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# STRUCTURAL AND ULTRASTRUCTURAL ANALYSIS OF GILLS AND GUT OF *HEXAGENIA RIGIDA* NYMPHS (EPHEMEROPTERA) IN RELATION TO CONTAMINATION MECHANISMS

Keywords: Hexagenia rigida, gut ultrastructure, gill ultrastructure, contamination mechanisms, Mercury compounds

ABSTRACT. In the context of an experimental approach to organic and inorganic mercury bio-accumulation by burrowing mayfly nymphs—*Hexagenia rigida*—, gills and different parts of the gut were investigated by light and electron microscopes. In the gut, two regions were studied: (1) mesenteron (midgut) characterized by cells with microvilli and a peritrophic membrane throughout this part, a lot of fungal hyphae being found on it; (2) proctodeum (hindgut) characterized by macrovilli, the apical face of cells being covered by a thick layer of chitin; many bacteria were observed at the bottom of macrovilli. The stomodeum (foregut) is extremely short. The gills are made up of six pairs of lamellae fringed with long filaments which are arranged on both sides of the long axis. Gills are constituted by tracheae and tracheoles, with a large canal for haemolymph circulation; muscle masses and nerve bundles were also observed. This structural approach shows that the nymph interface with the external environment, at gill and gut barrier levels, are quite different. Links between this structural and ultrastructural analysis and contamination mechanisms, via the trophic or direct route, are discussed; gills and gut could be transfer routes for mercury absorption, but also target organs for metal accumulation.

# Introduction

In the context of our research, we have carried out an experimental study of the bioaccumulation of mercury compounds (HgCl<sub>2</sub> and CH<sub>3</sub>HgCl) by the nymph of an aquatic intra-sedimentary insect—*Hexagenia rigida* (burrowing mayfly)—(Saouter *et al.*, 1989; 1991a; 1991b). Using optic and electronic microscopy we observed the principal biological barriers involved in the mechanisms of contamination by this metal. These mechanisms are based on the fixing and/or crossing of the different epithelia in direct contact

Received 14 June 1991. Revised 4 July 1991. with the external environment or ingested food; because the mercury is carried by the haemolymph, it is also able to accumulate in different internal organs and tissues. Hence, the structure and ultrastructure of these epithelia and also their physiological functions are very significant in relation to bio-accumulation processes and toxic effects which occur at different biological integration levels (cell, organ, organism).

From the ecotoxicological point of view, *Hexagenia rigida* nymphs present some particularly interesting biological and ecological characteristics: nymphal aquatic life of 10–24 months in natural conditions, depending on climate; constant ingestion of sediment, the nymphs being detritivores and living in burrows in the upper layers of freshwater lentic sediments; size of between 1 and 3 cm; large biomasses in lakes and rivers, providing trophic support for many predators (fish, amphibians, bats, birds, etc.); high tolerance

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to modifications in abiotic factors (temperature, dissolved oxygen, pH, etc.); mass culture initiated in the laboratory from eggs collected in the field during emergence periods (Craven and Brown, 1969; Zimmerman, 1977; Friesen, 1982; Flannagan and Cobb, 1984; Petersen et al., 1985). The nymphal stages of this species thus provide a very good model for experimental research in Aquatic Ecotoxicology into mercury bioaccumulation and transfer mechanisms, with the water or sediment compartments as initial contamination source and for the quantification of actions and interactions between different abiotic factors (temperature, pH, photoperiod, etc.) and contamination factors (Hg chemical forms and species, contamination levels, etc.) (Saouter, 1990; Saouter et al., 1991a).

Contamination of this burrowing benthic species may derive from metal present in the aquatic phase (water column, interstitial water in the sediment) via the cutaneous covering and the gill epithelium-direct route; it may also derive from ingested sediment (mercury present in the interstitial water and fixed on the different geochemical phases) via the gut-trophic route-(Saouter et al., 1991a). For most heterotrophic aquatic organisms, the two main routes via which metal contaminants enter the organism are the gills and the gut (Cunningham and Tripp, 1975; Ribeyre and Boudou, 1984; Boudou and Ribeyre, 1985; King and Davies, 1987). In our study we have therefore concentrated our structural and ultrastructural analysis on the trachea-gills and the gut of Hexagenia rigida nymph, linked with respiration and nutrition respectively, as these two epithelia show a high degree of absorption activity. We should also add that, to our knowledge, no other study of this type is to be found in current literature, even though several studies have been carried out on this organism in the fields of Ecology (Fremlin, 1968; McCafferty, 1975; Zimmermann, 1977) and Aquatic Toxicology (Friesen et al., 1983; Malueg et al., 1983; Henry et al., 1986; Landrum and Poore, 1988).

# **Materials and Methods**

### Hexagenia rigida nymphs

The nymphs used for microscopy analysis were at the end of the nymphal stage (8-10

months, 20–25 mm long and 60–70 mg fresh weight). Mass culture was initiated from eggs collected each summer from Lake Winnipeg (Freshwater Institute, Winnipeg, Canada) and stored at +4°C. The culture technique (Friesen, 1982) has been adapted in our laboratory (Saouter, 1991b). Hatching was carried out in dechlorinated tap water saturated with oxygen, by raising the temperature from 4 to 24°C in 4° stages, every 48 hr. Newly hatched nymphs were gently transferred to glass tanks, using a Pasteur pipette. Each tank contained a natural sediment layer, from the banks of the Garonne river, and was filled with dechlorinated tap water. Nymph density was about 1-2 nymphs per cm<sup>2</sup>. Food supplies—Tetra Min B—were added at the rate of 0.3-0.5 g per tank, twice a month during the first two months. At this stage, about one tenth of the original population was still alive. The nymphs-3-8 mm long—were then transferred to new tanks with an average density of 1 nymph per 3 cm<sup>2</sup>. Prior to emergence, the nymphs now 25-30 mm long-swam to the surface, shed their exuvia and emerged in the terrestrial subimaginal stage. The number and frequency of instars for *Hexagenia rigida* has not been determined, but numerous molts occur during postembryonic development.

### Light microscopy

The organisms were fixed for 48 hr in a solution of Bouin trichloracetic. The legs and trachea-gills were removed before fixing in order to improve fixative penetration. The sections were contrasted by Hemalum de Masson, Eosine.

# Transmission electron microscopy (TEM)

The samples were directly excised in the fixative solution: 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. After fixation for 1 hr under vacuum in order to facilitate fixative penetration, they were postfixed for 1 hr in 1% osmium tetroxide in the same buffer. They were then dehydrated through graded ethanols and propylene oxide before embedding in Epon 812. The ultrathin sections were contrasted by uranyl acetate and lead citrate (Reynolds, 1963). Observations were made with a JEOL 1200 Ex transmission electron microscope.



(1) a



Fig. 1. Structural analysis of different parts of gut of *Hexagenia rigida* (nymphal stage). (a) Diagram of *Hexagenia rigida* gut ( $\times$ 7). I. level of Figure 1b and 1c, II: level of Figure 2a, 2b and 2c, III: level of Figure 2d, 2e and 2f. (b) Transversal analysis in posterior head (I in Fig. 1a) ( $\times$ 120). Rc: Regenerative cells, Ep: Epithelium, Pm: Peritrophic membrane, L: midgut Lumen. (c) Longitudinal analysis between head and thorax ( $\times$ 100). Ep: Epithelium, Pm: Peritrophic membrane, Fm: Food material.

# Scanning electron microscopy (SEM)

Sampling and fixation were the same as described for transmission electron microscopy. The pieces were then dehydrated through graded ethanols and  $CO_2$  critical point dried. They were mounted on aluminium stubs, coated with a gold-palladium alloy by sputtering and then with carbon by vaporization on a rotating platform. Observations were made with a JEOL840A scanning electron microscope.

#### **Results and Discussion**

# Structural and ultrastructural analysis of the gut

The gut takes up most of the nymph's hoemocelian cavity. It is in the form of a straight tube of varying diameter: it is a very short, narrow canal in the cephalic part of the organism, then enlarges considerably into the midgut lumen, at the point where head and thorax join (Fig. 1a). The diameter then remains constant throughout the abdominal area up to the point where the Malpighian tubes are inserted. In the hindgut, the epithelium thickens and the internal diameter increases at the part corresponding to the rectum.

Histological examination of the different parts of the gut enables us to dinstinguish a mesenteron and a proctodeum; it appears that the stomodeum is extremely short and localized in the anterior part of the cephalic canal. The epithelium in the posterior cephalic canal has the same features as that in the mesenteron: it consists of cylindrical cells with a large nucleus, crypts and regenerative cells (Fig. 1b). Moreover, the fact that the peritrophic membrane is continuous between the posterior cephalic canal and the thorax confirms that both form part of the mesenteron (Fig. 1c). This anatomical feature can be attributed to the 'archaic' nature of this species, with the Hexagenia rigida nymph having a digestive tract which is fairly undifferentiated. In insects, the stomodeum is lined with cuticle, sometimes sculptured to tear ingested food material in order to facilitate the action of digestive enzymes produced in the mesenteron. In Hexagenia rigida, however, ingested food is comminuted by grinding carried out mainly by the large quantity of solid particles ingested (mostly grains of sand-Richardson and Gaufin, 1971; Zimmerman, 1977); in fact, more than 10% of the intestinal contents is made up of non-organic material (Clifford and Hamilton, 1987). Crystalline structures can also be clearly seen in the intestinal contents.

The characteristic feature of the gut of Hexagenia rigida nymph is that it consists mainly of the mesenteron, with the proctodeum making up barely one quarter of the whole intestinal length. The apical face of the epithelial cells is covered with microvilli (Fig. 2a, b, c,), with deep digitations at the base. The presence of many mitochondria indicates intense metabolic activity. These are resting on connective tissue  $0.5-1 \,\mu m$ thick, made up of 5-8 layers; on the intestinal lumen side they are covered by the peritrophic membrane, which is most clearly visible using scanning electron microscopy (Fig. 2a). The periotrophic membrane envelops all the food material in the mesenteron. Its role is to protect the intestinal epithelium from attack by the non-organic, sedimentary particles that have been ingested. The presence of this membrane, which is not present in all insects, means that food material must be made soluble before being absorbed through the mesenteric epithelium; the porosity of this membrane is generally quite low, being only a few  $\mu m$ (Kerkut and Gilbert, 1984). In different parts

Fig. 2. Structure and ultrastructure of mesenteron (midgut) and proctodeum (hindgut) of *Hexagenia rigida* nymphs. (a) Apical face of midgut (SEM. ×2500). Pm: Peritrophic membrane, Mv: Microvilli, F: Fungal hyphae. (b) Magnification of Figure 2a: apical face of microvilli (SEM, ×39,000). (c) Midgut, in the apical cell (TEM, ×18,000). Mv: microvilli, Mi: Mitochondria, Zo: Zonula occludens, Cm: Cell membrane, Er: Endoplasmic reticulum L: midgut Lumen. (d) Apical face of proctodeum showing macrovilli and two grooves (SEM, ×1300). Mav: Macrovilli. (e) Magnification of Figure 2d, space between two macrovilli (SEM, ×9000). B: Bacteria. (f) Apical face of proctodeum (TEM, ×9000). C: Chitin, Ci: Chitin invagination into plasma membrane folds, Mi: Mitochondria, L: midgut Lumen.



of the midgut, we were unable to distinguish any areas that were anatomically or cytologically different one from another.

The point where the Malpighi tubes were inserted, slightly above the pyloric valvule, marks the beginning of the proctodeum. In the *Hexagenia rigida* nymph this is relatively short and the proctodeal epithelium (point III, Fig. 1a) has a very different appearance from that of the midgut. It consists of elongated, very ornamented macrovilli (Fig. 2d). The apical face of the cells is covered with a layer of chitin,  $0.5 \,\mu$ m thick, which incorporates the indentations of the plasmic membrane (Fig. 2f).

In the midgut, a considerable growth of fungal hyphae was observed on the surface of the peritrophic membrane (Fig. 2a). This was due to the ingestion by the organism of spores which soon found a favourable environment in which to develop. Such observations are often made in insects (Hames and Hopkin, 1989). In the hindgut, many bacteria were observed at the bottom of folds separating the macrovilli (Fig. 2e). The absence of any bacteria in direct contact with the microvilli of the mesenteron was due to the presence of the peritrophic membrane (Mercer and Day, 1952; Brandt *et al.*, 1978).

This structural and ultrastructural study of the digestive tract of Hexagenia rigida nymph has given us a much better understanding of the mechanisms of nutritional prehension which lead to the forming of a bolus from ingested sediment (Fig. 1c), and thus confirms the hypotheses put forward by Browns (1960) and Zimmerman et al. (1975). This mode of nutrition requires the ingestion of water, in order to make the subsequent dilution of the bolus easier. From an ecotoxicological point of view, contamination of organisms via the trophic route derives from mercury bound to the particulate phase of ingested sediments, but it also derives from dissolved mercury in the interstitial water and/or the water column, by means of the current created in the galleries by the more or less constant pulsing of the gills. We should mention that many authors are of the opinion that chemical species of mercury in the dissolved phase are more bio-available than complex forms associated with the particulate phase (Luoma, 1983; Campbell et al., 1988; Salomons and Forstner, 1984); studies on model membranes also show that chemical speciation reactions for mercury in the dissolved phase, which are closely dependent on the physicochemical characteristics of the environment (especially pH and pCl), have a very important role to play in the fixation of metal on membrane ligands and transmembrane flux (Bienvenue et al., 1984; Delnomdedieu, et al., 1989; Delnomdedieu, 1990; Boudou et al., 1991).

# Structural and ultrastructural analysis of the gills

The gills are made up of hollow, thin-walled evaginations, containing tracheae and tracheoles, which branch from abdominal trachean stems; they are external filamentous lamellae, carried on the first six segments of the abdomen. The gills of burrowing mayflyes constitute an important exchange area as they are inversely proportional to the hydrogen pressure of the environment, showing clearly how this species has adapted to environments with poor oxygen supplies (Dodds and Hisaw, 1924). They are extremely efficient in the respiration process, as for species similar to Hexagenia, 40-70% of oxygen consumed is provided *via* the gills, the rest is directly absorbed by the cutaneous layer (Wingfield, 1939; Eriksen, 1963). On either side of a principal axis, several hundreds of secondary ramifications are arranged, in regularly decreasing size from the base to the apex (Fig. 3a). The principal trachea has an average diameter of  $100 \,\mu m$ at the base. It has many outgrowths directed

Fig. 3. Gills of *Hexagenia rigida*. nymphs. (a) One gill lamella (SEM,  $\times$ 50). T: Trachea, C: Canal for heamolymph circulation, St: Secondary trachea. (b) Basis of gill lamella (SEM,  $\times$ 250). T: Trachea, C: Canal for haemolymph circulation. (c) Interior face of trachea showing chitin outgrowths (SEM,  $\times$ 4000). T: Trachea. (d) Secondary trachea (TEM  $\times$ 89,000). M: Microtubules. C: Chitin, R: Regenerative layer, H: Haemolymph. (e) General view of secondary trachea (TEM,  $\times$ 6000). Te: Tracheole, C: Chitin, R: Regenerative layer, N: Nucleus, Cm: Cell membrane, Cr: Crystals, H: Haemolymph. (f) Magnification of gill cell (TEM,  $\times$ 14,200). N: Nerve bundle, Cr: Crystals.



towards the tracheal lumen, and these increase considerably the exchange surface area (Fig. 3c). Alongside the trachea is an inter-cellular space through which the haemolymph circulates (Fig. 3b). The secondary ramifications become more and more simple in structure from the base to the tip. They have a central trachea (average diameter  $6\,\mu\text{m}$ ), bathed in haemolymph (Fig. 3e). Around the outside, one or two layers of cells separate it from a chitin envelope showing several lavers of different density: the outer denser layer, with an average thickness of  $0.1 \,\mu\text{m}$ ; the inner clearer layer, close to the regenerative cells, is thicker  $(0.5 \,\mu\text{m})$ . The cells hardly differ one from another and contain many microtubules, all with average orientation from the trachea towards the external chitin covering (Fig. 3d). Very dense granules with an average diameter of  $0.5 \,\mu m$ build up in places (Fig. 3f), similar to the accumulation of metal described in several studies (Hopkin and Martin, 1984; Seidman et al. 1986). In parallel research by X-ray energy dispersion spectrometry, however, no heavy metals were detected; only a strong osmiophily was observed. Note that the nymph used in this structural and ultrastructural study came directly from the hatching tanks and had not been exposed to any form of mercury contamination. Observation of the trachea-gills under an electron microscope revealed nerve bundles crossing the gill axis in various places (Fig. 3f). Muscle masses were also observed mainly localized at the base of these organs. Thus the pulsing of the gills, which brings about the almost constant water current within the galleries, is due to the contraction of these muscle masses in conjunction with abdominal movement by the nymph.

This structural and ultrastructural analysis of barriers in the gills and gut of Hexagenia rigida allows us to draw certain ecotoxicological conclusions relating to metal fixation and absorption. Both organs have very large surface areas for exchange to take place (microvilli in the midgut and secondary ramifications in the trachea-gills), but the characteristics of the areas of interface with the external environment are quite different. When contamination is via the food material, the presence of the peritrophic membrane, which envelops ingested sedimentary material, means that mercury must be dissolve in order to pass through this barrier and reach the apical face of the enterocytes. Hence, high concentrations of metal in the interstitial water of the sediment or transfers between the particle phase and this compartment, in relation to, for example, the degree of lability of chemical liaisons with different ligands, may make the interface area between the intestinal lumen and the gut wall more accessible to mercury. These problems of mercury accessibility are minimized in the case of the gill lamellae, which are in direct contact with the surrounding environment. This facilitates mercury transfer, especially when the metal is in the dissolved phase. Moreover, the muscle masses, situated as they are at the base of the main body of the gill lamellae, and in close contact with the haemolymph, are ideally placed for the fixation of mercury in this organ. Clearly, the gills are not only a route for the mercury to enter the organism, via the trachea system and the haemolymph; they are also a target organ for the bioaccumulation and organotropism of the metal. Parallel studies have shown that, when contamination is via the direct route, although the relative weight of trachea-gills is very small (about 6% of nymphs' fresh weight), they can contain very large quantities of metal, as much as 50-60%of total mercury. When contamination is *via* the trophic route, it is the gut, particularly the midgut, that contains the largest amount of accumulated metal in the organism (Saouter et al., 1991b; Hare et al., 1991). These two organs are not only barriers between the organism's external and internal environments, but they are also important accumulation sites.

# Conclusion

Whatever the biological complexity of living beings, all contamination processes must necessarily be based, at the outset, on interactions between toxic products and the biological barriers which separate organisms from their environment. In the research programme that we are currently developing in Aquatic Ecotoxicology, *Hexagenia rigida* nymphs are being used as a biological model to quantify, at laboratory level, transfers of mercury compounds from 'water column' and 'sediment' contamination sources. In this context we have studied the two main bio-

logical barriers involved in contamination via the direct and trophic routes: the tracheagills and the gut. This structural and ultrastructural study forms part of a vast amount of data collected from the various biotic and abiotic compartments of the experimental systems, in order to analyse the different ecotoxicological mechanisms involved: partitioning of the mercury compounds in the biotopes (e.g. sequential extraction techniques and dialysis cells for the sediment compartment); chemical transformations of the two mercury forms initially added to the contamination sources (methylation and demethylation reactions); direct and indirect effects of several abiotic factors (temperature, pH, photoperiod, organic content of natural sediment, etc.) on metal transfers and bioaccumulation; mercury organotropism in the nymphs), using <sup>203</sup>Hg radioisotope; etc.

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