Pre-validation study for dioxin and dioxin-like compound analysis in WWTP sludge with the CALUX bioassay - Emphasis on obtaining extracts suitable for both chemo- and bio-analytical determination.

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Introduction

Polychlorinated dibenzo-*p*-dioxins, -furans and biphenyls form a group of ubiquitous environmental contaminants that persist in time, accumulate in a non-polar matrix as well as biomagnify in the trophic chain[1]. Due to their hydrophobicity, they tend to accumulate in river sediment and in the sludge of waste water treatment plants[2] by processes of atmospheric deposition, accidental spills and subsequent run-off. It is therefore of particular interest to verify the contamination in sewage sludge and alike, that may be used as a biosolid for land application on soils intended for agricultural use.

Current legislation is scarce concerning the limit values of PCDD/Fs and dioxin-like PCBs in sludge. Major EC regulations tend to focus on heavy metal pollution for these types of matrices. Although a 3rd working draft document[3] exists (EC 2000), which proposes a limit of 100 ng I-TEQ kg^{-1} (dry weight). More recent publications[4,5,6] report values that either comply with this limit for all samples[4] or comply on average[5,6], the latter have maximum values going up to 250 pg PCDD/F's TEQ g^{-1} (dry weight) (TEF scheme not mentioned).

The focus of this paper is oriented towards a combined clean-up and extraction procedure for both bio-analytical and chemo-analytical methods. A modified extraction and clean-up procedure will be used in conjunction with the CALUX (Chemically activated luciferase gene expression) bioassay and evaluated for suitability with future GC-HRMS analysis. To accomplish a combined extraction and clean-up for chemo- and bio-analytical methods, certain issues inherent to both methods will need to be addressed. For the CALUX determination, solvent toxicity is a major source of concern, since impurities within the solvent may lower or increase dioxin-related effects[7] and thus bias the results. For GC-HRMS, elemental sulfur (as this is readily present and extracted from soil, sediment and similar matrices) will cause chromatographic interferences in the determination of PCBs[8] and needs to be removed from the final extract, prior to GC injection.

Materials en methods

Sample matrices used were sediment from the Zenne and Scheldt Rivers, waste water treatment plant sludge as well as NIST SRM 1944 for evaluation of the applied modifications of existing protocols.

Extraction

The US EPA method 4435[9] for toxic equivalent determination with the CALUX bioassay was used as a starting point for the assessment of dioxin-like activity in the previous mentioned matrices. Freeze-dried sediment/WWTP sludge (2g) was extracted with a 20/80 methanol/toluene mixture, toluene and passed over a celite filter column. This extraction method can be referred to as a soft extraction, as opposed to more harsh extraction conditions faced with ASE, PLE or Soxhlet extraction. Hence we may assume that such extraction method can provide information

regarding dioxin and dioxin-related compounds in a sample fraction that can be considered bio-available under average weather(ing) conditions.

Purification and separation

Extracts were redissolved in 5mL n-hexane and completed with 2.5mL concentrated sulfuric acid[10] to break down any acid-labile compounds. The n-hexane layer was transferred to a three stage column series along with three 2mL n-hexane rinses of the sample extract. Extract and rinses first passed through a sulfuric acid silica column[9,11] followed by an activated copper column[12] (20% HCl solution for activation). Target compounds were thereafter trapped in an X-CARB column[9] and differentially eluted in a dioxin-like PCB and dioxin/furan fraction. Dioxin and dioxin-like PCB extracts were redissolved in 4mL of n-hexane and stored awaiting CALUX analysis.

CALUX analysis and data-processing

The Chemically activated luciferase gene expression method uses a wild type Hepa1c1c7 cell that has been stably transfected with a luciferase gene under control of the Aryl hydrocarbon receptor, allowing induction of luciferase by dioxin and dioxin-like compounds in a time-, dose-, ligand- and AhR-dependent manner[13]. The cell line(s) used are the H1L6.1c3 and H1L7.5c1 cell line, both are recombinant mouse hepatoma cells that are, respectively, transfected with the pGudLuc6.1- and 7.5 vectors. The latter is termed a 3rd generation (G3) luciferase bioassay and can distinguish lower amounts of TCDD from basal levels (compared to the 2nd generation bioassay), hence shifting the sensitivity of the bioassay to a lower TCDD concentration range[14,15,16].

The CALUX protocol has been described in detail elsewhere[16]. Sample extracts are dosed as dilution curves (multiple serial dilutions for a single sample extract). Triplicate measurements are performed for the individual sample points as well as for the TCDD-standard curve. DMSO and media controls are included on every plate as negative controls.

Statistical analysis and BEQ quantification[17] involves fitting the four parameter Hill equation or the newer slope ratio method[18] using Excel. Briefly, the four parameter Hill function allows inverse prediction of single extract points based on the sigmoid curve plotted through the TCDD standard data points, where the parameters of the sigmoid fit are optimized by minimizing the sum of the squared errors via the solver add-in build in Excel. This has repercussions for the uncertainty assessment of the predicted BEQ-value due to multiplicative error propagation when switching from log-transformed data to a linear scale. Effective concentration (EC) ratios can also be applied. However, this requires full dose sample curves which may not always be obtained, as well as parallelism and equal efficacy of the sample relative to TCDD.
If sample curves deviate from the above conditions, a range of ECs can be given (EC20 to EC80) or a slope ratio method can be applied which utilizes Box-Cox transformations (linearization) and represents BEQ-values as the ratio of the sample to TCDD slope. This new data-analysis method[18] may help in precise analysis of dose potency since it does not require full sample dose curves, often an issue with low-contamination samples.

Results and discussion
Substantial differences from validated protocols and EPA methods are the addition of sulfuric acid directly to the extract and insertion of a copper column. The extra acid step is to facilitate break down of acid-sensitive compounds prior to transferring extracts onto the acid silica column and thus prevent clogging due to heavily contaminated samples (high organic load). From Figure 1 we can see that the treatment with sulfuric acid provides slightly higher BEQ-values (only the dioxin fraction is shown for individual experiments), but that this difference is not statistically significant (two-sided paired t-test of 0.106 and 0.917 for Scheldt and SRM 1944 (results not shown) respectively). Blanks for this treatment showed no elevated response.

Sample extracts (Scheldt River, Zenne River and SRM; see right part of Figure 1) were analyzed for evaluation of copper on the BEQ-result (acid-pretreatment occurred for all these samples). Results can be seen in Figure 1 (right, dioxin fraction only) and indicate no clear influence of the addition of copper in the clean-up process (no statistical significance with p-values of 0.03 up to 0.71 using a two-sided paired t-test).

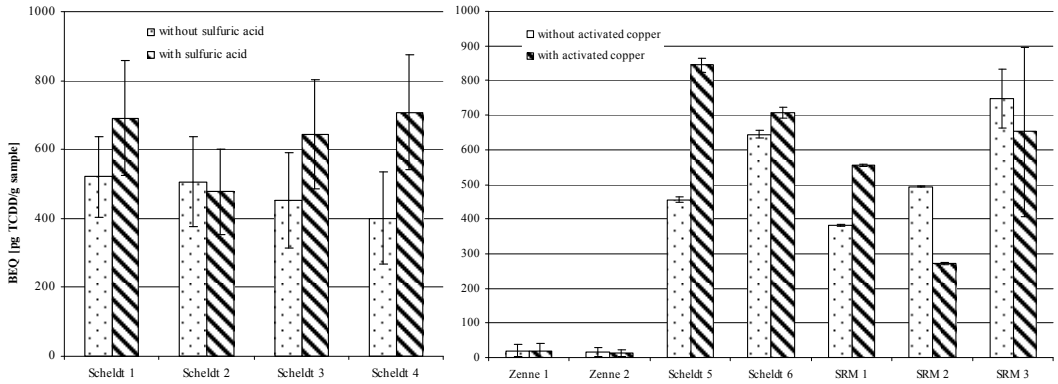

Figure 1: BEQ-values (pg TCDD/g sample) based on the slope-ratio method for the dioxin fraction of selected samples treated with or without sulfuric acid prior to clean-up [Left] and with or without copper in the experimental set-up for clean-up [Right]. Error bars indicate the expanded uncertainty, extracts were dosed to the H1L7.5c1 cell line.

Scheldt River sediment ranged from around 400 to 770 pg TCDD/g sample (both with and without sulfuric acid addition) for Scheldt [1-4] (Figure 1, left) and had values of 455-855 pg TCDD/g sample for Scheldt [5-6] (all acid pre-treatment, with or without copper, Figure 1, right).

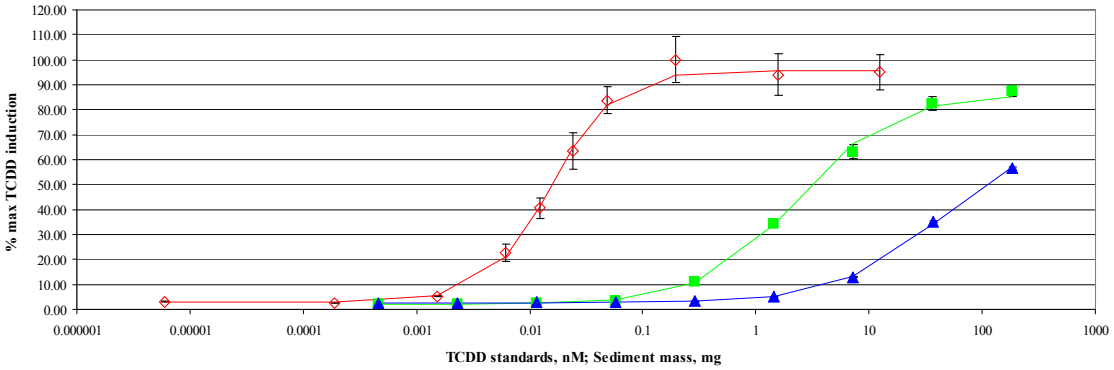

Figure 2: Analysis result of a WWTP sludge sample with TCDD standard curve (nM, ◊), sludge dioxin fraction (mg, ■) and sludge PCB fraction (mg, ▲) using the H1L6.1c3 CALUX cell line (values on the x-axis depicted on a log scale).

As a preliminary result, a WWTP sample (undisclosed source, Figure 2) was measured by analyzing a 9-point dilution curve (factor of 5 for both the dioxin and PCB fraction) and resulted in 188 pg TCDD/g sample (slope ratio method adjusted for different efficacy) or 317, 198 and 124 pg TCDD/g sample (Hill four parameter adjusted for different efficacy; EC20, 50 and 80 values) for the dioxin fraction and 5 pg TCDD/g sample (Hill four parameter not adjusted for efficacy; EC50) for the PCB fraction. It is expected that a plateau will be reached (this specific sample)

for the dioxin fraction if the more sensitive H1L7.5c1 cell line is used and the dilution factor adapted. From the EC20-80 values it is already clear that the sample dilution curve is not parallel to the TCDD standard curve. This shows the importance of multiple points in the linear part of the sample curve to assess this effect.
We were not able to determine the PCB fraction using the slope ratio method due to a low amount of points in the linear part of the curve; this too could be resolved by applying a lower dilution factor.
Overall, the adapted clean-up protocol shows that the method does not interfere with previously validated protocols and that an efficient way was found to eliminate copper from the extract for GC-MS analysis without compromising CALUX results. Future work will include the GC-MS analysis of these extracts for confirmation of the CALUX results.

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References:
1. Van Larebeke N, Covaci A, Schepens P, Hens L. (2002) *Journal of Epidemiology & Community Health* 56: 828-830.
2. Eljarrat E., Caixach J., Rivera J. (2003) *Chemosphere* 51: 595-601.
3. EU (European Union). Working document on sludge, 3rd draft; 2000.
4. Martinez K., Abad E., Palacios O., Caixach J., Rivera J. (2007) *Environment International* 33: 1040-1047.
5. Bright D.A., Healey N. (2003) *Environmental Pollution* 126: 39-49.
6. Moon H-B., Yoon S-P., Jung R-H., Choi M. (2008) *Chemosphere* 73: 880-889.
7. Van Wouwe N., Windal I., Vanderperren H., Eppe G., Xhrouet C., Massart A-C., Debacker N., Sasse A., Baeyens W., De Pauw E., Sartor F., Van Oyen H., Goeyens L. (2004) *Talanta* 63: 1157-1167.
8. US EPA Method 8082, Polychlorinated biphenyls (PCBs) by gas chromatography. *United States Environmental Protection Agency* (1996) Revision 0.
9. US EPA Method 4435, Method for toxic equivalents (TEQs) determinations for dioxin-like chemical activity with the CALUX bioassay. *Unites States Environmental Protection Agency* (2008) Revision 0.
10. Croes K., Van Langenhove K., Elskens M., Desmedt M., Roekens E., Kotz A., Denison M.S. and Baeyens W. *Chemosphere* 82: 718-724.
11. US EPA Method 3630C, Silica gel cleanup. *Unites States Environmental Protection Agency* (1996) Revision 3.
12. US EPA Method 3660B, Sulfur cleanup. *Unites States Environmental Protection Agency* (1996) Revision 2.
13. Seidel S.D., Li V., Winter G.M., Rogers W.J., Martinez E.I., Denison M.S. (2000) *Toxicological Sciences* 55: 107-115.
14. Denison M.S., He G., Baston D.S., Tsutsumi T. (2008) Organohalogen Compounds 70: 772-775.
15. He G., Tsutsumi T., Zhao B., Baston DS., Zhao J, Heath-Pagliuso S., Denison MS. (2011) Toxicological Sciences *submitted*.
16. Van Langenhove K., Croes K., Denison M.S., Baston D.S., Elskens M., Baeyens W. (2010) *Organohalogen Compounds* 72: 1092-1096.
17. Goeyens L., Hoogenboom R., Eppe G., Malagocki P., Vanderperren H., Scippo M.-L., Windal I., Baeyens W., Denison M.S. and Hädrich J. *Organohalogen Compounds* 72: 964-976.
18. Elskens M., Baston D.S., Stumpf C., Haedrich J., Keupers I., Croes K., Denison M.S., Baeyens W. And Goeyens L. *Submitted.*