MULTIVARIATE ANALYSIS OF ECOTOXICOLOGICAL DATA USING ORDINATION: DEMONSTRATIONS OF UTILITY ON THE BASIS OF VARIOUS EXAMPLES

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ABSTRACT

This paper describes the usefulness of the group of multivariate techniques belonging to ordination for the analysis of ecotoxicological data sets. It is argued that although ecotoxicologists often gather multivariate data sets, they usually do not evaluate them with techniques that can handle multivariate data. Ordination techniques enable the researcher to extract an underlying structure out of a data set (eg. differences in composition of macro-invertebrate community between sites) and, if measured, relate this structure to explanatory variables (eg. concentrations of toxicants at the same sites). Five example data sets are presented to illustrate the underlying theory and the possibilities of ordination techniques. Two methods are presented, one based on weighted summation (eg. Principal Component Analysis, PCA) and one on weighted averaging (eg. Correspondence Analysis, CA). These techniques differ in the shape of the modelled response (linear versus unimodal) as the type of data they model (absolute versus relative). Results of these two methods are illustrated using a data set comprising levels of different PCB congeners measured in the blood of Adélie penguins in three periods. After this the constrained forms of PCA and CA are discussed, ie. constrained means that they are able to optimally display the differences in species composition (here levels of PCB congeners) due to explanatory variables (here period). Further examples illustrate the use of covariables, continuous and nominal explanatory variables, supplementary explanatory variables and forward selection of explanatory variables. Finally, two examples of Principal Response Curves (PRC) analyses are given. PRC is a technique that is especially developed to analyse time-series in which a control or reference is present. The PRC results are discussed for a designed experiment and a monitoring data set. The paper ends with a discussion focussing on the comparison between ordination techniques and other multivariate techniques used in the field of ecotoxicology.

Keywords: multivariate analysis, principal component analysis, redundancy analysis, principal response curves, ordination.

INTRODUCTION

To study the influence of abiotic environmental variables on the biotic composition of ecosystems, multivariate techniques have been used for many years in ecology (Legendre and Legendre 1998). Dissimilarities in community composition between sampled sites are analysed and the differences detected are related to measured or observed variables such as pH, habitat quality and dissolved oxygen. In particular ordination has proved to be a very useful technique for this purpose because it results in a diagram (biplot or triplot) displaying both the sites and the species and, if measured, the environmental variables in a reduced space (Ter Braak 1995). It therefore enables the researcher to evaluate differences in species composition between sites and to identify the environmental variables responsible for these differences in a single analysis. This property of ordination is the main advantage over other multivariate techniques such as, for instance, clustering and similarity analysis.

In ecotoxicology, multivariate analyses have been used in recent years to display differences in community composition among treatments or sites and to relate these differences to imposed chemical treatment or measured chemical stress (see for instance, Van Wijngaarden *et al*. 1995; Shaw and Manning 1996; Sparks *et al*. 1999; Kedwards *et al*. 1999a, b). The multivariate analysis of designed experiments with artificial ecosystems (microcosms and mesocosms) became more informative by the new ordination technique Principal Response Curves (Van den Brink and Ter Braak 1999). The use of multivariate analysis, however, is not widely accepted yet in ecotoxicology (Maund *et al*. 1999). In this paper we try to demonstrate that multivariate techniques can be valuable in the analysis of a variety of ecotoxicological data. In this paper we will restrict ourselves to ordination techniques that operate on the original data set for its analysis and thus allow a direct interpretation in terms of the original variables, in most cases species (Ter Braak 1994 1995). These techniques are more direct than techniques that operate on (dis)similarity indices (eg. similarity analysis, clustering and multidimensional scaling). The techniques used in this paper and those based on (dis)similarity indices will be compared in the discussion section. Ordination techniques are capable of summarising very complex responses because they are not restricted to a single dimension (as for instance (dis)similarity analysis). When combined with Monte Carlo permutation testing not only is a graphical summary of the structure present in the data set obtained, but also the statistical significance of hypothesised differences (Ter Braak and Šmilauer 2002). This paper will present the analysis of five example data sets to illustrate the value of multivariate methods for the analysis of ecotoxicological data. We go beyond the traditional example in which a sample by species matrix is compared with a sample by environmental/explanatory variables matrix. In our examples, toxicity values, contaminant concentrations and physico-chemical parameters play the part of the species; and time, geographical position, chemical treatment and molecule characteristics play the part of explanatory variables.

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BACKGROUND OF ORDINATION

Scientific investigation does not always allow the collection of species and environmental data simultaneously, often only species data is gathered to monitor changes in time. The data gathered can be comprised of samples containing either chemical or biological species or both. Ordination is able to express differences in species composition between samples without the use of measured environmental or explanatory variables. In such an analysis, ordination constructs imaginary, latent explanatory variables which maximise the variation in species composition between sites, ie. which best represent the underlying structure in the data set (Ter Braak 1995). The first latent variable is constructed in such a way that it explains the largest part of the total variance, the second one the largest part of the remaining variance etc. The first two latent variables are normally used to construct an ordination diagram of which they form the axes. Samples and species are represented in the diagram by points (or arrows) plotted at the scores (values) they have on the latent variables (see for instance Figure 1). Samples with nearly identical species composition lie close together in the diagram, while samples that lie far apart have very different species composition. In biplots, arrows (for species or environmental variables) point in the direction of higher values. In the example section a precise interpretation of ordination diagrams will be given.

When explanatory variables are measured, they can be included in the analysis in two ways. First, the explanatory variables can be laid over the ordination diagram using their values at the different sites. After the ordination analysis of the species by sample matrix they are simply regressed upon the latent variables. This analysis is called an indirect or unconstrained analysis with supplementary explanatory variables, ie. the explanatory variables do not play an active role within the analysis. The analysis can also be constrained to the part of the variance that is captured by the explanatory variables. The analysis is performed using this explained variance only, and the latent variables constructed are a linear combination of the measured explanatory variables. This analysis is called a direct or constrained analysis. The row entries of Table 1 summarise the distinction between unconstrained and constrained analysis.

Sometimes the effects of some explanatory variables can a priori be expected but one is not interested in the effects of these variables. On the one hand, these variables are important leading factors influencing the data set, on the other hand one does not want these variables to dominate the analysis. The variance explained by such variables can be excluded from the analysis. The resulting analysis is called a partial ordination or an ordination with covariables. Covariables can be used in both unconstrained and constrained ordination.

We discuss two groups of methods: methods based on Principal Component Analysis (PCA) and methods based on Correspondence Analysis (CA). PCA is based on a linear response model relating species and environmental variables, whereas CA can be derived from a unimodal response model (column entries of Table 1). In

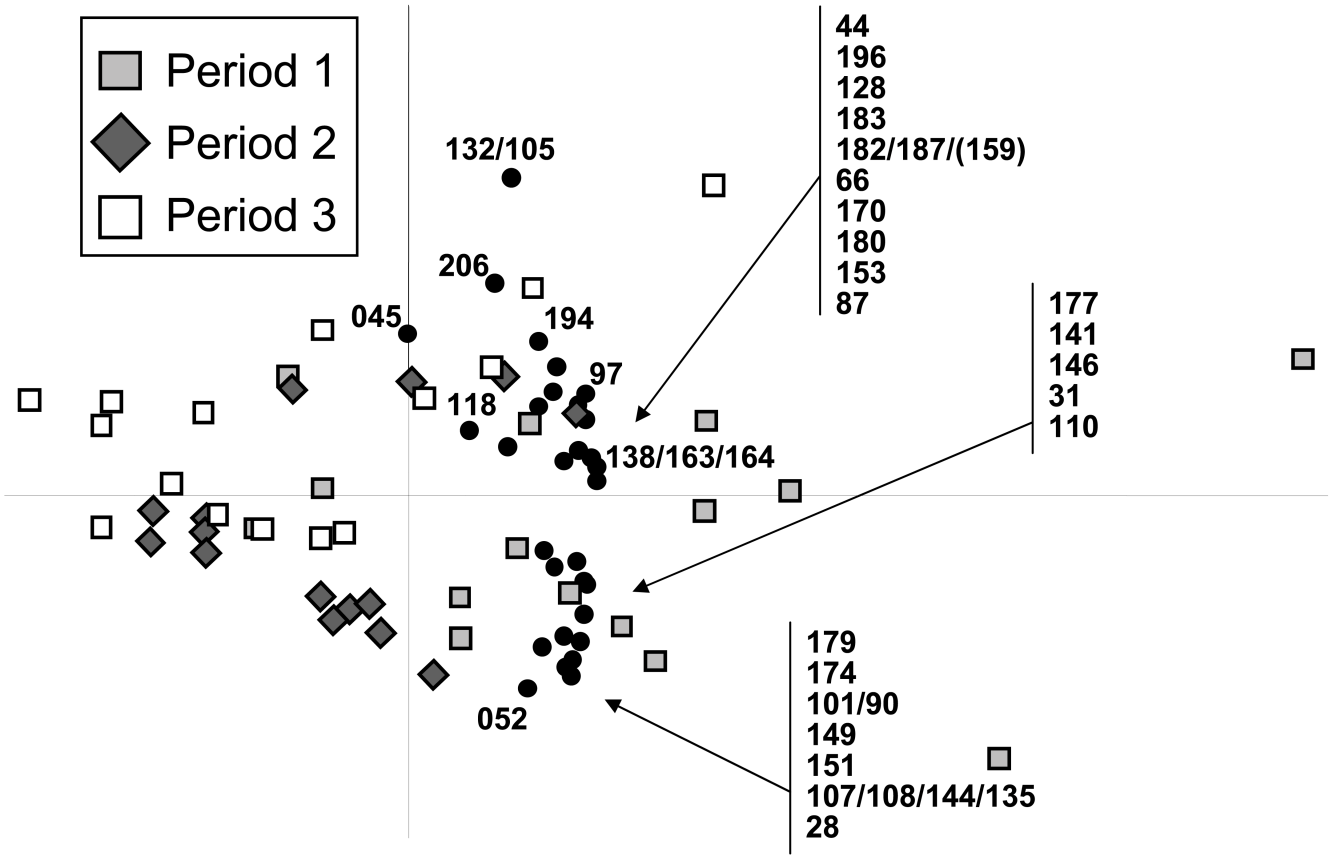

Figure 1. *PCA biplot, with focus on samples, showing the absolute differences in concentrations of PCB congeners in the different penguin blood samples. The different numbers refer to the different PCB congeners, eg. the number 177 refers to the PCB177 congener. Of all the variation in concentration of PCB congeners, 55% is displayed on the first axis, and another 12% on the second one. For clarity samples taken at period 1, 2 and 3 are represented by different symbols.*

Table 1. *Overview of multivariate methods used in this paper arranged by their characteristics*

	Weighted summation – linear response model – analysis of absolute differences	Weighted averaging – unimodal response model – analysis of compositional differences
Unconstrained analysis – analysis of all species variation – indirect analysis of species – environment interaction	Principal Component Analysis (PCA) with or without supplementary explanatory variables	Correspondence Analysis (CA) Detrended Correspondence Analysis (DCA)
Constrained analysis – analysis of species responses to explanatory variables – direct analysis of species – environment interaction	Redundancy Analysis (RDA), Principal Response Curves (PRC)	Canonical Correspondence Analysis (CCA) Detrended Canonical Correspondence Analysis (DCCA)

ecology mainly CA is used because it fits the concept of niche-space partitioning with species rising and falling along an ecological gradient (Ter Braak 1995). In this model each species can be characterised by its position (optimum or centre) along the gradient. This position is estimated in CA by weighted averaging, whereas the score of a species in PCA is estimated by linear regression, which in the case of PCA simplifies to a weighted summation (Table 1; Ter Braak 1995; Legendre and Legendre 1998). The linearity of PCA must not be misinterpreted, as PCA is well able to show non-linear patterns (eg. Figure 1). Sometimes the sampled sites represent only a small part of this ecological gradient, eg. when only sites with a pH value between 7.0 and 7.5 are sampled. In these cases a linear model is used because it describes the rise (or fall) along a short gradient better than a unimodal one. In ecotoxicology it may be expected that a species normally does not have an optimum along the gradient of a stressor. Therefore attempts to use multivariate techniques in ecotoxicology mainly focussed on linear methods (Van Wijngaarden *et al.* 1995; Kedwards *et al.* 1999a and b).

The choice to either use PCA or CA can be based on two characteristics, the length of gradient present in the data set and the type of data gathered. The length of gradient is a measure for the degree of unimodality of a latent variable (ordination axis). If a length of gradient of the latent variable is short, PCA is the model to choose, and when it is long CA is best (Table 1). This length of gradient can be calculated using Detrended Correspondence Analysis (DCA), a method we will not discuss in this paper because of its limited use for ecotoxicology. For a description and the background of the DCA method is referred to Ter Braak (1995). A general rule of thumb is that when the length of gradient is longer than 4 Standard Deviations (SD), the species data clearly show a unimodal response along the latent variable and CA is preferred. Another difference between CA and PCA is that CA models relative abundance instead of the absolute abundance, which is modelled using PCA (Ter Braak and Šmilauer 2002). The methods we discuss are cross-classified in Table 1.

INDIRECT AND DIRECT ORDINATION: THE PENGUIN EXAMPLE (1)

The dynamics of the concentrations of PCB congeners in the blood of Adélie penguins were studied during the breeding season (Table 2). From 15 birds, blood samples were collected during three periods: 1) egg laying period, a period prior to breeding in which the birds starve for a prolonged period on the nest; 2) egg-hatching period in which parental birds takes shifts on the nest while the other goes out to sea foraging and 3) the crèche-stage when chicks are left by the parents, although each parent returns regularly to feed the chicks (Van den Brink *et al.* 1998). This resulted in 45 blood samples (15 individuals times 3 periods) which were analysed for concentrations of 30 different PCB congeners by GC-electron capture detection (ECD). All procedures and the univariate evaluation of the data are described in detail by Van den Brink *et al.* (1998). The concentrations were ln-transformed before analysis. All multivariate analyses in this paper were carried out using the Canoco for Windows package, version 4.5 (Ter Braak and Šmilauer 2002). In Canoco, one can choose between predominantly interpreting relationships among samples or among species from the ordination diagram. In our case scaling was focussed on inter-sample distances because differences between periods were of interest, for all other questions the default options were chosen.

Since the length of gradient of the data set was very short (0.6 SD), the data are analysed by PCA. Figure 1 shows the ordination diagram resulting from the PCA analysis of the 45 samples by 30 PCB species matrix. In this diagram, the blood samples are represented by squares and species (different PCB congeners) by circles. (In a linear biplot, species could be represented by arrows by connecting the points with the origin, but because many species are analysed simultaneously this would have resulted in a cluttered biplot.) Figure 2 gives an example of the interpretation of linear biplots. In Figure 2 the circle denoting the species point for the grouped congener species 138/163/164 is enlarged a bit and not filled for clarity. For the interpretation a help line is drawn through this species point and the origin of the plot. When all sample points are projected perpendicularly onto this line, as is shown for a few samples as an example in Figure 2, the fitted concentration of the PCB congener can easily be ordered from high to low. Sample A has the highest

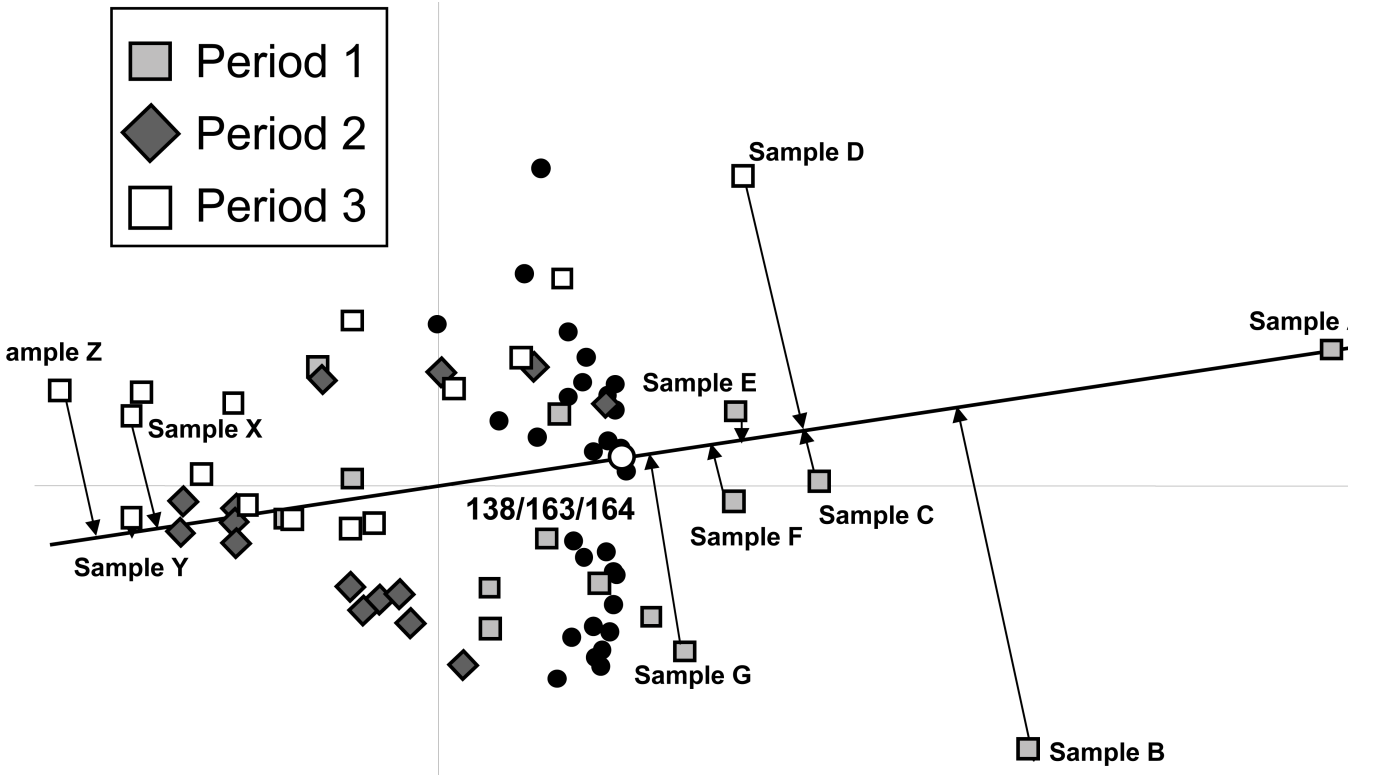

Figure 2. *PCA biplot, with focus on samples, that shows an example on how to interpret biplot ordination diagrams. The numbers refer to the different PCB congeners, ie. the 138/163/164 point represents the summated levels of the congeners PCB138, PCB163 and PCB164. The interpretation is only shown for a few example "sample points" for clarity. For further explanation see text.*

Table 2. *Overview of data sets analysed*

Example	Methods	Species	Samples	Explanatory
Penguin	PCA/RDA/CCA	PCB congeners	samples	period
Leces	Partial RDA	species	samples	Factory; date as covariable
Toxicity	PCA	toxicity and BCF	chemicals	characteristics
Carbendazim	PRC	species	samples	treatment
STP effluent	PRC	physico-chemical	samples	position

fitted concentration, sample B the next highest fitted concentration and so on for the samples C, D, E, F and G (Figure 2). Low fitted concentrations occur at the opposite end of the line (samples X, Y and Z), with the lowest concentration in sample Z. The distance between the species point and the origin of the diagram is a relative measure for the magnitude of the differences in concentrations between the samples, as indicated by the biplot. Thus, the further away the species point is from the origin, the larger the differences in PCB concentrations between the samples, as indicated by the biplot, are.

The diagram can be evaluated in terms of the percentage variance displayed. The first principal component (latent variable, ie. horizontal axis of the biplot) displays 55% of the total variance in concentrations of PCB congeners between the samples, the second one (vertical axis) 12% (Figure 1), so together the axes display 67% of the variance. The third axis explained only 8% of the total variation, and is therefore left out of consideration. Hence by applying PCA the "species by sample" matrix is reduced from 30 (species) dimensions into 2 dimensions, retaining three-quarters of its variance. Most PCB congeners are placed on the right side of the origin of Figure 1, which shows that the concentrations of most congeners are somewhat collinear with each other. The concentrations tend to be high in samples placed on the right side of the origin and low in those placed on the left side. Figure 1 also shows differences between periods. Samples taken in period 1 are placed somewhat at the right side of the diagram, those taken in period 2 to the left-down and those taken in period 3 left-upper part of the diagram. This arrangement indicates that concentrations of most PCB congeners were generally higher in the samples taken in period 1 compared to those taken in periods 2 and 3. The concentrations of PCB congeners placed in the right-upper quadrant had especially low concentrations in samples taken in period 2, while those placed in the right-down quadrant are indicated to have the lowest concentrations in samples taken in period 3.

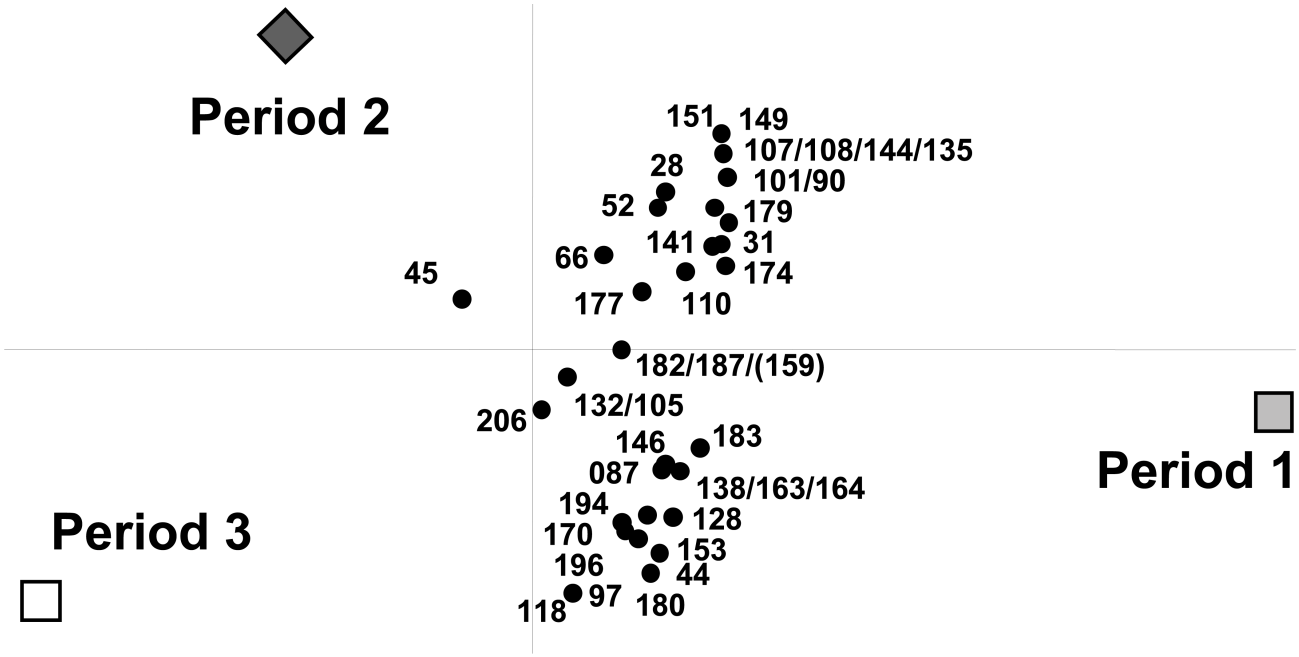

Figure 3. *A RDA biplot, with focus on samples (represented by periods), showing the absolute differences in concentrations of PCB congeners between the different periods. The numbers refer to the different PCB congeners. Of all variation in concentrations of PCB congeners in the penguin blood samples, 24% was explained by the nominal explanatory variables representing the periods. Of this explained variance, 84% is displayed on the first axis, the remaining 14% on the second one. For clarity the explanatory variables representing period 1, 2 and 3 are represented by different symbols. Indicated differences are significant between all periods ($p < 0.05$, Monte Carlo permutation tests).*

Although Figure 1 indicates differences in concentrations of PCB congeners among the periods, these differences are not optimally displayed. This data set contains variation due to differences between periods, individual penguins and measurement error, although the latter can not be distinguished from the second because no replicate samples were taken. The PCA biplot focuses on the differences in concentrations of PCB congeners between samples. The information on the period in which a sample was taken is added post-hoc, ie. after the analysis has been carried out. This is called indirect or unconstrained ordination. The interpretation of the indicated differences between samples takes place in an indirect way, after the analysis has been performed. Redundancy Analysis (RDA) is the direct form of PCA that enables the researcher to focus the analysis on that particular part of the variance that is explained by external explanatory variables (Table 1). In our example we used three nominal (1/0) variables denoting the period in which a sample was taken as explanatory variables. The resulting RDA biplot (Figure 3) focuses on the differences in concentrations of PCB congeners between periods. For clarity, the samples are not displayed, their important feature, ie. the period in which they were sampled, is represented by the placement of the three explanatory variables denoting the periods. As in the PCA biplot, higher concentrations of most PCB congeners are indicated for period 1, the egg-laying period, and lower ones for periods 2 and 3. This can be explained by the atrophy of the pectoral muscle during period 1 (Van den Brink *et al*. 1998). This muscle is a sink of PCBs, which are released into the blood during atrophy. In period 2 this muscle recovers due to the foraging in the sea so concentrations of PCBs go down. In period 3 a part of fat-reserve is consumed but Van den Brink *et al*. (1998) argue that this is only a small sink for PCBs, compared to the large pectoral muscle, so the concentrations do not change that much during that period. Of the total variance almost a quarter could be explained by the indicator variables denoting period (Figure 3). Of this variance the majority is displayed on the first axis (84%), the remaining part on the second one. RDA can be followed by Monte Carlo permutation tests to test 1) whether PCB congener concentrations differ significantly between periods; 2) whether the first axis of the RDA biplot displays a significant part of the between period variation; and 3) whether differences between the individual periods are significant. All periods were tested against each other and all tests resulted in p-values < 0.01. The results of the PCA and RDA analyses of this data set show that ordination can provide a clear summary of the underlying structure of the data set. It also enables the researcher to focus on that part of the variance that is of interest, in this case the differences between periods. When constrained ordination is combined with Monte Carlo permutation tests, one can also test the significance of indicated differences. The results of the multivariate analyses are comparable to the univariate one, with the results of multivariate analysis showing more details for individual congeners.

RDA VERSUS CCA: THE PENGUIN EXAMPLE (2)

As mentioned in the background section, PCA and its constrained counterpart RDA focus on the absolute differences in 'species' abundances (here PCB levels) between the samples, whereas CA and its constrained counterpart CCA focus on relative differences. This difference between the two classes of methods is illustrated by Figures 3 and 4. Figure 4 shows the CCA biplot of the same data set as Figure 3. The interpretation of the diagram is analogous to the RDA biplot (since biplot scaling was used, see Ter Braak and Verdonschot 1995 for details). In the CCA analysis the nominal variables denoting the periods explained 14% of the total variance, of which approximately 50% is displayed on the first, horizontal axis and the remaining part on the second, vertical one. Again, we

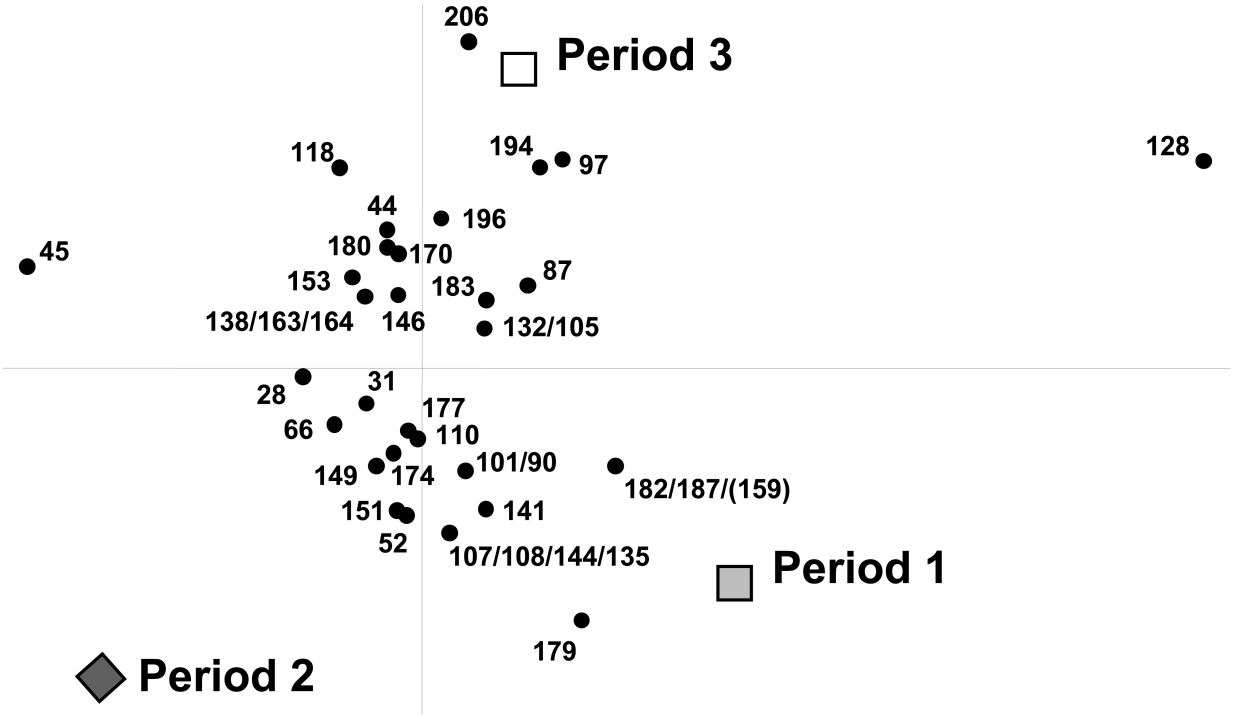

Figure 4. *A CCA biplot, with focus on samples, showing the compositional differences of PCB congeners between the different periods. The numbers refer to the different PCB congeners. Of all variation in levels of PCB congeners in the penguin blood samples, 14% was explained by the nominal explanatory variables representing the periods. Of this explained variance, 59% is displayed on the first axis, and the remaining 41% on the second one. For clarity the explanatory variables representing period 1, 2 and 3 are represented by different symbols. Indicated differences are significant between all periods (p < 0.05, Monte Carlo permutation tests).*

see a separation of the three periods; period 1 and 2 are placed at the bottom of the diagram, period 3 at the top. We also see that in this diagram, in contrast to the RDA biplot, not all congeners are placed on one side of the diagram, but scattered around the origin. This is a result of the fact that CCA displays proportional, relative differences. The figure indicates that the relative levels of some congeners are higher in period 1 and others in period 3. This could be a result of differences in susceptibility to metabolisation between the congeners. Compared to period 1, it may be that congeners most susceptible to metabolisation increased during period 2 due to feeding (foraging in the sea) and decreased during period 3 due to metabolisation. The same Monte Carlo permutation tests were performed as described in the RDA section and again all tests yielded p-values < 0.05. It is notable that a RDA using centring by samples and by species on the log-concentrations (ie. a log-ratio analysis; Aitchison 1990; Ter Braak and Šmilauer 2002) yielded almost identical results as the CCA analysis (results not shown).

The results of the RDA and CCA analyses show that ordination successfully detected both absolute and compositional (or relative) differences in levels of PCB congeners between the samples taken in the three periods.

CONTINUOUS AND NOMINAL EXPLANATORY VARIABLES AND COVARIABLES: THE LECES EXAMPLE

The Leces data set consists of abundance of macro-invertebrate species on five locations of the Leces River (East Java, Indonesia, Table 2). The community was sampled from the riverside three times between September and November 1991 at each location using the "kicking method", together with several physico-chemical parameters. Sampling station 1 was the most upstream station, sampling station 5 the most downstream one. Between sampling station 2 and 3 is a paper mill factory, the effluent of which was discharged into the river. The macro-invertebrate abundance data were ln(2x+1) transformed prior to the analysis (see Van den Brink *et al*. 2000 for rationale). Since the differences between sampling dates were not of interest, three nominal variables denoting the three sampling dates were introduced as covariables, ie. the part of the variance captured by these variables was excluded from the analysis. Sampling date explained 14% of the total variance in abundance values of the macro-invertebrate community between the samples. The remaining 86% can be attributed to differences between sites. Figure 5 displays the first two axes of a PCA of this remaining variation. Figure 5 shows clear differences between sites 1 and 2 on the one side (placed on the left side of the diagram) and 3, 4 and 5 on the other side (right side), suggesting effects of the effluent of the paper mill factory. For interpretation we also included nominal variables denoting the five sites, two indicating whether a sample was taken upstream or downstream of the factory, and the continuous variables pH, current velocity, conductivity, temperature, dissolved oxygen, hardness, chemical oxygen demand (COD) and ammonium concentration. In the resulting biplot, nominal variables

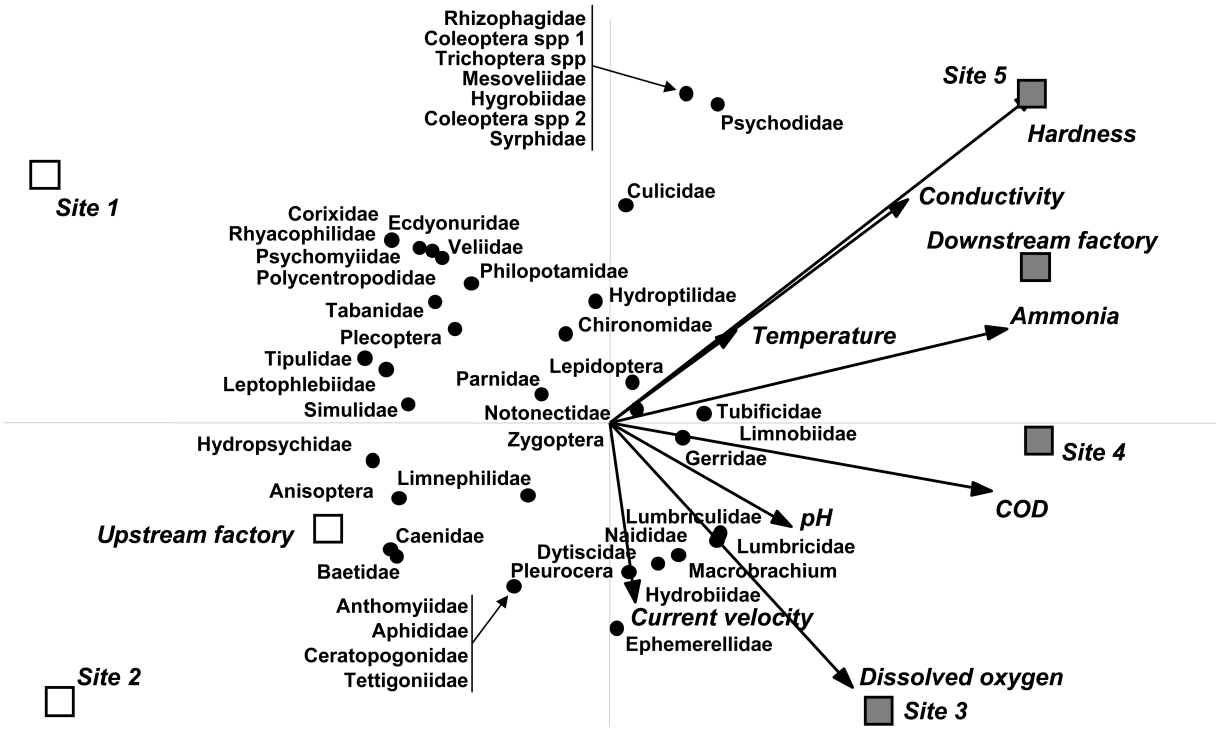

Figure 5. *A PCA biplot, with focus on samples, of species composition showing the within-date differences in species composition between samples taken upstream and downstream of the wastewater outlet in the Leces River. In this analysis, sampling date is defined as covariable and explained 14% of the total variance in species composition. Of the remaining variance, 41% is displayed on the first axis, and another 15% on the second one. The third axis (not shown) explained another 11% but was not related to the outlet. Also the relation with the supplementary variables sites and several physico-chemical parameters is displayed. For clarity the explanatory variables representing upstream and downstream factory are depicted by different symbols than those representing the different sites.*

are represented by points, whereas continuous variables are represented by arrows. These variables are supplementary as they do not influence the PCA itself. The biplot (Figure 5) suggests that most taxa (especially those belonging to Trichoptera, ie. Psychomyiidae, Hydropsychidae, Polycentropodidae, Philopotamidae, Rhyacophilidae and Limnephilidae) are negatively affected by the effluent and that the levels of indicators of pollution (COD, ammonia, hardness, conductivity) are indicated to have increased due to the effluent. Carrying out a partial RDA by defining the supplementary variables as true explanatory variables yielded exactly the same result as the previous partial PCA. The reason is that the number of explanatory variables, including covariables, is larger than the number of samples so that the explanatory variables do not really constrain the analysis (Ter Braak and Šmilauer 2002).

To test the statistical significance of the effluent of the mill factory, we carried out a RDA with factory as the only explanatory variable, and sampling date as nominal covariable. A Monte Carlo permutation test using random permutation indicated that the place relative to the factory has a significant influence on the macro-invertebrate community ($p < 0.05$). Random permutation takes the samples as independent whereas in practice there may be correlation of parameter values in space (along the river) and time. When these are taken into account (in a split-plot design with cyclic permutation of sites and of times, (see Ter Braak and Šmilauer 2002) the statistical evidence of an effect evaporates ($p > 0.40$). Note that the lack of statistical significance is not solely due to the reduced number of permutations in this advanced permutational scheme as the number yielding different F-ratios is still over 1500.

The example shows that partial ordination enables the researcher to exclude a part of the variance that is not of interest. It allows the researcher to yield an optimal summary of structures in the remaining variation and relate this structure to nominal and measured explanatory variables simultaneously.

CORRELATION TRIPLOT, SUPPLEMENTARY EXPLANATORY VARIABLES AND FORWARD SELECTION: THE TOXICITY EXAMPLE

Deneer *et al.* (1987; 1989) performed toxicity tests with an alga (*Chlorella pyrenoidosa*), crustacean (*Daphnia magna*), fish (*Poecilia reticulata*) and a bacterium (*Photobacterium phosphoreum*) with 13 mono-nitro compounds together with a bioconcentration experiment with the fish (Table 2). The goal of these experiments was to relate the toxicity of mono-nitro compounds to two physico-chemical parameters of these compounds, ie. to determine a Quantitative Structure – Activity Relationship (QSAR). The two parameters were the $\log(K_{ow})$ (octanol-water partitioning coefficient) and Hammett σ constants (a measure for the reactivity of the compound, for its transformation into the active n-hydroxy form). The toxicity was expressed by eight variables (EC50-, LC50- and/or LOEC-values for the test species and a BCF value for the fish). A PCA analysis was performed in which the compounds play the role of samples, the toxicity measures the role of species and the two physico-chemical parameters the role of supplementary explanatory variables (see background section). All toxicity and BCF values were ln-transformed and centred and standardised (giving mean = 0 and SD = 1 for each variable) within Canoco for Windows, and the scaling is focussed on variables, yielding a correlation bi- or triplot.

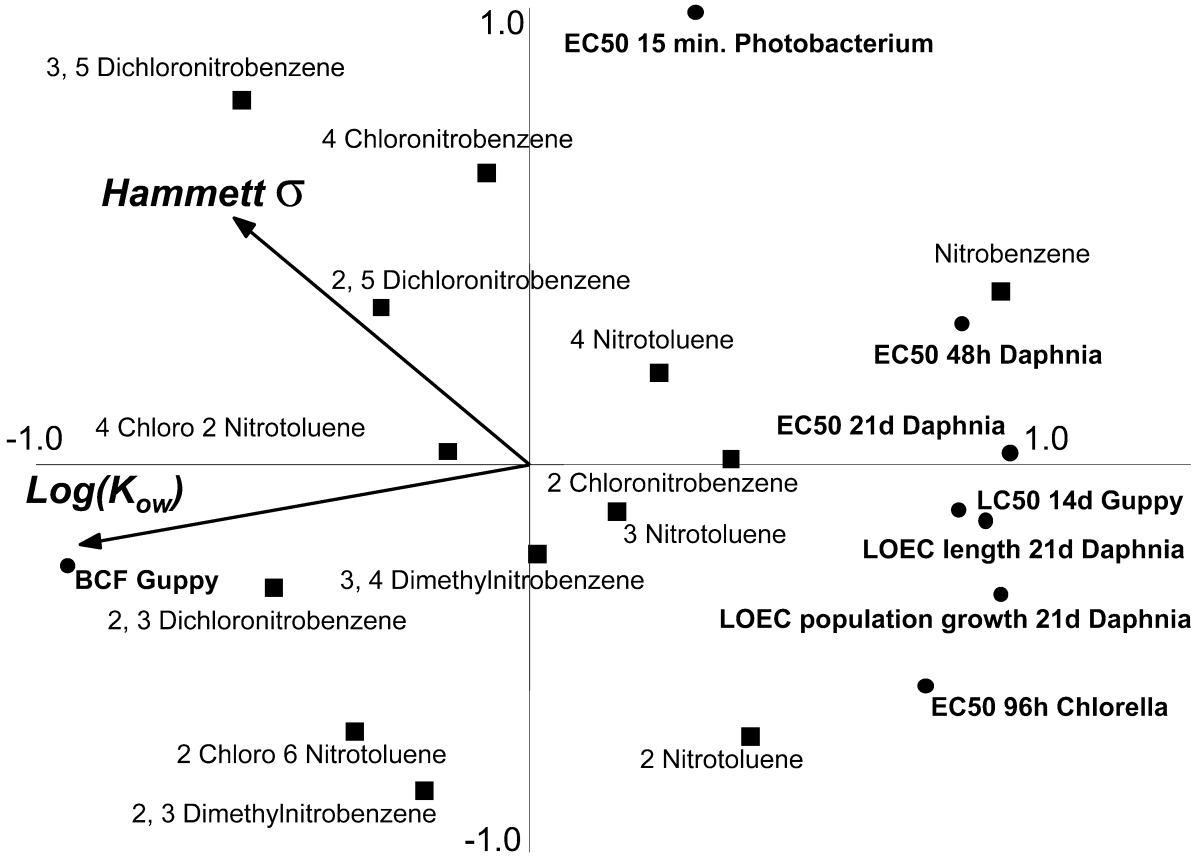

Figure 6. *PCA correlation triplot showing the relation between different mono-nitro compounds and their laboratory toxicity to several aquatic organisms and BCF for Guppy. Also the relation between two physical parameters and the toxicity is indicated. Of all variation in toxicity, 71% is explained by these two parameters, of which 89% is displayed on the first axis, another 10% on the second one. Of all variance, 73% is displayed on the first axis, another 15% on the second one. Both physical parameters explain a significant part of the variation in toxicity between the compounds (p < 0.05, Monte Carlo permutation test).*

Standardisation treats all variables equally important regardless of their variability in the data. In a PCA analysis without standardising the endpoint with the largest variability would influence the analysis most.

The triplot (Figure 6) shows that all toxicity values, except the EC50 of *Ph. phosphoreum*, have a high positive correlation with each other and, as expected, a negative one with the BCF of the fish. The log(K_{ow}) parameter has, as expected, a high positive correlation with BCF and herewith a negative one with most toxicity values. Also the Hammett σ constants have a negative correlation with all toxicity values except for *Ph. phosphoreum*; a high Hammett σ constant is associated with a low toxicity value (eg. a low EC50) and therefore a high toxicity. The different placement of *Ph. phosphoreum* might indicate that mono-nitro compounds do not only have an anaesthetic mode of action to this bacterium. The toxicity of mono-nitro compounds towards fish, *D. magna* and *C. pyrenoidosa* is somewhat higher than expected for compounds acting solely though an anaesthetic mode of action. This excess toxicity is probably caused by the formation of reactive N-hydroxy metabolites. The rate of transformation of these metabolites is probably related to the Hammett σ constant, which explains the significance of this variable. For a complete evaluation of this data set (and more), the reader is referred to Deneer *et al.* (1987; 1989). Together the two parameters explain 71% of the total variance in toxicity and BCF values between the compounds, of which 99% is displayed in the triplot.

Within Canoco, forward selection can be used for the ranking of the explanatory variables in importance for determining the species data. In this way a large set of environmental variables can be reduced to a meaningful small one. When forward selection in combination with Monte Carlo permutation tests is performed under the RDA option, first log(K_{ow}) is added to the model, explaining a significant 61% of the total variance ($p < 0.05$). After the inclusion of log(K_{ow}), the Hammett σ constant is added to the model explaining another significant 10% of the total variance ($p < 0.05$). Since the Hammett σ constant alone explains 31% of the total variance, both parameters share 21% of the total variance. So for this example both log(K_{ow}) and the Hammett σ constant explain a significant part of the toxicity and BCF values, although the two parameters share a large part of explained variance. Forward selection is an important technique to limit the number of explanatory variables to a set that best explains the variation in the species community (here being toxicity and BCF values). One should be aware of two problems (Legendre and Legendre 1998). First, the type I error is far greater than the nominal 5% level, because forward selection involves multiple testing, Bonferroni adjustment of the significance level may be useful. Second, forward selection may lead to other results than stepwise selection or backward elimination of explanatory variables; there is thus no guarantee that forward selection finds the best model. Despite these problems, forward selection of explanatory variables has great data-analytic utility.

PRC ON A DESIGNED EXPERIMENT: THE FUNGICIDE EXAMPLE

Principal Response Curves is a relatively new technique that is designed for the analysis of microcosm and mesocosm experiments (Van den Brink and Ter Braak 1999). It was first applied to the results of ecotoxicological experiments evaluating the effects of pesticides on freshwater ecosystems (eg. Van den Brink *et al*. 2000) but applications to terrestrial ecosystems (eg. Smit *et al*. 2002) and the analysis of ecological field experiments (eg. Frampton *et al*. 2001) followed rapidly. The Principal Response Curves method was developed to overcome very cluttered biplots when the information of many sampling dates and many treatments is displayed in one diagram and time is not expressed as a single direction in the biplot (see Kersting and Van den Brink 1997 as an example). In this section the application of PRC and the interpretation of first and second PRC diagrams will be discussed using an experiment described in Cuppen *et al*. (2000) and Van den Brink *et al*. (2000).

The semi-field experiment for the evaluation of chronic exposure to carbendazim consisted of indoor microcosms (1 m^3), which represented macrophyte-dominated drainage ditches. The systems were treated chronically for four weeks with 0, 3.3, 33, 100, 330 or 1000 µg/L carbendazim, with two replicates per concentration. The species composition of the phytoplankton, periphyton and invertebrate communities were monitored in time, together with chlorophyll-a content, various physico-chemical parameters and macrophyte biomass. For a more detailed presentation and evaluation of the results, see Cuppen *et al*. (2000) and Van den Brink *et al*. (2000). In this paper we use the macro-invertebrate data set, consisting of 86 different taxa, as an example (Table 2).

No consistent NOECs lower than 3.3 µg/L were recorded on the species level. Direct effects on macro-invertebrates became manifest following a treatment with 33 µg/L. Several "worm-like" taxa belonging to the groups of flatworms, leeches and oligochaete worms showed altered abundance values, together with two crustacean taxa. At this treatment concentration, indirect effects in the form of increases of several snail taxa, were also observed, indicating food-web changes due to increase of food resources of these species.

The effects of the carbendazim treatment at the macroinvertebrate community level were analysed by the Principal Response Curves method (PRC). The PRC method is a multivariate technique specially designed for the analysis of data from microcosm and mesocosm experiments and can be obtained using RDA (Van den Brink and Ter Braak 1998; 1999). The model for the first PRC is:

$$y_{d(j)tk} = y_{0tk} + b_k c_{dt} + \xi_{d(j)tk,}$$

where $y_{d(j)tk}$ is the log-abundance of species k in replicate microcosm j of treatment d at time t, y_{0tk} is the mean log-abundance of species k in week t in the control (d = 0), c_{dt} is the score of the d^{th} treatment at time t, b_k is the weight of the k^{th} species, and $\xi_{d(j)tk}$ is an error term with mean zero and variance σ_k^2. Note that by definition $c_{0t} = 0$ for every t. The model is fitted to data by an RDA, using nominal variables denoting sampling date as covariables and the product of sampling date and treatment levels as nominal explanatory variables. This RDA yields least-squares estimates of the treatment scores $\{c_{dt}\}$ and species weights $\{b_k\}$. See Van den Brink and Ter Braak (1998) and Ter Braak and Šmilauer (2002) for details.

PRC results in a diagram showing the sampling weeks on the x-axis and the first Principal Component of the variance explained by treatment on the y-axis (see c_{dt} values in Figure 7A for an example). This yields a diagram showing the deviations, in time, of treatments compared to controls. For instance, Figure 7A indicates that for the period after the start of the treatment, the greatest deviations from the controls occurred at the two highest treatment concentrations, while smaller deviations were found at the intermediate treatment concentrations. It also indicates minor differences relative to the controls at the lower treatment concentrations. The species weights shown on the right side of the diagram can be interpreted as the weight of each species for the response given in the diagram. Thus, the flatworm *Dugesia tigrina*, which has the highest weight in the diagram, is shown to have decreased most at the higher treatment concentrations. The negative weight of the snail *Lymnaea* juvenile in the diagram indicates that its numbers increased at the higher treatment concentrations. In quantitative terms, multiplying the weight b_k of species k by the regression coefficient c_{dt} of a treatment d at a particular sampling date t yields the fitted change on a log-scale of this species relative to the controls. In terms of abundance, taking the exponential of this quotient yields the relative abundance compared to the controls. For instance, the relative abundance of *Dugesia tigrina* indicated by the first PRC (Figure 7A) for the microcosms with the highest treatment concentration in week 3 is exp(4.14*-1.25) = 0.57% of the abundance in the controls.

Figure 7A shows the first PRC, expressing the most dominant effects of carbendazim on the composition of the macro-invertebrate data set. It shows clear deviations from the control for the four highest treatments concentrations (33 µg/L and higher). Between the treatment concentrations a clear dose-response was present, the higher the treatment concentrations the larger the deviations from the control. No indication of recovery was demonstrated. For all post treatment sampling dates a significant influence of the treatment regime as a whole was found ($p < 0.05$). This was tested by Monte Carlo permutation performed for each sampling date using ln-transformed treatment levels. For the sampling dates 1, 5, 7 and 9 post start of the treatment a $NOEC_{community}$ of 3.3 µg/L was calculated (Williams ANOVA test applied on first principal component, see Van den Brink *et al*. 1996 for more details). Monte Carlo permutation tests permuting whole time series indicated that the first PRC diagram displayed a significant part of the treatment variance ($p < 0.05$, see Van den Brink and Ter Braak 1999 for more details). The second PRC also displayed a significant part of the treatment variance, the third did not. This means that no single dose-response type is present in the data set but several sub-dominant ones. The model for two PRC components is:

$$y_{d(j)tk} = y_{0tk} + b_{k1} c_{dt1} + b_{k2} c_{dt2} + \xi_{d(j)tk,}$$

where the scores $\{c_{dt1}\}[t = 1\ldots T]$ represent the first principal response curve (PRC) for treatment d, ie. course of treatment d in time relative to the controls, the scores $\{c_{dt2}\}[t = 1\ldots T]$ represent the second principal response curve for treatment d, b_{k1} is the weight of species k on the first PRC, and b_{k2} is the weight of species k on the second PRC.

The second PRC is shown in Figure 7B and displays the most important deviations from the first PRC present in the data set. It shows relatively large deviations from the control for the 33 and

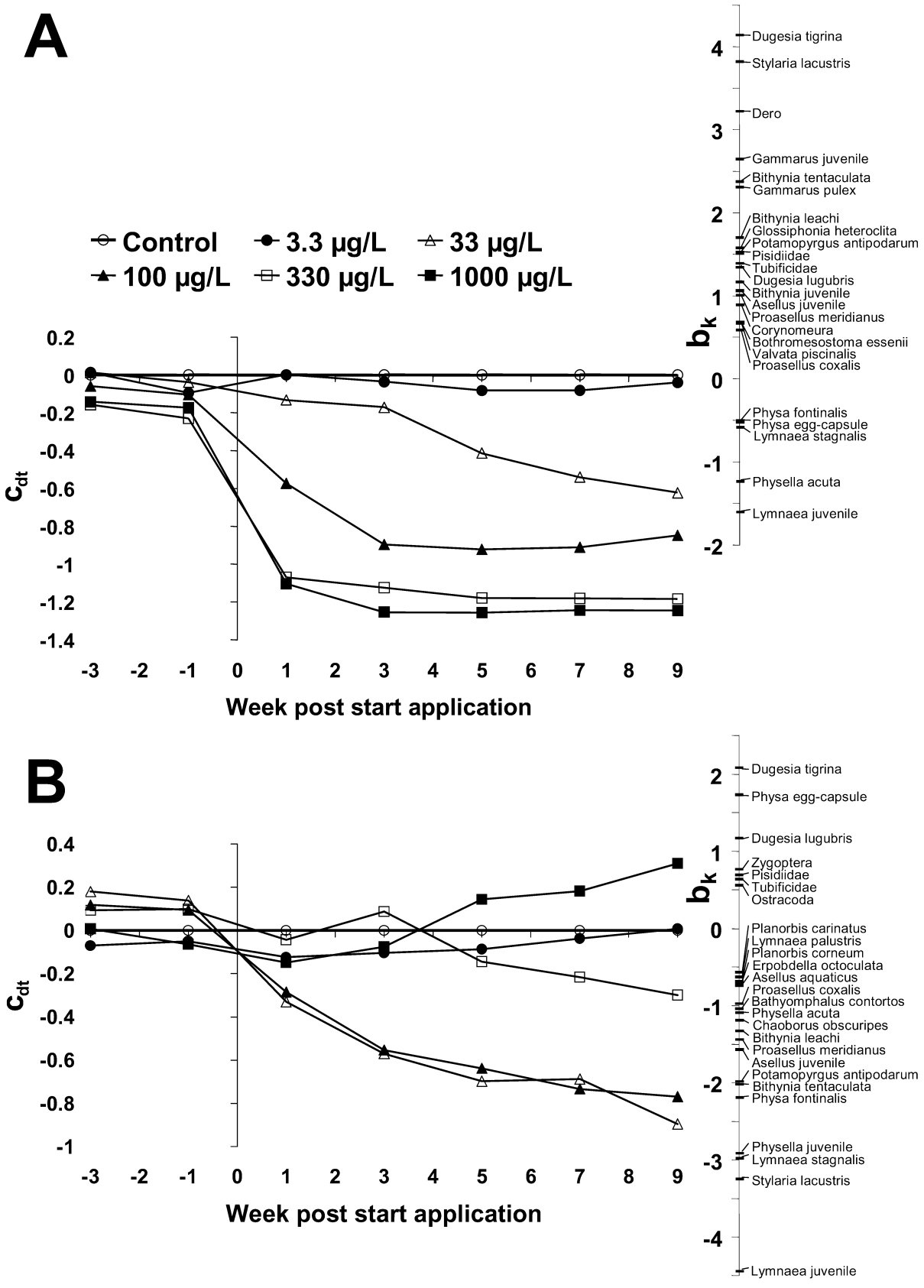

Figure 7. *First (A) and second (B) Principal Response Curves indicating the effects of the fungicide carbendazim on the macro-invertebrate community. Of all variance, 30% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-nine percent of all variance could be attributed to treatment, the remaining variance (21%) is between replicate variation. Of the treatment variance, 44% is displayed on the vertical axis of the first PRC (A), and another 17% on the vertical axis of the second PRC (B). The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the Principal Response Curves (c_{dt}). Taxa with a species weight between 0.5 and -0.5 are not shown for clarity. The treatment explained a significant part of the total variance, of which also a significant part is displayed in the first and second PRC ($p < 0.05$, Monte Carlo permutation test with permuting whole time series only). The third PRC did not display a significant part of the treatment variance and is thus not shown ($p > 0.05$).*

100 µg/L treatment concentrations. The second PRC thus differentiates between taxa showing a response at the four highest treatment concentrations and those showing a response at the two highest treatment concentrations only. For instance, *Dugesia tigrina* has a positive weight with both diagrams. When both diagrams are considered in the interpretation, the indicated response for this taxon is a sum of both diagrams, in which the diagrams are weighted by the species scores of *D. tigrina* (Van den Brink and Ter Braak 1998). This indicates that this taxon suffered more severely from the 33 and 100 µg/L carbendazim treatment than was indicated by the first PRC diagram alone (Figure 8, see Cuppen *et al*. 2000 for its real response). On the other hand *Stylaria lacustris* has a positive weight with the first and a negative weight with the second diagram. To deduce the indicated response of this taxon from the two PRC diagrams one has to subtract the second PRC from the first PRC diagram. The result of this subtraction indicates that this taxon only suffered from the carbendazim treatment in the two highest treatment concentrations (Figure 8). By doing so all taxa can be grouped on the basis of the shape of their response to the carbendazim treatment (see Figure 8 for a graphical representation). For instance, all taxa with a positive response with both diagrams (eg. *Dugesia lugubris*, *Dugesia tigrina*, Pisidiidae) are indicated to have decreased in all but the lowest treatment levels; all taxa with a positive weight with the first PRC and a small weight with the second one (eg. *Gammarus* juvenile, *Gammarus pulex* and *Dero* sp.) are indicated to have strongly decreased in the two higher treatment concentrations and only moderately in the intermediate ones; and all taxa having a positive weight with the first and a negative one with the second PRC (eg. *Stylaria lacustris*, *Potamopyrgus antipodarum*, *Bithynia tentaculata* and *Proasellus meridianus*) are only indicated to show effects for the two highest treatment concentrations. This grouping can be done for all combination of weights (Van den Brink and Ter Braak 1998). This example shows that PRC is able to show the response of a whole community into an easy to read diagram (Figure 7A), its outcome is representative for the most sensitive species ($NOEC_{community}$ = 3.3 µg/L), it can easily be combined with multivariate statistical testing (eg. Monte Carlo permutation test), and it is able to summarise very diverse response patterns when the second PRC is also taken into account.

PRC ON MONITORING DATA: THE SEWAGE TREATMENT PLANT EXAMPLE

PRC has only been applied to experimental data except in a study by Leonard *et al*. (2000). Leonard *et al*. (2000) took samples at several sampling dates at several sites of a river, some of which were influenced by endosulfan exposure. The non-exposed sites served as the undosed control, to which the endosulfan influenced samples were contrasted. In this way the experimental design needed for PRC (treatment and control) was imposed on the monitoring data.

Often, however, no undosed control is present, but a reference site can be assigned within the monitoring scheme. In this section, PRC will be applied to a monitoring data set is which a reference is assigned. Coad (2001) measured several physico-chemical parameters weekly at five sites: 1) 300 m upstream of a sewage treatment plant (STP) outlet, 2) 100 m upstream of the STP outlet, 3) in the STP outlet 4) 100 m downstream of the STP outlet and 5) 1 km downstream of the STP outlet (Table 2). The upstream part of the river (sites 1 and 2) was located in an urban area. In total 795 samplings were performed at the five sites in the period 1994 through 2002 to evaluate the performance of the STP. All details are described in Coad (2001). It is clear when this data set is analysed with PCA or RDA and its results are displayed in a biplot, a very cluttered and crowded diagram would be the result of the presence of many samples. Figure 9 shows the results of the PRC analysis using sampling month as covariable and the product of sampling month and site as explanatory variables. Site 3 (the outlet of the STP) was used as the reference because it has the most complete time-series. Figure 9 indicates the largest differences from the STP outlet for the two upstream sites. For these sites, compared to the outlet, lower levels of NOx, total nitrogen, conductivity, salinity, total phosphorus and temperature are indicated together with higher levels of turbidity and faecal coliforms. For the two downstream sites smaller differences are indicated, but in the same direction. From the PRC analysis it is clear that the outlet of the STP lead to an increase of concentrations of nitrogen and phosphorus, temperature and associated measures as conductivity and salinity in the river. After the outlet, values of these parameters decrease in the downstream sites, but not as low as in the upstream sites. The STP seems very successful in reducing faecal coliforms, their level is even lower in the outlet compared to the upstream sites which are slightly contaminated due to urbanisation. No trend in time is apparent, indicated differences are relatively stable in time with a few outliers. This example shows that PRC can also be used for the evaluation of (bio)monitoring data, even when no apparent control is present. PRC results in an easy to interpret overview of differences, even when the sites are sampled very often, because it displays time in a single direction in the diagram.

DISCUSSION

In the ecotoxicological literature, Canonical Variate Analysis (CVA also called Discriminant Analysis, DA) is often used to elucidate which combination of variables discriminate best between different treatment groups. Technically, CCA (Table 1) is a generalisation of CVA. To obtain a CVA through CCA, variables denoting groups are introduced as "species" data and characteristics of these groups (eg. contaminant data) as "explanatory" variables (Ter Braak and Šmilauer 2002). Bernet *et al*. (2001) used CVA to elucidate which fish-serum parameters explained the differences between control and sewage impacted sites. Their ordination diagram shows the size of group differences. The diagram could have been more informative if arrows for the fish-serum parameters had been added to the diagram, as the resulting biplot would allow one to interpret the group differences in terms of the fish-serum parameters. Aptula *et al*. (2002) used CVA in quite a different context. They tried to explain the differences in a priori assigned mechanisms of toxic mode of action on the basis of their molecular descriptors. This analysis results in a Canonical Discriminant Function (CDF) in which the explanatory variables are weighted on the basis of their ability to predict the classification the best. It is interesting to compare these uses of CVA with the methods we discussed. In our penguin and Leces examples (RDA and CCA) the characteristics (biological or chemical "species') are the responses to be explained by groups, a nominal explanatory variable (eg. denoting period or place relative to the factory). As mentioned above, in CVA it is the other way round. Because characteristics are explanatory variables in CVA, CVA is hampered by multicollinearity among the

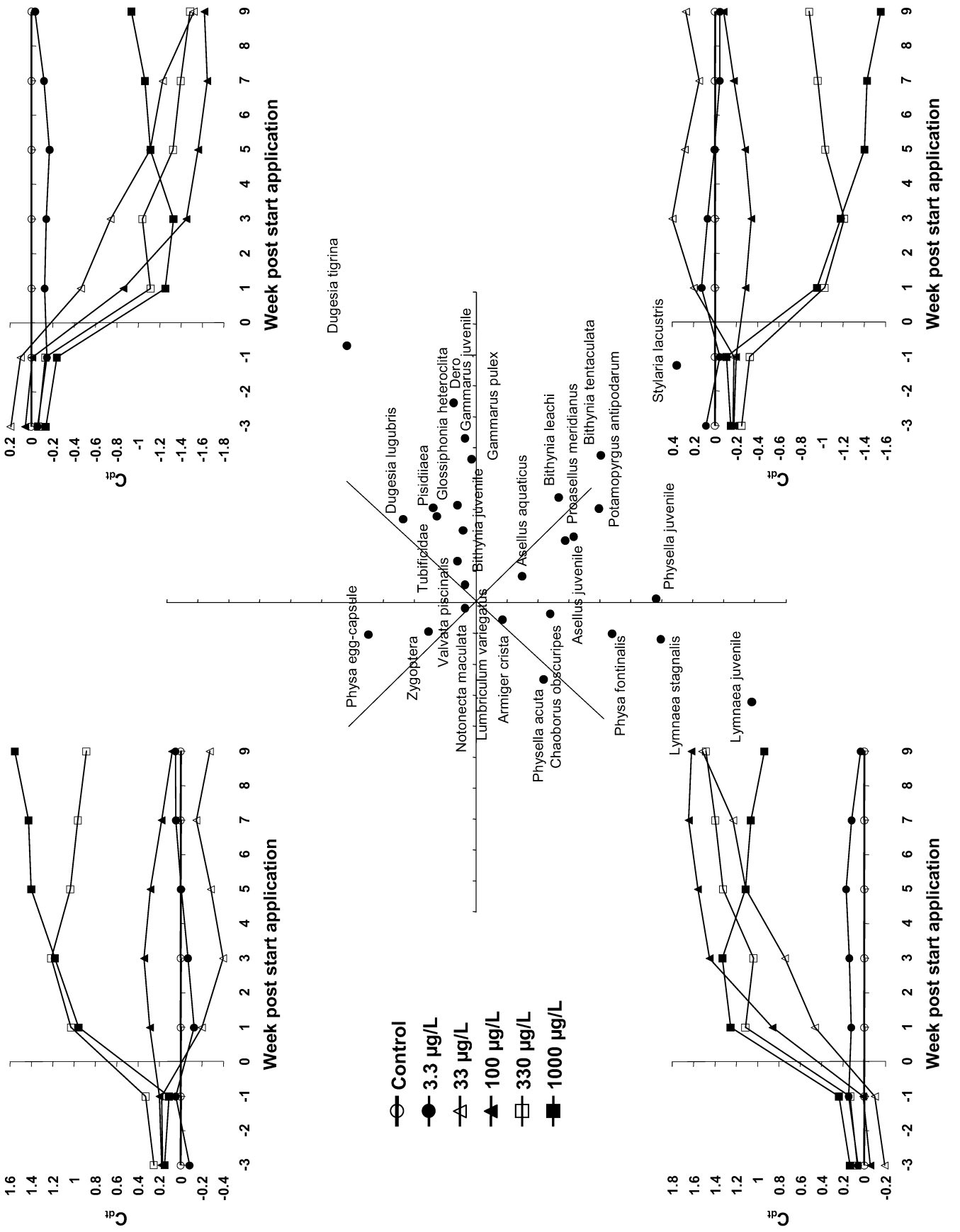

Figure 8. *Two dimensional plot of the weights of the invertebrate taxa on the first and second PRC, as given in Figure 7. The four diagrams in the corners apply to the taxa that have equal weights on the two PRCs (except from the sign).*

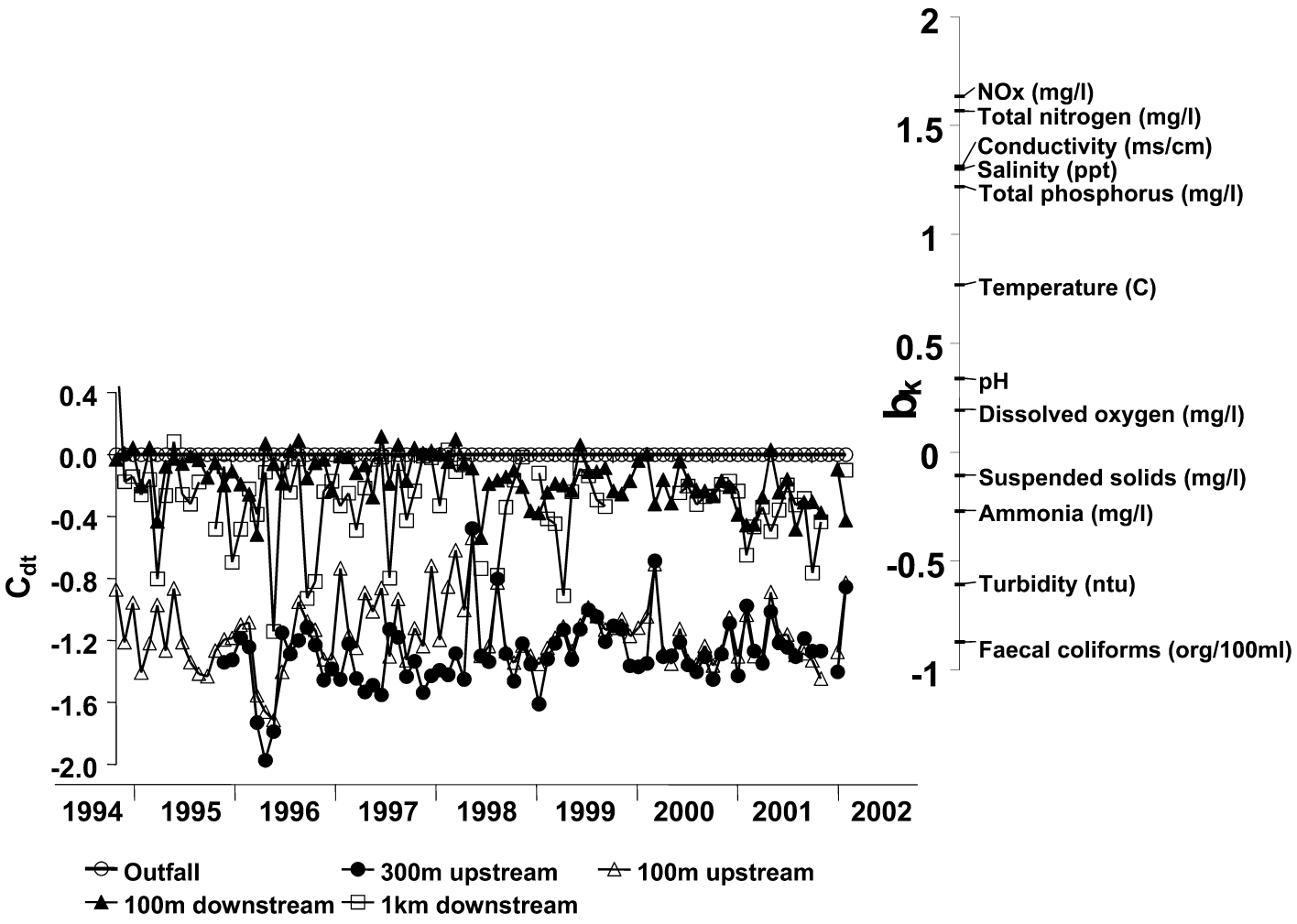

Figure 9. *Principal Response Curves indicating the effects of the outlet of a sewage treatment plant on some monthly averages of physico-chemical characteristics of a river. Of all variance, 24% could be attributed to between month variation; this is displayed on the horizontal axis. Fifty-seven percent of all variance could be allocated to between site differences, the remaining 19% to within month variation. Of the between site variation, 58% is displayed on the vertical axis. The lines represent the course of the sites in time with respect to the outlet. The weight of the physico-chemical variables (b_k) can be interpreted as the affinity of the variables with the Principal Response Curves (c_{dt}).*

characteristics, in particular when the number of characteristics is greater than or of the same order of magnitude as the number of samples (Ter Braak 1995). For this reason, users of CVA apply stepwise selection methods to reduce the number of characteristics. This was not needed in our examples.

Which method is the method of choice in a particular application, will naturally depends on the research question. In many QSAR studies multivariate Partial Least Squares is used (PLS2; eg. Eriksson *et al*. 2000; Drew *et al*. 1999). PLS2 is similar to RDA, but differs in that PLS2 guards automatically against multicollinearity among the explanatory variables whereas RDA does not (see Ter Braak and De Jong 1998 where RDA is termed reduced rank regression, and Ter Braak and Verdonschot 1995). In RDA the problem of multicollinearity (when there are many explanatory variables) would be tackled by forward selection of variables (c.f. CVA above). No other applications of CA and related methods could be found. The reason for the low use of CA and related methods in ecotoxicology could be that the researcher is interested in logistic dose-response relationships between a stressor and the absolute abundance of species, which is better modelled by PCA and derived methods than CA (Van den Brink and Ter Braak 1997). We note that the logistic version of RDA can be fitted to presence/absence (1/0) species data by the recent RR-VGLM software of Yee and Hastie (2003).

In the ecotoxicological literature we found many applications of PCA to link biological data (eg. benthic communities) to chemical composition of the habitat (eg. sediment). Some of them use ordination diagrams for the presentations of the results of the analyses (Vogt 1990; Berggrena *et al*. 1999; Carr *et al*. 2000, DelValls *et al*. 1998, Pedersen *et al*. 1999), but sometimes only tables with the site and species scores of the different principal components are provided (Riba *et al*. 2002; DelValls *et al*. 2002; Boluda *et al*. 2002). The reason for this could be that one wants to consider more than two principal components. It must be stated that when using biplots often only the first two principal components are taken into consideration without stating reasons why the third is not. It is recommended to use biplots because (cor)relations between species and sites stand out more in biplots than in tables. Nevertheless a proper reasoning must be provided why a succeeding axis is not taken into consideration.

We also found many applications of Non-metric Multi-Dimensional Scaling (NMDS) in the ecotoxicological literature. The main difference between PCA and NMDS is that PCA uses the original "species by sample" matrix to extract principal components based on Euclidean distance measure, whereas NMDS estimated distances between samples out of a derived "sample by sample" matrix. This "sample by sample" matrix is obtained by transforming the original "species by sample" matrix using a (dis)similarity measure. NMDS thus has the advantage over PCA that it is not restricted to Euclidean distance measure but any (dis)similarity measure can be used, which can also relax the requirement of normality of data. Another advantage is that NMDS can better deal with missing data because the (dis)similarity between samples can be calculated from the measured variables only, whereas PCA needs a complete "species by sample" matrix. On the other hand, the fact that PCA uses the original "species by sample" matrix has some advantages. Kraufvelin (1999) for instance uses NMDS to visualise variability in mesocosms within and between years and compare them with natural systems. He shows many NMDS diagrams, but because a "sample by sample" matrix is used for the analysis, only samples can be displayed in the diagram, a direct interpretation back to the species level is not possible (but see the SIMPER procedure for an indirect interpretation; Clarke 1999). Because NMDS does not use the original matrix also an evaluation in terms of displayed percentage variance (eigenvalues) is not possible. This becomes particularly important if replicated, designed experiments are evaluated. Kreutzweiser *et al*. (2002) evaluated a mesocosm experiment studying the effects of an insecticide on the zooplankton community with the use of NMDS. Because NMDS does not have a constrained counterpart all data are averaged per treatment level before analysis. This has the disadvantage that the experiment can not be evaluated in terms of partitioning the total variance into parts explained by sampling date, treatment and variation between replicates, which is possible when using the constrained form of PCA, RDA or even PRC (see fungicide example). Another obvious difference between PRC and the Kreutzweiser *et al*. paper is that when using PRC, time is displayed as a single direction in the PRC diagram, whereas in Kreutzweiser *et al* (2002) the time trajectory is quite non-linear, which hampers an easy interpretation of community effects. An example of the difficulty of linking species with explanatory variables using NMDS is given by Pedersen *et al*. (1999). They studied the effect of a copper gradient on a microarthropod field community and analysed their data using NMDS. In the NMDS they superimposed the copper and humus concentrations on the sites like we did in the PCA of the penguin example (Figure 1). Because the information of the explanatory variables (in this example copper and humus) is superimposed afterwards, their correlation and relation with the arthropod community is not optimally displayed; a direct or constrained analysis would overcome this problem.

Remarkably, all these papers use a very indirect way to connect the degree of contamination with the data on communities on the same sites. Whereas most authors analyse the chemical and biological data sets separately and only link them qualitatively by combining the results visually (e.g. DelValls 1998) some authors use more quantitative approaches. Vogt (1990) for instance reduced the chemical data set to three principal components using PCA and reduced the biological data set to one dimension using a diversity measure. After this the principal components are used in Polynomial Principal Component Regression (PPCR) analysis to construct a model that predicts the species diversity the best. Carr *et al*. (2000) uses a similar approach. They reduced the biological, toxicity and physico-chemical data set gathered on the same sites to two principal components using PCA. After that they performed a regression and correlation analysis on the principal components of the three data sets to evaluate relations between the three. The approaches discussed above have the disadvantage that the individual variables may be partitioned over more than three principal components. If this is the case, information of interest is left out of the correlation analysis following the PCA analyses. A more direct way of combining these data sets is using the original species by site matrix as species data and the contaminant by site matrix as explanatory variables in a constrained analysis like is done in the "penguin" and "Leces" example. In this way one is able to focus on that part of the variance that is of interest, namely that part that is captured by the explanatory variables. When combined with Forward Selection a meaningful set of contaminant variables can be retained and their relation with the biological data displayed in a triplot. Also their significance can be obtained using Monte Carlo permutation tests.

We also like to mention recent progress to generalize RDA to other than Euclidean distance measures. First, Legendre and Gallagher (2001) provide several transformations of the species data that are useful in ecological ordination. The transformations are chosen in such a way that the Euclidean distance between samples after transformation is identical to (for example) their Hellinger distance before transformation. Secondly, the lack of a constrained form of NMDS has led to distance-based redundancy analysis (db-RDA; Legendre and Anderson 1999). The essential idea is simple: choose an appropriate (dis)similarity measure, calculate a principal coordinate analysis (metric multidimensional scaling) on the sample-by-sample matrix of (dis)similarities and use the resulting components as response variables in an RDA instead of the original species data matrix. This approach, which allows Monte Carlo permutation testing, is made available in Canoco 4.5 (Ter Braak and Šmilauer 2002). McArdle and Anderson (2001) and Anderson (2001) show how to avoid the initial principal coordinate analysis; their approach to db-RDA works directly on the dissimilarity matrix.

From the above it is clear that multivariate analysis in general and ordination is particular, may be of great value for the field of ecotoxicology. The methods are, however, not used routinely although the development of software (eg. Canoco for Windows, PRIMER, ADE-4 and PC-ORD) facilitates their implementation into ecotoxicology. The potential of multivariate techniques is not yet exploited to the full because of their relative complexity and steep learning curve. To obtain a full exploitation we need education, guidance for use, and communication between ecotoxicologists and statisticians.

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