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Comparative Studies on the Biological Glue of Some Opportunistic Adult Marine Macro-Fouling After Dislodgement and Construction of Temporary Faunal Conglomerations

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Abstract

This study aimed to elucidate the composition of the biological glue of the sea anemone *Metridium senile*, the tubeworm *Serpula vermicularis*, the acorn barnacle *Balanus amphitrite*, the gastropod limpet *Patella aspera* and the sea squirt *Styela plicata* and to analyze these biological glue in the different seasons of the year. Furthermore, to propose antifouling strategies that can be developed from the present results. The sessile macro-fouling have the capability to dislodge and crawl searching for new suitable substrata for attachment. A total of 30 taxa representing five phyla were sampled every season across Northern Khobar estuarine beach of the Arabian Gulf. The proximate compositions of protein, lipid and carbohydrate in the basal discs or attachment integuments were measured using the Folin-Ciocalteu Phenol method of 66 Lowry et al., the chloroform-methanol method according to Folch et al. and the phenol-sulfuric acid method of Dubois et al. respectively. The total fat, protein, carbohydrate, and moisture contents ranged from 1 ± 0.57% -2 ± 0.98%, 23 ± 1.60 -23 ± 0.80, 0,2 ± 0,10 -1,98 ± 0,20 and 71 ± 0.61% -72 ± 2.07% by weight, respectively. The indispensable and conditional amino acids predominated in tissues were estimated. A high-pressure chromatographic separation was applied for amino acid analysis of biological glue. The most predominant amino acids were Aspartic acid (Asp D), Glutamine (Gln Q), Glycine (Gly G), Methionine (Met M), Phenylalanine (Phe F), Serine (Ser S) and Valine (Val V). Proteins in biological glue of the five species were resolved by SDS-PAGE and digested with trypsin. The majority of the resolved protein spots were within the molecular size ranging from 250 kDa to less than 30-kDa. The total lipid was estimated by chloroform-methanol method according to Folch et al. Capillary gas chromatography was applied to measure the fatty acid profiles of the biological glue. The lipid content of the biological glue of the five species studied were roughly similar but in different quantities. It was found that lipids of biological glue were predominant by C18:1 V-5, C18:2 V-6, C19:1 v- 8, C20:1 V-9, C24:1 V-6, C19:2 V-6. The estimation of total carbohydrate content of tissue in the basal discs or attachment integuments of the five macro-benthos ranged from 2.5 to 8.7 mg carbohydrates g^{-1} wet weight. The carbohydrate content of the biological glue was apparently dependent partly on species and partly on the season of settlement. All data obtained were statistically analyzed using One-way analysis of variance (ANOVA) of means with significance at $P<0.05$ with R squared variable; Newman-Keuls Multiple Comparison Test Mean Difference at $P>0.05$; Tukey's Multiple Comparison Test Mean Difference at $P>0.05$, $P<0.01$ and $P<0.001$; Bartlett's test for equal variances Mean Difference at $P<0.05$; Bonferroni's Multiple Comparison Test at variable Mean Difference; Dunnett's Multiple Comparison Test at $P<0.01$ and $P>0.05$.

Keywords

Biological glue; Macro-fouling; Basal disc; Attachment integument; Protein; lipid; Carbohydrate; ANOVA; Newman-Keuls multiple comparison test; Tukey's multiple comparison test

Introduction

To adhere on immersed substrata in their aquatic habitats, many sessile marine invertebrates secrete biological glue such as mucus consisting primarily of a network of polysaccharides and proteins entangled to form a weak sticky material containing more than 95% water [1-5]. Fouling community conglomeration may differ temporally and spatially in different gradients such as latitudinal, inshore to offshore or on a finer scale between habitats based on breeding times and larval transport as well as with changes in water quality [6]. Many marine benthic organisms are equipped with adhesive organs, the secretions of which allow them to attach to the substratum. Three types of adhesion may be distinguished: permanent adhesion involving the secretion of a cement, transitory adhesion permitting simultaneous adhesion and movement along the substratum and temporary adhesion allowing an organism to attach strongly but momentarily to the substratum [7-9]. Marine bio-fouling can be defined as the growth of sessile undesirable accumulation of organisms on the surface of artificial structures immersed in sea water [10,11]. Biofouling is a complex problem irrespective of the species. It occurs on a variety of structures and materials, and has serious economic implications for a number of industries, including shipping, aquaculture and other industries which use seawater (and freshwater) for cooling or other purposes [12]. At the same time, biofouling is a major pathway for the introduction of invasive species, with all the additional problems inherent to that issue. Among the different bio-fouling species, cnidarians, tubeworms, barnacles, gastropods, and ascidians that resemble a specific threat as they are difficult to remove, able to damage fouling release coatings and increase the drag force of ships. Fouling communities are mostly marine [13,14]. Many species have been classified as invasive [15,16]. The potentially catastrophic effects of introduced organisms on local ecology are one of the major threats to global biodiversity. Drake and Lodge [17] studied the hull-fouling communities of intercontinental species carried on transoceanic ships and concluded that the number of species invasively introduced, and abundance of species represented a greater risk than those living in the native water. The ecological impacts of an invasive species are complex and occur as a result of changes to the local biodiversity and/or alteration of ecological processes caused by that species. While the initial impacts may be minor and near-invisible,

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as the population increases over time, the impacts will increase in severity. They may include: 1- competing with native species for space and food, 2- preying upon native species, 3- altering habitat of other species, 4- altering environmental conditions (e.g. decreased water clarity), 5- altering the food web, 6- displacing native species, reducing native biodiversity and even causing local extinctions. In relation to biodiversity, there have been losses of individual species at least in certain localities, and a general tendency towards homogenization of species composition and loss of unique traits of particular communities [18]. The Mediterranean, for example, which has a high level of endemism and unique communities, has a high proportion of introduced species [19,20]. When a structure is immersed in seawater, it is rapidly covered by unavoidable fouling. This growth is a complex phenomenon and much remains to be understood. In marine environments, over 4000 organisms [21] are related to fouling problems. Organisms may be divided according to their size into micro-organisms (or so called biofilm, slime, micro-fouling) and macro-fouling. The succession of fouling states is generally considered in five main stages: 1- the first event is the adsorption of organic and inorganic macromolecules immediately after immersion, forming the primary film; 2- second, the transport of microbial cells to the surface, and the immobilization of bacteria on the surface; 3- in the third stage, the bacterial attachment to the substratum is consolidated through extracellular polymer production, forming a microbial film on the surface; 4- the fourth stage corresponds to the development of a more complex community with the presence of multicellular species, microalgae, debris, sediments, etc. on the surface; 5- the last stage is the attachment of larger marine invertebrates such as barnacles, mussels and macro-algae.

Most cnidarians, including corals, zooids and sea anemones are capable of secreting a mucus-based surface layer essential for a number of processes such as holdfast, feeding, protection against pathogens, desiccation, and a number of environmental stresses. Mucus production may account for as much as 40% of the net daily fixed carbon in the coral *Acropora acuminata* [22]. Other uses that should be considered are protection from aggression and as an offensive weapon. The coral *Lobactis* (*Fungia*) *scutaria* in response to contact with other corals or rough human handling secretes mucus containing cytotoxic molecules to other corals. A highly active cytolysin as well as aliphatic-antibiotic compounds has been isolated from the mucus secretion of the sea anemone *Heteractis magnica* [23]. In spite of the multitude of ecological and physiological roles played by the cnidarian mucus, relatively little is known about the link between biochemical structures and functions. Tissue extracts of *A. equina* has been long investigated for their peptide and protein toxins [24,25]. The sabellariids are gregarious obligate tube-dwelling marine polychaetes [26]. The sabellariids have an unusual strategy for constructing their mineralized tubes. Rather than synthesizing a complete mineralized structure by the controlled precipitation of concentrated ions with matrix proteins, like many other tube or shell-dwelling marine invertebrates [5,27,28], the sabellariids gather the mineral phase adventitiously as preformed particulates from the water column, usually sand and bits of calcareous shell of the right size, and secrete only a biological glue for joining the particles. Captured particles are conveyed along the tentacles of the crowns to the building organ, a U-shaped invagination near the mouth, where they are held, turned, and evaluated for size, shape, and composition. The sabellariids are commonly called sandcastle worms because individual tubes with their resident worm are honeycombed together into large reef-like mounds, although the mounds more-closely resemble proletarian apartment buildings than castles. Their gregariousness is due to a component of the tube cement that induces larvae to settle, metamorphose and build a new tube on existing conspecific tubes [29-31]. Barnacles (cirripedians) have a unique larval form - the cyprid. This life history stage is adapted to locate a spot on which to permanently settle, develop, grow, and survive for the rest of its life. Barnacles have a worldwide distribution and various lifestyles, from parasitic species on the gills of decapod crustaceans to free-living groups. The free-living groups are adapted to permanently attach via cement onto other living organisms, rocks or man-made materials, and barnacle “fouling” on marine installations and cargo ships is increasingly of economic concern [32]. Within the free-living barnacles, a further division is recognized between acorn (Order Sessilia) and stalked (Order Pedunculata) forms. Certain stalked species are termed “pleustonic” due to a lifestyle at the air/water interface [33]. Barnacles use glue to perform underwater adhesion in marine habitats. Temporary adhesion is used for exploration by the cyprid prior to committing itself permanently to one spot [34-36]. Once a spot has been chosen by the cyprid, permanent glue is produced. The main innovation in natural underwater biological glue, compared with synthetic alternatives, is the ability of natural biological glue to displace the bound water layer on the substratum and to maintain a stable bond under various levels of humidity. Other important processes for adhesion underwater are spreading, coupling with various materials, curing, and resisting biodegradation [36-38]. Understanding the mechanism(s) involved is important since this could potentially lead to interesting surgical applications, or alternatively, could be used to prevent fouling. The ingenuity of barnacle glue is, above all, demonstrated by the huge range of materials with different surface properties to which they attach. Gastropods secrete viscoelastic mucous gels with functions that include feeding, protection, reproduction, locomotion, lubrication, defense, and adhesion [39]. While the functional demands of such disparate tasks obviously vary widely, there has been little work on the biochemical variations and different secretory structures that give rise to these functional differences. The general structure and mechanics of gastropod mucus have been reviewed [39,40], and the biochemical structure of some adhesive gels has been analyzed [41]. Among gastropods and probably most other animals, the term mucus is used for any viscous secretion from an epithelium [42]. Such secretions can be created by any polymers that form giant complexes. Ascidians are a good model system for research on permanent underwater adhesion strategies. Ascidians are noted for their cosmopolitan distribution and opportunistic behavior. Population outbreaks of these sea squirts have caused substantial bio-fouling problems for aquaculture operations in Southern Africa [43], New Zealand [44], Chile [45] and Scotland [46]. Although reported in Canadian waters prior to 1900 (Stimpson 1852), it is only recently that this cryptogenic species has been observed in high densities. In terms of the Mediterranean there are records for the east coast of Spain, Italy, Greece, Turkey, and Egypt [47]. Fouling animals are believed to have been spread widely throughout all temperate regions by shipping activities, particularly as a hitchhiker on the hulls of vessels [48,49]. In the available literature, some authors concerned to study some aspects of marine pollution on fouling communities including ascidians inhabiting the Arabian Gulf [50-58].

Many studies also focused on biofouling communities, describing community development in marine environments [59]. In these studies, succession is thought to be mainly dependent on the unique life-history characteristics of particular species in a specific habitat

and physical factors such as salinity, temperature, light and flow rate. Because these factors tend to differ strongly among seasons, seasonality is thought to be an important factor in the development of marine fouling communities in temperate regions [60-62]. The larvae of fouling invertebrates are planktonic and are thus 'forced to wander' the sea until they become competent to metamorphose and locate a site suitable for settlement. Particular chemical cues then promote larval settlement and subsequent metamorphosis by competent larvae of many species. These chemical cues may indicate the presence of appropriate food, conspecific adults to mate with, or other environmental factors that signal the suitability of a site to live in for juveniles and adults [59].

This study aimed to elucidate the composition of the biological glue of the sea anemone *Metridium senile* Linnaeus, 1761; the calcareous tubeworm *Serpula vermicularis* Linnaeus, 1767; the acorn barnacle *Balanus amphitrite* Darwin, 1854; the gastropod limpet *Patella aspera* Lamarck, 1819 and the sea squirt *Styela plicata* Lesuer, 1823; to compare the adhesives of the five species and to analyze the biological glue in the different seasons of the year. Another purpose of this study is to develop antifouling strategies and to describe the various safety applications that are developing from the present results. The approach to anti-fouling is to prevent the initial settlement of fouling species by repelling or killing them; to inhibit their growth or reducing their adhesion ability; and to remove the fouling through cleaning. Identification of the five species of macrobenthos was done according to Wilson and Markhaseva [63,64].

Materials and Methods

Field sites and survey of fouling adults

A total of 30 taxa representing five phyla were sampled every season across Northern Khobar estuarine beach of the Arabian Gulf. These faunae are considered as macro-zoobenthos and live in the intertidal zone (Figures 1-5). Phylum Cnidaria comprised the smallest percentage with nearly 20% of all benthic invertebrates and represented by the anthozoan sea anemone *Metridium senile*, followed by Annelida which comprised over 30% of all invertebrates and represented by the tubeworm *Serpula vermicularis*, followed by Cirripede *Balanus amphitrite* which comprised nearly 25% of all invertebrates. Gastropoda which comprised nearly 40% of all invertebrates and were represented by the limpet *Patella aspera*. Sea squirts comprised nearly 20% of all invertebrates and were represented by the ascidians *Styela plicata*. Adult distribution and associated circumstances were quantified. The number of individuals of the five species and the number of individuals per clump were counted. Due to the aggregating nature of sessile organisms and because they were often covered by algae or other fouling organisms, clumps were removed and brought to the laboratory where they could be cleaned and sorted to count the number of individuals precisely.

Estimation of Crude materials of basal discs and attachment integuments

The proximate compositions of protein, carbohydrate and lipid in the basal disc and integuments of the five macrobenthos under investigation were determined by using standard methods and values were expressed as percentage of wet weight (mg/100mg) tissue. Total protein in the tissue was estimated by the Folin-Ciocalteu Phenol method of Lowry et al. [65]. The estimation of total carbohydrate content of tissue, phenol-sulfuric acid was followed according to Dubois et al. [66]. The total lipid was estimated by chloroform-methanol method according to Folsch et al. [67].

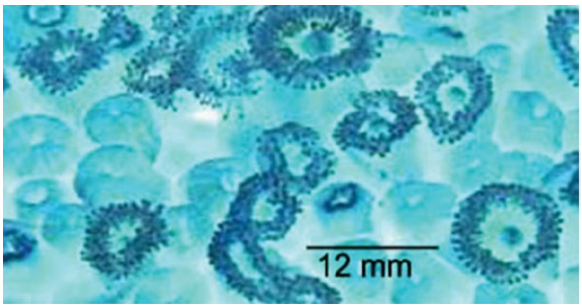

Figure 1: Photomacrograph of a whole mount of the sea anemone *Metridium senile*.

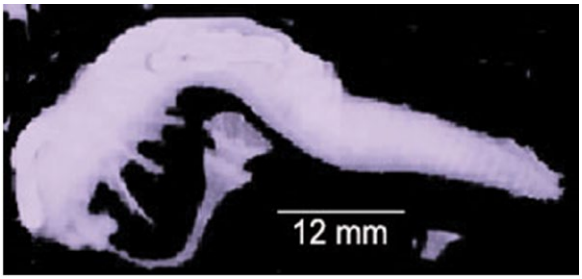

Figure 2: Photomacrograph of a whole mount of the calcareous tubeworm *Serpula vermicularis*.

Figure 3: Photomacrograph of a whole mount of the acorn barnacle *Balanus Amphitrite*.

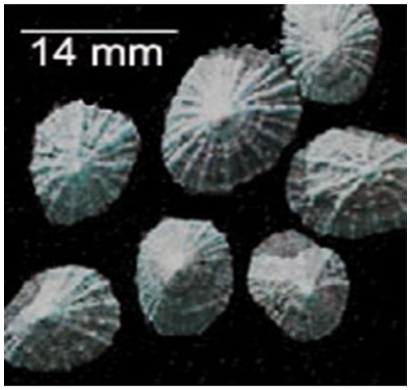

Figure 4: Photomacrograph of a whole mount of the limpet *Patella aspera*.

Figure 5: Photomacrograph of a whole mount of the sea squirt *Styela plicata*.

Protein analysis

SDS-PAGE was applied [68]. Proteins were extracted from the biological glue of the five macro-fouling through Dounce homogenizer for 5 min then were centrifuged at 1500 x g for 10 min in 1.5M Tris-HCl buffer (pH 8.5) for disruption. Protease inhibitors were applied to avoid protein degradation during protocol. These protease inhibitors were 1 µg/ml Aprotinin and 1 mM EDTA. Proteins in the extract were separated according to size using polyacrylamide gel electrophoresis (PAGE). A gel was prepared by the polymerization of bisacrylamide and acrylamide. Sodium dodecyl Sulphate (SDS) added to the gel binds to proteins, giving each a negative charge proportional to its mass (SDS-PAGE). The gel was placed in an electrophoresis tank filled with buffer that would carry current. Prior to loading on the gel, protein samples of the biological glue were boiled in 1× Novex SDS Tricine loading buffer containing 2% (v/v) 2-mercaptoethanol at 95°C for 7 min. SDS used as a denaturing detergent. Tris-Tricine 1.5M (pH 8.5) buffer was used to maintain a proper pH at 125 V. The negatively charged proteins migrated away from the anode. The proteins were transferred electrophoretically to a membrane, where they became immobilized using 90mM Tris-borate, 2.5mM EDTA, 0.1% SDS, and 25% methanol as transfer buffer. The gel and membrane were placed between filter paper and sponge pads. Voltage was applied in a buffer tank and proteins moved from the gel to the membrane. The membrane was blocked to prevent nonspecific binding of antibodies, and then incubated with a polyclonal primary antibody directed against phosphorylated proteins. This polyclonal primary antibody was diluted with 1.5M Tris-HCl buffer in a ratio 1:10000 and incubation at room temperature (20°C) for an hour. Following incubation with the primary antibody, the blot was washed with 10mM phosphate buffer saline. Following several washes, a labeled goat-anti-rabbit HRP-conjugated secondary antibody 500 µl bound to the primary and provided a means of detection. After washing the membrane to remove unbound antibody, antibody bound protein on the blot was detected.

Protein assay

To reduce phosphatase activity during protein phosphorylation, sodium pyrophosphate 10 mM was applied to the protein extracts. For chemiluminescent detection, the secondary antibody was conjugated to horseradish peroxidase (HRP), addition of an HRP substrate led to an enzymatic reaction with light as a product. The light was detected using digital imaging.

Amino acid analysis

Biological glue of sea anemones, tubeworms, barnacles, gastropod and sea squirt were extracted manually from their basal discs or integuments where they settle on the substrata. The discharge of biological glue was induced mechanically by pinching the adults with forceps. The expelled biological glues were collected in clean glass Petri dishes (diameter 15 cm) filled with filtered seawater (0.22 mm). The Petri dishes were then thoroughly rinsed in distilled water and freeze-dried. The lyophilized biological glue print material was scraped off using a glass knife and stored at -20°C. For amino acid analysis, a high-pressure chromatographic separation was used. This technique generates mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument has postcolumn derivatization capability. Analytical reagents are changed routinely using only high-pressure liquid chromatography (HPLC) grade solvents (116). Purification of protein and peptide samples was performed through buffer components containing salts and urea. After complete purification, the protein was precipitated from the buffer using acetone as an organic solvent. Postcolumn derivatization of the amino acids was applied.

Hydrolysis of protein and peptide samples was necessary for amino acid analysis of these molecules. To clean glass hydrolysis tubes, the tubes were boiled for 1 hour in 1 N hydrochloric acid or soaked in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes were rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. To avoid complete or partial destruction of several amino acids through this technique, application of adequate vacuum (≤ less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel is used to reduce the level of oxidative destruction.

Amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours was often employed to analyze the starting concentration of amino acids that might be partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. During the hydrolysis time course, the analyst would observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

Postcolumn ninhydrin detection

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. Na-based cation-exchange system was used for the amino acid mixtures obtained with protein hydrolysates. Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient was often employed to enhance separation. Acid hydrolysis using hydrochloric acid containing phenol was the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine. Hydrolysis solution applied was 6 N hydrochloric acid containing

0.1% to 1.0% of phenol. Liquid phase hydrolysis was applied by placing the protein or peptide sample in a hydrolysis tube. The sample was dried so that water in the sample will not dilute the acid used for the hydrolysis. Adding 200 µL of hydrolysis solution per 500 µg of lyophilized protein. The sample tube was frozen in a dry ice-acetone bath, and flame sealed in a vacuum. Samples were typically hydrolyzed at 110 °C for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (48 and 72 hrs) are investigated if there is a concern that the protein is not completely hydrolyzed.

Fatty acid analysis

Total lipids of biological glue were extracted using a chloroform-methanol solution (2:1, v/v) according to the method described by Peng et al. [69]. Capillary gas chromatography (GC) was used to determine the fatty acid profiles using an HP6890 (FID detector) and a SPTM-2380 column (30 m × 0.25 mm × 0.20 µm). Nitrogen was the gas carrier and the column temperature was programmed to increase from 140 °C to 240°C at a rate of 4°C /min (with holds of 5 and 10 min at 140°C and 240°C, respectively). The fatty acids were detected at 260°C using a split injector (50:1) and identified by comparing their retention time to a chromatographic Sigma standard. The peak areas were determined using Varian software.

Carbohydrate analysis

Biological glue was first wet weighed and then placed into centrifuge tubes containing 3 ml of KOH solution (30%). The centrifuge tubes were kept in a hot water bath for 20 min. Then 0.5 ml of saturated Na2SO4 and 3 ml of ethyl alcohol (95% pure) were added, followed by boiling for a further 15 min. After being cooled, all samples were centrifuged at 3500 rpm and the supernatants were discarded. The precipitations in the tubes were dissolved in 2 ml of distilled water followed by the addition of 2.5 ml of ethyl alcohol (95% pure). The tubes were then centrifuged at 3500 rpm for a further 10 min and the supernatants were discarded. The final precipitations in the tubes free of lipid and protein were then dissolved in 2 ml of HCl (5M) and neutralized with 0.5M NaOH followed by dilution to 50 ml with distilled water before analysis). The total carbohydrate levels in all samples were analyzed by the Phenol-sulphuric acid method of 65 Dubois et al. [66]. Glucose (1 μgmL^{-1}) was used as a reference standard at concentrations of 20, 40, 60, 80 and 100 µg μL^{-1}. The absorbance was measured at 490 nm in a spectrophotometer (HITACHI-220S UV). All data were treated with One-way analysis of variance (ANOVA) followed by Bartlett's test for equal variances.

Statistical analysis

Analysis of variance (ANOVA) is a broad group of techniques for identifying and measuring different sources of variation within the data set. It consists of a set of procedures by which a variance of the random variable is broken down by certain sources of variation of its value. With the components of variance, depending on the sources, one can conclude if there is a significant difference between the values of dependent variable for different levels of the observed factor variables. If the above-mentioned assumptions for ANOVA are not met, dislodge was used for determining whether four or more independent samples give a clear cut difference. When this test leads to significant results, at least one sample differs from the others. A principal component analysis is a standard tool in modern data analysis. It is a simple, nonparametric method for extracting relevant information out of confusing data sets. Principal component analysis is concerned with the interpretation of the variance and covariance structure of the original set of variables through a small number of their linear combinations. The general objectives of principal component analysis are data reduction and interpretation.

Results

The concern of this study was to elucidate the composition of the biological glue secreted by five benthic species of sessile marine invertebrates included within five phyla. The cnidarian *Metridium senile*, the tubeworm *Serpula vermicularis*, the barnacle *Balanus amphitrite*, the gastropod *Patella aspera* and the ascidian *Styela plicata* (Figure 1-5) have life cycles that rely on their attachment on suitable substrata underwater for successful survival. This biological glue is a complex mixture of proteins and polysaccharides forming a viscoelastic sticky material. Sessile adult invertebrates have the capability to dislodge and crawl searching for a new suitable place for attachment in response to structural support, defense against enemies, heterotrophic feeding, evading desiccation, parasites, and environmental stresses. Therefore, sessile adult invertebrates secrete this biological glue at need. Mobile benthic invertebrates frequently found in fouling communities include zooids, polychaete tubeworms, barnacles, limpets, sea squirts. Macro-invertebrates constitute the backbone of marine faunal communities in benthic ecosystems. Many macro-invertebrates have an important role in benthic ecosystem, ranging from enhancing habitat complexity to provide food sources for other organisms including humans. Very few studies have attempted to describe the mechanism of adhesion and settlement of benthic macro-invertebrate conglomerations across Mediterranean and Red Seas and there is a lack of study concerning this natural phenomenon in Arabian Gulf. Sea-anemone lacks a calcareous skeleton and bears an adhesive disc (Figure 1). Mucus secretion is necessary to forbid sedimentation, lessen drag force, facilitate adhesion, minimize water loss, and aid in locomotion. To build their tube, sabellariids collect sand grains or mollusc shell fragments in their surroundings, dab them with spots of biological glue, and assemble them into a rigid composite tube (Figure 2). The biological glue is produced by epithelial exocrine glands which lie in the anterior part of the animal and which open at the level of a specialized building organ made up of two lobes located near the mouth. Barnacles depend heavily on their ability to adhere to substrates underwater and are considered as macro-fouling (Figure 3). They are adapted to adhere via biological glue onto other living organisms, rocks, or man-made materials. Barnacles use biological glue to perform temporary adhesion in marine habitats and for exploration. Limpets adhere strongly to most surfaces using a biological glue secretion (Barnacles depend heavily on their ability to adhere to substrates underwater and are considered as macro-fouling (Figure 4). This renders them almost immovable in the face of predators and crashing waves. Sea squirts are marine sessile invertebrates commonly known as ascidians (Figure 5). The adult stage lives in the shoreline, often fixed to the walls of harbors, to the keels and sides of boats and ships. The adult stage is preceded by a swimming larval stage. Adult stage can dislodge attachment and search for a suitable substratum.

The Crude materials of basal discs and attachment integuments were estimated using standard measurement protocols. Total protein in the tissue was estimated by the Folin-Ciocalteu Phenol method of 65 Lowry et al. [65]. During spring, the adhesive basal discs or attachment integuments of the five species studied consisted contain 71 ± 0.61-72 ± 0.45% moisture, 22 ± 3.50-24 ± 0.21% protein, 1 ± 0.04-2 ± 1.13% fat, and 0.2 ± 0.10-1,98 ± 0.20% carbohydrate

(Table 1). Statistically, no differences in moisture, protein, fat, and carbohydrate contents were observed among the five species at P>0.05. In sea anemone, the total carbohydrate, the total fat, protein, and moisture contents were 1,98 ± 0,20, 1 ± 0.57%, 23 ± 1.60 and 71 ± 0.61% by weight, respectively. In tubeworm, the total carbohydrate, the fat, protein, and moisture contents were 0,2 ± 0,10, 1 ± 0.04%, 22 ± 3.50 and 72 ± 0.45% by weight, respectively. In barnacle, the total carbohydrate, the total fat, protein, and moisture contents were 1,2 ± 0,30, 1 ± 1.06%, 23 ± 0.20 and 71 ± 1.23 % by weight, respectively. In gastropod, the total carbohydrate, the total fat, protein and moisture contents were 1,7 ± 1,20, 2 ± 0.98%, 23 ± 0.20 and 70 ± 0.04% by weight, respectively. In sea squirt, the total carbohydrate, the total fat, protein, and moisture contents were 1,01± 0,10, 2 ± 1.13%, 23 ± 0.80 and 72 ± 2.07% by weight, respectively.

Crude amino acid analysis of the basal discs or attachment integuments

Dry samples of the basal discs or attachment integuments of the five species in winter showed that the indispensable and conditional amino acids predominated in tissues are especially Aspartic acid (Asp D), Glutamine (Gln Q), Glutamic acid (Glu E), Glycine (Gly G), Methionine (Met M), Phenylalanine (Phe F), Serine (Ser S), Threonine (Thr T), Tryptophan (Trp W) and Valine (Val V). Aspartic acid (Asp D) ranged from 8 ± 3 to 22 ± 6 % of the total dry weight of tissues; Gln Q ranged from 13 ± 4 to 14 ± 9 % of the total dry weight of tissues; Glutamic acid (Glu E) ranged from 4 ± 3 to 17 ± 2 % of the total dry weight of tissues; Gly G ranged from 12 ± 1 to 15 ± 1% of the total dry weight of tissues; Met M ranged from 12 ± 4 to 14 ± 8 % of the total dry weight of tissues; Phe F ranged from 11 ± 6 to 15 ± 1% of the total dry weight of tissues; Ser S ranged from 9 ± 6 to 13 ± 1% of the total dry weight of tissues; Thr T ranged from 12 ± 3 to 13 ± 9% of the total dry weight of tissues; Trp W ranged from 12 ± 9 to 14 ± 1 % of the total dry weight of tissues and Val V ranged from 12 ± 4 to 16 ± 1% of the total dry weight of tissues (Table 2, Figure 6-10).

Crude total lipid analysis of the basal discs or attachment integuments

The total lipid was estimated by chloroform-methanol method according to Folsch et al. [67]. The total monounsaturated fatty acids concentrations ranged from o.1- 2 % at P<0.05. Dry samples of the basal discs or attachment integuments of the five species showed that the fatty acids predominated in tissues are especially C18:1 V-5, C18:1 v- 7, C18:2 V-3, C18:2 V-6, C18:2 v- 33, C20:1 V-7, C19:2 V-6, C20:5 V-3, C22:1 V-7, C22:1 V-9. The rest of lipid contents of biological glue are considered negligible. C18:1 V-5 ranged from 0.1 ± 2 to 2 ± 4 % of the total dry weight of tissues; C18:1 V-7 ranged from 0.1 ± 1to 22 ± 6% of the total dry weight of tissues; C18:2 V-3 ranged from 0.2 ± 4 to 7 ± 2 % of the total dry weight of tissues; C18:2 V-6 ranged from 0.1 ± 2 to 5 ± 6% of the total dry weight of tissues; C18:2 V-33 ranged from 1 ± 3to 6 ± 2% of the total dry weight of tissues; C20:1 V-7 ranged from 0.4 ± 5 to 21 ± 2% of the total dry weight of tissues; C19:2 V-6 ranged from 2 ± 1to 15 ± 1% of the total dry weight of tissues; C20:5 V-3 ranged from 0.1 ± 1to 12 ± 4% of the total dry weight of tissues; C22:1 V-7 ranged from 0.2 ± 2 to 12 ± 2 % of the total dry weight of tissues; C22:1 V-9 ranged from 1 ± 4 to 14 ± 2 % of the total dry weight of tissues (Table 3). In all seasons of the year, there were slight fluctuations in the percentage of the most abundant fatty acids in basal disc of sea anemone. During spring, there were C18:1 v-7 (7.2 %); C16:2 v-6 (4.1 %) and C20:5 v-3 (4.6 %); in the integument of tubeworm during summer C20:1 v-7 (12.3 %); C22:1 v-7 (5.4 %); C22:1 v-9 (3.9 %); C19:2- v-6 (5.7 %) was found. In attachment integument of barnacle during autumn C20:1 v-7 (13.4 %); C22:1 v-7 (3.9 %), C22:1 v-9 (4.2 %) and C19:2- v-6 (6.7 %) was detected. In attachment integument of gastropod during autumn C20:1 v-7 (13.7 %), C22:1 v-7 (5.7 %); C22:1 v-9 (4.6 %) and C19:2- v-6 (7.8 %) was detected. In attachment mantle of sea squirt during winter C20:1 v-7 (5.3 %); C22:1 v-7 (3.9 %), C22:1 v-9 (3.7 %) and C19:2-v- 6 (4.1 %) was observed (Figures 11-15).

Table 1: Proximate composition of the crude materials of the basal discs or attachment integuments (%, wet bases).

Parameters	sea anemone	tubeworm	barnacle	gastropod	Sea squirt
Crude carbohydrate	1,98 ± 0,20	0,2 ± 0,10	1,2 ± 0,30	1,7 ± 1,20	1,01 ± 0,10
Crude protein	23 ± 1.60	22 ± 3.50	23 ± 0.20	24 ± 0.21	23 ± 0.80
Crude lipids	1 ± 0.57	1 ± 0.04	1 ± 1.06	2 ± 0.98	2 ± 1.13
Moisture	71 ± 0.61	72 ± 0.45	71 ± 1.23	70 ± 0.04	72 ± 2.07

Table 2: Amino acids composition of the basal discs or attachment integuments (%, dry bases).

Amino acids	sea anemone	tubeworm	barnacle	gastropod	sea squirt
Asp D	8 ± 3	10 ± 1	15 ± 7	22 ± 6	13 ± 4
Gln Q	13 ± 4	14 ± 6	13 ± 7	14 ± 1	14 ± 9
Glu E	17 ± 2	14 ± 1	4 ± 3	6 ± 5	6 ± 2
Gly G	15 ± 1	13 ± 5	12 ± 1	16 ± 2	13 ± 5
His H	2 ± 6	1 ± 2	2 ± 9	3 ± 2	4 ± 1
Leu L	2 ± 7	3 ± 1	4 ± 8	2 ± 9	3 ± 4
Lys K	5 ± 9	4 ± 3	4 ± 9	3 ± 6	4 ± 9
Ile I	4 ± 1	6 ± 1	6 ± 1	4 ± 8	4 ± 9
Met M	12 ± 4	13 ± 1	13 ± 7	14 ± 4	14 ± 8
Phe F	14 ± 2	11 ± 6	13 ± 2	15 ± 1	13 ± 5
Ser S	13 ± 1	12 ± 3	10 ± 4	9 ± 6	12 ± 2
Thr T	12 ± 7	12 ± 3	13 ± 9	12 ± 9	13 ± 5
Trp W	12 ± 9	13 ± 4	14 ± 4	13 ± 6	14 ± 1
Tyr Y	3 ± 5	4 ± 1	4 ± 7	3 ± 7	2 ± 8
Val V	16 ± 1	14 ± 7	15 ± 1	15 ± 3	12 ± 4

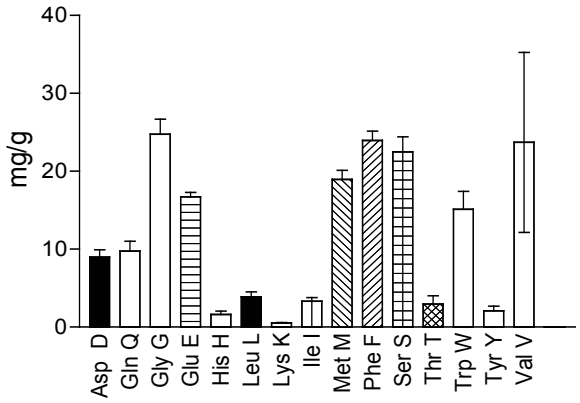

Figure 6: Amino acids analysis of adhesives in sea anemone.

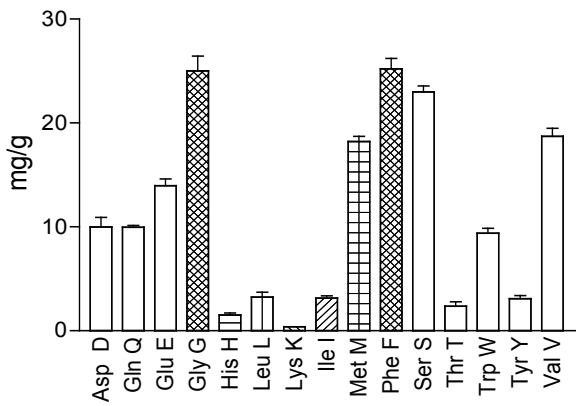

Figure 7: Amino acids analysis of adhesives in tubeworm.

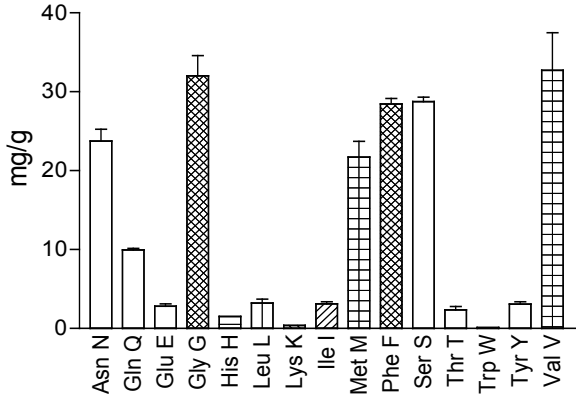

Figure 8: Amino acids analysis of adhesives in barnacle.

Carbohydrate analysis of the basal discs or attachment integuments

For estimation of total carbohydrate content of tissue, phenol-sulfuric acid method was followed according to Lowry et al. [65]. Glycogen is the main bulk of carbohydrate in marine invertebrates, representing about 50% of total carbohydrate content. It is the basic form of energy reserve especially in settlement process of sessile marine fauna. Glycogen undergoes fast catabolism that provides instant energy under hypoxic or anoxic conditions. The varying amounts of carbohydrates were apparently dependent partly on species and partly on the season of settlement. In the basal discs or attachment integuments the following percentage quantities were observed: in the basal disc of sea anemone, 2.5 mg carbohydrates g^{-1} wet weight; in the integument of tubeworm, 0.9 mg carbohydrates g^{-1} wet weight; in the basal disc of barnacle and gastropod, 3 & 5.5 mg carbohydrates g^{-1} wet weight respectively; in the basal mantle of sea squirt, 1.5 mg carbohydrates g^{-1} wet weight (Figure 16).

Amino acids analysis of biological glue

A high-pressure chromatographic separation with Postcolumn Ninhydrin Detection has been applied for amino acid analysis. Fifteen amino acids were identified in the biological glue of the five species studied during the four seasons of the year. The most predominant amino acids were Aspartic acid (Asp D), Glutamine (Gln Q), Gly G, Met M, Phenylalanine (Phe F), Ser S and Val V (ranging from 30 to 90 mg/g in sea anemone (Figure 17); 20 to 100 mg/g in tubeworm; 20 to 120 mg/g (Figure 18); in barnacle (Figure 19); 30 to 180 mg/g in gastropod (Figure 20) and 10 to 90 mg/g in sea squirt (Figure 21). Of these amino acids, Gly G, Phe F and Val V were the most predominant. Gly G represented 65-160 mg/g and Phe F represented 90-120 mg/g and Val V represented 60-170 mg/g. One-way analysis of variance (ANOVA) of amino acids analysis of biological glue in sea anemone showed means Significant Difference at $P<0.05$ with R squared 0.9029 and Newman-Keuls Multiple Comparison

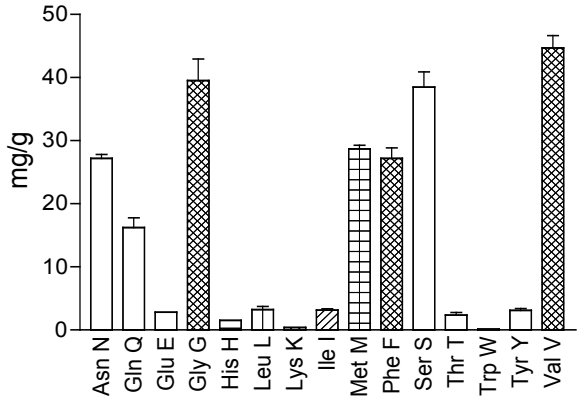

Figure 9: Amino acids analysis of adhesives in gastropod.

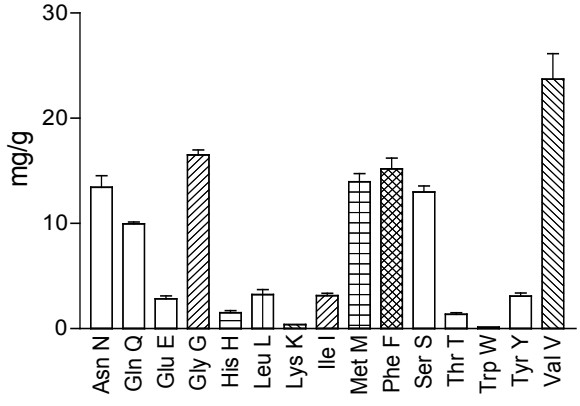

Figure 10: Amino acids analysis of adhesives in sea squirt.

Table 3: Fatty acids composition of the basal discs or attachment integuments (% of total fatty acids).

Fatty acids	sea anemone	tubeworm	barnacle	gastropod	sea squirt
C18:1 v- 5	2 ± 4	0.1 ± 1	0.4 ± 1	0.1 ± 1	0.1 ± 2
C18:1 v- 7	22 ± 6	0.1 ± 1	0.1 ± 1	0.1 ± 2	0.1 ± 1
C18:1 v- 9	4 ± 6	1 ± 4	1 ± 2	1 ± 4	0.1 ± 5
C19:1 v- 8	4 ± 1	2 ± 7	1 ± 5	0.2 ± 1	0.2 ± 3
C20:1 v- 5	3 ± 1	3 ± 2	2 ± 9	2 ± 1	5 ± 4
C20:1 v- 6	2 ± 7	0 ± 4	1 ± 6	3 ± 2	6 ± 1
C20:1 v- 7	0.4 ± 5	15 ± 8	18 ± 1	21 ± 2	13 ± 3
C20:1 v-9	0.3 ± 7	0.1 ± 6	0.4 ± 6	4 ± 2	0.3 ± 5
C22:1 v- 7	0.2 ± 2	12 ± 2	10 ± 3	6 ± 1	12 ± 2
C22:1 v- 9	1 ± 4	14 ± 2	12 ± 1	7 ± 2	11 ± 4
C24:1 v- 3	2 ± 1	2 ± 1	1 ± 1	3 ± 3	0.1 ± 2
C24:1 v- 6	0.1 ± 6	1 ± 2	2 ± 2	2 ± 1	1 ± 1
C24:1	0.2 ± 8	3 ± 1	2 ± 1	2 ± 7	2 ± 3
MUFAs	0.1 ± 1	3 ± 5	4 ± 2	6 ± 5	1 ± 5
C16:2 v- 6	0.1 ± 1	0.2 ± 1	0.1 ± 1	2 ± 4	2 ± 5
C18:2 v- 3	0.2 ± 4	7 ± 2	6 ± 1	2 ± 3	1 ± 8
C18:2 v- 6	0.1 ± 7	6 ± 1	5 ± 6	2 ± 1	2 ± 6
C18:2 v- 32	0.1 ± 2	2 ± 1	1 ± 8	1 ± 4	3 ± 2
C18:2 v- 6	14 ± 1	0.1 ± 1	0.6 ± 8	3 ± 5	4 ± 2
C18:2 v- 33	6 ± 2	2 ± 3	3 ± 2	2 ± 3	1 ± 3
C19:2- v- 6	7 ± 6	11 ± 4	13 ± 5	15 ± 1	2 ± 1
C20:2 v- 6	5 ± 3	0 ± 1	0.1 ± 2	2 ± 2	13 ± 1
C20:3 v- 6	2 ± 8	0.1 ± 1	0.2 ± 1	2 ± 3	2 ± 4
C20:4 v- 6	3 ± 7	0.1 ± 2	0.9 ± 2	1 ± 5	1 ± 1
C20:5 v- 3	12 ± 4	0.1 ± 1	0.2 ± 1	0.1 ± 3	7 ± 3

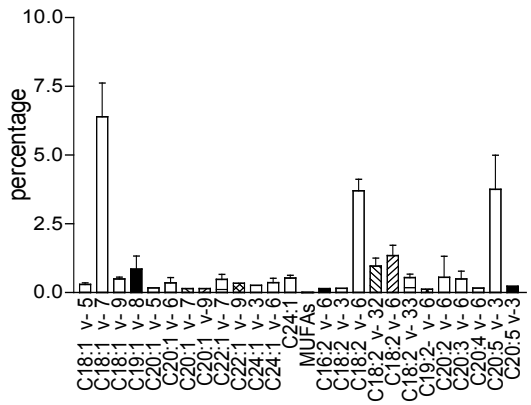

Figure 11: Fatty acids analysis of adhesives in sea anemone.

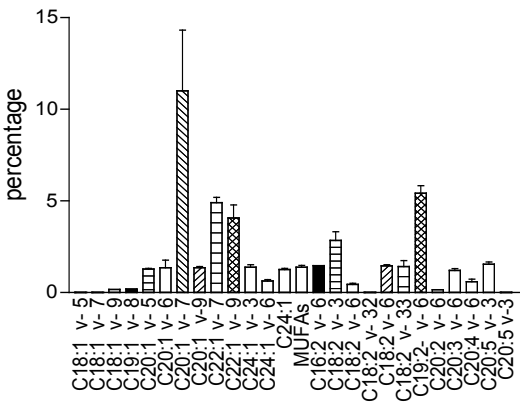

Figure 12: Fatty acids analysis of the integument in tubeworm.

Test proved the lowest value of Mean Difference -0.25 of Val V vs Phe F at P>0.05. The highest value of Mean Difference -24.25 was observed in Lys K vs Gly G. In case of tubeworm ANOVA showed means Significant Different at P<0.05 with R squared 0.9831 and Newman-Keuls Multiple Comparison Test proved the lowest value of Mean Difference -24.82 of Lys K vs Phe F at P<0.001 whereas the highest value of Mean Difference -0.025 was found in Gln Q vs Asp D. In case of barnacle ANOVA showed means Significant Difference at P<0.05 with R squared 0.9556 and Tukey's Multiple Comparison Test proofed the lowest value of Mean Difference -30.34 in Thr T vs Val V whereas the highest value of Mean Difference 31.88 was found in Gly G vs Trp W. In case of gastropod ANOVA showed means Significant Difference at P<0.05 with R squared 0.9788 and Newman-Keuls Multiple Comparison Test proved the lowest value of Mean Difference -44.58 in Trp W vs Val V at P<0.001 whereas the highest value of Mean Difference -1.005 at P>0.05 in Ser S vs Gly G. With the exception of Met M in the gastropod, no significant differences among amino acids in the five species was found in the other amino acids at P>0.05. The percentages of the essential amino acids (EAAs) to the total amino acids in the amino acids of the five species were 46.25% and 43.57% respectively. As shown in Figures 6-10 Val V was the EAA with the highest concentration in gastropod (45.60 mg/g protein) and in barnacle and sea squirt (35.2-35.8 mg/g protein). Lys K was the EAA with the lowest concentration. The concentrations of all other EAAs were higher than the reference standard concentration [70].

Protein analysis of the biological glue

Proteins in biological glue of the five species were resolved by SDS-

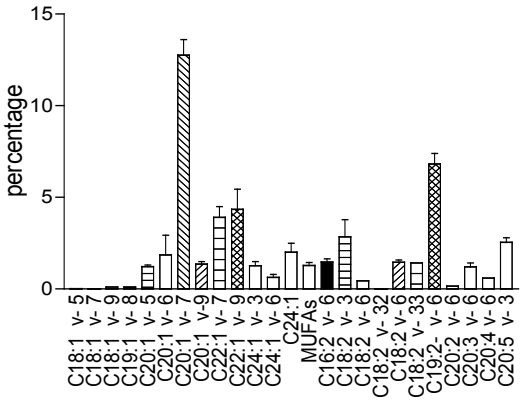

Figure 13: Fatty acids analysis of adhesives in barnacle.

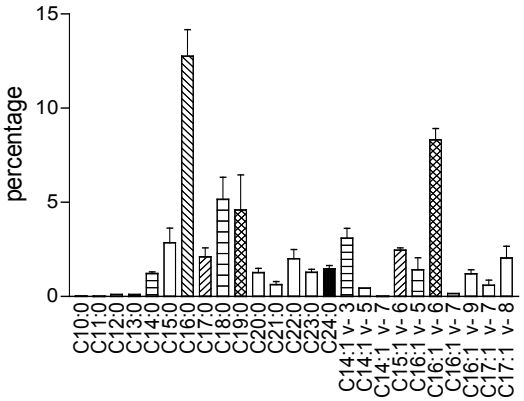

Figure 14: Fatty acids analysis of adhesives in gastropod.

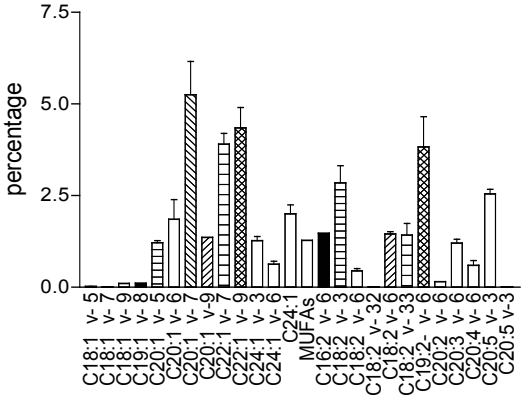

Figure 15: Fatty acids analysis of adhesives in sea squirt.

PAGE and digested with trypsin. A peptide with the same sequence was obtained from protein bands of different apparent molecular masses. The biological glue containing protein was first broken down mechanically using a homogenizer. In the electric field the polypeptide molecules moved with negative charge to the positive pole. SDS was applied as an anionic detergent that denatured non-disulfide-linked tertiary structures, and applied a negative charge to each polypeptide molecule in proportion to its mass. The loaded particles migrated in the solution under the influence of an electric field at different speeds. The rate of migration of the polypeptide molecules relied on various factors such as charge density of the molecules (acceleration) and the size of the molecules (friction resistance) in which smaller molecules traveled faster (and thus wider) than larger ones. As the voltage was applied, the molecules separated according to charge and size; suction currents; evaporate buffer; electro-osmosis and adsorption

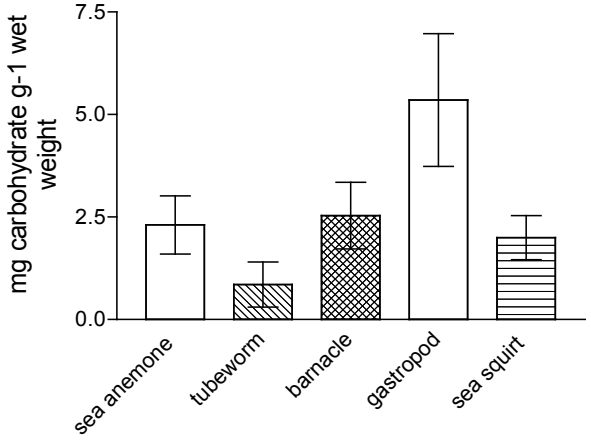

Figure 16: Carbohydrate analysis of the pedal disc, integument and basal mantle.

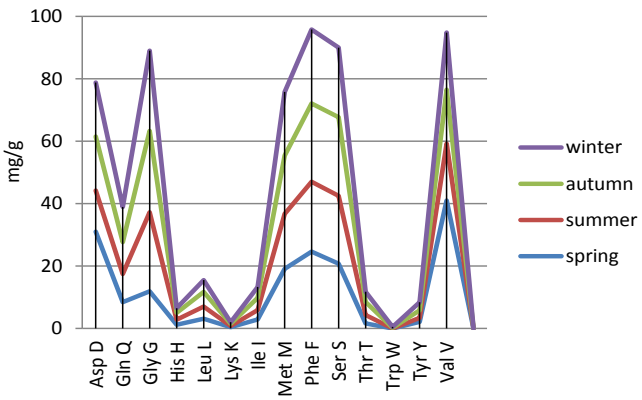

Figure 17: Amino acids analysis of the biological glue in sea anemone.

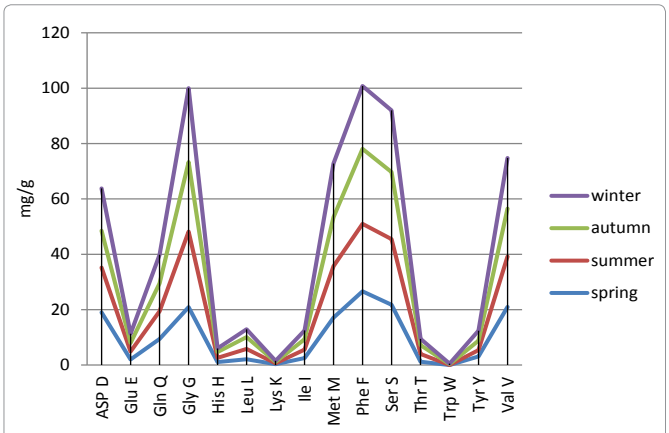

Figure 18: Amino acids analysis of the biological glue in tubeworm.

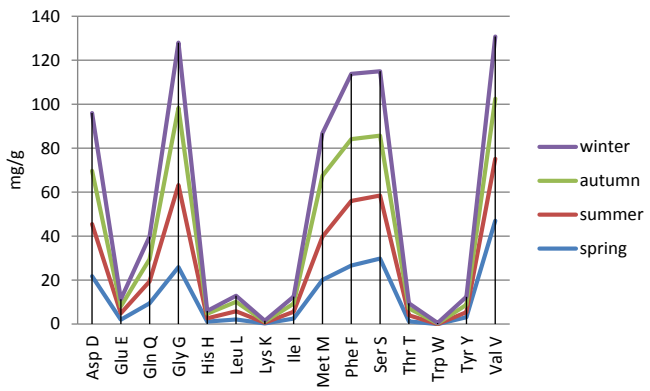

Figure 19: Amino acids analysis of the biological glue in barnacle.

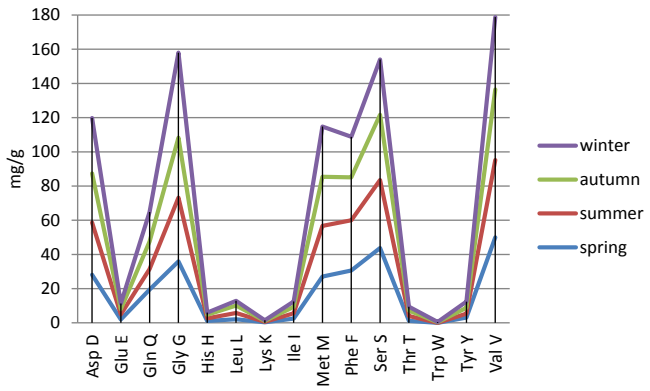

Figure 20: Amino acids analysis of the biological glue in gastropod.

analytes to carriers. Sodium dodecyl sulfate (SDS) was applied as an anionic detergent to the biological glue containing protein to impart a negative charge to linearized proteins (SDS-PAGE). This analysis revealed that the biological glue contained about 15 different proteins. Their apparent molecular masses were approximately 30, 35, 40, 45, 50, 65, 70, 75, 80, 85, 100, 140, 180, 200 and 250 -kDa. There was some variability between the five protein extracts of the five species, and little variability of protein contents were found within the same species. Two proteins were especially inconsistent in extraction, the 30-kDa and the 250-kDa proteins. There was also a component that was too large to enter the running gel >300-kDa (Figure 22). The proteins that were analyzed in the five species had similar amino acid compositions. They were all rich in glycine Gly G (22-29%) and in acidic residues (18-22%). The majority of the resolved protein spots of the five species had pI values between pH 4 and pH 12 and the molecular size of the pattern ranged from 250 kDa to less than 30 kDa. Approximate 70% of the proteins ranged from 140 kDa to 30-kDa. Around 200-50 spots were detected in the five gels, which were compared for their intensity to identify differentially expressed spots. After their separation by SDS-PAGE, proteins of the biological glue of the five species were blotted onto PVDF membranes and probed for phosphoproteins using the monoclonal anti-phosphorylated amino acid antibodies. Several labeled bands were present in the biological glue from the five species. Among them, the most strongly labeled are a protein of 70-85 kDa and a protein located just above the migration front. SDS-PAGE facilitated the process of separating the polypeptide molecules in the acrylamide gel which were successfully transferred to a stable PVDF membrane. Immunochemical techniques were applied to visualize the transferred molecules, as well as the increases or decreases of the polypeptide molecules of interest.

Lipids analysis of the biological glue

Total lipids were extracted using capillary gas chromatography. This test revealed that the lipid contents of biological glue of the five species studied were roughly similar but in different quantities. It was found that lipids of biological glue were predominant by C18:1 V-5, C18:2 V-6, C19:1 v- 8, C20:1 V-9, C24:1 V-6, C19:2 V-6. It was found that the amount of lipids present in the biological glue of the five species studied was roughly variable and differed according to seasons of the year. In sea anemone, the amount of C18:1 V-5 varied from 20%-70% in spring-winter respectively (Figure 23). In tubeworm 0.1%-85% (Figure 24); in barnacle, gastropod and sea squirt C18:2 V-6 varied from 20%-90% (Figures 25-27). In sea anemone C18:2 V-32 varied from 30%-85%, in barnacle, gastropod and sea squirt. C19:1 v-8 varied from 5 %-75% in sea anemone, 25%-85% in tubeworm, 40%-85% in barnacle, gastropod and sea squirt. C20:1 V-9 varied from 20%-65% in sea anemone and 30%-85% in tubeworm, barnacle, gastropod and sea squirt. C24:1 V-6 varied

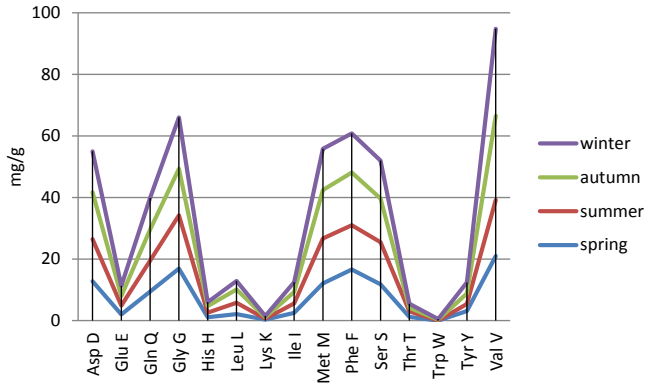

Figure 21: Amino acids analysis of the biological glue in sea squirt.

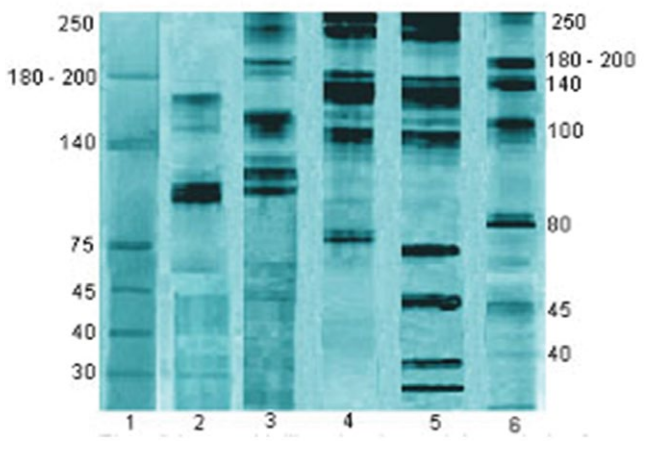

Figure 22: Immunoblotting showing protein analysis of the biological glue in the five macro-fouling. **(1)** ladder **(2)** sea anemone **(3)** tubeworm **(4)** barnacle **(5)** sea squirt.

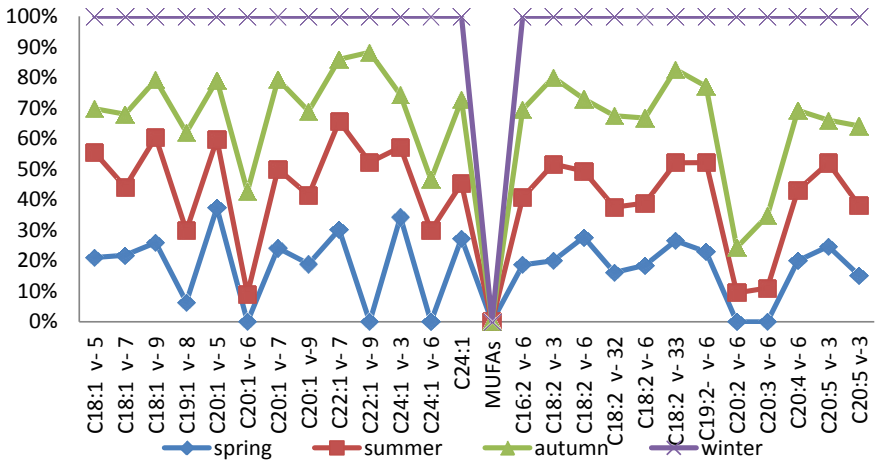

Figure 23: Percentage of fatty acids of the biological glue in sea anemone.

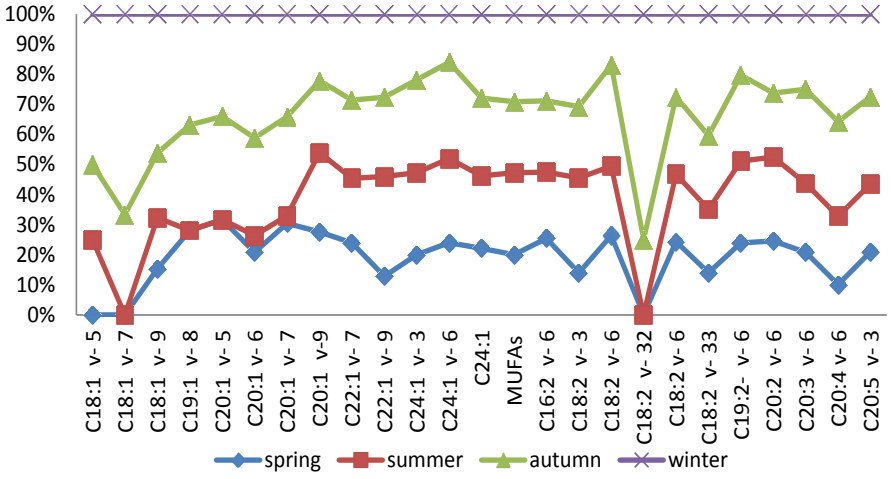

Figure 24: Percentage of fatty acids of the biological glue in tubeworm.

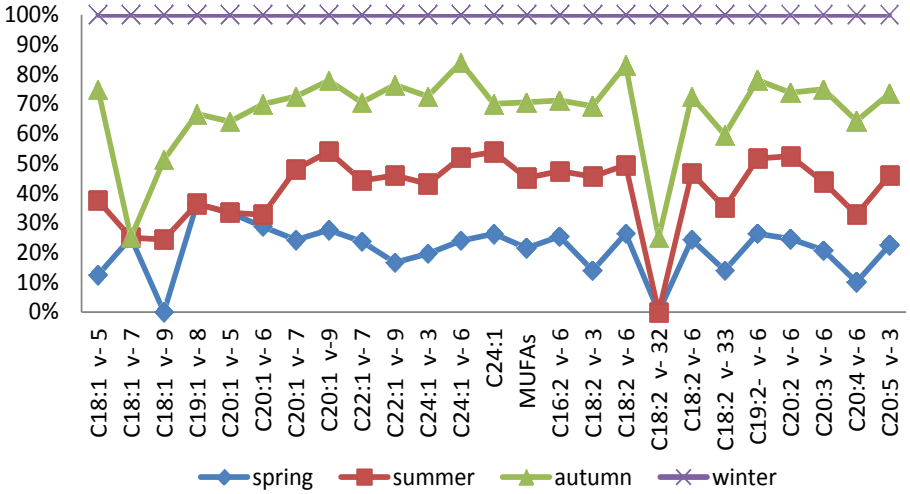

Figure 25: Percentage of fatty acids of the biological glue in barnacle.

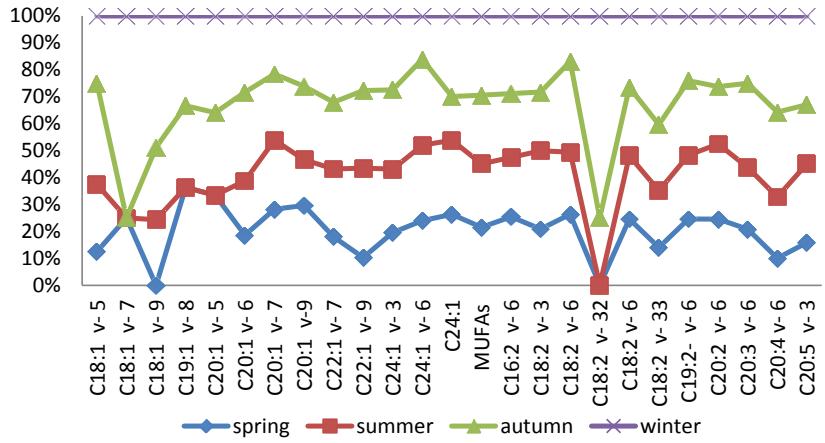

Figure 26: Percentage of fatty acids of the biological glue in gastropod.

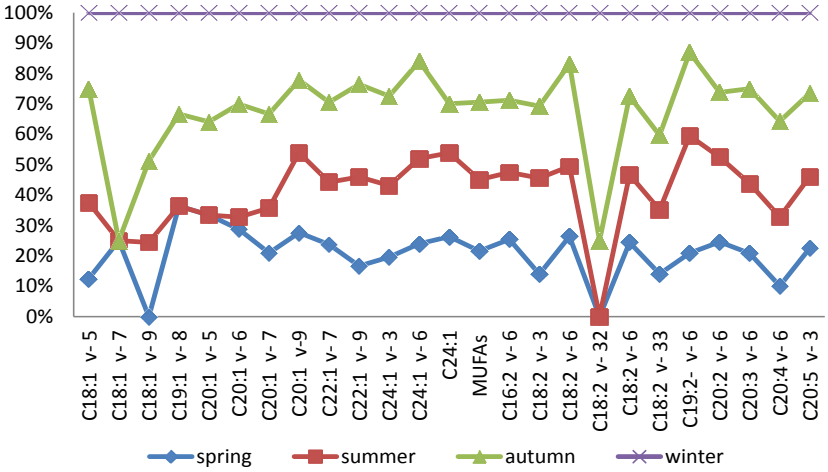

Figure 27: Percentage of fatty acids of the biological glue in sea squirt.

from 0%-45% in sea anemone and 25%-85% in tubeworm, barnacle, gastropod and sea squirt. C19:2 V-6 varied from 25%-85% in sea anemone, tubeworm, barnacle, gastropod and sea squirt. Generally, the amount of lipids secreted by the five species studied showed a maximal concentration in winter and gradually decreased to be the minimal concentration in summer. One-way analysis of variance (ANOVA) showed means Significant Difference at $P<0.05$ among the fatty acids in the five species. Dunnett's Multiple Comparison Test for fatty acids of sea anemone showed lowest Mean Difference -6.097 between C18:1 v-5 vs C18:1 v- 7 and highest Mean Difference 0.2975 between C18:1 v-5 vs MUFAs at $P>0.05$. Newman-Keuls Multiple Comparison Test for fatty acids of tubeworm showed lowest Mean Difference -11 between C18:1 v- 7 vs C20:1 v- 7 and highest Mean Difference 10.0 between C18:1 v-5 vs C18:2 v-32 at $P>0.05$. Tukey's Multiple Comparison Test for fatty acids of barnacle showed lowest Mean Difference -6.723 between C18:1 v-9 vs C19:2-v- 6 and highest Mean Difference 1.207 between C20:1 v-5 vs C18:2 v-32 at $P>0.05$. Tukey's Multiple Comparison Test for fatty acids of gastropod showed lowest Mean Difference -8.305 between C10:0 vs C16:1 v-6 and highest Mean Difference -1,207 between C11:0 vs C14:1 v-7 at $P>0.05$. Dunnett's Multiple Comparison Test for fatty acids of sea squirt showed lowest Mean Difference -4.33 between C12:0 vs C14:1 v-5 and highest Mean Difference 001 between C10:0 vs C11:0 at $P>0.05$.

Carbohydrate analysis of the biological glue

The biological glue of sea anemone contained 2,3.7,4.1 & 5.2 mg carbohydrates g^{-1} wet weight in spring, summer, autumn, and winter respectively. The biological glue of tubeworm contained 1,2,2.1 & 3.5 mg carbohydrates g^{-1} wet weight in spring, summer, autumn, and winter respectively. The biological glue of barnacle contained 4,2,5.1 & 6.3 mg carbohydrates g^{-1} wet weight in spring, summer, autumn, and winter respectively. The biological glue of gastropod contained 5,5.5,7.1 & 8.7 mg carbohydrates g^{-1} wet weight in spring, summer, autumn, and winter respectively. The biological glue of sea squirt contained 3,3.7.4 & 6 mg carbohydrates g^{-1} wet weight in spring, summer, autumn, and winter respectively (Figure 28). One-way analysis of variance (ANOVA) showed means Significant Different among the five species at $P<0.05$ with F 9.28 and R 0.403. Bartlett's test for equal variances at $P<0.05$ showed variances differ Significant among the five species and Bonferroni's Multiple Comparison Test showed the lowest Mean Difference -1.629 between sea anemone vs gastropod at

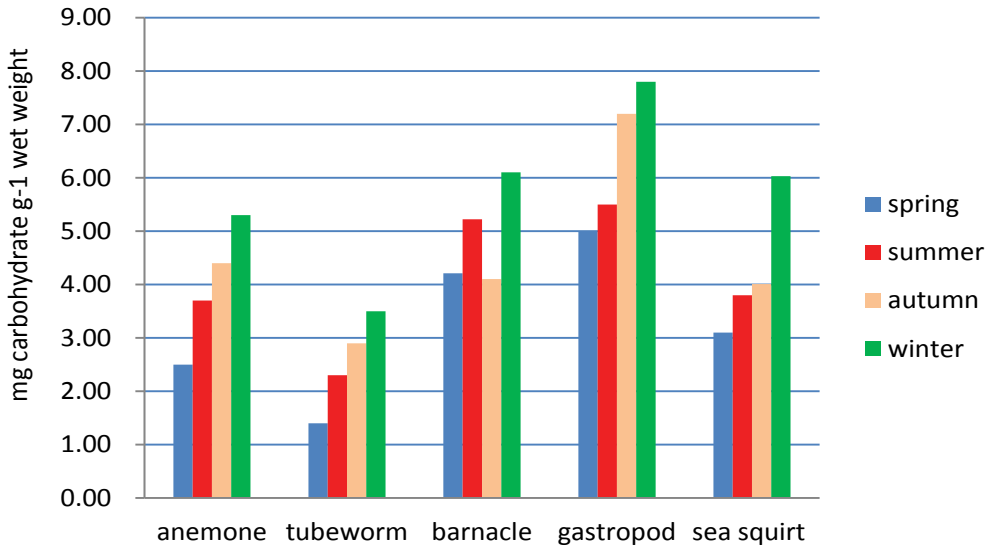

Figure 28: Carbohydrate analysis of the biological glue.

P<0.001whereas the highest Mean Difference 1.858 was shown between gastropod vs sea squirt at P<0.001.

Discussion

Marine fouling communities such as tubeworms, sea anemones, barnacles, gastropod limpets, and sea squirts form rich food sources for fish and humans. Sessile zoobenthos dislodge from substrata and search for suitable place in response to food resources, environmental stresses, predators, parasites, and pathogens. The concern of this study was to elucidate the composition of the biological glue secreted by five benthic species of sessile marine invertebrates included within five phyla. The cnidarian *Metridium senile*, the tubeworm *Serpula vermicularis*, the barnacle *Balanus amphitrite*, the gastropod *Patella aspera* and the ascidian *Styela plicata* which have life cycles that rely on their attachment on suitable substrata for successful survival. Inadequate studies have attempted to describe the mechanism of adhesion and settlement of larvae that belong to benthic macro-invertebrate conglomerations across the Mediterranean and Red Seas and there is a lack of study concerning this natural phenomenon in Arabian Gulf. Settlement of sessile zoobenthos and secretion of biological glue occur naturally in a wide range of substrata, either natural or man-made [71,72], and plays an important role in organisms' life cycles and survival [9,13]. Biological glue, secreted by marine zoobenthos enable permanent or temporary adhesion, the mechanisms that are not entirely studied. It is thought that two main stages control the interaction between the biological glue, secreted by zoobenthos, and the type of substratum [71]. The first stage is wetting of the substratum by fatty acids [58]. Previous studies mentioned that lipids, secreted first, possibly displace water from the surface interface and create a conducive environment for introduction of phosphorous glycoproteins while simultaneously modulating the spreading of the protein phase and protecting the nascent biological glue from bacterial biodegradation [8,73,74]. The two distinct stages are noticed within the cyprid cement glands of the barnacle *Balanus amphitrite*.

In the present study, the total protein in the basal discs and the attachment integuments of the five species was estimated by the Folin-Ciocalteu Phenol method [65]. During spring, these tissues contained 0.2 ± 0.10-1,98 ± 0.20% carbohydrate, 1 ± 0.04-2 ± 1.13% fat, 22 ± 3.50-24 ± 0.21% protein, 71 ± 0.61-72 ± 0.45% moisture. In sea anemone, the total carbohydrate, the total fat, protein, and moisture contents were 1,98 ± 0,20, 1 ± 0.57%, 23 ± 1.60 and 71 ± 0.61% by weight, respectively. In tubeworm, the total carbohydrate, the fat, protein, and moisture contents were 0,2 ± 0,10, 1 ± 0.04%, 22 ± 3.50 and 72 ± 0.45% by weight, respectively. In barnacle, the total carbohydrate, the total fat, protein, and moisture contents were 1,2 ± 0,30, 1 ± 1.06%, 23 ± 0.20 and 71 ± 1.23 % by weight, respectively. In gastropod, the total carbohydrate, the total fat, protein, and moisture contents were 1,7 ± 1,20, 2 ± 0.98%, 23 ± 0.20 and 70 ± 0.04% by weight, respectively. In sea squirt, the total carbohydrate, the total fat, protein, and moisture contents were 1,01 ± 0,10, 2 ± 1.13%, 23 ± 0.80 and 72 ± 2.07% by weight, respectively. In a previous study on *K. opima* [75] and on *Thais mutabilis* [76], observations regarding carbohydrate, the total fat, protein, and moisture contents were analyzed. Wang et al. [77] reported that the maximum protein storage in the edible gastropod snail *Bellama bengalensis* was observed during spawning period. In another study, it was mentioned that male and female *F. ficoides* have maximum protein content in foot and gill organs during the season of monsoon and minimum amount of protein recorded during the season of post monsoon [78].

Dry samples of the basal discs or attachment integuments of the five species in winter showed that the indispensable and conditional amino acids predominated in tissues are especially Aspartic acid (Asp D), Glutamine (Gln Q), Glutamic acid (Glu E), Glycine (Gly G), Methionine (Met M), Phenylalanine (Phe F), Serine (Ser S), Threonine (Thr T), Tryptophan (Trp W) and Valine (Val V). The percentage of these amino acids varied in tissues of attachment integuments as well as in the biological glue of the five species studied. Fifteen amino acids were identified in the biological glue of the five species studied during the four seasons of the year. It was found that the amount of amino acids present in the biological glue of the five species studied was roughly variable and differed according to seasons of the year. In another study on the biological glue secreted by the larvae of sea squirts *Ciona intestinalis*, *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia nigra* [58] found out that Glycine ranged from 21.91

% to 25.70 % and Serine ranged from 12.78 % to 14.30 % of the amino acid content of the biological glue of the four studied ascidian larvae. There were differences in the percentage of amino acids in the four species studied with the exception of Cysteine, Lysine, and Tryptophan.

SDS-PAGE was applied to identify proteins the of biological glue. Proteins in different samples of the five zoobenthos were resolved by SDS-PAGE and digested with trypsin. A peptide with the same sequence was obtained from protein bands of different apparent molecular masses. This analysis revealed that the biological glue contained about 15 different proteins. Their apparent molecular masses were approximately 30, 35, 40, 45, 50, 65, 70, 75, 80, 85, 100, 140, 180, 200 and 250 -kDa. There was some variability between the five protein extracts of the five studied species, and little variability of protein contents were found within the same species. Two proteins were especially inconsistent in extraction, the 30-kDa and the 220-kDa proteins. There was also a component that was too large to enter the running gel >250-kDa. The proteins that were analyzed in the five species had similar amino acid compositions. They were all rich in Phenylalanine (25-30%) and in acidic residues (13-22%). The majority of the resolved protein spots of the five species had pI values between pH 4 and pH 12 and the molecular size of the pattern ranged from 250 kDa to less than 30 kDa. Approximate 70% of the proteins ranged from 140 kDa to 30 kDa. Around 250-30 spots were detected in the five gels, which were compared for their intensity to identify differentially expressed spots. After their separation by SDS-PAGE, proteins of the five species were blotted onto PVDF membranes and probed for phosphorous proteins using the monoclonal anti-phosphorylated amino acid antibodies. Several labeled bands were present in the biological glue from the five species. Among them, the most strongly labeled are a protein of 40 kDa and a protein located just above the migration front.

Biological glue protein motifs have been identified in phylogenetically different marine invertebrates [79,80]. DOPA is considered as one such motif [81,82]. Dopa has been discovered in the biological glue of *Bdelloura candida* [83] and in the blood fluke *Schistosoma mansoni* [84]. Dopa-containing protein has been identified from the blood cells of *Pyura stolonifera* [85]. Dopa-containing peptides have been isolated from the blood cells of *Halocynthia roretzi* [86-88]. Another modified amino acid, phosphoserine (pSer), is an important motif in biological glue [89]. Protein phosphorylation occurs in marine biological glue in the form of serine phosphorylation and has been described in *Mytilus edulis* and *Mytilus galloprovincialis* and in one tube-worm *Phragmatopoma californica* [90-94].

Dry samples of the basal discs or attachment integuments of the five species showed that the fatty acids predominated in tissues are especially C18:1 V-5, C18:1 v- 7, C18:2 V-3, C18:2 V-6, C18:2 v- 33, C20:1 V-7, C19:2 V-6, C20:5 V-3, C22:1 V-7, C22:1 V-9. The rest of lipid contents of biological glue are considered negligible. The percentage of these fatty acids varied in tissues of attachment integuments as well as in the biological glue of the five species studied. It was found that lipids of biological glue were predominant by C18:1 V-5, C18:2 V-6, C19:1 v- 8, C20:1 V-9, C24:1 V-6, C19:2 V-6. It was found that the amount of lipids present in the biological glue of the five species studied was roughly variable and differed according to seasons of the year. In a previous study on the biological glue secreted by the larvae of sea squirts *Ciona intestinalis*, *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia nigra* [57] found out that the total lipid content of biological glue of the four species studied were roughly similar but in different amounts. It was found that lipids of biological glue were represented by C14:0; C18:2 V-32; C20:1 V-9; C18:1 V-7; C16:1 V-6 and C18:1 V-5. The rest of lipid contents of biological glue are considered negligible. Knowledge of the lipid contribution in settlement of zoobenthos will inspire development of synthetic and environmentally benign antifouling coatings [13]. However, a great number of abiotic parameters may influence the degree of adhesion. Although temperature can be the first factor, adhesion of zoobenthos is succeeded in tropical areas. Any warm waters and especially near-surface substrata, heated by the sun, are potential areas for adhesion of zoobenthos. Favorable conditions for settlement differ substantially among sessile invertebrates [21,95]. The amount of carbohydrates in the basal discs or attachment integuments and in the biological glue of the five species apparently varied partly on species and partly on the season of settlement. In a previous study on the biological glue secreted by the larvae of sea squirts *Ciona intestinalis*, *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia nigra* [58] found out that no significant differences in the total carbohydrates of biological glue. Glycogen is the main carbohydrate content in marine fauna, representing about 50% of total carbohydrates [96]. Glycogen is considered as a form of energy reserve particularly in settlement process of ascidian larvae because of fast glycogen catabolism that provide instant energy under hypoxic or anoxic conditions [97,98].

Another purpose of this study was to elucidate the composition of the biological glue with emphasis on discovering a radical solution for antifouling strategies. In the available literature, it was noticed that the mucus secreted by marine invertebrates *Calliosioma zizyphinhtm* [99] and *Mytilus* spp. [100] is a complex mixture of proteins and polysaccharides forming a weak watery gel. It is necessary for vital activities including movement, navigation, support, feeding and defense against environmental stresses, predators, parasites, and microbes. The term mucus, however, is broadly used for any slimy secretion from an epithelial surface [42]. There are three sorts of biological polymers that are basically used to form gels: glycoproteins/proteoglycans, polysaccharides, and proteins. These build gels at the same concentrations, but the underlying mechanics can widely vary. Gel-forming proteoglycans and glycoproteins are long molecules consisting of a protein with attached carbohydrate side chains. They typically have masses larger than a megadalton, and they are often linked together into even larger complexes. They are so long that they form gels solely by entangling [101-103]. The carbohydrates typically account for up to 90% of the mass [104]. Proteoglycans are common gel-forming polymers. In this case, polysaccharides such as glycosaminoglycans are attached to a protein. Denny [39] suggests that most invertebrate mucus polymers may fit on a continuum between mucin and proteoglycans. Gelatin consists of collagen fragments extracted from animal connective tissues. The majority of the protein in gelatin is roughly 100-200 kDa, and it is these fragments that contribute the most to the mechanics of the gel [105]. While gelatin is well-known from the food industry, it also has a long history of use as an adhesive. Until the early 1900s, the most common biological glue was gelatin and starch [106].

During settlement, barnacle and tunicate larvae respond to stimulatory [107,108] and inhibitory signals [109]. Chemical cues adsorbed to body surfaces as well as those free in sea water serve to transmit specific information in marine environments. Such cues are important in the settlement, attachment, and metamorphosis of sessile marine invertebrates [110-113]. Chemical cues that influence

settling have been isolated both from within the species [114] and from other organisms [115,116]. Pheromones released from living adult *Balanus* spp. have been reported to mediate conglomeration by settling larvae [71,117]. Adult barnacle peptides affect the behavior and induce metamorphosis of larvae in the settling stage [118].

Recommendations

Lipids are secreted first possibly to displace water from the surface interface and protect the nascent biological glue from biodegradation. It was found that lipids of biological glue were represented by C18:1 V-5, C18:2 V-6, C19:1 v-8, C20:1 V-9, C24:1 V-6, C19:2 V-6. Fatty acids contain double bonds, hydroxyl groups, or other functional groups. Search for a potential benign chemical, to be added in the paint as antifouling agent that dissolve this lipid moiety and thus prevent settlement is a useful strategy. This can be done through: 1- Suspension Hydrogenation: adding a fine powdery copper catalyst. The excess hydrogen serves to circulate the reaction mixture. The product mixture splits into a gas phase and a liquid phase. 2- Bashkirov Oxidation: adding of boric acid ester which scavenges the hydroperoxides that are formed as intermediates. Hydrolysis leads to a statistical distribution of secondary alcohols in which the hydroxyl function may occupy any position on the carbon chain. 3- Industrial oxidation: using a nitrogen- air mixture containing approx. 3.5 % of oxygen. Finding out a technique to prevent post-translational modifications of amino acids mainly Aspartic acid, Glutamine, Glycine, Methionine, Phenylalanine, Serine, and Valine with glycogen for the biological glue and cohesive function. Polymeric Betaine (DPAB) with high protein resistance can be used in as marine paint to prevent attachment of marine organisms. Sulfobetaine polymer brushes may show strong resistance to the attachment of fouling organisms. Betaine polymers can be designed based on the main chains or the backbones of the polymers, the linking groups that connect betaine moieties to the backbones. The distribution pattern of individual carbohydrate reflects the various stages of the biological glue secretions, and the biological and abiotic processes controlling the formation and alteration of dissolved organic matter.

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