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Biodegradation capabilities of acclimated activated sludge towards glyphosate: Experimental study and kinetic modeling

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HIGHLIGHTS

- Complete glyphosate removal has been obtained by acclimated activated sludge.
- C-N cleavage confirms the use of glyphosate as carbon source.
- Monod model fitted experimental data of glyphosate biodegradation kinetics with a maximum growth rate of 0.34 h^{-1} .
- A biodegradation pathway was deduced from kinetic results.

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ABSTRACT

The acclimation process of activated sludge from a wastewater treatment plant for degradation of glyphosate and its biodegradation kinetics were studied in a batch reactor. The parameters monitored included the concentrations of glyphosate, as well as aminomethylphosphonic acid (AMPA), its main metabolite, total organic carbon (TOC), pH, dissolved oxygen (DO) and biomass concentration. At the end of the acclimation process, glyphosate removal efficiency of the acclimated sludge was compared to the fresh sludge one. The results showed that the acclimation process highly increased degradation efficiency. Complete glyphosate removal has been achieved during kinetics experiments. Glyphosate removal kinetic of the acclimated sludge was modeled by Monod model that accurately fitted the experimental results with a maximum growth rate (μ_{max}) of 0.34 h^{-1} and half-saturation constant (K_s) of 1600 mg L^{-1} . Finally, a biodegradation pathway of glyphosate used as carbon source was proposed.

1. Introduction

Glyphosate (N-(phosphonomethyl)glycine), a synthetic phosphate compound, is a broad-spectrum, post-emergent, and non-selective systemic herbicide used to eliminate grasses and herbaceous plants [1–3]. Glyphosate is one of the most widely used herbicides in the world against annual and perennial weeds in agriculture, urban areas, domestic gardens and silviculture [4,5]. Glyphosate acts on plants by inhibiting the activity of enolpyruvyl shikimate-3-phosphate synthase, an enzyme for aromatic amino acid biosynthesis in the shikimate pathway [6]. The massive use of this molecule has been reported to weaken plant defense systems [7], disturb the metabolism [8] and cause DNA or liver damage [9] both for terrestrial and aquatic animals [10,11]. In 2015, the International Agency for Research on Cancer of World Health Organization (WHO) classified glyphosate as “probably

carcinogenic to humans” based on epidemiological, animal and in vitro studies.

Glyphosate contaminates the aqueous environment from various sources, such as industrial effluents and agricultural runoff [9]. Xing et al. [12] reported that glyphosate concentration in waste stream could achieve up to 2560 mg L^{-1} . Thus, it is necessary to remove glyphosate from the Environment. There are several processes used for the removal of glyphosate, e.g. biodegradation, photodegradation, ozone oxidation, adsorption, membrane processes, flocculation and filtration [13]. Among these technologies, biological treatment is considered as an easy, eco-friendly and cost effective process [14]. Many microorganisms were reported to be able to utilize glyphosate as carbon, nitrogen or phosphorus source [15–19]. The two main degradation pathways in glyphosate-degrading microorganisms are conversions to aminomethylphosphonic acid (AMPA) through the cleavage of C–N bond,

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catalyzed by an oxidase, and to sarcosine through the direct cleavage of C–P bond, catalyzed by C–P lyase [3]. Single AMPA or sarcosine pathway or both pathways have been frequently found in glyphosate-degrading microorganisms using glyphosate as a phosphorus source. Little information was reported for glyphosate as carbon or nitrogen source. However, microorganisms' growth demands much bigger carbon than nitrogen or phosphorus. Thus, using glyphosate as carbon source for microorganisms may be a smart technology to increase its removal efficiency at high concentrations.

Moreover, most of the research on the biodegradation of glyphosate focuses on pure microbial cultures, but very few studies are available on the application of mixed cultures, such as activated sludge. Nonetheless, mixed cultures are more likely able to completely degrade contaminants compared to pure culture due to the various available enzymes [14]. Furthermore, due to the high requirements of pure culture, mixed culture processes are more suitable for industrial applications. So, there is a need to study mixed culture ability to remove glyphosate from waste streams.

The above-cited studies have focused on the biodegradability of glyphosate and its degradation pathways. However, little attention has been paid to the acclimation process. Generally, acclimation denotes the adjustments made by microorganisms in response to changes in the environment commonly applied to activated sludge [20]. The adaptive mechanism involving acclimation process include mutation, enzyme formation, modification of cell structure, and composition changes of the population due to multiplication, death, or shifts in predominance [20]. It is known that degradation capacities of activated sludge can be enhanced by the acclimation of the culture [21]. Most studies focused on the isolation and identification of the glyphosate-degrading microorganisms, whereas information on kinetics of glyphosate biodegradation still remains poor. These kinetics could be modeled by Monod or Haldane models. Monod expression was reported by Nourouzi et al. [14] to describe glyphosate consumption kinetics by mixed culture isolated from soil with a specific maximum growth rate of $0.18 - 0.87 \text{ h}^{-1}$. Haldane model was used to describe the glyphosate inhibition to biomass growth with a low ratio of self-inhibition and half-saturation constants [14,22].

In aerobic activated sludge process, oxygen is an important substrate used for growth, maintenance and some metabolic routes of bacteria [23]. The oxygen uptake rate (OUR) is a physiological characteristic of culture growth, which can be used to evaluate the performance of aerobic activated sludge process [23,24]. OUR monitoring is crucial for the assessment of the viability of the microorganisms.

Therefore, the objective of this paper is to investigate the performance of the acclimation process of activated sludge for glyphosate biodegradation and to study its biodegradation kinetics. For that purpose, we carried out acclimation experiments with glyphosate as C source. The biodegradation kinetics of glyphosate were monitored at three concentrations levels of synthetic wastewater and fitted by Monod model. The removal efficiencies of glyphosate and TOC of the acclimated sludge were compared to the fresh sludge performances.

2. Material and methods

2.1. Chemicals

Glyphosate (powder, purity > 95%) was purchased from Leap Labchem Co., Limited, China. AMPA (powder, purity of 99%) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Ninhydrin (crystals) and sodium molybdate (powder) of analytical grade were obtained from Sigma-Aldrich (France). All other chemicals and solvents used were also obtained from Sigma-Aldrich. Aqueous stock solutions were prepared in polypropylene bottles, as well as standards and injection solutions.

2.2. Acclimation process of activated sludge

Acclimation experiments were conducted using a 5-L fed-batch reactor. The raw activated sludge was collected from an urban wastewater treatment plant in Aix-en-Provence city, France. The 5 L-stirred reactor was filled with 4.5 L of raw activated sludge. The initial biomass concentration was 5.35 g L^{-1} MLSS (mixed liquor suspended solids). The basic composition of medium was: $(\text{NH}_4)_2\text{SO}_4$ 223 mg L^{-1} , K_2HPO_4 27 mg L^{-1} , KH_2PO_4 21 mg L^{-1} , $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$ 160 mg L^{-1} , CaCl_2 20 mg L^{-1} , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 1 mg L^{-1} , NaMoO_4 2 mg L^{-1} and MnCl_2 1 mg L^{-1} , pH 7 ± 0.05 . Every 24 h (72 h for the weekend), the bioreactor received a pulse feeding of synthetic substrate-glyphosate mixture. Every week, the medium was refreshed with increasing glyphosate concentration from 100 to 1000 mg L^{-1} (glyphosate solubility of 1.2 g per 100 mL) and decreasing glucose concentration from 480 to 0 mg L^{-1} . The TOC concentration of the daily added solution is kept constant (213 mg L^{-1}) during the whole acclimation process. An aeration system was used to maintain dissolved oxygen (DO) concentration above 3 mg L^{-1} . The experiments were conducted at room temperature. The acclimation process was divided into three stages. Each stage was conducted with increasing glyphosate concentration to 1000 mg L^{-1} and decreasing glucose concentration to 0 mg L^{-1} . The acclimation process lasted 280 days.

2.3. Experiments with acclimated and fresh sludge

In order to study the acclimated sludge degradation benefits, experiments were performed simultaneously with acclimated and fresh sludge during acclimation process. 500 mL of synthetic wastewater with only glyphosate as carbon source were prepared with concentrations of 1000 mg L^{-1} of glyphosate, respectively, in addition to the micro-nutrients specified in Section 2.2. These experiments were performed at room temperature in stirred and aerated 1L-reactors. MLSS concentrations were kept constant in acclimated and fresh sludges. The hydraulic residence time (HRT) was set to 24 h. Glyphosate biodegradation was monitored by regularly collecting 10 mL-samples to perform glyphosate and TOC analyses.

2.4. Analytical methods

During the experimental processes, different parameters were followed: MLSS (mixed liquor suspended solids), pH, Total Organic Carbon (TOC), Dissolved Oxygen (DO), Oxygen Uptake Rate (OUR) [25] and the concentrations of glyphosate and its possible by-products.

2.4.1. Effluent composition analysis

pH was determined by the mean of a pH-meter (HACH Sension + PH3). TOC concentration was calculated from the difference between total carbon concentration (TC) and inorganic carbon concentration (IC) determined using a TOC-L SHIMADZU analyzer. The procedure for the measurement of MLSS is outlined in *Standard Method 2540 D* [26].

The concentration of glyphosate was firstly monitored by absorbance measurements using a spectrophotometer (photoLab® 6600 UV-vis) during the preliminary acclimation process in comparative experiments with acclimated and fresh sludges. This method is based on the reaction ability of glyphosate with ninhydrin and sodium molybdate to produce *Ruhemann's* purple dye with maximum absorption at 570 nm [27].

Glyphosate biodegradation kinetics were investigated in 500 mL-flasks at initial concentration of 200, 500, and 1000 mg L^{-1} , respectively, using the procedures described in section 2.3. For these experiments, the concentration of glyphosate and its metabolites, AMPA and glyoxylic acid, were analyzed by liquid chromatography/high-resolution mass spectrometry technique (LC/HRMS). LC/HRMS analyses were conducted using an Agilent 1290 Infinity system coupled to an Agilent

6530 Q-TOF tandem mass spectrometer equipped with a jet stream ion source. Mass Hunter workstation software B4.00 was used to perform instrument control, data analysis, and processing. The analysis method was performed according to Yoshioka et al. [28]. The separation column was an Obelisc N column (150 mm x 2.1 mm I.D, 5 µm) distributed by SIELC Technologies (Interchim, Montluçon, France). Mobile phase was acetonitrile/water (20/80, v/v) acidified with 0.1% formic acid at a flow rate of 0.2 mL min⁻¹. The column was kept at 40°C in the column oven. After LC separation, the sample was introduced into the atmospheric pressure ionization source and ionized by electrospray in negative ion mode (ESI) leading to the formation of the [M - H]⁻ ions of the analytes. Working conditions were as follows: fragmentor 140 V, capillary 3000 V, skimmer 65 V. Nitrogen was used as the drying (350 °C, 10 L.min⁻¹), nebulizing (30 psi) and sheath (350 °C, 8 L min⁻¹) gas. Scanning was performed from *m/z* 50–1000 amu with 10 038 transients per spectrum. For quantification of the two targeted analytes, Extracted Ion Current (EIC) chromatograms were applied. Retention times, mass of deprotonated molecules and EIC target molecular weight ranges shown between brackets were as follows: glyoxylic acid (3.400 min, 72.9943 amu, [72.8–73.2 amu]), AMPA (3.695 min, 110.0032 amu, [109.8–110.2 amu]) and glyphosate (7.455 min, 168.0111 amu, [167.8–168.2 amu]). The quantification of glyphosate, AMPA and glyoxylic acid concentrations was based on linear regression (R² > 0.999) obtained by injecting glyphosate, AMPA or glyoxylic acid standard solutions in a range of concentrations of 5–200 µg mL⁻¹. Instrumental QC was performed by regular analyses of solvent blanks and random injection of standards. Measured values were not deviating more than 15% of the theoretical values.

The other possible byproducts, sarcosine, formaldehyde, PO₄³⁻, NH₄⁺, and NO₃⁻ [3], were also measured by UV-vis spectrophotometry (photoLab® 6600 UV-vis). Sarcosine was detected by a specific test kit (Biovision kit Sarcosine, K636–100) with maximum absorption for a wavelength of 570 nm. HCHO is measured by Spectroquant® test kit (1.14678.0001, Merck Chemicals) with maximum absorption at 565 nm. PO₄³⁻ was quantified through using Spectroquant® test kit (1.00798.0001, Merck Chemicals) with maximum absorption at λ = 690 nm. NH₄⁺ was detected by UV-vis spectrophotometry through using Spectroquant® test kit (1.14752.0001, Merck Chemicals) with a maximum wavelength of 690 nm. NO₃⁻ was measured by UV-vis spectroscopy (Spectroquant® test kit 1.09713.0001, Merck Chemicals) with maximum absorption at 340 nm.

2.4.2. OUR measurements

OUR was measured during the biodegradation process by the mean of an open respirometer technique. The open respirometer consisted in a 500 mL-reactor. A sludge sample without glyphosate was introduced into the reactor at room temperature. The sludge was continuously stirred, and an air pump was used to keep aeration conditions in the reactor. The DO concentration was recorded as a function of time (*t*) using a DO probe (HACH, HQ 40d). In actual biodegradation processes, endogenous OUR (*OUR_{en}*) and exogenous OUR (*OUR_{ex}*) should be taken into account [29]. The apparent oxygen mass transfer coefficient (*k_La*) and the *OUR_{en}* were obtained using a dynamic method based on an unsteady-state procedure [30]. By stopping the aeration, the DO concentration decreases due to the oxygen consumption by the microorganisms; with restarting the aeration, the DO concentration increased to reach a steady-state value [30]. *k_La* and *OUR_{en}* can be obtained through the monitoring of DO concentration evolution. *OUR_{ex}* can be calculated according to the DO mass balance by Eq. (1) [31].

$$OUR_{ex} = k_L a (C_s - C) - OUR_{en} - dC/dt \quad (1)$$

where *C_s* and *C* are the saturated and the instantaneous DO concentrations (mg L⁻¹), respectively.

OUR_{ex} is associated with glyphosate aerobic biodegradation and can be written as Eq. (2) [29].

$$OUR_{ex} = \frac{1}{Y_{S/O}} \frac{dS}{dt} \quad (2)$$

where *S* is the glyphosate concentration (mg L⁻¹), *Y_{S/O}* is the glyphosate yield on oxygen (mg-glyphosate (mg O₂)⁻¹).

2.4.3. Kinetic model

Growth kinetics of microorganisms in activated sludge processes can be expressed by Monod model [32], which describes the growth of culture on the utilization of single substrates.

The Monod model can describe the cell growth rate as follows:

$$dX/dt = \mu X \quad (3)$$

Monod described the specific growth rate of bacteria depending on a specific maximum growth rate and a limiting substrate concentration:

$$\mu = \mu_{max} \frac{S}{K_s + S} \quad (4)$$

The rate of glyphosate consumption by bacteria could be described by the Monod expression in a batch reactor (Eq. (5)).

$$-\frac{dS}{dt} = \frac{\mu_{max} S X}{Y(K_s + S)} \quad (5)$$

where *X* is the bacteria concentration (i.e. MLSS, g L⁻¹); *dX/dt* is the bacteria growth rate (g L⁻¹ h⁻¹); *μ* and *μ_{max}* is the specific bacteria growth rate and maximum specific bacteria growth rate (h⁻¹), respectively; *S* is the concentration of glyphosate (mg L⁻¹); *dS/dt* is the glyphosate utilization rate (mg L⁻¹ h⁻¹); *K_s* is the half-saturation constant (defined as the substrate concentration at half of the maximum specific bacteria growth rate, mg L⁻¹); *Y* is the bacteria cell yield (mg mg⁻¹).

3. Results and discussion

3.1. Acclimation process

Biomass acclimation process to hardly biodegradable organic compounds is a critical step to induce microbial selection and physiological transformations of the metabolic pathways during the biodegradation process [33]. Biomass concentration has an important effect on the biological treatment performance of activated sludge [34], described by MLSS in this study.

The concentration of MLSS (Fig. S1) began to slowly rise in the later period of the second stage, indicating that the bacteria began to adapt themselves to generate enzymes to degrade glyphosate. This trend was confirmed in the third stage which explains that the performance of this latter stage was mainly discussed below.

In parallel to measurements of bacterial activity, TOC and glyphosate removal were followed. The results obtained for a batch of 7 days showed a global decrease of the TOC and glyphosate in the third stage (Fig. 1).

Fig. 1 shows that for different initial glyphosate concentrations, glyphosate removal efficiencies generally decreased with time, possibly caused by an inhibition linked to the accumulation of by-products from glyphosate. Glyphosate removal efficiencies at different initial glyphosate concentrations were in the range of 30–60%. These results confirmed that glyphosate has been used by bacteria as carbon source for their metabolism.

3.2. Comparative performances of fresh and acclimated sludges for glyphosate removal

In order to evaluate the benefits of the acclimation process, comparative experiments of glyphosate biodegradation between acclimated and fresh sludge were conducted during acclimation process. After 24 h, for acclimated sludge, the MLSS of 2.75 g L⁻¹ at initial glyphosate

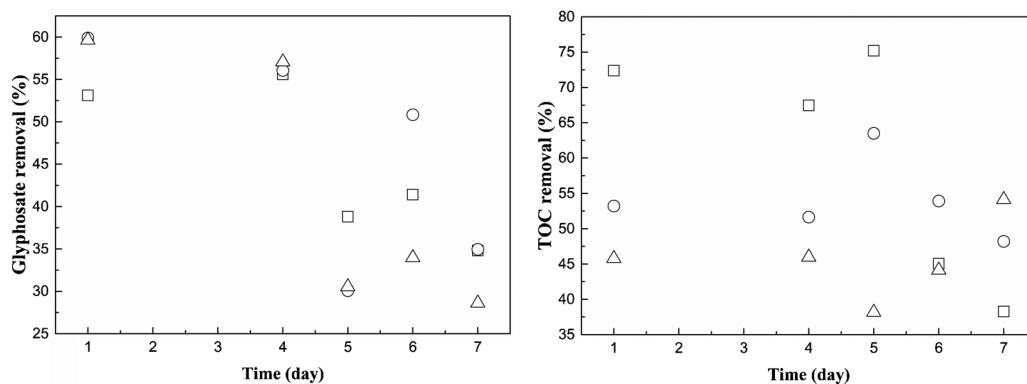


Fig. 1. Glyphosate and TOC removal with time in the third stage of the acclimation process: (1) □ 200 mg.L⁻¹ glyphosate + 426 mg.L⁻¹ glucose; (2) ○ 500 mg.L⁻¹ glyphosate + 266 mg.L⁻¹ glucose; (3) △ 1000 mg.L⁻¹ glyphosate.

concentrations of 1000 mg L⁻¹, was nearly equal to the initial MLSS ($3 \pm 0.39 \text{ g L}^{-1}$), demonstrating the stability of the bacterial activities in acclimated sludge. While for fresh sludge, the MLSS was significantly decreased to 1.59 g L^{-1} after 24 h as compared to the initial MLSS ($3 \pm 0.1 \text{ g L}^{-1}$). This indicates that glyphosate inhibited the growth of biomass in the fresh sludge.

Removal profiles of glyphosate and TOC in the acclimated and fresh sludge for 9 h at initial glyphosate concentration of 1000 mg L⁻¹ are illustrated in Fig. 2. It shows that for acclimated sludge, glyphosate removal first increased fast, and then slowly increased until achieving the equilibrium at about 42%. For the initial concentration of 1000 mg L⁻¹, equilibrium was not achieved after 9 h. Thus, the glyphosate concentrations measured after 24 h showed a removal efficiency up to 60%

In the case of non-acclimated sludge, glyphosate concentration after 24 h was almost the same as the initial concentration of glyphosate, showing almost no biodegradation. The same differences were found for TOC removal by acclimated and non-acclimated sludge (Fig. 2). After 24 h, TOC removal in acclimated sludge reactor was about 60%, which indicated the formation of organic intermediates during glyphosate biodegradation process. In the case of non-acclimated sludge, TOC reduction was almost zero. This can be explained by an inhibition of glyphosate on bacterial growth. These results validated the acclimation process of activated sludge to glyphosate and demonstrated the ability of selected bacteria to use glyphosate as carbon source.

3.3. Kinetics of glyphosate biodegradation

Once the acclimation process was performed and efficient, degradation kinetics of glyphosate by acclimated sludge were experimentally studied.

3.3.1. Glyphosate and TOC reduction

Fig. 3 shows that glyphosate was totally removed after 3 h by the

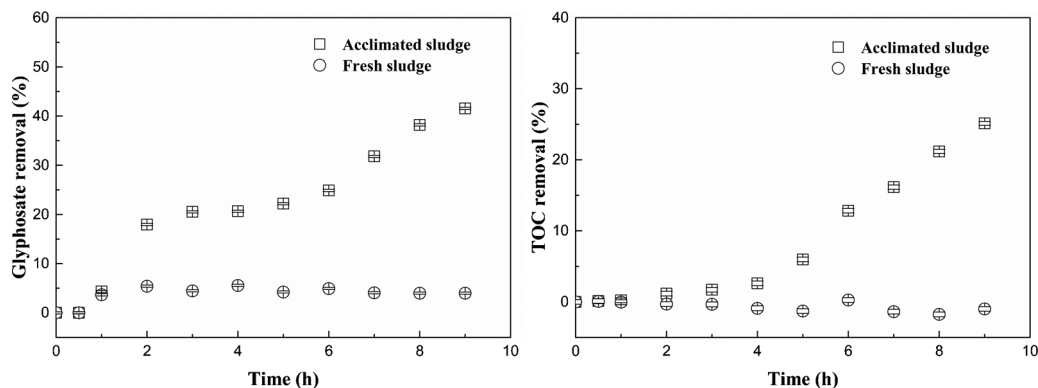


Fig. 2. Glyphosate and TOC removal in (□) acclimated and (○) non-acclimated sludge.

acclimated sludge at the three initial glyphosate concentrations of 200, 500 and 1000 mg L⁻¹, respectively. At the same time, Fig. 3 showed that AMPA is produced with a stoichiometric ratio of 1:1 compared to glyphosate consumption. This result gives an important clue on the involved metabolic pathways. However, TOC reductions were 52.6, 58.1 and 57.8% after 24 h for initial glyphosate concentrations of 200, 500 and 1000 mg L⁻¹, respectively, which was still not sufficient due to the formation of organic intermediates, which remained in the solution and were not further metabolized.

3.3.2. Respirometric experiments

Bacterial activity was confirmed by DO measurements. Respirometer experiments were performed to evaluate the effects of initial glyphosate concentration on the total OUR (OUR_T), which represented the sum of the exogenous OUR (OUR_{ex}) and endogenous OUR (OUR_{en}), calculated from Eq. (1). The results are shown in Table 1. It shows that the oxygen mass transfer coefficient obtained for the acclimation process was $0.14 \pm 0.03 \text{ min}^{-1}$, which is in the range of those found in the literature [29,35]. Moreover, it depicts that the OUR_{en} of the acclimated activated sludge was $0.11 \pm 0.02 \text{ mgO}_2\text{L}^{-1} \cdot \text{min}^{-1}$, which was defined as the oxygen consumption by microorganisms in the absence of substrate. When glyphosate was added to activated sludge, the OUR_T values increased with the increase of initial glyphosate concentrations, revealing that glyphosate was metabolized by bacteria. The glyphosate yield on oxygen ratio ($Y_{S/O}$) significantly increased with the increase of glyphosate concentrations from 200 to 500 mg L⁻¹. This phenomenon could be explained by the high value of half-saturation constant (K_s), which is discussed below.

As in the case of oxygen transfer, increasing glyphosate concentration in the bulk to a value exceeding glyphosate uptake rate of growing cell could result in the increase of glyphosate transfer rate [36]. Here this glyphosate transfer rate is considered by the glyphosate yield on oxygen ratio ($Y_{S/O}$). Indeed this increase of the values of $Y_{S/O}$ with the increase of the concentration of glyphosate in the bulk show that

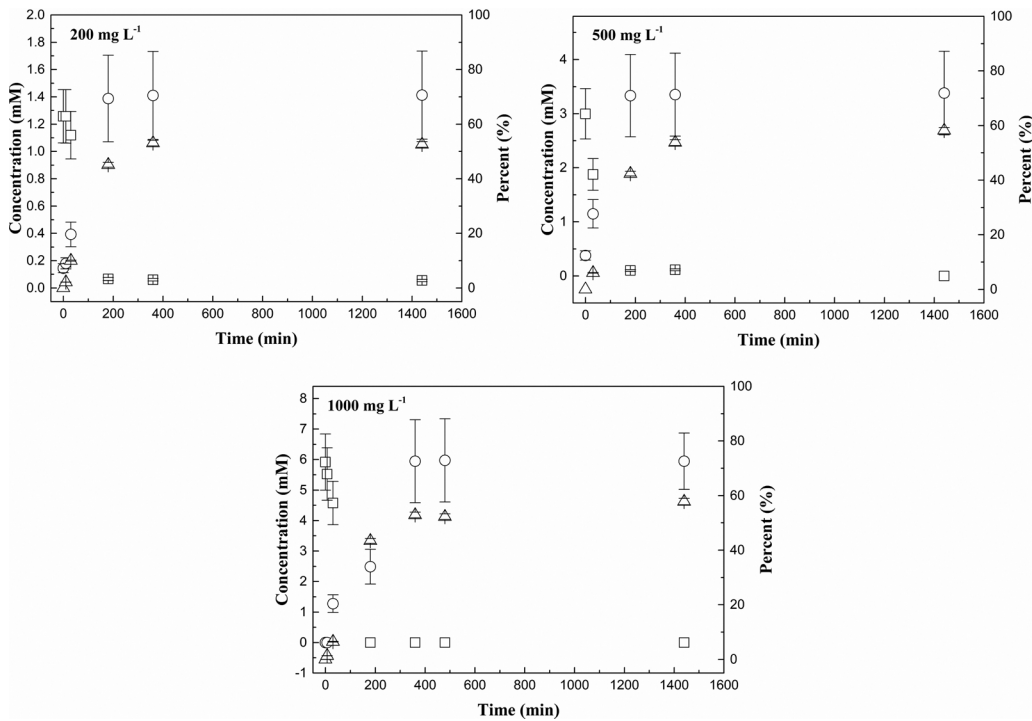


Fig. 3. Glyphosate and AMPA concentrations and TOC reduction during the kinetics experiments under three initial glyphosate concentration: (□) glyphosate concentration; (○) AMPA concentration; (△) TOC reduction.

200 mg L⁻¹ is not sufficient to equal the glyphosate uptake rate of growing cells in the case of this acclimated sludge.

3.3.3. Kinetic model

The Monod's system of differential equations (Eqs. (3)–(5)) was solved with the ode's Matlab solver through using the fourth-order Runge–Kutta method to obtain the time evolution of *S* and *X*. An optimization procedure through coupling the *fsolve* function of Matlab with the Levenberg–Marquardt optimization algorithm has been also implemented to obtain the optimal parameters *Y*, μ_{max} and *K_s* that lead to the best quadratic error between experimental and computed values for *S*. Time evolution and comparison with the experimental data is represented in Fig. 4. It shows that Monod model well fitted the growth kinetic of bacteria using glyphosate as limiting substrate. Bacteria cell yield (*Y*) predicted by the model was 0.69, which is in the range of values reported for other organic compounds biodegradation by activated sludge [33,37]. The maximum growth rate (μ_{max}) was found at 0.34 h⁻¹ for the acclimated sludge, which is in the middle range of those (0.05–0.87 h⁻¹) found in the literature [14,22] and lower than that for glucose (0.79 h⁻¹) [38]. This latter difference can be explained by several factors, such as the differences in biodegradability between glyphosate and glucose, in metabolism pathways, and in microbial species selected during the acclimation process. Moreover, during the glyphosate biodegradation process, some organic metabolites generated could act as inhibitory substrates for microorganisms. The half-saturation constant (*K_s*) was 1600 mg L⁻¹, which is much higher than that of glyphosate biodegradation by mixed culture (33–181 mg L⁻¹) [14] and non-acclimated sludge (340 mg L⁻¹) [22]. Literature shows that the difference of kinetic parameters is caused by several factors, such as inoculum history (acclimation), changes in the predominating

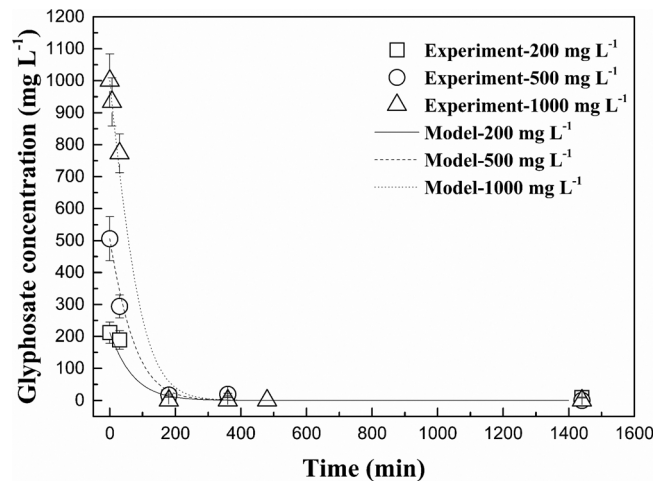


Fig. 4. Time evolution of glyphosate concentration using Monod model and comparison with the experimental data.

microbial species during the assays, and environmental factors (pH, temperature) [31]. Furthermore, during the acclimation process, the conditions changed, such as substrate concentration, dilution rate, or substrate to microorganism ratio, which can modify the population metabolic state and also affect kinetic parameters [39].

Finally, modeling results showed that glyphosate removal kinetics could be fitted by a Monod model after acclimation of the sludge. This confirmed that glyphosate was used as carbon substrate by acclimated bacteria as well as a non-inhibiting substrate.

Table 1

OUR in the kinetics process under different initial glyphosate concentrations.

Glyphosate concentration (mg L ⁻¹)	OUR _{en} (mg O ₂ L ⁻¹ min ⁻¹)	<i>k_La</i> (min ⁻¹)	OUR _T (mg O ₂ g ⁻¹ min ⁻¹)	<i>Y_{s/o}</i> (mg mg ⁻¹ O ₂)
200	0.11 ± 0.02	0.14 ± 0.03	0.97 ± 0.14	0.89
500			1.36 ± 0.12	5.70
1000			1.51 ± 0.20	5.48

Table 2

The concentration of possible byproducts of glyphosate before and after 24 h treatment.

Glyphosate concentration (mg.L ⁻¹)	Before treatment (mM)				After 24 h treatment (mM)			
	HCHO	PO ₄ ³⁻	NH ₄ ⁺	NO ₃ ⁻	HCHO	PO ₄ ³⁻	NH ₄ ⁺	NO ₃ ⁻
200	0.007	0.16	2.79	0.04	0.038	0.19	–	0.24
500					0.045	0.20	–	0.32
1000					0.091	0.24	–	0.52

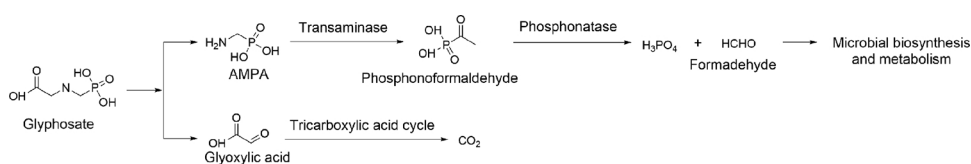
3.3.4. Proposed biodegradation pathway

Two pathways have been already proposed for glyphosate biodegradation in the literature: (1) the conversion of glyphosate to AMPA and glyoxylic acid through the C-N cleavage by the presence of glyphosate oxidoreductase; (2) the formation of sarcosine through the C-P cleavage catalyzed by C-P lyase [40]. In order to elucidate the glyphosate pathway in the acclimated sludge, potential byproducts of glyphosate biodegradation were analyzed. Sarcosine was never detected in the samples. However, AMPA was detected by LC-MS analysis with the *m/z* of 110.0032 amu (Fig. 4). AMPA concentrations increased with time under the three initial glyphosate concentrations tested. The stoichiometric ratio observed is almost 1:1 revealing the only one C-N cleavage pathway involved in glyphosate biodegradation in our case. These findings are consistent with literature [18,41]. Concerning glyoxylic acid, it was not detected in any samples. This could be explained by a quick integration of this molecule in the tricarboxylic acid cycle [19,42].

Furthermore, the presence of other by-products of glyphosate metabolism were measured by UV-vis spectrophotometry (Table 2). It is interesting to note that PO₄³⁻ slightly increased with the increase in glyphosate concentration. Since glyphosate was almost completely degraded into AMPA, PO₄³⁻ was not directly converted from glyphosate, as mentioned in previous studies [43,44]. AMPA is more probably transformed into PO₄³⁻ by C-P lyase [45,46]. The concentration of formaldehyde (HCHO) increased with the increase in glyphosate concentration and seems to be directly linked to the amount of PO₄³⁻. This suggests the existence of phosphonatase pathway, which was also proposed in the literature [19,47]: (1) the transamination of AMPA to phosphonoformaldehyde catalyzed by an aminotransferase; (2) the formation of formaldehyde and PO₄³⁻ from C-P bond cleavage of phosphonoformaldehyde. However, the phosphonatase pathway of glyphosate biodegradation remains a hypothesis as its enzymes have never been isolated nor characterized [19]. Although there is no direct evidence to confirm this pathway, the existence of such a pathway of glyphosate mineralization is the most congruent explanation.

Finally, NH₄⁺ was not detected after biological treatment, since it is probably used as nitrogen source for the synthesis of bacterial cell substances or nitrogen-containing metabolites. However, the concentration of NO₃⁻ was increasing with glyphosate concentration after 24 h treatment. This is possibly due to the nitrification process.

According to the above evaluation of the byproducts of glyphosate, a possible biodegradation pathway was proposed in Fig. 5. First, the acclimated sludge was able to utilize glyphosate as the sole carbon source with the stoichiometric formation of AMPA and glyoxylic acid through the cleavage of C-N bond by the presence of glyphosate oxidoreductase. Glyoxylic acid may be incorporated very quickly in the tricarboxylic acid cycle [19,42]. AMPA was further metabolized to phosphonoformaldehyde through the transamination catalyzed by an

**Fig. 5.** Proposed biodegradation pathway of glyphosate.

aminotransferase and then transferred to formaldehyde and PO₄³⁻ from C-P bond cleavage of phosphonoformaldehyde. The resulting formaldehyde can enter the carbon metabolic pathways of cellular growth [22].

4. Conclusions

The results obtained in this study suggested that an acclimated process promote the biodegradation of glyphosate by activated sludge through using glyphosate as sole carbon source. Complete glyphosate removal was achieved by acclimated sludge, whereas it cannot be obtained by non-acclimated one. Conventional Monod model was successfully used to adequately describe both biomass growth and glyphosate degradation profiles at various glyphosate initial concentrations. With the help of these results, a biodegradation pathway of glyphosate by acclimated sludge was proposed and confirmed the pre-eminence of the C-N cleavage. This study opens up important engineering implications for the biological treatment of real glyphosate-containing wastewater.

CRedit authorship contribution statement

Dan Feng: Investigation, Visualization, Writing - review & editing. **Laure Malleret:** Methodology, Conceptualization. **Guillaume Chiavassa:** Methodology, Conceptualization. **Olivier Boutin:** Supervision, Funding acquisition. **Audrey Soric:** Validation, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bej.2020.107643>.

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