between metals and metalloids in sediment and *U. laetevirens* in LV. (Bold values are significant at $p < 0.05$, $|r| \geq 0.82$)

	As - *Ulva*	**Cu - *Ulva***	**Hg- *Ulva***	**Mn - *Ulva***	**Ni - *Ulva***	**Pb - *Ulva***	**V- *Ulva***	**Zn- *Ulva***
As-sed	0.25	0.80	0.75	**0.91**	-0.33	**0.96**	**-0.84**	**0.99**
Cu-sed	0.44	**0.90**	**0.87**	0.80	-0.50	**0.89**	**-0.93**	**0.99**
Hg-sed	**0.99**	**0.84**	**0.90**	-0.02	**-0.95**	0.16	**-0.83**	0.47
Mn-sed	-0.25	0.43	0.34	**0.99**	0.15	**0.95**	-0.47	**0.82**
Ni-sed	**0.97**	**0.83**	**0.89**	-0.01	**-0.99**	0.17	**-0.83**	0.47
Pb-sed	**0.89**	**0.95**	**0.99**	0.29	**-0.89**	0.46	**-0.96**	0.72
V-sed	**0.99**	**0.83**	**0.88**	-0.06	**-0.95**	0.11	-0.80	0.42
Zn-sed	0.62	**0.95**	**0.95**	0.66	-0.67	0.78	**-0.99**	**0.94**

(sed: sediment)

Arsenic was highly accumulated in *U. laetevirens* tissues at PM and in a minor extent at SG, whereas the accumulation recorded at SMM in the whole seasons was low (**Fig. 32**). The highest As concentration was measured in spring at PM (14.6 ± 0.25 µg/g) whereas it decreased in the other seasons. Significant correlations were obtained between As in *U. laetevirens* and Hg, V and Ni (r = 0.99, 0.99 and 0.97, respectively) in surface sediments (**Table 13**).

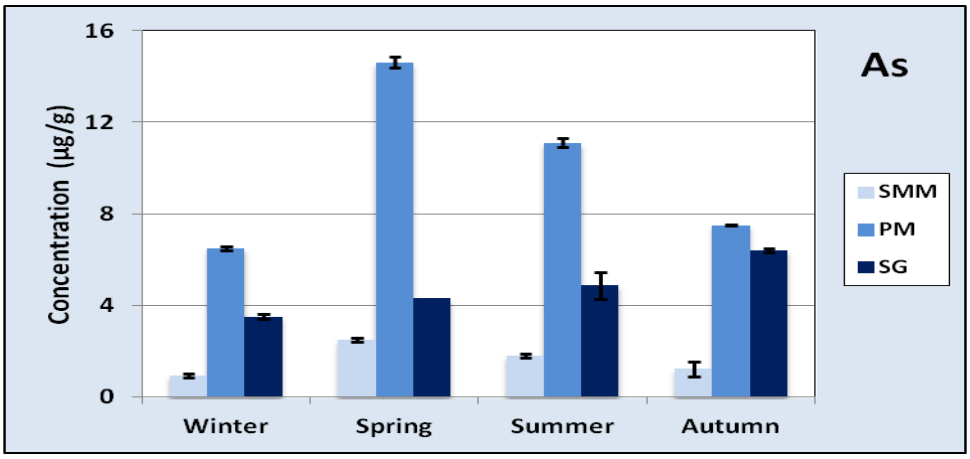

Fig. 32: As concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

Cupper was highly accumulated during spring and summer at PM and SG (**Fig. 33**). The highest accumulation was measured at PM in summer (73.7 ± 0.85 µg/g). The macroalgae tissues at SMM showed similar rate of accumulation during all seasons except spring. Cu accumulation in *U. laetevirens* was correlated with Cu in surface sediment (r = 0.90), furthermore, positive correlations were observed between Cu in *U. laetevirens* and Hg, Ni, Pb, V and Zn in surface sediments (**Table 13**).

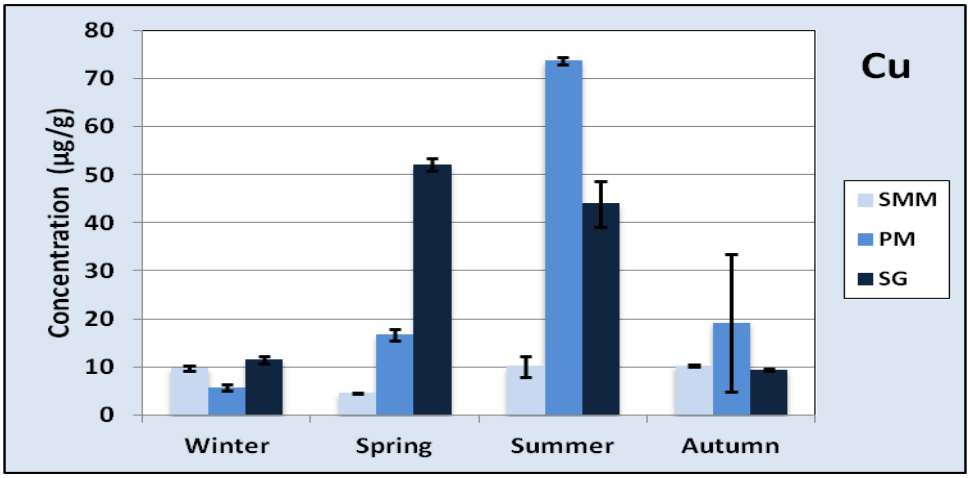

Fig. 33: Cu concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

The accumulation of **Hg** in *U. laetevirens* was similar during seasons (**Fig. 34**). The tissues of *U. laetevirens* accumulated higher amounts of Hg at SG during all the seasons, except in autumn; the highest accumulation was recorded in spring (0.34 ± 0.01 µg/g). Low accumulation of Hg was recorded at SMM during all the seasons (0.08 – 0.15 µg/g). Significant correlations were found between Hg in *U. laetevirens* and sediment (r = 0.90), and between Hg in *U. laetevirens* and Cu, Ni, Pb, V and Zn in sediment (**Table 13**).

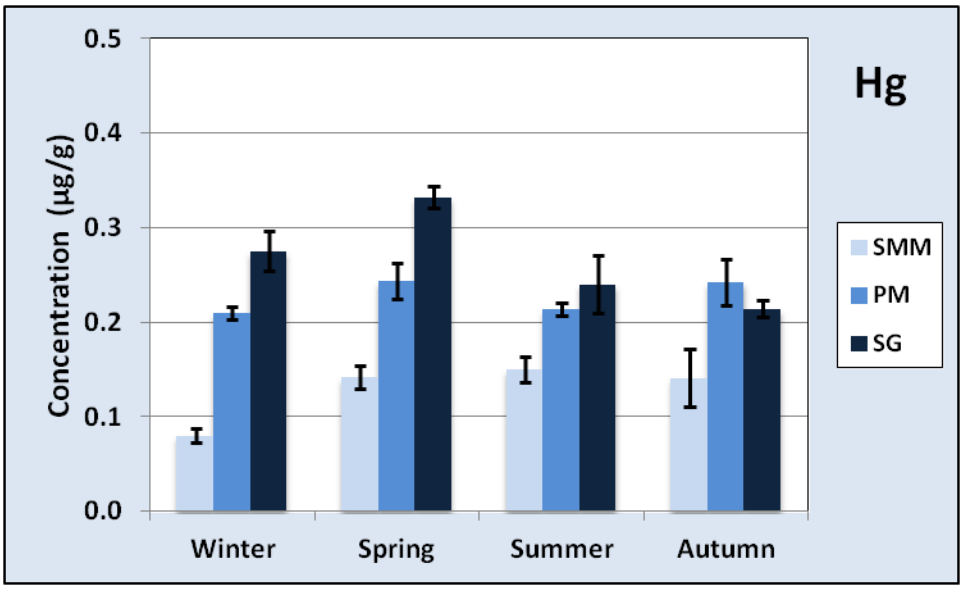

Fig. 34: Hg concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

The highest accumulation of **Mn** was found at SG during spring (**Fig. 35**). The sites SMM and PM exhibited quite similar accumulation during spring and summer. In all the seasons, except autumn, SG exhibited the highest accumulation of Mn (52.1- 400.4 µg/g). The concentration of Mn in *U. laetevirens* was strongly correlated with Mn (r = 0.99) and As (r = 0.91) in surface sediments (**Table 13**).

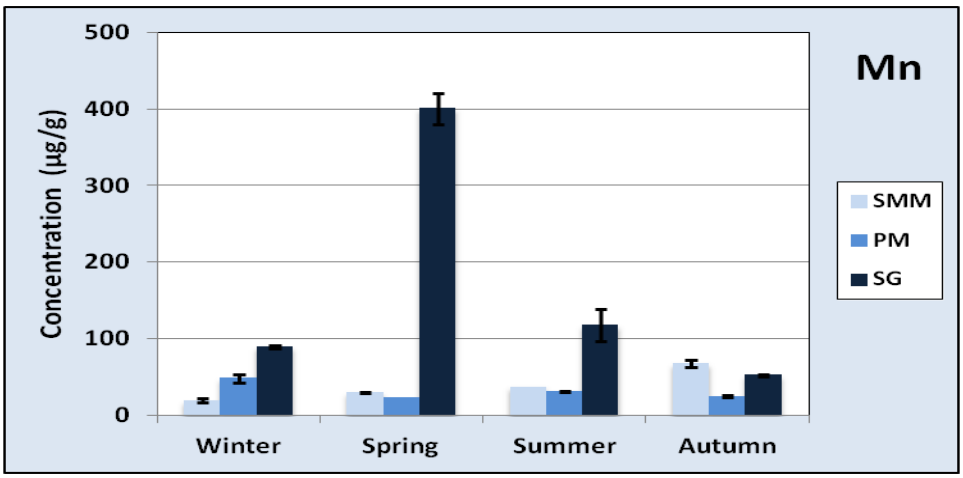

Fig. 35: Mn concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

Low concentrations of **Ni** were generally recorded in *U. laetevirens* during all seasons (**Fig. 36**). The highest accumulation of Ni was observed in autumn at SMM (3.5 ± 0.2 µg/g). Ni was accumulated in *U. laetevirens* at SMM (1.2 - 3.5 µg/g); whilst the lowest measured value was at SG (average of 1.94 ± 0.3 µg/g during seasons). Ni concentrations in *U. laetevirens* were negatively correlated with Hg (r = 0.95), V (r = 0.95), Pb (r = 0.89) as well as Ni (r = 0.99) in surface sediment (**Table 13)**.

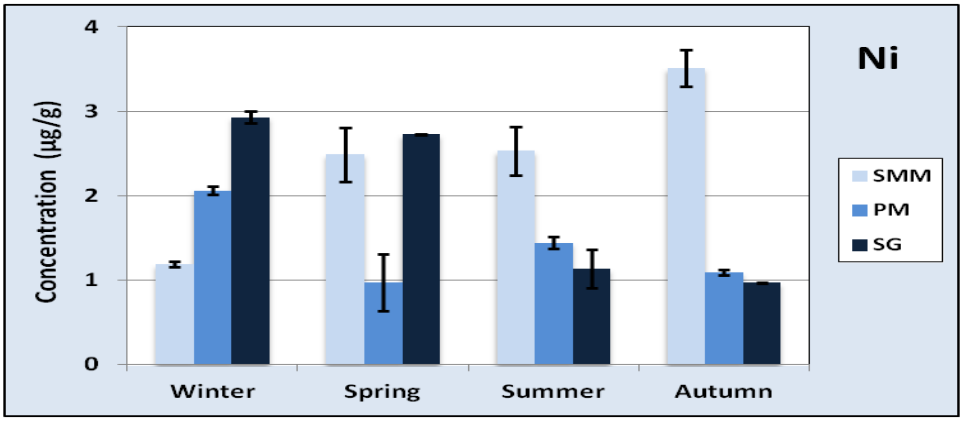

Fig. 36: Ni concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

Low levels of **Pb** accumulation in *U. laetevirens* were observed comparing to the level of Pb in sediment. The highest concentrations were measured at SG in all seasons, with the highest accumulation in spring (4.23 ±1.06 µg/g) (**Fig. 37**). Extremely low concentrations of Pb were measured at SMM in spring and summer (0.06, 0.04 µg/g, respectively). Positive correlations were obtained with As (r = 0.96), Mn (r = 0.95) and Cu (r = 0.89) in surface sediments (**Table 13**).

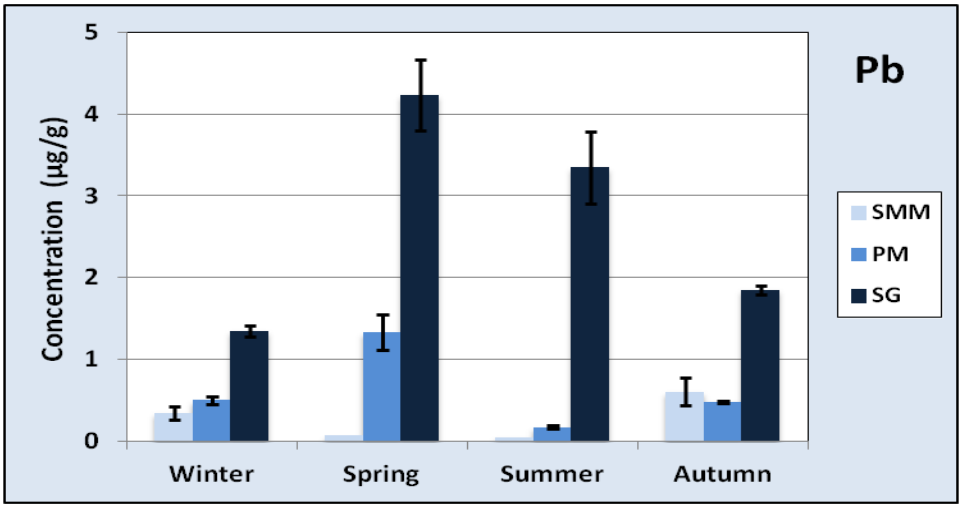

Fig. 37: Pb concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

Vanadium in *U. laetevirens*, contrarily to the other metals was highly accumulated at SMM (**Fig. 38**). The highest accumulation was found in autumn (23.4 ± 0.70 µg/g). The accumulation of V at PM and SG in autumn was very low, likewise, in the other seasons the concentrations of V were always lower than 0.28 µg/g at these sites. As, Cu, Hg, Ni and Zn in surface sediment were negatively correlated with V in *U. laetevirens* (**Table 13**).

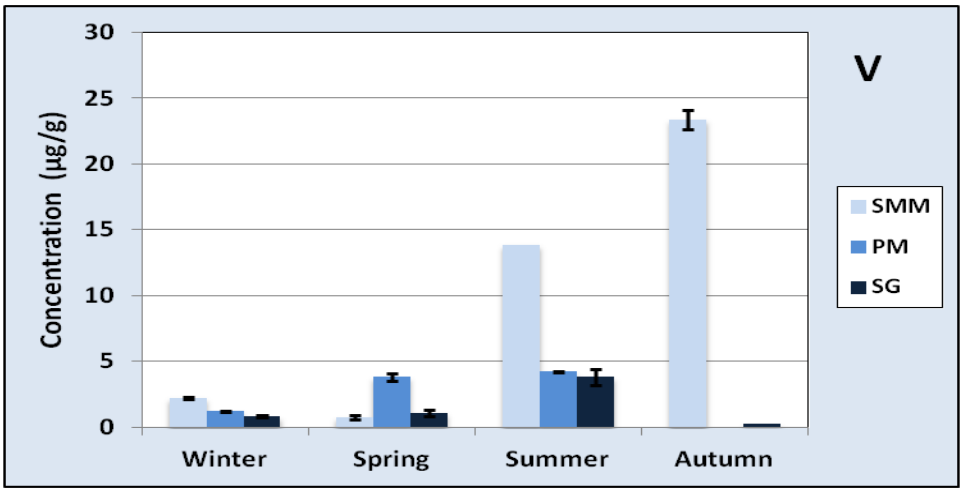

Fig. 38: V concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (n=3)

Zinc was highly accumulated at SG during all seasons (**Fig. 39**). The uncontaminated site (SMM) showed the lowest level of accumulation among all seasons. The highest concentration was measured during spring at SG (93.2 ± 0.03 µg/g). The average accumulation level during the whole seasons in *U. laetevirens* was higher at both PM and SG (33.3, 56.2 µg/g, respectively, appendix D). Zn in *U. laetevirens* was found highly correlated with both As and Cu (r = 0.99), Zn (r = 0.94) and Mn (r = 0.82) in surface sediments (**Table 13**).

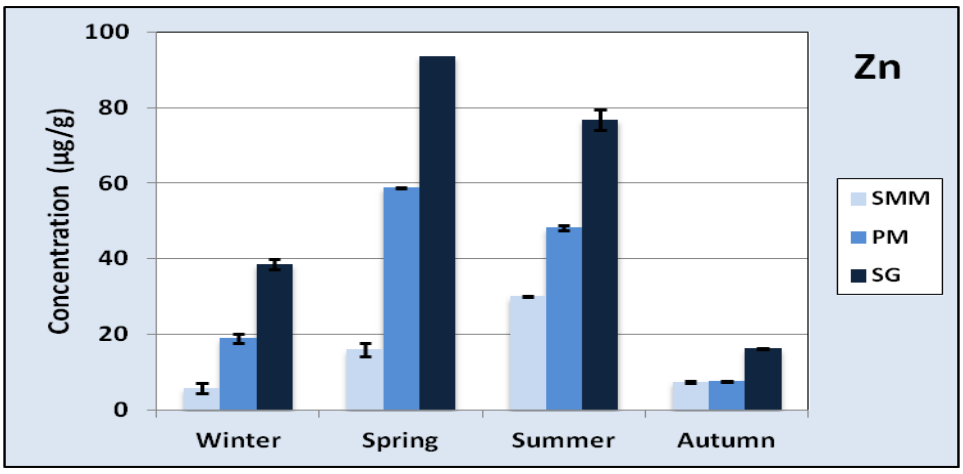

Fig. 39: Zn concentration (µg/g, dw) in *U. laetevirens* collected from LV. Data are Mean ± SD (*n=3*)

3.7.3 Oxidative Stress Biomarker (LPO)

The level of LPO (expressed as MDA content) in tissues of *U. laetevirens* homogenate is mostly used as biomarker for oxidative stress cytotoxicity. The maximum levels of LPO were recorded at PM and SG in spring (32.0 and 55.3 µM/g, respectively). Moreover, the level of LPO at PM showed low seasonal fluctuations (25.7 ± 5.1 µM/g). The level of LPO (7.88 ± 4.30 µM/g) was significantly lower (one-way ANOVA, $p < 0.05$) in the uncontaminated site (SMM) compared with the levels obtained from the other stressed sites during all sampling seasons (**Fig. 40**). The levels of LPO at PM and SG during winter and spring were significantly higher (Kruskal-Wallis one-way ANOVA, $p < 0.05$) than the uncontaminated site (SMM). Furthermore, the Tukey`s Pairwise test indicated high significant differences between SG and SMM ($p < 0.01$).

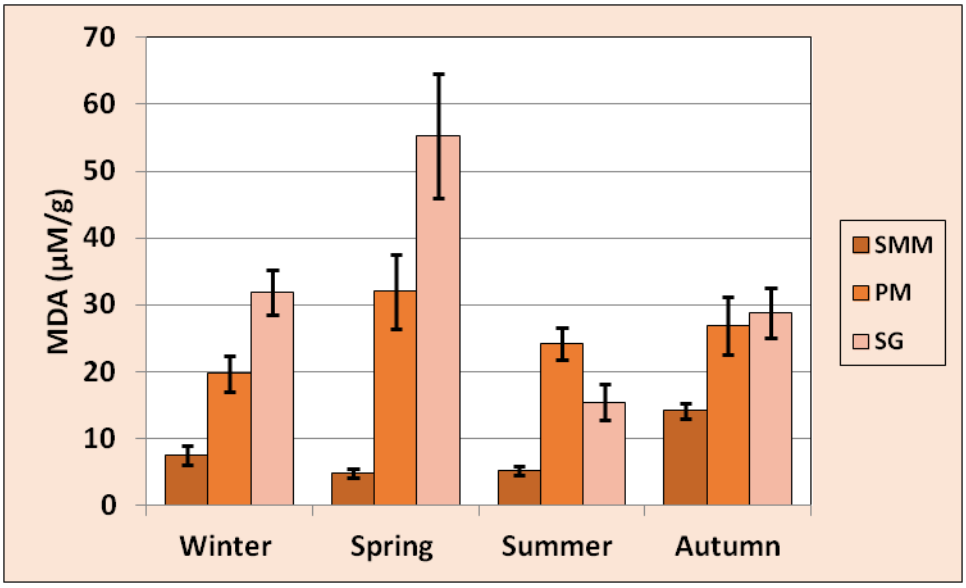

Fig. 40: Level of LPO (as MDA concentration) in *U. laetevirens* from LV. Data are Mean ± SD (*n=8*)

The LPO levels in *U. laetevirens* varied proportionally with the level of metals and metalloid in the macroalgal tissues of the corresponding sites indicating a close relationship between LPO and metals. The overall concentration of metals in *U. laetevirens* (MPI) was found to be correlated with LPO levels (R^2= 0.625, $p < 0.005$). Furthermore, As ($p < 0.005$) and Mn ($p < 0.05$) showed the highest significant correlations with LPO. Similarly, LPO was correlated with the metal content in surface sediments and SPM (Kruskal-Wallis one-way analysis of variance on equal median, (PM, $p < 0.01$; SG, $p < 0.05$). This indicates the close relationship between LPO levels and metals in *U. laetevirens*. The data of statistical analyses are included in appendix (E).

3.8 Metals Analyses (Gulf of Aqaba)

3.8.1 Metals concentration in surface sediments

The concentrations of metals from surface sediments in GA are listed in **Table 14**. Metal (Cd, Cr, Ni, Mn and Zn) concentration in sediments from the contaminated sites (IC and PB) was considerably higher (Tukey`s test, $p < 0.05$) than those from the reference site (MSS). Conversely, Pb concentration was found higher at MSS (3.47 ± 1.0 µg/g). No significant variation of Cu concentration among sites was recorded. The concentration of Fe at IC was significantly higher than in the other sites (80,640 ± 42.0 µg/g).

The application of the Metal Pollution Index (MPI) to compare the degree of metal pollution in surface sediments at MSS, PB and IC returned values of 8.8, 11.2, and 13.2, respectively. These MPI values reflected the contamination status in the three sites.

Table 14: Metals concentrations (µg/g dw) in surface sediments collected from GA. Data are Mean ± SD (*n=3*). Statistically significant differences among areas are represented by "a"

Sites	Cd	Cr	Cu	Ni	Mn	Pb	Zn	Fe
				µg/g				
MSS	0.91 ± 0.10	23.7 ±1.50	2.10 ±0.80	25.4 ±8.00	147 ±10.0	3.47 ±1.00	22.3 ±11.6	27,947 ± 38.0
PB	1.72 ± 0.70[a]	67.6 ±2.10[a]	3.50 ±0.40	70.9[a] ±3.00	181 ±4.00	1.47 ±0.90	87.7[a] ±14.9	6,890 ± 20.0
IC	1.90 ± 0.21[a]	71.7 ±1.70[a]	3.80 ±0.30	77.4 ±7.30[a]	198[a] ±8.50	1.80 ±0.80	93.6 ±18.4[a]	80,640[a] ± 42.0

3.8.2 Metals concentration in *Ulva laetevirens*

Total metal (Cd, Cr, Cu, Mn, Ni, Pb, Zn and Fe) concentrations were measured in *U. laetevirens* collected from the three sites in the GA (**Fig. 41**). The detectable levels of metals in the decreasing order were: Cd< Cu<Pb< Cr<Mn< Ni< Zn< Fe. No significant differences were obtained between the reference site (MSS) and the other sites (PB and IC) (Mann- Whitney pairwise test, $p > 0.05$). The accumulation of Mn, Ni, Zn and Fe was slightly higher in *U. leatevirens* tissues from IC contaminated site. The data of statistical analyses are included in appendix (F).

The relative abundance of metals in *U. laetevirens* reflected the seaweed uptake according to the level of metals measured in surface sediments. The concentrations of metals in surface sediments reflected the degree of contamination in the water column. Therefore, it is not surprising that there was a significant correlation between metals in sediment and metals in macroalgae. Statistical correlations ($p < 0.05$) demonstrated a direct dependence of metals (Mn, Ni, Pb, Zn

and Fe) in the two matrices (**Table 15**) except for Pb which showed negative correlations.

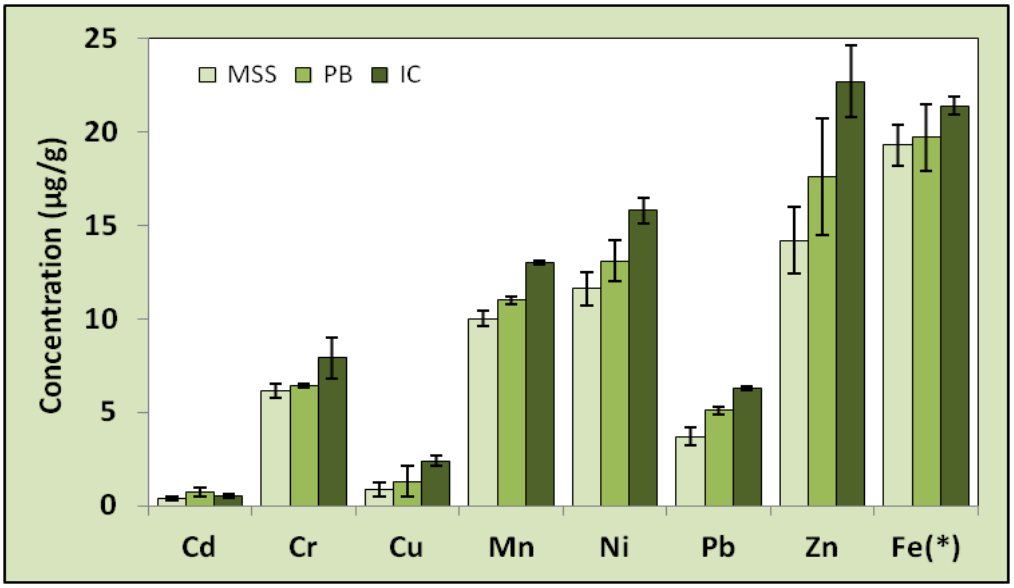

Fig. 41: Concentration of metals (µg/g dw) in the *U. laetevirens* tissues from GA sites. (Data are Mean ± SD (*n=3*) * Fe column represent 0.1 Fe concentrations.

Statistical significant correlation were recorded between the concentration of Mn, Ni in *U. laetevirens* and surface sediments (r = 0.86, 0.79, respectively), whereas no significant correlation were found for the other metal pairs. Furthermore, Statistical significant correlations were also recorded between Pb, Ni, Cu and Mn in *U. laetevirens* and the metals in surface sediments. For instance Mn, Ni and Pb in *U. laetevirens* were significantly correlated with the metals (Cd, Cr, Cu, Mn, Ni, Zn and Fe) from sediment with different degrees. Conversely, the concentration of Pb in sediment wasn't significantly correlated with the metals accumulated in the tissues of *U. laetevirens.*

Table 15: Pearson coefficient of correlation between metals concentration in *U. laetevirens* and sediment (In **bold** correlations are significant at $p < 0.05$, $|r| \geq 0.66$)

	Cd-*Ulva*	**Cr-*Ulva***	**Cu-*Ulva***	**Mn-*Ulva***	**Ni-*Ulva***	**Pb-*Ulva***	**Zn-*Ulva***	**Fe-*Ulva***
Cd-sed	0.05	0.44	**0.69**	**0.7**	**0.73**	**0.76**	0.47	**0.71**
Cr-sed	0.36	0.53	0.65	**0.79**	**0.77**	**0.89**	0.53	0.58
Cu-sed	0.13	0.48	0.52	**0.67**	**0.83**	**0.77**	0.51	**0.74**
Mn-sed	0.21	0.58	**0.71**	**0.86**	**0.88**	**0.91**	0.51	**0.75**
Ni-sed	0.32	0.49	0.63	**0.78**	**0.79**	**0.87**	0.48	**0.66**
Pb-sed	-0.55	-0.47	-0.39	-0.54	-0.37	-0.65	-0.47	-0.02
Zn-sed	0.22	0.55	0.59	**0.72**	**0.79**	**0.83**	0.6	0.62
Fe-sed	-0.24	**0.74**	**0.7**	**0.81**	**0.74**	**0.65**	0.5	0.58

(sed: sediment)

3.8.3 Oxidative Stress Biomarker (LPO)

The level of lipid peroxidation (measured as MDA level) in the tissues of *U. laetevirens* collected from the three sites of GA are showed in **Fig. 42**. The highest LPO level was measured in the stressed site (IC) with 49.2 ± 2.3 µM/g MDA. Statistical significant differences between the reference and the contaminated sites were obtained (Kruskal – Wallis test, $p < 0.05$). The data of statistical analyses are included in appendix (F).

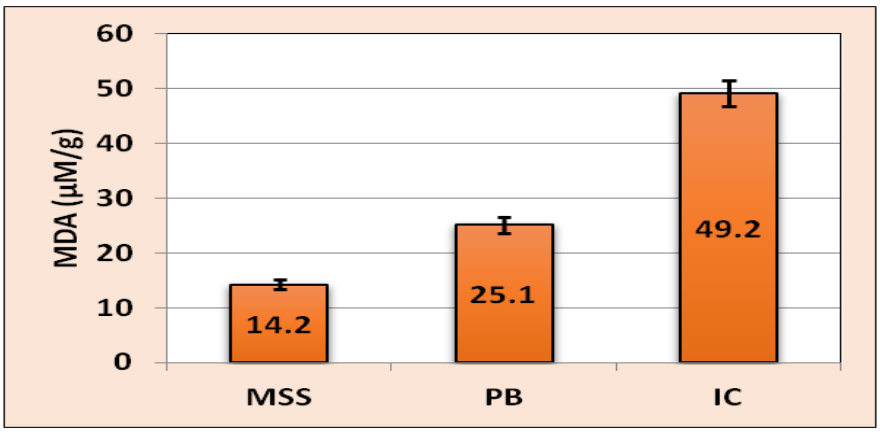

Fig. 42: Level of LPO (as MDA concentration) in *U. laetevirens* from GA sites. Data are Mean ± SD (*n=8*)

Significant correlations were obtained between the metals in both *U. laetevirens* and sediment and LPO level. Cr, Cu, Mn, Ni and Fe exhibited significant correlation with the LPO, the concentration of metals in *U. laetevirens* tissues and the sediment collected from the corresponding sites. Conversely, Cd in both algae and sediment was found not significantly correlated with the level of LPO. The data of statistical analyses are included in appendix (F).

3.9 Multivariate analysis (PCA)

Results from multivariate analyses are shown in **Fig. 43**. PCA analysis describes the variance of macrophyte variables, metals accumulation and environmental parameters in both LV and GA sampling sites. The first two components explained the 92.4% of the total variance; PC1 accounted for the 78.5 % of the total variance. Sites were sorted into approximately two parts; LV sites (SMM, PM, SG) in the right (with slight deflection of SMM to left) and GA sites (MSS, PB, IC) to the left. Aquatic angiosperms and Rhodophyta were clearly associated with the site of high ecological conditions (SMM at LV and MSS at GA). A second group that represent to the contaminated sites in LV (PM and SG)

was characterized by the prevalence of Chlorophyta, high nutrient concentration in water (DIN, RP and SIO_4), high Chl-*a*, particulate matter (FPM) and amount of fine sediment. In addition, this group was characterized by the accumulation of some metals (As, Cu, Mn and Zn) in *U. laetevirens* tissues. A third group was represented by the contaminated sites of GA (PB and IC) which were characterized by high salinity and water temperature, high concentrations of total phosphorus and the accumulation of other metals in *U. laetevirens* tissues (Pb, Ni). These last two groups (contaminated sites in both LV and GA) were also associated with the oxidative stress index (LPO) confirming our results on the presence of several potentially toxic elements that affect the health status in these contaminated sites.

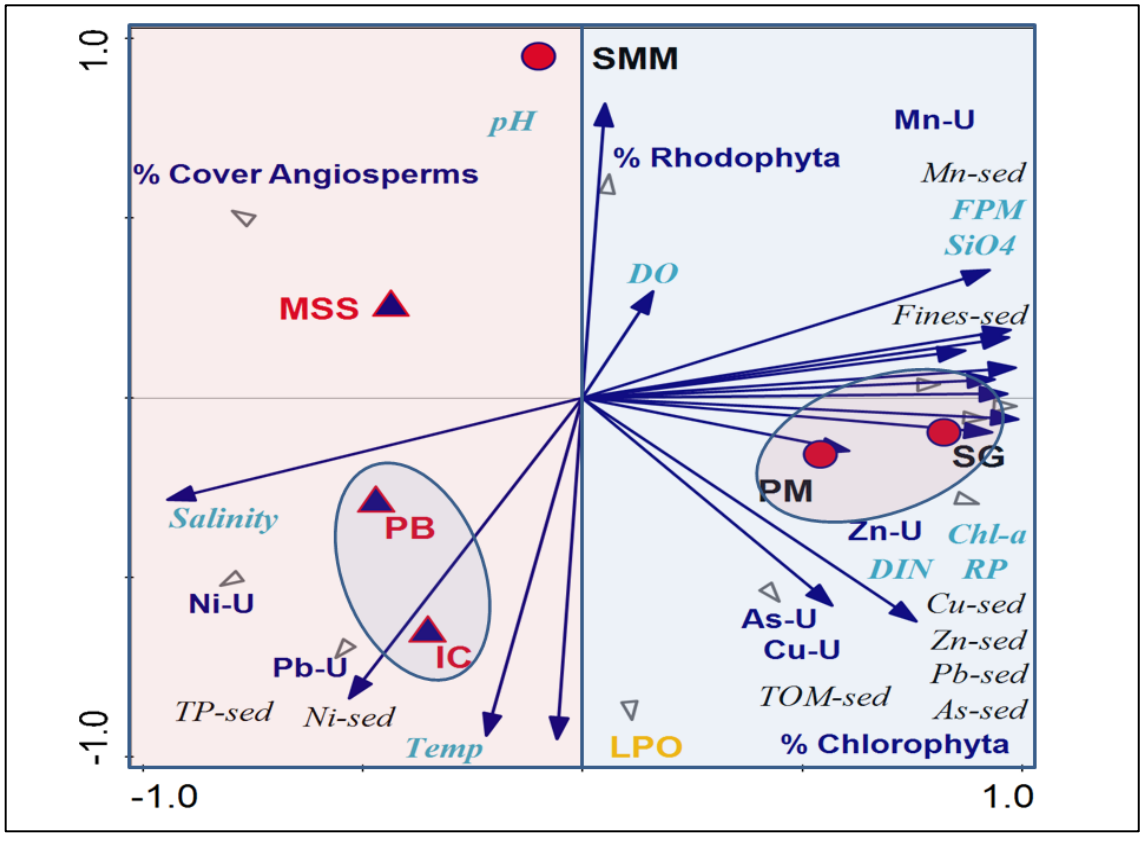

Fig. 43: Multivariate PCA analysis of macrophyte variables in response to environmental parameters and metals contamination for both LV and GA sites. Sampling sites are in **bold** (SMM, MSS, PM, SG, IC, PB); *Italics* represents water and sediment environmental parameters and metals in sediment (sed: sediment); metal **U** represent metals in *U. laetevirens* tissues.

Part IV: Microbial community

The microbial communities associated with *U. laetevirens* in both LV and GA aquatic environment were investigated using the NSG technique in order to explore the diversity of microbial community in response of environmental and anthropogenic stressors in aquatic ecosystems.

3.10 Overview on sequencing output

The raw output of the Illumina® NextSeq resulted in a total of 4.7 M reads, comprising all samples sites during the corresponding seasons. The processing of sequences with DADA2 pipeline resulted in a total of 2,253,643 reads, assigned to a total of 6935 ASVs (singletons) after removing the chloroplast and mitochondrial reads which represented ca.19.4 and 8.7% respectively, of the total ASVs reads (**Fig. 44**). The average number of usable reads for samples was 102, 43 reads, where the number of reads ranged between 97,846 – 105,679 reads. The ASVs for each sample were normalized for the selected threshold in order to standardize the differences in the sampling efforts among the samples; the subset ASVs was trimmed to 3960. The total numbers of ASVs and α-diversity indices for all samples are shown in appendix (G).

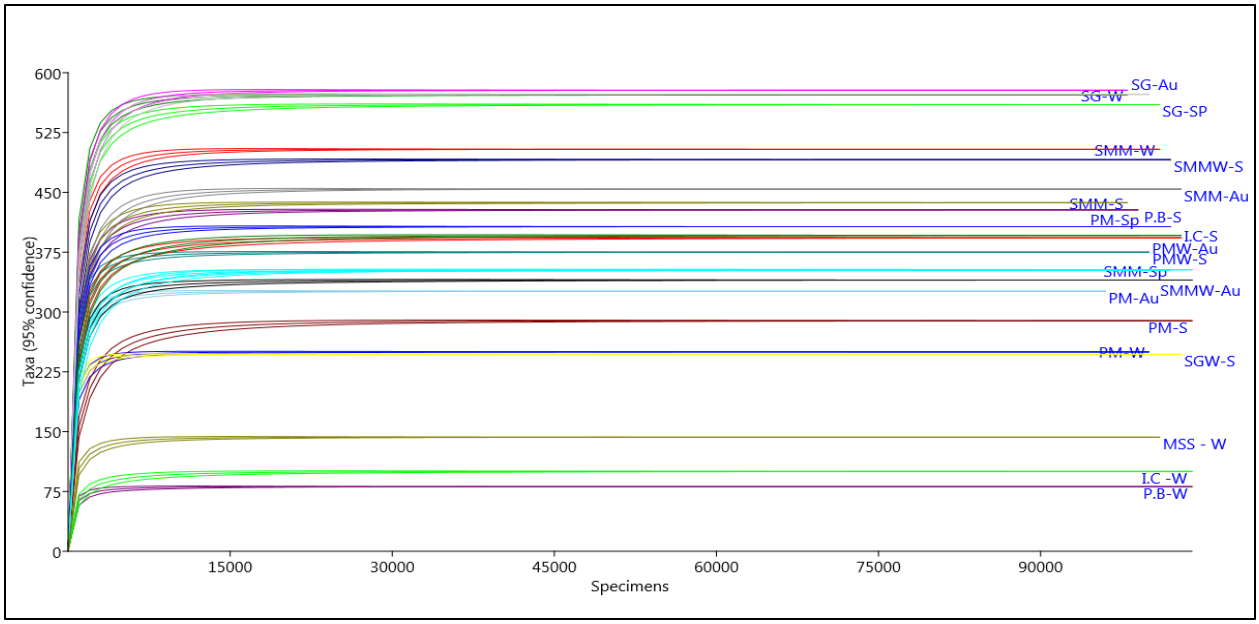

Fig. 44: Rarefaction curves of the microbial communities associated with the *U. laetevirens* and with seawater in the sampling sites: (SMM, PM & SG) (LV) and (MSS, PB & IC) (GA).Specimens represent the No. of sequences, Taxa represent No. of ASVs. (**W**: Winter, **Sp**: Spring, **S**: Summer, **Au**; Autumn)

3.11 Microbial community (Lagoon of Venice)

3.11.1 Distribution of microbial community

The distribution of bacterial phyla in LV sampling sites during the four seasons is illustrated in **Fig. 45**. The classified ASVs belonged to 15 phyla among all sites. The dominant phyla included Proteobacteria and Bacteroidetes which averagely accounted for 51 taxa, ca. 88% of the total sequences among all sites. The relative abundance of each phylum changed among sites and seasons. The 7 less abundant phyla accounted for ca. 0.8 % of the total sequences. They included: Acidobacteria, Fusobacteria, Deinococcus-Thermus, Chloroflexi, Thaumarchaeota, Tenericutes, Kiritimatiellaeota. Some phyla were highly abundant during specific seasons; Epsilonbacteraeota for instance, was most abundant in winter and autumn in all sites. The relative phylum abundance also varied in the same site during different seasons; this was clearly evident by comparing the proportion of each

phylum in the same site during the sampling seasons. For instance, the abundance of Cyanobacteria increased during summer in all the investigated sites (1.3, 8.0 & 2.6%) of the total sequences at SMM, PM& SG, respectively. Moreover, the phylum Planctomycetes increased significantly during spring at SG (11.8% of the total sequences) and Verrucomicrobia at SMM during the same season (18% of the total sequences).

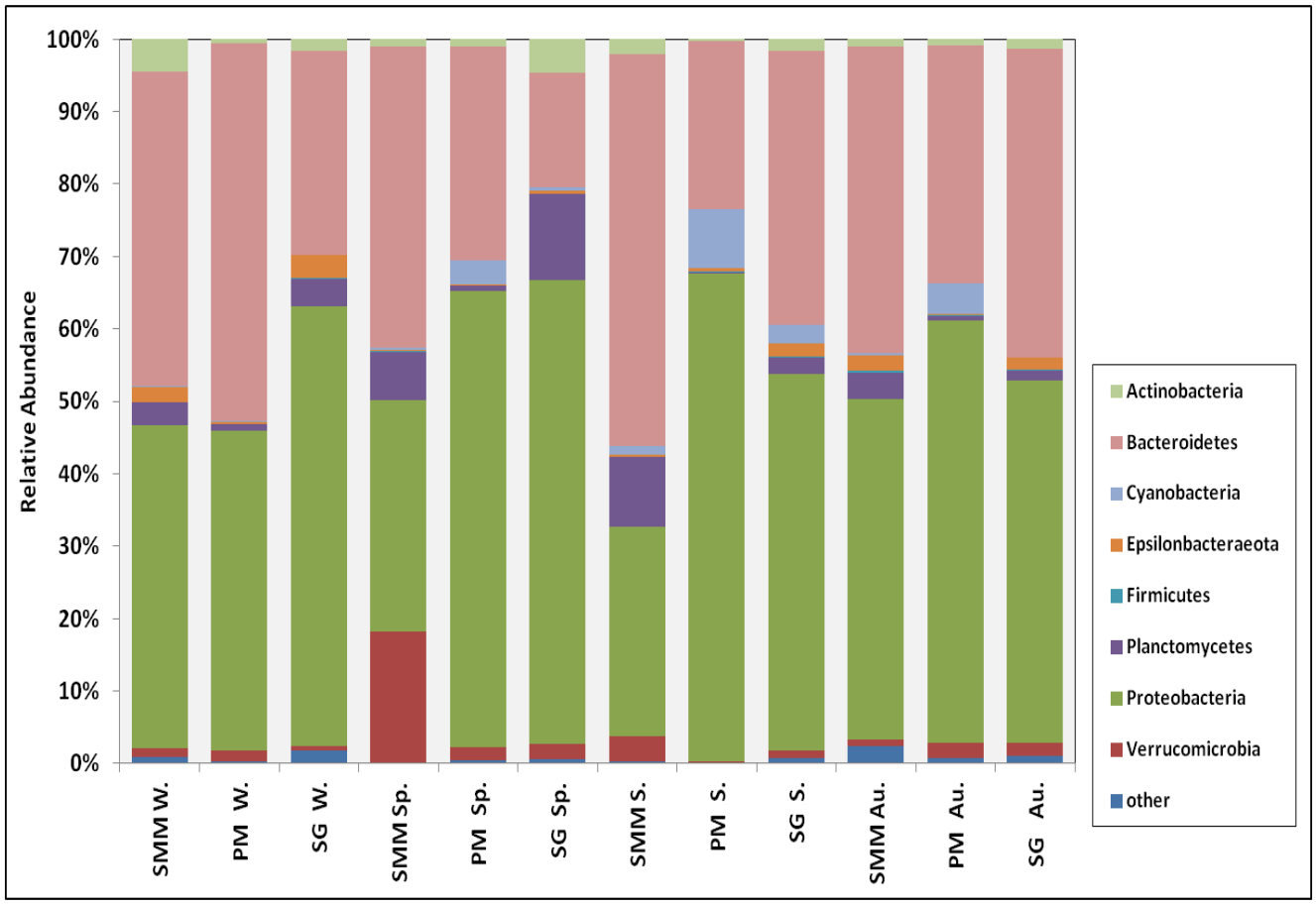

Fig. 45: Distribution of *U. laetevirens* associated microbial community at phylum level for LV sampling sites. (**W**: Winter, **Sp**: Spring, **S**: Summer, **Au**; Autumn)

Considerable differences between sites during seasons were also evident at finer levels of phylogenic resolution. At the class level, differences in the relative abundance were obtained (**Fig. 46**) among all sites. Three classes dominated the

algae surface: Alphaproteobacteria, Bacteroidia, Gammaproteobacteria which accounted for about 29, 21 and 37% of the total sequences, respectively. The remainder 21 classes accounted for only ca. 11% of the total sequences considering all samples. The distribution of classes among the same sites also clearly changed during different sampling periods. The SIMPER test analysis (similarity percentage test) showed low dissimilarity (22.4%) between SMM and PM with six bacterial classes: Gammaproteobacteria, Planctomycetacia, Alphaproteobacteria, Bacteroidia, Oxyphotobacteria and Verrucomicrobiae contributing to > 81% of the differences. Lower dissimilarity (16.8%) was showed between SMM and SG where these classes contributed to > 79.0% of the differences.

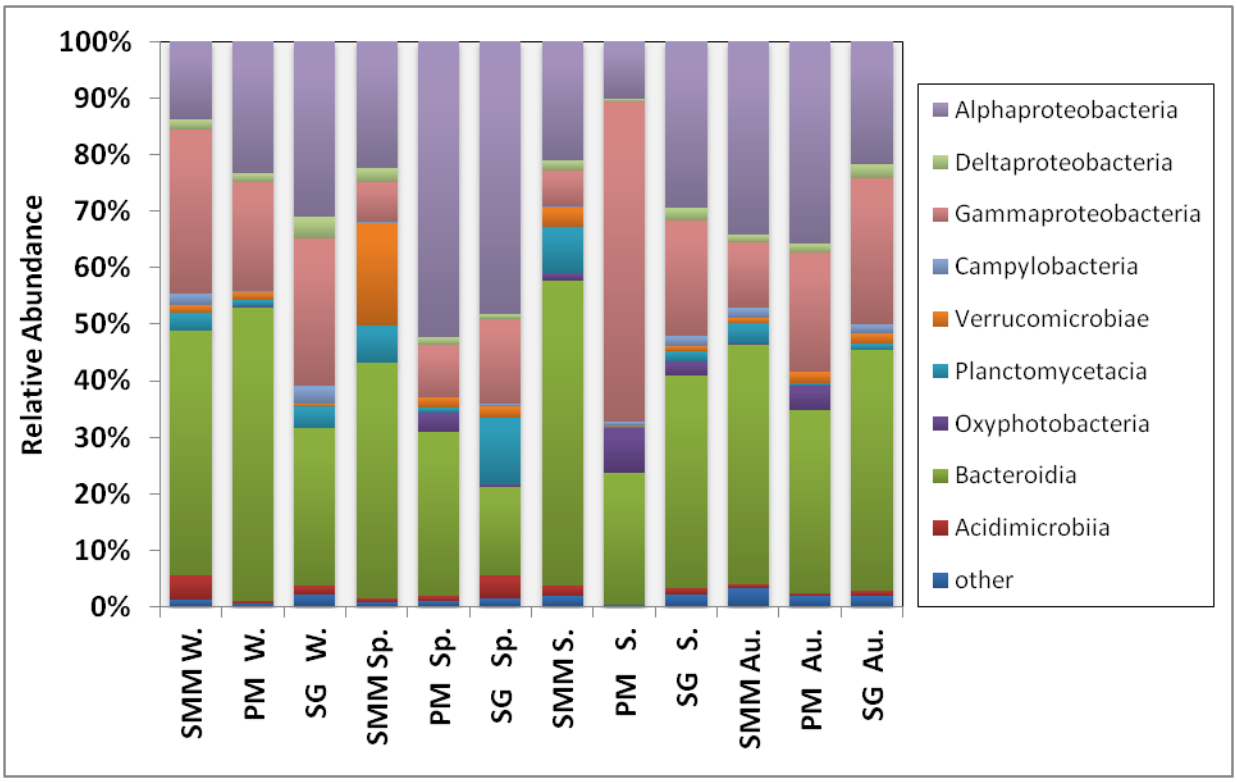

Fig. 46: Distribution of *U. laetevirens* associated microbial community at class level for LV sampling sites. (**W**: Winter, **Sp**: Spring, **S**: Summer, **Au**; Autumn)

3.11.2 Distribution of shared and non-ubiquitous ASVs

The variation in the shared and non ubiquitous Amplicon Sequence Variant (ASVs) in the LV sampling sites during the four seasons is illustrated in Venn diagrams (**Fig. 47**). The diagram showed a low number of classified ASVs in the sites during all seasons at PM (849 ASVs) while the highest number was found at SG (1397 ASVs). A total of 1241 ASVs were shared among all the three sites during the study period. The highest percentage of shared ASVs was, obviously, obtained at SG with ca. 40% of the total ASVs observed in the site, while the lowest was found at PM (32%).

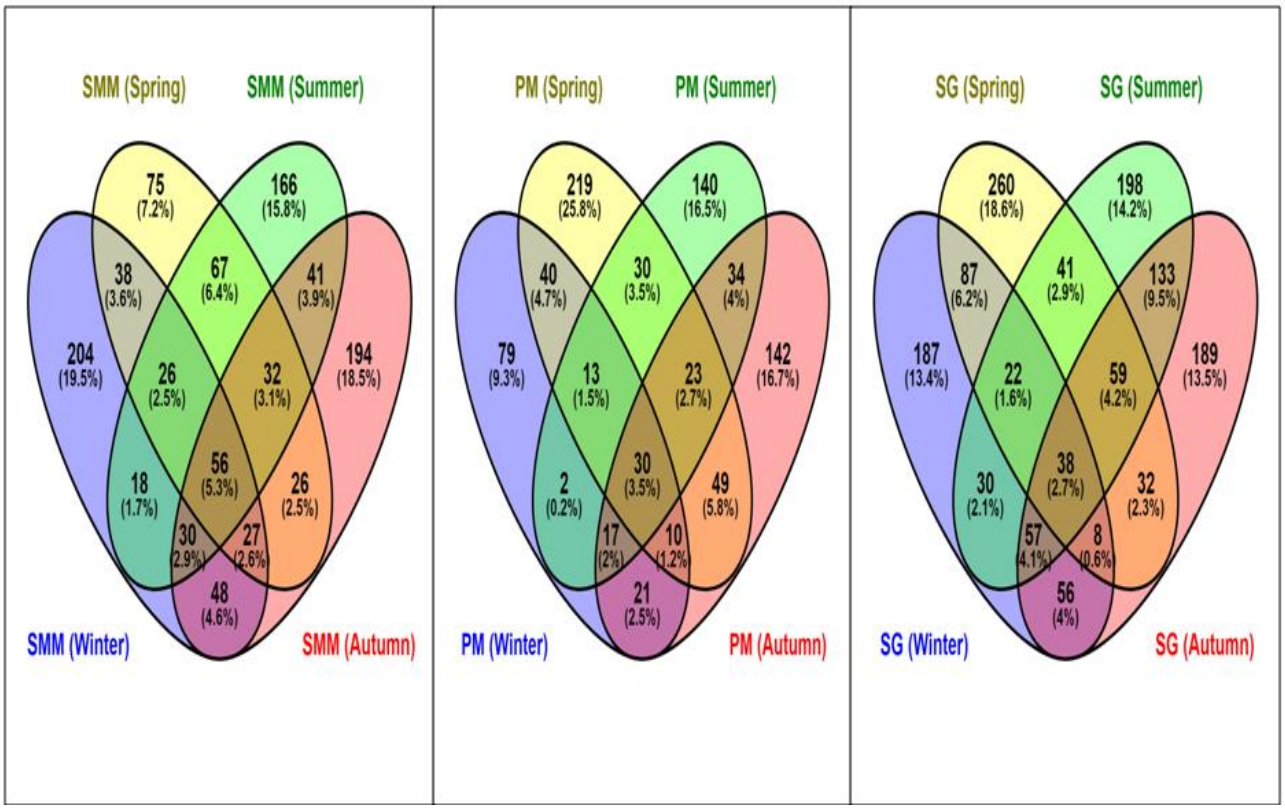

Fig. 47: Venn diagram of ASVs from *U. laetevirens* associated microbial community for each site of LV during all seasons.

The SIMPER analysis was used to measure the similarity percentage between microbial community within seasons and sites in LV samples. The highest dissimilarity was obtained between SMM and PM (83.9%) while the lowest one was between PM and SG (77.7%). Among the seasons, the highest dissimilarity was noticed between winter and summer samples (80.7%), whereas summer and autumn showed the lowest dissimilarity (74.9%).

During the same season, the number of non-ubiquitous ASVs varied among sites (**Fig. 48**). An average of ca. 71% of the total ASVs in each season was belong to non-ubiquitous ASVs for sampling sites. In winter the highest percentage of non-ubiquitous ASVs was found at SMM, while in spring it was at SG (33.7%). In summer and autumn the percent of site specific ASVs were comparatively equal for both seasons.

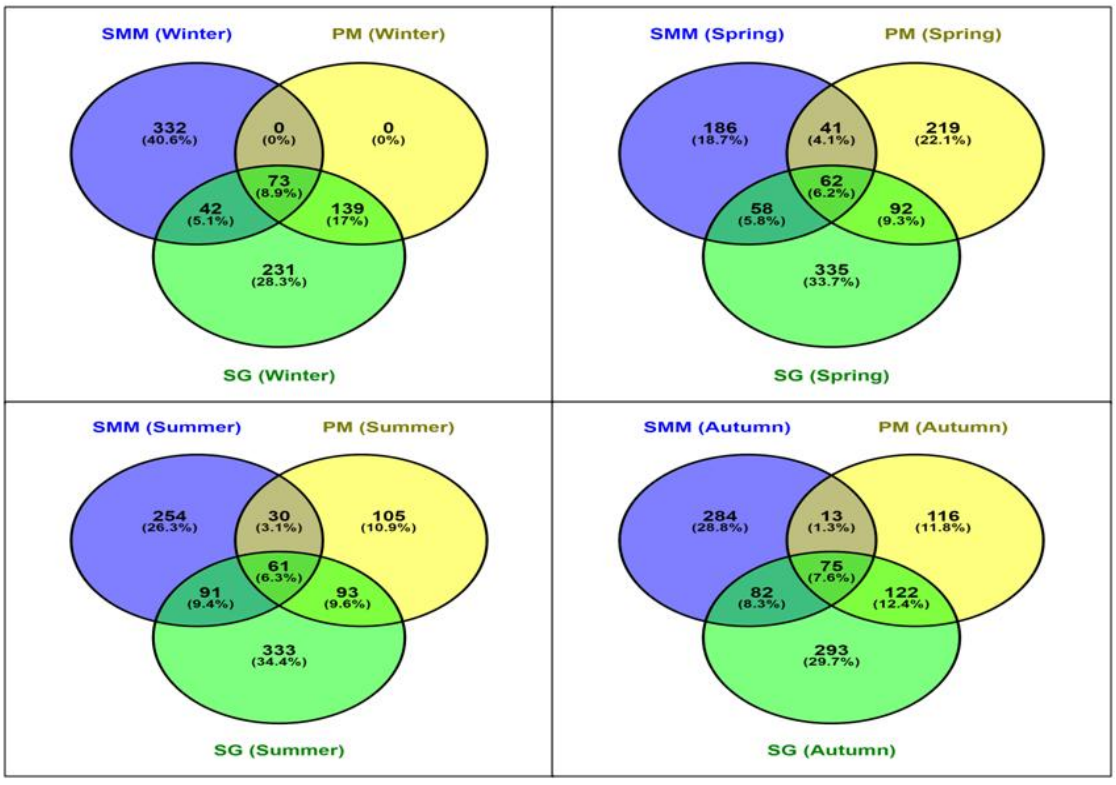

Fig. 48: Venn diagram of ASVs from *U. laetevirens* associated microbial community at LV sampling sites during the different seasons.

A comparison was also obtained between ASVs associated with *U. laetevirens* and those isolated from adjacent seawater during summer and autumn in LV sites (**Fig. 49**). In summer season the number of shared ASVs between seawater and *Ulva* associated community was higher at SG (143 ASVs). SIMPER analysis indicated an average dissimilarity of 89.8% between the two matrices (*Ulva* and seawater) for all sites. SMM showed the lowest percentage of shared ASVs, ca. 13.5% of the total number of ASVs. At PM, 15.4% ASVs was shared between seawater and *Ulva* associated ASVs, whilst at SG the percentage was the highest of 21.3% of the total ASVs. Surprisingly, high percentage of non-ubiquitous *Ulva* associated ASVs (ca. 64.9%) was noticed at SG sampling site.

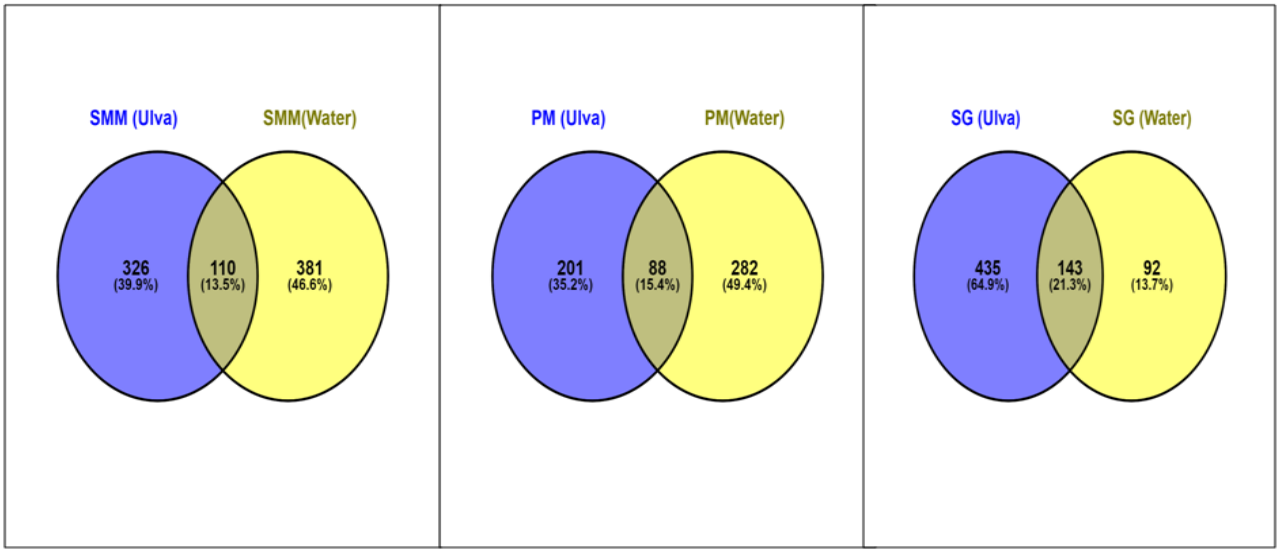

Fig. 49: Venn diagram of ASVs from *U. laetevirens* associated microbial community and from adjacent seawater during summer season at LV sampling sites.

3.12 Microbial community (Gulf of Aqaba)

3.12.1 Distribution of microbial community

High variability in the composition of microbial communities between the two seasons was noticed in GA samples. The observed ASVs belonged to 11

phyla; among them 3 phyla (Bacteroidetes, Firmicutes, and Proteobacteria) accounted for ca. 97.5% of the total sequences of the *U. laetevirens* associated microbial community (**Fig. 50**). Furthermore, the less abundant phyla (Chloroflexi, Epsilonbacteraeota, Tenericutes, and Thaumarchaeota) accounted for less than 0.07% of the total sequences. Bacteroidetes and Proteobacteria relative abundance reduced during winter by 46 and 42%, respectively. The phylum Planctomycetes showed a higher abundance (4.2%) in summer at IC.

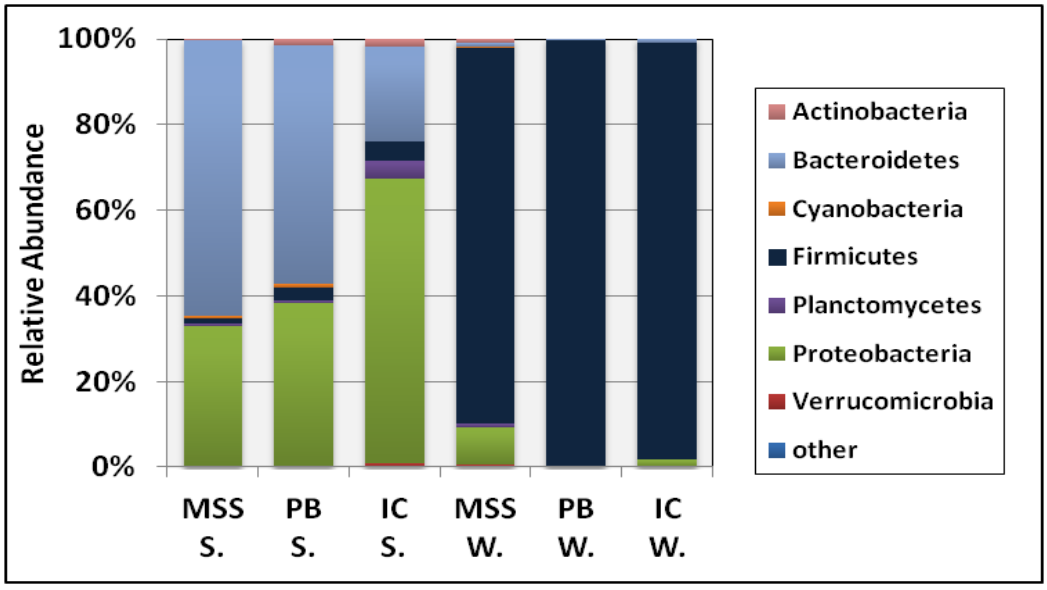

Fig. 50: Distribution of *U. laetevirens* associated microbial community at phylum level for GA sampling sites. (**W**: Winter, **S**: Summer)

The differences of microbial phylotypes were deeply verified at lower taxonomic levels (**Fig. 51**). On the total, 17 classes were classified in all the sampling sites during winter and summer. The most abundant classes (Alphaproteobacteria, Bacilli, Bacteroidia, and Gammaproteobacteria) accounted for ca. 97% of the total sequences. From winter season, the class Bacilli was notably dominant at all sampling sites, and accounted for ca. 95% of the total

sequences. Seasonal variation between sites was clearly evident especially for the less abundant classes. Oxyphotobacteria, for instance, showed a higher abundance at MSS and PB during summer.

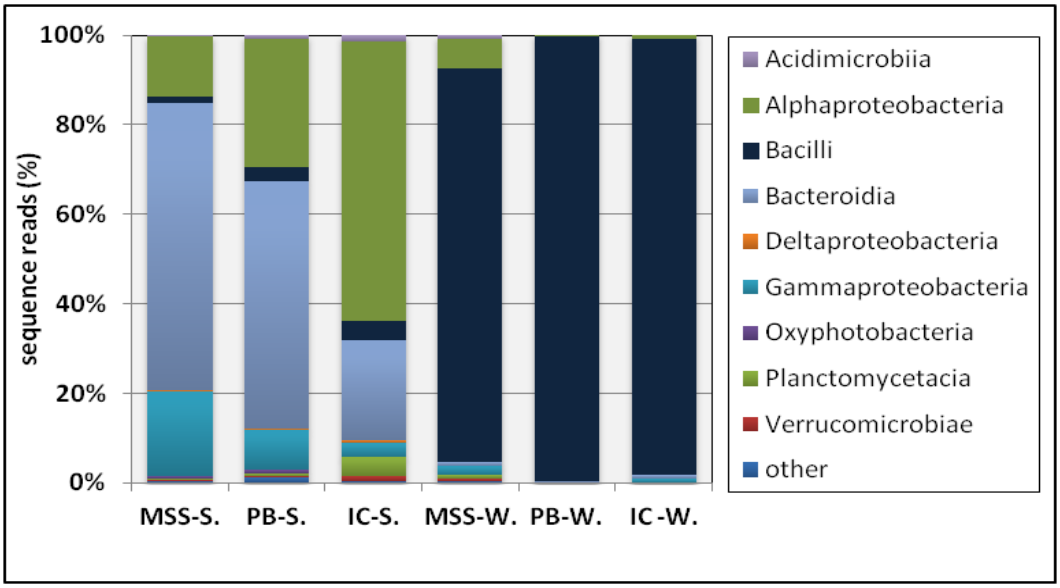

Fig. 51: Distribution of *U. laetevirens* associated microbial community at class level for GA sampling sites. (**W**: Winter, **S**: Summer)

3.12.2 Distribution of shared and non-ubiquitous ASVs

The variations in the distribution of shared and specific ASVs among sites during the different seasons are showed by the Venn diagram (**Fig. 52**). In summer, a higher percent of shared ASVs between sites was observed (54% of the total ASVs present in summer), while in winter this percentage was reduced to 46%. The highest number of non-ubiquitous ASVs for summer samples belonged to IC (144 ASVs) and was reduced to 20 ASVs during winter.

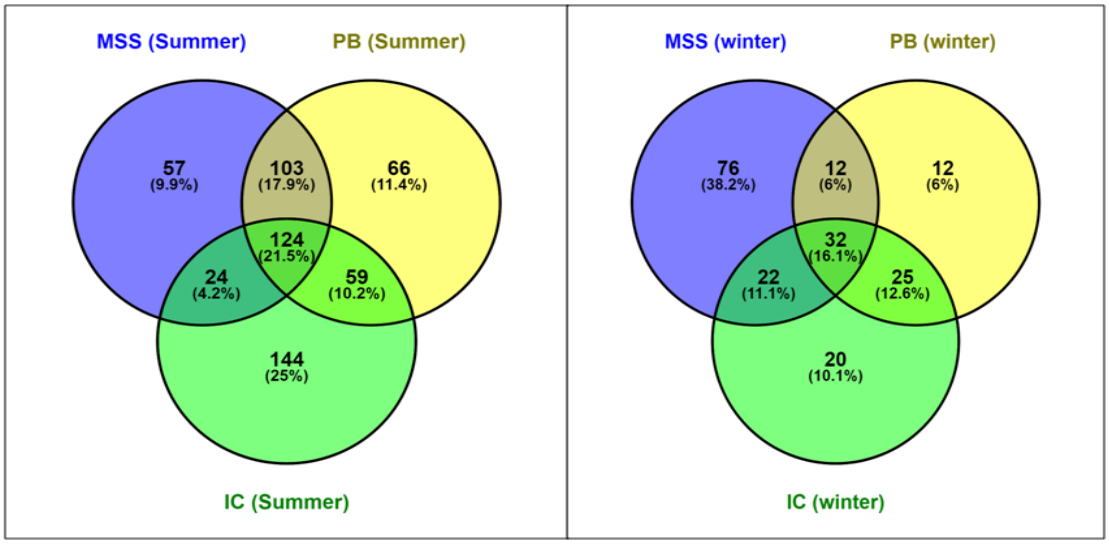

Fig. 52: Venn diagram of ASVs from *U. laetevirens* associated microbial community at GA sampling sites during different seasons.

The average calculated similarity between GA samples was 56.7% of the total ASVs (SIMPER analysis). The dissimilarity between seasons (winter and summer) for all sampling sites was higher (ca. 88.1 %). By comparing the sites of the GA, SIMPER analysis indicated that a similar level of dissimilarity was obtained between sites (averagely 66 % of the total ASVs).

3.13 Microbial community diversity

3.13.1 Alpha (α) diversity indices for microbial communities

The diversity of microbial community was estimated using different diversity indices (Shannon, Simpson, Chao1 and Fisher) after normalization of the dataset (**Fig. 53**). Regarding the LV, Shannon diversity index was higher at SG (5.85 ± 0.15) (among all seasons), whereas the lowest diversity was observed at PM (Shannon index = 3.96) during summer. This reduction was corresponding with the decrease of ASVs number (289 ASVs). At SMM, the highest diversity

was reached in winter (5.7), reducing in the other seasons. Similarly, the Simpson diversity index confirmed the results obtained by the application of the Shannon diversity index. The higher values were observed at SG among all seasons (0.991 ± 0.002). The lowest diversity value was obtained at PM in summer (0.911).

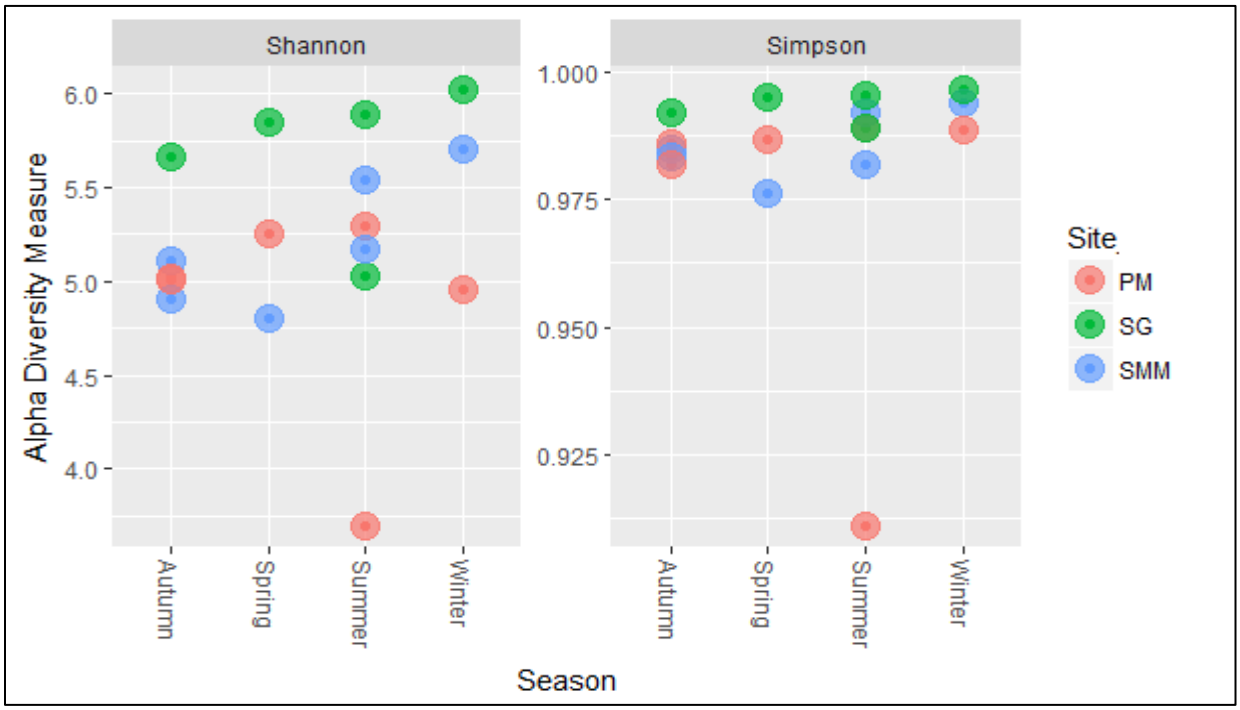

Fig. 53: α- Diversity indices (Shannon & Simpson) of microbial communities in LV samples.

Chao 1 index was additionally used to evaluate the diversity of microbial communities. This index was applied for dataset after extrapolating out the number of rare (less abundant) ASVs. The diversity of SG samples was the highest and consistent during all seasons (570 ±8). SMM diversity was reduced in spring (352) and increased in summer and autumn. The diversity indices were also used to evaluate seawater samples diversity collected from LV. The highest diversity of seawater microbial communities was evaluated for SMM during summer season (chao 1 = 491) which reduced to 340 during autumn season. The obtained results are included in appendix (G).

For the GA samples the diversity indices were higher in summer for all the sites **(Table 16)**. The highest diversity was observed at PB during both seasons. This was highlighted by the three selected diversity indices (Shannon, Simpson and Chao 1) with values of 5.27, 0.988 and 407, respectively. Conversely, the diversity recorded in winter and based on the listed diversity indices was extremely low compared to the summer samples and to LV samples.

Table 16: Alpha (α) diversity indices (Shannon, Simpson and Chao1) for *U. laetevirens* associated microbial community at GA sampling sites.

Sample	Season	# ASV	Diversity index		
			Shannon	**Simpson**	**Chao1**
MSS	Summer	353	4.68	0.976	353
PB		407	**5.27**	0.988	407
IC		396	5.05	0.986	396
MSS	Winter	143	3.5	0.931	143
PB		81	2.8	0.868	81
IC		100	**2.52**	0.815	100

3.13.2 Beta (ß) diversity for microbial communities

Non-metric multidimensional (NMDS) scaling was performed on the total microbial community structure to determine the distribution and differences among communities during different seasons and sites (**Fig. 54**). Clear differences were recorded between the seawater and the microbial communities associated with *U. laetevirens* which were clustered in the left part. Similarly, GA samples were clustered in different parts in comparison to the LV sites. Moreover, in LV the samples of SMM were clustered together and were different from the other sites (PM and SG). The variation of distribution was confirmed by one-way ANOSIM

(Analysis of similarities) test which was used to examine the similarities between samples. Significant differences were obtained between sites ($r = 0.513$, $p < 0.005$), whereas, no significant correlations were obtained for microbial community in GA sampling sites ($r = -0.5$, $p > 0.30$).

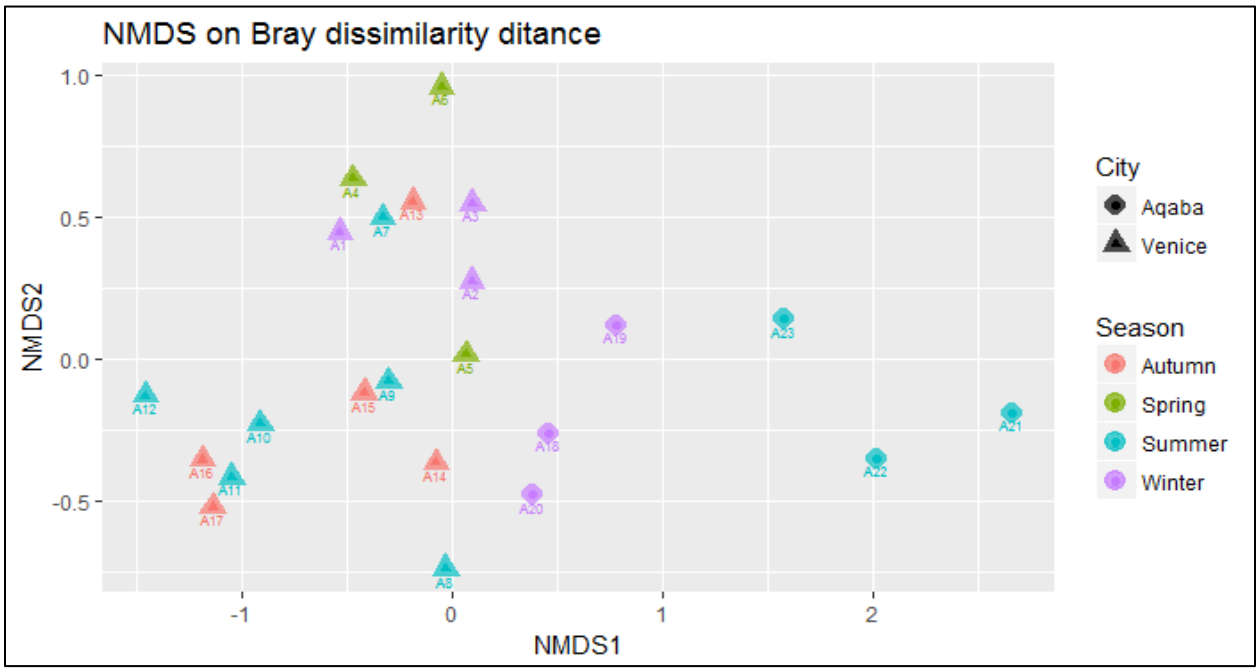

Fig. 54: Non-metric multidimensional scaling plot of microbial community structure in LV and GA sites based on Bray –Curtis dissimilarity distance of square-root transformed relative abundance ASVs. (Ax: sample code, appendix H). The lowest stress is **0.14**.

The differences among bacterial communities were also assessed by hierarchical clustering (UPGMA). The method used the relative abundance of each ASV to calculate the similarity between samples using the Bray-Curtis dissimilarity distance (**Fig. 55**). The variability between seawater and the bacterial communities associated with *U. laetevirens* was confirmed by the clustering of seawater samples which are separated from the other samples. Site differences were also noticed for SMM in comparison to the other sites confirming the variability of bacterial communities between sites. This variability was also

statistically assessed using PERMANOVA test based on Bray-Curtis measures of square root transformed relative abundance of ASVs. The variation was found highly significant (Psudo- F = 2.897, $p < 0.005$, Monte Carlo 9999 Permutations). Similarly, the variation of microbial community was significantly different between LV and GA samples (PERMANOVA test, Psudo- F= 3.03, $p < 0.005$, Monte Carlo 9999 permutations).

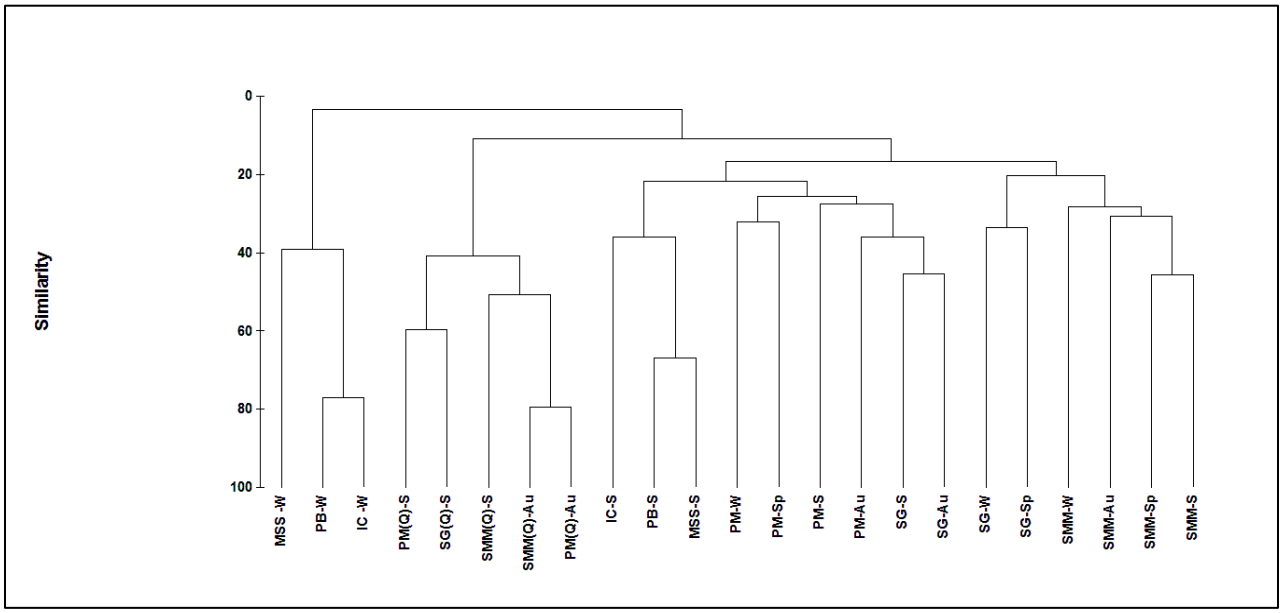

Fig. 55: Hierarchical (UPGMA) clustering of *U. laetevirens* and seawater associated microbial community. (**W**: Winter, **Sp**: Spring, **S**: Summer, **Au**: Autumn, Q: seawater microbial community)

3.13.3 Relationship between bacterial community structure and environmental variable (LV)

The correlation of environmental variables and the microbial community associated with *U. laetevirens* in LV using db-RDA analysis is shown in **Fig. 56**. The first two axes expressed 38% of the total variations. The samples of SMM were clustered together with indication of slight seasonal variation across the seasons. The microbial community associated with *U. laetevirens* in contaminated sites (PM and SG) were distributed in different pattern. The samples of PM were

clustered closed together and also showed slight variation across seasons. SG samples were more dispersed with slight variation between winter and spring samples separated from the other two seasons. Environmental parameters appeared to affect significantly the associated microbial community at SG and PM. Salinity affected the associated microbial community at SMM and PM. Toxic metals affected the associated microbial community at SG and PM across the whole seasons.

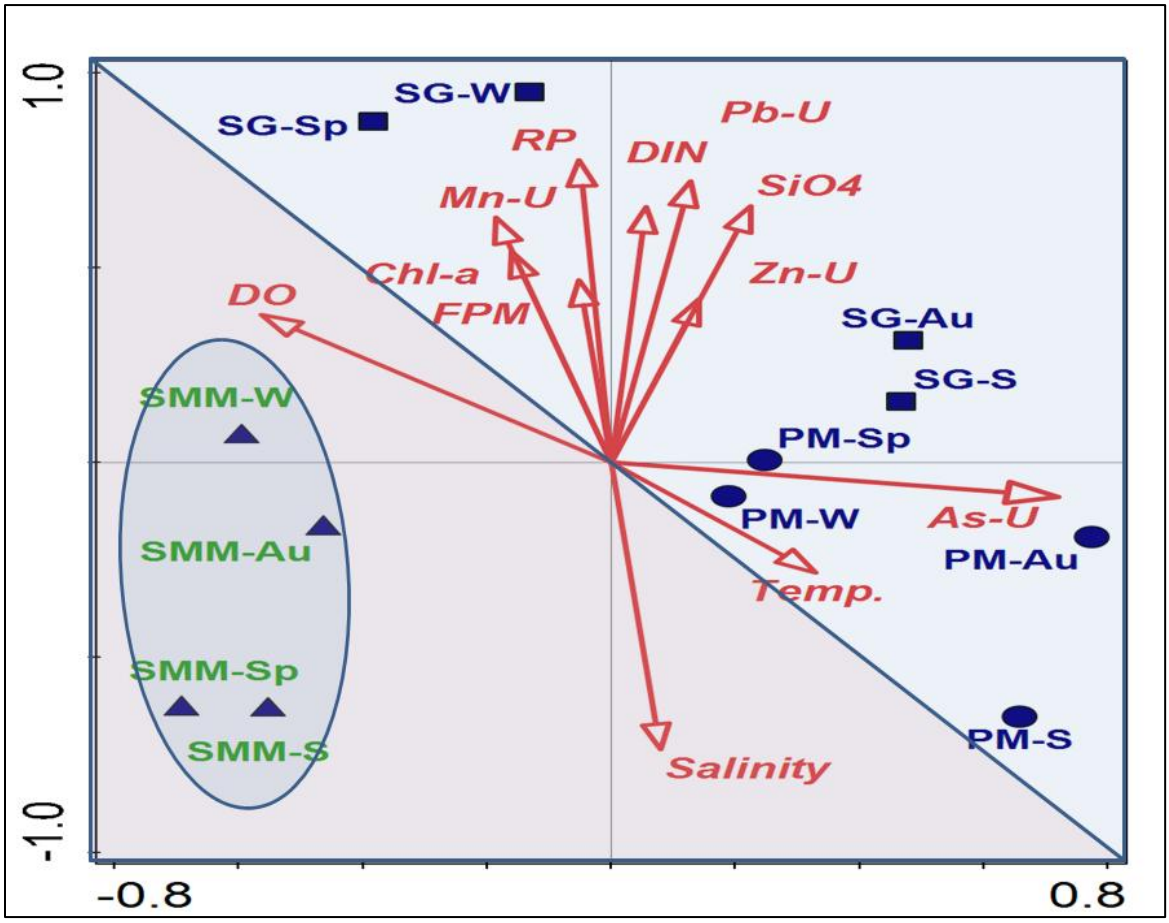

Fig. 56: db-RDA analysis for *U. laetevirens* associated microbial community in response to environmental variables at LV sites. Sampling sites are in **bold** (SMM, PM, SG) with the corresponding season (W: winter, Sp: spring, S: summer, Au: autumn); *Italics* represents environmental variables.

3.13.4 Relationship between the bacterial community structure and environmental variables (GA)

The correlation between the microbial community associated with *U. laetevirens* and the environmental variables recorded in GA sites across the two seasons are shown by db-RDA analysis (**Fig. 57**). The first two axes of db-RDA explained 78.0% of the total variation where 55.2 % of the total variation was explained by the first axis. The microbial community from summer samples was clustered in opposite side of winter samples. DIN and Chl-*a* appeared to affect the majority of the microbial community associated with *U. laetevirens* during summer. Vice versa, temperature of water column and RP were correlated with ASVs of the community of winter samples.

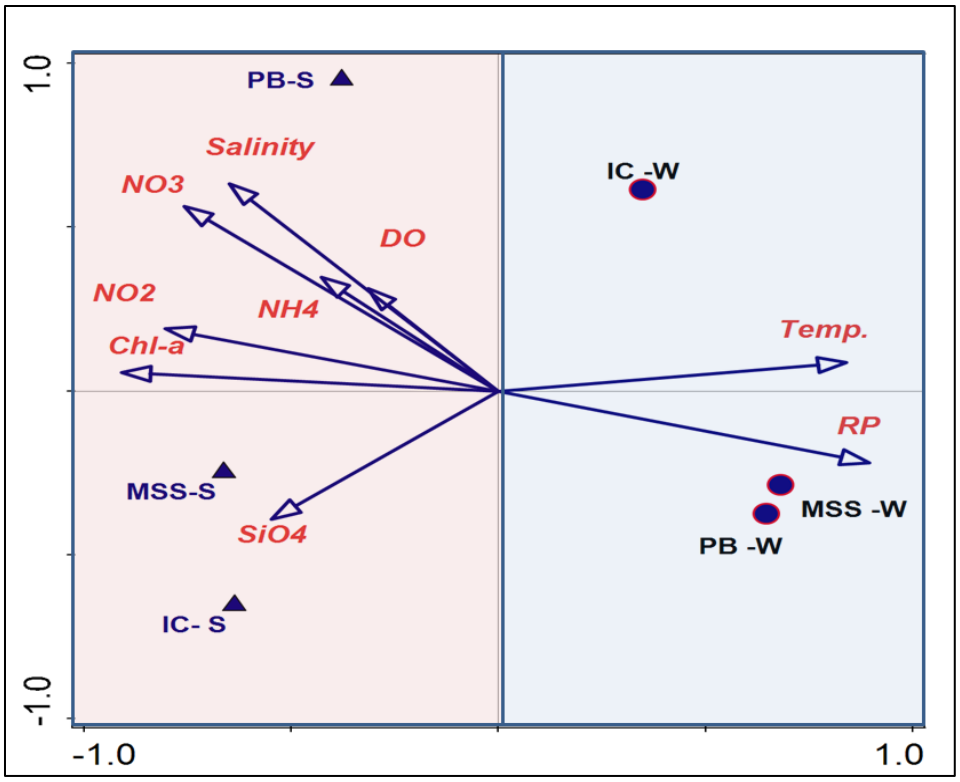

Fig. 57: db-RDA analysis for *U. laetevirens* associated microbial community in response to environmental variables at GA sites during sampling seasons. **Bold** represent environmental variables.

4. DISCUSSION

Part I: Health Status and Macrophyte Communities

4.1 Macrophyte diversity in the Lagoon of Venice

The perturbations of anthropogenic pressures in aquatic environments have extensively studied in the last decades. Macrophytes (macroalgae and angiosperms) are one of the fundamental BQEs used within the WFD employed to assess the health status of aquatic environments affected by various anthropogenic stressors. Macrophytes reflect the changing of the ecological conditions in coastal and transitional water bodies due to their rapid response to anthropogenic stressors (Sfriso et al., 2017). In order to assess the health status of transitional lagoons affect by anthropogenic stressors, several ecological indices based on macrophyte assemblages were developed (ex. Sfriso et al., 2014). Among them, MaQI was validated to assess the ecological status of Italian marine transitional environments (Sfriso et al., 2009, 2014). MaQI is characterized by a wide range of application, from pristine environmental conditions, dominated by macroalgal sensitive species and/or angiosperms, to a degraded status, with the dominance or almost absence of opportunistic species (Sfriso et al., 2014).

The structure of macrophyte assemblages across the studied stations in LV reflected the variation of the ecological conditions. SG and PM were particularly colonized by thionitrophilic and opportunistic macroalgal species (mainly Ulvaceae and Gracilariaceae), and two non indigenous species: *Agardhiella subulata* (C. Agardh) Kraft and M.J. Wynne and *Solieria filiformis* (Kiitzing) P.W. Gabrielson, whereas no aquatic angiosperms were recorded. The increase of eutrophication (mainly at SG) enhanced the fast growing opportunistic taxa that

replaced angiosperms and sensitive macroalgae which decreased the biodiversity and the vegetation quality (Buosi and Sfriso, 2017). By application of MaQI, which was designed for application in transitional water bodies, these stations were assigned to POOR ecological status (**Table 6,** page **83**). SG was characterized by high nutrient (DIN, RP and SiO_4), Chl-*a* and suspended matter concentrations, especially during winter and spring. The high concentrations of nutrients at SG came mainly from freshwater inputs by Osellino and Dese rivers (Sfriso et al., 2008). In addition, the river outflows conveyed pollutants such as metals and pesticides (Maroli et al., 1993). Indeed, the concentrations of heavy metals in sediment and SPM were found significantly high at both SG and PM (**Table 12,** page **93**). Furthermore, a considerable increase of pollutants was discharged into the lagoon by the industrial activities of PM (Masiol et al., 2014; Zonta et al., 2007).

Conversely, the best ecological conditions were found at SMM which was characterized by the highest macroalgal species diversity with a high percentage of sensitive species (32%), as well as the presence of *Cymodosa nodosa* and *Zostera noltei*. In fact, this area was characterized by clear waters (low turbidity) and low nutrient concentrations in water column. These ecological conditions agree with the characteristics of pristine environments reported by Sfriso et al., (2009). Overall, this ecological status assessment based on macrophyte assemblages was consistent with previous investigations carried out in LV (Sfriso et al., 2009; 2014).

This ecological status assessment by MaQI showed significant correlations with the most common environmental parameters affected by anthropogenic disturbances (i.e. nutrient concentrations in the water column and surface

sediments, Chl-*a* concentrations, water turbidity, etc.). Hence, the changes of these parameters are strongly correlated to the ecological status of the considered environments. The significant correlations between nutrients (DIN, RP and SiO_4) and Chl-*a* concentration of water column and macrophyte variables were also confirmed by the PCA multivariate analysis that grouped the parameters according to their variance (**Fig. 43,** page **107**). The high nutrient concentrations of water at PM and SG (DIN: 9.0 - 85.0 µM; RP: 0.3 - 3.15 µM; Chl-*a*: 1.0 – 30.0 µg/L) were positively correlated with the presence of Chlorophyta (mainly Ulvaceae). An excess of nutrients and high water turbidity favour the growth of opportunistic macroalgae (Viaroli et al., 2008) due to their high growth rates and need for nutrients (Thompson and Valiela, 1999). In addition, the mono/bi-layer structure of opportunistic macroalgae, where all the cells have chloroplasts, requires lower light quantity for growth than perennial multilayer macroalgae (Hemminga and Duarte, 2000). The correlation of nutrients and water turbidity with tolerant taxa was confirmed by Sfriso et al. (2017a, b) for the transitional environments of the Mediterranean Sea and coastal areas of the Adriatic Sea, as well as by Orfanidis et al., (2003) for the Greek lagoons. In contrast, low nutrient concentrations and clear waters favour the dominance of aquatic angiosperms and sensitive macroalgae resulting in a reduction of opportunistic species.

The ecological status assessment based on macrophyte assemblages is also well supported by the strong correlations with the total organic content of sediments and SPM from the respective stations. In fact, the accumulation of organic nutrients in sediment or SPM was related to the increased level of these nutrients in water column beside other sources. These correlations were also in agreement with the findings of Sfriso et al., (2009) for investigation on the Italian lagoons. Similarly, the high percentage of fine sediments was correlated with the

percentage of angiosperms and MaQI. This was supported by the fact that in the studied areas angiosperms grew on sandy sediments. Beside, the release of heavy metals into aquatic environments can cause direct toxic effects including impairment of sensitive species (Fleeger et al., 2003). The presence of heavy metals at PM and SG notably affected the ecological status of these stations. Toxic metal concentrations resulted from agricultural and industrial activities were considerably higher in these sites (Sfriso et al., 2008; Zonta et al., 2007). This was particularly highlighted by the significant positive correlation between some toxics (Pb, As) and the poor ecological status (**Fig. 43,** page **107**) and was in consistence with the study by Sfriso et al., (2009) about the evaluation of the ecological status by using MaQI.

4.2 Macrophyte diversity in the Gulf of Aqaba

More than 500 macroalgal taxa were recorded in the Red Sea (Head, 1987) and ca. 9% of them are endemic. The diversity of macroalgal associations in the northern part of Red Sea (Gulf of Aqaba and Gulf of Suez) is generally low. This is due to oligotrophic conditions and lower temperatures in comparison to the southern part of the Red Sea (Walker, 1987). These results are confirmed by PCA analysis and the negative correlation between nutrients in the water column and macroalgal diversity. Indeed, the number of macroalgal taxa in the northern Red Sea accounted for 8-40% of the total macroalgae recorded in the Red Sea (Walker, 1987).

In the Jordanian coast of Gulf of Aqaba macroalgae occurs on the shallow flat reef and other hard substrata and some subtidal soft-bottom habitats. The macroalgal community in the selected stations was composed of 35 species collected at a depth of 1-3 meters. The species belonging to green macroalgae

(Chlorophyta) were laminar Ulvaceae, some filamentous Cladophoraceae, and calcareous Halimedaceae. The brown algae (Ochrophyta: Phaeophyceae) consisted primarily of some Sargassaceae and Dictyotaceae in the shallow flat reef, and the turf macroalga *Sphacelaria tribuloides* Meneghini. Moreover, several species of Rhodomelaceae and filamentous Ceramiaceae were present.

The macroalgal cover and biomass at GA stations during the sampling periods was considerably low. This is confirmed by Badran (2001) who indicated that the lowest macroalgal cover was generally detected (especially in summer) in the northern part of Red Sea. This is related to the lack of water mixing during summer (Manasrah et al., 2006) which is the main source of dissolved nutrients in water column (Al-Zibdah and Damhoureyeh, 2006). The rocky substratum and the grazing pressure by fish and other herbivores are the main factors that reduced the cover of macroalgae (Benayahu and Loya, 1977).

The macroalgal associations recorded at IC were characterized by the dominance of some Sargassaceae (*Cystoseira myrica* (S.G.Gmelin) C.Agardh and *Turbinaria ornata* (Turner) J.Agardh). Moreover, the presence of the green calcareous macroalga *Halimeda tuna* (J. Ellis and Solander) J.V. Lamouroux was exclusively observed at IC station. This species is known to grow under low light intensity and grazing pressure and in the presence of higher nutrient concentrations (Vroom et al., 2003). In fact, high phosphorus concentrations at IC may result from industrial activities (fertilizer industry) and the dust release from phosphate transportation (Rasheed et al., 2005). In addition, the species diversity recorded at IC does not agree with previous investigations of Al-Zibdah and Damhoureyeh (2006), highlighting the effects of additional anthropogenic pressures in this area.

The creation of a new harbour in an adjacent area may be the cause of this environmental degradation.

The diversity of red macroalgae (Rhodophyta) was noticed to be higher at both MSS and PB. Rhodophyta grew in an aggregate way and filled the spaces among branches of dead stony corals as previously noticed by Haas et al., (2010). Some calcareous macroalgae such as those belonging to genus *Jania* are among the most common species in these stations. The macroalgal biodiversity recorded in these stations was in agreement with that recorded during other investigations in Jordanian coasts of GA (Lundberg and Popper, 1996; Al-Zibdah and Damhoureyeh, 2006). However, intense touristic activities and domestic wastewater discharges, as well as boat navigations at PB could affect macroalgal diversity. Similarly, macroalgal turfs (e.g. *Cystoseira* sp., *Sphacelaria* sp., *Titanophycus* sp.) are the dominant reef habitats at MSS. This habitat reached the highest development in the partially exposed areas of the flat reef, growing on the dead coral skeletons (Haas et al., 2010). One of the possible causes of relative higher macroalgal biodiversity at MSS can be related to the navigation activities (passenger's ship harbour) in the vicinity of the station. Ballast waters and hull fouling of these ships may have contributed to the transfer of macroalgae from other parts of the Red Sea.

The investigation of seagrass distribution reveals that *Halophila stipulacea* was the dominant species colonizing all the stations of GA at low depth (1-3 m). Similar results were observed by Hulings, (1979) and Al-Rousan et al., (2011). These authors reported high densities of *H. stipulacea* in the GA at depths of 1-2 m, suggesting that this species can flourish in the presence of high irradiance. *H. stipulacea* was found to grow within a wide range of sediment grain-size (sand or

silt) through the coral rubble (Angel et al., 1995). This was in consistence with the strong correlation obtained between the percentage of sandy sediment and the cover and biomass of seagrass at GA. Indeed, a high content of TOM was measured in the sediments of the sampling station (MSS and IC) that may stimulate the growth of seagrass. Wahbeh (1982) concluded that physical factors such as light intensity and wave action were the main factors that influenced the seagrass distribution. On the other hand, extensive human activities, including swimming and boat navigations as well as domestic waste discharge, may reduce the seagrass cover as found at PB. These activities have resulted in an increasing sedimentation and turbidity that affected the growth of seagrass (Al-Rousan et al., 2005).

These preliminary results on macrophyte biodiversity in the Gulf of Aqaba can be considered a base for future investigations on macrophyte assemblages in the coasts of the Red Sea. Further surveys and intensive macrophyte sampling should be performed in order to set up a complete check-list of the macrophyte associations present in the Jordanian coasts of GA. This would allow the application of indices based on macrophyte assemblages to assess the health status of coastal areas as currently happens in The Mediterranean Sea and other European coasts.

Part II: Health Status and Oxidative Stress Biomarker

4.3 Metal levels in surface sediments and environmental availability

The present study investigated the use of metal bioaccumulation in macroalgae and the oxidative stress biomarker responses in order to assess the health status of coastal and transitional water bodies. Metal accumulation was generally originated from extensive anthropogenic activities that deteriorate the health status of LV and GA environments.

The current findings show that the availability of metals in sediment of contaminated sites at LV (PM and SG) and GA (PB and IC) varied considerably from the uncontaminated control sites selected in LV (SMM) and GA (MSS). In particular, the most significant differences were recorded for As, Cd, Cu, Pb and Zn.

The comparison of metal concentrations in surface sediment of the LV sampling sites with the reference values reported from Ministry for the Environment (1993) indicated that the sediment from PM and SG presented values exceeding the LCC for Cu, Hg and Pb and values above MCC for Zn (at SG). Metal concentrations were higher in the fine sediments at PM and SG due to their affinity to organic matter and fine particles, as already reported elsewhere (Sfriso et al., 2008; Pereira et al., 2009). High organic matter content in fine sediments of these sites is related to the urban effluents, agricultural runoff and industrial activities, combined with the relatively long residence time of waters (especially at SG) that can reach up to 60 days (Guerzoni and Tagliapietra, 2006), promoting the settlement of organically enriched particles. Metal concentrations in surface sediments of the Venice lagoon were in agreement with those of previous investigations (Sfriso et al., 2008; Masiol et al., 2014). On the other hand, the

characteristics of sediments (low moisture and sandy grain-size), beside the low nutrient concentration in water column agrees with the lower metal concentrations recorded in the sediment at the uncontaminated site (SMM).

Similarly, metal (Cd, Cr, Ni, Mn and Zn) concentrations in sediments from the contaminated sites at GA (IC and PB) were considerably higher than those from the reference site (MSS). The concentrations of metals from sediments in the GA was compared with the values of "Effects Range-Low" (ERL) (i.e. 46, 34, 81, 21, 1.2 µg/g for Pb, Cu, Cr, Ni, Cd, respectively) reported in the sediment quality guidelines proposed by Long et al., (1995). The contaminated sites (IC and PB) presented concentrations above ERL for Cd and Ni and with a minor extent for Cr. Conversely, metal concentrations in MSS were largely below ERL. Industrial and transportation activities were known to cause an increase of metal concentration at IC and BP (Wahsha et al., 2017; Al-Absi et al., 2016). Metal concentrations in sediments were comparable to those found by Al-Rousan et al., (2016) in GA area.

4.4 Metal levels in *U. laetevirens* as exposure biomarker

The macroalga *U. laetevirens* collected from contaminated sites at both LV and GA showed a bioaccumulation of metals (Cu, Mn, Pb and Zn) and metalloid (As at LV), reflecting the efficiency of this species as bioindicator for metal contamination.

In the **Lagoon of Venice**, spatial variations revealed that Cu, Hg, Mn, Pb and Zn bioaccumulation in *U. laetevirens* was higher at SG and PM, in agreement with the corresponding metal concentrations measured in surface sediments. These associations were statistically supported by Pearson correlation analysis and previous studies that recorded metal concentrations in macroalgae from polluted

sites ca. 3 – 10 fold higher than samples collected from less polluted ones (Olivares et al., 2016; Vasconcelos and Leal, 2001). A significant higher bioaccumulation of Mn, Pb and Zn and As was measured at SG during spring, this reflects the environmental bioavailability of these metals in surface sediments and SPM. Contrary to expectations, the concentrations of Ni and V were higher at SMM (uncontaminated site) which reflects the bioavailability of these metals from other sources. On the whole, except for Pb, the concentrations of metals found in *U. laetevirens* were in accordance with the results in LV macroalgae reported by Caliceti et al., (2002).

The variation of the metal bioaccumulation level in macroalgae was related to the bioavailability of metals from the surrounding waters and the capacity of algae to accumulate metals (Karez et al., 1994). Besides, the distribution of metals occurs in a variety of association forms, differing in the intensity of metal-matrix bonds, each of these forms exhibits different bioavailability and different potential for remobilization (Argese and Bettiol, 2001).The high levels of Cu, Mn and Zn in *U. laetevirens* in the polluted site reflected the high bioavailability of these metals and their capacity to be bioaccumulated from the surrounding polluted environment. The degree of metal accumulation in algal tissues from surrounding water is mostly related to the density of surface functional sites and the binding capacity of intracellular ligands (Levy et al., 2008). Moreover, the metal bioaccumulation ability of macroalgae under environmental stressors is attributed to the formation of thiols and peptides in their tissues which enhance the absorption capacity from the surrounding environment (Haritonidis and Malea, 1999).

The seasonality of metal accumulation for macroalgae is a conflicting matter. Notably, spring and summer seasons demonstrated higher bioaccumulation

of metals in *U. laetevirens* (compared by MPI). Villares et al., (2001) demonstrated that metal accumulation decrease during the period of growth and increase in the dormant winter season. Contrarily, Catsiki and Papathanassiou (1993) concluded that the higher metal accumulation was during summer growth period for *Ulva* sp.. This was in accordance with the findings of this study for Cu. High accumulation is explained by the higher rates of photosynthesis and respiration, which favouring the assimilation of metals. This concept is disagreeing with Giusti (2001) who suggested that the dilution resulted from macroalgal growth during summer season lead to low bioaccumulation of metals. Current results revealed seasonal variations at SG, where accumulation of the metals (Mn, Pb and Zn) occurred mainly in spring. Manganese, for instant, is an essential metal that plays a major role in photosynthesis, respiration and activation of several enzymes (Marschner, 1995). Therefore, macroalgal regulation of Mn uptake and retention is driven by environmental bioavailability of this metal in sediment and water. Conversely, Ni showed to be accumulated during winter at PM in accordance with Riget et al., (1995) and Villares et al., (2001) results. Conversely, As in this site was generally higher during all seasons. Zonta (2006) reported high As concentrations in the freshwater discharged into PM area, mainly in a bioavailable form (soluble or adsorbed by particulate matter).

Metal accumulation in biota is a complex process which does not only depend on the metal bioavailability in the environment (Villares et al., 2002). In fact, particular attention should be given to endogenous and exogenous factors such as nutrient availability. Significant correlations were recorded between Pb and Hg accumulation in *U. laetevirens* and DIN and RP concentrations in water column. This is in agreement with the fact that higher metal bioaccumulation occurs with increasing nutrient availability from the water column as previously demonstrated in laboratory for *Ulva fasciata* by Lee and Wang (2001).

Furthermore, the patterns of bioaccumulation in *U. laetevirens* in different sites can be explained taking into account the metals specific behaviour: Zn is a particularly mobile metal, present in the LV in bioavailable forms (Sfriso, 2013; Breda, 2017). Moreover, As, Cu, and Ni mainly occur in potentially bioavailable forms, associated with carbonates, Fe/Mn oxide/hydroxides and organic matter (Breda, 2017).

Referring to the limits established by the EU for certain contaminants in the food stuff (EC, 1881/2006), its noteworthy observing that toxic metal (Pb) at SG site exceeded this limit during spring and summer. This increment can be explained by the pollution resulting from the Osellino river effluents and from the boats that are extensively present in this area, as well as by the long residence time for waters, as previously mentioned.

In the **Gulf of Aqaba**, Cd, Cr, Cu, Mn, Ni, Pb, Zn and Fe concentrations were measured in *U. laetevirens* collected during summer. The detectable levels of metals in the decreasing order were: Cd< Cu<Pb< Cr<Mn< Ni< Zn< Fe. This trend was consistent with the previous reported data (e.g. Haritonidis and Malea, 1999). The mean concentration of all metals (except Ni and Pb) was, in general, comparable to that found in the whole area of the GA (Marsa-Alam, Egypt) as determined by Abdallah et al. (2006). These authors concluded that, the high level of metals was related to the anthropogenic influence due to recreation and tourism activities, land-filling, solid waste disposal and phosphate pollution. These results were also confirmed by El-Moselhy and Gabal (2004) who found that the highest level of metals were found in the sites strongly influenced by sewage and industrial effluents in the Gulf of Suez area. The concentrations of Cd, Cr, Ni recorded in GA sites were higher than the concentrations found in the Arabian Gulf (Basson and

Abbas, 1992). Haritonidis and Malea (1999) found that the concentrations of Pb and Fe in *Ulva rigida* C. Agardh tissues in the area of Aegean (Greece) were 5-times less than the concentrations recorded in the GA area. Moreover, in comparison to LV, low accumulation of the metals (Cu, Mn and Zn) was observed in the *U. laetevirens* collected from GA sites.

The variability of metals bioaccumulation in *U. laetevirens* was related to the degree of contamination of surface sediments. In fact, low levels of metal accumulation at MSS were related to the low level of anthropogenic activities of this reserved area (Wahsha et al., 2017). For instant, the higher level of Ni and Pb are related to the high level of anthropogenic pressures in both polluted areas at IC and PB. On the other hand, the level of Cd, Cr, Pb and Zn in *U. laetevirens* was 5 to 10-fold higher than the values reported by Laib and Leghouchi (2012) in *U. lactuca* from Algeria (Mediterranean Sea) reflecting the metal contamination degree in the GA area.

High concentrations of Mn and Zn in *U. laetevirens* reflected the high bioavailability of these metals in the study area, and its capacity to be accumulated from the surrounding environment (Karez et al., 1994). Furthermore, the highest level of Fe may be attributed to the established need of Fe as essential micronutrient for the primary productivity in the aquatic waters (Di Tullio et al., 1993), and the ability of algal tissues to magnify Fe from the surrounding environment (Eisler, 1981).

The toxic metal (Cd) concentration in the *U. laetevirens* exhibited the lowest concentration and variability over all metals with an average value of 0.54 µg/g dw. These concentrations were in the range of values recorded by El-Moselhy and

Gabal (2004) in the area of Gulf of Suez. The accumulation of Cd is enhanced by the water concentration of N and P. These nutrients are incorporated by phytoplankton that, after completing its life cycle, sink to the bottom where the biogenic materials along with cadmium are transported by upwelling process into the water column and uptaken by macroalgae (Bruland et al., 2001). The concentration of Cd didn't exceed the EU limit for the food stuff (EC, 1881/2006). Whereas the concentration of Pb exceeded the maximum limits of the EU levels for contaminates in food stuff (EC, 1881/2006). Considerable concentrations of Pb are released from boats and ships and industrial waste effluent discharge into the gulf water (Al-Rousan et al., 2012; Wahsha et al., 2017). The elevated level of Pb in *U. laetevirens* can be related also to the higher rate of photosynthesis and respiration during summer which improves the assimilation of metals (Catsiki and Papathanassiou, 1993).

4.5 Oxidative stress and metals exposure

The relationship between environmental variables, metal accumulation and oxidative stress biomarker (LPO) was analyzed through the PCA analysis to assess the health status of aquatic environments. The PCA analysis (**Fig. 43,** page **107**) evidenced that metals availability and accumulation were associated with the high level of LPO measured in the contaminated stations in both LV and GA. The significantly higher LPO levels in the *U. laetevirens* collected from the contaminated stations (SG, PM, PB and IC) when compared with the control sites (SMM and MSS), indicated an overproduction of ROS in *U. laetevirens* which reinforcing the existence of oxidative damage. This induction is in agreement with the spatial differences of accumulated metals (Cu, Pb and Zn) and metalloid (As) in *U. laetevirens*. The high levels of LPO were recorded in the contaminated stations (SG, PM, PB, and IC) in agreement with the environmental availability of

metals in these stations. This result is supported by the significant correlation between LPO and metals in sediment and SPM, reflecting the degree of stress in the environment, thus confirming the use of LPO in the assessment of heath status for aquatic environments.

The industrial activities and domestic discharges increased markedly the level of LPO in *U. laetevirens* collected from those stations. The significant increase of LPO level was in accordance with previous investigation of Olivares et al., (2016) and Pereira et al., (2014) who found significant increases in the LPO level in the tissues of *Ulva* collected from sites characterized by high level of anthropogenic disturbances. Under laboratory conditions, Lin et al., (2016) found a significant increase in the LPO levels under high exposure of metals (Cu, Pb and Cd) in the leaves of the seagrass *Zostera japonica*. Generally, current data evidence a linkage between external levels of exposure, bioaccumulation and oxidative stress response (LPO) when Cu, Pb, Cr and Zn are considered.

The level of LPO was found higher at winter and spring seasons for all stations in LV. This was correlated with the metal accumulation in *U. laetevirens* during these seasons. For instance, the metals: Cu, Pb and Zn and the metalloid As were accumulated at higher concentration during spring in agreement with the elevated level of LPO measured in *U. laetevirens*. Low temperature during winter reduces the metabolic rates and the enzymatic activities (Vidal et al., 2002), in consistence with the current temporal variations in the level of LPO that showed an enhancement in the colder seasons (winter and spring).

Besides the obvious and significant relationship between metals and oxidative stress induction in macroalgae, the role of environmental parameters should also be considered. In facts, the PCA analysis indicated a strong association

between LPO and nutrient (DIN, RP, and SiO4) concentration in the water column that are grouped in the same biplot quadrant. This positive association was particularly significant with ammonium in water column in autumn, winter and spring and agree with Pereira et al., (2009) who found similar results for *Ulva* sp. in sites characterized by high trophic conditions. At SG the LPO level in *Ulva* was high during all seasons. Indeed, the toxicity of ammonium has been described in plants (Nimptsch and Pflugmacher, 2007), explained by the decrease of pH resulting from the excess of intracellular H^+, due to the high rate of proton extrusion from the plant into the surrounding medium (Taylor and Bloom, 1998). Furthermore, the involvement of ammonium on oxidative stress can be explained by the synergistic interaction with metals, and the interference with metal accumulation and macroalgal growth. On the other hand, *Ulva* sp in shallow environments with nutrient limitation in summer periods showed an increased level of oxidative stress (Malta et al., 2003). This may explain the increase of LPO level at PM during summer. The negative correlation of some metals with the LPO level despite their bioavailability in the environment and accumulation in *U. laetevirens* is not surprising (e.g. Mn). In fact, this can be explained by multiplicity of factors preceding the occurrence of peroxidative damage, including the complex antioxidant system and metal sequestering mechanisms (e.g. phytochelatins), which can impair the perception of such linear association (Pereira et al., 2009).

Finally, the level of LPO in the samples of *U. laetevirens* collected from areas affected by anthropogenic pressures were comparable to those measured in laboratory under induced stress with silver nanoparticles, which increased the toxicity in *Ulva rigida* in term of oxidative damage (LPO) (Sfriso et al., manuscript in preparation).

Part III: Health Status and Microbial Communities

The surface of macroalgae hosts a rich microorganism biodiversity with functions related to health and defence of the host. The investigation of the responses of associated microbial communities plays a key role to understand the anthropogenic perturbations in marine ecosystem. This study assessed the shift of the structure and diversity of microbial community associated to the sessile macroalga *U. laetevirens* across different seasons and sites impacted by anthropogenic stressors in Venice Lagoon (Italy) and Gulf of Aqaba (Jordan). Here, next generation sequencing (NSG) analysis of 16S rRNA sequence was used to provide extensive characterization of *U. laetevirens* associated microbial community.

The microbial communities associated with *Ulva* showed differences in structure between the LV and GA, based on the abundance and phylogenetic relationships among the two areas which are highlighted by cluster analysis (**Fig. 55,** page **121**). This variation of microbial community could be explained by a combination of environmental variables linked to different anthropogenic pressures in the distinct areas. Meanwhile, water microbial community in different seasons was different from the microbial community associated with *U. laetevirens*, thus supporting the selectivity of macroalgal surface for microorganisms. This difference is in agreement with the findings of Michelou et al., (2013) who found similar results studying the kelp *Macrocystis pyrifera* and adjacent seawater microbial community.

4.6 Macrophyte associated microbial community (Lagoon of Venice)

Across the Venice lagoon, the microbial communities associated with *U. laetevirens* showed different diversity, richness, structure and community

composition among sites during the whole seasons. Overall, the highest microbial diversity and richness was recorded at SG. These results were obtained by Alpha diversity indices, and may be associated with the availability of nutrients in the turbid water column and Chl-*a* concentration that increases the level of metabolic activities in the surface of *U. laetevirens*. This is suggested by the high abundance of bacterial opportunistic phyla, *Proteobacteria* and *Bacteroidetes*, which represent about 87% of the total sequences in this site, as this phylotypes are known to reproduce under increased influx of organic nutrients (Teira et al., 2010) and high levels of Chl-*a* (Witt et al., 2012). PM showed the lowest species richness and diversity across the seasons. This can be explained by the increased abundance of toxic Cyanobacteria (Order Nostocales) which are noticed to significantly alter the structure of the native community and to modify ecosystem functioning due to a variety of toxic compounds that some species produce (Sukenik et al., 2012; Ferber et al., 2004). This reduction of diversity was also proved by the high dissimilarity between SMM and PM obtained by SIMPER analysis. The lowest species richness and diversity was recorded in summer both at PM and SG, and may be related to the reduction of some nutrients and DO saturation in water column during that period. SMM exhibited consistent changing in diversity and species richness among the seasons in agreement with the slight variability of nutrient and Chl-*a* concentration and DO saturation in water column (Cloern, 2001).

Seasonal variability, however, was noticed between summer and winter; the high dissimilarity between microbial communities (81%) may be significantly driven by the variation of temperature in the water column which affected the diversity of microbial community. This is explained by environmental changes found in the LV during summer, as the significant modification of dissolved nutrients and water temperature, together with microalgal blooms, may lead to the

dominance of few bacterial taxa. The same results were also obtained by studying several microbial communities associated with *U. laetevirens* during the warmer season (Ladau et al., 2013; Luria et al., 2016). In addition, the physiological changes occurred in *U. laetevirens* during the growth season (spring and summer) may depend on the great energy demand as discussed by Singh (2013). It's worthy to state that macroalgal polysaccharides are a crucial source of carbon and energy for numerous marine bacteria (Hehemann et al., 2012) that produced several molecules facilitating the formation of macroalgal-bacterial associations (Lachnit et al., 2013). For instance, the genus *Roseobacter* is known to enhance the zoospore settlement and induce the morphogenesis and growth especially in Ulvaceae (Spoerner et al., 2012). The abundance of this genus notably increases during spring and summer in all the sampling stations.

The overall microbial community associated with *U. laetevirens* was mainly composed by Alphaproteobacteria, Bacteroidia and Gammaproteobacteria (**Fig. 45,** page **110**), and appears similar to that are described in other *Ulva* sp. (Tait et al., 2009) and the red alga *Amphiroa anceps* (Huggett et al., 2006). Likewise, the predominance of the classes Planctomycetacia and Bacteroidia was already described in *Ulva australis* (Burke et al., 2011; Lachnit et al., 2011). The microbial community on *U. laetevirens* was also composed by several marine bacterial taxa such as Alteromonadaceae, Flavobacteriaceae, Hyphomonadaceae, Rhodobacteraceae which are described as symbionts of aquatic organisms on *Ecklonia radiata*, Marzinelli et al., 2018).

The abundance of the genus *Catenococcus* (Family Vibrionaceae) notably increased during summer at SG and PM. This genus is originated from urban waste discharged; hence, it's frequently isolated from polluted sediments in LV (Borin et al., 2009). Additional taxa represented by Cyclobacteriaceae (Class Bacteroidia)

were presented within the microbial community at PM and SG during the whole seasons. These taxa are found to grow in habitats characterized by a wide range of temperature and salinity, and the ability to degrade lipids and polysaccharides (Pinnaka et al., 2014). Similarly, Saprospiraceae are the most abundant Bacteroidetes (9% of the total sequences). This common macroalgal associated bacteria are characterized by their ability to degrade the associated complex of nutrients and hydrolyze common polymers in the surface of macroalgae (Burke et al., 2011). Interestingly, some of the *U. laetevirens* associated bacterial species that were more abundant, belonged to taxa associated with macroalgal diseases. For instance, during autumn at SG, the increase of the genus Pseudoalteromonas (Class Gammaproteobacteria) was observed. This genus which accounted for about 7% of the total sequences was related to symptoms of hole-rotten disease in *Laminaria japonica* (Wang et al., 2017).

In terms of holobiont adaptation at the microbial level, the analysis of the distribution patterns of individual ASVs, found highly specialized ASVs that are restricted to certain site or season. The most widely shared ASVs, with high abundances for all sites across the seasons, were members of Caulobacterales, Flavobacteriales and Rhodobacterales. They composed the structural part of the *U. laetevirens* associated microbial community. Conversely, the non-ubiquitous ASVs strongly contributed to the observed differences between sites. For example the non-ubiquitous ASVs belonging to the family Rubritaleaceae was particularly abundant at SMM during spring. At PM, the non-ubiquitous ASVs recorded during summer belonged to the order Oceanospirillales and contributed to 52% of the total sequences of this site. Interestingly, these bacteria were isolated from seafloor sediment after the 2010 in the deep water horizon spill in the Gulf of Mexico

(Yang et al., 2017). This fact seems to suggest that the presence of these bacteria can be related to accidental oil spill at PM or petroleum factories in this area.

By comparing ASVs between *U. laetevirens* and seawater column microbial communities, it's clearly that at SG the ASVs in *U. laetevirens* were higher than seawater ASVs. One of the possible explanations of this result was the presence of essential morphogenetic and beneficial nutritive factors for *U. laetevirens* or other macroalgal species (Miranda et al., 2013), which assimilated from the surrounding water column.

4.6.1 Microbial community and environmental parameters (Lagoon of Venice)

Anthropogenic activities have detrimental impact on marine microbial communities (Nogales et al., 2011). Different sources of stress impacted the *U. laetevirens* associated microbial community composition in LV. Indeed, it changed in response of anthropogenic perturbations affecting the polluted sites PM and SG, especially high nutrient loads from river runoff and Chl-*a* which increased the microbial diversity, mostly at SG. Recent investigations highlighted nutrient scavenging and iron utilization by microbial communities associated to macroalgae (Burke et al., 2011b).

The members of bacterial orders Campylobacterales, Desulfobacterales, Rhodobacterales, Sphingomonadales and Thiotrichales were more abundant at SG and PM. The occurrence of these taxa was consistent with a putative role of higher nutrient loads and chemical contamination, as all these taxa are frequently associated to polluted environments. Some of these taxa consumed oxygen and nitrate favouring sulphate reduction as investigated by Zhang et al., (2017). The members of Planctomycetales were found to include interesting marine bacteria that are able to oxidize ammonia in the absence of oxygen, playing important role

in nitrogen cycling (Fuerst, 2010). These bacteria were more abundant at SG where higher DIN concentrations were measured. However, bacteria of the genus *Arcobacter* (Order Campylobacterales) were abundant at SMM. These bacteria are characterized by their ability to oxidize sulphides (Maugeri et al., 2000). The occurrence of this genus at SMM may be related to an increase of sewage effluents (especially during winter) which may transfer these bacteria into water column. However, the presence of this genus in polluted areas is well documented (Levican et al., 2016) and their unhealthy effects suggest its potential role as opportunistic pathogen (Olsen et al., 2014).

Species of the order Rhizobiales are abundant in polluted sites and are utilized as novel driver for functional remediation of heavy metals in polluted soil (Teng et al., 2015). A high abundance of this bacterial order was detected at SG and PM where high concentrations of metals were accumulated in the tissues of *U. laetevirens*. Likewise, species of the order Alteromonodales have also been found at PM and SG sites that affected by urbanization and eutrophication, this is in agreement with Gomes et al., (2008) and Zeng et al., (2010) results. Some species of the order Alteromonodales are metal-resistant species capable of binding Cu^{2+} and Zn^{2+} cations, thereby reducing their toxicity (Vincent et al., 1994). The toxic effect of heavy metals in macroalgae, and other environmental stressors, appears to be related to the production of ROS which triggered oxidative stress on the cell of macroalgae (Wahsha et al., 2017). These findings are highlighted by the significant occurrence of Rhodobacterales and Rhizobiales (heavy metal resistant bacteria) and the higher LPO level in the LV polluted sites.

The members of family Halomonadaceae were found at PM and SG during winter. This family characterized by their ability to grow in saline sites

contaminated with crude oil (e.g. Zhao et al., 2011), and are able to degrade toxic compounds (Margesin and Schinner, 2001). *Pseudomonas* spp. are among the mostly investigated microorganisms of Halomonadaceae that able to degrade pesticides (Mollaeia et al., 2010; Wasi et al., 2011). Furthermore, *Pseudomonas* spp. are considered among the most promising bioremediating species for the detoxification of highly contaminated water and/or industrial wastewater (Nawab et al., 2003; Wasi et al., 2011). As well as, these species have been proposed as polyaromatic hydrocarbon (PAH)-degrading bacteria (Bello-Akinosho et al., 2016; Nkem et al., 2016). Indeed, heavy metals, PAH and pesticides represent a significant contamination source for both PM and SG areas (e.g. Bellucci et al., 2002; Sfriso et al., 2013; Zonta et al., 2007).

4.7 Macrophyte associated microbial community (Gulf of Aqaba)

The microbial community on *U. laetevirens* in GA sites showed less variability in comparison with LV. The diversity of microbial community varied with environmental conditions and season. Generally, the diversity, richness and structure of the community increased during summer in all sites. The results obtained by applying the Shannon index were comparable with those of the microbial community associated with *H. stipulacea* in GA area (Rotini et al., 2017). Highest diversity and richness at PB could be related to the increasing water temperature during summer. The extensive swimming activities in this area may also to be a source of nutrients that stimulate the growth of specific types of bacteria on the surface of *U. laetevirens*. However, the decrease of diversity and richness of microbial community associated with *U. laetevirens* during winter in all the stations can be explained by the variability of the biological, physical and chemical properties of macroalgal surface (Egan et al., 2013). Since the

environment displays variable conditions due to seasons and nutrient availability (Hellio et al., 2004), it is not surprising that the associated bacterial community can change accordingly. The dominant microbial core on *U. laetevirens* encompassed different phylotypes belonging to Alphaproteobacteria, Bacteroidia and Bacilli orders. This was similar to the core microbial community composition reported by Mejia et al., (2016) and Rotini et al., (2017) for the microbial community associated with *H. stipulacea* in GA area. High microbial community biodiversity was observed at PB during summer. This periodic shifting in the associated microbial community was similar to those described for other macroalgae (Campbell et al., 2015a). The dominance of the class Alphaproteobacteria on *U. laetevirens* in GA may be related to its natural abundance in the water column and its adaptation to multiple environmental stressors, especially in oligotrophic coastal waters (Witt et al., 2012). Interestingly, the family Rhodobacteraceae was very abundant at PB (57% of the total reads) during summer. This family is significantly correlated with the variation of salinity, oxygen saturation, pH, nitrate and temperature of the water column (Buchan et al., 2005; Campbell et al., 2015b). Similarly, the family Rubritaleaceae of the class Verrucomicrobiales was very abundant at PB. The members of this family have a potential role in the polysaccharide degradation and are able to adapt to nutrient changes (He et al., 2017). The relative high abundance of the family Xenococcaceae (Order Oxyphotobacteria) was found in summer, supporting the evidence that Cyanobacteria generally thrive at higher temperatures (Butterwick et al., 2005; Jöhnk et al., 2008).

As expected from the alpha diversity of *U. laetevirens* microbial community, the highest shared ASVs was observed during summer. ASVs belonging to the genus *Alteromonas* were predominant at IC during summer (6.7% of the total

sequences). These free living bacteria grow in Mediterranean seawater and are known to take advantages of the sporadic inputs of organic matter that appear in marine environment (López-Pérez et al., 2016). Moreover, the non-ubiquitous ASVs found in summer and belonging to genus *Algibacter* was interestingly isolated from the green macroalgae *Ulva fenestrata* Ruprecht (Nedashkovskaya, 2004).

4.7.1 Microbial community and environmental parameters (Gulf of Aqaba)

The microbial community on *U. laetevirens* exhibited different pattern in community composition hosting different communities in the seasons. Higher diversity was found during summer in the presence of high temperatures and in combination with relatively higher concentrations of DIN and Chl-*a*. This indicates that the microbial communities associated to *Ulva* are stressed by touristic and industrial activities of the nearby area. The temporal changes of the microbial communities are highlighted by the results obtained by db-RDA analysis (**Fig. 57,** page **123**). The variability of these microbial communities in the polluted stations (PB and IC) indicates that they were already adapted to the polluted environment and maintained their biodiversity by various resistance mechanisms (Aires et al., 2016). This suggests that the microbial communities on *U. laetevirens* might be more influenced by the environment rather than the host. Overall, it's worthy to state that the effect of ecosystem disturbances is complex, often multiple stressors (natural or anthropogenic) coexist in the stressed area affecting the response of microbial communities (Nogales et al., 2011).

5. CONCLUSION

In this thesis, the integrated approach illustrates the combination of ecological indices (MaQI), oxidative stress biomarkers (lipid Peroxidation), and the microbial communities associated with the surface of macroalgae from the genus *Ulva* to assess the changes of macrophyte ecosystems in response to various anthropogenic pressures. In fact, macrophyte ecosystems were strongly affected by anthropogenic stressors which are primarily related to industrial and agricultural activities.

The contaminants, which were released by industrial and agricultural activities discharged into the lagoon of Venice, deteriorated significantly the health status of macrophyte ecosystems by the dominance of these thionitrophilic and opportunistic macroalgae. The "Poor" ecological status obtained by the application of MaQI in two (SG and PM) of the three stations was in agreement with the increase of nutrient concentration and water turbidity because of the presence of several anthropogenic pressures which affected these areas. On the other hand, the dominance of seagrasses and sensitive macroalgal species characterized the high ecological status of the third area (SMM) which showed low anthropogenic disturbances.

Similarly, the macrophyte diversity in the Gulf of Aqaba was clearly affected by the intensive industrial activities and the disturbances related to the touristic activities along the coasts of the gulf. These anthropogenic stressors, beside temperature and oligotrophic conditions, may be considered the main factors which reduced macrophyte biodiversity in the gulf area.

Despite the heterogeneity of transitional water bodies and the complexity of the processes that affected the metal uptake in macrophytes, their bioaccumulation in *Ulva* systematically reflect their occurrence in the environment. Therefore, the results demonstrate the significant use of *Ulva* as bioindicator of metal contamination (mainly for As, Mn and Pb).

A Clear evidence of toxicity (LPO) was observed in the *Ulva* which were collected from the stations impacted by anthropogenic stressors. In fact, the oxidative stress response identified the areas that are mostly impacted by anthropogenic stressors. The responsiveness of oxidative stress by LPO in *Ulva* showed its ability as an early signal to detect the changes in the health status of aquatic environments, and highlighted the effectiveness of LPO test. In this sense, these findings reinforce the importance of biomarkers to detect the effects of metal bioaccumulation.

Nutrient concentrations in the water column and the sensitivity to environmental chemicals affect the composition and diversity of the microbial communities associated with *Ulva*. The spatial and temporal variations of these microbial communities suggest that they may be essentially impacted by anthropogenic stressors. The effect of industrial and agricultural activities was clearly evidenced by the dominance of taxa which are involved in the xenobiotic degradation, and the ability to adapt to environmental stressors. Thus, indicating the functional relationship between macrophytes (*Ulva*) and their associated microbial community.

This study recommends a new multi-level approach to assess the stresses of aquatic environments in order to reach a better interpretation of the environmental

health status. Additionally, microbial community's analyses play a key role as putative biomarkers for the deterioration of aquatic environments.

Future studies should take into consideration the metagenomic functional profile as a tool to understand the biological and physico-chemical factors that affect the composition of microbial communities associated with macrophytes. This will help understand the host-microbial community interactions in response to the environmental changes.

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7. Appendices

Appendix (A)

Seasonal measurement of environmental parameters at Lagoon of Venice

Site	Season	RP µM	Nitrite µM	Nitrate µM	NH_4 µM	DIN µM	Silicate µM	Chl-a µg/L	pH	Eh mv	DO (%)	Salinity PSU	Temp °C	FPM mg/L
SMM	**W**	0.16	0.75	5.31	0.67	6.74	7.47	2.08	8.37	298	159	24.9	14.2	18.4
	Sp	0.18	0.29	0.57	4.19	5.05	3.03	0.75	8.40	259	159	22.2	20.5	20.0
	S	0.11	0.28	4.10	2.38	6.75	9.90	0.60	8.32	265	158	32.5	25.2	34.8
	Au	0.16	0.48	2.62	0.57	3.67	5.89	0.75	8.10	312	142	27.0	19.0	42.8
PM	**W**	0.60	3.54	9.12	1.53	14.2	15.4	1.39	8.15	307	157	25.4	15.8	30.8
	Sp	0.76	0.93	3.42	7.40	11.7	5.69	1.50	8.33	219	169	23.7	25.5	33.4
	S	0.39	0.39	3.86	4.70	8.95	15.1	3.29	8.10	300	108	27.2	30.6	31.0
	Au	0.32	0.59	6.03	1.16	7.78	8.82	1.50	8.01	300	113	22.5	20.0	40.0
SG	**W**	1.40	1.91	24.3	58.5	84.7	43.7	2.08	8.12	346	136	6.40	14.0	20.4
	Sp	3.15	2.78	1.07	8.32	12.2	14.4	29.9	8.73	222	258	17.5	26.1	121.5
	S	0.81	0.58	2.92	8.64	12.1	21.7	3.59	8.11	570	125	26.5	29.8	35.0
	Au	0.65	2.02	14.6	10.1	26.7	19.0	1.80	7.91	297	110	26.6	19.3	47.8

(W: winter, Sp: spring, S: summer, Au: autumn)

Appendix (B)

Chick list of macroalgae from the sampling sites of Lagoon of Venice

(0 = opportunistic, 1 = indifferent, 2 = sensitive species)

	SMM	PM	SG
OCHROPHYTA			
Phaeophyceae			
Cystoseira barbata (Stackhouse) C. Agardh	1		
Dictyopteris polypodioides (A.P. De Candolle) J.V. Lamouroux	2		
Dictyota dichotoma (Hudson) J.V.Lamouroux var. ***dichotoma***			
Dictyota dichotoma (Hudson) J.V. Lamouroux var. ***intricata*** (C. Agardh) Greville	1		
Dictyopteris polypodioides (A.P. De Candolle) J.V. Lamouroux	2		
Cladosiphon zosterae (J. Agardh) Kylin	2		
CHLOROPHYTA			
Bryopsis plumosa *(Hudson) C. Agardh*	1		
Bryopsis muscosa *J. V. Lamouroux*	1		
Chaetomorpha linum (O. F. Müller) Kützing	2		
Cladophora fracta *(O. F. Müller exVahl) Kützing*	1		
Cladophora glomerata (Linnaeus) Kützing	1		
Cladophora hutchinsiae		1	
Cladophora laetevirens *(Dillwyn) Kützing*	0		
cladophora sericea	0		
Ulva fasciata Delile	0	0	0
Ulva intestinalis Linnaeus	0	0	0
Ulva intestinalis Linnaeus, fo. ***cornucopiae*** *(lyngbye)* Sfriso *et* Curiel, 2007	0	0	
Ulva laetevirens Areshoug	0	0	0
Ulva linza Linnaeus	0	0	
Ulva prolifera O. F. Müller			0
Ulva rigida C . Agardh	0	0	0
Ulva rotundata Bliding		0	
RHODOPHYTA			
Alsidium corallinum C. Agardh	2		
Acrochaetium savianum (Meneghini) Nägeli			
Agardhiella subulata (C. Agardh) Kraft *et* M. J. Wynne	1	1	
Aglaothamnium feildmenniea		1	1
Anotrichium tenue			
Bangia atroporpurea (Roth) C. Agardh		1	
Callithamnion corymbosum (J. E. Smith) Lyngbye			
Caulacanthus ustulatus *(Turner) Kiitzing*	1	1	

Centroceras gasparinnii	1		
Centroceras clavulatum	2		
Cerandum ciliatum (J. Ellis) Ducluzeau var. ciliatum	2		
Ceramium siliquosum	1		
Ceramium polyceras (Kützing) Zanardini	1		
Chondracanthus acicularis *(Roth) Fredericq*	2		
Callithamnion corymbosum			
Chylocladia verticillata (Lightfoot) Bliding	2		
Chondria capillaris (Hudson) M. J. Wynne	1		
Chondria coerulescens (J. Agardh) Falkenberg	2		
Dasya baillouviana (S. G. Gmelin) Montagne	1		
Dasya punicea (Zanardini) Meneghini *ex* Zanardini	2		
Gracilaria bursa-pastoris (S.G. Gmelin) P.C. Silva	1	1	1
Gayliella flaccida (Harvey ex Kützing) T. O. Cho et L. McIvor	2		
Gracilaria gracilis (Stackhouse) Steentoft *et al*.	0	0	
Gracilaria vermiculophylla (Ohmi) Papenfuss			
Gracilariopsis longissima (S. G. Gmelin) Steentoft *et al*.		0	0
Grateloupia filicina *(J. V. Lamouroux) C. Agardh*	2		
Grateloupia viridis	0		
Hypnea flexicaulis Yamagishi & Masuda	1		
Hypnea musciformis (Wulfen) J. V. Lamouroux	2		
Neosiphonia harveyi (J. W. Bailey) M. S. Kim *et al*.		1	
Polysiphonia breviarticulata *(C. Agardh) Zanardini*	1		
Polysiphonia denudata	1		
Polysiphonia morrowii *Harvey*	1		
Rhodophyllis divaricata *(Stackhouse) Papenfuss*	1		
Rhodymenia ardissonei *J. Feldmann*	1		
Rhodymenia ligulata *Zanardini*	1		
Solieria filiformis (Kützing) P. W. Gabrielson	0	0	0

Appendix (C)

Chick list of macroalgae from the sampling sites of Gulf of Aqaba

	MSS	PB	IC
OCHROPHYTA			
Phaeophyceae			
Cystoseira myrica (S.G.Gmelin) C.Agardh	√		√
Cystoseira trinodis (Forsskål) C.Agardh	√		
Colpomenia sinuosa (Mertens ex Roth) Derbès & Solier		√	
Dictyota dichotoma (Hudson) J.V.Lamouroux			√
Lobophora variegata (J.V.Lamouroux) Womersley ex E.C.Oliveira			√
Hydroclathrus clathratus (C.Agardh) M.Howe		√	
Turbinaria ornata (Turner) J.Agardh	√		√
Padina pavonica *(Linnaeus) Thivy*	√		
Sargassum sp.		√	
Sphacelaria tribuloides Meneghini	√	√	√
CHLOROPHYTA			
Chaetomorpha linum (O. F. Müller) Kützing	√		
Cladophora lehmanniana (Lindenberg) Kützing	√		
Caulerpa serrulata (Forsskål) J.Agardh	√		√
Halimeda tuna (J.Ellis & Solander) J.V.Lamouroux			√
Ulva laetevirens Areschoug	√	√	√
Ulva intestinalis Linnaeus		√	
Ulva flexuosa Wulfen	√		
RHODOPHYTA			
Acanthophora spicifera (M.Vahl) Børgesen	√	√	
Aglaothamnium sp.	√	√	
Caulacanthus ustulatus *(Turner) Kiitzing*	√		
Centroceras clavulatum (C.Agardh) Montagne		√	
Ceramium sp.	√		
Coelothrix irregularis (Harvey) Børgesen		√	
Chondria capillaris (Hudson) M. J. Wynne		√	
Dasya sp.	√		
Gelidium pusillum (Stackhouse) Le Jolis		√	
Herposiphonia tenella (C.Agardh) Ambronn	√		
Hypnea anastomosans Papenfuss, Lipkin & P.C.Silva		√	
Jania adhaerens J.V.Lamouroux		√	
Neosiphonia harveyi (J. W. Bailey) M. S. Kim et al.		√	
Laurencia papillosa (C.Agardh) Greville			√
Pterocladiella sp.		√	
Polysiphonia sp.	√		

Sarconema filiforme (Sonder) Kylin	√	√	
Titanophycus validus (Harvey) Huisman, G.W.Saunders & A.R.Sherwood	√		

Appendix (D)

Average concentration of metals (µg/g, dw) in *U. laetevirens* collected from Lagoon of Venice

	As	V	Ni	Mn	Zn	Cu	Pb	Hg	MPI
SMM-W	0.9	2.2	1.2	18.6	5.7	9.7	0.3	0.1	1.7
PM-W	6.5	1.2	2.1	47.9	18.8	5.6	0.5	0.2	3.1
SG-W	3.5	0.8	2.9	89.2	38.5	11.5	1.3	0.3	4.3
SMM-sp	2.5	0.7	2.5	29.5	15.9	4.5	0.1	0.1	1.7
PM-sp	14.6	3.8	1.0	22.1	58.7	16.7	1.3	0.2	5.0
SG-sp	4.3	1.1	2.7	400.5	93.5	52.1	4.2	0.3	8.7
SMM-S	1.8	13.8	2.5	36.8	29.9	10.1	0.0	0.2	2.9
PM-S	11.1	4.2	1.4	30.9	48.1	73.7	0.2	0.2	4.7
SG-S	4.9	3.8	1.1	117.3	76.7	43.9	3.3	0.2	7.0
SMM-Au	1.2	23.4	3.5	67.2	7.3	10.2	0.6	0.1	3.8
PM-Au	7.5	0.0	1.1	24.5	7.5	19.1	0.5	0.2	3.2
SG-Au	6.4	0.2	1.0	52.1	16.1	9.4	1.8	0.2	2.9

*****MPI: metal pollution index

Appendix (E)

Statistical analysis of metals and LPO for Lagoon of Venice samples

LPO differences between sites

Test for equal means

	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	1317.13	2	658.565	6.171	**0.02054**
Within groups:	960.524	9	106.725	Permutation p (n=99999)	
Total:	2277.65	11	0.01441		

Components of variance (only for random effects):

Var(group):	137.96	Var(error):	106.725	ICC:	0.563827

omega2: 0.4629

Levene´s test for homogeneity of variance, from means p (same): 0.1868

Levene´s test, from medians p (same): 0.2724

Welch F test in the case of unequal variances: F=14.47, df=5.411, p=0.006739

	SMM	PM	SG
SMM		0.03098	0.01079
PM	0.03098		0.6949
SG	0.01079	0.6949	

Kruskal-Wallis test (Metals accumulation and LPO level)

Metal	P value
As	**0.001**
V	**0.069**
Ni	**$3.2*10^{-5}$**
Mn	**0.01**
Cu	**0.525**
Pb	**$3.2*10^{-5}$**
Zn	**0.326**
Hg	**0.235**

Appendix (F)

Statistical analysis of metals and LPO level of Gulf of Aqaba samples

	MSS	PB	IC
MSS		0.1797	0.00729
PB	0.1797		0.1797
IC	0.00729	0.1797	

Kruskal-Wallis test for equal medians

H (chi2): 7.2

Hc (tie corrected): 7.2

p (same): **0.02732**

There is a significant difference between sample medians

Coefficient of correlation (R^2) between LPO level and metals in *U. laetevirens* and sediment in GA. * significant ($p<0.05$); n.t: not significant.

	Cd	Cr	Cu	Mn	Ni	Pb	Zn	Fe
U. laetevirens	$0.139^{n.t}$	0.547*	0.637*	0.955*	0.834*	0.850*	$0.278^{n.t}$	0.444*
Sediment	$0.362^{n.t}$	0.545*	0.453*	0.754*	0.561*	$0.116^{n.t}$	0.456*	0.661*

Appendix (G)

Alpha diversity indices of *U. laetevirens* associated microbial community at Lagoon of Venice.

Sample	Season	# ASV	Diversity index		
			Shannon	**Simpson**	**Chao1**
SMM	winter	504	5.7	0.994	504
PM		250	4.96	0.988	250
SG		572	6.03	0.996	572
SMM	Spring	352	4.8	0.976	352
PM		428	5.24	0.986	428
SG		560	5.85	0.995	560
SMM	Summer	437	5.17	0.982	437
PM		289	3.69	0.911	289
SG		578	5.88	0.995	578
SMM	Autumn	454	5.11	0.984	454
PM		326	5.02	0.985	326
SG		573	5.66	0.991	573
SMM	Summer	491	5.54	0.992	491
PM		375	5.29	0.989	375
SG		247	5.03	0.988	274
SMM	Autumn	340	4.91	0.983	340
PM		393	5.01	0.981	393

Appendix (H)

Codes of sampling sites of Lagoon of Venice and Gulf of Aqaba

SampleID	Season	Matrix	Site	City
A1	Winter	*Ulva*	SMM	Venice
A2	Winter	*Ulva*	PM	Venice
A3	Winter	*Ulva*	SG	Venice
A4	Spring	*Ulva*	SMM	Venice
A5	Spring	*Ulva*	PM	Venice
A6	Spring	*Ulva*	SG	Venice
A7	Summer	*Ulva*	SMM	Venice
A8	Summer	*Ulva*	PM	Venice
A9	Summer	*Ulva*	SG	Venice
A10	Summer	Water	SMM	Venice
A11	Summer	Water	PM	Venice
A12	Summer	Water	SG	Venice
A13	Autumn	*Ulva*	SMM	Venice
A14	Autumn	*Ulva*	PM	Venice
A15	Autumn	*Ulva*	SG	Venice
A16	Autumn	Water	SMM	Venice
A17	Autumn	Water	PM	Venice
A18	Summer	*Ulva*	PB	Aqaba
A19	Summer	*Ulva*	IC	Aqaba
A20	Summer	*Ulva*	MSS	Aqaba
A21	Winter	*Ulva*	PB	Aqaba
A22	Winter	*Ulva*	IC	Aqaba
A23	Winter	*Ulva*	MSS	Aqaba

English

Studente: **Abdul-Salam Fayiz Ahmed Juhmani** matricola: **956263**

Dottorato: **scienza ambientale**

Ciclo: **31**

Titolo della tesi: **Ecological and Molecular Analyses of Macrophyte Ecosystems for the Assessment of the Environmental Health of Coastal and Transitional Areas**

Abstract:

Degradation of coastal and transitional water quality by anthropogenic contaminants is an important concern worldwide. Identifying the patterns of biological response to contamination in the aquatic environment is essential for reliable evaluation of ecosystem health. The present study aims to use macrophytes to assess the ecological health status for marine transitional (Lagoon of Venice) and coastal (Gulf of Aqaba) environments employing an integrated approach based on ecological indices and biomarkers as well as macrophyte associated microbial communities.

The results obtained by the application of Macrophyte Quality index, oxidative stress biomarker and the diversity of macroalgal associated microbial community indicate that the macrophyte ecosystems were strongly affected by anthropogenic stressors which are primarily related to industrial and agricultural activities. Thus, deteriorate the ecological health status of caostal and transitional areas.

This study recommends a new multi-level approach to assess the stresses of aquatic environments for better interpretation of the environmental health status. Furthermore, microbial community's analyses playing a key role as putative biomarkers for the degradation of aquatic environments.

Firma dello studente

Estratto per riassunto della tesi di dottorato

Studente: **Abdul-Salam Fayiz Ahmed Juhmani** matricola: **956263**

Dottorato: **Scienze Ambientali**

Ciclo: **31°**

Titolo della tesi: **Ecological and Molecular Analyses of Macrophyte Ecosystems for the Assessment of the Environmental Health of Coastal and Transitional Areas**

Abstract:

La degradazione della qualità delle acque costiere e di transizione da parte di contaminanti antropogenici è una preoccupazione importante in tutto il mondo. Identificare i modelli di risposta biologica alla contaminazione dell'ambiente acquatico è essenziale per una valutazione affidabile della salute dell'ecosistema. Il presente studio mira a utilizzare le macrofite per valutare lo stato di salute ecologica degli ambienti marini costieri (laguna di venezia) e di transizione (golfo di aqaba) utilizzando un approccio integrato basato su indici ecologici e biomarcatori, nonché su comunità microbiche associate alle macrofite.

I risultati ottenuti dall'applicazione dell'indice di qualità delle macrofite, del biomarcatore di stress ossidativo e della diversità della comunità microbica associata alle macroalghe indicano che gli ecosistemi a macrofite sono fortemente influenzati da fattori di stress antropogenici, principalmente correlati alle attività industriali ed agricole che peggiorano lo stato di salute ecologica delle aree costiere e di transizione.

Questo studio raccomanda un nuovo approccio a più livelli per valutare gli stress degli ambienti acquatici per una migliore interpretazione dello stato di salute ambientale. Inoltre, le analisi della comunità microbica associata svolgono un ruolo chiave come presunti biomarcatori del degrado degli ambienti acquatici.

Firma dello studente

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nat ... a Ibb (prov. ...) il 14/05/1984

residente a mestre in Via Cindi n. 21/2

Matricola (se posseduta) 956263 Autore della tesi di dottorato dal titolo:

Ecological and Molecular Analyses of Macrophyt Ecosystems for the Assessment of the Environmental Health of Coastal and Transitional Areas

Dottorato di ricerca in Environmental Sciences

(in cotutela con .....)

Ciclo 31°

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