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The dynamics of the yeast community of the Tagus river estuary: testing the hypothesis of the multiple origins of estuarine yeasts

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Abstract Yeasts are common inhabitants of different types of aquatic habitats, including marine and estuarine waters and rivers. Although numerous studies have surveyed yeast occurrence in these habitats, the identification of autochthonous populations has been problematic because several yeast species seem to be very versatile and therefore mere presence is not sufficient to establish an ecological association. In the present study we investigated the dynamics of the yeast community in the Tagus river estuary (Portugal) by combining a microbiological study involving isolation, quantification, and molecular identification of dominant yeast populations with the analysis of hydrological and hydrographical data. We set out to test the hypothesis of the multiple origins of estuarine yeast populations in a transect of the Tagus estuary and we postulate four possible sources: open sea, terrestrial, gastrointestinal and the estuary itself in the case of populations that have become resident. *Candida parapsilosis* and *Pichia guilliermondii* were correlated with *Escherichia coli*, which indicated an intestinal origin. Other cream-colored yeasts like *Debaryomyces hansenii* and *Candida zeylanoides* had similar dynamics, but no association with *E. coli* and quite distinct ecological preferences. They might represent a group of resident estuarine populations whose primary origin is diverse and can include marine, terrestrial, and gastrointestinal habitats. Another major yeast population was represented by *Rhodotorula mucilaginosa*. The cosmopolitan nature of that species and its moderate association with *E. coli* point to terrestrial sources as primary habitats.

Keywords Yeasts · Estuaries · Molecular identification · Microbial ecology

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Introduction

Yeasts are fungi of the Ascomycota and Basidiomycota that exhibit a marked reduction of the characteristic fungal filamentous structures. The unicellular habit favours dispersal and the exploitation of liquid or moist environments (Lachance and Starmer 1998). Yeasts are heterotrophic and therefore depend entirely on the availability of organic carbon sources in the environment. However, their nutritional versatility combined with a simplified lifestyle and the evolution of different physiological adaptations have allowed the colonization of a wide range of habitats, either terrestrial or aquatic. The versatility and resilience of

yeasts even allows the colonization of extreme environments like, for example, cold habitats (Starmer et al. 2005), the deep sea (Bass et al. 2007; Nagahama et al. 2001), and marine hydrothermal vents (Gadanho and Sampaio 2005).

The study of yeasts found in the marine and estuarine systems was initiated long ago and had important contributions in the 1950s, 1960s, and 1970s. It was then observed that the density of the yeast populations was highest in fresh waters and decreased in marine waters with increased depth and increased distance from land (van Uden and Fell 1968). As reviewed by Hagler and Ahearn (1987), yeast counts in open ocean are typically below 10 cfu l^{-1} whereas open waters of clean lakes have up to 100 cells per litre. Coastal and estuarine waters tend to have higher counts, typically in the range of 100–1000 cfu l^{-1}, and pollution can increase substantially these numbers. However, in spite of this knowledge of the densities of the different communities, studies on the ecology of aquatic yeasts have been sporadic and mostly descriptive. Consequently, even today, the knowledge of the forces that promote yeast distribution in water bodies is limited and our understanding of the autochthonous and allochthonous yeast populations and their role in the aquatic ecosystems is scarce.

The Tagus river estuary has been used as a study site early in the 1960s by van Uden and co-workers who reported yeast counts of 1228 cfu l^{-1} and the isolation of 14 yeast species (Taysi and van Uden 1964). In a review of studies on yeasts occurring in estuaries, van Uden (1967) proposed that the estuarine yeast community could be divided into three main groups: (i) the pink (carotenoid-producing) yeasts that are abundant in estuaries but occur also frequently in open sea and inland waters; (ii) the intestinal yeasts that are present in estuaries due to sewage pollution; and (iii) yeasts from terrestrial substrates that tend to be the dominant forms in estuaries but are rare in the open sea. More recently, Gadanho and Sampaio (2004) studied the same estuary and monitored the yeast community with Temperature Gradient Gel Electrophoresis (TGGE), a culture-independent method for the molecular detection of yeasts, and compared this approach with cultivation-dependent techniques. The combined analysis of culture-dependent and culture-independent methods indicated that typical estuarine yeasts (sensu Hagler and Ahearn 1987, Table III), like *Rhodotorula mucilaginosa* (pink yeast), *Debaryomyces hansenii*, and *Pichia guilliermondii* were present and that the first two species belonged to the dominant fraction of the community. In another study centred on the Tagus river estuary, Almeida (2005) addressed the issue of the primary origin of estuarine yeasts. This study employed a modified approach combining biological, hydrographical, and geophysical data and long-term sampling in an attempt to give a more meaningful ecological perspective. It was found that yeast occurrence did not depend on the tidal cycle, but river discharge did influence the density and diversity of the community. Whereas *Rhodosporidium diobovatum*, a pink yeast, was linked to a marine origin, the occurrence of *Candida parapsilosis* was correlated with that of *Escherichia coli* and therefore with fecal contamination. The incidence of *P. guilliermondii* and *Candida catenulata* suggested a possible association with recent rainfall and therefore with contamination from the margin and consequently a terrestrial origin. *D. hansenii*, a yeast found in coastal and oceanic environments (Ahearn and Crow 1980; Gadanho et al. 2003), showed a different population dynamics. This yeast was regularly present in the Tagus estuary but disappeared when the river discharge increased. Such a pattern could indicate a distinct group of estuarine yeasts not directly associated with a marine or terrestrial origin nor with fecal contamination. *D. hansenii* could exemplify a fourth type of estuarine yeast that is capable of some degree of residence in the estuary. Such populations are found consistently but seem to be temporary as they can be eliminated or severely reduced in winter when river discharge increases.

In the present study we set out to investigate the dynamics of the yeast community in the Tagus estuary under this quadripartite model of yeast origin. For this we combined a microbiological study with an analysis of hydrological and hydrographical data. Moreover, whereas in the 2005 study samples were taken from the shore and only at the surface, here a research vessel was employed and the entire water column was analysed across a section of the estuary. We aimed at correlating the dominant yeast populations occurring in the estuary with one of the four possible classes discussed above. Six sampling campaigns were carried out in 2006 and 2007. In each

campaign the same transect of the estuary was studied and the same fixed positions (stations) were monitored at different depths. Most samples were collected when water was leaving the estuary (during ebb) as we reasoned that except for marine yeasts this period of the tidal cycle would maximise the recovery of distinct populations. In parallel with the monitoring of the density and composition of the yeast community, we also quantified *E. coli*, a bacterial indicator of fecal pollution, and assessed the community of filamentous fungi. The data were used to test the hypothesis of the multiple origins of estuarine yeasts.

Materials and methods

Study site and sample collection for microbiological analysis

The Tagus estuary comprises an area of 325 km^2 and has three main components: a delta-like part, a shallow and wide basin and a channel that connects the estuary to the sea (Bettencourt 1990). This estuary displays a semi-diurnal tide regime, with a tidal prism of 600×10^6 m^3 against an estuary volume of 1900×10^6 m^3 and an average tidal amplitude of 2.6 m (Ferreira et al. 2001). For this study we defined a transect of 1.6 km in the estuary channel and seven stations, i.e., sampling positions, along it (Fig. 1). Stations were designated with numerals, station no. 1 being the closest to the right bank and station no. 7 the closest to the left bank. In each station, measurements and water samples were taken at different depths: surface and bottom (stations 1–3 and 7) and surface, middle of water column, and bottom (stations 4–6) (Fig. 1). As can be observed in Fig. 1 the river floor is asymmetrical—shallow in the right bank, deeper in the left bank. This asymmetry accounts for distinct hydrodynamics along the right and left banks. The right (shallow) margin is mostly associated with the passage of freshwater and suspended sediment. Although urban effluents from the city of Lisbon (located on the right bank) are treated, minor emissions (especially from the densely populated right bank) cannot be discounted. Therefore fecal contamination originating upstream in the right margin would be travelling to the sea along the same margin. Seawater, which is denser, enters the estuary

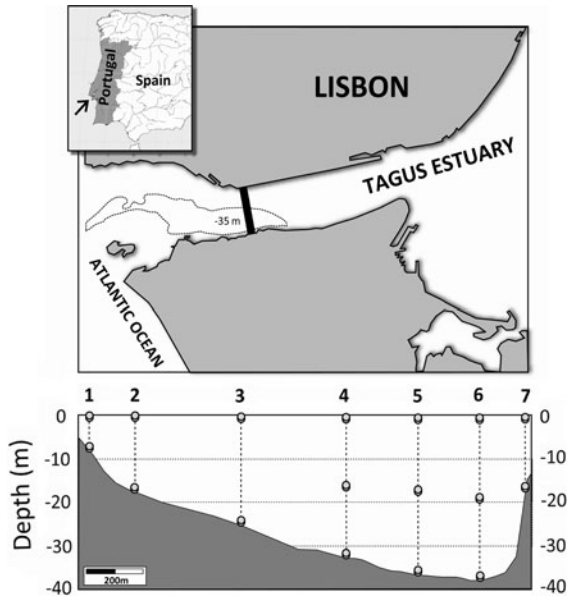

Fig. 1 The Tagus estuary and stations used for collecting samples. The localization of the estuary channel and the studied transect (*black thick line*) is shown in the *upper part* of the figure. The seven stations defined along the transect are depicted in the *lower part* of the figure. Stations were numbered from the *right* (northern) to the *left* (southern) banks. For each station the depths at which samples were taken are represented by *dots*

during flood, close to the bottom and through the left margin. This water mass exits the estuary during ebb, mostly close to the right margin. Therefore in the prevalent conditions of low river discharge associated with the Mediterranean climate, the estuary is filled mainly with seawater, although there is always some flow of freshwater at the top layer and close to the right margin. During the course of this study, rainfall was lower than usual, thus contributing to the confinement of the freshwater flow to the right shore. However, during periods of more intense rain, sporadic increases in river discharge, which is controlled by several dams located upstream, alter this pattern. In these cases the flow of freshwater can spread towards the left margin.

Six campaigns were carried out in January (C1), May (C2), July (C3) and December (C4) of 2006, and January (C5) and April (C6) of 2007. In each campaign each station was visited twice. Samples were collected during the ebb phase of the tide, except in April 2007, when observations covered a full tidal cycle. At each station an *Idronaut Ocean*

Seven 316 CTD (Conductivity, Temperature, and Depth instrument) coupled to a nephelometer was lowered to the bottom, operating in self-recording mode. Lowering was stopped when a depressor placed 0.5 m below the CTD touched the bottom and caused slackening of the rope. Then, 1–2 m of suspension wire were hauled in and a messenger sent down the wire to close a 5-l Niskin bottle connected to the lowering cable 1 m above the CTD. At the same time, another Niskin bottle, operated by hand from the surface, was closed via another messenger. A second cast was performed whenever a sample was needed at an intermediate depth (stations 4–6, see Fig. 1). After collection, the samples (1 l) were immediately transferred to sterile polypropylene bottles for transport to the laboratory. Depending on the length of the campaign the yeast isolation procedures started on the same day or on the next day, in which case the water samples were kept refrigerated at 4°C overnight. Conductivity, temperature, depth, and turbidity data were downloaded upon completion of each station and post-processed ashore.

Hydrographical, hydrological and meteorological data

CTD and turbidity data were corrected for differences in sensor response times and depth inversions and further averaged in 1 m intervals. Temperature, salinity, density and turbidity fields were constructed for each cross-estuary section. Tagus flow data were obtained for Ómnias (Santarém) from the web page of the Portuguese Institute for Water (http://snirh.pt). Meteorological data were obtained from the coastal station operated by Instituto Hidrográfico near Ferrel, some 100 km north of Lisbon. In spite of the latitude difference, the station is believed to represent rather well the forcing off the central west coast of Portugal.

Yeast isolation and quantification

Volumes of 100 ml of water were filtered through sterile nitrocellulose membranes of 0.45 μm pore size and 47 mm diameter (Gelman Science) and the membranes were placed on MYP agar (malt extract 0.7% w/v, yeast extract 0.05% w/v, soytone 0.25% w/v and agar 1.5% w/v) supplemented with 500 ppm of chloramphenicol. The process was replicated three times for every set of incubation conditions (25 and 37°C). Yeast colonies were counted at the earliest possible stage (approx. 3 days) through digital photograph analysis. When yeast counts were not too high (number of colonies less than 80) or when moulds were absent or scarce, all yeast colonies were purified for further characterization. In the other cases, a representative number of colonies (30–60 colonies) was randomly selected and transferred. Purification was performed on the same medium without chloramphenicol. The total number of cultures purified and studied further with molecular methods was 399.

Isolation and quantification of *E. coli*

E. coli was detected and quantified according to the rapid variant of the membrane filtration method prescribed by the ISO 9308-1 standard. This method involves a culture step on a dual layer medium—tryptone bile agar on tryptone soy agar—with successive incubations at 36 and 44°C. After the incubation period, *E. coli* colonies were revealed by UV irradiation in the presence of an indole-revealing reagent. This test was done in triplicate.

DNA extraction

Two loopfuls of MYP agar grown cultures were suspended in 500 μl lysing buffer (50 mmol l^{-1} Tris, 250 mmol l^{-1} NaCl, 50 mmol l^{-1} EDTA, 0.3% w/v SDS, pH 8) and the equivalent to a volume of 200 μl of 425–600 μm glass beads (Sigma) was added. After vortexing for 3 min, the tubes were incubated 1 h at 65°C. The suspensions were then centrifuged for 10 min. Finally, the collected supernatant was diluted 1:750 and 5 μl were directly used in the PCR. The remaining supernatant was immediately conserved at −20°C.

MSP-PCR fingerprinting

The microsatellite primers $(GTG)_5$ and $(GAC)_5$ were used as previously described (Gadanho and Sampaio 2002). All PCR reactions were performed in 25 μl reaction volumes containing 1× PCR buffer (GE Heathcare), 2 mmol l^{-1} of each of the four dNTPs (GE Heathcare), 0.8 μmol l^{-1} of primer, 5 μl of the diluted supernatant containing the genomic DNA and

1U of *Taq* DNA polymerase (GE Hearthcare). Amplification was performed in a Uno II Thermal Cycler (Biometra), consisting of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 45 s at 93°C, 60 s at 50°C and 60 s at 72°C and a final extension step of 6 min at 72°C. A negative control in which DNA was replaced by sterile distilled water was also included. Amplified DNA fragments were separated by electrophoresis in 1.4% (w/v) agarose gel (GIBCO, BRL), in 0.5× TBE (Tris–Borate–EDTA) buffer at 90 V for 3.5 h and stained with ethidium bromide. On each gel, a molecular size marker was used for reference (λ DNA cleaved with *Hind*III and ΦX174 DNA cleaved with *Hae*III—Fermentas).

DNA banding patterns were visualized under UV transillumination (Bio-Rad Universal Hood II) and images were acquired with Gel Doc EQ using the software Quantity One (4.5.0) (Bio-Rad). All fingerprints obtained were grouped by similarity using Gel Compar 4.1 (Applied Maths 1998) and the Pearson correlation coefficient. A visual confirmation of each group was performed and minor adjustments were made. Finally each group was assigned in a distinct MSP-PCR class.

rDNA sequence analysis

Total DNA was extracted using the method described above and amplified using rDNA primers ITS5 (5′GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5′CGC CAG TTC TGC TTA CC). Cycle sequencing of the 600–650 base pair region D1/D2 at the 5′ end of the 26S rDNA domain employed forward primer NL1 (5′GCA TAT CAA TAA GCG GAG GAA AAG) and reverse primer NL4 (5′TCC TCC GCT TAT TGA TAT GC). For identification, the obtained sequences were compared with those of all known yeast species, available at the GenBank database.

Results

Yeast isolation and formation of macromorphological groups

Two incubation temperatures were employed for yeast isolation: 25 and 37°C. At 25°C we aimed to monitor broad communities, whereas at 37°C we hoped to trace yeast populations associated with the gastrointestinal tract of humans and other warm-blooded animals. At 25°C two main types of yeast colonies appeared. One type corresponded to the pigmented yeasts, which formed colonies with different shades of pink/orange coloration. The other type had different shades of cream-colored colonies. Based on particular details of pigmentation and of colony texture, the colonies of pink yeasts were divided into four groups and the cream-coloured colonies were divided into six groups. At 37°C only cream-colored colonies developed. These were divided into two groups. Therefore, 12 groups were formed based on the combination of macromorphological types and incubation temperatures. These groups were used in the yeast quantification study performed directly on the filter disks used for yeast isolation. The total number of yeast colonies counted on the filters approached 5000. As this number was too high for subsequent analyses involving culture purification, DNA extraction, typing and identification down to species level, only a fraction of the yeasts that grew on the filters (399 isolates, see "Materials and methods" section) were characterized by molecular fingerprinting.

Molecular fingerprinting and yeast identification

Because of our previous experience with the technique of MSP-PCR for molecular fingerprinting (e.g. Gadanho et al. 2003) we were expecting the number of MSP-PCR groups that was obtained during the study of 399 isolates to be higher than that of the macromorphological groups initially established. This was indeed the case (26 MSP-PCR groups compared to only 12 macromorphological groups), indicating that each macromorphological group might include more than one yeast species. Moreover we also anticipated that for some species intraspecific variability might result in more than one profile.

For identification, two or three strains of each MSP-PCR group were arbitrarily selected for sequence analysis of the D1/D2 domains of the large subunit (LSU) rRNA gene. As this sequence is species-specific and the complete database of virtually all yeast species is publicly available and searchable, this sequencing approach is currently the most adequate method for species identification. Our method for yeast isolation

and species identification allowed us to detect 29 yeast species, 9 of which belonged to the ascomycetous genus *Candida*. Table 1 depicts the list of yeast species found in the Tagus river estuary and their relative frequency in relation to the group of 399 isolates that was subjected to molecular characterization. Most species had a low incidence and were therefore difficult to trace in a quantitative ecological study. Therefore, we decided to base the more detailed analyses on a restricted group of six species that together represented 68.2% of the strains studied by molecular methods. These species were *C. parapsilosis, Candida zeylanoides, Clavispora lusitaniae, D. hansenii, P. guilliermondii and Rh. mucilaginosa* (Table 1).

Table 1 Yeast species identified in the Tagus estuary and their approximate relative frequency

Species	Frequency (%)
Candida boidinii	1.25
Candida fermentati	1.75
Candida glabrata	1.00
Candida oleophila	2.76
Candida palmioleophila	0.75
Candida parapsilosis	**9.27**
Candida pseudointermedia	2.26
Candida pseudolambica	1.50
Candida zeylanoides	**8.77**
Clavispora lusitaniae	**5.51**
Coniochaeta sp.	0.25
Cryptococcus albidus	0.75
Cryptococcus laurentii	2.26
Cryptococcus oeirensis	0.5
Debaryomyces hansenii	**11.53**
Filobasidium uniguttulatum	0.25
Issatchenkia orientalis	1.00
Kluyveromyces lactis/marxianus	0.5
Pichia anomala	0.5
Pichia guilliermondii	**8.27**
Pichia sp.	0.5
Rhodosporidium babjevae	1.5
Rhodosporidium diobovatum	0.75
Rhodosporidium kratochvilovae	1.5
Rhodotorula glutinis	1.25
Rhodotorula mucilaginosa	**24.81**
Saccharomyces cerevisiae	2.01
Sporobolomyces roseus	0.25
Yarrowia lipolytica	1.00
Unidentified species of *Candida, Cryptotoccus, Rhodotorula* and *Sporobolomyces*	5.76

The group of six most abundant species that was studied further is given in bold emphasis

Macromorphological groups and yeast identification

Yeast quantification and yeast identification were separated in time. Yeast populations were quantified according to macromorphological groups immediately after the filtration and incubation procedures, but yeast identification required much more time and was completed at a later stage. Moreover, because different yeast species can be undistinguishable in colony morphology, we did not expect that each macromorphological group contained only a single and unique (to our study) yeast species. As mentioned earlier, we defined 12 groups based on macromorphological characteristics and incubation temperatures. As shown in Table 2, and taking into consideration the 399 isolates characterized by molecular methods, five macromorphological groups included 87.5% of the isolates subjected to molecular typing. Group A consisted mostly (76%) of three species—*C. parapsilosis, C. zeylanoides* and *D. hansenii*. Group B was the most heterogeneous group and besides representatives of the three species present in group A it included also, as major species, *C. oleophila* and *C. boidinii*. Therefore group A and B, which generally correspond to cream-colored yeasts isolated at 25°C, are difficult to distinguish in terms of species composition and, combined, they represent 42% of the yeasts studied by molecular methods. Group L, which contains yeasts isolated at 37°C, has a distinct composition, containing as major species *P. guilliermondii* and *Cl. lusitaniae*. However, *C. parapsilosis*, which was present in groups A and B, was also present in group L. Finally, groups G and I contain pink yeasts isolated at 25°C and both groups have, as dominant species, *Rh. mucilaginosa*. In the analyses that follow we combine the macromorphological groups defined in the beginning of the study with the identifications at the species level that were performed subsequently and we explore associations of yeast populations with hydrologic parameters and traditional fecal indicators.

Table 2 Yeast species composition of the dominant macromorphological groups (all values are in percentages)

Macromorphological group (temperature of isolation, color of colonies)	Frequency ($\Sigma = 87.47$)	Major species	Species frequency within each group	Σ
A (25°C, Cream)	20.5	*Debaryomyces hansenii*	37.5	76.25
		Candida zeylanoides	22.5	
		Candida parapsilosis	16.25	
B (25°C, Cream)	21.55	*Candida zeylanoides*	19.77	60.47
		Debaryomyces hansenii	16.28	
		Candida oleophila	11.63	
		Candida parapsilosis	6.98	
		Candida boidinii	5.81	
L (37°C, Cream)	18.55	*Pichia guilliermondii*	33.78	77.03
		Candida parapsilosis	24.32	
		Clavispora lusitaniae	18.92	
G (25°C, Pink)	13.78	*Rhodotorula mucilaginosa*	89.09	
I (25°C, Pink)	13.53	*Rhodotorula mucilaginosa*	72.22	

Correlation between sampling site and yeast populations

We analysed the incidence of groups A and B (that together represent 92% of the cream-coloured yeasts isolated at 25°C), groups G and I (representing 78% of the pink coloured yeasts) and group L (representing 96% of the cream-coloured yeasts isolated at 37°C) in the seven sampling sites that were considered in our study. These results are shown in Fig. 2. Higher yeast counts seem to be concentrated on stations 1 and 2, i.e. on the right bank. This pattern is especially evident for groups A and B although the remaining groups have also higher counts in stations 1 and 2. As indicated in the Methods section, freshwater flows mainly through the right margin. Therefore it seems that the higher yeast counts observed in stations 1 and 2 can be associated with freshwater dynamics. It should be noted that freshwater carries not only suspended sediment but also microorganisms associated with terrestrial runoffs and microbial contaminants from upstream sewage discharges, especially from contamination sources located on the right margin. Therefore, these particular factors of the Tagus river dynamics contribute to making stations 1 and 2 substantially different from the other stations. It can be observed that yeast counts have, in general, high standard deviations. This is particularly evident for groups A and B and is the result of practical limitations, especially the relatively small volume of water that was filtered, which in turn resulted in a marked variation of the number of yeast colonies. Another problem was the suspended sediment. In some cases, water turbidity tended to occlude the filters, thus precluding the employment of larger sample volumes. Finally the intermittent occurrence of high numbers of filamentous fungi hampered yeast counting in some cases.

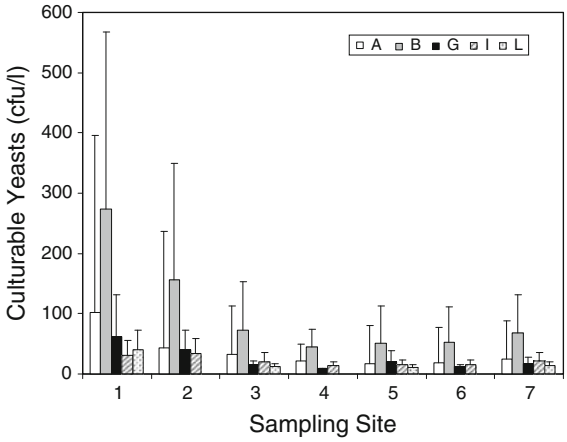

Fig. 2 Distribution of major yeast groups (*A*, *B*, *G*, *I* and *L*) along the seven sampling stations. Columns represent average occurrence values, and *error bars* are proportional to their standard deviation

Correlation between water depth and yeast populations

We evaluated the variation in yeast community as a function of the depth of the estuary (Fig. 3). Because

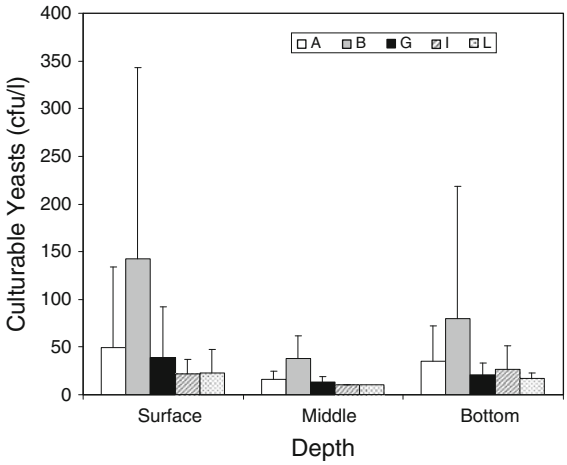

Fig. 3 Distribution of major yeast groups (*A*, *B*, *G*, *I* and *L*) according to depth. Columns represent average occurrence values, and *error bars* are proportional to their standard deviation

freshwater (less dense) tends to flow near the surface and seawater (more dense) tends to flow closer to the bottom, we reasoned that if different yeast populations occur in different water types—freshwater and seawater—their distribution in the water column should be distinct. River flow was negligible, usually below 50 $m^3\ s^{-1}$, in all campaigns except C4 (December 2006), when the average measured flow was 2000 $m^3\ s^{-1}$. Even if hydrological stratification is not a major factor, the distribution of yeast occurrence plotted in Fig. 3 suggests that the relative composition of this community differs between the upper and intermediate to lower levels of the water column. The variation is more evident for Groups A and B (cream-colored yeasts isolated at 25°C), which are more abundant near the top of the water column than at the other water depths. The pink yeasts (groups G and I) seem to be present in more or less equivalent numbers through the entire water column. An important aspect affecting this representation of yeast incidence is the asymmetry in the distribution of depths sampled across the transect: whereas surface and bottom samples represent the entire river width, the middle depth class concerns only the left bank (Fig. 1).

Correlation between fecal contamination and yeast populations

We analysed the association of the incidence of the five major yeast groups with the incidence of *E. coli*. The correlation analyses are shown in Fig. 4. Whereas the incidence of the yeasts isolated at 37°C, (*P. guilliermondii, C. parapsilosis* and *Cl. lusitaniae*), was strongly correlated with the incidence of *E. coli*, no such association was found for groups A and B, which together contain five dominant species (*D. hansenii, C. zeylanoides, C. parapsilosis, C. oleophila* and *C. boidinii*). Similarly, no association was found between *E. coli* and groups G and I, which that correspond to pink yeasts isolated at 25°C, a group composed mostly by *Rh. mucilaginosa*. The observed differences in the correlation value (Pearson coefficient) between the occurrences of the three groups of yeasts against *E. coli* may explain their history in the estuary. Yeasts isolated at 37°C showed a strong correlation ($r = 0.802$) with fecal pollution, and it is likely that they came with urban domestic wastewater discharged from the right bank. On the other hand, cream-coloured yeasts cultivated at 25°C showed no meaningful correlation with fecal pollution ($r = 0.183$). Although several yeasts species in this group can be isolated from contaminated waters, their true origin should be assigned to a different source—probably the sea or the estuary itself. The group of pink yeasts shows an intermediate relationship with fecal contamination because although the degree of correlation is low ($r = 0.670$), it is far from null. These yeasts seem not to be primarily associated with the gastrointestinal environment. In fact, *Rh. mucilaginosa*, the dominant pink yeast found in this study, can be found in association with soil and vegetation (Babjeva and Sadykov 1980; di Menna 1971; Maksimova and Chernov 2004; Ruinen 1963; Slávikóvá and Vadkertiová 2000). The moderate correlation between this yeast and *E. coli* could be due to the sharing of the same entry routes in the estuary which would then result in a similar dispersion pattern.

Occurrence and incidence of filamentous fungi

Since the colonies of molds could be easily distinguished from yeast colonies during the initial step of colony counting on the filtration disks, we monitored the occurrence and incidence of filamentous fungi. Although, filamentous fungi belong to multiple species, in this study they were counted as a single group because we were merely aiming at complementing the yeast community dynamics study. The filamentous fungi that were detected probably result

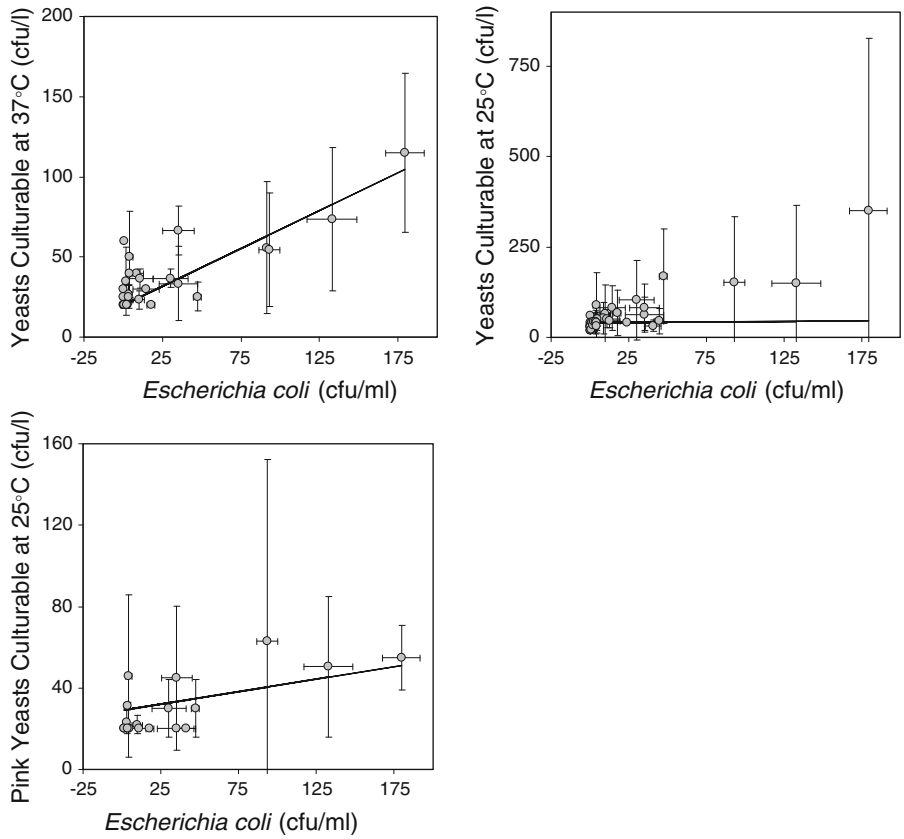

Fig. 4 Weighted linear correlations between yeast groups and *E. coli*. Group L—*cream*-colored yeasts isolated at 37°C (*top left*) shows a very good correlation ($r = 0.8018$) whereas groups A + B—*cream*-colored yeasts isolated at 25°C (*top right*) have a low correlation ($r = 0.1829$) with fecal contamination. Groups G + I—*pink* colored yeasts (*bottom*) have an intermediary correlation ($r = 0.6701$). *Error bars* are proportional to the observed standard deviation

from the germination of conidia that were initially present in the water. Given that these structures tend to be produced in high numbers, the quantification of filamentous fungi is always problematic. Our counts of filamentous fungi ranged between 0 and 400 l^{-1}. It is conceivable that fungal conidia are formed mainly in terrestrial environments and that they reach the estuary through wind dispersal, water runoffs, and other sources. In principle fungal spores should remain viable in water for long periods of time. As expected, filamentous fungi were not associated with *E. coli*, which confirmed that the routes of entrance in the estuary are different for these two groups. Likewise, the incidence of filamentous fungi was not correlated with the different yeast populations monitored (data not shown). Although some yeast populations, like for example those of the pink yeasts of groups G and I, should share with filamentous fungi similar entrance routes, other factors like for example survival times, adsorbance to particles in suspension and, hydrophobicity might affect the dynamics of fungal spores once in the estuary.

Discussion

It is well known that yeasts can be found in aquatic habitats ranging from river and lake systems to marine and estuarine environments (reviewed in Hagler and Ahearn 1987; Nagahama 2006). To date most studies have focused on describing yeast diversity and density in different types of aquatic habitats. From the extensive literature on this subject a few general rules can be drawn: (i) although a considerable number of yeast species have been isolated from water, only a limited number can be

considered prevalent (Fell 1976; Kohlmeyer and Kohlmeyer 1979; Hagler and Ahearn 1987 and references therein); (ii) yeast populations in freshwater have higher cell numbers than in marine water and distance from terrestrial environments reduces yeast density; and (iii) human pollution increases total yeast counts (Hagler and Mendonça-Hagler 1981; Hagler et al. 1982; Soares et al. 1997). Being at the interface between the marine and freshwater systems, estuaries might be viewed as complex and transition systems that can harbour distinct yeast populations, some primarily associated with marine open waters, others initially associated with terrestrial environments and a last group associated with the gastrointestinal systems of man and other warm-blooded animals. The complexity of the estuarine environment results not only from the interplay of multiple physicochemical factors but also from additional aspects such as the influence of the surrounding terrestrial environment and/or the effects of human activities. In this study we were interested in analysing the occurrence and incidence of distinct yeast populations in a transect of the Tagus river estuary and in connecting their origin with four possible sources: open sea, terrestrial environments, the gastrointestinal system and the estuary itself in the case of resident populations.

Yeasts that have the open sea as their primary source were difficult to detect in this study because most of the water samples studied were collected during ebb. For example, *Sakaguchia dacryoidea*, a pink yeast autochthonous to the marine environment, was absent from our study although it was repeatedly isolated from coastal waters in the South of Portugal (Gadanho et al. 2003). We reasoned that because the large majority of our samples were collected when the water was flowing into the sea, the group of pink yeasts, which are known to include some autochthonous marine species, contained in our study only species that are not primarily associated with the ocean. Indeed whereas *R. diobovatum* was previously found by in the Tagus river estuary and linked to a marine origin (Almeida 2005), in the present study this yeast was basically absent as only three strains (frequency of 0.75%) were isolated (Table 1). On the other hand the cosmopolitan pink yeast *Rh. mucilaginosa* had a frequency of 24.8%. This yeast has been isolated from terrestrial sources as well as freshwater, estuaries and seawater (Hagler and Ahearn 1987) and was by far the dominant pink yeast in our study. As we did not study samples from the flood we suppose that *Rh. mucilaginosa* entered the estuary from a source other than the ocean or the gastrointestinal system (note that we did not find a strong correlation between the occurrence of groups G and I, both dominated by *Rh. mucilaginosa*, and *E. coli*). The moderate correlation between *Rh. mucilaginosa* and *E. coli* mentioned earlier can be viewed as the result of a similar dispersal pattern from the margin—whereas *Rh. mucilaginosa* would enter the river through terrestrial runoffs, *E. coli* would enter the river through multiple and relatively minor sewage discharges. In the present study we did find yeasts associated with the gastrointestinal environment. Species like *P. guilliermondii* and *C. parapsilosis* have been reported in the literature as being associated with polluted water and with sewage (Hagler and Ahearn 1987) and *Cl. lusitaniae* has been found in clinical specimens and in the intestine of a pig, among other substrates (Lachance and Phaff 1998; Lachance et al. 2003). These three species are able to grow at 37°C or higher temperatures and were the dominant members of a group of cream-coloured yeasts that was isolated at 37°C (group L). Moreover, the occurrence of these yeasts was strongly correlated with the occurrence of *E. coli*. We conclude that these yeasts enter the estuary through sewage discharges and could therefore be viewed as additional indicators of fecal pollution. The yeasts of groups A and B showed the lowest correlation values with *E. coli* and therefore we discard a strong association with fecal pollution. Morphological differences of colony appearance did not allow a clear separation of species of group A and B and as a consequence *D. hansenii, C. parapsilosis,* and *C. zeylanoides* were present in the two groups. It is difficult to recognize common ecological specificities in the dominant species of groups A and B. Whereas *D. hansenii* could have a marine origin (van Uden and Fell 1968; Ahearn and Crow 1980; Yamasato et al. 1974), *C. parapsilosis* and *C. zeylanoides* have been found in association with the human niche (e.g., sputum, throat, nail, skin, feces) as well as with other environments including seawater, rotten wood and foodstuffs like meet, sausages, vegetables and pickles (Meyer et al. 1998; Hagler and Ahearn 1987). The remaining species of groups A and B, *C. boidinii* and *C. oleophila*, seem to be mostly related with terrestrial habitats like

different parts of plants and soil (Meyer et al. 1998). We hypothesize that the yeasts of groups A and B have become resident populations in the estuary. Their primary origin might be diverse and could include the human mycobiota in the case of *C. parapsilosis* and *C. zeylanoides*, terrestrial habitats, as exemplified by *C. boidinii* and *C. oleophila*, or the marine environment as typified by *D. hansenii*. In our model these yeasts have somewhat adapted to the estuarine environment and have established resident populations that may have different temporal horizons depending on the characteristics of each species and also on the particular environmental conditions.

The model for the multiple origins of the yeasts found in the Tagus river estuary is based mostly on the results included in this report and also on those of Almeida (2005). We suggest that estuarine yeasts can have four distinct origins (Fig. 5). The first contributor is the sea and an exemplary species is *R. diobovatum* (Almeida 2005). The occurrence and prevalence of such type of yeasts in the estuary is necessarily related with entry of seawater and consequently with the tidal cycle and river flow. Estuarine yeasts can also originate from the gastrointestinal system and exemplary species are *P. guilliermondii, C. parapsilosis* and probably also *Cl. lusitaniae*. The third source of estuarine yeasts is the terrestrial environment that surrounds the estuary and the upper river. Water runoff from the shore would carry terrestrial yeasts into the estuary and the entry points of these yeasts might not differ substantially from the entry points of gastrointestinal yeasts. Examples of terrestrial yeasts present in the estuary are the pink and cosmopolitan yeast *Rh. mucilaginosa*. Finally a fourth source of estuarine yeasts could be the estuary itself. We postulate that certain yeast species associated with the gastrointestinal, terrestrial, and marine environments are compatible with the estuarine environment and thus become resident, probably during a relatively short period of time. These resident but, in the long term, ephemeral populations, are probably dependent on the seasonal availability of organic matter and on the intensity of river flow that would tend to wash them from the estuary as previously discussed by Almeida (2005). According to our model, *C. boidinii* and *C. oleophila* might constitute resident populations whose primary origin is terrestrial, whereas *D. hansenii* might be also resident in the estuary although its primary origin is marine. Finally *C. parapsilosis* and *C. zeylanoides* would exemplify the third component of the resident community: intestinal yeasts that have adapted to the estuary. We note that for *C. parapsilosis* two populations were detected: one strongly associated with the gastrointestinal environment (included in group L) and another one less linked with this environment and more associated with the estuary which is included in groups A and B. Such model is probably incomplete and needs to be tested in other estuaries. We think, however, that it provides the conceptual framework for more detailed investigations on the ecology of estuarine yeasts.

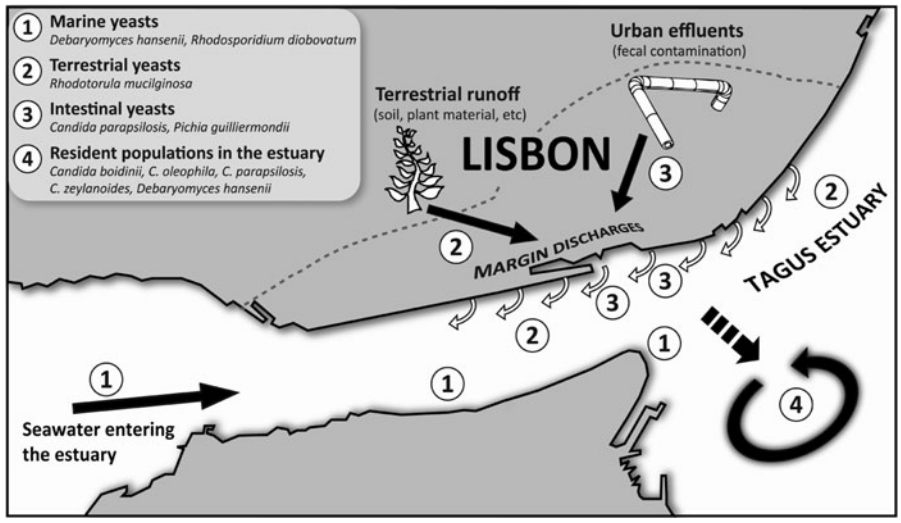

Fig. 5 A model for the multiple origins of estuarine yeasts in the Tagus estuary. The four possible origins of estuarine yeasts are indicated in the *top left panel*

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