# The Giant Freshwater Pearl Mussel *(Margaritifera auricularia)* Handbook

# Volume 2 – Technical Manual: Monitoring, artificial reproduction, rearing techniques, and suggestions for habitat conservation

coordinated by Karl M. Wantzen and Rafael Araujo

with contributions by Joaquin Soler, Catherine Boisneau, Nina Richard, Philippe Jugé, Yann Guerez, Laure Morisseau, Michèle De Monte, Keiko Nakamura and Vincent Prié

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**This publication should be cited as follows:**

Wantzen K. M. and Araujo R. (eds, 2018): The Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) Handbook Volume 2 – Technical Manual: Monitoring, artificial reproduction, rearing techniques, and suggestions for habitat conservation with contributions by Karl M. Wantzen, Rafael Araujo, Joaquin Soler, Catherine Boisneau, Nina Richard, Philippe Jugé, Yann Guerez, Laure Morisseau, Michèle De Monte, Keiko Nakamura and Vincent Prié
University of Tours, France, CNRS UMR CITERES, LIFE+ project 13BIO/FR/001162 „Conservation of the Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) in Europe". Tours (France) 109 pp.
Frontispiece: *Margaritifera auricularia*, photographed (c) by Philippe Jugé

This publication was conducted within the scope of the LIFE project 'LIFE13 BIO/FR/001162 Conservation of the Giant Pearl Mussel in Europe' with the contribution of the financial instrument LIFE of the European Union. This paper has been produced under the auspices of the UNESCO Chair "Fleuves et Patrimoine / River Culture".

**Outline:**

The Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) is one of the rarest invertebrate species worldwide. This two-volume book aims to bring together all the so far available information on the species. Both volumes are independent books, although they can be seen as complimentary, giving scientific and technical information. They result from work by the authors in the context of the LIFE+ project 13BIO/FR/001162 „Conservation of the Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) in Europe“ and include additional work by the contributors from other conservation and research projects.

Volume 2 is a manual that focuses on the practical aspects. It delivers hands-on information on how to find then animals in the field, how to identify them, how to study their habitats, how to prepare and run a laboratory for artificial reproduction and rearing, informs about release techniques, as well as descriptions of methods how to reinforce populations by infesting and releasing alternative host fish, or how to preserve mussel habitats. For a review of the literature on the species, please refer to Volume 1.

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## Preface:

The animals we are dealing with here have colonised planet Earth very long time before the advent of the human species or even before the first primates. The Family of Margaritiferidae is considered an ancestral group of freshwater mussels and apparently diverged from Unionidae at a minimum of 230 million years (Curole & Kocher, 2002, see vol. 1), while the eldest primate fossils date about 44 million years and our own species, *Homo sapiens*, occurred only 0.2 million years ago. Currently, we are undergoing the 6$^{th}$ wave of mass extinction, which has an unprecedented pace and which is definitively caused by the human species (Ceballos et al. 2017). With few exceptions (mostly humans and domesticated mammals and birds), practically all larger animal species are going towards extinction.

The Giant Freshwater Pearl Mussel (*Margaritifera auricularia*, abbreviated GFPM), is attained by this trend twofold: It is a large species (among the largest and heaviest known continental invertebrates), and depends on generally large and migratory fish species for its reproductive cycle. Previously common in all large European Rivers, it has become one of the rarest invertebrate species worldwide (Prié et al. 2018). Most of the extant populations are composed by residual, old specimen, living in highly degraded habitats. Population numbers are shrinking at a terrifying pace, and the sturgeon populations, one of the best host fish of *M. auricularia* (see below and Volume 1), are almost extinct. The yet-surviving adults still release glochidia (for a detailed review on the ecology of the species, refer to Volume 1), but natural reproduction hardly ever takes place. Even with detailed field studies, no recent juveniles smaller than 5 cm shell length were found.

The need to preserve the species from final extinction is obvious and mandatory for the current (human) generation. As long as there is no sign of natural reproduction, efforts have to be taken to produce viable juveniles, that may found future generations. However, it is also obvious that artificial reproduction is only an auxiliary measure to actions to re-establish environmental conditions, under which this flagship species for large European rivers and streams can thrive naturally. We have to gain time. The extant populations are quite old, and according to current knowledge on the age structure of the populations and longevity of about 80 years, we estimate that individuals of the currently existing populations may survive another 10-20 years. Water and habitat quality is continuously improving in European rivers, thanks to enormous efforts made in the context of the Water Framework Directive, although pesticides and emerging substances are still worrying (Malaj et al. 2014) and hydromorphological dynamics and

environmental flows definitively need to be improved. The re-establishment of fish populations after restoration in Europe is encouraging (Thomas et al. 2015), but it is variable for known and potential host fish of *M. auricularia*: for some species such as the sea lamprey it is quite successful (Beaulaton et al. 2008) but still in its infancies for the sturgeon species (Elvira et al. 2016). Thus, there is hope that if we make it to help this species survive and to provide new generations of juveniles for the next 2 or 3 decades, there is good reason to assume that the Giant Freshwater Pearl Mussel will continue to survive.

What do we have to do now? First, we have to know the requirements of the different stages of the life cycle of the Giant Freshwater Pearl Mussel in detail. This helps us to identify the weakest phases of the life cycle, which is currently the reproduction and recruitment of viable juveniles, but also the situation of the host fish populations. Volume 1 of this Handbook is dedicated to this task. Then, actions have to tackle these points. This booklet, a result of the LIFE+ project 13BIO/FR/001162 „Conservation of the Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) in Europe“, including the University of Tours and the Departement of the Charente-Maritime, in close cooperation with the Museo de Ciencias Naturales-CSIC in Madrid, the consulting agency Biotope, and the people working with the Giant Freshwater Pearl Mussel in Zaragoza, Spain, and the Freshwater Pearl Mussel (*Margaritifera margaritifera*) projects in France, Spain, and Germany, aims to summarise actions suggestions by the experts and to give practical advices how to perform them. We hope that the techniques described here will soon be improved by further research and active conservation projects that aim to maintain this impressive animal species alive on our planet.

Each chapter is introduced with a short contextualisation, then we deliver a description of the methods and give planning support about materials and time effort needed. Considering the urgency of the situation, the low number of yet available animals, the lack of information on the biological requirements of the species, and the low chance to repeat experiments (animals reproduce only once a year), we have set a special focus on avoiding drawbacks and mistakes, therefore, there is an own chapter on “pre-troubleshooting” and we have added a section on caveats in each chapter.

# 1. Conservation status of *Margaritifera auricularia* and legal restrictions to manipulate, sample, or transport animals

The conservation status of *M. auricularia* is CR (critically endangered, criterion A2ac), worldwide and in Europe it is listed on the Red Lists of IUCN as of 2010. The species is also listed on Appendix IV of the EEC Habitats Directive, which includes animal and plant species of European interest requiring strict protection, and on Appendix II of the Bern Convention, which includes strictly protected animal species and their habitats. It is protected by law in France (arrêté du 23 avril 2007 ; article 2) under the name *Pseudunio auricularius*. In Spain is protected under the Catálogo Español de Especies Amenazadas "en peligro critic de extinction".

As a consequence of this status, it is strictly forbidden to disturb or sample juveniles or adults of the species, or to modify their habitats, without previous permission. This has important consequences for all kinds of conservation actions, as any kind of contraventions against the conservation laws is subject to punishments, and will preclude the involved person or institution from future permits. Permits need to be demanded at the regional authorities for environmental conservation. These are in France, the Direction Départementale des Territoires (DDT), Service de l'Eau et des Ressources Naturelles and the Direction Regional de l'Environnement de l'Aménagement et du Logement (DREAL), or the Préfecture Départementale. In Spain the permits should be demanded to the Regional Governments: Gobierno de Aragón (DGA) through the Instituto Aragonés de Gestión Ambiental (INAGA), the Gobierno de Navarra Gobierno de La Rioja and the Generalitát de Cataluña. These authorities emit forms (in France they are called CERFA), which require a due explanation, how and why, e.g., adults of the species have to be sampled, how many of them in relation to the estimated total population size, how the animals will be further treated, and how and when they will be reintroduced. In our case, it was helpful that we could show that marked animals that had been temporarily used as glochidia-donors the year before, had not suffered any mortality (which is, of course, only possible from the second year onwards). Note that the permission procedure is also required for the transport of organisms (e.g. for infestation of electro-fished host fish, see chapter on this procedure), as well as the reintroduction of the reproduced juveniles into nature. Genetic analysis of the populations of these mussels are often done by extracting body liquid with a syringe, or by making biopsy from the foot muscle. These manipulations, too, require a full permission process. In the practice, permits are less and less often given, due to the fact that e-DNA samples can be taken without harming the animals. However, e-DNA samples contain degraded DNA, i.e. short fragments, that are generally not suitable for phylogenetic or population genetics, therefore,

sampling on living animals cannot always be avoided. Lastly, even dead parts of the mussels (shells) are protected by law, therefore even the demonstration of dead shells for environmental education or museum exposures requires permits.

Further note that the entire procedure to demand permits is very time-consuming, as the responsible evaluation teams often do not decide *ad hoc*, but rather wait for regular meetings, therefore, permits should be requested at least 6 months or better a year before the planned action. This may be difficult, as the precise moment of the sampling depends on the actual hydro-climatic conditions (we observed variation of about 3 weeks in France and 4 in Spain for glochidia release). Therefore we recommend to include an observation phase (e.g. diving observation of the maturity status of the adults) prior to the real sampling. This should be mentioned when demanding a permit.

Due to the fact that the species is considered as „extinct" in other countries (e.g., Germany), in case of a potential re-introduction, the species would need to be analysed prior to re-introduction in the same way as a non-native species, which may be quite a laborious procedure.

Theoretically, all habitats in which species belonging to the Appendix IV of the EEC Habitats Directive occur, are automatically protected. Both in France and in Spain the species is considered threatened with extinction by inclusion on the National Endangered Species List. A strict protection regime must be applied across their entire natural range within the EU, both within and outside Natura 2000 sites. In practice, this is not always the case, and landowners and communities are rarely informed. There is an option to protect mussel occurrence sites (at least, periodically during the reproduction season) by regional regulations, e.g., in France the so-called APPB (Arrêté Préfectoral de Protection de Biotope), however, we recommend to proceed very carefully here. Cases were reported, in which angry fisherman pulled out mussels (in these cases, shells of other species than *M. auricularia*) from a protected site in order to get rid of the legal constraints brought by the protected species. Moreover, the official declaration of occurrence sites of rare mussels may attract collectors and curious people who may inflict damages on the mussels (e.g. just by pulling them out of their habitat).

Glochidia developing in fish gills are theoretically protected, too, but they will hardly be detected in gills of caught fish. In so-called „Arch" projects, i.e. activities by which a subpopulation of mussels is held in a natural or nature-like river stretch, where fish are exposed to the glochidia under natural conditions, special care has to be taken to protect these fish from fishing.

## 2. Conservation status of host fish of *Margaritifera auricularia* and legal restrictions to manipulate, sample, or transport fish, including rules for manipulations with vertebrate animals

**Introduction:** Care must be taken when performing artificial reproduction of *M. auricularia* using host fish. The "classical" host fish species are threatened species by themselves and special regulations apply concerning capture, transport, manipulation or even killing of these fish (see previous chapter concerning procedures). The Atlantic Sturgeon (*Acipenser sturio* Linnaeus, 1758) is listed on Washington Convention Washington, ann. I, which strictly prohibits the trade of animals or plants listed in this annex, on Appendix II and IV of the EEC Habitats Directive, which includes animal and plant species of European interest requiring strict protection, and on Appendix II and IV of the Bern Convention, which includes strictly protected animal species and their habitats. It is also critically endangered according to the IUCN Red List for Europe (http://www.iucnredlist.org/details/230/0) and protected by law in France (Arrêté du 25/01/82). The same is true for "alternative" host fish, such as the sea lamprey (*Petromyzon marinus* Linnaeus, 1758), which is of least concern, but still regionally protected.

But even if the host fish were not protected by law, restrictions apply concerning their manipulation. All manipulations on vertebrates including fish require the preliminary evaluation of the planned experiments by an ethic commission for animal experimentation, then an authorisation for experiments must be demanded by the Ministry of Research (in France). In the case of experimental research, the establishment of an animal-testing facility (french: *agrément pour l'expérimentation animale*, German: *Tierversuchsanlage*) is mandatory. This establishment must dispose of a permit for hosting the experiments, which may be demanded and given by the Ministry of Agriculture. Cephalopods are so far the only invertebrates for which ethical restrictions for laboratory manipulations exist. Officially acknowledged fish-breeding stations may be exempted from establishing an official animal-testing facility, as they already possess the necessary permits. The legal framework on the European level is the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. European Union L276, 34–79. This framework finds its reflections in national legal procedures, e.g. in France the articles R214-87 à R214-137 of the Code Rural, which has been updated by the décret 2013-118 and five arrêtés published by the 7 February 2013 (the

responsible ministry is that of Agriculture), in Germany the Tierschutzgesetz (TierSchG) as of 13.7.2013. In Spain, local state laws apply.

**Method description**: These regulations have a series of consequences for artificial reproduction of *M. auricularia*:

a) All planned experiments must be described in detail in a document (in France: *Dossier d'évaluation éthique et d'autorisation de projet*, short DAP), which will be analysed by the reference institution (in France: *Comité d'éthique en Experimentation Animale local et Ministère de la Recherche*). In the DAP, the number of animals which will be used and the species of those animals (and explanations about the choice of the species) must be indicated. Experiments must follow the "3 R rule": *Replace* (it must be proven that no alternative procedure that does not harm is available), *Reduce* (it must be declared that the experiments are performed in a minimum number of animals), *Refine* (it must be shown that all procedures are adapted to the biological requirements of the experimental species in the best way possible). For some scientists, this procedure may counterintuitive to their experimentation routine, if they are used to adapt the experimental procedure during a series of experiments. The relatively long time required to plan experiments in advance (about 2 months) does not permit adaptations of the procedures according to recent results or insights from other work groups. Plans have to be made long time in advance, and they cannot be changed beyond the described procedures.

b) Origin of the animals (here: host fish). It must be assured that the animals come from a licenced breeder who warrants a sanitary quality of the animals. This is relatively easy when Siberian Sturgeon (*Acipenser baerii*) are used, as this species is commonly produced for caviar production (although we currently have found difficulties in finding bred sturgeon, due to infestation of several breeding stations with herpes-like viruses). Specific permit is needed if species are protected but exceptions exist, e.g., in France, sea lamprey is legally used for food and you can buy it by officially from fishermen.

b) Legitimacy of the experiments. It must be assured that the experiments are really necessary, and that no alternative options exist to replace them. Justifications include human or animal healthcare, legal medicine, or environmental protection, the latter of which applies in the case of *M. auricularia*. The number of manipulated and the number of killed animals must be reduced to a minimum, and the least disturbing/painful methods must be applied. Whenever possible, anaesthetics should reduce pain (which is not possible in the case of *M. auricularia*). This principle means in the case of host fish infestations with glochidia, that the fish gills are not overcharged with glochidia, or that aquaria must not be stocked

with too many animals. See protocols below for stocking and dosage recommendations. Even though the effects of glochidia infestation on fish are relatively well understood (Filipsson et al. 2017), it is still discussed whether infestations with glochidia is a painful procedure for the fish or not. A critical issue for work with freshwater mussels in general is that fish need to be starved before the mussel juvenile excyst from their gills. Starvation is considered a painful experience for the fish. The better the developmental time (in degree-days) is known the more precise the starving period can be set, i.e. to keep the fish under this stressful conditions as short as possible. Also concerning the period of the ending of the starvation period (and the ending of the juvenile collection), variations are possible. For experimental studies at the beginning of the routine work, it is important to cover the entire excystment period of several weeks. Once the dynamics of excystment (which follow a Gaussian distribution) are known, the collection of juveniles may be restricted to the peak period of excystment.

c) Licensing of the institution. Both breeders and animal-testing facilities must be previously licensed. This includes that adequate hygienic conditions are provided, that the personnel is sufficiently trained (both for conceptualising and for executing projects including manipulation of animals), and that a veterinary and a commission for the well-being of the animal have been appointed. In France, licensing is given by the ministry of Agriculture and a proven training of the executing technicians and scientists is needed to manipulate the animals.

d) Licensing of the experiments and regular inspections. The veterinarian appointed to the animal testing site and the ethical commission verify if experiments have been performed and documented according to the previously submitted and agreed-upon experimental plans.

e) Laboratory routines include that all experiments (and all visits in the animal-testing facility) are well-documented, high hygienic standards are maintained (fully desinfectable rooms, disinfestation of shoes when entering, use of gloves, laboratory coat, specific waste disposal and clean-up), and that emergency plans exist (e.g. which treatments may be used in case that fish become ill). This latter case is specifically difficult as the ethical rules (well-being of the fish) override the interest of the project (save as many lives of juvenile mussels) even if fish have to be killed at the end of the experiment (which can be avoided if an adequate structure, e.g. a public aquarium, takes over the fish).

Checklist of items that have to be informed in a DAP form:

- General information (name, duration and general description of the project, explanation how the “3R rule” has been considered)

- Administrative information (name and number of the licensed institution, name of the responsible(s) of experimentation and of the project, training status of the personnel)
- Description of the project (scientific justification, working protocol for the current period, species of the animals, numbers, explanation if, why and how animals become killed at the end of the experiment, description of the species names and their evtl. conservation status, information about origin of the animals (details about the breeder), number of animals used.
- Description of the experimental procedures (described individually, in our case: a) infestation with glochidia, b) starvation at the end of the metamorphosis of the juveniles, c) anaesthesia and gill check-up for identifying the developmental status, d) experiments that analyse factors to increase the encystment or the survival rates of the glochidia in the fish gills). The number of concerned fish is an important detail here. If fish breeders cannot deliver animals of the desired size, the relationship of fish number to aquarium/basin will be changed. This may have severe consequences for the resulting number of juvenile mussels. Moreover, fish species not described in the protocol must not be used (e.g., as surrogates).
- Each procedure must be classified concerning the impacts suffered by the animals (no incidence, light, moderate, or severe impact)

**Time effort:** As with the permits of sampling or manipulating protected animal species, obtaining the permits for laboratory experiments, training of personnel, and additional paperwork (e.g., documentation) are very time consuming. In our case, a full new animal-testing facility had to be implemented, which meant that 5 persons were trained for 1 or 2 weeks, a voluminous document had to be delivered, and for each candidate fish species a detailed worksheet had to be developed. The training occurs only once a year, and participants for the courses must be available full day during the training (which may be given in an institution far away, so travel efforts have to be envisaged). For future projects with *M. auricularia*, it is strongly advised to check the local rules and requirements several years prior to the planned experimentation. In the case of *M. margaritifera* projects, artificial reproduction and manipulations with fish are often linked to existing fish breeding stations that have previously been licensed.

## 3. Monitoring techniques to find *Margaritifera auricularia* in the field: where to find them? How to observe them in wadeable and deep running water systems? How to use e-DNA techniques? How to mark adults in the field?

**Preliminary remark:** All kind of interaction with the Giant River Mussel is subject to the conservation legislation (see chapter 1). None of the procedures described in the following may be performed without a special permit.

**Introduction:** *M. auricularia* is – in spite of its often impressive size – an extremely inconspicuous species. It thrives in deep and often turbid water in turbulent-flowing rivers. Contrary to its sister species *M. margaritifera*, the Giant River Mussel rarely occurs in dense "mussel beds" (this may have been the case prior to mass exploitation of mussels for mother-of-pearl in the 19$^{th}$ century), today, they are rather well distributed in the sediments, or occur in smaller groups. Due to their sedentary life style, the black mussel shells often become overgrown by biofilms, and the siphons and other visible soft parts have a brown colour, thus, the adult animals are well camouflaged. Juveniles are even more difficult to observe, as they pass a phase of yet unknown duration in the hyporheic interstitial of the sediments, which makes it almost impossible to observe them without applying sediment sampling/coring strategies. Adults, too, hide themselves in the sediments temporarily, resulting in strongly variable counts of animals from one year to another (R. Araujo, V. Prié, K. Nakamura, pers. obs.). In Spain, the smallest juvenile found on the surface was 5 cm long (K. Nakamura, pers. obs.). The behaviour of the animals to penetrate the hyporheic interstitial deserves further studies. We do not know yet to which extent it is limited by sediment grain size in relationship to body size, by which environmental cues it is triggered, and if it serves as a mechanism to shear off biofouling from algae or *Dreissena polymorpha* mussels, which have been shown to kill other unionoid mussels by clogging and starving them.

Under these circumstances, it is no wonder that many populations have passed unperceived for many years, or have become extinct without being registered! The most conspicuous part of *M. auricularia* is its whitish inner shell from dead animals, which can be easily identified in the field due to its impressive size, its thickness and singular shape (see chapter on identification in Volume 1). To find live animals, we propose 3 different techniques (see below). For a comparison of the methods and examples of their application if differently sized rivers see Prié et al. (2018). Once the animals have been identified, they should be carefully marked if a follow up of the population monitoring is envisaged.

## 3.1 Field observation using the Aquascope (valid for shallow waters)

**Method description**: An aquascope is a bucket-like device with a glass bottom that allows to visualise the sediment surface of shallow (up to 1,5 m, depending on the turbidity of the water) water bodies. In a preliminary visit, the precise position of the sampling area should be recorded with a precision GPS, and a detailed map should be prepared (and sealed in plastic). The aquascope can be hand-held while walking or sitting in a boat (fig. 1). Its disadvantage is that the breadth of the observed bottom strip is very limited (ca 1m), thus either a single person needs extensive working time, or several persons observe the stream bottom in line. Walking in streams and their riparian zone causes environmental impact, and should be limited to a minimum. Inattentive persons risk to crush mussels, rip off vegetation and destroy habitats.

When the Aquascope is used while walking on the sediments, extreme care has to be taken not to disturb the sediments or the riparian vegetation by trampling. The walking direction should always be against the current (i.e. field workers have to return to the lower end of the studied section by walking outside the stream, also taking care not to disturb the riparian zone), as mobilised fine sediments blur the underwater view at the downstream sites. We suggest to make line transects across the stream with 1-2 m distance of 10-20m long sectors to be checked. Safety instructions apply (protection against cold using neoprene suits or waders, sun protection, buoyancy devices and safety ropes if the current is strong). The operator bias in identifying mussels at the stream bottom is very high between more or less experienced persons, therefore training and/or repeated observations with changing personnel is recommended. The position of the mussels is noted on prepared field protocols and on a plasticized map.

For additional habitat analyses, it is recommended to record water depth, water velocity (surface, 60% depth, near bottom), sediment structure (estimation of grain sizes according to Wentworth classes), photographs (evtl. computerized grain size analysis), or even selected sediment sampling (see chapter on habitat structure in Vol. 1).

**Material needed:** 1 Aquascope per person, Neoprene suit or waders, safety gear, metal sticks (ca. 1,5 m) to mark the line transects, hammer, metric tape, plastic rope with knot marks every meter, precision GPS, detailed, plasticized map, waterproof pens, waterproof notice board. Aquascopes cost 30-50€ per unit and can be purchased online. They are also offered as “bathyscope”. Alternatively, they can be built from plastic tubes (diameter 30 cm) and Plexiglas disks.

**Time effort:** Strongly depends on experience of the operators, water turbidity and velocity. A rough estimate is 30 seconds per meter of checked stream bottom plus preparation time.

**Figure 1 Sampling *M. auricularia* at the Ebro River (Photo: R. Araujo)**

## 3.2 Field observation using SCUBA devices (valid for deeper waters)

**Description**: Diving allows to observe *M. auricularia* in deeper rivers with a longitudinal visibility of at least 1 m. Depending on the current, only downstream or downstream/upstream observations are possible. Safety instructions are even more important here, Manuals for Diving Safety in Scientific Diving (e.g., https://scripps.ucsd.edu/sites/scripps.ucsd.edu/ files/basic-page-scidive/field_attachment/ 2014/scidive-siopub-manualfordivingsafety-2012.pdf) should be consulted prior to planning the search strategy. A minimum rule is to have two divers under water, another safety diver ready to interfere on the boat, and one pilot (even if the boat was anchored!). Permits for diving must be obtained previously from the local environmental agencies *and* from the harbour authorities prior to operation, considering that anchoring and diving may interfere with navigation. The diving site must be duly signalised. Each country has its own legislation about diving at work. In France, only professional, licensed divers can go under water during working time. In the case of tour LIFE project, we made a partnership with diving associations, excepted for the wide-ranged survey conducted in France from 2015 to 2018, for which the private consultancy in charge of the project provided professional divers.

We suggest line transects of ca 20-50 m length at a distance of ca 2.5 meters, going along previously fixed plastic ropes on the river bottom (special care with the rope: avoid trapping the divers!). If the current is low, one diver can go upstream on the right side of the rope, then downstream on the opposite side, while a second diver does the opposite. The position of the mussels is noted on prepared

field protocols and on a plasticized map. Values are averaged. If large operator bias occurs, the observation has to be repeated. This procedure has proven to be successful in repetitive tests.

**Material needed:** 1 diving equipment per person, safety gear, metal sticks (ca. 0,5 m) to mark the line transects, hammer (short handle for under-water use), metric tape, plastic rope with knot marks every meter and every 5 meter, detailed, plasticized map, waterproof pens, waterproof notice board.

**Time effort:** Strongly depends on experience of the operators, water turbidity and velocity. A rough estimate is 30 seconds per meter of checked stream bottom plus preparation time.

## 3.3 e-DNA sampling

**Description**: Environmental DNA is becoming an increasingly common method to record the presence of even cryptic species, even in deep and turbid waters. The method has proven to be a successful tool to analyse presence/absence of *M. auricularia.* For a detailed review, refer to Bohmann et al. (2014). Basically, water sampling has to be made in a river section that is representative of the water flow of the entire river channel (in broader rivers, several samples may be taken in a cross section). Below tributary confluences, there is uncertainty about the origin of the genetic signal. Special care must be taken not to contaminate the samples (use of gloves and DNA-free glassware). Water can be filtered, we used 50 litres of water per sample. Ideally, water is sampled into a new, disposable plastic bottle. If water samplers are used, they need to be made DNA free prior to use (UV radiation, bleach or specific DNA removal kits), which must be removed themselves prior to action in order to avoid DNA destruction in the sample to be analysed. Autoclave treatment is not recommendable as DNA fragments remain. Samples must be stored on ice and shipped quickly to specialized laboratories, where they will be analysed by high-throughput DNA sequencing methods. Bohmann et al. (2014) suggest to collect 15 ml of water into a sample tube, add 1.5 ml of NaOAc & 33ml ethanol, and to store samples at -20 °C, then to extract and amplify DNA in the laboratory with Qiagen's QIAmp Tissue Extraction Kit and qPCR (quantitative polymerase chain reaction). DNA metabarcoding uses previously studied DNA libraries to determine what organisms are present (e.g. BLAST). Several enterprises are specialised in eDNA analysis. The SpyGen protocol consists in sampling about 25 litres of water, sampled using a peristaltic pump, repeated twice (see Valentini et al. 2016) for a similar protocol targeting fish species). For sampling, a DNA-free tube is fixed on the peristaltic pump, with one hand in the water and the other fixed on a specific DNA filtering capsule. The capsule is then sent to the lab and DNA is extracted from the filter and amplified in a rare DNA lab. A specific reference library was established for European bivalves (Prié et al. *in prep*.) allowing metabarcoding of all bivalve species from any sample.

**Material needed:** Gloves, water sampler (may be lowered from a bridge), ice box, sodium acetate, pure ethanol. Gloves, DNA filtering capsule, DNA-free sampling tube, peristaltic pump.

**Time effort:** The field work is quick (water sampling), however, when planning a sampling trip, consider that even ice-cooled samples must be transported quickly to the next freezer providing -20 °C. Water sampling takes 30 minutes (may be longer if the water is very turbid) and is repeated twice. Altogether, sampling a single site with only one pump takes about one hour and a half.

## 3.4 Marking techniques

**Preliminary remark:** For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume.

**Introduction:** Simple tags consist of simple marks that cannot be confused with natural erosion. Complex tags require special adhesion or application techniques. All specimens collected of *M. auricularia* will be identified with a plastic label with an unique number. This is the only way to know if we use different specimens for the laboratory propagation each year. Tag types include glue dots (for small juveniles), Hallprint tags and PIT tags (Patterson et al. 2018).

This technique is also useful for tagging juveniles released to the wild in order to differentiate natural juveniles for those released from the laboratory (see below). Juveniles can be tagged when they are larger than two cm.

Specimens can also be marked by engraving, both manually and mechanically. For this, the periostracum is engraved by using from basic knives to Dremel tools and laser engraving machines. For this engraving, remove the periostracum and enter the nacre but not so deep to perforate the shell. We recommend making these marks near the umbo where the shell is thickest.

**Method description**: Plastic tags (e.g., Hallprint or Adasa) can be purchased in the market. Use coloured superglue or tags marked with numbers and letters. We normally put them in the dorsal part of the left valve near the umbo. Alternatively, labels can be put in the central area towards the anterior part of the shell, where the shell is flat. If possible, you can stick labels on both sides of the shell in case one of them is lost. Once the label has been put on the shell, it is necessary to wait 1-2 minutes until the label is fixed.

If the shell is covered by dirt or algae, clean the area where the tag will be glued with rub and water and then dry the shell before placing the glue; do not use chemicals. For Dremel use a 3/32 to 1/8 inch

spherical burr bit. Laser engraving machines are flat-bed dot-matrix printers that can be adjusted to burn a groove in the shell, one dot at a time. A guide is used to fix the focal point of the laser in a particular plane (Patterson el al., 2018).

Code: Contrary to bird and mammal marking projects, there are no standardized rules yet. We suggest a 3-letter-code for the river (e.g., EBR for Ebro), followed by a four-digit number. These codes should be thoroughly noted, communicated to the responsible environmental agencies, and made available to other researchers. This helps to compare data (e.g. growth data) from different sites.

**Procedure details:** We normally use Super-glue which is a fast drying glue based in the cyanoacrylate, which is the best fixative with the water.

**Material needed:** Forceps. Gloves. Super-glue. Plastic labels with different numbers and/or letters. It is good to use different plastic colours for the different water sheds. For engraving: Dremel and/or laser engraving machines.

**Time effort:** The time needed to put a label is about 2-4 minutes.

**Caveats:** Although there are several adhesives in the market, we recommend the use of cyanoacrylate. Some adhesive labels can be unfixed with the time although normally they can stay for years. If adhesive tags are lost during our work, is preferable to use Dremel or laser engraving. PIT tags (passive integrated transported), although expensive, can be also used. For the PIT tags, the bibliography recommends epoxy glue. We have used cyanoacrylate to attach the transponders and many of them have been lost. Be careful in acidic waters where the shells can lose the periostracum. In this case, avoid marking by engraving.

4. Preliminary considerations about artificial reproduction and rearing of freshwater mussels, with special reference to *Margaritifera auricularia*

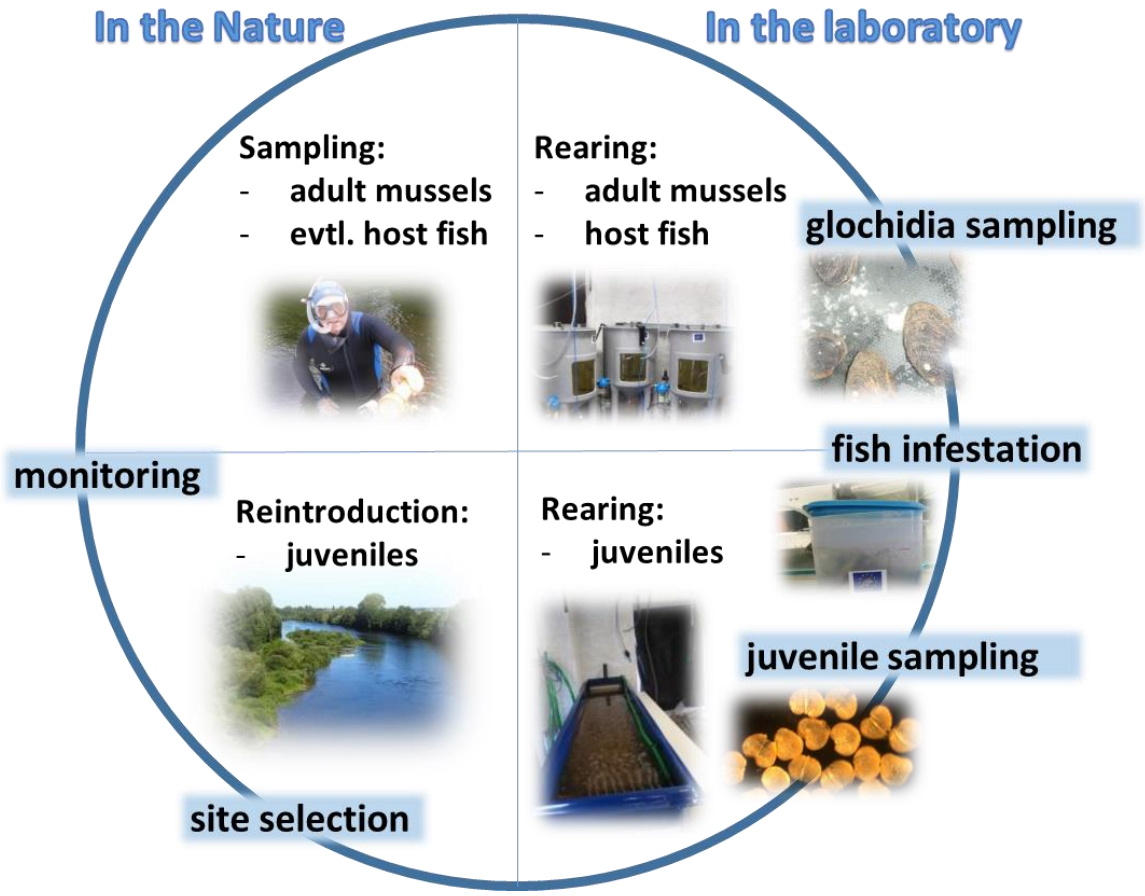


**Figure 2 Reproduction cycle in the laboratory, sampling and reintroduction of juveniles (Graph: C. Boisneau & K. M. Wantzen)**

**Remark:** Raising of juvenile mussels in the laboratory is a challenging task. The largest part of the life cycle of the animal needs to be copied (see fig. 2, Table 1), and all environmental parameters that are artificially introduced must correspond to the natural needs of the organism, concerning quality, quantity, and timing. A single mistake may set the success for a whole reproductive year at stake. Therefore, in the following, we prepare a table including all the phases of the reproductive cycle and their respective parameters (and details about mistakes to be avoided). Detailed information about the procedures is given in the following chapters.

Remember that not all published techniques for culture and propagation of freshwater mussels should work with all species. Every propagation facility is different, so a culture system that works well at one facility may not work well in another. Each mussel species also will have different culture requirements (food, flow, substrate,….), so a culture system that works well for one species may not work for another (Patterson et al. 2018).

**Table 1: Pre-trouble shooting: potential sources of non-success of artificial rearing of *M. auricularia* and suggestions how to avoid mistakes**

| **Life cycle stage/possible problems** | | **Possible solutions, parameters to control** |
|---|---|---|
| Gravid mussels... | | |
| | ... do not produce glochidia in time | Know the gamogenetic cycle of the species in order to optimise timing of sampling, make field survey before sampling,<br>record degree-days prior to glochidial release every year. Consider latitudinal differences (In the Ebro, Spain, glochidia release occurs in March-April, in the Charente, France it is April-May). Winter temperatures and the onset of spring may influence this time. Field observations help to find the best moment for sampling adults for reproduction.<br>Climate change (global warming) may be advancing the period of liberation of glochidia in the natural habitat. |
| | ... do not produce glochidia at all / in bad quality | Glochidia are released in a bell-shaped time pattern. Too early or too lately produced glochidia are of worse quality.<br>Prefer younger specimen. In Spain a release of glochidia has been observed in mussels beginning with a shell size of 10-11 cm length. Mark donor adults, record release of glochidia per donor over the years.<br>If only unfertilized eggs are recorded, instead of glochidia, some infertility problems may be occurring. |
| | ... eject pre-mature glochidial mass | Avoid stress (transport animals in moist tissues, careful cooling (no contact with ice, control temperature), offer substrate for donor adults so they can live in a natural (upright) position. Store adults been stored in water of their original habitat (transport and aquarium rearing) or if it is not possible, you should use water with similar origin and physicochemical parameters. Manipulate adults as little as |

| | | |
|---|---|---|
| | | possible. Avoid disturbances: sounds, lights and thermal stress (control day-night rhythm and maintain temperatures similar to the habitat of the species). |
| | | |
| Glochidia quality | | |
| | Glochidia of bad quality that are later not able to encyst in fish | Be careful with possible fungal infestation of the glochidia. This can be avoided by absolute cleanliness when sampling glochidia and by using only fresh glochidia. Control temperature: should be environmental temperature, do not store glochidia in the cold, minimum temp = 15°C and with continuous aireation in order to maintain them resuspended in the water. The use of the glochidia must be done the first 24 hours after being released. Up to a maximum of 48 hours they can be used but the more time passes, the capacity and effectiveness of the glochidia to get hooked on the fish host quickly decrease. Use new water and new glochidia for each fish batch. Otherwise, old glochidia attach to floating mucus and cannot be used any more. |
| | | Improve sampling method of glochidia (ideally, maintain the mussels in aquaria, the least possible time). There are two options to gain glochidia (see detailed method description in 6.3 Sampling of glochidia from gravid adults) |
| | | It is important not to feed the adult mussels while the glochidia release process lasts, because the incorporation of phytoplankton increases the densities of predators and parasites in natural water and also affect the glochidia quality. In fact a high density of these organisms can completely consume a batch of glochidia in less than 24 hours. |
| | | |
| Water problems in tanks of adult mussels and of host fish | | |
| | Too high nitrite/ammonia values | Reduce density, improve filter, exchange 1/4-1/3 of the water volume, let filter run at least 10 days before experimentation (“feed the filter” with fish food so that the bacteria responsible for denitrification can establish or add batches of nitrifying bacteria (available in aquarium stores)) |

| | | |
|---|---|---|
| | | Thumb rule: The water should ideally be the water of the natural environment (filtered by 75-100 µm before). |
| | Lack of oxygen | Ensure the oxygen saturation in the water is near saturation to avoid the premature release eggs in formation or immature glochidia.<br>In fish, symptoms such as taking puffs of air on the surface of the water or moving the gills very fast clearly indicate lack of oxygen in water. Use external aeration or place water pumps that produce a cascade effect. |
| | Not enough water in stock | Anticipate rain/flood events that might cause too high values of suspended solids or solutes. |
| | | |
| Infestation efficiency problems | | |
| | Infested fish absorb or eject cysts before metamorphosis of the juveniles | Fish develop immune responses to glochidial infestation, so ideally work with "virgin" fish that were never infested (not possible if working with wild specimen but you can fish in areas or rivers without freshwater mussels). |
| | Low rate of infestation | After the first infestation attempt, the gills of the host fish should be checked. If it has been unsuccessful (e.g. bad quality of glochidia) you can re-infest the same fish with a new batch of glochidia provided that the period between the first infestation and the second is short (one or two days).<br>Ensure that fish they have not been infested before discarding a possible immunity to glochidia.<br>Observe the motility of glochidia using a microscope. Add drops of NaCl solution to a small subsample of glochidia and observe the movement. If they are not active (intensive movement due to the chemical stimulus), discard the entire batch of glochidia. |
| | | |
| Problems with infested fish when maintained after infestation | | |
| | Fish diseases | It is possible that with the rise of the spring temperature, the white dot disease *(Ichthyophthirius multifiliis)* appears in the blenny river (*Salaria fluviatilis*) or other host fish. Appropriate medications should |

| | | |
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| | | be applied to eliminate protozoa or salt baths (5 gr to 2-3 litres of water) if the fish were not yet infested with glochidia. (See chapter 7) |
| | | Be sure that treatments do not affect the metamorphosis of the glochidia. |
| | | Maintain good water quality and a good filter system |
| | | Try to use healthy fish for the infestation |
| | | If possible, the fish used for glochidial infestation should be maintained at the lab one month before the infestation as quarantine. |
| | | It must be taken into account that the fish should be very well fed during the month prior to the infestation because then they will spend a period of starvation to avoid the mix of faeces with the juveniles when they are born, in this way it ensure that the juveniles are born clean. |
| | Too high nitrite/ammonia values | Reduce density, improve filter, exchange 1/4-1/3 of the water volume |
| | | |
| Problems occurring when young excysted mussels are collected after metamorphosis | | |
| | Juveniles are “dirty”, mixed with other detritus | Starve fish one to (better) two weeks prior to the expected date for the anticipated excystment, to avoid collecting debris from non-ingested food and fish faeces. Clean the juvenile catching device (netting) as often as possible.<br>If juveniles are still mixed with organic detritus, you may clean juveniles using 2 needles under the microscope (this is very time-consuming, and risky for juveniles), as juveniles are very fragile.<br>Alternative cleaning treatments: A) Put the juveniles in a sieve, according to their size (for newborns it should be 120 microns) and wash them carefully with natural water from a watering can, while gently moving the sieve. B) Put the juveniles in a little container with sand and water and stir to cause friction between them. |
| | Juveniles with broken shells | They should eliminated and not be used for captive breeding |

| | | |
|---|---|---|
| Problems occurring with the rearing system (artificial flumes with sediment) | | |
| | Bacterial growth on the sediment surface (biofilms) | Carefully (!!!) stir sediment surface to a depth of 0,5 cm, using a plastic spatula.<br>Change the water. Clean and change the sediment. |
| | Anoxia (black and grey zones) in the sediments | Carefully (!!!) transpose sediment containing juveniles (without touching the black and grey zones in the sediments) into a round recipient, suspend (elutriate) mussels by stirring and shaking, and collect them with a 200 µm net, then transfer them into a clean raising device (substratum should have been in place at least one week time prior to use in order to allow bacterial growth). Regularly, change the water, and clean and change the sediment. |
| | Clogging of sediments due to carbonate precipitation | Avoid carbonate rock as substrate |
| | Substrate size | The better is to use calibrated sediment of the same size, normally smaller than the juveniles. In this way, juveniles can be separated from the sediment by sieving. As the juveniles grow, we can change the calibre of the sediment. |
| | Imbalance in the physicochemical parameters: decrease of oxygen, increase of ammonium and nitrites, or increase of conductivity. | Periodic water changes (once a week 50% and if the parameters are stable you can extend the period every 15 days). Add extra aeration. Cover the tank, completely or partially, to avoid excessive evaporation which may produce a rapid increase in conductivity. |
| | | |
| Problems occurring with the food for rearing | | |
| | Juveniles feed, but do not grow | The exact food of the juvenile mussels is yet unknown, but current experiences show the need of natural detritus in the food mixture, either for substances that are not available in the commercial algal food, or for delivering symbiotic bacteria that have to grow in the digestive tracts of the juveniles to help them digest the food. Uneven stoichiometry of nutrients may also be the reason for ineffective |

| | | |
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| | | growth (e.g., diet is unbalanced vs. carbon), or the stoichometry PUFAs (polyunsaturated fatty acids) is unbalanced? (See e.g. recent review by Evans-White & Halvorson, 2017)). Thus, add natural detritus. Refer to specialized literature or make own analyses of food quality and of growth effects. Make feeding experiments with different kinds of diet.<br>Increase the doses of food or increase the number of times it is fed. Try using phytoplankton species with a high levels of PUFAs such as *Neochloris oleabudans* (Chlorophyceae). |
| | Juveniles receive a sufficient amount of food, but do not feed/grow | Set up feeding protocol before experimentation: how is it given? (continuous, pulsed, automated), how often is it given (times per week)? Possibly, the food ends up in the filter before it has been ingested. Reduce flow of filter to almost zero while feeding. |
| | | |
| Other possible causes for mortality of early juveniles | | |
| | Fungi, parasites, other animals. | Avoid feeding of host fish at least one week prior to excystment. Reasonable starving duration depend on fish size. With larger host fish, e.g. *Acipenser baeri* (50 cm, in length) two weeks are possible (K. Nakamura, pers. comm.). This reduces the production of unwanted organisms along with juveniles. Clean (separate) juveniles with a pipette from detritus. Control dosage of food and oxygenation of sediments (does a grey/black layer develop, indicating reduced conditions) and transpose juveniles if this happens. |
| | Juveniles with broken shells | An alternative methodology to separate the juveniles is the elutriation (i.e. separation of particles according to their density) similar to the methodology described by Lavictoire et al. (2016). |
| | Negative effects by other macroinvertebrates or protozoa in the culture | Copepods and water fleas may compete with juveniles for phytoplankton in the culture. Protozoa such as *Vorticella* sp. can invade the entire outer surface of the juvenile and cause death. Filter the water exhaustively, filter water samples and analyse them under the microscope. |
| | | |

| | | |
|---|---|---|
| Possible causes for mortality of larger juveniles | | In good conditions of food, this it rarely happens. Juveniles normally survive when they have attained more than 1 mm in length. |
| | Imbalance in the physicochemical parameters: decrease of oxygen, increase of ammonium and nitrites, or increase of conductivity. | Periodic water changes (once a week 50% and if the parameters are stable you can extend the period every 15 days).<br>Add extra aeration.<br>Cover the tank, completely or partially, to avoid excessive evaporation that leads to rapid increase in conductivity. |
| | | |
| Problems occurring with reintroduction to the nature | | |
| | Clogging of reintroduction cages | According to Gum et al. (2011), this is one of the most common reasons for lacking success. Clean the outer netting regularly. |
| | | If juveniles are small, they can get lost. |
| | | |
| Possible causes for mortality of larger juveniles | | |
| | Fish predation | This was observed with *Unio ravoisseri* in Spain, animals were eaten by carps. Keep juveniles longer, let them grow bigger. Check fauna of potential predators at the reintroduction site beforehand. |

# 5. Technical advices for building up and running a laboratory for artificial rearing of *M. auricularia*

**Preliminary remark:** Several options exist to foster natural populations of freshwater mussels, including "arc" projects, where adult mussels are displaced towards habitats, where the chances for reproduction are higher [not dealt with here, see Gum et al. (2011) and (Patterson et al. 2018) for a revision], artificial reproduction and rearing in the laboratory (this chapter), and infestation and release of host fish (see chapter 13, this volume). For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume. All laboratories have to be built and run in accordance to ISO/IEC 17025:2017 norm, which specifies the general requirements for the competence, impartiality and consistent operation of laboratories.

**Introduction:** Planning and establishing a laboratory unit is a laborious task. During the planning phase, there is an intensive exchange between scientists (who are thinking about results) and technicians/ engineers (who think about dimensions and materials to be used), and it may be a difficult task to converge the expectations of both groups. We highly recommend to contact several other laboratories working on the same issue during the early planning phase, and specifically to ask them about their drawbacks, in order to avoid them. A lot of detail questions concerning the origin of used water, electric power supply and the placement of mobile labs have to be considered (see checklist in "Caveats", above and below).

**Method description**: A lab for artificial rearing should be composed of several elements:

- A **water storage unit** (to store river water from the origin or destination of the mussels), equipped with aeration devices and options to eliminated decanted sediments. If the rearing station is close to a river that delivers the water, great care has to be taken for providing sufficiently efficient filtering equipment. Small sediment particles and flocculated, previously 'dissolved' organic matter may clog the pore space in the rearing devices very quickly. Temporary pollution of the river water could also happen. Ventilation and air renewal is very important to prevent a humid situation and mold development.
- A fully acclimatized **unit for keeping adult mussels and fish** before during and after infestation, equipped with all necessary elements of an animal-testing facility (see chapter 2), including an entry zone where shoes can be changed or sterilized, cupboard for laboratory coats, sterilisable laboratory tables for the infestation procedure, aquaria and cylindroconics (see below) for

keeping fish and gaining excysted juveniles, filtering devices, cupboard for safe storage of chemicals, food, refrigerator, freezer etc.

- A fully acclimatised **unit for rearing juvenile GFPM** during earliest life stages, including aquaria and artificial flumes or other rearing units (see below) for juveniles, filtering devices, water reservoirs, and enough room for handling and storage. This unit may include a unit for keeping adult mussels before and after sampling of glochidia (otherwise, an additional aquarium room is necessary).
- A **laboratory** equipped with binocular and standard devices to observe excysted juveniles from the collector (see below), to transfer them into the rearing unit, and to prepare food solutions etc.
- Additionally, a **unit for the production of algal food** can be added (not necessary if commercial food is used)
- Additional outdoor rearing facilities for larger juveniles have been successfully employed in projects with other mussel species than *M. auricularia*.

**Procedure details and material:**

*Description of the laboratory facilities of the LIFE+ M. auricularia project in Chinon, France*

*Water supply:* Natural river water is pumped from the nearby Vienne River and transported with a trailer with a 1500 L container that has been equipped with an electric pump and a generator (figs. 3,4). Water is stored in 2 x 650 L tanks plus one 1 $m^3$ polyethylene tank. All tanks are covered from sunlight to avoid algal growth. Tanks are situated in a nearby house (outside the mobile labs) with a natural climate (thick stone walls) that maintains the water temperature between 10 and 18 °C. Water is aerated with an air pump commonly used in fish-ponds and filtered. Decanted sediments can be removed via a lower water outlet. The 650 l tanks are connected to those the mobile rearing units with an integrated system that refills them automatically. Additionally, water can be drawn from the 1 $m^3$ polyethylene tank by flexible polyethylene tubes when necessary; flow is initiated by a hand-pump. They are slightly elevated to facilitate water flow when pumps do not work. The average use of the rearing unit is 500 L per week, i.e. one tank is for current use, the other one is a reserve tank. This allows to run the equipment for 2 weeks in case that refilling was not possible due to a flood event.


**Figure 3. Polyethylene tanks with the stored water (Photo R. Araujo)**

**Figure 4. Water transport trailer and containers of the mobile laboratories (Photos R. Araujo)**

*Container labs*

Each of the two containers has external dimensions of 600 x 230 cm (length x depth) x 220 cm (height). They are insulated and equipped with a powerful (and energy-consuming) air conditioning to maintain inner temperatures between 15 and 25 degrees even under summer conditions.

In both container labs, water quality is measured continuously (temperature, oxygen content), with a probe that is connected to an alarm system, which may be connected to a cell phone. This is very important, specifically for the rules of animal experimental facilities, which require short-interval surveillance of water quality. Week-end and holiday shifts may then be exempt from being present at the station, provided that they can arrive there within 20-30 minutes. Chemical analysed are also done every day.

*Container lab 1: Fish rearing unit*

The internal part of the container lab is depicted in fig. 5 (below), its outer appearance in fig 4 (above). On the left part of the figure, there is an entrance with a hygiene lock, where shoes can be changed or sterilized (on the bottom, there is a basin filled with a formaldehyde-free disinfection solution), and a cupboard for laboratory coats, as well as a small table, with a record book for visitors of the animal testing site (mandatory). On the right end (opposite to the entrance), there are shelves for aquaria of different size to keep the host fish prior to the infestation cycle (adaptation to water quality, quarantine procedures). On the back side, there is a 190 x 60 cm stainless steel laboratory bench including a sink and a warm/cold water tap. Above the bench, there are cupboards for glassware. Due to lack of place, another air-conditioned cupboard for safe storage of chemicals as well as refrigerator and freezer are placed outside the container lab (in the nearby laboratory building). Refrigerators can be placed in the container, but keeping them outside avoids additional heating of the lab and disturbance of the fish due to the vibrations of the compressors. On the bottom, a rubber mat absorbs the shock waves caused by walking. The light can be dimmed and timed from a central control unit.

After infestation, fish are reared in so-called *cylindroconics*, i.e. rounded water containers with a conical bottom (see fig. 5), dimensions: 66cm inner diameter, height of the cylindrical part 70 cm, height of the conical part 30 cm. Cylindroconics are made of artificial raisin, and have an inserted Perspex control window of 30 x 50 cm. Four of them are on the front side, three on the back. A detailed description is found in the next chapter. Filtering is performed with commercially available UV and biological filters (one per cylindroconic) that are situated on the bottom of the lab. Each cylindroconic has a separate filter, in order to avoid cross-infestation in case of diseases. A compressor (situated outside) delivers air for aeration, which comes from tubes that are connected to the compressor via the ceiling.

The dimensioning of *cylindroconics* and aquaria for fish is key for the output of the reproduction. The bigger and the more basins can be used, the more juveniles per year can be produced. Considering the high juvenile mortality, an ideal number would be above 100.000 juveniles per year. In our case, the dimensioning of the container lab permitted the installation of 8 *cylindroconics*, each one with a hosting potential for 15 small sturgeons. Assuming an average juvenile production of 1000 juveniles/fish, this would account for 105,000 juveniles per year. If larger fish are used (consider that they can survive starving better, allowing a longer starvation period before excystment of the juveniles, and cleaner juveniles), larger cylindroconics have to be implemented (with a lower number of units per laboratory).

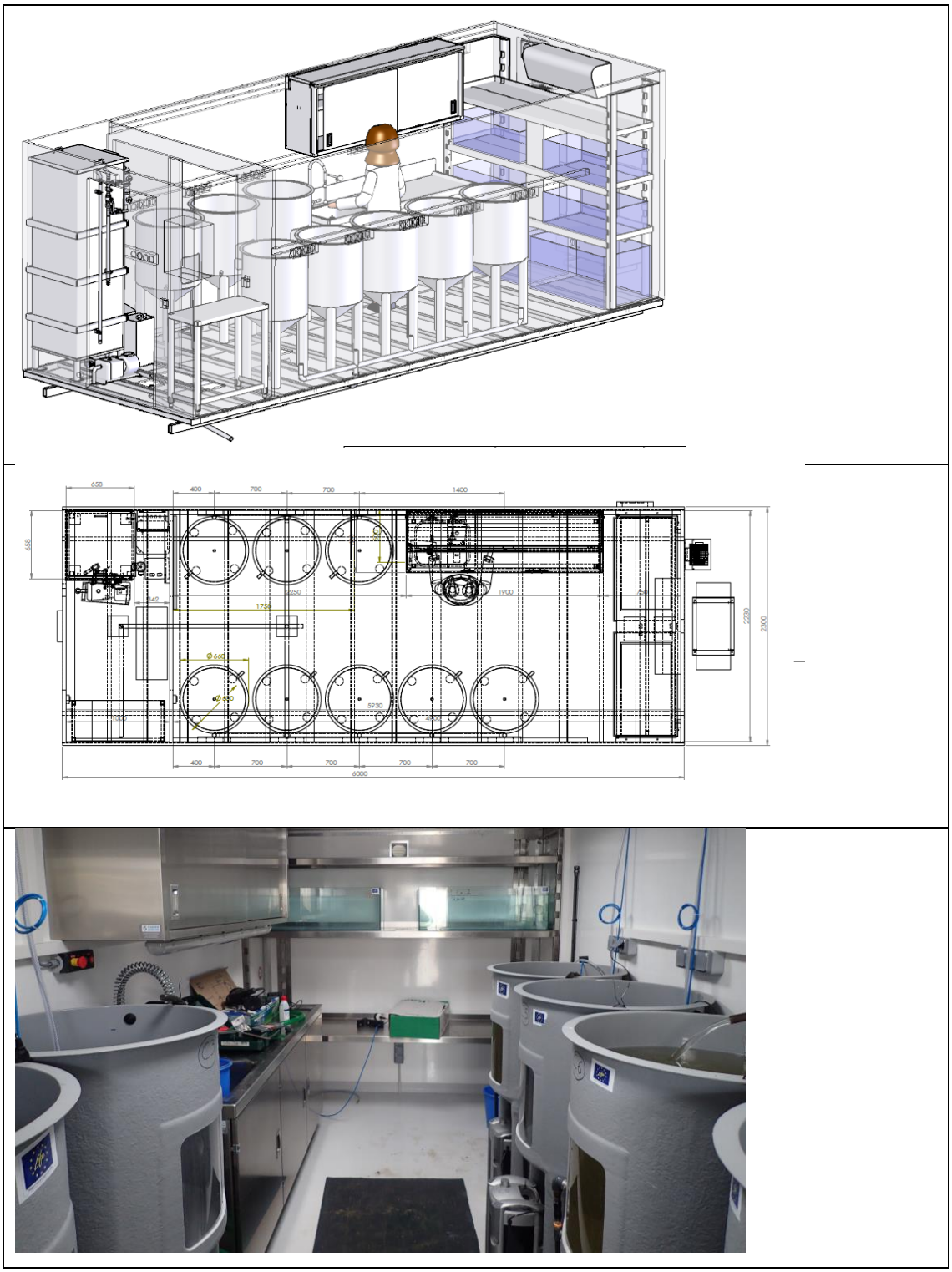


**Fig. 5: Above: lateral view, middle graph, plan view of the mobile fish laboratory (design: CASTEX). Below Photograph of the container lab, taken from the entrance (c) M. Sicot).**

*Container lab 2: Mussel rearing unit*

This container has the same outer dimensions and the positioning of the bench and the shelves for aquaria as the previous one, however it lacks the hygiene lock at the entrance. At the long sides, shelves support three vertical rows of artificial flumes (front side, 2 shelves, back side, 1 shelf), totalling space for 9 flumes (fig. 6).

Outside installation: In order to prevent loss of electricity during blackouts of the local energy supply, an electrical generator was needed for keeping oxygenation and water circulation in aquaria.

**Time effort:** *Establishment of the laboratories:* Estimate 9 months for conceptualising the mobile laboratories, including several visits in other mussel projects, and meetings with engineers. Many details are important such as the number of electrical outlets, the specific electric power requirements of the installation, the system of water supply, treatment and filtration, oxygenation and ventilation of the laboratory etc. We recommend to involve experienced aquariologists to make a first design and then to discuss the project with construction firms. Some other projects have employed design offices in order to build their laboratory. Estimate another 6 to 9 months for constructing and assembling the laboratory. Public institutions have to publish calls for tenders, which have their specific delays (3-6 months). Non-reply by the firms (e.g., because the contract volume is too small for such a specific project or the demand is too specific for a firm) may further delay the project. Considering the local situation and the specific regulations valid for the chosen site for placement of the mobile labs (e.g. architectural heritage sites nearby, or flood protection reglementations), two years should foreseen for the establishment of the labs. (For the routine running of the laboratories see next chapter).

**Caveats:** Unexpected drawbacks may occur, which may delay the project, or block the procedures and we suggest the following checklist.

*Purchase or construction of a mobile laboratory?* Laboratories as described in this chapter are offered "ready to use" by different firms specialized, e.g. in oyster reproduction, or less specialized models are offered by firms working with mobile laboratories, however we recommend a very critical discussion between experienced mussel breeders (from other projects) and the laboratory builders before ordering. Some specificities with animals, experimentation or water might not be incompatible with "ready to use" system. In the end, details may arise that had not been planned. Therefore, any laboratory has to be planned in a way that modifications (e.g. variable size and numbers of cylindroconics or juvenile rearing

devices) can be easily made. In addition to the planning of sufficient personnel for running the laboratory, nearness to a technical unit or skilful craftsmen is highly recommended.

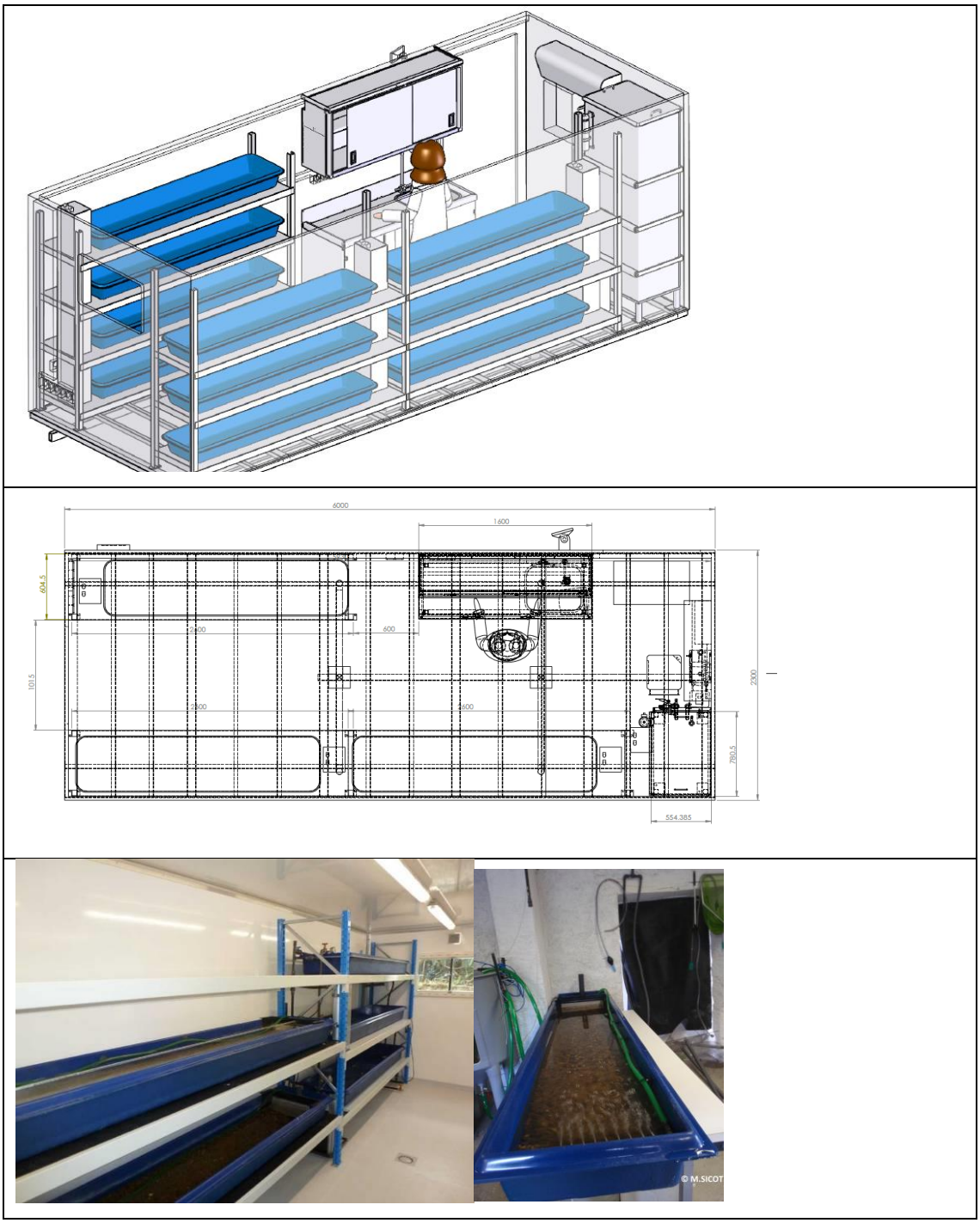



**Fig. 6: Above: lateral view, centre: plan view of the mobile MUSSEL laboratory (design: CASTEX). Below: artificial flumes. Photographs: left: J. Soler, right: M. Sicot.**

*Water supply*. Is the site sufficiently near to the water source? If water was transported to the lab in tanks, is a sufficiently strong car (strong engine, 4x4 drive) available to pull the trailer with the tanks? Is there a ramp nearby that allows to pump water? Are permits needed and provided to pump water from the river? Is there enough storage place for the water container, will there be sufficient temporary storage (floods and droughts, or pollution spills may make natural water sources temporarily unavailable) in a place that keeps the water at a cool temperature? Is there enough time for decantation of sediments before use? If water is deviated from a nearby stream: Make detailed and quantitative analyses of suspended sediments and developing flocculates. These may clog the pore-space of the sediments in the artificial flumes or rearing pots. Some projects (e.g., the LIFE *Unio* project in Clairvaux, Luxemburg) have employed a several-step cleaning unit, including a self-cleaning, centrifugal filter and a sand bed filter to pre-clean the water. Attention if nearby groundwater was used: Water supply may cease in dry summer months, moreover groundwater may have very low oxygen conditions and may contain dissolved iron ions that precipitate on all tubes and glassware etc.

*Placement of the mobile containers*: Is the soil, where the containers should be placed, sufficiently consolidated, to avoid that the wheels might sink into the ground? Is the place possibly in a floodable part of the city (then, provisions for evacuation have to be proven)? Is the place possibly near architectural heritage (then, the responsible architects employed by the city must agree on the outer appearance of the containers; in France these “Architectes des Bâtiments de France” change regularly, and only the person currently in charge has the final say, which means that contradictory statements may be made by different persons)? Is the doorway wide enough and sufficiently solid to transport the container to its final position? How are the fluids (water and electrical current) provided and how is waste water connected to the sewer system? How are the fluids accounted for (are there separate gages/counters/telephone lines) if they are financed by a specific project, and not by the overall laboratory costs? Is the type of biologically and chemically polluted waste water of the laboratories in agreement with the local regulations? Are the containers exposed to the sun, or are they shaded (this may be important during heat waves when the air-conditioning system is at the limits of performance)? Is there a risk that trees fall on the roof during storms? Are the containers placed in an inconspicuous way, not visible from the next road (currently, there is increasing vandalism on animal experimental sites, even if they were employed to preserve rare species)?

# 6. Breeding period, sampling of gravid adults, transport and sampling of glochidia in the laboratory

**Preliminary remark:** Knowing the precise period when mussels are "gravid" i.e. when they carry and release glochidia, is essential for artificial reproduction projects. The quality and quantity of the glochidia varies considerably between early and late release, the best glochidia are generally those of the central release period. When reproducer adults have been sampled, they may abort masses of not yet developed glochidia under stress conditions. Therefore, care must be taken to sample mussels and glochidia correctly. For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume. Due to the fact that *M. auricularia* is a protected species, permissions from the competent authorities are needed to collect the specimens. (This must be done sufficiently long in advance!)

## 6.1. Assessment of breeding period and glochidial release

**Introduction:** In the Ebro River (Spain) *M. auricularia* is a female hermaphrodite and gametogenesis occurs from December to March (Grande et al. 2001). Drift net sampling in Spain indicated *M. auricularia* glochidia release during March and April (fig. 7).

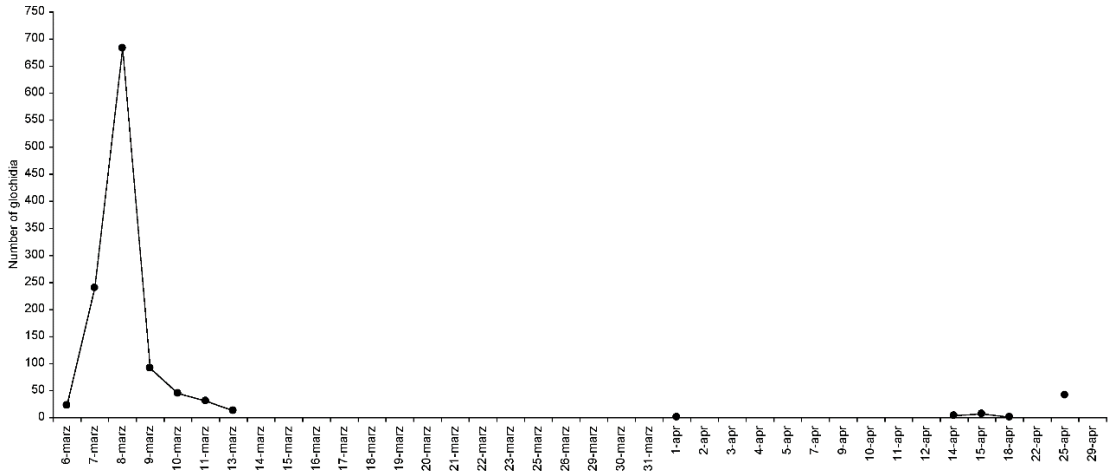



**Figure 7. Daily density of *M. auricularia* glochidia (No/30 ml) in stream drifts (from Araujo et al. 2000)**

Ova and developing embryos from the French Charente and Creuse river populations were found in March, with glochidial release occurring in April. Development from first cleavage to glochidial maturity took 25–37 days. Compared with the Spanish populations, the reproductive period begins some weeks later.

Previous knowledge of the reproductive season of *M. auricularia* is based on Ebro Basin populations, which are gravid in February (see above) and release glochidia in March (Araujo et al. 2000). Field and laboratory observations of two French populations carried out in the Charente and Vienne/Creuse rivers from 2015 to 2017 showed that mussels had developing embryos through March and that the beginning of glochidial release occurred in early to mid-April. Taken together, this indicates a difference in the timing of glochidial release between Spanish and French populations and, indeed, even between the two French populations, with glochidial release in the more northern River Creuse population delayed by several days. The estimated number of glochidia per gravid mussel was around 2,000,000 (Soler et al. 2018b).

**Method description**:

Presence of glochidia released into the natural running water system can be checked with drift nets (mesh size 100 µm, opening ca 50 x 20 cm, net length ca 100 cm, fig. 8). These nets are positioned with metal rods (in smaller streams), or held by hand (with a rod) from boats (in rivers), so that the upper rim of the net protrudes some centimetres above the water surface. The exposure time depends on the saturation (clogging) of the net, maximum ca. 30-60 minutes.

**Figure 8. Drift net used in the Canal Imperial for collect *M. auricularia* glochidia (Photo R. Araujo)**

Measurements should occur from mid-March to mid-April. If the average water velocity in the net opening is measured, and the discharge of the stream is known, then the drift rate of the entire stream can be assessed (calculation: set the “discharge” inside the net (velocity x net opening dimensions) in proportion to the discharge of the stream, and multiply the number of individuals found in the net accordingly). Interval measurements may permit identifying the preferential release time.

For the assessment of the maturity of the glochidia (presence of ova, developing embryos, or glochidia), adults need to be sampled. It is very important that adult mussels are placed back to the precise site where they were taken from by using site markers for the individual mussels (e.g., small metal rods with a coloured plastic rope). These site markers carry the same number as an elastic "sleeve" (e.g., a cut piece of a sock), which will be pulled over the mussel immediately after removal. With this method, the mussel and the marker display the same number. When returning the mussel after analysis/glochidia release, they can be placed in the correct place. Use a small scoop to dig a hole before placing the mussel back. (The procedure can be visualised in the film on the LIFE project). We suggest that each year 1-2 animals are resampled from the previous year, in order to proof that the sampling procedure is not harmful for them. However, in order to maintain a higher genetic diversity among the reproduced juveniles, the larger part of the sampled mussels should vary from year to year.

*How to know if mussels are gravid*. It is important to minimize stress on gravid female mussels. To know if they are gravid (glochidia in the gills) they can be inspected by opening slightly the two valves with a reverse plier along the anterior ventral margin of the shell (to be done very carefully and slowly to avoid muscle rupture) or with the support of a syringe and looking inside to see if they have eggs (yellow colour) or glochidia (grey masses) stored in the gill (figs. 9, 10, see also Patterson et al. 2018). Using a lamp should be helpful. This should only be done by expert hands, the intervention should be kept as short as possible to avoid abortion.

**Fig. 9 The use of a syringe to check for the gravidity on *M. auricularia* (Photo R. Araujo)**

Once it has been confirmed that they are gravid, the mussels should be transferred to the aquaria at the laboratory. Transport should be made in well-pampered thermos boxes (avoid vibrations), which are cooled with ice (mussels must not get in contact with the ice!). Mussels are kept in moist tissues. Try to keep transport times as short as possible.

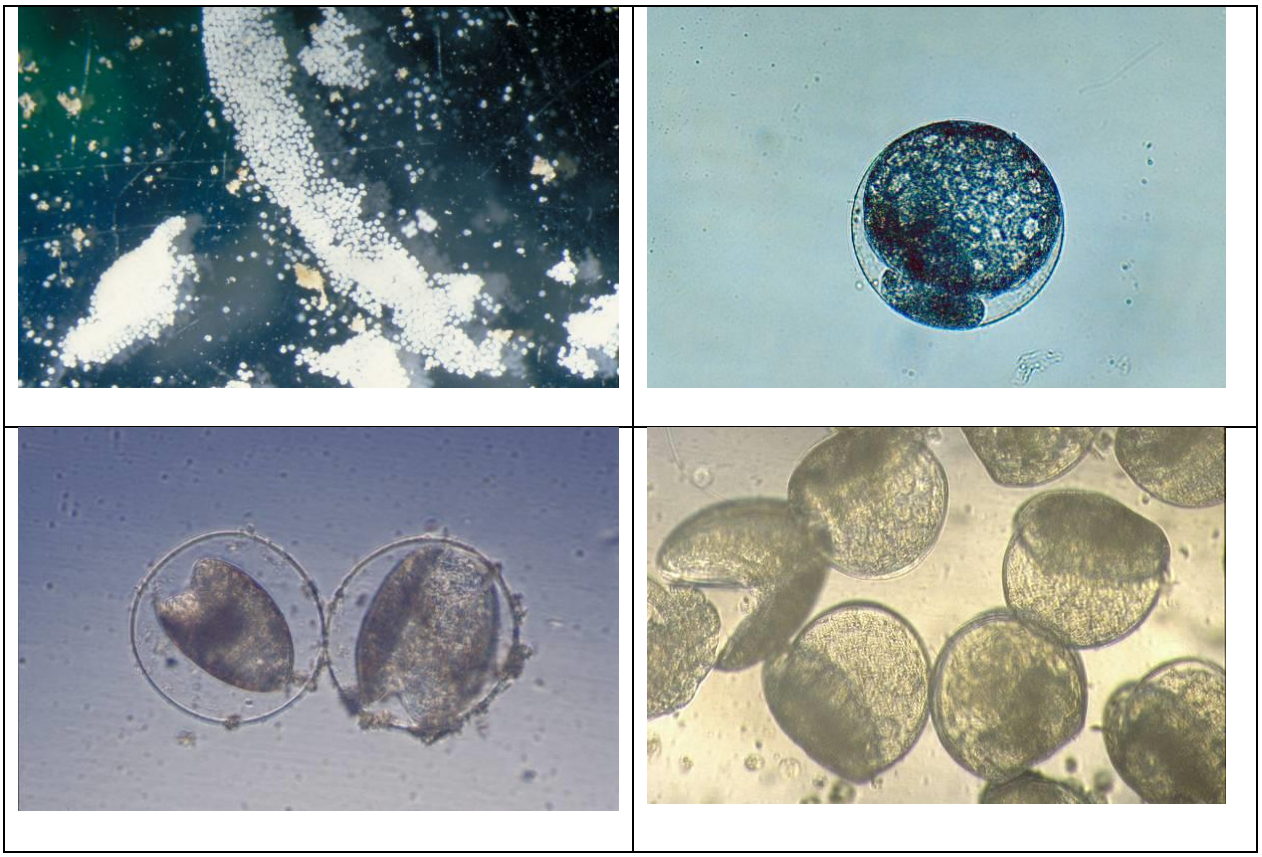


**Figure 10: Above: Conglutinate of eggs and embryo of *M. auricularia*. Below: Encapsulated (still developing) and mature glochidia of *M. auricularia* (Photos: R. Araujo)**

**Material needed:** *For assessing the release period using natural drift:* Drift nets with adequate support, wash bottles, flasks, thermos box, microscope. *For assessing the maturity in sampled adults:* Aquascope observation or diving equipment (see chapter 3), site markers (rods, sleeves, see above), thermos box, shovel or scoop, microscope, ice, water from the river, reverse plier, syringe, needle.

**Time effort:** *Drift sampling*: Transport time for approaching, ca. 20 min. for preparations, 60 min. drift measurements and sample storage, ca. 60 – 100 min. per sample for microscopic analysis. Sampling should occur over several (6-10) weeks. *Mussel in situ analysis for developmental stage of embryos*: Sampling: Aquascope or SCUBA sampling (see chapter 3), check-up of the mussels, ca. 2 minutes per animal.

**Caveats:** Mussels may be stressed by the procedure and abort their eggs or embryos. This procedure of sample the mussel gills should only be performed by experienced researchers, specifically the use of reverse pliers and syringe to check the stage of maturity of the mussel gills (see Patterson et al., 2018).

The best is to know the reproductive cycle of the species and to transport the specimens to the lab two-three days before glochidia release.

In an extreme case when mussels do not release glochidia in the aquarium (for instance for abortion), female specimens can be fertilized by mature males in the aquarium. The problem in this case is that mussels should be maintained until releasing of the new glochidia (25-37 days).

## 6.2. Sampling gravid adults and transport to the laboratory

**Method:** Gravid specimens will be collected using an aquascope in wadeable streams and by scuba diving in deeper water bodies. All collected specimens will be identified with a plastic label with a unique number, in this way we will try to use different specimens each year (fig. 11).

**Figure 11. Adult French specimen of *M. auricularia* with the numbered red tag (Photo: J. Soler)**

**Material:** Aquascope or SCUBA sampling (see chapter 3), ice boxes, container to transport at least 100-200 litres of water from the original river

**Time effort:** For the check-up of the mussels, ca. 5 minutes per animal.

**Caveats:** The date of collecting in the French rivers has been done in the month of March because we expected that glochidial release occurred the first days in April. Nevertheless, we took some samples

beginning from the month of February (especially after warm winters) to check for evtl. changes in the gravidity period.

In order to keep the glochidia inside the mussels and to avoid abortion, gravid mussels must be transported with ice in the shortest possible time. For the travel between the river and the laboratory the mussels will be stored without water, just enveloped in a moist cloth and over a bed of ice inside a foam box, avoiding direct contact between mussels and ice.

At the moment of the mussel collecting, it is of the main importance to collect at least 100-200 litres of water from the original river in order to fill the aquaria where the mussels will be maintained.

## 6.3. Sampling of glochidia from gravid adults in the laboratory

**Introduction:** Once the gravid adults have been carefully transported to the laboratory, glochidia can be sampled. Glochidia should be used as soon as possible, but they can be stored at 12 degrees in filtered water from their environment for maximum 1-2 days, however it is much better to use fresh glochidia for infestation, and to keep gravid adults in aquaria until glochidia are needed. Be careful with the appearance of fungus in the glochidia. Once fungi appear, discard the entire batch of glochidia. To keep losses low, keep glochidia batches from each donor mussel separated, and mix them only just before infestation.

**Method:** At the laboratory, it is very important to maintain the gravid mussels in the same conditions (water and temperature) they were in the original river. Each week, we will change a third of the water from the aquaria by the original water from the river. If the original water runs out before the glochidia release, we can use water from a nearby river.

There are two alternatives to gain the glochidia:

A) If no glochidia sampling unit (see below) was available and place is restricted, mussels are kept without sediments in order to get clean glochidia using a syringe. This requires one aquarium of 100 litres for 8-10 specimens of 10-12 cm. This method produces clean glochidia, however, some uncertainties about the developmental status of the glochidia remain, and the adults remain in an uncomfortable, lateral position. In the aquaria the mussels will be kept without sediment in order to obtain clean glochidia from the

aquarium bottom with a pipette (fig. 12). Then analyse the white mass of larvae at the binocular in order to use only mature glochidia.

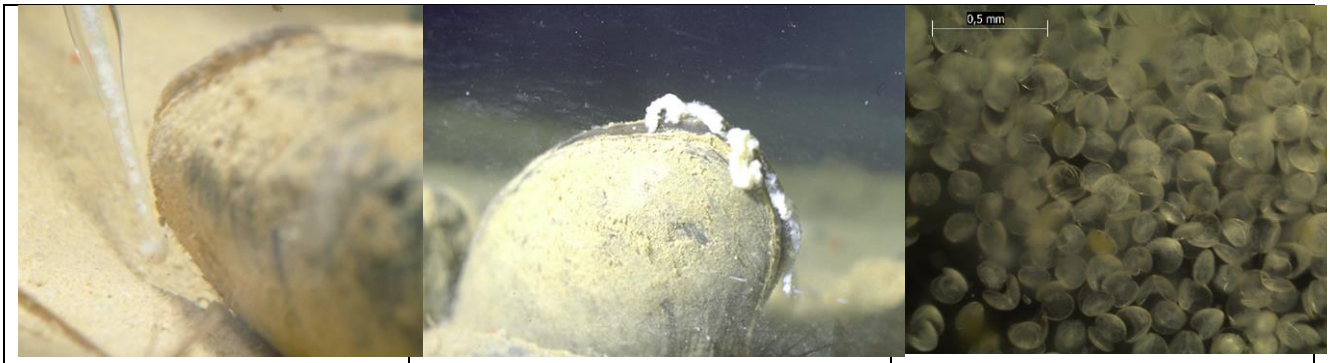



**Figure 12. Left: Sampling of released glochidia using a pipette. Middle: Releasing of glochidia. Right: A mass of glochidia (Photos R. Araujo)**

Normally, in 10-30 days the mussels release enough glochidia to make the fish infestation experiences (fig. 12). Aliquots of glochidia should be sampled with a 100 µl Pipette and counted with a Sedgewick Rafter chamber under the binocular (magnification = 70-100X).

B) Alternatively, adults are kept in plastic boxes (suggestion: 200 L for 8 adults), with sediments, and an automatic collection system (ACS) for glochidia is used (Nakamura, 2018b and unpublished) placed above a 1000L reservoir (fig. 13). This allows that the mussels maintain their natural position, which avoids disturbing the adults, or releases of immature glochidia. Moreover, it keeps the mussels in a large volume of water, which avoids ammonium peaks, nitrites and stresses due to changes in temperature or physical-chemical parameters (pH, conductivity, oxygen). It is recommended to filter the natural water to avoid macroinvertebrates like water flea (*Daphnia* sp., *Moina* sp.), copepods, that can negatively affect on the glochidia. The continuous filtering system eliminates impurities and glochidia predators. When the mussels start to release the glochidia, this filter is stopped and replaced by a sampling device for glochidia, similar to that of juveniles (see figure 13, and see chapter 8). Water from the plastic boxes containing the mussels is transported by gravitation into a sieve (75-100µm), which is placed in a plastic tray, and - from there – runs into a 1000L tank, then it is pumped back into the aquarium. The sieve is checked several times a day by replacing one sieve with another, i.e. water flows without interruption.

**Figure 13. Sampling unit for released glochidia (Photo: Nakamura)**

**Material**: Aquarium for adult naiads maintenance (avoid keeping the mussels a long term in the lab). Camping fridge for the trip with the adult mussels, 2 aquaria 150 litres. Sufficient amount of river water to renovate it every 5 days. Aeration (aquarium air bubbler), 5 containers of 30 litres for water transport, 20 petri dishes 5 cm diameter, labels with sequential numbers, superglue and etiquettes (for marking animals that were used for artificial reproduction, use different colour or number codes each year), 2 floating thermometers, siphon for pumping out water, digital calliper, Syringe and needles

**Time effort:** Once the gravid adult mussels are settled in the laboratory, glochidia can be sampled. Depending on the experience of the operator, count 20-30 minutes per animal to obtain glochidia. Glochidia should be used as soon as possible.

**Caveats:** The glochidia released from one specimen of *M. auricularia* may be sufficient to infest 100 *Acipenser baeri* at a size of 15 cm body length, however, in order to diversify the genetic pool, care should be taken to use glochidia from as many mussels as possible, even if not all glochidia of each mussel are used (in nature, only a minor part of the glochidia actually reach the fish for infestation).

We usually used 10-20 gravid specimen of *M. auricularia*, to infest 100-200 sturgeons of the species *Acipenser baeri* at a size of 15 cm body length.

## 7. Maintenance and infestation of host fish in aquaria

**Preliminary remark:**. For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume.

**Introduction:** Fish will be kept in different stages: First, they are kept in quarantine, then they will be infested and subsequently kept in basins while being regularly fed. Lastly they are kept in so-called "cylindroconic" tanks, which allow the sampling of the excysted juveniles of the mussels.

Maintaining a large population of infested fish is the limiting factor for the production of large quantities of juvenile naiads. This method has to be optimised to two different ends. The legal restrictions require a minimum number and a minimum disturbance of the fish, specifically considering starvation. The demands for conservation require a maximum output of clean and healthy mussel juveniles. An optimum time for keeping fish without food prior to the excystment of the juveniles has to be found. One leverage between both ends is done via the *infestation dosage* and the fish size. Smaller fish can take only lower amounts of glochidia, but more individuals of fish can be kept on the same place. Larger fish can carry more juveniles per specimen, may support a longer starvation phase, but represent a higher risk if one individual fails due to diseases. Another leverage is *number and volume of aquaria and "cylindroconic" tanks*, in which fish are kept prior to the release (excystment) of juvenile mussels.

Fish must be held in ideal health conditions. The fish used for glochidial infestation should be maintained at the lab one month before the infestation as quarantine. However, they should not be fed prior to the release of the juveniles, in order to avoid mortality of juveniles due to "pollution" with fish faeces. Fish may fall ill, and must be treated against diseases according to the rules for the animal well-being, but treatments should not affect the mussel juveniles.

Lastly, the question arises what to do with the fish after the infestation cycle was finished. As fish develop immune responses, they cannot be used twice for infestation, except in the case of further investigations. After excystment, fish are either kept in captivity or will be killed. Fish used for animal experiments are not supposed to be returned into the wild. In some cases, official, licensed fish keepers (such as public aquaria) may take these fish back and , they have to be fed again to reach a good condition and kept again in basins.

All these details need to be mentioned beforehand in the experimental protocols.

## 7.1 Preparing aquaria and filters for keeping host fish

**Preliminary remark:** Refer to professional fish keepers about rearing conditions and specific requirements of the individual fish species. These must be listed in the "DAP" or other protocols which are part of the licensing procedure for the animal hatchery. The establishment of an animal-testing facility and the training of the personnel are very time-consuming and should be completed 1 year before the practical work begins (see chapter 2). For the routine work of the animal-testing facility, a mid to long-term personnel strategy should be employed, including plans "B" and "C" in case of, e.g., a fish breeder is not able to deliver, the only veterinarian licensed to analyse fish retires, or a technician moves to another unit. Specifically for the week-end and holiday shifts in the animal-testing facility (daily visit is mandatory), trained personnel must be accounted for (including elevated salaries for week-end shifts) in the planning. As long as fish are present, their permanent survey must be warranted..

**Introduction:** This short introduction cannot replace a course in aquariology. We suggest specific literature and discussions with professionals, and mention only the essential elements for maintaining fish at good health, which are: (i) adequate water quality, (ii) adequate food, (iii) stress-poor habitat conditions for fish.

*Water quality* depends on the water that is used (i.e. the dissolved ions, gases, and organic compounds, pH value, inoculum with natural bacteria etc.), and the functioning of the filters. The main actors on water quality are bacteria, which increase their activities with increasing water temperatures. If the aquaria are exposed to light, algae, which consume oxygen during night time, will grow. Unconsumed food and fish faeces that has not been eliminated may quickly result in fast increasing nitrite or ammonium values (nitrite is an intermediate product of protein degradation, ammonium results from excretion by fish) and decreasing oxygen concentrations (as a result of bacterial respiration when degrading organic matter, and of fish respiration, which increases with temperature, and solubility of oxygen, which decreases with increasing temperatures), all of which can quickly cause lethal conditions for fish. Even though UV-cleaning units in aquarium filters remove many bacteria and split up organic matter, aquaria are by no means and should not be sterile. Freshly set-up aquaria generally display fast change of water quality parameters due to variable growth of different microbial assemblages. Thus, before the fish enter the system, an equilibrium should have established. The larger the volume of an aquarium, the easier it is to keep water quality parameters stable, as e.g., a local focus of mineralisation of organic matter will have a lower impact on the oxygen budget of a larger water body.

*Food types* are well described for each species. Especially for fish bred and kept in captivity, commercial food sources are available. The amount and period of feeding (during the day/night cycle, and the feeding rhythm per day/week) is very important, as residual food may alter the water quality. Fish will not take up food when they are stressed or when food is given while they are resting.

Fish are sensitive organisms. *Stress-poor habitat conditions* involve a day/night rhythm of illumination, good water quality and feeding, hiding places for benthic species (e.g., catfish), accident-protected inner walls of basins (in our case: the glass windows of the cylindroconic were placed in a way that sturgeon could not hit their snouts when circling along the inner wall of the basin). Fish also feel uncomfortable if the bottom colour of the aquarium differs from their body colour as they sense an increased predation risk. Other stressors to be avoided are: (i) radical change of water (differences in water temperature and water quality...), (ii), stress by visitors or untrained persons (noise, flashing cameras, knocking on aquaria,...), (iii) temperature changes, etc.

**Method description**: The aquaria and tanks should run one month prior to the arrival of the fish. During this time, water quality parameters should be checked regularly, and checked for evtl. sedimentation and flocculation, so that these can be eliminated. The bacterial community needs to develop in the filter, and should therefore be "fed" with small dosages of fish food (no live food). Aeration needs to be installed, too. In our case, each of the "cylindroconics" was pre-run, using a biological and UV filter.

Food needs to be provided, feeding schemes set up, and intervention schemes in case of diseases should be prepared (including clarifying the sources of medicaments for the fish, and information about their interference with juvenile mussel development).

## 7.2 Maintenance of fish prior to infestation

**Method description**: The fish used for glochidial infestation should be maintained at the lab one month before the infestation as quarantine (their duration has to be noted in the DAP document for the animal experimentation site). This period of adaptation is necessary for wild fish but it can be shortened (minimum: two weeks) if the fish come from a certified aquaculture.

Fish should either be ordered from licensed fish breeders (who will deliver guarantees about fish health), or – if wild specimen are needed (e.g. for test for additional alternative host fish) – fish caught by

professional fishermen, or by electrofishing (specific permits are needed). Fish from aquaculture are used to the type of food they were brought up with, so this food should be ordered along with the fish. However, aquacultures usually feed fish to gain weight quickly, so the dosage can be kept lower. It also should be excluded that the food contains antibiotics or hormones, which could be harmful for the mussels. We recommend to inquire about the usually used fish food and possible replacements beforehand.

Wild fish are much more delicate, they often carry or catch diseases when kept in captivity, and they are not used to artificial food, and adapting them takes additional time.

Ideally, the fish used each year should be maintained in rounded plastic outdoor pools or large indoor basins where they should be fed and treated against diseases and other possible infestations. Five days before the infestation, fish will be transferred from the outdoor pools to the indoor aquaria or tanks. Here they will pass another period of adaptation to the indoor conditions. From these tanks, fish will be transferred to the infestation containers. In other mussel projects, fish are kept in aquaria until 10 days prior to the calculated excystment date, and only then they are transferred to the "cylindroconic" tanks. In our case (no outdoor space available, and to simplify the procedure), we kept fish in large (500 l) indoor tanks prior to infestation, and kept them in the "cylindroconics" directly after infestation. Daily water exchanges mainly to compensate evapotranspiration (10%) warranted a high quality of water parameters.

It is important to apply a strict hygiene protocol of disease prevention, including the sterilisation of all equipment used in the aquaria. Nevertheless, it should first be assured that the treatments do not affect the development of metamorphosis or the future viability of juveniles. (Additional fish specimens for these tests must appear in the laboratory protocol prior to demanding permission). For animal experimental routine, a number of animals (in our case: 3 out of 15) need to be sacrificed and analysed as "control", i.e. these fish cannot be accounted for juvenile production.

Check and record water quality regularly, daily for oxygen, temperature, pH, and twice a week for conductivity, nitrite (precision tests exact to 1mg/L). Be alerted when nitrite values increase, then increase measuring intervals. Mitigation of increasing nitrite values: exchange 10% to 25% of the water in the basins, reduce food dosage, check raw water quality, improve filter activity, add a filtering unit, e.g. with charcoal.

**Material needed:** Basins in a sufficient size for the fish used. The thumb rule is "1 Litre of aquarium volume per 1cm of fish", for example, we regularly used 15 sturgeons of 15cm length (sum of lengths = 225 cm) in 230L basins with good results. Of course, dimensions of basins must match with fish size, the diameter (or minimum length) of the basins should be at least twice the length of the fish. In the mobile labs, we kept sturgeons in the "cylindroconic" tanks throughout the infestation period to save space and to reduce manipulations with fish.

Water quality probes: oxygen, temperature, pH should be hourly recorded and thresholds are set to trigger alerts directly on a specific phone.
Conductivity, nitrite (precision tests exact to 1mg/L).

**Time effort:** Consider 1 month for aquaria preparation, another month for running the fish keeping system without fish. Usual time for quarantine is 4 weeks. If the quarantine aquaria were set up 1 week prior to the quarantine, the 4 weeks of quarantine can be run in parallel with the 4 weeks of running the fish keeping system without fish. Water quality is a very essential parameter of success for fish keeping so the duration of this step has to be respected.

**Caveats:** Attention with fish diseases. We currently encountered problems in getting healthy sturgeon in France and Italy, due to herpes-like virus infestations. Getting fish from abroad may cause considerable additional effort of time and money. Size of the fish and period of needed could be also problematic: young fish for a specifically class of size (in our case: fish of 25cm total length) are not available all the year, depending of the uses of the fish farms.

Consider sufficiently long preparation time when working with wild fish. It may happen that mussels are already in the breeding mood, but wild fish have not yet left their winter habitats due to a cold spring (this happened to us with *Silurus glanis* catfish), so prepare this by interviewing experienced fisherman, and in the worst case (if the probability is high that wild fish will not available some weeks earlier to mussel reproduction), keep fish from the previous year in captivity.

Be informed about the maintenance (or natural) conditions of fish, allow sufficiently long time for adaptation/quarantine. Check regularly for diseases, and separate sick fish asap., do not use them for infestation. Wild fish and natural fish food is an additional source for infestations. Consult the veterinarian beforehand to be prepared to cure fish if necessary with adequate medicaments. Consider that some medicaments are not compatible with mussel breeding. If possible, eliminate infected fish as soon as possible and discard them for mussel reproduction.

All fish jump, and eel-like fish (e.g., lampreys) are able to squeeze themselves through the tiniest openings. Cover fish tanks therefore with adequate material and fix it tightly, but not air-proof, e.g., with 5mm mesh net that is tightly fixed to the upper rim of the fish container. Air current above the water surface must be warranted to avoid fungal growth, therefore airproof lids should be avoided (or an additional aeration installed).

## 7.3 Infestation of host fish

**Preliminary remark:** For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume. Remember when making calculations about potential juvenile output, that some fish (in our case: 3 out of 15 per cylindroconic) need to be used for efficiency monitoring and/or being sacrificed and analysed as witnesses for fish well-being. These fish will not deliver excysted juveniles. If fish are to be anesthetised (see alternative infestation procedure), this must be mentioned in the DAP protocol.

**Introduction:** During infestation, fish are exposed to water containing high concentrations of glochidia for a short time in a small container. With their respiration, water flows to the gills, to which glochidia can attach themselves. The procedure as such is not complicated, but care has to be taken that (a) glochidia are of good quality (not stored for more than max 2 days prior to use, clean and not infested by fungi), (b) number of living glochidia in the stock solution is known so that fish receive the correct dosage of glochidia. Too low numbers would result in less juveniles than expected, too high numbers may lead to stress (and eventually be fatal) for host fish. In nature, fish gills are found to be infested by very few juveniles, in experiments, fish can be infested by 100-500 juveniles per gram fish,.

All the fish infested during two days belongs to “one infestation process” and can be maintained together in the same tank for the rest of the metamorphosis. We have used 8 cylindroconic tanks, where the infested fish were maintained until the collection of the excysted juveniles (Figures 7, 14). The principle of this tank is to keep the fish under comfortable conditions, and allow the development of the encysted glochidia in their gills. A “cylindroconic tank” is composed by a large cylinder attached to a funnel on its lower end, allowing the collection of particles (i. e. juveniles) at the lower end (see chapter on laboratory construction).

In these cylindroconic tanks, that have a filter to clean the water, the fish will be fed daily until five to seven days before the releasing of glochidia (longer starvation periods are possible with larger fish). In this moment, the filter system will be changed for the juvenile collecting system (see below).

The cylindroconic tanks deposits are funnel-shaped at the bottom, at the lower end of which they have a hole provided with a plug or tap directed to the filtering or collecting system. Juvenile mussels detaching from the fish fall to the bottom and may be picked up at the tap.

A grid (mesh 0,5 cm) must be installed at the bottom of the tank to prevent fish preying on juveniles or passing through the hole into the filter or collection system.

**Figure 14. Cylindroconic tanks to maintain the infested fish. They are equipped with a filter and/or a juvenile collector (Photo: R. Araujo)**

**Method description**:

*Calculation of the correct dosage for infestation:*

The glochidia just released by the mussels (or stored in the refrigerator at 12°C), will be prepared for infestation as follows: check regularly (every day before infestation and immediately before infestation) their quality under the binocular (observe a subsample under the microscope: When adding a small drop of NaCl solution, the valves should move vividly, when glochidia are in a good health state. Do not use

this subsample for infestation any more. If this reaction does not occur, do not use this batch of glochidia. For glochidia of good quality, estimate their density from a subsample in a Sedgewick Rafter chamber and transfer the amount corresponding to 100 glochidia per gram fish into the infestation container. In order to calculate the doses, we will first weight the batch of fishes to be infested. The number of glochidia to add to the volume of water will be calculated by using the following formula:

N = D x W

Where N is the number of glochidia to add to volume of water, D is the desired dose (100, 300 or 500 glochidia per fish gram) and W is the weight of the batch of fishes to be infested in g (Soler, 2017). For example, to infest a 20 g sturgeon we used an aliquot to 2000 glochidia to 1 L of water for infestation, to obtain an infestation of 100 glochidia per gram fish weight. For bigger fish increase the doses of glochidia accordingly.

*Preparation of the infestation container:*

It has been found that infestation of fish in small containers is very effective. We use covered plastic containers of 1 litre (for 15 cm fish) or buckets (Figure 9) filled with the same water, in which the fish were kept before. An air diffuser is installed beforehand to provide water movement. Aeration is important to keep the glochidia in suspension and to maintain high oxygen concentrations (fish breathe vividly during the infestation).

*Infestation procedure:*

The infestation is performed in “batches” of 10 small (15 cm) or a lower number of larger fish. All fish of one infestation batch will later be placed into the same cylindroconic tank.

Ideally, two persons work almost simultaneously. Person A keeps freshly quantified glochidia solution at hand, and is prepared to add them to the clean infestation container (e.g., a bucket), which is pre-filled with river water. Person B catches fish carefully using two hand nets. Try to avoid stress. Do not use sedated fish to avoid collateral effects by the anaesthetic on the glochidia. Place the fish into the infestation container, immediately after Person A having dispersed and suspended the glochidia (fig. 15).

Keep fish in the container for 2-5 minutes. In the case of large fish that move very intensively (e.g. 1m long sea lamprey), the method has to be adapted. We used a moist cloth to hoist the fish, which was then placed into an elongate container that allowed very little fish movements. It is very important to use new water and new glochidia for each infestation, that is, change the water and add new mature glochidia for each lot of 10 fish. This is because the glochidia from the anterior infestation process can already be attached to the floating mucus being not effective for ulterior infestations. After this infestation, fish are brought into the "cylindroconic" tanks.

*Alternative infestation procedure by perfusing glochidia containing suspensions directly into the fish gill:*

This method is available for larger fish (e.g., sturgeons of 50 cm), which allow that gill openings or opercula can be lifted. Catch fish using 2 hand nets, place them on a laboratory bench on a plastic tray, and perfuse glochidia solution of the desired concentration directly into the gills, using a syringe equipped with a soft silicone hose. Take care not to damage the gills. Leave the fish in a bucket with clean water and with extra aeration for 10 minutes to ensure the good attachment of the glochidia to the gill. Then, return fish into the basin or cylindroconic. Sturgeons can be handled without anaesthetics. If fish species are used that fight vividly, a very short termed fish sedation (using 100 % w/w tricaine methane sulphonate) can be envisaged, but side-effects of the pharmaceutical substances have to be assessed. If such protocol is used this must be mentioned in the DAP protocol.

To get an idea of the success of the infestation, the percentage of adhering glochidia can be calculated. Knowing the initial number of glochidia injected in the gill and then recovering the remaining in the cube, they are sieved and counted again. In Spain, the first approximations show a 30% of hooking of glochidia to the gill of *Acipenser baeri,* in the first attempt and the result was satisfactory, the fish was well infested.



**Figure 15. Buckets with fish and glochidia (Photos: N. Richard)**

**Material needed:** Sedgewick Rafter chamber , 4 Tupperware containers of 2 litre volume for fish infestation (sturgeon of max. 25 cm length), aeration bubbler, 2 plastic jars (0,5 litres), homogenizer, dipstick

**Time effort:** The process itself takes about 10-15 minutes and should be helped with an air diffuser. But time is necessary for solution preparing, depending of the batches of glochidia ant their quality.

**Caveats:** It is very important to use new water and new glochidia for each infestation, that is, change the water and add new mature glochidia for each fish lot (about 10 fish, that will be set into the same container). The remaining glochidia from the anterior infestations can attach to the floating mucus and they are not effective for infestation any more.

## 7.4 Maintenance and water quality control of host fish in cylindroconics until excystment of juvenile *M. auricularia*

**Preliminary remark:** For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume.

**Introduction:** Maintaining a large population of infested fish is the limiting factor for the production of large quantities of juvenile naiads. Success at this stage depends largely on the overall performance of the captive breeding program (Araujo et al. 2015). The method below corresponds to the infestation of sturgeons, but it is similar for other fish species (Araujo et al. 2003, Patterson et al. 2018, Soler et al. 2018a).

The duration of the developmental process of metamorphosis (i.e. until glochidia will fall from the fish gills) depends on the temperature; within a given range, animals develop faster when it is warmer. Therefore, we will use here the "day/degree" parameter, that is, the number of days of the process multiplied by the temperature degrees. In order to predict the "mussel fall" (excystment date) in the best way, it is important to work with a stable temperature and to log the temperature development continuously.

In Spain the metamorphosis of *M. auricularia* takes 635-700 degree-days: 30 days at 23– 24° C or 31 days at 20– 21° C (Araujo and Ramos 2000, Araujo et al. 2002, Araujo et al. 2003) (fig. 16), but other studies seem to indicate that more time is required for metamorphosis: close to 1,000 degree-days (the temperature fell to 18° C in the second half of the process and the last sturgeon of the experiment died on day 51 when juveniles were close to being released), and close to 1,100 degree-days (65 days at 16–17° C). This suggests that metamorphosis accelerates when water temperature is raised to 20° C. However, raising the temperature several degrees above this does not appear to further accelerate metamorphosis and may have a negative impact on the animals. In a similar experiment with *Salaria fluviatilis*, juveniles were released in 42 days at a mean temperature of 19° C (798 degree-days) (Araujo et al. 2001).

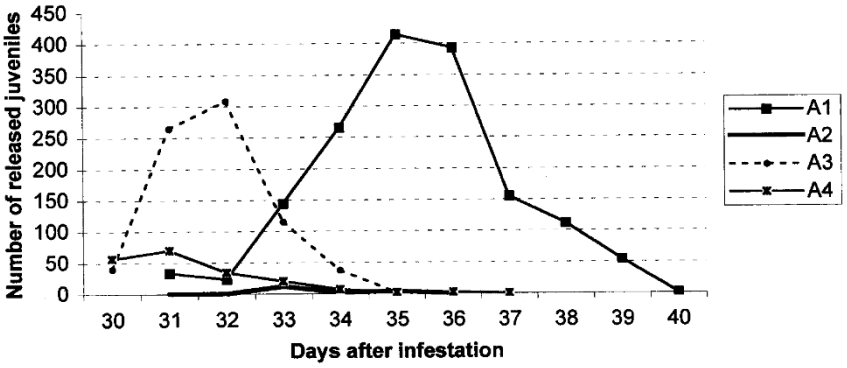



**Figure 16. Release of glochidia from infested fish. A1, A2: blenny. A3, A4: sturgeon (from Araujo et al., 2003)**

In the experiments made in France, in 2016 metamorphosis took more than 39 days (see Table 2). In 2017, the first juvenile fall occurred on May 13th, 29 days could be due to a better management of the temperature that was more and more stable. Prediction of this date is important so that fish in the cylindroconics can be kept

without food at least 3 days (better: one week) before the first day of juvenile appearing. In this moment, the filter system will be changed for the juvenile collecting system (see below).

Table 2. Duration of the juvenile metamorphosis in the sturgeon inoculated with glochidia at the Chinon laboratory .

| | N tanks | Duration of metamorphosis (DD) | Min - Max (°C) |
|---|---|---|---|
| 2016 | 8 | 572 - 669 | 11,8 - 19,4 |
| 2017 | 4 | 531 - 614 | |
| 2018 | 6 | 436 - 460 | 17,8 - 20,6 |

**Method description and time effort**: The process of the metamorphosis of the juveniles depends on the water temperature but is around 20-40 days. Measure and log water temperature of fish permanently during maintenance. Calculate possible excystment date, beginning from the basic hypothesis of at least 450 degree-days and take notes (local populations may differ in their respective developmental speed). Cease feeding 3 days before that date and prepare collection of juveniles (next step, see below). Sacrifice some fish (e.g., one shortly after infestation, then 1 fish per week), and check their gills under the dissecting microscope and analyse the developmental stage of the juveniles in order to monitor the process of infestation.

**Material needed:** In addition to the material listed in 7.1-7.3: thermometer with data-logger.

# 8. Sampling and cleaning of *Margaritifera auricularia* juveniles when excysting from host fish gills

**Preliminary remark:** For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume. This procedure is an optimisation between work effort, fish well-being and number of collected juveniles. At the beginning of the project, we recommend to get permits to starve the fish for a longer period, in order to produce degree-day vs. juvenile release number curves. Check for the presence of juveniles from the cylindroconic tanks until no further juveniles are found for two days. As soon as the optimum degree-day date is known, the fish starving period may be reduced to 3-5 days prior to the degree-day date plus 10-15 days of covering the period of the highest release. Counting and cleaning the juveniles is very labour-intensive, and sufficient man-power of skilled persons should be foreseen for this period. If the juveniles are sufficiently clean (i.e. no pollution by fish faeces), this step can be omitted. Counting can be reduced by just counting aliquota and upscaling the results.

**Introduction:**

The principle of the method is to gain excysted juveniles by filtering the water from the fish containers with a set of different mesh sieves, and wash them from the sieves into a container, from which they are cleaned, counted, and placed into the rearing unit. Juveniles are extremely thin-shelled and small, thus fragile, therefore all activities must be done with great care. Once juveniles have left the fish, every moment counts. The yolk reserves are small due to the minute size (170-200 µm) of the juveniles. The sooner they start feeding, the better, otherwise the mortalities will be dramatic. Avoid any kind of thermal or osmotic shock. Juveniles may die immediately when temperature rises abruptly etc. Keep handling times as short as possible and transfer juveniles asap. into their rearing unit.

**Procedure:** Three (minimum) to seven days before reaching the 600 degree-days date, stop feeding the fish. A 0.5 cm mesh will be installed on the bottom of the tank to prevent fish feeding on the juveniles or to pass through the hole in the filter or collection system. The filters to clean the water will be disconnected and the systems of collection of juveniles will be installed. Every day, 1/3 of the water of each cylindro-conical will be renewed. However, the biological filters will be kept in recirculation mode to ensure their equilibrium (switching them off would cause anoxia in the filters). The filters will be reconnected to cylindro-conical at the end of the juvenile collection process

*How to build a juvenile collector system*

Once the metamorphosis is complete, juveniles sediment to the bottom of the “cylindroconic” tanks, and are drifted into the collectors in each cylindro-conical tank. These collectors have three sequential meshes (500, 250 and 100 μm) to clean the juvenile samples, and they will to clean the water three to five days before the juveniles are born. There are no commercially available juvenile collectors, but they are easy to build (fig. 16). The working principles is that juvenile-containing water runs through (at least) 2 nets, the wider meshed holds back unwanted particles and the 100 μm holds back the juveniles. We used simple funnels (cut-off plastic bottles), with nets fixed with rubber bands to pieces of cut plastic tube (10 cm diameter for the wider mesh, 12 cm diameter for the 100 μm mesh), which allows fast handling. Alternatively nets can be placed in container boxes containing water, in which the water flows laterally across the nets.

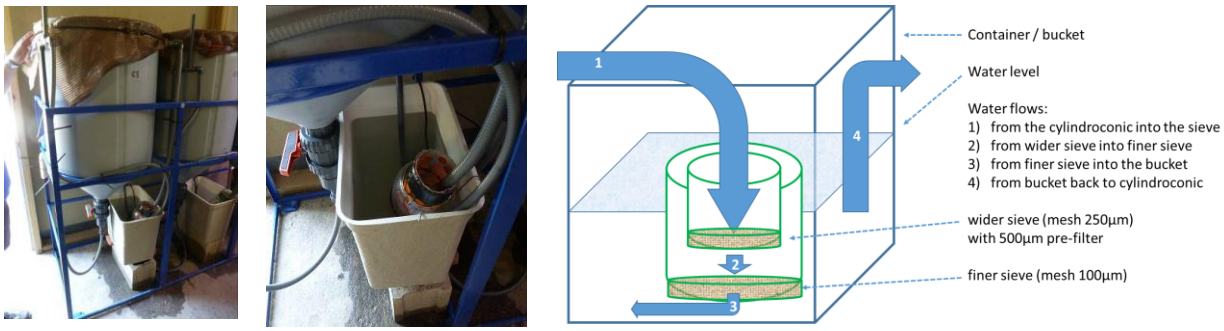



**Figure 16: Juvenile collector system in the cylindroconic tanks (Photos R. Araujo, graph: K. M. Wantzen)**

Water pressure must not be high, rather allow the water to flow slowly across the nets, following the hydrostatic gradient (use a hose clamp to regulate flow). Filtered water is collected and pumped back into the cylindro-conical tank.

The juveniles will be collected in the meshes, then cleaned and separated. This work must be done under a good binocular (the juveniles have a size of about 200 μm).

*How to clean juveniles:* If juveniles are still mixed with organic detritus, you may clean juveniles using 2 needles under the microscope (this is very time-consuming, and risky for juveniles), as juveniles are very fragile. Alternative cleaning treatments: A) Put the juveniles in a sieve, according to their size (for newborns it should be 120 microns) and wash them carefully with natural water from a watering can, while gently moving the sieve. B) Put the juveniles in a little container with sand and water and stir to cause friction between them.

Once the juvenile collection is finished, the fish are returned to basins or the outdoor pools, and are fed normally again. The counting/cleaning procedure includes the following steps (see fig. 17):

1. prepare the replacement filtering unit (containing nets of 250 and 100 µm, and 500 µm pre-filter)
2. carefully remove the filtering unit in use and replace it
3. remove the nets individually, placing the 100 and 200 µm nets in separate glass bowls containing filtered water from the cylindroconic, these contain the juveniles
4. 500 µm nets are washed with the jet of a laboratory bottle, discard content.
5. transport glass bowls with the nets the into the laboratory, wash them carefully and thoroughly with the jet of a laboratory bottle (always use filtered water from the river, this water will also be used for the rearing of the juveniles, avoid thermal or osmotic shocks!) into a 500 ml beaker
6. suspend the juveniles in the beaker and transfer them to a smaller sieve of 100 µm mesh size; cut-off piece of plastic tube, with 100 µm mesh glued to the "bottom" (reduce volume of sample to be analysed). From there, transfer the juveniles into graduated rectangular petri dishes for counting/cleaning.
7. using a magnification of 50-100x under the binocular (cold light is mandatory!), count juveniles (hand counter) and separate them from surrounding debris, using 2 dissecting needles, then transfer them into another small beaker using a 200 µl (yellow tip) pipette, from where they will be transferred into the rearing system. Start with the 100 µm net, then the 250 µm net (you will find juveniles there only if the net has started to clog). Note counting per mesh size, and mesh of individual cylindroconic, and observations (e.g. amount of detritus, presence of other animal species(, we often observed micro invertebrates such as rotifers.
8. All the juveniles from each cylindroconic tank will be kept in petri dishes with river water until they go to the breeding system. The temperature of the water during this process must be maintained.


**Figure 17: Filter cleansing, juvenile separation and counting (Photos: R. Araujo). Upper left : Juvenile collecting unit. Upper right : dismantling the collecting unit and cleaning the nets, Middle left : cleaning the nets, middle right : Cleansing/counting of the juveniles, Lower left : hand-held counter, lower right : juvenile *M. auricularia***

**Material needed:** Juvenile collection system (can be made from plastic bottles, or from plastic tubes of 12 and 10 cm diameter. Nets are fixed with rubber bands. Nets of 100 µm, 200 µm, 250 µm and 500 µm (ca 1m² of each size). Water from the river (filtered to 100 µm before use). Plastic tubes with glued mesh to sieve the glochidia. Binocular, 3 counters , 10 plastic trays (30 cm long), 5 automatic micropipettes (1000 µm (blue tip), 200µm (yellow tip) + tips, filter paper , 4 siphons, 2 buckets, 4 boxes of latex gloves, 20 reticulated glass petri dishes of 10 cm diameter. Laboratory bench with sink and tap with spray hose. Hand paper, 10 permanent markers,1000 Eppendorf tubes, 10 plastic pipettes, wash-bottles, 2 dissecting scissors, 4 normal tweezers (thin tip) , 4 Soft tweezers

**Time effort:** Construction of the collection units: ca 1h/unit. Sample collection (exchange of collection unit): 5 to 10 min/unit. Cleansing and counting juveniles: depends on the degree of pollution (presence of debris in samples) and experience of worker, and the density of the juveniles. Estimate 2-3 hours work at the binocular to separate and count 300 juveniles. To save time, once the peak phase of juvenile excystment is known by experience, focus on counting aliquots during this period and calculate the numbers accordingly. Pre-and post “peak phase” juveniles should also be used, but do not necessarily need to be counted.

**Caveats:** Make tests for the functionality of the collecting unit before starting to work with fish in the cylindroconics. Avoid all kind of shocks resulting from the treatments with water (filtering, transfer...) always use the same type of water. Cleanliness of the juveniles is essential. The less they are in contact with mucus and debris the better. This depends on the period when fish began to be starved (faeces pollute the juvenile samples) and the frequency of filter cleaning (during the peak period, possibly exchange filters twice a day). Consider cleaning of juveniles with small amounts of sediments or by washing them in the 100 µm sieve (control efficiency of the method with the microscope). Remember to transfer juveniles asap to a container, in which food can be given to the juveniles (ideally, put them step by step into the rearing unit, as soon as they have been counted).

# 9. Different rearing techniques for juveniles of *Margaritifera auricularia* : detritus boxes, flumes vs. multiple beakers, substrates, diets, feeding cycles, stirring sediments, observation of juveniles.

**Preliminary remark:** For legal regulations concerning the manipulation of mussels and fish, see chapters 1 and 2, this volume.

**Introduction:** Contrary to the pearl mussel *M. margaritifera*, the ideal raising protocol for *M. auricularia* is still not found. The rearing of juvenile freshwater mussels at the lab is the most difficult tasks in the propagation processes. The loss rates are still too high for a sustainable, large scale reintroduction of *M. auricularia*. Very different rearing systems exist for diverse other mussel species (see Patterson et al., 2018) for a review of rearing techniques). So far, the success in the captive breeding of *M. auricularia* juveniles in France was limited to a survival no longer than 100 days, while experiments with juveniles from the Ebro river (Spain) obtained about 50 juveniles kept alive from the cohorts of 2014, 2015, 2016 and 2017. (Nakamura et al. submitted). The largest exceeds 2,5 cm shell length (Nakamura et al. 2018b) and the first experimental system are being prepared in semi-captivity to test whether these juveniles, born and fed in captivity, are able to start feeding on their own under semi-natural conditions (Gobierno de Aragón. Unpublished data).

Based on a morphological analysis of newly formed juveniles of *M. margaritifera* (Margaritiferidae) and *Unio mancus* (Unionidae), it has been demonstrated that a second metamorphosis, consisting of drastic morphological changes, occurs that leads to suspension feeding in place of deposit feeding by the ciliated foot (Araujo et al. 2018). This suspension feeding, in these two species, improves due to a gradual development of several morphological features like the contact between cilia of the inner gill posterior filaments, the inner gill reflection, the appearance of the ctenidial ventral groove and the formation of the pedal palps. Regardless of the presence of available food, a suspension feeding mode replaces deposit feeding, and juveniles unable to successfully transition morphologically or adapt to the feeding changes likely perish. The transition from pedal feeding to filter feeding occurs around 150-200 days post-emergence in *M. margaritifera* and around 70 days in *U. mancus*, after juveniles are greater than 1 mm in length, which coincides with the timing of high mortality (Araujo et al. 2018). Once this feeding metamorphosis is complete, juvenile mortality decreases. Of course these morphological and ecological changes do not occur at the same time in all freshwater mussels (Patterson et al., 2018) but we suppose a similar pattern in *M. auricularia*.

Here we suggest two different types, which may be combined, A) the detritus box or multiple beaker system and B) the artificial flumes.

The advantage of keeping mussels in smaller containers are, that they can be better observed, oxygenation and filtering are not needed, the disadvantage is that there is no water flow that would transport the food to the juveniles. In artificial flumes, due to the minute size of the juveniles, they may end up in a filter by themselves. Protection of juveniles against being collected by the filter is given by fine mesh gauze, which, in turn reduces through-flow. Therefore we recommend the box or beaker system for the earliest stages (until 1 mm size of juveniles), then the artificial flumes for larger animals.

In all cases, the important parameters to consider are (i) water quality (origin of water, if and how filtered) (ii) sediment size and chemical state (specifically, how to avoid anoxic/hypoxic layers), (iii) food quality and dosage, (iv) methods to observe the success of the breeding by measuring vital parameters of the juveniles.

We suggest to maintain the juveniles in captivity until they reach at least 3-4 cm length, when they will be used for natural repopulation. Further research is needed to improve the efficiency of the system.

## 9.1. "Detritus boxes" and Multiple beakers (drop by drop systems)

**Method description**: For detritus boxes (see Eybe et al., 2013, for method details), practically any type of transparent plastic or glass container can be used. Due to the large surface and short diffusion pathways, oxygenation from the water surface is generally sufficient. Water movement by stirring is recommended when food is applied.

For the preparation of the containers, they become filled with a small amount of sterilized river sediments (less than 1 cm high), and river water (less than 5 cm high) and are pre-colonized with an inoculum of river bacteria for at least 2 weeks prior to use. Control colonisation (microscope) regularly to avoid presence of other competing or predatory invertebrates. Freshly excysted juveniles are carefully transferred into the boxes (be sure that there is no difference in water quality and temperature) using a beaker glass. Feeding occurs regularly (daily or once in 3 days), avoiding overdosage, which would result in excessive bacterial development and anoxia. Exchange (decant) ¼ of the water regularly. The development of anoxic or low oxidized zones (indicated by dark zones in the sediments) and of bacterial

films at the water surface must be controlled regularly, if this occurs, larger parts of the water have to be exchanged, or the juveniles placed into another box. To avoid the development of thick biofilms on the sediment surface, scratch it very carefully with a plastic spatula. Juveniles can be separated from the sediments by elutriation (carefully stirring up the sediments, then pouring the supernatant in sieves), the devices for juvenile collection can be used for this. Water quality should be logged permanently (at least, temperature and oxygen), and nitrite values checked regularly (every 2$^{nd}$ day).

The culture of *M. auricularia* juveniles in Aragon, Spain was based on the "detritus boxes" methodology developed by Eybe et al. (2013) for *M. margaritifera* and that will soon be published. Presence of substrate, detritus, phytoplankton and use of river water are the main factors for a successful culture for *M. auricularia* juveniles. Others variables like density, feeding rate and type of container may influence in the survival of juveniles. A low density is recommended (100 juveniles per box), glass container for greater hygiene and a daily instead of weekly feeding (Nakamura and Elbaile, 2017).

For the rearing experiments made at Chinon in 2017 (Soler, unpublished report 2017), we maintained for 100 days a population of 1000 juveniles of *M. auricularia*. We used circular glass boxes (14 cm in diameter) but we did not used pulsed flow-through system for feeding. Each box was loosely closed with a cover to allow air exchange and stored at a constant temperature in a conditioning cabinet (Liebherr WK201, Germany) of 18 °C. Each box contained a thin layer of 3 mm of fine substrate (80-650 microns), 200 young mussels in 400 mL of river water (from River Vienne) that was previously treated with biomechanical and ultraviolet light filters and kept at 18ºC.

In order to avoid bacterial growths of whitish films on the water and in the sediment of the containers, which would have endangered the survival of the juveniles, we cleaned the water and sediment 3 times a week. For this cleaning, the empty containers were washed with diluted bleach and then rinsed thoroughly with river water. The content of the box were slightly removed by gentle circular movements and the supernatant was passed through a 100 µm mesh filter. Then the box was filled with river water. After repeating this operation three times, the material retained in the filter was transferred with the aid of a wash bottle to a petri dish and the juveniles recovered with a 1 ml micropipette under the stereomicroscope. The rest of the sediment that remained in the box was transferred to the 100 µm filter and washed with 500 ml of river water applied with moderate pressure, with the help of a wash bottle. Once clean, the sediment was transferred to a new clean box with renewed water and the corresponding food dose. Every 15 days juveniles were counted and measured and the dead specimens removed. The measurements were made under a stereomicroscope.

The pulsed flow-through system is based in toxicological studies and was developed for mussels by Dr. Chris Barnhart (Missouri State University. USA, (Barnhart 2006). Although we have not used it, it may be useful for juvenile rearing of *M. auricularia*. The juveniles in the container, with sediment, are fed with a pulse of flow every 60-90 minutes to exchange water, remove waste and introduce food. The system is used for up to 1000 juveniles in a container of 250 ml (Patterson et al., 2018). Beakers containing juveniles (without sediments) are placed on racks (fig. 18), and receive food a food-water mixture. On the upper shelf of the rack, a water container and acclimatised food container are placed. The food stock solution has to be freshly prepared, suspended (stirred) and cooled. The amount of liquid from both containers is controlled by solenoid valves. A peristaltic pump pumps the food-water mixture via manifolds to the beakers containing the juvenile mussels. Overflowing water runs from the beaks of the beakers into plastic trays, and from there into the recipient container on the lower part of the rack (Barnhart, 2006)

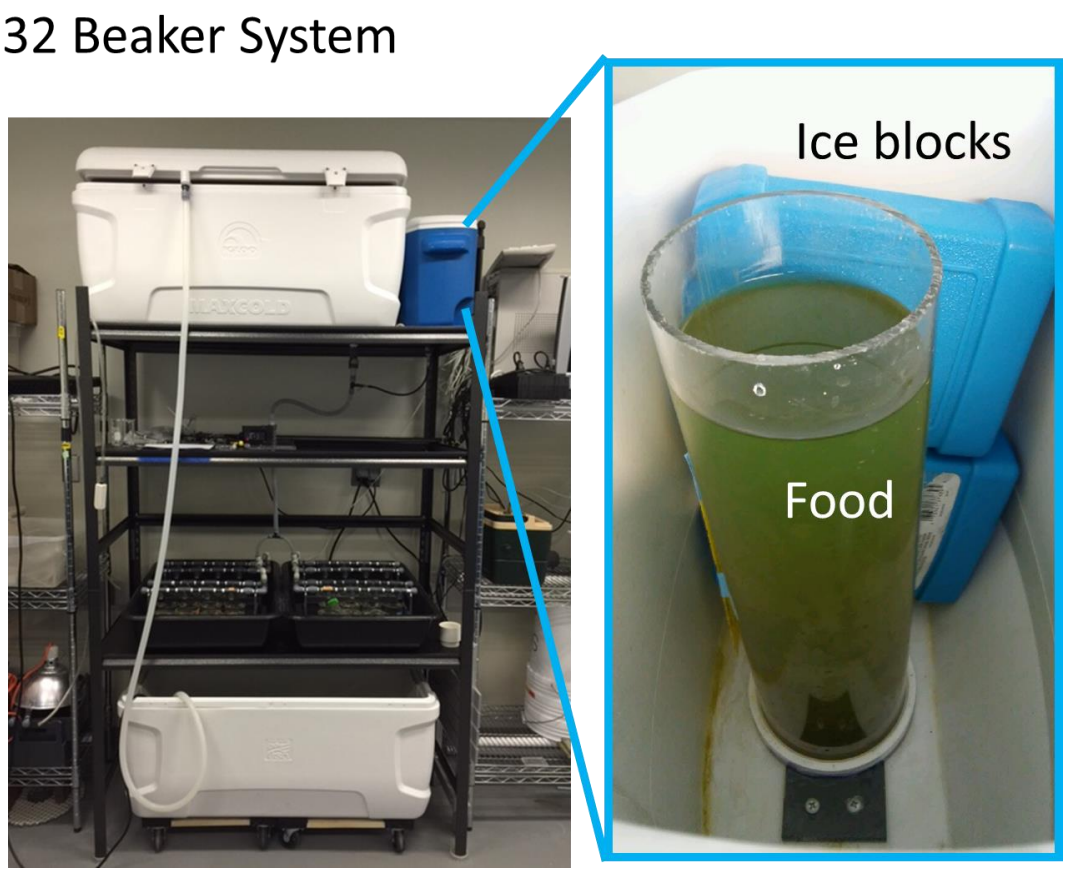



**Fig. 18 A 32-beaker mussel juvenile rearing system developed by Dr. Barnhart (photos)**

**Material needed:** Plastic containers. Climate chamber or conditioning cabinet. River water. Shower faucet. Bleach (to sterilize equipment). Food. Calibrated sediment. Stereomicroscope. For the pulsed flow-through system: Rack, beakers, peristaltic pump(s), manifolds, solenoid valves, water and food containers, plastic trays.

**Time effort:** Plan sufficient time for sieving and purchasing sediments, natural and artificial food. Regular (daily, or every 2nd to 3rd day) activities include: preparing food solution and feeding, controlling water quality, clean the sediment. The precise time effort depends on the number of rearing containers.

**Caveats:** As juveniles are filter- and/or benthic particle feeders (naturally feeding on debris suspended in the river water, or naturally produced in the rearing system), the cleansing of the water is a difficult task. Use of unfiltered natural river water risks to clog pore space in the sediments quickly). Filtering may be necessary, but means removal of food particles. Avoid sharp-edged sand (which is often sold in garden markets), river sediments are rounded and better for use. In drop-by-drop systems with a closed loop, the risk of cross-infestations is high. River water is a permanent source of hygienic problems. Unwanted organisms (other invertebrates that compete with, or prey upon juvenile mussels) may occur in the culture. Therefore, the system proposed by Dr. Barnhart works with tap water, or sterilized river water, in a flow-through system. Due the small size of the recent born *M. auricularia*, the monitoring of the rearing systems is very difficult.

## 9.2. Artificial flumes

**Method description:** Inspired by other projects working on pearl mussels, we have designed some types of artificial flumes to maintain the juveniles (Figures 19,20). Our flumes have outer dimensions of 250cm length, 60 cm breadth, and 20 cm height (a verifier). In the mobile lab, we managed to place 3-4 channels above each other in 1-2 columns (see chapter mobile lab).

Each flume has an individual water circulation and filtering system, which allows manipulation of sediment and filtered vs. unfiltered water from the river. At the “downstream” end of the flume, a 150 µm mesh net prevents juveniles from being collected by the filter. Due to the spray effect of the water inlet (little water jets that pass the air, in order to oxygenate the water), substantial volumes of water can be lost and are immediately replaced by coupling the flumes to the water storage via a gauge-meter that can trigger a pump. Additionally, each week 30-100% of the water of each flume should be renovated.

The large breadth allows to split each channel in the middle, e.g. to vary substrate type, but maintain the same water. While in some trays external food is not added, and juveniles may grow with natural food coming from the natural river and sediment (Araujo et al. 2015) in other trays several external food (algae, detritus,…) and artificial sediment according to the experimental design are added until the optimum conditions have been identified. For these experiments it is possible to use compartmentalized trays for different types of substrates and/or extra food. We will experience with each of these designs to see which is best for breeding of the juveniles.

There are many reports about the change of indoor to outdoor systems because in outdoor systems the growth is always better (see Patterson et al., 2018). In the case of *M. auricularia* it would be good to develop natural channels in outdoor laboratories to maintain the populations of juveniles. In the case that these channels had natural water from the river, the juveniles could use natural food from the "wild water" (see Patterson et al., 2018).

If we maintain indoor rear systems and artificial feeding for the juveniles, the best chance is to mix commercial algae with natural detritus using continuous feeding. Mussels in the wild ingest particles less than 28 µm in diameter (algae, detritus and bacteria mixed). In our indoor systems the better is to fed the juveniles with particles between 1-10 µm in diameter. The algal doses (see point 9.3) can be doubled after the first month and tripled after 6 months.

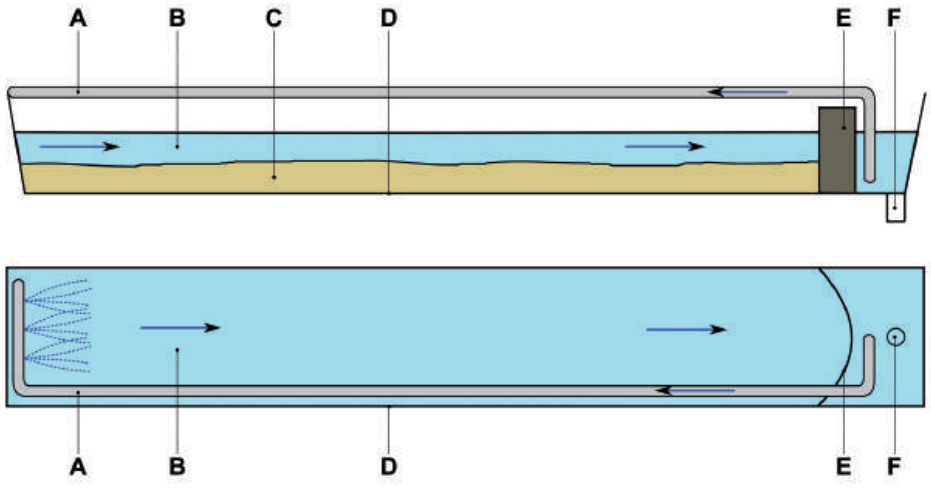



**Figure 19 Artificial flumes. Above, side view; below, top view (A: water return pipe fed by a pump; B: water filtered at 36 µm; C: aquarium substrate; D: plastic tray; E: grille holding the substrate to one side; F: trough purging system). (Source: Freshwater Pearl Mussel Conservation in the Armorican Massif. Programme Report, 2010–2016)**


**Figure 20. Pictures of the artificial flumes used at Chinon (photos: Araujo)**

In the following, we describe an experimental approach taking place in June 2016 (Soler 2016).

Between the end of May and the beginning of June 2016 28,292 juveniles of *M. auricularia* were collected at the Chinon lab. The average size of the new-born juveniles was 144 µm in length and 149 µm in height. The live juveniles were placed equitably in 4 rearing systems.

In each of the rearing systems, the following 4 different treatments were applied:

1. Natural water (Vienne River, without filtration) + natural sediment
2. Natural water (Vienne River, without filtration) + sieved sediment
3. Water (Vienne River) filtered and UV sterilized + natural sediment + algae
4. Water (Vienne River) filtered and UV sterilized + sieved sediment + algae

Where:

Natural water means water from the Vienne River, without filtration.

Natural sediment means sediment from the river without sieving (Figure 16).

Sieved sediment means sediment from the river sifted by 2 mm and sterilized by heating at 80ºC during 24h (fig 21).

Water (Vienne River) filtered and UV sterilized means water from the Vienne River filtered at 90 µm and sterilized with UV light.

The “algae” treatment was a mixture of commercially-sourced(ReedMariculture©) microalgae. A combination of Shellfish Diet (1,4 ml) + Nannochloropsis 3600 (0,6 ml) where applied weekly (fig. 21).


**Figure 21: Above Natural (left) and sifted sediment (right) used in the rearing systems. Below (left) commercial shellfish diet (right) *Nannochloropsis* used in the rearing systems (Photos: J. Soler)**

The systems were installed in a room without air conditioning. The temperature of the water in the systems was monitored by means of probes that collected data every 30 minutes.

In order to control the growth and survival of juveniles, in each breeding system a proportion of juveniles were placed in a sector delimited by visual marks made on the edges of the channel. For these revisions, a sediment sample from the delimited sectors was obtained from the breeding systems and placed on a petri dish. The juveniles found were photographed under a stereomicroscope coupled to a camera and measured with an image processing software.

The first revision was made on 07/18/2016 when the juveniles had about a month and a half of life (Table 3, fig. 22). In this review, numerous live juveniles were found. The mean size in system 2 (Natural

water (Vienne River, without filtration) + sieved sediment) was 274,66 µm in length (n = 18) and 271,19 µm in height (n = 21), which is a growth of 90% and 81% respectively. The average size in system 4 (Water (Vienne River) filtered and UV sterilized + sieved sediment + algae) was 310,69 µm in length (n = 16) and 316,44 µm in height (n = 26) which it represents a growth of 115% and 112% respectively. These results suggest that the growth was greater in the treatment with algae.

Table 3. Results of the measurements of the *M. auricularia* juveniles in two rearing systems in 2016 after 45 days of culturing in the flumes

| Date | Rearing system | Live juveniles | Average length | Average height |
|---|---|---|---|---|
| 18/07/2016 | 4 | 27 | 310,69 (n=16) | 316,44 (n=26) |
| 18/07/2016 | 2 | 22 | 274,66 (N=18) | 271,19 (n=21) |

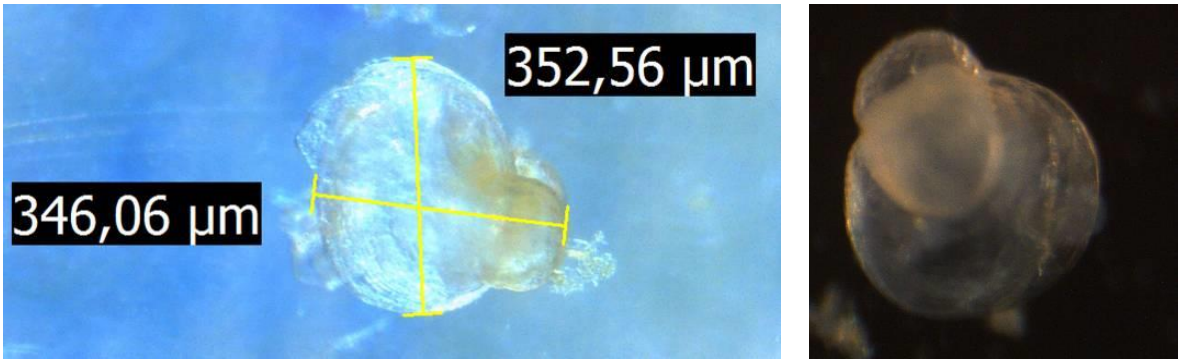



**Figure 22. Juvenile specimens measured on 07/18/2016 (Photos: J. Soler)**

The next revision was made on 08/29/2016. On that occasion no juveniles were found alive. Similarly, in the following revisions, carried out on 10/18/2016 and 10/01/2017, no juvenile was found alive in the breeding systems.

The breeding systems were maintained regularly. On 11/05/2017 System 2 was drained (with sieved sediment) and all the sediment was screened through a 2 mm sieve. All material superior to that size was reviewed but no juveniles were found. The remaining systems were emptied in September 2017. System 4, also with sieved sediment, was processed in the same way as system 2. In the rest of the systems, we

used sieves of 1 cm, 4 mm and 2 mm. Material greater than one centimetre was washed with river water under pressure and the detached material was passed to white plastic trays for review under hand magnifiers. The rest of the material was sieved between 2 and 4 mm and this material was also passed to white plastic trays and analysed under hand magnifiers. No more juveniles were found.

**Material needed:** Plastic flumes including pumping and filter system. Sieves. Filtered river water, automatic water supply from a reservoir. River sediment of defined size (2-4 mm is recommended). Stereomicroscope for the observation of juveniles.

**Time effort:** Ideally, the entire system is running one year before the first glochidia are produced. Similar to preparing fish tanks, consider 4 weeks of "empty running" of the flumes, prior to establishment of the mussels. Plan sufficient time for sieving and purchasing sediments, natural and artificial food. Regular activities include: preparing food solution and feeding (1x 15 + 1x 30 min/day), controlling water quality (nitrate: depending on device, ca 30 min per flume per 48h), clean the sediment, look for live juveniles.

**Caveats:** River water is a permanent source of problems. Unwanted organisms (fungi, parasites, other benthic organisms) may occur in the culture. During dry seasons, the pumping station may be too low to be reached, during floods, very high amounts of sediments need to be filtered, but then the river water does not contain natural food items any more (exception: bacteria). When establishing the system, take care with the weight of the sediment and water filled flumes, these must be placed on heavy duty supports. Due the small size and the interstitial life style of the early juvenile stages of *M. auricularia*, the monitoring of the rearing systems is very difficult, almost impossible. We therefore recommend this rearing method for animals larger than 1mm.

## 9.3. Types of diets

**Introduction:** Apart from the process of fish infestation, the provision of an adequate diet for juveniles is a key problem that must be solved. The success of Lefevre & Curtis (1912), Coker et al. (1921) and Howard (1922, all cited in Hruska 1999) in rearing juveniles of several species was dependent on the use of the water, food and sediment present in the natural ecosystems of the mussels. The idea that these elements were necessary was confirmed many years later in Europe by Hruska (1999), who hypothesized that the food required by *M. margaritifera* juveniles comes from a healthy rhizosphere of the riparian vegetation. This means that eutrophication, contamination and silting of the immediate environment were deemed responsible for the absence of available habitat, juvenile food and the recruitment of

young mussels. The success of Hruska (1999), who grew juveniles of pearl mussel to >5 cm, relied on river bank restoration and a semi-captive breeding system that provided for natural feeding.

It is important to mention the experience of Preston et al. (2007) who used a semi-natural system for rearing *M. margaritifera* using natural water and sediment. Although they did not recover all the bred juveniles, they had very good result in the growth and survival of 2 years-old juveniles.

In Spain, Comas and Valls (2007) grew juvenile *Unio mancus* Lamarck, 1819 to reproductive age in a system involving minimum management that made use of natural water and sediment - but not from the river were the mussels normally lived - without any extra nutrients. Other authors have developed more controlled systems (with more or less success) inspired by systems used in marine bivalve aquaculture, providing extra food in the form of algae (Araujo et al. 2015). These more controlled systems have inspired the main cultivation programmes for *M. margaritifera* in Europe (Gum et al. 2011, Eybe et al. 2013). Although the use of algae has sometimes been successful in the rearing of presumably healthy juveniles, Nichols & Garling (2000, 2002) report the main dietary source of carbon for naiads living in rivers and lakes to be bacterial. Algae do, however, appear to provide key nutrients such as vitamins and phytosterols. Much remains to be learned about the diet of juvenile naiads in natural environments and in captivity. The cultivation systems connected to the natural habitat of naiads could provide the unknown natural food required by the juveniles (Preston et al., 2007; Araujo et al., 2015).

Good results with other unionoid species were attained with 20,000-30,000 cells (algae) per ml, but also with doses between 100,000-500,000 cells per ml (Patterson et al., 2018). Working with *M. margaritifera* during one year, Araujo et al., (2018) used the following method based on other authors: 400 juveniles were maintained in a box filled with 475 ml of river water and 25 ml of detritus. Juveniles were fed algae once weekly during a water exchange. The algae consisted of 120 µl of Shellfish Diet 1800 (*Isochrysis sp., Pavlova sp., Thalossiosira weissflogii* (Grunow) and *Tetraselmis* sp., with a diameter of 4-20 µm) and 200 µl of *Nannochloropsis* sp. (1,5-2 µm) suspended in 10 l of river water (Eybe et al. 2013). The boxes were kept in a conditioning cabinet at a constant temperature of 17 ºC. The algal diet was doubled after the first month and tripled after 6 months.

**Method description:** For the rearing experiments made in 2017 (Soler, unpublished report 2017), the mussels were fed with different food diets three times per week during water exchange that consisted of algae, detritus, algae + detritus, egg yolk and bacteria. The presence of substrate is of great importance since it facilitates the cleaning of the valves of the juveniles. The survival was greater in the algae +

detritus treatment followed by the detritus treatment, which indicate that the detritus is of great importance for the maintenance of good physical-chemical conditions in the culture medium. The highest growth rates were in the treatments, in which algae were used as a food source (algae and algae + detritus treatments), suggesting that the algae provide a combination of nutrients suitable for the development of juveniles. However, in the egg treatment, growth rates were comparable to those reached in the treatments that included algae up to 75 days after the excystment. This suggests that the egg yolk is potentially a food resource with which juveniles can develop.

The detritus box methodology (Eybe et al. 2013) is currently used in Spain with juveniles of *M. auricularia* obtaining significant advances in the captive breeding for the first time (Nakamura et al. (2018a), Nakamura et al. (2018b)). A multifactorial experiment was carried out since 2014, with different treatments ("detritus boxes") depending on the combination of adding substrate, detritus, phytoplankton or extra aeration to filtered river water. Live juveniles born in 2014 -2015 and reached 2,5 cm in length, they are still maintained. In the same way, around 40 juveniles born in 2016 and 2017 are keeping up and growing at a good pace. The percentage of final survival using this methodology is not high, as stated elsewhere (Lavictoire et al. 2016), so it must be continued to test new combinations that allow a greater survival in the detritus boxes (Eybe et al. 2013) and the next phases (Nakamura et al. 2018a, Nakamura et al. 2018b).

**Material needed:** In addition to the rearing system (see 9.1, 9.2), different types of diet should be tested. Stereomicroscope for the observation of juveniles.

**Time effort:** Plan sufficient time for sieving and purchasing sediments, natural and artificial food. Regular activities include: preparing food solution and feeding (1x 15 + 1x 30 min/day), controlling water quality (nitrate: depending on device, ca 30 min per flume per 48h), clean the sediment, check for live juveniles.

**Caveats:** The ideal food composition is still one of the least known details about captive breeding of *M. auricularia*. Experimental studies in Chinon (Soler et al. unpublished) and Aragon (Nakamura et al. submitted) have evidenced the need to add natural detritus, and have proven independently that commercial algal food can be used. The efficiency of the different diet elements and their proportions requires further studies, not only concerning body size, but also concerning fitness parameters such as fatty acid or protein contents of the juveniles, and – on the long term – to check if artificially reared animals reproduce successfully, and which influence the rearing conditions have on their reproductive success. Further studies are urgently needed.

## 9.4. Monitoring of captive juveniles

**Introduction:** Monitoring of juveniles is another important step in the propagation process before releasing them into the wild. *M. auricularia* has among the smallest known juveniles among the Unionoidea (less than 200 µm), and the juveniles have a much shorter residence time in fish gills than in *M. margaritifera* (4 weeks vs. 8-9 months). As a result, freshly excysted juveniles of the GFPM are extremely sensitive. In addition to their high sensitivity to environmental parameters such as temperature, oxygen content etc., risks include i) smashing during manipulation or transfer between containers, ii) to become overgrown by bacteria and biofilms, iii) to become glued to substances (they easily get stuck in the extracellular polysaccharides and mucus produced by bacteria and oligochaetes), iv) infestations, e.g. by aquatic fungi, v) predation by or competition with other invertebrates, vi) starvation (the body reserves are minimal, animals have to eat from the first moment onwards), and vii) problems occurring during the (yet little known) secondary metamorphosis from pedal feeding to filter-feeding.

**Method description**: Juvenile growth and survival of the juveniles must be monitored regularly. This can serve to compare with obtained results in other facilities. To evaluate the success, it is important to record how many of juveniles that were placed into the rearing container survived. Measure shell length every 2-4 weeks or at least, with consistent intervals. At the same time if possible, measure also the survival or mortality of each system. The sampling of the reared juveniles depends on the rearing system used. In small containers, the content of the entire container is elutriated and sieved (fig. 23) Increase mesh size according to growth). In the case of artificial flumes, the area where the juveniles were released are marked, and aliquots of sediments can be sampled with a spoon, or subsamples of juveniles are placed in Buddensiek cages (see below: release techniques), that are regularly checked.

**Figure 23. Cleaning of the container with juveniles of *M. auricularia* (Photos: R. Araujo).**

**Material needed:** Stereomicroscope. Pipettes. Petri dishes. Spoon. Siphon. Plastic jars. Nets: 200 µm, 100 µm.

**Time effort:** Estimate 30 minutes to check for juveniles in one small container or one cage inside an artificial flume. Growth should be checked in monthly intervals for small stages, and 3-monthly when they have reached 1 cm in size. Maintain observations at least until juveniles attain 2-4 cm in length.

**Caveats:** Monitoring of *M. auricularia* captive juveniles should only be done by expert hands. Juveniles are very small and fragile. Small changes in the environment of the juveniles can easily cause mortality. Too frequent sampling in monitoring can affect negatively growth and survival. Therefore, only subsamples should be monitored, and effects of monitoring should be tested.

# 10. Identification of reintroduction sites

**Preliminary remark:** Remember that transport and release of juveniles requires special permits by the regional and national authorities for conservation of biodiversity (in France: DREAL-DDT) and that preliminary analysis would be needed in countries where the species is considered extinct for long (Bundesamt für Naturschutz). This may cause delays. This chapter rather describes the methods of identification, due to the fact that currently juveniles with a shell length less than 6 cm are hardly found in nature. Thus, future studies may add further information.

**Introduction:** Should freshly excysted juveniles of pearl mussels land on clean gravel or sand substrate, they will likely survive and start to grow. But if they land in unfavourable substrates, such as mud or silt, they probably die. Juveniles are also likely to perish if they have not developed to a sufficient size in the host fish. High levels of ammonia, nitrate, phosphate, sodium, potassium, calcium, and magnesium - all of which are chemicals commonly introduced to rivers by humans - also have a negative impact on juvenile survival. For reasons that are yet not fully understood, juvenile pearl mussels growing in locations with sizable aquatic insect (Chironomidae) populations are more likely to survive (Degerman et al. 2006).

Finding the ideal reintroduction site is very difficult. We have currently no clear idea about the habitats of the earliest life stages of *M. auricularia* in nature (probably they live in the hyporheic interstitial), nor do we know about the potential movements of juveniles between habitats (but we suppose that they do not move more than 10-20 meters). Therefore, as a general rule, we propose to release juveniles to places that have uncolmated, well oxygenized, at least 50 cm deep, and well mixed substrates, found at sites with a relatively low probability of strong sediment turnover. Sites below dam removal projects, sites with constrained layers of uncolmated sediments, or clay or above concrete channels should be avoided.

Following to what has been proposed for other freshwater mussels, the criteria in the selection of suitable sites for young mussel reintroduction should include the following:

- *Wild population functional / viable*
- *Reach(es) with suitable water quality*
- *Site(s) with suitable macrohabitat*
- *Spot(s) with suitable microhabitat*
- *Water quality, fish populations and microhabitat remain within the tolerance range for 3-5 years*

*Margaritifera auricularia* mainly lives in the lower sections of rivers and large streams. Surveying this habitat is challenging because it is often deep, turbid, strongly flowing and navigable. For this reason, there has been little work done on characterizing the micro and macro habitat of adults, and even less on that of juveniles.

In the LIFE project, the habitat requirements for *M. auricularia* were assessed, water quality average data include average values for Temperature (15,2°), pH (8), dissolved oxygen (9.6 mg/l), conductivity (438µS/cm), total phosphorous (0,07 mg/L), nitrates (12,5 mg/L), nitrite (0,04 mg/L), orthophosphate (o,1 mg/L), ,calcium (64 mg/L), organic carbon (3,4 mg/L), and the sediment structure in more than 70% was sandy-gravellish or gravellish, with moderate flow velocities (LIFE report, unpublished). Araujo and Ramos (2000) described the habitat of the Spanish populations on the Ebro Basin (Spain) this population in terms of water quality and substrate. Although more thorough studies are needed on habitat, the granulometry data suggest that *M. auricularia* numbers fall when substrates with a predominance of finer materials (gravel, sand, mud) increase and there is a relative decrease in pebbles. Regarding the water quality, the Ebro population lives in waters which are generally subsaline (having moderate conductivity), basic, well oxygenated and mesotrophic with moderate levels of phosphorus and organic matter (BOD5). This contrasts clearly with the conditions favoured by *M. margaritifera*, which are typically fast flowing, clean and oligotrophic rivers with low calcium levels.

Additionally, the nature of both the sediment and interstitial water impact greatly on the health of mussel populations and the possibility of recruitment. According to Geist and Auerswald (2007) they are the best physical parameters to describe mussel habitat. The stage during which young mussels burrow completely into the sediment is probably the most critical life cycle phase for this species so it is important that the sediment be relatively free of organic matter, permitting exchanges between free-flowing and interstitial waters.

The current velocity in low-water periods should be strong enough to ensure water oxygenation and to prevent the substrate from becoming clogged by particle settling. In the case of *M. margaritifera*, for the low-water period, Moorkens and J. Killeen (2014) described an optimal current speed of around 0.30m/s near the riverbed. During high water periods, with a rise in flow (and current speed), mussels tend to burrow more deeply into the substrate; however, under certain conditions, some individuals may be dislodged by the current. Several authors have indicated that critical shear stress could be useful in evaluating sediment stability.

**Method and site description**: As we currently have very little information about the habitat for small *M. auricularia*, we will include a "evolutive" strategy for selection on the release sites. To support this information, a field campaign was conducted in September 2017 to characterize the habitat of *M. auricularia* in a river section where young animals were observed. Given the difficulty of working in environments of great depth and current velocities like those of the Charente, it was decided to conduct this study in the Vienne River.

The study has focused on station 29-Sauvegrain, located on the river Vienne downstream of Chinon (fig 24). During surveys carried out at the Vienne and Creuse Rivers in the summer of 2016, the exact position of specimens of *M. auricularia* was located using a centimetre GPS (fig. 25). In this station, specimens of *M. auricularia* were located and many of them had a size less than 11 cm. Although not considered juveniles, their age have been estimated to be 15-20 years old. Interestingly, the specimens located in this station (as in most of the stations surveyed in the rivers Vienne and Creuse), were distributed in an aligned way close to the shoreline in coarse grained substrates and no specimen was found beyond 25 m from the left bank. The hypothesis to verify is that these zones have the following characteristics: 1) the substrate is stable (not mobilized by floods); 2) not affected by summer drying; 3) are subject to intermediate stream velocities (not too high so that the substrate is stable and not too low so that no fine particles settle that can clog the substrate).

**Figure 25. left Downstream view of the study area. Right: Upstream view of the study area (Photos: J. Soler)**

**Figure 25. Centimetre GPS used for precise location of mussels. (source bs-tech)**

**Methods**:

The characterization of the study area was carried out in 3 levels as shown in fig. 26:

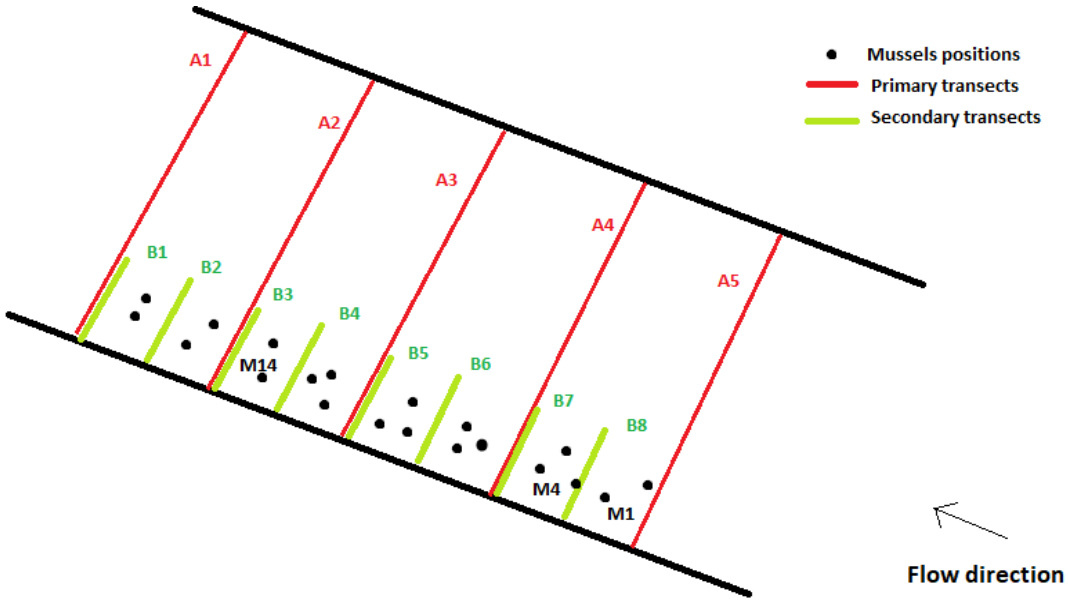



**Figure 26. Schematic representation of the three study levels (graph: J. Soler)**

1- Primary transects :

A 200 m section of the river containing the mussels was studied. The stretch was divided by 5 primary transects which were placed to cover the entire width of the river (ca 100 m). Along each primary transect, sampling was carried out systematically, with stations placed at intervals of 5 m. On each of the profiles, every 5 m the depth (h), the shear stress and the sediment type were measured during the low water period.

To estimate shear stress, the velocity of the current was measured at the bottom level and at 80% of the water depth. For this purpose, an Acoustic Doppler Velocimeter (ADV) current meter was used (fig. 27).

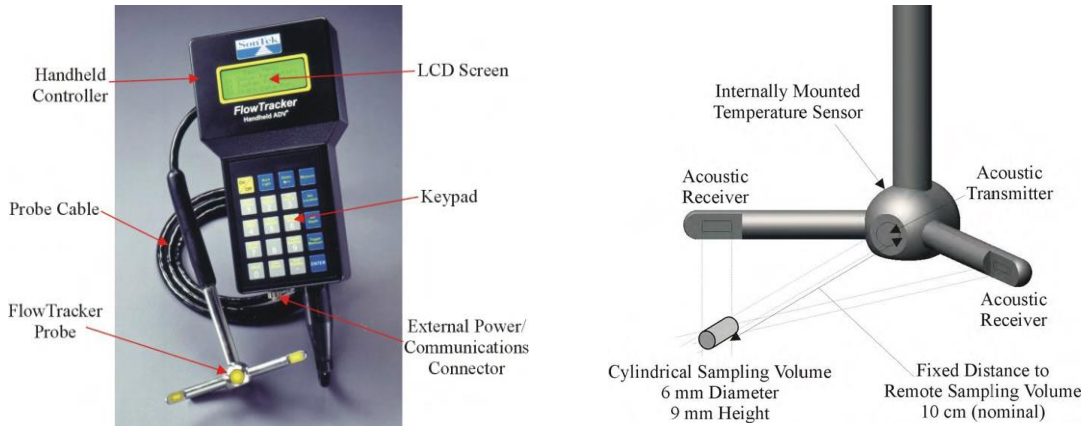



**Figure 27. ADV current meter used for measure water velocity (source: NovoTec)**

To characterize the type of sediment, an underwater photograph was taken by means of a camera anchored to a pole associated with a grid of known dimensions (fig. 28). Additionally, a surface sediment sample of approximately 1.5 kg was obtained with a hand net with 200 µm mesh size (fig. 29). In order to characterize the sediments, they were photographed and analysed visually beforehand and a selection of samples will be analysed in the laboratory. Sample selection was based on stratified sampling.

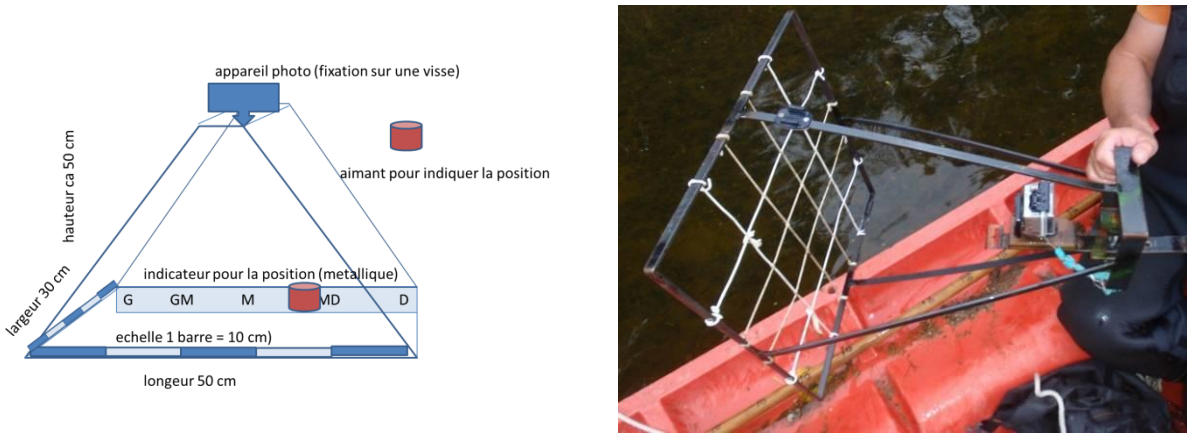



**Figure 28. positioning of the standardised photo-grid. Graph: Karl M. Wantzen, Photo: J. Soler**

**Figure 29. Sediment sampling Photo: J. Soler**

2- Secondary transects:

In the left bank area, where the mussels were geolocated in 2016, 8 secondary transects of 20 meters (from the river bank and perpendicular to the current) were established every 20 m. Along each secondary transect, sampling was done systematically with stations placed at intervals of 2.5 m. During the low water period, sediment type, depth and shear stress were measured following the same procedure as that used in the primary transects. However, all the sediment samples were preserved for subsequent granulometric analysis.

Additionally, in each station the redox potential of the water (20 cm above the bottom) and that of the sediments (at 5 and 10 cm depth) were analysed. Likewise, measurements of pH and conductivity in the water column and interstitial water obtained at 10 cm depth were taken. To measure redox potential, a WTW 3110 pH meter was used together with a platinum probe1 and a control Ag/ AgCl probe (fig. 30). A HI 9828 multiparameter meter was used to measure conductivity and pH. A syringe attached to a plastic hose with a metal tube at the end was used to sample interstitial water.

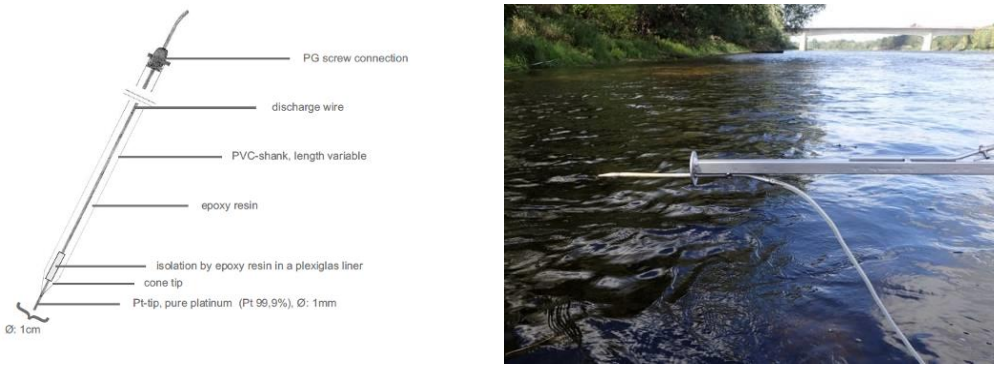



**Figure 30. Device used for Redox Potential (left) and interstitial water measurements (right) Photo: J Soler**

3- Substrate and current velocity near mussels:

At the location of the geolocated mussels, a 1-2 kg substrate sample was taken. In order to minimize the impact on the mussels, the samples were taken 15 cm downstream from the position of the mussels. Measurements of current velocity (at the bottom and 0.8 * h), depth, conductivity , pH and red-ox potential were taken following the same procedure as described for secondary transects (fig. 31).

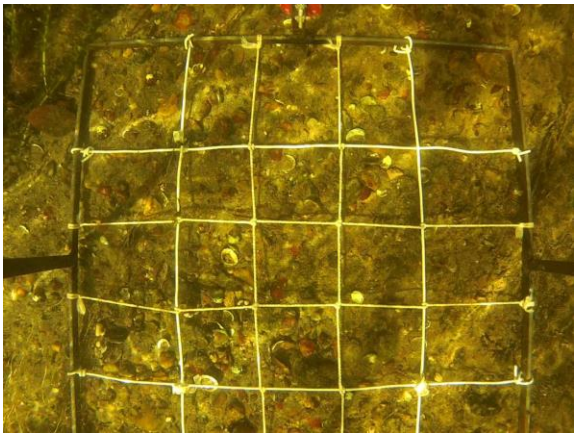
**Figure 31. Left: Water speed measurement. Right: Water quality parameter measurements. Photos: J. Soler, P. Jugé**

**Preliminary results:**

1- Primary transects:

The mean water speed at the bottom was 0,32 m/s with a maximum value of 0,53 m/s and a minim of 0,007 m/s. As a general pattern, it was more elevated at the centre of the river and lower near the banks.

According to the visual characterization of the sediments, on the left bank they consisted of gravels and ridges up to a distance of approximately 20 m from the shore (fig. 32). From this distance, the substrate was composed of sands up to a few meters before reaching the right bank, where gravel was again found.



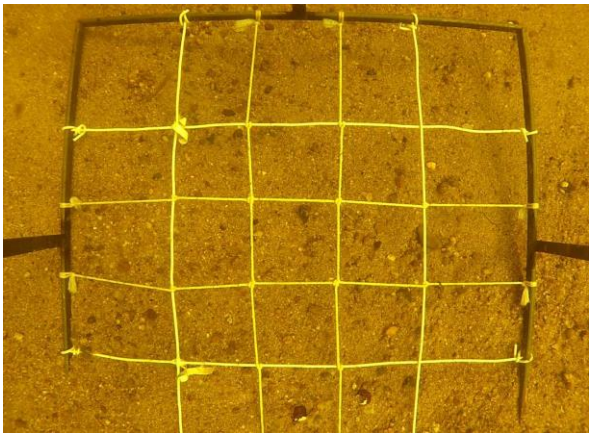


**Figure 32. Sediment at 5 m from left bank in Primary Transect A1 Figure 17. Sediment at 65 m from left bank in Primary Transect A1 Photo: J. Soler**

The mean water speed at the bottom was 0,108 m/s with a maximum value of 0,262 m/s and a minim of 0,139 m/s.. Regarding the water quality parameters, a summary of the values obtained in the secondary transects is given in Table 4.

**Table 4. Summary of water quality parameters measured in the mussels position stations (+20 cm = above the mussels, -10 cm = in 10 cm sediment depth)**

| | Conductivity (+20 cm)(µS/cm) | pH (+20 cm) | Conductivity (-10 cm)(µS/cm) | pH (-10 cm) |
|---|---|---|---|---|
| Mean | 290,6 | 8,2 | 373,3 | 7,6 |
| Max | 297 | 8,45 | 483 | 7,7 |
| Min | 251 | 7,98 | 307 | 7,45 |

**Material needed:** *For field analyses/sampling.* Boat, safety equipment, precision GPS, probes for water quality (pH, conductivity,...), sediment redox meter, underwater camera equipped with frame for standardized photography of sediments, ADP current meter, probes or echographs for analysing depth, sampling equipment for sediments, plastic bags,

*For laboratory analyses/data analysis.* drying oven, balance, shaker, and sieve-set for granulometric analysis, muffle furnace and precision balance for analysing organic matter content in sediments, software for "photo-sieving" and for calculation of shear stress.

**Time effort:** Campaign planning ca. 2 days (include geomorphologists, hydrologists, biologists and ecologists for discussion), preparation of the sampling points, ca. half a day, measurements and sampling, another half a day. Sediment sieving, ca 2 weeks for one person to sieve 50 samples.

**Caveats:** The main difficulty encountered was related to the redox potential measurements of the relatively coarse sediments. The values obtained, even without having been corrected for water temperatures, were very variable even in adjacent stations.

# 11. Different reintroduction techniques for juveniles of *Margaritifera auricularia*

**Preliminary remark:** Consider legal restrictions, see chapter 1

**Introduction:** So far, no reintroduction of artificially reared, larger juvenile *M. auricularia* has taken place. We so far released juveniles that had recently excysted from the host fish. These juveniles are too small to be monitored concerning their success. Thus, this chapter is based on theory and from experiences with the restoration of the habitats for reintroduction of juveniles of freshwater pearl mussels (Bolland et al. 2010, Taeubert et al. 2010, Gum et al. 2011, Araujo et al. 2015, Denic et al. 2015, Horton et al. 2015, Simon et al. 2015).

There are some essential questions that should be considered for all unionoid reintroduction projects. Juveniles live first in the hyporheic interstitial, and larger animals hide themselves in the sediments during adverse conditions. So, the chosen site should have a low to moderate average flow velocity, have well oxygenated sediments that have a relatively low disturbance rate, a moderately good water quality, still providing good amounts of phytoplankton, and show the presence (or expect to have) of host fish (see chapter 10 for details). If the same relationships between juveniles and riparian vegetation shown for freshwater pearl mussels (Hruska 1999) were true for *M. auricularia*, then a well-developed riparian forest would be needed.

Concerning the genetic composition of the original and the reintroduced population, it should be considered to use juveniles that are genetically as close as possible to the nearest population.

The greatest technical problem that has to be overcome with the reintroduction of *M. auricularia* is (to our knowledge) the oxygen content and the flow conditions inside the reintroduction device. The dilemma is that the juveniles are very small and that narrow-meshed grids clog very quickly, causing anoxia, and not allowing interstitial water to flow inside, which reduces also the access of food particles. The smaller the juveniles, the narrower the mesh and the greater is this problem. We therefore recommend the release of animals of at least 1 cm shell length, which is, however coupled to their maintenance in captivity for one year.

In smaller stream systems, and in rivers where the fine sediment pollution is coming from a distinct source (e.g. an erosion gully), steps can be taken to hold these sediments back either by an upstream dam construction (Altmüller and Dettmer 2006) or by restoring the riparian zone (Wantzen et al. 2006).

If the objective was to release recent born juveniles to the river, they should be seeded in the same river where the glochidia come from and, if possible, near the area where adult specimen of *M. auricularia* live. Concerning the timing of release, we recommend late spring, when the risk of frost events is low, and the river ecosystem is highly productive. The water quality in the rearing devices should be stepwise adapted to that of the mussel-receiving river.

For the reintroduction actions, we recommend the following methods:

- the establishment of benthic cages and/or concrete structures with sediment and juveniles
- the use of boats to suspend benthic cages with juveniles
- the use of Buddensiek (1995) boxes and curlers with juveniles submerged in the river bottom
- direct injection of juveniles into the sediments via tubes
- the release of native host fish infested with *M. auricularia* glochidia to the river. By now, the results obtained with the sea lamprey (*Petromyzon marinus*) recommend the use of this species for repopulation.

**Method description**: For reintroduction actions with juveniles, different method can applied:

*Use of benthic cages with sediment for recently excysted juveniles:* Since the juveniles are born in May, they will have a very small size (200 µm). The main problem is to find the way to protect the juveniles against predators and other incidences. For this, we will use cages to maintain the juveniles in the river bottom (fig. 33). Cages should have a metal frame, mesh walls and a wood or plastic tray in the bottom. This tray should have a small flange 3 cm high to retain the sediment and the juveniles, and to avoid that they were washed out of the cage. The sediment size is the same used for the juvenile breeding experiments (600-1500µm). The ideal is that the mesh size of the cage walls is smaller than that of the juveniles, but in our case this will not be possible, as small mesh sizes do not permit water exchange and will lead to anoxic conditions in the cages. So, a mesh of 0,5 x 0,5 cm should be used, with cages with plastic or metal mesh. The juveniles should be seeded in the cage by a team of divers once the cage is already submerged in the river. Placement of a cage already filled with juveniles will would result in great losses. These cages will have the following measures: 50 x 25 x 25 cm; plastic or metal mesh 0,5 x 0,5 cm. Use 5.000 to 10.000 juveniles per cage.

In order to avoid clogging of the mesh during spring and summer, the cages with the juveniles should be checked several times during the year (intervals have to be tested), in order to remove the growing algae

and avoid other dangerous incidents for the survival of the juveniles. All these cages will be installed in the bottom river, fixed to tree roots. This method may also be used for larger juveniles.

**Figure 33. Different kinds of benthic cages for the *M. auricularia* juvenile restocking. Photos: R. Araujo**

*Use of boats with hanging benthic cages:* Cages will be similar to the ones described above (Figure 19) with a plastic tray in the bottom. Due that the cages are suspended from the boat, it will more easy the inspection of the cages. The cages used should be hanged from a boat into the river. It is important that they are placed in slow-flowing areas, and that a grid is placed upstream to retain drifting algae or leaves. Only juveniles larger than the mesh size can be used here, smaller animals would quickly be washed out. Both the cages and the protective grids have to be checked regularly. The cages may have the following measures: 30 x 15 x 15 cm; plastic mesh of 0,5 x 0,5 cm. For 1.000 juveniles per cage.

*Use of concrete silos:* This portable cage system was developed for deployment of small groups of juvenile mussels in rivers (Patterson et al. 2018). Each silo consists of a concrete hemisphere weighing approximately 10 kg and containing a 5 cm diameter inner chamber with screen ends (fig. 34). Water flow over the hemisphere creates a Bernoulli effect that draws water through the chamber. Silos must be used on coarse substrate so that water can enter from underneath. Silos are stable during spates. They permit excellent growth in suitable conditions. Silos should be inspected regularly. In each silo we can put 20-50 juveniles with the same sediment used in the cages. It would be good to seed juveniles after 2-3 years of growing at the laboratory.

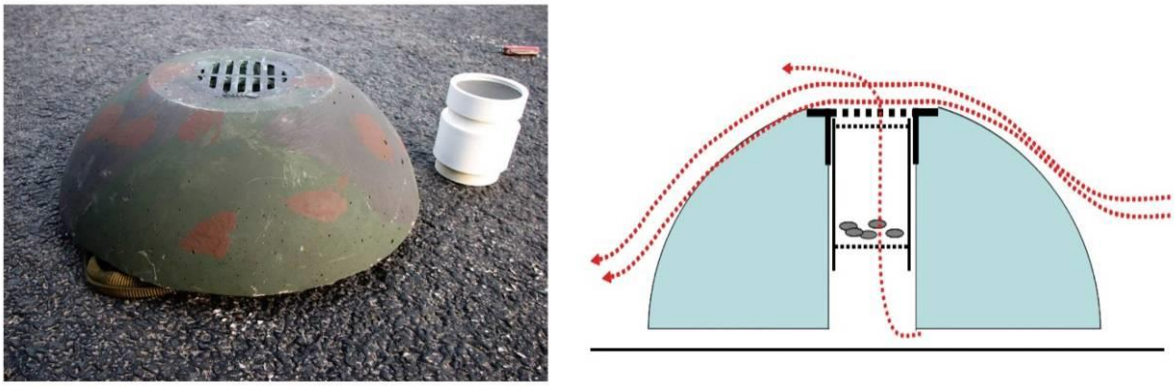


**Figure 34. Concrete silos for rearing of *M. auricularia* juveniles is the river bottom (Photo and Graph: Barnhart)**

*Use of Buddensiek cages and curls*

Metal *Buddensiek* boxes are currently used for *M. margaritifera* (Buddensiek, 1995) (Figure 22) in small streams with a relatively low load of fine sediments. If these devices may work with the juveniles of *M. auricularia* depends on the sediment structure of the receiving river. They did not work in the Ebro River (K. Nakamura, pers. obs.), because the fine sediments clogged the mesh too quickly. As with the cages and silos, Buddensiek boxes with the juveniles will be planted in the same river where the glochidia come from and, if possible, near the area where the adult specimens of *M. auricularia* live. Buddensiek boxes and curls (figs. 35, 36) should be used in gravel bottoms at water depths less than one meter. They will be checked several times during the year. In the Buddensiek boxes we can put up to 5 juveniles in each cell, and in of each curls we can put 25 juveniles. Use the same sediment that in the cages.


**Figure 35. Buddensiek boxes (Buddensiek, 1995) used in several LIFE Project for M. margaritifera (Photos and graphs: LIFE09NATFR583)**

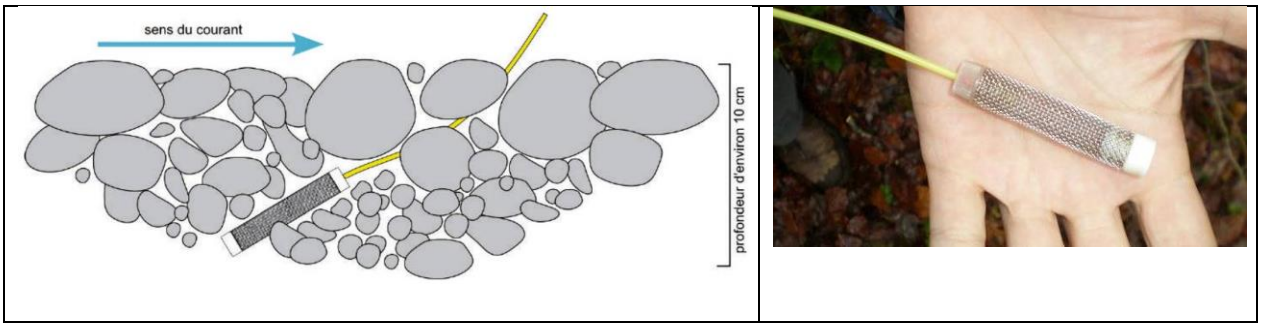



**Figure 36. Disposition of the curlers used in several LIFE projects for M. margaritifera (Graph and Photo: LIFE09NATFR583)**

*Direct injection of juveniles into the sediments:* In rivers with high loads of suspended fine sediments, such as the Ebro (K. Nakamura, pers. obs.) and the Rhine (Wantzen 1992), fine mesh cages are not recommendable as they clog too quickly, resulting in anoxic zones in the sediments. For the release of small juveniles, it is therefore recommended to penetrate the upper centimetres with a tube, and to inject the juveniles directly into the substrate via this tube. The tube is first pierced into the sediments, then filled with a water sample containing the juveniles (fig. xx), then put into an upright position, so that the juveniles can settle down. It has to be withdrawn very slowly, so that the juveniles remain in the sediments. This device can be run from a boat (fig. 37) or from land (K. Nakamura, in prep.)

**Figure 37. Release of juveniles into the sediments of the river using a tube (Photo : Morisseau)**

*Release of infested fish:* We can also use the infestation of native host fish and release to the river, which is the cheapest and most natural way to repopulate rivers with endangered freshwater mussels. Although it is also difficult to be monitored, there are several projects in which this technique had given very good results: with *M. margaritifera* (Altmüller and Dettmer 2006) and with *U. mancus* and *U. ravoisieri* in ditches around the Banyoles Lake (Araujo et al. 2015). In the first case, several juveniles were found in the river, and in the second, hundreds of juveniles of the two *Unio* species were found in ditches that previously did not have juveniles. For method details, see chapter 13.

**Material needed:** Plastic cages. Curls. Buddensiek cages. Concrete silos. Material to fix the reintroduction devices. Diver team (for deeper zones), otherwise waders. Aquascope. Boats. Rope.

**Time effort:** The work will depend on the staffing, on the type of reintroduction devices and the reintroduction site. Calculate two hours to equip one cage frame with the netting. Depending on travel time and preparative work, one site could be repopulated within one day. In order to avoid clogging of the mesh of the cages during spring and summer, the cages with the juveniles will be checked several times during the year, in order to take out the growing algae and avoid other dangerous incidents for the survival of the juveniles. The frequency of maintenance strongly depends on the water quality and the current, begin with frequent checks, later the intervals can be adapted.

**Caveats:** Considering the rareness of the animals and their sensitivity, the reintroduction devices should be testes without animals inside one year before, under identical or similar conditions. Keep in mind that conditions may change quickly, for example, in the Loire river system, we had very strong occurrence of floating macrophytes from one year to another. Rising water levels after droughts may release large amounts of floating debris and filamentous algae.

## 12.Monitoring of released juveniles of *Margaritifera auricularia* in the field

**Preliminary remark:** See chapter 1 for legal constraints.

**Introduction:** It is practically impossible to control the success of a reintroduction if the juveniles were freely released into the wild. Animals that are not found again could have suffered mortality from different causes (predation, parasitism, suffocation, starvation due to resource competition with invasive filter feeders, etc.), or they could just have drifted down some meters, and settle successfully. Only long-term monitoring will deliver solid statements about the success of the reintroduction.

Like in the rearing systems, the control of the success (survival and growth of the juveniles) in the wild should be regular, however sampling may harm the juveniles. Therefore . Maintaining a parallel population of juveniles in the laboratory can help to have control of mortality and growth rates in captivity vs. natural conditions, and thus be able

to compare them. Tag numbers or passive tags (PIT) on the shell of the juveniles will help in future monitoring, but care has to be taken not to damage the small animals.

**Method description**: The cages, silos and curls should be monitored at least two times each year, if environmental conditions were good. Check them more regularly in the beginning, especially if drifting algae or seston can be anticipated. Ideally, place an (inconspicuous) data logger nearby to record water quality and current. Juveniles should be transported to the lab in order to be measured, or can be measured with a binocular in the field. If juveniles are sized enough, they can be transported in the same water of the river where are they going to release. A simple cooler with a portable aerator may suffice.

Cages, silos and curls in deeper zones need to be collected by the diving team. Collect the sediment (and juveniles) from the cages and transport them to the lab to collect the (small) juveniles. Monitor the water quality and temperature during the transport of the juveniles. Make sure to have similar conditions in the lab than in the river (bring sufficient river water along). Limit this procedure to a minimum to avoid stress, select a subset of cages to be monitored in detail, in the remainder, just clean the cages. If the juveniles were bigger than 0,5 cm they can be collected one by one and measured in the field. It is necessary to identify areas of highest monitoring interest, because it is rarely feasible to monitor all the sites. We also recommend to maintain some reintroduction site free from impacts (including monitoring) during the first years.

Monitoring of the reintroduction success from the release of juveniles via a tube can be done ca. 1-2 years after the release, by carefully taking surber samples or underwater observations. Release sites should therefore be precisely recorded with a precision GPS. We strongly discourage marking the release sites to avoid the curiosity of passers-by. Monitoring of the reintroduction success from the release of infested fish is almost impossible. Only if ca. 5 years later large juveniles are found in a distance of some meters to several hundreds of km from the release site, a success can be noted.

**Material needed:** Dive team. Portable cooler. Containers for the sediment and the juveniles from the cages. Binocular.

**Time effort:** Depends on travelling time and team size. Calculate about 1 day for sampling, Maintain the system until juveniles attain the length to be used for repopulation (< 5 cm).

**Caveats:** During handling many juveniles will be lost, specifically the small ones. Monitoring of *M. auricularia* captive juveniles should only be done by expert hands. Juveniles are too small and too fragile. Small changes in the environment of the juveniles can easily lead to death. Oversampling in monitoring can affect negatively growth and survival. In order to avoid clogging of the mesh of the cages during spring and summer, the cages with the juveniles will be checked several times during the year, in order to take out the growing algae and avoid other dangerous incidents for the survival of the juveniles.

# 13. Search for alternative host fish and infestation and release of electro-fished, alternative host fish as an alternative to artificial rearing

**Preliminary remark:** Three new host fishes have recently been described as a result from the LIFE project (Soler et al. 2018a, Soler et al. submitted). Please, refer also to these publications. See chapters 1 and 2 for legal constraints.

**Introduction:** Recent, albeit weak, natural reproduction of *M. auricularia* , long time after the extirpation of the "classical" host fish, the European sturgeon, indicates that there must be other fish capable of carrying glochidia of the mussel until their full development and excystment. A critical revision of the so-far known host fish is given in Soler et al. (2018a). There is an essential difference between the "physiological host", i.e., a fish species that may be successfully infested and carry glochidia until full development under laboratory conditions, and the "ecological host", i.e., a species that is a physiological host and it also co-occurs at the same habitat and period with the gravid adult mussels. While physiological hosts may be used for catch-infest-and release techniques (only if they naturally occur in the respective river section, to avoid alienation of the local fish community), only ecological hosts may provide a sustainable, natural reproductory success, and should be target of habitat and species protection actions.

We hypothesised that *M. auricularia* has several host fish and that they have a marine origin and or amphidromous migratory fish (Soler et al. submitted) and suggest to follow this line of investigation (e.g., by testing eel, *Anguilla anguilla* or river lamprey) in the future. So far, the best candidates for this technique are *Salaria fluviatilis* for the Ebro, and *Gasterosteus aculeatus* and *Petromyzon marinus, the sea lamprey* for the french river systems project (Soler et al. 2018a, Soler et al. submitted).

## 13.1 Test for natural infestation

**Method description**: We recommend to test first if fish are naturally infested. To do so, fish can be caught by electro-fishing or net-fishing, and analysed in the field, if opercula allow to observe the gills. Fish are anesthetized in small containers with Eugenol 4% prior to gill inspection under a binocular microscope. Stunned fishes are collected with a dip net and maintained in plastic tanks with permanent oxygen supply (fig. 38). After that, most caught fishes can be directly released at the sampling sites. It is not possible to accurately determine the infestation rates of *A. anguilla* without harming them, the infestation of this species and of all the specimens that offered doubts in the field are observed from preserved samples in the laboratory.


**Figure 38. Left: Field laboratory to analyse fish for infestation by glochidia of unionoid mussels. Right: Situs of a living fish, the operculum is lifted so that the gills can be checked for infestations (photos: C. Boisneau)**

## 13.2 Identification of the glochidia on the gills:

**Method description**: The morphological characteristics in order to help their recognition to the genus level are the following table 5 and figure 39. The pictures are taken from Araujo et al. (2009).

Table 5: Identification key for glochidia

| Species | Length (microns) | Shape | Hook |
|---|---|---|---|
| *Anodonta sp* | 350 | Triangular | Yes |
| *Margaritifera auricularia* | 130 | Spoon shaped | No |
| *Potomida littoralis* | 210 | Spoon shaped | No |
| *Unio sp* | 210 | Triangular | Yes |

The glochidia of *M.auricularia* could be confused with those of *Potomida littoralis* except for the size.

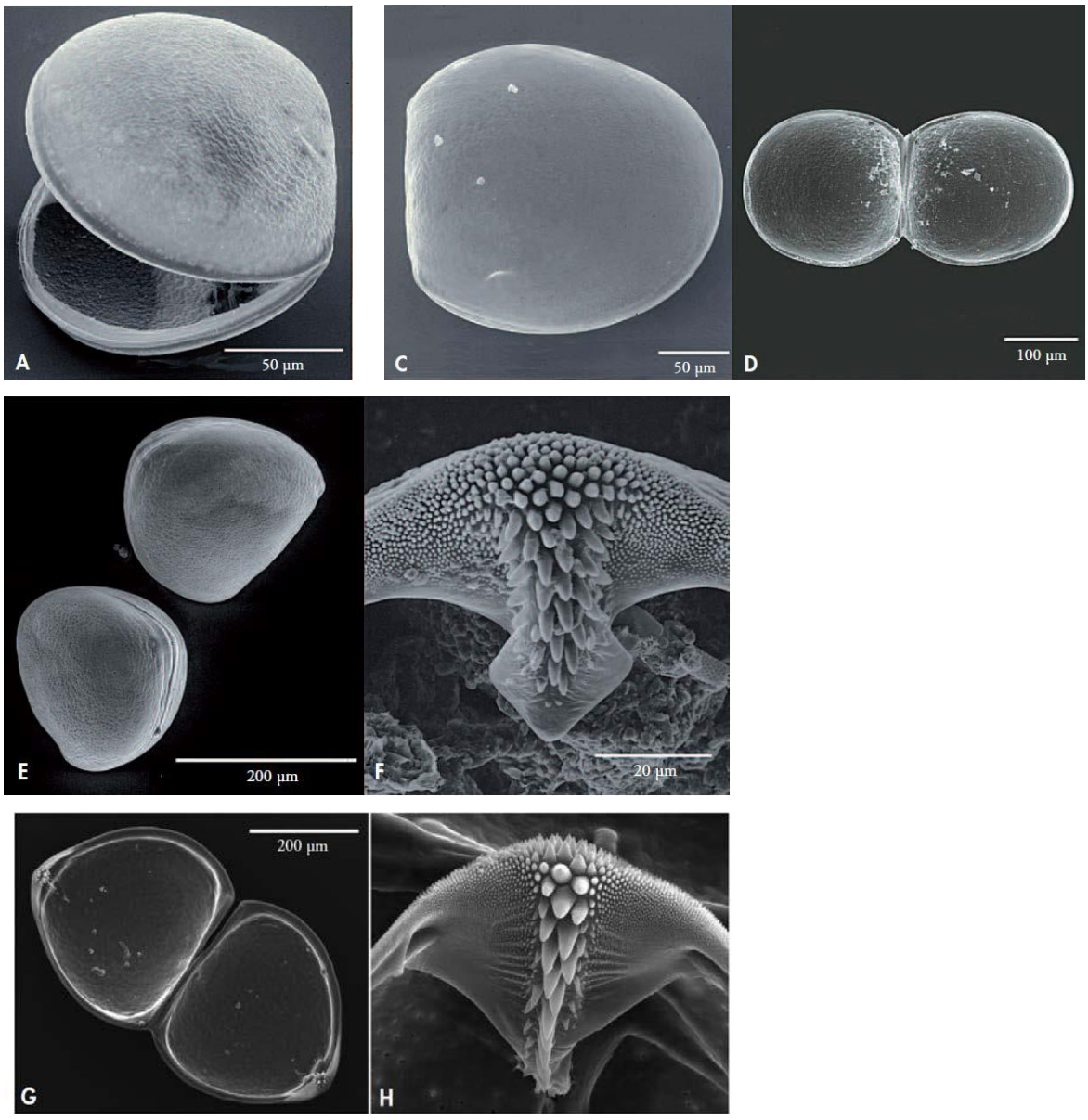



**Fig 39: Photographs of glochidia of different unionoid mussels, from Araujo et al. (2009). A = *M. auricularia*, C +D = *Potomida litoralis,* E+ F = *Unio mancus,* G+ H = *Anodonta anatina***

*Margaritifera auricularia:*

The glochidia of *M. auricularia* are 140 x 130 x 60 µm, white or light-coloured and very thin. The general shape is similar to the D- shape of other bivalve larvae (i.e. *Corbicula fluminea*) and the glochidia of *M. margaritifera*: with a straight hinge and a very rounded ventral margin. No hooks were observed on the margins of the valves, but minute teeth, covered by the rim of the periostracum, could be seen under a light microscope.

*Potomida litoralis:*

They measure 0.21 mm, are spoon shaped and are equipped with tiny cuticular spines on the edge of the valves, but without the strong typical ventral hook other unionoids.

*Unio mancus :*

They are off-white and triangular and have a strong hook in the middle of ventral edge, which appears armed with numerous spicules. The average size is 216 microns in length, 193 µm tall and 162 µm wide.

*Anodonta anatina :*

They are yellowish brown, and they are 350- 360 µm in length and 340-360 µm in height

## 13.3. On site infestation and release of host fish

**Method description**: The method described here is basically the same as that for sturgeon (see above). In the laboratory, we successfully tested three-spine stickleback, *Gasterosteus aculeatus* (Soler et al. 2018a), sea lamprey (*Petromyzon marinus*) and Wels (*Silurus glanis*) recently (Soler et al. submitted). For infestation at the river side, fish can be caught by electro-fishing or net-fishing (special permits are needed), or they can just be purchased from professional fisherman.

Glochidia have to be gained from fertile mussels (see 6.3). They are extremely sensitive from the moment they leave (or are withdrawn from) the adult mussel. Once this happened, the countdown is running, as quality of glochidia decreases continuously. After 48 hours, glochidia cannot be used any more. Two alternative exist: a) sample and maintain the mussels in the laboratory, and gain glochidia there either by sampling or by extraction with a syringe, then transport the cooled glochidia to the infestation site), or b) maintain adult mussels, bring them to the infestation site (or nearby), extract glochidia with a syringe, and use the fresh glochidia for infestation. While option a) is generally recommended (less effort in the field and certainty about the maturity of the glochidia), option b) is recommended when transport pathways are too long, e.g. if French or Spanish populations were used to repopulate rivers in Germany or England. A combination of both options can be made, i.e. to bring gravid adults near the infestation site to a laboratory, gain glochidia, and transport them from there to the infestation site.

For the infestation procedure, see 7.3.

Once fish are infested, they are kept for half an hour in a shaded container with river water, where they can calm down and glochidia can settle. If anaesthetics were used, fish have to be observed until they are fully awake. Then they are carefully released to the river.

Additionally, fish can be equipped with active radio-transmitters to be monitored (Kenward 2001). It would be interesting to identify the locality where they likely deposit the juveniles (the time of release can be estimated from degree-day analysis of laboratory experiments).

**Material needed:** See also chapters 7 and 8. For electro-fishing, specific gear and a boat is required. For field observation of glochidial infestation of fish, you need a tent or camping bus (rain protection, shadow), electricity source or battery power pack, dissecting microscope with lamps and graduated objectives for size determination, and camera equipment, petri dishes, tweezers, plastic trays etc., calliper and measuring devices for fish.

**Time effort:** Calculate several months for obtaining permits, one week for preparation and 1-3 days for field sampling. Laboratory run times see chapter 7.

**Caveats:** Electro-fishing is quite limited in deeper rivers. Sea lamprey are extremely good at escaping from their basins, so that solid coverage of the containers must be provided. The size of the infestation bucket, too, has to be adapted (and the amount of glochidia for infestation).

# 14. List of known and potential stressors to populations, and protective measures to preserve *Margaritifera auricularia* habitats

**Preliminary remark:** As long as the mechanistic causes for the reduction of the populations of the GFPM are not known, the suggestions made here follow a logic of general river habitat conservation. Beyond the known general decline (Prié et al. 2017), we recently observe a dramatic decline of the extant Spanish populations (Nakamura et al. 2018b) and French populations of the Vienne/Creuse system (Soler et al. in preparation), which deserve a decent analysis. Possibly, the interactions of multiple stressors, such as the long age of the animals, long-term accumulation of persistent pollutants, repetitive heat waves and droughts in the past years, competition with invasive filter feeders (Dreissena, Corbicula, mysid shrimps, etc.) as well as diseases carried by new vectors such as invasive *Corbicula* clams, are responsible.

**Introduction:** A range of causes of decline of *M. auricularia* populations has been suggested, and the list of risks and stressors can be seen as a task list for nature conservation. All of the problems mentioned in the following need to be reversed to assure survival not only of the GFPM, but also of other sensitive aquatic species and lastly human beings, which depend on a clean and functional, aquatic environment (Vörösmarty et al. 2010). It has to be stressed that conservation efforts must not be limited to the

individual habitat where the mussels occur, rather a catchment perspective must be applied, considering the entire river system and its tributaries, as well as the land use within the river basin.

The *decline of the host fish populations* of *M. auricularia* is often named as the prime cause for the shrinking populations, although alternative host fish species have been identified (Soler et al. 2018 and submitted).

*M. auricularia* is a filter feeder. Each animal pumps a great amount of water across its gills (about 50-100L/day), which means that not only particles suspended in the water, but also dissolved substances impair the animals. *Water pollution* is therefore a strong stressor, including organic substances (human and agricultural waste water), detergents (remember that the due to the long life of the mussels, they also have witnessed the massive phosphate pollution in the 1950ies to 1980ies), point sources of industrial pollution with toxic substances including persistent pollutants such as heavy metals and PAH, diffuse pollution with all types of pesticides from agricultural catchments, and, more recently, large amounts of pharmaceutical substances and endocrine disruptors, which are still not eliminated by waste water treatment plants.

Among the *suspended particles and suspensoids*, the increasing pollution with micro plastics (Blettler et al. 2018) and nanoparticles from cosmetics, abrasives etc. represent a double problem, as they may impair the feeding process and carry toxic substances. Even natural substances such as precipitating humic acids (very common in the Vienne/Creuse system) accumulate pollutants, e.g. heavy metals (Steinberg 2003). Food particles of GFPM include suspended organic matter such as planktonic and benthic algae and bacteria. Reduction of the food particle by competition from other mussel species, specifically the invasive *Corbicula* and *Dreissena* species, may have a dramatic impact on all native mussel species.

*M. auricularia* juveniles and adults live in the *sediments of rivers*, the juveniles live inside the hyporheic interstitial zone (Marmonier et al. 2012), whereas the adults dig themselves to about 1/3 into the sediment surface, but can penetrate the sediments by peristaltic movements of their foot. Their lateral movements are quite limited to ca. 1 m a day. This biological trait makes the GFPM specifically sensitive to human impacts on sediment structure and dynamics. Most rivers are canalized, incised, and / or impounded, so that the sediments are lacking, remain stable (undynamic), and/or have unnatural erosion/sedimentation patterns. Colmatation (clogging) of sediments by fine particles from agricultural erosion and/or from precipitating organic matter or carbonates, often combined with armouring of river

beds due to lacking sediment dynamics leads to impenetrable substrates, which become anoxic due to lacking exchange with the surface water. Moreover, no fresh organic matter may arrive in the habitats for the juveniles. In the Charente River, the largest remaining population of the species is severely threatened by coverage from sediments accumulating above the St. Savignien dam. The regional government (Conseil Général Charente-Maritime, now: Région Nouvelle Aquitaine) has planned a dredging of these sediments and to improve the management of the weirs in order to reduce this problem many years ago.

Due to *Global Warming*, European rivers encounter increasing average *water temperatures* but also heat waves and longer periods with temperatures above 25 or even 30° C. Even if *M. auricularia* adults may sustain relatively high water temperatures, we have no information about the consequences for longevity, fertility, and the survival rate of juveniles. Climate Change also increases the stochasticity of discharge including very short and strong flood events, which may be deleterious for GFPM populations (specifically in rivers with disturbed sediment regime, see above). Droughts and low water levels may be even worse, as mussel habitats may fall dry, or be subject to extreme hot or cold temperatures, all of which are deleterious.

We are only beginning to understand the impact of invasive species on large unionoids. From our experience, the known clogging of unionoid shells by *Dreissena polymorpha*, well-known for lakes, may not be an acute problem for large specimen of GFPM. Maybe they can shear off the zebra mussels when penetrating the hyporheic zone. The competition for food particles by invasive *Corbicula fluminea/fluminensis* and *Dreissena polymorpha,* and, more recently *D. bugensis* species definitively is a stressor, as the invasive mussels occur in very large numbers, have a high filtering rate, and a wider spectrum of particle sizes that they can ingest than the native species. *Limnoperna fortunei*, an Asian mytilid invading Latin American rivers, can be anticipated to arrive in the next years to Europe. We have no information yet about the predation (including filtration of glochidia) by other invasive invertebrates. It is known that the recent spread of *Rhodeus amarus*, a fish that oviposits in the gills of unionoids, represents a serious threat for the already weakened mussel populations (Soler et al., accepted). Invasive plants such as *Ludwigia* sp. may alter mussel habitats by producing enormous amounts of organic mud, reducing water velocity and carrying biofilms that filter food particles (Lambert et al. 2010).

**Method description**: All conservation measures have to be seen in a catchment context, but we limit the method description here to the specific work needed to restore mussel habitats of extant populations or to prepare habitats for reintroduction. These measures are a tentative and putative approach. None of them has yet been realised, thus no recorded experiences on their success exist.

In a first step, the structure and dynamics of the sediments in representative habitat patches *near* the mussel habitat should be analysed. Careful removal of individual stones of the sediments near the mussels may help to get an impression about the precise habitat of the animal, and then similar patches at a distance of 2-3 m downstream the lowermost individual can be analysed. Repetitive observations of the populations (see chapter 3) should be made to avoid that adults that are dug into the sediments are overseen, and become impaired by the measures. At these patches, water velocity should be identical. The measurement procedure is identical to that of the habitat structure, i.e. in equidistant line transects, sediment samples should be analysed and compared with those of the mussel habitats. These data serve as a baseline for habitat structure improvement methods. In the following table 6 we make suggestions of which encountered problems could be mitigated by which measure.

**Table 6: List of environmental stressors and suggestions for habitat restoration for *M. auricularia***

| **Type of problem encountered** | **Potential measures** | **Potential difficulties** |
|---|---|---|
| Sediments are armoured | Armouring can be removed with a caterpillar | Disturbance of other species, low sustainability |
| Sediments are clogged | Increase natural sediment dynamics | Risk of siltation |
| Sediments are anoxic | Increase natural sediment dynamics | Risk of siltation |
| Sediments are too shallow | Increase natural sediment dynamics | Risk of siltation |
| Sediments accumulate large amounts of organic matter | Identify source of organic pollution, improve waste water treatment, or (in case of diffuse pollution), build artificial (denitrifying, phosphate-capturing) wetlands | Great financial effort |
| Siltation | Construction of small dams in agricultural catchments | Disturbance of other species |

**Material needed:** Water velocity meter, equipment identical to a) population analysis and b) habitat analysis

**Time effort:** Tentative time table: One year for obtaining the permissions and planning, one year for the habitat analysis and detailed planning of the measurements

**Caveats:** Late summer is generally the best period for measurements and for realizing field work, but remember the annual and multiannual hydrological regime of the river. If possible, observations during flood events should be included into the planning.

All restoration measures have to be made with great care in order to avoid collateral damage for the existing population. Specifically when sediments become moved (dredged etc.), clogging of the sediments has to be avoided. We therefore suggest to restore habitats downstream of extant populations so that mussels can migrate to them (or colonize them anew), but clogging of the actual habitat is avoided.

In a worst case, adult animals can be temporarily removed during the measures, this is the case in the Canal Imperial in Aragon, Spain, which becomes completely dredged every five years.

In the context of mussel habitat restoration, dam removal is a critical issue. From pearl mussel habitats it is known that dams above the populations retain fine sediments from agricultural erosion. On the other hand, dam removal is the best option for restoring river sediment dynamics and passability for migratory (thus: host) fish species.

## 15. Appendix: Questionnaire for unionoid mussel raising

The questionnaire (table 7) has been developed to compare studies between European or International mussel projects, during the LIFE Unio meeting in 2015. It may help to streamline the activities of different projects in the future.

**Table 7 Questionnaire**

| | | |
|---|---|---|
| Your name | | |
| Institution | | |
| Project name | | |
| email | | |
| homepage | | |
| Country | | |

| | | |
|---|---|---|
| | | |
| | | |
| 1. Target species | | |
| | Margaritifera m. | |
| | *M. auricularia* | |
| | Unio crassus | |
| | U. other1 | |
| | U. other2 | |
| | U. other3 | |
| | Potomida littoralis | |
| | Anodonta anatina | |
| | A. cygnea | |
| | | |
| 2. Country | | |
| | | |
| 3. Hydrographic information | | |
| | basin name | |
| | river name | |
| | river size | |
| | lake name | |
| | lake size | |
| | | |
| 4. Population information | | |
| | population size | |
| | area of pop. Occurrence | |
| | further info | |
| | | |
| 5. Causes of decline/threats | | |
| | causes 1 | |
| | causes 2 | |
| | causes 3 | |
| | | |
| 6. Host fish species | | |
| | good species1 | |
| | good species2 | |
| | good species3 | |
| | | |
| | tested bad sp. 1 | |
| | tested bad sp. 2 | |
| | tested bad sp. 3 | |
| | | |

| | | |
|---|---|---|
| 7. Gravidity period of mussel | | |
| | Start | |
| | End | |
| | Comment | |
| | | |
| 8. How are glochidia sampled and stored? | | |
| | how to open mussel | |
| | flushing of gills? | |
| | how do you keep glochidia before infestation? | |
| | maximum time to keep glochidia before infestation? | |
| | | |
| | | |
| | | |
| 9. Water | open or closed system | |
| | Source | |
| | how filtered | |
| | | |
| 10. Infestation | | |
| | in situ (cage) | |
| | in vitro (bucket) | |
| | in vitro (other) | |
| | Details | |
| | | |
| 11. How infested fish are maintained during infestation | | |
| | cylindroconic tank | |
| | outdoor tank | |
| | fed with what | |
| | dosage g per fish/day | |
| | how fed | |
| | how often cleaned | |
| | | |
| | | |
| 12. How young mussels are collected after metamorphosis? | | |
| | | |
| | | |
| | | |
| | | |
| 12. Rearing system | | |
| | Box | |
| | cage in outdoor channel | |
| | cage in indoor channel | |

| | | |
|---|---|---|
| | hydrostatic beaker system | |
| | | |
| | dimensions (mm, L x B x H) | |
| | water renewal time (x/day) | |
| | | |
| | sediment source and type | |
| | sediment size (is it mixed) | |
| | do you mix up sediments, how and how often? | |
| | | |
| | | |
| 13. food for rearing | | |
| | commercial, which type | |
| | natural, which type | |
| | how is it given? (continuous, pulsed, automated) | |
| | how often is it given (times per week) | |
| | dosage (g food/day/g animal) | |
| | | |
| | | |
| 14. how long are young animals reared? | | |
| | until size = x mm | |
| | yy years | |
| | | |
| 15. Secondary rearing device (after growth) | | |
| | at which size do you transfer? | |
| | what system? | |
| | different food? | |
| | | |
| 16. Reintroduction to the nature | | |
| | into site of extinct population | |
| | boosting existing population | |
| | do you apply a habitat protocol before? | |
| | | |
| 17. Reintroduction technique | | |
| | Silo | |
| | Buddensiek box | |
| | small mesh tubes | |
| | | |
| 18. Success control | | |
| | survival rate? | |
| | how | |
| | how often | |

| | | |
|---|---|---|
| | | |
| | growth? | |
| | how | |
| | how often | |
| | | |
| 19. Monitoring habitat of reintroduction | | |
| | How long | |
| | at which intervals | |
| | Water quality (parameters)? | |
| | Sediment quality | |
| | Ecotoxicology | |
| | | |
| 20. Tagging of released mussels? | | |
| | plastic tag | |
| | PIT-tag | |
| | Laser imprint | |
| | other (describe) | |
| | None | |

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