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Kinetic responses of activated sludge to individual and joint nickel (Ni(II)) and cobalt (Co(II)): An isobolographic approach

Petros Gikas *

Ministry of Environmental Planning and Public Works, General Secretariat of Public Works, Special Service of Public Works for Greater Athens Sewerage and Sewage Treatment, Varvaki 12, Athens 11474, Greece

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Abstract

The effects of Ni(II) and Co(II) on the activated sludge growth rate have been assessed for a batch growth system, for a range of concentrations between 0 and 320 mg L^{-1}. The activated sludge was not acclimatized to the above metallic species, while a synthetic rich growth medium was used as substrate throughout out the experimental trials. Ni(II) and Co(II) have been found to stimulate microbial growth at concentrations approximately below 27 and 19 mg L^{-1}, with maximum stimulation concentrations 10 and 5 mg L^{-1}, respectively. The lethal concentrations (zero growth) for both species have been found to lie between 160 and 320 mg L^{-1}, with Co(II) identified as more potent growth inhibitor compared to Ni(II). The behaviour of activated sludge was also tested at the presence of three Ni(II) and Co(II) quotas, at various concentrations (75%Ni–25%Co (w/w), 50%Ni–50%Co (w/w) and 25%Ni–75%Co (w/w)). All the mixtures stimulated more drastically the activated sludge growth at relatively small concentrations, compared with the stimulation of equal concentrations of single species, whilst they also acted as more potent inhibitors at relatively high concentrations. Based on the isobole method, the data indicated that Ni(II) and Co(II) acted synergistically at the increasing stimulation and at the intoxication zones, whilst an antagonistic relation determined at the decreasing stimulation zone. Under the light of the present study, it is obvious that interactions (particularly synergism) between different metallic species should be taken into account in the methodologies used to establish criteria for tolerance levels in the environment.

Keywords: Activated sludge; Nickel; Cobalt; Heavy metals; Growth rate; Toxicity; Inhibition; Stimulation; Synergy; Isobole; Isobologram

1. Introduction

Heavy metals are commonly encountered in municipal and industrial effluents, usually causing negative effects to the efficiency of the treatment plants [1]. However, trace amounts of selected heavy metals have often beneficial effects on the biodegradation of liquid wastes [2,3], whilst addition of small amounts of heavy metals has been practiced in order to enhance the biodegradability of weak wastewaters, such as graywater [4]. Thus, some heavy metals (like Fe, Cu, Co, Ni, Zn) are considered as "essential" elements for microbial growth, whilst no biochemical role has been assessed, up to now, for other ones (like Cd, Hg, As, Ag, Au), which are considered as "nonessential" elements [5]. The "essential" heavy metals often function as protein stabilizers, as catalysts for biochemical reactions, as gene expression regulators, and as osmotic balance controller, across various microbial membranes [6]. The resistance of bacteria to heavy metals has been proved to be genetically oriented, either due to chromosomal or due to plasmid determinants [7]. "Essential" metal resistance mechanisms are usually chromosome-based, and more complex than plasmid-based, which usually are encoded systems for the efflux of toxic concentrations of metals [8]. Thus it is obvious that the resistance of a mixed microbial population, such as the activated sludge, to a particular heavy metal is determined by the specific resistance of each microbial species and by the interactions between the populations of the existing species. Additionally, it has been proved that the response of microorganisms to the exposure on multiple heavy metals may vary, compared with their response to single heavy metals. The combined effect of more than one heavy metal to a microbial population can be greater than the sum of the effects of each metal individually, according to a phenomenon called synergism [9,10], or vice versa, when antagonistic relation exists between the heavy metals [11,12]. Relations that are

* Tel.: +30 210 6444410; fax: +30 210 6452753.
E-mail address: petrosgikas@yahoo.gr.

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neither synergistic nor antagonistic and whose effect is the sum of the effects when the microorganisms are exposed to each metal alone are called additive interactions [13,14]. Thus, the occurrences of interactions between heavy metals (particularly synergism) have to be taken into account for the establishment of the tolerance levels of metals in the environment.

A number of methods have been proposed to characterize the type of the effect on a biological system, which is produced due to the simultaneous presence of more than one substance, in relation to the effects, which are produced due to presence of individual substances. The method of effect summation (according to which the effects of specified doses of two individual substances is compared with the response yielded by the combined doses of the same substances) has been extensively used to assess the joint effects of heavy metals on microorganisms [10,15]. However, this method can be successfully applied only if the dose response curves of the individual substances follow a linear pattern (which is the exception in toxicity situations). Failure to check this assumption (which is a common practice) may lead into elusive conclusions [16]. This problem may be overcome, by comparing the equi-effective concentrations (i.e. the concentrations which yield the same result) of the individual substances and their mixtures. Such equi-effective concentrations are used in the isobole method, which was originally introduced as a graphical tool by Fraser [17,18], and was further developed by Loewe and Muischnek [19], Loewe [20] and Berenbaum [21]. According to the isobole method, the effect of a substance when it is applied jointly with other substances can be estimated by dividing the concentration of the substance in the mixture with the concentration of the same substance when it is applied singly, that yields the same effect as the mixture. The main advantage of the method is its ability to compare the effects of substances with dissimilar response curves [16].

A number of studies [22,23] have indicated that the effects of the concentration of the "essential" heavy metals on the microorganisms can be divided into three zones: the zone of increasing stimulation, the zone of decreasing stimulation, and the toxicity zone. The determination of the critical heavy metal concentration beyond of which growth stimulation gives place to growth inhibition is of great significance for all those who work with microbial cultures at the presence of heavy metals. However, the above-mentioned critical point is not just a function of the particular heavy metal (or of the combination of heavy metals) and of the type of microorganisms, since microorganisms have the ability to adapt with time to relatively higher concentrations of heavy metals, according to a phenomenon called acclimation. Acclimatized microorganisms often activate alternative biochemical pathways, which allow them to continue growing [24]. However, if the concentration of heavy metals to the cell environment increases beyond to a point, cell metabolism can be totally contained [25,26].

A number of methods have been proposed for measuring metal toxicity in activated sludge systems, the more commonly used ones include the measurement of enzymatic activity [27,28], the measurement of respiratory rate [29,30], the influence on the micro-organism growth parameters [23,31,32], and the use of fluorescent and bioluminescence methods [33,34].

Nickel and cobalt are both used for the production of strong and chemically inert, iron based, metallic alloys. They are also used in the electroplating industry, for the production of green (nickel) and blue (cobalt) paints and pigments, and as catalysts in the chemical industry. The above uses illustrate the extensive use of both metals, which consequently leads to unintentional contamination of the environment. On the top to the anthropogenic contamination, nickel and cobalt enter into the aquatic environment due to leakages from naturally occurring minerals. Regardless of the source of pollution, it is the rule, that nickel and cobalt are encountered together in the aquatic environment, and thus any study on the effects of the above metals to the microorganisms should not ignore the joint effects of both species. A confirmative factor of the interrelation of nickel and cobalt stands the fact that the microbial resistance genotypes for both species are usually present in the same plasmid of the microorganisms [35,36]. Also, similar biochemical transportation mechanisms through microbial membranes have been identified for both species [37].

Both metals are encountered in aqueous solutions as di-valent (Ni(II), Co(II)), whilst cobalt can occasionally be uncounted in complex forms as tri-valent (Co(III)) [38]. The last (Co(III)) can be biochemically reduced into the di-valent form within the cells [39,40].

Both nickel and cobalt belong to the so-called "essential" metals [24]. To date nickel has been identified as a component in a number of enzymes, participating in important metabolic reactions, such as: ureolysis, hydrogen metabolism, methane biogenesis and acitogenesis [41–43]. Cobalt is an important cofactor in Vitamin B_{12}-depended enzymes [44], and an indispensable component in a number of enzymes [45]. It has been proved that at relatively small concentrations both species can stimulate aerobic and anaerobic microbial growth, however, despite their positive role in microbial growth, both metals have toxic effects at relatively high concentrations [2–4,46–50].

A number of studies have been carried out to investigate the effects of nickel and cobalt on the behaviour of activated sludge [4,27,30,48,50–52]. However, only a few studies deal with the effects of both species on activated sludge, and thus, since activated sludge characteristics exhibit significant variations from study to study, it is hard to draw a clear conclusion about the relative toxicity of the examined metallic species. Even less work has been performed to reveal the effects of the simultaneous presence of both nickel and cobalt on activated sludge, which is the common situation in activated sludge wastewater treatment plants. This study aims to investigate the joint effects of Ni(III) and Co(II) on unacclimatised activated sludge, with ultimate target being the identification of the type of interaction (synergism, antagonism or addition) between the above species on activated sludge growth.

2. Materials and methods

2.1. Experimental set-up and growth medium composition

The batch growth system has been described in detail elsewhere [23]. In brief, the cultures were grown in 30 borosilicate

Table 1
Composition of growth medium used in all trials with the addition of calculated amounts of Ni(II) or Co(II) in the form of $Ni(NO_3)_2$ and $Co(NO_3)_2$, respectively

Constituent	Concentration
Peptone (mg L^{-1})	7500
Dextrose (mg L^{-1})	5000
Yeast extract (mg L^{-1})	5000
KH_2PO_4 (mg L^{-1})	210
K_2HPO_4 (mg L^{-1})	180
NH_4SO_4 (mg L^{-1})	300
$MgSO_4{\cdot}7H_2O$ (mg L^{-1})	100
$NaNO_3$ (mg L^{-1})	25
$Ca(NO_3)_2{\cdot}4H_2O$ (mg L^{-1})	100
$FeSO_4{\cdot}7H_2O$ (mg L^{-1})	50
Triton X-100 (mL L^{-1})	0.1

cylindrical tubes with working volume of 50 mL each. The tubes were attached in a specifically designed rack, and were partially submerged in a 30 °C water bath, while air was pumped via 30 small air pumps (Resun AC-2600, E.U.). Approximately 20 bubbles of air per minute were dosed in each tube, via flexible PVC hoses, a sufficient flow to maintain saturated oxygen conditions in the growth medium, and simultaneously to keep the biomass in suspension.

The composition of the rich growth medium is shown in Table 1. Dextrose, peptone and yeast extract are the carbon providing sources. Nitrogen and phosphorus are provided by peptone and yeast, and are supplemented by ammonium and phosphate salts. Sufficient quantities of iron, magnesium and calcium are also provided, whilst yeast extract serves as micronutrient provider. Biomass flocculation was successfully controlled by the addition of a small quantity (0.1 mL L^{-1}) of Triton X-100 (Union Carbide Chemicals Co. Inc., Germany). The concentration of Ni(II) and Co(II) in the fermentation tubes was adjusted with the addition of calculated amounts of $Ni(NO_3)_2$ and $Co(NO_3)_2$, respectively. The pH of the growth medium was adjusted just after inoculation to 7.0, with the addition of HNO_3.

2.2. Analytical techniques

Microbial growth measurements were performed using a U-2000 Hitachi (Japan) spectrophotometer at 600 nm. 0.5 mL of mixed liquor was withdrawn from each tube every hour, and after appropriate dilution it was transferred to the spectrophotometer for the determination of the optical density (ABS). The corresponding mixed liquor suspended solids (MLSS) concentration was calculated by means of a calibration curve (Eq. (1)), which was obtained experimentally as follows: one litre of growth medium was equally distributed in five conical flasks with nominal volume of 500 mL, and a 5 mL activated sludge inoculum was added. The flasks were then placed in a shaker at 30 °C, and when sufficient growth was observed the optical densities and the dry weight concentration of the flask content was determined according to the following procedure: every hour (for the next 4 h), 30 mL of mixed liquor were extracted from each flask; the optical density was measured using 1 mL of the sample, whilst the rest was immediately filtered through pre-weighed filter paper with a pore size of 0.2 μm. The filter was washed twice with 100 mL of deionised water and then it was weighted, after drying at 105 °C. Optical densities (ABS) were plotted against dry weight, and the line which is determined by the least square method is described by Eq. (1):

$$\mathrm{MLSS} = 1.1203\ \mathrm{ABS} + 0.0819 \qquad (R^2 = 0.987) \tag{1}$$

where MLSS is expressed in mg L^{-1}.

The maximum specific growth rate (μ_{max}) for each growth curve was determined by applying Eq. (2) for the "linear" part of each logarithmic growth curve.

$$\ln\frac{\mathrm{MLSS}_i}{\mathrm{MLSS}_0} = \mu_{max}(t_i - t_0) \tag{2}$$

where $MLSS_i$ is the MLSS (mg L^{-1}) at time equal t_i, $MLSS_0$ the MLSS (mg L^{-1}) at the end of lag phase, μ_{max} the maximum growth rate (h^{-1}), t_i the time at the point of measurement, and t_0 is the time at the end of lag phase. μ_{max} was calculated by linear regression of all measured values.

2.3. Activated sludge inoculum and experimental procedures

Activated sludge inoculum was obtained from the aeration tank of the Athens municipal wastewater treatment plant in Psyttalia. The plant serves approximately 3,500,000 people from the Greater Athens (capital of Greece) area, and has an average hydraulic load of about 800,000 $m^3\ d^{-1}$. During the sampling from the plant, the sludge age was approximately 12 days, and the sludge density was measured to be 4.2 g L^{-1}. The inlet to the primary clarifier concentrations of Ni(II) and Co(II) have been measured (using a 3100-Perkin-Elmer, USA, atomic absorption apparatus with graphite furnace) as 38 and 11 μg L^{-1}, respectively, while the respective concentrations at the entrance of the aeration tank (the exit from the primary clarification) have been measured as 25 and 9 μg L^{-1}.

29.5 mL of rich growth medium among with 0.5 mL of activated sludge inoculum were placed in each try tube. The concentration of heavy metals in each tube was adjusted before seeding by the addition of nitrate salts of Ni and Co. Two series of trials were carried out using single heavy metal (Ni(II) or Co(II)) addition, and three more series of trials were contacted using combination of (Ni(II) and Co(II)) at the following quotas (w/w): 75%Ni–25%Co, 50%Ni–50%Co and 25%Ni–75%Co. Table 2 summarises the concentration of heavy metals in each batch growth. Three tubes were used for each heavy metal concentration, making the total number of trials: $5 \times 9 \times 3 = 135$, plus the blank (three tubes). Following inoculation the tubes placed in the water bath, and aeration started immediately. One sample was withdrawn every hour from each tube and the optical density was measured using the spectrophotometer described above. The total duration of incubation for each set was 22 h.

Table 2
The concentration of heavy metals (Ni(II) and Co(II)) in each batch growth test

Set	Tube set: 1		Tube set: 2		Tube set: 3		Tube set: 4		Tube set: 5		Tube set: 6		Tube set: 7		Tube set: 8		Tube set: 9	
	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)	Ni ($mg L^{-1}$)	Co ($mg L^{-1}$)
First	1	–	5	–	10	–	20	–	30	–	40	–	80	–	160	–	320	–
Second	–	1	–	5	–	10	–	20	–	30	–	40	–	80	–	160	–	320
Third	0.75	0.25	3.75	1.25	7.5	2.5	15	5	22.5	7.5	30	10	60	20	120	40	240	80
Fourth	0.5	0.5	2.5	2.5	5	5	10	10	15	15	20	20	40	40	80	80	160	160
Fifth	0.25	0.75	1.25	3.75	2.5	7.5	5	15	7.5	22.5	10	30	20	60	40	120	80	240

Three tubes were used for each combination of heavy metal concentrations, and three tubes were used as blank. Thus the total number of trials was $5 \times 9 \times 3 + 3 = 138$.

2.4. Statistical screening of data

The Student's *t*-distribution, with a 0.05 level of significance, was used to reject the statistically extreme values of absorbance, considering each single measurement. The statistical analysis indicated that six measurements out of 138 had to be withdrawn.

2.5. Isobolograms

The type (synergism, antagonism or addition) of the joint effects of Ni(II) and Co(II) species on the growth rate of activated sludge, has been evaluated using the isobole method. An isobole is a contour line that represents equi-effective quantities of two substances and their mixtures. The line itself is drawn by extrapolating the equi-effective points on the isobologram. The location of the isobole line on the isobologram, in relation to the theoretical straight line of additivity, indicates the type of joint effects of the examined substances. For comprehensive analysis of the method the reader may refer to the review articles by Wessinger [53] and Gessner [54].

3. Results and discussion

3.1. Effects of Ni(II)

Fig. 1 depicts the growth curves of activated sludge growing at different Ni(II) concentrations. As the concentration of Ni(II) increased, the lag time of the culture increased, with the exception of the growth curve obtained at Ni(II) concentration of 1 $mg L^{-1}$, where a slight decrease in lag time was observed, compared with the blank. The growth curves for Ni(II) concentrations up to 10 $mg L^{-1}$ lied quite close each other, but they were not identical. Significantly prolonged lag times were observed for Ni(II) concentrations over 40 $mg L^{-1}$. This phenomenon has been ascertained by Cobet et al. [55], who reported extension of the lag time of the bacterium *Arthobacter marinus*, from 3 to 72 h at the presence of 0.4 mM (=23.5 $mg L^{-1}$) Ni(II). In the present study, a weak biomass growth was observed at the presence of 80 and 160 $mg L^{-1}$, after approximately 13 and 16 h of incubation, respectively; however, growth was not sustained at Ni(II) concentration of 320 $mg L^{-1}$ (at least during the first 22 h after inoculation). Limited research on activated sludge systems has been carried out at relatively high Ni(II) concentrations (over 50 $mg L^{-1}$). However, a number of studies have identified nickel as trace element in various biological systems [41,56], while, some microorganisms (like the cyanobacterium *Oscillatoria* sp. [57]) demonstrate an absolute metabolic requirement for nickel. Duxbury [58], has proposed that bacterial strains able to grow at Ni(II) concentrations higher than 1.70 mM ($\cong$100 $mg L^{-1}$) may be characterized as Ni-tolerant species. Schmidt and Schlegel [59], have isolated bacterial strains from metal processing wastewater treatment plants, capable of growing at $NiCl_2$ concentrations up to 20 mM (1174 mg(Ni) L^{-1}), they also managed to enrich bacterial strains from ordinary soil, able to grow in media containing up to 1 mM Ni(II) (=58.71 mg(Ni) L^{-1}). Spingael et al. [60], reported that they managed to construct (by plasmid transfer) several stains of *Alcaligenes eutrophus*

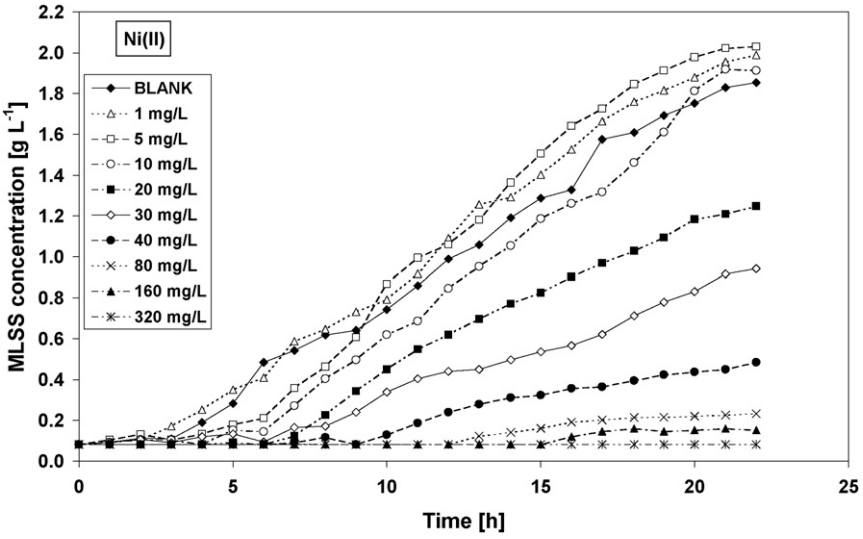

Fig. 1. Growth curves (MLSS concentration vs. time) for activated sludge growth at different Ni(II) concentrations. The total duration of the trial was 22 h.

able to degrade haloaromatic compounds in a growth medium containing 1 mM Ni(II) (=58.71 mg(Ni) L^{-1}). Otth et al. [61], have experimentally estimated the minimal inhibition concentration of Ni(II) on 49 strains of *Arcobacter butzleri* to lie just below 4 mM (=236 mg L^{-1}), while for one strain it was found to be just below 8 mM (=472 mg L^{-1}). In accordance with the results in the present work, Ainsworth et al. [9], who worked with batch cultures of *Klebsiella pneumoniae*, concluded that an increase of the Ni(II) concentration resulted, both, to prolonged lag times, and to a decrease of the final MLSS concentration. More specifically, they reported a reduction on the MLSS from 41.2 to 15.9 mg L^{-1} when the Ni(II) concentration in the growth medium was increased from 300 to 500 mg L^{-1}. They also reported a 50% reduction in microorganism survival, by the use of viability counts on agar plates, at a Ni(II) concentration of 6.65 mg L^{-1}. Babich and Stotzky [10], who investigated the effects of Ni(II) concentration towards the growth of heterotrophic microorganisms, reported that statistically significant inhibition was occurred above 5–10 mg(Ni) L^{-1} for *Bacillus subtilis*, *Nocardia corallina* and *Candida krusei*, above 10–25 mg(Ni) L^{-1} for *Aspergillus flavipes* and above 25–50 mg(Ni) L^{-1} for *Enterobacter aerogenes*, however, all the examined microorganisms were able to grow, at reduced rates, at significantly higher Ni(II) concentrations. The same researchers [62] reported complete growth containment between 20 and 30 mg(Ni) L^{-1} for *N. corallina*, at 30 mg L^{-1} for *Bacillus brevis*, between 40 and 50 mg L^{-1} for *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Nocardia rhodochrous* and *Asticcacaulis excentricus*, at 50 mg L^{-1} for *Serratia marcescens*, and over 100 mg L^{-1} for *Caulobacter leidyi*.

Fig. 2 depicts the maximum growth rate of biomass (μ_{max}) versus Ni(II) concentration. μ_{max} appears to increase when the Ni(II) concentration increases from 0 to 10 mg L^{-1}, thereafter decreasing gradually to nil (at concentrations higher than 160 mg L^{-1}). Based on linear regression, and according to Fig. 2, the μ_{max} of the blank (μ_{max0}) is smaller compared with the μ_{max} values observed at the presence of approximately up to 27 mg L^{-1} of Ni(II) (the μ_{max} at Ni(II) concentration of 30 mg L^{-1} is marginally smaller compared to the blank). The above data indicate that Ni(II) stimulates the aggregate activated sludge growth at concentrations below approximately 27 mg L^{-1}, while at higher concentrations it acts as a growth intoxicator. Yetis and Gokcay [51], and Gokcay and Yetis [50], who studied the effect if Ni(II) on the performance of a continuous activated sludge system, reported duplication of the MLSS after gradual increase of Ni(II) concentration from 0 to 5 mg L^{-1}, while a further increase of Ni(II) to a concentration to 10 mg L^{-1} resulted to an MLSS concentration slightly higher than the one measured at zero Ni(II) concentration. Sujarittanonta and Sherrard [27], who worked with a continuous bioreactor set-up found that addition of Ni(II) at concentrations between 1 and 5 mg L^{-1} enhanced, both, the maximum biomass yield and the maintenance coefficient of activated sludge. They attributed the above effects either to a shift in microbial species with different physiological properties, or to the stimulatory effects of nickel to microbial activity. Gikas and Romanos [52], who worked with activated sludge growing in a batch system, reported stimulation with Ni(II) concentrations up to approximately 40 mg L^{-1} (beyond of which the Ni(II) was acted as growth inhibitor), with maximum stimulation concentration of about 20 mg L^{-1}. The above results are in general agreement with the results obtained in the present study, taking into account that most of those have been obtained in continuous systems, which are vulnerable to increases of the concentrations of growth inhibitors due to washing out. On the other hand, in batch systems, like the one, which used in the present study, a weak growth (small μ_{max} values) can be sustained at higher heavy metal concentrations (despite the prolonged lag phase). The stimulatory effects of nickel have been widely acknowledged in anaerobic growth [63]; however, the present work has been primarily focused on results obtained during aerobic growth processes, which are closer related to the activated sludge process.

A number of researchers have reported only growth inhibition due to the presence of Ni(II) in the environment of the

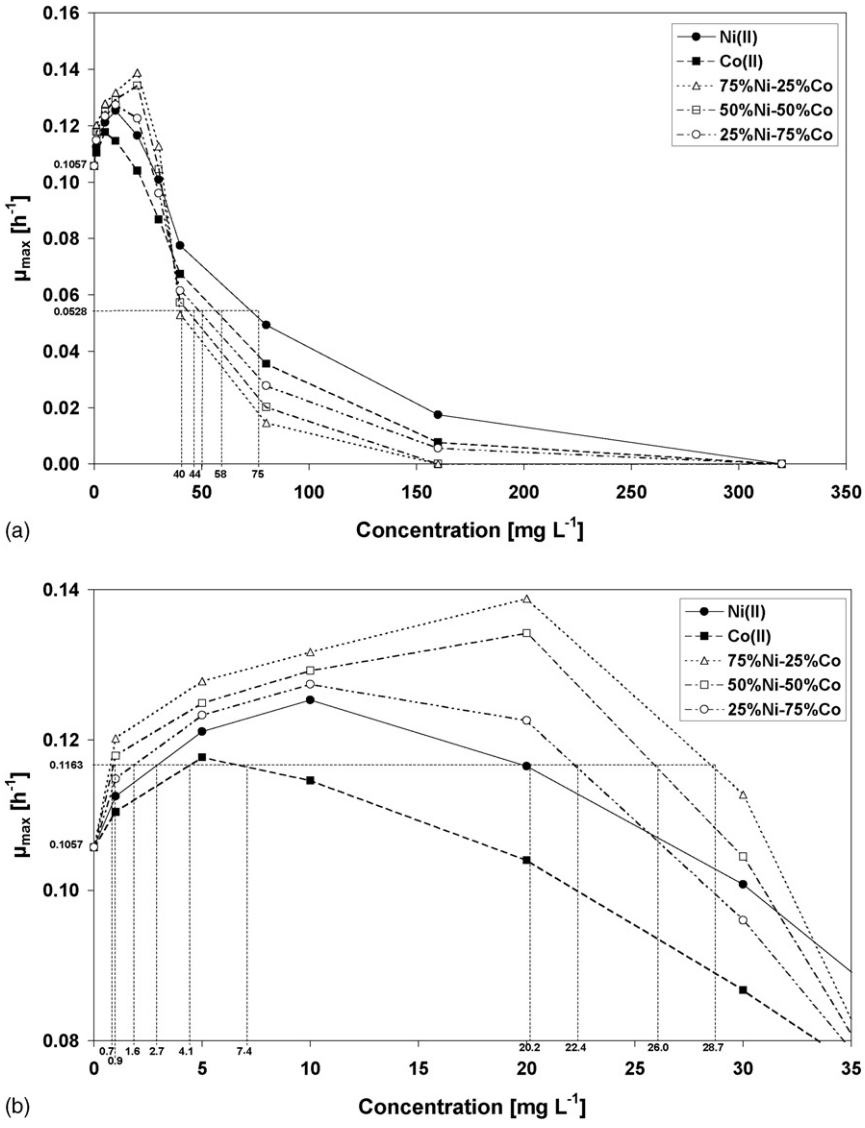

Fig. 2. (a) Calculated values of μ_{max} vs. the concentrations of Ni(II), Co(II) and mixtures (w/w): 75%Ni–25%Co, 50%Ni–50%Co, 25%Ni–75%Co. (b) Detail for concentrations up to 35 mg L^{-1}. The equi-concentratios (which produces the same effects at each studied case) and the relative responses appear with small fonts at the *X* and *Y*-axes.

microorganisms. Amor et al. [64], who studied the effects of several heavy metals on *Bacillus* sp. growing on toluene, did not observe stimulatory effects for Ni(II) concentrations between 0.4 and 1.0 mM (≅24–60 mg L^{-1}), however, they did not carried out any trials at smaller Ni(II) concentrations. Kelly et al. [34], determined the EC_{50} values for activated sludge at the presence of Ni(II), by the use of bioluminesence, to lie over 100 mg L^{-1}, while the same value was measured as 76 mg L^{-1}, by the use of the SOUR (specific oxygen uptake rate) method. Cobet et al. [55], reported that 0.1 mM of $NiCl_2$ (=5.9 mg(Ni) L^{-1}), slightly affected the growth of the marine bacterium *Arthrobacter marinus*, while a more pronounced effect was induced by the addition of 0.4 mM (=23.5 mg(Ni) L^{-1}), and finally, the addition of 0.5 mM (=29.3 mg(Ni) L^{-1}), resulted to sustention of cell division. They also reported increase of the lag phase with the rise if Ni(II) concentration (from 3 to over 70 h at the presence of 0.4 mM Ni(II)), and transfiguration of the cells into megalomorhic type (the cell size increased up to 250 times the normal size after 10 h of incubation at the presence of 0.4 mM Ni(II)). McDermott et al. [65], reported that the overall efficiency of a continuous activated sludge plant was significantly affected by the presence of 2.5, 5 and 10 mg L^{-1} of Ni(II) in the feed stream, while the system was able to withstood the continuous presence of 1 mg L^{-1}. They also reported that a slug dose of 200 mg L^{-1} caused serious reduction in the treatment efficiency for a few hours, but within 40 h the plant had returned to its normal performance. Geslin et al. [66], who experimented with a pure culture of *Escherichia coli* growing in a batch system, measured the Ni(II) minimal inhibitory concentration to be 0.2 mM (=11.74 mg L^{-1}). Finally, Mowat [30], who measured the respiratory activity of activated sludge at the presence of 1, 5, 10 and 20 mg(Ni) L^{-1} reported a reduction of 22.6, 44.6, 57.3 and 62.7%, respectively.

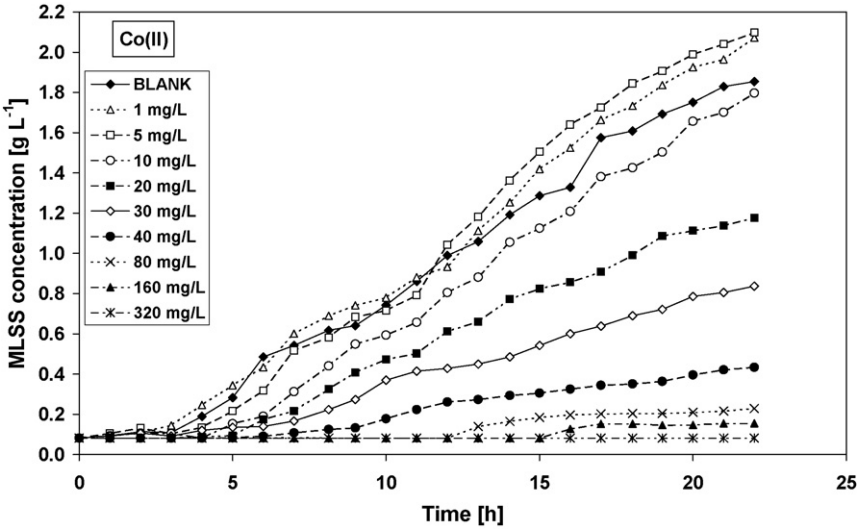

Fig. 3. Growth curves (MLSS concentration vs. time) for activated sludge growth at different Co(II) concentrations. The total duration of the trial was 22 h.

3.2. Effects of Co(II)

The effects of Co(II) on the growth curves of activated sludge are depicted in Fig. 3. A similar trend with the growth curves obtained at the presence of Ni(II) can be observed. The lag time was particularly prolonged for Co(II) concentrations over 40 mg L^{-1}, whilst a weak increase in MLSS could be observed, after approximately 17 h from inoculation, at Co(II) concentration of 160 mg L^{-1}. No growth was observed at Co(II) concentration of 320 mg L^{-1}. The effects of cobalt on the growth of microorganisms have been studied to a lesser extent compared with the relative work on the effects of nickel, which may be probably attributed to the fact that nickel has more industrial applications compared to cobalt. In agreement with the above results, Ainsworth et al. [9], who worked with *K. pneumoniae*, reported, both, increase of lag time, and decrease of final MLSS concentration with the increase of Co(II) concentration in the growth medium. Cobalt has been identified as a trace element by a number of researchers [2,4], but only a few microorganisms are able to grow at relatively high Co(II) concentrations. Evans and Mason [67] have reported 120% increase of the activity of the bacterial clostridiopetidase-A with the addition of Co(II). Duxbury and Bicknell [68] have isolated, from metal-polluted soil, a bacterial stain able to resist to the presence of up to 3 mM of Co(II) (=176.8 mg L^{-1}). Schmidt and Schlegel [59] have isolated, from metal processing wastewater treatment plants, bacterial strains able to grow at $CoCl_2$ concentration of up to 20 mM (=1178.6 mg L^{-1}). Otth et al. [61] have reported that *A. butzleri* was able to grow at the extreme Co(II) concentration of 80 mM (=4714.4 mg L^{-1}). Similarly to nickel, cobalt is also an anaerobic growth microbial stimulant [69], but for the reason stated above, this study does not give particular weight on anaerobic growth situations.

Fig. 2 illustrates the effects of Co(II) concentration on the maximum specific growth rate (μ_{max}) of activated sludge. μ_{max} appeared to rise with the increase of Co(II) concentration for concentrations up to 5 mg L^{-1}, while Co(II) concentration of 20 mg L^{-1} resulted to a μ_{max} value slightly smaller than the one of the blank (μ_{max0}) (linear regression indicated that the decreasing concentration–toxicity crossover point was approximately 19 mg L^{-1}). Further increase of Co(II) concentration resulted to severe growth inhibition, while, μ_{max} reached zero at 320 mg(Co) L^{-1} (at least for the incubation time allowed during the present trials). In accordance with the above results, Jefferson et al. [4], have reported aerobic growth stimulation with the addition of cobalt in a graywater treatment system. More specifically, they stated that addition of Co(II) at a concentration of 5 mg L^{-1} resulted to 30% increase of COD removal rate. Similarly, Sathyanarayana Rao and Srinath [48] have reported remarkable stimulation of activated sludge growth with the addition of 5 mg(Co) L^{-1}.

Some studies have failed to determine stimulatory effects to microbial growth due to the presence of small amounts of Co(II). Norberg and Molin [70] who experimented with the bacterium *Zoogloea ramigera* (a common aerobic microorganism in sewage treatment plants) reported a slight reduction in microbial growth when Co(II) was added at concentrations up to 5 mg L^{-1}, while Co(II) concentration of 10 mg L^{-1} resulted to significant increase of lag time with parallel reduction of the MLSS concentration, while, no growth was observed with the addition of 50 mg(Co) L^{-1} (at least during the duration of the trial, which was 32 h). Chen et al. [71] have estimated the EC_0 (maximal "no-response" concentration), EC_{20} and EC_{50} values for *P. aeruginosa* growing in a batch system to be 1.15, 27.1 and 150 mg(Co) L^{-1}, respectively. Finally, Mowat [30] reported reduction of the respiratory activity of activated sludge by 16.2, 50.2, 54.7 and 58.2% with the addition of Co(II) at concentrations of 1, 5, 10 and 20 mg L^{-1}, respectively.

3.3. Effects of joint Ni(II) and Co(II)

The effects of simultaneous presence of Ni(II) and Co(II) to the maximum growth rate of activated sludge are depicted in Fig. 2. Three quotas (w/w) of Ni(II) and Co(II) were tested for the range of concentrations shown in Table 2. The growth patterns

were similar to the patterns of the single ions, with somehow more prolonged lag phases (data not shown).

(i) 75%Ni–25%Co: According to Fig. 2, μ_{max} appeared to increase by approximately 31.3% of the μ_{max0} (=0.1057 h^{-1}) (maximum growth rate of the blank) value, at a joint concentration of 20 mg L^{-1}, followed by a drastic decrease at joint concentrations higher than 30 mg L^{-1}, whilst, no growth was observed (during the time of the experiments) at a joint concentration of 160 mg L^{-1}.

(ii) 50%Ni–50%Co: This mixture increased the μ_{max} by approximately 27.0% of the μ_{max0} value, at a joint concentration of 20 mg L^{-1}. Further increase of the joint concentration resulted to a decrease of μ_{max}, but at a reduced rate compared to the 75%Ni–25%Co mixture. Growth was also nil at a joint concentration of 160 mg L^{-1}.

(iii) 25%Ni–75%Co: μ_{max} rose by approximately 20.5% of the μ_{max0} value, when the joint concentration reached 10 mg L^{-1}, followed by gradual decrease for joint concentrations up to 30 mg L^{-1}, while it was further decreased at higher concentrations, and reached zero at a joint concentration of 320 mg L^{-1}.

From Fig. 2 it is obvious that, in all cases, the simultaneous presence of Ni(II) and Co(II) stimulated the activated sludge growth to a higher degree compared with the stimulations due to the presence of equal amount of sole Ni(II) or Co(II). For the mixtures: 75%Ni–25%Co and 50%Ni–50%Co, μ_{max} achieved its maximum at a joint concentration of 20 mg L^{-1} (which is a higher concentration compared with the ones for sole Ni(II) (=10 mg L^{-1}) or for sole Co(II) (=5 mg L^{-1})), while the maximum stimulant concentration for the mixture 25%Ni–75%Co was measured as 10 mg L^{-1}. From Fig. 2, can be concluded that a given increase of μ_{max} may be achieved at two different doses of a particular mixture of Ni(II)–Co(II) (including the case of single species): a smaller one at the zone of increasing stimulation and a relatively higher at the zone of decreasing stimulation. This given increase of μ_{max}, at the zone of increasing stimulation demands smaller concentrations of Ni(II)–Co(II) mixture, compared with the corresponding concentrations of the sole species, indicating synergy between Ni(II) and Co(II). This is graphically illustrated by the use of the isobole method, for an increase of μ_{max} by 10% of the μ_{max0} value (0.1057 h^{-1} × 1.10 = 0.1163 h^{-1}), in Fig. 4. The concentrations of the metal species, which produce the same effect to μ_{max} have been estimated by linear regression between the nearest points (see Fig. 2). All the points of mixed species appear to the left of the theoretical straight line of additivity on the isobologram, which is the characteristic pattern of synergy. The dashed isobole line, which represents the equi-concentration has been drawn arbitrary, since more data are needed for a precise draw.

The opposite phenomenon is observed at the zone of decreasing stimulation, since in this case, for a given rise of the μ_{max}, higher quantities of joint concentrations demanded for mixed species, compared to the corresponding equi-concentrations of the sole species. Fig. 5 shows the isoboles, which correlates with

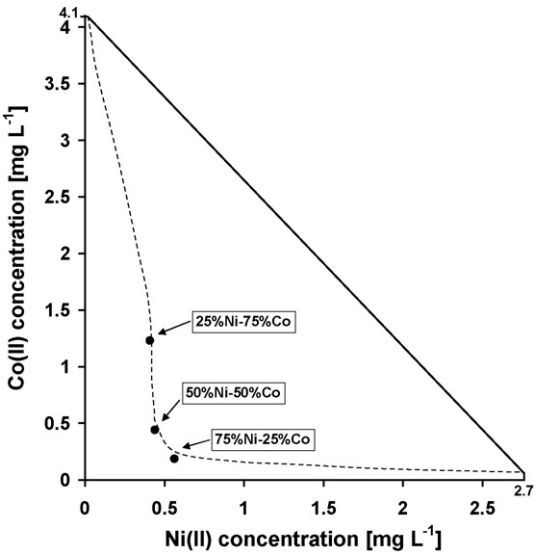

Fig. 4. Isobologram showing the equi-effective concentrations of individual species and mixtures for 10% increase of μ_{max}, compared to the specific growth rate of the blank (μ_{max0}), at the zone of increasing stimulation. The equi-effective concentration for Ni(II) and Co(II) have been estimated as 2.7 and 4.1 mg L^{-1}, respectively. The interaction between Ni(II) and Co(II) characterized as synergic, since all the isobole points lie at the left of the theoretical straight line of additivity. (The isobole line has been drawn arbitrary.)

an increase of the μ_{max} by 10% of the μ_{max0} value, at the zone of decreasing stimulation. All the equi-concentration points of the mixture are well to the right of the theoretical straight line of additivity, indicating antagonistic relation between Ni(II) and Co(II).

Fig. 6 depicts the isobolographic situation for a 50% reduction of the μ_{max} (0.1057 h^{-1} × 0.50 = 0.0528 h^{-1}), in relation to the μ_{max0} value (at the toxicity zone). All the equi-concentration

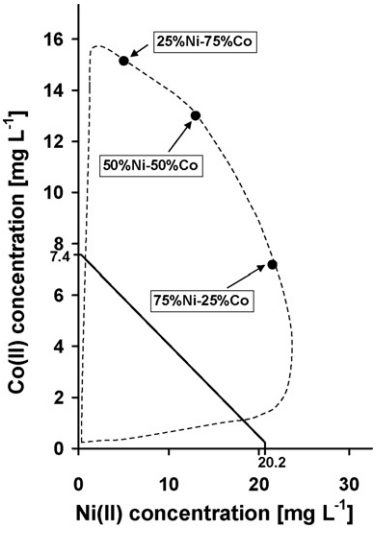

Fig. 5. Isobologram showing the equi-effective concentrations of individual species and mixtures for 10% increase of μ_{max}, compared to the maximum specific growth rate of the blank (μ_{max0}), at the zone of decresing stimulation. The interaction between Ni(II) and Co(II) characterized as antagonistic, since all the isobole points lie at the right of the theoretical straight line of additivity. The equi-effective concentration for Ni(II) and Co(II) have been estimated as 20.2 and 7.4 mg L^{-1}, respectively. (The isobole line has been drawn arbitrary.)

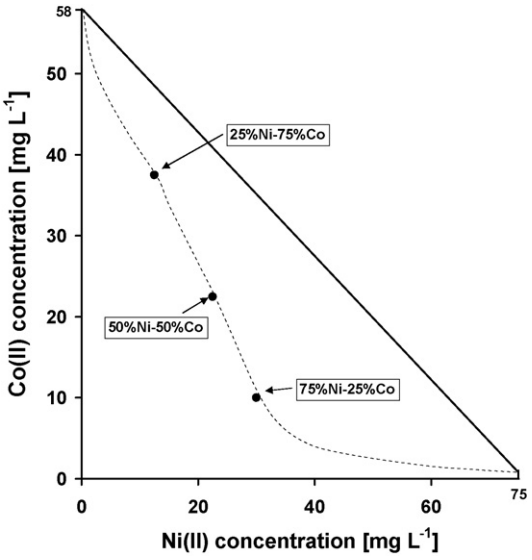

Fig. 6. Isobologram showing the equi-effective concentrations of individual species and mixtures for 50% decrease of μ_{max}, compared to the specific growth rate of the blank (μ_{max0}), at the toxicity zone. The interaction between Ni(II) and Co(II) characterized as synergic, since all the isobole points lie at the left of the theoretical straight line of additivity. The equi-effective concentration for Ni(II) and Co(II) have been estimated as 75 and 58 mg L^{-1}, respectively. (The isobole line has been drawn arbitrary.)

points of the mixtures lying to the left of the theoretical straight line of additivity, indicating synergy between Ni(II) and Co(II).

From the above investigations it is obvious that the characterization of the type (synergy, antagonism, additivity) of the joint effect of two substances on the maximum growth rate may not be derived just by simple test at one concentration level, since at a different level the relationship may be inverted. In the present situation, and based on the shape of the μ_{max}–concentration curves (Fig. 2), the categorization into three individual zones appears reasonable. It is wise noting, that the range of concentrations between approximately 30 and 40 mg L^{-1} acts as a transition zone between synergism and antagonism, thus any attempt to characterize the Ni(II)–Co(II) relation in this zone is particularly precarious.

It is worth noting, that the use of the effect summation method instead of the isoble one, can lead into different estimations regarding the type of the effects of Ni(II) and Co(II) on activated sludge growth, however, the use of the effect summation method can often lead into false conclusions [16], particularly if the dose response curves are not linear (which is the case in the present study).

A number of works has been published on the type of the effects of heavy metals to the growth of microorganisms, which range from strong synergism to strong antagonism. However, very little work has been published on the joint effects of Ni(II) and Co(II) on microorganisms. Ainsworth et al. [9], have characterized the effect of Ni(II) and Co(II) to the growth of *K. pneumoniae*, as additive, based on viable counts on petri dishes. On the other hand, Cross et al. [72], who investigated the joint effect of Ni(II) and Co(II) to the growth of cultured epithelial cells, reported a strong synergy among the above species. More specifically, they measured the LD_{50} concentration for Ni(II) and Co(II) as 5.7 mM (=334.6 mg L^{-1}) and 1.1 mM (=64.8 mg L^{-1}), respectively, whilst, the effect of a mixture of 0.75 mM Ni(II) (=44.0 mg L^{-1}) and 0.75 mM Co(II) (=44.2 mg L^{-1}), reduced cell viability by more than three times the value predicted by the additive approach.

3.4. Overall activated sludge behaviour at the presence of Ni(II) and Co(II)

According to Fig. 2, Ni(II) appears as a more potent growth stimulator compared to Co(II), at relatively small concentrations, whilst at larger concentrations Co(II) appears to inhibit activated sludge growth more drastically than Ni(II). The last is in agreement with studies by Wu et al. [73], and Alu et al. [74], who worked with *E. coli* and *Pseudomonas putida*, respectively, and reported that Co(II) is a stronger bacterial growth intoxicator. Schmidt and Schlegel [59] have also reported that bacterial resistance to Co(II) is somehow lower compared with the resistance to Ni(II). Bhattacharya et al. [75], who tested a large number of *Vibrio parahaemolyticus* strains, reported that 75% of the stains were Ni(II) resistant, while only 37% were Co(II) resistant. On the other hand, Mowat [30] suggested that both species have more or less similar effect on the respiratory activity of activated sludge. Ainsworth et al. [9], who experimented with *K. pneumoniae*, found that Ni(II) is more inhibitory than Co(II), with respect to the duration of the lag times in batch cultures; however, they reported equivalent toxicity of both species with respect to viable counts on agar plates. Finally, Otth et al. [61], reported that *A. butzleri* is more sensitive to Ni(II) than to Co(II).

Fig. 2 also shows that the maximum growth stimulation occurred when a relatively small amount of Ni(II) (25%, w/w) was replaced by the equivalent amount of Co(II). The same mixture was also the most toxic compared with all the other mixtures and with the individual species. The above observation suggests that relatively small amounts of Co(II) in a Ni(II) contaminated growth medium may alter the growth pattern of activated sludge more drastically than the opposite. It is however wise to note that nickel and cobalt have similar chemical and physicochemical properties, since they appear side by side at the eighth column of the periodic table of the elements, and their electronic structure differs by one electrode in the inner electronic shell ($[Ar]3d^8 4s^2$ for Ni; $[Ar]3d^7 4s^2$ for Co). They have similar electronegativities (1.9 for both species at the scale of Pauling, 1.75 for Ni and 1.70 for Co, at the scale of Allred and Rochow), while the effective radii of the hydrated ions at 30 °C have been calculated (no direct data exist) as 4.21 and 3.80 Å for Ni(II) and Co(II), respectively.

The investigation of the stimulation–intoxication mechanisms is out of the scope of the present study, however, is should be noted that a number of environmental factors can determine the intention of the effects of the studied metallic species on the microorganisms. A positive correlation between Ni(II) and Co(II) toxicity and pH has been determined [10,62], while reduction of the toxic effects of the above species with the increase of Mg(II) concentration has been referred [9,76]. To avoid the influence of such environmental factors all trials were

performed under the same initial conditions, however, it was expected an decrease of the pH with the progress of microbial growth. Another important factor, which can affect the growth pattern at the presence of heavy metals is the fact that activated sludge is a mixed culture, thus the overall effects may also be attributed to shifts of microbial species populations.

4. Conclusions

The effects of Ni(II) and Co(II), as single species and in mixture, to the growth pattern of unacclimatized activated sludge, growing on a synthetic rich medium, have been studied for a batch growth system. The experimental data indicated that:

(i) Ni(II) and Co(II) stimulate activated sludge growth up to approximate concentrations of 27 and 19 mg L^{-1} respectively, exhibiting maximum stimulation at 10 and 5 mg L^{-1}, respectively.
(ii) Ni(II) is a more potent activated sludge growth stimulator compared with Co(II).
(iii) Co(II) is more toxic than Ni(II), at relatively high concentrations.
(iv) The mixtures of Ni(II) and Co(II) are on the one hand more potent growth stimulators (at relatively small concentrations), and on the other hand more toxic (at relatively high concentrations), compared with the equivalent concentrations of the single species.
(v) Replacement of small amount of Ni(II) by equivalent amount of Co(II) maximised, both, growth stimulation and growth inhibition.
(vi) Based on the isobole method, a synergic effect between Ni(II) and Co(II) on activated sludge growth has been identified for the zones of increasing stimulation and intoxication, whilst at the zone of decreasing stimulation the above species were acted antagonistically.

Under the light of the present study, it is obvious that interactions (particularly synergism) between different metallic species should be taken into account in the methodologies used to establish criteria for tolerance levels in the environment.

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