Microbiology – Part I

Microbiological water quality and DNA-based quantitative microbial source tracking

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Introduction

Escherichia coli (*E. coli)* and intestinal enterococci are used worldwide as sensitive indicators for the assessment of faecal pollution in the aquatic environment. Faecal indicators are excreted by humans and warm blooded animals in high concentrations and survive for a certain time (being indicative for days to months) in aquatic systems. Faecal pollution can be caused by point sources like discharges of sewage, from human sources or livestock enterprises and by non-point sources like pasture, urban and agricultural run-off or water fowl (KIRSCHNER ET AL. 2004). Faeces frequently contain pathogenic microorganisms like bacteria, viruses and parasites. Therefore intestinal indicator bacteria like *E. coli* and enterococci indicate the potential presence of pathogens.

Surface water, especially lotic systems, can act as highly efficient transport media for pathogenic microorganisms. Contamination with pathogens, their spread in the aquatic environment and finally their oral ingestion can cause severe outbreaks or may contribute to the background rate of endemic diseases (FARNLEITNER ET AL. 2007). For example, enterococci concentration has been shown to be a reliable factor in explaining rates of gastrointestinal illness in swimmers exposed to faecally contaminated water (U.S. ENVIRONMENTAL PROTECTION AGENCY, 1986).

Because of the hazard to humans caused by aquatic faecal pollution, strict quality regulations for water intended for irrigation, water-contact recreation (e.g. bathing), aquaculture and water for human consumption exists (e.g. BATHING WATER QUALITY DIRECTIVE, 2006/7/EC). According to the WATER FRAMEWORK DIRECTIVE (2000) protected bodies of water - like recreational waters, including areas nominated as bathing waters - can be designated. Bathing waters have to fulfil the requirements of the EU-Bathing Water Quality Directive. Measures for restoration of protected areas within common measures are necessary if not compliant to quality targets.

Faecal pollution and microbiological contamination from anthropogenic sources have been shown to be a crucial problem throughout the Danube River Basin (KAVKA & POETSCH, 2002) and impose a threat to all kinds of water uses. The river and its tributaries receive incompletely treated waste water e.g. from urban areas, animal farms and pasture leading to serious debasement of water quality. Thus, detailed knowledge on the extent and the origin of microbial faecal pollution is crucial for watershed management activities in order to maintain safe waters according to their quality targets (FARNLEITNER ET AL., 2001). However, standard faecal indicators, such as *E. coli*, do not give information about the source of faecal input (FARNLEITNER ET AL., 2006). In this respect, DNA-based quantitative microbial source tracking (QMST) techniques have shown great potential to specifically quantify human faecal pollution and thus will allow more target oriented measures in the Danube catchment area (REISCHER ET AL., 2008).

Aims and Goals

Microbiological data were collected during the Joint Danube Survey 2 (2007) along the longitudinal stretch of the River Danube from the upper section (km 2600) to the Delta (km 7) for the following goals:

- Analysis of the extent and variation of faecal pollution by microbiological standard indicators (E. coli, intestinal enterococci) along the longitudinal stretch of the River Danube, in side arms and main tributaries. Total coliforms, not entirely specific for faecal pollution, have been determined because of historical reasons (comparability to JDS1).
- Identifying hot spots of faecal pollution of the Danube River basin.
- Assessment and description of the microbiological water quality in the sections of the river and tributaries. Data were compared and water quality determined according to the faecal pollution classification scheme of KAVKA ET AL. (2006), showing 5 micro-biological water quality categories. In this Water Framework Directive harmonised system, faecal pollution levels of quality class I and II are below but quality classes III and above exceed the faecal pollution threshold level for good quality according to the EU Bathing Water Directive 2006.
- Generating an actualised microbiological water quality map based on the one-out – all-out principle
- To evaluate comparability of on board analysis, data collected were compared with data from three reference laboratories which received simultaneously taken parallel samples.
- Comparison of the data collected during JDS 2 with the data of the JDS 1 (2001).
- Application of a DNA based quantitative microbial source tracking method for all tributaries and side arms to detect and quantify (point) sources of human impact.
- Specification of recommendations and general conclusions.

Methods

Sampling and Storage

Water samples were collected from the ship in sterile 1 L Schott-flasks from all JDS 2 sampling stations with a sampling rod at a water depth of approximately 20 to 30 cm. Samples were immediately processed in the on-board laboratory, i.e. within 0.5 hours. Parallel samples (250 ml sterile one-way plastic flasks) for the three reference laboratories were taken simultaneously and stored at 4°C until use (2 h – 24 h).

The three reference laboratories were:

Germany (JDS 1 – JDS 7): Bayerisches Landesamt für Umwelt, Referat 77 - Biotestverfahren, mikrobielle Ökologie (Dir. Dipl. Biol. Willi Kopf, Dr. Margit Schade)

Austria (JDS 8 – JDS 12): Medical University Vienna, Clinical Institute of Hygiene and Medical Microbiology – Water Hygiene (Univ. Prof. Dr. Regina Sommer)

Slovakia (JDS13 – JDS 19): Water Research Institute Bratislava, Slovak National Water Reference Laboratory (RNDr. Miloslava Prokšova, RNDr. Jarmila Makovinská)

Escherichia coli / Total Coliforms [MPN/100ml] Colilert 18

Detection method: Colilert 18 (IDEXX, Ludwigsburg, Germany): Most probable number technique, 3 dilutions: 100 ml, 1 ml 0.01 ml

Incubation temperature / time: 35 ± 0.5°C / 18 - 22 hours

Analysis: UV-cabinet: blue chambers are positive for *E. coli* (**Fig. 1**)

Simultaneous detection of Total Coliforms: yellow chambers are positive for Total Coliforms under visible light

Intestinal Enterococci [MPN/100ml] ISO 7899-1, 1988

Detection method: MU/SF Microtiter plates (BIORAD, Vienna, Austria): Most probable number technique, 4 dilutions: 200µl of 1:2, 1:20, 1:200; 1:2000 sample dilutions

Incubation temperature / time: 43 ± 1°C / 36 - 40 hours

Analysis: UV-cabinet: blue chambers are positive for Enterococci

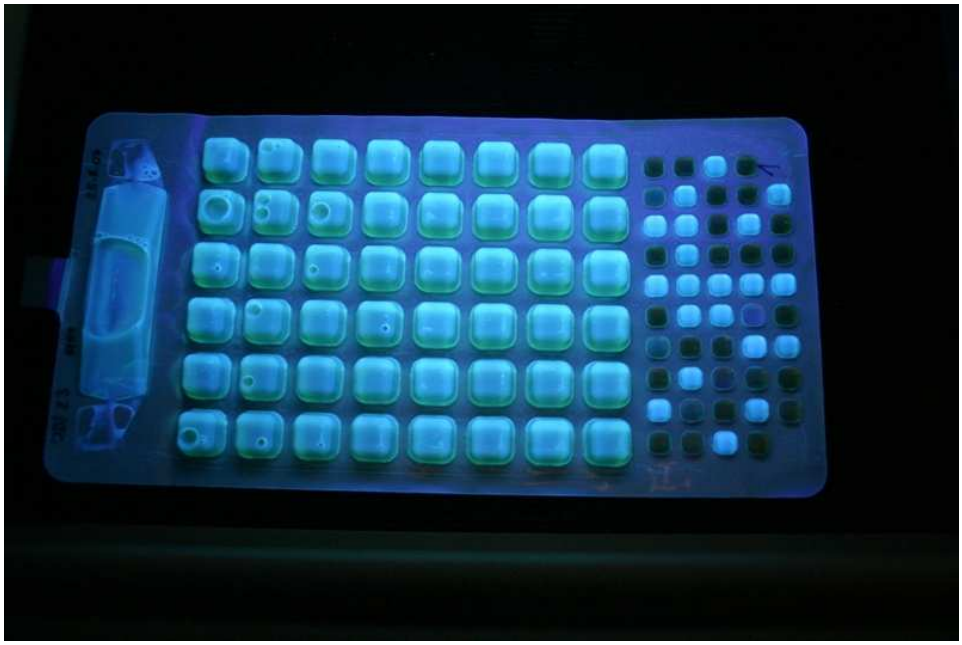

Fig. 1: Example of *E.coli* detection under UV light

Classification System

To facilitate the interpretation of data, faecal indicators were classified by a system presented in **Table 1**. The classification system of KAVKA ET AL (2006), showing 5 microbiological water quality categories and the EU BATHING WATER QUALITY DIRECTIVE 2006 were taken into consideration in this scheme in the way that faecal pollution levels of quality class I and II are below but quality classes III, IV, V exceed the faecal pollution threshold level for good bathing water quality.

Table 1: Microbiologically based classification system of water quality according to faecal pollution

Classification of faecal pollution		Class				
		I	II	III	IV	V
Parameter	Faecal pollution	little	moderate	critical	strong	excessive
Escherichia coli EC	in 100ml water	≤ 100	> 100 - 1 000	> 1 000 - 10 000	> 10 000 - 100 000	> 100 000
Intestinal Enterococci ENT	in 100ml water	≤ 40	> 40 - 400	> 400 - 4 000	> 4 000 - 40 000	> 40 000
Total Coliforms TC	in 100ml water	≤ 500	> 500 -10 000	> 10 000 - 100 000	> 100 000 - 1 000 000	>1 000 000

Quantitative microbial source tracking (QMST)

For QMST analysis, 22 tributary/side arm samples were taken from 19 official JDS sites and 3 additional tributaries 10 to 70 km upstream of their confluent site with the Danube (Drava, Tisza and Sava). The same samples as for the determination of faecal indicators were used. A volume between 125 and 250 ml was filtered through 3.0 µm polycarbonate filters and the resulting filtrate was again filtered through a 0.2 µm polycarbonate filter. All filters were individually acid rinsed (0.1 M HNO_3) and autoclaved before use. Filters for both fractions were stored at -20°C during the rest of the cruise, transferred to the laboratory and stored at -80°C. DNA was extracted from the filters and BacH quantitative real-time PCR (qPCR) was performed on DNA extracts (REISCHER ET AL. 2008). BacH is a Bacteroidetes specific marker specific for human origin and has previously been tested for tributary samples from JDS 1 as well as for a variety of samples from a karstic alpine region in Austria (REISCHER ET AL. 2007). Resulting marker copy numbers from both size fractions were summed up, related to the original filtration volume and expressed as marker equivalents per L of sample (ME L^{-1}).

Results and Interpretation

Variation of faecal indicator bacteria along the longitudinal stretch of the River Danube, in side arms and main tributaries

Three standard bacteriological determinands (*E. coli*, Enterococci, Total Coliforms) were studied along the longitudinal stretch of the Danube River and the main tributaries and important side arms. During the Joint Danube Survey, 96 sampling sites from Germany (Neu-Ulm, Danube km 2600) to the Black Sea (St. George Arm, km 7) were investigated. Variation of microbiological parameters along the course of the Danube are shown in **Fig. 2-4**

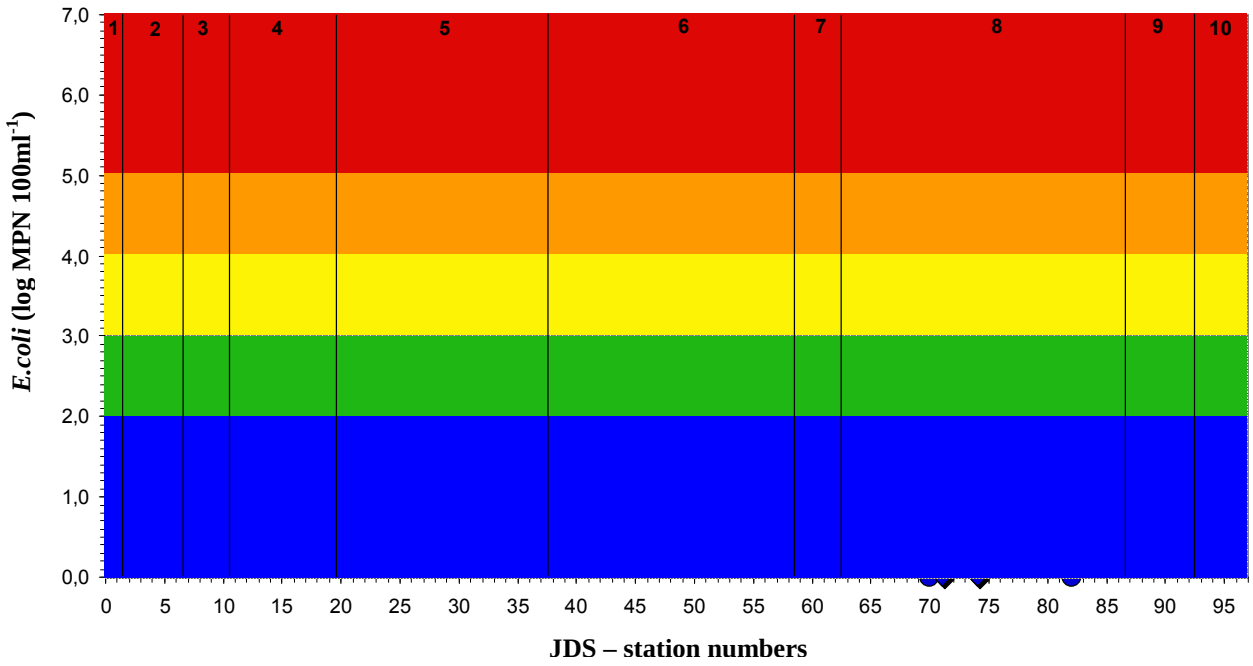

Fig. 2: Variation of *E. coli* [log (MPN 100ml^{-1})] along the longitudinal course of the Danube River (circles) and its main tributaries and side arms (diamonds). Numbers designate the 10 different Danube section types, colours refer to the five water quality classes (**Table 1**).

E. coli numbers ranged from over 6 orders of magnitude from undetectable to 1.5 x 10^6 MPN 100ml^{-1} (**Fig. 2**). Critical to excessive faecal pollution (> 1000 MPN 100ml^{-1}) was found in 33% of all sampling sites. As the most polluted sites the tributaries or side arms Arges (1.5 x 10^6), Russenski Lom (2.4 x 10^5), Rackeve-Sorocksar Arm (9 x 10^4) and Moson Danube (9 x 10^4) were identified. 25% of all sampling stations showed excellent water quality according to *E.coli* determination (low pollution < 100 MPN 100 ml^{-1}).

In the case of Enterococci, values ranged from undetectable (detection limit: 38 MPN 100 ml^{-1}) to 3.5 x 10^5 MPN 100 ml^{-1} (**Fig. 3**). 11% of all sites showed critical to excessive faecal pollution according to **Table 1** (> 400 MPN 100 ml^{-1}). The most polluted sites were Arges (3.5 x 10^5) and Russenski Lom 1.1 x 10^5). 50% of all sampling stations showed excellent water quality with low pollution levels (< 40 MPN 100 ml^{-1}).

Total Coliform numbers ranged from undetectable to 10.5 x 10^6 MPN 100 ml^{-1} (**Fig. 4**). 21 % of the samples exceeded the quality class II of 10.000 MPN 100 ml^{-1}. Highest pollution values were recorded for the tributaries or side arms Arges (10.5 x 10^6), Russenski Lom (5.3 x 10^5), Rackeve-Sorocksar Arm (6.7 x 10^5) and Moson Danube (2 x 10^5).

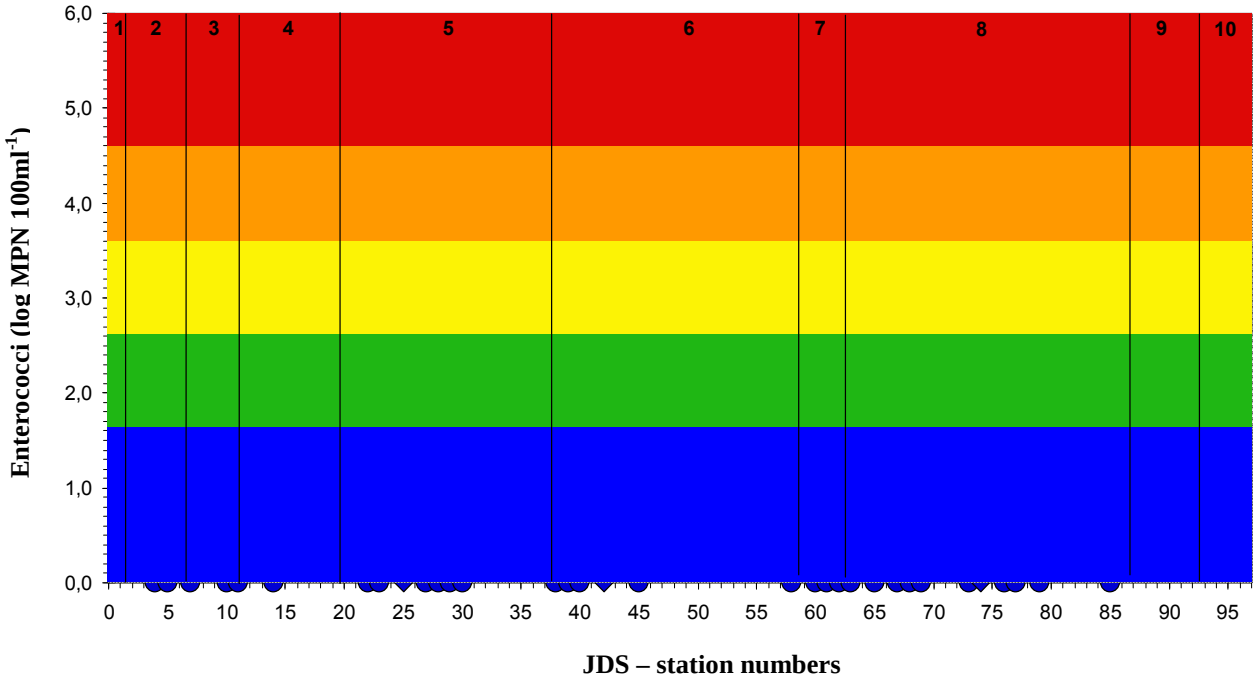

Fig. 3: Variation of Enterococci [log (MPN 100ml^{-1})] along the longitudinal course of the Danube River (circles) and its main tributaries and side arms (diamonds). Numbers designate the 10 different Danube section types, colours refer to the five water quality classes (**Table 1**).

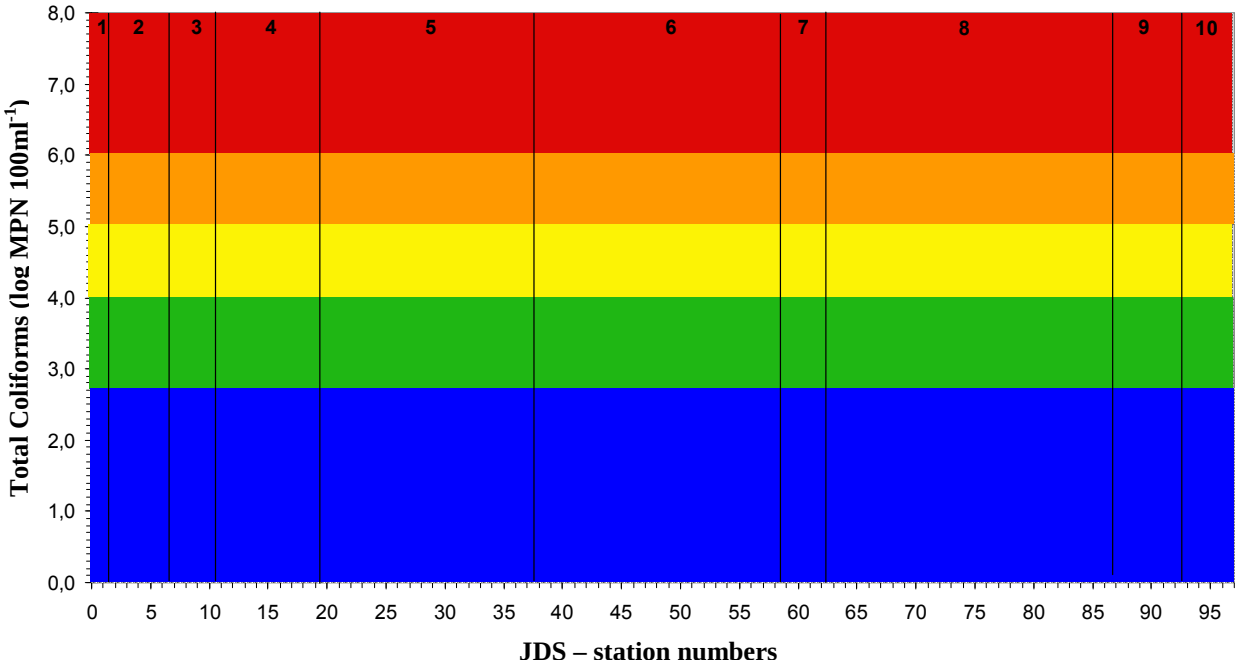

Fig. 4: Variation of Total Coliforms [log (MPN 100ml^{-1})] along the longitudinal course of the Danube River (circles) and its main tributaries and side arms (diamonds). Numbers designate the 10 different Danube section types, colours refer to the five water quality classes (**Table 1**).

Comparison between *E. coli* and Enterococci

There was a highly significant correlation between *E. coli* and Enterococci concentrations over the whole length of the Danube ($r = 0.62$, $p < 0.001$). When methodically caused zero-values of Enterococci were removed from the data set the correlation coefficient increased markedly ($r =$

0.84, $p < 0.001$) showing that by the application of both parameters a reliable estimation of faecal pollution in the Danube was possible. The ratio of *E. coli* to Enterococci has often been used as an indicator of the origin of faecal pollution. According to GELDREICH AND KENNER (1969), *E. coli* to Enterococci ratios in fresh faeces of < 1.5 should indicate pollution by waterfowl while ratios of > 4 should be typical for anthropogenic pollution. A high ratio should thus indicate a higher impact of human urban point-source pollution, while a lower ratio should indicate faecal pollution from (non-point) animal sources like waterfowl or rural agriculture. However, this concept was called into question (e.g. KIRSCHNER ET AL. 2004) who argued that this ratio rather reflects the specific survival characteristics of both groups which is dependent on the varying environmental constraints (salinity, temperature, biological removal of cells).
As the data in **Table 2** shows, no difference between the ratio in tributaries and in the Danube River itself was observed. Values varied strongly between 0.03 and 91.2 without detectable pattern with similar median and average values for tributaries and the main river.

Table 2: *E. coli* to Enterococci ratio in the Danube and tributary (side arm) samples. Data were ignored when one of the two parameters was zero.

	JDS total (n = 61)	Tributaries (n = 17)	Danube (n = 44)
Median	5.77	3.29	5.97
Average	9.49	10.9	8.95
Min	0.03	0.47	0.03
Max	91.2	91.2	63.4

Assessment of the microbiological water quality for the sections of the River Danube and major tributaries

Section type 1: Upper course of the Danube (rkm 2786: confluence of Brigach und Brege – rkm 2581: Neu Ulm)

There was only a single sampling point within this uppermost stretch of the Danube which showed moderate faecal pollution in case of Enterococci and Total Coliforms (class II), while in case of *E. coli* values were above the limit of 1000 MPN 100 ml^{-1} for class II. A possible influence of the high water situation during the first days of the JDS 2007 cannot be ruled out and may have led to elevated levels of faecal pollution in this stretch.

Section Type 2: Western Alpine Foothills Danube
(rkm 2581: Neu Ulm - rkm 2225: Passau)

The second section with 5 sampling stations was characterized by low to moderate faecal pollution (class I and II). Classes I and II were never exceeded by any of the three investigated parameters. Also the sample taken from the major tributary Inn at the end of this stretch showed moderate pollution values.

Section Type 3: Eastern Alpine Foothills Danube
(rkm 2225: Passau - rkm 2001: Krems)

Within the third section two stations downstream Linz and Ybbs revealed critical levels of faecal pollution (class III) according to *E. coli* measurements. Enterococci and Total Coliforms showed moderate faecal pollution.

Section Type 4: Lower Alpine Foothills Danube
(rkm 2001: Krems - rkm 1789.5: Gönyű / Klizská Nemá)

A heterogeneous situation was observed for section 4 of the Danube, ranging from the Wachau until the Moson Danube side arm downstream Bratislava. From low to moderate pollution levels (class I and II), the concentration of faecal indicators increased downstream Vienna and the confluence of the Danube channel, receiving the treated waste water from the Viennese waste water treatment plant. All parameters indicated critical faecal pollution (class III). After a decrease at Hainburg (15 km downstream) the tributary Morawa again showed critical levels of faecal contamination, which was most probably due to the high water situation of this river during sampling time. From Bratislava throughout the Gabcikovo channel faecal pollution was low to moderate (class I and II) according to all three parameters. Moson Danube however, turned out to be one of the most polluted side arms/tributaries of the Danube with critical to strong faecal pollution levels (class III and IV), which are most probably caused by the city of Györ. This sampling station was identified as a “hot spot”.

Section Type 5: Hungarian Danube Bend
(rkm 1789.5: Gönyű / Klizská Nemá - rkm 1497: Baja)

18 stations were investigated within section 5 which was characterized by a two completely contrasting stretches. Upstream Budapest mostly low to moderate levels of faecal pollution (class I and II) were observed. Also the tributaries Hron and Ipoly showed a good microbiological water quality. Downstream the capital of Hungary critical to strong faecal contamination (class III and IV) and was faced, especially in the Rackeve-Sorocksar side arm. Both sampling stations in this side arm were identified as “hot spots”. The tributary Sio was faecally only low contaminated.

Section Type 6: Pannonian Plain Danube
(rkm 1497 : Baja - rkm 1075 : Bazias)

In section 6 most stations had critical levels of faecal contamination according to *E. coli* determination. Downstream station 48 (upstream Tisza) Danube faecal pollution levels started to decline gradually over approximately 100 km, a trend continuing in section 7. In contrast to *E. coli*, Total Coliforms indicated mostly moderate pollution and Enterococci were often below detection limit. The capital of Serbia, Belgrade, did not lead to a significant increase in faecal indicators. The main tributaries Sava and Tisza were much less polluted than the Danube River itself with excellent water quality parameters (class I). Drava and Velika Morava on the other hand exhibited critical pollution, with the exception of Enterococci in the Drava River.

Section Type 7: Iron Gate Danube
(rkm 1075: Bazias - rkm 943: Turnu Severin)

The whole section 7 (Iron Gate Danube) showed the best microbiological water quality of all sections. All *E. coli* and Enterococci values and 50 % of Total Coliform samples were within class I.

Section Type 8: Western Pontic Danube
(rkm 943: Turnu Severin - rkm 375.5: Chiciu/Silistra)

The longest Danube section (> 550 km) was characterized by a mostly excellent to good microbiological water quality with low to moderate levels of faecal pollution (class I and II). Low faecal contamination was also observed in the main tributaries Timok, Iskar, Olt and Jantra. On the other hand, the most polluted side-arm (Russensky Lom) and tributary (Argus) showed with 2.4×10^5 and 1.5×10^6 MPN 100 ml^{-1} *E. coli* a 2500 – 3500 fold level of faecal contamination than observed at the upstream sampling point of the Danube. These sampling sites were identified as “hot spots”. Surprisingly, in the Arges River, which collects the untreated waste water from the capital of Romania, absolutely no increase in the faecal indicator concentrations in the Danube was observed at the downstream station. Arges has approximately 1 % of the discharge of the Danube, and thus an at least 25 fold increase of faecal indicators would be expected. Most probably, sampling in the middle of the Danube at the station 3 km downstream Arges failed to trace the sewage plume at the left river bank, and the impact of the waste water from Bucharest was manifested during JDS 2 only in a 3 fold increase at station 86 (50 km downstream).

Section Type 9: Eastern Wallachian Danube
(rkm 375.5: Chiciu/Silistra - rkm 100: Isaccea)

With the exception of one *E. coli* value (sampling point 88) all stations within Danube section 9 were moderately polluted with *E. coli*, Enterococci and Total Coliforms. Also the two tributaries Siret and Prut showed good water quality (class II).

Section Type 10: Danube Delta
(rkm 100: Isaccea - rkm 20 Chilia arm, rkm 19 Sulina arm and rkm 7 St. Gheorghe arm)

In the last section, the Danube delta, faecal pollution was low to moderate (class I and II) in all branches of the river.

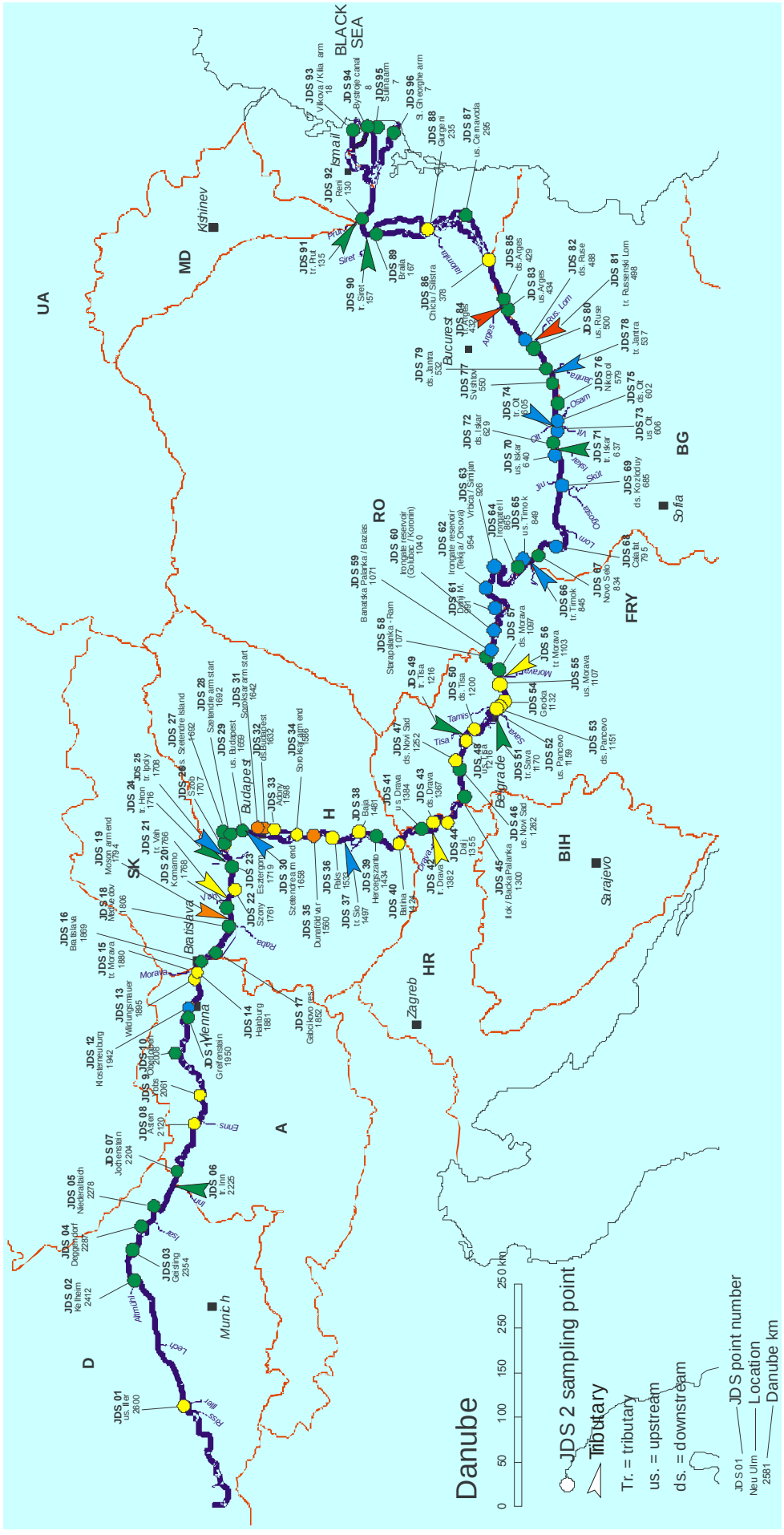

Fig. 5: Microbiological map of faecal pollution; classes of faecal pollution assessed by the concentrations of either E. coli or Enterococci; the worse classification is represented in the map (one–out – all–out principle; classification system see **Table 1**; blue coloured symbols: class 1 - little faecal pollution; green: class II- moderate faecal pollution; yellow: class III-critical faecal pollution; orange: class IV-strong faecal pollution; red: class V-excessive faecal pollution.

Water quality map according to faecal pollution levels

The microbiological water quality classification according to faecal pollution levels was carried out by data based on both *E. coli* and Enterococci concentrations (classification system see **Table 1**). The worse classification, considering both parameters for each sampling station, is represented in the map (**Fig. 5**). Because the various microbiological parameters used have a different environmental persistence and explanatory power, the parameter indicating the highest pollution level is used for water quality assessment (one-out – all-out principle). However, it should be kept in mind, that a single measurement per location, as performed during the course of the JDS 2, is not sufficient to make a definitive and final assessment of compliance with the EU Bathing Water Directive 2006.

Comparability and reliability of the chosen parameters

To evaluate the comparability of the results measured on board of Argus during the course of JDS 2, samples from selected locations were simultaneously analysed by three reference laboratories. Data for the 19 parallel *E. coli* and Faecal Coliform measurements, respectively, are shown in **Fig. 6**.

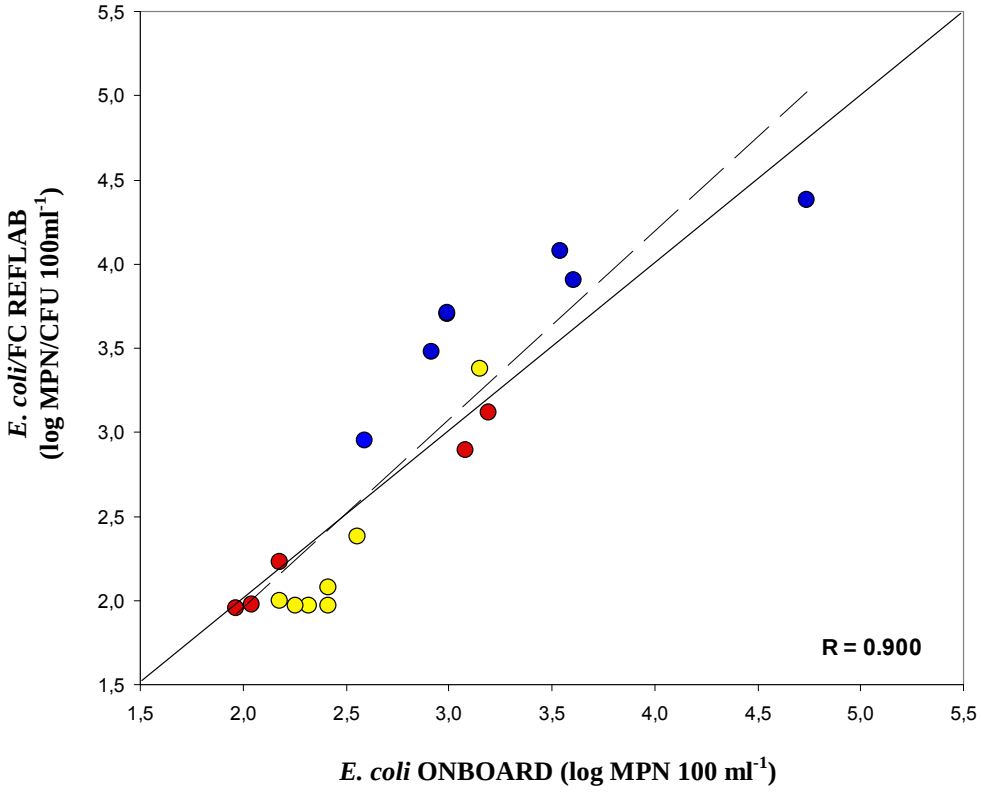

Fig. 6: Comparison of the *E. coli* determination via Colilert on board of Argus with the results from the reference laboratories. Yellow: German reference laboratory, Faecal Coliform (FC) detection via MPN (Bundesgesundheitsblatt 10/1995); Red: Austrian reference Laboratory, Membrane filtration on TBX Agar. Blue: Slovakian reference laboratory, membrane filtration (EN ISO9308-1). The dashed line indicates the regression line and the solid line the 1: 1 relationship.

A highly significant correlation between the data was achieved ($R = 0.90$, $p<0.001$, $n = 19$) and the regression line is close to the 1: 1 relationship, indicating that the data obtained on board with the chosen methodology yielded highly comparable data of *E. coli* detection.

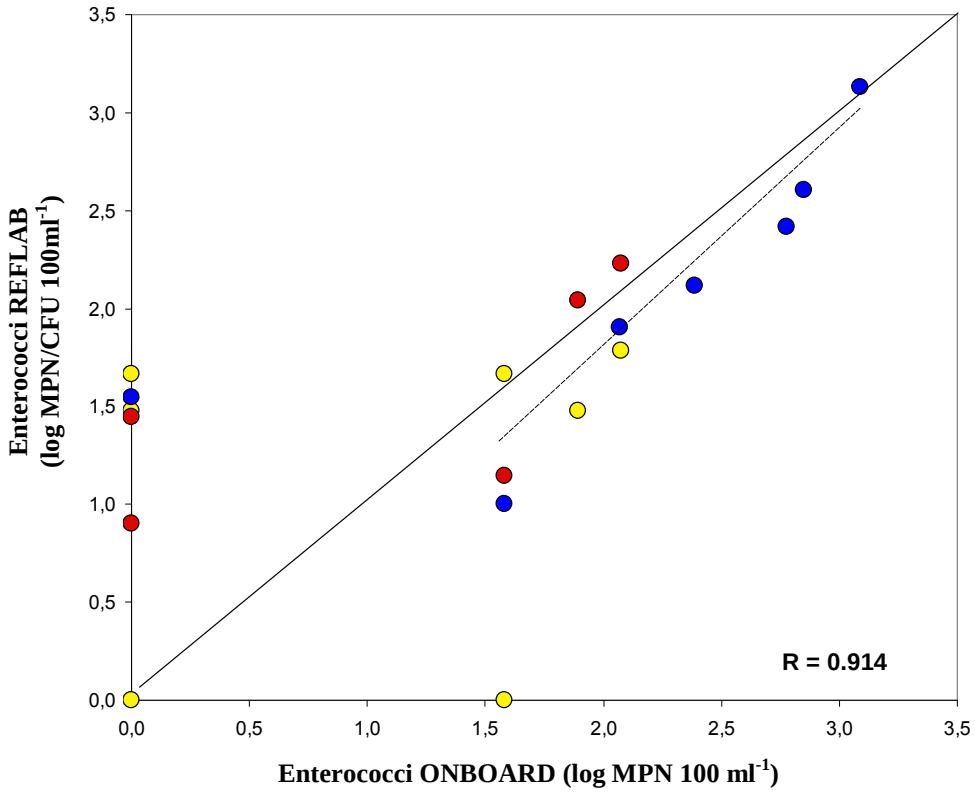

Fig. 7: Comparison of the Enterococci determination via microtiter plate technique on board of Argus with the results from the reference laboratories. Yellow: German reference laboratory, same microtiter plate technique; Red: Austrian reference Laboratory, Membrane filtration on Slanetz-Bartley Agar. Blue: Slovakian reference laboratory, membrane filtration. The solid line indicates the 1: 1 relationship.

For Enterococci (**Fig. 7**), the correlation between the ship and the reference lab for the total selected data set was low because of the high number of values below detection limit (< 38 MPN 100 ml^{-1}). If these data are excluded, a highly significant correlation ($r = 0.91$, $P < 0.001$, $n = 12$) very close to the 1:1 line was achieved. Thus the comparability of the Enterococci data measured on board can be considered as high, if values are above detection limit of the method.

Comparison of the data with JDS 2001

Due to financial constraints and change of the legal basis (the old EU BATHING WATER QUALITY DIRECTIVE 76/160/EEC, 1976 has to be replaced by the new BATHING WATER QUALITY DIRECTIVE 2006/7/EC, 2006 by the member states in 2008), partly different parameters were measured and different methods were applied for the 3 faecal indicators.

E. coli

E. coli values, measured with the Colilert method in 2007, were compared with the Faecal Coliform data from 2001, because it is well known that in surface waters faecal coliforms and *E. coli* determined with membrane filtration methods show a high 1:1 correspondence (e.g. KIRSCHNER ET AL 2004). Correlation analysis showed a highly significant relationship ($r = 0.82$, $p < 0.001$, $n = 86$) with slightly lower *E. coli* values in 2007 (**Fig. 8**). Because of several limitations, a quantitative comparison of the data between the two years is not admissible. First, it has to be kept in mind that the methods used during both cruises were different and there is no reliable information about the correspondence between Faecal Coliform determination via membrane filtration and *E. coli* determination via the Colilert MPN approach available for the

Danube River. Second, it has to be taken into consideration, that also differences in natural conditions and changing impacts can influence the monitoring results, especially when only one single measurement per sampling point is available. And third, the comparisons presented here are only based on a comparison of concentrations. A more reliable comparison has to be based on load values, but unfortunately, no discharge data for JDS 1 are available. However, the high correspondence between the two years corroborated the localization of hot spots of faecal pollution in the Danube River basin including its major tributaries.

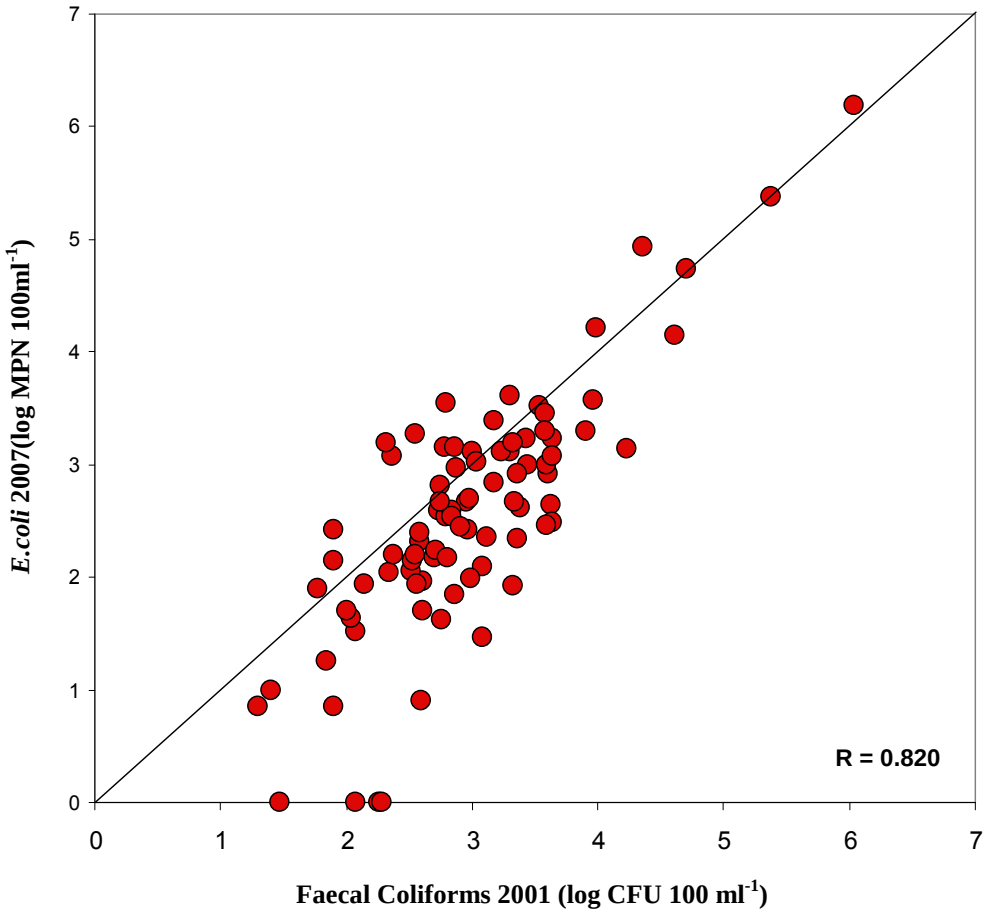

Fig. 8: Correlation between *E. coli*, determined via Colilert in 2007 (y-axis) and Faecal Coliforms (FC) determined via membrane filtration on MFC Agar in 2001 (x-axis). Data indicate a decreasing trend from 2001 towards 2007 by a downwards directed deviation from the 1:1 line (green arrows).

Enterococci

The comparison between the Enterococci determined in 2007 via microtiter plate technique and in 2001 via membrane filtration on Slanetz-Bartley agar is shown in **Fig. 9**. Due to the rather high detection limit of the first method (38 MPN 100 ml^{-1} = log 1.58), the many zero-values (n = 35) obstruct to a certain degree a reliable correlation analysis. It should be stated again that (i) because of the different methods used, (ii) because data were collected at only 1 sampling time and (iii) discharge data for JDS 1 would be necessary to calculate absolute loads, it is not admissible to make a comparative quantitative assessment between the two years.

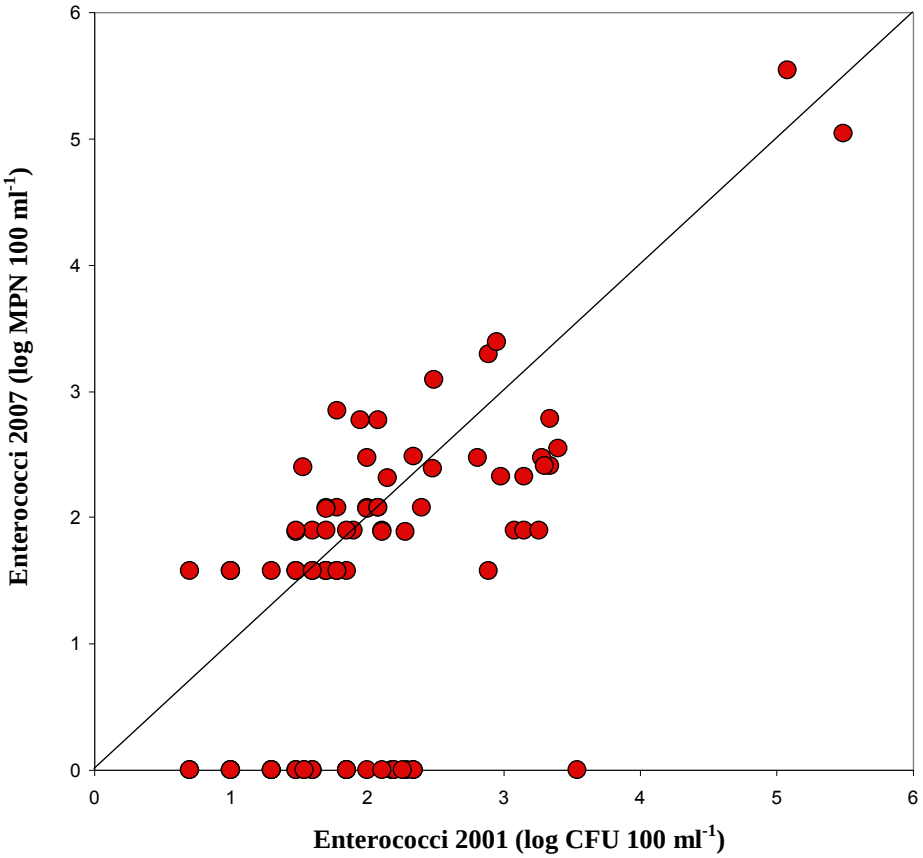

Fig. 9: Correlation between Enterococci determined in 2007 via microtiter plate technique (y-axis) and in 2001 via membrane filtration on Slanetz-Bartley agar (x-axis). A high proportion of the samples measured in 2007 were below detection limit of 38 MPN 100 ml^{-1} (log 1.58).

Total Coliforms

Also in the case of Total Coliforms methodical differences (Colilert 2007 vs. membrane filtration on ENDO agar 2001) complicate a direct comparison of the two cruises. It is well known that Colilert tends to overestimate Total Coliform numbers, because their identification relies simply on one enzymatic reaction (beta-galactosidase) in contrast to the Endo agar method which is based on a broader variety of identification parameters. **Fig. 10** shows the relationship of Total Coliforms between the two surveys. A highly significant correlation was observed ($R = 0.78$, $p < 0.001$; $n = 86$).

Microbial Source Tracking

The human marker BacH was detectable in 82% (18 of 22) of the samples included in this investigation. Interestingly, there was no clear trend towards higher marker concentrations in one of the two size fractions investigated (**Fig. 11**). Thus the results from both fractions were subsequently combined in all analysis. The maker concentrations ranged from 1.4×10^2 ME L^{-1} to 5.8×10^7 ME L^{-1} with a median concentration of 8.9×10^4 ME L^{-1}. A clear correlation of the human specific BacH marker concentrations in the tributaries to the *E. coli* concentration became evident (**Fig. 12**). The coefficient of determination in this relationship was 0.80, signifying that 80% of the variation in *E. coli* levels is explained by variation in QMST BacH. This indicates that the investigated tributaries were strongly influenced by human faecal pollution.

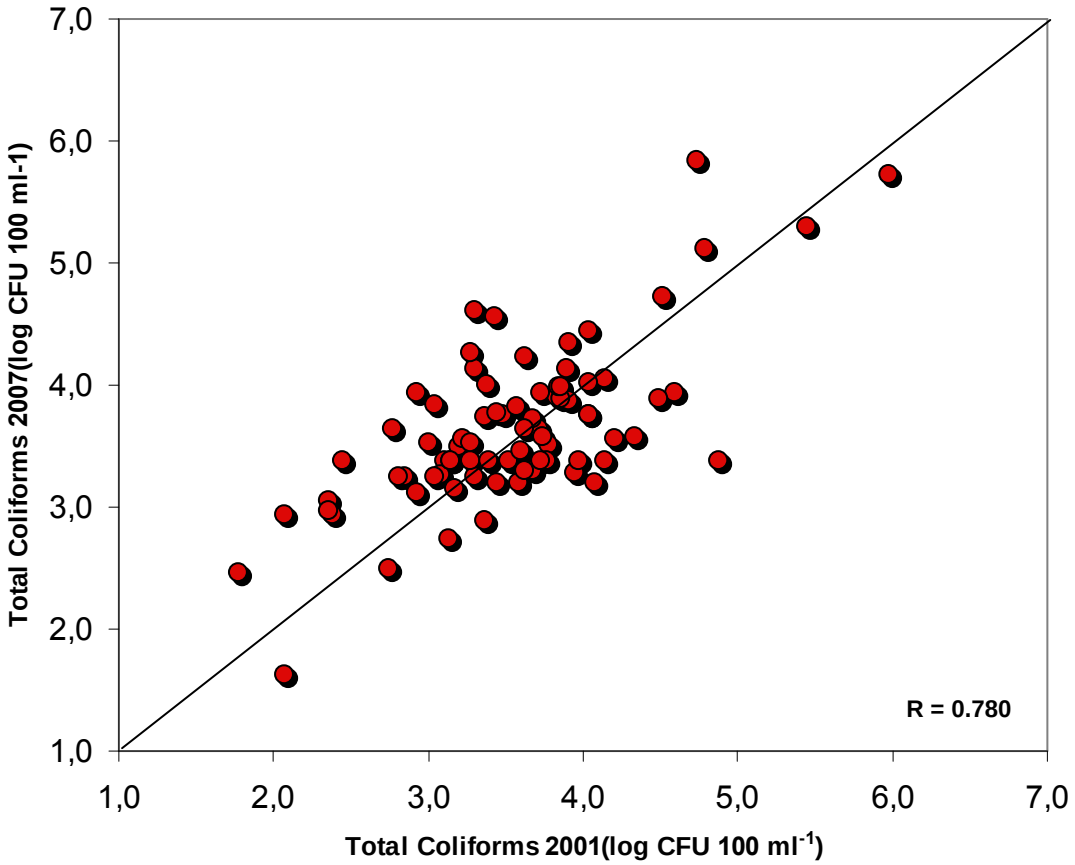

Fig. 10: Correlation between Total Coliforms determined in 2007 via Colilert (y-axis) and in 2001 via membrane filtration on ENDO agar (x-axis). The solid line depicts the 1:1 relationship.

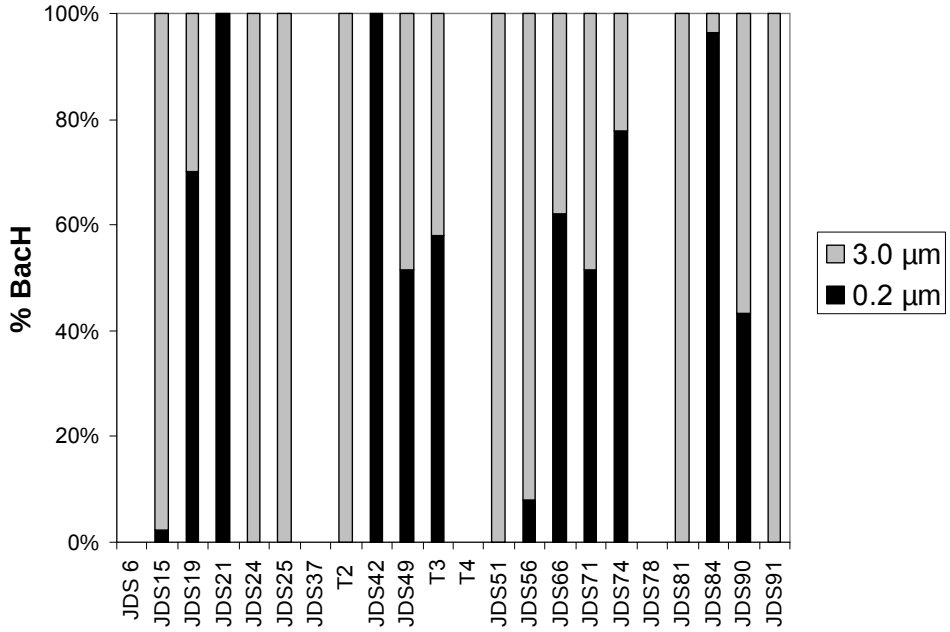

Fig 11. Distribution of the BacH marker between the two filtration fractions (3 µm and 0.2 µm) for the investigated tributaries. Samples without bars had no detectable BacH marker.

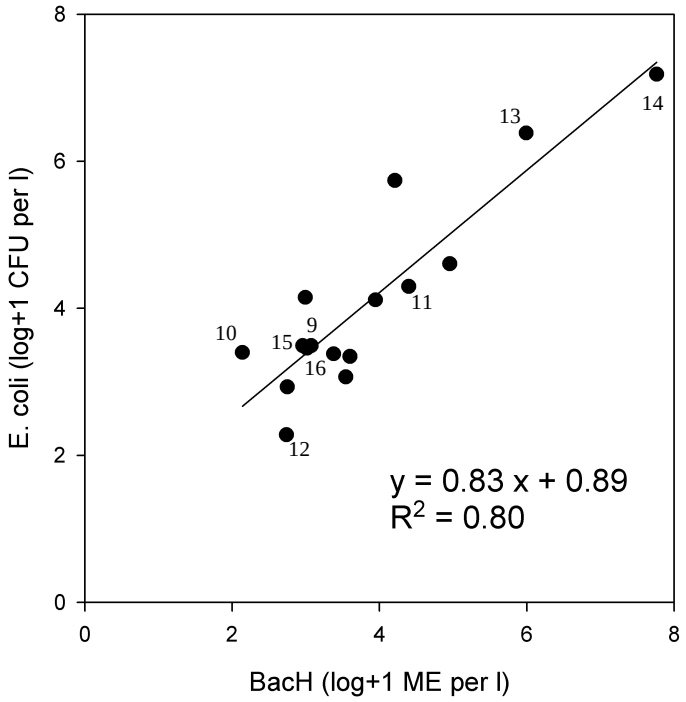

Figure 12. Regression between QMST BacH and *E. coli* concentrations in Danube tributaries. Tributaries: 1, JDS15-Morava; 2, JDS19-Moson Danube; 3, JDS21-Vah; 4, JDS24-Hron; 5, JDS25-Ipoly; 6, T2-Drava upstream; 7, JDS42-Drava; 8, JDS49-Tisza; 9, T3-Tisza upstream; 10, JDS51-Sava; 11, JDS56-Velika Morava; 12, JDS66-Timok; 13, JDS81-Russenski Lom; 14, JDS84-Arges; 15, JDS90-Siret; 16, JDS91-Prut.

Summary and Conclusions

- The longitudinal study of the entire course of the Danube River and its tributaries by applying uniform methods in an on-board laboratory allowed for a reliable quantitative estimation of the presence of faecal indicators and thus faecal pollution levels.

- The use of the Colilert system for *E. coli* detection was the most appropriate and robust parameter to predict faecal contamination. Enterococci measured with the microtiter plate technique with 4 dilutions showed a too high detection limit. Total Coliform detection with the Colilert system most probably overestimated faecal pollution because of methodical reasons. Moreover, Total Coliforms are not entirely specific for faecal pollution and are thus not longer recommended for faecal pollution monitoring.

- The comparison of the on-board data with three national reference laboratories revealed a good correspondence with the exception of methodically caused detection limits of some of the MPN based approaches (especially ENT microtiter plate technique).

- Through the application of a “5-level” classification system, which is harmonised with the limit values for good bathing water quality according to the EU BATHING WATER QUALITY

DIRECTIVES (1976 and 2006; KAVKA ET AL. 2006), the assessment of the microbiological water quality regarding faecal pollution became possible. However it has to be kept in mind that a single determination at one sampling point is not enough to classify a sampling point as "of sufficient" or "of poor" water quality with respect to the EU Bathing Water Directive and such classification was thus not taken into consideration.

- Thirty-one JDS sampling sites (22 Danube samples and 9 tributaries/side arms) were classified as critically, strongly or excessively polluted. As sources of highest contamination the tributary Arges and the side arms Russenski Lom, Rackeve-Soroksar and Moson Danube were identified as "hot spots". Lower levels of faecal pollution were found in the uppermost stretches in Germany and Austria, between Estergom and Budapest in Hungary, in the Iron Gate Reservoir in Serbia and the Western Pontic Danube until Russenski Lom in Romania and the Danube Delta itself. Highest contamination levels in the Danube River itself were found in the stretch between Budapest and Belgrade.

- The comparison of the pooled data set with the preceding JDS 2001 showed a high correspondence for *E.coli* and Total Coliforms. For Enterococci the correlation was obstructed by several zero-values obtained in 2007 due to the high detection limit of the chosen protocol. Due to several methodical reasons no quantitative comparison between the two years is admissible. However, the high correspondence between the two years corroborated the localization of hot spots of faecal pollution in the Danube River basin including its major tributaries. Especially, no changes in water quality was observed for the three most polluted tributaries/side arms (Rackeve-Soroksar Arm, Russenski Lom and Arges) indicating that an implementation of new water treatment plants will have a great potential for a further reduction of faecal pollution in the Danube River basin.

- The application of the quantitative microbial source tracking BacH marker (QMST BacH) for all tributary and side arm samples demonstrated impressively the importance of human faecal contamination of these water sources by explaining more than 80 % of the variation of *E. coli* concentrations. Thus, the QMST - technique has shown great potential to specifically quantify human faecal pollution and will allow more target oriented measures in the Danube catchment area.

- Methodical recommendations for a third Joint Danube Survey explicitly imply sampling in the middle and at both river banks to improve significantly the detection probability of faecal contamination from smaller sources. Moreover, a more sensitive protocol for Enterococci detection has to be considered (2 dilutions for most of the sampling points, membrane filtration technique). The integration of more reference laboratories from other countries should be enforced. For a better comparison the use of the same methodology for *E. coli* and Enterococci in the next JDS should be prerequisite, optimally paralleled by on board measurements of these two parameters with standardised membrane filtration methods. Quantitative microbial source tracking (QMST) proved to be very useful to determine the extent of faecal pollution and should therefore be extended also to other possible sources if available (e.g. ruminants).

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References

EDBERG S.C., E.W. RICE, R.J. KARLIN AND M.J. ALLEN (2000) *Escherichia coli:* the best biological drinking water indicator for public health protection. *J. Appl. Microbiol. 88:(Suppl.):106-116.*

EU BATHING WATER QUALITY DIRECTIVE (1976) EU Bathing Water Quality Directive 76/160/EEC

EU BATHING WATER QUALITY DIRECTIVE (2006) EU Bathing Water Quality Directive 2006/7/EC

FARNLEITNER A.H., KIRSCHNER A.K.T., ZECHMEISTER T.C. AND MACH R.L. (2001) Untersuchungstechniken in der mikrobiologischen Analyse von Wasser und Gewässern: Staus Quo und Perspektiven*; pp. 125-154. Austrian Association of Water and Waste Management. ÖWAV Schriftenreihe Heft 150; ISBN 3-902084-11*

FARNLEITNER A.H., REISCHER G.H., WINTER C., HEIN T., MACH R.L., KAVKA G.G. (2006) Progress in aquatic microbiology: introducing molecular biological approaches on a River Danube scale from Germany to the Black Sea. *Bulletin of the International Association for Danube Research 13-14: 8 - 12*

FARNLEITNER A.H, MACH R.L, REISCHER G. AND KAVKA G.G. (2007) Mikrobiologisch – hygienische Risiken trotz Kläranlagen am Stand der Technik? *Wiener Mitteilungen* 201: 209-242

GELDREICH E.E. AND KENNER B.A. (1969) Concepts of fecal streptococci in stream pollution. J. Water Pollut. Control Fed. 41:336–352.

KAVKA G.G. AND POETSCH E. (2002) Joint Danube Survey 2001 - Microbiological Results. In: *Joint Danube Survey – Technical Report of the International Commission for the Protection of the Danube River. 138 – 150 pp*

KAVKA G.G., KASIMIR G. D. AND A.H FARNLEITNER (2006) Microbiological water quality of the River Danube (km 2581 – km 15): Longitudinal variation of pollution as determined by standard parameters. In: *Proceedings 36th International Conference of IAD. Austrian Committee Danube Research / IAD, Vienna. ISBN 13: 978-3-9500723-2-7. pp 415-421.*

KIRSCHNER A.K.T., ZECHMEISTER T.C., KAVKA G.G., BEIWL A., HERZIG A., MACH R.L., FARNLEITNER A.H. (2004) Integral strategy to evaluate the faecal indicator performance in bird influenced saline inland waters. *Appl. Environ. Microbiol. 70: 7396-7403*

REISCHER G.H., KASPER D.C., STEINBORN R., FARNLEITNER A.H., MACH R.L. (2007) A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. *Letters in Applied Microbiology 44, 351–356*

REISCHER G.H., KAVKA G.G., KASPER D.C., WINTER C., MACH R.L., FARNLEITNER A.H. (2008) Applicability of DNA based quantitative microbial source tracking (QMST) on a large scale in the Danube River and its important tributaries. *Fundamental and Applied Limnology, Supplement 162: 117-125*

U.S. ENVIRONMENTAL PROTECTION AGENCY (1986): Ambient water-quality criteria for bacteria – 1986: Washington, D. C. . Office of Water Regulations and Standards , 17 p.

EU WATER FRAMEWORK DIRECTIVE (2000) EU-Water Framework Directive 2000/60/EC

Appendix

Table A1: Basic microbiological results of all JDS sampling stations; tributaries, side arms marked blue; n.d.: not detectable

			EC	ENT	TC
JDS nr.	Sampling site	river km	MPN/100 ml	MPN/100ml	MPN/100ml
1	Upstream Iller	2600	1410	119	5380
2	Kelheim	2412	210	78	2400
3	Geisling power plant	2354	260	38	1733
4	Deggendorf	2287	180	n.d.	1733
5	Niederalteich	2278	360	n.d.	2850
6	Inn	2225	260	38	1600
7	Jochenstein	2204	150	n.d.	1733
8	Abwinden-Asten	2120	1200	78	3350
9	Ybbs-Persenbeug	2061	1550	119	8570
10	Oberloiben	2008	150	n.d.	920
11	Greifenstein	1950	110	n.d.	770
12	Klosterneuburg	1942	93	38	550
13	Wildungsmauer	1895	3500	706	13300
14	Hainburg	1881	820	n.d.	5650
15	Morava	1880	4040	1228	36500
16	Bratislava	1869	980	245	7490
17	Gabcikovo reservoir	1852	980	117	7850
18	Medvedov / Medve	1806	390	38	1850
19	Moson Danube	1794	54700	600	198600
20	Komarom	1768	435	38	2420
21	Vah	1766	1410	299	9880
22	Iza / Szony	1761	1730	n.d.	5650
23	Esztergom	1719	345	n.d.	2420
24	Hron	1716	115	119	6900
25	Ipoly	1708	84	n.d.	3600
26	Szob	1707	220	38	1870
27	US Szentendre Island	1692	225	n.d.	3270
28	Szentendre Iskand arm	1692	125	n.d.	1990
29	US Budapest	1659	175	n.d.	3130
30	Budapest end of side arm	1658	42	n.d.	1410
31	RSD arm start	1642	86600	1960	677000
32	Budapest downstream	1632	14140	2420	130000
33	Adony / Lorev	1598	3790	395	24800
34	RSD arm end	1596	1840	250	4350
35	Dunaföldvar	1560	16160	255	51700
36	Paks	1533	3350	204	27500
37	Sio	1497	70	38	41000

38	Baja	1481	2420	n.d.	13700
39	Herczegszanto	1434	690	n.d.	6630
40	Batina	1424	1300	n.d.	8550
41	Upstream Drava	1384	460	77	2420
42	Drava	1382	1300	n.d.	22500
43	Downstream Drava	1367	2880	305	11370
44	Dalj	1355	1300	119	7660
45	Backa Palanka	1300	650	n.d.	3550
46	US Novi Sad	1262	920	78	18500
47	DS Novi Sad	1252	1730	300	10200
48	Upstream Tisza	1216	5760	1950	16600
49	Tisza	1216	240	38	2420
50	DS Tisza / UP Sava	1200	4800	1110	21870
51	Sava	1170	250	78	1733
52	Upstream Pancevo	1159	3720	299	8600
53	DownstreamPancevo	1151	1990	78	7800
54	Grocka	1132	1550	584	9600
55	Upstream Vel. Morava	1107	1050	38	4400
56	Velika Morava	1103	1990	584	9600
57	DS Velika Morava	1097	390	38	
58	Starapalanka-Ram	1077	140	n.d.	2420
59	Banatska Palanka	1071	86	38	2400
60	Iron Gate Reservoir	1040	33	n.d.	1300
61	Donji Milanovac	991	15	n.d.	387
62	IGR Tekija/Orsova	954	7	n.d.	291
63	Vrbica/Simijan	926	10	n.d.	866
64	Iron Gate II	865	105	38	1046
65	Upstream Timok	849	78	n.d.	866

66	Timok	845	18	38	2420
67	Pristol / Novo Salo	834	140	n.d.	1120
68	Calafat	795	43	n.d.	921
69	Downstream Kozloduy	685	50	n.d.	308
70	Upstream Iskar (Bajkal)	640	1	38	4
71	Iskar	637	n.d.	78	n.d.
72	Downstream Iskar	629	345	78	1987
73	Upstream Olt	606	7	n.d.	42
74	Olt	605	n.d.	n.d.	n.d.
75	Downstream Olt	602	8	38	9
76	Downstream Nikopol	579	160	n.d.	1733
77	Downstream Svistov	550	160	n.d.	2420
78	Jantra	537	96	38	16700
79	Downstream Jantra	532	50	n.d.	2420
80	Upstream Ruse	500	86	78	6000
81	Russenski Lom	498	242000	110340	530000
82	Downstream Ruse	488	n.d.	38	n.d.
83	Upstream Arges	434	460	119	3300
84	Arges	432	1530000	351840	10470000
85	Downstream Arges	429	150	n.d.	1553
86	Chiciu / Silistra	378	1400	208	2420
87	Upstream Cernavoda	295	410	77	3800
88	Giurgeni	235	1200	208	5700
89	Braila	167	460	77	2420
90	Siret	157	310	255	3800
91	Prut	135	285	350	1553
92	Reni	130	820	78	5300
93	Vilova / Kilia Arm	18	280	119	2400
94	Bystroye canal	8	280	38	1733
95	Sulina arm	7	490	38	4400
96	St.Gheorge arm	7	29	117	2900