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Biochemical Content (fatty acids, sterols, total protein, lipophilic vitamins) and Antioxidant activity (GSH, MDA) of Cladocerans (*Daphnia* sp., *Diaphasoma branchyrum*)

Fatma CAF[1*], Nurgül ŞEN ÖZDEMİR[1], Zehra GÖKÇE[2]

[1]Department of Veterinary Medicine, Vocational School of Technical Sciences, Bingöl University, 12000, Bingöl, Turkey
[2]Department of Health Menegemant, Yusuf Serefoglu Faculty of health Science, Kilis 7 Aralik University, 79000, Kilis, Turkey
*e-mail: fcaf@bingol.edu.tr

ABSTRACT

This paper presents biochemical content such as fatty acids, sterols, total protein, lipophilic vitamins, and antioxidant parameters of mixed cladoceran samples (*Daphnia* sp., *Diaphanosoma brachyurum*) in the freshwater ecosystem. Results indicated that predominant fatty acids were 20:5n3 (19.85%), 16:1n7 (17.29%), 16:0 (13.1%), 24:0 (12.55%), 18:0 (10.68%) and 18:1n9 (7.99%). Cholesterol was the principal sterol (194.1 µg/g) in cladocerans. Total protein was calculated as 52.26 µg/g in cladocerans. α-tocopherol (vitamin E) was the most important vitamin (4.6 µg/g) among lipophilic vitamins. Glutathione (GSH) was quite high (450µg/g) than MDA (11.5 nmol/g).

KEYWORDS: EPA, cholesterol, α-tocopherol, GSH.

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1. Introduction

Zooplankton are considered an important compartment of aquatic ecosystems because of act as primary and secondary links in all of the aquatic food webs (Chari et al., 2013). They have also a major role in nutrient cycling in an aquatic ecosystem. Zooplankton species are commonly utilized for environmental changes because they respond quickly to environmental pollution as a whole community (Whitman et al., 2004; Altındag et al., 2009; Kaya et al., 2010; Sellami et al., 2010). Moreover, they are frequently used as indicators of water quality due to the close relationship between environmental factors and species composition. Cladocerans are major components of the zooplankton of freshwater environment and especially significant in the food web of stagnant waters (Mansour et al., 2005) and one of the most abundant primary consumer groups in lentic freshwater ecosystems. They are good indicators of environmental changes (e.g. water quality and/or historical differences), especially regarding xenobiotic effects (Pereira et al., 2009; Vidal et al., 2012). Most species of zooplankton are filter-feeders as cladocerans. They are generally reproduced by cyclical parthenogenesis. During these processes the fatty acids (FA) are essential for the survival and growth of animals (Mansour et al., 2005) because of transferred to higher trophic levels in aquatic food webs (Desvilettes et al., 1997) by converting phytoplankton and bacteria into animal protein and making it available to higher animals such as fish (Willis et al., 1995). However, different species have variations in their FA composition and levels. The variation in FA of fish is due to diet consumed, reproductive cycle, temperature, season, and geographical location (Özparlak 2013). In freshwater pelagic food webs, zooplankton FA composition is an association with diet quality closely, especially the long-chained, polyunsaturated fatty acids (PUFA) (Bourdier and Amblard, 1989; Sekino et al., 1997; Ahlgren et al., 1990; Muller-Navarra 1995a, b; Muller-Navarra et al., 2000). Additionally, temperature effects on the fatty acids in aquatic webs (Downer and Kallapur, 1981). Because the variations in temperature, both diurnal and seasonal are a natural phenomenon in aquatic ecosystems, their influence on the nutritional value of phytoplankton may have a remarkable ecological relation to herbivorous zooplankton (Smirnov, 2014). Zooplankton growth and egg production were strongly related to the primary producer 20:5n-3 to carbon ratio. This indicates that the limitation of zooplankton production by this essential fatty acid is of central importance at the pelagic producer-consumer interface (Müller-Navarra et al., 2000).

Sterols are another class of lipids and seem to differ in their allocation to somatic growth and reproduction. Thus, structural differences of dietary sterols have pronounced effects on the life-history traits of cladocera (Martin-Creuzburg and Von Elert, 2004).

Lipid peroxidation is a major contributor to the loss of cell function under oxidative stress (Marnet, 1999). Malondialdehyde (MDA) is one of the final products of PUFA peroxidation in the cells because of its facile reaction with thiobarbituric acid (TBA). MDA level has been commonly used for many years as a good biomarker of oxidative stress and the antioxidant status or lipid peroxidative damage to cell membranes (Pryor, 1989, Esterbauer and Cheeseman, 1999; Gil et al., 2002; Gawel et al., 2004). MDA was determined as the measure of thiobarbituric acid reactive substances (TBARS) (Jain et al., 1989). TBARS are naturally present in biological specimens and include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress (Armstrong and Browne 1994, Botsoglou et al., 1994).

Reduced glutathione (GSH) is the most abundant intracellular nonprotein thiol in cells among intracellular antioxidant molecules (Anderson, 1998). GSH plays an important role in protecting cellular proteins against oxidative damage. Reactive oxygen species (ROS) oxidizes proteins both through the formation of protein-free radicals and oxidation of protein thiols. GSH reduces protein free radicals yielding glutathione free radicals. In the case of oxidation of protein thiols, the activity of GSH is mediated by specific enzymes called thiol transferases, which acts as catalyzed, and are involved in the reduction of protein thiols by GSH, then transformed to an oxidized glutathione form (GSSG) (Bandyopadhyay and Chattopadhyay 2006; Naito and Yoshikawa, 2006).

α-tocopherol is a lipid-soluble free-radical scavenger that presumably protects cells from oxidative damage. α-tocopherol is an important antioxidant in biological systems (Kostner et al., 1995). It is present in lipid bilayers of biological membranes and may play a structural role in the membranes (Gurr and Haryvood, 1991). It not only inhibits peroxidation of membrane lipids but also plays an important role in protecting plasma lipoproteins against oxidative modification (Kostner et al., 1995)

Most of the studies, on Cladocera researched to evaluate the influence of species, size, developmental stage or environmental factors (e.g. temperature, quality and quantity of food), taxonomy and distribution (e.g. Hessen, 1990; Manca et al., 1994; Forro et al., 2008). Recent studies were focused on identifying and importance the biochemical composition and antioxidant activity of Cladocerans in freshwater ecosystems (e.g. Von Elert 2002, Von Elert et al., 2003, Barata et al., 2005; Sperfeld and Wacker 2014; Makhutova et al., 2014; Gladyshev et al., 2015; Gama-Flores et al., 2015). However, these researches were done in vitro or familiar freshwater areas. The sampling area in the study is a special lake because these areas have been a few in the World (e.g. Ingle lake, Zacaton lake, Italy Lago Della Regina region). Known biological studies very limited in these areas. In the Sampling area, these were two different studies about fatty acids of zooplankton (Şen Özdemir and Caf, 2015a) and zooplankton fauna (Şen Özdemir and Caf, 2015b). However, we think that it is important to investigate the different parameters in different organism groups in terms of determination of the ecological structures of the lake. Thus, we aimed to obtain general information about the biochemical parameters of some cladoceran species (*Daphnia* sp., *D. brachyurum*). Also, Daphnia is the most important herbivore zooplankton in freshwater environments (Von Elert et al., 2003). We used mixed cladoceran samples, including *Daphnia* sp. and *D. brachyurum* because they were the most abundant species in the sampling season.

2. Material and Methods

2.1. Study Area and Sampling Sites

Zooplankton samples were collected from Bingöl Turnalar Lake location, the floating Islands (Figure 1) in 2012 winter. The samplings were conducted a mix (side and center of the lake). The study includes only mixed cladoceran species (*Daphnia* sp.-80% and *D. brachyurum*-20%), does not include other species from other groups of Zooplankton.

The lake has three floating Islands surface of the water. The floating Islands can independently move the water surface of the lake. Bingöl Floating Islands declared as a natural monument by the Ministry of Environment and Forest, General Directorate of Nature Protection and National Parks in 2005. The formation of floating islands where this place is connected to an old landslide masses. Although the sampling area (lake) in the past was more than 2000 m^2, it is now 600 m^2. Now, the old lake surface is a swamp (1200 m^2) (Bulut, 2012). Its depth is about 5m and thick of floating islands changes 85-192cm (Doğan Demir et al., 2013).

Figure 1. Photograph of Bingol Floating Islands in Turnalar Lake, Turkey

Zooplankton samples were collected with a vertical haul from 5 m depth up to surface water. The haul had a 200 µm mesh and 110 cm mesh Hydro-Bios net with a mouth diameter of 110 cm. The cladoceran species immediately separated under leica brand stereo microscope from the others zooplankton. Then, they transferred in the glass vials and were kept in the freezer (-80°C) until biochemical analyzes. The water temperature was measured with a digital thermometer.

2.2. Lipid Extraction

Cell pellets were homogenized with 3/2 (v/v) hexane-isopropanol. The homogenate was centrifuged at 4500 rpm for 10 minutes at 4 ºC. The cell pellet residue was precipitated. The supernatant part was used in fatty acid analysis (Hara and Radin, 1978).

2.3. Derivatization of Fatty Acid Methyl Esters (FAME)

2 % H_2SO_4 (5 ml) prepared in methanol was added in the aliquot taken from the supernatant part. The mixture was vortexed and kept at 50 ºC for 18 hours. 5 % $NaClO_2$ (5 ml) was added and vortexed. FAME were extracted with 2x5 ml hexane. FAME were treated with 2 % $KHCO_3$ solution (5 ml). The hexane phase of the samples was evaporated by the pure nitrogen (N_2) flow. 1 ml hexane was added to dry lipid. The samples were taken to autosampler vials. The vials were capped under N_2 (Christie, 1990).

2.4. Protein Extraction

A modified step by the Folin-phenol reagent method (Lowry et al., 1951) was used using BSA (bovine serum albümin) as standard protein in protein extraction.

2.5. Derivatization of Reduced Glutathione (GSH)

GSH was measured according to the method of Ellman (1959). Cladocera quantitated in homogenates of GSH was calculated based on the calibration curve. The absorbance was recorded against a blank at the same wavelength. The concentration of GSH was calculated using the standard curve.

2.6. Derivatization of Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation was determined by the thiobarbituric reactive species (TBARS) TBARS measures the production of malondialdehyde (MDA) that reacts with thiobarbituric acid (Ohkawa et al., 1979). The concentration of TBA-malondialdehyde (MDA) was shown as nmol MDA cladocera g^{-1} and nmol MDA cldocera^{-1} was calculated using the standard curve.

2.6. Analysis of Fatty Acid Methyl Esters (FAME)

FAME were analyzed with the Gas Chromatography (GC) (SHIMADZU GC 17 Ver. 3-Kyoto, Japan). The capillary column was 25m long (Machery-Nagel-Germany), 0.25µm inner diameter and 25µ film thickness was used. The column temperature was 120-220 ºC, injection temperature was at 240 ºC and the detector temperature was at 280 ºC during the analysis. The column temperature program was adjusted from 120 ºC to 220 ºC and the temperature increase was determined as 5 ºC/min until 200 ºC and 4 ºC/min from 200 ºC to 220 ºC. It was kept at 220 ºC for 8 minutes. The total duration was set as 35 min. Nitrogen was used as the carrier gas. Standard FAME mixture was used and the residence time of each fatty acid was determined. After the necessary programming was made, fatty acid amounts were determined as percent (%) of determined fatty acids (Christie, 1990).

2.7. Analysis of Lipophilic Vitamins and Sterols

5 ml supernatant was taken and 5% KOH solution was added. After this mixture was vortexed, it kept at 85°C for 15 min. 5 ml pure water was added above the sample and mixed. Lipophilic molecules were extracted with 2×5 ml hexane. The hexane phase was evaporated with N_2 flow. 1 ml (50 + 50%, v vG1) acetonitrile/methanol mixture was added in the sample was put to autosampler vials for analysis.

The analysis was made with the HPLC (SHIMADZU). LC-10 ADVP UV-visible pump,

SPD-10AVP detertor, CTO- 10ASVP column oven, SIL-10ADVP autosampler detector, DGU-14A degasser unit and Class VP software (Shimadzu, Kyoto Japan) were used. The mobile phase was the acetonitrile/methanol (60+40% v vG1) mixture. UV detector and the Supelcosil LC 18 (15×4.6 cm, 5 µm, Sigma, USA) column were used. 326 nm for vitamin A, 202 nm for vitamin E, 265 nm for vitamins D, K (Katsanidis and Addis, 1999).

3. Results and Discussion

Mixed cladoceran samples were used in all biochemical analyzes, including *Daphnia* sp. at 80% and *Diaphanosoma brachyurum* at 20% of total samples because they were the most abundant species in the sampling season (winter). It is known that the season is the coldest season of the region during the year. In the sampling season, the water temperature was measured on average 0.5 °C.

Figure 2 shows prominent fatty acids (% of total FA) in cladocerans. The present study showed that eicosapentaenoic acid (20:5n3, EPA) the most important fatty acid with 19.85 % in cladocerans. The others most important fatty acids were 16:1n7 (17.29%), 16:0 (13.1%), 24:0 (12.55 %), 18:0 (10.68 %) and 18:1n9 (7.99 %). Long-chain highly unsaturated fatty acids (HUFA) such as EPA and docosahexaenoic acid (22:6n3, DHA) have an essential physiological importance for all animals (Muller-Navarra, 1995a; Arts et al., 2001; Wacker and Von Elert, 2001; Copeman et al., 2002; Garg et al., 2006; Gladyshev et al., 2006; 2008). Animals can generally convert the parent acid of the n3 family, alphalinolenic acid (ALA, 18: 3n3) into EPA and DHA. However, the rate of conversion is very low level (Goulden and Place 1990, Plourde and Cunnane, 2007) and many animals must obtain EPA and DHA from food (Lands, 2009; Wall et al., 2010). EPA and DHA are significantly synthesized at large amounts by phytoplankton (Harwood, 1996; Tocher et al., 1998), and these essential acids are transferred through food chains to higher trophic levels such as fish (Gladyshev et al., 2009). Zooplankton are a secondary link for this transport where aquatic ecosystems are the main source of essential HUFA in the aquatic food webs (Chari et al., 2013; Gladyshev et al., 2009). Therefore, these fatty acids are very important fatty acids in aquatic organisms. Our study showed that DHA was not among predominant fatty acids of cladocerans. Gladyshev et al. (2011) indicated that zooplankton from cold lakes had higher levels of DHA than from warm lakes in the freshwaters. However, whereas cladocerans from warm waters have a considerable higher percentage and contents of 20:0 and 22:0 than cladocerans from cold water bodies, cladocerans from cold waters have a higher percentage of EPA than those from warm waterbodies (Gladyshev et al., 2015). Our samples was taken in the cold season (in winter) and the water temperature was low (0.5ºC). In our study, 24:0 was among the major saturated fatty acid (SFA) with 12.55%. Gladyshev et al. (2015) pointed out that high percentages of 20:0 and 22:0 in cladocerans from cold waters were correlated with homeoviscous adaptation and cladocerans probably syntheise these fatty acids. 24:0 as highly SFA may take on the similar functions.

The ability of cladocerans to synthesise EPA de novo is very limited (Goulden and Place 1990; Weers et al., 1997; Taipale et al., 2011). Therefore; EPA must be obtained from the food of them. Also; selective accumulation of EPA by Cladocera from food with little PUFA is well known (Weers et al., 1997; Taipale et al., 2011; Masclaux et al., 2012; Hartwich et al., 2013; Koussoroplis et al., 2012). Thus; EPA was not the only fatty acid responsible for temperature adaptation of Cladocera (Martin-Creuzburg et al., 2012; Gladyshev et al., 2015). Cladocerans might need other factors together with dietary EPA for temperature adaptation (Gladyshev et al., 2015). Additionally; many study showed that copepods have higher levels of DHA rather than cladocerans (Desvillettes et al., 1997; Ballantyne et al., 2003; Persson Vrede 2006; Hessen and Leu, 2006; Smyntek et al., 2008; Brett et al., 2009; Kainz et al., 2009; Burns *et al.*, 2011; Makhutova et al., 2014; Gladyshev et al., 2015). Makhutova et al., (2014) showed that EPA was the most important PUFA with 9.83 %; 16:0 was the most important SFA with 19.78% and 18:1n9 was the most important MUFA with 9.39 % in *D. galeata*. They showed similar results in *D. brachyurum*. They found that DHA was in very small quantities in the both of cladoceran species (0.44-1.01%). EPA and arachidonic acid (20:4n6; ARA) are essential fatty

acids (EFA) and they are precursors for the eicosanoid groups of hormones that control reproductive processes (Funk, 2001). Gama-Flores et al. (2015) found that ARA was high quantities (1.7%) in neonates of *Moina macrocopa* (Cladocera) produced under diurnally variable temperature regimes. In the study; ARA was among prodominant PUFA at 1.64% in cladocerans. A high quantity of ARA could be used for future reproduction when the neonates were mature into adults of cladocerans (Gama-Flores et al., 2015).

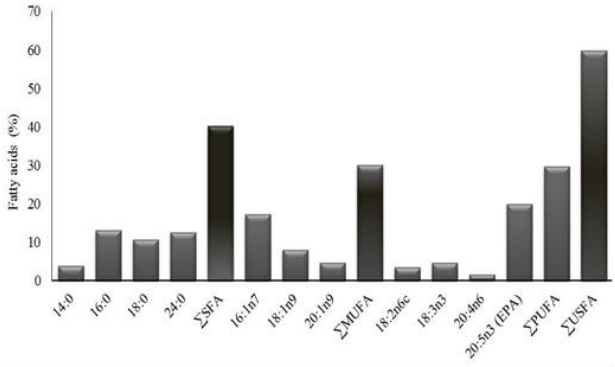

Figure 2. Prominent fatty acids (% of total FA) in cladocerans

Sterols are a lipid class such as fatty acids (Volkman, 2003). Sterols can play a constrained role in the carbon transfer, especially between cyanobacteria and the herbivore *D. galeata* (Von Elert et al., 2003). Crustaceans are incapable of synthesizing sterols de novo. Therefore, they must obtain essential nutrients from their diet. Cholesterol is the principal sterol in crustaceans. In addition, it is a key in food-web dynamics and zooplankton life history (Hassett, 2004; Martin-Creuzburg et al., 2005). The herbivorous daphnids can not rely on a dietary source of cholesterol because only trace amounts are found in phytoplankton species (Nes and Mckean, 1977). However, eukaryotic phytoplankton is rich in various phytosterols. Some phytosterols are suitable precursors for cholesterol synthesis (Martin-Creuzburg and Von Elert, 2004). For example, Von Elert et al. (2003) determined that in *D. galeata* feeding on green alga *S. obliguus* who has phytosterols but no cholesterol, cholesterol was the major sterol, with the time feeding on the eukaryotic algae. They emphazised that the cholesterol content of the animals was significantly affected by the diet. Similarly, we found that cholesterol was the major sterol (194.1 µg/g) in cladocerans and we thougt that it could depend on the dietary source of the environment (Table 1).

Table 1. Lipophilic vitamin and sterol content (µg/g) of cladocerans

Vitamins	
K_1	0.1±0.03
D_2	0.1±0.03
α tocopherol	2.02±0.77
α tocopherol acetate	0.1±0.001
D_3	0.2±0.00
Sterols	
Cholesterol	194.1±21.4
Stigmasterol	6
β-sterol	34.11±1.17
	0.3±0.055

Protein has immensely important functions in biological processes of living beings, such as enzymatic processes, transport and storage, mechanical support, immune protection, generation and transmission of nerve impulses, and control of growth and differentiation (Zaia et al., 1998). In the study, total protein was calculated and it was found as 52.26 µg/g. Protein is obtained by Cladoceran from algae and seston. Indeed, it was showed with reference to *Daphnia obsuta* that the amounts of released nitrogen and phosphorus are generally proportional to the amounts ingested with food algae (Sterner and Smith, 1993).

In the present study, α-tocopherol (vitamin E) was the most important vitamin (4.6 µg/g) among lipophilic vitamins, following D_3 (0.2 µg/g), D_2 (0.1 µg/g) and K_1 (0.1 µg/g) (Table 1). α-tocopherol is an important antioxidant, and antioxidants play an important role in reducing or preventing lipid peroxidation (Aitken et al., 1989). Its main role is to protect unsaturated fatty acids against oxidative stress (Hamre et al., 1998). α-tocopherol is a very important dietary factor of animal nutrition for reproduction. It has an accelerating effect on reproduction (Kahn-Thomas and Enesco 1982; Shalaby et al., 2004). This effect can be possibly derived from the antioxidant effect of α-tocopherol (Shalaby et al., 2004). Rice and Kennedy (1988) demonstrated that the amount of α-tocopherol present in tissues is related to the PUFA concentration. α-tocopherol probably reflects a higher degree of antioxidant protection required n-3 PUFA-rich organisms (Hamre and Lie, 1995).

Free radicals create the lipid peroxidation in an organism (Gawel et al., 2004). Lipid peroxidation is the main molecular mechanisms involved in the oxidative damage to cell structures and in the toxicity process that cause to death of cells. Lipid peroxidation is the consequence of toxic metabolites. These metabolites produce highly reactive species, disruption of the intracellular membranes and cellular damage (Dianzani and Barrera, 2008). In some crustacean species, oxidative damage increases with increasing age, concomitant with decreasing levels of antioxidant defenses (Viarengo et al., 1999; Mourente and Diaz-Salvago 1999; Correia et al., 2003). Another factor affecting oxidative damage is the level of target molecules such as PUFA, which are easily oxidized by ROS to lipid peroxides (Di Giulio et al., 1995) and account for an important percentage of membrane phospholipids in *Daphnia* (Goulden and Place, 1990). Thus, the effect of lipid peroxide may principally depend on the level of antioxidant enzymes and the fatty acids composition of the organisms but variations with age in *Daphnia* affects lipid peroxidation (Bychek and Gushchina 1999). In the cell, malondialdehyde (MDA) is the final product of PUFA peroxidation. MDA level is a marker of oxidative stress and the antioxidant status. Overproduction MDA is based on an increase in free radicals in cells (Gawel et al., 2004). We found that MDA was quite low (11.5 nmol/g) and GSH was 450 µg/g in cladoceran. GSH is a major antioxidant in the cell because of its central role in maintaining the cell's redox state. It protects cells from free radicals as oxygen radicals which are highly reactive substances. GSH is maintained in the reduced form by the enzyme glutathione reductase and in order to reduce other metabolites and enzyme systems, such as ascorbate in the glutathione-ascorbate cycle, glutathione peroxidases, and glutaredoxins, as well as reacting directly with oxidants (Meister and Larsson, 1995). Additionally, GSH is a very important detoxifing agent and plays a key role in maintaining a balance between oxidation and anti-oxidation. This regulates many vital functions in the cell like the synthesis and repair of DNA, the synthesis of proteins and the activation, maintaining the essential thiol status of protein, immune function, regulate nitric oxide homeostasis, modulate the activity of neurotransmitter receptors and regulation of enzymes (Oja et al., 2000; Dalle-Donne et al., 2007). The lower level of GSH is related to different physiological and biochemical disturbances (Catala, 2012). Fidan et al. (2008) showed that GSH level was the same as MDA due to the increase and decrease of oxidative stress in *Carassius carassius* (fish) in Eber Lake. MDA level increases when the natural antioxidant system is insufficient (Şahan et al., 2003). We may think that the naturel antioxidant system of Cladoceran was sufficient because GSH was quite higher than MDA. The increase of the MDA level in winter can be because of the cold conditions of the fish environment, and the lipid catabolism occurs in cold seasons to get much more energy for their metabolisms. MDA levels vary during the seasons

and the highest level is in winter and the lowest level is in spring and summer seasons and also the highest GSH level is in the winter season (Fidan et al., 2008).

Consequently, biochemical parameters were only determined in the winter season. High GSH level may depend on winter season and cladocerans may resist against oxidative stress, especially in cold seasons. However, to compare all season will be useful for all the biochemical parameters. It is known that diet has important effective changes of biochemical parameters together with environmental factors. DHA was not among predominant fatty acids in cladocerans although it was among dominant fatty acids in zooplankton. We may think that the naturel antioxidant system of Cladoceran was sufficient because GSH was quite higher than MDA. High GSH level may depend on winter season and cladocerans may resist againgst oxidative stress, especially in cold seasons. However, to compare all season will be useful. However, we thought that the study would contribute to future studies about biochemical content and antioxidant activity of cladocerans because it is first known record together biochemical content and antioxidant activity of cladocerans in a nature freshwater environment.

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