EFFECT-BASED SCREENING OF PERSISTENT ENVIRONMENTAL POLLUTANTS AND ENVIRONMENTAL SAMPLES USING AN AUTOMATED PANEL OF CALUX REPORTER ASSAYS

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Introduction

Since the potential risks related to various persistent pesticides and other pollutants that could accumulate in the environment gained attention among the larger public half a century ago, the use of many of these compounds has been banned or limited in large parts of the world. Nevertheless, the ongoing presence or even *de novo* accumulation of persistent chemicals from anthropogenic sources forms an ongoing hazard for human and ecological health worldwide. The screening for adverse chemical compounds in environmental matrices is typically performed by chemical analysis for a set of compounds that have been prioritized for their frequent occurrence and / or their associated health risk. Although this approach may be efficient for the identification of the majority of polluted hotspots, it has its limitations as a standalone method for hazard identification since an important part of the pollutants may not be identified in the standard chemical analytical screens, or be poorly characterized with respect to their toxicological traits. In addition, it has become clear that chemicals can act in concert to exert harmful toxicological effects. An alternative or complementary approach for the identification of environmental samples that could form a health risk is effect-based testing. Whereas individual reporter assays for specific effects have been successfully used for various applications, BioDetection Systems now performs screenings with a CALUX panel that comprises multiple effects. The use of this human cell line-based set of reporter assays allows for the high throughput generation of effect profiles of pure compounds as well as complex mixtures. We could demonstrate this approach both for a set of persistent environmental pollutants (pesticides, PAHs, heavy metals, organometals) as well as on a set of soil samples from a pesticide dump.

Materials and methods

Chemicals and samples

Analytical grade compounds were obtained from various commercial suppliers. Soil samples were obtained from a pesticide dump from Tajikistan, central Asia, to which the last official depositions were made over a decade ago. Extracts of the dry samples were made by accelerated solvent extraction using a dichloromethane / acetone extraction. Samples and compounds were dissolved in DMSO for exposure to the CALUX cells.

CALUX reporter assays

CALUX reporter assays for the human ERα (estrogen receptor α), ERβ (estrogen receptor α), AR (androgen receptor), PR (progestin receptor) (1), PPARα (peroxisome proliferator activated receptor α; (2), PPARγ (peroxisome proliferator activated receptor γ (3) are all based on the human osteosarcoma cell line U2OS to which an expression construct for the respective nuclear receptor and construct with a luciferase reporter under the control of a multimerized response element for the respective receptor were stably introduced. Two cell lines for the detection of activity mediated by the dioxin receptor, the DR CALUX (4) and the PAH CALUX (5), were based on the rat hepatome H4IIE cell line. In addition, reporters to assess transcriptional activation by the p53 protein; p53 CALUX line (6), the oxidative stress responsive nrf-2 pathway; Nrf2 CALUX, the endoplasmic reticulum stress pathway; ESRE CALUX, the activator protein 1 pathway; AP1 CALUX, and the hypoxia-inducible factor induced pathway; HIF1α CALUX were added to the screen. Based on previous experience it was decided to focus on antagonistic activities for the AR- and the PR CALUX, by measuring reduction in receptor mediated activity due to the test sample or compound.

The CALUX assay were performed using an automated platform in 384-well plates as described before (7). The amount of light produced when substrate is added to the lysed cells after the incubation period is proportional with the effect and is measured with a luminometer.

Data analysis
Luminometer data from the CALUX analyses were processed and analyzed as previously described (7). The signals induced by the test compounds were expressed in % of maximum reference signal. Then, the concentration where the test compound reached 10% of the maximum effect of the reference compound was determined with Graphpad Prism software (log agonist binding curve fit, extrapolate unknowns). This concentration, designated PC10, was used to rank the potencies of the compounds.
Relative potencies (REPs) were obtained by dividing the EC10 of the reference compound for the assay, by that of the PC10 of the concerning compound. The expected bioactivity equivalents (BEQ) in the different assays for the pesticide dump samples mixtures were obtained by multiplication of the concentrations (M) of the individual compounds (available from chemical analysis) with their REP value, and subsequent adding up of the resulting individual BEQs.
Prior to hierarchical clustering, –log10(PC10) values for the different compounds were range-scaled for which the –log10(PC10) of the reference compound on the respective assay was set as the maximal value.

Results and discussion
Screening of pure compounds
A primary impression of the data was generated by hierarchical clustering and indicates a wide diversity of effects at the cellular level (Fig. 1). Although a more thorough analysis of the results is ongoing, several trends can already be observed: Major part of the tested pesticides showed an anti-androgenic, anti-progestogenic and /or estrogenic effect. These findings correspond with previous reports that indicate endocrine as a predominant effect of many pesticides. Notably, this may be linked to known adverse health effects of these compounds among which carcinogenicity and developmental disorders (8). The observation that most of the pesticides that have been associated with carcinogenicity do not have an effect on the p53 CALUX may be explained by their non-genotoxic carcinogenic mode of action. The dioxin-like PCBs and the PAHs that were tested all showed an effect on the assays for DR-receptor mediated expression. This receptor has been implicated as an important mediator of the toxic effect associated with these compounds (9).The effects evoked by the heavy metals and the organometals confirm that they are very diverse with respect to their toxic effect. From the screened compounds, 14 compounds showed a response in the p53 CALUX assay indicative of genotoxic effects (6). Genotoxicity is confirmed in scientific literature for 11 of these compounds. The good predictive value of the p53 has previously been indicated in a validation study in which the assay was used in combination with *in vitro* metabolism using S9-liver fractions (6). In the current screening mode S9 was not included, and further work will be done to confirm activities using the in ref 6 described validated method.

Screening of pesticide dump samples
Soil extracts from 2 different spots within the pesticide dump site polygon as well as 3 reference samples from the direct surrounding area were screened on the same reporter cell line panel as the pure compounds. Quantitative chemical analysis data were available from a standard screening for a set of 25 pesticides. From these pesticides 10 were detected in one or more of the samples at concentrations > 0.10 mg / kg dry weight. Based on these data, the expected response in the bioassays could be calculated using the relative potencies for the individual compounds. Both the expected and the observed responses (expressed in equivalents of the reference compounds for the assays) are depicted for the five assays for which responses were observed in Fig. 2: The ERα CALUX, the anti-AR CALUX, the anti-PR, the AP1 CALUX, the p53 CALUX. Clear differences in the detected responses on ERα-, anti-AR-, and anti-PR CALUX' can be observed between the dump samples and the samples from the surrounding region. A very good correspondence between the expected and the measured values on the ERα CALUX could be observed for both samples from the pesticide dump itself. Remarkably, while the measured values are conform with what was expected on all assays for dump sample 2, large differences can be observed for the measurements of dump sample 1 on expected activity measured using the anti-AR, anti-PR, AP1, and p53 CALUX'. These data demonstrate that the CALUX assays show good performance with respect to the identification and quantification of additive biological effects in soil extracts.

Moreover, there is a very strong suggestion that the bioassay panel detected abundant activities on anti-AR, anti-PR, AP1, and p53 CALUX from compounds that were not identified by chemical analysis. Chromatographic fractionation of dump sample 1 in combination with CALUX analyses should result in isolation of the compound(s) responsible for this combined effect.
These results clearly show that the CALUX panel is not only successful in the detection of effects of pure toxicants at the cellular level but also that it is very suitable for effect-based screening of complex mixtures.

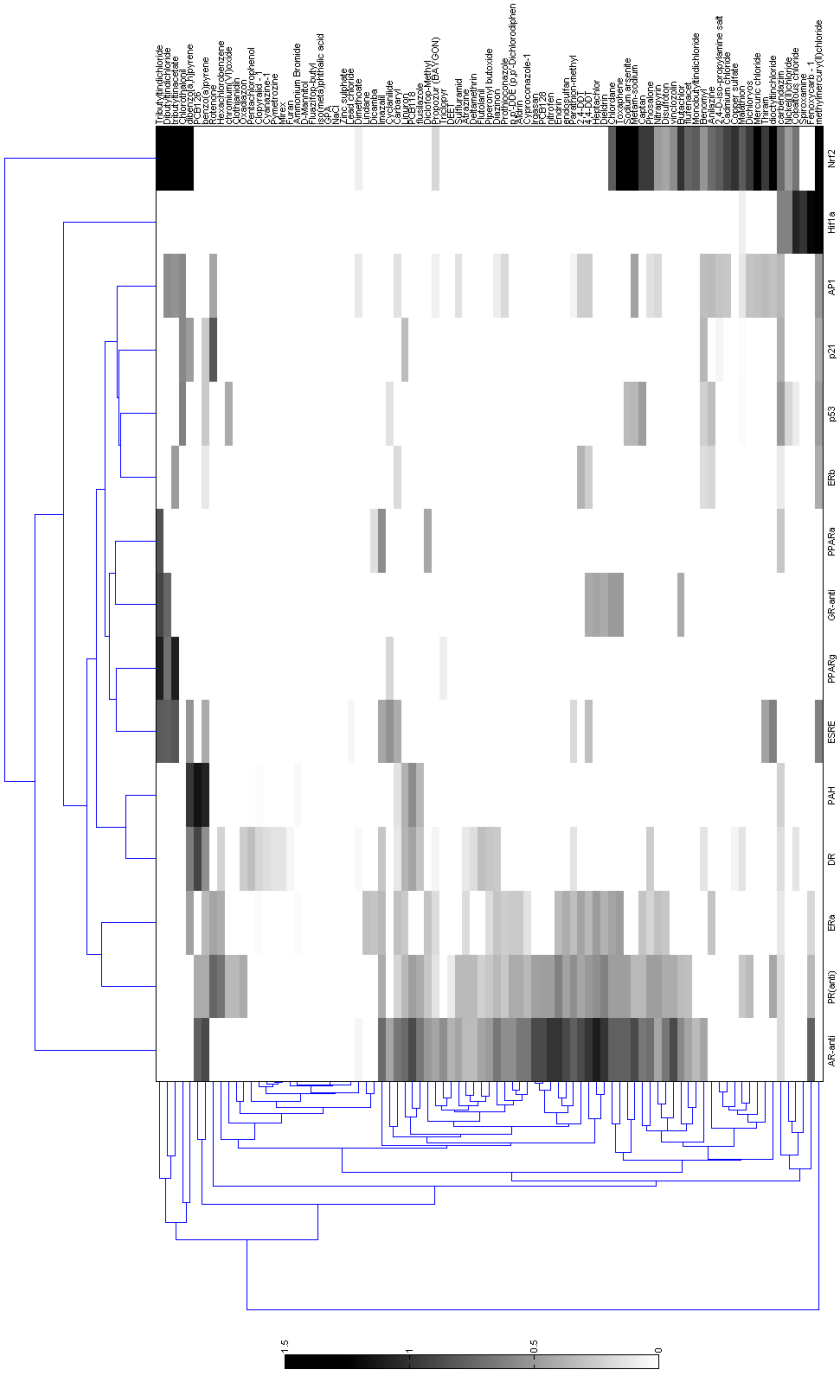

Fig 1. Visual representation of hierarchical clustered data of the responses of the CALUX assay panel towards a set of persistent environmental toxicants. The strength of signal is represented by the amount of shading which increases with stronger effects

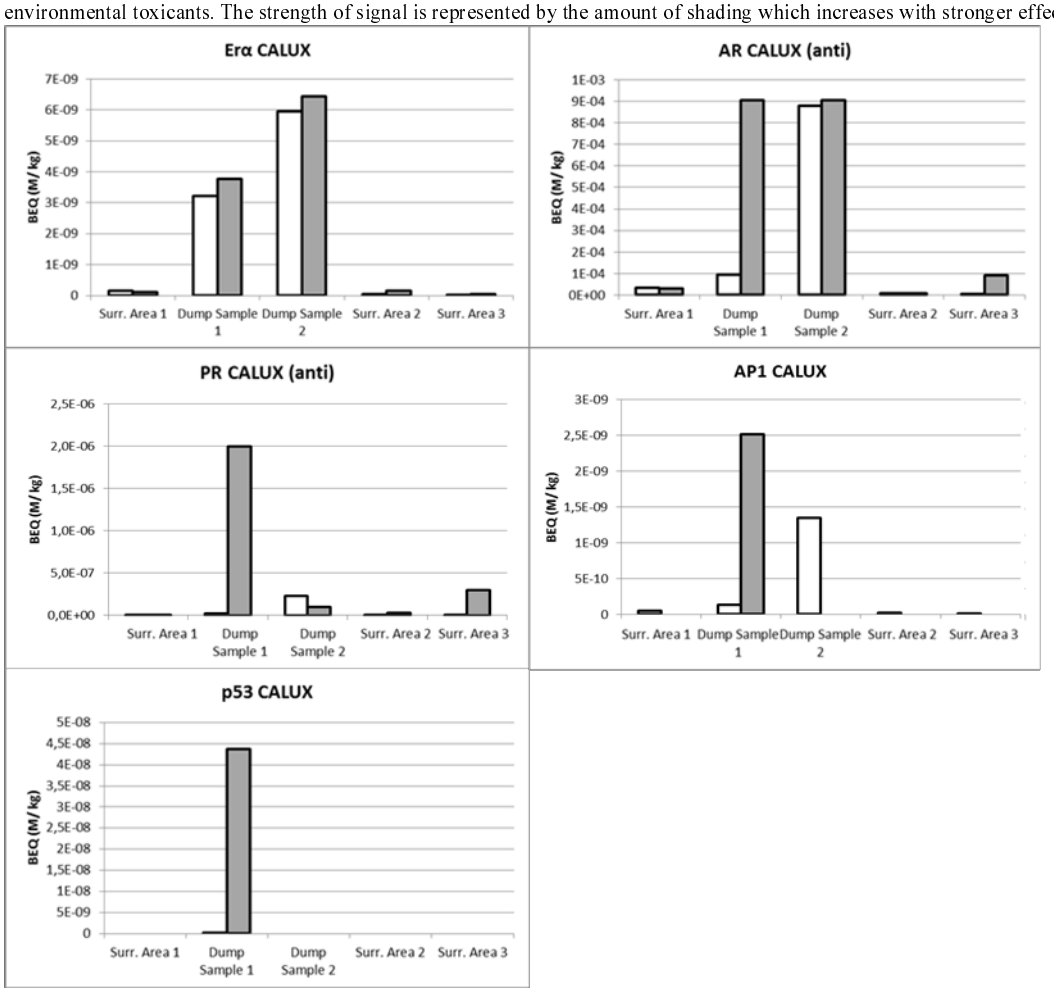

Fig 2. Biological activities in extracts from the pesticide dump and surrounding are expressed in equivalents of the reference compounds: 17β-estradiol (ERα CALUX), flutamide (AR CALUX-anti), ru486 (PR CALUX-anti), 12-O-tetradecanoylphorbol-13-acetate (AP1 CALUX) , actinomycin D (p53 CALUX). White bars represent the expected activities based on chemical analytical data, Grey bars represent the data from the CALUX measurements.

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