Background:
Over the last decade hospital-acquired infections due to Acinetobacter baumannii are increasing worldwide. Clinical isolates of A. baumannii are usually multi-drug resistant (MDR), with resistance to carbapenems increasing drastically in Croatia, from 10% in 2008 to 82% in 2014 [1]. The most important mechanism of carbapenem resistance in A. baumannii is the enzymatic hydrolysis mediated by oxacillins encoded by blaoXA genes [2].

Although A. baumannii has been isolated from patients and hospitals during outbreaks, how this pathogen is introduced into the hospital environment remains incompletely understood. Crucial questions regarding the epidemiology of A. baumannii are not known: are the infected patients and hospital environment the only sources of A. baumannii, at which extent A. baumannii are released from hospitals in nature, do they survive or even multiply in nature, do they have natural habitat outside hospitals.

Here we report the finding of MDR carbapenem resistant A. baumannii in treated municipal wastewater which is related to clinical isolates.

Material/methods:
The isolate was recovered from the effluent of the secondary type of wastewater treatment plant of the City of Zagreb (Fig. 1) where the municipal wastewater treated consists of domestic, industrial, hospital and storm waters. The composite 24h sample of the effluent wastewater was collected on April 2014 in sterile glass bottle and analysed within 2h. The wastewater sample was concentrated on sterile membrane filters of pore size 0.45µm after dilution in sterile pentone water.

Results:
By phenotypical analyses and Vitek 2 system the isolate named EF2 was confirmed as A. calcoaceticus- baumannii complex. MALDI-TOF MS analysis gave the reliable score value of 2.352 identifying it as A. baumannii (Table 1). Phylogenetic analysis of the rpoB gene fragment confirmed the identity as A. baumannii and showed 100% sequence ID to the clinical isolates (Fig. 3).

Isolate was susceptible to amikacin, trimethoprim- sulfamethoxazole and colistin, but resistant to carbapenems (imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin), amnoglycosides (gentamicin, tobramycin) and therefore could be classified as MDR (Table 2).

Amplification of blaoXA genes by multiplex PCR and sequencing confirmed the presence of intrinsic blaoXA-46 and acquired blaoXA-46 genes. Phylogenetic analyses of blaoXA genes from environmental isolate showed association with those previously described from clinical isolates (Fig. 4).

Isolate multiplied in water up to 50 days of monitoring at 16.7°C when its number was 9% higher than initial number (Fig. 5).

Table 1. Bruker Daltonik MALDI Biotyper classification results for analyte EF2.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Phenotypic Pattern</th>
<th>ScoreValue</th>
<th>NCBIIdentifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii (ATCC 19606)</td>
<td>++</td>
<td>3042</td>
<td>NC_007240.1</td>
</tr>
<tr>
<td>Acinetobacter baumannii LMG 994</td>
<td>++</td>
<td>2.352</td>
<td>470</td>
</tr>
<tr>
<td>Acinetobacter baumannii DSM 30007T</td>
<td>++</td>
<td>1.674</td>
<td>470</td>
</tr>
<tr>
<td>Acinetobacter baumannii UFL 1323</td>
<td>++</td>
<td>1.802</td>
<td>42</td>
</tr>
<tr>
<td>Acinetobacter baumannii KV 470</td>
<td>++</td>
<td>2.224</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. MIC values of tested antibiotics for the A. baumannii isolate EF2. *MEM, meropenem; IPM, imipenem; LUX, levofloxacin; CP, ciprofloxacin; TOB, tobramycin; GEN, gentamicin; AKM, amikacin; SXT, trimethoprim-sulfamethoxazole; CST, colistin.* resistant according to EUCAST criteria.

<table>
<thead>
<tr>
<th>MEM (mg/L)</th>
<th>IPM (mg/L)</th>
<th>LUX (µg/mL)</th>
<th>CP (µg/mL)</th>
<th>TOB (µg/mL)</th>
<th>GEN (µg/mL)</th>
<th>AKM (µg/mL)</th>
<th>SXT (µg/mL)</th>
<th>CST (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;32</td>
<td>&gt;8</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

Material of antibiotics (mg/L)

Acknowledgements:
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References:

1 University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia; 2 Institute of Public Health of Split and Dalmatia County, Split, Croatia; 3 Zagreb Wastewater - Management and Operation Ltd., Zagreb, Croatia.

Conclusion:
MDR A. baumannii recovered from treated municipal wastewater is most probably of clinical origin and is able to survive in environment outside hospital.

Figure 1: Municipal wastewater treatment plant of the City of Zagreb.

Figure 3: Phylogenetic tree (NJ method, number of differences) constructed on the basis of rpoB gene representing the molecular identification of A. baumannii isolate. GenBank accession numbers are given next to the name of each strain. Moraxella catarhalis rpoB gene sequence was used as an outgroup to root the tree. Black dots represent the sequences of strains analysed in this study.

Figure 4: Unrooted phylogenetic tree (NJ method, number of differences) constructed on the basis of blaoXA genes encoding OXA-type carbapenemases. GenBank accession numbers are given next to the name of each strain. Gene sequences of blaoXA-46 type are marked with black dots while the black triangles denote blaoXA-23 type gene sequence.

Figure 2: Pure culture of presumptive A. baumannii grown on CHROMagar Acinetobacter. Colonies were large, circular, convex, smooth, red with a paler central area.

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Figure 5: Survival of A. baumannii isolate EF2 recovered from effluent wastewater in the autoclaved effluent wastewater during 50 days. Average values and standard deviations of triplicate measurements are presented.

Figure 6: Pure culture of presumptive A. baumannii grown on CHROMagar Acinetobacter. Colonies were large, circular, convex, smooth, red with a paler central area.