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Original Research

Evaluation of pond water after the culture of ostracods (*Heterocypris incongruens*): associating toxicity with risk assessment

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ABSTRACT:

Protections of aquatic lives from pollution associated hazard are critical to the sustenance of fish pond ecosystem. To determine whether valuable substances such as pond water can pose unacceptable level of risk to fish is the purpose of this investigation. Research has shown that the adverse outcomes of life threatening conditions originate from micro-level events. The use of whole organisms was an alternative approach to integrate and understand the biological substances that can cause toxicity to aquatic organisms. In this research, *In vivo* Early Life Stage (ELS) testing was conducted on the whole organism in the pond water prior to the independent feeding of ostracod (*Heterocypris incongruens).* The growth inhibition was also studied to effectively monitor the onset of toxic metabolites in the stationary fish pond. For sampling purpose, fish pond water was collected for five different days after the replacement of old water. It was noted that no further serial dilution was done as the number of days were adopted as dilution one to dilution five. The fish pond has the dimensions of 10×8×7 m, containing 500 liters of water and 200 fish. Fish pond water samples collected from days 1–4 showed average mortality 42 – 47%, while day 5 showed 77% ostracods mortality rate. These results suggested that the onset of fish pond water toxicity starts from day 2 – 5. This implied that toxic metabolites in fish pond water at day five and over can progressively impaired the proper growth and development of fish and other lower organisms.

Keywords:

Fish-pond-water, Risk, Toxicology, Ostracods-growth-inhibition, Ecotoxicology and Hazard monitoring.

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INTRODUCTION

A pond is a stationary water body that is natural or constructed by man; it usually contains shallow water with marsh of aquatic plants and animals (Blanken *et al*., 2016).Several factors can be used in combination with others to determine life in pond water. They range from water level regime (particularly depth and duration of flooding), nutrient levels, presence or absence of shade by trees, presence or absence of streams, grazing by animals and salinity (Keddy, 2010). Fish farming involves raising controlled populations of fish in a healthy environment; ponds have been used extensively for this purpose, but given their stagnant nature there is the tendency for toxicants to partition or bioaccumulate in the organisms' overtime due to metabolic wastes and organic decomposition of cells and nutrients FAO (2014). The metabolite toxicity is considerably significant because the sustainability and growth of aquatic life is a factor of water quality (Kim *et al*., 2015). According to EPA (1992), evaluation of water quality is by chemical method of analyses of pollutants.

However, chemical analyses of pollutants are grossly insufficient to establish pond water quality, thus, the need for toxicity testing (Latiff and Licek, 2004). Results from toxicity assessment characterize the health of aquatic and terrestrial ecosystems, with the efficacy of environmental services, providing basis for decision making and risk management (Tinsley *et al*., 2004). Through this process, obvious gross morphological effect (such as weight loss, visible lesions, death) or more subtle biochemical marker of exposure (an indicator of internal dose, such as a metabolite in urine) or biomarkers of effect (an indicator of a health effect - enzyme activity) can be monitored. The type of toxicity monitored determines the duration of the exposure, from short-term acute effects (96 hrs or less), sub-acute (a couple of days), sub-chronic (a couple of weeks) through to the chronic effects (a significant portion of the organism's life expectancy) (ENHEALTH, 2012). Using selected organisms such as ostacoda shown in Figure 1, with ecologically relevant sensitivity to toxicants and a reliable toxicity test can be performed. If the maxim is considered "the dose makes the poison" is a concept of the *in vivo* toxicity assessment and is a most relevant predictor of human health effects. This is because, *in vivo* testing measures absorption, distribution, metabolism and excretion, all of which encompasses modulating toxicity of the sample.

Ostracoda is a benthic bivalve micro-crustacean that lives in small water bodies of temperate climates (De Cooman *et al*., 2015). *Heterocypris incongruens* spends time in open water but is considered a true benthic organism, lying on the sediments and periphyton (Valls *et al*., 2014).

It's biological life cycle shifts to the production of dormant eggs (cysts). It reproduces mainly by parthenogenesis producing dormant eggs that resist desiccation. The eggs (cysts) hatch as minute nauplii, with a size of about 150µm to 200µm, and pass through eight larval and pre-adult stages before reaching adulthood within few weeks De-Cooman *et al*. (2015). Ostracods appear to be filter-feeders (Morrone, 2001), though their feeding behaviour does include scavenging, predation, ecto-parasitism and cannibalism (Rossi *et al*., 2011). They prey on small crustaceans such as Cladocera, Copepoda as well as feed on carcasses of fish, water birds and amphibians (Ottonello and Romano, 2011). Thus, this investigation was designed to monitor the toxic build-up in fish pond water within five days using

Figure 1. *Heterocypris incongruens* (Crustacea, Ostracoda) De Cooman *et al*. (2015)

the ostracod (*Heterocypris incongruens)* as test organisms.

MATERIALS AND METHODS

Collection of test samples

Test samples were collected for five consecutive days from a fish pond of dimension 10×8×7m, containing 500 liters of water and 200 fishes each. The collection was initiated every day and the old water was replaced with fresh water; samples were taken in every 24 h interval. Their pH were determined after collection and stored in a refrigerator until use.

Ostracodtoxkit F^{tm}

This bioassay involves six days and ostracod was in direct contact with the test samples. Neonates of the benthic crustacean *Heterocypris incongruens*, were hatched forth from the cysts and, are utilized to identify and evaluate the toxicity of the test sample. Towards the end of six days contact with the test sample, the percentage mortality and the growth of the crustacean are recorded and contrasted with the results of the reference (non-toxic) sample .

Experimental design

The water samples collected from the fish pond were tested for toxicity effects on the ostracods as outlined below:

Day 1: Samples collected on day 1

Day 2: Samples collected on day 2

Day 3: Samples collected on day 3

Day 4: Samples collected on day 4

Table 1. pH values of the fish pond water samples

S. No	Days	Number of samples	pH Values
1	Day 1	3	6.60 ± 0.010
2	Day 2	3	6.66 ± 0.015
3	Day 3	3	6.95 ± 0.010
4	Day 4	3	7.26 ± 0.021
5	Day 5	3	7.34 ± 0.020

Data were expressed as mean ± SD.Mean values with $p < 0.05$ were considered significant

Day 5: Samples collected on day 5

Day 6: (Control –reference sediment for the ostracod test obtained from the ostracod toxkit)

Toxicity tests

A battery of microbio tests (ostracod) at the primary tropic level was used for this study. Ostracod (*Heterocypris incongruens*) test was a six-day measure, performed in 12 cup-multiwell plates with 10 organisms per cup and six replicates following the International Standard for Organization guideline 14371 modifications (De Cooman *et al*., 2015). .

Preparation of algal feed suspension for feeding the ostracods

The storage medium of one of the algal bead tubes was poured out and 7ml dissolving matrix was added to the tube. The tube was capped, shaken on a vortex mixer for 15 minutes, and centrifuged at 3000 rpm for 10 minutes. The supernatant was decanted, 10 ml standard freshwater was added, and the tube was capped and shaken. After the re-suspension of the algae, the algae suspension was transferred to a 25ml volumetric flask and standard freshwater to the mark. The cap was capped and shaken to thoroughly re-suspend the algae to obtain a homogenous algal suspension (ISO, 2012; De Cooman *et al*., 2015).

Addition of algal feed to the ostracods

This operation was performed with one test plate for the reference sediment, and one for the test sediment. Each well of a multi-well test plate was added with 2 ml of standard freshwater. Two spoonfuls (1000µl) of sediments were taken from one of the sediment pots, struck with a spatula to remove excess sediments, and transferred into the well at the top left side of the multiwell plate. Similar operations were performed and 1000µl sediment was transferred to the five different wells (De Cooman *et al*., 2015). Keeping the multiwell horizontally, it was shaken tenderly to circulate the sediment uniformly over the base surface of the test cups. After five minutes, the flasks with the algal sus-

Table 2. Ostracods mortality rate

S. No	Days	N	Initial number of organisms	Final number of organisms	Mean	% Mortality
1	Day 1	6	60	35	5.500 ±0.547	42
2	Day 2	6	60	33	5.500±0.547	45
3	Day 3	6	60	33	5.333±0.516	45
4	Day 4	6	60	32	2.333±0.516	47
5	Group 5	6	60	14	9.500±0.547	77
6	Day 6 (Control)	6	60	57	5.833±0.752	5

Data are expressed as mean ± SD. Mean values with $p < 0.05$ were considered as significant; Initial number (Organisms at the start of the experiment); Final number (Organisms at the end of the experiment)

pension was shaken altogether and 2ml was transferred from it into each well of the test plate.

The lid of the hatching petri-dish was filled with 5 ml of standard freshwater and placed on the stage of the dissection microscope. Using the glass micropipette, part of the ostracod neonates were transferred into the lid of the hatching petridish. The lid of the hatching petri-dish was now laced on the stage of the dissection microscope and with the glass micropipette, ten ostracods were transferred into each well of the test plate (De Cooman *et al*., 2015)

Preparation of standard freshwater (moderately hard synthetic water, USEPA formula)

Eleven volumetric flasks were filled with approximately 800ml of deionized water. The five vials containing concentrated salt solutions were uncapped and their contents were emptied into the flask. Deionized water was added up to1 liter mark and the resulting mixture was shaken to homogenize the medium. The standard freshwater was aerated for 15 minutes before using it for the hatching of the cysts and preparation of the algal feed suspension (De Cooman *et al*., 2015)

Hatching of the ostracod cysts

Hatching of the cyst was done prior to toxicity testing; a petri-dish was added with 8 ml of standard freshwater. One vial with cysts was emptied into it; the vial was rinsed twice with 1ml of standard freshwater to ensure complete transfer of the cysts. The hatching petri-dish was covered with lid and incubated at 25^0C for 52 h under continuous illumination (light source of min. 3000 - 4000 lux) (De Cooman *et al*., 2015)

Pre-feeding of the freshly hatched ostracods

After 48 h of the incubation of the cysts, one of the tubes containing the *Spirulina* powder was filled with the standard freshwater, stoppered, its content was thoroughly shaken to disperse the *Spirulina* particles. The *Spirulina* suspension was poured into the hatching petri-dish and the freshly hatched ostracods were allowed to incubate for another four hours (De Cooman *et al*., 2015).

Length measurement of the freshly hatched ostracods

At 52 h incubation, ten ostracods were picked from the hatching petri dishes using a glass micropipette and transferred into one cup of the "thin-bottom" multi-well. One drop of lugol was added to the cup with the ostracods and allowed for five minutes till the organisms were immobile. The micrometer slip was positioned in the middle of the glass plate at the bottom stage of the dissection microscope and verified at high magnification, where the intersection of the two perpendicular micrometer lines are exactly in the center of the visual field. Both sides of the micrometer slip were fixed to the glass plate with a transparent tape. The multi-well was placed on the stage of the microscope, and the cup containing the ostracods was positioned at the

Table 3. Ostracods growth inhibition

S. No	Days	N	Initial mean length (µm)	Final mean length (µm)	% Growth inhibition
1	Day 1	6	185.00±0.00	223.81±23.41	82
2	Day 2	6	185.00±0.00	224.72±24.51	82
3	Day 3	6	185.00±0.00	223.06±22.72	82
4	Day 4	6	185.00±0.00	221.94±21.87	83
5	Day 5	6	185.00±0.00	218.06±14.35	85
6	Day 6 (Control)	6	185.00±0.00	399.17±8.58	nil

Data were expressed as mean ± SD. Mean values with $p < 0.05$ were considered as significant

top of the micrometer slip. The multi-well was rotated for each ostracod to have its length axis exactly on the top of one of the two micrometer lines, until the length of the ten ostracods was measured (De Cooman *et al*., 2015).

Incubation of the test plate with ostracods

The test plates were covered with a piece of Parafilm and closed with the lid. The multi-wells were placed in darkness at 25 ºC for six days (De Cooman *et al*., 2015).

Recovery of the surviving ostracods from the test plate with reference sediment

The multi-well was placed on the stage of the dissection microscope. Using the glass micropipette, all the living ostracods were picked up from the first well of the test plate and transferred into the second cup of the top row of the length measurement multi-well (De Cooman *et al*., 2015). The living ostracods were also recovered from five other wells of the test plate and put into the next five cups of the length measurement multi-well. A drop of Lugol fixative was added to each cup of the length measurement multi-well containing live ostracods to immobilize them. The number of ostracods in each glass was counted and scored on the result sheet A - for mortality (De Cooman *et al*., 2015).

Recovery of the surviving ostracods from the test plate with fish the pond water sample

With a large mouth micropipette, the sediment in the first test cup was blended with the water layer on the sediment top. Some portion of the sediment suspension was sucked up and transferred into the micro-sieve. The contents of the micro-sieve were flushed with tap water until the point when all the fine sediment particles were washed out. Stepwise exchange of the sediment suspension from the well to the micro-sieve was done, trailed by washing with tap water until the point when the greater part of the sediment have been exchanged from the well of the micro-sieve. A 1ml of standard freshwater was added to the cup, blended it with the rest of the sediment and exchanged the latter to the micro-sieve for flushing, and this activity was repeated a few times to ensure that all the sediment and ostracods have been transferred (De Cooman *et al*., 2015). The micro-sieve was turned upside down on top of a small petri-dish and the content of the micro-sieve was rinsed back into the petri-dish with standard freshwater. The petri dish was put on the stage of the dissection microscope and, with the aid of the glass micropipette, all the living ostracods were transferred into the second cup of the third row of the length measurement multi-well. A drop of Lugol fixative was added to each cup of the length measurement multi-well containing live ostracods to immobilize them. The number of ostracods in each glass was counted and scored on the result sheet of a – mortality. A similar rinsing and transfer activities were performed for the five other cups with test sediment (De Cooman *et al*., 2015).

Determination of the percentage mortality of the ostracods

The information filled out in the result sheet A

were utilized to determine the total number of surviving ostracods in the six test cups. The total number of surviving ostracods was ascertained as A (De Cooman *et al*., 2015).

The total (A) was subtracted from 60 = 6 × 10 where 60 is equivalent to the 6 (samples) × 10 (ostracods) inoculated at the start of the assay; to obtain the total number of dead ostracods. (B = 60 - A)

The percentage mortality in the reference sediment and the fish pond sediment were determined using the equation 1:

% Mortality = B/60 × 100 Equation 1

Determination of the percentage growth inhibition

Growth inhibition is the second effect criterion of the ostracods (microbiotest) which allows the evaluation of the sub lethal effect. The growth inhibition is analyzed by comparing at the length of the surviving ostracods in the test sediment with that in the reference sediment toward the finish of the test (Jarosław *et al*., 2016).

Validity criteria for the ostracod test

Two validity criteria proposed by the test procedure for the assay were as follows:The percentage mortality of the ostracods in the reference sediment should not be higher than 20%. The mean length of the ostracods in the reference sediment after 6 days should have increased by a factor of 1.5 to the mean length of the organisms at the start of the test[14].

Statistical analysis

Data obtained were expressed as mean ± SD and tests of statistical significance were carried out using two-way analysis of variance (ANOVA). Mean values with $p < 0.05$ were considered significant.

RESULTS

Table 1 shows the pH of the collected fish pond water samples increased from day one to day five. The elevation in pH levels was significant at $p < 0.05$. the pH of day one sample was 6.60 ± 0.010, followed by 6.66 ± 0.015 for day two, while 6.95 ± 0.010, 7.26 ± 0.021 and 7.34 ± 0.020, respectively represent day three, four and five, differently.

Percentage ostracods mortality rate

Table 2 shows the mortality of ostracods, *H. incongruens* of the fish pond water samples. Fish pond water samples collected from days one to four showed average of 42% - 47% mortality, day five gave 77% mortality and 5% mortality for the reference sediment (control).

Table 3 shows the growth inhibitions of ostracod, *H. incongruens* in fish pond water samples. Non-significant ($p > 0.05$) growth inhibitions were observed among the ostracods incubated in fish pond water samples. The initial mean length, final mean length and percentage growth inhibition was calculated. Result shows that at day one, the initial length mean of the ostracod was 185.00±0.00 µm and a final mean length of 223.81±23.41µm with 82% of growth inhibition. Similar trend was observed in day two and three. But at the four and five, the percentage growth inhibition increased from 83% to 85%, with initial and final mean length of 185.00±0.00µm and 221.94±21.87µm at day four. At day five, 185.00±0.00µm and 218.06±14.35µm were recorded.

DISCUSSION

Fish takes in nutrients - nitrogen and phosphorous and excrete ammonia as a by-product of metabolism (Randall and Tsui, 2002). The release of ammonia into the pond water by fish accompanied by bacterial decomposition of organic matter was probably the reason for the elevation in the pH of the pond water as presented Table 1. Research has shown that nutrients flow in fish pond via fish feed and excretion (Sipaúba-Tavare *et al.*, 2013). The assimilation and metabolism of nutrients release metabolic products, causing deterioration and decolouration of the pond water. The decomposition of organic matter by bacteria, due to bio-oxidation,

clearly contributed to the progressive deterioration of pond water quality day after day (Hari *et al.*, 2006). Indeed, this may not be novel as it is well anticipated. The idea is at this point extend do these contaminants and begin to decrease organism's survival value. The direct exposure of ostracods (*H. incongruens*) to fish pond water had a mean length after 52 h hatching period. This result is agreed with the report averred by De Cooman *et al*. (2015), about the size of newly hatched ostracods to be 150µm to 200µm.

Some authors have argued that adopting mortality rate of test organisms could be the very first validity criteria used to determine toxicity at the end of the six days exposure of the ostracods to toxicants. According to ISO 8692 (2012), the percentage mortality of the ostracods in the reference sediment should not be higher than 20% and the mean length of the ostracods in the reference samples should have increased by a factor 1.5 to the mean length of the organisms at the start of the test. Considering this result, the mortality of the ostracods in the control (Day 6) was within the stipulation by ISO (2012). The mortality rates in the test samples were higher than 30% hence indicating lethal toxicity see Table 2. This observed lethal effect on the ostracods was caused by accumulation of contaminants in the fish pond water. As stated by (Hari *et al*., 2006), fish feed supplies nutrients in excess of 75% which becomes trapped in the sediments, and mineralization of these nutrients and caused the formation of toxic metabolites. Similarly, the assimilation and metabolism of nutrients lead to the excretion of metabolic waste which contaminates the fish pond water further (Sipaúba-Tavare *et al*., 2013).The increase in lethal effects of the pond water may be suggestive of a progressive deterioration as a result of increased formation of toxic metabolites, especially from the fifth day (Sipaúba-Tavare *et al*., 2013).

The calculated mortality rate of test samples was higher than 30%. Based on this reason, the determination of growth inhibition was no longer necessary since the toxicant had already exhibited high lethal effect (ISO 8692, 2012). However, in this study it was carried out in order to evaluate the sub-lethal toxicity of the samples, and also determine the growth increment of the ostracods in the control which is the second validity criterion for the assay. The mean length of the ostracods in the test samples increase by a factor - 1.2 to the mean length of the organism at the start of the test. For the control (Day 6), the mean length of the ostracods increased by a factor of 2.2 to the mean length of the organisms at the start of the test, and this was in accordance with the (ISO 14371, 2012) guidelines. Increase in length of the ostracods as a result of growth was expected because the organisms were fed with algae during the six days incubation period. Ostracods have a feeding behaviour that ranges from filter-feeding to scavenging, predation, ectoparasitism and cannibalism (Rossi *et al*., 2011).There were measured differences among the increases in mean lengths of the ostracods in the test groups (Day 1-5), however these were not significant at $p<0.05$. Hence it was assumed that the differences in the level of contamination of the fish pond water from day one to day five were negligible. The contamination from nutrition and metabolism from day one to day five was not sufficient enough to cause significant differences in the growth of the ostracods in the test groups (Day 1-5). However, the differences among the mean growths in the test groups showed a significant ($p<0.05$) decrease compared to the control day six. Given that the control contained reference sediment with no toxicant, growth inhibition which obviously appeared in the test samples was clearly an evidence of pollution.

CONCLUSION

This investigation revealed changes in water quality of fish pond within five days, in terms of toxicity, caused lethal and growth inhibition effects to the ostracods (*H. incongruens*). The ostracod bioassays could serve a useful purpose for fish farmers and agri-

culturist in the area of management, monitoring and quick assessment of toxicity build-up in stationary fish ponds.

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