LIPOFUSCIN AS
PHYSIOLOGICAL INDICATOR OF HEAVY METAL STRESS

6.1. INTRODUCTION.

Heavy metals form a dangerous group of potentially hazardous pollutants, which are released into the marine realms threatening the very existence of aquatic biota (Bryan, 1984). The response of marine animals to a pollutant can be detected at different levels of organization and responses. All species are tolerant to a certain amount of environmental variation. However, beyond the tolerant limits, characteristic biological responses related to the ultimate survival or death of the individual are elicited (Blackstock, 1984; Bayne et al., 1985). The biological responses include physiological, biochemical, morphological, genetic and behavioural responses of organisms to stress (Widdows, 1985).

Most organisms are able to concentrate heavy metals in their body. This holds true for bivalves too. These organisms concentrate heavy metals at very high levels in the different tissues. However, it has been seen that they are able to survive and apparently reproduce normally, which indicates that they have evolved control or tolerance
mechanisms at the cellular level (Akberali and Trueman, 1985). The toxic action of heavy metals is generally attributed to the inhibitory effect on the enzyme system.

In general, high concentration of heavy metals have harmful effects on living organisms, although in some cases very low concentration (for copper and mercury) are toxic to some organisms. Since sublethal concentration of metals manifest many physiological changes in the animal, they are considered to be more deleterious and harmful than lethal concentration. It can ultimately affect the population as a whole without the danger being noticed.

Copper is one of the trace elements required by many marine organisms for normal growth and development (Bryan, 1971). Its absorption from food or water, in which the organisms live, can be regulated by different mechanisms in different animals. At lower concentration, copper acts as an essential element (Villarreal et al., 1986; William et al., 1987), whereas at higher concentration, it becomes inhibitory and toxic and can be used as a molluscid (Simkiss and Mason, 1985).

Many pollutants exert their effect on biological system by inducing the production of free radicals (Halliwell, 1981; Halliwell and Gutteridge, 1985). According to Pisanti et al. (1988), all transition metals were found to induce
lipofuscinogenesis. The transition metals can be considered as free radicals since they have one or more unpaired electrons (Halliwell and Gutteridge, 1985). Therefore they undergo variations in their oxidative state tending towards the donation or acquisition of electrons inorder to couple the unpaired electrons. Due to this character, transition metals react with oxygen, producing dangerous free radicals such as superoxides and hydroxyl radicals. Thus, copper being a transition metal, may act as a catalyzer of peroxidative reaction. Following are the two possible mechanisms by which copper can induce the formation of free radicals (Sreekumar et al., 1978).

\[ \text{Cu}^+ + \text{O}_2 \rightarrow \text{Cu}^2 + \text{O}_2^- \]

\[ \text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH} + \text{OH}^- \]

The reaction of such radicals with biological macromolecules like lipoproteins causes peroxidative phenomenon leading to lipofuscin production (Sohal, 1981; Aloj and Pisanti, 1985; Aloj et al., 1986b; Marzabadi et al., 1988; Pisanti et al., 1988).

The interference of copper in the histogenesis of lipofuscin granulation may be explained hypothetically in two ways:

1) a direct induction of peroxidation of the poly-unsaturated fatty acids of membranes (Sreekumar et al., 1978).
(2) an indirect action via a removal of thio groups which are indispensable for the metabolic role of glutathione dependent enzymes and some metallothioneins which are known to have the capacity for binding the excess heavy metals found in the tissues (Piccinni and Coppellotti, 1982; Viarengo, 1985).

Thus lipofuscin, a granular pigment of wear and tear, is considered to be a marker of cell damage. It is present particularly in post mitotic cells like neurons, which reveal the life history of an individual. They can be considered as an organelle, serving the function of a depot or store house of indigestible, unexcreted cellular wastes, primarily consisting of intracellular membranes (Sohal and Wolfe, 1986).

Massive accumulation of residual bodies in kidney is reported as a metal induced response (George et al., 1982). Copper exposure in the neurons of Torpedo sp. has resulted in a significant increase in lipofuscin granules with extensive damage to mitochondria (Aloj and Pisanti, 1985; Aloj et al., 1986b; Enesco et al., 1989). Presence of transitional metals like copper, iron, chromium, vanadium etc. in the culture medium induces a rise in the lipofuscin production in marine mycete Corollospora maritima (Pisanti et al., 1988). Increased lipofuscin accumulation in the digestive cells have been reported in M. edulis and Littorina littorea exposed to copper and hydrocarbons (Pipe and Moore, 1986; Moore, 1988) and in
*P. viridis* exposed to copper and mercury (Krishnakumar *et al.*, 1990).

Heavy metals were reported as the most potent inhibitor of mitochondrial respiration and oxidative phosphorylation (Zaba and Harris, 1976; Akberali and Earnshaw, 1982; Somasundaram *et al.*, 1984; Tort *et al.*, 1984 a, b; Babu and Rao, 1985; Crespo and Sala, 1986; Krishnakumar, 1987). Upon exposure to heavy metals, molluscs tightly close their valves resulting in a decreased rate of respiration. The copious secretion of mucus further reduces the efficiency of gaseous exchange across the gills thereby subjecting the animal to an anaerobic state. This hypoxia leads to the activation of terminal oxidative system of lipofuscin granules (Karnaukhov *et al.*, 1972; Karnaukhov, 1973b). The carotenoids being a component of the lipofuscin granule can provide the cells with energy required under hypoxia (Karnaukhov, 1973b; 1990).

Karnaukhov *et al.* (1977) observed an increase in the population of molluscs having high concentration of carotenoids, in the polluted area of Black Sea. At the same time, population with low concentration of carotenoid content decreases with pollution. A correlation has been observed by Krishnakumar (1987) in *Perna viridis*, wherein the mussels having high carotenoid concentration in their body were found to be able to withstand acute mercury, zinc and copper toxicity. The increase in the carotenoid concentration in the
body of the mussel, *P. viridis* was found to be linear with the increment in the metal concentration in the ambient medium.

The bivalve molluscs, are found to accumulate and concentrate most of the pollutants within their tissues to concentration significantly above the ambient level in the environment, thus facilitating accurate chemical analysis and assessment. The present study aims at elucidating the effect of sublethal exposure of copper on both the carotenoid concentration and the lipofuscin accumulation.

6.2. MATERIALS AND METHODS.

Details regarding the materials and methods have been described in Chapter-2.

6.3. RESULTS.

The sublethal levels of 2ppm and 7.5ppb copper taken for *S. scripta* and *P. viridis* respectively, were based on the previous copper toxicity studies conducted on these two species (Thampuran *et al.*, 1982; Krishnakumar, 1987). The sublethal effect of copper (2ppm) on the carotenoid concentration of *S. scripta* (35-45mm) has been presented in Table 6.1a. From the table, it could be seen that there is significant difference (*P*<0.01) between the time intervals (0h and 48 h). The readings at the 48h exposure were significantly
higher than that of the control (Fig. 6.1).

Table 6.1b represents the variations in the carotenoid concentration of *P. viridis* (55-65mm) exposed to sublethal levels of copper (7.5ppb). It has been graphically represented in Fig. 6.2. A significant difference (*P*<0.01) in the carotenoid concentration between the control (0h) and 48h of exposure has been noticed.

A morphological examination of lipofuscin granules in the hepatopancreatic tissues of *S. scripta* at 0h and 48h revealed characteristic features which are given in Table 6.2a. In comparison to control (0h), 48h copper exposed animals exhibit more pigmentation with increased granular size and number. At 48h of exposure, heterogenous granulations could be noticed due to high aggregative nature (Fig. 6.3a and 6.3b).

Characteristic morphological features of lipofuscin granules of *P. viridis* in the hepatopancreatic tissues have been described in Table 6.2b. Corresponding carotenoid concentration has been illustrated in Fig. 6.2. The main contrast depicted in the lipofuscin granules between *P. viridis* and *S. scripta* is in the size of the granules (Fig. 6.4a and 6.4b). In the former species, granular size remains the same at 0h and 48h of exposure, whereas in latter, an increase in granular size has been observed at 48h of exposure, resulting in huge heterogenous granulations due to aggregation. The total
carotenoid concentration and morphological characteristics of lipofuscin granules at 48h of heavy metal exposure is similar to that observed at 48h of hypoxic exposure in both *S. scrippta* (Table 5.1a and 5.3a; Table 6.1a and 6.2a) and *P. viridis* (Table 5.2a and 5.3b; Table 6.1b and 6.2b).

Tables 6.3a and 6.3b represents the number of lipofuscin granules/cm² in *S. scrippta* and *P. viridis* respectively under heavy metal exposure. In both the species, a significant difference (*P*<0.5) in the number of lipofuscin granules has been observed between the control and the copper exposed animals. Exposed animals gave more number of granules /cm² in comparison to non-exposed in both the species.

6.4. DISCUSSION.

The data presented here demonstrate that the exposure to sublethal levels of copper produces remarkable biochemical changes at the cellular level which has been indicated by an elevated carotenoid concentration and lipofuscin accumulaton. In both *S. scrippta* and *P. viridis*, high carotenoid concentration at 48h of exposure in comparison to control (0h) seems to be consistent with that observed in *P. viridis* and Villorita cyprinoides var cochinensis exposed to acute mercury, zinc and copper and sublethal levels of mercury and copper respectively
(Krishnakumar, 1987; Krishnakumar et al., 1987; Sathyanathan et al., 1988).

In both *P. viridis* and *S. scripta*, lipofuscin granules displayed marked morphological changes after 48h of heavy metal exposure in comparison to the control. The control (0h) exhibits fewer number of granules, scattered in the cytoplasm with less pigmentation and non aggregative nature in both the species. At 48h of exposure, more pigmentation with increased clustering resulting in heterogenous granulations have been observed. In *P. viridis*, the granular size remains unaltered at 0h and 48h of exposure, whereas in *S. scripta*, an increase in the granular size has been noticed at 48h of exposure.

A similar change in the lipofuscin granule has been observed in both the species at 48h of hypoxic exposure (Chapter-5). In *P. viridis*, as in heavy metal stress, 48h of hypoxia exhibits more pigmentation with increased granular number and aggregation, resulting in heterogenous granulations. The granular size remains the same for 0h and 48h of hypoxic exposure. Regarding *S. scripta*, during hypoxic exposure as well as in heavy metal exposure, more pigmentation with increased size and aggregation have been observed. Hardly any difference in the granular number between 0h and 48h of hypoxia has been noticed on statistical analyses. But during heavy metal exposure, an increase in the granular number has been noticed at 48h of exposure. The increased granular size noticed in
S. scripta may be due to the high clustering or aggregative nature exhibited by the lipofuscin granules. The characteristic differences in the lipofuscin granules of both S. scripta and P. viridis need further study.

Since carotenoid is a constituent of the lipofuscin granules (Karnaukhov et al., 1972; Karnaukhov, 1973b; 1990), at 48h of exposure, concomitant with the increase in lipofuscin accumulation, an enhancement of the total carotenoid concentration has been observed in both the species. In both the stressed condition, a rise in the total carotenoid concentration and lipofuscin accumulation were noticed at 48h of exposure.

Regarding S. scripta, the total carotenoid increase during 48h of hypoxic exposure as well as at 48h of heavy metal exposure have been found to be nearly 40% greater than that of the control value. In the case of P. viridis, the carotenoid build up during 48h of heavy metal stress is nearly 95% greater than that of the control (0h), whereas at 48h of hypoxic exposure, the increase is found to be nearly 87% higher when compared to the control (0h).

Both carotenoid and lipofuscin accumulation are taken as an index of heavy metal stress in molluscs (Krishnakumar et al., 1990; Moore, 1988). When the animals were exposed to heavy metals, they were found to be subjected to
hypoxic stress. The lowering of the metabolic rate with a reduced oxygen uptake rate had been reported in both *P. viridis* (Krishnakumar, 1987) and *S. scripta* (Thampuran, 1986) during heavy metal exposure. Both partial and complete shell valve closure during heavy metal exposure is known to reduce the heart beat rate in molluscs (Bayne *et al.*, 1976a). Reduction in the heart rate will bring about reduction in ventilation within tissues, thus influencing the oxygen uptake. The magnitude of the carotenoid increase during 48h hypoxic exposure is very much similar to that observed at 48h of heavy metal stress. Characteristic features in the accumulation of the lipofuscin granules at 48h of hypoxia were found to be similar to that occurring at 48h of heavy metal exposure. As the quantitative changes in the carotenoid content and lipofuscin accumulation under hypoxia and heavy metal stress are of the same magnitude, it is reasonable to presume that the increase in carotenoid concentration and lipofuscin accumulation expressed by bivalves under heavy metal stress can be due to the indirect effect of hypoxia.
Sublethal effect of copper on total carotenoid concentration (mg 100g⁻¹ wet wt)

Table 6.1a *Sunetta scripta* (35-45mm) (2 ppm)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Total carotenoid mg 100g⁻¹ wet wt</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.600 ± 0.141</td>
<td>**</td>
</tr>
<tr>
<td>48</td>
<td>2.250 ± 0.071</td>
<td>12.748</td>
</tr>
</tbody>
</table>

Table 6.1b *Perna viridis* (55-65mm) (7.5 ppb)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Total carotenoid mg 100g⁻¹ wet wt</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.197 ± 0.001</td>
<td>*</td>
</tr>
<tr>
<td>48</td>
<td>0.385 ± 0.054</td>
<td>3.487</td>
</tr>
</tbody>
</table>

* - P<0.1 
** - P<0.01
Total carotenoid concentration during sublethal exposure of copper

![Chart 1](image1)

**Fig. 6.1** *S. scripta* (35-45 mm)

![Chart 2](image2)

**Fig. 6.2** *P. viridis* (55-65 mm)
Morphological features of lipofuscin granules observed in control and copper exposed animals

Table 6.2a *Sunetta scripta* (2ppm) (35-45mm)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Characteristics of lipofuscin granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Less pigmentation, small spherical bodies with no aggregation.</td>
</tr>
<tr>
<td>48</td>
<td>More pigmentation, high clustering resulting in heterogenous granulations with increase in the granular size.</td>
</tr>
</tbody>
</table>

Table 6.2b *Perna viridis* (7.5ppb) (55-65mm)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Characteristics of lipofuscin granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Mainly scattered, spherical granules fewer in number with less pigmentation and non aggregation.</td>
</tr>
<tr>
<td>48</td>
<td>More pigmentation, granular size same as that of control. Increased number with more clustering resulting in heterogenous granulations.</td>
</tr>
</tbody>
</table>
Comparison of the number of lipofuscin granules of control and copper exposed (sublethal) animals

Table 6.3a *Sunetta scripta* (35-45mm)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>No: of lipofuscin granules/cm² (± SD)</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.208 ± 2.70</td>
<td>***</td>
</tr>
<tr>
<td>48</td>
<td>4.083 ± 5.21</td>
<td>1.1042</td>
</tr>
</tbody>
</table>

Table 6.3b *Perna viridis* (55-65mm)

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>No: of lipofuscin granules/cm² (± SD)</th>
<th>t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.750 ± 2.49</td>
<td>***</td>
</tr>
<tr>
<td>48</td>
<td>3.875 ± 3.79</td>
<td>0.8592</td>
</tr>
</tbody>
</table>

a = no: of lipofuscin granules/cm² was ascertained on micrographs (200X)

*** - p<0.5
Effect of sublethal exposure of copper (2ppm) on lipofuscin accumulation (X200)

*Sunetta scripta* (35-45mm)

Fig. 6.3a Control (0h)

Fig. 6.3b Exposed (48h)
Effect of sublethal exposure of copper (7.5 ppb) on lipofuscin accumulation (X200)

*Perna viridis* (55-65mm)

Fig. 6.4a Control (0h)

Fig. 6.4b Exposed (48h)