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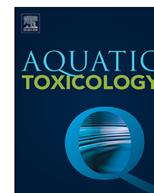

Retrospective analyses of archive phytotoxicity test data can help in assessing internal dynamics and stability of growth in laboratory duckweed cultures

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A B S T R A C T

High growth potential of duckweed species (Lemnaceae family) has been utilized in wide range of research and practical applications. Based on literature data, however, it can be assumed that duckweed populations maintain constant growth rates only when short periods are considered but can vary over longer time scales. This intrinsic instability in growth can affect the interpretation of growth data. Duckweed phytotoxicity tests are usually performed according to highly standardized protocols. Therefore the archive data provide an opportunity for retrospective comparisons. In the present study we collected growth (frond number- and frond area-based relative growth rates) and morphology (average frond and colony sizes) data from control treatments of phytotoxicity tests. All the analyzed tests were carried out with the same *Spirodela polyrhiza* (L.) Schleid. (giant duckweed) clone (RDSC ID No. 5501) under the same experimental conditions over more than four years. We aimed to assess the overall variability of the above parameters and to test if intrinsic growth patterns affect growth data in short-term. In general, the results reflected high stability of the measured parameters in long term but also indicated that some temporal variability is inevitable which can bias the comparability of growth tests. The frond area-based relative growth rate resulted in smaller coefficient of variation than the usually preferred frond number-based one. The results also revealed a negative correlation between mean growth rates and their coefficients of variation. Therefore, it would be advisable to introduce higher minimal growth rates and/or maximized tolerable coefficients of variation for control cultures into the standard duckweed growth inhibition tests. Analyses of growth data aggregated on seasonal basis indicated faster growth and larger mean frond size in laboratory duckweed cultures from mid-autumn till mid-spring than during summer and early autumn. But, in shorter term (~50 days) we did not observe distinct trends in growth suggesting that the successive frond generations have no effect on growth traits within this time-scale. Our results point to the importance of assessing intrinsic growth dynamics in duckweed cultures and also to the re-usability of the already collected phytotoxicity data in addressing new research questions.

1. Introduction

Duckweed species (members of the Lemnaceae family) have become widely used plants in both laboratory and practical applications. Duckweed growth inhibition tests use duckweed cultures to predict potential risks of chemicals and contaminants for aquatic macrophytes in natural ecosystems (Mkandawire et al., 2013). Duckweed pond systems have been introduced to waste water management (Ziegler et al., 2016) and to production of economically valuable biomass (Cheng and Stomp, 2009; Cui and Cheng, 2014). The common basis of all these applications is the astonishingly high and constant growth rate that duckweeds can perform in suitable environments. Under laboratory conditions the fastest growing duckweed clones easily double their biomass in less than 2 days and maintain this multiplication rate until crowding or nutrient limitation takes place (Ziegler et al., 2014). Such a rapid growth can be explained by the small size and predominantly vegetative way of propagation. Each plant (usually referred as frond) reaches its full size within days and rapidly forms successive descendant fronds by its meristematic region(s). During its ~1 month long lifespan each frond can potentially produce 7–15 daughter fronds (Claus, 1972; Lemon and Posluszny, 2000) which also start to produce their descendants usually before detaching from the parental frond; this way of

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frond production results in exponential growth of the population size.

Growth properties of duckweeds have been long studied at both individual (frond) and population levels. In laboratory studies endogenous frond-level growth cycles have been reported for duckweed cultures. Ashby et al. (1949) and Ashby and Wangermann (1951, 1954) observed that the successively formed offspring of *Lemna minor* L. fronds exhibit decreasing size and shorter lifespan. These observations were later confirmed by Claus (1972) and it was also demonstrated that the cycle is reset in every frond generation (Barks and Laird, 2015, 2016). From an ecological aspect this oscillation in frond traits over consecutive generations can be considered as a strategy to increase phenotypic variability in genetically homogenous duckweed populations (Abley et al., 2016; Mejbel and Simons, 2018).

At population level the distribution of different frond generations becomes constant in longer term (Lehman et al., 1981) masking the possible effects of frond-level cycles. Yet, some papers described considerable variations (Wang, 1987; Jan et al., 2015) or even a definite pattern (Tillberg et al., 1979; Scherr et al., 2008) in growth of laboratory duckweed cultures. The latter reports also suggested a seasonal oscillation in growth rates despite constant environment. Growth can be considered as a proxy which indicates in an integrated manner the overall physiological state of plants; the lower the growth rate, the greater the vulnerability of plants to stressors (Jan et al., 2015). Thus, instability in growth or other physiological traits of model plants can bias the interpretation of the results in ecotoxicology and plant physiology applications. Knowledge of intrinsic growth characteristics is especially important when several clones are tested in order to select the fastest growing or most tolerant one. Different duckweed species/ecotypes are usually compared on short-term laboratory growth tests (Ziegler et al., 2014, Sree et al., 2015). These projects have revealed large variability in growth potential and stress tolerance of different duckweed genotypes. Due to the cyclic nature of growth, on the other hand, Leng (1999) pointed to the fact that short-term experiments could give misleading results. Studies focusing on long-term growth dynamics of duckweed cultures are, however, time-consuming and laborious and thus they are constrained by laboratory capacity.

Standardized experimental protocols for *Lemna*-tests (e.g. ISO, 2005; OECD, 2006) aim to overcome several factors potentially causing inhomogeneous growth data. Uniform culturing and test conditions support intra- and inter-laboratory comparability of test results. The most cardinal point is the selection (choice) of test endpoint. The most basic test endpoint applied in duckweed test protocols is the frond number-based growth rate of test cultures (for a comparative summary of different test methods see Environment Canada, 2007). It can be determined non-destructively and counting fronds does not need highly trained staff or expensive apparatus (Sims et al., 1999). Additional test endpoints (e.g. frond area, fresh or dry weight, root length, chlorophyll or nitrogen content, etc.) are also suggested or mandatory in various test protocols (Environment Canada, 2007). Since different endpoints differ in their sensitivity (Naumann et al., 2007, Oláh et al., 2016), the question arises if the frond number is indeed the most appropriate one. When an image analysis technique is used for counting frond number in duckweed cultures also the frond area can be defined. The latter growth parameter has been reported to be more accurate in estimation of biomass change than frond number (Mkandawire et al., 2006). Frond area also gives lower calculated effective concentrations of a toxicant as compared to the frond number (Oláh et al., 2015).

The aim of the present study was to demonstrate how the already accumulated growth inhibition test data can be utilized to improve the performance of duckweed tests and to assess whether the growth of cultures would remain stable over long-term. Our hypothesis was that highly standardized experimental design of duckweed tests allows not only inter-laboratory comparisons of phytotoxicity data but also offers the possibility of retrospective analyses if sufficient amount of growth data is available in the same laboratory. For this purpose we gathered and analyzed archive growth data for a selected duckweed clone which was extensively used in phytotoxicity research in our laboratory. The specific aims were i) to analyze the consistency of growth rates and morphological parameters over several years, ii) to compare suitability of growth rates calculated on frond number and frond area basis for toxicological testing, and iii) to assess if seasonal or shorter-term trends can be found in the studied parameters.

2. Materials and methods

2.1. Experimental conditions

Growth and morphology data analyzed in the present paper were obtained from negative control cultures of duckweed growth inhibition tests performed to assess phytotoxic effects of various toxicants between 2013 and 2017 (see e.g.: Oláh et al., 2014; Oláh et al., 2015; Hepp et al., 2016). All tests were conducted with axenic cultures of the *Spirodela polyrhiza* (L.) Schleid. clone registered under No. 5501 in the RDSC database. Culturing and experimental conditions were in accordance with the OECD (2006) guidelines and followed exactly the same design in all experiments. This experimental design was reported in detail in the above listed papers (see e.g. Oláh et al., 2015). Briefly, axenic stock cultures were maintained in modified Steinberg's medium (pH 5.5 ± 0.2, Environment Canada, 2007), under continuous warm white irradiation (58 ± 4 μmol m^{-2} s^{-1}) in the plant tissue culturing room of the Department of Botany, University of Debrecen (Hungary). The temperature of the growth room was set to constant 24 ± 2 °C. Since late May of 2017 a Hobo Temp/RH logger (Onset Computer Corporation, Pocasset, USA) has been installed which records the actual temperature and relative humidity of the specific shelf used for duckweed growth tests. Based on those data the average ambient temperature during the recorded 5 months was 26 ± 3 °C. The test plants were obtained from 7 to 10 days old stock cultures. These stock cultures were always initiated with 2-2 colonies which were transferred from the previous subcultures and grew exponentially at the beginning of the experiments. Growth tests were performed in 80 mm crystallizing dishes containing 100 ml of modified Steinberg's medium (pH 5.5 ± 0.2) always freshly prepared at the beginning of tests. The starting inoculum consisted of 2 colonies with 8–12 healthy fronds; the overall mean was 10.5 ± 2.2 fronds with 2.22 ± 0.45 cm^2 total frond area. The growth tests were conducted with either 3 (25 experiments) or 4 (40 experiments) parallel control cultures for 7 days. Digital images of the cultures were taken on the 0th and 7th days of the tests using a camera positioned orthogonally above the test vessels. The test vessels (control and treated ones together) were positioned in a completely randomized design on the shelf where the experiments were conducted. The position of the vessels was re-mixed randomly twice (on the 3rd and 5th days) during the growth tests due to mitigate possible differences in the microclimate.

2.2. Measured parameters

In order to ensure a uniform image processing protocol over the entire study period all images were reassessed for the present study by means of ImageJ (Abramoff et al., 2004) and using the same settings. The following parameters were measured in all cultures: total frond area of cultures (as cm^2), total frond number of cultures (all distinguishable fronds were counted) and colony number of cultures (all distinguishable colonies were counted), respectively. Based on these data the following parameters were calculated:

relative growth rates of frond area (RGR_{FA}) and frond number (RGR_{FN}): the growth of the population was calculated using the formula (OECD, 2006): $RGR_X = (\ln(X_7) - \ln(X_0)) / 7$

where X_0 and X_7 are the respective data (frond area or frond number) on test days 0 and 7.

Doubling times for frond number and frond area in the cultures was calculated according to the formula (OECD, 2006): $T_d = \ln 2 / RGR_x$

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