BACHELORARBEIT

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Vorkommen und Diversität von Hefen im Blütennektar von *Helleborus viridis* L.

Occurrence and Diversity of Yeasts in Floral Nectar of *Helleborus viridis* L.

von

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1 Introduction

Yeasts are frequent in floral nectar of animal-pollinated plants. Since nectar is the most frequent reward plants offer to their pollinators and therefore represents the key link in the plant-pollinator-mutualism (Simpson and Neff, 1983), a vast amount of studies investigating its composition, spatial and temporal secretion patterns, as well as the adaption of these features to the characteristics of pollinators, has been carried out (e.g. Baker and Baker, 1982; Vesprini et al., 1999; de Vega et al., 2009). However, little information is known about the yeasts that occur in floral nectar, even if their existence has been known to microbiologists for more than a century (Boutroux, 1884; Grüß, 1917).

Floral nectar is thought to be initially sterile and nectarivorous yeasts are apparently introduced by pollinating insects (Grüß, 1917; Brysch-Herzberg, 2004). This finding is corroborated by several studies, where nectar remained sterile, if pollinators were experimentally excluded from plants (e.g. Schoellhorn, 1919; Herrera and Pozo, 2010). It could be shown that blossom honey contains yeast species that are typical of floral nectars (Seijo et al., 2011), which confirms at least the transportation of yeasts by pollination vectors. However, the plant-pollinator mutualism was traditionally believed to be a binary system, where insects are rewarded for their pollination service by plants through the provision of rewards, such as nectar. Recent studies have revealed that nectar yeasts have a major impact on this mutualism and therefore should be regarded as a third player in this interaction (e.g. Canto et al., 2007, 2008; Herrera et al., 2008, 2009). However, their exact influence on the plant-pollinator mutualism is not clarified yet.
In many cases, nectar yeasts could be interpreted as mainly parasitic towards plants and hence towards pollinators. It could be demonstrated that they modify the composition of floral nectar (Herrera et al., 2008): total sugar concentration declines with increasing density of yeast cells in nectar. Furthermore sucrose is hydrolysed into the monosaccharids glucose and fructose, which are then metabolised by the yeasts. Because the proportions of these monosaccharids in modified nectar are highly nonstoichiometric, it is supposed that glucose is preferably metabolised compared to fructose (Herrera et al., 2008). This modification in nectar composition implies a degradation of the nectar's nutritional value and therefore probably also a loss of its attractiveness to pollinators. A study revealed that bumblebees, which are frequent pollinators, are very sensitive to sugar concentration in nectar and quickly disregard flowers with dilute nectar if

flowers with more concentrated nectar are available (Cnaani et al., 2006). However, in this study, nectar concentration was directly associated to a flower type with a specific odour. Nectarivorous yeasts cause fermentation odours, which are known to play an important role in signal mediation between plants and pollinators (Pozo et al., 2009), but the concrete effects on the mutualism are not known here. It has to be further investigated, if pollinators can associate these odours with lower floral reward and, furthermore, if they can perceive these odours already before landing on a flower, i.e. before pollination service. This would most probably result in a lower visitation frequency of affected flowers by pollinators and hence to a reduced reproductive success of the plant. Another negative impact yeasts evoke towards their hosts is that their occurrence in nectar may inhibit pollen germination, if it normally takes place in the nectar (Eiskowitch et al., 1990).
On the other hand the occurrence of yeasts in floral nectar may also have some positive effects on the plant-pollinator mutualism. A recent study revealed that nectar yeasts warm the flowers of *Helleborus foetidus*, a winter-blooming plant. This effect was tested by experimentally excluding yeasts from flowers or adding yeasts to flowers, respectively. Thermal differences lay between 1 °C and 2 °C (Herrera and Pozo, 2010). Floral warming can have positive impacts on the plant-pollinator mutualism, as foraging insects prefer warmer flowers. However, this tendency could only be shown to be significant, if thermal difference was 4 °C or more (Dyer et al., 2006). Furthermore, as floral warming via yeasts is caused by the yeasts' metabolism and therefore is associated with a degradation of nectar sugar concentration, the positive effect on the plant-pollinator mutualism may be compensated depending on the circumstances (Herrera and Pozo, 2010).
Concluding one can say that nectar yeasts probably are predominantly parasitic exploiters of plant-pollinator mutualisms; to what extend they may also have positive effects on it has to be further investigated.

Yeasts are frequent in floral nectar of many plant species, irrespective of continent or habitat type (Herrera et al., 2009). Studies investigating their frequency of occurrence report that yeasts could be found in about 40 % (Jimbo, 1926; Sandhu and Wairach, 1985; Herrera, 2009) to about 70 % (Sandhu and Waraich, 1985) of all nectar samples of all examined plant species combined in each case. For individual plant species, the frequency of occurrence ranged between 0 % and 100 % (Jimbo, 1926; Herrera et al., 2009). Occurrence of yeasts in floral nectar depends on several

environmental factors and is influenced by the yeasts' physiological possibilities, as well as by the pollinator visitation frequency.

Chemical and physical nectar properties are of great importance here. Nectar yeasts occupy a broad niche in floral nectar and can grow on most sugar compositions, sugar concentrations and pH-values naturally occurring in floral nectar so that these properties do not directly influence yeast proliferation in most cases. However, as nectar features strongly affect the visitation frequency of pollinators and thus the dispersal of yeast inocula, they indirectly play a major role in determining yeast occurrence in floral nectar (Brysch-Herzberg, 2004).

Temperature is another important aspect here, as yeasts show higher growth rates with higher temperatures. This explains the seasonal fluctuations with most nectar yeasts found in summer, whereas the question of where they survive through winter remains open. Pollinators, which are more active and numerous at higher temperatures, reinforce this temperature effect (Brysch-Herzberg, 2004).

Since occurrence of yeasts depends on the yeasts' physiological possibilities, as well as on pollinator visitation (see above), it is suggested that yeasts may show certain distribution patterns within flowers. However, studies analysing distribution patterns of nectar yeasts are scarce. High variance in yeast occurrence could be demonstrated at the intra-plant level, when single flowers were assessed as sampling units. Flowers are discrete, island-like habitats. The distribution patterns of yeasts are non-random here and are probably caused by spatially non-random foraging by pollinators, which is strongly related to territoriality and the quality of nectar (Belisle et al., 2011). Other studies rather analysed variation of nectar composition on different scales (among plants of the same species, among flowers of the same plant, among nectaries of the same flowers) (Herrera et al., 2006; Canto et al., 2007). While variance among plants was rather negligible, variance among flowers of the same plant and among nectaries of the same flower were high. It is hypothesised that this variation in nectar composition is caused by variable yeast contamination at the different levels but experimental studies corroborating this assumption are lacking (Canto et al., 2007).

Yeasts can reach extremely high densities in floral nectar: cell densities in the order of 10^3 - 10^4 cells mm^{-3} are commonplace and densities over 10^5 cells mm^{-3} are not unusual (Herrera et al., 2008, 2009). Yeast cell size may be crucial in limiting the maximum densities due to simple

physical constraints. This is corroborated by the fact that the highest cell densities ever reported in nectar (up to 3.6 x 10^6 cells mm^{-3}) were found for rather small yeasts (de Vega et al., 2009).

The main components of nectar are sugars, mainly sucrose, glucose and fructose (Baker and Baker, 1982). It therefore may be regarded as a highly favourable habitat for yeasts. However, nectar also contains secondary metabolites, such as alkaloids and phenols as a protection against nectar robbers (Baker and Baker, 1982), even if it is assumed that plants only produce few metabolites that can favour pollinators and discourage nectar exploiters at the same time (Kessler and Baldwin, 2007). Furthermore floral nectar is characterized by very low nitrogen content (Nicolson et al., 2007). All these aspects make a combination of osmotolerance, tolerance or resistance to secondary compounds and efficient nitrogen use essential for its colonisation. Hence, nectar acts as a "filter": while pollinators bear inocula of a vast amount of different yeast species to the flowers (the potential yeast species pool), only a small, phylogenetically clustered, subset of these species can also be found in floral nectar (the realised community) (Herrera et al., 2009). This illustrates the fact that only few specialists are able to colonise floral nectar. Moreover, strong priority effects between different yeast species limit the per-flower species richness. Here the arrival order of yeasts is crucial and negative growth effects are especially strong between closely related species. Strong priority effects may also lead to positive feedback where abundant species become more abundant, consequently excluding other species and leading to impoverished yeast communities (Peay et al., 2011). A survey reported that 97 % of colonised flowers had only one fungal species (Belisle et al., 2011); in another survey each nectar sample contained 1.3 ± 0.6 yeast species (Pozo et al., 2010).

The majority of nectar yeasts belongs to the ascomycetes. The most common nectar yeast species by far are *Metschnikowia reukaufii* PITT & M.W. MILL. and *Metschnikowia gruesii* GIM.-JURADO (e.g. Brysch-Herzberg, 2004; Pozo et al., 2011), which were considered as one up to 1992 (Giménez-Jurado, 1992). They differ in their cell configuration with *Metschnikowia gruesii* typically showing an airplane cell configuration (Giménez-Jurado, 1992) that allows it to stick more easily and in greater numbers to the fine hairs of insects than other yeasts (Grüß, 1917). Both *Metschnikowia* species are highly specialised in the plant-pollinator mutualism and live in the nectars of numerous plant species. As nectars represent a patchy and highly heterogeneous habitat (see above), phenotypic plasticity is essential for these yeasts. A recent study revealed that environmentally induced DNA methylation polymorphisms enabled genotypes to exploit such a

broad niche (Herrera et el., 2011). Other ascomycetous nectar yeasts, like *Candida spec.* and *Zygosaccharomyces spec.*, are less frequent in floral nectar and are not restricted to the plant-pollinator mutualism (Brysch-Herzberg, 2004). Basidiomycetes, like *Cryptococcus spec.*, may also be found in nectar. However, as they are much more frequent on plant surfaces and the distances between some plant surfaces and nectaries are very short, it is suggested that they belong to phyloplane and not to nectar. Furthermore it seems that only ascomycetous yeast cells multiply well in nectar, while basidiomycetous yeasts lack this ability (Brysch-Herzberg, 2004).
Concluding you can say that nectar "filtering" and priority effects result in a very low yeast diversity in floral nectar and that extremely high phenotypic plasticity allows *Metschnikowia spec.* to be "jack of all nectars (and) master of most" (Herrera et al., 2011).

Occurrence and diversity of yeasts in floral nectar have been investigated on several plant species, including *Helleborus spec.* (e.g. Herrera et al., 2009; Pozo, 2009). The genus *Helleborus* belongs to the Ranunculaceae. Plants are herbaceous deciduous perennial hemikryptophyts or chamaephyts that flower from late winter to early spring (Rothmaler, 2011). Seed dispersal is myrmecochor. As it is the case for many other Ranunculaceae, petals of *Helleborus* are modified to nectaries that consist of an epidermis and a nectar producing parenchyma and secrete nectar continuously for about 20 days. They are green and show considerable photosynthetic activity (Vesprini et al., 1999).
Helleborus viridis L., the species probed in the present study, is endemic to western and central Europe (Flora Europaea). Its distribution area embraces submeridional (montane) to temperate zones (BiolFlor). In Germany it is particularly protected and not threatened; it is dispersed in central and southern parts of the country, few neophytes can also be found in the east. *Helleborus viridis* occurs in mesophilous deciduous forests in association with Querco-Fagetea BR.-BL. et VLIEGER. It grows on semi-humid calciferous soils (Rothmaler, 2011). *Helleborus viridis* measures between 15 cm and 40 cm; leaves are digitate and scleromorphic. The hermaphroditic plant is protogynous and the main flowering period lies between March and April. The perianth, consisting of five sepals and a variable number of nectaries, is green and outstretched (Rothmaler, 2011). Nectar of *Helleborus viridis* is completely hidden and the main pollinators are Hymenoptera, typically bumblebees and bees, as well as some Diptera (BiolFlor).

The aims of the present study are (i) to investigate the frequency of occurrence of yeasts in floral nectar of *Helleborus viridis*, (ii) to assess if yeast distribution patterns within flowers are related to nectary position or nectar volume, (iii) to determine yeast species and (iv) differences in their abundance in floral nectar, as well as (v) in their to distribution patterns within flowers and, furthermore, (vi) to assess the yeast's α-diversity and β-diversity on different scales.

2 Material and Methods

2.1 Laboratory Equipment

2.1.1. Devices

Centrifuge 5424	Eppendorf, Hamburg, Germany
DNA Engine Peltier Themal Cycler	Bio-Rad Laboratories, Inc., Munich, Germany
shaker SM-30	Edmund Bühler GmbH, Hechingen, Germany
Thermomixer Comfort/Compact	Eppendorf, Hamburg, Germany
UV transilluminator	Vilber Lourmat, Marne-la-Vallée, France
Vortex-Genie ® 2	Scientific Industries, Bohemia, USA

2.1.2. Chemicals

agar	AplliChem, Darmstadt, Germany
agarose	Bio-Budget Technologies GmbH, Krefeld, Germany
boracic acid	AplliChem, Darmstadt, Germany
bromophenol blue	Merck, Darmstadt, Germany
buffer Y	Peqlab Biotechnologie GmbH, Erlangen, Germany
chloroform	J. T. Baker, Deventer, The Netherlands
EDTA	Merck, Darmstadt, Germany
ethanol	Sigma-Aldrich, St. Louis, USA
fructose	AplliChem, Darmstadt, Germany
glucose	AplliChem, Darmstadt, Germany
hydroxyquinoline	Merck, Darmstadt, Germany
dNTPs	Bio-Budget Technologies GmbH, Krefeld, Germany
ethidium bromide	Fisher Scientific GmbH, Schwerte, Germany
lactat	Roth, Karlsruhe, Germany
malt extract	Merck, Darmstadt, Germany
NaCl	J. T. Baker, Deventer, The Netherlands
peptone/soytone	Roth, Darmstadt, Germany
primers	Sigma Genosys, The Woodlands, USA
RNase	Sigma-Aldrich, St. Louis, USA
SDS	AplliChem, Darmstadt, Germany
sucrose	AplliChem, Darmstadt, Germany
Taq-Polymerase	Peqlab Biotechnologie GmbH, Erlangen, Germany
tris base	Sigma-Aldrich, St. Louis, USA
tris-HCl	AplliChem, Darmstadt, Germany
triton x-100	Roth, Darmstadt, Germany
yeast extract	AppliChem, Darmstadt, Germany
yeast nitrogen base	Fluka, Sigma-Aldrich, St. Louis, USA

2.1.3. Solutions, Buffers and Media

0.8 % / 2 % agarose:	0.8 % / 2 % agarose 1x TBE buffer	Heat in the microwave until agarose is dissolved.
6x loading buffer:	50 % (w/v) sucrose ca. 0,1 % (w/v) bromophenol blue	Prepare with TE-buffer-water and sterile-filter. Store at 4 °C.
5x TBE buffer:	440 mM tris base 440 mM boracic acid 2 % (v/v) 0,5 M EDTA, pH 8	Prepare with seradest water.
TE-phenol/chloroform:	1 part of phenol 0.32 % 8-hydroxyquinoline 1 part of chloroform	Add 8-hydroxyquinoline to phenol, until colour turns yellow. Add chloroform. Centrifuge at 4000 rpm for 15 min. Store at 4 °C.
TE-RNase:	1 % (w/v) RNase	Solve in TE-buffer pH 8.
usti lysis buffer:	10 mM TrisHCl pH 8 100 mM NaCl 1 mM Na_2-EDTA 1 % SDS 2 % (v/v) Triton x-100	Fill up with seradest water up to according volume. Do not autoclave. Store at room temperature.
YM medium (fixed):	1 % (w/v) glucose 0.3 % (w/v) yeast extract 0.5 % (w/v) peptone/soytone 0.3 % (w/v) malt extract 2 % (w/v) agar 0.1 % (w/v) lactat	Prepare with distilled water in wide neck bottle, add agitator. Autoclave for 5 to 20 min at 121 °C.
YM medium (liquid):	1 % (w/v) glucose 0.3 % (w/v) yeast extract 0.5 % (w/v) peptone/soytone 0.3 % (w/v) malt extract	Prepare with distilled water in wide neck bottle. Autoclave for 5 to 20 min at 121 °C.
YNB + 3 S medium:	0.67 % (w/v) yeast nitrogen base 1 % (w/v) glucose 1 % (w/v) sucrose 1 % (w/v) fructose	Prepare with distilled water in wide neck bottle, add agitator. Autoclave for 5 to 20 min at 121 °C.

2.2 Nectar Sampling and Yeast Cultivation

Samples were collected at two sites in Hagen (Germany) during late March to early April. Both sites, Hasselbachtal and Weißenstein, are nature reserves, which vegetation is mainly characterised by *Fagus sylvatica* L. Streams and meadows are nearby.

All nectaries (7 to 13) of five flowers of *Helleborus viridis* were probed at two different points of time at each site. Each nectary represented a single sampling unit resulting in a total of 162 samples (table 1). Only flowers being in a rather late (masculine) sexual phase were chosen. Nectar was taken using a 1 µl capillary; nectary position and nectar volume were noted. 100 µl of YNB + 3 S medium (10x) were added to each nectar sample and the mixture was thermomixed at room temperature and 600 rpm for 24 hours for yeast enrichment. Each sample was then plated on YM-media and stored at 4 °C, 16° C or room temperature (depending on growth rapidity of yeasts and moulds) until yeast colonies became visible.

As a negative control for the enrichment, nectar samples of an additional flower of each population were given into 100 µl of H_2O and directly plated. Furthermore a control for the sterility of the YNB + 3 S medium was established by plating 100 µl of the pure medium on YM-medium.

Liquid cultures of every identified yeast strain of each plate were established: cultures were transferred to 2000 µl of liquid YM-medium and mixed on a shaker for at least 24 h at 200 rpm at room temperature until cloudy.

Table 1: Sampling design. Numbers represent the number of samples collected at the corresponding date and site. * marks the negative control for the enrichment. A, B, C and D will be used as nomenclature for the corresponding samplings in the further analysis.

site	Hasselbachtal	Weißenstein
date		
16.3.12	A: 48 + 9*	
20.3.12		B: 39 + 8*
29.3.12	C: 38 + 8*	
4.4.12		D: 37 + 8*

2.3 Yeast Identification

DNA was isolated via phenol-chloroform-extraction: 1000 µl of yeast culture and 200 µl of sterile glass beads (∅ 0.25-0.5 mm) were centrifuged at 14680 rpm for 2 min and the supernatant was removed. Samples were frozen for at least 30 min. After defrosting, 500 µl of usti lysis buffer and 500 µl of TE-phenol-chloroform were added to each sample, which was then vortexed and centrifuged, both at full speed and for 15 min. 400 µl of the upper phase and 1000 µl of ethanol (100 %) were mixed and centrifuged at 14680 rpm for 15 min for precipitation. The supernatant was removed and 50 µl of TE-RNase (pH 8.0; 10 µg/ml) were added. Samples were then thermomixed at 55 °C at 800 rpm for 15 min to solve the pellet.

DNA extraction was controlled via gel electrophoresis and subsequent gel documentation. Each gel consisted of 40 ml of agarose (0.8 %) and 2 µl of ethidium bromide. 2 µl of isolated DNA and 5 µl of loading buffer were given into each slot and the gel was put into a gel electrophoresis chamber filled with 1x TBE-buffer. A voltage of 130 V was applied for 30 min. DNA bands were then made visible under UV light.

To reduce the number of samples to sequence, MSP-PCRs were carried out. Two microsatellite primers (5'-$(GTG)_5$ and 5'-$(GAC)_5$) were used for this purpose. Reactions were carried out in a 27 µl volume containing 2.5 µl of Buffer Y (10x with 20 mM $MgCl_2$), 19.9 µl of ddH_2O, 0.5 µl of dNTPs, 0.5 µl of each primer (10 pmol/µl), 0.1 µl of *Taq*-polymerase and 3 µl of diluted DNA (dilution ranged between 1:1 to 1:1000 depending on the intensity of DNA bands on the DNA extraction control gel documentation). MSP-PCRs were conducted as follows: initial denaturation step of 96 °C for 2 min; 34 cycles with denaturation at 96 °C for 20 s, anealing at 52 °C for 50 s and extension at 72 °C for 90 s; final extension step of 72 °C for 7 min; storage at 10 °C.

Gel electrophoresis and gel documentation were then accomplished as described above, but using 40 ml of agarose (2 %) and 3 µl of ethidium bromide for each gel and giving 8 µl of MSP-PCR product and 2 µl of loading buffer into each slot. Samples showing identical electrophoretic profiles were considered as conspecific and only one of each representative was further proceeded.

The D1/D2 domains of the large subunit nuclear rRNA, which are commonly used for yeast identification (e.g. Kurtzmann and Robnett, 1998; Fell, 2000; Brysch-Herzberg, 2004) were amplified with the primers ITS1-f (5'-CTT GGT CAT TTA GAG GAA GTA) and NL4 (5'-GGT CCG TGT TTC AAG ACG G). PCR reactions were performed in a 50 µl volume using 5 µl of

Buffer Y (10x with 20 mM $MgCl_2$), 40.8 µl of ddH_2O, 1 µl of dNTPs, 1 µl of each primer (10 pmol/µl), 0.2 µl of *Taq*-polymerase and 1 µl of extracted DNA. PCRs were conducted as follows: initial denaturation step of 96 °C for 2 min; 34 cycles with denaturation at 96 °C for 20 s, anealing at 52 °C for 50 s and extension at 72 °C for 90 s; final extension step of 72 °C for 7 min; storage at 10 °C.

To control PCRs, gel electrophoresis and subsequent gel documentation were carried out as described above, but using 40 ml of agarose (2 %) and 2 µl of ethidium bromide for each gel and giving 5 µl of PCR product and 2 µl of loading buffer into each slot.

PCR products were purified using DNA Clean and Concentrator -5 Kit (Zymo Research) following the manufacturer's instructions. According to the intensity of DNA bands in the gel, purified PCR products were solved in 10 µl to 20 µl of ddH_2O. PCR products were then sequenced and sequences were contingated and edited in Sequencher 5.0 (Gene Codes Corporation). Yeasts were identified using the BLAST algorithm (NCBI). The first hits showing the same ΔE value and a maximum identity value differing by less than 2 % were chosen for the further analyses.

2.4 Statistical Analyses

Statistical analyses were executed in Microsoft Excel (Microsoft Corporation) and R: A Language and Environment for Statistical Computing (R Development Core Team, 2011).

α-diversity was assessed using the Shannon-Index, as well as the Evenness. β-diversity was calculated with the Jaccard-Index.

3 Results

3.1 Occurrence of Yeasts in Floral Nectar of *Helleborus viridis*

3.1.1 Frequency of Occurrence of Yeasts

Yeast species were found in 50 of the 162 nectar samples, corresponding to about 30,86 % colonised nectaries. The frequency of occurrence of yeast species in samples increased with the sampling date, ranging from 8,3 % in the first sampling day to 81 % in the last respectively (fig. 1). However, this tendency was reversed in the negative control for the enrichment (fig. 1). The plated YNB + 3 S control did not show any yeast growth, demonstrating the sterility of the medium.

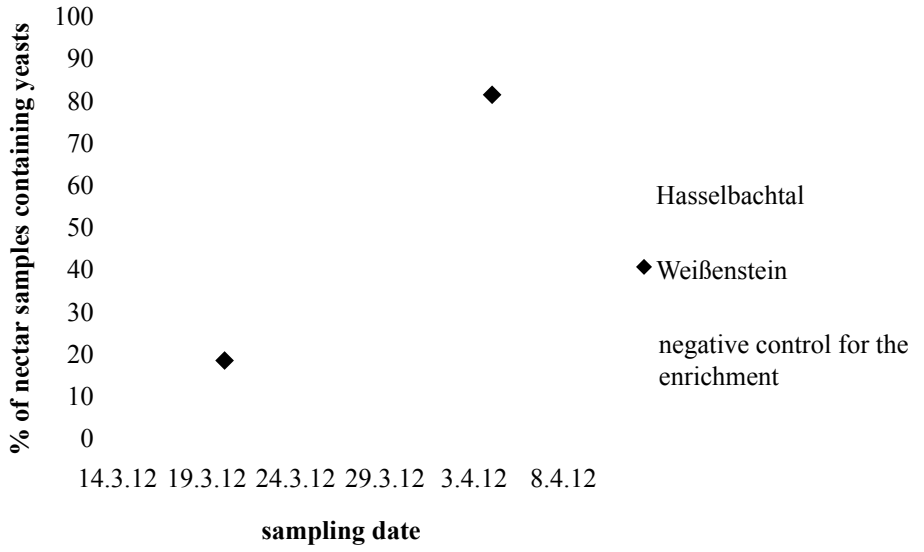

Fig. 1: Frequency of occurrence of yeasts (%) in nectar samplings. X-axis shows sampling date, y-axis gives the percentages for the two populations (Hasselbachtal and Weißenstein). Crosses mark the frequency of occurrence of yeasts in the negative control for the enrichment .

Considering the samplings B, C and D, average yeast cell densities after the enrichment constantly grew from the first to the last sampling date, rising from calculated values of 2441 cells/µl in sampling B, to 13798 cells/µl in sampling C, to 61540 cells/µl in sampling D (fig. 2). Yeasts in sampling A, reached an average cell density of 50000 cells/µl.

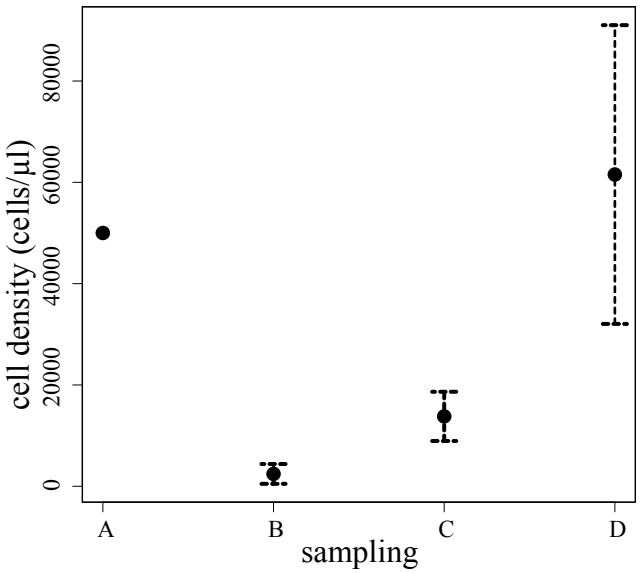

Fig. 2: Average yeast cell density per sampling. Dashed lines mark standard errors. Negative controls for the enrichment and four other samples with undetermined cell density are not included. Therefore only one sample is contained in sampling A.

3.1.2 Distribution Patterns of Yeasts within Flowers

Yeasts were more frequent in the upper nectaries of flowers than in the lower ones (fig. 3). However, when flowers were divided into halves (in case of an unpair number of nectaries, the remaining nectary was counted to the upper half), the upper nectaries did not contain significantly more yeasts than the lower ones ($p = 0.3$, two-tailed exact binomial test).

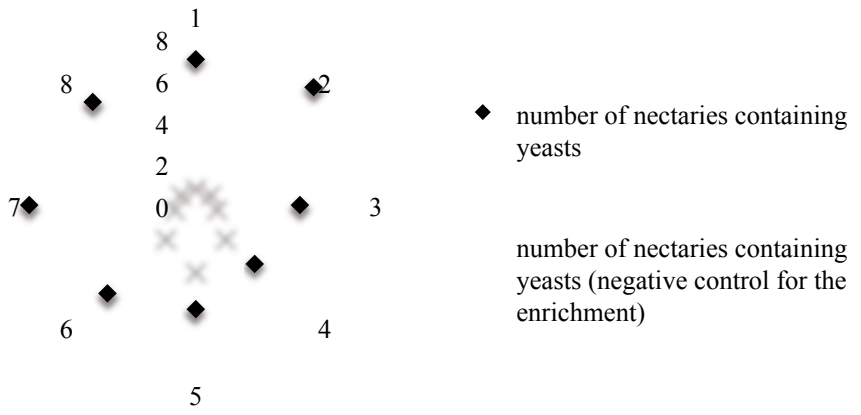

Fig. 3: Number of nectaries containing yeasts according to nectary position. X-axis indicates the flower eighths calculated as follows: (nectary number / total number of nectaries of the flower) x 8. Y-axis shows the number of colonised samples originating from the corresponding flower eighth. Crosses mark the negative control for the enrichment.

Average nectar volumes reached similar values in all flower eighths, independently of nectary position. It was lowest in part 8 and highest in part 1 (fig. 4). Differences between average and median nectar volume were rather big, ranging up to 0.07 µl (fig. 5).

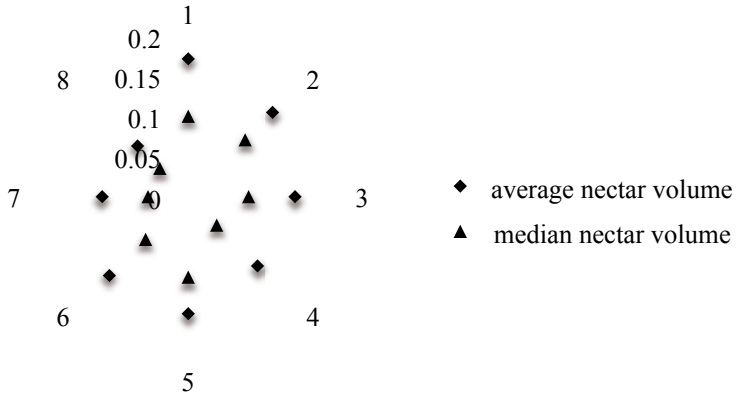

Fig. 4: Average and median nectar volume per flower eighth. X-axis indicates the flower eighths calculated as follows: (nectary number / total number of nectaries of the flower) x 8. Y-axis shows the average and median nectar volume of all samples taken from the corresponding flower eighth. 22 nectar samples are not included as nectar volume could not be determined for them. Negative controls for the enrichment are included.

As shown in fig. 5, yeasts occurred in flower eighths with high average nectar volume (e.g. 0.18 µl for flower eighth 7), as much as in flower eighths with low average nectar volume (e.g. 0.04 µl for flower eighth 3).

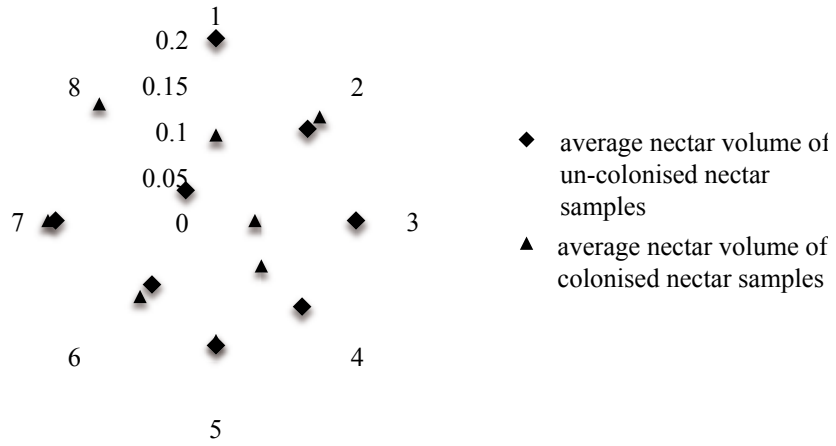

Fig. 5: Average nectar volume per flower eighth for colonised and un-colonised samples. X-axis indicates the flower eighths calculated as follows: (nectary number / total number of nectaries of the flower) x 8. Y-axis shows the average nectar volume of colonised and un-colonised samples taken from the corresponding flower eighth. 22 nectar samples are not included as nectar volume could not be determined for them. Negative controls for the enrichment are included.

3.2 Diversity of Yeasts in Floral Nectar of *Helleborus viridis*

3.2.1 Yeast Species

Considering all nectar samples, a total of 28 yeast taxa could be identified: 18 yeast species belonging to 12 different genera could clearly be determined. Further 5 samples could only be identified up to the genus, and 5 samples had ambiguous BLAST results (table 2). 43 % of identified yeasts belonged to the ascomycetes, 57 % belonged to the basidiomycetes (samples of the negative control for the enrichment excluded). *Metschnikowia reukaufii* was the most frequent species by far, followed by *Starmerella bombicola* and *Cryptococcus victoriae* (table 2). Three yeast taxa could not be identified and are therefore not included in the further analysis.

Table 2: Yeast Taxa identified using the BLAST algorithm. Multiple taxa are listed if the BLAST result was ambiguous. The first number in the right column indicates the total number of nectar samples containing to the corresponding taxon. The following numbers in the right columns A, B, C and D indicate the number of nectar samples containing the corresponding taxon for each sampling. * indicates that the taxon came from negative controls for the enrichment.

			total	A	B	C	D
Ascomycota	Pezizomycotina	*Hormonema* aff. *prunorum*	1				1
	Saccharomycotina	*Candida bombi* MONTROCHER	1		1		
		Candida xestobii YARROW & S.A. MEY. *Pichia caribbica* VAUGHAN-MART., KURTZMANN, S.A. MEY. & E.B. O'NEILL *Pichia guilliermondii* WICK.	1				1
		Debaryomyces maramus DI MENNA	1		1		
		Metschnikowia gruessii GIM.-JURADO *Metschnikowia reukaufii* PITT & M.W: MILL	1			1	
		Metschnikowia reukaufii PITT & M.W. MILL.	14			7	7
		Starmerella bombicola C.A. ROSA & LACHANCE	7				7
	Taphrinomycotina	*Taphrina spec.*	1		1*		
Basidiomycota	Agaricomycotina	*Cryptococcus festucosus* GOLUBEV & J.P. SAMPAIO	6				6
		Cryptococcus laurentii (KUFF.) C.E. SKINNER *Cryptococcus nemorosus* GOLUBEV, GADANHO, J.P. SAMP. & N.W. GOLUBEV *Cryptococcus rajasthanensis* SALUJA & G.S. PRASAD	1				1
		Cryptococcus spec.	3		1		2
		Cryptococcus taibaiensis F.Y. BAI & Q.M. WANG	1				1
		Cryptococcus tephrensis VISHNIAC	1				1
		Cryptococcus victoriae M.J. MONTES, BELLOCH, GALLANA, M.D. GARCÍA, C. ANDRÉS, S. FERRER, TORR.-RODR. & J. GUINEA	6	1	1	1	3
		Cystofilobasidium capitatum (FELL, I.L. HUNTER & TALLMAN) OBERWINKLER & BANDONI	1		1		
		Dioszegia rishiriensis M. TAKASHIMA, B.H. VAN, K.-D. AN & M. OHKUMA	1	1			
		Dioszegia spec.	3	1			2
		Mrakia spec.	1				1

		Rhodotorula spec.	2		1	1	
	Pucciniomycotina	*Erythrobasidium hasegawianum* HAMAM., SUGIY. & KOMAG.	1				1
		Rhodotorula minuta (SAITO) F.C. HARRISON	1				1
		Rhodotorula slooffiae E.K. NOVÁK & VÖRÖS-FELKAI	3	1	1	1*	
		Rhodotorula spec. *Zymoxenogloea spec.*	1		1		
		Sporobolomyces clavatus F.Y. BAI & Q.M. WANG	1		1		
		Sporobolomyces coprosmae HAMAM. & NAKASE	1				1
		Sporobolomyces gracilis DERX	1		1		
	Ustilaginomycotina	*Pseudozyma prolifica* BANDONI	10	9*	1*		
		Rhodotorula hinnulea (R.G. SHIVAS & RODR. MIR.) RODR. MIR. & WEIJMAN *Rhodotorula phylloplana* (R.G. SIVAS & RODR. MIR.) RODR. MIR. & WEIJMAN	1	1			

The phylogentic reconstruction of the identified taxa resulted in the following phylograms. They show the genetic distances between the sequences generated in this study and the assigned Genbank-sequences and therefore the probability of correct identification.

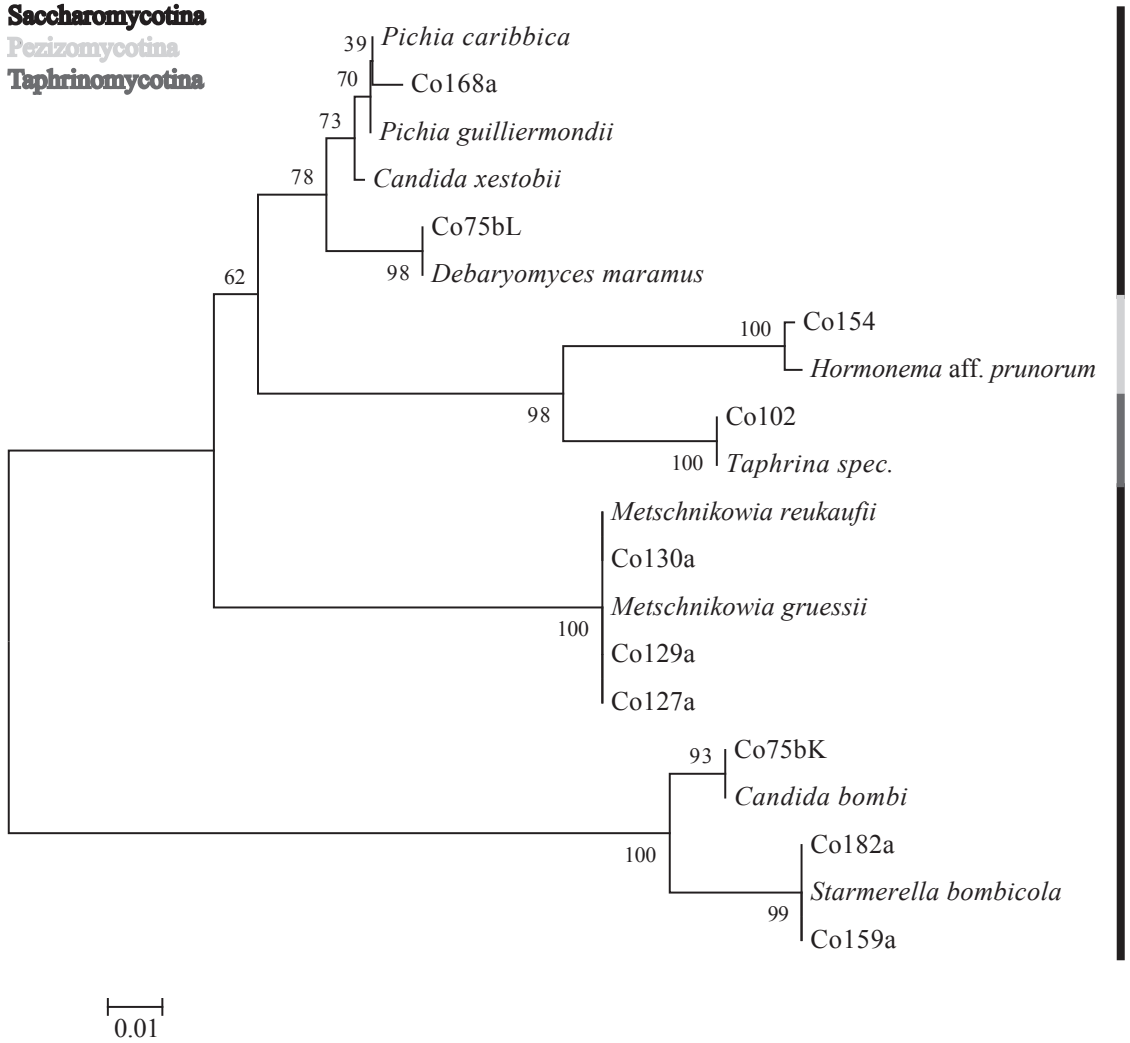

Fig. 6: NJ-phylogram containing all identified ascomycetes and the corresponding first BLAST hits. ITS-sequences were aligned with MUSCLE and cut with Gblocks. Numbers represent bootstrap values (500 replicates). Scale bar indicates 0.01 nucleotide subtitutions per site.

The phylogram shows that the generated sequences of *Metschnikowia spec., Candida bombi* and *Starmerella bombicola* are almost identical to the corresponding Genbank-sequences. The other generated sequences and their corresponding Genbank-sequences have more base substitutions.

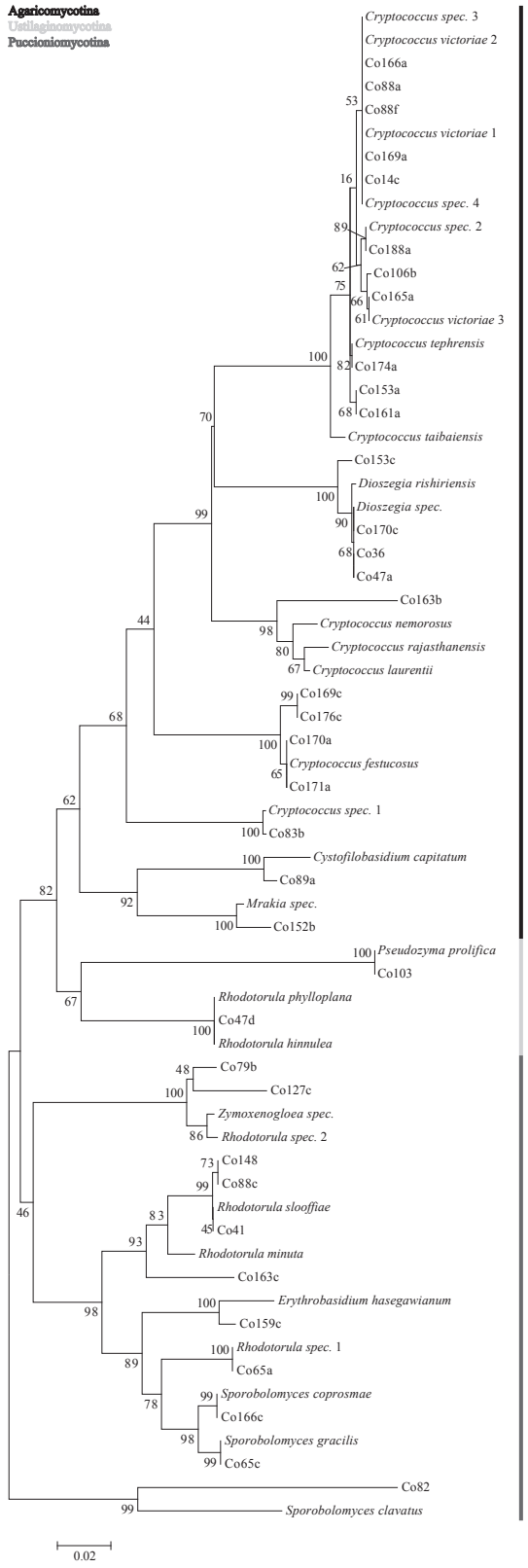

Fig. 7: NJ-phylogram containing all identified basidiomycetes and the corresponding first BLAST hits. ITS-sequences were aligned with MUSCLE and cut with Gblocks. Numbers represent bootstrap values (500 replicates). Scale bar indicates 0.01 nucleotide subtitutions per site.

From the colonised nectar samples containing yeasts, only one yeast species was found in 72 % of the cases, two yeast species in 24 % and three yeast species in 4 %, corresponding to an average of 1,32 ± 0,55 yeast species per sample. Table 3 gives the percentages of nectar samples containing none, one, two or three different yeast species for each sampling and for all samplings together.

Table 3: Percentage of nectar samples containing none, one, two or three yeast species for each sampling and for all samplings combined.

number of yeast species per sample	0	1	2	3
sampling(s)				
A	91.67	4.17	4.17	0
B	82.05	10.26	5.13	2.56
C	76.32	21.05	2.63	0
D	21.62	56.76	18.92	2.7
all	**69.14**	**22.22**	**7.41**	**1.23**

It can be seen that the percentage of nectar samples without yeasts constantly decreases during sampling progress. Nectar samples containing only one yeast species constantly increase from the first to the last sampling date. In contrast the percentage of nectar samples showing more than one yeast species does not show any temporal, but rather a local tendency, with higher values in the Weißenstein population.

3.2.2 Yeast Abundance in Floral Nectar per Species

Metschnikowia reukaufii reached the highest yeast cell densities by far (fig. 8). Cell densities for the following two species, *Starmerella bombicola* and *Cryptococcus victoriae* were about seven times lower and lay around 20000 cells/µl. All other species had yeast densities under 2500 cells/µl.

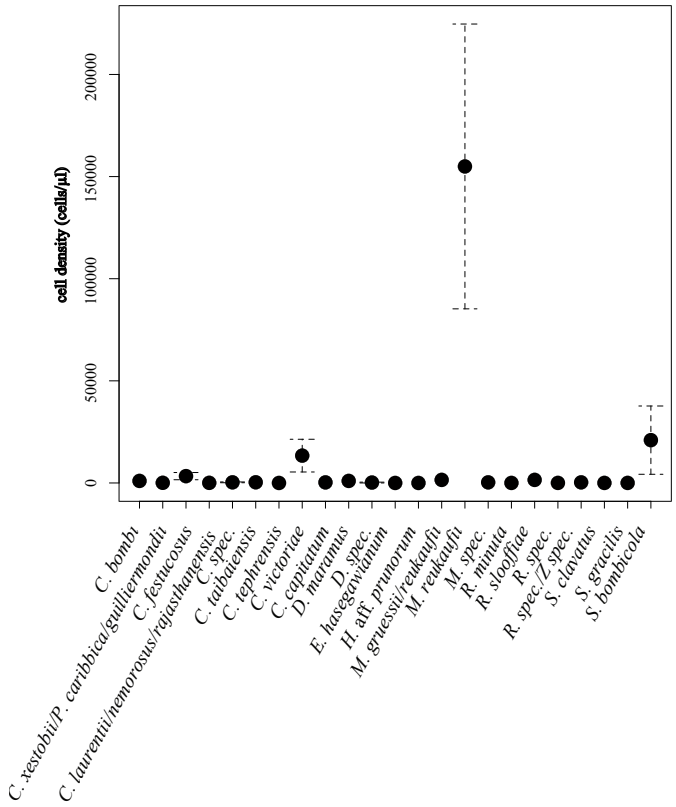

Fig. 8: Average yeast cell density per yeast species. Dashed lines mark standard errors. Negative controls for the enrichment and four other samples with undetermined cell density are not included.

3.2.3 Distribution Patterns of Yeast Species within Flowers

Different yeast species showed different distribution patterns among nectaries. In most cases, each yeast species, if present in a flower, occurred only in one nectary. *Metschnikowia reukaufii, Stramerella bombicola* and *Cryptococcus festucosus*, however, if present in a flower, appeared in an average of 60 %, 50 % and 38 % of nectaries, respectively (fig. 9).

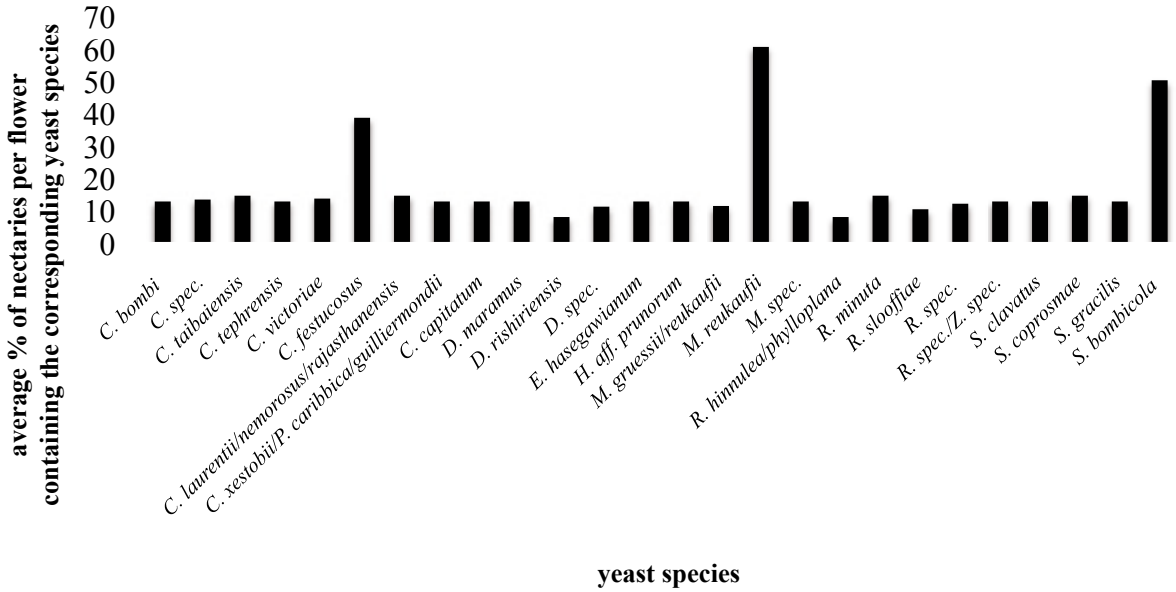

Fig. 9: Average percentage of nectaries per flower colonised by different yeast species. Species present only in the negative control for the enrichment are not included. Values between 7 % and 10 % indicate that only one nectary per flower was colonised by the corresponding species, the fluctuation being due to the variable number of nectaries per flower.

3.2.4 Diversity Indices

To assess the degree of the α-diversity, the Shannon-Index was calculated (table 4).

Table 4: Shannon-Indeces (H) and Evennesses (E) for all samplings combined, for each sampling separately and for different combinations of two samplings. Negative controls for the enrichment are not included in the analysis. $H = \sum_{i=1}^{s} \frac{n_i}{n} \ln \frac{n_i}{n}$ and $E = \frac{H}{\ln s}$ with s = number of species in the sampling; n_i = number of individuals belonging to species I; n = total number of individuals in the sampling

	sampling(s)	Shannon-Index	Evenness
	all	2.78	0.85
	A	1.61	1.00
	B	2.30	1.00
	C	0.94	0.68
	D	2.36	0.87
temporal comparison	A+B (early)	2.52	0.98
	C+D (late)	2.31	0.82
local comparison	A+C (Hasselbachtal)	1.71	0.82
	B+D (Weißenstein)	2.78	0.89

The Shannon-Index for all samples combined was 2.78, the Evenness was 0.85. Considering the samplings separately, those collected at the Weißenstein had a higher Shannon-Index (B: 2.3, D: 2.36) than those collected at the Hasselbachtal (A: 1.61, C: 0.94). The combination of the two Weißenstein samplings also showed a higher Shannon-Index (2.78) than the combination of the two Hasselbachtal samplings (1.71). Combining the two early and the two late samplings respectively, resulted in similar Shannon-Indices (table 4).
The Evenness was lowest for sampling C (0.68), indicating the dominance in the frequency of occurrence of one or few species in this sampling. It was highest for sampling A and B (1.00), where all species found within the sampling showed the same frequency of occurrence. For all other samplings and sampling combinations Evenness lay between 0.82 and 0.98.

The similarity of diversity between samplings was compared using the Jaccard-Index. Results are shown in table 5.

Table 5: Similarity matrices indicating the Jaccard-Indeces (J) of different combinations of samplings. Negative controls for the enrichment are not included in the analysis. $J = \frac{c}{a+b+c}$ with a/b = number of species present in sampling 1/2; c = number of species present in sampling 1 and 2.

	A	B	C	D
A		0.15	0.12	0.11
B	0.15		0.15	0.09
C	0.12	0.15		0.12
D	0.11	0.09	0.12	

temporal comparison		
	AB	CD
AB		0.15
CD	0.15	

local comparison		
	AC	BD
AC		0.19
BD	0.19	

The Jaccard-Index was highest between samplings A and B and between B and C and lowest between B and D, indicating they had many or few common species, respectively. The Jaccard-Index was lower when early and late samplings were compared, than if the two different populations were compared (table 5).

4 Discussion

In the present study, yeasts occurred in about 30 % of all nectar samples. However, this percentage was not equally distributed over the four samplings and clearly showed a temporal dependence with the more yeasts occurring in samples the later they were taken. This is consistent with the results of an earlier study conducted in Germany, where 28 % of all nectar samples hosted yeasts in the first half of April, while this percentage was 88 % for the period from May to September (Brysch-Herzberg, 2004). This suggests that the climatic season, and its associated temperature, plays a major role in determining nectar yeast occurrence. Higher temperatures allow higher yeast growth rates and, furthermore, entail a higher pollinator activity resulting in a more frequent transfer of yeasts between flowers (Brysch-Herzberg, 2004).

Compared to earlier studies conducted in other climatic regions, the frequency of occurrence of nectar yeasts found in this study is rather low. Herrera et al. (2009), who carried out a quantitative survey of nectar yeasts in three different climates (hot Mediterranean, Spain; cool Mediterranean, Spain; seasonally dry tropical, Mexico) found yeasts in 31,8 %, 42,3 % and 54,4 % of samples, respectively; Sandhu and Wairaich (1985) reported a percentage of 68 % colonised plants (semiarid climate, India).

However, yeast occurrence is not only determined by climatic factors, but certainly depends on the host plant, too. Brysch-Herzberg (2004), who sampled nectar from numerous plant families, found different frequencies of occurrence of nectar yeasts for the different taxa: while nectar was colonised in almost 90 % of the samples for Lamiaceae, this percentage was under 50 % for Ranunculaceae and 0 % for Fumariacae. Other studies (Jimbo, 1926; Herrera et al., 2009) came to similar results with yeasts occurring in 0 % to 100 % of samples for individual plant species. Differences between pollinator species and nectar composition probably are determining factors here.

Summarising, this study is in agreement with numerous previous studies reporting high frequencies of occurrence of yeasts in nectar of insect-pollinated plants. Especially regarding the fact that *Helleborus viridis* is an early-blooming plant, meaning that rather few pollinators are foraging during its flowering period, the found percentage of colonised nectar samples was to be expected.

Yeast cell density after the enrichment also showed a temporal tendency with higher values, the later samples were taken, if sampling A was excluded (fig. 2). Possible reasons are the temperature, as well as the increasing number of ascomycetes, which are thought to have a better ability to reproduce in nectar than basidiomycetes (Brysch-Herzberg, 2004). Sampling A, reaching a cell density almost as high as the last sampling, does not fit this temporal tendency. However, since only one sample was included here, its informative value is low.

Within flowers, yeasts were more frequent in the upper nectaries than in the lower ones (fig. 3). A possible explanation for this are foraging patterns of pollinators: Some bumble-bees were followed while visiting *Helleborus viridis* and it was observed that the insects landed in the upper part of the flower and then turned into either direction while drinking the nectar (and transferring the yeasts into the nectaries). However, since the described distribution patterns of yeasts within flowers were statistically not significant and, furthermore, only few foraging pollinators could be observed, this hypothesis will have to be tested by further experiments.

Nectar yeast occurrence does not seem to be associated to nectar volume (fig. 5). This is plausible, as nectar volume is highly variable and nectar yeasts would not be able to survive for long, if they were dependent on a certain nectar volume. When pollinators drink floral nectar, its volume within the nectary is drastically reduced, but the remaining nectar film is sufficient for a few yeast cells to survive. Furthermore, it was shown for *Helleborus foetidus* that nectar is continuously secreted by nectaries for about 20 days, after which the nectaries fall off (Vesprini et al. 1999). This corroborates the assumption that nectaries always contain enough nectar for yeasts to survive, reasoning the non-existence of a correlation between nectar volume and yeast occurrence. Nonetheless, their seems be a correlation between nectar volume and yeast cell density, at least for *Metschnikowia reukaufii* (fig. 10). Nectar volume mirrors the frequency of pollinator visits up to a certain degree: More pollinator visits result in a decreasing nectar volume, and, at the same time in an increasing yeast transfer.

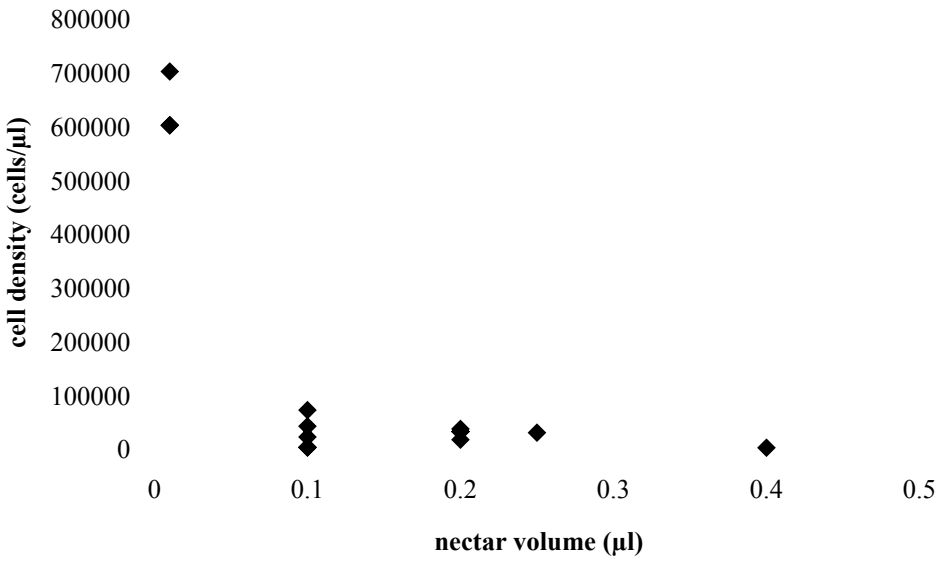

Fig. 10: Cell density of *Mestchnikowia reukaufii* depending on nectar volume. X-axis indicates nectar volume (µl), y-axis indicates calculated cell density (cells/µl). Trend line is logarithmic.

About 43 % of colonised samples contained ascomycetes, 57 % contained basidiomycetes (samples of the negative control for the enrichment excluded) (table 2). This is a rather low percentage of ascomycetes compared to other studies, which found that ascomycetes were much more frequent in floral nectar than basidiomycetes (e.g. Brysch-Herzberg, 2004; Pozo et al., 2010). It was even suggested that basidiomycetes belong to the phyloplane and only "accidentally" occur in nectar due to the short distances between the two habitats (Brysch-Herzberg, 2004). The percentage of ascomycetes was not equally distributed over the four samplings and was higher in the late samplings (C and D) (52 %) than in the early samplings (A and B) (13 %) (table 2). The first samplings rather contained ubiquitous basidiomycteous yeast species, which belong to the genera *Cryptococcus* and *Rhodotorula*, while ascomyceteous nectar specialists were found later. A probable reason for this is the scarce pollinator visitation of the flowers up to the early samplings. Plant-associated basidiomycetes, mainly belonging to the phyloplane (see above), may find different ways (e.g. raindrops) to cover the small distances between plant surfaces and nectaries (Brysch-Herzberg, 2004). "Typical" nectar yeasts, in contrast necessitate pollinators for their distribution. At this point, the question where nectar yeasts come from, i.e. where they survive in winter arises once more.

The most frequent yeast species found in nectar samples was *Metschnikowia reukaufii*. This is in agreement with numerous previous studies (e.g. Brysch-Herzberg, 2004; Herrera et al., 2009,

2010; Pozo et al., 2010). However, in this study *Metschnikowia reukaufii* only occurred in 28 % of the colonised samples (negative controls for the enrichment excluded), which is extremely low in comparison to the other studies, reporting a percentage up to over 90 %. Such a high proportion (80 %) of *Metschnikowia reukaufii* was only assessed for the late Hasselbachtal sampling considered separately (table 2). *Metschnikowia gruesii*, which was reported to come second regarding its frequency of occurrence in nectar, was found only once. Nevertheless it has to be taken into consideration that both *Metschnikowia* species only occurred in the late samplings, of which they represented 33 % of all yeast species found and that this percentage would probably have risen with further (later) samplings. This is corroborated by another study conducted in Germany that reported that *Metschnikowia reukaufii* was present in 48 % of nectar samples if the whole sampling period was considered, but that it was 64 % if only the time of maximum frequency and abundance of yeasts in nectar was included (Brysch-Herzberg, 2004).

Starmerella bombicola was present in seven of 50 colonised nectar samples. The species may be regarded as a "typical" nectar yeast, as it was isolated from the nectary region of flowers for its first description (Rosa and Lachance, 1998). However, it is far less frequent than *Metschnikowia reukaufii* (Brysch-Herzberg, 2004) and all nectar samples containing it came from the same flower.

Several *Crytococcus* species were present in 36 % of colonised samples. Herewith it was the most frequent basidiomyceteous genus in this study, which is concordant with the results of Brysch-Herzberg (2004).

Pseudozyma prolifica was frequently found in the negative control for the enrichment. This may lead to the conclusion that the species frequently occurs in floral nectar of *Helleborus viridis* but does not survive the enrichment process. However, this can be excluded, as *Pseudozyma prolifica* was found in a pre-experiment comprising exactly the same treatment. The species was only found in two flowers: in all nectaries of a flower from the first sampling and in a nectary of an additional flower from the second sampling (table 2). It is therefore likely that the occurrence of *Pseudozyma prolifica* in the negative controls was rather due to the chosen flower, than to the difference in the treatment of the samples. This might also be an explanation for the negative controls from the last sampling, which did not show any yeast growth. As a result it is suggested that negative controls should rather be taken from one nectary of each probed flower, than from all nectaries of a different flower, as it was done in this study.

The two other yeast species occurring in the negative controls for the enrichment were *Rhodotorula sloffiae* and a *Taphrina* species. The fact that *Rhodotorula sloffiae* was also present in treated samples is in favour of the enrichment, demonstrating that the species could survive it. Contrarily, *Taphrina spec.* was only found in the negative control. Whether it does not survive the enrichment, or simply only occurred in this single sampling, cannot be decided.

Considering only colonised samples, each sample contained an average of 1.32 ± 0.55 yeast species. This low species richness per nectar sample has been reported as characteristic of nectar as a habitat by several studies before (e.g. Belisle et al., 2011, Pozo et al., 2010). One important reason for it is nectar composition, which makes this ecological niche hardly colonisable. Furthermore low species richness is reinforced by strong priority effects (Peay et al., 2011).

Most yeast species had average cell densities under 2500 cells/µl after the enrichment, probably showing they could not reproduce well in nectar. *Metschnikowia reukaufii*, however, reached an average cell density more than 60 times higher. This illustrates the specialisation of the species, making it "jack of all nectars (and) master of most" (Herrera et al., 2011).

Metschnikowia reukaufii, *Starmerella bombicola* and *Cryptococcus festucosus* often occurred in several (adjacent) nectaries of a single flower (fig. 9). In contrast, all other species, if present in a flower, occurred only in one nectary. This phenomenon could mirror the way yeasts had been transported into the flowers: yeasts occurring in adjacent nectaries of one flower may have been distributed by pollinating insects, which show characteristic foraging patterns (see above), while yeasts found only in one nectary per flower may have got into the nectary another way, e.g. via smaller insects, that only come into contact with few nectaries of a flower, or via raindrops or wind.

The calculated Shannon-Indices and Evennesses (table 4) indicate that yeast diversity was lowest in the first sampling (A). Five yeast species, each occurring only once, were found in these samples (table 2). This low diversity is probably due to the early sampling date and the associated scarce previous pollinator visitation of the probed flowers. The early Weißenstein sampling (B) shows a higher diversity, possibly resulting out of the later sampling date and/or the different site. The second samples taken at the Hasselbachtal (C) show a very low yeast diversity: the Shannon-

Index was 0.94, even though species abundance was far from being equally distributed over all occurring species (Evenness: 0.68). In this sampling *Metschnikowia reukaufii* was the most frequent yeast species by far, occurring in 80 % of colonised samples. Only two samples contained basidiomycetes. This very low diversity with *Metschnikowia reukaufii* being the dominant species coincides with several previous studies (e.g. Herrera et al., 2010, 2011; Peay et al., 2011). The much higher diversity in the late Weißenstein sampling (D) may be due to the late sampling date and the different sampling site. Another, explanation for this high diversity may be the fact that it had been raining in the night before the collection of these last samples, while the other samples were taken under dry conditions. It is possible that raindrops transported epiphytic yeast species into the nectaries, resulting in this high (especially basidiomyceteous) species diversity.
Comparing the two samplings sites, samples taken at the Weißenstein population showed a higher diversity than those taken at the Hasselbachtal (Shannon-Indeces: 2.78 vs. 1.71 with similar Evennesses). On the one hand, this may be due to the fact, that samples at the Weißenstein were collected a few days later than those at the Hasselbachtal, probably allowing more flowers to be visited by pollinators before the sampling. On the other hand, the different plant communities found at both sites may represent a possible reason for these differences, too.

The calculated Jaccard-Indices, being rather low (table 5), indicate that the four samplings were rather different concerning their species composition. The two Weißenstein samplings (B and D) showed the greatest differences here, which is in agreement with the high yeast diversity already demonstrated via the Shannon-Index. The two Hasselbachtal samplings (A and C) are more alike and show a smaller species diversity. Temporal comparison (early samplings vs. late samplings) resulted in a lower Jaccard-Index than local comparison (Hasselbachtal vs. Weißenstein), suggesting that the composition of yeast communities is more importantly influenced by the time, than by the site.

As a conclusion of this study one can say, that nectar yeasts are frequent in floral nectar of *Helleborus viridis* from a certain point of time in spring (probably depending on pollinator activity).

Metschnikowia reukaufii was confirmed to be a real specialist in nectar, being the most frequent species found by far and reaching cell densities more than 60 times higher than most other found species. Its distribution patterns suggest that it was distributed by pollinators.

Starmerella bombicola was found to be well adapted to nectar, too, even if in a far lower degree than *Metschnikowia reukaufii*. It came second in frequency of occurrence, as well as in reached cell densities and showed similar distribution patterns within flowers.

The remaining yeast species appeared far less specialised, and it is suggested that they mainly get into the nectar via other ways than pollinators and cannot reproduce well in this habitat, resulting in the very low species diversity found in this study.

5 Abstract

5.1 Abstract

Yeasts are frequent inhabitants of floral nectar of animal-pollinated plants. According to several previous studies, they have to be considered as a third player in the plant-pollinator mutualism; however the influence they have on this interaction is not clarified, yet. Since nectar is hardly colonisable due to its composition, yeast species diversity was found to be very low with only few specialists being able to survive and reproduce well in this habitat.

In this study, we investigated the occurrence and diversity of yeasts in floral nectar of *Helleborus viridis*, an early-blooming plant. To allow temporal, as well as spatial comparisons, four nectar samplings were carried out at four different points of time and two different plant populations. Yeasts were then identified using molecular methods.

Yeasts were found to be frequent in floral nectar of *Helleborus viridis*. Their frequency of occurrence showed a clear temporal increase with under 10 % of nectar samples containing yeasts for the first sampling date (middle of March) to over 80 % for the last sampling date (beginning of April).

Results of this study suggest that the occurrence of nectar yeasts is not associated to a certain nectar volume. However, it is probably linked to foraging patterns of pollinating insects, since these act as vectors for nectar yeasts. For example, yeasts were more frequent in the upper nectaries than in the lower ones, and if a yeast species was present in several nectaries of a single flower, these usually were adjacent. Both findings are suggested to reflect foraging patterns of pollinators.

The most frequent yeast species in collected nectar samples was *Metschnikowia reukaufii*. Its frequency of occurrence, as well as its high cell densities within nectar, are in agreement with several previous studies denoting *Metschnikowia reukaufii* as the dominating and most specialised nectar yeast. *Starmerella bombicola* came second in this study. Species of the genus *Cryptococcus* and other basidiomycetes were frequent as well, but since they did not show as high cell densities, nor the typical distribution patterns (e.g. occurrence in several nectaries per flower) mentioned above, it is suggested that these (mostly ubiquitous) species occur in floral nectar rather "accidentally".

In total, the low yeast species richness reported by several previous studies for nectar of different plant species, could also be demonstrated for floral nectar of *Helleborus viridis*.

5.2 Zusammenfassung

Hefen sind häufige Bewohner des Blütennektars insektenbestäubter Pflanzen. Aufgrund ihrer großen, wenn auch nicht vollends geklärten, Auswirkungen auf die Symbiose zwischen Pflanzen und ihren Bestäubern, kamen zahlreiche vorangegangene Studien zu dem Schluss, dass Nektarhefen als drittes Glied in dieser Interaktion angesehen werden müssen. Da Nektar aufgrund seiner Zusammensetzung nur schwer besiedelbar ist, wurde eine sehr geringe Hefeartendiversität, mit nur wenigen Spezialisten, in diesem Habitat erfasst.

In dieser Studie wurde das Vorkommen und die Diversiät von Hefen im Blütennektar von *Helleborus viridis*, einer frühblühenden Pflanze, untersucht. Um temporäre, sowie lokale Vergleiche zu ermöglichen, wurden Nektarprobennahmen zu vier verschiedenen Zeitpunkten aus zwei verschiedenen Pflanzenpopulationen entnommen. Die darin enthaltenen Hefen wurden mittels molekularer Methoden identifiziert.

Hefen waren häufig im Blütennektar von *Helleborus viridis*. Ihr Vorkommen zeigte einen klare zeitliche Abhängigkeit: beinhalteten bei der ersten Probennahme (Mitte März) unter 10 % aller Nektarproben Hefen, so waren es bei der letzten Probennahme (Anfang April) über 80 %.

Die Ergebnisse dieser Studie weisen darauf hin, dass das Vorkommen von Nektarhefen nicht an ein bestimmtes Nektarvolumen assoziiert ist. Es ist jedoch von Besuchen bestäubender Insekten abhängig, die als Vektoren für Nektarhefen agieren. Beispielsweise waren Hefen in den oberen Nektarien tendenziell häufiger, als in den unteren, weiterhin waren einzelne Hefearten, wenn sie in mehreren Nektarien einer Blüte vorhanden waren, meist in benachbarten Nektarien aufzufinden. Beide Beobachtungen könnten Besuchmuster bestäubender Insekten widerspiegeln.

Die häufigste Hefeart war *Metschnikowia reukaufii*. Das häufige Vorkommen, sowie die hohen Zelldichte dieser Art im Nektar, stimmen mit vorangegangenen Studien überein, die *Metschnikowia reukaufii* als die dominierende und am höchsten spezialisierte Nektarhefe kennzeichnen. *Starmerella bombicola* kam in dieser Studie an zweiter Stelle. Verschiedene Arten der Gattung *Cryptococcus* und andere Basidiomyceten waren ebenfalls häufig vorzufinden. Allerdings zeigten sie weder die oben erwähnten hohen Zelldichten, noch die typischen Verteilungsmuster (z.B. Vorkommen in mehreren Nektarien einer Blüte), sodass vermutet wird, dass diese (vor allem ubiquitären) Hefearten eher "zufällig" im Blütennektar vorkommen.

Insgesamt konnte eine geringe Hefeartendiversität, wie sie bereits für den Nektar anderer Pflanzenarten beschrieben wurde, auch für den Blütennektar von *Helleborus viridis* festgestellt werden

6 List of Abbreviations

A	adenine
BLAST	Basic Local Alignment Search Tool
C	cytosine
ddH_2O	double distilled water
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleotide triphosphate
EDTA	ethylenediamine tetraacetate
G	guanine
ITS	internal transcribed spacer
$MgCl_2$	magnesium chloride
MSP-PCR	microsatellite-primed polymerase chain reaction
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
T	thymine
Taq	*Thermus aquaticus*
TBE	TRIS-borate-EDTA
TE	TRIS-EDTA
TRIS	Tris(Hydroxymethyl)aminomethane
YM	yeast media
YNB	yeast nitrogen base

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9 Erklärung

Hiermit erkläre ich, dass ich die heute eingereichte Bachelorarbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie Zitate kenntlich gemacht habe. Bei der vorliegenden Bachelorarbeit handelt es sich um in Wort und Bild völlig übereinstimmende Exemplare.
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Bochum, den 29.05.2012