Intrabody TTX distribution and possible way of its migration in ribbon worms *Cephalothrix* cf. *simula*

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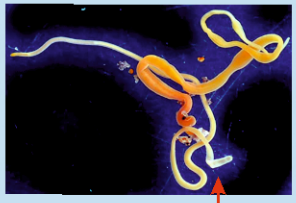
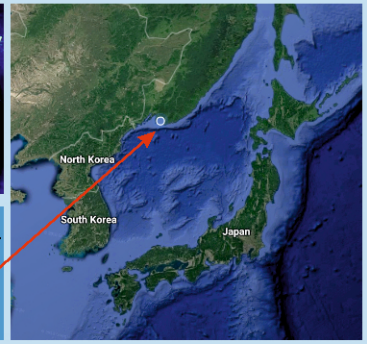
The data of tetrodotoxin and its analogs (TTXs) distribution in highly toxic animals is of great interest due to its contribution to the understanding of entering ways of the toxin in animal's body, toxin migration and accumulation in tissues and cells, and its functions. In 2004 Tanu with colleagues (Tanu et al., 2004) investigated TTX's intrabody distribution in nemerteans on unidentified species of the *Cephalothrix* genus for the first time. They studied the toxin's intrabody distribution only in the foregut region and described only TTX-positive cells, not defining all cell types of TTX-positive tissues and organs. In the current work, four regions of toxic *Cephalothrix* cf. *simula* were studied: (1) the precerebral region, (2) the foregut region, (3) the middle and (4) the posterior intestine regions.





Representatives of *C.* cf. *simula* were collected in the rhizoids of the brown algae *Saccharina* sp. in Spokoinaya Bay, Peter the Great Bay, in the Sea of Japan (42.7090° N, 133.1809° E) in August 2020.

For TTX identification and quantification high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) was used.

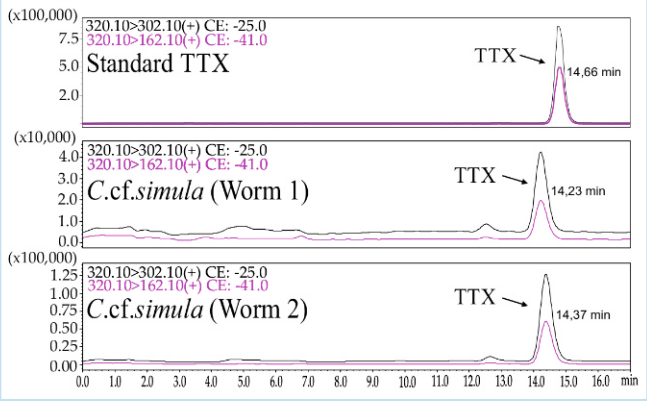


Figure 1. High-performance liquid chromatography tandem mass spectrometry (HPLC MS/MS) chromatograms of standard tetrodotoxin (TTX) and TTX obtained from whole-body extracts of two worms of *Cephalothrix* cf. *simula*.

At the light-optical level, all cell types of TTX-positive tissues (namely cephalic gland, integument, intestinal epithelium, glandular epithelium of proboscis, blood and nephridial systems) were described. Using confocal laser scanning microscopy with anti-TTX antibodies and cryosections, cell types accumulating the toxins were identified. In the current study, we obtained new data on toxin localization. An intense label was found in the glandular epithelium of the proboscis, protonephridia, epidermis, and intestinal epithelium. The medium intensity label was detected in the cephalic glands, lateral nerve, and oocytes. Weak TTX-like immunoreactivity was observed in the musculature of the body wall and proboscis and the endothelium of blood vessels.

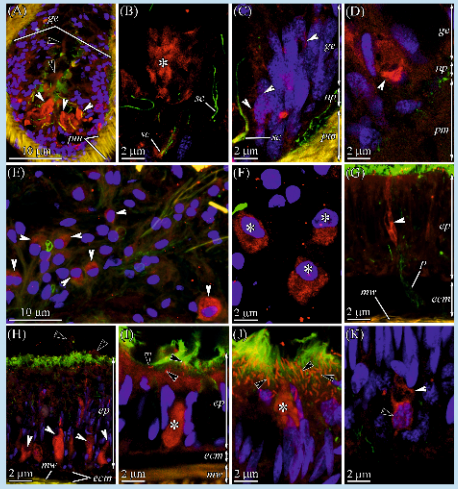


Figure 2. TTX-like immunoreactivity of the everted proboscis (A–D), cephalic gland (E–G), and integument (H–K) of *Cephalothrix* cf. *simula*. The confocal laser scanning micrographs show substacks of transverse sections. Red, TTX-like immunoreactivity; green, α-acetylated tubulin immunoreactivity; blue, nuclei (DAPI); yellow, musculature, phalloidin-positive. (A) Panoramic view of proboscis; white arrowheads point to TTX-positive cells, and black arrowheads point to TTX-positive slime on the apical surface of the glandular epithelium and in the proboscis lumen. (B) TTX-positive granules of type II gland cells (asterisk). (C) The bodies of cells with TTX-positive structures; arrowheads point to elongated sickle-shaped structures. (D) TTX-positive sickle-shaped structure of the epithelial cell. (E) Cephalic gland with TTX-positive mucoid cells (arrowheads). (F) TTX-positive cell bodies of mucoid cells (asterisks). (G) TTX-positive ducts/lacunar extensions (arrowhead) of the mucoid cell passing through the pore of extracellular matrix (ecm) and integument. (H) Panoramic view showing TTX-positive granules of serous cells (white arrowheads) and TTX-positive secretions (black arrowheads) onto the surface of the epidermis (ep). (I) The TTX-positive granule of the serous cell (asterisk) and TTX-positive microvilli of ciliary cells (arrowheads). (J) The distal region of the epidermis with apical extension (asterisk) filled by TTX-positive spherical granules. Arrowheads point to the TTX-positive microvilli of ciliary cells. (K) The proximal region of the epidermis with TTX-positive filamentous structures surrounding the nucleus (black arrowhead) and occupying the perinuclear region (white arrowhead). ecm, extracellular matrix; ep, epidermis; ge, glandular epithelium of proboscis; mw, musculature of body wall; np, basiepithelial nerve plexus; p, pore; pm, proboscis musculature; sc, sensory cell.

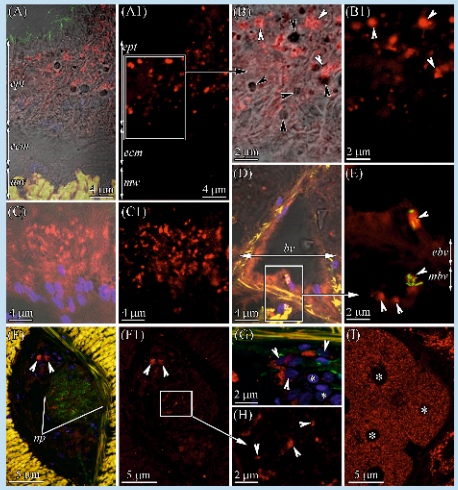


Figure 3. TTX-like immunoreactivity in the foregut (A–B1, D–I1) and intestine (C, C1, I) regions of *Cephalothrix* cf. *simula*. The confocal laser scanning micrographs show substacks of transverse sections. Red, TTX-like immunoreactivity; green, α-acetylated tubulin immunoreactivity; blue, nuclei (DAPI); yellow, musculature, phalloidin-positive. (A) Transmission image of intestinal epithelium (epi) with underlying body-wall muscles (mw) of the body wall united with immunostaining. (B) Transmission image of the intestinal epithelium united with TTX-positive (white arrowheads) and TTX-negative (black arrowheads) phagosomes. (C) Transmission image of intestinal epithelium in anterior intestinal region. (D) Transmission image of the lateral blood vessel united with immunostaining. (E) Terminal organ of protonephridium (arrowheads). (F) The lateral nerve with TTX-positive bodies of nerve cells (perikaryons) (arrowheads). (G) TTX-positive (arrowheads) and TTX-negative (asterisks) perikaryons of nerve cells (arrowheads). (H) TTX-positive nerve trunks (arrowheads). (I) Oocytes (asterisks). bv, blood vessel; ebv, epithelium of blood vessel; ecm, extracellular matrix; epi, epithelium; mw, musculature of body wall; mbv, muscles of blood vessel; np, neuropil.

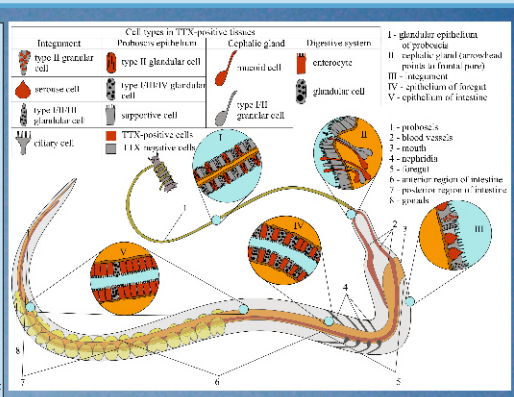


Figure 4. Schematic illustration of tetrodotoxin (TTX) distribution in the proboscis, cephalic gland, integument, and digestive system of *Cephalothrix* cf. *simula*.

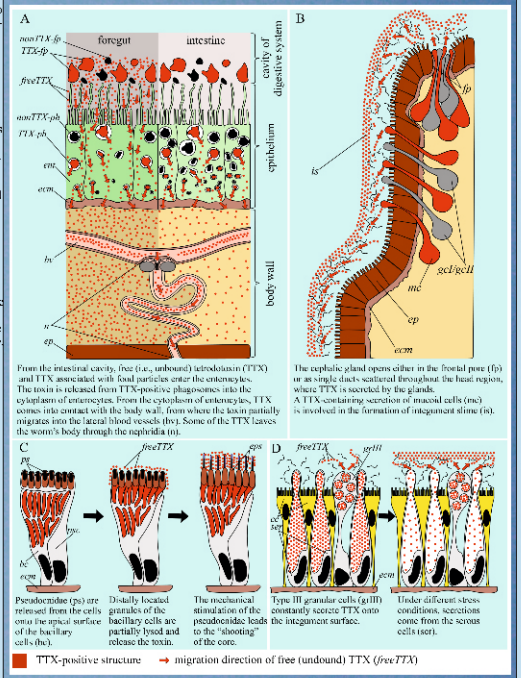


Figure 5. Schematic diagrams illustrating the use of tetrodotoxin (TTX) by a *Cephalothrix* cf. *simula*. (A) TTX uptake by the digestive system. (B) Constant release of the toxin by the cephalic gland. (C) Use of the toxin by the glandular cells of the proboscis. (D) Use of the toxin by the integumentary cells of the body wall. bc, bacillary cell (type II glandular cell); bv, blood vessel; cc, ciliary cell; ecm, extracellular matrix; ent, enterocyte; ep, epidermis; eps, extruded pseudocnida; FreeTTX, free TTX; fp, frontal pore; gcI/gcII, type I/II gland cell; grIII, type III granular cell; is, integumentary slime; mc, mucoid cell; nonTTX-fp, non TTX-positive food particle; nonTTX-ph, non TTX-positive phagosome; pn, protonephridial system; ps, pseudocnida; psc, pseudocnidae-containing cell; ser, serous cell; TTX-fp, TTX-positive food particle; TTX-ph, TTX-positive phagosome.

According to the data obtained, TTX in *C.* cf. *simula* enters the intestine with food. Absorption of the main amount of TTX occurs in the foregut, where free toxin enters the intestinal cells. The structural features of the circulatory system allow the toxin to migrate mainly to the organs of the anterior part of the worm, in particular to the glandular systems producing epidermal mucus, effectively protecting nemerteans from predators. At the same time, cells accumulating TTX contribute to toxicity in different ways: some types of cells can maintain a constant concentration of toxin in the epidermal mucus, releasing granular secretions in one granule, while others can rapidly secrete large amounts of TTX-containing serous mucous secretions in response to stress. In the current study, we revealed TTX-like compounds in granules of bacillary cells contained in the glandular epithelium of proboscis. Bacillary cells associated in pairs with pseudocnid-containing cells form a glandular system taking part in prey retention. That is, the "sticky" component, which is part of the secretion of bacillary cells, can enhance the adhesion of pseudocnidae to the surface of the victim's body, while the toxic component has an immobilizing effect on the victim.