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Automated system for sampling, counting, and biological analysis of rotifer populations

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Abstract

Zooplankton organisms with short generation times, such as rotifers, are ideal models to study general ecological and evolutionary questions on the population level, because meaningful experiments can often be completed within a couple of weeks. Yet biological analysis of such populations is often extremely time consuming, owing to abundance estimation by counting, measuring body size, or determining the investment into sexual versus asexual reproduction. An automated system for sampling and analyzing experimental rotifer populations is described. It relies on image analysis of digital photographs taken from subsamples of the culture. The system works completely autonomously for up to several weeks and can sample up to 12 cultures at time intervals down to a few hours. It allows quantitative analysis of female population density at a precision equivalent to that of conventional methods (i.e., manual counts of samples fixed in Lugol solution), and it can also recognize males, which allows detecting temporal variation of sexual reproduction in such cultures. Another parameter that can be automatically measured with the image analysis system is female body size. This feature may be useful for studies of population productivity and/or in competition experiments with clones of different body size. In this article, I describe the basic setup of the system and tests on the efficiency of data collection, and show some example data sets on the population dynamics of different strains of the rotifer *Brachionus calyciflorus*.

Introduction

There is a long tradition of using zooplankton, such as rotifers, as experimental models in limnological and ecological research. Their small body size and fast population growth, and the ease of keeping large populations in a relatively small space, make them ideal for experimental approaches in the laboratory, especially at the population level. Various general questions have been addressed using laboratory populations of rotifers, for example, assimilation efficiency and productivity of populations (Boraas 1983; Rothhaupt 1985; Walz 1993), food chains (Van Der Stap et al. 2007; Verschoor et al. 2004), resource competition (Boraas et al. 1990; Ciros-Perez et al. 2001; Rothhaupt 1988; Stelzer 2006), population dynamics (Fussmann et al. 2000; Kirk 1998; Yoshinaga et al. 2001), nutrient limitation (Rothhaupt 1995), and evolutionary change (Fussmann et al. 2003; Yoshida et al. 2003, 2007). Experimental investigations at the population level are usually constrained by the time-consuming counting of the samples. In practice, populations have to be sampled at least once per day to achieve an adequate temporal resolution. After fixation, samples are concentrated in sedimentation chambers and analyzed by microscopic examination under an inverted microscope (e.g., Fussmann et al. 2000). If adequate replication is used in such experiments and/or if populations are studied for several weeks, the work effort quickly reaches levels that are barely manageable. Moreover, the sampling interval is usually constrained to 24 h, which may be too long to resolve fine patterns of population dynamics.

Automation of sampling and counting of experimental populations is clearly a desirable goal. One of the first steps in this direction was taken by Boraas (1983), who used electronic particle counters (EPCs) (e.g., the Coulter Counter®) to study the population dynamics of the rotifer *Brachionus calyciflorus*. With EPCs, rotifers are detected by the changes in conductance when individuals pass a narrow channel in the measuring unit of the device. In addition to counting, EPCs give estimates on the body size distribution, as the strength of the electrical signal is proportional to body volume (Boraas 1983). There are several drawbacks to this method, however: (1) the

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culture sampling has to be done manually, which is time consuming and presents a continuing risk of contamination; (2) operation of an EPC may be faster than "manual counting," but it is still time consuming, e.g., loading the samples, reading data, cleaning, and routine maintenance of the counting device; (3) the sample has to be diluted in a special electrolyte buffer, which lowers the detection limit if only few rotifers are present; (4) the data output of EPCs usually cannot be directly transferred to other computer programs (e.g., Excel); and (5) EPCs are expensive to buy and maintain.

Recent technological advances in digital imaging sensors have opened alternatives for automatic analysis of rotifer populations. Briefly, in digital image analysis, samples are drawn from a culture vessel into a flat optical chamber (similar to a flow-through cuvette). Digital pictures are taken by a camera and sent/analyzed automatically in a PC. Live individuals, which are continuously swimming, can be detected by subtraction of two digital images that have been taken in short temporal sequence. This principle of "background subtraction" is commonly used to detect moving objects, e.g., estimating urban traffic in cities (Cucchiara et al. 2003), but has also been exploited in biological context, e.g., in population size estimation of bacteria in microchemostats (Balagadde et al. 2005). The proof-of-principle paper with rotifers was presented by Alver et al. (2007), who developed an automated system to determine the density of rotifers in first feeding tanks in fish aquaculture. They showed that image analysis can give good estimates of the rotifer concentration in such tanks.

Here I describe a sampling and image analysis system that was designed for studies of aquatic ecology, i.e., where rotifers are used as model organisms to investigate general ecological questions. The image analysis system allows in-depth analysis of rotifer population, including abundance estimation, detection of males (i.e., episodes of sexual reproduction), and measurement of body size distributions. The sampling system includes several new features to efficiently work with a large number of replicates and prevents cross-contamination between measurements of different cultures. It works completely autonomously and can sample up to 12 independent rotifer populations in short time intervals (down to a few hours) and over several weeks. In this article, I describe the basic setup of the system and tests on the efficiency of data collection, and show some example data sets on the population dynamics of different strains of the rotifer *Brachionus calyciflorus*.

Materials and procedures

General setup—The main components of the automated sampling and image analysis system are shown in Fig. 1. The

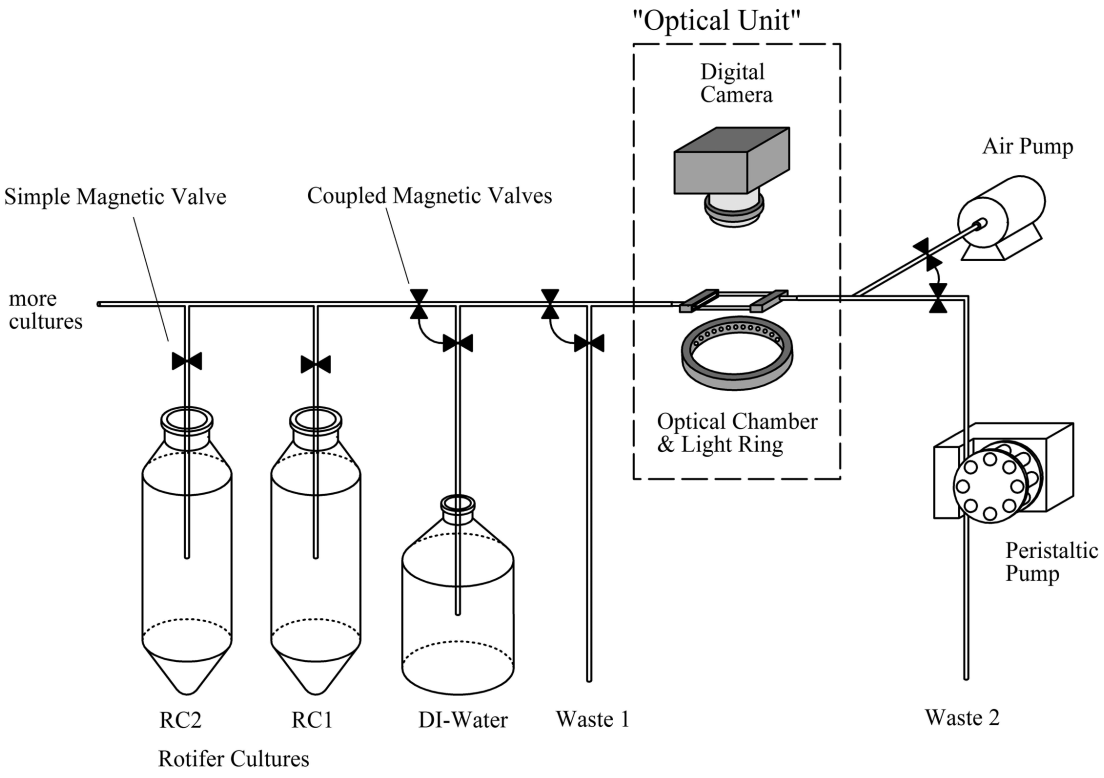

Fig. 1. Schematic drawing of the sampling and image analysis system. For simplicity, only two rotifer cultures are displayed (the system can handle up to 12 cultures). The tubing connecting to these cultures was 120 cm in length, measured from the outlet of the culture to the dedicated simple magnetic valve (not true to scale in Fig. 1). All electrical parts, i.e., magnetic valves, air pump, peristaltic pump, camera, and illumination, can be controlled by a PC (PC and wiring not shown). *Simple magnetic valves* are either opened or closed (default: closed); *coupled magnetic valves* can switch between the states: valve 1 open/valve 2 closed and vice versa.

system is composed of parts dedicated to the automated sampling (silicone tubing, magnetic valves, air pump, peristaltic pump) and parts associated with image acquisition (optical unit in Fig. 1: camera, optical chamber, illumination). All electrical parts were controlled by a PC: The digital camera was controlled through a Firewire connection, the peristaltic pump through a serial RS232 connection, and some parts were indirectly controlled through a Quancom® USBREL64 relay interface (magnetic valves, LED illumination unit). For clarity, the computer and the connecting cables were omitted from Fig. 1.

The heart of the image acquisition system is the optical unit, consisting of optical chamber, ring illumination, and digital camera (Fig. 1). The optical chamber consists of two glass plates separated by a frame to form a chamber with the inner dimensions of 18 × 24 × 3 mm (i.e., approximately 1.2 mL volume). Two stainless-steel tubes attached at the lower left and upper right end of the chamber provide in- and outflow of the culture medium. The chamber is fixed in a metal frame, which itself is mounted onto a commercially available LED darkfield illumination unit (CCS LDR2-74SW2-LA). The images are taken with an industrial monochrome digital camera with a resolution of 6 megapixels (PixeLINK® PL-B781F). As optics, I use a Schneider-Kreuznach APO-Componon 45mm lens with two 10-mm macro distance rings. One pixel corresponds to a square with approximately 9 µm length. All optical parts, chamber, illumination unit, and camera are mounted on a metal support so that the field of view of the camera exactly matches the size of the chamber (not visible in Fig. 1).

Sampling system—Up to 12 rotifer cultures are connected via silicone tubing with the optical chamber (Fig. 1). The length of these connections is 120 cm, measured from the outlet of the culture to the dedicated simple magnetic valve (not true to scale in Fig. 1). The magnetic valves (FluidConcept S104) of each culture only open during sampling events (controlled by the PC). Individual cultures are sampled using the following sequence of events:

(1) Filling the chamber. The magnetic valve connecting to the focal rotifer culture is opened and the peristaltic pump is switched on. This creates suction in the silicone tubing, which causes the culture medium to flow into the optical chamber. When the chamber is filled with the rotifer culture to be sampled, the pump stops and the digital camera takes two pictures and sends them to the PC, where they are digitally subtracted, stored, and analyzed.
(2) Emptying the chamber. Used samples are expelled from the optical chamber with the membrane air pump: the two magnetic valves directly flanking the chamber are switched and the air pump is turned on. Note that these two magnetic valves are coupled (one channel is opened while the other is closed and vice versa). This allows changing the direction of flow so that the used culture medium is expelled in reverse direction into a waste reservoir (Fig. 1). The change in flow direction is essential, because otherwise the chamber cannot be emptied completely.
(3) Rinsing the chamber. Between samplings from two different rotifer cultures, or at the end of sampling, the optical chamber is rinsed with deionized (DI) water from a separate reservoir (Fig. 1) to prevent cross-contamination. To initiate the rinsing cycle, the magnetic valve connecting to the DI reservoir is opened (connecting the DI water bottle but disconnecting the rotifer cultures) and the peristaltic pump sucks DI water into the chamber. The DI water is expelled in the same way as described in step 2.

With this sequence of events, each rotifer culture can be sampled, and afterward the system can be paused by filling the chamber with DI water until the next sampling event (to prevent fouling within the chamber). To avoid dilution with residual DI water, the optical chamber is emptied before each sampling event with the membrane air pump (step 2). Additionally, old culture medium and DI water in the tubing are removed by filling the chamber once before each measurement (step 1) and immediately emptying it without taking pictures (step 2). The complete sampling procedure for 12 rotifer cultures and three samplings per culture, including all rinsing cycles, takes less than 90 min.

All events of the sampling procedure are controlled by a program written in Labview® 8.5 and NI Vision (National Instruments). Figure 2 displays a schematic depiction of the program's general structure. The actual Labview code is available from the author on request. The program represents a simple "state machine" that switches between two states: a waiting state and a sampling state (Fig. 2). Within the waiting state, the program reads the current time and compares it to the preset sampling times, which may be one or several different time points during a day. When the time of a sampling event is reached, the program switches to the sampling state, which causes all cultures to be sampled in sequential order. After sampling is completed and image analysis is done, the program switches back to the waiting state. Due to its modular structure, the program can be adjusted quickly to accommodate a wide range of variation in experimental designs: number of cultures sampled, number of samplings per day, number of samples per culture (within one sampling).

Image analysis—Images are automatically processed using NI Vision, running within the Labview programming environment. Briefly, the two pictures from each sample are loaded into the memory of the PC and one image is digitally subtracted from the other. After subtraction, the thresholding algorithm of NI Vision generates a binary image (i.e., an image consisting of black and white pixels only). Shape recognition algorithms are used on the binary image to detect rotifers and classify them. The detection criteria for female and male rotifers are summarized in Table 1. These criteria are empirical estimates and are optimized for the lightning conditions provided by the light ring and settings of the camera (brightness, gain, exposure time). I found that a relatively long exposure time of 100 ms and a waiting time of 1 s between the two pictures gave best

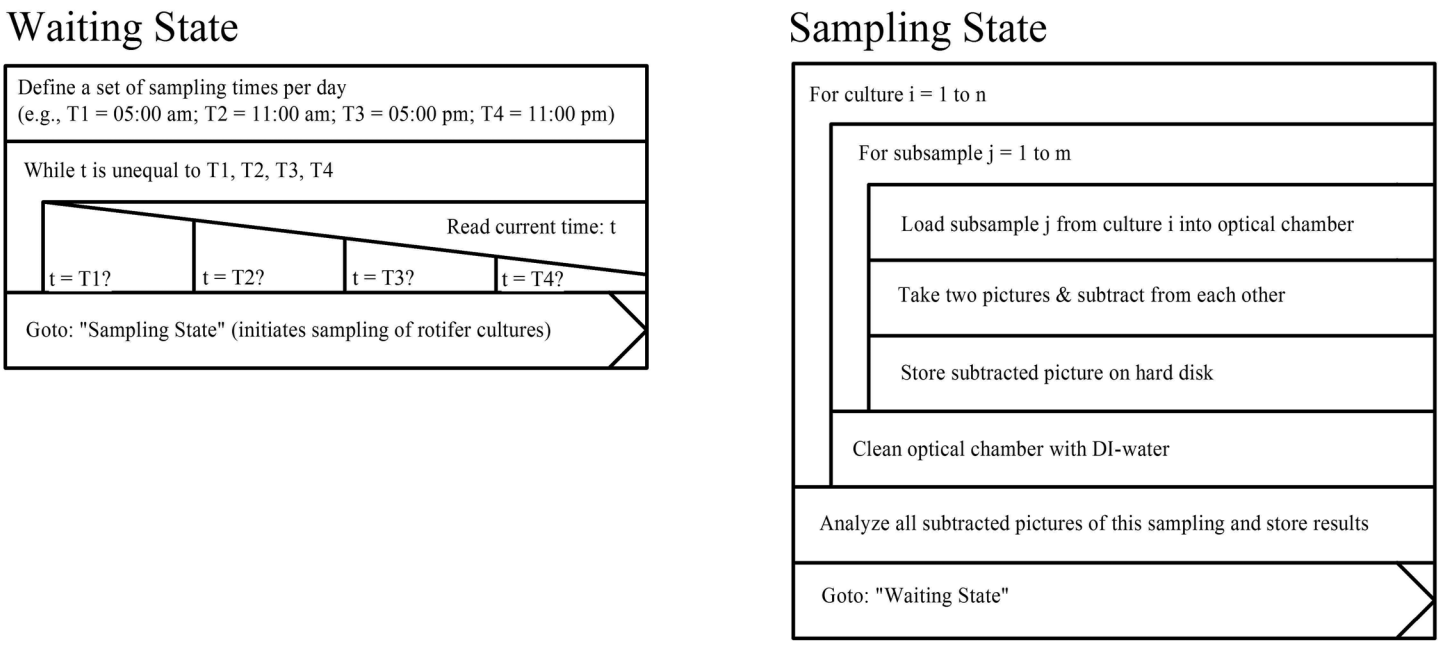

Fig. 2. Simplified structure of the program that controls sampling, image acquisition, and image analysis (Nassi-Shneidermann diagram). Note that the program switches between two fundamental states, a waiting state (for the times between two samplings) and a sampling state. The switch between these two states would continue indefinitely, unless the program is interrupted or terminated by the user.

Table 1. Detection criteria of females and males in the image analysis program.

	Command/detection criteria	Parameter range
Female preprocessing	1. Erode picture (removes random noise)	
	2. Set threshold[a]	50–255
	3. Fill holes (of particles)	
Female detection	Particle area	80–2000 pixels
	Elongation factor[b]	1–4.5
	Equivalent ellipse minor axis	8–30 pixels
Male preprocessing	1. Erode picture	
	2. Set threshold	50–150
	3. Exclude particles with holes	>50 pixels
	4. Fill holes (of remaining particles)	
Male detection	Elongation factor	>6
	Particle area	80–200 pixels
	Equivalent ellipse major axis	20–40 pixels
	Equivalent ellipse minor axis	4–7 pixels

[a]Thresholding transforms a grayscale picture to a binary picture: All pixels meeting the inclusion criteria are set to white; all remaining pixels are set to black. After thresholding, particles can be identified as a group of white pixels directly adjacent to each other.
[b]Elongation factor was defined as a particle's largest intercept divided by the mean perpendicular intercept.

results for detecting both female and male rotifers: Individual females look like rice grains, whereas the fast-swimming but much smaller males leave more narrow traces, reminding of eyelashes (Fig. 3). These two shapes are clearly distinct and can be reliably separated by the exclusion criteria listed in Table 1. Note that due to the long exposure time of 100 ms, rotifers tend look a bit more elongated than their natural shape, because of the distance they swim during exposure.

Rotifer cultures—The performance of the sampling and image analysis system was assessed using laboratory cultures of the rotifer *Brachionus calyciflorus*. All *Brachionus* lines used in this study were descendants of two strains from Florida and Georgia, respectively, and were originally provided by J.J. Gilbert (Dartmouth College, Hanover, NH, USA). They have been maintained in my laboratory since autumn 2006. Clonal lines of these strains differed with respect to body size and

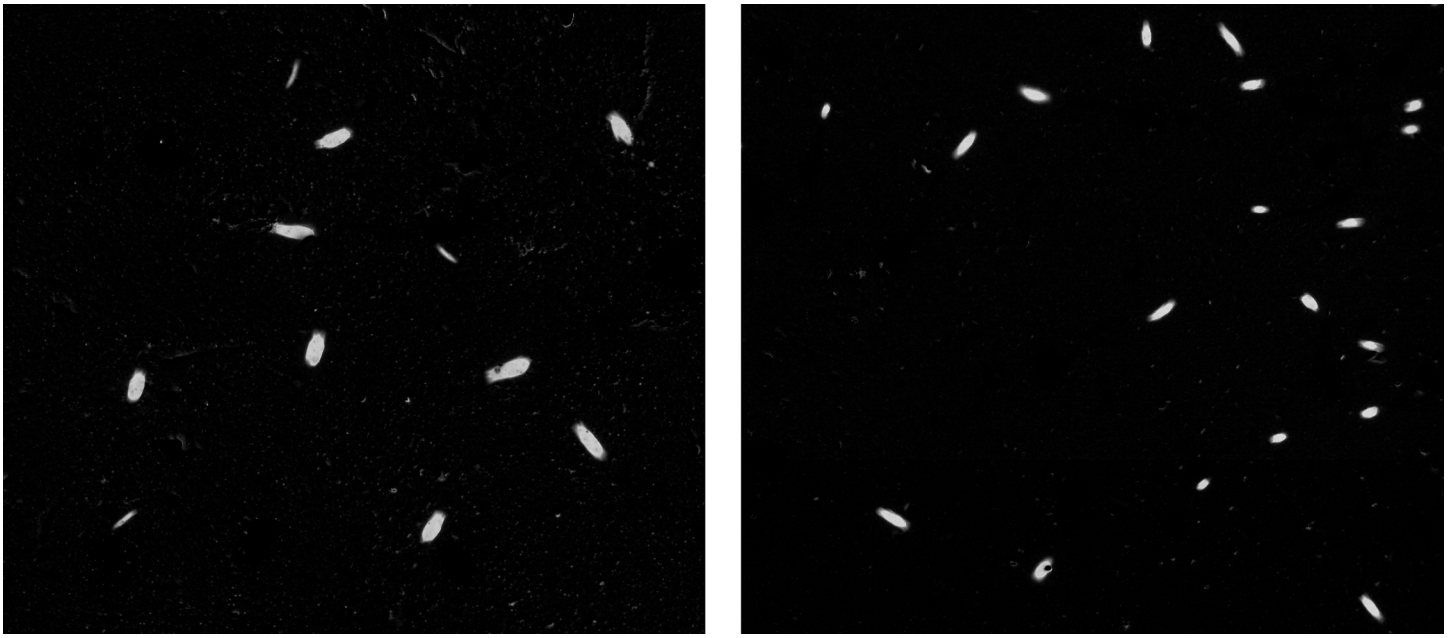

Fig. 3. Photographs of two different rotifer cultures (taken by the image analysis system). Right: Cyclical parthenogenetic clone. Visible are eight females and three males. Left: Obligate parthenogenetic clone. Visible are 19 females. The photographs show only a small section of the actual picture used by image analysis (approximately 13%).

their ability to induce sexual reproduction: Some of them were cyclical parthenogens and others were obligate parthenogens that had lost the ability to reproduce sexually (Stelzer 2008). The rotifers were cultured in Combo medium (Kilham et al. 1998) with the unicellular algae *Chlamydomonas reinhardii* as food source (strain SAG11-32b, Sammlung fuer Algenkulturen). The rotifer populations used in this study were cultured in chemostats, i.e., flow-through cultures that were continuously diluted with food suspension harvested from a separate algal culture. Algae in this algal chemostat grew at a dilution rate of 0.89 d^{-1} and had entered a steady state with a relatively constant biovolume of 1.2×10^8 femtoliters mL^{-1} (approximately 750,000 cells mL^{-1}). This algal suspension was diluted 1:1 with fresh culture medium before being distributed among the rotifer chemostats. In the experiments, I used chemostats with 380 mL volume and a dilution rate of 0.56 d^{-1}. All experiments were done in a temperature controlled room at 24°C. For further details on culturing methods, *see* Stelzer (2008).

Assessment

Detection of females and males—To find out if the image analysis system reliably recognizes and counts female and male rotifers, a quantitative test of the accuracy of the system was done by comparing counts based on samples fixed in Lugol solution with estimates from image analysis from the same time points. These measurements were done on different chemostat cultures and different *Brachionus* clones. Samples of 15 mL each were taken manually from these chemostats, fixed with Lugol solution, and enumerated in sedimentation chambers. At least 60 rotifers, but usually between 200 and 300, were counted for each fixed sample. Because the image analysis system was drawing samples from the chemostats every 6 h, it provided a whole time series around the manual sampling event. To reduce random fluctuations within this time series, local exponential smoothing was applied within Sigmaplot® (Jandel Scientific Software). Parameters chosen were a polynomial degree of 1; the option "remove outliers" was selected; and the sampling proportion was adjusted to 0.05 and 0.1, depending on the length of each dataset, so that the smoothing was applied to four consecutive samplings (i.e., 24 h). Finally, the microscopic count was compared to the smoothed value from the image analysis system of the same time point.

Overall there was a strong positive correlation between manual counts and image analysis counts for females (Spearman rank correlation, $n = 52$, $\rho = 0.951$, df = 50, $P < 0.0001$). The slope of the regression line, using manual counts as predictor variable, was close to 1, which suggests that image analysis reliably counts all females in the sample. Likewise, the male counts with both methods showed a strong positive correlation ($n = 16$, $\rho = 0.953$, df = 14, $P < 0.0001$). However, the slope of the regression line was 0.28, which indicates that image analysis only detects 28% of all males in the samples (Fig. 4, lower graph). This was probably due to the much smaller size of males and correspondingly weaker signals for image analysis. A typical male is 60 µm in body length and produces a signal of around 120 pixels (area), whereas the larger females can produce signals of up to several hundred pixels. Thus males that are not exactly in the focal plane may not be recognized. In the larger females, this is hardly a problem: females not exactly in

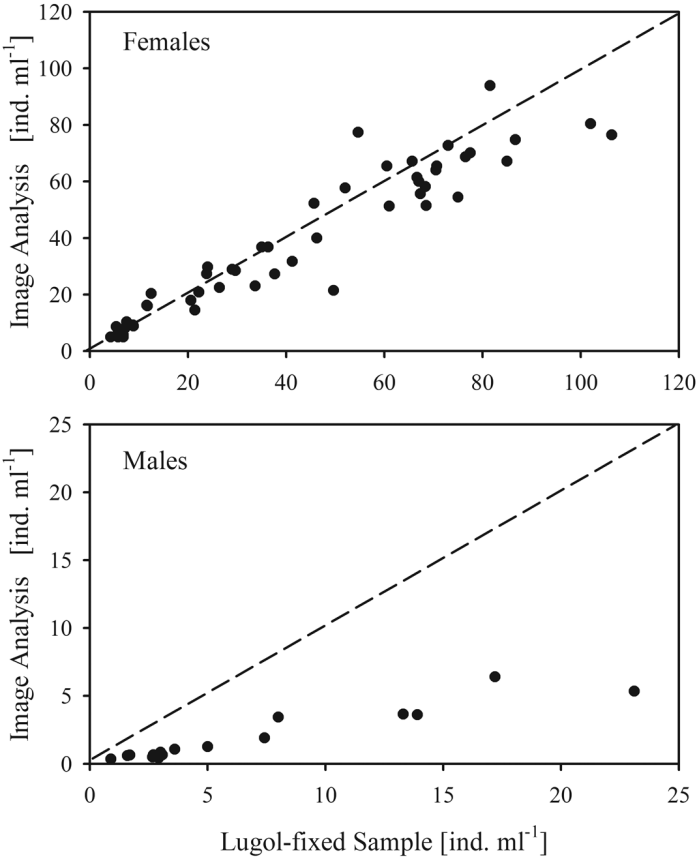

Fig. 4. Comparison of rotifer counts with the image analysis system versus a conventional counting method (Lugol-fixed samples counted under an inverted microscope). Top graph, female detection; bottom graph, male detection. Dashed line represents the expectation if both methods yield exactly equivalent results (1:1 ratio).

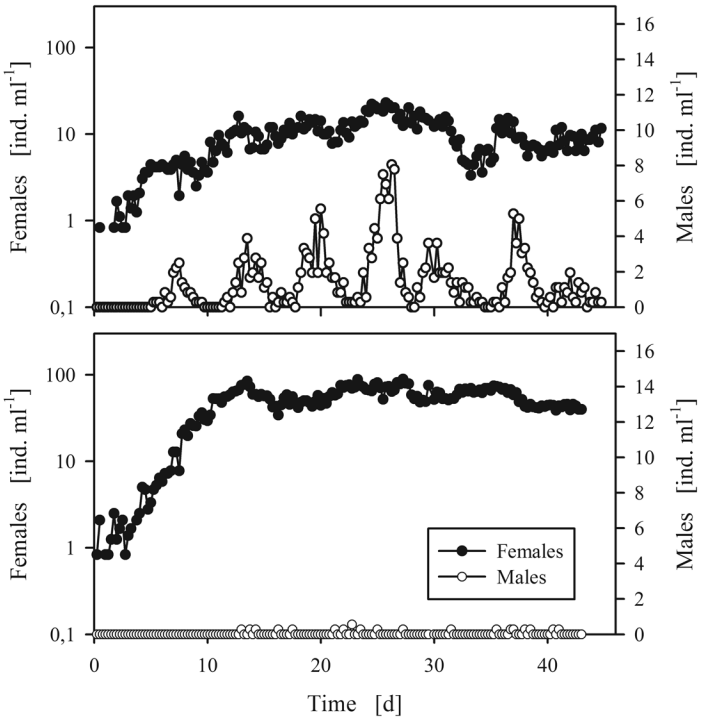

Fig. 5. Example of a long-term run with the image analysis system. Population dynamics of a cyclical parthenogenetic clone (top) and an obligate parthenogenetic clone (bottom). Note that the different scaling at the *y* axis for females and males.

the focal plane may give a weaker signal (i.e., reduced body size), but are still detected. Nevertheless, the detection sensitivity for males was sufficient for recording highly dynamical patterns of male production in rotifer cultures (*see* "Long-term operation").

Long-term operation—The reliability of the sampling system was assessed during a 45-day chemostat experiment. The population dynamics of two chemostat cultures are displayed in Fig. 5. The first culture contained a cyclical parthenogenetic clone of *Brachionus calyciflorus* (Fig. 5, upper graph), i.e., a clone that produces males at high population densities. The high temporal resolution due to the short sampling intervals (6 h) revealed a dynamic pattern of male production. Male abundances were not constant, but rather showed cyclic oscillations with several peaks of male concentration, followed by collapses to values near the detection limit. By contrast, the second chemostat culture (Fig. 5, lower graph) contained an obligately parthenogenetic clone of *B. calyciflorus*, which has completely lost the ability to produce sexual stages, including males. The population dynamics of the females was typical for a two-stage chemostat culture: an initial period of exponential growth was followed by dampened oscillations toward a stable equilibrium (at around 60 females per mL).

Even though this rotifer culture was completely asexual, the image analysis system sporadically scored males. Closer examination of these pictures suggested that that a few optical signals were wrongly scored as males. Turbulences created by swimming females seemed to have caused movement of nearby small detritus particles and created a signal similar to that of a swimming male. However, those instances were extremely rare (*see* Fig. 5, lower graph). Further support for the notion that obligate parthenogenetic clones do not produce males at all comes from microscopic counts of such cultures. During the experiments of this study, more than 300 mL Lugol-fixed samples were enumerated under an inverted microscope: In cultures of cyclical parthenogenetic clones, a total of 2488 females and 1117 males were found, whereas in obligately parthenogenetic clones a total of 4473 females, but not a single male, were detected. Similarly, live examinations of cultures of obligately parthenogenetic clones did not reveal any indications for the presence of males, such as male-producing females or diapausing eggs. It thus seems that the few male signals visible in the lower graph of Fig. 5 represent the background noise of the male detection in the image analysis system.

Body size distributions—The image analysis system may be used to estimate body size distributions within populations. This feature was assessed in cultures of two *Brachionus* clones,

which had clear differences in body size (approximately 2-fold by body volume; Stelzer, unpubl. data). These two clones were cultured in eight chemostat populations, with four replicate chemostats for each clone. The populations were grown until they reached equilibrium at densities ranging from 30 to 85 females mL^{-1}. Samples were taken manually, fixed with Lugol solution, sedimented, and measured under an inverted microscope at 200-fold magnification. Body size was measured for 40 randomly selected females for each chemostat population. These measurements included three distance measurements: total length, maximum breadth, and breadth at the anterior end. To make these measurements comparable to the data obtained by the image analysis system, I calculated the area covered by a female (in μm^2), assuming the shape of an ellipse with body length as the major axis and the mean of the two breadth measurements as the minor axis. The measurements of the image analysis system were adjusted by multiplying female size (in pixels) by a factor of 81 (one pixel was a square of approximately 9 µm in length). In this way, Lugol measurements and estimates from the image analysis system could be directly compared.

The image analysis system was able to detect differences in body size, since both clones showed distinguishable body size distributions (Fig. 6). In addition, a nested ANOVA with chemostat number as nested factor showed that these two clones can be distinguished by measurements with the image analysis system (n = 1211, F = 422.34, df = 1, $P < 0.0001$). Nevertheless, there was significant overlap between the two curves, which indicates that in many cases it would not be possible to assign individual females to one of the two clone types. The size distribution curves obtained with the image analysis system were broader than those obtained by microscopic measurements (Fig. 6). This was also evident from the coefficients of variance (displayed in Table 2): Measurements based on image analysis generally had larger coefficients of variation than those based on microscopic measurements (approximately 44% versus 23%). Image analysis also gave slightly larger estimates of body size than microscopic measurements (*see* "Grand means" in Table 2). This was likely a consequence of the long exposure times (100 ms), which led on average to a slightly increased body length. In addition, animals that were not swimming exactly 90 degrees relative to the observation axis, which leads to a weaker optical signal, might have caused the larger variation of body size. Nevertheless, the ratios of body size between clones were almost identical: the large clone was 2.4 times larger than the small clone based on image analysis, and it was 2.3 times larger based on microscopic measurements (values calculated from the grand means in Table 2). This suggests that the image analysis system is suitable to track changes in relative body size in rotifer cultures.

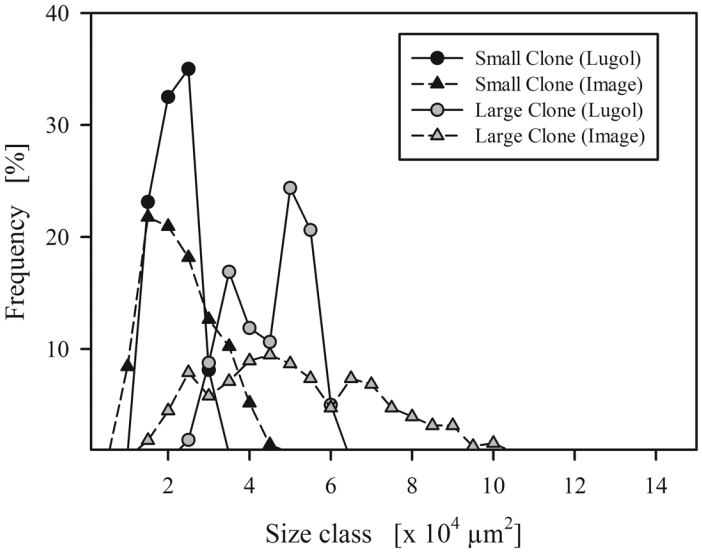

Fig. 6. Comparison between body size distributions with image analysis system (Image) versus microscopic measurements on samples fixed in Lugol solution (Lugol). Two different clones of *Brachionus calyciflorus* were used: a clone with large and small body size.

Discussion

The automated system for sampling and analysis of rotifer populations allows detailed investigations of experimental rotifer populations for up to several weeks. Its biggest benefits lie in the reduced time effort for counting rotifers and in the high temporal resolution (i.e., sampling in "inconvenient" time intervals, such as every 6 h). Such high temporal resolution should allow new insights into the population dynamics of rotifers. In the rotifer cultures used in this study, the work effort for manual counting of Lugol-fixed samples was estimated to be about 40 min per sample (taking the sample, fixation and sedimentation, counting, and cleaning and sterilizing of sampling equipment). For comparison, the image analysis system needed only 7.5 min to sample and analyze one culture (12 cultures in 90 min) and did this autonomously. Thus, if the image analysis system were to be run continuously, sampling intervals down to 1.5 h would be possible (for 12 cultures), or up to 48 parallel cultures could be sampled (in a 6-h sampling interval). In Fig. 5, I present the data from two of a total of eight chemostats that were simultaneously run for 45 days and sampled every 6 h, i.e., 1440 samples in total. Manual sampling and counting would have taken 960 h (note that this only considers simple counting, not size measurements). It is thus clear that data sets as displayed in Fig. 5 can be obtained only by automated sampling and counting.

The total costs for the sampling and image analysis system were about €10,000, with the most expensive parts being camera, optics, illumination, and software. Most components were of industrial-grade quality and are commercially available from a variety of manufacturers. Only few components were self-made, e.g., the optical chamber, which was crafted in the workshop of our institute. Nevertheless, similar constructions could be ordered custom-made from manufacturers of flow-through cuvettes (e.g., Hellma). Thus, building and implementing this

Table 2. Comparison of body size measurements with image analysis versus microscopic measurements.

		Image analysis system			Microscopic measurement		
Rotifer clone	Replicate	n	Mean body size, 10^6 µm³ ± SD	CV, %	n	Mean body size, 10^6 µm³ ± SD	CV, %
Small body size	1	155	2.05 ± 0.90	43.9	40	1.82 ± 0.44	24.2
	2	219	2.12 ± 0.92	43.5	40	1.76 ± 0.41	23.4
	3	206	2.07 ± 0.88	42.7	40	2.04 ± 0.49	23.8
	4	251	2.17 ± 0.94	43.5	40	1.95 ± 0.51	21.1
	Grand mean		2.11 ± 0.92			1.89 ± 0.45	
Large body size	1	91	4.62 ± 2.04	44.1	40	4.19 ± 0.95	22.6
	2	66	5.49 ± 2.41	43.9	40	4.13 ± 0.93	22.4
	3	124	5.08 ± 2.22	43.8	40	4.51 ± 0.97	21.6
	4	99	4.98 ± 2.21	44.4	40	4.21 ± 0.91	21.6
	Grand mean		5.02 ± 2.22			4.26 ± 0.94	

image analysis system does not require any specialized infrastructure and can be financed within a regular research grant.

The key principle of this image analysis system was background subtraction, a widely used method in industrial image processing, in which two pictures are taken sequentially and one picture is subtracted from the other. Live rotifers, which are always moving, can easily be distinguished from nonmoving particles (algae clumps, detritus, dead rotifers). This principle has also been exploited in the rotifer counter of Alver et al. (2007), which was designed to measure the quantity of rotifers (as food) in fish tanks. The system described here was developed with different aims. In addition to a counting of females, it should allow quantifying several biological properties of the rotifer populations, such as the intensity of sexual reproduction and body size distribution. In addition, this system contains several improvements for working with a large number of replicates, such as a flush routine of the optical chamber (with DI water from a separate reservoir), which prevents cross-contamination between different cultures. In that sense, the system described here is designed for studies that use rotifers as model organisms for studying general ecological and evolutionary questions.

The accuracy of the measurements with the image analysis system should be adequate for most applications. The precision of female counts at concentrations of 1–100 females mL^{-1} was equivalent to microscopic counts of Lugol-fixed samples. If working at very low female densities, e.g., <1 female per mL, the accuracy of image analysis counting could likely be improved by sampling a larger volume (e.g., by using 10 subsamples of each culture). The accuracy of male counts was somewhat lower than that of females, presumably because of the small size of males. If compared to microscopic counts, image analysis recognized roughly 30% of all males in the sample. Nevertheless, this was sufficient to track the dynamics of sexual reproduction in rotifer cultures, as can be seen in Fig. 5. However, for detecting extremely low numbers of males, e.g., determining thresholds for mictic reproduction, further modifications may be necessary (*see* "Comments and recommendations"). Size measurements with the image analysis contain a larger error than microscopic measurements, yet the mean body size of a population and/or relative differences in mean size could be accurately estimated. This should allow measurement of competitive interactions between differentially sized clones in mixed cultures: For instance, if a small clone were a better competitor than a large clone, a mixed culture of both clones would decrease in mean body size until it stabilizes at the mean body size of a pure culture of the small clone. In recent experiments, I have obtained data sets that strongly indicated such competitive replacements, and the competitive replacement could be confirmed by independent measures of the clonal composition of the population (Stelzer, unpubl. data). Measurements of the body size distribution may also be useful for studies of productivity, i.e., where total biovolume concentration is the variable of interest. Biovolume can be calculated from the measurements of body area (the output data of the image analysis system), assuming that the shape of rotifers is an ellipsoid of rotation. It should be kept in mind that the body volumes measured with the image analysis system were slightly biased upwards (*see* Table 2). This was presumably due to a motion blur caused by the long exposure times. For relative comparisons of biovolume production, this bias is probably irrelevant, since it would affect all treatments in the same way. However, if absolute values of biovolume were needed, it would be necessary to correct for this bias (e.g., by calibration with body volume measurements by other methods).

In conclusion, the automated sampling and image analysis system described here should be a highly useful and efficient tool for population studies in rotifers. Even though the establishment of such a system requires significant efforts in terms of time and money, such efforts will soon pay off via future savings in the work effort. In addition, automated analysis of rotifer populations should allow completely new insights into population dynamics due to an unprecedented high temporal resolution with sampling intervals of only a few hours.

Comments and recommendations

The sampling and image analysis system is ideal for long-term population studies running over several weeks to months. Yet long-term operation may give rise to problems not encountered in shorter runs. For instance, as all sampling and image processing is controlled by a PC, the system is sensitive to power failures, which would lead to a complete shutdown. To avoid this, I installed an uninterruptible power supply, which allows normal operation during power failures for up to 2 h. In case of long power failures, the system would have to be rebooted manually.

Contamination is a problem in chemostat studies, especially during longer runs. The sampling system described here cannot prevent this problem completely, since it is inherent to the chemostat design. However, the automated sampling system can at least reduce the danger of contamination—that is, because the magnetic valves connecting to the culture are opened only for the few seconds and, when they are opened, the culture medium is flowing away from the culture toward the image analysis system. By contrast, conventional sampling of chemostats requires using sterile techniques, which are time-consuming and risky. In my experience, contamination is the only reason that prevents experimental runs longer than 2 months.

The detection of males could probably be improved by the use of an additional "male detection unit" in the image analysis system, e.g., an additional smaller and flatter optical chamber with a separate camera and higher-magnification lens. Although technically straightforward, this would also increase the costs of the complete system, as the optical parts (camera, lens) comprised about 30% of the total cost. In conclusion, it depends on the application whether the currently available semiquantitative estimation of male abundance is sufficient, or whether exact male counts are necessary (e.g., for an estimation of thresholds of mictic reproduction).

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