ECOTOXICITY TESTING USING ALGAE AND DAPHNIDS

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KICK-OFF MEETING

Integrated evaluation of aquatic organism responses to metal exposure: gene expression, bioavailability, toxicity and biomarker responses (BIOTOXMET)

Zagreb, 11th October 2021

ECOTOXICITY TEST

- For ecotoxicological research we used:
  - cultures of ***Daphnia magna*** Straus and
  - cultures of freshwater green algae ***Pseudokirchneriella subcapitata*** (Korshikov) Hindak

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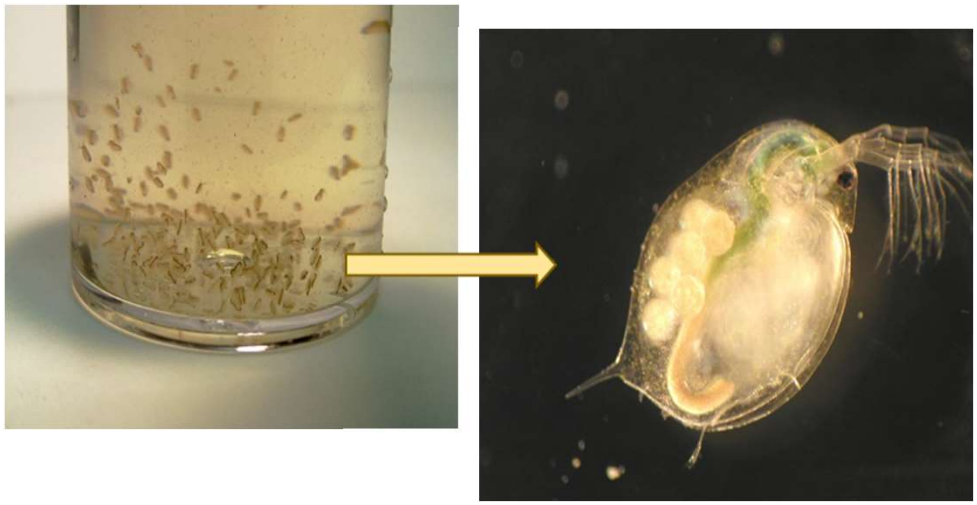
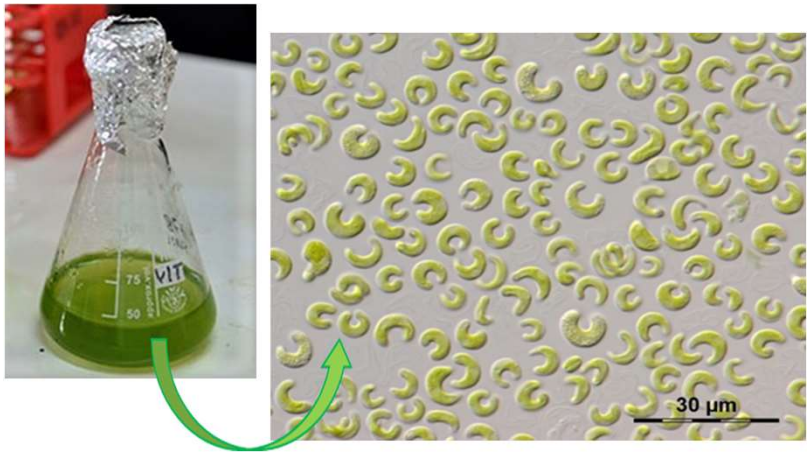


Daphnia magna Straus



Pseudokirchneriella subcapitata
(Korshikov) Hindak

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Daphnia magna immobilization test

- *Daphnia magna* Straus used in the study was obtained from the Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium.
- Tests were carried out with hatched neonates (<24 h) according to the standard operating procedure Daphtoxkit F magna (1996), which is in accordance with ISO 6341 (2012).
- Hatching of the ephippia were initiated 3-4 days prior to the start of the toxicity tests in petri dishes with medium at 20-22 °C under a constant illumination of 6000 lx.

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Daphnia magna immobilization test

Preparation of effluent dilution

- the test concentrations ranging
  - from 6.25% to 100% were set by dilution of wastewater effluents and
  - from 25% to 100% for surface waters.

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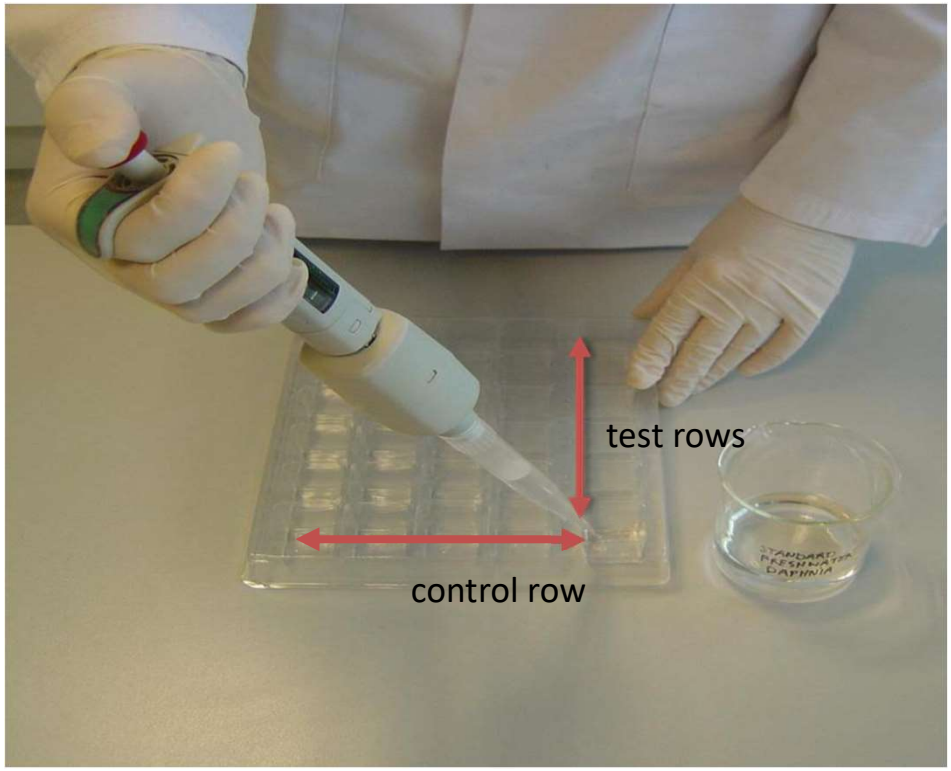
Daphnia magna immobilization test

Filling of the test plate

- Transfer 10 mL standard freshwater into each well of the control row
- Transfer 10 mL effluent dilution into each well of the corresponding rows (test rows)



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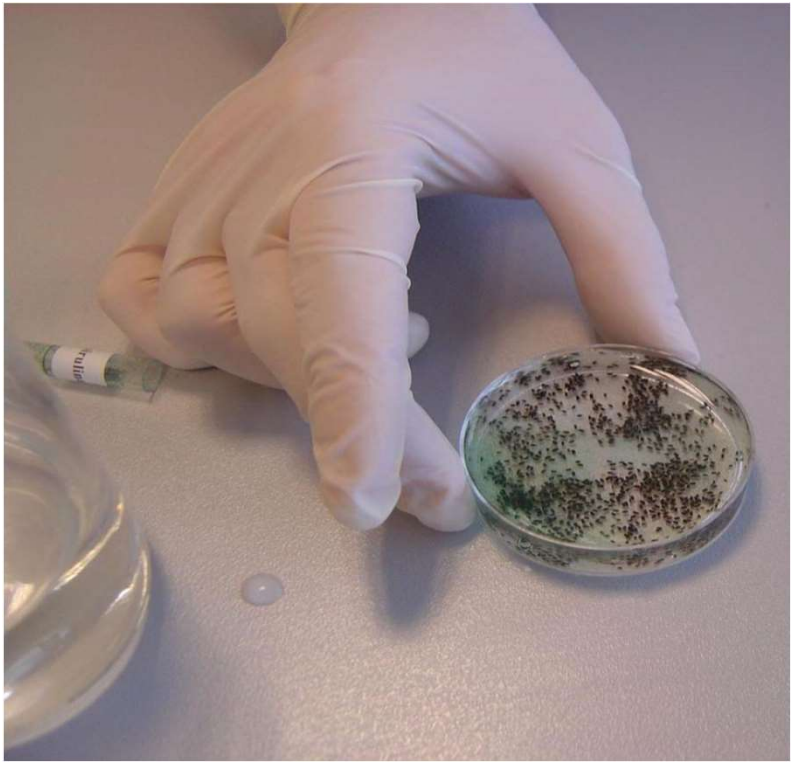
Daphnia magna immobilization test

- 2h “pre-feeding” is applied with a suspension of *Spirulina* microalgae



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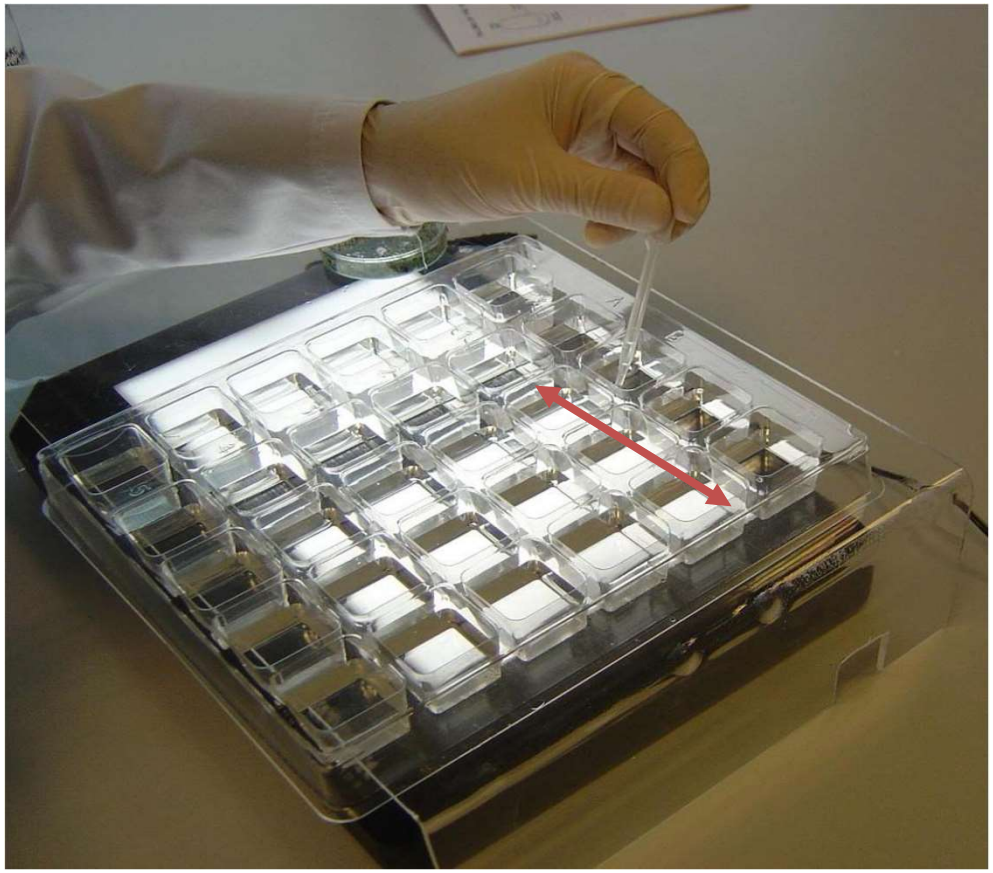
Daphnia magna immobilization test

- Exactly 5 daphnias were transwered into the 4 wells of the corresponding row.

- So, five individuals (less than 24 h old) were exposed to each dilution sample in 4 replicates.



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Daphnia magna immobilization test

- All tests for immobilisation of daphnids were carried out under controlled conditions in an incubator at 20 °C in darkness for 24/48 hours.

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Daphnia magna immobilization test

- After 24 h and 48 h incubation at 20 ± 1 °C, the number of immobile daphnids was recorded.
- The Lowest Ineffective Dilution, for which at least 90% of the daphnids are mobile, is given as test result (LID_D-value).
- Hereby, the LID corresponds to the dilution factor or the reciprocal value of the volume proportion of the wastewater.
- The calculations were done by probit analysis (Probit Programme which is available in USEPA (USEPA, 2002)).

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Algae inhibition test

- The planktonic freshwater algae species *Selenastrum capricornutum* (first renamed as *Raphidocelis subcapitata* and presently as *Pseudokirchneriella subcapitata*) are exposed to a dilution series of the reference substance or environmental sample;
- performing the standard operating procedure Algaltoxkit F™ (1996) that complies with ISO 8692 (2012).
- First we prepared **algal culturing medium** and then **concentrated algal inoculum**.

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Algae inhibition test

- three replicates for each test concentration and
- six control replicates without tested sample were prepared
- then they were transfered into the corresponding long cells

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Algae inhibition test

- All microalgal growth inhibition tests were conducted under controlled conditions in an incubator:
  - at 23±2°C
  - with continuous illumination of 10,000 lx,
  - during 72 h.

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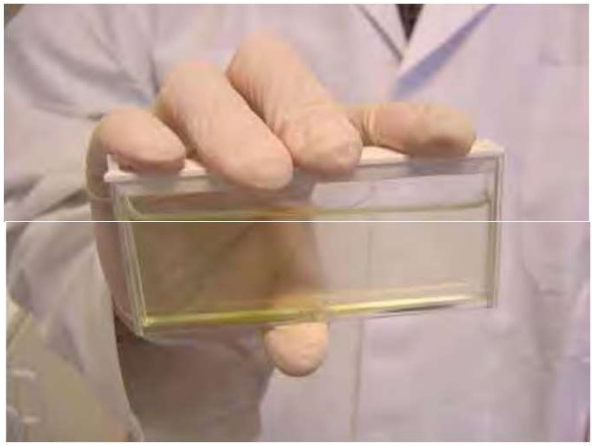
- Growth of the algae were analysed after 24, 48 and 72 h incubation by measuring optical density at 670 nm.



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- The test endpoint, inhibition of growth, is calculated by comparing the logarithmic increase in biomass during the exposure period (average specific growth rate) for each test concentration with the growth rate in the control batch.
- Dose-effect relationships are calculated by means of regression analysis and from this the 50% effect concentration regarding growth (E_rC_{50}) is determined (for reference toxicant).

- When testing waste / surface water by means of a graduated dilution, the test medium with the highest concentration at which an inhibition <20% is observed is termed the lowest ineffective dilution (LID) and reported as final result of the test (LID_A-value).

- In order to check the physiological state of the test organisms as well as the reliability (reproducibility) and reproducibility of the test procedures, tests were also performed with the reference chemical.
- Our studies were conducted with potassium dichromate, $K_2Cr_2O_7$, as a reference toxicant, and the values obtained for freshwater algae *Pseudokirchneriella subcapitata* were ErC_{50} = 0.73 mg /L.
- For *Daphnia magna* obtained values were EC_{50} = 0. 78 - 0.89 (24 h), EC_{50} = 0, 78 - 0.89 (48 h) which is in accordance with the prescribed requirements of the norm.

Samples analyzed in 2021

- Five samples were analyzed in May and July 2021:
  - (Krka River source (KRK),
  - Tributary Orašnica River (TOR),
  - Krka River Knin (near municipal outlet of the Town of Knin) (KRK),
  - Tributary Butišnica River (TBU) and
  - industrial wastewater (near screw factory) (IWW))

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Samples analyzed in 2021

- For samples analyzed in May 2021 the maximum LID values were observed with the IWW sample (algae LID_A = 32 (72 h), daphnia LID_D = 16 (24 h) and LID_D = 18 (24 h)).

- Testing on algae showed that for all other samples (KRS, TBU and TOR) LID_A = 1 were obtained. The only exception is the KRK sample for which the LID_A = 2 was observed.
- Testing on daphnia showed that for samples KRS, TBU, TOR and KRK, LID_D = 1 (24 h) values were observed. After 48 hours, values of LID_D = 2 were observed for TOR and KRK samples while for samples KRS and TBU obtained values remained the same LID_D = 1.

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Samples analyzed in 2021

- Testing on daphnia in July showed that in samples KRS, TBU, TOR and KRK also LID_D = 1 (24h and 48 h) were obtained.
- The only exception is the IWW sample for which the value LID_D = 2 was obtained both after 24 and 48 h. Thus, this sample showed significantly lower toxicity compared to May when the values were LID_D = 16 (24 h) and LID_D = 18 (48 h), respectively.

- Testing on algae in July showed that in samples KRS, TBU and TOR LID_A = 1 (72 h) were obtained.
- The only exception is the KRK sample for which the value LID_A = 2 was obtained and IWW sample for which the value LID_A = 16 was obtained after 72 h. Thus, IWW sample showed lower toxicity compared to May when the value was LID_D = 32 (72 h).

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CONCLUSIONS:

- Statistically significant increase, meaning a high LID value, was observed for IWW, pointing to toxic influence of the industrial wastewater from the screw factory and the importance of proper purification before discharging it in the environment.
- Therefore, strict and continuous biomonitoring plans must be established if serious consequences want to be avoided on the whole ecosystem, biota and the national park itself.

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