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New data on endohelminth communities of barbel *Barbus barbus* from the Bulgarian part of the River Danube

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Summary

Species diversity and composition of the parasite communities of barbel (*Barbus barbus*) at the infracommunity and component community levels were studied in the Lower Danube River, Bulgaria. During the two-year investigations, five parasite species have been found in 92 host fish: *Bathybothrium rectangulum* (Cestoda), *Acanthocephalus anguillae* and *Pomphorhynchus laevis* (Acanthocephala) and larval stages of *Contracaecum* sp. and *Raphidascaris acus* (Nematoda). *Bathybothrium rectangulum* and *R. acus* found in barbel represented new host records in Bulgaria. Parasite communities of barbel were species-poor and highly unbalanced. *Pomphorhynchus laevis* represented the dominant (core) species (prevalence 98.9 %), the second most frequent component parasite was *Contracaecum* sp. (P = 14.1 %) and remaining three species occurred only accidentally in barbels. Differences in species richness, prevalence, intensity of infection and ecological indices between individual seasons (spring, summer, autumn) were statistically significant, but considerably affected by unequal species structure of communities with highly prevailing *P. laevis*. Low parasite species diversity of barbel and low values of most ecological indices, when compared with previous studies in this area (or other Bulgarian parts of the River Danube) might indicate that environmental conditions are impaired and thus, not favourable for the development of barbel parasites (primarily to their intermediate host survival) in the Lower Danube River of Bulgaria.
Keywords: *Barbus barbus*; helminths; parasite community structure; seasonality; Danube River; Bulgaria

Introduction

The River Danube is a longest river of the Central and Eastern Europe, which flows 2,860 km through ten countries or touches their borders. Its stream use to be divided into three broad sections with the delta representing a separate and unique system. The Upper Basin extends from the source in Germany, to the Devín Gate (Austria/Slovakia border), the Middle Section to Iron Gate dams (the Serbia/Romania border), and the Lower Section to the entrance of the Delta, with the whole section of the Bulgarian river bank (for review see Tubic *et al*., 2013). The barbel, *Barbus barbus* (Linnaeus, 1758) is indigenous to the ichthyofauna of the Danube Basin. As the potamodromous and benthopelagic species, it feeds chiefly on benthic invertebrates such as small crustaceans, insect larvae, molluscs, mayflies and midge larvae, but also small fish and occasionally algae (Froese & Pauly, 2018). Parasite fauna of this fish reflects these food habits and local ecological conditions.

The fish community in the shoreline zone of the Danube River in Bulgaria was investigated by Polačik *et al.* (2008). In this study, they identified 44 fish species, barbel, represented rarely occurring and low abundant fish species.

Fish and parasite communities from the lower section of the Danube River, including the Bulgarian part of the river, were often studied (Margaritov, 1959, 1966; Kulalovskaya & Koval, 1973; Kakacheva-Avramova, 1977, 1983; Kakacheva *et al*., 1978; Nedeva *et al*., 2003; Polačik *et al*., 2008,; Atanasov, 2012; Kirin *et al*., 2013, 2014 etc.), but only Nachev & Sures (2009) and Nachev (2010) examined specifically barbel.

The aim of this paper was to find the diversity of endoparasites of barbel and evaluate the structure of their communities in the Bulgarian part of the Lower Danube River.

Materials and Methods

During spring, summer and autumn of 2015 and 2016, a total of 92 specimens of barbel were collected from the Lower Danube River near the village Vetren, Bulgaria (44°133'N, 27°033'E). The village is situated on the riverside, in the north-eastern part of the Danube Valley.

Barbels were caught using gill nets and by angling under a permit issues by the Ministry of Agriculture and Food of the Republic of Bulgaria. The scientific and common names of fishes are used according to the FishBase database (Froese & Pauly, 2018).

The fish were weighed (a mean weight was 445.9 ± 40.1 g and ranged between 220 – 788 g) and measured (mean standard body length 36.1 ± 1.0 mm, range 290 – 450 mm). Corresponding values for individual seasons are shown in the Table 1. The sample size can yield to reliable estimates the parasite abundance (Shvydka *et al*., 2018). The fish were immediately after their capture examined for gastrointestinal and tissue helmiths (an incomplete parasitological study) using standard techniques. Helminths were cleaned in a saline solution and fixed in 70 % ethanol. Cestodes were stained with acetic carmine and mounted as permanent slides in a Canada balsam according to Georgiev *et al.* (1986) and Scholz & Hanzelová (1998). Acanthocephalans and nematode larvae were examined as temporary slides in ethanol-glycerine and glycerine, respectively, and identified by use of keys by Petrochenko (1956), Bykhovskaya-Pavlovskaya (1985), Khalil *et al.* (1994) and Moravec (2013) and Lab Compound Microscope XS-213. In total, 6,408 adult or larval helminth specimens were collected (Table 2).

The helminth community structure was studied during three seasons at component community and infracommunity levels. Ecological terms were used according to Bush *et al*. (1997); a prevalence (P, %), mean abundance (MA) and mean intensity of infection (MI). The component data were determined by the total number of species, Shannon diversity index (H'), Pielou's evenness index (E) and Berger-Parker dominance index (d) according to Magurran (2004). The dominance of the component helminths within communities was determined according to the prevalence criterion (P) proposed by Kennedy (1993) as accidental (P<10), component (10<P<20) and core (P>20) species. The infracommunity data were calculated using the mean number of species, mean number of individual helminth specimens, Brillouin diversity index (HB), (Magurran, 2004; Kennedy, 1993, 1997). The quantitative similarity between parasite communities during three seasons was determined by the Sorensen index (Percentage similarity index, Ics), (Krebs, 1999).

The significance of seasonal changes in the prevalence and MI was evaluated by the Chi-square (χ^2) and Two-sample t-test, respectively. A one-way ANOVA was used to compare the mean number of helminth species and the Brillouin diversity index within infracommunities, same as mean number of *P. laevis* with mean number of specimens of all other species. All statistical tests were performed using statistical software programs Quantitative Parasitology, version 3, Rozsa *et al.* 2000; STATISTICA 6.0 program and Microsoft Excel/Windows® XP Home Edition.

Ethical Approval and/or Informed Consent

This research carried out on fish has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

Results

Helminth community structure of barbel

All barbels examined (92/92; P = 100 %) were infected and following parasite species were identified: the tapeworm *Bathybothrium rectangulum* (Bloch, 1782), thorny-headed worms *Acanthocephalus anguillae* (Müller, 1780) and *Pomphorhynchus laevis* (Zoega in Müller, 1776), and nematode larvae of *Contracaecum* sp. and *Raphidascaris acus* (Bloch, 1779). All but one are generalists, *B. rectangulum* is specific parasite to *Barbus* spp. (Protasova, 1977; Moravec *et al.*, 1997). Adult stages of the cestode *B. rectangulum* and acanthocephalans *A. anguillae* and *P. laevis* (all autogenic) occurred in a host intestine. Allogenic nematode larvae were found either encapsulated in various internal organs and an intestine serosa, or free in abdominal cavity of infected fish.

Infracommunities of barbel helminth endoparasites

Species richness in helminth infracommunity of barbel ranged from one to three parasite species. A total of 74 fish individuals were infected with a single helminth species (80.43 %); 16 barbels harboured two helminths (17.40 %); namely, 11 barbels were parasitized by *P. laevis* and *Contracaecum* larvae, two barbels by *P. laevis* and *B. rectangulum*, and three of them by *P. laevis* and *R. acus* larvae. Only two fish (2.17 %) were infected with three parasite species (*P. laevis*, *Contracaecum* sp. and *B. rectangulum*).

A maximum of 354 helminth specimens per fish host was detected. The average species richness (mean number of species in fish specimen) in infracommunities of barbel was 1.22 ± 0.46, the average abundance (mean number of helminth specimens in

Table 1. Basic characteristics of *Barbus barbus* examined from the River Danube within individual seasons.

Season	Number of fish	Mean body length and range (mm)	Mean body weight and range (g)
Spring	33	350.5 (290 – 450)	415.2 (220 – 788)
Summer	28	361.0 (290 – 445)	431.3 (285 – 635)
Autumn	31	371.0 (310 – 420)	491.3 (395 – 745)

fish) was 69.6, Brillouin diversity index HB = 0.075±0.079 (range 0.008 – 0.166). A comparison of the mean HB and the mean number of helminth species showed significant seasonal differences (Two-sample t-test, p = 0.00). The mean number of specimens of *P. laevis* was significantly higher than the number of specimens of all other species in each season (One-way ANOVA, p = 0.03).

Component community of helminth endoparasites of barbel

The vast majority (6,340 specimens) of the parasite component community of *B. barbus* composed two acanthocephalan species, followed by nematodes (49) and cestodes (19), with *P. laevis* as the most prevalent, core species (P = 98.9 %). *Contracaecum* sp. was proved as the second most frequent species (P = 14.1 %), while *B. rectangulum* (P = 4.4 %), *R. acus* (P = 3.3 %), and *A. anguillae* (P = 1.1 %) represented accidental species of the component community (Table 2).

The maxima of the mean intensity and mean abundance were detected in *P. laevis* in all seasons and thus, it represents the dominant species of the component community (Table 3). Significant differences were found between the number of *P. laevis* and the summary number of all other parasites in each season (Two-sample t-test, p_{spring} = 0.002, p_{summer} = 0.03, p_{autumn} = 0.002). The prevalence of *P. laevis* and other parasite species also differed significantly (One-way ANOVA, $p_{P. laevis/B. rectangulum}$ = 0.001, $p_{P. laevis/A. anguillae}$ = 0.002, $p_{P. laevis/Contracaecum\ sp.}$ = 0.006, $p_{P. laevis/R. acus}$ = 0.002). The same results were found for mean intensity of infection (One-way ANOVA, $p_{P. laevis/B. rectangulum}$ = 0.03, $p_{P. laevis/A. anguillae}$ = 0.02, $p_{P. laevis/Contracaecum\ sp.}$ = 0.01, $p_{P. laevis/R. acus}$ = 0.02).

Contracaecum larvae showed much lower infection values throughout seasons with the highest prevalence and intensity of infection in summer (Table 3). Remaining parasites occurred solely in summer and autumn (*B. rectangulum*), only in summer (*A. anguillae*) or only in autumn (*R. acus*), always with a low infection (Table 3).

The highest component species diversity was found in summer and autumn, when four parasite species regularly occurred in barbels (Table 3). In spring, only two parasite species were detected; all barbels were infected with *P. laevis* and the only fish harboured two *Contracaecum* larvae. Above two species also occurred in *B. barbus* in all investigated seasons. The only fish specimen free of *P. laevis* occurred in summer, but it was infected with *A. anguillae*. The differences between the seasonal patterns of the *P. laevis* prevalence were not significant, similarly as between the intensity of infection, which culminated in spring (MI = 78.9). In *Contracaecum* sp. nematodes, significant seasonal differences were observed in both, the prevalence (χ^2, p=0.04) and mean intensity of infection between spring and summer (Two-sample t-test, p = 0.00), spring and autumn (Two-sample t-test, p = 0.00) and summer and autumn (Two-sample t-test, p = 0.01). The mean intensity of infection of *B. rectangulum* was significantly higher in summer than in autumn (Two-sample t-test, p = 0.07).

Taking into account all parasites except for *P. laevis*, significant differences were found between their mean intensity of infection and mean number of parasite specimens in summer and autumn (Two-sample t-test, p = 0.02 and p = 0.008, respectively).

Shannon diversity index and Pielou's evenness index were relatively low, with the lowest values in the spring period and the highest in summer (Table 4). On the contrary, Berger-Parker dominance index was the highest in spring (0.999) and lowest in summer (0.972). The Percentage similarity index (Ics) showed the highest similarity of helminth component communities of barbel between the spring and autumn periods (Ics = 0.989), followed by that between summer and autumn (Ics = 0.981) and summer and spring (Ics = 0.973), but the differences were not significant.

Table 2. The prevalence (P) and intensity values of helminth parasites detected in 92 barbels *Barbus barbus* from the River Danube.

Helminth species	Number of fish infected by individual parasite	P (%)	Number of specimens	Mean abundance ± SD	Mean intensity ± SD (range)
Bathybothrium rectangulum	4	4.4	19	0.2 ± 1.1	4.8 ± 2.9 (1 – 9)
Acanthocephalus anguillae	1	1.1	5	0.05 ± 0.5	5.0 (5)
Pomphorhynchus laevis	91	98.9	6,335	68.8 ± 67.1	69.6 ± 67.1 (2 – 354)
Contracaecum sp. larvae	13	14.1	43	0.5 ± 1.6	3.3 ± 3.0 (1 – 12)
Raphidascaris acus larvae	3	3.3	6	0.06 ± 0.4	2.0 ± 0.8 (1 – 3)

Table 3. Species diversity of helminth parasites of *Barbus barbus* from the River Danube within three seasons (N - number of examined fish, P – prevalence (%), MI – mean intensity).

Helminth species	Spring (N = 33)		Summer (N = 28)		Autumn (N = 31)	
	P	MI (range)	P	MI (range)	P	MI (range)
Bathybothrium rectangulum	–	–	3.6	9.0 (9)	9.7	3.3 (1 – 5)
Acanthocephalus anguillae	–	–	3.6	5.0 (5)	–	–
Pomphorhynchus laevis	100.0	78.9 ± 67.8 (2 – 341)	96.4	60.1 ± 68.6 (2 – 354)	100.0	68.0 ± 63.5 (2 – 237)
Contracaecum sp. larvae	3.0	2.0 (2)	21.4	5.3 (3 – 12)	19.4	1.5 (1 – 2)
Raphidascaris acus larvae	–	–	–	–	9.7	2.0 (1 – 3)

Discussion

Even though the parasitofauna of various fish species from the Lower Danube River, including its Bulgarian part, had been relatively often investigated (see the Introduction), data on helminths of barbel *B. barbus* were infrequently recorded. Summary data of the Table 5 show that helminth communities vary more or less in individual years. For instance, the cestode *B. rectangulum* (Bothriocephalidea) had so far been reported only from *Barbus meridionalis* and *Barbus cyclolepis* in Bulgaria (Kakacheva-Avramova, 1983). We have found this parasite in the *B. barbus* that represents the new host record in the Lower Danube River and even in Bulgaria.

Compared to *B. rectangulum*, acanthocephalans *A. anguillae* and *P. laevis* (both Echinorhynchida) have much broader fish host spectrum in the Danube River. *Acanthocephalus anguillae* is a euryxenous parasite having a wide host range, which includes at least 40 fish species (Moravec, 2001). In the Lower Danube River, this species was commonly referred in *B. barbus* by Margaritov (1959, 1966), Kakacheva-Avramova (1977), Kakacheva *et al*. (1978), Nachev & Sures (2009), Djikanovic *et al*. (2010) and Atanasov (2012), but it also often occurs in the Upper and Middle Danube sections (Moravec 2001; Moravec *et al*., 1997; Djikanovic *et al*., 2012).

The other common thorny-headed worm *P. laevis* has often been reported from various fish hosts including barbel, through the whole Danube River flow (e.g. Molnár, 1970; Moravec et al. 1997; Moravec, 2001; Schludermann *et al*., 2003; Ondračková *et al*., 2005; Djikanovic *et al.* 2010, 2012). In Bulgarian part of the Danube River, *P. laevis* was found in as many as 20 fish species including *B. barbus* (Nedeva *et al*., 2003). More recently, Nachev & Sures (2009) and Nachev (2010) also showed *P. laevis* as very frequent species in this location.

Acanthocephalans of the genus *Pomphorhynchus* have been intensively studied during the past decades. In Europe, *Pomphorhynchus* species have shown a certain degree of variability in their morphological characteristics and behaviour, which caused difficulties with their correct identification. Nowadays, the comprehensive molecular study on the phylogeography of European populations of *P. laevis* and *P. tereticollis* (Rudolphi, 1809) has been done by Perrot-Minnot *et al*. (2018) and it appears that only *P. laevis* occurs through the main flow of the River Danube. The study additionally showed rare co-occurrence of these species in the same habitats, several small rivers and a lake from the Danube River Basin. In this respect, the recent findings of *P. tereticollis* in *Abramis brama* from the Bulgarian lake Srebarna, adjacent to the Danube River (Kirin *et al*., 2013; 2014) is of greatest interest and needs additional study (Špakulová, personal communication). The coexistence of *P. laevis* and *P. tereticollis* had also been documented both in fish and intermediate gammarid hosts in different localities of Europe (Perrot-Minnot, 2004; Westram *et al*., 2011; Perrot-Minnot *et al*., 2018).

Nematode larvae were rarely referred in fishes of the Lower Danube; except of the recently found *Contracaeum* and *Raphidascaris* representatives, only *Eustrongylides* sp. and *Hysterothylacium* sp.

Table 4. Comparison of seasonal diversity of helminth communities of *Barbus barbus* from the River Danube.

Season	Spring	Summer	Autumn
Number of helminth species	2	4	4
Number of helminth specimens	2606	1669	2133
H' (Shannon, diversity index)	0.006	0.149	0.076
E (Pielou, evenness index)	0.009	0.107	0.055
d (Berger-Parker, dominance index)	0.999	0.972	0.988
Dominant species	*P. laevis*	*P. laevis*	*P. laevis*

Table 5. Overview of helminth species of *Barbus barbus* recorded in the lower section of the River Danube.

Authority / Helminth species	Margaritov (1959)	Margaritov (1966)	Kakacheva-Avramova (1977)	Nachev & Sures (2009)	Atanasov (2012)	This study
Cestoda						
Bathybothrium rectangulum						●
Caryophyllaeus laticeps	●	●			●	
Caryophyllaeus fennica		●			●	
Monogenea						
Dactylogyruscarpathicus			●			
Dactylogyrusmalleus	●		●			
Dactylogyrussphyrna			●			
Diplozoon sp.			●			
Trematoda						
Metagonimus yokogawai larv.				●	●	
Diplostomum spathaceum larv.				●	●	
Diplostomum pseudospathaceum larv.					●	
Posthodiplostomum cuticola larv.				●		
Nematoda						
Rhabdochona hellichi			●	●	●	
Rhabdochona denudata					●	
Rhabdochona sulaki (=gnedini)		●				
Rhaphidascaris acus larv.						●
Pseudocapillaria tomentosa				●		
Eustrongylides sp. larv.				●		
Contracaecum sp. larv.						●
Hysterothylacium sp. larv.				●		
Acanthocephala						
Pomphorhynchus laevis	●	●	●	●	●	●
Acanthocephalus anguillae				●	●	●
Leptorhynchoides plagicephalus				●		

were found by Nachev & Sures (2009) and Nachev (2010). In Europe, larvae of the ascaridoid genus *Contracaecum* sp. (Rhabditida) often occurred in internal organs of a wide spectrum of mainly cyprinid and perciform fishes. Up to ten *Contracaecum* species are available at the moment, but the systematics of their larval stages is difficult and little elaborated (Moravec, 2013). Therefore, supplementary molecular analyses of our *Contracaecum* samples would be beneficial to confirm species determination. We suppose, however, that it could be *Contracaecum microcephalum* (Rudolphi, 1809), because it was repeatedly reported from several other fish species in the River Danube in Bulgaria (Shukerova, 2006; Shukerova *et al*., 2010; Chunchukova *et al*., 2016).

Raphidascaris acus larvae (Rhabditida) occur in many fish species of different families of wide Holarctic distribution, most often in cyprinids (Moravec, 2013). In Bulgarian part of the Danube River and the lake Srebarna, larvae of *R. acus* were to date found in three cyprinids, *Abramis brama*, *Alburnus alburnus, Squalius cephalus* and *Perca fluviatilis* (Shukerova 2010; Shukerova *et al*., 2010; Chunchukova *et al*., 2017). In Upper and Middle Danube sections, *R. acus* was found frequently in various fishes including barbels (e.g. Moravec *et al*., 1997; Moravec, 2001; Ondračková *et al.*, 2005). Regarding Bulgaria, the recent report of *R. acus* larvae in barbel represents the new geographic and host record.

Information about seasonal changes of the fish helminth community structure is of great value for any studies on fish parasites and ecological assessment of freshwater habitats. Considering the seasonal changes in the component community structure, it comprised four parasites in summer (*B. rectangulum*, *A. anguillae*, *P. laevis* and *Contracaecum* sp.) and in autumn (*B. rectangulum*, *P. laevis, Contracaecum* sp. and *R. acus*) and only two helminths (*P. laevis* and *Contracaecum* sp.) in spring. Only *Contracaecum* larvae were found in all three seasons round, being accidental in spring (3.0 %), core in summer (21.4 %), and component species in autumn (19.4 %). Numerous, mainly cyprinid fish including barbel (genera *Abramis, Alburnoides, Alburnus, Barbus, Gobio, Rutilus*), represent intermediate or paratenic hosts of *Contracaecum* nematodes (Moravec *et al*., 1997).

In the present study, *P. laevis* was the most abundant (core) species during all studied seasons of two years. It dominated significantly all other helminths in the number of specimens, prevalence and mean intensity of infection. Seasonal differences between these indices were not significant, which partly coincide with a life cycle mode of *P. laevis*, which is not seasonally dependent, same as a composition of the food of barbel. Potential decline of infection rates of the parasite might be related to availability of gammarid intermediate host in preferred habitats. It should be remembered that this parasite, in high numbers, may significantly affect the health status of the host fish and can cause substantial loss and even destruction of its populations, including in aquaculture (Moravec et al., 1997; Gettová *et al.*, 2016).

The stenoxenous *B. rectangulum* was classified as the parasite of barbel with an accidental incidence. Its mean intensity increased in summer, the maximum prevalence was quoted during autumn, but was completely absent in the study site in spring. In other Danube habitats, however, it occurred during the whole year including spring seasons (Scholz & Moravec, 1996; Moravec *et al.,* 1997). These authors described a seasonal cycle of this tapeworm and explained that worms with ripe eggs left the fish hosts from May to July. Reproduction cycle of *Bathybothrium* might be modified by specific local environmental conditions, which could the lack of the species recorded by us in spring. The host specificity of *B. rectangulum* discussed Kakacheva-Avramova (1983), who found this species also in *Gobio gobio* or *S. cephalus*, but the cestode had never reached sexual maturity in these fish hosts.

In the lower Danube, some helminth species seems to be rather rare. Only five specimens of the acanthocephalan *A. anguillae* were found in a single fish host screened in summer, and only six nematode larvae the *R. acus* were found in three barbels screened in autumn. The number of studies (for review see Moravec *et al*., 1997) showed clear seasonal dynamics in occurrence of this nematode with maximum in autumn, decrease in winter, second maximum in spring and minimum in summer. At the same time, significant variations were reported by the habitats, fish size and age and specific fish host diet.

Taking into account all parasites except of *P. laevis*, their mean intensity of infection and mean number of parasite specimens were significantly higher in summer comparing with autumn. The Brillouin diversity index (HB) was significantly higher in summer than in autumn, while the maximum values of Shannon diversity and the Pielou's evenness indices were documented higher in summer but contrary to HB, they were lowest in spring. These results indicate better biotic and environmental conditions for a majority of barbel parasites in summer seasons (Hudson *et al*., 2006). Evaluating the complex information including *P. laevis* data, most indices have changed significantly due to the unbalanced number of individual parasite species (significantly prevailing *P. laevis*) in the examined data set in all seasons. The Shannon diversity and the Pielou's evenness indices were low, while the Berger-Parker dominance and Sorensen similarity indices were relatively high (see Table 4). Berger-Parker dominance index was the highest in the spring period and lowest in summer, in accordance with the dominance of *P. laevis* in the data set.

Our study of endohelminth community of *B. barbus* from the Lower Danube (biotope Vetren) corresponds only partially with previous surveys by Margaritov (1959, 1966), Kakacheva-Avramova (1977), Nachev & Sures (2009), and Atanasov (2012) (Table 5). The spectrum of barbel parasites differed depending on the study site, number of examined fish, and various ecological factors like fish feeding habits and water quality. The number of helminth species, reported by above papers, ranged between three (Margaritov, 1959) and 10 taxa (Nachev & Sures, 2009). Caryophyllidean cestodes, acanthocephalans (mainly the dominating *P. laevis*) and nematodes (the obligate *Rhabdochona* spp.) were most common, while trematodes (e.g. *Diplostomum* sp. larvae) often absent and monogeneans were most probably rarely checked. Significant differences in the parasite diversity of barbel have been found between our study, other Danube River sites or areas geographically close to the Danube (Moravec *et al.,* 1997; Schludermann *et al*., 2003; Nachev, 2010). Relatively low parasite species diversity of barbel recorded during two years, and low values of most indices might indicate some negative environmental conditions in the studied area, which should be subsequently reviewed.

Conflict of Interest

Authors state no conflict of interest.

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