https://doi.org/10.26651/allelo.j/2019-46-1-1195 0971-4693/94 Euro 20.00
Allelopathy Journal 46 (1): 17-24 (January, 2019) *International Allelopathy Foundation* 2019
Table: 1, Figs : 2

Allelopathy experiments with Chara algae model: Histochemical analysis of the participation of neurotransmitter systems in water inhabitation

V.V. Roshchina, N.K. Prizova and L.M. Khaibulaeva
Lab. Microspectral analysis of cells and cellular systems, Institute of Cell Biophysics,
Institutskaya str. 3, Pushchino, Moscow region, Russia, 142290.
E. Mail: roshchinavic@mail.ru

(Received in revised form: November 20, 2018)

ABSTRACT

We studied the participation of neurotransmitters acetylcholine and biogenic amines in allelopathic water relations on the model of water contacts between algae *Chara vulgaris* and worm - planarian *Girardinia tigrina*. Colour and fluorescent histochemical reactions were considered in order to locate the neurotransmitters in *Chara vulgaris* cell. Azo dyes Fast Red TR salt and DTPDD (Red analogue of Ellman reagent) demonstrated colour reactions to show the location of cholinesterase as marker of acetylcholine. Fluorescent reactions for the observation catecholamines and histamine *in situ* were studied using glyoxylic acid and o-phthalic aldehyde, relatively. It demonstrated the occurrence of cholinesterase activity and the biogenic amines on surface of cells (cell wall and plasmalemma) and in their excretions. The testing of neurotransmitters on the planarian motility showed the negative influence of high concentrations of dopamine and histamine in dense population of algae.

Key words: Acetylcholine, biogenic amines, cholinesterase, contacts, dopamine, glyoxylic acid, histamine, luminescence microscopy, neurotransmitters, o-phthalic aldehyde

INTRODUCTION

Interactions of organisms in biocenosis are connected with the excretions from both plants and animals. But till now special studies of allelopathic chemical communication with a participation of compounds acetylcholine and biogenic amines, known earlier as neurotransmitters in similar contacts, were rare (19). Suitable model system of signaling between the aquatic inhabitant organisms may be, in particular algae and animal.

Today we have some data about the neurotransmitter location in algae and their excretions as well as interactions with animal organisms. Similar contacts of algae excretions enriched in histamine (27,28) or dopamine (29,30) that acted on animal inhabitants have been described in 21^{st} century, although first information about occurrence of histamine in red algae was in paper of Blackwell (1). Possible marker of the presence of acetylcholine is cholinesterase enzyme degraded the neurotransmitter, whose activity is found in river- and lake- living *Nitella* algae (4) and in marine algae (7). Histochemical methods based on the use of special dyes or reagents making visible the presence and location of the neurotransmitter compounds are not widely used on plant cells yet. Many approaches may be applied by the plant biologists too using both assays from neurological field, although there are few examples of the studies on plant cells.

First histochemical research for coloration of cholinesterase in animal cells is based on the colour reactions with some azo compounds (14,15). Some authors applied azodye Fast Red TR salt to determine the spot (band) of cholinesterase reacting with β- naphthyl

---

*Correspondence author

acetate as substrate in gel-electrophoresis (8) or bacteria analysis (9). The histochemical staining was also used in plant cells and isolated organelles (20). Main mechanisms of the dye Fast Red TR salt staining and β-naphthyl acetate as substrate is based on the transfer of colourless object to red color. Besides, DTPDD [2,2-dithio-bis-(p-phenyleneazo)-bis-(1-oxy-8-chlorine-3,6) -disulfur acid] in form of sodium salt as red colour analogue of Ellman reagent is also used (16,22). In this case the red reagent binding with thiocholine, as a product of acetylthiocholine hydrolysis, forms the blue product (16). This method is used for extracts from plant objects, especially from pollens (16, 22). Some experiments are done with colouring on cholinesterase of worm planarians that also releases the enzyme from the body to river water (17).

Histochemical determination of biogenic amines in plants (10,11,21,23,24) has originated from the studies on animal samples. The assays are based on the drying of cellular samples after the treatment with glyoxylic acid (2) for the analysis of catecholamines (dopamine) or o-phthalic aldehyde for the analysis of histamine (3). The characteristic fluorescence in blue or blue-green shows the presence of dopamine or histamine.

We have no data about the histochemical analysis of the presence of neurotransmitters in algae *Chara vulgaris* L. that is wide spread in nature and in aquatic ecosystem. This object may serve as model in such experiments to analyze the presence and excretion of cholinesterase or biogenic amines from the algae cells that, as told above, can be of great ecological importance for water-living plants and animal of the biocenosis. The work aimed to use the histochemical approach to similar analysis.

MATERIALS AND METHODS

Object. Algae *Chara vulgaris* L. was grown in aquarium at room temperature. Preparation of samples for histochemical analysis was on the subject glasses placed in Petri dish and poured in reagents**.**

Cholinesterase activity: For the experiments to observe the cholinesterase activity in algae cells we used dye Fast Red TR which in the presence color-less α- or β- naphthyl acetate colours in red (20). All experiments on cholinesterase activity were done at 20-22^{o}C temperature. Samples of slide treated with optimal for hydrolytic reaction substrate concentration - 10^{-3} M β- naphthyl acetate (0.05 ml) dissolved in 0.05 M potassium phosphate buffer pH 7.25-7.5. Enzyme activity was estimated based on some publications (9,20) by incubation of algae sample (0.5-1 cm) with α- or β-naphthyl acetate (10 mg dissolved in 0.5 ml dimethylsolfoxide or acetone and then diluted to 3 ml by 50 mmol L -1 phosphate buffer pH 7.0) at 20-22 ^{o} C during 30 min were incubated in 350 μL of at 22 °C in presence of Fast Red TR salt (50 μL;10 μmol.L^{-1} in 50 mmol.L^{-1} phosphate buffer, pH 7.0). Activity was assayed at 560 nm by measuring the degree of substrate hydrolysis. Spontaneous, non-enzymatic substrate hydrolysis was minimal. Pre-treatment (before adding a substrate) for 20-30 min with cholinesterase inhibitors neostigmine or physostigmine (10^{-6}-10^{-4} M) showed the enzyme activity or not in the sample. All reagents for the assay of cholinesterase activity were from Sigma Company (USA). The most complete inhibition of colouring was observed at concentrations 10^{-4}M, as described in the work (5,12) using electron microscopy. DTPDD, red analogue of Ellman Reagent

(Khimanalyt, Russia) was also tested for the histochemical staining as described earlier (16,22). The images of cells were analyzed and photography under transmitted light of luminescence microscope Leica DM 6000 B. The absorbance spectra of samples were recorded by Specord UV-VIS M-40 (Karl Zeiss, Germany). The absorbance intensity was measured in relative units in 3-4 samples of algae body per variant. Results were expressed as mean ± SEM (shown graphically as upper horizontal lines on the figure histograms). The relative standard deviation (RSD) was 5-6% (P =0.95).

Assay of biogenic amines: Treatment of samples for histochemical analysis of biogenic amines poured in drops of 1% aqueous solutions of glyoxylic acid to determine the catecholamines described in (13,21) or 1 - 0.5% solution of o-phthalic aldehyde (21). Samples after 10-20 min staining were dried at 50-80^{o} C. Fluorescence of living cells and isolated organelles was observed and photographed by luminescence microscope Leica DM 6000 B like on intact pollens and isolated organelles (18). The emission spectra were recorded by Perkin-Elmer Spectrofluorimeter 350. Registration of fluorescence intensity was expressed in relative units in 3-4 samples per one variant.

Observation of worm behavior: Influence of biogenic amines, present in the algae excretion was studied, on neighbour water animals in model experiments on the behavior of water worms - planarians *Girardinia tigrina*. Small life water volume 200 ml in vessel per one worm included various concentrations of dopamine or histamine in range from 10^{-8} M to 10^{-2} M. The 6 vessels for every variant were used. In the work all reagents from Sigma Company (USA) were used.

Statistical analysis: Results were expressed as mean ± SEM (shown graphically as upper horizontal lines on the figure histograms). The relative standard deviation (RSD) was 5-6% (P = 0.95).

RESULTS AND DISCUSSION

Cholinesterase. Staining with dye Fast Red TR salt for the cholinesterase assay demonstrated the red coloration of algae cells after the exposure with colorless substrate. Red colour was seen in thick cellular wall and in plasmalemma as well in excretions (Fig.1) in regimes of transmitted light (2) and phase contrast (3) of the microscope. Its appearance prevented by the cholinesterase inhibitors neostigmine or physostigmine. After the preliminary exposure of the samples in the inhibitors the evidence of the cholinesterase activity confirmed by the decrease or lack of coloration, like it is illustrated for neostigmine (4). By spectrophotometer the changes in the absorbance at 560 nm have been registered on the algae body (Fig. 2). For conformation of the effects with Fast Red TR Salt we also stained the samples with DTPDD reagent if used acetylthiocholine as a substrate (16,20). Unlike the variant with Fast Red, blue product formed on the surface of the cells was lesser noticed by the eye under the microscope due to intensive bluish-green native colour of the algae. It was better registered as absorbance of algae body at 620 nm by spectrophotometry (Fig. 2).

Among the species of Characeae the cholinesterase activity was found only in *Nitella* (4). Earlier location of the cholinesterase in plasmalemma of cells from some terrestrial species was demonstrated with electron microscopy (5-7) or usual light microscopy after vital coloration with azo dyes such as DTPDD (16,22) or Fast Red TR salt (20). Presence of cholinesterase as a marker of acetylcholine on the surface and excretions of *C. vulgaris* shows the possibility of the algae communications with other water organisms (both animals and plants) which able to release this neurotransmitter (biomediator).

Biogenic amines. Unlike cholinesterase, biogenic amines were studied by fluorescent methods with special histochemical reagents that resulted in the formation of fluorescent products. The reactions for catecholamines with glyoxylic acid were considered for animal cells by some authors (2) as well as reaction for histamine with o-phthalic aldehyde (3). Kimura M (10) was first who applied this achievement to study catecholamines in plants histochemically and later Barwell (1) determined histamine in the red alga of *Furcellaria* by similar approach. Later subcellular localization of alkaloids and dopamine in different vacuolar compartment of *Papaver bracteatum* was studied using fluorescent method with glyoxylic acid and gas-chromatography (11). Fig. 1 demonstrated blue-fluorescent images of *C. vulgaris* after reactions under excitation of UV-light occurred after the treatment with glyoxylic acid or o-phthalic aldehyde. The emission maxima of the product with catecholamines - 460-470 nm, while product with phthalic aldehyde 455-460 nm. Autofluorescence of untreated algae bodies was weak and strengthened (Figs.1 and 2) after the staining with the reagents. More clear fluorescent reaction under luminescence microscope (Fig.1) was seen for catecholamines. Fluorescence in samples consisting from nodes and internodes was of different intensity (Fig. 2). Catecholamines prevailed (about 1.5 folds) in nodes, while for histamine the difference was not so marked. To keep in mind that the amount of biogenic amines in terrestrial plants appears to increase at stress (18) the variability appears to be not constant. It seems important for the water inhabitants of biocenosis.

Observation of worm behavior as recipient of neurotransmitters in water. Mdeling of the biogenic amines influence on the behavior of water-living worms - planarian *Girardinia tigrina* has been done too (Table 1). In concentrations 10^{-8} - 10^{-2} M of pure dopamine or histamine showed the sensitivity of planarian as animal object to high amount of the biogenic amines (>10^{-4}M): the worm loosed its motility and moved to the bottom. Acetylcholine had no visible effects on the worm motility, perhaps due to active cholinesterase seen on the surface of the planarian after the histochemical staining with DTPDD (17) which hydrolyzes the neurotransmitter. In all variants were similar results.

Table 1. The planarian motility after 24 h living in water with exogenous biogenic amines and acetylcholine. The 6 vessels with worms were used for each concentration of neurotransmitter studied.

Dopamine Conc.	Behavior of worm	Histamine Conc.	Behavior of worm	Acetylcholine Conc.	Behavior of worm
10^{-8} -10^{-5} M	No visible change in moving	10^{-8} -10^{-5} M	No visible change in moving	10^{-8} -10^{-5} M	No visible change in moving
10^{-4} -10^{-2} M	Lying on the bottom without moving	10^{-4} -10^{-2} M	Lying on the bottom without moving	10^{-4} -10^{-3}M	No visible change in moving

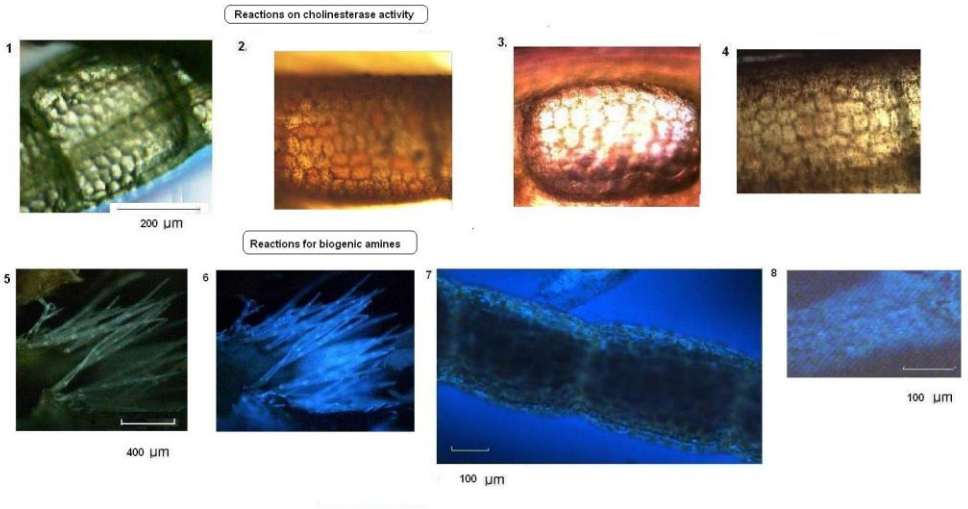

Figure 1. The images of *Chara vulgaris* cells stained with histochemical reagents for the reactions for cholinesterase activity and presence of biogenic amines (catecholamines and histamine) that were observed under transmitting light (1-4)or actinic light 360 -380 nm (5-8) of luminescence microscope DM 6000 Leica (USA-Germany). 1 - cell of the node without any treatment; 2 - red coloration in excretion and on the surface after the addition of Fast Red reagent; 3- the same with addition of the phase contrast regime ; 4 -the absence of the red colour if the sample before the substrate addition was 20 min treated with 10^{-4} M neostigmine; 5 - common image of the plant that weakly fluoresces under excitation by light 360-380 nm; 6 and 7 - the same sample as a whole and separate cells with intensive blue fluorescence excited with the UV-light seen after the treatment with glyoxylic acid , relatively; 8 - cell weaker blue emission excited with the UV-light after the treatment with o-phthalic aldehyde.

One of mechanism of the effect on the motility may be explained from data described in some experiments (26), where the interaction of dopamine with model membranes, isolated G-actin and living cells, such as Mauthner neurons and fibroblast-like BHK-21 cells has been studied. It was found that *in vitro* dopamine passes through the phospholipid membrane and directly polymerizes G-actin due to incorporation into threads as their integral part. According to van Alstyn with co-workers (29,30) the dopamine effects of algae may be related to oxidized derivatives of the compounds. During first 1 hour dopamine is oxidized to free superoxide anionradical and semiquinone and then the formation of red pigment (absorbance maxima 480 nm) aminochrome is observed. Following oxidation led to the appearance of black pigment melanin, which is polymerized (quinones as monomers) products formed with participation of polyphenoloxidases.

Recently possible role of plant systems included neurotransmitters such as acetylcholine (25) or biogenic amines (21) has been discussed for terrestrial allelopathic relations. In this way, to our opinion, cells of algae *Chara vulgaris* may serve as a suitable model in ecology for the analysis of contacts between various living inhabitants of water biocenosis due to secretions of neurotransmitters contacting with biosensors such as cholino- or aminoreceptors as well as cholinesterase.

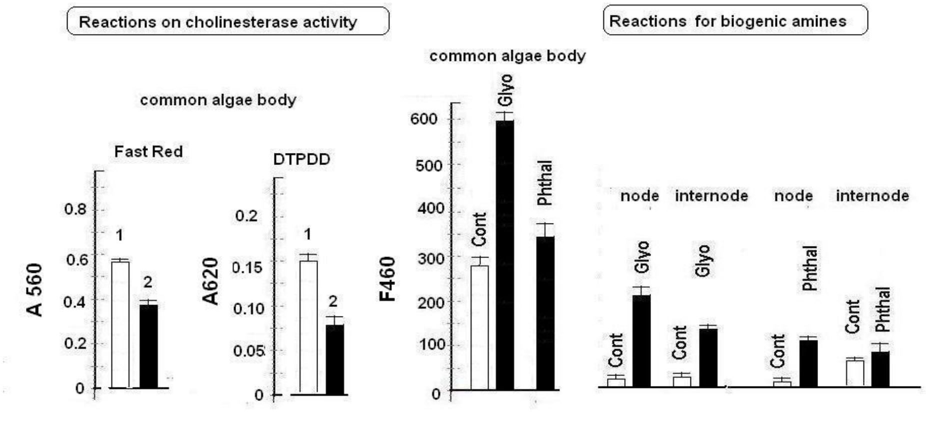

Figure 2. The absorbance and fluorescence of the *Chara vulgaris* bodies stained with histochemical reagents for the reactions for cholinesterase activity under transmitting light and presence of biogenic amines (catecholamines and histamine) excited by actinic light 360 -380 nm. Reactions for cholinesterase 1 and 2 -without and with neostigmine. A 560 and A 620 - absorbance (units of optical density) at 560 and 620 nm. Reactions for biogenic amines. Cont - control, without any treatment, Glyo and Phthal - after the treatment with glyoxylic acid and 0-phthalicaldehyde, relatively. F460 – the fluorescence intensity at 460 nm (relative Units).

CONCLUSIONS

The use of colour and fluorescent histochemical reactions at the studies of the neurotransmitters in plant cell opens new possibilities for the intercellular and intracellular signaling within biocenosis that directly relates to allelopathically interactions. Applicating reagents on cholinesterase activity (marker of the presence of acetylcholine) or reagents for catecholamines and histamine assays one could estimate the location of neurotransmitter in alga, in particular on suitable model such as *Chara vulgaris.* A possibility of the exogenous neurotransmitters in water medium to influence on the worm motility has been demonstrated in our experiments.

ACKNOWLEDGMENTS

Authors thankful to Dr. Kreshchenko for the help and consultation during the experiments with worm.

REFERENCES

1. Barwell, C.J. (1979). The occurrence of histamine in the red alga of *Furcellaria lumbricalis* Lamour. *Botanica Marina* **22:** 399-401.
2. Bjorklund, A., Lindvall, O. and Svensson, L.A. (1972). Mechanisms of fluorophore formation in the histochemical glyoxylic acid method for monoamines. *Histochemie* **32:** 113-131.

3. Cross, S.A.M., Even, S.W. and Rost, F.W.D. (1971). A study of the methods available for the cytochemical localization of histamine by fluorescence induced with o-phthalaldehyde or acetaldehyde. *Histochemical Journal* **3**: 471-476.
4. Dettbarn, W.D. (1962). Acetylcholinesterase activity in *Nitella*. *Nature* **194**: 1175-1176.
5. Fluck, R.A. and Jaffe, M.J. (1974). Cholinesterases from plant tissues, VI. Distribution and subcellular localization in *Phaseolus aureus* Roxb. *Plant Physiology* **53:** 752-758.
6. Gorska- Brylass, A., Rascio, N. and Mariani, P. (1990). Cytochemical localization of cholinesterase in the thylakoids of chloroplasts of *Marchantia polymorpha*. *Cell Biology International Reports* **14** (Abstr Suppl): 208.
7. Gupta, A., Vijayaraghavan, M.R. and Gupta, R. (1998). The presence of cholinesterase in marine algae. *Phytochemistry* **49:** 1875-1877.
8. Harris, H., Hopkinson, D.A. and Robson, E.B. (1962). Two-dimensional electrophoresis of pseudocholinesterase components in normal human serum. *Nature* 1**96** (4861): 1296-1298.
9. Kakariari, E., Georgalaki, M., Kalantzopoulos, G. and Tsakalidou, E. (2000). Purification and characterization of an intracellular esterase from *Propionibacterium freudenreichii* ssp. freudenreichii ITG 14. *Le Lait* **80**: 491-501.
10. Kimura, M. (1968). Fluorescence histochemical study on serotonin and catecholamine in some plants. *The Japanese Journal of Pharmacology* **18**: 162-168.
11. Kutchan, T.M.., Rush, M. and Coscia, C.J. (1986). Subcellular localization of alkaloids and dopamine in different vacuolar compartment of *Papaver bracteatum*. *Plant Physiology* **81**: 161-166.
12. Maheshwary, S.C., Gupta, R. and Gharyal, P.K. (1982). Cholinesterases in plants. In: *Recent Developments in Plant Sciences*: *SM Sircar Memorial Volume.* (Ed. S.P. Sen), pp. 145-160, Today and Tomorrows, New Delhi.
13. Markova, L.N., Buznikov, G.A. and Kovačević, N. (1985). Histochemical study of biogenic monoamines in early (Prenervous) and late embryos of sea urchins. *International Journal of Developmental Neuroscience* **3**(5): 493-499.
14. Menten, M.L., Junge, J. and Green, M.H. (1944). A coupling histochemical azo dye test for alkaline phosphatase in the kidney. *Journal of Biological Chemistry* **153**: 471-477.
15. Nachlas, M.M. and Seligman, A.M. (1949). The histochemical demonstration of esterase. *Journal of National Cancer Institute* **9:** 415-425.
16. Roshchina, V.V. (2001). *Neurotransmitters in Plants Life*. Science Publishers, Plymouth, USA. 283 p.
17. Roshchina, V.V. (2014). *Model Systems to Study Excretory Functions of Higher Plants*. Springer, Dordrecht, Heidelberg.
18. Roshchina, V.V. (2016). The fluorescence methods to study neurotransmitters (Biomediators) in plant cells. *Journal of Fluorescence* **26**: 1029-1043.
19. Roshchina, V.V. (2016). New tendency and perspectives in evolutionary considerations of neurotransmitters in microbial, plant and animal cells. *Advances in Experimental Medicine and Biology* **874**: 25-77.
20. Roshchina, V.V. (2018). Cholinesterase in secreting cells and isolated organelles. *Biological Membranes* (Russia) **35**: 143-149
21. Roshchina, V.V. and Yashin, V.A. (2014). Neurotransmitters catecholamines and histamine in allelopathy: Plant cells as models in fluorescence microscopy. *Allelopathy Journal* **34**: 1-16
22. Roshchina, V.V., Melnikova, E.V., Kovaleva, L.V. and Spiridonov, N.A. (1994). Cholinesterase of pollen grains. *Doklady Biological Sciences* **337**: 424-427
23. Roshchina, V.V., Yashin, V.A. and Vikhlyantsev, I.M. (2012). Fluorescence of plant microspores as biosensors. *Biochemistry* (Moscow) Supplement Series A: *Membrane and Cell Biology* **6**(1): 105-112
24. Roshchina, V.V., Yashin, V.A. and Kuchin, A.V. (2016). Fluorescence of neurotransmitters and their reception in plant cell. *Biochemistr*y (Moscow) Supplement Series A: *Membrane and Cell Biology* **10** (3): 233- 239.
25. Sharma, R. and Gupta, R. (2019). Role of acetylcholine system in allelopathy. In: *Neurotransmitters in Plants: Perspectives and Applications.* (Eds., A. Ramakrishna and V.V. Roshchina), pp. 243-270, CRC Press, Boca FL. Raton.
26. Shubina, V.S., Lavrovskaya, V.P., Bezgina, E.N., Pavlik, L.L., Moshkov, D.A. (2011). Cytochemical and Ultrastructural characteristics of BHK-21 cells exposed to dopamine. *Neuroscience and Behavioral Physiology* **41**: 1-5.
27. Swanson, R.L., Williamson, J.E., De Nys, R., Kumar, N., Bucknall, M.P. and Steinberg, P.D. (2004). Induction of settlement of larvae of the sea urchin *Holopneustes purpurascens* by histamine from ahost alga. *The Biological Bulletin* **206**: 161-172.

28. Swanson, R.L., Marshall, D.J. and Steinberg, P.D. (2007). Larval desperation and histamine: How simple responses can lead to complex changes in larval behaviour. *Journal of Experimental Biology* **210**: 3228-3235.
29. Van Alstyne, K.L., Anderson, K.J, Winans, A.K. and Gifford, S.A. (2011). Dopamine released by the green algae *Ulvaria obscura* after simulated immersion by incoming tides. *Marine Biology* **158:** 2087-2094.
30. Van Alstyne KL, Harvey, E.L. and Cataldo, M. (2014). Effects of dopamine a compound released by the green-tide macroalge *Ulvaria obscura* (Chlorophyta) on marine algae and invertebrate larvae and juveniles. *Phycologia* **53**: 195-202.