1. Adams M. I. Bacteriophages: Interscience Publishers, New York, 1959. 2. Beck N., Callahan K., Nappier S., Kim H., Sobsey M. and Meschke J. Development of a spot-titer culture assay for quantifying bacteria and viral indicators// Journal of Rapid Methods and Automation in Microbiology. – 2009. – Vol. 17, № 4. – P. 455-464. 3.Bergh O., Borsheim K. Y., Bratbak G., Heldal M. High abundances of viruses found in aquatic environments // Nature – 1989. – Vol.340. – P. 467–468. 4.Muniesa M., Jofre J. Factors influencing the replication of somatic coliphages in the water environment // Antonie Van Leeuwenhoek J. Microbiol. – 2004b. – №86. – P. 65–76. 5. Osawa S., Furuse K., Watanabe I. Distribution of ribonucleic acid coliphages in animals // Appl. Environ. Microbiol. – 1981. – №41. – P.164–168.

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ANALYSIS OF WATER SAMPLES FOR VIRUSES INFECTING FRESHWATER ALGAE FROM *CHLOROPHYTA* DIVISION

У статті розглядаються основні підходи детекції вірусів прісноводних водоростей у прісних водоймах. Для детекції використовувалися методи біотестування і електронної мікроскопії з попереднім диференційним центрифугуванням.

In this article main approaches to detection of viruses infecting freshwater algae are discussed. For detection we used bioassay and transmission electron microscopy with previous differential centrifugation of water samples.

Introduction. Studies on ecology of algae viruses are important for both fundamental and applied science. Nowadays the issues of food shortage and energy deficit are regularly raised, and microalgae can provide solutions to both these problems as they are considered as perspective source of nutrients and biofuel. In turn, viruses of microalgae are viewed as the major cause of loss of basic raw material and hence as the primary reason for commercial losses [3]. That is why significant attention of researches has been drawn to algae viruses.

Today, the nomenclature of viruses invading single cell microalgae is a fast-developing branch of taxonomy. Basing on the first reports approved by the International Committee on Taxonomy of Viruses (ICTV), this group of viruses included about 60 representatives divided into four genera comprising the only *Phycodnaviridae* family. During the recent years many new algae viruses have been described [1; 4] and the family is currently under revision with several new genera being introduced. RNA-containing algae viruses have been described and consequently allotted into separate *Marnaviridae* family which in turn has been ascribed to the order *Picornavirales* [2].

Despite recent review, complexification and advances in the taxonomy structure of algae viruses, it is still rather questionable as establishing a new RNA virus family did not help with 'artificial' allotment of all DNA-containing viruses into single family. As of today the taxonomy structure of *Phycodnaviridae* family is deemed obsolete and unreasonable (for instance, only 9 homologous genes have been found for large full genomic sequences of three different viruses belonging to this family [8]). Phylogenetic analysis of family representatives based on full sequences of DNA polymerase gene also points on its extreme heterogeneity (Fig.1).

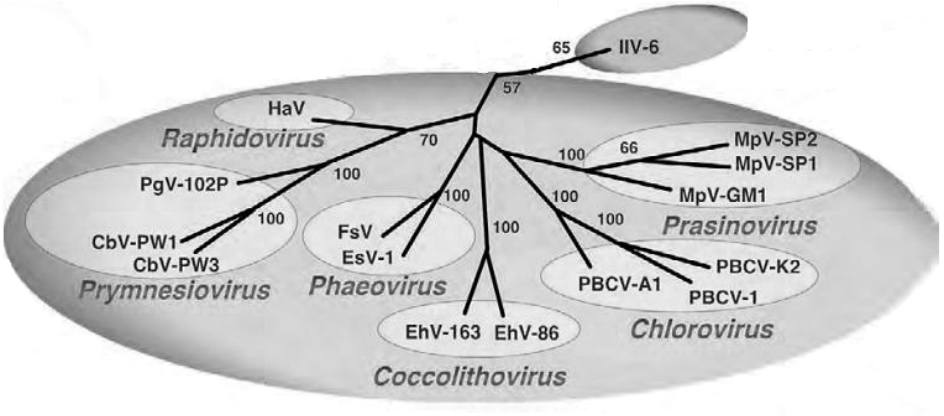

Fig.1. Phylogenetic tree showing evolutionary relationships among representatives of different genera of *Phycodnaviridae* family based on DNA polymerase gene [7]

Such attention to algae viruses is not surprising as they were demonstrated to take an active part in global biological processes of the oceans. The primary reason for studying some algae viruses lies with their crucial role in controlling the abundance of phytoplankton in water ecosystems. Ability of algae viruses to induce lysis of more than 25% of phytoplankton blooming [3; 1] is highly important from economic point of view as this phenomenon may be practically employed for such control eliminating the unavoidable water contamination with toxins and metabolic products. This would favor preservation and growth of aquatic fauna. From the other side, the algae (and especially microalgae) form the base of pyramid of numbers in water reservoirs and are the major primary producers in water food chains [1]. Hence the tentative applied use of algae viruses was another incentive for their fundamental research.

However we should note that recent advances in our knowledge of biological, morphological, genetic and molecular characteristics of these viruses are owing to research of marine microalgae. Few representatives of 'properly' described and systematized viruses infecting green freshwater algae (*Chlorophyta* division) belong to *Chlorovirus* genus. Several dozens of recent reports on isolation and description of viruses or virus-like particles from freshwater algae have not been evaluated and approved by the ICTV yet [8].

Starting from the lack of available sound information on viruses of freshwater algae, we focused on the identification of viruses able invading species of *Chlorophyta* microalgae.

Materials and Methods. Test (indicator) cultures of the following microalgae have been generously provided by the colleagues from the Department of Botany (Taras

Shevchenko' Kyiv National University): *Planophila sp., Chlorella sp., Carteria crucifera, Chlorococcum sp., Radiosphaera sp., Chlamydomonos heterogama, Scenodesmus sp., Borodinella sp.,* and *Oocystis sp*.

Approximately 40 water samples have been collected from various water reservoirs in different regions of Ukraine for the initial virus screening. The reservoirs were characterized with different level of anthropogenic pressures varying from negligibly low to moderate (city water ponds/lakes). To remove all cells from the samples these have been filtered via bacterial filter (millex GV 0,22).

Bioassay (microalgae indicator cultures) and transmission electronic microscopy after differential centrifugation of the samples were used for virus indication [5].

Algae cultures were grown following standard recommendations [7] in liquid and agar Bold's Basal Medium (BBM) medium. 2ml of algae culture suspension and 2ml of the medium were added in the tube. The active growth of the culture is normally expected in 5-7 days. Fresh suspension of algae cells then may be used again for subcultivation (in this case 2 ml of algae culture suspension and 2-4 ml of the medium are mixed). However the cultures rarely survive more than 10-15 days when employing such approach for algae cultivation.

We have also revealed that test cultures grew poorly in the flasks of relatively large volume (0.25-0.50l). After 2-3 days of active growth the algae cells either formed sediments at the bottom of such flask or became concentrated at the surface. The described effects probably reflect poor aeration of the medium in the flasks of large volumes highlighting the need for occasional mixing of the content with culture suspension for its better aeration.

Despite this modified technique was shown adequate for accumulation of algae cultures, it was also demonstrated to be inefficient for long term 'maintenance' of the cultures in active state. For this, the cultures needed to be cultivated on solid agar medium. Samples of 5-7 days' old algae cultures were loop-plated onto slants with 1.5% solid agar BBM. Normally the culture growth could be seen in 3-4 days. In such state the cultures could be maintained active for 3-4 weeks without additional passage or addition of fresh medium.

It should be said that algae viruses infect only those algae cultures which are at active growth stage or at a certain stage of the life cycle [3]. Keeping this in mind, the microalgae cultures required preliminary 'preparation' before commencing biotesting assay. Lawn scrapes of test cultures (previously cultivated on solid agar medium for 10-14 days) were deposited into plastic tubes with 1ml of BBM medium. Afterwards the tubes were kept in the dark for 12 hours for culture's synchronization and then cultivated under normal light conditions after adding equal portion of fresh medium. Occurrence of monad forms typical for active growth stage of the culture was monitored via light microscopy. The outcomes of biotesting assays have been registered by 7-10 days of cultivation [5].

Using biotesting we attained sufficient volumes of cultures' lysates for subsequent isolation and concentration of virus isolates. Concentration of virus isolated have been performed via differential centrifugation (low speed centrifugation stage at 5000 rpm for 30 min, then high speed centrifugation stage at 32000 rpm for 3 hours, and low speed centrifugation stage at 5000 rpm for 30 min) [5].

Virus morphology was studied employing transmission electronic microscopy (TEM) with positive contrasting technique (2% of sodium uranyl acetate (UA)). TEM grid with previously applied virus preparation was deposited into a drop of 2% UA and incubated for 1-10 min.

For biotesting, 2ml of collected water samples (filtered via bacterial filter) and 2ml of previously 'prepared' microalgae cultures (*Planophila sp., Chlorella sp., Carteria crucifera, Chlorococcum sp., Chlamydomonos heterogama, Scenodesmus sp., Borodinella sp., Oocystis sp.*) were added into tubes. According to literature sources [3, 5], algae viruses are normally abundant in natural habitats of microalgae and hence the lysis of algae culture may be expected by 7-10 days [5]. We have also conducted the biotesting assay on solid agar medium. 1ml of algae culture was plate into Petri dish and spread with sterile glass rod over the surface of the medium. After the uniform lawn is made, the bottom of the dish is divided into 10 sectors and 0.1ml of filtered water samples are separately plated on each of them [5].

Results and Discussion. Sample biotesting on solid medium provided no clear results, probably because of small volume of water samples applied onto the lawn of test cultures.

However, biotesting assay in liquid medium has shown that the samples collected in Kyiv from Opechen lakes of Obolon region (Verbne lake (No.1) and Velykyi Opechen lake (No.2)) and from Rylskyi Park' lakes (No.6) were capable of lysing test cultures of *Planophila sp*. and *Chlamydomonos heterogama*. In turn, water samples taken from the reservoir near the city of Slavutych and from Siverka River near Vita-Pochtova village lysed test cultures of *Chlorella sp*. and *Radiosphaera sp*. (Table 1). All samples capable of inducing lysis of test cultures were subsequently subcultivated. For this, 2ml of synchronized cultures and 1ml of respective lysat were added into a tube. The recurring effect of lysis was typical only for water samples collected from the reservoir near the city of Slavutych.

Table 1. **Results of biotesting assay for water samples on test algae cultures**

	Planophila sp.	Chlorella sp.	Carteria crucifera	Chlorococcum sp.	Radiosphaera sp.	Chlamydomonos heterogama	Scenodesmus sp.	Borodinella sp.	Oocystis sp.	Control
Verbne lake	-	+	+	+	+	-	+	+	+	-
Velykyi Opechen lake	-	+	+	+	+	-	+	+	+	-
Rylskyi Park' lakes	-	+	+	+	+	-	+	+	+	-
Siverka river near Vita-Pochtova village	+	+	+	+	-	+	+	+	+	-
Water reservoir near city of Slavutych	+	-	+	+	+	+	+	+	+	-
Control	+	+	+	+	+	+	+	+	+	

Notes: '-' lysis of test culture (no growth);
'+' normal growth of test culture.

Test cultures of *Carteria crucifera* and *Chlorococcum sp*. have demonstrated spontaneous lysis by 3 weeks of cultivation when no water samples were added.

Further, the water samples capable of inducing lysis of algae test cultures and culture samples demonstrating autolysis were subjected to TEM studies. In the autolysis

sample of *Chlorococcum sp*. culture we have identified spherical virus (or virus-like) particles of 50±2nm in diameter (Fig.2). However we have been able to demonstrate presence of virus particles in the lysates of other algae cultures. We deem that this is connected with low concentration of the particles in the sample not allowing their easy isolation and concentration.

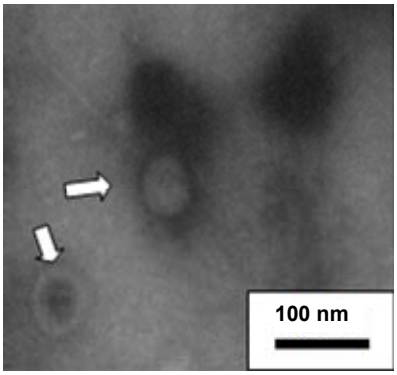

Fig.2. TEM image of virus-like particles in the autolysate sample of *Chlorococcum sp.* algae culture

Further studies will involve analysis of new water samples, as well as isolation and concentration of identified virus isolates.

In conclusion, biotesting assay of water samples collected in Verbne and Velykyi Opechen lakes, and from Rylskyi Park' lakes (Kyiv) were able to lyse *Planophila sp*. and *Chlamydomonos heterogama* algae test cultures. Respectively, water samples collected from the reservoir near the city of Slavutych and from Siverka River near Vita-Pochtova village lysed algae test cultures of *Chlorella sp*. and *Radiosphaera sp*. Using differential centrifugation of the samples, we purified and concentrated virus isolate(s) from the samples of autolysis of *Carteria crucifera* and *Chlorococcum sp*. algae cultures. TEM studies of such virus isolate(s) confirmed presence of spherical virus (or virus-like) particles of 50±2nm in diameter. Further work will be focused on studying molecular properties of the virus(es).

1. Bidle K.D., Haramaty L, Barcelos e Ramos J, Falkowski P.Viral activation and recruitment of metacaspases in the unicellular coccolithophore, Emiliania huxleyi // Proc Natl Acad Sci U S A. – 2007. – Vol. 104, No 14. – P. 6049-6054. 2. Bischoff, H. and Bold, H.C. Phycological Studies. IV. Some soil algae from Enchanted Rock and related algal species // University of Texas Publication. – 1963. – No. 6318. – P. 1–95 pp. 3. Castdery T. Algal viruses: Characteristics and ecological effects. – PY: Dep.of Micr.,University of Bergen, 2001. 4. Nagasaki K., Tomaru Y., Katanozaka N. et all Isolation and Characterization of a Novel Single-Stranded RNA Virus Infecting the Bloom-Forming Diatom Rhizosolenia setigera // Applied and Environmental Microbiology. – 2004. – Vol. 70, No. 2. – P. 704-711. 5. Stepanova O.A. Ecology of allochthonic and autochthonic viruses in the Black Sea. – Sevastopol, 2004.6. Van Etten J.L., Graves M.V., Muller D.G., Boland W., and Delaroque N.. Phycodnaviridae – large DNA algal viruses // Archives in Virology. – 2002. – Vol. 147; No 8. – P. 1479-1516. 7. Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses / C.M. Fauquet, M.A. Mayo, J.Maniloff, U.Desselberger, L.A.Ball – NewYork: Elselver Academic Press., 2005. 8. Wilson W. Giant algal viruses: lubricating thegreat engines of planetary control // Microbiology today. – 2002. – Vol 29. – P.180-182.

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SOIL-BORNE VIRUSES DETECTED IN SUGAR BEET IN LITHUANIA AND UKRAINE

Проведено обстеження зразків цукрового буряку отриманих на території Литви та України на наявність вірусу некротичного пожовтіння жилок буряку (ВНПЖБ), ґрунтового вірусу буряку (ҐВБ) та Q вірусу буряку (ВБQ). Тестування проводили методами ІФА, в модифікації DAS і TAS, та мультиплексною ЗТ-ПЛР (мЗТ-ПЛР), що дозволяє одночасно ідентифікувати ВНПЖП, ҐВБ та ВБQ. Зразки коренеплодів цукрового буряку, з симптомами ризоманії, відбирали на плантаціях Литви та України та тестували на наявність ВНПЖБ за допомогою DAS-ELISA. Для детекції ҐВБ, методом TAS-ELISA, використовували лише ті зразки, що позитивно прореагували з антисироваткою до ВНПЖБ. У литовських зразках цукрового буряку не було відмічено випадків змішаної інфекції ВНПЖБ і ҐВБ або ВПQ. На противагу в українських зразках (відібраних в Рівненській, Львівській, Івано-Франківській та Хмельницькій областях) крім ВНПЖБ була доведена наявність ҐВБ. Ідентифікацію ҐВБ підтверджували за допомогою мЗТ-ПЛР, в усіх випадках були отримані специфічні продукти для ВНПЖБ (545 п.о.) та ҐВБ (399 п.о.). ВПQ не було виявлено в жодному з досліджених зразків.

Tests for detection Beet necrotic yellow vein virus (BNYVV), Beet soil-borne virus (BSBV) and Beet virus Q (BVQ) in sugar beet samples from Lithuania and Ukraine were conducted using direct DAS-ELISA, direct TAS-ELISA and multiple RT-PCR (mRT-PCR), allowing simultaneous identification BNYVV, BSBV and BVQ. Sugar beet root samples with suspicious symptoms of rhizomania were collected in Lithuanian and Ukrainian sugar beet growing areas and analyzed by DAS-ELISA for the presence of BNYVV. For BSBV detection by TAS-ELISA only samples with positive reaction to BNYVV were used. Mixed infections of BNYVV and BSBV or BVQ in sugar beet samples in Lithuania were not detected. Meanwhile, in some Ukrainian sugar beet samples (from Rivno, Lviv, Ivano-Frankivsk and Khmelnytskyi regions) along with BNYVV, presence of BSBV was detected. Identification of BSBV was confirmed by mRT-PCR. In all cases specific BNYVV (545 bp) and BSBV (399 bp) products were obtained. BVQ was not found in any of investigated samples.

Introduction. Several soil-borne viruses are known to infect sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) worldwide. Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV; genus *Benyvirus*) (Tamada and Baba, 1973), is one of the most destructive diseases of sugar beet. Losses of sugar beet root yield can reach 50-60 % (Henry, 1996). Rhizomania was originally described in Italy (Canova, 1959) but now it is present in sugar beet areas all over the world. BNYVV has a multipartite single-stranded RNA genome with all natural isolates containing four RNA species, although some isolates have a fifth RNA (Tamada et al., 1989). The larger RNA1 and RNA2 contain the housekeeping genes of the virus and are always required for infection, whereas the smaller RNAs are involved in pathogenicity and vector transmission. RNA5-containing isolates are restricted to Asia and some parts of Europe and these isolates tend to be more aggressive (Richards and Tamada, 1992; McGrann et al., 2009). BNYVV is transmitted and preserved in the soil by *Polymyxa betae* Keskin. Like BNYVV, the plasmodiophorid has a host range mainly restricted to the roots of *Chenopodiaceae* spp. (Barr and Asher, 1992) and is present in most sugar beet growing countries (Payne and Asher, 1990). The virus can survive in *P. betae* cystosori for more than 15 years (Abe and Tamada, 1986; McGrann et al., 2009). According to the alert list of European and Mediterranean Plant Protection Organization (EPPO), BNYVV has been detected in many European, Asian countries (Austria, Belgium, Bulgaria, China, Croatia, Czech Republic, Denmark, Federal Republic of Yugoslavia, France, Germany, Greece, Hungary, Iran,