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Article

Nitrate Removal from Wastewater through Biological Denitrification with OGA 24 in a Batch Reactor

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Abstract: Nitrates pollution of waters is a worldwide problem and its remediation is a big challenge from the technical and the scientific point of view. One of the most used and promising cleaning techniques is the biological treatment of wastewaters operated by denitrifying bacteria. In this paper we begin a thorough study of denitrifying performances of the bacterium *Azospira* sp. OGA 24, recently isolated from the highly polluted Sarno river in the south of Italy. Here, the kinetics of nitrates consumption operated by bacteria in a specifically devised batch bioreactor, in anoxic condition and with acetate as the organic substrate, has been characterized. Experimental data were then used in a simplified model of a real wastewater treatment plant to find that OGA 24 can clean water with efficiency up to 90%. The denitrifying performances of OGA 24 match the requirements of Italian laws and make the bacterium suitable for its employment in treatment plants.

Keywords: bacterial denitrification; *Azospira* sp. OGA 24; wastewater treatment plants; CSTR

1. Introduction

The discharge of nitrogen components in the environment is hazardous for both human and animal health and the nitrate pollution and remediation is at the same time a worldwide problem and a challenge. Nitrates cause the most serious problems when dispersed in water, since they cause the depletion of aquifers and the eutrophication of rivers. Sources of nitrate comprise natural cycle and human activities, mainly from uncontrolled land discharges of treated or raw domestic and industrial wastewaters, landfills, and animal wastes predominantly from animal farms [1,2]. Therefore, several studies focused on the nitrates removal from wastewater in order to achieve an acceptable concentration in treated waters to be discharged into the environment. Complementary techniques are often required for obtaining a free nitrate stream in treatment plants, being the most common methods either physicochemical or biological and sometimes a combination of both [3–5]. The biological denitrification is recommended for the removal of relatively low concentration of nitrogen components and it is operated by the so called denitrifying bacteria in anoxic conditions, where they use nitrates as electron acceptors during their respiratory process in the place of the oxygen. Nitrates are efficiently removed when an external organic carbon source, generally methanol, ethanol or acetic acid, is added [4,6–8]. As demonstrated by the most recent literature, new bacterial strains are continuously isolated and tested for their NO_3^- removal abilities [9–13]. Depending on their characteristics, different bacteria are employed in different waste treatment facilities, with a preference towards those microorganisms capable of combined heterotrophic nitrification and aerobic denitrification. However, other characteristics are often desirable, for example bacteria with a marked resistance to high salinity, are generally employed in the treatment of polluted seawater [14] and strains isolated from critically polluted environments are used for the treatment of special industrial wastes, such as tannery wastewater [15].

In a previous work [16] we studied an enrichment sample of a bacterial colony coming from a contaminated site and capable of reducing perchlorates and nitrates. The colony was isolated from the water of Sarno River, located in the south part of Italy and known to have high levels of both organic and inorganic pollutants, mainly due to the presence of tomato industries and leather tanneries [17]. The high resistance of the bacterial colony to extreme conditions and its ability to reduce both perchlorate and nitrates simultaneously, motivated a deeper study in order to employ bacteria in wastewater remediation. From the original colony a beta proteobacterium was isolated, which in batch experiments in anoxic conditions was able to efficiently use nitrates as electron acceptors and acetates as electron donors and carbons source. This strain was identified as *Azospira* spp., *Azospira* sp. OGA 24, because from molecular analysis based on 16S rRNA sequence it showed 99.7%–100% identity with this genus (NCBI accession number, GU294119.1).

With this work we start a thorough characterization of the denitrifying properties of the *Azospira* sp. OGA 24. By using a specifically devised experimental setup, a sterile and anoxic environment was obtained where both the nitrates consumption and the bacterial growth could be easily monitored in real time. In our experimental conditions, the denitrification kinetics was found to be compatible with a zero- and a first-order kinetics, both reliable when an excess of nitrates and of organic substrate are present in the reactor. Moreover, an assessment of the denitrification potential of OGA 24 when used in real wastewaters treatment plants was done. A simple theoretical model was used to simulate the

behaviour of OGA 24 during the anoxic stage of a denitrification process and the results were found to meet with the Italian legislation requirements.

2. Materials and Methods (or Experimental)

2.1. Bacteria and Media

Azospira sp. OGA 24 was isolated from an enrichment sample obtained from a highly polluted river (Sarno river, Italy) by tube to plate method [16]. It was identified molecularly by 16S rRNA sequence analysis. The ribosomal sequence was deposited at NCBI database with accession number GU294119.1. For assays of denitrification bacterium was grown anaerobically on minimal VG medium modified by the addition of B vitamins [16], containing 10 mM acetate as the sole carbon source and electron donors and 10 mM nitrate ($NaNO_3$, analytical grade, Carlo Erba) as electron acceptors. To obtain rapid population expansion, the strain was grown aerobically in rich Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl). For both growth conditions, the bacterium was incubated at 37 °C. For assays of nitrate respiration, frozen (−80 °C) cultures in 20% glycerol were revived by incubating 24 h in LB medium at 37 °C with constant shaking at 150 rpm. Cells were collected by centrifugation at 2.500 × g at room temperature, washed twice with water saline solution (0.9% NaCl) and pre-incubated in VG medium with 10 mM NO_3^- and 10mM acetate at concentration corresponding to the optical density at $\lambda = 600$ nm (OD_{600}) of 0.02 A.U. ($OD_{600} = 1$ A.U. ~1×10^9 individuals/mL). The growth was carried out in 100 mL flasks sealed with rubber stoppers to ensure anaerobic conditions at 37 °C for 24 h without shaking. After pre-incubation cells were harvested and inoculated at similar concentration in fresh VG medium for bioreactor assays.

2.2. Experimental Set Up

Experiments on nitrates reduction were run by transferring the cell suspension in a bioreactor containing a VG medium supplemented with $[NO_3^-]_0 = 350$ mg/L (total volume $V_0 = 250$ mL).

The bioreactor (see a sketch in Figure 1) consisted in a three necked round bottom flask ($V_{tot} = 400$ mL) and equipped with several probes: dissolved oxygen (DO, Vernier Software and Technology, Beaverton, OR, USA), pH (Vernier Software and Technology) and Nitrate Ion Selective Electrode (NO_3^- ISE, Vernier Software and Technology). All the probes were connected to a LabQuest2 data logger (Vernier Software and Technology) for data acquisition and storage.

Three disposable needles (STERICAN BRAUN), with 0.45 μm filters placed at the luer inlets, were also inserted in one of the necks. A needle was used for sampling the solution in order to check possible contamination and for injecting nitrate solutions. The two other needles (one inlet and one outlet) were connected to a peristaltic pump (REGLO MS 2/8, ISMATEC) in line with a UV-VIS spectrophotometer (Vernier Software and Technology) and arranged in a closed loop for a continuous monitoring of the bacteria population in solution (OD at $\lambda = 600$ nm).
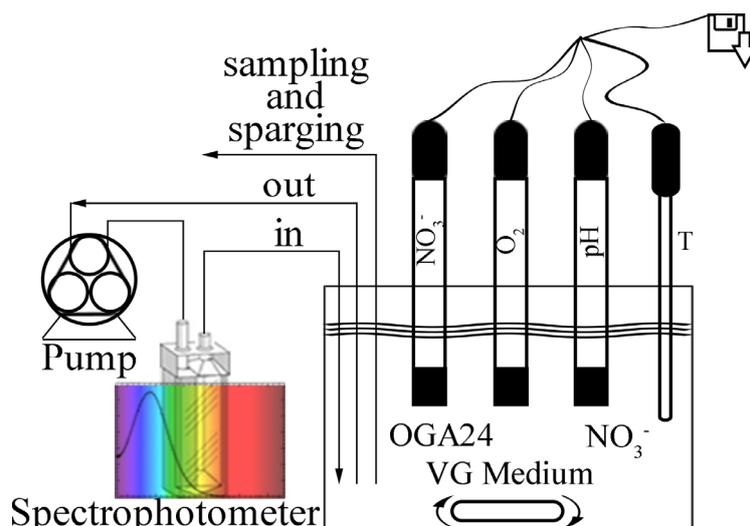
Figure 1. Skecth of the bioreactor used in experiments.

In order to obtain anaerobic conditions, before data acquisition the solution was sparged with a constant flux of Argon, injected through the sampling needle, until [O_2] ~0 mg/L (~20 min). The bioreactor was placed in an air thermostat at a temperature of 30 ± 1 °C and stirred continuously at 200 rpm. Phosphate salts in the VG medium acted as buffer keeping the pH around 7 during the whole course of the experiments. All the experiments were performed in triplicate and the data reported represent their average values.

The NO_3^- ISE was calibrated by using 1 mg/L and 1000 mg/L standard solutions of KNO_3 (Carlo Erba, Analytical grade) and a series of intermediate solutions obtained by dilution. We checked the correct functioning of our experimental apparatus by performing a simple semi-batch experiment. We infused in a glass reactor containing a known volume of nitrate solution (V_0 = 250 mL, [NO_3^-]$_0$ = 10 mg/L) a second nitrate solution ([NO_3^-]$_{in}$ = 13.6 g/L) by a syringe pump (NE-300, New Era Pump Systems) at a constant flow rate (F_{in} = 2.11 µL/min). We then monitored over time the concentration of nitrate ions in solution ([NO_3^-](t)) by using our calibrated NO_3^- ISE and we compared the experimental values with the theoretical curve Equation (1), which describes the nitrates evolution in time. Here the nitrate ions do not undergo any reaction and the only variation is due to the input of the known amount of nitrates and to the dilution effect. The comparison between the experimental and the theoretical data reported in Figure 2 reveals a very good response of the NO_3^- ISE. The same results were obtained in the presence of a VG medium having the same composition as that used in the bioreactor.

$$\left[NO_3^- \right](t) = \frac{\left[NO_3^- \right]_0 V_0 + \left[NO_3^- \right]_{in} F_{in} t}{V_0 + F_{in} t} \tag{1}$$

Finally, in order to further check the reliability of the ISE, random samples were collected during the course of the experiments and analyzed by means of an ion chromatograph (DIONEX DX-120, Dyonex, Sunnivale, CA, USA) equipped with an anion exchange column (IONPAC AS14, Dyonex, Sunnivale, CA, USA). The difference in [NO_3^-] determined with the two methods was found to be lower than 2%.

All the stock solutions used both for the bacteria culture and for the batch experiments where carefully prepared by weight with an error smaller than 1%.
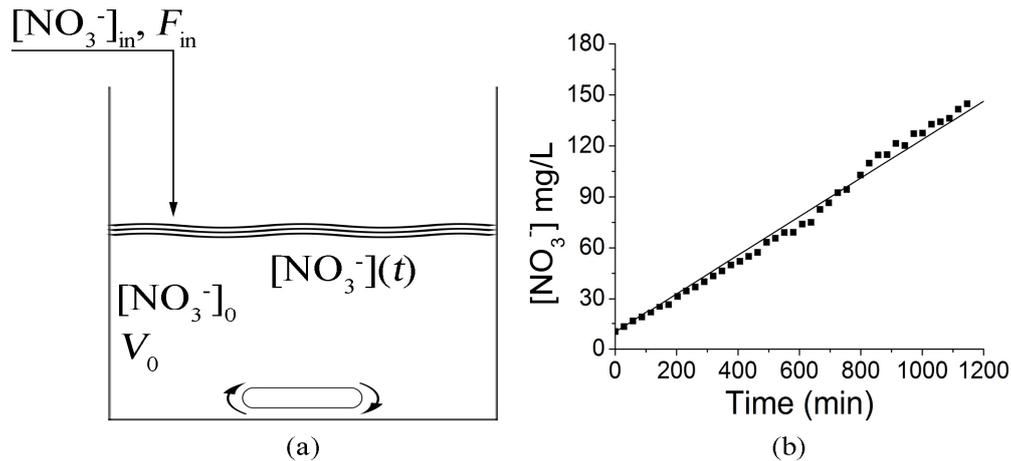
Figure 2. (**a**) Sketch of the Semi-batch reactor used for the experimental setup tests; (**b**) Comparison between the experimental data (black squares) and the theoretical curve (black line) of $[NO_3^-](t)$ in a semi-batch reactor where V_0 = 250 mL, $[NO_3^-]_0$ = 10 mg/L, $[NO_3^-]_{in}$ = 13.6 g/L and F_{in} = 2.11 μL/min.

3. Results and Discussion

Aerobically grown up to an optical density of 0.02 A.U. at 600 nm (roughly equal to 2×10^7 individuals/mL), *Azospira* sp. OGA 24 was then inoculated in the batch bioreactor in the presence of the VG medium, acetate as the organic substrate (electron donor) and $[NO_3^-]$ = 350 mg/L (electron acceptor). The bioreactor was previously sparged with argon in order to obtain anaerobic conditions, so that the bacteria could use nitrates as the only final electron acceptors for their respiratory functions. Thanks to the *ad hoc* experimental setup and to the analytical methods employed, the kinetics of the nitrates consumption and of the bacterial population growth could be recorded simultaneously and with a continuous sampling rate. Figure 3 shows a typical time series of the nitrate consumption operated by OGA 24 (black trace, right axis) and the corresponding growth curve of the bacteria (red trace, left axis). The whole dynamics could be divided into three main regions for both the curves. The first region represents a rather long (0–~1800 min) acclimation period (A in the figure) where bacteria adapt to the anaerobic condition and to the high concentration of nitrates and their population slightly increases (OD 0.02→~0.3 A.U.); correspondingly the concentration of nitrates slowly decreases from 350 mg/L to ~320 mg/L. The quite long acclimation period could certainly be reduced by starting experiments with a larger population of bacteria, *i.e.*, by increasing the duration of the cells culture. The acclimation period is followed by the exponential region (B in the figure, ~1800–~2500 min) where nitrates are rapidly depleted until their concentration reaches a value of about 0 mg/L. The rapid consumption of the electron acceptor species corresponds to a fast increase of the bacteria population (OD ~0.3→~1.2 A.U.). Finally, a plateau region (C in the figure) indicates that the system reached a steady state where nitrates are completely consumed and the number of bacteria is stationary in time (~1.2×10^9 individuals/mL).
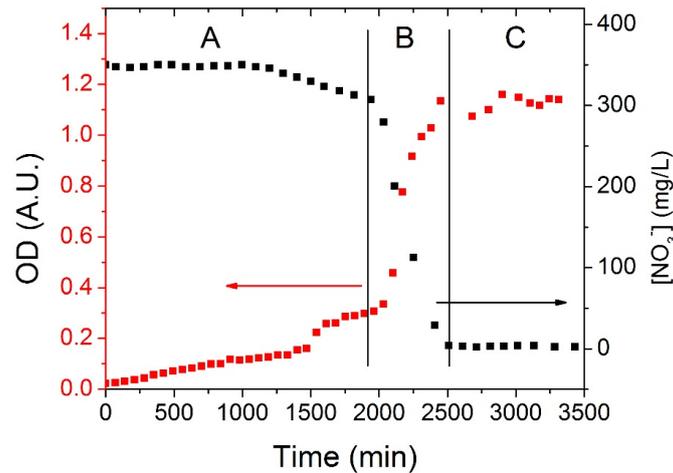
Figure 3. Experimental curves of nitrates reduction operated by the azospira OGA 24. Black squares represent nitrates concentration over time, measured by means of the NO₃⁻ ISE; red squares show the increase of the solution optical density over time, *i.e.*, the growth of the bacteria population. Three different regions can be identified: **A**: acclimation zone; **B**: exponential consumption of nitrates (increase of the bacterial population); **C**: steady state.

In order to understand the potentiality of the *Azospira* sp. OGA 24 as denitrifying bacterium in wastewater treatment plants, the kinetics of the exponential region B was characterized. The bio-denitrification process is generally described by a Monod or Michaelis-Menten type kinetics [18,19], however, when the initial concentration of nitrates is high (generally above 200 mg/L) and the organic substrate is in large excess, the nitrates consumption dynamics can be approximated to a linear zero- or exponential first-order kinetics [18,20–23].

Figure 4a shows the detail of the exponential region B relative to the [NO₃⁻] time series of Figure 3, where the rate of consumption of the electron acceptor is maximum. In order to characterize the kinetics of the denitrification process, data from different experiments where fitted both to a linear equation (blue dashed line, fitting interval 2000–2400 min) having the form

$$[NO_3^-](t) = -k_d^0 t + [NO_3^-]_0 \tag{2}$$

where k_d^0 (mg L⁻¹·min⁻¹) is the kinetic constant of the zero-order kinetics, and to an exponential equation (red line, fitting interval 2000–2800 min) having the form

$$[NO_3^-](t) = [NO_3^-]_0 e^{-k_d^1 (t-t_0)} \tag{3}$$

where k_d^1 (min⁻¹) is the kinetic constant of the first-order kinetics. Data where fitted by means of a Levenberg–Marquardt [24] nonlinear regression algorithm, which yielded a value of $k_d^0 = 0.63 \pm 0.03$ mg L⁻¹ min⁻¹ and $k_d^1 = 4 \pm 0.5 \times 10^{-3}$ min⁻¹. For both the fitting procedures the value of the R-squared index was higher than 0.99, meaning that both the mechanisms proposed for the denitrification process were plausible.
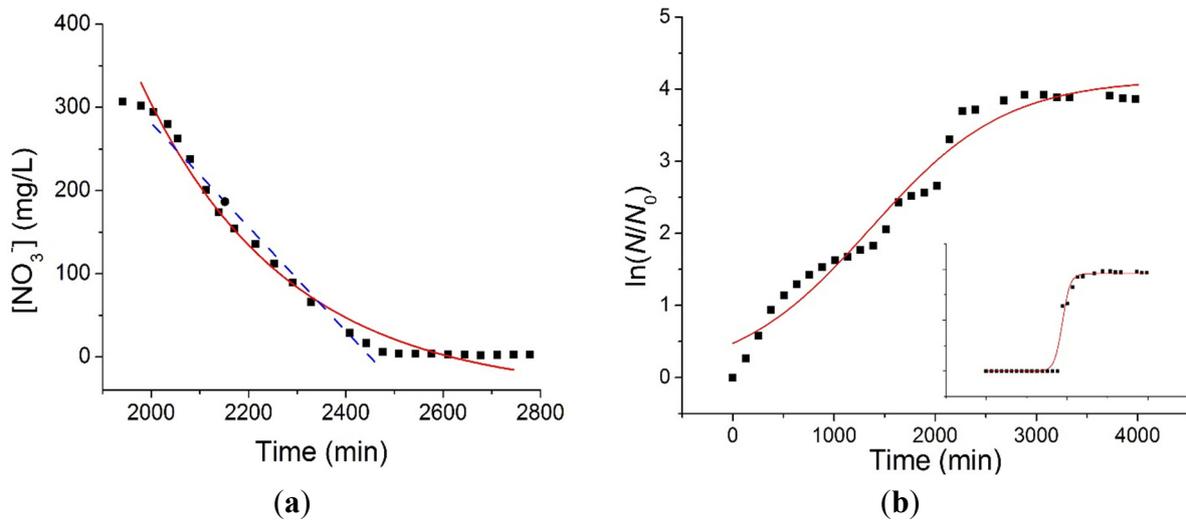
Figure 4. (**a**) Exponential fitting (red line, Equation (3)) and linear fitting (blue dashed line, Equation (2)) of the [NO₃⁻] consumption during the exponential period; (**b**) Fitting of the bacterial growth curve by means of the logistic Equation (4). The inset shows the bacterial growth curve when the bacterial population is considered constant during the acclimation period.

The growth dynamics of the bacterial population was also characterized by fitting OD data (Figure 4b) to a logistic equation having the form

$$\ln(N/N_0) = \frac{A}{1+\exp\left[\dfrac{4\mu_m}{A}\left(\lambda - t\right) + 2\right]} \tag{4}$$

where N/N_0 is the relative bacterial population size, $A = \ln(N_\infty/N_0)$ is the maximum value reached, λ is the lag time, *i.e.*, the time of the acclimation period (region A in Figure 3) and μ_m is the maximum specific growth rate [25]. The slight increase of the population size during the acclimation period is amplified in the logarithmic scale and it makes difficult a good fitting to the Equation (4), as showed in the main graphic of Figure 4b. The values of the fitting parameters were found to be $A = 4.14 \pm 0.04$ (which roughly corresponds to a population increase of 60 times, *i.e.*, 10 times larger than the value obtained from OD measurements), $\mu_m = 1.6 \pm 0.04 \times 10^{-3}$ min⁻¹ and an unrealistic lag time $\lambda = 28 \pm 10$ min, with R-squared = 0.95. In order to obtain a better fitting and a more realistic value for the lag time, the population size was considered as constant during the acclimation period (inset in Figure 4). In this case, the fitting procedure yielded $A = 3.83 \pm 0.04$ (which roughly corresponds to a population increase of 50 times in accordance with the OD measurements), $\mu_m = 9.9 \pm 0.4 \times 10^{-3}$ min⁻¹ and $\lambda = 1689 \pm 12$ min, with R-squared = 0.99. The specific growth rate differs by almost one order of magnitude in the two cases and it is difficult at this stage to obtain a proper characterization of the growth dynamics. However, the value of k_d^1 was found to be in between these two extremes, therefore the value of μ_m can be reliable in both cases.

In order to assess the performances of the *Azospira* sp. OGA 24 for its employment in a real wastewater treatment plant, the kinetic data obtained in the batch bioreactor were used in a simple theoretical model of the denitrification process. For the sake of simplicity only the anoxic stage, which is

generally the core of the biological wastewater treatments [6,8,26], was considered. In this stage the bacteria are continuously supplied with an organic substrate and they reduce the nitrates to nitrogen in the absence of oxygen. The process can be modeled by considering a CSTR (Continuously Stirred Tank Reactor) with an excess of organic substrate and with a flux of incoming wastewater, F_{in}, equals to the outgoing denitrified water, F_{out}, *i.e.*, the volume of the reactor, $V(t)$, is in stationary state: $F_{in} = F_{out} = F$ (m^3/d), $V(t) = V_0$ (m^3). Moreover, the bacterial population is constant and in the exponential growth stage.

Figure 5 shows a scheme of the CSTR where [NO$_3^-$]$_{in}$ is the concentration of nitrates in the wastewater, [NO$_3^-$]$_0$ is the initial concentration of nitrates at time $t = 0$, R_d represents the kinetics of the denitrification reactions and [NO$_3^-$](t) is the concentration of nitrates in time, which corresponds to the concentration of nitrates in the outgoing wastewater. When we consider the zero-order kinetics for the nitrates consumption, the system is described by a differential equation having the form

$$\frac{d[NO_3^-]}{dt} = \underbrace{[NO_3^-]_{in}\frac{F}{V_0}}_{\substack{\text{incoming} \\ \text{wastewater}}} - \underbrace{[NO_3^-]\frac{F}{V_0}}_{\substack{\text{outgoing treated} \\ \text{wastewater}}} - \underbrace{k_d^0}_{\substack{\text{denitrification} \\ \text{reaction}}} \tag{5}$$

where [NO$_3^-$] $\equiv$ [NO$_3^-$](t). Being both F and V_0 constant, $k_0 = F/V_0$ (d^{-1}) can be defined as the reciprocal of the mean residence time of a molecule in the reactor and the integral form of the Equation (5) yields the variation in time of nitrates

$$[NO_3^-] = [NO_3^-]_{in} - \frac{k_d^0}{k_0} + \left([NO_3^-]_0 + \frac{k_d^0}{k_0} - [NO_3^-]_{in}\right)e^{-k_0 t} \tag{6}$$

The stationary state value of nitrates [NO$_3^-$]$_{ss}$, *i.e.*, the concentration in the outgoing wastewater when the system is in continuous working regime, can be found by Equating (5) to zero or by calculating the limit of Equation (6) for t $\rightarrow \infty$ and obtain

$$[NO_3^-]_{ss} = [NO_3^-]_{in} - \frac{k_d^0}{k_0} \tag{7}$$

We then substituted in the equation the value for k_d^0 found in the experiments ($\sim$ 907 mg·L^{-1}·d^{-1}) and the values for k_0 and [NO$_3^-$]$_{in}$ of a real wastewater treatment plant with a potential of < 100,000 A.E. (F = 210 m^3/d, V_0 = 93 m^3, k_0 = 2.25 d^{-1}) and [NO$_3^-$]$_{in}$ = 450 mg/L to find that [NO$_3^-$]$_{ss}$ ~47 mg/L which corresponds to a reduction of nitrate of about 90%. This value meets the requests of Italian legislation that requires a minimum reduction of nitrate of 70% for this kind of treatment plant. The process was also modeled considering a first-order kinetics and using k_d^1 in the place of k_d^0, in this case

$$[NO_3^-]_{ss} = \frac{k_0[NO_3^-]_{in}}{(k_0 + k_d^1)} \tag{8}$$

and the efficiency of the process was found to be about 70%. A comparison with the literature confirms that the performance of the *Azospira* sp. OGA 24 are in line (sometimes better) of the most common bacteria employed in the biological treatment of polluted waters [7,12,23,27–29].
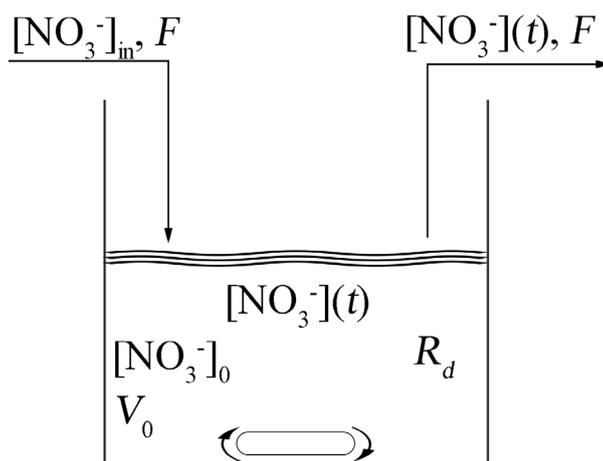
Figure 5. Scheme of the anoxic denitrification process in a Continuously Stirred Tank Reactor (CSTR).

4. Conclusions

With this work we started a thorough investigation of the denitrifying properties of the bacterium *Azospira* sp. OGA 24, recently isolated from the highly polluted waters of the Sarno river in the south of Italy. Thanks to the specifically devised bioreactor, it was possible to follow in real time both the concentration of nitrates, through an ion-selective electrode, and the optical density of the solution (a measure of the bacteria population size) through an in-line flow-thru spectrophotometer. The fitting of the experimental data allowed us to extract important parameters, which characterize the denitrification ability (kinetics constants) and the population growth (maximum specific growth rate) of the bacteria in anoxic conditions. In particular, it was found that in the presence of an excess of the organic substrate and at a relatively high $[NO_3^-]$, the stage with the maximum rate of nitrates consumption can be described by both a zero- and a first-order kinetics. The main motivation of the present work was to assess the effectiveness of OGA 24 as denitrifying agent; therefore a better characterization of the kinetic mechanism will be done in future works.

Kinetic constants were finally used in a simplified model of the anoxic stage of a real wastewater biological treatment plant, in order to assess the denitrifying capability of OGA 24 in the presence of a continuous influx of wastewater with a high concentration of nitrates. In particular, we looked for the stationary state solutions of the model where the characteristic parameters were taken from a real treatment plant and from our experiments. Despite many simplifications we introduced, our simulations could give a clear idea of the effectiveness of OGA 24 in depleting the nitrates concentration in polluted wastewater and the stationary state solutions were found to match the Italian law requirements, both when a zero- and a first-order denitrification kinetics were considered. Although many bacteria or consortium are known and used for the denitrification process, our results might provide an alternative microbial resource for biological removal of nitrogen compounds from wastewater. In particular, the extreme conditions where the bacteria colony was isolated favor the employment of the strain in those environments that generally inhibit biological treatments, such as tannery wastewater. Moreover, the OGA 24 effectiveness in reducing both nitrates and perchlorates simultaneously, make this strain very versatile and useful in several environmental conditions. Further

investigations are still in progress to study the influence of bacterial concentration, temperature and pH variations on the denitrification efficiency of OGA 24.

Acknowledgments

Author Contributions

Federico Rossi devised the experimental strategy, performed experiments and simulations, wrote the paper and contributed to the paper revisions. Oriana Motta and Antonio Proto devised the experimental strategy, wrote the paper and contributed to the paper revisions. Simona Matrella contributed to the biological part of the experiments. Giovanni Vigliotta contributed to the Biological part of the experiments and provided the purified OGA 24 strain.

Conflicts of Interest

The authors declare no conflict of interest.

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