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Analysis of the seasonal variation in biochemical composition of *Daphnia magna* Straus (Crustacea : Branchiopoda : Anomopoda) from an aerated wastewater stabilisation pond

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Keywords : *Daphnia magna*, lipids, proteins, carotenoids, chitin, biochemical composition, waste stabilisation pond, aquaculture.

The biochemical composition of *Daphnia magna* Straus, the dominant planktonic crustacean of the waste stabilisation pond of Differdange (Grand-Duchy of Luxembourg), was quantitatively determined from October 1993 to July 1994. Over the sampling period, the average composition (mean ± S.D.) was 271 ± 64 mg proteins.g^{-1} dry weight (DW), 100 ± 28 mg lipids.g^{-1} (DW), 96 ± 58 µg carotenoids.g^{-1} (DW), 49 ± 14 mg chitin.g^{-1} (DW) and 125 ± 78 mg ash.g^{-1} (DW). The seasonal variations of the biochemical composition were related to several ecological variables (water temperature, dissolved oxygen concentration, pH, water transparency, chlorophyll *a* concentration and *D. magna* biomass). The chitin content was positively correlated to the water temperature as a result of the strong influence of this later variable on the moulting rate of the daphnids and, subsequently, on the chitin synthesis by these organisms. The carotenoid content was positively correlated to the water transparency as a result of their photoprotective role in daphnids. The fluctuations of the lipid, protein and ash levels in *D. magna* depended to the food availability. Despite a seasonal variation in the biochemical composition, *D. magna* appeared to have adequate lipid and protein levels to be used in aquaculture. Its carotenoid content is similar to fish meals used to color salmonid flesh and these organisms could be used for this purpose. The prospect of using *D. magna* for chitin extraction is worth considering with respect to its significant chitin content, especially if highly valuable applications are aimed.

Analyse de la variation saisonnière de la composition biochimique de *Daphnia magna* Straus (Crustacea : Branchiopoda : Anomopoda) vivant dans un étang de lagunage aéré

Mots-clés : *Daphnia magna*, lipides, protéines, caroténoïdes, chitine, composition biochimique, étang de lagunage, aquaculture.

La composition biochimique de *Daphnia magna* Straus, le crustacé planctonique dominant dans l'étang de lagunage de Differdange (Grand-Duché de Luxembourg), a été déterminée quantitativement d'octobre 1993 à juillet 1994. Pendant cette période, la composition moyenne a été caractérisée par des teneurs s'élevant à (moyenne ± écart-type) 271 ± 64 mg protéines.g^{-1} masse sèche (MS), 100 ± 28 mg lipids.g^{-1} (MS), 96 ± 58 µg caroténoïdes.g^{-1} (MS), 49 ± 14 mg chitine.g^{-1} (MS) and 125 ± 78 mg cendres.g^{-1} (MS). Les variations saisonnières de la composition chimique des daphnies ont été mises en relation avec plusieurs variables écologiques (température de l'eau, concentration en oxygène dissous, pH, transparence de l'eau, concentration en chlorophylle *a* et biomasse de *D. magna*). La teneur en chitine s'est avérée positivement corrélée à la température de l'eau en raison de l'influence marquée de celle-ci sur le rythme de mue des daphnies et, par conséquent, sur la synthèse de chitine chez ces organismes. Le contenu en caroténoïdes s'est révélé positivement corrélé à la transparence de l'eau en raison du rôle de photoprotection de ces pigments. Les fluctuations des teneurs en lipides, protéines et cendres chez *D. magna* dépendent de la disponibilité en nourriture dans le milieu. Malgré les variations saisonnières de sa composition biochimique, *D. magna* s'avère avoir une teneur en lipides et en protéines adéquate pour son utilisation en aquaculture. Sa teneur en caroténoïdes est similaire à celle des régimes utilisés pour colorer la chair des salmonidés. Les daphnies peuvent donc être utilisées à cet effet. La perspective d'utilisation de *D. magna* comme source commerciale de chitine est particulièrement prometteuse compte tenu de sa teneur en chitine, en particulier si on envisage des applications à haute valeur ajoutée.

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1. Introduction

Due to its ability to survive in highly eutrophic conditions, *Daphnia magna* Straus is commonly found in waste stabilisation ponds (Dinges 1973, Dor et al. 1987, Hathaway & Stefan 1995). In such ponds, this planktonic species is thought to improve the biological quality of the effluent by extensive grazing on suspended bacteria, algae and detritus. The standing crops of *Daphnia magna* are often considerable, notably because of the absence of predators, and its annual production reach from 100 to 500 g dry weight.m^{-3}. $year^{-1}$ in some facilities (Sevrin-Reyssac 1992, Cauchie et al. 1995). At present, these crustaceans are mainly used, alive or preserved, as food for fish in aquaculture (Edwards & Pullin 1990, Sevrin-Reyssac 1992).

In 1988, *Daphnia magna* was introduced in the waste stabilisation pond of Differdange (Grand-Duchy of Luxembourg) in order to enhance the wastewater treatment. Since 1992, large amounts of daphnids have been regularly harvested in spring and in summer to be sold as aquarium fish food. The aim of this study was to determine the seasonal variation of the daphnid biochemical composition in order to evaluate the prospect of using them for commercial applications such as fish meal preparation or chitin extraction. Chitin, poly-β-1,4-D-glucosamine, and its deacetylated derivative, chitosan, have recently found numerous applications in various fields with a great added value, including pharmaceutical and biomedical sectors, cosmetics and wastewater treatment (Muzzarelli 1996). The basic biochemical composition of *Daphnia magna* (proteins, lipids, chitin, ash and carotenoids) was investigated from October 1993 to July 1994. The seasonal variation of the composition was analysed in relation to physico-chemical variables in a redundancy analysis.

2. Material and methods

2.1. Site description

The studied waste stabilisation pond is located near the town of Differdange in the Grand-Duchy of Luxembourg (49°32'N - 5°55'E). It is a roughly rectangular pond (59,000 m^2, 2.3 m mean depth) equipped with two surface aerators (35 kW capacity each) collecting the waste waters of the town (15,000 inhabitant-equivalents) after primary treatment. In terms of biomass, the zooplankton community is dominated by crustaceans, namely the anomopod *Daphnia magna* (Cauchie et al. 1995).

2.2. Biochemical analysis of *Daphnia magna*

From July 1993 to June 1994, zooplankton samples were collected on fourteen occasions. Crustaceans were concentrated by pumping and filtering large volumes of water over a Nylon net (mesh size = 100 µm). At the laboratory, 2 mm long *D. magna* specimens were selected, killed with 4 % formaldehyde and immediately rinsed during 10 minutes in distilled water. In order to prevent the alteration of the carotenoids, the daphnids were freeze-dried in the dark. The samples were kept at 4°C until biochemical analysis.

For protein and chitin analyses, the freeze-dried material was first decalcified by means of HCl 0.5 N during 4 hours. Proteins were then extracted in the residue using a NaOH 0.5 N solution at 100°C for 6 hours and were assayed by the Lowry method (Lowry et al. 1951) as modified by Schacterle & Pollack (1973). The amount of protein removed by the HCl treatment was estimated spectrophotometrically in HCl extracts by the method described by Scopes (1974). Total protein content was estimated by adding HCl-soluble protein estimates to NaOH-soluble protein concentrations. Chitin was enzymatically determined according to Jeuniaux (1963, 1965) in the insoluble residue remaining after the successive HCl and NaOH treatments. The residue was incubated in a solution of purified chitinase (Sigma 6137; 1 $mg.ml^{-1}$ distilled water) at 37°C during 8 hours. The supernatant was then incubated in a solution of N-acetylglucosaminidase (lobster serum diluted ten times) during 4 hours at 37°C. The N-acetylglucosamine monomers liberated by the enzymatic hydrolysis of chitin were measured by the colorimetric method of Reissig et al. (1955).

Total lipid extraction was performed on freeze-dried daphnids using a chloroform : methanol (2 :1) mix (Bligh & Dyer 1959). The chloroform layer was separated and dried at 50°C under dry nitrogen flow. Lipids were assayed colorimetrically in the residues using the sulfophospho-vanilline method (Barnes & Blackstock 1973).

The total carotenoid content of the daphnids was determined following a method similar to that of Herring (1968a). The pigments were extracted in 90 % ethanol during 24 hours at 20°C in the dark. Absorption scans of the supernatant were performed between 350 and 700 nm with a precision of 1 nm using a Beckman DU 650 spectrophotometer. The total carotenoid content was calculated using the average OD of the supernatant between 450 and 480 nm assuming an extinction coefficient of 2,500 as an approximate value for carotenoids (Zagalsky et al. 1967). The wavelengths corresponding to maximal absorbance were identify in or-

der to determine, if possible, the nature of the carotenoids on the basis of their specific absorbance profiles in 90 % ethanol (Davies 1976).

The ash content was estimated by determining the weight loss after the incineration of freeze-dried material at 550°C for 24 hours in a muffle furnace. At this temperature, $CaCO_3$ sublimation is negligible (Paine 1971).

2.3. Ecological variables

Ecological variables were measured twice a month from October 1993 to July 1994. Temperature, dissolved oxygen concentration, pH and water transparency were measured using a WTW oximeter equipped with an oxygen probe and a thermistor, a WTW pHmeter and a Secchi disk, respectively. Chlorophyll *a* concentrations were measured using the methods described by Lorezen (1967). 100 ml of pond water were filtered through a Gelman Nylon membrane (pore size = 0.45 µm) and acetone (90 %) was used as solvent. Zooplankton samples were collected using a 3-liter Van Dorn bottle, filtered on Nylon net (mesh size = 80 µm) and preserved in 4 % formaldehyde. *Daphnia magna* was enumerated and measured to the nearest 50 µm using a dissecting microscope equipped with a micrometer. Its biomass was calculated using length-weight regressions established by Dumont et al. (1975).

2.4. Data analysis

The biochemical composition of *D. magna* was related to the ecological variables in a Redundancy analysis (RA) using the program CANOCO for Windows (ter Braak & Smilauer 1998). Ecological variables were standardised to zero mean and unit variance (ter Braak & Prentice 1988). A Monte-Carlo permutation test was realised to determine whether the biochemical variables were significantly related to the ecological variables (Verdonschot & ter Braak 1994).

3. Results

3.1. Biochemical composition of *Daphnia magna*

The variations in the biochemical composition of *D. magna* are presented in Fig. 1. The total lipid content of *D. magna* varied between 101 and 171 $mg.g^{-1}$ (DW) from October to December 1993 and between 65 to 107 $mg.g^{-1}$ (DW) from February to July. Protein content increased steeply from 186 to 334 $mg.g^{-1}$ (DW) from October to December 1993. From February to July 1994, the protein content varied between 217 and 396 $mg.g^{-1}$ (DW).

From October to March, the chitin level of *D. magna* varied, on the whole, from 30 to 50 $mg.g^{-1}$ (DW). It increased then steeply in April and reached 67 $mg.g^{-1}$(DW) at the beginning of May. It varied between 60 and 70 $mg.g^{-1}$ (DW) in June and July.

Carotenoid level in *D. magna* fell from 156 $\mu g.g^{-1}$ (DW) in October to 63 $\mu g.g^{-1}$ (DW) at the beginning of December. In February and March, it was lower than 50 $\mu g.g^{-1}$ (DW). A peak of carotenoid content was then observed in May with a maximum value of 246 $\mu g.g^{-1}$ (DW). On the fourteen sampling dates, the maximum absorbance of the ethanol extract lay between 460 and 466 nm.

The ash content of *Daphnia magna* was quite low (< 100 $mg.g^{-1}$ (DW)) from October to December 1993. In 1994, it increased steeply at the end of April to reach a maximum value of 327 $mg.g^{-1}$ (DW). From the end of May to the end of July, the ash content varied from 100 to 200 $mg.g^{-1}$ (DW).

3.2. Ecological variables

Variations in the ecological variables are shown in Fig. 2. Water temperature varied from 1.7°C in December to 25.2°C in July. Dissolved oxygen concentration was higher than 8 mg $O_2.l^{-1}$ from December to March and then sharply decreased under 4 mg $O_2.l^{-1}$ at the beginning of May. In June, a peak of oxygen was observed with a maximum value of 10 mg $O_2.l^{-1}$. pH was quite constant over the sampled period, ranging from 7.8 to 9.0. Maximum values were observed in March (pH = 9.0) and in June (pH = 8.6). Highest water clarity was observed in January and May when the disk was still visible below a depth of 2 meters. Chlorophyll *a* concentration was relatively low (< 5 $mg.m^{-3}$) during the sampling period, except in March and in June when it reached 283 and 160 $mg.m^{-3}$, respectively. *Daphnia magna* biomass decreased from about 0.9 g (DW).m^{-3} in November to virtually zero in December. It remained nil until the end of February. At the beginning of March, *D. magna* biomass peaked to 1.7 g (DW).m^{-3}. From March to July, four successive peaks of daphnid biomass were observed. Maximum values ranged from 1.4 to 1.7 g (DW).m^{-3}

3.3. Relationships between biochemical and ecological variables

On the basis of the RA ordination (Fig. 3), several correlations were detected between biochemical and ecological variables. It was inferred that the chitin content and the carotenoid content were correlated with the water temperature and the water transparency, respectively. The chlorophyll *a* concentration, dissol-

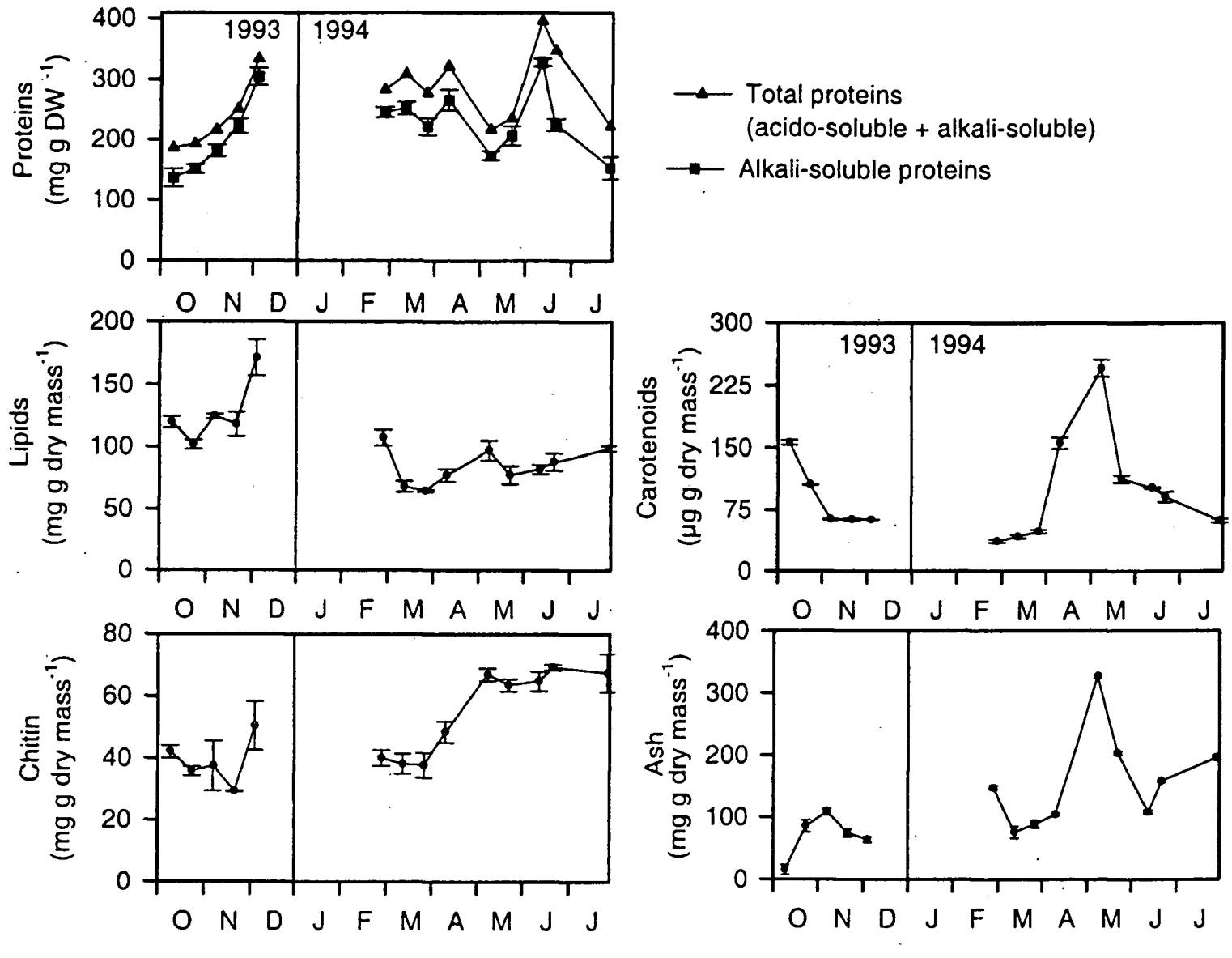

Fig. 1. Seasonal variations of the biochemical composition of *Daphnia magna* in the waste stabilisation pond of Differdange from October 1993 to July 1994.

Fig. 1. Variations saisonnières de la composition biochimique de *Daphnia magna* dans l'étang de lagunage de Differdange d'octobre 1993 à juillet 1994.

ved oxygen concentration and pH were positively correlated with each other and with the protein content. On the other hand, these three variables were negatively correlated with the water transparency. The lipid content was negatively correlated with *D. magna* biomass. The ash content was not clearly correlated with any of the studied variables.

4. Discussion

Lipid levels measured in *D. magna* at Differdange are, on the whole, in good agreement with other data found in the literature (Table 1). On the contrary, protein levels are globally lower than those measured by Blazka (1966), McKee & Knowles (1987) and to a certain extend by Elendt (1989). Differences in methodology may account for this divergence. Blazka (1966) used the Kjeldahl method to determine proteins. This method indeed lacks specificity since it assays not only protein-nitrogen but total organic nitrogen. The total protein content may thus be overestimated. McKee & Knowles (1987) did not measure the initial dry mass but estimated it from summation of the residual mass after all the extraction and the concentration of the assayed compounds. This means that biomolecules such as some soluble sugars and ions that have been extracted but not assayed were not accounted for in the calculation of the dry mass. The total dry mass was thus presumably underestimated and the protein content was overestimated. Elendt (1989) used the method of Bradford (1975) which often yields results comparable to those obtained by the method of Lowry. However, the dependence of the Bradford procedure on protein amino acid composition (Dunn 1993) can sometimes produce significantly different results from those obtained with the Lowry method.

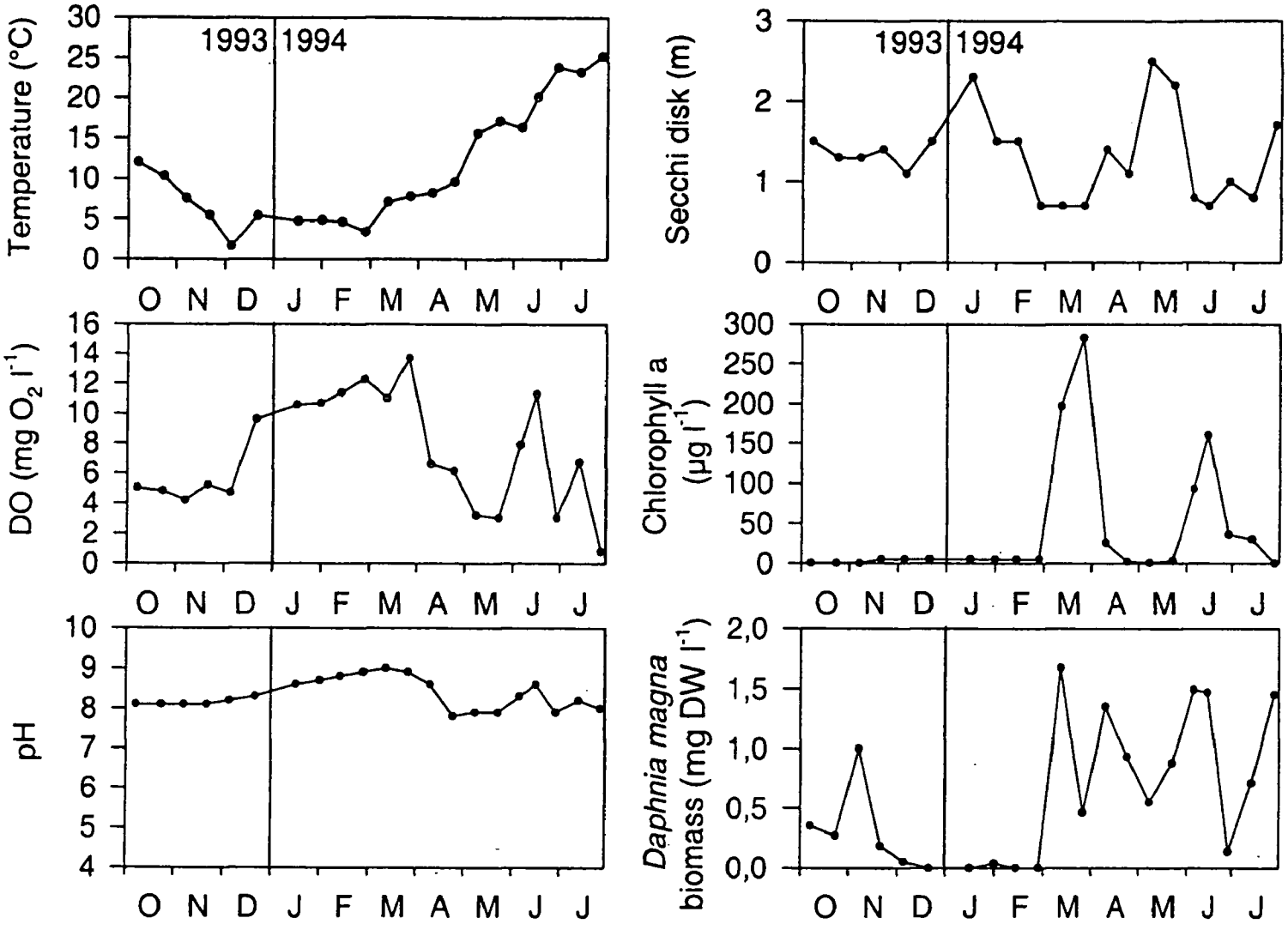

Fig. 2. Seasonal variations in water temperature, dissolved oxygen concentration (DO), pH, water transparency (Secchi disk), chlorophyll *a* concentration and *Daphnia magna* biomass in the waste stabilisation pond of Differdange from October 1993 to July 1994.

Fig. 2. Variations saisonnières de la température de l'eau, de la concentration en oxygène dissous (DO), du pH, de la transparence de l'eau (disque de Secchi), de la concentration en chlorophylle *a* et de la biomasse de *Daphnia magna* dans l'étang de lagunage de Differdange d'octobre 1993 à juillet 1994.

Table 1. Protein, lipid and carotenoid levels in *Daphnia magna.*

Tableau 1. Teneurs en protéines, lipides et caroténoïdes de *Daphnia magna.*

References	Proteins ($mg.g^{-1}$)	Lipids ($mg.g^{-1}$)	Carotenoids ($\mu g.g^{-1}$)
Farkas, 1958 *	223	346	—
Blazka, 1966 *	680	131	—
Czeczuga, 1984	—	—	4
Partali *et al.*, 1985	—	—	25 – 237
McKee & Knowles, 1987	477 – 620	64 – 179	—
Elendt, 1989	263 – 566	57 – 128	—
De Meester & Beenaerts, 1993	—	—	250 – 457
This study	186 – 397	65 – 171	37 – 246

* cited in Vijverberg & Frank, 1975

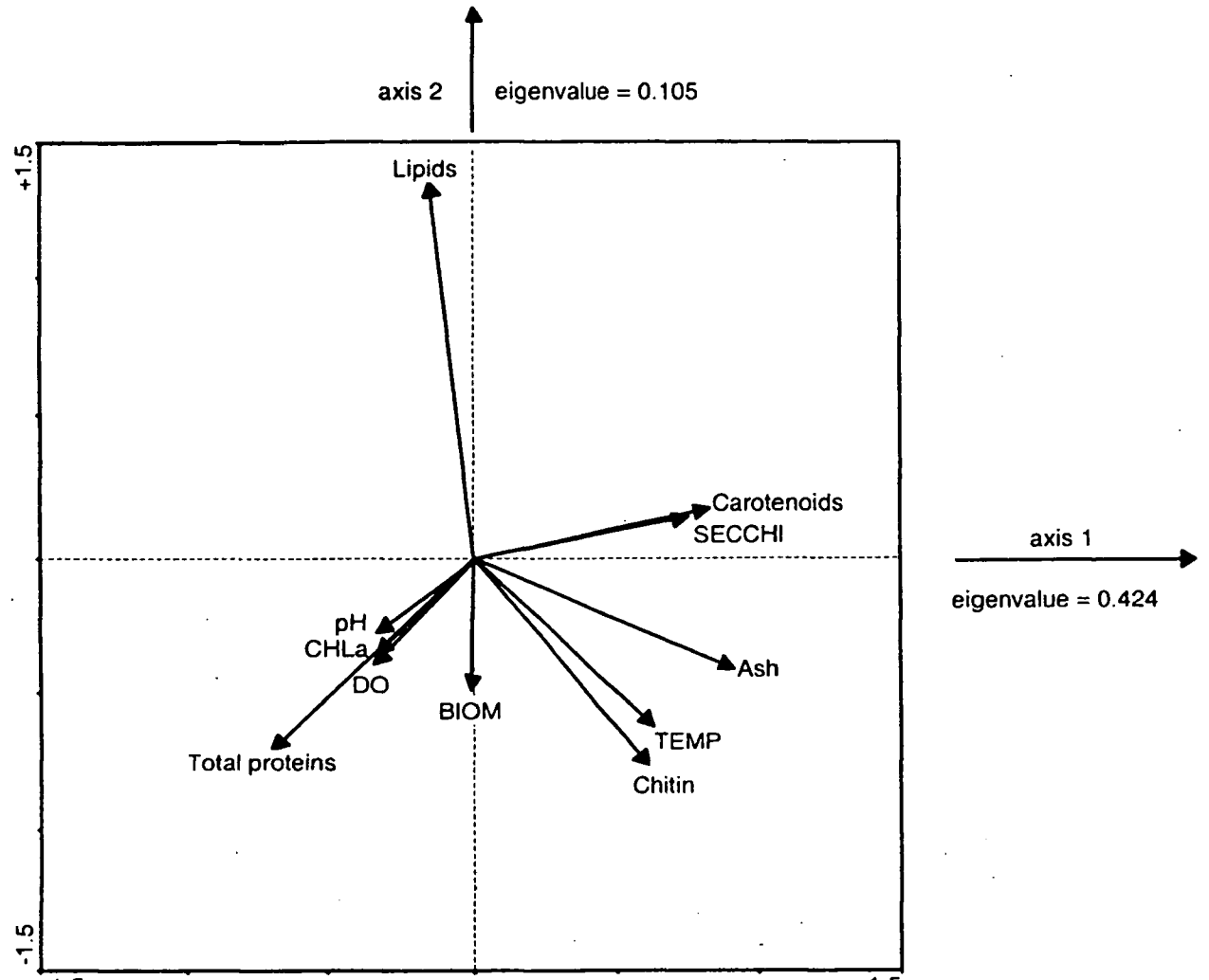

Fig. 3. Redundancy analysis ordination diagram of biochemical (in lower case letters) and ecological variables (in upper case letters). Abbreviations : TEMP = water temperature ; DO = dissolved oxygen concentration ; SECCHI = water transparency measured with the Secchi disk ; CHLa = chlorophyll *a* concentration ; BIOM = *Daphnia magna* biomass.

Fig. 3. Diagramme de l'ordination par analyse de redondance des variables biochimiques (lettres minuscules) et écologiques (lettres majuscules). Abréviations : TEMP = température de l'eau ; DO = concentration en oxygène dissous ; SECCHI = transparence de l'eau mesurée à l'aide du disque de Secchi ; CHLa = concentration en chlorophylle *a* ; BIOM = biomasse de *Daphnia magna*.

In cladocerans, lipids and proteins are considered to be good indicators of the nutritive state (Tessier & Goulden 1982, Guisande et al. 1991). At low food concentration, lipid reserves, mainly triacylglycerides, are metabolised while proteins are only catabolised under severe starvation (Elendt 1989). Under laboratory conditions, the lipid content of *D. magna* ranges from 128 to 197 $mg.g^{-1}$ (DW) in well-fed animals and drops to about 60 $mg.g^{-1}$ (DW) in starved, hatching or senescent specimens (McKee & Knowles 1987, Elendt 1989). The ash content is also used as an indicator of the zooplankton nutritive state (Lemcke & Lampert 1975, Berberovic 1990). Under starvation, the relative ash content has been found to increase because the absolute ash content remains constant while the total dry weight is decreasing as a result of lipid and protein catabolism.

In the waste stabilisation pond of Differdange, high lipid levels and low ash levels were recorded in daphnids during autumn. This indicates that, despite the absence of algae, daphnids were not starved and probably fed on other food sources such as bacteria, protozoa or detritus (Porter et al. 1983, Jürgens 1994). On the other hand, the lipid content was low (< 100 $mg.g^{-1}$ (DW)) from February to July. Lipids were particularly depressed when chlorophyll *a* and daphnid biomass were high in March and June. This is probably due to the increasing reproductive rate during daphnids blooms. The lipid level of females is indeed lowered when releasing lipid-rich neonates (Elendt 1989). Contrary to lipids, proteins was positively correlated with algal food resources. The depression of proteins and the steep increase of ashes in May probably highlighted a real depression in the food resources

which led to starvation and a steep decrease of *D. magna* biomass.

Data about chitin content in crustaceans are scarce and, regarding anomopods, seem to be restricted to our study. Comparisons with chitin levels in other branchiopods have been made elsewhere (Cauchie et al. 1997). Contrary to proteins, lipids or carotenoids, chitin is localised nowhere else than in the cuticle. Therefore, at the individual level, an increasing chitin content indicates that the animal is in pre- or post-moulting stage. Considering that moulting is not synchronised in the population, an increasing chitin level in our samples reflects an increase of the proportion of animals that are in a moulting stage. As the moulting frequency is closely related to temperature (Bottrell 1975), this may explain why a positive correlation was found between chitin level in *Daphnia magna* and temperature.

The synthesis of carotenoid in cladocerans and copepods is generally regarded as a way of protecting against the deleterious effects of U.V. radiation (Hairston 1976, Siebeck 1978). In the pond of Differdange, the positive correlation between the carotenoid content of *D. magna* and water transparency, coupled with the fact that the pond is shallow and that no light refuge would exist, seems to confirm this hypothesis.

The carotenoid levels measured at Differdange are lower than those reported by De Meester & Beenaerts (1993) from 28 *D. magna* clones (from 250 to 457 $\mu g.g^{-1}$ (DW)) (Table 1). These differences are certainly due to the difference in algal abundance between our study and theirs. Indeed, zooplankton can not synthesise body carotenoids *de novo* but needs phytoplankton β-carotene as precursors for this synthesis (Herring 1968a, b, Castillo et al. 1982). Therefore, a reduced availability of algae lowers the maximum quantity of carotenoids that zooplankton can synthesise. This has been confirmed by Partali et al. (1985) who observed a reduction of the carotenoid content from 237 to 25 $\mu g.g^{-1}$ (DW) in *D. magna* when individuals were reared for several generations on a yeast diet alone. However, the dependence of the carotenoid synthesis on the algal availability does not imply that the carotenoid body level and chlorophyll *a* concentration vary parallely. In the pond of Differdange, the carotenoid concentration reaches minimal values in spite of high chlorophyll *a* concentration because the need for photoprotection is almost non-existent at this time as the water transparency was low as a consequence of the high algal abundance. Our results must, however, be taken cautiously because the carotenoid content of the algae that were present in the gut of the assayed animals may have biased to a certain extent the estimation of the body carotenoid content of *D. magna*.

The presence of a maximum absorbance between 460 and 466 nm in *D. magna* has already been noted by De Meester & Beenearts (1993). It suggests that echinenone, which has a maximum absorbance at 462 nm when extracted in ethanol (Davies 1976), could be one of the major carotenoid in *D. magna*.

Despite a marked seasonal variation in biochemical composition, *Daphnia magna* developing in the waste stabilisation pond have sufficient levels of proteins and lipids to be used for the nutrition of aquacultured fishes (Macartney 1996, Oberle et al. 1997). Moreover, *Daphnia magna* has a similar carotenoid content to fish meals used to color salmonid flesh (Simpson 1978) and could thus be used for this purpose. On the other hand, the chitin content of the daphnids is significant and comparable or slightly lower than the current source of chitin for the industry, i.e. crabs and shrimps (Sandford 1989). On the basis of an annual secondary production of 101 $g.m^{-3}.year^{-1}$ (Cauchie et al. 1995), the annual chitin production would reach about 5 g $chitin.m^{-3}.year^{-1}$, that is to say a total chitin production of 690 $kg.year^{-1}$ for the whole waste stabilisation pond. The chitin production by *D. magna* appears higher than most of other crustaceans, especially as far as marine species are concerned. (Cauchie 1997). The source of chitin studied in the present paper is therefore worth considering for commercial applications. Further chemical characterisation of the chitin isolated from daphnids is, however, needed to complete the evaluation of the prospect of using these crustaceans as a commercial source of chitin.

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