Thermochimica Acta, 72 (1984) 95–102
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

MICROCALORIMETRIC STUDIES OF THE MODE OF ACTION OF ENVIRONMENTAL CHEMICALS ON CONTINUOUS MICROBIAL CULTURES

Peter Weppen and Dieter Schuller
University of Oldenburg, P.O. Box 25 03, 2900 Oldenburg, F.R.G.

ABSTRACT

Direct calorimetry is a superior technique in the field of bioenergetics. It is used to measure the metabolic heat production rate of chemostat-cultures of the bacterium Acinetobacter calcoaceticus. The heat production of these cultures normally remains constant as a consequence of steady-state conditions. The constancy is disturbed if poisonous substances are added to the culture. This effect disappears some time later and the initial steady state is recovered.

The application of the direct calorimetric technique, to observe and to quantify toxic properties of environmental chemicals on chemostat cultures in relation to the substances $HgCl_2$, 2-Nitrophenol, 4-Nitrophenol, 2.4-Dinitrophenol, Phenol, and Pentachlorophenol is demonstrated. Aspects of the toxicological test procedure outlined here are discussed concerning its ecotoxicological reliability as well as deficites of the concepts of microbial growth kinetics with respect to transient states.

INTRODUCTION

Bioenergetic investigations which should be most important in the field of the assessment of harmful properties of substances in ecotoxicology (ref. 1) are closely related to the applicability of the direct calorimetry in biology because there is scarcely another method to analyse metabolic activities possessing such a general validity as the calorimetry (ref. 2).

Calorimetric investigations of the effects of harmful substances on microbial metabolism have been carried out mainly by using batch cultures and ampoule or flow calorimetry (ref. 3). The energetics of chemostat-cultures under steady-state conditions were studied calorimetrically and confirm to a large extend the theoretical concepts of the Chemostat as outlined by Monod (ref. 6) and modified by Pirt (ref. 7) (ref. 8).

One of the most important problems of toxicity tests using batch-cultures of microbes arises from the restricted reproducibility of the inoculum. Chemostat-cultures being self-balancing systems according to the theory (ref. 6) in contrast can be reconstituted exactly as a consequency of the determination of its steady-state by a few easily reproducible parameters.

The introduction of a batch dose of a particular chemical may cause a response of the heat production rate if it affects the microbial metabolism or growth dynamics. The transient variances induced by a chemical can be analysed qualitatively and quantitatively to describe the behaviour of the substance in the microbial population.

0040-6031/84/$03.00

The practical application and the reliability of the calorimetric technique for testing ecotoxicity of water soluble chemicals are demonstrated.

Calorimetrically observed toxicity data of the substances $HgCl_2$, 2-Nitrophenol, 4-Nitrophenol, 2.4-Dinitrophenol, Phenol, and Pentachlorophenol are discussed in relation to data from simple screening tests. Besides this the calorimetric data are investigated in relation to modern concepts of microbial growth dynamics (ref. 9) (ref. 10).

THE ENERGY-FLOW OF THE CHEMOSTAT

The balance of a chemostat-culture related to its energetics under steady-state conditions can be described by simple equations describing (1) the substrate and the biomass (2) equilibrium and the steady-state heat production rate (3)(ref. 6)(ref. 5):

$$\hat{S} = K_S \frac{D}{\mu_m - D} \tag{1}$$

$$\hat{X} = Y (S_R - \hat{S}) \tag{2}$$

$$\dot{Q} = D \left[(S_R - \hat{S}) \Delta H_{cS} - \hat{X} \Delta H_{cX} - \sum_{i=1}^{n} S_F \Delta H_{cF} \right] \tag{3}$$

Within these equations the symbols mean: $\hat{S}$ substrate concentration in the culture at equilibrium, K_S substrate affinity, D dilution rate being the quotient of the medium feeding rate and the volume of the culture vessel, μ_m the maximum specific growth rate of the culture, $\hat{X}$ biomass-concentration at equilibrium, Y yield-coefficient; ΔH_{cS}, ΔH_{cX} and ΔH_{cF} are the standard values of the heat of combustion of the substrate, the biomass and the metabolic products, respectively.

Concerning ecotoxicological effects of chemicals, it seems to be important that at least variances of the values of μ_m, K_S, Y or the metabolic pathway of the catabolism will cause changes of the heat-production rate $\dot{Q}$.

MATERIAL AND METHODS

Calorimetry

A heat-conduction-twin-calorimeter specially designed (ref. 11) and tested by us (ref. 12) allows to maintain chemostat-cultures of chemoorganotrophic aerobic bacteria within the calorimetric measuring cells. The cultures were aerated and fed with culture medium as outlined in Fig. 1. The measuring range normally used was 0 - 100 mW with a long-term noise level of 1 - 2 mW related to a working volume of 140 ml per measuring cell.

Thermogram data were processed on a Siemens PC 100 Microcomputer to recover

the original heat-production-rate $\dot{Q}$ (t) according to the Tian-equation as described by Marti et. al. (ref. 13).

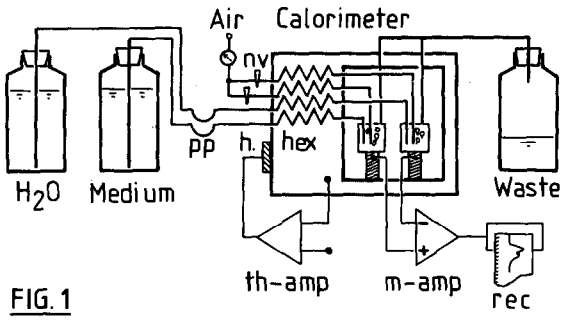

FIG. 1

The experimental design used to investigate chemostat-cultures by direct calorimetry.
The culture inside the measuring cell and the reference cell were fed with medium or water by a peristaltic pump pp. Needle valves served to adjust the airflow from a compressed air supply. Heat exchangers He and a thermostat equipped with a regulating amplifier th-amp and a heating element he protected the measuring unit from thermal disturbances in the working room.
The calorimetric values are captures by transistor-sensors being inserted into the bottom of the measuring cells, amplified by a microvolt-amplifier m-amp and documented by a recorder.

Culture medium and organisms

A synthetic culture medium containing 11.2 g $Na_2HPO_4 \cdot 2\ H_2O$, 4.65 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 400 mg $MgSO_4 \cdot 7\ H_2O$, 40 mg $CaCl_2 \cdot 2\ H_2O$, traces and Fe-EDTA-Citrat-Complex according to Rippka et. al. (ref. 14) and 6 mMol Na-Acetat (all values per litre) has been used in our experiments. Sodiumacetate served as the limiting factor of the chemostat-culture.

The bacterium Acinetobacter calcoaceticus was isolated from activated sludge of a municipal wastewater treatment plant.

Standard values of the growth kinetics of Acinetobacter

The dilution-rate D being equal to the specific growth rate μ of a chemostat-culture was set to a value of 50 % of the critical specific growth rate μ_c. The critical specific growth rate has been defined to be the value of u, if $S = S_R$. Table 1 presents an overview of the kinetic constants of Acinetobacter and the experimental parameters used here.

TABLE 1

Growth kinetics and experimental parameters of the calorimetric toxicity text

Parameter	Symbol	Value		
Limiting Substrate Na-acetate	S_R	6	mMol l^{-1}	
Maximum specific growth rate	μ_m	1.22	h^{-1}	(ref. 12)
Critical specific growth rate	μ_c	1.08	h^{-1}	
Substrate affinity	K_S	0.9	mMol l^{-1}	(ref. 12)
Molar Yield Coefficient	Y	19	g mol^{-1}	(ref. 12)
Dilution rate in the experiments	D	0.54	h^{-1}	

EXPERIMENTAL RESULTS

Reproducibility of the chemostat-culture

Variances between independent experiments on chemostat cultures depend on the reproducibility of the limiting substrate concentration and of the long-term behaviour of the medium supply to the culture. The precision of the heat flowmeasurement is subjected to the stability of the calorimetric unit.

A series of ten independent runs of chemostat cultures resulted in a mean steady state heat production rate $\dot{Q}$ of 465 mW l^{-1} and a biomass-concentration of 86 mg l^{-1}. The heat production varied for 4.5 %. Within a five days run we never found a variance exceeding 2.5 %.

Effects of toxic chemicals on the heat production of the Chemostat-culture

An example of the transient states, which are caused by toxic chemicals, Fig. 2 presents the heat-production versus time curves after the addition of 2-Nitrophenol.

The dependency of the heat flow response on the initial concentration of a toxicant can be described phenomenologically using the relation between the $\dot{Q}$ when the transient state reaches its maximum and the steady state value of $\dot{Q}$ as demonstrated in Fig. 3.

In contrast to the simple inhibiting effects of 2-Nitrophenol several chemicals gave rise to increased values of $\dot{Q}$ value, e.g. Phenol, 4-Nitrophenol, and 2.4-Dinitrophenol, which are correlated to the initial concentration of the chemical. Above a critical concentration the effect changed to an inhibition of the heat production rate.

Additions of $HgCl_2$ and 2-Nitrophenol caused a slow decrease of the relative heat production rate reaching the minimum minutes to hours after the addition. The other substances tested here decreased the heat production in a fast step.

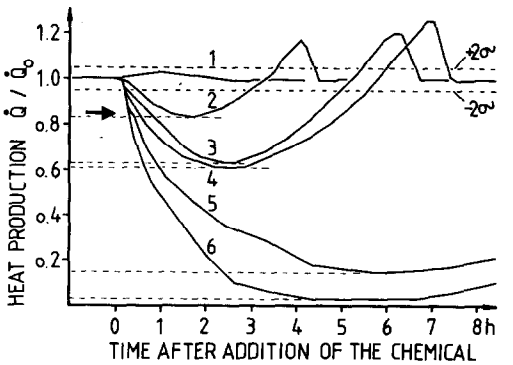

Figure 2: Response of the heat production rate of Acinetobacter calcoaceticus maintained as chemostat-culture after addition of various amounts of 2-Nitrophenol . Dotted lines indicate the significiance-levels and the readings of the relative heat production rate. 1) 3.6 ppm, 2) 5.4 ppm, 3) 7.1 ppm, 4) 11 ppm 5) 51 ppm, 6) 71 ppm initial concentration of the substance.

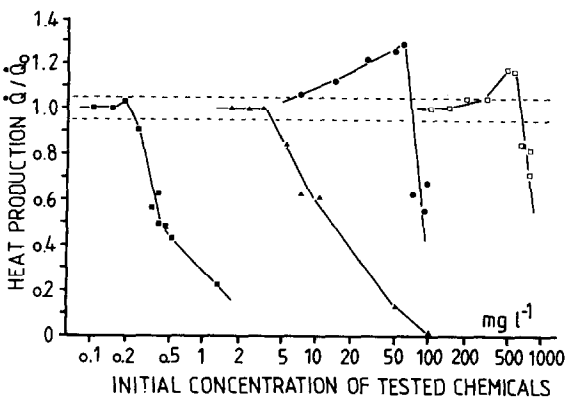

Figure 3: The relative heat-production rate as a function of the concentration of the toxicant. Concentrations are scaled logarithmically.
■ $HgCl_2$, ▲ 2-Nitrophenol, ● 2.4-Dinitrophenol, □ Phenol

Determination of standard values of toxicity

Standard values of toxicity of chemicals in the calorimetric test procedure can be defined similar to standard methods:

(i) MEC = Minimum Effective Concentration raises up the $\dot{Q}/\dot{Q}_0$ ratio by 2 .

(ii) MIC = Minimum Inhibitory Concentration decreases the $\dot{Q}/\dot{Q}_0$ ratio by 2 .

(iii) IC 50 = Inhibiting Concentration causing a 50 % decrease of the heat production rate.

Data concerning the chemicals that have been used in this study were calculated assuming a linear fit of the data presentation in Figure 3 using the least square method. Results of the calorimetric determinations and comparable data from simple screening tests (ref. 12) are listed in Table 2.

TABLE 2

Toxicological standard values from
(I) Calorimetric determinations
(II) Respirometric growth rate inhibition test (ref. 12)
(III) Short term respiration test (ref. 12)

Substance:	MEC (I)	MIC (I)	IC 50 (I)	MIC (II)	IC 50 (II)	MIC (III)	IC 50 (III)
Phenol	240	640	830	340	660	430	1300
2.4-Dinitrophenol	6.5	68	105	19	80	70	360
4-Nitrophenol	25	65	80	ne	ne	80	120
2-Nitrophenol	*	3.5	15	2.5	11	1.7	80
Pentachlorophenol	*	0.17	1.8	0.44	3.3	0.12	4.5
$HgCl_2$	*	0.22	0.45	0.02	0.16	0.02	0.09

All data are given as concentrations in mg l^{-1}
ne = not examined
* = effect does not occure

DISCUSSION

The chemostat-culture for evaluating toxic properties

The chemostat-culture of chemoorganotrophic microorganisms combined with a direct calorimetric monitoring is suitable to produce biological targets of ecotoxicological tests with a high degree of reproducibility and provides a simple data aquisitation. Problems concerned with the time dependent variations of the physiological state of batch cultures were overcome by the principle of the chemostat.

The evaluation of toxic properties using the simple ratio of $\dot{Q}/\dot{Q}_0$ as outlined above results in values of toxicity data comparable to standard methods. The MEC demonstrates shifts of the bioenergetic balance which can not be analysed quantitatively by simple screening tests.

The increase of the substrate conversion to heat must be interpreted as a result of a decreased efficiency of the coupling between catabolic pathways and

ATP-genesis (Y_{ATP}). The shift of the energy conservation rate to lower values, that means the turnover of substrate to heat is increased, seems to be important when ecological effects of a chemical are discussed in relation to food chains and food webs.

In contrast to the screening tests the calorimetry enhances the accuracy of the determination of the physiological activity of the cultures to a variance of 2.5 % compared with 10 % of the screening tests.

According to the data of Table 2 the calorimetric determination of toxicity results in lower as well as higher values of the standard data within the range of variances of toxicity determinations by different laboratory using one standard method except for Hg^{2+}.

The Hg^{2+} toxicity data from the calorimetric tests may be distorted due to electrochemical interactions of the mercury and the metal parts of the calorimetric setup.

Ecological aspects

The chemostat culture was chosen as a simple analogue of the chemoorganotrophic microbiocoenosis of the activated sludge excluding problems concerned with the interactive species diversity of the real activated sludge.

The low values of the substrate concentration (organic carbon in the medium) and the low substrate turnover rate as well as the attainable precision of the calorimetric monitoring in our investigations demonstrates the applicability of the calorimetry to investigate eutrophicated ecosystems as it has been stated by Fortier et. al. (ref. 16) and Tiefenbrunner (ref. 17).

Direct calorimetry on continuous cultures demonstrates a fast response technique to determine toxic properties of chemicals and seems to be qualified for a feed foreward control strategy within biological sewage treatment. So calorimetry could help in saveguarding of our environment by improving the performance and the operational safety of wastewater treatment plants.

Concepts of modellisation

The experimental data clearly show a lack in interpreting the transient states following the addition of a harmful substance to the chemostat-culture. The classical theory of Monod (ref. 6) describes the microbial growth kinetics as a Michaelis-Menten enzyme Kinetic assuming steady-state conditions. It is not designed to modellise transient-states. Modern concepts as outlined by Chase (ref. 9) and especially the"unified theory" of Daigger and Grady (ref. 10) give rise to approaches of a modellisation of transient states and could be modified to depict a concept of toxic effects within the chemostat.

A sufficient modellisation of toxic effects on the chemostat culture must take into consideration several mechanisms of interactions between the toxicant and a

living cell, e.g. uptake kinetics of the noxious substance its action on energy metabolism, on synthesis of biomolecules and their assemblation to functioning parts of the cell.

The theory of Daigger and Grady seems to be the most convenient with this respect because it describes the microbial growth kinetics by interactive functional compartments of the cell.

The results presented here should help to develope a better understanding of microbial growth dynamics and the multitude of toxic effects of chemicals on microbial metabolism.

This work was supported by the Bundesminister für Forschung und Technologie (F.R.G.), Grant. No. 03 7240.

REFERENCES

1) J. Cairns Jr., Water Research, 15 (1981) pp. 941 - 952
2) E. Gnaiger, in E. Marti, H.R. Ostwald, H.G. Wiedemann (Eds.), Angewandte chemische Thermodynamik und Thermoanalytik. Vorträge des Rapperswieler TA-Symposiums, 18. - 20. April 1979 Birkhäuser Verlag, Basel, Boston, Stuttgart, pp. 155 - 165
3) B.Z. Chowdhry, A.E. Beezer & E.U. Greenhow, Talanta 30 (1983) pp. 209 - 243
4) J.M. Cardoso-Duarte, M.J. Marinho & N. van Uden, 1976) in A.C.R. Dean, D.C. Ellwood, C.G.T. Evans & J. Melling (Eds.), Continuous Culture 6: Applications and new fields. Ellis Horwood LTD, Chichester Chapter 3, pp. 40 - 48
5) R. Brettel, I. Lamprecht, B. Schaarschmidt, Thermochimica acta 49 (1981) pp. 53 - 61
6) J. Monod, Ann. Inst. Pasteur. 79 (1942) pp. 390 - 410
7) S.J. Pirt, Proc. Roy. Soc. B, 163 (1965) pp. 224 - 231
8) S.J. Pirt, Arch. Microbiol. 133 (1982) pp. 300 - 302
9) M. Chase, Biotechnol. Bioeng. 19 (1977) pp. 1431 - 1447
10) G.T. Daigger & C.P.L. Grady, Water Res., 16 (1982) pp. 365 - 382
11) D. Schuller, J. Kahle, H. Riemann, P. Weppen & I. Wolke IN: Journées de calorimetrie et d'analyse thermique, Université de Nancy I, 14 - 15 Mai 1981, Vol. XII 18 pp., special paper
12) P. Weppen, Diploma Thesis, University of Oldenburg, 1983, 195 pp.
13) E. Marti, A. Geoffroy, J. Affolter & A. Kabay, in E. Marti, H.R. Ostwald & H.G. Wiedemann (Eds.): Angewandte chemische Thermodynamik und Thermoanalytik Vorträge des Rapperswieler TA-Symposiums, 18. - 20. April 1979 Birkhäuser Verlag, Basel, Boston, Stuttgart, pp. 184 - 199
14) R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman & R.Y. Stanier, J. gen. Microbiol., 111 (1979) pp. 161
15) D. Schuller & P. Weppen, Calorimetrie et analyse thermique Journées de la Gaillarde, Association Française de Calorimetrie et d'Analyse thermique, Vol. 14 (1983) pp. 264 - 266
16) J.L. Fortier, B. Reboul, P. Philip, M.-A. Simard, P. Picker & J. Jolicoeur, Journal WPCF, 52 (1) (1980) pp. 89 - 97
17) F. Tiefenbrunner, in I. Lamprecht & B. Schaarschmidt (Eds.) Applications of calorimetry in life sciences, Walter de Gruyter, Berlin (1977) pp. 305 - 314