Video Article

Bioindication Testing of Stream Environment Suitability for Young Freshwater Pearl Mussels Using *In Situ* Exposure Methods

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Abstract

Knowledge of habitat suitability for freshwater mussels is an important step in the conservation of this endangered species group. We describe a protocol for performing *in situ* juvenile exposure tests within oligotrophic river catchments over one-month and three-month periods. Two methods (in both modifications) are presented to evaluate the juvenile growth and survival rate. The methods and modifications differ in value for the locality bioindication and each has its benefits as well as limitations. The sandy cage method works with a large set of individuals, but only some of the individuals are measured and the results are evaluated in bulk. In the mesh cage method, the individuals are kept and measured separately, but a low individual number is evaluated. The open water exposure modification is relatively easy to apply; it shows the juvenile growth potential of sites and can also be effective for water toxicity testing. The within-bed exposure modification needs a high workload but is closer to the conditions of a natural juvenile environment and it is better for reporting the real suitability of localities. On the other hand, more replications are needed in this modification due to its high-hyporheic environment variability.

Video Link

The video component of this article can be found at https://www.jove.com/video/57446/

Introduction

The exposure of experimental organisms *in situ* with the subsequent evaluation of their condition is one possible way to get information about the environmental quality and (especially) the site suitability for a species. Within animals, such a bioindication is applicable primarily for small invertebrates which are able to live in a limited bounded space. Young stages of bivalves (Bivalvia) are one such suitable organism group[1].

Bivalves of the family Unionidae are a very important component of aquatic ecosystems[2]. However, these species are often critically endangered, especially in streams and rivers. Some of them are characterized as 'umbrella species' whose conservation is closely related to the conservation of the whole stream biotope and which require a comprehensive approach[3]. These animals have a life cycle associated with many environment components, from water chemistry[4,5] to changes in the populations of fish which serve as mussel larvae hosts[6]. Because mussel juveniles often represent a critical phase of the mussel life cycle, the site suitability for their development at this stage is crucial for a successful species population development in a locality.

The freshwater pearl mussel (FWPM, *Margaritifera margaritifera;* Unionida, Bivalvia) is a critically endangered bivalve occurring in oligotrophic European streams. Their numbers have fallen drastically during the 20th century across the occurrence area. It seems that the current decline in species reproduction in the majority of the central European populations is primarily caused by very low to zero survival of juveniles during the first few years of their life. It is assumed that juvenile FWPMs live for many years in the shallow hyporheic zone[7], of which the conditions and their variability are still not well described. Moreover, until their second year of life, the juveniles only have a dimension of up to about 1 mm, so they are very difficult to find in large volumes of sediment under natural conditions[8]. Therefore, experiments with captive juveniles are necessary for the study of their ecology.

Within the Czech Action Plan for Freshwater Pearl Mussel[9], there are thousands of juveniles rising every year from a semi-natural breeding program. Nevertheless, there is a question of which localities and habitats are suitable for successful population support by these juveniles or for eventual species reintroduction. *In situ* bioindications present a way of finding the answer.

Despite the fact that inconsistent survival rates of juvenile mussels in exposure cages were observed in some earlier works that questioned the suitability of juvenile mussels as bioindicators[10], several recent studies have confirmed the applicability of juvenile exposure methods for water

quality testing[11,12,13]. Additionally, it has been demonstrated that several factors need to be considered when interpreting the results of these particular studies, such as the stock origin[14] and the persisting effects of larval conditions[15].

The question arises of how to install experimental juveniles in tested localities and how to most effectively evaluate their condition. The first rigorous application of *in situ* exposure methods with juvenile FWPMs was published by Buddensiek[16]. Juvenile FWPM individuals were kept in sheet cages, exposed in the free-flowing water of streams, and their survival and growth were quantified after several weeks of exposure. The approach was originally developed as a semi-artificial breeding method, but the author also highlighted its applicability for the assessment of habitat requirements and water quality. Although the FWPM juvenile survival is naturally very low on a scale of months/years and only a very small number of animals will survive, the survival rate can be a good marker of the environmental effect on a scale of several weeks[16]. Over years of research, exposure methods were developed further to hold experimental juvenile mussel in-stream habitats and to evaluate their growth and survival rates; these include sandy boxes[17], mussel silos based on an upwelling principle[18], and various other exposure cages (summarized by Gum and colleagues)[11]. Because juveniles occur naturally in shallow hyporheic zone[7], the application of experimental devices within the stream bottom is very desirable.

In our article, we describe the use of two exposure devices for FWPMs: i) modified Buddensiek sheet cages ("mesh cages") also enabling bioindication testing in hyporheal conditions; and ii) Hruška sandy boxes ("sandy cages"). The protocol describes the application of both methods in open water and hyporheic conditions (*i.e.*, four variants of exposure are described). The methods were gradually modified and expanded over more than 15 years of application within the Czech Action Plan for Freshwater Pearl Mussel[9] and verified by a set of experiments.

Protocol

1. Mesh Cage

Note: See **Figure 1**.

1. **Prepare material**
    1. Prepare the material for the in-laboratory part of the experiment: ~1 - 2 L of river water per mesh cage, mesh cages (1 main plastic body, 2 plastic covers, 2 sheets of special technical sieves with 340 µm pores, 4 bolts and 4 nuts per cage), pliers, a spanner, Pasteur pipettes, a strainer, a digital camera, a trinocular dissecting zoom stereo microscope, a calibration grid (microscope equipment), 5 Petri dishes of 50 mm diameter, beakers, 2 plastic dishes (~25 cm x 15 cm x 3 - 5 cm), and a plastic box.
    2. To perform the hyporheal installation, prepare a rubber hose and a 100-µm-pore mesh, and a squirt bottle. For the construction of the device, see **Supplementary File 1: S.1. Mesh cages construction**.

2. Assemble the bottom and central part of the mesh cages. Assemble the part of the cage that holds the individuals. Insert one plastic cover first, then one sheet of the plastic sieve, and finally the main body on top. Use four bolts to secure it.
3. **Prepare biological material**
    1. Put the mesh cage into the plastic dish containing river water. Ensure that the chambers are half full. Take the FWPM juveniles (see **Supplementary File 1: S.6. Biological material**) out of the thermally-insulated box and put them in the Petri dish.
    Note: Ensure that sudden temperature changes do not exceed ~2 °C.
    2. Using a squirt bottle and strainer, sift through the juveniles to clear the detritus.

4. Set up the microscope and camera. Perform a calibration of the instruments (see **Supplementary File1: S. 5. Microscope and phototechnics**). Place a Petri dish containing a little water under the microscope.
5. **Put the juveniles into cages (experimental laboratory work)**
    1. Use a Pasteur pipette to remove one individual from a Petri dish and carefully place it in the Petri dish under the microscope.
    2. Check the individual's fitness by looking into the eyepiece (~40X magnification).
    Note: "Good" fitness signifies that the individual moves, rotates from side to side, pushes the foot out of the shell, *etc.* Remove dead or low fitness individuals with a Pasteur pipette and place them in a separate Petri dish (FWPM juveniles with an opened shell, no movement, the foot is not pulled out, a fragmented shell, juveniles who float uncontrollably in the water, a visible decomposition of the shell, partial decalcification).
    3. Take two photographs of an FWPM individual showing good fitness using a constant magnification of ~80X. See **Supplementary File 1: S.5. Microscope and phototechnics**. Save the photos.
    Note: For a good measurement of its length, the juvenile must be laid lengthwise (lateral view). The main goal is to take a high-quality picture of the maximum shell length good enough to enable a picture analysis afterward.
    4. Insert the juvenile into the appropriate chamber in the cage as soon as the pictures are taken. Record the numbers of the pictures and the chamber.
    5. Repeat this step with each individual for all the used chambers in the mesh cage.
    Note: see **Supplementary File 1: S.1. Mesh cages construction**.
    6. Once all the used chambers have pearl mussels, put the plastic sieve on the cage, then gently put the plastic cover on and secure all parts together with the nuts.
    7. In the case of an installation into a hyporheic zone, pass one of the hose ends through one of the chambers and fix it in this position, then take the anti-clogging mesh and bind it on the bottom end (see **Supplementary File 1: S.1. Mesh cages construction**).

6. **Store juveniles**
    1. Put the cage into the plastic box with the river water, so that the juveniles are fully immersed, and keep it in the thermobox. Before the installation, let the juveniles adapt to the *in situ* river water temperature at the place of installation (gradual cooling, max. 5 °C in 24 h).

7. **Install mesh cages**

1. Prepare the field material including the mesh cages with the juveniles, steel spikes, bolts and metal nuts, a spanner, field temperature dataloggers (see **Table of Materials** and **Supplementary File 1: S.4.2. Water measurement**), a string, a camera, the field protocol, a hammer, and a spade.
2. Transport the FWPM juveniles to the site in a field thermobox (insulated box), keeping a stable water temperature with variations < ~2 °C. Put the thermobox with the mesh cages into the river on the site to let the juveniles adapt to the local environmental conditions (pH, conductivity, *etc.*).
3. Install the mesh cage.
    1. Remove the mesh cage from the field thermobox. Provide it with two steel spikes and fasten the field datalogger. Anchor the cage into a habitat with conditions typical for FWPMs in the study area (*e.g.*, at the edge of the main stream flow, not in direct water flow, not in standing water, not in direct sunlight).
        1. For open water, using a pair of the steel spikes, fix the cage to the river bottom; lay it on its side and level with the river bottom, downstream at an angle of 45° to the river flow, towards the center of the river. The lower horizontal edge should be about 10 - 15 cm above the river bottom surface. Maintain a minimum distance of 2 m between each cage at one locality (see **Supplementary File 1: S.4. Cages maintenance**).
        2. For the hyporheic zone, dig the cages into the river bottom in a perpendicular landscape position, perpendicular to the stream of water, so that the upper horizontal edge of the cage is parallel to the river bottom surface and the chambers are located at the hyporheic depth which should be tested. Take out the upper end of the rubber hose above the bottom surface for the possibility of water sampling during the experiment (see **Supplementary File 1: S.4.2. Water measurement**).
        Note: It is recommended to perform regular checks and maintenance on the cages (see **Supplementary File 1: S. 4. Cages maintenance**).

8. Uninstall the cages and transport the juveniles after the exposure. For this, pull the cages out of the water, clear them of fine sediment as well as of drifted material and put them into the field thermobox filled with river water. Transport the cages immediately to the laboratory and start the mortality and growth rate evaluation.
Note: See **Supplementary File 1: S.3. Exposure duration**. In the case of a temperature difference of more than 5 °C between the cages and the laboratory environment, it is first necessary to let the temperature equalize.
9. Evaluate the experiment by checking the life/fitness of each juvenile (see steps 1.5.2 and 1.5.3) and take 2 images of each live juvenile in a Petri dish using a constant magnification of ~80X. Record the fitness and the numbers of the pictures and chambers.
10. **Complete the experiment (common to all methods)**
    1. Perform the measurements in image analysis software. Use image analysis software for the body size determination of every evaluated juvenile on both the input images (step 1.5.3) and on the output images (step 1.9). Use the maximum total shell length recorded in both photographs as body size values in both input and output.
    2. Insert the measured values into the table processor and calculate the growth increment (%) for each surviving juvenile.
    3. Estimate the survival rate (%) per mesh cage using the ratio of the number of surviving individuals to all experimental individuals in the mesh cage.
    Note: After the experiment, return the survivors to the breeding program (see **Supplementary File 1: S.6. Biological material**).

2. Sandy Cage

Note: See **Figure 2**.

1. **Prepare material**
    1. Prepare the material for the in-laboratory part of the experiment: 2 Petri dishes (diameter ~8.5 cm), Pasteur pipettes, a strainer, 25 L of river water, a plastic box, sieves (mesh size 1 and 2 mm), a big plastic box (25 L), a sandy cage (see **Supplementary File 1: S.2. Sandy cages construction**), a digital camera, a trinocular dissecting zoom stereo microscope, a calibration grid (microscope equipment), sorted river sand from the study area (see step 2.1.3), and the protocol. See **Table of Materials** and **Supplementary File 1: S. 2. Sandy cages construction**.
    2. Prepare the material for the isolation process: round containers (1 for each cage plus 1 extra), 2 Petri dishes (diameter ~14 cm), a Pasteur pipette, magnifying glasses, and 1 L of river water.
    3. Sift the river sand through a 2-mm sieve and then through a 1-mm sieve to get a grain size of 1 - 2 mm. Dry the sand and save it in a dry form until required.

2. Take the juveniles (see **Supplementary File 1: S.6. Biological material**) out of the thermobox and put them in the Petri dish. Using a squirt bottle and strainer, sift through the juveniles to clear the detritus.
3. Set up the microscope and camera (see **Supplementary File 1: S.5. Microscope and phototechnics**).
4. **Put juveniles into cages (experimental laboratory work)**
    1. Place the sandy cage in the plastic box. Scatter the sorted sand (see step 2.1.3) up to one-third of the height of the sandy cage. Pour water into the box. Ensure that the sand surface is about 10 mm below the water level. Insert the sandy cage into the 25 L box of river water and expose it to the same temperature as the juvenile FWPMs (see **Supplementary File 1: S.6.2. Storage of the biological material**) for 12 h. Avoid any exposure of the sand to sunlight.
    2. Take the Petri dish with the prepared FWPM juveniles.
    3. Check the individuals' fitness by looking into the eyepiece (see step 1.5.2).
    4. Perform the photographic documentation as follows. Take one picture of all individuals discovered (see step 1.5.3) and choose 10 of the largest individuals. Alternatively, take pictures of all juveniles together with low magnification (~40X) for a bulk evaluation and choose the 10 largest individuals. Save all the photos and record their numbers.

5. Using a squirt bottle, move the FWPM juveniles into the prepared sandy cage.

5. **Store juveniles**
   1. Put the cage into the big plastic box with river water so that the cage is fully immersed and keep it in the thermobox. Let the juveniles adapt to the *in situ* river water temperature (gradual cooling, max. 5 °C for 24 h) before the installation.

6. **Install sandy cages**
   1. Prepare the material for the field installation: sandy cages, a ~25-L field thermobox, a flat stone (minimal weight 1 kg), a net (mesh size 10 x 10 mm), a squirt bottle, field temperature dataloggers (see **Table of Materials** and **Supplementary File 1: S.4.2. Water measurement**), a spade, and the field protocol.
   2. Transport the cages with the juveniles to the site in the field thermobox, keeping a stable water temperature (~2 °C change). Put the field thermobox with the sandy cages into the river at the field site to let the FWPM juveniles adapt to the local environmental conditions (pH, conductivity, *etc.*).
   3. Install the sandy cages into habitats with conditions typical for FWPMs (*e.g.*, at the edge of the main stream flow in a meander, not in direct water flow, not in standing water, not in direct sunlight).
      1. For open water, fasten the sandy cages to a flat stone using a net and place it on the river bottom. Ensure that the larger side of the cage forms an angle of 45° with the flow.
      2. For Hyporheal, dig the cages into the river bottom perpendicular to the flow of water so that the cage lid is level with the river bottom surface.
         Note: It is recommended to perform regular checks and maintenance on the cages (see **Supplementary File 1: S. 4. 1. Site checks**).

7. **Uninstall cages and transport juveniles after exposure**
   Note: see **Supplementary File 1: S.3. Exposure duration**.
   1. Pull the cages out of the water, clear them of drifted material and put them into the field thermobox filled with river water.
   2. Transport the cages to the laboratory and start the mortality and growth rate evaluation.
      Note: In the case of a temperature difference of more than 5 °C between the cages and the laboratory environment, it is necessary to let the temperatures equalize.

8. **Separate FWPM juveniles from sand**
   1. Prepare a round container with a water depth of 50 mm (for each cage separately) and one extra round container. Transfer the sand from the cage into the round container. Use a swirling motion to wash out the lighter particles into an extra container.
   2. Sample the content from this container gradually and search for juveniles step-by-step using a Pasteur pipette and a magnifying glass. Put the juveniles in the Petri dish using the Pasteur pipette. Repeat this step until the last juvenile has been found and then another 10x after the first negative finding. After each wash step, add clean river water to the original container with sand.
      Note: Especially after the first washing out, properly examine the content and clean it of ballast such as fine sediment and other alluvia.

9. **Evaluate the experiment**
   1. Check the fitness of each juvenile (see steps 2.4.3 and 1.5.2) and count the number of survivors.
   2. Take a picture (see step 2.4.4.) of each individual separately, although this means there is no clear identity of each individual. Alternatively, take bulk photos and choose a subset of the 10 best-grown individuals from the final results.
      Note: Both possibilities have a similar reporting value. Possibility 1 has a limitation of a higher workload but also the benefit of the highest photo magnification and thus also greater accuracy.

10. **Complete the experiment**
    1. Perform measurements in image analysis software. Complete the experiment as done in the mesh cages (see step 1.10) with the following exception: do not evaluate the growth rate (%) of each juvenile but evaluate the group as a whole in the sandy cage experiment.
       Note: After the experiment, the survivors should be returned to the breeding program (see **Supplementary File S.6.1. Selection of a biological material**).

Representative Results

The four bioindication methods (open water sandy cages, within-bed sandy cages, open water mesh cages, and within-bed mesh cages) were applied to investigate the environment condition suitability for FWPMs in the upper Vltava River Basin (Bohemian Forest, Czech Republic). This river represents one FWPM residual locality within central Europe[19]. Here, we present a specially selected set of results illustrating the most important aspects of the four methods. Further details are described in a comprehensive study by *Černá et al.*[13].

The river environment was studied at two levels:

(I) A longitudinal river profile was represented by main stream localities (sites A - E) and tributaries of different pollution stages (sites R and V). The localities were tested both by sandy cages and by mesh cages installed in free-flowing water. In addition, a gravel hyporheic zone was tested by within-bed sandy cages in localities B, C, and D.

(II) A hyporheic environment was tested in the selected locality C. The suitability of different substrates (sand, gravel, stones) was tested by within-bed mesh cages.

The growth rate and survival rate of >1-year-old juveniles (see **Supplementary File 1: S.6. Biological material**) were tested. The experiment was carried out to its full extent in the summer of 2014 and was repeated to a smaller extent at some localities in the summer of 2015. Within level (I), 2 - 6 sandy cages with a minimum of 100 juveniles and 6 (2014) or 4 (2015) mesh cages with 6 juveniles were applied at each locality tested by the appropriate method. Within level (II), 7 mesh cages with 6 juveniles were installed in every tested environment. The exposure time was one month for the mesh cages and three months for the sandy cages.

The statistical analysis was conducted in R, version 3.1.0[20]. Kruskal-Wallis, Kruskal-Nemenyi, and Wilcoxon-Mann-Whitney tests were used. For data with a normal distribution, linear or quadratic regression was carried out.

The localities can be clearly distinguished based on the growth rate in the open water mesh cages despite the high within-cage variability, even in different growth-favorable periods (**Figure 3**). In the more growth-favorable exposure in 2015 (growth rate 19.3 - 41.8%), a significant trend was discovered in the longitudinal profile where the growth rate increased downstream (Kruskal-Wallis test, $p < 0.001$). Importantly, the survival rate was equivalently high in both seasons (from 83%) (**Figure 4A**).

On the other hand, the open water sandy cages showed a different trend between the main stream localities in 2014: the growth rate increased downstream from locality A (52%) through the middle locality C (153%), and thereafter decreased again until locality E (46%) (a quadratic regression of absolute growth values: $r^2_{adj} = 0.77$, $F_{2,13} = 25.66$, d.f. = 16, $p < 0.001$). This trend was also confirmed in 2015 when the greatest growth rate was recorded at the middle locality C again. Also, the absolute growth rate values did not differ much between 2014 and 2015. On the other hand, the survival rate differed between the years, being much higher in 2015 (from 48% to 72%) than in 2014 (about 25%) (**Figure 4B**).

An effect of two different exposure methods is also clearly visible in the polluted tributary (locality V). The sandy cages exposed here during the three months showed 0% survival, while an 83% survival rate with some growth was recorded by open water mesh cages exposure here during the 30 days.

Results from the within-bed sandy cages illustrate different conditions in the hyporheic environment in comparison to open water in the relevant localities. The growth rate was always lower in the hyporheal sites than in open water, and the survival rate was much more variable (from almost 50% to 0%, **Figure 4B**).

A study of hyporheic microhabitats using the within-bed mesh cages showed a significant effect of the substrate composition on juvenile survival. The best conditions were recorded from the oxygen-saturated stony bottom (a survival rate close to 100%) while the worst (a < 40% survival rate) were indicated in poorly oxygenated sand where a very high variability in surviving was also detected. Hyporheic water oxygenation, which was repeatedly measured during the experiment, explains this trend (**Figure 5**).

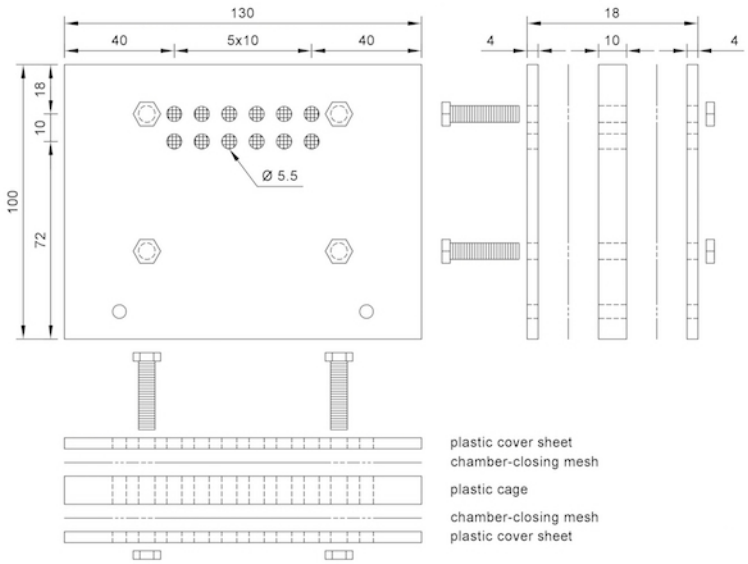

Figure 1. Bioindication mesh cage with individual chambers. See **Supplementary File 1** for further details. Please click here to view a larger version of this figure.

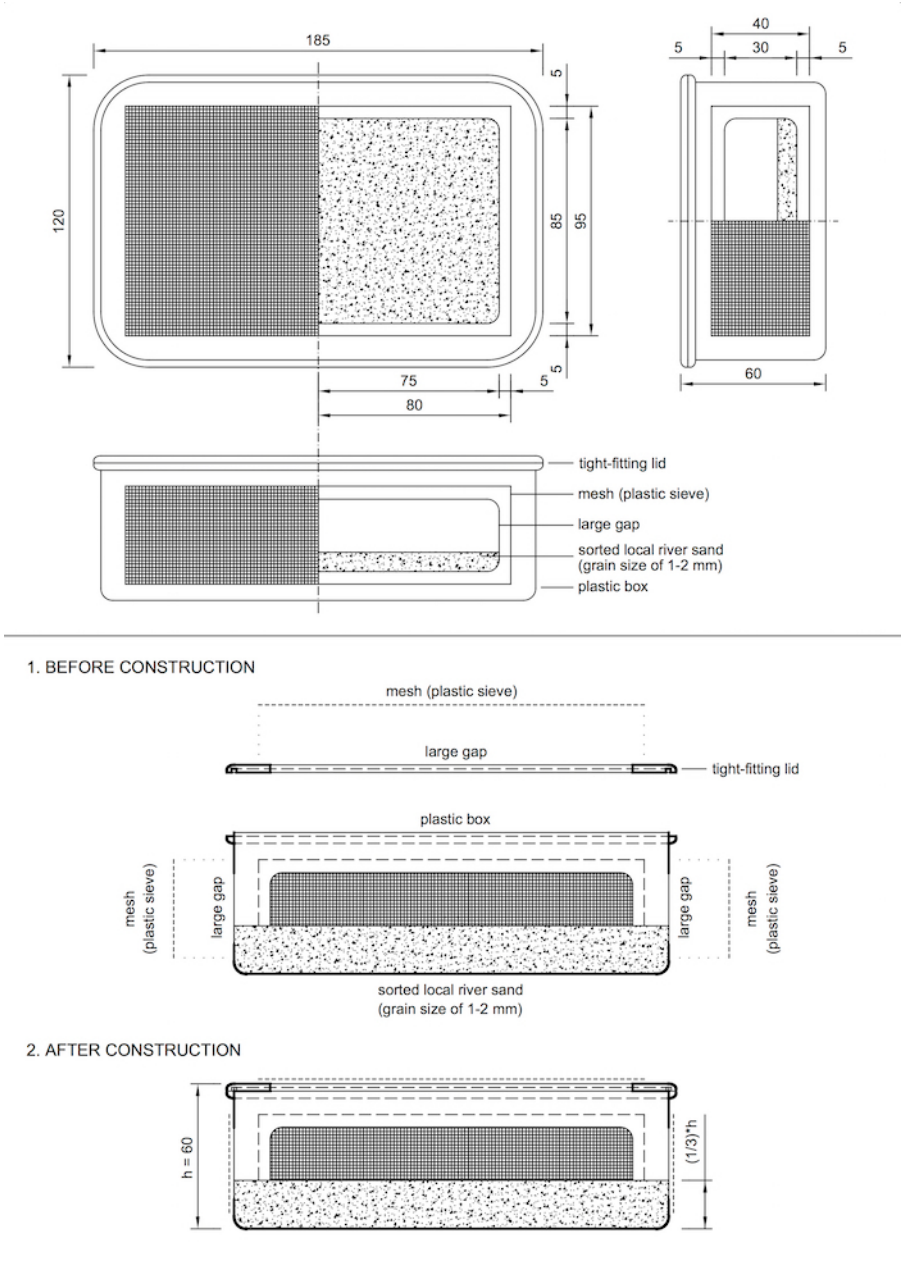

Figure 2. Bioindication sandy cage. See **Supplementary File 1** for further details**.** Please click here to view a larger version of this figure.

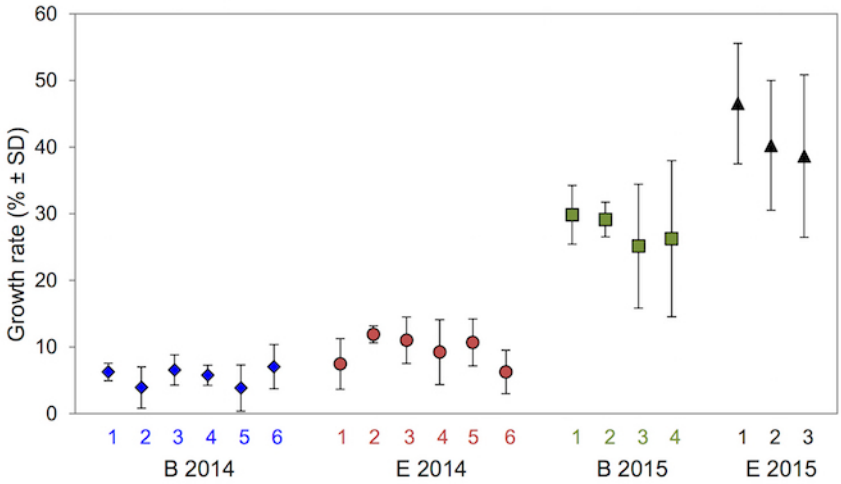

Figure 3. Individual variability in juvenile growth rate recorded by open water mesh cages in localities B and E during two seasons. The means and standard deviation are described for every mesh cage. The values are based on the measurement of 6 juveniles (or 4 - 5 juveniles if the mortality rate > 0%) in every mesh cage.

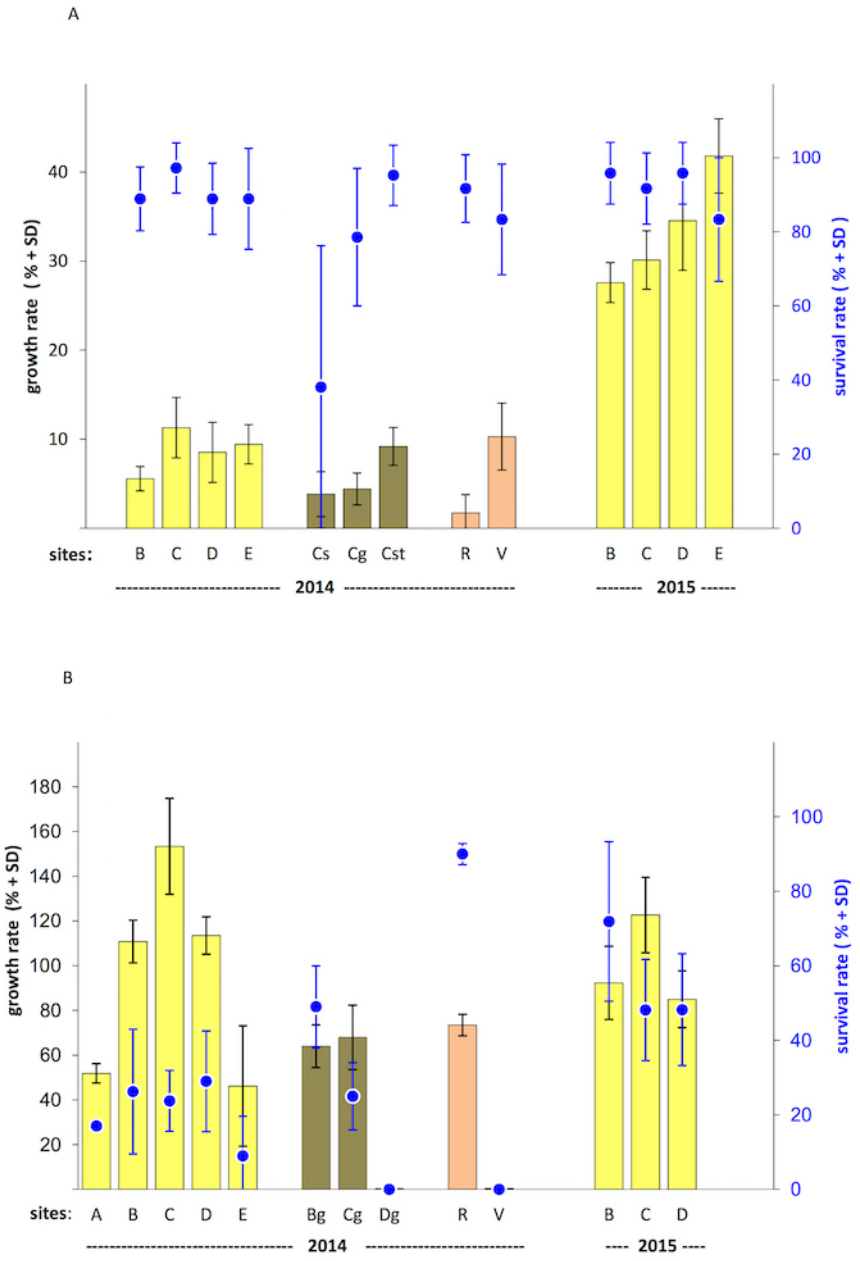

Figure 4. Example results from a field bioindication experiment with mesh and sandy cages. (**A**) This panel shows example results from a field bioindication experiment with mesh cages. A total of 6 localities (B, C, D, E, R, and V)within the Vltava River catchment were tested on 2 separate occasions (in 2014 and 2015). The exposure time was 30 days during the summer season. The Localities B - E represent (in order) a longitudinal profile of an approximately 20-km stretch of the river's main stream. Localities R and V represent profiles of 2 tributaries. Capitals mark the same locality both in panel (**A**) and (**B**). All localities were tested with open water mesh cages. In addition, locality C was also tested using within-bed mesh cages installed in 3 different types of river bed (Cs = sand, Cg = gravel, Cst = stones) in 2014. The cages were installed in 4 - 7 replications at every site. 6 freshwater pearl mussel juveniles of 1+ year old were used per mesh cage. The average growth rates are marked for the 3 largest individuals (3 MAX) from every tested mesh cage (columns, left axis) and the average survival rate per mesh cage (blue points, right axis). (**B**) This panel shows example results from a field bioindication experiment with sandy cages.A total of7 localities (A, B, C, D, E, R, and V)within the Vltava River catchment were tested over on 2 separate occasions (in 2014 and 2015). The exposure time was 3 months during the summer season. Sites A - E represent (in order) a longitudinal profile of an approximately 30-km long stretch of the river's main stream. Sites R and V represent profiles of 2 tributaries. Capitals mark the same locality both in this and in the previous panel. All localities were tested with open water sandy cages. In addition, localities B, C, and D were also tested using within-bed sandy cages installed in gavel river bed substrate (Bg, Cg, and Dg) in 2014. The cages were installed in 2 - 4 replications at every site. At least 100 freshwater pearl mussel juveniles were present in every sandy cage. The average growth rate for the 10 largest individuals (10 MAX) from every tested sandy cage (columns, left axis) and the average survival rate per sandy cage (blue points, right axis) are marked. Please click here to view a larger version of this figure.

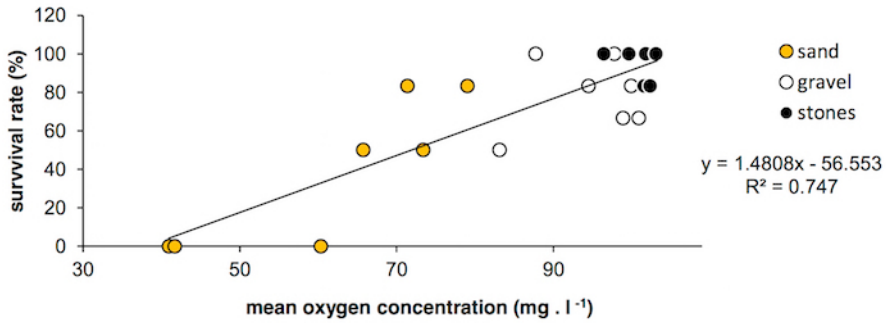

Figure 5. Oxygen saturation. This panel shows the relationship between minimal values of oxygen saturation over 30 days of mesh cages exposure and the surviving rate per cage in within-bed mesh cages exposed in different bed microhabitats in 2014. Please click here to view a larger version of this figure.

	2014		2015	
locality	**3-month exposure of sandy cages**	**1-month exposure of mesh cages**	**3-month exposure of sandy cages**	**1-month exposure of mesh cages**
A	13.9	-	-	-
B	14.4	13.4	13.9	17.5
C	15	13.8	14.4	18.3
D	15	13.8	14.3	18.3
E	15.5	14	-	18.7
R	13.5	12.8	-	-
V	14	13.2	-	-

Table 1. Average surface water temperature (°C) at the localities during exposure in 2014 and 2015.

Discussion

Exposure time:

Even one-month exposed mesh cages show a visible growth reflecting differences between localities (**Figure 3**), so they are very usable for the quick and easy detection of a locality characterization. Nevertheless, the relevance of the results depends on the short-term state of the conditions, which can oscillate. In particular, short rainfall events can play a role. In contrast, unpredictable episodic pollution may not always be recorded. In locality V (**Figure 4A**), water chemistry analysis detected a short wave of strong ammonium increase[13]. This was probably responsible for the mortality in the three-month exposed sandy cages but did not affect the 30-day exposed mesh cages.

Temperature fluctuations can also affect short-term exposure results. The one-month average temperature during mesh cage exposure differs between the years (**Table 1**). The growth rate also varies where higher temperatures were accompanied by higher growth rates (Kruskal-Wallis test $p < 0.001$). On the other hand, the average water temperature at the same localities during the three-month sandy cage exposure was very similar in both years (**Table 1**) and the growth rate did not differ significantly (**Figure 4B**).

Benefits and failings of the described methods:

An open water exposure is relatively easy to perform but is of limited value for habitat bioindication. The method of open water mesh cages is relatively old[16] and has been repeatedly used with minor modifications[10,11,12,13,21,22,23]. Nevertheless, these cages are not limited by oxygen, whose deficiency is probably responsible for many juvenile deaths in hyporheic conditions. Thus, open water mesh cages can show good development even in localities with increased mortality and a declining growth rate in open water sandy cages (locality E) or a 100% death rate in within-bed sandy cages, as at locality D in 2014 (**Figure 4B**). Apparently, the open water mesh cages show locality growth potential, but this may not be realistic as it is dependent on the real availability of hyporheic microhabitats within a locality. Because the open water mesh cages have the ability of high survival (**Figure 4A**), even up to a 100% survival rate[13], they can serve well for the bioindication of chronic toxicity (or acute toxicity if it is expected at a given time). Also, they can be a useful food source presence testing to some extent.

As a new and uncommon method, the open water sandy cages better simulate hyporheic habitat conditions. Movement of the juveniles between sand grains is possible in this apparatus, which helps to reduce the biofilm growth on the juvenile shell. A hyporheic oxygen deficiency can be caused by the activity of microbes colonizing the sand grains; this effect can also partially occur in cages placed above a river bottom. Nevertheless, due to the necessary periodic cleaning of clogging drifting material from a cage, fine sediments are also removed and thus the conditions are changed in comparison to the natural hyporheic habitat. So, the growth rate can also be considered as locality growth potential in open water sandy cages. However, this is closer to real locality suitability than in open water mesh cages. Therefore, the longitudinal growth rate gradients recorded by sandy cages (**Figure 4B**) also seem to be more plausible and indicate a more suitable river stretch. Moreover, in sandy cages, the possibility of juveniles and subadults breeding up to sexual maturity is verified[9], so sandy cages can serve as a safe breeding and biomonitoring method simultaneously.

Sandy cages and mesh cages placed in the within-bed position are closest to the real conditions in a shallow hyporheal. By allowing a juvenile's movement, sandy cages, in particular, offer them both a vertical and horizontal gradient of several centimeters in scale. This ability to move could be very important for escaping from temporary oxygen-deficient micro-zones. This possibility is absent in the within-bed mesh cages. Therefore, a relatively high number of bioindication units is necessary, because the hyporheic conditions are very variable[13,24] (**Figure 5**) and losses due to an unsuitable location are common.

In summary, the bioindication methods used in this research correspond with presumed juvenile natural conditions in the following order:
1. open water mesh cages,
2. open water sandy cages,
3. within-bed mesh cages,
4. within-bed sandy cages.

The workload per unit increases in the same order. Moreover, the juvenile number required for a statistical testing of the results obtained increases in within-bed exposures too. It seems that the within-bed sandy cages represent a more expensive but accurate bioindication method. This new method needs more testing in the future and comparison with other types of hyporheic studies based on the piezometer measurements[25,26]. In particular, there is a need to study the degree of similarity using a direct probe measuring physicochemical conditions in the cages and in the surrounding hyporheic environment.

The number of individuals measured in one cage:

Compared to mesh cages, it is not possible to measure the growth increment of specific juveniles in sandy cages as there is no information on which individual from the input set is which one in the output. It is necessary to work with an average value. If counted for all the individuals, this value can be very low due to a number of very slowly growing specimens; however, a couple of individuals can grow very quickly (growth jumpers). Such uneven growth is typical for mussels[27]. The growth variability among juveniles rises with increasing exposure time and big differences can occur, especially in growth-favorable seasons. Also, a long exposure leads to major mortality rates in the mesh cages (for a review see Lavictoire, Moorkens, Ramsey, Sinclair, and Sweeting[28]), so we can work with a significantly lower number of individuals at the end of the experiment compared to the input juveniles' set. Measuring only the several best-growing juveniles is a possible method.

The experience of FWPM breeding within the Czech Action Plan for Freshwater Pearl Mussel[9,29], as well as the results from experiments on sea bivalves[30,31], suggests that growth-deficient juvenile bivalves have a high mortality rate, and there is only a negligible chance of their living to maturity. In contrast, growth jumpers have a higher survival rate and they are crucial for a population recovery. The parameter 10 MAX (the 10 most-quickly growing individuals) takes the growth jumpers into account and can increase the informative value of the experiment, even if high mortality takes place (**Figure 4B**, season 2014). It should be noted that the growth estimate obtained by this method cannot be a false positive. It may only be slightly underestimated because many of the largest juveniles at the end of the experiment would have grown a little bit more in this case. Also, the workload is less if only 10 individuals are evaluated. Similarly, a measurement of three maximally growing individuals (3 MAX) proved to be appropriate in mesh cages, eliminating the influence of slowly growing, non-perspective individuals, which could bias the real image of site growth potential.

Disclosures

The authors have nothing to disclose.

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References

1. Goldberg, E.D. The mussel watch-a first step in global marine monitoring. *Marine Pollution Bulletin.* **6** (7), 111-114 (1975).
2. Vaughn, C.C. Ecosystem services provided by freshwater mussels. *Hydrobiologia.* In Press (2017).
3. Lopes-Lima, M. *et al.* Conservation status of freshwater mussels in Europe: state of the art and future challenges. *Biological Reviews.* **92** (1), 572-607 (2017).
4. Strayer, D.L., Malcom, H.M. Causes of recruitment failure in freshwater mussel populations in southeastern New York. *Ecological Applications.* **22** (6), 1780-1790 (2012).
5. Douda, K. Effects of nitrate nitrogen pollution on Central European unionid bivalves revealed by distributional data and acute toxicity testing. *Aquatic Conservation: Marine and Freshwater Ecosystems.* **20** (2), 189-197 (2010).
6. Modesto, V. *et al.* Fish and mussels: importance of fish for freshwater mussel conservation. *Fish and Fisheries.* In Press (2017).
7. Bauer, G., Wächtler, K. Ecology and evolution of the freshwater mussels Unionida. *Ecological Studies.* **145**, 1-394 (2001).
8. Neves, R. J., Widlak, J.C. Habitat ecology of juvenile fresh-water mussels (Bivalvia, Unionidae) in a headwater stream in Virginia. *American Malacological Bulletin.* **5,** 1-7, (1987).
9. Švanyga, J., Simon, O. P., Mináriková, T., Spisar, O., Bílý, M. *Záchranný program pro perlorodku říční v ČR (Action plan for the endangered freshwater pearl mussel in the Czech Republic).* (in Czech). NCA CR. Prague, Czech Republic (2013).
10. Schmidt, C., Vandré, R. Ten years of experience in the rearing of young freshwater pearl mussels (*Margaritifera margaritifera*). *Aquatic Conservation: Marine and Freshwater Ecosystems.* **20** (7), 735-747 (2010).

11. Gum, B., Lange, M., Geist, J. A critical reflection on the success of rearing and culturing juvenile freshwater mussels with a focus on the endangered freshwater pearl mussel (*Margaritifera margaritifera L.*). *Aquatic Conservation: Marine and Freshwater Ecosystems.* **21** (7), 743-751 (2011).
12. Denic, M., Taeubert, J. E., Lange, M., Thielen, F., Scheder, C., Gumpinger, C., Geist, J. Influence of stock origin and environmental conditions on the survival and growth of juvenile freshwater pearl mussels (*Margaritifera margaritifera*) in a cross-exposure experiment. *Limnologica.* **50**, 67-74 (2015).
13. Černá, M., Simon, O.P., Bílý, M., Douda, K., Dort, B., Galová, M., Volfová, M. Within-river variation in growth and survival of juvenile freshwater pearl mussels assessed by *in situ* exposure methods. *Hydrobiologia.*, In Press (2017).
14. Denic, M., Taeubert, J. E. , Geist, J. Trophic relationships between the larvae of two freshwater mussels and their fish hosts. *Invertebrate Biology.* **134** (2), 129-135 (2015).
15. Douda, K. Host-dependent vitality of juvenile freshwater mussels: implications for breeding programs and host evaluation. *Aquaculture.* **445**, 5-10 (2015).
16. Buddensiek, V. The culture of juvenile freshwater pearl mussels *Margaritifera margaritifera L.* in cages: a contribution to conservation programmes and the knowledge of habitat requirements. *Biological Conservation.* **74** (1), 33-40 (1995).
17. Hruška, J. Experience of semi-natural breeding program of freshwater pearl mussel in the Czech Republic. *Die Flussperlmuschel in Europa: Bestandssituation und Schutzmaßnahmen.* Kongressband. WWA Hof. Albert-Ludwigs Universität: Freiburg, 69-75 (2001).
18. Barnhart, M.C. Buckets of muckets: a compact system for rearing juvenile freshwater mussels. *Aquaculture.* **254** (1), 227-233 (2006).
19. Simon, O. P., Vaníčková, I., Bílý, M., Douda, K., Patzenhauerová, H., Hruška, J., Peltánová, A. The status of freshwater pearl mussel in the Czech Republic: several successfully rejuvenated populations but the absence of natural reproduction. *Limnologica.* **50**, 11-20 (2015).
20. R Core Team. *R: A language and environment for statistical computing.* [<https://www.r-project.org/>]. R Foundation for Statistical Computing. Vienna, Austria (2013).
21. Hastie, L. C., Yang, M. R. *Conservation of the freshwater pearl mussel I: captive breeding techniques.* Conserving Natura 2000 Rivers Conservation Techniques Series No. 2, Natural England. Peterborough, UK (2003).
22. Hruška, J. Nahrungsansprüche der Flußperlmuschel und deren halbnatürliche Aufzucht in der Tschechischen Republik. *Heldia.* **4** (6), 69-79 (1999).
23. Scheder, C., Lerchegger, B., Jung, M., Csar, D., Gumpinger, C. Practical experience in the rearing of freshwater pearl mussels (*Margaritifera margaritifera*): advantages of a work-saving infection approach, survival, and growth of early life stages. *Hydrobiologia.* **735** (1), 203-212 (2014).
24. Braun, A., Auerswald, K., Geist, J. Drivers and spatio-temporal extent of hyporheic patch variation: implications for sampling. *PLoS ONE.* **7** (7), e42046 (2012).
25. Franken, R. J. M., Storey, R. G., Williams, D.D. Biological, chemical and physical characteristics of downwelling and upwelling zones in the hyporheic zone of a north-temperate stream. *Hydrobiologia.* **444,** 183-195 (2001).
26. Roley, S. S., Tank, J. L. Pore water physicochemical constraints on the endangered clubshell mussel (*Pleurobema clava*). *Canadian Journal of Fisheries and Aquatic Sciences.* **73** (12), 1712-1722 (2016).
27. Larson, J. H., Eckert, N. L., Bartsch, M. R. Intrinsic variability in shell and soft tissue growth of the freshwater mussel *Lampsilis siliquoidea. PLoS ONE.* **9** (11), e112252 (2014).
28. Lavictoire, L., Moorkens, E., Ramsey, A. D., Sinclair, W., Sweeting, R. A. Effects of substrate size and cleaning regime on growth and survival of captive-bred juvenile freshwater pearl mussels, Margaritifera (Linnaeus, 1758). *Hydrobiologia.* **766** (1), 89-102 (2015).
29. Hruška, J. Experience of semi-natural breeding programme of freshwater pearl mussel in the Czech Republic. In: *Die Flussperlmuschel in Europa: Bestandssituation und Schutzmassnahmen.*, Ergebnisse des Kongresses vom 16.-18.10.2000 in Hof, 69-75. Wassereirtschaftsamt Hof, Germany (2000).
30. Bayne, B. L. Physiological components of growth differences between individual oysters (*Crassostrea gigas*) and a comparison with *Saccostrea commercialis. Physiological and Biochemical Zoology.* **72** (6), 705-713 (1999).
31. Tamayo, D., Azpeitia, K., Markaide, P., Navarro E., Ibarrola, I. Food regime modulates physiological processes underlying size differentiation in juvenile intertidal mussels *Mytilus galloprovincialis. Marine Biology.* **163** (6) (2016).

