# Evolution of the microbial activity during the acclimation and deacclimation (starvation) of activated sludge to 4-chlorophenol

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**Abstract:** The variation of the microbial activity during the acclimation and deacclimation process in the degradation of 4-chlorophenol (4CP) was studied in a sequencing batch reactor. The evolution of the kinetics curves and the respiratory activity of a mixed culture of microorganisms was follow. The microbial activity was followed by measuring the substrate uptake rate and the specific oxygen uptake rate. The influence of initial toxic concentration on acclimation of activated sludge from a municipal wastewater treatment plant was studied. The experimental design considered three different sets of initial concentration of 4CP: 50, 100 and 200 mg/L. After acclimation, the biomass was exposed to different periods of starvation to study the deacclimation. The results showed a reduction in the degradation time as the acclimation process occurred. Degradation time was reduced from 40 h to 50 min, in the case of 50 mg 4CP/L and from 52 to 1.16 h, for the case of 100 mg/L. In both cases this process was accomplish in less than 125 h. As the acclimation took place, it was found that the affinity of the consortia to biodegrade the toxic increased, whereas the ability to biodegrade acetate decreased. There exist a diminution on a toxic metabolite production as the bacteria adapted to the 4CP degradation. Starvation generates a decrease on the microbial activity. A decrement from 21 to 44 % was observed on the removal rate, and from 26 to 35 % of activity lost measured as specific oxygen uptake rate. The extent of the deacclimation of starved microorganisms seems to be affected by the history of the culture. Degradation rates during the acclimation and deacclimation process followed an exponential model.

## Introduction

Many of the industrial processes generating wastewater containing toxic compounds are characterized by their variability. In the chemical, pharmaceutical, plastic, and petrochemical industries, for some cases, production processes are in batch. Because of the high variations in flow and concentration of contaminants in industrial wastewater, treatment processes do not obtain satisfactory removal efficiencies. Besides, due to its toxicity, biological treatment of the industrial wastes containing high concentration of phenols is difficult. The first step to biodegrade toxic substances in a wastewater treatment plant is the acclimation of the microorganisms. When microorganisms are put in contact with toxic compounds, in a favorable environment, acclimation to these compounds may occur (Aelion *et al.* 1989). Different phenomena have been described to explain the acclimation phase. Wiggings *et al.* (1987) suggested that there is a selection and a multiplication of specialized microorganisms during this phase and physiological transformations in the metabolic system of the microorganisms, i.e., alterations in the enzymatic level, regulation and production, mutations, etc. In aerobic microbial communities, the acclimation periods typically range from several hours to several days (Wiggings *et al*. 1987). However, not much information is available concerning the activity evolution of a microbial consortium during the acclimation to toxic wastewater.

On the other hand, it has been shown that the acclimation is not permanent (Buitrón and Moreno, 2002). The exposition of the acclimated population to prolonged periods of starvation produces a decrease of the bacterial activity and, even the death of some of them (Coello *et al.*, 2003). Buitrón

*et al.* (1994), found a negative effect of starvation periods on 4-chlorophenol (4CP) degradation for an activated sludge in a sequencing batch reactor (SBR) system. In that study, the aeration was extended 20 to 23 hours after the toxic degradation had been completed. It was found that the degradation time increase 6 times (from 0.7 to 4.5 h) as a result of starvation period. This loss in the microbial degradation capacity was attributed to a decline in both the enzymatic activity and the viability of the suspended cells. Although this loss of activity of the microorganisms under toxic starvation has been reported, this variable is not taken into account on the operation of biological wastewater treatment plants. In order to monitor and control the biological WWTP with a certain confidence it is necessary to consider the acclimation and deacclimation processes to avoid the reactor malfunctioning, traduced in loss of efficiency.

The present work describes the evolution of the kinetics curves and the respiratory activity of a mixed culture of microorganisms during the acclimation and deacclimation by starvation during the degradation of 4CP in a sequencing batch reactor.

## Methodology

An aerobic automated Sequencing Batch Reactor (SBR) system with a capacity of 7L and an exchange volume of 57% was used (fig 1). The airflow rate was 1.5 liters per minute and the temperature was maintained at 20 ºC inside the reactor. The reactor was inoculated with microorganisms coming from a municipal activated sludge treatment plant. A synthetic wastewater containing 4CP was used as a sole source of carbon and energy. Nutrients such as nitrogen, phosphorus, and oligoelements were added following the techniques recommended by ANFOR (1985). The SBR was operated under the following strategy: preaeration time (15 min) filling time (5 min), reaction time (variable depending on the necessary time to reach a removal efficiency of 4-CP of 99%), settling time (12 to 30 min) and draw time (1 min). Degradation time was followed using the dissolved oxygen concentration present in the reactor (Buitrón *et al.*, 2003).

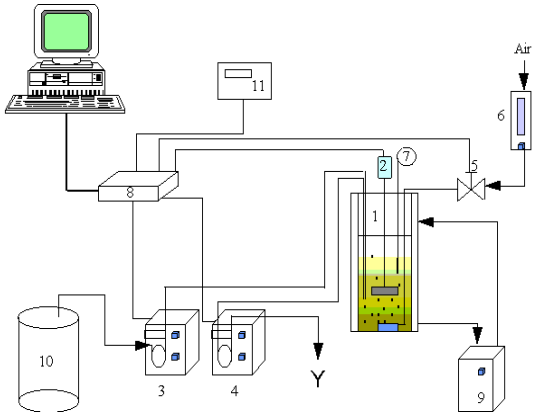



**Figure 1** Experimental assembly. Reactor (1), mixer (2), feeding pump (3), drawing pump (4), flow controller (5), flowmeter (6), thermometer (7), interface (8), flow-through heater (9), storage tank (10), oxymeter (11)

The substrate concentration was measured taking samples and processing them offline using the colorimetric technique of the 4-aminoantipyrine (Standard Methods, 1992). Total and volatile suspended solids (TSS and VSS) analyses were determined according to the Standard Methods (1992). Dissolved organic carbon (DOC) was determined with a Shimadzu TOC-5050 and Chemical Oxygen Demand (COD) according to Standard Methods (1992). Theses analyses were performed to evaluate the 4CP mineralization. The metabolite (5-chloro-2hydroxy-muconique acid semialdehyde) formed by an alternate degradation route of 4-CP by the microorganisms, and that

can be inhibitory for the microorganisms, was also determined by spectrophotometry at 380 nm using a HACH spectrophotometer.

In order to follow the respirometric activity, the specific oxygen uptake rate (SOUR) was measured by placing 10 mL of the mixed liquor of the SBR harvested just after a degradation cycle in a mini-reactor of 160 mL. An oxygen-saturated solution with nutrients and substrate (acetate or 4CP) was added and dissolved oxygen measured was recorded. Endogenous respiration was measured adding only nutrients. SOUR was computed from the slope of the reprogram divided by the VSS concentration.

### Acclimation

The reactor was inoculated with activated sludge from a municipal wastewater treatment plant containing 2000 mgVSS/L. The biomass was acclimated using a variable cycle strategy, i.e., the reaction phase duration was variable and stopped when the removal of 4CP was equal or greater than 95%. The experimental design considered three different sets of initial concentration of 4CP (50, 100 and 200 mg/L) named AC50, AC100 and AC200, respectively. Each experiment was initiated with fresh non-acclimated sludge, except for the case of AC200, in which the previously 100 mg/L acclimated sludge, was used as inoculum.

### Deacclimation (Starvation)

Acclimated biomass was exposed to different starvation periods. Starvation was introduced by keeping the aeration the necessary time after the degradation of 4CP was completed, i.e. microorganisms were maintained under endogenous conditions. For each acclimated biomass, sets of starvation periods were studied. Thus, for the acclimated biomass to 50, 100 and 200mg/L (ST50, ST100 and ST200), starvation times were as follows. For ST50: 8, 12 and 24h, ST100: 12 and 24 h, and ST200: 12, 24 and 36 h. For each experiment the kinetics of degradation and the SOUR were followed before and after the starvation. Each set of conditions was made in the same reactor. Once a starvation time was studied, the biomass was allowed to recover its capacities before starvation by feeding the reactor and proceeding as in the acclimation phase in order to reach the degradation time observed before the starvation.

## Results and discussion

### Acclimation process

The degradation kinetics for the acclimation process was followed. For the two initial concentrations of 4CP used, acclimation was obtained in 10 degradation cycles. During the acclimation, the 4CP was degraded with efficiencies higher than 99% as 4CP and 95% as DOC and COD. During the acclimation of the activated sludge, the relationship between residual 4CP concentration and incubation time gradually changed, and then reached steady. In the case of AC50, degradation time was reduced from 40 h to 50 min, after 75 h (from cycle 1 to cycle 10)(Fig. 2A). For AC100 (Fig. 2B) degradation times were reduced from 52 to 1.16 h, after 125 h (Cycle 1 to 10). It was observed that there is a proportional increment on the acclimation period when the initial concentration of the toxic is increased from 50 to 100 mg/L (75 vs 125 h).

When the pre-acclimated sludge AC100 was exposed to an increment of 100% of the initial concentration, degradation was only slightly affected. First degradation cycle for AC200 took only 2.5 to remove 100% of the initial 4CP. After 5h, degradation times were reduced to 1.75 h (from cycle 1 to 6). This result indicated that during the AC100 acclimation the required microorganisms reproduced and they also developed the necessary enzymatic activity. Doubling the initial concentration only generates a proportional increment on the degradation time.

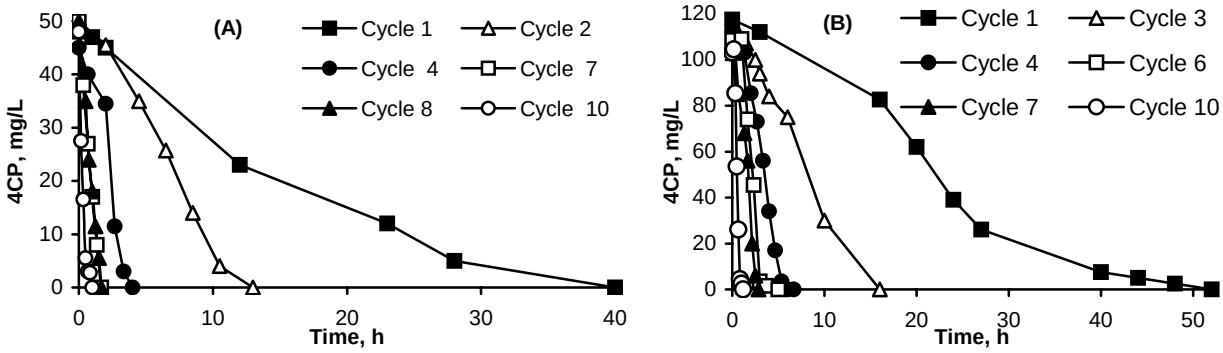



**Figure 2** Degradation kinetics of during the acclimation process. A) acclimation to 50 mg4CF /L and b) acclimation to 100 mg/L

Once the biomass was completely acclimated to the 4CP, when being added the compound again, the configuration of the degradation curve was similar, independently of the initial acclimation concentration. It has been observed that the acclimated microorganisms could degrade concentrations of 4CP up to 1400 mg/L (Buitrón *et al.,* 2003).

## Microorganism activity in the acclimation phase

Figure 3 presents the evolution of the SOUR computed during the acclimation of 4CP to microorganisms. Two parallel experiments were carried out. First, the SOUR was measured feeding the mini-reactor with an easy-to-biodegrade substrate, namely acetate. A second experiment was conducted using 4CP as substrate. The substrate for each case was the sole source of carbon and energy, and for both cases, endogenous respiration was evaluated and took into account for calculations. As acclimation took place, an increment in the SOUR against the 4CP was observed. On the contrary, a decrease on the SOUR occurred for the case of acetate. In figure 3, a crossing point is observed at 60 h and 80 h for the case of AC50 and AC100, respectively. We can consider that after this point affinity of the consortia is higher for the toxic compound than for an easy-to-biodegrade substrate as the acetate. Figure 4 presents the specific substrate uptake rate, $q_x$, as a function of the acclimation time. The evolution of $q_x$ during the acclimation period was highly correlated with an exponential relationship. It is possible to observe that after the crossing point discussed before, the $q_x$ is increasing exponentially (see the case for the acclimation to 100 mg/L). Thus, there is a point when the affinity of microbial community shifts towards the toxic compound, and after, the degradation rate increases rapidly and stabilizes.

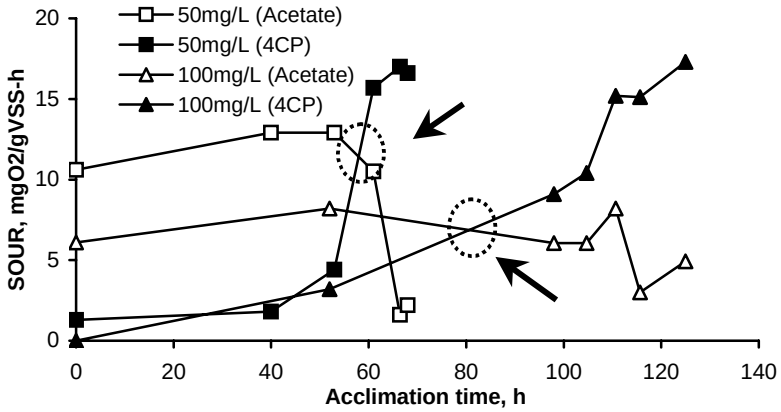



**Figure 3** Evolution of the SOUR for the microorganisms acclimated to 4CP. Arrows indicate the crossing point when the activity to the consumption of 4CP is higher than the activity of acetate consumption.

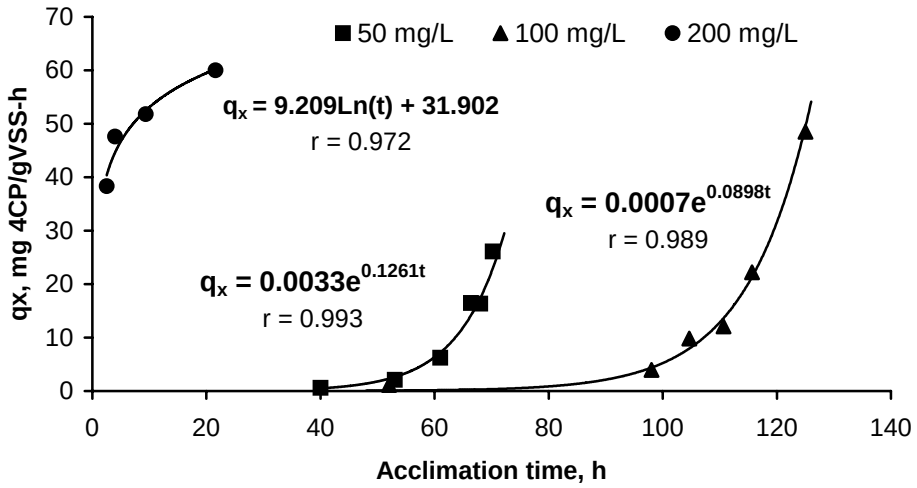



**Figure 4** Evolution of the specific degradation rate, $q_x$, as a function of the acclimation time. Degradation rate vas evaluated dividing the concentration of 4CP by the time needed to degrade more than 99% of the original concentration and divided by the VSS present in the reactor.

When a previously acclimated biomass was exposed to a higher concentration of 4CP, the acclimation time was lower than the time needed by a non-acclimated sludge, as is shown in figure 4 for $q_x$ obtained for the case of AC200. In this case, a logarithmic relationship was obtained to explain the evolution of the $q_x$. This observation indicated that after the acclimation process was completed the $q_x$ is not more increasing exponentially, but it seems that it stabilized in a maximal value. Care must be taken when acclimation is conducted, since this is done at an elevated concentration of the toxic compound (beyond the inhibition concentration) there will be problems and the acclimation process could not be successful. Moreno and Buitrón (2003) observed that inhibition is not only function of the initial substrate concentration, but also of the initial biomass concentration. In general, a low biomass concentration will produce a greater inhibition. For this reason the acclimation of microorganisms is preferable to be done at lower initial substrate to microorganisms' ratio.

## Metabolite production in the acclimation phase

The metabolite 5-chloro-2hydroxy-muconic acid semialdehyde (Comandeur and Persons, 1990) is a compound formed by an alternate degradation route of 4-CP by the microorganisms, and this can be inhibitory at very low concentrations. Figure 5A shows the metabolite production during the acclimation experiments. To take into account the metabolite production, it was computed the absorbance generated during the degradation cycle (area under the curve), thus results are expressed as absorbance units multiply by hours. It is observed that higher quantities of metabolite were generated at the beginning of the acclimation. As the bacteria adapted, the toxic metabolite production diminished from 0.053 to 0.016 (for AC50) and from 0.110 to 0.018 absorbance units-hour. It has been observed that the apparition of this metabolite indicated an operation problem, thus a deacclimation of the microorganisms (Buitrón *et al.*, 2003).

## Sludge Volumetric Index in the acclimation phase

As the acclimation took place the sludge volumetric index (SVI) decreased (Figure 5B). It is clear that filamentous bacteria found in the inoculum used for the AC100 experiment were eliminated by the toxic. In general, the microorganisms degrading the 4CP presented excellent settling proprieties. Even for a problematic inoculum, SVI decreased from 500 to 240 mL/g after 10 cycles, and to 70 after 20 more cycles (see the values for 200 mg 4CP/L).

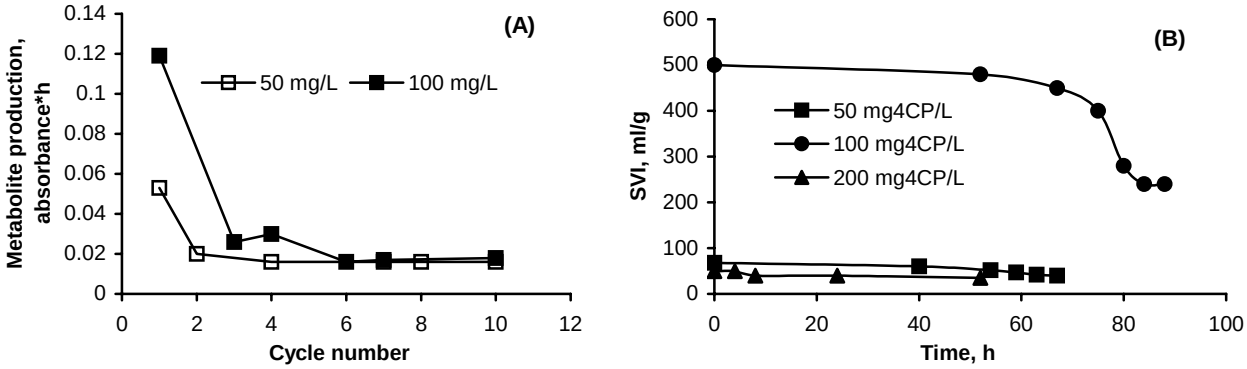



**Figure 5** (A) Metabolite production during acclimation; (B) Evolution of the SVI during the acclimation to 4CP

## Deacclimation induced by starvation

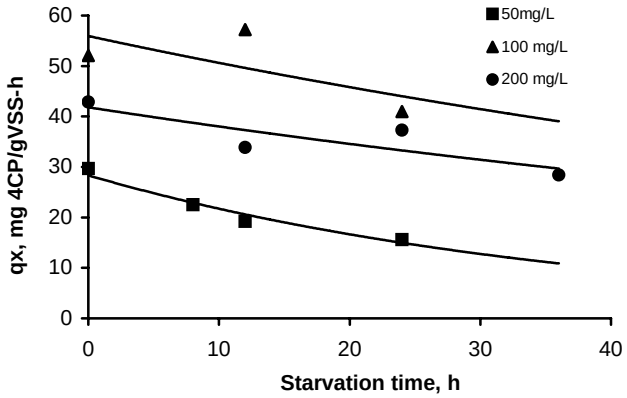
Many of the industrial processes generating wastewater containing toxic compounds are characterized by their variability. In the chemical, pharmaceutical, plastic and petrochemical industries, for some cases, production processes are in batch. Because of the high variations in flow and concentration of contaminants in industrial wastewater, treatment processes do not obtain satisfactory removal efficiencies. The first step to biodegrade toxic substances in a wastewater treatment plant is the acclimation of the microorganisms. Nevertheless, it has been shown that this acclimation is not permanent (Buitrón and Moreno, 2002). In effect, for instance, when the containers and reactors are cleaned, a peak in the concentration of toxic substances (shock load) is found in the wastewater treatment plant. After this sudden increase, there is a decrease of the toxic concentration. These variations of the toxic substrate or starvation in relation to the toxic compound have an important effect on the sludge activity. This is the case for intermittent industrial operation, which sometimes generates sudden increase of toxic substrate concentration (concentration peaks).

Figures 6 and 7 present the influence of starvation on the specific removal rate, qx and on the SOUR. As it is possible to note starvation gradually decreases the microbial activity. In general, a decrement from 21 to 44 % was observed on $q_x$ due to the introduction of starvation periods, and from 26 to 35 % of activity lost measured as SOUR (Figure 7). It was found that the substrate uptake rate was highly correlated with the negative exponential of starvation time, independently of the originally acclimated concentration (figure 6).

It is interesting to point out that the strategy to conduct the experiments of starvation influence the results obtained. Buitrón *et al.*, (1994) starved the biomass in the same reactor applying several and consecutives starvation periods of 24 h. The degradation time increases up to 6 times from the initial value generating 80% of reduction on $q_x$. In the present study, the biomass in the reactor was acclimated, and then the starvation experiments were conducted for each initial concentration. Between each starvation experiment several cycles were conducted, in order to recover the initial activity of the biomass and then, the next starvation time was applied. This procedure generated a history in the culture. Apparently, by using this operation mode the deacclimation effect is diminished. In Figure 7 for the case of ST100 and 200 it is possible to observe that after a cycle of starvation, there exist even an increase in the $q_x$ value indicating a reduction of the degradation time. Thus, a positive influence of starvation is observed.

Previous studies have examined effects of pre-starvation on the survival of inoculant cells; an improved survivability has been reported (Van Elsas et al., 1994), while another study has documented that pre-starvation exerted no significant effects on the survival (Van Overbeek et al., 1995). The inconsistency may have been due to the different starvation conditions used in those

studies. The point to be considered is that during starvation there exists a decrease in the population density, respiration rate, dehydrogenase activity and in the phenol-oxygenating activity that may affect the performance of a wastewater treatment plant (Watanabe et al., 2000). In addition, there are some physiological changes occurred to starved cells. Microscopic observations of the microorganisms revealed a reduction in cell size, a change in cell shape, and a loss of the motility during starvation. Watanabe et al. (2000) also demonstrated an increase in cell surface hydrophobicity and floc adhesion capacity due to starvation. Consequently, it is possible to consider that the microorganisms, subject to cycles of starvation-activity recovery periods-starvation, changed in such a way to protect themselves. There is a minimal reduction in the microbial activity in this case. Nevertheless, in practice, the industrial wastewater treatment plants are subject to random changes of toxic concentration increasing the risk of deacclimation by an important activity variation of the microorganisms.




$$q_x = q_o e^{-kt}$$

| 4CP mg/L | $q_o$ mg/gVSS-h | k $h^{-1}$ | r |
|---|---|---|---|
| 50 | 28.3 | 0.027 | 0.979 |
| 100 | 55.9 | 0.01 | 0.697 |
| 200 | 41.8 | 0.01 | 0.851 |

**Figure 6** Influence of starvation time on the specific substrate removal rate. Results after starvation are compared with the initial condition before the perturbation. The model is represented in the figure and the coefficients are shown in the table of the right side.

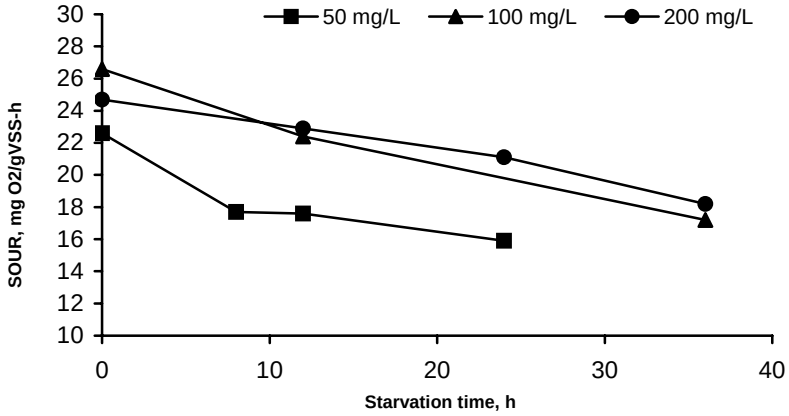



**Figure 7** Influence of starvation time on the specific substrate removal rate for SOUR. Results after starvation are compared with the initial condition before the perturbation.

## Conclusions

The results showed a reduction in the degradation time as the acclimation process occurred. In the case of 50 mg 4CP/L, degradation time was reduced from 40 h to 50 min, after 75 h and from 52 to 1.16 h, after 125 h. It was observed that there is a proportional increment on the acclimation period when the initial concentration of the toxic is increased from 50 to 100 mg/L. As the acclimation took place, it was found that the affinity of the consortia to biodegrade the toxic increased, whereas the ability to biodegrade acetate decreased. There exist a diminution on the toxic metabolite production as the bacteria adapted to the 4CP degradation. Acclimation to 4CP increases the settling capacity of the sludge.

Starvation generates a decrease on the microbial activity. In general, a decrement from 21 to 44 % was observed on qx due to the introduction of starvation periods, and from 26 to 35 % of activity lost measured as SOUR. The extent of the deacclimation of starved microorganisms seems to be affected by the history of the culture. The effect of starvation on the degradation rate is less significant when microorganisms are starved, recovered and then starved again, than in the case where starvation is cyclically presented without recovery period. In any case, there exist an activity decrement generated by the variation of the toxic and thus a deacclimation of the previously acclimated bacteria that could explain the variations on the performance of treatment of toxic industrial wastes. It was observed that the specific degradation rates during the acclimation (increase) and deacclimation (decay) process followed an exponential model.

## Aknowledgements

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