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Primary screening of potentially bio-active substances in the lyophilisate of *Pectinatella magnifica* biomass

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ABSTRACT: The purpose of this research was to examine potential bio-active substances in the lyophilisate of *Pectinatella magnifica* biomass and its focus was on primary screening of the biomass. Extracts of lyophilisate were applied to murine cells and their effects on cell population growth, cell morphology and cell distribution were monitored. The inhibition of cytoskeleton repair reached its maximum after a 24-h exposure of the cells to the extract prepared at a temperature of 37 °C, whereas the 1-h application of the extract leached at a temperature of 37 °C resulted in quick repair of cytoskeletal function. When compared with the control, in which no extract was applied, the application of all treatments of the extract reduced the time needed for the duplication of cells. The lowest growth rate was found in the cells in the extract prepared at 37 °C for 24 h. The mitotic activity reached its maximum when using the extract prepared at 37 °C for 1 h. Under such conditions, the process of cell duplication was even faster than in the control sample, while it was the slowest using the extract prepared at 37 °C for 24 h. The study of potential biologically active substances of *Pectinatella magnifica* is highly topical due to the frequent occurrence of this organism in the water-supply and recreational reservoirs.

Keywords: bryozoan; cell line L929; spreading; specific growth rate; cell proliferation; time-lapse cinemicrography; bio-active substances

Pectinatella magnifica (Leidy 1851) is a colonial freshwater bryozoan originating from North America. In Europe it was first found in the Billa River (a tributary of the Labe River) near Hamburg in 1883. In the Czech Republic this bryozoan species was observed in the Labe and Vltava Rivers already in the first quarter of the 20th century, namely in the Labe River near Litomerice in 1922. Subsequently, the organism was continuously detected in the Vltava and Labe Rivers and also in the Kninicska dam in the river-basin of the Danube River (Opravilova 2005). Recently, it has become a common species e.g. in freshwater bodies of the Protected Landscape Area (PLA) and Biosphere Reserve (BR) Trebonsko (South Bohemia, Czech Republic). *P. magnifica* colonies differ from colonies of other freshwater bryozoan species especially in size. Their fresh weight can reach up to 70 kg (Balounova et al. 2011) or up to 1 m in diameter (Rodriguez and Vergon 2002). Regarding its location, it can be found in lakes, ponds and rivers with gentle water flow (Opravilova 2005) and its colonies are mostly found attached to willow branches under the water surface, a piece of timber submerged in water, stones or technical devices.

Some species of marine bryozoans have the ability to produce certain bio-active substances of which the macrolide lactones (bryostatins), indole alkaloids (often bromine derivatives), isoquinoline quinones, sterols (steroids) and other carbohydrates are the most monitored metabolites. The antibacterial activity and/or cytotoxicity are regarded as the most significant properties of these substances. Also, dermal allergy, anthelmintic activity or prevention of cell division had been identified in some substances (Sharp et al. 2007). Bryostatins considered as the most important bio-active substances were first discovered in the marine bryozoan *Bugula neritina* (Davidson et al. 2001; Manning et al. 2005; Schmidt 2005; Sun and Alkon 2006; Mayer and Gustafson 2008)), and

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bryostatin 1 has been proposed as a potential drug for treatment of leukaemia, lymphomas, melanomas and solid tumours (Davidson and Haygood 1999; Davidson et al. 2001), traumatic brain injury (Zohar et al. 2011), depression, Alzheimer's disease and other CNS disorders (Sun and Alkon 2006; Paul et al. 2007). In addition, it can activate innate immunity (Ariza et al. 2011).

Many bio-active substances are produced by commensal/symbiont microorganisms of bryozoans: bacteria, cyanobacteria and algae; the source of bryostatins is the symbiont of the bryozoan *Bugula neritina* (Paul et al. 2007). This symbiont is the bacterium *Candidatus Endobugula sertula,* living in all life stages of its host *Bugula neritina* (Lopanik et al. 2004).

In addition, numerous symbionts, bacteria, cyanobacteria and algae are found in the colonies of freshwater bryozoan *P. magnifica*.

Blue-green algae – Cyanophyta, dominate on surfaces with the colonies of *Pectinatella magnifica* and in the matrix inside colonies (90%), while on the surfaces of the base without colonies of *Pectinatella magnifica* mixed communities of diatoms (78%), Cyanophyta (12%), and green algae (11%) are formed (Joo et al. 1992).

Some observations indicate that colonies of *Pectinatella magnifica* contain such kinds of bioactive substances which have not been described and identified yet and which not only show antibiotic and anti-predatory features, but at the same exhibit long-term stability, which is also supported by the fact that the necrotic gelatinous corm, so-called matrix, can persist in water reservoirs from August until late March of the following year. It is also interesting that there is evidence suggests that the bryozoan *Pectinatella magnifica* is mainly found in mesotrophic or oligotrophic waters, often used for recreational purposes. The presence of this species can be easily observed if the colonies spread and reach large proportions so that it comes to a mass occurrence. Provided that this happens, then the issues of safety and potential health risks, as well as the issue of impact of mass occurrence of *Pectinatella magnifica* on the fish population, especially during the summer months, which is not yet known, will become a topic of frequent discussion.

The presence (or the activity) of so far unidentified substances was examined and studied in a biological test carried out *in vitro* on animal cells. It is hoped that the results of this research will form a basis for follow-up studies on these substances.

MATERIAL AND METHODS

Standard methods were used to determine the cytotoxicity to animal cells (Standard CSN EN ISO 10 993-5 in accordance with Standard CSN EN ISO 17 025). The treatments were carried out on a permanent heteronuclear murine fibroblast line L929 from the European Collection of Cell Cultures – ECACC, which is generally and commonly used for similar treatments mainly because of its reactions to foreign substances and cell locomotion. The biological test was based on adding aqueous extract of the corm lyophilisate (i.e. the whole colonies including matrix, zooids and released statoblasts as taken from the sites) into the nutrient medium of cell cultures. The experimental cultivation itself was carried out in Petri dishes (under standard cultivation conditions: in a Thermo Scientific BBD 6220 incubator with CO_2 (5%) and temperature (37 °C) regulation, in MEM medium with addition of antibiotic and antifungal agents). First, the cell population was dissociated from the cultivation dish via trypsin or versen, then separated in a centrifugal machine and mixed with the appropriate lyophilisate extract treatment from the bryozoan colony. The next step was to inoculate the cells into a cultivation dish so that the resulting density was about 30 to 50 cells in the microscopic field of vision. The process of subsequent cultivation was monitored and scanned using Nikon Biostation (a compact automatic monitoring device which allows observation of cells under optimal conditions in Petri dishes. Moreover, its recording device allows monitoring of up to seven fields of vision from one dish at the same time), using the method of time-lapse cinemicrography which enables recording of data from several different places in the Petri dish containing living cells. The observations in the first treatments were carried out in three fields of view at × 20 magnification using phase contrast. The outputs were time-lapse photos (the interval between individual exposures (photos) was 2 min) and the process was repeated three times. The data were recorded within 72 h, which means a period of about three successive cell duplications.

Extract preparation from the *P. magnifica* colonies lyophilisate. The impact of four lyophilisate extraction protocols varying in temperatures and extraction times were examined and tested. Two temperatures were used: 37 °C and 5 °C, both for two periods, 1 h and 24 h. According to these parameters the samples were marked: 37/24, 37/1,

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5/24, 5/1; the first number in the fraction represents degrees of Celsius, the second number the cultivation period. The control sample, which contained medium without serum and lyophilisate, was cultivated at 37 °C for 24 h and designated as 37/24c. All of the extracts were leached into MEM growth medium without the addition of serum and antifungal agents while being systematically stirred using a thermo scientific multi purpose rotator (60 oscillations/min). The batch size was 1g of lyophilisate per 100 ml of medium and the resulting solutions were applied directly without any further dilution. The control medium was treated in the same way, but without the addition of any other substances. Finally, the extracts were subjected to a bacterial filtration and supplemented with 5% of foetal calf serum that had been applied during inoculation).

The scheme of the treatment. The extract was subjected to a bacterial filtration and then inoculated with the cells so that the resulting density of about 30 to 50 cells in a microscopic field of vision was achieved. A video camera was turned on immediately after inoculation had been performed and the interval between individual exposures (photos) was set at 2 min.

The treatments were carried out three times, each time under the same cultivation conditions, and the behaviour of cells was recorded using the cinemicrography method.

Evaluation. To determine the biological impact, the following parameters were examined and tested:

(1) Spreading – showing the restoration rate of the cell cytoskeleton and return to homeostatic conditions after inoculation.
(2) Specific growth rate – showing whether the cell population growth was affected.
(3) Morphological cell and population abnormalities during the growth period and monitoring of cell division using the frequency of mitosis.

(1) Spreading: For the purpose of this analysis a series of consecutive images (at 10-min intervals 0, 10, 20, 30, 40, 50 and 60 min) was prepared.

The data shown in the tables and charts represent the number of dilated and non-dilated cells, from which the total number of cells was calculated.

The dilatation index was determined for each interval (i.e. each evaluated scan) according to the following formula:

$$\text{Index } R = (\text{number of dilated cells/total number of cells}) \times 100\ (\%)$$

The evaluated records were those ones recorded 60 to 120 min after the treatment had been started. After this period the dilatation was finished and the phase of common cell cycle followed, or the cycle was stopped and the cells withered. In the control treatments the cell population was usually fully dilated after the 50-min period (Figure 1.)

(2) Specific growth rate (SGR), resp. average cell doubling times (ACDT): We aimed to determine the number of cells in subsequent fields of view using the method of quantitative analysis of records which consisted in determining the number of cells in the chosen scans, i.e. in the first scan, taken at the zero time point, and in the last scan, taken after 72 h. Afterwards, the resulting number of cells was logged and according to the varying number of cells the specific growth rate was determined, resp. the average cell doubling time (ACTD) (Figure 2).

$$SGR = (\ln Nt - \ln N0)/\Delta t$$

$$ACTD = \ln 2/SGR$$

(3) Mitotic activity: The last of the examined phenomena was the aspect of mitotic activity. The monitoring of cell division using the frequency of mitosis also comprises the frequency of occurrence of morphological cell and population abnormalities during the growth period. The exact time of each cell division was determined and recorded as well as the scan number (field), in which mitoses (cell division) occurred, and the number of divided cells in the individual scans.

The scan (field) number was multiplied by two to obtain the exact time and after examining all the recorded scans the total number of mitoses was established (Figure 3).

For the purpose of statistical evaluation the Kolmogorov-Smirnov test was used (Bastinec 2009).

RESULTS

Spreading

The maximum inhibition of cytoskeleton repair was observed for the extract prepared at a temperature of 37 °C over 24 h. By comparison, the extract leached at the same temperature but within only 1 h, resulted in rapid repair of cytoskeletal function. Furthermore, significant inhibition of cytoskeletal repair was also noted for the extract prepared at a low temperature of 5 °C within only 1 h (Table 1,

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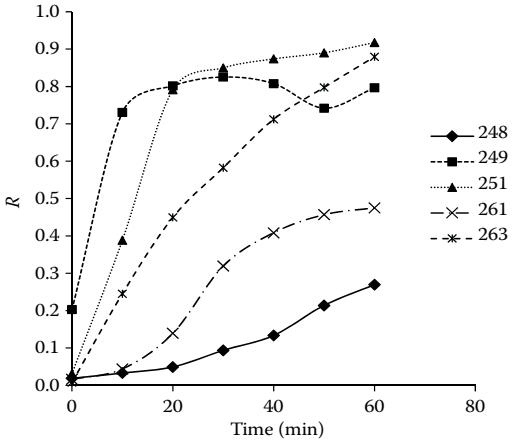

Figure 1. Spreading within 1 h (60 min). Inhibition of cytoskeleton repair in the heteronuclear murine fibroblast line L929 in samples of extract prepared at 5 °C or 37 °C for 1 h or 24 h; x axis = time (min), y axis = index *R* (number of dilated cells/total number of cells × 100%)

Figure 1). Further, slow cytoskeleton repair was observed for the extract leached at a temperature of 5 °C over 24 h. The control cultivation showed signs of depression after a 40-minute period, which was caused by the initiation of cell division.

Impact on the growth rate

In all of the experimental treatments the cell doubling time was longer, i.e. the control cells showed the maximum specific growth rate. The extract 37/1 (1 h and 37 °C) slightly inhibited the cell growth rate and the extract 5/1 (1 h and 5 °C) significantly slowed the cell growth rate. A slightly higher growth rate was observed in the 5/24 extract. As could have been expected, the lowest growth rate was observed for the 37/24 extract (24 h and 37 °C) (Table 2, Figure 2).

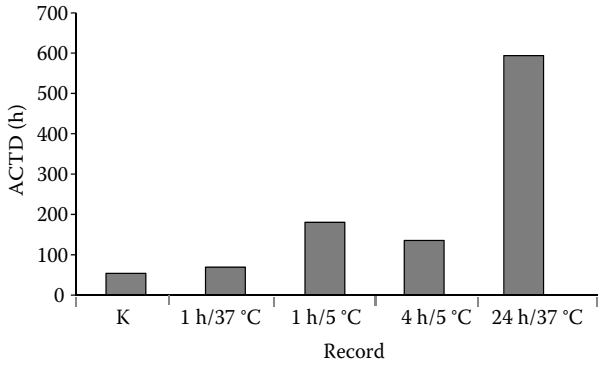

Figure 2. Average cell doubling time (ACTD, h) of heteronuclear murine fibroblast line L929. x axis = samples, K = control, y axis = ACTD (h)

Impact on mitotic activity

The mitotic activity reached its maximum in the 37/1 extract (1 h and 37 °C) and the number of cell divisions was even higher than in the control sample. The lowest number of cell divisions occurred in response to the 37/24 treatment. Compared to the control sample, the 37/1 treatment (1 h and 37 °C) had a stimulating effect on mitotic activity. All the other treatments (containing the lyophilisate leached at both higher and lower temperatures and over shorter or longer time periods) either suppressed or inhibited mitosis (treatment 37/24, i.e. leaching time 24 h and temperature 37 °C), see (Figure 3).

DISCUSSION

This work presents the first results of testing carried out on material from *Pectinatella magnifica* and can thus be considered as a pilot study in the area. The maximum inhibition of cytoskeleton repair was observed in the extract prepared at a temperature of 37 °C for 24 h. Strong inhibition

Table 1. Spreading – inhibition of cytoskeleton repair of the heteronuclear murine fibroblast line L929 in samples of extract prepared at 5 °C or 37 °C for 1 h or 24 h

Sample/Time (s)	37 °C/24 h	37 °C/24 h control	37 °C/1 h	5 °C/1 h	5 °C/24 h
0	0.018868	0.202899	0.031268	0.016164	0.007576
10	0.033025	0.730788	0.388462	0.043994	0.245226
20	0.049295	0.801678	0.791258	0.139184	0.449428
30	0.093712	0.825292	0.849891	0.319433	0.582700
40	0.133676	0.80778	0.873911	0.408442	0.712100
50	0.213966	0.742088	0.890154	0.456855	0.797527
60	0.269661	0.796377	0.917578	0.475253	0.879365

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Table 2. Specific growth rate and average doubling time of the heteronuclear murine fibroblast cell line L929

Sample	37 °C/24 h	37 °C/24 h control	37 °C/1 h	5 °C/1 h	5 °C/24 h
Specific growth rate (h)	0.001181	0.012898	0.010092	0.004203	0.005158
Average doubling time (h)	593.69	53.75	68.93	180.42	135.23

of cytoskeleton repair was found also for the extract prepared at a low temperature of 5 °C over only 1 h. Low cytoskeleton repair was observed in the extract leached at a temperature of 5 °C over 24 h, and a rapid repair of the cytoskeleton was achieved at in response to treatment with extract leached at a temperature of 37 °C over 1 h. These results indicate the presence of substances affecting subcellular structures.

We consider it highly interesting that the shortest average cell doubling time was observed in the control cultivation (without serum and lyophilisate). This finding may indicate a certain inhibitory effect of *Pectinatella magnifica* lyophilisate on cell proliferation. This seems to correspond to the result that showed the lowest number of cell divisions in the 37/24 treatment. In contrast, the 37/1 treatment (1 h and 37 °C) had a stimulating effect on cellular mitotic activity. These observations also support the aforementioned possibility of the presence of substances acting on cell cultures and the need for their additional testing, and in particular the necessity of characterisation of these substances. The possible presence of bio-active substances in the colony can be among others related to the activity of algae and cyanobacteria in the matrix of *Pectinatella magnifica* colonies, as it is known that a broad spectrum of algae and cyanobacteria species can be found in the matrix corm (Joo et al. 1992).

The primary testing of biological material in cell culture is an essential step for detecting the presence of potentially bio-active substances. The next steps and procedures should be aimed at confirming the presence of bio-active substances in subsequent treatments, their possible identification and finally, comparing their effects with substances known from marine Bryozoa (among many sources e.g. Davidson and Haygood 1999; Davidson et al. 2001; Sharp et al. 2007; Ariza et al. 2011).

CONCLUSION

The bioassays conducted here indicate biological activity in substances present in the lyophilisate of matrix freshwater bryozoans *Pectinatella magnifica*. Lyophilisate which served as leachate in the growth medium influenced the growth of the murine fibroblasts cell line L929, as well as cell morphology and cell spreading after inoculation.

Following these results, further and more detailed testing and identification of potentially bio-active substances is needed. In Europe, *Pectinatella magnifica* shows invasive behaviour. It spreads to different types of waters which are used not only for fishing, but also for recreation and often as a source of drinking water. This makes the knowledge of its

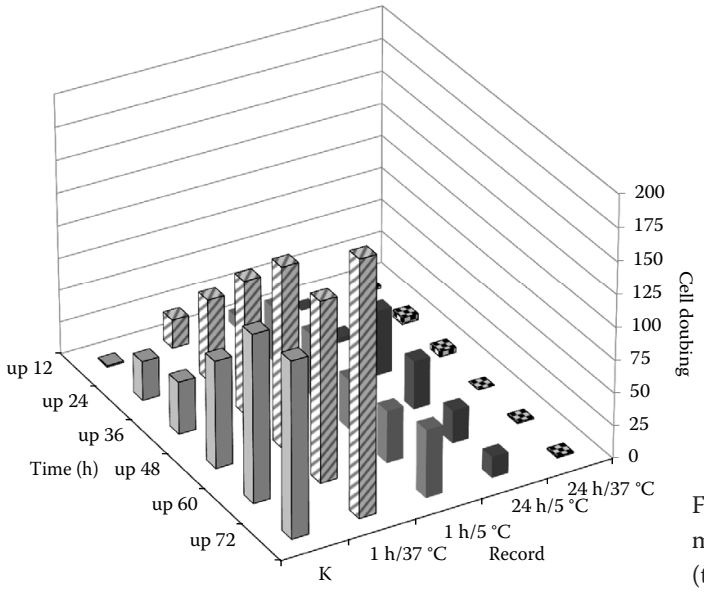

Figure 3. Mitotic activity of the heteronuclear murine fibroblast line L929 within 12-h intervals (total monitoring time: 72 h)

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potential impact on other organisms and on aquatic ecosystems highly topical.

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