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Abstract: The purpose of this paper is to compare various indicators of microbiological contamination in coastal areas near estuaries around the world. A major factor contributing to the presence of fecal coliforms is the geomorphology of the area, and, more particularly, whether the area concerned is an open or enclosed bay, the climate of the area, its tourism development, and, lastly, the urban, agricultural, livestock, or industrial activities carried out near river mouths. The aim was also to attempt to systematize the microbiological contamination of the above – mentioned estuaries around the world for broader applicability. The method used and the experimental apparatus for the isolation of bacteria was the membrane filtration method described in ISO 7704:1985. For the growth and enumeration of total coliforms and Escherichia coli the method described in ISO 9308-1 was used, while for enterococcus the one described in ISO 7899-2:2001. No statistically significant difference was found at many points in Greece and Spain, for either of the two pathogenic bacteria. In this work, the documentation of the microbiological contamination of river deltas was achieved in similar areas (Greece and Spain) and could be applicable throughout the Mediterranean.

Keywords: Fecal Indicator Bacteria (FIBs); *Total coliforms (TC)*; *Enterococci*; *Escherichia Coli (E. Coli)*; Marine recreational water; Greece

1. Introduction

The contamination of ecosystems has either direct or indirect impact on human health [1]. Poor treatment of waste and its discharge into the sea leads to the contamination of coastal recreational areas with microorganisms. Almost all epidemiological studies conclude that there is a significant risk to the health of people swimming in coastal areas where estuaries are found [2–8]. The increase of human activities for the purpose of either tourism or recreational activities is evidenced by the fact that two-thirds of the world's population resides in coastal areas, and this number tends to increase even more [9,10]. A number of years ago, the quality of bathing waters were a matter of concern for the EU, which had to be safeguarded in the interests of public health and tourism. In this respect, the EU adopted Directive 2000/60/EC [Water Framework Directive (WFD)], which is a landmark. The geomorphology of the soil and the high tourist presence on the beach are factors contributing to the increase of the number of FIBs [7,11–16]. The total number of bacteria present in sewerage is estimated to be 106/ml to 107/ml, of which approximately 105/ml or, according to others, 106/ml to 108/100 ml belong to the E. coli group and constitute 1% of the biomass in the large intestine [17,18]. An average of 1,95x109 coliforms are produced from human feces per day [19]. The bacterium Escherichia coli – irrespective of whether it originates from animal or human feces – is the best biological indicator of fecal contamination of the water [20], indicating the possible contamination of the water with other pathogenic microorganisms. It can be easily and rapidly counted in recreational waters, as several studies have shown that its presence is associated with the incidence of gastrointestinal disorders in swimmers [21–28]. The prevalence of diarrheal diseases caused by E. coli strains in coastal recreational areas is increasing due to the increased presence of human waste [29,30]. The same

results were also confirmed by a more recent (2016) study on diarrhea symptoms in children [31]. Fecal enterococci are part of the normal flora of humans and warm-blooded animals, are always present in feces [32], resistant to environmental conditions, and their possible presence in water combined with the absence of the bacterium E. coli indicates an old fecal contamination of the water. Enterococci are the best bacterial indicator for assessing fecal contamination of marine waters as well as their association with waterborne diseases [11,33–35]. The major source of fecal bacteria on a beach are humans [36–38] and animals. Increased phosphorus concentrations in agricultural waste lead to an increased presence and abundance of both fungal and bacterial pathogens [39–42]. The main threats to health associated with waterborne pathogenic bacteria are attributable to human feces as well as to domestic waste waters, industrial and hospital sewage, and urban or rural runoff [40,43–45]. It is noteworthy that increased concentrations of enterococci and total coliforms are observed following rainfall and storms [32,46–48]. Total coliforms can proliferate in nature even in the absence of sewage, when the E. coli group, fecal coliforms and enterococci are in warm environments. In addition to the intestinal flora of humans and warm-blooded animals, however, coliforms can also be found in many species of cold-blooded animals [49–51], and they constitute a useful tool for assessing recreational waters quality in case they are contaminated with waste water. In recent years the effort of scientists has been focused on trying to predict contamination in similar areas with innovative and intelligent methods [52].

2. Materials and Methods

The method used for the isolation of bacteria was the membrane filtration method described in ISO 7704:1985 [53]. For the growth and enumeration of total coliforms and Escherichia coli the method described in ISO 9308-1/2014 [54] was used, while for enterococcus the one described in ISO 7899-2:2001 [55].

2.1. Samling, Agar and Chemicals

The sampling was conducted in the morning hours and the water samples were transported to the laboratory in an isothermal container in order to preserve the microbial population under the best possible conditions and minimize the effect of external factors, such as heat. The analysis of the samples was performed within 8 hours. The sample volume was 500 ml. The sampling frequency was during the four seasons of the year. The nutrients used are, as follows:

A. The agar Harlequin® Chromogenic Agar of the manufacturer Neogenusa (CCA referred to in ISO 9308-1) was used for the enumeration of Escherichia coli and coliforms. CCA was made by dissolving 28.7 grams in 1 liter of water.

B. The substrate m–Enterococcus agar (Slanetz-Bartley Medium (referred to in ISO 7899-2:2001) of the same manufacturer was used for enterococcus. Slanetz-Bartley Medium was made by dissolving 43.5 grams in 1 liter of water.

The method involves the filtration of a sample of 100 ml through a membrane filter; the membrane filter is subsequently placed on a CCA to allow the growth of coliforms and E. coli, and on an Slanetz-Bartley Medium to allow the growth of enterococci. The incubation was performed at a temperature of 37°C for 24 ±2 hours. The confirmatory methods which were used for the enumeration of E. coli and coliforms following the use of Harlequin® Chromogenic Agar are, as follows: First confirmation test using Tryptone Soy Agar (TSA). About 10 red/pink colonies were picked from the CCA plate and then each sample was plated on TSA. Finally, an incubation was performed at 35-37°C for 24 ±2 hours. Second confirmation test using oxidase strips. Test performed on TSA colonies using oxidase strips. Samples are oxidase positive when the color on the strip is changed to blue – light purple. The medium Bile Esculin Agar was used for the detection and enumeration of enterococci. The procedure followed for all samples is, as follows: a membrane was placed on a Slanetz-Bartley Medium plate and then transferred to the Bile esculin agar medium plate. Subsequently, the plate was incubated in inverted position at 44±0.5 °C for 2 hours. Furthermore, according to ISO 9308-1: "total coliforms bacteria are the sum of oxidase negative colonies with pink to red color and all dark-blue violet colonies". The laboratory research was carried out in the Pineios

Delta area, very close to the city of Larisa, in the geographical region of Thessaly, and involved the coastal areas of this region. Samplings were conducted throughout the year.

3. Results

It should be noted that the samples collected in November 2023 and February 2024 are of particular importance as they allow us to draw useful data for the study areas following the Storm Daniel, which affected the wider area of Thessaly, Greece, an area which is crossed by Pineios River and into which its tributaries empty. Thus, conclusions for the water contamination prior and following the said storm can be drawn.

3.1. Study Area

Pineios Delta (Figure 1, Supplementary Figure) occupies an area of approximately 8.585 m² (2.121 acres) and is located in the Municipality of Tempi of the Regional Unit of Larisa of the Region of Thessaly. It is an extensive deltaic system sourced from the largest river in Thessaly. The total length of Pineios River along with its dispersions and meanderings is approximately 262 km. The climate of the area in question is Mediterranean on the eastern side and continental on the central and western sides, with two landscape variations – mountainous and lowland landscape – depending on the geological conformation. The temperature differences in the area between the seasons are large due to the fact that winters are cold while summers particularly hot. The coldest months are January, February, and December, while the warmest are July and August [56]. Pineios River is the recipient of the greatest amount of pollutants in the area. The major sources of pollution include treated urban and industrial waste and the intense agricultural and livestock activity that takes place in the Thessalian plain [57]. The delta area is of great ecological and socio-economic significance due to its rich biodiversity and natural beauty. The Pineios Delta area has been registered in the CORINE Biotopes database (1988) and designated as a Special Protection Area for the conservation of wild birds in accordance with Directive 79/409/EEC. Moreover, it belongs to the Natura 2000 network (code number GR1420002) and has wetlands, habitats, wild olive and olive groves, pastures, coastal and riverside forests, sand dunes, and sandy moors [58].

Cullera Bay is a coastline of approximately 6 km on the Mediterranean coast. Jucar River (with a length of 509 km), which carries sewage and waste from the urban and industrial areas it crosses, flows therein. Cullera Bay is delimited to the north by the cape of the same name and to the south by open sea. The city of Cullera is situated 38 km south of the city of Valencia. The study covers a period from July 2002 to April 2003. As mentioned in the literature, the samples were collected from 11 sites on the Cullera coastline. The bacteria which were studied are Enterococcus and Escherichia coli. There is a high influx of tourists in the area during summer months (21,500 residents during winter while this figure is 150,000 during summer [59], while the direction of the winds also affects the pollution of the area [60,61]. Furthermore, in Cape Cullera a notable increase of bacteria considered that driven by seasonal winds. Three sampling campaigns were conducted between July 2002 and April 2003 at 11 sites described in the search article. As concerned, E.Coli the values were far above the maximum limits on site 1 (1600 cfu/100ml) in July were the bay is more enclosed. On the said site enterococcus values were exceeded the permitted limits according to the same samples (166 cfu/100ml). The results of the September 2002 samples showed that E.Coli values were exceeded the permitted limits on site 2(991 cfu/100ml). An outbreak of E. Coli was observed in April 2023 on site 3(2021 cfu/100ml) the highest values that mentioned. Enterococcus values were within the permitted limits on all sites according to this sample. The results on the southern site, where the sea is open, showed that E.Coli and Enterococcus values were in the permitted limits.

Gonzaguinha and Ilha beaches, Brazil. The results revealed that at Gonzaguinha beach E. coli values were far above the maximum limits with 2640 cfu/100ml. At Ilha beach the maximum limits were not exceeded.

Miami Beach – Florida, USA. Enterococci concentrations were particularly high during the wet season. At area level, the concentrations of microorganisms showed differences regarding the distance from the coast. The concentrations were high in coastal areas and low in offshore areas. At

Grandon Beach the number of enterococci was 95% less than that at Hobie Beach. The two count sites at Hobie Beach (sites H1 and H2) showed differences in the concentrations of all microbes, with site H2 having a 95% higher concentration than site H1. The study revealed increased concentrations of enterococci on the shore during a tide, with the maximum value reaching 306 cfu/100 ml on the west and east sides of the beach.

3.2. Legislation

The legislative frameworks on the parameters in the study areas are summarized in Tables 1,2, where the bathing water quality classification is shown. Microbiological status of the waters in accordance with Framework Directive 2006/7/EC. Table 2 shows the limits (desirable and maximum) for bathing waters. The evaluation of all data will be carried out in accordance with the current European legislation. The samples were collected during the months of May, August, November 2023, and February 2024. Table 3 (supplementary material) presents the results of the microbiological analyses in detail.

The number of colony forming units is expressed in cfu/100 ml. Some of the analysis results are given in Figure 1.
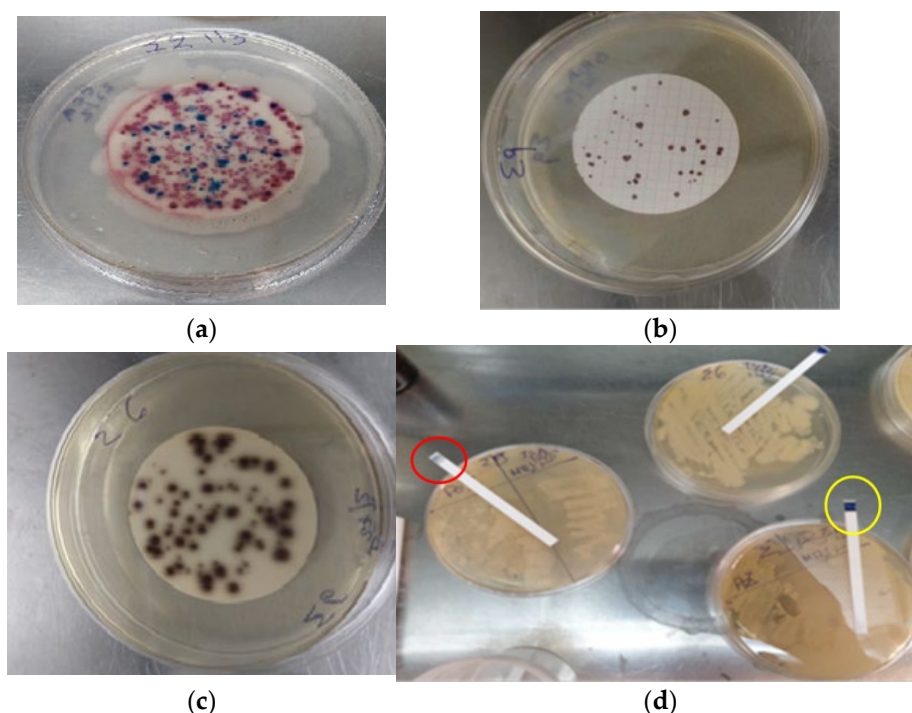

(**a**)   (**b**)
(**c**)   (**d**)

Figure 1. Examples of microorganisms growth in various nutrient substrates in Figures 2a-d. **a**: Total coliforms growth on Coliform Count Agar (CCA); **b:** Enterococcus growth on Slanetz and Bartley agar; **c**: Confirmation test for enterococcus using Bile Esculin Azide agar (BEA); **d**: Confirmation test using Tryptone Soy Agar (TSA) using oxidase strips. Grey color is negative on the left and black color is positive on the right.

The study results of May 2023 samples showed that Total Coliforms values were within the desirable limits on all coastal sites in Thessaly, Greece. Regarding the E. coli bacterium, the results demonstrated that its values were above the desirable limits only in Nea Messagala (280 cfu/100ml) in the samples of the same month and in Spain exceeded the legal limits (Figure 3, Supplementary Figures). Enterococcus values were also above the maximum limit in Nea Messagala (600 cfu/100ml) and USA (Figure 4, Supplementary Figures). As far as August 2023 samples are concerned, the results showed that E. coli values exceeded the desirable limits in Agia Paraskevi (369 cfu/100ml), Nea Messagala (373 cfu/100ml), and in Spain and Brazil exceeded the legal limits (Figure 5, Supplementary Figures). Enterococcus values were within the permitted limits on all these sites in the aforementioned samples. As for November 2023 samples, the results revealed that Total Coliforms values exceeded the desirable limits in Alexandrini (519 cfu/100ml), Stomio – Faros area

(621 cfu/100ml), Nea Messagala (528 cfu/100ml) (Figure 6, supplementary Figures). E. coli values were also above the desirable llimits in Nea Messagala (360 cfu/100ml) according to the samples of the same month and in Spain exceeded the legal limits (Figure 7, Supplementary Figures). Enterococcus values were within the permitted limits on all sites in the samples in question. The results of February 2024 samples showed that Total Coliforms values exceeded the desirable limit in Alexandrini (1446 cfu/100ml), Stomio – Faros area (1410 cfu/100ml), and Nea Messagala (1347 cfu/100ml) (Figure 8, Supplementary Figures). E. coli values were above the desirable limits in Alexandrini (432 cfu/100ml) according to the same samples (Figure 9, Supplementary Figures). Enterococcus values exceeded the maximum legal limits in Alexandrini (399 cfu/100ml), Stomio – Faros area (225 cfu/100ml), and Nea Messagala (426 cfu/100ml) (Figure 10, Supplementary Figures). Following the completion of the microbiological analyses during all seasons of the year, the times series for all microbiological parameters for the period from May 2023 to February 2024 were generated (Figure 11 to 14, Supplementary Figures). In Alexandrini an increase in Total coliforms and E. coli is observed following the extreme floods in Thessaly, Greece, at the end of 2023; this is due to the fact that Pineios River empties into this area. In Agia Paraskevi an outbreak of Total Coliforms (657 cfu/100 ml) and E. coli is observed during August 2023 and February 2024 (Total Coliform count: 384 cfu/100 ml) (Figure 12, Supplementary Figures). The increase in those values during August is due to the fact that it is summer vacation period and, therefore, the influx of tourists is higher. Furthermore, this is due to the geomorphology of the area and the fact that the bay is enclosed. In Stomio – Faros area an increase in Total Coliforms and E. coli is observed during summer months due to the high influx of bathers. This Total Coliforms increase was even more pronounced following the flood events occurred in the Region of Thessaly in Greece in the autumn of 2023. Moreover, enterococcus values exceeded the permitted limits in February 2024. In Nea Messagala an outbreak (i.e. values three times higher than the maximum limits) of enterococcus was observed in May 2023. E. coli values exceed legal limits during summer (and 373 cfu/100 ml). A rapid increase of Total coliforms was observed following the floods of 2023, with their value being 1347 cfu/100 ml in February 2024.

3.3. Statistical Analysis

SPSS package was used and Mann – Whitney Test was performed. No statistically significant difference was found before and after the flood events, for any of the two (E. coli P=0,263>0,05 and Enterococcus P=0,938>0,05) pathogenic bacteria (Table 4 – Supplementary material). No statistically significant difference was found at all points in Greece (except Agia Paraskevi – Stomio) and Spain, for either of the two pathogenic bacteria (Table 5). All P values ranged from 0,065 till 0,221.

4. Discussion

In this work, the microbiological contamination of river deltas was achieved in similar areas (Greece and Spain, Table 5 Supplementary material) and could be applicable throughout the Mediterranean. Therefore, the present study can be applied to areas with similar characteristics throughout the Mediterranean. The limitations of the research are that it can be applied to areas with the same geomorphology, climate temperature, winds and whether they are tourist resorts. The detailed analysis of the study areas shows that the areas of Florida, Miami, Hobie beach, USA, and Gonzaguinha beach in Brazil are enclosed bays with poor water circulation. Furthermore, the climate in Florida, Miami, Grandon beach in the USA, and Ilha beach in Brazil is tropical and subtropical, and the said areas are located in open ocean waters. Thus, these four areas cannot be compared to the other study areas. The only areas that can be compared to each other are the Pineios Delta areas and the Jucar River areas in Spain. The above areas have common natural, geomorphological and climate characteristics. In addition, both areas are near city centres – Larissa and Valencia, respectively –, and both areas are a magnet of tourists during summer months, and, thus, recipients of treated wastewater and urban and industrial waste. Prior to comparing the microbiological analysis results, we should take into account that the length of Pineios River is half the length of Jucar River, on the one hand, and that a flooding occurred in Greece in September 2023, resulting in higher coliform values in those areas, on the other hand. No statistically significant difference was found before and

after the flood events in Greece, for E. coli and Enterococcus bacteria (Table 4, Supplementary material). The microbiological analysis results indicate that in summer 2023 Escherichia Coli value reached 373 cfu/100ml in Greece and 1600 cfu/100ml in Jucar River (Figure 5). The analysis results for autumn (October) of the same year in Greece (360 cfu/100ml in Sbuka Beach Bar in Nea Messagala) and Spain (991 cfu/100ml) are proportionate to each other. Moreover, the water pollution with the Escherichia Coli bacteria remained at the same levels as that of summer months caused by the influx of tourists in Greece due to the floodings occurred in October 2023. In contrast, the pollution of the Jucar River waters reduced in October 2023, as no tourist activities have taken place, which could lead to water pollution. No statistically significant difference was found at all points in Greece (except Agia Paraskevi – Stomio) and Spain, for E. coli and Enterococcus pathogenic bacteria (Table 5). It is noteworthy that the number of Total coliforms in February 2024 in Greece reached the same levels as E. coli in Spain. Additionally, the only checkpoint in Greece that meets the requirements of Directive 2006/7/EC is Agia Paraskevi. A future research improvement could be the modeling the two rivers flow in Greece and Spain.

5. Conclusions

A key achievement of the research is that the contamination correlation of river deltas was achieved in similar areas (Greece and Spain). Continuous monitoring of contamination in sensitive delta areas throughout the Mediterranean Sea is a key issue for the protection of society, public health and environment.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, K.K.; Methodology, L.M.; software, K.K.; validation, K.K.; formal analysis, K.K.; investigation, K.K.; resources, K.K.; data curation, B.D.; writing—original draft preparation, K.K. and L.M.; writing—review and editing, K.K. and L.M.; visualization, K.K.; supervision, K.K.; project administration, K.K.; All authors have read and agreed to the published version of the manuscript.".

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