

Fitting dose-response curves from bioassays and toxicity testing

by Johannes Ranke

Introduction

In attempts to quantify detrimental effects of chemical substances on biological organisms, the responses of these organisms to different doses¹ are often observed in a quantitative way. Examples of the observed variables (endpoints of toxicity) are the number of surviving individuals, the length of wheat seedlings after being exposed to different concentrations of the chemical substance for a defined time interval, the activity of luminescent bacteria, the ability of cell cultures to reduce a specific dye, the growth rate according to number of individuals or biomass, the number of viable offspring and many others.

These observed variables have in common that a reference magnitude for healthy and viable organisms can be defined (normalised response level $r = 1$), and that the magnitude of the variable (response) is limited by a zero response ($r = 0$) where the maximum of the effect is observed. In between, there is either a continuum of possible responses, or, if only the presence/absence of a specific quality of n individual organisms is observed (commonly life/death), then only $n + 1$ response levels are possible.

Such dose-response relationships can generally be expressed as

$$r = f(d, \vec{p}) + \epsilon \quad (1)$$

where r is the normalised response at dose d , $f(d, \vec{p})$ is the model function with parameter vector \vec{p} , and ϵ is the error variable describing the variability in the observations not explainable by the model function $f(d, \vec{p})$.

This article shows how different model functions $f(d, \vec{p})$ can be conveniently fitted to such dose-response data using the R package **drfit**, yielding the vector of parameters \vec{p} that gives the description of the data with the least residual error. The fitting can be carried out for many substances with a single call to the main function `drfit`.

The results that the user will probably be most interested in are the doses, at which a response of 50 % relative to healthy control organisms is to be expected (termed ED_{50}), as this is a very robust parameter describing the toxicity of the substance toward the organism investigated.

The **drfit** package internally uses the R function `nls` from the recommended **stats** package for nonlinear regression analysis as detailed by Bates and Watts

(1988).

drfit defines a dose-response data representation as a special case of an R dataframe, facilitates fitting standard dose-response models (probit, logit, weibull and linearlogit at the time of this writing), and a function to produce different types of plots of the data as well as the fitted curves.

Optionally, the raw data can be kept in an external database connected by **RODBC**. This has proven to be useful if the data of a large number of dose-response experiments have to be evaluated as e.g. in bioassays based on microtiter plates.

Recently, the R package **drc** containing similar functionalities to **drfit** has been uploaded to CRAN. Unfortunately, I have noticed the existence of this package only during the preparation of this article, after having maintained **drfit** on CRAN for almost one and a half years. Maybe in the future it will be possible to join forces.

In this introductory article, it is explained how the input data must be formatted, how dose-response curves are fitted to the data using the `drfit` function and in what ways the data and the models can be plotted by the `drplot`. Since the package was actively developed during the preparation of this article, the reader is advised to upgrade to the latest `drfit` version available. Note that $R \geq 2.1.0$ is needed for recent `drfit` versions.

Collecting dose-response data

The `drfit` function expects the dose-response data as a data frame containing at least a factor called 'substance', a vector called 'unit' containing the unit used for the dose, a column 'response' with the response values of the test system normalised between 0 and 1 and a column 'dose' with the numeric dose values. An example of such data can be easily obtained from the built-in dataset `XY`.

```
> library(drfit)
> data(XY)
> print(XY, digits=2)
  nr.  substance dose unit fronds response
1   1   Control   0 mg/L   174    1.050
2   2   Control   0 mg/L   143    0.973
3   3   Control   0 mg/L   143    0.973
4   4 Substance X  10 mg/L   147    0.983
5   5 Substance X  10 mg/L   148    0.986
6   6 Substance X  10 mg/L   148    0.986
7   7 Substance X 100 mg/L    63    0.651
```

¹ The term dose is used here in a generalised way, referring to doses in the strict sense like mg oral intake per kg body weight as well as to measured concentrations in aquatic toxicity tests or nominal concentrations in cell culture assays.

| | | | | | |
|----|----|-------------|-----------|-----|-------|
| 8 | 8 | Substance X | 100 mg/L | 65 | 0.663 |
| 9 | 9 | Substance X | 100 mg/L | 55 | 0.598 |
| 10 | 10 | Substance X | 300 mg/L | 20 | 0.201 |
| 11 | 11 | Substance X | 300 mg/L | 22 | 0.238 |
| 12 | 12 | Substance X | 300 mg/L | 25 | 0.288 |
| 13 | 13 | Substance X | 1000 mg/L | 13 | 0.031 |
| 14 | 14 | Substance X | 1000 mg/L | 16 | 0.113 |
| 15 | 15 | Substance X | 1000 mg/L | 16 | 0.113 |
| 16 | 16 | Control | 0 mg/L | 153 | 0.999 |
| 17 | 17 | Control | 0 mg/L | 144 | 0.975 |
| 18 | 18 | Control | 0 mg/L | 163 | 1.024 |
| 19 | 19 | Substance Y | 10 mg/L | 20 | 0.201 |
| 20 | 20 | Substance Y | 10 mg/L | 19 | 0.180 |
| 21 | 21 | Substance Y | 10 mg/L | 21 | 0.220 |
| 22 | 22 | Substance Y | 100 mg/L | 13 | 0.031 |
| 23 | 23 | Substance Y | 100 mg/L | 12 | 0.000 |
| 24 | 24 | Substance Y | 100 mg/L | 13 | 0.031 |
| 25 | 25 | Substance Y | 300 mg/L | 12 | 0.000 |
| 26 | 26 | Substance Y | 300 mg/L | 12 | 0.000 |
| 27 | 27 | Substance Y | 300 mg/L | 14 | 0.061 |
| 28 | 28 | Substance Y | 1000 mg/L | 12 | 0.000 |
| 29 | 29 | Substance Y | 1000 mg/L | 12 | 0.000 |
| 30 | 30 | Substance Y | 1000 mg/L | 12 | 0.000 |

Normalisation of the response data is not done within the **drfit** package. It can either be carried out with a typical spreadsheet file, with some extra lines of R code, or by an external procedure, while/before the data is read into a database.

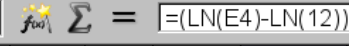
If the data is collected and normalised using MS Excel, it can be easily transferred to R by saving it in CSV format, and reading it in using the R function `read.csv2`. If OpenOffice.org Calc is being used, and the default values are used for exporting the data in CSV format, the function `read.csv` is very helpful.

Figure 1 shows a possible spreadsheet layout for capturing dose-response data including both the observed endpoint (number of fronds in this case) and the response value calculated by dividing the growth rate for each data point μ_i by the growth rate of the appropriate controls μ_c .

This preprocessing ensures that the response data is normalized to the interval from 0 to 1. Total growth inhibition is in this case the natural lower limit of the response and will be zero if the number of duckweed (Lemna minor) fronds stays at the initial level n_0 during the observation time. The natural reference for the healthy organisms (response=1) is in this case given by the growth rate of the controls μ_c , calculated by

$$\mu_c = \frac{\ln(\bar{n}_c) - \ln(n_0)}{t - t_0} \quad (2)$$

where \bar{n}_c is the mean number of fronds in the control experiments after the observation time.

F4 

| | A | B | C | D | E | F |
|----|---|-------------|------|------|--------|----------|
| 1 | Concentration-response data for the Lemna growth test | | | | | |
| 2 | | | | | | |
| 3 | nr. | substance | dose | unit | fronds | response |
| 4 | 1 | Control | 0 | mg/L | 174 | 1.0496 |
| 5 | 2 | Control | 0 | mg/L | 143 | 0.9726 |
| 6 | 3 | Control | 0 | mg/L | 143 | 0.9726 |
| 7 | 4 | Substance X | 10 | mg/L | 147 | 0.9834 |
| 8 | 5 | Substance X | 10 | mg/L | 148 | 0.9861 |
| 9 | 6 | Substance X | 10 | mg/L | 148 | 0.9861 |
| 10 | 7 | Substance X | 100 | mg/L | 63 | 0.6509 |
| 11 | 8 | Substance X | 100 | mg/L | 65 | 0.6631 |
| 12 | 9 | Substance X | 100 | mg/L | 55 | 0.5976 |
| 13 | 10 | Substance X | 300 | mg/L | 20 | 0.2005 |
| 14 | 11 | Substance X | 300 | mg/L | 22 | 0.2379 |
| 15 | 12 | Substance X | 300 | mg/L | 25 | 0.2881 |
| 16 | 13 | Substance X | 1000 | mg/L | 13 | 0.0314 |
| 17 | 14 | Substance X | 1000 | mg/L | 16 | 0.1129 |
| 18 | 15 | Substance X | 1000 | mg/L | 16 | 0.1129 |
| 19 | 16 | Control | 0 | mg/L | 153 | 0.9991 |
| 20 | 17 | Control | 0 | mg/L | 144 | 0.9754 |
| 21 | 18 | Control | 0 | mg/L | 163 | 1.0240 |
| 22 | 19 | Substance Y | 10 | mg/L | 20 | 0.2005 |
| 23 | 20 | Substance Y | 10 | mg/L | 19 | 0.1804 |
| 24 | 21 | Substance Y | 10 | mg/L | 21 | 0.2197 |
| 25 | 22 | Substance Y | 100 | mg/L | 13 | 0.0314 |
| 26 | 23 | Substance Y | 100 | mg/L | 12 | 0.0000 |
| 27 | 24 | Substance Y | 100 | mg/L | 13 | 0.0314 |
| 28 | 25 | Substance Y | 300 | mg/L | 12 | 0.0000 |

Figure 1: Data structure for a typical toxicity test in OpenOffice Calc. Note that the response column is calculated (see text).

If the spreadsheet from Figure 1 (which can be found at <http://www.uft.uni-bremen.de/chemie/ranke/data/drfit/>) would be exported by writing a CSV file, this file could be processed by something like

```
> d <- read.csv('sampledata.csv', skip=2, dec=',')
```

depending on the path to the CSV file, the number of lines before the column headings and the decimal separator used.

Fitting and plotting

A quick result for a compatible dataframe can usually be obtained by a simple call to `drfit`

```
> rXY <- drfit(XY)
```

The contents of the dataframe `rXY` containing the results of the fitting procedure are shown in Figure 2. Each fitted dose-response model (usually only one per substance) produces one line. The number of dose levels `nd1` is reported, the total number of data points used for the model fitting `n`, the decadic logarithms of the lowest dose `l1d` and the highest dose `l1d` tested.

The next column contains the type of the dose-response model fitted (probit, logit, weibull or linlogit) or, if not applicable, a classification of the substance data as "active" (if the response at the lowest dose is < 0.5), "inactive" (if the response at the highest dose is > 0.5) or "no fit".

The log ED_{50} is given with the standard deviation as calculated by the `nls` function. This only works if the log ED_{50} is one of the model parameters. Therefore, in the case of the weibull model, no standard deviation of this parameter is given. Giving the argument `conf=TRUE`, a confidence interval for the log ED_{50} is given for an two-sided α of 5 % instead of the standard deviation.

Finally, the residual sum of squares `sigma` is listed and the fitted parameters `a` and `b`, or, in the case of the three parameter model `linlogit`, the parameters `a`, `b` and `c` are listed.

Once the `drfit` function has been successfully called and the result assigned a name (`rXY` in this case), dose-response plots for the fitted data can easily be created using the `drplot` function. The following example produces a single graph (`overlay=TRUE`) with the fitted dose-response curves and raw data with standard deviation bars for all substances and fitted models in dataframes `XY` and `rXY` using color (`bw=FALSE`) as shown in Figure 3.

```
> drplot(rXY,XY,overlay=T,bw=F)
```

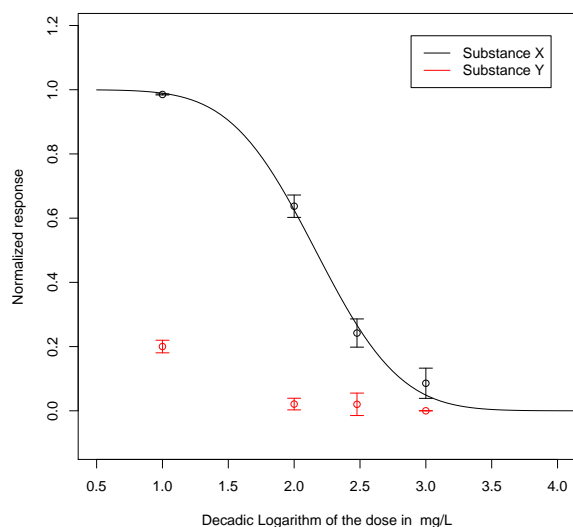


Figure 3: Output of the `drplot` function for the sample data from the package.

If the user prefers to view the raw data, the argument `dtype` can be set to "raw". The default is "std" for showing standard deviations as in Figure 3, another possible value is "conf" for showing confidence intervals.

Additionally, the scatter of the responses in control experiments can be displayed, by setting the argument `ctype` to "std" or "conf":

```
> drplot(rXY,XY,overlay=T,bw=F,
ylim=c("auto",1.3),dtype="raw",ctype="conf")
```

This results in a slightly customized plot as shown in Figure 4

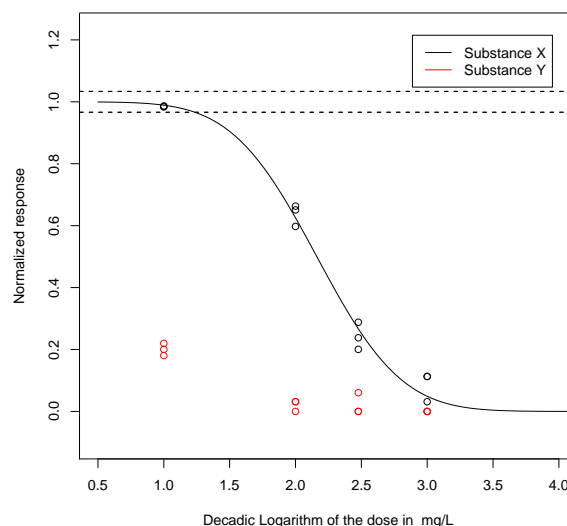


Figure 4: Output of the `drplot` function for the sample data from the package.

In the following, the analysis of a somewhat more complicated, but also more interesting example is illustrated, which has been published by Ranke et al. (2004) before the basic `drfit` code was packaged.

First, dose-response curves with the default settings of `drfit` are generated as shown in Figure 5.

```
> data(IM1xIPC81)
> dIM <- IM1xIPC81
> rIM <- drfit(dIM)
> drplot(rIM,dIM,overlay=T,bw=F)
```

```
> print(rXY,digits=2)
  Substance ndl n  lld  lhd  mtype logED50  std unit sigma  a  b
1   Control  1 6 -Inf -Inf inactive    NA    NA mg/L    NA  NA  NA
2 Substance X  4 3   1   3  probit   2.2 0.024 mg/L 0.041 2.2 0.51
3 Substance Y  4 3   1   3  active    NA    NA mg/L    NA  NA  NA
```

Figure 2: Contents of the dataframe containing the results from the fitting procedure for example data from the package (see text for explanations).

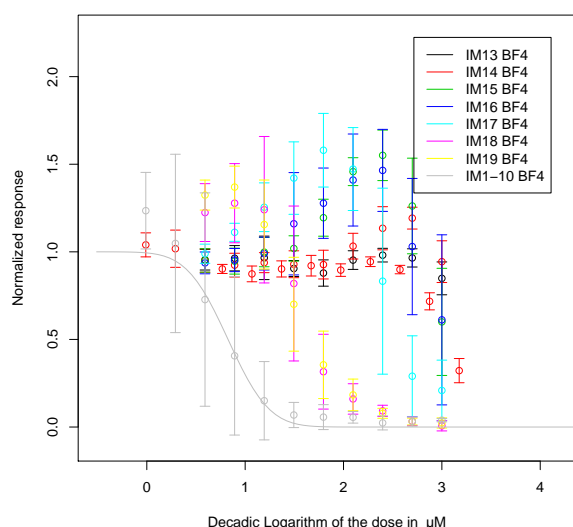


Figure 5: Dose-response plot showing the toxicities in a homologous series of compounds and the fitted probit model for IM1-10 BF4.

The graph shows that only one dose-response curve is fitted with the built-in default arguments of the `drfit` function and that the legend is interfering with the data. It is obvious that for almost all substances in this data, response values > 1 are caused in a certain dose range, a phenomenon which is called hormesis. In order to properly model such data, the so-called linear-logistic dose-response model has been introduced by Brain and Cousens (1989). The `drfit` package makes use of it in the parameterization suggested by van Ewijk and Hoekstra (1993), which allows for a convenient calculation of confidence intervals of the ED_{50} .

To include the linear-logistic model (`linlogit` in `drfit` terminology) in the fitting procedure and list the results including confidence intervals one simply calls

```
> rIM2 <- drfit(dIM,linlogit=TRUE,conf=TRUE,
chooseone=FALSE)
```

First, the `linlogit` argument causes the `linlogit` model to be additionally tried. Then, the argument `chooseone=FALSE` leads to reporting one line

for each fitted model. If the argument `chooseone` is set to `TRUE` (default), only the first convergent dose-response model (probit and `linlogit` in this case) from the somewhat arbitrary sequence `linlogit > probit > logit > weibull` is reported.

The dataframe with the results shown in Figure 6 accordingly lists all instances of fitted models, and gives confidence intervals for the $\log ED_{50}$ values.

Then, a customized plot can be generated:

```
> drplot(rIM2,dIM,overlay=T,bw=F,
xlim=c("auto",5))
```

The `xlim` argument to `drplot` fixes the interference between legend and data. Furthermore, the plot produced in the above example shown in Figure 7 shows two fitted dose-response curves for the substance IM1-10 BF4 (grey lines), one for the probit and one for the `linlogit` model.

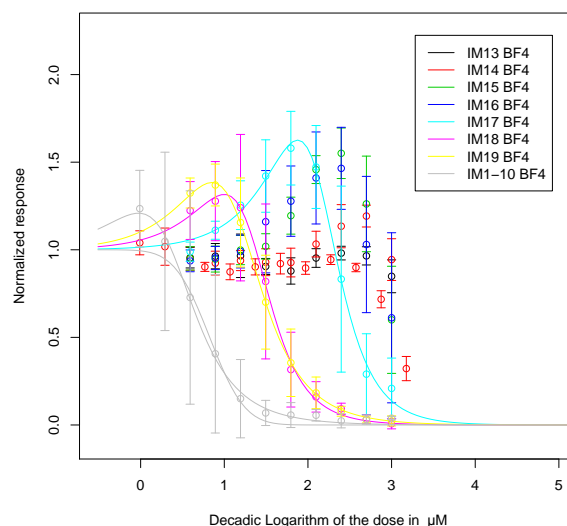


Figure 7: Dose-response plot showing the dose-response curves for a homologous series of compounds and all fitted `linlogit` and probit models.

External databases

Certain screening bioassays can be carried out with relatively low investments of time and money,

```
> print(rIM2,digits=2)
```

| | Substance | ndl | n | lld | lhd | mtype | logED50 | conf | unit | sigma | a | b | c |
|---|------------|-----|-----|--------|-----|----------|---------|-------|------|-------|------|------|-------|
| 1 | IM13 BF4 | 9 | 81 | 0.592 | 3.0 | inactive | NA | NA | µM | NA | NA | NA | NA |
| 2 | IM14 BF4 | 20 | 216 | -0.010 | 3.2 | no fit | NA | NA | µM | NA | NA | NA | NA |
| 3 | IM15 BF4 | 9 | 135 | 0.592 | 3.0 | inactive | NA | NA | µM | NA | NA | NA | NA |
| 4 | IM16 BF4 | 9 | 108 | 0.592 | 3.0 | inactive | NA | NA | µM | NA | NA | NA | NA |
| 5 | IM17 BF4 | 9 | 81 | 0.592 | 3.0 | linlogit | 2.58 | 0.076 | µM | 0.24 | 2.58 | 2.30 | 0.015 |
| 6 | IM18 BF4 | 9 | 135 | 0.592 | 3.0 | linlogit | 1.68 | 0.059 | µM | 0.23 | 1.68 | 2.24 | 0.057 |
| 7 | IM19 BF4 | 9 | 81 | 0.592 | 3.0 | linlogit | 1.65 | 0.052 | µM | 0.15 | 1.65 | 1.98 | 0.110 |
| 8 | IM1-10 BF4 | 11 | 162 | -0.010 | 3.0 | linlogit | 0.77 | 0.082 | µM | 0.30 | 0.77 | 1.94 | 0.458 |
| 9 | IM1-10 BF4 | 11 | 162 | -0.010 | 3.0 | probit | 0.83 | 0.085 | µM | 0.31 | 0.83 | 0.33 | NA |

Figure 6: Contents of the dataframe containing the results from the fitting procedure for the chain length data IM1xIPC81 from the package (see text for explanations).

so large volumes of dose-response data can built up (high-throughput screening/high-content screening). The drfit package allows to retrieve data stored in databases accessible by ODBC using the RODB package internally. Since RODB works both on Windows, Mac OS X and Unix platforms, the code is platform- and database independent to a high degree.

For storing cytotoxicity data in a MySQL database, the following minimum database definition is advisable:

```
CREATE TABLE 'cytotox' (
  'pk' int(11) unsigned NOT NULL auto_increment,
  'plate' int(11) NOT NULL default '0',
  'experimentator' varchar(40) NOT NULL
    default '',
  'substance' varchar(100) NOT NULL default '',
  'celltype' varchar(20) NOT NULL default '',
  'conc' float NOT NULL default '0',
  'unit' set('unit1','...') default 'unit1',
  'viability' float NOT NULL default '0',
  'performed' date NOT NULL
    default '0000-00-00',
  'ok' set('not ok','ok','?', 'no fit')
    default '?',
  PRIMARY KEY ('pk'),
)
```

The pk and the performed data field are not interpreted by the package, databases with any other columns missing have not been tested.

The column called viability contains the normalised response that has been calculated at the time of the data import into the database. Of course, the Data Source has to be configured to be a valid and accessible ODBC DSN on the platform used, e.g. by installing and configuring unixodbc and myodbc under Linux or MyODBC under Windows. This also involves setting up the MySQL server to listen to network connections, if it is not located on the local computer, and adequate MySQL user privileges.

With such a setup, the drdata function from the package can be used to conveniently retrieve data

from the database and evaluate it with the drfit and drplot functions:

```
> s <- c("Sea-Nine", "TBT", "ZnPT2")
> d <- drdata(s, experimentator = "fstock",
  whereClause="performed < 2006-04-04")
> r <- drfit(d, linlogit=TRUE)
> drplot(r, d, dtype="none", bw=F, overlay=T)
```

The whereClause argument to the drdata function allows for flexible selection of data to be used for the analysis, e.g. by using comparison operators on columns containing dates as illustrated in the above example.

Additionally, the use of the argument dtype="none" to the drplot function is shown, which leads to the display of the fitted models only, without the data, as shown in Figure 8.

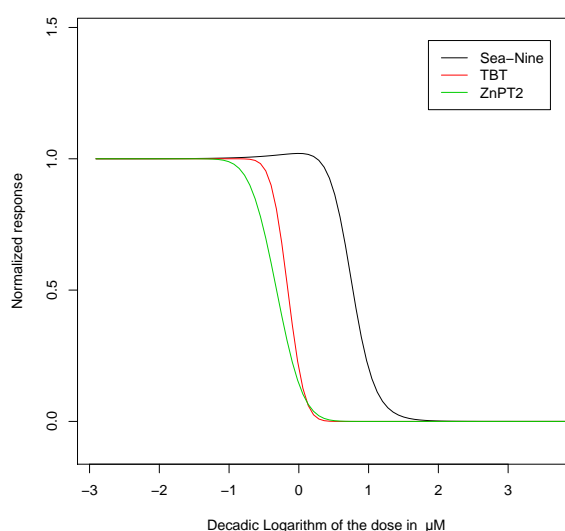


Figure 8: Dose-response plot showing the fitted dose-response curves for three antifouling biocides in the cytotoxicity assay fitted with the linlogit model.

In the UFT Center of Environmental Research and Technology, we use the *drfit* package for regular batch-processing of all our dose-response data from several bioassays for a substance library of more than 200 compounds. The results are in turn written to a database, and the *drplot* function is used to create updated dose-response plots every time the raw data has been augmented. The whole process of fitting all data and producing the plots takes less than 5 minutes on an 950 MHz AMD Duron PC for all the cytotoxicity data we have, provided that the new data has been checked by the *checkplate* and *checksubstance* functions, which allow for an easy validation of experimental dose-response data generated by plate-reader bioassays stored in a *drfit* conformant MySQL database.

The whole system provides the basis for analysis of the toxicity data, e.g. by (Quantitative) Structure-Activity Relationships (SAR/QSAR), which are the topic of other contributions to this issue.

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Johannes Ranke

Department of Bioorganic Chemistry

UFT Center for Environmental Research and Technology

University of Bremen jranke@uni-bremen.de