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1 **Distribution of *Asellus aquaticus* and microinvertebrates in a non-chlorinated**
2 **drinking water supply system – effects of pipe material and sedimentation**

3

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8

9

10 **Abstract**

11 Danish drinking water supplies based on ground water without chlorination were investigated for
12 the presence of the water louse, *Asellus aquaticus* and microinvertebrates (< 2 mm). In total 52
13 water samples were collected from fire hydrants at 31 different locations, and two elevated tanks
14 (6,000 and 36,000 m³) as well as one clean water tank at a waterworks (700 m³) were inspected.
15 Several types of invertebrates from the phyla: arthropoda, annelida (worms), plathyhelminthes
16 (flatworms) and mollusca (snails) were found. Invertebrates were found at 94 % of the sampling
17 sites in the piped system with *A. aquaticus* present at 55 % of the sampling sites. Populations of *A.*
18 *aquaticus* were present in the two investigated elevated tanks but not in the clean water tank at a
19 waterworks. Both adult and juvenile *A. aquaticus* (length of 2-9 mm) were found in tanks as well as
20 in pipes. *A. aquaticus* was found only in samples collected from two of seven investigated
21 distribution zones (zone 1 and 2), each supplied directly by one of the two investigated elevated
22 tanks containing *A. aquaticus*. Microinvertebrates were distributed throughout all zones.
23 Comparisons with data from samples collected in 1988-89 showed that the distribution pattern of *A.*
24 *aquaticus* had not changed considerably over 20 years. Centrifugal pumps have separated the
25 distribution zones during the whole period and may have functioned as physical barriers in the
26 distribution systems, which large invertebrates such as *A. aquaticus* could not pass alive. Another
27 factor characterising zone 1 and 2 was the presence of cast iron pipes. The frequency of *A.*
28 *aquaticus* was significantly higher in cast iron pipes than in plastic pipes. *A. aquaticus* caught from
29 plastic pipes were mainly single living specimens or dead specimens being transported passively
30 through by the water flow, while cast iron pipes provided an environment suitable for relatively large
31 populations of *A. aquaticus*. Sediment volume for each sample was measured and the study
32 described for the first time that the correlation between presence of living *A. aquaticus* and
33 sediment volume is not simple but rather expressed by a minimum sediment value of approximately

34 100 ml/m³ sample. Presence of *A. aquaticus* was not correlated to turbidity of the water.
35 Measurements by ATP, heterotrophic plate counting and Colilert® showed that the microbial
36 quality of the water was high at all locations with or without animals. Four other large Danish
37 distribution companies were additionally sampled (nine pipe samples and one elevated tank), and
38 invertebrates were found in all systems, three of four containing *A. aquaticus*, indicating a
39 nationwide occurrence.

40

41 Key words: invertebrates, microbial quality, distribution system, cast iron, water storage tank

42

43 **1. Introduction**

44 Invertebrate animals are present in drinking water distribution systems worldwide. In tropical and
45 subtropical countries, some species of invertebrates can act as secondary hosts for parasites and
46 thereby pose a serious health risk to consumers (Evins 2004). In temperate areas, the presence of the
47 animals is largely regarded as an aesthetic problem (van Lieverloo et al. 2002). However, previous
48 studies have shown that invertebrates such as crustaceans and nematodes can harbour bacterial
49 pathogens and potential pathogens e.g. *Escherichia coli* (indicator organism for faecal
50 contamination) (Bichai et al. 2009), *Salmonella livingstone* (Levy et al. 1984) and *Campylobacter*
51 *jejuni* (Schallenberg et al. 2005) and may play a role in the survival of these organisms in drinking
52 water systems. The Danish water supply systems are based solely on ground water without
53 chlorination, which may increase the risks of growth of bacteria and biofilm formation in the water
54 pipes (Martiny et al. 2003) that may serve as a food supply for animals in the system. The absence
55 of hygienic barriers between waterworks and consumers in terms of chlorination increases the focus
56 on any potential carrier of pathogens such as e.g. invertebrates.

57 The abundance of invertebrates in distributed drinking water is a source of consumer
58 complaints and the supply companies highly desire to control the invertebrate abundance. Well
59 established sampling methods have been developed in the Netherlands to assess the abundance of
60 most invertebrate taxa in distribution systems, and a two-year survey has confirmed the wide
61 abundance of invertebrates (van Lieverloo et al. 2004). However, studies on the controlling
62 parameters for the distribution of invertebrates on full scale distribution systems are still lacking. In
63 order to obtain and distribute biostable drinking water, biostable materials are needed (van der
64 Kooij et al. 1999) and it has therefore been suggested that pipe material may influence the
65 occurrence of invertebrates (van Lieverloo et al. 2002). This hypothesis has not been tested on a full
66 scale distribution system, nor has the correlation to sedimentation in the pipes and turbidity of the
67 water. Van Lieverloo et al. (2002) suggest that multiplication of invertebrates in distribution
68 systems depends on the presence of biofilms and sediment and it is known that keeping the pipes
69 clean by e.g. flushing diminishes the amount of invertebrates in the system (Levy 1990, van
70 Lieverloo et al. 1998). The water consumption in Odense has dropped approximately 40 % since
71 1990 with a similar tendency nationwide, which enhances the risk of high sedimentation rates in
72 water pipes constructed for higher flows.

73 The water louse, *Asellus aquaticus*, is present in water distribution systems globally
74 (Australian Government 2004, Gauthier et al. 1999, Gray 1999), which often causes consumer
75 complaints (Walker 1983 and pers. obs.) due to its size, which makes it visible to the naked eye.
76 Another nuisance is discoloration of the water by the faeces of *A. aquaticus* (pellets). A survey from
77 the Netherlands showed that though *A. aquaticus* was not the most abundant of invertebrates
78 present in water distribution systems, most of the invertebrate biomass (86%) was formed by *A.*
79 *aquaticus* (van Lieverloo 1998).

80 The aims of this study were, a) to implement methods to examine the distribution of
81 invertebrates in a drinking water system with special emphasis on *A. aquaticus*, b) to investigate the
82 spatial distribution of *A. aquaticus* in different pressure zones and c) to identify factors influencing
83 or being influenced by the presence of *A. aquaticus* with special emphasis on pipe materials,
84 sedimentation, turbidity and microbial water quality.

85

86 **2. Material and methods**

87 *2.1. Locations*

88 The investigated water supply system in Odense, Denmark supplies approximately 150,000 people,
89 via a distribution system with 1,000 km of pipes and a total pipe volume of 40,000 m³. The supply
90 company distributes slightly more than 10 million m³ per year with an average flow velocity in the
91 pipes of 0-0.5 m/s. Hence the average residence time is two days but varies from 1 to 14 days. The
92 majority of pipes are PVC pipes (46%) or PE/PEM pipes (33%), while 20 % of the pipes are
93 concrete, asbestos cement or ductile iron pipes (Table 1). The remaining cast iron pipes (1 %) are
94 currently being replaced by plastic pipes. The supply system is divided into 11 pressure zones of
95 which zones 1-8 were sampled. Although connected, the pressure varies in the different zones,
96 which are separated by centrifugal pumps. The supply network is constructed after a finger
97 principle, which means that it is branched and has a unidirectional flow, hence terminating at the
98 consumers. The transmission network on the other hand is designed as a ring system in order to
99 obtain security of supply. The raw water is ground water treated only by aeration/stripping and
100 biological rapid sand filtration, and distributed without the use of chlorination. Main water quality
101 parameters are presented in Table 2.

102

103 *2.2. Sampling from pipes and in clean water tanks*

104 Water samples from pipes were collected by flushing from above ground fire hydrants. Before each
105 sampling, the part of the hydrant above the water main was flushed with tap water to remove
106 terrestrial animals living in the water free part of the hydrant. Clean water (10-20 L) was poured
107 into the hydrant and pumped out through a drainage valve with a manual pump. For sampling a
108 flowmeter and a fire hose were attached to the hydrant and the water was flushed directly into
109 transparent single-use plastic bags in 1 m³ containers. The flowmeter was cleansed after each
110 sampling and a fresh pre-rinsed fire hose was used at each site. No water was discharged by pre-
111 flushing in order to be able to detect invertebrates inhabiting dead ends by the hydrants.

112 At each site samples were obtained by flushing 1 m³ at maximum obtainable flow
113 (turbulent flow). The sampled volume was measured per time unit in order to calculate the flow
114 velocity, and the Reynolds numbers were reported. Samples were obtained from 31 locations. To
115 avoid public interest, filtration on the street at the sampling point was not used but all samples were
116 transported to the waterworks and slowly filtered (5-10 L/min) successively through two nets with
117 mesh sizes of 500 and 100 µm. To avoid contamination from one sample to another the nets were
118 cleansed with tap water at high flow.

119 Reproducibility was investigated at three locations where sampling was repeated one
120 or two times with varying time intervals (Table 3).

121 Three water tanks: one 700 m³ clean water tank of a waterworks and two elevated tanks (elevated
122 tank 1 containing 36,000 m³ and elevated tank 2 containing 6,000 m³) were emptied and the floors
123 were carefully inspected. In the elevated tank 1, 20 random samples (each covering 0.35 m²) were
124 taken on the floor in half of the tank. In the other half of the tank the flush channel (30 m²) in the
125 length of the tank was sampled by sucking up the animals with 10 ml pipettes.

126 *Asellus aquaticus* was easily visible in the 500 µm net samples, while 1-5 ml sediment per
127 sample from 100 µm net samples and samples from clean water tanks were examined by stereo

128 microscopy with a Protec digital camera (16x11.3x0.63-4.0 magnification). Invertebrates were
129 identified, counted and measured (head to tail).

130 In order to investigate whether the occurrence of invertebrates in the drinking water
131 supply was nationwide, additional samples were taken from four large Danish water supply systems
132 during March - December 2009. Three times three samples were obtained from cast iron pipes
133 (Aarhus Water Ltd, Aalborg Supply, Water Ltd and TRE-FOR Water Ltd) by flushing and one
134 sample was collected by visual inspection in an empty elevated tank (Copenhagen Energy Ltd).

135

136 *2.3. Validation of sampling from pipes*

137 Prior to the main sampling rounds, sampling efficiency was studied at varying flow velocities, with
138 swabbing applied, with cut out pieces of pipes and filtration with various mesh sizes. Up to three
139 samples were taken at low laminar flow (Reynolds numbers $< 2,100$) as well as up to three samples
140 at maximum obtainable flow (turbulent flow, Reynolds numbers $> 2,100$) at each locality. After
141 sampling, 150 meters of plastic pipe were swabbed with a foam sponge and finally two meters of
142 pipe were cut out for visual inspection. Swabbing was not possible in cast iron pipes due to scaling
143 but two meters of pipe were cut out for visual inspection. Four mesh sizes were tested for filtration
144 of the water samples (500, 100, 20 and 10 μm).

145

146 *2.4. Analyses*

147 **Bacterial analyses:** Biofilm samples were collected from the inner pipe surfaces of the cut out pipe
148 pieces by scraping of biofilm from 10 cm^2 with a cotton bud. Three scrapes were taken from the
149 plastic pipe (one before and two after swabbing with a sponge). Three samples were taken from two
150 pieces of one meter cast iron pipes (one from the end, one from the middle and one from a vent).
151 Each cotton bud was kept cold in 10 ml sterile water until 50 μl of the suspension was spread on

152 R₂A and 1 ml was spread on yeast extract agar plates within 24 hours and incubated 14 days at 20°
153 C and 22° C. Regular bacterial control measurements by HPC (heterotrophic plate counts on yeast
154 extract agar) at 22° C and 37° C as well as Colilert® on the supply system were conducted by
155 Eurofins. Sediment samples from the 36,000 m³ elevated tank 1 were investigated for bacterial
156 numbers by R₂A colony count 20° C, yeast colony count 22° C and 1 – 5 *Asellus aquaticus* per
157 sample at randomly chosen samplings were crushed with a mortar and analysed for *Escherichia coli*
158 and other coliform bacteria by Colilert®. ATP measurements on the sediment were conducted on an
159 Advance Coupe (Celsius, Landgraaf, The Netherlands) with a Celsius kit.

160 **Iron and Manganese:** Sediment from the elevated tank 1 was analysed for content of iron and
161 manganese by absorption flame spectrometry after acid digestion with 14M HNO₃ and filtration
162 (DS259 2003).

163 **Turbidity:** After settling for a minimum of two hours, 5 liters of sample were transferred to a
164 plastic container. Following 5 sec. of shaking, turbidity was measured in triplicates on a Hach
165 2100N Laboratory Turbidimeter. Repeated measurements were made on all samples when only 200
166 L of water sample remained in the 1 m³ container. Turbidity readings on the initial water were in
167 accordance with the repeated measurements.

168 **Sediment volume:** Sediment remaining in the 100 and 500 µm filters and sediment scraped from
169 the 1 m³ plastic bags were stored in glass bottles. After sedimentation for a minimum of seven days,
170 the total sediment volume of all three fractions was measured.

171

172 Statistical analyses were performed using R software (R Development Core Team 2010).

173

174 **3. Results and discussion**

175 *3.1. Validation of sampling methodology*

176 Sampling at different flow rates revealed that only microscopic invertebrates and oligochaete
177 worms were flushed out at laminar flow (Reynolds numbers $< 2,100$). Highly turbulent flow
178 (Reynolds numbers $>25,000$) was necessary to flush out *Asellus aquaticus*. When a pipe was
179 swabbed with a sponge following sampling, it was revealed that even after flushing at highly
180 turbulent flow both *A. aquaticus* and microscopic invertebrates were still present in the pipes. In a
181 previous study with flushing at 1.0 m/s, the removal efficiencies of different invertebrate groups
182 varied between 30 % and 75 % assuming a complete removal by extensive cleaning (high velocity
183 flushing and swabbing with 3 consecutive swabs) after sampling. Mains couplings though, proved
184 to be hide-outs for *A. aquaticus* out of reach for practical sampling methods (van Lieverloo et al.
185 2004). In the present study additional invertebrates were not found in the cut out piece of plastic
186 pipe nor in the cast iron pipe but this may be due to the time consuming process of cutting the pipes
187 during which the animals may escape.

188 In studies operating with fixed flows (e.g. van Lieverloo et al. 2004), the sampling procedure is only
189 applicable on pipes within a certain interval of pipe diameters since flow velocities depend on the
190 main diameters. In this study pipes with diameters from 63 to 500 mm were sampled. In order to
191 apply the method to all pipe sizes a novel approach using Reynolds numbers was adopted, which
192 allows for expressing the actual turbulence that the invertebrates experience while the pipes are
193 being flushed.

194 The 10 μm mesh clogged instantly, and the 20 μm mesh net clogged frequently and were only used
195 in the methodology studies. Van Lieverloo et al. (2004) found that 100 μm nets retained 53 – 100 %
196 of the taxa with copepod larvae and nematodes being the hardest to retain. A 20 μm mesh could be
197 used to obtain greater accuracy on the quantification on microinvertebrates but for the purpose of
198 this study, processing of more samples was favored. After implementation of the methodology, all
199 subsequent sampling was done at maximum obtainable flow. Sampling size of 1 m^3 was chosen as

200 the standard sample size due to prioritisation of the quantity of sampling localities, though this
201 volume is most likely to be too small to identify all positive samples. This is in accordance with a 2-
202 year survey in the Netherlands, where a sample volume of 1 m³ was recommended due to
203 applicability (van Lieverloo et al. 2004). The low filtration rate of 5-10 L/min minimised injuring
204 the invertebrates but damage during sampling may had led to an underestimation of the number of
205 samples containing living *A. aquaticus*.

206 Random sampling in the first half of the 36,000 m³ elevated tank 1 yielded only one *A.*
207 *aquaticus* in total from 20 random samples covering a total area of 7 m². *A. aquaticus* was not
208 randomly distributed on the floor of the tank but gathered in remaining pools of water. In the second
209 half of the tank >200 *A. aquaticus* were sampled from an area of 30 m² in the flush channel cutting
210 transversely through the tank with remaining water. The optimal sampling method in tanks was
211 inspection of the entire floor, which was done in the 700 m³ and the 6,000 m³ tanks. When size does
212 not allow this method samples should be collected from flush channels and similar low lying areas
213 with water remaining.

214

215 3.2. Reproducibility of flushing pipes

216 Three locations were sampled two or three times (Table 3). At site 1, no *Asellus aquaticus* was
217 found during the first sampling, though 3 m³ were flushed out at highly turbulent flow (Reynolds
218 number: 100,000, flow: 1.1 m/s). Microscopy of the flushed out sediment revealed a high number of
219 *A. aquaticus* pellets. When sampling at the same site approximately one year later, two *A. aquaticus*
220 were caught in 1 m³ of flushed out water, which indicates that *A. aquaticus* was present or had been
221 present recently at site 1 during the first sampling and that the population size remained low over
222 time. At the sites 9 and 15, *A. aquaticus* were caught at all samplings at higher as well as lower
223 numbers per m³ than at the previous sampling. At a sampling conducted less than two months after

224 the first sampling at site 9 the caught number of *A. aquaticus* was raised from 9/m³ to 16/m³, hence
225 there was no indication of *A. aquaticus* being removed from the location on a long term scale by
226 sampling at maximum obtainable flow (Reynolds number of 84,000).

227

228 3.3. Occurrence of invertebrates in pipes and clean water tanks

229 Invertebrates within the phyla: arthropoda, annelida (worms) and plathyhelminthes (flatworms)
230 were found in the drinking water distribution system (Fig. 1). The observed invertebrates are all
231 commonly found in drinking water distribution systems (Evins 2004, van Lieverloo et al. 2002). A
232 land slug was observed on the wall of a clean water tank. The water louse, *Asellus aquaticus*, was
233 found at 55 % of the investigated sampling points, while 94 % of the samples contained animals
234 when microscopic invertebrates (< 2 mm) and annelida were included. The highest concentrations
235 of microinvertebrates observed were 9000 specimens/m³ sample with an average of 800
236 specimens/m³ sample. Levels of 0-959 invertebrates/m³ in drinking water leaving the water works
237 were measured in a German groundwater based supply (DVGW 1997). The concentration of *A.*
238 *aquaticus* in the positive samples varied between 1 and 14 specimens/m³ with an average of 4/m³.
239 This is slightly higher than observed in the German survey, where 1-10 *A. aquaticus*/m³ with an
240 average of 2/m³ were observed. Compared to observations decades ago these concentrations are
241 relatively low, e.g. another survey from Germany reports concentrations of *A. aquaticus* of 5-30
242 specimens/m³ (Schwarz et al. 1966).

243 *A. aquaticus* varied in size from 2 to 9 mm, which is small compared to *A. aquaticus*
244 from fresh water ponds, which can reach 20 mm. *A. aquaticus* sampled in this study were brown
245 with small eyes (Fig. 1). Characteristic *A. aquaticus* pellets (DVGW 1997, Walker 1983) were
246 observed in many sediment samples (Fig. 2) and could be used as an indication of the presence of *A.*
247 *aquaticus* populations.

248 The highest occurrence of *A. aquaticus* in the clean water tanks was found in the
249 36,000 m³ elevated tank 1. The average of *A. aquaticus* in the flush channel in half the tank was
250 7/m². In the elevated tank 2 of 6,000 m³, an equivalent of 0.1 *A. aquaticus*/m² was found on the
251 floor of the tank. *A. aquaticus*, annelida and microinvertebrates were found in both elevated tanks
252 but not in the clean water tank of the waterworks, and the water supply company had never
253 observed *A. aquaticus* nor their trails (Fig. 2) during previous controls in clean water tanks of any
254 waterworks. In a German drinking water supply system, partially supplied by ground water, *A.*
255 *aquaticus* was also found in 50 % of the samples from the distribution system, while no *A.*
256 *aquaticus* could be found at the waterworks (DVGW 1997).

257 Both of the investigated elevated tanks contained a layer of fine grained sediment.
258 There was no sediment in the 700 m³ clean water tank at the waterworks and the bacterial
259 concentration in the water in this tank was 23 CFU/ml water (HPC 22° C). The sediment from the
260 elevated tank 1 had a high content of iron (5 mg/g wet weight), manganese (1 mg/g wet weight) and
261 bacteria (76,000 +/- 2,700 pg ATP/ml wet sediment and 140,000 CFU/ml wet sediment by HPC 22°
262 C. ATP measures of water leaving the two elevated tanks before and after the periods of sampling
263 were low, varying between 1 and 6 pg ATP/ml (Corfitzen and Albrechtsen 2010).

264 Samples taken from four additional large Danish distribution companies, nationwide,
265 showed the presence of invertebrates in all investigated systems. *A. aquaticus* was found in three of
266 four systems.

267

268 3.4. Distribution between pressure zones

269 Pressure zone 1 with the elevated tank 1 contained the majority of the caught *Asellus aquaticus* (68
270 % positive samples in zone 1, Fig. 3), while microinvertebrates were present in all parts of the
271 investigated distribution system (94 % positive samples) (Fig. 4). Pressure zone 2 with the elevated

272 tank 2 had a few *A. aquaticus* positive samples, with only one living *A. aquaticus* and only an
273 average of 1 specimen per positive sample. No *A. aquaticus* were caught in the remaining zones;
274 zone 3 – zone 8 (Fig. 3).

275 Samples from 1988-89 covering the same area showed a similar distribution pattern:
276 46 % of the samples in zone 1 were positive of *A. aquaticus* while only 5 % of the samples in zones
277 2 - 8 were positive and only containing dead *A. aquaticus* (Fig. 3). Hence, the distribution of living
278 and dead *A. aquaticus* in the samples from 2008-09 was consistent with the samples from 1988-89
279 ($p = 1.000$, Fisher's exact probability test for 2x2 tables). This indicates that the populations are
280 quite stable once established or that newly entered specimens have similar habitat preferences as
281 prior populations. Previous studies conclude that the establishment of breeding populations are
282 responsible for the greatest number of invertebrates in distribution systems (Evins 2004). DVGW
283 (1997) pointed at a pipe leakage 30 years prior to the investigations as the way of entry for *A.*
284 *aquaticus*, and Small and Graves (1968) identified species in several distribution systems in the
285 1960s that according to Evins (2004) had not been recorded from natural water since the 1920s.

286 The repeated samplings (Table 3) showed that the occurrence of *A. aquaticus* was
287 independent on the season of the year. In nature, *A. aquaticus* breed between February and October
288 (Gledhill et al. 1993), while this was not the case in the investigated drinking water distribution
289 system since we found juvenile *A. aquaticus* all year round. *A. aquaticus* is known to adapt to
290 changing environments over a small spatiotemporal scale (Hargeby et al. 2004). Our observations
291 showed that populations in the drinking water system were able to increase their life span since
292 natural populations in northern Europe are recorded a life span of up to 1 year (Gledhill et al. 1993)
293 while the *A. aquaticus* collected in this study survived in culture (10° C, darkness, on sediment
294 collected from water pipes and on maple leaves) for up to 2½ years.

295 Zone 1 contained above 70 % of the cast iron pipes of the system (Table 2) and was
296 furthermore the earliest constructed zone (starting in the 19th century), which would provide plenty
297 of time for the populations to establish. Zone 2 contained the remaining cast iron pipes (Table 2). It
298 is likely that *A. aquaticus* over time has entered the distribution system in other zones than zone 1
299 and 2 but have not been able to establish breeding populations. Since zone 1 hosted a larger
300 percentage of both cast iron pipes and *A. aquaticus* than zone 2, pipe material may have the greatest
301 impact on the distribution of *A. aquaticus*. Previous literature states that a species like *A. aquaticus*
302 is recruited into the system infrequently and in small numbers but reach high numbers by successful
303 establishment and breeding (Smalls and Greaves 1968). Alternatively, the elevated tanks in zone 1
304 and 2 may have functioned as sources for *A. aquaticus* but since the 36,000 m³ elevated tank 1 has
305 been emptied, chlorinated and hosed down one year prior to sampling breeding populations may
306 also exist in the pipes. The presence of both juvenile and adult *A. aquaticus* (2-9 mm) in tanks as
307 well as in pipes supports the presence of breeding populations in both systems. Finally, a factor
308 which could inhibit migration between zones was the centrifugal pumps, which separated the zones,
309 and may have functioned as physical barriers by killing larger invertebrates with the fast rotating
310 blades.

311

312 3.5. Sedimentation

313 The availability of food plays a great part in the ability of *Asellus aquaticus* to survive and establish
314 breeding populations. The number of living *A. aquaticus* was not directly correlated to the sediment
315 volume in the samples (Pearson's test for correlation), however the vast majority of samples with
316 living *A. aquaticus* contained a substantial volume of sediment (typically more than 100 ml
317 sediment/m³ sample) (Fig. 5).

318 All samples were collected at highly turbulent flows (Reynolds numbers > 24,000). At
319 these velocities, sediment volume was not correlated to flushing flow velocities or Reynolds
320 numbers (R-values below 0.22), hence the correlation between sediment volume and *A. aquaticus*
321 positive samples cannot be explained by higher catchment rates due to more efficient flushing.
322 Regular flushing of pipe systems can reduce the occurrence of *A. aquaticus* (van Lieverloo et al.
323 1998) but, to our knowledge, no quantitative correlations have been made before. Repeated
324 sampling at three localities showed that sediment volume varied from sampling to sampling and
325 neither the sediment nor *A. aquaticus* were eliminated by sampling at maximum obtainable flow
326 (Table 3). Flushing larger water volumes than 1 m³ at maximum obtainable flow may reduce the
327 sediment to values below the threshold of approximately 100 ml sediment/m³ sample, where living
328 *A. aquaticus* was found to occur, and hence reduce their occurrence.

329

330 3.6. Pipe materials

331 To investigate the importance of pipe materials we compared samples from cast iron and plastic
332 pipes in zone 1. Although present in both pipe types significantly more samples from cast iron pipes
333 than from plastic pipes contained *Asellus aquaticus* (100 % positive samples versus 45 % positive
334 samples) ($p = 0.018$, Fisher's exact probability test for 2x2 tables) (Fig. 6). Five samples were taken
335 at localities within a 300 m radius with the same source of water supplying all five points. Three of
336 the sampled pipes were plastic pipes and the remaining two were cast iron pipes. Only the cast iron
337 pipes contained *A. aquaticus*. This indicates that cast iron pipes provide an environment suitable for
338 breeding populations of *A. aquaticus* while *A. aquaticus* caught from plastic pipes are mainly single
339 living specimens or dead specimens, which may have been transported passively through by the
340 water flow. The average concentration of *A. aquaticus* was also higher in cast iron pipes (6
341 specimens/m³) than in plastic pipes (1.6 specimen/m³) ($p = 0.037$, Mann-Whitney U-test) (Fig. 6).

342 There was no difference between the median value nor the mean value of sediment/m³
343 sample in cast iron and plastic pipes on a 5 % level of significance (Mann-Whitney U-test and a t-
344 test with log transformation of the data), hence the amount of sediment was similar in the two pipe
345 types. High sediment volumes (>100 ml sediment/m³ sample) were obtained from plastic pipes in
346 45 % of the samples but only 40 % of the fraction with high sediment volumes contained *A.*
347 *aquaticus*. Therefore the pipe type itself had a large influence on the occurrence of *A. aquaticus*,
348 which was not just caused by one pipe type accumulating more sediment than the other.
349 There may be several factors involved in making cast iron pipes a preferable habitat for *A.*
350 *aquaticus*: They provided many hiding places due to corrosion and scaling. More food, e.g. from
351 iron-oxidising and nitrite-oxidising bacteria may be available in cast iron pipes (Martiny et al.
352 2005). Finally, the cast iron pipes were old pipes (up to 90 years) providing an undisturbed
353 environment. Since all cast iron pipes were more than 62 years old at the time of sampling, there
354 was no basis for studying the effects of pipe age of cast iron pipes. For plastic pipes, the samples
355 taken in 2008-09 containing *A. aquaticus* were all but one from pipes older than 32 years. In the
356 1988-89 samples all *A. aquaticus* positive samples were from pipes, which were 17-19 years old at
357 the time of sampling. The common characteristics of these positive samples were that the pipes
358 originated from around 1970. Hence, it may merely be due to factors correlated to the specific
359 period of the construction of the system in 1970 than the pipe age itself.

360

361 3.7. Turbidity

362 The abundance of *Asellus aquaticus* did not correlate with turbidity. This was probably because
363 high turbidity values were often measured due to red iron or black manganese colloidal particles,
364 which did not sediment in spite of days of settling of the samples. Hence, since turbidity does not

365 simply reflect the amount of sediment, turbidity cannot be used for prediction of the presence of *A.*
366 *aquaticus*.

367

368 3.8. Microbial water quality

369 Over the two years of sampling heterotrophic plate counts (HPC 37° C) did not exceed 5 CFU/ml at
370 any control measurement at the sampling points. Neither were any *Escherichia coli* or other
371 coliform bacteria detected at any sampling location or in the analyses of crushed *Asellus aquaticus*.
372 This is contrary to land slugs intruding clean water tanks, which have been observed to cause
373 measurable concentrations of coliform bacteria (unpublished results).

374 Scrapes from biofilm (not sediment) in the cut out pieces of pipes showed low levels of
375 heterotrophic bacteria (below an average of 190 CFU/cm², HPC 22° C) in cast iron as well as
376 plastic pipes. At 80 % of the sampling locations, bacterial numbers measured prior to and after
377 sampling did not exceed 10 CFU/ml (HPC 22° C). The Danish guideline value of 200 CFU/ml
378 (HPC 22° C for water at the consumers tap) was exceeded at two locations. The two exceedings
379 were measured after sampling at the two sites, where pipes had been cut out and were most likely
380 generated by the pipe work. Bacterial concentrations increased from 3 CFU/ml before sampling to
381 210 CFU/ml after sampling, and from 4 CFU/ml before sampling to 220 CFU/ml after sampling.
382 There was no correlation between the distribution of *Asellus aquaticus* and heterotrophic bacteria
383 based on the regular control measurements and the microbial quality of the water in the distribution
384 system was good in the investigated zones over the two years of sampling, including locations
385 where *A. aquaticus* was caught repeatedly.

386

387 4. Conclusions

388 In conclusion, this first investigation of invertebrate occurrence in a Danish drinking water
389 distribution system showed that:

- 390 • Flushing at highly turbulent flow (Reynolds numbers > 24,000) and preferably swabbing
391 was necessary to sample *Asellus aquaticus* from drinking water pipes, but swabbing injured
392 the animals
- 393 • Juvenile and adult invertebrates (*A. aquaticus* or microinvertebrates) were present in 94 %
394 of the samples, both in the distribution system in pipes and in the clean water tanks
- 395 • Microinvertebrates were present in all parts of the distribution system, while the occurrence
396 of *A. aquaticus* was influenced by the location in the distribution system (percentage of cast
397 iron pipes, separation by centrifugal pumps)
- 398 • Data from 1988-89 samples showed that the distribution pattern of *A. aquaticus* had not
399 changed considerably over 20 years.
- 400 • Microinvertebrates were present in cast iron as well as plastic pipes
- 401 • *A. aquaticus* was present mainly in cast iron pipes and in higher concentrations than in
402 plastic pipes
- 403 • The number of living *A. aquaticus* in the samples was not directly correlated to sediment
404 volume in samples but the vast majority of samples that were positive with living *A.*
405 *aquaticus* contained a substantial volume of sediment (approximately 100 ml sediment/m³
406 sample)
- 407 • The microbial quality of the investigated drinking water distribution system was high and
408 without correlation to the presence of *A. aquaticus*

409

410 **Perspective**

411 Despite various attempts over time, total removal of invertebrates from drinking water supply
412 systems have shown close to impossible. A great nuisance to consumers is caused by larger animals
413 like *Asellus aquaticus*. The knowledge obtained from this study can be applied to control the
414 presence of *A. aquaticus* by replacing cast iron pipes with plastic pipes in areas with high
415 concentrations of *A. aquaticus*. Sediment threshold values in supply system can be used to
416 determine a feasible level of cleaning of the pipes in order to control *A. aquaticus* populations.

417

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428

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484 **Table 1. Characteristics and number of sampling sites in the various distribution zones**

| Zone | Area [km ²] | Pipes [km] | Resident pop. # | Revenue water [m ³] | Pipe material [%] | | | Samples taken # | |
|-------|----------------------------|---------------|--------------------|------------------------------------|-------------------|-----------|-------|-----------------|-----------|
| | | | | | Plastic | Cast iron | Other | Plastic | Cast iron |
| 1 | 78 | 463 | 93,567 | 5,971,911 | 74 | 2 | 24 | 11 | 8 |
| 2 | 78 | 383 | 54,467 | 2,871,174 | 81 | 1 | 18 | 5 | 2 |
| 3 | 23 | 43 | 1,624 | 83,474 | 99 | 0 | 1 | 1 | 0 |
| 5 | 16 | 22 | 1,557 | 79,535 | 96 | 0 | 4 | 1 | 0 |
| 6 | 7 | 8 | 281 | 11,040 | 93 | 0 | 7 | 1 | 0 |
| 7 | 4 | 12 | 1,805 | 84,525 | 100 | 0 | 0 | 1 | 0 |
| 8 | 2 | 5 | 208 | 9,616 | 100 | 0 | 0 | 1 | 0 |
| Total | 208 | 936 | 153,509 | 9,111,275 | 79.2 | 1.4 | 19.4 | 21 | 10 |

485
486
487**Table 2. Main water quality parameters of the supply system in Odense, Denmark**

| Water quality parameter | Measured values in Odense | Danish guideline values |
|-------------------------|---------------------------|------------------------------|
| Oxygen | 9.0-9.3 mg/l | Min. 5 mg/l |
| NVOC | 1.3-2.0 mg/l | Max. 4 mg/l |
| Temperature | 5-16°C | Max. 12°C (recommended) |
| Conductivity | 57-79 mS/m | Min. 30 mS/m (recommended) |
| Total hardness | 14-21 H degrees | 5-30 H degrees (recommended) |
| pH | 7.4-7.6 | 7.0-8.5 |
| Iron | <0.01-0.02 mg/l | Max. 0.1 mg/l |
| Manganese | <0.005 mg/l | Max. 0.02 mg/l |
| Ammonium | <0.01-0.06 mg/l | Max. 0.05 mg/l |

488
489**Table 3. Repeated samplings**

| Sample locations | Dates | 1 st sampling | | 2 nd sampling | | 3 rd sampling | |
|------------------|--------------------------------|--------------------------------|------------------------------------|--------------------------------|------------------------------------|--------------------------------|------------------------------------|
| | | <i>Asellus</i> /m ³ | sediment vol. [ml/m ³] | <i>Asellus</i> /m ³ | sediment vol. [ml/m ³] | <i>Asellus</i> /m ³ | sediment vol. [ml/m ³] |
| 1 | 07.01.08 + 24.03.09 | 0 | 180 | 2 | 200 | - | - |
| 9 | 23.10.08 + 15.12.08 + 10.06.09 | 9 | 5 | 16 | 60 | 5 | 20 |
| 15 | 15.12.08 + 16.03.09 | 9 | 170 | 3 | 300 | - | - |

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494 **Figure 1. A) Adult and juvenile *Asellus aquaticus* (Malacostraca) B) Seed shrimp (Ostracoda) C) Flatworm**
495 **(Turbellaria) D) Land slug from a clean water tank E) *Cyclops* sp. (Maxillopoda) F) *Tubifex* sp. (Clitellata) G)**
496 **Springtail (Entognatha. Photos: S.C.B. Christensen.**

497
498 **Figure 2. Traces of *Asellus aquaticus*. A) Trails on sediment in empty elevated tank. B) Pellets (faeces). The**
499 **characteristic transverse fissure is seen on some pellets. Photos: S.C.B. Christensen.**

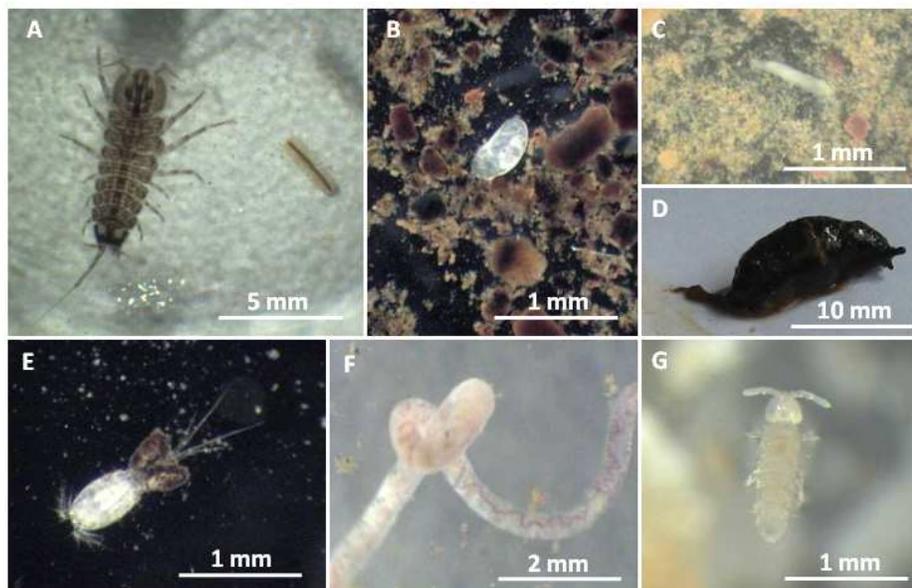
500
501 **Figure 3. Distribution of *Asellus aquaticus* in pressure zones 1-8. The distribution of living and dead *A. aquaticus***
502 **in the samples from 2008-09 was consistent with the samples from 1988-89 ($p = 1.000$, Fisher's exact probability**
503 **test for 2x2 tables). The elevated water tanks in zones 1 and 2 contained *A. aquaticus*, while none was observed in**
504 **the clean water tank in zone 8. Living *A. aquaticus* were observed in zone 1 covering a wide area while living *A.***
505 ***aquaticus* in zone 2 was found at only one sampling location. No *A. aquaticus* was observed in zones 3-8. Numbers**
506 **refer to sampling locations.**

507
508 **Figure 4. Samples containing invertebrates in distribution zone 1 and distribution zones 2-8.**

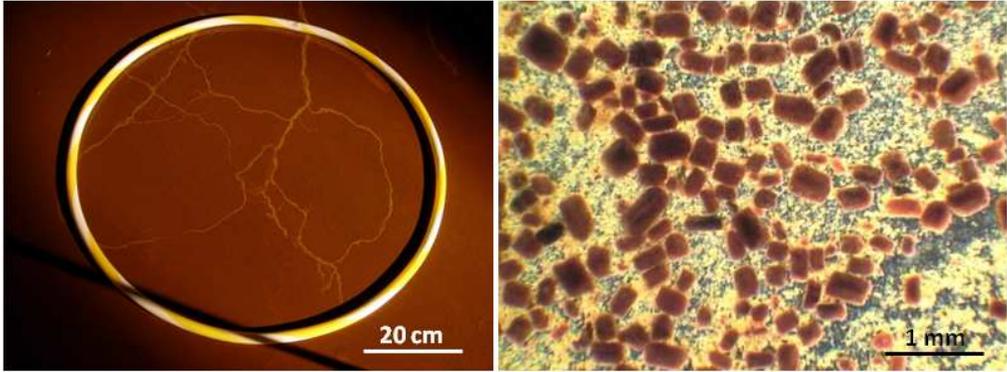
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510 **Figure 5. Numbers of living *Asellus aquaticus* and the connection to sediment volume per sample. Pointed bars**
511 **show values above 2500 ml sediment or above two *A. aquaticus*/m³ sample. The proportion of *A. aquaticus* in**
512 **samples containing >100 ml sediment/m³ sample (53%) was significantly higher than in samples containing <100**
513 **ml sediment/m³ sample (10%) ($p = 0.008$, Fisher's exact probability test for 2x2 tables)."**

514
515 **Figure 6. The distribution of samples with living *Asellus aquaticus* and dead *A. aquaticus* from 8 cast iron pipes**
516 **and 11 plastic pipes from zone 1. *A. aquaticus* was present in a significantly higher number of samples from cast**
517 **iron pipes than plastic pipes (100 % positive samples versus 45 % positive samples) ($p = 0.018$, Fisher's exact**
518 **probability test for 2x2 tables). There was a significantly higher concentration of *A. aquaticus* in cast iron pipes**
519 **6.0/m³ than in plastic pipes 1.6/m³ ($p = 0.037$, Mann-Whitney U-test).**

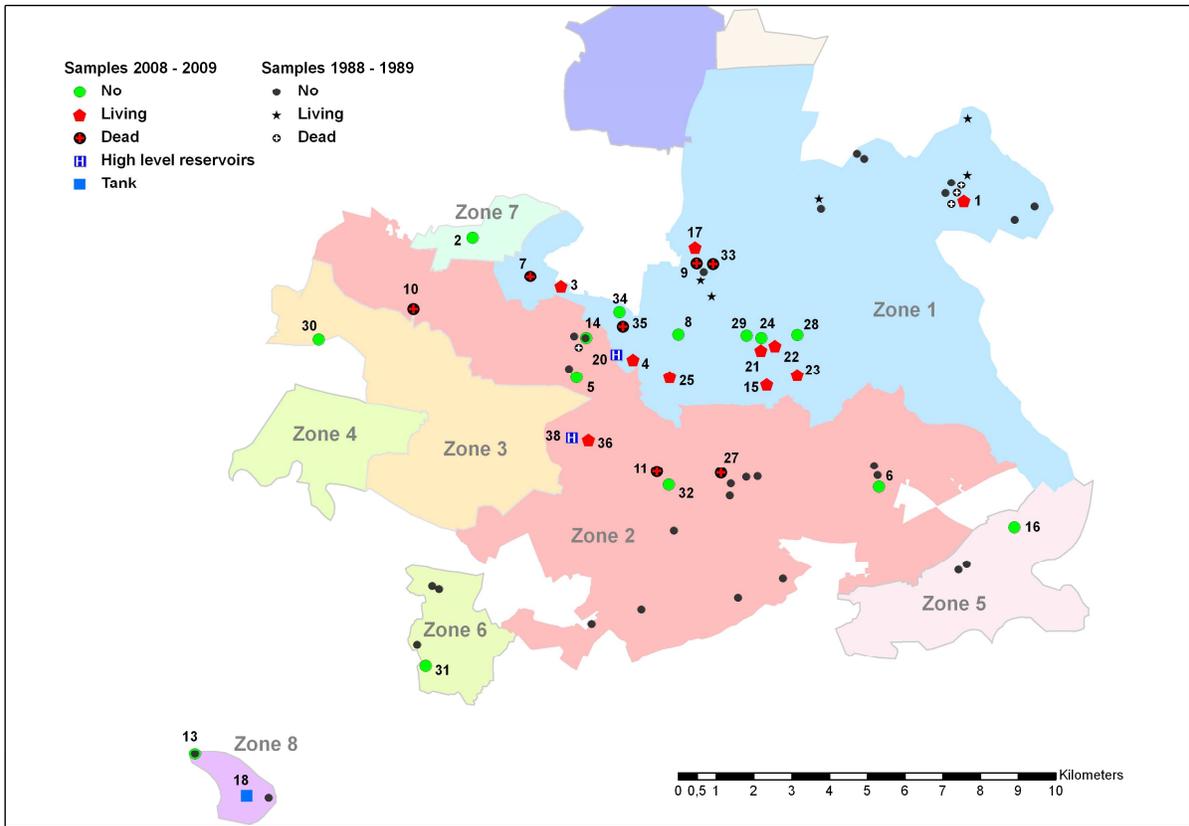
520 **Replicate samplings are removed. Dead *A. aquaticus* may be present in samples with living *A. aquaticus*.**
521



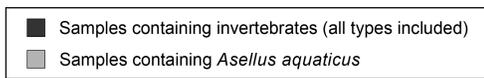
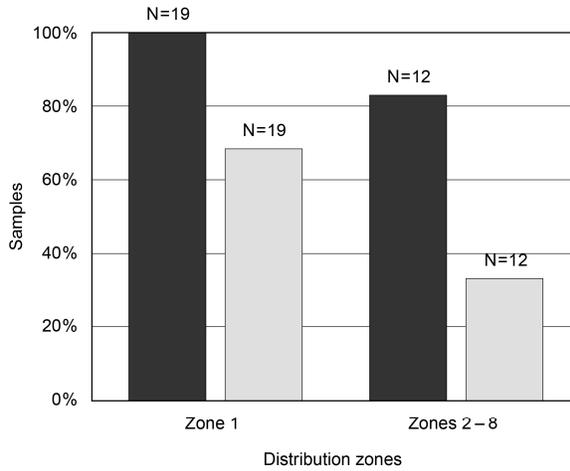
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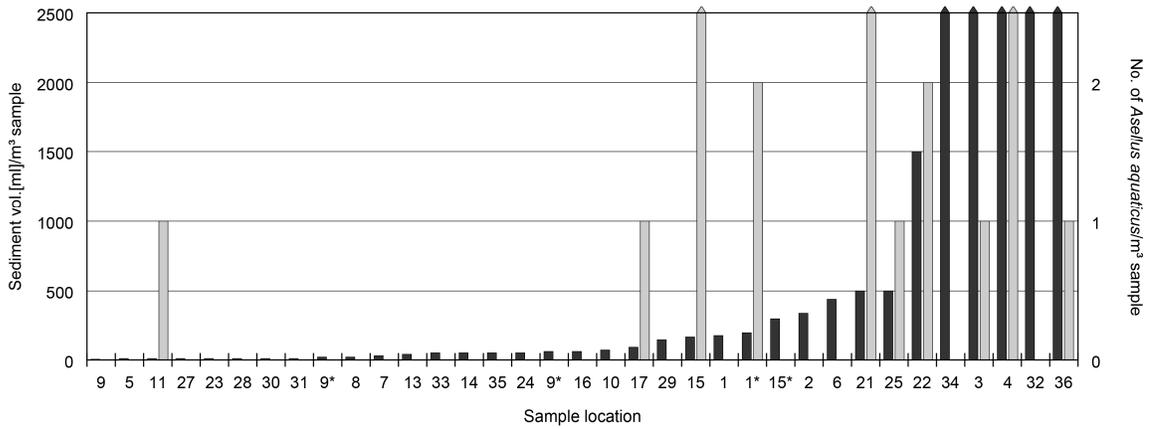
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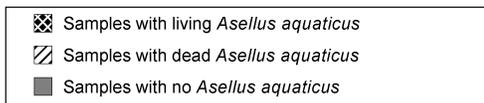
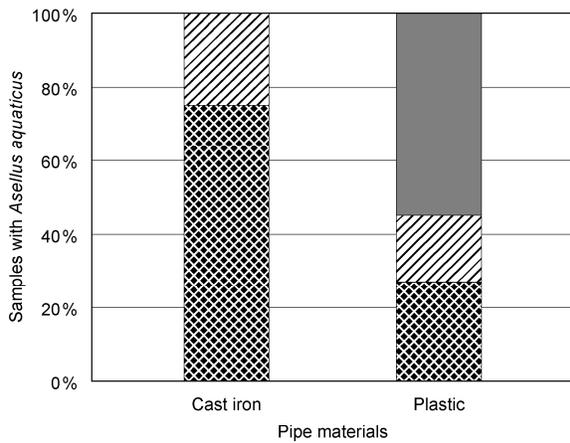
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Supplementary material

Sampling data, invertebrate concentrations and bacterial values

| Sample location | Sampling date | Pressure zone | Pipe material | Age 2010 [years] | Pipe diameter [mm] | Pipe vol./m [L] | Flow velocity [m/s] | Flow rate [L/min] | Reynolds number | Sediment volume [ml] | Turbidity [NTU] | Living <i>A. aquaticus</i> /m ³ sample | Dead <i>A. aquaticus</i> /m ³ sample | Microinvertebrates [# /ml sediment] | Before sampling HPC (Kim22) [CFU/ml] | After sampling HPC (Kim22) [CFU/ml] | Before sampling HPC (Kim37) [CFU/ml] | After sampling HPC (Kim37) [CFU/ml] | Before sampling Coliform37 [MPN/ml] | After sampling Coliform37 [MPN/ml] |
|-----------------|---------------|---------------|---------------|------------------|--------------------|-----------------|---------------------|-------------------|-----------------|----------------------|-----------------|---|---|-------------------------------------|--|---|--|---|---|--|
| 22eri | 16.03.09 | 1 | CI | 81 | 80 | 5.03 | 0.50 | 150 | 39789 | 1500 | 31 | 2 | 0 | 1 | 2 | 1 | <1 | <1 | <1 | <1 |
| 32dal | 10.06.09 | 2 | CI | 80 | 200 | 31.4 | 0.31 | 590 | 108461 | 5000 | 50 | 0 | 0 | 0 | 10 | <1 | 1 | <1 | <1 | <1 |
| 17 hav | 15.12.08 | 1 | CI | 79 | 100 | 7.85 | 1.06 | 500 | 106103 | 90 | 15 | 1 | 13 | 0 | <1 | <1 | <1 | <1 | <1 | <1 |
| 21øst | 16.03.09 | 1 | CI | 76 | 100 | 7.85 | 0.51 | 240 | 50930 | 500 | 58 | 4 | 1 | 1 | 2 | 1 | <1 | <1 | <1 | <1 |
| 35 møl | 10.06.09 | 1 | CI | 73 | 150 | 17.7 | 0.24 | 250 | 35368 | 50 | 68 | 0 | 1 | 5 | 8 | <1 | <1 | <1 | <1 | <1 |
| 9 hav | 23.10.08 | 1 | CI | 72 | 100 | 7.85 | 0.72 | 340 | 72150 | 5 | 40 | 0 | 9 | 0 | <1 | <1 | <1 | <1 | <1 | <1 |
| 9 rep | 05.12.08 | 1 | CI | 72 | 100 | 7.85 | 0.85 | 400 | 84883 | 60 | 23 | 0 | 16 | 0 | <1 | <1 | <1 | <1 | <1 | <1 |
| 9 rep | 10.06.09 | 1 | CI | 72 | 100 | 7.85 | 0.85 | 400 | 84883 | 20 | 16 | 0 | 5 | 0.5 | 3 | 5 | <1 | <1 | <1 | <1 |
| 25 hed | 16.03.09 | 1 | CI | 71 | 100 | 7.85 | 0.53 | 250 | 53052 | 500 | 86 | 1 | 5 | 1 | 1 | 3 | <1 | 1 | <1 | <1 |
| 27 mar | 16.03.09 | 2 | CI | 70 | 150 | 17.7 | 0.50 | 530 | 74980 | <10 | 25 | 0 | 1 | 2 | 4 | 4 | 2 | <1 | <1 | <1 |
| 4 got | 25.06.08 | 1 | CI | 67 | 100 | 7.85 | 0.66 | 310 | 66000 | 4000 | 16 | 3 | 0 | 0 | 3 | 210 | <1 | <1 | <1 | <1 |
| 15 vis | 15.12.08 | 1 | CI | 63 | 100 | 7.85 | 1.17 | 550 | 116714 | 170 | 23 | 9 | 0 | 0 | 9 | <1 | <1 | <1 | <1 | <1 |
| 15 rep | 16.03.09 | 1 | CI | 63 | 100 | 7.85 | 0.57 | 270 | 57296 | 300 | 13 | 0 | 3 | 1 | 2 | 1 | <1 | <1 | <1 | <1 |
| 23la ri | 16.03.09 | 1 | PVC | 52 | 100 | 7.85 | 0.74 | 350 | 74272 | 10 | 5 | 1 | 2 | 1 | 2 | 1 | <1 | <1 | <1 | <1 |
| 5 roes | 11.06.08 | 1 | BON | 49 | 500 | 196 | 0.05 | 540 | 23000 | <10 | 35 | 0 | 0 | 0 | 4 | 130 | <1 | 1 | <1 | <1 |
| 14 brø | 15.12.08 | 2 | PVC | 47 | 90 | 6.36 | 1.44 | 550 | 129682 | <50 | 97 | 0 | 0 | 5 | 18 | 40 | <1 | 5 | <1 | <1 |
| 10 kal | 23.10.08 | 2 | PVC | 45 | 110 | 9.5 | 0.99 | 520 | 100316 | 70 | 54 | 0 | 1 | 2 | 17 | 45 | 5 | <1 | <1 | <1 |
| 33gøt | 10.06.09 | 1 | PVC | 43 | 110 | 9.5 | 1.09 | 620 | 119607 | Na | Na | 0 | 1 | Na | 3 | 5 | <1 | <1 | <1 | <1 |
| 34 hav | 10.06.09 | 1 | PVC | 41 | 90 | 3.36 | 1.36 | 520 | 122608 | 3000 | 90 | 0 | 0 | 1.5 | 8 | <1 | <1 | <1 | <1 | <1 |
| 1 dalt | 07.01.08 | 1 | PVC | 40 | 90 | 6.36 | 1.1 | 420 | 99030 | 180 | 31 | 0 | 0 | 1 | 5 | <1 | <1 | 3 | <1 | <1 |
| 1 (rep) | 24.03.09 | 1 | PVC | 40 | 90 | 6.36 | 0.89 | 340 | 80167 | 200 | 26 | 2 | 0 | 1 | <1 | 4 | 5 | 2 | <1 | <1 |
| 7 spa | 23.10.08 | 1 | PVC | 40 | 90 | 6.36 | 1.21 | 460 | 108461 | 30 | 39 | 0 | 1 | 1 | 27 | 20 | 3 | <1 | <1 | <1 |
| 3 stef | 11.06.08 | 1 | PVC | 39 | 110 | 9.5 | 1 | 570 | 110000 | 3000 | 18 | 1 | 1 | 3 | 4 | 220 | <1 | <1 | <1 | <1 |
| 13 nev | 15.12.08 | 8 | PVC | 34 | 90 | 6.36 | 0.71 | 270 | 63662 | 40 | 4 | 0 | 0 | 1 | 5 | <1 | 4 | <1 | <1 | <1 |
| 36 mik | 10.06.09 | 2 | PVC | 33 | 90 | 6.36 | 1.47 | 560 | 132040 | 6100 | 60 | 1 | 0 | 1 | 10 | <1 | 1 | <1 | <1 | <1 |
| 6 vægt | 23.10.08 | 2 | PVC | 32 | 90 | 6.36 | 1.31 | 500 | 117893 | 440 | 84 | 0 | 0 | 1 | 12 | <1 | 1 | <1 | <1 | <1 |
| 16 bir | 15.12.08 | 5 | PVC | 29 | 90 | 6.36 | 2.1 | 800 | 188628 | 60 | 13 | 0 | 0 | 3 | 4 | 10 | <1 | <1 | <1 | <1 |
| 2 sand | 14.01.08 | 7 | PEM | 20 | 200 | 31.4 | 2 | 750 | 400000 | 340 | 2 | 0 | 0 | 4 | <1 | <1 | <1 | <1 | <1 | <1 |
| 24 chr | 24.03.09 | 1 | PEM | 20 | 110 | 9.5 | 0.44 | 250 | 48229 | 50 | 16 | 0 | 0 | 1 | 2 | 1 | <1 | <1 | <1 | <1 |
| 28 ejb | 24.03.09 | 1 | PE | 20 | 110 | 9.5 | 0.75 | 430 | 82953 | 10 | 11 | 0 | 0 | 1 | 2 | 1 | <1 | <1 | <1 | <1 |
| 30 dyr | 24.03.09 | 3 | PVC | 18 | 75 | 4.42 | 0.87 | 230 | 65077 | 10 | 172 | 0 | 0 | 1 | 1 | 13 | <1 | 1 | <1 | <1 |
| 8 ørst | 23.10.08 | 1 | PE | 12 | 90 | 6.36 | 1.18 | 450 | 106103 | 20 | Na | 0 | 0 | 1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 11 bir | 23.10.08 | 2 | PE | 10 | 63 | 3.12 | 2.89 | 540 | 181891 | <10 | 26 | 0 | 1 | 1 | 1 | 10 | <1 | 1 | <1 | <1 |
| 31 bry | 24.03.09 | 6 | PE | 9 | 90 | 6.36 | 0.39 | 150 | 35368 | 10 | 10 | 0 | 0 | 0 | 13 | 19 | <1 | <1 | <1 | <1 |
| 29geo | 24.03.09 | 1 | PE | 8 | 90 | 6.36 | 1.15 | 440 | 103745 | 150 | 17 | 0 | 0 | 2 | 2 | 1 | <1 | <1 | <1 | <1 |

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The samples are sorted primarily by pipe material and secondly by age. CI: cast iron, BON: bonna (concrete) PVC: poly vinyl chloride, PE: polyethylene, PEM; polyethylene medium density.