# Removal and biodegradation of polycyclic aromatic hydrocarbons by immobilized microalgal beads

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## Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic, carcinogenic and/or mutagenic organic compounds, which must be removed from contaminated environments such as wastewater. The present study aims to explore the potential of a fresh water green microalga, *Selenastrum capricornutum*, immobilized in alginate beads to remove mixed PAHs and determine the optimal density for an effective removal. Results demonstrated that the immobilized microalgal beads were effective in removing and biodegrading mixed PAHs at their dissolved concentrations, namely phenanthrene (PHE, 1 mg $l^{-1}$), fluoranthene (FLA, 0.25 mg $l^{-1}$) and pyrene (PYR, 0.1 mg $l^{-1}$). These abilities were significantly affected by cell density, ranging from $2.5x10^5$ to $5x10^6$ cells $bead^{-1}$. At the optimal cell density of $2.5x10^6$ cells $bead^{-1}$, 92% PHE and all FLA were removed in 168 hours, and it only took 24 hours to completely remove PYR. In 168-hour treatment, 73% PHE was biodegraded while FLA and PYR were completely degraded. The removal and biodegradation of PAHs were further enhanced by increasing the number of beads used and the optimal bead density was 20 beads $ml^{-1}$ wastewater. These findings suggested that PAHs were quickly adsorbed onto cells and the alginate matrix, absorbed within cells and further degraded by live cells immobilized in alginate beads.

*Keywords: algae, alginate, persistent organic pollutant, PAHs, Selenastrum, wastewater treatment.*

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# 1 Introduction

Polycyclic aromatic hydrocarbons (PAHs), a large class of persistent, carcinogenic and/or mutagenic organic compounds consisting of two or more fused aromatic rings in a linear, angular or cluster structure, are generated from both natural and anthropogenic sources. With increasing human activities, PAHs have been widely contaminating our environment and have attracted increasing concern. Bioremediation by bacteria and fungi has been suggested as an alternative means to remove PAHs [1]. Relatively little work has been done on PAH removal by microalgae, although this microbial group has been reported to be effective in removing toxic metals and other organic pollutants, such as tributyltin [2-4]. One of the major problems of employing microorganisms, including microalgae, to remove pollutants from wastewater is the difficulty in separating them after treatment. Immobilization techniques by embedding microbial cells into a matrix to make big particles could solve the harvesting problem. Matrices, such as alginate, *k*-carrageenan, agarose, chitosan, polyacrylamide, etc., have been suggested [5-7]. Among these, alginate has been very popular and is a commonly used matrix because of its reliability, stability and simplicity of preparation, and relatively non-toxic to microorganisms. Growth of *C. vulgaris* immobilized in alginate was not affected and its efficiency to remove nitrogen and phosphorus was even higher than the free cell counterpart [6]. Travieso *et al*. [7] reported that the original shape and structure of alginate pellets of *C. vulgaris*, *C. kessleri* and *Scenedesmus quadricauda* were still maintained, but the *k*-carrageenan pellets of *S. quadricauda* disintegrated with cell leaking during the ten-day nutrient removal experiment. The alginate immobilized *C. vulgaris* could remove and regenerate nickel for at least ten cycles without losing any removal capacity, indicating the superior stability of alginate [8].

The performance of immobilized microalgae in removing contaminants is affected by the cell density embedded within the matrix, and the number of beads employed for treatment, that is, bead density. Pradhan and Rai [9] reported that the removal percentages of copper(II) by a cyanobacterium, *Microcystis* sp., immobilized in alginate increased when biomass increased from 0.016 to 0.064 g but further increase of the biomass did not enhance the removal percentages. Similar to free cells, a higher biomass would provide more cell surface, volume and enzymes for adsorption, absorption and degradation of the contaminant. However, competition of resources, such as nutrients and light, self-shading and limitation in the mass transfer of contaminants, will occur when the cell density is too high [10]. Tam and Wong [11] found that the optimal bead density for the removal of ammonium and phosphate from wastewater by alginate immobilized *C. vulgaris* was 12 beads $ml^{-1}$. Although alginate immobilized microalgae have been optimized for the removal of nutrients and heavy metals from wastewater [5-8, 11, 12], knowledge on treating PAH-contaminated wastewater using immobilized microalgae is very limited. The present study aims to determine the optimal initial cell density inside each bead, as well as the bead density, on the removal and degradation of a mixture of phenanthrene (PHE), fluoranthene

(FLA) and pyrene (PYR) by alginate-immobilized algal beads. *Selenastrum capricornutum* was chosen as a model microalgal species because of its ubiquitous occurrence and ease of cultivation. The free cells of *S. capricornutum* were also found to have a higher ability to degrade PAHs than other green microalgae, such as *Chlorella* [13, 14].

## 2 Materials and methods

### 2.1 Preparation of immobilized beads

The fresh water unicellular microalga, *Selenastrum capricornutum*, was purchased from Carolina Biological Supply Company (USA). Cells cultured in modified SE medium under axenic condition were harvested after 10-12 days [15]. The algal suspension was mixed with heat-dissolved sodium alginate to form 3% algal alginate mixture, dropped into 2.5% calcium chloride solution by a peristaltic pump (Cole-Parmer Instrumental Company Masterflex L/S model 7519-06) to produce immobilized algal beads, each with a diameter of 3 mm. Blank beads were prepared in the same way with sterilized de-ionized water instead of cell suspension.

### 2.2 Effects of initial cell densities per bead on PAH removal

A total of 90 sterilized conical flasks (250 ml), each with a stopper consisting of a cotton wool plug wrapped by aluminium foil containing 100 ml SE medium dissolved with 1 mg $L^{-1}$PHE, 0.25 mg $L^{-1}$ FLA and 0.1 mg $L^{-1}$ PYR, were prepared. Microalgal beads at initial cell densities of 0 (without cells, blank bead control), $2.5x10^5$, $1.25x10^6$, $2.5x10^6$ and $5x10^6$ cells $bead^{-1}$ were examined. 400 alginate-immobilized microalgal beads were inoculated to each of the flasks (equivalent to the bead density of 4 beads $ml^{-1}$). The flasks were shaken at 150 rpm and were illuminated by cool fluorescent light (50 μE $s^{-1}$) with a 16/8 hour light/dark cycle. At 1, 24, 48, 72, 96, and 168 hours, triplicate flasks from each treatment were retrieved. The beads, after separated from the medium, were dissolved by 80 ml sodium citrate (0.2 M). The dissolved alginate matrix was separated from the cells by centrifugation at 2,400 g for 15 min. The PAH concentrations in the medium, in alginate matrix and taken up by cells were extracted by ethyl acetate following the standard method [15], and determined by a Hewlett Packard 5890 gas chromatography (GC) equipped with a flame ionization detector (FID). The fused capillary column (J & W Scientific HP-5MS) was 30 m long with 0.25 mm internal diameter and was coated with methyl silicone of 0.25 μm film thickness. The temperature program was maintained at 120°C for 1 min, increased to 214°C at a rate of 2.5°C $min^{-1}$, then to 222°C at a rate of 1°C $min^{-1}$ and maintained at 222°C for 1 min. The injector and detector temperatures were 280°C (splitless for 1.5 min) and 300°C, respectively. The carrier gas was helium.

The PAH removal percentage was calculated by: (Amount of PAH added – Amount remained in the medium) / Amount of PAH added x 100%. The PAH

degradation percentage was calculated: (Amount of PAH added – Amount of PAH remained in medium – Amount of PAH adsorbed on alginate matrix – Amount of PAH taken by cells) / Amount of PAH added x 100%.

### 2.3 Effect of a different number of microalgal beads on PAH removal

The same experimental set-up was used to determine the effect of bead density. The number of beads applied to each flask was 12, 20 and 40 beads $ml^{-1}$ with bead-to-medium volume ratios of 0.9:5, 1.5:5 and 3:5, respectively. Only one initial cell density, $2.5x10^6$ cells $bead^{-1}$, was used. Triplicate flasks from each treatment were retrieved at regular time interval to determine the removal and degradation of PAHs as described above.

## 3 Results

### 3.1 Effects of cell density per microalgal bead on PAH removal

The removal of all three PAHs increased significantly from blank beads (no microalgae) to microalgal beads of higher initial cell densities (Figure 1). In the first hour of treatment, the density of $2.5x10^6$ cells $bead^{-1}$ had the highest PHE removal (61%) when compared to the other densities. From 24 hours onwards, the removal percentages at the two highest densities ($2.5x10^6$ and $5x10^6$ cells $bead^{-1}$) were comparable, increased from 80% in 24-hour to 92% PHE removal in 168-hour treatment. At the lowest density, $2.5x10^5$ cells $bead^{-1}$, the removal percentages increased from 42% in 1-hour to 56% in 24-hour, and only 60% PHE was removed at the end of the experiment (164-hour). Similar removal patterns were observed for FLA and PYR but with different amplitudes. Same as PHE removal, the initial density of $2.5x10^6$ cells $bead^{-1}$ had the highest FLA removal (70%) in the first hour, the removal then increased to 90% in 24-hour and there was no significant difference between this density and the highest one ($5x10^6$ cells $bead^{-1}$) from there onwards. At the lowest density, $2.5x10^5$ cells $bead^{-1}$, only 76% FLA was removed in 24 hours. After 168 hours, FLA was completely removed by beads at the two highest densities while 95 and 83% FLA removal were achieved by beads at $1.25x10^6$ and $2.5x10^5$ cells $bead^{-1}$, respectively. For the removal of PYR, $2.5x10^6$ cells $bead^{-1}$ treatment had 73% removal in the first hour while its initial removal at the other cell densities was lower, ranging from 50 to 55%. All PYR was removed by immobilized microalgae at the three highest densities in 24 hours. At the lowest density ($2.5x10^5$ cells $bead^{-1}$), PYR removal was 78% in 24 hours and increased to 85% in 168 hours (Figure 1).

### 3.2 Effects of cell density per microalgal bead on degradation of PAHs

The effects of cell density on PAH degradation by immobilized *S. capricornutum* (Figure 2) was very different from that of PAH removal (Figure 1). The percentages of PHE, FLA and PYR degraded by blank beads

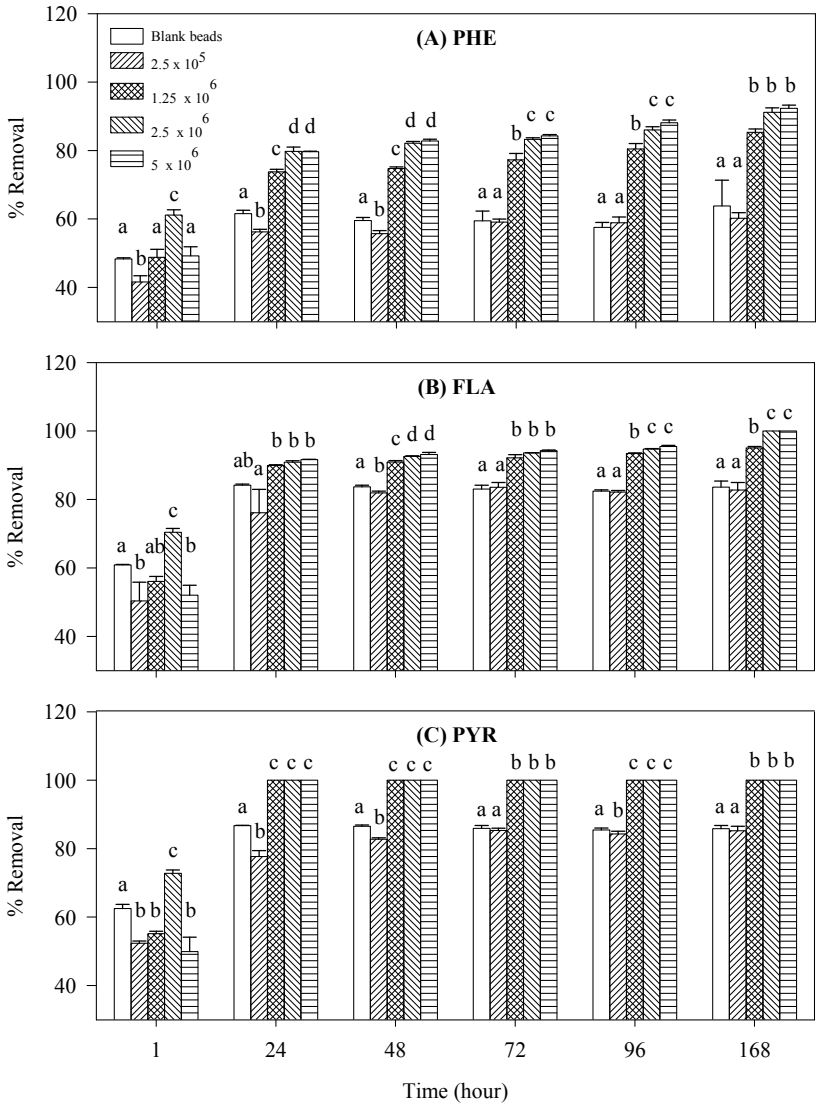


Figure 1: The removal of (A) PHE, (B) FLA and (C) PYR by immobilized *Selenastrum capricornutum* at various initial cell densities (blank beads, $2.5x10^5$, $1.25x10^6$, $2.5x10^6$ and $5x10^6$ cells bead$^{-1}$) during 168-hour treatment (the bead density for all the treatments was 4 beads ml$^{-1}$; mean and standard deviation of three replicates are shown; significant differences between cell densities at each treatment time are shown by different lowercase letters according to one-way ANOVA test at $p \leq 0.05$).

(without cells) were 18, 11 and 12% at all treatment times, respectively (Figure 2), indicating that degradation without algae was minimal. For the microalgal beads, the percentages of PAH degradation increased significantly with treatment time and the effects of cell density were PAH-dependent. At the end of 168 hours, PHE degradation increased with cell densities, from 14% at the lowest density to 55% at the second lowest density, and the two highest densities had comparably high degradation percentages (>65%). Similarly, the lowest density ($2.5x10^5$ cells $bead^{-1}$) only achieved 1% FLA degradation, while FLA was completely degraded at $2.5x10^6$ cells $bead^{-1}$ in 168 hours. All PYR was also degraded by immobilized beads at the two highest densities ($2.5x10^6$ and $5x10^6$ cells $bead^{-1}$) in 168 hours, while only 12% PYR was degraded at the lowest cell density. The small degradation found at the lowest initial cell density suggested that a certain number of cells were needed for an efficient degradation of PAHs. It is clear that the second highest density ($2.5x10^6$ cells $bead^{-1}$) not only had the best removal, it also achieved the best degradation percentages for PHE, FLA and PYR, and was the optimal cell density for the removal and degradation of mixed PAHs.

### 3.3 Effects of bead density on the removal and biodegradation of PAHs

When compared to the PAH removal by the immobilized *S. capricornutum* at a bead density of 4 beads $ml^{-1}$ (Figure 1), initial removal of PAHs, as well as the removal efficiency, were significantly enhanced at high bead densities (Figure 3). The removal also increased gradually with time and reached the maximum removal within 6 to 12 hours, depending on the bead density and the types of PAH. For PHE removal, the bead density at 12 beads $ml^{-1}$ could remove 79% PHE in the first hour while 84% PHE was removed at the two highest bead densities, 20 and 40 beads $ml^{-1}$. In 96-hour treatment, PHE was completely removed at 20 beads $ml^{-1}$ while the removal at 12 and 40 beads $ml^{-1}$ was 96 and 99%, respectively. These PHE removal percentages were much higher than that at 4 beads $ml^{-1}$ (Figure 1). Similarly, almost 168 hours were needed for complete removal of FLA when the bead density was only 4 beads $ml^{-1}$(Figure 1), but FLA was completely removed in 24 hours at a bead density of 12 beads $ml^{-1}$ and the time reduced to 6 hours at a higher bead density (20 and 40 beads $ml^{-1}$). In the first hour, PYR was completely removed at 20 beads $ml^{-1}$, while it took 6 hours to remove all PYR at bead densities of 12 and 40 beads $ml^{-1}$ (Figure 3).

PAH degradation by immobilized *S. capricornutum* at high bead densities increased significantly when compared to that at the low bead density of 4 beads $ml^{-1}$ (Figures 1 and 4). The degradation of PAHs by immobilized *S. capricornutum* increased with time (Figure 4). For PHE degradation, the 12 beads $ml^{-1}$ treatment was higher than that at 20 beads $ml^{-1}$. However, the reverse was found for FLA and PYR with more degradation at a bead density of 20 beads $ml^{-1}$. In 96-hour treatment, FLA was completely degraded at 20 beads $ml^{-1}$ while only 88% FLA degradation was recorded in the 12 beads $ml^{-1}$ treatment. All PYR was degraded in 48 hours at 20 beads $ml^{-1}$ but it took 96 hours when the bead density decreased to 12 beads $ml^{-1}$ (Figure 4) and almost 168 hours at the

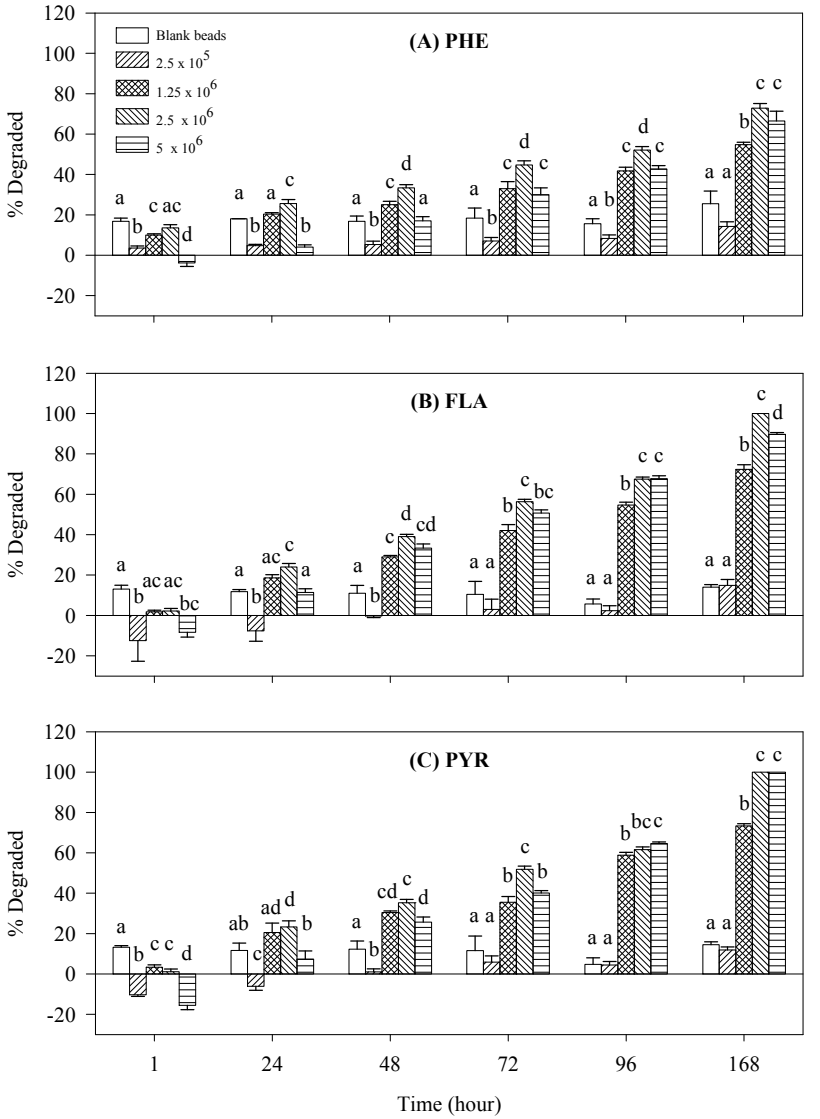


Figure 2: Degradation of (A) PHE, (B) FLA and (C) PYR by immobilized *Selenastrum capricornutum* at various initial cell densities (blank beads, $2.5x10^5$, $1.25x10^6$, $2.5x10^6$ and $5x10^6$ cells bead$^{-1}$) during 168-hour treatment (the bead density for all the treatments was 4 beads ml$^{-1}$; mean and standard deviation of three replicates are shown; significant differences between cell densities at each treatment time are shown by different lowercase letters according to one-way ANOVA test at $p \leq 0.05$).

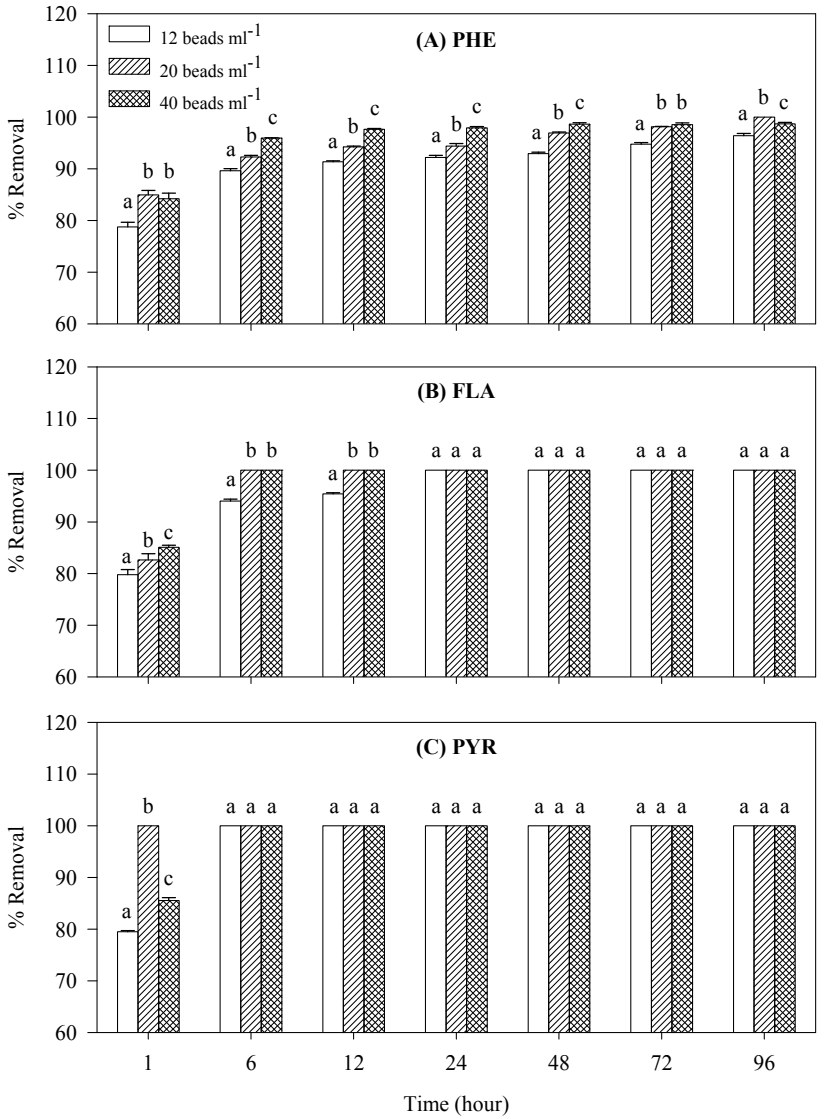


Figure 3: Removal of (A) PHE, (B) FLA and (C) PYR by immobilized *Selenastrum capricornutum* at various bead densities (12, 20 and 40 beads $ml^{-1}$) during 96-hour treatment (mean and standard deviation of three replicates are shown; significant differences between bead densities at each treatment time are shown by different lowercase letters according to one-way ANOVA test at $p \leq 0.05$).

low bead density (4 beads $ml^{-1}$) as shown in Figure 2. The bead density of 20 cells $bead^{-1}$ had the best removal rate of PYR and the best degradation performance on the more toxic and persistent PAHs, FLA and PYR, and was the optimal bead density for PAH removal and degradation.

## 4 Discussion

A mixture of PAHs, including PHE, FLA and PYR, were successfully removed and biodegraded by alginate-immobilized *S. capricornutum* in the present study. PAHs in the medium were quickly adsorbed onto alginate matrix and cell surfaces, passed through cell walls and cell membranes, accumulated inside cells and gradually degraded by live cells immobilized in alginate beads. Lee *et al*. [16] found that both alginate-immobilized and free cells of a mixture of bacteria could efficiently biodegrade phenol at low concentrations but only immobilized cells could maintain their degradation efficiency at a phenol concentration of 300 mg $l^{-1}$. The nickel(II) biosorption capacity of *C. sorokiniana* was increased by 25.3% after loofa sponge immobilization [17]. However, the effects of immobilization on the removal and degradation of pollutants by microorganisms are not always positive. A microalga, *Prototheca zopfii*, was found to be less efficient in degrading *n*-alkanes ($C_{14}$ to $C_{16}$) after polyurethane foam immobilization and complete degradation of hydrocarbons by the microalgae was delayed from 210 to 290 hours after immobilization [18].

The removal efficiency of immobilized beads, same as free cells, was related to the amounts of biomass used. Macfie *et al*. [19] reported that higher cell density or biomass provided more surface area and cell volume for adsorption and absorption of pollutants leading to a better removal at a shorter period of time. Pradhan and Rai [9] also showed that the removal of copper(II) by alginate-immobilized *Microcystis* increased with increasing biomass up to 0.064 g but further increase from 0.064 to 0.128 g did not enhance removal percentages. The removal and degradation of PAHs by immobilized *S. capricornutum* in this study was found to increase with initial cell density per bead, from $2.5x10^5$ to $2.5x10^6$ cells $bead^{-1}$, but a further enhancement effect was not observed at the highest initial cell density ($5x10^6$ cells $bead^{-1}$). The cells entrapped in immobilization matrix would compete for space, nutrients, carbon dioxide and light supply, similar to or even more severely than that in free cells, especially when the cell density became very high [10]. The mass transfer of nutrients to the cells inside the immobilized bead was also limited.

Another possible mean to enhance the removal and degradation of contaminants by immobilized microorganisms is to increase bead density (number of beads used). The removal of ammonium by alginate-immobilized *C. vulgaris* at 8 and 12 beads $ml^{-1}$ were significantly higher than that at other densities, 4, 16 and 20 beads $ml^{-1}$, while the phosphate removal was not affected by bead density [11]. Dense microalgal beads would limit the light penetration and enhance the self-shading problem among beads which in turn reduced cell growth and metabolic activities [20]. Self-shading might also affect the mass

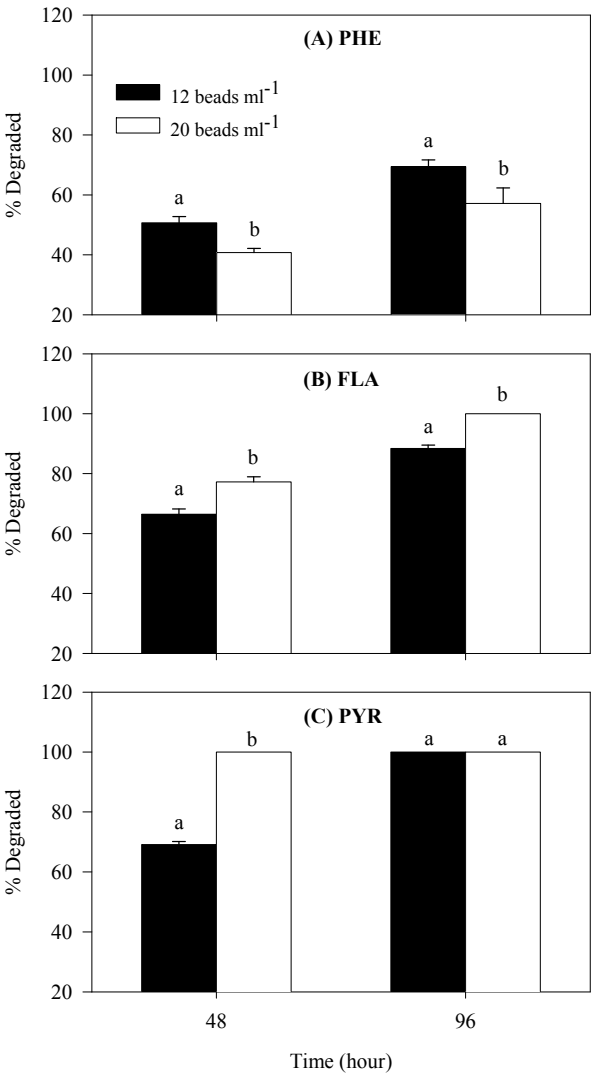


Figure 4: Degradation of (A) PHE, (B) FLA and (C) PYR by immobilized *Selenastrum capricornutum* at bead densities of 12 and 20 beads $ml^{-1}$ during 96-hour treatment (mean and standard deviation of three replicates are shown; significant differences between bead densities at each treatment time are shown by different lowercase letters according to *t*-test at $p \leq 0.05$).

transfer of the contaminants to the microalgal beads. This explained why the removal and degradation performance of PAHs in the present study increased significantly from 4 to 20 beads $ml^{-1}$ but a further enhancement effect was not obvious when the bead density was increased to 40 cells $bead^{-1}$.

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