

Dear Macrostromum Co-workers,

Here is a set of guidelines which we try to follow in our lab. If you have any questions, doubts or suggestions, please communicate them to me.

Thank you.

Dita

Macrostromum Lab Work Guidelines

by Dita B. Vizoso, last update: May 2013

Worm Cultures.

The worm cultures are the heart of our lab. They are not only important for your current experiments: their well-being is indispensable for all future work. We favour the use of glass containers whenever possible, as we have observed strong effects of plasticware on growth and reproduction of algae and worms. All our cultures are kept in **glass** petri dishes (100 by 20 mm).

Proceedings (How to maintain the cultures).

The worms are kept in 32‰ f/2 to allow for the growth of algae, at 20° C, with a 14:10 light:dark cycle. A few days after the generation time (e.g. four weeks for the *M. lignano* outbred cultures, or five weeks for line DV1) a new generation is founded as follows:

1. **Clean well ALL the working space**, including the stereomicroscope and all places that may get in contact with your hands (e.g. microscope knobs, pipettes), with the boxes and dishes (e.g. trolleys, benches), etc. with 96% Ethanol *purum* (although 70% Ethanol is favoured by molecular biologists, it is not enough against many eukaryotes, and the moisture left behind is favourable for fungi, spores of algae, flagellates, etc.). If you are experiencing contamination problems, or the space is used for culturing other organisms, use bleach (~1.5%. Most commercial bleaches are about 5%).

2. Prepare the fresh glass petri dishes: discard the old medium and add fresh 32‰ f/2. **The interface between the dish and the lid should remain dry**, so don't use too much liquid (half-full at most), and dry well both lid and rim of the dish after discarding the liquid. Do this away from possible sources of contamination such as sinks (unless you have a separate sink for clean work), corners with cultures or samples, etc. To discard liquid while working on a bench, you can use a container for liquid waste.

3. Transfer about 100 (the optimal number may depend on species, line, and conditions: keep a log to find your own optimum) **hatchlings** or **juveniles** into

a small petri dish with 32‰ ASW, in order to wash them. Afterwards, transfer 100 worms to the fresh glass petri dishes. The dishes with the parents are kept as a back-up (preferably in an independent incubator), and the oldest back-up (it may vary with culture- ask for your particular one) is discarded.

4. Transport with care so that the lids don't get wet (see point 2 above). We've repeatedly seen that dishes that have been "sealed" with water contain worms that look unhealthy. We presume it may be oxygen depletion.

Culture Guidelines.

Ideally, worms and diatoms should be the only eukaryotes present in the cultures. However, lots of other creatures can grow in such a rich culture medium. Bacteria are abundant, but so far have not been a problem. Other eukaryotes, on the other hand, have previously destroyed important cultures and experiments. As we do not work under sterile conditions, extra care should be taken to prevent the contamination of cultures with undesired creatures. Here is a series of guidelines which have worked well in our lab:

1. Quality of materials.

Always use fresh media (see Media section). Always use good-looking algae: they should be not too old (exact age will depend on the method of culturing, see Algae section), without transparent patches, without different species of algae (unless specifically desired). A brief check under the binocular should be made before using the algae. In case of doubt: ASK BEFORE USING. Always use clean pipette tips (avoid using pasteur pipettes for culturing!).

Plasticware can strongly slow down development and decrease reproduction of *M. lignano* (Vizoso & al. unpub.). We avoid using plastic for culture maintenance, and treat the multiwell-plates we need for experiments extensively before first use (see "Well Plates" section).

2. Quality of worms: always check and only keep healthy worms.

Before collecting worms from a dish, check for parasites, flagellates, different algae species, or anything out of the ordinary. Please ask for instructions on how to check worms (we use bright field light to check the worms, and dark field light to check for flagellates).

If the worms of one dish look sick, swollen, dissolving, or strange in any way, please communicate that immediately, i.e. write an email to everyone in your group so that people are alerted of the issue, and don't use worms from that culture. Avoid transferring from suspect dishes, and by no means mix hatchlings from potentially contaminated dishes with hatchlings from good-looking dishes. Keep suspect dishes in quarantine or, even better, discard them immediately (and restart from the backup if necessary).

3. Avoid contaminations.

Never use the same pipette tip for more than one dish. Even if you are going to mix the worms later, the original plates should not be cross-

contaminated, as they should remain as back-up (and may be necessary if the current culture crashes). Rinse the collected hatchlings in plenty of fresh ASW, of the appropriate salinity (see Media section), before transferring them to the new dishes.

Try to avoid working with more than one culture on the same day. If you cannot avoid that, separate them as much as possible: spatially and/or in time. Clean everything very well in between.

Keep field samples and any other cultures or possible sources of organisms (e.g. media, agar plates, dirty dishes and bottles) as far away from the culture as possible.

Clean the working space thoroughly before and after working. This includes the knobs of the stereomicroscope and any other surface you may have touched.

4. Using the "worm-pump".

Just like for the normal transfers, **never use the same pipette tip for more than one dish.** To add a new tip, cut the back rim of a white tip and insert it in the tube. It is useful to prepare the tips beforehand (it works very well with a cutter –Stanley knife or carpet knife– but please do not do it directly onto the table).

When you finished with one tube, rinse it with distilled water (inside the tube!) and place it in the plastic box beside the sink.

Media.

Media like ASW and especially f/2 are used to culture a wide range of organisms. This means that things do grow very well in these media. I recently trashed some different ASW and f/2 media ranging from 1 to six months old, which all contained some sort of organisms, including some beautiful green algae, and some small flagellates, and loads of bacteria. Many of these eukaryotes can disperse via desiccation-resistant spores, so having accidental cultures of them around greatly increases the chances of having them in our cultures, even if those media were not used.

1. Location

Keep the media (f/2, ASW, MgCl₂) storage area clean, and do not place any containers with worms / old media / toxic stuff / etc. nearby. Keep your media far from the place where dirty dishes and bottles are stored.

2. Freshness

Whenever you need ASW, do it fresh, and use it within one week. Try to prepare the right amount and trash the rest. It is not only your experiments which are at risk, but the cultures and experiments of everyone. If you use f/2 and there's only 100-200 mL left in the bottle which you won't use in the following days, trash it.

For washing cultures, ALWAYS use fresh ASW. You can use small bottles: 200 mL should be enough for washing 12 dishes of worms, so a 250 L bottle should suffice. Make sure to always measure the salinity.

Rinse the salinometer with distilled water after use, and never use the same pipette to measure the salinity of different bottles or dishes.

3. Recipe

A detailed recipe for f/2 can be found in our website:

<http://evolution.unibas.ch/people/dita/lab/media.html>

Note that an old recipe for f/2 from Innsbruck has an 10X excess of Thiamine and a 1000X excess of Biotin and Cyanocobalamin (which gave the medium its characteristic pink colour) with respect to the original f/2 medium. We use the original recipe since 2005 and had very good results (and a lower amount of bacteria in a test for the different media performed in that year).

Likewise, between 2005 and 2010, we reduced the amount of Silicium to half, as we did not find any differences in the algae or worm cultures, and a substantial amount of silicium is filtered out before use. For consistency between labs, we are now using the unmodified f/2 medium from Andersen (i.e., with full amount of silicium)

Algae.

We use *Nitzschia curvilineata* (originally from the SAG in Göttingen <http://epsag.uni-goettingen.de/>), and keep two kinds of cultures: agar stocks and cultures in glass petri dishes.

The protocols for keeping the cultures are available in our lab website:

<http://evolution.unibas.ch/people/dita/lab/algae.html>

Some important points:

Plasticware can strongly slow down development and decrease reproduction of *Nitzschia* (Vizoso & al. unpub.). We avoid using plastic for algae maintenance, and treat the multiwell-plates we need for experiments extensively before first use (see "Well Plates" section).

Have a good routine for cleaning the petri dishes and tubes, dry them at >100 °C, and keep the storage place clean. Clean the containers for the dishes (we use polystyren transparent boxes, 31x22x5 cm) with ≥96% Ethanol before storing the dishes.

Algae quality and age affect the fecundity of *M. lignano*. A continuous production of algae dishes is thus indispensable for the proper culturing of the worms. Agar stocks grow slowly and we use them exclusively to seed the algae petri dishes. We don't use, as in other labs, bottles to grow the diatoms before distributing to petri dishes, but in emergencies prepare a solution from the petri cultures to seed "fast" dishes. Using two dishes for one box will allow a medium algae cover in about one week. Repeatedly seeding from petri dishes has shown a decrease

in algae quality (our own experience, and reports from Johannes Achatz), so we avoid using "fast" dishes to seed (maybe a point to consider for those who use the bottle-approach and have problems with the algae).

Light is important. We use Osram Lumilux Biolux T8, two tubes per shelf, at about 20-25 cm distance from the boxes (Osram 12/950 are also a possibility).

Dishes seeded from an agar tube (one tube for one box) take about two-three weeks to produce a dense cover (speed will likely depend on particular lab conditions). They are usually still good after 8 weeks, but preferably should be used within 2-6 weeks under our chamber conditions. If the algae look bright orange, or there are many empty frustules (the silica cell walls of diatoms), the culture is probably too old. Note that worms can be fed with old algae, but their reproduction tends to decrease (Sandner *et al.* in prep.)

To seed agar tubes it is best to simply inoculate from a mature tube directly, without making an algae suspension.

Pipetting.

Make sure you know how to use a pipette before using them to work with the cultures. Notice that pipettes are an excellent vehicle for the spread of contaminants.

In order to reduce transmission of undesired contaminants (including worms), care should be taken that the pipette itself does not get into contact with the liquid. In the case of 200 μ L and 1000 μ L pipettes (we use Eppendorf, but the same applies to several other brands: check how it looks in your own lab), the tips can be filled with medium, so liquid can touch the pipette when using them at full capacity if they are not used properly.

I find it easier to work with small volumes (10-20 μ L) when transferring worms. With a bit of practice one can easily transfer >50 worms in 20 μ L. This also reduces the amount of old medium transferred. If you prefer to work with larger volumes, make sure the volume set in the 200 μ L pipettes is no larger than 100 μ L.

When using ANY pipette, but especially the 200 μ L and 1000 μ L, for anything, try to suck up the liquid gently and always check if liquid touched the pipette. If so, clean with EtOH immediately, and dry with Kimwipe paper. If liquid with any contaminants gets in touch with the pipette, please report that to the person in charge of the lab, and "isolate" the pipette. It should be autoclaved.

When refilling the pipette-tips dispensers, clean well the surface with EtOH or Bleach, and clean your hands or use gloves. A piece of clean kitchen paper as a table cloth is also a good solution. If you're experiencing contamination, autoclave the tips (or buy sterilized tips).

Well plates

Due to detrimental effects of plastic on the performance of both the algae and the worms, we treat the multiwell-plates before first use, as follows:

Plates are soaked in de-ionized water at 55°C for at least one week, with daily changes of water (a minimum of 6 changes). They are then machine-washed using a standard plastics washing cycle (60°C) and dried at 40-65°C.

After being used, plates are soaked in a 1% bleach solution for at least one hour (usually overnight), then rinsed once with water, machine-washed using a standard plastics washing cycle (60°C) and dried at 40-65°C.

General lab good practice.

Please calculate the cleaning time (2-10 minutes) in your schedule, and **always clean before and after work.**

Trash the disposable plastic items, like plastic petri dishes, plates, pipette tips, etc. daily (we have table bins which should be emptied into garbage containers). If there are pipette tips stuck in the bottom of the table bin, please remove them, rinse the bin in the sink, and place on the dish rack to dry. Clean such table bins regularly (bleach and/or $\geq 96\%$ ethanol)

Keep things to wash orderly and separated from the working space. Don't leave dishes or plates with worms or medium laying about.

Don't use items that you're not sure are clean.