Enterotyping Danish Wastewater Treatment Plants

Exploring patterns in the microbial community composition using ordination

Master Thesis in Biotechnology by:

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Tak AAU, MA, PHN

Preface

This is a report about microbes and ordination. Yes, it is!

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Abstract

The preface pretty much says it all.

Second paragraph of abstract starts here.

1. Introduction

The importance of access to clean drinking water for human civilisation is without doubt essential. A report by the World Health Organisation (WHO) in 2015 showed that roughly 663 million people still lack improved drinking water resources.

1.1 Wastewater treatment

detflot

1.2 16S amplicon sequencing

hej

2. Ordination in Microbial Ecology

To visualise very large and complex multidimensional data as obtained with next-generation sequencing of sometimes hundreds of samples each containing hundreds of different microorganisms, perhaps the most suited method is ordination. In essence, ordination seeks to reduce the dimensionality of a contingency table into a few, usually 1-3, more important dimensions (hence it is also termed dimensionality reduction techniques) to ease interpretation which makes it perfectly suited for complex ecological data. Through 'dimensional yoga' one obtains $n - 1$ new dimensions, where n is the total number of objects, or samples in the case of ecology, each containing a part of the total inertia in the data, whether it be (co)variance, (dis)simililarity, distance, correlation or any other statistical property. The first axis will then display the most inertia, the second axis the second most, the third axis the third most etc, and plotting the first, usually two, axes can then reveal interesting patterns between the samples, simply by interpreting the distances between the points.

It can be difficult for the human mind to grasp more than 3 dimensions, because this is something that only exists in math, and the complex math behind the scenes lies beyond the scope of this report. However, there are various different types of ordination, each suited for a particular purpose and understanding the key differences between them, which to use when, and why is important. The most commonly used ordination methods in microbial ecology will be described below.(P. Legendre & Legendre, 2012)

2.1 Exploratory vs. Explanatory

The most commonly used ordination methods can generally be divided into two groups based on their purpose. The first group is the *exploratory* analyses, also known as *unconstrained* or *indirect gradient* analyses, which are suited for identifying global patterns between the objects (samples) based on the distribution or (dis)similarity of the values of multiple variables (species abundances) associated with them. The exploratory analyses do not take environmental variables (fx sample location, pH, temperature, nutrient concentrations etc, both qualitative or quantitative) into account and thus do not explain the revealed patterns directly. However it is still possible to color or shape the points by known environmental variables (see Figure 2.1), but the scores (coordinates) on the ordination axes remain the same. The most commonly used exploratory methods in microbial ecology are Principal Components Analysis (PCA), *non-Metric* Multidimensional Scaling (nMDS), Principal Coordinates Analysis (PCoA/*metric* MDS) and Correspondence Analysis (CA).(Ramette, 2007)

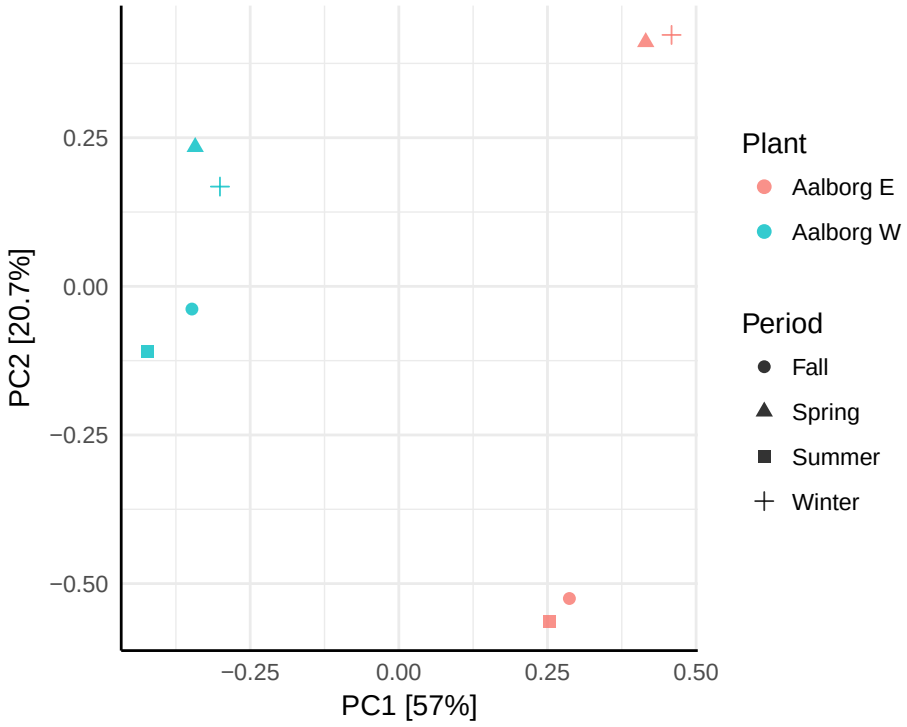


Figure 2.1: A minimal example of exploratory data analysis using Principal Components Analysis. 8 samples from two wastewater treatment plants, Aalborg East and Aalborg West have been analysed and the points have been shaped by when the samples were taken in 2012.

The second group is the *explanatory* analyses, also known as *canonical*, *constrained* or *direct gradient* analyses, which show only the variation in the data that can be explained by *known* environmental variables and not all the variation in the data as with unconstrained analysis. The response variables (species abundances) are thus concidered to be the result of gradients along the environmental variables, or a combination of them. These gradients are called environmental gradients and the constrained ordination methods mainly differ in how they mathematically hypothesise the distribution of the response variables along the environmental gradient(s) to be, either linear or unimodal. Currently the two main constrained

ordination methods used in microbial ecology are Redundancy Analysis (RDA) and Canonical Correspondence Analysis (CCA), which are concidered extensions of Principal Component Analysis (PCA) and Correspondence Analysis (CA), respectively. RDA (and PCA for unconstrained analysis) is the optimal choice for purely linear distributions along the, preferably short, environmental gradient(s). CCA (and CA for unconstrained analysis) is the optimal choice for unimodal distributions along longer gradients where many double-zeros occur (more about double-zeros in Chapter 2.2), but in most cases CCA also performs well with short and linear gradients, it will just show a more qualitative representation of the samples (Braak & Prentice, 1988). Both RDA and CCA are eigenanalyses and calculate their constrained/canonical axes by introducing a linear combination of the response variables and the environmental variables as an additional step in the procedure. Otherwise the procedure is identical to that of PCA (when performing RDA) or CA (when performing CCA).(Braak & Prentice, 1988; P. Legendre & Legendre, 2012)

Table 2.1: A classification of the most used ordination methods in microbial ecology. Based on (Braak & Prentice, 1988; Ramette, 2007)

Unconstrained analyses	**Constrained analyses**
Eigenanalysis-based	
Principal Components Analysis (**PCA**)	Redundancy Analysis (**RDA**)
Correspondence Analysis (**CA**)	Canonical Correspondence Analysis (**CCA**)
Distance-based	
non-metric Multidimensional Scaling (**nMDS**)	
Principal Coordinates Analysis (**mMDS/PCoA**)	

2.2 Niche theory and the double-zero problem

In reality there are often many, known or unknown, environmental variables affecting the presence of species and the gradient is then concidered a complex environmental gradient. As niche theory states, species have ecological preferences

and are present under a set of optimal environmental conditions, including the presence of other species (Hutchinson, 1957). The theory also predicts that species have unimodal distributions along environmental gradients, illustrated in Figure 2.2, so that they are found in greater abundances at some intervals along the major environmental gradients and gradually less present away from that optimal set of thriving conditions, ultimately absent (Whittaker, 1967). This has the consequence that community composition data typically contain many zeros, which can pose a problem for some ordination methods, specifically those using a Euclidean distances like PCA and RDA.(P. Legendre & Legendre, 2012)

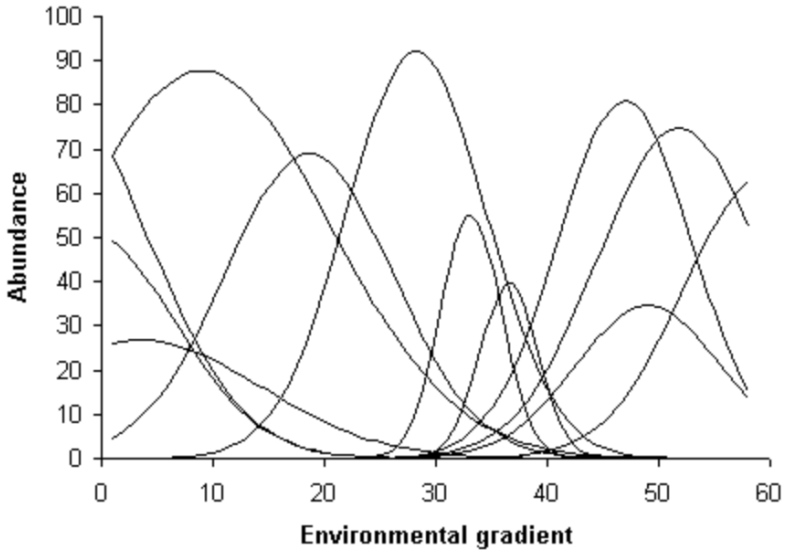


Figure 2.2: Species response (or abundance-) curves in most cases show a unimodal distribution along an environmental gradient. Adapted from (Whittaker, 1967)

The goal of using ordination is often to represent patterns or differences between samples based on species abundances to draw ecologically meaningful conclusions about the sampling site(s) and their corresponding β- or γ-diversity. The species abundances are concidered to be response variables in the sense that they are indicators of their nearby environment. Variation in the environment is expected to be reflected in the relative productivities or abundances of the species (Whittaker, 1972). If a species is present at two sites, this means that the sites share conditions

that are favorable for the species, indicating a similarity between the sites. On the contrary, if a species is present at one site and not the other, this indicates that the ecological conditions at the sites most likely are dissimilar. However, if a species is absent from both sites, it provides no valuable ecological information since both sites either have ecological conditions outside the niche of the species, and these conditions may be very similar or very different, or the niche has been occupied by another species prefering the same conditions. Including double zeros in the comparison of sites thus results in a higher similarity between the sites than there is ecologically, and this phenomena is called the *double-zero problem*. It is therefore important to concider whether there is relevant ecological information in double-zeros in the particular study, but in most cases it is preferable not to conclude anything ecologically about them and avoid including them in the analysis. This is called asymmetric analysis as double presences are treated differently than double absences and is done in practice when the distance coefficients between sites are computed, either implicitly or explicitly during ordination. Choosing the correct ordination method and, for some, also a distance metric therefore depends on whether this information is meaningful for the study. If linear ordination methods like Principal Components Analysis or Redundancy Analysis is used, then they should first be subjected to appropriate data transformation (more on transformation in Chapter 2.5) or manual curation of the data to correct for the problem and reflect the ecological differences more correctly.(P. Legendre & Legendre, 2012)

2.3 Distance-based ordination

Fundamental to any ordination method is not only dimensional reduction, but more importantly also how it does so and how it displays and calculates distances between objects (sites) or variables (species). As stated in Table 2.1, ordination methods can be classified by whether they are distance-based or not, however they could just as well have been classified by whether they use a metric explicitly or implicitly to calculate distances, respectively (P. Legendre & Legendre, 2012). As

such, eigenanalysis-based ordination methods use a metric of their own as part of their algorithm, while distance-based ordination can only be performed on a distance- or (dis)similarity matrix, which has to be calculated first using one of many distance- or (dis)similarity metrics. When calculated, a symmetrical matrix is then obtained containing *distance coefficients*, or more generally *association coefficients*, between all pairs of sites. This gives the ecologist flexibility as to how it would be ecologically meaningful to represent the differences between sites or species, however it also implies the importance of knowing how to interpret the results based on the particular metric and choosing it wisely (more on metrics in Chapter 2.3.3).

Currently the most used distance-based ordination methods are non-Metric Multidimensional Scaling (nMDS) and Principal Coordinates Analysis (PCoA), also called *metric* Multidimensional Scaling (mMDS). There is also Polar Ordination, however it is rarely used and will not be detailed in the following.(Ramette, 2007)

2.3.1 Principal Coordinates Analysis

Principal Coordinates Analysis (PCoA) is very useful at exploring microbial ecology data because it can represent relationships between samples measured by any distance coefficient in Euclidean space (also called metric space, fx a Cartesian coordinate system). Therefore PCoA is also called *metric* Multidimensional Scaling, because it can represent the *metric* properties of the distance- or (dis)similarity matrix. When the distance metric is Euclidean, the results obtained by PCoA is identical to that of PCA.

Since the choice of metric directly influences the result, it has to be done with care. However, this is also one of the advantages of using PCoA because it is then possible to better deal with ecological problems like the *double-zero problem* while still using Euclidean mapping. The choice of metric also influences how the results are to be interpreted, as the original data is then a function of the chosen metric, which may be non-Euclidean, and does not always allow for a true representation in Euclidean space. Furthermore, it is possible to obtain negative eigenvalues of the

resulting axes, especially when a semi-metric is used, and this should be corrected for if they occur on the main axes (ie the axes being plotted). The eigenvalue of an axis is an indication of its contribution of inertia to the total inertia in the data, which is therefore also obscured by the choice of metric and its value should not be directly refered to when performing PCoA. PCoA is partly based on eigenanalysis, however it is more appropriate to classify it as a distance-based analysis since it is highly dependent on the chosen distance metric.(Ramette, 2007)

2.3.2 non-Metric Multidimensional Scaling

Non-Metric Multidimensional Scaling (nMDS) is similar to PCoA in that it is also performed on a distance- or (dis)similarity matrix calculated using a specific metric. However, nMDS is different from PCoA on numerous aspects. PCA aswell as PCoA both try to maximise a linear correlation (of course dependent on the metric used in the case of PCoA) of species abundances along the environmental gradient, which will often result in an artifact called *the horseshoe effect* when the response variables are the result of a non-linear or long gradient (Podani & Miklós, 2002). This results in an arch shaped and incorrect pattern of the points leading to false conclusions. nMDS eliminates this by only preserving the ranked order of the distance coefficients between samples. As the name suggests, this makes the procedure *non-metric*, and the distances between points is therefore not to be interpreted numerically.

nMDS does not try to explain as much variation in the data as possible as with PCA or PCoA, but more the discontinuities in the data. It is a very robust technique that can handle missing values aswell as multiple data types at once. It has no distributional assumptions about the data compared to all other ordination methods, where the data is assumed to have for example linear or unimodal distributions as with PCA/RDA and CA/CCA, respectively. nMDS is therefore the ordination method of choice when the nature of the data is unknown.(Buttigieg & Ramette, 2014; P. Legendre & Legendre, 2012)

nMDS is furthermore very different from other ordination methods by how it is computed. nMDS is an iterative procedure where the number of dimensions is

chosen *a priori* (before analysis) and the algorithm tries to find a solution from either random starting points or from the results of a PCoA on the same data provided by the user. The solution is not unique as with all other ordination methods and it is therefore recommended to run it multiple times to validate the result, preferably with different numbers of dimensions. During the algorithm, a stress value is calculated to express the goodness-of-fit of the solution and the procedure is repeated many times (20+ is not unusual) using the solution of the previous cycle as the starting point until the stress value does not change significantly and reach an acceptable, low value. A stress value is concidered good when below 0.05, while below 0.1 is acceptable. A stress value above 0.2 is suspect and the results should not be trusted. Generally choosing more dimensions will lower the stress value.(Ramette, 2007)

One of the major drawbacks of nMDS is that it is very computationally demanding. However, modern computers are getting increasingly more powerful, so this is less of a concern compared to the time during which the method was developed by the psychometricians Kruskal and Shepard at the Bell Telephone Labs in the 1960's.(Kruskal, 1964; Shepard, 1966)

2.3.3 Distance- and (dis)similarity metrics

As mentioned, choosing a distance- or (dis)similarity metric that makes ecological sense is crucial for the analysis and subsequent interpretation of the ordination. A distance metric is basically a mathematical function with which to calculate distances between objects or variables in the data. There are many, many ways (60+) of calculating distance/association coefficients between objects and/or variables, however only the general concepts and most important differences will be covered in the following. For in-depth knowledge of how to calculate association coefficients using all metrics, semi-metrics, non-metrics and their exact formulae, refer to Chapter 7 in (P. Legendre & Legendre, 2012).

For association coefficients to be concidered metric, the four properties listed below have to be satisfied. When this is true, the coefficient is called a distance

coefficient or a metric coefficient, since it can be fully represented in Euclidean (metric) space:

The four metric properties

1. The distance between identical objects is 0, which is the lowest possible value:
   if $a = b$, then $D(a, b) = 0$

2. When the compared objects are not identical, the coefficient, and the distance, has a positive value:
   if $a \neq b$, then $D(a, b) > 0$

3. Symmetry: the distance from A to B is the same as the distance from B to A:
   $D(a, b) = D(b, a)$

4. Triangle inequality: The sum of two sides of a triangle of points in Euclidean space is equal to or longer than the third side. In other words, the shortest distance between two points in Euclidean space is a straight line:
   $D(a, b) + D(b, c) \geq D(a, c)$

When one or more of the four properties are not satisfied, in most cases the fourth property (triangle inequality), the coefficients calculated are not concidered to be *distance* coefficients, they are then termed *semi-metric* coefficients. When this is the case, distances cannot be ordinated (at least reliably) in Euclidean space and nMDS is the optimal ordination method. Therefore concidering whether the chosen metric is suitable for the ordination method used is important, if not then interpretation of the result should be done with caution. When all four properties are met, both nMDS and PCoA can be used. Dissimilarity and similarity coefficients can be both metric or semi-metric. They are often just termed similarity coefficients because it is straight forward to convert dissimilarity coefficients (D) to similarity coefficients (S) and vice versa: $S = 1 - D$.(P. Legendre & Legendre, 2012)

LIST OF COMMON METRICS: BRAY-CURTIS, KULCZYNSKI, JACCARD, MANHATTAN/CITYBLOCK METRICS ETC

2.4 Eigenanalysis-based ordination

The eigenanalysis-based ordination methods have more specific purposes than the distance-based methods, because they are limited to represent the distances only by the capabilities of their implicit distance function (metric), where distance coefficients are not first calculated manually by the user. They all have a few things in common, however:

- There is always one unique solution to the data
- Each axis is an eigenvector associated with an eigenvalue expressing the axis' contribution to the inertia in the data
- The axes are ranked by (and plotted by) the eigenvalues, highest to lowest
- The axes are orthogonal to eachother, thus uncorrelated and express their own 'unique' inertia (P. Legendre & Legendre, 2012)

Normally, the two axes with the highest eigenvalues are plotted in a Cartesian coordinate system, where the highest eigenvalue axis is represented by the first axis and the second highest on the second axis. The most inertia in the data is therefore always represented by the first (x-)axis, which can, for example in the case of two distinct groups of sample points as seen with the example in Figure 2.1, often be interpreted as 'between-group variation'. The secondmost inertia is expressed by the second (y-)axis and can then be interpreted as 'within-group variation'.

It is important to examine all eigenvalues obtained for each axis to confirm that the axes being plotted are significant and represent a large portion of the inertia in the data. To do this, a simple plot called a *scree plot* can be made where all axes are plotted on the first axis ordered by eigenvalue in decreasing order and their corresponding eigenvalue on the second axis, as illustrated in Figure 2.3. Optimally, the first two axes represent more than half of the inertia in the data and have high values compared to the rest of the axes, where the latter should make up a slightly

decreasing, straight line. The worst case scenario is when all the values make up a nearly horizontal, straight line. In this case, the ordination is either failing at representing the inertia in the data and a different ordination method may be better suited for the data or there is simply no inertia to represent at all. The sum of all eigenvalues is always equal to the total inertia in the data and the corresponding eigenvalues of the axes plotted are often shown as a percentage of the total inertia on the axis labels. Below, the remaining four ordination methods listed in 2.1 will be explained briefly.(Ramette, 2007)

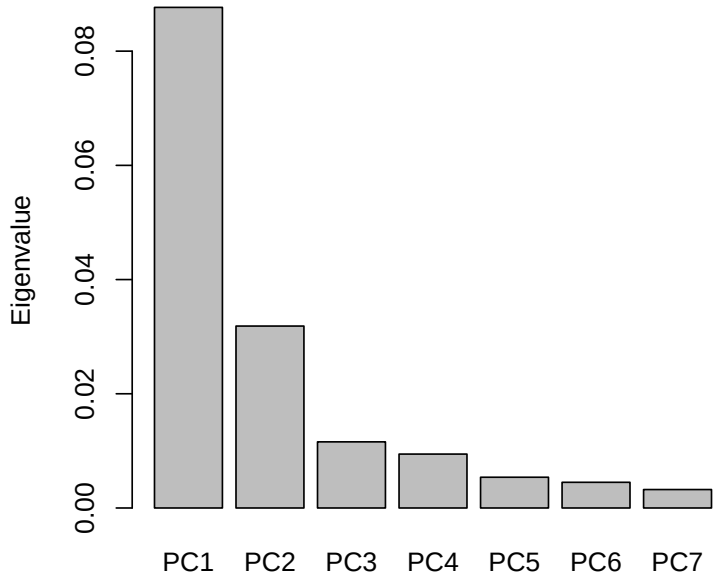


Figure 2.3: A scree plot of the eigenvalues of the 7 axes obtained from the PCA ordination seen in Figure 2.1.

2.4.1 Principal Components Analysis

Principal Component Analysis (PCA) is the oldest ordination method and still also the most used, perhaps due to its simplicity (Ramette, 2007). It has its roots all the way back to 1901 when Karl Pearson explained how to represent objects by the

'best-fitting' line or plane (Pearson, 1901). The simplest example of dimensional reduction is the representation of 2-dimensional data in 1 dimension, which is normally called linear regression. It is simply a straight line, drawn through the *centroid* of the data, see Figure 2.4.

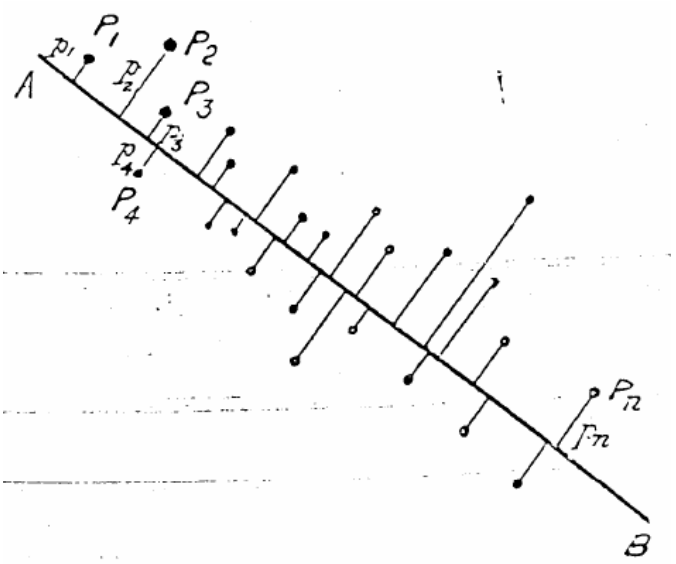


Figure 2.4: The main structure of two-dimensional data can often be described in one dimension, by positions on a straight line. Adapted from (Pearson, 1901)

Pearson described how this is done mathematically, which formed the concepts of how to describe the overall structure of complex data. In 1933, Harold Hotelling (Hotelling, 1933) applied the concepts on multidimensional data and explained how to represent the data by its *principal components*, which in turn formed the fundamental of PCA.

The goal of PCA is simple: express the maximum amount of variation in the data. This is done by generating new, synthetic axes, which are synonymous to eigenvectors, where the first axis is alligned so that it represents the most inertia in the data. Inertia in the case of PCA is specifically: *variance*. PCA is therefore concidered more of a quantitative ordination method, as it excels at highlighting differences between samples based on numerical differences in species abundances and is most reliable when the same species are present in most or all of the samples. As mentioned in Chapter 2.2, this is rarely the case with ecological data, and the

double-zero problem thus has a major impact on the results obtained by PCA, questioning its usefulnes without appropriate data transformation. Of course, this is not a problem when analysing α-diversities since there will be no zero-abundances.(P. Legendre & Legendre, 2012)

PCA is a linear method because it represents the linear correlation or covariance of species abundances between samples using Euclidean distances. Both the sample scores (points) and species scores (arrows) are usually plotted together to form a biplot, where arrows indicate the linear gradients of species abundances and the relative right-angle projections of the samples onto the arrows then approximate their corresponding abundance of the particular species. This makes PCA suited to answer questions like for example: "How are samples different in terms of the abundances of species X and Y?".(Ramette, 2007)

Fundamental to PCA (and Redundancy Analysis) is the Euclidean distance, which is calculated by the following formula, equivalent to the pythagorean theorem:

The euclidean distance

$$D_{Euclidean}(x_1, x_2) = \sqrt{\sum_{j=1}^{p} \left(y_{1j} - y_{2j}\right)^2} \tag{2.1}$$

where $Y = [y_{ij}]$ is a species abundance table of the size $(n \times p)$ with sites (rows) $i = [1...n]$ and species (columns) $j = [1...p]$.(P. Legendre & Gallagher, 2001)

2.4.2 Redundancy Analysis

To answer a question like: "How do species abundances correlate with a measured carbon source concentration?", PCA would be insufficient. To answer that question, Redundancy Analysis (RDA) is the perfect choice. RDA is concidered an extension of PCA, or the constrained version of PCA, which can directly explain the observed patterns based on known environmental variables. This is done by making a linear combination of the species abundance matrix and a matrix containing information

about one or several known environmental variables. These variables are then hypothesised to be able to explain a portion of the observed variance in the data and the resulting constrained axes are plotted. Just as with unconstrained ordination, the two axes with the highest eigenvalues are plotted and their contribution to the total variance in the data are usually indicated on the axis labels to give an indication of the significance of the constrained variable(s). When doing constrained analysis, both with RDA and CCA (Chapter 2.4.4), a plot with not only sites and species are made, but also environmental vectors are plotted together in one plot called a triplot. This is a very convenient way to visualise all three types of information at once to easily draw ecological conclusions about the sites.

Because RDA is based on Euclidean distances just like PCA, it is only suited for the analysis of short, linear environmental gradients with few or no species absences, which can limit its use in ecology.(Ramette, 2007)

2.4.3 Correspondence Analysis

Perhaps the most appropriate ordination method for ecological data is Correspondence Analysis (CA) because it represents the differences between sites hypothesising a unimodal distribution, which fits the species niche theory well, as discussed in Chapter 2.2. As the name suggests, CA tries to represent the *correspondence* between samples and species by testing how "this species correspond to that site". CA is thus concidered more of a qualitative representation of the data and is based on the Pearson chi-squared statistic of the form $\chi^2 = \sum \frac{(observed-expected)^2}{expected}$.

CA is developed by several authors independently and has several different names, for example *contingency table analysis*, *reciprocal averaging*, *weighted averaging*, *dual scaling* or *homogeneity analysis*. Fundamentally, CA calculates the weighted averages of the sample scores as defined by the χ^2-metric described below, where the weights are, with community composition data, specifically species abundances. This means that species abundances to some degree contribute to the calculated distances, however they are also calculated relative to the average abundances and therefore does not influence the results as much as with the Euclidean-based

ordination methods. This has the consequence that low abundant species may have an unduly high influence on the results, because the abundances of common species contribute less to the calculated distance compared to low abundant species, which are often numerous. With CA, and CCA, it is therefore important to concider the importance of low abundant species in the particular study and give less weight if needed (Braak & Prentice, 1988; P. Legendre & Gallagher, 2001).

guldkorn:

Since the distances between the samples are calculated as weighted averages of the species, absent species in samples have exactly zero weight.

awesome for β-diversity

during the procedure, the result is matched with a random solution and a P-value can be extracted to reject the NULL-hypothesis of no association

a site with more species than another will have higher chi-squared distances

- low abundances has an unduly high influence, use transformation
- eigenvalues are correlation coefficients between samples and species

Total inertia The total inertia of a CA solution conveys the degree to which the values of rows and columns correspond to each other. More specifically, it reflects the degree to which rows and columns deviate from the null hypothesis of "no association", according to the logic of the Pearson's χ^2 statistic.

- Eigenvalues in CA are not equivalent to those of PCA and should not be interpreted in terms of "variation" but "inertia".

The chi-squared distance

$$D_{\chi^2}(x_1, x_2) = \sqrt{y_{++}} \sqrt{\sum_{j=1}^{p} \frac{1}{y_{+j}} \left(\frac{y_{1j}}{y_{1+}} - \frac{y_{2j}}{y_{2+}} \right)^2} \tag{2.2}$$

where $Y = [y_{ij}]$ is a species abundance table of the size $(n \times p)$ with sites (rows) $i = [1...n]$ and species (columns) $j = [1...p]$, with row sums y_{i+}, column sums y_{+j} and total sum y_{++}.(P. Legendre & Gallagher, 2001)

2.4.4 Canonical Correspondence Analysis

Constrained version of CA

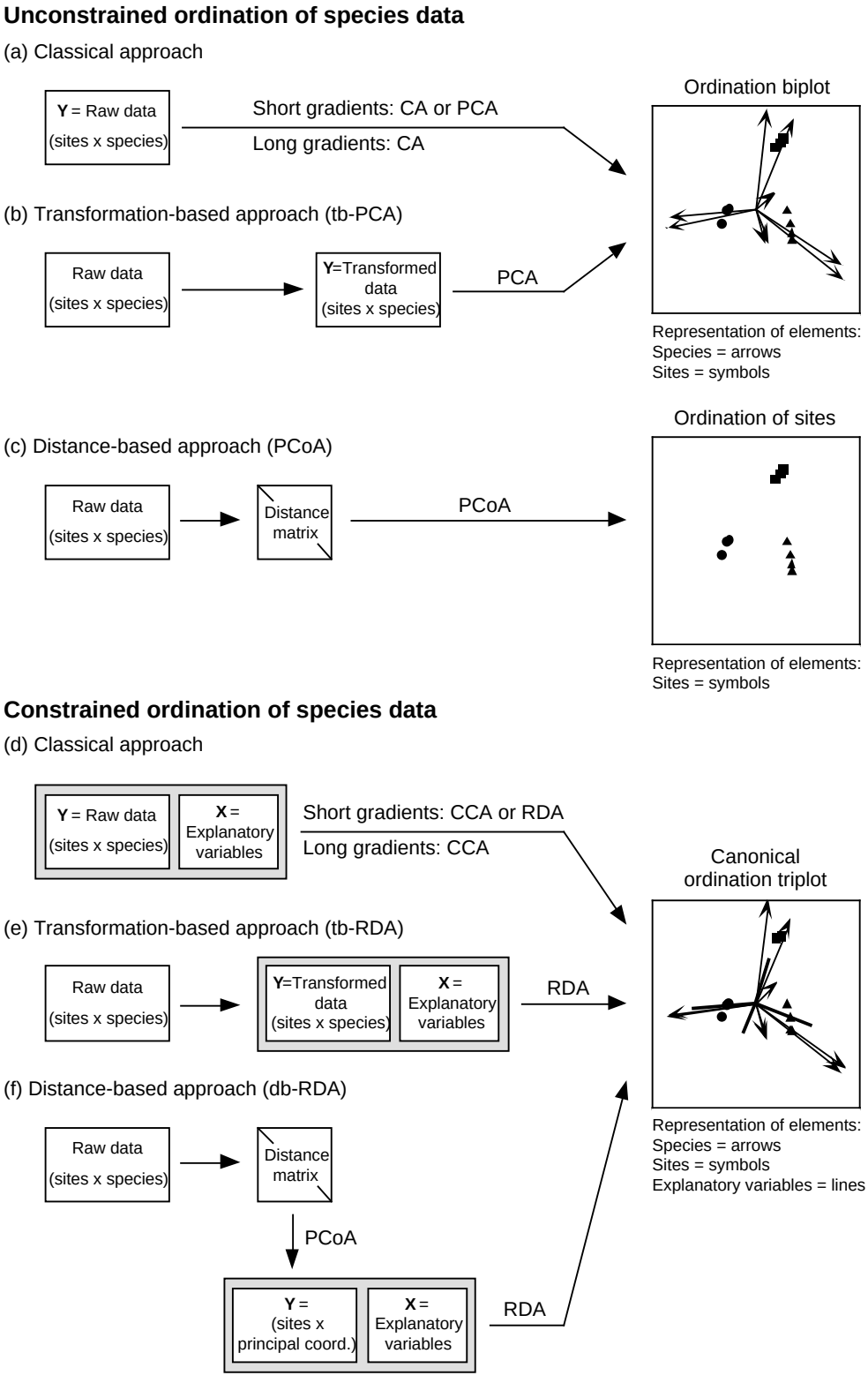


Figure 2.5: Principles of unconstrained analyses

2.5 Data transformation

Important, weighting low/high abundant species

$$D_{Hellinger}(x_1, x_2) = \sqrt{\sum_{j=1}^{p} \left(\sqrt{\frac{y_{1j}}{y_{+j}}} - \sqrt{\frac{y_{2j}}{y_{2+}}} \right)^2} \tag{2.3}$$

(P. Legendre & Gallagher, 2001)

3. Aims

- What are the key differences in the microbial community compositions of danish Wastewater Treatment Plants?
- What causes the differences?
  - Underlying environmental gradients
  - Known physical-/operational parameters
  - Weather conditions
  - Plant design
  - Influent microbial community composition
  - Other. . .
- Is the microbial community composition stable over time and how can it be related to plant performance?
- Are the abundances of microbes with similar function(s) correlated?
- Are microbes present in the influent also present in the activated sludge and in the same amount? (Odense, Janni's data)

4. Materials and Methods

4.1 Sampling

Samples were taken at danish wastewater treatment plants mostly more than two times a year from 2006 to 2015. BLA BLA

4.2 Library preparation

Prepared by: (or refer to a protocol?) ### DNA extraction Beadbeating+DNA extraction kit xxyy+centrifuge BLA BLA

4.2.1 Polymerase Chain Reaction

Temperature scheme? Primer region V1-1234 BLA BLA

4.2.2 Library pool cleanup

BLA BLA

4.3 DNA sequencing and bioinformatics

Usearch9, MiDAS database version xx samples checked with rarefaction curves, filtered which? Only looking at WWTPs with many samples

4.3.1 Filtering

OTU's at or below 0.1% abundance in all samples have been removed.

4.4 Data visualisation

Done in R, wrote my own functions. Made a shiny app for quick, brief analysis.

Both available at github: `https://github.com/knaldhat`

FILTRATION: <=0.1% REMOVED WHICH RESULTED IN 32 WWTPs

5. Results Part 1: Exploring the Microbial Communities

Before filtering, the 622 samples from 32 Wastewater Treatment Plants (WWTPs) contained a total of 21728 different OTUs. After filtering the low abundant OTUs (<0.1%) the size of the data reduced remarkably with a total of 2366 different OTUs in all the samples (mean per sample: 1078, SD: 291.7). Because this is still a very large amount of data to visualise (even when using ordination), the following plots may be better viewed in the online *bookdown* version of this report, where it is possible to zoom in the plots and hover the points. It is available at `https://github.com/KasperSkytte/MasterThesis`.

In this chapter the WWTPs will be described using mainly exploratory/unconstrained ordination methods to get an overview of the differences between the WWTPs with respect to their microbial communities. In Chapter 6 these differences will be explained using also the explanatory/constrained ordination methods based on information about the WWTPs and how they are designed. The differences are represented by the distance between sample points if not noted otherwise. The points are colored by a unique color for each WWTP, but because there are 32 different WWTPs it can be difficult to distinguish between the colors and the corresponding name of the WWTPs are therefore written at the approximate center of all the sample points from the particular WWTP. The same colors listed in the legend in Figure 5.1 will be used in subsequent ordination plots in the chapter. Furthermore, the eigenvalues of the axes plotted are indicated by the axis titles as a percentage of the total sum of eigenvalues. Scree plots can be found in Appendix C.

5.1 Overview of the differences between the WWTPs

Clearly, there seem to be many similarities between the WWTPs when using Principal Components Analysis (Hellinger transformed) as the groups of samples tend to overlap, see Figure 5.1. However, it is possible to identify global patterns by groups of WWTPs (groups of groups), which seem to be similar at least partly. The largest dissimilarities observed in an ordination plot are points positioned diagonally of one another (when there is large variation on both axes), which is the case with for example the Ribe and Bjergmarken WWTPs. Now, imagine a line from the Ribe label towards the Bjergmarken label. Two groups of similar WWTPs can then be identified by separating the line with a perpendicular line roughly in the direction of Fornaes-Haderslev, where the WWTPs on either side of this perpendicular line can be concidered two different groups. Of course, this is a coarse way of clustering the WWTPs, but along the Ribe-Bjergmarken diagonal seems to be the greatest variation in species composition and/or abundances (the response variables) between the WWTPs. Large variation between the samples within individual WWTPs can also be observed, for example within Bjergmarken, whose samples cover a broad area in the top group of WWTPs. This is the case with many of the WWTPs and these differences are mostly evident on the first axis. It is worth noting that the eigenvalues of the two axes plotted are nearly identical (10.1% vs 9.2%). This further highlights that the differences between the samples are just as large within the WWTPs as they are between them. If there would have been a clear difference between the WWTPs, they would have been positioned horisontally with few overlaps, the first axis would have had a much greater eigenvalue than the second axis, and the within-group variation would have been evident mostly on the second axis.

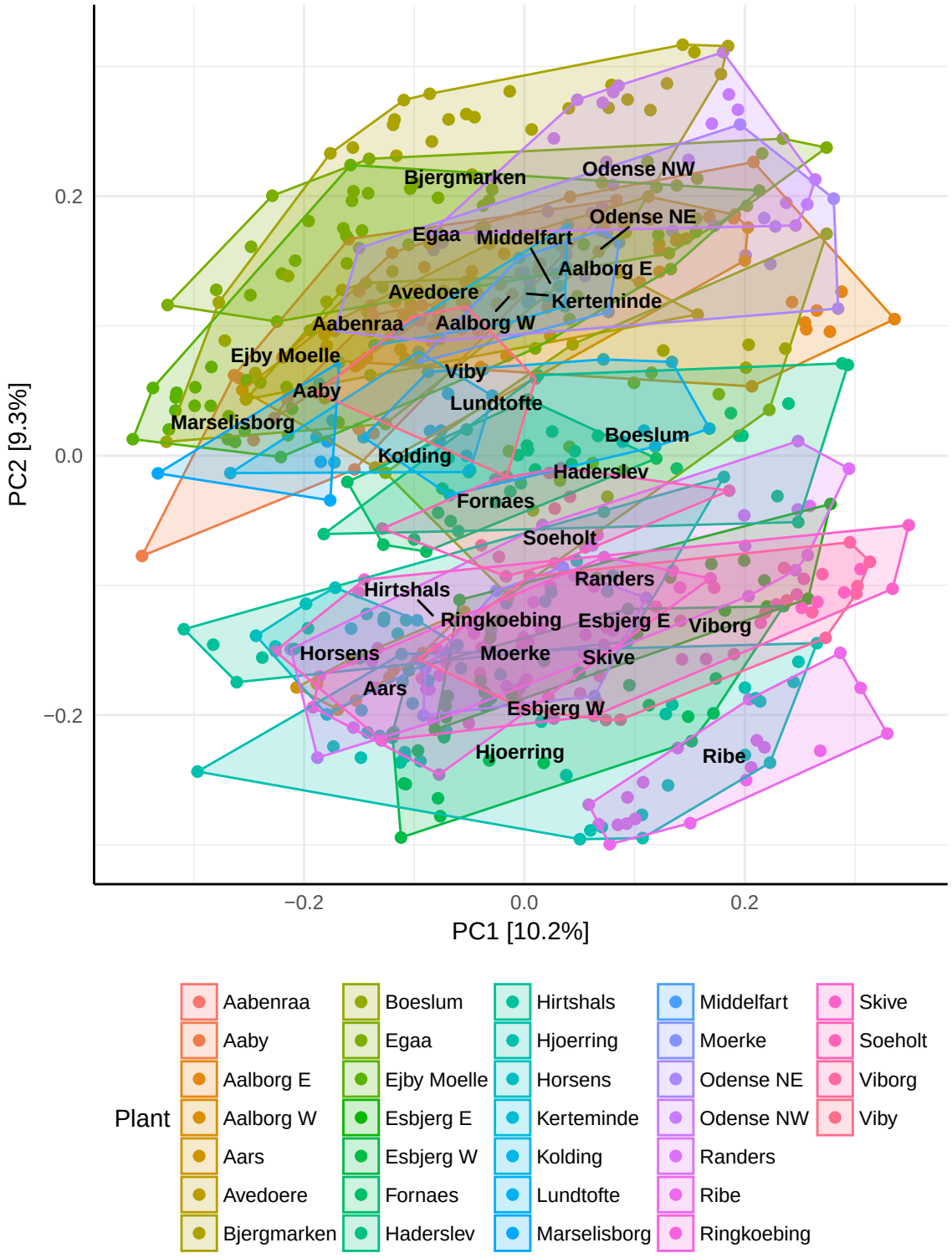


Figure 5.1: Principal Components Analysis of samples from the 32 WWTPs. The data has been transformed using the Hellinger transformation. Each WWTP has been assigned a unique color as indicated by the legend and labels have been positioned approximately at the center of the points.

With PCA the abundances of the species (aka the weights) contribute conciderably to the distances between the samples. To represent the differences between the WWTPs where the species abundances have less of an impact on the distances, the widely used Bray-Curtis Dissimilarity index (BCD) is appropriate. With this measure the abundances have less of an impact because the species abundances are relativised to the sum of the species in the two samples being compared. As BCD is a semi-metric (does not satisfy the triangle inequality property), Principal Coordinates Analysis has to be used and the result can be seen in Figure 5.2. The relative positions of the sample points on the axes are not always in the same orientation between different ordination methods and reversing the first axis (mirroring the plot vertically) reveals relative positions similar to those in the Principal Component Analysis, Figure 5.1 (symmetric procrustes sum-of-squares value: 0.80). Again, the groups are overlapping and are not well separated on the first axis, indicating higher variation within the WWTPs than betweem them, but there is a slightly clearer separation of the two (top and bottom) groups observed with PCA (Figure 5.1). The fact that abundances contribute less to the distances when using the BCD index and the result is similar to that of PCA shows that species abundances are not the primary cause of the differences and that the WWTPs must have many species in common. The eigenvalues of the axes are not ideal, however, and using non-Metric Multidimensional Scaling with the BCD index had a bad stress value of 0.247 (see Appendix B, Figure B.1), which confirms that all the variation in the data cannot be fully represented in two dimensions.

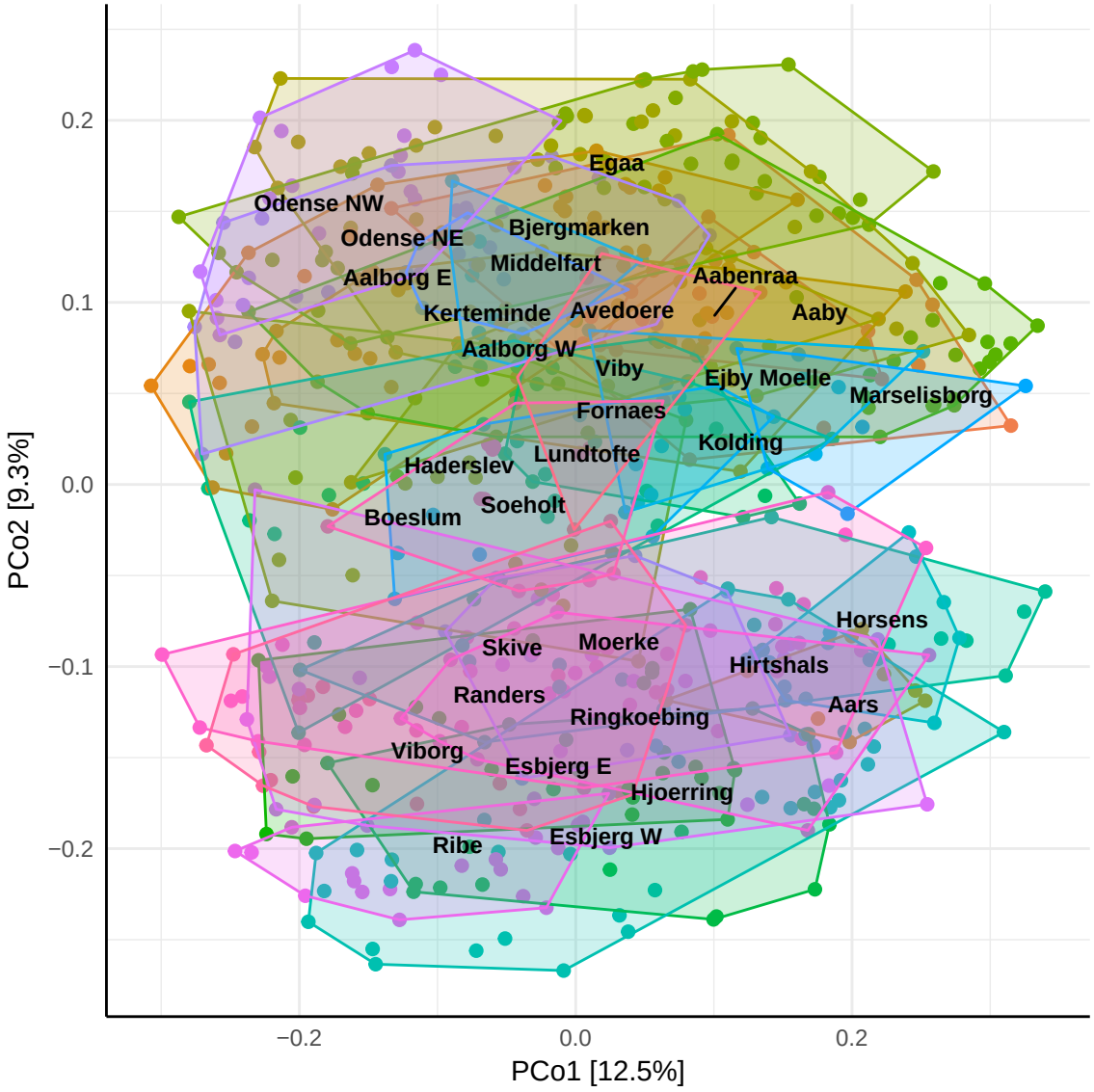


Figure 5.2: Principal Coordinates Analysis of samples from the 32 WWTPs using the Bray-Curtis Dissimilarity (BCD) index. Each WWTP has been assigned a unique color as indicated by the legend in Figure 5.1 and labels have been positioned approximately at the center of the points.

As mentioned in Chapter 2, species are most likely present when a set of optimal environmental conditions are met at the sampling site resulting in a unimodal abundance distribution across samples. The ecological differences between the WWTPs are therefore expected to be reflected by both unique species and to a lesser extend abundances of shared species. To compare the WWTPs more in terms of their species distribution, the Pearson χ^2-statistic used in Canonical Correspondence Analysis (CCA) is more appropriate than the measures of PCA and PCoA (with BCD), as it better reveals the unique species that would correspond to each WWTP. As seen in Figure 5.3, CCA (Hellinger transformed) shows that Esbjerg E, Esbjerg W and Ribe seem to be significantly different from the rest of the WWTPs. In general the sizes of the groups are smaller, more distinct and the overlaps are less prevailing. When interpreting a CCA plot it is important to note that the sample points positioned closest to the center of the plot (0,0) have the highest *probabilty* of containing the most common species across all samples and the samples closer to the edges of the plot have a higher probability of containing unique species. This means that the Esbjerg E+W and Ribe WWTPs must either contain several unique species which the rest of the WWTPs are highly unlikely to contain, or the opposite; common species in the other WWTPs are of low abundance or completely absent in these 3 WWTPs (this will be investigated in the following Chapter 5.2). Except for these 3 WWTPs, the overall groupings of WWTPs observed with PCA and PCoA are also evident with CCA. Concidering only the relative positions of the text labels, the differences between the WWTPs seem to be relatively similar to that observed with PCA and PCoA, but now on the primary axis. It can be difficult to see in the figure, but other than the Esbjerg E+W and Ribe WWTPs, there are a few additional WWTPs that are (almost) not overlapping with other WWTPs, namely Lundtofte, Marselisborg and Moerke, indicating that their distribution of species are different from the rest of the WWTPs. They are closer to the center, however, indicating that the differences are most likely due to differences in abundances of shared species and not due to unique species.

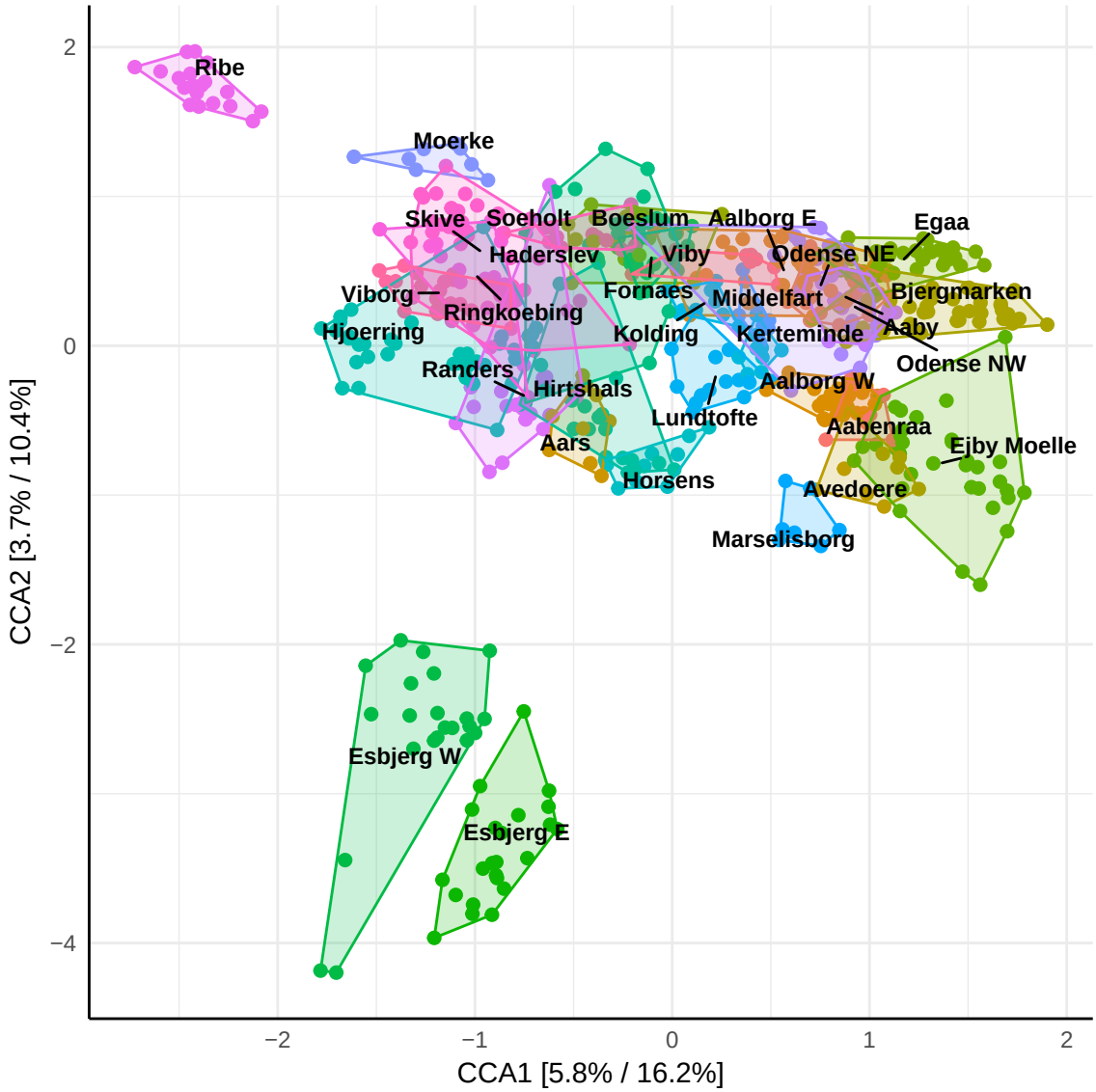


Figure 5.3: Canonical Correspondence Analysis of samples from the 32 WWTPs constrained to the WWTP where samples were taken. Hellinger transformed. Each WWTP has been assigned a unique color as indicated by the legend in Figure 5.1 and labels have been positioned approximately at the center of the points. The percentages indicated on the axis titles are (left): the eigenvalue of the axis relative to the total sum of eigenvalues and (right): the eigenvalue relative to the total sum of only the constrained eigenvalues.

Again, the eigenvalues of the axes are low, but this is expected since the WWTPs must have many species in common concidering the fact that there are an average of 1078 different OTUs in each sample, and only 2366 different OTUs in all the 622 samples.

5.2 How does the microbial community composition describe the WWTPs?

Describing the differences between the WWTPs with respect to their microbial communities is not an easy task. As the differences are the result of variation in the presences and/or abundances of 2366 different species, it is impossible to provide an extensive overview while covering all important aspects of the differences. The heatmap shown in Figure 5.4 is a good example of the challenge of visualising the complex microbial communities characteristic of the individual WWTPs. The most abundant species are often of most interest, however. The heatmap shows an overview of the 40 most abundant genera in *all* WWTPs. Noticably, there seem to be only a few species in high abundance in almost all the WWTPs, namely *Tetrasphaera*, *Candidatus Microthrix*, *Trichococcus*, *Rhodobacter*, *Rhodoferax* (the top 5). Specifically *Tetrasphaera* is the only species abundant in all WWTPs while other species are (at least nearly) absent in at least one of the WWTPs. It is also clear that there are several species which are only abundant in one or a few WWTPs, for example *Gordonia* at the very bottom of the figure is abundant mostly only in Moerke.

- few highly abundant shared
- many unique species in each plant
- remember to comment esbjerg+ribe

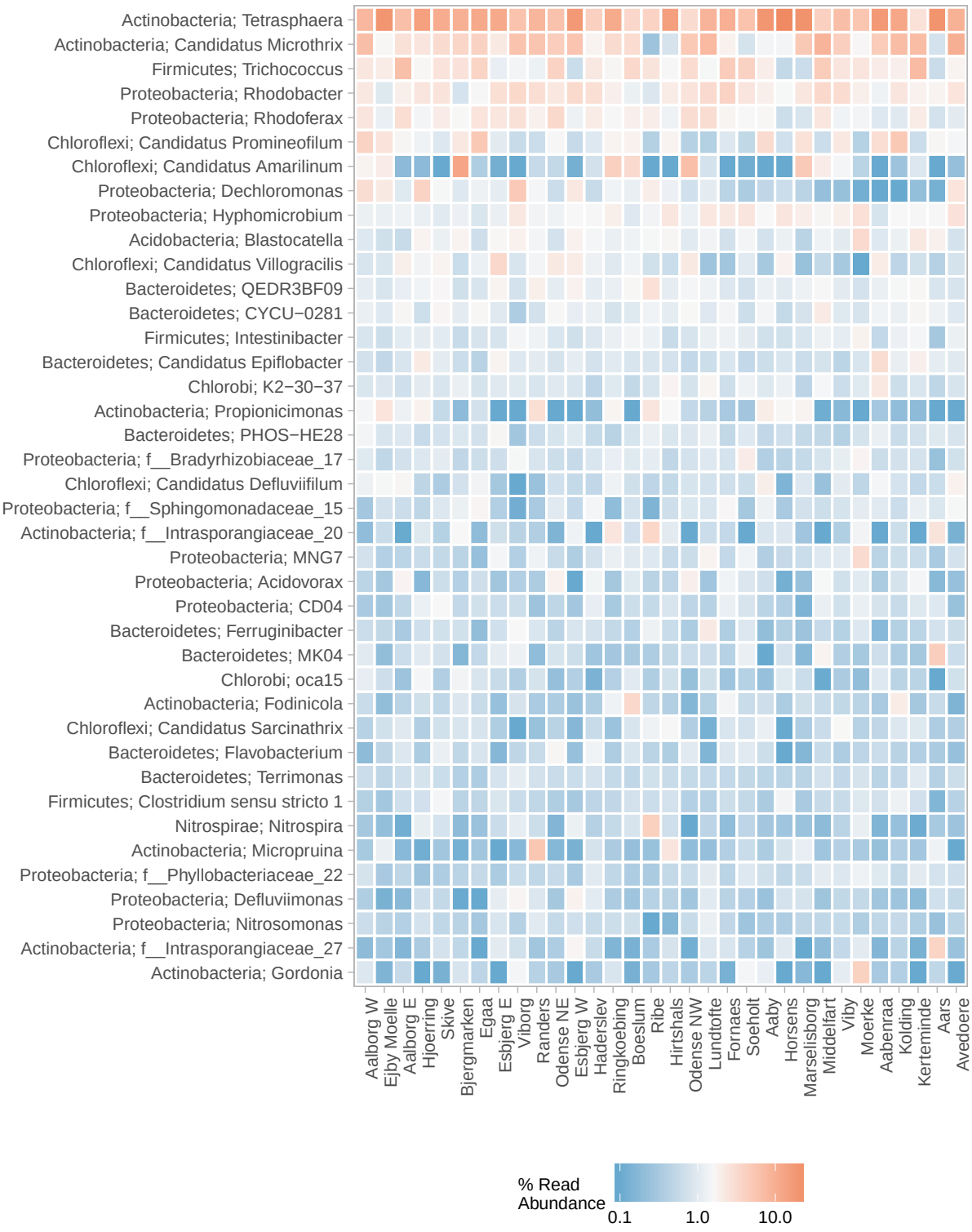


Figure 5.4: A heatmap of the 40 most abundant genera in all the samples grouped by the average in each WWTP. The WWTPs are ordered by similarity. The relative abundances are indicated by a color gradient according to the legend. The corresponding phylum of each genus is written to the left of the semi-colon: 'phylum; genus'.

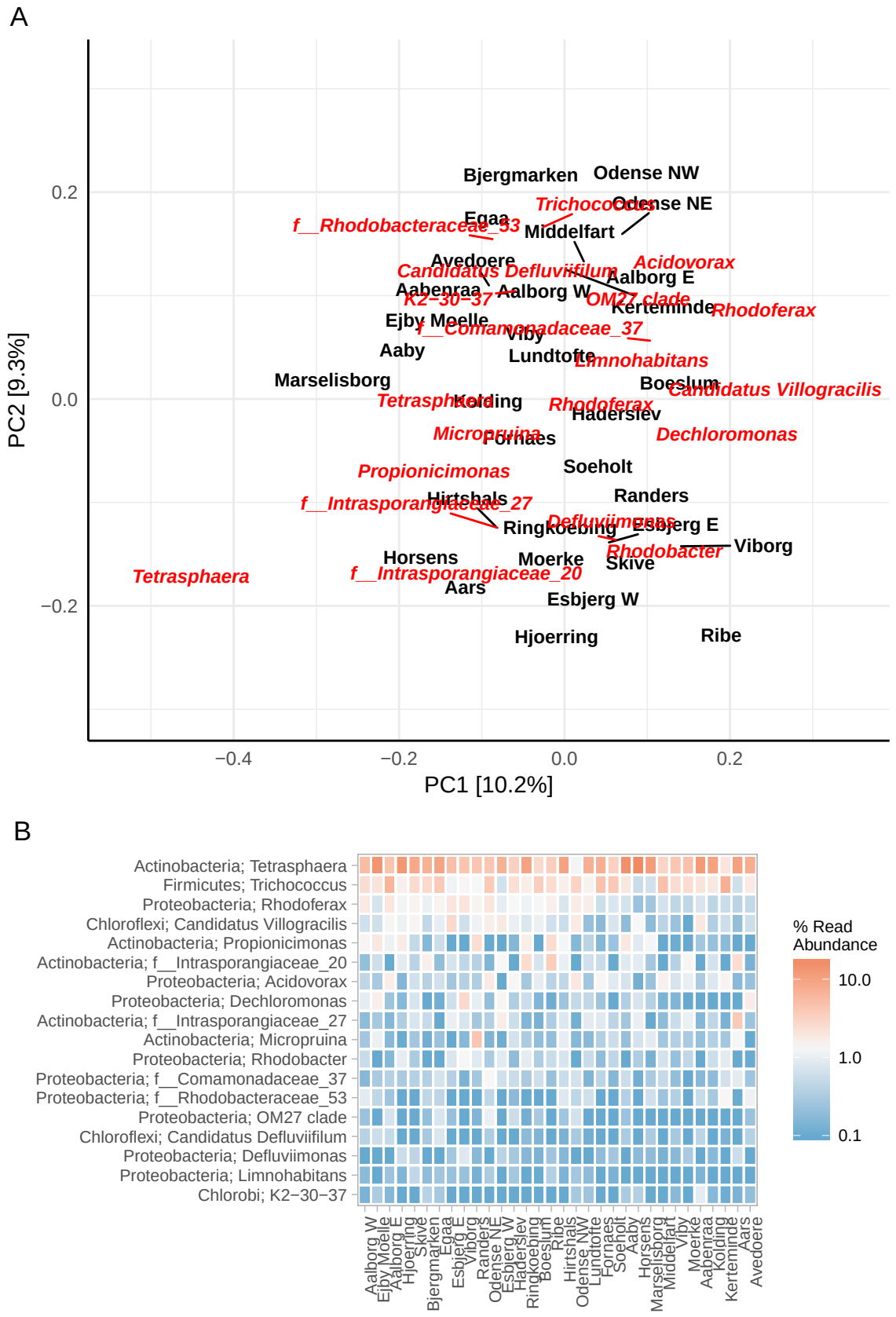


Figure 5.5: Overview species PCA, hellinger - updated 14/3-15:20

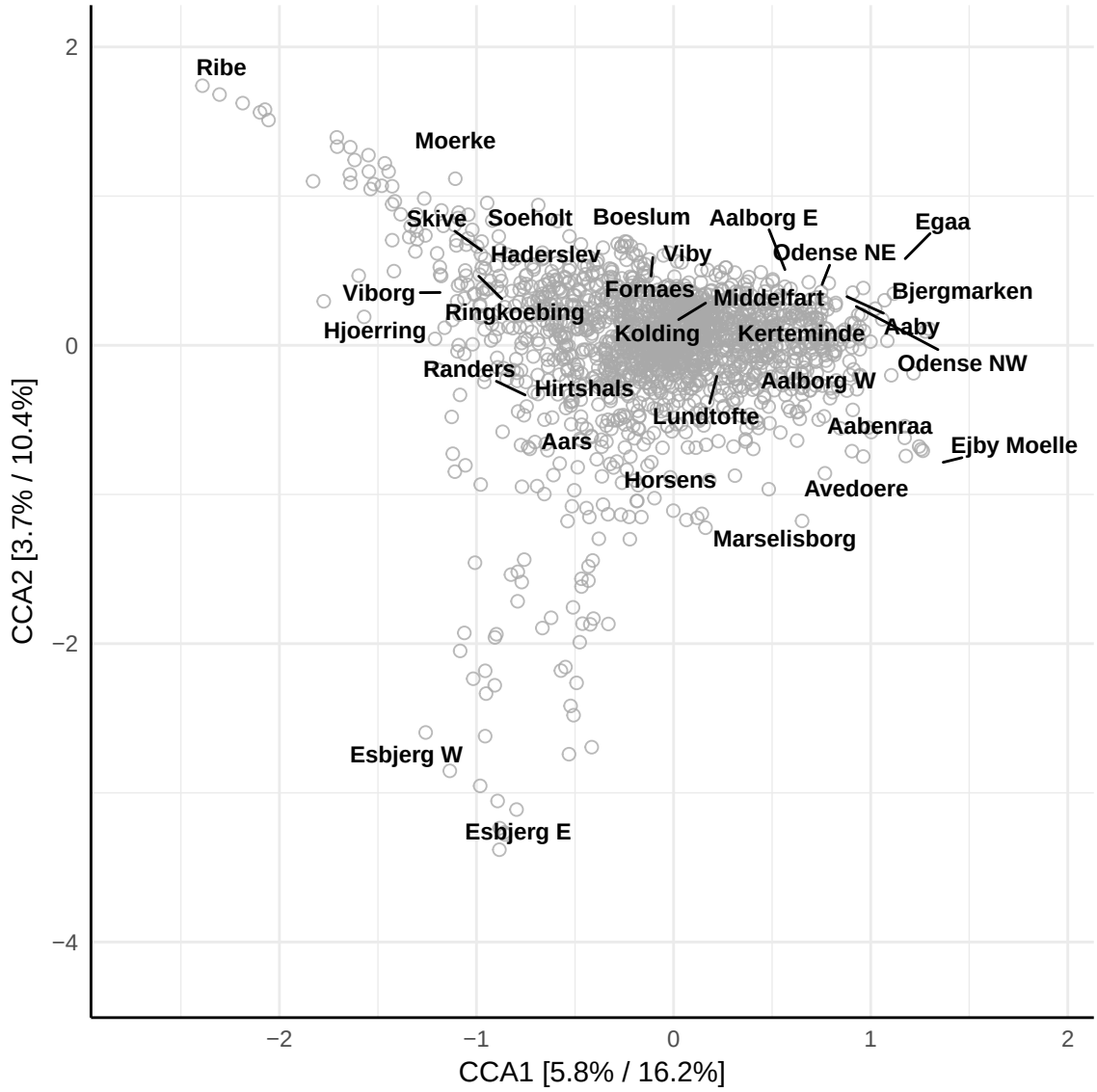


Figure 5.6: overview CCA, hellinger - updated 14/3-15:20

6. Results Part 2: Explaining

Soeholt: Digester fra summer 2009 Viborg: RSS og EBPR fra early 2012

6.1 The effect of plant design on the microbial community composition

6.1.1 Configuration

```
[1] "Eigenvalue of RDA1: 1.2%"
```

6.1.2 Enhanced Biological Phosphorus Removal (EBPR) vs Biological Nutrient Removal (BNR)

```
[1] "Eigenvalue of RDA1: 0.9%"
```

6.1.3 The effect of primary setling

```
[1] "Eigenvalue of RDA1: 1%"
```

6.1.4 The effect of industral inflow water

```
[1] "Eigenvalues of RDA: 1.1%, 0.3%"
```

6.2 Plant stability over time

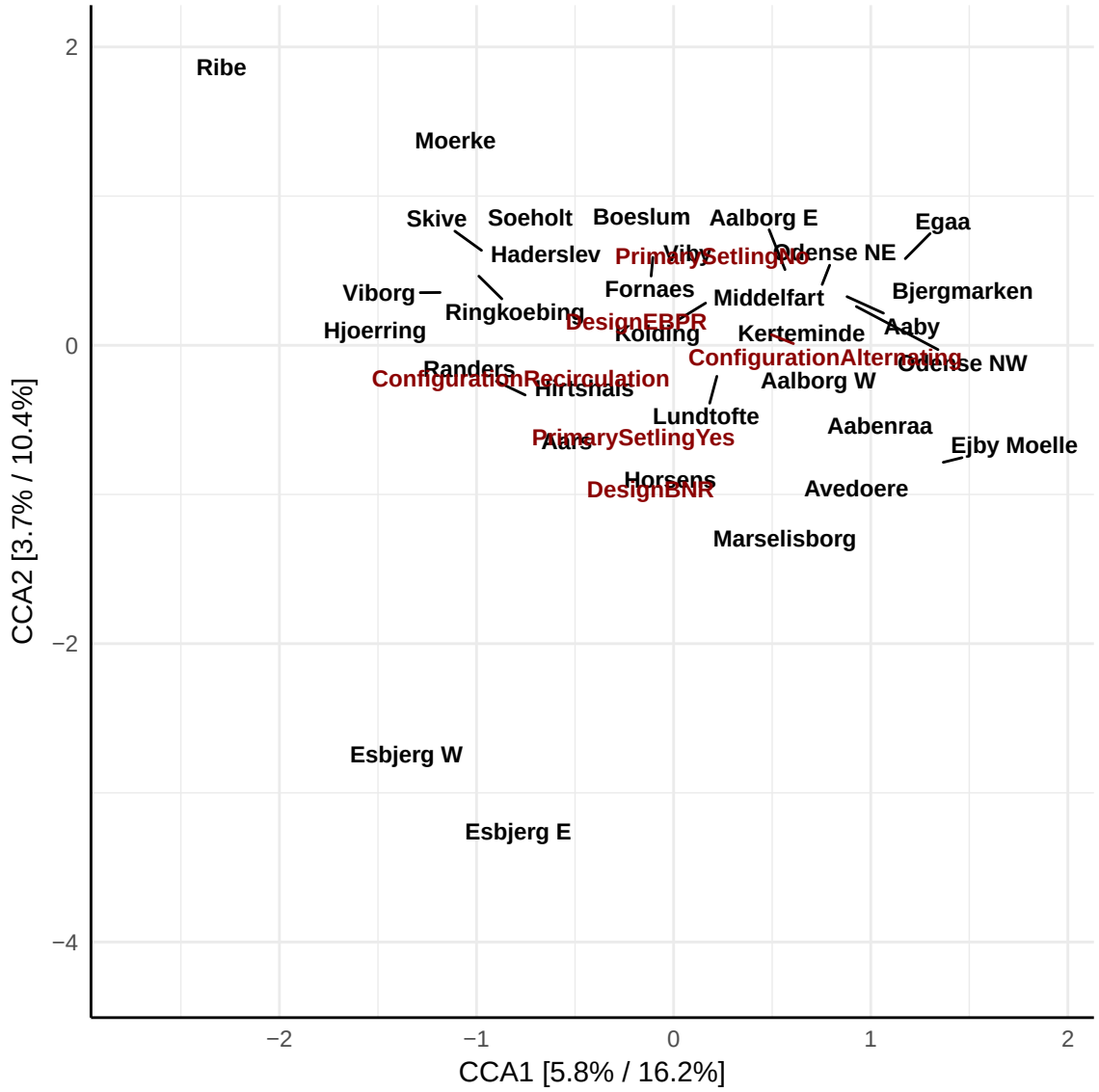


Figure 6.1: EV fit CCA, hellinger - updated 14/3-15:20

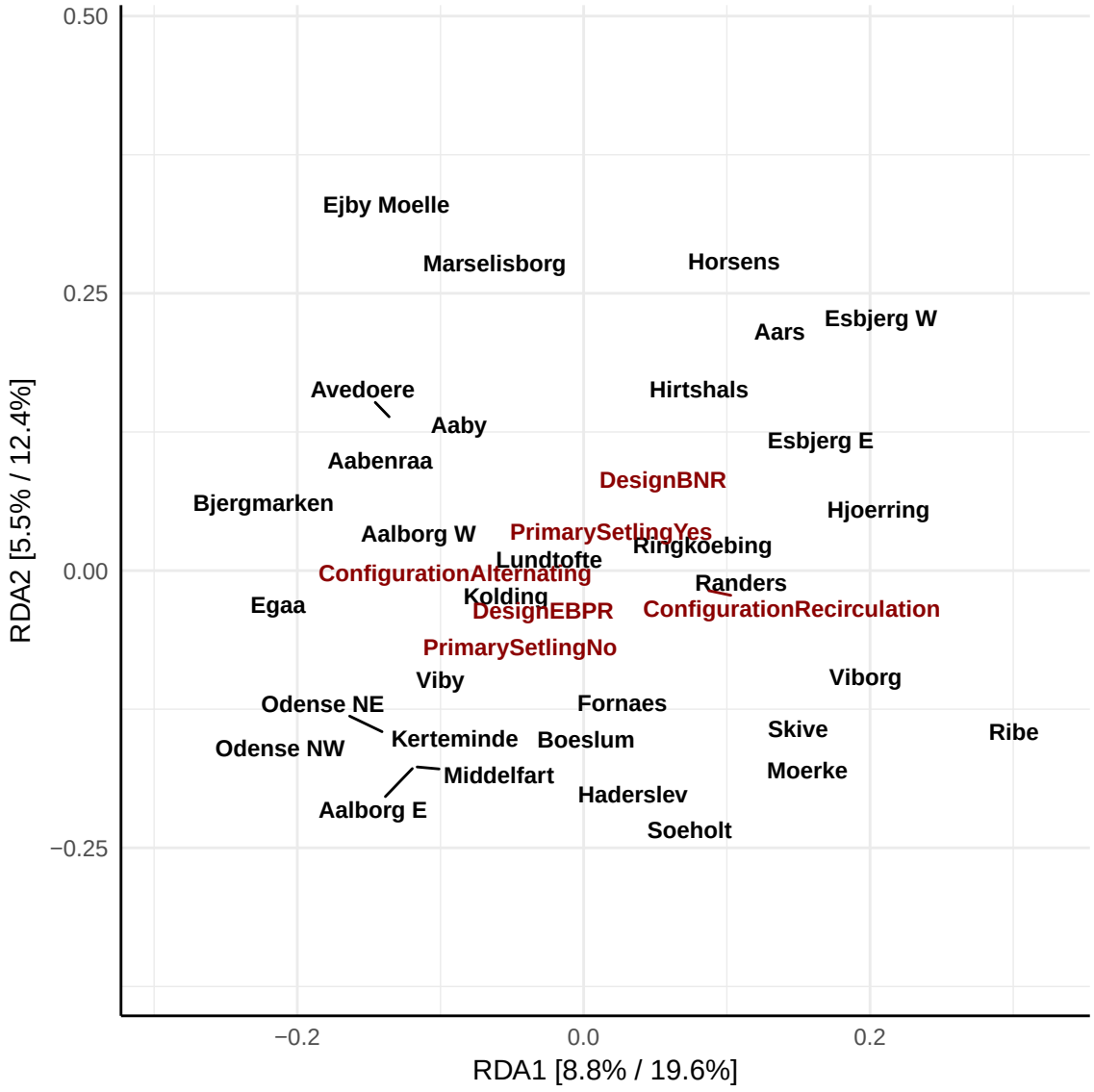


Figure 6.2: EV fit RDA, hellinger - updated 14/3-15:20

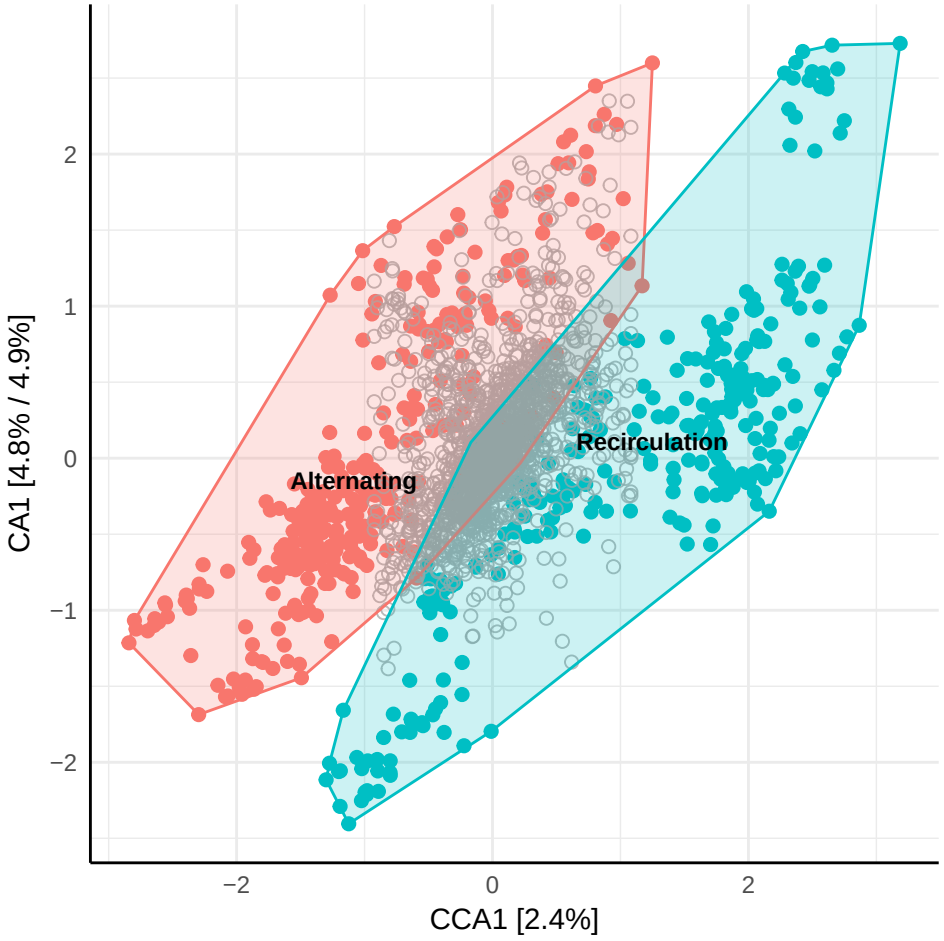


Figure 6.3: Plant design, hellinger - updated 14/3-15:20

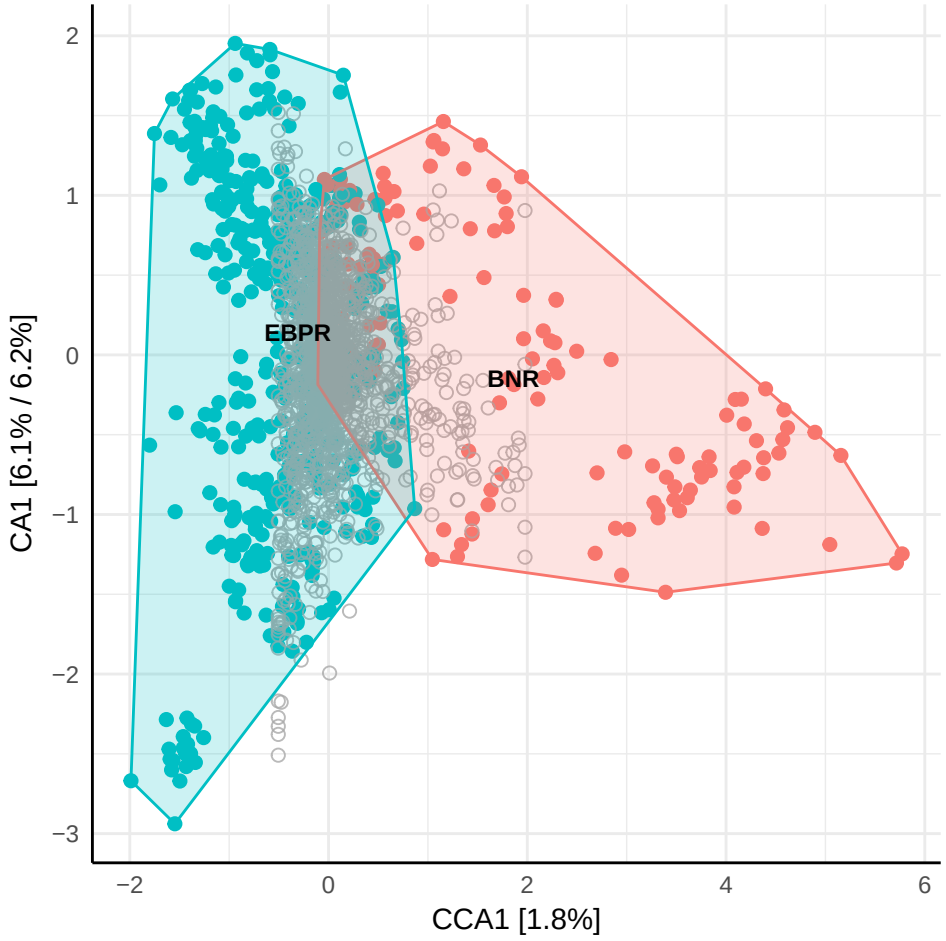


Figure 6.4: EBPR vs BNR, hellinger - updated 14/3-15:20

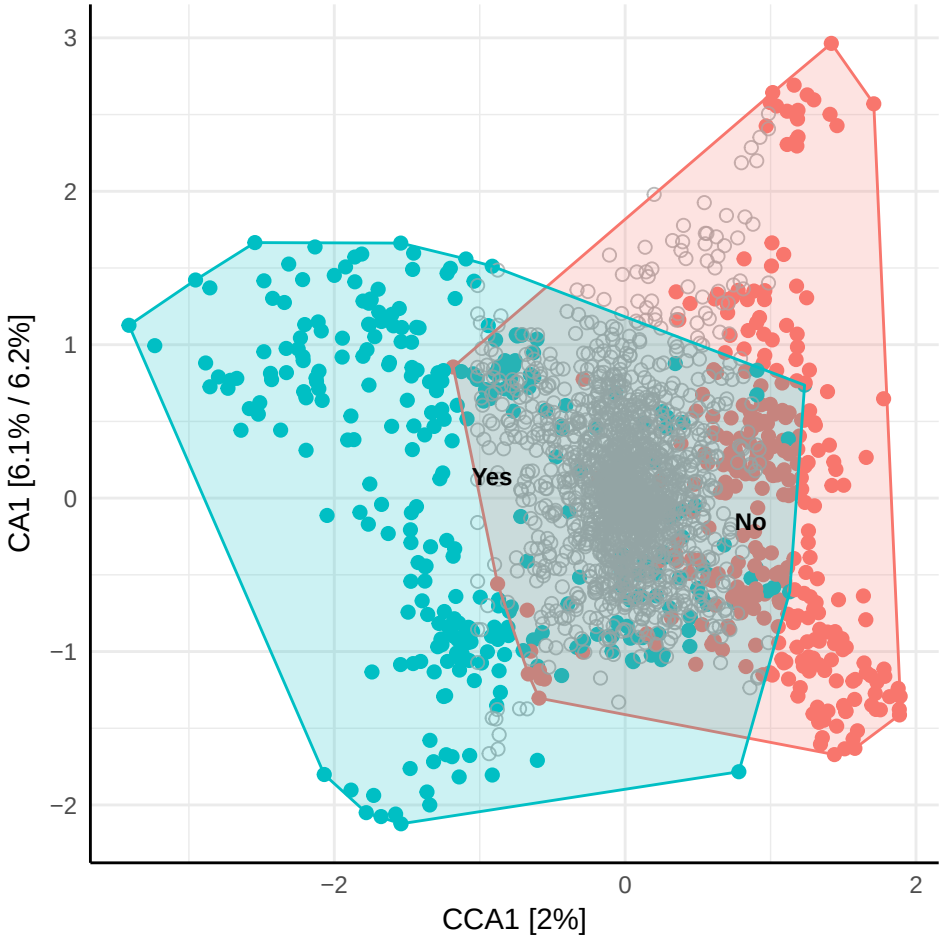


Figure 6.5: Primary setling, hellinger - updated 14/3-15:20

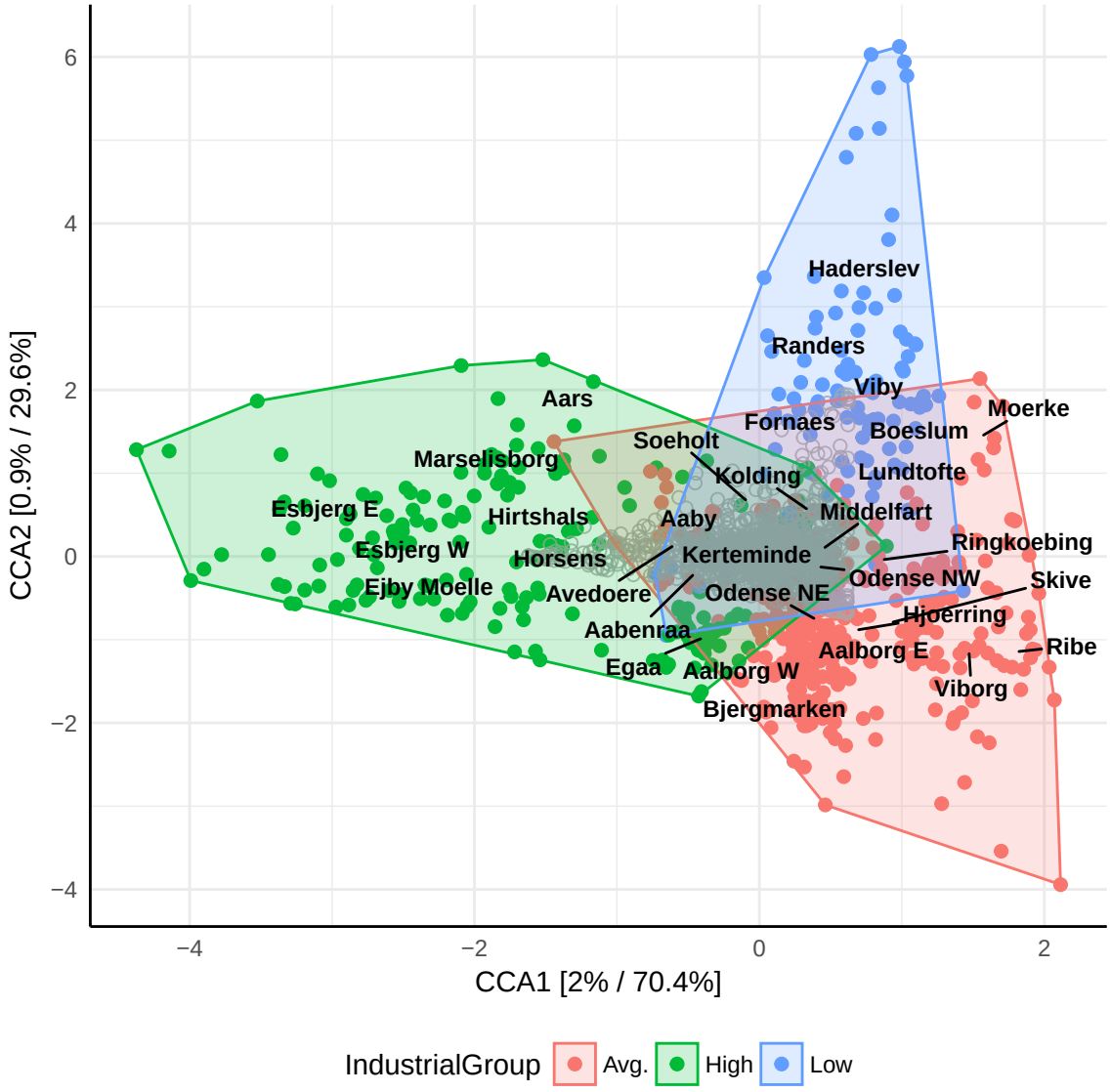


Figure 6.6: Industrial, hellinger - updated 14/3-15:20

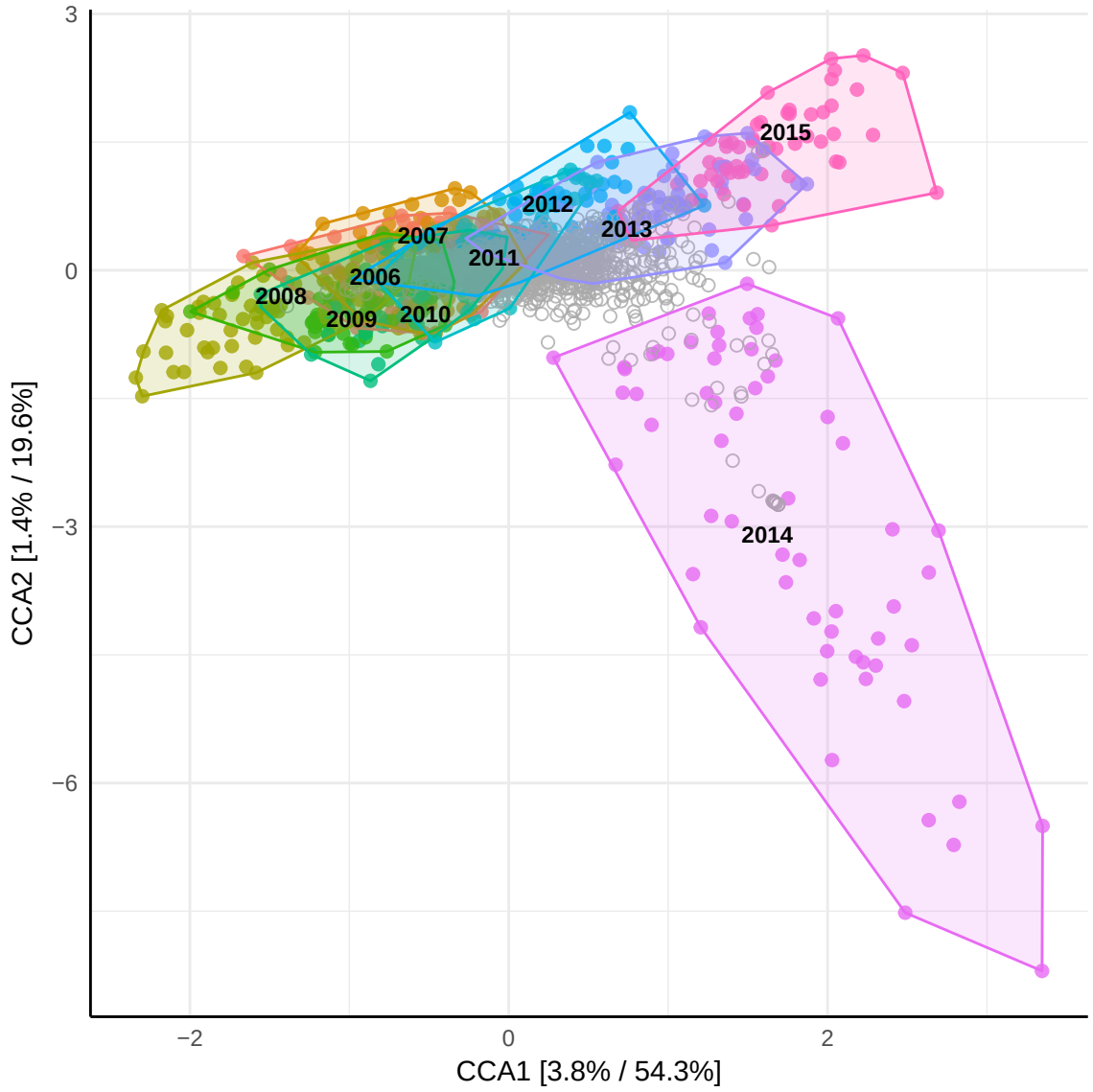


Figure 6.7: Stability

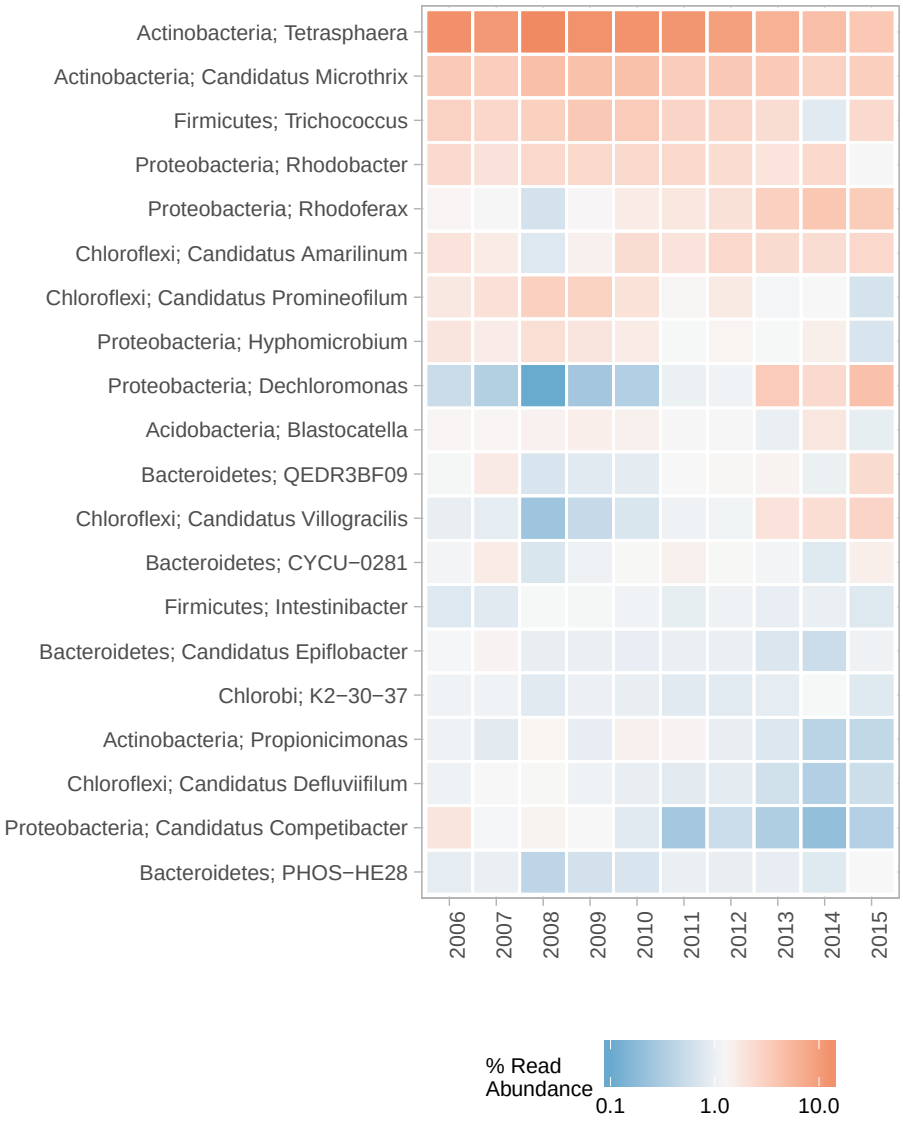


Figure 6.8: Stability

7. Discussion

- Procrustes SS MDSbray + MDSjsd = 0.04089
- nMDS bray-curtis and JSD stress value around 0.24, TERRIBLE
- rJSD, lower numbers because sqrt, procrustes affected
- hellinger DISTANCE is possible, but hasn't been tested
- Movement in the water, sample position not the same
- subsetting ribe+esbjerg makes ejby moelle stand out in CCA, highlights that more data means smaller distances on the plot
- removing 2014 didn't make a difference

8. Conclusion

If we don't want Conclusion to have a chapter number next to it, we can add the `{-}` attribute.

More info

And here's some other random info: the first paragraph after a chapter title or section head *shouldn't be* indented, because indents are to tell the reader that you're starting a new paragraph. Since that's obvious after a chapter or section title, proper typesetting doesn't add an indent there.

A. Characteristics of the WWTPs

Table A.1: Summary of the design of the 32 wastewater treatment plants. EBPR: Enhanced Biological Phosphorous Removal, BNR: Biological Nutrient Removal, RSS: Return Sludge Sidestream, Industrial Inf.: Industrial Inflow

Plant	Configuration	Design	RSS	Primary Setling	Digester	Industrial Inf.
Aabenraa	Alternating	EBPR	RSS	No	Yes	Low
Aaby	Alternating	EBPR	NO	No	Yes	Avg.
Aalborg E	Alternating	EBPR	RSS	No	Yes	Avg.
Aalborg W	Alternating	EBPR	RSS	Yes	Yes	Avg.
Aars	Alternating	BNR	NO	No	No	High
Avedoere	Alternating	BNR	NO	No	Yes	Avg.
Bjergmarken	Alternating	EBPR	NO	No	Yes	Avg.
Boeslum	Recirculation	EBPR	NO	No	No	Low
Egaa	Recirculation	EBPR	RSS	No	No	High
Ejby Moelle	Alternating	EBPR	NO	Yes	Yes	High
Esbjerg E	Recirculation	BNR	NO	Yes	Yes	High
Esbjerg W	Recirculation	BNR	NO	Yes	Yes	High
Fornaes	Recirculation	BNR	NO	No	Yes	Low
Haderslev	Alternating	EBPR	RSS	No	No	Low
Hirtshals	Alternating	EBPR	NO	No	No	High
Hjoerring	Recirculation	EBPR	NO	Yes	Yes	Avg.

Horsens	Recirculation	BNR	NO	Yes	Yes	High
Kerteminde	Recirculation	EBPR	NO	No	No	Avg.
Kolding	Alternating	EBPR	NO	Yes	Yes	Avg.
Lundtofte	Alternating	EBPR	NO	Yes	Yes	Low
Marselisborg	Alternating	BNR	NO	Yes	Yes	High
Middelfart	Recirculation	BNR	NO	Yes	Yes	Avg.
Moerke	Alternating	EBPR	NO	No	No	Avg.
Odense NE	Alternating	EBPR	NO	Yes	Yes	Avg.
Odense NW	Alternating	BNR	NO	Yes	Yes	Avg.
Randers	Recirculation	EBPR	RSS	Yes	Yes	Low
Ribe	Recirculation	EBPR	RSS	No	No	Avg.
Ringkoebing	Alternating	EBPR	RSS	Yes	Yes	Avg.
Skive	Recirculation	EBPR	RSS	No	No	Avg.
Skive	Recirculation	EBPR	RSS	No	No	High
Soeholt	Alternating	EBPR	RSS	Yes	Yes	High
Viborg	Recirculation	EBPR	RSS	Yes	Yes	Avg.
Viby	Recirculation	EBPR	RSS	No	Yes	Low

B. Supplementary plots

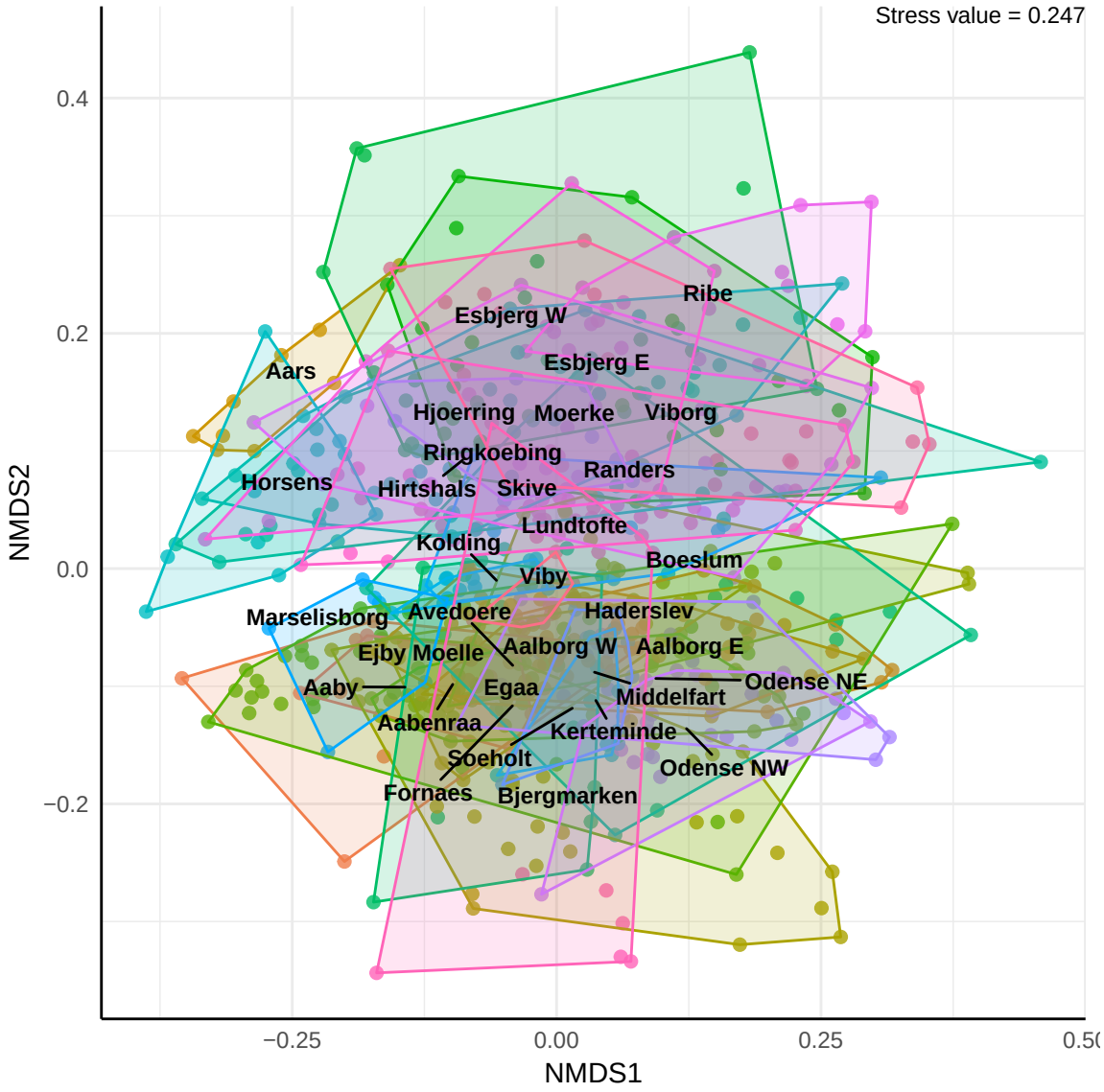


Figure B.1: non-Metric Multidimensional Scaling, Bray-Curtis Dissimilarities. No Transformation.

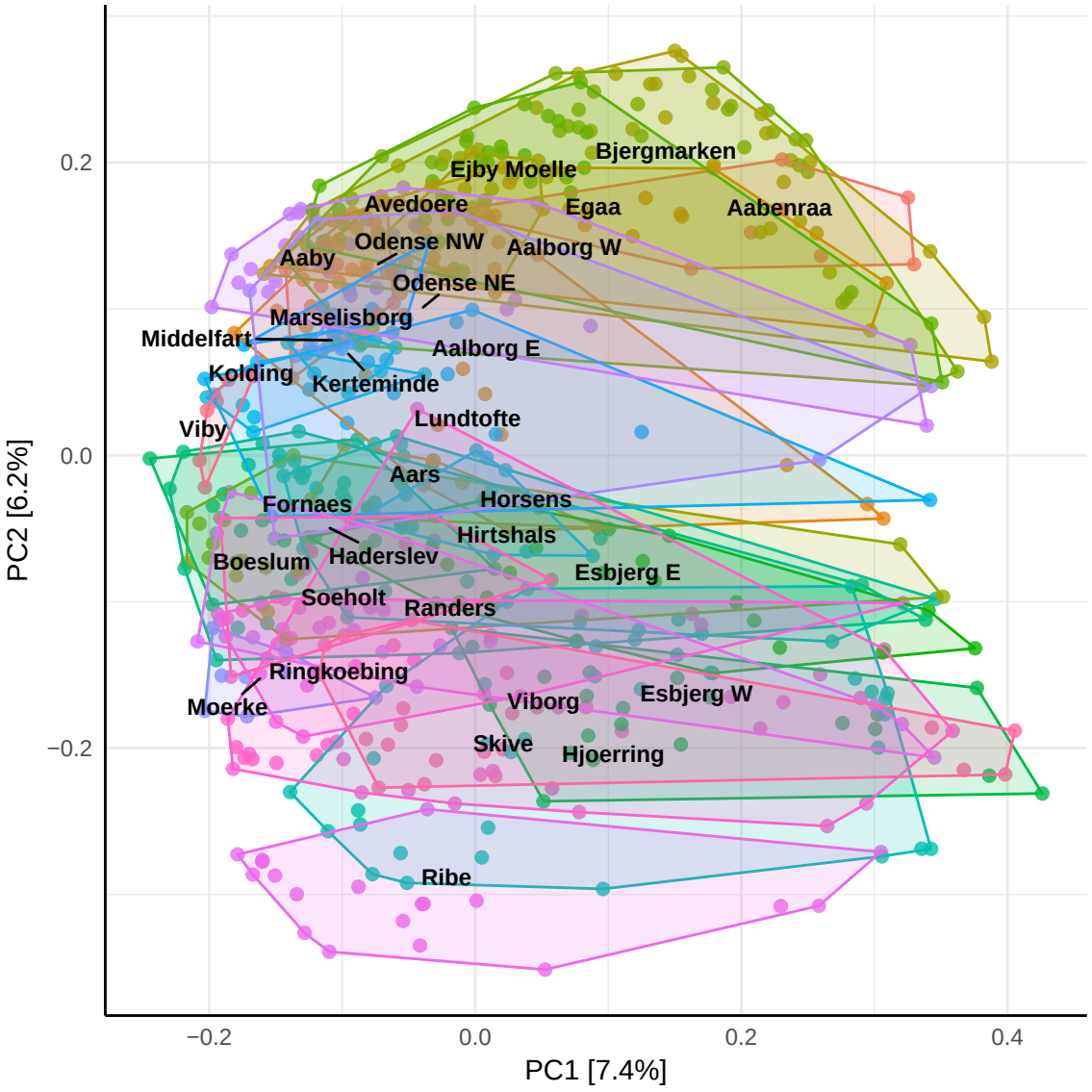


Figure B.2: Principal Components Analysis where all positive abundances have been set to 1. Hellinger Transformed.

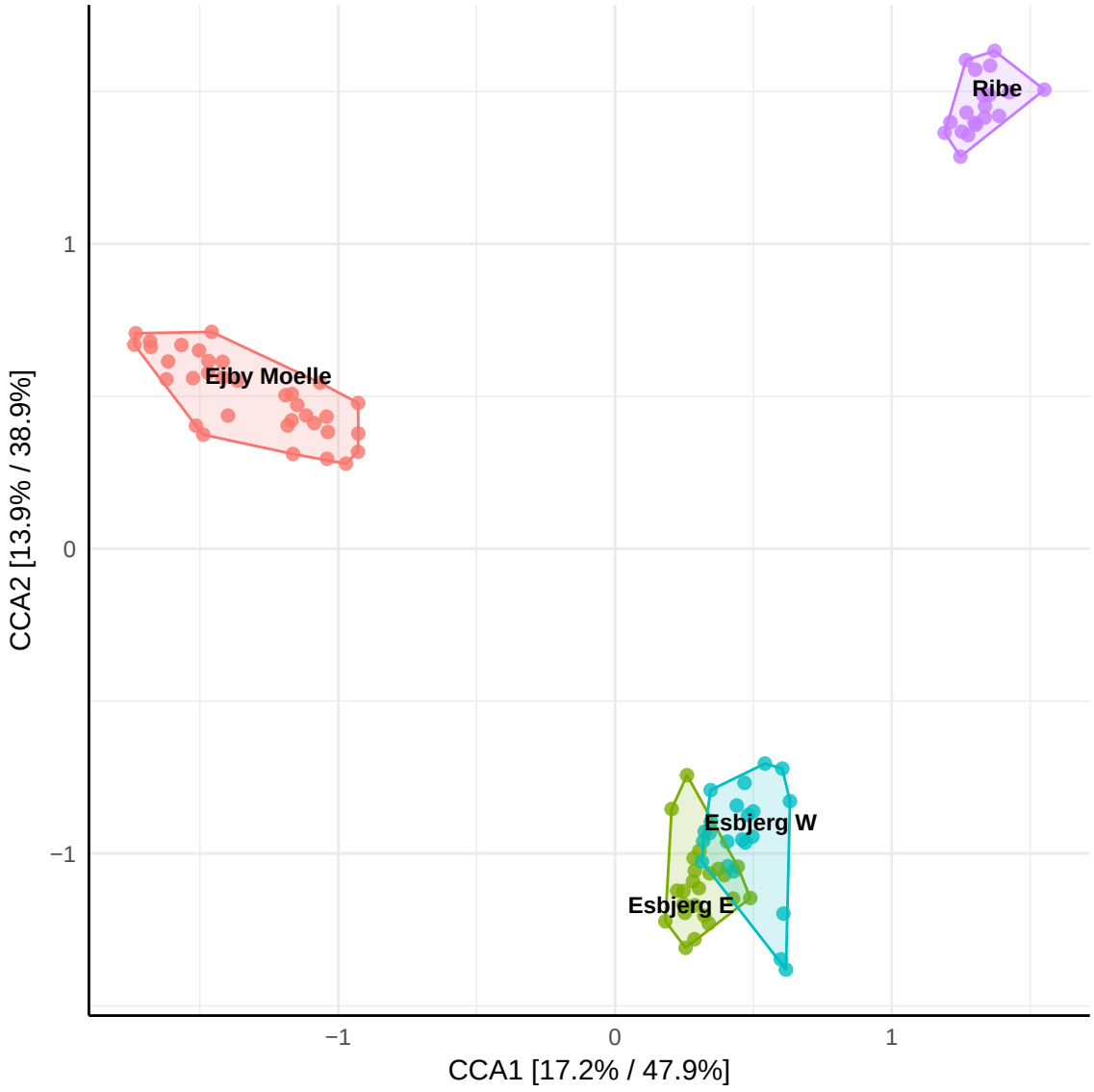
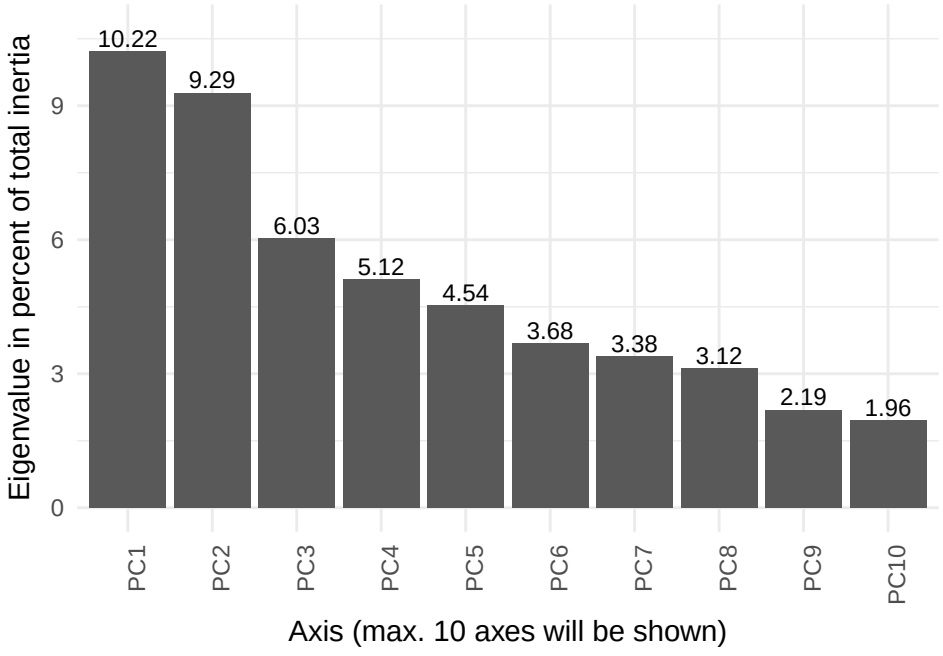


Figure B.3: Canonical Correspondence Analysis of samples from 4 of the WWTPs. Hellinger transformed.

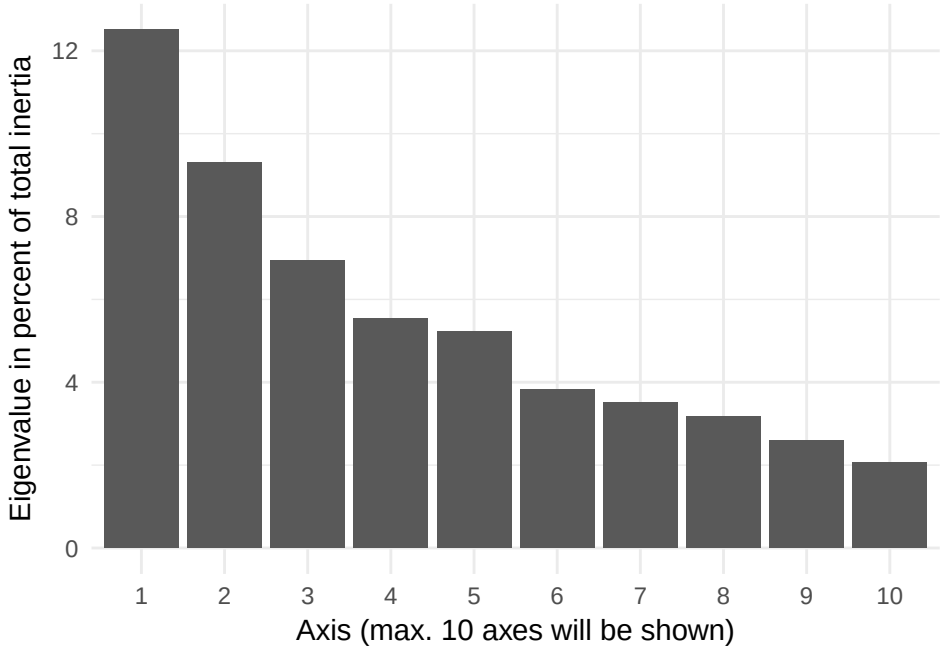
C. Scree plots

Scree plots for all plots in the two results chapters are shown here.

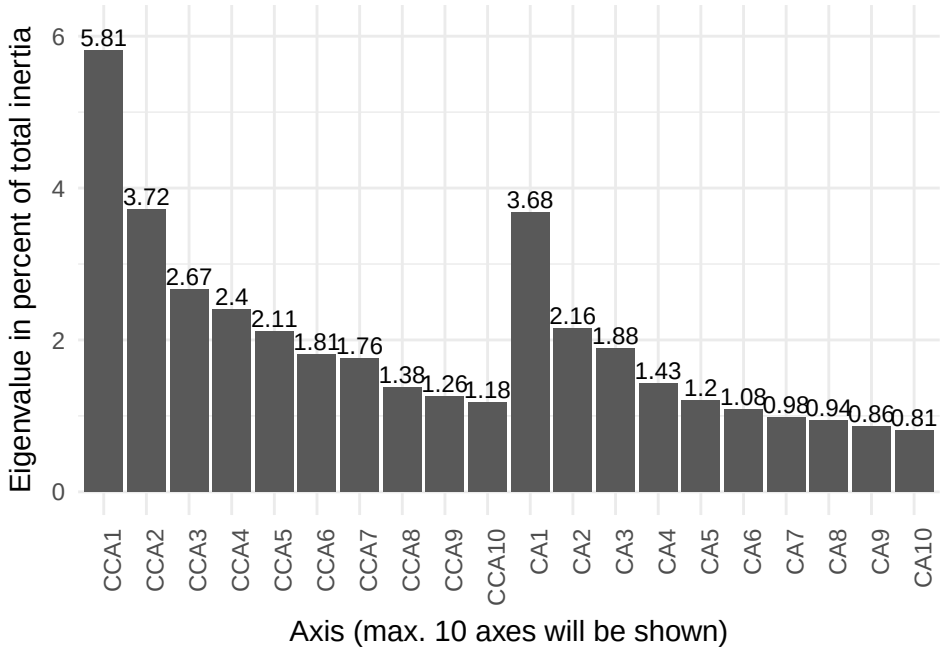
C.1 Scree plot of Figure 5.1



C.2 Scree plot of Figure 5.2



C.3 Scree plot of Figure 5.3



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