Adsorption and degradation effects of three PAHs by Chlamydomonas reinhardtii

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Abstract

In this study, adsorption and degradation experiments are carried out on three polycyclic aromatic hydrocarbons (PAHs) contaminants (naphthalene, anthracene, benzo[a]pyrene) that are commonly present in the natural environment by using Chlamydomonas reinhardtii, a model laboratory organism. In laboratory culture conditions, the Chlamydomonas reinhardtii was acclimated with 1 mg/L, 10 mg/L and 20 mg/L PAHs, and then cultured with three different PAHs in liquid medium (1 mg/L each) for 14 d. Ten d later, the levels of three PAHs were determined by HPLC, thereby deriving the PAHs adsorption and degradation rates of Chlamydomonas reinhardtii. Samples were collected every 24 h, and the changes in chlorophylls a, b, and total chlorophyll were measured with UV/Vis spectrophotometer, while the algal density was detected by flow cytometer. Experiment revealed that the chlorophyll contents of Chlamydomonas reinhardtii were lower for various PAHs groups than the control group, of which benzo[a]pyrene exhibited less effect on chlorophylls, while naphthalene greater effect. Regarding the growth of Chlamydomonas reinhardtii, all three PAHs produced certain inhibitory effects on the algal growth, among which benzo[a]pyrene was less effective, while naphthalene and anthracene were analogous in effect. In terms of the PAHs adsorption and degradation, Chlamydomonas reinhardtii exerted certain removal effects on all three PAHs, with degradation rates of 85.5%, 89.5% and 16.9% (naphthalene, anthracene, benzo[a]pyrene), respectively; and absorption or adsorption rates of 13.1%, 9.8% and 82.0%, respectively. This suggests that Chlamydomonas reinhardtii removes low-ring PAHs (naphthalene and anthracene) mainly through degradation, while eliminating high ring PAHs (benzo[a]pyrene) primarily by adsorption. The results of this experiment have a certain role in guiding the environmental removal of PAHs with Chlamydomonas reinhardtii.

Keywords

Chlamydomonas reinhardtii, PAHs, adsorption, degradation .

1. Introduction

PAHs are a class of teratogenic, mutagenic, carcinogenic and toxic organic substances that are present extensively and stably in the eco-environment (Bao et al., 2003). In general, they comprise two or more benzene rings that are connected in linear, curvy or cluster arrangements (Zhou, 1990). The production and sources of PAHs are classified into two major categories: Natural production and anthropogenic production. Natural factors mainly include the extensive combustion of organic matters following forest and pasture fires, the oil leakage in the lower formations, the volcanic eruptions and the tree exudates. Anthropogenic sources of PAHs are mainly the substantial combustion of petroleum and other fuels, the tar resulting from industrial coal combustion, the massive burning of wood, the pollution of white garbage, the waste lubricating oil and filter (C.P. Kaushik et al., 2006), the incineration of urban solid waste, the oil spills and emissions, as well as the atmospheric pollution, sewage irrigation, sludge farmland, etc. in agricultural production (KUMAR et al., 2014).

The topmost thing in the repair of PAHs-contaminated ecological environment is to reduce the environmental pollution caused by PAHs in the ecosystem. Incineration, alkali-catalyzed

dechlorination, UV oxidation, fixation and solvent extraction, as several common physical and chemical treatments for eliminating PAHs contamination, have gained wide practical application (Norris et al., 1993; Gan et al., 2009). However, these traditional techniques are not only exorbitant, but also incapable of removing PAHs contaminants completely, which merely transfer them from one environment or form to another. To tackle this urgent problem, researchers have designed an efficient and green cleaning technology, i.e. bioremediation, which has been increasingly perfected through research. It is also recognized as the most effective way to eliminating PAHs pollution at present. Utilizing the detoxification capacity of organisms, the bioremediation technology can transform harmful organic wastes, including PAHs isomers, into harmless substances such as carbon dioxide and water (Cerniglia & Heitkamp, 1989; Mueller et al., 1996; Bamforth & Singleton, 2005; Johnsen et al., 2005). Compared to physical and chemical methods, it has a huge cost advantage. Hence, bioremediation has become the most effective measure for eliminating various organic contaminations, including PAHs (Young & Cerniglia, 1996; Juhasz & Naidu, 2000; Kastner, 2000; Lovley, 2001; Andreoni & Gianfreda, 2007; Jorgensen, 2007; Megharaj et al., 2011; Abdel-Shafy & Mansour, 2016).

The majority of previous works on the PAHs degradation have focused on the research of bacteria and fungi. Most bacteria are capable of degrading low-ring PAHs (2-3 rings) like naphthalene and phenanthrene, while some bacteria can degrade both low- and high-ring PAHs (Juhasz et al., 1997; Kim et al., 2003). Ligninolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) can be secreted by some fungi (e.g. white-rot fungus), which can thus degrade multiple organic contaminants including PAHs (WU et al., 2013).

According to a few studies, some algae also have the capacity to remove PAHs through adsorption, absorption, enrichment and degradation processes, and the degradation ability varies among microalgae for different PAHs and their transformation products, which is often species-specific. For instance, *Skeletonema costatum* and *Nitzschia* can adsorb phenanthrene and fluoranthene rapidly (Hong et al., 2007). *Selenastrum capricornutum* and *Chlorella vulgaris* remove 48%-78% of fluoranthene and pyrene in culture medium (Lei et al., 2007). Brown, red, green algae and some charophyte species produce certain bioaccumulation, migration and transformation effects on benzo[a]pyrene, and such abilities differ somewhat among microalgal species. Clearly, algae play an important role in the environmental degradation of PAHs, which need further exploration (Kirso et al., 1998).

As a model organism commonly used in laboratories, *Chlamydomonas reinhardtii* is characterized by simple culture conditions, short growth cycle, high photosynthetic efficiency, and more importantly, clear genetic background and structure (Zhang et al., 2011). These traits facilitate further research of PAHs' toxicity mechanism on algae by combined molecular biological approach. Investigating the PAHs bioaccumulation and degradation abilities of *Chlamydomonas reinhardtii* can deepen the theoretical foundation on the migration, transformation and biodegradation of PAHs in the water environment, thus providing reference data for ecological risk assessment of the PAHs in aquatic ecosystems.

2. Materials and Methods

2.1 Materials and Reagents

The materials and reagents used in this study are shown in the tables below(Tab. 1, Tab. 2):

Tab. 1 Reagents used in this study

Reagent name	Grade/specification	Manufacturer
Naphthalene	HPLC	Macklin Biochemical Co., Ltd., Shanghai
Anthracene	HPLC	Aladdin Biochemical Technology Co., Ltd., Shanghai
Benzo[a]pyrene	Standard	Sigma-Aldrich Corp., USA

Acetonitrile	HPLC	ANPEL Laboratory Technologies (Shanghai) Inc.
Acetone	HPLC	Aladdin Biochemical Technology Co., Ltd., Shanghai
Dichlormethane	HPLC	ANPEL Laboratory Technologies (Shanghai) Inc.
Anhydrous ethanol	Analytical	Yongda Chemical Reagents Co., Ltd., Tianjin
Formaldehyde	Standard 50% solution	Xilong Scientific Co., Ltd.
Dimethyl sulfoxide	HPLC	Aladdin Biochemical Technology Co., Ltd., Shanghai
Sep Pak C18 cartridge		Waters Corporation, USA
BG11 medium (powder)		HopeBio Technology Co., Ltd., Qingdao
Organic phase nylon syringe filter		ANPEL Laboratory Technologies (Shanghai) Inc.
Nitrogen		Yingxin Gases Co., Ltd., Guangzhou
Ethyl acetate	HPLC	Aladdin Biochemical Technology Co., Ltd., Shanghai

Tab. 2 Instruments used in this study

Instrument	Manufacturer
Artificial climate incubator	Xutemp Temptech Co., Ltd., Hangzhou
High-speed refrigerated centrifuge	Thermo Fisher Scientific, USA
Microscope	Olympus Corp., Japan
HSC-12A nitrogen evaporator	Hengao Technology Development Co., Ltd., Tianjin
Shaking incubator	New Brunswick Scientific, Canada
Electronic balance	Sartorius, Germany
UV-Vis spectrophotometer	Shimadzu Corporation, Japan
Ultraclean bench	Sujie Purification Equipment Co., Suzhou
Mili-Q integral water purification system	Milipore Corp., USA
101A-2E electric blast drying oven	Anting Scientific Instrument Co., Ltd., Shanghai
Shimadzu LC-20AD HPLC system	Welch Materials, Shanghai
PAHs columns	Welch Materials, Shanghai
BDC6 flow cytometer	BD Biosciences, USA
Vortexer	Miu Instruments Co., Ltd., Hangzhou
Ultrasonic cleaner	BRANSONIC, USA
Cell sonifier	Autoscience Instrument Co., Ltd., Tianjin

2.2 Alga cultivation

Chlamydomonas reinhardtii used in this study was from the Research Center of Hydrobiology, Jinan University (Guangzhou, Guangdong, China). The alga was cultured at 20 °C in 100 ml of sterilized BG-11 medium under an illumination intensity of 100 μmp L·m-2·S-1, and a light-dark cycle of 12 h:12 h. The algal growth curve was plotted, and the log phase was determined as the experiment needed to use log phase alga. During the alga cultivation and experimentation, the culture flask was shaken on a shaker every day to allow uniform mixture between the microalgae and the medium.

The BG-11 medium was prepared with BG-11 powder (1.70 g of the BG-11 powder was added per 1,000 ml of RO water, heated, dispensed and autoclaved at 121 °C for 20 min for later use). Tab. 3 below lists the components of the prepared BG-11 medium:

Tab. 3 BG-11 medium formula

No.	Molecular formula (or name)	Concentration
1	K2PO4•3H2O	4.0g/dl
2	MgSO4•7H2O	7.5g/dl
3	CaCl2•2H2O	3.6g/dl
4	Citric acid	0.6g/dl
5	Ferric ammonium citrate	0.6g/dl
6	EDTA•2Na	0.1g/dl
7	Na2CO3	2.0g/dl
8	A5	
	H3BO4	2.86g/l
	MnCl2•4H2O	1.81g/l
	ZnSO4•7H2O	0.222g/l
	Na2MoO4•2H2O	0.39g/l
	CuSO4•5H2O	0.08g/l
	Co（NO3）2•6H2O	0.49g/l

2.3 Bioassay.

In a 150 ml sterilized conical flask, 100 mL of *Chlamydomonas reinhardtii*-containing medium was prepared, in which the initial concentration of *Chlamydomonas reinhardtii* was approximately 8 x 10^4 cells/ml. Three PAHs were added into the BG-11 medium at a 1 mg/L concentration and co-cultured with *Chlamydomonas reinhardtii*. Other conditions were the same as those for algal cultivation.

During co-cultivation of PAHs and *Chlamydomonas reinhardtii*, 1 mL of sample was collected every 24 h. After sample treatment, the absorbance of photosynthetic pigments in algal solution was measured at 645 nm and 663 nm with an UV-Vis spectrophotometer, and recorded. The variations of chlorophyll a, chlorophyll b and total chlorophyll in the samples were calculated according to the following formula (Dai et al., 2004):

$$\text{Chlorophyll a (mg/L)} = 12.7 \cdot OD_{663} - 2.69 \cdot OD_{645} \quad (1)$$

$$\text{Chlorophyll b (mg/L)} = 22.9 \cdot OD_{645} - 4.68 \cdot OD_{663} \quad (2)$$

$$\text{Total chlorophyll (mg/L)} = \text{Chlorophyll a} + \text{chlorophyll b} = b = 20.2 \cdot OD_{645} + 8.02 \cdot OD_{663} \quad (3)$$

Collecting additional 1 mL of sample was necessary every 24 h, which was fixed by adding 40 μl of 2% formaldehyde solution, shaken uniformly and counted by flow cytometry within 7 d. When measuring the cell density on a flow cytometer, high-speed sample loading was chosen to record the data, which was precisely the microalgal density.

On the 10th day of co-cultivation, 2 mL of sample was collected. After treatment, the residual amounts of PAHs in the algal solution were measured by HPLC, as well as the contents of PAHs adsorbed and absorbed by the microalgal cells, thereby calculating the PAHs degradation and adsorption rates by the microalga.

3. Sample preparation

3.1 Extracting chlorophylls from algal solution

Commencing from the 1st day of the experiment (referred to as day 0), 1 ml of algal solution was collected every 24 h, centrifuged for 15 min at 5000 r/min, discarded of the supernatant, and then added with 1 ml of leaching solution (acetone: ethanol = 1:1), shaken well and dark treated in a 4 °C refrigerator for 24 h. Afterwards, the solution was taken out and centrifuged for 15 min at 5000 r/min.

The supernatant was collected for absorbance measurement at 645 nm and 663 nm against the blank leaching solution control.

3.2 Extracting the residual PAHs in medium and the PAHs adsorbed on and absorbed by cells

Commencing from the 1st day of the experiment (referred to as day 0), 1 ml of algal solution was collected every 24 h and centrifuged for 15 min at 5000 r/min. Then, the supernatant was passed through a 0.22 μm organic phase syringe filter, and the resulting filtrate was placed in a sterilized 2 ml UP tube to serve as the sample for testing the amount of residual PAHs in algal solution.

Commencing from the 1st day of the experiment (referred to as day 0), 1 ml of algal solution was collected every 24 h and centrifuged for 15 min at 5000 r/min. The supernatant was discarded, and the precipitate was freeze-thawed in a -20 °C refrigerator for 5 h four to five times until cell wall rupture was observed microscopically. Afterwards, the resultant was dissolved in 1 ml of EA, sealed and ultrasonicated in an ultrasonic cleaner for 1 h until disruption of most cells under microscopic examination, followed by centrifugation at 5000 r/min for 15 min. The supernatant was taken and passed through a 0.22 μm organic phase syringe filter, and the resulting filtrate was placed in a sterilized 2 ml UP tube to serve as the sample for testing the amount of PAHs adsorbed on and absorbed by algal cells.

3.3 Solid phase extraction of PAHs

Prior to sample loading, the C_{18} solid phase extraction column was activated sequentially with 10 ml of dichloromethane, 10 ml of methanol and 10 ml of RO water while making sure that the liquid level was not lower than the height of filler. Meanwhile, the column bed should not be drained dry during activation and conditioning of the extraction column, in order to prevent crack formation in the packing layer from causing reduced recovery. Sample was loaded at a flow rate of 10 ml/min. After loading, the C_{18} column was blown with a nitrogen evaporator for 60 min to dryness, and then eluted at 1 ml/min with 10 ml of dichloromethane twice each in a centrifuge tube. Then, the eluates were collected in a centrifuge tube and blown to dryness with nitrogen at 40 °C for approximately 60 min. At this point, PAHs powder appeared at the bottom of the centrifuge tube, which was reconstituted by adding 1 ml of acetonitrile, sealed and ultrasonicated in an ultrasonic cleaner for 60 min to facilitate dissolution. Subsequently, the sample was taken with a syringe, passed through a 0.22 μm organic phase syringe filter, and the resulting filtrate was placed in a brown vial for later testing. The recovery with the present extraction method is higher than 90%, indicating validity of the method.

4. HPLC analysis

Since PAHs are only slightly soluble in water and ordinary organic solvents, DMSO was used as the experimental solvent. The Nap, Ant and Bap standards were dissolved in DMSO to prepare a mother solution having a concentration of 1 g/l. On the basis of the mother solution, 1 mg/l, 0.5 mg/l, 0.1 mg/l, 50 μg/l, 10 μg/l single and mixed standard solutions were prepared separately and stored in the dark at 4 °C until use.

Among the 16 priority PAHs controlled by the US EPA, 15 substances can produce rather strong fluorescence except for acenaphthene, which does not produce fluorescence. Accordingly, UV detector is used for acenaphthene detection, while fluorescence detector is used for detecting the remaining 15 substances, in order to attain the best sensitivity. The three PAHs studied in this experiment were detected with a fluorescence detector.

HPLC conditions for PAHs were as follows: Ultimate PAHs column (4.6 mm × 250 mm, inner diameter 5.0 μm); extracted samples were made up to volume with acetonitrile; mobile phase acetonitrile: water (90:10); flow rate 1.0 ml/min; column temperature 35 °C; injection volume 10 μl; excitation wavelength 270 nm; and emission wavelength 324 nm (after scanning the optimal excitation and emission wavelengths of each PAHs component, we found that various components show sharp, symmetrical peak shapes, good separation effect and fine repeatability at an excitation wavelength of 270 nm and an emission wavelength of 324 nm, so these two wavelengths were selected to set the wavelength program).

Determination was performed using an online HPLC system. Five sets of mixed PAHs solutions as shown above were used for HPLC analysis, and the standard curves of three PAHs were plotted based on the correlation between peak area and concentration. The curve equations for the three PAHs were derived separately as follows:

$$\text{Nap, } y=9\times10^{-5}x+0.2052,\ R^2=0.9999; \quad (4)$$

$$\text{Ant, } y=1\times10^{-5}x+0.2031,\ R^2=0.9999; \quad (5)$$

$$\text{Bap, } y=8\times10^{-6}x+0.3182,\ R^2=0.9999. \quad (6)$$

The contents of PAHs in various samples can be determined using the standard curve equations.

5. Results and Discussion

5.1 Effects of various PAHs on the chlorophyll contents in Chlamydomonas reinhardtii

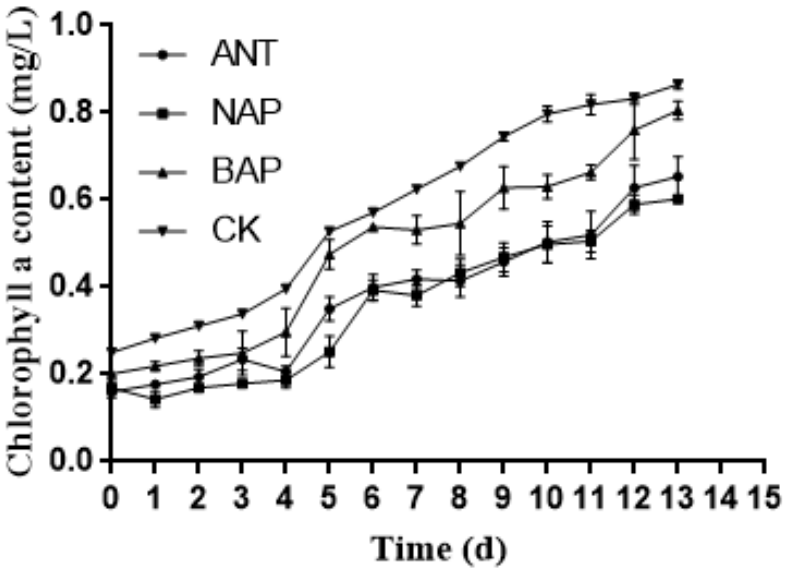

Fig. 1 Effects of different PAHs on the content of chlorophyll a in Chlamydomonas reinhardtii

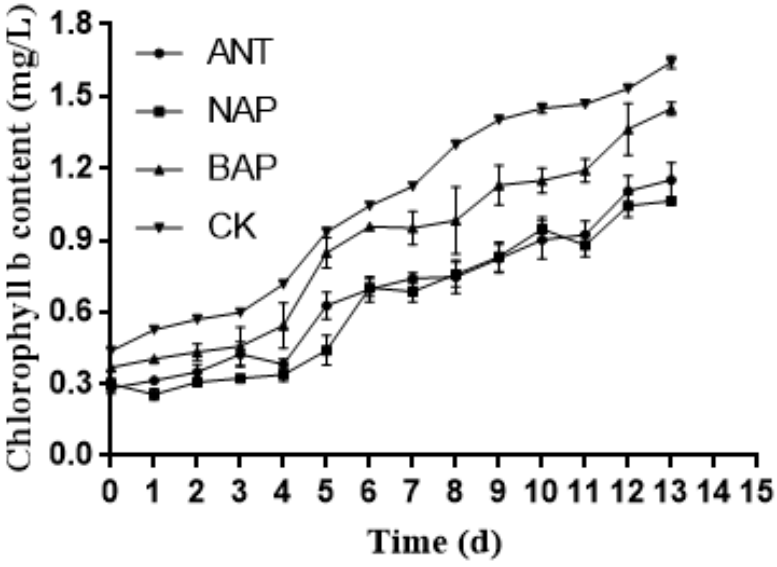

Fig. 2 Effects of different PAHs on the content of chlorophyll b in Chlamydomonas reinhardtii

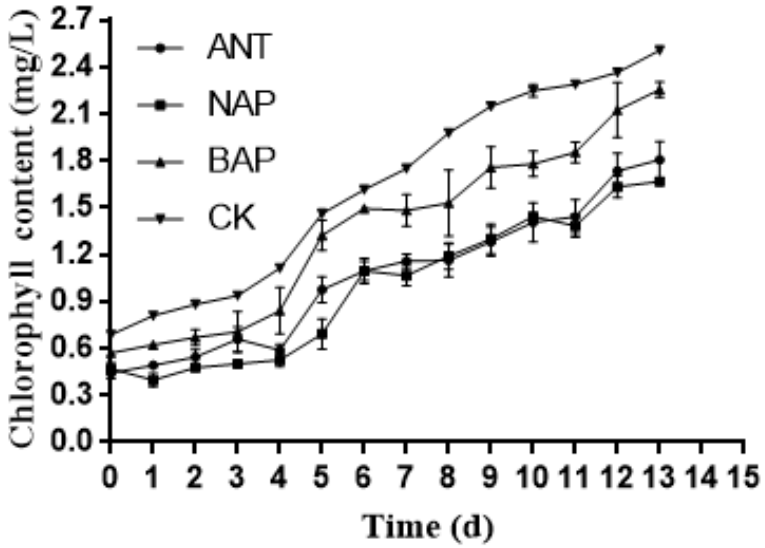

Fig. 3 Effects of different PAHs on the content of chlorophyll in Chlamydomonas reinhardtii

According to the above three curves(Fig. 1, Fig. 2, Fig. 3), the contents of chlorophylls a, b and total chlorophyll contents of Chlamydomonas reinhardtii in the PAHs-containing media are all less than the control group. Moreover, the Bap addition group exhibits minimal effect on the chlorophyll contents, while the Nap and Ant addition groups present rather small effects on the chlorophyll contents. It is thus clear that all the three PAHs may affect the chlorophyll biosynthesis to varying extents, thereby influencing the photosynthesis and growth of Chlamydomonas reinhardtii to varying degrees. To determine whether the PAHs' effects on the chlorophyll contents of Chlamydomonas reinhardtii are merely affecting chlorophyll contents or affecting the chlorophyll contents by suppressing the algal growth, we examined the effects of various 1 mg/L PAHs on the Chlamydomonas reinhardtii growth, as shown in Fig. 4.

5.2 Effects of various PAHs on the growth of Chlamydomonas reinhardtii

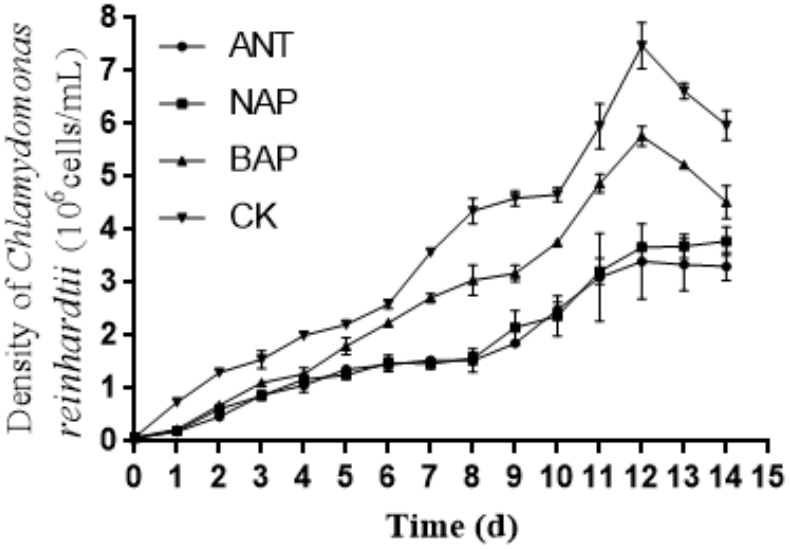

Fig. 4 Effects of different types of PAHs on the growth of Chlamydomonas reinhardtii

From the Fig. 4 , it can be seen that the effects of different PAHs on the growth of *Chlamydomonas reinhardtii* are almost consistent with their effects on the chlorophyll contents in the alga. In other words, various PAHs exert influences on *Chlamydomonas reinhardtii* by affecting its chlorophyll contents, which in turn affects the accumulation of organic matters during its photosynthesis. The decline in the productivity of Chlamydomonas *reinhardtii* may lead to its density reduction. That is, although *Chlamydomonas reinhardtii* acclimated with various PAHs becomes somewhat more

tolerant to PAHs, the presence of PAHs still exerts certain influences on it. The slight difference is that although the density of *Chlamydomonas reinhardtii* decreased to varying degrees on the 12th d of co-cultivation with PAHs, the chlorophyll contents did not decline significantly on that day. Thus, the following two possibilities are speculated: Firstly is under PAHs stress, the microalga may offset the weakening overall photosynthesis production resulting from density decline by increasing the chlorophyll contents when the algal density began to decrease on the 12th d. Secondly is after the death of microalga, a part of chlorophylls may not degraded immediately. That is, the death of algal cells and the degradation of chlorophylls are unsynchronized. Surely, these assumptions need to be verified in subsequent experiments.

As a common laboratory model organism, how *Chlamydomonas reinhardtii* is influenced by the presence of PAHs can reflect the responses of other microalgae to PAHs. Thus, the results of the above curves can explain that PAHs affect the productivity of ecosystem by exerting certain influences on the metabolic activities of unicellular microalgae.

5.3 Degradation and adsorption of various PAHs by Chlamydomonas reinhardtii

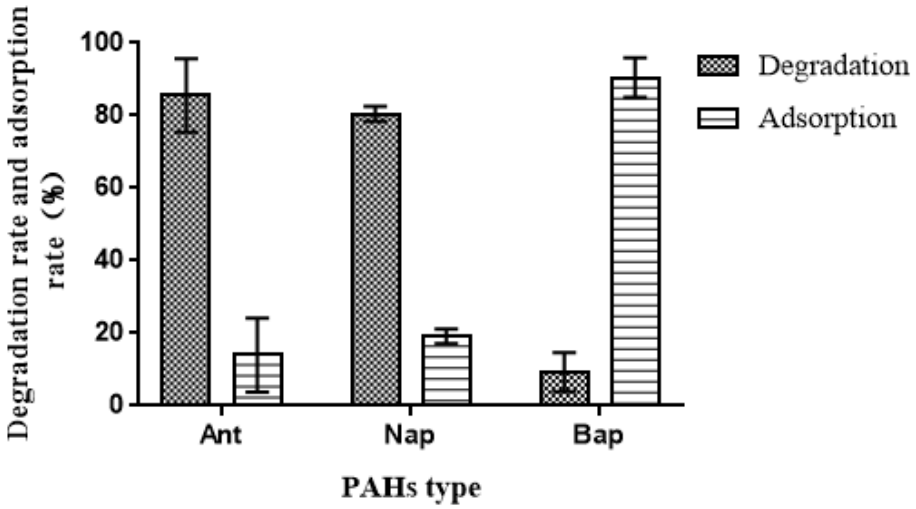

Fig 5 Degradation and adsorption of different PAHs by Chlamydomonas reinhardtii

From the above Fig. 5 , it can be seen that Chlamydomonas reinhardtii has significant, preferable effects on removing all the three PAHs types. For Ant and Nap, Chlamydomonas reinhardtii removes them mainly by degradation. As for Bap, elimination is achieved mainly through absorption and adsorption by the algal cells. There are several probable reasons for such effect disparity: First of all, Nap and Ant have two and three benzene rings, respectively, small relative molecular masses and simple molecular structures. The low-ring PAHs degradation process is rather simple for both bacteria and fungi. Thus presumably, *Chlamydomonas reinhardtii* has similar pathways for degrading PAHs as bacteria and fungi. Of course, further identification of the PAHs degradation products is required to prove this assumption. Secondly, due to the selective permeability of cell membranes and the selective expression of intracellular genes, different microalgae differ in the absorption amount of the same substances and in the intracellular gene expression of enzymes that degrade the same PAHs, while the same microalga species differ in the absorption amount of different PAHs and in the intracellular gene expression of enzymes that degrade different PAHs. Surely, to prove this assumption, expressions of the cell membrane proteins and the relevant intracellular genes need to be analyzed at the molecular level. This will also be our later stage task. Thirdly, based on a combination of Fig. 3 and Fig. 4, we found that Bap exerts weaker effects on the density and chlorophyll contents of Chlamydomonas reinhardtii than Ant or Nap, which is somewhat associated with its predominant absorption and adsorption by the alga rather than degradation. It can thus be inferred that *Chlamydomonas reinhardtii* may sacrifice part of matters or energy to compensate for the PAHs

degradation process. To prove this point, further molecular analysis is needed to determine the effects of different PAHs on the relevant gene expressions of *Chlamydomonas reinhardtii*.

6. Conclusion

By researching the degradation and adsorption of three PAHs by *Chlamydomonas reinhardtii*, we find that both the chlorophyll contents and algal density of *Chlamydomonas reinhardtii* somewhat decrease under the stress of PAHs, of which the high-ring PAHs exhibits weaker influences than the low-loop PAHs. Chlamydomonas *reinhardtii* is capable of removing the three typical PAHs preferably, which eliminates low-loop PAHs primarily by degradation, and eliminates high-ring PAHs mainly through adsorption and absorption. This paper preliminarily speculates on the way whereby *Chlamydomonas reinhardtii* eliminates PAHs pollution in the course of degrading PAHs: Is it via transformation of PAHs into other substances, or via absorption and adsorption by cells? This has a certain correlation with the ring number of PAHs. Further molecular biology research is needed, in order to clarify the overall mechanism.

Acknowledgments

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