

## Lettuce grown in silver laden soil at two different activity levels of soil microorganisms

Anneli Pedersen Brandt, Laila M. Karlsson, Uno Wennergren

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### Summary

Lettuce biomass, silver accumulation in lettuce, and effect of activity of soil microorganisms on these items, were studied in a series of experiments. Lettuce was cultivated in two kinds of soil with different organic matter concentrations. Initially the soil was either sterile or non-sterile, and had been supplied with different silver nitrate concentrations. Lettuce growth was significantly negatively affected by silver, especially in initially sterile soil with a lower organic matter content. There was also a significantly enhanced silver accumulation at larger silver supply in initially sterile soil with the lower organic matter content, otherwise there was no enhanced silver accumulation. There was a significant difference in respiration rate after harvest between the initially sterile soil and the non-sterile soil. In soil with the lower organic matter content, microorganism activity was inhibited by silver. In conclusion; silver accumulation increased and growth decreased in the lettuce grown in soil containing silver when the microorganism community in the soil had been affected by sterilization. The negative effects of silver on both lettuce and microorganisms were more distinct when the soil had a lower organic matter content.

### Introduction

Plants (HIRSCH, 1998) and mammals (RATTE, 1999) do not seem to accumulate silver to any large extent, and are therefore not affected by low levels in the surroundings. On the contrary, to microorganisms silver ions,  $\text{Ag}^+$ , are even more harmful than cadmium ions (RATTE, 1999). The cell walls of microorganisms, in natural environments, are negatively charged and metal cations bind readily to them (BRIERLEY et al., 1989). Microorganisms can, for example, adsorb more cadmium than the same amount (dry weight) of sand or clay (KUREK et al., 1982). Metals are also transported through the cell walls (SILVER and PHUNG, 1996). There seems to be differences in metal accumulation between living and dead cells of microorganisms. The cell walls of *Saccharomyces cerevisiae* have been shown to bind less silver in relation to their mass than intact cells do (SIMMONS and SINGLETON, 1996). The amount of silver accumulated can be large in some species without any toxic effects being noticed, while other species can react strongly already from minor amounts (RATTE, 1999). Depending on what particular activity of the microorganisms is being studied, the effect of metals can be impossible to detect even if the microorganisms are in reality affected (WITTER, 1992).

In sludge, as well as in soil and sediment, silver is found mostly as silver sulphide, which is sparingly soluble and therefore comparatively harmless (NATURVÅRDSVERKET, 1996). Since the silver compounds in sludge are relatively immobile, repeated fertilizing with sludge results in an accumulation of silver in soil (RATTE, 1999). However, metals that are complex bound to organic substances can be released when microorganisms are using the organic ligand as a carbon or energy source (BRYNHILDSEN, 1991). Bacteria oxidizing reduced sulphur compounds can participate in the release of metals (MCBRIDE et al., 1997). Availability of metals also depends on soil

characteristics, as pH and content of organic matter (GHORAYSHI, 1989). A small but important fraction of free silver ions can therefore exist in soil. The toxicity of silver depends mainly on the concentration of free silver ions (RATTE, 1999).

Using sewage sludge is a way to reuse nutrients and close resources cycles. While sewage sludge has organic matter and phosphorous, it also has organic pollutants and heavy metals (e.g. SAMSØE-PETERSEN, 2003; STERNBECK and ÖSTLUND, 1999). Hence there is a potential risk that using sludge as fertilizer could lead to negative impact on soil microorganisms or deteriorated quality of crop. One method to determine the activity of a microorganism community is to measure the amount of  $\text{CO}_2$  produced per unit of time and amount of soil, which reflects the respiration rate of the microorganisms (SCHNURER et al., 1985).

The objective of this work was to investigate if soil microorganisms affect the impact on lettuce, *Lactuca sativa* L., from silver in soil, and if this affection differs between two soils with different soil characteristics. Our hypothesis was that silver concentration in the green parts of lettuce, in relation to the amount of harvested biomass, would increase and the yield of the crop decrease, with increasing silver input to the soil. Furthermore, we assumed that this effect would be larger when the soil was initially sterile and the activity of microorganisms was therefore reduced. To determine the activity of the microorganism community we measured the respiration rate of the microorganisms. We expected this protective effect to be larger – and the microorganisms more inhibited – in soil with a lower concentration of organic matter.

### Materials and methods

#### Soil

Two types of soil (both classified as brown earth), hereafter called sandy soil and humus-rich soil, respectively, were collected in spring 2001 from the upper 10 cm of a ploughed field belonging to the farm Siggantorp (58° 20' N, 15° 40' E) in southern Sweden. This land has not received pesticides for 15-20 years, has not had artificial fertilizer added during the last ten years and sludge has never been added to it (Farmer S. A. CARLSSON, personal communication, 2001). The soil was sieved (6 mm mesh size), and autoclaved (200 kPa, 120°C) for 30 min. All soil was autoclaved in order to avoid a bias due to a possible change of soil characters. To restore the original microorganism community half of the soil of each of the two soil types was then inoculated with an amount of fresh soil corresponding to 2 %. Both the inoculated, here denoted non-sterile, and the sterile soil were incubated ten days at 20°C in a temperature controlled (ADU 200, Styrprojektering AB, Sweden) isolated room (Ki-PANEL, Huurre Sweden AB, Sweden) equipped with light 14 h per day. Light tubes gave 31-67  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (photometer: SKP 200, sensor SKP 215, Skye Instruments Ltd, Wales, measure precision 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 660/730 nm photon fluence ratio 5.9-8.3 (photometer: SKR 100, sensor SKR 110, Skye Instruments Ltd, Wales).

The soil (sandy and humus-rich, sterile and non-sterile) was examined concerning water content and concentration of organic matter (three

replicates). The soil was weighed, dried at 105°C for three days, and then weighed again (DW). Soil was heated at 600°C for 2 h and was then allowed to cool down in an exicator and weighed to reveal the concentration of organic matter. To determine the pH of the soil before plantation, 100 mL plastic mugs were filled with soil packed up to 2 cm (two replicates). Then 0.2 M KCl was added in an amount enough to stand 2 cm above the surface of the soil and the soil samples were put in a shaking machine for 2 h. The solution was then allowed to settle and the pH was determined in the water column.

### Cultivation

Plastic pots, volume 120 mL, upper area 30 cm<sup>2</sup>, were filled either with sterile sandy soil, non-sterile sandy soil, sterile humus-rich soil, or non-sterile humus-rich soil, 16 pots each soil type. The amount of soil in each pot corresponded to 100 g soil DW.

Salt solutions of AgNO<sub>3</sub> and/or NaNO<sub>3</sub> in different concentrations were added to the soil. This resulted in an amount of silver equivalent to 0, 5, 50, or 100 mg Ag kg<sup>-1</sup> soil DW in each pot. The solutions of NaNO<sub>3</sub> were added to balance the nitrogen amount and resulted in an amount of inorganic nitrogen corresponding to 13 mg N kg<sup>-1</sup> soil DW in all treatments. Four replicates were used to each treatment of soil; the total number of pots was thus 64.

At least 2 h after preparation of the soil, 12 germinated seeds of lettuce were planted in each pot. The seeds had been sown on moistened filter paper two days earlier. The lettuce was cultivated at 20°C, with a relative humidity of 75 %, in the temperature controlled room equipped with light 14 h per day. The pots were watered with tap water, the same amount in all pots, and with deionised water if any pot needed more water. All water used had been sterilized by boiling for 10 min.

A couple of days after the planting out, two pots with initially sterile sandy soil and the highest silver concentration were damaged and were therefore removed. Two new pots with planted lettuce were then prepared in the same way as before.

Twenty-three days after planting the germ materials (13 days for those pots replacing the destroyed ones), each pot was supplied with a commercial solution of nutrients (Weibulls Rika, Hydro Agri AB, Sweden) resulting in a nutrient supply equivalent to 23 mg N kg<sup>-1</sup> soil DW, 3.6 mg P kg<sup>-1</sup> soil DW, and 17 mg K kg<sup>-1</sup> soil DW.

### Biomass

The lettuce was harvested one month after planting. In some of the pots one plant had died, and the smallest plant from the other pots was therefore removed to get eleven from each pot. Roots were removed, and stalk and leaves were washed before they were dried at 105°C. After four days the dry biomass was weighed (DW).

### Respiration rate

During the week after the plants had been harvested, the soil in the pots was watered and then put in 500 ml glass bottles. After 11-12 days of incubation at 14°C in the temperature controlled room equipped with light 14 h per days, vial flasks containing 4 ml 0.5 M NaOH were placed in each bottle containing soil and also in four empty bottles (blind samples).

The bottles were hermetically sealed and incubated at 14°C in darkness for two days. The CO<sub>2</sub> absorbed in NaOH was bound with 2.5 ml 1 M BaCl<sub>2</sub>. The remaining NaOH was titrated with 0.1 M HCl. Phenolphthalein was used as pH indicator.

The amount of CO<sub>2</sub> from the blind samples constituted the content

of CO<sub>2</sub> in the air enclosed in the bottle. The mean value from these bottles was deducted from the result for each bottle containing soil, thus giving a value for the amount of CO<sub>2</sub> respired from the microorganisms in the soil.

After the measurement of the respiration rate, soil from the bottles was dried for three days at 105°C and weighed. The amount of respired CO<sub>2</sub> per hour could then be related to the amount of soil DW.

### Silver concentration

The dried lettuce was weighed and put in acid-washed bottles (Swedish standard method SS 02 81 50). The four replicates of lettuce from the pots treated with the highest concentration of silver (100 mg kg<sup>-1</sup> soil DW) were put together to form a single sample for each soil type (sterile sandy soil, non-sterile sandy soil, sterile humus-rich soil and non-sterile humus-rich soil). This was done because the amount of biomass was very small.

To the bottles was then added 14 M HNO<sub>3</sub> (65 %, quality suprapur, Merck) in amounts adjusted to the quantity of biomass – 100 mL per g biomass. In a few cases, the amount of biomass was small and therefore 300 mL per g biomass was used. The bottles were sealed and autoclaved (200 kPa, 120°C) for 30 min. The samples were diluted with four parts of milli-Q water, and were then analysed by ALcontrol AB, Linköping, Sweden, with ICP-MS (Induced Coupled Plasma Mass Spectrometry). The detection limit with this method is 1 µg Ag L<sup>-1</sup>.

### Statistical analyses

Analysis of variance, multi-way between-groups ANOVA, and Tukey's HSD-test for mean comparison were performed. For all testing, an  $\alpha$  risk of 0.05 was selected for statistical significance.

## Results and discussion

### Type of soil

The sandy soil had a higher pH than the humus-rich soil, while the water content and organic matter content were higher in the humus-rich soil (Tab. 1). Differences between the two soil types concerning pH and content of organic matter were statistically significant. The soil with a higher organic matter content had a lower pH than the sandy soil, which was expected since organic acids acidify the soil (VAN BREEMEN, 1991). When pH is below 6, the activity of the microorganisms normally decreases, but, on the other hand, organic matter has a positive effect on both activity of microorganisms (VAUGHAN and ORD, 1985) and growth of plants (VAUGHAN and MALCOLM, 1985). The results indicate that in this experiment pH

**Tab. 1:** Characteristics of the soil. pH, n=2. Organic content and water content in fresh soil, mean (SD), n=3.

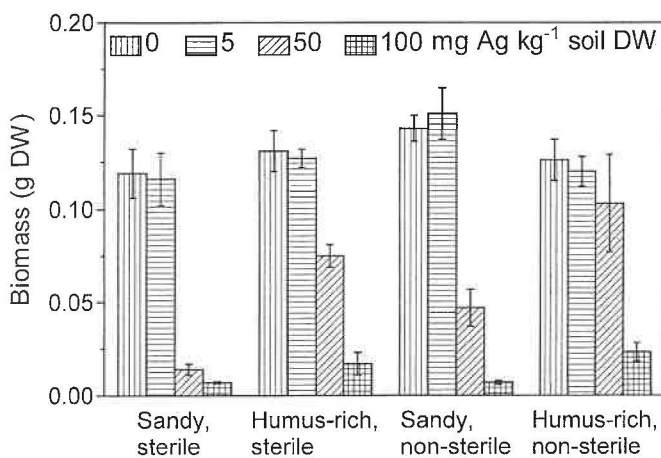
Soil type	Treatment	pH	Organic content (%)	Water content (%)
Sandy soil (brown earth)	Non-sterile	6.3	3 (0.07)	16 (0.16)
	Sterile	6.2	3 (0.04)	16 (0.12)
Humus-rich soil (brown earth)	Non-sterile	5.8	8 (0.02)	21 (0.02)
	Sterile	5.6	8 (0.07)	21 (0.20)

had a minor importance compared to the concentration of organic matter, since cultivation in the humus-rich soil, in spite of its slightly lower pH, generally resulted in a larger volume of biomass and a lower concentration of silver in that biomass than did cultivation in the sandy soil (Fig. 1, 2).

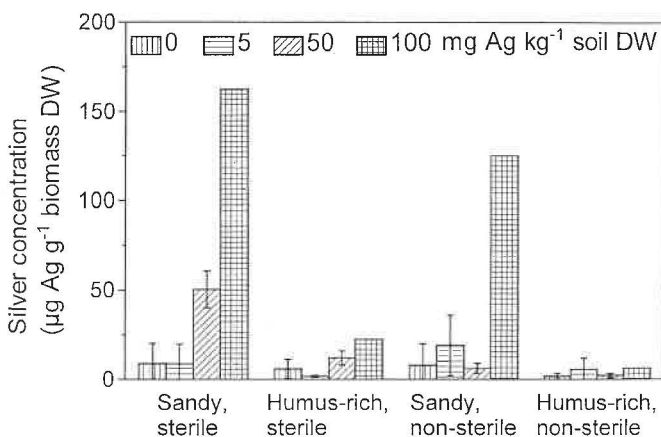
### Respiration rate

When silver had not been added to the soil, the respiration rate differed significantly only between the initially sterile sandy soil and the non-sterile sandy soil. The respiration rate was generally lower when silver had been added or when the soil was sterile from the beginning (Fig. 3).

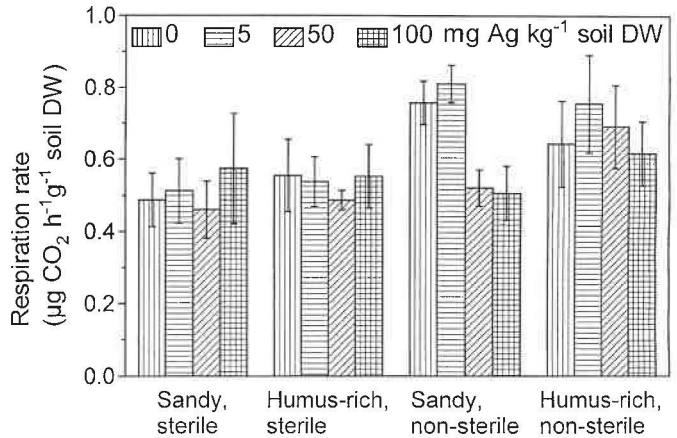
When the soil had a living microorganism community during the entire experiment, silver had a significantly negative impact on res-



**Fig. 1:** Biomass of lettuce, dry weight (DW), per pot in relation to type of soil, soil sterility, and silver supply to the soil. Mean values with 95 % confidence range (n=4). Soils used were both brown earth from Sweden. "Sterile" and "non-sterile" refers to soil autoclaved and kept as clean as possible, and soil containing the original microorganism community, respectively.



**Fig. 2:** Silver concentration in biomass of lettuce, dry weight (DW), in relation to type of soil, soil sterility, and silver supply to the soil. Mean values with 95 % confidence range (n=4). Soils used were both brown earth from Sweden. "Sterile" and "non-sterile" refers to soil autoclaved and kept as clean as possible, and soil containing the original microorganism community, respectively.



**Fig. 3:** Respiration rate in soil after harvest in relation to type of soil, soil sterility, and silver supply to the soil. Mean values with 95 % confidence range (n=4). Soils used were both brown earth from Sweden. "Sterile" and "non-sterile" refers to soil autoclaved and kept as clean as possible, and soil containing the original microorganism community, respectively.

piration rate in the sandy soil but not in the humus-rich soil (Fig. 3). It is likely that the humus-rich soil, with its higher concentration of organic matter, had bound silver to a higher degree than the sandy soil and thereby lowered the metal's toxicity to the microorganisms (CLAPP et al., 1986).

In the initially sterile soil, there was no significant difference in respiration rate between the different silver concentrations or between the two soil types (Fig. 3). However, the respiration rate was not negligible in the soil sterilized at the start of the experiment, in spite of the care that had been taken not to contaminate it with microorganisms during the experiment. Microorganisms entering the soil after sterilization probably originated from airborne particles and from the seeds (LYNCH, 1983).

Measurement of the respiration rate showed the activity of the microorganisms at the time of measurement and not how it had been during the period the lettuce was growing in the soil. Microbial activity should have been comparatively continuous in the non-sterile soil unladen with silver. In the initially sterile soil, the amount of living microorganisms, and therefore the respiration rate, was at first absent but then gradually increased. Furthermore, when silver was added, the activity of microorganisms during cultivation was probably varying depending on the amount of silver and the microorganisms' response to it (RATTE, 1992; WITTER, 1992). As time went on, more and more silver ions presumably became bound to living and dead cells and organic substances, whereby the negative effect of the silver decreased (COLLINS and STOTZKY, 1989). In this way, the type of soil and treatment in the different pots probably influenced the amount and composition of microorganisms.

Note that a change in composition of the community during a maintained total activity would not have been seen in the respiration rate measurement. JOHANSSON (2000) found that denitrification bacteria were very sensitive to silver and that their denitrification activity was affected at a concentration of 0.40 mg Ag (as silver sulphate) kg<sup>-1</sup> soil DW. In contrast, total respiration only became negatively influenced after a silver concentration of 15 mg kg<sup>-1</sup> soil DW had been reached. Metal-resistant microorganisms, that have competitive advantages in relation to microorganisms sensitive to metals, may respire more than normal since many resistance mechanisms are energy dependent. Therefore, the total respiration rate can even increase slightly under a moderate metal influence (WITTER, 1992).

### Biomass

Type of soil did not affect biomass when silver was not added (Fig. 1). The silver supplied had a significant importance in the growth of the lettuce. The negative effect of silver on the yield of lettuce was more pronounced in the sandy soil and when the soil was initially sterilized (Fig. 1). The amount of biomass collected from sandy soil (both initially sterile and non-sterile), and from initially sterile humus-rich soil, decreased when the silver concentration was enhanced from 5 to 50 mg Ag kg<sup>-1</sup> soil DW. In non-sterile sandy soil and in initially sterile humus-rich soil, the growth decreased even more when the silver concentration increased to 100 mg Ag kg<sup>-1</sup> soil DW. In non-sterile humus-rich soil, the size of the yield did not decrease significantly until the silver concentration was 100 mg Ag kg<sup>-1</sup> soil DW (Fig. 1).

With a silver supply of 5 mg Ag kg<sup>-1</sup> soil DW, lettuce growth in sandy soil was higher when living microorganisms were present in the soil from the start, compared to when they were initially absent. In humus-rich soil, microorganisms also had a positive impact on growth, but first at 50 mg Ag kg<sup>-1</sup> soil DW (Fig. 1). Thus, the silver-binding capacity of microorganisms seems to have been of more benefit to plants in the sandy soil than in the humus-rich soil. This may follow from a higher content of metal-binding components in the humus-rich soil (CLAPP *et al.*, 1986). However, microorganisms had no impact on growth in either of the two soil types when 100 mg Ag kg<sup>-1</sup> soil DW had been added (Fig. 1). Either the large amount of silver damaged the microorganisms or the direct impact the silver had on the plants superseded any beneficial effect from the activity of the microorganisms.

A poor yield can be the result of a nutrient deficiency in the soil, and metals can inhibit the mineralization of organic matter. This does not necessarily depend on toxic effects on subverting microorganisms; instead, it can be due to the fact that complexes formed between metal and organic substances are hard to break down (BRYNHILDESEN, 1991). However, it is not likely that nutrient deficiency was occurring in this experiment, since nutrients were added. The plants were probably negatively affected by the silver and hence disturbed in their growth.

The fact that silver affects certain plants has been shown earlier. HIRSCH (1998) found that lettuce and cabbage cultivated in soil fertilized with sludge amended with silver sulphide corresponding to 15, 68, or 155 mg Ag kg<sup>-1</sup> soil DW respectively, resulted in a lower amount of biomass than when the sludge contained no silver. Furthermore, the presence of silver sulphide in sludge-amended soil, at a concentration of  $\geq 14$  mg Ag kg<sup>-1</sup> soil DW, was shown to cause a significantly lower harvest of cabbage, lettuce, and spinach, but not of corn, wheat, and soybean.

### Silver concentration

This study focused on silver content in the crop and not the possibilities of remediating soils by means of plants (phytoremediation). Therefore, silver concentration in biomass was related to the weight of biomass instead of the quantity of plants; so also did HIRSCH (1998) in her investigations of silver impact on different crops.

Both type of soil, activity of the microorganisms (soil sterilized or not), and silver supply had a significant effect on the silver concentration in the lettuce. The silver accumulation was generally larger from sandy soil than from humus-rich soil (Fig 2). Silver accumulation from initially sterile sandy soil with a silver concentration of 50 mg Ag kg<sup>-1</sup> soil DW significantly exceeded all other treatments, except treatments with 100 mg Ag kg<sup>-1</sup>, which could not be included in analysis. Otherwise, there were no significant differences between mean values. The silver accumulation was not affected

by microorganisms in the humus-rich soil even at a silver content of 50 mg Ag kg<sup>-1</sup> soil DW; whereas, in the sandy soil, microorganisms had an effect on silver accumulation at this silver concentration in soil but not at the lower concentrations (Fig. 2).

Concerning the silver accumulation in the lettuce grown in soil with the highest silver concentration, 100 mg Ag kg<sup>-1</sup> soil DW, there was only one replicate belonging to each treatment (type of soil and initially sterile/non-sterile), since the four replicates had been lumped together; consequently, no statistics exist for this concentration. Each value for the highest silver concentration, 100 mg Ag kg<sup>-1</sup> soil DW, was divided by four since they each came from four samples. Silver content was much higher in the biomass from sandy soil than from humus-rich soil. Within both soil types, the silver content was higher when the soil had been initially sterile, especially in sandy soil (Fig. 2).

Metal concentrations can sometimes be high in humus-rich soils. Metals can be effectively bound by some organic substances, and are therefore not readily leached out of the system. Yet, because the metals are strongly bound and therefore not taken up by the plants to any large extent, the metal content in crops grown in humus-rich soils is lower compared to other soils (HELLSTRAND and LANDNER, 1998).

In our experiment, the silver concentration in soil that caused significantly higher silver concentration in lettuce than in the control was 50 mg Ag kg<sup>-1</sup> soil DW (initially sterile sandy soil). This can be compared to the results from laboratory experiments performed by HIRSCH (1998), where the silver concentration in crops was examined. The leaves of lettuce grown in sludge-amended soil, with a silver content of 68 and 155 mg Ag kg<sup>-1</sup> DW respectively, contained significantly higher concentrations of silver than did the controls. During another of her experiments, 'laboratory-produced' sludge with a certain concentration of silver was used; the amount of sludge and thereby the amount of silver added to the soil was varied. The leaves of lettuce grown in soil containing 5 mg Ag kg<sup>-1</sup> DW and 120 mg Ag kg<sup>-1</sup> DW had significantly higher silver content than the control plants.

When silver is taken up in plants through the roots, only a minor fraction is transported to other parts of the plant (RATTE, 1999). In lettuce grown in soil without any silver addition, HIRSCH (1998) measured 0.024  $\mu$ g Ag g<sup>-1</sup> biomass DW in the leaves and almost six times more silver (0.142  $\mu$ g Ag g<sup>-1</sup> biomass DW) in the roots. The silver content in lettuce cultivated in soil without added silver was remarkably high in our investigation. The mean value of these samples, no matter what soil type or whether it had been sterilized or not, was 6.07  $\mu$ g Ag g<sup>-1</sup> biomass DW (SD 8.37). This could indicate that the natural background concentration of silver in the soil at Siggartorp was rather high, in spite of the fact that this soil has not been amended with sewage sludge.

### Conclusions

In nature, microorganisms are adapted to some variation in the environment. Both in soil initially sterile and in non-sterile soil, a more or less active microorganism community existed at the end of the experiment, even if its composition was probably by then different from that at the start. Because of the sterilization, there would be dead microorganisms that were capable of binding silver in both soil types. In spite of that, it was when living microorganisms existed when silver was added that the negative effects of silver on the plants decreased (Fig. 1, 2), especially when the content of organic matter was low. The activity of the microorganisms was also more affected by the silver in sandy soil than in humus-rich soil (Fig. 3). The results indicate that living microorganisms and a high organic matter content in the soil protected against a decreased growth of

lettuce in the presence of silver. In soil with a low concentration of organic matter, the microorganisms also reduced the silver accumulation in the lettuce. The organic content in silver laden soil seems to be important both to the status of the microorganisms and to how dependent the plants are on the buffering capacity of the microorganisms. Sandy soils may therefore be particularly inappropriate for fertilizing with sludge. It is likely that the activity of microorganisms could have an effect on silver accumulation and growth of plants even in natural systems – to what extent, however, depends on a great variety of factors which are all interacting with each other.

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Address of the authors:

Anneli Pedersen Brandt, Laila M. Karlsson (corresponding author, email: laika@ifm.liu.se), Uno Wennergren, Department of Biology – IFM, Linköping University, SE-581 83 Linköping, Sweden