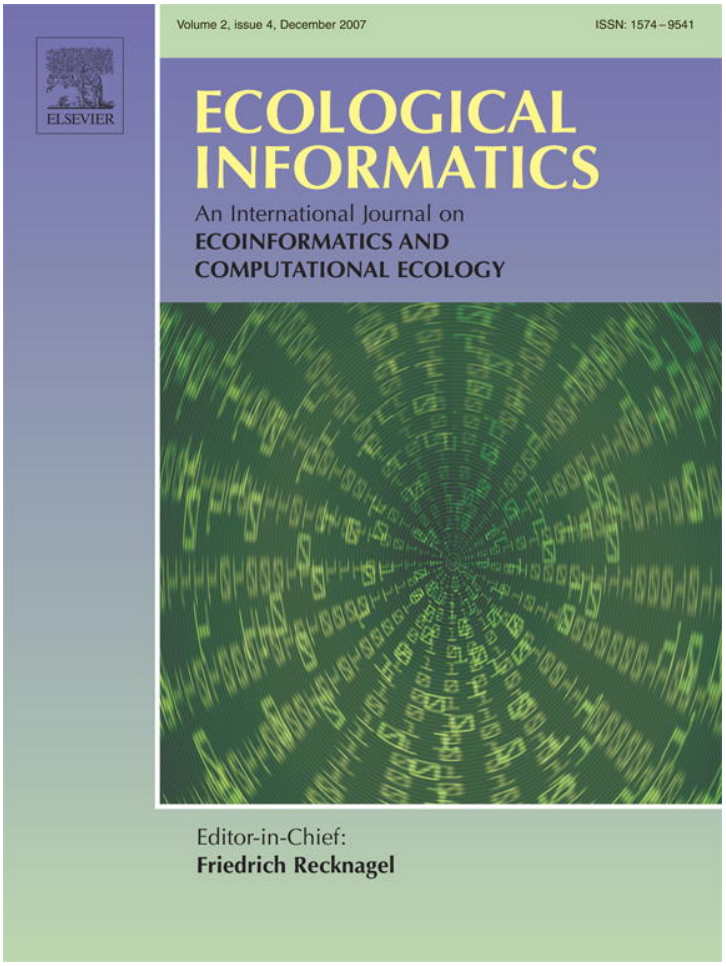

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Does digital analysis of micro image data improve understanding of reality? Contradictions–Challenges

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ABSTRACT

Understanding of reality means among many others the ability to recognize, to predict reliably behaviours and properties of certain entities, and to describe. Besides verbal, often metaphorical descriptions, models are frequently used to formalize this understanding. In science the validity of models (hypotheses) has to be proven by experiments. Model parameters like start values, constraints and boundary conditions are often extracted from image data. Qualitative terms like shape, surface, structure (density, texture) and arrangement (spatial relationship) are explored and their possible measurements are outlined. They are also related to underlying experimental questions and intuitive understanding. Particular emphasis is given to properties without a commonly accepted quantitative counter part or which pose difficulties in perception and discrimination.
Image analysis in methodologies and results from eco- and geobiology are presented and discussed. Namely, measurements of bacteria in biofilm and in the rhizosphere, characterization of biofilm growth, bacterial impact on surfaces, and automated phytoplankton analysis are outlined and iconographically related.

1. Introduction

Image or more general data gathering devices allow the generation of regularly gridded data. In the 2-D case these data can be considered and displayed as images. Higher dimensional data result from digitization of depth, time or spectral information. Although images (2-D regularly gridded data with intensity or colour (spectral channels) are the most common way of information exchange, the quantitative description of image entities is still a problem (Gombrich, 1960). The content of images is often not completely clear for observers in cases of new or unexpected properties. Even worse, such properties may not be described and hence not recognizable for the observer (Eco, 2000). Consequently most data gathered nowadays are merely utilized as markers or indicators for certain (expected) events or states, with all possible errors in attribution. The Sufi story: *The elephant and the blind men* illustrates this dramatically (Appendix A.1).

The problems become much worse in cases of higher dimensional data produced by, for example, confocal laser scanning microscopy (CLSM), electron tomography (ET) and other high resolution scanning devices. Besides the difficulties mostly surface oriented observers have in relating perception with understanding, the presentation of such data is itself a problem. The relatively simple example of the surface of a sand grain saves to illustrate this. Different representation schemes (Fig. 1) could be variously interpreted and pose challenges to recognize or to unveil hidden properties or

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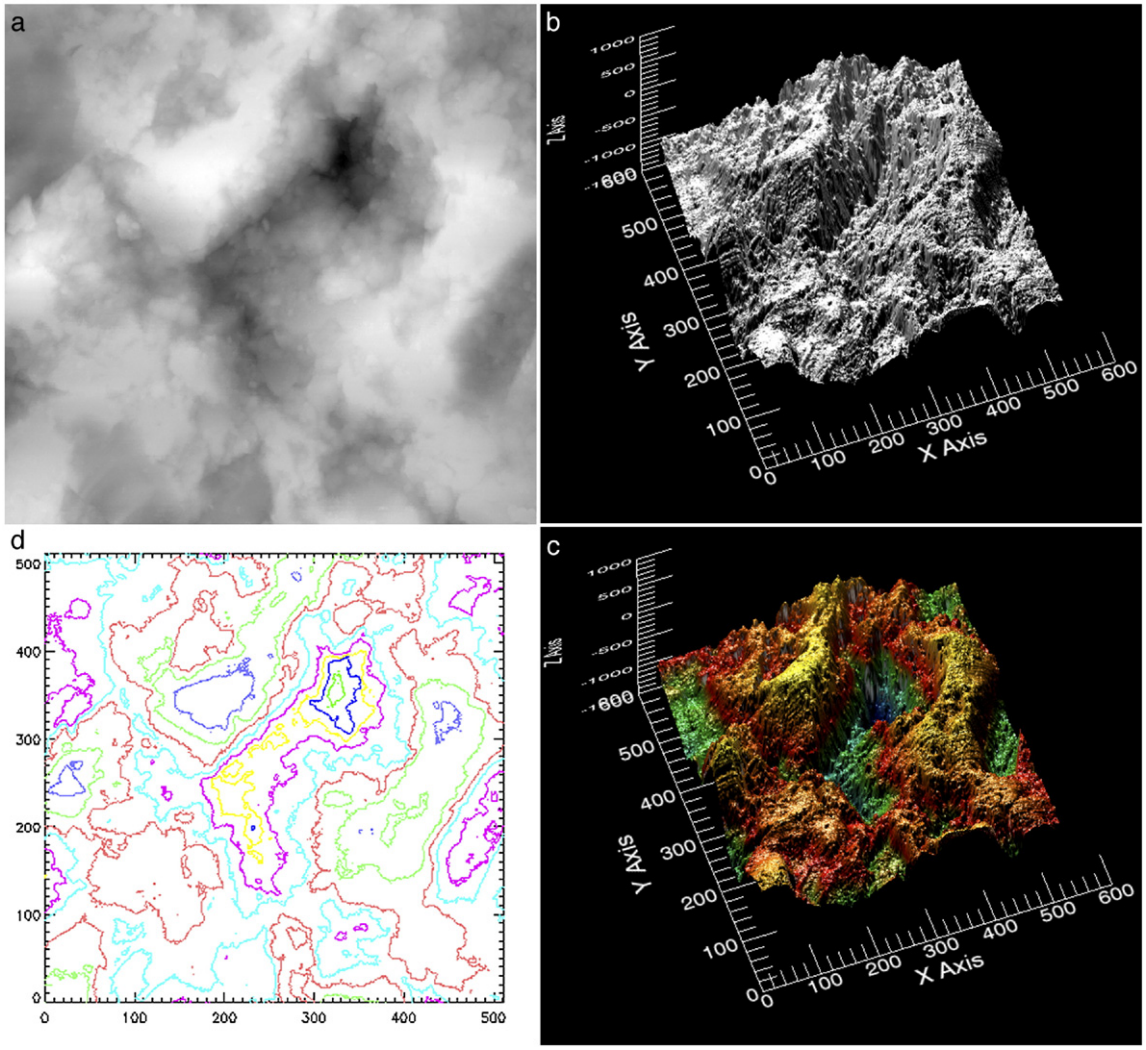

Fig. 1 – Geomorphology: topographic imaging of quartz sand grain surfaces using Atomic Forces Microscopy (see Appendix A.5). Without knowing the context different interpretations could be given to the images for example: (a) A cloudy morning; (b) landscape on Moon or (c) Mars; (d) a poorly described weather map?

relationships. The display of images derived or extracted from higher dimensional data as sections or projections are always a balancing act between intension and pretension. There is no natural or canonical way of image generation.

1.1. *Improving understanding of reality*

Images are mostly used to show what objects, themselves look like. A slightly more complex use of images is in comparison of objects, for example comparing the resemblance of a child with its parent. A slightly more complicated step in understanding is to use images to order objects by certain properties, for example a person as a baby, an adolescent and an adult. This is the way we learn to see, to build internal models, and to understand our environment by pictorial representations (Gregory, 1970). We also have to establish descriptions for transfer of information via non-visual channels (verbal, textual, functional, numeral), which requires the choice of different ways of coding information. My working hypothesis is: *The better we can describe the better we can perceive and recognize*. Hence improving understanding of reality requires the development of adequate descriptions, as illustrated by Thompson (1966).

1.2. *Contradictions–challenges*

Today's laboratory equipment allows production of high quality, high resolution and multi-spectral, hence multi-dimensional digital data. However, the analytical tools available for these types of data are not adequate. Qualitative, mostly visual observation, at best (pseudo-) quantitative methods are applied for feature detection and extraction. Data from multi-dimensional origin are rarely interrogated. Additionally, possible interpretations are rarely explored. As well-known in multi-dimensional statistical analysis, data allow a multitude of different views. It is unlikely to find by chance the best view or interpretation. The dichotomy of more or less intuitive understanding and quantitative characterization of data initiates creative processes. Still the development of adequate descriptions is the base to establish adequate models relating the functional with the perceivable.

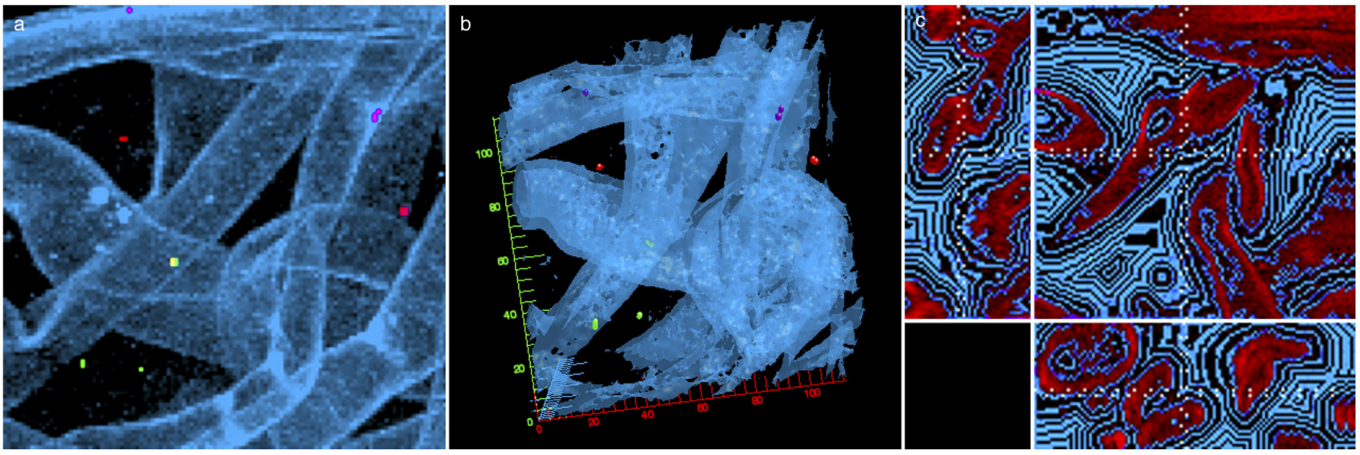

Fig. 2 – Rhizosphere: scanned by confocal laser scanning microscope (CLSM), sub-volume of size $72 \times 72 \times 30\ \mu m^3$; (a) bacteria in red, green and yellow, root in blue, volume in max. projection; (b) snapshot of 3-D representation; (c) orthogonal sections of 3-D distance transform of root environment, iso-distance surfaces in blue, root in red. (see Appendix A.2 for experimental detail).

1.3. *Goals*

Here I will explore some qualitative terms, illustrated by some biological and ecological experiments (rhizosphere, biofilm, geobiology, algae taxonomy), and try to establish connections between perception (image), qualitative description and modelling via quantitative characterization. The methods should serve as spur to extend, improve and creatively use (multi-dimensional, gridded) digital data.

2. Methods for describing images

In this section I will explore qualitative terms and measurements, with examples from general biology and specifically, I will consider the frequently used qualitative terms *spatial relationship* in rhizosphere and biofilm, to characterize the *impact* of bacteria on *surfaces* and to group algae in fresh water phytoplankton populations by *shape*.

Images from any experiment show often differences either difficult to describe or not recognizable at all. A way out of this dilemma is (i) the careful or even meticulous selection of regions (sets of points, pixel, voxel) inside the data, (ii) the seemingly excessive quantification of these regions (Rodenacker and Bengtsson, 2003) and finally (iii) the thoroughly analysis of the resulting features by statistical methods related to the experimental question, the visual representation of the (image) data and the respective algorithmic concept of the feature extraction method. Typically this is a highly creative exchange process between experimenter and image analyst.

Resulting numerical data reflect the experimental question. In general such numbers arrange the set of images (regions) into an ordered sequence. The observer can recognize this order if he has already conceptualized the respective measurement. Vice versa, a found order, a quantitative feature, can be used for training purposes of observers by presentation of image sequences. Unfortunately concepts of properties like spatial relationship, surface or shape are hardly developed and, even worse, the quantitative representation can be done by arbitrarily many different methods.

2.1. *Spatial arrangement and relationship*

Characterization of groups of objects in space, e.g. bacteria as outlined in Appendices A.2 and A.3, Figs. 2 and 3, are governed by distance and the similarity. Bacteria are considered as *related*

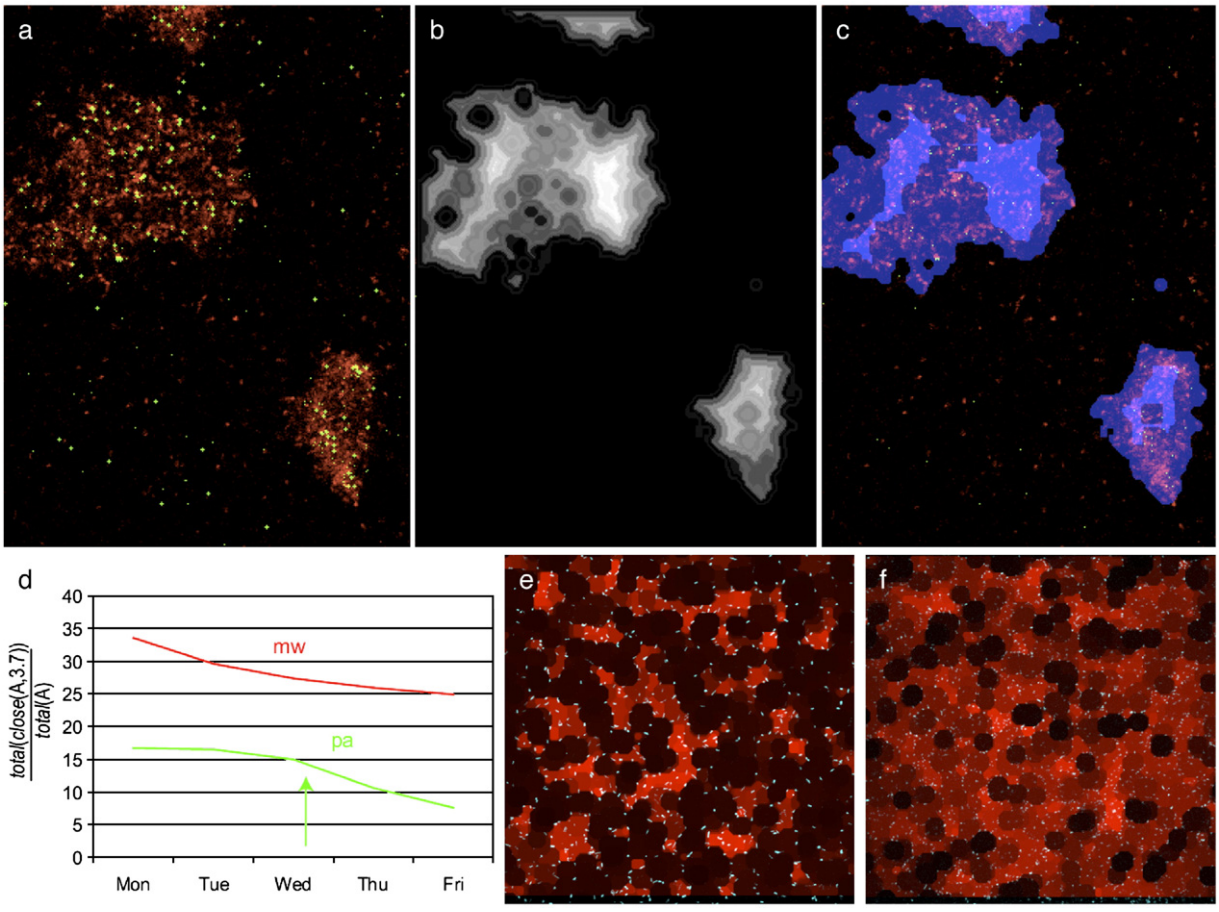

Fig. 3 – Biofilm: (a) Recipient (red) and donor (green) bacteria; (b)clusters of recipient bacteria with distance to the cluster border; (c) bacteria with clusters and cluster centres overlayed. (d) Area density (percentage of by radius 3.7 μm closed area) of wild-type (pa) and mutant (mw); (e) wild-type and (f) mutant bacteria after four days of growth and morphological closure (see Appendix A.3 and A.4).

if their distance is small enough. This relationship is refined by intra- and interrelationship of different bacteria. The latter is differentiated by marker fluorescence or any other feature. Statistical estimators of distance distributions of different related groups deliver a quantitative description of the relationship. A distance transformation from a binary data set of segmented bacteria (Fig. 2c) serves as a visualization of the spatial distribution and delivers easily the distance distribution of one (related) group of objects to another (used for the distance transformation). The extracted quantities have than to be related to external properties of the objects under examination.

A similar concept is adopted to characterize the arrangement of bacteria and their existence or modification by a possible gene transfer from other (in the data set differently marked) bacteria. The local density of bacteria is used to define clusters. The occurrence of modified bacteria in such clusters show their ability to penetrate, although such clusters are embedded into extra cellular polymerized substance (EPS). The latter is considered as a stabilizer and protector of biofilm.

To quantify growth characteristics of bacteria in biofilm grown in a flow chamber (Fig. 3d–f) daily measurements over one week were performed. The 3-D data are difficult to understand. Possible measures are the (bacterial) volume and the surface occupied. The latter showed a small difference after three days between wild- and mutant type bacteria (Fig. 3d). Seemingly wild-type bacteria are inhibited more by contact than were the mutant type.

2.2. *Surface characterization*

Geomorphology for analysis of rock and mineral surface modifications (see Appendix A.5 and Fig. 1) comprises the analysis of small scale surface deviations. The underlying idea of surface topography is the composition of large scale variations which are superimposed by small scale variations. Height maps from an atomic force microscope (AFM) are smoothed by a non-linear Gaussian (*nlg*) and a morphological opening-closing (*of*) of defined size. The difference between the original and the smoothed version may show: (1) a strong effect of smoothing; (2) the existence of many small scale variations; or (3) little effect, because surface was already smooth. The *nlg* smoothing guarantees a preservation of edges and hence a reduced influence of larger structures. The watershed segmentation derived from mathematical morphology (Serra, 1982) allows the differentiation of the surface into *regions* of depression and elevation. Quantitatively the amount of variation, e.g. the standard deviation of the difference, reveals the small scale variability of surface regions. It can be shown that sand grains in water are smoother than those in air. The increased smoothness is related to elevation regions. However, with these features a smoothness of depressions can be defined too. Biologically treated, overgrown by biofilm, grains are smoother than those in water. They show similar smoothness in regions of depression and elevation.

2.3. *Shape*

As with surface characterization, the shape of phytoplankton (see Appendix A.6 and, Fig. 4) is considered as a combination of *global* or large-scale shape properties combined with *local* or small-scale additions. Global shape properties are typically those with standard or simple shapes, such as circles or ellipses. However, only geometrically defined shapes or properties, such as symmetry, are quantitatively comparable. An example of quantification an object is provided by the well-known shape parameter P2A as the ratio of the area of a circle with object perimeter P and object area A:

$$P^2A = \frac{A_{\text{circle}}}{A} = \frac{r^2\pi}{A} = \left(\frac{P}{2\pi}\right)^2 \pi/A = \frac{P^2}{4\pi A}.$$

Some other possible features are outlined in Fig. 4b and in Fig. 4c. The *maximum inscribable* circle or the *deficiency* derived from the convex hull allow interesting characterizations for different shapes. Again the lack of descriptive tools is the largest drawback for computerized shape discrimination compared to the richness of visual shape discrimination and recognition. Shape metaphors have to be unravelled prior to transfer to quantitative descriptions.

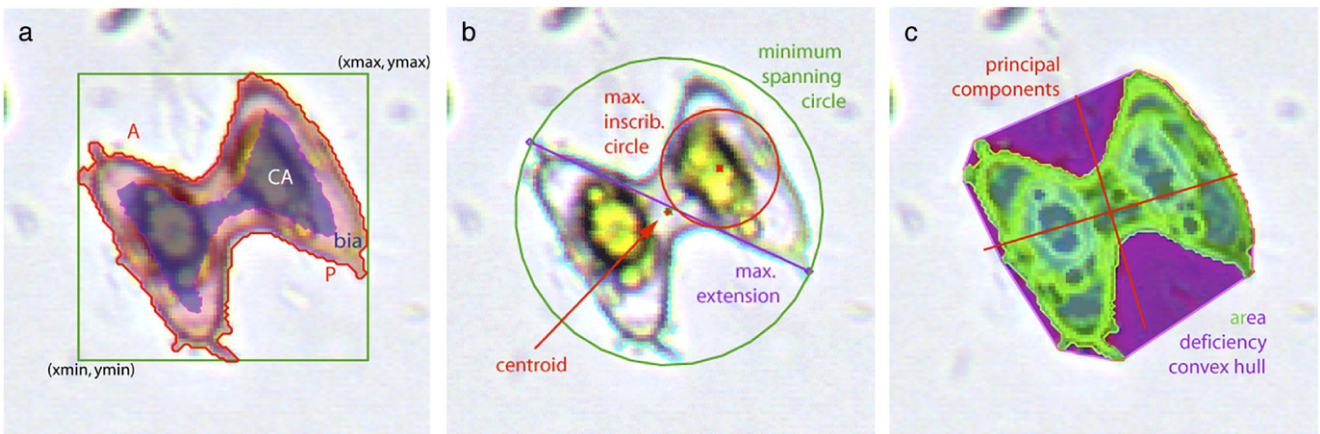

Fig. 4 – Phytoplankton: illustration of some shape features.

2.4. Current status

Interpreting images intuitively and based on experience (knowledge based) provides suggestions for characterization and hence quantification strategies. However, learnt or hypothesized attributes have to be re- (or back-) translated into algorithmic procedures for implementation into software. This translation is considerably limited by the bad overlap of visual and algorithmic descriptions. Descriptors applicable (for the field of application of the data, the type of model and the data) and acceptable by the observer must be found. This is essential for the transfer of findings to others.

Qualities such as *arrangement*, *surface* and *shape* lead to descriptive fields including well-known *measures* and *metrics*. (Meijster et al., 2000; Rodenacker and Bengtsson, 2003), but also terms *for topology*, *neighbourhood*, *relationship*, and *hierarchy* (Rodenacker and Bischoff, 1990). This recalls the terms *knowledge engineering* and *expert systems*, which are now out of favour. Although the expectations that arose from such terminology were never fulfilled, the methodologies hidden behind them help us to structure and describe visual findings.

To overcome the aforementioned unknown we start from a more or less vague verbal description consisting of words for *objects* and *entities*. The words for objects help to identify the *regions of interest*. The words describing entities help to translate from perception and intuition to *procedural fields*, e.g. size — measures of sizes, colour — spectral measures, neighbourhood — definition of a topology and/or relationship, distance — distances of related objects. Typically this process is repeated for refinement. The foregoing serve as a hint to objectify qualitative terms and attributes in this permanent process of improvement.

3. Conclusions

Multi-dimensional pictorial data can be used adequately and efficiently for determining existence, in other words for counting, e.g. organisms. However, it is clear from the examples presented, that is is not easy to extract features and even harder to describe them. We often encounter the problem of finding a way (channel) and a language (code) to transmit visual experiences to another observer (Kepes, 1965; Gordon, 1973; Eco, 1984, 2000). Unfortunately, computers are helpless at interpretation (*understanding*!) visual input. Indeed, this is true for all observers trying to transmit *qualitative*, only vaguely perceived or presumed properties.

To reformulate the starting question we could ask: Do we learn from the exaggerated use of highly resolved gridded digital data? Is the learning effect related to the effort? Both answers are clearly *yes* in the case of classical science e.g. hypothesis testing by experiments. However, it is a different situation when we try to focus on properties not fully defined, neither understood nor formulated. These properties include *growth properties*, relationships between *form* and *function* or *signal* and *function*, and (in our understanding) *weak changes* hidden in complex environments with all their consequences. An actual example is the influence of cell phones. They might cause an accident by electronic malfunction of a nearby car or plane. However, we have *not learnt* to locate influences in the much more complex living organisms.

The possibilities today to generate image data in huge amounts pose a challenge to improve in several areas:

(1) *Observation*: to sharpen the descriptive skills and hence to improve understanding and insight as well as the ability to map observations into analysis protocols and discrimination function.
(2) *Differentiation*: conscious and descriptive definition of properties such as shape, surface, structure and spatial relationship.
(3) *Logical clearness*: for the relationships of observation, model and simulation in terms of proof, test, goodness (quality) and rejection.

Acknowledgement

All the work and hence all these examples could only be presented through the input and work of the many colleagues and co-workers cited in the Appendix.

Appendix A. Descriptions of examples

A.1. The elephant and the blind men

Six blind men heard of a giant wondrous animal called the elephant. Since they were blind they could only feel the form of an elephant. A circus was passing and the blind men went to the circus and asked the elephant keeper if they could feel his great beast. The keeper agreed and each felt the elephant. They then described the elephant. The first likened it to a pillar, the second to a great big wall, the third to a leather fan, the fourth a great saber, the fifth a piece of rope, and the sixth to a fire hose. The elephant keeper explained that they were all right, and yet all wrong. Each had felt part of the elephant, but not all. The leg, is like a pillar, the body like a wall and then the ear, the tusk, and the trunk. (From http://www.spiritual-teachers.com/stories/mullah.htm, shortened)

A.2. Rhizosphere: Quorum sensing in bacteria; BA HENSE[a], K RODENACKER, M ROTHBALLER[b], A HARTMANN[b]; [a]GSF-IBB, [b]GSF-AMP

Quorum sensing (QS), a regulation mechanism by bacterial density dependent signal substances, e.g. via N-acylhomoserine lactones (AHL) in gram-negative bacteria, is regarded as a major path for bacterial cell-to-cell communication (Hense et al., 2007). AHL producing bacterial species occur in the rhizosphere, where AHLs additionally play a role in bacteria–plant interaction. Information exists about molecular mechanisms of QS *in vitro*, mostly obtained from mono-species cultures. However, knowledge about the function in more complex , environments e.g. rhizosphere, is poor. The objective of our study was to quantify information about the QS activation threshold bacteria density influences of heterogeneous bacteria distribution and root matrix.

We analyzed AHL communication distances of *Pseudomonas putida* colonizing wheat roots grown in a monoxenic quartz sand system. Two *P. putida* strains were constructed. The AHL producing strain (Iso F) was chromosomal tagged by a *gfp*-gene (green fluorescent protein), which was constitutively expressed. The AHL-deficient detector strain (F117) constitutively produced RFP (red fluorescent protein). Additionally, it contained a plasmid (pKR-C12) bearing a *gfp*-gene regulated by an AHL-controlled promoter. The expression of GFP reported the presence of a certain amount of AHL at the colonization site and together with the constitutive RFP labelling resulted in a yellow staining of the respective cells, while not induced cells remained red. With this reporter system the spreading behaviour of AHL in the rhizosphere could be assessed.

Root fibres were scanned by optical sectioning with a confocal laser scanning microscope (CLSM). Each stack of image data represented a volume of (Δx, Δy, Δz)=(325, 325, 30) μm^3 with 512×512×30 voxel (pixel). In Fig. 2c a 3-D distance transform is displayed by orthogonal sections with the region of interest (ROI) in red and iso-distance surfaces in blue. Segmentation of the digital data delivers root surface and the bacteria. In a first sweep we estimated distributions (frequency) of bacteria relative to the shortest distance to the next root surface (bacteria~root) and relative to the shortest distance to another differently marked bacterium (bacteria~bacteria). These distributions enabled us to estimate the probabilities of bacterial incidence for simulations.

A.3. *Biofilm: Horizontal gene transfer; S WUERTZ[a], M HAUSNER[b], K RODENACKER; [a]UC, DAVIS, CA 95616, [b]NU, EVANSTON, IL 60208*

Biofilms are spatially organized accumulations of microbial communities and extracellular polymeric substances (EPS) at interfaces. Conjugative transfer of plasmids or transposons has been reported in bacterial biofilm from various environments (Wuertz et al., 2001). It is considered a significant factor in spread of antibiotic resistance genes in intestinal, urogenital, and respiratory tracts. The current paradigm states that transfer of plasmids by planktonic donor cells is limited to surface biofilm layers in contact with the surrounding bulk fluid. With quantitative microscopic and image analysis techniques we show that the success of conjugation in model biofilm is determined by the specific biofilm architecture consisting of microbial cells and EPS.

CLSM volume data (pixel sizes were (Δx, Δy, Δz)=(0.3 or 0.5, 0.3 or 0.5, 1) μm^3) with recipient bacteria, donor and transconjugant bacteria were analysed. Bacteria clusters were located and the occurrence of transconjugant bacteria was estimated relative to their location inside the clusters, reflecting bacterial penetration. We could show that donor cells penetrated both the biofilm matrix and recipient clusters in biofilm layers near the attachment surface, and initiated plasmid transfer. The implications are that the spread of antibiotic resistance and other plasmid-borne genes in biofilm is not limited to recipient cells near the biofilm–liquid interface. Donors and released transconjugants can move through a biofilm, mediating horizontal and vertical transfer of plasmids (see Fig. 3a–c).

A.4. *Biofilm: Differentiation of wild and mutant bacteria Pseudomonas aeruginosa by growth behaviour; M HAUSNER[a], K RODENACKER; [a]NU, EVANSTON, IL 60208*

Biofilms were grown on cover slips in continuous flow-through stainless steel cells. Flow cells were inoculated with *P. aeruginosa* strains PA01 and MW1 (Davies et al., 1998). The strains were labelled with the red fluorescent protein (dsRed) using a pUT delivery plasmid containing a dsRed cassette carrying a gentamycine resistance marker. The biofilm was irrigated with a mineral medium with 0.2% glucose as carbon source. Biofilm development took place at room temperature for 5 days with a continuous supply of the medium at a flow rate of 0.016 ml min^{-1}. The biofilm was scanned on 5 consecutive days directly in the flow cell with a LSM410 confocal laser scanning microscope, using a 63×1.2 NA water-immersion objective resulting in daily stacks of dsRed conferred fluorescence intensity images. Daily a volume of (Δx, Δy, Δz)=(1561.6, 156.16, 20) μm^3 with 5120×512×20 voxel (pixel) (10 stacks) along the flow channel was captured.

To characterize the bacteria several measurements were performed (Rodenacker et al., 2003). A cluster was found if sufficient bacteria were located in a small neighbourhood. The following sub-volumes were measured: whole cube, slice (per depth), cluster, and cluster centre (per cluster). Measured features were volumes, centroid co-ordinates of the clusters. Besides these cluster features substrate occupation area and density, mean growth height and spatial occupation (Rodenacker et al., 2000) were estimated under spatially sensitive transformations derived from mathematical morphology (Serra, 1982). The temporal changes were compared at feature level (see Fig. 3d–f).

A.5. *Geomorphology: Rock and mineral surface modifications — Chemical, mechanical and biological; AA GORBUSHINA[a], A KEMPE[b], K RODENACKER, RW STARK[b], WE KRUMBEIN[a]; [a]CVO UNIV. OLDENBURG ICBM, [b]LMU MÜNCHEN TNMG*

Three mechanisms (1) mechanical attack by force of wind and water (2) chemical solution, and (3) mechanical (bioerosion) and chemical (biocorrosion) changes produced by biofilm growth contribute to the wear-down process of mineral and rock surfaces under different environmental conditions. It is, however, difficult to attribute the surface changes to a specific environment or process.

Quartz sand grains that have been exposed to sub-aerial or sub-aquatic conditions were analysed for traces of Aeolian, aquatic and biological wear-down. Topographic imaging of the grain surfaces by Atomic Force Microscopy (AFM) under standardized experimental conditions was done (see Fig. 1). Field width was (Δx, Δy)=(20,20) μm^2, elevation $z \approx 2400$ nm with a pixel size of 0.06 μm. Quantitative topographical parameters of surface variations were extracted by non-linear methods derived from digital image analysis. Namely, the surface maps were smoothed by non-linear Gaussian and morphological opening and closing (Serra, 1982) to extract the small scale variations relative to the strength of smoothing transformation. From the differences between the original and the smoothed version the total variations and the variations of

the positive and negative portions were estimated. These parameters were examined by multi-variate statistics, yielding three well distinguished groups. This way it was possible to differentiate the surface changes dominated by sub-aerial, sub-aquatic and biological impact. The method may also be used for the detection of Aeolian, aquatic, and even biological modification of extant and former extraterrestrial rock sites (Kempe et al., 2005, and in prep.).

A.6. Phytoplankton: automatic recognition of fresh water algae for phytoplankton profiling; BA HENSE[a], U JÜTTING[a], P GAIS[b], K RODENACKER; [a]GSF-IBB, [b]GSF-PATH

An automatic microscope image acquisition, evaluation and recognition system was developed for the analysis of Utermöhl plankton chambers in terms of taxonomic algae recognition. The system called PLASA (PLAnkton Structure Analysis) comprises (1) fully automatical archiving (optical fixation) of volatile specimens as digital bright field and fluorescence images, (2) phytoplankton analysis and recognition and (3) training facilities for the new taxa. It enables characterization of populations of aquatic specimens (Rodenacker et al., 2006).

Plankton chambers are scanned by sizeable grids, divers objective(s) and up to four fluorescence spectral bands. Acquisition positions are focussed and digitized by a TV camera and archived on disk. The image data sets are evaluated by numerous quantitative features. Some shape features are illustrated in Fig. 4. Automatic classifications for a number of organisms was developed and embedded.

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