10. SUMMARY

Biofouling is a unique phenomenon that can influence the surfaces of living organisms. The living organisms, especially sessile forms, are more susceptible to fouling and predation. The fouling may be deleterious to seaweeds, which are also very much prone to herbivory that can alter their community structure and dynamics. Hence, most of the seaweeds have evolved a number of physical and chemical strategies to counter this problem. Most of the seaweeds though lack visible epibionts on their surface, harbour epiphytic bacteria, which produce inhibitory compounds through symbiotic or mutualistic association, to augment the host's defense. Though antibacterial properties of many seaweeds have been documented, little indication is available on the extent of their ecological role.

The rich seaweed resources in Gulf of Mannar, crowned with diverse coral reef and sea grass habitat and associated fauna and flora, may provide an insight into chemical defense strategies, which hitherto has not been subjected to comprehensive study. So, this study was undertaken to investigate and elucidate the chemical defense in seaweeds including seasonal variations, giving emphasis to the ecological role of the seaweed compounds.

Antibacterial assay against 20 biofilm bacterial strains (Four strains each of *Vibrio* sp. and *Pseudomonas* sp.; three strains of *Micrococcus* sp.; two strains each of *Enterobacter* sp. and *Cytophaga* sp. and one each of *Escherichia coli*, *Flavobacterium* sp., *Aeromonas* sp., *Bacillus* sp. and
Corynebacterium sp.), isolated from panels deployed in the sea, was used to assess the activity in methanol, acetone, butanol, ethyl acetate, toluene, chloroform and diethyl ether extracts of 43 seaweeds belonging to Chlorophyta (37%), Rhodophyta (37%) and Phaeophyta (26%). Higher activity was observed in Rhodophytes (30.9%) followed by Chlorophytes (29.5%) and Phaeophytes (23.6%). The Rhodophytes and Chlorophytes showed maximum percentage of inhibition against 90% of biofilm bacteria and the Phaeophytes showed a maximum of 75%. The diethyl ether extract showed overall higher range of activity (0 to 90%) followed by acetone (10 to 85%) and methanol (10 to 70%).

The 11 seaweeds (Ulva lactuca, U. reticulata, Chaetomorpha aerea, Udotea flabellum, Enteromorpha compressa, Gracilaria edulis, G. folifera, Acanthophora spicifera, Sargassum wightii, Stoechospermum marginatum, Padina tetrastomatica) have exhibited seasonal variation in antibacterial activity during 2005 to 2006. Higher activity was observed in post-monsoon, summer and pre-monsoon seasons and lower activity during monsoon season. The red seaweeds showed higher activity followed by green and brown seaweeds. The red and brown seaweeds showed a marked seasonal variation. Among the green seaweeds, Enteromorpha compressa showed higher activity during all seasons of 2005 and 2006. It showed wide spectrum (100%) activity during summer (2005 and 2006) and pre monsoon (2005).

Epiphytic bacteria were observed in all the 11 seaweeds, in spite of their exhibition of antibacterial activity against biofilm bacteria. But, their
epiphytic bacterial load (19 to 142 X 10^2 CFU/cm^2) was lower than the surrounding seawater (110 x10^4 CFU/ml). Higher number was noticed during summer, pre-monsoon and post-monsoon with lower density in monsoon season. Phaeophytes showed higher average density during 2006 followed by Chlorophytes and Rhodophytes and in 2005, it showed low average density followed by Chlorophytes and Rhodophytes. In cross-streaking method, 13 out of 110 epiphytic bacterial strains from 11 seaweeds showed antagonistic activity against biofilm bacteria. The percentage of activity ranged from 40 to 100%. The U2 (Ulva lactuca) strain showed wide spectrum of activity. Interestingly, these epiphytic isolates were insensitive against crude extracts of their hosts indicating the possibility that they may play a symbiotic role by helping the seaweeds in their defense against fouling. The epiphytic bacteria may be responsible for higher antibacterial activity observed in summer as the seaweeds showed higher antimacrofouling and feeding deterrent activity in pre-monsoon season.

Wide spectrum antibacterial activity was noticed in the supernatants of U2 (Ulva lactuca) (4 to 9 mm) and E1 (Enteromorpha compressa) (2 to 10 mm) strains. The E1 strain showed enhanced activity from 50% observed in cross streaking method to 100% in supernatant. The ethyl acetate extract of the U2 strain showed wide spectrum activity (3 to 6 mm) and that of the E1 strain against 90% of biofilm bacteria. The 100% of ethyl acetate column fraction of U2 strain and 25% Hexane: 75% ethyl acetate fraction of the strain E1 showed wide spectrum activity. The strains U2 and E1 were identified as Pseudomonas sp. and Vibrio sp. The Pseudomonas sp. showed potent
antagonistic activity and also produced extra-cellular metabolite that inhibited all biofilm bacterial strains. The *Vibrio* sp., though showed antagonistic activity against 50% biofilm bacteria, produced metabolites which inhibited all the 20 biofilm bacterial strains.

In preliminary antimacrofouling screening, the byssal production and attachment in the mussel *Perna indica* was inhibited by all the twenty seaweed extracts and the activity was concentration specific. The brown algae exhibited highest inhibition followed by red and green algae. The 11 seaweed extracts (five from Chlorophyta, three each from Rhodophyta and Phaeophyta) showed higher inhibition during summer and pre-monsoon seasons of 2005 and 2006. In general, all the three groups of seaweeds invariably showed seasonal variation with higher activity during pre-monsoon season. The seasonal variation in activity indicated that the secondary metabolites produced by the seaweeds may have an ecologically relevance. The higher activity in pre-monsoon and low activity in monsoon could be linked to the seasonal abundance of mussels and presence of herbivores. The minimum inhibitory concentration ranged from 202±12.5 (*Sargassum wightii*- pre-monsoon-2006) to 654±5.4 (*Gracilaria edulis*-post-monsoon-2005) μg/ml.

Secondary metabolites were considered as responsible deterrent factor against micro and then macrofouling. The Phaeophytes, which exhibited moderate level of activity in antibacterial assay, interestingly, exhibited high level of activity in mussel bioassay than red and green seaweeds. The brown
algae *Sargassum wightii* exhibited most prevalent inhibition of byssal thread formation and attachment at very low concentration. The higher activity in brown algae could be linked to the presence of phenolic compounds. The low activity observed in green seaweeds suggested that the green algal chemical defenses are targeted mainly towards antifeeding. The less EC$_{50}$ variation between 2005 and 2006 could be ascribed to the constant inhibitory activity of seaweeds on all seasons, which could be considered as deterrent and not toxic. The low EC$_{50}$ values than LC$_{50}$ values and the recovery of mussels within 24 hours of transfer to the extract free seawater indicated the non-toxic nature of the seaweed extracts.

The methanol, acetone, butanol, ethyl acetate, toluene, chloroform and diethyl ether extracts of 20 seaweeds showed herbivore deterrent activity against the sea angel *Monodactylus kottelati* in the preliminary screening. The brown algae showed average higher deterrence activity (85.9%) followed by green (78.7%) and red algae (77.7%). *Caulerpa scalpelliformis*, among Chlorophytes, exhibited 100% of deterrence followed by *Udotea flabellum*, *Enteromorpha compressa* and *Ulva lactuca* with 95.3±3.2, 88.5±3.2 and 85 ±2.5% respectively. In Rhodophyta, *Acanthophora spicifera* exhibited highest deterrence (96.6±2.4) followed by *Gracilaria crassa* and *Portieria hornemanni* with 95.5±2.5 and 92.3±3.6%. In Phaeophyta, the *Sargassum wightii* exhibited 98.2±0.6% of activity followed by *Stoechospermum marginatum* and *Lobophora variegata* with 88.5±2.3 % and 87.2±2.7% of deterrence.
Invariably, all the 11 seaweed extracts (five Chlorophytes, three each of Rhodophytes and Phaeophytes) showed marked variation with higher deterrence during pre-monsoon season followed by summer. Phaeophytes showed higher activity followed by Chlorophytes and Rhodophytes during both the years of study. The higher activity coincided with the abundance of fishes along Tuticorin coast during the pre-monsoon period (July-September). The deterrence was found to be dose dependent as the percentage increased with increasing extract concentration. The seasonal variation indicated the ecological significance of deterrents with the abundance of herbivores and fouling organisms.

The partitioned ethyl acetate phase of *Enteromorpha compressa* showed wide spectrum activity followed by butanol phase that showed 75% activity. In *Acanthophora spicifera*, both ethyl acetate and butanol phases showed wide spectrum activity. The wide spectrum activity in ethyl acetate phase indicated the non-polar nature of the active component. Further, the 75% and 100% activity observed in butanol phase of *E. compressa* and *A. spicifera* and some activity in water phase indicated the presence of more than one compound in the seaweeds.

Out of 12 fractions (1E to 12E), the fraction 2E (Hexane 75%: 25% diethyl ether) of *Enteromorpha compressa* showed wide spectrum activity. Further elution of the fraction 2E led to six fractions (1 EC to 6 EC) and of which, the 2EC showed wide spectrum activity against biofilm bacteria. Further elution of 5A (100%diethyl ether) fraction of *Acanthophora spicifera*,
which showed highest activity, led to five fractions (1AS to 5AS) and 3AS fraction showed wide spectrum activity. The analytical HPLC of active column fraction (2EC) of seaweed *E. compressa* showed mixture of two compounds with the prominent fraction 2EC-1. The 2EC-1 and 3AS fractions were subjected to antibacterial assay, mussel bioassay and antifeedent assay.

The minimum inhibitory concentration of 2EC-1 against biofilm bacterial strains was 25 µg/disc. This fraction exhibited 100% of feeding deterrence at 60±3.2 µg concentration. In mussel bioassay, the minimum inhibitory concentration (MIC) was found to be 160±8.5 µg/ml and EC<sub>50</sub> and LC<sub>50</sub> values were found to be 80±5 µg and 180±5 µg/ml. The Minimum inhibitory concentration of 3AS fraction was 30 µg/disc. It showed 100% feeding deterrence at 95±5 µg concentration. In mussel bioassay, the MIC was found to be 162±10.5 µg/ml and the EC<sub>50</sub> and LC<sub>50</sub> values were 88±4.5 µg/ml and 240±10 µg/ml. The active fraction 2EC-1 was characterized

The herbivore deterrent activity observed in *E. compressa* at very low concentration indicated that the green seaweed secondary metabolites predominantly play an important role in chemical defense against herbivory. Enhancement of activity in fractions (2EC-1 & 3AS) was noticed and *E. compressa*, the crude extract of which showed 100% deterrence at 750 µg, exhibited 100% deterrence at 60±3.2 µg fraction concentration. The low LC<sub>50</sub> values for 2EC-1 & 3AS than EC<sub>50</sub> values denoted their inhibitory nature and can be considered as non-toxic. The toxicity assay with the mussels indicated the non-toxic nature of the fractions as they did not cause any irreversible
change in mussels and 100% recovery was observed in all the mussels within 24 hours of transfer to extract free seawater.

The molecular weight of the compound (2EC-1) was found to be 390 (Dl) (M\(^{+}\)). The IR values have given a presumption that the compound might be having CH and -C-O functionalities and the NMR spectrum showed the possible presence of CH, O-CH, CH\(_2\)-CO, O-CH\(_2\), CH-Cl and CH\(_3\) functionalities. The elemental analysis indicated the presence of Carbon, Hydrogen, Oxygen and Chlorine and the compound may belong to Indole chloro acid derivative. The structural characteristics of the isolated compound could be confirmed through further studies. After confirmative studies, computer modeling can then be used to generate chemical analogues of this antibacterial compound and to examine the structure function relationships.

The column fractions of Enteromorpha compressa (2EC) and Acanthophora spicifera (3AS) inhibited the settlement of biofilm bacteria and the percent cover of foulers with increasing concentration in the field assay. The percent cover increased steadily over the 30 days period. The inhibitory effect indicated that the algal compounds play an important role in regulating the bacterial load and are not toxic. The sustained action of the compounds could be evidenced from the prominent antifouling activity exhibited by the fractions even after 30 days. The inhibition of fouling in the field along with laboratory based antifouling and herbivore deterrent activity indicated the ecologically significant adaptive value and multiple roles of these seaweed metabolites.