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Detection of heavy metals in bacterial biofilms and microbial flocs with the fluorescent complexing agent Newport Green

S Wuertz[1], E Müller[1], R Spaeth[1], P Pfleiderer[1] and H-C Flemming[2]

[1]*Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, D-85748 Garching, Germany;* [2]*Institute of Water Quality Chemistry and Water Technology (IWW), Moritzstr 26, D-45476 Mülheim/Ruhr, Germany*

The complexing agent Newport Green fluoresces upon binding of nickel, zinc or cobalt. It was used to detect nickel or zinc in MOPS buffer, in gel-like matrices, and in natural biofilms and microbial flocs cultivated in the laboratory. The response curves for increasing nickel concentrations indicated an equimolar binding capacity of Newport Green for nickel in MOPS buffer, whereas zinc fluorescence reached saturation in the presence of a 10-fold excess of zinc ions relative to Newport Green molecules. The maximum fluorescence intensity as determined by luminometry was 8-fold and 4-fold above background for nickel and zinc, respectively. The response of Newport Green to either nickel or zinc in the presence of the other metal is consistent with a different binding affinity of Newport Green for the two metals. Zinc binds more strongly to the complexing agent than nickel but it leads to a weaker fluorescent signal which was detectable by luminometry but not by confocal laser scanning microscopy (CLSM). Newport Green was able to complex nickel in the presence of 1% gelatin or agarose as determined by CLSM and image processing. Its application to fully hydrated bacterial biofilms or microbial flocs revealed the presence of nickel outside of cells. The results suggest that in addition to cellular sorption, metals are bound extracellularly by extracellular polymeric substances in intact and undisturbed microbial aggregates. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 116–123.

Keywords: biofilms; confocal laser scanning microscopy; Newport Green; nickel; zinc; sorption; extracellular polymeric substances

Introduction

The environmental fate of heavy metals is strongly influenced by sorption processes. Organic matter, which is composed of humic substances, debris and microbial biomass, accounts for a considerable proportion of sorption capacity in soils, sediments and water [9]. Both suspended and immobilized bacterial, fungal and algal cells, as well as wastewater flocs and biofilms have been used in bioremediation efforts to remove heavy metals from contaminated soil and water [6,7,35,37], but less attention has been paid to the importance of biofilms in natural systems. Biofilms occur at solid–liquid, liquid–liquid or liquid–gas interphases. They consist of cells, entrapped particles of both organic and inorganic origin, extracellular polymeric substances (EPS), and water. Biofilms partially mask solid materials including minerals and particulate material in aqueous systems and, consequently, are involved in sorption/desorption processes in natural and technical systems. They are involved in the chemistry of world rivers [19] and in mineral genesis [22]. It is important to understand the underlying mechanisms of metal sorption to biofilms as they can release metal ions upon decomposition leading to remobilization of pollutants.

Apart from cell walls, the EPS also contain numerous potential binding sites for metals including carboxyl, phosphoryl and sulphate groups suggesting a high cation exchange potential [34]. EPS may play a pivotal role in sorption processes in sewage treatment plants. Most studies, however, have been conducted on extracellular polymers extracted from bacterial cultures [10,17,25] or wastewater systems [11,16,28,29]. Extraction procedures of EPS may lead to changes in physico-chemical properties [31]. For example, destruction of the hydrated gel matrix may lead to the formation of additional sorption sites [29]. Little is known about the actual binding capacity of EPS for metals in fully hydrated undisturbed biofilms.

Noninvasive techniques for the *in situ* study of biofilms include microscopic [2,38], spectrochemical such as Fourier-transform-infrared spectroscopy [30,33], electrochemical such as microelectrodes for O_2, pH or NO_3^-, and piezoelectric approaches [27]. Among these, confocal laser scanning microscopy (CLSM) has received widespread acclaim because of its usefulness in resolving the population structure and architecture of biofilms based on the application of fluorescently labeled rRNA-directed oligonucleotide probes [1,23] and EPS-specific fluorescent stains or lectins [26], respectively. Biofilm penetration by fluorescent beads as a marker for particulate substrates [32] and local diffusion coefficients of fluorescein, phycoerythrin and fluorescent dextrans have also been determined by CLSM [5,21].

In this study we investigated the use of the commercially available complexing agent Newport Green, which fluoresces upon binding Ni, Zn or Co. Its fluorescent properties were studied as a function of Ni or Zn concentration in a buffer solution, in a viscous solution containing the protein

Correspondence: S Wuertz, Institute of Water Quality Control and Waste Management, Technical University of Munich, Am Coulombwall, D-85748 Garching, Germany. E-mail: stefan.wuertz@bv.tu-muenchen.de
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gelatin, in a gel-like matrix containing the polysaccharide agarose, and in natural biofilms and microbial flocs cultivated in the laboratory. Our goal was to differentiate between sorption to cell walls and to other extracellular constituents of biofilms as a prerequisite to further studies of sorption and desorption mechanisms.

Materials and methods

Chemicals and media

Stock solutions of $NiSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ were 10 mM in 5 mM MOPS buffer at pH 7.0 and 8.0. In biofilm studies, 1 mM $NiSO_4 \cdot 7H_2O$ was added directly to samples. Stock solutions of Newport Green and SYTO 17 (both from Molecular Probes, Eugene, OR, USA) were 100 μM and 5 μM, respectively. All metal solutions were sterilized by passing them through a 0.2-μm pore size disposable filter (Schleicher and Schuell, Dassel, Germany). Mineral medium used in rotating annular reactors and activated sludge cultures was autoclaved at 121°C for 20 min and contained per liter: 1 g KH_2PO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 2 g Na gluconate. Fresh gelatin and agarose solutions were prepared in 1 × PBS (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g NaH_2PO_4, pH 7.0) for each experiment. Gelatin was dissolved by heating the suspension to 60°C. For agarose the suspension was heated in a microwave to dissolve the agarose, left to cool at room temperature, and poured into a rectangular device. Individual slices of this gel were transferred to a coverslip for microscopic analysis.

Cultivation of biofilms

Biofilms were grown in a rotating annular reactor, which consisted of an external cylinder and an internal solid drum [12]. The drum was fitted with four uninterrupted tubes located at an 80° angle. Its rotation facilitated an optimal vertical exchange of fluid in the reactor system. This allowed free passage of medium through the tubes leading to an internal recirculation. The internal side of the exterior drum contained 12 removable polycarbonate slides to which additional coupons could be attached. The slides and coupons were removed for biofilm analysis through individual openings in the reactor lid without interrupting the operation of the system.

Hence biofilms were grown under defined hydraulic conditions leading to a homogenous distribution of biomass along the length of slides or coupons. The reactor was operated on a continuous basis with tap water at a flow rate of 1.5 L h^{-1} and mineral medium at a flow rate of 70 ml h^{-1} as nutrient input. The rotational speed of the drum was 250 rpm.

Luminometric measurements

The fluorescence of Newport Green in the presence of Ni and Zn was measured with a Perkin Elmer Luminescence Spectrometer LS50B. Excitation was at 488 nm and the scanning speed was 12000 nm min^{-1}. The excitation and emission slit widths were set at 2.5 nm. Background fluorescence of noncomplexed Newport Green was measured using concentrations ranging from 0.1 to 10 μM. To test the effect of Ni or Zn on fluorescence, the concentration of Newport Green (Figure 1) was kept constant at 1 μM; the pH was 7 or 8. Added Ni concentrations ranged from 0 to 12 μM, and added Zn concentrations ranged from 0 to 48 μM. When the effect of two metals was tested, either the Ni or the Zn concentration was kept constant at 0.48 and 4.8 μM, or 6.0 and 48 μM, respectively.

Microscopic measurements

Images in the horizontal *xy*-register in selected optical planes were obtained with a CLSM 410 confocal laser scanning microscope coupled to an AXIOVERT 135 M inverse microscope (both instruments from C Zeiss, Jena, Germany). The system utilized a motorized computer-assisted device to control the vertical positioning during optical sectioning of the biofilm. Light excitation was performed using the 488-nm and 543-nm laser lines for Newport Green and SYTO 17, respectively. Light emission by SYTO 17 was detected using the dichroic filter 560 and longpass filter 590. Newport Green fluorescence was detected using either longpass filter 515 or bandpass filter 515–525. Images were obtained with a 100×/1.3 NA or 40×/1.3 NA Plan-Neofluar oil immersion lens. Digital image processing and analysis were performed with a QUANTIMET 570 computer system (Leica, Cambridge, UK) as previously described [20]. Biofilm images were further processed using Adobe Photoshop 3.0.

For detection in buffer solution, a 2-μM Newport Green solution containing various concentrations of Zn or Ni was equilibrated for 15 min, placed on a coverslip, and viewed using an inverted LSM 410 microscope. A series of images in the *z*-direction (*z*-series) was obtained using the dialogue and menu facilities of the Zeiss CLSM software. The vertical step interval Δz was 2 or 8 μm resulting in a total depth resolution within the drop of 26 μm or 104 μm,

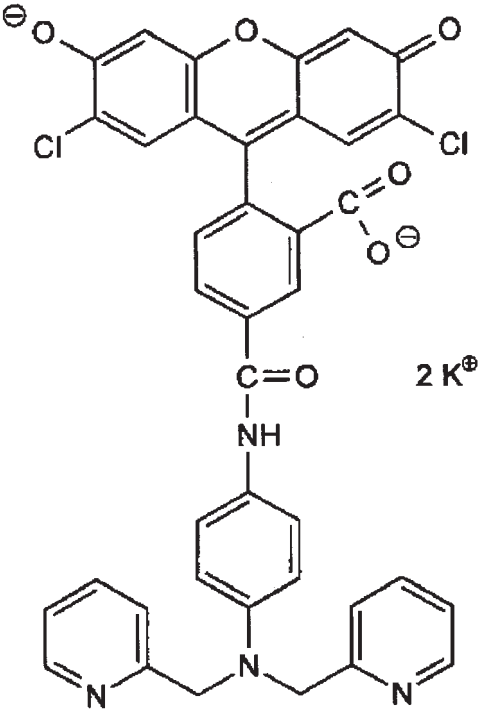

Figure 1 Chemical structure of Newport Green [15].

respectively. These steps were repeated at least five times at different *xy* positions. The mean grey value of the resulting digitized images was determined using a QUANTIMET 570 computer system (Leica, Cambridge, UK).

The procedure was similar for viscous 1% gelatin solutions. In the case of 1% agarose gels Newport Green was added afterwards; the agarose slices were left in the dark for 15 min to facilitate diffusion of Newport Green into the matrix. Before microscopic analysis, samples were mounted in Citifluor (Citifluor Ltd, London, UK).

Biofilms on 1×3 cm^2 coupons removed from reactor slides were exposed to 100 μl of 1 mM Ni solution for 1 h. Afterwards they were washed twice with 100 μl of sterile distilled water and resuspended in 100 μl of a 20-μM SYTO 17 solution. They were left in the dark for 15 min. The coupon was thoroughly rinsed three times with sterile distilled water before the addition of 100 μl of 2.4 μM Newport Green. The solution was left in the dark for 15 min and analyzed by CLSM and image processing.

To test the response of Newport Green to increasing concentrations of Ni in a biological system, a mixed bacterial culture was grown by inoculating mineral medium with activated sludge and shaking the mixture at room temperature for 48 h. Five hundred microliters of bacterial suspension were harvested by centrifugation at $2000 \times g$, washed with 5 mM MOPS buffer (pH 7.0), and resuspended in 250 μl MOPS buffer containing various concentrations of Ni. The suspension was incubated for 1 h at room temperature, centrifuged at $2000 \times g$ for 2 min, and washed with 100 μl sterile distilled water to remove any unbound metal ions. The pellet was resuspended in 100 μl of 2.4 μM Newport Green and incubated in the dark at room temperature for 15 min. Afterwards CLSM analysis was performed. Background fluorescence due to noncomplexed Newport Green was eliminated by choosing an appropriate threshold level.

Results

Detection of metal complexation in aqueous solution by luminometry

The cell-impermeable complexing agent Newport Green was tested for its fluorescent properties in the presence of the heavy metal ions Zn^{2+} and Ni^{2+}. Response curves were determined for varying metal concentrations in MOPS buffer at pH 7.0 and 8.0 using a luminometer. The Newport Green concentration was 1.0 μM as recommended by the manufacturer [15]. Higher concentrations generally led to fluorescence values in the presence of added metals ions which exceeded the range of the luminometer.

The highest relative fluorescence intensity for the Ni-Newport Green complex was found at pH 8 with a maximum value of 550 (Figure 2). The linear range was very narrow with saturation occurring at 2–3 μM Ni. For Zn much lower relative fluorescence intensities were obtained up to values of 230. A higher pH also had a positive effect. The linear range extended to approximately 25 μM Zn. This indicates that the binding capacity of Newport Green for Ni^{2+} is about equimolar, whereas zinc binding reached saturation only at a 10-fold excess of zinc ions relative to Newport Green molecules. A comparison of

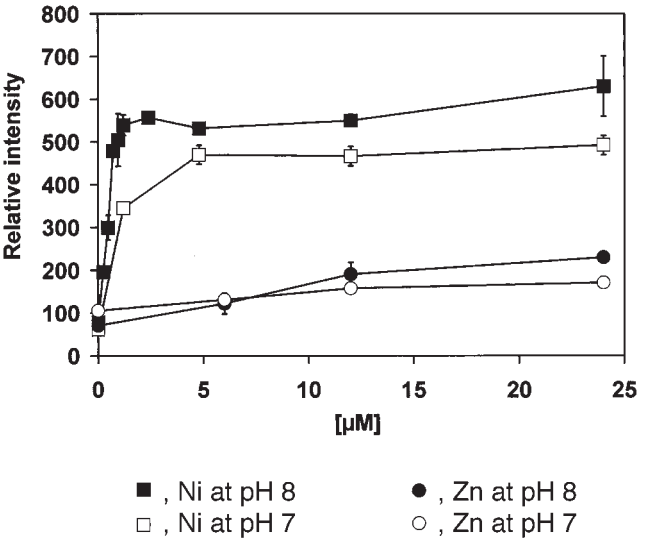

Figure 2 Fluorescence of complexed Newport Green as a function of nickel or zinc concentration. Light intensities were measured in solution using a luminometer. Values represent the mean of three determinations; error bars refer to the standard deviation.

fluorescence intensity in the absence of added metal and the intensity in the region of saturation reveals an 8-fold increase for Ni and a 4-fold increase for Zn. Quantitation of metals in solution by luminometry is possible only in the linear concentration range (Figure 2).

The response of Newport Green to either Ni or Zn in the presence of the other metal was tested using two concentrations of the competing metal, one giving a low fluorescence signal and one at near-saturation level. The addition of 0.48 μM Ni led to an increase in fluorescence intensity in the presence of varying concentrations of Zn (results not shown). There was competition between Ni and Zn for binding sites of Newport Green since Ni alone increased fluorescence to a relative intensity of 300. Increasing levels of Zn^{2+} only led to a very gradual increase in fluorescence intensity, which is in line with the lower degree of fluorescence enhancement due to Zn compared to Ni.

In the reverse situation the presence of 6.0 μM Zn had a lesser effect on the fluorescence intensity of the Ni-Newport Green complex, which remained in the same range as in the absence of Zn (Figure 3). This suggests that only a minor amount of Zn was present in the complex. A higher concentration of Zn (4.8 μM) further increased the basal level of fluorescence, as expected. The addition of Ni did not lead to the same degree of fluorescence intensity as in the presence of 6.0 μM Zn, suggesting that at the higher Zn concentrations the Zn-Newport Green complex was favored.

Detection of metal complexation in solution by CLSM

Background fluorescence of Newport Green in MOPS buffer was detectable with a confocal laser scanning microscope (CLSM). There was a linear relationship between Newport Green concentration and emitted fluorescence signal (results not shown). The optimal concentration for CLSM studies was between 2 and 3 μM. CLSM analysis

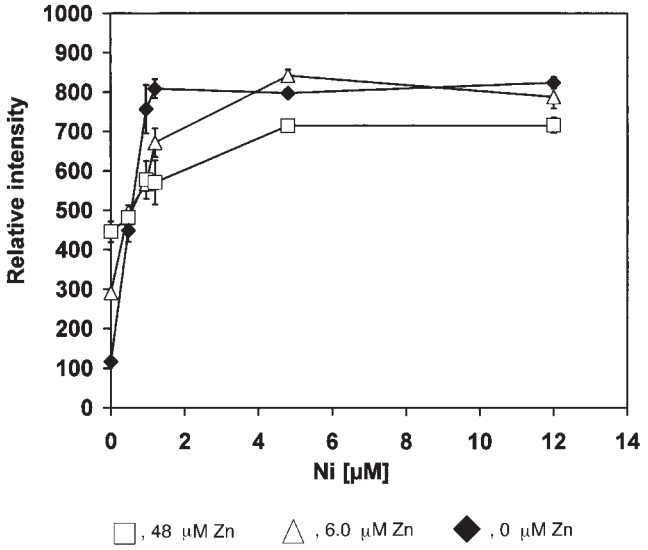

Figure 3 Effect of zinc on the fluorescence of Ni-Newport Green complex. Newport Green was present at 1 μM, the pH was 8.0. Light intensities were measured in solution using a luminometer. Values represent the mean of three determinations; error bars refer to the standard deviation.

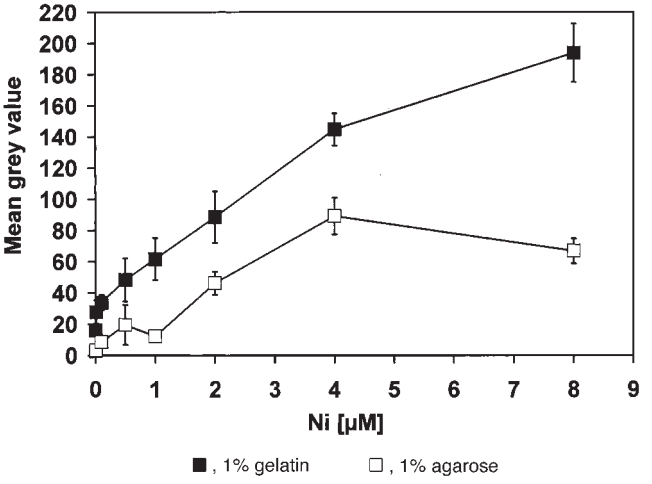

Figure 4 Microscopic determination of fluorescence of complexed Newport Green as a function of Ni in the presence of gelatin or agarose. Images were obtained with a Zeiss LSM 410 confocal laser scanning microscope. Grey values were determined with a Leica QUANTIMET 570 image analysis system. Values represent the mean of five determinations; error bars refer to the standard deviation.

of Newport Green fluorescence in the presence of Ni mirrored the results obtained by luminometric measurements. There was a rapid change in fluorescence intensity between 0.1 and 1 μM Ni with mean grey values increasing from 5 ± 0.4 to 24 ± 5. Higher nickel concentrations did not result in further enhancement of fluorescence. In contrast to the results obtained for Ni, 1–200 μM Zn had no discernible effect on the fluorescence of 2 μM Newport Green in 5 mM MOPS buffer at pH 8.0 (results not shown). These results were not due to a pH effect and contradict the luminometric data on Zn-induced fluorescence of Newport Green in aqueous solution (Figure 2).

Detection of metal complexation in gelatin solution and agarose gel by CLSM

As a first approximation to microbial cells and EPS in biofilms we investigated the effect of Ni and Zn in a viscous solution of $1 \times$ PBS at pH 7.0 containing 1% gelatin and in 1% agarose gels. As in the case of Newport Green in solution there was a linear increase in background fluorescence with increasing concentrations of Newport Green (results not shown). The mean grey values at 2 μM Newport Green were 26.1 ± 1.5 and 2.2 ± 0.3 for 1% gelatin and 1% agarose, respectively. The addition of Ni led to an enhanced signal for both gelatin- and agarose-containing matrices (Figure 4). In the case of gelatin there was a linear increase for all concentrations measured up to 8 μM Ni; for agarose, saturation was reached at 4 μM Ni. This indicates that Newport Green is able to complex Ni even when other potential sorption sites in the form of a viscous or semisolid matrix are present. Compared to complexation in MOPS buffer only (results not shown), the linear range of fluorescence due to the complexation of Ni by Newport Green was extended in the presence of gelatin and agarose. The overall fluorescence intensity also increased from a mean grey value of 25–35 to 220 for 1% gelatin and 70–80 for 1% agarose (Figure 4). The addition of zinc to 1% gelatin solution or 1% agarose gel did not lead to enhanced fluorescence at concentrations ranging from 1 to 160 μM (results not shown).

Detection of metal complexation in biofilms and flocs by CLSM

The usefulness of Newport Green for the microscopic detection of nickel and zinc in biofilms was investigated. Biofilms were grown on removable polycarbonate slides and coupons in an angular rotating reactor [12] fed with tap water and mineral medium. These coupons were then exposed to varying concentrations of Ni.

To determine whether metals were exclusively bound to cell walls, test coupons were exposed to Newport Green and stained with SYTO 17. Filamentous organisms, which dominated this biofilm, did not act as sorbent for heavy metals (Figure 5). Most of the metals appeared to have adsorbed to cell walls of rod-shaped bacteria. A significant amount was not associated with cells. This metal fraction was either bound to extracellular polymeric substances or dissolved in water within the biofilm. We explored the value of Newport Green for a semi-quantitative analysis of nickel in a mixed bacterial culture that had been grown in a mineral medium in the absence of added nickel and zinc. Bacterial aggregates emitted a low background signal after addition of Newport Green (Figure 6). Increasing concentrations of Ni led to higher fluorescence intensities. There was no discernible difference in the level of fluorescence between dissolved Ni and Ni bound to cell walls. Intracellular Ni was not detected since Newport Green is cell-impermeable.

Discussion

According to the manufacturer, fluorescence intensity of Newport Green increases upon binding Zn^{2+}, Ni^{2+} or Co^{2+}. At a concentration of 1 μM Newport Green at 22°C in 5 mM MOPS, pH 7.0, the relative fluorescence has been

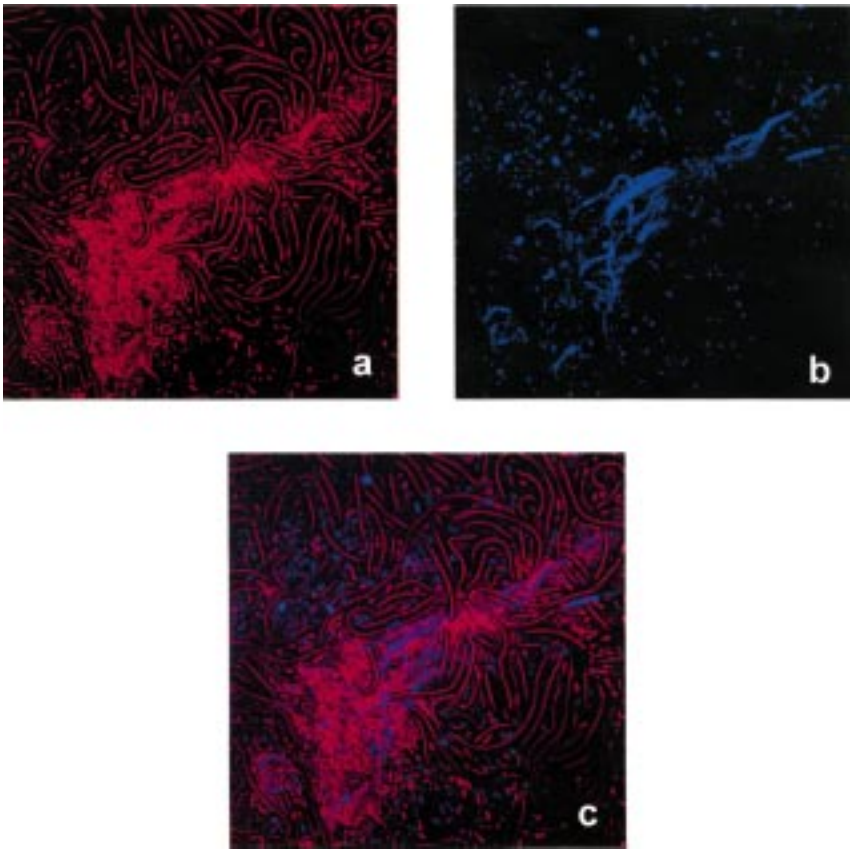

Figure 5 Confocal laser scanning microscopic image of a biofilm containing Zn and Ni. Panel (a): the red color shows cells labeled with the nucleic acid stain Syto 17; panel (b): the blue color shows metals complexed by Newport Green; panel (c): panels (a) and (b) were superimposed; the pink color shows metals adsorbed to cells. Magnification: 1000×.

reported to increase 16-fold in the presence of 25 μM Ni and 10-fold in the presence of 25 μM Zn^{2+} or Co^{2+}. Other tested cations including Cu^{2+}, Cu^{+}, Hg, Pb^{2+}, Fe^{2+}, Fe^{2+}, Ca^{2+} (100 μM) and Mg^{2+} (35 mM) had no effect [15]. Our results do not support these claims with respect to fluorescence intensity in the presence of nickel and zinc. Under the given experimental conditions the maximum increase in fluorescence as measured by luminometry was 8-fold for Ni and 4-fold for Zn. Furthermore, these values were obtained at a higher pH of 8 compared to the manufacturer's results which pertain to pH 7.0 [15]. Our data are in agreement with a different binding affinity of Newport Green for the two metals. Although Zn binds more strongly ($k_d = 1.0$ μM) than Ni ($k_d = 1.5$ μM), it produces a less fluorescent complex [15]. Hence the addition of Ni to solutions containing Newport Green plus Zn at varying concentrations resulted in an enhanced fluorescent signal. The opposite occurred when Zn was present in excess (48.0 μM) and Ni was varied. Due to the higher stability of the Zn-Newport Green complex the equilibrium was moved towards the more weakly fluorescing Zn complex. Owing to these competing reactions there is no clear concentration dependency of fluorescence in the presence of both metals.

The discrepancy between luminometric and microscopic measurements with respect to Newport Green fluorescence in the presence of Zn can be explained by the difference in sensitivity of the two instruments used. It was not due to daily variations since the experiments were repeated several times over a period of days. The observed increase in Ni-Newport Green fluorescence in the presence of gelatin or agarose may be due to a mechanism activating the formation of Ni-Newport Green-protein and Ni-Newport Green-sugar complexes. Possibly this involved polynuclear complexes with several Newport Green molecules binding more than an equimolar amount of Ni^{2+} ions. The extended linear range of fluorescence intensity as a function of Ni concentration was not based on unavailability of Ni to the complexing agent since fluorescence intensity was also higher.

In situ analysis by CLSM revealed a heterogeneous distribution of Ni in biofilms and bacterial flocs. In the biofilms studied the dominant microbial fraction consisted of filamentous organisms that did not bind nickel. Fluorescent Ni-Newport Green complex was also detected extracellularly. It is possible that this fraction represented Ni^{2+} in solution, although the biofilm had been thoroughly rinsed to remove any unbound Ni before applying Newport Green. Biofilms contain up to 85–95% water based on their wet weight [3]. EPS in biofilms usually account for 1–2% (w/w), a concentration which allows the formation of gels similar to agarose or gelatin. A mixture of galactomannan and xanthan at 0.8% (w/w) can lead to gel formation [4]. Hence Ni may have been complexed in a gel-like EPS

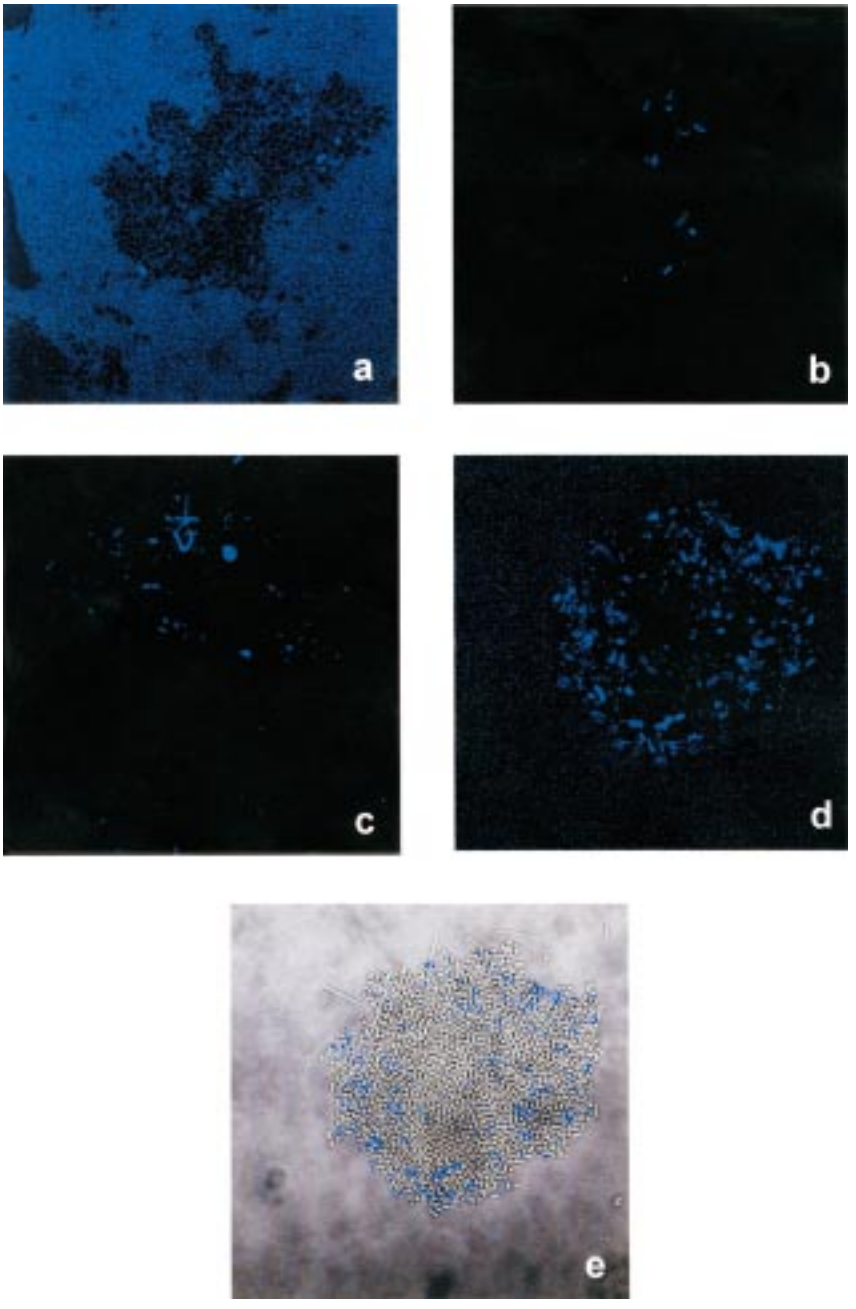

Figure 6 Mixed bacterial culture exposed to increasing concentrations of Ni as viewed by confocal laser scanning microscopy. Panel (a): no added Ni; panel (b): 0.1 μM Ni; panel (c): 1.0 μM Ni; panel (d): 5.0 μM Ni; panel (e): superimposition of panel (d) with a transmission image. The fluorescence based on noncomplexed Newport Green shown in panel (a) was suppressed in subsequent images by choosing an appropriate threshold. The figure shows that not all cells bind Ni. Magnification: 1000×.

environment. The addition of Newport Green as a complexing agent may have released the ions or led to the formation of a complex including the EPS components. A weakening of gel matrices by chelating agents has been reported for ethylene glycol-bis-(2-aminoether)-tetra acetic acid (EGTA) [36].

It was shown in the case of sewer biofilms that equimolar concentrations of even strongly complexing agents like EDTA or HEDP with stability constants for metal complexes of up to 10^{18} [14] resulted in only negligible remobilization of bound metal ions at neutral pH [13]. Hence, we suggest that the observed Ni-specific fluorescence in biofilms in this study was associated with EPS and was not the result of remobilization and complexation of originally cell-bound metals by the action of Newport Green.

Sorption of Ni^{2+} and Zn^{2+} to EPS may be explained as an ion exchange mechanism. EPS contain a great number of negatively charged functional groups such as carboxyl, phosphate and sulfate groups [34]. In bacterial cultures 20–50% of polysaccharides in EPS may be glucuronic acids

[18]. Metals can replace Mg^{2+} and Ca^{2+} as bridging agents for polymers, for example, poly-L-guluronic acid sequences in alginates [9]. Binding of metals via ion exchange is strongly dependent on pH. Lower pH values result in fewer negative charges on functional groups like carboxylate and phosphate. The opposite occurs at neutral or alkaline pH [8]. Previous studies have shown that EPS extracts can complex metal ions. Different microorganisms tend to have different affinities of EPS for metal ions, with stability constants ranging from 10^5 to 10^9 [17,29].

Apart from pH, other factors like E_h, competing ions, metal-specific attributes including heat of hydration and charge density [24], as well as temperature [10] and the presence of other surfaces may influence the sorption behavior of EPS. The degree of acetylation of polymers can affect the binding capacity for cations. Acetylated polysaccharides appear to be more selective for divalent cations like Mg^{2+} and Ca^{2+}, whereas nonacetylated polysaccharides preferentially bind monovalent cations like Na^+, K^+ or Li^+ [10]. Clearly, Newport Green can be used in a qualitative manner to demonstrate the presence of the heavy metals Ni and Zn in defined systems. It will be of interest to combine knowledge about the local binding of heavy metals in native biofilms with information regarding the nature of constituents of EPS. In this way, Newport Green may be used to confirm the metal-binding properties of polysaccharides and proteins extracted by physical and chemical techniques.

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