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Automated Nanocosm test system to assess the effects of stressors on two interacting populations

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ABSTRACT

There is a great need in environmental research for test systems that include ecologically important factors and that are also easy to use. We present here the automated test system Nanocosm, which is composed of populations of *Daphnia magna* and *Culex pipiens molestus*. The Nanocosm system allows the investigation of stressed populations in the presence of interspecific competition, which is a very important factor involved in the dynamics of ecosystems. With the Nanocosm system, the abundance and size structure of populations of both species are quantified by image analysis. The technique enables a time-efficient, non-invasive and reliable long-term monitoring of interactions between two aquatic populations. We recommend the Nanocosm system as a novel tool for the simplified integration of competition into environmental and ecotoxicological research as well as for the assessment of risk due to stressors.

1. Introduction

Competition is a very important factor involved in the development of populations and communities in the field. Therefore, it can be assumed that competition strongly influences the population and community responses to stressors such as toxicants. It is well known that the impairment or mortality of sensitive species after exposure to toxicants may result in a release from competition with positive effects on the development of non-sensitive species (reviewed by Fleeger et al., 2003; Preston, 2002; Relyea and Hoverman, 2006). However, only little is known about toxic effects and recovery in the presence of high intra- and interspecific competition. For example, after exposure to toxicants, competing field populations may show unexpected long-term alterations in size structure (Driskell et al., 2001). Using a test system under laboratory conditions, similar effects of toxicants on the size structure of populations could be directly observed. The test system comprised of populations of *Daphnia magna* that were pulse-exposed to an insecticide and monitored by image analysis. The *Daphnia magna* population responded by a long-term increase of the abundance of small individuals that could be explained by high intraspecific competition (Liess and Foit, 2010; Liess et al., 2006).

At the community level, field investigations have shown unexpected long-term alterations in the community structure of macroinvertebrates following exposure to insecticides (Liess and von der Ohe, 2005; Schäfer et al., 2007). The mechanisms responsible for such long-term effects have yet to be revealed. Common test systems at the community level, such as pond mesocosms or artificial streams, often involve a multitude of biological species and therefore include complex interspecific interactions (Hanazato, 1998; Kennedy et al., 1995). This complexity impedes the identification and characterization of the individual mechanisms that are acting upon the community, and requires the time-consuming identifying and counting of large numbers of individuals. Hence, there is a great need for test systems that involve ecologically important factors and that are easy to use. To the best of our knowledge, a test system that allows two populations to be monitored non-invasively, in a time-efficient manner, and with detailed resolution of population size structures is not yet available.

The aim of this paper is to describe the novel test system Nanocosm, which can be used for monitoring two competing species by image analysis. The Nanocosm system is composed of populations of the cladoceran *Daphnia* (*Daphnia magna*) and the mosquito *Culex* (*Culex pipiens molestus*). Both species are primarily filter feeders with a strong overlap of natural diets (DeMott, 1982; Merritt et al., 1992). Hence, *Daphnia* and *Culex* larvae compete for the same food resource which results in a negative relationship, as already observed in outdoor microcosm experiments (Duquesne et al., 2011; Stav et al., 2005).

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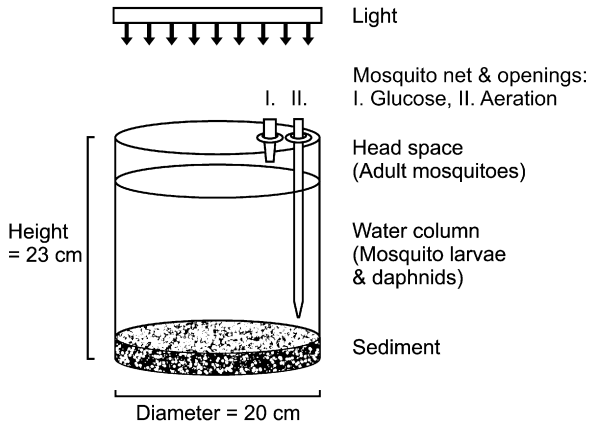

Fig. 1. Schematic illustration of the test system, which is composed of populations of *Daphnia magna* and *Culex pipiens molestus*.

A first version of the monitoring technique enabled the automated detection of *Daphnia* (Liess et al., 2006). The current version can detect and differentiate between both species. The appropriateness of the image analysis technique for the monitoring of population abundance and size structure is determined. The main interest of the Nanocosm system is its use in ecology and ecotoxicology. The Nanocosm system allows the monitoring and analysis of the effects of stressors and of recovery processes over several generations in the presence of interspecific competition.

2. Methods

2.1. Aquatic populations

We established five replicates of the test system Nanocosm, which were observed for a period of 103 days. Each Nanocosm system was initiated with 15 neonates of *Daphnia*, clone B (obtained from Bayer CropScience, Monheim, Germany), and 15 first-instar *Culex* larvae (obtained from the Federal Environment Agency, UBA, Berlin, Germany). The populations were cultured in 5.5 L cylindrical glass vessels (Harzkristall, Derenburg, Germany; Fig. 1). The glass vessels were filled with 4 L of Elendt M7 medium (OECD, 1997). Each glass vessel contained 500 g of washed aquarium sand (diameter, 1–2 mm) at the bottom, which served as a support for bacteria to promote self-purification of the test system. The populations were fed three times a week with a constant food concentration. The food was given as a suspension of ground dog biscuits (Hd-H biscuits, obtained from ssniff Spezialdiäten GmbH, Soest, Germany) mixed with stinging nettle (*Folia Urticae*, obtained from Caesar & Loretz GmbH, Hilden, Germany; weight ratio, 1:1; total carbon content, 0.9 mg/L). The populations adapted to the food supply by reaching the carrying capacity. The test vessels were covered with a net (polyester, 0.5 mm mesh size, obtained from Brettschneider, Heimstetten, Germany) to prevent the escape of adult mosquitoes. Two holes of 1 cm in diameter were made in the net to enable access to the populations (Fig. 1). Opening I was used to feed adult mosquitoes above the water surface. We closed the opening with a rolled-up pad of cotton wool that was soaked in a saturated solution of glucose and was replaced three times a week. Opening II was used to aerate the culture water three times a day for 15 min via silicone tubing (14 cm below the surface of the water; diameter, 4 mm; tapered end, 0.5 mm). The studies were performed at 20 °C. The photoperiod was controlled (16:8 h light:dark), and lighting was provided by a 70 W, cool-white fluorescent tube that was situated 10 cm above the test vessels. The biofilm on the front window of the test vessel was removed once a week with a magnetic aquarium cleaner. The water quality was measured every month. The concentrations of ions were such that no negative effects on *Daphnia* or *Culex* larvae would be expected (NH_4^+, 0.01 ± 0.04 mg/L [mean ± SD; $n = 20$]; NO_2^-, 0.004 ± 0.006 mg/L; pH 7.7 ± 0.33; dissolved O_2, 6.4 ± 0.46 mg/L; temperature, 20.5 ± 0.1 °C; electrical conductivity, 810 ± 58 μS/cm)

2.2. Photography of the populations

The populations of the Nanocosm systems were monitored three times a week by non-invasive image analysis. The populations were photographed using a digital camera (Camedia C-4000 Zoom; Olympus, Melville, NY, USA). In order to obtain a high-quality image that was free from reflections, the camera was fixed to one end of a rectangular lightproof box (length, 0.7 m), whereas the opposite end of the box was fitted against the front surface of the test vessel. The organisms were illuminated from above (light intensity below net cover, ~46,400 lux). To increase the contrast of the illuminated organisms, a black plastic film was taped to the back of the test vessels.

To improve the distribution of individuals within the image, we photographed the two species one after another and influenced their distribution within the vessel as follows. First, we attracted individuals of *Daphnia* to the upper front side of the water column by utilizing their positive phototaxis, that is, their movement toward a weak light source (intensity below 1500 lux). After individuals of *Daphnia* had gathered at the upper front side of the water column, we dispersed them by inducing negative phototaxis. The *Daphnia* swam downwards and three consecutive, time-lapsed (0.4 s) images were taken. When the *Daphnia* individuals reached the bottom of the test vessel, *Culex* larvae tended to leave the area. The *Culex* larvae swam upwards, gathered below the water surface, and another three time-lapsed photos were taken. The digital camera had the following settings: image resolution 2048 × 1536 pixels, shutter speed 1/30 s, aperture F2.8, photosensitivity ISO 400, 3× optical zoom, and focal depth in the middle of the test vessel.

2.3. Image analysis – overview

The photographs were evaluated by image analysis. The image analysis technique consisted of two parts. In Part I, *Daphnia* and *Culex* larvae were detected as moving objects while swimming with algorithms adapted from Liess et al. (2006). In Part II, *Culex* larvae that had gathered in a motionless state below the water surface for breathing were detected. For the image analysis, we used the free software *ImageJ* (1997–2009).

2.3.1. Image analysis – Part I: Daphnia and Culex larvae as moving objects

The detection of moving objects was performed separately for each species and consisted of the following four steps (Fig. 2). Step 1: the three consecutive images of each species were converted to grayscale (8-bit, pixel values from 0 to 255) and subtracted from each other. After subtraction, only moving objects remained in the image and were distinguished from the dark background by setting a threshold (accepted pixel values > 45; Liess et al., 2006). Step 2: owing to potentially high densities of *Daphnia*, we separated overlapping and touching individual *Daphnia* (watershed algorithm, *ImageJ*). Step 3: the two species were distinguished according to their measured length/width ratio (i.e. the ratio of the primary to secondary axes of the best-fitting ellipse): *Daphnia* were defined as round objects with a length/width ratio of <3.5; *Culex* larvae were defined as elongated objects with a length/width ratio of >3.5. Using these rules, individuals of the respective species not under analysis were identified and removed from the image. Step 4: the individuals of the analyzed species were counted and their lengths were measured.