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Review

Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms: A review

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ABSTRACT

Nickel (N(II)) and cobalt (Co(II)) are often encountered in wastewaters. As conventional wastewater treatment may only partially remove nickel and cobalt, a large fraction of the above metals is released to the aquatic environment. Both metals have been identified as micronutrients, at trace concentrations; however, they are both microbial growth inhibitors, at relatively high concentrations. On the other hand, the combined effects (e.g.: growth stimulation or toxicity) of the above metals have been found to differ from the summation of the effects which occur when the metals are applied individually. Moreover, a number of environmental factors (e.g.: pH, biomedium composition, biomass concentration, presence of other heavy metals) can affect the microbial toxicity of the above metallic species. The present review discusses, in a systematic way, the individual and joint effects of the above heavy metals to the growth of microorganisms grown under aerobic conditions, with focus on the growth of activated sludge. Data on multi-metal toxicity are particularly useful in establishing criteria for heavy metal tolerance levels in the environment.

Contents

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1. Introduction

Zero-valent heavy metals are considered as having no biological activity, however, simple or complex forms of ionized heavy metals can dramatically affect the performance of biological systems. Trace amounts of so called "essential" heavy metals (such as Fe, Zn, Ni, Cu, Co) have been found to stimulate microbial growth, while no beneficial biochemical role has been assessed, up to now, for other ones (like Hg, Ag, Cd, As, Au), which are considered as "non-essential" substances [1]. The main biochemical roles of the "essential" heavy metals comprise in the: (i) catalysis of biochemical reactions, (ii) stabilization of proteins, (iii) regulation of gene expression and (iv) control of osmotic pressure gradients across various microbial membranes [2]. The presence of some heavy metals is indispensable for the evolvement of particular biochemical pathways [3], while some microorganisms fail to grow at the absence of selected heavy metals [4,5]. The beneficial effects of trace amounts of heavy metals on the biodegradation of various wastewaters has been demonstrated by a number of researchers [6,7], while addition of selected heavy metals has been practiced for the enhancement of the bio-treatment of "weak" wastewaters, such as graywater [8]. On the other hand, increased concentrations of either "essential" or "non-essential" heavy metals have as a result the reduction of the biological activity of the microorganisms, and finally the total containment of the microbial growth [9–12].

The relative response of microorganisms to the presence of heavy metals has been graphically demonstrated quite elegantly by McCarthy [13] (Fig. 1), who proposed the categorization of the effects into three zones, with respect to the heavy metal concentration: (i) zone of increasing stimulation, (ii) zone of decreasing stimulation and (iii) toxicity zone. The significance of the identification of the growth response curve as a function of heavy metal concentration is obvious; however, a number of environmental factors, such as pH [14,15], metal speciation [16], mixed liquid suspended solids (MLSS) concentration [17], age of the culture (activated sludge age) [17,18], presence and concentration of other heavy metals [11,12,19,20] or of other active or phenomenaly inert substances [21,22] can influence the shape of the curve.

Another factor which can affect the shape of the above curve is the "history" of the microbial culture with respect to contact with the particular heavy metal. Ginn et al. [23] have proposed that the toxic effects of heavy metals to the growth of microbial cultures are not just a function of the heavy metal concentration, but they are dependant on the cumulative contact time. However, prolonged heavy metal-microorganism contact times may result into microbial acclimation to the particular heavy metal; thus, acclimatized microorganisms can ultimately grow at significantly higher heavy metal concentrations, compared with the same unacclimatized microbial strains [24–26]. Repeated sub-cultivations of a microorganism to increased concentrations of heavy metals may allow the isolation of heavy metal-tolerant mutants [20]. The adaptation of acclimatized microorganisms to relatively high heavy metal concentrations has often been attributed to the activation of alternative biochemical pathways which allow cells to continue growing [27]. Barndt et al. [28] have proposed a mathematical model to simulate microbial adaptation to unfavourable environmental conditions, which could be modified to describe microbial adaptation to the presence of heavy metals.

It is worth mentioning that the resistance of microorganisms to heavy metals has been proved to be genetically oriented, either due to chromosomal or more commonly due to plasmid determinants [29–31]. "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 [32].

1.1. Assessment of metal toxicity

A number of methods have been proposed for measuring metal toxicity in microbial systems [33], the more commonly used include the measurement of the enzymatic activity [34,35], the measurement of the respiratory rate [36,37], the assessment of various growth parameters [11,38–40], the measurement of cell viability via plate counting [41,42] and the use of fluorescent and bioluminescence methods [43,44]. The above methods usually indicate the same qualitative trends, regarding the effects of particular heavy metals on microbial growth, however, the rule is that generally they are not in agreement with respect to the quantitative effects [44]. Codina et al. [45], who investigated the toxicity of several heavy metals on *Pseudomonas fluorescens*, concluded that the toxic doses (EC_{50} and EC_{20} values) are a strong function of the toxicity test utilized. Thus, particular care should be exercised when comparing data obtained by the use of different methods, even if they are referred to the same microbial system.

A number of environmental factors may influence the effects of a particular heavy metal on a microbial system. Apart from physical parameters, such as temperature [46], which can obviously affect the toxicity of heavy metals, the chemical background often affects significantly the biological actions of heavy metals. The quantity [34] and type [47,48] of substrate, the presence of specific chemical substances [49,50], the heavy metal speciation [51,52] and the presence of other heavy metals (see next paragraph) can influence considerably the effects on the microbial system.

1.2. Multi-metal toxicity

The pattern of the effect of any biologically active substance is usually also a function of the presence of other biologically active substances at the environment of the microorganisms. Thus, apart from the distinct case according to which the effect of two combined substances is the sum of the effects of each substance individually applied, termed the "additive" case [20,53], two other type of interactions may occur. Either the combined effect is greater than the sum of the effects of the substances applied individually (synergism) [12,54], or vice versa (antagonism) [55,56]. Understanding the type of interaction between two (or more) heavy metals with the microbial system is of vital importance for all those who are dealing with the growth of microbial cultures at environments contaminated with heavy metals [57], such as aquatic systems, soils or biological treatment plants. It is obvious that heavy metal interactions (particularly synergism) have to be assessed for the establishment of the tolerance levels of metals in the environment. However, the relative environmental legislations, in most countries, have set the maximum acceptable heavy metal concentrations in the aquatic environment for each heavy metal alone,

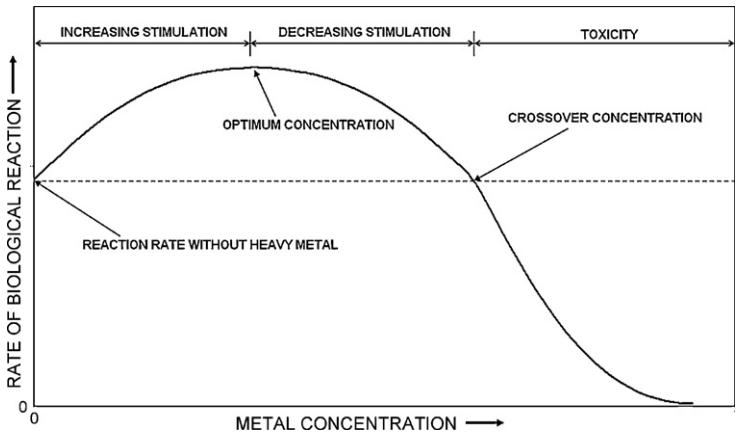

Fig. 1. The effects of heavy metal concentration to the microbial growth (adapted from McCarthy [13]).

regardless of the presence of other metals at the examined aquatic environment. This is unfortunate because metals as contaminants rarely occur in isolation. A bright exemption comes from Australia and New Zealand, which have already established basic criteria for multiple toxicity [58], assuming additive effects. However, mainly due to the lack of data, the legislation has a long way to go before it enacts on the assessment of multi-metal toxicity, by taking into account the interactions of multiple metals with (micro)organisms.

Several methods have been proposed to predict the effects of multiple substances on a biological system, the most popular of which are the method of concentration addition (or effect summation) and the method of response addition. According to the first, the effects of combined doses of more than one biologically active substances is compared with the effect of each substance applied individually to the examined biological system [54,59,60]. Despite the popularity of the above method, it can be correctly applied only if the dose–response curves of the individual substances follow a linear pattern (which is not the norm in biological systems) [61]. The second of the above-mentioned methods is based on the comparison of the equi-effective concentrations (i.e.: the concentrations which yield the same effect) of the individual substances and their mixtures, which leads to more realistic conclusions [62,63]. This approach is utilized by the isobole method, which has been extensively applied in pharmacological studies [64,65], but can successfully describe multi-metal toxicity in microbial systems [12]. The isobole method was originally introduced as a graphical tool by Fraser [66,67], and was further developed by Loewe and Muischnek [68], Loewe [69] and Berenbaum [70].

1.3. Nickel and cobalt in the environment

Both, nickel and cobalt are used in the metallurgical industry, for the production of high quality iron-based alloys. They are also used extensively as catalysts in the chemical and food industry, as prime materials for the production of paints and batteries, and in the electroplating industry [71]. Due to the extensive use of nickel and cobalt, it is likely that a considerable amount of the above metals will find its way to the aquatic environment or to biological wastewater treatment plants. Leakages from naturally occurring minerals, rich in the above metals, comprise an extra source of nickel and cobalt to the aquatic environment. It is the rule that the above metals are occurring jointly in the environment [72,73], thus data on the joint toxicity of nickel and cobalt are considered particularly useful.

Both metals are encountered in aqueous solutions as di-valent (Ni(II), Co(II)), while they can occasionally be encountered in complex forms as tri-valent (Ni(III), Co(III)) [74]. The last can be reduced biochemically (usually under anaerobic conditions) into the di-valent species within the cells [75–77]. The effects of the trivalent ions to microorganisms are not assessed by the present review.

1.4. The biological role of nickel and cobalt

Both nickel and cobalt belong to the so called “essential” metals [27,78,79]. Up 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 [80–84]. Nickel has also been identified as a component of a superoxide dismutase protein [85]. A number of studies have identified nickel as trace element in various biological systems [80,86], while some microorganisms (like the cyanobacterium *Oscillatoria* sp. [4]) have demonstrated an absolute metabolic requirement for nickel. Nickel has also been identified as an indispensable element for the chemilithotropic growth of a number of microorganisms, like *Pseudomonas flava* [87].

Cobalt is an important co-factor in vitamin B_{12}-depended enzymes [88,89], and an indispensable component in a number of enzymes [90], and particularly in the nitrile hydratases [91]. Cobalt has also been found to stimulate the activity of some microbial enzymes, such as clostridiopetidase-A [92].

Both, nickel and cobalt have been identified as trace elements in anaerobic biochemical reactions [93–95], however, anaerobic microbial growth is not discussed in the present review article.

Nickel and cobalt resistant genes are often encountered in the same plasmid of nickel–cobalt resistant microorganisms [96–98]. It is worth to note that similar types of biochemical transportation mechanisms have been identified for both metal species [99].

Like all the essential elements, nickel and cobalt often stimulate the microbial growth, at relatively low concentrations; however, both metals are toxic at relatively high concentrations [7,8,12,95,100–103].

1.5. Scope

The present review aims in presenting in a systematic way the published research on the effects of individual and joint nickel (Ni(II)) and cobalt (Co(II)) on the growth of microorganisms growing under aerobic conditions, with emphasis on activated sludge systems. The review also deals with the effects of nickel and/or cobalt on particular aerobic microbial species, in conjunction with other environmental factors. The present review does not emphasise on either the biochemical mechanisms for nickel or cobalt

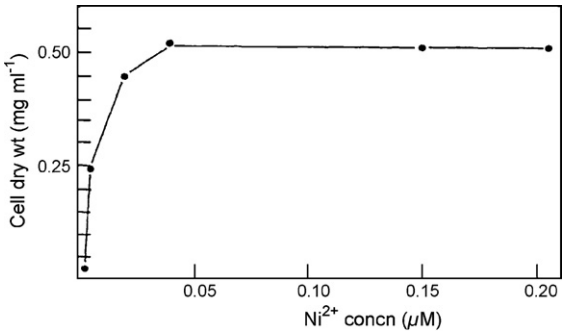

Fig. 2. The effects of Ni(II) concentration to the growth of the cyanobacterium *Oscillatoria* sp. No growth was sustained at the absence of nickel (adopted from Van Baalen and O'Donnell [4]).

tolerance by the microorganisms, nor on the phenomenon of bioaccumulation/bioremediation of the above metal species by microbes.

2. Effects of nickel

Nickel has been identified as a trace element (see Section 1.4). This is reflected in a number of experimental studies, which indicate microbial growth stimulation at relatively low nickel concentrations. An extreme example has been reported by Van Baalen and O'Donnell [4], who reported that the cyanobacterium *Oscillatoria* sp. was not able to grow at the absence of nickel (see Fig. 2). Nickel has also been identified as an indispensable element for the chemolithotrophic growth of a number of microbial species [87,104,105].

2.1. Effects of Ni(II) on activated sludge

Nickel is commonly present in municipal wastewater in trace concentrations. Results reported by Maeda and Azumi [106] indicate that nickel concentration in an activated sludge wastewater treatment plant varied between the detection limit and 0.08 mg g^{-1}(MLSS) (mixed liquid suspended solids), with average value and standard deviation 0.04 mg g^{-1}(MLSS) (same value). Gikas [12] measured the concentration of nickel in raw municipal wastewater and at the overflow after primary clarification as 0.038 and 0.025 mg L^{-1}, respectively. However, the above values are based on grab samples, and potentially they may not reflect the average nickel concentrations.

In small concentrations (usually below 5 mg L^{-1}), nickel has been found to enhance the growth of activated sludge and the organic carbon removal efficiency. Higher concentrations are usually having severe effects on the performance of the activated sludge systems, which can lead to complete containment to microbial growth.

Research carried out by Sujarittanonta and Sherrard [34], who worked with activated sludge growing in a continuous bioreactor set up, indicates that addition of 1 or 5 mg(Ni(II)) L^{-1}, enhanced, both, the maximum biomass yield (Y_m) and the maintenance coefficient (b) of activated sludge. They also reported that the values of the above parameters (Y_m and b) are a function of the inlet COD:Ni(II) concentration ratio (where chemical oxygen demand (COD) represents substrate). However, no significant effect to the COD removal efficiency due to the addition of nickel was recorded, while the nitrification process was significantly suppressed even by the addition of 1 mg(Ni(II)) L^{-1}, for low COD:Ni(II) ratios. They attributed the above effects either to the stimulatory of the microbial activity, or to changes in the microbial populations in activated sludge. The above researchers have proposed an empirical equation to predict the degree of nitrification in activated sludge at the presence of nickel. However, the validity of the proposed equation is questionable, as it is based only in data obtained using three COD:Ni(II)) ratios.

Table 1
Biokinetic parameters and mixed liquid suspended solids (MLSS) concentration for activated sludge growing in a continuous system, at different inlet Ni(II) concentrations (from Yetis and Gokcay [107])

Ni(II) inlet conc. (mg L^{-1})	μ_{max} (h^{-1})	K_s (mg L^{-1})	MLSS conc. (mg L^{-1})
0.0	0.31	85	400
5.0	0.67	97	950
10.0	0.32	105	600
25.0	Unstable operation		

Yetis and Gokcay [107], and Gokcay and Yetis [103], also studied the effects of Ni(II) addition on the performance of a continuous activated sludge system. The experimental results are summarized in Table 1. From Table 1, is obvious that addition of 5 mg(Ni(II)) L^{-1}, results in dupling of the MLSS concentration and in significant increase of the maximum growth rate (μ_{max}) with the increase of nickel concentration from nil to 5 mg L^{-1}, while further increase of the inlet Ni(II) concentration to 10 mg L^{-1}, resulted to a μ_{max} value close to the baseline value (zero Ni(II) concentration). Additionally, they noted improved sludge settling characteristics (compared to the blank) when the inlet Ni(II) concentration was 5 mg L^{-1}. They did not provide an explanation about the last observation, but this probably was due to the selective toxic effects of nickel to the growth of filamentous microorganisms (see reference [108]). Finally, they observed unstable growth when the inlet Ni(II) concentration was increased to 25 mg L^{-1}. However, despite the comprehensive analysis by the above researchers, they did not check the performance of the system at the reverse order (e.g.: starting with 10 mg(Ni(II)) L^{-1}, followed by 5 mg(Ni(II)) L^{-1}, and finally by nickel free feed). However, they effectively controlled wall growth, which can affect the microbial growth behavior in such types continuous systems [109].

Gikas [12] who studied the effects of Ni(II) on the maximum growth rate (μ_{max}) of activated sludge, growing in a modified batch system, reported growth stimulation for Ni(II) concentrations up to approximately 27 mg L^{-1}. Further increase of the Ni(II) concentration resulted to growth inhibition, while no growth (during the time of the experiment) was observed for Ni(II) concentrations higher that 160 mg L^{-1}. Maximum growth stimulation was achieved at approximately 10 mg(Ni(II)) L^{-1}, at which point the maximum specific growth rate (μ_{max}) was measured approximately 18.5% higher, compared with the baseline. The effects of Ni(II) concentration on activated sludge are graphically depicted in Fig. 3a and b. Gikas [12] also reported a significant increase in lag times for Ni(II) concentrations higher than 40 mg L^{-1}, while a small decrease in lag time (compared to the blank) was observed for Ni(II) concentration of 1 mg L^{-1}. Working with a similar system, but with activated sludge from a different source, Gikas and Romanos [110] reported increased activated sludge μ_{max} values for Ni(II) concentrations up to about 40 mg L^{-1}, while a weak growth was observed even at the presence of 320 mg(Ni(II)) L^{-1}. Batch systems have in general higher resistance in toxic loads, since they are not susceptive to washing out (like continuous suspended cell systems). Moreover, the above researcher used a modified batch growth system, with continuous aeration, which may be the reason for the relatively high nickel concentrations tolerated by the mixed population.

Arican and Yetis [111] examined the effect of increasing Ni(II) concentration on activated sludge growing in a "once-through completely mixed tank reactor", at a dilution rate 0.11 h^{-1}. They observed significant increase of the COD removal rate, MLSS concentration and observed biomass yield, with a stepwise rise of

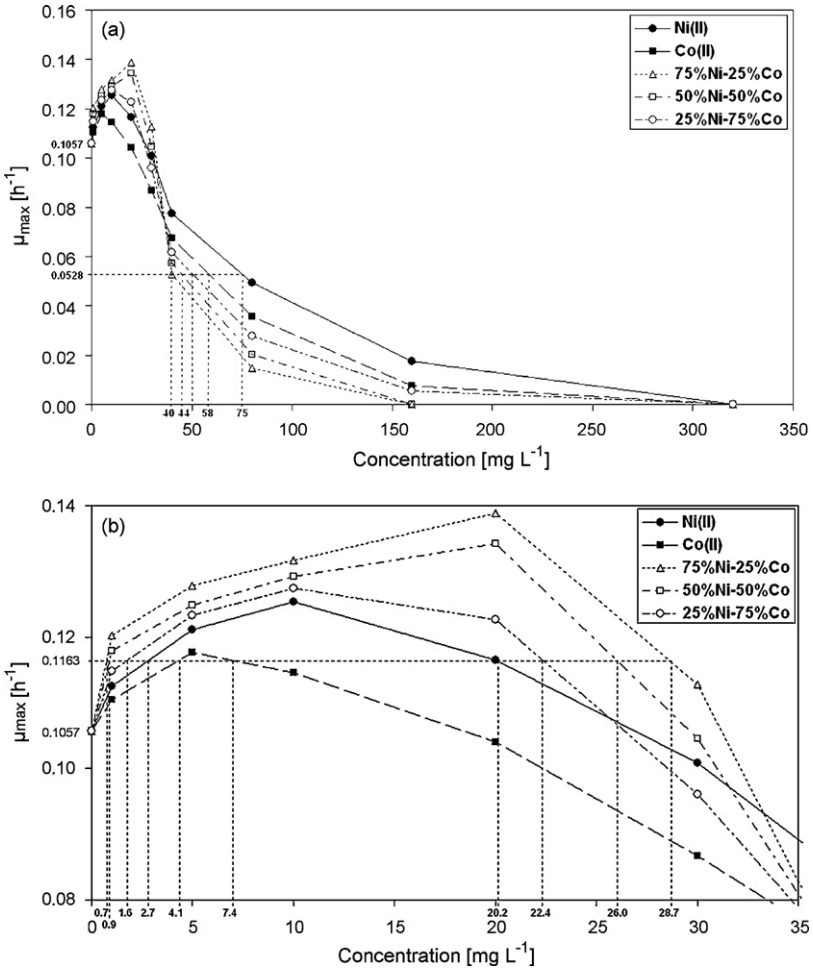

Fig. 3. (a) Calculated values of μ_{max} vs. the concentrations of Ni(II), Co(II) and mixtures: 1:3, 1:1 and 3:1, Ni(II):Co(II) (w/w); (b) detail for concentrations up to 35 mg L^{-1}. The equi-concentrations (which produce the same effects at each studied case) and the relative responses appear with small fonts at the *X*- and *Y*-axes (adopted from Gikas [12]).

Ni(II) concentration from 0 to 85.2 μmol L^{-1} (=5 mg L^{-1}). They also reported a significant increase in nickel sorption by the biomass, with the increase in the inlet Ni(II) concentration. The primary aim in the above research was the study of nickel sorption by activated sludge, and not the investigation of the effects of nickel concentration to the growth of activated sludge. The conclusions would have been more powerful if the reverse order of nickel concentrations were also contacted (from high to low nickel concentrations), to investigate if adaptation to higher nickel concentrations would alter the posterior effects of smaller nickel concentrations.

On the other hand, a number of researchers have reported only inhibition to the growth of activated sludge, due to the presence of Ni(II), as opposed to enhancement of growth at small nickel concentrations. Research carried out by McDermott et al. [112] indicates that a continuous mode activated sludge plant was able to withstand the addition of 1 mg(Ni(II)) L^{-1} in the feed solution, however, addition of 2.5, 5 or 10 mg L^{-1} of Ni(II) resulted to up to 5% reduction of the biochemical oxygen demand (BOD) removal efficiency. They also reported that a slug dose of 200 mg L^{-1} caused serious reduction to the treatment efficiency for a few hours, but within 40 h the system had returned to its normal performance.

Research performed by Ong et al. [108] indicated that the specific oxygen uptake rate (SOUR) of activated sludge, growing in a continuous system, reduced by 20%, 30% and 55%, compared to the baseline, with the acute addition of 10, 35 and 90–150 mg(Ni(II)) L^{-1}, respectively. The above researchers [108] also operated a sequence batch reactor (SBR) for activated sludge growth, reporting 23% and 22% reduction in the specific oxygen uptake rate and in the total organic carbon (TOC) removal rate, respectively, with the addition of 5 mg(Ni(II)) L^{-1} in the inlet steam. SOUR and TOC values, respectively, reduced by 57% and 51%, at 10 mg(Ni(II)) L^{-1}. In all cases, they reported complete recovery of the system with the suspension of the Ni(II) supply. Microscopic examination of the sludge indicated the elimination of filamentous microorganisms with the addition of Ni(II) (which appear to be more sensitive to the presence of nickel, compared with other microorganisms), which macroscopically reflected with improved sludge volume index (SVI) values. The main advance of the above experimental set up was the use of an identical reactor (without the use of nickel) which was operated in parallel with the primary reactor. The results are supported by electron microscopy, while the performance of the system was also checked after the termination of nickel dosing.

Wong et al. [113], who measured the respiration rate of activated sludge at the presence of nickel, reported a steady reduction from 0.218 to 0.105 mg(O_2) L^{-1} min^{-1}, with increasing Ni(II) concentration from 0 to 23 mg L^{-1} (which corresponds to 51.92% growth inhibition) (see Fig. 4). However, the main aim of the above research was the evaluation of a novel heavy metal toxicity methodology (they experimented with six heavy metal ions), rather than the study of the effects of nickel concentration to activated sludge. The EC_{50} values for activated sludge at the presence of Ni(II), have been

Table 2a
Effect of Ni(II) concentration to the biochemical oxidation (expressed as percentage of control BOD_5) of municipal wastewater (from Heukelekian and Gellman [114])

Time (days)	Nickel concentration (mg L^{-1})				
	0	5	10	25	50
1	57	8	8	8	5
1.25	63	23	11	8	5
2	80	62	48	8	5
3	90	70	61	8	5
4	96	74	65	8	5
5	100	76	68	8	5

Table 2b
Effect of Ni(II) concentration to the biochemical oxidation (expressed as percentage of control 22 h-BOD) of municipal wastewater, inoculated with 2 mg(MLSS) L^{-1} of activated sludge (from Heukelekian and Gellman [114])

Time (h)	Nickel concentration (mg L^{-1})					
	0	5	10	25	50	100
1	9	7	6	6	5	5
2	15	12	10	9	8	8
4	31	24	19	16	13	12
6	40	33	24	18	14	13
22	100	88	68	42	27	21

estimated by Kelly et al. [44]. They [44] reported EC_{50} value over 100 and 76 mg L^{-1}, by the use of bioluminescence and SOUR method, respectively. This discrepancy indicates the influence of the measuring procedure to the quantification of the toxic effects of heavy metals to microbial growth (similar variations were also reported by the same manuscript for other heavy metals).

Heukelekian and Gellman [114] monitored the effects of several heavy metals to the biodegradation of sewage, using a Warburg apparatus (manometric respirometer), which allowed the measurement of oxygen consumption with incubation time. The relative experimental results are summarized in Table 2a. They [114] also examined the effect of Ni(II) addition to a mixture of sewage and activated sludge. In the last case they observed microbial growth at significantly higher Ni(II) concentrations (see Table 2b). They attributed the above observed differences on the greater concentration of organisms and organic residues in the later experimental set up. Despite the fact that the above study conducted more than 50 years ago, it has examined the effects of nickel (as well as the effects of other heavy metals) to sewage microorganisms in a systematic way. The comparison between seeded and raw wastewater is particularly useful, indicating the role of microbial concentration on the microbial intoxication by heavy metals. More recently, the EC_{50} value of activated sludge, due to the addition of Ni(II), has been measured respirometrically as 33 mg(Ni(II)) L^{-1} [115]. However, the same research team has reported that the EC_{50} value of activated sludge is a strong function of the type of substrate, as it has been found to increase from 33 mg(Ni(II)) L^{-1} for growth on peptone-meat extract to 145 and 180 mg(Ni(II)) L^{-1} when starch-acetic acid or glucose are used as carbon sources, respectively [48]. Finally, Mowat [37], who measured the respiratory activity of activated sludge at the presence of 1, 5, 10 and 20 mg(Ni) L^{-1}, reported an approximate reduction by 22.6%, 44.6%, 57.3% and 62.7%, respectively.

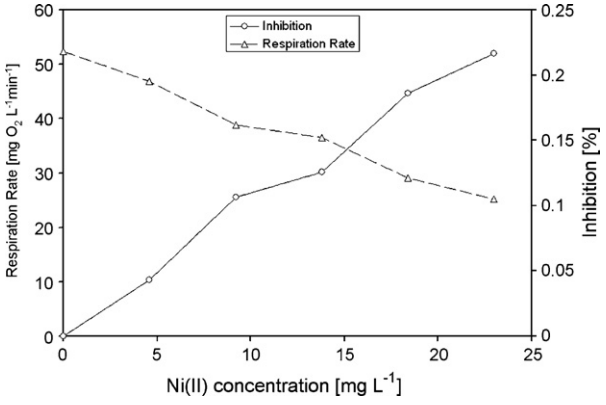

Fig. 4. The effect of nickel concentration on the respiration rate (mg O_2 L^{-1} min^{-1}) and to the percentage growth inhibition for activated sludge (Wong et al. [113]).

It is thus obvious that a number or published reports have identified nickel as an activated sludge stimulator (at relatively small concentrations), while a significant fraction of reports have solely identified nickel as activated sludge intoxicator. However, the last may be due to a failure to check the effects of even smaller doses of nickel to the examined activated sludge systems. The effects of nickel to activated sludge systems, as they have been reported by the above works, are summarized in Table 3.

Heavy metals have also been found to affect the growth of protozoan communities in activated sludge [116–118]. Nitrifying [119,120] and denitrifying [121] microorganisms are also particularly sensitive to the presence of heavy metals. Sujarittanonta and Sherrard [34] have reported that the nitrification processes is significantly more sensitive to the presence of nickel, compared to the biochemical oxidation of carbonaceous substrate (see above in this section). Ammonia nitrification takes place in two stages: ammonia oxidation to nitrite, followed by nitrite oxidation to nitrate. The first of the above processes is generally more sensitive to the presence of nickel [122,123]. 1 mM (=58.7 mg L^{-1}) of nickel applied on a mixed nitrifying culture caused 30% reduction in ammonia oxidation, with no significant inhibition to nitrite oxidation; however, the strength of inhibition has been found to be influenced by nickel speciation [122]. Exposure time to nickel also affects nitrification: nitrification inhibition increased from approximately 35% to 60–65% with the increase of exposure time to nickel from 1 to 8–25 h [124]. Growth mode has also been found to affect the first stage of nitrification (ammonia oxidation), thus 0.2 mM Ni(II) (=11.7 mg(Ni(II)) L^{-1}) caused 30% inhibition on ammonia oxidation when a mixed nitrifying microbial culture was growing in a batch system, while inhibition over 95% was occurred when the same culture was growing in a continuous system [123]. Adaptation of nitrifying microorganisms to nickel has also been reported, thus the EC_{50} value of nitrifying microorganisms has been doubled when previously exposed microbial populations were exposed to the same concentrations of nickel, compared with unexposed microorganisms [125]. Finally, denitrifying microorganisms have been found to be affected more severely compared to nitrifyiers. Lawrence et al. have reported that 0.5 mg(Ni(II)) L^{-1} inhibited nitrification by a mixed microbial population, with no apparent effects on the nitrification process [50].

2.2. Effects of Ni(II) on particular aerobic microorganisms

A number of reports are dealing with the estimation of the minimum inhibition concentration (MIC) (MIC is defined as the lowest concentration of metal at which no colony forming units (CFU) are observed), of Ni(II) on various microorganisms, utilizing a range of assays. However, the effects of Ni(II) concentration on microbial growth (and consequently, the reported MIC values) are often apparently affected by the type of the assay used. Codina et al. [45] investigated the effects of Ni(II) to *P. fluorescens* growing either in a buffer solution or in raw sewage, using both a spectrophtometric test and the commercial toxicity test Microtox® (based on microbial bioluminescence). They reported the EC_{50} values for the spectrophotometric assay as 763 and 1301.2 mg(Ni(II)) L^{-1}, in buffer solution and in wastewater, respectively. For the Microtox®

Table 3
Effects of nickel to various activated sludge systems

Reference	Type of growth	Effects of nickel to activated sludge
Arican and Yetis [111]	Continuous	Addition of 5 mg(Ni(II)) L^{-1} results to increase in COD removal rate, MLSS concentration and observed biomass yield
Cokgol et al. [48]	Batch	Growth inhibition, EC_{50} value depends on type of substrate: 33 mg(Ni(II)) L^{-1} for growth on peptone-meat extract, 180 mg(Ni(II)) L^{-1} for growth on glucose, 145 mg(Ni(II)) L^{-1} for growth on starch-acetic acid
Gikas [12]	Batch, continuously aerated	Growth stimulation up to 27 mg(Ni(II)) L^{-1} (maximum stimulation at approximately 10 mg L^{-1}), no growth at concentrations above 160 mg(Ni(II)) L^{-1}, progressive increase of lag time above 40 mg(Ni(II)) L^{-1}
Gikas and Romanos [110]	Batch, continuously aerated	Growth stimulation up to 40 mg(Ni(II)) L^{-1}, vestigial growth was observed even at 320 mg(Ni(II)) L^{-1}
Guclu et al. [115]	Batch	Growth inhibition with EC_{50}: 33 mg (Ni(II)) L^{-1}
Heukelekian and Gellman [114]	Batch	Raw sewage: no growth above 25 (Ni(II)) mg L^{-1}, raw sewage plus activated sludge: remarkable growth even at 100 (Ni(II)) mg L^{-1}
McDermontt et al. [112]	Continuous	Wastewater treatment plant performance: not affected with the addition of 1 mg(Ni(II)) L^{-1}, severely affected with the addition of 2.5–10 mg(Ni(II)) L^{-1}
Mowat [37]	Batch	1–20 mg(Ni(II)) L^{-1} resulted to reduction in respiration rate by 22.6–62.7%, respectively
Ong et al. [108]	Continuous	Progressive reduction of the SOUR with the addition of Ni(II) (up to 55% reduction at the presence of 90–150 mg(Ni(II)) L^{-1}.
Sujarittanonta and Sherrard [34]	Continuous	1–5 mg(Ni(II)) L^{-1} improved the maximum biomass yield and the maintenance coefficient. No significant effect to COD removal efficiency
Wong et al. [113]	Batch	51.92% reduction of the respiration rate with the addition of 23 mg L^{-1}
Yetis and Gokcay [107]	Continuous	Duplication of μ_{max} with the addition of 5 mg(Ni(II)) L^{-1} μ_{max} was not affected with the addition of 10 mg(Ni(II)) L^{-1}, unstable growth with the addition of 25 mg(Ni(II)) L^{-1}

assay, they reported 55.2 and 170.8 mg(Ni(II)) L^{-1}, in buffer solution and in wastewater, respectively. It is thus obvious, that in the above case the measured nickel toxicity is a strong function of the type of the assay.

The minimum inhibition concentration for *Escherichia coli* has been reported as 1 mM (=58.7 mg L^{-1}) [79], and by other researchers as 0.2 mM (=11.74 mg L^{-1}) [126]. Experimental results reported by Babich et al. [127] on the same microorganism (*E. coli*), indicate unobstructed growth at 5 mg(Ni(II)) L^{-1}, significant growth reduction at 10–20 mg(Ni(II)) L^{-1} and no growth at 40 mg(Ni(II)) L^{-1}. Studies on the effects of several heavy metals on *Bacillus* sp. growing on toluene [128], indicated 55–62% growth rate reduction for Ni(II) concentrations between 0.4 and 0.8 mM (=23.5–47 mg L^{-1}), while no growth was observed for Ni(II) concentrations over 1 mM (=58.7 mg L^{-1}). Experiments carried out on batch cultures of *Klebsiella pneumoniae* [41], indicated that increase of the Ni(II) concentration results, both, to prolonged lag times, and to reduced MLSS concentrations (MLSS dropped from 41.2 to 15.9 mg L^{-1} when the Ni(II) concentration in the growth medium increased from 300 to 500 mg L^{-1}). The same researchers [41], also reported 50% reduction in microorganism survival, by the use of viability counts on agar plates (at 6.65 mg(Ni(II)) L^{-1}). The above work is particularly interesting as it demonstrates the effect of the type of the growth media to nickel-induced toxicity. According to Visca et al. [129], small concentrations of nickel stimulate the growth of *Pseudomonas aeruginosa* (10 μM(Ni(II)) (≅0.6 mgNi(II) L^{-1}) reduced the generation time of the microorganism by 12%). Babich and Stotzky [54], 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 [130] have measured the nickel concentrations for incipient growth and for complete growth containment for a number of filamentous fungus, eubacteria, actinomycetes and yeasts. The growth of the fungus *Monoascus ruber* was reduced by 50% with the addition of 10 mg(Ni(II)) L^{-1}, while no growth was observed at approximately 50 mg(Ni(II)) L^{-1} [131]. Cobet et al. [132] reported that 0.1 mM of $NiCl_2$ (=5.9 mg(Ni(II)) L^{-1}), slightly affected the growth of the marine bacterium *Arthrobacter marinus*, some growth inhibition was occurred at 0.4 mM (=23.5 mg(Ni(II)) L^{-1}), while no growth was observed at 0.5 mM (=29.3 mg(Ni(II)) L^{-1}). They [132] also reported increase of the lag phase with the rise of Ni(II) concentration (from 3 to over 70 h, at 0.4 mM(Ni(II))), and transfiguration of the cells into megalomorphic 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) (=23.5 mg(Ni(II)) L^{-1})).

Nickel has been identified as a stimulant to the growth of a number of cyanobacteria and blue-green algae. Growth stimulation has been observed for the alga *Scenedesmus abliquus* and for the diatom *Nitzschia perminuta* at concentrations up to 1 and 2 mg(Ni(II)) L^{-1}, respectively [133]; however, the growth was inhibited with further increase of Ni(II) concentration [133]. Angadi and Mathad [134] reported that 0.1 mg(Ni(II)) L^{-1} stimulated, the growth (OD), percentage survival (viability), chlorophyll-*a* and -*b* production, and DNA, RNA and protein content of the green algae *Scenedesmus quadricauda*, while 0.2 and 0.4 mg(Ni(II)) L^{-1} were also stimulated the growth and percentage survival (but not other parameters) of the above microorganism; however, all the measured parameters were inhibited at higher nickel concentrations. Rai and Raizada [135] reported that the growth, nitrogenase activity, and the CO_2 uptake rate of the cyanobacteria *Nostoc muscorum* were stimulated at the presence of 0.01, 0.025 and 0.05 mg(Ni(II)) L^{-1} (maximum growth stimulation (43%) occurred at 0.0105 mg(Ni(II)) L^{-1}); however, growth inhibition was occurred at higher nickel concentrations [136].

On the other hand, some experimental works report just algal growth inhibition by the addition of even small amounts of nickel. Thus, the growth of the green algae *Chlorella pyrenoidosa* 251, was not affected by the addition of 0.5 mg(Ni(II)) L^{-1}, while nickel concentrations above 1 mg(Ni(II)) L^{-1} resulted to growth reduction [137]. Asthana et al. [138] found that the increase of the nickel concentration from 0 to 5 and 10 μM (=0.3 and 0.6 mg L^{-1}), resulted to a small, but progressive, reduction of the growth of the cyanobacterium *N. muscorum* ISU, while the growth was severely affected by 15 μM(Ni(II)) (=0.9 mg(Ni(II)) L^{-1}).

2.3. Ni(II) tolerant microorganisms

Nickel tolerant microorganisms have been isolated from nickel polluted sites, or from natural sites lying close to nickel mineral

deposits. Alternatively, nickel-tolerant strains have been "created" by the transfer of nickel tolerant genes encountered in plasmoidal DNA. According to Duxbury [139], a bacterial strain may be characterized as nickel tolerant, if it is able to grow at Ni(II) concentrations higher than 1.70 mM (≅100 mg L^{-1}).

Wastewater treatment plants, treating industrial effluents from the metal processing industry, are places where nickel tolerant species may be encountered. Schmidt and Schlegel [140] have isolated a bacterial strain from such a plant, capable of growing at $NiCl_2$ concentrations up to 20 mM (1174 = mg(Ni(II)) L^{-1}). They [140] also applied the same isolation protocol to ordinary soil, however, no bacterial strains were able to grow in media containing more than 1 mM Ni(II) (=58.71 mg(Ni(II)) L^{-1}). Genetically engineered strains of *Alcaligenes eutrophus* have been reported to grow at the presence of 1 mM Ni(II) (=58.71 mg(Ni(II)) L^{-1}) [141]. Otth et al. [142] 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}). The MIC of heterotrophically grown *A. eutrophus* CH34 has been experimentally estimated to be 2.5 mM (=147 mg L^{-1}) [143]. The wild strains of the fungus *Aspergillus niger* can tolerate nickel at concentrations up to 0.2–1.6 mM (=11.7–93.9 mg L^{-1}); however, an isolate from metal contaminated soil was able to grow at 6.5 mM Ni(II) (=381.5 mg L^{-1}) [144]. A nickel-tolerant strain of *E. coli* (strain V48) has been isolated from the municipal wastewater treatment plan of Vilnius [145]. The MIC for the above strain was measured as 5 mM Ni(II) (=293.5 mg (Ni(II)) L^{-1}), which, according to the authors, is 50 times greater than the MIC of the nickel-non-tolerant strain of *E. coli* JM101. Kaur et al. [146] have isolated, from an anaerobic digester, the aerobic microorganism *Alcaligenes denitrificans* strain (4a-2), which is able to grow heterotrophically at Ni(II) concentrations up to 20 mM (=1174 mg L^{-1}).

Natural ecosystems rich in nickel, like serpentine soils, are often home to exceptionally nickel tolerant microorganisms, as these microorganisms have been acclimatized to grow at high nickel concentrations for centuries or more. Pal et al. [147] have isolated from nickel-rich serpentine soil, from the Andaman islands, *Pseudomonas* sp. with MIC greater than 400 mg(Ni(II)) L^{-1}. *Pseudomonas* sp., from serpentine soils of central Italy, able to grow at 10 mM(Ni(II)) (=587 mg(Ni(II)) L^{-1}) have also been isolated [148]. Nickel-tolerant strains, isolated from the vicinity of the roots of nickel-accumulating plants, in serpentine soils from New Caledonia, are able to grow at the presence of 20 mM(Ni(II)) (=1174 mg (Ni(II)) L^{-1}) [149]. Research by Hashem and Bahkali [150] indicated that a strain of the fungus *Fusarium solani* (isolated from Saudi Arabian soil), was able to grow at the presence of up to 300 mg(Ni(II)) L^{-1}. Schmidt et al. [151], and Stoppel and Schlegel [152] reported that the bacterial strains *Alcaligenes xylosoxydans* 31A and *A. eutrophus* KT02, which were isolated from ecosystems heavily polluted with heavy metals, are able to grow at Ni(II) concentrations up to 50 mM (=2935 mg L^{-1}). Finally, Congeevaram et al. [153] have reported an approximately linear reduction of biomass concentration for *Aspergillus* and *Micrococcus* species with the increase of Ni(II) concentration; while both species were able to grow even at the presence of 500 mg (Ni(II)) L^{-1}.

The phenomenon of adaptation to nickel has been studied by Thomas et al. [154] who exposed *P. aeruginosa* and *Pseudomonas putida* to various concentrations of Ni(II). They found that, both, lag time and cell doubling time were decreased with the adaptation of *P. aeruginosa*, while no difference was observed for *P. putida*, which is obviously more tolerant to nickel (see Table 4).

Genetic manipulation has also been practiced to "create" nickel tolerant species. Thus, *E. coli* has been genetically transformed to grow at 100 times higher Ni(II) concentration, compared to the wild strain [155].

2.4. Effects of Ni(II) on the chemolithotrophic growth of microorganisms

Nickel has been identified as a trace element in the chemolithotrophic growth of microorganisms, which is reported in the present manuscript separately, as it is a distinct case of aerobic growth. More specifically, nickel catalyzes the synthesis of hydrogenase [156], while in some autotrophic bacteria it has been identified as a constituent of the hydrogenase in both the cytoplasmic NAD-reducing and in the membrane-bound forms [157,158].

Two *Hydrogenomonas* strains (H1 and H16) have been found to depend on nickel for chemolithotrophic growth (estimated optimum nickel concentration is 3×10^{-7} M (=18×10^{-6} mg L^{-1}) [104] Similarly, *A. eutrophus* [105], *Xantobacter autotrophicus* and *P. flava* [87] are also unable to grow chemolithotrophicaly at the absence of nickel. Moreover, it was found that the growth of *A. eutrophus* was increased eight times by the addition of 7.5×10^{-7} M(Ni(II)) (=44×10^{-6} mg(Ni(II)) L^{-1}) [159]. However, growth inhibition was occurred above 1×10^{-6} M (=58.7×10^{-6} mg L^{-1}) [159].

A number of works have been published on genetically manipulated chemolithotrophic microbial species, to enhance nickel tolerance, particularly on *A. eutrophus* ([82,160]).

2.5. Combined effects of Ni(II) with other environmental factors on aerobic microorganisms

The pH of the growth media affects the speciation of nickel, as Ni^{2+} forms complexes with OH^-, according to the sequence:

$$Ni^{2+} \xrightarrow{OH^-} NiOH^+ \xrightarrow{OH^-} Ni(OH)_2 \xrightarrow{OH^-} Ni(OH)_3^- \xrightarrow{OH^-} Ni(OH)_4^{2-}$$

However, the hydroxylated species of nickel are formed in appreciable amounts only at pH above 9.5 [74]. Consequently, the decrease of nickel toxicity with the increase in pH cannot attributed to the formation of those species. It is possible that this behavior can be attributed to a more efficient competition between Ni^{2+} and H^+ for binding sites of nitrogenous organics in the growth media, thus, at relatively higher pH (lower H^+ concentration) nickel complexes with soluble nitrogenous organics to form less toxic compounds compared to free Ni^{2+} [161].

Babich and Stotzky [130] who quantified the toxicity of nickel to a number of microorganisms found that, for all the examined microbial strains, nickel toxicity was potentiated with the reduction of pH. The same researchers have reported reduction of nickel toxicity at increased pH values for the microorganisms *Penicillium vermiculatum*, *Rizopus stolonifer*, *Trichoderma viride* and *Gliocladium* sp. [162]. The growth of the yeast *Saccharomyces cerevisiae* B11842, is also significantly reduced at pH lower than 5, if nickel is present [163]. Finally, in situ reduction of pH (from 6.8 to 5.3) to the water of a lake has been found to increase the toxicity of 75 mg(Ni(II)) L^{-1} to the aquatic microorganisms *Seratia marcescens* and *Nocardia rhodochrous* [164].

The opposite behavior has been reported by Gimmler et al. [165] who found that the growth of the acidotolerant filamentous fungus *Bispora* sp. was inhibited by 50% at the presence of 2×10^{-5} M(Ni(II)) (=1.17 mg(Ni(II) L^{-1}), at neutral pH (=7.0), while this fungus was growing with no signs of inhibition at Ni(II) concentrations higher than 3×10^{-3} M (=176.1 mg L^{-1}), at pH 1. The growth of the metal resistant bacterium *Burkholderia cepacia* has been found to decrease with pH at the presence of nickel [166,167]. On the other hand, the same research team [167] has reported that pH changes did not affect the growth of the metal tolerant species *Ralstonia metallidu-*

Table 4
The effect of adaptation to either nickel or cobalt, to lag and to doubling time for *Pseudomonas aeruginosa* and for *Pseudomonas putida*

Type of adaptation (metal concentration: 5 mmol L^{-1})	*Pseudomonas aeruginosa*		*Pseudomonas putida*	
	Lag time (h)	Doubling time (h)	Lag time (h)	Doubling time (h)
Cells non-adapted to Ni(II)	40	31	6	6
Cells adapted to Ni(II)	20	16	6	6
Cells non-adapted to Co(II)	47	55	75	10
Cells adapted to Co(II)	22	47	20	24

The above data indicate that the behavior of the system depends, both, on the type of the heavy metal and on the type of microorganisms (from Thomas et al. [154]).

rans, at the presence of Ni(II), which implies that induced changes in nickel toxicity by pH alterations is a microorganism-dependant property.

Synthetic chelators, such as ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA), and natural chelators, such as citrate, aspartate and 2,6-pyridinedicarboxylic acid (PDA) bind with free nickel, thus reducing or eliminating the toxicity of nickel to microorganisms [41,49,50,161,168]. Lee and Lustigman [49] investigated the combined effects of nickel and EDTA on the cyanobacteria *Anacystis nidulans*; 10 mg(Ni(II)) L^{-1} severely inhibited the growth of the above microorganism, while the toxic effects of nickel were almost lifted by the addition of 0.1% (w/v) EDTA, due to the binding of free Ni(II) with EDTA. However, the toxic effects of 25 mg(Ni(II)) L^{-1} could not be reversed by EDTA addition. Similarly, Rai and Raizada [168] concluded that the toxic effects of nickel to the cyanobacterium *N. muscorum* reduced by the addition of either EDTA or calcium, with respect to survival (viable counts), growth and carbon fixation. They also reported that nickel concentrations up to 2.1 μM (=0.12 mg L^{-1}) stimulated both carbon fixation and nitrogenase production. Ainsworth et al. [41] found that addition of either, EDTA, aspartate or citrate ameliorated the toxic effects of nickel to the growth and viability of *K. pneumoniae*, while a similar effect to *N. rhodochrous* by the addition of either EDTA, PDA or NTA has been reported by Babich and Stotzky [161].

The hydrostatic pressure has been found to increase the nickel toxicity to marine microorganisms [169]. However, other reports have indicated a neutral effect [170].

Simultaneous interactions between more than one heavy metals with the microorganisms may have either synergic, antagonistic or additive effects (see Section 1.1). The mechanism of such interactions may be particularly complex, and unique, depending on the combinations of heavy metals and microbial strains. Babich and Stotzky [54] reported synergic effect among nickel and copper to the growth of a number of heterotrophic microorganisms. On the other hand, Mg(II) or Fe(III) have been found to act antagonistically with Ni(II) to the growth of the fungus *A. niger* [171]. Synergism between nickel and either copper or hexa-valent chromium, with respect to growth and chlorophyll-*a* synthesis by the algae *C. pyrenoidosa* 251, has been reported by Wong and Chang [136]. Zinc, lead and cadmium have been found to antagonize nickel with respect to the toxic effects to *Saccharomycopsis lipolytica* [172], *Saprolegnia* sp. [173], *Achyla* sp. [173], and *Ankistrodesmus falcatus* [56]. Finally, synergism between the antibiotic erythromycin and nickel on the growth of a number Gram-negative and Gram-positive microorganisms has been reported by Sultana et al. [174].

3. Effects of Co(II)

The effects of cobalt on microbial growth have been studied to a less extent, compared with relative studies on the effects of nickel. Even less work has been contacted on the effects of cobalt on activated sludge systems, possibly because the industrial applications of cobalt are not as wide as those of nickel.

3.1. Effects of Co(II) on activated sludge

The theoretically optimum cobalt concentration for unrestricted performance of activated sludge systems has been estimated to lie between 20×10^{-3} and 50×10^{-3} mg L^{-1} [6]. Maeda and Azumi [106] reported that cobalt concentration in an activated sludge treatment plant varied between the detection limit and 0.14 mg(Co) g^{-1} (MLSS) (mixed liquid suspended solids), with average value and standard deviation of 0.03 and 0.05 mg(Co) g^{-1} (MLSS), respectively. The cobalt concentration values reported by Wood and Tchobanoglous [6] and Maeda and Azumi [106] are in good agreement (based on the MLSS values reported by Maeda and Azumi [106], the average per volume cobalt concentration is calculated as 34×10^{-3} mg L^{-1}). The concentration of cobalt at the raw wastewater and at the overflow of the primary clarifier were measured, in grab samples, by Gikas [12] as 11×10^{-3} and 9×10^{-3} mg L^{-1}, respectively; which indicates that only a small fraction of cobalt is removed by primary clarification.

Graywater often lacks nutrients, thus addition of trace elements may be needed for the removal of the organic carbon with biological means. COD removal rate in graywater has been enhanced by 30% with the addition of 5 mg(Co(II)) L^{-1} [8].

Trace amounts of cobalt have been reported to stimulate the biodegradation process of municipal wastewater. The synthesis of vitamin B12 has been found to increase by 50% with the addition of 1 mg(Co(II)) L^{-1} in activated sludge systems [102]; however, the observed increase in vitamin B12 production was not accompanied by increase in the COD or BOD removal rates. Co(II) concentrations higher than 3 mg L^{-1} resulted to reduced microbial activity, however, growth was sustained even at the presence of 50 mg(Co(II)) L^{-1} [102].

The respiration rate of activated sludge has been found to increase with the addition of 1 mg(Co(II)) L^{-1}, accompanied by a slight reduction of the COD removal rate [7]. Moreover, addition of 5 mg(Co(II)) L^{-1} in a batch activated sludge system has been found to increase the COD removal rate by 30% [175].

Gikas [12] who studied the effects of a range of cobalt concentrations on activated sludge growing in a batch system, reported increase of the maximum specific growth rate (μ_{max}) for Co(II) concentrations up to approximately 19 mg L^{-1}, with maximum stimulation at 5 mg L^{-1}. However, further increase in Co(II) concentration resulted to gradual decrease in growth rate, while complete elimination of the growth was occurred at cobalt concentrations over 160 mg L^{-1} (Fig. 3a and b). According to Gikas [12], μ_{max} was measured 11.3% above the baseline, at the maximum growth stimulation induced by Co(II). The same researcher has also reported a significant increase in lag times for growth at Co(II) concentrations over 40 mg L^{-1} [12].

Some studies have reported only inhibition to the growth of activated sludge at the presence of cobalt. Mowat [37] reported reduction of the respiratory activity of activated sludge by approximately 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. Significant increase of the initial retardation time, during the biological oxi-

Table 5a
Effect of Co(II) concentration to the biochemical oxidation (expressed as percentage of control BOD_5) of municipal wastewater (from Heukelekian and Gellman [114])

Time (days)	Cobalt concentration (mg L^{-1})				
	0	5	10	25	50
0.75	48	18	8	4	5
1	58	30	17	5	5
2	80	56	47	8	5
3	89	64	56	25	5
4	95	70	60	42	5
5	100	76	64	50	6

Table 5b
Effect of Co(II) concentration to the biochemical oxidation (expressed as percentage of control 23 h-BOD) of municipal wastewater, inoculated with 2 mg(MLSS) L^{-1} of activated sludge (from Heukelekian and Gellman [114])

Time (h)	Cobalt concentration (mg L^{-1})					
	0	5	10	25	50	100
1	12	11	11	10	9	7
4	33	31	31	27	22	16
6	46	43	42	36	29	24
8	59	54	53	45	36	24
12	73	68	67	60	50	34
23	100	89	89	80	72	55

dation of raw sewage at the presence of 5–25 mg(Co(II)) L^{-1} has been reported by Heukelekian and Gellman [114], while growth was not sustained at 50 mg(Co(II)) L^{-1} (Table 5a). Addition of activated sludge to the raw sewage resulted to a significant increase of the overall microbial activity, eliminating the intoxication effects due to the presence of cobalt (Table 5b).

Based on the majority of the references quoted above, cobalt is likely to act as activated sludge growth stimulant, at trace concentrations. Table 6 summarizes the effects of cobalt to the various activated sludge systems, reported above.

To the knowledge of the author, the effects of cobalt to the activated sludge nitrification and denitrification processes have not been widely assessed by the international literature.

3.2. Effects of Co(II) on particular aerobic microorganisms

A number of reports indicate microbial growth stimulation with the addition of small amounts of cobalt, which is in agreement with the biological role of cobalt (see Section 1.4). 0.1 mg($CoCl_2$) L^{-1} (=0.062 mg(Co(II)) L^{-1}) stimulated the maximum specific growth rate (μ_{max}) and biomass yield coefficient (Y_b) of an aerobic bacterium isolated from activated sludge (strain CIP I-2052), by 78% and 200%, respectively [176], while growth was contained at 0.5 mg L^{-1} (=0.312 mg(Co(II)) L^{-1}). The growth of *Streptomyces coelicolor* increased by approximately 50% with the addition of 300 mg (Co(II)) L^{-1}, however, at this cobalt concentration the production of antibiotic (actinorhodin), by the fungi, was not sustained [177]. Falih [178], who studied the effects of cobalt to the growth of *Phanerochaete chrysosporium*, concluded that the growth (biomass production) of the above fungi was significantly stimulated by the presence of 100 mg(Co(II)) L^{-1}, while addition of 200 mg(Co(II)) L^{-1} resulted to growth inhibition. Research carried out by Visca et al. [129], indicated that 0.01 mM(Co(II) (=0.59 mg(Co(II)) L^{-1}) increased the growth of *P. aeruginosa* by 21%. Finally, Sayed et al. [179] reported stimulation to the growth of an actinobacteria species (*Frankia* sp.), by selected concentrations of cobalt.

The stimulant effects of cobalt in a batch system may not be obvious right after inoculation. Thus, for some time after inoculation the blank may exhibit higher growth rates, while later on, the cobalt supplemented culture may take over. This has been demonstrated by Barabasz et al. [180] who reported growth stimulation of *E. coli* with the addition of 5 or 25 mg(Co(II)) L^{-1} after 3–4 days of incubation.

Growth stimulation by cobalt has been also reported for algae and diatoms: Graneli and Risinger [181] found that cobalt is essential for the growth of the dinoflagelated algae *Chrysochromulina polylepis*, reporting that the final number of the cells, during batch cultivation, increased when cobalt was present at concentrations between 1 and 3 nM Co(II) (=58.9×10^{-6}–76.7×10^{-6} mg(Co(II) L^{-1}). Both, the growth of the green alga *S. abliquus* and of the diatom *N. perminuta* were stimulated by 1 and 1.5 mg(Co(II)) L^{-1}, respectively [133], however, further increase of the Co(II) concentration resulted, in both cases, to growth inhibition. Poskuta [182], reported that 20 mM (=1178 mg L^{-1}) of Co(II) stimulated, both, the respiration rate and the rate of photosynthesis of the green alga *C. pyrenoidosa*. Finally, El-Naggar et al. [183] found that 0.01 mg(Co(II)) L^{-1} stimulated, by approximately 20%, the growth of the cyanobacterium *N. muscorum*, while 0.15 mg(Co(II)) L^{-1} resulted to approximately 69% growth reduction.

On the other hand, Co(II) has been solely identified as growth inhibitor by many experimental studies. Increased lag time and deceased final MLSS concentration has been reported for *K. pneumoniae*, with the increase of Co(II) concentration in the growth medium [41]. 5 mg(Co(II)) L^{-1} slightly affected the growth of *Zoogloea ramigera*, 10 mg(Co(II)) L^{-1} resulted to significant increase of lag time with parallel reduction of the MLSS concentration, while no growth was observed at 50 mg(Co(II)) L^{-1} [184]. Chen et al. [185] 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(II)) L^{-1}, respectively. The growth of *B. cepacia* $PR1_{301}$, is inhibited by 68.7% and 80.5% at pH 5.0 and 6.0, respectively, at the presence of 4.25 mg(Co(II)) L^{-1}, while no growth is sustained at pH 7.0 [167]. The MIC for *E. coli* has been measured as 1 mM (=58.9 mg L^{-1}) [79]. Guha and Mookerjee [186] who experimented with the same microorganism, found that 300 μM(Co(II)) (=17.7 mg(Co(II)) L^{-1}) resulted to a 2.5 times fold slow down of the synthesis of both, messenger RNA and stable RNA, which implies that, both, the process of transcription and the process of translation are affected by the presence of cobalt. The growth of *Thiobacillus ferooxidans* is not affected by cobalt concentrations up to 0.1 M (=5.9 mg L^{-1}), while it is slightly inhibited at 0.25 M(Co(II)) (=14.7 mg(Co(II)) L^{-1}) [187]. The lag phase of *P. aeruginosa*, was doubled with the introduction of 20 mg(Co(II)) L^{-1}, while no growth was observed over 80 mg(Co(II)) L^{-1} [188]. Thomas et al. [154] reported that prolonged exposure to cobalt resulted to the reduction of lag time for *P. aeruginosa* and for *P. putida* (see Table 4).

Lee et al. [189] measured the growth of the unicellular cyanobacterium *A. nidulans*, using both direct counts and a hemocytometer. They observed complete growth reduction above 30 mg(Co(II)) L^{-1}, while smaller concentrations resulted to a relatively prolonged lag phase, compared to the baseline. A remarkably good agreement between the two microbial growth measuring methods (direct counts and hemocytometry) was observed. 50% reduction on the growth rate of *S. quadricauda* at 4.49 mg(Co(II)) L^{-1} has been reported [190]. 10 mg(Co(II)) L^{-1} resulted to slight decrease of the growth of the green algae *Chlamidomonas reinhardtii*, while no growth occurred at 30 mg(Co(II)) L^{-1} [191]. Kostiaev [192] based on measurements of the rate of photosynthesis, concluded that cells at the beginning of the exponential growth phase were more sensitive to cobalt, compared with cells towards the end of exponential growth phase.

Research carried out by Adiga et al. [171] indicated that approximately 110 mg(Co(II)) L^{-1} reduced by 50% the growth and the glucose consumption of the fungi *A. niger*. Al-Sarrani [131], reported

Table 6
Effects of cobalt to various activated sludge systems

Reference	Type of growth	Effects of cobalt to activated sludge
Burgess et al. [7]	Batch	1 mg(Co(II)) L^{-1} increases of the respiration rate, with parallel slight decrease of the COD removal rate
Fang et al. [175]	Batch	5 mg(Co(II)) L^{-1} increases by 30% the COD removal rate
Gikas [12]	Batch, continuously aerated	Growth stimulation up to 19 mg(Co(II)) L^{-1} (maximum stimulation at approximately 5 mg L^{-1}) No growth at concentrations above 160 mg(Co(II)) L^{-1} Progressive increase of lag time above 40 mg(Co(II)) L^{-1}
Heukelekian and Gellman [114]	Batch	Raw sewage: slight growth inhibition up to 25 mg(Co(II)) L^{-1}, no growth above 50 mg(Co(II)) L^{-1} Raw sewage plus activated sludge: remarkable growth even at 100 mg(Co(II)) L^{-1}
Jefferson et al. [8]	Batch	Graywater: 30% increase in COD removal rate with the addition of 5 mg(Co(II)) L^{-1}
Kelly et al. [44]	Batch	EC_{50} = 100 mg(Co(II)) L^{-1} (based on bioluminescence measurements) EC_{50} = 76 mg(Co(II)) L^{-1} (based on SOUR measurements)
Mowat [37]	Batch	1–20 mg(Co(II)) L^{-1} resulted to reduction in respiration rate by 16.2–58.2%, respectively
Sathyanarayana Rao and Srinath [102]	Batch	1 mg(Co(II)) L^{-1} resulted to increase in vitamin B12 synthesis by approximately 50%, with no effect on the COD and BOD removal rates Higher cobalt concentrations stimulate the production of vitamin B12, but severely affect the microbial activity. Limited growth even at 50 mg(Co(II)) L^{-1}
Wood and Tchobanoglous [6]	–	Theoretical calculation: 20×10^{-3}–50×10^{-3} mg(Co(II)) L^{-1} are demanded for unrestricted growth

that the growth of the fungus *Monoascus rubber* was inhibited by approximately 40% at the presence of 10 mg(Co(II)) L^{-1}, while no growth was observed at 100–200 mg(Co(II)) L^{-1}. Finally, the growth of the soil bacterium *Rhizobium* GN1 reduced by 70% at 20 μM(Co(II)) (=1.18 mg(Co(II)) L^{-1}) [193].

The MIC (minimum inhibition concentration) of cobalt for several strains of *Pseudomonas* sp. and *Proteus* sp. have been found to lie between 0.4 to 0.8 mM (=23.5–47 mg L^{-1}), while for *E. coli* it has been measured as 0.2 mM (=11.8 mg L^{-1}) [194]. The same research team [195] reported that the cell production rate of *P. aeruginosa* was marginally affected by the presence of cobalt at concentrations up to 1 mM (=5.9 mg L^{-1}); while just 0.1 mM (=0.6 mg L^{-1}) of cobalt reduced the synthesis of proteins by approximately 24%. Venkateswerlu and Stotzky [196] found that cobalt affected the cell wall composition of the fungus *Cunninghamella blakesleeana*, while the MIC for the above strain was measured as 3.5 mg(Co(II)) L^{-1}.

3.3. Co tolerant microorganisms

Cobalt resistant microbial strains have been isolated from, both, naturally and anthropogenically cobalt contaminated sites. The bacterial strains *A. xylosoxydans* 31A and *A. eutrophus* KT02, isolated from metal contaminated sites, have been found to grow at the presence of up to 20 mM Co(II) (=1178 mg(Co(II)) L^{-1}) [151,152]. Similarly, Sajani and Maruthi Mohan [197] obtained a cobalt-tolerant strain of the fungus *Neurospora crassa*, by repeated sub-cultivations of the wild type on cobalt-containing agar medium. The MIC of the mutant was measured as 8 mM (=471.2 mg L^{-1}), 20 times higher that the one of the wild strain. 80% of the cobalt in the culture containing the mutant cells was bound on proteins, while this fraction in the wild strain culture was approximately 25% [198]; indicating that cobalt is part of the proteinic structure of the cobalt tolerant cells. Hashem and Bahkali [150] reported that the fungus *F. solani*, which was isolated from soil in the Arabian peninsula, was capable to grow at Co(II) concentrations above 300 mg L^{-1}.

Mergeay et al. [199], isolated from a zinc processing factory a bacterial strain of *Pseudomonas palleroni*, able to grow at the presence of 5 mM(Co(II)) (294.5 mg(Co(II)) L^{-1}) (the same strain was also resistant to zinc, cadmium, copper and mercury, up to 20, 6, 5 and 5 mM, respectively). Genetically engineered strains of *P. palleroni*, sensitive to the above metals, were able to grow at high metal concentrations, after successful transfer of the extrachromosomal DNA from the metal resistant strain [199]. Similarly genetically engineered *E. coli*, was able to grow in environments with 100 times higher Co(II) concentration, compared to the wild stain [155].

3.4. Effects of Co(II) on the chemolithotrophic growth of microorganisms

Cobalt has been identified as a trace element for the chemolithotrophic growth of microorganisms, as it is a component of indispensable enzymes [90]. Repaske and Mayer [159], have identified cobalt as an essential nutrient for the chemolithotrophic growth of *A. eutrophus*, the growth of which was stimulated significantly by the addition of 2×10^{-7} M(Co(II)) (=11.8×10^{-6} mg(Co(II)) L^{-1}), however, further increase of the Co(II) concentration resulted to growth inhibition [159]. Mergeay et al. [143], have calculated the MIC for the chemolithotrophic growth of *A. eutrophus* as 20 mM (=1178 mg L^{-1}). Finally, the MIC of genetically manipulated *A. eutrophus* CH34 has been reported to increase from 0.1 mM(Co(II)) (=5.9 mg(Co(II) L^{-1}) for the wild strain to 9 mM(Co(II)) (=530.1 mg(Co(II) L^{-1}) for the mutant [82].

3.5. Combined effects of Co(II) with other environmental factors on aerobic microorganisms

Cobalt (II) speciation [74]:

$$Co^{2+} \xrightarrow{OH^-} CoOH^+ \xrightarrow{OH^-} Co(OH)_2 \xrightarrow{OH^-} Co(OH)_3^- \xrightarrow{OH^-} Co(OH)_4^{2-}$$

is quite similar to that of nickel (see paragraph 2.5), thus based on the proximity of the ionic structure of Co^{2+} and Ni^{2+}, it is likely

that Co^{2+} also competes with H^+ for binding sites with nitrogenous organics in the growth media. However, the limited experimental data, that exist, suggest a more complex relation between cobalt toxicity and pH.

pH increase from 1 to 7 resulted to the decrease of the D_{50} value of *Bispora* sp. (an extreme acidotolerant filamentous fungus) from 3×10^{-3} M (=0.18 mg L^{-1}) to 5×10^{-5} M Co(II) (=2.9×10^{-3} mg L^{-1}) [165]. The growth of the yeast *S. cerevisiae* B11842 is highly sensitive to pH changes, as it fails to grow at the presence of 1.2 mM (Co(II)) (=70.9 mg(Co(II)) L^{-1}) at pH below 5.0, or above 6.5 [163]. Schmitt et al. [200], who worked with a wild strain of *S. cerevisiae*, reported increase of the MIC from 14.3 to 19.8 mg(Co(II)) L^{-1} when the pH was reduced from 6.4 to 4.75.

Cobalt toxicity in marine microorganisms has been found to increase with hydrostatic pressure [169], or to be independent of it [170].

The effects of cobalt to particular microbial species can be significantly altered by the presence of other metallic species (see paragraph 1.2). Sajani and Maruthi Mohan [197] reported reversion of the intoxication by cobalt, in a wild and in a cobalt-tolerant strain of the fungus *N. crassa*, by the addition of either Mg(II) or Fe(III). The Co(II):Mg(II) ratio and the Co(II):Fe(III) ratio for complete reversion of the Co(II) toxic effects was 10:1, for the wild strain, while the cobalt-tolerant strain required a ratio of 1:1. The antagonism between Co(II) and either Mg(II) or Fe(III) has been primarily attributed to the reduced cobalt binding to mycelia and to cell walls, due to the competition by manganese or iron ions. Essential metal substitution (particularly zinc) by cobalt has also been reported: Price and Morel [201] have proved experimentally that cobalt can promote the growth of zinc limited phytoplankton, by substituting zinc in some metallo-enzymes. More specifically, the cyanobacterium *Thalassiosira weissflogii* was practically unable to grow at the absence of zinc, while addition of cobalt (in zinc free media) resulted in growth at 60% level, compared to the zinc rich media. Similarly, Sunda and Huntsman [202] reported that zinc is an essential nutrient for the growth of the diatoms *Thalassiosira pseudonana* and *Thalassiosira oceanica*, however, they found that the need for zinc can largely be met by cobalt. Finally, Sultana et al. [174] reported that cobalt has a synergic effect with the antibiotic erythromycin, on the growth inhibition, in a number Gram-negative and Gram-positive microorganisms.

4. Comparative effects of individual Ni(II) and Co(II) on the same microbial systems

Nickel and cobalt have similar chemical and physicochemical properties, since they appear side by side at the 8th 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 cells are also utilizing almost identical biochemical mechanisms for dealing with those elements [79], while the same genes, or genes on the same plasmid are often encoding the biochemical behaviour of the cells at the presence of nickel and cobalt [27]. However, despite the above-mentioned physicochemical and biological interaction similarities, it has been found that the above heavy metals usually have different effects on similar biological systems. The comparative biological activity of nickel and cobalt has been assessed by a number of experimental works; however, the results are often controversial, as a clear conclusion about the comparative stimulation/toxicity of nickel and cobalt cannot be reached. It is wise to underline that the biological activities of particular heavy metals can be compared only between identical biological systems; otherwise, false conclusions may be derived.

4.1. Comparative effects of Ni(II) and Co(II) on activated sludge

No clear conclusions can be derived for the comparative effects of Ni(II) and Co(II) on activated sludge systems.

Heukelekian and Gellman [114] who measured the biological activities of both nickel and cobalt on activated sludge have reported that nickel is more toxic than cobalt (see Tables 2a, 2b, 5a and 5b). On the contrary, Gikas [12] reported that cobalt is more potent intoxicator than nickel, to activated sludge, at concentrations higher than approximately 20 mg L^{-1}. However, at smaller concentrations nickel stimulates microbial growth to a greater extent than cobalt. More specifically, Gikas [12] has reported that 50% growth inhibition occurs at approximately 75 mg(Ni(II)) L^{-1} or at 58 mg(Co(II)) L^{-1} (see Fig. 3a and b). Finally, Mowat [37] suggested that both species have more or less similar effect on the respiratory activity of activated sludge.

4.2. Comparative effects of Ni(II) and Co(II) on particular aerobic microorganisms

A non-clear picture of the comparative effects of individual Ni(II) and Co(II) on particular aerobic microbial systems does exists, as for activated sludge systems.

Yang and Ehrlich [170] have reported that nickel stimulates more efficiently than cobalt the growth of a marine rod, at concentrations up to 5 mg L^{-1}, while at 10 mg L^{-1} nickel appears more toxic than cobalt. This is in broad agreement with the study by Barabasz at al. [180], according to which, the growth of *E. coli* is stimulated by the presence of up to 25 mg(Co(II)) L^{-1}, while no significant effect was observed by the same amount of Ni(II). According to Falih [178], 100 mg(Co(II)) L^{-1} were stimulatory to the growth of *P. chrysosporium*, while the same amount of Ni(II) acted as growth inhibitor.

Cobalt has been found to be more toxic than nickel to *Flavobacterium* sp. and *Pseudomonas* sp. [203], and to *S. cerevisiae* [163], On the other hand, Adiga et al. [171] have reported that nickel is more toxic than cobalt on the growth of *A. niger*, reporting 50% reduction to the growth of the above fungus at the presence of either 40 mg(Ni(II)) L^{-1} or 110 mg(Co(II)) L^{-1}.

F. solani can grow on either nickel or cobalt at concentrations up to 300 mg L^{-1}, however, the overall behaviour of the fungus indicates that nickel is more potent growth inhibitor than cobalt [150]. Similarly, Al-Sarrani [131] reported that the growth of the fungus *M. ruber* was inhibited to a significantly greater extent by nickel, compared with the inhibition occurred by cobalt.

Cobalt is more potent inhibitor than nickel to the growth of *E. coli* [204] and *P. putida* [205]. Schmidt and Schlegel [140] who studied the effects of Ni(II) and Co(II) on a number of bacteria isolated from metal contaminated soils, have also reported that bacterial resistance to Co(II) is somehow lower compared with the resistance to Ni(II). *A. butzleri* has been reported to be more sensitive to Ni(II) than to Co(II) [142]. Bhattacharya et al. [206], who tested a large number of *Vibrio parahaemolyticus* strains, reported that 75% of the strains were Ni(II) resistant, while only 37% were Co(II) resistant. On the other hand, Ainsworth et al. [41], who experimented with *K. pneumoniae*, found that Ni(II) is more toxic 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. Research carried out by Collard et al. [82], on a number of facultative autotrophic strains, did not indicated clearly which of the two metals is more toxic. Finally, Losi and

Table 7
Comparative toxicity of nickel and cobalt to various microbial strains and cultures

Nickel more toxic than cobalt		Cobalt more toxic than nickel	
Reference	Microorganism	Reference	Microorganism
Adiga et al. [171]	*Aspergillus niger*	Ivanov et al. [205],	*Pseudomonas putida*
Ainsworth et al. [41]	*Klebsiella pneumoniae* (lag time only)	Bhattacharya et al. [206]	*Vibrio parahaemolyticus*
Al-Sarrani [131]	*Monoascus ruber*	Chandy [203]	*Flavobacterium* sp., *Pseudomonas* sp.
Barabasz at al. [180]	*Escherichia coli*	Gikas [12]	Activated sludge
Falih [178]	*Phanerochaete chrysosporium*	Pearse and Sherman [163]	*Saccharomyces cerevisiae*
Hashem and Bahkali [150]	*Fusarium solani*	Schmidt and Schlegel [140]	Various bacteria isolated from metal contaminated sites
Heukelekian and Gellman [114]	Activated sludge	Wu et al. [204]	*Escherichia coli*
Otth et al. [142]	*Arcobacter butzleri*		
Yang and Ehrlich [170]	Gram-negative marine rod		

Frankenberger [207] who studied bacterial isolates from chromium Cr(VI) contaminated sites, reported that cells able to grow at Cr(VI) concentrations up to 500–1000 mg L^{-1}, were also able to tolerate equally single nickel or cobalt up to a concentration of approximately 50 mg L^{-1}.

The comparative toxicity of nickel and cobalt to various microbial strains and cultures are summarized in Table 7.

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

A limited number of works has been carried out to investigate the joint effects of nickel and cobalt on microbial systems. Even less work has been performed on the combined effects of nickel and cobalt on activated sludge systems, despite the fact that these two metals are often encountered together in the aqueous environment.

5.1. Effects of joint Ni(II) and Co(II) on activated sludge

Gikas [12] has published an extensive study on the individual and joint effects of nickel and cobalt on activated sludge. The individual effects of the above metals have been discussed above (Sections 2.1 and 3.1). Gikas [12] investigated the effects of three quotas of Ni(II) and Co(II): 1:3, 1:1 and 3:1, Ni(II):Co(II) (w/w) for a range of concentrations, and reported that their growth patterns were similar to the growth patterns of either Ni(II) or Co(II) applied individually, with somehow more prolonged lag phases and with some alterations on the maximum growth rate (μ_{max}). A graphical illustration of the effects of joint Ni(II) and Co(II) to the growth rate of activated sludge is shown in Fig. 3a and b.

According to Gikas [12], for the quota 3:1, Ni:Co (w/w), μ_{max} increases by approximately 31.3% compared to 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}, while no growth was observed (during the time of the experiments) at a joint concentration of 160 mg L^{-1}. For the quota 1:1 Ni:Co (w/w), μ_{max} increases by approximately 27.0% compared to the μ_{max0} value, at a joint concentration of 20 mg L^{-1}. Further increase of the joint concentration resulted to a decrease of the μ_{max}, but at a reduced rate compared to the 3:1 Ni:Co mixture. Growth was also nil at a joint concentration of 160 mg L^{-1}. Finally, the quota 1:3 Ni:Co (w/w) increased the μ_{max} by approximately 20.5% in relation to 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} (see Fig. 3a and b).

Gikas [12] used the isobolographic method to characterize the interaction between nickel and cobalt on the growth rate of activated sludge. According to his report, nickel and cobalt acted synergistically both at the zone of increasing stimulation and at the zone of toxicity, while an antagonistic effect was observed at the zone of decreasing stimulation Fig. 5a–c. Thus, according to Gikas [12], the maximum growth stimulation, in activated sludge, 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, at relatively higher concentrations (at the toxicity zone). 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.

5.2. Effects of joint Ni(II) and Co(II) on particular aerobic microorganisms

Antagonism between nickel and cobalt on the toxic effects on the growth of a Gram-negative rod has been reported by Yang and Ehrlich [170] who measured that 10 mg(Co(II)) L^{-1} had no effect on the growth of the above microorganism, while 10 mg(Ni(II)) L^{-1} resulted in approximately 40% reduction of the growth rate. However, simultaneous presence of 10 mg(Co(II)) L^{-1} and 10 mg(Ni(II)) L^{-1}, resulted to approximately just 15% reduction of the growth rate.

The effect of Ni(II) and Co(II) to the growth of *K. pneumoniae*, has been characterized as additive, based on viable counts on agar plates [41]. On the other hand, Cross et al. [208] 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, while the effect of a mixture of 0.75 mM(Ni(II)) (=44.0 mg(Ni(II)) L^{-1}) and 0.75 mM(Co(II)) (=44.2 mg(Co(II)) L^{-1}), reduced the cell viability by more than three times the value predicted by the additive approach.

Barabasz et al. [180] who worked with *E. coli* reported growth stimulation by the addition of 5-25 mg(Co(II)) L^{-1}, while addition of similar amounts of nickel had no effect on the growth of *E. coli*. However, according to the above researchers [180], simultaneous addition of even small amounts of both nickel and cobalt was absolutely lethal to the growth of the above microorganism.

Finally, Repaske and Repaske [209] reported approximately 180% stimulation to the chemolithotrophic growth of *A. eutrophus* by the addition of trace amounts of nickel and cobalt, however, no significant growth stimulation was observed by the addition of sole nickel or cobalt. The above indicates strong synergy between nickel and cobalt.

The type of the effects of nickel and cobalt to various microbial strains and cultures are summarized in Table 8.

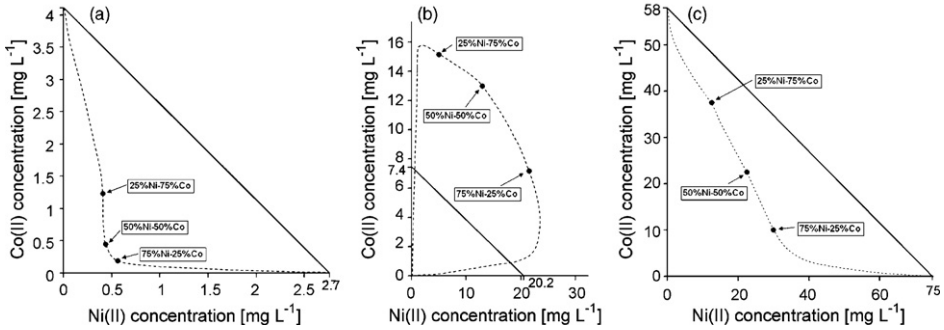

Fig. 5. Isobolograms depicting the equi-effective concentrations of mixtures of nickel and cobalt to the growth of activated sludge. (a) Zone of increasing stimulation, synergic effect, (b) zone of decreasing stimulation, antagonistic effect and (c) toxicity zone, synergic effect (adopted from Gikas [12]).

Table 8
Type of joint effects of nickel and cobalt to various microbial strains and cultures

Reference	Microorganism	Type of Joint Effects
Ainsworth et al. [41]	*Klebsiella pneumoniae*	Additivity
Barabasz et al. [180]	*Escherichia coli*	Synergism
Cross et al. [208]	Cultured epithelial cells	Synergism
Gikas [12]	Activated sludge	Synergism (increase stimulation and toxicity zones), antagonism (decreased stimulation zone)
Repaske and Repaske [209]	*Alcaligenes eutrophus*	Synergism
Yang and Ehrlich [170]	Gram-negative marine rod	Antagonism

6. Summary: literature evaluation

Nickel and cobalt have been found to inhibit the microbial growth at relatively high concentrations, by all the published works. However, a significant number of works has indicated that both metals stimulate microbial growth at trace concentrations, while some reports supported that some microorganisms failed to growth at the absence of the above metals. Some studies have failed to report microbial growth stimulation by either nickel or cobalt, at trace concentrations. This may be attributed either to the fact that the above metals were acted solely as growth inhibitors, or to the fact that the researchers did not test the behavior of the microbes at low enough metal concentrations. In a similar manner, the effects of nickel or cobalt to the growth of mixed microbial populations, such as activated sludge, can be attributed either to effects of the above metals to the "average" growth of the mixed cultures, or to shifts in the microbial populations.

The impacts of both, nickel and cobalt, to microbial growth have been found to be affected on a case per case basis, by a number of environmental parameters, such as: pH, biomedium composition, presence of other heavy metals or active substances, or even hydrostatic pressure.

A general rule on the relative nickel and cobalt toxicity cannot be derived from the published data, as they are often contradictive, even for similar type of microbial systems. It should emphasized that useful comparisons on the relative toxicities can only be done among almost identical microbial systems, since the sensitivity to external environmental factors may affect their behavior more dramatically than the metal concentrations itself. Similarly, a clear line on the joint effects of the above metallic species cannot be drawn, as all three possible interactions (synergy, additivity and antagonism) have been reported.

The importance of understanding multi-metal toxicity, in relation with the chemical background, in environmental applications is obvious, as the toxic effects to microorganisms usually do not follow the rule of additivity. As multi-metal toxicity has become an issue of investigation, particularly for setting the maximum allowable metal concentrations in the aquatic- and in the geo-environment, more research has to be performed. It is important, however, to standardize the microbial systems used for such research purposes, as collection of more data from various systems and under various conditions is not expected to yield the desired information.

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