Int. J. Environ. Sci. Technol. (2016) 13:649–662
DOI 10.1007/s13762-015-0906-y

ORIGINAL PAPER

Understanding the simultaneous biodegradation of thiocyanate and salicylic acid by *Paracoccus thiocyanatus* and *Pseudomonas putida*

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Received: 9 June 2015 / Accepted: 7 October 2015 / Published online: 29 October 2015

Abstract Phenolic and cyanide compounds, which frequently appear mixed in several industrial effluents, are difficult to be biodegraded under certain conditions. In this work, salicylic acid (SA) and thiocyanate (SCN^-) were selected as model pollutants of these two families and experiments of biodegradation with specific microorganisms were developed. It was found that the best well-known bacteria able to biodegrade each one of these pollutants, *Pseudomonas putida* for SA and *Paracoccus thiocyanatus* for SCN^-, do not biodegrade the other one. Therefore, the co-culture was required, producing interesting interaction phenomena. When both pollutants were simultaneously biodegraded, a commensalism effect was observed improving SCN^- removal. Experimental data for SCN^- and SA removals were successfully fitted to zero reaction kinetic orders, with induction time in the case of SCN^-, and substrate dependences were fitted to Tessier models. A flow cytometry method was developed and employed to obtain the evolution of the viable, damaged and dead cells for different substrate concentration and the degree of agglomeration in the co-culture experiments.

Keywords Biodegradation · Co-culture · Flow cytometry · Salicylic acid · Substrate inhibition · Thiocyanate

Electronic supplementary material The online version of this article (doi:10.1007/s13762-015-0906-y) contains supplementary material, which is available to authorized users.

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Introduction

Activated sludge plants are the most widespread solution for the treatment of industrial wastewaters. Nevertheless, effluents from industrial plants such as petrochemical industries, coke-processing plants, metal finishing units or pharmaceutical factories, are difficult to be biodegraded, due to the toxic properties of some pollutants usually present in them (Banerjee 1996; Chaudhari and Kodam 2010; Huang et al. 2013). Besides, if these toxics are not degraded in situ, they can affect the performance of centralized industrial wastewater treatment plants, which receive the effluents from various industrial parks (Fall et al. 2012; Lei et al. 2010). The biodegradation of a mixture of pollutants from different industrial sources by means of an activated sludge system is very complex and analysis of the interactions that occur between substrates and bacteria helps to get a general comprehensive approach of the entangled relations occurring during the activated sludge treatment.

The physical, chemical or biological degradation of the most of toxic compounds usually present in industrial wastewaters is well documented (Collado et al. 2009, 2010; Kim et al. 2008; Li et al. 2011; Marañón et al. 2008). Particular importance has been devoted to the biological removal of phenolic and cyanide compounds by activated sludge systems, either together or separately (Banerjee 1996; Huang et al. 2013; Juang and Tsai 2006; Kwon et al. 2002; Sharma et al. 2012). These two families of compounds, which frequently appear simultaneously in wastewaters from different industrial sectors, such as the pharmaceutical or iron and steel industries, are strictly regulated worldwide because of their extreme toxicity (Li et al. 2011). This has caused the development of several researching works investigating the biodegradation of these kinds of compounds. In some of these works, a real

wastewater and a bioaugmented or native mixed culture are used (Kim et al. 2008; Li et al. 2011; Marañón et al. 2008; Staib and Lant 2007), focusing on the global yields and rates obtained during the treatment or on possible changes in reactor configuration or operational conditions. Due to the high variability in the activated sludge biocenosis and even in the wastewater composition, the extrapolation of conclusions is very complicated and the interactions between bacteria and pollutants are complex and frequently not studied. So, the biological reactors in these studies were frequently considered as "black boxes" difficult to predict and control (Herrero and Stuckey). Others woks use model pollutants or synthetic wastewaters and pure cultures (Agarry et al. 2009; Agarry and Solomon 2008; Arutchelvan et al. 2005; Combarros et al. 2014, 2015; Juang and Tsai 2006; Kim and Katayama 2000; Kwon et al. 2002). The aim of these studies is usually to define the capacity of the bacterium to degrade a specific compound, the biodegradation pathway and the effect of the culture conditions on the bacterial activity. Pure cultures usually show lower resistance to stress conditions than mixed ones, amplifying the response of the system to perturbations. The removal of phenol or cyanide compounds in biological wastewaters processes using pure cultures of *Pseudomonas*, *Klebsiella*, *Ralstonia*, *Thibacillus* or *Paracoccus* has been analyzed (Agarry et al. 2009; Agarry and Solomon 2008; Combarros et al. 2014, 2015; Juang and Tsai 2006; Kim and Katayama 2000; Kwon et al. 2002). The main limitation of these studies is that they provide too simple scenarios, which usually do not reflect the complexity of the interactions.

Finally, a third group of papers employ co-culture, mixed culture or bioaugmentation. It is very common that the target contaminant can only be degraded by a very specific mixture of microorganisms that cooperate in a synergistic way. Therefore, a simplified model of the activated sludge can be built for the treatment of specific pollutant, including only the strains involved in their degradation (Chaudhari and Kodam 2010; Fuentes et al. 2014; Huang et al. 2013; Ibáñez et al. 2014; McGenity et al. 2012; Mikesková et al. 2012). The aim is to obtain a system sufficiently complex to simulate a real sludge but, at the same time, simple enough to allow the study of the interactions between species (Mikesková et al. 2012). Works using this approach are focused on the study of the degradation of a single or more compounds by two or more bacteria. In these works, persistent symbiotic interactions between bacteria or co-metabolism phenomena between compounds are frequently reported (Mikesková et al. 2012).

The bacteria species most commonly used for the biodegradation of phenolic and cyanide compounds are *Pseudomonas* and *Paracoccus*. Surprisingly, as far as we know, there are no studies dealing with the simultaneous biodegradation of cyanide and phenolic compounds using a co-culture of both species. This information would be very useful to determine synergetic effects between species comparing with results obtained using mixed cultures (Herrero and Stuckey; McLaughlin et al. 2006; Shivaraman et al. 1985). Besides, this kind of studies is also necessary as previous step for investigating the potential benefits that may be obtained by adding selected pure cultures to a mixed one in order to improve the degradation of these xenobiotics (*bioaugmentation*) (Herrero and Stuckey; McGenity et al. 2012; McLaughlin et al. 2006).

Taking into account these considerations, the aim of the present work has been to evaluate the simultaneous biodegradation of thiocyanate (as model of cyanide pollutant) and salicylic acid (as model of phenolic pollutant) by a co-culture of *P. thiocyanatus* and *P. putida*. For this purpose, the individual and simultaneous biodegradations of different concentration of thiocyanate and salicylic acid by a co-culture of both bacteria have been monitored. In basis of experimental results, the synergy/inhibitory phenomena between species are determined and a kinetic model defined. Additionally, a flow cytometry procedure by using double staining (cFDA/PI) has been developed and tested with the aim of determining the evolution of the physiological status of the co-culture bacteria and the influence of parameters considered.

Materials and methods

Media composition

Initial biodegradability tests of each pollutant by each bacterial species were carried out using the mineral medium recommended by Combarros et al. (2014, 2015) (PpMM for *Pseudomonas putida* and PtMM for *Paracoccus thiocyanatus*). For co-culture experiments, a combination of both mineral media was selected (CCMM), its final composition being: 0.5 g L^{-1} K_2HPO_4, 0.3 g L^{-1} $(NH_4)_2SO_4$, 0.05 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.01 g L^{-1} $FeCl_3{\cdot}6H_2O$, 0.01 g L^{-1} $CaCl_2{\cdot}2H_2O$, and 0.05 g L^{-1} tryptone and 10 mL L^{-1} of trace solution. The trace solution was composed of 8 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 4 mg L^{-1} H_3BO_3, 4 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 4 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 4 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 4 mg L^{-1} $CoCl_2{\cdot}6H_2O$. As can be observed, CCMM medium contained all components of individual media, selecting the higher concentration when a compound was present in both media.

Microbial strain and culture conditions

A previously isolated *Paracoccus thiocyanatus* [BCCM, Belgian Coordinated Collections of Microorganisms (LMG 24666)] and *Pseudomonas putida* [Leibniz Institute DSMZ-German Collection of Microorganism and Cells Cultures, Germany (DSM 4478)] were chosen for degrading thiocyanate and salicylic acid, respectively.

P. thiocyanatus colonies, which had been grown in growth medium (PtGM) agar for 4 days at 30 °C, were inoculated into 250-mL Erlenmeyer flasks containing 50 mL of PtGM. PtGM composition was 5 g L^{-1} peptone, 5 g L^{-1} beef extract, 5 g L^{-1} yeast extract, 2.5 g L^{-1} NaCl, 0.1 g L^{-1} K_2HPO_4 and 0.05 g L^{-1} $MgSO_4{\cdot}7H_2O$ (Combarros et al. 2015). After incubation at 28 °C and 250 rpm for 24 h, an aliquot was taken and used as an inoculum for subsequent experiments. In the same manner, *P. putida* colonies, which had been grown in growth medium (PpGM) agar for 1 day at 30 °C, were inoculated into 250-mL Erlenmeyer flasks containing 50 mL of PpGM. PpGM composition was peptone 5 g L^{-1}, meat extract 3 g L^{-1}, KH_2PO_4 0.422 g L^{-1}, K_2HPO_4 0.375 g L^{-1}, $(NH_4)_2SO_4$, 0.244 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.05 g L^{-1}, $C_6H_{11}NO_7Fe$ 0.054 g L^{-1}, $CaCl_2{\cdot}12H_2O$ 0.015 g L^{-1} and NaCl 0.015 g L^{-1} (Combarros et al. 2014). After incubation at 30 °C and 150 rpm for 16 h, an aliquot was also taken and used as an inoculum for subsequent experiments. In both cases, the inocula were centrifuged at 10,160×*g* for 10 min and the supernatants were eliminated so that only bacterial cells were added to the corresponding mineral medium to avoid introducing compounds from the growth medium.

For the biodegradations using an only bacterium, the culture conditions for each bacteria were chosen as recommended for an optimum growth, i.e., 200 rpm and 30 °C in 250-mL Erlenmeyer flasks containing 100 mL of PpMM for *P. putida* and 250 rpm and 28 °C in 500-mL Erlenmeyer flaks containing 100 mL of PtMM for *P. thiocyanatus* (Combarros et al. 2014, 2015). The biodegradation conditions selected for the co-culture were 30 °C, 250 rpm and 100 mL of CCMM in 500-mL Erlenmeyer flasks. In all cases, initial cells concentrations were 10^7 cfu mL^{-1} of each bacterium.

Biodegradation experiments

Table S1 shows the experiments carried out in this work in order to determine the biodegradation potential and the effects of the pollutants on the pure cultures (rows 1–6), the effect of composition medium (rows 8 and 10), and the culture conditions (rows 7 and 9) on SCN^- or SA biodegradations using pure cultures and the interactions between species during the simultaneous biodegradation of SA and SCN^- (rows 11–15).

Flow cytometry analysis

Staining procedures

Samples from cultures were harvested by centrifugation at 16,000×*g* for 5 min. Before staining, cells were washed twice in phosphate-buffered saline (PBS; pH 7.4, sterile and filtered at 0.22 μm) and were then held in the "hot spot" of a sonication bath for 2 s to prevent bacterial aggregation before flow cytometric analysis (Hewitt and Nebe-von-Caron 2004). Propidium iodide (PI; Invitrogen), and carboxyfluorescein diacetate (cFDA; Invitrogen) were used as fluorescent dyes in a dual-staining procedure (cFDA/PI) in order to evaluate cell physiological status. Staining procedures were performed as previously reported Alonso et al. (Alonso et al. 2012). Total counts of cells were determined by staining with SYBRgreen (SYBRgreen; Invitrogen), a green fluorescent cell-permeable DNA probe (Foladori et al. 2010; Nielsen et al. 2009), to differentiate the microorganisms from other particles in samples (background). Fluorescent microspheres (Perfect Count; Cytognos, Spain) were used as internal standards in each sample, following the supplier's recommendations for ratiometric counting (Quirós et al. 2007). In order to obtain a significant number of cells to ensure the efficiency of the test, 2000 microspheres were acquired in each analysis. Analyses were performed in triplicate for each dye, including an un-stained sample as a control.

Stock solutions were prepared as follows: PI was made up to 1 mg mL^{-1} in distilled water (0.22 μm filtered) and maintained at 4 °C, whereas cFDA and SYBRgreen were prepared in dimethyl sulfoxide (Sigma-Aldrich) at a concentration of 1 mM and stored at −20 °C. PI staining was prepared by diluting the stock solution in sterile distilled water and adding this work solution to the cell suspension at a final concentration of 5 μg mL^{-1}. This mixture was then incubated for 30 min in the dark at room temperature. Working solutions of cFDA were made up to 10 μM in PBS containing 1 mM EDTA. PI-stained samples were subsequently incubated with 0.1 μM cFDA for 15 min in dark at room temperature (Alonso et al. 2012). SYBRgreen work solution was prepared by diluting the stock solution in PBS containing 1 mM EDTA at final concentration of 0.01 mM. The SYBRgreen work solution was added to the cell suspension at a final concentration of 0.1 μM, and the mixture was incubated for 15 min in dark at room temperature (Foladori et al. 2010).

Multi-parameter flow cytometry

Flow cytometry measurements were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with a 488 and 633 nm excitation light source

from an argon ion laser. Green fluorescence from samples (corresponding to cFDA or SYBRgreen-stained cells) was collected on the FL1 channel (530 nm), whereas PI fluorescence was registered on the FL3 channel (610 nm). Each analysis was performed in duplicate at a low flow rate setting (4000 events s^{-1}). Data acquisition was carried out using Cytomics RXP software (Beckman Coulter). Gates and quadrants were established according to staining controls. For cFDA/PI dual-parameter flow cytometric analysis, data collected from 200,000 events were analyzed using Kaluza® Flow Analysis Software, Beckman Coulter. The fluorescent microspheres presented two types: A microspheres, excitable at 506 nm, and B microspheres, excitable between 365 and 650 nm.

Fluorescence and confocal microscopy

Stained samples were examined under a Leica TCSSP2-AOBS confocal laser scanning microscope (Leica Microsystems Inc., Heidelberg, Germany) at excitation wavelengths of 488 and 568 nm with an emission wavelength of 530 (green fluorescence) or 630 nm (red fluorescence) (Alonso et al. 2012), to check that the staining procedure was suitable for both bacteria (images not shown).

Analytical methods

Determinations of SA and SCN^- in the samples were performed by HPLC (Agilent 1200) using a Mediterranean sea18 column (5 µm × 25 cm × 46 cm, plus a reverse-phase column from Waters) combined with a UV detector. The limit of detection is up to 5 mg L^{-1}, so the calibration curves of both compounds were performed from 5 to 1000 mg L^{-1}, obtaining a correlation coefficient of 0.999 in both cases. The wavelengths used for detection of SA and SCN^- were 214 and 280 nm, respectively. Prior to HPLC analysis, all the samples were filtered through 0.45-µm PVDF filters. The mobile phase consisted in a mixture of two solutions: acetonitrile (solution A) and 0.4 % phosphoric acid in water (solution B) (Hsu et al. 2004). The method employed comprised a binary gradient from 30 % solution A to 95 % solution B at a constant flow rate of 0.7 mL min^{-1}. The column was maintained stable at a temperature of 40 °C during all the analyses. Retention times observed for SA and SCN^- were 5.5 and 9.4 min, respectively.

In the case of pure cultures, cell concentrations in the medium were measured by two techniques: spread plate counting on GM-agar and optical density measurement at 660 nm (Shimadzu, UV 1203). Data were converted into cell dry weight (g L^{-1}) using the corresponding calibration curves. Cell concentrations of co-culture experiments were measured by optical density at 660 nm and by flow cytometry, thus also obtaining the physiological status of the co-culture.

All experiments were carried out at least in duplicate, and each sample was analyzed in triplicate. Standard deviations (SD) obtained are shown as vertical lines in figures.

Results and discussion

Salicylic acid and thiocyanate biodegradation using pure cultures

First of all, the ability of each bacterium to remove both contaminants together or individually was studied.

Effect of SCN^- on the degradation of SA by P. putida

The biodegradation of SA acid by *P. putida* in absence or presence of SCN^- was studied in order to determine possible inhibitory effects between pollutants. So, an inoculum of *P. putida* was added into three PpMM containing 500 mg L^{-1} of SA, 500 mg L^{-1} of SA and 500 mg L^{-1} of SCN^- or 500 mg L^{-1} of SCN^-, the biodegradation conditions being maintained at 200 rpm, 30 °C and 100 mL of PpMM in 250-mL Erlenmeyer flasks. Figure 1 shows the evolution of salicylic acid and thiocyanate concentrations (Fig. 1a, b, respectively) and cell concentration (g L^{-1}) of *P. putida* (Fig. 1c) for the individual or simultaneous biodegradation runs of both pollutants.

Figure 1a shows that in absence of thiocyanate, 500 mg L^{-1} of SA was removed from the medium in just 22 h, reaching a specific and volumetric degradation rates of 2.5 mg SA (h mg cell)$^{-1}$ and 31 mg SA $(Lh)^{-1}$, respectively. The cellular growth (Fig. 1c) achieved a μ value of 0.2459 h^{-1}. However, when 500 mg SCN^- L^{-1} was added to the mineral medium, only 73 % of the initial SA concentration was removed. In addition, the removal of the SA occurred only during the first hour, remaining constant for higher times. In presence of thiocyanate, the maximum specific degradation rate, which was reached in the early hours of the process, was 0.92 mg SA (h mg cell)$^{-1}$ and then decreased. Nevertheless, the average volumetric degradation rate has a value of around 6.25 mg SA $(Lh)^{-1}$. Moreover, *P. putida* growth was not observed under these conditions (Fig. 1c). So, the presence of SCN^- in the medium negatively affects the SA removal by *P. putida*, since it completely inhibited the growth of the bacterium, reducing by half the specific SA biodegradation rate.

A similar behavior was described by Arutchelvan et al. (2005) for the biodegradation of phenol by the strains *P.*

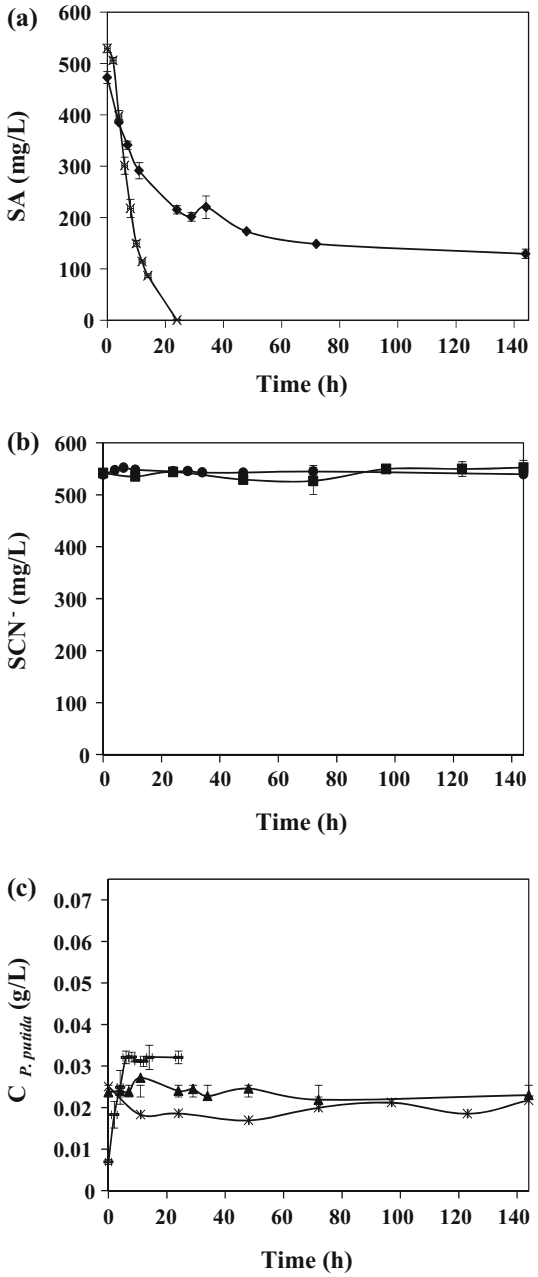

Fig. 1 Evolution of salicylic acid and thiocyanate concentrations and growth curves of *Pseudomonas putida* in PpMM. **a** Evolution of SA concentration in absence (*cross*) or presence (*diamond*) of SCN^-. **b** Evolution of SCN^- concentration in absence (*square*) or presence of SA (*circle*). **c** Growth curves for *Pseudomonas putida* in PpMM with SCN^- (*asterisk*), SA (*line*) or both (*triangle*). In all cases: initial SA concentration: 500 mg L^{-1}, initial SCN^- concentration: 500 mg L^{-1}, 200 rpm, 30 °C, 100 mL in 250-mL Erlenmeyer flasks

cepacia and *B. brevis* in presence of thiocyanate in Erlenmeyer flasks. These authors pointed out that thiocyanate concentrations up to 1000 mg L^{-1} did not inhibit the phenol degradation by *P. cepacia* and *B. brevis*, whereas, for concentrations of thiocyanate higher than 1500 mg L^{-1}, the rate of degradation was reduced considerably for *P. cepacia*. In the case of *B. brevis,* this inhibitory effect of the thiocyanate on the phenol biodegradation was more marked.

Furthermore, SCN^- biodegradations by *P. putida* in presence and absence of SA are compared in Fig. 1b. Findings revealed that the removal capacity of SCN^- by *P. putida* (Fig. 1b) of mineral medium was negligible; after 150 h, there were no changes in SCN^- concentration in either presence or absence of SA in the mineral medium. Regarding the bacterial growth, a decrease in the biomass concentration was observed at the beginning of the biodegradation when only SCN^- was in the mineral medium, while the biomass was remaining relatively constant when SA was also present in the process (Fig. 1c).

Effect of SA on the degradation of SCN^- by P. thiocyanatus

In a similar way, the influence of SA presence in SCN^- biodegradation by *P. thiocyanatus* was also studied. For this purpose, an inoculum of *P. thiocyanatus* was added into PtMM containing 500 mg L^{-1} of SCN^-, or 500 mg L^{-1} of SCN^- and 500 mg L^{-1} of SA or 500 mg L^{-1} of SA, with the biodegradation conditions being always maintained at 250 rpm, 28 °C and 100 mL of PtMM in 500-mL Erlenmeyer flaks. Figure 2 shows the evolution of the concentrations of SCN^-, SA and *P. thiocyanatus* obtained during the biodegradation by *P. thiocyanatus* for the three experiments.

As shown in Fig. 2a, when SA was not present in the medium, 500 mg L^{-1} of SCN^- was completely removed in just 48 h, reaching a maximum specific degradation rate of 0.32 mg SCN^- (h mg cell)$^{-1}$ and a volumetric degradation rate of 20.70 mg SCN^-(h L)$^{-1}$ (not considering the initial induction time). The cellular growth (Fig. 2c) achieved a maximum μ value of 0.0314 h^{-1}. The presence of an initial stage of around 20 h where the SCN^- concentration did not change, corresponding probably to the acclimation of *P. thiocyanatus* to the new conditions, is remarkable.

However, when the SA was added to the mineral medium, *P. thiocyanatus* needed 144 h to completely remove the SCN^-. In the same manner, the acclimation of the bacteria required 48 h, twice the time needed in absence of SA. In this case, the maximum specific degradation rate was 0.35 mg SCN^- (h mg cell)$^{-1}$ [the volumetric degradation rate was 6.7 mg (SCN^-(h L^{-1}))], this value being very similar to that obtained in absence of SA. Therefore, it can be concluded that the SA had a negative impact on the initial acclimation of the *P. thiocyanatus*, but not on its final SCN^- assimilation rate. With regard to *P. thiocyanatus* growth curves, the presence of SA also had an adverse effect, considerably decreasing the growth rate of the bacterium. So, a slight cellular bacterial growth

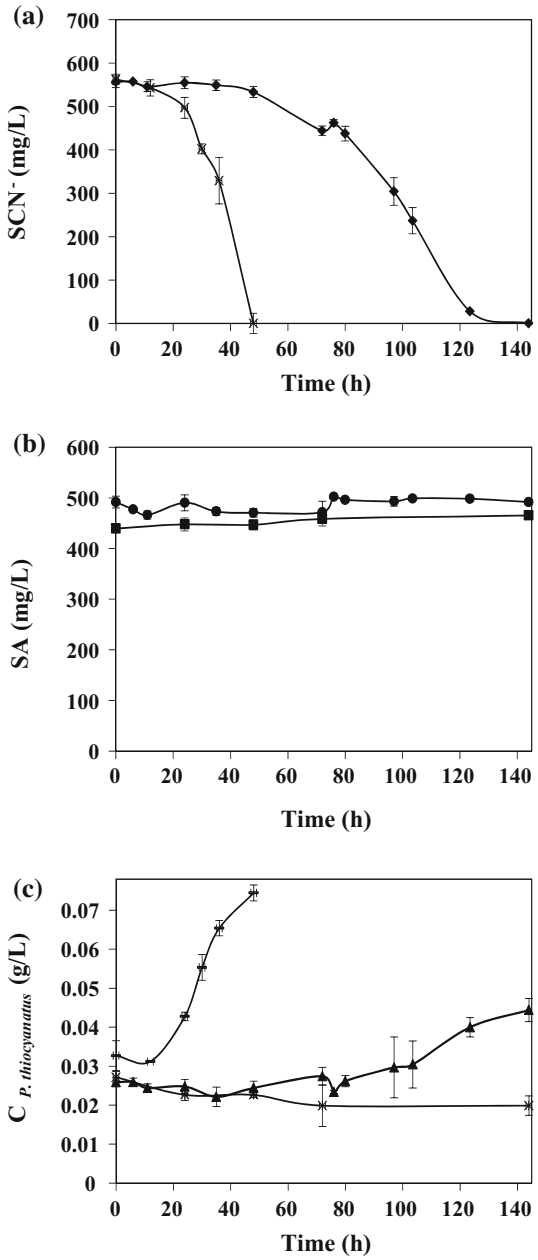

Fig. 2 Evolution of thiocyanate or salicylic acid concentrations and growth curves of *Paracoccus thiocyanatus* in PtMM. **a** Evolution of SCN^- concentration in absence (*cross*) or presence (*diamond*) of SA. **b** Evolution of SA concentration in absence (*square*) or presence of SCN^- (*circle*). **c** Growth curves for *Paracoccus thiocyanatus* in PtMM with SA (*asterisk*), SCN^- (*line*) or both (*triangle*). In all cases: initial SCN^- concentration: 500 mg L^{-1}, initial SA concentration: 500 mg L^{-1}, 250 rpm, 28 °C, 100 mL in 500-mL Erlenmeyer flasks

(Fig. 2c) was observed, obtaining a μ value of 0.0093 h^{-1}. It can be also noted that the bacterial growth and the pollutant degradation started at the same time. Comparing these results, it can be confirmed that SA presence increased the acclimation period of the *P. thiocyanatus,* adversely affecting SCN^- removal.

Kwon et al. (Kwon et al. 2002) also studied the removal of thiocyanate by *Acremonium strictum* and the inhibition by secondary toxicants in shake-flask cultures. In that research, 1200 mg L^{-1} of SCN^- was completely degraded in presence of 312 mg L^{-1} of phenol although the complete degradation of thiocyanate was delayed from 23 h without the addition of phenol to 60 h. For a phenol concentration of 625 mg L^{-1}, thiocyanate degradation was seriously inhibited and only 33 % of thiocyanate was removed after 60 h. Shivaraman et al. (Shivaraman et al. 1985) showed that phenol and cyanide had a negative influence on thiocyanate degradation.

As seen in Fig. 2b, the removal capacity of SA by *P. thiocyanatus* was checked too. The SA removal by *P. thiocyanatus* was negligible in either absence or presence of SCN^-. So, after 150 h, the SA did not change in both cases. A progressive decrease in biomass was observed when only SA was present in mineral medium. However, the biomass slightly increased at the end of the process when SA and SCN^- were simultaneously present in mineral. In view of the results, it can be stated that during the simultaneous SA and SCN^- biodegradation by a co-culture of *P. thiocyanatus* and *P. putida*, each bacterial strain degrades only SCN^- or SA, respectively.

Effect of the culture conditions on P. putida degradation

The effects of biodegradation conditions and medium composition were tested. An inoculum of *P. putida* was introduced into PpMM or CCMM, containing 500 mg L^{-1} of both contaminants and maintaining the biodegradation conditions selected for *P. putida* or co-culture shown in *Materials and methods* section. Figure 3 shows the effect of the biodegradation conditions or culture medium on SA (Fig. 3a) and SCN^- (Fig. 3b) removal and cell growth of *P. putida* (Fig. 3c).

In SA and SCN^- biodegradation by *P. putida* in PpMM using the co-culture conditions, the results show a clear improvement in the biodegradation rate, (S1) when compared with those obtained selecting the pure culture conditions. However, *P. putida* was unable to metabolize SCN^- from the medium. It must be mentioned that the only difference between both experiments is the stirring speed, 200 rpm or 250 rpm for the initial or the co-culture conditions, respectively. The modification of the stirring speed supposed doubling of the degradation rate of *P. putida* [6.25–13.6 mg SA $(L\ h)^{-1}$]. This change was mainly due to a significant increase in the biomass growth (0.0007–0.2338 h^{-1}) since the specific degradation rate remained constant. This fact suggests that higher concentration of dissolved oxygen in the medium improves the SA removal capacity of *P. putida* reducing correspondingly the convection resistance (Díaz et al. 1996). Hadibarata and

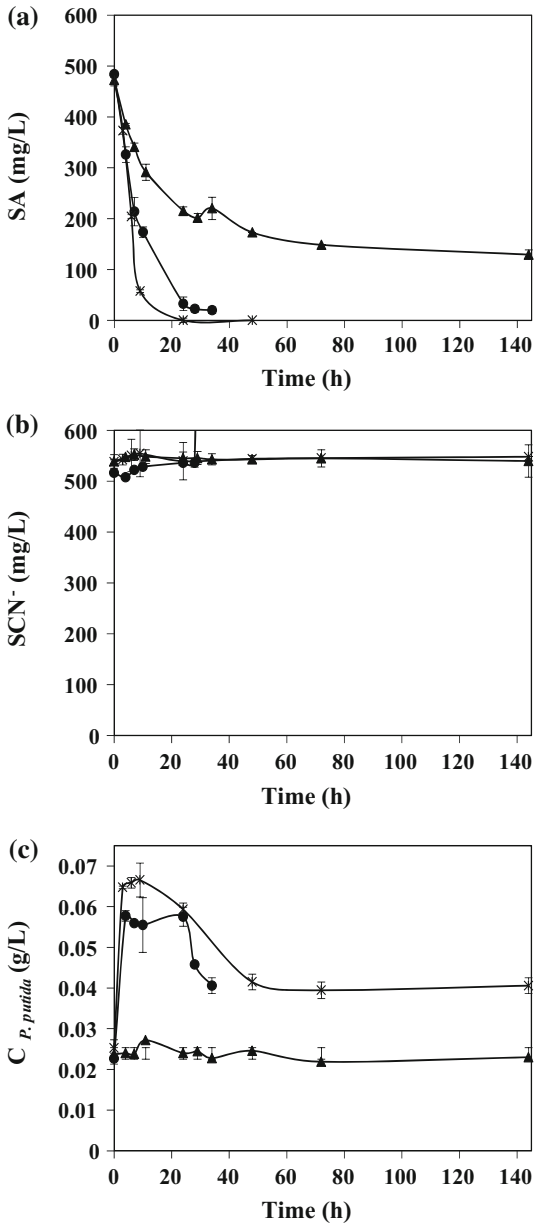

Fig. 3 Evolution of salicylic acid (**a**) or thiocyanate (**b**) concentrations and growth curves (**c**) of *Pseudomonas putida* under different biodegradation conditions: recommended conditions and medium for pure culture (*triangle*), recommended medium for pure culture but co-culture conditions (*circle*) or co-culture medium and conditions (*asterisk*). In all cases: initial SA concentration: 500 mg L^{-1}, initial SCN^- concentration: 500 mg L^{-1}, 30 °C

Teh (Hadibarata and Teh 2014) show that agitation ensures the supply of nutrients and oxygen in the system.

The SA biodegradation can even be more improved using also the CCMM instead of PpMM (see data in S1). Nevertheless, either the new conditions or the new medium did not allow removing SCN^-. In this regard, there was a more marked increase in cell growth (μ value of 0.3137 h^{-1}), with the consequent increase in the degradation rate [0.86 (mg SA (h mg cell)$^{-1}$] when the recommended medium was replaced by the co-culture one. This fact is probably due to the presence of the trace elements solution in the composition of the co-culture medium. In this sense, Qiao and Wang (2010) also observed a similar behavior; they proved that trace mineral elements were the essential substrates for pyridine degraded effectively by *Paracocccus* sp strain KT-5.

Effect of the culture conditions on P. thiocyanatus

As in the case of *P. putida*, the effects of biodegradation conditions and medium composition on *P. thiocyanatus* were also evaluated. In this case, an inoculum of *P. thiocyanatus* was introduced into PtMM or CCMM, containing 500 mg L^{-1} of both contaminants and maintaining the biodegradation conditions recommended for *P. thiocyanatus* or selected for co-culture. Figure 4 shows the evolution of the SCN^- (Fig. 4a) and SA (Fig. 4b) concentrations and the *P. thiocyanatus* growth curves (Fig. 4c) obtained using the aforementioned conditions and media.

Summarizing the results of the effect of experimental conditions in PtMM, the change in two degrees in the incubation temperature is barely noticeable for either the specific degradation rate of SCN^- or cellular growth values (see the result in S1). The increase in the temperature either had effect on the SCN^- removal; its concentration remained constant either at 28 or at 30 °C. Cai et al. (Cai et al. 2013) show that the optimal temperature range for *Paracoccus* sp. is between 30 and 35 °C, range in which a small change in temperature marks a very slight shift in cell growth.

Regarding the effect of the medium composition, a decrease in the lag phase (30 h) and an increase in cell growth (0.0186 h^{-1}) were obtained by adding tryptone as additional source of N. The results in this case were similar to the values obtained in individual SCN^- biodegradation by *P. thiocyanatus*. However, SA was again not removed from the mineral medium. This suggests that the additional nitrogen sources were utilized for biomass production. Similar behavior was observed by Chaudhari and Kodam (2010) with co-culture of *Klebsiella pneumoniae* and *Ralstonia* sp. and by Combarros et al. (2015) with *P. thiocyanatus*.

Simultaneous biodegradation of thiocyanate and salicylic acid by co-culture

Once the effect of the pollutants on each bacterium has been evaluated and the best operating conditions for co-culture have been selected, the simultaneous biodegradation of SA and SCN^- in CCMM by *P. putida* and *P. thiocyanatus* co-culture was tested. The biodegradation conditions were 30 °C, 250 rpm and 100 mL of CCMM in

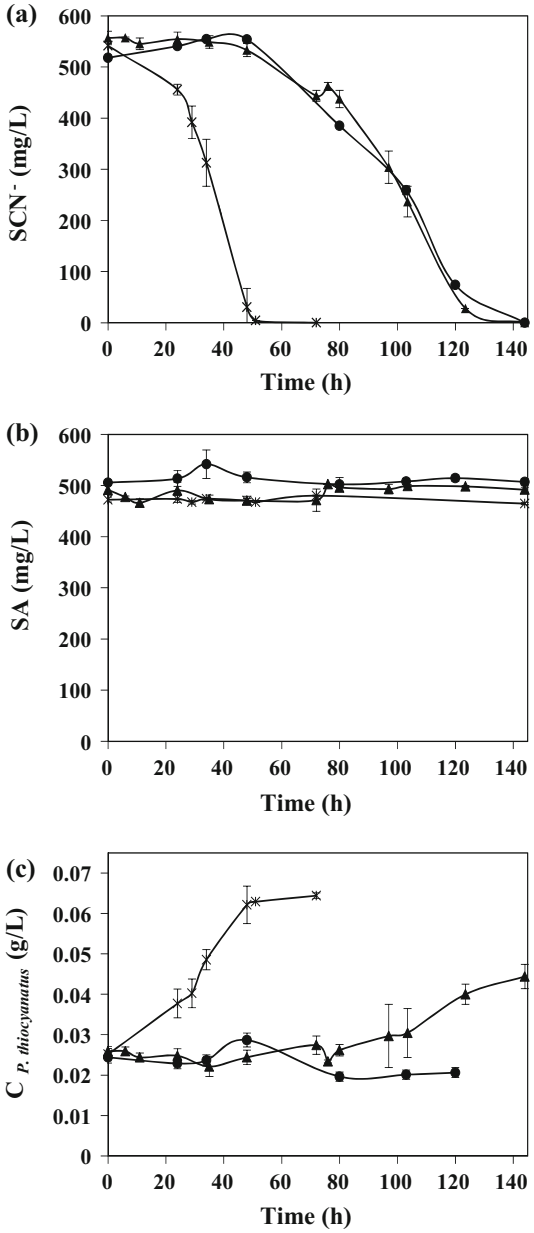

Fig. 4 Evolution of thiocyanate (**a**) or salicylic acid (**b**) concentrations and growth curves (**c**) of *Paracoccus thiocyanatus* under different biodegradation conditions: recommended conditions and medium for pure culture (*triangle*), recommended medium for pure culture but co-culture conditions (*circle*) or co-culture medium and conditions (*asterisk*). In all cases: initial SCN^- concentration: 500 mg L^{-1}, initial SA concentration: 500 mg L^{-1}, 250 rpm, 100 mL in 500-mL Erlenmeyer flasks

500-mL Erlenmeyer flasks. Initial cells concentrations of 10^7 cfu mL^{-1} were inoculated into de CCMM for each bacterium.

First, the behavior of the co-culture in a medium containing only SA or SCN^- was studied (see graph S2 in supplementary data). For the co-culture in presence of only SA, the SA removal was not affected by the presence of *P. thiocyanatus*. However, for the co-culture containing only SCN^-, the presence of *P. putida* involved a worsening of the SCN^- biodegradation process by *P. thiocyanatus* since the time required for SCN^- depletion was increased in 10 h. This effect was particularly marked during the acclimation period, increased from 11 to 17 h by the presence of *P. putida*.

Afterward, the effect of the initial concentrations of both contaminants (between 250 and 4000 mg L^{-1}) on the co-culture was evaluated. Figure 5 shows the biodegradation of both contaminants (thiocyanate and salicylic acid) and the cell growth for six different initial concentrations. In all cases, the medium pH was between 6.70 and 7.20.

The first thing to be pointed out is the complete removal of either SA or SCN^- for initial concentrations of both pollutants up to 2000 mg L^{-1}. However, each pollutant showed a different behavior during the biodegradation. SA removal occurred at the beginning of the process following a constant degradation rate, whereas thiocyanate needed an acclimation period to begin decreasing. This behavior was also observed for pure culture of *P. thiocyanatus*.

Obviously, the higher the initial concentration of the pollutant, the higher the time required for its complete removal. So, complete thiocyanate degradation was achieved after 25, 29 and 46 and 170 h with initial concentrations of 250, 500, 1000 and 2000 mg L^{-1}, respectively. In a similar way, 9, 12, 34 and 110 h were necessary for the complete depletion of 250, 500, 1000 and 2000 mg L^{-1} of SA, respectively. As can be seen, the SA disappeared rather faster than the SCN^-. In fact, Fig. 5a–d suggest that the biodegradation of SCN^- only starts when the SA is exhaust in the medium. Moreover, an induction period in thiocyanate removal was also observed, this lasting until the SA was completely removed from the mineral medium. This sequential removal probably is due to the double inhibitory effect of SA on the SCN^- biodegradation and on the lag phase of the *P. thiocyanatus*. Therefore, when the SA is removed by *P. putida*, its inhibitory effect on *P. thiocyanatus* also disappears and the bacterium recovers its activity. Banerjee (1996) observed a similar behavior during the biodegradation of a synthetic wastewater by activated sludge in a four-stage across-the-flow RBC reactor. According to these authors, phenol was mostly removed in the first two stages, whereas thiocyanate removal was greater in the last two stages, where inhibition due to the presence of phenol was much less. In the work of Li et al. (2011), in which a coke-oven wastewater containing phenolic compounds and thiocyanate was treated with activated sludge in a MBBR reactor, it was also observed that thiocyanate degradation rate had two different values, being smaller when the concentration of phenolic compounds in the medium was higher, while this rate increased as the phenolic compounds concentration decreased.

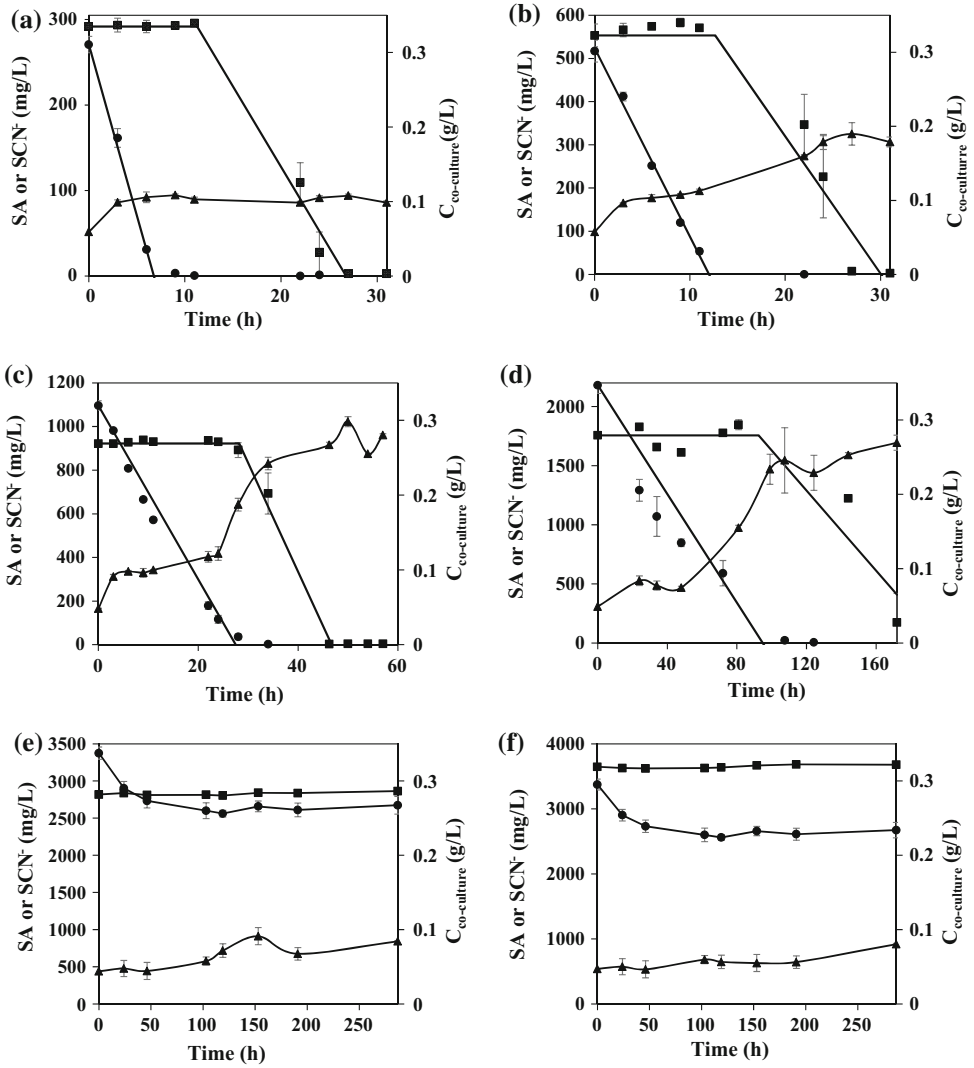

Fig. 5 Evolution of salicylic acid (*circle*) or thiocyanate (*square*) concentrations and growth curves (*triangle*) during the simultaneous biodegradation of both contaminants by a co-culture of *Paracoccus thiocyanatus* and *Pseudomonas putida* using different initial SCN^- and SA concentrations **a** 250 mg L^{-1}, **b** 500 mg L^{-1}, **c** 1000 mg L^{-1}, **d** 2000 mg L^{-1}, **e** 3000 mg L^{-1}, **f** 4000 mg L^{-1}. In all cases: CCMM, 30 °C, 250 rpm, and inoculum size of (1×10^7 cfu mL^{-1} of both bacteria). *Solid lines* in figures denote fitting of thiocyanate and salicylic acid biodegradation to a pseudo-zero-order kinetics model

When the initial concentrations were 3000 or 4000 mg L^{-1}, no removal of thiocyanate was observed. At the same time, salicylic acid concentration decreased slowly during the first 50 h and then it maintained a constant value until the end of the experiment. Thus, only 23 and 11.5 % of the initial SA amount was eliminated when the initial concentrations were 3000 and 4000 mg L^{-1}, respectively.

Figure 5 also reveals that the SA removal by *P. putida* is faster when *P. thiocyanatus* is also present in the medium. This behavior was attributed to the formation of one or several intermediates during the degradation of SA, which can be easily assimilated by both species. In this respect, the presence of *P. thiocyanatus* establishes a competence with *P. putida* for these intermediates. The lower bioavailability of these compounds, in turn, causes that *P. putida* focused its activity on the biodegradation of the parent compound, forming more intermediates and increasing also the removal rate of SA. Additionally as result of consumption of these intermediates, *P. thiocyanatus* is metabolically more active, decreasing Lag time and increasing the specific degradation rate of SCN^-. (See S1, row 10 and 15).

To validate this theory, *P. putida* was initially inoculated in CCMM containing 500 mg L^{-1} of SA and, after periods of time of 4.5, 10 or 22 h, the media were centrifuged and filtered through 0.22-μm filters in order to remove *P. putida*. Afterward, *P. thiocyanatus* was inoculated in these media. As can be seen in figure S3, *P. thiocyanatus* grew in the three media, corroborating the formation of intermediates during SA biodegradation, which can be easily assimilated by *P. thiocyanatus*. The growth was more marked with the medium obtained after 10 h of biodegradation. This can be explained if the concentration of intermediates is maximum in this case. It is logical to think that for 4.5 h, the concentration was lower

due to a lower degree of degradation of SA. For 22 h, the concentration of intermediates is lower because SA was completely removed and the intermediate is the only available carbon source, this being assimilated by *P. putida*. This theory also was validated by measuring the TOC of the samples of the SA–SCN^- biodegradation by co-culture; the results show that the intermediates TOC ($TOC_{Intermediates} = TOC_{Total} - 7 \times C_{SA} - C_{SCN}^{-}$) increase until 11 h of around 204 mg CL^{-1}; after that the TOC concentration declines gradually with time. The TOC measurements also reveals that, although the SCN^- and SA were completely eliminated from the medium, there is always a remnant of about 10 % of the TOC at the end of the experiments (final inert products, non-biodegradable by *P. putida* and *P. thiocyanatus*).

Figure 5 also shows the evolution of cell concentration in mg L^{-1} for each of the initial SCN^- and SA concentrations. An increase in biomass concentration was observed for all initial contaminants concentrations. This increase was more marked for initial concentrations of 500, 1000 and 2000 mg L^{-1}. It is also noticeable that the growth curves showed two phases. The first one, with a faster cell growth, coincides with the degradation of SA, and it is probably due to the growth of *P. putida*. In turn, the second phase of growth and the SCN^- biodegradation occurred at the same time, suggesting the growth of *P. thiocyanatus*. It can be noted that specific growth rates for *P. putida* are higher than for *P. thiocyanatus*.

As can be observed in Fig. 5, the evolution of the concentrations of either SA or SCN^- was successfully fitted to a zero-reaction-order model including term for the induction time (t_i):

$$C_i = C_{i,0} - k(t - t_i) \quad (1)$$

where i is the pollutant (SA or SCN^-) and k is the pseudo-zero-order kinetic constant, which is the same as the volumetric degradation rate. The obtained values of t_i and k during the fitting are summarized in Table 1. In view of the results for the SCN^-, the higher the initial concentration, the longer the period of induction, as might be expected. With SA, the kinetic constant initially increased with the initial concentration, and then it showed a maximum at an initial concentration of around 500 mg L^{-1} (43.6 mg $(L\ h)^{-1}$) and finally decreased, reaching a value of zero for initial concentrations higher than 2000 mg L^{-1}. The SCN^- kinetic constant also showed this behavior except that the maximum was obtained for an initial concentration of 1000 mg L^{-1} (49.8 mg$(L\ h)^{-1}$), indicating that *P. thiocyanatus* has higher tolerance for SCN^- than *P. putida* for SA. Degradation rates between 1 and 87 mg$(L\ h)^{-1}$ were reported for the biodegradation of thiocyanate by other microorganisms (Chaudhari and Kodam 2010; Kim and Katayama 2000; Kwon et al. 2002; Sorokin et al. 2004) similar to the degradation rates obtained in this work. Furthermore, Kwon et al. (Kwon et al. 2002) confirmed that the presence of phenol reduced the thiocyanate degradation rate from 52 mg$(L\ h)^{-1}$ to 6.6 mg$(L\ h)^{-1}$ when phenol concentration increased from 0 to 625 mg L^{-1}. Degradation rates between 3 and 23 mg$(L\ h)^{-1}$ were reported for the biodegradation of salicylic acid by other microorganisms (Juang and Tsai 2006; Loh and Yu 2000), the values lower than those obtained in this work. Sharma et al. (Sharma et al. 2012) studied the effect of the presence of cyanides on the biodegradation of phenolic compounds by a mixed culture: 800 mg L^{-1} of phenol was eliminated at a rate of 32 mg$(Lh)^{-1}$, but when CN^- was added to the medium, the degradation rate decreased to 10.4 mg$(Lh)^{-1}$.

Comparing these results from Figs. 3 and 4, it can be concluded that the presence of *P. thiocyanatus* has no effect on the SA bioremoval rate by *P. putida*. Concerning SCN^-, the time required for its complete depletion by *P. thiocyanatus* when *P. putida* was present or not in the medium was 29 h or 51 h, respectively. As explained earlier, this increase in removal rate of SCN^- is due to the reduction in the inhibitory effect of the SA, which is progressively assimilated by *P. putida*. In the case of co-culture, the value of μ was 0.0295 h^{-1}, considering the total optical density, a value that lies between (0.3137–0.0186) h^{-1} for the pure cultures of *P. putida* and *P. thiocyanatus*.

Regarding the specific degradation rates in the co-culture, it was observed for all the initial concentrations that it increased during the first minutes, achieving a maximum, and then decreased due to the depletion of the substrates. With respect to the effect of the initial concentrations of the pollutants, the specific degradation rate in the case of SA had a maximum value of 0.61 (mg SA(mg cell h) $^{-1}$) when initial SA and SCN^- concentrations were 1000 mg L^{-1},

Table 1 Induction periods and pseudo-zero-order kinetic constants obtained at different initial concentrations of thiocyanate and salicylic acid by a co-culture of *Paracoccus thiocyanatus* and *Pseudomonas putida*

SA_0 (mg L^{-1})	SCN_0^- (mg L^{-1})	SA		SCN^-	
		t_i (h)	k [mg/(L h^{-1})]	t_i (h)	k [mg/(L h^{-1})]
270.7	291.9	0	39.9	11.3	18.8
516.9	553.1	0	43.6	12.7	31.8
1096.4	921.7	0	39.8	28.2	49.8
2180.9	1756.2	0	14.0	92.4	16.9

whereas the maximum specific degradation rate for SCN^- was 0.40 (mg SCN^-(mg cell h)$^{-1}$), being achieved when the initial concentrations were 500 mg L^{-1}. In all cases, the specific degradation rate obtained during simultaneous SA and SCN^- biodegradation by the co-culture is an order of magnitude higher than that obtained by Banerjee (Banerjee 1996). Phenol-specific degradation rate value obtained was 0.021 mg SA(mg VSS h)$^{-1}$ in RBC (rotating biological contactor reactor) first stage, while SCN^--specific degradation rate was 0.026 mg SCN^- (mg VSS h)$^{-1}$ in the final stage of RBC reactor.

As it was previously explained, the growth curves showed two faster increments. The first one was due to the growth of *P. putida* during the biodegradation of the SA, whereas the second one coincided with the biodegradation of SCN^-, so it was attributed to the growth of *P. thiocyanatus*. In order to corroborate this assumption, *P. putida* growth using the co-culture medium and conditions was compared in presence and absence of *P. thiocyanatus* (S4). As can be seen, the growth of a pure culture of *P. putida* coincided exactly with the initial growth observed in the co-culture, so this fact suggests the sequential growth of the bacteria. In the same way, *P. thiocyanatus* growth using the co-culture medium and conditions in presence and absence of *P. putida* were compared (S4). The bacterial growth in co-culture is greater than in the pure culture of *P. thiocyanatus*, probably due to the fact that SA had been previously removed from the medium by *P. putida*. A summary of the different tests and results obtained is given in Table S1 in the supplemental data.

So, assuming a separated growth, the growth curves for each of bacteria during the co-culture experiments can be estimated. Using these data, the specific growth rates can be calculated for both bacteria: μ_{Pp} and μ_{Pt}. The specific growth rates (μ_i) versus salicylic acid or thiocyanate initial concentrations obtained from co-culture experiments are shown in S5. At this point, it is important to note that *P. putida* grows in presence of SA and SCN^-, whereas only SCN^- was present in the medium when *P. thiocyanatus* started to grow rapidly. For this reason, a substrate and toxic inhibition model (Eq. 2) was proposed for the fitting of the specific growth rate of *P. putida* in the co-culture. This model consists of a modification of Tessier model, in which a term defining the toxic inhibition by SCN was added (S5):

$$\mu = \left(\mu_{max} \cdot e^{-S/K_i} - e^{-S/K_s}\right) \cdot \left(1 - \frac{P}{P^*}\right)^n \quad (2)$$

where P is the concentration of toxic (SCN^-), P^* is the concentration of the toxic that slows the reaction absolutely and n is the reaction order. In our case, P^* has a value of 4000 mg SCN^- L^{-1}, as can be observed in Fig. 5.

The next fitting parameters were obtained for the growth of *P. putida* in co-culture with *P. thiocyanatus*: $\mu_{Pp\text{-}max} = 0.052\ h^{-1}$, $K_s = 552.3$ mg L^{-1}, $K_i = 2570.13$ - mg L^{-1}, $n = 0.54$; $r^2 = 0.99$. In a previous work (Combarros et al. 2014), the specific growth rates were obtained for a pure *P. putida* culture with Haldane equation for SA concentrations between 0 and 530 mg L^{-1}. The values obtained were μ_{max} of 0.25 h^{-1}, K_s of 61.25 mg L^{-1} and K_i of 704.93 mg L^{-1}. Comparing these results, the value of μ_{Ppmax} in the co-culture is an order of magnitude lower than for the pure culture, while both K_s and K_i are higher, indicating that *P. putida* cells show a higher affinity and tolerance to SA in co-culture form.

In the case of the growth of *P. thiocyanatus*, where the SA was not present in the medium, the experimental data were successfully fitted to a Tessier model (S5). The next fitting parameters were obtained $\mu_{Ptmax} = 0.08\ h^{-1}$, $K_s = 329.2$ mg L^{-1}, $K_i = 755.5$ mg L^{-1} with a $r^2 = 0.97$. In a previous work, Combarros et al. (2015) reported the next parameters for Tessier model using a pure *P. thiocyanatus* culture and SCN^- as only substrate: $\mu_{max} = 0.334\ h^{-1}$, $K_s = 1150$ mg L^{-1} and K_i of 1730 mg L^{-1}. These results suggest that the substrate inhibitory effect on the *P. thiocyanatus* is more marked in the co-culture than in the pure culture.

Physiological status of co-culture of *P. thiocyanatus* and *P. putida* during shake-flask biodegradation

Figure S6 in supplementary data shows the changes in the physiological status of co-culture in terms of metabolic activity and membrane integrity during shake-flask biodegradation. The different panels of S6 illustrate the dual flow cytometric assessment of green fluorescence versus red fluorescence (cFDA/PI). The cFDA is cleaved by the esterase activity inside the living cells, thus releasing a polar fluorescent portion which is unable to pass through the intact membrane. The PI can only cross the plasmatic membrane if it is permeabilized, corresponding to those cells whose membranes are compromised. Taking this into account, the upper left gate (viable and active) of dot plots in S6 shows cells with esterase activity and membrane integrity (cFDA(+), PI(−)). Damaged cells (cFDA(+)/PI(+)) are shown in the upper right gate (damage), being bacteria active but no viable. Likewise, dead cells (cFDA(−), PI(+)) are shown in the lower gate.

Results based on esterase activity and membrane integrity suggest that the loss of metabolic activity was progressive, leading first to an intermediate "damaged" cell state and then irreversibly to cell death. Cytograms show that in the case of the lower SA and SCN^- concentrations, there was a cellular growth and these cells remained within the viable cells gate (Fig. S6A, S6B and

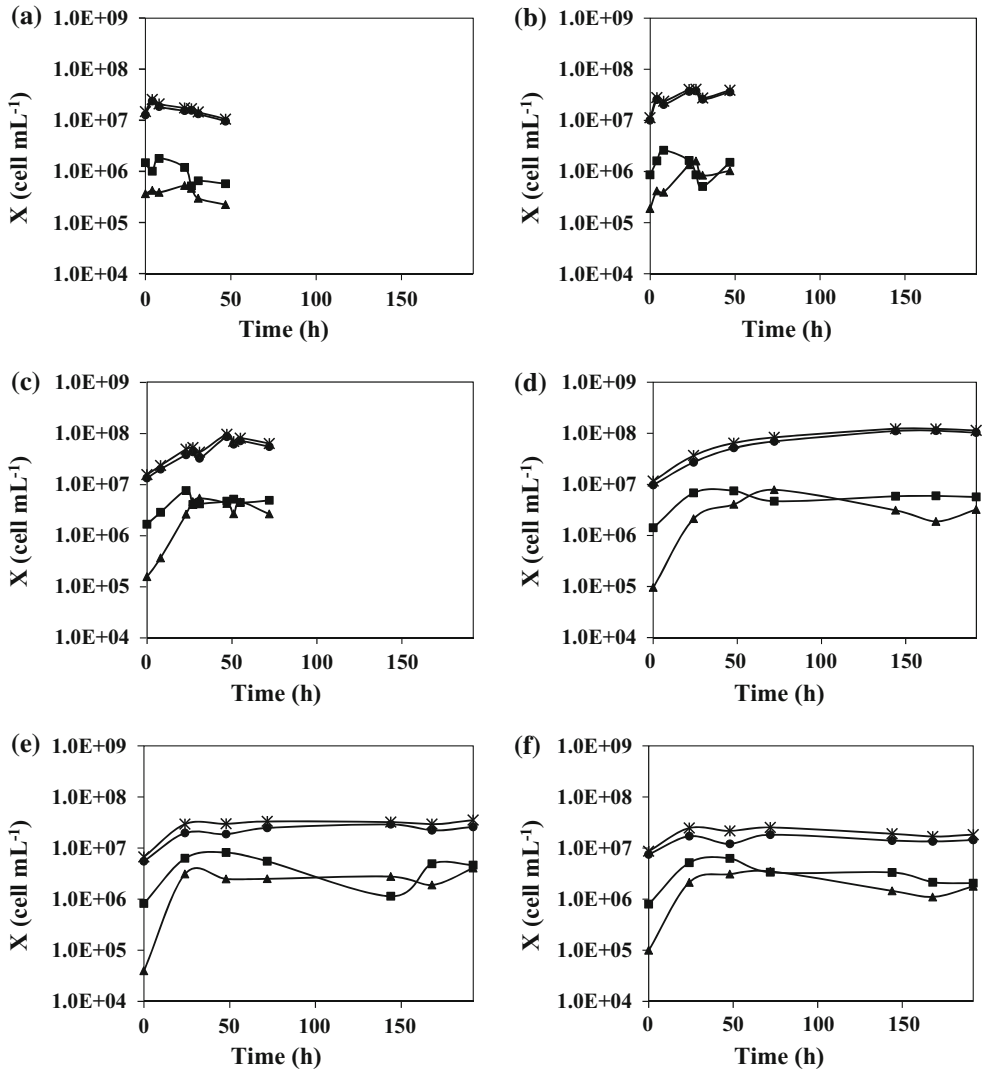

Fig. 6 Cell subpopulations concentration of *Paracoccus thiocyanatus* and *Pseudomonas putida* co-culture throughout the shake-flask of thiocyanate and salicylic acid biodegradation under different initial SCN^- and SA concentrations. **a** 250 mg L^{-1}, **b** 500 mg L^{-1}, **c** 1000 mg L^{-1}, **d** 2000 mg L^{-1}, **e** 3000 mg L^{-1}, **f** 4000 mg L^{-1} (*circle* viable cells, *triangle* damage cells, *square* dead cells, *asterisk* total cells). In all cases: CCMM, 30 °C, 250 rpm, and inoculum size of 1×10^7 cfu mL^{-1} of both bacteria

S6C). The increase in SA and SCN^- initial concentrations caused an increase in total cell concentration but, at the same time, provoked an increase in the percentages of dead and damaged subpopulations (Fig. S6D, S6E and S6F). Finally, for 3000 mg L^{-1} the growth decreased, whereas no cell growth was observed for the concentration of 4000 mg L^{-1} and cells were moved into dead and damaged gate, these results corroborating the no biodegradation of the pollutants.

In supplementary data, Fig. S7 shows representative dot plot diagrams of side scatter (SSC) versus forward side scatter (FSC) signal obtained during simultaneous biodegradation of 3000 mg L^{-1} of both salicylic acid and thiocyanate. As can be seen in these cytograms, an increase in the intensity of the FSC was evidenced in the flow cytometric dot plots during the experiment. Cells of co-culture exhibited both higher relative cell size (forward scatter, FSC) and cell complexity signals (sid scatter, 90°-angle scatter of blue laser light, SSC) with age for all the initial concentrations used. Therefore, examination of these cytograms (Figs. S6 and S7) reveals that shake-flask cultivation displayed marked physiological heterogeneity in terms of metabolic status, membrane integrity and cellular size. A similar behavior was observed in the work of Alonso et al. (Alonso et al. 2012) during the production of lactobionic acid by *P. teatrolens.*

In Fig. 6, the subpopulation concentrations are shown depending on initial SCN^- and SA concentration. Comparing total and viable cells concentrations, it can be noted that both subpopulations had a similar behavior; the

bacterial concentrations (cells mL^{-1}) increased in the early stages of SA and SCN^- biodegradation, reaching a maximum value, and then this maximum value was kept constant until the end of the experiment (Fig. 6a–c). This finding demonstrates that low concentrations of SA and/or thiocyanate in the co-culture medium do not involved a loss in the viability of the mixed population. Up to concentrations of 2000 mg L^{-1} (Fig. 6e, f), the higher the SA and SCN^- initial concentration, the higher the population of viable cells. However, for initial concentrations above 2000 mg L^{-1} (Fig. 6d), this trend was reversed and an increase in the initial concentration caused a reduction in the total bacteria and, at a time, in the proportion of the viable subpopulation. It is remarkable that ups and downs in the viable cells concentration were observed. These changes in viable cells tendency coincide with the moment in which the SA was completely assimilated and SCN^- began to be removed, that is, when *P. putida* stopped growing and *P. thiocyanatus* growth began. Regarding the damaged subpopulation, its concentration (Fig. 6a) remained constant at the beginning and decreased at the end of the process when the initial concentration was 250 mg L^{-1}, probably due to cellular lysis phenomena. However, with higher SA and SCN^- concentrations, a significant increase in damage cells concentration was observed during the first 24 h, this value remaining constant during the rest of the process. The higher the initial concentrations, the more marked the increase in the proportion of damaged bacteria. Concerning the dead bacteria, its concentration in the experiments with 250 and 500 mg L^{-1} of SA and SCN^- (Fig. 6a, b) firstly increased and then slightly decreased, probably also due to the cellular lysis. For concentrations higher than 1000 mg L^{-1} (Fig. 6c) the cellular death makes more significant; in fact, the dead subpopulation when initial concentrations were 3000 or 4000 mg L^{-1} (Fig. 6e, f) was around the 15 and 17 % of the total bacteria. In all cases discussed so far, the increase in dead cells occurred mainly during the removal of SA, when both pollutants are present at high concentrations.

To obtain a deeper knowledge about the effect of high concentration of pollutant on the viability and activity, the next test was conducted: A co-culture was initially exposed during 120 h to a medium that contained 4000 mg L^{-1} of both pollutants, afterward, the biomass was recovered by centrifugation and subsequently inoculated into a new CCMM containing 500 mg L^{-1} of both SA and SCN^-, and the evolutions of both pollutants were followed over time (S8). The thiocyanate was not degraded during the first 50 h, whereas in the case of SA, an induction time was observed. In addition, the previous exposure of the co-culture to high concentrations involved a decreased in the specific degradation rate from 0.53 (mg SA(mg cell h)$^{-1}$ to 0.35 (mg SA(mg cell h)$^{-1}$). These findings corroborate that the exposure to high concentrations of both pollutants had a significant effect on the physiological status of *P. putida*.

In addition, cytometry samples were analyzed by confocal laser microscope to corroborate the results obtained. The observation of stained samples under fluorescence microscopy also confirmed that *P. putida* and *P. thiocyanatus* co-culture cells were predominantly metabolically active at the initial time of biodegradation, whereas cells were transformed into damage and dead cells at the end of the process. Moreover, aggregates of dead cells were detected during the later stages of cultivation. Interestingly, the observed increase in light scattering properties during flow cytometry analysis (S7) could be ascribed to this phenomenon, as was previously reported by Alonso et al. (2012) for *Pseudomonas taetrolens* cultures.

Conclusion

A complete removal of either SA or SCN^- by co-culture was observed for initial concentrations up to 2000 mg L^{-1} showing a behavior defined as commensalism. The growth of *P. thiocyanatus* was successfully fitted to a Tessier model ($r^2 = 0.97$). To modelize the growth of *P. putida*, a SCN^- inhibition term had to be included ($r^2 = 0.99$). The initial concentrations of phenolic and/or cyanide compounds had a marked effect on the physiological heterogeneity of the co-culture, in terms of metabolic status, membrane integrity and cellular size.

Acknowledgments R. G. Combarros wishes to express gratitude for a research grant from the Government of the Principality of Asturias (Severo Ochoa Programme).

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