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Ralf Pätzold · Maike Keuntje · Angelika Anders-von Ahlften

A new approach to non-destructive analysis of biofilms by confocal Raman microscopy

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Abstract Confocal Raman microscopy (CRM) of biofilms enables one to determine the distribution of different microorganisms and other substances inside physiological intact microbial communities. These biofilms are of outstanding interest for biological wastewater treatment. In contrast to invasive techniques, such as fluorescent in situ hybridization (FISH), we were able to identify anaerobically ammonium-oxidising (anammox) bacteria without pretreatment processes of the samples just by its Raman vibrational signature. The presented results provide new insights into the complex interactions of different organisms in microbial communities without interfering with them.

Keywords Confocal Raman microscopy · Biofilm · Microbial communities

Introduction

Spectroscopic techniques that detect molecular vibrations have been used for analysing and identifying microorganisms for several years [1–3]. This promising approach provides the potential to clearly classify a bacterium in a shorter time and by using a lower quantity [4, 5] in contrast to conventional biological determination methods. The popular IR spectroscopy has some drawbacks concerning sample preparation and spatial resolution that limit in situ and non-invasive applications in the micro regime. Raman scattering is superior to IR spectroscopy with respect to these issues, especially for aqueous solutions. Single microorganisms can be identified by applying Raman microscopy [6–9]. The deployment of a confocal Raman microscope allows the three-dimensional distribution of substances to be recorded with high spatial resolution by scanning a tightly focussed laser beam over the sample [10].

Here we demonstrate the non-invasive detection of a wastewater-related microorganism, *Brocadia anammoxidans*, directly in its natural environment. The anammox bacteria [11, 12] perform the complete autotrophic nitrogen-removal over nitrite in cooperation with other bacteria [13]. Additionally, we show the microbial composition of a biofilm without destroying it. Although the biological part of wastewater treatment is well established, it still represents a "black box" [14, 15]. In particular, little is known about the interactions between the different types of microorganisms [16]. In principle, it is possible to identify different microbes that are involved in the decomposition process using FISH, but this represents a destructive interference to the microbial community. Analysis of the complex interactions of the different microorganisms in biofilms requires a technique that not only determines the internal composition but also the spatial distribution of the community. This requirement necessitates the application of a non-invasive microscopic method. CRM has been demonstrated to be suitable for microbial analyses in several recent investigations [4, 5, 7, 8, 17]. It provides not only a specific spectral fingerprint for unique identification, but also the distribution of different microorganisms inside the biofilm—without destroying it. The subject of our investigation, Candidatus *Brocadia anammoxidans*, was discovered 10 years ago [11]. This bacterium belongs to the planctomycetes group [18] and is capable of anaerobically oxidizing ammonium (anammox) as well as some other bacteria recently discovered (e.g. Candidatus *Kuenenia stuttgartiensis* and Candidatus *Scalindua sorokinii*). These microorganisms are mainly involved in the one-stage deammonification process, which is of outstanding interest for wastewater treatment plants [19]. Due to limitations in enrichment of anammox microorganisms [20], the establishment of an efficient community represents an active research target [21]. Knowledge about such communities permits a deeper insight into the complex biofilm system, allows an assessment of their interactions

R. Pätzold (✉) · M. Keuntje · A. Anders-von Ahlften
Institut für Biophysik, Universität Hannover,
Herrenhäuser Str. 2,
30419 Hannover, Germany
e-mail: paetzold@biophysik.uni-hannover.de
Tel.: +49-511-7622607
Fax: +49-511-7625916

and will therefore optimise the wastewater treatment process of the future [14].

Experimental

Spectroscopic instrumentation

All Raman spectra were recorded by using a commercial confocal Raman microscope (CRM 200, WITec Ltd.). For excitation two different single-mode laser systems could be applied to the CRM: a diode-pumped solid state laser (λ=532 nm; max. 13 mW on the sample; WG-SLM-020) and a diode laser (λ=785 nm; max. 230 mW on the sample; XTRA, Toptica). Both of lasers were frequency and intensity stabilised. Laser radiation was coupled into the microscope via a single-mode fibre. Optical components such as filters, gratings and objectives were adapted to the appropriate wavelength.

In the experiments the laser light was focussed into the sample by two different microscope objectives. The usage depended on the investigated sample: for samples in aqueous solutions a 60×, numerical aperture (NA) 1.0 water immersion microscope objective (Nikon CFI Fluor) was used. Samples prepared on a microscope slide were analysed by a 100×, NA 1.25 oil immersion microscope objective (Nikon CFI achromat). The same objectives collected the inelastic scattered light (Raman effect) which is separated from the intensive elastic scattered light by an edge filter.

The pinhole which is essential for the confocal principle in order to suppress out-of-focus light was realised by means of a multi-mode fibre with 50-μm diameter. In combination with the microscope objectives this fibre provided an axial resolution of 1.5 μm for the 60×, NA 1.0 and an axial resolution of 1.2 μm for the 100× objective, respectively (full width at half maximum of the 520 cm^{-1} band of silicon in reflection, excitation wavelength 532 nm). Lateral resolution was diffraction limited (ca. 350 nm). The ellipsoid sample volume from which Raman spectra were recorded was below 1 μm^3. That means that the surrounding matter of a bacterium (1 to 1.5 μm) which resides in the focus effects the Raman spectrum to a negligible degree.

The light was spatially dispersed by a spectrometer (SP-308, Acton) with a 600 lines/mm grating (spectral resolution 12 cm^{-1}; spectral accuracy 2 cm^{-1}). The spectra were recorded by a backthinned CCD camera (DU401-BR-DD, Andor) which is thermo-electrically cooled to −70 °C. Additionally to this array detector a single-photon counting avalanche photodiode (APD) was attached to the spectrometer. A mirror selected between both detectors. The 'faster' APD provided a first overview of a specific Raman band (normally the CH-stretching mode) of the specimen (max. 200 × 200 μm^2). From this so-called fast image, the measuring site was chosen and spectrally resolved with the array detector.

The CRM 200's scanning unit consisted of a capacitively controlled piezoelectric scan table with a lateral scan width of 200 μm (accuracy 3 nm). Axial movement was implemented by a stepper motor that moved the whole microscope body over some centimetres with an accuracy of 50 nm. In the measurements the largest z-scan range was 100–200 μm.

Laser power was always adjusted to provide a non-destructive treatment of the specimen. Typical values were about 10 mW on the sample. The exposure time for spectra recording was in the range of 1–10 s. Due to different reasons (bleaching, reasonable overall measurement time), as will be explained below, the exposure time was always set to 1 s. Recorded spectra were pre-treated by the internal software of the CRM, such as spatial intensity distribution of Raman bands, baseline correction, etc. A hierarchical cluster analysis was performed using OPUS software including OPUS/IDENT (Bruker Optics Ltd.).

Bacterial cultures

Biofilms

The biofilms in which we non-invasively detected the anammox bacteria were grown in sequencing batch reactors (SBR) from the Institute of Communal Wastewater and Treatment (ISAH), University of Hannover. The 10-L reactor was built-up as a pilot engineering plant of an anammox SBR. Detailed information is given elsewhere by Gaul et al. [22]. The biofilms had a size of approximately 2 mm.

In order to get a clear identification of anammox bacteria, the spectra were compared with reference bacteria. Therefore, enriched cultures of *Brocadia anammoxidans* from three different reactors of the Kluyverlaboratory for Biotechnology, Delft University of Technology, were used. Two reactors were fed with synthetic wastewater containing the substrates ammonium, nitrite and bicarbonate for ideal nutrition conditions [23], whereas in another reactor ammonium was replaced by urea. The sequencing batch reactors were operated at 38 °C under limited oxygen concentrations [20]. Under these conditions the anammox bacteria were enriched to more than 88% of the biomass.

Microorganisms

In order to identify spectra of other bacteria which are supposed to be in the biofilm, cultivated nitrifying organisms were measured in suspension with the CRM. The nitrifying microorganisms were provided by the Institute of Botany, University of Hamburg (*Nitrosomonas europeae* Nm 50, *Nitrosomonas eutropha* Nm 53, and *Nitrosomonas eutropha* Nm 57) and by the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig (*Nitrobacter hamburgensis* DSM 10229).

The *Nitrosomonas* strains were inoculated from a stock solution, which we received from the University of Hamburg, to a working solution. This medium and the cultivation conditions were described previously [24]. A

1-mL aliquot of the working solution was centrifuged in an Eppendorf tube. The supernatant was removed and the bacterial pellet was resuspended in phosphate-buffered saline (PBS). The last two steps were done twice to remove the highly fluorescent pH indicator of the working medium.

The cultivation medium for *Nitrobacter hamburgensis* can be found at the website of the DSMZ (medium 756)[1]. Cultivation conditions can be found in the literature [25]. The preparation of the bacterial sample for CRM measurements was the same as for *Nitrosomonas*.

CRM measurements

All described biofilms were grown as granules. The biofilms and the microorganisms were put between a microscope slide and a cover slip for the measurements. The biofilms were prepared with a drop of its growing medium, whereas the bacteria were suspended in PBS. Biofilms were analysed in microscopic scanning mode with an integration time of 1 s as a compromise between measuring time and Raman intensity. The reference spectra of the nitrifying bacteria were achieved in solution without scanning. In this case the tightly focussed laser beam acted as optical tweezers.

Results and discussion

Limit of detection (LOD)

In preliminary experiments it could be shown that the LOD of our CRM system is 10 mM for nitrate. These investigations were performed with an excitation wavelength of 785 nm, a laser power of 100 mW in the sample, an integration time of 10 s, and using a 100-μm fibre. These parameters are not reasonable for high resolution CRM of biofilms because of the high laser power which destroys the biofilm and because of the long measurement times: an area of the biofilm sampled with 100×100 pixel would take more than 1 day of measuring time.

Additionally, this result concludes that the Raman intensities of dissolved mineral nutrients (<1 mM) are more than 3 orders of magnitude lower than the signals of the microorganisms.

Excitation wavelength

In our preliminary experiments, a near-IR diode laser (785 nm) was used for two reasons: first, as commonly stated in literature [26, 27], fluorescence excitation in the sample volume should be avoided; second, because the anammox bacteria are supposed to be located in deeper layers of the biofilm (anaerobic zone), laser penetration into the specimen was required to be as deep as possible (>100 μm). By applying the diode laser as excitation source it was possible to measure Raman spectra up to150-μm depth (ca. 50 mW). But the quality of the spectra excited with 785 nm was quite poor: The few Raman bands were broad and not very rich in detail. Much better results from the specimen were obtained with the green 532-nm excitation source. Despite the assumption of fluorescence excitation, the spectra of our samples showed rather little fluorescence activity. Edwards et al. reported similar results when investigating cyanobacterial gypsum [28]. They also got the best Raman spectra by using a green wavelength, 514 nm.

Penetration depth

Due to ν^4-dependency of the scattered light, with the same laser output power (10 mW), different penetration depths into the biofilms were achievable using the laser at 532-nm or 785-nm wavelengths, respectively. With the NIR laser diode (785 nm) it was possible to acquire Raman spectra of the bacteria up to 150 μm, whereas with the frequency-doubled solid-state laser (532 nm) the bacteria could be detected up to 70-μm depth. As a compromise of higher penetration depth and better signal quality (see above) we chose the solid-state laser in our further experiments.

Raman spectrum of an anammox bacterium

Since there is no comprehensive database for bacterial substances at present, the first goal was to record spectra from bacteria involved in water clarification processes. Figure 1 shows Raman spectra of different microorganisms. Spectra a and b are obtained from bacteria, which were found in a biofilm of the SBR from ISAH. Spectra c–f were recorded from enriched or pure cultures. A unique feature of anammox bacteria is the membrane construction of the central organelles, the so-called anammoxosome. The membrane lipids consist of ladderanes, concatenated cyclobutane rings [29]. Despite this unique molecular feature, the non-resonant Raman process which is used in CRM is estimated to be to weak in detecting them (see LOD). This especially applies in this research which uses short exposure times of 1 s for recording a spectrum.

Nevertheless, the obvious difference between Raman spectra of anammox bacteria (spectra a and c in Fig. 1) and spectra of other microorganisms (b, d, e and f in Fig. 1) is an unambiguous, broad band at 3,180 cm^{-1}. The corresponding stretching vibration ν(NH) can be assigned to ammonium, hydrazine and hydroxylamine. These substances are involved in the main reaction which takes place in the anammoxosome. By oxidizing ammonium with nitrite to nitrogen gas via the intermediates hydrazine and hydroxylamine the anammox bacteria gain the needed energy [21]. Distinct bands δ_s(NH) and δ_a(NH) at 1,494 cm^{-1} and 1,620 cm^{-1}, respectively, are also seen in spectra a and c in Fig. 1, which confirms the high concentration of the abovementioned substances with NH functional groups. Therefore, spectrum c from Fig. 1 represents the

[1] http://www.dsmz.de/media/med756.htm

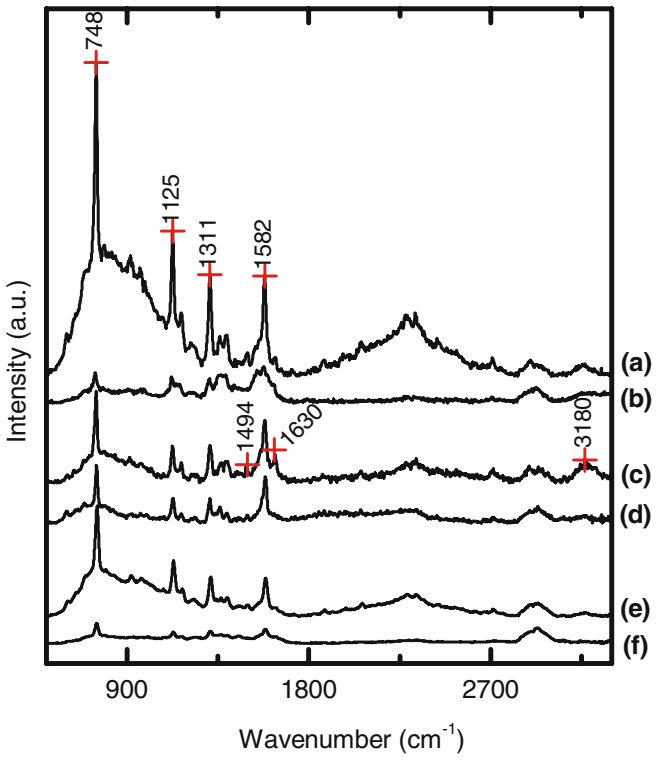

Fig. 1 Raman spectra of different microorganisms: **a**, **b** averaged spectra from different colonies in a biofilm (see Fig. 2) of a SBR that performed the one-stage deammonification process, **c** enriched culture of Candidatus *Brocadia anammoxidans*, **d** *Nitrobacter hamburgensis* DSM 10229, **e** *Nitrosomonas eutropha* Nm 57, **f** *Nitrosomonas europeae* Nm 50. All spectra were averaged over several measuring sites of the organisms, normalized to the CH content (2,820–3,010 cm^{-1}), and background corrected (laser power 10 mW, excitation wavelength 532 nm)

reference spectrum for anammox bacteria. By using this reference we also identified anammox bacteria in the pilot engineering SBR of the ISAH (see below).

In order to supply anammox organisms with nitrite in a one-stage deammonification process, ammonium-oxidizing bacteria, like *Nitrosomonas*, should be detected in the biofilm. Curves d–f in Fig. 1 show the associated spectra of two strains of *Nitrosomonas* as well as one strain of *Nitrobacter*, which is a nitrite-oxidising bacterium. The organisms were obtained under culture conditions. All spectra in Fig. 1 have four distinct and characteristic Raman bands at 748 cm^{-1}, 1,125 cm^{-1}, 1,311 cm^{-1} and 1,582 cm^{-1}. The substance(s) could not be correlated with a literature or a database query.

Microorganisms in biofilms

When displaying the spatial distribution of specific Raman bands, the so-called chemical imaging, it is possible to get an overview of microbial communities. In Fig. 2a the distribution of the characteristic Raman band at 748 cm^{-1} is shown. The spectrally resolved areas of highest intensities form a micro-colony of bacteria with several gaps between them which build ideal pathways for feed. The spectrum of the microbes is displayed in Fig. 1a. Averaging 100 spectra with highest intensities of the 748 cm^{-1} band, a hierarchical cluster analysis assigns the averaged spectrum to enriched *Brocadia anammoxidans* (spectrum c in Fig. 1).

Figure 2b displays the distribution of the CH-stretching mode ν(CH) at 2,900 cm^{-1}. The colony of these unidentified bacteria is represented by the averaged spectrum of Fig. 1b. Till now, it was not possible to assign the spectrum to a known bacteria due to the limited amount of reference data.

Investigations of the enriched culture of *Brocadia anammoxidans* reveal further compartments in those biofilms: the lateral CRM scan of a 15 μm × 15 μm area of such an enriched culture is displayed in Fig. 3. In addition to enriched anammox bacteria, other substances could also be detected in the biofilm which is grown with urea as nutrition medium. Spectral analysis of this area with the CRM shows, for instance, a strong stretching mode of the phosphate group ν(PO) at 988 cm^{-1} in the lower left corner (Fig. 3a). A database query gives as result of calcium phosphate for this crystal. Further spectral evaluation as well as the size (ca. 15 μm) indicates the presence of algae in the biofilm. In Fig. 3b a typical Raman spectrum of lipids [4] is shown and the spectrum in Fig. 3c can be assigned to carotenoids on which the accessory pigments of algae are based.

Bleaching effect

In order to get reference spectra from known bacteria, Raman spectra from cultivable microorganisms like *Nitrobacter* and *Nitrosomonas* were recorded. The four characteristic bands (748 cm^{-1}, 1,125 cm^{-1}, 1,311 cm^{-1} and 1,583 cm^{-1}) of Brocadia anammoxidans, *Nitrobacter hamburgensis* DSM 10229, *Nitrosomonas europeae* Nm 50 and *Nitrosomonas eutropha* Nm 57 in Fig. 1, could also be found in other bacteria (*Staphylococcus cohnii* DSM 6669) [30]. Harz et al. investigated bulk material as well as single microorganisms by micro Raman spectroscopy. They discovered the same four bands in bulk material of *Staphylococcus cohnii* DSM 6669 but not in the spectra of a single bacterium. This is due to their long exposure time of 60 s whereby the characteristic Raman bands are photobleached.

In our experiments a spectrum of *Nitrosomonas eutropha* 57 suspension was taken each second with 10-mW laser power. As shown in Fig. 4 bleaching effect of the four bands takes place immediately when the bacterium occur in the laser focus. In Fig. 4 the integral intensity of each band is displayed. In order to compare the signal decrease they are normalised to the maximum when the bacterium had “entered” the laser focus and was caught by the tightly focussed laser beam from the high-NA objective (100×, 1.25 NA oil immersion). Due to the asymmetric curve, the decrease of the signal is not caused by a

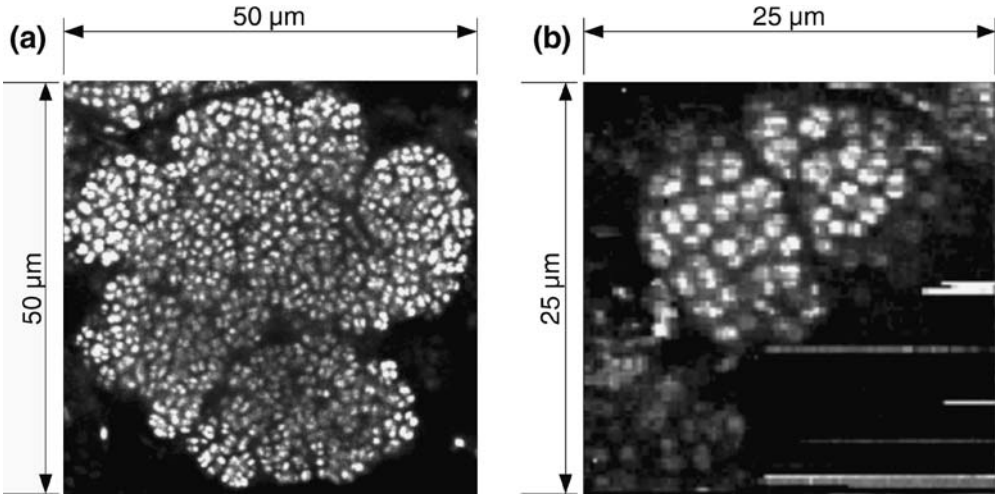

Fig. 2 Two colonies of different microorganisms in the same biofilm from a SBR that performed the one-stage deammonification process: lateral distribution of the 748 cm^{-1} band in a depth of 10 µm below the biofilm surface (**a**) and 2,900 cm^{-1} in 8-µm depth (**b**). Associated spectra are displayed in Fig. 1 (**a** and **b**, respectively; laser power 10 mW, excitation wavelength 532 nm)

movement of the microorganism out of the laser focus. In fact, the bacterium is captured by the laser beam in optical tweezers. This is proved by the intensity of the ν(CH)-stretching mode which remains constant over time.

This result concludes that a spectrum which is achieved in 1 s looks different to a spectrum which was recorded for 10 s. This circumstance has to be taken into account if reference spectra for identification purposes are recorded. Therefore, the integration time of the measurement has to be treated as a parameter for the reference database.

Minerals in biofim

In addition to organic components of the biofilm community, inorganic substances could also be found. Figure 5 shows a cut through a biofilm which was grown on a cylindrical carrier. Coloured spots are due to different minerals which can be retrieved in a Raman database. Spectra a and b in Fig. 5 represent common minerals (iron oxide and titanium dioxide) which are typically found in wastewater. The barite (barium sulfate, shown in spectrum C) is less common.

Remarks and conclusion

We demonstrated that CRM is capable of recording the spatial distribution of microorganisms in their natural environment without interfering with the sensitive microbiota. In contrast to other techniques such as FISH or confocal laser scanning fluorescence microscopy (CLSM)

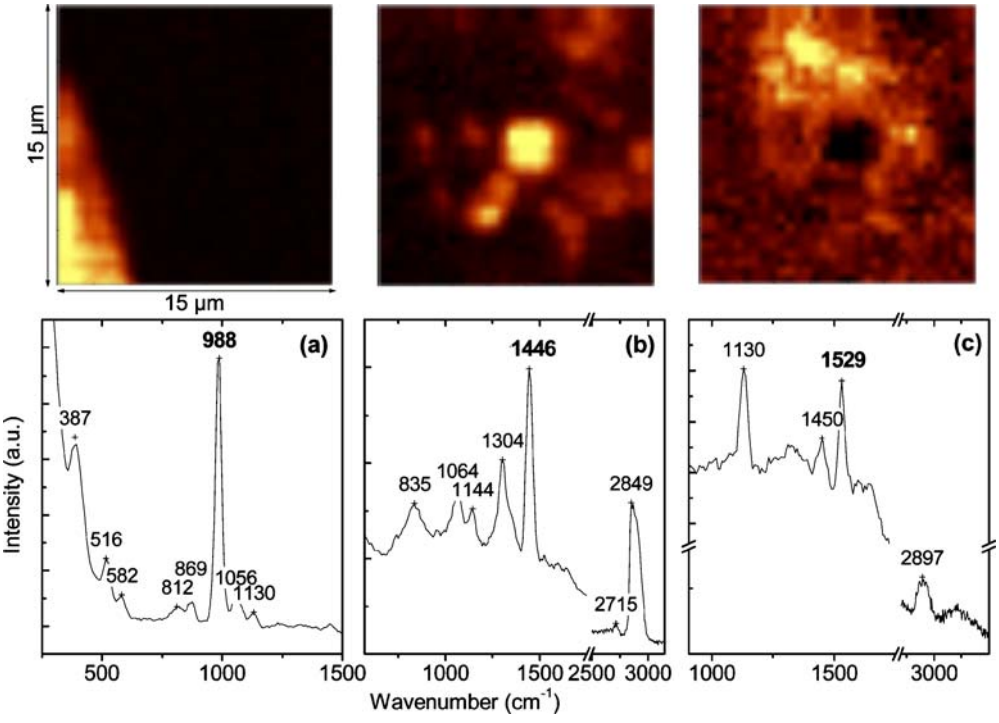

Fig. 3 Lateral distribution of three different Raman bands in the same area of a biofilm in a depth of 5 µm. Components which can also be found in biofilms of enriched cultures of *Brocadia anammoxidans*: **a** Raman band 988 cm^{-1}, crystalline calcium phosphate, **b**, **c** parts of an alga due to lipids (Raman band 1,446 cm^{-1}) and pigments (Raman band 1,529 cm^{-1}), respectively; (laser power 30 mW, excitation wavelength 785 nm; spectra were averaged over several pixels)

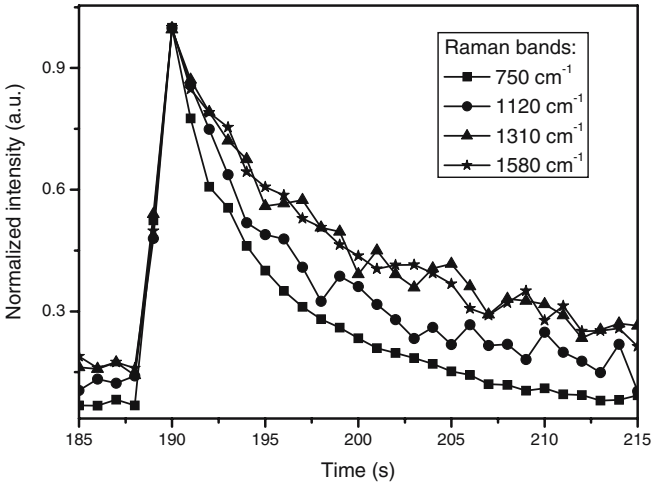

Fig. 4 Bleaching effect of Raman bands of *Nitrosomonas eutropha* Nm 57 suspension. Extract of a time-dependent series. Integration time was 1 s; measurement site was fixed; laser power 10 mW; excitation wavelength 532 nm. See text for further information

which are also used to explore the cellular architecture, the distribution and production of extracellular polymeric substances, or to prove mathematical biofilm models, CRM provides these insights into the complex construction of biofilms without destroying them (FISH) or putting (toxic) fluorescent markers into them (CLSM). An online survey of microbial communities is possible with CRM. As a result of this new approach, it is much easier to acquire better strategies for the establishment of an efficient biofilm for (a) wastewater clarification in general, and for (b) the promising one-stage deammonification process (complete autotrophic nitrogen-removal over nitrite) in particular. So, for instance, the effects of different parameters on the microbial community, like nutrition or temperature, can easily be monitored. Of course, this method is not limited to biofilms in wastewater, but also allows one to get an idea of the biofilm formation and microbial distribution on surgical instruments, medical equipments, or even anywhere communities of microorganisms occur.

The low detection volume (ca. 1 μm^3) of the confocal approach and the discrimination of out-of-focus light means that a single spectral signal is dominated by the whole microorganism. Raman signals from the surroundings of the bacteria are negligible. The difficulty with actual experiments where microbes are identified by their vibrational signature is mainly the lack of a spectral database of microorganisms. Therefore, such a database has to be constructed first by measuring pure strains of wastewater bacteria in order to determine them in the biofilm later on. At the moment, the task of creating a database of vibrational spectra from microorganisms is being performed by different groups (e.g. Popp et al. and Naumann et al.) [2, 7]. Since the spectral signal of a given bacterium depends on its state (growing phase, inactive, fission, budding, dead or alive) [7, 30], the database has to include spectra of these states as well. Additionally, the applied computer-based identification method (principal component analysis, artificial neural networks, support vector machines, etc.) also has to pay attention to the bacterial-state-dependent spectrum. And finally, long exposure time of seconds up to minutes are needed to record a significant Raman spectrum due to the weak process. Dynamic processes in the second regime or faster cannot be detected. Optical tweezers may help to keep the bacterium in the laser focus during the measurement [6, 31]. But these long-term measurements at a single point limit the size of the sample volume for which recording by sequential scanning is reasonable. Measurements of $10 \times 10 \times 10$ μm^3 in some hours are typical.

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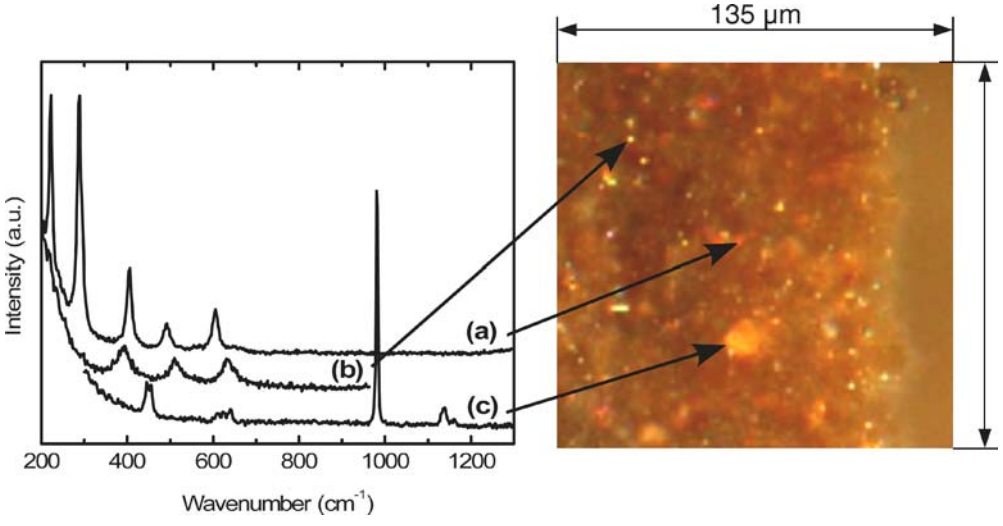

Fig. 5 Raman spectra of minerals measured in a biofilm grown in a moving bed reactor: **a** iron oxide *red spots*, **b** titanium dioxide *bright white spots*, **c** barium sulfate *orange area* (laser power 30 mW, excitation wavelength 785 nm)

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