SHORT TERM SCIENTIFIC MISSION (STSM)
SCIENTIFIC REPORT

This report is submitted for approval by the STSM applicant to the STSM coordinator

Action number: CA18221
STSM title: PEsticide RIsk AssessMent for Amphibians and Reptiles
STSM start and end date: 27/01/2020 to 07/03/2020
Grantee name: Gledjan Caka

PURPOSE OF THE STSM:

The main goal of the present STSM was to assess and compare the effects of different groups of chemicals (metals and pesticides) on aquatic life stages (embryos and tadpoles) and cell lines [kidney epithelial cells (A6) and fibroblast cells (XTC-2)] of the anuran model species *Xenopus laevis*. To attain this main goal, four specific objectives were set to be reached during the period from 27/01/2020 to 07/03/2020:
1 - Acquire knowledge and practical experience on the methodologies used to maintain healthy laboratorial cultures of adults (reproductive males and females) of *X. laevis*.
2 - Learn the methodologies used to reproduce *X. laevis* in laboratory, in order to obtain viable eggs and tadpoles to be used in experimental procedures.
3 – Perform acute ecotoxicity assays with embryos and tadpoles of *X. laevis*, to assess their sensitivity to four chemicals (potassium chloride, copper sulphate, acetamiprid and the commercial formulation of the insecticide Epik®).
4 – Carry out cytotoxicity assays to assess the sensitivity of two *X. laevis* cell lines to the same three chemicals tested in the acute ecotoxicity assays (specific objective 3)

DESCRIPTION OF WORK CARRIED OUT DURING THE STSMS

Specific objective 1
The work carried out in the lab consisted of several steps. Firstly it consisted of taking care of the *X. laevis* well being and cleaning their environment in order to have optimal culture conditions. Tanks were cleaned every two days, removing the stagnant water and putting new fresh dechlorinised water. Feeding the *X. laevis* was done three days per week by feeding the females with 2 pellets (XE 40) and seven *Tenebrio molitor* worms, whereas males were fed 1 pellet and 5 worms to keep them in good health and well fed. The *X. laevis* were put individualy per aquarium and kept at a constant temperature of 23±1 ºC and a photoperiod of 14:10 hours light:dark.

Specific objective 2
In order to obtain eggs and tadpoles to carry out the ecotoxicity assays, the reproduction of pairs of *X. laevis* was induced by injecting, both the female and male, with the human hormone chorionic gonadotropin. The females and males were injected in the dorsal region with 500UI and 150UI of the hormone, respectively. Afterwards, the pair was transferred to a reproduction container, with a plastic net in the bottom, and left there overnight.
The next day the eggs were collected with a plastic pipette and transferred into a new recipient with freshly FETAX prepared medium (composition: NaCl, $NaHCO_3$, KCl, $CaCl_2$, $CaSO_4.2H_2O$, $MgSO_4$, with pH adjusted to 7.5–8.5). Eggs were then all checked, under a stereomicroscope, for their viability. All the viable and

healthy eggs were set aside to be used either in the embryo assays or delevop to be used in the tadpole assays.

Specific objective 3
Three chemicals, with different modes of action, were selected to perform the ecotoxicity assays: potassium chlorid (KCl), copper sulphate ($CuSO_4$) and the commercial formulation of the insecticide Epik® (a.i. acetamiprid). To run the assays with embryos and tadpoles the Embryo Teratogenicity assay (ASTM, 2012) and the Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians (ASTM, 2002) guidelines were followed, respectively, with some minor modifications.
For the embryo assays we prepared 7 different concentrations of the three selected chemicals. Several assays were run per chemical and life stage; the concentrations tested for KCl ranged from 0.78mg/L to 90mg/L; for $CuSO_4$ concentrations ranged from 38.5µg/L up to 1152µg/L; for acetamiprid concentrations varied from 100µg/L to 1139µg/L, and for EPIK the range of concentrations went from 64.2mg/L as high as up to 487.5mg/L a.i..
For the embryos assay, were prepared three replicates for each concentration and five replicates for the control. In each replicate were introduced 20 viable embryos at NF stage 6-8 and 20ml of test solution, exposure occurred for 96 hours. After 48 hours of exposure the medium of each replicate was changed and fresh one was added.
For the tadpole assays, which were at NF stage 46 at the beginning of the assay, we prepared 7 different concentrations. For each concentration we had 5 replicates and the same number was used for the control. Each test vessel used for the assay had 4 larvae in 200ml freshly prepared medium. After 48 hours the medium was changed. After 96 hours of exposure the following endpoints were monitored: mortality, malformations and morphometry.
At the end of the assays, both the live embryos and larvae were introduced in Eppendorf tubes filled with formal 3% to fix the organisms. Afterwards, embryos and larvae were placed in a petri dish and pictures were taken at the stereomicroscope with a camera to be later measured.

Specific objective 4
Cultures of A6 and XTC cell lines were put to grow for two weeks in order to run *in vitro* cytotoxicity assays with the same chemicals tested in the ecotoxicity assays with embryos and tadpoles, but though two attempts were made to grow the cultures they turn out to be contaminated and for that reason it was not possible to run the planned epxeriments.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

Embryo assays:
In the preliminary assay performed with embryo exposed to KCl (concentrations ranged from 18.7g/L to 90g/L) a 100% mortality occurred at all tested concentrations. Another assay was carried out by testing concentrations ranging from the range of concentrations 0.39 to 25 mg/L. The two highest concentrations resulted in 100% deaths for the embryos just after 24 hours of exposure, whereas 6.25 and 3.12 had 50% mortality after 72 hours and total deaths after 96 hours. The remaining concentrations had very few deaths, less or up to 10% of the total population. Aiming to compute and LC50, the assay was repeated by adjusting the range of concentrations to 3.12 to 6.25 mg/L. However, an all-or-nothing response was again obtained, with very low mortality at concentrations up to 1.56g/L and very high mortality at concentrations higher than 3.12g/L. At the concentrations where low mortality occurred, malformations (e.g. curved tail, gut malformations) were observed in the alive larvae. The percentage of these malformations was below 10% of all organisms that had mutations which were observed at concentrations were total mortality was not achieved.
Though malformations were observed, no effects were observed in the developmental stage; at the end of the assays all alive larvae were at developmental stage NF46.

The preliminary assay performed with copper sulphate (38.5, 50, 65, 84.5, 110, 140, 182µg/L of Cu) induced no mortality in the embryos of *X. laevis*, and few malformations were observed in the copper exposed organisms. Higher copper concentrations were tested in another assay (182, 236.6, 307.6, 399.9, 519.8, 878 and 1141.4µg/L), though the highest registered mortality was less than 10%. However, the percentage of malformations was high, reaching 36% at the highest concentrantion 1141.4µg/L, 30% at 878µg/L, more than 15% at concentrations above 399.9 µg/L and less than 10% in concentrations lower than 307.6µg/L. After the 96 hours exposure, larvae were at an earlier stage of development comparatively to the control (NF46) at concentrations above 182µg/L (stage NF23-27).

Regarding assays with the a.i. acetamiprid, a preliminary assay was run with concentrations ranging from 100µg/L to 1139µg/L, using a dilution factor of 1.5. After 96 hours of exposure no mortality was observed at all tested concentrations, but severe malformations (namely tail curled) were registered at all concentrations. At all concentrations we had mutations in less than 10% of the larvae.

Tadpoles assays:
In order to check the effects of KCl on Xenopus we also ran the assays in tadpoles. The tadpoles were not fed one day prior to the experiment and were subjected to the same concentrations as the embryos, namely 0.39g/L to 6.25g/L. After 96 hours the results showed that apart from 0.39 and 0.78mg/L KCl, the other concetrations had a 100% mortality rate for the larvae. From this we can conclude that *X. laevis*larvae are more sensitive than the embryos.

For the tadpole assays we used 462.96, 555.55, 666.66, 800, 960 and 1152µg/L Cu concentrations. After the 96 hours were over the results showed that 1152 and 960 ug/L had total mortality and there were many deaths at 800ug/L. The lower concentrations had very few or no deaths which allowed us to compute the LC50 for the assays and we got a concentration of 821ug/L for 100% mortality with a 95% confidence level.

Since we did not get any deaths with acetamiprid we then switched to using the main compound which is the pesticide EPIK. We used a ten-fold concentration for the tadpoles as opposed to what we used for the embryos and after 72 hours we had total mortality in all concentrations. These results forced us to lower the concentrations to 12.7, 19.2, 28.5, 42.8, 64.2, 96.3 and 144.5 mg/L but since we didn't get 100% deaths in any concentration it was impossible to compute a LC50. Because of that we increased the concentrations again with 64.2 being the lowest and 487.5 being the highest. We had total deaths in the 3 highest concentrations (216.7, 325, 487.5 mg/L respectively), total deaths in 3 replicates of the 144.5mg/L and 50% deaths in the remaining replicates. This made it possible to compute the LC50 which was set at 132mg/L with a 95% confidence level.

FUTURE COLLABORATIONS (if applicable)

After the completion of the short training and discussion with supervisor Isabel Lopes we agreed on working at a later time with different compounds which we previously worked and also to start new assays with an in vitro approach. We will try to establish a good assay by using *X. laevis* cell lines and more specifically epithelial and kidney fish cells. Since it was not possible to run the experiments with the cell lines during the STSM training, it was agreed to run them later by also involving students from the University of Aveiro. By using both in vivo and in vitro assays it is intended to compare the sensitivity of the two methods to chemicals in order to establish associations and infer on the possibility to use in vitro assays as surrogates on in vivo assays for early stages of amphibian risk assessment.