Limnol. Oceanogr., 44(8), 1999, 1894–1903
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Urea excretion by *Daphnia*: A colony-inducing factor in *Scenedesmus*?

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Abstract

It is known that the zooplankter *Daphnia* induces colonies in the alga *Scenedesmus.* As *Daphnia* grazes on *Scenedesmus,* it has been postulated that colony formation represents an algal defense mechanism. This induction of *Scenedesmus* coenobia/colonies by *Daphnia* could be associated with a substance exuded by the animals that acts as a specific infochemical (kairomone) for *Scenedesmus.* However, the chemical nature of such a substance is still unknown. Because coenobia can be formed in the absence of *Daphnia* kairomone, as a result of different N concentrations in the algae and media, we checked different nitrogen excretory products of *Daphnia* as potential candidates. In this paper we test the hypothesis that ammonia and urea excreted by zooplankters (*Daphnia*) may induce coenobia formation in *Scenedesmus.* Using experiments with dialysis separation of *Daphnia* and algae we show that one of the excretory products of zooplankters, urea, can induce colony formation in *Scenesdesmus,* whereas ammonia has no effect. Although the effect of urea on coenobia formation may simply be a nutrient effect, this does not exclude the possibility that it results in grazing protection of the algae. Hence, urea produced by zooplankton may serve as a colony inducer to algae.

The induction of colonies in the alga *Scenedesmus* by the zooplankter *Daphnia* has been the subject of many studies. This is mainly due to the original work by Hessen and van Donk (1993), which that showed that when *Daphnia magna* and *Daphnia* water were introduced to single-celled cultures of *Scenedesmus subspicatus,* the algae formed coenobia (the term coenobium is often used for *Scenedesmus* colonies, although strictly speaking the definition of coenobium is "a colony of unicellular organisms having a definite form and organisation, which behaves as an individual and reproduces to give daughter coenobia" [Lawrence 1997]) and that spine formation was also enhanced. Lampert et al. (1994) followed up this work and showed that coenobia were induced by adding *Daphnia* water to single-celled cultures of *Scenedesmus acutus* (now known to be *Scenedesmus obliquus,* strain SAG 276–3a, Göttingen, Hegewald). Both sets of authors showed that *Daphnia* had to be fed in order for colony induction to occur.

As *Daphnia* is a grazer on *Scenedesmus,* it has been postulated that colony formation represents an algal defense mechanism (Hessen and van Donk 1993). However, the extensive work of van Donk et al. (1999) showed that colony induction of *Scenedesmus* is not specific to *Daphnia magna.* Indeed most cladocerans, a copepod (*Eudiaptomus gracilis*), and some rotifers seem to induce colony formation in *S. acutus/obliquus.* In terms of the postulated algal defense mechanism, this is rather intriguing, as some of these organisms are not known to consume *Scenedesmus.* Other incongruities have been revealed by Lürling and others (Lürling and van Donk 1996, 1997; Lürling et al. 1997) in studies on the differences between single-celled and multicelled *Scenedesmus* in terms of food quality for ingesting *Daphnia.* Although the smaller *Daphnia cucullata* seem to grow less well on colonies, the larger *Daphnia* seem to be able to cope quite well with the *Scenedesmus* colonies. Thus, it may be of little use to *Scenedesmus* to form colonies as a defense mechanism in the presence of large ingesting zooplankters.

Although the induction of *Scenedesmus* coenobia by *Daphnia* could, upon first examination, be attributed to the fact that the animals probably exude a substance that acts as a specific infochemical or kairomone (according to Hederson's dictionary of biological terms [Lawrence 1997], a kairomone is "a chemical messenger or pheromone emitted by one species which has an effect on a member of another species, sometimes to the detriment of the transmitter") for *Scenedesmus* (Lampert et al. 1994), the identification of such a substance has so far largely remained illusive. Extensive efforts by Franck (1995) and the recent publication of von Elert and Franck (1999) have facilitated a broad classification of possible functional groups (possibly an olefinic carboxylic acid) and the molecular size cut-off point, *see* Lampert et al. (1994). Studies on the induction of different *Scenedesmus* ecomorphs (coenobia–unicell interactions) are actually made very difficult due to the extreme phenotypic plasticity evinced by this alga. Indeed, a very large body of literature exists on the effect of different growth conditions and nutrient levels on *Scenedesmus* morphology (for detailed review *see* Trainor [1998]).

In our work on the physiological differences between unicells and *Daphnia*-induced multicelled *Scenedesmus,* it was important to investigate the potential importance of the influence of excreted nutrients on induction of colony formation, especially given the sloppy feeding habits of *Daphnia* (Lampert 1978). From Sterner and Hessen's (1994) work we know that *Daphnia* have a relatively high P requirement and that they, therefore, tend to excrete low amounts of this nutrient. Thus, we chose to ignore phosphate as an induction candidate in the first instance. In addition, because *Scenedesmus* coenobia formation has been shown to be influenced by different N concentrations (*see* Trainor et al. 1976), we looked first to nitrogen excretory products as potential can-

Acknowledgments

We thank G. Fußmann and C. Schöps who kindly provided the 1993 and 1998 Schöhsee zooplankton data. A. Möller, D. Albrecht, and S. Krohn cheerfully carried out a lot of the analyses and helped perfect the techniques. Many thanks also to B. Meyer for all the stimulating discussions and M. Boersma who was, as always, an unfailing source of advice.

didates. Textbook knowledge (Parry 1960) seems to be that crustaceans excrete almost all their nitrogen as ammonia. However, this is not reconciled with the large body of literature also reporting the fact that urea is found in association with grazing zooplankters in aquatic systems (Remsen 1971; McCarthy 1972; Berman 1974; Mitamura and Matsumoto 1981; Kaufman et al. 1983; Turley 1985; Mitamura and Saijo 1986; Metzler et al. 1997). Therefore, in this paper we tested the hypothesis that ammonia and urea excreted by zooplankters (*Daphnia*) can induce coenobia formation in *Scenedesmus.*

Methods

Three different sets of experimental examinations formed the backbone of this research. These were 1) the investigation of urea production by *Daphnia,* (2) experiments with *Scenedesmus* and ammonia and urea, and (3) experiments on interactions of *Scenedesmus, Daphnia,* and urea. The experiments are described under each heading separately below.

In addition, we carried out field investigations also described as separate entities. These were investigations into the relationship between the induction capacity of natural lake water and zooplankters (4), and urea production by zooplankters in the field (5).

The methods common to all experiments are presented as a general methods preamble.

General methods—The alga used for all of our investigations was *S. obliquus.* This is the alga formerly known as *S. acutus* (strain SAG 276-3a algal collection Göttingen), an organism used, under this older name, by Lampert et al. (1994) and Lürling (1999). The algae were cultured in chemostats (Lampert 1988) and the culture medium was inorganic Chu 12 (Müller 1972). Algal growth rate in the chemostats was 0.7 d^{-1}. These algae are mainly single-celled (usually 1 and 2% of the cells are found as colonies). The cell shape is ovoid, spineless, and they have an average spherical diameter of 6 μm. The algae and their morphological variants (1–8-cell aggregates) were counted under the light microscope before, during, and at the end of each experiment. In addition, the algal densities and their size distributions (mean biovolume in spherical units) were measured throughout, using a CASY particle analyzer with a 60- or 150-μm capillary. The upper and lower particle size cutoff points were 3 and 20 μm, respectively. The dilution of the CASY samples was set so that the maximum particle counts were 4,000.

The *Daphnia* used in our experiments were held in filtered lake water (from the Schöhsee) at densities of approximately 100 animals per liter. The species used was *D. magna* Straus. They were fed daily with a suspension of *Scenedesmus* at concentration of around 1 mg C L^{-1}.

In those experiments where we held *Daphnia* and *Scenedesmus* together, e.g., the dialysis experiments, we introduced Z4 (Zehnder and Gorham 1960) to the algal Chu 12 medium (1 : 1) to elevate the salt concentration of the media so as to prevent osmotic stress in the *Daphnia.* This worked very well and meant that we also had a good medium in which we could grow the algae and *Daphnia* at the same time.

Ammonia was determined in the samples with the Technicon autoanalyzer system at our institute. The method was based on the Berthelot reaction and photometric detection of a sodium nitroprusside color complex (Albrecht and Overbeck 1969). There is no interference with urea in this method.

Urea was determined using two methods. For quick analyses we used the Merck urea test based on the breakdown of urea into ammonia and carbon dioxide via urease, the nitroprusside color reaction, and subsequent colorimetry. We corrected for ammonia contents. For the exact quantitative investigations we used a modified version of the diacetylmonoxim colorimetric method as described by Kakáč and Vejdĕleck (1974) and Koroleff (1983). Although this method is highly specific for urea and thiourea derivatives, we checked that it was indeed urea that we were analyzing using urease. Thus, the analyses were carried out with and without the addition of 20 μl of urease to 5 ml of sample (urease in glycerin with an activity of 500 U mg^{-1} of urea). There was never a color reaction in samples were urease had been added. We also checked for the unlikely reaction with ammonia and indeed found that the reaction with ammonia was zero with no color production.

Ammonium and urea excretion by Daphnia—In order to investigate urea excretion by *Daphnia* we incubated 10–10.5 mg dry weight (equivalent to approximately 200 individuals depending on their size) of *Daphnia* per liter for 48 h in filtered lake water, artificial zooplankton media, and in a mixture of Chu 12 : Z4 media (1 : 1). The incubations were carried out in triplicate and repeated nine times.

Experiments with Scenedesmus, *ammonia, and with* Scenedesmus *and urea*—In order to determine the influence of urea or ammonia on the development of coenobia by *Scenedesmus,* we set up simple experiments in six-welled culture plates. Each well was filled with 10 ml of, in this case, over 80% single-celled algae (chemostat cultured) in inorganic Chu 12 medium. The algal concentrations were approximately 4×10^6 cells ml^{-1}. The concentrations of urea and ammonia-N in the wells were 0, 50, 150, 300, and 600 $\mu g\ L^{-1}$. The lowest concentration was based upon the approximate maximum amount of ammonia excreted by 200 *Daphnia* in 1 liter over 24 h and a subsequent dilution factor of 25 (number of animals and dilution according to Lampert et al. [1994]). Thus, the lowest concentration (50 $\mu g\ L^{-1}$) was approximately 1.5 times the standard *Daphnia* factor water used to induce *Scenedesmus* (Lampert et al. 1994). The concentrations were made up in replicates of three. Each well was stirred gently using a magnetic stirrer throughout the experiment and subjected to 150 $\mu mol\ m^{-2}\ s^{-1}$ of continuous fluorescent light in the daylight spectral range. The culture temperature was 20°C. Each day, at the same time, a 1-ml sample was removed and the relative number of cells per colony counted, as a percentage of the total cells, under the light microscope.

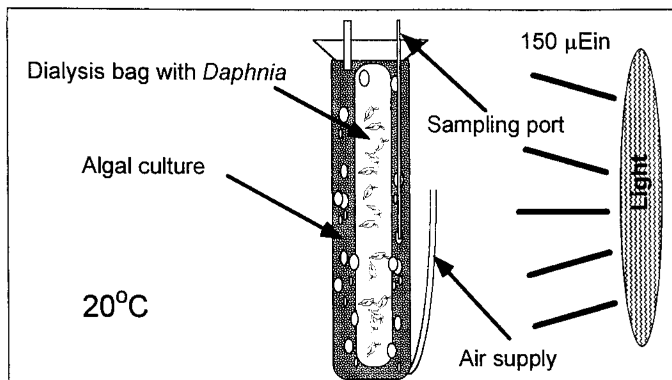

Fig. 1. The experimental setup using a dialysis membrane to separate *Daphnia* from *S. obliquus.*

Experiments with Daphnia, Scenedesmus, *and urea*—The methods used for investigating the effect of urea on the induction of *Scenedesmus* colonies by *Daphnia* involved placing the *Daphnia* in washed dialysis membrane sacks and hanging these within long glass tubes containing cultures of single-celled algae. The treatment design consisted of three replicates each of:

1) the controls containing only algae (C),
2) algae with urea (U),
3) algae with starved *Daphnia* (D),
4) algae with starved *Daphnia* and urea(DU),
5) algae with fed *Daphnia* (DF), and
6) algae with fed *Daphnia* and urea (DFU).

The experimental apparatus is depicted in Fig. 1. It consisted of glass culture tubes, 30 cm long, with a volume of 300 ml, and with an air inlet at the base. These were placed in front of a light bank in such a manner that the inside of each tube received 150 μmol m^{-2} s^{-1} of continuous fluorescent light in the daylight spectral range. Temperature of the culture room was set at 20°C. Twenty-four hours before the start of the experiment subadult *Daphnia* (size range 1.5–2 mm) were isolated from our stock cultures and held at the same light and temperature conditions in a mixture of Chu 12/Z4 media (1 : 1). Twelve hours before the experiments half of the animals were placed in food-free media (again Chu 12 : Z4). The algae for experiments were also taken from the chemostats and held in a mixture of the same Chu 12/Z4 media (1 : 1) under the same conditions for 24 h. Upon setting up the experiments 75 ml of the algal suspension was poured into the tubes (5.2×10^4 cells ml^{-1}). Then the dialysis bags were filled with 75 ml of media (Chu 12/Z4) with or without 65 *Daphnia,* depending on the treatment. The end concentration per tube was 240 animals per liter. Urea was added to the relevant tubes making up an end concentration of 300 μg L^{-1} urea (=140 μg N L^{-1}). The tubes were then stoppered leaving a sampling port and, where appropriate, a *Daphnia* feeding port. The air was turned on and bubbled gently through the tubes at a rate of 150 ml min^{-1}. This served to keep the cells in suspension and to ensure adequate mixing and CO_2 delivery. The pH of the tubes was monitored for stability every time a sample was taken and there was no difference between the tubes. The urea content was measured on a present or absent basis in the tubes using the Merck test. The sampling was carried out every 24 h and the particle size was usually monitored using the CASY instrument as well as the mean number of cells per colony counted under the microscope (data not shown).

Relationship between lakewater induction of coenobia and zooplankters in the field—From 19 May to 28 September 1993, we attempted to determine if *Scenedesmus* colonies could be induced with natural lake water from the mesotrophic Schöhsee in front of the Max Planck Institute of Limnology. Surface water for the tests was sampled weekly and zooplankton biomass determined concurrently. Zooplankton was sampled with a Hensen plankton net (100 μm mesh with an opening diameter of 10 cm). Vertical tows were made at a fixed station from near bottom (12 m) to the surface. Live zooplankters were immediately transported to the laboratory and, within 30 min, filtered onto small preweighed discs of 100 μm mesh. The discs were dried at 60°C overnight and weighed again. Dry biomass of zooplankton was calculated from the weight difference and the volume sampled by the plankton net.

Algal tests were run according to the protocol described in Lampert et al. (1994), except that the treatments consisted of lake water instead of medium inoculated with *Daphnia* culture water. Each week we ran three replicates of three treatments. We added 3 ml of *Scenedesmus* inoculum to 47 ml of, (1) CHU 12 medium (controls), (2) membrane-filtered (0.2 μm) lake water, and (3) membrane-filtered (0.2 μm) lake water enriched with nutrients in order to obtain the same concentration as in CHU 12 medium. Nutrient addition was necessary as concentrations in the Schöhsee are very low during summer and *Scenedesmus* would only have grown very slowly in pure Schöhsee water. Size distributions of *Scenedesmus* were measured after incubation for 48 h, as in the standard tests for colony induction.

Urea production by zooplankters in the field—The discovery that urea induced coenobia formation in *Scenedesmus* under laboratory conditions and that *Daphnia* also produced urea in appreciable quantities led to the question as to whether urea could be found in the field. We, therefore, analyzed the water taken with a 30-liter Schindler Trap, from different depths (1, 5, 9, and 12 m) on 21 May 1998, 3 July 1998, 14 July 1998, and 12 August 1998. The zooplankters in 30 liters of water were preserved and subsequently identified and counted under the microscope. Their biomass was calculated according to Bottrell et al. (1976).

Results

Ammonia and urea excretion by Daphnia—Animals that were fed on *S. obliquus* were found to produce between 0.13 and 1.1 μg mg^{-1} h^{-1} of urea (0.06 and 0.5 μg N); mean, 0.36 μg mg^{-1} h^{-1}; standard error, 0.1. Starved animals only produced between 0.06 and 0.1 μg mg^{-1} h^{-1} of urea. In comparison, the ammonia produced by fed *Daphnia* was between 0.31 and 1.20 μg mg^{-1} h^{-1} (N); mean, 0.76 μg mg^{-1} h^{-1}; standard error, 0.06. Starved *Daphnia* liberated between 0 and 0.5 μg mg^{-1} h^{-1} (N); mean, 0.45 μg mg^{-1} h^{-1}; stan-

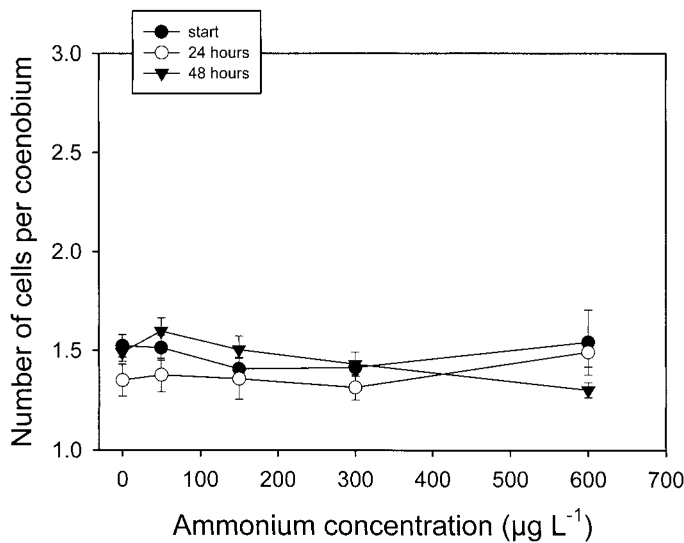

Fig. 2. The effect of ammonia (N) on coenobial induction in *S. obliquus.* Standard errors given. See Table 1a for statistics.

dard error, 0.12. In addition, the production rate of urea by the *Daphnia* seemed not to be dependent on the media but was found to vary depending on the batch of *Daphnia* used and probably also their physical condition.

Experiments with Scenedesmus, *ammonia, and with* Scenedesmus *and urea*—The effect of ammonia on the production of colonies in a culture of single cells is shown in an example in Fig. 2. The summary results of a repeated-measurement analysis of variance (ANOVA) are given in Table 1a. With the nutrient concentrations as the fixed factor, and time as the repeated measurement factor, no significant difference between any of the ammonium treatments was found. Indeed, it was irrelevant whether ammonia was present or not. At 48 h there was some indication that the higher concentrations of ammonium resulted in more unicells.

Unlike ammonia, urea repeatedly resulted in significant effects for all the treatments. An example of the effect that urea had on colony formation in a well experiment is given in Fig. 3. The number of cells per counted unit are presented. The summary results of a repeated-measurement ANOVA are shown in Table 1b, with nutrient concentrations as the fixed factor, and time as the repeated measurement factor. Unlike for ammonium, every effect was significant. Colonies were formed to a significantly higher degree after 48 h when urea was present in the culture media. Although the lowest treatment presented here is 150 μg L^{-1}, this experiment was repeated with 50 μg L^{-1}, and then we observed that for fast-growing algae (over 1.5 d^{-1}) there was a significant difference between the controls and the treatments ($F_{1,10}$ = 8.24; P = 0.02) even after 24 h. Posthoc analyses showed that there was no difference between the levels of treatments. All treatments showed a significant effect relative to the control without urea.

Experiments with Daphnia, Scenedesmus, *and urea*—The effect of urea in association with *Daphnia* on the size of the cell aggregates as measured in the dialysis experiment is demonstrated in Fig. 4. The summary results of a repeated-measurement ANOVA are shown in Table 2, with the treatments (urea, *Daphnia,* fed/unfed) as the fixed factors, and time as the repeated measurement factor. For every sampling, identical letters indicate homogenous groups after Tukey posthoc comparisons. After 24 h there was no difference between any of the treatments. After 48 h, however, a significant difference between the mean volume of the control algal coenobia and those of the treatments was found. It could be seen from the light microscope counts that this was a result of the formation of 2-, 4-, and some 8-celled coenobia. In addition the *Dactylococcus*-like *Scenedesmus* morph (Chodat 1926) was noted in the DU and DFU treatments. The fact that all treatments showed a significant effect relative to the control without urea was rather surprising with regard to the tubes in which the *Daphnia* had not been fed and in which between 20 and 30% of the animals seemed to have died due to starvation. However, in these tubes it was evident from the quick urea test that there was urea

Table 1. Summary tables of repeated-measurement ANOVAs with nutrient concentration as the fixed factor and time (0, 24, 48 h) as the repeated-measurement factor, for (a) ammonia, and (b) urea. Df, degrees of freedom; MS, mean squares; eff, effect; err, error.

Effect	Df eff.	MS eff.	Df err.	MS err.	*F*	*P*-level
a) Ammonia						
Concentration	4	0.015	9	0.008	1.91	0.190
Time	2	0.054	18	0.020	2.71	0.090
Concentration × time	8	0.013	18	0.020	0.63	0.740
b) Urea						
Concentration	3	0.194	6	0.017	11.34	0.007
Time	2	1.858	12	0.023	80.57	<0.001
Concentration × time	6	0.186	12	0.023	8.05	0.001

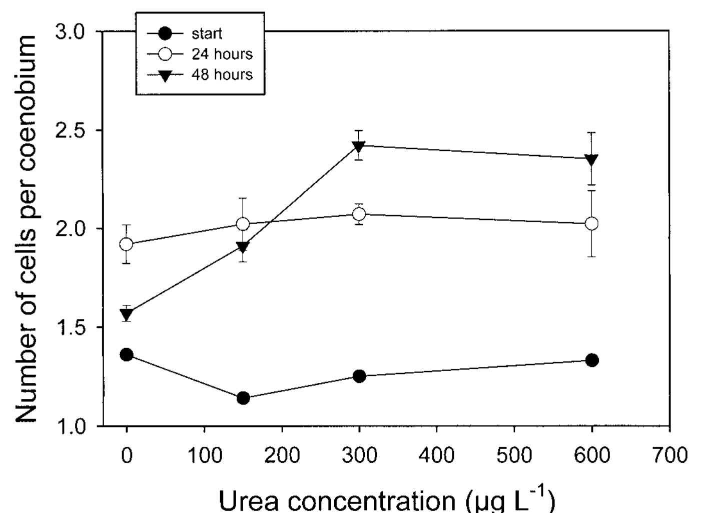

Fig. 3. The effect of urea on coenobial induction in *S. obliquus.* Standard errors given. See Table 1a for statistics.

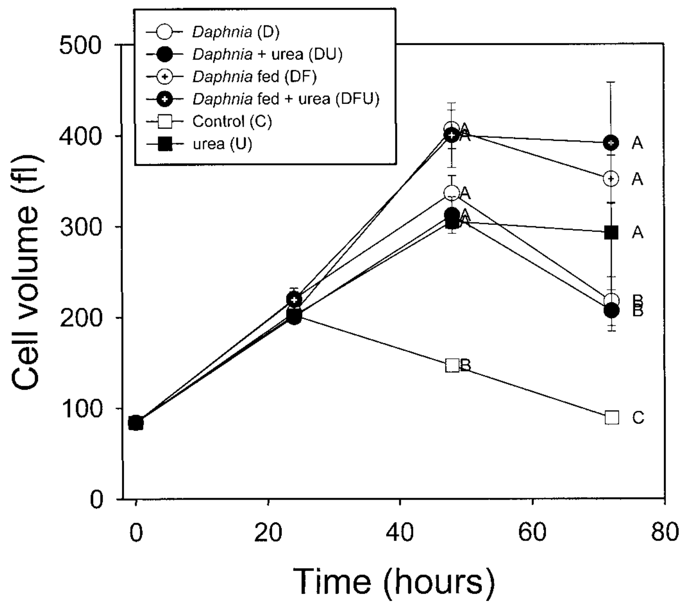

Fig. 4. The mean volume of algae as a result of the formation of 2-, 4-, and 8-celled coenobia in a dialysis experiment with *Daphnia, Scenedesmus,* and urea. Standard errors given. For every sampling, identical letters indicate homogenous groups after Tukey posthoc comparisons. See Table 2 for statistics.

present in the system in appreciable quantities. From the data it is evident that there was no significant difference in particle volume when urea was added to a system where the *Daphnia* were being fed and producing it anyway. Only after 72 h a nonsignificant tendency for the DFU treatment to form larger coenobia was observed. There was no significant difference between the growth rates (biovolume) of the different treatments.

Relationship between lakewater induction of coenobia and zooplankters in the field—Nutrient-enriched Schöhsee water induced colonies in *Scenedesmus,* but the effect varied with season. The largest differences in colony size were found in spring, when zooplankton showed a biomass maximum (consisting largely of *Daphnia galeata* × *hyalina*). The effect was smaller in July and August, when zooplankton biomass was low and consisted of fewer *Daphnia* (including small *D. cucullata*) and *Eudiaptomus.*

Averaged over the sampling period (N = 20), the mean (±1 standard deveiation [SD]) particle sizes of *Senedesmus* were 138 (±39) μm^3 in the controls, 118 (±33) μm^3 in Schöhsee water, and 244 (±89) μm^3 in nutrient-enriched Schöhsee water. A Mann–Whitney U-test showed that the treatments with nonenriched lake water did not differ significantly from the controls (P = 0.086). However, particle sizes in enriched lake water differed significantly from those in both controls ($P < 0.001$) and nonenriched Schöhsee water ($P < 0.0001$). Hence, Schöhsee water contains a colony-inducing factor or induces larger cells. This factor is only active if *Scenedesmus* has enough nutrients to grow.

Although seasonal data suggest a correlation between zooplankton biomass and the capacity of Schöhsee water to induce colonies, the relationship is not very strong. We calculated the induction effect as the difference between mean particle sizes in controls and enriched Schöhsee water. These differences are plotted versus zooplankton biomass in Fig. 5. There is a trend of increasing induction capacity with increasing zooplankton biomass, but the regression (Y = 0.298X + 33.6; n = 19; r^2 = 0.2012) is only marginally significant (P = 0.054).

Table 2. Summary table of a repeated-measurement ANOVA with treatment (urea, *Daphnia,* fed/unfed) as the fixed factor and time (0, 24, 48, 72 h) as the repeated-measurement factor.

Effect	Df eff.	Ms eff.	Df err.	MS err.	F	P-level
Treatment	5	32,523	11	2,298	14.16	<0.001
Time	3	169,548	33	1,015	167.02	<0.001
Treatment × time	15	12,314	33	1,015	12.13	<0.001

It should be noted that at the time of the above experiments we were unaware that urea (our work) and other colony-inducing substances (Lürling 1999) may leak from membrane filters and we did not run the controls with 0.2-μm-filtered medium. However, as we always filtered the same amount of lake water through one filter, a possible filter leaching effect could not have resulted in the slope and would have been eliminated by plotting the volume difference.

Urea production by zooplankters in the field—The urea concentrations found at the different depths were found to be significantly, positively correlated with the number of zooplankters on 21 May 1998, 03 July 1998, and 14 July 1998. The numbers of zooplankters that consisted of *Daph-*

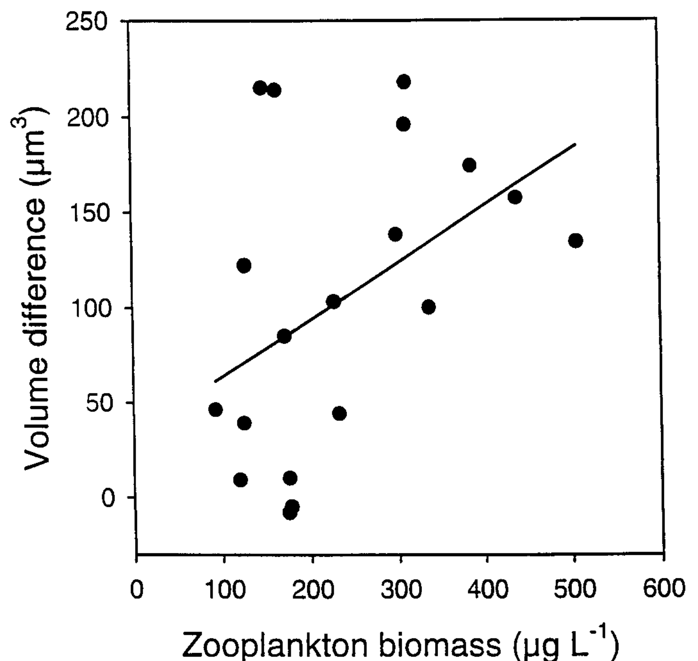

Fig. 5. The correlation between Schöhsee coenobia induction capacity and total zooplankters. Y = 0.298X + 33.6; n = 19; r^2 = 0.2012; P = 0.03.

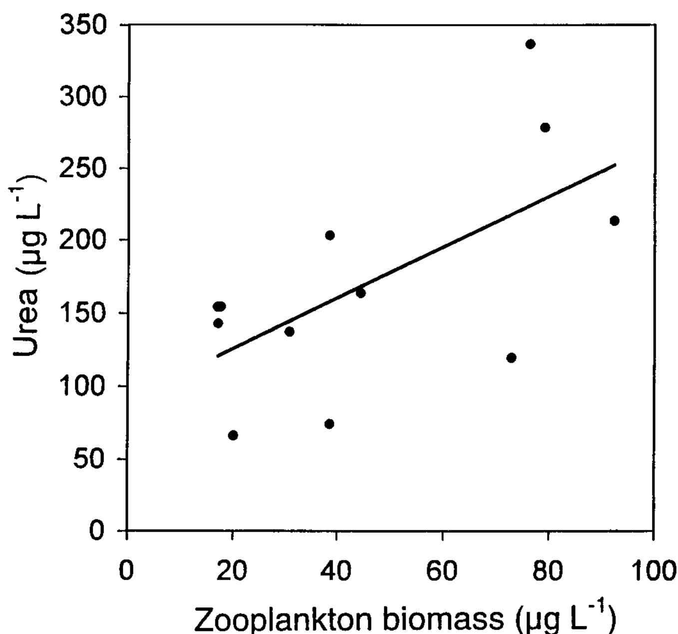

Fig. 6. The relationship between urea concentrations in the Schöhsee and total zooplankters. $Y = 1.749X + 90.967$; $n = 12$; $r^2 = 0.3799$; $P = 0.054$.

nia, calanoid and cyclopoid copepods, *Bosmina,* and *Ceriodaphnia* were converted into biomass units and these plotted against urea (Fig. 6). A significant correlation was found, i.e., $P = 0.03$ where $Y = 1.749X + 90.967$; $n = 12$; $r^2 = 0.3799$. When, however, the data obtained for 12 August 1998 are added, this correlation becomes weaker. In these samples a substantial zooplankton biomass and little urea were present. This is doubtless due to the fact that on 12 August 1998 there was a massive phytoplankton bloom. The secchi disk depth had dropped from 5.2 m on 14 July 1998 to 2.4 m in the August sample. Pigment data and microscopic examination of the sample suggest that this could have been caused by the dinoflagellate, *Ceratium* sp. and a cryptomonad. Dinoflagellates are known to utilize urea very well as a nutrient source, whereas cryptomonads do not grow well on urea. The cryptomonads would have been a good food source for the zooplankters in the water (up to 80 μg L^{-1}, consisting mainly of calanoid copepods and *Daphnia*), whereas the *Ceratium* would have been a poor food source. Any urea produced by the zooplankters is likely to have been removed rapidly by the dinoflagellates.

Discussion

The genus *Scenedesmus* is known as one of the best algal manifestations of phenotypic plasticity (Egan and Trainor 1991). Under in vivo conditions, *Scenedesmus* is usually a colonial alga forming colonies of between 2 and 16 cells. It also sometimes produces unicells resembling *Chodatella* at certain stages of its life cycle in nature (Egan and Trainor 1989; Trainor 1998). It has been postulated that the unicellular morph may be important in recolonization of waters in spring (Egan and Trainor 1989; Trainor 1998). In general it is the production of unicells from colonies (coenobia) that seems to be considered an important controlling step in the life-cycles of *Scenedesmus* (*see for example* Egan and Trainor 1989) and not the step from unicells to colonies that has been concentrated on by the zooplankton researchers, e.g., Hessen and van Donk (1993). The induction of the different coenobial ecomorphs and particularly of unicells in vitro has been studied intensively by many authors, unfortunately most of the work carried out has been with spined strains. From the work of Lürling (1999) we know that the same principles may not necessarily apply to nonspiny strains. The work by Siver and Freeda (1982) and Sêtlík et al. (1972), for example, suggests that the number of coenobial cells is related to the amount of energy and protoplasm in the original parent cell. Unicells are dominant in young cultures (Swale 1967; Egan and Trainor 1989), at low cell numbers, and in dilute media (Egan and Trainor 1989). Temperatures over 20°C can also promote unicell production (Trainor 1993). Low temperatures are thought to result in coenobia (Trainor 1993). Coenobia also dominate in old cultures (Swale 1967; Trainor 1979) and according to Gavis et al. (1979) could possibly be a result of high growth rates (for opposite opinion, *see* Siver and Trainor [1981] and Trainor [1998]).

Steenbergen (1975) has shown that sudden changes of culture conditions (e.g., the light regime) can also affect coenobial numbers and size classes. However, according to Egan and Trainor (1989), the most important controlling aspect in unicell and coenobia transformations is the nutrient status of the algal growth medium. They state that the formation of colonies is merely an expression of physiological state. Trainor and Roskosky (1967), demonstrated that excess nitrogen (ammonia) resulted in unicell formation, and Shubert and Trainor (1974) showed that at low total salt and phosphate concentrations coenobia were formed. Overbeck and Stange-Bursche (1965) demonstrated that the formation of coenobia/unicells can be dependent on nutrient adaptation conditions of the algae and that in *Scenedesmus quadricauda* the unicell to coenobia relationships were controlled by phosphate. Trainor (1964) showed the induction of colonies upon the addition of glucose in *S. obliquus,* although in a recent study by Lürling (1999) this was not found.

In the face of these examples it is clear that *Scenedesmus* ecomorphs can be induced by a wide variety of conditions. Those that are of importance to this study involve nutrient and organic addition, and it was from this starting point that we worked when considering which compounds in *Daphnia* water might induce colony formation.

The substance that is excreted in the greatest quantities by *Daphnia* is ammonia. We found this to be up to 1.2 μg mg^{-1} h^{-1} (N) for adult *Daphnia* fed on *Scenedesmus.* Starved animals produced maximally 0.5 μg mg^{-1} h^{-1} (N), and this was probably due to the residual voidance of the gut. These values are concomitant with values found in the literature for production of ammonia by *Daphnia* (Parry 1960). We also measured the amount of nitrate, nitrite, and phosphate excreted and found this to be negligible.

From the detailed work of Siver and Trainor (1981), we knew that it was the nitrogen levels, ammonia in particular, that influenced the formation of unicells. These authors

showed that when 7.8 mg L^{-1} of ammonium chloride were added to the culture medium at 22°C and 5,400 lux unicells predominated, particularly when an organic source was added. When all of the nitrogen (including nitrate) was removed from the cultures coenobia dominated. The concentrations of ammonia-nitrogen used in our study were very much lower (factors of between 4 and 40) than those used by Siver and Trainor (1981), and yet our unicells were maintained and coenobia were not formed compared with the controls. It is, therefore, highly unlikely that the ammonia excreted by *Daphnia* would induce colony formation. Indeed, this has been corroborated by Lampert et al. (1994) who used concentrations of up to 1 mg L^{-1} and also by the thesis work of Lürling (1999).

Urea, was also found to be liberated into the holding water, in appreciable amounts, when *Daphnia* were present, i.e., between 0.06 and 0.5 μg (N) mg^{-1} h^{-1} in fed animals. In the initial well experiment described above, we found that urea concentrations as low as 50 μg L^{-1} (23 μg N L^{-1}) induced the formation of colonies in *Scenedesmus* and that there was no significant difference between the lowest and highest levels of urea treatments. This could suggest that minimum threshold levels of induction exist and that these had not been reached in our experiments. It also could point to the involvement of enzymatic processes in the induction of coenobia. The fact that absolute cell size difference, as a reaction to urea can be rather low when compared to the *Daphnia* water effect, could indicate that urea is merely one substance in a mixture of inducing substances.

It is well known that marine phytoplankters can utilize a wide variety of organic nitrogenous compounds including urea as their sole nitrogen supply effectively (McCarthy 1972; Rees and Syrett 1979; Goldman and Dennet 1983). Consequently, urea is considered to be a very important nutrient in marine systems (Remsen 1971; Tamminen and Jrmisch 1996). Freshwater phytoplankters have also been shown to utilize urea as a nitrogen source (Syrett 1962) and even as a carbon source (Mitamura and Matsumoto 1981). Algae often take up urea in preference to nitrate and sometimes in preference to ammonia. The Chlorococcales and, indeed, *Scenedesmus* can utilize urea very efficiently as a nitrogen source. Green algae that are ammonia starved are known to take up urea readily (Morris 1974).

The uptake mechanism of urea by algae is generally accepted to be either with the enzyme urease or with an amidolyase-catalyzed ATP-dependent cleavage of urea into carbon dioxide and ammonia (Syrett 1962; Morris 1974). The latter is likely to be important in the Chlorophyceae (Morris 1974), and it is probably inducible, whereas urease is always present (Bekheet and Syrett 1979). However, little is known about the control of urea uptake in algae and in particular how this could be related to changes in cellular morphology or the production of extracellular products.

Although it is unclear exactly why urea should induce colonies, it is known that the relationship of nitrogen to carbon in the cell probably is the single most important factor determining the morphology of *Scenedesmus* (Siver and Trainor 1981). Thus, the cell's nutrient history is vital to ecomorph induction. *Scenedesmus* grown on media with different nutrient concentrations will have a varying morphology. In addition to this, they will react differently to nutrient pulses, such as the addition of N (*see* Siver and Trainor 1981).

In general, from the work by Siver and Trainor (1981), it seems that cells grown with an excess of carbon will form colonies, whereas cells grown with an excess of nitrogen form unicells. Here the cellular internal nitrogen levels affect the uptake rates of ammonia in a culture and thus the ongoing morphology. However, the actual cellular nitrogen (resulting from the culture conditions before a nutrient pulse) seem to determine the morph upon the pulse of nutrients. If a cell has large nitrogen reserves (over 4%), it will produce colonies, given enough carbon and ammonia. Low internal nitrogen reserves (under 3%) and low carbon often evince slow nutrient uptake that also results in coenobia. On the other hand, cells with low nitrogen reserves but lots of available carbon have a large N requirement. Thus, they can take it up rapidly (as ammonia) and form single cells.

The *Scenedesmus* used in our study from the chemostat culture in Chu 12 nutrient concentrations had large carbon reserves. CHN analyses showed that algae had an atomic carbon to nitrogen ratio of 9:1, $n = 3$, standard error 0.43. This is well above the Redfield ratio. In addition, although the unicellular algae used in our experiments from the chemostats were grown with low amounts of nitrate, they were ammonia starved. They will, upon having been introduced to the experimental batch cultures, have been capable of taking up ammonia quickly to produce unicells. This is the relevant scenario for the controls without *Daphnia* or urea and indeed fewer colonies formed. This was not observed when *Daphnia* or urea were in the systems.

Siver and Trainor (1981) found that, when the internal reserves of carbon were high and when cells had a high nitrogen requirement, unicells formed when large amounts of ammonia or even nitrate were taken up quickly upon a nutrient pulse. When the uptake of ammonia was slow, then colonies formed. In our treatments with *Daphnia* and urea more colonies formed than in the control. Thus, it could be postulated that something was inhibiting the uptake of ammonia.

Urea in the concentrations used in our experiments have been known to inhibit the uptake of ammonia and nitrate (Molloy and Syrett 1988*a*). However, in general it is the reverse—ammonia in concentrations over 1 mM is known to inhibit the uptake of urea in algae (Molloy and Syrett 1988*b*). The latter is unlikely to have happened as the concentrations of ammonia were too low in our experiments. Thus, it could be postulated that the unicell effect of ammonia could have been negated by urea and is taken up instead.

How well urea, relative to ammonia, is taken up by algae is very dependent on the C:N contents of the cell. This situation is compounded by the fact that ammonia uptake by algae requires a large amount of intracellular carbon to detoxify the ammonia (Siver and Trainor 1981), whereas urea does not seem to require this. It could be postulated that if the uptake of urea by algae requires less internal carbon, particularly as urea has its own carbon source. This in itself could also result in the formation of colonies.

In their work, Lampert et al. (1994) found no effect of

urea on colony formation with concentrations similar to, and lower than, those used here. It is clear that in the future a greater spectrum of concentrations should be checked particularly in the light of the fact that urea uptake is an enzymatic process. In work carried out recently Lürling (1999) and von Elert (pers. comm.) tested the effect of urea in WC and Bristol's medium and found no effect on colony formation. However, in Medium 7 Lürling (1999) actually shows a seemingly significant increase in cell volume when urea is added, although the cell count changes are not significant due to a high variability. His results are difficult to compare with ours as the actual preconditioning of the algae is unclear in that work.

In our experiments with *Daphnia* and urea we repeatedly observed the formation of giant colonies known as the *Dactylococcus*-like ecomorph (Chodat 1926). These cells have vaguely been associated with the presence of organic material in media. It is not stated whether this could include organic nitrogen. Whatever the reason for the formation of coenobia in the presence of urea, the mechanisms are unclear and there is little evidence in the literature to support this.

In our experiments we took great care to eliminate any other secondary morphological inducers, such as temperature, light, phosphate, and pH. We used a weak medium as a starting medium, with low salts. From the paper by Siver and Trainor (1981) it seems possible that our results might have been completely different had we used a richer medium such as WC or Z4. At this point, the study on a nonspiny species (*S. obliquus*) by Lürling (1999) on the role of different N : C relationships on the production of coenobia, in rich WC medium, should be mentioned. Unlike Siver and Trainor (1981), who worked on a spiny species, he found no correlation between either the carbon or N (as nitrate) concentrations and coenobial formation. In cells grown on WC medium, there obviously was no relationship between N (as nitrate) and carbon concentrations and *Daphnia* water induction. The question remains as to whether the inoculate cells were conditioned to slow nitrate uptake and whether they, therefore, formed coenobia and unicells in equal amounts. If, however, they were to have taken up carbon from the *Daphnia* water they could have formed more colonies. Indeed in his work, Lürling (1999) hypothesizes that small molecules of carbon such as glucose might be important, although he, unlike Trainor (1964), was unable to show this for his spineless *Scenedesmus.*

In future investigations on the effects of *Daphnia* on *Scenedesmus* morphology, the shift of the N : C relationships of cells cultured with urea and *Daphnia* water should be investigated thoroughly, with great emphasis on the condition of the cells before they are used in the experiments.

The importance of nutrients in the *Daphnia* factor induction of colonies is further high-lighted by the fact that, in our tests for lakewater induction, we found that nutrients had to be added to mesotrophic lake water before induction took place. Lake water on its own did not induce colony formation.

The production of urea in areas with high zooplankton grazing activity is well known in aquatic systems (Remsen 1971). In the euphotic zone of Lake Biwa, Mitamura and Saijo (1986) estimated that the maximum daily regeneration rate of urea (sum of bacterial mineralization and zooplankton excretion) was 12.3 μg N L^{-1} d^{-1}. The amount of urea in the total zooplankton excretion was as much as 30%. Although the authors presented no real data for the actual zooplankter composition, it was stated that they were herbivorous zooplankters consisting mainly of copepods. They calculated that they excreted several times in excess of their body nitrogen. We have shown that even relatively low numbers of zooplankters in the Schöhsee were associated with high concentrations of urea in the water phase. However, in the presence of algae, such as dinoflagellates, that utilize urea as a nutrient, this relationship could breakdown, a situation that we may have seen in the August 1998. Such a scenario, as well as other sources of urea, might also explain why the seasonal relationship of lake water induction relative to zooplankton is not very strong. Unfortunately, we had no suspicions about urea when the lakewater induction experiment was carried out in 1993, hence urea was not measured in the water (Fig. 5). We ran urea analyses in the 1998 work when zooplankton data were again available (Fig. 6). Thus, the two graphs are not directly related. The biomass values presented were rather different for 1993 and 1998. Part of this discrepancy may be due to the different sampling techniques and biomass estimation methods (direct in 1993 and calculated in 1998). However, the zooplankton data presented in Fig. 6 for 1998 are actually unusually low, while the data presented in Fig. 5 are typical of the Schöhsee. Indeed, the reduced grazing pressure was evident from the lack of a clear-water phase in 1998 (C. Schöps pers. comm.). Moreover, from the graphs it is also clear that an extrapolation of urea concentrations to higher zooplankton biomass results in field concentrations consistent with the effective concentrations found in the laboratory.

Urea is a substance found in appreciable quantities in aquatic systems with grazing zooplankters and it is known to play an important role in nitrogen recycling and regeneration. We found that, relative to ammonia, urea was liberated in quite high amounts in our *Daphnia* cultures, i.e., as 18–45% of the total ammonia and urea (as N) excreted. It is difficult to put these urea amounts into context, as there have been practically no studies on urea liberation by freshwater Crustacea and we certainly have found none on *Daphnia.* However, according to Claybrook (1983), Crustaceae are generally only supposed to liberate between 1 and 12% of their excreted N as urea. It should be noted that these values are based mostly on marine organisms and only one or two freshwater crustaceans. In a freshwater lake, Mitamura and Saijo (1986) estimated that 30% of the total excretory products of their small copepods was urea. Thus, our measured urea concentrations in *Daphnia* water are possibly slightly on the high side of the correct order of magnitude. The ammonia amounts liberated by the *Daphnia* are within the normal ranges found for *Daphnia* and freshwater zooplankton in the literature (Regnault 1987). Although, as we fed our *Daphnia* during our experiments and as the *Daphnia* water is not axenic, it is likely that substantial amounts of ammonia were removed by algae and bacteria in the system.

It seems that even quite low numbers of zooplankters in aquatic systems produce substantial amounts of urea and that this alone is an important N source for phytoplankters.

Conclusions

In this paper we have shown that under certain conditions urea induces coenobia formation in *Scenedesmus* in the laboratory. We have also shown that *Daphnia* liberate urea and that it is correlated to grazing zooplankton in vivo. We thus postulate, that at least one of the reasons why *Daphnia* cause coenobial induction is because *Daphnia* water contains large amounts of urea. Trainor et al. (1976) suggested that some of the various morphological forms evinced by *Scenedesmus* could act as a grazing deterrent. Hessen and van Donk (1993) showed that *Daphnia* induced coenobial formation in cultures of unicellular *S. subspicatus.* Lampert et al. (1994) followed this work up and showed the same effect for *S. obliquus/acutus.* It was postulated that this may be a response of the algae creating a grazing deterrent. However, it has been demonstrated by Van Donk et al. (1999) that only very small *Daphnia* have problems with *Scenedesmus* colonies and that even zooplankters that do not graze on *Scenedesmus* induce coenobia. Lürling (1999) has covered almost every aspect of the role of *Daphnia* in the induction of coenobia. He has shown, on the one hand, that *Daphnia* water can increase algal growth rates but, on the other hand, also that it probably is not merely a simple nutrient effect. From his work and our own work it would seem that *Daphnia* water acts something like a carbon and salt cocktail, possibly containing an olefinic carboxylic acid (von Elert and Franck 1999) that when introduced to a *Scenedesmus* culture acts as a colony inducer. It would also seem likely that several single substances could work in synergy. Because we have shown here that an excretory product of zooplankters, urea, can induce coenobia in *Scenesdesmus* we have tried to build a connection between all this *Daphnia* induction work with the many studies on *Scenedesmus* phenotypic plasticity. Our conclusion is that, although it would seem that urea induces coenobia and that this may simply be a nutrient effect, this does not exclude the possibility that this results in protection of the algae against predation. Indeed, both Trainor (1998) and Lürling (1999) also are of this opinion. If the products of grazing zooplankters favor the formation of large cells, coenobia or cells with tough cell walls, this can be seen as advantageous to the alga and thus as a grazing defense mechanism.

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Received: 25 January 1999
Accepted: 4 August 1999
Amended: 25 August 1999