



## Review

# Omics in algae: Paving the way for a systems biological understanding of algal stress phenomena?

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## ABSTRACT

The last decade, the biological and biomedical scientific landscape has seen the increase in use and applications of “omics” technologies. These technologies provide methods that allow for a comprehensive description of nearly all components within the cell. Microalgae not only play an important ecological role, but are also of commercial importance and therefore call for an in depth knowledge of basic biological functions. Knowledge of separate algal subsystems has gradually become available, but the challenge remains to integrate data obtained from these subsystems and from different levels of biological organization. Systems biology is a discipline that aims at this integration.

In this paper, the current status of “omics” in algae is reviewed. At the lowest level, genome studies and the use of microarrays seem to have found widespread acceptance in algal research. At higher level such as the proteome and metabolome, however, very few *omic* studies have been carried out in algae so far. Moreover, the need arises for the construction of computer databases to store obtained information in a systematic way. To illustrate the use and especially the future needs of algal “omics” in a systems biological context, a case study is presented in which a freshwater alga was subjected to heavy metal stress and toxicity endpoints were monitored on different levels of biological organization.

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## 1. Introduction

Despite the “omics” being very young technologies, they have taken up a very important position in the biological and biomedical scientific landscape during the last decade. They provide high data content generating methods that allow for a comprehensive description of nearly all components within the cell. Inside the cell, DNA is first transcribed to mRNA and translated into proteins, which

can catalyze reactions that act on and give rise to metabolites. The genome refers to the total DNA (comprising the genes) of a cell. Analogously, the transcriptome, proteome and metabolome refer to the pool of RNA transcripts, proteins and metabolites in a cell, respectively. The “omics” methods provide tools for the study of these biomolecules.

Early “omic” studies were focused mainly on bacterial and mammalian organisms because of the direct importance of the information gained to human health and biomedical applications. However, other – often less well known or common – systems play equally important roles and it is in this perspective that these systems have gradually been subjected to “omic” research as well.

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Microalgae, being at the base of the food chain, are critical components of many habitats on the planet and are major producers of oxygen. Moreover, they are of commercial importance in the food industry and aquaculture as a natural source of high-value products such as carotenoids, fatty acids, steroids and algal toxins (Cardozo et al., 2007). Algal transgenics may lead to the use of microalgae as green factories of recombinant products such as vaccines and other products with pharmaceutical relevance (Siripornadulsil et al., 2007; Griesbeck et al., 2006). Furthermore, algae are currently being grown and studied for the production of biofuels, consuming CO<sub>2</sub> in the process (Chisti, 2007).

All these and future applications demand a thorough knowledge of the basic biological functions in microalgae, a knowledge which over the years has gradually been made available partly due to the “omic” technologies. An important next step in turning data obtained at different levels of biological organization into biologically relevant knowledge is the integration of these data and the creation of a general view of what is happening in the whole system rather than in its individual compartments. It is this integration which is the main aim and focus of a systems biological approach. It is difficult to pin down systems biology to one definition, for we seem to be dealing with a cluster of partially overlapping concepts rather than a single well-defined field. Systems biology can refer to a field of study (dealing with the interaction between components of biological systems), to a paradigm (systems biology as opposed to the reductionist paradigm; integration rather than reduction), or to a collection of operational protocols used for performing research and the experimental techniques used therein (a cycle of theory – computational modelling – proposition of testable hypotheses – experimental validation and refinement of the model, making use of “omics” techniques). In any case, the recurring theme in all approaches seems to be the development of a comprehensive and consistent knowledge of a biological system by investigating the behavior of and interaction between its individual components. This requires a substantial amount of mathematical modeling, since not only qualitative knowledge on the structure of the system is necessary, but also quantitative knowledge on the state of the system, i.e. its dynamics. Research in systems biology can be divided in two major groups or disciplines that are complementary. On the one hand, research is needed on tools and algorithms for system-level studies, on the other hand research on the system properties is required, using the tools and algorithms developed (for more extensive reviews on the definition, scope and issues of systems biology, the reader is referred to Kirschner, 2005; Ideker et al., 2001; Kitano, 2002).

In this paper, the current status of algal “omic” research will be reviewed. To illustrate the use and especially the future needs of algal “omics” in a systems biological context, a case study will be presented in which a freshwater alga was subjected to heavy metal stress and toxicity endpoints were monitored on different levels of biological organization.

## 2. Genomics

Genomics is often viewed as the generation and analyses of nucleotide sequences of the full or near-full genome as well as cDNA collections. From sequence information, individual genes and repeat elements are identified, the organization and arrangement of genes are analyzed and comparisons are made among genomes with respect to gene arrangement and sequence identity or similarity (Grossman, 2005). At base level, the genomic study of an organism simply tells us something about the structure and organization of the genome of that organism. It is only in a further stage, in functional genomics, that information regarding the function of a gene can be obtained. Knowledge of the function of a gene (or a

set of genes), is the first step towards solving the puzzle that is a metabolic pathway. Comparison of the genomes of different organisms (comparative genomics) assists in phylogenetic analysis and can moreover lead to the discovery of conserved genes (Hardison, 2003).

In the 1980s, already the sequencing of a number of viral genomes had been undertaken. Today, numerous prokaryotic and eukaryotic genome sequences are publicly available (<http://www.genomesonline.org>). In the case of algae, several organellar genomes have been sequenced, and for a limited number of species, the whole genome sequence has been generated or is currently being generated in one of the algal genome projects.

The algal species probably best studied in “omic” is *Chlamydomonas reinhardtii*, a unicellular freshwater chlorophyte. A *Chlamydomonas* genome project was set up, with the genome draft sequence completed in 2003 and the whole genome sequence published in 2007 (Merchant et al., 2007). Around this period, several articles were published on the structure and organization of both *C. reinhardtii* organelles' genomes and its whole genome (Lefebvre and Silflow, 1999; Maul et al., 2002; Grossman et al., 2003; Kathir et al., 2003). *C. reinhardtii* genomic information has moreover been coupled to technologies for mRNA expression analysis of genes (a *C. reinhardtii* microarray has been developed) and is being used as a foundation for proteomic and metabolomic approaches (see below). The functional annotation of several of the genes in *C. reinhardtii* has led to the elucidation of the regulation of the nitrate assimilation pathway (Gonzalez-Ballester et al., 2005), to a better understanding of photosynthesis (Dent et al., 2005), to the assembly of the gene set involved in cell cycle regulation (Bisova et al., 2005) and to the identification of numerous other metabolic pathways (for a review, see Grossman et al., 2007).

Concerning genomics in algal species other than *C. reinhardtii*, Scala et al. (2002) have described the genome properties of the diatom *Phaeodactylum tricornutum*. Along the same line, Ohta et al. (2003) and Nozaki et al. (2007) have studied the plastid and whole genome, respectively, of the red alga *Cyanidioschyzon merolae*. In the same alga, functional and comparative genomics have led to the affirmation of a common origin of all plastids, deriving from a single endosymbiotic event (McFadden and van Dooren, 2004). Other comparative studies include the comparison of two closely related red algae, *Galdieria sulphuraria* and *C. merolae*, revealing significant differences in carbohydrate metabolism of both algae (Barbier et al., 2005), and the comparison of a *P. tricornutum* nonredundant gene set to the proteomes of *C. reinhardtii*, *C. merolae* and the diatom *Thalassiosira pseudonana*, aiming at the discovery of differences in general cell metabolism (Montsant et al., 2005). Furthermore, comparative genomics of a number of flagella and basal body related genes of *C. reinhardtii* to the human genome led to the identification of the human BBS5 gene, a novel gene for Bardet-Biedl syndrome (Li et al., 2004).

## 3. Transcriptomics

One step beyond the raw sequence data which are provided in genomics, transcriptomics provide information on the presence and relative abundance of RNA transcripts and thus offer a better view of the active components in the cell than a genomic approach. Techniques for gene expression profiling (in here gene expression profiling indicates only transcriptional responses) can be divided into two broad categories: (i) those techniques for which no *a priori* knowledge of the genes in question is necessary, such as differential display, suppression subtractive hybridization (SSH) and SAGE (serial analysis of gene expression, actually gene transcription would be more appropriate name), and (ii) techniques which do require *a priori* knowledge of gene sequences, such as microarrays.

Full descriptions of all these techniques are beyond the scope of this article (for a good review of transcriptomic technologies, the reader is referred to Bhadauria et al., 2007); for the purpose of this review only microarrays will be discussed. A microarray is a glass microscopy slide onto which gene fragments are spotted, in the form of cDNA fragments (cDNA microarray) or *in situ* synthesized oligonucleotides (oligonucleotide microarray). The principle of microarray studies is based on the ability of an mRNA molecule to hybridize to its original DNA sequence spotted on the array. Messenger RNA is extracted from samples such as control organisms and test organisms. The mRNA is reverse transcribed to cDNA and labeled with a fluorescent label. One sample is labeled with a green fluorescent dye (Cy3), whereas the cDNA from the other sample is labeled with a red fluorescent dye (Cy5). Cy3 and Cy5 labeled samples are mixed together in equal quantities and hybridized to the microarray. The array is then scanned using laser emission. Software is used to visualize the expression levels of mRNAs of the genes and the amount of each labeled target bound to each spot on the array is quantified. By determining the ratio of signal intensities between control and test cDNA, identification of induced, repressed or unchanged mRNA expression becomes possible. Hegde et al. (2000) wrote a review on the technical aspects of microarray fabrication, hybridization and analysis. For a discussion on the applications of the microarray technique in a toxicological context, the reader is referred to Lettieri (2006).

Looking at the applications of transcriptomics in microalgae, one can conclude that of the two major areas – transcriptomics as an actual identification or detection tool or transcriptomics as a way to expand our fundamental knowledge and understanding of certain metabolic pathways or biological processes – at present the latter clearly has the upper hand. A microarray has been developed for the green unicellular freshwater alga *Chlamydomonas reinhardtii* containing 10,000 oligonucleotide sequences – each sequence representing a unique gene – and covering nearly the full genome of the alga (<http://www.chlamy.org>). Because of the ready availability of this microarray, most of the existing microarray studies to reveal the underlying transcriptional mechanisms of certain pathways are carried out with *C. reinhardtii*. Using this microarray, the transcription of light-regulated genes and the involvement of phototropin therein have been studied in *C. reinhardtii* (Im et al., 2006), as well as the specificity of a chloroplast RNA stability mutant (Erickson et al., 2005). Quite a few transcriptomic analyses have been carried out applying certain stressors or stimuli to algae to unravel the underlying metabolic pathways which are involved at the transcriptional stage. In this perspective, *C. reinhardtii* has been exposed to copper and microarray analysis was carried out to look at the differential gene transcription upon exposure. mRNA levels of both the glutathione peroxidase gene and a probable glutathione S transferase gene were found to be upregulated. Moreover, several genes in the thioredoxin system were differentially transcribed (Jaspers et al., 2006). These are all genes involved in oxidative stress defense mechanisms, indicating that the microarray analysis was able to identify those genes playing a role in the metabolic processes which are involved in defense against copper toxicity. Microarrays were likewise used for *C. reinhardtii* to test the alga for its ability to acclimate to specific forms of oxidative stress (Ledford et al., 2007), to understand the response to phosphorus deprivation (Moseley et al., 2006) and survival during sulfur starvation (Zhang et al., 2004), to profile the mRNA expression patterns of genes after exposure to the explosive 2,4,6-trinitrotoluene (TNT) (Patel et al., 2004), to study anoxic mRNA expression of genes after anaerobic acclimation (Mus et al., 2007) and to survey the plastid and mitochondrial transcriptomes for changes in RNA profiles as a response to certain biotic and abiotic stimuli (Lilly et al., 2002).

Concerning organisms other than *C. reinhardtii*, dos Santos Ferreira et al. (2007) studied the mRNA expression profiles of

genes in *Euglena gracilis* under different stress conditions. To this end, they developed their own microarray by constructing a non-normalized cDNA library from *E. gracilis* and sequencing 1000 cDNAs. Microarrays were generated by spotting those ESTs whose sequence showed similarity to either Plantae or Protista genes. Similarly, a cDNA microarray was developed to screen differentially transcribed genes in the unicellular green alga *Haematococcus pluvialis* under astaxanthin-inducible culture conditions (Eom et al., 2005). For the unicellular red alga *Cyanidioschyzon merolae* a plastid DNA microarray was created to investigate the transcriptional regulation of the red algal plastid genome (Minoda et al., 2005).

Despite the fact that with the advent of microarray technology other techniques such as differential display and SAGE have been used less frequently, they are still being used to a certain extent. Dyhrman et al. (2006) used long serial analysis of gene expression for gene discovery and transcriptome profiling in the marine coccolithophore *Emiliana huxleyi*, while Im and Grossman (2001) and Rubinelli et al. (2002) used differential display to identify and study transcriptional induction of genes by high light and cadmium stress in *C. reinhardtii*, respectively.

As an actual tool transcriptomics tend to be used most in the context of biosensor development for the detection and enumeration of toxic algal species. The sandwich hybridization assay (SHA) is a regularly used method for this application. A capture probe is attached onto a solid support and functions to immobilize (usually ribosomal) RNA extracts of algal species of interest. Next, a labeled signal probe is hybridized to the rRNA, forming a sandwich hybrid complex. These hybrids are then detected by an anti-label antibody conjugated to an enzyme, which generates a signal. Depending on the enzyme used (e.g. horseradish peroxidase or an enzyme catalyzing a redox reaction) the signal will differ (e.g. colorimetric product or electrical current). The SHA has been used for monitoring the toxic algal species *Heterosigma akashiwo* (Tyrrell et al., 2002; Ayers et al., 2005), *Cochlodinium polykrikoides* (Mikulski et al., 2008) and *Alexandrium* sp. (Metfies et al., 2005; Diercks et al., 2008a) as well as for the development of multiprobe chips for the simultaneous detection of several toxic species (Diercks et al., 2008b). Ahn et al. (2006) developed a fiber-optic microarray for the simultaneous detection of multiple harmful algal bloom species. They describe a specific sandwich hybridization assay that combines fiber-optic microarrays with oligonucleotide probes to detect and enumerate different harmful algal bloom species. Along the same line, Ki and Han (2005) developed a low-density oligonucleotide array for the simultaneous detection of several harmful algal bloom species. Species specific oligonucleotides were designed based on the partial LSU rDNA (large subunit ribosomal DNA) sequence and applied to a glass slide. The array produced unique hybridization patterns for each harmful species investigated and allowed for the differentiation between closely related species.

Although the development of these tools is gradually moving in the direction of true high throughput systems allowing the simultaneous identification and enumeration of numerous species, to the present day the number of species which can be detected is still quite limited.

#### 4. Proteomics

The field of proteomics is complementary to genomics and transcriptomics in that it provides additional information on gene expression and regulation. Proteomics include the determination of protein expression levels and protein–protein interaction studies. Moreover, proteomics studies aim to identify posttranslational modifications of proteins, as well as the organization of proteins in multi-protein complexes and their localization in tissues (for a general review on proteomics and its technologies, see de Hoog and Mann, 2004).

Technologies enabling proteomic studies have been in development for several decades now. A proteomic experiment involves a number of typical steps, which can be taken using different technological platforms (for a review on technologies and applications in proteomics, see [Molloy and Witzmann, 2001](#)). A crucial, first step is sample preparation and fractionation, which traditionally is being done using two-dimensional gel electrophoresis (2-DE). Proteins are separated based on their electrical charge (by means of isoelectric focusing, IEF) in the first dimension and based on their molecular weight (by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE) in the second. The dynamic range of 2-DE can be improved by enriching the sample for certain desired proteins. Membrane proteins, for example, can be enriched by a sequential extraction based on the solubilities of proteins in different solutions. After sample separation, the next step is protein identification. Proteins of interest can be excised from a 2-DE gel, digested and identified by mass spectrometry. Peptide mass data obtained for each sample are queried against a peptide database with known peptide masses of unique proteins ([Gevaert and Vandekerckhove, 2000](#)).

Regarding proteomics in algae, it is again the freshwater chlorophyte *C. reinhardtii* that most studies have focused on. [Stauber and Hippler \(2004\)](#) published a review on *C. reinhardtii* proteomics, discussing mainly studies of cellular compartments and functional groups of proteins. Other, similar, investigations were carried out analyzing the eyespot and centrioles in *C. reinhardtii*, respectively ([Schmidt et al., 2006](#); [Kelle et al., 2005](#)) and the cell wall proteins of the green alga *Haematococcus pluvialis* ([Wang et al., 2004a,b](#)). [Vener \(2007\)](#) recently published a review focusing on environmentally modulated phosphorylation and dynamics of proteins in photosynthetic membranes in plants and *C. reinhardtii*.

As in transcriptomic approaches, proteomics can also be used for the elucidation of certain metabolic pathways. In comparative proteomics, a large fraction or the entire proteome of one sample is compared to that of another sample, kept under different environmental conditions. Proteins differentially expressed due to the environmental stimuli can be identified, as well as the metabolic pathway they are part of. Compared to the proteomic analysis of specific subcellular compartments or specific protein classes, comparative proteomic studies have so far not been reported that often. [Förster et al. \(2006\)](#) established a partial 2-D reference map from wild type *C. reinhardtii*, representing primarily the soluble subproteome. They subsequently used this map to investigate high light-induced changes to the proteome and at the same time compared the response of both the wild type and a mutant. Similarly, [Naumann et al. \(2007\)](#) used a comparative quantitative proteomic approach to study iron deficiency in *C. reinhardtii* and found the induction of stress response proteins, such as peroxiredoxin and a stress-induced light-harvesting protein, as well as a number of proteins of unknown function. In studying the effects of cadmium exposure on *C. reinhardtii*, [Gillet et al. \(2006\)](#) found a decrease in abundance of both large and small subunits of the ribulose-1,5-bisphosphate carboxylase/oxygenase, in correlation with several other enzymes involved in photosynthesis. Cadmium-induced protein profile alterations were also investigated in the marine alga *Nannochloropsis oculata* ([Kim et al., 2005](#)). In *Haematococcus pluvialis* a proteomic approach was employed to elucidate the alga's response to oxidative stress ([Wang et al., 2004a,b](#)) and in the halotolerant alga *Dunaliella salina* the molecular basis of salinity tolerance was clarified by proteomics ([Liska et al., 2004](#)). Finally, in the context of harmful algal bloom prediction, [Chan et al. \(2004\)](#) constructed proteome reference maps for several algal species and tested them for their ability for species recognition. Species specific 2-DE protein profiles were observed for all species tested and even distinction between closely related species was possible.

## 5. Metabolomics

Metabolites are the end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes. Analogous to the terms 'genome', 'transcriptome' and 'proteome', the metabolome refers to the set of low molecular weight metabolites present in a cell ([Fiehn, 2002](#)). The size of the metabolome varies greatly depending on the organism studied. [Förster et al. \(2003\)](#) reconstructed the metabolic network of *Saccharomyces cerevisiae* using genomic, biochemical and physiological information and found that the network contains 584 metabolites. In contrast, [Fiehn \(2002\)](#) reports on an estimated number of 200,000 metabolites in the plant kingdom. Even within the metabolome of one organism, many different kinds of metabolites exist, with different chemical and physical properties. Moreover, the levels at which various metabolites are present within a cell can cover several magnitudes of concentration. The vastness of the metabolome and its diversity make it technologically impossible to analyze all an organism's metabolites in a single analysis. Different strategies and methodologies have therefore been developed (for a review on current analytical platforms and methodologies, see [Fiehn, 2002](#), and [Dunn and Ellis, 2002](#)). Metabolite target analysis is restricted to one or a few metabolites related to a specific metabolic reaction and as such does not constitute a high throughput approach. This strategy is mainly used for screening purposes. To elucidate the function of a metabolic pathway, the metabolite profiling approach identifies and quantifies a selected number of predefined metabolites, which belong to a certain class of compounds or to a specific pathway. Metabolic profiling is often used in the context of drug research to study drug candidates. Finally, in metabolic fingerprinting, a rapid, global analysis is performed for sample classification according to origin or biological relevance. Quantification and metabolic identification are generally not necessary, allowing for a higher throughput of samples.

In algae, most metabolic analyses have so far been focused on the quantification and identification of secondary metabolites with economical value in food science, pharmaceutical industry and public health, among others. Fatty acids, steroids, carotenoids, polysaccharides, lectins, polyketides and algal toxins are among the algal products being studied (for an extensive covering of species of high potential and their valuable secondary products, see [Richmond, 2004](#)). Environmental metabolomic studies, in contrast – as the application of metabolomics to characterize the metabolic response of an organism to environmental stimuli or stressors – have to date only rarely been carried out in algae. [Bölling and Fiehn \(2005\)](#) report on the metabolite levels of *C. reinhardtii* under nutrient deprivation. They describe a procedure for cell preparation and metabolite extraction. In extract chromatograms of standardly grown algae (sufficient nutrients), more than 800 metabolites could be detected, with Ala, pyruvate, Glu, glycerolphosphate and adenosine 5'-monophosphate being among the most prominent peaks. When cells were grown under nutrient deficient conditions (depletion of nitrogen, phosphorus, sulfur or iron), highly distinct metabolic phenotypes were observed.

## 6. Future needs: integrating “omics” in systems biology

Thanks to the availability of complete genome sequences and the advent of high data content measurement techniques for transcripts, proteins and their interactions and metabolites, a new level of understanding of cells and organisms has become possible. As already mentioned in the introduction, in systems biology, the main goal is to develop a comprehensive and consistent knowledge of a biological system by investigating the behavior of and interac-



tion between its individual components. One of the key steps in this process involves modeling. Once the structure of the system is unraveled, mathematical algorithms allow its dynamics to be modeled. Mathematical models describe the system, but also allow the prediction of the system's response to perturbations. Ideker et al. (2001) formulated a framework for systems biology studies, involving several distinct steps. In a first step, understanding of the structure of the system is required. This involves the identification of the elements of the system, such as gene networks, protein interactions and metabolic pathways. This knowledge is used to construct an initial model of its behavior. Depending on how much is known about the different components of the system the modeling can be carried out at several levels. Steady-state studies can be done even when knowledge on certain parameters is lacking. Using the available knowledge on the system's structure theoretical upper and lower limits are calculated, as well as an optimal operation point of the system in steady-state. Other types of analyses allow for an understanding of the dynamic properties of a system, but require knowledge of certain parameters. Secondly, the system is perturbed (genetically or using environmental stimuli) and corresponding responses are measured using high throughput measurement tools. Data observed at different levels of biological organization are integrated with each other and with the current model of the system. Following, the model is adapted in such a way that the experimentally observed phenomena correspond best with the model's predictions. A new set of perturbations is selected and applied to the model, to distinguish between multiple model hypotheses. These steps are continually repeated, thereby expanding and refining the model until the model's predictions reflect biological reality.

It is obvious that a systems biology analysis requires collective efforts from multiple research areas, such as molecular biology, computer science and control theory, and is therefore not easy to accomplish. Recent and ongoing studies putting the above systems biology framework into practice have focused on bacterial chemotaxis in *E. coli*, sugar metabolism in *S. cerevisiae* and embryo development in the sea urchin, among others (for a more thorough sampling and discussion of systems biology approaches, see Ideker et al., 2001). For algae, no systems biology studies with extensive computational modeling efforts have been reported so far to our knowledge. However, more and more knowledge of the components of algae has become available and the first results on the integration of observations on different levels of biological organization are being published. Mus et al. (2007) used metabolite, genomic and transcriptomic data to provide genome-wide insights into the regulation of the metabolic networks utilized by *Chlamydomonas reinhardtii* under anaerobic conditions associated with  $H_2$  production. During acclimation to anoxic conditions the hydrogenase activity, photosynthesis, cellular respiration and organic acid accumulation of algal cells were monitored. In conjunction with the formation of fermentation products the levels of transcripts encoding proteins associated with the various fermentation pathways were analyzed using real time PCR. After establishing that the algae were acclimated to the anoxic conditions microarray analyses were carried out to gain insight in the effects of dark anaerobiosis on transcript abundance in a genome-wide context. Results showed that congruent with elevated  $H_2$  production following exposure of the algae to anaerobic conditions was the accumulation of various fermentation products. These findings on metabolome level were augmented with real time PCR analysis. Results obtained by microarray analysis were in agreement with the real time PCR data. In addition, genes encoding proteins involved in other metabolic pathways as well as encoding transcription/translation regulatory factors were differentially transcribed. Although this study cannot be considered a true systems biology approach yet – rather than relating individual elements quantitatively to each other and to the covering system, a qualitative analysis is made – here we see the

onset of the integration of data gathered on different levels of biological organization to improve our understanding of (a metabolic network of) a biological system.

## 7. Case study—a systems biological approach to heavy metal stress in algae

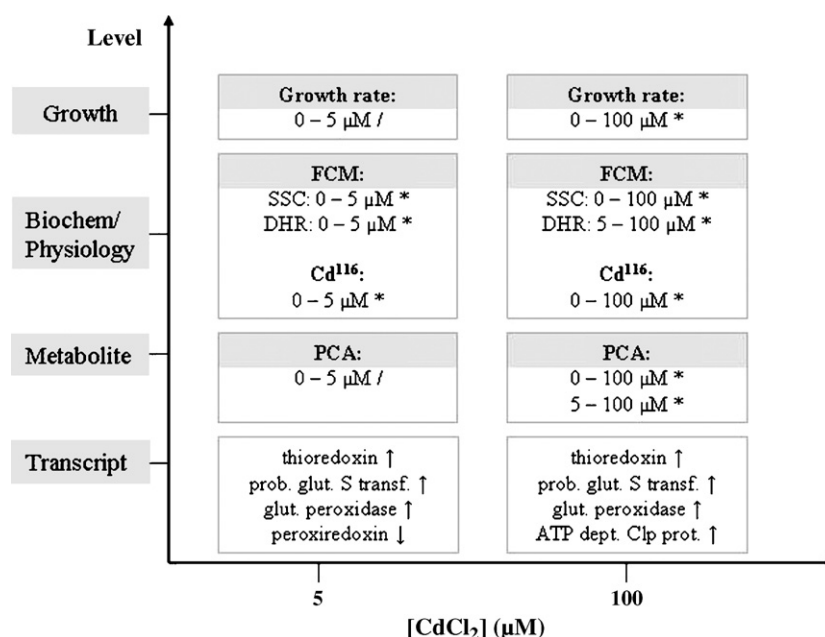
To illustrate the use and future need of “omics” in algae in a systems biological framework, results are presented here of an experiment we conducted in which the green freshwater alga *C. reinhardtii* was exposed to the trace metal cadmium. Different end-points were followed up at different levels of biological organization and an attempt was made to relate the data to each other qualitatively so as to develop a more comprehensive view of the effects and toxicity mechanisms of this potentially toxic compound.

During 72 h, *C. reinhardtii* was exposed to either a low (total nominal concentration of 5  $\mu\text{M}$  – actual concentration of  $5.5 \pm 1.16 \mu\text{M}$ , as determined with atomic absorption spectroscopy) or a high concentration of  $\text{CdCl}_2$  (total nominal concentration of 100  $\mu\text{M}$  – actual concentration  $107.03 \pm 23.9 \mu\text{M}$ ). A control culture (no  $\text{CdCl}_2$  added – actual concentration  $0.007 \pm 0.01 \mu\text{M}$ ) was included. The following effects were assessed: at organism level growth and growth rate were followed up. At mRNA expression level of genes microarrays were carried out to investigate which mRNAs were differentially expressed. Similarly, at the metabolome level differences in metabolite pools due to exposure were looked for. Finally, at physiological and biochemical level flow cytometry was used to analyze the viability of the algal cells as well as the presence of reactive oxygen species. Moreover, Cd uptake kinetics was monitored using stable isotopes. Since practically it was impossible to perform all these measurements in one experiment, separate exposures were set up. For details on the experimental conduct, the reader is referred to the specific related articles (Jaspers et al., 2006, submitted for publication).

Fig. 1 gives an overview of the setup and the results observed at different levels of biological organization. Whereas no significant differences in growth were found between control algae and algae exposed to 5  $\mu\text{M}$   $\text{CdCl}_2$ , algae grown in a medium with 100  $\mu\text{M}$   $\text{CdCl}_2$  did show a significantly reduced growth compared to both the control and 5  $\mu\text{M}$  cultures.

Microarray hybridizations revealed that in algae exposed to 5  $\mu\text{M}$   $\text{CdCl}_2$  mRNAs of a total number of 364 unique genes were differentially expressed; for the 100  $\mu\text{M}$  cultures this number came to 469. Of all the genes with differential mRNA expression, 108 were functionally annotated. The mRNA expression of certain genes involved in oxidative stress defense mechanisms and the redox regulation of the cell such as thioredoxin, a probable glutathione S transferase and glutathione peroxidase was strongly influenced by the exposure of the algae to  $\text{CdCl}_2$ . Since cadmium is known to cause its toxic effects in part by the generation of oxidative stress, it was not unlikely to encounter transcriptional induction of these genes. From the technical point of view, however, these results at the same time also do show that with the use of microarray hybridizations it is possible to identify possible mechanisms of toxicity.

At metabolite level, principal component analysis (PCA) of nuclear magnetic resonance (NMR) measurements revealed that – all cultures considered – separation of the different exposure cultures was possible. However, it seemed to be the algae exposed to 100  $\mu\text{M}$   $\text{CdCl}_2$  that contributed most to this separation. To test for this, PCAs were conducted comparing cultures one by one. We observed that the 0  $\mu\text{M}$  and 5  $\mu\text{M}$  cultures did, indeed, not separate out from each other, whereas a clear separation could be found for 0  $\mu\text{M}$  and 100  $\mu\text{M}$  and 5  $\mu\text{M}$  and 100  $\mu\text{M}$  cultures. Analyses of gas chromatography coupled to mass spectrometry (GC–MS) measurements yielded essentially the same outcome as the PCAs



**Fig. 1.** Setup and results of case study. \* = significant differences between indicated cultures were found. / = no significant differences between indicated cultures were found. The transcript level only presents a selection of differentially expressed mRNAs of genes. Abbreviations:  $\uparrow$  = upregulation;  $\downarrow$  = downregulation; prob. glut. S transf. = probable glutathione S transferase; glut. peroxidase = glutathione peroxidase; ATP dept. Clp prot. = ATP dependent Clp protease proteolytic subunit; PCA = principal component analysis; FCM = flow cytometry; SSC = side scattering signal; DHR = dihydrorhodamine 123; Cd<sup>116</sup> refers to the stable isotope tracer experiment.

carried out with the NMR results. This led us to conclude that a very small metabolic difference exists between control algae and algae exposed to 5  $\mu\text{M}$  CdCl<sub>2</sub>.

Flow cytometric measurements revealed a significant increase in side scattering with increasing cadmium concentration, reflecting increased internal complexity and granularity. With increasing cadmium concentration we also found a significant increase in the mean fluorescence intensity of dihydrorhodamine 123 (DHR123), reflecting intracellular reactive oxygen species (ROS) generation. These findings not only agree with the general knowledge that heavy metals exert their toxicity through the generation of ROS, but also confirm our results at mRNA expression level of genes.

Results of the stable isotope (Cd<sup>116</sup>) tracer experiment showed that the exposure concentration gradient is nicely reflected in the internal cadmium content, with algae exposed to 5  $\mu\text{M}$  Cd<sup>116</sup> and 100  $\mu\text{M}$  both clearly exhibiting a higher metal content than the control culture – indicating that cadmium was indeed taken up in the cells – and algae exposed to 100  $\mu\text{M}$  Cd<sup>116</sup> in their turn accumulating more cadmium than the 5  $\mu\text{M}$  culture. A strong accumulation was observed in the first 3 h of exposure, after which cadmium concentrations steadily declined, suggesting a regulatory mechanism being at work. However, at the end of the exposure period, differences in metal content between the three cultures persisted, indicating that neither of the exposed cultures succeeded in removing the metal completely.

With this case study, we aim at illustrating the intrinsic potential of “omics” data in a systems biology framework. When connecting the “omics” results with each other, we find both confirmation and complementation over the different levels of biological organization and see that results at each level have their own added value to contribute to the bigger picture. As an example of complementation, we saw at transcript level – in contrast with the growth results, where no significant differences were found between the control culture and algae exposed to 5  $\mu\text{M}$  CdCl<sub>2</sub> – that exposure to a low concentration already did cause mRNAs of a considerable number of genes to be differentially expressed, among which were genes play-

ing a role in oxidative stress defence mechanisms, such as a probable glutathione S transferase and glutathione peroxidase (see above and Fig. 1). This means that even though at a higher level of biological organization no effects are observed, at a lower level already a number of changes are taking place. These changes are probably situated in traditional homeostasis phenomena. Interestingly, we moreover found that – although the difference between the 5  $\mu\text{M}$  and 100  $\mu\text{M}$  cultures was outspoken at growth level – there was only a 14% increase in the number of differentially expressed mRNAs of genes in the algae exposed to 100  $\mu\text{M}$  CdCl<sub>2</sub> compared to the 5  $\mu\text{M}$  exposed cultures. Besides function, one might say that it is not the number of differentially expressed mRNAs of genes that matters, but the degree of induction or repression. However, in the function or in the number of differentially expressed mRNAs, only small but no marked differences could be noticed between the two exposed cultures.

Compared to the results obtained with the microarray hybridizations, the outcome of the metabolomics analyses more closely resembled – and seemed to confirm – our observations at growth level with no apparent effect of cadmium on algae when exposed to a low concentration (5  $\mu\text{M}$ ), but a clear difference between control algae and algae exposed to a high concentration (100  $\mu\text{M}$ ). Flow cytometric observations proved to give insight in the mechanisms of toxicity and at the same time confirmed microarray results. Side scattering signals (SSCs) showed an increased granularity and complexity of exposed cells, corresponding to observations found in literature (Reiriz et al., 1994; Franklin et al., 2001a). Results of DHR123 staining, furthermore, indicated a higher presence of ROS with increasing cadmium concentration, shedding light on the mechanism of toxicity. Finally, internal cadmium concentrations in the algae showed that in both exposed cultures cadmium was indeed taken up. However, in algae exposed to 100  $\mu\text{M}$  cadmium this concentration was much higher than in algae exposed to 5  $\mu\text{M}$  cadmium. Taken together with our observations at growth level this once again points to 5  $\mu\text{M}$  being a concentration still within the homeostatic range of *C. reinhardtii*, whereas 100  $\mu\text{M}$  cadmium clearly elicits toxic effects.

## 8. Conclusions—future perspectives

The last decade, the use of “omics” techniques has expanded enormously. “Omics” technologies have been applied in many different organisms and research areas. In this paper, the current status of “omics” in algae was reviewed. Moreover, a case study was discussed in which effects of a low and high concentration of cadmium on *C. reinhardtii* were studied at different levels of biological organization and an attempt was made to integrate them and eventually create a systems biological view of what happens in the algal cell. Based on the results, we concluded that the low cadmium concentration still fell within the homeostatic range of the algae, whereas the high concentration clearly caused toxic effects. Moreover, the study revealed that both confirmation and complementation were found for data acquired at different levels of organization. Each level provides an information building block needed to solve the puzzle of the structure of the whole system. At the same time, putting together all the data allows for a better insight into the mechanisms of cadmium toxicity. This case study demonstrates the value of approaching a scientific question from different angles so as to generate a more comprehensive data set of which the separate components can be linked to each other making a more in depth knowledge of the problem under study possible. However, even though a considerable number of levels of organization were touched upon in this case study, no modeling-based systems biological approach was conducted yet.

More generally, for algae, the challenge in the future will firstly be the further expansion of our knowledge of the basic components of different algal systems. On genome level, steady progress is being made with the ever increasing number of algal genome projects being set up or completed. Likewise, the use of microarrays has found widespread acceptance in algal studies. On higher levels, however, very few studies have been carried out so far analyzing the whole proteome or metabolome or large parts thereof. Concurrent with fundamental research, attention should be given to the construction of computer databases to store obtained information in a systematic way and computer models should be developed in which data are assimilated such that predictions regarding network behavior will become possible.

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