CHARACTERISATION OF DIOXIN-LIKE COMPOUNDS IN ANAEROBICALLY DIGESTED ORGANIC MATERIAL BY BIOASSAY-DIRECTED FRACTIONATION

Helena Olsman[1], Helén Björnfoth[1], Bert van Bavel[1], Gunilla Lindström[1], Anna Schnürer[2], Magnus Engwall[1]

[1]Man-Technology-Environment Research Centre, Dep. of Natural Sciences, Örebro University, 701 82 Örebro, Sweden
[2]Department of Microbiology, Swedish University of Agricultural Science, PO Box 7025, 750 07 Uppsala, Sweden

Introduction

Environmental samples contain a broad mixture of substances, including known and unknown substances. Chemical separation of a sample extract, combined with bioassay testing, may be a useful tool to identify biologically active substances[1]. By this means, identification of new environmental contaminants and transformation products is possible. In this study, we apply this approach to samples of anaerobically digested, organic household waste. A previous study has shown that anaerobic digestion of this material lead to an increase in dioxin-like content[2]. It is important to identify the contributing compounds, to understand the mechanisms behind this increase in dioxin-like toxicity.

Material and methods

Digestion

The material used in this study is sub-samples from 12 tons of sorted organic household-waste, collected from 12 000 households in Uppsala, Sweden. The material ground to a maximum particle size of 13 mm. The organic material was digested in two semi-continuous anaerobic digesters, one mesophilic (37ºC) and one thermophilic (55ºC), temperatures commonly used in large scale waste-treatment with anaerobic digesters. Samples were collected from the reactors during four weeks. They were pooled and frozen (-20°C) until extraction.

Extraction and cleanup

Dried samples were extracted with acetone and cyclohexane. Extracts were washed with NaCl (0.2 M) and cyclohexane/diethylether (9:1), and volume-reduced using low-pressure rotary evaporation and N_2(g). Half of each extract was subjected to chromato-graphic cleanup on an open silica column (10% H_2O) to remove macromolecules, and the other half was treated with concentrated sulphuric acid to remove non-persistent compounds.

HPLC fractionation

Samples were fractionated by HPLC (normal phase) into three fractions: aliphatics/monoaromatics, diaromatics, and polyaromatics[3]. The separation was carried out on an amino column (uBondapack, 125 Å, 10 um, 7.8x300 mm HPLC column) used in an HPLC-system (Hewlett-Packard, model HP 1100) with a UV-DAD detector. n-Hexane (Riedel-de Haën) was used as mobile phase. To obtain the polyaromatic fraction as a narrow band, the system was backflushed.

Carbon column fractionation

To separate planar and non-planar compounds, the HPLC-fractions were further fractionated on a carbon column (Carbopack C)[4]. Carbon on celite (1:9) (Celite 545, Fluka, 20-45um) was washed with toluene in a Soxhlet device (24h) and dried in 130°C. 1.5 g of the adsorbent, and a layer of Na_2SO_4, was packed in glass columns. The column was washed with hexane before application of the sample. The sample was eluted into four subsequent fractions with 10 ml of hexane (non-planar compounds), 10 ml, 30 ml and 80 ml of toluene (coplanar compounds). Data for the three toluene fractions is presented as a sum for coplanar compounds.

Bioassay analysis

The bioassay used for detection of dioxin-like substances is based on 7-ethoxyresorufin-*O*-deethylase (EROD) induction in chicken (*Gallus domesticus*) embryo livers[5]. DMSO was used as a vehicle for transfer of samples into the culturing medium. Livers were dissected from 8-days-old embryos. Four livers were exposed to each concentration tested (four concentrations for HPLC fractions and two for carbon column fractions), in a volume of 2 ml. In each assay, one dose of 2,3,7,8-TCDD (0.33 nM), known to give maximum EROD induction, was used as a positive reference. The EROD activity in each liver piece was determined after 48h exposure, by measurement of the deethylation of 7-ethoxyresorufin into the product resorufin. The fluorescence of resorufin was measured in a multiwell-plate reader (Wallac 1420, Victor^2)[6]. For HPLC-fractions, bioassay determined TEQs (bioTEQs) were calculated from the EC_{25}-value of the dose-response curve. As only two doses were tested for carbon column fractions, these bioTEQs were estimated by relating to a 2,3,7,8-TCDD standard curve ($1x10^{-9}$ to $1x10^{-12}$ M).

Results and discussion

Di- and polyaromatic fractions from both mesophilic and thermophilic treatment did induce EROD-activity (fig. 1). No quantifiable activity was induced by the monoaromatic fractions. The composition of compounds contributing to the dioxin-like activity was different after mesophilic digestion, compared to thermophilic. The dioxin-like activity in the mesophilic residue was dominated by diaromatics, of which about half was acid-resistant, whereas the thermophilic residue was dominated by acid-degradable polyaromatics.

Further fractionation of the four acid-resistant, biologically active HPLC-fractions showed that a diverse mixture of compounds might be contributing to the dioxin-like activity in the samples (fig. 2). Mesophilic and thermophilic samples displayed an overall similar pattern of dioxin-like activity for the different fractions. However, the mesophilic diaromatic fractions induce a higher dioxin-like activity compared to the analogous thermophilic fractions. The activity in the non-planar fractions may be induced by mono-*ortho*-PCBs, whereas in the planar fraction, the inducers are likely non-*ortho*-PCBs and/or PCDD/Fs.

Interestingly, the polyaromatic fractions of both mesophilic and thermophilic samples contained compounds with dioxin-like activity in the same order of magnitude to that seen in the diaromatic fractions. This activity may be caused by acid-stable halogenated or non-halogenated polycyclic aromatic hydrocarbons (PAHs), e g polychlorinated naphtalenes (PCNs). Depending on degree of chlorination, PCNs exist as planar or non-planar molecules[7].

The mesophilic and thermophilic samples appear more similar after the second fractionation than what might be expected from the results from the HPLC-fractions. Negative interactions in the HPLC-fractions may have occurred, yielding apparently lower TEQ-values, compared to the carbon column fractions. As can be seen, the sum of TEQs detected after carbon column fractionation is higher, suggesting a lower degree of inhibition of EROD activity.

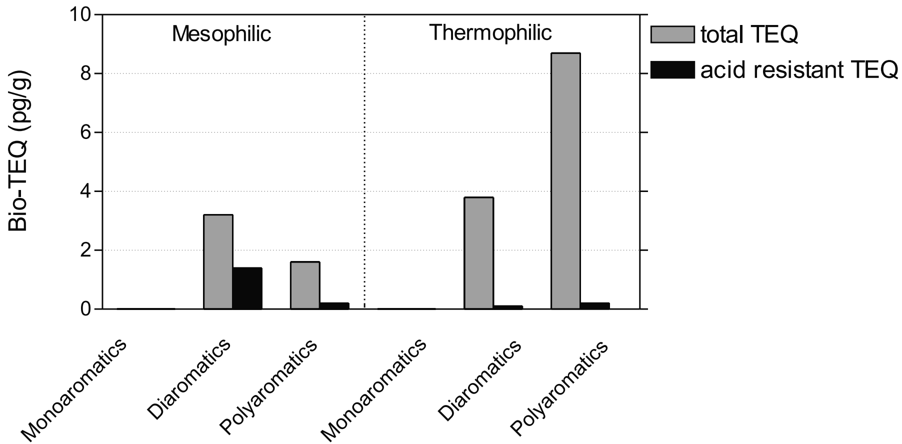

Figure 1. Dioxin-like content, presented as bioassay-derived TCDD-equivalents (BioTEQs), in fractionated residues of organic waste after mesophilic (37°C) or thermophilic (55°C) anaerobic digestion.

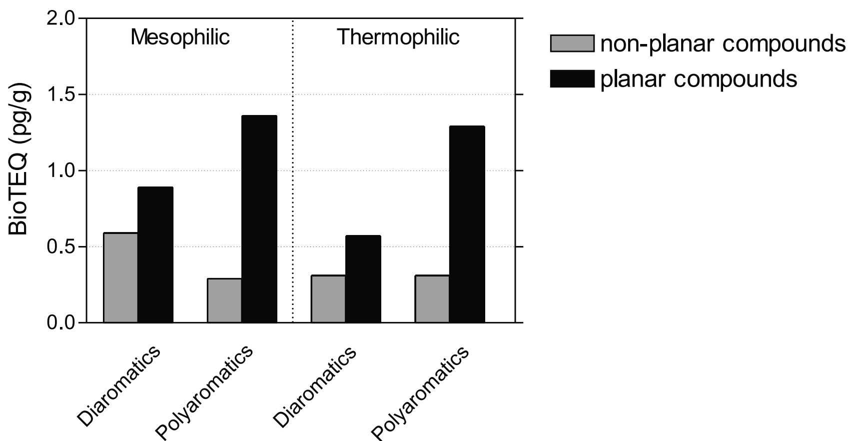

Figure 2. Acid-resistant dioxin-like content, presented as bioassay-derived TCDD-equivalents (BioTEQs), in fractionated residues of organic household-waste after anaerobic mesophilic (37°C) or thermophilic (55°C) digestion.

Conclusion

We found that very complex extracts are difficult to test using the bioassay approach. In order to obtain reliable results, an extensive cleanup and fractionation procedure may be necessary. Due to the removal of potential inhibitors during cleanup, the results may be difficult to interpret, as TEQ-values after different fractionation steps are strongly depending on the presence of inhibitors. Thus, an extensively cleaned sample may result in much higher TEQs, compared to a raw extract.

In the studied samples, di- and polyaromatics contribute to the dioxin-like activity. No monoaromatic fractions induced any dioxin-like activity. The acid-resistant, diaromatic fractions of the mesophilic sample show higher EROD-inducing potency, and differs in composition of non-planar and planar compounds. These results indicate that the previously reported[2] increase of dioxin-like compounds during mesophilic, anaerobic digestion, is due to an accumulation of different dioxin-like compounds. Further studies will be conducted to elucidate the mechanisms behind the accumulation.

Acknowledgement

This study was financially supported by the Swedish Council for Forestry and Agricultural Research (SJFR) and the Knowledge foundation (KK-stiftelsen).

References

1. Engwall M., Broman D., Ishaq R., Näf C., Zebühr Y. and Brunström B. (1996) Environ Toxicol Chem. 15(2), 213-222.
2. Engwall M. and Schnürer A. (2002) Sci Tot Environ. In press.
3. Zebühr Y., Näf C., Bandh C., Broman D., Ishaq R. and Pettersen H.(1993) Chemosphere. 27, 1211-1219.
4. Lindström G. and Rappe C. (1990) Chemosphere 20(7-9), 851-856.
5. Brunström B., Engwall M., Hjelm K., Lindqvist L. and Zebühr Y. (1995) Environ Toxicol Chem. 14(5), 837-842.
6. Brunström B. and Halldin K. (1998) Comp Biochem Physiol C. 121, 213-219.
7. Ishaq R. (1999), Fractionation and analysis of chlorinated and non-chlorinated aromatic compounds in biotic and abiotic environmental samples (Thesis), Stockholm University, ISBN 91-7153-954-9