Raman Microspectroscopy for Environmental Analysis

Raman-Mikrospektroskopie in der Umweltanalytik

Habilitationsschrift zur Feststellung
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“Everything in Life is Vibration”

Albert Einstein

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Contents

List of Abbreviations

AF4	asymmetrical flow field-flow fractionation
BC	black carbon
CF3	centrifugal field-flow fractionation
CRT	continuously regenerating traps
DPF	diesel particulate filters
EC	elemental carbon
FPA	focal plane array (detector)
FT-IR	Fourier transform infrared (spectroscopy)
HA	humic acids
HOPG	highly ordered pyrolytic graphite
HRTEM	high-resolution transmission electron microscopy
ICP-MS	inductively coupled plasma mass spectrometry
LOD	limit of detection
MALS	multi-angle light scattering
MP	microplastics
MPSS	Munich Plastic Sediment Separator
OC	organic carbon
PA	polyamide
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PLS	partial least-squares
PE	polyethylene
PMMA	polymethyl methacrylate
POPs	persistent organic pollutants
PP	polypropylene
PS	polystyrene
PSD	particle size distribution
PVC	polyvinyl chloride
Pyr-GC-MS	pyrolysis gas chromatography mass spectrometry
RM	Raman microspectroscopy
SEM	scanning electron microscopy
SERS	surface-enhanced Raman scattering
SIRM	stable isotope Raman microspectroscopy
srswor	simple random sample of units selected without replacement
TED-GC-MS	thermo-extraction and desorption coupled with gas chromatography mass spectrometry
TERS	tip-enhanced Raman spectroscopy
TPO	temperature-programmed oxidation

1. Introduction

Raman microspectroscopy (RM) has been recognized as a powerful analytical tool in science and industry. RM is a nondestructive analytical technique which is based on the effect of inelastic light scattering by molecules, providing characteristic vibrational fingerprint spectra with the spatial and depth resolution of a confocal optical microscope. The intensity of the Raman signal is directly proportional to the concentration of the analyte. The RM analysis requires no or limited sample preparation and can be performed *in situ* and *in vivo* without interference of water. However, the potential of this technique for the identification and structural characterization of different (environmental) matrices/systems, ranging from biofilms, microplastic and nanoplastic particles in the environment and food to atmospheric aerosol particles and (bio)diesel soot has not yet been systematically explored. Furthermore, RM can open possibilities for the nondestructive and quantitative analysis of stable isotope tracer incorporation in (in)organic and (micro)biological samples. Additionally, the sensitivity of the technique can be significantly improved (by a factor of 10^3 – 10^6) by employing the surface-enhanced Raman scattering (SERS) effect, e.g. for studies of microorganisms and biofilms.

In this work, new application fields for Raman microspectroscopy will be presented. The feasibility and limitations of the method will be discussed, with the focus on the analysis of soot, microplastic and nanoplastic particles as well as microorganisms and biofilms. RM has been shown to be an efficient technique for the characterization of the nanostructure of combustion aerosol particles, and hence is suited for the prediction of the structure-related reactivity of e.g., (bio)diesel soot samples. Another anthropogenic pollutant – microplastics and nanoplastics – has been found in the environment and food, but the degree of contamination remains uncertain. The further development of (automated) RM-based analysis can enable the reliable quantification of plastic particles down to 1 µm and even below. Furthermore, RM and SERS in combination with the stable isotope approach are shown to be an emerging tool for the nondestructive 2D and 3D characterization of the molecular and isotopic composition of microorganisms on the single-cell level, which can enable *in situ* investigations of ecophysiology and metabolic functions of microbial communities.

2. Raman microspectroscopy (RM) for the characterization of soot

Carbonaceous aerosols, or soot particles, result from incomplete combustion of fossil fuels or biomass burning. Soot is of high importance for the climate as well as the environment and human health, as it interacts with clouds or the earth's radiation, causes respiratory diseases, or transports and converts substances [1-3]. Soot consists mostly of carbon and is composed of agglomerated primary particles with diameters of 10 – 50 nm that comprise nanocrystalline (sp^2-bonded graphite like carbon) and amorphous (sp^3- and sp^2-bonded carbon) domains. The amorphous domains are disordered mixtures of polycyclic aromatic hydrocarbons (PAHs) and other (in)organic components [1,4].

Soot which is present in diesel engine exhaust is particularly important, as it is classified to be carcinogenic to humans (Group 1) by the World Health Organization (WHO) [5]. Despite continuous optimization, combustion engines cannot avoid inhomogeneities in the internal combustion chamber, leading to the formation of soot nanoparticles (NP) [1]. Therefore, continuously regenerating traps (CRT) or diesel particulate filters (DPF) are applied in order to remove soot particles from diesel engine exhaust. These systems, however, have to be continuously (CRT) or periodically (DPF) regenerated by oxidation and gasification of the deposited soot. The behavior of this regeneration step is strongly influenced by the structure and reactivity of the deposited soot particles [1,6-8]. Furthermore, depending on the combustion conditions, fuel, and lubricant composition, the emitted exhaust contains particles of complex composition: soot particles can be internally or externally mixed with minerals and coated with adsorbed semi-volatile compounds or sulfuric acid [1,9].

Despite the enormous effort made in developing electromobility, the predominance of combustion engines will remain for at least several decades [1]. Therefore, the comprehensive characterization of soot structure and reactivity is essential to meet future low-emission standards [1] and also to understand impact of soot on the environment [1-3].

The reactivity of soot is usually determined by temperature-programmed oxidation (TPO). The emitted carbon oxides (CO_2 and CO) are quantified by Fourier transform infrared (FT-IR) spectroscopy or mass spectrometry [1,6-8]. To investigate the soot structure, high-resolution transmission electron microscopy (HRTEM) is usually applied [7,10]. It has been shown that differences in the oxidation behavior of soot are

associated with different nanostructures [10]. However, TPO and HRTEM measurements are too demanding for routine analysis. Therefore, we explored the potential of Raman spectroscopy for the characterization of soot structure and reactivity.

Raman spectroscopy was first applied for the characterization of graphite-like carbon in soot by Rosen and Novakov in 1977 [11]. Since then, many studies have reported and discussed the correlation of Raman spectroscopic parameters with the structure of soot and related carbonaceous materials (see reviews [1,4] and references therein, as well as [3] (**PI**).

Figure 1 shows Raman spectra of soot and related carbonaceous materials. For highly ordered pyrolytic graphite (HOPG), only one strong sharp peak around 1580 cm^{-1} (G – "Graphite" peak) can be observed. However, already for graphite powder the Raman spectrum exhibits two peaks – a strong sharp G peak around 1580 cm^{-1} and a weak peak around 1350 cm^{-1} (D – "Defect" or "Disorder" peak). Spectra of soot samples (and humic-like substances) consist of two strong overlapping G and D peaks. In order to get more detailed information on Raman spectroscopic parameters, different fitting procedures can be applied [12]. Figure 2 illustrates the commonly used five-band fitting procedure proposed by Sadezky et al. in 2005 [13].

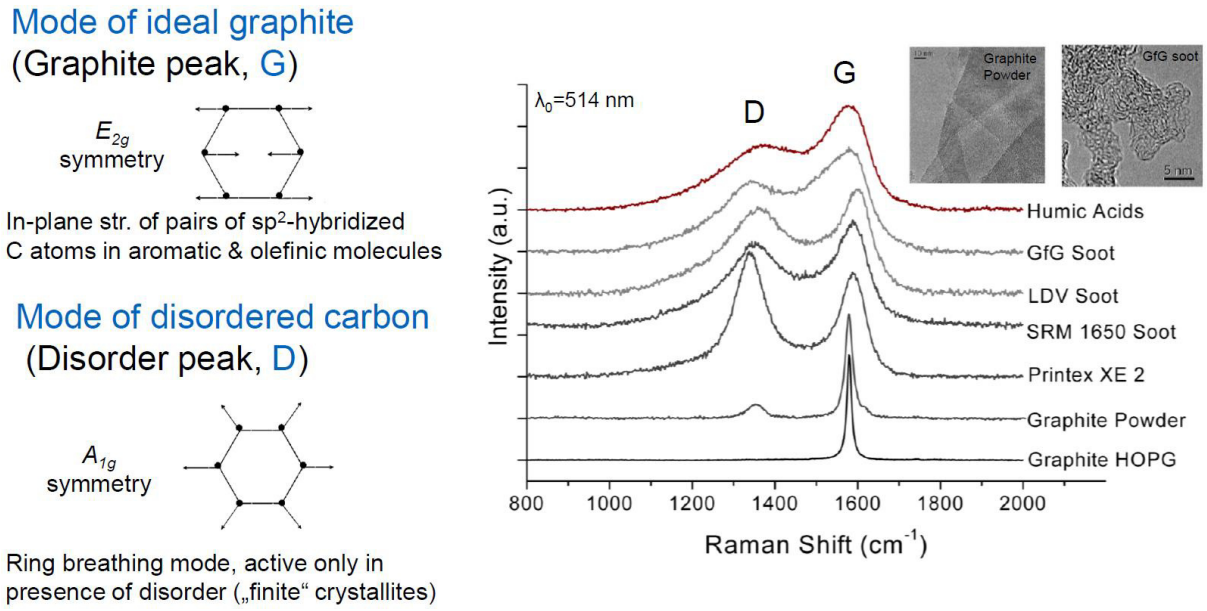

***Figure 1**: Main vibrational modes in Raman spectra of carbonaceous materials (left) and spectra of different soot samples and related carbonaceous materials (right): highly ordered pyrolytic graphite (HOPG), graphite powder, Printex XE 2 industrial soot, SRM 1650 diesel soot, light-duty diesel vehicle (LDV) soot, spark discharge (GfG) soot and humic acids. The spectra are offset for clarity. Inserts: HRTEM images of graphite powder and GfG soot. Adopted from Ivleva et al. [12] and Knauer et al. [7].*

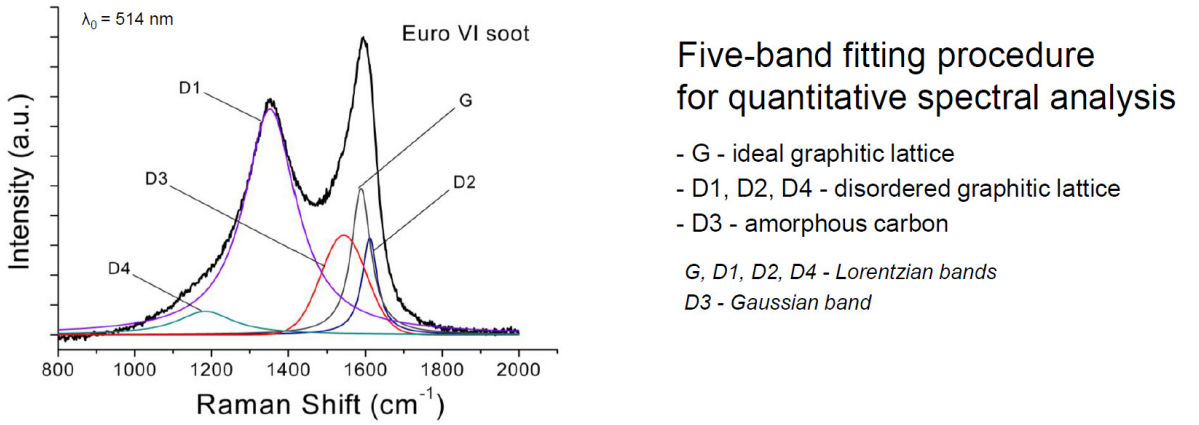

***Figure 2**: Raman spectrum of untreated EURO VI soot with five-band fitting procedure according to Sadezky et al. [13]. Adopted from Knauer et al. [7].*

Obviously, Raman spectroscopic parameters – such as peak positions, widths, and intensity ratios – differ significantly for different soot samples and related carbonaceous materials, and therefore can be applied for structural characterization. Furthermore, since the oxidation behavior of soot depends on the nanostructure of soot, Raman spectroscopy has a potential for the prediction of soot reactivity, which was first demonstrated in 2007 by Ivleva et al. [6]. As shown in Figure 3, the differences in the soot reactivity determined by temperature-programmed oxidation (increase from the least reactive graphite powder through EURO IV and EURO VI soot to the most reactive GfG soot) are in a very good agreement with the differences in soot nanostructure measured by Raman spectroscopy (increase in the D1 width from graphite powder through EURO IV and EURO VI soot to GfG soot which exhibits the highest degree of disorder).

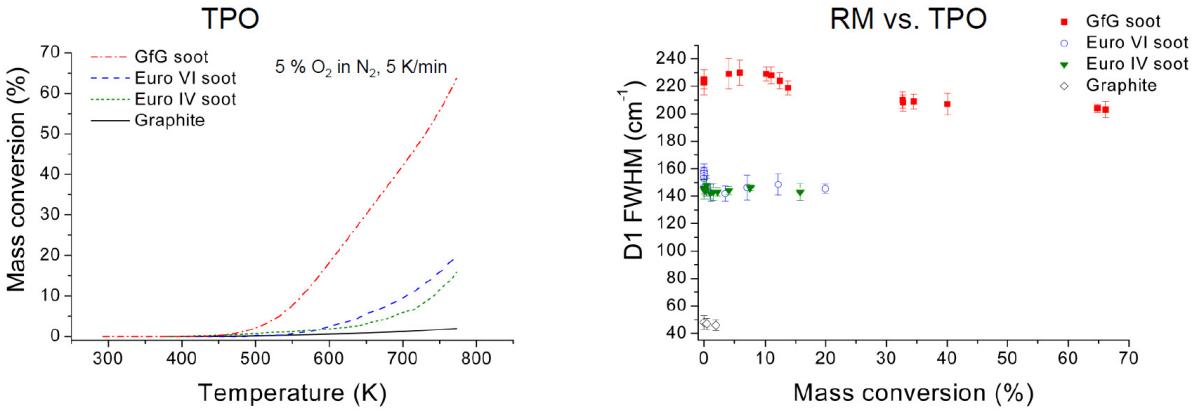

***Figure 3**: Mass conversion versus temperature by oxidation up to 773 K, heating rate 5 K/min (left). Changes in full width at half-maximum (FWHM, cm^{-1}) of D1 band for GfG soot, EURO VI soot, EURO IV soot, and graphite powder during oxidation versus mass conversion (right). Adopted from Knauer et al. [7].*

Generally, the five-band fitting procedure can be successfully used for the analysis of the structure of a large variety of different diesel soot samples and related carbonaceous materials [1,7]. However, for some soot samples, unusual signals in the D4 area (spectral region around 1200 cm^{-1}) caused by organic carbon and/or inorganic impurities were observed, making this fitting procedure inapplicable. This issue can be resolved by applying multiwavelength Raman microspectroscopy [8]. This method is based on the dispersive character of the carbon D mode in Raman spectra (i.e., red shift and increase in intensity at higher excitation wavelength, λ_0). For soot nanoparticles, the classic rule of the invariance of Raman shift at different λ_0 is not valid because of the broken symmetry. This leads to a so-called double-resonant Raman process, which (for a given laser energy and phonon branch) selectively enhances a particular phonon wave vector and phonon frequency [14].

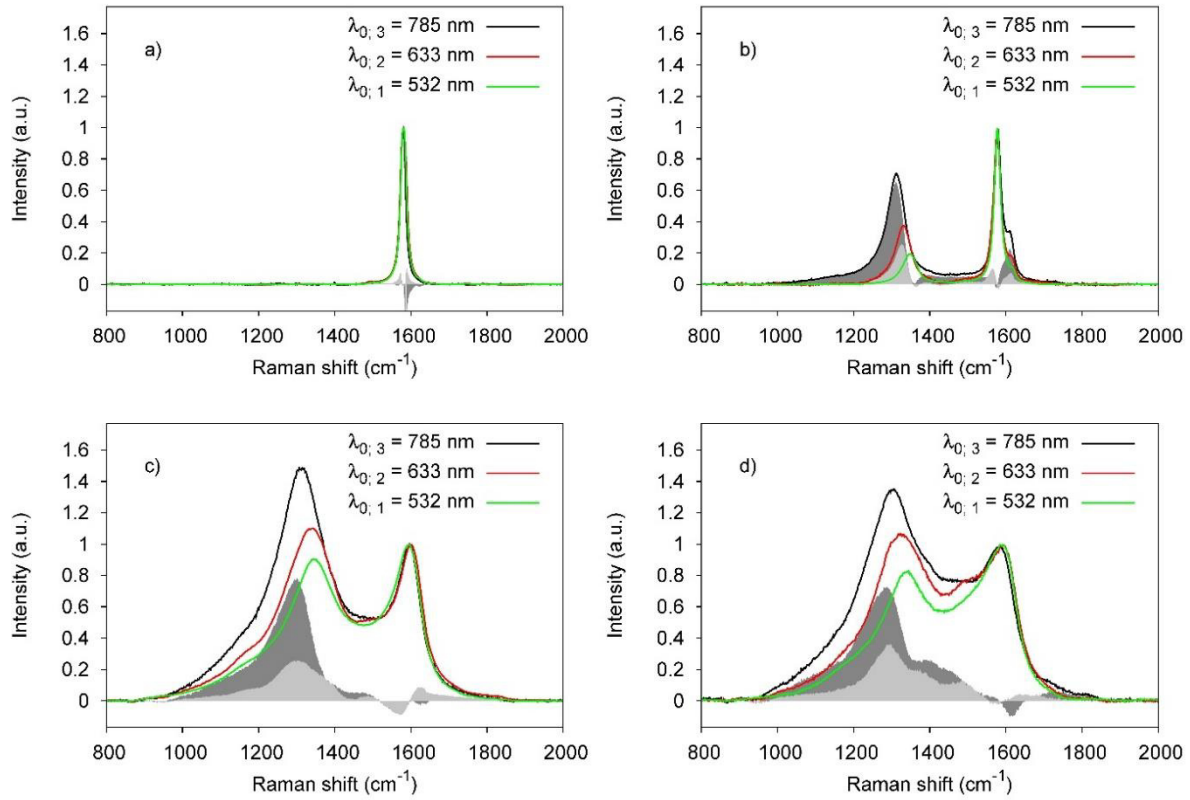

***Figure 4**: Raman spectra of (a) HOPG, (b) graphite powder, (c) DS 12, and (d) GfG soot (sorted by increasing structural disorder) measured at different excitation wavelengths ($\lambda_{0;1}$ = 532 nm, $\lambda_{0;2}$ = 633 nm, $\lambda_{0;3}$ = 785 nm). The grayish areas are the result of the subtraction of the $\lambda_{0;1}$ spectra from the $\lambda_{0;3}$ spectra for dark gray and the $\lambda_{0;2}$ spectra for light gray, resp. From Schmid et al. [8].*

The applicability of the multiwavelength approach was proven by investigating various diesel soot samples and related carbonaceous materials at different λ_0 (785 nm, 633 nm, 532 nm and 514 nm). As shown in Figure 4, only HOPG sample exhibits the invariance of the Raman shift at different λ_0 (G peak at 1580 cm^{-1}). However, already for graphite powder and for all studied soot samples the dispersive character of the D peak can clearly be observed. Additionally, the changes become more pronounced

with increasing nanostructural disorder. Furthermore, good correlation between these Raman values and the corresponding TPO data was found (Figure 5).

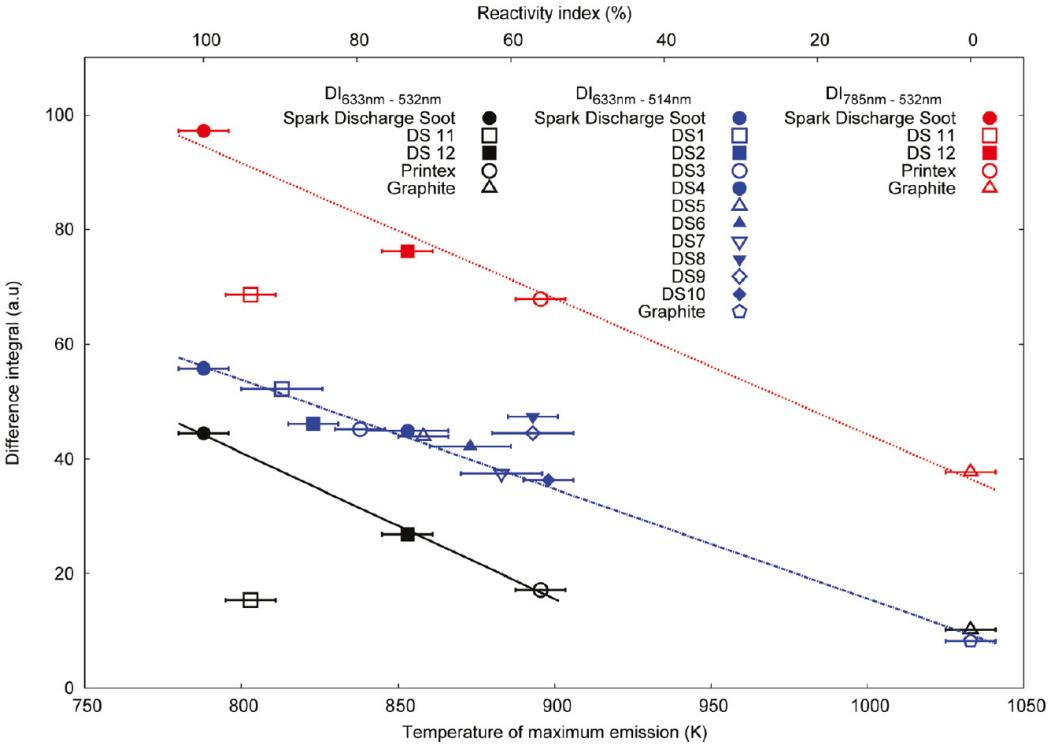

***Figure 5**: TPO results (maximum emission temperature) and the reactivity index (GfG soot and graphite powder represent higher and lower reactivity limits, resp.) versus the difference integral for various soot samples and carbonaceous materials. From Schmid et al. [8].*

The production of biodiesel fuels has been increasing continuously in the last decade, since the EU demands the use of renewable energy sources. Hence, the information on the structure and reactivity of biodiesel soot is of high interest. However, very contradictory results can be found in the literature. Therefore, we have studied the reactivity of soot produced by a diesel engine operated with fuels of different biodiesel content [15] (**PII**). TPO results indicate an increasing reactivity with increasing biofuel ratio. This implies that soot generated with 100% biofuel (consisting of rapeseed oil methyl ester) is more reactive than soot generated with commercial gasoline fuel containing up to 7% biodiesel, while soot from fossil fuel is even less reactive. Surprisingly, RM analysis yields very similar spectra for the samples, indicating that all investigated soot samples possess a similar graphitic nanostructure [15] (**PII**). However, we found that the reactivity of biodiesel soot increases with decreasing size of soot agglomerates as well as with increasing content of Fe, Zn, and Cu in the soot, which was determined by inductively coupled plasma mass spectrometry (ICP-MS). Thus, the soot reactivity is not determined by a single parameter, but by a combination of many soot properties, such as nanostructure, particle size and/or inorganic components (impurities or additives) [16-18].

The potential of Raman microspectroscopy was further tested for the analysis of soot with different organic carbon (OC) content. Carbonaceous aerosols are often characterized by black carbon (BC), elemental carbon (EC) and/or OC content. The term BC is linked with the strong absorption properties of aggregates of small carbon spheres with predominantly graphite-like nanostructure. The term BC is often used equally with elemental carbon (EC) [19], although BC and EC are operationally defined. EC is a carbonaceous fraction that is inert and nonvolatile in the atmosphere [20]. EC and OC [21] are determined operationally by thermal-optical reflectance and thermal-optical transmission techniques [19,22]. But describing a soot composition by its EC/OC content may be afflicted by errors and not comparative with the findings of others as EC and OC which are defined by the used method. Hence, the separation of EC and OC can be ambiguous [19,22]. Thus, the information on the relation between OC content and soot properties, including the structure and reactivity, is highly desired.

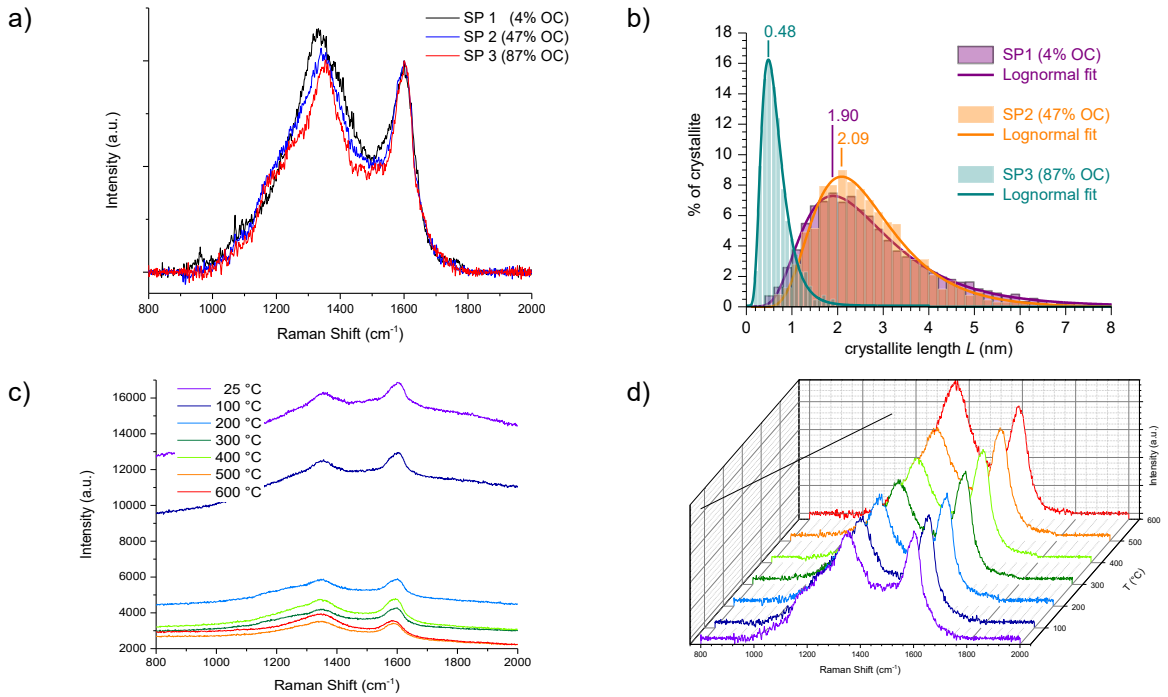

***Figure 6**: Raman spectra of untreated soot samples with different OC content (a). Length's distributions of soot nanocrystallites from HRTEM images (b). Raman spectra without baseline correction and normalization of the sample with 87% OC (c). Evolution of the Raman spectra of the soot sample with 87% OC content with increasing temperature (d). From Ess et al. [3] (**PI**).*

We have applied RM in combination with HRTEM and FT-IR spectroscopy for the characterization of soot with different organic carbon (OC) content (4%, 47% and 87%) [3] (**PI**). The FT-IR analysis of the samples revealed their organic composition by showing aromatic compounds for the samples with 47% and 87% OC additional to the aliphatic compounds and ketones/aldehydes present also in the sample with 4% OC. According to the RM data and in agreement with HRTEM analysis, the nanostructural order was high for the soot with 4% of OC and low for the soot with 87% of OC (Figure

6a,b). Furthermore, we have performed *in situ* RM analysis during the soot oxidation at temperatures up to 600 °C in air using a heating stage. The (fluorescent) organic components were evaporated/transformed or oxidized with increasing temperature (up to 500 °C), and the soot nanostructure changed significantly (Figure 6c,d). At 600 °C the chemical heterogeneity vanished and the structural order increased, since the organic components as well as amorphous carbon were oxidized by that time [3] (**PI**). Thus, significant differences in the structure and reactivity of soot with different organic carbon (OC) content were revealed. These results can help in understanding the relation between the OC content in the soot and its structure, reactivity and impact on the environment.

Thus, RM provides information on the soot nanostructure and allows for the prediction of soot structure-related reactivity. Hence, it can be applied together with other methods, e.g., HRTEM, FT-IR and TPO for the comprehensive characterization of carbonaceous aerosols in order to get better understanding of their properties and impact on the environment and human health. Furthermore, RM is an efficient tool for the structural characterization of graphene nanoarchitectures (e.g., produced by photo-induced C-C reactions in insulators [23]) or for the determination of graphene doping (induced, e.g., by organic solid-solid wetting deposition) [24] (**PIII**).

3. Analysis of microplastics and nanoplastics by RM-based methods

Synthetic polymer (usually termed plastic) materials have become an inherent part of our everyday life. Being lightweight, durable and corrosion-resistant, they offer remarkable technological and medical benefits. Plastic production grows, reaching 64.4 million metric tons (Mt) in Europe and 348 Mt globally in 2017 [25]. Unfortunately, only 73% of plastic is recovered through recycling (42%) and energy recovery (31%). The remaining 27% of the plastic waste are transported to landfills [25], and a part of it is carried away by winds. Along with carelessly discharged materials, plastic waste continuously enters the environment. Despite the general durability of synthetic polymers, a combination of mechanical abrasion, UV irradiation, and (micro)biological degradation in the environment causes the formation of tiny plastic fragments – secondary microplastic (MP). Apart from these, the so-called primary MP particles are designed and produced on purpose (e.g., virgin plastic pellets or MP for industrial cleaners and personal care products) and can also enter the environment by different pathways. Therefore, the contamination of the environment with plastic, and especially with MP is of increasing scientific and public concern. MP is defined as synthetic polymer particles (including fragments, spheres, films and fibers) in the size range of 1 µm – 1 mm [26,27]. Plastic particles with sizes between 1 mm – 5 mm are called large MP [27]. Recently, it has been proposed that also smaller plastic particles, the so called submicro- (100 nm – 1 µm) and nanoplastic (<100 nm) are discharged into the environment or/and are formed from larger (micro)plastic debris [28-30]. Most studies on the chemical composition of MP in aquatic systems have reported polyethylene (PE), polypropylene (PP), polystyrene (PS), and, less frequently, polyamide (PA), polyvinyl chloride (PVC) and polymethyl methacrylate (PMMA) [31] (**PIV**).

Undoubtedly, (micro)plastic as global anthropogenic contaminant represents a big aesthetic problem. It is also assumed that MP could have negative impact on public health and on the environment. Since small-size MP can be ingested by different aquatic organisms, MP could enter the food chain and accumulate at higher trophic levels [32]. In particular, the negative impact of plastic debris on living organisms could be related to the leaching of monomers and additives, some of which have been proven to be toxic, carcinogenic, or endocrine-disrupting [32]. Furthermore, due to the large surface-to-volume ratio and the nature of the MP surface, it can enrich persistent organic pollutants (POPs, for example, polychlorinated biphenyls (PCBs), polycyclic

aromatic hydrocarbons (PAHs)), or toxic metals from aquatic environments [33,34]. In addition, it has been shown that MP particles can act as a vector or carrier (for the ecosystem) of foreign species and potentially pathogenic microorganisms [35,36]. However, the reported results on the MP impacts are very contradictory, ranging from detrimental (including lethal) through no-effects up to detoxification (when the initial concentration of pollutants in organisms was higher than in ingested MP) [37]. It is noteworthy that, in most experiments, very high MP concentrations were used. Therefore, it is important to investigate the effects of MP under environmentally relevant conditions [31] (**PIV**).

However, the degree of MP contamination of the environment remains uncertain. Depending on sampling, processing, and especially identification methods, reported values for number concentrations span ten orders of magnitude (10^{-2} – 10^{8} items/m^3 across individual samples and water types [31,38]. Therefore, prominent efforts are being undertaken in Germany [39], Europe [40] and worldwide to improve and harmonize methods for representative sampling and sample preparation, identification and quantification of MP in different environmental matrices.

3.1 Identification and quantification of microplastics

The identification and quantification represent the crucial step in MP analysis [31] (**PIV**), [41]. The commonly applied visual sorting can lead to a high level of false (positive and/or negative) results (up to 70% [33]), especially for particles <500 µm (e.g. quartz particles are frequently mistaken for MP). Detailed information on the polymer type and additives of MP can be achieved by thermoanalytical methods – pyrolysis gas chromatography mass spectrometry (Pyr-GC-MS) and thermo-extraction and desorption coupled with gas chromatography mass spectrometry (TED-GC-MS) [41,42]. For example, TED-GC-MS can provide valuable data on the mass fraction of different polymer types in environmental samples [41]. However, since bulk samples are measured, information on the particle size distribution is lost. In contrast, spectroscopic methods – attenuated total reflection (ATR)- Fourier-transform infrared (FT-IR) spectroscopy, micro-FT-IR spectroscopy and Raman microspectroscopy (RM) are appropriate for the analysis of single particles [31] (**PIV**), [41]. While ATR-FT-IR is applied for the detection of MP particles larger than 500 µm, micro-FT-IR enables the

(automated) detection of particles down to 10 – 20 µm [43-45]. As shown in Figure 7, RM is suitable for the analysis of MP in the entire size range (1 µm – 5 mm) [46-49], [50] (**PV**), [51] (**PVI**). Figure 8 shows examples for Raman spectra of common polymers.

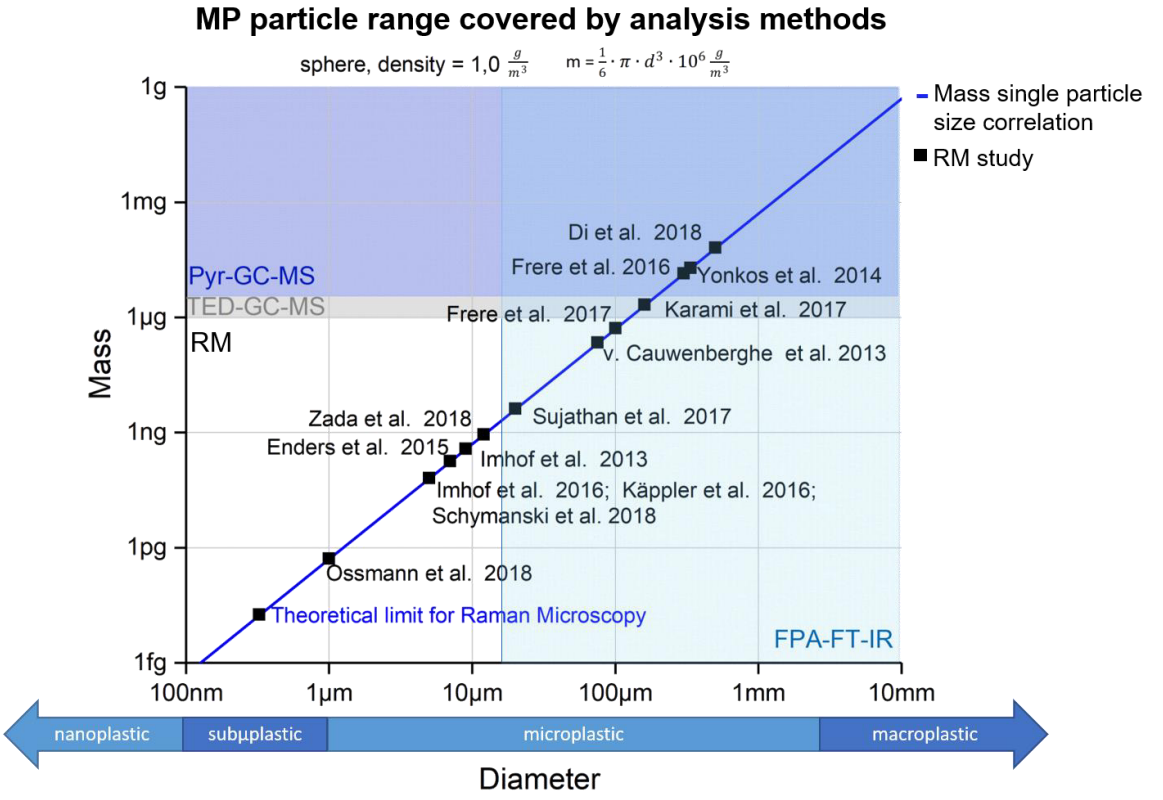

***Figure 7**: Mass to diameter correlation of spherical MP particles with a density of 1 g/cm³ (dark blue line). Analytical range of TED-GC-MS (gray) and Pyr-GC-MS (dark blue) for PE, as the most commonly found MP. As well, the limit for focal plane array detector (FPA)-FT-IR (light blue) leaving the niche for Raman microspectroscopy (white). Points indicate smallest reported MP. From Anger & von der Esch et al. [51] (**PVI**).*

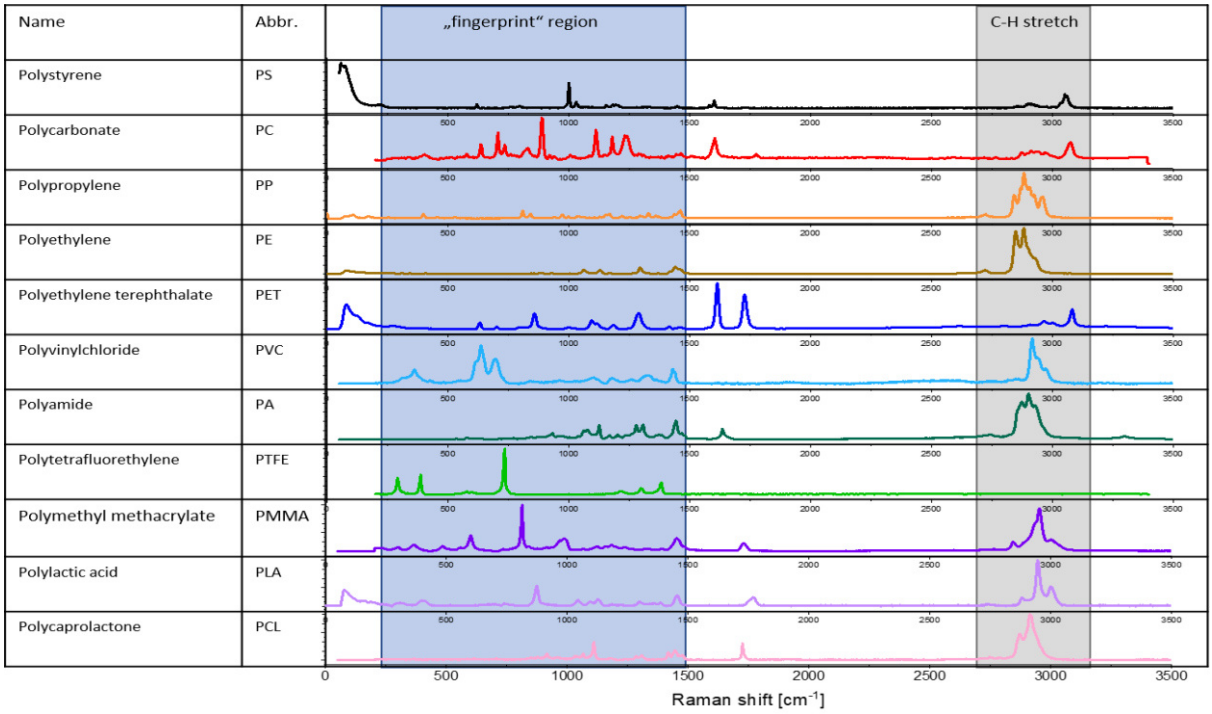

***Figure 8**: Raman spectra of relevant polymers. "Fingerprint" region and region for C-H stretching modes of alkyls, alkenes and aromatic protons are highlighted. From Anger & von der Esch et al. [51] (**PVI**).*

Although RM is very efficient for the identification of synthetic polymers, the analysis of environmental samples can be hampered by the interference of fluorescence from (micro)biological, organic (e.g. humic substances), and inorganic (e.g., clay minerals) contaminations. Therefore, the samples should undergo purification before Raman analysis. Additionally, the choice of appropriate acquisition parameters (laser wavelength, laser power, photobleaching, measurement time, magnification of objective lens, confocal mode) is important to circumvent the problem of strong fluorescence background [31] (**PIV**).

We have reported the first investigations (in cooperation with Prof. Dr. C. Laforsch, University Bayreuth) on the microplastic contamination of freshwater ecosystems in Europe (the subalpine Lake Garda, Italy, was chosen as example) in 2013. We applied RM for the analysis and showed that these systems act, at least temporarily, as sinks for plastic particles. In samples from beach sediments of Lake Garda we found primarily low density polymers, namely PS (45.6%), PE (43.1%) and PP (9.8%). However, in the size class of very small microplastic particles (9 – 500 µm), also PA and PVC were identified [47]. In a follow-up study [50] (**PV**), we have focused on a qualitative and quantitative RM analysis of microparticles of different size classes from sediment samples in Lake Garda. For the separation of plastic particles from sediment

we used the Munich Plastic Sediment Separator (MPSS) [46] which was developed (in cooperation with Prof. Dr. C. Laforsch, University of Bayreuth) and built at our institute. In the sediment samples we identified about 600 microplastic particles with a diameter down to 4 µm. Apart from plastic particles, a large number of pigmented (non)plastic particles were detected. ICP-MS analysis showed that pigmented particles can contain large amounts of (toxic) heavy metals. The number of these particles (Figure 9) increases with decreasing size, which suggest that even smaller pigment particles might be present (down to the nm-range).

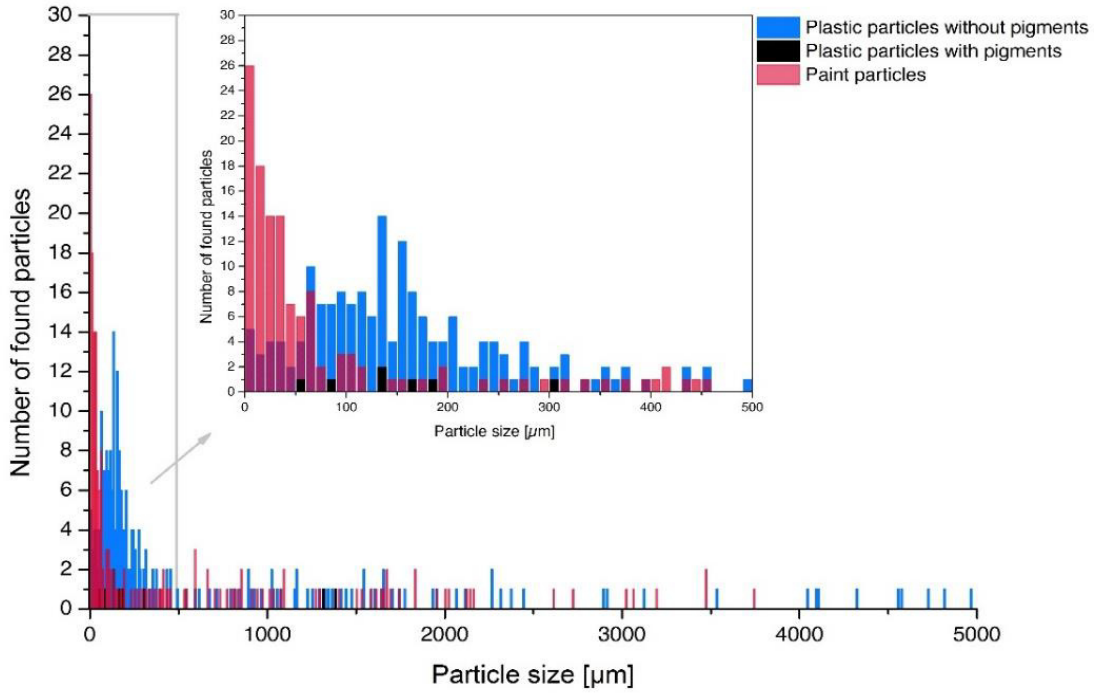

***Figure 9**: Size distribution of the particles from Lake Garda beach sediment. For plastic particles the maximum is located at around 130 µm. The amount of paint particles increases with a decrease in size. This is highly pronounced in the size class below 50 µm. From Imhof et al. [50] (**PV**)*

Even though RM has greatly advanced in recent years, to become a useful tool for the detection of MP in the environment, there is a room for significant improvement and development of this technique. Especially, the MP particles <20 µm provide a niche for RM [51] (**PVI**). The most urgent challenges are to establish representative measurements and to automate the procedure. However, how many particles need to be analyzed to get a statistically meaningful result? In order to answer this question, we have suggested a random sampling approach – *simple random sample of units selected without replacement* (*srswor*). The *srswor* is an unbiased sub sampling technique which helps us to determine the number of particles needed for the analysis. It also acts as a virtual mixing, so that aggregation of particles similar physical

properties within the sample does not lead to a pronounced grouping and segregation error the analysis. Depending on a total number of particles on the filter, an estimated MP fraction and acceptable margin of error. Our calculations show that for, e.g., a filter with 10^6 particles, MP fraction of 5% and the margin of error of 10%, the analysis of around 5000 particles will be sufficient. It is not necessary to analyze all particles on the filter in order to obtain statistically reliable results and more importantly there is a limit, where measuring more particles will lead to significantly higher measurement time, but the margin of error will not significantly improve. However, even if the number of particles which need to be measured is reduced down to several thousands, it is very difficult and extremely time consuming to perform such analysis manually. Therefore, automation of the entire procedure is required, including i) recognition and localization of particles deposited on a filter, ii) their morphological characterization (size/size distribution and shape), iii) calculation of the number and random selection of particles that need to be measured, iv) their chemical characterization by RM, and v) spectral identification of particles and summary the results. Furthermore, it is important to develop advanced automated particle recognition and characterization which are appropriate for all MP shapes (spheres, fragments and fibers) without miscalculation of particle sizes and size distributions. Altogether, this will enable morphological and chemical characterization of microplastic particles and fibers measured by RM (and, additionally, morphological characterization of non-microplastic particles and fibers recognized on the filter). Although some commercial programs for one or several steps are available, none of them is currently suitable and validated for this five-step MP analysis. Therefore, we are working on our own automated procedure. For the particle recognition and morphological characterization (steps i and ii) we have already implemented *Otsu's* algorithm (which is an automatic thresholding algorithm that splits pixels in two groups (bright and dark) by minimizing the between-class variance of the two groups) [52]. Based on the already quite successful characterization of microplastic particles with the first program, a second more advanced characterization tool (TUM-ParticleTyper, TUM-ParTy) is in preparation. TUM-ParTy features the localization of particles visualized by optical, fluorescence, as well as SEM analysis, which makes the program suitable for various MP detection protocols. Furthermore, this program is equipped with an image calibration tool, which enables users to automatically find a suitable parametrization for new samples and new device settings. By analyzing a new set of images with the optimal

parametrization, the detection limit and localization error can be estimated [von der Esch & Kohles et al., in preparation]. Statistical sample size reduction and automation of the MP detection, identification and quantification is expected to significantly accelerate the overall analysis, leading to a higher sample throughput and, simultaneously, providing high analytical accuracy for MP analysis.

RM can be applied not only for the analysis of MP on the filter, but is also well suited for the 2D and 3D visualization of MP in biota samples, e.g. of MP incorporated in tissues or ingested by aquatic organisms (Figure 10). Especially the analysis of particles in the lower µm-range is of high importance for the assessment of environmental risks associated with MP (e.g., because it can be translocated in tissues).

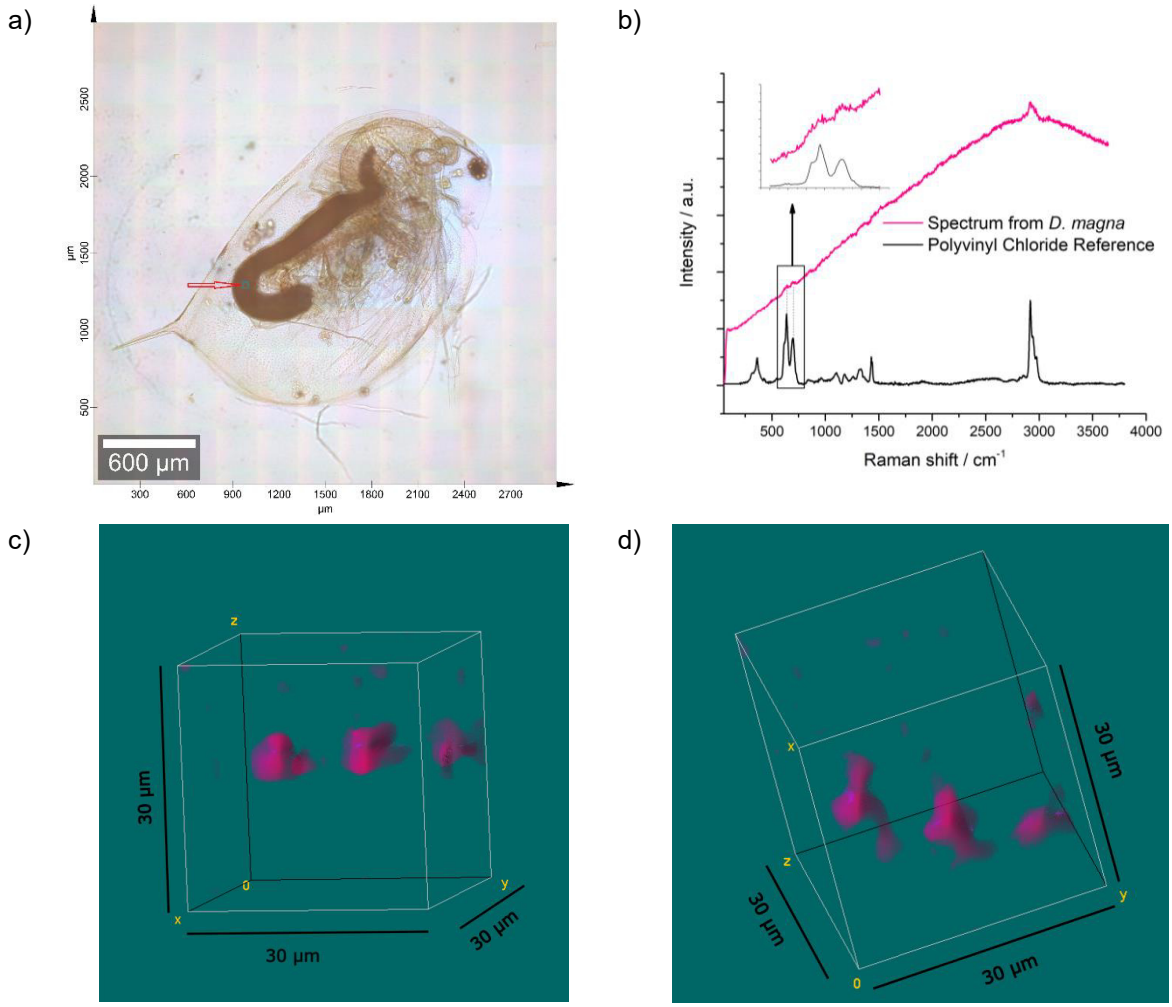

***Figure 10**: Microscopic image of Daphnia magna fed with PVC (a), corresponding Raman spectra (b) and 3D Raman images (c and d; magenta: PVC particles) of the marked part in the microscopic image (sample preparation by Dr. H. K. Imhof, analysis by P. M. Anger).*

3.2 Detection of plastic particles smaller than 1 µm

Recently, questions concerning even smaller particles, so-called nanoplastics, have emerged and became of pressing interest, especially since they have been detected in facial scrubs [53] and in marine surface waters [30]. Often, plastic particles below 1 µm are called nanoplastics. However, since particles <100 nm are already defined as nanoparticles by the International Union for Pure and Applied Chemistry (IUPAC) and the International Organization for Standardization (ISO), the particles in the size range 100 nm – 1 µm can be assigned to subµ-plastics [26,31].The topic of subµ-plastics and nanoplastics, thereby, creates a cross-section with nanoparticle science, since nanoplastic particles are in principle polymeric nanoparticles [54]. It is, however, well placed in the field of environmental plastic analysis, since it is part of the whole plastic contamination problem [55] (**PVII**). It is worth to note that the mass of the particle decreases with the third power of its diameter *d*. Therefore, one 100 µm plastic particle (m = 1 mg) is equivalent to a thousand of 10 µm, a million of 1 µm, a billion of 100 nm and a trillion of 10 nm particles. Thus, subµ- and nanoplastic can constitute high particle numbers but, at the same time, low masses in a sample and, therefore, analytical techniques have to provide low particle size detection limits and/or low LODs in terms of mass to detect these plastic particles.

Surely, by the analysis of the plastic particles smaller than 1 µm we are facing a methodological gap (Figure 11). When entering the nanometer size range, a new approach in the analytical methodology must be taken. This concerns specific characteristics, such as the particle size distribution (PSD) or morphology and the chemical identity, for which techniques that detect particles in the nanometer range will be needed. In addition, an appropriate sample treatment, especially, a preconcentration and also a separation step to properly isolate the particles, will be an essential part of the required protocol [55] (**PVII**).

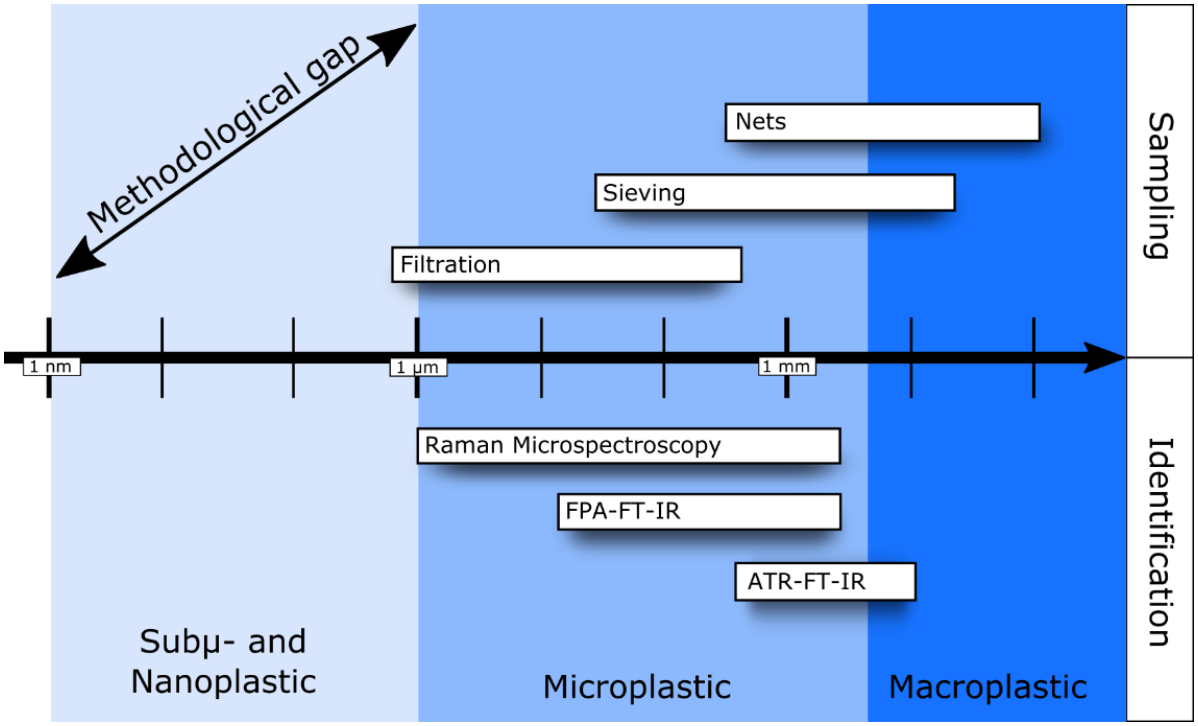

***Figure 11**: The analysis of MP is established for particles down to 1 µm. Below, there is a methodological gap. From Schwaferts et al. [55] (**PVII**).*

The established methods for MP analysis, however, have a potential to be adapted for the analysis of subµ- and nanoplastic particles, by combining them to other techniques. Very promising is a combination of Raman microspectroscopy and scanning electron microscopy (SEM). Here, the RM can provide diffraction-limited (down to around 300 nm) chemical information on subµ-plastic particles at the single-particle level, while SEM can be applied to verify the size of analyzed particles and to get further information on their morphological characteristics. We have found that the combination of RM and SEM analysis enables reliable characterization of PS particles down to 500 nm (Figure 12) and even 250 nm [Schwaferts et al., in preparation].

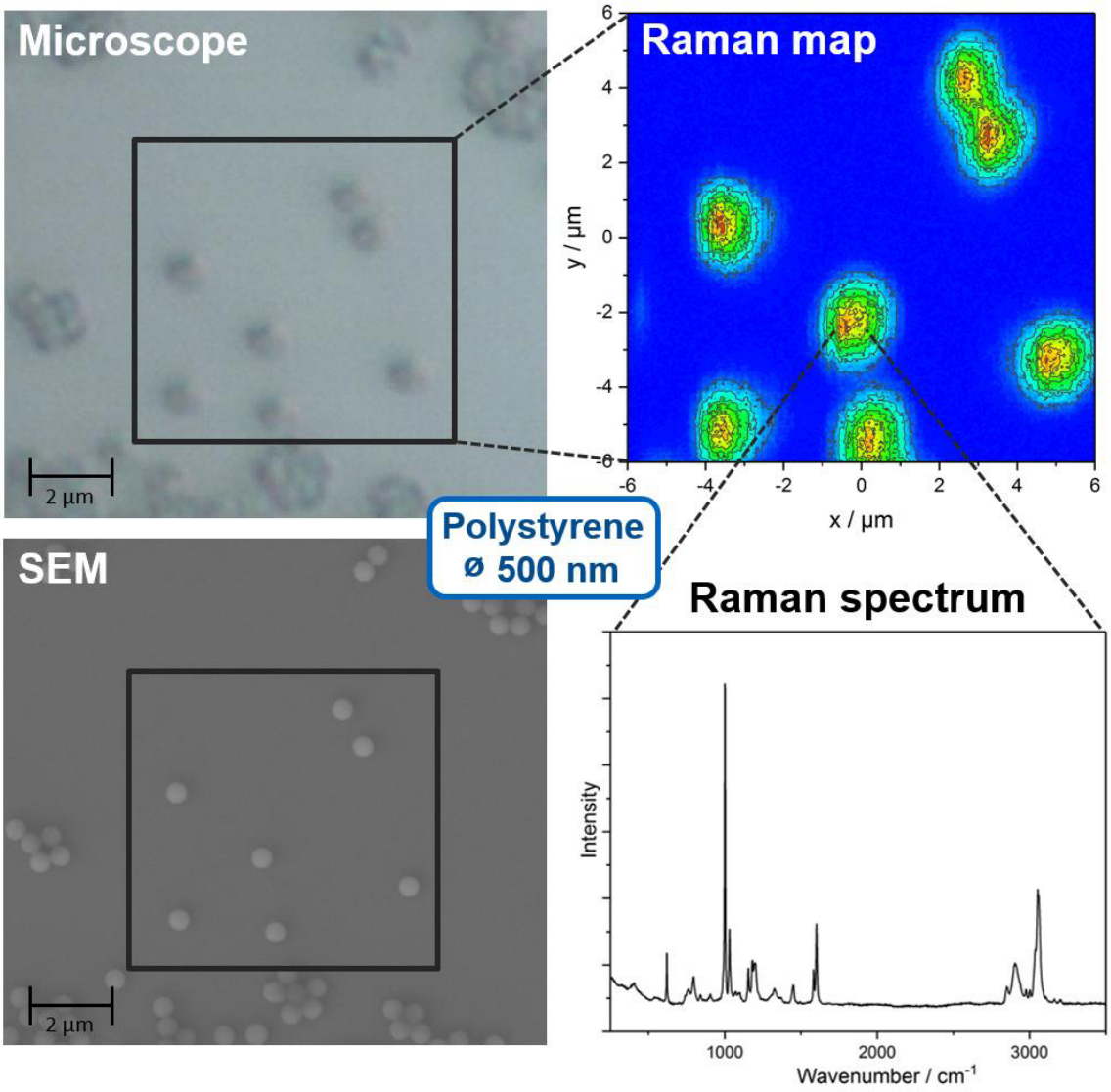

***Figure 12**: Optical image, Raman microspectroscopic image and Raman spectrum as well as SEM image of 500 nm PS particles on Al-coated slide. Sample preparation and RM-SEM analysis by C. Schwaferts.*

Although the combination of RM and SEM yields very valuable information, this approach only allows us to analyze individual particles and is very time consuming. Therefore, alternatively, RM analysis of bulk samples can be applied for different size fractions of subµ-particles, e.g., by fractionation methods such as asymmetrical flow field-flow fractionation (AF4) and centrifugal field-flow fractionation (CF3). These methods can be extended by using UV-visible absorption and multi-angle light scattering (MALS) detectors [56-58] for the characterization of concentration and size distribution of particles, respectively. We have already combined these fractionation methods for the particle separation and size characterization with RM for offline and also for online chemical identification of subµ-particles. For the online analysis, a Raman flow cell has been designed [59] and applied. This cell utilizes 2D optical tweezers for particle trapping, in order to increase the efficiency of the Raman analysis. Particles of different materials in the size range from 200 nm to 5 µm, with

concentrations down to 10 µg/L (e.g., for 600 nm PS particles) can then be identified [Schwaferts et al., in preparation].

Thus, among the available methods, Raman microspectroscopy is best suited for the identification and quantification of different types of plastic and pigment particles down to 1 µm and even below. Implementation of the statistical sample-size reduction and automation will facilitate an overall faster procedure and higher sample throughput, simultaneously providing high analytical accuracy of MP analysis. Additionally, RM can be applied not only for the analysis of MP on filters. This method allows for the visualization and characterization of microplastic particles in biota samples by 2D and 3D Raman imaging. Since RM enables the analysis of the particles in the lower µm-range within tissues samples (or even entire small organisms, e.g., *Daphnia magna*), it can provide valuable data for the assessment of environmental risks associated with MP. Furthermore, RM in the combination with SEM yields diffraction-limited (down to around 300 nm) chemical information on subµ-plastics on the single-particle level. Finally, RM has a potential for a high throughput offline and online chemical characterization of subµ-plastic particles by the combination with fractionation techniques (e.g., AF4 and CF3) and, hence, can enable the reliable analysis of subµ-plastics and nanoplastics from real samples in the future.

4. Stable isotope Raman microspectroscopy (SIRM) in analytical chemistry

Stable isotope-based analytical methods gain increasing relevance in different scientific fields. Although mass spectrometry-based (MS) methods enable sensitive analysis of bulk samples (e.g., isotope ratio mass spectrometry, IRMS) [60,61] or provide a spatial resolution down to 50 nm (e.g., nanoscale secondary ion mass spectrometry, NanoSIMS) [62,63], these methods are destructive and require time-consuming sample preparation. Here, a combination of Raman microspectroscopy (RM) with the stable isotope approach – stable isotope Raman microspectroscopy (SIRM) – can extend the capabilities of the well-established techniques with a nondestructive, quantitative and spatially-resolved analysis. SIRM provides characteristic fingerprint spectra of samples with the spatial resolution of a confocal optical microscope, containing information on stable isotope-labeled substances and the amount of a label (based on red shift of bands of the labeled substances). Simultaneously, these spectra deliver information on the chemical composition and structure of samples. Furthermore, this method requires no or limited sample preparation, and can be performed *in situ* and *in vivo* without spectral interference of water [64-69], [70] (**PVIII**).

4.1 SIRM for quantitative analysis of organic substances

To put further approaches on a firm basis, in a first step we have performed the analysis of stable isotope-labeled reference compounds, in order to reveal the feasibility of the SIRM technique for the quantification of isotope ratios and absolute concentrations. To this end, $^{12}C/^{13}C$-phenylalanine, $^{12}C/^{13}C$-glucose and $^{12}C/^{13}C$/D-sodium acetate were mixed in different proportions to create standards representing different labels of stable isotope tracer (e.g., 1 – 99% of ^{13}C). The ratios of the intensities for ^{13}C- and ^{12}C-related peaks as well as a multivariate calibration method, called partial least-squares (PLS), were used to determine the ^{13}C-content. A more sensitive LOD of 2.8% ^{13}C-content (for Phe) was calculated for the SIRM approach. Additionally, the minimal absolute amount of the ^{13}C-compound detectable in the laser spot was determined. With acquisition times of 100 s per spectra, 0.148 ± 0.008 and 0.327 ± 0.017 pg ^{13}C-glucose can be detected for the 532 nm laser (8.4 mW at the sample) and the 633 nm laser (3.7 mW at the sample), respectively [71].

At the next step, we have examined the potential of SIRM for the evaluation of differently enriched ^{13}C-labeled humic acids (HA) as model substances for soil organic matter. Using glucose and urea as educts for synthesis, artificial HA with known isotopic compositions were produced and analyzed. By performing a controlled burning (pregraphitization using 532 nm excitation laser), a suitable analysis method was developed to cope with the high fluorescence background. The results were verified against IRMS (in cooperation with Prof. M. Elsner, Institute of Groundwater Ecology, Helmholtz Zentrum München, now director of IWC-TUM). The limit of quantification

was determined as 2.1 × 10^{-1} $^{13}C/C_{tot}$ when evaluated from all points of the calibration and 3.2 × 10^{-2} $^{13}C/C_{tot}$ for a linear correlation up to 0.25 $^{13}C/C_{tot}$ (Figure 13). Complementary, NanoSIMS analysis (in cooperation with Prof. Dr. I. Kögel-Knabner and PD Dr. C. W. Müller, Chair of Soil Science, TUM) indicated good qualitative agreement, but small-scale heterogeneity within the dry sample material. Our study shows that SIRM is well-suited for the analysis of stable isotope-labeled HA. This method requires no specific sample preparation and can provide information with a spatial resolution in the µm-range [72] (**PIX**).

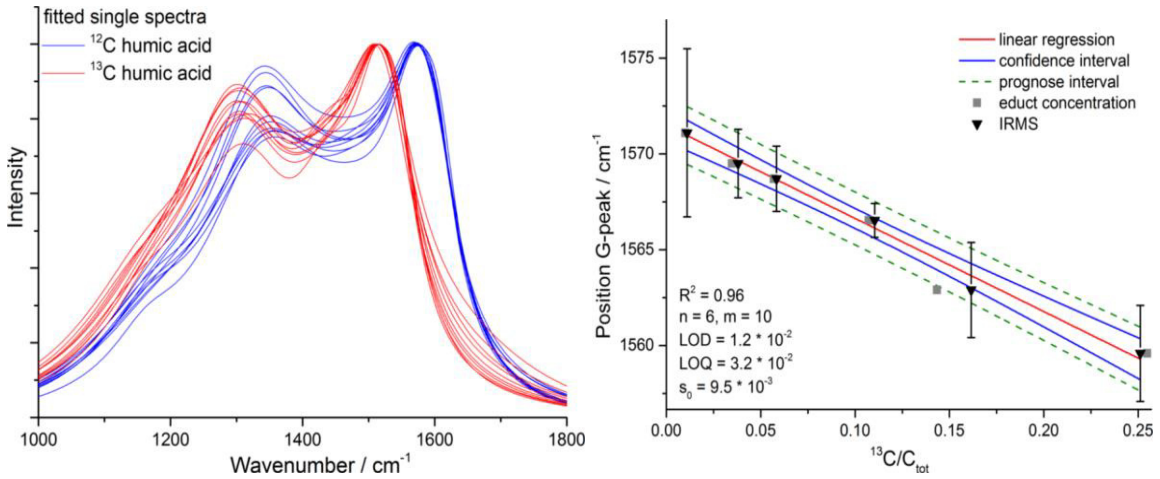

***Figure 13**: Fitted and baseline-corrected Raman spectra of ^{12}C- and ^{13}C-labeled HA with G (graphite) and D (defect) peaks at ca. 1600 cm^{-1} and 1350 cm^{-1}, resp. (left). Linear regression of the relative amount of $^{13}C/C_{tot}$ (C_{tot} = total amount of carbon) and Raman shift of G-peak of the fitted spectra for HA samples up to 25% of ^{13}C-content. From Wiesheu et al. [72] (**PIX**)*

4.2 SIRM for the analysis of microorganisms and biofilms

In environmental chemistry, RM and especially SIRM have a high potential for the analysis of microbial communities (biofilms) and their metabolic functions. Microorganisms living in diverse natural environments usually form biofilms, where cells are embedded in a hydrogel matrix of extracellular polymeric substances (EPS). RM was shown to be suited for the characterization of entire biofilms, including microbial constituents and EPS matrix [70] (**PVIII**). Biofilms are essential for global biogeochemical cycles and, especially, for the biodegradation of pollutants that are related to water quality. Here, SIRM can provide information about metabolic pathways and carbon flows together with “whole-organism fingerprints” at the single cell level [64-68], [70] (**PVIII**).

Raman band shifts in isotope-labeled bacterial cells were first reported by Huang *et al.* in 2004 [64] for *Pseudomonas fluorescens* grown in media containing different ratios of ^{12}C-glucose and ^{13}C-glucose as the sole carbon source. Red-shifts of many different Raman peaks were assigned to proteins, lipids and nucleic acids. Furthermore, in 2007 Huang *et al.* [65] showed the possibility to combine SIRM with an *in situ* identification method (fluorescent *in situ* hybridization, FISH), for the simultaneous determination of

^{13}C-incorporation into biomass by RM and the identification of cells by FISH. An almost linear correlation between the known ^{13}C-content of the cultivated microorganism and the phenylalanine (Phe) peak ratio was found (i.e., the ratio of the Phe band at 966 cm^{-1} in bacteria grown in 100% ^{13}C-glucose compared to the band of 1003 cm^{-1} in ^{12}C-cultivated bacteria). A minimum labeling of only 10% ^{13}C-content was sufficient to discriminate between labeled and unlabeled cells.

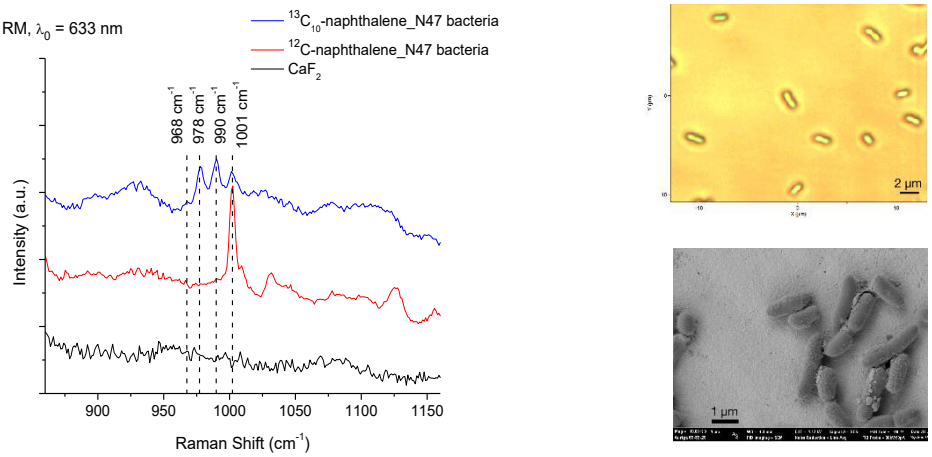

***Figure 14**: Raman spectra of N47 cells cultivated with either ^{12}C-napthalene or ^{13}C-naphthalene and the characteristic red-shift of the Phe band (left). The four highlighted peaks were assigned to four different isotopologues of Phe (with 0, 2, 4 or 6 ^{13}C-atoms). Optical microscope and SEM images of single cells of strain N47 (right). From Kubryk et al. [71].*

We have applied SIRM for the analysis of the *Deltaproteobacterium* strain N47 (a strictly anaerobic sulfate-reducer, that degrades naphthalene, an environmental pollutant) and showed the applicability of the sharp Phe band as a marker for the characterization of: i) the naphthalene degradation process and ii) the incorporation of stable isotope-labeled compounds into microbial biomass (Figure 14) [71].

4.3 Improvement of SIRM sensitivity by resonance and SERS effects

One major problem with RM is its limited sensitivity, caused by the low quantum efficiency of the Raman effect (typically $10^{-8} – 10^{-6}$). This usually leads to long acquisition times, especially for the analysis at the single cell level. Fortunately, there are strategies to amplify the Raman signal. One of them is resonance Raman scattering. The wavelength of the excitation laser is so that the incident photon energy is equal or close to the energy of an electronic transition of an analyte. This results in an increase of the Raman scattering intensity by a factor of $10^2 – 10^6$. The sample must contain substances that are resonance Raman active (e.g., a chromophore containing molecules such as carotenoids [73], cytochrome *c* [74], or flavin nucleotides [75]). In this context, we have explored the potential of resonance SIRM for the analysis of microorganisms containing cytochrome *c* [71]. A clear differentiation between ^{13}C-labeled and unlabeled *Geobacter metallireducens* cells was possible with a laser excitation wavelength of 532 nm (4 mW at the sample) and acquisition times as short as 1 s.

If the application of resonance SIRM for a specific sample is not possible (e.g., due to the absence of chromophore containing molecules), surface-enhanced Raman scattering (SERS) is an alternative to improve the sensitivity of RM. Raman signals of analytes can be significantly enhanced if they are located close to or are attached to nanometer-sized metallic structures (Ag or Au). Furthermore, the fluorescence – which often hampers RM measurements of organic and (micro)biological samples – can be effectively quenched by SERS. Enhancement factors of the Raman signal in the range of 10^3 – 10^{11} can be achieved, because of electromagnetic ("localized surface plasmon resonance") and chemical ("charge transfer") enhancement effects [76-80]. Furthermore, when Raman analysis with a spatial resolution down to 20 nm is desirable, tip-enhanced Raman spectroscopy (TERS) can be applied [81]. The distance (d) between the analyte and the SERS-active surface is essential, since the SERS intensity (I) decreases dramatically with distance ($I \sim d^{-12}$) in the case of electromagnetic enhancement. Hence, almost no enhancement can be achieved for d ≥10 nm. The chemical enhancement requires direct contact between the SERS-active surface and the analyte. Furthermore, the so-called hot spots can provide extra field amplification, resulting in enhancement factors of up to 10^9 – 10^{11}, and allow single-molecule detection [78]. But such high amplifications are mostly expected in very restricted areas, and hence are hardly reproducible [82]. Therefore, in the SERS analysis of bacteria, which started twenty years ago [83], averaged spectra are commonly used. However, this would contradict the required approach of analyzing single cells, based on stable isotope-induced red-shift(s) of SERS band(s). Hence, highly reproducible SERS spectra are necessary prerequisites for successful combination of the stable isotope approach with SERS. In this context, the choice of an appropriate SERS substrate which provides reproducible SERS spectra of microorganisms with good enhancement factors is an important and difficult task.

The enhancement factor depends on the metal, on the nanoparticle or nanostructure size and shape as well as on the excitation and the Raman scattered wavelengths. Furthermore, the affinity of different components to Ag or Au surfaces and, hence, the associated enhancement is different. This results in the selectivity of SERS analysis. Because of different optical properties, different excitation wavelengths are optimal for diverse metal nanoparticles or nanostructures; for example, gold plasmons are red-shifted by about 100 nm compared to silver plasmons, and therefore show a stronger excitation in the red and near IR (λ >600 nm) [84]. Silver, however, is plasmonically more active, and its SERS enhancement outperforms that of gold. Therefore, Ag nanoparticles allow ultrasensitive analysis and are used more often than gold (which is, however, characterized by better biocompatibility).

The first application of SERS for the *in situ* analysis of a complex multi-species biofilm matrix has been presented by Ivleva *et al*. in 2008 [85]. Colloidal AgNP produced by reduction of silver nitrate with hydroxylamine hydrochloride were applied as the SERS medium. Because of good reproducibility and an enhancement factor of up to a hundred, it was possible to sensitively characterize different components of the biofilm matrix. Follow-up studies [86,87] reported on the feasibility of SERS imaging for microbial biofilm analysis, including the detection of different constituents and their spatial distribution in a biofilm at the initial growth phase and also in the mature matrix.

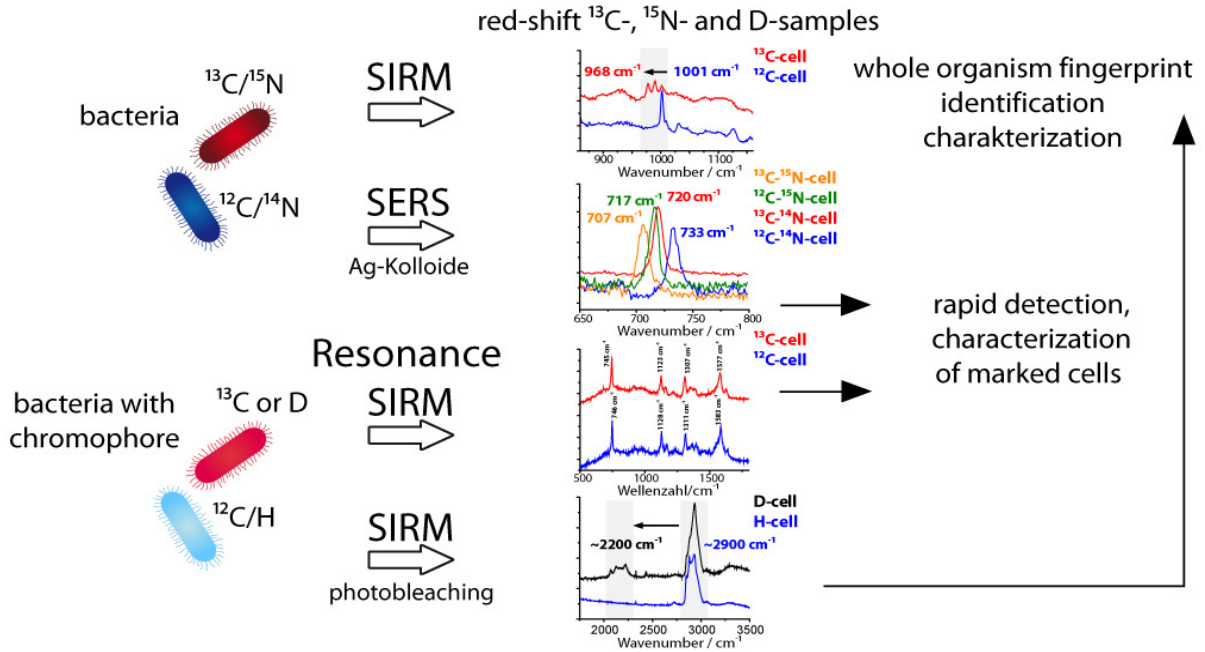

Figure 15: *Scheme of the SIRM studies with the focus on the nondestructive quantitative and spatially resolved analysis of the incorporation of the stable isotope-labeled compounds into microbial biomass. Adopted from Kubryk et al. [71].*

Our group was the first who demonstrated the feasibility of SERS for the analysis of stable isotope-labeled microorganisms on the single-cell level [71]. For this, we have applied an *in situ* AgNP preparation procedure, which has been recently developed at our institute [88]. *E. coli* cultivated with ^{12}C- or ^{13}C-glucose was used as a model organism for SERS analysis with a laser wavelength of 633 nm. A reproducible red-shift of an adenine-related marker band in the SERS spectra for ^{13}C-labeled cells was observed. The further research of our group on stable-isotope labeling, using partially and fully ^{13}C- and ^{15}N-labeled cells [89], allowed to identify purine bases as the major origin of SERS spectra. Recently, Premasiri *et al*. confirmed this finding, by studying several microorganisms with known differences in the metabolic pathway of purine at the bulk level (using an Au substrate and 785 nm excitation wavelength) [90]. They assigned these bands to purine bases and biochemically relevant derivatives, e.g., adenine, guanine, hypoxanthine, xanthine. Figure 15 summarizes SIRM studies with the focus on the nondestructive quantitative and spatially resolved analysis of the incorporation of the stable isotope-labeled compounds into microbial biomass.

Additionally, our recent study indicated that SERS signals of microorganisms are strongly influenced by the metabolic activity of the cells [91] (**PX**). We have found that different physiological conditions (e.g., storage or deuterium-labeling) have a significant impact on the release of nucleotides and/or their degradation products and, hence, on the intensity of SERS signals which they cause. These results suggest that SERS in combination with SIRM is a promising approach for the analysis of environmental samples (biofilms), which can decipher metabolic activity of microorganisms.

The combination of SIRM with SERS can allow us to perform sensitive, spatially resolved analysis of microorganisms in environmental samples. Therefore, we have tested the *in situ* AgNP synthesis as a way to accomplish a 3D detection of bacteria. Figure 16 displays the 3D SERS image of an artificial biofilm prepared with unlabelled and ^{13}C-labelled *E. coli* cells embedded into an agarose gel. The distinct signal at around 730 cm^{-1} enables the visualization of bacteria as well as discrimination between labelled and unlabelled cells [91] (**PX**).

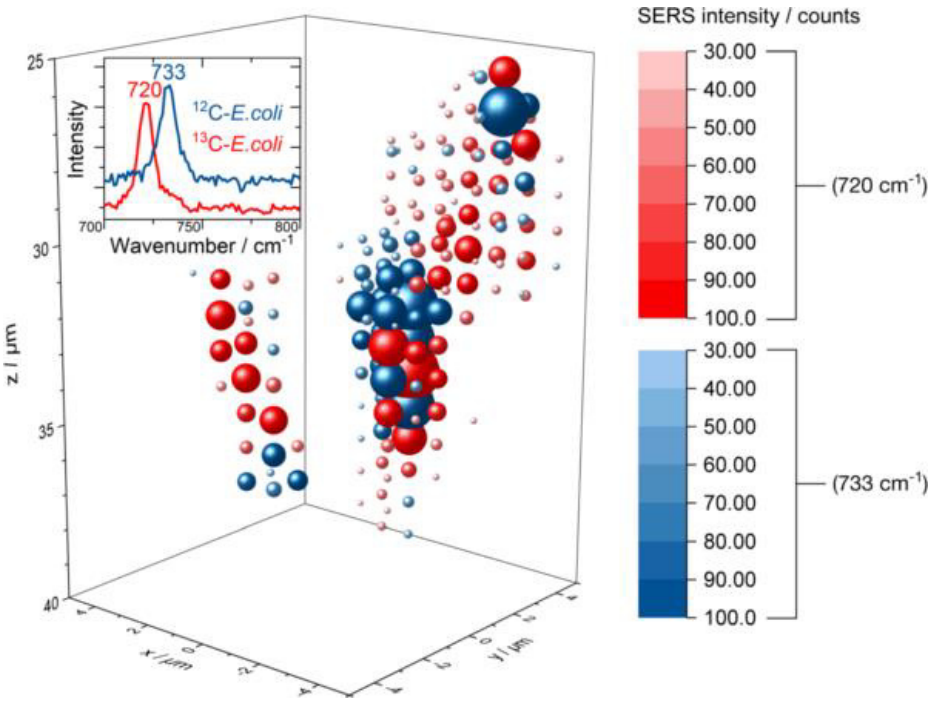

Figure 16: *3D SERS image of ^{12}C/^{13}C-labeled E. coli cells embedded into an agarose matrix. Not shifted and red-shifted SERS signal are drawn at each grid position in blue and red spheres respectively, the size and hue represent the intensity. From Weiss et al. [91] (**PX**).*

Furthermore, we have applied *in situ* SERS technique for the sorting of bacterial cells by laser tweezer Raman spectroscopy (LTRS, in cooperation with Prof. Dr. M. Wagner and M. Palatinszky, Division of Microbial Ecology, Department of Microbiology and Ecosystem Science, University of Vienna, Austria). It was possible to trap and analyze *E. coli* cells by SERS at acquisition times as short as 100 ms. The LTRS experiments performed with a mixture of unlabeled and fully ^{13}C-labeled bacteria proved that ^{13}C-isotope incorporation into trapped microbial cells can be detected based on the red-

shifted SERS signal (shift from 733 cm^{-1} to 720 cm^{-1}, Figure 17) [91] (**PX**). Thus, trapping and sorting of stable-isotope labeled bacteria can be facilitated by SERS.

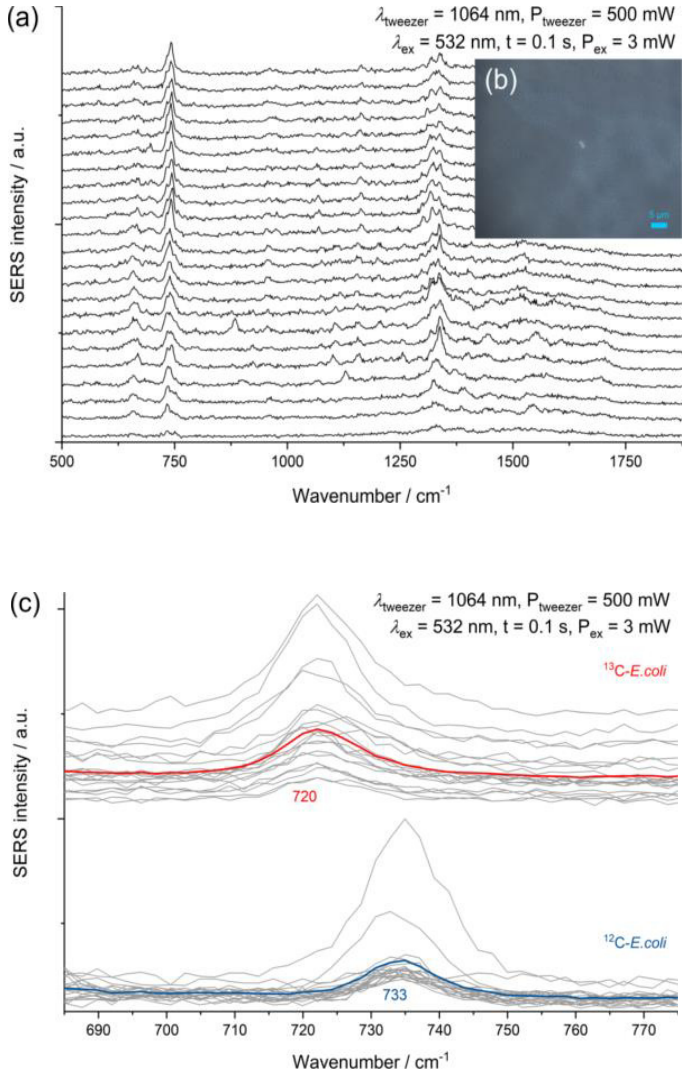

Figure 17: *(a) Continuously acquired spectra of AgNP@E. coli agglomerate inside of laser focus. (b) Microscopic image during sorting by optical tweezing with AgNP@E. coli agglomerate inside of laser focus (b). Consecutive SERS spectra of ^{13}C-E. coli (red mean spectrum) and ^{12}C-E. coli (blue mean spectrum) inside of the same sample with activated optical tweezer laser. Associated spectra are shifted for a better visualization (c). From Weiss et al. [91] (**PX**).*

Thus, SIRM (in combination with resonance and SERS effects) has a high potential for the nondestructive, quantitative and spatially resolved analysis of different environmental samples and especially, biofilms. It can provide information on the carbon metabolism/flow, cell activity, and cell interactions in microbial communities. In the future studies we plan to explore the feasibility of SIRM for the characterization of environmental microbial communities, in particular for the analysis of microbial degradation of microplastics and nanoplastics.

5. Concluding remarks

In the last fifteen years Raman microspectroscopy became a very efficient analytical technique in science and industry. The remarkable variety of applications (e.g., in inorganic and organic chemistry, pharmacology, microbiology, medicine, process control and quality control) reflects key advantages of RM, making this technique favorable compared to e.g., IR spectroscopy: i) insensitivity to water and, hence, suitability for the characterization of aqueous samples as well as (micro)biological systems *in situ* and *in vivo*; ii) a broad range of excitation wavelengths, helping to minimize the fluorescence problem and to improve the spatial resolution. Additionally, a combination of RM with stable isotope approach (SIRM) enables characterization of the molecular and isotopic composition of different samples down to µm-range. Furthermore, the sensitivity of RM and SIRM can be significantly improved by utilizing resonance or/and SERS effects.

The present work summarizes studies on the applicability of RM for the environmental analysis, performed at our institute with the focus on i) characterization of nanostructure of carbonaceous materials and prediction of their structure-related reactivity; ii) identification and quantification of microplastic and nanoplastic particles; and iii) SIRM and SERS analysis of microorganisms and biofilms.

In the future, automation of the entire RM analysis, including the recognition and localization of particles or microbial cells followed by their morphological and chemical characterization, will facilitate a higher sample throughput together with high analytical accuracy of studies. Furthermore, a combination of RM with other techniques, providing better spatial resolution (e.g., SEM, NanoSIMS) or fractionation of nanoparticles (by e.g., AF4 or CF3), can extend the applicability of RM below diffraction limit. The combination of RM with the stable isotope approach can give unique insights into the carbon metabolism/flow, cell activity, and cell interactions in microbial communities. Altogether, this should open new possibilities for comprehensive analysis of complex environmental matrices and for better understanding of processes occurring on µm-scale or on the single-cell level.

6. Literature

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7. Appendix A – Complete list of publications

current WoS h-index: 27 (before habilitation: 38 papers, WoS h-index: 17)

58) P. M. Anger, L. Prechtl, M. Elsner, R. Niessner & **N. P. Ivleva***, Implementation of an Open Source Algorithm for Particle Recognition and Morphological Characterisation for Microplastic Analysis by Means of Raman Microspectroscopy. *Analytical Methods* **2019**; DOI: 10.1039/c9ay01245a.

57) C. Schwaferts, R. Niessner, M. Elsner & **N. P. Ivleva***, Methods for the Analysis of Submicrometer- and Nanoplastic Particles in the Environment. *Trends in Analytical Chemistry* **2019**, 112, 52-65 (invited review)

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55) R. Weiss, M. Palantinszky, M. Wagner, R. Niessner, M. Elsner, M. Seidel & **N. P. Ivleva***, Surface-Enhanced Raman Spectroscopy of Microorganisms: Limitations and Applicability on the Single-Cell Level. *Analyst* **2019**, 144, 943-953

54) P. M. Anger, E. von der Esch, T. Baumann, M. Elsner, R. Niessner & **N. P. Ivleva***, Raman Microspectroscopy as a Tool for Microplastic Particle Analysis. *Trends in Analytical Chemistry* **2018**, 109, 214-226 (invited review)

53) J. Domogalla-Urbansky, P. M. Anger, H. Ferling, F. Rager, A. C. Wiesheu, R. Niessner, **N. P. Ivleva*** & J. Schwaiger*, Raman Microspectroscopic Identification of Microplastic Particles in Freshwater Bivalves (*Unio pictorum*) Exposed to Sewage Treatment Plant Effluents under Different Exposure Scenarios. *Environmental Science and Pollution Research* **2018**, 26/2, 2007-2012

52) C. Massner, F. Sigmund, S. Pettinger, M. Seeger, C. Hartmann, **N. P. Ivleva**, R. Niessner, H. Fuchs, M. H. de Angelis, A. Stelzl, N. L. Koonakampully, H. Rolbieski, U. Wiedwald, M. Spasova, W. Wurst, V. Ntziachristos, M. Winklhofer & G. G. Westmeyer, Genetically Controlled Lysosomal Entrapment of Superparamagnetic Ferritin for Multimodal and Multiscale Imaging and Actuation with Low Tissue Attenuation. *Advanced Functional Materials* **2018**, 28/19, 1706793 (1-10)

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48) A. C. Wiesheu, R. Brejcha, C. W. Mueller, I. Kögel-Knabner, M. Elsner, R. Niessner & **N. P. Ivleva***, Stable-Isotope Raman Microspectroscopy for the Analysis of Soil Organic Matter. *Analytical & Bioanalytical Chemistry* **2018**, *16th Anniversary Issue*, 410, 923-931

47) A. Nistler, C. Hartmann, C. Rümenapp, M. Opel, B. Gleich, **N. P. Ivleva**, R. Niessner & M. Seidel, Production and Characterization of Long-Term Stable Superparamagnetic Iron Oxide-Shell Silica-Core Nanocomposites. *Journal of Magnetism and Magnetic Materials* **2017**, 497-503

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45) **N. P. Ivleva***, P. Kubryk & R. Niessner, Raman Microspectroscopy, Surface-enhanced Raman Scattering Microspectroscopy, and Stable-isotope Raman Microspectroscopy for Biofilm Characterization. *Analytical & Bioanalytical Chemistry* **2017**, 409, 4253-4375 (invited review)

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43) A. C. Wiesheu, P. M. Anger, T. Baumann, R. Niessner & **N. P. Ivleva***, Raman Microspectroscopic Analysis of Fibers in Beverages. *Analytical Methods* **2016**, 8, 5722-5725

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41) M. N. Ess, D. Ferry, E.D. Kireeva, R. Niessner, F.-X. Ouf & **N. P. Ivleva***, *In Situ* Raman Microspectroscopic Analysis of Soot Samples with Different Organic Carbon Content: Structural Changes During Heating. *Carbon* **2016**, 105, 572-585

40) P. Kubryk, Niessner & **N. P. Ivleva***, On the Origin of the Band at around 730 cm^{-1} in SERS Spectra of Bacteria: Stable Isotope Approach. *Analyst* **2016**, 141, 874-2878

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* corresponding author

Further Publications

N. P. Ivleva, How to detect microplastics. *Analytica Pro* **2018**, 1, 14-18

N. P. Ivleva & R. Nießner, Kunststoffpartikel in Süßwasser. *Nachrichten aus der Chemie* **2015**, 63, 46-50

8. Appendix B – Selected publications cited in this work

PI M. N. Ess, D. Ferry, E.D. Kireeva, R. Niessner, F.-X. Ouf & **N. P. Ivleva***, *In Situ* Raman Microspectroscopic Analysis of Soot Samples with Different Organic Carbon Content: Structural Changes During Heating. *Carbon* **2016**, 105, 572-585 (IF: 7.466)
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PII M. Ess, H. Bladt, W. Mühlbauer, S. Seher, C. Zöllner, S. Lorenz, D. Brüggemann, M. Nieken, **N. P. Ivleva** & R. Niessner, Reactivity and Structure of Soot Generated at Varying Biofuel Content and Engine Parameters. *Combustion & Flame* **2016**, 163, 157-169 (IF: 4.494)
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PIX A. C. Wiesheu, R. Brejcha, C. W. Mueller, I. Kögel-Knabner, M. Elsner, R. Niessner & **N. P. Ivleva***, Stable-Isotope Raman Microspectroscopy for the Analysis of Soil Organic Matter. *Analytical & Bioanalytical Chemistry* **2018**, *16th Anniversary Issue*, 410, 923-931 (IF: 3.307)
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9. Appendix C – Scientific *Curriculum Vitae*

Dr. NATALIA P. IVLEVA

Senior scientist, Institute of Hydrochemistry (IWC),
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PERSONAL DATA

Name:	Natalia P. Ivleva (Porollo)
Affiliation:	Institute of Hydrochemistry, Chair of Analytical Chemistry and Water Chemistry, Technical University of Munich, Marchioninistr. 17, D-81377 Munich
Home address:	Lyonel-Feininger-Str. 21, 80807, Munich
Date of birth / place:	02.06.1971 / Rostov-on-Don, Russia
Citizenship:	German (since 2016)
Family status:	Married: PD Dr. Alexei V. Ivlev, theoretical physicist Child: Anna Ivleva (1998)

EDUCATION

1988 – 1993: Diploma in Chemistry & Biology (with distinction); Southern Federal University, Rostov-on-Don, Russia

1993 – 1997: Ph.D. in physical chemistry: “Synthesis and reactivity of metal-containing monomers. Salts of unsaturated dicarboxylic acids”; Institute of Chemical Physics, Russian Academy of Sciences (RAS), Chernogolovka, Russia

since 12/2015: Habilitation candidate: "Raman microspectroscopy for environmental analysis", Department of Chemistry, TUM

PROFESSIONAL EXPERIENCE

10/1997 – 12/1998: Postdoc at the Biochemistry Laboratory, Institute of Chemical Physics, RAS, Chernogolovka, Russia

1999 – 2003: Parental leave

since 03/2003: Research scientist at the Chair of Analytical Chemistry, Institute of Hydrochemistry (Director Prof. Dr. Reinhard Niessner), TUM, Germany

since 02/2013: Head of the Raman Group at the Chair of Analytical Chemistry, Institute of Hydrochemistry, TUM, Germany

since 04/2017: Head for the Raman & SEM Group at the Chair of Analytical Chemistry and Water Chemistry, Institute of Hydrochemistry (Director Prof. Dr. Martin Elsner), TUM, Germany

RESEARCH INTERESTS

Application of Raman microspectroscopy (RM), stable isotope Raman microspectroscopy (SIRM) and surface-enhanced Raman scattering (SERS) for the nondestructive chemical 2D & 3D-analysis of various environmental matrices/pollutants, ranging from biofilms and microorganisms through microplastic and engineered nanoparticles to soot and atmospheric aerosols.

MEMBERSHIPS

Gesellschaft Deutscher Chemiker (GDCh)

Deutscher Arbeitskreis für Analytische Spektroskopie (DAAS), GDCh

Wasserchemische Gesellschaft (WG), GDCh; WG-Fachausschuss „Mikroplastik“

DIN-Normenausschuss NA 054-01-06 AA „Kunststoffe und Umweltaspekte“

ISO/TC 61/SC 14 "Plastics and Environment" / WG 4 „Microplastics“ (DIN Expert)

TEACHING ACTIVITIES

- Advanced Analytical Methods. Part: Trace Analysis/Separation Methods (Fortgeschrittene Analytische Verfahren Teil: Spurenanalytik/Trennmethoden), Lectures. Responsible: Prof. Dr. Elsner, PD Seidel, Prof. Dr. Haisch. For Bachelor Students of Chemistry and Food Chemistry (4 h).
- Bioanalysis of Organic Traces & Particle Measurement Technique, Lectures (LV2131). Responsible: Prof. Dr. Elsner, PD Seidel, Prof. Dr. Haisch, Dr. Ivleva. For Master Students of Chemistry and Food Chemistry (6 h).
- Practical course in Analytical Chemistry - Organic Trace Analysis (LV2132). Responsible: Prof. Dr. Elsner, PD Seidel, Prof. Dr. Haisch, Dr. Ivleva: ELISA, Microarrays, HPLC-MS, GC, Raman-Microspectroscopy and FT-IR. Part of the module Analytical Chemistry - Organic Trace Analysis. For Master Students in Chemistry.
- Research lab course (LV 2009). Responsible: Prof. Dr. Elsner, PD Seidel, Prof. Dr. Haisch, Dr. Ivleva. Module as part of the module Analytical Chemistry - Organic Trace Analysis. For Master Students in Chemistry.
- Practical IR and Raman Spectroscopy by Prof. Mink, Part: Vibrational Spectroscopy in Analytical Chemistry: Focus on Applicability of Raman Microspectroscopy, Lecture. Course for Master and PhD Students of Chemistry (4 h), SoS 2019.
- Raman & SEM group meeting (weekly)

SUPERVISION OF BSc/MSc- AND PhD WORKS

- Bachelor theses (before habilitation / during habilitation) 4 / 1
- Master theses (before habilitation / during habilitation) 6 / 3
- PhD theses (co-supervision before habilitation / habilitation) 5 / 1
- PhD theses (supervision before habilitation / during habilitation) 2 / 6

INVITED LECTURES

Raman Microspectroscopy in Analytical Chemistry, *Universitäres Zentrum für Gesundheitswissenschaften am Klinikum Augsburg - UNIKA-T*, 7.6.2019, Augsburg, Deutschland

Applicability of Raman Microspectroscopy for Environmental Analysis, *Universität Tübingen*, 5.6.2019, Tübingen, Deutschland

Mikroplastik in der Umwelt, *6. E.O. Fischer-Seminar „Umwelt und Energie“ in Burghausen Aventinus Gymnasium Burghausen, TUM*, 04.10.2018, Raitenhaslach, Deutschland

Microplastic in Environmental Samples: Focus on Raman Microspectroscopic Analysis, *University of Vienna, Department of Environmental Geosciences*, 26.06.2017, Vienna, Austria

Raman Microspectroscopy for Nondestructive 2D and 3D Analysis of Environmental Samples, *Department of Bioengineering and Electrical and Computer Engineering, Northeastern University*, 09.05.2017, Boston, USA

Identification and Quantification of Microplastic in Environmental Samples, *International Summer School “Microplastic in Aquatic Environment”*, 27.09.2016, Lake of Haltern, Germany

Microplastics in Aquatic Ecosystems: Focus on Raman Microspectroscopic Analysis, *Karlsruhe Institute of Technology, KIT*, 15.7.2016, Karlsruhe, Germany

Raman Microspectroscopy and SERS for Analysis of Biofilms, *Hong Kong University of Science and Technology, HKUST*, 8.6.2016, Hong Kong, China

Raman Microspectroscopy and SERS for Environmental Analysis, *Summer School "Experimental Analytical Techniques for Characterization of Nanoparticles and their Effects" (InterNano)*, 12.-16.10.2015, Landau in der Pfalz, Germany

Raman Microspectroscopy for Characterization of Environmental Matrices: Focus on Stable Isotope Technique, Chair of Hydrology, TUM, 26.11.2014, Munich, Germany

CURRENT RESEARCH PROJECTS

DFG Project (IV 110/2-2): Stable Isotope Raman Microspectroscopy (SIRM) for Quantitative and Nondestructive 2D & 3D Analysis: Microbial Degradation of Microplastics / StaR_Mic2; **PI: Dr. Ivleva**

BMBF Project (02WRS1378C): Microplastic in Water Cycle - Sampling, Sample Handling, Analysis, Occurrence, Removal and Validation; Subproject: Microplastic in Environmental Samples: Identification and Quantification by Raman Microspectroscopy / MiWa; **PI: Dr. Ivleva**

BMBF Project (02WPL1443A): Tracking of (Sub)Microplastics of Different Identity – Innovative Analytical Tools for the Toxicological and Process-Technical Evaluation / SubµTrack; Subproject IWC-TUM **PI: Dr. Ivleva**

BMBF Project LegioTyper (13N13698) Part: Raman Microspectroscopy for Non-invasive, Three-dimensional Analysis; PI: PD Dr. Seidel; **Responsible: Dr. Ivleva**

Bavarian Research Fund (Bayerische Forschungsstiftung, BFS, AZ-1258-16): Microparticles in the Aquatic Environment and in Food – Are Biodegradable Polymers a Feasible Solution for the “Microplastic Problem”? / MiPAq; Subproject IWC-TUM **PI: Dr. Ivleva**

TUM International Graduate School of Science and Engineering (IGSSE) Project: Engineered Biomagnetic Interfaces for Non-invasive Molecular Control / BIOMAG; Co-PIs: Prof. Dr. Niessner, Prof. Dr. Westmeyer; **PTL: Dr. Ivleva**

IWC-TUM Project: Development of Bioorthogonal Noncanonical Amino Acid Tagging – Surface-enhanced Raman Scattering (BONCAT-SERS) to Visualize Active Bacteria Responsible for Degradation of Organic Pollutants; **Co-PI**s: Prof. Dr. Elsner, **Dr. Natalia Ivleva**

SELECTED FORMER RESEARCH PROJECTS

DFG Project (IV 110/2-1): Stable Isotope Raman Microspectroscopy (SIRM) for Quantitative and Nondestructive 3D Analysis: Investigation of SIRM Potential for Applications in Environmental Analysis / STARAMM; **PI: Dr. Ivleva**

DFG Project (LA 2159/7-1 & NI 261/29-1): Microplastic Particles in Aquatic Systems; **Co-PI**s: Prof. Dr. Laforsch, Prof. Dr. Niessner, **Dr. Ivleva**

DFG Project (NI 261/26-1): Internally Mixed Soot Aerosols; **Co-PI**s: Prof. Dr. Niessner, Dr. Popovicheva, **Dr. Ivleva**

DFG Project (NI 261/21-1 & SCHL 332/10-1): Analysis of Changes in Structure and Reactivity of Soot Undergoing Oxidation by Raman Microscopy; Co-PIs: Prof. Dr. Niessner, Prof. Dr. Schlögl; **Responsible: Dr. Ivleva**

DFG Project (HA3507/2-1 & HO1910/7-1): Non-destructive Analysis of Biofilms by Raman Microscopy; Co-PIs: Prof. Dr. Haisch, Prof. Dr. Horn; **Responsible: Dr. Ivleva**

Water Alliance, Helmholtz Zentrum München Project: „Dynamic Processes and Filter Functions in Groundwater“, Subproject: Raman Microscopic Studies on Accumulation of Pollutants by Biofilms in Aquatic Systems; PI: Prof. Dr. Niessner; **Responsible: Dr. Ivleva**