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Effect of chlorine on filamentous microorganisms present in activated sludge as evaluated by respirometry and INT-dehydrogenase activity

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Abstract

Activated sludge technology is more used than any other for biological treatment of wastewater. However, filamentous bulking is a very common problem in activated sludge plants, chlorine being the chemical agent normally used to control it. In this work the effect of chlorine on microorganisms present in activated sludge flocs was assessed by a respirometric technique (oxygen uptake rate, OUR) and by the INT-dehydrogenase activity test (DHA) measured by two techniques: spectrophotometry (DHA_a) and image analysis (DHA_i). Both DHA tests were optimized and correlated with the respirometric technique (OUR) using pure cultures of a filamentous microorganism (*Sphaerotilus natans*) under chlorine inhibition. Using these correlations the tested methods were applied to determine the action of chlorine on respiratory activity in activated sludge. The OUR and the DHA_a quantifies the action of chlorine on the total respiratory activity (RA) of flocs (filamentous and floc-forming bacteria); in contrast, the DHA_i test evaluates specific action of chlorine on the RA of filamentous microorganisms.

In activated sludge flocs containing filamentous microorganisms, a chlorine dose of 4.75 mg Cl_2 $(gVSS)^{-1}$ with a contact time of 20 min reduced about 80% of the RA of filamentous bacteria while affecting only 50-60% of the total RA of flocs. Besides, a chlorine dose of 7.9 mg Cl_2 $(gVSS)^{-1}$ produced the total respiratory inactivation of filamentous microorganisms after 10 min contact, however, with this dose the total RA of activated sludge flocs was reduced only about 45–65%; controlling filamentous bulking without affecting too much floc-forming bacteria.

At the tested chlorine concentrations the inhibition of filamentous microorganisms was higher than in the whole activated sludge. Although floc-forming microorganisms were demonstrated to be more susceptible to chlorine than filamentous in pure cultures, results obtained in the present work confirmed that it is the location of the filamentous microorganisms in the flocs and the presence of extracellular polymer substances which largely determines their higher susceptibility to chlorine; consequently this feature plays a critical role in bulking control.

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Keywords: Filamentous bulking; Chlorination; *Sphaerotilus natans*; INT-dehydrogenase activity; Respirometry; Image analysis

1. Introduction

Filamentous bulking is a frequent problem in many activated sludge plants which leads to poor settling and packing of activated sludge solids. One of the first methods for controlling filamentous bulking in the activated sludge was chlorination [1].

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Nomenclature

A_{490}	absorbance of the dye colorant extracted at 490 nm (UA, unit of absorbance)
A_c	area occupied by intracellular INT-formazan crystals
A_T	total area of the filamentous microorganisms
C	biomass concentration ($gVSS\,l^{-1}$)
D	dilution rate (h^{-1})
DHA	INT-dehydrogenase activity
DHA_a	INT-dehydrogenase activity measured by spectrophotometry (UA l $(gVSS)^{-1}\,h^{-1}$)
$DHA_{a(chlorine)}$	INT-dehydrogenase activity measured by spectrophotometry in chlorinated samples (UA l $(gVSS)^{-1}\,h^{-1}$)
$DHA_{a(control)}$	INT-dehydrogenase activity measured by spectrophotometry in untreated samples (UA l $(gVSS)^{-1}\,h^{-1}$)
DHA_i	INT-dehydrogenase activity measured by image analysis (dimensionless)
$DHA_{i(chlorine)}$	INT-dehydrogenase activity measured by image analysis in chlorinated samples (dimensionless)
$DHA_{i(control)}$	INT-dehydrogenase activity measured by image analysis in untreated samples (dimensionless)
FR	bacterial respiratory activity fraction (dimensionless)
FR_{DHA_a}	bacterial respiratory activity fraction obtained by spectrophotometry (dimensionless)
FR_{DHA_i}	bacterial respiratory activity fraction obtained by image analysis (dimensionless)
FR_{OUR}	bacterial respiratory activity fraction based on respirometric technique (dimensionless)
j	correction factor for the dilution caused by formaldehyde addition
OUR	oxygen uptake rate (respirometry) ($mg\,O_2\,l^{-1}\,h^{-1}$)
$OUR_{chlorine}$	oxygen uptake rate in chlorinated samples ($mg\,O_2\,l^{-1}\,h^{-1}$)
$OUR_{control}$	control oxygen uptake rate ($mg\,O_2\,l^{-1}\,h^{-1}$)
q_{O_2}	specific oxygen uptake rate ($mg\,O_2\,(gVSS)^{-1}\,h^{-1}$)
RA	respiratory activity
SOUR	specific oxygen uptake rate ($mg\,O_2\,(gVSS)^{-1}\,h^{-1}$)
t	incubation time with INT (h)
v	volume of the solvent acetone/tetrachloroethylene (ml)
V	volume of the INT-treated sample (ml)

Although chlorine is a very potent inactivating agent of filamentous microorganisms, it is not selective, affecting floc-forming organisms as well [2]. Therefore, its efficiency depends on a delicate balance to ensure that the beneficial effects of chlorine on bulking control are not offset by its undesirable action on nutrient removal [3], and COD biological removal [4].

Lakay et al. [4] reported doses of 8 mg Cl_2 $(gVSS)^{-1}$ to control filamentous bulking, while Jenkins et al. [5] recommended doses between 1 and 15 mg Cl_2 $(gVSS)^{-1}$.

In a previous work, Caravelli et al. [6] analyzed chlorine inactivation of floc-forming and filamentous microorganisms in pure cultures as a function of time using respirometric tests and the results showed that floc-forming microorganisms were more sensitive to chlorine than filamentous when pure cultures were individually treated with this oxidant.

Respirometry is a simple technique that indicates the overall respiratory activity (RA) in the sludges given by filamentous and floc-forming microorganisms [7], but it does not distinguish one culture from another. An additional method used to measure the effect of toxic agents on activated sludge is that which follows the dehydrogenase activity of the electron transport system (ETS) [8]. The ETS is a common component to metabolic pathways of virtually all bacteria, including lithotrophs and methylotrophs, facultative and strict anaerobes, and aerobic heterotrophs [9]. The ETS activity can be measured by the use of artificial electron acceptors, such as the tetrazolium salts which, once reduced, allow the proportion of cells having metabolic activity in a sample to be microscopically distinguished from non-respiring cells. The electron transport system activity was shown to be related to respiration [10].

The tetrazolium salts have been extensively used for measuring the RA of microorganisms in natural waters, drinking water, sediments and activated sludge [11]. In particular, INT (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride) [12,13] and CTC (5-cyano-2,3-di-4-tolyl-tetrazolium chloride) [14,15] have been used to microscopically determine the proportion of metabolically active cells in a sample. In the case of INT, the technique comprises INT reduction to INT-formazan (INTF) by active bacterial electron transport systems, a test known as INT-dehydrogenase activity (DHA). INTF is deposited in active cells as dark intracellular crystals that are quantified by spectrophotometric techniques after extraction in proper solvent, or by microscopic observation of prepared specimens coupled with image analysis of INTF crystals.

In activated sludge both the DHA test that is spectrophotometrically quantified (DHA_a) and the respirometric technique based on oxygen uptake rate (OUR) are able to determine the overall RA of activated sludge and so it requires a microscopic examination of prepared specimens to evaluate the effect of chlorine on the various microorganisms forming the activated sludge flocs.

Bitton and Koopman [16] have developed a tetrazolium reduction test for determining the physiological activity of filamentous microorganisms within activated sludge. Logue et al. [17], by microscopic observation of prepared specimens have measured the proportion of active filamentous bacteria by comparing the total length of active filaments with reference to the total length of active and inactive filaments. Kim et al. [8] compared the INT-dehydrogenase activity (DHA) test with the OUR and ATP methods under chlorine and hydrogen peroxide inhibition. CTC was also used for determining metabolically active bacteria in activated sludge. However, the dehydrogenase activity determined by epifluorescence microscopic enumeration did not correlate with cumulative measured activity as determined by formazan extraction [18]. Although the direct counting technique [8,17] has been employed to evaluate chlorine efficiency for controlling filamentous bulking, it does not consider the specific RA of the respiration sites, as far as the determination of inhibition indexes is concerned. Mauss et al. [19] have used an image analysis system to locate and characterize respiration sites in filamentous bacteria after incubation with INT; however, scarce bibliographical support is still available on image analysis techniques to quantify respiration sites of filamentous microorganisms in activated sludge. The image analysis technique consists basically of a light microscope and a photo-camera connected to a PC that runs an appropriate image analysis software.

The general objective of the present work was to analyze the effect of chlorine on activated sludge, especially its influence on filamentous and floc-forming bacteria by using different techniques that evaluate the RA of the microorganisms.

Considering that the measurement of the OUR and the absorbance based INT-dehydrogenase activity test (DHA_a) do not distinguish between the RA of filamentous and floc-forming microorganisms present in the activated sludge, one of the objectives was to apply an image analysis based method to evaluate the action of chlorine on filamentous microorganisms in the flocs.

The specific objectives were:

(1) To optimize the INT-dehydrogenase activity (DHA) tests both by spectrophotometry and image analysis working with pure cultures of filamentous microorganisms (*Sphaerotilus natans*).
(2) To correlate both DHA tests with the respirometric technique (OUR) following chlorination.
(3) To evaluate the effect of chlorine concentrations and contact times on the total RA of activated sludge in comparison to that of the filamentous microorganisms by applying simultaneously the DHA optimized techniques and OUR.

2. Materials and methods

Sphaerotilus natans ATCC #29329 was obtained from the American Type Culture Collection. Pure culture experiments were performed in a chemostat apparatus, at $30 \pm 0.2°C$ and pH = 7.0; aeration was enough to keep dissolved oxygen concentration above $2\,mg\,O_2\ l^{-1}$. Dilution rates tested ranged between 0.19 and $0.21\,h^{-1}$.

The following culture medium was used: monohydrate citric acid $3480\,mg\,l^{-1}$, $(NH_4)_2SO_4$ $1000\,mg\,l^{-1}$, $MgSO_4 \cdot 7H2O$ $400\,mg\,l^{-1}$, $CaCl_2 \cdot 2H_2O$ $50\,mg\,l^{-1}$, KH_2PO_4 $250\,mg\,l^{-1}$, $Na_2HPO_4 \cdot 12H_2O$ $1000\,mg\,l^{-1}$, vitamin B12 $100\,\mu g\,l^{-1}$, $FeSO_4 \cdot 7H_2O$ $15\,mg\,l^{-1}$, $ZnSO_4 \cdot 7H_2O$ $5\,mg\,l^{-1}$, $MnSO_4 \cdot H_2O$ $3\,mg\,l^{-1}$, $CuSO_4 \cdot 5H_2O$ $0.75\,mg\,l^{-1}$, $CoCl_2 \cdot 6H_2O$ $0.15\,mg\,l^{-1}$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ $0.5\,mg\,l^{-1}$, BO_3H_3 $0.1\,mg\,l^{-1}$, IK $0.1\,mg\,l^{-1}$. The medium was kept in a retort at 121°C for 45–60 min. Vitamin B12 was sterilized by membrane filtration (0.45 μm Millipore HA) and then added to the sterilized medium.

Experiments with activated sludge were conducted at $20 \pm 2°C$ on a laboratory-scale continuous sludge reactor (3.5 l), the hydraulic residence time being 36 h, and the cellular residence time of 21 days. The influent consisted of a wastewater model system of a potato processing plant (DQO = $1000\,mg\,l^{-1}$, pH = 6) obtained by immersing potato cubes 1 cm side in water at room temperature for 24 h [20].

2.1. Chlorination tests in Sphaerotilus natans pure cultures and activated sludge

Chlorination assays were carried out using *S. natans* pure cultures and activated sludge. Samples of 180 ml from the *S. natans* pure culture at steady state, were placed in a magnetically stirred container, at a controlled temperature of 30°C, and then treated with sodium hypochlorite doses ranging between 6.97 and $11.75\,mg\,Cl_2\,l^{-1}$, equivalent to the range 6.2–11.1 $mg\,Cl_2\,(gVSS)^{-1}$.

For activated sludge the chlorination tests were conducted under the same conditions but the samples were washed twice with phosphate buffer (0.03 M, pH = 7). The concentration range of sodium hypochlorite was: 3.3–6.0 $mg\,Cl_2\,l^{-1}$, corresponding to 4.75–7.9 $mg\,Cl_2\,(gVSS)^{-1}$. Contact times with chlorine ranged between 1 and 30 min with *S. natans* pure cultures, and 1–25 min in activated sludge. The residual chlorine was

removed with sodium thiosulfate (0.02% w/v, final concentration).

The RA of the bacteria was determined by three methods: the INT-dehydrogenase activity by spectrophotometry (DHA_a), the INT-dehydrogenase activity by image analysis (DHA_i), and a respirometric technique (OUR).

2.2. Determination of INT-dehydrogenase activity (DHA tests)

The INT-dehydrogenase activity of respiring bacteria in *S. natans* pure cultures and activated sludge was measured by DHA_a and DHA_i.

2.2.1. Spectrophotometric method (DHA_a)

This is a modified version of a technique proposed by Logue et al. [17]. The INT incubation tests were carried out at 20°C and pH 7 in 1.5 ml microcentrifuge tubes in the presence of an oxidizable exogenous substrate. Citric acid for *S. natans* pure cultures and a model wastewater from a potato processing plant in the case of activated sludge were used as exogenous substrates. Buffer phosphate 0.03 M (pH 7) was added to fill microcentrifuge tubes and to minimize any oxygen interference during incubation. After this period, the reaction was stopped by addition of formaldehyde 40% and, subsequently, 0.5 ml of the mixture treated with INT was centrifuged at 14 000 rpm for 5 min; the supernatant was discarded. The INT-formazan crystals were extracted by adding either 1.5 ml (*S. natans* pure culture) or 1 ml (activated sludge) of an acetone/tetrachloroethylene solution (1.5/1 v/v), the concentration of dye being measured by absorbance at 490 nm. Extraction of INT-formazan was done at 20°C for 30 min. Absorbance measurements were then used in a slightly modified version of the equation proposed by those authors [17]:

$$DHA_a = \frac{A_{490} v}{VCtj}, \quad (1)$$

where DHA_a is the INT-dehydrogenase activity measured by spectrophotometry, A_{490} is the absorbance of the dye colorant extracted at 490 nm (UA), v = solvent volume (ml), V = the volume of the INT-treated sample (ml), C is the *S. natans* or activated sludge biomass concentration (gVSS l^{-1}) according to the experiment, t = incubation time (h) and j is a correction factor that accounts for the dilution caused by formaldehyde addition.

In activated sludge, the bacterial respiratory activity fraction (FR) obtained from the spectrophotometric technique in chlorination experiments, (FR_{DHA_a}), was evaluated as follows:

$$FR_{DHA_a} = \frac{DHA_{a(chlorine)}}{DHA_{a(control)}}, \quad (2)$$

where $DHA_{a(chlorine)}$ represents the spectrophotometrically measured INT-dehydrogenase activity in chlorine treatments while $DHA_{a(control)}$ corresponds to the untreated samples.

The use of the spectrophotometric technique in activated sludge gives an overall result of RA without distinguishing the contributions of filamentous and floc-forming microorganisms to the total INT-dehydrogenase activity. For that reason a microscopy image analysis method was also employed to discriminate these contributions.

2.2.2. Microscopy image analysis (DHA_i)

This method is a modified version of a technique by Logue et al. [17]. Smears were made from the INT-incubated mixtures once the reaction was stopped with formaldehyde and allowed to dry. The dried smears were covered with a 0.05% (w/v) aqueous solution of malachite green for a period of 15 s. The excess of malachite green was removed from the slides by using distilled water and the system was dried again. Then, the preparations were observed by bright field microscopy at 1000x under a drop of concentrated fructose solution [21] to prevent solubilization of INT-formazan crystals into the immersion oil [22]. Observations were done using a Leica DMLB microscope with a built-in camera. Images taken were logged with a Leica DC 100 Version 2.51 software and analyzed with the Global Lab *Image* Version 2.10. The image analysis method was used in *S. natans* pure cultures and in activated sludge to measure RA of filamentous microorganisms. The following parameters were determined: the area occupied by intracellular INT-formazan crystals in filamentous microorganisms (A_c), the total area of the analyzed filamentous microorganisms (A_T) and the ratio, $DHA_i = A_c/A_T$ (dimensionless) that was considered as the INT-dehydrogenase activity of the filamentous microorganisms measured by image analysis.

In activated sludge, the bacterial respiratory activity fraction (FR) of filamentous microorganisms during chlorination assays obtained by the proposed image analysis technique (FR_{DHA_i}), was calculated as follows:

$$FR_{DHA_i} = \frac{DHA_{i(chlorine)}}{DHA_{i(control)}}, \quad (3)$$

where $DHA_{i(chlorine)}$ and $DHA_{i(control)}$ correspond to treated and untreated samples, respectively.

2.3. Optimization of the DHA tests using S. natans pure cultures

Optimization of DHA tests consisted in searching proper conditions for spectrophotometric and image analysis determinations; they were conducted with *S. natans* pure cultures obtained from a continuous aerobic

reactor with dilution rates ranging between 0.19 and $0.21\,h^{-1}$, at 30°C and pH = 7.0. Operating conditions were selected to favor INT reduction by active bacteria, to permit relatively large INT-formazan intracellular deposits to be detected. Thus, various INT concentrations ($0.005–1.25\,g\,l^{-1}$) and contact times (10–50 min) were tested in *S. natans* pure cultures. All DHA tests were carried out in 1.5 ml microcentrifuge tubes. Samples of 0.4 ml of *S. natans* pure cultures, together with 0.2 ml of the oxidizable substrate (citric acid) neutralized with NaOH (pH 7), were incubated with several volumes (4–1000 μl) of an INT solution (3.95 mM) to produce concentrations ranging from 0.005 to $1.25\,g\,l^{-1}$. Tubes were incubated at 20°C. DHA tests were stopped by using 0.1 ml formaldehyde 40%. Adequate operating conditions were those that increase INT-dehydrogenase activity of living bacteria while favoring its measurement by spectrophotometry and image analysis.

2.4. Respirometry (oxygen uptake rate)

The global RA of both *S. natans* pure cultures and activated sludge were measured by OUR. Respirometry is based on the relationship between oxygen uptake rate (OUR) and the concentration of respiratory biomass (X_v):

$$\mathrm{OUR} = q_{O_2} X_v, \tag{4}$$

where q_{O_2} is the specific oxygen uptake rate ($mg\,O_2\,(gVSS)^{-1}\,h^{-1}$).

Assuming that q_{O_2} is constant, the respirometry technique determines the effect of chlorine on the biomass by means of the following equation:

$$\mathrm{FR_{OUR}} = \frac{\mathrm{OUR_{chlorine}}}{\mathrm{OUR_{control}}} = \frac{X_v}{X_{vo}}, \tag{5}$$

where FR_{OUR} is the bacterial respiratory activity fraction based on OUR determinations; $OUR_{control}$ is the control oxygen uptake rate, X_{vo} is the control active biomass (before chlorination) and $OUR_{chlorine}$ and X_v correspond to the values after chlorination.

The specific rates of oxygen uptake (SOUR) were expressed per unit of suspended volatile solids. Respirometric assays were conducted with *S. natans* pure cultures and also with activated sludge. The equipment included a vessel containing a polarographic oxygen probe (YSI Incorp., OH, USA), an aerator, magnetic stirring and temperature control (30°C). The output from the oxygen probe was connected to a computer. For *S. natans* pure cultures, oxygen uptake rate (OUR) was measured by placing 20 ml of the bioreactor liquid in the respirometer. A volume of 1 ml of citric acid solution (3.5%, pH = 7) was added as oxidizable substrate and, after 1 min of contact, the system was aerated. After stopping aeration, a linear decrease of dissolved oxygen concentration with time was found, the slope being the OUR. As described above, after chlorine treatment of *S. natans* pure cultures (contact times 1–30 min), 20 ml were placed in the respirometer and the residual chlorine was removed with sodium thiosulfate. A volume of 1 ml of citric acid solution (3.5%, pH = 7) was added and after 1 min contact time, the sample was aerated to determine OUR after chlorine treatment ($OUR_{chlorine}$). Before aeration, 0.4 ml of *S. natans* were transferred to microcentrifuge tubes to conduct DHA tests. Values of OUR for control and chlorine-treated samples were expressed per unit of suspended volatile solids as specific oxygen uptake rate (SOUR) and correlated with DHA tests.

In activated sludge, $OUR_{control}$ and $OUR_{chlorine}$ were measured under the same conditions described before for pure cultures but substituting citric acid by raw wastewater (model wastewater of a vegetable processing plant) as oxidizable substrate.

2.5. Correlation between respirometric technique and DHA tests using S. natans pure cultures

Respirometry (OUR), INT-dehydrogenase activity determined by spectrophotometry (DHA_a) and image analysis (DHA_i) were correlated using chlorination experiments on *S. natans* pure cultures; chlorine doses ranged between 6.97 and $11.75\,mg\,Cl_2\,l^{-1}$ (corresponding to $6.2–11.1\,mg\,Cl_2\,(gVSS)^{-1}$) and contact times between 1 and 30 min. Correlations between these techniques were tested in pure cultures with the purpose of applying them in activated sludge. A satisfactory correlation between OUR and DHA_a would permit the utilization of both techniques to evaluate the effect of chlorine on the global RA of sludges. A good correlation with DHA_i test allows to validate this procedure as a methodological instrument to determine the RA of filamentous microorganisms in activated sludge.

2.6. Effect of chlorine on activated sludge

The optimized DHA_i and DHA_a techniques together with the OUR were applied to activated sludge containing filamentous and floc-forming microorganisms to asses the effect of chlorine in the flocs, where filamentous are located outside and the floc-forming bacteria in the inner zone of the floc. The objective was to determine the response of filamentous bacteria to the oxidizing agent and to compare with the inhibition produced on the whole metabolic activity of activated sludge.

3. Results and discussion

3.1. DHA test optimization

Optimization of the DHA tests was done using several concentrations of INT (0.005–1.25 $g\,l^{-1}$) and contact times (10–50 min) in pure cultures of *S. natans*. Results shown in Fig. 1 correspond to micrographs obtained by light microscopy of *S. natans* pure cultures treated at several concentrations of INT and 50 min contact time. At concentrations of INT ranging between 0.08 and 0.15 $g\,l^{-1}$, the INT-formazan granules were found to be considerably large and well defined, making their microscopical detection easier (Fig. 1a). Higher concentrations of INT (0.6–1.25 $g\,l^{-1}$) increased the number of extracellular formazan deposits while intracellular ones were seen blurred, with little sharpness (Fig. 1b).

Fig. 2a shows INT-dehydrogenase activity by spectrophotometry (DHA_a) as a function of INT concentration. None of the set of operating conditions using the acetone/tetrachloroethylene solution led to total extraction of intracellular INT-formazan possibly because *S. natans* sheath acts as a resistance to the extraction of intracellular reduced INT.

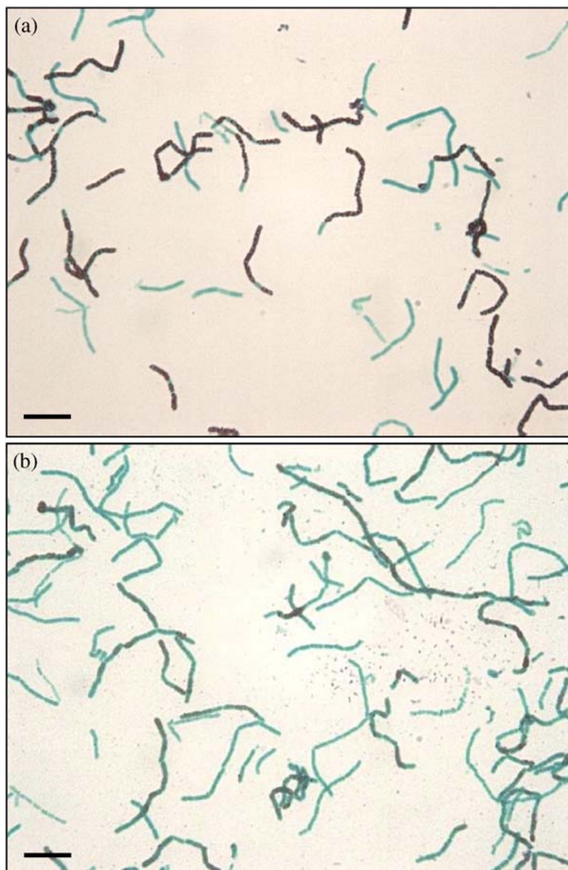

Fig. 1. Micrographs of pure *S. natans* cultures after incubation with INT and counterstain with malachite green (1000x) for different testing conditions : (a) 0.125 g INT l^{-1}, incubation time, 50 min. (b) 1.25 g INT l^{-1}, incubation time, 50 min.(— 10 μm).

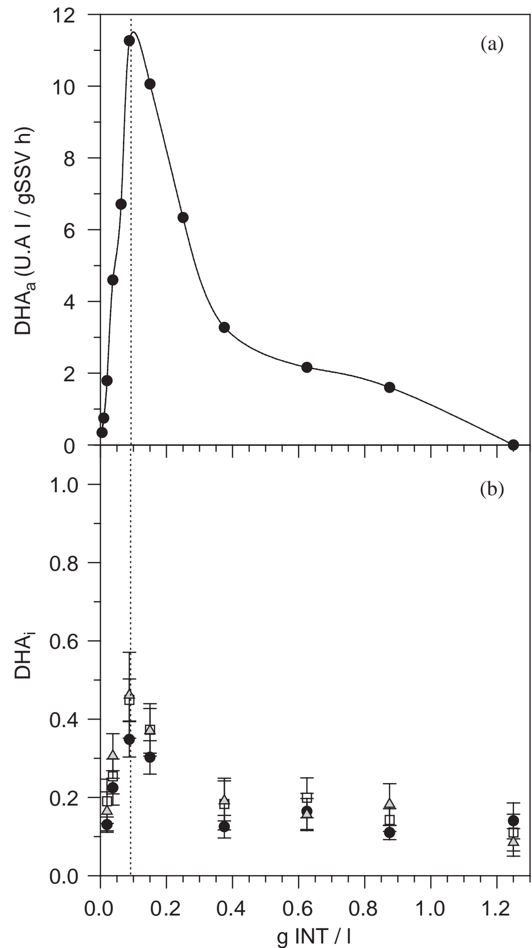

Fig. 2. Optimization of INT-dehydrogenase (DHA) test in *S. natans* pure cultures (a) spectrophotometrically determined INT-dehydrogenase activity (DHA_a) as a function of INT concentration, (b) INT-dehydrogenase activity, as determined by image analysis, (DHA_i) as a function of INT concentration for different contact time (●) 10 min, (□) 30 min, (△) 50 min. The optimal INT concentration is 0.0875 $g\,l^{-1}$ (– – – – –).

Fig. 2b exhibits INT-dehydrogenase activity by image analysis (DHA_i) as a function of INT concentration for several contact times. The most favorable range of INT concentrations for both DHA methods was 0.08–0.15 $g\,l^{-1}$ (Fig. 2a and b) because of the relatively high INT reduction rates (Fig. 2a, DHA_a). Besides, this concentration range led to considerably large formazan granules (Fig. 1a), thus increasing the ratio of the area occupied by INT-formazan crystals to the total area of filamentous microorganisms (Fig. 2b, DHA_i); this

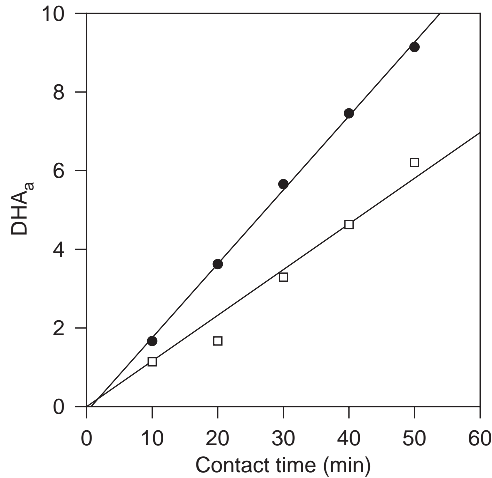

Fig. 3. DHA_a (determined spectrophotometrically) as a function of contact time for different INT concentrations ($g\,l^{-1}$): (●) 0.0875, (□) 0.125, in *S. natans* pure cultures.

facilitates microscopical detection of microorganisms, as well as its subsequent image analysis. For contact times between 30 and 50 min, the increase in the total area of INT-formazan granules was not important, nor it was the ratio of the area occupied by INT-formazan granules to the total area of filamentous microorganisms (Fig. 2b). However, the total amount of INT reduced by the active bacteria (measured as DHA_a) did increase linearly with contact time at different INT concentrations (Fig. 3). This suggests that the INT-formazan increases optical density in the granules formed, without contributing to enlarge their size and therefore the area they occupy (A_c). Thus, the optimum contact time based on DHA analysis by both techniques was 50 min and the adequate INT concentration range was: 0.08–0.15 $g\,l^{-1}$.

3.2. Correlation between respirometric technique and DHA test

Respirometry (OUR) is commonly used to quantify the effect of toxic agents on microorganisms. The feasibility of a correlation of DHA tests with the specific rates of oxygen uptake (SOUR) was evaluated in chlorination tests with *S. natans* pure cultures.

Linear correlations were found between DHA_a and SOUR ($r^2 = 0.904$, Fig. 4a) and between DHA_i and SOUR ($r^2 = 0.903$, Fig. 4b). In the last case the correlation was valid for DHA_i greater than 0.1 and SOUR higher than 10 mg O_2 $(gVSS\,h)^{-1}$. A possible explanation of these results is that for rather low respiratory activities of filamentous microorganisms, INT-formazan granules were mostly small and with low optical density, thus making microscopical identification and further image analysis more difficult; therefore, at

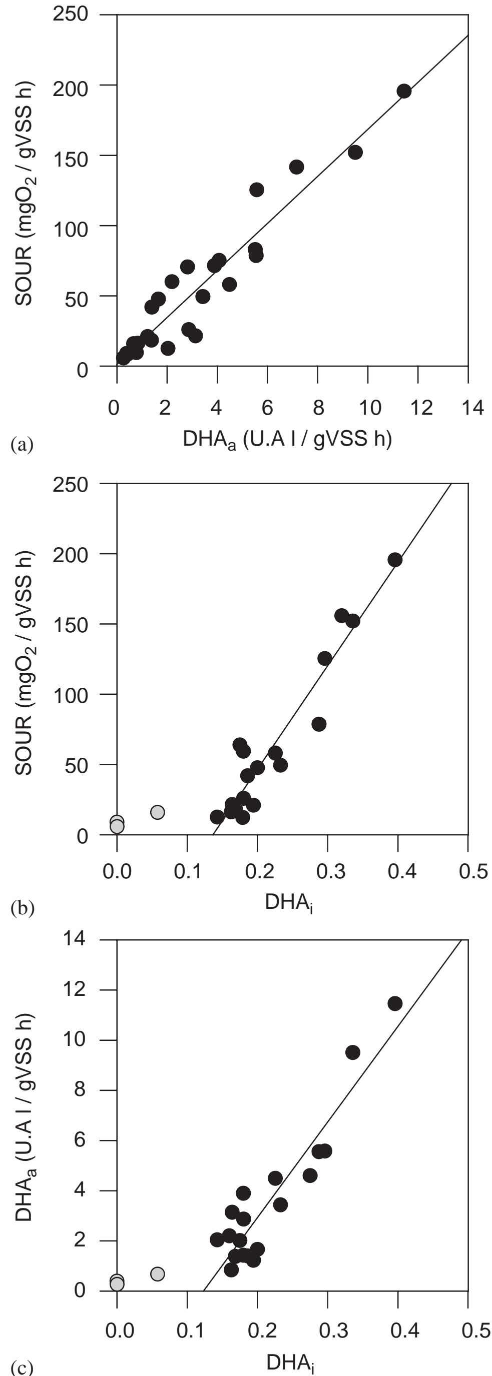

Fig. 4. Correlation between the specific oxygen uptake rate (SOUR), as determined by respirometry, and INT-dehydrogenase activity quantified both by spectrophotometry (DHA_a) and image analysis (DHA_i) in *S. natans* pure cultures: (a) SOUR as a function of DHA_a, $r^2 = 0.904$; (b) SOUR as a function of DHA_i, $r^2 = 0.903$; (c) DHA_a as a function of DHA_i, $r^2 = 0.855$.

SOUR values below $10\,mg\,O_2\,(gVSS\,h)^{-1}$, the image analysis technique becomes unreliable to determine INT-dehydrogenase activity, and no correlation was found with the SOUR.

From the chlorination tests on *S. natans*, the relationship between the DHA_a and DHA_i was also evaluated. A good correlation was obtained for $DHA_i > 0.1 (r^2 = 0.855)$ (Fig. 4c); for $DHA_i < 0.1$, no definite correlation was found because, both microscopical detection and image analysis quantification of INT-formazan granules was difficult as explained above.

Similar results were reported by Kim et al. [8] who studied the correlation between respirometry and DHA tests for pure cultures of filamentous microorganisms as well as for activated sludge exposed to chlorine and hydrogen peroxide. In the work by Kim et al. [8] spectrophotometric DHA was determined by extraction technique and a direct counting technique in a pure culture of *S. natans* and type 021 N filamentous microorganisms was also tested. The differences with our work are that: (a) Kim et al. [8] have used the technique proposed by Bitton and Koopman [16], consisting of microscopic observation of prepared specimens and expressed as a percentage of the total length of active filaments (containing INT-formazan crystals) to the total filament length, whereas in the present work we considered the degree of metabolic activity of active filaments, as indicated by the extent of INT reduction in their respiration sites (DHA_i); (b) they have used an INT concentration of 0.36 mM and an incubation time of 20 min, while in the present work INT concentration was 0.247 mM and the contact time chosen was 50 min; (c) they have employed dimethyl sulfoxide (DMSO) as the organic solvent instead of acetone/tetrachloethylene solution; (d) microorganisms have been resuspended in phosphate buffer (pH 7.6) and then the OUR was determined whereas in the present work a substrate pulse was added to ensure exogenous respiration by the microorganisms.

3.3. Analysis of the effect of chlorine on activated sludge flocs

The tests using *S. natans* pure cultures allowed the OUR to be correlated with both DHA tests, in order to validate the image analysis technique as a method for testing RA of filamentous microorganisms that could be applied to monitoring control procedures for filamentous bulking.

In activated sludge both the OUR and DHA_a test provided the total RA of the sludges, without differentiating the individual contributions of filamentous and floc-forming microorganisms. In contrast, by using DHA_i the specific inhibitory effect of chlorine on filamentous microorganisms can be assessed. For the chlorination tests in activated sludge, the optimum conditions established earlier for DHA tests were applied: INT concentration of $0.15\,g\,l^{-1}$ and 50 min contact time. Fig. 5 shows micrographs of activated sludge obtained by light microscopy; Fig. 5a corresponds to a control sample and Fig. 5b to a sample after a contact time of 10 min with a dose of sodium hypochlorite: $7.9\,mg\,Cl_2\,(gVSS)^{-1}$. As observed in Fig. 5b, INT-formazan granules are absent in the filamentous microorganisms of flocs because chlorine affected their RA. The application of the image analysis technique to floc-forming microorganisms present in activated sludge was not possible because the size, shape and distribution of these microorganisms in the floc, did not allow visualization of INT formazan crystals. Thus DHA_i evaluates only chlorine action on filamentous microorganisms that are visible in activated sludge (i.e. extend beyond the floc structure) and not in floc-forming bacteria.

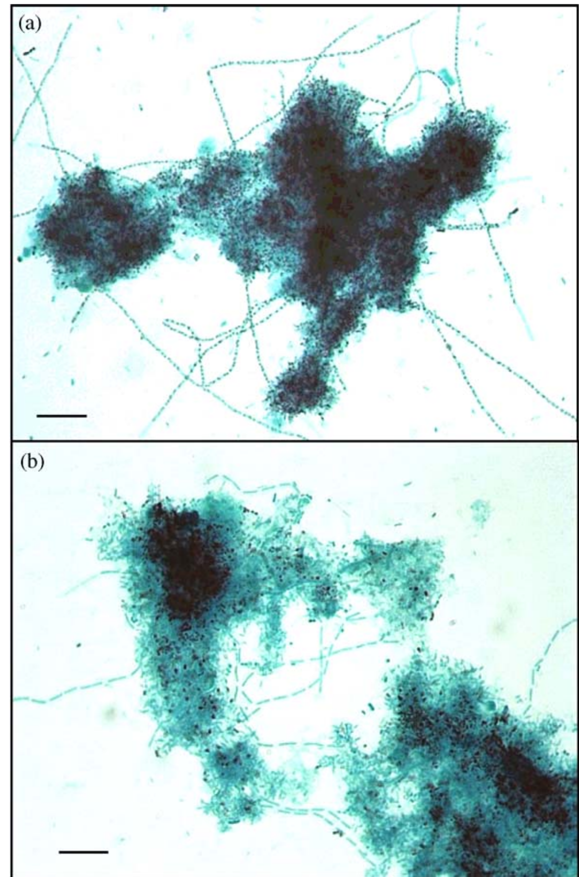

Fig. 5. Micrographs of activated sludge after treatment with INT and counterstain with malachite green for different tests (a) control sample (no chlorine added), (b) chlorine treated sample, with $7.9\,mg\,Cl_2\,(gVSS)^{-1}$ for 10 min. (—10 μm). (1000x).

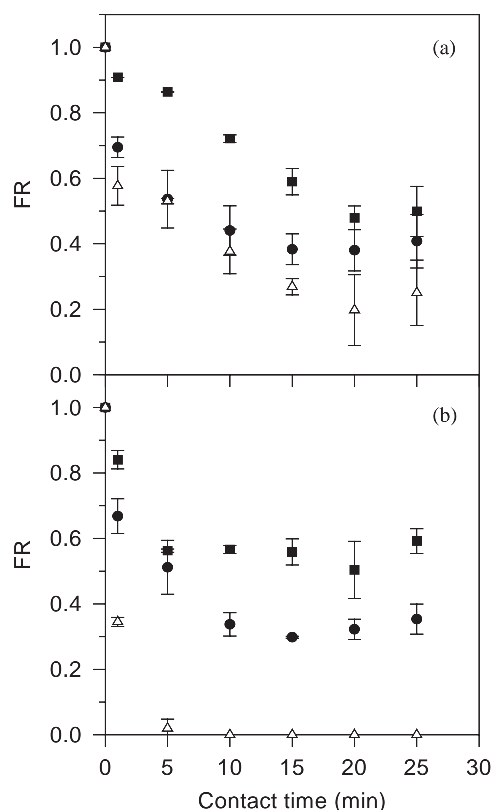

Fig 6. Effect of chlorine doses and contact times (min) on bacterial respiratory activity fraction (FR) in activated sludge. Chlorine doses (mg Cl_2 $(gVSS)^{-1}$): (a) 4.75, (b) 7.9. (●) FR_{OUR}, bacterial respiratory activity fraction as determined by respirometry in activated sludge, (■) FR_{DHA_a}, bacterial respiratory activity fraction based on spectrophotometric INT-dehydrogenase activity in activated sludge, (△) FR_{DHA_i}, bacterial respiratory activity fraction based on INT-dehydrogenase activity determined by image analysis in filamentous microorganisms present in activated sludge.

Fig. 6 (a and b) shows the effect of chlorine doses and contact time on FR in activated sludge measured by different methods: the respirometric technique (FR_{OUR}), the spectrophotometric DHA test (FR_{DHA_a}) and the image analysis DHA test (FR_{DHA_i}).

Two chlorine concentrations were tested: 4.75 and 7.9 mg Cl_2 $(gVSS)^{-1}$ based on the values recommended by Lakay et al. [4], who reported a concentration of 8 mg Cl_2 $(gVSS)^{-1}$ to control filamentous bulking and by Jenkins et al. [5] who used doses between 1 and 15 mg Cl_2 $(gVSS)^{-1}$.

For a chlorine concentration of 4.75 mg Cl_2 $(gVSS)^{-1}$ and 20 min contact time (Fig. 6a), the $FR_{OUR} = 0.4$ and $FR_{DHA_a} = 0.5$, while the value corresponding to filamentous microorganisms in activated sludge measured by image analysis FR_{DHA_i} was about 0.22. These results show that chorine inhibited the global RA of activated sludge by 50–60%, and under the same conditions the RA of filamentous microorganisms was specifically reduced by 80%. However, for a dose of 7.9 mg Cl_2 $(gVSS)^{-1}$ (Fig. 6b), the FR_{DHA_i} values were found to be zero after a contact time of 10 min; therefore, the RA of filamentous microorganisms was completely inhibited meanwhile the FR_{OUR} and FR_{DHA_a} representing the RA of the entire floc were of 0.35 and 0.55, respectively. In both cases the image analysis-based FR_{DHA_i} (which indicates the bacterial RA fraction of filamentous microorganisms present in the flocs) were considerably lower than those determined by the overall RA of flocs by respirometry (FR_{OUR}) and spectrophotometric technique (FR_{DHA_a}).

In a previous work Caravelli et al. [6] proposed a mathematical model to predict chlorine decay and inactivation using individual tests with pure cultures of filamentous (*Sphaerotilus natans*) and floc-forming (*Acinetobacter anitratus*) microorganisms as a function of time. Critical chlorine doses (the lowest initial dose leading to total microorganism inactivation) were experimentally determined to be 11.9 mg Cl_2 $(gVSS)^{-1}$ for *S. natans* and 4.5 mg Cl_2 $(gVSS)^{-1}$ for *A. anitratus*. These values indicate that in pure cultures, floc-forming microorganisms were more susceptible to chlorine action than filamentous ones. However, in this work we showed that during chlorination of activated sludge the filamentous microorganisms present in the flocs suffered a higher reduction in their RA in comparison to that of the whole flocs. By comparing the results from the previous work with those obtained in the present research, it can be concluded that filamentous microorganisms being located on the outer zone of the flocs become more exposed to the action of oxidizing chemical agents. Besides, it must be considered that in activated sludge flocs and biofilms, living cells, lysed decaying cells, non-biodegradable cell debris and influent solids are enclosed in extracellular polymer substances (EPS) forming aggregates [23]. The EPS have two different origins, from metabolism or lysis of microorganisms (proteins, DNA, polysaccharides and lipids) and from the wastewater (humic acids, cellulose etc.) [24]. The EPS may protect cells against environmental changes in pH, water and salt content [25]. The limited penetration of chlorine in biofilms was attributed to the diffusion of the biocide in the matrix consisting of cells and EPS [26]. Biocides may not reach the floc-forming bacteria as a result of diffusional resistance of the floc or neutralization of the biocide inside the matrix floc. Consequently, experimental results confirmed that the relative position of the filamentous microorganisms in the floc and the presence of extracellular polymer substances are determinant factors for the chlorine efficacy in the bulking control and not the intrinsic sensitivity of each microorganism to this oxidant.

4. Conclusions

The INT-dehydrogenase activity test measured by spectrophotometry (DHA_a) and image analysis (DHA_i) can be applied concurrently to determine chlorine doses sufficient for controlling filamentous bulking without affecting seriously the overall metabolic activity of activated sludge.

Both DHA tests were optimized through assays carried out on pure cultures of *S. natans*, a typical filamentous microorganism present in activated sludge. The optimum INT concentration range was found to be between 0.08 and $0.150\,g\,l^{-1}$, with an incubation time of 50 min. Such conditions favor INT reduction and, therefore, the evaluation on INT-dehydrogenase activity by spectrophotometry as well as by image analysis both in pure cultures and activated sludge.

Good correlations were obtained between the oxygen uptake rate (OUR) and the DHA tests using *S. natans* pure cultures under chlorine inhibition. These findings validate the DHA test coupled with image analysis as a methodological tool to measure and monitor the respiratory activity of filamentous microorganisms present in activated sludge during the control treatment of filamentous bulking with oxidizing chemical agents such as chlorine.

By applying OUR and DHA tests, the effect of chlorine on activated sludge was evaluated. At the concentrations tested, 4.75 and $7.9\,mg\,Cl_2\,(gVSS)^{-1}$, the inhibition of filamentous microorganisms was higher than in the whole activated sludge. Although floc-forming microorganisms were demonstrated to be more susceptible to chlorine than filamentous in pure cultures, results obtained in the present work confirmed that in activated sludge, the location of the filamentous microorganisms in the flocs and the presence of extracellular polymers, largely determine their higher susceptibility to chlorine; consequently this feature plays a critical role in bulking control.

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