

## Use of an isothermal microcalorimetry assay to characterize microbial oxalotrophic activity

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### Abstract

Isothermal microcalorimetry (IMC) has been used in the past to monitor metabolic activities in living systems. A few studies have used it on ecological research. In this study, IMC was used to monitor oxalotrophic activity, a widespread bacterial metabolism found in the environment, and particularly in soils. Six model strains were inoculated in solid angle media with K-oxalate as the sole carbon source. *Cupriavidus oxalaticus*, *Cupriavidus necator*, and *Streptomyces violaceoruber* presented the highest activity (91, 40, and 55  $\mu\text{W}$ , respectively) and a maximum growth rate ( $\mu_{\text{max}} \text{h}^{-1}$ ) of 0.264, 0.185, and 0.199, respectively, among the strains tested. These three strains were selected to test the incidence of different oxalate sources (Ca, Cu, and Fe-oxalate salts) in the metabolic activity. The highest activity was obtained in Ca-oxalate for *C. oxalaticus*. Similar experiments were carried out with a model soil to test whether this approach can be used to measure oxalotrophic activity in field samples. Although measuring oxalotrophic activity in a soil was challenging, there was a clear effect of the amendment with oxalate on the metabolic activity measured in soil. The correlation between heat flow and growth suggests that IMC analysis is a powerful method to monitor bacterial oxalotrophic activity.

### Keywords

isothermal microcalorimetry (IMC); oxalotrophic activity; oxalotrophic bacteria.

### Introduction

Oxalic acid ( $\text{H}_2\text{C}_2\text{O}_4$ ) and oxalate minerals are widely distributed among plants, animals, fungi, and bacteria present in soils (Tamer *et al.*, 2002). Oxalic acid is often accumulated as a metabolic end product in plant tissues or is released by root systems as a free organic acid or mineral salts such as calcium, iron, or magnesium oxalate (Cailleau *et al.*, 2005). The oxalate released in soils can play important roles, for example by increasing the availability of phosphorous and other micronutrients for plant uptake (Franceschi & Nakata, 2005). The release of calcium oxalate has also been involved in the formation of calcite ( $\text{CaCO}_3$ ) deposits in otherwise acidic carbonate-free soils (Braissant *et al.*, 2004; Cailleau *et al.*, 2004; Dupraz *et al.*, 2009). This process, also called the oxalate–carbonate pathway (Cromack *et al.*, 1977; Verrecchia, 1990; Verrecchia *et al.*, 2006), relies on the biological degradation of oxalate and has been pointed out

as an underestimated long-term carbon sequestration mechanism (Braissant *et al.*, 2002). The biological degradation of oxalate is in agreement with the fact that accumulation of metal oxalates has not been observed in geological records (Schilling & Jellison, 2004; Verrecchia *et al.*, 2006), except in some very specific settings such as hydrothermal springs (Hofmann & Bernasconi, 1998) and septarian concretions (Hyde & Landy, 1966). Normally, a microbiologically mediated process is assumed to be the main oxalate degradation process in natural environments, leading to the precipitation of calcium carbonate or other forms of carbonate depending on the oxalate mineral sources (Robbel & Kutzner, 1973).

Bacteria using oxalate as a source of carbon and energy are called ‘oxalotrophic bacteria’ (Sahin, 2003). Oxalotrophic bacteria can be considered ‘generalists’ when they are able to metabolize other substrates or ‘specialists’ when they use oxalate as their sole carbon and energy sources (Trinchant & Rigaud, 1996). There are still many questions

regarding the specific metabolic pathways involved in the use of oxalate. The first metabolic studies on oxalate-consuming bacteria started a long time ago (Quayle & Keech, 1959; Blackmore & Quayle, 1968; Dijkhuizen *et al.*, 1977; Aragno & Schlegel, 1991). For its use as carbon and energy sources, in *Cupriavidus oxalaticus* (Vandamme & Coenye, 2004), oxalate is activated to oxalyl-CoA before a part of it is oxidized to CO<sub>2</sub> (energy generation) and the remaining part is reduced to glyoxylate for biosynthesis (Jayasuriya, 1955; Blackmore & Quayle, 1970). Biosynthesis is thought to occur either via the serine or the glycolate pathways (Tamer & Aragno, 1980; Sahin, 2003). Because of the fact that oxalate is a highly oxidized substrate, bacterial growth yield is low (about 2.5 g mol<sup>-1</sup>) (Braissant *et al.*, 2002). In addition to the direct assimilation of oxalate, this organic salt has been implicated in pH regulation and aluminum detoxification in nonoxalotrophic bacteria (Friedrich *et al.*, 1979).

Previous studies have described various methods for measuring oxalotrophic activity. These methods imply either the disruption of the cells, allowing measurements of enzymatic activity (Quayle & Keech, 1960; Quayle, 1963; Milardović *et al.*, 2000), or colorimetric assays in order to measure pH changes linked to oxalate consumption (Cromack *et al.*, 1977). More recently, a new molecular marker has been identified as specific to oxalotrophic bacteria (gene *frc* codifying for the enzyme formyl-CoA transferase) (Khammar *et al.*, 2009). This opens up the possibility of detecting oxalotrophic activity through transcriptomic studies. However, there are some oxalotrophic bacteria that do not have a functional *frc* gene, but are able to grow *in vitro* using selective media with calcium oxalate (false negatives), or bacteria that contain the *frc* gene, but do not use oxalate as a carbon or an energy source (false positives; e.g. *Escherichia coli* K12; D. Bravo *et al.*, unpublished data). Therefore, alternative approaches are needed to estimate oxalotrophic activity and to improve our understanding of oxalate metabolism by bacteria. Isothermal calorimetry could fill such a gap by allowing the measurement of oxalotrophic activity in real time over a wide variety of conditions.

The term isothermal microcalorimetry (IMC) is commonly used to refer to the measurement of heat production in the microwatt range under essentially isothermal conditions (Wadsö & Goldberg, 2001). Several types of isothermal microcalorimeters exist, allowing nearly isothermal conditions through phase transition, power compensation, or heat conduction. Nowadays, heat conduction microcalorimeters (also called heat flux microcalorimeters) are most commonly used (van Herwaarden, 2000; Braissant *et al.*, 2010b). Isothermal titration calorimetry (ITC) – a particular application of IMC – has been widely used to characterize the binding affinities of various ligands to protein and macromolecules (Cooper, 2003). In contrast to ITC, IMC has been less commonly used in biological sciences

compared with other areas. However, IMC can be used to measure oxic and anoxic metabolisms, with reproducibility and long-term baseline stability, and it is therefore pertinent for measuring microbial activity in soils (Vor *et al.*, 2002; Rong *et al.*, 2007; Wadsö, 2009). In this way, every process in soil can be recorded as a specific metabolic activity as a function of heat production. The importance of calorimetric analysis is supported by the fact that the results can be expressed as ‘life intensity’ or ‘biological response’ (Mortensen *et al.*, 1973). In addition, results can be expressed in terms of the thermodynamic or the kinetic properties of bacterial activity and are directly related to other experimental conventional techniques (see the review in Braissant *et al.*, 2010a). Despite its potential for measuring exothermic reactions associated with the consumption of oxalate, at present, there are no precedents on the use of IMC to study oxalotrophic activity, and in particular, in soils.

In this study, the growth of oxalotrophic bacteria is characterized for the first time using IMC. The aim of this study is to describe the oxalotrophic activity of model strains and in a model soil. Three different types of experiments were performed. First, several model oxalotrophic strains were grown in potassium oxalate in order to compare their respective heat release during oxalate consumption. Second, a group of selected strains was used to compare oxalotrophic activity in the presence of different oxalate sources (K, Ca, Cu, and Fe). Finally, a model soil was used to test the potential of IMC to measure oxalotrophic activity in soils. In this context, the present study contributes to the understanding of the microbial ecology of oxalotrophic activity in soil bacteria.

## Materials and methods

### Bacterial strains and growth conditions

Four generalistic strains were used to test the pertinence of IMC to measure oxalotrophic activity. *Methylobacterium extorquens*, *C. oxalaticus*, *Streptomyces violaceoruber*, and *Cupriavidus necator* have been reported as oxalotrophic bacteria (Tamer *et al.*, 2002; Sahin, 2003). In addition, *E. coli* was used as a negative control. *Escherichia coli* has been reported as a non-oxalate-oxidizing bacteria (Turroni *et al.*, 2007). The strain BV1M3 corresponds to a *Streptomyces* sp. and was isolated from a tropical soil in Bolivia. This soil was collected near the oxalogenic tree *Terminalia oblonga*. All cultures were maintained in nutrient broth or nutrient agar (NB/NA) media (Difco) for regular transfers. All the strains used in this study are summarized in Table 1.

### Growth conditions for calorimetric assays in pure cultures

For growth in the microcalorimeter, all strains with the exception of *E. coli* were preincubated on solid Angle media

**Table 1.** Oxalotrophic strains used in the study

Strain	Collection number	Strain number
<i>Escherichia coli</i> K12	NEU 1007	K12
<i>Methylobacterium extorquens</i>	NEU 44	TA3
<i>Cupriavidus oxalaticus</i>	NEU 1047	OX 1
<i>Streptomyces violaceoruber</i>	NEU 1225	–
<i>Cupriavidus necator</i>	NEU 2073	NS2
<i>Streptomyces</i> sp. BV1m3	BV1m3	BV1m3

All these strains are deposited in the culture collection of the Laboratory of Microbiology, University of Neuchâtel (LAMUN).

(Angle *et al.*, 1991) supplemented with  $4\text{ g L}^{-1}$  calcium oxalate as the carbon source. *Escherichia coli* was preinoculated on NA. Replicate cultures for all strains were performed in 4 mL microcalorimetric ampoules filled with 2 mL of slanted solid Angle's medium to which  $4\text{ g L}^{-1}$  potassium oxalate (pH=7.0) was added. Inoculation was performed with an inoculation loop, ensuring that bacteria would grow as a lawn. Previous studies have shown that decreasing the inoculum concentration resulted in a lower maximum activity (Braissant *et al.*, 2010a, b). The measurements of oxalotrophic activity were performed in an isothermal heat conduction microcalorimeter (TAM III, Waters/TA Instruments, Delaware) equipped with 48 channels. The temperature of the microcalorimeter thermostat was set for the growth of environmental bacteria at  $25\text{ }^{\circ}\text{C}$ . After stable temperature conditions were obtained, each measuring channel was calibrated using a built-in electrical heater of known power. All the individual microcalorimeters (i.e. measuring channels) also have a built-in aluminum reference with a heat capacity and conductivity approximately equal to that of a 4-mL glass ampoule containing 3 mL of water. This reduces the equilibration time and improves the stability of the heat-flow rate measurements. The baseline was obtained from ampoules containing sterile medium only. Therefore, any heat production by the media (i.e. chemical heat) could be subtracted from the signal in the ampoules inoculated with the strains. However, such subtraction was not necessary because measurement using sterile media remained within the background noise level. The measurements obtained in the microcalorimeter were recorded after a two-step thermal equilibration procedure recommended by the manufacturer that lasted 1 h. Briefly, during the first 15 min, the samples were placed in the equilibration position to achieve preliminary thermal equilibration. Samples were then placed in the measuring position. However, 45 additional minutes were necessary to achieve fine thermal equilibration and start measurements.

Three strains were selected for the second experiment. The strains were inoculated in an inclined Angle agar medium, supplemented with different insoluble metal-oxalate sources. Calcium, copper, and iron oxalate were

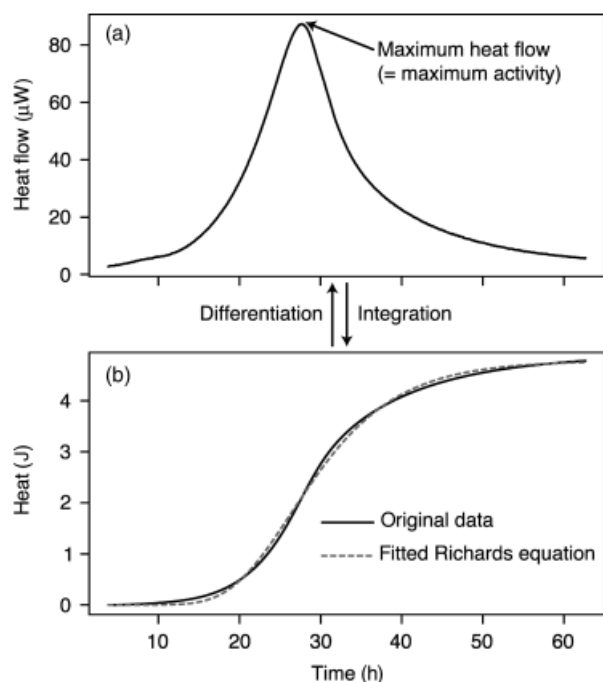
selected because they are representative sources available in a soil (Cromack *et al.*, 1977). Copper oxalate was produced following the protocol of David (1960) from copper sulfate and oxalic acid. Iron oxalate was obtained using a protocol developed by the Académie de Bordeaux. Briefly, ferrous ammonium sulfate was dissolved in acidified water at  $40\text{ }^{\circ}\text{C}$  (4% 2 M sulfuric acid) and mixed with a solution of oxalic acid. Copper and iron oxalates were prepared at the Laboratory of Mycology, University of Neuchâtel. The sources were tested as the sole carbon source at  $4\text{ g L}^{-1}$ . The isothermal microcalorimeter was filled with replicates of each carbon source per strain. The baseline was obtained from ampoules with a sterile medium. The measurements obtained in the microcalorimeter were recorded after the recommended two-step thermal equilibration procedure that lasted 1 h as well.

### Microcalorimetric assay of soil

The soil selected as a model for the assays was used in previous studies and its physicochemical parameters have been characterized extensively (Milleret *et al.*, 2009). The soil corresponds to the organo-mineral horizon of an Anthrosol collected at the botanical garden of Neuchâtel (Switzerland). The soil is a carbonated loamy soil (45.3% sand, 28.0% silt and 26.7% clay), containing 20.7% w/w carbonates and 2.0% w/w total organic carbon and having a  $\text{pH}_{\text{KCl}}$  of 7.8. The cation exchange capacity was  $21.3\text{ cmol}_c\text{ kg}^{-1}$ . Soil was sieved in the laboratory to remove root fragments and large particles. Four treatments were applied to the soil: (1) no additions; (2) spiked with  $10^9\text{ CFU mL}^{-1}$  of *C. oxalaticus*; (3) addition of Angle liquid medium (800  $\mu\text{L}$ ) with  $4\text{ g L}^{-1}$  potassium oxalate; and (4) spiked with  $10^9\text{ CFU mL}^{-1}$  of *C. oxalaticus* and Angle liquid medium (800  $\mu\text{L}$ ) with  $4\text{ g L}^{-1}$  potassium oxalate. Two grams of soil were added in each 4 mL microcalorimetric ampoule. For the treatment without additions (1), the soil was homogeneously humidified with 800  $\mu\text{L}$  of liquid sterile angle media without a carbon source. All treatments were run at  $25\text{ }^{\circ}\text{C}$ . As for the cultures on solid media, the measurements obtained in the microcalorimeter were recorded after the recommended two-step thermal equilibration procedure described above.

### Data analysis

Analysis of the heat flow (thermograms) was used for calculating the kinetic parameters. Because  $1\text{ }\mu\text{W} = 1\text{ }\mu\text{J s}^{-1}$ , the maximum heat flow (Fig. 1a) was used to calculate the maximum oxalotrophic activity assuming the following reaction:  $2\text{H}_2\text{C}_2\text{O}_4 + \text{O}_2 \rightarrow 4\text{CO}_2 + 2\text{H}_2\text{O}$  with a reaction enthalpy of  $\Delta H^{\circ}$  of  $-499\text{ kJ mol}^{-1}$  (standard enthalpies of formations of reactants and products were obtained from Dean, 1999). This calculation assumes that the heat



**Fig. 1.** Sketch showing the relationship between actual calorimetric measurements (heat flow; a) that corresponds to the microbial activity and the evolution of heat (b), which is a proxy for products resulting from oxalotrophic activity (i.e. biomass or  $\text{CO}_2$  for example). The maximum heat flow (arrow) is directly proportional to the maximum oxalotrophic activity of the strain.

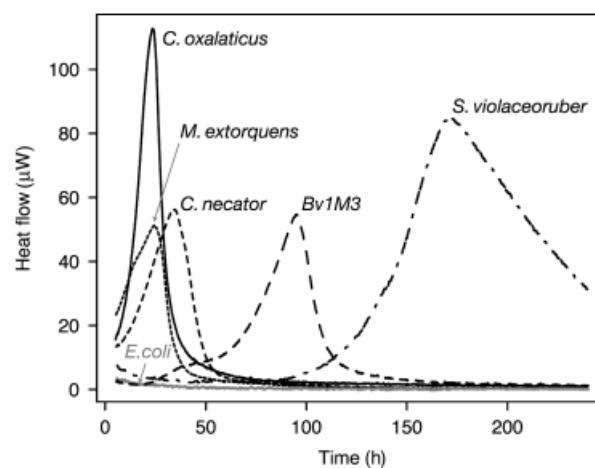
produced by biomass formation is ignored because the biomass yield on oxalate is very low. However, it must be noted that models exist to estimate the amount of heat released during biomass production (Heijnen *et al.*, 1992). The heat over time curve was obtained by integrating the heat flow data (Fig. 1). Using the heat over time curve, the net growth rate was calculated by fitting an exponential model ( $Q_t = Q_0 e^{\mu t}$ , where  $Q$  represents the heat) over the exponential part of the curve. Similarly, the maximum growth rate was calculated by fitting the modified Richard's equation (see Zwietering *et al.*, 1990) to the complete heat over time curve. For every strain, these calculations were performed over three to six replicates, allowing the calculations of the mean and SD. The thermograms of each strain were analyzed in terms of the thermodynamic consumption of oxalate as a function of oxalotrophic activity and oxalate source. To calculate the accuracy of IMC in measuring oxalotrophic activity, the degradation rates of oxalate calculated by IMC were compared with degradation obtained by HPLC using the same strains and medium (in this case in liquid). The chromatograms were obtained using an HPLC 110 (Agilent Technologies) with a diode array detector (210 nm UV) and a column RPC18. The method carried out was according to previous studies for oxalogenic fungi (Schilling & Jellison, 2004), with the following modifica-

tions for oxalotrophic bacteria: the samples were centrifuged at 10 000  $g$  for 2 min. Five hundred microliters from the supernatant was dissolved in 800  $\mu\text{L}$  of 20mM  $\text{H}_2\text{SO}_4$  HPLC grade. The solution was mixed vigorously by pulsed vortexing for 20 s. The samples were incubated for 2 h at room temperature. Before HPLC analysis, the samples were filtered with Whatman membrane cellulose filters of 0.45  $\mu\text{m}$  pore size in 1.5-mL vials closed with septum caps.

## Results

### Oxalotrophic activity measured by IMC in pure cultures

The aim of this first experiment was to compare oxalotrophic activity between strains and select those with higher efficiency on the degradation of the carbon source to be tested with alternative metal-oxalate sources. Potassium oxalate was selected as a substrate to measure oxalotrophic activity in four known oxalate-consuming bacteria and one environmental strain. In addition, *E. coli* was used as a negative control. Representative thermograms obtained for each one of the strains are shown in Fig. 2. In the case of the negative control (*E. coli*), even after 280 h of incubation at 25  $^\circ\text{C}$ , no change in the baseline signal was observed. In contrast, in all the other strains (known to consume oxalate), a clear peak of activity was observed. For *C. oxalaticus*, *M. extorquens*, and *C. necator*, the maximum heat flow was observed before 50 h of incubation. In the two strains of actinobacteria (*S. violaceoruber* and *Streptomyces* sp. BV1M3), the maximum heat flow was recorded considerably later between 100 and 200 h.



**Fig. 2.** Representative thermograms recorded for *Cupriavidus oxalaticus*, *Methylobacterium extorquens*, *Cupriavidus necator*, *Streptomyces violaceoruber*, *Sptreptomyces* sp. BV1M3, and *Escherichia coli* inoculated in Angle medium with potassium oxalate as the sole carbon source.

The parameters of biological activity derived from the heat flow (Table 2) show that *C. oxalaticus* has the highest activity on potassium oxalate (measured as the consumption rate), followed by *C. necator* and *M. extorquens*. In all the cases, there was a linear correlation between the maximum oxalate consumption rate and the net (Fig. 3a) and maximum (Fig. 3b) growth rates. Values slightly deviating from the linear trend might be due to maintenance effects for the different strains.

When the same analysis was carried out in data obtained from growth in NA (Table 2b), both *E. coli* and *C. oxalaticus* showed higher maximum heat flow and growth than the other strains. For generalist strains such as *E. coli* and *C. oxalaticus*, more nutrients represent more activity and a higher growth rate. Other strains do not benefit from additional nutrients and no increase in the growth rate is observed. This is the case for *C. necator* and *M. extorquens*, which are specialized in C1 and C2 compounds. In nature, they play key roles in the global cycling of methane, methanol, halogenated methanes, methylated sulfur compounds, and organic acids in litter (Guo & Lidstrom, 2008; Toyota *et al.*, 2008).

### Comparison of different metal-oxalate as carbon sources

*Cupriavidus oxalaticus*, *C. necator*, *S. violaceoruber*, and *Streptomyces* sp. BV1M3 were selected to test the effect of different metal-oxalates as carbon sources on growth and metabolic activity. In addition to potassium, calcium, copper, and iron-oxalate were assayed. Figure 4 shows the results obtained for *C. oxalaticus* in the different oxalate sources. Heat flow in potassium and calcium oxalate showed a clear peak corresponding to the activity in these substrates.

This was also the case for *S. violaceoruber* and *Streptomyces* sp. BV1M3 in the same substrates (data not shown). For copper and iron-oxalate, the heat flow was undistinguishable from the baseline (Fig. 4), and this was the case for all four strains (data not shown). These results indicate that these strains were unable to use pure copper and iron-oxalate as substrates for growth.

The biological activity derived from the heat flow for calcium oxalate (Table 3) produces results very similar to those obtained in potassium-oxalate. Once again, the highest activity was observed in *C. oxalaticus*. Interestingly, for *C. necator* and *Streptomyces* sp. BV1M3, the consumption rates on calcium oxalate were about 20% higher than those observed in potassium oxalate.

Although the cultures were performed in liquid media, the consumption rates for calcium-oxalate determined by HPLC were very similar to those obtained by IMC. For example, the consumption rate for *C. oxalaticus* determined using the HPLC measure was  $5.5 \mu\text{M h}^{-1} \text{mL}^{-1}$ , and with microcalorimetry, the consumption rate was  $3.3 \mu\text{M h}^{-1} \text{mL}^{-1}$ . These results allow the elucidation of the sensibility and power resolution of the IMC technique to measure oxalotrophy.

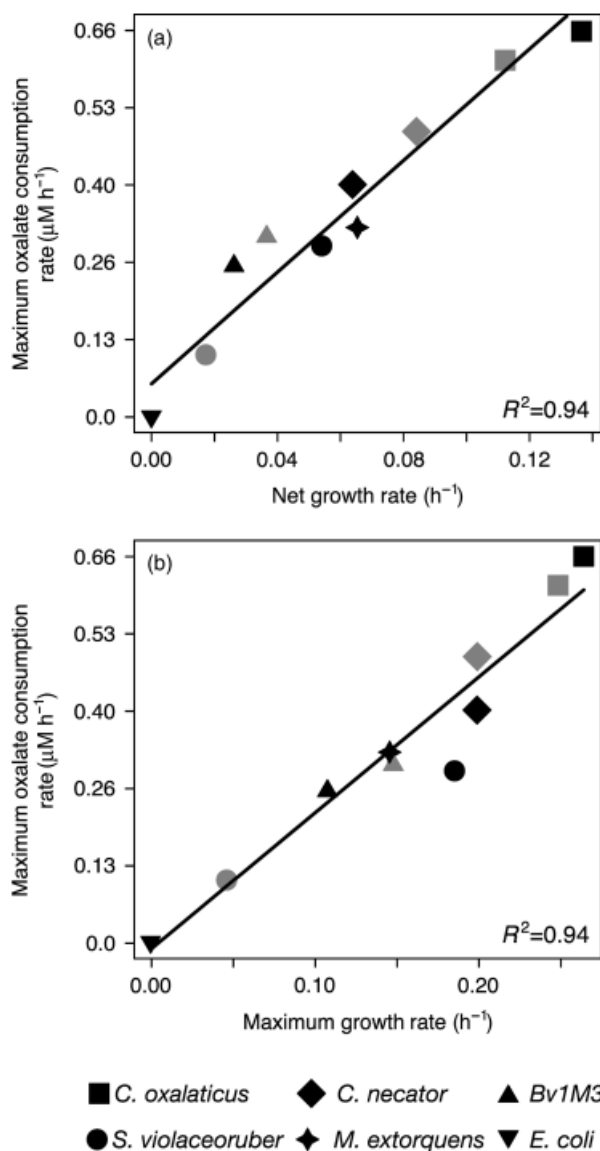
### Oxalotrophic activity measured in soil

The possibility of using IMC to measure the potential oxalotrophic activity directly in soil samples was assayed as well. Although four treatments were performed in soil (see Materials and methods), the results were grouped only according to the addition or not of potassium oxalate. The amendment with *C. oxalaticus* did not have an effect on the activity. In contrast, the addition of potassium oxalate had an effect on the heat produced in soil (Fig. 5a), compared

**Table 2.** Kinetic parameters obtained after the analysis of heat flow thermograms obtained in Angle medium with potassium oxalate (A) and nutrient agar (B)

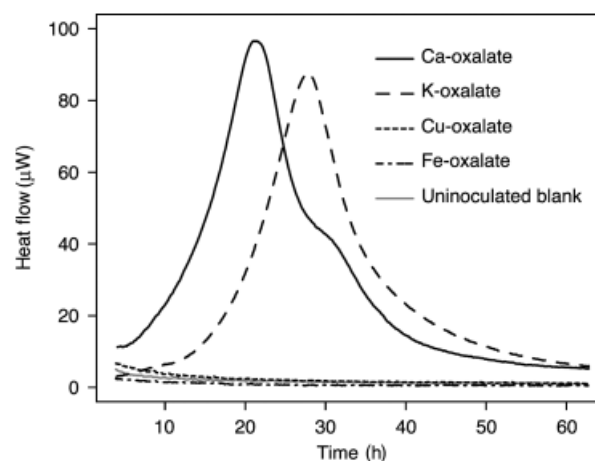
Name	$\mu \text{ h}^{-1}$	$\mu_{\text{max}} \text{ h}^{-1}$	Maximum heat flow (mW)	Consumption rate ( $\mu\text{mol h}^{-1}$ )	<i>n</i>
(A)					
<i>Cupriavidus oxalaticus</i>	$0.14 \pm 0.02$	$0.26 \pm 0.04$	$0.09 \pm 0.01$	$0.66 \pm 0.10$	6
<i>Methylobacterium extorquens</i>	$0.07 \pm 0.02$	$0.15 \pm 0.02$	$0.04 \pm 0.01$	$0.32 \pm 0.06$	5
<i>Streptomyces violaceoruber</i>	$0.05 \pm 0.02$	$0.19 \pm 0.09$	$0.04 \pm 0.03$	$0.29 \pm 0.20$	5
<i>Cupriavidus necator</i>	$0.06 \pm 0.02$	$0.20 \pm 0.03$	$0.05 \pm 0.01$	$0.40 \pm 0.07$	6
<i>Streptomyces</i> sp. BV1M3	$0.03 \pm 0.02$	$0.11 \pm 0.05$	$0.04 \pm 0.02$	$0.26 \pm 0.13$	3
<i>Escherichia coli</i> K12	$0.00 \pm 0.00$	$0.00 \pm 0.00$	$0.00 \pm 0.00$	$0.00 \pm 0.00$	3
(B)					
<i>Cupriavidus oxalaticus</i>	$0.23 \pm 0.05$	$0.92 \pm 0.20$	$0.24 \pm 0.04$	NA	3
<i>Methylobacterium extorquens</i>	$0.03 \pm 0.00$	$0.22 \pm 0.04$	$0.06 \pm 0.01$	NA	3
<i>Streptomyces violaceoruber</i>	$0.07 \pm 0.04$	$0.52 \pm 0.17$	$0.13 \pm 0.04$	NA	3
<i>Cupriavidus necator</i>	$0.03 \pm 0.00$	$0.16 \pm 0.03$	$0.05 \pm 0.01$	NA	3
<i>Streptomyces</i> sp. BV1M3	ND	ND	ND	NA	NA
<i>Escherichia coli</i> K12	$0.16 \pm 0.08$	$1.01 \pm 0.10$	$0.21 \pm 0.04$	NA	3

To convert the maximum heat flow into the maximum consumption rate, the following enthalpy of reaction value was used:  $\Delta H^{\circ}$  of  $-499 \text{ kJ mol}^{-1}$ . *n*, number of replicates; ND, not determined; NA =, not applicable.



**Fig. 3.** Data showing the correlation between the net growth rate ( $\mu$ ) (a), maximum growth rate ( $\mu_{\text{max}}$ ) (b), and the maximum oxalate consumption rate for the six strains tested. The black symbols are for data obtained using potassium oxalate and the gray symbols are for data obtained using calcium oxalate. The correlation coefficient is indicated at the bottom of each panel.

with the unamended soil. The difference in the total heat released in the presence and absence of oxalate was equivalent to the heat produced by a culture with *C. oxalaticus* growing in the presence of the same amount of oxalate. This suggests that the community in the soil indeed used the oxalate added to the soil. The heat flow resulting from comparing the data obtained for the difference in activity between amended and unamended soil (Fig. 5b) showed two different activity peaks: the first one at about 50 h and the second one closer to 200 h of incubation.



**Fig. 4.** Thermogram curves for *Cupriavidus oxalaticus* in four metal-oxalate substrates. Data from Cu- and Fe-oxalate are indistinguishable from the baseline. The IMC method was able to distinguish differences in metal-oxalate source consumption.

## Discussion

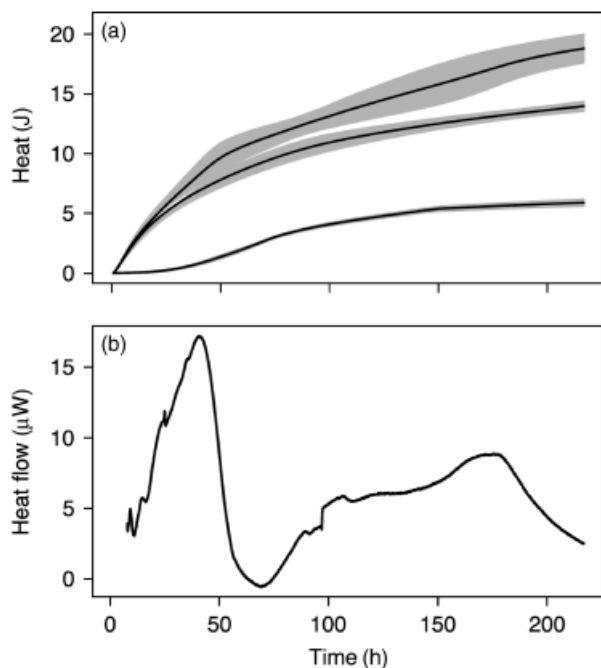
IMC was a useful method to identify oxalotrophic activity in pure cultures. Characteristic curves of heat flow were related to the oxalotrophic activity in the five oxalate-consuming strains. Differences were already clearly visible in the raw heat flow data (maximum heat flow for example), which could be compared between the strains. In addition, other biological parameters were obtained from these data. The biological activity measured as heat release could be analyzed to obtain the growth parameters in potassium and calcium oxalate substrates (Fig. 2 and 4). In addition, the maximum heat flow can be related to the maximum degradation rates of oxalate. The maximum degradation rates can be compared for the different strains and oxalate salts. This is certainly an important feature when new strains are being screened for their metabolic capabilities, because it offers a quantitative means of comparison among different substrates. In addition, IMC offers the opportunity to work with a solid-state medium and relatively insoluble substrates, which are extremely difficult to approach with other conventional microbiological techniques.

The biological activity measured by IMC can also contribute towards understanding the metabolism of an organism. For example, *E. coli* is known to contain a copy of a gene homolog to *frc*, coding for the formyl-CoA transferase (Gruez *et al.*, 2004), which mediates the metabolism of oxalate in *C. oxalaticus* and the anaerobic bacteria *Oxalobacter formigenes* (Sidhu *et al.*, 1997). In addition to this, experiments carried out with the purified protein homolog to *frc* show that it can actually catalyze the synthesis of oxalyl-CoA from formyl-CoA and oxalate, as inferred based only on the genome annotation (Toyota *et al.*,

**Table 3.** Kinetic parameters obtained after the analysis of heat flow thermograms obtained in Angle medium with calcium oxalate

Name	$\mu \text{ h}^{-1}$	$\mu_{\text{max}} \text{ h}^{-1}$	Maximum heat flow (mW)	Consumption rate ( $\mu\text{mol h}^{-1}$ )	N
<i>Cupriavidus oxalaticus</i>	$0.11 \pm 0.02$	$0.25 \pm 0.03$	$0.08 \pm 0.01$	$0.61 \pm 0.09$	3
<i>Streptomyces violaceoruber</i>	$0.02 \pm 0.00$	$0.05 \pm 0.01$	$0.01 \pm 0.00$	$0.11 \pm 0.02$	3
<i>Cupriavidus necator</i>	$0.08 \pm 0.00$	$0.20 \pm 0.03$	$0.07 \pm 0.01$	$0.49 \pm 0.07$	3
<i>Streptomyces</i> sp. BV1M3	$0.04 \pm 0.00$	$0.15 \pm 0.02$	$0.04 \pm 0.01$	$0.31 \pm 0.04$	3

To convert the maximum heat flow into the maximum consumption rate, the following enthalpy of reaction value was used:  $\Delta H^{\circ}$  of  $-499 \text{ kJ mol}^{-1}$ .



**Fig. 5.** Heat production over time in samples from soil incubated in the presence (upper line) and absence (middle line) of potassium oxalate (a). The heat produced by *Cupriavidus oxalaticus* is presented as a comparison (bottom line). Gray areas correspond to the SD. The differential of the difference between the curves with and without oxalate is presented in (b). (b) Two peaks of activity were observed in the presence of oxalate, most likely corresponding to oxalotrophic activity.

2008). With the identification of a formyl-CoA : oxalate CoA transferase, a question that arises is the extent and importance of oxalate-related metabolism in *E. coli*. Although *E. coli* has been implicated in the biomineralization processes leading to the formation of calcium oxalate crystals (Chen *et al.*, 2007), growth measurements in anaerobic media containing 5 mM of sodium oxalate suggest that *E. coli* does not degrade this compound (Turroni *et al.*, 2007). The IMC experiments performed for degradation and consumption rates of oxalate in the presence of *E. coli* produced consistent results. For this strain, no oxalotrophic activity as a function of heat flow signal was found. After an equilibration period, no heat production was observed during the total incubation time, indicating that *E. coli* is unable to aerobically use oxalate as an energy or a carbon source, at least under the concentra-

tions used (approximately 20 mM). In *E. coli*, the absence of growth was confirmed in the vials after the experiment. The results obtained in the presence of potassium oxalate were clearly due to the carbon source selected, because considerable growth was observed for the experiments carried out in NB ( $\mu_{\text{max}} = 1.01 \pm 0.09 \text{ h}^{-1}$  and maximum heat flow =  $0.20 \pm 0.04 \text{ mW}$ ). These results, the absence of growth in poor medium and active growth in a complex medium, appear to confirm previous observations indicating that *E. coli* needs enriched conditions to grow and develop (Boe & Lovrien, 1990).

The effect of different oxalate sources on metabolic activity could also be assessed by IMC. The results showed that even though copper and iron oxalates are widespread in natural soils (Cromack *et al.*, 1977), they are poor substrates for bacterial growth, at least for the strains tested. This is in agreement with previous statements indicating that different metals might be toxic for oxalotrophic bacteria (Sahin *et al.*, 2002). In our study, these metals were released directly from the oxalate salts and the maximum concentration of  $[\text{Cu}^{2+}]$  and  $[\text{Fe}^{2+}]$  ions in the media could be estimated at  $1.73 \times 10^{-4}$  and  $4.4 \times 10^{-4} \text{ mol L}^{-1}$  (solubility values), respectively. On the other hand, potassium and calcium oxalate were consumed at similar rates for most of the strains tested. The only exception was *S. violaceoruber*, which showed a twofold decrease.

In the experiments with soil, the most influential factor affecting the evolution of heat was the addition of potassium oxalate (Fig. 5a). Interestingly, the difference between the curves with and without potassium oxalate was almost the same as the difference between the baseline and *C. oxalaticus* heat over time curve. Because the heat difference caused by the addition of oxalate (independent of the amendment with bacteria) is equal to the heat released by *C. oxalaticus*, this suggests that soil activity is independent of the addition of bacteria. This result reflects a clear response to oxalate that most likely is a function of the potential oxalotrophic activity in soil. The low impact of amendments with *C. oxalaticus* is probably likely to occur because of the lesser importance of populations from this strain relative to the global metabolic activity from autochthonous soil populations. It is important to emphasize that the composition and nature of the oxalotrophic communities in soil was not the

focus of this study, but rather a ‘proof of concept’ for the utilization of IMC directly on soil samples.

In the differential heat flow between soil with or without oxalate, two peaks of activity (also independent of the addition of bacteria) were observed (Fig. 5b). The first peak corresponds most likely to direct oxalate consumption, as potassium oxalate will be a major carbon source added to the soil. The second peak could represent either the use of oxalate by bacteria with slower growth rates (e.g. *Streptomyces*-like) or the consumption of a less accessible pool of oxalate. In fact, in unsaturated porous media such as soils, food, or leaves, the distribution of environmental factors is very patchy and limits both bacterial dispersion and access to the substrate (Zhou *et al.*, 2002, 2004; Wang & Or, 2010). Alternatively, the consumption of a product derived from the metabolism of oxalate could also explain the appearance of a second activity peak. Several intermediate metabolic products have been identified from the metabolism of oxalate, such as CO<sub>2</sub>, formate, or malate (Quayle *et al.*, 1961). However, both the variability of microbial communities’ access to oxalate at different rates and the impact of metabolic end-products need to be assessed in future experiments.

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## Authors’ contribution

D.B. and O.B. contributed equally to this work.

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