Histol Histopathol (2010) 25: 1519-1529
DOI: 10.14670/HH-25.1519

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Gill alterations as biomarkers of chronic exposure to endosulfan in *Bufo bufo* tadpoles

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Summary. Endosulfan sprayed on agricultural fields accumulates in temporary pools due to surface runoff or sediment transport and may result in high water concentrations in spring and summer, coinciding with breeding and crucial stages of amphibian larval development. In the present study, *Bufo bufo* tadpoles were exposed to three different concentrations of endosulfan (0.01, 0.05 and 0.1 mg/L) until they reached complete metamorphosis. The aim of the study was to investigate the effects of endosulfan, at environmentally relevant concentrations on gill morphology and ultrastructure.

Modifications in ultrastructure and cell composition were observed at all concentrations after 96 h. The main gill effects recorded in treated animals were: mucous secretion, the appearance of tubular vesicles cells (TVC) and a degeneration phenomenon.

Comparing these results with our previous findings in which we used growth, developmental rate and behaviour as endpoints, we also demonstrated that the first effect of endosulfan on *Bufo bufo* was gill alteration, thus supporting the role of a morphological approach in toxicological studies.

This study provides additional information on the role of morphological studies in demonstrating the effects of exposure to environmental pollutants. In this context, the use of amphibian gills, as effective biomarkers, is a valuable approach in evaluating exposure to agrochemicals.

Key words: Endosulfan, Gills, Ultrastructure, Amphibian decline, Water pollution

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Introduction

The causes of the decline in amphibians are under investigation. However, emerging evidence indicates that decreased species richness and reduced populations reported in agroecosystems may be linked to the extensive use of pesticides and fertilizers, acting singly or in combination with other stressors (Berrill et al., 1994, 1997; Knutson et al., 2002; Houlahan and Findlay, 2003; Bridges et al., 2004; Davidson, 2004; Mann et al., 2009). This group of vertebrates has been used extensively in standardized toxicological tests to assess the toxic effects of pesticides (Venturino et al., 2003). In fact, many amphibian species breed and develop in ponds, streams, and ephemeral aquatic environments immersed or surrounded by agricultural areas; hence such environments receive spray drift and runoff from lands where fertilizers and pesticides are applied and, at normal application rates, the concentration of agrochemicals may reach high levels without substantial dilution (Bishop et al., 1999; Mann and Bidwell, 2001; Hamer et al., 2004; Jergentz et al., 2005).

Agrochemicals can seriously affect local populations and the community structure of amphibians, reducing survival, altering feeding and swimming activity, causing a high incidence of deformities and decreasing growth and development of larvae (Semlitsch et al., 1995; Bonin et al., 1997; Bridges, 1999, 2000; Boone et al., 2001; Rohr et al., 2003; Relyea, 2005; Taylor et al., 2005; Peltzer et al., 2008).

Endosulfan is a broad spectrum organochlorine insecticide and acaricide which has been in commercial use for over 50 years (EFSA, 2005). Due to its persistence, potential toxicity to humans and its role as an endocrine disruptor (German Federal Protection Agency, 2007) this pesticide has become the centre of a

highly controversial debate and there is a worldwide warning against the use of this product. Recently, the scientific review committee of the Stockholm Convention on Persistent Organic Pollutants (POPs) voted unanimously to consider the addition of endosulfan to the Treaty's list of chemicals to be phased out (UNEP, 2008). Nevertheless, endosulfan is still extensively used in the USA, Australia and emerging countries.

The literature on endosulfan in both field and laboratory toxicity studies shows that when endosulfan is applied for agricultural purposes near aquatic ecosystems, these ecosystems can exhibit the effects of the pesticide long after application, and may cause adverse effects in non-target organisms, especially those inhabiting aquatic environments (Murty, 1986; Carriger and Rand, 2008). Moreover, the effects of endosulfan, like various other pesticides, exerts its damaging effects mainly on juvenile organisms (Dutta, 1995; Pan and Dutta, 1998).

The toxicity of endosulfan to amphibians is supported by new evidence of its effect on different amphibian species (Jones et al., 2009; Shenoy et al., 2009). Recently, we also demonstrated that endosulfan may affect growth, development, and survival in *Bufo bufo* tadpoles and cause severe malformations after chronic exposure at environmentally relevant concentrations ranging from 0.001 to over 0.1 mg/L (Brunelli et al., 2009). The Anuran gill apparatus is a multifunctional organ responsible for respiration, osmoregulation, and acid-base balance. As widely demonstrated in fish, this organ is sensitive to chemicals in water, providing a large surface area for direct and continuous contact with contaminants in water. Our earlier short-term toxicity study revealed interesting changes in gill morphology following exposure to a sublethal concentration of endosulfan (0.2 mg/L), whereas mortality and gross anatomy were unaffected, thus suggesting an important role for this organ as a marker of contamination in amphibian species (Bernabò et al., 2008).

A wide range of biological responses can be useful as biomarkers, ranging from the molecular to community structure (Peakall, 1992). As outlined by Au (2004), histo-cytological responses are relatively easy to determine, and can be related to health and fitness of individuals which, in turn, allow further extrapolation to population/community effects.

On this basis, we conducted further investigations on *Bufo bufo* tadpoles by exposing them to environmentally realistic concentrations of endosulfan (0.01, 0.05, and 0.1 mg/L) during larval development and evaluated the specific effects of endosulfan exposure on the gills.

We analysed the histopathological and sub-cellular alterations induced by chronic exposure to endosulfan with the aim of further elucidating the role of morphological studies in demonstrating the effects of exposure to environmental pollutants.

Materials and methods

Test organism

The common toad (*Bufo bufo*) is an opportunistic species which lives in a variety of habitats including agricultural lands and is one of the most widely distributed European anuran amphibians. In recent decades, a marked decrease in *Bufo bufo* populations has been observed in Europe (Pavignano and Giacoma, 1990; Hilton-Brown and Oldham, 1991). This animal spawns in ponds and shallow water bodies in the spring, coinciding with the application of pesticides. It has been suggested that intensive agricultural practices and/or alterations in breeding habitats have contributed to the decline of this species (Carrier and Beebee, 2003).

Bufo bufo tadpoles were collected as newly oviposited egg masses from a permanent pond in a regional natural reserve located near Cosenza (16°00'E, 39°33'L) which has never been exposed to pesticides. Eggs were then transported to the Laboratory of Zoomorphology, at the University of Calabria in Southern Italy, and maintained at 20°C in glass tanks with aerated dechlorinated tap water.

Exposure conditions

After hatching, all animals were held under controlled laboratory conditions of 12:12 h light-dark cycles, water temperature of 22±1°C, and median pH of 7.3. Water quality parameters (pH, dissolved oxygen, conductivity, alkalinity, and hardness) were monitored before and following renewal of the test solutions in all tests. Tadpoles were staged following the table proposed by Gosner (1960). The experimental period was approximately 48 days. Animals were exposed throughout the larval period from Gosner stage 25 until complete tail reabsorption. Tadpoles were fed with organic boiled lettuce or spinach *ad libitum* three times weekly throughout the exposure period until the start of metamorphosis (Gosner stage 41). When the front legs emerged (Gosner stage 42), the tadpoles were transferred from exposure tanks to 5 L plastic tanks containing both wet (with treatment solution) and dry areas; the tadpoles were not fed until the tail had been reabsorbed (metamorphosing tadpoles live off fat stored in their tails).

The experimental design was previously described (Brunelli et al., 2009). Briefly, exposure solutions were prepared by dissolving commercial-grade endosulfan (purity 99%, Chem Service Inc., West Chester, PA, USA) in dechlorinated tap water to obtain the following nominal concentrations: 0.01, 0.05, and 0.1 mg/L (referred to as low, medium, and high, respectively). Control tadpoles were kept in tap water.

Each treatment was replicated twice (30 larva/per replicate); glass tanks (30 L) were cleaned, water changed, and treatments renewed every 3 days (static-

renewal exposure system). Since endosulfan has an aqueous half-life of several weeks, nominal concentrations were expected to remain constant for 72 h (Miles and Moy, 1979; Broomhall 2004). The endosulfan concentrations were chosen based on our previous studies with *Bufo bufo*, where we demonstrated an LC50-96 h value of 0.43 mg/L and the effects of environmentally relevant concentrations on life-history traits (Bernabò et al., 2008; Brunelli et al., 2009).

Morphological analysis

After 96 h, 8, 14 and 20 days, three exposed and three control tadpoles were randomly selected and analysed at each time point using SEM and TEM studies, respectively. The animals were euthanized with 2-4 g/L MS-222 (tricaine methanesulfonate, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and the gills were removed using a dissecting microscope. The specimens were fixed in 3% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) for 2 h at 4°C and then post-fixed in 1% osmium tetroxide in the same buffer for 2 h at 4°C (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The specimens were contrasted in block with an aqueous solution of 5% uranyl acetate for 2 h, dehydrated in acetone, and then embedded in Epon-Araldite (Fluka Ag, Buchs, Switzerland). Ultrathin sections (2 μm), cut on a LKB Nova ultramicrotome, were stained with uranyl acetate and lead citrate, and then coated in Edwards EM 400. Observations were made using a Zeiss EM 900 transmission electron microscope. Samples for the SEM study were dehydrated and dried according to the critical point method, covered with gold, and observed using a Zeiss DSM 940 scanning electron microscope. All analyses were conducted blindly, without knowledge of which exposure the tadpole had been subjected to.

Results

The general structure of the gills in control *Bufo bufo* tadpoles was similar to that of other Anuran species and only a brief general description relevant to the present paper will be given. Each gill was supported by four branchial arches from which arise dorsal gill filters and ventral gill tufts, which represent the respiratory organs (Fig. 1A). The dorsal segment of the gill has the function of a "food-trap" maintaining the mucous cordon which absorbs food particles (Brunelli et al., 2004) and is constituted by a main axis from which primary and secondary branching filter rows are found laterally (Fig. 1A). With further enlargement we observed gill tufts which were composed of numerous ramifications branching out from a carrier stem (Fig. 1B).

The gill tufts are highly vascularized with a thin bilayered epithelium which lies on large capillaries (Fig. 1C). The inner layer is made up of flattened basal cells, in contact with the basal lamina, whereas the external layer is mainly composed of pavement cells (PVC) equipped with short microridges (Fig. 1C) and few scattered mitochondria rich-cells (MRC) (Fig. 1D).

The ultrastructural analysis of MRC demonstrated the presence of numerous large secretory granules which form a continuous layer under the apical plasmalemma; the electron-dense cytoplasm is filled with a large number of mitochondria mainly located in the apical portion, whereas the wide nucleus is generally located in the basal position (Fig. 1D). MRC surrounded by polygonal PVC equipped with microridges are easily recognizable under SEM due to the presence of a long tuft of slender microvilli on their apical surface (Fig. 1E). The organization of gill filter epithelium is very simple and it is mainly composed of pavement cells and underlying basal cells (Fig. 1F). Cubic cells (CUC) with a high nuclear-cytoplasmic ratio could be seen in the distal portion of the filter, thus forming an intermediate layer (Fig. 1F).

Exposure to endosulfan induced several changes in the gill apparatus. After 96 h of exposure, the first alterations in the gill tufts were observed in all exposed groups. The tufts under SEM examination showed an irregular surface and the PVC were infolded at several points; this phenomenon was evident in the distal region in the low concentration group (Fig. 2A), and in the whole tuft in both the medium and high concentration groups (Fig. 2B).

Ultrastructural observations revealed that external layers lost contact with inner basal cells at several points, thus forming wide lacunae (Fig. 2C). In all experimental groups, a modification of cell composition was detected, with the appearance of elongated cells with abundant rough endoplasmic reticulum profiles and tubular vesicles in their cytoplasm, recognizable as tubular vesicles cells (TVC) (Fig. 2D). Gross morphology and ultrastructure of gill filters were not different to those of the control group.

After 8 days of exposure SEM observations showed further morphological changes involving the gill filters, which appeared thinner and more dehydrated (Fig. 3A) than the control specimens. The gill tufts had undergone an extensive shrinking (Fig. 3B), and in the highest concentration group the apical parts of the tufts were often collapsed (Fig. 3C). High magnification showed obvious folding of PVC and hypertrophy of the microridges of apical pavement cells (Fig. 3D). TEM observations confirmed the results obtained by SEM, and the gill damage was more pronounced compared with the 96 h group. Nuclear degeneration was detected mainly in apical cells along with the appearance of lamellar bodies; the epithelial lacunae were large and the relationship between adjacent cells was completely lost (Figs. 3E, F). Ultrastructural modifications also extended to the endothelium, and hypertrophy of endothelial cells and alterations in the inner profile of capillaries were observed (Fig. 3F).

The severity of these morphological changes increased after 14 days of endosulfan exposure. Long mucous cords covered the tuft surface (Fig. 4A) and

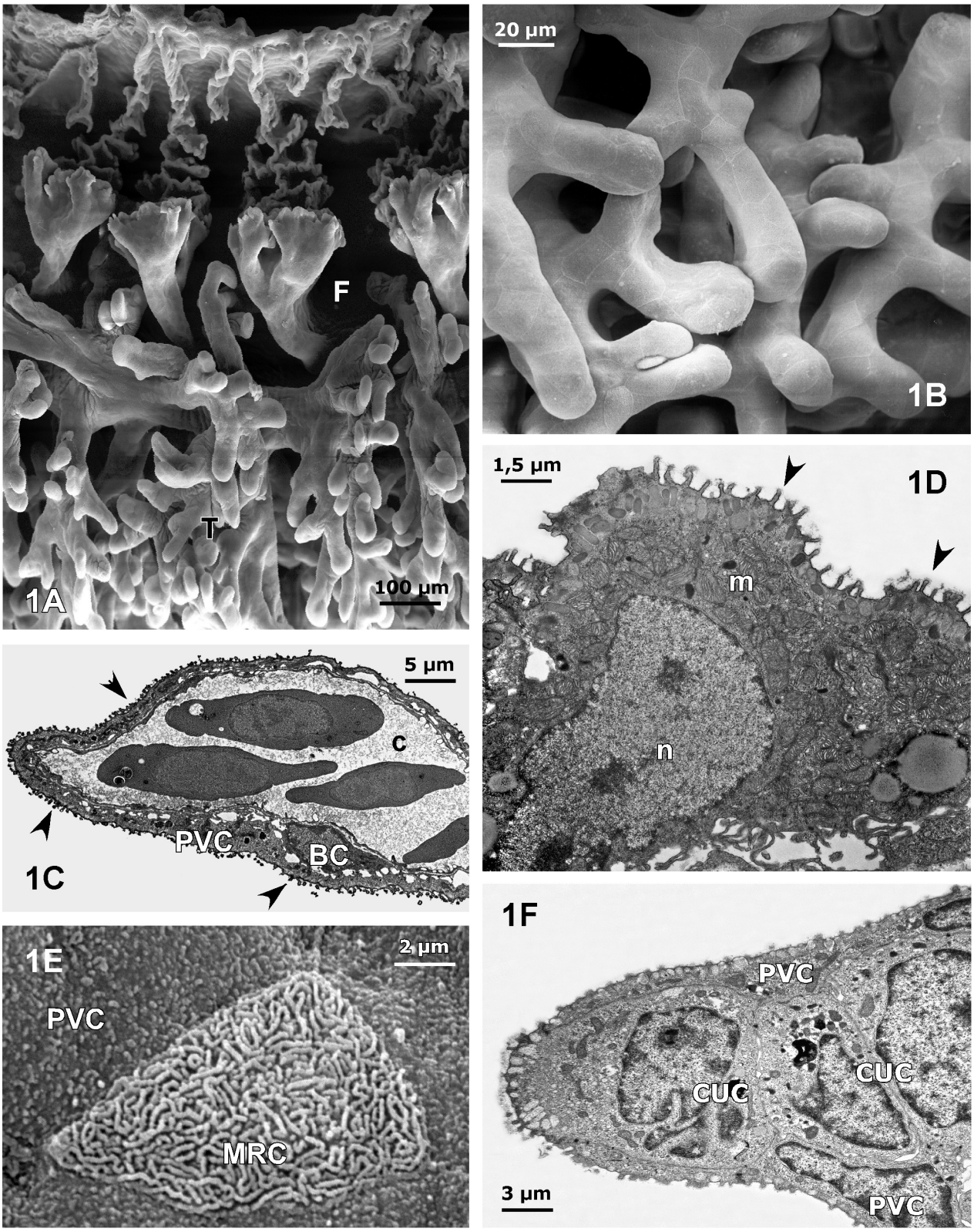

Fig. 1. *Bufo bufo* gill apparatus under control conditions. **A.** SEM image of general view of the gills with ventrally the tufts (T) and dorsally the filters (F). **B.** High resolution showing gill tuft ramifications. **C.** TEM image of gill epithelium in correspondence of capillary (c) composed by basal cell (BC) and flattened pavement cell (PVC) equipped with short microridges (arrowheads). **D, E.** Mitochondria-rich cell (MRC) characterized by an electron-dense cytoplasm filled by numerous mitochondria (m) and microvilli on the apex surface (arrowheads). **F.** Ultrastructure of the gill filter epithelium organization. Apical pavement cells (PVC) and underlying cubic cells (CUC) with a high nuclear-cytoplasmic ratio could be seen.

numerous large secretory vesicles were scattered throughout the entire cytoplasm (Fig. 4B).

After 20 days of endosulfan exposure, these alterations were very intense at all tested concentrations, and it was impossible to recognize the morphological organization typical of the control group. Both gill tufts and filters were shortened and ramifications of the tufts disappeared, leaving the stem without lateral branches (Fig. 5A). The epithelium was shown to have a squamous surface (Fig. 5B). Under TEM, the epithelium appeared to be composed of an external layer of degenerated cells which resulted in loss of integrity of

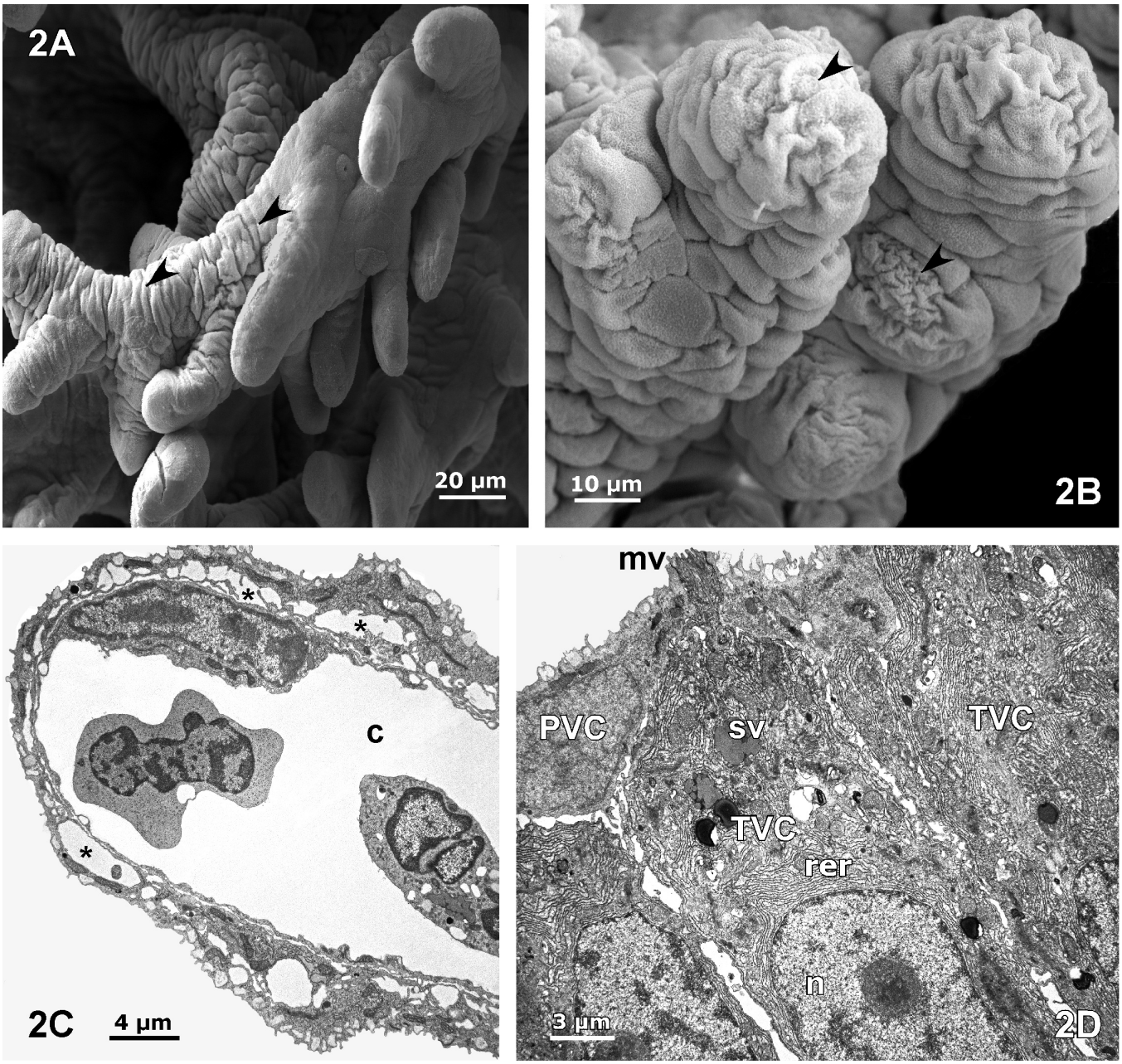

Fig. 2. *Bufo bufo* gill apparatus after 96 h. SEM micrographs of gill tufts in treated tadpoles exposed to 0.01 mg/L **(A)** and 0.05 mg/L **(B)** showing an irregular appearance and surface epithelium infolded at several points (arrowheads). **C.** Samples treated at the higher concentration of endosulfan display the appearance of large lacunae (*). **D.** TEM micrograph. Tubular vesicles cell (TVC) in a sample exposed at the lower endosulfan concentration: note a narrow apical surface with microvilli (mv), secretory vesicles (sv) and a well developed rough endoplasmatic reticulum (rer); (n: nucleus).

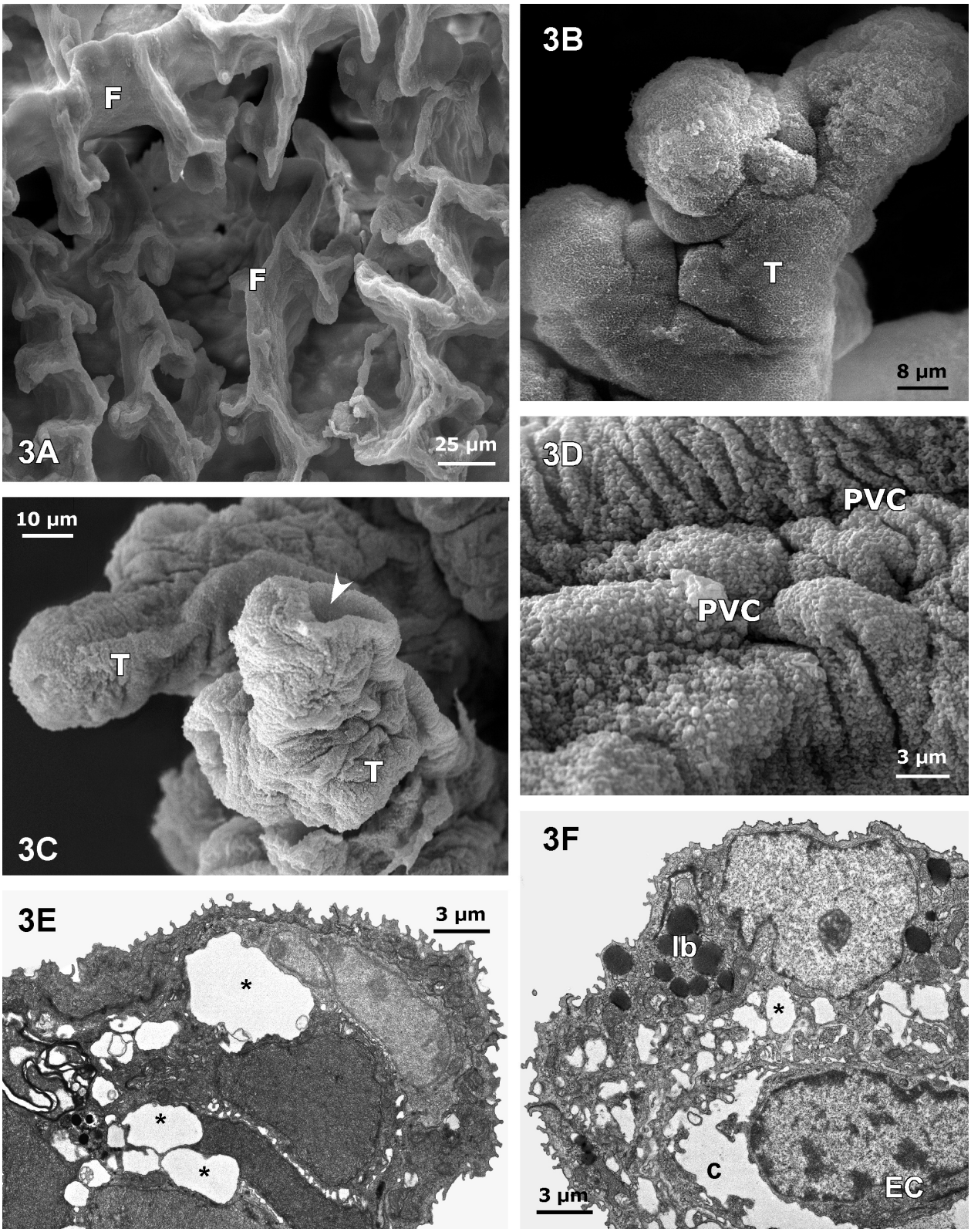

Fig. 3. *Bufo bufo* gill apparatus after 8 days of exposure. SEM images **(A-D)** showing **(A)** marked alterations in filters (F) and tufts (T) of tadpoles from the low concentrations group **(B)**; Samples exposed at the high concentration show tufts (T) heavily dehydrated, particularly in the apical portion which undergoes a collapse in several points (arrowhead) **(C)**; higher magnification of a tuft (T): note hypertrophy of microridges of pavement cells (PVC) **(D)**. Ultrastructural alterations **(E-F)** after exposure to the high and low concentrations group respectively: the widening of the intercellular spaces with the appearance of large lacunae (*) may be observed. Some cells show nuclear degenerations **(E)** along with appearance of numerous lamellar bodies (lb) and hypertrophy of endothelial cells (EC) **(F)**. c: capillary.

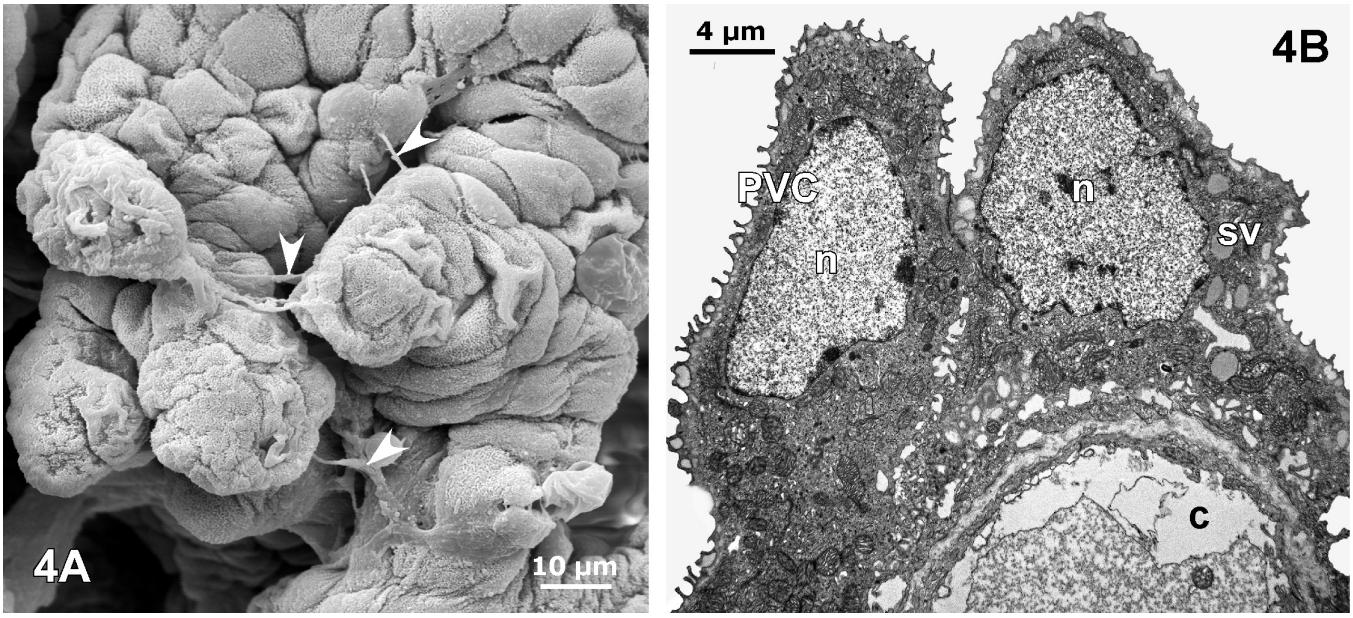

Fig. 4. Samples exposed at the highest concentration after 14 days. **A.** SEM micrograph showing increased presence of mucus (arrowheads). **B.** Large secretory vesicles (sv) scattered through the cytoplasm observed at TEM (n: nucleus, c: capillary).

Table 1. Comparative summary of developmental (Brunelli et al., 2009) and gill alterations (present paper) caused by a chronic exposure to three concentrations of endosulfan in *Bufo bufo* tadpoles.

Target	Effects		
	0.01 mg/L	0.05 mg/L	0.1 mg/L
Gills	4 d	4 d	4 d
Behaviour and swimming activity	-	4 d	4 d
Deformity	-	8 d	8 d
Body weight	9 d	9 d	9 d
Developmental rate	-	8 d	8 d

the natural tissue arrangement (Fig. 5C). However, some TVC (Fig. 5D) along with MRC (Fig. 5E) preserved their ultrastructural features. In all concentration groups, macrophages could be seen (Fig. 5F) and in the high concentration group capillary lumen was often obstructed by hypertrophic endothelial cells (Fig. 5G).

Discussion

Endosulfan concentrations found in surface water and in runoff from agricultural areas range from 0.001 to over 0.1 mg/L although levels of up to 0.7 mg/L have been reported in water bodies 10 m away from the application sites (Ernst et al., 1991; Wan et al., 1995; Schulz et al., 2001a,b; Mersie et al., 2003).

The toxic effects of endosulfan have been studied in mammals and fish (Paul and Balasubramaniam, 1997; Dutta and Arends, 2003; Giusi et al., 2005) and it has been shown that one of the main target organs is the central nervous system. Several authors have tested different endosulfan concentrations (ranging from 0.47 to 4,700 μg/L) using both acute and chronic tests and different parameters to assess the effect of this pollutant on amphibians (i.e., growth, survival, metamorphosis, and behaviour) (Berrill et al., 1998; Harris et al., 1998, 2000; Bernabò et al., 2008; Brunelli et al., 2009; Jones et al., 2009; Relyea, 2009; Shenoy et al., 2009; Sparling and Fellers, 2009). To date, there have been no studies examining how environmentally relevant concentrations of pesticides affect the morphological features of amphibian gills in long-term experiments.

The present study was successful in showing that environmentally relevant concentrations of endosulfan negatively affected gill apparatus in *Bufo bufo* tadpoles.

Examination of *Bufo bufo* gills after long-term exposure to endosulfan showed remarkable effects, resulting in modifications in ultrastructure and cell composition. These results are consistent with our previous findings (Bernabò et al., 2008) concerning the morphological alteration pattern during acute exposure. Endosulfan exerts its influence by firstly inducing a general defensive response (mucous secretion), then a more specific epithelial reaction with the appearance of TVC, and finally the appearance of a typical degeneration phenomenon (macrophages and large epithelial lacunae).

We recently demonstrated that gill damage was strongly correlated with exposure time (Bernabò et al., 2008). It is not surprising that to reach the extent of the modifications observed in this study after 96 h of exposure to 0.2 mg/L endosulfan, at least 8 days would be needed at a concentration of 0.1 mg/L. More

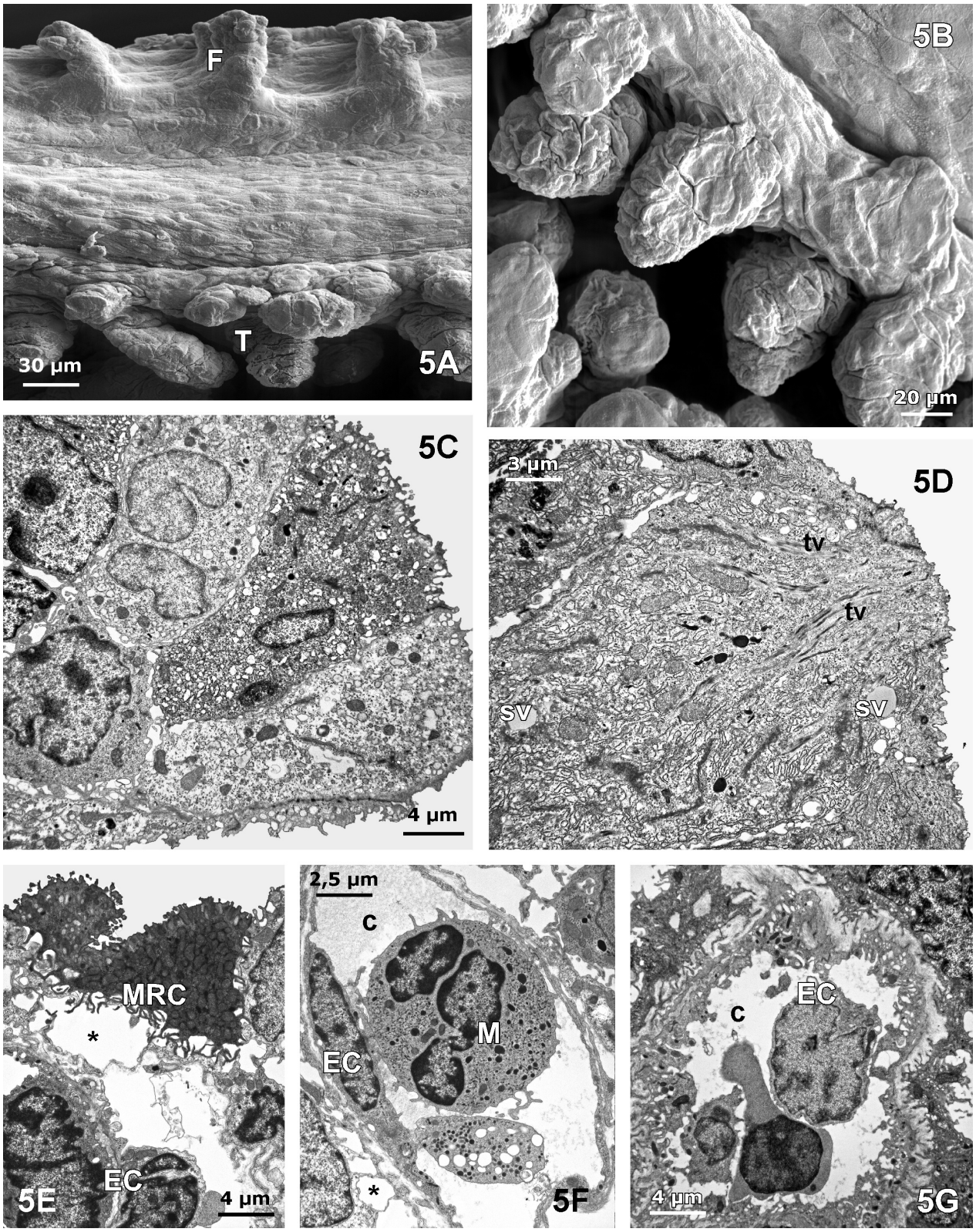

Fig. 5. SEM micrographs of *Bufo bufo* gills after 20 days of endosulfan exposure showing a severe reduction of the entire structure **(A)** and the squamous and dehydrated surface **(B)**. TEM micrographs of samples **(C-G)**. **(C)** Samples exposed at all endosulfan concentrations showed in the tuft epithelium the co-presence of degenerated cells; **(D)** preserved tubular vesicles (tv) cell and MRC with an evident enlargement of the intercellular space (*) **(E)**. **F.** Numerous macrophage (M) are observed. **G.** Note the hypertrophy of EC causing occlusion of the capillary lumen and the uneven profile of endothelium.

interesting is the histological evidence showing the early (precocious) appearance of gill alterations (96 h) in the low exposure group, and the increase in histological and ultrastructural pathology with an increase in pollutant concentration. Time dependence (Bernabò et al., 2008) was confirmed in this study, along with a dose response relationship, thus supporting the role of amphibian gill morphology as a good biomarker following pesticide exposure.

In our previous study (Brunelli et al., 2009), we showed by using the same concentrations tested here, that a long-term exposure to endosulfan impairs development, growth, and swimming patterns (inducing hyperactivity, convulsive swimming and body twisting followed by temporary paralysis). These results are summarized in Table 1 and highlight the time of appearance of different noxious effects. After 96 h of exposure (4 days) to low concentration of endosulfan (0.01 mg/L) swimming activity, growth and body weight showed no differences compared to the control group, whereas morphological alterations were already detectable at the gill level. Our results provide direct evidence that the first effect of exposure to endosulfan in amphibians is alterations to gill morphology. It is interesting to note that even if a very low concentration of endosulfan (0.01 mg/L) did not affect tadpole behaviour (and one might suppose would not affect either of the nervous systems) this concentration is high enough to induce gill alterations.

The literature review on endosulfan surface water concentrations in the field and the results of aquatic laboratory and field toxicity studies highlight that when endosulfan is applied for agricultural purposes near aquatic ecosystems, it may cause adverse effects on non-target aquatic life (Carriger and Rand, 2008). Although the U.S. Environmental Protection Agency does not require testing on amphibians to determine pesticide safety, toxicity of endosulfan to amphibians is supported by new evidence of its effect on different amphibian species (Jones et al., 2009; Shenoy et al., 2009). Several studies have been published on the effects of pesticide on fish and amphibians, but only a few have considered gill morphology and ultrastructure. The objective of the present paper was to provide valuable information for a more comprehensive understanding of their effects on and potential risks to the non-target organisms in aquatic and agricultural ecosystems.

It is concluded that by using a morphological approach it is possible to detect early alterations following exposure to pesticide concentrations which could occur in the environment. As suggested for fish, histological examination could represent a useful tool to assess the effects of xenobiotics (Bernet et al., 1999; Dezfuli et al., 2006).

Acknowledgements. We thank Enrico Perrotta for technical assistance. This study was supported in part by a grant provided by "Regione Calabria – Assessorato Ambiente".

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Accepted May 21, 2010