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Title: Diversity and antibiotic resistance of *Acinetobacter* spp. in water from the source to the tap

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Abstract

Acinetobacter spp. are ubiquitous bacteria in the environment. *Acinetobacter* spp. isolated from a municipal drinking water treatment plant and from connected tap water were identified to the species level, on the basis of *rpo*B gene partial sequence analysis. Isolates were typed to assess intra-species variation, based on the analysis of the partial sequences of housekeeping genes (*rpo*B, *gyr*B and *rec*A). Antibiotic resistance was characterized, using the disk diffusion method, and classified as wild or non-wild type, according to the observed phenotype.

The strains of *Acinetobacter* spp. were related to 11 different validly published species, although four groups of isolates, presenting low *rpo*B sequence similarities with previously described species, may represent new species. Most of the isolates were related to the species *A. johnsonii* and *A. lwoffii*. These two groups, as well as others related to the species *A. parvus* and *A. tjernbergiae*, were detected in the water treatment plant and in tap water. Other strains, related to the species *A. pittii* and *A. beijerinckii*, were isolated only from tap water. Most of the isolates (80 %) demonstrated wild type to all of the 12 antibiotics tested. Non-wild types were observed for tetracycline, meropenem and ceftazidime, among others, in water-treatment plant samples or in tap water samples. Although, in general, this study suggests a low prevalence of acquired antibiotic resistance in water *Acinetobacter* spp., the potential of some species to acquire and disseminate resistance via drinking water is suggested.

Introduction

Members of the genus *Acinetobacter* spp. are Gram-negative, obligate aerobic, heterotrophic bacteria with wide-spread distribution in natural environments (Juni 2005). Given their metabolic versatility and general physiological characteristics, *Acinetobacter* spp. can easily settle in anthropogenic habitats, including waste-water treatment facilities. In these habitats, the roles of *Acinetobacter* spp. on the removal of phosphorus or in the degradation of polycyclic aromatic hydrocarbons are well known (Kim 1997; Thangaraj 2008). However, in these same habitats, *Acinetobacter* spp. may be subjected to selective pressures capable of promoting antibiotic resistance acquisition (Zhang 2009). The tendency noticed world-wide for *Acinetobacter* spp. to develop antibiotic resistance (Henwood 2002; Kuo 2010; Marshall 2007; Tognim 2004; Xu 2008), may, in part, be attributed to the transient colonization of waste-waters and other habitats subject to anthropic pressures.

Acinetobacter spp. are frequently found also in unpolluted sites, such as ground-, surface- or tap water (Hoefel 2005; Mckeon 1995; Pavlov 2004; Who 2008). These habitats constitute important sources of bacteria with potential to infect humans, particularly immune-compromised hosts, for example, via water baths and room humidifiers (Who 2008). In this respect, the ability of some *Acinetobacter* spp. to form biofilms is documented to be important (Bhargava 2010; Simões 2010). This ability of some *Acinetobacter* spp., to form biofilms, have recognized them a status as important nosocomial agents (Henwood 2002; Idzenga 2006; Medina 2007; Regalado 2009; Tognim 2004). *A. baumannii* is the most frequently reported (Towner 2009). *Acinetobacter* species, such as those of the *A. calcoaceticus-A. baumannii* complex, have been reported also as being potentially hazardous for human health. Additionally, members of the species *A. haemolyticus*, *A. lwoffii*, *A. junii* or *A. radioresistans*, with widespread distribution in the environment, have been referred to as “agents of clinical

79 relevance" (Dimopoulou 2003; Idzenga 2006; Kappstein 2000; Poirel 2008; Quinteira
80 2007; Regalado 2009; Spence 2002; Tognim 2004). Given the ubiquity of these species,
81 their capabilities to cross between different water compartments (waste-, surface-,
82 ground-) and their capacity to infect humans, the presence of *Acinetobacter* spp. in tap
83 water may represent a potential health risk. In such situations, the severity of the
84 associated risks will depend, among other factors, on the species and the occurrence of
85 acquired antimicrobial resistance. The hypothesis of this work was that drinking water
86 represents a vehicle for *Acinetobacter* spp. dissemination, in which the hazardous
87 potential could be aggravated by reduced antimicrobial susceptibility. To study this
88 hypothesis, the current study was designed to characterize: i) the diversity of
89 *Acinetobacter* spp. in water sources (drinking water treatment plant and taps) at the
90 species and sub-species levels; and ii) the non-wild type susceptibility to antibiotics of
91 different classes.

Materials and methods

Sampling

The bacterial strains examined in this study were isolated from a water treatment plant (WTP) and distribution system, from tap water in 11 houses and in one health care facility (Fig. 1). The houses and health care facility are served by the WTP studied and are located within an area of 25 km^2. Samples from the WTP were collected at eight different locations along the production process: surface water (W1); alluvial wells (W2); after the ozonation step (W3); after the chlorination point (W4; preceded by flocculation and activated carbon filtration); and at four points downstream in the bulk supply distribution system (W5-W8) (Fig. 1). These samples were collected in November 2007 and in September 2009, at sampling points used for routine control of water quality. Tap water samples were collected in April, July and October 2009 from 11 household taps (T1-T11; from 11 different houses) with low usage (1-4 times a month). The tap water from the health care facility (H) was collected in June 2008, from a tap used regularly, located in the sterilization unit. Water samples were collected and processed as described before (Vaz-Moreira 2011c).

Bacterial enumeration and isolation

Cultivable bacterial enumeration and isolation were based on the membrane filtration method, using the culture media, R2A (Difco, Le Pont de Claix, France), *Pseudomonas* Isolation Agar (PIA, Difco) and Tergitol 7-Agar (TTC, Oxoid, Hants, UK) as described before (Vaz-Moreira 2011c) Bacterial isolates for purification were selected after visual examination of culture plates with countable numbers of colonies. The criteria for further isolate purification were: all colonies of morpho-types represented by less than five colonies; half of the colonies of morpho-types represented by five to 10 colonies; and approximately one third of the colonies of morpho-types represented by more than

10 colonies. Isolates were purified and preserved as described before (Vaz-Moreira 2011c).

Preliminary identification of presumptive *Acinetobacter* spp.

After the preliminary characterization, as described in,Vaz-Moreira (2011c) of a total of 2833 bacterial isolates, a group of 323 Gram-negative cocco-bacilli, oxidase-negative and catalase-positive isolates, assumed presumptively as possible *Acinetobacter* spp., were selected for further analysis. This set of isolates was screened for presence of the *Acinetobacter* spp. 16S rRNA signature, as described by Vanbroekhoven et al. (2004). PCRs were carried out with the following programme: initial denaturation at 95 ºC for 5 min; 30 cycles at 95 ºC for 15 sec, 58 ºC for 30 sec, 72 ºC for 40 sec and a final extension at 72 ºC for 4 min. The DNA template of *A. baumannii* CCUG 19096^{T}, *E. coli* ATCC 25922 and *Ps. aeruginosa* DSM 1117, were used in each amplification reaction as positive and negative controls.

Identification at the species level and determination of intra-specific variation

Acinetobacter isolates identification was based on analysis of the sequence of the gene for RNA polymerase beta subunit (*rpo*B) (La Scola 2006). Additionally, two other house-keeping gene sequences, recombinase A (*rec*A) and gyrase beta subunit (*gyr*B), were analyzed. The combined multi-locus sequence analysis (MLSA) of the three gene sequences was used to assess the intra-species genetic variation.

The partial sequences of *rpo*B (902 bp) and *gyr*B (909 bp) were amplified with the primers and conditions described before (La Scola 2006; Vaz-Moreira 2011b), and *rec*A (425 bp) was amplified with the primers described previously (Nowak and Kur 1995) in reactions of 50 µL, with 1.23 U of Taq PCR MasterMix Kit (Qiagen, Hilden, Germany), 0.5 µM of each primer, RA1 and RA2, and 10 µL of bacterial genomic DNA. After initial

144 denaturation at 95 ºC for 2 min, 35 amplification cycles were performed according to the
145 following format: 30 sec at 95 ºC; 1 min at 55 ºC; 2 min at 72ºC; and a final extension
146 of 10 min at 72 ºC. PCR products were purified, using the Qiagen DNA Purification Kit
147 (Qiagen, Hilden, Germany), according to the supplier's instructions, and sequenced,
148 using a 3130XL Genetic Analyzer (Applied Biosystems, California, USA).
149 The partial *rpo*B, *rec*A and *gyr*B nucleotide sequences were edited manually, using the
150 software BioNumerics (ver. 6.1, Applied Maths, Sint-Martens-Latem, Belgium). To
151 determine species affiliations, *rpo*B nucleotide sequences were compared with the *rpo*B
152 sequences of the type strains of all *Acinetobacter* species, available in the GenBank
153 database (http://www.ncbi.nlm.nih.gov) or determined in this study. To assess the intra-
154 species variation the nucleotide sequences of *rec*A and *gyr*B also were examined.
155 Dendrograms were constructed, based on the model of Jukes & Cantor (1969), using the
156 neighbour-joining, maximum parsimony and maximum likelihood methods. In the
157 analysis 828, 852 and 363 nucleotide positions of the *rpo*B, *gyr*B and *rec*A sequences,
158 respectively, were used. For strain discrimination, the nucleotide sequences of each of
159 the three genes were compared within each species (as determined by *rpo*B sequence
160 analyses). Isolates which sequences differed in, at least, one nucleotide position were
161 considered to represent distinct sequence types (ST).
162 For simplicity, comparison of the isolates was represented in a dendrogram constructed
163 based on 2043 positions of the concatenated partial sequences of *rpo*B, *rec*A and *gyr*B
164 genes (Fig. 2). One representative of each sequence type of the genes *rpo*B, *rec*A and
165 *gyr*B was deposited in the GenBank database with the accession numbers (JN903770-
166 JN903895).
167
168 Antibiotic resistance phenotypes

169 Antibiotic resistance phenotypes were determined for all the 244 isolates that gave a
170 positive result for the 16S rRNA signature, using the disk diffusion method, according
171 to standard recommendations (Clinical and Laboratory Standards Institute 2007). The
172 antibiotics tested were: amoxicillin (AML, 25 µg); gentamicin (GEN, 10 µg);
173 ciprofloxacin (CIP, 5 µg); sulphamethoxazole/trimethoprim (SXT, 23.75/1.25 µg);
174 tetracycline (TET, 30 µg), cephalothin (CP, 30 µg); meropenem (MER, 10 µg);
175 ceftazidime (CEF, 30 µg); ticarcillin (TIC, 75 µg); colistin sulphate (CT, 50 µg);
176 sulphamethoxazole (SUL, 25 µg); and streptomycin (STR, 10 µg) (all Oxoid, Hants,
177 UK). Cultures were incubated for 24 h at 37 °C, except for isolates most related with the
178 species *A. parvus*, which being unable to grow at that temperature, were incubated at 30
179 ºC. In each experimental set were included the reference strains, *E. coli* ATCC 25922
180 and *P. aeruginosa* DSM 1117, incubated at temperatures 30 ºC and 37 ºC. The
181 inhibition zones were measured. For reference strains, an average deviation of 1-2 mm
182 was observed. For each antibiotic, the epidemiological cut-off value was determined,
183 based on box-plot representations of the data for the inhibition diameters observed in
184 this study or, for the antibiotics ciprofloxacin, meropenem and
185 sulphamethoxazole/trimethoprim, complemented with data available in public databases
186 (http://mic.eucast.org/Eucast2/). Based on box-plot representations of the inhibition
187 zone diameters, outliers were identified and classified as non-wild types (non-WT).
188 Thus, isolates with wild-type (WT) tolerance to an antibiotic presented an inhibition
189 zone diameters which fell inside the box-plot bar whereas non-wild-type (non-WT)
190 were outliers.
191
192 Statistical analyses
193 Bacterial richness, diversity and evenness indices were determined to compare the
194 occurrence of ST:s of cultivable *Acinetobacter* spp. over the different sampled sites. The

Diversity [H' = - Σ p_i ln(p_i)] and Evenness [J=H'/ln(Hmax)] were calculated, using the Shannon's (Shannon and Weaver 1949) and Pielou's indices (Pielou 1966), respectively. Species and sequence types (resultant from the analysis of the three housekeeping genes) were used as the taxonomic units for these calculations, which included all of the isolates (n=244), even when recovered on the same date, place or culture medium.The analyses of the distribution of antibiotic inhibition zones were supported by box-plots supplied by SPSS software (version 18.0). The epidemiological cut-off values were defined as the values below the lower limits of the normal distribution of inhibition zones.

Results

Diversity of *Acinetobacter* spp.

Cultivable *Acinetobacter* spp. were isolated from samples with total heterotrophic counts ranging from 10^1-10^3 CFU mL^{-1} in pre-treated surface water in the WTP, 10^{-2}-10^2 CFU mL^{-1} in treated water in the WTP and 10^1-10^4 CFU mL^{-1} in tap water (Table 1). Using PCR and *Acinetobacter*-specific 16S rRNA gene primers (Vanbroekhoven 2004), 244 isolates out of the 323 presumptive *Acinetobacter* spp. (Gram-negative coco-bacilli, oxidase-negative and catalase-positive), were indicated to be members of this genus. Given the criterion for bacterial isolation (explained in the Methods section) some repetitive isolates could be expected. In order to avoid the artefact of the overrepresentation of some sequence types or non-wild antibiotic resistance phenotypes, repetitive isolates were removed from further analysis. Thus, when two or more bacterial isolates from the same place, same date, and same culture medium exhibited identical *rpo*B, *gyr*A and *rec*A sequence types and antibiotic resistance patterns, they were considered replicas of the same isolate and were excluded from further analysis. This procedure led to the establishment of a collection of 118 *Acinetobacter* isolates

which were compared for their antibiotic resistance types and genotypic diversity. Twenty-four per cent of these isolates (28/118) were from the WTP and 76 % (90/118) were from taps. From the WTP, 22 isolates were collected in November 2007 and six in September 2009. From the taps, five of the isolates were collected in April, 46 in July, 38 in October of 2009 and one in June 2008. None of isolates recovered from three of the four sampling sites located after chlorination points in the WTP (Table 1) were identified as *Acinetobacter* spp..

In an attempt to identify the *Acinetobacter* isolates to the species level, the *rpo*B partial sequences were compared with those of the type strains of all validly published *Acinetobacter* spp., (by what date?) as recommended by previous studies (La Scola et al., 2006; Gundi et al. 2009). On the basis of analysis of the partial sequence of *rpo*B, the closest neighbours of the *Acinetobacter* isolates were members of 11 distinct species (Table 2). Nevertheless, considering the conclusions of La Scola *et al*. (La Scola 2006) that *rpo*B sequence similarity values below 95 % indicate distinct species, it is hypothesised that 7 isolates recovered during this study represent three novel species. The closest neighbours of these 7 isolates comprised the species *A. gerneri* (one isolate, 85% similar *rpo*B sequence from W5), *A tandoii* (two isolates, 94.2% similar *rpo*B sequence, from W1) and *A. tjernbergiae* (five isolates, 94-94.4% similar *rpo*B sequence, from W3, T2 and T5) (Table 2). In general, the analysis of the *gyr*B and *rec*A sequences gave concordant species affiliations with those determined on the basis of *rpo*B nucleotide sequences. Exceptions were observed for the strains identified as or closest related to *A. baylyi*, *A. gerneri*, *A. parvus* and *A. tjernbergiae*.

Isolates with high *rpo*B sequence similarity, and probably members of the same species, were often detected in a single site or sampling date. For instance, this was observed in isolates most closely related to the species *A. baylyi*, *A. calcoaceticus*, *A. gerneri*, *A. junii* and *A. tandoii*, which were isolated only from WTP samples (Table 2). Others,

247 closely related to *A. pittii* and *A. beijerinckii* were isolated only from tap water. In
248 contrast, isolates related with the species *A. johnsonii*, *A. lwoffii*, *A. parvus* and *A.*
249 *tjernbergiae* were obtained from both the water treatment plant and tap water. From
250 alluvial wells, in which human impact is supposed to be lower than in surface water
251 samples, isolates identified as six different species were observed, *A. baylyi*, *A.*
252 *calcoaceticus*, *A. junii*, *A. johnsonii*, *A. lwoffii* and *A. parvus* (Table 2).
253 In an attempt to determine intra-species strain diversity and investigate possible clonal
254 relationships between isolates from different sampling sites or dates, concatenated
255 partial nucleotide sequences of the genes *rpo*B, *gyr*B and *rec*A were compared (Table 3,
256 Fig. 2). This analysis supported the definition of 39 sequence types. Isolates most
257 related with the species *A. pittii* and *A. beijerinckii*, which were represented by more
258 than one isolate but included a single sequence type, had origins in different locations
259 and sampling dates. In the same way, isolates sharing identical sequence types, related
260 with the species *A. johnsonii*, *A. lwoffii* and *A. tjernbergiae*, were observed in different
261 taps. This observation could indicate a possible common origin of these strains (Table
262 3). The occurrence of different lineages was evidenced for isolates which closest
263 neighbours were members of the species *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. parvus*, *A.*
264 *tandoii* and *A. tjernbergiae*, represented by up to 13 different sequence types. Most of
265 these STs were isolated from distinct taps or sampling dates. In other cases, different
266 sequence types were observed in the same tap (for example *A. lwoffii* ST lw2, lw4, lw5,
267 lw7 and lw8 in taps 1, 5, 8 and 10; and *A. johnsonii* ST jo2, jo3, jo5, jo7-14 in taps 3, 5,
268 7 and 10). This pattern may suggest different episodes of colonization by *Acinetobacter*
269 in the same tap.
270 No particular sequence type was ever observed in both water treatment plant and in tap
271 water samples (Fig. 2, Table 3). Similarly, within the water treatment plant, unique
272 sequence types were detected for each sampled site, with the exceptions of the ST ju6 of

A. junii and ST pa6 of *A. parvus*. Strains with these STs were isolated from alluvial wells and also from samples collected downstream from the chlorination point, hinting at the possible survival of these bacteria during the disinfection process. This can be explained by the fact that, in this treatment facility, water from alluvial wells is not subjected to the treatment stages prior to chlorination, due to its supposed pristine character.

According to the criterion established for bacterial isolation and purification, the set of isolates collected was representative of the variety of cultivable *Acinetobacter* spp. in each location. Based on this, a comparative analysis of the diversity and evenness indices was made. The comparison of the sequence type diversity and evenness in the different sites did not reveal dramatic differences between the WTP and tap water samples (Fig. 3). Apparently, ozonation, more than chlorination, imposed a marked reduction on the diversity of sequence types. In general, the diversity observed was higher in pre-treated (W1-W2) and in tap water than immediately after disinfection (W3, W5). Evenness was higher in ozonated water (W3) than in water from alluvial wells (W2) or surface water (W1) and, in general, it was lower in tap water than in the water treatment plant.

In contrast to the general trend in the WTP, in tap water, the same sequence type was detected in samples from different taps. For instance, the sequence type lw8 of the species *A. lwoffii* was detected in tap 10 and in taps 5, 8, 9 and 10 with an interval of three months, suggesting a continuous supply of this bacterium in water, or that they live and proliferate in household pipes. The temporal persistence of a specific sequence type in the same site of isolation could be inferred also from its presence at different sampling dates (Fig. 2). Such persistence could be hypothesized for the isolates most related with the species *A. beijerinckii*, *A. junii* and *A. lwoffii*. An interesting example

298 was given by isolates of *A. junii*, with the same sequence type being isolated from
299 alluvial wells almost two years apart, suggesting the stability of this habitat.
300
301 Antimicrobial susceptibility
302 The term epidemiological cutoff (ECOFF), proposed by the European Committee on
303 Antimicrobial Susceptibility Testing (EUCAST), corresponds to the lower limit of the
304 inhibition zone in a wild type (WT) population distribution. According to the EUCAST
305 definition, a microorganism is defined as wild type (WT) if it does not exhibit acquired
306 and mutational resistance mechanisms to a given drug. This definition has no
307 correspondence to the clinical response to antimicrobial treatment and does not vary by
308 changing circumstances. ECOFF values have been published for numerous bacterial
309 groups and antimicrobial agents (http://www.eucast.org) and allow a reliable
310 differentiation between wild bacteria and those which acquired any antibiotic resistance
311 mechanism. Nevertheless, for some bacterial groups, ECOFF values databases are still
312 under construction. One of such examples is the genus *Acinetobacter* spp., for which the
313 available data on inhibition zones is scant. Indeed, from the 12 antibiotics used in this
314 study, the ECOFF value was defined only for gentamicin. This fact motivated the
315 calculation of epidemiological cutoff values based on the data obtained in the current
316 study, whenever possible, complemented by data available in EUCAST database (Table
317 4). Isolates were classified as WT or non-WT, although the limited number and origin
318 of strains may have introduced some bias on the data analysis. For instance, in the case
319 of gentamicin, for which an ECOFF value was defined, the value estimated based on
320 our data was ≥ 18 mm while EUCAST recommends a value > 15 mm. When EUCAST
321 data was included in our dataset, the determined cutoff value for meropenem was
322 lowered from 23 to 20 mm and the value varied for sulfamethoxazole/trimethoprim
323 from 18 to 16 mm. In contrast, for ciprofloxacin the same value of 20 mm was obtained

using only our dataset or including also the EUCAST inhibition zones data. Cephalothin, for which most of the isolates (> 75 %) presented inhibition zones of zero mm, was excluded from this analysis.

In general, non-wild types were rare among the studied *Acinetobacter* spp. from both the WTP and tap water samples, with 80 % of the isolates showing a wild type of susceptibility to all the antibiotics tested. Nevertheless, with exception for ciprofloxacin, non-WTs were observed for every antibiotic tested. Non-WT for gentamicin, meropenem, streptomycin, sulphamethoxazole/trimethoprim, sulphamethoxazole and ticarcillin were detected exclusively among the tap water isolates. In contrast, non-WTs for ceftazidime and colistin sulphate were observed only in WTP. Additionally, non-WTs for tetracycline were significantly more prevalent ($p<0.05$) among the WTP isolates than in tap water (Table 4). Some non-WTs were observed preferentially in some *Acinetobacter* groups. For instance, non-WTs for streptomycin and tetracycline were restricted to isolates closely related with the species *A. tjernbergiae* and *A. pittii* or *A. parvus* and *A.johnsonii*, respectively (Table 3). This distribution may suggest the tendency of members of these lineages to acquire those resistance phenotypes.

Discussion

Numerous studies focusing on the diversity of *Acinetobacter* spp., published over the last years, refer to the potential of some members of this genus to act as opportunistic pathogens, develop antibiotic resistance or discuss their role in wastewater bio-treatment systems. Although drinking water can harbour different species of *Acinetobacter* (Leclerc and Moreau 2002; Norton and Lechevallier 2000; Who 2008), to our knowledge, the diversity and dynamics of members of this genus in potable water has not been addressed in literature. Some studies characterizing the bacterial community through cultivation-independent approaches do not report the detection of

350 *Acinetobacter* spp. in these habitats (Hong 2010; Li 2010; Revetta 2010), while others
351 do confirm the relevance of *Acinetobacter* spp. in potable water (Vaz-Moreira 2011a;
352 Vaz-Moreira unpublished). In the present work, cultivable *Acinetobacter* spp. reached
353 as much as 10^4 CFU mL^{-1} of tap water (Table 1), a density which, in some
354 circumstances, may deserve attention.
355 The selection of the genetic loci used to type *Acinetobacter* spp. was supported by
356 previous publications. The gene *rpo*B, given the low resolution of species detected by
357 the 16S rRNA gene, has become an important and recognised tool for *Acinetobacter*
358 species delineation (La Scola 2006). Species identification, based on the analysis of the
359 partial sequence of the gene *rpo*B, allowed for the differentiation of 11 species groups,
360 three of which include presumably members of novel species. The other two gene
361 sequences analysed, *gyr*B and *rec*A, constituting part of the recommended protocol for
362 multi-locus sequence typing (MLST) of *A. baumannii*, have also been reported to
363 discriminate *Acinetobacter* species (Bartual 2005; Krawczyk 2002; Nowak and Kur
364 1995; Yamamoto 1999). In this study, the inconsistency of *Acinetobacter* identifications
365 based on the *rpo*B and *gyr*B sequences, previously referred to (La Scola 2006) was
366 confirmed, mainly for strains with the highest *rpo*B sequence similarity to the species *A.*
367 *baylyi*, *A. gerneri* and *A. parvus*. Nevertheless, the combination of these three loci
368 (*rpo*B, *gyr*B and *rec*A) allowed intra-species differentiation for most of the
369 *Acinetobacter* groups studied in this work. For the group of isolates under study
370 (including the type strains included in Fig. 2), the resolving power of the three
371 nucleotide sequences could be ranked as *rpo*B< *rec*A< *gyr*B, with ranges of sequence
372 similarity, after Jukes & Cantor correction, of 72.8 %–98.9 %, 74.7 %–90.6 %, 68.8 %–
373 88.9 %, respectively.
374 Most of the species identified in this study have a recognized environmental
375 distribution. *Acinetobacter* spp. have been isolated from contaminated soils

(Vanbroekhoven 2004), freshwater fish (Gonzalez 2000), raw milk (Hantsis-Zacharov and Halpern 2007) or human skin (Seifert 1997). Isolates most related with the species *A. lwoffii*, *A. johnsonii*, *A. parvus* and *A. tjernbergiae* were those detected in both the WTP and in tap water. Of the species detected in tap water, *A. lwoffii* and *A. pittii* are those more commonly associated with opportunistic infections (Dimopoulou 2003; Idzenga 2006; Regalado 2009).

Although alluvial wells (W2) were the sampled sites with the highest number of different species (n=6), taps 1, 3, 5, 7 and 11 also presented a high diversity of species and/or of sequence types (Table 3 and Fig. 3). Even when isolates with high *rpo*B sequence similarity were present in both WTP and in tap water, these were always represented by distinct ST (Fig. 2), suggesting a dynamic character of the *Acinetobacter* spp. populations throughout the water circuit and/or the entrance of *Acinetobacter* spp. strains downstream the water treatment plant. The inclusion of amoebae-resistant *Acinetobacter* spp. by free-living amoebae may be also an important factor influencing the dynamics of members of this genus in disinfected water (Thomas 2008; Thomas 2010); (Loret and Greub 2010). Nevertheless, a similar distribution of diversity and evenness indices in the water treatment plant and in tap water deny a hypothetical founder effect due to water disinfection (Fig. 3).

Most of the isolates (80 %) were WT for the antibiotics tested, suggesting low rates of acquired resistance mechanisms. Nevertheless, in both WTP and tap water, isolates with non-WT to different antibiotics were observed. For some antibiotics, non-WT were observed only among tap water isolates, suggesting that tap water *Acinetobacter* spp. do not originate from the WTP or that resistance acquisition to some antibiotics may take place throughout the water distribution. The first hypothesis, which would suggest that *Acinetobacter* spp. detected in tap water enter the system downstream the WTP, is supported by the genotypic and taxonomic analysis. In turn, the fact that tap water

402 isolates with the same ST could be differentiated in WT and non-WT, suggests some
403 kind of resistance emergence (mutation or horizontal gene transfer) downstream the
404 WTP. The relevance of tap water as a potential source of antimicrobial resistant bacteria
405 has been suggested in different studies and bacterial groups (Vaz-Moreira 2011c; Vaz-
406 Moreira 2012; Xi 2009). Studying cultivable sphingomonads and *Pseudomonas* spp.,
407 which are known to occur frequently in drinking water worldwide, Vaz-Moreira et al.
408 (2011c; 2012) showed that these bacteria are relevant potential reservoirs of antibiotic
409 resistance in drinking water. Also Xi et al. (2009) observed the re-growth of bacteria in
410 drinking water distribution systems and concluded that most antibiotic resistance genes
411 studied, conferring resistance to beta-lactams or sulfonamides, were more abundant in
412 tap water than in finished disinfected water or source water. Nevertheless, such
413 differences may be species or even strain-dependent, as suggested before for
414 sphingomonads and *Pseudomonas* spp. (Vaz-Moreira 2011c; Vaz-Moreira 2012),
415 recovered from the same drinking water system. The fate of bacteria from the source to
416 the tap, depends also on the taxonomic group as was demonstrated comparing the
417 current study with another one on aeromonads (Figueira 2011), which density was
418 reduced after water disinfection, being below the detection limit in tap water. The
419 ability of bacteria to form or adhere to biofilm structures may be determinant, among
420 other factors, for their capability to colonize tap water. Indeed, *Acinetobacter* spp. have
421 such a capacity (Simões 2010), a fact that may explain the prevalence of these bacteria
422 in tap water and also the liability to acquire antibiotic resistance determinants.
423 Drinking water quality is influenced by several factors, including geography and climate
424 conditions. Nevertheless, the major bacterial groups present? are similar worldwide
425 (Eichler 2006; Hoefel 2005; Kormas 2010; Poitelon 2009; Revetta 2010; Thomas 2006;
426 Xi 2009). For this reason, it is expected that studies on bacterial diversity and antibiotic
427 resistance conducted in drinking water treatment plants or tap water worldwide share

some general trends. Additionally, these studies are also relevant to allow further inferences on the differences of resistance patterns observed worldwide. *Acinetobacter* spp. are ubiquitous bacteria with the potential to rapidly adapt to the hospital environment and behave as nosocomial pathogens (Gundi 2009; Van Looveren 2004; Visca 2011). The ability to develop antibiotic resistance is part of such an adaptive process and give to some *Acinetobacter* species the character of clinically relevant environmental bacteria (Bergogne-Berezin and Towner 1996; Montealegre 2012). These arguments motivated the current study, conducted in a water supplying system feeding about half a million of inhabitants and several health care facilities.

In this study the highest prevalence of non-WT was observed for the antibiotics amoxicillin, tetracycline, colistin and streptomycin (Table 4). Some of these resistance phenotypes were previously observed in environmental bacteria of this genus (Dhakephalkar and Chopade 1994). For cefatzidime, tetracycline and colistin, non-WT were only observed or were more prevalent in the WTP. This is relevant for instance for colistin which is one of the antibiotics commonly used for the treatment of *Acinetobacter* infections (Fishbain and Peleg 2010) and was not detected in tap water. It is also noteworthy that isolates related with *A. pitti* were the major harbors of streptomycin non-WT, which was only observed in tap water. Remarkably, *A. pittii* is among the major causes of *Acinetobacter* infection in humans and is a recognized host for new antibiotic resistance determinants (Gundi 2009; Montealegre 2012; Visca 2011).

This study confirms the ubiquity of some species of *Acinetobacter* in water, including in tap water and emphasizes the fact that tap water may represent a vehicle of clinically relevant environmental bacteria to humans. Although multiple sources of colonization, other than the water supply system, may explain the presence of *Acinetobacter* spp. in tap water, their presence and antibiotic resistance patterns deserve attention. Non-WT

isolates were rare, but nevertheless, more frequent and diverse in tap water than in WTP, suggesting that the entry of *Acinetobacter* spp. harbouring acquired resistance or the acquisition of resistance after water disinfection are likely processes.

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642 selective increase of antibiotic resistance among *Acinetobacter* spp. Sci Total Environ
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644 Table 1. Total heterotrophic counts (CFU mL^{-1}) and percentage (%) of *Acinetobacter*
645 representatives in the different samples sites

Local of isolation		Range of Total Heterotrophs counts (CFU's mL^{-1})			% of *Acinetobacter* spp. isolated (nº *Acinetobacter* / total nºof isolates)
		Minimum	Mean value	Maximum	
WTP	W1	$2.5x10^3$ - $3.6x10^3$	$3.0x10^3$	$3.6x10^3$	3.6 % (7/196)
	W2	$1.4x10^1$ - $2.1x10^1$	$1.8x10^1$	$2.1x10^1$	42.7 % (56/131)
	W3	$2.0x10^1$ - $1.2x10^3$	$4.4x10^2$	$1.2x10^3$	1.4 % (2/145)
	W5	$9.7x10^0$ - $2.0x10^2$	$1.1x10^2$	$2.0x10^2$	9.0 % (17/189)
TAP	T1	$2.3x10^2$ - $1.6x10^3$	$7.5x10^2$	$1.6x10^3$	11.1 % (14/126)
	T2	$3.6x10^2$ - $1.1x10^3$	$6.3x10^2$	$1.1x10^3$	3.3 % (4/122)
	T3	$6.0x10^1$ - $5.7x10^2$	$2.7x10^2$	$5.7x10^2$	22.6 % (26/115)
	T4	$2.0x10^2$ - $2.0x10^3$	$8.6x10^2$	$2.0x10^3$	9.0 % (12/133)
	T5	$3.3x10^1$ - $3.3x10^2$	$1.6x10^2$	$3.3x10^2$	16.1 % (24/149)
	T6	$1.5x10^3$ - $6.3x10^3$	$3.9x10^3$	$6.3x10^3$	7.2 % (10/139)
	T7	$2.5x10^2$ - $1.8x10^3$	$1.1x10^3$	$1.8x10^3$	18.7 % (26/139)
	T8	$7.9x10^3$ - $7.7x10^4$	$3.4x10^4$	$7.7x10^4$	5.5 % (8/146)
	T9	$2.9x10^3$ - $1.4x10^4$	$9.1x10^3$	$1.4x10^4$	0.7 % (1/148)
	T10	$2.2x10^3$ - $9.9x10^3$	$4.8x10^3$	$9.9x10^3$	15.4 % (23/149)
	T11	$1.4x10^3$ - $1.3x10^4$	$8.3x10^3$	$1.3x10^4$	6.4 % (8/125)
	H	$3.0x10^1$ - $1.3x10^3$	$6.5x10^2$	$1.3x10^3$	4.3 % (6/140)

646
647 WTP, water treatment plant; W1, surface water; W2, alluvial wells; W3, after ozonation; W5, before a re-
648 chlorination point; T1-T11, household taps; H, health care unit tap.
649 Acinetobacter spp. could not be isolated from the WTP sampling point W4 (146 isolates, collected after
650 the chlorination), and in three points of the bulk supply distribution system: W6-W8 (395 isolates).

651 **Table 2.** Closest neighbours of bacterial isolates based on the *rpo*B sequence analysis,
652 per site of isolation

Closest related species (type strain)	% of *rpo*B sequence similarity (accession number)	Site[a] (number of isolates)
A. baylyi (CIP 107474)	99.4 (DQ207472)	W2 (1)
A. beijerinckii (NIPH 838)	98.3 (EU477124)	Taps (18)
A. calcoaceticus CIP 81.8)	97.0 (DQ207474)	W5 (1)
A. gerneri (CIP 107464)	85.4 α (DQ207482)	W2 (1)
A. johnsonii (CIP 64.6)	98.2-99.9 (DQ207485)	W2 (5); Taps (31)
A. junii (CIP 64.5)	98.2-98.8 (DQ207486)	W1 (2), W2 (5), W5 (2)
A. lwoffii (NIPH 512)	98.1-99.7 α (EU477111)	W2 (1); Taps (23)
A. parvus (CIP 108168)	97.8-98.2 (DQ207488)	W1 (3), W2 (3), W5 (1); Taps (5)
A. pittii (NIPH 519)	99.7 (EU477114)	Taps (9)
A. tandoii (CIP 107469)	94.2 α -97.5 (DQ207491)	W1 (1), W3 (1)
A. tjernbergiae (CIP 107465)	94.0-94.4 α (DQ207492)	W3 (1); Taps, (4)

653 α, *rpo*B sequence similarity values < 95 %, suggest that these isolates may represent
654 novel species
655 [a]W1, surface water; W2, alluvial wells; W3, after ozonation; W5, downstream from the
656 chlorination tank (see figure 1 for details).
657

658

Table 3. Diversity of *Acinetobacter* spp. sequence types according to isolation site and antibiotic resistance type, classified as WT or non-WT

			Site[a] (n)												
			WTP (28)	Taps (90)											
Closest neighbour	**Sequence Type (n)**	**Phenotype[b]**	**0[c]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**H**
A. baylyi	by2(1)	AML	W2 (1)												
A. beijerinckii	be2(18)	WT		3			5		5		2			3	
A. gerneri(85%)	ge2(1)	CT CEF	W5 (1)												
A. calcoaceticus	ca2(1)	AML	W2 (1)												
A. johnsonii	jo2(1)	WT						1							
	jo3(3)	WT			1	1									
		TET						1							
	jo4(1)	CT	W2 (1)												
	jo5(3)	WT				3									
	jo6(4)	WT	W2 (4)												
	jo7(1)	TIC				1									
	jo8(1)	WT						1							
	jo9(1)	AML MER SXT SUL											1		
	jo10(4)	WT				3		1							
	jo11(7)	WT		1		3				2				1	
	jo12(3)	WT				1				2					
	jo13(4)	WT				1				3					
	jo14(3)	WT								2			1		
A. junii	ju2(1)	WT	W1 (1)												
	ju3(1)	WT	W5 (1)												
	ju4(2)	WT	W2 (2)												
	ju5(1)	WT	W1 (1)												
	ju6(4)	WT	W2 (3) / W5 (1)												
A. lwoffii	lw2(1)	WT						1							
	lw3(1)	WT	W2 (1)												
	lw4(3)	WT		1						2					
	lw5(1)	CT CEF									1				
	lw6(1)	WT													1
	lw7(3)	WT		1					1					1	
	lw8(14)	SUL											1		
		WT						3			2	1	7		
A. parvus	pa2(1)	WT	W1 (1)												
	pa3(5)	WT				4									
		GEN						1							
	pa4(1)	WT	W1 (1)												
	pa5(1)	WT	W1 (1)												
	pa6(4)	TET	W2 (2) / W5 (1)												
		WT	W2 (1)												
A. pittii	pi2(9)	STR		3					1	1				1	
		AML STR							1					2	
A. tandoii(94%)	ta2(1)	WT	W3 (1)												
	ta3(1)	WT	W1 (1)												
A. tjernbergiae(94%)	tj2(4)	STR			2			2							
	tj3(1)	WT	W3 (1)												

[a]Site – 0 refers to WTP; 1-11 refers to household taps; H refers to health care unit tap

[b] WT, wild type; the indication of antibiotic means that a non-wild phenotype was observed; AML, amoxicillin; GEN gentamicin; CIP, ciprofloxacin; SXT sulphamethoxazole/trimethoprim; TET, tetracycline; CP, cephalothin; MER, meropenem; CEF, ceftazidime; TIC, ticarcillin; CT, colistin sulphate; SUL, sulphamethoxazole; and STR, streptomycin

[c] W1, surface water; W2, alluvial wells; W3, after ozonation; W5, before a re-chlorination point.

Table 4. Percentage of WTP and tap water isolates with non-wild phenotypes for the different antibiotics tested

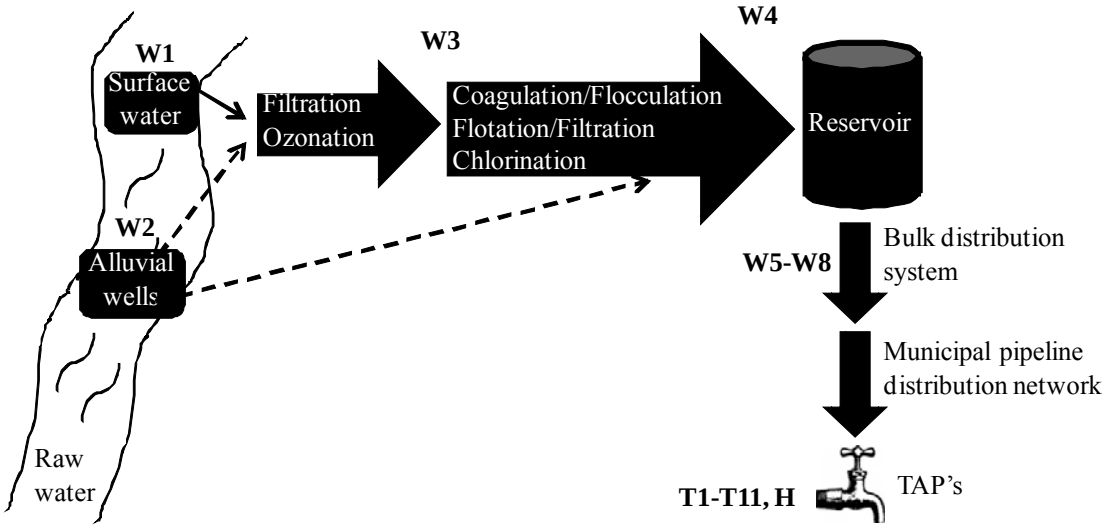
	Epidemiological cutoff values (mm) (n independent determinations)										
	AML <12mm£ (244)	**GEN <15mm* (135)**	**CIP <20mm£ (370)**	**SXT <16mm£ (370)**	**TET <18mm£ (244)**	**MER <20mm£ (370)**	**CEF <14mm£ (244)**	**TIC <19mm£ (244)**	**CT <13mm£ (244)**	**SUL <15mm£ (244)**	**STR <15mm£ (244)**
Site (n.º isolates)	**Percentage of non-WT (%)**										
WTP (28)	$7.1^{9\text{-}11}$	0	0	0	$10.7^{10\text{-}15}$	0	3.6^{0}	0	$7.1^{10\text{-}12}$	0	0
Tap (90)	$4.4^{9\text{-}11}$	1.1^{14}	0	1.1^{0}	1.1^{14}	1.1^{17}	0	1.1^{12}	0	3.3^{0}	$14.4^{8\text{-}11}$

£, value determined in this study; * EUCAST value;

AML, amoxicillin; GEN gentamicin; CIP, ciprofloxacin; SXT sulphamethoxazole/trimethoprim; TET, tetracycline; CP, cephalothin; MER, meropenem; CEF, ceftazidime; TIC, ticarcillin; CT, colistin sulphate; SUL, sulphamethoxazole; and STR, streptomycin

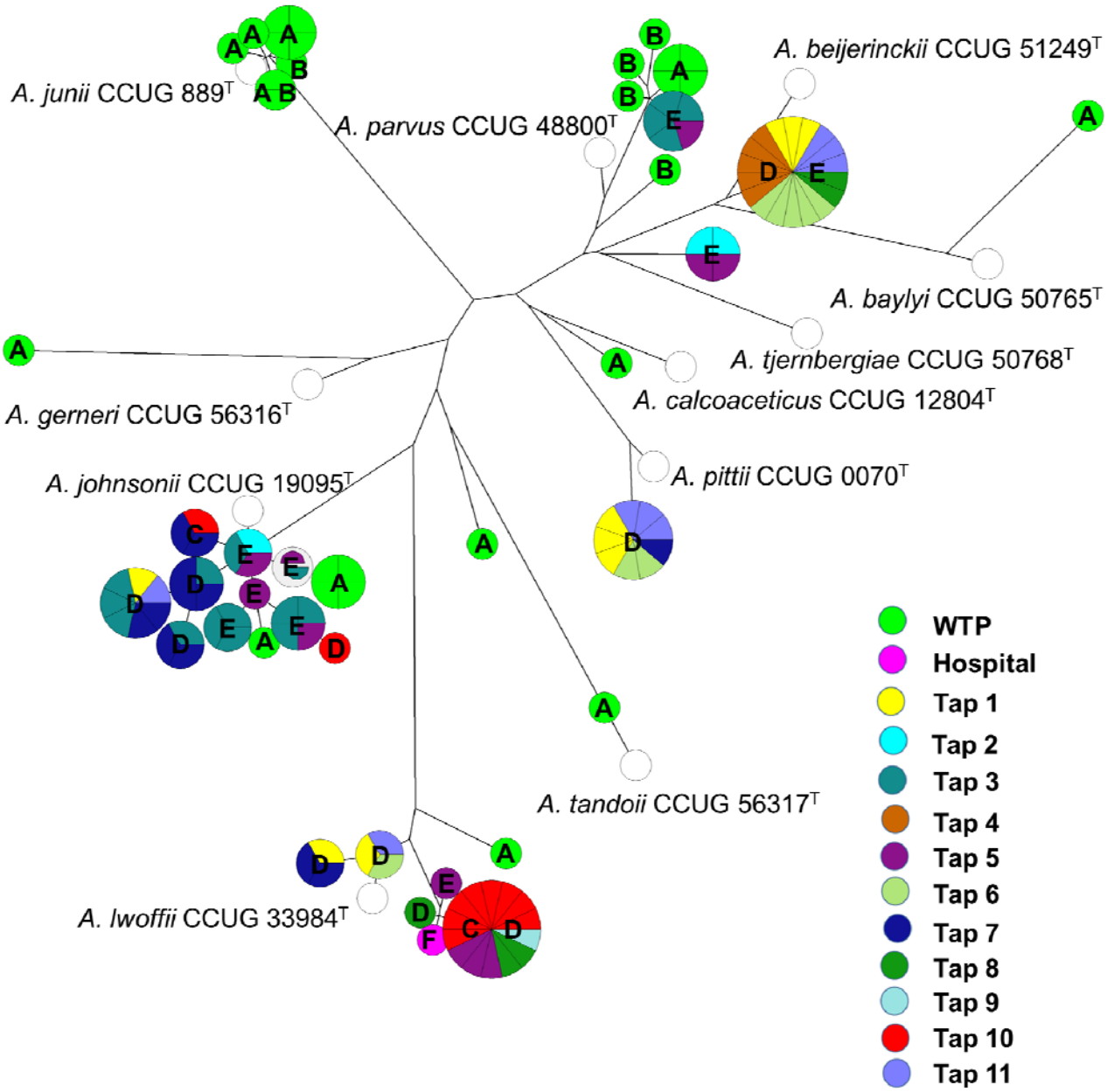
Superscripts refer to the range of non-wild inhibition zones values observed (mm).

Shadowed cells correspond to significant differences ($p<0.05$)



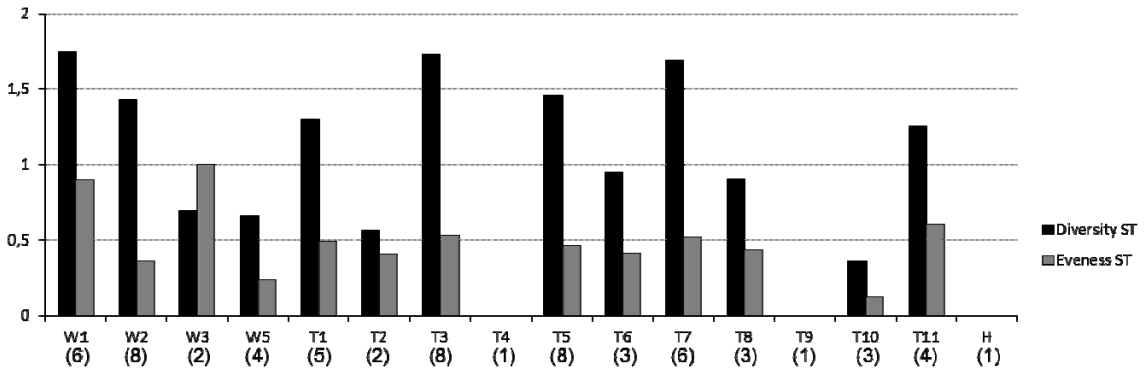
678

679 **Fig. 1** Schematic representation of the process of drinking water production and
680 distribution analysed in this study. The locations of water sampling are indicated as W1-
681 W8 (water treatment plant and distribution system) and T1-T11 and H (taps).



682
683 **Fig. 2** Maximum likelihood tree of the water isolates and the type strains of
684 *Acinetobacter* spp., constructed on the basis of concatenated *rpo*B, *rec*A and *gyr*B
685 sequences with the isolation site indicated. Each circle represents a different ST. The
686 circle size is proportional to the number of isolates with that ST. White circles indicate
687 the type strains. The letters within the circles refer to the sampling date: **A** = November
688 2007; **B** = September 2009; **C** = April 2009; **D** = July 2009; **E** = October 2009; **F** =
689 June 2008.

690



691

692 **Fig. 3** Diversity and evenness indices, assuming each sequence type as an operational
693 taxonomic unit. The values of richness (number of different sequence types) is indicated
694 below the legend of the sample. Legend details as in Table 3