



4TH SULPHYTON

5th - 8th September 2013
Athens, Greece



Welcome to the 4th Sulphyton Workshop on the Sulphur Nutrition of plants

Plant Sulphur Network

4th Sulphyton Workshop

5th - 8th September 2013

Stanley Hotel, Athens, Greece

**Organised by the Agricultural University of Athens
Plant Physiology Laboratory**

Cover photos

Upper – The sulfidic metallurgical processing wastes of Komobil, with the characteristic yellow colour during the summer; in the winter the colour turns red purple, especially after raining. These tailings which have been produced between 1953 and 1963, are characterised by high levels of Ag, As, Bi, Co, Fe, K, S, Sb, Sn and V and are weathered to a variable extent. (photo: Dimitris Bouranis)

In the background – The theatre of Thorikos above the mine at Velatouri hill, one of the most ancient theatres in Greece, along with the reconstructed washery. Thorikos constitutes a unique ancient mining centre worldwide; the first underground excavations began around 3000 BC, a fact that establishes Thorikos mines among the oldest in Europe. Mining provided silver containing lead sulfide or argentiferous galena. (photo: Eleni Papazoglou)

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**and it is a joint project with JKI,
Braunschweig, Germany**

Thursday, 5th September 2013

17:00 – 19:00 Welcome and registration

Friday, 6th September 2013

Oral presentations

Session I – Chairperson: Malcolm Hawkesford

- 09:00** **Dimitris Bouranis**
Opening of the 4th Sulphyton Workshop
- 09:05** **Rainer Hoefgen**
Towards systems biology of plant sulfate metabolism: The OAS module
- 09:30** **Anna Wawrzyńska**
Arabidopsis cytoplasmic serine acetyltransferase interacts with transcription factor SCL11
- 09:45** **Anna Znój**
In search of UP15 function, a protein encoded by gene induced during sulfur and nitrogen deficiency
- 10:00** **Sarah J. Whitcomb**
Using metabolic engineering to improve the nutritive quality of rice
- 10:15** **Philippe D’Hooghe**
Alterations of seed yield and quality in sulphur-limited *Brassica napus* L.
- 10:30** **Coffee break**

Session 2 – Chairperson: Mario Malagoli

- 11:00** **Elke Bloem**
How abiotic stress affects glucosinolate biosynthesis in plants
- 11:15** **Tahereh Aghajanzadeh**
Impact of pedospheric and atmospheric sulfur nutrition on glucosinolate metabolism in *Brassica* species

- 11:30 Elise Sorin**
Effect of S-limitation on osmotic potential components in oilseed rape leaves: towards the development of early indicators?
- 11:45 Assylay Kurmanbayeva**
Elucidating the molecular components that allow *Salicornia* and *Sarcocornia* to thrive in high sulfate and sulfide levels
- 12:00 Dmitry Yarmolinsky**
The role of sulfite reductase in sulfite homeostasis
- 12:30 Lunch**

Session 3 – Chairperson: Rüdiger Hell

- 15:00 Robert Hänsch**
Regulatory Network of SO₂ detoxification
- 15:30 Elmien Heyneke**
The arbuscular mycorrhizal symbiosis influences sulfur starvation responses of *Medicago truncatula*
- 15:45 Bernd Zechmann**
Compartment-specific changes in glutathione and ascorbate levels during high light stress in Arabidopsis
- 16:00 Nora Luschin-Ebengreuth**
The role of antioxidant metabolism during dark-induced senescence in Arabidopsis
- 16:15 Barbara Koffler**
Cadmium sensitivity of *pad2-1* and *vtc2-1* mutants is correlated to lower subcellular glutathione rather than ascorbate contents
- 16:30 Coffee break**

Session 4 – Chairperson: Agnieszka Sirko

- 17:00 Luit J. De Kok**
Metal toxicity and sulfur metabolism in Chinese cabbage
- 17:30 Martin Reich**
Using H⁺- selective microelectrodes to determine sulfate fluxes at roots of intact plant seedlings

- 17:45 Annekathrin Weese**
Metabolic coordination of sulfur metabolism in *Brassica napus* by clock-controlled genes
- 18:00 George Diliias**
The effect of the continuous light in combination with sulfur deprivation on the chlorophyll levels and carotenoids in young maize leaves
- 18:15 Filippa Maniou**
Aerenchyma formation in maize leaves during sulphate deprivation
- 18:30 Fayeze Arabi**
Functional analysis of Arabidopsis SDI1/2
- 18:45 Chat**
- Dinner**

Towards systems biology of plant sulfate metabolism: The OAS module

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O-acetyl-L-serine (OAS) provides the carbon backbone for cysteine synthesis and is assumed to positively regulate sulfur deficiency-responsive genes as its amount increases upon sulfur limitation. Therefore, mRNA accumulation of a number of sulfur responsive genes is apparently controlled by OAS. Recently this hypothesis was strengthened by global co-expression analysis under conditions of variable OAS levels with constant sulfur status, such as light dark shifts, oxidative stress and, obviously, also under sulfate starvation. This led to the identification of a stably co-regulated gene cluster, the OAS-module.

Arabidopsis cytoplasmic serine acetyltransferase interacts with transcription factor SCL11

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Cysteine biosynthesis in higher plants involves a bienzyme complex, the cysteine synthase complex (CSC), which consists of serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OAS-TL) enzymes. The formation of CSC alters the activities of both enzymes and serves SAT activation but OAS-TL deactivation. This reversible association of both proteins controls cellular sulfur homeostasis. There are different isoforms of both enzymes in cytosol, plastids and mitochondria, however, the relevance of CSC formation in each compartment for flux control of cysteine synthesis remains controversial. In this study, we report the application of a yeast two-hybrid method that used the entire Arabidopsis cytoplasmic CSC to search for interacting proteins. This approach identified, among other putative partners of the CSC, a transcription factor SCL11. Interestingly, the formation of the CSC is not a prerequisite for the interaction as more detailed studies revealed that SCL11 binds to SAT5 but not to OAS-TLA1. Additionally, the interaction domain is likely present in a structurally conserved region of SAT because SCL11 was also able to interact with other SAT isoforms. The interaction was next confirmed by BiFC method, revealing the cytoplasmic localization. The possible biological role of SAT5-SCL11 interaction will be discussed.

Acknowledgments: This work was supported by the Polish Ministry of Science and Higher Education (project No 344/N-DFG/2008/0) and the National Science Centre (project No 2011/03/B/NZ1/01243).

In search of UP15 function, a protein encoded by gene induced during sulfur and nitrogen deficiency

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Many regulatory aspects of plants adaptation to sulfur deficiency are still poorly understood. Investigation of the proteins of unknown function encoded by the genes strongly regulated during sulfur limiting conditions may help to clarify these processes. In our laboratory, by the application of SSH method, we identified several genes from tobacco as “up” or “down” regulated during sulfur deficiency. We concentrated on defining the function of genes encoding proteins of unknown function. One of these genes is *UP15*, strongly induced by sulfur deficit. *UP15* encodes a small protein (168aa) with a Gly- and His-rich regions and one possible nuclear localization signal (NLS) located at the C-terminus. However, despite the fact that UP15::GFP fusion protein shows a nuclear localization, UP15 fused to the double GFP::GUS-tag was observed only in cytoplasm, when transiently expressed in *Nicotiana benthamiana* leaves. Therefore, the functionality of NLS signal is still unclear. Strong regulation of *UP15* expression by sulfate availability or by nitrogen limitation was confirmed by northern blots and RT-PCR. The highest level of *UP15* transcript was observed in young and mature leaves of tobacco while in roots the gene expression was rather low. Sequence similarity and expression patterns allowed us to select four putative homologs of *UP15* from *Arabidopsis thaliana* (At2g05580, At2g05510, At1g64730, At5g46730). Their expression in response to low sulfur or low nitrogen stress was checked by RT-PCR. Based on these results At2g05510 was chosen for further studies. Our preliminary results suggest that UP15 may play a role in adaptation of plant metabolism to the imbalanced nitrogen/sulfur supply. However the precise role of UP15 in this regulatory network remains to be uncovered.

Using metabolic engineering to improve the nutritive quality of rice

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Rice is a staple food for 3 billion people and can account for a significant proportion of their dietary protein, especially in the developing nations of Asia. However rice protein is deficient in several amino acids essential in animal diets, including lysine, tryptophan, and methionine. Unless the diet is supplemented with other protein sources, these amino acid deficiencies can result in significant health consequences for humans and significantly stifled growth in animals. Traditional breeding programs have developed high yielding varieties with higher seed protein levels (e.g. IR64), but these varieties are still deficient in several essential amino acids. A more targeted approach to improve amino acid balance in rice is seed-specific expression of exogenous storage proteins rich in essential amino acids. Despite high exogenous storage protein expression in the seed, overall levels of essential amino acids remained nearly unchanged, perhaps due to limited free amino acid pools in these plants. Similarly, increased biosynthesis of these essential amino acids in leaves also failed to sufficiently improve seed protein quality. Since neither manipulation of sink nor source strength proved to be sufficient on its own, we are combining these two approaches in one rice line. We anticipate that this “Push plus Pull” approach will result in rice with greatly improved nutritive quality for human and animal consumption.

Alterations of seed yield and quality in sulphur-limited *Brassica napus* L.

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Sulphur (S) limitation reduces seed yield and quality of various crops such as cereals, mainly used for producing flour, or oilseed rape, a high S demanding plant cultivated for its nutritional value for animal and human feeding. In this work, we describe the impacts of S restriction applied at the bolting (LS32), early flowering (LS53) or start of pod filling (LS70) stages on *Brassica napus* seed composition. For this purpose, lipids were analysed by Near-infrared spectroscopy and two-dimensional electrophoresis were performed on total proteins extracted from mature seeds. The major S compounds of mature seeds were also determined. The reduction of protein quality observed for all LS seeds was related to a reduction of S-rich seed storage protein (SSP) accumulation (as Cruciferin Cru4) at benefit of S-poor SSP (as Cruciferin BnC1). Through this adaptive response, the protein contents of LS70 and LS53 seeds were not affected, but it was reduced for LS32 seeds. The reduction of lipid content in LS53 and LS32 seeds was primarily associated with a reduction of C18 derivatives. The $\omega 6/\omega 3$ ratio was increased in LS53 and LS32 seeds. Modulations of proteins associated with lipid storage and carbohydrate metabolism (reduction of caleosins, glyoxysomal malate synthase, thiazole biosynthetic enzyme THI1; accumulation of citrate synthase) could be involved in the alteration of lipid composition of LS53 and LS32 seeds. The accumulation of proteins associated with stress response and a lower level of glutathione in LS53 and LS32 seeds may decrease seed resistance to biotic/abiotic stresses during conservation and germination.

How abiotic stress affects glucosinolate biosynthesis in plants

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The glucosinolates (GSL) content is an important quality parameter in crops such as mustard and nasturtium. Both plants are grown for their taste and medical purposes. The impact of different stress parameters on the GSL content and growth of *Tropaeolum majus*, *Sinapis alba* and *Brassica juncea* was investigated in greenhouse trials. Drought stress (soil water content of 4-8 Vol% in comparison to 12-18 Vol% in the control), salt applications (2 mg per day with irrigation water until evapotranspiration was reduced effectively) and methyljasmonate (MeJA) (spray application of 4mL of a 200µmol/L MeJA solution) were applied. Different parameters were recorded to classify the impact of the treatments on plant performance: evapotranspiration, biomass development, specific leaf weight, plant pigments, plant thiols and the GSL content. Plants were harvested three times during the vegetation period.

In all three plant species drought stress and the application of MeJA increased the content of major GSLs on a dry weight basis: sinalbin in *S. alba*, sinigrin in *B. juncea* and glucotropaeolin in *T. majus*. Salt applications did not affect the GSL content consistently. It can be concluded that drought stress and MeJA are suitable to increase the GSL content on production fields. However, drought stress reduces biomass production whilst increasing the GSL content so that total GSL yield proved to be comparable to the control or even lower. A high GSL concentration in the dry mass together with a reasonably high yield was only achieved after application of MeJA.

Impact of pedospheric and atmospheric sulfur nutrition on glucosinolate metabolism in *Brassica* species

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In addition to sulfate taken up by the roots, *Brassica* species are able to utilize atmospheric sulfur gasses, viz. H₂S or SO₂ taken up by the leaves as sulfur source for growth. In current study the impact of atmospheric sulfur deposition on glucosinolate metabolism was studied in two *Brassica* species characterized by a high (*B. juncea*) and low (*B. rapa*) glucosinolate content. At an ample sulfate supply, exposure of both species to atmospheric sulfur gasses hardly affected content and composition of glucosinolate. H₂S and SO₂ exposure resulted in a slight decrease in expression of APS reductase expression whereas that of APS kinase and ATP sulfurylase remained unaffected. Sulfate-deprivation of plants resulted in a decreased biomass production and glucosinolate content. Expression of APS reductase was strongly enhanced in sulfate-deprived plants but expression of both APS kinase and ATP sulfurylase was unaffected. When sulfate-deprived plants were exposed to H₂S or SO₂, plant growth was restored, however, the glucosinolate content remained lower than that of sulfate-sufficient plants. Moreover, the expression of APS reductase was partially downregulated again, whereas expression of APS kinase and ATP sulfurylase remained unaffected.

Effect of S-limitation on osmotic potential components in oilseed rape leaves: towards the development of early indicators?

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Oilseed rape (*Brassica napus*) is a S demanding crop, S-limitation resulting in a reduction of yield and of nutritional quality of seeds. Optimization of S fertilization requires to identify indicator of S nutrition that could be used early in the growth cycle.

In this study, we examined the effects of S-limitation on osmotic potential components of oilseed rape leaves. Plants were grown at vegetative stage and submitted to S-limitation (S+: 508.7 $\mu\text{M SO}_4^{2-}$ or S-: 8.7 $\mu\text{M SO}_4^{2-}$) and were harvested at 0, 1, 2, 3, 7, 13, 24 and 34 days of S-limitation. Each plant was sampled as old leaves, new leaves emerging after S-limitation, roots and petioles.

Plant growth was maintained during the first 13 days of S-limitation as a result of massive internal sulfate mobilization mostly from leaves and its subsequent assimilation. This was at least partly compensated for by an accumulation of malate, nitrate, chloride and phosphate in leaves and to a lesser degree in roots. More surprisingly, leaf osmotic potential decreased after two days of S-limitation. Other compounds such as amino acids, soluble sugars and cations will be quantified in order to evaluate their contribution to the leaf osmotic potential. Additional data (¹⁵N-nitrate uptake, nitrate reductase activity, transcript levels of sulfate and nitrate transporters) show that under S-limitation, osmotic potential is affected earlier than growth or N metabolism suggesting that field measurements of leaf ion contents, acting as osmoticum, could be used as early indicators for S fertilization management.

Elucidating the molecular components that allow *Salicornia* and *Sarcocornia* to thrive in high sulfate and sulfide levels

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Salicornia and *Sarcocornia* are extreme halophytes that grow on sea water with high sulfide and sulfate levels and have been recently introduced as new crops for extreme salt conditions such as in the level of Dead Sea water. We are exploring the mechanism/s that allows these plants to cope with sulfate and sulfide levels which are toxic to many other plants. We grew the plants at different NaCl, sulfate and sulfide levels and scanning for relevant genes and proteins that might be related to plant resistance to those stresses.

The role of sulfite reductase in sulfite homeostasis

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Using *Arabidopsis* and tomato plants with modified SiR expression, we investigated the role of SiR in various metabolic processes in plants. We observed that resistance to ectopically applied SO₂/sulfite was a function of SiR expression levels and that plants with reduced SiR expression exhibited higher sensitivity than the wild type. The sulfite sensitive mutants accumulated sulfite and showed a decline in glutathione levels. In contrast, mutants that over-express SiR were more resistant to sulfite toxicity; exhibiting little or no damage. Resistance to high sulfite application was manifested by fast sulfite disappearance and increase in glutathione levels. The notion that SiR plays a role in the protection of plants against sulfite was supported by the rapid up-regulation of *SiR* transcript and activity within 30 min of sulfite injection into *Arabidopsis* and tomato leaves. Our results indicate that, in addition to participating in the sulfate assimilation reductive pathway, SiR also plays a role in sulfite homeostasis together with sulfite oxidase and the other members of the sulfite network.

Regulatory Network of SO₂ detoxification

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Abiotic stress occurs when plants are exposed to high concentrations of sulfur dioxide (SO₂) and its derivative sulfite. Physiological studies suggested sulfite oxidase (SO) as one important component of SO₂ detoxification. However, it is unknown to which extend plant fumigation with SO₂ triggers a specific transcriptional response. To address this question, we compared Arabidopsis wildtype (WT) and SO knock-out lines (SO-KO) facing the impact of 600 nL L⁻¹ SO₂ using RNAseq to quantify absolute transcript abundances. These transcriptome data were correlated to sulfur metabolism related enzyme activities and metabolites obtained from the same samples in a physiological study. SO-KO plants showed first symptoms of leaf injury after fumigation that were not detectable in fumigated WT plants. Analogously, SO-KO exhibited remarkable and broad regulative responses at the mRNA level, especially in transcripts related to sulfur metabolism enzymes but also of those related to stress response and senescence. Our data provide evidence for a highly specific co-regulation between SO and sulfur related enzymes like APS reductase. Involvement of other sulfite detoxification mechanisms will be discussed within the talk.

The arbuscular mycorrhizal symbiosis influences sulfur starvation responses of *Medicago truncatula*

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Arbuscular mycorrhizal (AM) symbiosis is a mutualistic interaction that occurs between the large majority of vascular plants and fungi of the phylum Glomeromycota. In addition to other nutrients, sulfur compounds are symbiotically transferred from AM fungus to host plants; however, the physiological importance of mycorrhizal-mediated sulfur for plant metabolism has not yet been determined. We applied different sulfur and phosphate fertilization treatments to *Medicago truncatula* and investigated whether mycorrhizal colonization influences leaf metabolite composition and the expression of sulfur starvation-related genes. The expression pattern of sulfur starvation-related genes indicated reduced sulfur starvation responses in mycorrhizal plants grown at 1mM phosphate nutrition. Leaf metabolite concentrations clearly showed that phosphate stress has a greater impact than sulfur stress on plant metabolism, with no demand for sulfur at strong phosphate starvation. However, when phosphate nutrition is high enough, mycorrhizal colonization reduces sulfur stress responses, probably as a result of symbiotic sulfur uptake. Mycorrhizal colonization is able to reduce sulfur starvation responses in *M. truncatula* when the plant's phosphate status is high enough that sulfur starvation is of physiological importance. This clearly shows the impact of mycorrhizal sulfur transfer on plant metabolism.

Compartment-specific changes in glutathione and ascorbate levels during high light stress in Arabidopsis

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Excess light conditions induce the generation of reactive oxygen species (ROS) which are detoxified by antioxidants such as ascorbate and glutathione. In this study compartment specific changes in antioxidant levels and related enzymes were monitored among *Arabidopsis thaliana* Col-0 plants exposed to different light intensities (150 which was considered as control condition, 300, 700 and 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 4 h and 14 d. The results revealed that wild type plants reacted to short term exposure to excess light with the accumulation of ascorbate and glutathione in chloroplasts, peroxisomes and the cytosol and an increased activity of catalase in the leaves. Long term exposure led to an accumulation of ascorbate and glutathione mainly in chloroplasts. The accumulation of ascorbate in vacuoles indicates an important detoxification route for hydrogen peroxide which accumulated in the tissue especially along the tonoplast and inside vacuoles during the exposure to excess light in the short term. The results obtained in this study helped to draw a model for the compartment specific detoxification of ROS by ascorbate and glutathione during high light conditions in plants.

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The role of antioxidant metabolism during dark-induced senescence in Arabidopsis

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During senescence an oxidative burst can be observed and hydrogen peroxide (H₂O₂) is known to have important signaling functions in triggering the expression of many senescence related genes. The equilibrium between the production and the scavenging of reactive oxygen species is influenced by antioxidants as well as antioxidative enzymes. The aim of this study was to investigate the compartment specific importance of ascorbate and glutathione and related enzymes during dark induced senescence in Arabidopsis Col-0 and the ascorbate and glutathione deficient mutant lines *vtc2-1* and *pad2-1*, respectively.

After initiating leaf senescence by dark treatment a yellowing of the leaves could be observed which correlated with a strong accumulation of H₂O₂. This oxidative burst correlated with a significant decrease in pigment contents, a strong decrease in subcellular glutathione contents and a decrease in the activities of antioxidative enzymes such as dehydroascorbate reductase, catalase, and glutathione reductase. Only minor changes were observed in subcellular ascorbate contents, whereas ascorbate peroxidase showed a strong increase in all investigated plants.

Thus, we can conclude that the compartment specific decrease of glutathione rather than ascorbate contents contributes to the accumulation of H₂O₂ which is a well known signal for triggering senescence.

Acknowledgement: This work was supported by the Austrian Science Fund (FWF 22988-B16).

Cadmium sensitivity of *pad2-1* and *vtc2-1* mutants is correlated to lower subcellular glutathione rather than ascorbate contents

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Toxic levels of cadmium (Cd) can cause alterations of the chloroplast ultrastructure, disturbs the synthesis of chlorophyll and carotenoids, leads to CO₂ deficiency due to stomatal closure and can cause several other metabolic disturbances. Indirectly Cd can cause oxidative stress and thus leads to the accumulation of reactive oxygen species (ROS). Ascorbate and glutathione detoxify ROS through the ascorbate-glutathione-cycle which makes these two antioxidants essential during Cd-induced stress. Additionally, glutathione has the ability to bind to Cd directly and serves as a precursor of phytochelatins which forms complexes with Cd which are then deposited in vacuoles.

In this study changes in the compartment specific distribution of ascorbate and glutathione were analyzed over a time period of 14 days in Cd treated (50 µM and 100 µM) Arabidopsis Col-0 plants. To obtain a deeper insight into how possible limitations of ascorbate and glutathione contents affect the defense of plants against Cd we additionally analysed the importance of subcellular ascorbate and glutathione contents in mutants deficient in ascorbate and glutathione (*vtc2-1* and *pad2-1*, respectively).

The results revealed that both mutants showed higher sensitivity to Cd treatment than the wildtype (earlier and stronger development of Cd-induced symptoms) and that *pad2-1* mutants developed even stronger symptoms than *vtc2-1* mutants. Additionally, strongly reduced glutathione contents could be correlated with the development of symptoms in both mutants throughout the experiment. These data suggests that higher sensitivity of *pad2-1* and *vtc2-1* to Cd is related to low glutathione rather than low ascorbate contents.

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Metal toxicity and sulfur metabolism in Chinese cabbage

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Copper and zinc are essential nutrients for the plants; however, they become rapidly phytotoxic at elevated concentrations in the root environment. Exposure of Chinese cabbage (*Brassica pekinensis*) to Cu²⁺ or Zn²⁺ concentrations $\geq 5 \mu\text{M}$ resulted in leaf chlorosis and a decreased biomass production. The uptake and metabolism of sulfur and nitrogen were differentially affected at elevated Cu²⁺ or Zn²⁺ concentrations. Both Cu²⁺ and Zn²⁺ exposure resulted in an increased sulfate uptake by the roots and in enhanced total sulfur content of the shoot, which could be ascribed partially to an accumulation of sulfate. Moreover, exposure resulted in a strongly enhanced level of water-soluble non-protein thiols in the root, which only partially could be ascribed to a metal-induced synthesis of phytochelatins. The nitrate uptake by the root was decreased upon Cu²⁺ or Zn²⁺ exposure, demonstrating the absence of a mutual regulation of the uptake of sulfate and nitrate. There was no direct relation between the sulfur metabolite levels *viz.* total sulfur, sulfate and water-soluble non-protein thiols and the expression and activity of the sulfate transporters and the expression of APS reductase at elevated Cu and Zn concentrations. Apparently, the presumed signal transduction pathway involved in their regulation appeared to be overruled or bypassed at high tissue Cu and Zn levels. It is doubtful that the Cu²⁺ or Zn²⁺-induced effects on the uptake and metabolism of sulfate have any adaptive significance in the detoxification of these metals in Chinese cabbage.

Using H⁺- selective microelectrodes to determine sulfate fluxes at roots of intact plant seedlings

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Ion-selective microelectrodes are a useful tool to answer questions in various fields of plant physiology, e.g. growth regulation, calcium signaling, salt stress, calcium signaling, growth and defense responses to pathogens. In plant nutrition ion-selective microelectrodes have been used to study e.g. the uptake preference of plants for different nitrogen sources or the uptake of calcium and potassium by wheat seeds. However, determination of sulfate uptake by plant roots using ion-selective microelectrodes was not carried out until now, probably due to the lack of a liquid ion exchanger that is selective enough for sulfate over other anions. Here we present the possibility to properly estimate sulfate influx at plant roots by measuring the corresponding proton fluxes (sulfate is taken up by plant sulfate transporters via sulfate/proton-symport), using the non-invasive Microelectrode Ion Flux Estimation method (MIFE). In a case study roots of seedlings that were grown under sufficient nutrient conditions were compared with roots that had been sulfur deprived with the result that the latter showed a much higher proton influx in response to addition of MgSO₄. This strongly indicates that the activity of the sulfate uptake system can be determined directly by measuring proton fluxes using the MIFE technique. The impact of compounds that are potentially controlling sulfate transporter activity will be dissected subsequently. Because ion fluxes are recorded non-invasively at the root surface of intact plants and with a high resolution in time, direct biochemical effects on the transport system can be distinguished from effects on the gene expression of transporter proteins which are expected to set in later.

Metabolic coordination of sulfur metabolism in *Brassica napus* by clock-controlled genes

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Experiments done with *Arabidopsis* plants so far, such as results from microarrays, indicate that some key enzymes in sulfur metabolism are regulated diurnally and/or by the circadian clock. Understanding metabolic changes in plants and determining output-related function of clock genes could help in elucidating circadian-clock mechanisms underlying plant growth and development. The sulfur requirement of the plants fluctuate during plant development and vary between species differing in sulfur need for growth and the potential sink capacity of secondary sulfur compounds.

Several sulfur-containing metabolites are involved in pathogen defense mechanisms and are suggested as compounds to enhance defense (SED= sulfur enhanced defense). We analyzed whether in addition to genes involved in sulfur metabolism also the levels of sulfur-containing metabolites such as cysteine and glutathione were clock regulated. They could be released rhythmically to the apoplastic fluid or via the stomata and defend the plants against attacking pathogens.

Oilseed rape (canola) is one of the most important agricultural crop plants for oil and declared as renewable resource. Two different *Brassica napus* L. (oilseed rape) lines were analyzed on the transcriptomic and metabolomic level in light/dark and free-running conditions. Different sulfur fertilization was applied to analyze the effects on pathogenesis-related compounds. Results from these experiments could help to optimize the use of fertilizer and if applicable reduce the amount of fungicides/pesticides.

The effect of the continuous light in combination with sulfur deprivation on the chlorophyll levels and carotenoids in young maize leaves

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Light fulfils two significant roles in plant growth; it drives photosynthesis by providing energy and it is perceived by several photoreceptors, thus activating signal pathways. Continuous light changes plant physiology by affecting both roles, thus creating difficulties in identifying the factors that are responsible for injuries under such treatment. The effect of continuous light in combination with plant's nutritional status or nutritional deficiency on its physiology is poorly studied and there are no references with regard to sulfur deficiency. The responses of *Zea mays* plants to light environment in combination with nutrition were studied in four treatments; C: normal photoperiod & complete nutrient solution, Cc: continuous light & complete nutrient solution, -S: normal photoperiod & nutrient solution without sulfur, -Sc: continuous light & nutrient solution without sulfur. Plants were grown for seven days under normal photoperiod condition and then the treatment was applied for 3 weeks. The photon flux density was not modified during this period.

A two days delay was observed in laminas L₅, L₆, L₇ and sheaths S₂, S₃ during sulfur deficiency under normal photoperiod. No injuries were caused in laminas. The laminas' average Chla:Chlb ratio increased by 5.6%, whilst the average one in sheaths decreased by 10.4%. In laminas, the average of Car:Chl ratio increased by 13.6%, whilst in sheaths the corresponding average increased by 3.6%.

Under continuous light combined with sulfur deficiency, the appearance of organs took place at the same time as in control plants, with the exception of L₇ and S₂. This fact indicates that the deficiency counteracted the effect of continuous light. Aging and collapsing was observed at the oldest organs L₀, L₁, S₀, S₁. In laminas, the average value of Chla:Chlb ratio increased by 5.6%, whilst in sheaths the corresponding ratio decreased by 6.9%. At the end of the experiment, in laminas the average value of Car:Chl ratio increased by 22.7%, whilst in sheaths the corresponding one was as in control plants.

Aerenchyma formation in maize leaves during sulphate deprivation

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Aerenchyma is the term given to plant tissues containing enlarged gas spaces exceeding those commonly found as intracellular spaces. Nitrogen- or phosphorus- or sulphur deficiency leads to formation of aerenchyma in maize adventitious roots by lysis of cortical cells.

Seven-day-old maize plants were grown in a hydroponics setup for nineteen days under S deprivation conditions against plants under full nutrition. At day 17 and 26 from sowing (d10 and d19 of the deprivation respectively), samples from the fresh plant material had been taken, fixed and embedded in paraffin. Sections were received with microtome from the top, middle and base of the 2nd leaf and stained with safranin-fast green. In –S plants, the leaf showed enlarge gas spaces between the intermediate and small vascular bundles by lysis of mesophyll cells to a greater extent on the d17 compared to d26.

Under the deprivation, the –S leaf presented less amount of total sulphur by 48% and 63% than control at d17 and d26 respectively. The corresponding percentages of the sulphate were 74% at d17, whilst it was as in control at d26. These data indicate that aerenchyma in maize leaves may be formed in response to sulphur deficiency.

On d17, the S-deprived leaf presented less specific surface (g DM/cm² leaf surface) and more width by 18,5% and 12,2% respectively in relation to control. On d26, the S-deprived leaf's specific surface was as in control and it presented 12% less width compared with control. These results were in accordance with the reduced percentage of aerenchyma presented on d26.

Functional analysis of Arabidopsis SDI1/2

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O-acetyl-L-serine (OAS) provides the carbon backbone for cysteine synthesis and is assumed to positively regulate sulfur deficiency-responsive genes as its amount increases upon sulfur limitation. Therefore, mRNA accumulation of a number of sulfur responsive genes might be controlled by OAS. Recently this hypothesis was strengthened by global co-expression analysis under conditions of variable OAS levels with constant sulfur status. The function and the signaling cascade regulating the identified OAS cluster genes (*SDI-1*, *SDI-2*, *LSU1*, *LSU2*, *ChaC* and *SHM7*) are mostly unknown and need further elucidation. Functional characterization of SDI (sulfur-deficiency-induced 1-2) is the main part of our study as these candidate genes are prominently expressed upon sulfate deprivation and upon OAS accumulation. Our work will contribute to our understanding of signaling processes and the actual function of these genes. To this end, knock-out and over-expressor lines are being analysed by transcriptomic and metabolomic approaches and some promising results relating to the metabolite alternation could be observed.

Saturday, 7th September 2013

Scientific excursion

Departure from Stanley Hotel

During the ride to Lavrion, brief description of the excursion's background
(Dimitris Bouranis)

Visit to Thorikos place

Visit to Cultural – Technological Park of Lavrion

The excavations of Kamariza – Soureza

Ride through the Sounion National Park

Arrival at Assimakis's resort place

Swimming (optional)

Conference Lunch

Ride to Sounion

Arrival at Neptune's Temple in Sounion

Visit to the Temple

Chat and coffee

Arrival at Stanley Hotel

Sunday, 8th September 2013

Cultural sightseeing

Visit to Acropolis (optional)

End of Workshop

Making wealth out of sulfides and its consequences in Lavrion peninsula, Greece

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The history and the environmental issues of Lavrion are closely connected to sulfur and this connection was the subject of an excursion during the 4th Sulphyton Workshop. The ancient mining activities were aiming at the excavation of the silver-bearing galena of Lavrio deposits, the main mineral among the mixed sulphurous ores, such as PbS, ZnS, and FeS. Silver was extracted from galena and used to make silver coins. During the period 1864–1989 mining and metallurgical activities exploited the remaining deposits and the huge piles of mineral and metallurgical wastes, rich in Pb, Zn, Cu, Fe and other mineral elements. Enormous quantities of wastes from these activities, were deposited in heaps around Lavrio area, near the coastline, or dumped into the sea and heavily burdened the natural environment. Lavrion has many peculiarities with respect to its contamination characteristics. The local population in Lavrion is exposed to multiple hazardous pollution sources, including acid-generating sulfidic tailings, slags, contaminated soils and the carbonaceous tailings. Each specific pollution source contributes with a different intensity and through different pathways to the migration of contaminants and their final intake by humans. Extended pollution has been determined especially Pb, and As, whilst increased concentrations of heavy metals are present especially in the mineral excavation areas and in the processing and depositing waste areas. High concentrations of heavy metals are recorded in groundwaters of Lavrion, too. These facts reveal the coexistence of physical and anthropogenic pollution in these areas with obvious adverse effects on man, animals and plants.

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Introduction

A sulfide is an anion of sulfur in its lowest oxidation state of 2-. Many important metal ores are sulfides. Argentite (silver sulfide), cinnabar (mercury), galena (lead sulfide), molybdenite (molybdenum sulfide), pentlandite (nickel sulfide), realgar (arsenic sulfide), stibnite (antimony sulfide), sphalerite (zinc sulfide), pyrite (iron disulfide), and chalcopyrite (iron-copper sulfide). Iron disulfide (pyrite, FeS_2) on the other hand consists of S_2^{2-} , or S^{2-} - S^{2-} dianion, in association with divalent iron in the formal 2+ oxidation state (ferrous ion: Fe^{2+}). This ensemble of sulfides is present in Lavreotiki peninsula and constitutes the base of this excursion.

Lavrion was rich in argentiferous galena and the ancient Greeks developed a remarkable technology to extract this silver, which was the base of the wealth of the «golden» age of Athens during the 5th century BC. Actually it was the base of the significant cultural and scientific development in this period of history. The contemporary mining and metallurgical activity left the area with wastes heavily polluted the environment with heavy metals; among these wastes are also sulfidic ones.

In this review, we have summarised the mining characteristics and history of Lavrion, the emphasis given to sulfide ores, sulfidic tailings and the related environmental pollution due to the presence of a vast array of heavy metals. The original material that provided us with the various historical and scientific aspects presented below is given in the literature, which is a rather rich one. Our goal was to provide the participants of the 4th Sulphyton Workshop a brief and concise summary of the connection between sulfur and the various aspects of life in Lavreotiki through the years.

Lavrion and the area of Lavreotiki

Fifty five kilometers from Athens is Lavrion, a town inhabited by more than 10,000 people. Lavrion exhibits a rich history, which is reflected by its neoclassical buildings, the Town Hall, the monument of Serpieri, the Mineral and Archaeological Museum, the churches of Agia Paraskevi and Evangelistria, the Old Market (built in 1885-1887), the French Ore Loading Dock in the harbour (created in 1888), and picturesque alleys; all of them are worth-seeing sights during a visit to the town.

Lavrion is situated in the eastern part of a peninsula called Lavreotiki, i.e. the surrounding area of Lavrion, and it took its name after the ancient Greek word «lavra» which means narrow passage or tunnel, describing the characteristics of mining galleries. Lavreotiki is located in the southeastern tip of Attica, it covers a surface of approximately 200 square kilometers where the places of Thorikos, Kamariza, Plaka, Botsaris Valley, Cavity Soureza Valley, Megala Pefka, Dimoliaki etc. are situated. Most of these places are remarkable centres of cultural and scientific tourism with ancient mines and ore washing plants, the Ancient Theatre of Thorikos, the natural National Park of Sounion, and the temples of Neptune and of Athena at Sounion.

The landscape is characterized as hilly or semi-hilly with medium highs, from 0 to 372 m (the highest altitude is at Megalo Ribari site); it is crossed by numerous valleys, whilst small plains extend down to the coast where there are coves suitable for anchorage. The area runs along the western coast of the Aegean Sea. In some parts of the basin the relief becomes intense with the appearance of some ravines. To the north is located the Thorikos-Plaka basin which dominated by agricultural activities. To the south the area is surrounded from smaller basins with more intense relief. Lavreotiki ends in Cape Sounion, where the strategically important ancient fort was also located. Lavreotiki is rather dry, characterized by the typical Mediterranean climate of Attica and covered partly by pine trees. Access to it is normally through paved roads which are relatively easy to travel.

Lavreotiki today is an industrial over ground museum of ancient and 19th century metallurgy industry and a huge underground mineralogical and geochemical laboratory of nature. The total length of galleries is approaching 2000 km, whilst the presence of about 500 mineral species has been ascertained by geologists. The history of the area, its sights, its natural beauty, its proximity to Athens, and its touristic infrastructures render Lavreotiki peninsula a marvelous spot for visits.

Among the important sights is the Technological – Cultural Park of Lavrion, a unique monument of industrial archaeology and architecture. The site of the Technological – Cultural Park of Lavrion is an initiative of the National Technical University of Athens, which has undertaken the responsibility of marking, protecting and promoting the region. Thus, the Technological – Cultural Park of Lavrion contributes to the development of the area by providing a link between the scientific and technological research conducted in the National Technical University of Athens, and the needs and interests of businesses. It was created in 1992 at the place where the old French Mining Company headquarters used to be and it comprises of 41 buildings 25,000 sq.m overall, which have been designated as protected sites by the Ministry of Culture. (Brochure of Archaeological Museum of Lavrion, Theodossiou 2009, Demetriades 2010)

Ancient history and ancient mining activities of Lavreotiki

Lavrion and the wider area of Lavreotiki was the centre of considerable mining and smelting activities from ancient to recent times, due to its rich mineral wealth which consists of polymetallic sulphides and iron ore. Lavreotiki was rich in argentiferous mineral deposits, due to which the history of Lavrion mines is directly connected with the history of Greece.

Human presence in the area is confirmed in the Palaeolithic period (40,000 years ago) in the Cave of Kitsos, where there was also a large settlement in the Late Neolithic period (5300 – 4500 BC). From the Final Neolithic (4th millennium BC) onward, Lavreotiki became more densely populated. Exploitation of Lavreotiki's mineral wealth is attested from the Early

Bronges Age (3rd millennium BC) at Thorikos. This apparently continued uninterrupted and seems to have gradually intensified from the 6th c. BC.

The first mining and smelting activities in the Lavreotiki Peninsula began early in history. Intense mining and metallurgical activity was developed from 3500 BC reaching its peak in the 5th and 4th centuries BC. That activity brought about economic along with cultural development. The ancient Greeks exploited the minerals of Lavrion to extract mainly silver and lead. Mining galleries and shafts are exceptionally concentrated throughout Lavreotiki. The workshops for cleaning the argentiferous ores, the washeries, were in the valleys where there was water. In the valley of Soureza, a complex of such workshops has been turned into an archaeological site open to the public. In Sounion National Park, silver and lead were produced in the furnaces of the smelting workshops, of which only six have been found. There are also quarries that the area of Agrileza provided the marble for the temples at Sounion.

The mining history of Lavrion begins at Thorikos, a settlement to the north of Lavrion and one of the most ancient industrial areas in Europe. Thorikos is one of the important sites of Lavreotiki, which grew into a centre in the Late Bronges Age (Mycenaean period 1600-1100 BC), as the monumental tholos tombs and their finds on the Velatouri hill attest; the site continued to flourish during the ensuing Geometric, Archaic, and Classical periods (10th – 4th BC). A number of scattered farming installations and houses, accommodations for entrepreneurs and slaves who worked in the mines, cemeteries, and small sanctuaries have also come to light in Lavreotiki. Archaeological excavations at Thorikos confirmed the existence of mining activities as early as 3000 BC. The first underground excavations began around 3000 BC. This fact establishes Lavrion mines as the oldest in Europe. The ancient town extends from the foot of Velatouri hill to the plain and to the sea in the East, where the ancient port was. On the hill there are ruins of a Mycenaean acropolis (4800 BC), vaulted Mycenaean tombs of elliptical form, a settlement dating back to the geometric period, a temple of Demeter and Persephone, a sanctuary of Asclepius and Hygeia, a theatre, etc. The theatre of Thorikos is one of the most ancient theatres in Greece (end of the 6th c. BC). The excavations by the Belgian Archaeological School and archaeologist B. Stais unearthed an industrial town of 150,000 sq.m with houses, walls, towers, galleries, shafts, ore washing plants, cisterns, graves and port infrastructures. Some of the washing plants have been reconstructed and the oldest gallery above the Thorikos theatre has been partly reconstructed. Thus, Thorikos constitutes a unique ancient mining centre worldwide; it combined the economic, religious and artistic aspects of life, as was the rule in ancient times.

The peak of mining and smelting activities was between the 6th and 4th centuries BC, and especially during the 5th century BC, the Golden Age of Athens or Pericles. The production of silver was limited until 483 BC (the period of Greek-Persian wars), when the rich deposits of Maronia (present day Kamariza or Aghios Konstantinos) were discovered and the greatest mining period began. Mining galleries up to 120 m long were opened. There are the deeper deposits, in the contact zone between marble and schist. In 480 BC the proceeds from silver extraction were so remarkable that Themistocles proposed that they be used to build a fleet. The historians of the Classical Period wrote: «the silver is flowing like spring water». The Lavrion silver mines soon became the principal source of wealth for the Athenian State. Hundreds of tons of silver that had been initially stored in the Parthenon treasury were liquefied in order to erect several of the city's architectural monuments. The construction of an enormous war and trade fleet with a hegemonic presence in the Eastern Mediterranean region was financed. Fine Arts and Sciences (philosophy, mathematics, astronomy, drama, history etc.) flourished. The tetradrachmon, the currency of the Athenian State, which was constructed from the silver, became the dominant currency of the ancient world. The revenue from the mines was used first to build in 483 BC the large Athenian fleet, which defeated the

Persians at the naval battle of Salamis in 480 BC, and subsequently financed the construction of the renowned monuments seen today on the Acropolis of Athens, the Parthenon, the Erechtheum, etc. The mines belonged exclusively to the city of Athens, which granted concessions to exploit them to private citizens.

Eight municipalities were created through the reforms by Kleisthenes (507 BC). The most popular were the coastal municipality of Anaphlystos (modern Anavyssos) and the mountain municipality of Phrearrhioi (from the Greek «phrear» which means shaft). From the municipality of Sounion, part of the settlement at the front on the cape and the agora of the Salaminians at Limani Passa are known. From the municipality of Thorikos large portions of its neighbourhoods, sanctuaries, the theatre (with its unusual oval cavea), cemeteries, a washery, and a gallery on the western slope of the Velatouri hill are accessible to the public.

During the classical period (5th – 4th century BC), a system of collaboration was implemented involving the Athenian state, owner of the metalliferous land, and entrepreneurs. The mine or area to be mined was leased for a specific period to entrepreneurs on clear legal terms. At the same time, support enterprises developed for the sale and rental of slaves, the water trade, foodstuffs and wood for the furnaces, and for construction of mining installations by skilled artisans.

During and after the Peloponnesian War, Lavrion declines, mainly due to the discovery of rich silver and gold deposits in Thrace and Macedonia. The decline of the mines came during the Peloponnesian War, a 30 year civil war between Athens and Sparta. In 413 BC, the Spartans captured Lavrion and the excavations in the mines ceased. Some decades later, Athenian economy recovered and silver production recommenced. However, Macedonia under Philip II (Alexander's father) was now the dominant power in the Hellenic region and the silver Athenian tetradrachmon was surpassed by the golden Macedonian drachma. The works in Lavrion mines will continue, but with decreased rates, until the first Byzantine Period (6th century AD).

After the 4th century BC it appears that exploitation declined. Mining and smelting activities continued intermittently until the 1st century AD. During the Roman and Early Byzantine period (2nd century BC – 6th century AD) it was sporadic and of small scale. Lavrion area between the 6th and 19th century was abandoned, losing all of its old glory.

Excavations by various entities unearthed excavating sites, galleries and shafts, washing plants, cisterns and furnaces, thus leading to useful conclusions and considerable knowledge on the ancient mining and metallurgical art. As a matter of fact, the remnants of ancient technological plants (washing plants mainly) are almost intact, despite the passage of time. They are permanent constructions, either built or carved in the rock. Similar plants have not been found anywhere else on the planet, as other people used wooden constructions that did not stand the passage of time. (Brochure of Archaeological Museum of Lavrion, Theodossiou 2009, Demetriades 2010)

The geological traits of Lavreotiki

Lavreotiki is part of the Attic-Cycladic massif and is composed of metamorphic rocks such as schists, phyllites and marbles. The Attic-Cycladic massif in southern Attica consists of two main units (Marinos and Petrascheck 1956, Stamatis et al. 2001).

(a) The autochthonous unit: It consists of marbles, dolomites and schists with visible thickness greater than 1000 m. This unit comprises of three subunits, from bottom up: the lower marble (the thickness exceeds 600 m), the mica schist of Kamariza-Kaisariani (the

thickness is approximately 600 m), and the upper marble (the thickness is approximately 100 m).

(b) The overthrust nappe unit: It consists mainly of phyllites with marble intercalations, quartzites, prasinites and serpentinites.

Under high pressure and temperature conditions, the older basic igneous rocks were metamorphosed to prasinites, metabasites, which are found as intercalations inside the phyllites. After the metamorphism a granodioritic intrusion entered into both units; the granodiorite of Plaka. This intrusion caused intensive contact metamorphism in the surrounding area.

The mixed sulfurous ore-bearing of the broader area of Lavrio are composed of silver-bearing galena (the majority of which has been exploited), sphalerites and silica. The ores occur mainly inside marbles and close to their contact with schists. Ore is of hydrothermal apomagmatic origin. The metalliferous liquids have resulted from the granodiorite of Plaka (Marinos and Petrascheck, 1956).

The Quaternary deposits, located in the valleys, are composed mainly of clays, gravels and coastal coarse-grained sandstones alternations. In Thorikos basin unconsolidated sediments overlaid the mica schists to the NE and the upper marble to the SE, and their thickness is up to 10 m. In the northern part of the area the loose deposits overlaid phyllites and schists of the nappe unit and their thickness reaches 5 m. The lower marble forms an anticlinal structure, west of Lavrio with an almost N–S trending axis, traversing the hilly area of Sounio-Kamariza-Plaka. WNW–ESE to W–E trending folds have been also encountered across the above-mentioned large-scale structures. In the eastern part of the Lavrio area, the bedding planes of the nappe unit dip gently to the East.

The ore deposits of Lavreotiki

There are mainly two types of mineralisation in the substratum of Lavreotiki:

- a mineralisation of mixed sulfide ores of basic metals, such as lead, zinc, iron and copper, which has been intensely exploited
- and a metalliferous of Fe-Mn ores, the presence of which is limited in general.

The primary sulfide mineralisation of mixed sulfides, constitutes the main mineralisation of the area, characterised formerly by the presence of galena rich in silver, sphalerite and iron-pyrite. A number of other sulfide minerals (e.g. Cu, As) and a variety of sulfur salts also take part in the ore mineral suite of the area. The accessory minerals that prevail are fluorite, calcite, barite, quartz and dolomite. Sulfide mineralisation is mainly hosted within the carbonate formations of the «paraautochthonous» series of Lavreotiki or «Kamariza series» («lower marble», «upper marble», «transgressive limestone»). As regards the form of the sulfide ore, they relate mainly to vein-like, lens-shaped and stratiform forms (the last one near the contact of carbonate and schistose rocks). According to the prevailing view, the origin of the sulfide mineralisation is epigenetic, directly related to the Upper Miocene magmatic activity; it is of hydrothermal origin, with a temperature of formation of 280 °C and it belongs to the category of replacement type carbonate, hosted massive sulphide Pb-Zn-Ag ore deposits.

As a result of the extreme phenomena of oxidation and then displacement of the ingredients of primary mineralisation, large accumulations of secondary ores, such as zinc carbonate or smithsonite or «calamines» (ZnCO_3), argentiferous lead carbonate or cerusite (PbCO_3), were created in the wider area of Lavreotiki and underwent intense exploitation. In the area of

Plaka, a significant massive magnetite skarn type ore deposit is located. (Theodossiou 2009, Demetriades 2010)

The ancient technology of silver and lead extraction from galena

The ancient Greeks extracted mainly silver and lead from the minerals of Lavrion, and especially from galena ore, with remarkable techniques of underground exploitation, of hydrometallurgical processing and of smelting the low grade sulfide deposits. Galena is a lead sulphide plus silver. The extraction of silver and lead from galena ore in the ancient times was indeed a technological miracle, a process accomplished in four stages.

The **galena** ore was enriched in washing units by using water under pressure in order to remove the light and poor in metal parts of the ore.

The **enriched galena** was converted to the oxide form ($\text{PbO} + \text{Ag}$) by the process called *reduction roasting*. The $\text{PbO} + \text{Ag}$ chemical compound is called **litharge**.

Litharge was directed into a furnace, where it melted with carbon (a process called *reduction melting*) and produced a **PbAg alloy**.

The Pb-Ag alloy was next placed into a fireproof ceramic cup. The cup was heated in a specially constructed furnace where air was blowing under pressure. At 900 °C the alloy melted and lead was oxidizing whilst silver was not oxidizing. Then the alloy formed two separate layers, a heavy one containing silver in the bottom of the cup and a lighter containing litharge in the top. Litharge was removing and the process was continuing until the silver in the bottom was pure (over 99%). The precious metal was then ready for coins construction. (Konofagos 1980)

The mining activities in Lavrion in the modern era

In 1859, the Greek State (founded in 1830) sent the Greek metallurgist Andreas **Kordelas** to Lavrion with the mission to examine the possibility of reopening the mines. Kordelas detected the presence of extensive ancient mining residues, which could produce large quantities of lead and zinc, and he proposed the exploitation of the ancient slags. The excavations of these residues during 1860 unearthed the ancient mining plant. Zinc was an unknown metal in ancient world, whilst lead had limited applications and was produced in small quantities.

In 1863, the Italian I. B. **Serpieri** arrived in Lavrion and the following year he founded the Italian-German company Roux-Serpieri-Eressynet CA. A metallurgical industry with furnaces, small washing plants and machine shops was established and the production of argentiferous lead began in 1865, by means of the processing of slags and poor ores. In 1869, an underground tunnel was constructed for the first railway line in Greece, which was used to transfer the minerals to the port. In 1871, poor ores were proclaimed national property, due to the arbitrary appropriation of the poor ore and the claim of property on the land by the foreign company. This caused a conflict with the Greek State, which was the beginning of the «Lavreotiki issue» that shook Greece. The issue was solved when the foreign company granted its rights to the slags and the poor ores to the Bank of Constantinople (A. Syngros).

In 1873, the **Greek Company** of Lavrion Metallurgical Plants was founded, aiming at the exploitation of the ancient mining tailings. The mine exploitation rights were granted to the **French Company**, a new company founded by Serpieri in 1875 with main centres of extraction in Kamariza, Soureza and Plaka. The French Company was exploiting newly discovered underground ore deposits. Both companies rejuvenated the abandoned city of

Lavrion, which became one of the most important mining-metallurgical centres in Europe with thousands of workers. Silver, lead, zinc and arsenic were produced in vast amounts. This industrial activity became an important source of income for the Greek State. The ore floatation factory of the Greek Company in the beginning of 20th century was one of the biggest in the world. Unfortunately, a small part of it is saved in nowadays. The metallurgical process of ancient Greek metallurgists was followed, with no essential changes, until nowadays for silver production. Its efficiency was slightly improved in the late decades of 19th century with the discovery of flotation method for the ore enrichment and the Water-Jacket furnace for reduction melting. In 1917, the Greek Company interrupted its activity, due to a stock market scandal, and strikes by the workers.

The cessation of the French Company's activities, after the definite exhaustion of the ore deposits, as well as the reduction of the Greek Company's activities following the gradual reduction of the mining tailings brought economic decline in Lavrion leaving thousands of workers unemployed in the late 80s. The French Company was active until 1982 when it let its plants to the Greek company, whilst the latter closed down permanently in 1989. In 1992, the plants of the French company were bought by the Greek state and the National Technical University of Athens started the creation of a Technological – Cultural Park.

Thanks to the natural beauty of Lavreotiki and to the existence of archaeological monuments, a passenger's port was constructed and tourism was developed and the situation changed to new economic development in late 90s.

The consequent environmental issues of Lavrion

The ore beneficiation and smelting activities of the recent period produced large volumes of hazardous metallurgical processing wastes. Lavrion was developed close to the waste stockpiles. The industrial and urban development, particularly during the 1950s and 1960s, has led to the expansion of residential areas even on the top of these stockpiles. Nowadays, the wastes are used by the Municipality, the local inhabitants, and the various private companies for a variety of purposes, e.g. slag as hard core on roads, school yards, the port facilities, infilling of foundations of buildings, covering of dirt roads by sand-blast material made from slag, the flotation sands as building material for houses. Accordingly, houses, schools, parks, playgrounds, shops and roads are either situated on the wastes or they are very close to these wastes.

The comparatively dry climate for most months aids transportation of dust by wind. Dust is a major hazard in Lavrion; it is released from soil by the action of wind, with or without the assistance of mechanical disturbance. During loading, transportation and unloading of the metallurgical processing waste, fine dust is released into the ambient air, thus being available for transportation by wind. The winds transport fine- to medium-grained metallurgical processing wastes to distant places. Deposition of these materials and their subsequent mixing with residual and alluvial soil, caused the alteration of the chemical composition of surface soil.

It goes without saying that the industrial conditions in Lavrion were adverse and this fact stimulated several epidemiological and other medical studies. In the late 1980s, the Institute of Geology and Mineral Exploration began environmental impact studies in Lavreotiki, which provided evidence of widespread contamination of soil and stream sediment. In 1990, urban geochemical surveys started in Lavrion and Aghios Constantinos and lasted up to 1992. Samples of garden soil, road dust and house dust were collected from Lavrion and analysed for As, Cd, Cu, Fe, Pb, Zn, and Hg. In 1992, the urban geochemical surveys were extended

covering the whole Lavreotiki Peninsula with the collection of surface soil and rock samples. Finally, the most comprehensive environmental impact study ever carried in an urban–suburban environment was co-financed by the European Community and the Greek State through the LIFE programme with the title «Soil Rehabilitation in the Municipality of Lavrion». The project began in 1994 and was completed in 1999 with the submission of a massive report, as well as other site or subject-specific reports and papers, revealing the environmental issues of Lavrion (for details see Demetriades 2010 and references cited therein).

The characteristics of the metallurgical processing wastes

Metallurgical processing of ore-grade materials from 1864 to 1989 produced a considerable amount of waste materials, including pyrite, slag, flotation sand and other coarser materials. In other words, the metallurgical wastes in the Lavrion urban area which are the products of over hundred years of ore processing, accumulated in such a volume that ancient wastes that may have existed are completely masked. The metallurgical processing wastes cover a large part of the Lavrion urban area, and nowadays they are the major primary source of contamination of the surface environment.

Three main categories of these wastes occur in the Lavrion urban area: Slags, acid-generating sulfidic (pyritiferous) tailings and carbonaceous beneficiation tailings, exhibiting different geochemical and environmental characteristics. Differences in the mineralogy of processed ore and changes made in the metallurgical methods resulted in the production of wastes of different chemical composition. These wastes cover approximately 25% of the area. Their subsequent redistribution by aerial, fluvial and anthropogenic processes has caused the contamination of surface residual soil and house dust. As a consequence, each waste category represents different risks and requires the implementation of specific remedial measures in order to protect the wellbeing of the local population and prevent further contamination of adjacent soils and natural waters.

Especially, the area covered by sulfidic tailing is rather small, mostly the coastal area from Nichtochori-Komobil to Kiprianos and Kavodokanos. The sulfidic (pyrite) tailings are distinguished chemically into four broad categories:

- (i) The Nichtochori-Komobil tailings have been produced from 1953 to 1963, are characterised by high levels of Ag, As, Bi, Co, Fe, K, S, Sb, Sn and V and are weathered to a variable extent.
- (ii) The Kavodokanos-Kiprianos-Santorineika sulfidic tailings are characterised by high levels of Al, Ca, Cd, (Cr), Cu, Fe, (Hg), La, Li, Mn, (Ni), P, Sr and Zn; Cr is only enriched in pyrites of Kavodokanos and Hg and Ni are only enriched in pyrites of Kavodokanos and Kiprianos. The Kavodokanos sulfidic tailings are the most weathered.
- (iii) The Thorikos Gulf beach sulfidic tailings are characterised by high levels of As, Bi, Co, Fe, Mn and S.
- (iv) The Thorikos inland (Komobil) sulfidic tailings are characterised by high levels of Ag, Al, B, Ba, Cd, Cr, Hg, K, La, Li, Ni, P, Sb, Sn, Ti, V and Zn.

All pyrite tailings produce acid drainage; therefore, are hazardous for polluting groundwater resources. The mobility of cations in the largest part of Lavrion is in general comparatively low to moderate due to the alkaline pH of soil water. In the area of pyritiferous tailings the action of air and rainwater causes the oxidation of pyrite and generation of acid drainage which is very corrosive due to sulphuric acid. It leaches metals from the wastes, thus the acid

drainage contains high to extreme concentrations of toxic elements, which in turn could be harmful to humans, animals and plants. The acid drainage is also a hazard to groundwater supplies.

The metallurgical wastes are the major source of contamination of overburden materials, including residual soil, because they possess high concentrations of As, Be, Cd, Cu, Hg, Pb, Sb, and Zn. Residual soil has been contaminated to a variable degree, and constitutes today the greatest hazard, since it covers the greatest area in Lavrion. Uncontaminated soil is found only in a few places. The natural landscape has been altered considerably by the deposition of these wastes, by their subsequent moving about by the local inhabitants, and by the importation of garden soil from other parts of Greece. According to Demetriades, the local situation is described perfectly well by the term «overburden», which means any unconsolidated material overlying bedrock, either transported or formed in place, i.e. the loose soil, silt, sand, gravel, or other. Therefore, the restoration of the areas covered by metallurgical processing wastes is of first priority. (Kontopoulos et al. 1995; Mylona et al. 2000, 2005, 2007; Xenidis et al. 2002, 2003, 2007; Demetriades 2010)

Land use in Lavreotiki of today

The use of land is an important parameter in the exposure assessment to environmental contaminants, and also in the «soil» restoration plan. The Lavrion urban area extends from the residential part of the town into its outskirts, which are either agricultural or forested. Land use categories are classified into two broad groups: i) the residential area, tillage, forest and open space and ii) the industrial and commercial area.

Open space with or without bushes and scattered trees, areas occupied by pine forest, open space with trees, olive groves, vineyards, vegetables and wheat fields make up 70% of the total area. Vineyards are mainly grown over the flotation residues, as is a large part of the olive groves. All industries cover 16% of the total area, and only about 14% of the land is utilised for residential and recreational purposes. The agricultural part dominates the area, followed by the industrial area. The residential and recreational section of the town covers the smallest area, a large part of which is situated over contaminated soil, flotation residues and slag. Local inhabitants live, work and play in the most hazardous areas.

The contemporary hydrogeological traits of Lavreotiki

A relationship between the quality of groundwater and the geological environment has been established by a hydrogeochemical study (Alexakis and Kelepertsis, 1998) carried out in many sampling areas of eastern and southern Attica (Spata, Keratea, Thorikos and Aghios Konstantinos/Kamariza). It has also revealed the extent of pollution in these areas resulting from natural and man-made factors.

Another study (Stamatis et al. 2001) focussed on the hydrogeological conditions in relation to the quality of different aquifers developing in the Lavrio area. The heavy metals distribution inside the aquifers is particularly stressed and the extent of physical and anthropogenetic activity taking place in the historical area is evaluated.

The lithological composition of geological formations and the intense tectonism directly affect the hydrogeological conditions of Lavreotiki. The area is composed of geological formations with differing hydrogeological behaviours. There are similar hydrogeological properties among the massive rocks, schists and phyllites. These rocks are particularly impermeable, because their fracture system is blocked by finer-grained material. Weathered

subsurface zones of schists and phyllites form an unconfined aquifer exploited by the inhabitants, mostly in the southern area of Lavrio and Kamariza from shallow wells. The water supply from these wells is not adequate to cover the water demands and most of them dry up during prolonged periods of drought. The limited permeability, characterizing these formations, results in high runoff. This is reflected in the drainage pattern development, which is of the dendritic type.

In carbonate rock areas a parallel pattern is developed. Consequently, the distinction between the two prevailing hydrolithological units of the area is feasible. Carbonate formations, the lower and upper marbles, compose the main (karst) aquifer of the area. These rocks have high permeability due to intense fracturing and especially great karstification. The lower marbles crop out in restricted areas located South of Kamariza and southwest of Lavrion. On the contrary, in the western area of the Lavrio peninsula the development of lower marble is more extensive and constitutes the coastline. The lack of an impermeable horizon (baselevel) results in groundwater seeping under the lower marble towards sea level. The upper marble has overlaid the mica schists of Kamariza and is widespread in the area. Its thickness can reach as much as 100 m and varies either because the upper marble overlays the lower one, without any schist intercalation, or because it has undergone intense erosion and weathering. The infiltrating water following the general Eastward dip of the beds, decant partially lateral toward the loose quaternary deposits or they find a possible submarine outlet. In the alluvial deposits, which cover the greater part of the plain in the Thorikos basin and parts of the Lavrion basin, an unconfined aquifer has developed. This aquifer displays limited permeability and poor hydraulic characteristics due to fine grain compositions. The impervious bedrock in the Thorikos basin is formed by mica schists of Kamariza. In the Lavrion basin the impermeable bedrock consists of phyllites and schists of the overthrust nappe. The replenishment of unconfined aquifer in alluvial deposits is ensured, both from the direct infiltration of rainfall throughout the alluvial deposits and also from lateral seepage of karst water of upper marble and of groundwater from weathered schists.

The hydraulic connection of both karst and unconfined aquifer with the sea, in combination with their intensive exploitation for irrigation, have contributed to the deterioration of groundwater quality, due to seawater intrusion. The seawater intrusion is exceptionally intense in both the Thorikos and Lavrion basins.

For the hydrochemical traits of the Lavrion area, the following conclusions have been drawn: There is wide dispersion of values of Mn, Zn, Cd in groundwater and much smaller dispersion of Cu, Fe and Ni, mainly in alluvial unconfined aquifer. High concentrations of heavy metals are recorded at groundwater samples in Lavrio area, the highest from the unconfined aquifer (alluvial deposits and schists). The concentration of lead and cadmium is higher than the upper limit for drinking waters for 66 and 75% of the collected samples from both unconfined and karst aquifers, respectively. High zinc concentrations were detected in most groundwater samples from the unconfined aquifer of schists, related to oxidation of sulfide minerals and the leaching of schist. Nickel concentration is high in both the unconfined and karst aquifers. Quality deterioration of groundwater is documented and attributed to seawater intrusion and nitrate pollution due to intensive agricultural activities. A significant relationship has been observed between the major ions Ca–Na, Mg–Na, Na–Cl, Ca–SO₄ and Ca–Mg (karst aquifer). There is a close relationship between the elements participating in the sulfide (Pb, Zn, Cu, Cd) and iron-manganese mineralization and a strong relationship between Ni–Cr.

The intensive mining activities and the intensified exploitation of aquifers resulted in the groundwater pollution.

The presence of heavy metals in Lavrion

On the basis of the aforementioned traits and the results, which have risen from the environmental studies up to now, the broader area of Lavrion is classified, from an environmental point of view, as a high risk area (Kontopoulos et al. 1995; Demetriades 2010). The improper management of the metallurgical processing waste in the past resulted in the migration of heavy metals to the surroundings, contributing to widespread soil and water contamination. The overburden materials, including residual and alluvial soil, have been contaminated to an extremely high degree by the metallurgical processing activities. As a result, the local population is exposed to multiple hazardous pollution sources. The extreme multi-element soil contamination in Lavrion is a permanent feature, since the residence time of heavy elements in soil is very high. Three high-risk exposure routes for humans have been identified: direct contact, ingestion and inhalation of contaminated material. The epidemiological studies have shown very high concentrations of Pb and As in blood and urine and high concentrations of Pb, Cd and Zn in children's teeth. Lead poisoning is a common phenomenon in adults and elderly men (Eikmann *et al.*, 1991; Makropoulos *et al.*, 1991). These data from human tissues show, therefore, that Pb and As are in bioaccessible forms, and remedial actions must be taken to reduce exposure to acceptable levels. Apart from the extreme contamination of overburden materials and the residual soil, the house dust exhibits values varying from 500 to 18,600 ppm Pb and As from 130 to 3,820 ppm.

Site-specific Soil Action Levels (SAL) have been produced and used for the estimation of the contamination index for As, Ba, Be, Cd, Cr, Cu, Ni, Pb, V and Zn. The concentrations of Pb and As in all Lavrion overburden samples are well above their respective SALs of 500 ppm Pb and 25 ppm As. Barium, Ni and V have values below the estimated site specific Soil Action Levels for Lavrion. The other elements have a smaller proportion of samples exceeding their SALs. (Demetriades 2010)

High concentrations of heavy metals (lead, cadmium, zinc, nickel) are recorded in groundwater samples in the Lavrio area, too. The highest concentrations are recorded in the unconfined aquifer (alluvial deposits and schists). There is a relationship between the elements participating in the sulfide (Pb, Zn, Cu, Cd) and iron-manganese mineralization. Most of the water demands are covered from the exploitation of groundwater by numerous bored and dug wells. Due to the gradual impairment of groundwater quality from seawater encroachment, the water supply of the area is finally achieved by importing water. The industrial and animal rearing activities in and around the Lavrio area and the land is used locally for the cultivation of cereals, olives, vegetables and vineyards. Also, in the recent decades, industrial units located in the region have also affected groundwater quality. Therefore, it is easy to conclude that the effects on the health of the population from the long-term use of the local water sources may be serious due to high concentrations of heavy metals. In view of the alarming conditions an integrated water resources management should be applied for the confrontation of the environmental problem of Lavrion, too. (Stamatis et al. 2001)

Lavreotiki is a challenge for phytoremediation processes

The molecular links between metals in the environment and plant sulfur metabolism have been reviewed (Sirko and Gotor, 2007). To our knowledge, no phytoremediation actions have taken place in Lavreotiki. Plants are the most tolerant living organisms to pollution, a fact that is most profound in Lavreotiki. This fact emphasizes their utility for phytoremediation, a promising strategy for environmental cleanup. Many botanical families include multiple

species which exhibit a notable phytoremediation potential (Gawronski and Gawronska, 2007). Each of these species enjoys certain advantages, but at the same encounters some limitations with respect to its application as phytoremediant.

Careful selection of the appropriate family and genotype to match the particular pollutant and environment is crucial for successful phytoremediation and the Poaceae family seems to be one of the most important for this technology (Bouranis et al. 2012). Efficient phytoextraction techniques require large biomass yield, fast growth rate, easy harvest, metal accumulation ability and tolerance to heavy metal stress. *Arundo donax* (giant reed, Poaceae) is widely spread in Lavrion peninsula. It is a fast growing, robust, invasive perennial grass, wildgrowing in southern European regions and other Mediterranean countries. Giant reed can easily adapt to different ecological conditions and grows in all types of soils, it is a highly pest-resistant crop and it produces high biomass yield (32–37 tons of dry mass per hectare). Growth and photosynthesis of giant reed was not affected by high cadmium and nickel soil content (Papazoglou et al. 2005, 2007). For these reasons giant reed is proposed as a potentially high-yielding nonfood crop, which displays many attractive characteristics for the production of biomass, of pulp and paper and of activated carbons.

Concluding remarks

Sulfur and Lavreotiki seem to be interwoven to one another. From the sulphurous ores to the sulfidic tailings through the centuries, a long chain of mining and metallurgical activities has led to a seriously contaminated land, starting from wealth that produced history, culture, philosophy and science, beyond the amazing, slavery-based mining and metallurgical technology. In his detailed review on the environmental pollution of Lavrion, Demetriades (2010) stressed out that «although a detailed study was completed at the end of 1999, and a massive report submitted in 2000 to the Lavrion urban authorities and the Hellenic Ministry of Environment, with concrete cost-effective proposals for remediation of soil, not a single action was taken up to now. The situation remains the same. Houses and schools are built on metallurgical processing wastes, whilst children and adults alike are exposed to the same environmental contaminants.». As a concluding remark, Demetriades (2010) wrote that «We are obliged to offer better and healthier living conditions to the new generation; otherwise, in the near future we may resort to extreme solutions, because the remediation of “soil” would be more costly than moving the whole population of Lavrion and Lavreotiki Peninsula to another more suitable area».

The aforementioned concluding remark reflects the human point of view of course, because the natural vegetation seems to have adapted nicely after all. Ironically or thankfully, sulfur is a strong player in the detoxification and/or homeostasis mechanisms which are in charge of plant growth in such contaminated soils. Contrary to humans, the plants of the area, especially those of the natural vegetation, seem to have nicely worked out their own protection and, as it turns out, they are happily living in Lavreotiki.

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