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Spatial sequencing of microbial reduction of chromate and nitrate in membrane bioreactor

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Abstract Sequential reduction of chromate and nitrate, two competitive electron acceptors, has been demonstrated for strains of *Pseudomonas* genus for both planktonic cells and cells immobilised in agar layers on the surface of synthetic membrane. Denitrification occurs practically after chromate depletion. This order of reduction process is consistent with redox potentials of the respective reactions. In a membrane bioreactor, competitive inhibition results in nitrate transfer through the membrane without transformation. Thus the receiving phase is contaminated with nitrate. To address this problem, a membrane has been used for spatial sequencing of chromate and nitrate reduction. Bacterial cells were immobilised in two layers with each layer placed on opposing sides of the membrane. By this means, chromate reduction is localised into the layer contacting the feed phase while nitrate reduction occurs in the layer facing the receiving phase. As a result, only traces of the pollutants are detected in the receiving phase.

Keywords Membrane bioreactor · Chromate · Nitrate · Microbial reduction · Immobilisation

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Introduction

Microorganisms' ability to adapt to a wide range of environmental conditions and use various alternative electron acceptors for respiration enables involvement of various inorganic species into metabolic pathways. Hence microbial technologies have been widely considered in potential approaches for removal of inorganic contaminants from aqueous streams. Some applications, such as water denitrification, have matured to the industrial scale [1–4]. Another promising direction for applications of microbially mediated reduction is remediation of water contaminated with heavy metals. Dissimilatory reduction by some bacteria has been demonstrated for various metal and radionuclide species such as U(VI), Cr(VI), Co(III), and Tc(VII) [5–9]. It is generally believed that reduced forms of transition metals are, as a rule, less toxic or can be easily removed from the contaminated water streams due to their low solubility. Therefore, microbial metal reduction is considered as a feasible alternative or a complementary approach to conventional water treatment technologies for remediation of wastewater and natural water containing heavy metals.

Wastewaters or even natural waters are complex systems that contain various species able to act as terminal electron acceptors in microbial processes. The overall efficiency of contaminant removal can be significantly affected when several terminal electron acceptors are present in the system. For example, mutual competitive inhibition was observed for systems containing chromate and nitrate [10] or chromate and nitrite [11] as the competitive terminal electron acceptors during reduction by *Shewanella oneidensis* MR-1. Kinetic inhibition is dependant on concentrations of competing electron acceptors. These studies demonstrated that with sufficiently low

concentrations, both nitrate and chromate do not practically influence the reduction of the corresponding competing electron acceptor. However, high nitrate concentrations significantly reduce the rate of the chromate reduction, and high concentrations of Cr(VI) can completely inhibit nitrate reduction. Similarly, the rate of chromate reduction mediated by pseudomonad isolates was found to be dependant on the concentration of arsenate and Cu(II) [6].

Further progress in applications of dissimilatory reduction of inorganic species for bioremediation demands understanding of the process in multicomponent systems. Such knowledge not only assists the optimization of process parameters but can provide vital information for choice of ways for the process implementation. The simplest way to treat water containing several reducible inorganic contaminants is sequential exhaustive reduction of each component. However, this will result in lengthy processing time. Deployment of bacterial consortium with bacteria of different substrate specificity has been proposed in an attempt to reach simultaneous reduction of competing electron acceptors [12]. This approach provided simultaneous reduction of chromate, nitrates and sulphates during the treatment of groundwater in the area of a former electroplating factory using bacterial consortium isolated from contaminated soil. The bacterial consortium provided simultaneous reduction despite high concentrations of competing electron acceptors: 15, 150 and 320 mg l^{-1} for Cr(VI), nitrate and sulphate, respectively.

Another way to intensify microbial reduction in multicomponent system is a sequencing process that allows for reaching optimal conditions for transformations of a specific contaminant at a certain stage. Recently, there was a rise of interest for application of membrane technologies in design of sequencing bioreactors [13–15]. Integration of semi-permeable membranes in such kind of bioreactors further improves flexibility in control of operational conditions via selective mass transport. Moreover, membranes can be used as carriers of microorganisms. Previously, we have demonstrated chromate reduction in a membrane bioreactor with *Pseudomonas* cells immobilised on membrane surface [16]. Bacteria embedded into agar layer on the membrane surface provided cell protection from the excessive toxic action at high chromate concentration, allowing Cr(VI) reduction in solution containing up to 20 mg l^{-1} of Cr(VI). This paper investigates microbial Cr(VI) reduction in the presence of another electron acceptor, nitrate, using membrane bioreactor with bacteria immobilised on membrane surface via embedding into agar gel. We demonstrate the feasibility of spatial sequencing for microbial reduction of competing electron acceptors using membranes with bacteria immobilised on both surfaces.

Materials and methods

Microorganisms and media

Denitrifying bacteria of the genus *Pseudomonas* (*P. aeruginosa* P1, *P. fluorescens* var. *pseudo-iodinum* P11, *P. mendocina* P13 and *P. stutzeri* P19) were obtained from the collection of microorganisms at the Laboratory of Microbiology of the National University of Kiev-Mohyla Academy (Kiev, Ukraine). The biomass was grown on Nutrient Agar Standard I (Serva, Germany) at 28°C.

Microbial denitrification and chromate reduction have been studied using M9 broth (Fluka). The broth was mixed with 1 ml of microelement solution in order to obtain 1 l of the solution. Composition of microelement solution was as follows (in mg l^{-1}): $FeSO_4 \cdot 7H_2O$—25; $MnSO_4 \cdot 2H_2O$—5; $CoSO_4$—1; ZnSO4—1; $CuSO_4 \cdot 6H_2O$—0.1; H_3BO_3—0.1; Na_2MoO_4—25; $NiCl_2 \cdot 6H_2O$—0.1. Ethanol has been used as a carbon source with its concentration 1 g l^{-1}. KNO_3 and $K_2Cr_2O_7$ were introduced as source of nitrate and chromate, respectively.

Microbial reduction with planktonic cells was studied in 100-ml Erlenmeyer flacks at 28°C. Stock cultures were removed from the solid media and aseptically transferred into the flasks in order to reach cell concentration of 300 mg l^{-1}. Cell concentrations were determined from the calibration graphs against optical density. The latter was measured at wavelength of 540 nm using a 5-cm layer. Nitrate and chromate concentrations were 200 and 10 mg l^{-1}, respectively. In order to isolate the system from the air, a cultivation medium was covered by the 2-cm layer of sterile Vaseline oil. Aliquots for analysis were withdrawn using a sterile syringe and needles through the Vaseline oil layer.

Preparation of membranes with immobilised cells

Cells were entrapped into 1% agar films, which are formed on the surface of commercially available ultrafiltration membranes C005F (Microdin-Nadir, Germany) with molecular cut off 5 kDa.

1% agar solution was cooled to 40–50°C. This solution was mixed with equal volume of bacterial suspension in physiological solution containing 10^8 cell ml^{-1}. The resulting suspension was spread on the membrane surface using calibrated film-casting knife with 300-μm gap. Solidification of agar layer occurred in 10 min at 10°C.

Membrane bioreactor

Denitrification and chromate reduction were studied in a dialyze two-chambered membrane reactor. The volume of

each chamber was 130 ml. The membrane with immobilized bacteria was fixed between the chambers. The membrane area was 25 cm^2. Both chambers were filled with equal volumes of solutions based on M9 broth. One semi-cell considered as a feeding chamber was spiked with ethanol as electron donor, nitrate and chromate of variable concentrations. The dissolved oxygen was not removed from the medium before the start of the experiment. Surfaces of the solutions in each semi-cell were covered by 2-cm layer of sterile Vaseline oil. Solutions in both semi-cells were stirred using magnetic stirrers with the speed of 50 rpm, which did not disturb the layer of Vaseline oil.

Analytical methods

The chromate concentration was determined spectrophotometrically using the biphenyl carbazide method with measurement of optical density at 540 nm [17]. Nitrite and nitrate were analyzed by capillary electrophoresis using a Kapel 103P system (EuroLab, St Petersburg, Russia).

Results and discussion

Competitive Cr(VI) and nitrate reduction by planktonic bacteria of *Pseudomonas* genus

Microbial mediated reduction occurs through complex electron transport systems consisting of various functional-specific enzymes and coenzymes. For microorganisms adapted to the presence of different species, such a variety of redox mediators results in relatively low substrate selectivity. Both the ability of mediators to implement electron transport from/to various substrates and the competitive redox processes mediated by different highly specific mediators, contribute to the ability of microorganisms to use different electron acceptors and donors. As a result, readiness of substrates to accept electrons is expected to be dominant in microorganism selection of preferred electron acceptors. Therefore, standard reduction potential is used as indicative parameter to predict the sequence of microbial reduction of potential electron acceptors. The more potent oxidants, which are characterised by higher standard redox potential, are assumed to be preferred electron acceptors and reduced first.

However, many factors such as substrate/product toxicity, involvement of different mechanisms, etc. can affect the ability of micro-organisms to reduce potent oxidants. For example, the tellurite anion was reduced in preference to selenite by *Desulfovibrio desulfuricans* despite higher reduction potential of SeO_3^{2-}/Se couple than TeO_3^{2-}/Te couple (0.885 and 0.827 V, respectively) [7]. Moreover, many reduction processes occur via the formation of intermediate oxidants, which are characterized by different redox potentials. As a result, competitiveness of potential electron acceptors can change in the course of the reduction process. Thus sequences of microbial reduction in systems with more than one potential electron acceptors must be verified for each specific system.

It is generally accepted that microbial denitrification is a multi-step process with easy identification of nitrite as a stable intermediate. Therefore, for chromate/nitrate systems the following chemical transformations of the potential electron acceptors must be considered (in the order of decreasing redox potential) [18]:

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightarrow 2Cr^{3+} + 7H_2O \quad E_0 = 1{,}333\,\text{mV}$$
$$NO_3^- + 2H^+ + e^- \rightarrow NO_2 + H_2O \quad E_0 = 780\,\text{mV}$$
$$2NO_2 + 2e^- \rightarrow 2NO_2^- \quad E_0 = 880\,\text{mV}$$
$$2NO_2^- + 4H_2O + 6e^- \rightarrow N_2 + 8OH^- \quad E_0 = 410\,\text{mV}$$

Redox processes relevant to denitrification are characterised by significantly lower redox potentials compared with chromate reduction. One can assume that a limiting step for denitrification process is nitrite reduction, since its standard redox potential is about twice as lower than nitrate reduction to nitrogen dioxide or nitrogen dioxide transformation into nitrite. In fact, nitrate reduction is a strongly anaerobic process. The ability of all tested pseudomonad to switch to nitrate respiration has been confirmed by adapting the aerobically grown cultures in media containing only nitrate as a terminal electron acceptor without any electron donors. Internal energy that bacteria have acquired during aerobic growth was sufficient to start the use of nitrate as an alternative electron acceptor.

All the studied denitrifying *Pseudomonas* strains are able to reduce Cr(VI). For example, in media containing either nitrate or chromate as a single electron acceptor *P. fluorescens* var. *pseudo-iodinum* P-11 completely reduces nitrate in 2 days when initial nitrate concentration was 200 mg l^{-1}, while it takes 12 days to reduce chromate in media with Cr(VI) concentration of 10 mg l^{-1} (Fig. 1).

As can be seen from Fig. 1, when both chromate and nitrate are present in the media chromate reduction occurs in preference to nitrate. With *P. fluorescens* var. *pseudo-iodinum* P-11 as reducing bacteria, nitrate reduction begins only when Cr(VI) concentration decreased to 3 mg l^{-1} which was reached after 9 days. Thus there is a significant delay in nitrate reduction in comparison with the denitrification process if media contains only nitrate as terminal electron acceptor. When concentrations of competing chromate and nitrate are below 3 and 200 mg l^{-1}, respectively, both species are reduced simultaneously with completion of their reduction after 12 days.

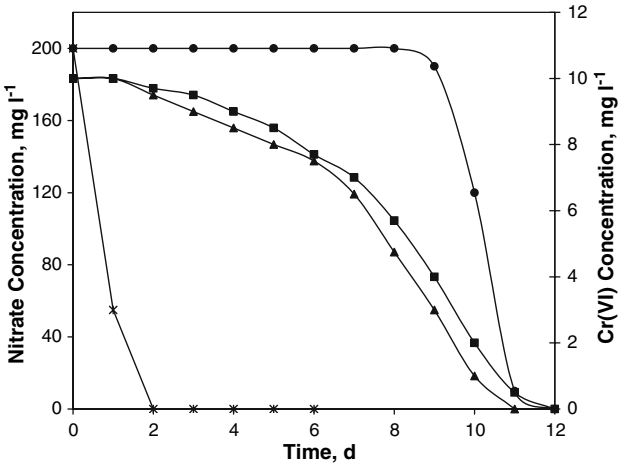

Fig. 1 Chromate and nitrate reduction by planktonic *P. fluorescens* var. *pseudo-iodinum*. Change of Cr(VI) concentration in the absence (*filled triangle*) and presence of nitrate (*filled square*). Change of nitrate concentration in the absence (*asterisk*) and presence of chromate (*filled circle*)

Similar behaviour was observed for other denitrifying bacteria. As can be seen from the Table 1, chromate concentration at the start of nitrate reduction is dependant on the strain used for the reduction. The lowest Cr(VI) concentration of 0.5 mg l^{-1} must reach to allow for nitrate reduction by *P. aeruginosa* P1 and *P. stutzeri* P19. However, this threshold chromate concentration is reached quicker for *P. aeruginosa* P1 than for *P. stutzeri* P19 (6 and 10 days, respectively). All strains demonstrate very similar denitrification kinetics after reaching threshold chromate concentration, with about 3 days to reach complete denitrification. Since longer periods to reach threshold chromate concentration were observed in case of *P. fluorescens var. pseudo-iodinum* P-11 and *P. stutzeri* P19, the overall duration for complete pollutant reduction is the longest for these strains. From the comparison of chromate reduction in the presence of nitrate, one can conclude that there is practically no influence of nitrate on the chromate reduction, especially at chromate concentrations higher than the threshold concentration. However, the complete chromate reduction is reached later in the presence of nitrate compared with the reduction of chromate alone. This might indicate that with chromate depletion nitrate/nitrite become increasingly competitive and slightly retard reduction of chromate traces. Thus, for studied planktonic pseudomonad chromate reduction is dominant when both oxidants are present in the solution. Such a sequence of the reduction processes is predicted by the standard redox potentials.

Table 1 Nitrate and chromate reduction by planktonic bacteria of *Pseudomonas genus* in solutions containing both pollutants

Strain	Threshold Cr(VI) concentration[a] (mg $l^{-1)}$	Duration of microbial reduction (days)	
		To reach threshold Cr(VI) concentration	To reach complete nitrate reduction
P. aeruginosa	0.5	6	9
P. fluorescens var. *pseudo-iodinum*	3	9	12
P. mendocina	2	7	10
P. stutzeri	0.5	10	12

[a] Threshold Cr(VI) concentration is defined as Cr(VI) concentration when nitrate reduction begins

Chromate reduction and denitrification in membrane bioreactor

In membrane bioreactors, with whole cells supported by a semi-permeable membrane, reaction occurs in a limited space, in a layer of immobilised biocatalyst. In this study, membrane bioreactor is based on a membrane element of a dialysis type. Substrates and products are transferred to or from the biocatalyst layer due to concentration gradients. Thus mass transfer rate depends on solute concentration in solutions separated by membranes and membrane permeability.

On the basis of the results presented in the previous section, it is highly probable that inhibition of nitrate reduction in the presence of chromate will also occur in membrane bioreactor. Therefore, the reactor operation was initially characterised in the denitrification process only. As Fig. 2 shows, during the treatment of media containing 600 mg l^{-1} of nitrate, complete denitrification (nitrate absence

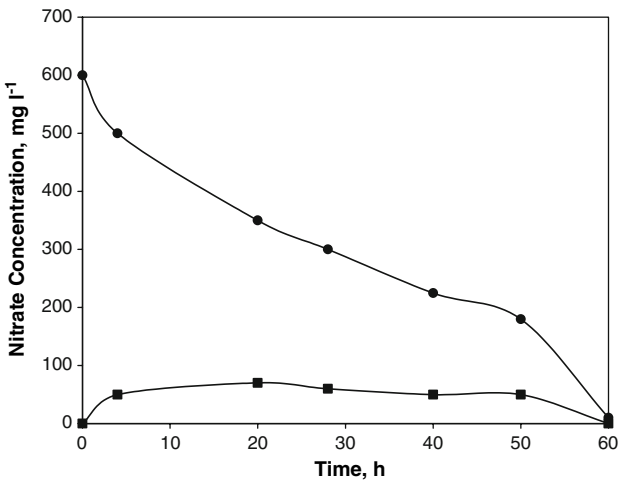

Fig. 2 Dynamics of nitrate concentration in the feed (*filled circle*) and receiving (*filled square*) phases during denitrification in membrane bioreactor by *P. fluorescens* var. *pseudo-iodinum* immobilised on the membrane surface. Biocatalytic layer faces a feed phase

in both feed and receiving phases) was observed after 60 h. It can be seen that nitrate is present in the receiving phase, however, its concentration does not exceed 50 mg l^{-1} in the course of the experiment. Therefore, the decrease in nitrate concentration in the feed phase is mostly due to nitrate reduction, which occurs in the layer of immobilised cells on the membrane surface.

In contrast, when both chromate and nitrate are present in the solution in the first 40 h, nitrate is transferred into the receiving phase through the biocatalytic membranes without reduction (Fig. 3a). Nitrate concentration in receiving phase is higher than 200 mg l^{-1}. The overall duration of nitrate removal in the presence of chromate increased up to 120 h, while complete denitrification for nitrate alone was finished in 60 h. Therefore, chromate causes strong inhibition of nitrate reduction when these two electron acceptors are present in the membrane bioreactor. In fact, notable nitrate reduction begins when chromate concentration reaches 4 mg l^{-1} (after 40 h of reactor operation).

At the same time, chromate reduction takes place from the very beginning of reactor operation (Fig. 3b). Practically linear decrease in chromate concentration was observed within 60 h of reactor operation. Chromate concentration in receiving phase does not exceed 0.5 mg l^{-1}, which indicates that the rate of chromate biotransformation in the layer of immobilised cells corresponds to the rate of mass transfer of chromate.

For reduction of chromate/nitrate pair in membrane bioreactor with immobilised pseudomonad the reaction sequence follows electrochemical sequence. As a result, an electron acceptor with a lower redox potential practically does not undergo reduction in the layer of immobilised cells and diffuses into the receiving phase. After chromate depletion, nitrate in receiving phase can be reduced but it demands nitrate diffusion back through the membrane to the layer of immobilised bacteria. Consequently, the overall time to reach complete contaminant reduction significantly increases.

In view of the fact that chromate reduction is practically complete in the layer of immobilised cells, chromate and nitrate reduction can be spatially separated. In order to localise reduction of two competing electron acceptors in different places, bacterial cells were immobilised on both sides of membrane. As can be seen from Fig. 4, two layers

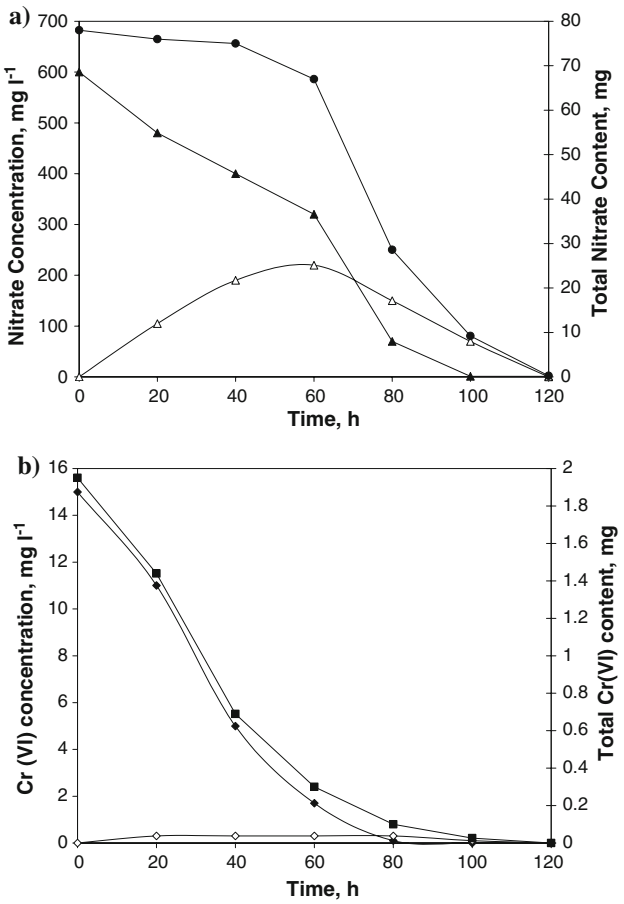

Fig. 3 Nitrate (**a**) and chromate (**b**) reduction by immobilised *P. fluorescens* var. *pseudo-iodinum* in membrane bioreactor with both contaminants loaded into a feed phase. Nitrate concentrations in the feed (*filled triangle*) and receiving (*open triangle*) phases. Dynamics of total nitrate content in feed and receiving solutions of membrane bioreactor (*filled circle*). Cr(VI) concentrations in the feed (*filled diamond*) and receiving (*open diamond*) phases. Dynamics of total Cr(VI) content in feed and receiving solutions of membrane bioreactor (*filled square*). One layer of immobilised cells was formed on one side of the membrane and faced a feed phase

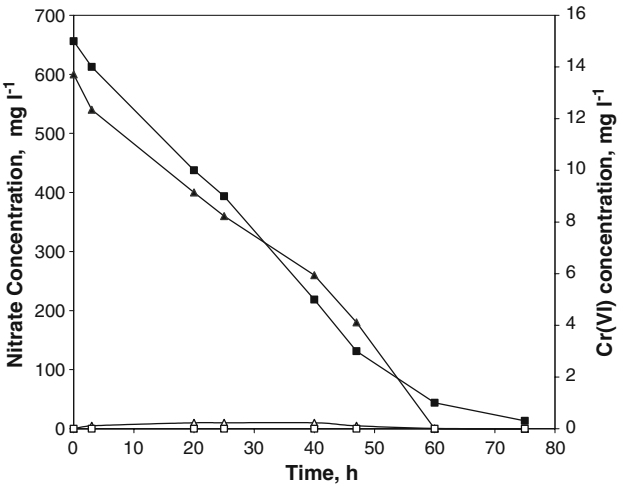

Fig. 4 Nitrate and chromate reduction by immobilised *P. fluorescens* var. *pseudo-iodinum* in membrane bioreactor with both contaminants loaded into a feed phase. Nitrate concentrations in the feed (*filled triangle*) and receiving (*open triangle*) phases. Cr(VI) concentrations in the feed (*filled square*) and receiving (*open square*) phases. *P. fluorescens* var. *pseudo-iodinum* embedded into two layers placed on opposing sides of membrane

of immobilised biocatalyst spatially separated by semi-permeable membrane enable simultaneous reduction of chromate and nitrate. As in the case of single layer, nitrate concentration in feed phase decreases with time due to diffusion through the biocatalytic membrane. However, only trace quantities of nitrate are detected in the receiving phase through the entire cycle of reactor operation. This indicates nitrate reduction in membrane bioreactor despite the presence of chromate. Nitrates pass the first biocatalytic layer without transformation and undergo reduction in the second layer of immobilised biocatalyst where only traces of competing chromate are present. Thus chromate and nitrate reduction occurs simultaneously in two spatially separated reaction zones. It is worth noting that both pollutants are practically absent in the receiving phase. This indicates that the rates of reduction of chromate and nitrate in the corresponding layers and mass transfer rates are synchronised for the chosen method of biocatalytic membrane preparation, bacteria, and operational parameters. To our best knowledge, this is the first example of the use of catalytically active membrane for spatial sequencing of competitive reactions.

Conclusions

Studies of microbial reduction of chromate and nitrate by four strains of planktonic pseudomonad demonstrated high inhibitory effect of chromate on the nitrate reduction when both contaminants are present in the solutions. The overall reduction is a sequential process with nitrate reduction commencing after virtually complete chromate depletion. Such a sequence is consistent with redox potentials of corresponding redox processes with more potent oxidant being consumed first as a terminal electron acceptor. Nitrates have low influence on chromate reduction; however, they become increasingly competitive when chromate concentration is low.

Competitive inhibition was also observed for pseudomonad immobilised on the surface of semi-permeable membrane by embedding into an agar layer. In membrane bioreactor with biocatalytic membranes, the inhibition of nitrate reduction results in transfer of nitrate without transformation into the receiving phase. Eventually, when concentration of chromate in feed solution is low (<4 mg l^{-1}), nitrate from both feeding and receiving streams is reduced, however, it requires nitrate diffusion from the receiving phase through the membrane to reach biocatalyst layer. This factor and sequential character of the process as a whole significantly increases duration of the process necessary for complete reduction of two contaminants.

Biocatalytic membranes with whole cells immobilised on both sides of the membrane allow for spatial sequencing of competitive reduction processes. In a layer of immobilised bacteria facing the feed phase with high chromate concentration, chromate reduction takes place and nitrates leave this layer without transformations. Their reduction occurs in the second layer of immobilised biocatalyst facing receiving phase where only traces of competing chromate are present. Thus chromate and nitrate reduction occur simultaneously in two spatially separated reaction zones. As a result, the overall duration for complete contaminant reduction drastically decreases. In membrane bioreactor with membrane supporting two biocatalytic layers, synchronisation of rates of reduction of chromate and nitrate in the corresponding layers and mass transfer rates has been achieved, thereby freeing the receiving phase of both pollutants during the entire process of microbial reduction.

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