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RSE

ASSESSMENT OF THE EFFECTS OF SULFATE AND NITRATE ON THE TEMPORAL EVOLUTION OF *KLEBSIELLA OXYTOCA* AND *STAPHYLOCOCCUS AUREUS* ABUNDANCE UNDER SHAKING CONDITIONS, IN AQUATIC MICROCOSM

Évaluation de l'influence du nitrate et du sulfate sur l'évolution temporelle d'abondance de Klebsiella oxytoca et Staphylococcus aureus sous conditions agitées en milieu aquatique en microcosme

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ABSTRACT

Most chemicals in natural aquatic media can be assimilated by bacteria. The impact of various environmental conditions on this microbial process is not always clear. This study aimed at investigating changes in the abundance of *Klebsiella oxytoca* and *Staphylococcus aureus* under different shaking conditions, in aquatic microcosms containing nitrate and sulfate. Sodium chloride solution (8.5 g NaCl•L^{-1}), and nitrate and sulfate solutions (0.005, 0.05, 0.5 and 5 g•L^{-1}) containing bacteria were supplemented with tryptic peptone at a final concentration of 10 g•L^{-1}. The solutions were incubated under shaking conditions (300, 350 and 400 rev•min^{-1}). Bacteriological analyses were performed hourly over a 6-h period. During the first 3 h of incubation, results showed that the highest values of the apparent cell growth rates (CAGRs) with K_2SO_4 in pure cultures, at a shaking speed 400 rev•min^{-1}, were 0.656 h^{-1} for *S. aureus*, and 0.364 h^{-1} for *K. oxytoca*. In mixed culture, the CAGR was 0.235 h^{-1} for *S. aureus*, and 0.388 h^{-1} for *K. oxytoca*, both recorded at 300 rev•min^{-1}. With KNO_3 in pure culture solutions, the CAGR was 0.353 h^{-1} for *S. aureus* at 300 rev•min^{-1}, and 0.367 h^{-1} for *K. oxytoca* at 350 rev•min^{-1}. In mixed culture it was 0.454 h^{-1} for *S. aureus* and 0.393 h^{-1} for *K. oxytoca*, both recorded at 350 rev•min^{-1}. The highest value of the apparent cell inhibition rate (CAIR) for *S. aureus* was 0.520 h^{-1} in K_2SO_4 (5 g•L^{-1}, 400 rev•min^{-1}), and 0.115 h^{-1} in KNO_3 (5 g•L^{-1}, 300 rev•min^{-1}). For *K. oxytoca*, it was 0.07 h^{-1} in K_2SO_4 in pure culture (0.05 g•L^{-1}, 300 rev•min^{-1}), and 0.044 h^{-1} in mixed culture (0.05 g•L^{-1}, 350 rev•min^{-1}). In KNO_3 it was 0.239 h^{-1} in mixed culture (5 g•L^{-1}, 300 rev•min^{-1}). The growth and inhibition potentials of different microbial species were impacted by the chemical concentrations and the movement speeds.

Key words: aquatic microcosm, shaking, sulfate, nitrate, bacteria evolution.

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RÉSUMÉ

L'impact de diverses conditions environnementales sur l'assimilation bactérienne dans l'eau, des composés chimiques est peu connu. La présente étude a visé l'évaluation de la dynamique d'abondance de *Klebsiella oxytoca* et *Staphylococcus aureus* sous diverses conditions, en milieu aquatique microcosme contenant du sulfate ou nitrate. Des solutions du NaCl (8,5 g•L^{-1}), sulfate et nitrate (0,005, 0,05, 0,5 et 5 g•L^{-1}) contenant des cellules ont été enrichies à la peptone trypsique (concentration finale 10 g•L^{-1}), puis incubées sous conditions dynamiques (300, 350 et 400 tr•min^{-1}). Les analyses bactériologiques ont été effectuées pendant 6 h. Il ressort qu'au cours des trois premières heures d'incubation, le taux de croissance cellulaire apparent (TCCA) le plus élevé en culture pure, contenant du K_2SO_4, est de 0,656 h^{-1} pour *S. aureus*, et 0,364 h^{-1} pour *K. oxytoca*, enregistrés à 400 tr•min^{-1}. En culture mixte, il est de 0,235 h^{-1} pour *S. aureus*, et 0,388 h^{-1} pour *K. oxytoca*, enregistrés à 300 tr•min^{-1}. Avec du KNO_3, en culture pure, le TCCA est de 0,353 h^{-1} pour *S. aureus* à 300 tr•min^{-1}, et 0,367 h^{-1} à 350 tr•min^{-1} pour *K. oxytoka*. En culture mixte, il est de 0,454 h^{-1} pour *S. aureus* et 0,393 h^{-1} pour *K. oxytoca*, enregistrés à 350 tr•min^{-1}. Le taux d'inhibition cellulaire apparent (TICA) le plus élevé de *S. aureus* est de 0,520 h^{-1} en présence du K_2SO_4 (5 g•L^{-1}, 400 tr•min^{-1}), et 0,115 h^{-1} en présence de KNO_3 (5 g•L^{-1}, 300 tr•min^{-1}). Pour *K. oxytoca*, il est de 0,07 h^{-1} avec du K_2SO_4 en culture pure (0,05 g•L^{-1}, 300 tr•min^{-1}), et 0,044 h^{-1} en culture mixte (0,05 g•L^{-1}, 350 tr•min^{-1}). Avec du KNO_3, il est de 0,239 h^{-1} en culture mixte (5 g•L^{-1}, 300 tr•min^{-1}). La croissance et l'inhibition des microorganismes sont affectées par les concentrations en sels et la vitesse de mouvements du milieu.

Mots clés : milieu aquatique en microcosme, agitation, sulfate, nitrate, évolution bactérienne.

1. INTRODUCTION

Natural aquatic environments harbor many bacterial species and numerous chemicals such as nitrates and sulfates. These chemicals can result from natural processes or from anthropological effects. Nitrates and sulfates can be assimilated by *Klebsiella* and *Staphylococcus* (PINAR and RAMOS, 1998). These bacteria can use nitrate and nitrite as sole nitrogen sources during aerobic growth. Nitrate assimilation takes place by three sequential steps: (i) nitrate transport into the cell by a specific nitrate permease; (ii) reduction to nitrite by assimilatory nitrate reductase; and (iii) further reduction to ammonium by assimilatory nitrite reductase. The resulting ammonium is then incorporated into central metabolism through the action of glutamine synthetase and glutamate synthetase (WU and STEWART, 1998). Nitrate sometimes favors the degradation of other compounds such as naphtalene under certain conditions (ROCKNE *et al.*, 1999). Degradation of numerous carbon molecules can be coupled to that of nitrates, but not often to sulfates (BAE *et al.*, 2002).

Most of aerobic aquatic bacteria are known to use assimilatory sulfate reduction to supply sulfur for biosynthesis, although many can assimilate sulfur from organic compounds that contain reduced sulfur atoms (COTTRELL and KIRCHMAN, 2000; TRIPP *et al.*, 2008). The assimilation of sulfate by bacteria is increased at the pH of the environment close to neutral (REIS *et al.*, 2005). In the presence of oxygen, sulfur metabolism is particularly energy costly. Sulfur and sulfate must first permeate the cell, usually against the intracellular electric potential which is usually strongly negative, then change from a highly oxidized state to a reduced state (SEKOWSKA and DANCHIN, 2009).

The biodegradation of sulfates and nitrates occurs in surface waters as well as during water infiltration through soil columns, and this process is sometimes impacted by the variation in oxygen concentrations in the environment (GANDHI *et al.*, 2002; ZINEBI *et al.*, 1994). Surface waters contain a variety of inorganic compounds and numerous bacterial species. These media are more often in movement. Along their trajectories, the quiet flow sections alternate with the fast ones which are more often agitated and are characterized by turbulence.

Microscopic organisms in aquatic environments are continuously exposed to a variety of physical and chemical conditions. Traditionally, it is accepted that due to their small size the physiology of microscopic organisms is not affected by the moving fluid at their scale (HONDZO and WUEST, 2009). The role of turbulence on the bacterial abundance evolution rate has been assessed by MOESENEDER and HERNDL (1995). They noted that bacterial growth rate is significantly modified by this factor with respect to the substrate. However, little data are available on the impact of this physical parameter when the medium contains the known chemicals and bacteria. Little is known of the impact of nitrates and sulfates on the microbial evolution rate in nutrient rich medium under shaking conditions. This study aims at assessing the impact of nitrates and sulfates compared with physiological solution on the temporal evolution of *Klebsiella oxytoca* and *Staphylococcus aureus* abundance under shaking condition, in aquatic microcosm environment. The two species are potentially human pathogen bacteria when using contaminated water. *K. oxytoca* is mostly isolated in urinary tract and *S. aureus* is mostly isolated in purulent wound on human skin (LE MINOR and VERON, 1989; WHO, 2003).

2. MATERIALS AND METHODS

2.1 Bacterial characteristics and storage

Staphylococcus aureus and *Klebsiella oxytoca* were isolated from stream, on Baird-Parker agar (Bio-Rad) and Hektoen agar medium (Biokard) respectively, using the membrane filtration method (MARCHAL *et al.*, 1991; RODIER, 1996). Their biochemical identification was performed according to HOLT *et al.* (2000).

For the preparation of bacterial stocks, a colony forming unit (CFU) of each strain from standard agar medium was inoculated into 100 mL of nutrient broth (Oxford) for 24 h at 37°C. After, cells were harvested by centrifugation at 8,000 rev•min^{-1} for 10 min at 10°C and washed twice with NaCl (8.5 g•L^{-1}) solution. Each pellet was re-suspended in 50 mL of NaCl solution and 500 µL was transferred in sterile tubes to be stocked. The stocks were then stored frozen.

2.2 Preparation of nitrate and sulfate solutions

Nitrate and sulfate solutions of concentration 5 g•L^{-1} each were prepared. From each of them, three diluted solutions 0.005, 0.05 and 0.5 g•L^{-1} respectively, were then prepared. At the same time, the NaCl solution (8.5 g•L^{-1}) was prepared. Then 1 mL of a trypsic peptone solution was added in each of the solutions above, at the final concentration of 10 mg•L^{-1} of trypsic peptone. This concentration value aimed to mimic the biodegradable oxygen demand (BOD_5) sometimes registered in surface water (DJUIKOM *et al.*, 2006, 2008). The pH was then adjusted to 7.0 using HCl (1 M) and NaOH (1 M). Series of 199 mL of each these final solutions (5, 0.5, 0.05, 0.005 g•L^{-1} and NaCl 8.5 g•L^{-1}) were transferred in non-chicanery glass flasks (Erlenmeyers 300 mL), and then sterilized.

2.3 Bacterial harvested and inoculation

Prior to the experiments, each of the stocks frozen vial containing *S. aureus* or *K. oxytoca*, was thawed at room temperature. Then 100 µL of the culture was transferred into 10 mL of nutrient broth (Oxford) in tube and incubated at 37°C. After 24 h, 100 µL of the suspension was added to 100 mL of the same nutrient broth and incubated also for 24 h at 37°C. Cells were then harvested by centrifugation at 8,000 rev•min^{-1} for 10 min at 10°C and washed twice with sterile NaCl solution (8.5 g•L^{-1}). The pellets were then re-suspended in 50 mL of sterilized solution containing either K_2SO_4 or KNO_3. After serial dilutions, 1 mL of the suspension was added to 199 mL of each sterilized salt solution containing peptone as indicated above. Based on our preliminary study, cell concentration was adjusted at 10^3 CFU•mL^{-1}. Experiments were done in two steps. In the first, only one bacteria strain was added in each solution in the flask and this was called the "pure solution". In the second, the two strains were added at the same time and this was called the "mixed solution". Flasks were then incubated.

2.4 Incubation of solutions and bacterial analysis

Incubations of solutions were done on an agitator GLF 3018, for 6 h at a room temperature (24 ± 1°C). For all the experiments, three shaking speeds were used: 300, 350 and 400 rev•min^{-1}. The three shaking speeds were chosen based on our preliminary results which showed that at shaking speeds higher than 400 rev•min^{-1}, cells can be destroyed. At shaking speeds lower than 300 rev•min^{-1}, the evolution of cell abundances was similar in most cases. Experiments were carried out under shaking conditions aiming to favor the biodegradation of organic compounds. For each concentration and each shaking speed, solutions were made in triplicate. Analyses to determine concentrations of cultivable bacteria in each solution were performed hourly. Six interval periods were thus chosen. Each analysis was performed from 1 mL sampled in each solution in the flask, and was done using plate count method on standard agar medium. Petri disks were then incubated at 37°C for 24 h and the CFUs were enumerated.

2.5 Data analysis

The hourly mean values of CFU were calculated. Curves of cell abundance evolution have been plotted. Those from the NaCl were inserted in all curves for a better visualization of the differences in bacterial evolution abundances. Because most of the curves are superposed, the standard deviations were not mentioned on the graphs. An overall comparison (ANOVA) of changes in bacterial abundance has been carried out considering the concentrations and the shaking speeds. The straight Ln (number of CFUs) lines against storage duration were plotted. Each straight line equation $y = ax + b$ has been calculated. The slope a of each regression line was considered as the apparent evolution rate of the abundance of each bacteria species at the third and sixth hour of incubation in each condition. This slope was then considered as the cell apparent growth rate (CAGR) when it was positive, or as the cell apparent inhibition rate (CAIR) when it was negative.

3. RESULTS

3.1 Evolution of S. aureus *abundance in pure solution*

When the shaking speed was 300 rev•min^{-1}, the abundance of *S. aureus* in NaCl solutions (8.5 g•L^{-1}) increased gradually from 10•10^2 to 43.1•10^2 CFU•mL^{-1}. The highest abundance was recorded after 6 h of incubation (Figure 1). In solutions containing K_2SO_4 at concentrations 0.05 and 0.005 g•L^{-1}, the highest abundances were recorded respectively after 2 and 5 h of incubation. At the concentrations 0.5 and 5 g•L^{-1}, they were recorded after 3 h of incubation (Figure 1). In solutions containing KNO_3, the highest cell abundances were 31.9•10^2, 33•10^2, 37•10^2 and 25.4•10^2 CFU•mL^{-1} at concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1} respectively (Figure 1).

When the solutions were shaked at 350 rev•min^{-1}, the highest cell abundance in NaCl was 67.5•10^2 CFU•mL^{-1} and was recorded after 5 h of incubation (Figure 1). In solutions containing K_2SO_4, the cell highest abundances were 77•10^2, 67.5•10^2, 39•10^2 and 31•10^2 CFU•mL^{-1} at concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1} respectively (Figure 1). In solutions containing KNO_3, the highest cell abundances were 28.9•10^2, 30.5•10^2, 33.9•10^2 and 34.3•10^2 CFU•mL^{-1} respectively recorded at concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1} (Figure 1).

At a shaking speed of 400 rev•min^{-1}, the highest cell abundance (87.2•10^2 CFU•mL^{-1}) was recorded after 5 h of incubation in the NaCl solution. When the solutions contained K_2SO_4 at concentrations 0.005 and 0.05 g•L^{-1}, it was 68•10^2 and 104•10^2 CFU•mL^{-1} respectively. Both values were recorded after 6 h of incubation (Figure 1). In solutions containing KNO_3 at the concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1}, they were recorded respectively after 5, 6, 3 and 2 h of incubation, and were 30.6•10^2, 28.7•10^2, 26.8•10^2 and 14.4•10^2 CFU•mL^{-1} respectively (Figure 1).

3.2 Evolution of K. oxytoca *abundance in pure solution*

At a shaking speed 300 rev•min^{-1}, the increase in cell abundances in solutions containing NaCl and K_2SO_4 was gradual during the second half of the incubation period. The highest cell abundances were recorded after 6 h of incubation (Figure 2). In solutions containing KNO_3, the shape of the cell abundance variations was similar to those observed in K_2SO_4 solutions and the highest cell abundances were also recorded after 6 h of incubation (Figure 2).

The increase in cell abundances was gradual in the second half of the incubation period at a shaking speed 350 rev•min^{-1}, with the exception of the NaCl solution in which the shape of cell abundance variations was hyperbolic. The highest cell concentrations were 86•10^2 CFU•mL^{-1} in NaCl solutions, and 44.1•10^2, 70.4•10^2, 29.5•10^2 and 26•10^2 CFU•mL^{-1} in those containing K_2SO_4 at concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1} respectively (Figure 2). In solutions containing KNO_3, the curves of cell abundance variations were hyperbolic at 0.005 g•L^{-1}, and sinusoidal in the others. At concentrations 0.5 and 5 g•L^{-1} of KNO_3, they were 19.2•10^2 and 32•10^2 CFU•mL^{-1} respectively, and were recorded after 4 and 3 h of incubation (Figure 2).

The evolution of the cell abundance was gradual in solutions containing K_2SO_4 at concentrations 0.005, 0.5 and 5 g•L^{-1} when the medium was shaked at 400 rev•min^{-1}, and the shape of the curve of cell abundance variations seems sinusoidal at 0.05 g•L^{-1} (Figure 2). In solutions containing KNO_3, all curves of cell abundance variations were sinusoidal. At the concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1}, the highest cell abundances were 30•10^2, 27.7•10^2, 24.6•10^2 and 21.4•10^2 CFU•mL^{-1} respectively (Figure 2).

3.3 Evolution of S. aureus *and* K. oxytoca *in mixed solutions*

When both cell species were present simultaneously, the curves of their abundance variation were hyperbolic in NaCl solution at a shaking speed of 300 rev•min^{-1} and 400 rev•min^{-1} for *K. oxytoca*, and at 350 rev•min^{-1} and 400 rev•min^{-1} for *S. aureus* (Figures 3 and 4). At 300 rev•min^{-1}, the highest cell abundance was 48.2•10^2 CFU•mL^{-1} for *K. oxytoca*; this value was noted just after 3 h of incubation. That of *S. aureus* was 32.1•10^2 CFU•mL^{-1} and was recorded after 6 h of incubation (Figures 3 and 4). When the solutions contained K_2SO_4, curves of cell abundance variations were sinusoidal, those of *S. aureus* except at concentrations 0.005 and 0.05 g•L^{-1} (Figure 3).

At a shaking speed 350 rev•min^{-1}, the highest abundance of each species was recorded at the end of fourth hour of incubation in a NaCl solution. It was 21.4•10^2 CFU•mL^{-1} for *K. oxytoca*, and 43.7•10^2 CFU•mL^{-1} for *S. aureus* (Figures 4). In solutions containing K_2SO_4, the abundances reached 28.6•10^2 CFU•mL^{-1} for *K. oxytoca*, and 30•10^2 CFU•mL^{-1} for *S. aureus* at concentration 0.005 g•L^{-1}, 20•10^2 CFU•mL^{-1} for *K. oxytoca* and 19.5•10^2 CFU•mL^{-1} for *S. aureus* at concentration 0.05 g•L^{-1}, 18.6•10^2 CFU•mL^{-1} for *K. oxytoca* and 21.7•10^2 CFU•mL^{-1} for *S. aureus* at 0.5 g•L^{-1}, and 14.3•10^2 CFU•mL^{-1} for *K. oxytoca* and 22•10^2 CFU•mL^{-1} for *S. aureus* at 5 g•L^{-1}. At this shaking speed, all highest cell

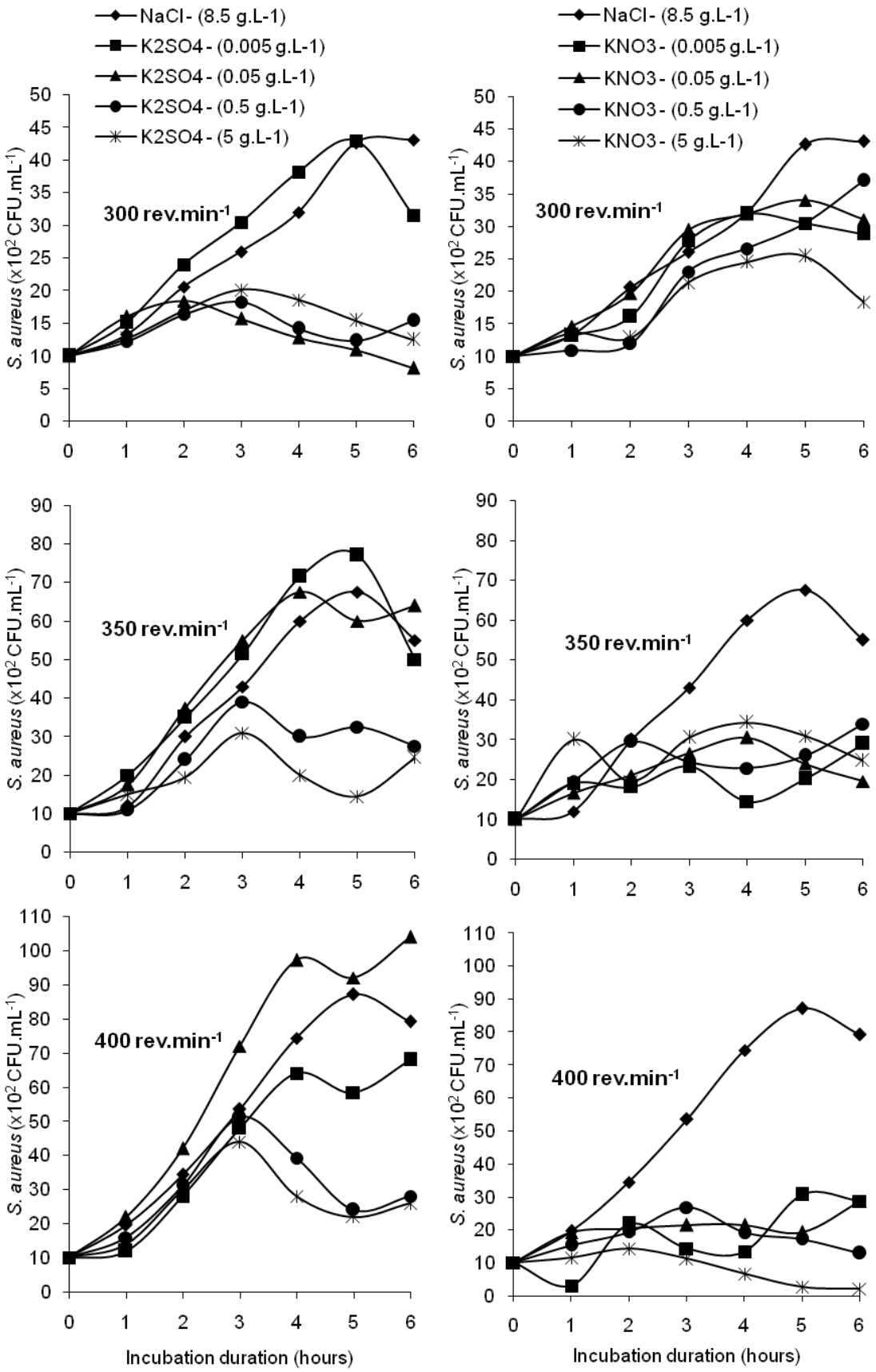

Figure 1. **Temporal variation of mean values of the *S. aureus* abundance in each of K_2SO_4 and KNO_3 solutions, when shaking at 300, 350 and 400 rev•min^{-1} respectively.**
Variation temporelle des valeurs moyennes des abondances de* S. aureus *dans les solutions de K_2SO_4 et KNO_3 aux vitesses d'agitation 300, 350 et 400 rev•min^{-1}, respectivement.

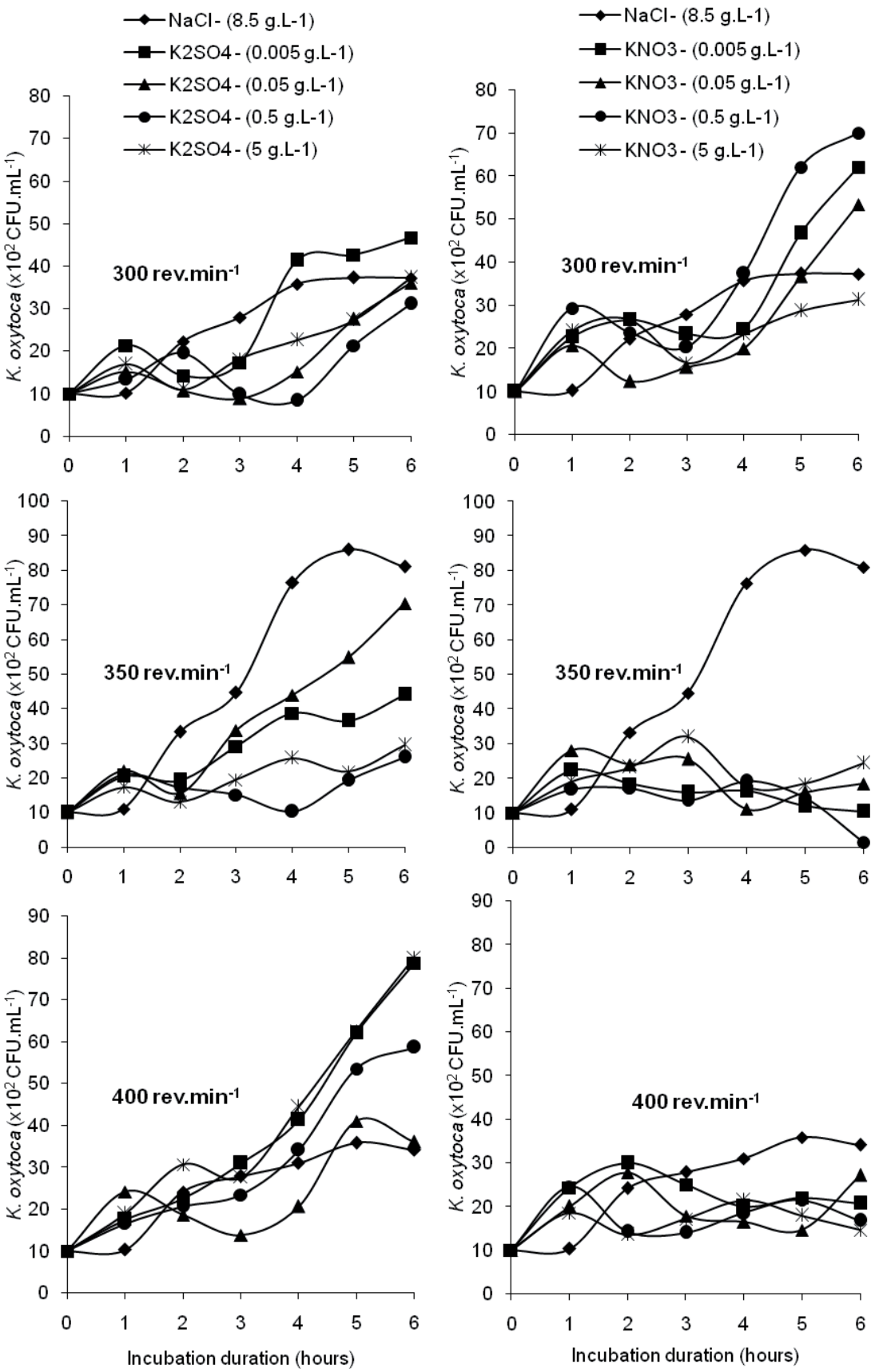

Figure 2. Temporal variation of mean values of the *K. oxytoca* abundance in each of K_2SO_4 and KNO_3 solutions, when shaking at 300, 350 and 400 rev•min^{-1} respectively.
Variation temporelle des valeurs moyennes des abondances de* K. oxytoca *dans les solutions de K_2SO_4 et KNO_3 aux vitesses d'agitation 300, 350 et 400 rev•min^{-1}, respectivement.

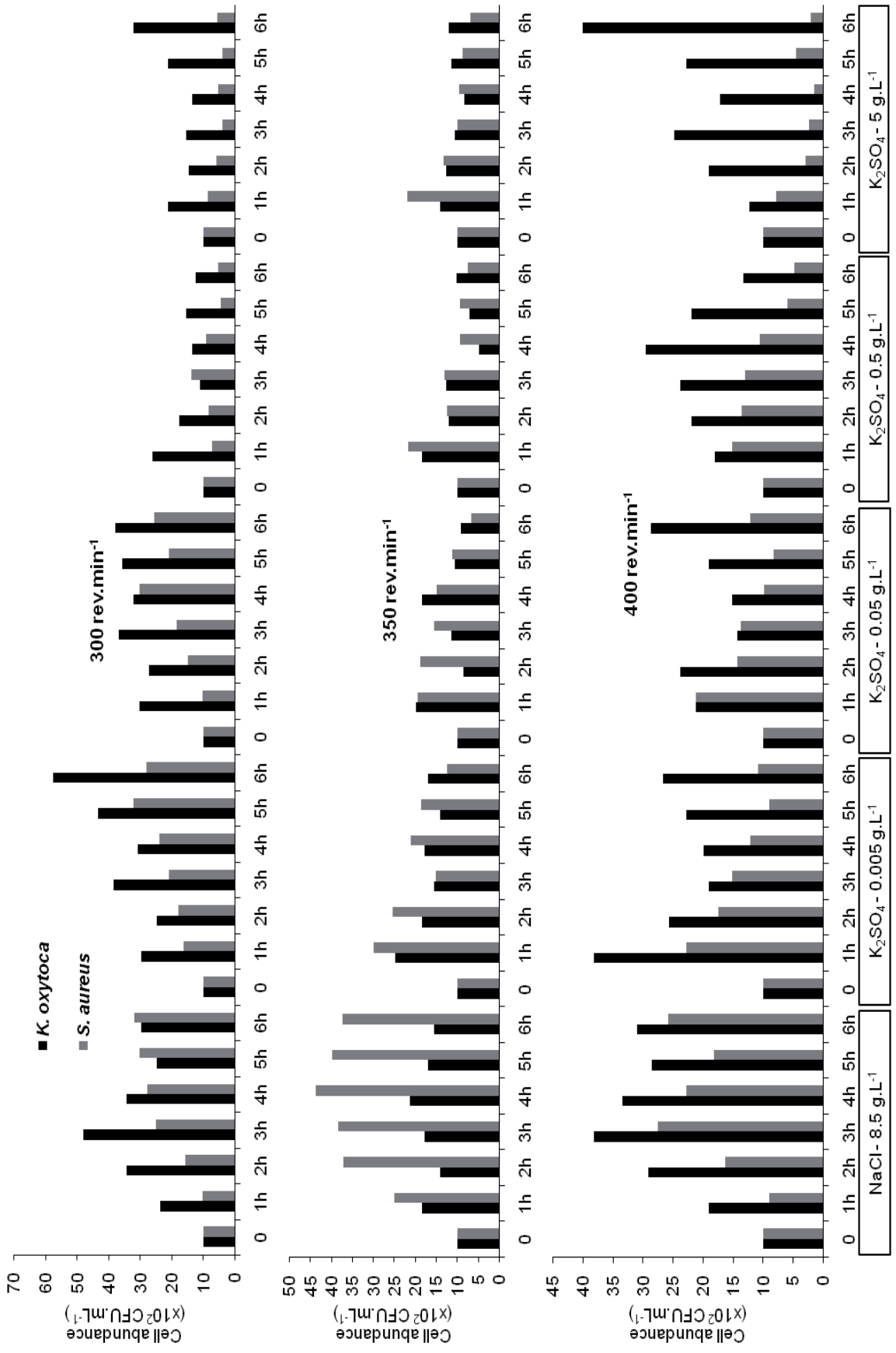

Figure 3. Temporal variation of mean values of the abundance of *K. oxytoca* and *S. aureus* in each K_2SO_4 solution, when shaking at 300, 350 and 400 rev•min^{-1} respectively.
Variation temporelle des valeurs moyennes de l'abondance de K. oxytoca *et* S. aureus *dans chaque solution de K_2SO_4, aux vitesses d'agitation 300, 350 et 400 rev•min^{-1}, respectivement.*

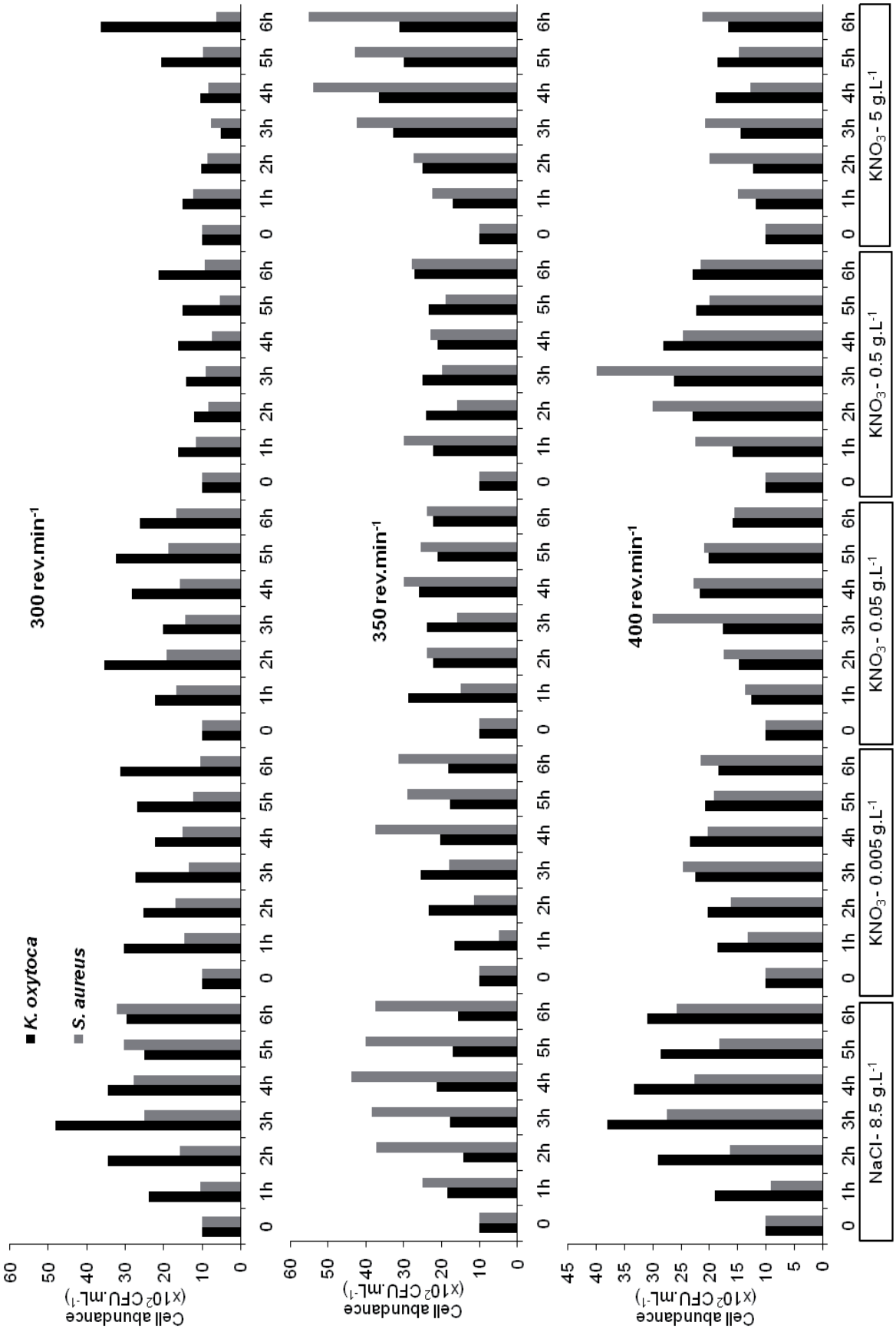

Figure 4. Temporal variation of mean values of the abundance of *K. oxytoca* and *S. aureus* in each KNO_3 solution, when shaking at 300, 350 and 400 rev•min^{-1} respectively.
Variation temporelle des valeurs moyennes de l'abondance de K. oxytoca *et S.* aureus *dans chaque solution de KNO_3, aux vitesses d'agitation 300, 350 et 400 rev•min^{-1}, respectivement.*

abundances were recorded just after 1 or 2 h of incubation (Figure 3).

At 400 rev•min^{-1}, the highest cell abundances of both bacterial species were noted at the end of the first 3 h of incubation in NaCl solutions. They were 38.1•10^2 CFU•mL^{-1} for *K. oxytoca* and 27.6•10^2 CFU•mL^{-1} for *S. aureus* (Figures 3 and 4). In the presence of K_2SO_4 at concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1}, the highest abundances of *K. oxytoca* were 38.1•10^2, 28.7•10^2, 29.5•10^2 and 40•10^2 CFU•mL^{-1} respectively. Those of *S. aureus* were 22.7•10^2, 21.2•10^2, 15.2•10^2 and 10•10^2 CFU•mL^{-1} respectively, and were recorded just after 1 h of incubation, except in the solution at 5 g•L^{-1} in which cell abundance declined during the incubation period (Figure 3).

When both cell species were present simultaneously in solutions containing KNO_3, the increase in *S. aureus* abundances was noted after 1 h of incubation in most cases at 300 rev•min^{-1} (Figure 4). At a shaking speed of 350 rev•min^{-1}, the highest abundances of *S. aureus* were 37.5•10^2, 25.5•10^2, 30•10^2 and 55•10^2 CFU•mL^{-1} at concentrations 0.005, 0.05, 0.5 and 5 g•L^{-1} respectively; those of *K. oxytoca* at the same concentrations were 25.5•10^2, 28.9•10^2, 27.2•10^2 and 56.7•10^2 CFU•mL^{-1} (Figure 4). When the medium was agitated at 400 rev•min^{-1}, the cell abundance variation also varied with the concentration of KNO_3, and the curves seem hyperbolic in most cases (Figure 4).

3.4 Comparison of the evolution of bacterial abundances

An overall comparison (ANOVA) of changes in bacterial abundances has been carried out considering the concentrations of salts used, for each shaking speed. It appeared that at all shaking speeds of the medium in mixed culture containing K_2SO_4, changes in abundances of *S. aureus* differed significantly ($P < 0.001$) from one concentration to another. Meanwhile, changes in abundances of *K. oxytoca* differed significantly ($P < 0.05$) from one concentration to another only at the shaking speeds of 300 and 350 rev•min^{-1} (Table 1). In mixed culture containing KNO_3 and in pure culture containing each of the two salts used, the magnitude of the differences among changes in bacteria abundances varied with the salt concentration (Table 1). An overall comparison of the evolution of cell abundances has also been carried out considering the shaking speeds of the medium. It has been noted that in mixed and pure cultures, changes in abundances of *S. aureus* differed significantly ($P < 0.001$) with a shaking speed when the medium contained KNO_3 at a concentration of 5 g•L^{-1} (Table 2). In other cases, the magnitudes of the differences are very variable.

3.5 Assessment of bacterial abundance apparent evolution rates

The slope a of each regression line was calculated as the apparent evolution rate of each bacteria species abundance at the third or sixth hour of incubation in each condition. This slope was then considered as the cell apparent growth rate (CAGR) when it was positive, or to the cell apparent inhibition rate (CAIR) when it was negative. It appeared that in NaCl solutions, the CAGRs of *S. aureus* in pure culture varied during the first 3 h of incubation, from 0.331•h^{-1} when the solutions were agitated at 300 rev•min^{-1}, to 0.559•h^{-1} when they were agitated at 400 rev•min^{-1}. Considering the full period of incubation (6 h), it was noted that the CAGRs in these solutions varied from 0.255•h^{-1} to 0.355•h^{-1} (Table 3). There was a decrease in the cell growth rate during the second half of the incubation period. In mixed culture, CAGRs varied from 0.317•h^{-1} to 0.444•h^{-1} during the first 3 h of incubation, and the greatest value was recorded when the solution was agitated at 350 rev•min^{-1}. However, when considering the full period of incubation, the CAGRs during this period varied from 0.162•h^{-1} to 0.221•h^{-1}, the highest value was also observed when the solution was agitated at 350 rev•min^{-1} (Table 3). This shaking speed seems to create the most favorable conditions to the *S. aureus* multiplication in the presence of *K. oxytoca* in NaCl solutions containing biodegradable organic matter.

In the presence of K_2SO_4 or KNO_3, there was a relative variability of *S. aureus* apparent evolution rates in pure cultures as well as in mixed cultures. For the whole, when considering the pure cultures, the highest CAGR of *S. aureus* during the first 3 h of incubation was 0.656•h^{-1} in the presence of K_2SO_4 (0.05 g•L^{-1}, 400 rev•min^{-1}), and 0.353•h^{-1} in the presence of KNO_3 (0.05 g•L^{-1}, 300 rev•min^{-1}) (Table 3). The CAGRs were lower after 6 h of incubation (Table 3). This would be due to the decrease in cell growth rates during the second half of the incubation period. When considering the mixed culture, the highest CAGR value during the first 3 h of incubation was 0.235•h^{-1} in the presence of K_2SO_4 (0.005 g•L^{-1}, 300 rev•min^{-1}), and 0.454•h^{-1} in the presence of KNO_3 (5 g•L^{-1}, 350 rev•min^{-1}). As in pure cultures, the CAGRs were also lower after 6 h of incubation (Table 3). Considering the full period of incubation in mixed cultures, it was noted that the cell apparent evolution rates were negative in the presence of K_2SO_4 at all concentrations, when the solutions were shaked at 350 and 400 rev•min^{-1}, and led to the CAIRs (Table 3). This would be due to a greater inhibition of *S. aureus* growth during the second half of the incubation period. The highest value of CAIR in the presence of K_2SO_4 considering the first 3 h of the incubation was 0.520•h^{-1} (5 g•L^{-1}, 400 rev•min^{-1}); in the presence of KNO_3, it was 0.115•h^{-1} (5 g•L^{-1}, 300 rev•min^{-1}). No CAIR was noted during this incubation period in pure cultures (Table 3).

Table 1. **Comparison (ANOVA) among the evolution of the bacterial abundances when considering the salt concentrations, with respect of the shaking speed.**

Tableau 1. ***Comparaison (ANOVA) entre l'évolution des abondances bactériennes en considérant les concentrations des sels, en fonction de la vitesse d'agitation du milieu.***

Salt used and culture conditions		Shaking speeds and bacteria species					
		300 rev•min^{-1}		350 rev•min^{-1}		400 rev•min^{-1}	
		K. oxytoca	*S. aureus*	*K. oxytoca*	*S. aureus*	*K. oxytoca*	*S. aureus*
M. culture	K_2SO_4	4.150**	8.775***	3.609*	11.036***	1.071	7.636***
	KNO_3	3.729*	9.015**	2.409	3.185	4.629**	1.336
P. culture	K_2SO_4	1.359	5.099**	3.324	2.654	0.983	2.628
	KNO_3	0.842	0.760	6.126**	3.088	1.855	8.481***

N= 28 observations; M. culture: mixed culture; P. culture: pure culture;
Significant difference (*: P<0.05; **: P<0.01; ***: P<0.001)

Table 2. **Comparison (ANOVA) among the evolution of the bacterial abundances when considering the shaking speeds, with respect of the salt concentration.**

Tableau 2. ***Comparaison (ANOVA) entre l'évolution des abondances bactériennes en considérant les vitesses d'agitation, en fonction de la concentration en sel du milieu.***

Salt used and culture conditions		Bacteria species and salt concentrations (g•L^{-1})							
		K. oxytoca				*S. aureus*			
		0.005	0.05	0.5	5	0.005	0.05	0.5	5
M. culture	K_2SO_4	4.668*	10.627**	4.532*	3.252	2.368	2.124	1.397	6.954**
	KNO_3	2.385	3.449	3.649*	4.080*	1.480	1.153	10.088**	13.088***
P. culture	K_2SO_4	0.645	2.525	3.230	3.407	1.314	6.601**	3.711*	2.657
	KNO_3	3.614*	0.530	5.700*	2.616	0.658	0.631	0.470	12.240***

N = 21 observations; M. culture: mixed culture; P. culture: pure culture;
Significant difference (*: P<0.05 ; **: P<0.01 ; ***: P<0.001)

Table 3. **Apparent evolution rate value of *S. aureus* (and regression coefficient) in pure culture (p) and in mixed culture (m), in each experimental solution during the first 3 h of incubation and during the full incubation period (6 h) at each shaking speed.**

Tableau 3. ***Valeurs du taux d'évolution apparent de* S. aureus *(et coefficient de régression) en culture pure (p) et en culture mixte (m), dans chaque solution expérimentale, durant les trois premières heures d'incubation et pour la période entière d'incubation (6 h), et à chaque vitesse d'agitation.***

Solutions	Cell apparent evolution rate (h^{-1})											
	300 rev•min^{-1}				350 rev•min^{-1}				400 rev•min^{-1}			
	3h-(p)	3h-(m)	6h-(p)	6h-(m)	3h-(p)	3h-(m)	6h-(p)	6h-(m)	3h-(p)	3h-(m)	6h-(p)	6h-(m)
NaCl - 8.5 g•L^{-1}	0.331	0.317	0.255	0.221	0.529	0.444	0.330	0.180	0.559	0.363	0.355	0.162
	(0.986)	(0.912)	(0.961)	(0.905)	(0.938)	(0.834)	(0.938)	(0.564)	(0.991)	(0.842)	(0.887)	(0.633)
K_2SO_4 - 0.005 g•L^{-1}	0.380	0.235	0.214	0.170	0.547	0.107	0.294	-0.16	0.555	0.098	0.347	-0.069
	(0.984)	(0.883)	(0.774)	(0.882)	(0.984)	(0.077)	(0.742)	(0.008)	(0.951)	(0.136)	(0.868)	(0.205)
K_2SO_4 - 0.05 g•L^{-1}	0.149	0.223	-0.062	0.174	0.587	0.130	0.307	-0.087	0.656	0.057	0.383	-0.059
	(0.527)	(0.931)	(0.211)	(0.795)	(0.985)	(0.300)	(0.798)	(0.252)	(0.992)	(0.058)	(0.861)	(0.172)
K_2SO_4 - 0.5 g•L^{-1}	0.210	0.111	0.042	-0.092	0.486	0.026	0.193	-0.099	0.556	0.068	0.147	-0.152
	(0.976)	(0.277)	(0.195)	(0.293)	(0.932)	(0 .010)	(0.606)	(0.394)	(0.995)	(0.253)	(0.334)	(0.581)
K_2SO_4 - 5 g•L^{-1}	0.239	-0.299	0.041	-0.115	0.365	-0.049	0.094	-0.115	0.520	-0.520	0.132	-0.226
	(0.991)	(0.960)	(0.126)	(0.549)	(0.990)	(0.029)	(0.300)	(0.462)	(0.976)	(0.928)	(0.329)	(0.496)
KNO_3 - 0.005 g•L^{-1}	0.326	0.101	0.197	-0.013	0.249	0.259	0.110	0.290	0.304	0.292	0.260	0.116
	(0.952)	(0.340)	(0.823)	(0.021)	(0.778)	(0.398)	(0.482)	(0.734)	0.217)	(0.979)	(0.505)	(0.648)
KNO_3 - 0.05 g•L^{-1}l	0.353	0.122	0.198	0.055	0.317	0.188	0.110	0.139	0.236	0.353	0.115	0.087
	(0.997)	(0.314)	(0.833)	(0.293)	(0.956)	(0.458)	(0.423)	(0.608)	(0.726)	(0.970)	(0.610	(0.273)
KNO_3 - 0.5 g•L^{-1}	0.258	-0.065	0.242	-0.067	0.307	0.145	0.142	0.090	0.319	0.444	0.035	0.066
	(0.776)	(0.334)	(0.937)	(0.341)	(0.710)	(0.167)	(0.596)	(0.277)	(0.987)	(0.923)	(0.050)	(0.113)
KNO_3 - 5 g•L^{-1}	0.221	-0.115	0.132	-0.067	0.291	0.454	0.119	0.253	0.058	0.247	-0.297	0.063
	(0.822)	(0.528)	(0.648)	(0.467)	(0.517)	(0.935)	(0.360)	(0.802)	(0.253)	(0.898)	(0.713)	(0.234)

For *K. oxytoca*, the greatest CAGR value in pure cultures in NaCl solutions after the first 3 h of incubation, at the shaking speed of 350 rev•min^{-1} was 0.558•h^{-1}. In the presence of *S. aureus*, its highest CAGR was 0.509•h^{-1}; it was recorded at the shaking speed of 300 rev•min^{-1} (Table 4). As observed in the case of *S. aureus*, the apparent evolution rates of *K. oxytoca* in the presence of K_2SO_4 or KNO_3 underwent a relative variability with respect to the shaking speed and the concentration of salts in solutions. Considering the first 3 h of the incubation, it was noted that in the presence of K_2SO_4 the highest CAGR value of *K. oxytoca* in pure cultures was 0.388•h^{-1} (0.005 g•L^{-1}, 300 rev•min^{-1}). In mixed cultures, it was 0.363•h^{-1} (0.005 g•L^{-1}, 400 rev•min^{-1}). In the presence of KNO_3, it was 0.367•h^{-1} in pure cultures and 0.393•h^{-1} in mixed cultures. Both values were noted at the concentration 5 g•L^{-1} and at a shaking speed of 350 rev•min^{-1} (Table 4). As observed in the case of *S. aureus*, the apparent evolution rates of *K. oxytoca* were relatively low when considering the full period of incubation. Its highest CAIR value during the first 3 h of incubation in the presence of K_2SO_4 was 0.07•h^{-1} in pure cultures (0.05 g•L^{-1}, 300 rev•min^{-1}), and 0.044•h^{-1} in mixed cultures (0.05 g•L^{-1}, 350 rev•min^{-1}) (Table 4). In the presence of KNO_3, it was 0.239•h^{-1} in mixed cultures (5 g•L^{-1}, 300 rev•min^{-1}). No CAIR was noted in the pure cultures during this incubation period (Table 4).

4. DISCUSSION

This study shows that at all shaking speeds in NaCl solution, the abundance of *S. aureus* or *K. oxytoca* increases with the increasing of the incubation duration. In pure cultures, the maximum abundances were recorded after 5 or 6 h of incubation. In mixed cultures, they were recorded after 3 or 4 h of incubation. Moreover, their evolution seems hyperbolic in most cases. However, in solutions containing K_2SO_4 or KNO_3, a temporal variability in evolution rates of cell abundances was noted from one concentration in salt to another for the same shaking speed, and from one shaking speed to another for the same salt concentration. This variability was also noted in pure and mixed cultures. Changes in evolution rates of cell abundance may be related to the accumulation of certain toxic chemicals which temporarily became toxic to the cells, and which would also decrease or inhibit their proliferation. Over time, the complexion of these products with others could neutralize them and allow an increase in cell growth rates. In aqueous solutions, sulfates might be transformed to sulfide with the reaction of biogenic hydrogen sulfide, extracellular substances and organic acid (ZHAO *et al.*, 2007).

Results showed that shaking speeds impacted the cell apparent evolution rates. The role of the turbulence of aquatic medium on the bacterial abundance evolution rate has also been assessed by MOESENEDER and HERNDL (1995). They noted that the bacterial growth rate was significantly modified by the turbulence with respect to the substrate. TALON *et al.* (1999) noted that in static conditions, the synthesis of nitrate reductase enzyme is maximal during cell exponential growth phases whereas in shaking condition, its synthesis is maximal at the beginning of the stationary phase. Agitation speed is also an important factor in the fermentation process since it will increase the amount of dissolved oxygen in the cultivation medium. DARAH and IBRAHIM (1998) reported that a maximum lignin peroxidase activity and a maximum fungal growth were achieved when the optimal agitation speed of 150 rev•min^{-1} was used. Some authors, when investigating on the activity of two *Bacillus* species noted that it was maximal at the outset of incubation under agitation speed of 300 rev•min^{-1} (EL-TAYEB *et al.*, 2007). This situation reflects an in vivo-limited functioning of most enzymes in bacteria. When investigating on the impact of hydrological factors on the microbial distribution in aquatic microcosm, PINHASSI *et al.* (2004) noted that agitation was a noninvasive means to induce phytoplankton; and changes in phytoplankton community composition were followed by shifts in bacterioplankton community composition. Other investigations showed that mixing during resuspension events may also have profound effects on the dynamics of resuspended benthic microbial food webs (GARSTECKI and WICKHAM, 2001).

Excessive agitation would produce greater mechanical forces or hydrodynamic shear stresses and this condition is known to damage fungal mycelia and pellets (PORCEL *et al.*, 2005), that lead to cell destruction thus lowering the enzyme production. PAPAGIANNI *et al.* (2001) also found that the enzyme production was strongly affected by the agitation. At higher agitation rates, the enzyme production dropped. Agitation speed of the culture broth has a variety of effects on microorganisms, including rupture of the cell wall, change in the morphology of filamentous microorganisms, variation in the efficiency and rate of growth and also variation in the rate of formation of the desired product (PORCEL *et al.*, 2005). However, it has been reported that a marine bacterium (*Vibrio splendidus*) was not affected by the turbulence of the medium, whereas a protistan predator (*Paraphysomonas sp*.) was significantly affected; bacterial grazing mortality by *Paraphysomonas sp*. was 1.3- to 2.5-fold greater in the turbulent than static treatments among all four temperatures, and the rates of cell-specific ingestion of bacteria by *Paraphysomonas sp*. was 2-fold greater at 10-15°C in the turbulent than in the static treatment (DELANEY, 2003). The environmental temperature can influence this factor. The effects of turbulence have been reported as small under cold conditions (temperature less than 2°C), but increase with the size of the organism (DELANEY and KNOECHEL, 2004).

Table 4. **Apparent evolution rate value of *K. oxytoca* (and regression coefficient) in pure culture (p) and in mixed culture (m), in each experimental solution during the first 3 h of incubation and during the full incubation period (6 h) at each shaking speed.**

Tableau 4. ***Valeurs du taux d'évolution apparent de* K. oxytoca *(et coefficient de régression) en culture pure (p) et en culture mixte (m), dans chaque solution expérimentale, durant les trois premières heures d'incubation et pour la période entière d'incubation (6 h), et à chaque vitesse d'agitation.***

Solutions	Cell apparent evolution rate (h^{-1})											
	300 rev•min^{-1}				300 rev•min^{-1}				300 rev•min^{-1}			
	3h-(p)	3h-(m)	6h-(p)	6h-(m)	3h-(p)	3h-(m)	6h-(p)	6h-(m)	3h-(p)	3h-(m)	6h-(p)	6h-(m)
NaCl - 8.5 g•L^{-1}	0.386 (0.886)	0.509 (0.940)	0.250 (0.855)	0.120 (0.275)	0.558 (0.900)	0.147 (0.451)	0.400 (0.885)	0.057 (0.255)	0.392 (0.867)	0.443 (0.965)	0.229 (0.815)	0.154 (0.533)
K_2SO_4 - 0.005 g•L^{-1}	0.124 (0.248)	0.388 (0.727)	0.252 (0.802)	0.222 (0.740	0.314 (0.832)	0.105 (0.127)	0.225 (0.876)	0.016 (0.016)	0.364 (0.967)	0.154 (0.124)	0.333 (0.989)	0.059 (0.097)
K_2SO_4 - 0.05 g•L^{-1}	-0.070 (0.157)	0.381 (0.711)	0.192 (0.617)	0.160 (0.548)	0.330 (0.682)	-0.044 (0.024)	0.311 (0.910)	-0.024 (0.025)	0.070 (0.057)	0.118 (0.151)	0.179 (0.597)	0.089 (0.298)
K_2SO_4 - 0.5 g•L^{-1}	0.031 (0.015)	-0.001 (0.001)	0.125 (0.310)	-0.023 (0.024)	0.102 (0.180)	0.032 (0.027)	0.077 (0.219)	-0.096 (0.240)	0.278 (0.911)	0.279 (0.849)	0.291 (0.975)	0.055 (0.103)
K_2SO_4 - 5 g•L^{-1}	0.137 (0.347)	0.094 (0.152)	0.201 (0.841)	0.122 (0.482)	0.171 (0.555)	0.010 (0.006)	0.156 (0.800)	-0.009 (0.015)	0.352 (0.807)	0.315 (0.984)	0.321 (0.948)	0.188 (0.787)
KNO_3 - 0.005 g•L^{-1}	0.270 (0.610)	0.282 (0.508)	0.244 (0.820)	0.108 (0.359)	0.121 (0.208)	0.315 (0.918)	-0.044 (0.099)	0.064 (0.209)	0.296 (0.601)	0.253 (0.797)	0.056 (0.121)	0.078 (0.344)
KNO_3 - 0.05 g•L^{-1}	0.083 (0.117)	0.257 (0.405)	0.237 (0.753)	0.122 (0.390)	0.266 (0.517)	0.235 (0.419)	-0.001 (0.001)	0.068 (0.184)	0.206 (0.393)	0.185 (0.991)	0.066 (0.160)	0.096 (0.595)
KNO_3 - 0.5 g•L^{-1}	0.191 (0.281)	0.075 (0.220)	0.278 (0.800)	0.086 (0.611)	0.094 (0.236)	0.283 (0.704)	-0.203 (0.255)	0.106 (0.460)	0.046 (0.026)	0.326 (0.951)	0.055 (0.160)	0.120 (0.525)
KNO_3 - 5 g•L^{-1}	0.163 (0.227)	-0.239 (0.479)	0.130 (0.505)	0.161 (0.313)	0.367 (0.937)	0.393 (0.976)	0.082 (0.243)	0.174 (0.673)	0 .130 (0.366)	0.116 (0.959)	0.054 (0.217)	0.103 (0.827)

In solutions containing K_2SO_4, the negative apparent evolution rates at shaking speeds of 350 rev•min^{-1} and 400 rev•min^{-1} were noted in most cases when considering the full period of incubation. This would result from interactions between cells in solutions. ARAYA *et al.* (2003) noted in stream water that community structures in planktonic *Proteobacteria*, *Cytophaga* and *Flabobacterium* changed continuously with time. At some agitation speeds or some salt concentrations in the medium, the evolution rate of cell abundance of the bacterial species seems opposite. Moreover, bacterial growth rate and production of quorum-sensing inhibitors constituted an attempt to identify attributes allowing bacteria to effectively interact and coexist in a water environment. SIMOES *et al*. (2007), when investigating on interactions amongst most species of *Methylobacterium*, *Sphingomonas*, *Burkholderia*, *Staphylococcus* and *Acinetobacter* genera, noted synergy/cooperation between some species, and antagonism and neutral interactions between others, the result of interactions undergoing temporal evolution. This would be one of the origins of variations in the changing of the evolution rates of cell abundances observed in this study. Moreover, mechanical stress gave greater impact on the cells and could alter the cell internal structures and also impact the enzyme activity. It has also been indicated that the small-scale turbulence on *Escherichia coli*, a single-celled motile heterotroph, and *Selenastrum capricornutum*, a single-celled nonmotile autotroph significantly modulates in different magnitudes the nutrient uptake and growth in comparison to still-water control; the rate of energy dissipation emerges as a physically based scaling parameter integrating turbulence across a range of scales and microscopic organism responses at the cell level (HONDZO and WUEST, 2009).

It was observed that the bacterial abundance undergone temporal fluctuations during the same shaking speed at each of the salt concentration. According to PURWANT *et al.* (2009), the agitation at the beginning could provide higher amounts of dissolved oxygen in the medium and might enhance the enzyme production. But for a long period, due to shear stress and forces, as well as abrasion, the cell morphology changes and the enzyme production decreases. It was noted in this study that the shapes of curves of the abundance variation of each bacterial species in pure cultures are different from those obtained in mixed cultures. This could be due to different products released in the medium by each cell species. In addition, according to STRATFOR and WILSON (1990), cells in aqueous suspensions may be regarded as colloidal. Negatively charged microbes attract positive ions from the medium, forming electric double-layers, and remain in suspension by mutual repulsion. However, many microbes form specific structures and substances with the aim of promoting interactions. These include cell-cell recognition phenomena and mating interactions. Moreover, agitation may also tear aggregates apart and cause surface damage to or disruption of individual cells. It lets out that in natural aquatic environment, the abundance evolution of a bacterial species resulted from complex processes.

5. CONCLUSION

At the same chemical concentration in the aquatic environment, cells are impacted by the speed of movement of the medium. The growth potential of a microbial species can be positively or negatively influenced by these chemical and physical factors, independently of other species. But in the presence of other species, the process seemed quite different.

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