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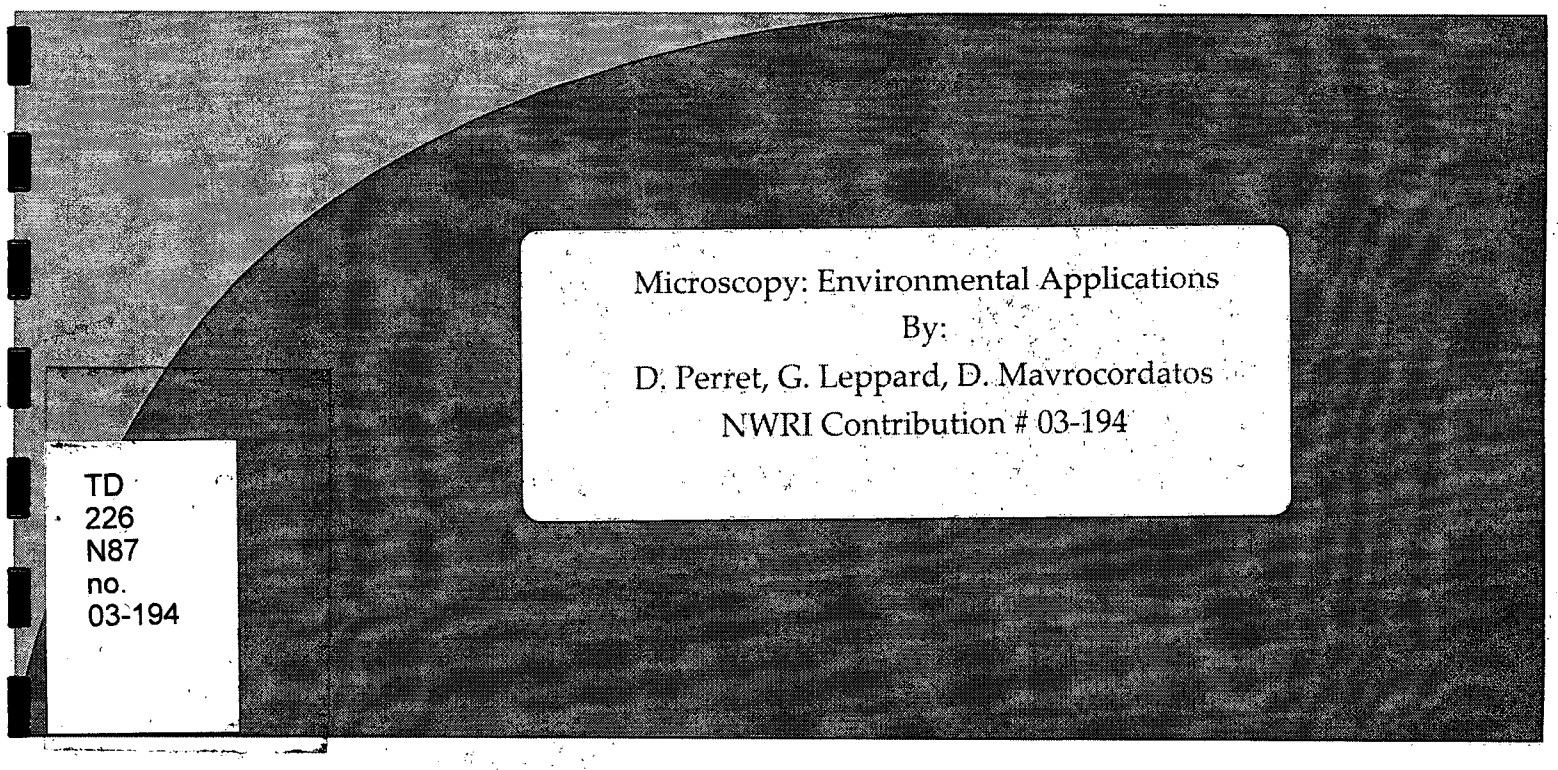

Microscopy: Environmental Applications

By:

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MICROSCOPY: ENVIRONMENTAL APPLICATIONS

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Abstract

Analytical electron microscopy and multi-scale correlative microscopy have recently emerged as powerful technologies for application in many fields of environmental science. These microscopes are currently being developed for routine use to demonstrate, characterize and quantify finely dispersed particles in natural waters, including toxic colloidal substances. Much of the international technology development and transfer activity is led by the three co-authors and their collaborators. This invited encyclopedia article focuses on (1) the optimization of specimen preparation methods for nanoscale examinations of environmental materials and (2) the extraordinary potential for electron microscopy to solve problems involving aquatic processes.

NWRI RESEARCH SUMMARY

Plain language title

Application of analytical microscopy to the solution of environmental problems

What is the problem and what do sicentists already know about it?

Colloids in aquatic ecosystems are responsible for much of the transport and transformations of toxic contaminants. Analytical electron microscopy, used correlatively in conjunction with related technologies that permit multi-scale structural and chemical analyses on a "per colloid species" basis, provides the sole means to detect, characterize and quantify the diverse individual colloid species important in transport and transformations. The importance of this evolving new high technology has been recognized internationally. Our article describes the new technology and how to apply it.

Why did NWRI do this study?

NWRI established much of the rules-of-the-game in developing and applying the new technology, as well as creating many of the initial case studies.

What were the results?

The results are an increasing use of analytical electron microscopy to solve scientific problems previously considered impossible to address by extant means.

How will these results be used?

The article is a review which features the new high technology. The results can be categorized as (1) major improvements to preparatory protocols, (2) the fine-tuning of sophisticated analytical instruments to make them user-friendly, (3) the generation of published case studies on aquatic environments to demonstrate applicability, and (4) the generation of exceptionally-generous grants to expand the research.

Who were our main partners in the study?

I have co-published the basis for this Encyclopedia research review with more than 80 different scientists over the past decade; these many scientists and their organizations and granting agencies are all partners. With regard to the two Swiss co-authors featured here, both have been funded generously by the Fonds National Suisse and both are former students of mine. Dr. Didier Perret is with the Swiss Federal Institute

of Technology in Lausanne, while Dr. Denis Mavrocordatos is with the Swiss Federal Institute for Environmental Science and Technology in Dubendorf (Zurich).

MICROSCOPIE: APPLICATIONS ENVIRONNEMENTALES

D. Perret, G.G. Leppard et D. Mavrocordatos

Résumé

La microscopie électronique analytique et la microscopie de corrélation à plusieurs échelles sont devenues récemment de puissantes technologies qui s'appliquent à un grand nombre de domaines touchant aux sciences environnementales. Ces microscopes font actuellement l'objet de mises au point qui permettront de déterminer, caractériser et quantifier de façon courante les particules finement dispersées dans les eaux naturelles, incluant les substances colloïdales toxiques. Les trois coauteurs et leurs collaborateurs dirigent la majeure partie du développement et du transfert de la technologie dans ce domaine. Cet article d'encyclopédie préparé sur demande fait le point sur (1) l'optimisation des méthodes de préparation des spécimens pour l'étude à l'échelle nanométrique des matières environnementales et (2) l'incroyable potentiel de la microscopie pour résoudre des problèmes liés à des processus propres aux milieux aquatiques.

Sommaire des recherches de l'INRE

Titre en langage clair
Application de la microscopie analytique à la résolution de problèmes environnementaux

Quel est le problème et que savent les chercheurs à ce sujet?
Les substances colloïdales dans les écosystèmes aquatiques jouent un rôle important dans le transport et les transformations des contaminants toxiques. La microscopie électronique analytique, utilisée en combinaison et de façon corrélative avec des technologies connexes qui permettent d'effectuer des analyses structurales et chimiques à plusieurs échelles selon l'espèce de colloïde considérée, s'avère le seul moyen de détecter, caractériser et quantifier les diverses espèces individuelles de colloïdes jouant un rôle important dans le transport et les transformations des contaminants. Cette nouvelle technologie de pointe évolue rapidement et son importance est reconnue à l'échelle internationale. L'article fait la description de cette nouvelle technologie et explique comment la mettre en application.

Pourquoi l'INRE a-t-il effectué cette étude?
L'INRE a fixé la plupart des règles de jeu en élaborant et en mettant en application la nouvelle technologie, ainsi qu'en mettant en œuvre la plupart des premières études de cas.

Quels sont les résultats?
Par conséquent, on assiste à une utilisation croissante de la microscopie électronique analytique pour la résolution de problèmes scientifiques auparavant impossibles à analyser par les moyens existants.

Comment ces résultats seront-ils utilisés?
L'article est une synthèse qui fait le point sur cette nouvelle technologie de pointe. Les résultats peuvent être regroupés sous les catégories suivantes : (1) amélioration importante concernant des protocoles préparatoires, (2) mise au point d'instruments analytiques sophistiqués de façon à en permettre une utilisation facile, (3) publication d'études de cas sur les milieux aquatiques démontrant les possibilités d'application, (4) l'octroi de subventions extrêmement généreuses permettant à la recherche dans ce domaine de prendre de l'expansion.

Quels étaient nos principaux partenaires dans cette étude?
J'ai copublié les fondements pour cet article de synthèse encyclopédique en collaboration avec plus de 80 chercheurs différents au cours de la dernière décennie ; ces nombreux chercheurs, les organisations auxquelles ils appartiennent ainsi que les organismes subventionnaires sont tous des partenaires. En ce qui concerne les deux coauteurs suisses de cet article, ils ont tous les deux reçu de généreuses subventions de la part du Fonds national suisse et font partie de mes anciens étudiants. Didier Perret travaille pour l'Insitut Fédéral de technologie suisse, à Lausanne, tandis que Denis Mavrocordatos travaille pour l'Institut fédéral de recherches pour l'aménagement, l'épuration et la protection des eaux à Dubendorf (Zurich).

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Aquatic particles

Remark:

Suggestions for cross-references to other articles within the Encyclopedia are marked <suggested cross-reference> in the text.
Suggestions for positioning Tables and Figures are marked <Table/Figure *i* near here> in the text.

BRIEF HISTORY

The invention of electron microscopy dates back to 1931 and ~~comes~~ is attributable to Ruska, who succeeded in identifying small biological objects with a Transmission Electron Microscope (TEM) <suggested cross-reference> operated at low voltage (50 kV) and exhibiting a resolution far beyond that of photon microscopy <suggested cross-reference>. Eventually, Ruska was lately awarded the 1986 Nobel prize for his fundamental work in electron optics, shared with Binnig and Rohrer for their invention of the Scanning Tunneling Microscope (STM) <suggested cross-reference> in the late 70's.

An approximate decade of developments in lenses and specimen preparation ~~had to be awaited since the invention~~ occurred prior to the first publications on the use of electron microscopy and interpretation of micrographs for the investigation of environmental samples. The establishment of the earliest Scanning-Transmission Electron Microscopes (STEM) <suggested cross-reference> in the late 30's, and of the first Scanning Electron Microscope (SEM) <suggested cross-reference> in the early 50's, followed by the development of high voltage microscopes, the incorporation of different detectors (Energy Dispersive X-Ray Spectroscopy, EDS; Electron Energy Loss Spectroscopy, EELS) <suggested cross-reference> and the development of brighter electron sources (LaB_6; Field Emission Guns, FEG) in the 60's and 70's, opened increasingly new opportunities for the study and the analysis of a broad range of environmental specimens with various thicknesses and compositions. The emergence of Environmental Scanning Electron Microscopes (ESEM) <suggested cross-reference> in the late 80's dramatically completed the palette of instruments amenable to the study of samples from the environment.

The earliest observation of soil particles by electron microscopy dates back to 1940, while a preparation scheme for the identification of airborne particles at magnifications up to 200'000× was initially published in 1946. It was however not until the mid-70's that the first papers dealing with suspended aquatic particulates finally appeared. Since then more than a

thousand publications have been reported to link the use of electron microscopes to environmental particles and colloids. Historically, electron microscopes have been exploited for the evidencing of the morphotypes, textures and sizes of particulate materials in environmental samples, and the majority of micrographs published had been used until recently for merely illustrative purposes. Nowadays, Analytical Electron Microscopes (AEM) have come out of their infancy in to the broad field of environmental science, and are used more and more frequently in order to demonstrate and quantify the presence of well characterised species down to the nanometer scale, and even to assess relationships between the formation or existence of specific types of particles or colloids at the microscopic scale, and the functioning of their natural milieu at the macroscopic scale. Indeed the present contribution focuses on the specimen preparation methods dedicated to scanning and transmission microscopies, and to the extraordinary potentialities of the latter for solving environmental situations and processes involving complex and heterogeneous species.

what is the former?

SAMPLING AND SPECIMEN PREPARATION

Unbiased electron microscopic investigation of particulate material, be it of living (bacteria, protozoa, algae, cellular fragments) or non-living nature (macromolecular organics, viruses, crystalline or amorphous mineral phases), requires a blend of dedicated sample collection schemes and specimen preparation techniques. Figure 1 serves as a rough guideline for the selection of the most adequate microscopic approach, from specimen preparation to particle characterisation.

<Figure 1 near here>

Whatever the investigation to be performed ~~on whichever sample~~, it must be kept in mind that every step of the protocol should be designed to avoid modifications of the native physico-chemical characteristics of the sample (e.g. precipitation/coagulation/dissolution due to [particle]/I/pH/redox/T shifts or uncontrolled dehydration of sample).

AEROSOLS AND ATMOSPHERIC PARTICLES

Atmospheric particles are probably the most straightforward to sample, as a variety of collectors have been devised and optimised for more than half a century <suggested cross-reference>, in which particulate material can eventually be size fractionated as a function of the operating conditions of the collector. ~~As opposed~~ In contrast to aquatic systems, sampling of particles in a gaseous milieu is less sensitive to physico-chemical changes, although biased size fractionation may occur due to particle hydrophilicity/hydrophobicity or surface charges (this should be concurrently checked by Scanning Mobility Particle Sizing; SMPS <suggested cross-reference>).

For indoor particles (e.g. asbestos fibers, industrial particles, soot or combustion smoke of health concern), direct sampling by air pumping through collecting membranes (smooth/flat neutron-impacted Nuclepore™-type filters) without size fractionation is best suited. Outdoor atmospheric or plume particles can be size fractionated and collected by cascade impactors, charged-particle collectors, thermal precipitators, or nephelometers. Particles will have to be transfered by contact from the sampling device to the specimen holder, be it a stub for SEM or a grid for TEM.

The collection of aerosols requires special procedures aimed at maintaining the original temperature and hydration status of the sample, in particular for studies involving wet or iced entities in which the mechanisms of cloud or ice formation are driven by the intimate combination of water, inorganic condensation nuclei, dissolved salts, and organics acting as hygroscopic agents; in these cases, microscopic examination of specimens with a cold stage should be planned within the shortest delay. Transmission (for the smallest colloids) and scanning (for larger particles) electron microscopes, in imaging or elemental analysis modes, are used indiscriminately for the characterisation of atmospheric entities, though the ESEM,

which allows imaging under various conditions of temperature and pressure, is especially befitting for water-containing particles larger than 50 nm.

AQUATIC PARTICLES

The study of aquatic particles (i.e. individual entities, and aggregates of these) encompasses a broad variety of matrices (marine and freshwaters; surface and groundwaters; gravitational and capillary water of soils), particle types (nanometer to millimeter; living and non living; organics, organo-mineral mixtures and minerals), and processes (structure-composition-function relationships; contaminant transport; mechanisms of formation and dissolution). Sampling of aquatic particles is thus a complex task which will systematically require adaptation of generic protocols to the specificities of the study. Whatsoever, the most relevant investigations are usually obtained by means of correlative electron microscopic approaches, i.e. schemes involving the use of scanning and transmission microscopes to study in parallel whole mounts and resin-embedded specimens (see below), in order to cover the broadest and most accurate physico-chemical fingerprint of particles.

Because of the rapid modifications of the physico-chemical and microbiological characteristics of natural water samples, initiated by changes in T, pH, depth, dissolved O_2 or CO_2, light, and convection, aquatic samples should be processed for EM within the shortest delays to avoid dissolutions, precipitations, coagulations, sedimentations, microbial growth, or shifts in chemical equilibria affecting colloids and particles, as it also prevails for conventional bulk chemical analyses. Pre-fractionation of aquatic particles and colloids into narrower classes can be performed by gravitational sedimentation in thermostated columns, by single or cascade centrifugations/ultracentrifugations, or by single or cascade filtrations/ultrafiltrations <suggested cross-references>. Both fractionation approaches are subjected to artifacts which can be minimised under carefully controlled conditions: Centrifugations must not be performed with highly concentrated suspensions to avoid problems of differential settling; similarly, tangential-flow filtration at low flow-rates usually

yields no or less polarisation concentration than uncontrolled/unstirred cross-flow filtration. Centrifugation and filtration are usually chosen to eliminate the fractions containing the largest particles, although they may be used instead to collect appreciable amounts of particulate material from poorly charged waters (e.g. groundwaters, pristine waters), with possible biases caused by apparent coagulation of particles.

Particulate material can also be sampled from natural waters by direct collection onto vertical or horizontal plates (glass, Teflon™, or other plastics) inserted ~~for a while~~ in the water column. Horizontal plates collect sedimenting particles without the drawbacks of conventional sediment traps (shifts in biological activity and redox conditions due to the absence of mixing at the bottom of the trap); vertical plates selectively collect those entities exhibiting a certain affinity for the plate (e.g. adhering bacteria, polysaccharides, Fe-oxyhydroxides).

Particles in soil water require different sampling approaches, depending on the type of water to be sampled. Gravitational water is better collected by means of tension-free lysimeters; these devices integrate over time ~~the sampled water~~, ~~which~~ should ~~thus~~ be recovered ~~within short delays~~ to avoid modifications in the size distribution or chemistry of the particles. It must however be underlined that tension-free lysimeters may exhibit fairly low collection efficiencies (as low as 10 % of the gravitational water, depending on soil texture and porosity). On the other hand, the capillary water of soils can be recovered by means of suction cups, which are made of a porous material (ceramic or plastic) inserted in the soil and connected to a syringe or pump. Due to their porosity (ca. 10 µm to 100 µm), these devices induce a pre-fractionation of the sampled particulate, and tend to clog with time.

SOIL AND SEDIMENT PARTICLES

These types of particles have been the subject of numerous publications related to the identification of the phases which constitute the soil or the sediment matrix, and to the formation of soils or sediments and their stratigraphic characteristics, but to a lesser extent to

the role of particles as carriers and scavengers of contaminants. For these reasons, sampling and specimen preparation protocols for soil or sediment particles are usually less sophisticated than protocols for aquatic particles, provided that one considers soil and sediment particles as static entities having no degree of freedom in their surrounding water.

While the majority of soil samples are characterised by slow reactivities and thus require fewer precautions, hydromorphic soils subjected to rapid hydration/dehydration processes are highly sensitive to redox changes and should be sampled with the greatest care to avoid precipitation of dissolved species (Fe^{2+}, Mn^{2+}) during accidental aeration. Otherwise, soils are usually dried, sieved and ground prior to be resuspended in various electrolytes for the selective isolation of certain constituents (e.g. organic matter, clays, silts, sands) by sedimentation or centrifugation. These approaches are indeed not recommended for the identification of trace metals, as drastic morphological and compositional modifications are expected at each step of the protocol. Because of their size, soil particulates are most frequently analysed by SEM, but the ultrastructural analysis of clay micelles or other finely divided components (e.g. iron oxides) requires TEM examination.

The particulate phases building up aquatic sediments represent an intermediate situation between aquatic particles and soil particles: Except for highly consolidated, deep sediments, their water content is large and their reactivity is comparable to, or even larger than the one of hydromorphic soils. Sediments are sampled by means of vertical corers, from which the different season- or event-dependent strata must be sub-sampled by slicing in a glove box under controlled atmosphere. Textural analysis of sediment particles can be performed by SEM, but more detailed investigations will require dilution of the samples in an electrolyte of composition similar to the interstitial water, prior to specimen preparation for TEM.

SPECIMEN PREPARATION

Specimen preparation for SEM requires collection of particulates onto stubs (either directly, or after pre-collection of entities onto Nuclepore™-type membranes), followed by post-

coating, most frequently with a Au or Pt film (for imaging) or C film (for analysis). The maximum achievable resolution limit that one can expect for SEM of complex heterogeneous environmental particles is ca. 10-50 nm, even for high intensity FEG-SEM.

<Figure 2 near here>

Cryotechniques offer an alternative and complementary preparatory technology to chemical fixation and embedding, producing aggregates whose 3D relationships are spatially "fixed" by a physical means such as vitrification. The most structurally faithful of the cryotechniques is freeze-etching which consists of freezing an aquatic sample rapidly enough to vitrify it, mechanically generating a fracture plane through it and then making a metallic replica of the fracture surface, all the while maintaining the vitrified sample below the recrystallization temperature. The product of the freeze-etch technique is a replica which presents a topographical image of a colloid or aggregate, untouched by chemical agents and amenable to analysis by TEM.

As compared to SEM, TEM offers a much broader palette of protocols for specimen preparation (see the most prominent procedures on Figure 2). Qualitative investigations shall favour the direct deposition of a suspension onto TEM grids (whole mounts), followed by evaporation; this rapid procedure may however induce (a) crystallisation of undesirable electrolytes (e.g. salt crystals for marine samples, which can be avoided by rapid rinsing of the grid in ultra-pure water), (b) shrinkage of aggregates of flexible organic materials (e.g. exocellular polymeric substances) or (c) coagulation of small colloids.

For the extraction of ultrastructural information (in particular with bacteria, algae and 3D-networks of organic flocs), large size entities (i.e. > 1 μm) should be visualised by TEM after preparation of thin sections obtained by resin embedding and ultramicrotomy <suggested cross-reference>. To avoid artifacts, the choice of an appropriate embedding media is crucial and should be directed toward a hydrophilic resin (Nanoplast™), rather than more conventional hydrophobic ones (e.g. Spurr™, Epon™, or Araldite™), because the latter require dehydration

steps of the sample in organic solvents (acetone, methanol, ethanol or propylene oxide); these steps are potentially disturbing, as they may cause dissolution of particulate organic moieties and modifications of the morphologies of complex 3D networks containing organic entities. As Nanoplast™ produces water molecules during the permeation and polymerisation step, it infiltrates readily in porous (e.g. loose and amorphous colloids) and biological (e.g. bacteria) entities, maintaining their fine morphological features.

Ultramicrotomy (see Figure 2) of the resin-embedded material should be performed exclusively with a diamond knife to overcome the hardness of mineral particles or biogenic minerals such as silica frustules, which quickly damage conventional glass knives.

Targeted sectioning can also be performed by high energy Focused Ion Beam (FIB) <suggested cross-reference>. This emerging technique shall prove highly valuable for environmental science in the near future. In principle, any material (hard inorganic or soft organic entities) is amenable to FIB sectioning.

Ultrastructural investigations require the preparation of ultra-thin sections (ca. 50-100 nm), while larger sections (much easier to obtain, but which should not exceed 150 - 200 nm) are still amenable to elemental analysis without important biases, even when performing Electron Energy Loss Spectroscopy, which theoretically requires the thinnest possible materials.

Probably the most interesting approach to the preparation of specimens for quantitative analysis by TEM is the direct ultracentrifugation of colloidal and particulate entities onto TEM grids. This procedure (see Figure 2) yields quantitative whole mounts with evenly distributed particles. For a given suspension, the final coverage of the grid can be finely tuned by varying the volume to be centrifuged, allowing a fairly accurate estimation of the particle concentration in the initial sample, yet avoiding too densely populated grids as is often the case for conventional whole mounts. Entities sensitive to dehydration or redox modifications can be post-protected by horizontally spinning an ultra-thin film of hydrophilic

Nanoplast™ resin. Indeed, the preparation of quantitative mounts can be coupled to sequential fractionation schemes to further narrow the types of particles that are collected onto the grids.

The choice of the grid type and the supporting film is critical for analytical TEM/STEM (AEM in EDS or EELS modes). One must indeed be reminded that the X-rays produced by the analyte under the focussed electron beam are emitted in a sphere of volume and will induce secondary X-rays of the materials they hit (e.g. the supporting grid or the pole pieces of the EM column) which may be emitted in the direction of the detector, generating artifactual peaks. While Formvar™- or Parlodion™-coated (10-50 nm; supporting film needed for strength/flexibility), carbon-sputtered (3-10 nm; needed for thermal/electrical conductivity) copper grids are the best alternative in price and ease of operation for imaging purposes, elemental analysis requires supporting materials which shall not mask the elements of interest: gold grids are preferred for the EDS analysis of trace transition metals; for the identification of carbon-rich entities prepared as whole mounts or quantitative mounts (i.e. without resin embedding), the supporting film must be substituted by a carbon-free 5-15 nm SiO film (not adapted for Si-rich entities) or Be film (expensive); large scale entities (e.g. 3D-networks or organic-mineral mixtures) can be collected on holey or Quantifoil™ (supporting film with controlled and repetitive holes) or Lacey carbon films for the unconstrained analysis of their unsupported portions, with the risk of a weaker mechanical and electrical stability under the electron beam. Whichever grid is used, the alpha-numeric styles shall be prefered in order to keep track of particles of interest over time.

Indeed, staining procedures designed for biological or medical applications <suggested cross-references> can be applied to environmental specimens (in particular in soils, sediments and natural waters) to either enhance the contrast of poorly electron-opaque organic material (salts of heavy elements, amongst which the most commonly employed are uranyl acetate, lead citrate, phosphotungstate, alcian blue), or selectively stain exocellular polymeric substances (multi-step reaction with silver proteinate to yield Ag grains onto

polysaccharides). To date, it is regretably not possible to clearly identify colloidal humic/fulvic entities, which constitute an important proportion of natural organic materials. On the other hand, highly sophisticated labeling techniques by means of ultra-specific markers (e.g. gold-lectins complexes) should prove in the near future greatly valuable to distinguish morphologically similar natural organic macromolecules (e.g. neutral vs. acidic polysaccharidic moieties produced by bacteria and algae). In many environmental situations, colloidal organic matter is naturally stained by the major ions present in the electrolyte, and thus requires no staining for simple visualisation purposes. It must however be noted that most existing staining protocols were not designed for environmental specimens (whole mounts or resin embedded) and necessitate careful optimisation prior to be used on a routine basis.

MICROSCOPIC INVESTIGATION

Electron microscopic investigation of environmental samples has been traditionally performed for years for merely illustrative purposes, although the lateral resolution power and the analytical capabilities of modern electron microscopes are in many situations unique features that can bring unequivocal qualitative and quantitative answers to complex problems in which colloids and particles play a central role. For ease, the qualitative and quantitative approaches of investigation are discussed independently in the following sections, but they should be considered as intimately intermingled within the frame of every cleverly planned correlative electron microscopic investigation, as would be the case for the idealised correlative investigation depicted in Figure 3.

<Figure 3 near here>

QUALITATIVE/SEMI-QUANTITATIVE INVESTIGATION

SEM and ESEM are particularly well suited for the evidencing of surface and textural features of large particulate entities (ca. > 1 μm), and for the rough estimate of the particle size distribution. Qualitative 3D morphological information is readily extracted from SEM operated either in secondary electron mode or in backscattered electron mode, and the extent of aggregation between particles (provided that it is not an artifact produced by overloaded specimen stubs) can be documented with a resolution down to ca. 50 nm without difficulty. Because of its ease of operation, SEM should be selected for the routine survey of samples, in particular for atmospheric and soil particles. Preliminary qualitative surveys should help the operator to focus on either the general trends (e.g. major classes of particle types or sizes or associations) or the significant specificities of the sample (e.g. characteristic aggregation between two types of particles, or prevalence of a narrow size class for a given type of entity).

When combined to conventional bulk experiments and analyses, qualitative SEM/ESEM may yield significant progress in the understanding of the studied ecosystem. For example, visualisation of the microscopic features of humic and fulvic substances subjected to preliminary changes in pH or ionic strength of the surrounding milieu have greatly contributed to the unequivocal appreciation of the conformational changes of these dominant organics in soils, ranging from spherocolloidal entities to flexible and extended fibrils or densely networked 3D systems. Likewise the effect of the intimate associations between clays and fibrillar polysaccharide networks on the stabilisation of soil structures has been assessed mainly by means of SEM. Nonetheless, such investigations require careful specimen preparation techniques (e.g. freeze-drying instead of air drying) aimed at minimising artifactual conformational changes at every step of the preparation.

Accurate qualitative or semi-quantitative investigations on colloidal entities (ca. < 1 μm) are better performed by means of TEM or STEM, with a resolving power down to the nanometer

scale, even for complex heterogeneous entities (while a claimed sub-Å resolution is achievable in TEM with aberration corrected electron optics). Whole mounts and quantitative mounts (see Figure 2) are appropriate for semi-routine TEM investigations on the presence of colloidal entities, provided that TEM grids are not too densely populated with large particles and aggregates. In addition, inorganic entities can be checked by qualitative electron diffraction (either in selected area mode, or in convergent beam mode) for their potential crystallinity; this is particularly useful for ill-defined particles (e.g. ferrihydrites or partly amorphous oxides) that may reveal locally ordered domains of their atoms, or to distinguish between particles exhibiting the same morphologies and rough composition but different stages of ageing.

Ultrastructural TEM characterisation of ultra-thin resin sections is recommended to reveal, e.g., tiny mineral deposits on bacterial cells, which would be obscured by the thickness-related opacity of bacteria prepared as whole mounts. Likewise, complex large-scale networks of fibrillar exopolymers shall retain their subtle conformation when embedded in the appropriate resin; the reconstruction of their 3D architecture is theoretically achievable by imaging of successive sections, but this requires tedious and extensive image analysis work.

QUANTITATIVE INVESTIGATION

Quantitative investigation of natural particulate material necessitates special requirements. In the first place, the specimen must be representative of its original milieu. For example, all particle types initially present in the natural sample must be represented in their right proportions, except if the sample has intentionally undergone controlled fractionation to remove several particle classes. The aggregates present in the specimen must not be artifactual expressions of an overloaded TEM grid or of a sample which would have experienced handling and preparation conditions favouring coagulation of existing entities or precipitation of dissolved species. In that respect, the preparation of quantitative mounts (see

Figure 2) by direct ultracentrifugation of suspended particles onto TEM grids is certainly the most appropriate approach for quantitative measurements, and most works performed on natural waters, sediments, or soils can be brought to this approach.

In a second place, quantitative investigations need to be performed on a statistically significant number of entities to yield sound measurements. Depending on the expected confidence in the final results, the measurements (size, composition) should be performed on a large set of particles, comprised between 100 and 10'000 entities (see Figure 3). When dealing with elemental composition, the measured EDS peak intensities must be calibrated against representative standards; these standards may have to be synthesized in the laboratory under the conditions encountered in the studied ecosystem, because the mechanisms of production of X-rays are influenced by the matrix of the studied material. Standardless analysis is feasible in EDS, but it requires the knowledge of accurate element- and microscope-related parameters. For EELS analysis, relative quantification (i.e. the ratio of one element to another one in the particle of interest) is readily obtained by calculation without the need for standards.

Quantitative particle size distributions (either of all types of particles and aggregates present in the specimen, or of a specific type of particle, identified either by morphological criteria or by routine EDS elemental mapping) must be performed on quantitative whole mounts; high-quality micrographs can then be digitised for mathematical morphometry using an image analysis software. The accurate determination of the particle size distribution of atmospheric, aquatic, soil or sediment entities may yield information on the processes driving their behaviour in the milieu (e.g. formation, dissolution, coagulation, sedimentation; see Figure 3). It is also possible to estimate the mechanisms of aggregation of colloids by means of the fractal dimension of aggregates. Provided that micrographs are obtained under carefully controlled conditions of illumination, estimates on the volume of non-spherical particles of

known composition can also be obtained by means of image analysis (thickness-dependant opacity of the particles).

Over the past two decades, the clever determination of the composition of unartifacted environmental particles and colloids by EDS analysis has definitely transformed electron microscopy into a powerful and unique analytical tool. Under optimal conditions, i.e. using an EDS detector equipped with an ultra-thin window, even carbon can be quantified, and the detection limits can be as low as 500-1000 mg/kg (0.05-0.1 $\%_w$), even for sub-micrometric particles, provided that the trace elements of interest are not overlapped by major or minor elements (see Figure 3). The method is particularly well suited for elements emitting K_α peaks in the sensitive 0-10 keV energy range, but L-emitting elements can also be measured in this range, provided that they are present as minor or major elements. Quantitative EDS analysis is nowadays the method of choice for the quasi-routine based detection of trace elements scavenged by particles, or the search for stoichiometric relationships between different elements in similar morphotypes.

Although the ability of EELS techniques (Electron Spectroscopic Imaging, ESI, in imaging mode; EELS in spectrum mode) to detect single atoms with energy resolutions as low as 0.2 eV in the useful 0-1000 eV energy range (in comparison, high resolution EDS is achieved at ca. 150eV) has been clearly demonstrated in materials science and even in biological science, EELS is regrettably not commonly used in environmental science. Indeed, the interpretation of spectra is not as clear-cut as in EDS, because (i) the technique is best suited for light elements (although transition metals yield valuable spectral information), (ii) the extraction of the EELS K- or L- or even M-edges of elements requires a careful stripping of the substantial background (in the form of a power law $I = a \cdot E^{-b}$), (iii) the region below ca. 100 eV (plasmons region) is difficult to model with accuracy, (iv) there remain uncertainties in the determination of the cross-sections of the M-edges used for absolute quantification purposes, and (v) EELS is theoretically dedicated to specimens with thicknesses below 20-50

nm. It has however been demonstrated that the technique can be used to quantify thicker (< 500 nm) environmental particles, although accuracy drops as specimen thickness increases.

In its simplest expression, EELS can be performed in Energy-Filtered (EF-TEM), or Electron Spectroscopic Imaging (ESI) mode, i.e. to acquire elemental maps, but EELS in spectroscopic mode is best suited for quantitative analysis, with a high energy and lateral resolution.

The main advantage of EELS over EDS is its ability to yield molecular information, in the form of specific features at and beyond the edges (see Figure 3). The features referred to as Energy Loss Near Edge Structure (ELNES; extending up to 50-100 eV beyond the edge; counterpart to X-Ray Absorption Near-Edge Structure, XANES, in X-Ray spectroscopy) are fingerprints that bring qualitative information about the molecular environment of the element giving rise to an EELS edge; for instance, the shape of an EELS-ELNES spectrum is different for aromatic, or aliphatic, or amorphous organic carbon centers, as well as for inorganic carbon centers. Likewise, EELS spectra acquired with a high energy resolution (i.e. < 0.5-1 eV) may reveal the electronic configuration of redox-sensitive elements (e.g. Fe^{2+} vs. Fe^{3+}, or mixtures of them in the same entity; Mn^{2+} vs. Mn^{3+} vs. Mn^{4+}). Although exemplary results have already been obtained on pure, crystalline iron minerals and manganese minerals, the approach is far from routine yet for the complex heterogeneous particles identified in soils, sediments and natural waters. Similarly, the use of EELS for the redox discrimination of elements of environmental concern such as Cr^{3+}/Cr^{6+}, Cu^{+}/Cu^{2+}, $As^{3-}/As^{3+}/As^{5+}$, $Sb^{3-}/Sb^{3+}/Sb^{5+}$, or $Se^{2-}/Se^{4+}/Se^{6+}$, has not been reported yet.

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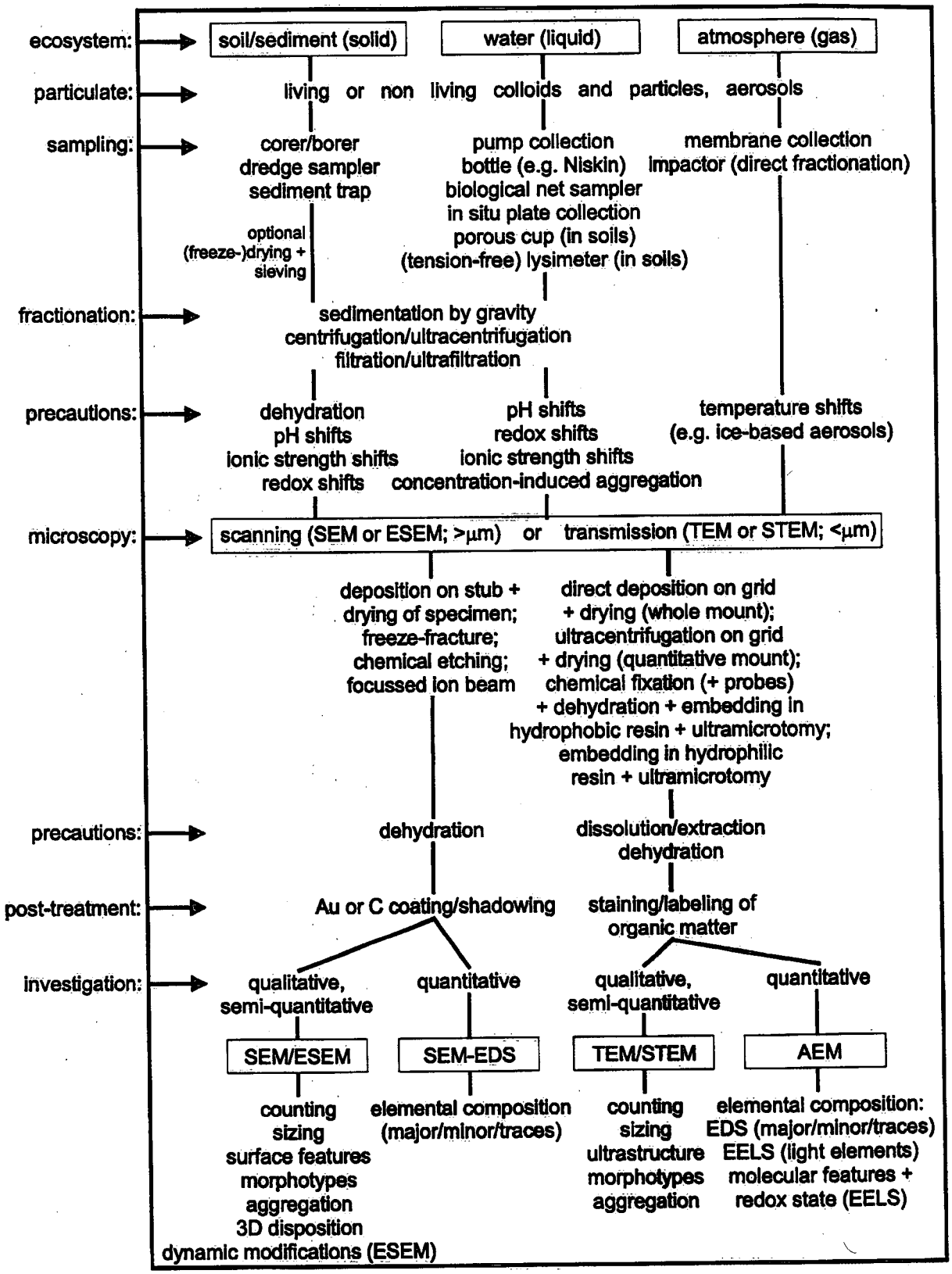
FIGURE CAPTIONS

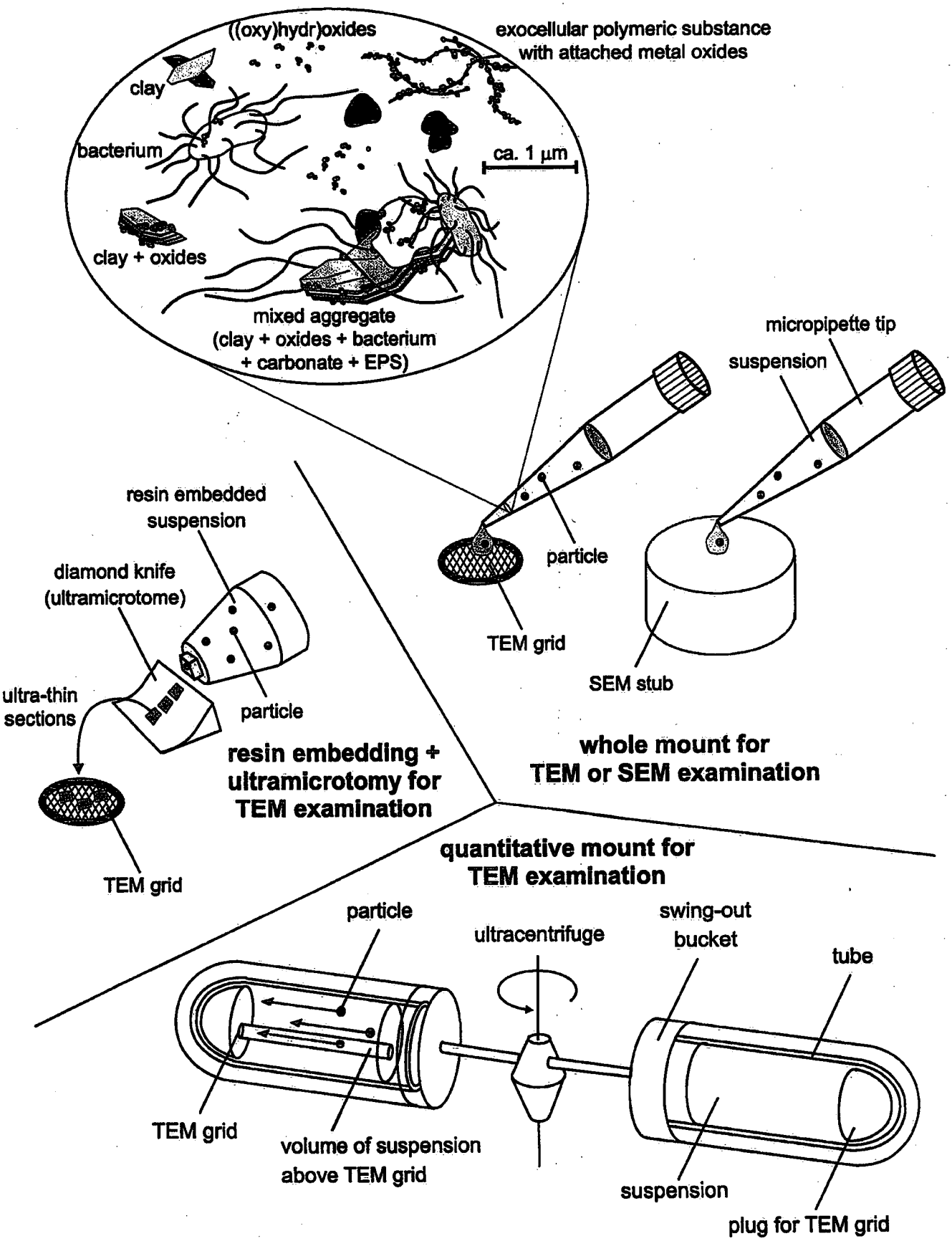
Figure 1: Overview of the recommended approaches, crucial steps and possible artifacts relevant to the physico-chemical characterisation of particulate material in environmental systems by electron microscopies. This scheme does not take into account precautions required for conventional bulk physico-chemical analyses.

Figure 2: Schematic of the most useful qualitative or quantitative specimen preparation schemes for SEM/TEM examination of colloids and particles in aqueous media.

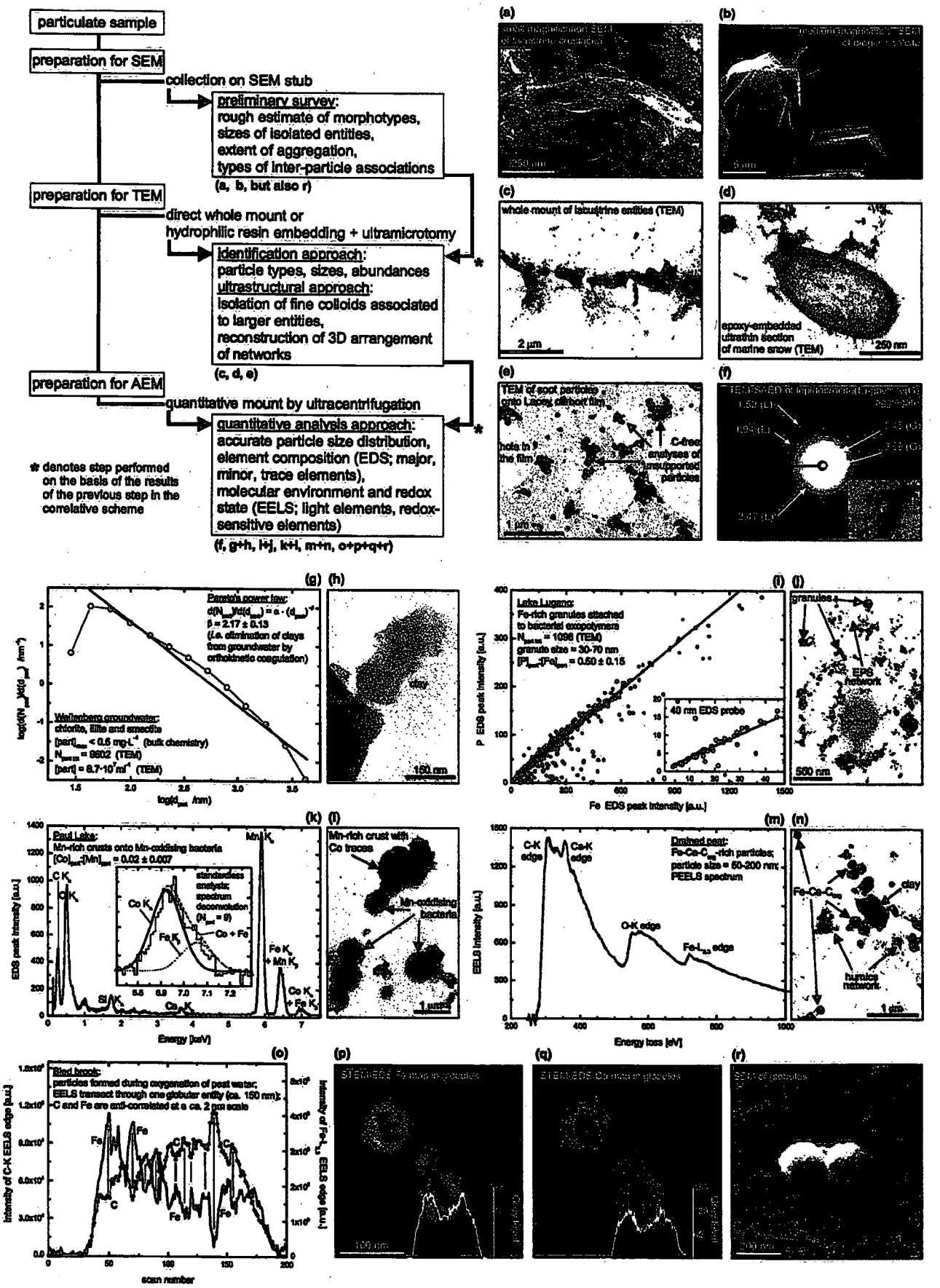
Figure 3: Idealised correlative electron microscopic procedure for the examination and physico-chemical characterisation of environmental colloids an particles. Micrographs, spectra and plots are extracted from published/unpublished work of the authors. **a**: adapted from Jackson T.A., Leppard G.G. (2002) Develop. Soil Sci 28A, 219-260. **c-d**: adapted from Lienemann C.-P., Heissenberger A., Leppard G.G., Perret D. (1998) Aquat. Microb. Ecol. 14, 205-213. **e**: adapted from Mavrocordatos D., Kaegi R., Schmatloch V. (2002) Atm. Environ. 36, 5653-5660. **f**: adapted from Mavrocordatos D., Fortin D. (2002) Amer. Miner. 87, 940-946. **g**: adapted from Couture C., Lienemann C.-P., Mavrocordatos D., Perret D. (1996) Chimia 50, 625-629. **i-j**: adapted from Lienemann C.-P., Monnerat M., Dominik J., Perret D. (1999) Aquat. Sci. 61, 133-149. **k-l**: adapted from Lienemann C.-P., Taillefert M., Perret D., Gaillard J.-F. (1997) Geochim. Cosmochim. Acta 61, 1437-1446. **n, p-q**: adapted from Mavrocordatos D., Mondi-Couture C., Atteia O., Leppard G.G., Perret D. (2000) J. Hydrol. 237, 234-247. **o**: adapted from Mondi C., Leifer K., Mavrocordatos D., Perret D. (2002) J. Microsc. 207, 180-190. All published material reproduced with permission. **b, h, m**: unpublished results.



ms390 Perret/Leppard/Mavrocordatos Figure 1



ms390 Perret/Leppard/Mavrocordatos Figure 2



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