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Measurements of Fungal Activity as a Function of Relative Humidity by Isothermal Microcalorimetry

Yujing Li*[a], Lars Wadsö*, Jonny Bjurman**

*Building materials, Lund University, Sweden
**Department of Conservation, Göteborg University, Sweden

[a] Corresponding author (yujing.li@byggtek.lth.se)

Abstract

The heat produced by the metabolism of a mould fungus (*Penicillium brevicompactum*) growing on softwood was measured by isothermal calorimetry under different relative humidity (RH) levels during desorption (from 95% to 65% RH) and absorption (from 65% to 95% RH). The humidification method was successful and it was possible to decrease and increase the relative humidity and moisture content in small steps. The calorimetric measurements were difficult to interpret as the thermal power was not constant at most levels. However, a general trend with lowered activity at lower relative humidity was seen. Calorimetry can possibly be used in mapping dynamic mould activity under changing environmental conditions and can therefore be applied in predicting the risk of mould in buildings.

Key words

Isothermal calorimetry, fungal activity, mould growth, softwood, relative humidity, *Penicillium brevicompactum*

1   Introduction

There is a need for building design tools which can take into account both microclimate and mould growth behaviour. Mould in buildings is of interest in relation to indoor air quality as there is evidence that human health can be influenced by indoor microbial activity (Gravesen et al. 1994). To prevent mould growth in building components it is essential to understand the correlation between physical parameters and mould growth.

Mould growth is influenced by many factors. The three most important in a building setting are probably nutrient availability (substrate), moisture state and temperature. However, in many constructions and most indoor environments, low levels of nutrients are always available in materials like wood and paper, or in organic dust, and the temperature is normally kept at around 20°C all year round. Therefore nutrients and temperature demands for growth of mould fungi are usually met in normal indoor environments. However, the relative humidity (RH) in constructions and in indoor environments can be very variable. Even if it is low in the air in a room, it can be high or even close to the dew point in/at certain building components, such as crawl spaces, cold parts of indoor surfaces, bathrooms, basement and attics. Therefore, RH is the most critical factor that one should look at when considering mould growth in constructions and indoor environments. In the following we will use the terms relative humidity and water activity as synonyms at equilibrium conditions, i.e. the water activity of a material or a mould equals the relative humidity of the surrounding air at equilibrium.

There has been a substantial amount of work quantifying the effect of water activity on mould growth, but most of this has been done on agar. One of the first such studies was made by Ayerst, who measured growth rates as a function of temperature and RH for a number of mould fungi growing on agar (1969). Some more recent example are (Dantigny et al. 2002; Pardo et al. 2004; Beyer et al. 2005). There is much less work done on "real" substrates like wood or foodstuffs, probably because it is quite difficult to quantify mould growth on, e.g. a cheese or a piece of wood. The studies that have been made on real materials have mainly used discrete subjective scales to grade the amount of mould growth (for example, from 0, no growth, to 5, covered with mould). Viitanen and Hukka made several such studies of mould growth on wood samples exposed to different temperatures and RHs (1994; 1999). Two similar, more recent studies, are Nielsen (2004) and Johansson (2006). Nevander and Elmarsson made an interesting compilation of experimental data into a diagram showing the probability of mould growth on wood as a function of temperature and RH (1991). However, in general, the precise quantitative growth pattern of mould fungi as a function of RH and temperature on different building materials is not well known.

Many methods can be used to assess the activity of fungi, and many different measures – biomass, hyphal elongation, radial growth, etc. – are used. We here use the measurement of thermal power produced by the fungal metabolism as a measure of fungal activity, and propose that this laboratory method can be used to assess mould growth as a function of

RH on building materials like wood. The thermal power (heat production rate) is measured by an isothermal microcalorimeter. Such results can possibly be used for modelling mould growth.

All biological processes are accompanied by heat production from their metabolic activities. This heat is continuously released to the surroundings. Most organisms rely on aerobic metabolism (respiration), which is essentially the combustion of organic nutrients and production of water, carbon dioxide and heat. The heat of combustion of most organic substances is proportional to the number of atoms of oxygen consumed (Thornton 1917; Battley 1999). From this follows that for organisms at steady-state, the metabolic enthalpy $\Delta_{met}H$ in units of joules per mol oxygen is also a constant. As an example, for glucose (a carbohydrate unit) we get:

$$\text{glucose} + 6O_2 \rightarrow 6H_2O + 6CO_2 + Q \qquad \Delta_{met}H = -469 \text{ kJ/mol } O_2$$
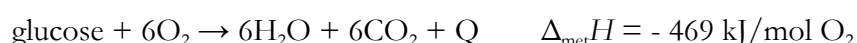
For every mol of O_2 consumed, about 469 kJ heat is produced. Hansen at al. (2004) give a practical value of -455 ± 15 kJ/mol(O_2). The heat per mol carbon dioxide produced is not a constant as the respiratory quotient – the ratio of oxygen consumption to carbon dioxide production – is different for different substrates.

Isothermal calorimetry has been used in biological studies in many different fields. Some examples of organisms studied are germinating seeds (Prat 1952), bacteria (Belaich 1980), yeast cells (Gustafsson and Larsson 1990; Gustafsson 1991), plant tissue (Hansen et al. 1997; Hansen et al. 1998), thermogenic flowers (Lamprecht et al. 1998), vegetable tissue (Wadsö et al. 2004), human cells (Beckman 1992), mammalian cells (Kemp 1991), insects (Schmolz et al. 2002), and fish (Van Ginneken et al. 1995). Only a few calorimetric measurements on mould fungi have been made (Wadsö and Bjurman 1996; Wadsö 1997; Wadsö et al. 2004; Li et al. 2007).

We prefer not to say that we are measuring fungal *growth*, but call it a way of measuring fungal *activity* (which may be a way to predict fungal growth). We do not say that it is the best way, as all methods have their advantages and disadvantages. Some advantages with using heat as a measure of mould growth are:

- Heat can be continuously and non-destructively measured on all types of samples.

- Heat can be measured on all types of samples, for example liquid and solid samples, or whether it is growing inside or on the surface of the sample.

There is, however, one important complication with heat measurements:

- Heat can also be produced by other processes, e.g. vaporization. Experiments must thus be designed so that only the biological heat production is measured.

Calorimetry is different from other measurement techniques in that heat is produced both by growth and by maintenance processes. It is probable that mould under stressed

conditions will produce heat mainly by maintenance, while it under optimal conditions will produce heat mainly from growth.

The aim of this study is to measure the fungal activity of a mould fungus growing on a typical building material, wood, at different relative humidities at 20°C in order to map the fungal activity as a function of relative humidity. A humidity generator was used in which the water activity of a sample can be modified in 2-4 hours. It is therefore possible to measure the activity of a mould sample at many different water activities (and temperatures) in a relatively short time. One of us has published two pre-studies with examples of such measurements (Markova and Wadsö 1998; Bjurman and Wadsö 2000).

2   Material and method

2.1   Biological material

In this study, *Penicillium brevicompactum* (CBS 119375) was used. This is a common indoor mould often found on wood materials (Bjurman 1994; Nielsen 2001). Spores of *P. brevicompactum* were collected from one week-old colonies growing on 2% malt exact agar (MEA) and dissolved in sterile deionized water. The spore suspension was evenly spread on the agar surface (2% MEA) in 80 ml glass Petri dishes, and then incubated at room temperature (about 20°C) in high humidity (about 100% RH) for one week.

2.2   Test specimen

Pine (*Pinus sylvestris*) sapwood was used. Samples were taken from a board with a yellowish surface, indicating that it was rich in nutrients (Theander et al. 1993). Thin wood samples (1 mm x 5 mm x 10 mm, dry mass about 50 mg) were cut so that one of their largest surfaces contained the drying surface. Before inoculation they were sterilized by 20 kGy γ-radiation.

After sterilization, the pinewood had a moisture content of about 0.1 (water mass/dry wood mass). Sterile deionized water was added to specimen before inoculation and the water content of the specimens was adjusted to be 0.3, which is equivalent to a water activity close to 1.0 for wood.

2.3   Inoculation and incubation

Two wood sticks were place on the top of the mould growth surface in the Petri dishes. Test specimens were placed on top of the wood sticks in order not to have direct contact with the mould surface or the agar (Fig. 1).

Petri dishes with test specimens were placed in a plastic box with a relative humidity controlled at close to 100% at room temperature (about 20°C) for two weeks before the measurement started. Three to four pieces of test specimens were placed in each 4ml glass calorimetric vial for measurements.
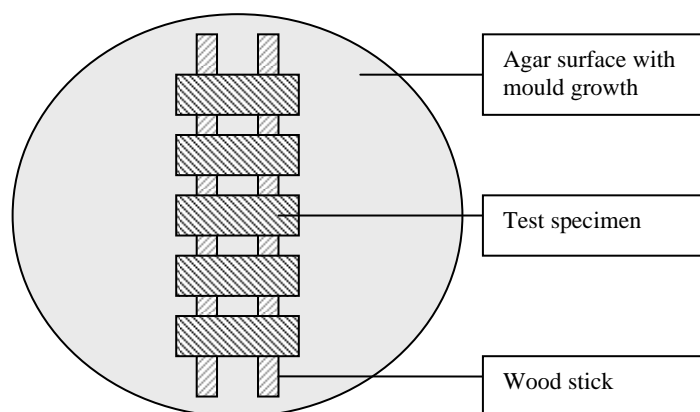
Figure 1. Schematic overview of the test specimen incubation.

2.4 Humidity modifier

A device to externally modify the RH of small microcalorimetric samples was developed based on the design by Wadsö (1997). The principle of the device is to create the desired RH by mixing different proportions of humidified air from two sources with different relative humidities (95% and 55%, respectively). The air flows through the calorimetric vials containing small samples at relatively high flow rate (about 2000 ml/h) so that the sample's water activity will be changed within a relatively short time. It should be noted that it is not possible to use such high flow rates while the sample is down in the calorimeter as this would disturb the thermal balance of the calorimeter.
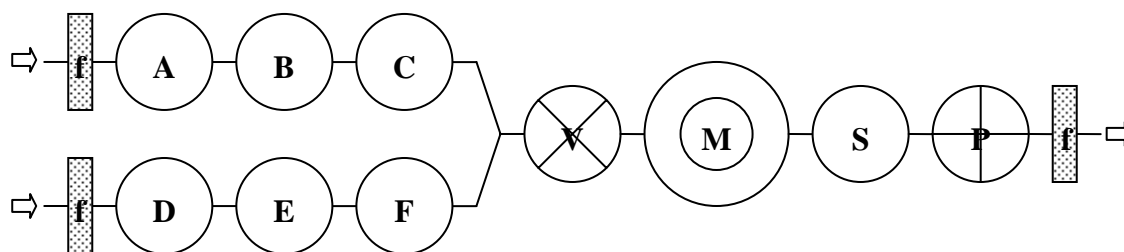

Figure 2. Overview of the humidity generator. A: Water-glycerol solution with 60-65% RH; B-C: Saturated Mg(NO₃)₂ solutions with 55% RH; D: Water; E-F: Saturated KNO₃ solutions with 95% RH; V: Valve; M: Humidity buffer volume; S: Microcalorimetric vial with sample; P: Pump; f: Millipore filters.

Figure 2 shows an overview of the humidity generator. A-C contain solutions that regulate the RH of the gas stream to 55% at 20°C. Glass tubes D-F contain solutions that regulate the air humidity to 95% RH at 20°C. Millipore filters prevent contamination of the solutions and the sample. A timer controls the three-way valve that governs the mean RH of the air stream by letting different proportions of air with the two relative humidities be mixed in a 1 meter long copper tube at M before entering the sample vial. All relative humidities from 55% to 95% can thus be generated. The humidified air continues to the sample (S) that is placed in the 4 ml calorimetric glass vial (Thermometic AB, Järfälla, Sweden) that later is taken to the TAM isothermal microcalorimeter for thermal power measurement. The air leaves the humidity generator through a Millipore filter to prevent spores or mould fragments from entering the indoor environment. A

peristaltic pump (P) draws air through the system. We have used four humidity generators placed in a temperature of 20°C during the experiment.

Note that because of different flow resistances in the 55% and 95% solutions, it may be difficult to predict the exact resulting RH. Therefore RH was measured after each calorimetric measurement. The humidification was also not run to completion, i.e. equilibrium between gas stream and sample, but was only run for a certain time to decrease or increase the water activity of the sample.

In the first half of the measurement, the substrates experienced desorption: water activities decreased from high (about 1.0) to low (about 0.65). During the second half of a measurement the sample absorbed water vapour as water activities increased from low (about 0.65) to high (about 0.95).

2.5 Isothermal calorimeter

The heat produced from fungal metabolism by the samples was measured by four isothermal microcalorimeters of the TAM-type in a Thermometric TAM thermostat (Thermometric AB, Järfälla, Sweden). The glass vials were sealed when in the microcalorimeter to prevent evaporation from the sample. The calorimeters were electrically calibrated and baselines were determined with 1 g sand in 4 ml glass vials, the same type of vials was also used as references. All data were Tian corrected for the thermal inertia of the instrument.

2.6 Relative humidity measurement

The relative humidities of the samples were measured by RH sensors (Vaisala, Finland) that were continuously logged. The RH sensors were calibrated in a calibrated Thunder 2500 two-pressure RH generator (Thunder Scientific Corportation, Albuquerque NM, USA) between 30 and 95% RH at 20°C.

2.7 Water content measurement

The water content of the specimens was measured by weighing the samples with a Sartorius AC211S balance after each measurement and comparing to their dry mass. When the experiment was finished, the specimens were dried at 105°C for 12 hours and the dry biomasses were determined.

2.8 Measurements

The measurements were carried out by the following steps:
  I. After incubation 3-4 wood pieces were placed into each 4 ml glass vial as one measurement sample. Four samples could be measure at the same time.
  II. The glass vials were sealed with silicon stoppers and placed half-way into the TAM for 15 minutes for temperature equilibration.

III. The samples were fully inserted into the calorimeters and the measurements started. A measurement normally would not take more than 4 h in order to avoid significant change in oxygen or carbon dioxide levels in the vials. Some measurements were continued for up to 12 h and the oxygen level never dropped by more than about 15%

IV. The silicon stoppers were removed after the calorimetric measurement and immediately exchanged to stoppers with RH sensors. The vials with the sensors were placed in the calorimeter channels to keep the temperature at the same level as during the measurement. The RH measurement lasted for about 30 minutes.

V. The mass of the samples were measured after the RH measurement.

VI. The vials were then connected to the humidity generators which were programmed for the next desired humidity level.

VII. Step II – VI were repeated for each humidity level during the desorption and absorption processes.

Three samples with inoculated specimens and one vial with sterile wood only (blank) were humidity modified and measured simultaneously. In total 9 samples were measured.

3   Results and discussion

In these measurements the wood samples were exposed to step-wise changing moisture conditions. At each step, both relative humidity and moisture content were quantified, and it is thus possible to construct a sorption isotherm for the wood material. This is shown in Fig. 3 for one of the samples from the second set of measurements. It is seen that the results follow a typical wood sorption isotherm, but that the moisture content was far up in the super-hygroscopic region when the measurement started. Essentially all water above about 30% (the fiber saturation point, (Skaar 1988)) is free, liquid water at close to a water activity of 1.0. The wood specimens must have absorbed liquid water during the incubation.

It is clear that the used RH-sensors could not register differences between very high RHs as all the super-hygroscopic values are about 97%. It is possible that the 30 min equilibration time for the RH sensors was not enough at very high RH and that they would have reached higher values if the RH measurement would have continued for a longer time.

The results in Fig. 3 shows that the method used to change the water state of the samples worked well. The measurements should, however, preferably be started with less excess of free water as it took a long time to remove this water. Note that in each drying in the super-hygroscopic range there is only very little response in the RH-sensors. Addition of water vapour is easier as there is no risk of absorbing any super-hygroscopic water in the humidification device used, as it cannot generate RHs higher than 95%.
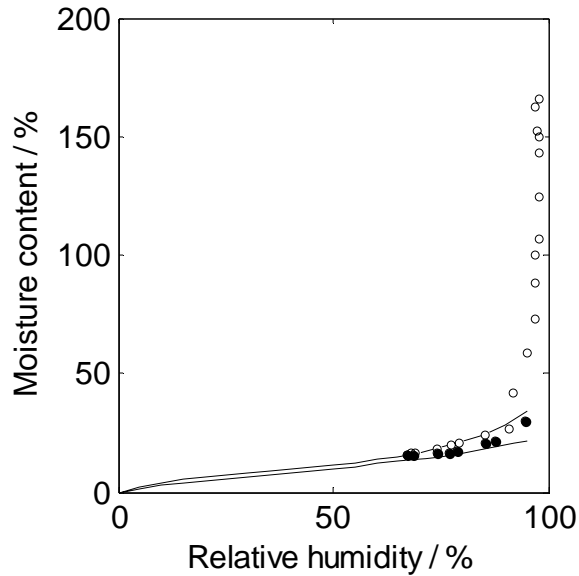
Figure 3. Sorption isotherms calculated from the present measurements (circles, white are for desorption, black are for absorption). The solid line shows results from measurements on spruce wood (Frühwald et al. 2007).

All thermal power measurements made showed qualitatively similar results. First high thermal powers at high moisture levels; a decreased thermal power as the moisture content was decreased; a significant transient increase at about 30% moisture content; low levels at low water activities; and finally an increase in thermal power at the end of the measurements when the water activity again was increased. The highest (at least transient) fungal thermal activity was at a water activity level close to 0.92 while the fungal activity was lower at higher water activity. This agrees with Grant and co-workers' finding that *P. brevicompactum* has its highest growth rate close to 0.88 but lower at higher levels when growing on malt extract agar (1989).

However, the results of many measurements were difficult to interpret as the measured thermal powers were not constant and some were even negative. Figure 4 shows three examples of results from the measurements. It is seen that the signals are not constant and that it is difficult to define a level of thermal power. Note that all results have been Tian-corrected to remove the influence of the thermal time lag of the calorimeter, so the results shown are the thermal powers produced in the sample. We have also seen negative thermal powers when measuring on humid wood and this could possibly be caused by rearrangement of absorbed water within the wood.
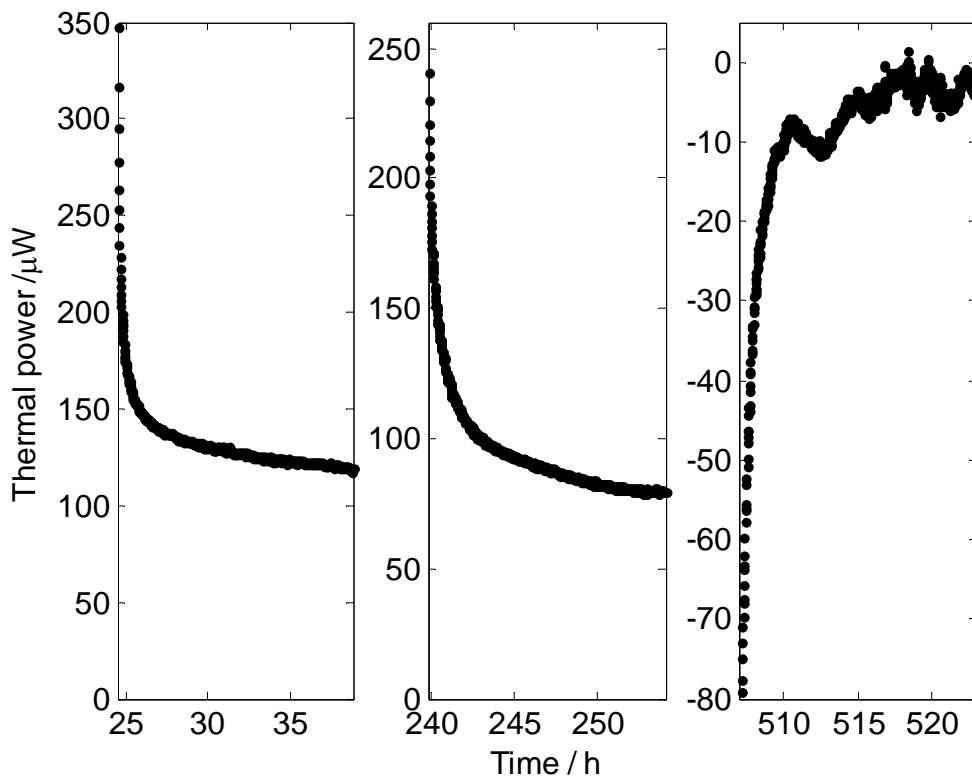
Figure 4. Three examples of results from growth of *P. brevicompactum* on pine wood. These examples correspond to measurements 1, 12 and 23 in Fig. 5.

An evaluation of the thermal powers was made by taking the mean value of the thermal power between 100 and 150 minutes after the start of each measurement (this is quite early in curves shown in Fig. 4). This was plotted together with relative humidity and moisture content in Figure 5. As the thermal powers in most cases were changing essentially exponentially towards some final value, we have also plotted these final thermal power values at the levels where the measurements were long enough so that curve fits could be made.

Because of sorption hysteresis – a phenomenon seen for most materials - the water content is higher during desorption than during absorption (at the same water activity). This might explain, at least partly, why the mould activities are lower during the absorption than during the desorption process, at the same RH level. This implies that biological activity is not only a function of water activity, but also of moisture content. A similar conclusion was reached by Labuza and co-workers for lipid oxidation in food-stuffs (Labuza et al. 1972). This is contrary to the present concept that water activity is the proper water quantity to use when investigating the relation between water state and reaction/growth rate (Scott 1957). It is probable that when looking at different samples (with different sorption properties) the water activity is the best parameter to use, but when looking at only one material the moisture content (influenced by hysteresis) is also of some importance.
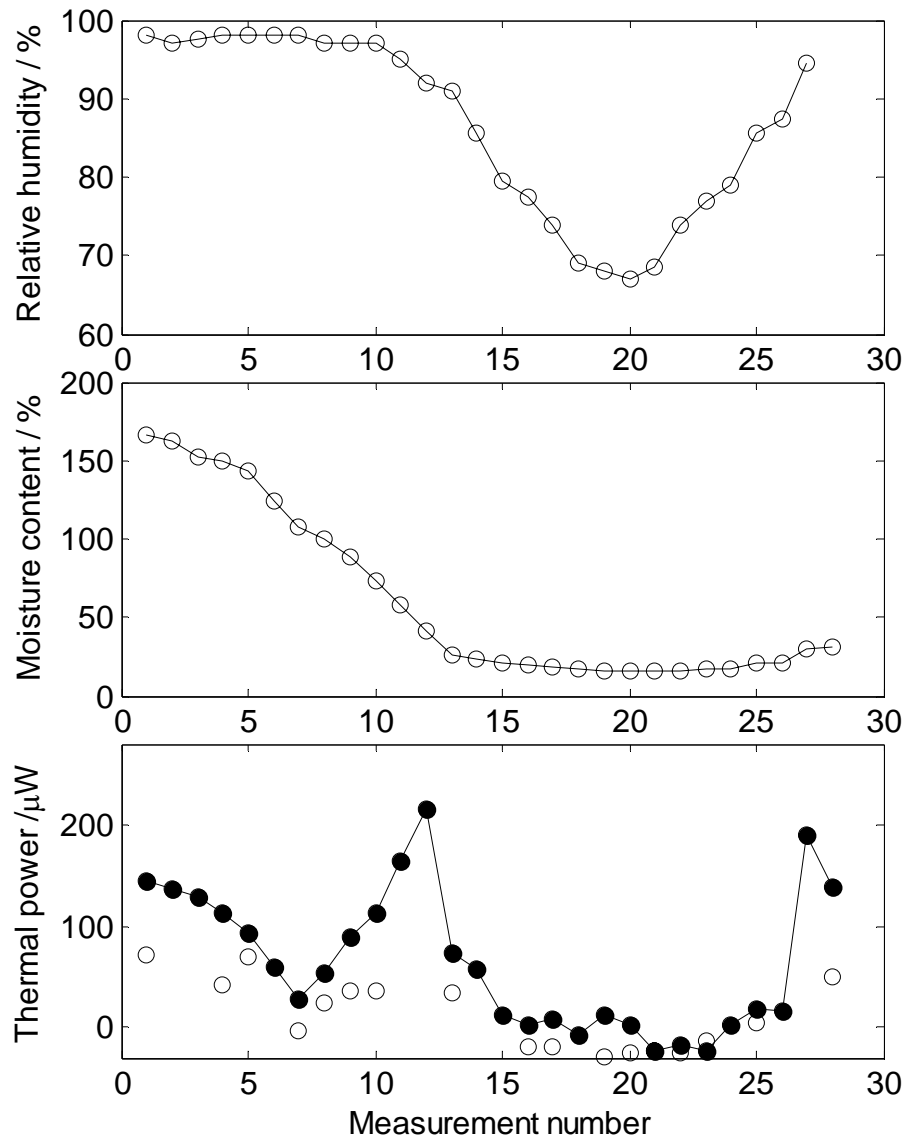
Figure 5. Overview of the results for one sample of the mould *P. brevicompactum* growing on wood during a desorption-absorption cycle. Each point corresponds to one humidity level. In the bottom figure the black markers are mean values between 100 and 150 min after a sample was charged into the calorimeter. The white markers are final values for the humidity levels where the measurement continued for such a long time that it was possible to evaluate a final constant thermal power level.

It should be noted that the above method based on calorimetry may seem to be limited by the fact that it is not possible to state how much mould (biomass) one will actually get. However, mould growth is a function of many complex factors, such as previous degrading light exposure of material surfaces, accumulation of dust/soil on surfaces (and the nutrient content of this), etc. It is enough challenge today to model the *relative* mould growth (activity).

The results of the measurements showed that isothermal calorimetry has a potential for measuring mould fungal activity as a function of RH (and then also of temperature). More measurements should be done before the generated data can be used in

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combination with numerical models of building microclimate for a more comprehensive modelling of the fungal growth in buildings.

It has since many years been standard practice to do calculations of mechanical strength of buildings and building components. At least in temperate climates energy calculations are also made. It is now also possible to calculate detailed temperature and relative humidity distributions in building components. Such data can in principle be used to predict the level of mould activity if one had a model of mould activity as a function of temperature and relative humidity.

What "mould property" should be used to model mould activity? This property should of course be relevant, but it should also be easy to measure so that one can find correlations with temperature and RH for different moulds on different materials in a reasonable time. Mould colony diameter has been a popular measure in for example food science, but it is mainly limited to growth on agar and other similar substrates. Growth on building materials is often diffuse and such materials have rough surfaces. Therefore it is difficult to measure its mycelia length or mass. Biomass quantified through measurements of the biomass proxy ergosterol may be used, but this is a destructive method that needs a large number of samples and long time of incubation, especially for the samples growing at low relative humidity. It would of course be ideal to use a property which is of interest in, e.g. the indoor environment, like mycotoxin production, but this is probably a quite difficult approach.

We do not have any definite answer to why the thermal powers are quite non-constant in many of our measurements. It is probable that if the measurement on each level would have continued for, e.g. 10 h, it would have been easier to evaluate steady-state thermal powers. It is possible that one could also have left the specimens in the temperature of the calorimeter to condition them before each measurement. One possible explanation to the changing thermal powers is that it is the redistribution of moisture within the samples that is responsible. Take the centre plot in Fig. 4 as an example. At this point (point 12 in Fig. 5) the water activity was about 0.95 and the change in moisture content from the previous step was about 7% for the 0.2 g sample. If the 10 h exponential decrease in thermal power seen, was caused by an exothermic process (if this process would not have been present, the thermal power would have reached about 80 µW directly after the start of the measurement). The heat produced by such a process is about 1 J (the area between 80 µW and the curve). It is not clear if this comparatively high heat can come from moisture redistribution within the sample.

The thermal power signal approached the final level from above for the initial and the final measurement steps. In between, the signals approached from below. It thus seems that high humidities give exothermic disturbances, while low humidities give endothermic disturbances.

4 Conclusion

We have worked with a method to change the moisture state of a sample of wood with mould growth, and measured the thermal power as a measure of mould activity. The humidification was successful and it was possible to decrease and increase the relative humidity and moisture content in small steps. The calorimetric measurements were difficult to interpret as the thermal power was not constant at most levels. However, a general trend with lowered activity at lower relative humidity was seen.

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