

## HISTOLOGICAL AND HISTOCHEMICAL ALTERATION IN CERTAIN BLACK SEA BIVALVES SUBMITTED TO ACCIDENTAL POLLUTION

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**Summary.** Due to their microphage capacity, two-valve shells have great biologic importance: they clean the environment of harmful microorganisms and decaying organic compounds which result in water purification, water filtering by means of adaptive structures of digestive system and, indirectly, by those of respiratory, circulatory and excretive system is of major significance. Waste materials derived from animal metabolism coalesce in larger aggregates which sink and deposit onto the bottom, thus enabling the bivalvular shells to contribute to developing and securing adequate conditions for the productive cycle of marine environment and even twining themselves into food for animals. Our study makes a histological, histochemical and histometric survey of branchia (ctenidia) of *Mytilus galloprovincialis* so as to enlarge the scant body of knowledge on the topic in reference literature [2, 11].

**Key words:** *Mytilus galloprovincialis*, branchial lamellae.

### INTRODUCTION

Marine water regularly contains a good deal of substances such as salts and organic compounds. Organic compounds decay is carried out by the action of saprophytic microflora algae, protozoa, vascular plants and certain animal organisms. Atmospheric oxygen plays its role in decaying organic compounds. Depending on its quantity it may quicken or slow down this process [8].

Assessing the quantity of costal organic matter is highly significant in point of measuring pollution levels [1]. Modifications of water salts concentration may be caused by a variety of factors. Hydrocarbon pollution is just one of them within certain limits.

Molluscs are able to adjust to variations of concentration levels in the outer environment. Trespassing of such tolerance limits will entail noxious consequences on the organism.

Oil spilling into sea water for different reasons and especially extended exposure of organisms to oil or oil compounds may trigger their morphological, physiological and biochemical alterations.

Such changes appear in mussels in consequence of oil contamination:

- tissular hyperplasia;
- necrosis of digestive and respiratory epithelia;

– gonad tumours.

Our study makes a histological, histochemical and histometric survey of branchia (ctenidia) of *Mytilus galloprovincialis* so as to enlarge the scant body of knowledge on the topic in reference literature [3].

## MATERIAL AND METHODS

### MATERIAL

We made an experimental study of two valve species *Mytilus galloprovincialis* of the Black Sea. The five-way acute intoxication (120 hours) was carried out on 5 batches the results of which were referred to the control batch.

Each batch consisted of 30 adult mussels:

Batch I was the control. It was administered seawater without any “mixture”.

Batch II (C1) was immersed in a tank with 10 mg “mixture”/l of seawater.

Batch III (C2) was immersed in a tank with 5 mg “mixture”/l of sea water.

Batch IV (C3) was immersed in a tank with 2.5 mg “mixture”/l of sea water.

Batch V (C4) was immersed in a tank with 2 mg “mixture”/l of sea water.

Batch VI (C5) was immersed in a tank with 1 mg “mixture”/l of seawater.

The “mixture” was dosed for 30 l seawater in each case.

### METHOD

Having examined the impact of the measured concentration of toxic mixture, we extracted the branchiae and the hepatopancreas as we considered it is at this level that the most decisive impact occurred.

The processing of the material was made by using the classic histological method: embedding in paraffin, microtome sectioning, staining with regular dyes such as Haematoxylin and Eosin, Goldner-Szekelly and Masson trichromic, as well as certain special staining methods (to highlight mucopolysaccharides) as are staining with Schiff periodic reagent or with Alcian blue.

Special inclusion techniques were also made use of in order to spot electron-microscopic structures, case in which 0.2 M buffer solution of cacodilate was used for fixing while for fixing 1% osmium tetroxid served for the same purpose. Using Leica - Ultracut R microtome, provided fine and semifine sections.

Thick sections were stained with toluidine blue, methylene blue, azure II and aniline blue.

Stained sections were examined on microscope in consequence of which fixing on special plane films were performed.

Pictures were obtained after development and printing on special paper by Epson printer.

Fine sections of 500–1200 Å thickness were collected on frames of fonvar film twice stained with alcoholic solution of uranile acetate and aqueous solution of lead acetate.

The specimens obtained were examined on microscope and the most characteristic images were recorded on the computer by means of a JVC video camera.

Pictures processing was performed with the help of Lucia G4.10 analysis program. The significant  $P < 0.05$  statistic coefficient assessed the value of statistic data obtained by Student-Fischer method. We highlight that the same measuring system for the morphometric processing of the pictures has been used for all batches.

The following conclusions have been reached in consequence of examining the histologic sections of *Mytilus galloprovincialis* – control batch held in seawater without added hydrocarbons. *Mytilus galloprovincialis* of the Romanian coastal waters of the Black Sea fall perfectly within the morphoanatomical and histological descriptions found in literature [2].

Mussels are ideal organisms to be used as biological control groups in certain polluting events. We opted for mussels (only one species) on the following counts:

- are widespread in the coastal areas of the Black Sea where they grow in dense population groups;
- being sessile they are able, through their sedentariness, to provide in future valuable data on the polluted zones;
- are readily accessible and adequate as experimentation material;
- have not yet been investigated enough histologically;
- can be easily processed for biological investigations.

## RESULTS

### ALTERATION IN THE HISTOLOGICAL STRUCTURE OF BRANCHIA IN *MYTILUS GALLOPROVINCIALIS*, EXPOSED ON CERTAIN HYDROCARBONS

#### *Results of morphometric processing of branchia*

Data concerning branchial lamina and lamella in all batches were put under scrutiny in order to assess the results acquired through mathematical processing of pictures:

- height of branchial lamina epithelium (Table 1, Figure 1, a and b – recognition index, EI);
- thickness of interlamellar connective tissue;
- height of lamellar epithelium;
- thickness of interlamellar septum (connective tissue);
- axial diameter of branchial lamina;

- transverse diameter of branchial lamina;
- density of branchial lamellae per surface unit (100 microns);
- number of granulations on a surface filled by 10 branchial lamellae.

*Table 1*  
Variation of epithelium height in branchial lamina - statistical data

|                          | EL.N     | EL.C1    | EL.C2    | EL.C2.5  | EL.C5    | EL.C10   |
|--------------------------|----------|----------|----------|----------|----------|----------|
| Mean                     | 15.462   | 16.225   | 13.905   | 26.93    | 24.55    | 22.138   |
| Std. Error               | 0.838492 | 0.808067 | 0.861745 | 0.985897 | 0.531975 | 1.330212 |
| Median                   | 15.67    | 16.585   | 14.105   | 27.2     | 24.76    | 22.435   |
| Std. Deviation           | 2.651544 | 2.555331 | 2.725078 | 3.117681 | 1.682254 | 4.2065   |
| Sample Var.              | 7.030684 | 6.529717 | 7.42605  | 9.719933 | 2.829978 | 17.69464 |
| Kurtosis                 | -0.12773 | 0.626665 | 3.216723 | 1.968342 | -0.27392 | 0.95669  |
| Skewness                 | -0.13621 | -0.181   | 1.231809 | -0.97366 | 0.28789  | -1.11915 |
| Range                    | 8.84     | 9.13     | 10.31    | 11.42    | 5.57     | 13.24    |
| Minimum                  | 10.89    | 11.59    | 9.95     | 20.08    | 22.05    | 13.22    |
| Maximum                  | 19.73    | 20.72    | 20.26    | 31.5     | 27.62    | 26.46    |
| Confidence level (95.0%) | 1.896802 | 1.827975 | 1.949405 | 2.230256 | 1.203413 | 3.009151 |

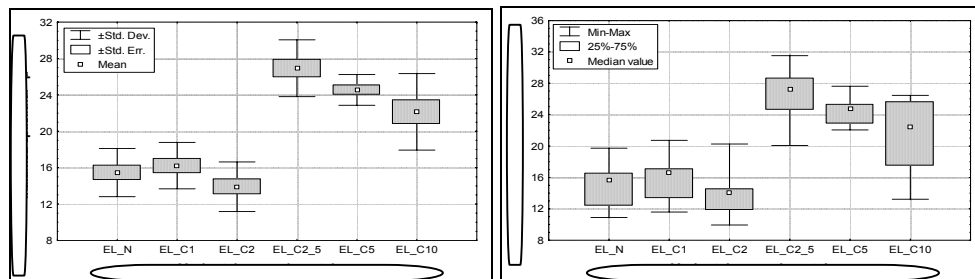


Figure 1 – a) Variation of height in branchial lamina (microns) – statistical analysis

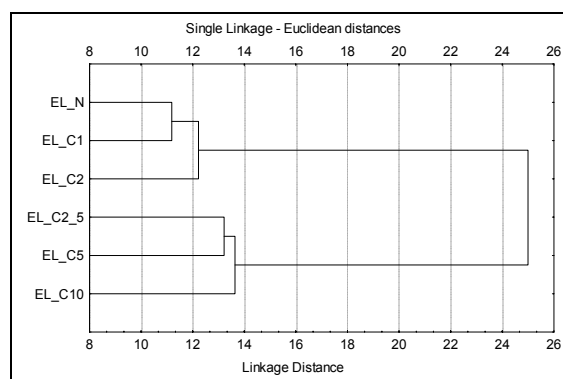


Figure 1 – b) Dendrogram of variations of branchial lamina epithelium in the 6 experimental variants

A progressive growth has been found from the control batch (measuring 15.46 microns through 16.22 microns) to the batch, which received the lowest concentration of pollutant mixture (C5). The growth was significantly higher – 22.85% in batch C4, followed by a highly marked growth – 26.93 microns in batch C3, followed by a significant decrease in batch C1 that received the highest concentration of mixture.

We ascertained that the height of branchial lamina undergoes adjusting alterations with growing concentrations of mixture. However, it does not change significantly with high concentrations.

The same system of analysis was used with the other parameters under discussion as the one described with the parameter “*Height of epithelium in branchial lamina*”.

#### ***Analysis of microscopic preparations of mussel branchiae***

As known, branchiae of bivalve molluscs lie on the sides of visceral mass. They are provided with cilia the action of which stimulates permanently refreshing water penetration into lamellar network (Figures 2 and 3).

#### **ALTERATION IN THE HISTOLOGICAL STRUCTURE OF HEPATO-PANCREAS IN *MYTILUS GALLOPROVINCIALIS* EXPOSED TO CERTAIN HYDROCARBONS**

#### ***Results of morphometric processing of mussel hepato-pancreas***

The following parameters are considered by morphometric processing of the hepato-pancreas pictures of batches under discussion by applying the same method used in morphometric processing of branchiae:

- height of duct epithelium (Table 2, Figure 4, a and b – recognition index, Ec);
- maximum diameter of ducts;
- epithelium height in secretion tubules (acinar appearance);
- maximum diameter of tubules;
- area fraction of tubules;
- area fraction of granules;
- area fraction of vacuoles.

The height of the epithelium in ducts is 54.56 microns on an average for the control group (Ec.N) and a variation interval of 9.12 microns. The contaminated batches react in two completely different ways as against this batch growing under “normal” conditions instead of progressively in step with the growing concentration.

Accordingly, the height of the epithelium is much greater in Ec.C1, Ec.C2 and Ec.C2.5 and presents a much wider variation scale than in Ec.N whereas in Ec.C5 and Ec.C10 this one is much lower.

*Table 2*  
Height of duct epithelium – data of statistical analysis

|                          | <b>Ec.N</b> | <b>Ec.C1</b> | <b>Ec.C2</b> | <b>Ec.C2.5</b> | <b>Ec.C5</b> | <b>Ec.C10</b> |
|--------------------------|-------------|--------------|--------------|----------------|--------------|---------------|
| Mean                     | 54.5575     | 63.2975      | 58.2575      | 63.11          | 29.49        | 25.02         |
| Std. Error               | 2.035208    | 18.21784     | 8.774725     | 8.159729       | 1.443324     | 1.205017      |
| Median                   | 54.115      | 49.01        | 57.37        | 63.92          | 30.055       | 24.42         |
| Std. Deviation           | 4.070417    | 36.43568     | 17.54945     | 16.31946       | 2.886647     | 2.410035      |
| Sample Var.              | 16.56829    | 1327.559     | 307.9832     | 266.3247       | 8.332733     | 5.808267      |
| Range                    | 9.12        | 79.15        | 42.85        | 34.36          | 6.51         | 5.64          |
| Minimum                  | 50.44       | 38.01        | 37.72        | 45.12          | 25.67        | 22.8          |
| Maximum                  | 59.56       | 117.16       | 80.57        | 79.48          | 32.18        | 28.44         |
| Confidence level (95.0%) | 6.476947    | 57.97735     | 27.92512     | 25.96792       | 4.593305     | 3.834906      |

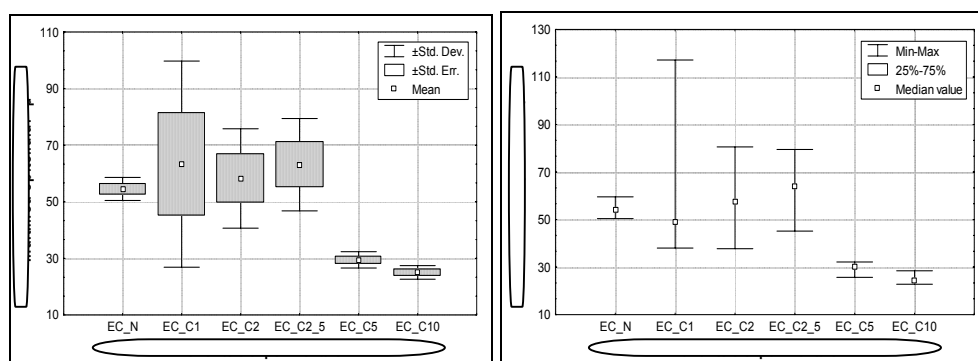


Figure 4 – a) Variation of epithelium height in ducts (microns) – statistical analysis

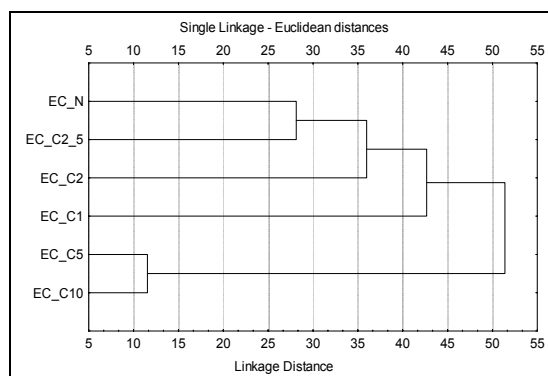


Figure 4 – b) Dendrogram of height variation in ducts with the 6 experimental variants

It is rather hard to account for such heterogeneous patterns of reaction, the low concentration batches are linked to the control group rather feebly, with which it constitutes a first grouping while those with high concentration are tightly packed into a second grouping.

The correlations are rather flimsy among various tested variants, the most significant values being Ec.N–Ec.C2.5–0.870 (attached to dendrogram), subsequently Ec.C2.5–Ec.C2–0.888, Ec.C2–Ec.C1–0.831, Ec.C2.5–Ec.C1–0.642, Ec.C1–Ec.C10–...–0.723 etc. (Figures 5 and 6).

Based on statistical data we consider that the lessening of hepato-pancreas epithelium in ducts of batches Ec.C5 and Ec.C10 is due to the alteration of functional capacities of these cells in consequence of the higher concentration of polluting agents in the experiment water.

The same analysis system as that described at parameter “*Height of epithelium in ducts*” was used with the other parameters under discussion.

### DISCUSSIONS

We are not able to extend the growth of death rate with batches C5 and C10 (which were given a “mixture” whose concentration exceeded the maximum permitted limit) along with the inherent alterations to mussels possible to be exposed to similar concentrations in case of disasters, as the experimental medium was different from the natural one. The data on the dynamics of parameters under discussion concerning branchiae reveal that mussels adjust themselves progressively up to maximum admitted concentration [10].

Even though death rate grows when maximum limits are surpassed (the raise being assessed by the presence of a higher death rate – 52% with batch C 2.5 – and by an increase of polluting mixture concentrations with batches C5 and C10, the surviving mussels raise their adjustment capabilities which is expressed by bringing the values of these parameters closer to the values of the control group. For our experiment the control group originates from non-polluted seawater [5, 6].

Due to the controlled laboratory conditions of the experiments it might be difficult for us to compare our results to those of natural conditions where the unconditioned ecological factors might modify the results. The subtle differences of the environmental factors such as temperature, air currents and food availability may modify the histologic results we obtained.

Since no other histological or histometric study as yet monitored the reaction of mussels exposed to their natural or laboratory environment (acute or chronic chemical stress) our results may be taken as a threshold for subsequent investigations. The maintenance of almost unmodified parameters with batches C2 and C1 (which were exposed to mixtures below the maximum admitted levels) and with which the survival rate was 10% can be accounted for by the fact that when the animals are for the first time exposed to stress inducing environment, the tissue is specifically inhibited and they become tolerant [5].

The correlation between the morphological alterations in mussel tissue and the oil content points to the fact that mussels are chemomonitors of the oil in the

environment being at the same time, the most sensitive monitors over short lengths of time (in case of acute explosives) [9]. Histometric surveys accompanied by statistical processing of several parameters in two organ systems which are highly involved in the filtering and feeding process of mussels (respiratory and digestive systems) mirror the correct substantiation of the effects of hydrocarbons administered in different amounts and intensity on the morphology of this organs.

Solely the qualitative assessment of these parameters may lead to inconclusive or erroneous conclusions. Therefore by correlating the results obtained through histological, histochemical and histometric methods piece together a more complete and correct picture of the alterations triggered by acute chemical stress [7].

### CONCLUSIONS

The following were ascertained in consequence of assessing the dynamics of branchiae parameters:

We find a significant alteration in the **height of branchial lamellae epithelium** with batch C2.5 that reaches 26.93 as against 15.46 with the control group. The dynamics of **growth of connective tissue of lamella** dwindles from 31.64 with the control batch to values oscillating from 22.94 with all other batches. This reduction is insignificant.

**The height of lamellar epithelium** changes significantly with batch C2.5 (36.65) this being the batch exposed to the mixture of polluting substances exceeding maximum allowed level (2.5 mg/l seawater). From this batches where survival rate was 48 the height of lamellar epithelium decreases gradually nearing the control batch (10.04). The growth of lamellar epithelium height is looked up on as an adaptation of cell aggregate so as to process the “mixture” of polluting substances.

In assessing the dynamics of the supporting shaft thickness of the lamellar epithelium in the individuals of batch C2.5, we distinguish that the connective tissue of the epithelium grows significantly (from 105  $\mu$  in batch C2.5) whereas with batches C5 and C10 it lessens gradually reaching 46.2  $\mu$  (with C10) but nevertheless staying far wide of the value of the control batch (9.84  $\mu$ ). The significant alteration of this parameter is triggered by the adjustment to the environment, the connective tissue shaft representing the morphologic support of the nutrient for the epithelium (as avascular tissue) [4].

We distinguish a significant increase of **axial diameter of the lamella** with batch C1 (211.09  $\mu$  as against N (59.38  $\mu$ ) followed by a more significant increase with batch C2 (353.72  $\mu$ ). This parameter decreases quite significantly with batch C2.5 (84.89  $\mu$ ) followed by slight increases with batches C5 (97.2  $\mu$ ) and C10 (99.18  $\mu$ ). After adjusting alterations with increasing values of this parameter



reaching a maximum value with batch C2 (353.72  $\mu$ ) there follow decreases with values closer to the control group. We consider that the axial diameter of the lamella with batch C2.5 reaches adjusting values faster than with the other batches.

**The transversal diameter of lamellae** grows significantly up to 94.85  $\mu$  with batch C2.5 to decrease significantly with batches C5 (60.2  $\mu$ ) and C10 (44.46  $\mu$ ) and getting closer to the value of the control group (15.99  $\mu$ ) but staying at a very marked difference. As for the **dynamics of density of branchial lamellae** per 100- $\mu$ m surface we distinguish a significant decrease with batch C2.5 from 1.49 lamellae as against 2.18 with the control group. The accumulation product expressed by **granulation presence** was assessed for a certain respiratory surface (this is expressed by the occupied surface by 10 lamellae batch).

Although the amount of granulations does not vary greatly (ranging between 10 and 13), their diameter changes significantly from 5.79  $\mu$ m with the control group to 8.78  $\mu$  with the batch which was administered the highest concentration of polluting mixture (C10). Granulations per examined surface could not be measured with all batches as the lamellae and the matrices were destroyed.

The significant lessening of branchial lamellae also entails the quantitative reduction of cilia along with their height, which brings along the capability of filtering water. With this parameter the most marked reduction of cilia height is again met with batch C2.5 from 10.24  $\mu$  with the control group to 8.79  $\mu$  with C2.5). Recent surveys reveal that mussels exposed to various polluting substances are less tolerant and cut down the survival time in proportion with the increasing hydrocarbon concentration. Up to batch C2.5 we distinguish insignificant variations as regards the dynamics of epithelium height in the ducts of hepatopancreas. This one decreases markedly as against the control groups from batches C5 and C10 onwards.

The decrease might be due to the alteration of functional capacities of cells exposed to high chemical stress. As to the maximum diameter of hepatopancreas ducts we find a significant growth with batch C1 (exposed to a low concentration of polluting mixture) while the duct diameter does not alter visibly with the batches exposed to high concentration of polluting substances (C2.5, C5, C10).

In assessing the height of hepato-pancreatic tubules we distinguish a significant growth with batch C1 (exposed to low concentrations polluting mixture) where as the tubule diameter does not alter markedly with the batches exposed to high concentrations. The maximum diameter of tubules grows significantly with all batches reaching increased values with C2 (94.89  $\mu$ m followed by batch C10 (92.24  $\mu$ ).

As for the morphometric measurements of area fractions of secretive tubules we witness and an increase of the surface with batches C5 and C10 (respectively 58% and 60%). The growth of tubule surface might be due to the more intense secretions so as to cope with the metabolic needs linked to the increase of polluting substances. In the control group the area function of granules highlights low values

of this parameter whereas from batch C1 onwards the values grow markedly. Morphometric evaluations of area function of vacuoles (lipidic aggregates) highlight a maximum surface value with batch C2.5 (17.70%) while with batches C5 and C10 it reaches 10.46% and 10.50% respectively.

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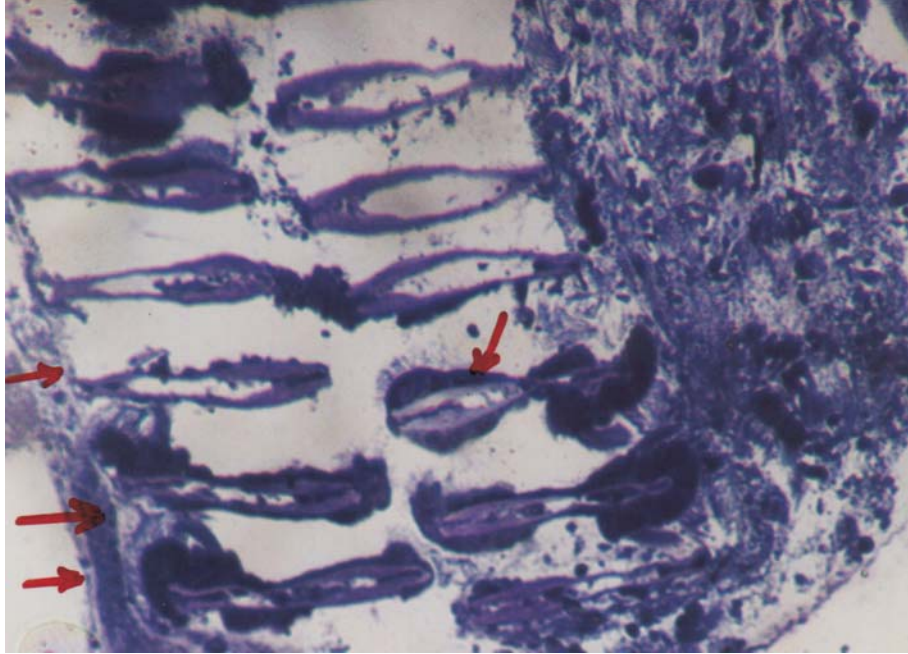


Figure 2 – Microscopic preparation of mussel branchiae

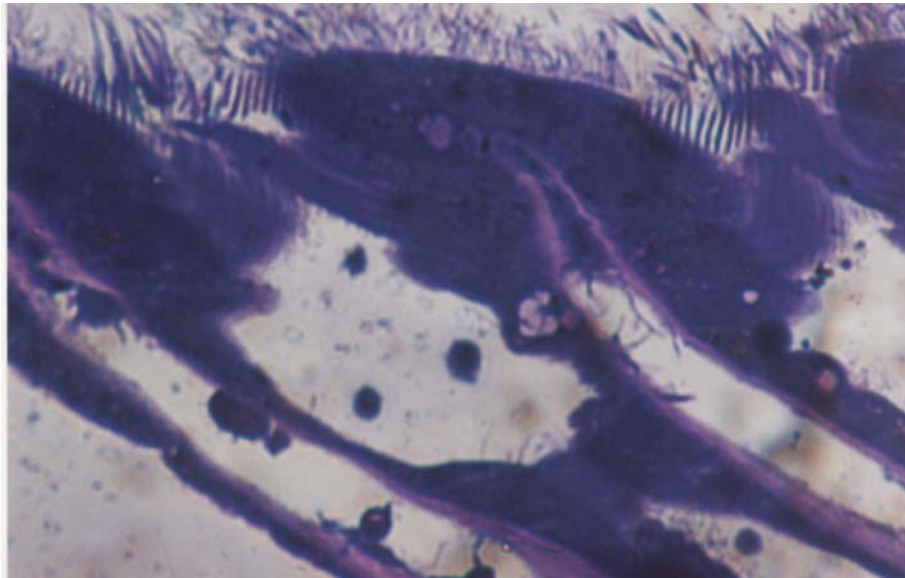


Figure 3 – Branchiae of bivalve molusc provided with cilia

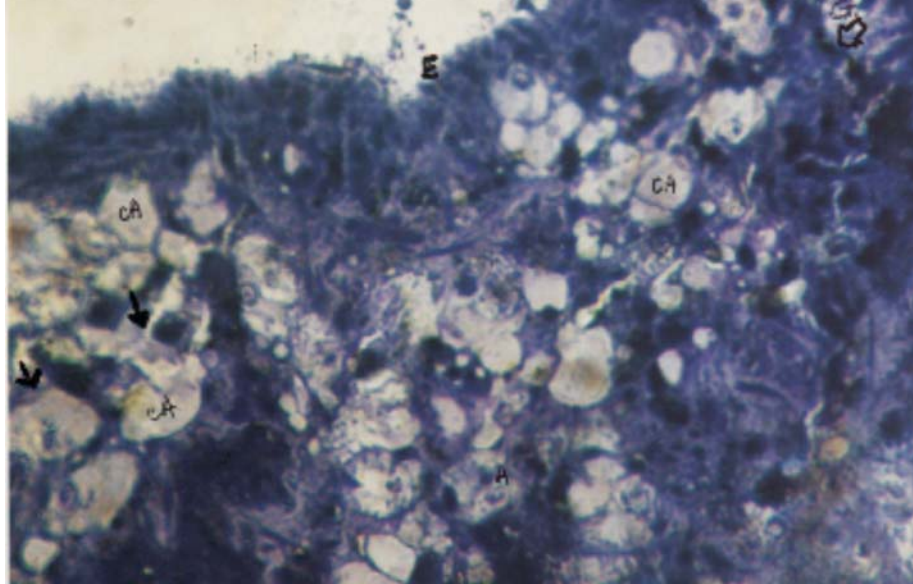


Figure 5 – Height of epithelium in hepato-pancreas ducts

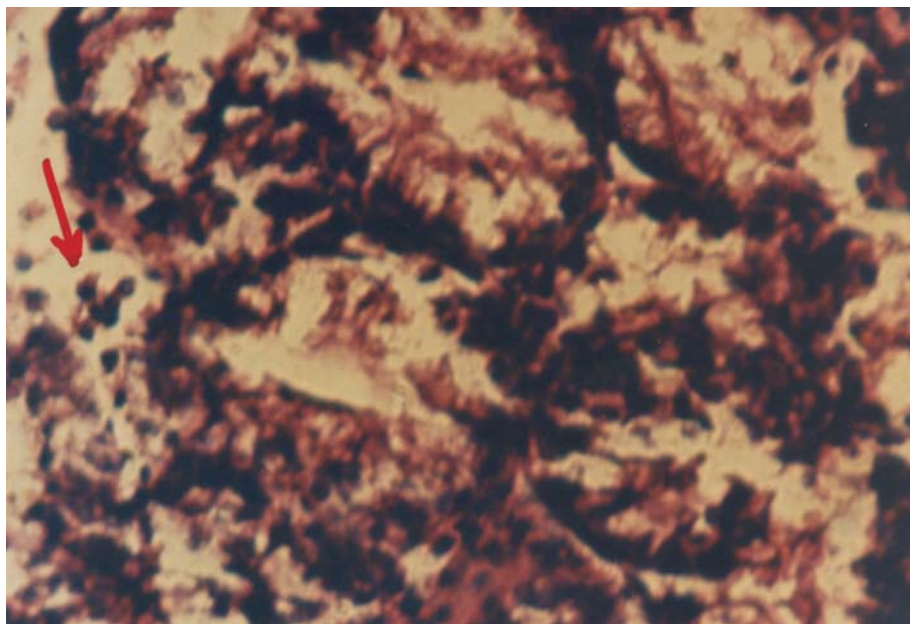


Figure 6 – Height of epithelium in ducts