

Daphnia can protect diatoms from fungal parasitism

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Abstract

Many phytoplankton species are susceptible to chytrid fungal parasitism. Much attention has been paid to abiotic factors that determine whether fungal infections become epidemic. It is still unknown, however, how biotic factors, such as interactions with zooplankton, affect the fungal infection process. Because the size of fungal zoospores is well within the preferred range of food particle size for *Daphnia*, we hypothesize that *Daphnia* will affect the fungal infection process by reducing the abundance of fungal zoospores. To examine the effects of zooplankton on the fungal parasitism of phytoplankton, we performed grazing experiments. These experiments revealed that *Daphnia* significantly decreased fungal parasitism by grazing on the fungal zoospores. *Daphnia* also had a small positive effect on fungal infection, probably by increasing the encounter rate between fungi and host phytoplankton cells. These results suggest that *Daphnia* can affect the seasonal succession of chytrids and their host phytoplankton species. In addition, these results imply that zoospore-producing fungi may play an ecological role as food sources for *Daphnia* in aquatic food webs.

Some chytrids are host-specific parasitic fungi of various phytoplankton species, such as diatoms, desmids, and dinoflagellates (Canter and Lund 1969; Sommer 1987; Van Donk 1989; Ibelings et al. in press). Chytrids often reduce phytoplankton blooms and can play a crucial role in phytoplankton succession (Canter and Lund 1948, 1969; Sommer 1987; Van Donk 1989). Abiotic factors such as light, temperature, and nutrients affect fungal infections and sometimes are critical in determining whether fungal infections become epidemic (Van Donk and Ringelberg 1983; Kudoh and Takahashi 1990; Bruning 1991a,b,c; Holfeld 2000; Kagami and Urabe 2002). It is still unknown, however, how other trophic levels such as zooplankton influence fungal infections.

Fungal parasites seem to be most common on large phytoplankton species that are fairly resistant to grazing by zooplankton (Sommer 1987). This suggests that fungal parasites and zooplankton mainly consume different size classes of phytoplankton. However, this does not necessarily mean that there is no interaction between zooplankton and parasitic fungi. Instead, we hypothesize that zooplankton may modify fungi-phytoplankton interactions through direct grazing on fungi. Specifically, large zooplankton may feed upon fungal zoospores and thus reduce the fungal infection rate of phytoplankton. Chytrids are characterized by free-swimming zoospores in their reproductive stage. The size of these fungal zoospores is 2–5 μm (Canter 1967; Kudoh 1990), which is well within the preferred range of food particle size for zooplankton like *Daphnia* (Geller and Müller 1981; Sterner 1989). In fact, flagellates in the size range of chytrid zoo-

spores are efficiently grazed by *Daphnia* (Knisely and Geller 1986; Sanders and Porter 1990; Pace and Funke 1991).

In Lake Maarsseveen, the Netherlands, the chytrids *Zygorhizidium planktonicum* and *Rhizophyidium planktonicum*, heavily infect the diatom *Asterionella formosa*, and, in some years, regulate the phytoplankton succession (Van Donk and Ringelberg 1983; Bruning 1991a). *A. formosa* in Lake Maarsseveen often has two distinct population maxima—the first in winter (January and February) and the second in summer (August) (Van Donk 1983). The prevalence of infection during winter commonly exceeds that during summer. In winter, water temperature is a crucial factor in determining whether fungal parasitism can regulate the dynamics of *A. formosa*. Specifically, during a long cold period ($<3^{\circ}\text{C}$), fungal parasitism on *A. formosa* is of minor importance, because fungal activity is inhibited by the low water temperature; thus, *A. formosa* becomes dominant in the phytoplankton. When only a short cold period occurs during the winter, *A. formosa* becomes heavily infected, and its population dynamics are strictly regulated by fungal parasites. As a result, other diatom species, such as *Stephanodiscus* and *Fragilaria*, form blooms (Van Donk 1983). However, it is still unknown why *A. formosa* can be an abundant species during summer even when infected by chytrids. In addition, as has been shown in laboratory experiments, the fungi that infect *A. formosa* grow well at higher temperature of 21°C (Bruning 1991b). Generation times of the fungus are even shorter at high temperature than at lower temperatures, such that the development time of the sporangia varied from 19.0 d at 2°C to 1.88 d at 21°C (Bruning 1991b). From those results, and taking the water temperature in Lake Maarsseveen during summer ($20\text{--}22^{\circ}\text{C}$) (Van Donk 1983) into account, summer seems to be more favorable for epidemics of these parasitic fungi than winter. We put forward that increased activity of zooplankton explains why fungal epidemics are reduced in summer, despite favorable conditions for the growth of the chytrids. During summer, zooplankters, like *Daphnia galeata* and *D. galeata hyalina*, are more abundant than during the winter and may influence fungal parasitism on *A. formosa* through grazing on fungal zoospores.

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To investigate the effects of zooplankton on fungal infection, we conducted two types of laboratory experiments using *D. galeata hyalina*, *A. formosa*, and the parasitic fungus, *Z. planktonicum*. All three were isolated from Lake Maarsseveen. First, fungal zoospores were incubated together with *Daphnia*, to test whether *Daphnia* could reduce the abundance of zoospores. Second, fungal zoospores and *Daphnia* were incubated together with *A. formosa*, to test whether *Daphnia* could reduce fungal infections. We hypothesized that zoospore abundance and fungal infections on *A. formosa* would be reduced in treatments where *Daphnia* was present, because zoospores would be lost through grazing.

Materials and methods

Standard cultures—*A. formosa* was isolated from Lake Maarsseveen I during 2001 (clone MS03301-1). The clone was maintained for 1 yr in nonaxenic batch cultures with modified Chu-10 medium (Stein 1973) at 40 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ with a 14:10 h light:dark cycle and a temperature of 18°C.

The chytrid fungus was isolated from Lake Maarsseveen I during 2001 (clone FMS34600). The fungus was identified as *Z. planktonicum* (Canter pers. comm.). An extensive description of the life cycle of *Z. planktonicum* can be found in Van Donk and Ringelberg (1983) and Doggett and Porter (1996). In short, the life cycle of a parasitic chytrid begins with the attachment of a motile zoospore to the surface of an algal host cell. Encystment is completed when a thickened wall is formed around the zoospore, which then enlarges and becomes a sporangium. The sporangium matures, and new zoospores are produced. The fungus was maintained with its host (*A. formosa*, clone MS03301-1) in the nonaxenic culture, according to the method of Bruning (1991a). Specifically, 150 ml 1-week-old *A. formosa* culture was infected with 10 ml of a 1-week-old *Z. planktonicum* culture. *D. galeata hyalina* was isolated from Lake Maarsseveen I in 1992. *D. galeata hyalina* was grown in filtered lake water from Lake Maarsseveen (<0.2 μm) and was fed with *Scenedesmus obliquus* cultured in WC (Woods Hole Chu-10) medium (Guillard 1975).

Experiments—Two types of experiments were performed in the laboratory in 2002. To test whether *Daphnia* can reduce the abundance of zoospores, fungal zoospores were incubated together with *Daphnia* (experiment 1). To test whether *Daphnia* can reduce fungal infections, fungal zoospores and *Daphnia* were incubated together with *Asterionella* (experiment 2).

Before starting the experiments, *D. galeata hyalina* (1.2 \pm 0.6 mm) was rinsed three times with Chu-10 medium and finally kept for 2 h in this medium to wash algae (*Scenedesmus*) away. Fungal zoospore suspensions, free of host cells, were obtained by filtration of a highly infected *A. formosa* culture over 33- μm mesh and subsequently a 5- μm filter. In the final filtrate, only fungal zoospores were found, and their swimming activity was not affected by the filtration procedure.

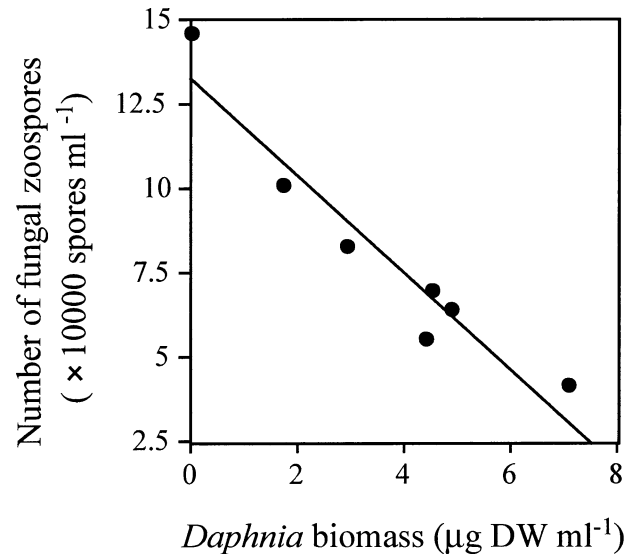


Fig. 1. Relationship between final zoospore abundance and *Daphnia* biomass after 6 h of incubation in experiment 1.

Experiment 1: Grazing experiment without host cells—At the start of the experiments, seven 100-ml flasks were inoculated with 70 ml of fungal zoospore suspension. Different numbers of *D. galeata hyalina* (0~12 individuals) were added to these flasks, to create a zooplankton biomass gradient with a >10-fold difference (Fig. 1). All flasks were incubated for 6 h under 40 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. During the 6-h incubation, any decrease in the zoospore density would only have been caused by grazing by *Daphnia*, because zoospores do not die within 6 h without host cells (Bruning 1991b), and they do not make any cysts without host cells (Doggett and Porter 1996).

Experiment 2: Grazing experiment with host cells—To initiate the experiments, 20 100-ml flasks were inoculated with 60 ml of uninfected *A. formosa* cells at a density of 20,000 cells ml^{-1} . In half of those flasks, different numbers of *D. galeata hyalina* (1~12 individuals) were added to create a zooplankton biomass gradient with a >10-fold difference (Table 1). After the addition of *D. galeata hyalina*, a 10-ml zoospore suspension (3,200 spores ml^{-1}) was added to all 10 flasks. These flasks served as *Daphnia* treatments. The other half of the flasks, those without *Daphnia*, received different concentrations of zoospore suspension (0~80% diluted zoospores with medium) and served as control treatments. The aim of the control treatments was to show that infection is dependent on the abundance of zoospores and that a reduction in zoospore abundance lowers infection rate.

All flasks were kept at an irradiance of 40 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ under a 14:10 h light-dark cycle at 18°C. Under these conditions, this fungus becomes epidemic. The flasks were shaken manually twice a day. After 1 d of incubation, all *D. galeata hyalina* were removed. Therefore, the effects of *Daphnia* on the infection processes were restricted to the stage in the life cycle during which motile fungal zoospores are searching for their host. One day is known to be enough for zoospores to find and attach to their host but not enough

Table 1. Initial abundance of *Daphnia galeata hyalina*, fungal zoospores, and *Asterionella formosa* in experiment 2.

Treatment	<i>Daphnia</i> ($\mu\text{g DW ml}^{-1}$)	Zoospores (spores ml^{-1})	<i>Asterionella</i> (cells ml^{-1})
<i>Daphnia</i> (single)			
1	0.43	457	20,000
2	1.55		
3	1.71		
4	2.87		
5	3.30		
6	3.36		
7	3.45		
8	4.11		
9	6.06		
10	6.77		
Control (duplicate)			
1	0	91	20,000
2		183	
3		274	
4		366	
5		457	

for them to mature and produce new zoospores (Bruning 1991b). Incubation of the algae and fungi was continued for 7 d, to enable quantification of the fungal infection. During the 7-d incubation, fungi may have three generations: the development time of sporangia has been shown to be 2 d (Bruning 1991b).

Analysis—At the end of the incubations, samples were fixed with glutaraldehyde (1% final concentration) and stored in a refrigerator until microscopic analysis. We applied two kinds of staining methods for counting chytrid fungi. One was for free-swimming zoospores (Kudoh 1990) and the other for fungi attached to host cells (Müller and Sengbusch 1983). To stain free-swimming zoospores, a new method was developed in which 1-ml samples were put into Utermohl counting chambers, to which $100 \mu\text{g L}^{-1}$ of Nile Red and $50 \mu\text{g L}^{-1}$ of 4',6-diamidino-2-phenylindole (DAPI) were added (both in final concentrations). The samples were then incubated for 30 min in the dark at room temperature. Nile Red stains the oil globule of the zoospores, emitting a characteristic orange fluorescence under green excitation (525 nm), and DAPI stains the nucleus emitting blue fluorescence under ultraviolet (UV) excitation (365 nm). After staining, the fungal zoospores were identified at $400\times$ magnification on the basis of their spherical shape ($3 \mu\text{m}$ in diameter), single flagellum (Canter 1967), and body containing a single oil globule and a single nucleus.

Fungi attached to algal cells (attached zoospores, sporangia, and empty sporangia) were stained with CalcoFluor White (Müller and Sengbusch 1983). Three drops of 10% KOH solution and three drops of 0.1% CalcoFluor White were added to 1-ml samples in Utermohl counting chambers, after which the samples were incubated for 10 min. CalcoFluor White binds to chitin, a cell wall component of chytrids. The number of *A. formosa* cells with and without attached fungi (attached zoospores, sporangia, and empty sporangia) and the number of fungi attached to algal cells

(attached zoospores and sporangia) were counted at $400\times$ magnification under a fluorescence microscope with UV excitation (365 nm) and also with visible light. The percentage of infected *A. formosa* cells was calculated by dividing the number of infected cells by the total number of host cells. We also calculated the mean number of fungi per host in a population, by dividing the total number of fungi attached to algal cells by the total number of host cells, to normalize the cell density among treatments. This value is referred to as the mean intensity of infection (Holfeld 2000), and it expresses how many fungi succeed in attachment to their host. To estimate the biomass of *D. galeata hyalina* in the experimental bottles, we measured the body length of each individual at $25\times$ magnification. The dry weights (DWs) of *D. galeata hyalina* were estimated according to the method of Bottrell et al. (1976).

The effects of *Daphnia* on the abundance of fungal zoospores in experiment 1 were assessed by simple regression analysis against *Daphnia* biomass. In experiment 2, the effects of *Daphnia* (*Daphnia* treatment) and the initial concentration of fungal zoospores (control treatment) on cell densities of *A. formosa*, percentages of infected cells, and the average number of fungi per host cell were assessed by simple regression analysis. Statistical tests were performed with StatView (version 5, SAS Institute).

Results

Experiment 1—The biomass of *Daphnia* in the flasks ranged from 0 to $7.09 \mu\text{g DW ml}^{-1}$ (Fig. 1). The initial abundance of zoospores was $146,000 \text{ spores ml}^{-1}$. After 6 h of incubation with *Daphnia*, the zoospore density varied between 146,000 and 41,800 spores ml^{-1} , depending on the treatment. There was a significant negative relationship between zoospore abundance (Z , spores ml^{-1}) and *Daphnia* biomass (D , $\mu\text{g DW ml}^{-1}$): $Z = -14,300D + 132,000$ ($r^2 = 0.913$, $P = 0.0008$; Fig. 1). The weight-specific clearance rate estimated from this equation was $57,200 \text{ spores } \mu\text{g DW}^{-1} \text{ d}^{-1}$. From the initial abundance of zoospores, $146,000 \text{ cells ml}^{-1}$, the weight-specific clearance rate was also estimated as $0.39 \text{ ml } \mu\text{g DW}^{-1} \text{ d}^{-1}$. This value is close to the median value of weight-specific clearance rates for cladocerans, $0.35 \text{ ml } \mu\text{g DW}^{-1} \text{ d}^{-1}$, established by Peters (1984).

Experiment 2—The initial abundance of zoospores in the experimental flasks was $457 \text{ spores ml}^{-1}$ (10 ml of 3,200 spores ml^{-1} with 60 ml of host cells). The biomass of *Daphnia* in flasks ranged from 0.43 to $6.77 \mu\text{g DW ml}^{-1}$ (Table 1, Fig. 2). After 7 d of incubation, cell densities of *A. formosa* reached $40,000 \text{ cells ml}^{-1}$, having started from 20,000 cells ml^{-1} in both *Daphnia* plus zoospores and the control treatments (zoospores only). No significant relationships were found between *Daphnia* biomass and cell densities of *A. formosa* in the *Daphnia* treatment ($P = 0.083$) and between initial zoospore abundance and cell densities of *A. formosa* in the control treatment ($P = 0.569$). Statistical analysis (*t*-test) showed that there was no significant difference in algal cell densities between control and *Daphnia* treatments ($P = 0.251$). These results mean that *D. galeata hyalina* did not graze on *A. formosa* clone MS03301-1.

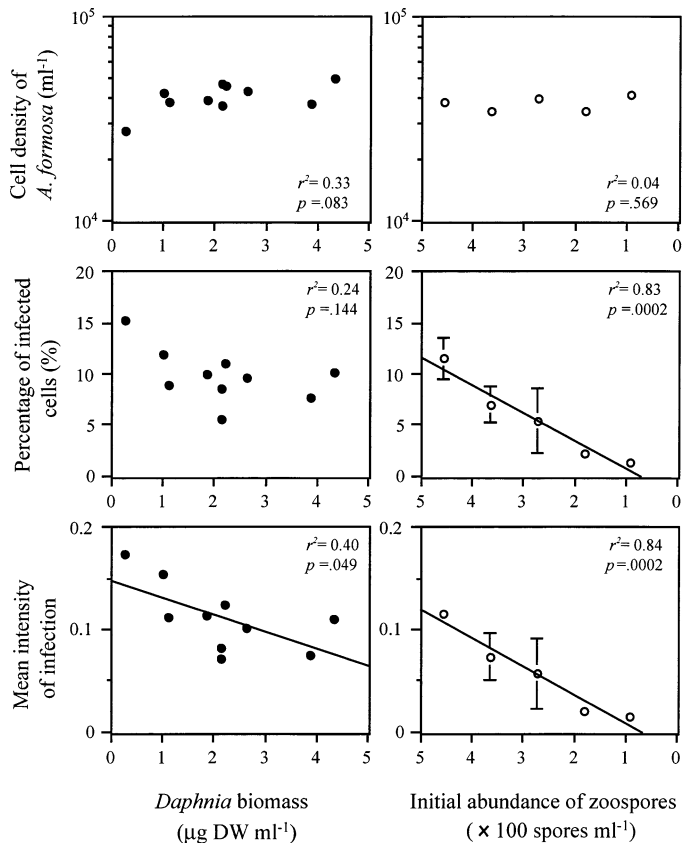


Fig. 2. Cell densities of *A. formosa*, the percentages of infected *A. formosa* cells, and the mean intensity of infection against *Daphnia* biomass in *Daphnia* treatments (filled circles) or against initial zoospore abundance in control treatments (open circles) after 7 d of incubation. Regression lines are shown if the relationships are significant at $P < 0.05$. Vertical lines are standard errors. Note that the scale of the zoospore abundance is reversed.

The percentages of infected *A. formosa* cells ranged from 1% to 13% in the control treatments and from 6% to 15% in the *Daphnia* treatment. In the control treatment, there was a significant positive relationship between percentages of infected *A. formosa* cells and the initial zoospore abundance ($P = 0.0002$, Fig. 2). In the *Daphnia* treatment, the percentages of infected *A. formosa* cells slightly decreased with *Daphnia* biomass but did not show a significant relationship ($P = 0.144$, Fig. 2). The mean intensity of infection showed significant relationships with initial zoospore density (positive) in the control treatment and with *Daphnia* biomass (negative) in the *Daphnia* treatment (Fig. 2). This means that *D. galeata hyalina* reduced the abundance of fungi, which is apparent in the mean intensity of infection but not in the percentages of infected cells.

Discussion

Our experiments showed that *D. galeata hyalina* significantly reduced the abundance of fungal zoospores (Fig. 1). *D. galeata hyalina* also reduced the mean intensity of infection in experiment 2 (Fig. 2). The reduction of the abundance of fungi by *Daphnia* can be explained by grazing on free-

swimming fungal zoospores. Free-swimming zoospores would be digested in the gut of *Daphnia*, because they do not have thick cell walls or sheaths (Beakes et al. 1988), which could protect them from digestion by *Daphnia* (Porter 1976; Van Donk et al. 1977). The percentage of *A. formosa* cells infected by fungi was slightly reduced by *D. galeata hyalina*, but not significantly (Fig. 2). After 7 d of incubation, fungal zoospores were often found to attack those cells that already had zoospores or sporangia attached to them. Such multiple infections may reduce the difference between treatments in the percentages of infected cells but increase the difference in the mean intensity of infection (Fig. 2). The lack of a significant relationship between *Daphnia* biomass and the percentages of infected cells does not necessarily mean that *D. galeata hyalina* would not reduce the percentages of infected cells in nature. In this experiment, we examined the effects of *D. galeata hyalina* on the stage during which free-swimming fungal zoospores are searching their host cells. Thus, *D. galeata hyalina* was allowed to graze on zoospores for a 1-d incubation only, whereas *A. formosa* and *Z. planktonicum* were incubated for 7 d, until fungal infection could be quantified. The long-term effects of *D. galeata hyalina* on fungal infection would be dependent on the relative rates of zoospore production and *Daphnia* grazing.

Of interest, positive effects of *Daphnia* on fungal infection were also suggested by some of the results in our experiments. On average, more fungi were attached to algal cells in flasks where *Daphnia* was present (*Daphnia* treatment) than in flasks where no *Daphnia* was present (control treatment, Fig. 2). In fact, the intercept of the number of fungi per cell in *Daphnia* treatments was significantly greater than the intercept in control treatments (one-tailed t -test, $P = 0.031$). Microscale turbulence is known to increase the encounter rate between zoospores and host cells (Kühn and Hofmann 1999). *Daphnia* could create such microscale turbulence through their feeding or swimming activities. This possibility indicates that negative effects on infection caused by grazing on zoospores may partly be compensated by positive effects via enhancement of the encounter rate between zoospores and host cells. However, the results cannot be related directly to in situ conditions. Positive effects through microscale turbulence may have little influence on the fungal parasitism in natural lakes, because there is much more turbulence than that created by *Daphnia*, such as wind-induced mixing and convection. Thus, under natural conditions, *Daphnia* may affect fungal infections primarily in a negative way by grazing on the zoospores.

In several lakes, *A. formosa* during summer is less severely infected by fungi than during winter (Canter and Lund 1948; Van Donk 1983; Kudoh and Takahashi 1990). Between winter and summer, environmental factors such as water temperature, light, nutrient concentrations, and zooplankton abundance are obviously different. In terms of water temperature and phosphorus concentration, it would be hard to explain the differences in fungal infection between winter and summer, because the moderately high temperature and relatively low phosphorus concentrations during summer would facilitate the development of fungal epidemics (Van Donk and Ringelberg 1983; Bruning 1991b,c). Kudoh and

Takahashi (1990) explained the low prevalence of fungal infection in the summer by low population densities of algal host cells, as has also been suggested by other authors (Reynolds 1973; Van Donk 1989). However, this explanation is not always applicable to the situation in Lake Maarsseveen, because, in some years, the host population density during summer was as high as that during winter, when host phytoplankton cells were heavily infected by fungi (Van Donk 1983). In Lake Maarsseveen, zooplankters, including *D. galeata* and *D. galeata hyalina*, are more abundant during summer than during winter (Van Donk 1983). The intensity of fungal parasitism may differ between seasons because of differences in grazing pressure on the free-swimming zoospores.

Our results are also relevant to nutrient cycling, because the results suggest that chytrids may be an additional food source for *Daphnia*. Phytoplankton species that are susceptible to fungal infection seem to be mainly large species, like *Asterionella* in the present study, which are less grazed by large zooplankton (Sommer 1987). Large phytoplankton species are believed to be lost by sinking from the euphotic zone instead of being grazed (Malone 1980; Legendre and Le Fèvre 1991; Kiørboe 1993). When those species are infected by fungi, however, nutrients within those cells are consumed by fungi, some of which in turn are grazed by *Daphnia*. Thus, we hypothesize that the fungus may act to transport nutrients from ungrazed large phytoplankton to *Daphnia* and may enhance the production and growth of *Daphnia*. Further experiments are planned to test this hypothesis.

In conclusion, our results suggest that *Daphnia* affects fungal parasitism negatively through reducing the abundance of fungal zoospores. *Daphnia* also had a small positive effect on fungal infection in the experiment, probably through increasing the encounter rates between fungi and host cells. The relative contribution of positive and negative effects should be quantified in the field to be able to understand the overall effects of *Daphnia* on fungal parasitism and seasonal succession of their host species in lakes.

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