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Allelopathic effects from the macrophyte *Myriophyllum aquaticum* on the population growth and demography of *Brachionus havanaensis* (Rotifera)

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ABSTRACT

We tested the allelopathic effects of *Myriophyllum aquaticum* on the survival and reproduction of a common brachionid rotifer, *B. havanaensis*. Total phenols of the lyophilized conditioned medium (CM) and the extract of *M. aquaticum* were 0.363 and 1.164 mg g^{-1}, respectively. We evaluated the demographic response of rotifers to 3- total phenols concentrations (2.0, 5.4 and 12.2 µg g^{-1} as equivalents of gallic acid) from the extract and three (0.26, 0.53 and 1.06 µg g^{-1}) from the CM. Rotifers exposed to total phenols from the extract at concentration of 12.2 µg g^{-1} did not survive beyond one week. Age-specific survival curves of *B. havanaensis* showed that those exposed to CM had lower mortality during the first 4 days, but afterwards there was a steep fall in survival. In contrast, those exposed to phenols from the extract, regardless of concentration, had a steep decline in survival, from day one. The reproduction of rotifers in controls was constant (2 offsprings day^{-1}) from day 3 -16. In the CM the rotifers produced higher numbers (3-4 day^{-1}) of offsprings, but only for a short duration (2-3 days). Depending on the source of allelochemicals, there were significant differences in the response of *B. havanaensis* in survival and reproduction related parameters. The average lifespan and life expectancy at birth were significantly reduced to 44-50 %, when exposed to allelochemicals from the plant extract or the CM. However, gross and net reproductive rates were affected by 28-32 % in CM, while the effect was more severe (53-65 %) with macrophyte extract. Some stimulatory (hormetic) effect on the rate of population increase per day was observed in treatments with macrophyte medium.

Keywords: Allelopathic effects, allelochemicals, *Brachionus havanaensis* conditioned-medium, hormesis, macrophyte, *Myriophyllum aquaticum* phenols, phytoplankton, population growth, rotifer, zooplankton.

INTRODUCTION

Myriophyllum aquaticum (Eurasian watermilfoil or parrot-feather), is a semi aquatic macrophyte, native to the State of Amazon (Brazil) but is invasive as it has spread all over world, including North America (23). Its presence in the tropics and subtropics is due to aquarium trade (25). This macrophyte species stabilizes the sediments and maintains clear-water phase (14), absorbs nutrients and heavy metals from waterbodies (13), but also competes with phytoplankton and inhibits their growth (21).

Aquatic ecosystems in Central Mexico are generally shallow and eutrophic, both of which favour the persistent presence of invasive macrophytes such as *Myriophyllum* and

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Ceratophyllum (33). Many species from these genera produce allelochemicals such as tellimagrandin II, gallic acid and elemental sulphur compounds (11). The allelochemicals produced by these macrophytes influence the density and diversity of phytoplankton and zooplankton (12), e.g. *Myriophyllum* adversely affects the phytoplankton by production of the alguicid and the tellimagrandin II (19). Since the abundance and diversity of zooplankton species depends on phytoplankton, any change in the latter strongly affects the former (20). The direct effects of allelochemicals from macrophytes on zooplankton are known to be positive or negative, depending on the species (29).

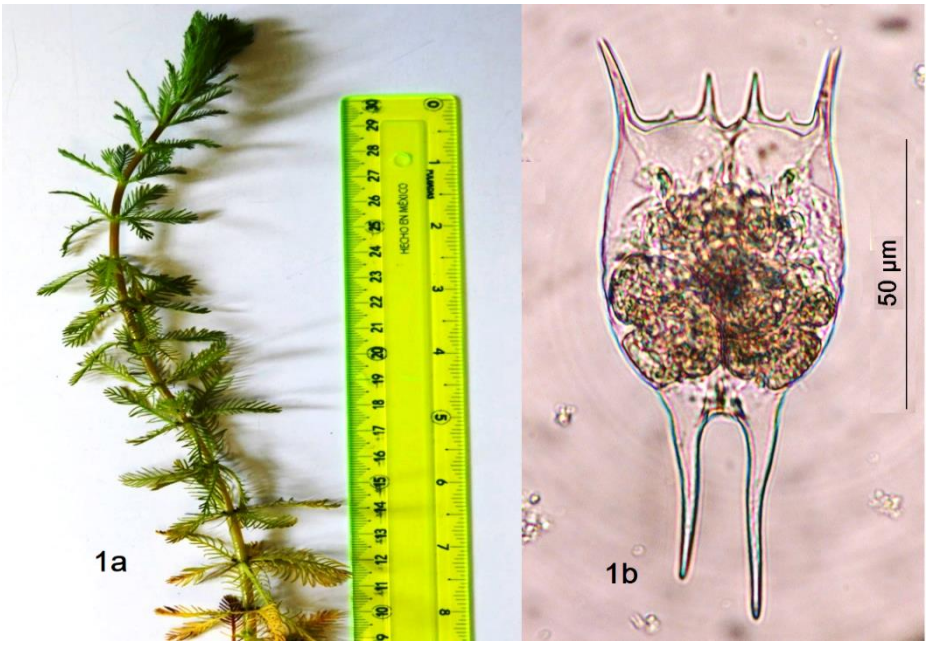

Figure 1: a. Macrophyte: *Myriophyllum aquaticum*; b. Rotifer: *Brachionus havanaensis*

In shallow lakes dominated by macrophytes there are many species of zooplankton, including rotifers (22). Periphytic rotifers are mainly associated with aquatic vegetation where, they feed on detritus and sedimented phytoplankton (28). However in shallow waterbodies, limnetic rotifer species are also associated with macrophytes, possibly to avoid competition from crustaceans and escape from fish predation (10). Allelochemicals from macrophytes exert their effects on these groups (4). For example, the interactions between the rotifers and certain macrophyte species (*Elodea canadensis* and *Potamogeton foliosus*) are antagonistic. Allelochemicals from *Elodea*, *Myriophyllum* and *Nitella* are harmful to both somatic growth and fecundity of crustacean zooplankton (*Daphnia magna* and *D. rosea*) (6,24). However, not all macrophyte-released allelochemicals are harmful to zooplankton. For example, allelochemicals from *Myriophyllum verticillatum* have positive effects on the demographic variables of *D. magna* (8). Zooplankton species associated with macrophytes in nature may simultaneously experience confounding effects of different biotic factors. To separate the effects of allelochemicals produced by

macrophytes from other factors (predation on the survival and reproduction of rotifers), lab. tests under controlled conditions are necessary.

Demographic traits of zooplankton are sensitive indicators of stress, hence, widely used to evaluate the allelopathic effects of different compounds (6,8). Population growth studies of zooplankton are used to test the impact of sublethal effects on many chemicals, both natural and man-made (28). The life table approach quantifies the effects caused by non-lethal stresses in an age-specific manner and is often used to test the effects of different stresses including the exposure to allelochemicals (9). The water-soluble secondary metabolites and extracts from tissues of macrophytes contain different groups of compounds, which influence the community composition of invertebrates but only few studies have deciphered their chemical profiles (30). This information helps to understand the allelopathic effects of macrophytes on the demographic responses of zooplankton.

In this study, we obtained the chemical profiles of exudates and extracts from *Myriophyllum aquaticum* (Figure 1a) and evaluated their effects on the demographic responses of a common brachionid rotifer, *Brachionus havanaensis* (Figure 1b).

MATERIALS AND METHODS

I. Macrophyte : *Myriophyllum aquaticum* was sampled from a perennial high-altitude shallow lake (Salazar Lake, Toluca, State of Mexico, 19° 18′26″ N 99° 23′20″ W; altitude 3000 m above sea level). The macrophyte species (30 shoots of about 30 cms length), collected randomly were kept in sterile plastic bags and transported to the laboratory at low temperature in an icebox. The plants were then washed using 10 % sodium hypochlorite solution to remove epiphytes and zooplankton and then rinsed several times with distilled water. Later, a part of material was dried in an oven at 30°C for 5 days. The remaining material was used to obtain conditioned medium. To produce enough conditioned medium, additional material was collected frequently.

II. Phytochemical analysis : To extract total phenols, flavonoids and terpenes, 0.5 g dry biomass of the apical part of *M. aquaticum* was suspended in 50 mL of 80 % methanol. Using a tissue homogenizer (IKA, Ultra-Turrax) the material was mechanically homogenized. Later it was stirred at 4 °C for 24 h to release the compounds from the plant. We centrifuged the suspension at 3000 rpm at 10 °C for 15 min. The supernatant containing the extract was used to quantify the chemical groups. This was done colorimetrically using standards (gallic acid, quercetin and ursolic acid) and units were expressed as mg g^{-1}. Freshly prepared macrophyte extracts were used in the experiments.

III. Preparation of test medium : For the conditioned medium (CM), pre-cleaned plants of *M. aquaticum* (approx 1 m length) were individually introduced into a glass jar containing 1 L of EPA medium at 23 ±1°C with a photoperiod of 12:12 light: dark. After 24 h, plants were separated from the medium. The medium was filtered using Whatman filters (0.45 µm) to eliminate the particulate organic matter. It was then lyophilized and resuspended in 80 % methanol to proceed with colorimetric methods to quantify total phenols, flavonoids and terpenes as described above. The concentration (mg g^{-1}) of total phenols was expressed as equivalent of gallic acid.

IV. Test concentrations : The extract obtained from the apical part of plant was used to prepare 3-concentrations : 2.0, 5.4 and 12.2 µg g^{-1} of total phenols as equivalents of gallic

acid. The required dilutions were prepared using EPA medium. The above undiluted filtered macrophyte-conditioned solution contained 1.06 µg g^{-1} total phenols as gallic acid equivalent, while, its two lower concentrations [50 % (0.53 µg g^{-1}) and 25 % (0.26 µg g^{-1})] were obtained by serial dilution using EPA medium. For the experiments with macrophyte exudates, we used freshly prepared conditioned medium.

V. Algal and Rotifer Cultures : *B. havanaensis*, originally isolated from Salazar Lake, was mass cultured from a single parthenogenetic individual in moderately hardwater (EPA medium) and using the single-celled green alga *Chlorella vulgaris* as diet. The EPA medium was prepared daily by dissolving 0.9 g $NaHCO_3$, 0.6 g $CaSO_4$, 0.6 g $MgSO_4$ and 0.04 g KCl in 1.0 L of distilled water (32). The alga was batch-cultured using Bold's basal medium in 2 L transparent bottles with continuous aeration and fluorescent illumination (3). *Chlorella*, harvested during the exponential phase, was allowed to sediment in refrigerator for 24 h and decanted. The sedimented alga was centrifuged at 3000 rpm for 5 min, rinsed and resuspended in small volume of distilled water. The concentrated alga was stored in a refrigerator for later use. The algal density was estimated using a Neubauer haemocytometer $0.5x10^6$ cells ml^{-1}. To ensure good algal quality, we used freshly harvested *Chlorella*.

VI. Population growth experiments : The treatments consisted of control and two factors: (i). Total phenols concentrations 3 (2.0, 5.4 and 12.2 µg g^{-1}) and (ii). condition medium Concentration 3 (0.26, 0.53 and 1.06 µg g^{-1}). The treatments were replicated 4-times. For the population growth study of *B. havanaensis*, we used total phenols from *M. aquaticum* and its conditioned medium as described earlier. The transparent jars (30 ml capacity) were used in this study, each containing 20 ml medium and 0.5 X 10^6 cells ml^{-1} of algal density, *Brachionus havanaensis* was introduced at initial density of 1 individual ml^{-1} under a stereomicroscope using a finely drawn Pasteur pipette. The test jars were kept in temperature-controlled chamber [23±1 °C and continuous diffused light]. The pH of medium in jars was 7.0-7.5. After every 24 h, the population density of rotifers from each jar was recorded by total count. When the density of rotifers in each jar was too high, they were divided in two aliquots of 1-5 ml each. After finding the rotifer density, the entire test medium was replaced with fresh EPA medium containing the chosen algal density and appropriate treatments (total phenols concentrations: 3, condition-medium concentrations: 3 and controls). The experiments were terminated, after 2-weeks, when the rotifer density began decreasing.

VII. Life table experiments : The life table demography experiments of *B. havanaensis* were done with the same treatments described above for the population growth study. However, here each jar received a cohort population of 20 parthenogenetic neonates (6±2 h of age) of *B. havanaensis*. From each jar, after every 12 h, the number of living individuals present and offspring produced, if any, were quantified. The newly born offspring and dead adults were discarded. The surviving individuals of original cohort were transferred daily to fresh jars containing the specified treatment with an alga. The experiments were terminated after 20 days, when all individuals of every cohort died. Data on survival and reproduction of rotifers were expressed on 24 h basis. The following life history variables were evaluated: survival (l_x), fecundity (m_x), average life expectancy (e_x), net reproductive rate (R_0), generation time (T) and rate of population increase (r) (17). Survival and fecundity curves were plotted as a function of the cohort age.

l_x = Proportion of surviving to start of age *x*
m_x = Offspring produced per female at age *x*

Life expectancy: $e_x = \frac{T_x}{n_x}$

Where T_x : Cumulative number of individuals for further life from age *x*
n_x : Number of living individuals at the initiation of age x (days)

Gross reproductive rate $m_x = \sum_0^\infty m_x$

Net reproductive rate $R_o = \sum_0^\infty l_x \cdot m_x$

Generation time $T = \frac{\sum l_x \cdot m_x \cdot x}{R_o}$

Population growth rate, $r = \sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1$, solved iteratively.

Statistical analysis : Differences in the life history variables *B. havanaensis* under different treatments were evaluated statistically using one-way ANOVA and for multiple comparisons, post hoc (Tukey) tests were conducted using (SigmaPlot 11).

RESULTS AND DISCUSSION

Chemical profiles : In *Myriophyllum aquaticum* the plant extracts and the exudates released into the conditioned medium contained the chemicals : alkaloids, phenols, flavonoids, saponins, tannins and terpenes as the main chemical groups. All these chemical groups were not present in two sources of macrophyte used. For example, apical plant tissue contained higher levels of phenols, flavonoids and tannins than conditioned medium (Table 1). Total phenols quantity present in the exudates (69 %) were much lower than in the plant tissues. Lyophilized medium also had a slightly higher level (7%) of terpenes, but flavonoids were at undetectable levels (Table 2).

Table 1. Qualitative analysis of the different chemical groups found in the plant extract and conditioned medium (CM) of *M. aquaticum*.

Material/ Technique used	**Chemical groups**					
	Alkaloids	Phenols	Flavonoids	Saponins	Tannins	Terpenes
Plant extract	-	++	++	-	++	+
Lyophilized CM	+	+	+	-	-	+
Standards						
Gallic acid		+++				
Quercetin			+++			
Soy saponin				+++		
β-Sitosterol						+++

Not present (-), not abundant (+), abundant (++) and very abundant (+++). CM : Conditioned medium.

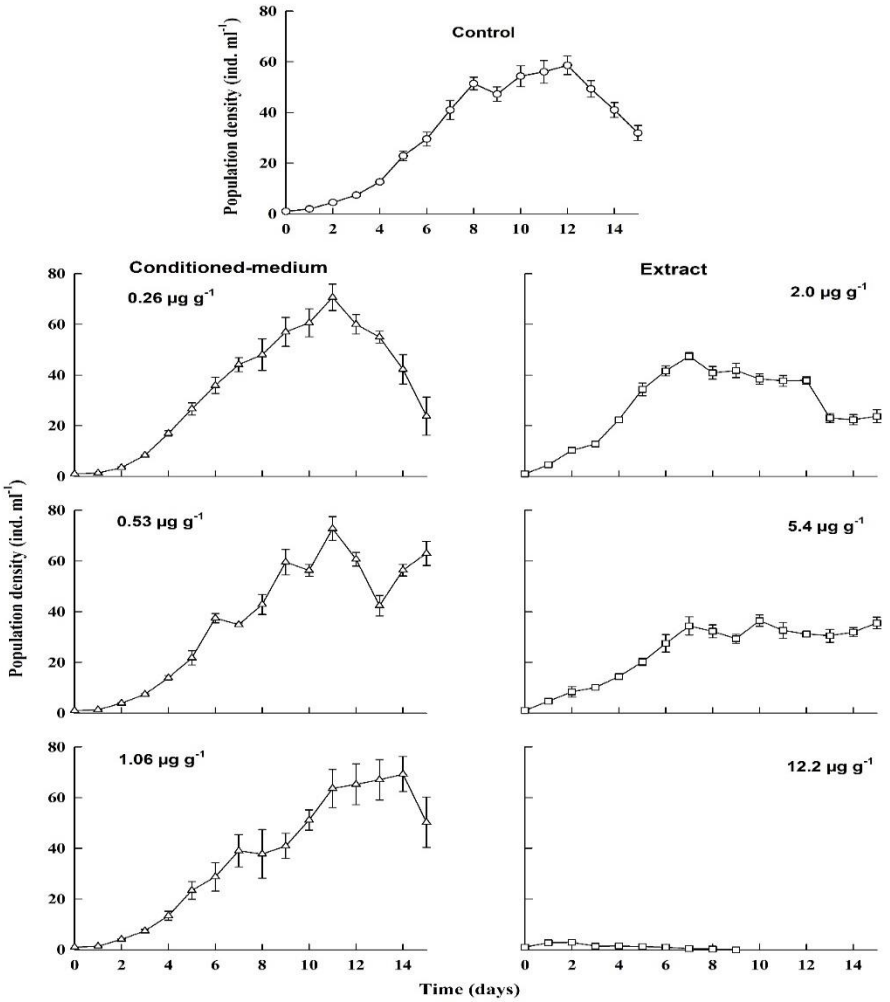

Figure 2. Effects of concentrations of total phenols (mg g^{-1} Gallic acid) from the plant tissue and conditioned medium from *M. aquaticum* on Population growth curves of *Brachionus havanaensis.* Shown are the mean ±standard errors based on four replicates.

Table 2. Quantitative analysis of some chemical groups (mg g^{-1}) in the plant *M. aquaticum* and its lyophilized conditioned medium (CM).

Material used	**Chemical groups**		
	Total phenols	Flavonoids	Terpenes
Plant extract	1.164	0.528	0.085
Lyophilized CM	0.363	Undetectable	0.091

Population growth : Population growth curves of *B. havanaensis* exposed to *M. aquaticum*- conditioned medium or its extract showed differences depending on the source of allelochemicals (Figure 2). In general, rotifers grown in the conditioned medium had higher population, than those exposed to plant extract and control. In the treatment containing CM, at lower concentrations (0.26 and 0.53 µg g^{-1}) the rotifers were able to reach peak abundances in about 10 days, while, those exposed to 1.06 µg g^{-1} needed 14 days to reach the peak value. Rotifers exposed to 12.2 µg g^{-1} of total phenols from the *M. aquaticum* extract did not survive beyond one week and during this period the population did not increase beyond the initial density of 1 individual ml^{-1}. In addition, those exposed to 2.0 or 5.4 µg g^{-1} had lower population abundances as compared to controls.

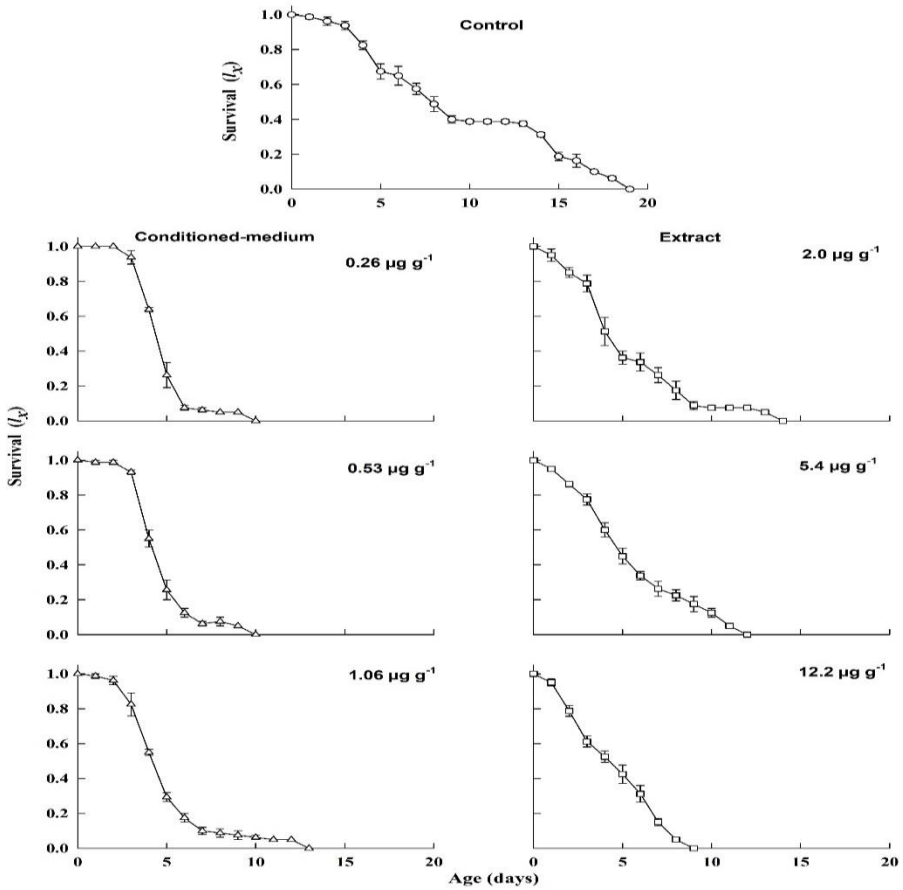

Figure 3. Effects of concentrations of total phenols (mg g^{-1} Gallic acid) from the plant tissue and conditioned medium from *M. aquaticum* on Age-specific survival curves (proportion of survival, l_x) of *B. havanaensis.* Shown are the mean ±standard errors based on four cohorts (replicates) of 20 individuals each.

Life table study : Age-specific survival curves of *B. havanaensis* showed that the rotifers had higher survival in controls, especially beyond 5 days than those exposed to CM or crude extract from *M. aquaticum* (Figure 3). Those exposed to CM had less mortality during the first 4 days, but afterwards there was a steep fall in survival of the cohort population. In contrast, those exposed to phenols from the extract, regardless of concentration, had a steep decline in survival from the day one.

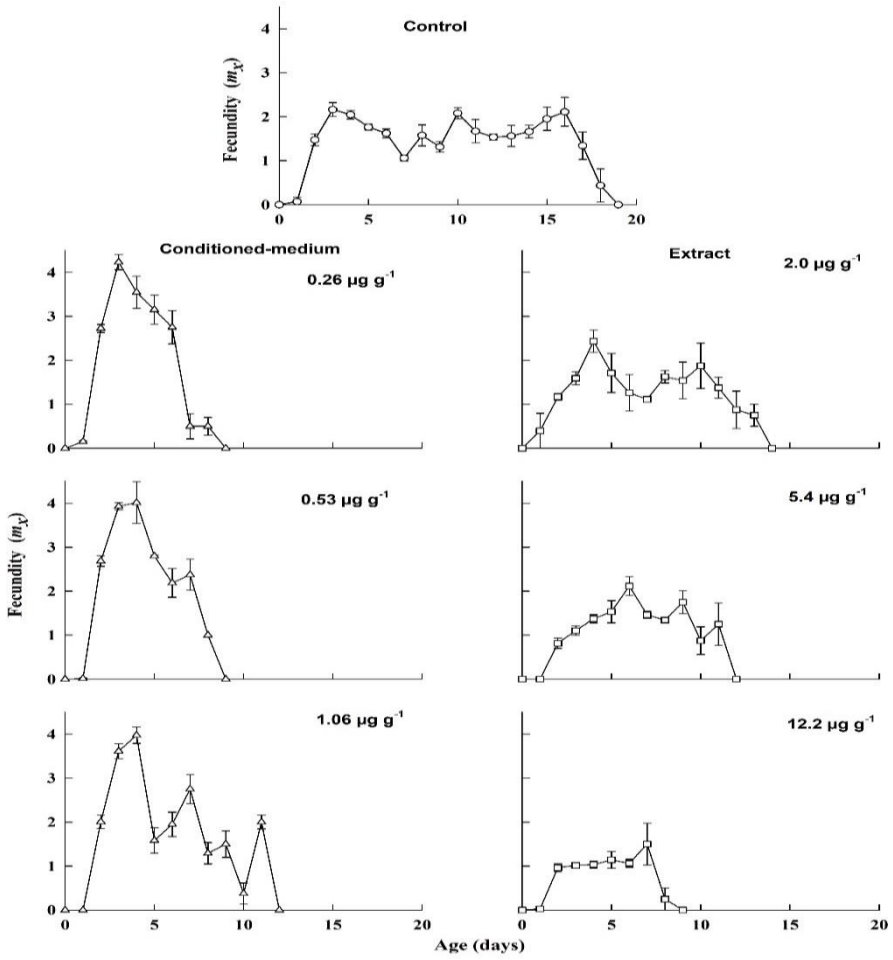

Figure 4. Effects of concentrations of total phenols (mg g^{-1} Gallic acid) from the plant tissue and conditioned medium from *M. aquaticum* on Age-specific fecundity (offspring female^{1}, m_x) of *B. havanaensis.* Shown are the mean ±standard errors based on four cohorts (replicates) of 20 individuals each.

In controls, the reproduction was nearly constant (about 2 offspring day^{-1}) from day 3 to 16 (Figure 4). While, in the treatments with conditioned-medium, the rotifers

produced more offspring (3-4 neonates per day), only for 2-3 days and later there was rapid decline in the neonate production. In treatments with crude extract, the offspring production was reduced with an increase in extract concentration. At the highest plant extract concentration (12.2 µg g^{-1} of total phenols), the neonate production lasted only 6 days.

Information on the selected life-history variables of *B. havanaensis*, showed significant differences in controls and treatments with allelochemicals. Gross and net reproductive rates and generation time of *B. havanaensis* in controls were significantly higher than those in treatments. The rate of population increase, *r*, in controls was 0.61 per day, which was significantly higher than treatment with extract. However, in treatments with a macrophyte-conditioned medium, the *r* values were significantly higher ($p < 0.05$, Tukey test) (Table 3).

Table 3. Effects of concentrations of total phenols (mg g^{-1} Gallic acid) from the plant tissue and conditioned medium from *M. aquaticum* on selected life history variables (ALS: Average Lifespan (days), E_0: Life Expectancy at birth (days), GRR: Gross Reproductive Rate (offspring female^{-1} lifespan^{-1}), Net Reproductive Rate R_0 (survival weighted offspring female^{-1} lifespan^{-1}), *T*: Generation Time (days) and *r*: Rate Of Population Increase per day) of cultured *B. havanaensis.*

Conc. (mg g^{-1})	Life history variables					
	ALS	E_0	GRR	R_0	T	r
Control	9.59±0.23^{a}	9.09±0.23^{a}	27.45±1.15^{a}	12.95±0.5^{a}	6.94±0.34^{a}	0.61±0.03^{a}
	M. aquaticum conditioned medium (CM)					
0.26	5.01±0.08^{de}	4.51±0.08^{de}	17.30±1.09^{de}	10.06±0.28^{de}	3.14±0.02^{de}	0.83±0.01^{ce}
0.53	4.96±0.14^{ce}	4.46±0.14^{ce}	18.50±1.35^{ce}	9.63±0.68^{ce}	3.23±0.07^{ce}	0.79±0.02^{be}
1.06	5.18±0.21^{be}	4.68±0.21^{be}	20.54±1.10^{be}	8.46±0.50^{be}	3.61±0.11^{be}	0.69±0.02^{de}
	M. aquaticum plant tissue					
2.0	5.60±0.33^{de}	5.10±0.33^{de}	17.71±1.56^{d}	5.78±0.27^{de}	4.32±0.32^{de}	0.55±0.07^{de}
5.4	5.81±0.08^{ce}	5.31±0.08^{ce}	13.62±0.58^{c}	4.87±0.17^{ce}	4.96±0.09^{ce}	0.38±0.01^{ce}
12.2	4.82±0.18^{be}	4.31±0.18^{be}	7.00±0.8^{b}	3.00±0.33^{b}	3.85±0.14^{be}	0.31±0.03^{be}

Data represent mean±standard error based on four cohorts (replicates) of 20 individuals each. For a given treatment, each variable carrying similar alphabets (as superscript) are not significant ($p > 0.05$, *Post hoc* test).

Like other macrophytes, *M. aquaticum* had common chemical groups (phenols, alkaloids, saponins) but many other allelopathic compounds are not identified (14). The test allelochemicals were obtained from *M. aquaticum* in two forms: (i). those released into the medium and those that are present in the macrophyte. The concentrations of test phenols in freshwaters and the median effective concentrations, EC_{50} for zooplankton are 10-42 µg g^{-1} (2). In this study, except for one, all other concentrations of phenols used are much lower than these EC_{50} values. Yet, these concentrations had significant allelopathic effects on the tested rotifer species.

Most research on the allelopathic evaluation of macrophytes considers either the effects of crude extract or the conditioned medium (9,18). In this study, we separately tested the effects of allelochemicals in both these forms and hence, we were able to compare the allelopathic effects on rotifers exposed to plant extracts and conditioned medium. Among the different chemical compounds present in the plants, phenols have allelopathic properties (15). In *M. aquaticum* phenols were present in both the conditioned

medium and in the plant extract. However, since the quantity of phenols in the conditioned medium was lower (nearly $1/10^{th}$ than plant tissue), its effect was not harmful to the population growth of *B. havanaensis*. Further, our results also showed that rotifers exposed to macrophyte extract at the highest tested concentration (12.2 µg ml^{-1}) could not reproduce and the entire population died after one week. This supports the fact that zooplankton exposed to phenols > 10 µg g^{-1} have low survival rates (2).

It is interesting to note that macrophyte conditioned medium had stimulatory effects (13-36 % compared to controls) on the rate of population increase in life table experiments, which was also reflected in higher population densities from the population growth experiments. However, the allelochemicals present in the conditioned medium significantly reduced all other life-history variables. Experimental design on the population growth and life table demography revealed different data, but is useful to understand the response of species to allelopathic stress (27). In growth experiments, the individuals of a population consist of various age groups of different generations and thus some of them adapted to the test concentration resistance. On the other hand, in the life table study, newly born individuals are removed at regular intervals and therefore, adaptation is not clear (28). However, the life table study gave data on age-specific survival and reproduction, which are helpful to understand the age-related resistance or sensitivity to stressors (16). Therefore, these two methods are complementary in evaluating the life history strategies of zooplankton.

Depending on the source of allelochemicals from the macrophyte, there were significant differences in the response of *B. havanaensis* on survival- and reproduction-related parameters. For example, average lifespan and life expectancy at birth were reduced to 44-50 % in both the sources of allelochemicals from *M. aquaticum*. On the other hand, gross and net reproductive rates decreased by 28-32 % in conditioned medium but the effects were more severe (53-65 %) from the macrophyte extract treatment. While the conditioned medium stimulated the *r* by about 26 %, it was actually reduced by 32 % by the extract. This suggests the macrophyte had higher levels of phenols, which were not released into the conditioned medium but were harmful to rotifer reproduction. This was also evident from our data of the quantitative analysis of chemical profiles. It is important to note that the plant extract also contain flavonoids, which could not be detected in the conditioned medium. Though many flavonoids have a protective role in organisms, it is possible that some of them could also be toxic (5) and may have allelopathic properties together with phenols as observed here in treatments with the plant extract.

It is known that reproduction-related parameters of zooplankton are more sensitive than survival variables (26). In this study, the survival parameters were equally affected (up to 52 %, than controls), while the gross and net reproductive rates were more adversely influenced but the variations were large (22 to 77 %) depending on the source of allelochemicals. These suggest that the life history variables respond differently depending on the nature of stress. Also, within the reproductive variables, the rate of population increase (*r*) is more sensitive, as it integrates both survival and reproduction (1). In this study, the '*r*' was stimulated due to the macrophyte conditioned medium but was negatively affected by the plant extract. The stimulatory response of rotifers to conditioned medium on the '*r*' suggests the involvement of some kind of hormesis, where under low stressful levels, the organisms overcompensate stress by enhanced reproductive output (7). Various stressful substances (heavy metals, pesticides, herbicides, natural toxins and

allelochemicals) elicit the hormetic responses (27,31). In this study, phenols present in low concentrations in the conditioned medium possibly caused the hormetic response.

CONCLUSIONS

Our study showed that both, conditioned medium and tissue extracts from *M. aquaticum* contained phenols, which significantly affected the population of *B. havanaensis*. The conditioned medium slightly stimulated the rate of population increase per day, but had the adverse effects on the survival and gross and net reproductive rates of *B. havanaensis*. Phenols from the plant extract were deleterious to both the survival and reproductive variables of *B. havanaensis*. More research is needed to confirm, if the observed patterns also exists in other zooplankton groups such as cladocerans, which coexist with rotifers in shallow ponds dominated by the macrophytes.

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